

MOLECULAR MECHANISM OF TUALANG HONEY
SUPPLEMENTATION IN THE ATTENUATION OF 12%
HIGH CHOLESTEROL DIET INDUCED NON-
ALCOHOLIC STEATOHEPATITIS IN RATS

BY

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ABSTRACT

The global surge in obesity, metabolic syndrome, and type 2 diabetes has driven a corresponding rise in non-alcoholic steatohepatitis (NASH), an advanced form of non-alcoholic fatty liver disease (NAFLD). Despite advancements in understanding its complex pathophysiology, NASH treatment options are limited. Current interventions primarily involve lifestyle changes, such as weight loss and exercise, and pharmacological agents which often present adverse effects, and no medication has been approved specifically for NASH. This highlights the urgent need for alternative therapeutic strategies. Tualang Honey (TH), a natural Malaysian product rich in antioxidants such as flavonoids and phenolic acids, represents a potential option for better NASH management. Previous studies have demonstrated TH's efficacy in improving liver enzymes and lipid profiles, particularly in animal models. However, the precise molecular mechanisms underlying its hepatoprotective properties remain poorly understood. This study investigated the molecular mechanisms underlying the attenuation of NASH through TH supplementation in a high-cholesterol diet (HCD)-induced NASH animal model. Twenty-five male Sprague Dawley rats were divided into five groups of 5 rats each: a normal control group (Group 1) fed with normal diet for 20 weeks and four groups fed a 12% HCD for 16 weeks to induce NASH, followed by four weeks of 12% HCD without TH (Group 2), 12% HCD and TH supplementation at doses of 1.2 g/kg/day (Group 3), moderate dose of 2.4 g/kg/day (Group 4), and high dose of 3.0 g/kg/day (Group 5). Histological examinations, biochemical assessment, immunohistochemical analyses, and gene expression profiling were conducted, followed by pathway analysis and validation of targeted gene expression. Statistical analysis was performed using one-way analysis of variance (ANOVA), followed by Tukey's least significant difference (LSD) post hoc test to identify group differences. The 12% HCD induced mild NASH, as evidenced by increased relative liver weight, presence of steatosis and inflammation in liver tissue samples, hypercholesterolaemia, hypertriglyceridaemia, as well as increased α -SMA expression and TUNEL assay positive staining in the liver sections of Group 2. TH supplementation at 2.4 g/kg/day dose improved liver gross morphology, significantly reduced total triglycerides, activated hepatic stellate cells and apoptotic liver cells. Microarray analysis revealed ten most significant upregulated and 10 most downregulated genes involved in oxidative stress, immune response, lipid metabolism, and cellular injury repair in the TH-treated animals. Further pathway analysis demonstrated three key mechanisms underpinning TH's hepatoprotective effects: (1) lipid and cholesterol metabolism (2) antioxidant and anti-inflammatory pathways, and (3) glucose and energy metabolism. Notably, the significant upregulation of the aldehyde dehydrogenase (*Aldh*) gene at 2.4 g/kg/day compared to the HCD group indicates enhanced aldehyde detoxification processes with TH supplementation. Meanwhile, a significantly reduced suppression at 2.4g/kg/day of the interferon regulatory factor 2 (*Irf2*) gene aligns with an attenuated inflammatory response. Conversely, high-dose TH (3.0 g/kg/day) exhibited paradoxical effects, as the histology, biochemical profiles and immunohistochemical analysis demonstrate findings resembling NASH pathology. In conclusion, this study demonstrates that the hepatoprotective effects of TH in a 12% HCD-induced NASH animal model were most pronounced at the moderate dose of 2.4 g/kg/day. Lipid and cholesterol metabolism, antioxidant and anti-inflammatory mechanisms, and glucose and energy metabolism pathways were identified as the primary molecular pathways supporting these effects, highlighting the therapeutic potential of TH at optimal dosage.

ملخص البحث

شهد العالم ارتفاعاً عالمياً في السمنة، ومتلازمة التمثيل الغذائي، ومرض السكري من النوع الثاني، مما أدى إلى زيادة موازية في التهاب الكبد الدهني غير الكحولي (NASH)، وهو شكل متقدم من مرض الكبد الدهني غير الكحولي (NAFLD) وعلى الرغم من التقدم في فهم الفيزيولوجيا المرضية المعقدة لهذا المرض، فإن خيارات علاج NASH لا تزال محدودة. وتشمل التدخلات الحالية تغييرات نمط الحياة، مثل فقدان الوزن وممارسة التمارين الرياضية، والعلاج الدوائي الذي غالباً ما يصاحبه آثار جانبية، كما أنه لم يتم الموافقة على أي دواء لعلاج NASH تحديداً. وهذا يبرز الحاجة الماسة إلى استراتيجيات علاجية بديلة. ويُعد عسل توالانغ (TH)، وهو منتج طبيعي ماليزي غني بمضادات الأكسدة مثل الفلافونويدات والأحماض الفينولية، خياراً محتملاً لإدارة NASH بشكل أفضل. وقد أظهرت الدراسات السابقة فعالية TH في تحسين إنزيمات الكبد والبروتين الدهني، خصوصاً في النماذج الحيوانية. ومع ذلك، لا تزال الآليات الجزيئية الدقيقة وراء خصائصه الواقية للكبد غير مفهومة بشكل جيد. تهدف هذه الدراسة إلى التحقيق في الآليات الجزيئية الكامنة وراء تخفيف NASH من خلال مكملات TH في نموذج حيواني مصاب بـ NASH ناتج عن نظام غذائي عالي الكوليسترول (HCD). تم تقسيم خمسة وعشرين جرذاً من نوع Sprague Dawley من الذكور إلى خمس مجموعات مكونة من 5 جرذان لكل مجموعة: مجموعة تحكم طبيعية (المجموعة 1) تم تغذيتها بنظام غذائي طبيعي لمدة 20 أسبوعاً، وأربع مجموعات تم تغذيتها بنظام غذائي يحتوي على 12% HCD لمدة 16 أسبوعاً لتحفيز NASH، تلتها أربعة أسابيع من 12% HCD دون TH (المجموعة 2)، و12% HCD مع مكملات TH بجرعات 1.2 غ/كغ/يوم (المجموعة 3)، وجرعة متوسطة 2.4 غ/كغ/يوم (المجموعة 4)، وجرعة عالية 3.0 غ/كغ/يوم (المجموعة 5). تم إجراء الفحوصات النسيجية، والتقييمات الكيميائية الحيوية، والتحليلات المناعية الكيميائية النسيجية، وتحليل تعبير الجينات، تلاها تحليل المسارات الحيوية والتحقق من تعبير الجينات المستهدفة. تم إجراء التحليل الإحصائي باستخدام تحليل التباين الأحادي (ANOVA)، تلاه اختبار Tukey LSD لتحديد الفروقات بين المجموعات. وقد تسبب نظام HCD بنسبة 12% في إحداث NASH خفيف، كما يتضح من زيادة وزن الكبد النسبي، ووجود التندس الدهني والالتهاب في عينات أنسجة الكبد، وفرط كوليسترول الدم، وفرط ثلاثي الغليسريد في الدم، بالإضافة إلى زيادة تعبير α -SMA وتلون TUNEL الإيجابي في مقاطع الكبد في المجموعة 2. أدى إعطاء TH بجرعة 2.4 غ/كغ/يوم إلى تحسين المظهر العام للكبد، وانخفاض كبير في مستويات ثلاثي الغليسريد الكلي، وتنشيط الخلايا النجمية الكبدية، وخلايا الكبد المبرمجة على الموت. كشفت تحليلات المصفوفات الدقيقة عن عشرة جينات مفعلة بشكل كبير وعشرة جينات مثبطة بشكل كبير مرتبطة بالإجهاد التأكسدي، والاستجابة المناعية، واستقلاب الدهون، وإصلاح إصابة الخلايا في الحيوانات المعالجة بـ TH. أظهر تحليل المسارات الحيوية ثلاثة آليات رئيسية تقف وراء التأثيرات الواقية للكبد لعسل (1) TH: استقلاب الدهون والكوليسترول، (2) المسارات المضادة للأكسدة والمضادة للالتهابات، و(3) استقلاب الجلوكوز والطاقة. ومن اللافت أن زيادة التعبير الجيني لإنزيم نازعة الهيدروجين الألدهيدية (Aldh) بجرعة 2.4 غ/كغ/يوم مقارنة بمجموعة HCD تشير إلى تعزيز عمليات إزالة سمية الألدهيد مع مكملات TH. في المقابل، فإن الانخفاض الملحوظ في تثبيط تعبير جين عامل تنظيم الإنترفرون 2 (Irf2) بجرعة 2.4 غ/كغ/يوم يتماشى مع استجابة التهابية مخففة. وعلى العكس من ذلك، أظهرت الجرعة العالية من TH (3.0 غ/كغ/يوم) آثاراً متناقضة، حيث أظهرت نتائج التحاليل النسيجية والكيميائية الحيوية والمناعية الكيميائية نمطاً يشبه مرض NASH. في الختام، تُظهر هذه الدراسة أن التأثيرات الواقية للكبد لعسل TH في نموذج NASH الناتج عن 12% HCD كانت أكثر وضوحاً عند الجرعة المتوسطة البالغة 2.4 غ/كغ/يوم. وقد تم تحديد استقلاب الدهون والكوليسترول، والآليات المضادة للأكسدة والمضادة للالتهابات، ومسارات استقلاب الجلوكوز والطاقة كآليات جزيئية رئيسية تدعم هذه التأثيرات، مما يبرز الإمكانات العلاجية لعسل TH عند الجرعة المثلى.

APPROVAL PAGE

I certify that I have supervised and read this study and that in my opinion, it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a thesis for the degree of Doctor of Philosophy in Medical Sciences.

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DECLARATION

I hereby declare that this thesis is the result of my own investigations, except where otherwise stated. I also declare that it has not been previously or concurrently submitted as a whole for any other degrees at IIUM or other institutions.

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Signature.....



Date..... 13/06/2025.....



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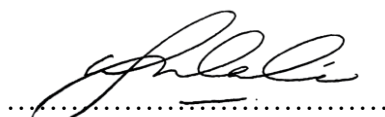
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DEDICATION



This thesis is dedicated to my wife and children.

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CHAPTER ONE

INTRODUCTION

1.1 BACKGROUND OF RESEARCH

Chronic liver disease (CLD) is a progressive deterioration of liver functions for more than six months, involving the continuous process of inflammation, destruction and regeneration of liver parenchyma, eventually leading to fibrosis and cirrhosis (Sharma, 2022). The most common aetiologies for CLD are chronic hepatitis B virus (HBV), hepatitis C virus (HCV), alcohol-related liver disease (ALD) and non-alcoholic fatty liver disease (NAFLD) (Moon et al., 2020). Chronic liver disease and its complications account for approximately 2 million deaths per year, comprising 3.5% of all deaths worldwide (Park et al., 2020). Making the most percentage of the causes of CLD, is the spectrum of NAFLD, amounting to 60% of all cases recorded (Cheemerla & Balakrishnan, 2021).

The spectrum of NAFLD progresses through three main stages from simple hepatic steatosis or non-alcoholic fatty liver (NAFL) to the more harmful non-alcoholic steatohepatitis (NASH), which refers to steatosis with necroinflammation, which could then lead to fibrosis, cirrhosis, and eventually hepatocellular carcinoma (HCC) (Abdul Sattar et al., 2021). As the name suggests, NAFLD is diagnosed when there is excess lipid accumulation in the liver of people who drink little or no alcohol, whereby definite diagnosis is only by liver biopsy (Friedman et al., 2018). NAFLD is currently the most common cause of liver disease in the United States, and is a large and growing worldwide public health problem, as the epidemic of metabolic syndrome spreads

(Koch & Yeh, 2018). From a recent review, it was reported that the worldwide prevalence of NAFLD stood at 37.8% (Riazi et al., 2022), seeing an increase from 21.9% back in 1991 (Le et al., 2022).

The progression from NAFLD to NASH is influenced by several factors including health conditions, such as type 2 diabetes and obesity, as well as genetic and environmental factors (Gabbia et al., 2021) including living an unhealthy lifestyle. It is estimated that roughly 40% of those diagnosed with NAFLD will progress to NASH, and 25% of adults with NASH develop cirrhosis later in life (Kleiner et al., 2019). Similarly, in Malaysia, with an increase in the prevalence of obesity and obesity-related diseases, it has been reported that the prevalence of NAFLD was at 37.4% among city-dwelling adults, and up to 86.7% in patients with diabetes mellitus (Khammas et al., 2019).

NASH is a complex disease involving several chains of events driving steatosis into four interconnected processes: deranged lipid metabolism, cell death, chronic inflammation, and wound healing (Wegermann et al., 2018). Histologically, NASH is categorised based on the severity of hepatic steatosis (5% and more), hepatocyte ballooning degeneration and hepatic lobular inflammation, with or without fibrosis (Sheka et al., 2020). It is noted that in patients diagnosed with NASH, liver fibrosis is the main determinant of mortality (Schwabe et al., 2020).

The mechanism leading to NASH appears to be multifactorial. There have been multiple hypotheses describing the pathogenesis of NASH, such as the ‘two hits’ hypothesis and most recently the ‘multiple hit’ hypothesis. In the ‘multiple hit’ hypothesis, it is suggested that the pathogenesis of NASH is limited through the triggering of excessive oxidative stress by lipotoxicity metabolites leading to hepatocyte damage (Fang et al., 2018). The injured hepatocytes in turn

release damage-associated molecular pattern molecules (DAMPs) that initiate an inflammatory response leading to direct recruitment of neutrophils, macrophages, and other components of the innate immune response (Farrell et al., 2018). The macrophages promote a proinflammatory microenvironment that causes activation of toll-like receptor (*TLR*), particularly TLR4 which in turn results in the release of monocyte chemotactic protein 1 (*MCP-1*), tumour necrosis factor- α (*TNF- α*), interleukin-1 β (*IL-1 β*), interleukin-6 (*IL-6*) and nuclear factor kappa-light-chain-enhancer of activated B cells (*Nf κ B*) (Hu et al., 2020). As a result, Cytochrome P450 2E1 (*CYP2E1*) expression is increased leading to further tissue damage through the generation of reactive oxygen metabolites (Harjumäki et al., 2021). The activated macrophages and damaged hepatocytes, especially ballooned hepatocytes, also bring about the release of profibrogenic cytokines of which platelet-derived growth factor (*PDGF*) and transforming growth factor beta (*TGF- β*) are the two best-described growth factors (Wada et al., 2019). The PDGF is involved in the signalling process required for hepatic stellate cell activation, while TGF- β promotes collagen production. Hepatic stellate cells (HSCs) proliferate and become myofibroblast-like that express alpha-smooth muscle actin (α SMA) in response to proliferative and fibrogenic cytokines (Liu et al., 2021).

Despite the increasing prevalence of NASH and being the most prominent cause of liver-related morbidity and mortality, the efficacy of current drug therapies is limited and there are no approved treatments for NASH (Muthiah & Sanyal, 2020). Currently, lifestyle modifications such as weight loss, physical activity and regular exercise remain the cornerstone approach for its management (Raza et al., 2021). It is to note that emerging therapies are currently being evaluated in clinical trials, such as pioglitazone, vitamin E and liraglutide. Presently, no single agent or combination has proven efficacy for patients with NASH and until the results of the ongoing

randomised, double-blind, placebo-controlled trials become available, lifestyle modifications and optimising metabolic risk factors are the best medical treatment options (Younossi, Loomba, Rinella, et al., 2018).

In recent years, demand for safe and effective natural products to treat chronic illnesses has been on the rise. This need has led to a re-evaluation of the therapeutic use of many plants and plant-based products, including honey. Among the many different types of honey, Tualang honey (TH) has shown some promise. TH is a Malaysian wild multifloral honey, produced by the rock bees (*Apis dorsata*) which build their hives on the tall branches of the Tualang trees. Various studies have thus far shown that TH has anti-inflammatory, antioxidant, antimicrobial, antidiabetic properties, and wound healing essential (Azman et al., 2021). Furthermore, when compared to several other local honey samples, such as Gelam and Indian forest, TH was found to have the highest content of antioxidants such as phenolics and flavonoids, as well as having the best free radical scavenging properties (Mohd Kamal et al., 2021).

In the context of NASH, TH has been shown to improve histological scoring, homeostatic model assessment for insulin resistance, liver function test and lipid profile, following a 4-week supplementation in a NASH animal model (Ghani et al., 2016). However, the molecular mechanisms of NASH attenuation following the Tualang honey supplementation have yet to be elucidated. This study aimed to investigate the molecular mechanisms involved in NASH following TH treatment in an animal model by examining the gene expression profile of markers of inflammation and fibrosis as well as the protein expression of hepatic stellate cell activation and apoptosis markers.

1.2 JUSTIFICATION OF RESEARCH

Non-alcoholic fatty liver disease (NAFLD) is a rapidly increasing and the most prevalent chronic liver disease that occurs in the absence of evident infection or significant consumption of alcohol. It is closely associated with metabolic syndrome particularly obesity and insulin resistance or type 2 Diabetes Mellitus. A study from Malaysia revealed a prevalence rate of 22.7% which was highest among Indians and Malays (Albracht-Schulte et al., 2019). It affects both adults and children. Non-alcoholic steatohepatitis (NASH) is the progressive form of NAFLD. Of concern is the fact that NASH carries the risk of progressing to cirrhosis with subsequent development of liver failure and or hepatocellular carcinoma (Karin, 2015). Because the prevalence of diabetes mellitus and obesity in Malaysia has reached epidemic proportion with half of whom (50%) have been diagnosed with NAFLD (Koh et al., 2016), it is essential that the possible treatment options for NASH must be explored as currently there limited established treatment for NASH beyond weight loss and associated metabolic co-morbidities management (Raza et al., 2021). To date, nutritional counselling and diet prescription to reduce bodyweight, coupled with physical exercise remains the first line of treatment. Drug therapy has typically been focused on the management of associated risk factors such as diabetes, obesity, hyperlipidaemia, inflammation and fibrosis (Banini & Sanyal, 2016). Effective treatment for NASH is therefore an area to be researched. Tualang honey which contains a complex mixture of sugars is known for its anti-inflammatory properties in addition to antioxidant effects. It has a potential application in attenuating NASH as shown by an earlier study in an animal model. However, there are no documented studies thus far that investigates the molecular mechanism of NASH attenuation with Tualang honey supplementation.

1.3 RESEARCH QUESTION

What are the molecular mechanisms by which Tualang honey supplementation attenuates non-alcoholic steatohepatitis (NASH) in an animal model?

1.4 RESEARCH OBJECTIVES

1.4.1 General Objective

To investigate the molecular mechanisms by which Tualang honey supplementation improves NASH in animal model.

1.4.2 Specific Objectives

1. To assess the effects of Tualang honey supplementation on relative liver weight, gross liver morphology and histological changes in the NASH animal model.
2. To determine the effects of Tualang honey supplementation on serum biochemistry profiles including liver function test, lipid profiles and homeostatic model assessment of insulin resistance (HOMA-IR) index in the NASH animal model.
3. To investigate the effects of Tualang honey supplementation on the protein expression profile of stellate cell activation (alpha-smooth muscle actin) and apoptosis markers (TUNEL) in the NASH animal model.
4. To investigate the effects of Tualang honey supplementation on gene expression profiles using microarray analysis in the NASH animal model.
5. To validate the upregulation and downregulation of selected microarray gene expression using RT-PCR in the NASH animal model.

1.5 RESEARCH HYPOTHESIS

- Tualang honey supplementation improves the relative liver weight, gross liver morphology and histological changes in the NASH animal model.
- Tualang honey supplementation improves the biochemistry profiles including liver function test, lipid profiles and homeostatic model assessment of insulin resistance (HOMA-IR) index in the NASH animal model.
- Tualang honey supplementation reduces the protein expression profile of stellate cell activation (alpha-smooth muscle actin [α -SMA]), and apoptosis markers (TUNEL assay) in the NASH animal model.
- Tualang honey supplementation modifies the gene expression involved in immune response, oxidative stress and cell apoptotic regulation in the NASH animal model.

1.6 CONCEPTUAL FRAMEWORK

Figure 1.1 illustrates the conceptual framework of molecular mechanism attenuation of TH supplementation in NASH animal model.

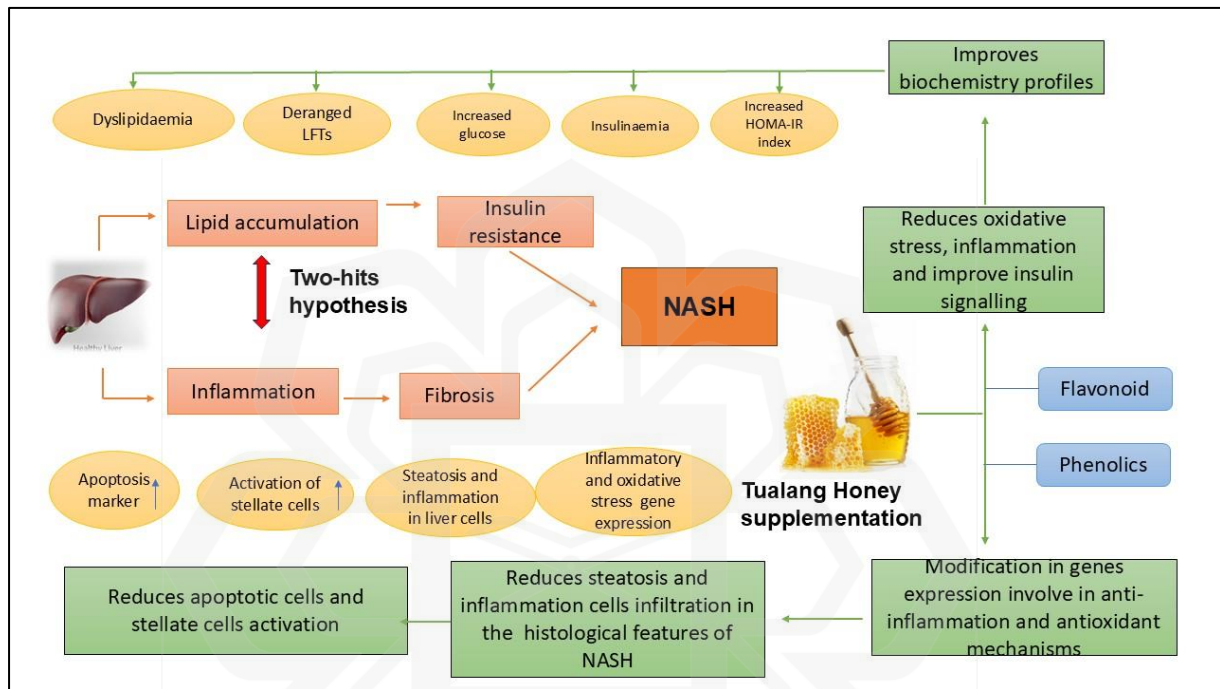


Figure 1.1 Conceptual framework of molecular mechanism attenuation of TH supplementation in NASH animal model.

CHAPTER TWO

LITERATURE REVIEW

2.1 CHRONIC LIVER DISEASE

Chronic liver disease (CLD) is characterised by the progressive decline in liver function over a period exceeding six months, involving the destruction and regeneration of liver parenchyma, which ultimately results in fibrosis and cirrhosis (Parel, 2021). Cirrhosis is an advanced stage of liver fibrosis that is accompanied by distortion of the hepatic vasculature that leads to portal hypertension and end-stage liver disease (Gines et al., 2021). The main aetiologies for CLD include chronic hepatitis B virus (HBV), hepatitis C virus (HCV), alcohol-associated liver disease (ALD) and non-alcoholic fatty liver disease (NAFLD). These diseases account for 99% of all CLD cases, while other liver diseases such as primary biliary cholangitis, primary sclerosing cholangitis, alpha-1-antitrypsin deficiency, Wilson's disease and autoimmune hepatitis account for 1% of cases (Cheemerla & Balakrishnan, 2021).

It is estimated that 1.5 billion cases of CLD were recorded globally, with viral hepatitis being the most common cause of liver deaths. At the same time, NAFLD is the most rapidly growing contributor to liver mortality and morbidity (Paik et al., 2020). The progression of fibrosis to cirrhosis is variable and is dependent on the cause of liver disease, environmental and host factors (Tanwar et al., 2020). This progression of disease does not occur in all patients with CLD, but when it does occur, the rate at which it happens varies from weeks to decades in certain cases (Gines et al., 2021). CLD complications account for 3.5% of all deaths worldwide, with cirrhosis causing

around 1.16 million deaths and liver cancer-causing 788,000 deaths (Shahin et al., 2021). This is an increase when compared to figures from the year 2000, when liver-related mortality accounted for 3% of all deaths worldwide (Asrani et al., 2019).

2.2 NON-ALCOHOLIC FATTY LIVER DISEASE SPECTRUM

Non-alcoholic fatty liver disease (NAFLD) encompasses a disease continuum from simple steatosis with or without fibrosis, to non-alcoholic steatohepatitis (NASH) which is characterised by necroinflammation and faster fibrosis progression than non-alcoholic fatty liver (Powell et al., 2021). Diagnosis is made when steatosis is seen in more than 5% of hepatocytes, in association with metabolic risk factors and the absence of excessive alcohol consumption (≥ 30 g per day for men and ≥ 20 g per day for women) or other chronic liver diseases (European Association for the Study of the et al., 2016). It was thought to be a benign condition but is now increasingly recognised as a major cause of liver-related mortality worldwide and is emerging as an important cause of end-stage liver disease, primary liver cancer and liver transplantation with a substantial health economic burden (Powell et al., 2021).

Approximately 2 billion adults worldwide who are obese or overweight and 400 million adults worldwide who have diabetes are at risk of NAFLD (Gines et al., 2021). Demographic factors, such as age, sex, and ethnicity, influence disease prevalence and progression. Men and postmenopausal women exhibit higher rates of advanced fibrosis, and ethnic disparities reveal heightened susceptibility among Hispanic populations and lower prevalence among African Americans (Bonacini et al., 2021). In Malaysia, the rapid increase in prevalence of obesity and obesity-related conditions has been accompanied by a reported NAFLD prevalence of 37.4%

among urban adults and as high as 86.7% among individuals with type 2 diabetes mellitus (T2DM) (Khammas et al., 2019).

NAFLD is often asymptomatic in its early stages, leading to underdiagnoses and delayed management (Farahat et al., 2022). Diagnosis relies on a combination of clinical evaluation, imaging modalities such as ultrasound and magnetic resonance imaging (MRI), and non-invasive fibrosis assessments, including elastography and serum biomarkers (Monelli et al., 2021). Liver biopsy remains the gold standard for differentiating NAFL from NASH and assessing fibrosis severity, though its invasiveness limits routine use (Sheka et al., 2020).

NAFLD has also been regarded as the hepatic manifestation of metabolic syndrome and is frequently associated with metabolic risk factors, including insulin resistance (IR), obesity, dyslipidaemia, hypertension, and diabetes (Chalasani et al., 2018). The pathophysiological relationship between NAFLD and insulin resistance (IR) is intricate and bidirectional; in fact, most extra-hepatic manifestations that are correlated with NAFLD share IR as a common etiological factor (Marušić et al., 2021). A recent meta-analysis of 19 studies with 296,439 individuals estimated that NAFLD patients had a 2-fold higher risk of T2DM development with respect to the general population, and that this risk was further increased (4.7-fold) in the presence of advanced fibrosis (Cuthbertson et al., 2021). In a study on 129 biopsy-proven NAFLD patients with a mean age of 51 years, the risk of T2DM development during a median follow-up time of 13.7 years was evaluated, showing that 78% of NAFLD subjects developed T2DM (Grecian, 2021). Established T2DM may further promote NAFLD progression to cirrhosis and the development of HCC. Moreover, the presence of T2DM is associated with increased all-cause and liver-related mortality in patients with NAFLD (Golabi et al., 2023).

The liver is one of the main organs involved in the alteration of glycaemic control during insulin resistance; in fact, patients with both NAFLD and T2DM were shown to have poor glycaemic control compared to patients with only T2DM (Colosimo et al., 2023). It was also noted that microvascular diabetic complications, such as retinopathy and nephropathy, were significantly more frequent in T2DM patients with concurrent NAFLD independent of confounding factors, including age, sex, body mass index (BMI), hypertension, smoking status, and medication use (Wen et al., 2022). More interestingly, these same findings were shown in type 1 diabetic patients with ultrasound-diagnosed NAFLD, confirming that NAFLD is involved in the pathogenesis of renal and retinal complications, probably through the release of pathogenic pro-inflammatory mediators such as advanced glycated end-products, reactive oxygen species (ROS), tumour necrosis factor- α (TNF- α), and transforming growth factor- β (TGF- β) (Orfanidou et al., 2023).

2.3 NON-ALCOHOLIC STEATOHEPATITIS

2.3.1 NASH Phenotype

Non-alcoholic steatohepatitis (NASH) represents a distinct phenotype within the spectrum of non-alcoholic fatty liver disease (NAFLD). It is characterised by histological, clinical, metabolic, and genetic features that distinguish it from simple steatosis and underline its progressive nature (Giashuddin & Alawad, 2022). Histologically, NASH is defined by hepatocellular fat accumulation (steatosis), lobular inflammation, and ballooning degeneration of hepatocytes (Peng et al., 2020). These features are often accompanied by varying degrees of fibrosis, which may advance to bridging fibrosis or cirrhosis. The hallmark of NASH is the presence of liver cell injury, which

contributes to its association with a higher risk of advanced liver disease and hepatocellular carcinoma (HCC) (Sheka et al., 2020).

Clinically, the NASH phenotype is closely linked to metabolic syndrome, characterised by insulin resistance, Type 2 diabetes, dyslipidaemia, and hypertension (Llovet et al., 2023). Obesity is a prominent risk factor, though lean NASH exists and underscores the heterogeneity of the disease. Lean NASH, more common in individuals with genetic predispositions, such as the 148 Isoleucine to Methionine protein variant (*I148M*) of patatin-like phospholipase domain-containing 3 (*PNPLA3*), presents diagnostic challenges due to the absence of overt obesity (Ahadi et al., 2021). In general, NASH is more prevalent in middle-aged individuals and postmenopausal women, with hormonal changes likely contributing to the sex-specific differences in disease progression (Younossi, Golabi, et al., 2023).

Genetic and molecular determinants play a pivotal role in shaping the NASH phenotype. Variants in *PNPLA3* and transmembrane 6 superfamily member 2 (*TM6SF2*) are strongly associated with hepatic fat accumulation and fibrosis, while protective variants such as hydroxysteroid 17-beta dehydrogenase 13 (*HSD17B13*) appear to reduce NASH risk (Paternostro et al., 2021). Epigenetic modifications and transcriptomic changes also contribute to the regulation of disease pathways, including inflammation and fibrosis. Elevated levels of pro-inflammatory cytokines, such as TNF- α and Interleukin-6 (IL-6), highlight the central role of chronic inflammation in disease progression (Tacke et al., 2023).

Recognising the NASH phenotype has significant clinical implications. It provides a framework for risk stratification, identifying individuals at high risk for disease progression. Moreover, phenotypic markers, including genetic variants, lipid profiles, and inflammatory

mediators, are being explored as tools for non-invasive diagnosis and monitoring. A deeper understanding of the NASH phenotype will be instrumental in advancing precision medicine, tailoring treatments to individual disease drivers, and improving outcomes in this complex and multifaceted disorder (Carlsson et al., 2020).

2.3.2 NASH Epidemiology

Non-alcoholic steatohepatitis (NASH) is a growing global health concern that affects 1.5% to 6.5% of the world's population, with significant regional and demographic variability (Kanwal et al., 2021). This variability is influenced by the prevalence of risk factors such as obesity, T2DM, and metabolic syndrome, as well as genetic predispositions in certain populations (Riazi et al., 2022). In North America, NASH prevalence is between 3% to 5% and approximately 12% among adults with NAFLD, reflecting the widespread presence of metabolic syndrome. Similarly, in the Middle East and Southeast Asia, NASH prevalence is high, correlating with rising rates of obesity and urbanisation (Teng et al., 2022). Conversely, regions like Africa and Central Asia report lower prevalence rates, although trends are increasing due to changes in lifestyle and diet (Younossi, Golabi, et al., 2023). Notably, the prevalence of NASH is increasing in East Asia, with a significant rise observed among non-obese individuals, likely driven by genetic influences (Table 2.1). NASH is not restricted to adults; its prevalence in children, particularly those with obesity, is growing, with rates ranging from 3% to 5% among paediatric populations in developed nations (Elizabeth & Schwimmer, 2021).

Demographically, NASH disproportionately affects middle-aged individuals, with peak prevalence typically occurring between 40 and 60 years of age (Dufour et al., 2021). Sex

differences are also evident; while NASH is slightly more common in men overall, postmenopausal women are at greater risk of developing advanced fibrosis, likely due to hormonal changes (DiStefano, 2020). Ethnic and racial disparities further highlight the complexity of NASH epidemiology (Table 2.1). Hispanic populations are at particularly high risk due to a combination of genetic susceptibility, such as the *PNPLA3 I148M* variant, and lifestyle factors (Bonacini et al., 2021). African Americans, despite exhibiting high rates of obesity and diabetes, tend to have a lower prevalence of NASH, possibly due to protective genetic factors similar to those observed in individuals of African ancestry (Younossi, Golabi, et al., 2023). Asian populations, particularly in East Asia, are witnessing a rapid increase in NASH prevalence, including among non-obese individuals, highlighting the significant influence of genetic and metabolic factors in the progression of the disease (Wong et al., 2023).

Table 2.1 Prevalence of NASH based on region

Region	Prevalence of NASH (% of population)
North America	3-5 % (lower in African Americans)
South America	2-4 %
Europe	1-4 %
Middle East	3-6 %
Africa	< 1-2 %
Central Asia	1-4 %
East Asia	2-6 % (rapid rise in non-obese individuals)
Southeast Asia	2-5 %
Australasia	2-4 % (higher in indigenous populations)

Note. This table represents approximate ranges, as exact data varies depending on study methods and population demographics. References (Dufour et al., 2021; Estes et al., 2018; Younossi, Golabi, et al., 2023)

The global burden of NASH is projected to increase significantly in the next decade. By 2030, it is estimated that the number of individuals with NASH will rise by more than 50% in many regions, leading to an increased burden of end-stage liver disease, liver transplantation, and HCC (Younossi, Paik, et al., 2023). In the United States, for example, NASH is estimated to account for up to 25% of cases of HCC (Motta et al., 2023). The growing demand for liver transplants due to NASH-related liver failure is placing a strain on organ transplantation programs, with a projected rise in both demand and associated costs over the next two decades. The systemic burden of NASH extends beyond liver disease, as it is closely linked to cardiometabolic comorbidities. Cardiovascular disease remains the leading cause of mortality in patients with NASH, highlighting the multi-systemic impact of the disease (Gutiérrez-Cuevas et al., 2021). Additionally, the presence of NASH exacerbates the progression of T2DM and increases the risk of chronic kidney disease, further complicating management and contributing to higher healthcare expenditures (Cheung & Ahmed, 2021). The disease's association with obesity also amplifies the burden, as both conditions often require extensive lifestyle interventions and long-term medical care.

Economic analyses reveal the high cost of managing NASH and its complications. Direct healthcare costs include diagnostic evaluations, treatment of liver disease complications, and transplantation expenses, while indirect costs stem from loss of productivity and reduced quality of life. In the United States and Europe, the annual economic burden of NASH is estimated to be in the billions, with further escalation expected as the prevalence of NASH rises (Witkowski et al., 2022). The global burden of NASH necessitates urgent attention from healthcare systems, researchers, and policymakers. Improved awareness, early diagnosis, and the development of effective therapeutic strategies are essential to mitigate its impact. Public health measures targeting

obesity, sedentary lifestyles, and metabolic syndrome are critical for addressing the root causes of the NASH epidemic. Meanwhile, advancements in non-invasive diagnostic tools and emerging pharmacological therapies offer hope for improved management and reduction of the long-term burden associated with this disease (Younossi, Paik, et al., 2023).

2.3.3 NASH Pathogenesis

The pathogenesis of NASH is complex and multifactorial, and it involves a combination of genetic, environmental, and lifestyle factors. The hallmark of NASH is the accumulation of fat in the liver, also known as hepatic steatosis. Hepatic steatosis results from an imbalance between the uptake, synthesis, and export of fatty acids in the liver. Excess accumulation of fatty acids leads to the formation of toxic lipid metabolites, which can trigger oxidative stress, inflammation, and cell death in the liver (Parthasarathy et al., 2020). In addition to hepatic steatosis, other histological features of NASH include lobular inflammation, hepatocyte ballooning, and perisinusoidal fibrosis. These features are thought to be the result of ongoing oxidative stress, inflammation, and damage to liver cells (Kobayashi et al., 2022). The degree of liver fibrosis is an important predictor of disease progression and is associated with an increased risk of cirrhosis, liver failure, and hepatocellular carcinoma.

Various mechanisms have been proposed to explain the development of NASH. One of the proposed mechanisms is the "two-hit" hypothesis, which suggests that the development of NASH requires a first hit, such as insulin resistance or lipid accumulation, followed by a second hit, such as oxidative stress or inflammation (Ota, 2021). Meanwhile, the "multiple-hit" hypothesis suggests that the development of NASH is the result of multiple hits that interact to cause liver damage.

These hits can include genetic factors, dietary factors, gut microbiota, and other environmental factors (Tilg et al., 2021).

The initial step in NASH pathogenesis is the accumulation of triglycerides and free fatty acids in hepatocytes, often driven by insulin resistance, which is a hallmark of metabolic syndrome. Insulin resistance promotes increased lipolysis in adipose tissue, leading to an influx of free fatty acids into the liver. Concurrently, *de novo* lipogenesis is upregulated, while fatty acid oxidation and export as very-low-density lipoproteins (VLDL) are impaired. This lipid overload results in hepatocellular steatosis, creating a substrate for further liver injury (Heida et al., 2021). Lipotoxicity, resulting from the accumulation of toxic lipid intermediates, induces mitochondrial dysfunction, endoplasmic reticulum (ER) stress, and hepatocyte apoptosis (Branković et al., 2022).

Oxidative stress plays a pivotal role in the progression of NAFLD to NASH. The excessive accumulation of fatty acids enhances mitochondrial β -oxidation and reactive oxygen species (ROS) production (Chen et al., 2020). ROS-induced damage to cellular macromolecules, such as DNA, proteins, and lipids, amplifies hepatocyte injury and activates inflammatory pathways. Oxidative stress also contributes to lipid peroxidation, which generates reactive aldehydes, further exacerbating liver damage and perpetuating a pro-inflammatory state within the liver (Parthasarathy et al., 2020).

Inflammation is central to the pathogenesis of NASH, as the injured hepatocytes release damage-associated molecular patterns (DAMPs) that activate Kupffer cells, resident macrophages of the liver (Xu et al., 2023). Activated Kupffer cells produce pro-inflammatory cytokines, such as tumour necrosis factor-alpha (TNF- α), interleukin-1 beta (IL-1 β), and interleukin-6 (IL-6), which recruit neutrophils and monocyte-derived macrophages to the site of injury (Ma et al., 2023). These

inflammatory mediators not only amplify hepatocyte damage but also activate hepatic stellate cells (HSCs), promoting fibrogenesis (Wang et al., 2022).

Fibrosis development is a critical determinant of NASH progression. Activated HSCs transform into myofibroblasts and secrete extracellular matrix components, including collagen. Fibrosis begins peri-sinusoidally and can progress to bridging fibrosis or cirrhosis over time (Zisser et al., 2021). Genetic factors, such as variants in the *PNPLA3* and *TM6SF2* genes, modulate susceptibility to fibrosis, while protective variants, like *HSD17B13*, can mitigate progression (Paternostro et al., 2021). Figure 2.1 summarises the pathogenesis of NASH which involve the intricate interplay of metabolic, inflammatory, and fibrogenic pathways. Understanding these mechanisms is critical for developing targeted therapies aimed at halting disease progression and improving outcomes for affected individuals.

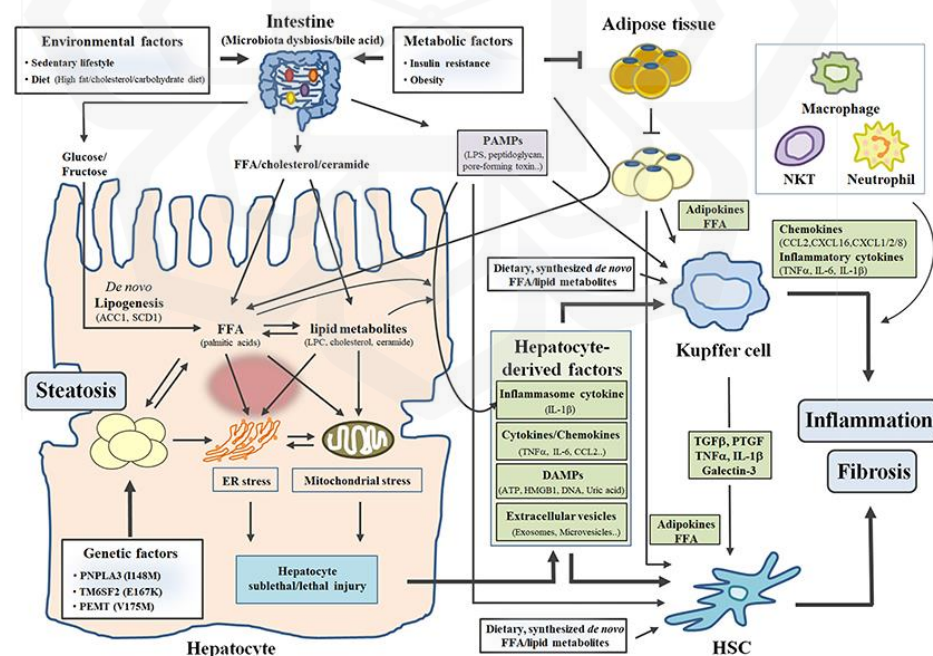


Figure 2.1 The intricate interplay of pathways involved in the pathogenesis of NASH
[Sourced from Kim and Lee (2018)]

2.3.4 NASH Diagnosis

The diagnosis of non-alcoholic steatohepatitis (NASH) can be challenging, as the disease often has no symptoms in its early stages. Diagnosis typically involves a combination of clinical, laboratory, and histological findings. Histological examination of liver tissue is considered the gold standard for the diagnosis of NASH (Piazzolla & Mangia, 2020).

The histological features of NASH are characterized by a combination of steatosis, inflammation, and hepatocellular injury. Steatosis, or the accumulation of fat in hepatocytes, is the hallmark feature of NASH (Giashuddin & Alawad, 2022). In NASH, steatosis is typically macrovesicular, meaning that the fat droplets are large and occupy most of the cytoplasm of the hepatocyte. Based on the NASH Clinical Research Network (CRN) criteria, steatosis is evaluated at low magnification and a visual estimate is made of the proportion of parenchyma involved in macrovesicular steatosis (Sanyal et al., 2024). Steatosis is graded as grade 0, 1, 2, and 3 with macrovesicular steatosis present in less than 5%, 5% to 33%, 34% to 66%, and more than 67% of the hepatocytes, respectively (Lai et al., 2022).

In addition to steatosis, NASH is characterised by inflammation, which is typically located in the perivenular and perisinusoidal regions of the liver lobule. The inflammatory infiltrate is composed of lymphocytes, macrophages, and neutrophils (Kage et al., 2020). The extent of inflammation is believed to play a significant role in the progression of NASH. In the early stages, portal tract inflammation is either absent or minimal, and bridging necrosis, commonly seen in viral hepatitis, is infrequent (Giashuddin & Alawad, 2022). As fibrosis progresses and the disease advances toward cirrhosis, severe inflammation becomes more apparent, particularly portal inflammation associated with interface hepatitis, marking a critical phase in the disease's evolution

(Lai et al., 2022). Finally, NASH is characterised by hepatocellular injury, which is manifested by ballooning degeneration of hepatocytes, which appear swollen and have a clear cytoplasm (Li et al., 2023). Hepatocytic ballooning is strongly linked to increased liver disease severity and a heightened risk of liver-related complications, cardiovascular disease, and chronic kidney disease. Liver-related mortality was more common in patients whose liver biopsies demonstrated hepatocytic ballooning, Mallory-Denk bodies (MDBs), or fibrosis (Singla et al., 2023).

The histological grading and staging of NASH are typically performed using the NAFLD Activity Score (NAS) and the Fibrosis-4 (FIB-4) score. The NAS is a composite score that combines the degree of steatosis, inflammation, and hepatocellular injury, and ranges from 0 to 8. A NAS score of 5 or greater is considered indicative of NASH (Pai et al., 2022). The FIB-4 score is a non-invasive marker of liver fibrosis that is calculated using age, AST, ALT, and platelet count. A FIB-4 score of less than 1.30 is considered indicative of no significant fibrosis, while a FIB-4 score of greater than 2.67 is considered indicative of significant fibrosis (Lee et al., 2021).

Liver biopsy remains the gold standard for the diagnosis of NASH, as it allows for the visualisation of the characteristic histological features of the disease (Heyens et al., 2021). However, liver biopsy is an invasive procedure that is associated with a risk of complications, such as bleeding and infection. As a result, non-invasive diagnostic techniques have been developed to aid in the diagnosis of NASH. Imaging techniques, such as ultrasound, computed tomography (CT), and magnetic resonance imaging (MRI), can be used to detect hepatic steatosis, but are not sensitive or specific enough to diagnose NASH (Troelstra et al., 2021). These techniques are useful in ruling out other liver diseases, such as cirrhosis and hepatocellular carcinoma (Piazzolla & Mangia, 2020).

Serum biomarkers play a pivotal role in the non-invasive assessment of non-alcoholic steatohepatitis (NASH), offering a practical alternative to liver biopsy for diagnosis and disease monitoring. These biomarkers are categorised into direct markers, reflecting hepatic injury, and indirect markers, indicative of metabolic dysfunction and systemic inflammation (Wang et al., 2022). Commonly used algorithms, such as the NAFLD fibrosis score (NFS), FIB-4 index, and aspartate aminotransferase-to-platelet ratio index (APRI), integrate routine laboratory parameters to estimate the risk of fibrosis (Zeng et al., 2022).

Additionally, cytokeratin-18 fragments, a marker of hepatocyte apoptosis, have shown promise in identifying NASH, although their sensitivity and specificity require further validation (Eguchi et al., 2022). Emerging biomarkers, such as adipokines, inflammatory cytokines, and oxidative stress indicators, provide insights into the underlying pathophysiology of NASH. Despite their potential, these markers lack the accuracy to differentiate simple steatosis from NASH reliably (Kjaergaard et al., 2023). Consequently, ongoing research aims to refine existing biomarkers and develop novel multi-omics approaches to enhance diagnostic precision, thereby reducing the dependence on invasive procedures.

2.3.5 NASH Treatment

Currently, there is no approved medication for the treatment of NASH. However, several drugs are being studied in clinical trials to evaluate their safety and efficacy in treating NASH. One class of drugs that is being studied for the treatment of NASH is insulin sensitizers. These drugs, such as pioglitazone and rosiglitazone, improve insulin sensitivity and reduce inflammation in the liver. They have been shown to improve liver function and reduce liver fat in individuals with NASH.

However, these drugs have side effects, such as weight gain and fluid retention, which limit their use (Smati et al., 2021).

Another class of drugs that is being studied for the treatment of NASH is GLP-1 receptor agonists. These drugs, such as liraglutide and semaglutide, stimulate the release of insulin and reduce inflammation in the liver. They have been shown to improve liver function and reduce liver fat in individuals with NASH. Nevertheless, the use of these drugs is constrained by their side effects, including nausea and diarrhoea (Rezaei et al., 2021). Antioxidants, such as vitamin E, have also been studied for the treatment of NASH. Vitamin E is a fat-soluble antioxidant that has been shown to reduce oxidative stress and inflammation in the liver. It has been shown to improve liver function and reduce liver fat in individuals with NASH. However, its usage is also limited by potential side effects, including an increased risk of bleeding and prostate cancer (Sumida et al., 2021). Farnesoid X receptor (FXR) agonists are a new class of drugs that are being studied for the treatment of NASH. These drugs, such as obeticholic acid, activate the FXR pathway, which regulates the metabolism of bile acids and lipids. FXR agonists have been found to enhance liver function, decrease liver fat, and lessen inflammation in individuals with NASH. Nonetheless, their use is associated with significant side effects, including pruritus and gastrointestinal discomfort (Kremoser, 2021).

Another approach to the treatment of NASH is the use of combination therapy. Combination therapy involves the use of two or more drugs that target different pathways involved in the development of NASH. For example, a combination of pioglitazone and vitamin E has been shown to improve liver function and reduce liver fat in individuals with NASH (Ratziu & Charlton, 2023). Although trials of combination therapies are currently underway, the regulatory pathway

regarding optimal design and choice of combination therapies is currently not established by the FDA (Dufour et al., 2020). The combination strategy can include two or more investigational drugs (such as in the ATLAS trial), an investigational drug with a previously approved drug for a different indication, or two (or more) previously approved drugs for a different indication as a novel combination therapy (Makri et al., 2022). It is anticipated that as the number of combination therapies and co-developed new investigational drugs increase, the study designs will need to accommodate more trial arms, and clinical trials will require larger numbers of patients. There will be a need to balance the level of evidence needed for approval with the challenges of patient recruitment and costs associated with the conduct of large clinical trials with multiple arms (Johnston et al., 2020).

In addition to medication, weight loss and lifestyle modifications remain the cornerstone of NASH treatment. Weight loss can improve insulin sensitivity, reduce inflammation, and decrease the accumulation of fat in the liver (Hydes et al., 2020). Exercise has also been shown to improve insulin sensitivity, reduce inflammation, and improve liver function in individuals with NASH (Semmler et al., 2021). A healthy diet that is low in saturated fat and high in fruits, vegetables, and whole grains can help reduce the accumulation of fat in the liver and improve metabolic health (Glass et al., 2020). Liver transplantation may be necessary in severe cases of NASH that progress to cirrhosis or liver failure. However, transplantation is limited by the shortage of donor organs, risks associated with the surgery and post-surgery complications (Burra et al., 2020). In summary, current treatment for NASH involves a combination of lifestyle modifications and medication. Lifestyle modifications, such as weight loss, exercise, and a healthy diet, remain the cornerstone of NASH treatment. Medications being studied for the treatment of NASH include insulin

sensitizers, glucagon-like peptide-1 (GLP-1) receptor agonists, antioxidants, FXR agonists, and combination therapy. However, these drugs have side effects that limit their use, and there is currently no approved medication for the treatment of NASH (Harrison et al., 2023). Ongoing research aims to identify effective therapies for NASH and to improve the management of this increasingly prevalent liver disease.

One of the many ongoing research projects in identifying effective therapies for NASH involve the use of a natural product native to Southeast Asia, a type of honey that is harvested from bee hives of the Tualang tree, aptly named Tualang Honey. It has been traditionally used for various medicinal purposes, including the treatment of liver diseases, cardiovascular conditions, cancer therapy and wound healing (Azman et al., 2024). Several studies have investigated the effects of Tualang honey on NASH and its associated metabolic complications. These studies have mainly been conducted on animal models, with limited clinical trials in humans. Nevertheless, the results of these studies suggest that Tualang honey may have beneficial effects on NASH (Taleuzzaman et al., 2020).

2.3.6 Microarray Studies in NASH

Microarray studies have significantly contributed to understanding the molecular mechanisms of NASH by revealing gene expression changes associated with lipid metabolism, inflammation, oxidative stress, and fibrosis. Frequently implicated pathways include PPAR signalling, NF- κ B activation, and mitochondrial dysfunction (Wang et al., 2022). Although results may vary due to sample and platform differences, microarrays remain a valuable tool for identifying potential biomarkers and therapeutic targets in NASH (Lee et al., 2021).

2.4 TUALANG HONEY

Tualang honey (TH) is a multi-floral honey produced by stingless rock bees (*Apis dorsata*) that build their hives on the high branches of Tualang trees (*Koompassia excelsa*) (Chew et al., 2018). TH is one the most treasured Malaysian natural products that has been used extensively for various purposes and it is well known for its high nutritional and medicinal values. This attribute is a result of the *Apis dorsata* bees that gather nectar from various species of wildflowers and herbs that contribute to the formation of high-quality honey (Saranraj & Sivasakthi, 2018). In Malaysia, the harvesting activity of TH by the authorised bee hunters as well as the authenticity of TH in the market is strictly supervised by the Malaysian Federal Agricultural Marketing Authorities (FAMA). It is characterised by its dark brown colour, pH ranging from 3.6 to 4.0, and a specific gravity of 1.34. It exhibits a slightly higher acidity compared to local Malaysian kinds of honeys like Kelulut and Gelam but shares a similar low pH profile with Manuka honey (Fuad et al., 2023).

2.4.1 Physicochemical Composition of Tualang Honey

The physicochemical property of honey varies according to the different types of honey. The variation in the physicochemical composition of honey is profoundly influenced by its geographical origin, floral resources of nectar and environmental climate (Cheng et al., 2019). A recent review comparing the physicochemical and medicinal properties of Tualang, Gelam and Kelulut honey reported that the water content of Malaysian TH varies from 17.53 to 26.51% (Mohd Kamal et al., 2021). As Malaysia is a tropical climate country with abundant rainfall throughout the year, the water content of local TH samples was found to be relatively higher than the maximum limit of 20% that was established by The International Honey Commission (IHC) for any honey

sample to be accepted for storage and commercialisation (Mohd Kamal et al., 2021). Since the water content is one of the most important quality parameters of honey as it has a direct effect on the glucose crystallisation and honey viscosity, the standard of procedure for TH preparation for storage includes the process of water evaporation to reduce the water content to achieve 20% and subsequently to prevent the spoilage of honey due to fermentation (Chew et al., 2018).

In general, the main constituent of honey is carbohydrates, comprising of fructose (38.2%), glucose (31.3%) maltose (7%) and sucrose (1.3%) (Masad et al., 2021). The minor components of TH include protein, enzymes and variable essential minerals such as sodium, potassium, magnesium and iron, accounting for about 1% of the honey constituents. TH also consists of active biological compounds mainly flavonoids (catechin, quercetin, kaempferol, luteolin, hesperetin, apigenin, 3,7,40-trihydroxyflavone, naringenin, chrysin, fisetin, vitexin, isoorientin, xanthohumol, pinobanksin-3-o-propionate, and pinobanksin-3-o-butyrate) and phenolic acids (gallic, coumaric, syringic, caffeic, cinnamic, benzoic, chlorogenic, salicylic, and ferulic acid) (Yap & Chin, 2021). The chemical properties of TH in comparison with another two common tropical rainforest honeys which are Gelam Honey and Kelulut Honey are demonstrated in Table 2.2.

Table 2.2 Chemical constituents of Tualang, Gelam and Kelulut honey
(Adapted from: Mohd Kamal et al., 2021)

Chemical constituent	Tualang Honey	Gelam Honey	Kelulut Honey
Total sugar content (g/100 g)	63.60 – 72.94	64.93-72.57	65.83 – 71.65
Reducing sugar (g/100 g)	61.94	62.17	No report found
Sucrose (g/100 g)	0.01-1.66	0.01 – 2.77	0.01 – 32.30
Glucose (g/100 g)	30.07-47.13	32.85-50.44	9.22-23.44
Fructose (g/100 g)	41.73-44.56	44.01-44.74	15.00-22.05
Maltose (g/100 g)	4.49	1.29	27.41
Protein content (g/kg)	3.6-6.6	3.14-7.00	3.9-8.5
Mineral content (mg/kg)	Tualang Honey	Gelam Honey	Kelulut Honey
Sodium	268.23-704.5	196.84-666.0	589.7
Potassium	976.9-1576.40	1132.2-1363.4	732.2
Iron	11.17-128.13	8.45-142.37	191.9
Magnesium	35.03-71.04	31.63-49.38	33.81
Biological compound (mg/kg)	Tualang Honey	Gelam Honey	Kelulut Honey
Total flavonoid (quercetin)	504.5	461.1	36.3
Total phenolic content (mg/kg)	251.7-1103.94	606.17-1597.43	477.30-614.7

Referring to the table on the previous page, TH possesses the highest composition of flavonoids (504.5 mg/kg) in comparison to Gelam (461.1 mg/kg) and Kelulut honey (36.3 mg/kg), being most abundantly present as a flavanol subgroup of quercetin. Other subgroups of flavonoids that are also present in TH are flavanones (naringenin, pinocembrin, and hesperidin), flavones (luteolin, apigenin, and chrysin), isoflavones (genistein), and anthocyanidins.

TH has also been reported to have a high content of phenolic acids. In a study by Kishore et al. (2011) TH was reported to have the highest total phenolic content, followed by Gelam, Indian forest, and pineapple honeys. This is supported by another study by Moniruzzaman et al., (2017) who demonstrated that TH had the highest percentage of phenolic acids compared to acacia, pineapple, Gelam, longan, Borneo rubber tree, sourwood honey, whereby it is most mainly existing as benzoic acids (75%). On the other hand, when compared to Kelulut honey, several studies documented Kelulut honey samples having a higher phenolic content than TH (Ranneh et al., 2018; Muhammad et al., 2021; Ismail et al., 2016). Nevertheless, the flavonoids and phenolics acid are the bioactive compounds that have been demonstrated by many studies that are accountable for the various medicinal properties of TH (Ranneh et al., 2018) (Yap et al., 2024) (Cheung & Ahmed, 2021)

2.4.2 Medicinal Properties of Tualang Honey

2.4.2.1 Anti-oxidant Property

One of the most well-known medicinal benefits of honey is its anti-oxidative properties. A study by Yaacob et al., (2020) reported that the methanolic fraction of Tualang Honey contains the highest concentration of flavonoids (189.53 ± 1.23 mg CEQ/100g) and phenolic (287.80 ± 0.85 mg CEQ/100g) compared to crude TH, TH irradiated and ethyl acetate fraction TH. In this study, the methanolic fraction of TH exhibited the highest antioxidant activities and there was a significant correlation between antioxidant contents and antioxidant activities, proposing flavonoids and phenolics are the main biologically active compounds for the antioxidant activities of TH (Yaacob et al., 2020). Both flavonoids and phenolic acids present with similar chemical structures (Figure 2.2 and 2.3) and exert antioxidative properties and radical scavenging effects by the donation of an electron or hydrogen atom. Figure 2.2 is the basic chemical structure of flavonoids consisting of a 15-carbon; C6–C3–C6 nuclear structure, including two benzene rings (A and B) linked by a heterocyclic pyran ring (C).

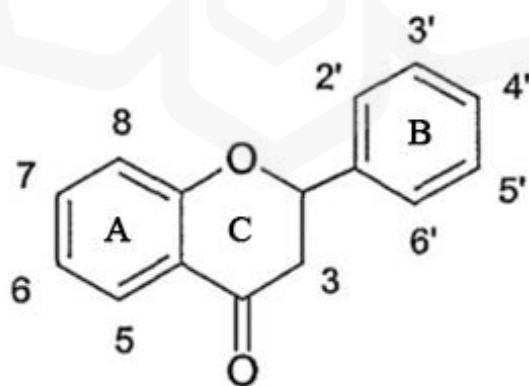


Figure 2.2 Basic chemical structure of flavonoids. Adapted from (Cianciosi et al., 2018).

The antioxidant activity of flavonoids relies on the number and position of hydroxyl groups and other substituents and the glycosylation of flavonoid molecules (Cheung et al., 2019). Another factor that affects the antioxidant activity of flavonoids is the substitution patterns in the A ring and B ring, and the 2,3-double bond (unsaturated) and 4-oxo group in the C ring (Cianciosi et al., 2018). Figure 2.2 shows a general chemical structure of phenolic acids that commonly categorised into two subgroups: hydroxybenzoic and hydroxycinnamic acids. Just like flavonoids, phenolic acids stabilise free radicals when they give off hydrogen from one of their hydroxyl groups; the degree of activity is related to the number of their hydroxyl groups.

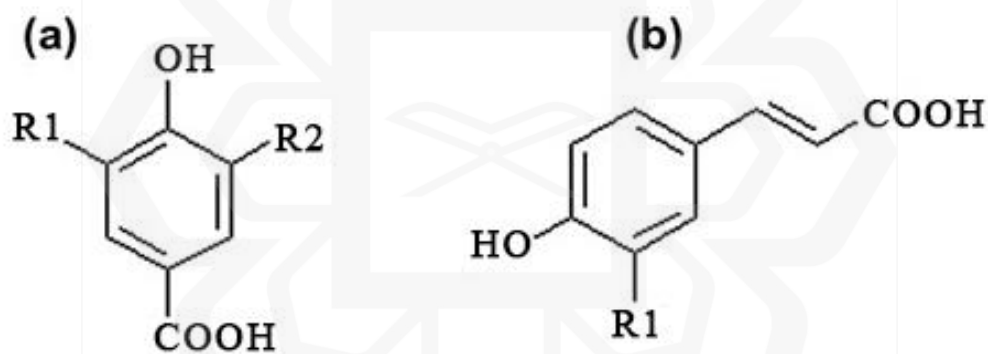


Figure 2.3 Chemical structure of phenolic acids. (a) Hydroxybenzoic acid: *p*-hydroxybenzoic, R1 = H, R2 = H; gallic acid, R1 = OH, R2 = OH. (b) Hydroxycinnamic acids: *p*-coumaric acid, R1 = H; caffeic acid, R1 = OH; ferulic acid R1 = OCH₃. (c). Adapted from (Shamsudin et al., 2022)

2.4.2.2 Anti-inflammation Property

Flavonoids and phenolic acids play key roles in the exhibition of anti-inflammatory properties of TH. As proposed by Masad et al. (2021), flavonoids are attributed to the anti-inflammatory property of honey via four anti-inflammatory mechanisms: free radical scavenging, inhibition of pro-inflammatory enzymes, modulation of pro-inflammatory cytokines and modulation of pro-inflammatory gene expression regulators. A study on a sample of TH phenolic-rich extract demonstrated that the phenolic acid improved the bioactivities of honey to scavenge free radicals and inhibit inflammation (Martinello & Mutinelli, 2021). TH has been reported to be equivalent to prednisolone in moderating the inflammatory pain of male Sprague Dawley rats by elevation of neutrophil counts and reduction of IL-6 (Azman et al., 2024). Another study documented the combination of TH and Docosahexaenoic acid (DHA)-rich fish oil administration reduced the concentration of TNF- α , IL-6 and interferon-gamma (IFN- γ) in the rat brain following exposure to chronic stress (Asari et al., 2022). Despite the positive correlation between anti-inflammatory activities and administration of TH that has been shown in animal studies, its clinical application in human remains inconclusive. A study on supplementation of TH for 12 weeks among 32 chronic smokers reported a significant reduction in the level of high-sensitivity C-reactive protein (hsCRP), yet there was a significant increase in the level of TNF- α post TH treatment (Navaei-Alipour et al., 2021). The authors suggested there is a need for further study on the anti-inflammatory effects of TH in humans and to include other inflammatory markers with a much larger sample size.

2.4.2.3 Anti-Cancer Property

TH is also widely researched for its anti-cancer properties in both vivo and vitro studies. The potential of TH as an anti-cancer agent as well as an adjuvant for chemotherapeutic agents is also proposed due to the rich content of flavonoids and phenolic acids (Masad et al., 2021). A study on oral carcinogenesis induced Sprague-Dawley rats reported that TH significantly reduced the incidence of oral squamous cell carcinoma (OSCC) and suppressed cancer cell proliferation via diminishing the expression of Cyclin D1, Epidermal growth factor receptor, and Cyclooxygenase-2 (Al-Koshab et al., 2020). Another study on non-squamous cell lung carcinoma cell lines proposed molecular mechanism of anti-cancer activity of TH were through modulation of relevant cancer-related proteins that are associated with cell proliferation, apoptosis inhibition and induction of angiogenesis (Amran & Abdul-Rahman, 2022). This is consistent with another study by Ahmed and Othman (2017) that showed administration of TH on female Sprague Dawley rats with breast cancer induced by carcinogen 1-methyl-1 nitrosoarea (MNU) 80mg/kg, increased the susceptibility of expression of proapoptotic proteins including Apoptotic protease activating factor-1 (APAF1), Caspase-9, IFN- γ , interferon-gamma receptor 1 (IFNGR1), and tumour protein p53 (p53). and decreased the expression of antiapoptotic proteins TNF- α , Cyclooxygenase-2 (COX-2), and B-cell lymphoma-extra-large (Bcl-xL) in its mechanism of action. The safety profile of the use of TH as a supplement in the treatment of breast cancer in humans has been examined by Zakaria et al. (2018) by prescribing supplementation of 20g/day of TH for 12 weeks to postmenopausal breast cancer patients. Subsequently a randomised control study by Hizan et al. (2018) proposed a combination of TH and anastrozole, that was found to be more effective than anastrozole alone in

decreasing breast background parenchymal enhancement in breast cancer patients with oestrogen receptor (ER)- positive breast cancer.

2.4.2.4 Anti-Microbial Property

There is an increasing number of studies on the anti-microbial properties of TH in response to the urgent need to discover alternative antibacterial agents to overcome the challenge of antibiotic resistance in bacterial infectious diseases. A study by Al-Kafaween et al. (2020) showed significant inhibition of *S.aureaus* cell growth and subsequent RT-qPCR analysis revealed gene expression of *S. aureus* essential gene was downregulated following exposure to TH. Another study reported the potential of TH to be an effective inhibitor of *P.euruginosa* and *S.pyogenes* characterised by loss of structural integrity, marked changes in cell shape and downregulation of virulence genes expression in both bacterial species (Al-Kafaween et al., 2023). The antibacterial mechanism of TH is attributed to its high osmolarity, acidity, and content of hydrogen peroxide and non-peroxide components. With a pH value of TH to be in the range of 3.14–3.80, the acidic nature of TH was of great importance for its antimicrobial properties since most bacteria grow in a neutral and mildly alkaline environment, whereas yeasts and moulds could grow in an acidic environment (Mohd Kamal et al., 2021).

2.4.2.5 Hepatoprotective Protective

Several studies have reported the hepatoprotective property of TH for the past decade. TH has been demonstrated to improve the liver function profile of streptozotocin-diabetic-induced rats by showing a significant reduction of serum Aspartate transferase (AST), Alkaline phosphatase (ALP)

and Alanine transaminase (ALT) following a 4-week administration of 1g/kg/day TH compared to diabetic control rats (Erejuwa et al., 2012). Another study by Mohamed et al. (2018) documented TH supplementation at a higher dose of 1.4g/kg/day for a 6-week duration, which resulted in an improvement of liver enzymes. However, the study revealed no changes in the dyslipidaemia and histopathological changes of the liver in the NASH Sprague-Dawley rat model induced by a 12% high cholesterol diet. Another study utilised the same animal model but administered a higher dose of TH of 2.4 g/kg/day and demonstrated significant improvement in both NASH grading and liver function enzymes profile. The ALT, low-density lipoprotein cholesterol (LDL-C), TC, triglyceride (TG), fasting insulin and Homeostatic Model Assessment of Insulin Resistance (HOMA-IR) levels were also reduced significantly (Rahim et al., 2019). However, the research on the exact molecular mechanism of the hepatoprotective effect of TH in reversing liver impairment in NASH remains unexplored.

The hepatoprotective properties of honey have also been studied in other types of honey. Acacia honey from Pakistan for instance, has been demonstrated to reduce body weight gain, serum lipids profile, glucose and liver steatosis in high-fat-diet-induced obesity Albino Wistar rats (Gohar et al., 2024). Another study utilised *A.cerana* honey from Qinling Mountains in China to evaluate their antioxidant and hepatoprotective effects against acute alcohol-induced liver damage and found a significant reduction in serum ALT, and AST, production of hepatic malondialdehyde (MDA) and a remarkable inhibition of TGF- β 1 in serum and the liver (Zhao et al., 2017). Meanwhile a recent study by Taleuzzaman et al. (2020) proposed flavonoids honey has positive effects on insulin resistance, lipid metabolism, inflammation, and oxidative stress that have a prominent pathophysiological pathway in NAFLD. A large cross-sectional study involving

21,979 adults aged 20-90 years of age, concluded that consuming honey 2-6 times per week was inversely associated with the occurrence of NAFLD (Zhang et al., 2021). One possible molecular mechanism explanation is that phenolic acids and flavonoids could have ameliorated NAFLD by activating the adiponectin-activated protein kinase (AMPK) pathway and suppressing the nuclear factor-kappa pathway (Ye et al., 2023). Flavonoids also prevent hepatic steatosis by increasing fatty acid oxidation in the liver, reduce caloric intake and decrease fat deposition in visceral tissues (Abtulov & Valcheva-Kuzmanova, 2021).

Considering the abundant presence of phenolic compounds, including flavonoids and phenolic acids, in Tualang Honey (TH), which contribute to its antioxidant and anti-inflammatory properties, it is valuable to investigate its potential role as an alternative or supplement in managing non-alcoholic steatohepatitis (NASH), a condition driven primarily by oxidative stress and inflammatory mechanisms. Despite extensive research into the various medicinal benefits of TH, studies focusing on its hepatoprotective effects in NASH animal models remain limited, particularly in uncovering the precise molecular mechanisms involved in the disease. There is an urgent need for evidence-based alternative treatments for NASH, and Malaysia's Tualang Honey presents a promising natural product that could serve as a viable option.

2.5 ANIMAL MODELS OF NASH

Clinical research into the mechanisms of NASH and its progression is limited by ethical concerns, particularly when it comes to obtaining human livers and other tissues. As a result, animal models are often preferred for liver tissue collection. The challenge lies in ensuring that the animal model accurately represents the liver pathology of NASH, including the context in which it develops. A good animal model of NASH must meet two key criteria. Firstly, the pathological pattern of liver injury must resemble human steatohepatitis, and the model must replicate the conditions under which human NASH develops (Gallage et al., 2022). Specifically, the animal model should exhibit liver pathology with steatosis, inflammation, hepatocyte ballooning, and progression to fibrosis, which are commonly observed in adult NASH. Recently, rats have emerged as the preferred animal model due to advancements in the establishment of a comprehensive genome sequence database and it has been demonstrated that rats and humans share a significant majority of their biochemical pathways at the genome level, highlighting the value of rats as a model organism for studying human biology and disease (Szpirer, 2020). Additional advantages of using rats include the availability of large sample sizes (e.g.: blood volume, liver size), ease of training, and the simplicity of performing surgical procedures to harvest the liver tissue (Nevzorova et al., 2020).

2.5.1 Methionine-choline deficient (MCD) dietary animal model

Methionine and choline are essential amino acids that serve as methyl-group donors and play a crucial role in lipid metabolism. The methionine and choline-deficient (MCD) diet model involves feeding rats a diet lacking these two amino acids, which are vital for hepatic beta-oxidation and very low density lipoprotein (VLDL) production. The deficiency of methionine and choline

disrupts phospholipid synthesis, and lipoprotein secretion, and contributes to oxidative stress and endoplasmic reticulum (ER) stress (Carreres et al., 2021). Sprague Dawley rats fed with MCD diet for 6–8 weeks developed severe steatosis, but exhibited minimal inflammation, with no hepatocyte ballooning or fibrosis (Takahashi et al., 2022). One of the main features of this dietary model is that it induces steatosis without the involvement of metabolic syndrome factors such as obesity and insulin resistance, making it particularly suitable for studies focused on liver-specific pathophysiology in the absence of these metabolic conditions.

2.5.2 High Fat Diet Animal Model

High-fat diet (HFD) animal models have been developed to reflect the detrimental eating habits typical of a modern diet, thereby mimicking the aetiology of NASH in humans. Emamat et al. (2020) demonstrated that Sprague Dawley rats fed with HFD; 60% of total calories from butter-derived fat for 7 weeks, developed metabolic syndrome, obesity, hypertriglyceridaemia, hyperinsulinaemia, and hyperglycaemia, all of which are associated with NASH development. Similarly, Mustika et al. (2023) reported that rats fed with HFD, primarily derived from lard (totalling 5210 kcal/kg) for 14 weeks, exhibited a NAS score greater than 5 and histological changes consistent with NASH, including large fat droplets, ballooning degeneration, and inflammatory cell infiltration. However, in a different study, the same diet (mainly from lard, 5210 kcal/kg) fed to Wistar rats for 12 weeks did not induce NASH (Fujita et al., 2018). While HFD models seem effective in inducing NASH in some animal models, challenges in reproducing consistent results across different laboratories limit their utility. Variability in responses to HFD among different rodent models can be attributed to genetic differences and environmental factors.

2.5.3 High cholesterol animal model

The liver is the primary organ responsible for cholesterol metabolism, with its homeostasis depending on cholesterol intake (both endogenous and exogenous through diet), transport, and catabolism. Cholesterol is often added to high-fat diets to create NASH animal models, with the amount typically ranging from 0.5% to 10%. For example, female Wistar rats fed a high-fat diet (HFD) with cholesterol consisting of 65% fat, 27% protein, 15% carbohydrates, 1% cholesterol, and 0.25% cholate for 16 weeks showed a NAFLD activity score (NAS) of 6.2, with maximum scores for steatosis and inflammation, but only a score of 0.7 for ballooning (Haldrup et al., 2018). In another study, researchers extended the feeding period to 24 weeks with a similar diet (45.2% fat, including 6.6% trans-fat from hydrogenated vegetable shortening, 2% cholesterol, calorie density of 4600 kcal/kg) and observed an NAS score of 6, with mild fibrosis in the animals (Cai et al., 2017).

While other studies used high-fat diets with lower cholesterol percentages, Ghani et al. (2016) used a high-cholesterol diet (12% cholesterol) added to a standard diet for 20 weeks and successfully induced NASH, as evidenced by abnormal liver biochemistry and histological changes typical of NASH, including altered HOMA-IR levels. As high cholesterol accumulates in the liver, inducing metabolic disturbances similar to those seen in human NASH, the high-cholesterol diet NASH model serves as a reliable and relevant model for studying the complex interactions between lipids, liver inflammation, and fibrosis in NASH. This model provides significant advantages in replicating human metabolic diseases, offering valuable insights into disease mechanisms and therapeutic opportunities.

CHAPTER THREE

METHODOLOGY

3.1 EXPERIMENTAL PROCEDURES

- Body weight and liver weight comparisons
- Gross morphological examination of the liver samples
- Histological procedures – H&E staining, Masson's Trichrome staining
- Biochemical analysis – Liver function test, lipid profile, fasting serum glucose
- ELISA for fasting serum insulin
- Immunohistochemistry – TUNEL assay and α -SMA staining
- Microarray analysis
- Reverse qPCR analysis for confirmation of microarray findings

3.1.1 Equipment

- WTB 2000 electronic weight scale (Radwag, Poland)
- NF 200 benchtop centrifuge (Nuve, Turkey)
- Scalpel and other surgical apparatus for liver extraction
- MDF-U4086S ultra-low temperature freezer (Sanyo, Japan)
- Automated tissue processor – Leica TP1020 (Leica Biosystems, USA)
- Embedding centre – Leica Model EG1160 (Leica Biosystems, USA)
- Semi-auto rotary microtome – Leica Model RM2245 (Leica Biosystems, USA)
- Warm oven – Memmert Model 100-800 (Micro Precision, USA)

- Laboratory Fume Hood (Method Enterprise, Malaysia)
- Glass microscope slides (Sail Brand, China)
- Light microscope – BX51 (Olympus, Japan)
- Olympus DP20 camera attached to the microscope (Olympus, Japan)
- AU680 Chemistry analyser (Beckman Coulter, USA)
- Microplate Reader Infinite 200 Pro 9 (Tecan, Switzerland)
- Rotor-stator homogenizer (Dyna-Passion, Malaysia)
- NanoVue™ Plus Spectrophotometer (Biochrom, United Kingdom)
- Agilent SurePrint G3 Human Gene Expression v3 8x60K - Design ID: 072363
- Agilent Microarray Hybridisation Oven & Chamber (Agilent, USA)
- Agilent SureScan Microarray Scanner (Agilent, USA)
- CFX96 Real Time Cycler (BioRad, USA)

3.2 SAMPLE SIZE CALCULATION

The study sample size was calculated using a formula based on Dell et al. (2002) with the following formula:

$$\text{Sample size (n)} = 1 + 2C (s/d)^2$$

C: constant based on alpha level, 0.5
s/d: standard deviation

According to Xu et al., (2010), standard deviation of 1.6 and the detectable effect size was 3.5 with a power of 0.8, and the calculated sample size was approximately 4 animals per group. Considering a 15% attrition rate to cater for unexpected animal loss, the sample size of 5 animals per group was applied in this study.

3.3 ETHICAL APPROVAL

The animal handling procedures, treatment and experimental protocols were approved by the Animal Care and Use Committee, International Islamic University Malaysia (IIUM/504/14/2/IACUC). This committee conformed to the guidelines for the Malaysian Code of Practice for Care and Use of Animals for Scientific Purposes.

3.4 EXPERIMENTAL DESIGN

A total of 25 male Sprague-Dawley rats were purchased from A-Sapphire Enterprise (Seri Kembangan, Selangor) with each rat weighing in around 200-250g. They were housed individually in polypropylene cages (43 cm x 27 cm x 15 cm) at $60 \pm 5\%$ relative humidity and $24 \pm 2^\circ\text{C}$, with a 12-hour light/dark cycle. They also had free access to water and food. Several enrichment items

such as pieces of cardboard, tissue rolls, and tissue papers were also provided regularly inside throughout the experimental period. The bedding was changed regularly to ensure the rats had an optimal environment for living and daily activities. Following two weeks of acclimatisation, these 25 Sprague-Dawley rats were randomly divided into five groups which consisted of 2 control groups and 3 treatment groups. The experimental animal grouping was as demonstrated in Table 3.1.

Table 3.1 Grouping of the animals (Abdul Rahim et al, 2018)

Animal group	Label	Treatment
Group 1	1	Normal control group. Rats were only given standard rat pellets* with no TH administration throughout the study period (20 weeks).
Group 2	2	Control for 12% HCD diet. Rats were given 12% HCD for 16 weeks. Continued 12% HCD and distilled water for four weeks.
Group 3	3	Rats were given 12% HCD for 16 weeks. Continued 12% HCD and 1.2 g/kg/day of TH for four weeks.
Group 4	4	Rats were given 12% HCD for 16 weeks. Continued 12% HCD and 2.4 g/kg/day of TH for four weeks.
Group 5	5	Rats were given 12% HCD for 16 weeks. Continued 12% HCD and 3.0 g/kg/day of TH for four weeks.

*Standard rat pellets contain crude protein (21-23%), crude fibre (5.0%), crude fat (3%), moisture (3.0%), calcium (0.8-1.2%), phosphorus (0.6-1.0%), nitrogen free extract (49%), vitamin A (10 M.I.U), vitamin D₃ (2.5 M.I.U), vitamin E (15g) and traces of vitamin K, vitamin B12, thiamine, riboflavin, pantothenic acid, niacin, pyridoxine, choline, santoquin, and microminerals.

3.4.1 Experimental flowchart

This study was divided into two phases: Phase 1 was an induction and Phase 2 was a treatment phase as shown in Figure 3.1. In Phase 1, the animals were given 12% HCD to induce NASH for 16 weeks while in the second phase, the animals were given TH at three different dosages.

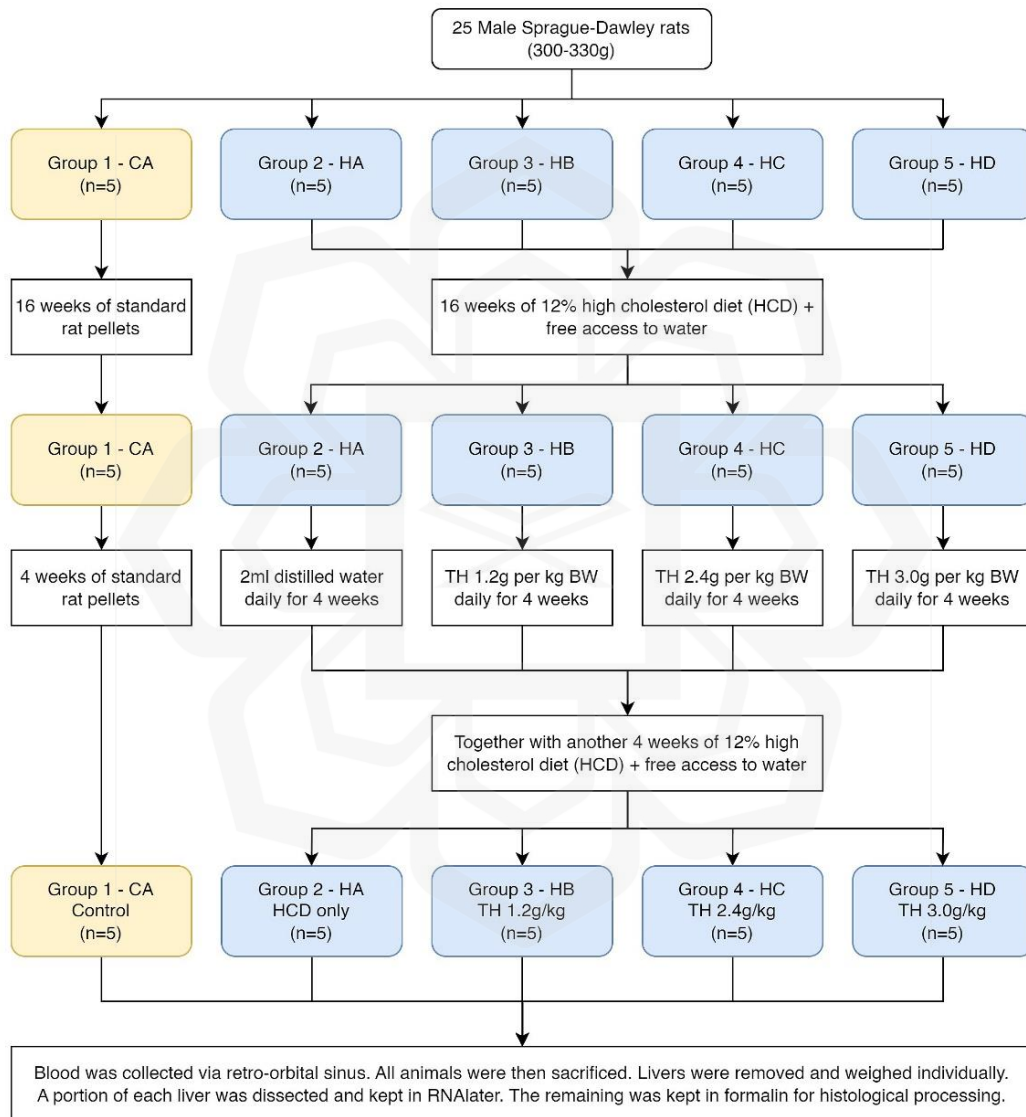


Figure 3.1: Experimental flowchart of the 20-week duration of study. TH: Tualang honey; BW: Body weight

3.5 HIGH CHOLESTEROL DIET

3.5.1 Materials

- Powdered commercial rat pellets.
- Analytical pure cholesterol powder (Purity: 99%)
- Cholic acid powder (Purity: 98%)

3.5.2 Preparation of 12% High Cholesterol Diet

The 12% high-cholesterol diet (HCD) was prepared by mixing 1 kg of powdered commercial rat pellets (Gold Coin, Malaysia) with 120 g of analytical pure cholesterol powder (Nacalai-Tesque Inc., Kyoto, Japan). Three grams of cholic acid (Nacalai-Tesque, Japan) was then added to the powdered mixture to enhance cholesterol absorption. In order to prevent oxidative modification of the cholesterol, the cholesterol/cholic acid mixture was prepared twice weekly.

The preparation was based on the modification of human cholesterol daily intake which was about 1-2% (Howell et. al, 1997) on average. The rat dose equivalent was calculated using the *Km* factor for rat. The *Km* factor of 6 as per Reagan-Shaw et al (2008) was chosen as shown in the following equation.

Cholesterol dose for rat:

= percentage of cholesterol daily intake in human x *Km* (Rat)

= 2% x 6

= 12% cholesterol daily for rat

3.6 TUALANG HONEY

3.6.1 Materials

Tualang Honey (AgroMas®) was provided by the Federal Agricultural Marketing Authority (FAMA), Ministry of Agriculture and Agro-Based Industry, Malaysia. It was harvested from *Apis dorsata* bees' nectar on Tualang trees in the Rain Forest of Kedah in Peninsular Malaysia in November 2019 and filtered to remove any solid particles. Fifty percent (w/v) stock solution of TH was prepared daily by weighing 50 g of TH and diluting it with 100 ml of distilled water. The solution was then vortex mixed for 5 minutes before being administered to the animals, once daily by oral gavage. The nutritional composition and specification of TH used in this study as shown below (Table 3.2):

Table 3.2: Nutritional composition and specifications of Tualang Honey used.

Parameter, Unit	Results	Standard
Reducing sugar, g/100g		>60.0
- Fructose	- 38.0	
- Glucose	- 36.9	
Sucrose, g/100g	Not detected (<0.01)	<10.0
Ash, g/100g	0.02	<1.0
Moisture, g/100g	23.1	<20.0

3.6.2 Preparation of Tualang Honey dosage and administration

The honey dose for rat was calculated by conversion of human equivalent dose (HED) to rat dose using *Km* factor (Jacob et al., 2022), applying the following formula:

Calculation of TH dosage based on HED:

$$\text{HED (g/kg)} = \text{Animal dose (g/kg)} \times \frac{\text{Animal } Km}{\text{Human } Km}$$

$$\text{Animal dose (g/kg)} = \text{HED(g/kg)} \times \frac{\text{Human } Km}{\text{Animal } Km}$$

On average, the daily intake of honey by a healthy adult (at an average weight of 60 kg) is about 1 teaspoon daily, which is equivalent to 12 g of honey daily. The consumption per kilogram is therefore 0.2 g (12g/60kg) for humans. Hence, the rat dose equivalent for rat using animal *Km* factor of 6, and Human *Km* of 37, is as shown below.

$$\begin{aligned} &= 0.2 \text{ g/kg/daily} \times \left(\frac{37}{6}\right) = 0.2 \text{ g/kg} \times 6.16 \\ &\sim 1.2 \text{ g/kg bodyweight daily} \end{aligned}$$

For this study, three doses of TH were prepared; low dose (1.2g/kg/daily), moderate dose (2.4g/kg/daily) and high dose (3.0g/kg/daily).

3.7 MEASUREMENT OF BODY WEIGHT

Body weight measurements of all the rats were taken using a digital weighing machine and documented twice a week (every Monday and Thursday). The administration of the TH dose was adjusted as needed based on changes in body weight.

3.8 BLOOD SAMPLE COLLECTION

At the end of the study period, the rats were fasted overnight, and 10 ml of blood were collected from each animal via the retro-orbital method, just before being sacrificed. The blood samples were collected into a plain tube with polymer gel for biochemical and insulin analysis, whilst a vacutainer tube containing sodium fluoride (antiglycolytic agent) was used for analysis of fasting blood glucose levels. The blood was centrifuged, and the samples were divided into several aliquots. Serum sample for basic biochemical analysis were sent to the Integrated Laboratory, Department of Pathology and Laboratory Medicine, Sultan Ahmad Shah Hospital (SASMEC) Kuantan to be analysed while serum for molecular analysis was stored in a -70 °C freezer until further analysis.

3.9 LIVER HARVESTING

All animals in this study were sacrificed for liver histological study after 20 weeks of experimental study. Prior to being sacrificed, each animal was fasted overnight and weighed for their final body weight. After shaving and disinfecting, the animal was anaesthetised and laid on its back with the tail pointing towards the researcher. The abdomen was cut open vertically and the liver was immediately removed, rinsed with ice-cold sterile physiologic 0.9 % sodium chloride, gently blotted on clean filter paper, and then weighed. The relative liver weights were determined using the following formula:

$$\text{Relative Liver Weight} = \frac{\text{Absolute liver weight}}{\text{Final body weight}} \times 100$$

3.10 GROSS EXAMINATION

The harvested livers were inspected for any visible abnormalities, and photographs were taken using a digital camera for documentation and to report any findings before the livers underwent histological processing.

3.11 HISTOLOGY PROCEDURES

3.11.1 Tissue slicing and fixation

The liver specimens were cut into smaller slices of less than 4 mm thickness. Some of the liver slices were immediately immersed into a container containing 10% neutral buffered formalin. A portion of the liver was dissected and kept in RNAlater solution and stored at -20 °C freezer for further analysis. RNAlater is a tissue RNA stabilisation and storage reagent that rapidly permeates tissues to stabilise and protect cellular RNA.

3.11.2 Tissue grossing

The liver slices for histological examination were dissected to produce representative samples from appropriate areas. The samples were placed in small cassettes and loaded onto a tissue processor for tissue processing in batches.

3.11.3 Tissue processing

Tissue processing aimed to remove water from the liver samples to allow paraffin wax impregnation. This procedure was performed by an automated tissue processor, Leica TP1020 (Leica Microsystem, Germany). The first involved a dehydration process using a series of alcohol

concentration; 50%, 70%, 80%, 95% for one hour respectively and twice in 100% for two hours each. The second step was known as clearing; replacing the dehydrating fluid with xylene with both the dehydrating fluid and embedding medium. Finally, the tissue was infiltrated with an embedding agent.

3.11.4 Tissue embedding

Following the tissue processing procedure, the samples were placed in the embedding centre (Leica Model EG 1160) and removed from their cassette to be transferred into wax filled moulds. The samples were placed carefully according to the orientation through which the samples would be sectioned. The cassettes used in tissue processing were placed on top of moulds prior to adding further wax. The sample blocks were placed on the cold surface to allow for the wax to solidify.

3.11.5 Trimming and sectioning

The liver tissue samples (embedded in paraffin blocks) were sectioned using a semi-auto rotary microtome (Leica, Model RM2245, Germany). Disposable steel blades were used with the microtome, which has a mechanism for advancing the block across the blade. Trimming and serial sectioning of tissue blocks were set at 4 μm thick ribbons. The ribbon sections were then floated out on the surface of warm water at 50°C to remove any wrinkles on the sample and subsequently mounted onto frosted glass microscope slides (Sail Brand, China). The glass slides were placed on a hot plate at 45 °C for drying and placed in a warm oven (Mettler, Model 100-800, Germany) at 37 °C overnight for the section to adhere to the slide surface. A total of 4 slides were prepared for each liver sample for two types of staining and two immunohistochemistry procedures.

3.11.6 Haematoxylin and Eosin (H&E) staining

H&E staining differentiates tissue components based on chemical affinity. Haematoxylin stains nuclei blue, while eosin stains cytoplasm and extracellular proteins pink. It is widely used to assess steatosis, inflammation, ballooning, and necrosis in NASH liver tissue.

3.11.6.1 Materials

- Xylene
- Alcohol (100%, 90%, 80%, 70% and 50%)
- SelectTech Haematoxylin 560 MX
- SelectTech Define concentration
- SelectTech Blue Buffer and Alcoholic Eosin Y 515
- Dibutylphthalate Polystyrene Xylene (DPX) Mountant

3.11.6.2 H&E staining procedures

The slides were deparaffinised by placing the slides in a xylene solution for 5 minutes to ensure the wax was removed from the liver sections. The slides were then rehydrated with another serial of alcohol concentrations; 100%, 90%, 80% 70% and 50% for 2 minutes respectively, prior to rinsing with distilled water for 5 minutes. Following hydration, the slides were stained by dipping the slides into haematoxylin solution for 15 minutes followed by washing in running tap water for 1 minute. Then, the slides were differentiated in working solution in a single dip and washed through running tap water for another 1 minute. Subsequently, the slides were immersed in blueing solution for 1 minute followed by washing with running tap water for 15 seconds. The slides were

then counterstained in eosin Y solution for 10 minutes. Next, the slides were rehydrated through a serial of alcohol concentrations; 70%, 80%, 90% and 100% for two minutes respectively prior to clearing twice with 2 changes of xylene for 5 minutes each. The slides were then carefully mounted with cover slips by using Dibutylphthalate Polystyrene Xylene (DPX) under the fume cupboard. The slides were viewed under light microscope (Olympus BX51) and analysed for any microscopic features seen. Photographs of microscopic features were captured using Olympus DP20 camera attached to the microscope.

3.11.7 Masson's Trichrome (MT) staining

MT highlights collagen by staining it blue, while nuclei appear black and cytoplasm red. This method is useful for evaluating liver fibrosis, allowing clear visualization of collagen deposition in NASH.

3.11.7.1 Materials

- Xylene
- Alcohol (100%, 90%, 80%, 70% and 50%)
- Bouin's fluid
- Weigert's haematoxylin
- Phosphomolybdic-phosphotungstic acid
- Masson's acid fuchsin
- Masson's Aniline blue
- Dibutylphthalate Polystyrene Xylene (DPX) Mountant

3.11.7.2 MT staining procedures

Liver sections were deparaffinised in two changes of xylene for 5 minutes each, followed by rehydration through graded alcohols (100%, 100%, and 90%) for 2 minutes each. Slides were rinsed under running tap water for 5 minutes and re-fixed in Bouin's solution at 60 °C for 1 hour to enhance staining quality, then washed under tap water for 15 minutes to remove residual yellow pigment. Staining was performed using Weigert's haematoxylin (15 min), Masson's acid fuchsin (15 min), and phosphomolybdic-phosphotungstic acid (10 min), with 5-minute water rinses between steps. Slides were then stained with aniline blue (10 min), briefly rinsed (15 sec), and dehydrated through 90% and 100% alcohol, followed by two xylene baths (2 min each). Slides were mounted with DPX and examined under a light microscope (Olympus BX51). Images were captured using an Olympus DP20 camera.

3.11.8 NASH grading, staging, and NAFLD activity score

The assessment of the grade (Table 3.3) and stage (Table 3.4) of NASH was performed based on the Brunt schema for grading and staging of NASH (Brunt et al., 1999). The slides were reviewed by two independent histopathologists. Brunt and colleagues categorized the stages of NASH into three grades: grade 1 (mild), grade 2 (moderate), and grade 3 (severe). This classification is determined by the extent of hepatocellular steatosis, ballooning, and disarray, as well as both intralobular and portal inflammation.

Table 3.3 Brunt schema for grading NASH (Giashuddin & Alawad, 2022)

Features	Mild (Grade 1)	Moderate (Grade 2)	Severe (Grade 3)
Steatosis	Predominantly macrovesicular involving less than 33%-66% of the lobules	Usually mix macrovesicular and microvesicular.	Typically, more than 66% (pan acinar) and commonly mix steatosis
Ballooning	Occasionally observed in zone 3 hepatocytes	Obvious and present in zone 3 hepatocytes	Marked ballooning and predominantly zone 3
Lobular inflammation	Mild acute inflammation (polymorph) and occasional chronic inflammation (mononuclear cells)	Polymorph may be noted with ballooned hepatocytes and pericellular fibrosis. Mild chronic inflammation may be seen.	Scattered acute and chronic inflammation. Polymorph may appear concentrated in zone 3 areas of ballooning and perisinusoidal fibrosis.
Portal inflammation	None or mild	Mild to moderate	Mild to moderate

Table 3.4 Fibrosis staging of NASH (Giashuddin & Alawad, 2022)

Stage	Description
0	None
1a	Mild zone 3 perisinusoidal fibrosis
1b	Moderate zone 3 perisinusoidal fibrosis
1c	Portal fibrosis only
2	Zone 3 perisinusoidal and periportal fibrosis
3	Bridging fibrosis
4	Probable or definite cirrhosis

The Non-alcoholic Steatohepatitis Clinical Research Network's NAFLD activity score (Table 3.5) (Younossi, Loomba, Anstee, et al., 2018) was also employed to evaluate the therapeutic effectiveness of TH in a rat model of NASH.

Table 3.5 NAFLD activity score (NAS) (Brunt et al., 2021)

Steatosis	Score
<5%	0
5% - 33%	1
>33% - 66%	2
>66%	3
Lobular inflammation	Score
None	0
<2 foci	1
2 – 4 foci	2
> 4 foci	3
Ballooning	Score
None	0
Few (or borderline) balloon cells	1
Many cells/prominent ballooning	2
NAS (Sum of Steatosis + Lobular Inflammation + Ballooning)	0-8

3.12 BIOCHEMICAL ANALYSIS

The biochemical parameters analysed include serum liver enzymes, glucose, total cholesterol (TC), triglycerides (TG), high-density lipoprotein cholesterol (HDL-C) and low-density lipoprotein cholesterol (LDL-C). The analysis was performed using the Beckman Coulter AU680 Chemistry analyser.

3.12.1 Aspartate aminotransferase (AST)

AST in the sample catalyses the transfer of an amino group between L-aspartate and 2-oxoglutarate to form oxaloacetate and L-glutamate. The oxaloacetate then reacted with nicotinamide adenine dinucleotide hydride (NADH), in the presence of malate dehydrogenase (MDH), to form nicotinamide dinucleotide hydride (NAD⁺). The AST was determined by measuring the decrease in absorbance carried out at 340 nm.

3.12.2 Alanine aminotransferase (ALT)

ALT in the sample catalyses the reaction between L-alanine and 2-oxoglutarate. The pyruvates formed was reduced by NADH in a reaction catalysed by lactate dehydrogenase (LDH) to form L-lactate and NAD⁺. The rates of the NADH oxidation are directly proportional to the catalytic ALT activity. The ALT was determined by measuring the decrease in absorbance carried out at 340 nm.

3.12.3 Alkaline phosphatase (ALP)

Measurement of serum ALP activity is based on colorimetric assay. It is based on the principle that in the presence of magnesium and zinc ions, the p-nitrophenyl phosphate is cleaved by

phosphatases into phosphate and p-nitrophenol. The p-nitrophenol activities are directly proportional to the catalytic ALP activity. The ALP was determined by measuring the increase in absorbance carried out at 409 nm.

3.12.4 Serum total cholesterol (TC)

Cholesterol was hydrolysed by cholesterol esterase to form free cholesterol and fatty acids. Free cholesterol produced is converted to cholet-4-en-3-one and hydrogen peroxide by oxygen and cholesterol oxidase. The hydrogen peroxide then reacted with 4-aminophenazone and phenol, catalysed by peroxidase forming the red dye named 4-(p-benzoquinone-monoimino)-phenazone. This red dye is directly proportionate to the cholesterol concentration and was determined by its wavelengths.

3.12.5 Serum triglyceride (TG)

Triglyceride was hydrolysed by lipoprotein lipase to form glycerol and fatty acids. This was followed by oxidation of glycerol to dihydroxyacetone phosphate and hydrogen peroxide. The hydrogen peroxide produced then reacts with 4-aminophenazone and 4-chlorophenol resulting in the formation of a red dye named 4-(p-benzoquinone-monoimino)-phenazone. This red dye is directly proportionate to the cholesterol concentration and was determined by its wavelengths.

3.12.6 High density lipoprotein cholesterol (HDL-C)

The quantification of HDL-C was based on enzymatic breakdown of cholesterol ester to free cholesterol and fatty acids via polyethylene glycol-cholesterol esterase. In the presence of oxygen,

cholesterol was oxidised by cholesterol oxidase to 4-cholestenone and hydrogen peroxide. The hydrogen peroxide reacts with 4-aminoantipyrine and sodium N-(2-hydroxyl-3-sulphopropyl)-3,5-dimethoxyaniline (HASDA) catalysed by hydrogen peroxidase leading to formation of blue quinonimine dye. The blue quinonimine dye is directly proportional to HDL-c concentration and was determined by its wavelengths.

3.12.7 Low density lipoprotein cholesterol (LDL-C)

LDL-C was calculated using Friedwald equation formula (Friedewald et al., 1972):

$$\text{LDL-C} = \text{TC} - \text{HDL-C} - \left[\frac{\text{TG}}{2.2} \right] \text{ mmol/L}$$

3.12.8 Fasting glucose

The principle of glucose measurement is based on the phosphorylation of glucose and adenosine triphosphate (ATP) by hexokinase catalysation to form glucose-6-phosphate and adenosine 5'-diphosphate (ADP). Glucose-6-phosphate is then converted to NADPH by glucose-6-phosphate dehydrogenase. The concentrations of NADPH formed is directly proportional to glucose concentration and measured at 340 nm.

3.12.9 Fasting Insulin

The concentration of serum insulin in each animal was measured using Colometric Sandwich ELISA kit (Proteintech).

3.12.9.1 Materials for ELISA analysis

- Microplates
- Standard solution
- Detection antibody (100x)
- HRP-conjugated Antibody (100x)
- Sample Diluent and Detection Diluent
- Extraction Reagent
- Washing Buffer Concentrate (20x)
- Tetramethylbenzidine Substrate (TMB)
- Stop Solution
- Plate Cover Seals
- Distilled water

3.12.9.2 Method for ELISA analysis

For standard working solution preparation, 2 ml of sample diluent was added to reference standard, labelled as SD7. The serial dilutions of standard working solution were prepared by carrying over 500 μ L of diluted standard into 500 μ L of fresh diluent for SD6, SD5, SD4, SD3, SD2 and SD1. The sample diluent without standard solution was the blank/zero standard, SD0. The mixture was inverted gently several times and mixed thoroughly between steps. Detection antibody working solution was prepared by dilution of the antibody with detection diluent with ratio of 10 μ L antibody + 990 μ L diluent. Concentrated HRP-conjugate working solution was prepared by dilution of 100x Concentrated HRP Conjugate to 1x working solution with Concentrated HRP

Conjugate Diluent. Wash buffer was prepared by dilution of an entire 30ml of wash water with 570 ml of distilled water. The solution was warmed to 37 °C and mixed gently to ensure no crystals remained in the concentrate.

For the assay procedure, the Standard working solution (100 µL) was pipetted into the first column of the pre coated ELISA microplate. Each concentration of the standard working solution was duplicated to one well each, side by side. Next, the serum samples (100 µL) were pipetted into other wells. After all samples were pipetted into each well, the plate was covered with a sealer provided in the kit. The plate was incubated at 37°C for 120 minutes.

Following incubation, the liquid was removed from each well using wash buffer 4 times, then dried against clean absorbent paper, after which, 100 µL of detection Antibody was added into each well. The plate was sealed with its cover and incubated at 37°C. After 40 minutes of incubation, the plate was washed again 4 times and dried against clean absorbent paper. About 100 µL of TMB substrate was then added into each well and incubated at 37°C for 20 minutes. Finally, 100 µl Stop Solution was pipetted into each well in the same order as TMB pipetting. The optical density (OD) value of each well was immediately determined using Microplate Reader Infinite 200 Pro 9 (TECAN, Switzerland) at 450 and 630 nm.

3.12.10 Homeostatic model assessment insulin resistance (HOMA-IR)

HOMA-IR is used to assess the physiological index of insulin resistance. HOMA-IR was calculated using the following formula.

$$\text{HOMA-IR} = [\text{Fasting Glucose (mmol/L)} \times \text{Fasting Insulin (mU/L)}] / 22.5$$

3.13 IMMUNOHISTOCHEMISTRY STAINING

3.13.1 TUNEL Assay Immunohistochemistry

The terminal deoxynucleotidyl transferase (TdT) dUTP Nick-End Labelling (TUNEL) assay is widely used for the detection of apoptotic events in paraffin-embedded tissue sections. The TUNEL assay identifies DNA breaks that arise during the final stages of apoptosis. This method involves the enzyme TdT, which adds deoxynucleotides to the 3'-hydroxyl terminus of DNA breaks. In this study, TUNEL Assay Kit-HRP-DAP ab206386 (Abcam, Cambridge, UK) was used.

3.13.1.1 Materials for TUNEL assay

- Tris-buffered saline (TBS)
- Proteinase K
- 30% hydrogen peroxide (H₂O₂)
- TdT Equilibration Buffer
- TdT Labelling Reaction Mix
- TdT Enzyme
- Stop Buffer
- Blocking Buffer
- 25X Conjugate
- DAB Solution 1 and DAB Solution 2
- Methyl Green Counterstain
- Ethanol 70% - 100%
- Xylene

3.13.1.2 Procedures for TUNEL assay

The paraffin embedded liver sections of all rats were first trimmed and sectioned (thickness of 4-6 µm). The thin sections were then mounted onto glass slides and left to dry for 24 hours at room temperature. The slides were first dewaxed using xylene (2 x 5-minute incubations) and rehydrated through a series of decreasing concentrations of alcohol (100%, 90%, 80% and 70%). Reagents were then added in the following order and incubated as below:

- Tris-buffered saline (TBS) solution for 5 minutes
- Dilute Proteinase K solution for 20 minutes (permeabilization of specimen)
- 3% H₂O₂ for 5 minutes (quenching – inactivation of endogenous peroxidase)
- TdT equilibrium buffer for 30 minutes (equilibration)
- TdT labelling reaction mix for 90 minutes (humidified chamber at 37°C)
- Blocking buffer for 10 minutes (blocking)
- 1X conjugate for 30 minutes (detection)
- Diluted Diaminobenzidine (DAB) solution for 15 minutes
- Methyl green counterstain solution for 5 minutes

Excess counterstain was then dried off with an absorbent towel, before dehydrating the specimen with 4 dips into 100% ethanol and 4 dips into 100% xylene. A glass coverslip was mounted and then stored carefully before viewing. The slides were reviewed by two independent histopathologists.

3.13.2 Alpha SMA Immunohistochemistry

The antibody kit used was Actin (smooth muscle) Dako Omnis purchased from Agilent Dako and performed using the automated Benchmark Ultra platform (Ventana Medical Systems, Roche, Switzerland). This antibody labels smooth muscle cells, myofibroblasts and myoepithelial cells. It was used as a marker of hepatic stellate cell activation.

3.13.2.1 Materials for alpha SMA

- Reaction buffer (Roche)
- Ultra Cell conditioning solution (CC1) (Roche)
- Antibody kit Actin Dako Omnis (Agilent Dako)
- UltraView universal DAB detection kit (Roche)
- Haematoxylin II (modified Mayer's haematoxylin) (Roche)
- Bluing reagent (Roche)
- Ethanol 70% - 100%
- Xylene

3.13.2.2 Procedures for Alpha SMA

The paraffin embedded liver sections of all rats were first trimmed and sectioned (thickness of 4-6µm). The thin sections were then mounted onto glass slides and left to dry for 24 hours at room temperature. The slides were first dewaxed using xylene (2 x 5-minute incubations) and rehydrated through a series of decreasing concentrations of alcohol (100%, 90%, 80%, and 70%). Reagents were then added and incubated in the following order (washed with reaction buffer after each step):

- Ultra cell conditioning (CC1) solution for 8 minutes at 95 °C
- UltraView inhibitor for 4 minutes (endogenous enzyme blocking)
- Primary antibody incubation (Dako SMA Clone 1A4) for 32 minutes at 37°C
- Secondary antibody incubation (HRP Multimer) for 8 minutes
- UltraView DAB H₂O₂ for 8 minutes
- UltraView Copper for 4 minutes
- Haematoxylin II (counterstain) for 4 minutes
- Bluing reagent for 4 minutes

Excess reagent was then dried off with an absorbent towel, before dehydrating the specimen with 4 dips into 100% ethanol and 4 dips into 100% xylene. A glass coverslip was mounted and then stored carefully before viewing. The slides were reviewed by two independent histopathologists.

3.13.3 Image processing for Immunohistochemistry

To analyse the immunohistochemistry liver-stained tissue samples images, the ImageJ, which is a free, open-source image processing program that can visualise, inspect, quantify, and validate scientific image data was used. To process the images, the files were opened in ImageJ software (National Institutes of Health). High-quality images of the stained liver tissues were acquired, ensuring proper exposure settings to avoid saturation. The images were converted to an 8-bit grayscale format to ensure consistency in analysis. Subsequently, image contrast was enhanced using the “Brightness/Contrast” adjustment to improve the visibility of these cells.

The next step involved thresholding the images to isolate targeted cells from the background. Thresholding was performed via the “Adjust Threshold” function, where either manual adjustments or automated methods were applied to set an optimal threshold level. A consistent threshold was applied across all images to maintain standardization. Once the threshold was determined, the images were converted into binary format using the “Make Binary” tool, rendering targeted cells as distinct black regions on a white background. This simplification facilitated particle analysis and minimised ambiguity during cell counting.

To quantify targeted cells, the “Analyse Particles” function in ImageJ was utilised. The TUNEL-positive index was calculated by dividing the number of TUNEL-positive nuclei by the total number of nuclei and multiplying by 100 to express the result as a percentage. While for α SMA expression, it was expressed as a percentage of the total tissue area or relative to counterstaining. Five randomly selected fields of view per sample were analysed to improve accuracy, and the results were averaged. All thresholding and analysis parameters were documented to ensure reproducibility. The data were then exported for further statistical analysis.

3.14 RNA EXTRACTION

A portion of the liver was dissected and preserved in RNAlater solution; a reagent designed for tissue RNA stabilization and storage that quickly permeates tissues to protect and stabilize cellular RNA. RNA was extracted from this preserved portion of the liver.

3.14.1 Materials for RNA extraction

- RNeasy Mini Spin Columns
- Collection tubes (1.5 ml)
- Buffer RTL, 45 ml
- Buffer RW1, 45 ml
- Buffer RPE (concentrate), 11 ml
- RNase-Free Water, 10 ml
- 96-100 % ethanol
- Microcentrifuge

3.14.2 RNA Extraction and Cleanup Procedure

Liver tissues were cut into small sections of not more than 30 mg and placed directly into a 2 ml microcentrifuge tube. The sample was then disrupted by adding 600 µl of RLT buffer (Qiagen) and immediately homogenised using a rotor-stator homogenizer for 30-40 seconds. The lysate was then centrifuged for 3 minutes at full speed. The resulting supernatant was carefully removed and transferred into a 1.5ml microcentrifuge tube. One volume of 50% alcohol was then added to the cleared lysate and mixed by pipetting.

A total of 700µl of the sample was transferred to a RNeasy spin column (placed in a 2 ml collection tube). This was then centrifuged at 10,000 RPM for 2 minutes. The resulting flow-through was discarded.

For the RNase-Free DNase (RFD) step, 350 µl of Buffer RW1 was added to the RNeasy spin column and placed in a new 2 ml collection tube. This was then centrifuged at 10,000 RPM for 1 minute. The resulting flow-through was discarded. An incubation mix of 10 µl DNase-1 stock solution with 70 µl of Buffer RDD was added to the RNeasy spin column. This mix was then incubated for 15 minutes, after which 350 µl of Buffer RW1 was added to the RNeasy spin column and centrifuged at 10,000 RPM for 1 minute. The resulting flow-through was discarded. Another 500 µl of Buffer RPE was added to the RNeasy spin column and centrifuged at 10,000 RPM for 1 minute. The resulting flow-through was discarded. The spin column was immediately centrifuged again for another 2 minutes at 10,000 RPM to wash the spin column membrane. The remaining flow-through and collection tube was discarded. Finally, the RNeasy spin column was placed in a new 1.5 ml collection tube and 30 µl of RNase-free water was directly added to it. This was centrifuged again for 1 minute at 10,000 RPM to elute the RNA. The resulting purified RNA (in the collection tube) was then stored at a -80 °C.

3.15 MICROARRAY ANALYSIS

3.15.1 Preparation

Microarray analysis involves the utilisation of gene chips capable of binding and quantifying thousands of distinct messenger ribonucleic acid (mRNA). Employing these chips to measure mRNA levels in various tissues or individuals subjected to different treatments, enables the identification of tens or hundreds of specific genes that exhibit variations in response to the tissue or treatment. The extracted RNA of the rats in the same group were pooled as a single sample due to financial constraints. Serial dilutions of the Spike Mix were first made according to table 3.6.

Table 3.6 Serial dilutions of Spike Mix

Serial dilutions	First (1:20)	Second (1:25)	Third (1:20)	Fourth (1:4)
Spike Mix volume to be used in each labelling reaction (μL)	2	2	2	10
Dilution buffer volume to be added into each labelling reaction (μL)	38	48	38	30

After mixing thoroughly on a vortex mixer and spinning down quickly in a microcentrifuge, 2 μL of this fourth dilution (40,000-fold final dilution) was added into 25 ng of each pooled sample RNA (that was diluted with water to become 1.5 μL in volume). For the labelling reaction, three master mixes from the One-Color Low Input Quick Amp Labelling Kit (Agilent, Valencia, Ca) were prepared and added to the sample in accordance with the steps mentioned in Table 3.7 as below.

Table 3.7 Preparation of master mixes

Steps	Mix	Components	Volume added	Incubated in circulating water bath
1	T7 Primer mix	- T7 Primer - Nuclease-free water	1.8 μ L	- 65°C for 10 minutes to denature primer - Incubated on ice for 5 minutes
2	cDNA Master mix	- 5x First Strand buffer - 0.1 M DTT - 10mM dNTP mix - Affinity Script RNase block mix	4.7 μ L	- 40°C for 2 hours - 70°C for 15 minutes to inactivate enzyme - Incubated on ice for 5 minutes
3	Transcription Master mix	- 5x Transcription buffer - 0.1 M DTT - NTP mix - T7 RNA Polymerase blend - Cyanine 3-CTP	6.0 μ L	- 40°C for 2 hours - Final volume: 16 μ L

3.15.2 Purifying the labelled/amplified RNA

Using the RNeasy Mini Kit (Qiagen), 84 μ L of nuclease-free water was added to the sample to make the total volume 100 μ L. A total of 350 μ L of Buffer RLT and 250 μ L of 96% ethanol was then added and mixed well by pipetting. The total 700 μ L of the complementary ribonucleic acid (cRNA) sample was transferred to a RNeasy spin column (placed in a 2ml collection tube). This was then centrifuged at 13,000 RPM for 30 seconds. The resulting flow-through was discarded. Buffer RPE (500 μ L) containing ethanol was added to the column and centrifuged at 13,000 RPM for another 30 seconds.

After discarding the flow-through, another 500 μL of buffer RPE was added to the column and centrifuged again at 13,000 RPM for 60 seconds. Purified cRNA was eluted by adding 30 μL RNase-free water onto the RNeasy filter column (placed in a new 1.5ml collection tube), and centrifuged at 13, 000 RPM for 30 seconds. The cRNA sample-containing flow-through was put on ice before hybridisation.

3.15.3 Hybridisation

After quantifying the cRNA using NanoVue™ Plus Spectrophotometer (GE Healthcare, UK), 0.825 μg of Cy3-labeled cRNA with specific activity ≥ 6 pmol Cy3 per μg CRNA for each of the samples, a fragmentation mix (containing 10x gene expression blocking agent, 25x fragmentation buffer and nuclease-free water) was added to the amplified cRNA, following the manufacturers' instructions. This was then incubated at 60 °C for exactly 30 minutes, and then immediately put on ice for 1 minute. To stop the fragmentation reaction, 250 μL of 2x Hi-RPM hybridisation buffer was added to the mix. This buffer was mixed well by careful pipetting and centrifuged at 13,000 RPM for 1 minute. The sample was then put on ice before being loaded onto the array.

The hybridisation sample was dispensed slowly onto the gasket well in a 'drag and dispense' manner from left to right. The array slide (Agilent SurePrint G3 Human Gene Expression v3 8x60K - Design ID: 072363) was then slowly put on to the gasket slide, making sure that the labelled barcode was facing down and the numeric barcode facing down. After putting on the chamber cover and sliding on the clamp assembly, the assembled chamber was vertically rotated to wet the gasket. The assembled chamber was then loaded onto the oven rotator rack and left to hybridise at 65 °C for 17 hours.



Figure 3.2 Agilent SurePrint G3 Human Gene Expression 8x60K slide (left) and Agilent Microarray Hybridisation Oven

3.15.4 Microarray Wash

At the 17-hour mark, the hybridisation chamber assembly was taken out from the oven. The array-gasket sandwich was then removed from the chamber and transferred to slide-staining dish #1 containing Gene Expression Wash Buffer 1 completely submerging it. While being submerged, the array slides were removed carefully from the gasket and transferred to slide-staining dish #2 that also contained Gene Expression Wash Buffer 1. Washing was done by adding a magnetic stir bar and stirred at setting 4 for 1 minute. The slides were then transferred to slide-staining dish #3 containing Gene Expression Wash Buffer 2, to be washed again using stir setting 4 for another 1 minute. Finally, the slides were removed from the slide-staining dish and put onto a slide holder with the labelled barcode facing up. Ozone-barrier slide covers were then put on top to further protect the slides.

3.15.5 Scanning and Feature Extraction

Slides were immediately scanned using Agilent SureScan Microarray Scanner (G4900DA) (Figure 3.3) with 3 μm resolution at wavelengths of 532 nm (Cy3) using the extended dynamic range (10–100%) setting. Normalised intensities were extracted using the Agilent Feature Extraction Software with protocol GE1_1200_Jun14 and all the data (in text format) files were obtained. The text files were then uploaded in Agilent GeneSpring Software for further microarray data analysis.

3.15.6 Microarray data analysis

Microarray data for this study were obtained from experimental assays comprising of 9 samples that represent HCD controls (2 samples from group 2) and treated samples from groups 3-5 (7 samples) under investigation. Raw data underwent pre-processing to ensure quality and consistency, including quality control steps such as visual inspections (e.g., box plots, density plots) to identify outliers and assess signal intensity distributions, followed by background correction using methods like Robust Multi-array Average (RMA).

Then, normalised intensities were extracted using the Agilent Feature Extraction Software with protocol GE1_1200_Jun14 and all the 9 data (in .txt format) files were obtained and summarised into single gene expression values using algorithms like median polish. Differential expression analysis identified differentially expressed genes (DEGs) between groups using statistical tests like the t-test with p-values adjusted via the Benjamini-Hochberg method to control the false discovery rate (FDR). Significant DEGs (e.g., $|\log_2\text{FC}| > 1$ and $\text{FDR} < 0.05$) were further analysed for functional enrichment through Gene Ontology (GO) and pathway analyses using tools like Kyoto Encyclopaedia of Genes and Genomes (KEGG) or Reactome.

Next, Clustering methods were applied, including hierarchical clustering and Principal Component Analysis (PCA), to visualise expression patterns and sample variation. While heatmaps of DEGs were generated to illustrate DEGs' profiles across conditions. Validation was performed using qPCR for a subset of DEGs, ensuring reliability by confirming concordance with microarray findings. Data integration with protein-protein interaction databases (e.g., STRING) enabled the construction of interaction networks and identification of hub genes. All analyses leveraged reliable software and tools, including Bioconductor packages for preprocessing, statistical tests in R, functional analysis via DAVID or GSEA, and visualisation tools such as ggplot2 and heatmap, adhering to best practices for high-throughput data analysis



Figure 3.3 Agilent SureScan Microarray Scanner

3.16 COMPLEMENTARY DNA SYNTHESIS

Before RT-qPCR analysis, the liver RNA samples needed to be converted into complementary deoxyribonucleic acid (cDNA). The QuantiTect Reverse Transcription Kit (Qiagen) was used.

3.16.1 cDNA Synthesis

cDNA synthesis is the process of converting RNA into complementary DNA (cDNA) using reverse transcriptase. The Quantiscript Reverse Transcriptase in QIAGEN's QuantiTect Reverse Transcription Kit is a proprietary blend of two recombinant enzymes: Omniscript and Sensiscript Reverse Transcriptase. Both enzymes are heterodimeric proteins expressed in *Escherichia coli* (*E. coli*). This unique combination provides high affinity for RNA templates, enabling efficient and sensitive cDNA synthesis across a wide range of RNA amounts (from 10 pg to 1 µg). Additionally, the blend includes an RNase inhibitor to protect RNA from degradation during the reverse transcription process.

3.16.2 Materials for cDNA Synthesis

- Sample RNA
- gDNA Wipeout Buffer
- Quantiscript® Reverse Transcriptase
- Quantiscript RT Buffer
- RT Primer Mix
- RNase-Free Water

3.16.3 cDNA Synthesis Procedure

The cDNA synthesis procedure started with thawing the RNA samples and all required solutions at room temperature. For each sample, up to 1 μg of total RNA was combined with 2 μL of gDNA Wipeout Buffer in a final volume of 14 μL , adjusted with RNase-free water. The mixture was incubated at 42°C for 2 minutes to degrade any contaminating genomic DNA, thereby preventing false-positive amplification in subsequent qPCR experiments. After incubation, the reaction was immediately placed on ice for 1 minute to halt the enzymatic activity and stabilise the RNA. This genomic DNA removal step was crucial to ensure that only cDNA, and not residual genomic DNA, would be amplified during PCR.

A reverse transcription master mix was prepared on ice to minimise enzyme degradation and maintain reaction efficiency. The master mix included 1 μL Quantiscript Reverse Transcriptase, 4 μL Quantiscript RT Buffer, and 1 μL RT Primer Mix for each reaction, totalling 6 μL . The Quantiscript RT Buffer contained magnesium ions, dNTPs, and optimized reaction components, while the RT Primer Mix included a blend of oligo-dT and random primers.

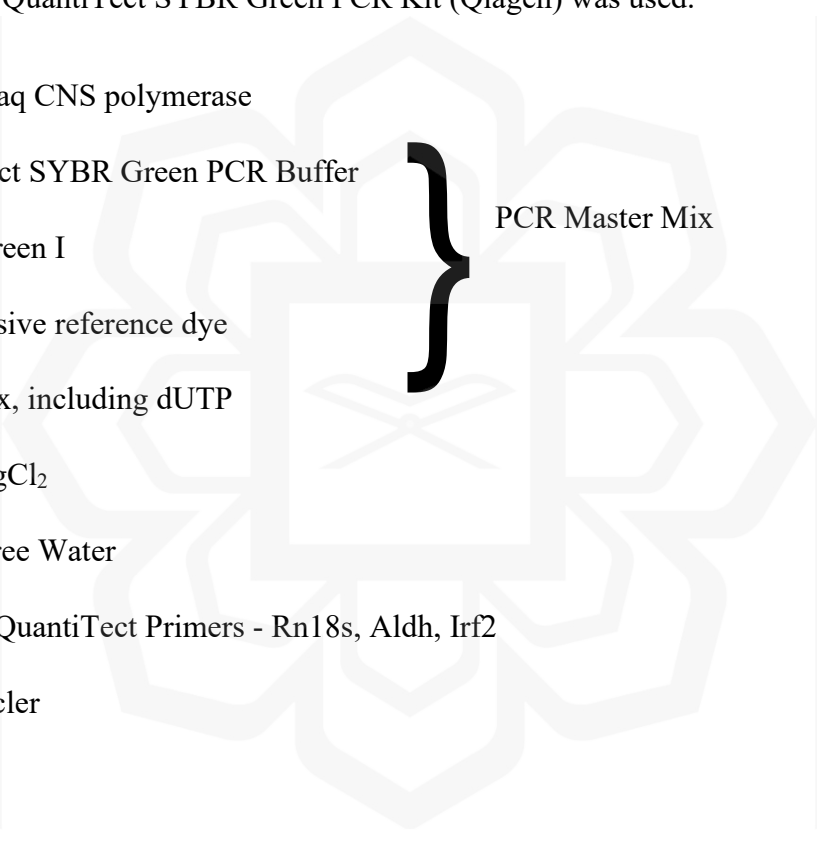
The reverse transcription reaction was assembled by adding the 6 μL master mix to the 14 μL RNA sample, resulting in a final volume of 20 μL . The reaction was incubated at 42°C for 20 minutes to allow for efficient cDNA synthesis. The relatively low incubation temperature and the enzyme's high affinity for RNA templates ensured robust synthesis even from limited or partially degraded RNA, which can occur in fatty liver tissues with oxidative stress and inflammation. After cDNA synthesis, the reaction was heated to 95°C for 3 minutes to inactivate the reverse transcriptase, stopping the reaction and preserving the cDNA integrity. The resulting cDNA was stored at -20°C for RT-qPCR.

3.17 RT-QPCR ANALYSIS

A reverse transcription quantitative polymerase chain reaction (RT-qPCR) is a laboratory technique that was used to validate the microarray results by comparison of cDNA abundance obtained by qPCR with selected pre-developed primer sets (Applied Biosystems).

3.17.1 Materials for qPCR

In this study, the QuantiTect SYBR Green PCR Kit (Qiagen) was used.

- HotStarTaq CNS polymerase
 - QuantiTect SYBR Green PCR Buffer
 - SYBR Green I
 - ROX passive reference dye
 - dNTP mix, including dUTP
 - 5 mM MgCl₂
 - RNase-Free Water
 - Selected QuantiTect Primers - Rn18s, Aldh, Irf2
 - qPCR cyclers
- 
- PCR Master Mix

3.17.2 qPCR Procedures

TE Buffer was added to Primers in microcentrifuge tubes and vortexed. The mixture was divided into several aliquots. The Master mix was incubated at 95 °C for 15 minutes to activate the HotStarTaq DNA polymerase. Then, the samples (template cDNA), master mix and RNase-free water were thawed. Next, the Real Time Cycler was programmed as shown in table 3.8.

Table 3.8 Initial activation steps

PCR Initial activation step	Time	Temperature
Denaturation	15 seconds	94°C
Annealing	30 seconds	55°C
Extension	30 seconds	72°C
Number of cycles	40 cycles	

Then, serial dilution of the undiluted sample was prepared for standard curve measurement. Later a reaction mix was prepared based on the following table.

Table 3.9 Preparation of reaction mix

Component	Volume/reaction	Final concentration
QuantiTect SYBR Green Master Mix	10 µl	1x
QuantiTect Primer Assay	5 µl	1x
Template cDNA/RNase-free water	5 µl	<100ng/reaction
Total volume	20 µl	

The above reagents were added to wells according to the following sequence: Master mix, Primers and then sample cDNA. Subsequently, the microplate was placed in the real-time cycler and the cycling program was commenced. Results were extracted from the raw data and reorganised in Excel sheets.

3.17.3 Gene expression analysis

Gene expression analysis was performed with normalisation and relative quantification to ensure accurate and reliable results. Expression levels were normalized to 18s RNA gene, serving as a reference to account for variability in cDNA input. Relative expression levels were calculated using the $2^{-\Delta\Delta Cq}$ method, where ΔCq represents the difference between the Cq values of the target and reference genes, and $\Delta\Delta Cq$ represents the difference between ΔCq of the sample and the control. Expression changes were expressed as fold changes relative to the control group.

Biological replicates and technical replicates were included to ensure robustness, and statistical analysis was conducted using ANOVA with significance set at $p < 0.05$. Results were reported as mean $\pm$ standard error of the mean (SEM). Quality control measures included melting curve analysis to confirm primer specificity by ensuring a single peak, and no-template controls (NTC) to rule out contamination or primer-dimer artifacts. Data analysis was carried out using Bio-Rad CFX Manager for qPCR data and IBM SPSS for statistical and graphical analysis.

3.18 STATISTICAL ANALYSIS

The data from this study was analysed using IBM® SPSS® Statistics software version 26.0.0.0. A statistically significant level was taken at a 95% confidence interval ($p < 0.05$). The numerical data were normally distributed and were presented as mean and standard deviation (SD). Statistical analysis was performed to compare among all the 5 experimental groups. The mean comparisons were performed using analysis of variance (ANOVA). The differences between each group were identified using Turkey's Least Significant Difference (LSD) post-hoc test.

CHAPTER FOUR

RESULTS

4.1 COMPARISON OF BODYWEIGHT GAIN, RELATIVE LIVER WEIGHT, LIVER GROSS MORPHOLOGY AND HISTOLOGICAL CHANGES

4.1.1 Percentage bodyweight gain (BWG)

Figure 4.1 shows the percentage of bodyweight gain in each experimental group. There were no significant differences in mean percentage BWG among the experimental groups.

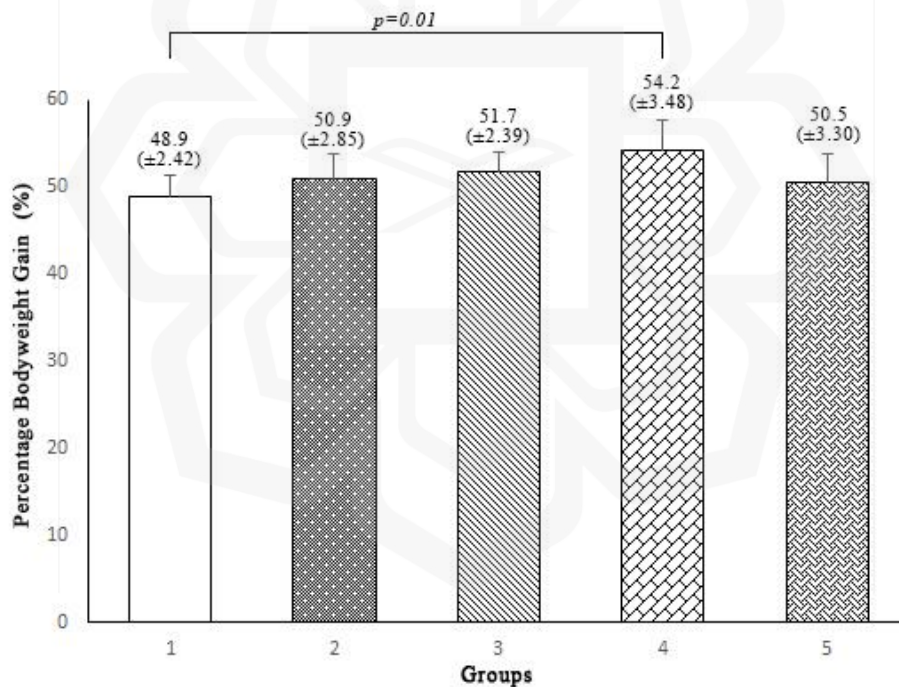


Figure 4.1 Percentage of body weight gain of experimental groups.

Note. 1=Control, normal diet; 2=12% HCD only; 3=12 % HCD with 1.2g/kg/day TH; 4=12% HCD with 2.4g/kg/day TH; 5= 12% HCD with 3.0g/kg/day TH. Results were represented as mean (standard deviation) and analysed using ANOVA. A p-value <0.05 was taken as statistically significant at a 95% confidence interval.

4.1.2 Relative liver weights

Figure 4.2 shows the mean relative liver weight (RLW) in each experimental group. The RLW was calculated as a percentage of absolute liver weight divided by the final body weight. The mean RLW was significantly increased ($p<0.01$) in the 12% HCD only animals (Group 2). Although the relative liver weight showed a reduced trend in the TH supplementation groups (Group 3, 4 and 5), the differences were not statistically significant.

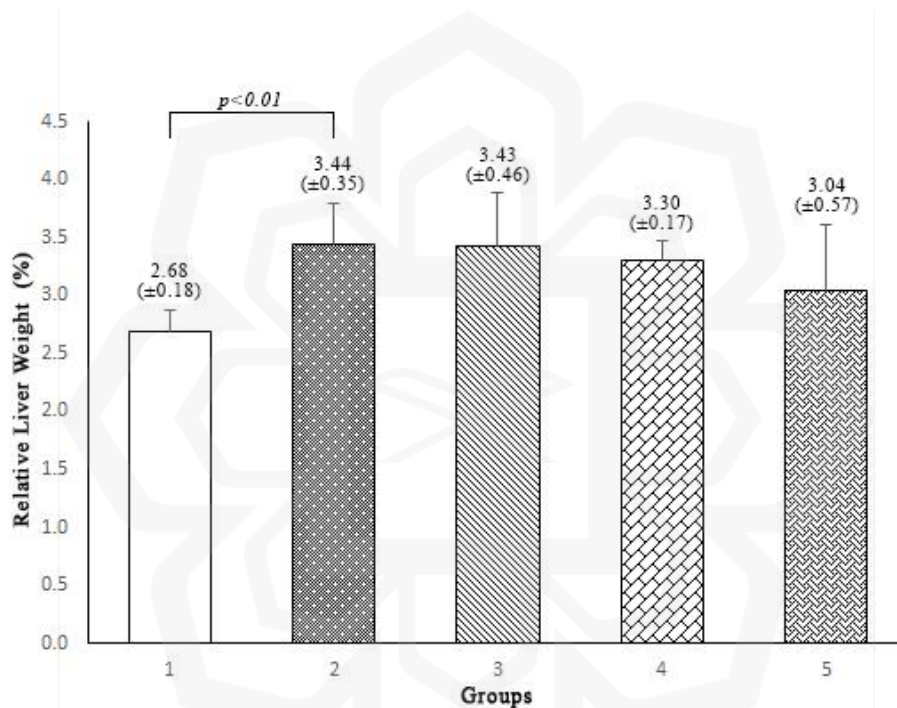


Figure 4.2 Relative liver weight of experimental groups.

Note. 1=Control, normal diet; 2=12% HCD only; 3=12% HCD with 1.2g/kg/day TH; 4=12% HCD with 2.4g/kg/day TH; 5=12% HCD with 3.0g/kg/day TH. Results were represented as mean (standard deviation) and analysed using ANOVA. A p-value <0.05 was taken as statistically significant at a 95% confidence interval.

4.1.3 Gross morphology

Figure 4.3 illustrates the gross morphology of rat livers across different experimental groups. In the control group (Group 1), the livers displayed normal liver gross morphology, maintaining their typical smooth surface, uniform reddish-brown colour, and normal architecture. Conversely, the livers in Groups 2, 3, 4, and 5 exhibited noticeable changes, including increased size and a yellowish discoloration, indicative of pathological alterations. Among these, the representative liver from Group 4 showed signs of improvement, with a reduction in liver size and partial restoration of smooth surface.



Figure 4.3 Gross morphology features of a representative liver from each experimental group

Note. 1=Control, normal diet; 2=12% HCD only; 3=12% HCD with 1.2g/kg/day TH; 4=12% HCD with 2.4g/kg/day TH; 5=12% HCD with 3.0g/kg/day.

4.1.4 Histological changes

4.1.4.1 Liver histology of normal control group (Group 1)

The H&E liver sections of rats in the control group showed tightly packed pink-staining hepatocytes within a normal liver architecture (Figure 4.4). The portal tracts which consist of a branch of the hepatic artery, portal vein, and bile duct, surrounded by connective tissue are seen. Figure 4.5 shows the central vein. No apparent steatosis or inflammation could be seen. The Masson Trichrome section did not exhibit any characteristic perivenular/pericellular fibrosis (Figure 4.6).

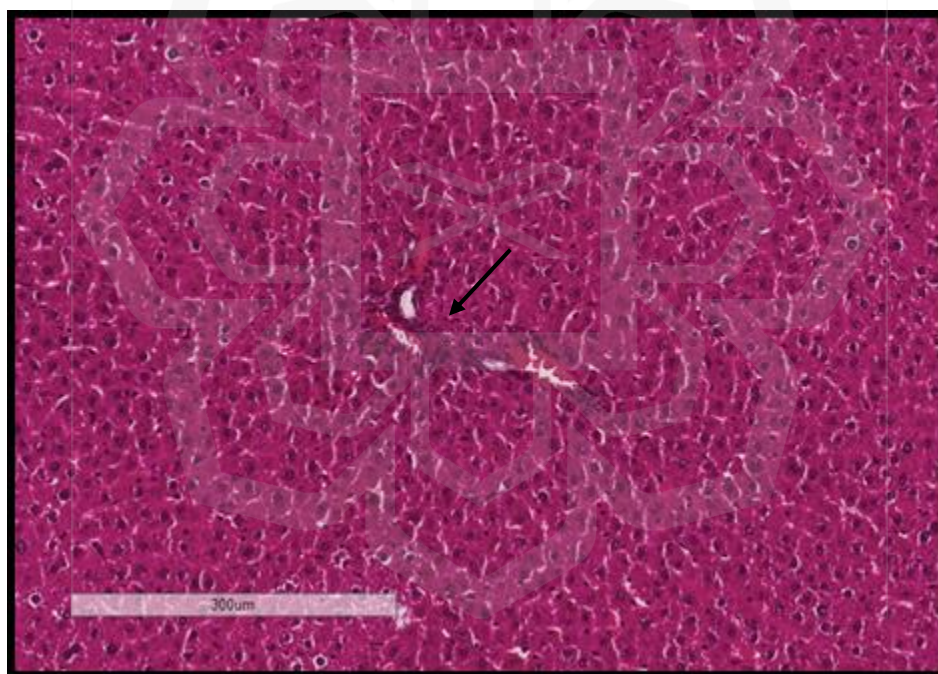


Figure 4.4 Photomicrograph of a representative liver section of Group 1-Normal control (H&E stain x10 objective) The section shows a normal liver histology. Arrow=portal tract area.

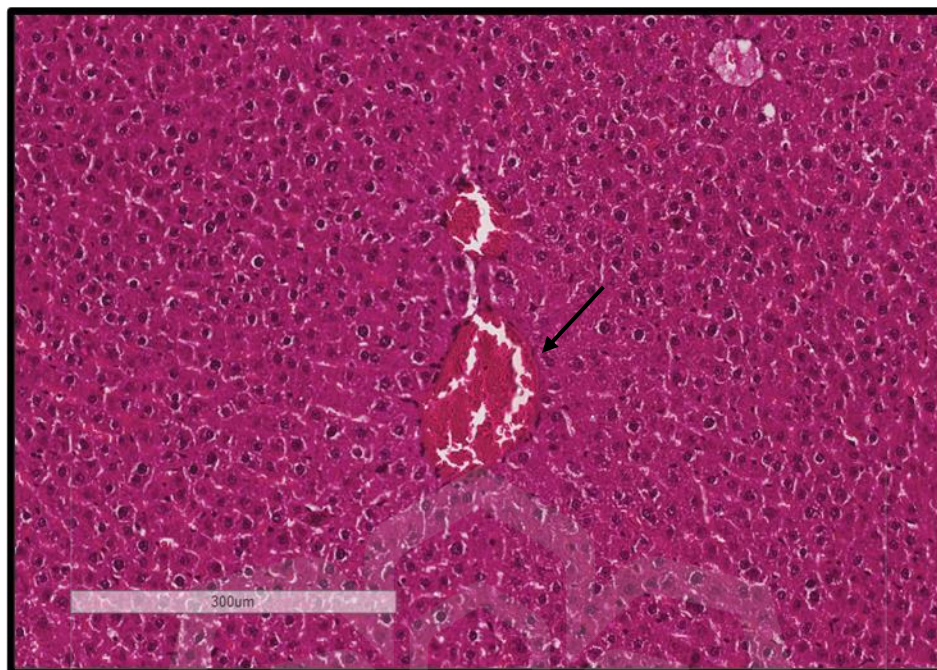


Figure 4.5 Photomicrograph of a representative liver section of Group 1- Normal control (H&E stain x10 objective) The section shows a normal liver histology. Arrow=central vein.

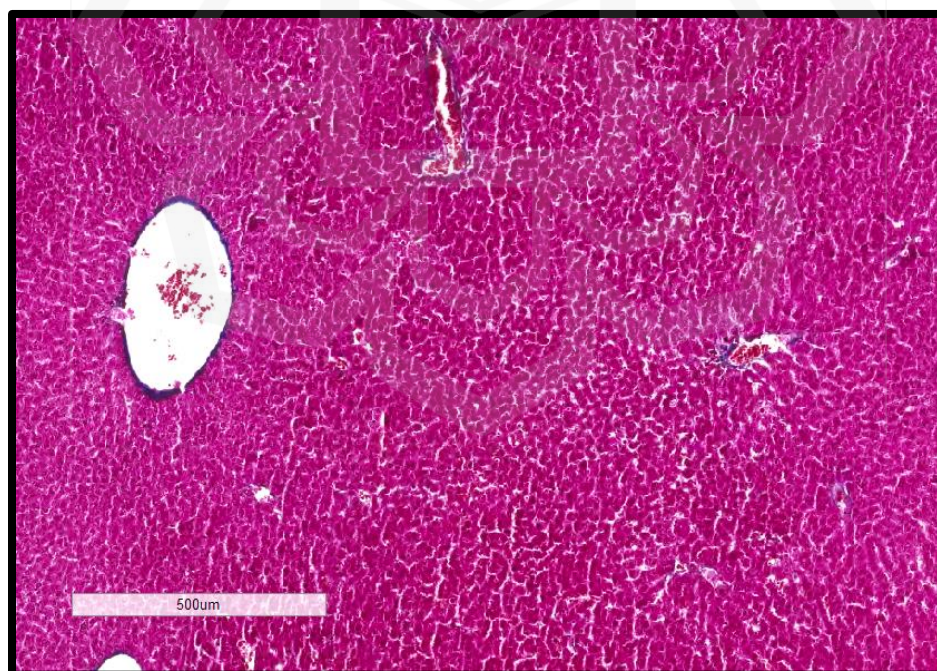


Figure 4.6 Photomicrograph of a representative liver section of Group 1-Normal control (MT stain x10 objective). The liver section shows no area of fibrosis.

4.1.4.2 Liver histology of 12% HCD group (Group 2)

The H&E sections of the liver for the rats in Group 2 showed extensive mixed steatosis (macrovesicular and microvesicular) (Figure 4.7). Lobular inflammation could be seen as the presence of neutrophil and mononuclear cell infiltration (Figure 4.7). Additionally, mild portal inflammation was also observed in some areas (Figure 4.8). Very occasional areas revealed ballooning degeneration of hepatocytes. The Masson Trichrome section did not show any area of fibrosis (Figure 4.9).

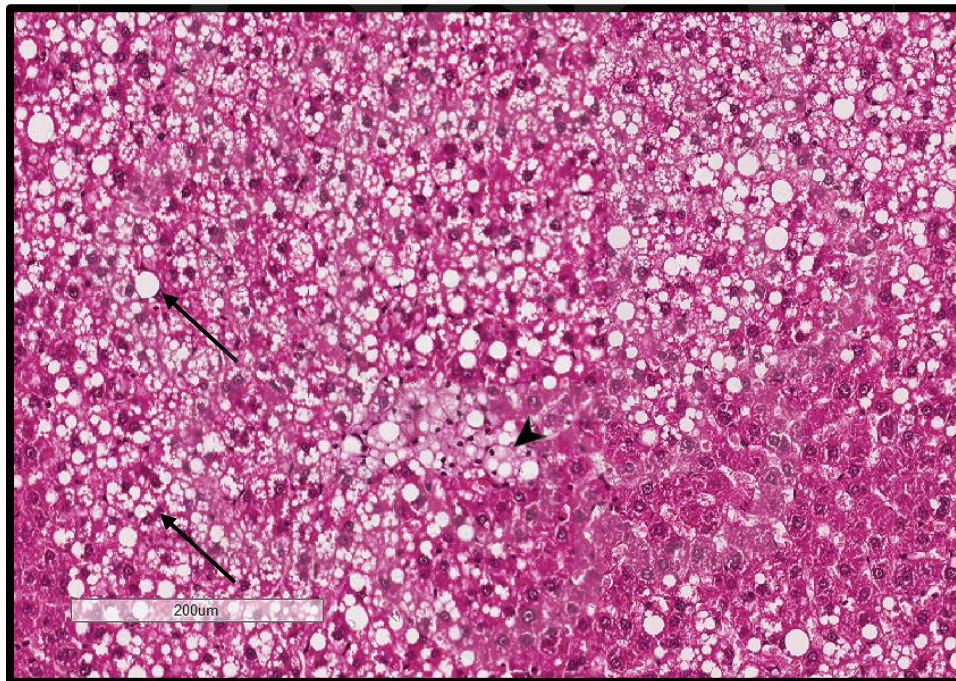


Figure 4.7 Photomicrograph of a representative liver section of Group 2 (H&E stain x10 objective): This section shows areas of steatosis with lobular inflammation. Arrow=steatosis; arrowhead=ballooning degeneration.

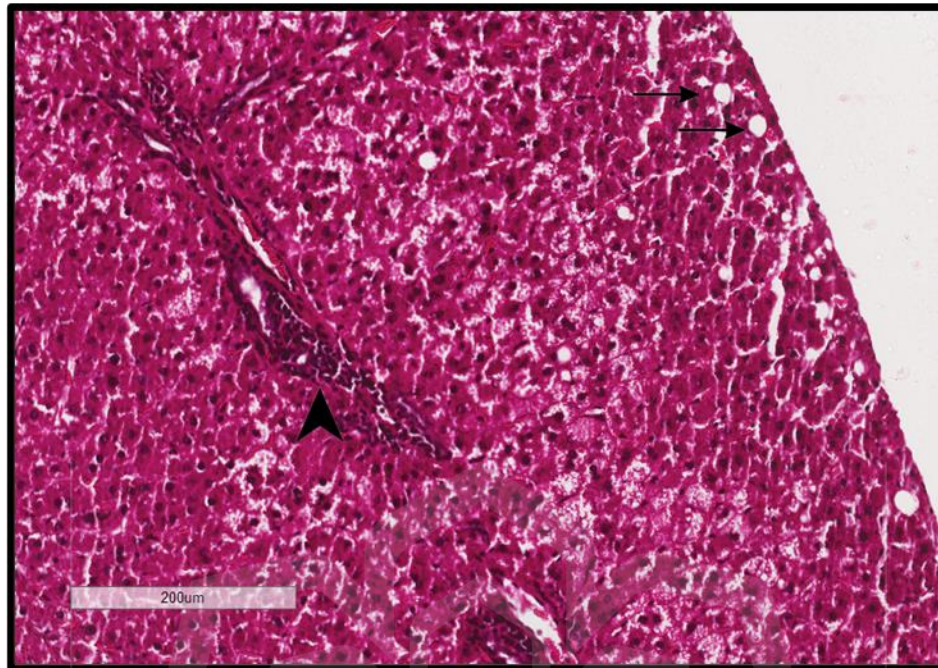


Figure 4.8 Photomicrograph of a representative liver section of Group 2 (H&E stain x10 objective): This section shows areas of steatosis with portal inflammation. Arrowhead=inflammation

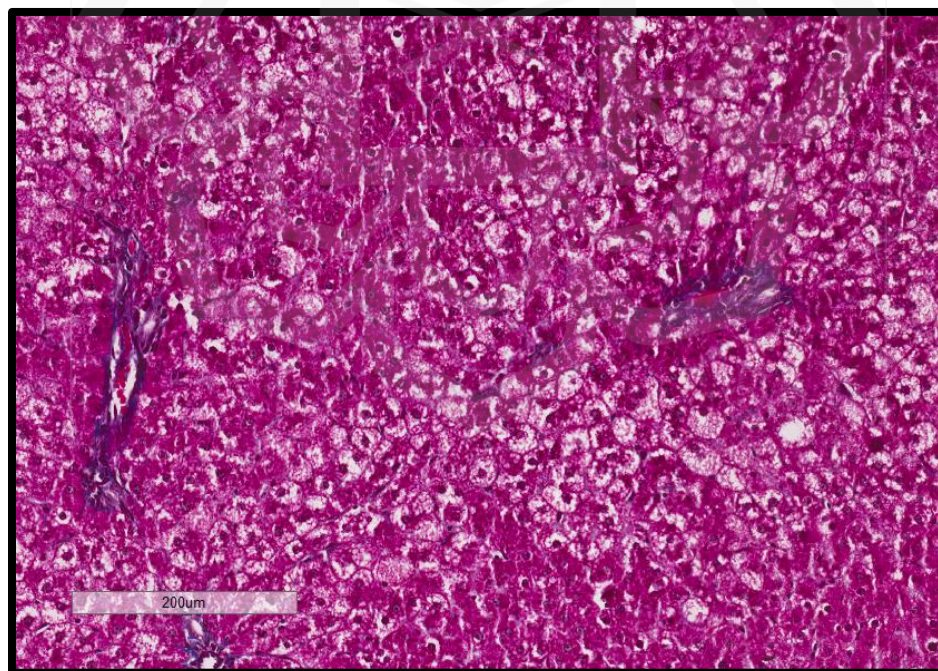


Figure 4.9 Photomicrograph of a representative liver section of Group 2 (MT stain x20 objective): The liver section shows no area of fibrosis.

4.1.4.3 Liver histology of 12% HCD with 1.2g/kg/day Tualang honey group (Group 3)

The liver specimens in Group 3 exhibited steatosis, encompassing both macrovesicular and microvesicular types (Figure 4.10). Some areas of lobular and portal inflammation were observed (Figure 4.10). Occasional ballooning degeneration of hepatocytes could also be seen (Figure 4.11). The Masson Trichrome section did not show any area of fibrosis (Figure 4.12).

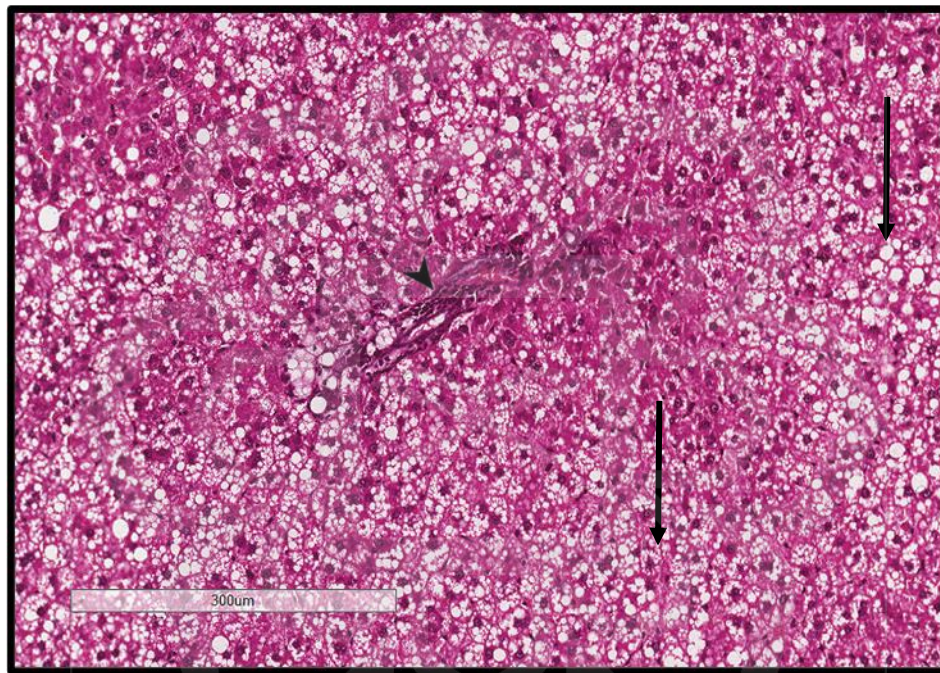


Figure 4.10 Photomicrograph of a representative liver section of Group 3 (H&E stain x10 objective): This section shows areas of steatosis with portal inflammation. Arrows=steatosis; arrowhead=inflammation.

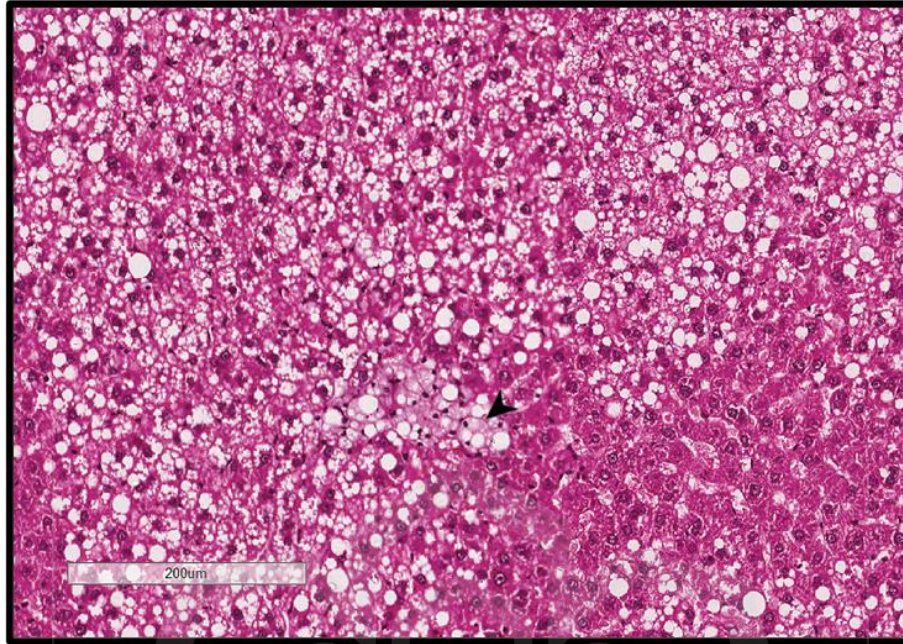


Figure 4.11 Photomicrograph of a representative liver section of Group 3 (H&E stain x10 objective): This section shows areas of steatosis with an area of ballooning degeneration. Arrowhead=ballooning degeneration.

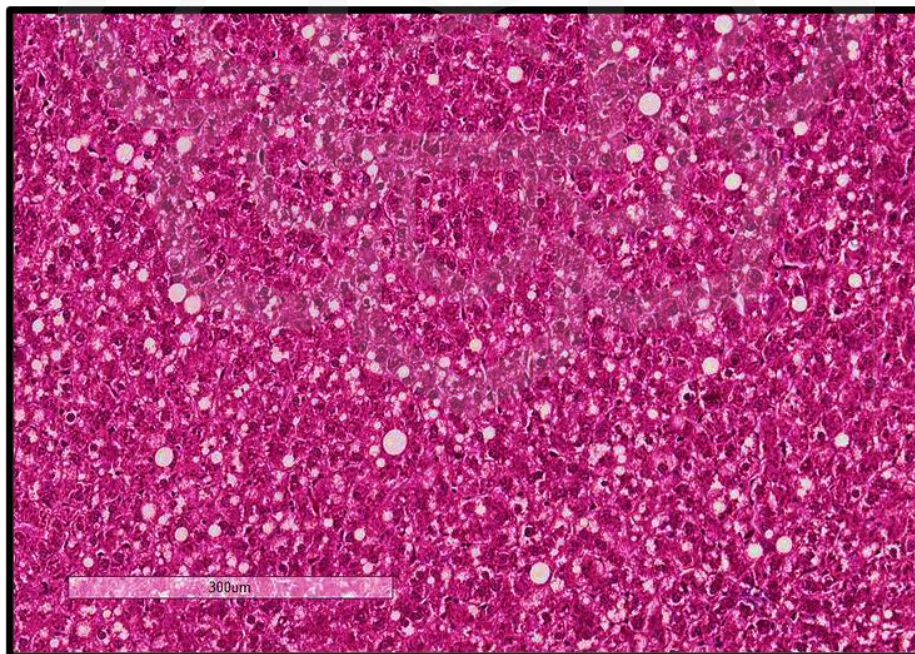


Figure 4.12 Photomicrograph of a representative liver section of Group 3 (MT stain x20 objective): The liver section shows no area of fibrosis.

4.1.4.4 Liver histology of 12% HCD with 2.4g/kg/day Tualang honey group (Group 4)

Figure 4.13 illustrates the H&E liver sections in Group 4, showing areas of steatosis, of both macrovesicular and microvesicular types (Figure 4.13). Mild portal and lobular inflammation could also be seen (Figure 4.14). Very occasional ballooning degeneration of hepatocytes were also observed. The Masson Trichrome section did not show any area of fibrosis (Figure 4.15).

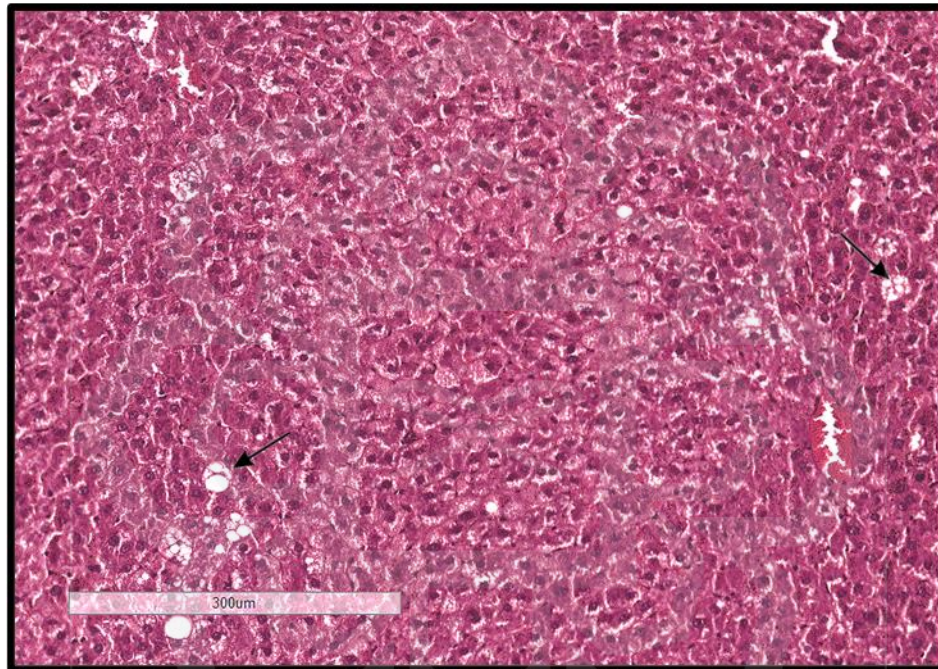


Figure 4.13 Photomicrograph of a representative liver section of Group 4 (H&E stain x4 objective): This section shows areas of steatosis. Arrow=steatosis

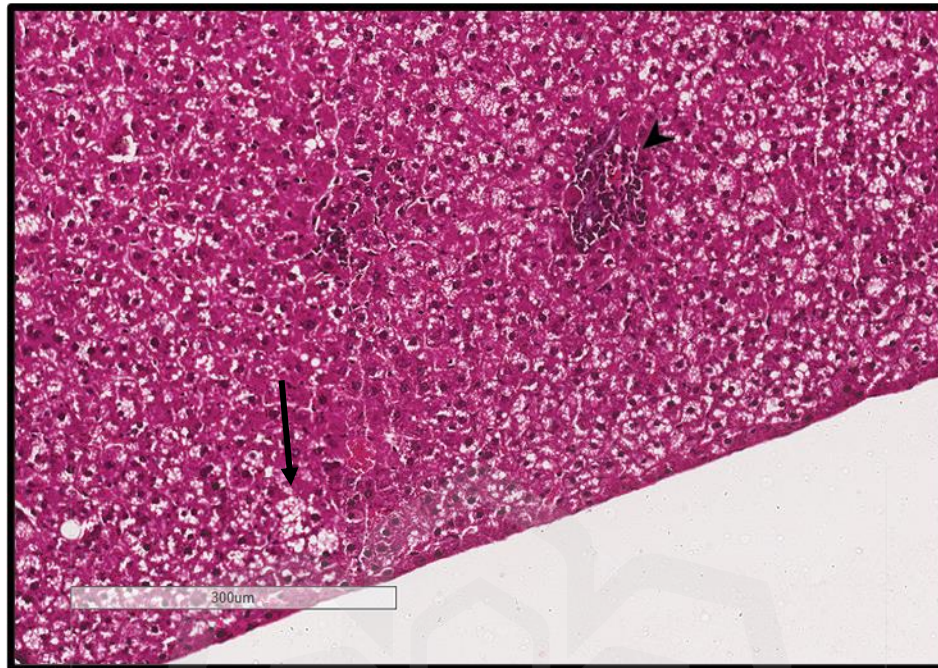


Figure 4.14 Photomicrograph of liver section of Group 4 (H&E stain x10 objective): This section shows areas of steatosis with lobular inflammation. Arrowhead=inflammation. Arrowhead=ballooning degeneration

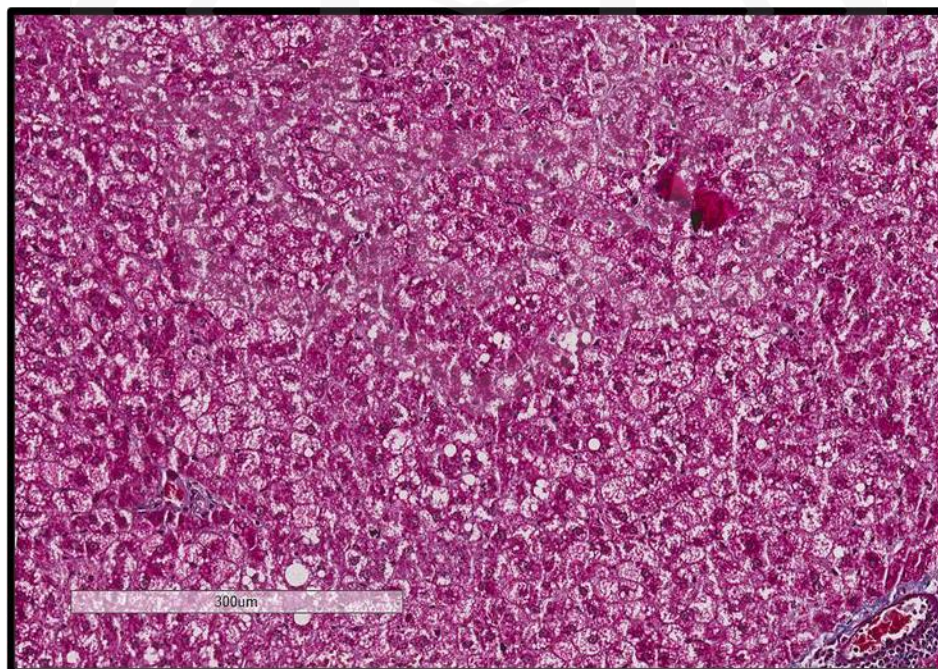


Figure 4.15 Photomicrograph of liver section of Group 4 (MT stain x10 objective). The liver section shows no area of fibrosis.

4.1.4.5 Liver histology of 12% HCD with 3.0g/kg/day Tualang honey group (Group 5)

The liver sections in Group 5 showed areas of steatosis, with hepatocyte ballooning (Figure 4.16). Areas of portal and lobular inflammation could also be observed (Figure 4.16 & Figure 4.17). The MT-stained section revealed areas of perisinusoidal fibrosis.

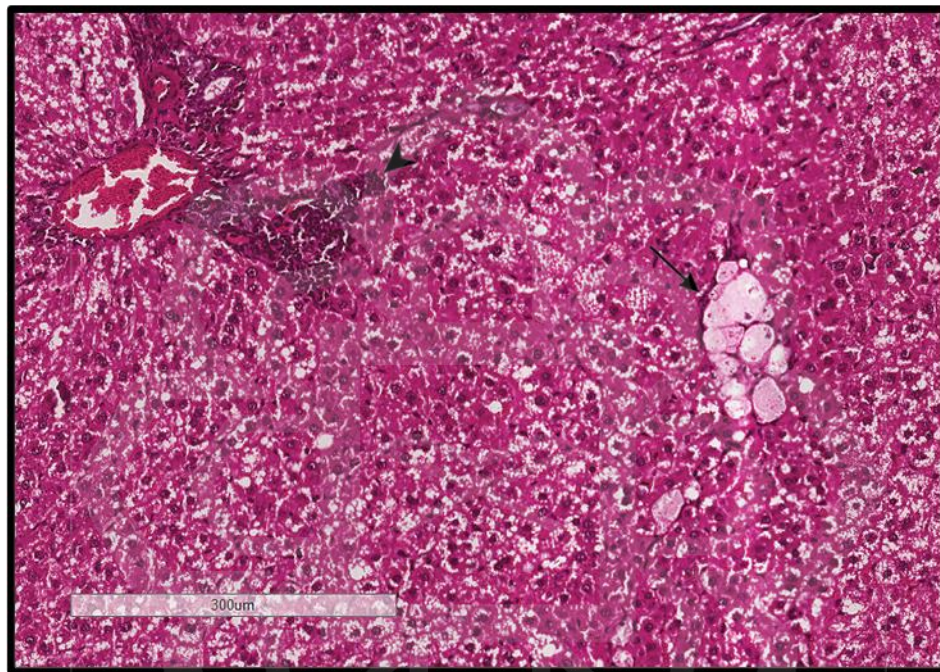


Figure 4.16 Photomicrograph of a representative liver section of Group 5 (H&E stain x4 objective): This section shows areas of steatosis with ballooning degeneration and portal inflammation. Arrow=ballooning degeneration; arrowhead=inflammation.

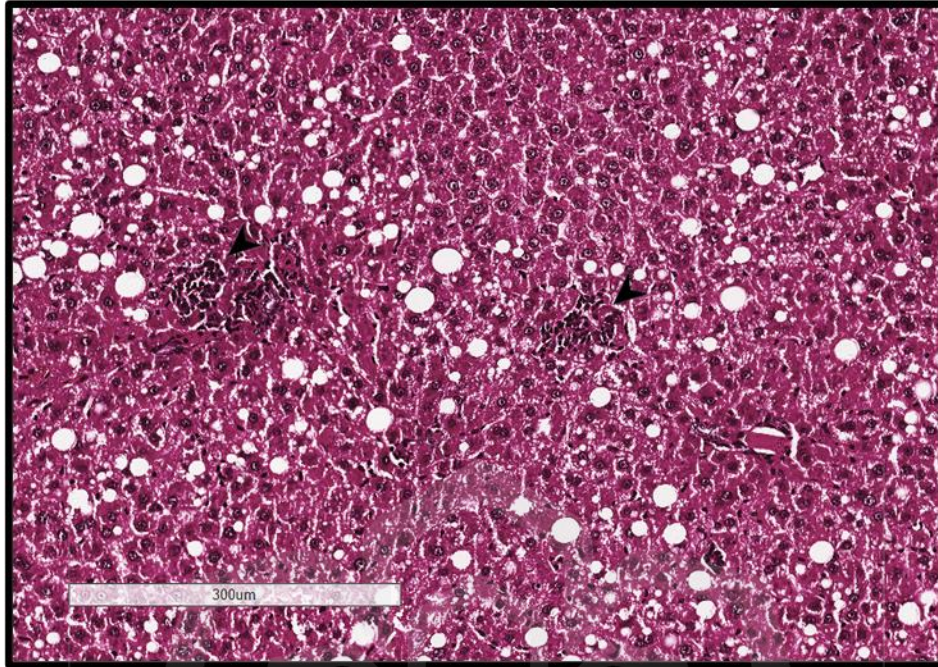


Figure 4.17 Photomicrograph of a representative liver section of Group 5 (H&E stain x10 objective): This section shows areas of steatosis and lobular inflammation. Arrowhead=inflammation.

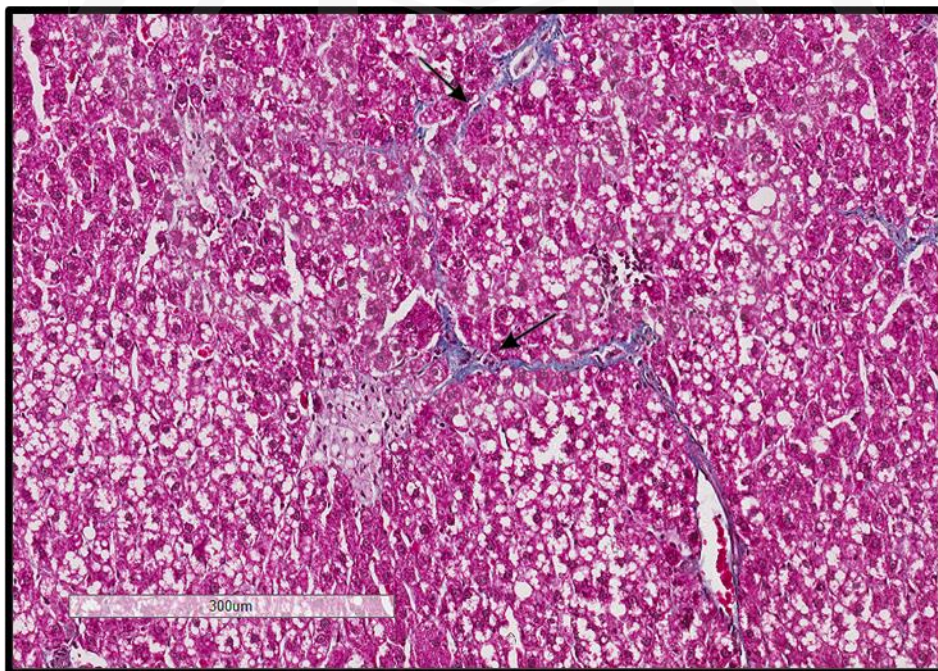


Figure 4.18 Photomicrograph of a representative liver section of Group 5 (MT stain x20 objective): This section shows areas of perisinusoidal fibrosis. Arrow=Fibrosis

4.1.5 NAFLD activity score

The NAFLD activity scores (NAS) were assessed to evaluate the histological impact of a 12% HCD and TH supplementation. Figure 4.19 shows there were no significant differences in NAFLD activity scores among the experimental groups.

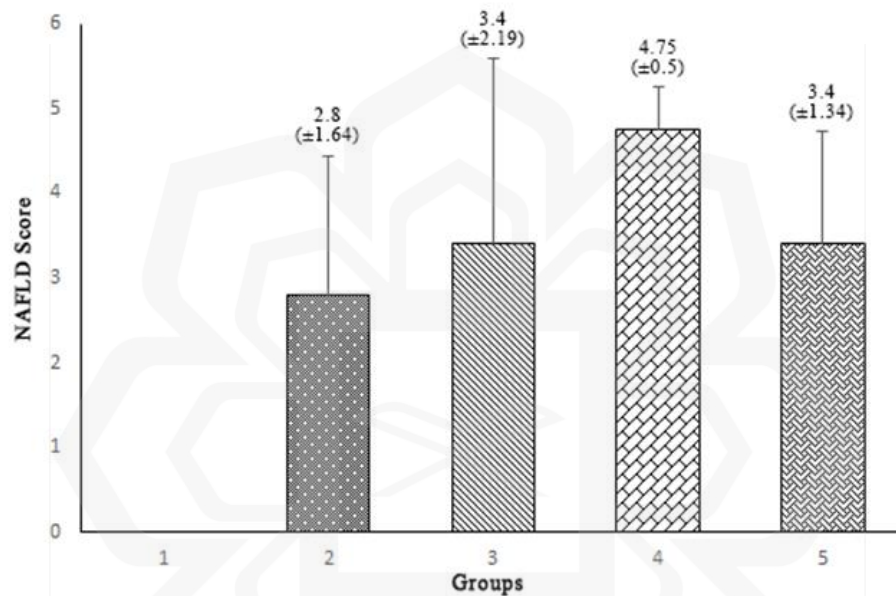


Figure 4.19 NAFLD Activity Score of the experimental groups.

Note. 1=Control, normal diet; 2=12 % HCD only; 3=12% HCD with 1.2g/kg/day TH; 4=12% HCD with 2.4g/kg/day TH; 5=12% HCD with 3.0g/kg/day TH. Results were represented as mean (standard deviation) and analysed using ANOVA. A p-value <0.05 was taken as statistically significant at a 95% confidence interval.

4.2 COMPARISON ON BIOCHEMICAL PROFILES

4.2.1 Serum aspartate transaminase (AST) levels

Figure 4.20 shows the serum AST levels in each of the experimental groups. There were no significant differences in serum AST levels among the experimental groups.

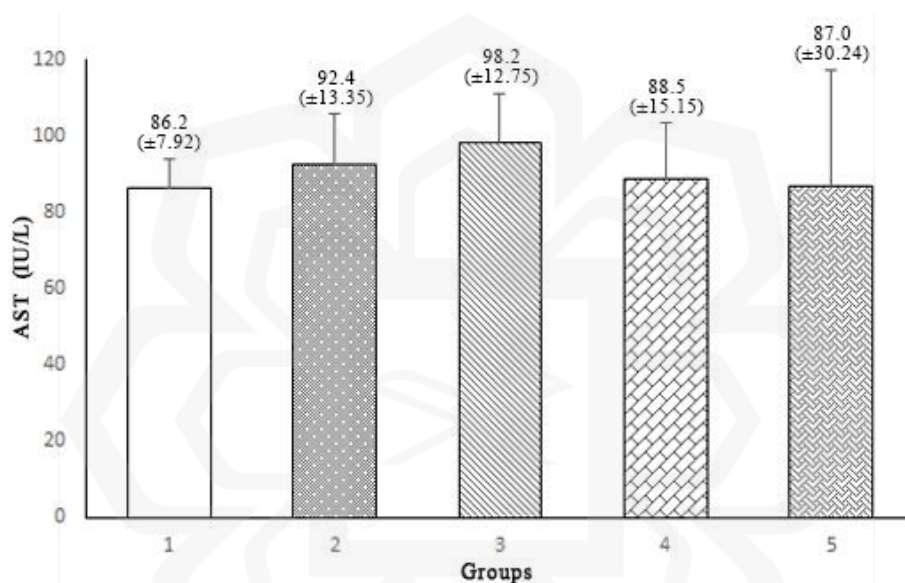


Figure 4.20 Serum AST levels of experimental groups

Note. 1=Control, normal diet; 2=12 % HCD only; 3=12% HCD with 1.2g/kg/day TH; 4=12% HCD with 2.4g/kg/day TH; 5=12% HCD with 3.0g/kg/day TH. Results were represented as mean (standard deviation) and analysed using ANOVA. A p-value <0.05 was taken as statistically significant at a 95% confidence interval.

4.2.2 Serum alanine transaminase (ALT) levels

Figure 4.21 shows the mean serum ALT levels in each of the experimental groups. There were no significant differences in serum AST levels among the experimental groups.

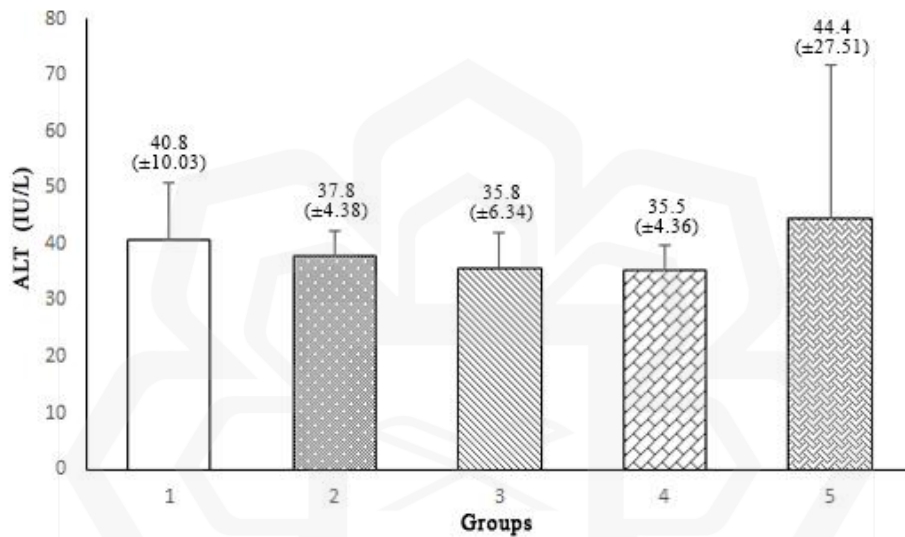


Figure 4.21 Serum ALT levels of experimental groups

Note. 1=Control, normal diet; 2=12 % HCD only; 3=12% HCD with 1.2g/kg/day TH; 4=12% HCD with 2.4g/kg/day TH; 5=12% HCD with 3.0g/kg/day TH. Results were represented as mean (standard deviation) and analysed using ANOVA. A p-value <0.05 was taken as statistically significant at a 95% confidence interval.

4.2.3 Alkaline phosphatase (ALP) levels

Figure 4.22 shows the serum ALP levels in each of the experimental groups. There were no significant differences in serum ALP levels among the experimental groups.

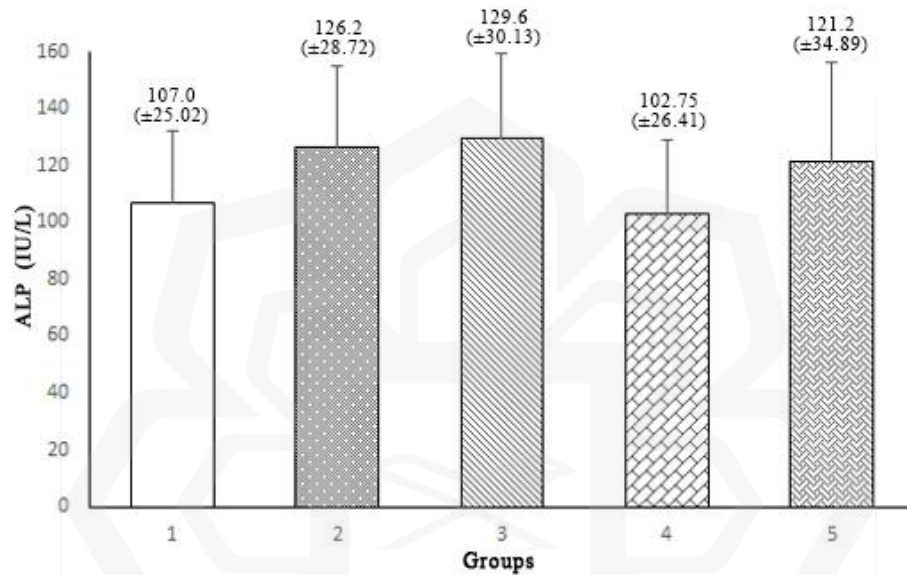


Figure 4.22 Serum ALP levels of experimental groups

Note. 1=Control, normal diet; 2=12 % HCD only; 3=12% HCD with 1.2g/kg/day TH; 4=12% HCD with 2.4g/kg/day TH; 5=12% HCD with 3.0g/kg/day TH. Results were represented as mean (standard deviation) and analysed using ANOVA. A p-value <0.05 was taken as statistically significant at a 95% confidence interval.

4.2.4 Serum total cholesterol (TC) levels

Figure 4.23 shows the serum TC levels in each of the experimental groups. The mean serum TC was significantly increased in 12% HCD group (Group 2) as compared to control (Group 1), $p < 0.05$. There were no significant differences in TC levels in animals given TH supplementation at all dosages (Group 3, 4 and 5) compared to Group 2.

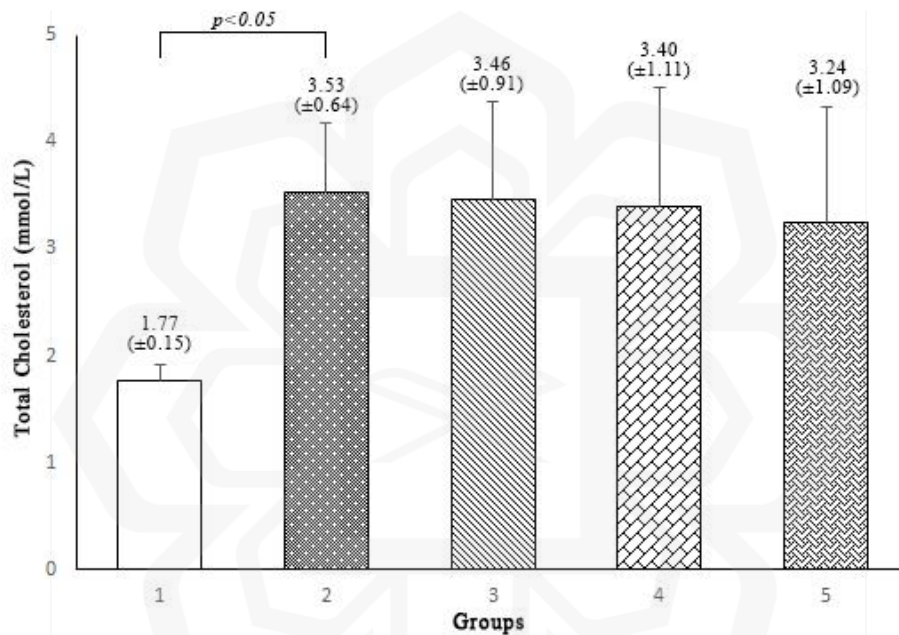


Figure 4.23 Serum total cholesterol levels in experimental groups

Note. 1=Control, normal diet; 2=12 % HCD only; 3=12% HCD with 1.2g/kg/day TH; 4=12% HCD with 2.4g/kg/day TH; 5=12% HCD with 3.0g/kg/day TH. Results were represented as mean (standard deviation) and analysed using ANOVA. A p-value < 0.05 was taken as statistically significant at a 95% confidence interval.

4.2.5 Serum triglyceride (TG) levels

Figure 4.24 shows the serum TG levels in each of the experimental groups. The mean serum TG was significantly increased ($p < 0.01$) in 12% HCD group (Group 2) as compared to the control group (Group 1). The groups with TH supplementation demonstrated a decreasing trend of the mean TG levels. The TG levels were significantly lower in all TH treatment groups than the 12% HCD (Group 2), $p < 0.05$.

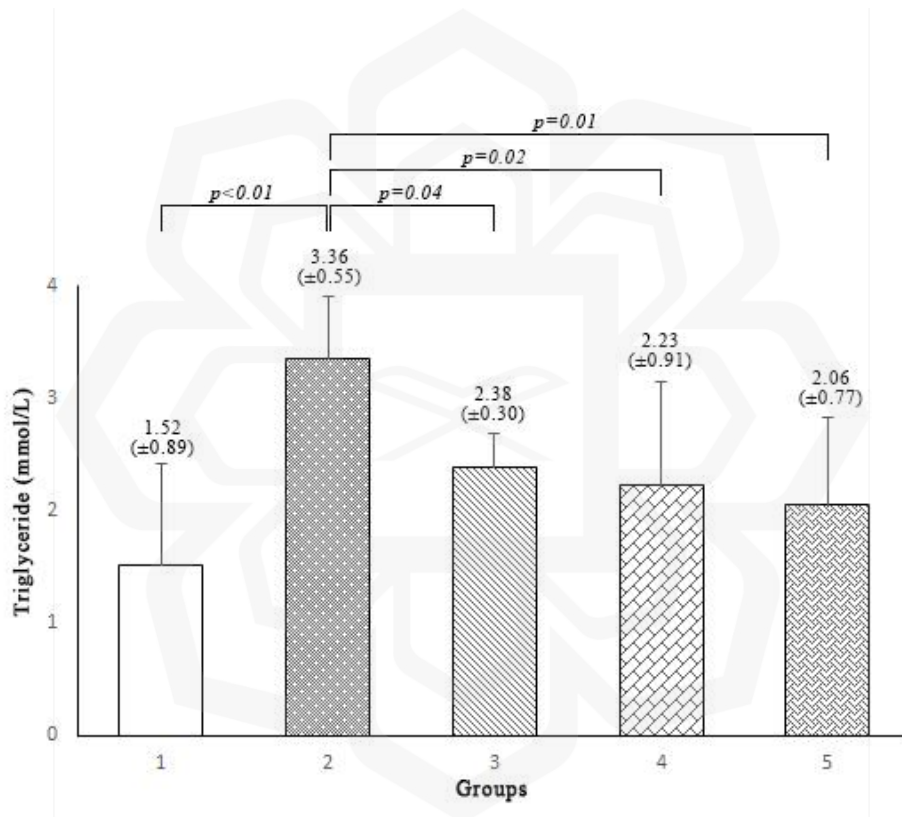


Figure 4.24 Serum total triglyceride levels in experimental groups.

Note. 1=Control, normal diet; 2=12 % HCD only; 3=12% HCD with 1.2g/kg/day TH; 4=12% HCD with 2.4g/kg/day TH; 5=12% HCD with 3.0g/kg/day TH. Results were represented as mean (standard deviation) and analysed using ANOVA. A p-value < 0.05 was taken as statistically significant at a 95% confidence interval.

4.2.6 Serum high-density lipoprotein cholesterol (HDL) levels

Figure 4.25 shows the serum HDL-c levels in each of the experimental groups. There were no significant differences in serum HDL-c levels among the experimental groups.

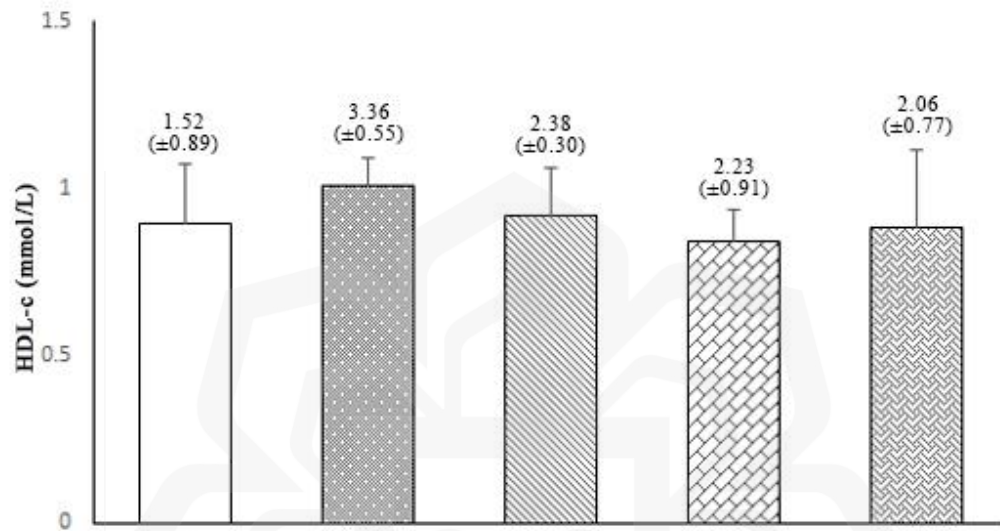


Figure 4.25 Serum HDL-c levels in experimental groups.

Note. 1=Control, normal diet; 2=12 % HCD only; 3=12% HCD with 1.2g/kg/day TH; 4=12% HCD with 2.4g/kg/day TH; 5=12% HCD with 3.0g/kg/day TH. Results were represented as mean (standard deviation) and analysed using ANOVA. A p-value <0.05 was taken as statistically significant at a 95% confidence interval.

4.2.7 Serum low-density lipoprotein cholesterol (LDL-c) levels

Figure 4.26 shows the serum LDL-c levels in each of the experimental groups. There were no significant differences in serum LDL-c levels among the experimental groups.

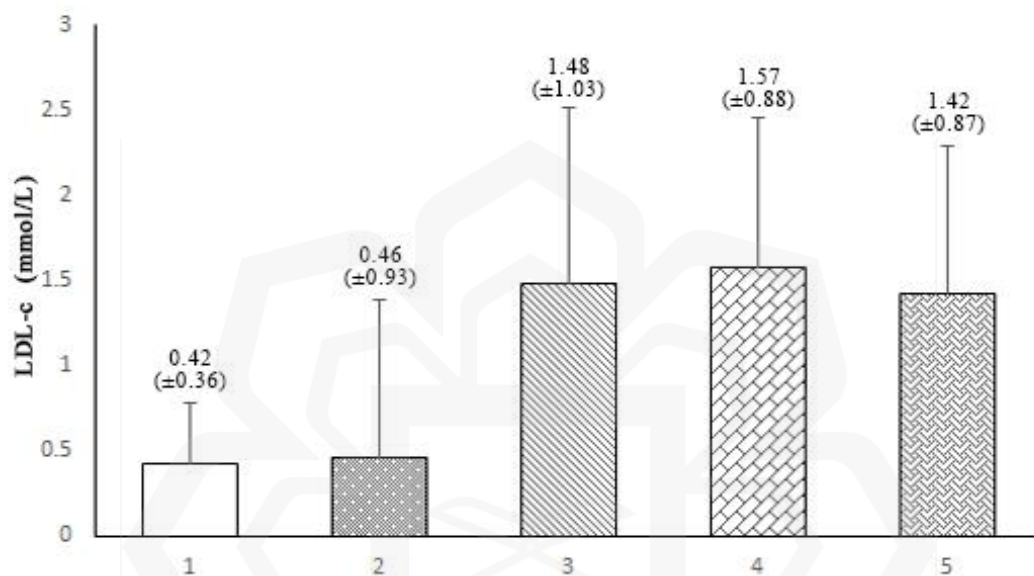


Figure 4.26 Serum LDL-c levels in experimental groups.

Note. 1=Control, normal diet; 2=12 % HCD only; 3=12% HCD with 1.2g/kg/day TH; 4=12% HCD with 2.4g/kg/day TH; 5=12% HCD with 3.0g/kg/day TH. Results were represented as mean (standard deviation) and analysed using ANOVA. A p-value <0.05 was taken as statistically significant at a 95% confidence interval.

4.2.8 Fasting insulin levels

Figure 4.27 shows the serum fasting insulin levels in each of the experimental groups. There were no significant differences in serum fasting insulin levels among the experimental groups.

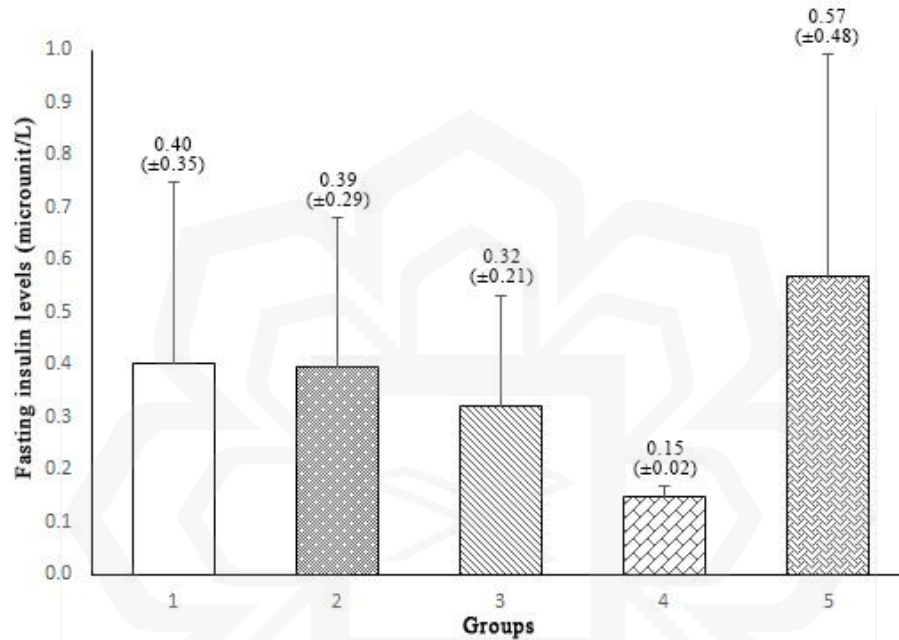


Figure 4.27 Fasting insulin levels in experimental groups.

Note. 1=Control, normal diet; 2=12 % HCD only; 3=12% HCD with 1.2g/kg/day TH; 4=12% HCD with 2.4g/kg/day TH; 5=12% HCD with 3.0g/kg/day TH. Results were represented as mean (standard deviation) and analysed using ANOVA. A p-value <0.05 was taken as statistically significant at a 95% confidence interval.

4.2.9 Fasting glucose levels

Figure 4.28 shows the fasting glucose levels in each of the experimental groups. There were no significant differences in serum fasting glucose levels among the experimental groups.

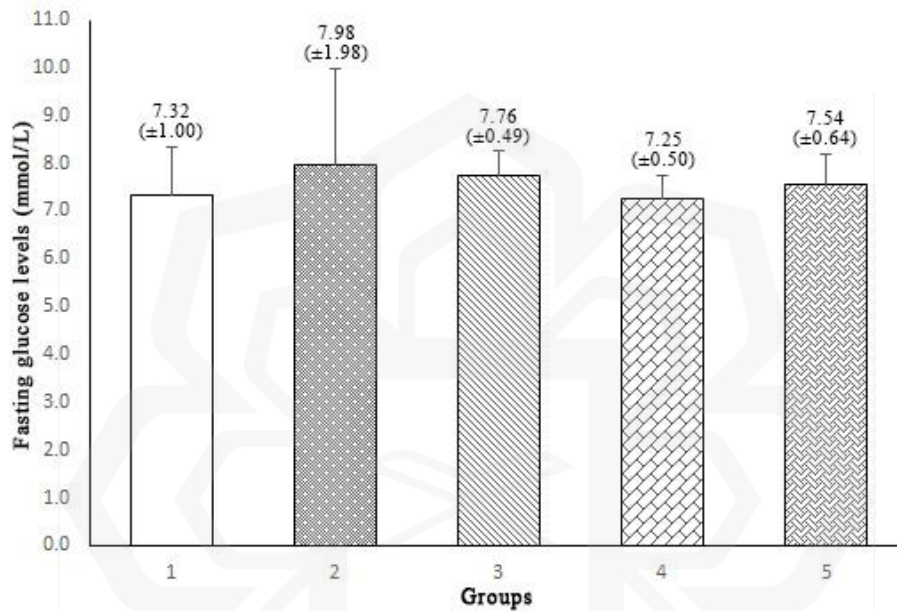


Figure 4.28 Fasting glucose levels in experimental groups.

Note. 1=Control, normal diet; 2=12 % HCD only; 3=12% HCD with 1.2g/kg/day TH; 4=12% HCD with 2.4g/kg/day TH; 5=12% HCD with 3.0g/kg/day TH. Results were represented as mean (standard deviation) and analysed using ANOVA. A p-value <0.05 was taken as statistically significant at a 95% confidence interval.

4.2.10 Homeostatic Model Assessment for Insulin Resistance (HOMA-IR)

Figure 4.29 shows HOMA-IR value in each of the experimental groups. There were no significant differences in HOMA-IR levels among the experimental group.

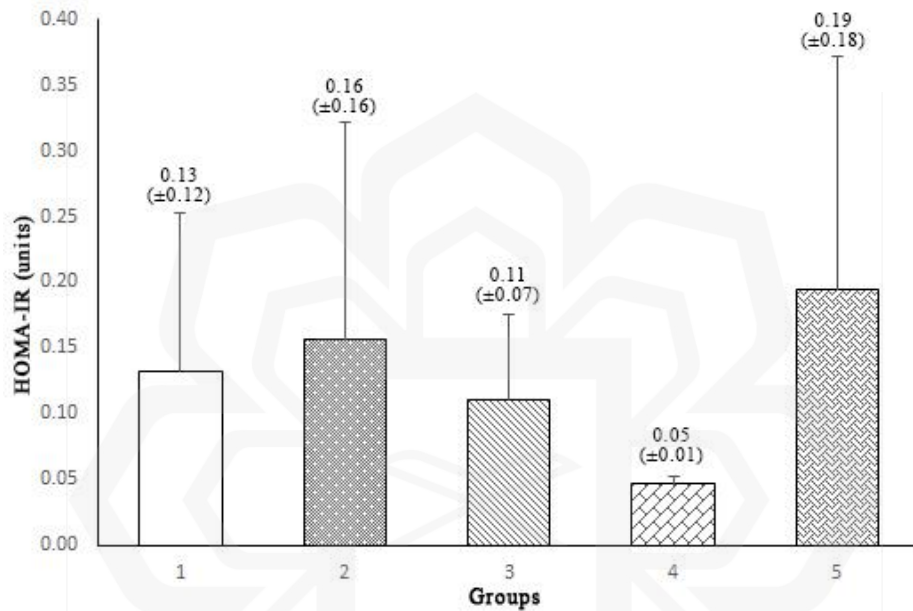


Figure 4.29 HOMA-IR values in experimental groups.

Note. 1=Control, normal diet; 2=12 % HCD only; 3=12% HCD with 1.2g/kg/day TH; 4=12% HCD with 2.4g/kg/day TH; 5=12% HCD with 3.0g/kg/day TH. Results were represented as mean (standard deviation) and analysed using ANOVA. A p-value <0.05 was taken as statistically significant at a 95% confidence interval.

4.3 IMMUNOHISTOCHEMISTRY ANALYSIS

4.3.1 Terminal deoxynucleotidyl transferase dUTP Nick-End Labelling (TUNEL) assay

The TUNEL assay was used to investigate the presence of apoptotic cells in the liver of experimental animals. Figure 4.30 shows the mean areas with TUNEL-positive staining relative to tissue area (percentage) in each experimental group. The percentage of TUNEL-positive staining was significantly increased in 12% HCD only group (Group 2) compared to normal control (Group 1) ($p<0.01$) and significantly reduced in Group 4 ($p<0.05$). Figure 4.31 illustrates a significant reduction of apoptotic cells noted in Group 4 compared to Group 2, however TUNEL-positive staining appeared to increase in Group 5 compared to Group 4 (Figure 4.31).

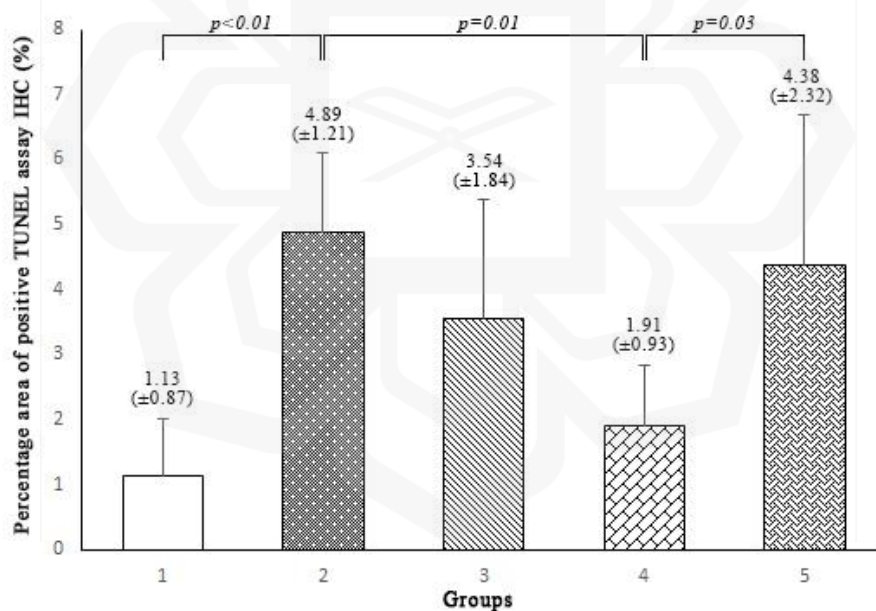


Figure 4.30 TUNEL assay percentage area of positive staining relative to tissue area.

Note. 1=Control, normal diet; 2=12 % HCD only; 3=12% HCD with 1.2g/kg/day TH; 4=12% HCD with 2.4g/kg/day TH; 5=12% HCD with 3.0g/kg/day TH. Results were represented as mean (standard deviation) and analysed using ANOVA. A p-value <0.05 was taken as statistically significant at a 95% confidence interval.

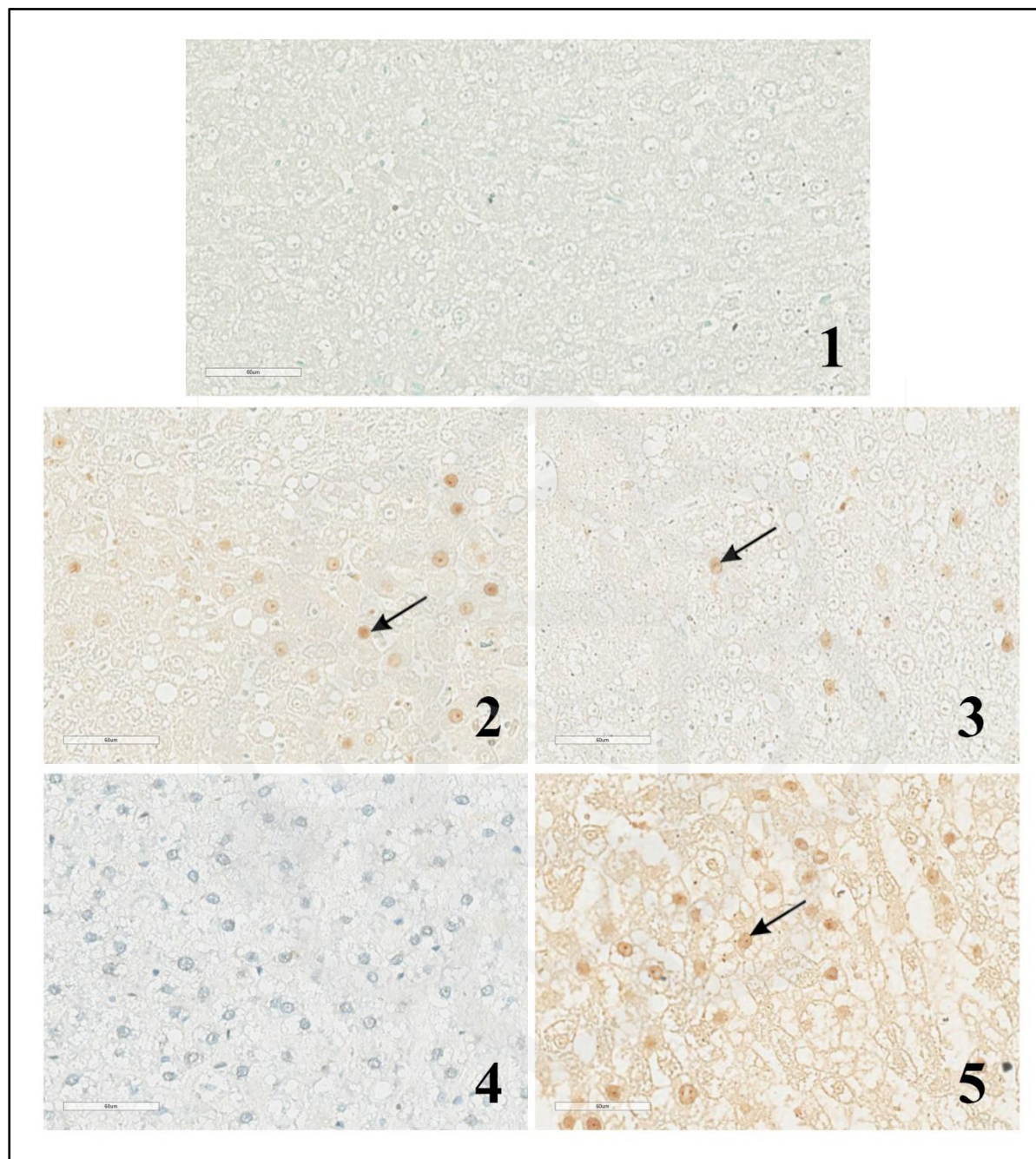


Figure 4.31 TUNEL assay of a representative liver in experimental groups. 1=Control, normal diet; 2=12% HCD only; 3=12% HCD with 1.2g/kg/day TH; 4=12% HCD with 2.4g/kg/day TH; 5=12% HCD with 3.0g/kg/day TH.

4.3.2 Alpha Smooth Muscle Actin (α -SMA) staining

The α -SMA staining was used to assess the percentage of alpha-smooth muscle actin-positive hepatic stellate cells (activated hepatic stellate cells). Figure 4.32 shows a significant increase of the percentage of the positive α -SMA staining in Group 2 ($p<0.01$) compared to normal control and the percentage significantly reduced in Group 3 ($p=0.01$), and the lowest in Group 4 ($p<0.01$). The positive α -SMA staining was significantly increased in Group 5 compared to Group 4 ($p<0.01$). Figure 4.34 demonstrates the corresponding changes in areas of the activated stellate cells across the experimental groups.

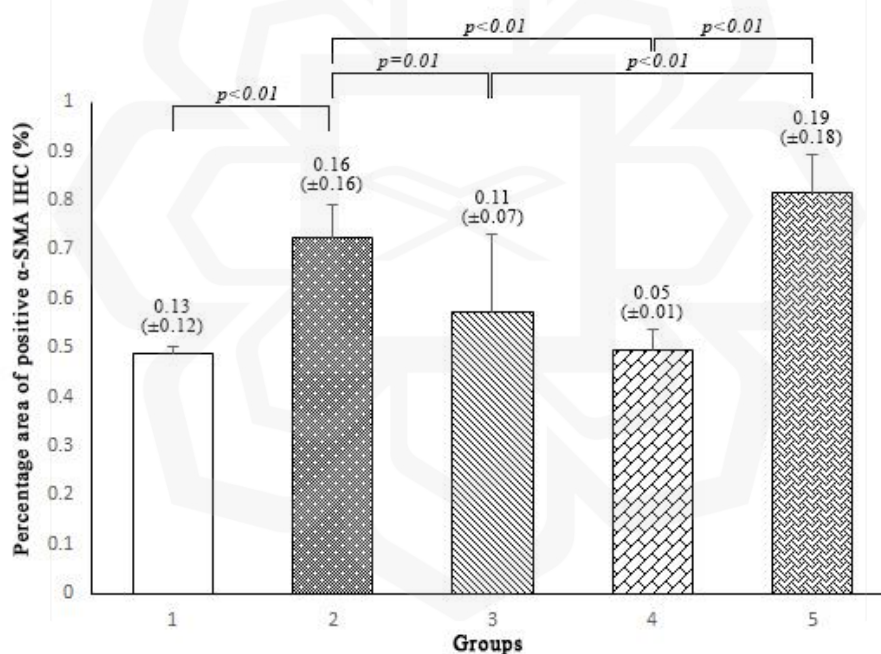


Figure 4.32 α -SMA percentage area of positive staining relative to tissue area.

Note. 1=Control, normal diet; 2=12 % HCD only; 3=12% HCD with 1.2g/kg/day TH; 4=12% HCD with 2.4g/kg/day TH; 5=12% HCD with 3.0g/kg/day TH. Results were represented as mean (standard deviation) and analysed using ANOVA. A p-value <0.05 was taken as statistically significant at a 95% confidence interval.

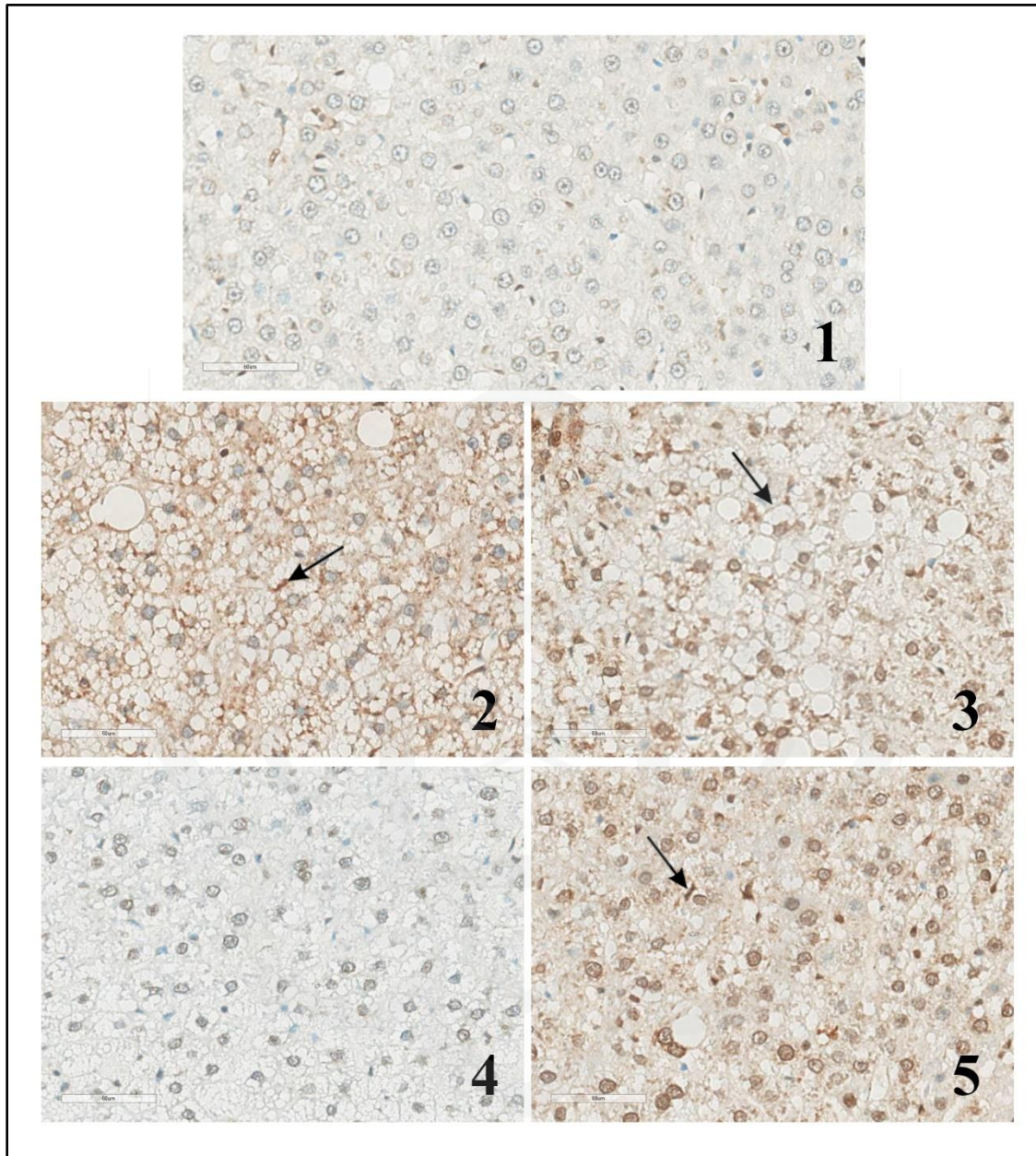


Figure 4.33 Immunohistochemistry staining for a α -SMA in a representative liver section in experimental groups. 1=Control, normal diet; 2=12% HCD control, 12% HCD only; 3=12% HCD with 1.2g/kg/day TH; 4=12% HCD with 2.4g/kg/day TH; 5=12% HCD with 3.0g/kg/day TH.

4.4 GENES PROFILING AND PATHWAY ANALYSIS

4.4.1 Differentially expressed genes

In this study, microarray analysis of RNA extracted from the rats was performed to compare gene expression profiles between animals in the HCD control group (group 2) and TH treated group (group 3, 4 and 5). Figure 4.34 demonstrates a volcano plot that provides a visual representation of gene expression changes in the TH-treated group. Positive values of the x-axis indicate upregulated genes, while negative values of the x-axis indicate downregulation. This study identified a total of 3062 differentially expressed genes (DEGs), including 2788 upregulated and 274 downregulated genes in TH-treated groups compared to the normal control.

Table 4.1 listed the 10 most significantly upregulated genes while Table 4.2 listed the 10 most significantly downregulated genes in TH treated group compared to the controls. These genes mainly are associated with pathways involved in lipid metabolism, immune response, oxidative stress, and cell apoptotic regulation.

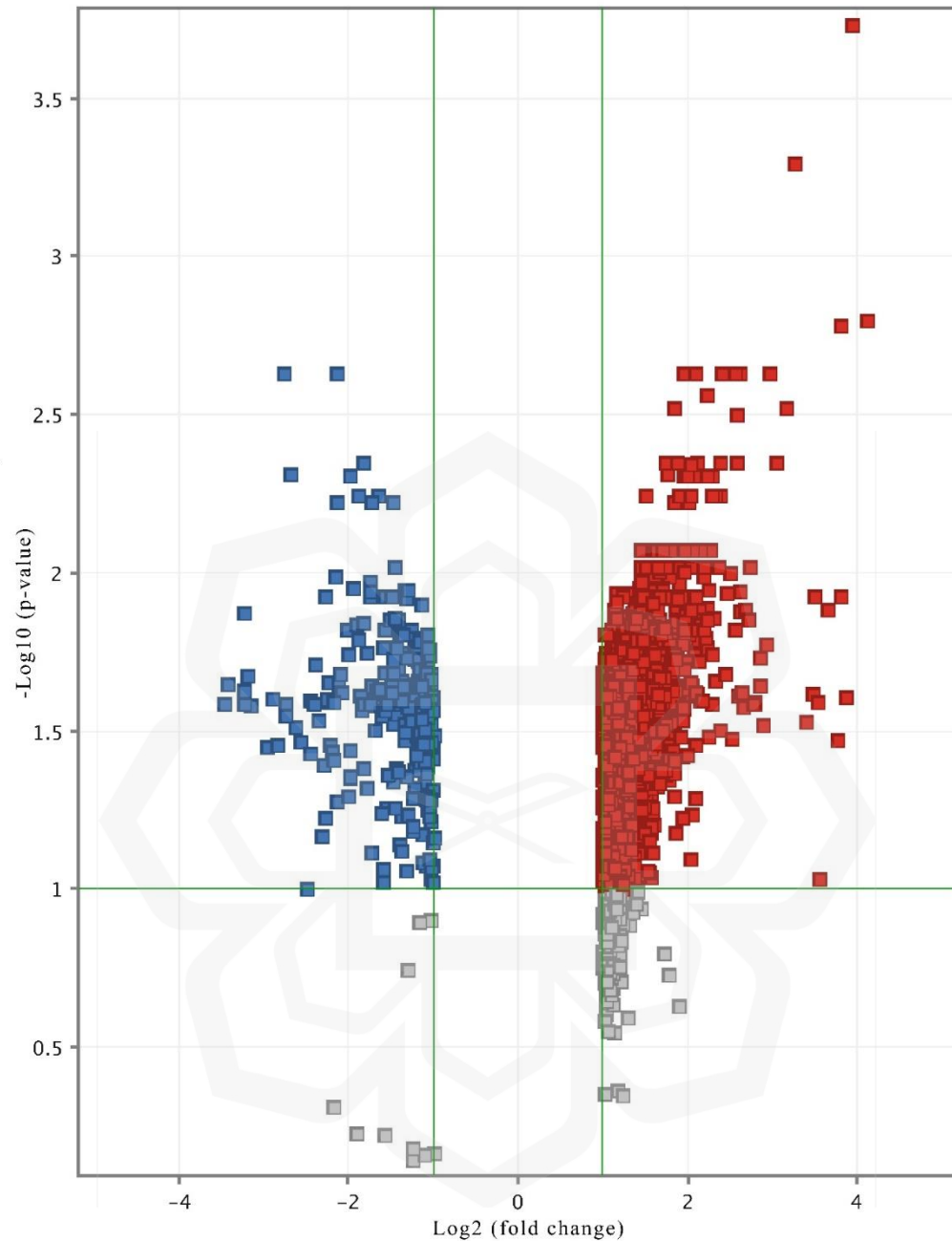


Figure 4.34 Volcano plot showing the differential expression of genes. The x-axis represents the log2 fold change in expression, and the y-axis shows the $-\log_{10}$ of the p-value. Red points represent significantly upregulated genes ($\log_2$ fold change $> X$ and p-value $< Y$), while blue points represent significantly downregulated genes ($\log_2$ fold change $< -X$ and p-value $< Y$). Grey points indicate genes that are not significantly differentially expressed. The green lines denote the thresholds for statistical significance in both p-value and fold change.

Table 4.1 The genes that were significantly upregulated in the animals treated with Tualang Honey compared to the controls.

No	Symbol	Gene name	Functions	Associated pathway	Fold change	p-value
1	<i>Aldh1a7</i>	Aldehyde dehydrogenase family 1, subfamily A7	Detoxifies aldehydes, critical for lipid embolism and cellular defence.	Lipid metabolism, oxidative stress response	122.2866	0.001
2	<i>Sqle</i>	Squalene epoxidase (Sqle)	Catalyses the first oxygenation step in sterol biosynthesis.	Cholesterol metabolic process	17.3504	0.05
3	<i>Duoxa1</i>	Dual oxidase maturation factor 1	Enable enzyme binding activity	Hydrogen peroxides metabolic process	14.7908	<0.05
4	<i>Syne4</i>	Spectrin repeat containing, nuclear envelope family member 4	Establishment of epithelial cell apical/basal activity, active in nuclear outer membrane.	Epithelial cell apical/basal polarity.	11.5368	<0.05
5	<i>Cyp4a2</i>	Cytochrome P450, family 4, subfamily a, polypeptide 2	Catalyses omega-1-hydroxylation; regulates fatty acid oxidation	Arachidonate metabolic process (a straight chain fatty acid)	13.9621	0.01
6	<i>Duox1</i>	Dual oxidase 1	Enable superoxide-generating NAD(P)H oxidase activity.	Reactive oxygen species metabolic process	12.4357	0.01
7	<i>Rgs16</i>	Regulator of G-protein signalling 16	Enable GTPase activator activity.	G protein couples receptor signalling pathway	10.6958	<0.05
8	<i>Cd209e</i>	CD209 antigen-like protein E	Recognizes and binds pathogens; modulates immune response	Regulation of T cells proliferation	11.0469	<0.05

9	<i>Nfx1</i>	Nuclear transcription factor, X-box binding 1	Enable DNS-binding transcription repressor activity.	Negative regulation of transcription by RNA polymerase II	14.5511	<0.05
10	<i>Fam111a</i>	FAM111 trypsin like peptidase A	Single-stranded DNA-binding serine protease that mediates the proteolytic cleavage of covalent.	DNA damage response	10.4274	<0.05

Note. List of the top 10 upregulated genes identified from the microarray analysis in the Tualang Honey-treated groups compared to controls. Fold changes represent the relative expression levels and P-values were calculated using the Limma package, with significance adjusted using the Benjamini-Hochberg method to control the false discovery rate. These genes are associated with pathways involved in lipid metabolism, immune response and oxidative stress.

Table 4.2 The genes that were significantly downregulated in the animals given Tualang Honey compared to the controls

No	Symbol	Gene name	Functions	Associated pathway	Fold change	P value
1	<i>Nid2</i>	Nidogen 2	Enable calcium ion binding activity.	Cell matrix adhesion	-10.3357	<0.05
2	<i>Apln</i>	Apelin	Endogenous ligand for the apelin receptor.	Apelin receptor signalling pathway	-10.6917	<0.05
3	<i>Irf2bp2</i>	Interferon regulatory factor 2 binding protein 2	Enable transcription corepressor activity	Immature B cell differentiation/immune response	-13.5409	<0.05
4	<i>Msl3</i>	Male-specific lethal 3 homolog	Enable DNA/methylated histone binding activity,	Positive regulation of DNA templated transcription	-10.0083	<0.05

5	<i>Cops9</i>	COP9 signalosome subunit 9	Cytoplasmic sequestering of protein	Positive regulation of cell population proliferation	-10.4501	<0.05
6	<i>Tubb2a</i>	Tubulin beta 2A class IIa	Forms beta tubulin heterodimers (microtubulin)	Phagocytosis pathway	-13.6747	<0.05
7	<i>Scd2</i>	Stearoyl-coenzyme A desaturase 2	Enable stearoyl-CoA 9 desaturase activity.	Fatty acid biosynthesis process	-10.3719	<0.05
8	<i>Hif1a</i>	Hypoxia inducible factor 1 alpha subunit	Enable DNA-binding transcription activator activity	Upstream of apoptotic process	-11.4394	<0.05
9	<i>Limk2</i>	LIM domain kinase 2	Enable AMP-activated protein kinase activity	Actin cytoskeleton organization	-15.6049	<0.05
10	<i>Hlf</i>	HLF transcription factor PAR bZIP family member	Enable DNA-binding transcription activator activity.	Positive regulation of transcription by RNA polymerase II	-11.1717	<0.05

Note. List of the top 10 downregulated genes identified from the microarray analysis in the Tualang Honey-treated groups compared to controls. Fold changes represent the relative expression levels and P-values were calculated using the Limma package, with significance adjusted using the Benjamini-Hochberg method to control the false discovery rate. These genes are associated with pathways involved in lipid metabolism, immune response and cell apoptotic regulation.

4.4.2 Pathway analysis

Following pathway enrichment analysis, 33 biological processes were significantly modified ($p < 0.05$) in the TH-treated group. Table 4.4 demonstrates the 10 most significantly modified biological pathways. Figure 4.34 shows that among the molecular pathways implicated in the effects of TH, cholesterol biosynthesis, and metabolism pathways are the most substantial molecular mechanisms involved in the hepatoprotective property of TH.

Table 4.3 Pathway analysis comparing the animals' continued high cholesterol diet given Tualang Honey and the normal controls.

No	Pathway name	Category	P value	-Log10 p-value
1	Biogenic Amine Synthesis	Antioxidant and inflammatory mechanism	0.012	1.918420806
2	Alanine and aspartate metabolism		0.035	1.451003191
3	Signalling Pathway		0.044	1.349497506
4	Cholesterol Biosynthesis	Lipid and steroid metabolism	2.11E^{-14}	13.67476095
5	Cholesterol metabolism		2.85E^{-10}	9.545573132
6	Steroid Biosynthesis		0.002	2.621756219
7	Beta Oxidation of Unsaturated Fatty Acids		0.049	1.302519049
8	Glycolysis and Gluconeogenesis	Glucose and energy metabolism	0.013	1.886019801
9	Fatty Acid Omega Oxidation		0.027	1.555613761
10	Insulin Signalling		0.034	1.461653815

Note. The value of the base 10 logarithm of the p-value is always negative because the p-value takes values between 0 and 1. For this reason, for the representation of positive values on the y axis, the value of the logarithm minus ($-\log_{10}(\text{p-value})$).

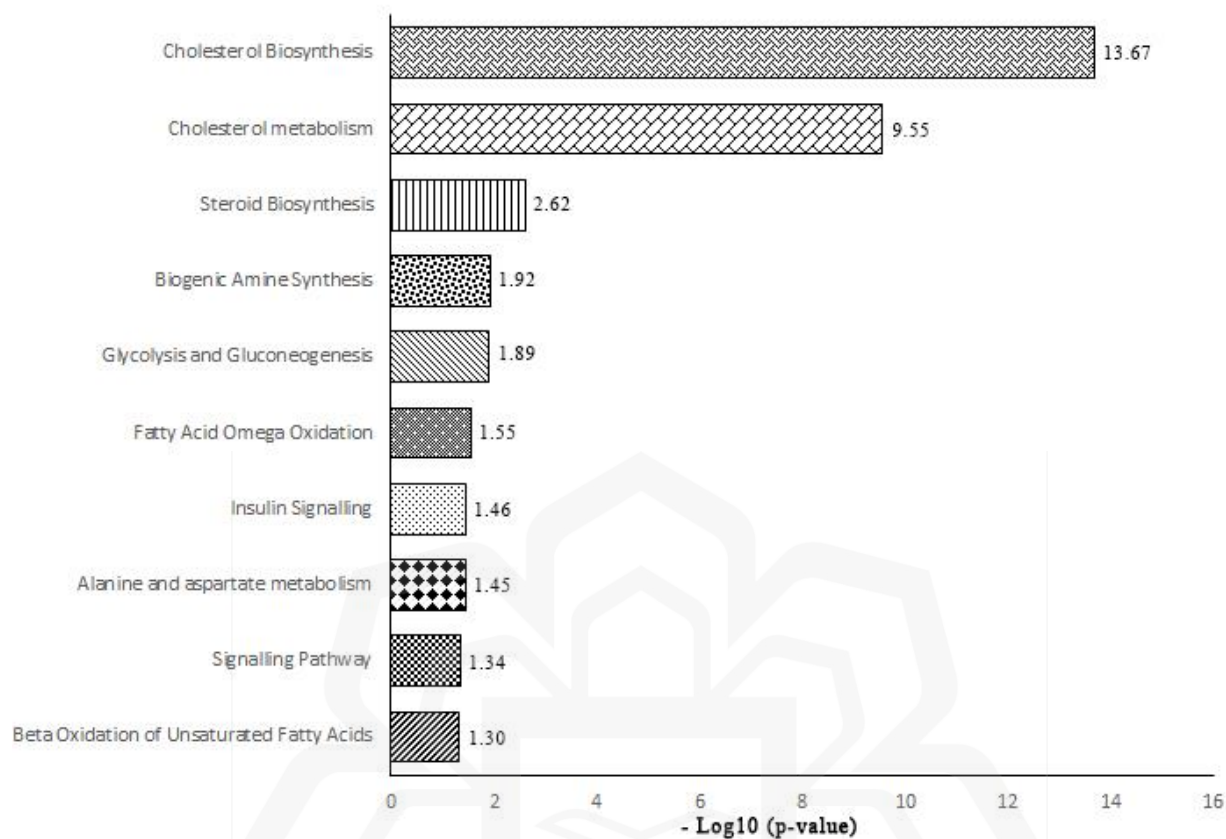


Figure 4.35 Pathway enrichment analysis of differentially expressed genes. The bar chart displays the top enriched pathways based on $-\log_{10}(\text{p-value})$, indicating the statistical significance of pathway enrichment. Pathways with higher $-\log_{10}(\text{p-value})$ values are more significantly enriched. Patterns in the bars represent different pathways for clear visualization.

4.5 VALIDATION OF SELECTED GENES EXPRESSION

The qRT-PCR validation confirmed the differential expression of *Aldh* (upregulated) and *Irf2* (downregulated) as identified in the microarray analysis, with expression levels quantified relative to the reference (housekeeping) gene 18s ribosomal RNA (*Rn18s*).

4.5.1 Aldehyde dehydrogenase (*Aldh*) gene

Table 4.4 demonstrates the expression of the *Aldh* gene was upregulated (relative to *Rn18s*) in the 12% HCD group (1.731×10^6 folds) compared to the normal control. *Aldh* gene expression is also seen to be significantly increased in Group 4 (2.4g/kg/day dose of TH) with 2.471×10^7 fold change and then reduced in Group 5 (3 g/kg/day dose of TH) with 2.486×10^4 fold change.

Table 4.4 Aldehyde dehydrogenase (*Aldh*) gene expression levels in all groups measured using SYBR-green PCR.

Group	Mean quantity of reference gene (18s)	Mean quantity of Target gene	Normalized target	Fold - Upregulated (Log10)	P-value
Group 1 (Control)	0.002	1.621E^{-5}	6.767E^{-3}	1.000 (0)	-
Group 2 HCD (no TH)	0.177	2.084E^3	1.171E^4	1.731E^6 (6.238)	$p < 0.01$
Group 4 HCD + 2.4 g	0.038	6.381E^3	1.672E^5	2.471E^7 (7.393)	$p < 0.01$
Group 5 HCD + 3.0 g	0.006	1.147	1.682E^2	2.486E^4 (4.396)	$p < 0.01$

Note. Normalized target (Mean q target gene/mean q reference gene); Fold (Normalized target each group/normalized target control). Group 3 (12% HCD + 1.2 g/kg/day TH) was not included as its result was inconclusive.

4.5.2 Interferon regulatory factor 2 gene

The *Irf2* gene exhibited significant downregulation in the 12% HCD group (Group 2) compared to the reference gene (5.916×10^{-1} folds). This suppression was partially reversed in animals treated with 2.4 g/kg/day of TH (2.63×10^{-5} folds) while further reduction in the expression was observed at the high dose of 3g/kg/day with 1.17×10^{-5} fold change, as shown in Table 4.5. Figure 4.36 is the visual representation of gene expression changes across the groups.

Table 4.5 Interferon Regulatory Factor 2 (IRF2) gene expression levels in all groups measured using SYBR-green PCR.

Group	Mean quantity of reference gene (18s)	Mean quantity of Target gene	Normalized target	Fold – Downregulated (Log 10)	P-value
Group 1 (Control)	0.002	3.227E^{-05}	1.345E^{-2}	1.000 (0)	-
Group 2 HCD (no TH)	0.177	1.415E^{-3}	7.957E^{-3}	5.916E^{-1} (0.228)	$p < 0.01$
Group 4 HCD + 2.4 g	0.038	4.496E^{-5}	5.554E^{-3}	8.759E^{-2} (1.058)	$p < 0.01$
Group 5 HCD + 3.0 g	0.006	4.871E^{-2}	0.714E^{-1}	1.843E^{-1} (0.734)	$p < 0.01$

Note. Normalized target (Mean q target gene/mean q reference gene); Fold (Normalized target each group/normalized target control). Group 3 (12% HCD + 1.2 g/kg/day TH) was not included as its result was inconclusive.

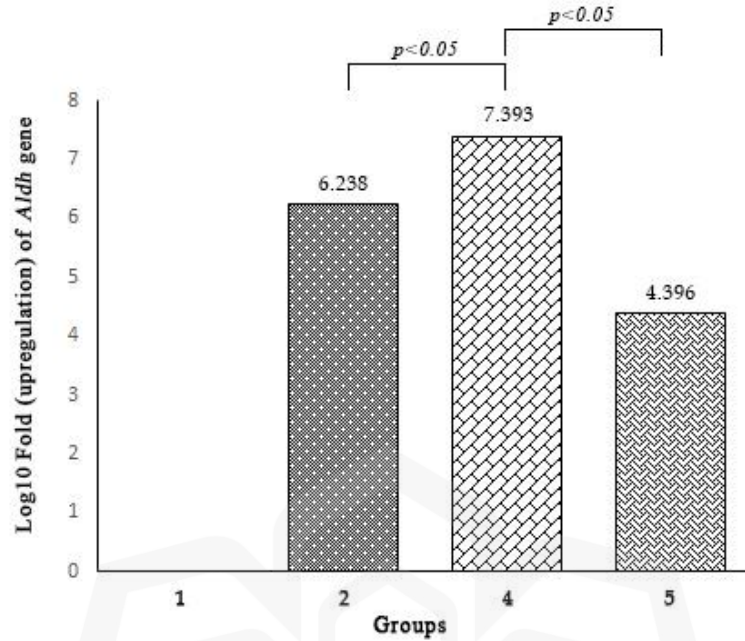
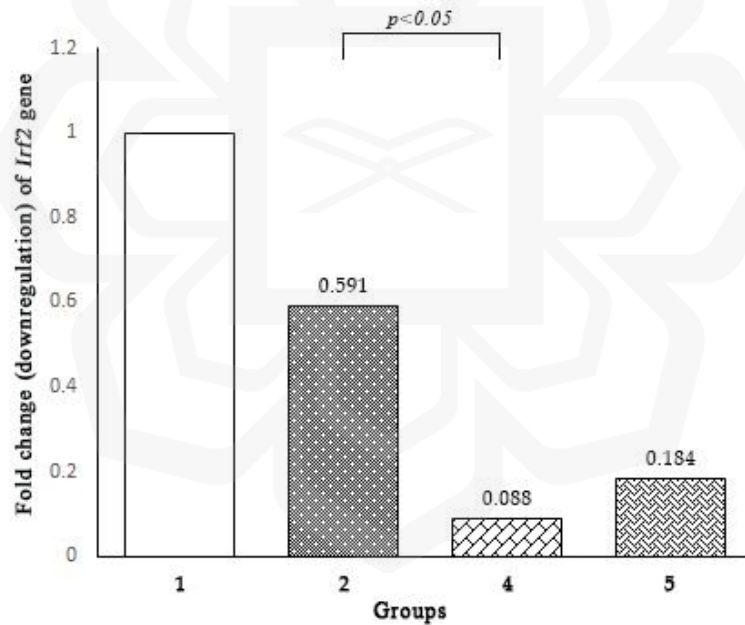
A**B**

Figure 4.36 Gene expression changes in the experimental groups. A: Log10 fold change of the upregulation of the *Aldh* gene expression. B: Fold change (normalised target expression) of the downregulation of the *Irf2* gene expression.

Note. The y-axis for each chart was chosen to better represent the changes seen between the groups. Group 1 for both charts is the reference point for both gene expression comparisons. A p-value < 0.05 was taken as statistically significant at a 95% confidence interval.

CHAPTER FIVE

DISCUSSION

5.1 OVERVIEW OF THE STUDY FINDINGS

This study investigated the effects of TH supplementation in the 12% HCD-induced NASH rat model. The evaluation included an assessment of the liver relative weight, gross liver morphology, histological structures, biochemical profiles, and the immunohistochemical staining of α -SMA protein for evidence of hepatic stellate cells activation and expression of apoptosis markers. Additionally, this study explored the molecular mechanisms underlying the effects of TH by analysing gene expression modifications, significantly associated pathways and validation of selected genes' expression. In this study, twenty-five male Sprague-Dawley rats were randomly assigned to five groups. The first group (normal control) was fed standard rat pellets for 20 weeks. The remaining four groups were fed a 12% HCD for 16 weeks to induce NASH, followed by either continued 12% HCD only (Group 2) or 12% HCD combined with TH supplementation at doses of 1.2 g/kg/day (Group 3), 2.4 g/kg/day (Group 4), or 3.0 g/kg/day (Group 5) for four weeks.

This study demonstrated the establishment of a mild NASH rats model using a 12% HCD administered over 20 weeks. This model was characterised by the significant increase in relative liver weight and histological evidence of steatosis, hepatic ballooning degeneration and inflammation of hepatocytes. There were also significant elevations of total cholesterol and triglycerides, in the animals given 12% HCD compared to normal controls. Immunohistochemistry analysis revealed increased α -SMA protein expression and percentage of TUNEL-positive area compared to control.

Supplementation with TH at various doses over four weeks yielded several key findings. Notably, a four-week TH supplementation at 1.2 g/kg/day, 2.4 g/kg/day and 3.0 g/kg/day significantly reduced total triglyceride levels in the respective TH-treated group. At the cellular level, TH supplementation markedly reduced the percentage area of TUNEL-assay positive at the moderate dose of 2.4 g/kg/day, indicating the reduction of apoptotic cells. Meanwhile, TH supplementation significantly reduced the percentage area of α -SMA staining at 1.2 g/kg/day and 2.4/kg/day, associated with the reduction of activated hepatic stellate cells compared to the 12% HCD group. Conversely, a high dose of TH supplementation 3.0 g/kg/day in group 5 significantly increased the percentage area of TUNEL-positive and α -SMA staining.

Microarray analysis revealed the 10 most significant upregulated and downregulated genes in the TH-treated animals were mainly associated with antioxidant, immune response, lipid metabolism, and cellular apoptotic pathways. Furthermore, pathway enrichment analysis of differentially expressed genes revealed cholesterol biosynthesis and cholesterol metabolism pathways are the most substantial molecular mechanisms involved in the hepatoprotective property of TH. Following validation by qPCR, the aldehyde dehydrogenase (*Aldh*) gene demonstrated significantly higher upregulation, whilst interferon regulatory factor 2 (*Irf2*) expression exhibited significantly reduced suppression in the animals given 2.4 g/kg/day of TH compared to 12% HCD, reflecting increased antioxidant and anti-inflammatory responses.

The key findings of this study suggest that a moderate dose of 2.4 g/kg/day appears as the optimal dosage for the hepatoprotective effects and therapeutic potential of TH. Molecular analysis indicates lipid and cholesterol regulation, along with antioxidant and immune modulation, are the primary molecular mechanisms underlying the mitigation of the NASH animal model.

5.1 COMPARISON OF RELATIVE LIVER WEIGHT, LIVER GROSS MORPHOLOGY AND HISTOLOGICAL CHANGES

5.1.1 Effects of TH Supplementation on Relative Liver Weight

In this study, we demonstrated a significant increase in the relative liver weight of rats which were fed a 12% HCD compared to the normal control group, suggesting the development of fatty liver as a result of high cholesterol intake. The relative liver weight is commonly used to indicate hepatic function and potential pathological changes in response to diet and interventions (Basalai et al., 2022). The present finding is consistent with several studies that have reported that HCD induces hepatomegaly due to lipid accumulation, steatosis, and inflammation (Shinohata et al., 2023; S. Zhang et al., 2021). Similarly, Rahim et al. (2019) revealed a significant increase in relative liver weight in the 12% HCD rats, attributing this to free cholesterol overload, resulting in hepatocyte steatosis, inflammation, and ultimately hepatomegaly.

A study by Pivtorak et al. (2022) that was investigating NASH induction in rats through a high-cholesterol diet reported an increase in relative liver weight up to 12.15% after 60 days and 15.12% after 90 days. The author proposed a novel theory suggesting that lipotoxicity acts as the primary trigger for hepatocyte dysfunction, ultimately driving NASH progression. This theory postulates that lipotoxic damage occurs concurrently with the formation of lipotoxic metabolites, which play a crucial role in liver disease progression and the development of insulin resistance.

5.1.2 Effects of TH supplementation on gross morphology and histology of the liver

In this study also, we demonstrated some changes in the liver gross morphology, liver histology structures and NAFLD activity score (NAS) across the experimental groups. The gross morphology

of rat livers that were fed a normal diet (Group 1) displayed a normal feature of healthy liver, a shiny smooth surface and reddish-brown in colour. Consistently, the microscopic histological findings of the normal liver in this study demonstrated tightly packed pink-staining hepatocytes with a normal liver architecture. The normal histology structure of the liver in rats include hepatocytes, biliary cells, Kupffer cells and stellate cells, along with less common cells like mast cells, lymphocytes and myeloid cells, all organised around the liver's vascular architecture (Masserdotti, 2023). Meanwhile the NAFLD activity score for the liver in group 1 was 0, which indicates the absence of steatosis, inflammation and fibrosis.

In contrast, rats on a 12% HCD (Group 2) exhibited significant adverse changes in liver morphology, including increased liver size and a yellowish discoloration. These changes align with literature findings on the hepatotoxic effects of a high-fat and high-cholesterol diet, which induce fat deposition and inflammation in the liver tissue (Alfarisi et al., 2020; Arab et al., 2018). Consistently, in this study, the 12% HCD group also exhibited marked microscopic histological changes, including the presence of steatosis (microvesicular and macrovesicular), inflammatory cell infiltration and hepatocyte ballooning degeneration which are hallmark features of NASH. These findings align with the observations of Hassan et al. (2018), who reported hepatocyte swelling, lipid vacuolisation, and immune cell infiltration in rodents fed a HCD, attributing these changes to cholesterol-induced oxidative stress and lipid peroxidation. Wang et al. (2020) further described that HCD leads to free cholesterol accumulation in hepatocytes, triggering endoplasmic reticulum stress and inflammation, as corroborated by this current study. The present findings are also in line with another research by Ichimura-Shimizu et al. (2022) that suggests such histological changes are representative of NASH.

Looking into the NAFLD activity score (NAS), the animals given a 12% HCD-only (Group 2) had a score of 2.8. The NAS is applied to assess the activity of NASH after the histologic diagnosis of NASH has been established as it quantifies the severity of liver inflammation and damage (Chandran et al., 2022). An elevation in the NAFLD activity score in the HCD animals suggests liver damage, consistent with the observed histological features, with the presence of steatosis, inflammation, and hepatocellular ballooning in the HCD group (Gao et al., 2023; Zhang et al., 2023). This is in line with a recent study that reported animals fed with a high-cholesterol diet for 16 weeks exhibit significantly elevated levels of hepatic triglycerides and cholesterol, leading to steatosis and liver damage (Taberner-Cortés et al., 2024). Additionally, a high-cholesterol diet has been reported to trigger an inflammatory response characterised by increased levels of immune cells, such as the cluster of differentiation 3 (CD3+) T cells and macrophages, which exacerbate liver inflammation (Hermanson et al., 2022; Zhang et al., 2023).

Interestingly, the present study demonstrated that TH supplementation at moderate dose of 2.4 g/kg/day appeared to partially ameliorate these effects, as evidenced by a slight reduction in liver size and a less pronounced yellowish discoloration compared to the HCD group. Mild improvement of gross morphology at the moderate dose of TH supplementation in this study is consistent with previous evidence showing that TH supplementation at the dose of 2.4 g/kg/day over a 4 week period improved gross liver morphology (Ghani et al., 2016). However, in this study, TH supplementation at all doses for four weeks did not exhibit significant microscopic histological and NAFLD activity scores improvement compared to the 12% HCD group.

The lack of improvement in the microscopic histology and NAS in animals supplemented with TH in this study can be attributed to several factors, including dosage, duration of

supplementation, and the inherent characteristics of the honey itself (Taleuzzaman et al., 2020; Xiao et al., 2016). While Al Farisi et al. (2020) found significant histological improvement in the liver of NAFLD rabbits fed 1% cholesterol when treated with lower doses of honey (0.3 g/kg/day and 0.6 g/kg/day), the duration was much longer (12 weeks).

It can also be seen in this study that a higher dose was utilised (3 g/kg/day) in Group 5. However, no significant morphological, microscopic histological, and NAS improvement was observed. The liver size and its yellowish coloration remained similar to those in Group 2. The present finding suggests increasing the dose of TH beyond 2.4 g/kg/day confers no additional benefits in terms of gross liver morphology appearance. The high dose (3.0 g/kg/day) may have overwhelmed hepatic metabolic pathways, exacerbating oxidative stress or immune activation, thereby negating potential benefits. Such paradoxical effects are documented in studies where excessive antioxidant or bioactive compound administration leads to counterproductive outcomes (Nweke et al., 2022; S. Zhang et al., 2021). Consistent with the liver gross morphology findings, the present study showed no improvement in the histological findings and NAFLD activity scores at a high dose.

The contradicted findings as demonstrated in the present study suggest although honey contains bioactive compounds like flavonoids and phenolic acids, the doses used and duration of treatment in this study may have been insufficient for the TH alone to counteract severe lipid accumulation and inflammation. Wang et al. (2022) highlighted that in conditions of extensive lipid accumulation and chronic inflammation, antioxidant therapies alone might not suffice unless combined with lipid-lowering agents. Additionally, the bioavailability of these antioxidants might be limited by poor absorption or metabolic degradation. Supporting this, Sadeghi et al. (2021)

observed that while honey improved inflammatory markers and liver enzymes in NAFLD rats, it did not result in significant histological changes in liver steatosis or inflammatory damage over 120 days of supplementation, indicating that the duration of treatment, dosage, and honey composition is critical to determine the hepatoprotective outcome.

In this study, the impact of continuous HCD in animals given TH supplementation seems to override the potential benefit of TH in improving the histological features of NASH, as reflected by inconclusive gross morphology, microscopic histological changes, and non-significant improvement in NAS across the treated groups. The composition of TH, which is rich in flavonoids as an antioxidant, may require adequate dosing and sufficient treatment duration to exert its full therapeutic potential. Insufficient dosing or inadequate treatment periods might limit its effectiveness in counteracting the severe dyslipidaemia induced by a high-cholesterol diet.

5.2 COMPARISON OF THE BIOCHEMICAL PROFILES

5.2.1 Effects of TH supplementation on the lipid profiles

In this study, we demonstrated that a 12% HCD increased total cholesterol and triglyceride levels in the NASH rat model compared to normal controls. Similarly, Ali et al. (2022) documented that a HCD for 10 weeks significantly increased total cholesterol and triglycerides levels in the rat model, indicating a hypercholesterolaemic state. This is due to the high saturated fat content of the diet, promoting lipid accumulation which disrupts lipid metabolism (Amirabadizadeh et al., 2017). HCD also influences the expression of key lipid metabolism genes, such as *Hydroxymethylglutaryl-CoA* (HMG-CoA) *reductase* and *Sterol regulatory element binding transcription factor 2* (SREBP-2), further exacerbating hyperlipidaemias (Wang et al., 2015).

The present study demonstrated TH supplementation at low (1.2 g/kg/day), moderate (2.4 g/kg/day), and high (3 g/kg/day) doses significantly reduced triglycerides in a dose-dependent manner, compared to a 12% HCD group. A reduction in triglyceride levels has been reported in other animal studies following TH supplementation, such as those by (Ghani et al., 2016), Mohamed et al. (2018) and Hasan et al. (2022) in the same 12% HCD animal model. Supporting the present findings, Alaebo and team documented a significant reduction in triglyceride levels in diabetic male Wistar rats supplemented with honey at doses ranging from 0.2 ml/kg to 0.8 ml/kg per day over three weeks (Alaebo et al., 2022). The bioactive compounds in TH, particularly flavonoids and phenolic acids, likely play a crucial role in its antioxidant and lipid-regulating effects.

TH has been shown to exhibit the highest composition of flavonoid (504.5 mg/kg) in comparison to Gelam (461.1 mg/kg) and Kelulut honey (36.3 mg/kg), most abundantly present as

flavanol subgroup of quercetin (Mohd Kamal et al., 2021). Other subgroups of flavonoids that are also present in TH are flavanones (naringenin, pinocembrin, and hesperidin), flavones (luteolin, apigenin, and chrysin), isoflavones (genistein), and anthocyanidins. TH has been found to contain the highest levels of phenolic acids compared to other honey types like Gelam, Indian forest, and pineapple honey, with benzoic acids being the most predominant (75%) (Kishore et al., 2011; Moniruzzaman et al., 2017).

Honey has been shown to effectively reduce triglyceride levels in rats through various mechanisms, primarily involving its antioxidant properties and modulation of lipid metabolism. Honey enhances antioxidant enzyme activity, such as superoxide dismutase (SOD) and catalase (CAT), which helps mitigate oxidative stress associated with hyperlipidaemia (Olukanni et al., 2020). The antioxidant properties of honey contribute to the reduction of lipid peroxidation, thereby improving overall lipid profiles (Erejuwa et al., 2018; Olukanni et al., 2020)

Another study by Moon et al. (2017) compared the hypolipidemic effects of natural honey with the standard anti-cholesterol agent, atorvastatin, in rats with hyperlipidaemia. The study demonstrated that honey reduced total cholesterol (TCL) and triglycerides (TG) similarly to atorvastatin (Moon et al., 2017). While atorvastatin had some side effects, natural honey is considered as a safe alternative. The results and observations of the present study supports a rationale for use of natural honey in the development of a new herbal medicine much needed for the reduction of serum triglyceride levels.

5.3 IMMUNOHISTOCHEMISTRY ANALYSIS

5.3.1 TUNEL assay analysis of the liver

In this study, we demonstrated that there was an increase in TUNEL-positive staining in the livers of 12% HCD-fed animals compared to controls. An increased TUNEL-positive staining in the livers of HCD-fed animals indicates elevated numbers of apoptotic cells. This finding concurs with Zheng et al. (2023), who demonstrated a similar observation as they compared the normal control, high-fat diet only, high-fat and high cholesterol diet to induce an experimental NASH animal model in rats. They reported that the apoptosis rate of the high-fat and cholesterol diet group was significantly higher than that of the control and high-fat diet alone, indicating the crucial role of cholesterol in promoting hepatocyte apoptosis (Zheng et al., 2023).

HCD has been shown to induce apoptosis in liver cells through interconnected mechanisms, including oxidative stress, endoplasmic reticulum stress, and the activation of pro-apoptotic pathways (Enriquez-Cortina et al., 2017; Yang et al., 2022; Shou et al., 2025). HCD has been reported to elevate reactive oxygen species (ROS) production, leading to oxidative damage in liver cells (Enriquez-Cortina et al., 2017). Additionally, cholesterol overload triggers the unfolded protein response (UPR), which has been linked to increased hepatic apoptosis (Ma et al., 2023; Yang et al., 2022). HCD also disrupts the balance between pro- and anti-apoptotic proteins, exacerbating cell death (Wang et al., 2019). Recently, (Shou et al., 2025) proposed that excessive free cholesterol accumulation contributes to hepatic apoptosis by increasing ROS levels, impairing lysosomal function, and disrupting lipophagy.

This study demonstrated TH supplementation at a moderate dose of 2.4 g/kg/day (Group 4) for four weeks significantly reduced TUNEL-positive areas compared to the 12% HCD group (Group 2). This finding suggests that TH provides a protective effect against liver damage induced by a 12% HCD at this dose. The reduction of apoptotic cells is likely mediated by TH antioxidant properties, which alleviate oxidative stress, and its anti-inflammatory effects (Talebi et al., 2020). Supporting this, Zakaria et al. (2021) reported that honey reduced oxidative stress parameters such as translocation of nuclear factor erythroid 2-related factor (Nrf2) to the nucleus, Kelch-like ECH-associated protein-1 (Keap1) expression, and inflammatory markers such as tumour necrosis factor-alpha (TNF- α), nuclear factor kappa B (NF- κ B) and monocyte chemoattractant protein-1 (MCP-1). These findings were further corroborated by histopathological improvement in steatosis, hepatocyte hypertrophy, inflammatory cell infiltration and collagen deposition, in a metabolic dysfunction-associated liver disease modelled by Zakaria and her team (Zakaria et al., 2021).

Interestingly, a paradoxical increase in TUNEL-positive cells was observed at 3 g/kg/day of TH supplementation (Group 5) compared to group 4 rats. In contrast, Ahrari Roodi et al. (2018) reported a reduction in apoptotic cells in a histopathological study investigating the protective effects of honey on acrylamide-induced liver injury. Their study, which administered honey at doses of 1.25, 2.5, and 5 g/kg body weight, found that the highest dose reduced tissue lesion severity. However, in that study, tissue injury and cell death were induced using acrylamide, a neurotoxin, meaning the observed hepatoprotective effects may not directly apply to liver damage caused by other factors, such as HCD in this study. Here, moderate doses of bioactive compounds in TH appear to optimize efficacy and safety by enhancing cellular defence mechanisms, whereas higher doses may trigger compensatory or toxic responses. (Martirosyan & Sanchez, 2022).

5.3.2 α -SMA staining analysis of the liver

In this study, the animals in a 12% HCD group exhibited increased α -SMA-positive stellate cells, indicating their activation to myofibroblast-like cells, compared to that of the normal control group. Expression of α -SMA staining is a hallmark of activated hepatic stellate cells, which play a central role in extracellular matrix deposition and fibrogenesis (Rauchbach et al., 2022). HCD led to significant activation of hepatic stellate cells which is evidenced by enhanced α -SMA staining around fibrotic areas, reflecting early fibrotic changes due to lipid accumulation and oxidative stress in hepatocytes, which activate pro-fibrotic pathways (Alfarisi et al., 2020; Ho et al., 2019; Rauchbach et al., 2022).

Cholesterol overload exacerbates endoplasmic reticulum stress and oxidative damage, promoting hepatic stellate cells activation and collagen deposition (Rauchbach et al., 2022). A study by Tsuchida et al. (2018) comparing between high-fat, high-cholesterol diet and chemical inducers like carbon tetrachloride (CCl_4) to induce NASH, documented amplified α -SMA expression and fibrosis, mimicking human NASH progression. Such models highlight the role of lipotoxicity and inflammation in hepatic stellate cells activation. As reported by Jiao et al., (2024), cholesterol is metabolised to 27-hydroxycholesterol (27HC), leading to increased expression of collagen (COL-1), α SMA, connective tissue growth factors (CTGF) and tissue inhibitors of metalloproteinases -1 (TIMP1), and promoting transforming growth factor beta ($\text{TGF}\beta$) signalling which mediates activation of hepatic stellate cell (Jiao et al., 2024). In another study, a 1% HCD increased free cholesterol levels, leading to DNA fragmentation and activation of apoptotic pathways, especially hepatic stellate cells, establishing liver fibrosis in a mice model of NASH (Rauchbach et al., 2022).

It can also be seen in this study that there were significant reductions in the α -SMA positive cells in the low dose (1.2g/kg/day) group and moderate dose (2.4g/kg/day) group, suggesting that TH has protective effects against stellate cell activation. The pronounced reduction at 2.4g/kg/day highlights the optimal therapeutic dose for leveraging TH's benefits. Consistent with this finding, honey has been reported to reduce α -SMA positive cells by inhibiting inflammatory cytokines and chemokines such as platelet-derived growth factor (PDGF), interleukin-4 (IL-4), transforming growth factor beta (TGF- β), and interleukin-13, which are involved in myofibroblast activation and proliferation, thus promoting extracellular matrix homeostasis and reducing fibrosis (Shoaib et al., 2020).

Meanwhile, in this study, the high dose of 3g/kg/day showed increased expression of α -SMA. The paradoxical increase of α -SMA expression at 3g/kg/day could be explained by the biphasic effect common to natural products. High doses of TH might have overstimulated the immune or metabolic pathways, exacerbating oxidative stress, or leading to the accumulation of honey's metabolic by-products, counteracting its beneficial effects (Akhbari et al., 2021). This phenomenon is often observed with bioactive compounds, including honey, due to complex interactions between the compound and biological systems.

5.4 EFFECTS OF TUALANG HONEY ON GENE EXPRESSION PROFILES AND PATHWAY ANALYSIS

5.4.1 Significant upregulated genes

In this study, the 10 most significantly upregulated genes in the animals treated with TH are Aldehyde dehydrogenase family 1 subfamily A7 (*Aldhla7*), CD209 antigen-like protein E (*Cd209e*), Cytochrome P450 family 4 subfamily a polypeptide 2 (*Cyp4a2*), Dual oxidase 1 (*Duox1*), Dual oxidase maturation factor 1 (*Duoxa1*), FAM111 trypsin like peptidase A (*Fam111a*), Nuclear transcription factor X-box binding 1 (*Nfx1*), Regulator of G-protein signalling 16 (*RGS16*), Spectrin repeat containing nuclear envelope family member 4 (*Syne4*) and Squalene epoxidase (*Sqle*). Generally, the top 10 dysregulated genes found in this study mainly are associated with pathways involved in lipid metabolism, oxidative stress, immune response and glucose metabolism.

One of the upregulated genes is *Aldhla7*, which plays a crucial role in detoxifying aldehydes and is involved in lipid metabolism and oxidative stress response (Chen & Li, 2024). Elevated *Aldhla7* expression enhances aldehyde detoxification, thereby reducing oxidative stress associated with lipid peroxidation (Duan et al., 2024). Another upregulated gene, *Sqle*, is also linked to lipid metabolism, as it encodes an enzyme (squalene epoxidase) responsible for catalysing the first oxygenation step in cholesterol biosynthesis (Yoshioka et al., 2020). The upregulation of *Sqle* may indicate increased sterol biosynthesis to meet cellular demands, such as membrane repair and bile acid synthesis (Sun et al., 2021). Elevated *Sqle* expression could represent a compensatory mechanism in the liver to regulate cholesterol levels, especially in response to stress from a high-cholesterol diet.

In addition, *Cyp4a2*, a member of the cytochrome P450 family, plays a key role in the omega-oxidation of fatty acids (Massart et al., 2022). The *Cyp4a2* gene encodes enzymes that hydroxylate both saturated and unsaturated fatty acids, enabling their conversion into dicarboxylic acids for further metabolism (Leahy et al., 2024). This process is essential for maintaining normal fatty acid levels and preventing excessive accumulation, which can lead to liver damage (Jarrar & Lee, 2021). The increased activity of *Cyp4a2* observed in animals treated with TH supplementation suggests enhanced fatty acid breakdown, thereby reducing lipid accumulation in the liver.

The *Duox1* and *Duoxa1* genes (dual oxidase and dual oxidase maturation factor 1), which are involved in modulating oxidative stress, were found to be significantly upregulated in the TH-treated group, suggesting their role in regulating oxidative stress responses. Wang et al. (2020) demonstrated that the knockdown of *Duox1* significantly increased cell proliferation, reduced reactive oxygen species (ROS) levels, and inhibited cell apoptosis by activating the Nuclear Factor kappa B (NF- κ B) signalling pathway. In contrast, the upregulation of *Duox1* observed in this study may reflect a tissue repair mechanism aimed at reversing tissue injury in the NASH animal model. Supporting this, another study highlighted *Duox1*'s dual role in melanoma, where it restrains proliferation but promotes cell growth (Pardo-Sánchez et al., 2023).

Meanwhile, the *RGS16* gene, a member of the Regulator of G-protein Signalling (RGS) family, functions as a GTPase-activating protein and plays a key role in regulating signalling pathways related to insulin, glucose metabolism, and inflammatory responses (Tian et al., 2022). The increased expression of *RGS16* observed in the TH-treated animals suggests an improvement in glucose homeostasis and insulin sensitivity, which may have implications for conditions such as non-alcoholic fatty liver disease (NAFLD) or diabetes (Tian et al., 2022).

5.4.2 Significant downregulated genes

In this study, the 10 most significant downregulated genes in TH treated animals were identified as Nidogen 2 (*Nid2*), Apelin (*Apln*), interferon regulatory factor 2 binding protein 2 (*Irf2bp2*), male-specific lethal 3 homolog (*Msl3*), COP9 signalosome subunit 9 (*Cops9*), Tubulin beta 2A class IIa (*Tubb2a*), stearoyl-Coenzyme A desaturase 2 (*Scd2*), hypoxia-inducible factor 1 alpha subunit (*Hif1a*), Limk2 LIM domain kinase 2 (*Limk2*), HLF transcription factor PAR bZIP family member (*HLF XI*). These genes are involved in various processes, including cell adhesion, immune response, lipid metabolism and cellular organization.

One of the downregulated genes is *Nid2*, which is involved in calcium ion binding and cell-matrix adhesion. Overexpression of *Nid2* has been associated with gastric cancer, promoting invasion and migration, and has been proposed as a novel biomarker for gastric cancer prognosis (Yu et al., 2019). The downregulation of *Nid2* in TH-treated animals may contribute to maintaining cellular structural integrity and extracellular matrix stability, potentially reducing fibrosis (Shoaib et al., 2020). Other downregulated gene is *Hif1a*, which functions as a DNA-binding transcription activator and plays a key role in the apoptotic process. While J. Zhang et al. (2021) reported that *HIF1a* overexpression exerts a protective effect on hepatic ischemia-reperfusion injury, its downregulation in TH-treated animals may suggest altered responses to hypoxia and regulation of apoptosis.

In this study, another gene involved in apoptosis regulation is *Cops9*, reported to be involved in cytoplasmic protein sequestration (Huang et al., 2021). Lei et al. (2013) demonstrated that hepatic deficiency of *Cops9* leads to ubiquitin-proteasome system impairment and Bim-

mediated apoptosis in murine livers. Its downregulation could potentially affect protein degradation and reduce cell proliferation in this context.

Another downregulated gene is an *Irf2bp2* gene, which encodes a transcription corepressor, plays a role in immature B cell differentiation and immune response (Fang et al., 2020). Knockdown of *Irf2bp2* has been shown to improve liver fibrosis by modulating TGF- β signalling pathways in hepatic stellate cells, enhancing hepatoprotective effects in CCl₄-induced liver injury models (Xu et al., 2022). However, Fang et al. (2020) reported that downregulation of *Irf2bp2* increases the transcription of ATF3, which contributes to the pathogenesis of NAFLD. These inconsistent findings highlight the need for further validation of this gene expression to verify its role in hepatoprotection, using targeted gene expression analysis such as qPCR.

Meanwhile, *Scd2*, a gene involved in lipid metabolism and oxidative stress response, encodes stearoyl-CoA 9 desaturase and plays a key role in fatty acid biosynthesis (O'Neill et al., 2020). Studies have shown that *Scd2* deficiency corrects hypometabolic phenotypes and hepatic steatosis in mice and attenuates fasting-induced liver steatosis (Dobrzyn et al., 2008). Moreover, the lack of *Scd2* expression improves insulin sensitivity in skeletal muscles and prevents diet-induced hepatic insulin resistance in mice (Dobrzyn et al., 2008). Additionally, inhibition of SCD-1 reduced triglyceride accumulation in the liver of NASH rats by 80% (Kurikawa et al., 2013). Consistently, the downregulation of *Scd2* in this study may contribute to the observed reduction in total triglyceride levels, as lower *Scd2* activity limits the synthesis of monounsaturated fatty acids needed for triglyceride formation, thereby decreasing lipid accumulation in the liver.

5.4.3 Associated Pathway Analysis

Based on the 10 most significant biological processes involved in the hepatoprotective effects of TH, this study identifies three key molecular mechanisms underlying the hepatoprotective effects of Tualang Honey: (1) regulation of lipid and cholesterol metabolism to reduce steatosis and maintain balance, (2) antioxidant and anti-inflammatory actions to protect hepatocytes, and (3) enhancement of glucose and energy metabolism to support recovery. Together, these pathways contribute to TH's protective effects on the liver as observed in this study.

5.4.3.1 Lipid and Cholesterol Metabolism

Lipid and steroid biosynthesis pathways contribute to the production of corticosteroids, which are anti-inflammatory (Charni-Natan et al., 2019). Corticosteroids help stabilize cell membranes and protect against oxidative stress (Chatuphonprasert et al., 2018). Additionally, they regulate bile acid production, improving lipid digestion and detoxification. Meanwhile, the cholesterol biosynthesis pathway is vital for maintaining cellular membrane integrity and producing bile acids for detoxification (Arguello et al., 2015). Enhanced cholesterol biosynthesis helps restore liver structure during injury as mentioned in a review by Sozen and Ozer (2017). Besides, the fatty acid omega-oxidation pathway aids in metabolizing unsaturated fatty acids, preventing lipid accumulation and reducing steatosis (Natarajan & Ibdah, 2018). Omega-oxidation supplements beta-oxidation during energy production, especially under oxidative stress. The significance of these pathways is for prevention of lipid accumulation, stabilization of cell membranes, and bile acid production for detoxification. The significance of this molecular mechanism is for reduction in ROS and inflammatory cytokines, promoting liver tissue repair.

5.4.3.2 Antioxidant and anti-inflammatory mechanism

The biogenic amine synthesis pathway is involved in the synthesis of biogenic amines such as serotonin and histamine which have roles in reducing oxidative stress and inflammation. These compounds can scavenge free radicals, reducing oxidative damage to hepatocytes (Reiter et al., 2008). They proposed that serotonin has been shown to have antioxidant properties, while histamine modulates immune responses to inflammation. Ommati et al (2023) by suggests biogenic amines decrease ROS and lipid peroxidation in hepatic cells (Ommati et al., 2023). Additionally, the interleukin-2 signalling pathway modulates the activity of T-cells and promotes tissue repair in the liver by reducing pro-inflammatory cytokines (Chen et al., 2016). These three pathways may help mitigate chronic liver inflammation, common in non-alcoholic fatty liver disease (NAFLD) and NASH.

5.4.4.3 Glucose and Energy Metabolism

Glycolysis and gluconeogenesis enhance glucose metabolism, ensuring hepatocyte energy demands are met during stress or injury. Alanine and aspartate metabolism pathway for instance, support amino acid catabolism and contribute substrates for gluconeogenesis, vital during periods of liver stress (Holeček, 2024). Enhanced alanine metabolism reduces nitrogen accumulation, alleviating hepatic encephalopathy risks, whilst β -oxidation of unsaturated fatty acids pathway includes beta-oxidation, breaks down fatty acids into energy, reducing lipid-induced oxidative stress and inflammation (Talley & Mohiuddin, 2023). This process protects against hepatic steatosis by preventing lipid overload. The significance of this pathway is for provision of energy during repair processes and sustained glucose levels during fasting or stress.

5.5 RT-PCR VALIDATION OF SELECTED GENES

In this study, two genes were selected for RT-qPCR analysis, to validate their respective role in hepatoprotective property of TH, namely aldehyde dehydrogenase (*Aldh*) and interferon regulator factor 2 (*Irf2*). Their expression levels were measured relative to a reference gene, 18s ribosomal RNA (*18s rRNA*). The use of 18s rRNA as a reference gene in microarray RNA analysis is widely accepted due to its stability and universal applicability across various species (Низкородова et al., 2022). While it is a common practice to use multiple reference genes for normalisation to enhance reliability and accuracy, this study employed a single reference gene. Despite this limitation, 18s rRNA was deemed a suitable choice because of its high stability and consistent expression across all samples analysed in the study.

5.5.1 Aldehyde dehydrogenase (*Aldh*) gene

In this study, we demonstrated that *Aldh* gene expression increased in the 12% HCD group compared to the control, and a significant increase in expression was observed at a moderate dose (2.4 g/kg/day) of TH supplementation. However, a reduction was noted at the high dose (3 g/kg/day) of TH. The *Aldh* enzyme plays a pivotal role in detoxifying aldehydes – highly reactive and toxic molecules generated during oxidative stress. The observed upregulation of *Aldh* gene in the 12% HCD group likely represents a compensatory mechanism by the liver to mitigate the oxidative stress and aldehyde production induced by the high-cholesterol diet. This finding aligns with previous studies, such as Yang et al. (2021), which reported that *Aldh* gene mutations exacerbate non-alcoholic fatty liver disease (NAFLD) in high-fat diet mice, highlighting the importance of *Aldh*-mediated detoxification in protecting against liver injury. Their study also

suggested that gut microbiota changes and sex hormones could also modulate the severity of liver damage, indicating that *Aldh* function may be influenced by multiple systemic factors.

The further increase in *Aldh* gene expression at the moderate dose (2.4 g/kg/day) of TH suggests that the increased dose enhances the detoxification pathways in the liver, possibly due to the antioxidant and polyphenol content of honey. Polyphenols are known to upregulate detoxification genes, as supported by Wimborne et al. (2019), who reported that *Aldh* activation accelerates aldehyde degradation and provides protection against mitochondrial dysfunction in acetaminophen-induced hepatotoxicity. This highlights the potential therapeutic value of targeting *Aldh* pathways to mitigate liver fibrosis and injury.

Interestingly, there was a reduction in the *Aldh* expression in group 5 compared to group 4, suggesting a potential saturation effect or dose-dependent inhibition. High doses of natural antioxidants, including honey, may exhibit diminishing or adverse effects, possibly due to metabolic saturation or the pro-oxidant activity of excessive sugar intake (Akhbari et al., 2021). This observation is consistent with previous immunochemistry parameters findings, indicating a biphasic response to natural supplements, where moderate doses provide maximum benefits, and higher doses may overwhelm metabolic pathways.

5.5.2 Interferon Regulatory Factor 2 (*Irf2*) gene

This study demonstrated the *Irf2* gene expression was significantly downregulated in a 12% HCD group compared to the normal control. Following a moderate dose (2.4g/kg/day) of TH supplementation, the gene was the least suppressed as the fold change is significantly reduced, indicating a potential dose-dependent improvement in immune response. *Irf2* is a transcriptional

factor involved in immune responses, particularly regulating interferon signalling and inflammation. Downregulation of *Irf2* in the 12% HCD group may reflect immune suppression due to the high cholesterol diet, which is known to induce systemic inflammation and oxidative stress. This is consistent with Zhang et al. (2022) in their review on the role of *Irf2* gene in NAFLD and NASH, highlighted that *Irf*s, as transcriptional factors, can regulate interferons, as well as other genes such as hypoxia-inducible factor 1-alpha ($\text{HIF1}\alpha$), which are associated with the pathogenesis of NAFLD and NASH (Woods et al., 2022). The roles of *Irf*-involved signalling pathways in hepatic inflammation, insulin resistance, and immune cell activation *Irf*s such as *Irf1* and *Irf4* are also involved in the polarization of macrophages that contribute to critical roles in NAFLD or NASH pathogenesis.

TH supplementation particularly at moderate and high doses shows a partial restoration of *Irf2* expression compared to the 12% HCD group. This supports the antioxidant and anti-inflammatory properties of TH in mitigating diet-induced stress. *Irf2* plays a multifaceted role in liver pathophysiology, including conditions like non-alcoholic steatohepatitis (NASH), by regulating key cellular processes and inflammatory pathways. *Irf2* has demonstrated anti-inflammatory properties, particularly in macrophages. It suppresses the activation of macrophages induced by lipopolysaccharides (LPS), partly by regulating hypoxia-inducible factor 1-alpha ($\text{HIF1}\alpha$)-dependent glycolysis (Woods et al., 2022). This suggests that *Irf2* may play a protective role in mitigating inflammation, a hallmark of NASH progression. Targeting *Irf2* and related signaling pathways holds promise for developing therapeutic strategies for NASH and related liver diseases. Modulators of *Irf2* signaling, such as certain microRNAs and anti-inflammatory agents, may prevent or slow the progression of liver inflammation and fibrosis.

5.6 LIMITATIONS AND RECOMMENDATIONS

There are some limitations in this study that should be addressed for future improvement. First, the treatment duration of four weeks may have been inadequate to completely assess the hepatoprotective effects of TH in all parameters in this study. This study demonstrated non-significance changes in percentage body weight gain, and microscopic histological markers, including NAFLD activity scores in TH-treated groups compared to a 12% HCD group. A longer treatment duration might be required to yield statistically significant changes in microscopic histological findings. Extended supplementation periods of 6-8 weeks have been shown to produce significant biochemical parameters such as liver enzymes (ALT, AST, ALP), HDL-c, LDL-c, glucose, insulin and HOMA-IR (Azman et al., 2024). Supporting this, Valdés García et al. (2020) highlighted that natural antioxidant interventions often require extended treatment periods to counteract the chronic effects of high-fat or high-cholesterol diets.

Another limitation of this study is the dose increments from moderate dose (2.4 g/kg/day) to high dose (3.0 g/kg/day) which resulted in inconsistent findings. Testing intermediate doses could provide more precise insights into the dose-response relationship and help identify the optimal dosing threshold in future studies. This is important as a natural bioactive compound including honey has complex interactions between compound and biological systems. Moderate doses of bioactive compounds often balance efficacy and safety by enhancing cellular defence mechanisms without triggering compensatory or toxic responses (Martirosyan & Sanchez, 2022). High doses may overstimulate immune or metabolic pathways, exacerbate oxidative stress, or lead to the accumulation of honey's metabolic byproducts, counteracting its beneficial effects (Akhbari et al., 2021).

For future projections, it is recommended to employ a more comprehensive analyses, such as transcriptomic, proteomic, and metabolomics approaches, to uncover detailed molecular pathways modulated by TH. These techniques could provide deeper insights into its antioxidant and anti-inflammatory mechanisms. Additionally, investigating the potential synergistic effects of TH with other therapeutic agents, such as lipid-lowering drugs or insulin sensitizers, or to couple TH supplementation with dietary modification may enhance the efficacy of interventions aimed at mitigating NASH pathology. As proposed by El-Haskoury et al. (2018), while honey has strong antioxidant activity, it alone may not produce substantial changes in certain metabolic and biochemical parameters, such as blood glucose levels and liver enzymes as seen in the present study. Combining treatments could amplify the therapeutic benefits and address multiple aspects of disease progression, potentially offering a more robust approach to NASH management in the clinical setting.

5.6 IMPACT AND NOVELTY OF THE STUDY

This study provides new insights into the dose-dependent hepatoprotective effects of Tualang Honey (TH) in an established animal model of diet-induced NASH, with clear evidence that a moderate dose (2.4 g/kg/day) yields the most significant benefits. Unlike previous research focusing mainly on general antioxidant properties, this work elucidates specific molecular pathways; lipid and cholesterol metabolism, antioxidant and anti-inflammatory responses, and glucose and energy metabolism, that underpin TH's therapeutic action. This integrative understanding advances the potential for TH as a natural, dose-optimized intervention for NASH, paving the way for further translational research and clinical evaluation.

CHAPTER SIX

CONCLUSION

This study demonstrated that a 12% high-cholesterol diet administered for 20 weeks induced mild non-alcoholic steatohepatitis (NASH) model in rats. This model was characterised by the significant increase in relative liver weight and histological evidence of steatosis, hepatic ballooning degeneration, and inflammation of hepatocytes. There were also significant elevation of total cholesterol and triglycerides, in the animals given a 12% HCD compared to normal controls. Immunohistochemistry analysis revealed increased α -SMA protein expression and percentage of TUNEL-positive area compared to normal controls.

1. Supplementation of TH had no significant changes in relative liver weight, gross morphology and microscopic histological findings.
2. Supplementation of TH at low dose (1.2 g/kg/day), moderate dose (2.4 g/kg/day) and high-dose (3 g/kg/day) significantly reduced the serum triglyceride levels compared to the 12% HCD group.
3. Supplementation of TH at moderate dose (2.4 g/kg/day) led to a marked reduction in activated hepatic stellate cells and apoptotic cells, highlighting its potential to attenuate cellular damage associated with NASH.
4. Microarray analysis revealed that TH-treated animals exhibited significant modulation of genes involved in lipid and cholesterol metabolism regulation, antioxidant and inflammatory pathways, and glucose and energy metabolism enhancement, reinforcing the hepatoprotective properties of TH.

5. In comparison to the 12% HCD group, the aldehyde dehydrogenase (*Aldh*) gene was significantly upregulated in the 2.4 g/kg/day group, signifying optimal antioxidant activity in the lipid metabolism process. Concurrently, interferon regulatory factor 2 (*Irf2*) gene expression was less suppressed at this dose, indicating enhanced anti-inflammatory responses.
6. High-dose supplementation of TH (3.0 g/kg/day) exhibited paradoxical effects with exacerbated NASH histological pathology appearance, increased α -SMA protein expression and TUNEL-assay positive area. These outcomes may be attributed to overstimulation of immune or metabolic pathways or the accumulation of metabolic by-products.

In conclusion, this study demonstrates that the hepatoprotective effects of TH in a 12% HCD-induced NASH animal model were most pronounced at the moderate dose of 2.4 g/kg/day. Lipid and cholesterol metabolism, antioxidant and anti-inflammatory mechanisms, and glucose and energy metabolism pathways were identified as the primary molecular pathways supporting these effects, highlighting the therapeutic potential of TH at optimal dosage.

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APPENDIX 1: PUBLICATIONS

JOURNAL ARTICLE:

Alias, Mohd Afzal, Roslina Abdul Rahim, Siti Aesah, Naznin Muhamad, Nor Zamzila Abdullah, Norlelawati A. Talib, and Khairunisa Ahmad Affandi. "Tualang Honey Supplementation Alleviates Obesity and Dyslipidaemia in High Cholesterol Diet Induced Non-Alcoholic Steatohepatitis Animal Model." *IJUM Medical Journal Malaysia* 21, no. 4 (2022).

ORIGINAL ARTICLE

Tualang Honey Supplementation Alleviates Obesity and Dyslipidaemia in High Cholesterol Diet Induced Non-Alcoholic Steatohepatitis Animal Model

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ABSTRACT

INTRODUCTION: Dyslipidaemia and obesity are two main features of non-alcoholic steatohepatitis (NASH). This study aimed to investigate the effects of Tualang honey (TH) supplementation on bodyweight, liver weight, and lipid profiles in high cholesterol diet (HCD) induced NASH animal model. **MATERIALS AND METHODS:** Sixteen Sprague-Dawley rats were given 12% HCD for 16 weeks to induce NASH. These animals were divided into 4 groups; Group 1 (continued HCD), Group 2 (changed to normal diet), Group 3 (normal diet and TH 1.2g/kg) and Group 4 (normal diet and TH 3.0g/kg) for the following 4 weeks. Bodyweight was measured daily. At the end of the study, blood was collected via retro-orbital bleeding and the rats were sacrificed to harvest their liver. **RESULTS:** The group 4 rats had significantly lower mean final bodyweight than rats in group 1, 2 and 3 (478.0 ± 24.4 vs. 641.5 ± 25.1 , 593.8 ± 29.3 , 552.0 ± 72.9 g, $p < 0.05$). Animals in group 4 were also found to have a significantly lower mean liver weight compared to groups 1 and 2 (12.9 ± 0.9 vs 20.1 ± 2.2 , 15.7 ± 1.2 g, $p < 0.05$). In comparison to controls, the mean concentration of total cholesterol was significantly lower in all the other groups and the lowest mean concentration of triglycerides was recorded in group 4 with significant difference when compared to the controls (0.9 ± 0.4 vs 3.6 ± 0.4 mmol/L, $p < 0.05$). **CONCLUSION:** The change from HCD to a normal diet coupled with TH supplementation has been shown to reduce bodyweight, liver weight, total cholesterol and triglyceride levels in the 12% HCD NASH induced animal models.

Keywords

Non-alcoholic steatohepatitis, obesity, tualang honey, dyslipidaemia, animal model

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INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) has been recognised as one of the leading causes of chronic liver disease worldwide.¹ NAFLD is an umbrella term describing excessive accumulation of fat in hepatocytes that progresses in the absence of secondary causes such as excessive alcohol intake, medications or certain inherited genetic disorders.¹ It is considered to be the hepatic manifestation of metabolic syndrome as individuals with components of metabolic syndrome, such as obesity, insulin resistance, and hyperlipidaemia, have an increased risk of developing NAFLD.² It is a multisystem disease, with several subtypes ranging from simple steatosis or

non-alcoholic fatty liver (NAFL) to non-alcoholic steatohepatitis (NASH), which is distinguished by inflammation and hepatocellular injury, in addition to the steatosis, with or without fibrosis.² Although NAFL commonly has a benign clinical course, NASH can gradually progress to cirrhosis, end-stage liver disease and has an increased risk of developing hepatocellular cancer (HCC).³

Prevalence of NAFLD worldwide is estimated at around 25% of the global population, bearing in mind that there are significant variations between countries and racial/

ABSTRACT PROCEEDING:

Alias, Mohd Afzal, Roslina Abdul Rahim, Nor Zamzila Abdullah, Naznin Muhammad, and Norlelawati A. Talib. 2021. "Lipid Profiles Changes In 12% High Cholesterol Diet Induced Nonalcoholic Steatohepatitis Animal Model Following Supplementation of Tualang Honey and Diet Modifications". International Journal of Allied Health Sciences 5 (1): 2164.



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LIPID PROFILES CHANGES IN 12% HIGH CHOLESTEROL DIET INDUCED NONALCOHOLIC TEATOHEPATITIS ANIMAL MODEL FOLLOWING SUPPLEMENTATION OF TUALANG HONEY AND DIET MODIFICATIONS

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ABSTRACT BOOK

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APPENDIX 2: AWARDS AND PRESENTATIONS

AWARDS:

1. First Place, Kulliyyah of Medicine (KOM) 3 Minute Thesis (3MT) Competition 2020.
Organised by Postgraduate Office, Kulliyyah of Medicine, IIUM.



2. First Place, University Level 3 Minute Thesis (3MT) Competition 2020. Organised by Centre for Postgraduate Studies, IIUM.



3. Gold Medal, Poster Presentation for Kuantan Research Day 2020. Organised by Research Management Centre, IIUM Kuantan Campus.

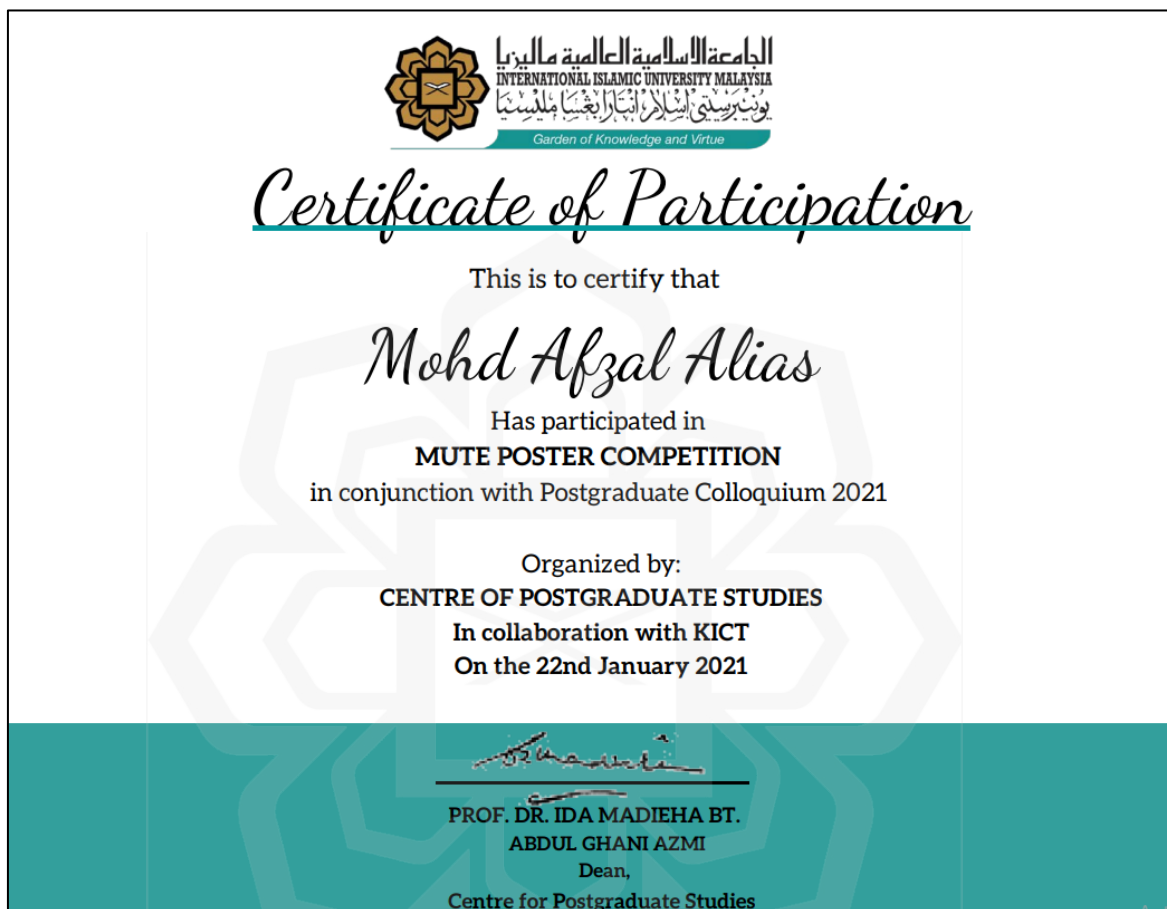


4. Commendation for Best Presentation (Poster Category), 34th MSPP Scientific Meeting 2021. Organised by the Malaysian Society of Pharmacology and Physiology.



PARTICIPATION – POSTER PRESENTATION:

1. Mute Poster Competition, Postgraduate Colloquium 2021. Organised by the Centre of Postgraduate Studies.



2. Poster Presentation for Monash International Health, Science and Technology Conference (MONASH INITIATE) 2021. Organised by the School of Pharmacy, Monash University Malaysia.



PARTICIPATION – ORAL PRESENTATION:

1. Online Oral Presentation, Medical Research Symposium 2021. Organised by the Office of Responsible Research and Innovation, Kulliyyah of Medicine.



APPENDIX 3: RAW DATA

DIFFERENTIALLY EXPRESSED GENES (DEG): UPREGULATED PROBE NAMES

A 43 P11832	A 44 P201139	A 44 P1140974	A 44 P1362734	A 44 P716655
A 44 P1108315	A 44 P684795	A 44 P1132933	A 44 P496972	A 44 P368032
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A 44 P1135099	A 44 P399966	A 64 P046135	A 44 P1365236	A 64 P073427
A 44 P1083098	A 44 P1121925	A 44 P1106662	A 64 P042611	A 44 P828792
A 44 P323773	A 44 P1120978	A 42 P636189	A 44 P1114865	A 44 P1361968
A 44 P269930	A 44 P1129145	A 44 P384066	A 44 P1144933	A 44 P107622
A 44 P1082289	A 44 P1127444	A 44 P478479	A 44 P1159211	A 64 P132044
A 44 P897781	A 44 P1123331	A 44 P1125929	A 44 P1122748	A 44 P1157322
A 64 P151071	A 64 P159300	A 44 P356513	A 64 P039576	A 44 P1113001
A 64 P165065	A 64 P087708	A 44 P1147747	A 44 P1365111	A 44 P1126054
A 44 P1148977	A 44 P1142032	A 44 P1148047	A 44 P1106312	A 44 P1108058
A 42 P524633	A 44 P1124877	A 44 P1098673	A 44 P1105677	A 44 P661373
A 64 P076638	A 43 P15381	A 44 P505440	A 44 P337991	A 44 P991924
A 44 P388973	A 64 P079900	A 44 P175364	A 64 P036174	A 64 P164429
A 44 P1035294	A 44 P1112751	A 64 P085946	A 44 P1368821	A 44 P1121938
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A 44 P956005	A 64 P083888	A 64 P061240	A 44 P1092061	A 44 P1106807
A 44 P514910	A 44 P275779	A 42 P794613	A 44 P221185	A 44 P1094825
A 64 P163708	A 44 P1137330	A 44 P1366014	A 44 P1164026	A 44 P1110308
A 64 P130683	A 64 P088382	A 44 P508386	A 44 P103966	A 44 P1151950
A 44 P1117326	A 64 P131395	A 44 P309202	A 44 P472919	A 44 P1120458
A 44 P1086350	A 44 P142025	A 44 P1158961	A 42 P822588	A 44 P786733
A 44 P168280	A 64 P127472	A 44 P484889	A 64 P067177	A 43 P14440
A 64 P142242	A 44 P1127352	A 64 P157446	A 44 P1101958	A 44 P1141466
A 43 P12366	A 64 P039809	A 44 P515825	A 44 P532249	A 44 P226363
A 43 P16774	A 44 P1151692	A 64 P021737	A 64 P000559	A 64 P089490
A 43 P18346	A 64 P036168	A 64 P078510	A 44 P416596	A 64 P098996
A 44 P1119806	A 44 P1155301	A 44 P339535	A 44 P208477	A 44 P1084410
A 64 P032863	A 44 P743875	A 44 P1363983	A 44 P1113170	A 44 P1098033
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A 44 P438718	A 43 P23155	A 44 P432965	A 44 P812776	A 44 P1137036
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A 44 P1138966	A 64 P116288	A 43 P13043	A 64 P031476	A 44 P1157613
A 44 P870880	A 44 P1152665	A 44 P1080810	A 44 P191021	A 44 P1366008
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A 44 P1084769	A 43 P13459	A 43 P13943	A 44 P1150972	A 44 P1135043
A 64 P083084	A 44 P463980	A 64 P006948	A 44 P1139493	A 44 P371635
A 44 P986792	A 44 P356027	A 64 P148225	A 64 P080598	A 44 P1103062
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A 44 P1083307	A 64 P078952	A 44 P250267	A 44 P1361170	A 42 P495355
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A 43 P13088	A 44 P1090529	A 44 P139836	A 44 P1118016	A 64 P073367
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A 64 P129316	A 64 P041054	A 44 P990998	A 44 P1027971	A 44 P1097843
A 44 P1120647	A 44 P1121855	A 64 P043811	A 44 P139415	A 44 P1121825
A 44 P1080066	A 44 P1370999	A 44 P1364101	A 44 P1134761	A 44 P1093627
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A 44 P1089976	A 44 P576733	A 44 P274762	A 44 P1128706	A 44 P241583
A 44 P1367764	A 44 P1368668	A 44 P676132	A 64 P134069	A 44 P1103405
A 64 P113231	A 44 P243295	A 64 P091148	A 44 P1146935	A 44 P1141770
A 44 P1112368	A 44 P425837	A 64 P033922	A 44 P1148815	A 64 P052159
A 44 P1141783	A 44 P1142936	A 64 P050921	A 44 P409739	A 44 P1140944
A 44 P1154852	A 43 P10241	A 44 P111816	A 44 P389448	A 44 P100758
A 44 P467055	A 44 P311136	A 44 P1093157	A 44 P1360422	A 44 P461251
A 44 P1095053	A 44 P1149120	A 44 P1157884	A 44 P1137498	A 44 P1102942
A 44 P1138994	A 44 P317993	A 44 P1134053	A 64 P145421	A 44 P264785
A 42 P629599	A 44 P1155910	A 44 P1139097	A 44 P423765	A 64 P148976
A 44 P384698	A 64 P131001	A 44 P531870	A 44 P1096993	A 44 P1104878
A 44 P1361891	A 44 P1081054	A 44 P374909	A 64 P085026	A 44 P635869
A 44 P1085764	A 44 P1132648	A 44 P1362433	A 44 P1093273	A 44 P1370185
A 44 P1112691	A 44 P1115557	A 44 P1114385	A 44 P304169	A 44 P1362888
A 44 P1115295	A 44 P1367704	A 64 P134401	A 44 P1099186	A 43 P11721
A 44 P422356	A 44 P1150218	A 44 P519935		

DIFFERENTIALLY EXPRESSED GENES (DEG): DOWNREGULATED PROBE NAMES

A 42 P753215	A 64 P002289	A 44 P1089741	A 64 P081053	A 44 P1124356
A 44 P1081420	A 44 P808264	A 44 P1125510	A 44 P522859	A 44 P1142324
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A 44 P1119392	A 44 P220861	A 64 P050084	A 44 P1018246	A 44 P231399
A 44 P425076	A 44 P1112542	A 44 P1160888	A 44 P1162830	A 44 P1111481
A 44 P501142	A 43 P13906	A 64 P028374	A 44 P1157066	A 44 P1150117
A 64 P153045	A 42 P794947	A 44 P958005	A 44 P743412	A 44 P548241
A 44 P445712	A 44 P1093736	A 64 P088792	A 44 P1111521	A 42 P606251
A 44 P1093504	A 43 P15139	A 44 P1115270	A 42 P518033	A 43 P12782
A 42 P802550	A 43 P17061	A 44 P699859	A 44 P1089851	A 44 P1163902
A 44 P381360	A 44 P238204	A 43 P12651	A 44 P1125644	A 44 P1368202
A 43 P16225	A 64 P048369	A 64 P103321	A 64 P076705	A 44 P1125033
A 44 P238424	A 44 P340477	A 44 P1090206	A 44 P1151493	A 44 P1366582
A 44 P379645	A 42 P754715	A 42 P573023	A 64 P100873	A 44 P113088
A 42 P749222	A 43 P10391	A 42 P837576	A 44 P1123436	A 44 P1119477
A 64 P102141	A 44 P1160209	A 44 P1083727	A 44 P1102458	A 43 P23124
A 64 P037035	A 44 P1127303	A 64 P084995	A 44 P1081575	A 44 P559041
A 44 P1113649	A 42 P494919	A 44 P1088186	A 44 P1108300	A 42 P704479

A 44 P1132089	A 42 P481849	A 44 P1117556	A 64 P147084	A 44 P173138
A 44 P1137473	A 64 P151592	A 44 P1094898	A 42 P618256	A 44 P401858
A 44 P178448	A 43 P11560	A 42 P530071	A 64 P081862	A 44 P1086859
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A 43 P14977	A 44 P1090201	A 42 P628409	A 44 P235978	A 44 P1084709
A 44 P135666	A 64 P015607	A 44 P1105592	A 44 P406825	A 42 P731541
A 43 P22409	A 64 P018116	A 44 P1148507	A 44 P1148712	A 42 P492882
A 44 P1113135	A 44 P1090480	A 44 P407204	A 44 P440392	A 43 P19789
A 44 P858108	A 44 P1085123	A 42 P623151	A 44 P198396	A 44 P1084980
A 44 P1137180	A 44 P496643	A 42 P579280	A 64 P107908	A 44 P184865
A 44 P1119109	A 42 P588785	A 44 P1105523	A 44 P1103800	A 44 P916237
A 64 P064108	A 44 P1096337	A 64 P094407	A 44 P1152134	A 44 P834980
A 44 P1157824	A 43 P11616	A 44 P1003212	A 44 P777710	A 64 P057402
A 64 P080817	A 44 P436942	A 44 P533992	A 43 P16959	A 43 P12274
A 43 P20274	A 64 P018497	A 44 P1030098	A 43 P22852	A 44 P1141703
A 44 P1082149	A 44 P776937	A 44 P1088586	A 44 P1090196	A 44 P100197
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A 42 P495895	A 64 P096267	A 42 P655706	A 64 P046577	A 44 P395709
A 64 P139947	A 44 P1101863	A 42 P618145	A 44 P332310	A 44 P1086684
A 44 P1057176	A 44 P1161805	A 44 P1116332	A 44 P1152863	DCP 1 2
A 44 P106883	A 44 P1108230	A 44 P1116318	A 44 P1098565	A 44 P1111041
A 42 P828102	A 44 P361857	A 64 P062288	A 64 P071556	A 64 P062230
A 42 P816529	A 44 P308530	A 44 P1159954	A 64 P091935	A 44 P130154
A 44 P1111766	A 64 P035152	A 44 P1154965	A 43 P13710	A 44 P1004323
A 44 P1083382	A 44 P1106232	A 64 P043164	A 43 P10424	A 64 P154082
A 64 P155920	A 64 P122489	A 44 P1083447	A 44 P1085819	A 43 P12805
A 43 P13648	A 42 P742084	A 44 P1102623	A 44 P367486	A 44 P197786
A 44 P1145320	A 64 P012977	A 42 P673212	A 44 P372215	A 43 P17945
A 44 P1117259	A 64 P086784	A 44 P1161270	A 64 P046761	A 64 P012198
A 44 P1118174	A 44 P1013667	A 44 P993464	A 64 P030347	A 44 P807238
A 42 P803752	A 44 P899831	A 44 P383596	A 44 P1364591	A 44 P1163846
A 43 P16623	A 44 P362981	A 44 P481892	A 44 P1141955	DCP 1 4
A 44 P438613	A 42 P789584	A 44 P1123587	A 44 P1120561	A 44 P450967
A 44 P1103212	A 43 P16131	A 44 P937648	A 64 P056861	

APPENDIX 4: ANIMAL ETHICS



الجامعة الإسلامية العالمية ماليزيا
INTERNATIONAL ISLAMIC UNIVERSITY MALAYSIA
بوتري بنتين إسلامبول
Compass No 131667-B

INSTITUTIONAL ANIMAL CARE & USE COMMITTEE (IACUC-IIUM)

Our Reference : IIUM/504/14/2/IACUC
Date : 16th June 2019

Asst. Prof. Dr. Roslina Abdul Rahim,
Department of Basic Medical Sciences,
Kulliyyah of Medicine
International Islamic University Malaysia,

Dear Dr,

السلام عليكم ورحمة الله وبركاته

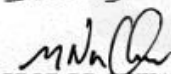
IACUC- IIUM APPROVAL

The Institutional Animal Care and Use Committee (I-ACUC) held its 2nd 2019 meeting and has reviewed and commented of the above research project.

ID	: IIUM/ IACUC- 2019 (9)
Study Title	: Molecular Approach of Attenuation Of Non-Alcoholic Steatohepatitis With Tualang Honey Supplementation In Animal Model
Study Site	: Kulliyyah of Medicine
Animal Details	: Rat (Sprague Dawley), Male. 2 weeks, 200gm
Animal Quantity	: 54
Principal Investigator	: Asst. Prof. Dr. Roslina Abdul Rahim
Expiry	: 30 th June 2020

Thank you.

والسلام


PROF. DR. MUHAMMAD NOR OMAR
Chairperson, I-ACUC

cc. Deputy Director RMC Kuantan

Appendix : Reviewing Committee list of IACUC Meeting 2/2019

DISCLAIMERS: This approval **ONLY** covers the Ethical aspect of your Study and **CANNOT** be use as permission/approval to use any facility (e.g. Animal Room) and authorization to store/handle any poison/chemical



SIRIM
CERTIFIED TO MS ISO 9001:2009
Registration No. AN 3074

Garden of Knowledge and Virtue

Office Address: Institutional Animal Care & Use Committee, International Islamic University Malaysia Kuantan Campus,
Jalan Sultan Ahmad Shah, Bandar Indera Mahkota, 25200 Kuantan, Pahang Darul Makmur
Tel: +609 570 4280 Fax: +609 571 6744 Email: iacuc@iiu.edu.my Website: www.iiu.edu.my/iacuc