METABOLOMIC ANALYSIS OF THE EFFECT OF TOCOTRIENOL-RICH FRACTION ON RAT HEPATIC CHANGES DURING AGEING

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METABOLOMIC ANALYSIS OF THE EFFECT OF TOCOTRIENOL-RICH FRACTION ON RAT HEPATIC CHANGES DURING AGEING

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THESIS SUBMITTED IN FULFILMENT FOR THE DEGREE OF MASTER OF MEDICAL SCIENCE

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ANALISIS METABOLOMIK KESAN FRAKSI KAYA TOKOTRIENOL TERHADAP PERUBAHAN HEPATIK TIKUS SEMASA PENUAAN

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TESIS YANG DIKEMUKAKAN UNTUK MEMPEROLEHI
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ABSTRAK

Penuaan merupakan penyumbang utama kepada disfungsi metabolik dan penyakit hati kronik. Oleh kerana hati berperanan sebagai hab metabolik utama yang mengawal metabolisme tenaga, detoksifikasi, dan homeostasis redoks, perubahan hati yang berkaitan dengan umur mempunyai implikasi kesihatan yang signifikan. Penuaan dikaitkan dengan perubahan progresif dari segi struktur dan metabolisme hati, yang menyumbang kepada tekanan oksidatif dan keradangan. Oleh itu, pemahaman mekanisme di sebalik penuaan hati adalah penting untuk membangunkan strategi bagi mengekalkan kesihatan metabolik semasa penuaan. Fraksi kaya tokotrienol (TRF) dilaporkan memiliki sifat antioksidan dan anti-radang, serta mampu memodulasi metabolisme tenaga, menunjukkan potensinya untuk menentang gangguan oksidatif dan metabolik hati yang berkaitan dengan penuaan. Walau bagaimanapun, hubungan antara tekanan oksidatif, keradangan, perubahan histologi, dan laluan metabolik semasa penuaan hati, serta peranan modulasi TRF, masih belum jelas. Metabolomik membolehkan profil menyeluruh metabolit bersaiz kecil, memudahkan pengenalpastian perubahan laluan metabolik yang berkaitan dengan umur. Kaedah ini memberikan pandangan mengenai bagaimana TRF mungkin mempengaruhi perubahan metabolik berkaitan umur dan melengkapkan penilaian biokimia serta histologi. Walau bagaimanapun, pendekatan ini belum banyak digunakan dalam konteks ini. Oleh itu, kajian ini bertujuan untuk mencirikan perubahan berkaitan umur dalam tekanan oksidatif hati, penanda keradangan, histologi, dan laluan metabolik, serta menentukan sama ada TRF memodulasi perubahan ini menggunakan pendekatan metabolomik tanpa sasaran (untargeted metabolomics). Tikus Sprague-Dawley berumur 3, 9, dan 21 bulan dibahagikan kepada kumpulan kawalan dan rawatan, menerima samada minyak sawit atau suplemen TRF. Tisu hati dianalisis menggunakan metabolomik tanpa sasaran melalui LC-MS/MS, berserta penilaian histologi dan penilaian biokimia bagi enzim antioksidan (SOD, CAT), penanda keradangan (IL-6, TNF-α dan penanda peroksidasi lipid (MDA). Aktiviti SOD menurun dengan ketara dengan peningkatan umur dalam kumpulan kawalan dan TRF tua ($p<0.05$), menunjukkan penurunan pertahanan antioksidan. Tiada perubahan ketara diperhatikan dalam CAT, IL-6, atau MDA antara kumpulan. Tahap TNF-α lebih tinggi secara signifi berbanding kumpulan TRF muda. Penilaian histologi secara kualitatif menunjukkan infiltrasi sel keradangan dan perubahan fibrotik seperti yang lebih jelas dalam hati kawalan, manakala hati yang dirawat dengan TRF menunjukkan perubahan yang lebih ringan. Profil metabolomik mengenal pasti perubahan berkaitan umur dalam metabolisme taurin dan hipotaurin, metabolisme pirimidin (uridin dan urasil), serta komponen kitaran TCA (malat dan glutamat). Penemuan ini menunjukkan bahawa TRF mungkin memodulasi perubahan hati berkaitan umur, terutamanya dari segi laluan metabolik dan ciri histologi, walaupun terdapat penurunan aktiviti antioksidan yang berterusan. Kajian ini memberikan pandangan mengenai laluan metabolik utama yang terlibat dan menyediakan asas untuk strategi terapeutik masa depan yang menyasarkan kesihatan hati semasa penuaan.

ABSTRACT

Ageing is a major contributor to metabolic dysfunction and chronic liver diseases. As the liver is a metabolic hub regulating energy metabolism, detoxification, and redox homeostasis, age-related hepatic alterations have health-related consequences. Ageing is associated with progressive structural and metabolic changes in the liver, contributing to oxidative stress and inflammation. Thus, understanding the mechanisms underlying liver ageing is essential for developing strategies to preserve metabolic health during ageing. TRF has been reported to possess antioxidant and anti-inflammatory properties, to modulate energy metabolism, and to counteract age-associated hepatic oxidative and metabolic disturbances. However, the relationship between oxidative stress, inflammation, histological alterations, and metabolic pathway changes during liver ageing, as well as the potential modulatory role of TRF, remain unclear. Metabolomics enables comprehensive profiling of small-molecule metabolites, facilitating the identification of age-related pathway-level alterations. This method provides insight into how TRF may modify age-related metabolic abnormalities and complements the biochemical and histological evaluations. Nevertheless, this platform has not been extensively applied in this context. Therefore, this study aimed to characterise age-related changes in hepatic oxidative stress, inflammatory markers, histology, and metabolic pathways, and to determine whether TRF modulates these alterations using an untargeted metabolomics approach. Sprague-Dawley rats aged 3, 9, and 21 months were assigned to control and treatment groups, receiving either palm olein or TRF supplementation. Liver tissues were analysed using untargeted metabolomics via LC-MS/MS, along with histological evaluation and biochemical assessments of antioxidant enzymes (SOD, CAT), inflammatory markers (IL-6, TNF-alpha), and lipid peroxidation markers (MDA). SOD activity declined significantly with age in both the control and TRF-treated old groups ($p<0.05$), suggesting reduced antioxidant defence with increasing age, whereas no significant changes were observed in CAT, IL-6, or MDA levels. TNF-α was significantly higher in the old TRF-treated group than in the young TRF-treated group. Histologically, TRF-treated livers displayed both milder fibrotic-like alterations and inflammatory cell infiltration than control livers. Metabolomics revealed age-related alterations in taurine and hypotaurine metabolism, pyrimidine metabolism (uridine and uracil) and TCA cycle components (malate and glutamate). These findings suggest that TRF might modulate age-related liver changes in morphology and metabolism, despite a persistent decline in antioxidant activity. It provides insight into key metabolic pathways involved, offering a foundation for future therapeutic strategies targeting liver ageing.

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α	Alpha
□	Beta
γ	Gamma
□	Delta
°C	Degree Celsius
µ □	Microlitre
µ □	Micromolar
µ g	Microgram
g	Gram
mg	Milligram
kg	Kilogram
mL	Millilitre
mm	Millimetre
nm	Nanometre
M	Molar
n	Number of samples

h	Hour
min	Minute
s	Second
%	Percent
±	Plus/minus (standard deviation or error)
pH	Potential of Hydrogen

LIST OF ABBREVIATIONS

UKM	Universiti Kebangsaan Malaysia
TRF	Tocotrienol-Rich Fraction
WHO	World Health Organization
LC-MS/MS	Liquid Chromatography□Mass Spectrometry/Mass Spectrometry
SOD	Superoxide dismutase
CAT	Catalase
MDA	Malondialdehyde
HDL	High-density lipoproteins
NASH	Non-alcoholic steatohepatitis
NAFLD	Nonalcoholic fatty liver disease
ROS	Reactive Oxygen Species
PEPCK	Phosphoenolpyruvate carboxykinase
G6Pase	Glu-6-phosphatase
FAO	Fatty acid oxidation
RCT	Reverse cholesterol transport
□ □ □	Superoxide radicals
OH	Hydroxyl radicals
NO	Nitric oxide
H2O2	Hydrogen peroxide
4-HNE	4-hydroxy-2-nonenal
GR	Glutathione reductase
Trx	Thioredoxins
MnSOD/SOD2	Manganese Superoxide Dismutase
Bcl-2	B-cell lymphoma-2
p21	Cyclin-dependent kinase inhibitor 1
p53	Tumour protein p53

Nrf2	Nuclear factor erythroid 2-related factor
NF-κ □	Nuclear Factor Kappa B
ELISA	Enzyme-Linked Immunosorbent Assay
GSH	Glutathione
H&E	Hematoxylin and Eosin
IL-6	Interleukin-6
TNF-alpha	Tumor Necrosis Factor-alpha
IL-□ □	Interleukin-1 Beta
IKK	Inhibitory-κ □kinase
SASP	Senescence-associated secretory phenotype
MAPK	Mitogen-activated protein kinase
T □ F □	Transforming growth factor beta
DCM	Dicromethane
MS	Mass Spectrometry
NADPH	Nicotinamide Adenine Dinucleotide Phosphate
PGC-□ α	Peroxisome proliferator-activated receptor gamma coactivator-1 alpha
PGC-□ □	Peroxisome proliferator-activated receptor gamma coactivator-1 beta
GLI1	Glioma-associated oncogene homolog 1
GLI2	Glioma-associated oncogene homolog 2
hTERT	Human telomerase reverse transcriptase
AMPK	Adenosine monophosphate-activated protein kinase
ER	Endoplasmic reticulum
mTOR	Mammalian Target of Rapamycin
AGEs	Advanced glycation end-products
mtDNA	Mitochondrial DNA
ATP	Adenosine Triphosphate
DNA	Deoxyribonucleic Acid

SOD1	Superoxide Dismutase 1
TLR	Toll-Like Receptor
CRP	C-reactive protein
BCAAs	Branched-chain amino acids
TMB	□ , □ -Tetramethylbenzidine
HRP	Horseradish Peroxidase
DPX	Dibutylphthalate Polystyrene Xylene
BSA	Bovine serum albumin
□ u □ □ 4	Copper (II) sulphate
KI	Potassium iodide
NaOH	Sodium hydroxide
LARU	Laboratory Animal Resources Unit
RBD	Refined, bleached, and deodorised
PBS	Phosphate-Buffered Saline
PCA	Principal Component Analysis
QC	Quality Control
SD	Sprague Dawley
SPSS	Statistical Package for the Social Sciences

CHAPTER I

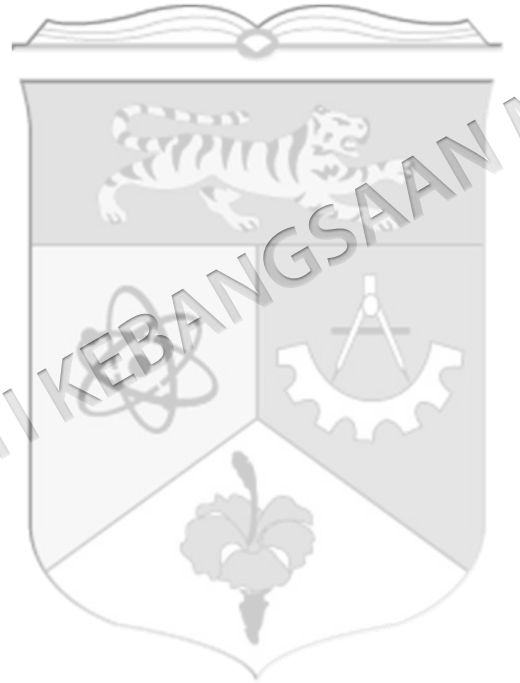
INTRODUCTION

1.1 RESEARCH BACKGROUND

Ageing is an inevitable process characterised by the gradual decline in biological functions and the accumulation of structural and molecular alterations in the body over time (Eggers et al. 2025). Currently, ageing represents the foremost demographic challenge both now and, in the years, to follow. The majority of people on earth are in their middle years or older, and the number of people over 60 is rising quicker than that of the rest of the population (Zulfah et al. 2021) (Blinkouskaya & Weickenmeier 2021)(Zulfah et al. 2021)In 2019, approximately 1 billion people worldwide were aged 60 years or older, a number projected to reach 1.4 billion by 2030 and 2.1 billion by 2050. This unprecedented demographic shift is expected to place substantial pressure on healthcare systems and individuals due to the increased prevalence of age-related frailty, chronic illnesses and cognitive decline (Arum et al. 2014a; Monamo et al. 2025).

Indeed, advanced age is a risk factor for a wide spectrum of conditions, including cardiovascular diseases (Zambon et al. 2009), metabolic disorders (Tomaru et al. 2012), and neurodegenerative diseases (Leary et al. 2000). This heightened susceptibility to disease is driven by interconnected processes,

including oxidative stress, chronic inflammation, metabolic dysregulation, and functional decline at molecular, cellular, and tissue levels (Elkordy 2025; Garrido et al. 2019; Tokarz et al. 2021). Given the multifactorial nature of ageing, studying isolated molecular changes is insufficient to fully capture its complexity. Therefore, high-t h r o u g h p u t a p p r o a c h e s s u technologies have become increasingly important for investigating ageing as a systemic and multifactorial phenomenon (Mora et al. 2022).



Among the 'omics' technologies, metabolomics, which focuses on low-molecular-weight metabolites including metabolic intermediates and end products, occupies a unique position (Eylem et al. 2022; Tokarz et al. 2021). Metabolites represent the downstream products of gene expression and protein activity, providing a direct functional readout of biological processes and reflecting real-time biochemical responses to physiological stimuli (Tzu-Ming Liu 2025). Thus, they are a crucial component of the metabolic signature that represents the overall state of the organism during the ageing process (Chan et al. 2019; Hoogerland et al. 2019). Furthermore, metabolites play essential roles in energy metabolism and cellular signalling, and metabolite profiling enables the characterization of physiological and pathological conditions by capturing a wide range of metabolites associated with biological processes (Yu et al. 2012). Systemic metabolic alterations are closely associated with ageing (Tang et al. 2023), reflecting disruptions across multiple interconnected pathways (F. Zhang et al. 2021). Therefore, untargeted metabolomics provides a comprehensive, systems-level snapshot of metabolic changes associated with ageing. In recent years, metabolomic profiling has become a valuable analytical technique for studying global metabolic changes in biological systems, identifying putative biomarkers and providing insights into age-related metabolic shifts. (Choi et al. 2025; Menni et al. 2013)

These metabolic changes are largely reflective of the performance of the body's core metabolic machinery (Yu et al. 2023). Central to this machinery is the liver. As a high-capacity metabolic hub, the liver is predisposed to progressive alterations in both structure and function throughout the lifespan (Hafez & Elbassuoni 2022; Luceri et al. 2018; Saleh et al. 2019). The liver preserves overall body homeostasis through the control of molecular biosynthesis, endobiotic and xenobiotic removal, and energy metabolism (Kalra et al. 2023). Due to its primary role in regulating metabolism, the liver is susceptible to cumulative molecular damage associated with ageing, making it a key target for metabolomic investigation (Ekeuku et al. 2024).

Additionally, with advancing age, the liver undergoes progressive structural and functional changes, including the development of cellular senescence and chronic low-grade inflammation, which collectively impair hepatic function (Huang et al. 2021;

Maeso-Díaz et al. 2019; Zoli et al. 1999). These impairments can also impact the metabolism of lipids, carbohydrates, and proteins, and their key metabolic pathways. Thus, are accompanied by numerous metabolic diseases, such as liver fibrosis, nonalcoholic fatty liver disease (NAFLD), and metabolic syndrome (Maeso-Díaz et al. 2019; Sabir et al. 2022; Yki-Järvinen 2014). For example, in NAFLD, the lipid metabolism is altered which can lead to the accumulation of triglycerides (Williams et al. 2011). In essence, the excess of fatty acids can elevate cellular stress levels, ultimately leading to cytotoxicity, which in turn leads to more age-related diseases, including type-2 diabetes, CVD, etc. (Fujita et al. 2018; Smith et al. 2020). Consequently, ageing is often characterized by upsurge of lipid synthesis and accumulation in liver (Hornburg et al. 2023; Mentha et al. 1992; Williams et al. 2011). At the cellular level, hepatocytes in aged mice not only accumulate more lipids but also exhibit increased uptake of both cholesterol and glucose compared to their younger counterparts, explaining the mechanism underlying lipid accumulation in the ageing liver (Seo et al. 2019). This coupling contributes to fluctuations in blood sugar levels and elevates the risk of insulin resistance, which is also related to NAFLD and type-2 diabetes in older age (Mitra et al. 2020).

Additionally, with ageing there are alterations in glucose metabolism (Gaspar et al. 2020), which are indicated by the elevated gluconeogenesis and increased expression of enzymes such as Glu-6-phosphatase and phosphoenolpyruvate carboxykinase ultimately leading to enhanced glucose production in the liver (Gaspar et al. 2020). The changes in metabolic pathways within the liver during ageing are further characterised by a relative decrease in components of the tricarboxylic acid (TCA) cycle and an increase in pyruvate levels, indicating a suppression of downstream pathways mediated by pyruvate (Roichman et al. 2021). These age-related alterations in liver function, including lipid accumulation, compromised glucose metabolism, and disrupted TCA cycle, can lead to various hepatic disorders such as steatohepatitis and cirrhosis, thereby affecting overall systemic glucose metabolism and potentially contributing to metabolic syndromes (Lanaspa et al. 2012; Mu et al. 2025; Zheng et al. 2023).

In metabolic syndromes and other age-related diseases a, heightened amount of reactive oxygen species (ROS) and oxidative stress were seen to be associated with

ageing (Banerjee et al. 2023; Chen et al. 2022). Increased oxidative stress contributes to elevated lipid accumulation within the liver with increased age thus causing inflammation and fibrosis (Maimunah et al. 2024). Conversely, reducing oxidative stress can have a lipid-lowering effect within hepatocytes (Hassan et al. 2014). and improved liver wellness (Arroyave-Ospina et al. 2021).

As individuals age, the gradual increase in ROS production can have detrimental effects on the liver. These ROS, including free radicals, hydroxyl radicals, superoxide radicals, nitric oxide and hydrogen peroxide, disrupt the body's biological equilibrium and cause oxidative damage (Liu et al. 2022). Antioxidant enzymes and small-molecule antioxidants regulate ROS levels within cells. Because of the disparity between ROS and biological antioxidant mechanisms, it results in oxidative stress. Antioxidant enzymes, such as catalase (CAT), glutathione peroxidase (GPX), glutathione reductase (GR), superoxide dismutase (SOD), and thioredoxins (Trx) reduce oxidative stress and reduce the ageing process (Yener et al. 2024). For example, to counteract the generation of superoxide, manganese-dependent superoxide dismutase (MnSOD) catalyses the dismutation of superoxide into hydrogen peroxide and oxygen (Harris et al. 2019). As in hepatic tissue, MnSOD, along with other antioxidant enzymes, declines with advancing age (Saleh et al. 2019). Additionally, the Bcl-2 apoptosis pathway, which involves Bcl-2 family proteins and apoptosis-related genes including p21 and p53, is influenced by SOD, CAT, and GSH-Px (Hu et al. 2019). These antioxidant enzymes have a vital role in the nuclear factor erythroid 2-related factor (Nrf2) pathway, which modulates the antioxidant protein expression during inflammation. Nrf2 regulates CAT, SOD, and a wide range of other antioxidant molecules (He et al. 2020). The release of ROS and oxidative stress activates the nuclear factor kappa B pathway (NF-kB) (Atia et al. 2021) and IKK/NF-kB signaling pathway has been suggested to play a crucial role in the regulation of the ageing process (Liu et al. 2020). NF-kB leads to the upregulation of proinflammatory cytokines-Alpha) and IL-6 (Interleukin-6), amplifying liver inflammation during the ageing process (Cavaliere et al. 2023; Ekeuku et al. 2024). Elevation of these cytokines contributes to oxidative damage, metabolic dysfunction and progression of age-related liver disorder(Kundu et al. 2020; Luo et al. 2019; Papatheodoridi et al. 2020; Saleh et al. 2019;;Wong et al. 2020). In parallel, excessive ROS induces lipid peroxidation through oxidative damage

to membrane lipids (Gao et al. 2020), which produces two primary products: 4-hydroxy-2-nonenal (4-HNE) and malondialdehyde (MDA). Studies have reported that these lipid peroxidation markers are elevated in ageing liver □ □ o -□rb ub a s et al. 1997; Uysal et al. 1989).

Furthermore, stress related damage in the liver cells can cause cellular senescence and cause age related steatosis (Ogrodnik et al. 2017; Xu et al. 2018). The oxidative stress results in the buildup of lipid peroxidation byproducts, along with disruptions in the regulation of signaling pathways associated with senescence (Huang et al. 2021). Substances released by senescent cells, known as, senescence-associated secretory phenotype (SASP), drive persistent inflammation and have the potential to trigger senescence in otherwise healthy cells (Xu et al. 2018). The senescent hepatocytes release pro-inflammatory substances such as IL-6 and TNF-alpha (Huang et al. 2021; Kundu et al. 2020; Papatheodoridi et al. 2020). Subsequently, liver cell senescence alters multiple metabolic pathways, including NF-κ □, □ □ □ □, -□ a n d T □ (Anerillas et al. 2020). Collectively, these age-related alterations highlight the liver as a critical organ in the progression of metabolic dysfunction during ageing.

Given the central role of the liver in regulating systemic metabolism, metabolomics provides a thorough approach to study hepatic ageing. As the liver governs key biochemical pathways involving lipids, carbohydrates, and amino acids, metabolomic profiling enables the comprehensive assessment of metabolic alterations occurring within this organ (Banerjee et al. 2023; Choi et al. 2025; Yang et al. 2015). Therefore, liver-focused metabolomic profiling provides an in-depth understanding of how ageing disrupts metabolic homeostasis and a valuable approach to characterise age-related biochemical changes and their association with oxidative stress and other physiological processes in the liver (Guo et al. 2024).

As mentioned before, a major contributor to these age-related hepatic changes is increased oxidative stress and chronic low-grade inflammation, which disrupt metabolic homeostasis (Bárcena et al. 2021; Cavaliere et al. 2023; Ekeuku et al. 2024; F. Zhang et al. 2021). Therefore, interventions that can attenuate oxidative stress and inflammation may play a crucial role in preserving liver function during ageing.

Given that oxidative stress and inflammation are key drivers of hepatic ageing, antioxidant-based interventions may play a protective role (Mustafa 2023). Vitamin E can serve as a fat-soluble antioxidant combating hepatic oxidative stress and inflammation (Ara et al. 2025). Research shows growing evidence that vitamin E, specifically its isomer tocotrienol, can prevent illnesses related to age and age-related metabolic disturbances, such as obesity (Al-Baiaty et al. 2021; Zhao et al. 2016)(Al-Baiaty et al. 2021; Zhao et al. 2016). There are eight different types of vitamin E, including four tocopherols and four tocotrienols (Sharif et al. 2025). Tocotrienols have homologs of delta □ □gamma □ γ beta, □ □and alpha □ α. According to previous clinical studies, tocotrienols have more potent anti-oxidative and anti-inflammatory qualities than tocopherols (Pervez et al. 2022) . Palm oil is a rich source of tocotrienols and is often referred to as the tocotrienol-rich fraction (TRF) (Al-Baiaty et al. 2021), comprising approximately 75% tocotrienols and 25% tocopherols (Zainal et al. 2019). TRF has demonstrated robust capabilities as an antioxidant and anti-inflammatory agent (In Het Panhuis et al. 2023;Mathew et al. 2023). Consistent with this, there is an increased focus in exploring the potential roles of tocotrienols in the prevention and treatment of chronic and age-related illnesses (Al-Baiaty et al. 2021; Makpol et al. 2011; Qureshi et al. 2001). TRF enhances endogenous antioxidant defences primarily through regulation of key enzymatic systems, including SOD and CAT (Abd Manan et al. 2012; Budin et al. 2009). Experimental studies have shown that tocotrienol treatment effectively attenuates oxidative stress by preventing lipid peroxidation and restoring antioxidant status (Budin et al. 2009; S. K. Wong et al. 2020). In animal models, tocotrienol administration reversed oxidative damage, as evidenced by reduced MDA levels alongside preservation of SOD and GPx activities and intracellular glutathione content, indicating improved redox balance (Abd Manan et al. 2012; Budin et al. 2009). In older individuals, reduced SOD activity following TRF supplementation has been attributed to diminished superoxide generation due to the radical-scavenging action of tocotrienols and tocopherols, whereas increased GPx activity reflects an adaptive response to enhanced hydrogen peroxide detoxification (Chin et al. 2011b). Concurrently, tocotrienols suppress inflammatory signalling by inhibiting NF-κ □ activation and downregulating pro-inflammatory cytokines, including TNF-α and IL-6 (Wu et al. 2008; Yam et al. 2009). This dual regulation of redox and inflammatory pathways is particularly relevant in ageing, where chronic low-grade inflammation and

oxidative stress are closely linked. TRF reduces oxidative damage to lipids and DNA within months of supplementation, highlighting its effectiveness in limiting cumulative oxidative injury associated with ageing (Kamat et al. 1997; Mutalib et al. 2003).

TRF is highly relevant to liver ageing due to its ability to modulate key biological processes that deteriorate with age, many of which are directly reflected in hepatic metabolomic profiles (Ekeuku et al. 2024; Fang et al. 2023). TRF supplements have been shown to promote healthy ageing (Goon et al. 2024), antioxidant properties and affect biochemical pathways, such as, bile acid and cholesterol mechanisms (Md Shahrulnizam et al. 2024). Although there have been studies on metabolites in ageing liver (Choi et al. 2025; Han et al. 2021), there have been no studies on metabolites altered in ageing liver with and without TRF supplementation, and metabolomic studies on TRF and ageing remain limited. Reversing the precise microenvironment in the ageing liver and effectively counteracting its consequences is a critical area of research, and there is a clear need to study metabolomic alterations in the liver with an antioxidant like TRF. In relation to TRF, metabolomics provides a systems-level perspective on TRF-mediated metabolic regulation in the ageing liver. Because TRF affects multiple interconnected metabolic pathways, metabolomics provides a powerful approach to capture the complexity of the ageing liver microenvironment and assess how TRF modulates age-associated hepatic metabolism. This is especially important as TRF not only exerts antioxidant and anti-inflammatory effects but also modulates key pathways involved in cellular homeostasis (Md Shahrulnizam et al. 2024). Indeed, studies using NAFLD animal models have demonstrated that TRF supplementation alters multiple metabolic pathways, including purine metabolism, fatty acid oxidation, bile acid metabolism, and lipid metabolism, highlighting its broad impact on hepatic metabolic networks (Goon et al. 2025). Therefore, metabolomics is an essential tool for comprehensively characterising the metabolic effects of TRF that cannot be fully captured by conventional methods. Besides, as metabolomics provides a snapshot of the biochemical state of the ageing liver, it is well-positioned to reflect the interaction between age-related hepatic alterations and interventions such as TRF that modulate ageing-associated metabolites, including BCAAs and TCA cycle intermediates, highlighting its influence on key metabolic processes related to ageing (Saud Gany et al. 2022; Selvaraju et al. 2014).

Despite advances in understanding liver ageing and the therapeutic potential of antioxidants such as TRF, the integrated effects of TRF on age-related metabolic alterations in the liver remain insufficiently explored at the metabolomic level (Azman et al. 2018; Han et al. 2021b; Wu et al. 2022). Combining liver-focused metabolomics with antioxidant intervention provides a comprehensive framework to simultaneously investigate metabolic dysregulation, oxidative stress, and inflammation. This integrated approach is essential for elucidating the complex interactions underlying hepatic ageing and for identifying potential metabolic targets for intervention.

Therefore, in our study, we aim to investigate the metabolomic changes associated with ageing in the livers of Sprague Dawley rats, a widely used model for studying ageing and metabolic disorders. By comparing young, adult, and old rats, we seek to identify key metabolites and metabolic pathways that are altered with age and to assess the relationship between these changes and the expression of oxidative stress markers. This research will contribute to a better understanding of the molecular mechanisms underlying age-related liver dysfunction. It may offer potential targets for therapeutic interventions to mitigate the effects of ageing.

Further, we will explore the role of TRF as an antioxidant in mitigating the adverse effects of ageing on the liver, shedding light on strategies to promote liver health throughout the ageing journey and utilising metabolomic analysis to relate metabolites and lipids to changes associated with ageing liver. Moreover, we will examine oxidative stress and inflammatory markers to investigate further the changes that occur in various pathways during ageing and how TRF affects these pathways in the liver. Understanding these multifaceted age-related liver changes is essential for addressing the unique health needs of older individuals and enhancing their overall well-being.

1.2 PROBLEM STATEMENT

The problem addressed by this study is the significant impact of age-related liver changes, characterised by oxidative stress, inflammation, and disrupted metabolic pathways, on the health of the ageing population. These changes can lead to various liver disorders, including liver fibrosis and NAFLD, posing substantial healthcare

challenges. Consequently, there is a critical need to explore potential interventions, such as TRF, as a therapeutic agent to alleviate age-related hepatic dysfunction. TRF has been reported to possess strong antioxidant and anti-inflammatory properties that may help mitigate oxidative stress and inflammatory responses associated with ageing. However, the potential role of TRF in modulating age-related hepatic metabolic alterations remains insufficiently understood. Therefore, further investigation is required to evaluate whether TRF can alleviate age-related liver changes and to develop new strategies to improve liver health in the ageing population, thereby potentially reducing the healthcare burden associated with age-related liver disorders.

1.3 OBJECTIVE OF RESEARCH AND SCOPE OF WORK

1.3.1 General Objective

To elucidate the hepatic changes associated with ageing through metabolomic profiling and their modulation by tocotrienol-rich fraction supplementation.

1.3.2 Specific Objective

1. To determine the changes in liver metabolites with ageing in Sprague Dawley (SD) rats by metabolomic profiling.
2. To evaluate the histological changes and expression of oxidative stress, lipid peroxidation and inflammatory markers in the liver of SD rats with ageing using H&E staining, enzyme assays and ELISA
3. To determine the modulation of tocotrienol-rich fraction (TRF) on hepatic changes with ageing in SD rats by metabolomic profiling, H&E staining, enzyme assays and ELISA.

1.4 HYPOTHESIS

This study hypothesizes that ageing in Sprague Dawley rats will induce alterations in hepatic metabolomic profiles, accompanied by histological changes, oxidative stress, lipid peroxidation, and inflammatory responses, and that tocotrienol-rich fraction (TRF) supplementation will modulate these age-related hepatic alterations.

CHAPTER II

LITERATURE REVIEW

2.1 THE LIVER AND THE HALLMARKS OF AGEING

Life expectancy has increased globally. Studies highlight that health expectancy has not kept pace, resulting in a widening gap between lifespan and healthspan (Chen et al. 2024; Islam et al. 2018). This divergence is further reinforced by reports showing that extended lifespan is often accompanied by a higher burden of chronic diseases (Chan et al. 2023; Garmany & Terzic 2024; Hu et al. 2022), suggesting that longevity does not necessarily equate to healthy ageing. In parallel, the rapid growth of the ageing population (Lee & Miller 2002), together with the strong association between age and healthcare expenditure (Mason & Miller 2018), underscores the increasing societal and economic impact of ageing. Therefore, understanding the underlying mechanisms of ageing has become increasingly important.

Ageing has been described from multiple complementary perspectives. While some studies define it as a progressive decline in physiological function (Erez et al. 2016; Manyilov et al. 2023), others emphasise it as a reflection of changes at the cellular, tissue, and organ levels (Nie et al. 2022; Phillip et al. 2017). The body's capacity to sustain a stable internal environment in the face of persistent internal and external stimuli shapes how we age (Forsberg et al. 2012). Important functions like immunological response, metabolic control, and cellular repair are supported by this equilibrium (Manyilov et al. 2023a; Ocampo et al. 2016; Salpeter et al. 2013). Extending this view, ageing is widely characterised as the progressive accumulation of molecular and cellular damage over time, ultimately leading to functional deterioration and increased disease susceptibility (Manyilov et al. 2023). These can cause physiological

deterioration when disturbed which manifests across organ systems: muscle mass and strength decline with age, contributing to sarcopenia (Dodds et al.

2017), which is associated with inflammation, mitochondrial dysfunction, and increased mortality risk (Newman et al. 2006; Xue et al. 2010).Cardiovascular disease, the leading cause of death in older adults, is similarly driven by age-dependent vascular remodelling and chronic inflammation (Zambon et al. 2009). Furthermore, age-related diseases are a leading cause of morbidity and mortality, imposing significant economic and social burdens (Fledsberg et al. 2023). These conditions include chronic conditions, including diabetes, neurodegenerative diseases, cardiovascular disease, musculoskeletal disorders, osteoporosis, and osteoarthritis, to name a few (Chen et al. 2022, 2024; Hasan et al. 2023; Jauhari et al. 2020; Wijsman et al. 2011; Xue et al. 2010). These can be influenced not only by intrinsic cellular damage but also by systemic factors (Ximerakis et al. 2023). While earlier views focused on cell-autonomous mechanisms, emerging evidence highlights the role of intercellular communication. Crucially, ageing is not purely cell-autonomous, research showed that exposing aged progenitor cells to a young systemic environment restores their function, suggesting that circulating factors actively contribute to age-related decline, which also support the role of systemic signals, such as metabolites, in shaping the ageing process (Ximerakis et al. 2023). Previous research suggest that ageing is not a single process, but rather the outcome of interconnected functional decline and cumulative biological damage, which speeds up ageing and makes people more vulnerable to age-related illnesses (Arum et al. 2014b; Faggioli et al. 2012; Manyilov et al. 2023b; Wijsman et al. 2011).

To organize these processes, a framework of hallmarks was proposed: telomere shortening, epigenetic modifications, compromised protein homeostasis, compromised mitochondrial function, disrupted nutrition sensing, cellular senescence, stem cell exhaustion, and disrupted intercellular communication chronic inflammation, disabled macroautophagy, and dysbiosis (Devesa-Peiro et al. 2022; Fraser et al. 2022; Ximerakis et al. 2023). Critically, these hallmarks do not operate in isolation, but they form an interconnected network of reinforcing feedback loops (Fraser et al. 2022). For instance, telomere dysfunction activates p53, which directly represses the mitochondrial regulators PGC-□α and□□, □□establishing a molecular attrition and mitochondrial dysfunction across liver, heart, and haematopoietic stem cells (Sahin et al. 2011). Same research also shows that mitochondrial dysfunction in turn increases reactive oxygen species production, which drives further DNA damage,

epigenetic alterations, and cellular senescence creating a self-amplifying cycle of deterioration. The liver is a natural focus for studying these processes, given its central role in metabolic homeostasis, detoxification, and immune regulation (Gao et al. 2020; Hamelin et al. 2007; Niu et al. 2022). It governs glucose, lipid, and protein metabolism through tightly regulated pathways, for instance, modulating glycogen storage versus gluconeogenesis in response to energy availability and clears both endogenous metabolites and exogenous xenobiotics (Asiwe et al. 2024; Cavaliere et al. 2023; Garrido et al. 2019). Age-related impairments in hepatic function therefore have widespread systemic consequences (Gao et al. 2020; Niu et al. 2022). The liver is particularly vulnerable to the interconnected decline (Sinha et al. 2025). The liver undergoes all the cellular hallmarks of ageing, driven by genomic and epigenomic alterations that dysregulate mitochondrial function and nutrient sensing, leading to cellular senescence and chronic low-grade inflammation (Cogger et al. 2003; Le Couteur et al. 2011; □ y s -g □ a d y st al. 2021; Schulz et al. 2012; Sinha et al. 2025). Consistent with these changes, a study showed that the composition of organelles and functional output are altered by liver ageing and aged hepatocytes accumulate d a ma g e d mi t o c h o n d r i a , l y g o s o m e s , s a n d e l i p Structural and metabolic impairments with hepatic ageing have also been reported, including lipid accumulation, collagen deposition, and reduced vitamin A metabolism (Cogger et al. 2003) At the molecular level, epigenetic studies further demonstrate age-associated remodelling in the liver. For instance, a study analyzed ~455,000 CpG sites in human liver, identified over 20,000 sites with age-associated altered DNA methylation, some of which overlap with systemic ageing signatures observed in blood (Bysani et al. 2017) Similarly, another study reported 8,823 age-associated differentially methylated CpG sites in human liver, where pathways such as Wnt signalling and epithelial□mesenchymal transition were implicated (Bacalini et al. 2019). When it comes to senescence and inflammation, cellular senescence connects to chronic inflammation through the senescence-associated secretory phenotype (SASP). Previous research developed a model of stress-induced hepatocyte senescence and characterised its unique secretory profile, showing that senescent hepatocytes release IL-8, IL-6, serum amyloid A4, and fibrinogen, and that conditioned medium from these cells promotes inflammatory macrophage migration.(Irvine et al. 2014)

These converging processes examine telomere-driven mitochondrial compromise, senescence-driven inflammation, and epigenetic erosion of metabolic gene regulation examine ultimately drive the spectrum of age-related liver diseases, from non-alcoholic fatty liver disease through fibrosis to hepatocellular carcinoma (Lara-Lemus et al. 2024; Niu et al. 2022; Zhou et al. 2016). The following sections examine in detail the key mechanisms examine oxidative stress, inflammation, and structural alterations examine through which these hallmarks manifest in the ageing liver.

Discussing the hallmarks of ageing is important in the context of this study because they provide the mechanistic framework underlying the hepatic alterations investigated. Several hallmarks, including mitochondrial dysfunction, altered nutrient sensing, cellular senescence, and chronic inflammation, are closely associated with the metabolic dysregulation, oxidative stress, lipid peroxidation, and inflammatory responses observed in the ageing liver (Devesa-Peiro et al. 2022; Fraser et al. 2022; Ximerakis et al. 2023). Since this study evaluates hepatic metabolomic, biochemical, and histological changes during ageing, understanding these interconnected ageing mechanisms is essential for interpreting how ageing contributes to liver dysfunction and how tocotrienol-rich fraction (TRF) may modulate these processes.

2.2 STRUCTURAL ALTERATION IN THE AGEING LIVER

As a key metabolic organ, the liver changes significantly in both structure and function in ageing organisms (Sinha et al. 2025). Hepatic tissue's ability to operate and maintain its structural integrity gradually deteriorates with age. At the macroscopic level, the liver undergoes a progressive reduction in volume with advancing age (Tajir & Shimizu 2013). This is partially explained by a decline in the number of hepatocytes and an increase in cellular senescence (Schmucker & Sachs 2002). Recent evidence identifies hepatocyte enlargement as a key structural feature of liver ageing, often associated with increased nuclear ploidy (Sinha et al. 2025). In addition to that, ageing also affects the relative quantities of hepatocyte organelles. As reported, age-related cytoplasmic accumulation of highly oxidized insoluble protein lipofuscin, also known as age pigment, is a common alteration in diagnostic liver biopsy results (Schmucker & Sachs

2002). This accumulation is indicative of a corresponding rise in the volume of the dense body compartment. Additionally, polyploid hepatocytes are the main source of physiological hepatocyte turnover during ageing, with no evidence of a major contribution from diploid liver stem cells, according to a study that used lineage tracing in Rosa-RGBow mice (Matsumoto et al. 2021). According to their RNA sequencing, polyploid hepatocytes upregulate genes related to mitochondrial function at baseline but significantly downregulate these genes as they age, indicating that polyploid cells may be more susceptible to mitochondrial malfunction as they age. Most recently, a study used single-nucleus RNA sequencing to show that polyploidisation pleiotropically buffers against age-related decline: tetraploid hepatocytes exhibit more robust transcriptional networks and can restore wild-type genome composition through non-random allelic segregation, while haploinsufficiency of master regulators (HNF4A, CEBPA) that promotes early tetraploidisation dramatically suppressed age-related steatosis tissue-wide (Yin et al. 2024). The deterioration of hepatic sinusoidal endothelial cells, which are crucial in order to preserve hepatic metabolic homeostasis, is one of the main causes of decline in the hepatic function as ageing advances (Hansen et al. 2002). Advanced glycation end-products (AGE), which have been linked to inflammation, oxidative stress, and fibrotic alterations in the liver tissue, accumulate due to the age-related reduction in liver sinusoidal endothelial cells function (Mousavi et al. 2007).

Furthermore, a reduction in autophagic activity, which is necessary for removing damaged organelles and protein aggregates, causes cellular debris to accumulate in the cytoplasm, which exacerbates hepatocellular dysfunction (Kim et al. 2021; Ravikumar et al. 2002). These cellular modifications eventually lead to noticeable structural changes, such as increased fibrotic deposition, sinusoidal capillarization, and lobular disarray. When combined, these structural and functional impairments provide a crucial basis for comprehending therapies meant to maintain liver health in ageing populations and explain the steady loss in hepatic performance with age (Cuervo et al. 1995; Schneider et al. 2014).

Together, these structural alterations and functional alterations creates a microenvironment increasingly vulnerable to oxidative stress and inflammation (Gan et al. 2011), which are explored in the following section.

2.3 ROLE OF OXIDATIVE STRESS IN AGE-RELATED HEPATIC DYSFUNCTION

2.3.1 Reactive Oxygen Species (ROS)

Early theories of ageing, such as the free radical theory, proposed that reactive oxygen species (ROS) are key drivers of cumulative molecular damage (Weydert & Cullen 2009). Reactive molecules, ROS, are produced naturally as byproducts of several metabolic activities, particularly when the mitochondria produce energy (Eggers et al. 2025). It serves essential signalling functions at low concentrations but becomes destructive when production exceeds antioxidant capacity and results in a state termed oxidative stress (Hassan et al. 2014). Therefore, even if the small quantities of these molecules are helpful for regular cell signalling but an overabundance harms vital cellular constituents like proteins, lipids, and genetic material (Christensen et al. 2024).

Expanding on this, protein carbonyls represent one of the many ROS-derived modifications and are recognised as key indicators of oxidative stress. They were found altered during liver ageing, reflecting oxidative damage and suggesting the presence of hepatic injury (Luceri et al. 2018). Additionally, mitochondria are both the principal source and primary target of ROS in hepatocytes, placing them at the centre of an age-related vicious cycle (Kivimäki et al. 2024). The genetic basis of this mitochondrial decline involves progressive damage to mitochondrial DNA (mtDNA). In a previous study, mtDNA copy number, cytochrome c oxidase (COX) transcript levels, and COX enzyme activity across tissues in young and aged rats were examined, and a striking 50% decline in hepatic mtDNA copy number with age was found, representing a larger reduction than in any skeletal muscle group (Barazzoni et al. 2000).

Subsequent studies extend this mitochondrial narrative of how ageing disrupts the electron transport chain, reducing ATP production while paradoxically increasing ROS generation (Bárcena et al. 2021; Dankel et al. 2023). Further studies explain that

oxidative stress-induced mitochondrial dysfunction can trigger apoptosis and necrosis, reinforcing a vicious cycle in which damaged mitochondria generate even more ROS (Bejma et al. 2000; Chen et al. 2022a; Mizota et al. 2021).

Beyond the mitochondrial respiration, ROS are also generated during inflammatory responses and immune activation, further amplifying the oxidative burden (Su et al. 2019). As reported, elevated ROS levels can induce widespread molecular damage and mutagenesis, ultimately impairing hepatocellular function (Gao et al. 2020). Additionally, oxidative DNA damage can spread to neighbouring cells through bystander effects mediated by gap junctions and diffusible ROS, while the SASP from senescent hepatocytes amplifies inflammatory signalling and further elevates local ROS production (Bárcena et al. 2021). This convergence of mitochondrial dysfunction, oxidative stress, and inflammation establishes the chronic low-grade oxidative state that characterises the ageing liver (Irvine et al. 2014).

□mportantly, the liver's role as the particularly vulnerable to oxidative stress as continuous exposure to endogenous and exogenous toxins further elevates ROS production (Shingina et al. 2019). At the same time, the antio□idant defence systems dec neutralise reactive species (Yang et al. 2015). This imbalance accelerates structural and functional deterioration, ultimately contributing to chronic inflammation, fibrosis, and carcinogenesis (Shingina et al. 2019).

2.3.2 Antioxidant Defence Mechanisms in the Ageing Liver

The liver is equipped with a coordinated antioxidant defence system, comprising both enzymatic and non-enzymatic components, to counteract oxidative stress (Yang et al. 2015). Superoxide dismutase, catalase, and glutathione-related system enzymes like glutathione peroxidase and glutathione peroxiredoxin are important enzymatic antioxidants that play central roles in detoxifying reactive oxygen species (Watanabe et al. 2014). To efficiently neutralise reactive species, these enzymes are divided into several cellular sites. Superoxide dismutase, for instance, comes in two isoforms: SOD-1, which is found in the cytosol, and SOD-2, which is found in the mitochondria. Superoxide radicals are converted by these enzymes to hydrogen peroxide, which is

then further broken down by mitochondrial enzymes, for example glutathione peroxidase and peroxiredoxins, as well as catalase. To maintain cellular homeostasis and stop oxidative damage to proteins, lipids, and DNA, this multilevel antioxidant system is essential. However, these antioxidant systems' effectiveness tends to decrease with age, which adds to the buildup of oxidative damage and structural degradation in hepatic tissue (Banerjee et al. 2023).

Consistent with this, multiple studies report age-related reduction in hepatic SOD and CAT activities, highlighting a weakened antioxidant capacity in the ageing liver (Bárcena et al. 2021; Périchon & Bourre 1995; Watanabe et al. 2014). Reports further support this decline, reinforcing the idea that impaired superoxide and catalase scavenging is a key feature of hepatic ageing (Bárcena et al. 2021; Yang et al. 2015).

The functional consequences of reduced SOD activity are evident in experimental models in the liver. For instance, Copper/zinc superoxide dismutase (SOD1)-deficient mice exhibit pronounced hepatic lipid accumulation and liver injury, even under conditions of low adiposity, indicating that oxidative stress rather than fat mass drives this phenotype (Kurahashi et al. 2015). Extending this, Sakiyama et al. (2016) demonstrate that SOD1 deficiency disrupts collagen homeostasis, promoting its accumulation and impairing degradation, thereby accelerating fibrotic progression in the liver. These findings collectively suggest that loss of SOD not only enhances oxidative damage but also contributes directly to metabolic dysfunction and fibrosis in liver (Sakiyama et al. 2016). Furthermore, reduced SOD2 expression has been observed in HCC tissues and is associated with ageing, tumour progression, and poorer patient outcomes, suggesting that compromised antioxidant capacity may contribute to both liver ageing and disease severity (R. Wang et al. 2016). Importantly, restoration or enhancement of antioxidant enzyme activity has been associated with improved hepatic outcomes. As reported, increasing SOD and CAT activities reduces lipid peroxidation, inflammation, and fibrosis in diet-induced liver injury, reinforcing the protective role of these enzymes against oxidative stress□mediated hepatic damage (Ulla et al. 2017).

There was a decline in antioxidant activity with age as catalase activity declined (Yang et al. 2015). Supporting the functional importance of SOD in maintaining hepatic

integrity, evidence showed that restoration of SOD1 expression specifically in the liver of deficient mice reduces oxidative damage and improves liver function, ultimately extending lifespan (Zhang et al. 2016). Additionally, there was a decline in key antioxidant genes, including mitochondrial SOD2, in aged livers, particularly under metabolic stress such as prolonged fasting. This reduction was accompanied by increased lipid peroxidation, indicating impaired oxidative defence and reduced adaptability in older organisms (Zhang et al. 2016). Collectively, these findings suggest that the imbalance between ROS detoxification pathways may contribute to increased oxidative damage, impaired metabolic adaptation, and heightened susceptibility to liver dysfunction with advancing age.

In addition to these defences, non-enzymatic antioxidants such as glutathione, ascorbic acid, retinol, and tocotrienols further support the scavenging of free radicals within hepatic cells (Rigobello et al. 2024). These molecules act as critical buffers against oxidative stress, particularly under conditions where enzymatic capacity is compromised. At the regulatory level, antioxidant defence is tightly controlled by transcriptional mechanisms of genes encoding antioxidants, which is governed mainly by nuclear factor E2-related factor 2 (Nrf2), a crucial component of antioxidant defence. Numerous genes that are responsible for maintaining the oxidation-reduction balance within cells have their expression modulated by this transcription factor. It was reported that Nrf2 protein content and the basal and inducible expression of Nrf2-dependent genes were significantly attenuated in hepatocytes from aged rats (Smith et al. 2015). It is increasingly recognised that activating the nuclear factor E2-related factor 2 pathway with exogenous drugs is a viable approach to enhance antioxidant capacity, particularly in ageing-related liver dysfunction (Atia et al. 2021).

2.4 INFLAMMATION IN HEPATIC AGEING

Oxidative stress and persistent low-grade inflammation, sometimes referred to as "inflammageing," are two crucial interrelated processes that frequently make the age-related decrease in cellular function and resilience worse (Yang et al. 2025). Inflammageing is a low-grade chronic inflammatory condition that arises from the

simultaneous impact of internal and external stimuli accumulated throughout life, as opposed to acute inflammation, which is a temporary reaction to illness or injury (Nguyen & Cho 2025). Prolonged immune pathway activation, elevated pro-inflammatory cytokine production, and immunological homeostasis dysregulation are characteristics of this process that all lead to the cumulative functional impairment that comes with ageing (García-Domínguez 2025).

In addition to oxidative stress, pro-inflammatory cytokines including IL-6 and TNF-α are produced at higher levels as people age (Minciullo et al. 2015b). Pro-inflammatory cytokines are implicated in the advancement of age-related diseases, according to recent meta-analytical evidence (Tylutka et al. 2024b). A meta-analysis, which included patients with age-related diseases and healthy controls, examined the levels of the pro-inflammatory cytokines. The study reported that IL-6 levels were significantly higher in elderly patients with diseases compared to healthy controls (Tylutka et al. 2024b).

This suggests that IL-6 may take part in the development of ageing related diseases. This is consistent with the more general idea of "inflammageing," which holds that long-term, low-grade inflammation plays a part in the liver's and other organs' functional decline (Gart et al. 2022; Jauhari et al. 2020). In older populations, higher IL-6 levels could be a useful biomarker for tracking disease progression and maximising treatment. Curiously, TNF-α levels were also discovered groups (Minciullo et al. 2015; Tylutka et al. 2024). Elevated TNF-α and IL-6 levels in aged livers have been linked with disrupted metabolic pathways and impaired mitochondrial function (Cavaliere et al. 2023). Their measurement, often via ELISA, is therefore vital in evaluating the extent of age-related inflammatory changes and the efficacy of therapeutic interventions. Clinical and population-based studies demonstrate that IL-6 is not only elevated in individuals with NAFLD, but is also independently associated with disease severity and related complications, suggesting a central role in linking hepatic inflammation to systemic pathologies (Simon et al. 2018). Similarly, increased circulating levels of IL-6 and TNF-α have been observed syndrome, reinforcing their contribution to chronic low-grade inflammation and metabolic dysregulation (Mohammadi et al. 2017).

Experimental models further support these findings by demonstrating that dysregulation of pro-inflammatory cytokines is closely associated with impaired lipid metabolism in the liver. For instance, elevated TNF-α levels have been correlate with lipid abnormalities in NAFLD models, indicating a mechanistic link between inflammation and hepatic metabolic disturbances (Toktogulova et al. 2021). In addition, interventions that attenuate IL-6 and TNF-α levels have been associated with reduced liver injury, oxidative stress, and fibrosis, highlighting the pathological role of these cytokines in mediating hepatic damage (Asiwe et al. 2024). Thus, IL-6 and TNF-α are not merely markers of inflammation b liver dysfunction.

The role of pro-inflammatory cytokines in hepatic ageing is further supported by both clinical and experimental evidence linking IL-6 and TNF-α to liver in fibrotic progression. Clinical studies in patients with chronic liver conditions, who are often older or exhibit age-related disease progression, demonstrate that circulating levels of TNF-α and IL-6 are positively associated with the severity of liver fibrosis, with TNF-α showing a particularly strong corr driving fibrogenesis (Maimunah et al. 2024). Complementing this, experimental models provide more direct evidence of age-dependent effects, where aged animals exhibit significantly elevated hepatic TNF-α levels and a heightened injury following physiological stress compared to younger counterparts, indicating impaired immune regulation with advancing age (Meng et al. 2023). The contribution of these cytokines to hepatic ageing is further reinforced by intervention studies. In a D-galactose-induced ageing model, which mimics accelerated ageing, it was demonstrated that attenuation of TNF-α levels was associated w stress, improved antioxidant capacity, and decreased fibrotic changes in the liver (Saleh et al. 2019). This suggests that TNF-α is not only elevated wi contributes to the progression of age-related hepatic damage.

Consistently, insulin resistance, fibrosis, and heightened vulnerability to liver disorders such as cirrhosis and NAFLD are influenced by this systemic inflammation (Gart et al. 2022; Jiang et al. 2020; Mak et al. 2012; Niu et al. 2022). In addition to

structural and functional deterioration, ageing of the liver also involves the accumulation of senescent cells, which are influenced by both internal ageing processes and extrinsic factors such as lifestyle, environmental exposure, and chronic liver diseases (Irvine et al. 2014). Liver ageing is primarily caused by cellular senescence, which is characterised by irreversible cell cycle arrest and the pro-inflammatory secretory profile known as SASP (Huang et al. 2021). Stressors such as oxidative stress, telomere shortening, and DNA damage cause senescent hepatocytes to accumulate (Barazzoni et al. 2000), and secrete cytokines, chemokines, and proteases that alter the tissue microenvironment (Zhu et al. 2014). These cells release inflammatory chemicals that impair liver function and continuously signal genomic stress. Senescent cells emit various secreted factors and signalling molecules that are included in SASP (Covarrubias et al. 2020). These chemicals can affect neighbouring cells and change the microenvironment. SASP initially promotes tissue healing and the immune system's removal of injured cells in response to oxidative stress or long-term DNA damage. Long-term SASP activation, on the other hand, promotes ageing-related illnesses such as cancer, fibrosis, and neurodegeneration, disrupts tissue homeostasis, and spreads senescence to neighbouring cells. These characteristics have the potential to disrupt liver function and promote the progression of liver disease (Zhu et al. 2014).

Oxidative stress and inflammation are not independent processes but are intricately connected. ROS can activate transcription factors such as NF-κ□, thereby pro the expression of pro-inflammatory cytokines. In turn, cytokines can further stimulate ROS production, creating a vicious cycle of damage (Masenga et al. 2023). This self-perpetuating loop is particularly detrimental in the ageing liver, where the regenerative capacity is already diminished. Therefore, breaking this cycle by targeting both oxidative and inflammatory pathways is a promising strategy for preserving liver health during ageing.

2.5 LIPID PEROXIDATION MARKER IN HEPATIC AGEING

Hepatic architecture and function gradually deteriorate with age, in part because of the cumulative effects of oxidative stress (Chin et al. 2011). Lipid peroxidation, or the oxidative breakdown of polyunsaturated fatty acids in cell membranes, is a defining feature of oxidative stress in biological systems in the liver. It results in organ dysfunction and hepatocellular damage (Su et al. 2019). As a key metabolic organ with many mitochondria and a role in lipid metabolism, the liver is especially vulnerable to lipid peroxidation as we age (Chin et al. 2011). Lipid peroxidation produces malondialdehyde (MDA), which is frequently employed as a biomarker for oxidative damage in hepatic tissues. Increased MDA levels are intimately associated with hepatocellular damage, inflammation, and mitochondrial dysfunction and have been regularly seen in ageing livers (Jiang et al. 2020; Varma et al. 2016).

Evidence from an early ageing study demonstrates that hepatic MDA levels are significantly elevated in older organisms, indicating increased lipid peroxidation with advancing [illegible] observations, a study provided a more comprehensive picture, in their mouse model, hepatic MDA levels, protein carbonyl content, and DNA oxidation were all significantly elevated in aged livers, and the apoptotic cells presented elevated levels of oxidised DNA, suggesting that ROS-induced apoptosis in these oxidative stress sensitive cells contributes directly to liver ageing (Colantoni et al. 2001).

Lipid peroxidation is caused by the production of ROS, which attack membrane lipids and start a chain reaction (Abd Manan et al. 2012). In the end, this mechanism leads to age-related hepatocyte apoptosis, steatosis, and fibrosis by impairing membrane fluidity and integrity (Abd Manan et al. 2012; Asiwe et al. 2024; Kiliç et al. 2022). Reduced antioxidant enzyme activity, including SOD and CAT, and histological signs of liver deterioration have been linked to elevated hepatic MDA concentrations in animal studies (Su et al. 2019). As animals age, these alterations show a shift in the redox equilibrium that leans towards oxidative damage (Petr et al. 2021). Supporting this, it was reported that elevated levels of MDA and protein carbonyls in aged mouse

liver were accompanied by activation of the Nrf2 pathway and its downstream antioxidant genes (Luo et al. 2022).

The impact of ageing on lipid peroxidation appears to be further exacerbated under pathological conditions. For instance, significantly increased hepatic MDA levels have been observed in aged type 2 diabetic models, indicating that metabolic dysfunction amplifies oxidative damage in the ageing liver(Fan et al. 2022). In line with this, it was demonstrated that ageing is associated with increased lipid peroxidation alongside elevated pro-inflammatory cytokines, including IL-6 and TNF-α ,and reduced antioxidant capacity (Albrahim & Alonazi 2022). This highlights a close interplay between oxidative stress and inflammation in driving hepatic dysfunction.

2.6 HISTOPSTHOLOGICSL CHANGES IN HEPATIC AGEING

A variety of histological changes that represent the liver's deteriorating structural integrity and functional ability are indicative of hepatic ageing (Elkordy 2025). As reported, histopathological assessment remains essential in liver research because it involves a systematic evaluation of changes in individual components of normal liver architecture (Cain & Hübscher 2019). Chronic low-grade inflammation, metabolic dysregulation, and cumulative oxidative stress cause the liver to gradually deteriorate morphologically as it matures (Conde de la Rosa et al. 2022). These alterations are visible under a microscope and are significant markers of liver degeneration in older models (Conde de la Rosa et al. 2022). In the context of ageing specifically, histology is indispensable because the liver's deterioration is not uniform: different cell types, zones, and structural compartments age at different rates and in different ways, and only microscopic examination can capture this heterogeneity (Hafez & Elbassuoni 2022).

For instance, histological changes in the ageing liver include pseudo capillarisation of the sinusoidal endothelium (McLean et al. 2003), demonstrating that ageing is associated with significant thickening of the sinusoidal endothelium. It was confirmed these findings extend across species, reporting similar pseudocapillarisation in old baboons (Cogger et al. 2003) and later validated in rat model (Warren et al. 2005).

Futhermore, the functional significance of these ultrastructural changes was elucidated in perfused rat livers to demonstrate that age-related pseudo capillarisation directly impairs hepatic transendothelial transfer of chylomicrons (Hilmer et al. 2005), providing a mechanistic link between sinusoidal ageing and the postprandial hyperlipidaemia commonly seen in elderly populations.

Beyond the sinusoidal endothelium, age-related changes in hepatic stellate cells (HSCs) have attracted increasing attention (Le Couteur et al. 2011). Evidence show that desmin-positive HSCs doubled in number in old mice and rats, with a substantial increase in the number and size of lipid droplets (Le Couteur et al. 2011). Increased fat accumulation within hepatocytes, known as hepatic steatosis, frequently coexists with age-related structural alterations. Steatosis is especially significant in elderly animals fed a diet high in fat or calories (Sabir et al. 2022). Furthermore, the liver's capacity to regenerate itself is compromised with age, which exacerbates the damage that gradually accumulates. Haematoxylin and eosin (H&E) staining usually shows periportal fibrosis, altered liver architecture, and increased cytoplasmic vacuolation in older mice (Hafez & Elbassuoni 2022). These characteristics, which include compromised detoxification and protein synthesis, are consistent with functional decline (Kiliç et al. 2022).

The spontaneous progression from age-related structural changes to overt fibrosis has been directly demonstrated in recent study (Qiu et al. 2023). Similarly, studies demonstrated that aged mice develop NASH phenotypes (steatosis, lobular inflammation, hepatocellular ballooning) more rapidly and severely than young mice on the same high-fat diet, with fibrosis emerging significantly earlier (Li et al. 2023a; Sagar et al. 2025,), underscoring why histological assessment at multiple time points is critical for capturing the accelerated disease trajectory in ageing models.

At the parenchymal level, the histological hallmarks of ageing include hepatocellular hypertrophy, nuclear enlargement, cytoplasmic vacuolisation, and lipofuscin accumulation (Kivimäki et al. 2024; Ocampo et al. 2016). A previous study documented these changes comprehensively using transmission electron microscopy in 24-month-old Wistar rats, observing sinusoidal collapse and congestion, endothelial thickening and defenestration, cytoplasmic vacuolisation, a significant

increase in the volume densities of nuclei, mitochondria and dense bodies, and marked lipofuscin accumulation (Mahmoud & Hegazy 2016). Numerous experimental models have shown that decreased antioxidant enzyme activity and increased oxidative stress indicators such as MDA are correlated with the degree of histopathological damage (Kiliç et al. 2022). In addition, ApoE-null mice as a model of accelerated liver ageing, showing that oxidative stress drove pseudocapillarisation, increased polyploidy, decreased hepatocyte number, and increased nuclear size in vitamin E deficient mice (Bonomini et al. 2013). This lends credence to the idea that oxidative damage is a significant factor in the age-related structural decline of the liver.

2.7 TOCOTRIENOL-RICH FRACTION (TRF) COMPOSITION AND BIOLOGICAL ACTIVITY

Tocotrienols occur naturally in select plant-based oils, with rice bran oil, annatto bean seeds, and palm oil containing the highest concentrations of tocotrienols (Drotleff et al. 2014; Vardanega et al. 2019). Palm oil, acknowledged as the most abundant natural source, is the primary source of the tocotrienol-rich fraction. Several variables, such as formulation, intestinal absorption methods, and dietary fat consumption, affect tocotrienol bioavailability. There are four forms of tocopherols and tocotrienols (Drotleff et al. 2014). Unlike tocopherols, tocotrienols possess an unsaturated farnesyl side chain, which confers distinct biological properties, including enhanced cellular uptake, superior membrane distribution and more potent biological activity in several tissues, including the liver (Atia, Salem Alra, & Abdullah 2021; Ayu Jayusman & Balkis Budin 2017). For example, this unsaturated tail enhances their lateral mobility within lipid bilayers, enabling rapid interaction with lipid radicals and contributing to more efficient termination of lipid peroxidation (Mutalib et al. 2003). Recent findings have highlighted that the enhanced biological activity of TRF may be partially attributed to its higher cellular uptake compared to tocopherols (Mohd Zaffarin et al. 2020). This difference is strongly mediated by albumin, a major serum protein that facilitates the transport of vitamin E analogues into cells. Evidence suggests that tocotrienols have a greater binding affinity to albumin than tocopherols, enabling more efficient uptake (Nakatomi et al. 2023). This process appears to occur primarily via facilitated diffusion rather than ATP-dependent mechanisms such as endocytosis. The structural differences,

particularly in the side chains of these compounds, contribute to variation in albumin-binding affinity, which in turn influences cellular transport efficiency. These mechanistic insights reinforce the notion that TRF's superior intracellular delivery may underpin its therapeutic advantage, especially in tissues vulnerable to age-related oxidative damage such as the liver. This structural variation contributes to the enhanced antioxidant capacity of tocotrienols compared to tocopherols (Maniam et al. 2008). For instance, TRF produced from palms is reported as a promising agent for liver protection since it tends to collect preferentially in hepatic tissue and exhibits greater antioxidant potential than tocopherols, which has been attributed to their unsaturated isoprenoid side chain that facilitates deeper distribution (Maniam et al. 2008; Mutalib et al. 2003)

Vitamin E, as a group, is renowned for its lipid-soluble antioxidant capacity, with the chromanol ring's phenolic group oxygen species (Ebhohimen et al. 2021). Tocotrienols, due to their unsaturated side chains, demonstrate superior biological activities in comparison to tocopherols, particularly in terms of anti-inflammatory, cholesterol-lowering, and antioxidant effects (Maniam et al. 2008; Nakatomi et al. 2023).

2.8 THERAPEUTIC EFFECTS OF TRF ON AGE-RELATED LIVER CHANGES

Tocotrienols have gained considerable attention due to their potent antioxidant and anti-inflammatory properties, which are particularly relevant in the context of ageing and age-associated liver dysfunction (S. K. Wong et al. 2020). Given that oxidative stress and chronic inflammation are central drivers of hepatic ageing, TRF has been widely investigated for its capacity to modulate these processes and restore cellular homeostasis (Grimm et al. 2016). One of the mechanisms underlying the therapeutic effects of TRF is its ability to improve systemic oxidative status (Fahami & Tajudin 2011). Clinical and experimental studies consistently demonstrate that TRF supplementation enhances antioxidant defence systems while reducing oxidative damage (Atia et al. 2021b; Lee et al. 2010). For instance, it was seen that in a human RCT involving healthy older adults, six months of TRF supplementation improved HDL-cholesterol, markedly decreased protein carbonyl content and advanced glycation end-product levels, and modulated SOD and CAT activities, indicating a restoration of

redox balance particularly relevant to the ageing population (Chin et al. 2011). In ageing mice specifically, long-term TRF supplementation increased SOD, CAT, and GPx activities in skeletal and cardiac muscle compared to untreated controls, suggesting a systemic effect on age-related antioxidant decline (Karim et al. 2015) .

Supporting these findings, mechanistic in vitro studies further highlight the superior antioxidant efficacy of TRF compared to α-tocopherol. For example, TRF was shown to significantly delay lipid peroxidation and reduce thiobarbituric acid reactive substances (TBARS) formation in oxidised low-density lipoprotein and endothelial cells, demonstrating stronger protection against oxidative damage at lower concentrations (Mutalib et al. 2003)Likewise, earlier work in liver microsomes demonstrated that TRF effectively inhibited both protein oxidation and lipid peroxidation, with tocopherol reinforcing its role as a potent chain-breaking antioxidant relevant to hepatic oxidative stress (Kamat et al. 1997) . At the cellular level, tocotrienols also appear to modulate ageing-related processes beyond oxidative damage. For example, evidence from fibroblast models shows that tocotrienols protect against oxidative stress-induced cellular ageing by preserving telomere length and enhancing telomerase activity, particularly when administered prior to oxidative insult (Makpol et al. 2010). This is further supported by recent clinical evidence demonstrating that tocotrienol supplementation in older adults improves antioxidant enzyme activity, reduces inflammatory markers such as TNF-α , and enhances telomerase activity, indicating improved genomic stability and resilience against ageing-associated stress (Sharif et al. 2025). Additionally, TRF supplementation has been shown to reduce lipid peroxidation (MDA) and DNA damage in older individuals, with some sex-specific differences observed, further highlighting its role in mitigating oxidative stress during ageing (Goon et al. 2017). Also, TRF supplementation significantly reduced systemic inflammatory mediators including TNF-α and IL-6 (Zainal et al. 2019).

Importantly, the hepatoprotective effects of TRF are particularly evident in models of liver injury. In experimental studies, TRF supplementation significantly attenuated oxidative liver damage by reducing hepatic MDA and protein carbonyl levels while restoring endogenous antioxidants such as glutathione (GSH), GPx, and ferric

reducing antioxidant power (FRAP) (Ayu Jayusman & Balkis Budin 2017) . These biochemical improvements were accompanied by reductions in liver enzymes and histological evidence of reduced hepatic damage, indicating direct protection against hepatocellular injury. Similarly, in xenobiotic-induced liver injury models, tocotrienols exerted cytoprotective effects by reducing reactive oxygen species, inhibiting lipid peroxidation, suppressing apoptotic pathways, and promoting markers of liver regeneration, further supporting their therapeutic potential in hepatic injury (Tan et al. 2015).

A key mechanism through which TRF exerts its hepatoprotective effects is the reinforcement of endogenous antioxidant defences. For example, in a chronic hepatotoxicity rat model, daily oral administration of palm TRF significantly enhanced liver CAT, SOD and glutathione peroxidase (GPx) activities, while simultaneously reducing hepatic thiobarbituric acid reactive substances (TBARS), triglycerides, and cholesterol levels (Lee et al. 2010). These findings align with work showing that TRF supplementation in stress-induced rats significantly reduced liver MDA levels and increased glutathione content, confirming its capacity to restore hepatic redox balance under oxidative challenge (Fahami & Tajudin 2011). More recently, TRF was shown to dose-dependently increase liver SOD3 gene and protein expression in mice (Atia et al. 2021) . As previously mentioned, the molecular basis for these antioxidant effects involve activation of the Nrf2 signalling pathway. It was demonstrated that TRF administration in mice initiated Nrf2 nuclear translocation in the liver with a concomitant dose-dependent increase in the expression of Nrf2-regulated cytoprotective genes (Atia et al. 2020). Taken together, these findings suggest that TRF engages the Nrf2-antioxidant response element axis to broadly enhance hepatic detoxification and antioxidant gene expression (Atia et al. 2018).

Beyond oxidative stress, TRF and its constituent tocotrienols have demonstrated potent anti-inflammatory properties relevant to hepatic ageing (Pervez et al. 2023; Sabir et al. 2022). This anti-inflammatory capacity was supported by evidence establishing that tocotrienols suppress NF-κB-dependent transcription of pro-inflammatory mediators including TNF-α, IL-1β, IL-6, and iNOS, with reductions ranging from 30% to 70% in macrophage models derived from both young and senescent mice (Qureshi

2022) . Supporting this, in vitro studies in monocytic and macrophage models revealed that TRF inhibits the production of nitric oxide, prostaglandin E2, and pro-inflammatory cytokines through downregulation of NF-κ□, i□□□, -2 a n d □ expression (Wu et al. 2008; Yam et al. 2009) . Notably, tocotrienols were found to exhibit stronger anti-i n f l a mma t o r y -tocopherol, highlighting their α therapeutic potential. Furthermore, in stress-induced rat models, tocotrienol supplementation significantly reduced hepatic MDA levels while restoring glutathione content, indicating effective attenuation of lipid peroxidation and oxidative stress within the liver (Azlina Mohd Fahami & Tajudin Abstrak 2011) .

The relevance of these antioxidant and anti-inflammatory properties is particularly evident in metabolic liver diseases such as NAFLD. In a rat model of NAFLD, tocotrienol treatment inhibited Toll-like receptor (TLR) activation, increased the anti-inflammatory cytokine interleukin-10, and reduced lipid peroxidation while raising hepatic antioxidant activities (S. K. Wong et al. 2020). Histopathological analysis in that study confirmed reduced steatosis, lobular inflammation, and hepatocyte ballooning with palm tocotrienol at 100 mg/kg (S. K. Wong et al. 2020).

The translational relevance of these preclinical findings has been corroborated by clinical studies (Magosso et al. 2013; Pervez et al. 2018, 2022, 2023). Additionally, α-tocopherol supplementation downregulated circulating miRNAs involved in hepatic steatosis, inflammation, and apoptosis (Pervez et al. 2022). Additional studies using modified TRF formulations also reported reduced NAFLD severity and lower NAFLD activity scores, supporting its role in slowing disease progression (Goon et al. 2022). Collectively, both preclinical and clinical evidence support the therapeutic potential of TRF in mitigating age-related liver changes. By simultaneously targeting oxidative stress, inflammation, and cellular ageing pathways, TRF emerges as a promising nutraceutical for preserving liver function during ageing. These multifaceted effects highlight its potential not only in preventing hepatic dysfunction but also in modulating the underlying mechanisms driving liver ageing.

2.9 METABOLOMICS IN LIVER AGEING RESEARCH

Despite the multifactorial nature of age-associated hepatic changes, conventional biochemical assays remain limited to the measurement of isolated markers, providing only a partial view of the underlying alterations (Petr et al. 2021). This limitation of single-marker approaches is further reflected in recent consensus frameworks. The Ageing Biomarker Consortium of China published a consensus framework in 2024 which emphasises that liver ageing biomarkers should span three dimensions: functional (cholesterol metabolism, coagulation), imaging (steatosis, blood flow), and humoral (hepatokines, enzymatic changes) (Jiang et al. 2024). This multi-dimensional framework acknowledges that no single conventional marker captures the ageing phenotype.

Multi-omics studies are now mapping what those conventional assays miss. A study integrated transcriptomics, proteomics, and metabolomics from baboon livers across the adult age span and identified ageing-correlated modules involving ER stress and xenobiotic metabolism pathways, changes invisible to standard biochemistry (Puppala et al. 2023). Untargeted and targeted metabolomics methods have been used in liver ageing research to identify significant metabolic alterations. These include changes in lipid metabolites, decreased antioxidant metabolites such glutathione (Jin et al. 2023) , and changes in the amounts of TCA cycle intermediates (Dankel et al. 2023). Additionally, branched-chain amino acids (BCAAs) and urea cycle intermediates are altered in aged livers, indicating dysregulation in amino acid metabolism and compromised nitrogen balance and protein catabolism (Wang et al. 2024b). This is further supported by metabolomic profiling across multiple organs, which demonstrates that ageing is characterised by widespread metabolic reprogramming. It identified both shared and tissue-specific metabolite alterations across several organs, including the liver, suggesting that ageing involves coordinated systemic and organ-specific metabolic shifts (F. Zhang et al. 2021).

Several metabolomics studies have now mapped the specific metabolic perturbations that characterise hepatic ageing (Petr et al. 2021; F. Zhang et al. 2021). Similarly, it was reported that an ageing liver exhibits significant alterations in key

metabolic pathways, including downregulation of glutathione, pyrimidine, and glycerophospholipid metabolism, alongside upregulation of arachidonic acid metabolism, indicating impaired antioxidant defence and enhanced inflammatory signalling (Jiangfeng et al. 2021). Extending this concept, metabolomic evidence also highlights the influence of the systemic environment on hepatic ageing (Han et al. 2021). It demonstrated that transplantation of young livers into aged recipients resulted in metabolic profiles resembling those of aged livers, indicating that circulating and whole-body factors can drive age-associated metabolic reprogramming in the liver. Pathway analysis further revealed alterations in lipid-related pathways, including glycerophospholipid and arachidonic acid metabolism, reinforcing the systemic regulation of hepatic ageing.

A previous study analysed hepatic metabolites across young and old rats and identified 25 key metabolites contributing to age-related separation, including lipid metabolites (glycerol-3-phosphate, lysophosphatidylcholines), energy metabolism intermediates (carnitine, acylcarnitines, creatine), and nucleic acid metabolites (hypoxanthine, xanthine) (Son et al. 2012). More recently, a metabolite profiling study in ageing rats confirmed that hepatic metabolites, phenylacetic acid, valine, isoleucine, and tyrosine were significantly altered with age, with pathway analysis indicating mitochondrial dysfunction and phenylalanine metabolism disorders as hallmarks of hepatic ageing (Choi et al. 2025). A multi-omics investigation combined metabolomics and phosphoproteomics in naturally aged mouse liver, identifying 150 associated metabolic pathways, with evidence of increased fibrosis, inflammatory cell accumulation, hepatocyte degeneration, lipid droplet deposition, and damaged mitochondria confirmed by histological examination (Tang et al. 2023).

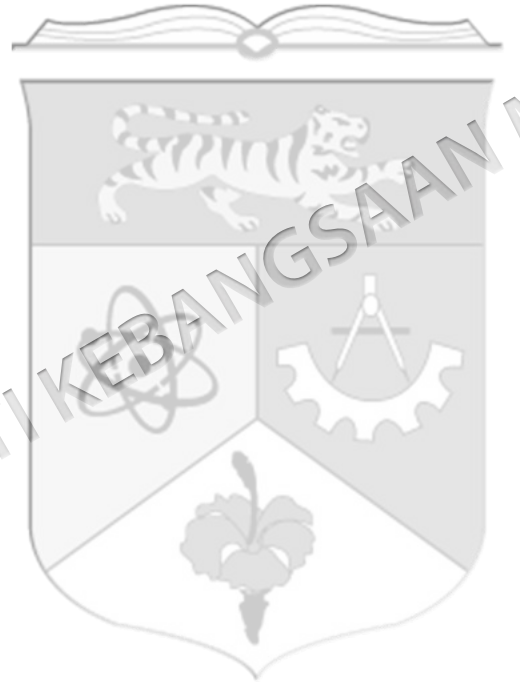
These metabolomic portraits of the ageing liver converge on several recurrent themes. Amino acid metabolism is consistently disrupted: a large human metabolomics study (n=1,030) identified aromatic amino acid catabolites and acylcarnitines as central hub metabolites of ageing, reflecting decreased mitochondrial oxidation and the accumulation of unsaturated fatty acids that may drive the oxidative damage and inflammation characteristic of ageing (Sol et al. 2023). Tissue-specific NMR metabolomics across six organs in young and old mice confirmed that the liver exhibits

distinct age-related metabolite alterations, with several metabolites altered across tissues suggesting potential universal biomarkers of ageing (Liu, et al. 2020). In the liver specifically, glutathione metabolism and pyrimidine metabolism have emerged as pathways critically impaired during ageing in metabolomics analysis (Liu, et al. 2020).

The metabolomic profile of the ageing liver directly intersects with clinically relevant disease states. A 2026 metabolomics study integrating machine learning in older adults with metabolic dysfunction-associated steatotic liver disease (MASLD) identified six optimal biomarkers, including phosphatidylcholine, porphobilinogen, and uridine, and demonstrated that phosphatidylcholine synthesis was significantly downregulated in both elderly human patients and aged mouse models (Jin et al. 2026). Critically for the present study, metabolomics has also demonstrated its power as a tool for evaluating the efficacy and mechanism of dietary interventions on hepatic ageing. Similarly, a study used untargeted metabolomic profiling to evaluate the effect of ginger supplementation on rat hepatic changes during ageing (Ekeuku et al. 2024). That study showed that metabolites involved in pyrimidine and purine metabolism, nicotinate and nicotinamide metabolism, glutathione metabolism, and arginine biosynthesis were altered by ageing, and that ginger supplementation reversed specific age-induced alterations in pyrimidine and glutathione metabolism (Ekeuku et al. 2024). This highlights the utility of metabolomics in evaluating dietary interventions, a principle that has also been applied to tocotrienol research.

In the context of tocotrienol research, an untargeted metabolomics study using UHPLC/MS demonstrated that tocotrienol supplementation in obese mice lowered serum levels of inflammatory and oxidative stress metabolites (trimethylamine N-oxide, kynurenate, oxidised glutathione) while increasing levels of essential amino acids (lysine, methionine), sphingomyelins, phosphatidylcholines, and B-vitamins (Shen et al. 2021). A clinical metabolomics study further confirmed that 12-week tocotrienol supplementation in postmenopausal women led to higher serum levels of lysophospholipids but lower acylcarnitines and tryptophan metabolites, a metabolic signature potentially linked to reduced inflammation and oxidative stress (Shen et al. 2021).

Thus, a metabolomic analysis of the effect of TRF on rat hepatic changes during ageing would not only validate the biochemical examinations done in this study at a systems level but would also identify novel metabolite biomarkers and metabolic pathways through which TRF exerts its hepatoprotective effects, potentially uncovering therapeutic targets that remain invisible to conventional analytical methods. The integration of such metabolomic data with histopathological and biochemical findings would provide a comprehensive assessment of TRF's capacity to mitigate age-associated hepatic decline.



CHAPTER III

MATERIALS AND METHODS

3.1 MATERIALS AND TOOLS OF STUDY

3.1.1 IL-6 Inflammatory Markers by ELISA

The materials used were IL-6 ELISA Assay Kit (ELK Biotechnology, Wuhan, #56731546) that included 500 pg/ml stock standard, standard diluent buffer, biotinylated antibody (100X), streptavidin-HRP (100X), biotinylated antibody diluent, HRP diluent, wash buffer (25X), TMB substrate solution, stop reagent, 96-well pre-coated microplates and plate covers.

3.1.2 TNF-Alpha Inflammatory Markers by ELISA

The materials used were TNF-alpha ELISA Assay Kit (ELK Biotechnology, Wuhan, #56100004) that included 1000 pg/ml stock standard, standard diluent buffer, biotinylated antibody (100X), streptavidin-HRP (100X), biotinylated antibody diluent, HRP diluent, wash buffer (25X), TMB substrate solution, stop reagent, 96-well pre-coated microplates and plate covers.

3.1.3 Malondialdehyde (MDA) by ELISA

The materials used were MDA ELISA Assay Kit (ELK Biotechnology, Wuhan, #37075210) that included 2000 pg/ml stock standard, standard diluent buffer, biotinylated antibody (100X), streptavidin-HRP (100X), biotinylated antibody diluent, HRP diluent, wash buffer (25X), TMB substrate solution, stop reagent, 96-well pre-coated microplates and plate covers.

3.1.4 Preparation for ELISA

a. 1X phosphate-buffered saline (PBS)

A 10X PBS (Cat. No. 6505-OP, Sigma-Aldrich, St. Louis, MO, USA)stock solution was diluted to 1X working concentration prior to use. The dilution was carried out by adding 100 mL of 10X PBS stock to 900 mL of distilled water in a clean 1 L volumetric flask, followed by thorough mixing until homogeneity was achieved.

The prepared PBS solution was stored at 4 °C until use.

b. Preparation of Tissue Homogenates

Tissue homogenates were prepared according to standard procedures, following the manufacturer's instructions provided in t modifications depending on tissue type. Liver samples were first rinsed in pre-cooled PBS to remove excess blood and subsequently weighed prior to homogenisation. The tissues were then minced into small pieces and homogenised in PBS at a tissue-to-buffer ratio of 1:9 (w:v), for example, 900 µL of PBS was added to 100 mg of tissue, and sonicated using an ultrasonic cell disrupter (Qsonica, USA) until the solution became clear. The homogenates were then centrifuged in a refrigerated centrifuge at 10,000 × g for 5 minutes, after which the supernatant was collected for immediate assay or stored in aliquots at -20 °C until further use. All procedures were carried out on ice to minimise protein degradation.

3.1.5 Metabolomic Analysis

a. Metabolites extraction

The chemicals used for metabolomics analysis were MS-grade unless specified otherwise. The materials used were probe sonicator (QSonica), water, methanol (MeOH) internal standards (SPLASH LIPIDOMIX Mass Spec Standard, Avanti Research, Alabaster, AL, USA), vacuum centrifuge (Eppendorf, Hamburg, Germany) and dichloromethane (DCM). The disposable materials used are 2 mL tubes, glass tubes, and glass serological pipettes.

The equipment used includes micropipettes, vortex mixers, vacuum centrifuges (Eppendorf, Hamburg, Germany), and a -80°C refrigerator.

b. Liquid Chromatography with tandem mass spectrometry (LCMS/MS)

The chemicals used for LCMS/MS are MS-grade water, isopropanol (Fisher Scientific, Hampton, NH, USA), and MS-grade methanol (Fisher Scientific, Hampton, NH, USA). The disposable materials used are 2 mL tubes.

The equipment used includes a micropipette and an LCMS/MS machine (Q Exactive HF Orbitrap, Thermo Scientific).

3.1.6 Histological Study

a. 10% neutral buffered formalin (NBF)

10% formalin was prepared in the fume hood by adding 100 mL of 40% formaldehyde (R&M Chemicals, Essex, UK) into a beaker containing 900 mL of deionised water. The solution was gently mixed and stored at room temperature for use in tissue fixation procedure.

b. Saline solution

0.9% NaCl solution was made by mixing accurately 0.9 g NaCl (EMSURE, Merck, Darmstadt, Germany) in 100 mL deionised water. The crystal was stirred using a magnetic stirrer set at 100 rpm until clear.

c. Materials for histology

Rat liver tissue processing was carried out using 10% neutral buffered formalin (NBF), graded ethanol (Supelco, Merck, Darmstadt, Germany) solutions (70%, 80%, 95%, and 100%), xylene, and paraffin wax. A Leica Tissue Processor ASP300 S (Leica Microsystems, Wetzlar, Germany) and a rotary microtome were employed for tissue preparation and sectioning. A floatation water bath was used for ribbon spreading.

Haematoxylin, eosin, 1% acid alcohol, and distilled water were used for staining procedures. Dibutylphthalate Polystyrene Xylene (DPX) (EMSURE, Merck, Darmstadt, Germany) mounting medium and glass coverslips were used for slide preparation. Microscopic examination was performed using a standard light microscope equipped with digital imaging software for histopathological documentation.

3.1.7 Protein Quantification for Hepatic Anti-Oxidant Activity Assays

For protein quantification for catalase and SOD experiments, the materials used included bovine serum albumin (BSA) (Biomax Scientific, Selangor, Malaysia) as the protein standard, copper □□□□ sulphate □ermany□, sodium potassium tartrate □□□ Darmstadt, Germany), potassium iodide (KI) (EMSURE, Merck, Darmstadt, Germany), sodium hydroxide (NaOH) pellets (EMSURE, Merck, Darmstadt, Germany), distilled water, and freshly prepared Biuret reagent according to the described method.

Glassware and plasticware were comprised of 15 mL Falcon tubes, 2 mL microcentrifuge tubes, volumetric flasks, beakers, and polyethylene storage bottles with tight-fitting caps. The equipment required included an analytical balance, a water bath maintained at 37 °C, adjustable micropipettes with disposable tips, a spectrophotometer capable of measuring absorbance at 540 nm, and magnetic stirrer.

3.1.8 Reagents for Catalase Assay

a. Phosphate buffer (50mM, pH-7.4)

Phosphate buffer (50 mM, pH 7.4) (Sigma-Aldrich, St. Louis, MO, USA) was prepared by dissolving □.□□□ g of potassium dihyd Merck, Darmstadt, Germany) and 1.775 g of disodium hydrogen phosphate □□a$_2$□□□$_4$□ (EMSURE, Merck, Darmstadt, Germany) separately in distilled water to a final volume of 250 ml each. The two solutions were then mixed in a 1:1.5 ratio □□□$_2$□a$_2$□□□$_4$□ to obtain the buffer solution. The pH was measured using a calibrated digital pH meter

and adjusted to the final desired value of pH 7.4 by the gradual addition of hydrochloric acid (HCl).

All solutions were prepared using an analytical balance, volumetric flasks, measuring cylinders, a magnetic stirrer, and beakers to ensure accuracy and homogeneity. The final buffer concentration was 50 mM.

b. 30% hydrogen peroxide □□₂□₂□

□ □□□ hydrogen peroxide □□₂□₂□ □□□□□R□, was prepared by measuring 0.34 ml of hydrogen peroxide using a micropipette and transferring it into a 100 ml volumetric flask. Phosphate buffer (50 mM, pH 7.4) was added up to the calibration mark to obtain a final volume of 100 ml. The solution was mixed thoroughly using a magnetic stirrer and stored in an amber bottle to minimise decomposition. A fresh solution was prepared before each use for the catalase assay.

3.1.9 Reagents for Superoxide Dismutase (SOD) Assay

a. Phosphate buffer (50mM, pH-7.2) with sucrose

Phosphate buffer was prepared by accurately weighing 0.68045 g of dipotassium hydrogen phosphate □□₂□□□₄□ □□□□□R□, □e analytical balance and dissolving it in 50 mL of distilled water in a beaker. The pH of the solution was measured using a calibrated digital pH meter and adjusted to pH 7.2 by the gradual addition of hydrochloric acid (HCl) (Supelco, Merck, Darmstadt, Germany) using a pipette. The solution was then transferred to a 100 mL volumetric flask, and the volume was made up to the mark with distilled water. 8.5575 g of sucrose □□₁₂□₂₂□₁₁□ □□□□□R□, □erc□, □armstadt, □e buffer solution, and the mixture was stirred using a magnetic stirrer until completely dissolved.

The prepared buffer was stored in a clean, airtight container at 4 °C until use.

b. Phosphate buffer (50mM, pH-7.8) with EDTA

Phosphate buffer (50 mM, pH 7.8) containing EDTA was prepared using standard laboratory apparatus. Solution A was prepared by weighing 7.098 g of disodium hydrogen phosphate □□a$_2$□□□$_4$□ □□□□□R□, □e analytical balance and dissolving it in 500 ml of distilled water in a 500 ml beaker. Solution B was prepared separately by weighing 3.90 g of sodium dihydrogen phosphate dihydrate □□a□$_2$□□$_4$and dissolving it in 250 ml of distilled water. For buffer preparation, 228.75 ml of Solution A was measured with a graduated cylinder and mixed with 21.25 ml of Solution B in a clean 1 L beaker. The mixture was transferred into a 500 ml volumetric flask, then distilled water was added to bring the volume up to 500 ml.

The pH of the solution was measured using a calibrated digital pH meter, and the value was adjusted to 7.8 by the gradual dropwise addition of hydrochloric acid (HCl) with a pipette. After pH adjustment, 0.015 g of ethylenediaminetetraacetic acid (EDTA) (EMSURE, Merck, Darmstadt, Germany) was weighed and added to the buffer solution, followed by stirring with a magnetic stirrer until fully dissolved. The prepared buffer was transferred into a labelled reagent bottle and stored at 4 °C until use.

c. Substrate solution for SOD assay (riboflavin–methionine–NBT assay)

The substrate solution for the superoxide dismutase assay was freshly prepared and protected from light. A phosphate buffer (pH 7.8, containing EDTA) was first measured to obtain 27.0 ml. Subsequently, 1.5 ml of L-methionine (Merck, Darmstadt, Germany) stock solution (0.3 g/10 ml), 1.0 ml of nitroblue tetrazolium chloride (NBT·2HCl) (Thermo Scientific; Waltham, MA, USA) stock solution (0.0141 g/10 ml), and 0.75 ml of Triton X-100 (Thermo Scientific; Waltham, MA, USA) stock solution (1% v/v) were sequentially added into an amber tube. The mixture was gently inverted to ensure homogeneity while avoiding bubble formation. All steps were carried out on ice, and the solution was wrapped in aluminium foil to minimise light exposure.

The preparation utilised volumetric flasks, graduated cylinders, micropipettes with tips, an amber container, and aluminium foil. The final mixture had approximate

concentrations of 10 mM L-methionine, 57 µM NBT, and 0.025% Triton X-100 in 45 mM phosphate buffer.

d. Riboflavin preparation

Riboflavin solution was prepared by accurately weighing 4.4 mg of riboflavin (Merck, Darmstadt, Germany) on an analytical balance and dissolving it in 100 mL of distilled water in a volumetric flas to obtain . solution was mixed thoroughly using a magnetic stirrer until completely dissolved. To prevent light-induced degradation, the solution was prepared under low-light conditions and stored in an amber glass bottle wrapped with aluminium foil. A calibrated micropipette was used for transfers, and the solution was kept refrigerated at 4 °C until use.

e. L-methionine preparation

L-methionine solution was freshly prepared by accurately weighing 0.300 g of L-methionine erc , armstadt , weighed powder was transferred into a clean beaker and dissolved in 10 mL of distilled water with gentle stirring using a glass rod until completely dissolved. The resulting solution, with a final concentration of 30 mg/mL (0.20 M), was transferred into a volumetric tube and immediately used for the assays.

f. NBT preparation

Nitroblue tetrazolium (NBT) solution was freshly prepared by accurately weighing . g of T l Thermo analytical balance. The weighed powder was transferred into a clean beaker and dissolved in 10 mL of distilled water d with continuous mixing using a glass rod until completely dissolved. The solution, with a final concentration of 1.41 mg/mL (2.3 mM), was transferred into a volumetric tube and stored in a dark container to protect it from light until use.

g. 1% Triton X-100 solution preparation

A 1% Triton X-100 solution was freshly prepared by pipetting 1 mL of Triton X-100 (Thermo Scientific; Waltham, MA, USA) using a micropipette and adding it to a volumetric flask containing approximately solution was mixed thoroughly using a magnetic stirrer until fully homogenised. Then distilled water was added to make the final volume to 100 mL with distilled water. The prepared solution was stored at room temperature until use.

3.2 RESEARCH METHODOLOGY

3.2.1 Experimental Design

The experimental design conducted in the study is shown in Figure 3.1.

The animal treatment, intervention, and tissue collection described in this section were conducted as part of a previously published study by Dr. Siti Liyana Gany (Saud Gany et al. 2022). In the present study, liver samples obtained from this work were utilised for further analyses, including metabolomic profiling and additional biochemical assessments. The experimental design is briefly described here to provide context and ensure transparency regarding the origin of the biological samples used.

A brief introduction to the experimental design is as follows: A total of sixty male Sprague Dawley (SD) rats were utilised in this study. The animals were divided into three age categories representing different life stages: young (3 months), adult (9 months), and old (21 months). Each age category comprised twenty rats, which were further subdivided into two groups (n = 10 per group). One group received tocotrienol-rich fraction (TRF) as the treatment, while the other group was maintained on refined, bleached, and deodorised (RBD) oil as the control (Saud Gany et al. 2022).

The intervention was carried out for three months. At the end of the treatment period, all rats were sacrificed, and liver tissues were harvested for subsequent investigations. These samples formed the basis for a range of analyses, including oxidative stress markers, antioxidant assays, inflammatory markers, histological

evaluation, and metabolomic profiling. The detailed procedures, analytical methods, and outcomes will be presented in the subsequent sections of this thesis.

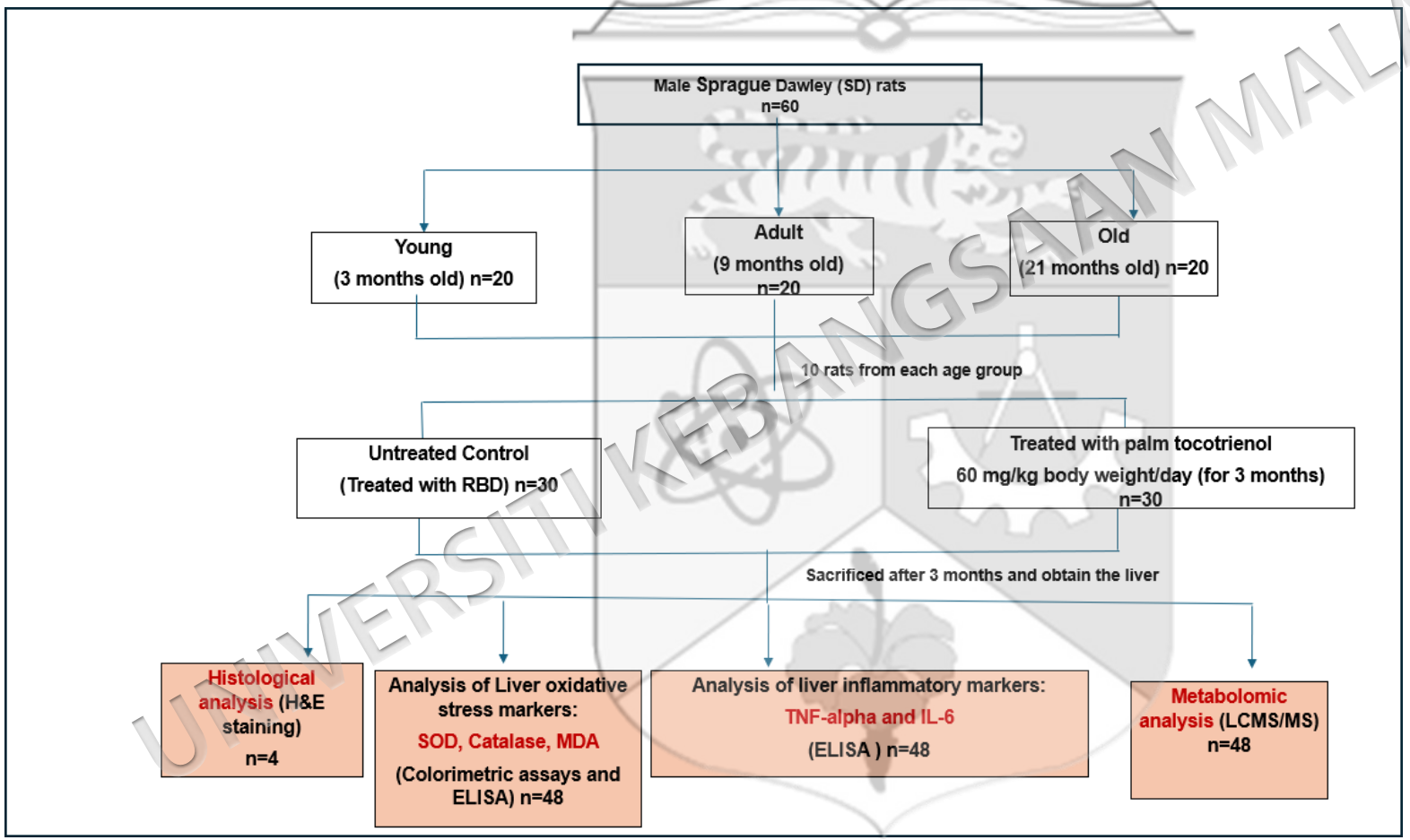


Figure 3.1 Study design to elucidate the untargeted liver metabolites profiling and oxidative stress expression in Sprague Dawley rats supplemented with TRF and its effect on hepatic changes during ageing.

3.2.2 Animal Model

Male Sprague Dawley (SD) rats aged three, nine, and twenty-one months were acquired from the Laboratory Animal Resources Unit (LARU) at the University Kebangsaan Malaysia. Three age categories were established: young (3 months), mature (9 months), and old (21 months).

Each group of rats was then subdivided into 10 rats each for the control and treatment groups. Rats were placed in cages one at a time and given unlimited access to food and water after a week of acclimatisation. Over the course of three months, rats in the control and treated groups received oral gavages of 60 mg/kg body weight/day of refined, bleached, and deodorised (RBD) palm olein (Sime-Darby Plantation Berhad) and 60 mg/kg body weight/day of tocotrienol-rich fraction (TRF) (Sime-Darby Plantation Berhad), respectively.

The rats were sacrificed and their organs, including the liver, were removed after three months of TRF treatment. Prior to additional analysis, they were kept at -80°C. All animal protocols utilised in this investigation were approved by the University Kebangsaan Malaysia ethical committee, and they were assigned an approval number BIOK/FP/2020/SUZANA/25-MAR/1099-MAR-2020-DEC.2022 (Saud Gany et al. 2022).

3.2.3 TRF Treatment

According to (Saud Gany et al. 2022), each gram of TRF is made up of a mixture of vitamin E isomers, including 27% alpha-tocotrienol, 24% alpha-tocopherol, 4% beta-tocotrienol, 32% gamma-tocotrienol, and 14% delta-tocotrienol. RBD palm olein has an iodine content of 64.71%, 0.043% moisture and contaminants, 0.054% free fatty acids, and 0.45% peroxide values. Each rat in the treated group received a dose of 60 milligrams of TRF per kilogram of body weight, and this treatment was administered

continuously for 3 months. Each week, in a dark room, TRF (2.4 g) were added to 40 ml of RBD palm oil in a Falcon tube, and the mixture was vortexed until it was well

combined. To shield it from the sun, the tube was covered in aluminium foil and stored at 4°C. Rats were placed in cages, one rat in each cage, and given access to food and water following a week of acclimatisation.

Over the course of three months, rats in each group (control and treated) received oral gavage. For the control groups, 60 milligrams of RBD palm olein per kilogram of body weight per day was given, and for the treatment groups, 60 milligrams of TRF per kilogram of body weight per day was given.

3.2.4 Euthanisation of Animals

All rats were fasted overnight on day 90 of the study before being killed for necropsy examination. The anaesthetic used in this trial was a mixture of ketamine, xylazine, and zoletil-50 (tiletamine and zolazepam), also referred to as KTX agents. Because the KTX agents are quick, efficient, and painless, they were injected intraperitoneally into the lower right quadrant of the abdomen. 0.1 ml/250 g of body weight KTX was given to each rat. After that, the rats were left for roughly half an hour so that the KTX agents could start to sedate them.

Clinical indications such as confusion and loss of consciousness, rapid or irregular breathing, depression of respiration, continuously decreasing blood pressure and heart rate, and changes in urine and excrement were used to observe this. A decapitator from Modiezhan Sdn. Bhd., Kuala Lumpur, Malaysia, was then used to decapitate the rats in order to end their lives (Saud Gany et al. 2022).

3.2.5 Collection of Organs

Internal organs such as the brain, heart, lungs, liver, spleen, kidneys, gastrocnemius muscle, and soleus muscle were dissected from the sacrificed rats. To avoid drying, the organs' weight was measured as soon as feasible (Saud Gany et al. 2022).

After being cleaned with 90% normal saline to get rid of any sticking tissue, they were weighed in accordance to the animals' body weight. After that, the organs were stored in a freezer set at -80°C.

3.2.6 Untargeted Metabolomic Profiling

a. Preparation of liver samples for untargeted metabolite extraction

Metabolic analyses were conducted using LC-MS to investigate the impact of TRF treatment on the metabolome of hepatic samples. The Folch extraction method was used to extract the polar metabolites. After thawing, the liver tissue sample was homogenized with 1 mL of cold water for every 1 g of tissue using a probe sonicator (QSonica). A total of 800 ul of methanol (MeOH) and 1 ul of internal standards (SPLASH LIPIDOMIX Mass Spec Standard) were added to 100 µL of tissue lysate. The mixture was then vortexed to ensure complete mixing, followed by addition of 1.6 ml of dicromethane (DCM) to the mixture. After shaking at 300 rpm for an hour at room temperature, the mixture was added with 10 min at room temperature. After that, the mixture was vortexed for 10 minutes at 1,000 × g at room temperature. Then, 1.0 ml of the upper phase (metabolites) was transferred to a new tube and dried in a vacuum centrifuge (Eppendorf, Germany) at room temperature. The dried metabolite extracts were kept at -80°C until use.

Untargeted metabolomic analysis of liver samples was performed using LC-MS/MS to enable sensitive and comprehensive profiling of metabolite changes associated with ageing and TRF treatment. The Folch extraction method was used to extract the polar metabolites because it provides efficient separation of metabolites from proteins and other tissue components, allowing recovery of the metabolite fraction for downstream analysis (Mok et al. 2024). After thawing, the liver tissue sample was homogenized with 1 mL of cold water for every 1 g of tissue using a probe sonicator (QSonica). A total of 800 ul of methanol (MeOH) and 1 ul of standards (SPLASH LIPIDOMIX Mass Spec Standard, Avanti Research, Alabaster, AL, USA) were added to 100 µL of tissue lysate to support quality control and improve analytical reliability

The mixture was then vortexed to ensure complete mixing, followed by addition of 1.6 ml of dicromethane (DCM) to the mixture. After shaking at 300 rpm for an hour

at room temperature, the mixture was added
incubated for 10 min at room temperature.

After that, the mixture was vortexed for 10 minutes at 1,000 × g at room temperature. Then, 1.0 ml of the upper phase (metabolites) was transferred to a new tube and dried in a vacuum centrifuge (Eppendorf, Hamburg, Germany) at room temperature. The dried metabolite extracts were kept at -80°C until use to maintain sample stability.

b. LCMS/MS analysis

Dried polar extracts were reconstituted
metabolite solubility while providing compatibility with LC-MS analysis, and filtered through a 0.2 µm regenerated cellulose membrane syringe filter (Phenomenex, Torrance, CA, USA), which was used to remove particulates, into LCMS vials with inserts.

A quality control (QC) sample was prepared by combining 10 µ aliquots from each sample, while the reconstitution buffer served as the solvent blank. This was done to assess analytical stability, monitor instrument drift, and support data quality assessment throughout the run, while solvent blanks were included to detect background contamination. Metabolomics data were acquired using LC-MS/MS on a UHPLC system (Dionex UltiMate 3000; Thermo Scientific, Waltham, MA, USA) coupled to an Orbitrap mass spectrometer (Q Exactive HF; Thermo Scientific) equipped with a heated electrospray ionization (HESI) source, following a previously described method with slight modifications (Tan et al. 2023).

Chromatographic separation was performed on a C18 column (2.1 mm ID × 100 mm L, 1.7 µm particle size, 100 Å pore size; Synchronis, Thermo Scientific) under the following conditions: column temperature 55°C, autosampler 10°C, injection volume 2 µL, and flow rate 0.45 mL/min. Mobile phase A consisted of water with 0.1% formic acid, and mobile phase B was acetonitrile with 0.1% formic acid. The 22-minute

gradient program was as follows: 0–1 min, 0.5% B; 1–16 min, linear increase to 99.5% B; 16–20 min, held at 99.5% B; and 20–22 min, returned to 0.5% B.

The HESI source was operated with the following parameters: sheath gas flow 50 arbitrary unit (AU), auxiliary gas flow 18 AU, sweep gas flow 0 AU, capillary temperature 320°C, S-lens RF level 55 %, auxiliary gas heater temperature 300°C, and spray voltage 3.5 kV (positive mode) and 3.0 kV (negative mode). Data were acquired using Xcalibur software (v4.2.47). Full MS scan (MS1) was acquired at 60,000 resolution with a scan range of m/z 100–1,000, AGC target of 1e6, and maximum injection time (IT) of 120 ms. Data-dependent MS/MS (MS2) was acquired at 15,000 resolutions with an AGC target of 5e4, maximum IT of 50 ms, loop count (top N) of 5, isolation window of 1.5 m/z, minimum AGC target of 8e2, intensity threshold of 1.6e4, dynamic exclusion 10.0 s, and stepped normalized collision energy of 20, 40, and 60 AU. QC samples were injected at the beginning of the run, after every 6 samples, and the end of the run. Sample injections between QC blocks were randomized to ensure balanced representation of all experimental groups.

LC-MS RAW files were processed using Compound Discoverer (v2.0), which included peak detection, alignment, and metabolite annotation. Peaks were detected with a minimum threshold of 2,000,000 AU. Alignment was performed with an MS1 tolerance of 5 ppm and features were grouped by retention time and molecular weight. The signal-to-noise ratio was set to <5, and gap filling was applied to minimize missing values. All annotated features were manually inspected by the author for MS1 peak quality, MS/MS fragment matching, and relative intensity. A LC-MS/MS was done to detect and compare a broad range of metabolites in the liver samples coefficient of variation (CV) cutoff of <30% was applied to QC samples to ensure data robustness.

3.2.7 Histological Study

Rat liver tissue samples from both the control and treatment age groups were put in a plastic cassette and left in a specimen container at room temperature for 24 hours in 10% neutral buffered formalin. A little sample, one centimetre in each direction, was taken after fixation. An automatic Leica Tissue Processor ASP300 S (Wetzlar, Germany) was used to slice and process them. The tissue processor, followed a

conventional procedure. As shown in Figure 3.2, all tissue was dehydrated for six hours in a sequence of graded ethanol (70%, 80%, 95%, and 100%), cleaned by passing through xylene for four hours and thirty minutes, and then embedded in paraffin wax for six hours. Then, using a microtome, paraffin-embedded tissues were sectioned at a thickness of 5 µm. This ensured that only single-layer cells made up the section, and because paraffin wax adheres to cells as they are cut, it creates a ribbon of section.

The paraffin sections then underwent "fishing," which involves flattening them by "floating out" on the surface of warm water in a floatation bath before being lifted onto microscope slides. Sections were stained with Haematoxylin and Eosin (H&E) for histological assessment. As shown in Figure 3.3, after deparaffinizing the slides in xylene twice for five minutes each, they were rehydrated using a series of decreasing ethanol concentrations (100%, 95%, 80%, 70%, and 50%) and rinsed with distilled water. After five minutes of haematoxylin staining, the sections were rinsed under running water, differentiated in 1% acid alcohol, and then rinsed once more. Eosin was used for counterstaining, followed by a final rinse with water. Following dehydration in increasing ethanol concentrations, the dyed sections were cleaned in xylene and mounted using DPX mounting media. To keep the sample safe, a coverslip was put on top. Using forceps, carefully tap the top of the coverslip to secure it and prevent bubbles. Digital imaging software was used to assess and record histopathological alterations on prepared slides as they were viewed under a light microscope at different magnifications.

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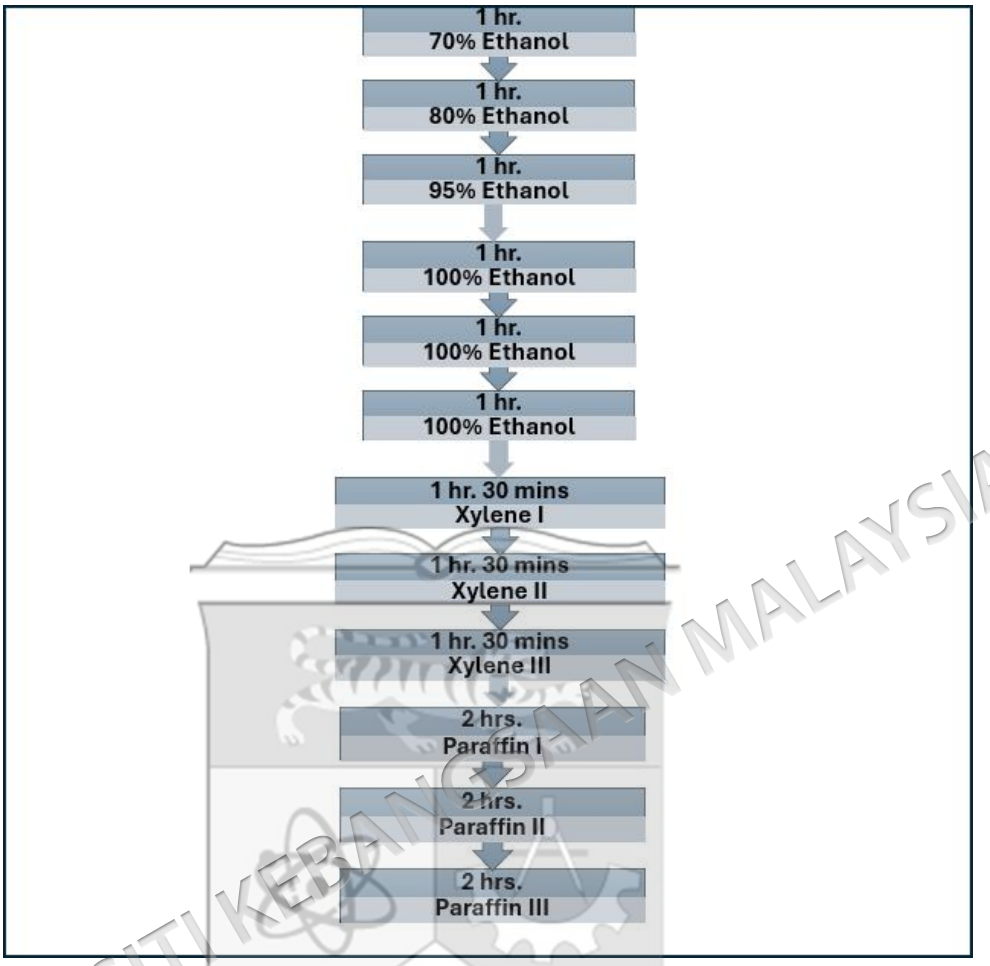
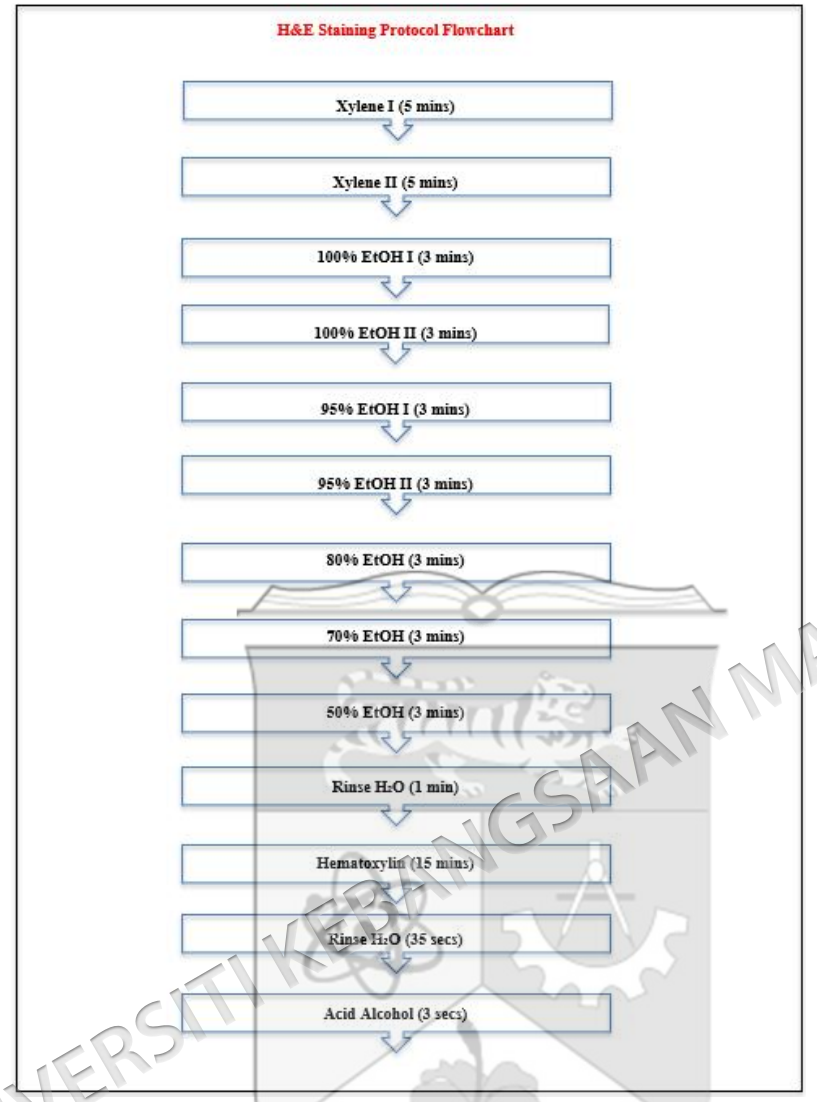


Figure 3.2 Automatic tissue processor protocol



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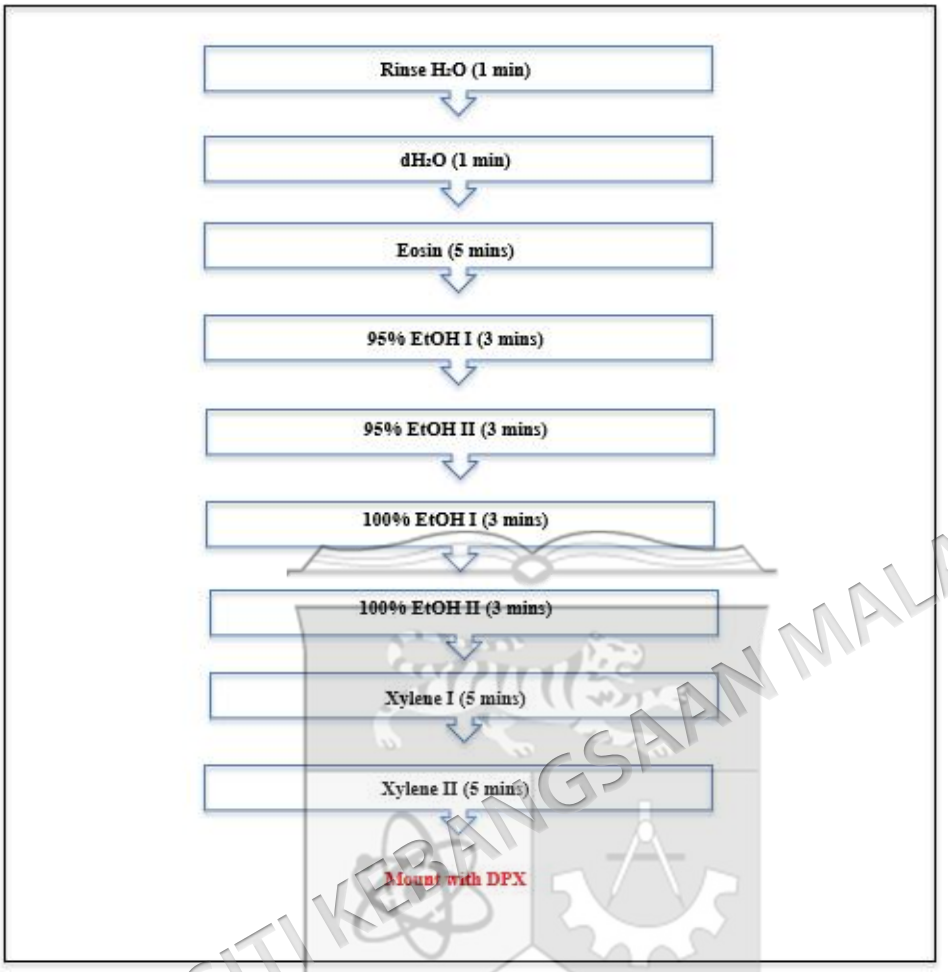


Figure 3.3 Automatic stainer protocol

3.2.8 Hepatic Anti-Oxidant Activity Assays

To prepare the liver homogenates for catalase, the protocol of (Sabir et al. 2022) with minor modifications. 10% w/v liver homogenate in phosphate buffer (50mM, pH-7.4).

0.15 g liver tissue sliced or minced in 1.5 ml buffer in a centrifuge tube and centrifuged at 10000 rpm, 4 °C for 10 mins. The supernatant was then collected. For the test cuvette, 0.1 ml of the homogenate sample was mixed with 0.4 ml of H_2O_2 and 2.5 ml of PBS. For the blank, 0.1 ml of the homogenate sample was combined with 2.5 ml of PBS. Both were run for 3 minutes.

Using the (Beers & Sizer 1952) method, the decrease in absorbance of H_2O_2 (E = 36 M-l cm-I) catabolised was measured at 240 nm to calculate the total activity of catalase spectrophotometrically. The quantity of enzyme that oxidises one umol of substrate per minute is known as enzyme activity (unit). This formula is used to determine catalase activity: catalase activity (Units/L) = (change in absorbance X total volume of assay)/(change in temperature x extinction coefficient x cuvette diameter x enzyme sample volume). For catalase, the extinction coefficient is 39.4 mM-1 cm-1, and the cuvette diameter is 1 cm. SOD was measured using the (Misra & Fridovich 1972) method , which was applied to assess the activity of SOD indirectly. Reduced nitroblue tetrazolium (NBT) was used to determine SOD activity (Asiwe et al. 2024).

3.2.9 Protein Quantification

Protein concentration in the samples was determined using the Biuret assay with bovine serum albumin (BSA) as the standard. Liver tissue homogenates were prepared as described previously, whereby an aliquot of 100 mg of rat liver tissue was homogenised in 1 mL phosphate-buffered saline (PBS) to yield a concentration of 100 mg/mL (Curti et al. 2019).

A standard calibration curve was prepared by serial dilution of the BSA stock solution (1 g in 10 mL distilled water). From this stock, six standards were prepared in

2 mL microtubes by sequentially diluting 1 mL of the preceding standard with 1 mL of distilled water to obtain final volumes of 2 mL. This yielded six concentrations of BSA standards, which were mixed thoroughly prior to use (Figure 3.4).

For the assay, test tubes (5 mL or 15 mL Falcon tubes) were prepared as follows: the reagent blank contained 50 µL distilled water and 2.5 mL Biuret reagent; the standard tubes contained 50 µL of each BSA standard and 2.5 mL Biuret reagent; and the sample tubes contained 50 µL of liver homogenate supernatant and 2.5 mL Biuret reagent. All tubes were mixed well and incubated in a water bath at 37 °C for 10 minutes. The absorbance of the standard solutions and samples was measured at 540 nm using a spectrophotometer. The concentration of soluble proteins in the liver homogenates was expressed as micrograms of soluble protein per millilitre of homogenate, based on the BSA calibration curve, and this value was subsequently used to normalise the quantification of antioxidant enzymes SOD and CAT.

The Biuret reagent was prepared freshly according to the following procedure. First, 6.0 M sodium hydroxide (NaOH) was prepared by dissolving 72 g of NaOH in 300 mL distilled water. To prepare 2.5 L of Biuret reagent, 7.5 g of copper (II) sulphate □□u□□$_4$□ was dissolved in □.□□ □ of distil
of sodium potassium tartrate □□□a□$_4$□$_4$□ and 12.5 g of □□□$_2$. The solution was stirred until all solids were fully dissolved, after which 250 mL of 6.0 M NaOH was added and the volume was made up to 2.5 L with distilled water. The reagent was stored in tightly capped polyethylene bottles at room temperature and remained stable for up to six months.

The protein standard was prepared by dissolving 1 g of BSA in 10 mL of distilled water (equivalent to 10 g in 100 mL stock). In addition, half-strength Biuret reagent was prepared by dissolving □.□□ g
by the addition of 11.25 g sodium potassium tartrate and 6.25 g KI. After complete dissolution, 125 mL of 6.0 M NaOH was added, and the solution was diluted to a final volume of 1.25 L with distilled water. This reagent was also stored in polyethylene bottles at room temperature for up to six months.

1. **Make a serial dilution for standard as follows: (in 2 mL microtubes)**

Standard	Stock	Distilled Water (1 mL)	Total Vol. (mL)
(in falcon tube) 1	1 g Bovine serum Albumin / 10 mL	–	–
2	1 mL from Standard 1	1 mL	2 mL
3	1 mL from Standard 2	1 mL	2 mL
4	1 mL from Standard 3	1 mL	2 mL
5	1 mL from Standard 4	1 mL	2 mL
6	1 mL from Standard 5	1 mL	2 mL

2. **Mix the/each solutions well.**

Figure 3.4 Protein concentration estimation by using the biuret method

3. **Prepare the test tube as follows: (5 mL or 15 mL falcon tube)**

Test tube	Reagent Blank	Standard (1 to 6)	Sample
dH_2O	50 µL	–	–
Standard	–	50 µL	–
Sample	–	–	50 µL
Biuret reagent	2.5 mL	2.5 mL	2.5 mL

4. **Mix well and incubate the test tubes for 10 minutes in waterbath at 37°C.**
5. **Measure and record the absorbance of the standard solution series and sample (X) at 540 nm by using spectrophotometer.**

3.2.10 Determination of Hepatic IL-6

The inflammatory markers in the rat livers of each group were examined to determine inflammation present in the livers with and without TRF in different groups. The levels of IL-6 were determined using ELISA kits (ELK Biotechnology, Wuhan).

a. Reagent preparation

All kit components and samples were brought to room temperature (25 °C) before use, ensuring that all reagents were fully dissolved and mixed. For each experiment, only the required number of strips and reagents were removed from storage, while the remaining components were kept under the The 25× Wash Buffer was diluted to 1× using double-distilled water.

The standard was centrifuged at 1000 × g for 1 minute and reconstituted with 1.0 mL of Standard Diluent Buffer. The solution was allowed to stand at room temperature for 10 minutes and mixed gently to avoid foaming. Seven serial dilutions were prepared in 0.5 mL of Standard Diluent Buffer to obtain concentrations of 500 pg/mL, 250 pg/mL, 125 pg/mL, 62.5 pg/mL, 31.25 pg/mL, 15.63 pg/mL, and 7.82 pg/mL. The final tube containing only Standard Diluent served as the blank (0 pg/mL). Fresh standard solutions were prepared for each experiment, with pipette tips replaced at every dilution step to prevent cross-contamination.

The Biotinylated Antibody and Streptavidin-HRP stock solutions were briefly centrifuged before use and diluted 1:100 with their respective diluents to obtain working solutions. The required volume of TMB Substrate Solution was aspirated with sterile tips, and unused solution was not returned to the stock vial.

b. Assay procedure

All standards, samples, and controls were assayed in duplicate. Initially, 100 µL of each standard or sample was added to the appropriate wells of the antibody-coated plate and

incubated at 37 °C for 90 minutes. After incubation, the wells were aspirated and washed three times with 1× Wash Buffer to remove unbound material. Subsequently, 100 µL of diluted Biotinylated Antibody working solution was added to each well, followed by incubation at 37 °C for 60 minutes.

The wells were washed three times before adding 100 µL of diluted Streptavidin-HRP working solution, which was incubated at 37 °C for 30 minutes. After another set of washes, 90 µL of TMB Substrate Solution was dispensed into each well and incubated in the dark at 37 °C for 15 minutes, resulting in blue colour development.

The reaction was terminated by adding 50 µL of Stop Solution, turning the colour to yellow. Optical density (OD) was measured at 450 nm using a microplate reader within 5 minutes of stopping the reaction. IL-6 concentrations in samples were calculated from the standard curve generated during the assay.

3.2.11 Determination of TNF-alpha

The inflammatory markers in the rat livers of each group were examined to determine inflammation present in the livers with and without TRF across increasing age. The levels of TNF-alpha were determined using their respective ELISA kits (ELK Biotechnology, Wuhan).

a. Reagent preparation

The TNF-alpha ELISA reagents were prepared by first allowing all kit components and samples to reach room temperature (18□25 °C), ensuring that all reagents were dissolved entirely and thoroughly mixed before use. When the kit was not used in a single run, only the required strips and reagents for the current experiment were removed. At the same time, the remaining materials were stored according to the manufacturer's instructions. The □-□ was distilled water.

The standard was centrifuged at 1000 × g for 1 minute and reconstituted with 1.0 mL of Standard Diluent Buffer, left at room temperature for 10 minutes, and gently mixed to avoid foaming. This produced a stock concentration of 1000 pg/mL. Seven

tubes, each containing 0.5 mL of standard diluent buffer, were prepared. A double dilution series was performed to obtain concentrations of 1000, 500, 250, 125, 62.5, 31.25, and 15.63 pg/mL, with an additional tube containing only standard diluent buffer serving as the blank (0 pg/mL). Fresh standard dilutions were prepared for each experiment, and pipette tips were changed between each dilution step to prevent cross-contamination.

The Biotinylated Antibody and Streptavidin HRP solutions were briefly centrifuged before being diluted 1:100 in their respective diluents. The required volume of TMB substrate solution was aspirated using sterile tips, and any remaining substrate was not returned to the original vial.

b. **Assay procedure**

All standards, samples, and controls were assayed in duplicate. Initially, 100 µL of each standard or sample was added to the appropriate wells of the antibody-coated plate and incubated at 37 °C for 90 minutes. After incubation, the wells were aspirated and washed three times with 1× wash buffer to remove unbound material. Subsequently, 100 µL of diluted Biotinylated Antibody working solution was added to each well, followed by incubation at 37 °C for 60 minutes.

The wells were washed three times before adding 100 µL of diluted Streptavidin-HRP working solution, which was incubated at 37 °C for 30 minutes. After another set of washes, 90 µL of TMB Substrate Solution was dispensed into each well and incubated in the dark at 37 °C for 15 minutes, resulting in blue colour development. The reaction was terminated by adding 50 µL of Stop Solution, turning the colour to yellow.

OD was measured at 450 nm using a microplate reader within 5 minutes of stopping the reaction. TNF-alpha concentrations in samples were calculated from the standard curve generated during the assay.

3.2.12 Hepatic Lipid Peroxidation Markers

The amount of MDA present in the liver determined the level of lipid peroxidation in liver cells. The levels of MDA were determined using their respective ELISA kits (ELK Biotechnology, Wuhan).

a. Reagent preparation

All kit components and samples were brought to room temperature (18□25°C) before use, ensuring that all reagents were fully dissolved and mixed thoroughly. For experiments where the kit was not entirely consumed in a single run, only the required strips and reagents were taken out, while the remaining components were stored as specified. The 25× Wash Buffer was diluted to 1× concentration using double-distilled water.

The standard solution was prepared by centrifuging the standard at 1000 × g for 1 minute, followed by reconstitution with 1.0 mL of Standard Diluent Buffer. This was left at room temperature for approximately 10 minutes and gently mixed to avoid foaming. The stock concentration of the standard was 2000 pg/mL. Seven tubes, each containing 0.5 mL of standard diluent buffer, were prepared for a two-fold serial dilution, yielding concentrations of 2000 pg/mL, 1000 pg/mL, 500 pg/mL, 250 pg/mL, 125 pg/mL, 62.5 pg/mL and 31.25 pg/mL, with the final tube containing only diluent serving as a blank (0 pg/mL). Fresh standard solutions were prepared for each experiment to ensure validity. During the dilution process from higher to lower concentrations, pipette tips were replaced for each step, and no solution was transferred into the blank tube from the preceding one.

Biotinylated Antibody and Streptavidin-HRP stock were briefly spun down before the experiment and diluted 100-fold with each of their diluents. The TMB substrate solution was aspirated in the required volume using sterile tips, and any residual solution was not returned to the original vial.

b. Assay procedure

The wells were designated for the diluted standard, blank, and samples, with seven wells allocated for the standards and one for standard working solution (prepared as described in the reagent preparation section) or the samples was added to the corresponding wells. The plate was covered with a plate cover and incubated at 37 °C for 80 minutes. Care was taken to add all solutions to the bottom of the micro ELISA plate wells, avoiding contact with the inner walls and minimising foaming. Following incubation, the liquid from each well was discarded, and the wells were washed with □□□ µ□ of remain in the wells for 1□2 minutes. Any remaining liquid was removed completely by snapping the plate onto absorbent paper. The washing process was performed three times in total. After the final wash, residual wash buffer was removed by aspiration or decanting, and the plate was inverted and blotted against absorbent paper. Throughout the washing process, care was taken to prevent the pipette tip from touching the well walls and to avoid cross-contamination between wells.

Subsequently, 100 µ□of the biotinylated antibody working solution was added to each well, the plate was covered, and incubation was carried out at 37 °C for 50 minutes. The aspiration and washing process, as previously described, was repeated three times. Thereafter, 100 µ□of the streptavidin-HRP working solution was added to each well, the plate was covered with a sealer, and incubation was again carried out at 37 °C for 50 minutes. The washing process was then repeated five times. Following this, □□ µ□ of T□□ substrate solution was with a new plate cover, and incubation was performed at 37 °C for 20 minutes in the dark (not exceeding 30 minutes). The addition of the TMB substrate solution caused the liquid to turn blue. The microplate reader was preheated for approximately 15 minutes before the optical density (OD) measurement.

The reaction was stopped by adding 50 µ□of stop reagent to each well, resulting in the liquid turning yellow. The plate was gently tapped to ensure thorough mixing, with the stop reagent being added in the same order as the TMB substrate solution. The bottom of the plate was wiped clean to remove any water droplets or fingerprints,

ensuring that no bubbles were present on the liquid surface. OD measurement was then performed immediately at 450 nm using a microplate reader.

The wells were designated for the diluted standard, blank, and samples, with seven wells allocated for the standards a either the standard working solution (prepared as described in the reagent preparation section) or the samples was added to the corresponding wells. The plate was covered with a plate cover and incubated at 37 °C for 80 minutes. Care was taken to add all solutions to the bottom of the micro ELISA plate wells, avoiding contact with the inner walls and minimising foaming. Following incubation, the liquid from each well was discarded, and the wells were washed with solution to remain in the wells for 1–2 minutes. Any remaining liquid was removed completely by snapping the plate onto absorbent paper. The washing process was performed three times in total. After the final wash, residual wash buffer was removed by aspiration or decanting, and the plate was inverted and blotted against absorbent paper. Throughout the washing process, care was taken to prevent the pipette tip from touching the well walls and to avoid cross-contamination between wells.

Subsequently, 100 µ of the biotinylated antibody working solution was added to each well, the plate was covered, and incubation was carried out at 37 °C for 50 minutes. The aspiration and washing process, as previously described, was repeated three times. Thereafter, 100 µ of the streptavidin-HRP working solution was added to each well, the plate was covered with a sealer, and incubation was again carried out at 37 °C for 50 minutes. The washing process was then repeated five times.

Following this, µ of TMB substrate plate was covered with a new plate cover, and incubation was performed at 37 °C for 20 minutes in the dark (not exceeding 30 minutes). The addition of the TMB substrate solution caused the liquid to turn blue.

The microplate reader was preheated for approximately 15 minutes before the optical density measurement. The rea reagent to each well, resulting in the liquid turning yellow. The plate was gently tapped to ensure thorough mixing, with the stop reagent being added in the same order as the

TMB substrate solution. The bottom of the plate was wiped clean to remove any water droplets or fingerprints, ensuring that no bubbles were present on the liquid surface.

OD measurement was then performed immediately at 450 nm using a microplate reader.

3.2.13 Statistical Analysis

Statistical analyses were carried out using MetaboAnalyst 5.0 (Pang et al. 2021), Data were normalized by log transformation and auto-scaling. Log transformation was applied to reduce skewness and stabilise variance. Auto-scaling was then performed to ensure equal contribution of all metabolites to multivariate analyses, preventing dominance by high-abundance metabolites. Principal component analysis (PCA) was conducted as an unsupervised multivariate method to visualise intrinsic clustering patterns, detect outliers, and assess variation between experimental groups. Differentially expressed metabolites (DEMs) were identified using one-way analysis of variance (ANOVA), with significance set at $p < 0.05$. Pathway analysis was subsequently performed using MetaboAnalyst 5.0 to identify biologically relevant metabolic alterations.

For univariate statistical analysis, data are presented as mean ± standard deviation (SD). Statistical comparisons between groups were performed using one-way □ □ □ □ □ followed by Tu□ey's post hoc test (GraphPad Software, San Diego, CA, USA). A p-value < 0.05 was considered statistically significant Normality was assessed by visual inspection of Q□Q plots of the residuals generated before performing one-way ANOVA.

CHAPTER IV

RESULTS

4.1 PRINCIPAL COMPONENT ANALYSIS (PCA) OF QUALITY CONTROL (QC) SAMPLES

Polar metabolite extracts from all experimental groups, along with pooled Quality Control (QC) samples, were analysed using Liquid Chromatography with Tandem Mass Spectrometry (LCMS/MS) (Q Exactive HF Orbitrap, Thermo Scientific) in both positive and negative ionisation modes. To assess the stability, reproducibility, and overall quality of the analytical platform, an unsupervised Principal Component Analysis (PCA) was conducted using all detected features from both ionisation modes.

As shown in Figure 4.1, the QC samples (green dots) exhibited relatively tight clustering in both PCA score plots. In the positive ion mode (Figure 4.1A), QC samples formed a moderately compact cluster at the centre of the score plot, indicating acceptable consistency of metabolite detection across runs. However, minor variability along the PC2 axis was observed. In contrast, negative ion mode analysis (Figure 4.1B) demonstrated even tighter clustering of QC samples, reflecting greater analytical stability under this ionisation condition.

These results confirm that the metabolomics data generated from this study are generally stable and reliable, and the observed metabolic variations across experimental groups can be attributed to biological differences rather than instrumental or technical drift.

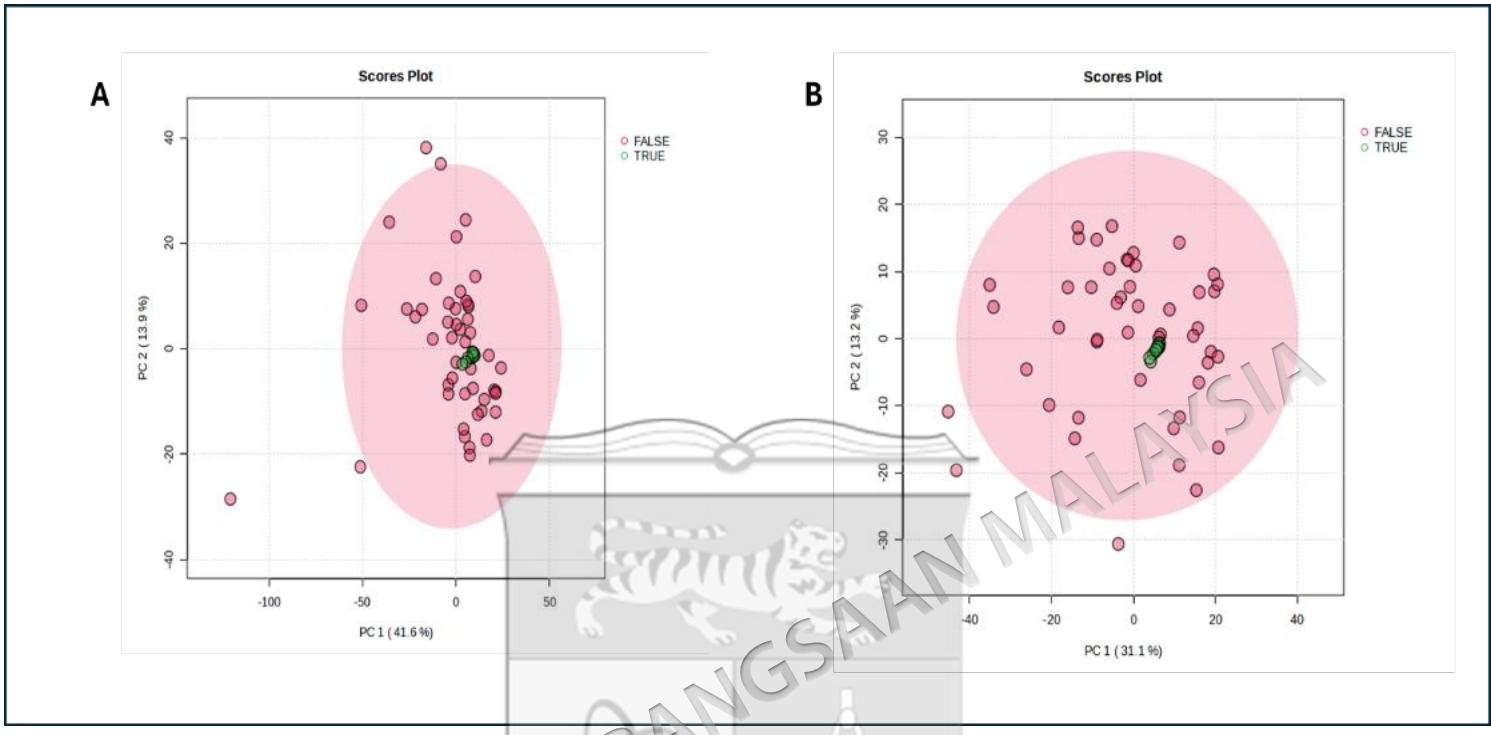


Figure 4.1 PCA score plots for QC samples in (a) positive and (b) negative ion modes. Each dot represents one sample. QC samples were labeled as □ T R □in green dot.

4.2 PCA AMONG ALL GROUPS

Prior to detailed metabolomic profiling, an unsupervised Principal Component Analysis (PCA) was conducted to assess the metabolic variations and treatment effects of TRF An unsupervised Principal Component Analysis was performed on all six experimental groups, including both young and old rats with and without TRF treatment. The PCA score plot (Figure 4.2) illustrates the distribution and clustering patterns, based on metabolic profiles, among all treatment and control groups across all age categories. We combined all groups into a single PCA model to visualise the overall metabolic variation and separation patterns across age and treatment simultaneously, allowing direct comparison.

The two principal components explained a cumulative variance of 54.9%, with PC1(first principal component) contributing 40.3% and PC2 (second principal component) contributing 14.6%. The PCA score plots indicated partial overlapping as well as separations among groups

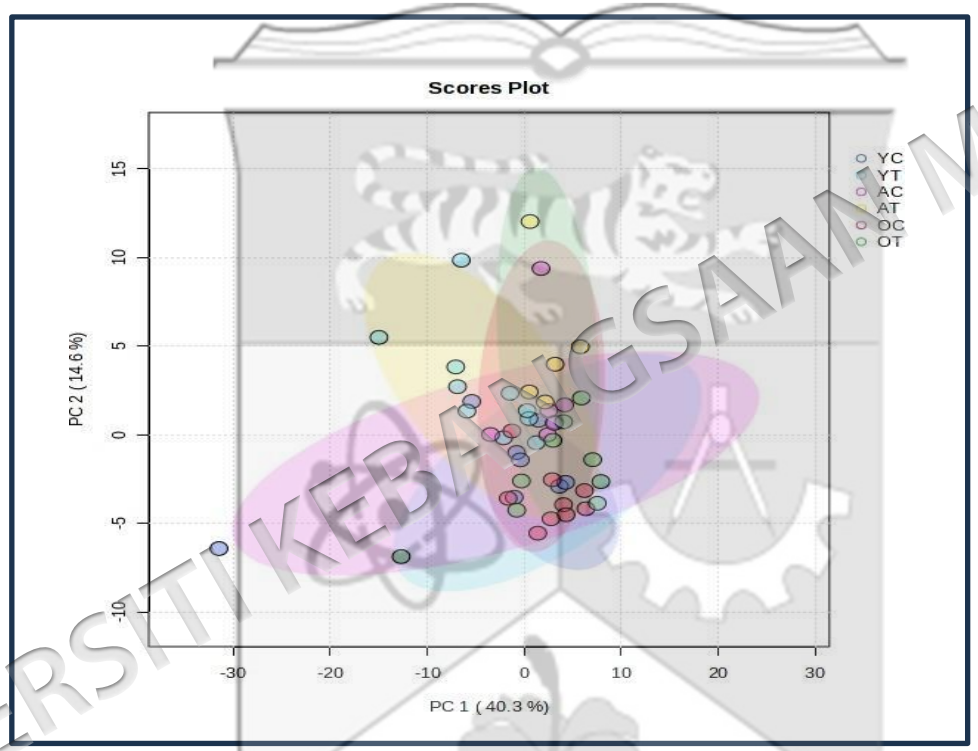


Figure 4.2 Distribution of samples for all groups and control groups based on PCA. Abbreviation: YC, young control; YT, young treatment; AC, adult control; AT, adult treatment; OC, old control; OT, old treatment. Metabolomic profile was represented by molecular features with annotation only combined from positive and negative ion modes. Each dot represents one sample.

4.3 PCA REVEALS AGE-RELATED METABOLIC SEPARATION AMONG CONTROL GROUPS

Next, to explore inherent metabolic differences across age groups, unsupervised PCA was performed on control samples (Figure 4.3). Both 2D and 3D score plots were generated to assess the spatial clustering and separation of groups based on principal components. PCA of metabolomic data showed different patterns among control groups. PCA was done to visualise the characteristics of different ageing groups where, PCA of YC and AC had PC1 score of 29.7%, PC2 score of 18.3% and PC3 score of 11.2%. PCA of YC and OC had PC1 score of 34.9%, PC2 score of 20.9% and PC3 score of 9.1%. PCA of AC against OC generated had the PC1 score of 30%, PC2 score of 21% and In the 2D PCA plot PC3 score of 12.1%.

PCA showed partial separation between adult control (AC) and YC groups (Fig. 4.3A and Fig. 4.3D). When comparing YC and OC, partial separation was observed between the young control (YC) and old control (OC) groups (Fig. 4.3B and 4.3E). PCA also showed some separation between the AC and OC groups, indicating that older age have some influences in the liver metabolic profile (Fig. 4.6C and Fig. 4.6F).

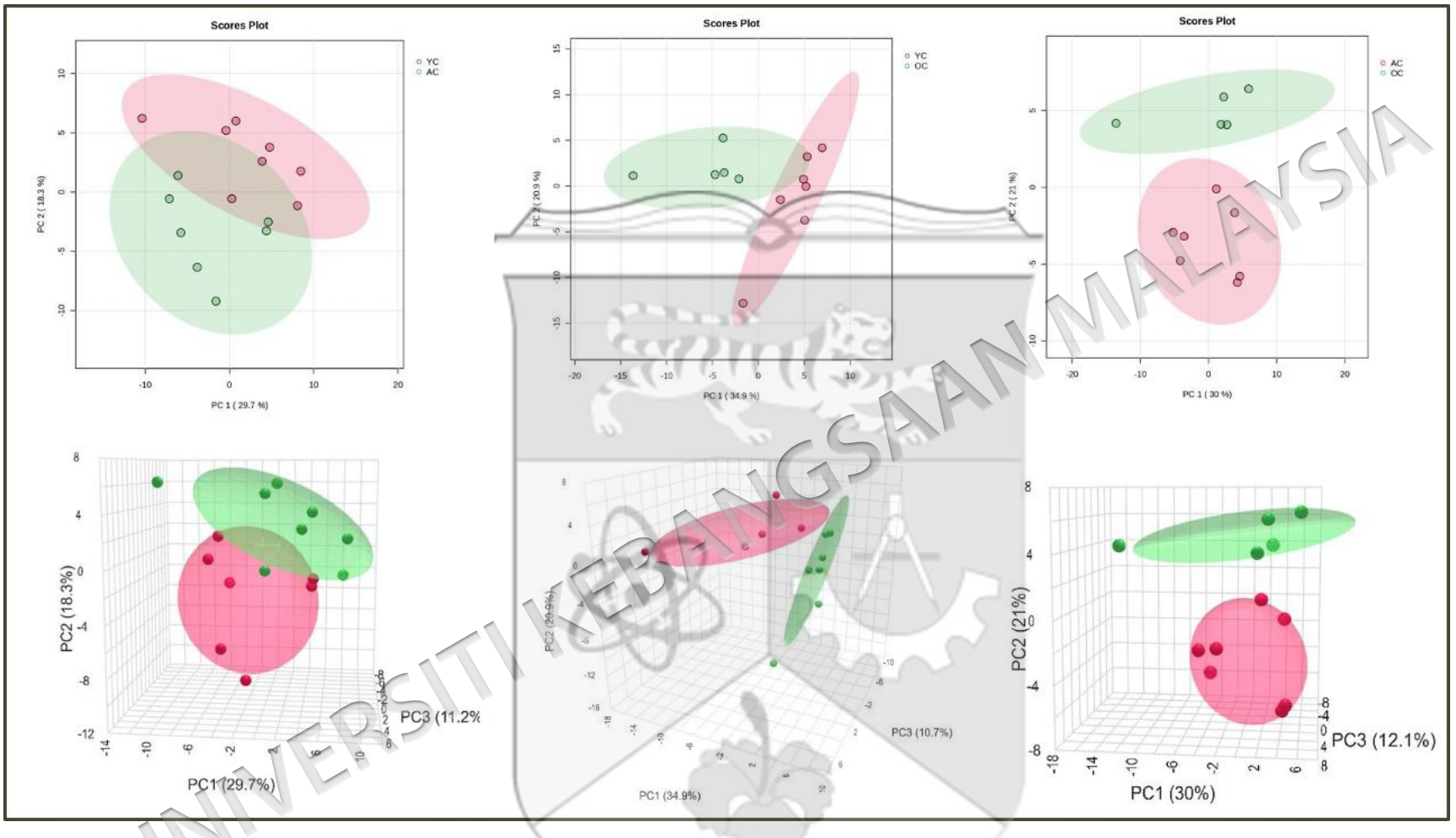


Figure 4.3 Distribution of samples between different control groups based on PCA. Each plot is shown in 2-dimensional and 3-dimensional views for clarity. PCA 2D score plots between YC and AC (A) YC and OC (B); AC and OC (C). PCA 3D score plots between YC and AC (D) YC and OC (E); AC and OC (F). Abbreviation: YC, young control; AC, adult control; OC, old control

4.4 PCA REVEALS AGE-RELATED METABOLIC SEPARATION AMONG CONTROL AND TREATMENT GROUPS

PCAs between the TRF-treated and control groups of different age groups were conducted as well to explore the liver metabolic shifts associated with tocotrienol-rich fraction (TRF) supplementation across various age groups. The comparison between the treatment groups and their respective age control groups revealed partial overlapping (Figure 4.4).

In the young group (YT and YC), PCA revealed some clustering (Figure 4.7A). Partial overlap was evident in the 2D score plot and the 3D PCA (PC1: 41.7%, PC2: 14.3%, PC3: 12.3%) (Figure 4.4D).

For the adult group (AT and AC), also partial separation was observed. The 2D PCA showed some clustering (Figure 4.4B), and the 3D PCA (PC1: 49.0%, PC2: 15.3%, PC3: 7.8%) confirmed this trend (Figure 4.4E).

Similarly, in the old group (OT and OC), PCA revealed partial overlap between groups (PC1: 31.6%, PC2: 23.8% PC3: 11.5%) (Figure 4.4C and Figure 4.4F). This pattern may suggest subtle to moderate metabolic alterations induced by TRF treatment across different aged groups in rats.

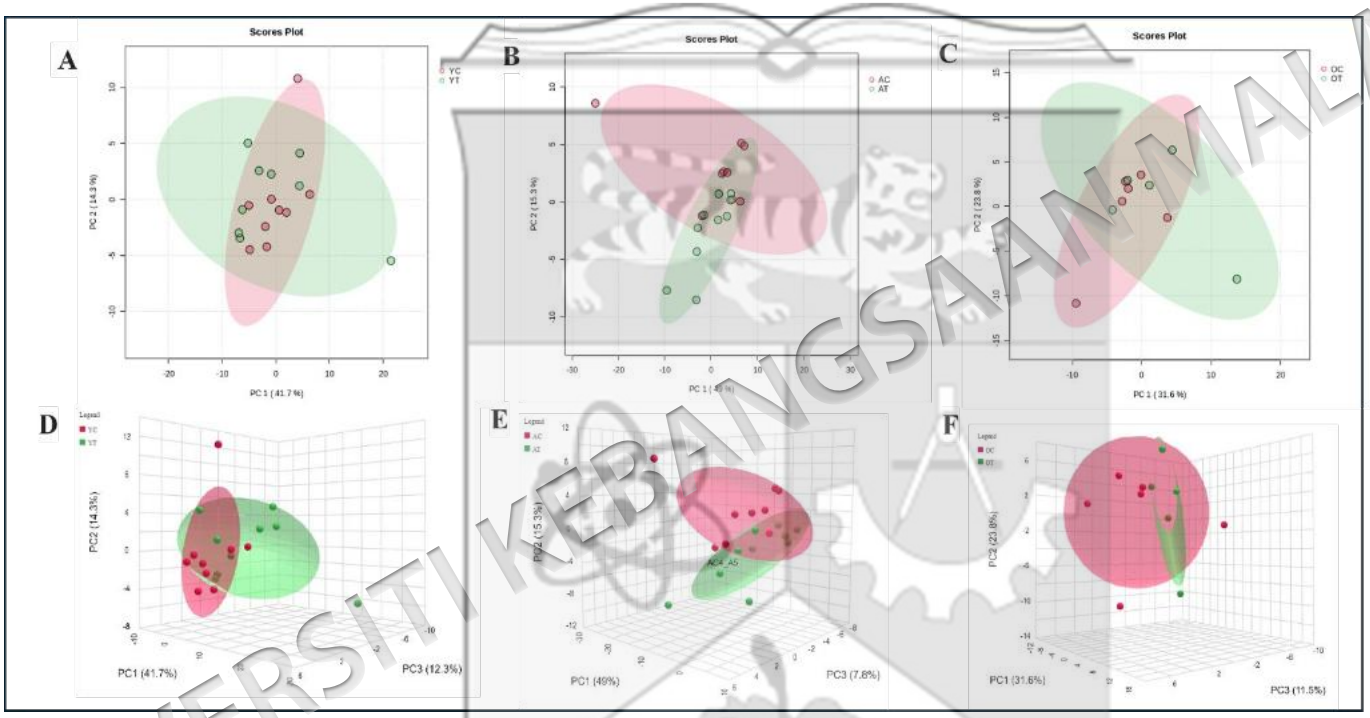


Figure 4.4 Distribution of samples for treatment groups versus control groups based on PCA. Each plot is shown in 2-dimensional and 3-dimensional views for clarity. Each dot represents one sample. PCA 2D score plots between YT and YC (**A**) AT and AC (**B**); OT and OC (**C**). PCA 3D score plots between YT and YC (**D**) AT and AC (**E**); OT and OC (**F**). Abbreviation: YC, young control; YT, young treatment; AC, adult control; AT, adult treatment; OT, old treatment.

4.5 DIFFERENTIAL LIVER METABOLITES IDENTIFIED IN CONTROL GROUPS

To investigate the impact of age on metabolite expression, control groups were compared among young, adult, and old groups (Table 4.1). A total of 111 metabolic features were obtained from the combined positive and negative ionisation modes following ANOVA and T u □ eHyon'ss Significant Difference (HSD) post hoc analysis ($p<0.05$). Among the control groups, a total of 13 metabolites were found to be significantly altered: 10 between AC and YC, 6 between OC and YC, and 3 between OC and AC.

As shown in Table 4.1, metabolites that were altered were: N acetyl alpha glucose amine 1 phosphate which was high in YC compared to AC and OC, sedoheptulose 7 phosphate which was more in YC than OC, isoleucine and pipecholate were more in YC than ac, uridine was more in YC than OC and AC more than OC, uracil was more in YC than OC, 4 guadinobutanatr was more in YC than AC and OC, glycoursideoxycholic acis more in OC than AC, trigonelinne more in YC than OC, acetyl arginine more in YC than AC and OC, methyl aminoisobutyric acid more in YC and AC, acetyl b methyl choline in YC than AC. In the AC and YC comparison, taurine (+3.27 FC) was elevated in adult controls. In contrast, N-acetyl-α-glucosamine 1-phosphate (-1.13 FC), 4-guanidinobutyric acid (-2.87 FC), glycoursodeoxycholic acid (-6.35 FC), trigonelline (□3.07 FC), acetylarginine (-6.26 FC), isoleucine (-7.83 FC), methyl aminoisobutyric (-1.86 FC), acetyl-□-methylcholine (-4.81 FC) and pipecholate (-2.20 FC) were decreased compared to young controls. These alterations suggest age-related shifts in metabolites, specifically amino acids, in adult livers. In the OC and YC group, uridine (-6.78 FC), N-acetyl-α-glucosamine 1-phosphate (-1.73 FC), uracil (-3.82 FC), 4-guanidinobutyric acid (-2.53 FC), acetylarginine (-4.79 FC), sedoheptulose 7 phosphate (-4.43 FC) were significantly decreased in old controls, indicating further metabolic changes with ageing, particularly in arginine and energy metabolism.

Similarly, in the OC and AC comparison, glycoursodeoxycholic acid (7.36 FC), remained elevated in old livers. In comparison, uridine (-3.68 FC) and Taurine (-2.91 FC) decreased, suggesting a potential age-associated decline in these metabolites.

Table 4.1 Significant liver metabolites in control groups

Mode	Metabolites	ID	MW	RT	P-value	AC vs YC (FC)	OC vs YC (FC)	OC vs AC (FC)
+	2-(Methylamino)isobutyric acid	HMDB0002141	117.079	0.619	0.000534	-1.863		
+	4-Guanidinobutyric acid	HMDB0003464	145.085	0.73	9.54E-06	-2.8732	-2.5297	
+	Acetylarginine	HMDB0004620	216.122	0.785	0.000221	-6.2582	-4.5693	
+	Acetyl-β-methylcholine	HMDB0015654	159.126	0.923	0.002589	-4.8151		
-	Glycoursodeoxycholic acid	HMDB0000708	449.313	8.912	1.38E-05	-6.3468		7.3572
+	Isomer: Leucine/Isoleucine	HMDB0000678/HMDB0000172/HMDB0013773	131.095	0.702	0.000284	-7.8302		
-	N-Acetyl-α-glucosamine 1-phosphate	HMDB0001367	301.055	0.544	2.58E-08	-1.1325	-1.7299	
+	Pipecolinic acid	HMDB0005960/HMDB0000070/HMDB0000716	129.079	0.821	0.003687	-2.2067		
-	Sedoheptulose 7-phosphate	HMDB0001068/HMDB0258206	290.04	0.532	0.000751		-4.4342	
+	Taurine	HMDB0000251	125.015	0.53	3.84E-05	3.2721		-2.9074
+	Trigonelline	HMDB0000875	137.048	0.656	0.000107	-3.0783		
+	Uracil	HMDB0000300	112.027	1.262	4.49E-06		-3.8204	
-	Uridine	HMDB0000296	244.069	1.273	8.21E-09		-6.7765	-3.6822

AC compared to YC, where YC, as denominator; OC and YC, YC, as denominator; OC and AC. AC, as denominator. Symbol: (+) increase; (-) decrease. Acronym: FC (fold change); m/w (molecular weight); RT (retention time). Potential metabolite identified by cross referencing with mzCloud data. ID are based on KEGG database (C) or Human Metabolome Database (HMDB).

4.6 DIFFERENTIAL LIVER METABOLITES IDENTIFIED IN CONTROL AND TREATMENT GROUPS

Treatment groups were compared based on fold change against untreated control groups of young, adult and old rats (Table 4.2), in which AT and AC showed significant metabolites, and YT and YC and OT and OC showed no significant metabolites. AT and AC comparison generated 7 identified metabolites from 111 metabolic features.

Most of the significantly altered metabolites in the AT group showed a decrease in relative abundance compared to the AC group, including Uridine (FC = -3.26), N-Acetyl-α-glucosamine 1-phosphate (FC = -1.49), Tryptophan (FC = -2.31), Phenylalanine (FC = -3.20), Glutamic acid (FC = -1.65), and Malic acid (FC = -1.50). These changes suggest a potential downregulation of these metabolites following treatment.

Conversely, Acetyl-□-methylcholine, detected in the positive mode, showed a significant increase in abundance (FC = 3.90) in the AT group. This metabolite is involved in choline metabolism, which may indicate treatment-induced modulation of the metabolite. These findings highlight specific metabolic responses to treatment in adult rats.

Table 4.2 Significant liver metabolites in different aged control and treatment groups

Mode	Metabolites	ID	MW	RT	P-value	YT vs YC (FC)	AT vs AC (FC)	OT vs OC (FC)
+	Acetyl-β-methylcholine	HMDB0015654	159.126	0.923	0.002589	~	3.9035	~
-	Glutamic acid	HMDB0000148/HMDB0003339/HMDB0060475	147.052	0.526	0.001244	~	-1.6497	~
-	Malic acid	HMDB0000156/HMDB0031518	134.02	0.668	0.00411	~	-1.503	~
-	N-Acetyl-α-glucosamine 1-phosphate	HMDB0001367	301.055	0.544	2.58E-08	~	-1.4914	~
-	Phenylalanine	HMDB0250791	165.078	3.184	0.000288	~	-3.1988	~
-	Tryptophan	HMDB0030396/HMDB0000929/HMDB0013609	204.089	3.67	1.30E-05	~	-2.314	~
-	Uridine	HMDB0000296	244.069	1.273	8.21E-09	~	-3.2634	~

YC compared to YT, where YC set as a denominator; AC andAT, where AC set as a denominator; OC and OT, where OC set as a denominator.(+) indicates increase; (-) indicates decrease. Acronyms: FC-fold change, MW-molecular weight, RT-retention time. Potential metabolite identified by cross referencing with mzCloud data. ID are based on KEGG database (C) or Human Metabolome Database (HMDB).

4.7 ANALYSIS OF BIOCHEMICAL PATHWAYS FOR LIVER METABOLOMES IN CONTROL GROUPS

Metabolomic pathway analysis was performed using MetaboAnalyst on the liver metabolome expression during ageing. A comparison of profiled metabolites between AC and YC was executed. Pathways were identified as significant with a *p*-value <0.05 and an impactor of >0.1. The significant and impactful pathways involved were Taurine and hypotaurine metabolism (Figure 4.5). Additionally, Valine, leucine and isoleucine biosynthesis were other significant pathways. This suggests a functional disruption in these pathways with ageing. Other pathways altered were lysine degradation, arginine and proline metabolism, valine, leucine and isoleucine degradation, amino sugar and nucleotide sugar metabolism, and primary bile acid synthesis (Table 4.3).

Though for AC and OC, there were not enough metabolites for analysis because too few significant metabolites were detected in this group. Metabolites profiled in OC compared to YC generated altered pathways, as shown in Figure 4.6.

The significant pathways involved in OC compared to YC were pyrimidine metabolism and other altered pathways were pantothenate metabolism, beta-alanine metabolism, pentose phosphate pathway, arginine and proline metabolism, and amino sugar and nucleotide sugar metabolism (Table 4.4).

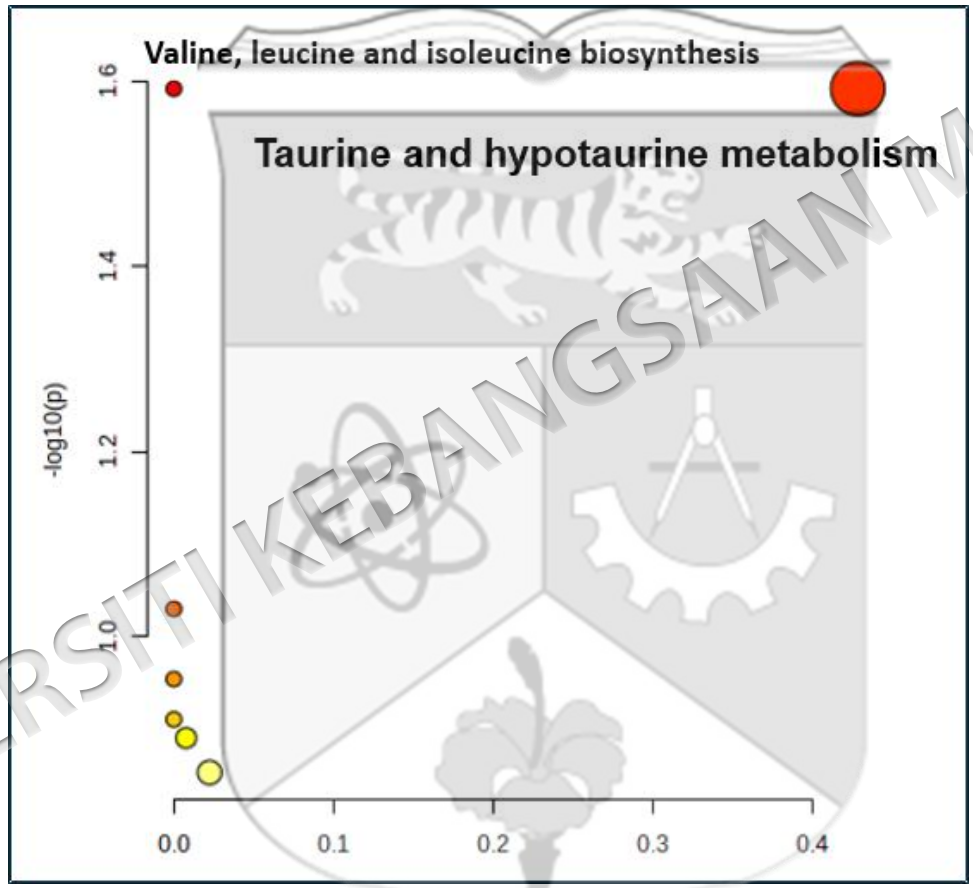


Figure 4.5 Biochemical pathway analysis of significant metabolites profile for YC and AC. The degree of colour saturation (from white to red) indicates a rise in statistical significance (p-value), while the size of the circle changes based on the impact of the pathway

Table 4.3 List of biochemical pathways of significant metabolites profiled for YC and AC

Pathway Name	P	Holm p	FDR	Impact
Valine, leucine and isoleucine biosynthesis	0.020532*	1	0.82128	0
Taurine and hypotaurine metabolism	0.020532*	1	0.82128	0.42857#
Arginine and proline metabolism	0.089912	1	1	0
Valine, leucine and isoleucine degradation	0.099514	1	1	0
Amino sugar and nucleotide sugar metabolism	0.10429	1	1	0.00771
Primary bile acid biosynthesis	0.11377	1	1	0.02239

*Considered as significant biochemical pathway, p-value <0.05 and impact value > 0.1.

Table 4.4 List of biochemical pathways of significant metabolites profiled for YC and OC.

Pathway Name	p	Holm p	FDR	Impact
Pyrimidine metabolism	0.0035957*	0.28765	0.28765	0.09246
Pantothenate and CoA biosynthesis	0.050735	1	1	0
beta-Alanine metabolism	0.05322	1	1	0
Arginine and proline metabolism	0.089912	1	1	0
Amino sugar and nucleotide sugar metabolism	0.10429	1	1	0.00771

*p-value < 0.05; and #impact >0.1 is regarded as significant.

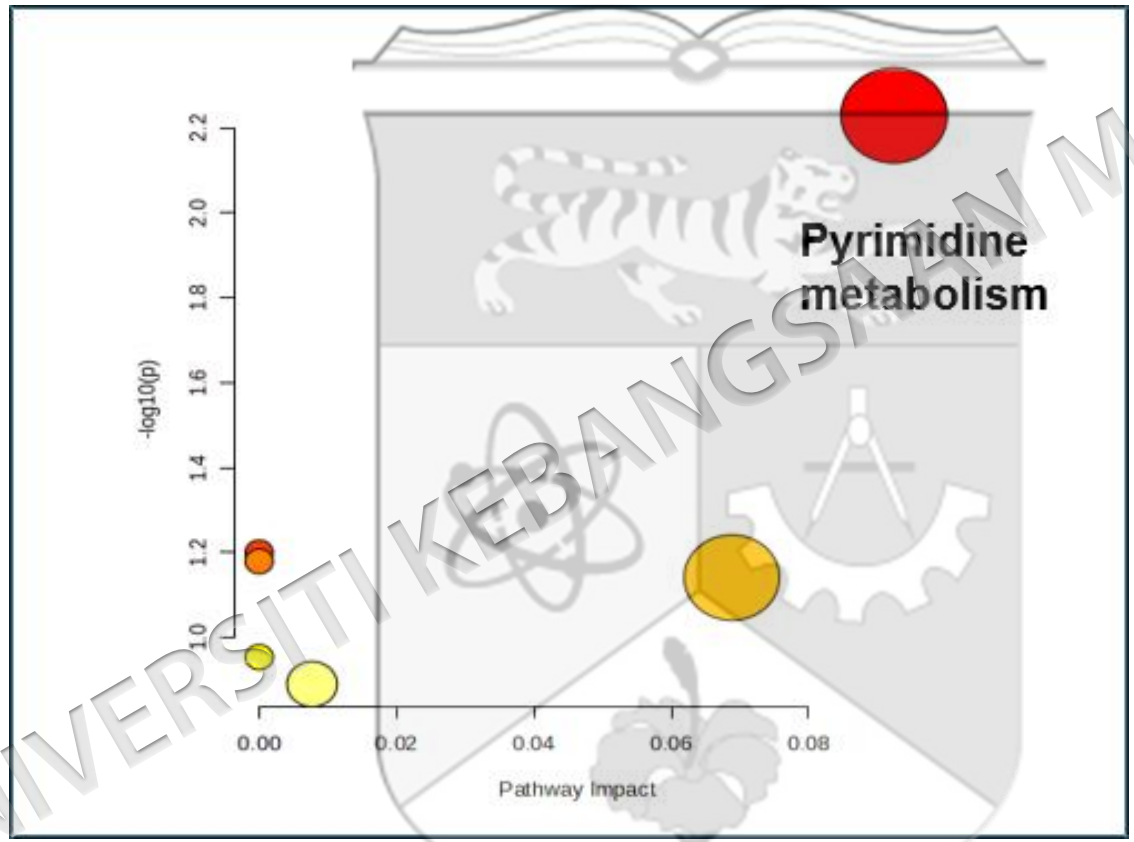
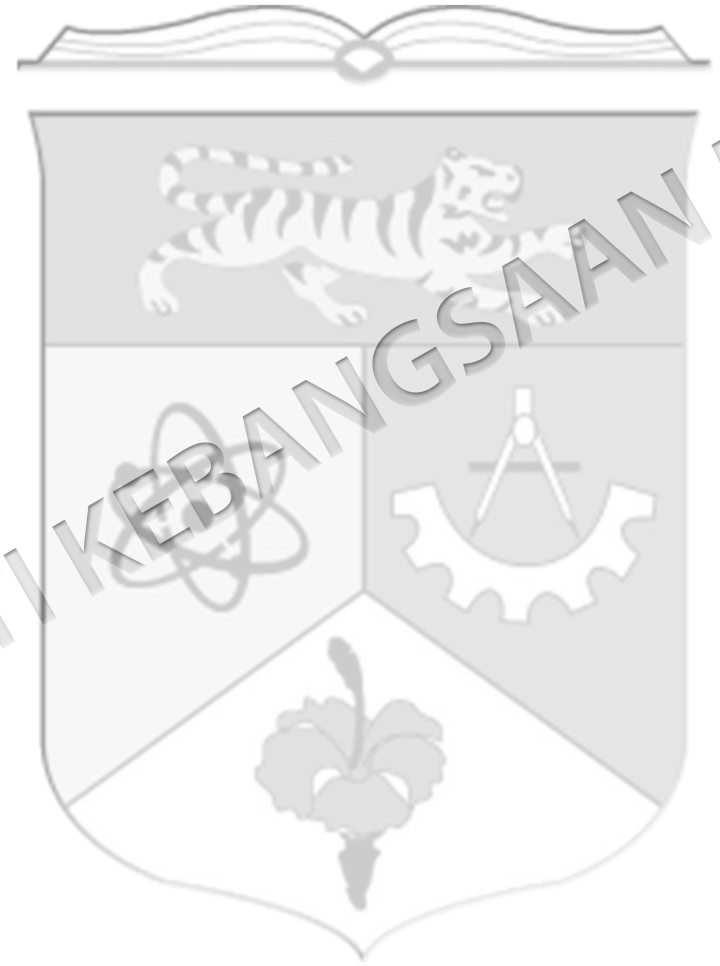


Figure 4.6 Biochemical pathway analysis of significant metabolites profile for YC and OC. The degree of colour saturation (from white to red) indicates a rise in statistical significance (p-value), while the size of the circle changes based on the impact of the pathway.



4.8 ANALYSIS OF BIOCHEMICAL PATHWAYS FOR LIVER METABOLOMES IN CONTROL GROUPS AND TREATMENT GROUPS

Pathway analysis was performed on liver metabolome expression during ageing to see the effect of TRF treatment on the metabolic pathway in ageing liver in comparison to the untreated control group (Figure 4.7).

Pathway analysis of the TRF-treated group revealed several metabolic pathways that were altered in response to treatment (Table 4.5). Among the affected pathways were glyoxylate and dicarboxylate metabolism, and nitrogen metabolism, suggesting potential changes in nitrogen handling and amino acid metabolism, and D-amino acid metabolism appeared on the pathway impact plot. Other observed alterations included butanoate metabolism, histidine metabolism, and the TCA cycle. Pathways such as pyruvate metabolism, glutathione metabolism, and alanine, aspartate, and glutamate metabolism were also altered.

Additional pathways like arginine and proline metabolism, pyrimidine metabolism, and tryptophan metabolism further suggest broader modulation of amino acid and nucleotide processes. These findings provide insights into the metabolic adaptations potentially mediated by TRF in the ageing model. Figure 4.8 shows the Integrated KEGG pathway of differentially expressed metabolites in the treatment and the control groups.

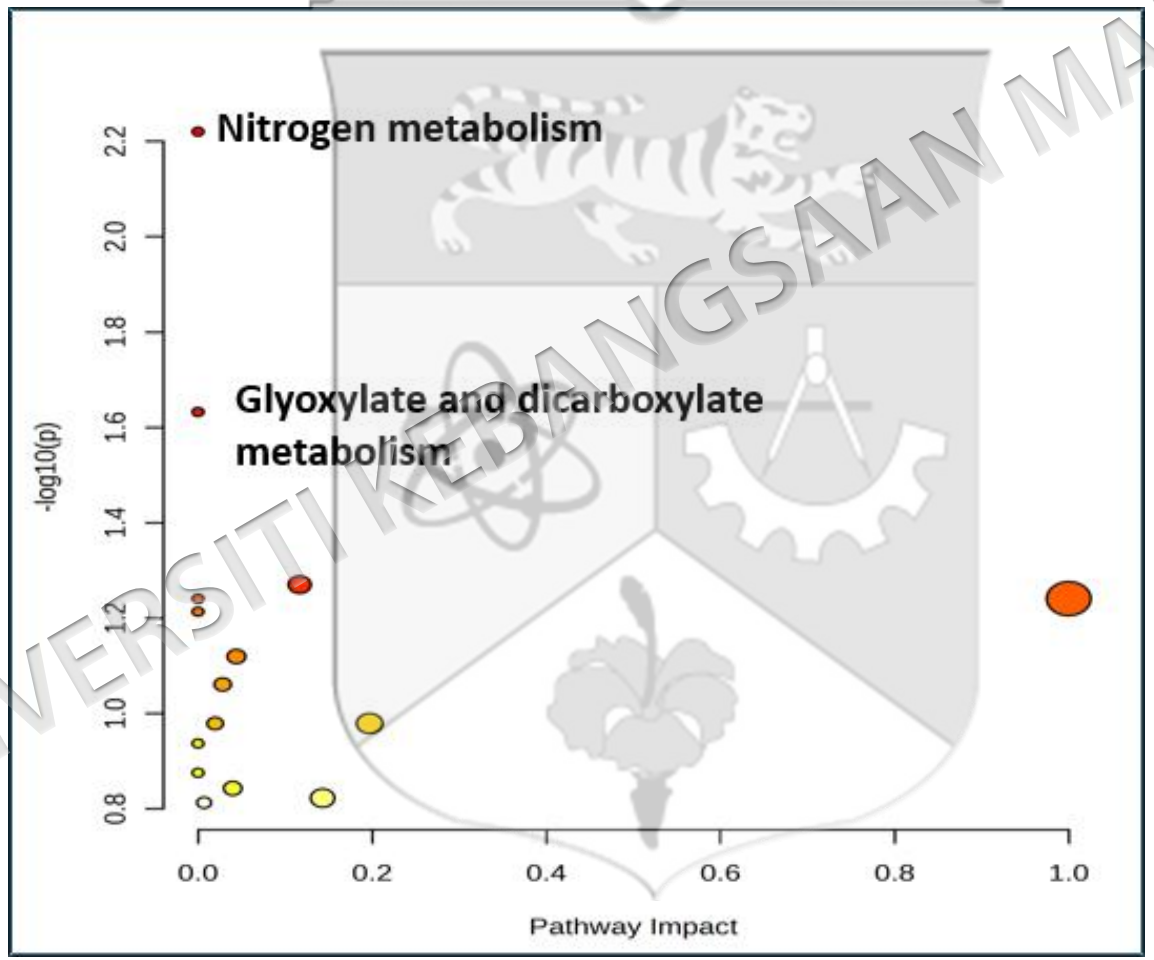


Figure 4.7 Biochemical pathway analysis of significant metabolites profile for AC and AT. The degree of colour saturation (from white to red) indicates a rise in statistical significance (p-value), while the size of the circle changes based on the impact of the pathway.

Table 4.5 List of biochemical pathways of significant metabolites profiled for AC and. AT.

Pathway Name	p	Holm p	FDR	Impact
Glyoxylate and dicarboxylate metabolism	0.0060194*	0.48155	0.482	0
Nitrogen metabolism	0.023308*	1	0.815	0
Arginine biosynthesis	0.053678	1	0.815	0.11675
Butanoate metabolism	0.057419	1	0.815	0
D-Amino acid metabolism	0.057419	1	0.815	1
Histidine metabolism	0.061147	1	0.815	0
Citrate cycle (TCA cycle)	0.075937	1	0.821	0.04412
Pyruvate metabolism	0.086901	1	0.821	0.0283
Glutathione metabolism	0.10493	1	0.821	0.01966
Alanine, aspartate and glutamate metabolism	0.10493	1	0.821	0.19712
Porphyrin metabolism	0.11561	1	0.821	0
Arginine and proline metabolism	0.13317	1	0.821	0
Pyrimidine metabolism	0.14356	1	0.821	0.03985
Tryptophan metabolism	0.15044	1	0.821	0.14305
Amino sugar and nucleotide sugar metabolism	0.15385	1	0.821	0.00698

*p-value < 0.05; and #impact >0.1 is regarded as significant.

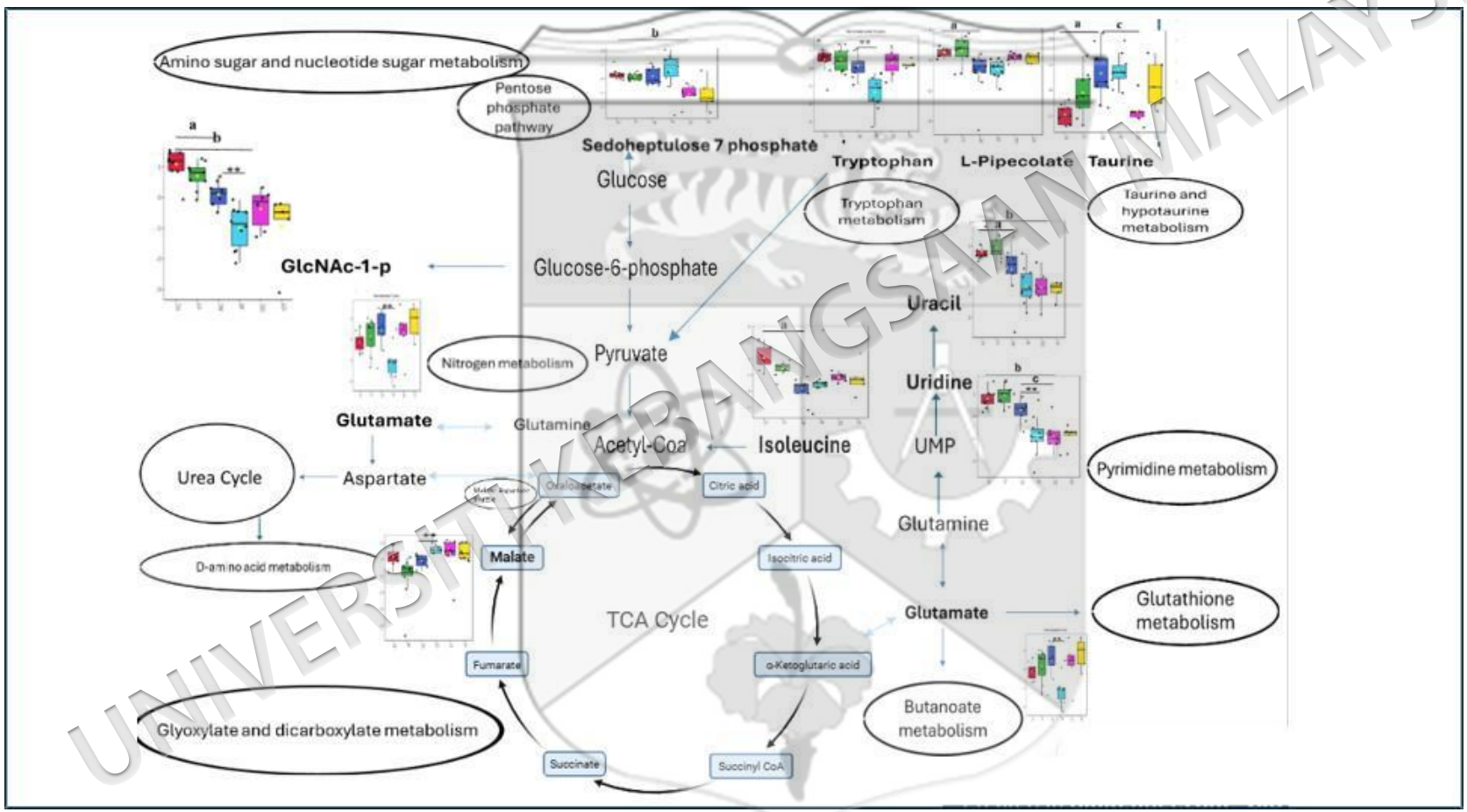


Figure 4.8 Summary of integrated KEGG pathways of differentially expressed metabolites in treated and control groups. Box and whiskers plots with 95% confidence intervals are presented. Analysis was performed using one-way Anova with T u □ epyst hoc analysis. *p < 0.05. * p < 0.05: significantly different for YC compared to YT group. a* p < 0.05: significantly different for YC compared to AC, b* p < 0.05: significantly different for YC compared to OC, ** p < 0.05: significantly different for AC compared to AT, c* p < 0.05: significantly different for AC compared to OC and *** p < 0.05: significantly different for OC compared to OT

4.9 HEPATIC HISTOLOGICAL ANALYSIS IN AGEING TISSUES AMONG CONTROL AND TRF TREATMENT GROUPS

The histological analysis of liver tissues was performed and interpreted with the assistance of Dr Tatiana, an expert from the Department of Anatomy. Hematoxylin and eosin (H&E) staining revealed distinct morphological differences among the groups, indicating varying levels of structural integrity and cellular organisation in the liver tissues (Figure 4.9).

Young control group liver sections displayed some fibrous cells, characterised by thickened spaces between cells, loss of the normal cellularpattern.Despite these features, hepatocyte morphology and organisation remained relatively normal, and the tissue maintained a preserved cellular structure with minimal changes. Adult control showed a greater area with fewer cellular structures, including less intact cell membranes and nuclei, and the presence of fibrotic-like areas were more compared to the young control group. Old control liver sections exhibited a further reduction in cellular structures compared to adult control and hepatocytes appeared more elongated.

The young treated group had better cellular integrity and organisation compared to the control. In adult treated group, there was a reduction in areas lacking cells compared to adult control group. The presence of infiltration was also lower in comparison, suggesting a possible protective effect of the treatment. Old treated liver sections showed regions devoid of cells, with distinct morphological differences compared to the younger groups. Fibrotic like regions were reduced, and fewer infiltration observed compared to old control groups. These histological observations highlight age-related changes in liver architecture and the potential effects of treatment in maintaining tissue structure and reducing fibrotic like alterations.

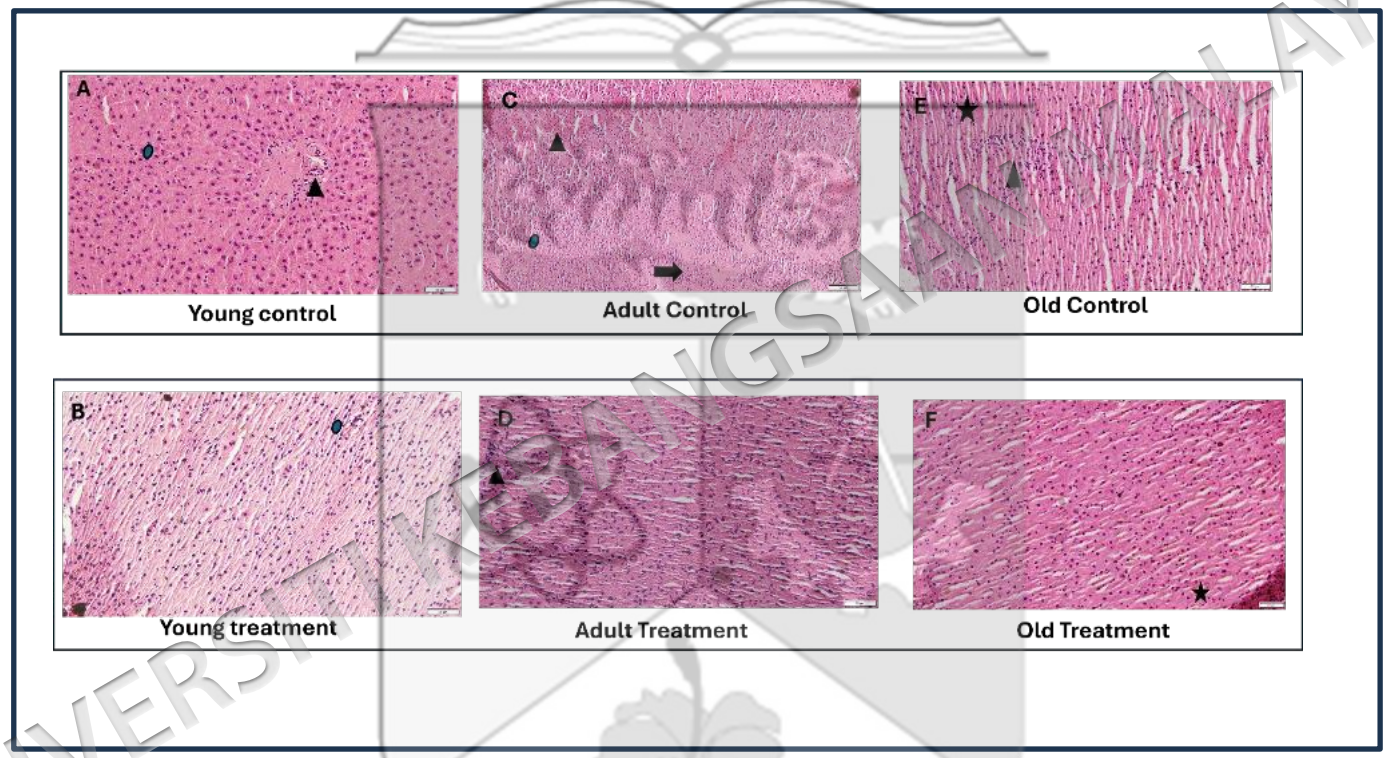


Figure 4.9 Histological differences in ageing liver tissues with and without TRF treatment. Histological images of the liver of a young control show: (A) Young control- some fibrous cells indicated by ▲. (B) Young treatment- shows some fibroblasts● and arrangements are better than control. (C) Adult control- more area with fewer cell structures than YT ➡ and fibroblasts are present. (D) Adult treatment- less area without cells, and with fewer fibroblasts. (E) Old control-less area of less cell structures, Hepatocytes - structures more elongated ★ (F) Old treatment- areas without cells, Morphology different than young. Less fibroblast or migrated macrophages than OC. Scale bar=100 µm.

4.10 ANTI-OXIDANT ENZYME ACTIVITY IN THE LIVER OF SD RATS ACROSS DIFFERENT AGE GROUPS

The SOD activity varied significantly across different age groups, indicating age-related changes in antioxidant enzyme function (Figure 4.10). A significant reduction in SOD activity was observed in the Old TRF group compared to the Young TRF group, suggesting that ageing reduces the effectiveness of TRF in maintaining antioxidant defence. Similarly, Adult TRF rats showed lower SOD activity than Young TRF rats, reinforcing the trend of declining enzymatic activity with age despite supplementation. In the control groups, Young Control rats exhibited significantly higher SOD activity than both Old and Adult Control rats, indicating a natural decline in antioxidant defence mechanisms with ageing. These findings highlight the impact of ageing on SOD activity and suggest that TRF supplementation may provide some protective effects.

Catalase activity in the liver of SD rats was assessed across different age groups with and without TRF supplementation (Figure 4.11). The groups included young control (n=9), young TRF (n=9), adult control (n=9), adult TRF (n=10), old control (n=6), and old TRF (n=5). The activity of CAT was expressed in Units/mg, where one □nit corresponds to the degradation of □ Statistical analysis was performed, with significance set at $p < 0.05$.

However, no significant differences in CAT activity were observed between any of the groups. This suggests that neither age nor TRF supplementation had a notable impact on catalase enzyme activity in the liver of SD rats under the conditions of this study.

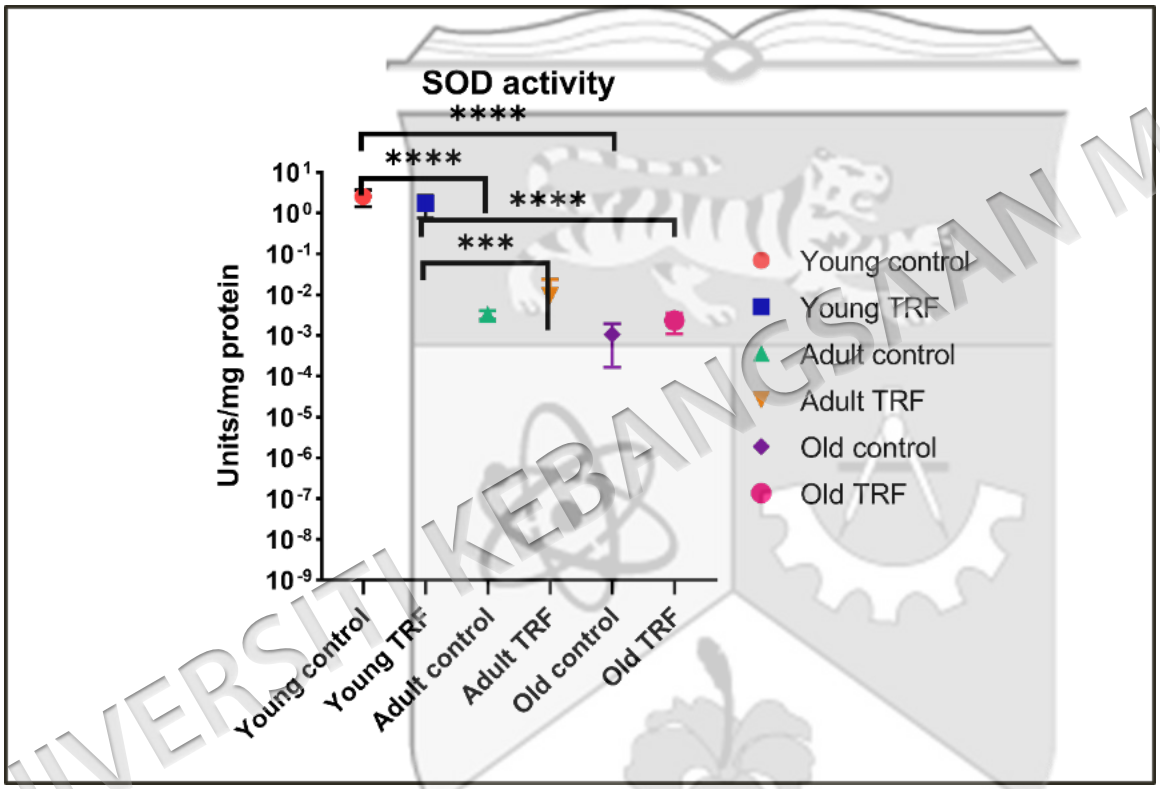


Figure 4.10 Superoxide dismutase (SOD) activity in the liver of SD rats of different age groups with and without TRF. The *** indicates a significant difference of p = 0.0002 and **** indicates a significant difference of p <0.0001 according to the Turkey post-hoc test. Each bar represents the mean and SD, *n* = 8. Values are considered significant at p < 0.05. SOD activity is expressed in U/mg of protein (the amount of enzyme needed to produce 50% dismutation of $O_2^{\bullet-}$ radical). The SOD activity values are plotted on a logarithmic scale to better visualise differences between age groups, but they represent actual enzyme activity. The SD values themselves are unchanged; only their visual representation is compressed to allow all groups to be compared on the same axis.

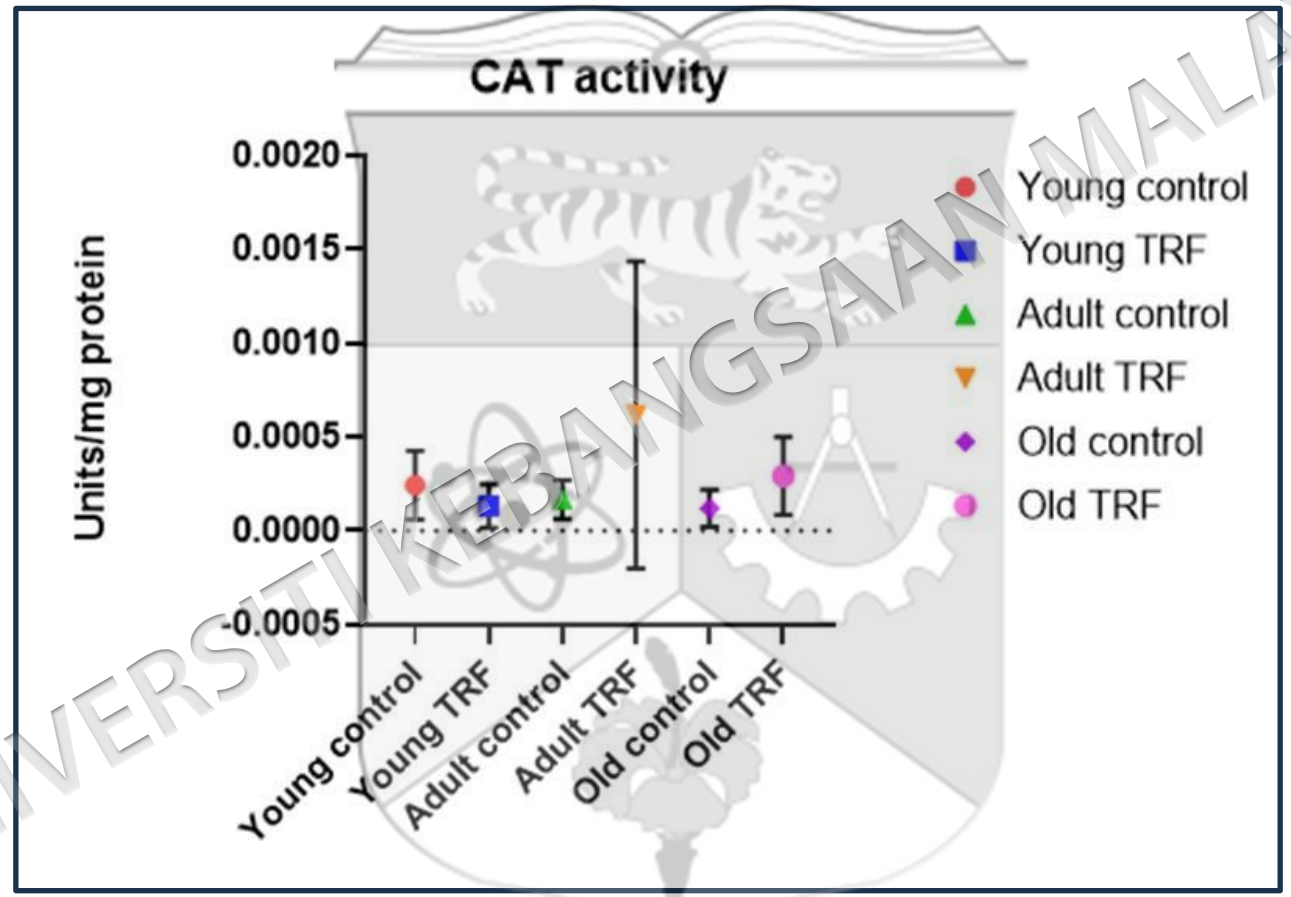


Figure 4.11 Catalase activity in the liver of SD rats of different age group with and without TRF: young control (n= 9), young TRF (n= 9), adult control (n= 9), adult TRF (n= 10), old control (n= 6) and old TRF (n=5). Each shape represents the mean and SD. Values are considered significant at $p < 0.05$. the activity of CAT is expressed in □n i t s □mg , w h e r e □n i t s i s e □u a l t o µ mo l □mg o f p r o t e i n □mi n □µ mo l o f □□□□ d e g r a d e d

4.11 INFLAMMATORY MARKERS IN THE LIVER OF SD RATS ACROSS DIFFERENT AGE GROUPS

The inflammatory cytokine Interleukin-6 (IL-6) in the liver of Sprague-Dawley (SD) rats was assessed across different age groups with and without tocotrienol-rich fraction (TRF) supplementation (Figure 4.12). The groups included young control (n=9), young TRF (n=9), adult control (n=9), adult TRF (n=10), old control (n=6), and old TRF (n=5). Statistical analysis was performed, with significance set at $p < 0.05$. No significant differences were observed between any of the groups. This suggests that neither age nor TRF supplementation had a notable impact on IL-6 in the liver of SD rats. However, there was a pattern in the young and adult groups that, with TRF, the IL-6 levels were lower than their normal counterparts.

The inflammatory cytokine TNF-alpha varied across different age groups, indicating age-related inflammation in the ageing livers (Figure 4.13). Statistical analysis was performed, with significance set at $p < 0.05$. A significant increase in TNF-alpha was observed in the old TRF group compared to the young TRF group, suggesting that TNF-alpha increases as the liver gets older.

Even though there was no significant difference in the other groups, we could see a pattern in the young and adult groups treated with TRF, the inflammatory TNF-alpha was lower than their normal counterparts. These findings highlight the increase in TNF-alpha with increasing age of livers.

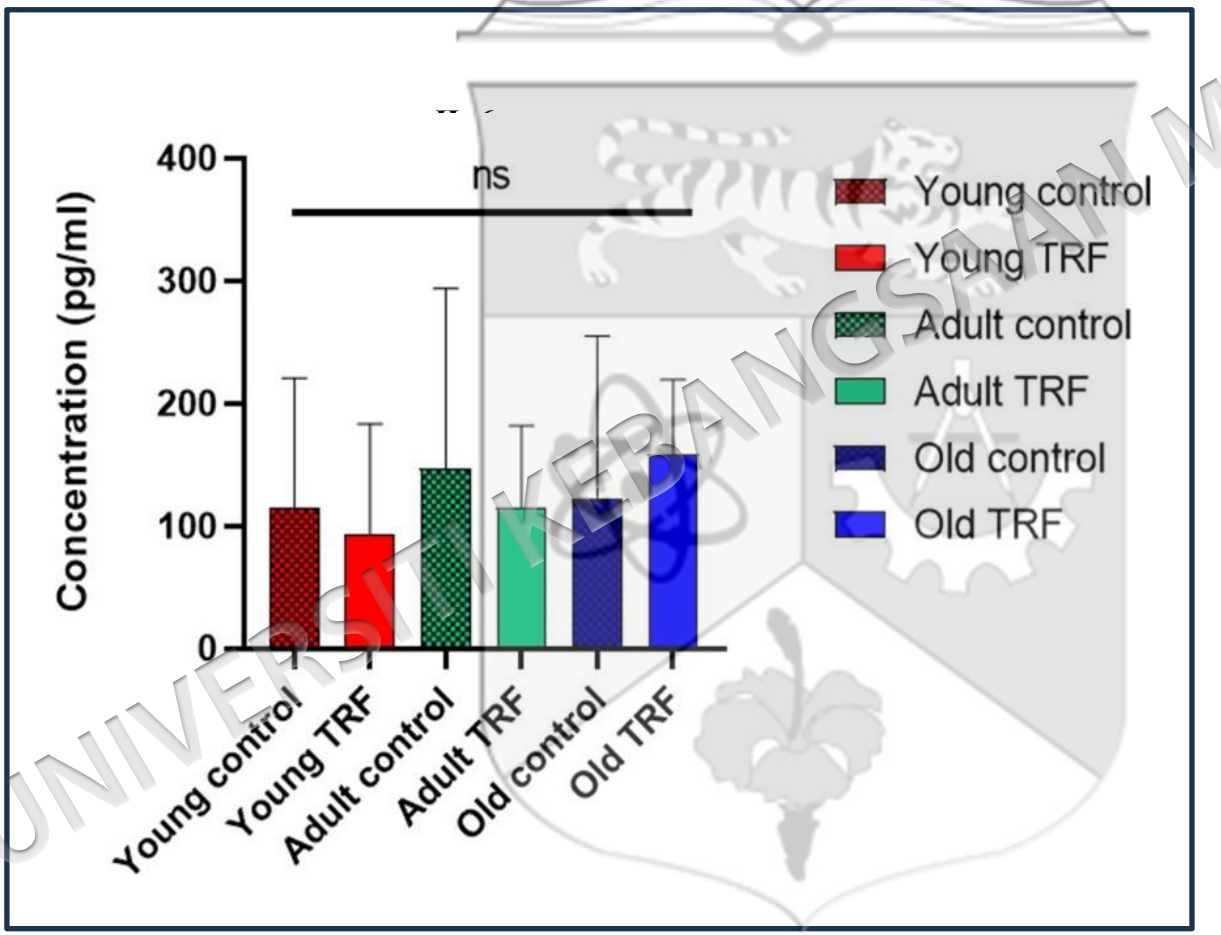


Figure 4.12 Hepatic IL-6 levels were determined by enzyme-linked immunosorbent assay in the liver of SD rats of different age group with and without TRF: young control (n= 9), young TRF (n= 9), adult control (n= 9), adult TRF (n= 10), old control (n= 6) and old TRF (n=5). Data are shown in pg/ml. Each bar represents the mean and SD. Values were considered significant at *p <0.05 according to the Turkey post-hoc test.

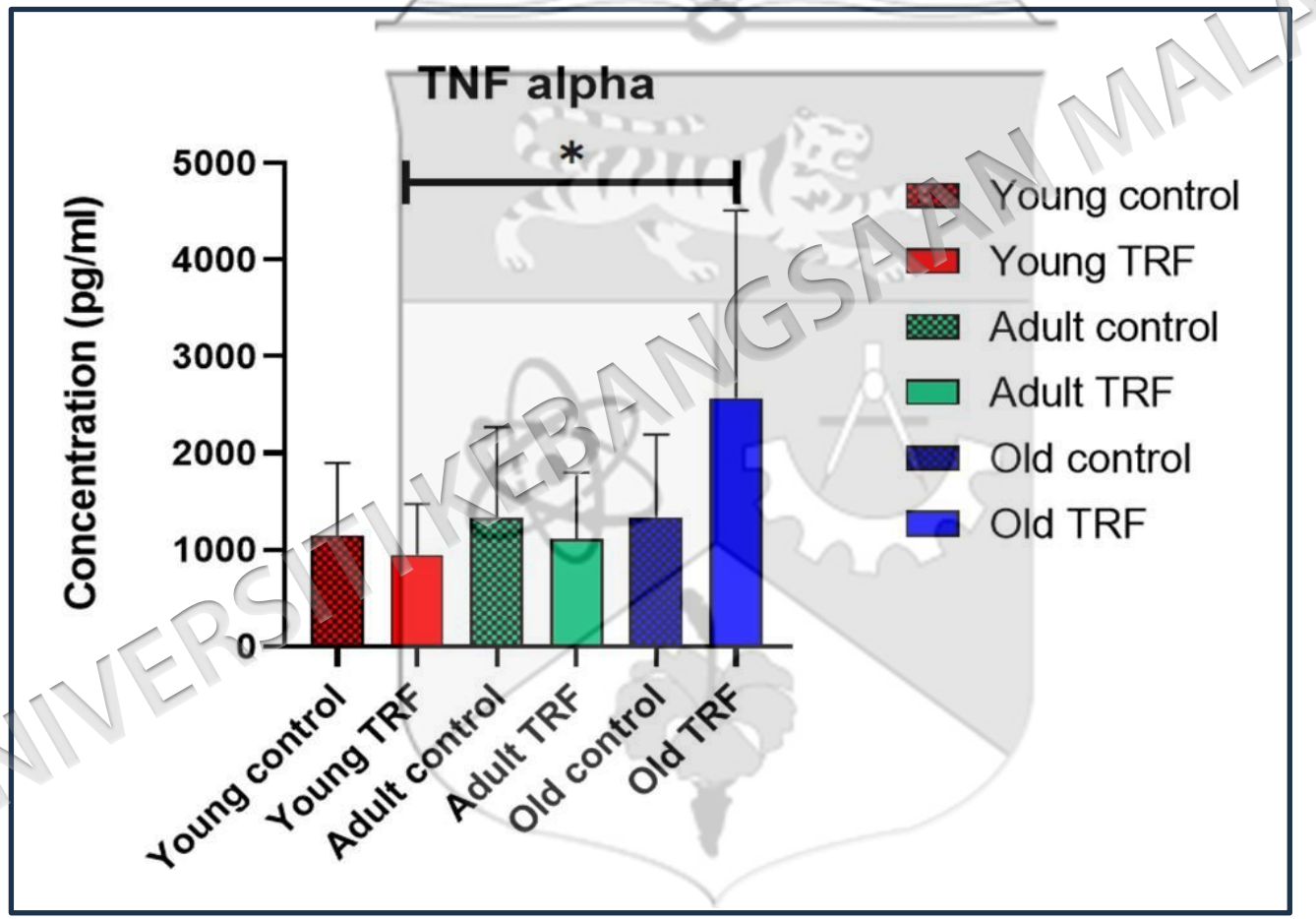
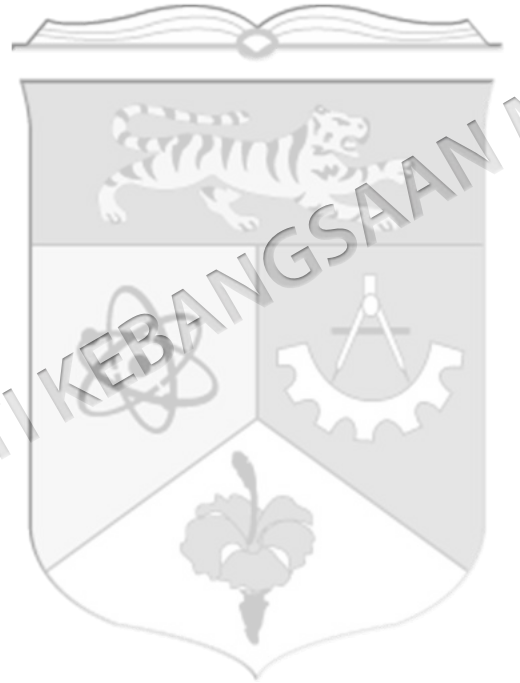


Figure 4.13 Hepatic TNF- alpha levels were determined by enzyme-linked immunosorbent assay in the liver of SD rats of different age group with and without TRF: young control (n= 9), young TRF (n= 9), adult control (n= 9), adult TRF (n= 10), old control (n= 6) and old TRF (n=5). Data are shown in pg/ml. Each bar represents the mean and SD. Values were considered significant at *p <0.05 according to the Turkey post-hoc test. A significant increase in TNF-alpha was observed in the old TRF group compared to the young TRF group, suggesting that TNF-alpha increases as the liver gets older.

4.12 LIPID PEROXIDATION MARKER IN THE LIVER OF SD RATS ACROSS DIFFERENT AGE GROUPS

The lipid peroxidation marker was measured in the ageing livers of SD rats, with and without TRF treatment (Figure 4.14). MDA in the liver of Sprague-Dawley (SD) rats was assessed across different age groups with and without tocotrienol-rich fraction (TRF) supplementation. The groups included young control (n=9), Young TRF (n=9), adult control (n=9), adult TRF (n=10), old control (n=6), and old TRF (n=5). Statistical analysis was performed, with significance set at $p < 0.05$. No significant differences were observed between any of the groups.



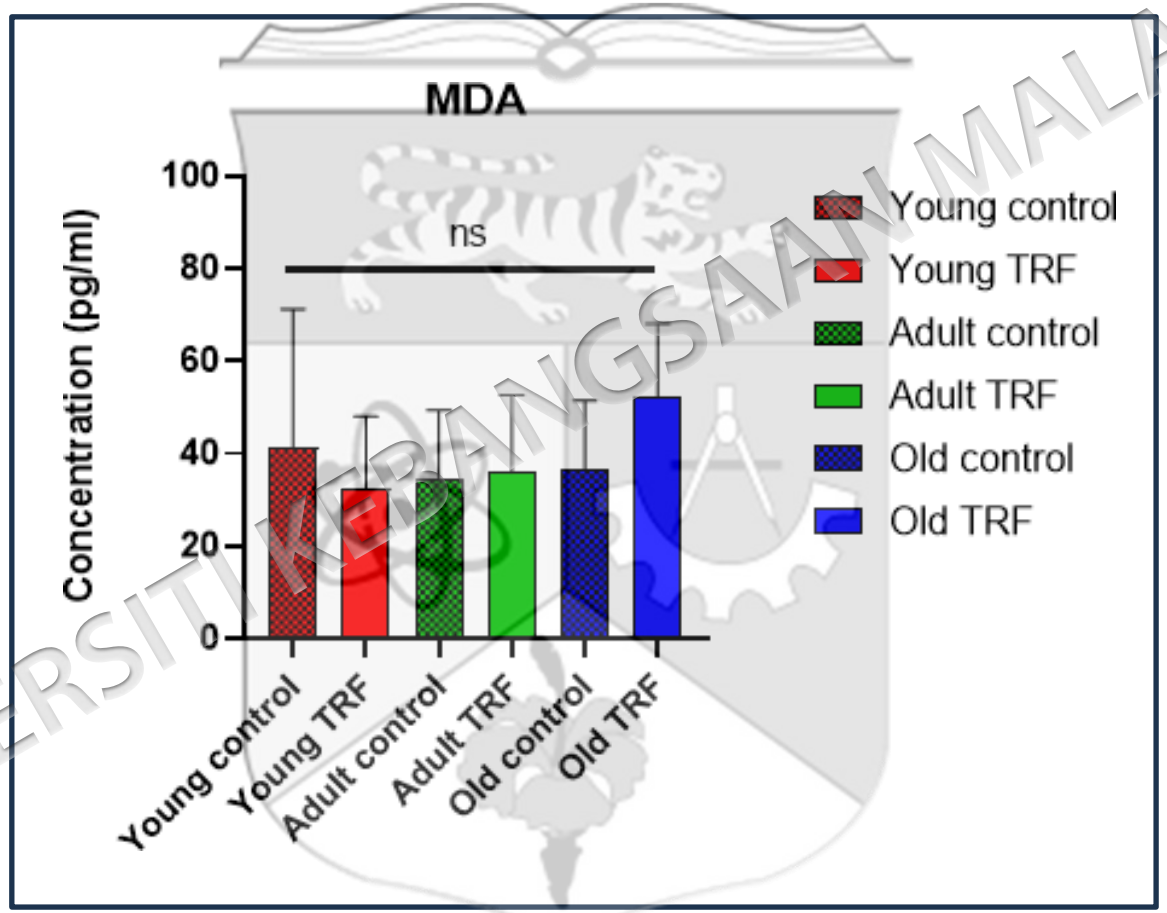


Figure 4.14 Hepatic MDA levels were determined by enzyme-linked immunosorbent assay in the liver of SD rats of different age group with and without TRF: young control (n= 9), young TRF (n= 9), adult control (n= 9), adult TRF (n= 10), old control (n= 6) and old TRF (n=5). Data are shown in pg/ml. Each bar represents the mean and SD. Values were considered significant at *p <0.05 according to the Turkey post-hoc test.

4.13 CORRELATION BETWEEN METABOLITES WITH OXIDATIVE, INFLAMMATORY AND LIPID PEROXIDATION MARKERS

To explore the relationships between liver metabolites and biochemical parameters, correlation analyses were correlation. The resulting heatmap represents correlation coefficients (r-values), thereby illustrating the strength and direction of associations. Only statistically significant correlations ($p < 0.05$) were considered for interpretation. The correlation between hepatic metabolites and, inflammatory markers (TNF-α , - 6), antioxidant enzymes (SOD, CAT), and lipid peroxidation markers (MDA) in overall regardless of age and treatment is shown in Figure 4.15. For SOD, several metabolites showed a positive correlation, indicating enhanced antioxidant defence. These included antioxidant and energy cofactors such as ergothioneine, flavin adenine dinucleotide (FAD), flavin mononucleotide F , , , and glutathione o i including glutamic acid, glutamine, histidine, ornithine, and valine, and nucleotides/nucleosides such as guanosine monophosphate, uridine monophosphate, and inosine. In contrast, adenine and pyroglutamic acid (5-oxoproline) were negatively correlated with SOD, suggesting reduced antioxidant activity linked to these metabolites.

For IL-6, negative correlations were observed with several metabolites that overlapped with the SOD-positive profile. Key metabolites negatively correlated with IL-6 included ergothioneine, FAD, FMN, glutamic acid, and glutamine, indicating that higher levels of these compounds are associated with lower inflammatory activity, and reduced SOD activity.

For MDA, positive correlations were primarily observed with adenine and pyroglutamic acid (5-oxoproline), which aligns with increased lipid peroxidation and reduced SOD. These metabolites represent potential markers of pro-oxidant states in the ageing liver.

Although no significant correlation was observed when the groups were classified into age control only and treatment only groups (Figure 4.16). Correlation analysis of metabolites with other parameters across individual age and treatment groups revealed notable shifts in association patterns, as shown in Figure 4.17. In the

young control group, malic acid exhibited a significantly negative correlation with CAT. In contrast, in the adult control group, malic acid demonstrated a significantly positive correlation with CAT. This shift indicates an age-dependent modulation of malate metabolism in relation to catalase activity. In the adult control group, creatinine and stearamide were significantly and negatively correlated with TNF-α . □ o n v e r s e l the correlation trends of these metabolites were significantly and positively correlated with TNF-α in the adult treated group. These findings suggest that TRF supplementation in adulthood may modulate these metabolites in response to inflammation.

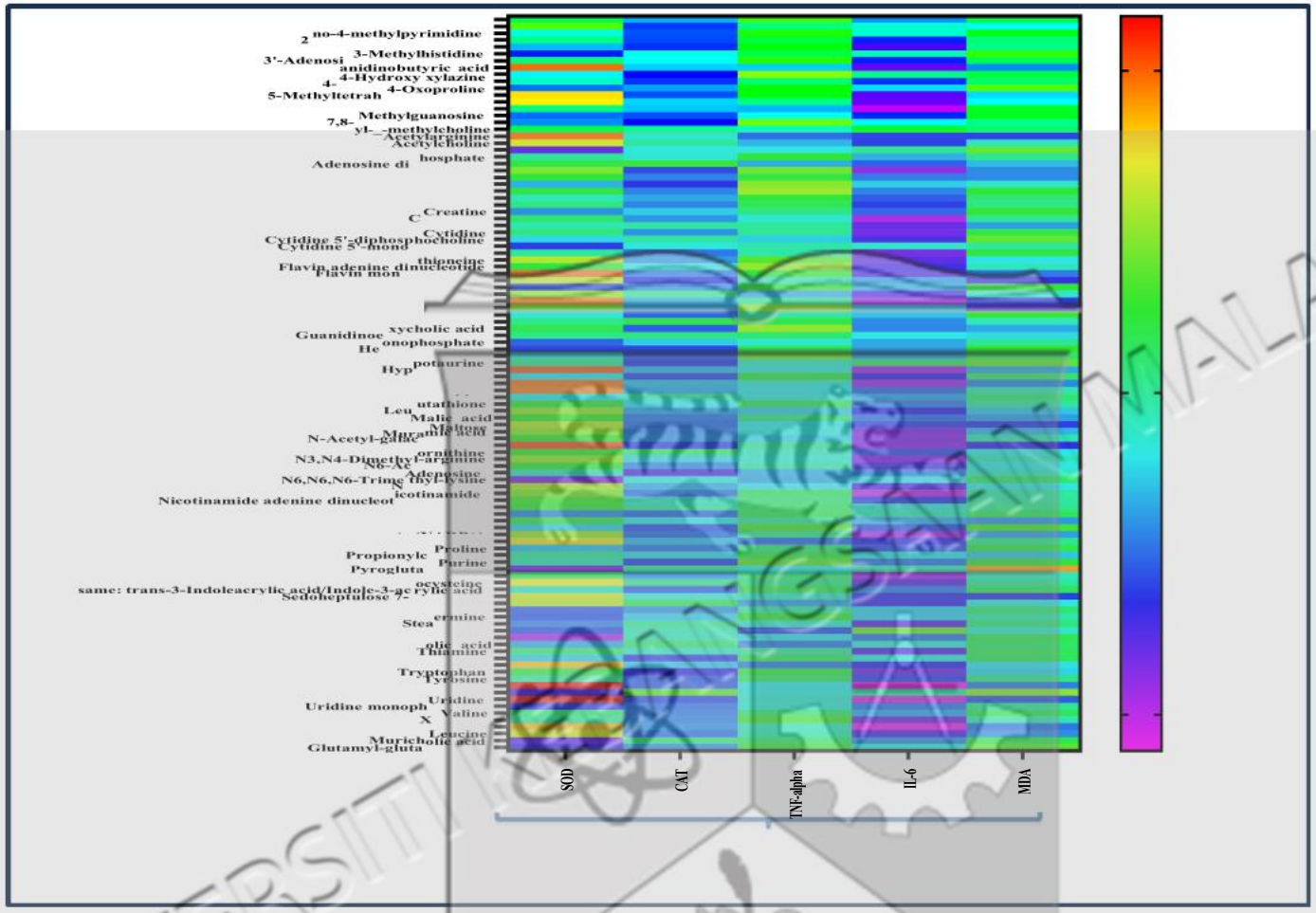


Figure 4.15 Heatmap illustrates the correlation between liver metabolites and parameters, including inflammatory markers (e.g., TNF-α , -6), antioxidant enzymes (e.g., □□□, □□T□, and lipid perо□idation mar□ers □e.g., □□□□on. The heatmap uses a rainbow colour coefficient gradient to represent correlation coefficients (r-values). Yellow to red hues represent strong positive correlations, while blue to purple tones indicate strong negative correlations. Green shades reflect weak or negligible correlations.

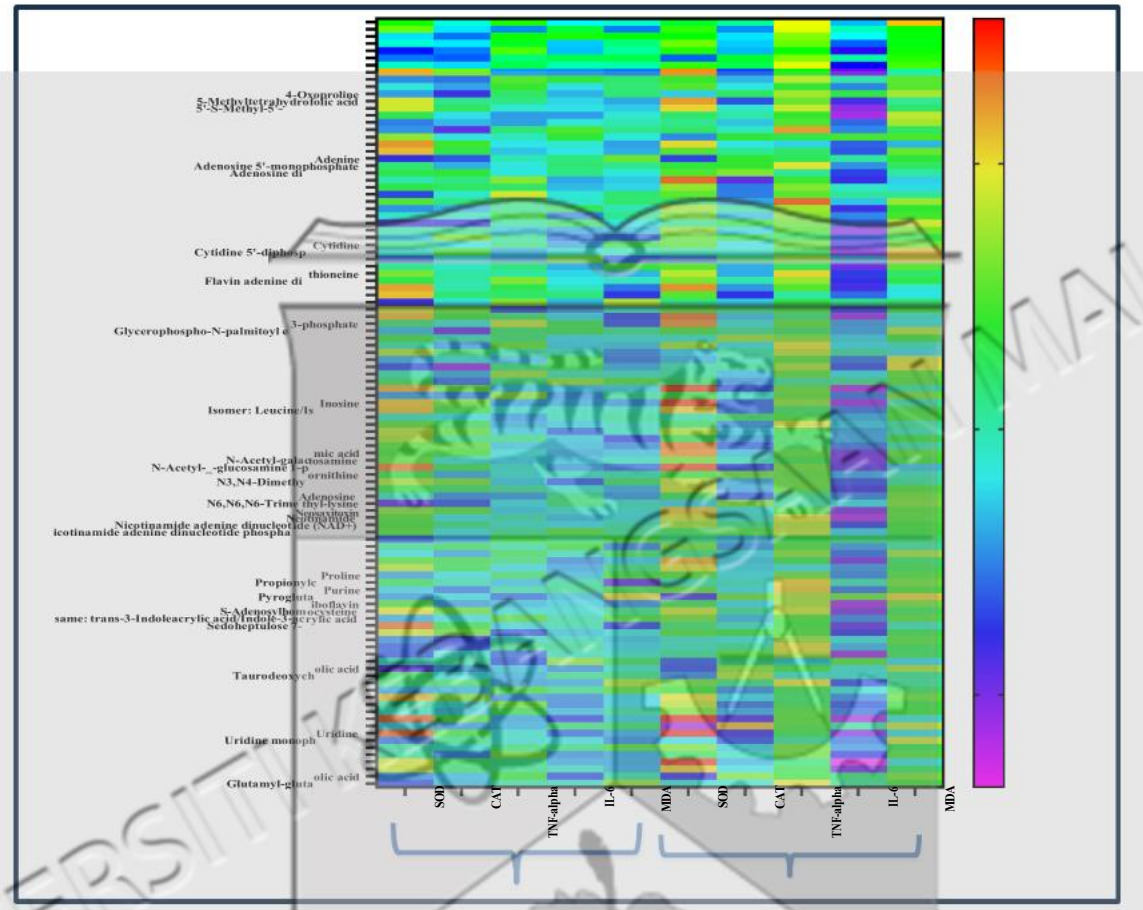


Figure 4.16 Heatmap of control and treatment-specific correlations between liver metabolites and all the parameters, including inflammatory markers (e.g., TNF-α, -6), antioxidant enzymes (e.g., SOD, CAT), and lipid peroxidation markers (e.g., MDA). Correlation coefficients were computed using pearman correlation. The heatmap uses a rainbow colour gradient to represent correlation coefficients (r-values). Yellow to red hues represent strong positive correlations, while blue to purple tones indicate strong negative correlations. Green shades reflect weak or negligible correlations.

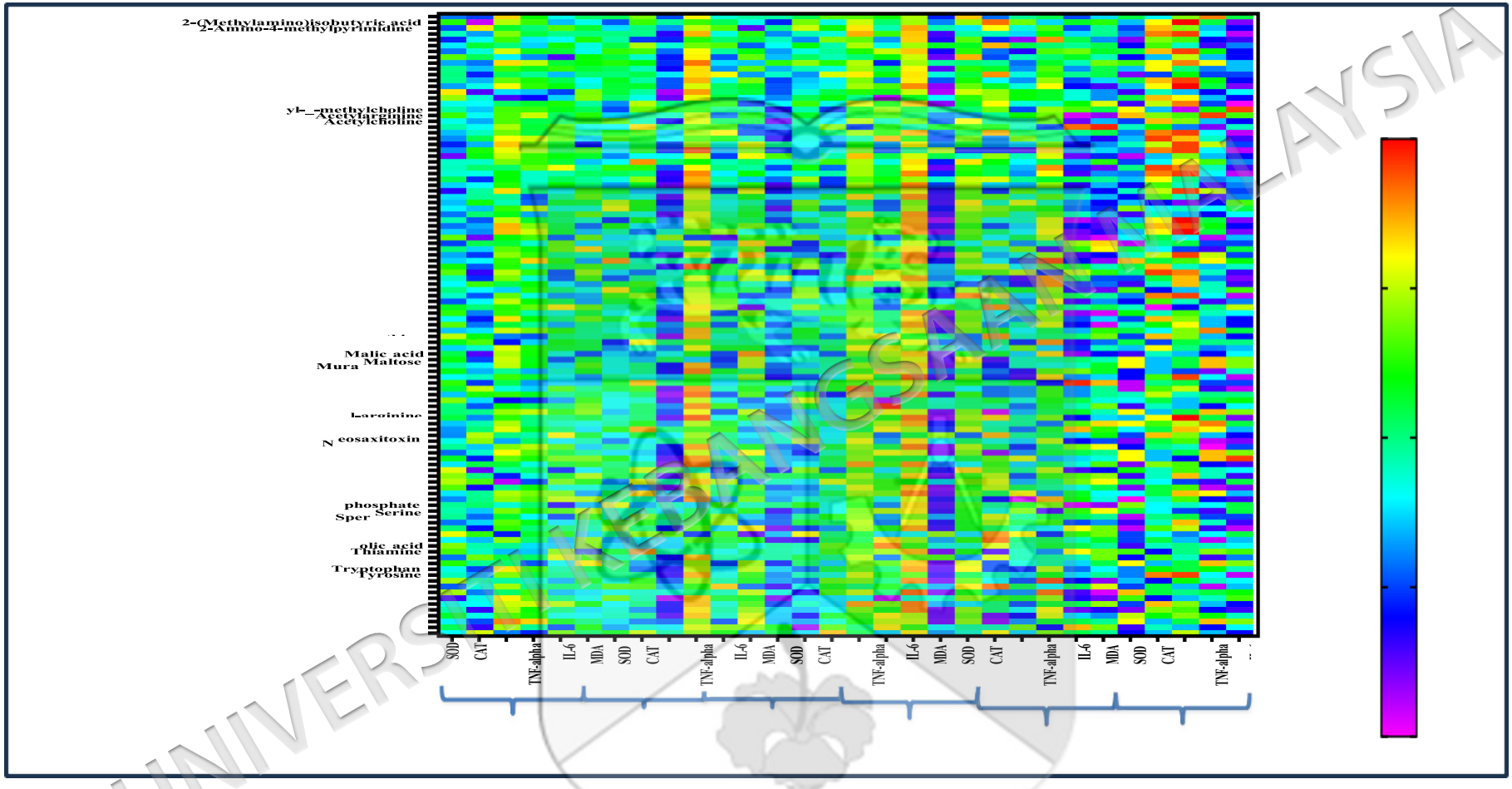


Figure 4.17 Heatmap of correlations between liver metabolites and all the parameters, including inflammatory markers (e.g., TNF-α, IL-6), antioxidant enzymes (e.g., □□□, □□T□, and lipid peroxidation markers (e.g., □□□□ rank correlation. The heatmap uses a rainbow colour gradient to represent correlation coefficients (r-values). Yellow to red hues represent strong positive correlations, while blue to purple tones indicate strong negative correlations. Green shades reflect weak or negligible correlations

CHAPTER V

DISCUSSION

5.1 UNTARGETED METABOLOMIC INSIGHTS INTO AGEING LIVER AND TRF SUPPLEMENTATION

PCA was utilised to assess metabolic shifts across age groups in control rats. The PCA score plot (Figure 4.5) illustrates the distribution and clustering patterns, based on metabolic profiles, among all treatment and control groups across all age categories. The PCA score plots revealed overlapping and some separations between the groups. The PCA of the metabolomic data showed (Figure 4.6) partial separation among control groups, indicating some influence in the liver metabolite profile during old age. This observation aligns with findings that early ageing stages are metabolically dynamic, with pronounced alterations in liver metabolites (Palmer & Jensen 2022) . PCA revealed age-dependent effects of TRF supplementation on liver metabolite profiles across all groups. Consistent with the control group, PCA of control groups and treatment groups also showed partial separations. This pattern may suggest subtle metabolic alterations induced by TRF treatment across different in aged groups in rats.

Our study showed dynamic metabolite changes in the liver with duirng adulthood, particularly between YC compared to AC and AC compared to OC. N-acetyl-α-glucosamine-1-phosphate, sedoheptulose-7-phosphate, isoleucine,

pipecolinic acid, uridine, uracil, 4-guanidinobutanoate, trigonelline, acetyl-arginine, methyl aminoisobutyric acid, and acetyl-□-methylcholine were seen to decrease with ageing liver, indicating that liver metabolism slows down with age. These metabolites are involved in various metabolic processes in the liver, including nucleotide metabolism, amino acid metabolism, and energy balance (Ling et al. 2023) .

An altered taurine and hypotaurine metabolism were seen in adult rat livers compared to young rat livers in the control groups. The pathway was both significant and impactful (Table 4.3). Taurine and hypotaurine metabolism in the liver plays a crucial role in protecting against oxidative stress and regulating lipid metabolism (X. Guo et al. 2024) . An important metabolite in the taurine and hypotaurine metabolism is taurine, which was altered in the control groups. Taurine levels were significantly higher in adult control in comparison with young control, upregulating the taurine and hypotaurine metabolism pathway in the adult control group. Research suggests that taurine and hypotaurine metabolism's protective role under stress conditions, can be altered (Ito et al. 2015; Mizota et al. 2021) . In acute liver failure, increased hypotaurine and taurine levels correlate with decreased glutathione, potentially serving as antioxidative mechanisms (Mizota et al. 2021) . Taurine is a non-essential amino acid as it can be made endogenously in the liver, which depends on whether methionine and cysteine (Baliou et al. 2021) are available. GSH biosynthesis affects taurine synthesis as well because cysteine can be used to synthesise GSH. In ethanol-induced liver injury, hepatic taurine levels increase (Hwang et al. 2025) . Even though, with age, there is a decrease in taurine biosynthesis (Yildirim & Kilic 2011) as corroborated with results of this study. These findings indicate that maybe an increased taurine and hypotaurine metabolism in adult liver may serve as a compensatory protective mechanism against various stressors.

Other significant pathway in the comparison of YC and AC included valine, leucine and isoleucine biosynthesis (Table 4.3). Furthermore, the significantly altered metabolites are isoleucine, pipecolate, acetyl-arginine, 4-guanidinobutanoate, methyl aminoisobutyric, which all decreased with ageing livers (Table 4.1). In previous report, these metabolites were seen to alter amino acid metabolisms, such as valine, leucine and isoleucine metabolism (Vanweert et al. 2022). The branched-chain amino acid Isoleucine can impair protein synthesis. Valine, leucine and isoleucine metabolism decrease in liver cirrhosis (Lo et al. 2022) and an elevated isoleucine and valine reduced steatosis in liver histology examination (Lo et al. 2021). Among the altered metabolites, isoleucine and pipecolate were related with lowering pro-inflammatory TNF-alpha in previous studies (Gart et al. 2022; Zhang et al. 2024). Additionally, the decline of methyl aminoisobutyric has been related to purine degradation and suggesting a slower

nucleotide recycling in ageing. Trigolnelline also decreased with ageing in rat livers. Trigonelline has been linked to NAD+ metabolism, energy production, and liver protection. The decline of this metabolite indicates reduced liver defence against ageing-related stress. As a NAD+ precursor, trigonilline enhances mitochondrial respiration and muscular performance as people age (Membrez et al. 2024). The ability of this metabolite to increase NAD+ levels is especially important because a decrease in NAD+ is linked to a number of age-related illnesses and metabolic dysfunctions (Covarrubias et al. 2020b). It also modulates oxidative stress, inflammation, and metabolic homeostasis (Xie et al. 2020) .

Ageing rat livers showed downregulation of uridine and uracil, indicating altered pyrimidine metabolism (Table 4.1). Consistently, pyrimidine metabolism was altered in our study (Table 4.4). Similar results were seen in a previous study, where glutathione metabolism and the immune function were also changed (Ekeuku et al. 2024) . As uridine and uracil decrease with age it indicates that RNA synthesis reduces as well in ageing livers (Xue et al. 2025) . Additionally, the liver repair and cellular energy balance was disrupted in the older rat livers. A decrease in uridine and uracil may indicate a slower hepatic regeneration in ageing (Ekeuku et al. 2024) and it was previously reported that uridine is altered in liver-related diseases (Xue et al. 2025) . By generating bioactive intermediates and preserving suitable pyrimidine pools, the pyrimidine metabolism pathway performs crucial biological role (Wang et al. 2021) . Because they are involved in many physiological processes, such as the synthesis of DNA, RNA, lipids, and carbohydrates, intermediates of pyrimidine metabolism are important biomolecules (Ekeuku et al. 2024) .

Besides that, the alteration of sedoheptulose-7-phosphate might affect the sugar and energy metabolism (Franceschi et al. 2022; Hoogerland et al. 2019) , as a metabolite of the pentose phosphate pathway (PPP). An intermediate of PPP is Sedoheptulose-7-phosphate (Franceschi et al. 2022) . This pathway contributes to NADPH and ribose-5-phosphate production (Qiao et al. 2025) and can regulate ROS in the liver and other ageing organs (Li et al. 2023a; TeSlaa et al. 2023a). Their decline suggests a reduction in oxidative stress defence and energy metabolism.

N-acetyl-α-glucosamine-1-phosphate (GlcNAc-1-P), a glycosylation pathway metabolite, declined with age, indicating altered sugar and energy metabolism (Petr et al. 2021) . GlcNAc-1-P is a vital precursor to several glycosylation processes in the liver, such as N-glycosylation (Paneque et al. 2023) , and the development of the Man-6-P marker on lysosomal enzymes (Zhang et al. 2021) and thus slower protein glycosylation, which is crucial for liver function. However, in TRF-treated adults we observed decreased hepatic uridine together with decreased N-acetyl-glucosamine-1-phosphate, contrasting with increased GlcNAc-1-P in young controls. It may reflect a metabolic coupling between pyrimidine nucleotide pools and hexosamine biosynthetic pathway (HBP) output (Le et al. 2013; Paneque et al. 2023) . The terminal step of the HBP consists of the conversion of GlcNAc-1-phosphate to UDP-GlcNAc, requires UTP as a co-substrate (Paneque et al. 2023) , establishing a direct biochemical dependency between uridine-derived nucleotide pools and HBP flux (McClain et al. 2010) . Accordingly, reduced hepatic uridine would limit UTP availability and thereby constrain the utilization of GlcNAc-1-P, potentially accounting for its decreased levels (McClain et al. 2010) . Exogenous uridine increases hepatic protein O-GlcNAcylation in mice, providing functional evidence that uridine availability is rate-influencing for HBP-dependent glycosylation (Urasaki et al. 2016) . Additionally, tocotrienols inhibit HMG-CoA reductase and modulate the mevalonate pathway, which shares acetyl-CoA as a precursor with the HBP competition for this common substrate under TRF supplementation could further limit flux into hexosamine biosynthesis (Schaffer et al. 2005) .

In comparison of AC and AT metabolites (Table 4.2), glutamate was found higher in AC than AT. In our study, reduced glutamate signals a major metabolic shift in the liver after TRF treatment. Importantly, glutamate is a key amino acid involved in multiple metabolic pathways, and its reduction suggests enhanced utilisation for glutathione synthesis, supporting the antioxidant effects of TRF. This metabolomic shift likely affects several interconnected pathways, including glutathione metabolism, alanine, aspartate, and glutamate metabolism, as well as arginine biosynthesis, all of which rely on glutamate as a precursor o fibrosis, the glutamine/glutamate ratio was disrupted, and levels of glutamate and TCA cycle intermediates increased as fibrogenesis progressed (Delgado et al. 2022) .

Additionally, lower glutamate levels may influence nitrogen metabolism and the urea cycle, indicating a negative nitrogen balance (Luczkowska et al. 2024) . This reduction also disrupts butanoate, histidine, and porphyrin metabolism pathways, which are tied to energy regulation and oxidative stress (Jin et al. 2023) .

Furthermore, lower glutamate affects the glutathione metabolism, alanine, aspartate and glutamate metabolism, arginine biosynthesis pathway, D-aminoadic metabolism, nitrogen metabolism, butanoate metabolism, histidine metabolism, porphyrin metabolism and arginine and proline metabolism in the liver. This indicates a significant metabolic shift in the liver after TRF treatment. Glutamate is a key amino acid which is involved in multiple metabolomic pathways, and its reduction suggests enhanced utilization for glutathione synthesis (Jin et al. 2023) , supporting the antioxidant effects of TRF.

There seems to be an energy balance shift after treatment in the adult groups. A reduction in malate and TCA cycle intermediates suggests that the liver may be adapting to a different energy metabolism state potentially more efficient □ □ o l 2023) e. Fatty acids can promote the production of malate and its movement from mitochondria. It implies that when the fatty acid decreased following treatment, so did the malate generation (Dankel et al. 2023) . However, in our study, we saw that uridine was higher in the control groups compared to the treated groups. Uridine synthesis may be regulated by glutamate levels indirectly as glutamate (Gong et al. 2023) . The downregulation of Uridine by TRF may be caused by decreased glutamate. Furthermore, uridine homeostasis disruption can cause liver steatosis (Zhang et al. 2020) .

The reduction also impacts butanoate metabolism, potentially reflecting altered energy regulation, and may affect histidine and porphyrin metabolism, both of which are linked to oxidative stress responses (Gao et al. 2021; Wang et al. 2024). These changes suggest that TRF promotes a shift toward antioxidant defence and metabolic efficiency in the liver. Overall, TRF supplementation was associated with significant metabolic alterations in adult rats, while no statistically significant metabolite changes

were observed in young or old groups, which suggesting an effect on liver metabolic profiles during adulthood.

5.2 HEPATIC ANTIOXIDANT ACTIVITY AND INFLAMMATORY AND LIPID PEROXIDATION MARKERS, AND HISTOLOGICAL ANALYSIS

To evaluate the antioxidant enzyme levels across different aged livers and with or without TRF treatment, we measured the liver SOD and CAT levels. We found that although CAT activity change was insignificant, but the SOD activity was significantly higher in young control groups than adult and old control groups. It is important to note that the SOD activity levels were low. Nonetheless some studies did show that in high oxidative damage liver SOD levels can be as low as 0.4–2.0 U/mg protein for liver tissue (Assady et al. 2011; Broide et al. 2000). Despite the lower absolute values, the observed trend aligns with existing literature demonstrating a decline in SOD activity with advancing age in the liver (Bárcena et al. 2021; Yang, et al. 2015). Similar trend as SOD was observed for the inflammatory cytokine TNF-alpha varied across different age groups, except that TNF-alpha levels were higher in old TRF-treated group compared to the young TRF-treated group indicating age-related inflammation in the ageing livers.

Both SOD activity and TNF-alpha results contributed to our metabolomics results in control groups, as we saw a decrease in SOD activity and an increase in TNF-alpha as the rat liver aged. Thus, supporting the notion that alteration of amino acid metabolism with ageing liver influenced oxidative stress and inflammation.

Antioxidant effects were more in the TRF-treated group compared to the AC, as evidenced by significant reductions in key metabolites such as glutamate, malate, and tryptophan. These changes may reflect a role of TRF to attenuate oxidative stress in hepatic tissue. Glutamate, a known excitatory amino acid and precursor for glutathione synthesis, tends to accumulate during oxidative damage due to its involvement in redox homeostasis (Hamelin et al. 2007). A reduction in glutamate levels in the TRF group may indicate enhanced glutathione activity or ROS production, both of which would contribute to improved antioxidant defence mechanisms in ageing liver cells (Hamelin

et al. 2007). Similarly, the decrease in malate, a key intermediate in the TCA cycle, may suggest a shift in mitochondrial metabolism □ □ o l 2023) e This could imply that TRF improves mitochondrial efficiency, thereby reducing the demand for TCA cycle activity and subsequently lowering mitochondrial-derived ROS. This is consistent with previous findings that tocotrienols can enhance mitochondrial function and bioenergetics under oxidative stress conditions (S. F. Chin et al. 2011).

Furthermore, changes in tryptophan and phenylalanine levels point to TRF's influence on inflammation. Tryptophan metabolism, particularly through the kynurenine pathway, is closely linked to immune regulation and chronic inflammation. A decline in tryptophan may suggest reduced activation of this pathway, potentially reflecting lower systemic inflammation (Reshetova et al. 2024). Likewise, alterations in phenylalanine, which can accumulate during oxidative or inflammatory stress, reinforce the anti-inflammatory potential of TRF (Durani et al. 2018). Collectively, these metabolomic shifts underscore T R F dual role in combating both oxidative stress and inflammation, two key hallmarks of hepatic ageing.

In the overall correlation heatmap, metabolite correlation with parameters, including inflammatory markers (TNF-α ,IL-6), antioxidant enzymes (SOD, CAT), and lipid peroxidation markers (MDA), were demonstrated. The correlation analyses were p e r f o r me d u s i n g □p e a r ma n ' s r a n □ c o r r e l a t i correlation coefficients (r-values) and statistically significant correlations ($p < 0.05$) were considered for interpretation. Metabolites such as ergothioneine, flavin cofactors (FAD, FMN), oxidized glutathione, glutamic acid, glutamine, histidine, ornithine, and valine were positively correlated with SOD and negatively correlated with IL-6, suggesting enhanced antioxidant defense and suppressed inflammatory signalling. The increase in these metabolites aligns with their known roles in redox homeostasis: ergothioneine and glutathione act as potent antioxidants, while flavins and NAD cofactors support enzymatic redox reactions and energy metabolism. Their negative association with IL-6, a pro-inflammatory cytokine, further supports their involvement in mitigating inflammageing.

In contrast, adenine and pyroglutamic acid (5-oxoproline) were negatively correlated with SOD and positively correlated with MDA, indicating increased lipid

peroxidation and impaired antioxidant capacity. Adenine accumulation reflects purine catabolism during oxidative stress, while elevated pyroglutamic acid suggests disruption of the γ-glutamyl cycle and glutathione recycling features commonly observed in oxidative liver injury. These findings imply that the rise in these metabolites may serve as biomarkers of redox imbalance in ageing livers. The correlation heatmap comparing control and TRF-treated groups revealed no significant differences in overall correlation trends.

However, when correlations were analysed within individual age and treated groups, notable shifts emerged. Malic acid demonstrated a negative correlation with catalase in the young control group but a positive correlation in the adult control group, suggesting an age-dependent shift in how malate metabolism links to antioxidant enzyme activity. This may indicate that malate contributes to antioxidant responses more prominently in adulthood as oxidative burden increases. Furthermore, in the adult group, creatinine and stearamide displayed opposite correlations with TNF-α between treated and control animals. In adult controls, both metabolites were negatively correlated with TNF-α, suggesting higher levels we inflammatory responses. Conversely, in adult-treated rats, these correlations shifted to positive, implying that TRF supplementation altered the relationship between these metabolites and inflammatory signalling. Such shifts may reflect TRF-induced metabolic remodelling rather than straightforward anti-inflammatory effects. In the old group, no substantial correlation changes were observed between control and treated animals, which may indicate that advanced age, with already established oxidative and inflammatory dysregulation, limits TRF's r

Histological evaluation using H&E staining demonstrated progressive age-related alterations in liver architecture, with corresponding differences observed following TRF treatment. The YC group largely maintained normal hepatic structure despite mild features such as fibrotic like cells and slight disruption of the honeycomb arrangement. In contrast, the AC and OC groups showed reduced cellular density, less distinct nuclei, increased fibrotic-like regions, and altered hepatocyte morphology, with more pronounced changes in OC. Comparison with treated groups suggests that TRF may contribute to structural preservation, as evidenced by improved hepatocyte

organisation in YT, reduced acellular regions and infiltration in AT, and decreased fibrotic-like areas and infiltration in OT relative to their respective controls. Although these findings indicate that TRF may attenuate age-associated histological changes, the effects were not complete, and further quantitative and molecular analyses are required to confirm these observations.

The comparative figure shows how TRF might help preserve liver tissue integrity in aged conditions in Figure 5.1.

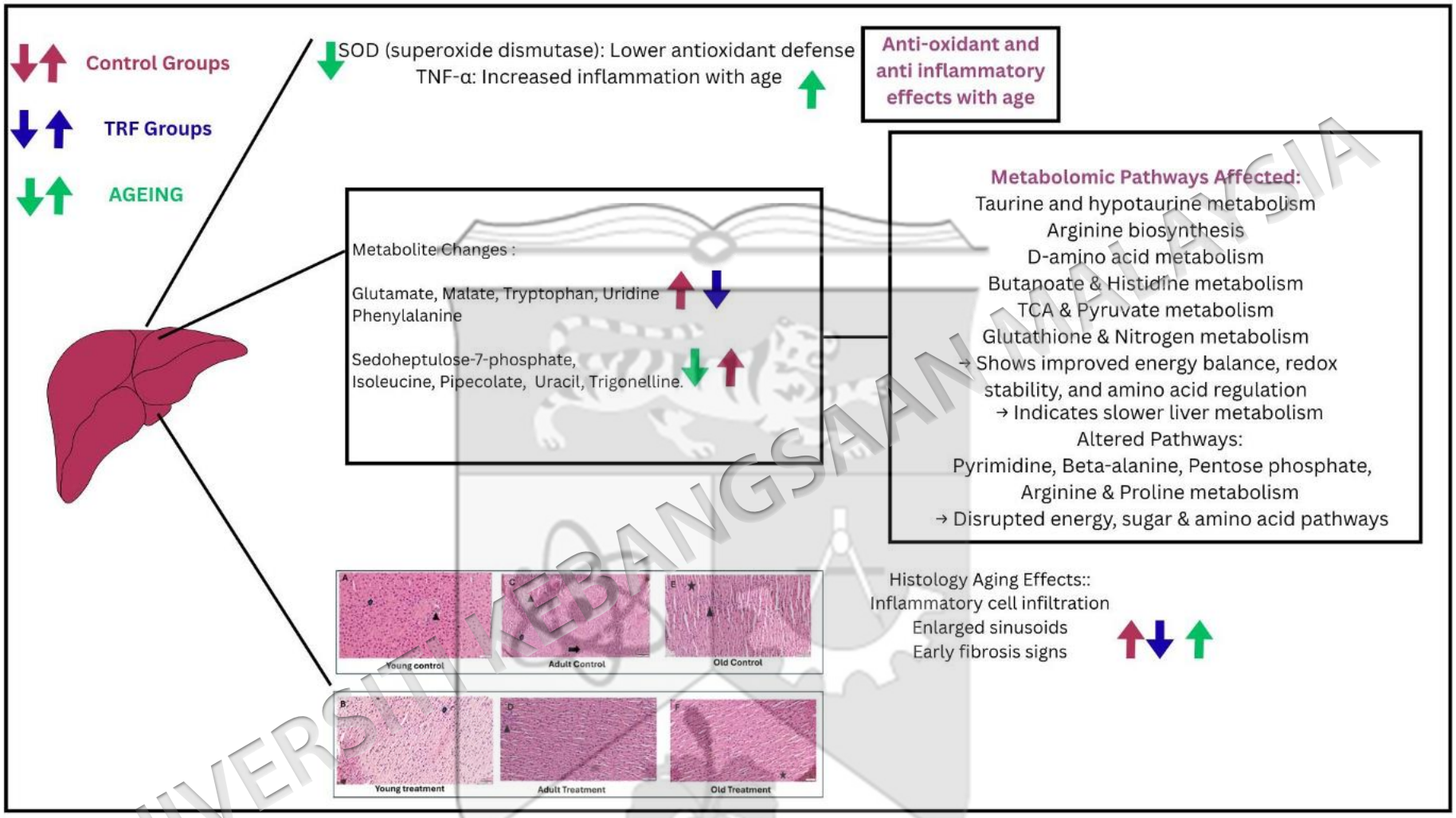


Figure 5.1 Summary illustration of the study key findings on the effects of TRF on ageing-related hepatic changes in rat

CHAPTERVI

CONCLUSION AND FUTURE WORKS

6.1 Conclusion of the Study

This study examined the impact of TRF on age-associated hepatic changes in Sprague Dawley rats by integrating histological observations, oxidative stress and inflammatory markers, and untargeted metabolomics profiling. This study comprehensively explored these by addressing three interrelated objectives.

In relation to the first objective, metabolomic profiling revealed that ageing was accompanied by alterations in hepatic metabolites spanning from pyrimidine metabolism (uridine, uracil), valine, leucine and isoleucine biosynthesis (isoleucine), taurine and hypotaurine metabolism (taurine). Furthermore, ageing alters the metabolites related to pathways, such as amino acid metabolism (isoleucine, pipecolate, acetyl-arginine), glycosylation intermediates (GlcNAc-1-P), and pentose phosphate pathway activity (sedoheptulose-7-phosphate). Collectively, these alterations indicate diminished nucleotide synthesis, reduced antioxidant capacity, and impaired energy homeostasis in older livers.

For the second objective, histological assessment demonstrated a progressive decline in liver structural integrity with advancing age, as evidenced by reduced cellular organisation, increased fibrotic features, and altered hepatocyte morphology from the YC to the OC groups. These structural impairments were accompanied by biochemical evidence of heightened

oxidative stress and inflammation, as indicated by elevated expression of TNF-α and decreased SOD activity.

Addressing the third objective, TRF supplementation in adult rats induced significant metabolic shifts, including reductions in glutamate, malate, uridine, and GlcNAc-1-P. The decrease in glutamate may reflect enhanced utilisation for glutathione synthesis, supporting the antioxidant properties of TRF, while the reduced malate suggests improved mitochondrial efficiency and lower production of TCA cycle-derived ROS. The concurrent decline in uridine and GlcNAc-1-P points to a metabolic coupling between pyrimidine nucleotide pools and hexosamine biosynthetic pathway output, potentially driven by TRF-induced alterations in nucleotide metabolism or substrate competition from tocotrienol glucuronidation. These metabolomic changes were consistent with the observed age-related decline in SOD activity and increase in TNF-alpha, and with histological evidence that TRF reduced hepatic fibrotic-like areas and preserved tissue architecture in treated groups.

Overall, these findings indicate that TRF supplementation may be associated with a modest modulation of age-related hepatic alterations at the structural, biochemical, and metabolic levels; however, further studies are required to confirm these effects and clarify the underlying mechanisms.

6.2 Limitations of The Study

Despite providing valuable insights into the hepatoprotective effects of TRF during ageing, this study has several limitations.

1. Although untargeted metabolomics allowed for the broad identification of altered metabolic pathways, the study lacked targeted validation of specific metabolites, which is essential for confirming biomarker relevance.
2. Pathway analysis is dependent on database mapping and may not fully reflect biological pathway activity in vivo
3. Furthermore, the histological assessment, while informative, was restricted to H&E staining and general inflammatory markers, without the inclusion of fibrosis-specific or cell-type-specific staining.

4. The absence of significant changes in IL-6 and CAT may reflect limited sensitivity of the selected markers to detect subtle inflammatory and antioxidant changes under the study conditions, rather than the absence of underlying biological effects.

6.3 FUTURE PERSPECTIVES

While the current findings support the efficacy of TRF in attenuating age-related hepatic alterations, further research is required to deepen our understanding of its mechanisms.

1. The altered metabolites observed in this study, for example, glutamate, taurine, etc., could be used for targeted quantification. This would confirm whether these metabolites can serve as reproducible biomarkers of hepatic ageing and TRF-mediated modulation.

2. Future studies could examine specific genes or proteins linked to the affected pathways identified in this study. Techniques such as qPCR can be used to validate whether TRF regulates these pathways at the molecular level.

3. A more detailed evaluation of liver tissue using fibrosis-specific staining, such as □asson's trichrome, can be conduct evidence of TRF's role in inflammation

4. Longitudinal studies evaluating different dosages and durations of TRF treatment across age groups are necessary to optimise its use and assess long-term outcomes.

5. Ultimately, translational studies in human populations will be essential to determine the applicability of TRF as a preventive or therapeutic intervention for liver ageing and age-related hepatic disorders.

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□a i n a l , □. , R a h i m, □. □. , □h a □a ' a i , □. □ Tocotrienol-Rich Fraction (TRF) and Carotenes in Ovalbumin (OVA)-Challenged Asthmatic Brown Norway Rats. *International journal of molecular sciences* 20(7).

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APPENDIX A CONFERENCE CERTIFICATES

Universiti Kebangsaan Malaysia
The National University of Malaysia

NARATIF BAHARU UKM
UNIVERSITI WATAN KITA

FACULTY OF MEDICINE
THE NATIONAL UNIVERSITY OF MALAYSIA

ICAMS

CERTIFICATE

OF POSTER PRESENTATION

THIS CERTIFICATE IS PROUDLY AWARDED TO

ANIKA TABASSUM

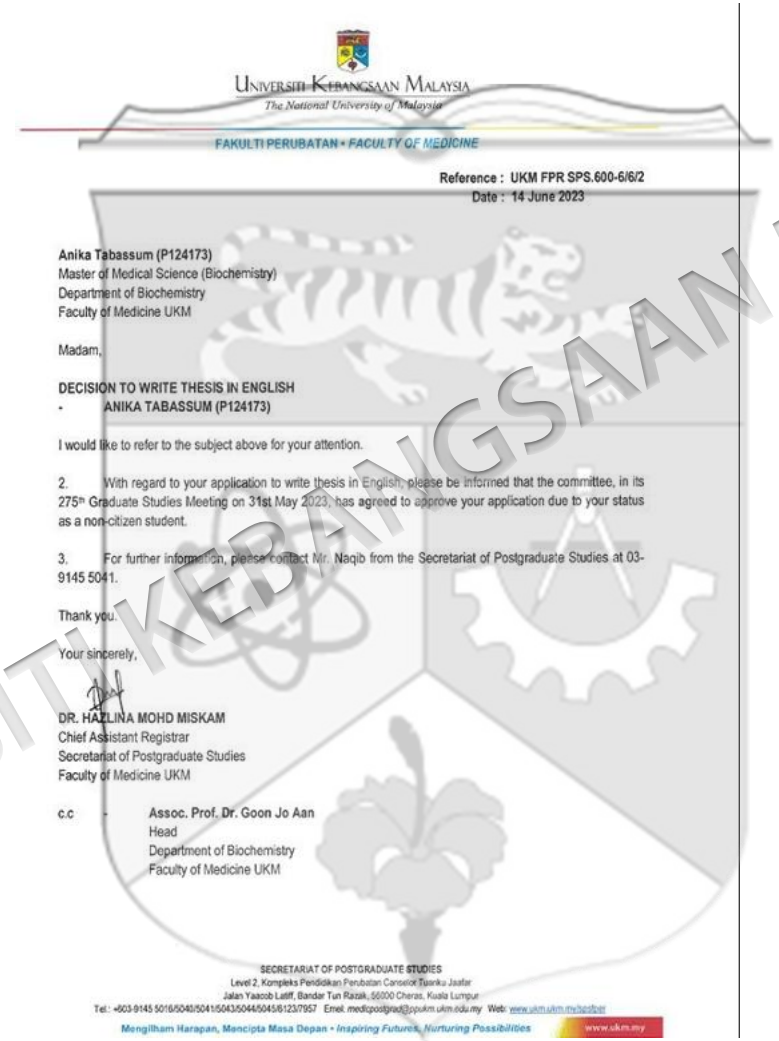
WITH THE PRESENTATION TITLE

Untargeted Liver Metabolites Profiling and Oxidative Stress and Inflammatory Markers Expression in Sprague Dawley Rats Supplemented with Palm Tocotrienol-Rich Fraction and its Effect on Hepatic Changes During Ageing

*during the **5th International Conference on Advances in Medical Science** on 29th & 30th April 2025 at Concorde Hotel, Kuala Lumpur, Malaysia*

Ts. Dr. Wong Sok Kuan
Chairperson

APPENDIX B APPROVAL LETTER TO WRITE THESIS IN ENGLISH



UNIVERSITI KEBANGSAAN MALAYSIA
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FAKULTI PERUBATAN • *FACULTY OF MEDICINE*

Reference : UKM FPR SPS.600-6/6/2
Date : 14 June 2023

Anika Tabassum (P124173)
Master of Medical Science (Biochemistry)
Department of Biochemistry
Faculty of Medicine UKM

Madam,

DECISION TO WRITE THESIS IN ENGLISH
- **ANIKA TABASSUM (P124173)**

I would like to refer to the subject above for your attention.

2. With regard to your application to write thesis in English, please be informed that the committee, in its 275th Graduate Studies Meeting on 31st May 2023, has agreed to approve your application due to your status as a non-citizen student.

3. For further information, please contact Mr. Naqib from the Secretariat of Postgraduate Studies at 03-9145 5041.

Thank you.

Your sincerely,

DR. HAZLINA MOHD MISKAM
Chief Assistant Registrar
Secretariat of Postgraduate Studies
Faculty of Medicine UKM

c.c - **Assoc. Prof. Dr. Goon Jo Aan**
Head
Department of Biochemistry
Faculty of Medicine UKM

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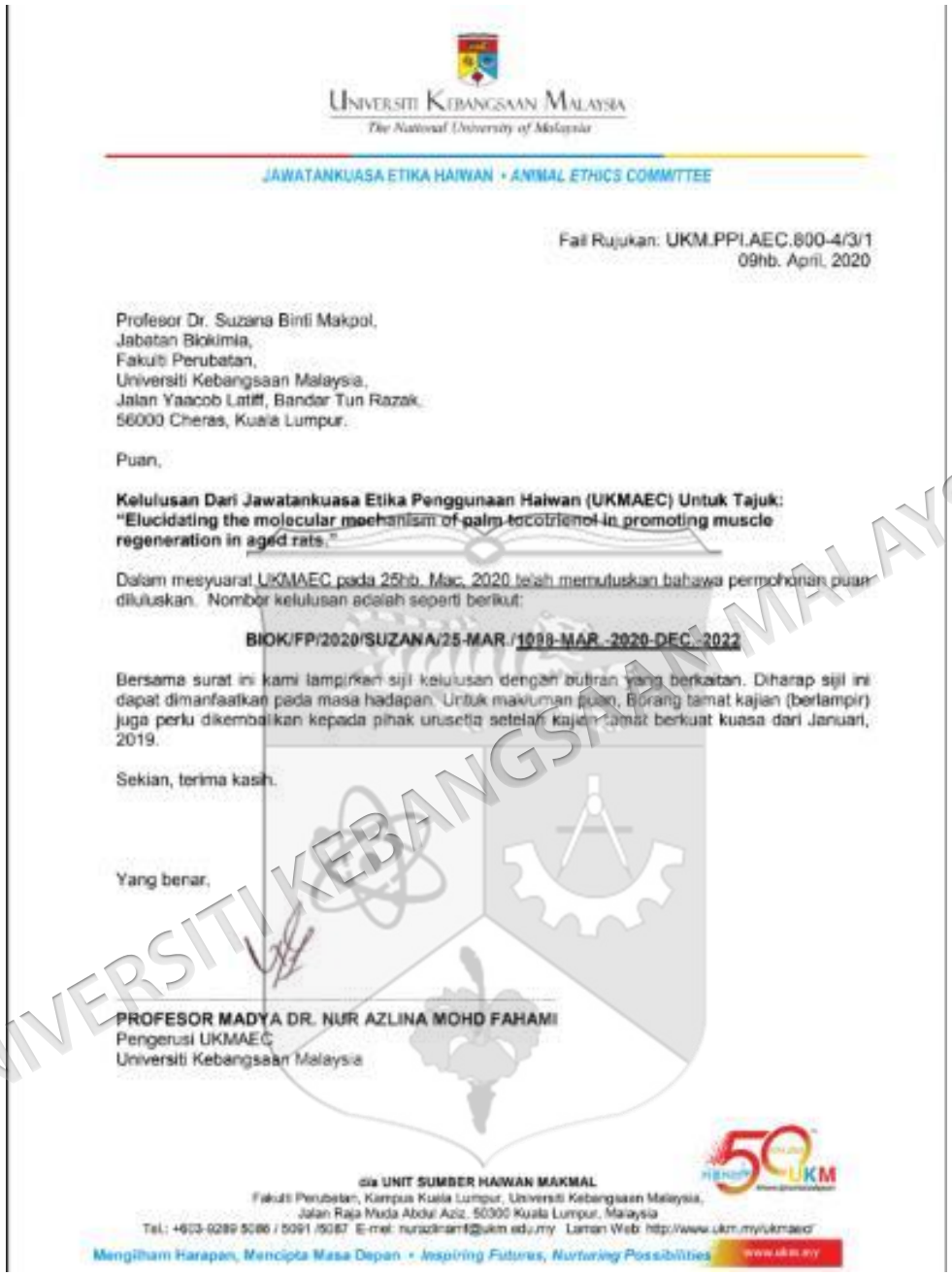
APPENDIX C ETHICS APPROVAL LETTER



UNIVERSITI KEBANGSAAN MALAYSIA
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JAWATANKUASA ETIKA HAIWAN • *ANIMAL ETHICS COMMITTEE*

Fail Rujukan: UKM.PPI.AEC.800-4/3/1
09hb. April, 2020

Profesor Dr. Suzana Binti Makpol,
Jabatan Biokimia,
Fakulti Perubatan,
Universiti Kebangsaan Malaysia,
Jalan Yaacob Latiff, Bandar Tun Razak,
56000 Cheras, Kuala Lumpur.

Puan,

Kelulusan Dari Jawatankuasa Etika Penggunaan Haiwan (UKMAEC) Untuk Tajuk: "Elucidating the molecular mechanism of palm tocotrienol in promoting muscle regeneration in aged rats."

Dalam mesyuarat UKMAEC pada 25hb. Mac, 2020 telah memutuskan bahawa permohonan puan diluluskan. Nombor kelulusan adalah seperti berikut:

BIOK/FP/2020/SUZANA/25-MAR./1098-MAR.-2020-DEC.-2022

Bersama surat ini kami lampirkan sijil kelulusan dengan butiran yang berkaitan. Diharap sijil ini dapat dimanfaatkan pada masa hadapan. Untuk makluman puan, Borang tamat kajian (berlampir) juga perlu dikembalikan kepada pihak urusetia setelah kajian tamat berkuat kuasa dari Januari, 2019.

Sekian, terima kasih.

Yang benar,

PROFESOR MADYA DR. NUR AZLINA MOHD FAHAMI
Pengerusi UKMAEC
Universiti Kebangsaan Malaysia

d/a UNIT SUMBER HAIWAN MAKMAL
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ANIMAL ETHICS COMMITTEE
c/o Laboratory Animal Resource Unit
Faculty of Medicine, UKM
Jalan Temerloh, 53200 Kuala Lumpur
Tel: 92895086, 92895091, 92895087

25th March, 2020

UKMAEC APPROVAL NUMBER:	**BIOK/FP/2020/SUZANA/25-MAR./1098-MAR.-2020-DEC.-2022**
CHIEF INVESTIGATOR:	**Prof. Dr. Suzana Binti Makpol**
DIVISION/DEPT. OF CHIEF INVESTIGATOR:	**Dept. of Biochemistry, Faculty of Medicien, Universiti Kebangsaan Malaysia.**
FUNDING INSTITUTION (S):	-
GRANT NUMBER (S):	-
PROJECT TITLE:	**Elucidating the molecular mechanism of palm tocotrienol in promoting muscle regeneration in aged rats**
CO-INVESTIGATOR:	**Dr. Tan Jen Kit** **Dr. Chin Kok Yong** **Dr. Amilia Aminuddin** **Nur Fathiah Abdul Sani** **Nazirah Ab Rani** **Nur Haleeda Hakimi**
STUDENT:	**Siti Liyana Saud Gany**
OTHER AUXILIARIES:	-
PROJECT CLASSIFICATION:	**C**
SOURCE OF ANIMALS:	**LARU, UKM.**
HOUSING-LOCATION OF ANIMALS DURING PROJECT:	**Level 8, Pre-Clinical Building, PPUKM.**
ESTIMATED DURATION OF PROJECT:	**From March 2020 to December 2022**

Assoc. Prof. Dr. Nur Adlina Mohd Fahami
Chairperson of UKMAEC
Universiti Kebangsaan Malaysia

APPROVED

Low Kiat Cheong
Secretary of UKMAEC
Universiti Kebangsaan Malaysia

APPENDIX D MANUSCRIPT

Sains Malaysiana 55(3)(2026): 475-489
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Liver Metabolite Profiles and Oxidative Stress in Ageing Rats and Their Modulation by Tocotrienol-Rich Fraction

(Profil Metabolit Hati dan Tekanan Oksidatif pada Tikus yang Semakin Tua dan Modulasinya oleh Fraksi Kaya-Tokotrienol)

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Received: 25 September 2025/Accepted: 19 February 2026

ABSTRACT

Ageing alters liver metabolism and function, accompanied by morphological changes in hepatic cells, including disrupted metabolite profiles and structural degeneration. These changes contribute to increased oxidative stress and inflammation. However, the connection between oxidative stress, inflammation, histological changes, and liver metabolism during ageing, and the potential of the tocotrienol-rich fraction (TRF) as a therapeutic agent, remains underexplored. This study investigates age-related hepatic changes and the effects of TRF using metabolomics. Sprague-Dawley rats aged 3, 9, and 21 months were assigned to control and treatment groups and received either palm olein or TRF supplementation. Liver tissues were analysed using untargeted metabolomics via LC-MS/MS, along with histological evaluation and biochemical assessments of antioxidant enzymes (SOD, CAT), inflammatory markers (IL-6, TNF-alpha), and lipid peroxidation markers (MDA). SOD activity declined significantly with age in both control and TRF-treated old rats ($p<0.05$), suggesting reduced antioxidant defence with increasing age, whereas no significant changes were observed in CAT, IL-6 or MDA levels. Histologically, TRF-treated livers displayed fewer fibroblasts and less inflammation than control livers. Metabolomics showed age-related alterations in taurine and hypotaurine metabolism, pyrimidine metabolism (uridine and uracil), and TCA cycle components (malate and glutamate). These findings demonstrate that TRF modulates age-related liver changes, particularly in morphology and metabolism, despite a persistent decline in antioxidant activity. This finding underscores the potential of TRF in mitigating liver ageing. It provides insight into key metabolic pathways involved, offering a foundation for future therapeutic strategies targeting liver health in the elderly.

Keywords: Hepatic ageing; liver; metabolomics; oxidative stress; tocotrienol-rich fraction

ABSTRAK

Penuaan mendorong perubahan dalam metabolisme dan fungsi hati, disertai dengan perubahan morfologi dalam sel hepatik seperti profil metabolit yang terganggu dan degenerasi struktur. Perubahan ini menyumbang kepada peningkatan tekanan oksidatif dan keradangan. Walau bagaimanapun, hubungan antara tekanan oksidatif, keradangan, perubahan histologi dan metabolisme hati semasa penuaan dan potensi fraksi kaya-tokotrienol (TRF) sebagai agen terapeutik masih kurang diterokai. Penyelidikan ini mengkaji perubahan hepatik yang berkaitan dengan usia dan kesan TRF menggunakan pendekatan omik lanjutan. Tikus Sprague-Dawley berumur 3, 9 dan 21 bulan dibahagikan kepada kumpulan kawalan dan rawatan, menerima sama ada olein kelapa sawit atau suplemen TRF. Tisu hati dianalisis menggunakan metabolomik yang tidak disasarkan melalui LC-MS/MS, bersama-sama dengan penilaian histologi dan penilaian biokimia enzim antioksidan (SOD, CAT), penanda keradangan (IL-6,TNF-alpha) dan peroksidasi lipid (MDA). Aktiviti SOD merosot dengan ketara dengan umur dalam kedua-dua kawalan dan tikus yang dirawat TRF ($p<0.05$), mencadangkan pengurangan pertahanan antioksidan dengan peningkatan umur, manakala tiada perubahan ketara diperhatikan dalam tahap CAT, IL-6 atau MDA. Secara histologi, hati yang dirawat TRF menunjukkan lebih sedikit fibroblas dan kurang keradangan daripada hati kawalan. Metabolomik mendedahkan perubahan berkaitan usia dalam metabolisme taurin dan hipotaurin, metabolisme pirimidin (uridin dan uracil) dan komponen kitaran TCA (malat dan glutamat). Penemuan ini menunjukkan bahawa TRF memodulasi beberapa aspek perubahan hati yang berkaitan dengan usia, terutamanya dalam morfologi dan metabolisme, walaupun penurunan antioksidan yang berterusan. Hasil kajian ini menggariskan potensi TRF dalam mengurangkan penuaan hati dan memberikan gambaran tentang laluan metabolik utama yang terlibat, menawarkan asas untuk strategi terapeutik masa hadapan yang menyasarkan kesihatan hati dalam kalangan warga tua.

Kata kunci: Fraksi kaya-tokotrienol; hati; metabolomik; penuaan hepatik; tekanan oksidatif;