ROLE OF THERMALLY OXIDIZED EDIBLE OILS ON METABOLIC FUNCTIONS AND ITS IMPLICATIONS IN CARDIOVASCULAR DISEASES

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(Faculty of Science)

By

SRUTHI P K

under the guidance of

Dr C. R. ACHUTHAN, Ph. D.

AMALA CANCER RESEARCH CENTRE,

THRISSUR, KERALA, INDIA

November 2024

Declaration

I hereby declare that the work presented in the thesis entitled "ROLE OF THERMALLY OXIDIZED EDIBLE OILS ON METABOLIC FUNCTIONS AND ITS IMPLICATIONS IN CARDIOVASCULAR DISEASES." is based on the original work done by me under the guidance of Dr. C R Achuthan, Associate Professor, Department of Biochemistry, Amala Cancer Research Centre, Thrissur and has not been included in any other thesis submitted previously for the award of any degree. The contents of the thesis have undergone a plagiarism check using iThenticate software at C.H.M.K. Library, University of Calicut, and the similarity index found within the permissible limit. I also declare that the thesis is free from AI-generated content.

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Dr. Achuthan C R
Supervising Guide

Dr. Achuthan C. Raghavamenon, Ph.D.
Associate Professor
Dept. of Biochemistry
Amala Cancer Research Centre
Amala Nagar, Thrissur- 680 555, Kerala

E-mail: office@amalacrcs.org | amalacancerresearch@gmail.com | Ph: 0487 230 7968, 0487 230 4190

Amala Cancer Research Centre Society
(A Society Registered T.C. Act, XII of 1955 Sl. No. 56 of 1984)
Amala Nagar- 680 555, Thrissur, Kerala, India

Ref.No. ACRC/REG(b1)/443/2024 Date: 21.11.2024

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Dr. C R Achuthan

Supervising Guide

Dr. Achuthan C. Raghavamenon, Ph.D.
Associate Professor
Dept. of Biochemistry
Amala Cancer Research Centre
Amala Nagar, Thrissur- 680 555, Kerala

E-mail: amalacancerresearch@gmail.com

Phone: 0487 2307968

Institutional Animal Ethical Committee

(Reg. No. 149/PO/Rc/S/1999/CPCSEA)

Amala Cancer Research Centre

RESEARCH DIRECTOR &
CHAIRMAN, IAEC : DR. RAMADASAN KUTTAN, Ph.D.

AMALANAGAR - 680 555, THRISSUR
KERALA, INDIA

Ref : Approval No: ACRC/IAEC/17(1)/P-02

Date: 22.12.2017

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Name of Chairman/Member Secretary IAEC

Dr.Ramadasan Kuttan, Ph D.

Name of CPCSEA nominee:

Prof. (Dr.) E Vijayan, Ph D.

22/12/17

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Name and signature of the Supervisor. Dr. Achuthan C. Raghavamenon, Ph.D.
Associate Professor
Dept. of Biochemistry
Amala Cancer Research Centre

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Sruthi P K

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List of Abbreviations

µL	microliter
ω-3	Omega-3
ω-6	Omega-6
AGE	Advanced Glycation End products
ALP	Alkaline Phosphatase
ALT	Alkaline Transferase
AST	Aspartate Transaminase
BSA	Bovaine Serum Albumin
CD 36 protein	Cluster Differentiation 36
CLA	Conjugated Linoleic Acid
CO	Coconut Oil
CRP	C-Reactive Protein
FABP	Fatty Acid Binding Protein
FBPase	Fructose 1,6 bisphosphatase
FBS	Fasting Blood Sugar
HDL-C	High-Density Lipoprotein Cholesterol
HOMA	Homeostasis Model Assessment
ICDH	Isocitrate Dehydrogenase
IL-10	Interleukin 10
IL-6	Interleukin 6
IR	Insulin Resistance
LCFA	Long Chain Saturated Fatty Acids
LDL - Cholesterol	Low Density Lipoprotein – Cholesterol

Lp-PLA_2	Lipoprotein Associated Phospholipase A_2
MCFA	Medium Chain Saturated Fatty acids
MCT	Medium Chain Triglycerides
MDA	Malonaldehyde
MDH	Malate Dehydrogenase
MO	Mustard Oil
MUFA	Monounsaturated Fatty acids
NAFLD	Non-Alcoholic Fatty liver Diseases
NCDs	Non-Communicable Diseases
OGTT	Oral Glucose Tolerance Test
PAH	Polycyclic aromatic hydrocarbons
PPAR α	Peroxisome Proliferator-Activated Receptor Alpha
PUFA	Poly Unsaturated Fatty Acid
SDH	Succinate Dehydrogenase
SFA	Saturated Fatty Acids
SO	Sunflower Oil
TCO	Thermally Oxidized Coconut Oil
TFA	Trans fatty Acids
TMO	Thermally Oxidized Mustard Oil
TPO	Thermally Oxidized Palm Oil
TSO	Thermally Oxidized Sunflower Oil
VCO	Virgin Coconut Oil

Abstract

The growing prevalence of cardiovascular diseases (CVD) and metabolic disorders such as diabetes and obesity, has spurred extensive research into the dietary factors contributing to these conditions. Many studies have examined the impact of unhealthy fats, high-fat diets, or high-fat diets combined with high sugar, which are known to impair metabolic functions and raise the risk of metabolic disorders. In addition, thermally oxidized lipids as a class of deleterious molecules have been emerged as the cooking practice involves repeated heating of oils. Several studies have addressed the effect of oxidized and peroxidized lipids in the aetiology of many metabolic disorders. However, no studies have yet explored the effects of the consumption of thermally oxidized oils at a normolipidemic (5%) dietary level. This study aims to fill that gap by investigating the impact of long-term consumption of 5% thermally oxidized coconut oil, mustard oil, palm oil, and sunflower oil on carbohydrate and lipid metabolism in Wistar rats over six months. The rats were grouped into eight, with six animals per group and fed either unoxidized and thermally oxidized forms of these oils. Various parameters including physiochemical characterization of oils, body weight, carbohydrate metabolism, lipid metabolism and inflammatory markers related to cardiovascular disorders were assessed. Physico-chemical analysis revealed that oils rich in unsaturated fatty acids, especially sunflower oil, mustard oil and palm oil were more prone to oxidation, resulting in increased levels of carbonyl compounds, lipid oxidative products, and short-chain fatty acids. Conversely, coconut oil with its medium-chain fatty acids showed higher resistance to oxidation. In this study, rats fed thermally oxidized palm and sunflower oils experienced reduced body weight gain, while those fed coconut and mustard oils did not. Coconut oil, whether oxidized or not, enhanced insulin sensitivity, whereas oxidized mustard and palm oils led to

hyperinsulinemia, dyslipidaemia and liver dysfunction. Thermally oxidized oils, particularly sunflower and palm oil promote hepatic steatosis, inflammation and elevated risk factors for cardiovascular diseases. In conclusion, prolonged consumption of thermally oxidized oil, especially sunflower and palm oil even at normal dietary levels, significantly increases the risk of metabolic disorders such as insulin resistance, obesity, non-alcoholic fatty liver, and cardiovascular diseases. Although thermally oxidized coconut oil appears to be less harmful, it still poses some inflammatory risks. These findings underscore the health risks associated with the consumption of thermally oxidized oils, even at normal dietary levels.

Keywords: thermally oxidized oils, lipid oxidation products, metabolic disorders, cardiovascular disorder, coconut oil, palm oil, mustard oil, sunflower oil

സംഗ്രഹം

ഹൃദയസംബന്ധമായ രോഗങ്ങളുടെയും പ്രമേഹം, അമിതവണ്ണം തുടങ്ങിയ ഉപാപചയഅസുഖങ്ങളുടെയും വർദ്ധിച്ചുവരുന്ന വ്യാപനം, ഈ അവസ്ഥകൾക്ക് കാരണമാകുന്ന ഭക്ഷണഘടകങ്ങളെക്കുറിച്ചുള്ള ഗവേഷണത്തിന് കാരണമായി. ഉപാപചയപ്രവർത്തനങ്ങളെ തടസ്സപ്പെടുത്തുകയും ജീവിതശൈലിരോഗങ്ങൾ ഉണ്ടാകാനുള്ള സാധ്യത വർദ്ധിപ്പിക്കുകയും ചെയ്യുന്ന അനാരോഗ്യകരമായ കൊഴുപ്പുകൾ, കൊഴുപ്പ് കൂടിയ ഭക്ഷണങ്ങൾ, ഉയർന്ന പഞ്ചസാര തുടങ്ങിയ ഭക്ഷണരീതികളെ കുറിച്ച് പല പഠനങ്ങളും നടത്തിയിട്ടുണ്ട്. കൂടാതെ, ആവർത്തിച്ച് ചൂടാക്കുന്ന എണ്ണകളുടെ ഫലമായുള്ള ഹാനികരമായ തന്മാത്രകളുടെ ഒരു വിഭാഗമായി ഓക്സിഡൈസ്ഡ് കൊഴുപ്പുകൾ ഉയർന്നുവന്നിട്ടുണ്ട് പല ഉപാപചയ വൈകല്യങ്ങളുടെയും രോഗഹേതുവായി ഓക്സിഡൈസ്ഡ്, പെറോക്സിഡൈസ്ഡ് കൊഴുപ്പുകളുടെ പ്രഭാവത്തെ പറ്റി നിരവധി പഠനങ്ങൾ അഭിസംബോധന ചെയ്തിട്ടുണ്ട്. എന്നിരുന്നാലും,——നോർമോലിപിഡിക് (5%) ഭക്ഷണതലത്തിൽ താപഓക്സികരണം നടന്ന എണ്ണകളുടെ ഉപയോഗം മൂലമുള്ള അനന്തര ഫലങ്ങളെ കുറിച്ച് പരിമിതമായ ഗവേഷണങ്ങളാണുള്ളത്. ആറ് മാസത്തിനിടെ വിസ്റ്റാർ എലികളിലെ ഉപാപചയത്തിലും അനുബന്ധ ആരോഗ്യ പ്രശ്നങ്ങളിലും ദീർഘകാലമായി 5% ഓക്സിഡൈസ്ഡ് വെളിച്ചെണ്ണ, സൂര്യകാന്തി എണ്ണ, കടുകെണ്ണ, പാം ഓയിൽ എന്നിവ കഴിക്കുന്നതിന്റെ ഫലങ്ങൾ ഈ പഠനത്തിൽ പരിശോധിച്ചു. ഇതിനായി എലികളെ എട്ട് ഗ്രൂപ്പുകളായി (ഒരു ഗ്രൂപ്പിന് n=6) വിഭജിക്കുകയും ഓരോ ഗ്രൂപ്പ് എലികൾക്കും ഓക്സിഡൈസ് ചെയ്യാത്തതും താപപരമായി ഓക്സിഡൈസ് ചെയ്തതുമായ എണ്ണകൾ ഭക്ഷണത്തിലൂടെ നൽകുകയും എണ്ണകളുടെ ഭൗതികരാസഗുണങ്ങൾ, അന്നജം, കൊഴുപ്പ് എന്നിവയുടെ ഉപാപചയത്തിലും ഹൃദയസംബന്ധമായ തകരാറുകളുമായി ബന്ധപ്പെട്ട കോശ ജ്വലന മാർക്കറുകളുടെയും പ്രധാന പാരാമീറ്ററുകൾ വിലയിരുത്തുകയും ചെയ്തു. അപൂരിത ഫാറ്റിആസിഡുകൾ, പ്രത്യേകിച്ച് സൂര്യകാന്തി എണ്ണ, കടുകെണ്ണ, പാം ഓയിൽ എന്നിവ ഓക്സിഡേഷന് കൂടുതൽ സാധ്യതയുള്ളതിനാൽ കാർബോണൈൽ സംയുക്തങ്ങൾ, ലിപിഡ് ഓക്സിഡേറ്റീവ് ഉൽപ്പന്നങ്ങൾ, ചെറിയ ചെയിൻ ഫാറ്റിആസിഡുകൾ എന്നിവയുടെ അളവ് വർദ്ധിക്കുന്നുവെന്ന് ഭൗതിക-രാസ വിശകലനം വെളിപ്പെടുത്തി. ഇതിനു വൈരുധ്യമായി ഇടത്തരം ചെയിൻ ഫാറ്റിആസിഡുകളുള്ള വെളിച്ചെണ്ണ ഓക്സിഡേഷനോട് ഉയർന്ന പ്രതിരോധം കാണിച്ചു. താപപരമായി ഓക്സിഡൈസ് ചെയ്ത പാം, സൂര്യകാന്തി എണ്ണ എന്നിവ എലികളുടെ ശരീരഭാരം കുറയ്ക്കാൻ കാരണമായി, അതേസമയം വെളിച്ചെണ്ണയും കടുക് എണ്ണയും ശരീരഭാരത്തിൽ വലിയ മാറ്റങ്ങളുളുണ്ടാക്കിയില്ല. വെളിച്ചെണ്ണ, ഓക്സിഡൈസ് ചെയ്താലും ഇല്ലെങ്കിലും ഇൻസുലിൻ സംവേദനക്ഷമത വർദ്ധിപ്പിച്ചു, അതേസമയം ഓക്സിഡൈസ്ഡ് കടുകെണ്ണയും പാം ഓയിലും ഹൈപ്പർഇൻസുലിനിമിയ, ഡിസ്ലിപിഡെമിയ, കരൾ അപര്യാപ്തത എന്നിവയ്ക്ക്

കാരണമായി. കൂടാതെ, താപപരമായി ഓക്സിഡൈസ് ചെയ്ത സൂര്യകാന്തി, പാം ഓയിൽ എന്നിവ കരൾ വീക്കം, ഹൃദയസംബന്ധമായ രോഗങ്ങൾ എന്നിവയുടെ അപകടസാധ്യത വർദ്ധിപ്പിച്ചു. ചുരുക്കി പറഞ്ഞാൽ, സാധാരണ ഭക്ഷണതലത്തിൽ പോലും താപ ഓക്സിഡൈസ്ഡ് എണ്ണകളുടെ ദീർഘകാല ഉപഭോഗം, പ്രത്യേകിച്ച് സൂര്യകാന്തി, പാം ഓയിലുകൾ, ഇൻസുലിൻ പ്രതിരോധം, അമിതവണ്ണം, നോൺ-ആൽക്കഹോളിക് ഫാറ്റിലിവർ രോഗം, ഹൃദയസംബന്ധമായ രോഗങ്ങൾ എന്നിവയുൾപ്പെടെയുള്ള ഉപാപചയ വൈകല്യങ്ങളുടെ അപകടസാധ്യത ഗണ്യമായി വർദ്ധിപ്പിക്കുന്നു. ദോഷകരമല്ലെങ്കിലും, താപപരമായി ഓക്സിഡൈസ് ചെയ്ത വെളിച്ചെണ്ണ കോശ ജ്വലന അപകടസാധ്യതകൾ ഉയർത്തുന്നുണ്ട്. ഈ കണ്ടെത്തലുകൾ സാധാരണ ഭക്ഷണതലങ്ങളിൽ പോലും താപ ഓക്സിഡൈസ്ഡ് എണ്ണകൾ കഴിക്കുന്നതിന്റെ ആരോഗ്യ അപകടങ്ങൾ അടിവരയിടുന്നു.

പ്രധാന വാക്കുകൾ: താപ ഓക്സിഡൈസ്ഡ് എണ്ണകൾ, ലിപിഡ് ഓക്സിഡേഷൻ ഉൽപ്പന്നങ്ങൾ, ഉപാപചയഅസുഖങ്ങൾ, ഹൃദയസംബന്ധമായ രോഗങ്ങൾ, വെളിച്ചെണ്ണ, പാം ഓയിൽ, കടുക് എണ്ണ, സൂര്യകാന്തി എണ്ണ

Chapter 1

Introduction

A rapid rise of non-communicable diseases (NCDs), which include diabetes, cardiovascular diseases, cancers, and chronic respiratory disorders, represents a significant global health challenge. As reported by the World Health Organization (WHO, 2023), NCDs constitute 74% of global mortality and significantly affect both developed and developing countries. The pathogenesis of these NCDs is influenced by a complex interplay of genetic, environmental and metabolic factors. Among these, metabolic factors driven by modern dietary habits have become a key contributor to the rising prevalence of these conditions. Modern diets are often characterized by high consumption of sugary beverages, excessive salt, unhealthy fats, processed and fried foods, and a lack of essential nutrients. These dietary patterns are linked to the onset of metabolic risk factors such as hypertension, dyslipidemia, hyperglycemia, and obesity. These metabolic risk factors are directly connected with the onset and progression of NCDs, including cardiovascular disorder, diabetes, non-alcoholic fatty liver (NAFLD) and many types of cancer. In this scenario, the importance of dietary fats and oils are major areas of concern and investigation of human nutrition, health, and diseases. The type and quality of dietary fats play an essential role in modulating metabolic pathways and influencing health outcomes. Therefore, understanding the impact of dietary fats, particularly within the framework of contemporary eating patterns, is crucial for addressing the global health concern posed by non-communicable diseases.

1.1 Lipids in human health

Lipids are essential biomolecules in human nutrition, encompassing fats, oils, phospholipids, glycolipids, and sterols. They have a significant role in various physiological functions including maintaining cell membrane integrity, regulating inflammation, brain function and supporting cardiovascular health and serving as a carrier for the uptake of fat-soluble vitamins A, D, E, and K. Lipids are broadly categorized into saturated and unsaturated fats according to the presence of double

bonds. Monounsaturated fatty acids (MUFA) have at least one double bond, polyunsaturated fatty acids (PUFA) have more than one double bond in the carbon chain and saturated fatty acids (SFA) are those that have no double bond between two carbon atoms. Among the various types of fats, omega-6 (ω-6) and omega-3 (ω-3) fatty acids are particularly important due to their essential roles in maintaining cellular function and overall health.

Dietary fats and oils are integral to human health due to their fatty acid content (Orsavova et al., 2015) and antioxidant compounds (Mazzocchi et al., 2021). Numerous studies have highlighted the adverse health effects associated with diets high in saturated fats. High intake of saturated fat (stearic acid, palmitic acid) raises serum cholesterol, and LDL cholesterol (LDL-C) and CVD risk (Hu et al., 1999, DiNicolantonio et al., 2016). Hooper et al., (2020) found that reduced consumption of saturated fat could decrease the incidence of CVDs. The consumption of mono and polyunsaturated fats has been linked to various health benefits, particularly with cardiovascular diseases. MUFAs are present in olive oil, sesame oil, mustard oil and peanut oil. Several studies have reported that MUFAs enhance insulin sensitivity and reduce obesity when replaced with saturated fats in the diet (Vessby et al., 2001, DiNicolantonio and O'Keefe, 2022). PUFAs include ω-3 and ω-6 fats, in which omega-3 fats are linked to a lower risk of CVDs, and improved lipid profiles (Ander et al., 2003). In contrast, excessive intake of omega-6 fats promotes inflammation and leads to chronic metabolic disorders.

Thus, the type of oil consumed has significant health implications, particularly concerning cholesterol levels, heart health, and overall well-being, emphasising the need to understand the impact of oils, especially when thermally oxidized oil, as part of a balanced diet. The repeated use of heated oils is common in cooking and the

commercial food industry. When oils are subjected to heat at high temperatures, thermal oxidation occurs which can lead to the deterioration of the oils. Thermally oxidized edible oil raises significant concern among nutritionists and researchers due to their cytotoxic compounds, loss of nutritive value, and antioxidative compounds such as polyphenols, and tocopherols. The unsaturated fatty acids in the oil are oxidized when exposed to oxygen, water, light and heat resulting in the formation of harmful compounds known as lipid oxidation products (LOPs), such as peroxides, aldehydes, ketones, polymers, and dimers (Choe and Min, 2006). Consumption of these LOPs in thermally oxidized edible oils can have deleterious biological effects. Some of these compounds, particularly certain hydroperoxides, aldehydes (such as acrolein, malondialdehyde (MDA), and 4-hydroxy-2-trans-nonenal (HNE)) and ketones are implicated in the development of oxidative stress, cytotoxicity, genotoxicity, and inflammation when consumed in significant quantities (Ambreen et al., 2020, Miyazawa, 2021). These events can potentially damage proteins, DNA, and tissues in the body and contribute to the pathogenesis of metabolic syndrome such as cardiovascular diseases, cancer, gastrointestinal disorders, and neurodegenerative disorders.

The intake of thermally oxidized oils has been elicited to deleterious health effects, particularly metabolic disorders like dyslipidaemia, obesity, diabetes, hypertension and cardiovascular disorders. In a diabetic rat model, the incorporation of heated coconut oil (8.4%) as a fat source in a high-fat diet (HFD) significantly contributes to fibrosis in the liver (Gopinath et al., 2021). Some studies have explored that intake of 15% of repeatedly heated palm oil induces the progression of cardiovascular risk like hypertension with reduced vascular relaxation response (Owu et al., 1997). Another study has also pointed out that long-term consumption of oxidized mixed edible oil induces oxidative stress and lipid peroxidation, which induce hepatotoxicity (Ambreen

et al., 2020). Research indicates that diets high in thermally oxidized oils show increased markers of oxidative stress and inflammation. This suggests that continuous consumption of such oils could exacerbate metabolic dysfunction, particularly in individuals with obesity and diabetes. Moreover, other studies have linked the dietary intake of repeatedly heated oils to a heightened risk of metabolic disorders, including hypertension, CVD and diabetes. In many of the above-mentioned studies, the levels of thermally oxidized oils used in rodent diets exceed the typical dietary intake, often surpassing 5%. Currently, most studies focus on the impact of fresh oils or oils subjected to extreme conditions, however, there is a gap when it comes to moderate consumption of thermally oxidized oils at normolipidemic levels in preclinical models. This thesis investigates the impact of the intake of thermally oxidized edible oils, even at normal dietary levels on the development of metabolic syndrome, an area that remains underexplored compared to the prevalent focus on high-fat diets, high-fat with high sugar intake. By examining the biochemical and physiological effects of these oils, this thesis aims to explore the impact of long-term consumption of normolipidic levels of thermally oxidized oil on metabolic processes. It is envisaged that the study may answer the question of whether dietary levels of thermally oxidized oils contribute to conditions such as insulin resistance (IR), obesity, dyslipidaemia, and inflammation as well as its influence on the emergence of metabolic disorders including cardiovascular diseases.

Scope of the Thesis

The health implications of the consumption of high-fat with high sugar diets, high-fat diets, and unhealthy fats such as trans fats, and heated oil have been extensively researched. These dietary patterns are known to contribute to metabolic disturbances, leading to conditions such as obesity, IR, dyslipidaemia, hypertension, diabetes, cardiovascular diseases, NAFLD, and cancer. In light of this background, the purpose

of this investigation is to examine the influence of the consumption of unoxidized and thermally oxidized oil at normolipidic dietary levels on carbohydrate and lipid metabolism and its implications in cardiovascular disorders. Based on fatty acid composition, coconut oil (MCTs), palm oil (LCTs), mustard oil (MUFAs), and sunflower oil (PUFAs) were selected for this study.

Chapter 2

Review of Literature

Cardiovascular disorders (CVD) are conditions that are related to the heart and blood vessels and represent a significant threat to global health. Over the years, the prevalence of cardiovascular disorders has emerged as one of the foremost causes of mortality worldwide. Globally, CVD is responsible for 32% of all fatalities, with ischemic heart disease and stroke being the primary contributors (WHO, 2021). In India, CVDs are also a major health burden, causing 27% of total deaths in 2017 (WHO, 2018, Bhatia et al., 2021). CVDs include various conditions such as hypertension, coronary artery disease, peripheral arterial disease, and arrhythmias. The most prevalent of them is coronary heart disease (CHD) also known as coronary artery disease (CAD) which affects the arteries that supply blood to the heart. When these blood vessels become narrowed or blocked due to calcified lipid lesions, it leads to a cardiac arrest. The primary cause of CVD is atherosclerosis, characterized by the build-up of fatty deposits in the arteries, leading to conditions such as CAD, stroke, and peripheral artery disease. It has multifactorial pathophysiology and is often interlinked from the combination of genetic predisposition and lifestyle factors. The behavioural risk factors associated with CVD are alcohol /tobacco consumption, lack of physical activity, and consumption of an unhealthy diet. However, the amount and types of fats and oils and their nutritional content of dietary fats playing a substantial influence on cardiovascular disease development.

Fat and oils are essential dietary components, aiding in the uptake of fat-soluble vitamins and providing a substantial source of energy. Nevertheless, inappropriate consumption of fats/oil can lead to various metabolic disorders such as hyperglycaemia, dyslipidaemia, obesity, hepatosteatosis, hypertension, cardiovascular diseases, and cancer (Morelli et al., 2017, Ismail et al., 2018). Fats, particularly oils are a key dietary fat component, and their quality and type can significantly influence

cardiovascular health. The findings of many studies on edible oils and their influence on cardiovascular health have yielded mixed results. Some studies demonstrate positive impacts, while others exhibit unfavourable effects. The fatty acid compositions found in various types of oils can have distinct effects on cardiovascular health. In addition to their fatty acid profiles, oils also contain several micronutrients, which may consequently help to protect the cardiovascular system.

This bioactive compound possesses anti-inflammatory, antioxidant, neuroprotective, hypolipidemic, anticancer and immunomodulatory properties (Kindernay et al., 2023, Tsamesidis and Kalogianni, 2023, Illam et al., 2017, Setyawati et al., 2023). Thus, the fatty acid profile as well as the micronutrient richness of the edible oil are important factors in their impact on cardiovascular health. The beneficial properties of edible oils can deteriorate upon repeated heating due to lipid oxidation, generating noxious compounds and diminished antioxidant properties. Free radicals and reactive oxygen species (ROS) are produced by this degradation process, aggravating oxidative stress and subsequently increasing the risk of CVD (Bester et al., 2010, Ng et al., 2014). The complex interaction between lipid and carbohydrate metabolism is central to the pathophysiology and progression of CVDs, involving metabolic alterations like insulin resistance (IR), dyslipidaemia, hyperglycaemia and lipotoxicity. Chronic hyperglycemia is caused by an imbalance in glucose metabolism that is promoted by IR. This persistent hyperglycemia induces oxidative stress and inflammatory reactions, culminating in cellular damage. Additionally, IR adversely affects systemic lipid metabolism, resulting in dyslipidaemia, characterized by elevated plasma concentrations of triglycerides and low-density lipoprotein cholesterol (LDL-C) while decreasing high-density lipoprotein cholesterol (HDL-C) concentrations. Dyslipidaemia increases the risk of atherosclerotic plaque formation and subsequent CVD events. The development of atherosclerosis is exacerbated by lipotoxicity, which

is caused by an excessive build-up of lipids in non-adipose tissues, hypertension, and CAD (Opie and Stubbs, 1976, Siri-Tarino et al., 2010, Ormazabal et al., 2018). This review intends to provide a comprehensive understanding of these metabolic pathways and their roles in the onset and progression of CVD. Oxidized edible oil consumption may significantly impact lipid and carbohydrate metabolism, driving the onset and progression of cardiovascular disorders.

2.1 Edible Oils

Edible oils, despite their bioactive components and beneficial nutritional value, play a vital role in human health (Rey et al., 2023). Based on the composition of their fatty acids, edible oils are divided into saturated, monounsaturated, and polyunsaturated fatty acids (PUFA) containing oils. Among the saturated fat-rich oils, medium-chain saturated fat (MCSFA) containing oil (coconut oil) and long-chain saturated fat-containing oil (palm oil) are present. Olive oil and mustard oils are rich in MUFAs. PUFA is again divided into omega-3 fats (fish and shark liver oil) and omega-6 fats (sunflower oil). Another type of fat in the oil is trans-fat, formed during food processing by hydrogenation of PUFA. Phytosterols, polyphenols, tocopherols (vitamin E), carotenoids, and squalene found in edible oils are known bioactive compounds, with varying compositions depending on the oils and their sources. These compounds have garnered significant attention owing to their potential antioxidant, anti-inflammatory, and other health-promoting effects. **Table 1** depicts different types of oil and its reported health benefits.

2.1.1 Coconut oil

Coconut oil (CO) is extracted from the kernel of mature coconut *(Cocos nucifera).* This oil is composed primarily of medium-chain saturated fats (MCFA), accounting for 90% of its fatty acid profile. Approximately 45-50% of its fatty acid content is lauric acid. CO has long been criticized for containing high amounts of saturated fatty acid (SFA)

and concerns about its link to heart disease and other health problems (Jayawardena et al., 2021). Studies indicate that incorporating cis-unsaturated fats instead of CO can reduce cardiovascular risk factors and improve blood lipid profiles (Eyres et al., 2016). Numerous studies have debunked the misconception surrounding CO and its previously perceived adverse effects due to its saturated fat content. Alternatively, virgin coconut oil (VCO), prepared by fermentation or hot processed method has the same fatty acid content and gained popularity due to its beneficial effects. VCO differ from refined CO for its polyphenol content. VCO has anti-inflammatory and antioxidant potential with a hypolipidemic effect (reduces total lipid and LDL-C in serum) (Illam et al., 2021, Nevin and Rajamohan, 2008a, Parathodi Illam et al., 2019). These studies have illuminated numerous health benefits associated with CO consumption.

2.1.2 Palm Oil

Palm oil (PO), is the most widely used vegetable oil globally, obtained from the fruit and seed of the palm tree (*Elaeis guineensis*). PO is rich in LCSFA, primarily palmitic acid, constituting approximately 44% of its fatty acid composition. Along with rich antioxidants such as carotenoids, lycopene, phytosterols, tocotrienols and tocopherols, it also contains monounsaturated and polyunsaturated fats. However, due to its high SFA content palm oil consumption has been linked to detrimental health effects, including raise in level of LDL-C and TG levels. Both of which are associated with an elevated risk of cardiovascular diseases, especially when consumed in excess and as part of a diet high in saturated fats (Go et al., 2015). Despite some studies indicate that PO may not significantly raise blood cholesterol levels. However, not all SFA cause an adverse impact on lipid profile and PO also contains other beneficial components like oleic and linoleic acids and tocotrienols (vitamin E), in appreciable quantities, that can hinder cholesterol synthesis. Palm oil has been extensively studied for its health impacts. Research indicates that palm oil contains phytonutrients like tocotrienols and

tocopherols that have anti-inflammatory and antioxidant potential which can benefit cardiovascular health (Aggarwal et al., 2010).

2.1.3 Mustard Oil

Mustard oil (MO) is an edible oil derived from seeds of mustard plants (*Brassica juncea and Brassica nigra*). It contains various volatile compounds that which give it a specific pungent taste and smell such as allellyl isothiocyanate responsible for the mustard taste. MO is used in traditional medicine and skin care due to its intended health benefits, in addition to its culinary uses. Mustard oil contains about 47-60% MUFA, 16-22% PUFA, and 4-10% saturated fats (Ostrikov et al., 2020, Sawicka et al., 2020). It contains antioxidants, vitamins A, D, E, and K as well as vital minerals including iron, calcium, and magnesium. It also contains omega-3 fatty acids, which are essential for maintaining heart function. Mustard oil should only be used sparingly, though, because it comprises erucic acid, which can be detrimental in excess quantities. Due to the presence of glucosinolates (sinigrin, sinalbin), isothiocyanates, phenolic compounds, phytic acid, and phytosterols, MO also has anti-inflammatory, anti-carcinogenic, and antimicrobial properties (Poyil et al., 2023).

2.1.4 Sunflower Oil

Sunflower oil (SO) is widely recognised as one of the most consumed edible vegetable oils produced from seeds of the sunflower plant (*Helianthus annuus L*). SO mostly contains high levels of linoleic acid (PUFA) and oleic acid (MUFA) (Petraru et al., 2021) and has negligible amounts of trans fats and saturated fats. Apart from fatty acids, SO also comprises minor components such as tocopherols (vitamin E), phospholipids, sterols, and trace amounts of other compounds. Research has shown that linoleic and oleic acids have the effect of cardioprotective agents by reducing arrhythmias (Desnoyers et al., 2018). SO, particularly, its linoleic acid content, has been associated with both anti-inflammatory and pro-inflammatory effects (Masi et al., 2012).

Table 2.1: Types of Edible Oils and Their Health Impacts.

Edible Oil	Source	Major Fatty acids	Phytocompounds	Biological impacts	References
Canola Oil (Rapeseed oil)	Seeds of Canola plant (*Brassica napus*)	Oleic Acid (MUFA) – 61% Linoleic acid (ω-6 PUFA) -21% Alpha-Linolenic acid (ω -3)11%	Sterols Tocopherol	↓cholesterol levels improve insulin sensitivity	(Lin et al., 2013) (Jones et al., 2014)
Coconut Oil	Fruit of coconut tree (*Cocos nucifera*)	Lauric Acid (SFA) -52% Myristic acid - 21% Palmitic acid – 10%	Polyphenols Tocopherols tocotrienols Phytosterols, and Flavonoids	↑HDL cholesterol levels associated with increased LDL cholesterol levels antimicrobial properties anti-inflammatory activities	(Chinwong et al., 2017, Intahphuak et al., 2010, Varma et al., 2019)
Corn Oil	Germ of corn kernels (*Zea mays*)	Linoleic Acid (PUFA) – 54-62% Oleic acid (MUFA)- 20-42% Palmitic acid (SFA) – 8-12%	Tocopherols, Carotenoids, phytosterols, and Polyphenols	↓ LDL cholesterol levels Potentially pro-inflammatory if omega-6/omega-3 ratio is high Antioxidant property	(Rouf Shah et al., 2016) (Maki et al., 2015)
Fish Oil	Mackerel (*Scomber scombrus*), Salmon (*Salmo salar*), Sardine (*Sardinella longiceps*)	Eicosapentaenoic Acid (EPA, PUFA) – 6-10% Docosahexaenoic Acid (DHA, PUFA)- 17-21% Oleic acid (MUFA)- 20-27% Palmitic acid (SFA) – 21-27%	Astaxanthin and Squalene Phospholipid	Supports heart health, reduces inflammation, Mental Health and Cognitive Function, and Anti-Inflammatory Effects	(Kelley et al., 2009, Sittiprapaporn et al., 2022, Liao et al., 2021)

Grapeseed Oil	Seeds of grape (*Vitis vinifera)*	Linoleic Acid (PUFA) – 70-80% Oleic Acid (MUFA) -15-20%	Tocopherols, phytosterols, and Polyphenols	Potentially pro-inflammatory due to high omega-6 content	(Gitea et al., 2023)
Olive Oil	Fruit of *Olea europaea*	Oleic Acid (MUFA) -75-80% Linoleic acid (PUFA) – 21% Palmitic acid (SFA) -15-20%	Carotenoids, Tocopherols, phytosterols, and Polyphenols	↓LDL-cholesterol levels Rich in antioxidants, Reduced risk of cardiovascular diseases, Anti-inflammatory properties	(Guasch-Ferré et al., 2014, Nocella et al., 2018)
Palm Oil	Fruit of the oil palm tree (*Elaeis guineensis*)	Palmitic Acid (SFA) -44-45% Oleic acid (MUFA) -39-40% Linoleic acid (PUFA) – 10%	Carotenoids Tocotrienols Tocopherols, phytosterols, and Squalene	↑ LDL cholesterol levels Obesity and Metabolic disorder	(Mancini et al., 2015)
Peanut Oil (Ground nut or arachis oil)	Fruit of peanut plant (*Arachis hypogaea*)	Oleic Acid (MUFA) – 50% Linoleic acid (PUFA) – 32-35% Palmitic acid (SFA) -15-20%	Resveratrol Tocopherol, polyphenols, and phytosterols	↓LDL cholesterol levels Antioxidant and anti-inflammatory properties.	(Parilli-Moser et al., 2022)
Mustard Oil	Seeds of mustard plant (*Brassica nigra*)	Erucic acid (MUFA) - 40-50% Oleic acid M (MUFA) – 20% Linoleic acid (PUFA) – 14%	Glucosinolates, phenolic compounds, and Flavonoids	Cholesterol-lowering properties, Anti-inflammatory, and Antimicrobial properties	(Tian and Deng, 2020)

		Alpha-Linolenic Acid (PUFA) - 10%		myocardial lipidosis	
Rice Bran Oil	The outer layer of rice grains (*Oryza sativa*),	Oleic acid (MUFA) – 38-44% Linoleic acid (PUFA) – 32-38% Palmitic acid (SFA) -15-20%	γ-Oryzanol Tocotrienols, Tocopherols, and phytosterols	Cholesterol-lowering, antioxidant, and anti-inflammatory properties.	(Saji et al., 2019, Junyusen et al., 2022)
Safflower Oil	Seeds of the safflower plant (*Carthamus tinctorius*)	Linoleic acid (PUFA) – 70% Oleic acid (MUFA) - 15% Palmitic acid (SFA) -10%	Flavonoids Tocopherols and phytosterols	↓ LDL cholesterol levels Anti-inflammatory, Antimicrobial properties, anti-diabetic effect	(Khémiri et al., 2020, Asp et al., 2011)
Sesame Oil	Sesame seeds (*Sesamum indicum*)	Oleic Acid (MUFA) -35-50% Linoleic acid -35-50% Palmitic acid (SFA) – 12%	sesamol and sesaminol lignans	↓ LDL cholesterol levels anticancer and anti-inflammatory properties	(Atefi et al., 2022, Oboulbiga et al., 2023)
Soybean Oil	Seeds of soybean plant (*Glycine max*)	Linoleic Acid (PUFA) – 57% Oleic acid (MUFA) – 27% Palmitic acid (SFA) – 13% Alpha-Linolenic Acid -11%	Isoflavones Tocopherols and phytosterols	↓ cholesterol levels anti-inflammatory and anti- cancerous activity	(Fan et al., 2022, Hsu et al., 2010)
Sunflower Oil	Seeds of the sunflower plant (*Helianthus annuus*)	Linoleic Acid (PUFA) – 74% Oleic acid (MUFA) - 34%	Tocopherols and phytosterols	↓ LDL cholesterol levels Excessive omega-6 PUFA may promote inflammation	(Guo et al., 2017, Hrádková et al., 2013)

2.2 Thermally Oxidized Edible Oils

2.2.1 Effects of Thermal Oxidation of Edible Oils.

Edible oils are essential in cooking and food preparation, providing flavour and texture, and are a medium for heat transfer. Heating of edible oils is more common in culinary preparation. However, the practice of reusing and repeatedly heating edible oil, whether in deep-frying or other cooking techniques, has raised health and safety concerns. Several physical and chemical changes are involved during the deep-frying process, temperature, atmospheric oxygen, and moisture content of oil make up a unique reaction system, where triglycerides and free fatty acids become the reactants thereby producing toxic compounds. Studies have shown that heating oils for prolonged periods or at high temperatures (160 – 200°C) can result in decreased levels of beneficial PUFAs like linoleic acid and alpha-linolenic acid while increasing the formation of harmful TFA and saturated compounds (Szabo et al., 2022). These deleterious molecules can also decrease antioxidant capacity, contributing to oxidative stress and potential health risks.

2.2.2 Mechanism and Products of Thermal Oxidation

During the heating of edible oils at high temperatures (160-200°C), several events can occur and products are formed due to the complex processes that affect the composition of the oils. The chemistry of edible oils during thermal oxidation has been extensively described by Choe and Min, (2007). Thermally-induced lipid oxidation of unsaturated fats is a complex process that occurs when oils are exposed to heat, with oxygen and various initiators such as free radicals. These complex chemical changes include hydrolysis, oxidation, polymerization, and isomerization. As a result, the chemical composition of the edible oils is altered, to form monoacylglycerides, diacylglycerides and polymeric triglycerides through the interaction between free fatty acids and free radicals. The primary products of thermal oxidation are peroxides and hydroperoxides;

which are unstable and further breakdown into various secondary products including volatile (such as alcohols and acids, aldehydes and ketones) and non-volatile compounds (such as carbonyls, dimeric, trimeric, polymeric and cyclic fatty acids). Some of these secondary oxidized products polymerize to form tertiary products. These products are considered polar in nature and grouped under total polar compounds (TPC). PUFA-rich oils are more susceptible to thermo-oxidative stress compared to saturated and monounsaturated fats, leading to higher concentrations of toxic lipid oxidation products (LOPs). According to Sadoudi et al., (2014), in addition to increasing the thermal oxidation of SO not only increases free fatty acids and peroxide value but also alters the composition of fatty acids by forming conjugated dienes. Bastida and Sánchez-Muniz, (2001) have reported that elevated levels of triacylglycerol polymers and dimers in these heated oils. Recently, Romero et al. (2006) have also analysed the content of cyclic fatty acid monomers (CFAM) as a major toxic component generated during the thermally oxidized edible oil, especially those that contain unsaturation. The Figure 2.1 depicted the oxidative product of oils. These chemical reactions include

(a) *Hydrolysis*: This process takes place due to the presence of moisture (water) from the foodstuff during heating, leading to the breakdown of the ester bond of TG in the oil, with subsequent release of fatty acids, monoacylglycerol (MAG), diacylglycerols (DAG) and glycerol (Dobarganes and Márquez-Ruiz, 2015). Glycerol can further break down into acrolein, a compound responsible for the characteristic acrid smell of overheated oil. This results in the increased acid value and polar content of the oil.

(b) *Oxidation*: Exposure to air and high temperature, unsaturated fatty acid undergoes oxidation and thermal alterations. This primarily leads to modifications in triacylglycerols (TAG), where at least one of the three fatty acyl chains becomes altered. Thermal oxidation has three phases: initiation, propagation, and formation of

oxidized products. Oxidation begins with the breakdown of chemical bonds in the fatty acids of the oil, resulting in the formation of free radical compounds. These free radicals interact with oxygen in the air to create peroxyl radicals (ROO^{-}). A chain reaction can be continued by these peroxy radicals in turn react with unsaturated fatty acids generating new free radicals. The free radicals react with oxygen to form lipid hydroperoxides. This chain reaction is the propagation phase. Hydroperoxides are comparatively unstable and can further break down into secondary oxidation products such as aldehydes, ketones, alcohols, and acids. It increases peroxide values, colour, viscosity, and polymers of triglycerides. Other than temperature, the presence of metals, reactive radicals, and light also increases the oxidative modifications in oils. Contrary to SFA-rich oils, unsaturated fatty acid-containing edible oils are more prone to oxidative modifications.

(c) *Polymerization and isomerization*: This process combines free fatty acids and free radicals and causes the fatty acid chains to link or cross-link, leading to polymerization. These leads to the generation of high molecular weight, more complex linear or cyclic compounds, dimerization (intra-strand cross-linking between two different fatty acids), and polymerization (cross-linking of two or more triglycerides in thermally oxidized oils). Usually, saturated fatty acid-rich oils undergo polymerization reactions during high-temperature treatments. As the oil continues to degrade, polar compounds form, which are more polar than the original non-polar triglycerides. These compounds increase the viscosity of oils and contribute to oil darkening and foaming.

Under prolonged high heat, oils can decompose, releasing volatile compounds and smoke. This smoke point indicates the temperature at which the oil degrades significantly, producing visible smoke and acrid odours. Smoke is often a sign of significant thermal oxidation and decomposition. Oxidation and polymerization lead to reduced oil quality, resulting in off-flavours, decreased nutritional value, and potential

health risks due to the formation of harmful compounds.

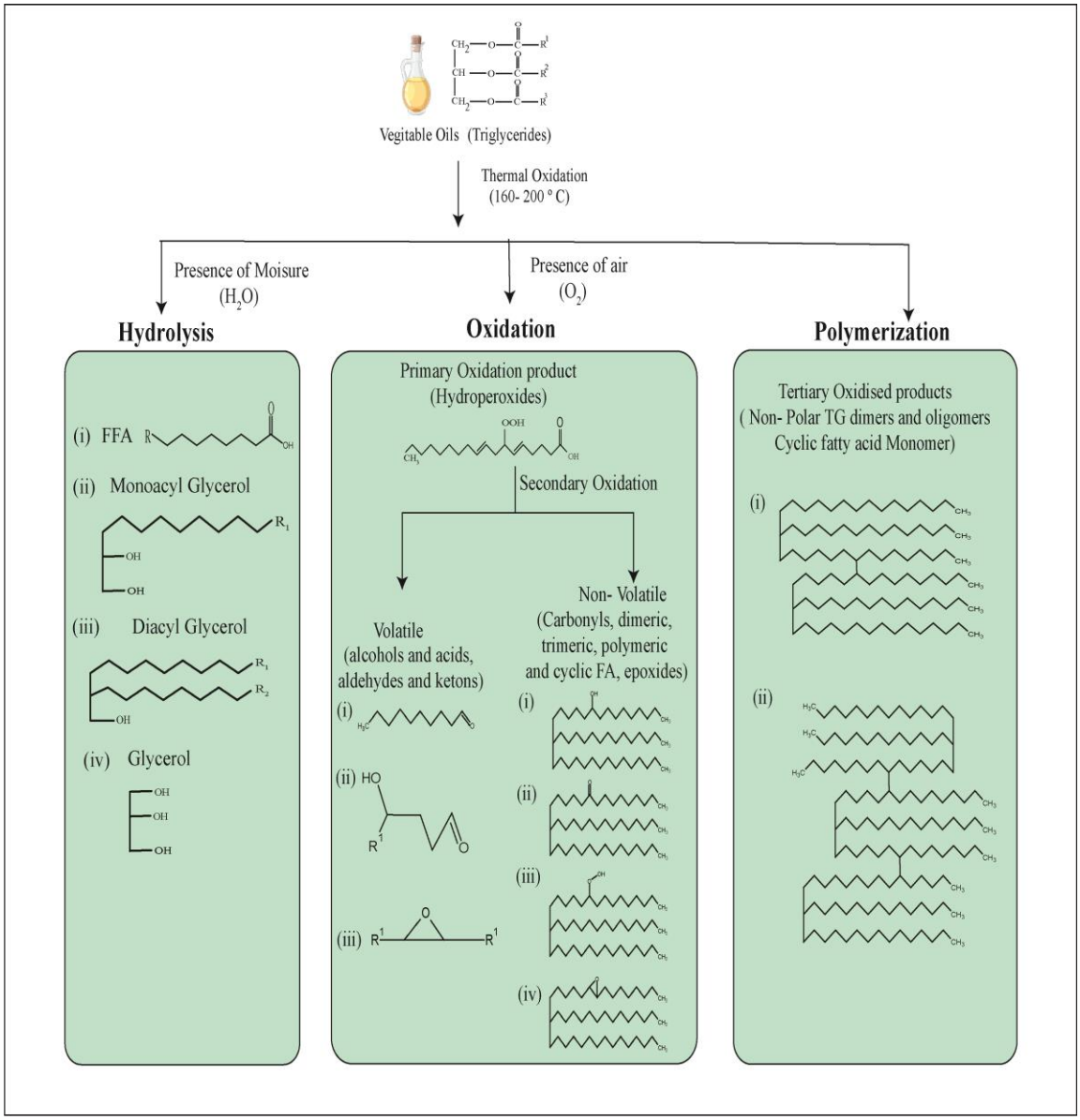

Figure 2.1: Oxidized Products Formed During Thermal Oxidation of Edible Oil

2.3 Changes in Antioxidant Compounds of Edible Oil

Edible oils with higher levels of polyphenols tend to be more resistant to heat-induced oxidative changes (Konsoula, 2010). However, during thermal oxidation, these oils experience a significant reduction in their phenolic content (Brenes et al., 2002, Gómez-Alonso et al., 2003). The extent of loss of phenolic compound depends on the fatty acid composition and oils high in polyunsaturated fatty acids (PUFAs) tend to lose more phenolics during thermal oxidation (Brenes et al., 2002). Additionally, some

phenolic compounds oxidize, resulting in the formation of oxidized phenolics. Among the various phenolic compounds, lignans are the most resistant to thermal oxidation, while simple phenolic acids and complex phenolics tend to undergo oxidative changes more quickly (Carrasco-Pancorbo et al., 2007). Tocotrienols, the antioxidant present in palm oil is destroyed in thermal oxidation (Sen et al., 2010, Xian et al., 2012).

Other bioactive components in oils, such as beta-carotene also undergo oxidative modifications during deep frying. According to Zeb and Murkovic, (2013) the thermal oxidation of corn oil can lead to beta-carotene oxidation, resulting in the formation of compounds like 8′-apo-β-carotenal, 5,6-epoxy-8′-apo-β-carotenal, β-carotene-2,2′-dione, and E-5,6-epoxy-β-carotene. These oxidation products can then accelerate the oxidation of triglycerides, leading to the formation of hydroxy bis-hydroperoxides and epi-deoxy bis-hydroperoxides.

2.4 Impact of Thermally Oxidized Products on Health

Thermal oxidation deteriorates the quality of edible oils by generating cytotoxic compounds, reducing antioxidant properties, and causing loss of nutrients like carotenoids, phenolics, and vitamins (Falade et al., 2017). Compounds such as trans-fatty acids, aldehydes (malonaldehyde, 4-Hydroxy-2-nonenal, hexanal), ketones (2-pentanone, 2-heptanone, 2 octanone), conjugated linoleic acid (CLA), epoxides, and dicarboxylic acids like azelaic acid formed as result of lipid peroxidative reaction, also can react with fried food containing amino acids and proteins to form additional compounds like polycyclic aromatic hydrocarbons (PAHs), advanced glycation end-products (AGEs), and acrylamide (Hidalgo and Zamora, 2000).

According to existing literature, it has been revealed that the consumption of high TFA leads to a heightened risk of developing CVD, inflammation, and metabolic disorders (Bhat et al., 2022, Szabo et al., 2022). A by- product formed during frying of peanut oil namely Trans-2,4 decadienal is found to exhibit genotoxicity by generating reactive

oxygen species (ROS) and reducing cellular glutathione content (Chang et al., 2005). PAHs and AGEs are carcinogenic compounds that can be generated during high-temperature cooking processes (Chiang et al., 1997). Studies conducted by Chiang et al., (1999), and Wu and Yen, (2004) indicates the presence of various toxic and carcinogenic molecules, such as benzo[a]pyrene and benzo[a]anthracene in safflower, olive, coconut, mustard and corn oils after thermal oxidation.

As discussed above, the physicochemical properties of edible oils and fats undergo significant changes during deep frying, leading to the formation of reactive and toxic molecules that can negatively impact health. While unoxidized oils are generally considered beneficial for various health aspects, the consumption of oils that have been deep fried is associated with adverse health effects due to these newly formed compounds and their metabolism in the body, which is being discussed in the coming sessions.

2.5 Consumption of Thermally Oxidized Oil and Metabolic Changes in the Body

Thus, from the literature, it is clear that intake of thermally oxidized oils and fats alters the normal metabolism of carbohydrates and lipids. Since metabolic alterations are closely correlated with the onset and advancement of various lifestyle diseases, it is possible that the prolonged administration of thermally oxidized oils may heighten the risk of developing various chronic degenerative diseases, especially metabolic disorders.

Carbohydrate and lipid metabolism are essential for maintaining energy balance and overall health, but their dysregulation can significantly contribute to cardiovascular disease (CVD). Disruptions in carbohydrate metabolism, particularly due to insulin resistance or diabetes, which promote endothelial dysfunction and inflammation, are two major risk factors for CVD. Conversely, lipid metabolism encompasses the processing of fats in the body, and imbalances such as high levels of LDL-C or

triglycerides can cause atherosclerotic plaques to build up in the arteries, heightening the risk of heart attacks and strokes. Therefore, effectively managing carbohydrate and lipid levels is crucial for preventing and mitigating cardiovascular diseases.

2.5.1 Insulin Resistance and Obesity

The consumption of thermally oxidized cooking oil has been shown to have detrimental effects on carbohydrate metabolism in the body. Carbohydrate metabolism involves the breakdown of carbohydrates into glucose, which is then utilized by cells to produce energy. Oxidized oils increase oxidative stress, which can impair pancreatic β-cell function and lead to decreased insulin secretion. This contributes to IR, a hallmark of metabolic disorders such as type 2 diabetes (Tangvarasittichai, 2015). Compounds formed during oil oxidation can trigger inflammatory responses. Chronic inflammation is associated with metabolic syndrome and can interfere with insulin signalling pathways, further exacerbating insulin resistance and disrupting normal carbohydrate metabolism. Consumption of oxidized oils can lead to unfavourable changes in lipid profiles, such as increased LDL-C and reduced HDL-C. These lipid imbalances can influence glucose metabolism and insulin sensitivity, further complicating carbohydrate metabolism.

Intake of thermally oxidized edible oils has been extensively investigated for their contribution to promoting obesity and insulin resistance (IR). Obesity, defined as the excess fat accumulation in the body, is closely associated with IR which promotes increased fat storage, disrupting the balance between fat storage and utilization, and contributing to metabolic dysregulation that favours weight gain. Hyperinsulinemia, characterized by elevated insulin levels, often precedes IR and is a major risk factor for the development of obesity, Diabetic Mellitus, CVDs, cancer and other metabolic disorders. IR occurs when cells become unresponsive to insulin produced by the pancreas or downregulate insulin receptors, leading to impaired glucose uptake and

hyperglycemia. Insulin plays a central role in metabolism by enhancing glucose uptake in skeletal muscles and adipocytes, facilitating glycogenesis in skeletal muscles, inhibiting gluconeogenesis in the liver and stimulating triacylglycerol synthesis while suppressing lipolysis in adipocytes (Dimitriadis et al., 2011). Furthermore, insulin enhances the expression of GLUT-4, a protein crucial for glucose transport in muscle cells (Furtado et al., 2002, Watson et al., 2004). Insulin has cytoprotective effects on vascular health through the activation of endothelial nitric oxide synthase (eNOs) through the PI3K/Akt signalling pathway leads to vasodilation (Muniyappa and Sowers, 2013). Conversely, IR disrupts these protective effects, causing vascular smooth muscle cell proliferation, vasoconstriction, and pro-inflammatory responses via the mitogen-activated protein kinase (MAPK) pathways, which promote a pro-coagulant and pro-atherogenic state (Cersosimo and DeFronzo, 2006, Muniyappa et al., 2020, Li et al., 2023, Fu et al., 2021, Zhou et al., 2010). These mechanisms underscore and highlight the intricate role of insulin in both protective and detrimental effects on vascular health. IR also promotes the release of free fatty acids from adipose tissue and inflammatory cytokines such as IL-6, TNF-α and leptin, leading to increased hepatic synthesis of triglycerides and VLDL-C, contributing to hypertriglyceridemia, which in turn leads to non-alcoholic fatty liver diseases (NAFLD).

Several investigations have demonstrated that oxidative stress induced by heated oils can impair insulin sensitivity in peripheral tissues, leading to insulin resistance. Experimental models using high-fat diets, such as those containing Indian vanaspati and coconut oil with fructose, have been developed to induce hyperlipidaemia and insulin resistance in rats, mimicking conditions relevant to coronary heart disease (Munshi et al., 2014). Fatty acids from fried oil can impact insulin secretion through PPARα signalling, affecting glucose tolerance (Shetty and Kumari, 2021). Long-chain unsaturated fatty acids, natural ligands of PPARs, and SFAs like palmitic acid (PA)

have been reported to affect insulin secretion by modulating the expression of enzymes involved in insulin secretion via transcription factors. Additionally, PPARα activation can lower muscle lipids, improve insulin sensitivity, and enhance muscle insulin signalling in high-fat-fed rat models of insulin resistance. Therefore, the consumption of fried oil by rats may lead to reduced insulin secretion and glucose intolerance mediated through PPARα signalling pathways (Fatehi-Hassanabad and Chan, 2007, Ye et al., 2001). Chiang et al., (2011) reported that oxidized frying oils may cause oxidative damage to pancreatic islet cells, impairing insulin secretion associated with vitamin E deficiency. Another study has shown that dietary administration of 10% of thermally oxidized coconut and mustard oils, along with fructose, altered glucose tolerance and increased hyperglycemia in rats (Narayanankutty et al., 2017a). Additionally, a study has also indicated that rats fed diets containing 10% and 20% avocado oil exhibited lower IR, highlighting the potential benefits of avocado oil in mitigating insulin resistance (Del Toro-Equihua et al., 2016).

2.5.2 Dyslipidaemia

Lipids play a crucial role in cellular function, serving as structural components of cell membranes, signalling molecules and energy storage molecules. Lipid metabolism comprises the synthesis, degradation and utilization of various lipid components. The interference of oxidized oils with lipid metabolism has significant health implications, contributing to metabolic disorders such as obesity, IR, and type 2 diabetes. Additionally, oxidized lipid products promote inflammation and oxidative stress, which are key factors in the progression of cardiovascular diseases and non-alcoholic fatty liver disease (NAFLD). Oxidized lipids, especially, oxidized fatty acids can directly inhibit key enzymes critical for lipid metabolism, including those involved in TG synthesis and degradation, cholesterol synthesis carnitine palmitoyl transferase 1 (CPT1), a crucial enzyme for transporting fatty acids into mitochondria for β-oxidation.

This inhibition impairs fatty acid oxidation and results in unoxidized fatty acids build up in tissues. This accumulation can lead to lipotoxicity, where excess fatty acids and their derivatives cause cellular dysfunction and death, contributing to conditions like IR and type 2 diabetes. Zhang et al., 2023 found that feeding of oxidized corn oil to broiler chicken led to lipid deposition by upregulating the expression of CPT, FABP, CD36 protein and PPARα, which are involved in lipid metabolism. Furthermore, Enzymes such as glycerol-3-phosphate acyltransferase (GPAT) and hormone-sensitive lipase (HSL) are particularly affected, leading to altered triglyceride levels in tissues. This disruption promotes lipid accumulation in adipose tissue, contributing to obesity and related metabolic disorders. Phospholipid biosynthesis is critical for maintaining cellular membrane integrity and function. Oxidized oils can disrupt the enzymes involved in phospholipid synthesis, leading to compromised membrane structure and function. Additionally, oxidized lipids disrupt cholesterol homeostasis by modulating the expression of key proteins involved in cholesterol synthesis and uptake, such as HMG-Co. A reductase and LDL receptors. This dysregulation can lead to altered cholesterol levels and an elevated risk of atherosclerosis.

Obesity and IR are often linked with serum and hepatic dyslipidaemia, characterized by abnormal levels of lipids such as cholesterol, triglycerides, free fatty acids and lipoprotein in the blood. Dyslipidemia, particularly elevated levels of LDL-C and decreased levels of HDL-C, is strongly related to the increased risk of atherosclerosis a condition where cholesterol as well as other lipids accumulate in the arteries, leading to plaque formation and potential arterial blockages (Hernáez et al., 2019). The pathogeneses of dyslipidaemia associated with obesity and IR begin with the inability of adipose tissue to effectively store triglycerides. Due to this impairment, blood levels of circulating free fatty acids (FFA) were elevated, which contributed to hypertriglyceridemia. Hypertriglyceridemia, in turn, enhances the activity of

cholesteryl ester transfer protein (CETP), a crucial enzyme in lipid metabolism by facilitating the transfer of cholesteryl esters from HDL-C to VLDL-C. This CETP-mediated transfer reduces HDL-C levels, which are critical for the reverse transport of cholesterol from peripheral tissues to the liver. Thereby contributing to the progression of atherosclerosis. Increased flux of FFAs to the liver stimulates the hepatic production of triglycerides and VLDL-C. The overproduction of VLDL-C is also a contributor to hypertriglyceridemia. Similarly, CETP also transfers triglycerides from VLDL-C to LDL-C, forming TG-rich LDL particles. These triglycerides in LDL are subsequently hydrolysed by hepatic lipase, converting LDL into smaller, denser particles. These small, dense LDL particles are particularly atherogenic due to their higher susceptibility to oxidation, enhanced ability to penetrate the arterial wall and a longer plasma half-life than larger LDL particles. Consequently, dyslipidaemia significantly contributes to the progression of atherosclerosis and associated CVD risk.

Research exploring the impact of thermally oxidized oils on lipid profiles has yielded inconsistent findings, with various factors contributing to the onset of dyslipidaemia. Rats who received a diet comprising 10% thermally oxidized sunflower oil (TSO) for 42 days of dietary intervention had significantly higher serum cholesterol, malondialdehyde (MDA), and HDL-C levels compared to those fed fresh oil. However, when the oxidized oil was supplemented with pectin, serum cholesterol and MDA levels were significantly lower (Shafaeizadeh et al., 2011). Another study found feeding rats different forms of sunflower oil including fresh and heated oil subjected to various heating durations of 30,60,120, and 240 minutes over 45 days promoted the formation of active aldehydes, which was accompanied by a significant elevation in serum triglycerides, LDL-C, as well as VLDL-C along with a reduction in HDL-C levels. Leptin resistance, fatty liver, and dyslipidaemia might be linked with daily ingestion of such oxidized oils, (Nili-Ahmadabadi et al., 2022). Despite being rich in

saturated fats, coconut oil did not considerably raise LDL-C levels when compared to butter in a randomized control experiment, which involved 200 healthy men and women after feeding 50g of coconut oil, olive oil and butter for 4 weeks (Khaw et al., 2018).

2.5.3 Endothelial Function and Inflammation

Vascular health depends on the endothelium, a thin layer of cells that lines blood vessels and regulates blood flow, vascular tone, and immune function. Any disruption in endothelial function can lead to CVDs, including atherosclerosis, hypertension and thrombosis. Studies have shown that consuming thermally oxidized oils increases oxidative stress biomarkers such as malonaldehyde (MDA), and reduces the bioavailability of nitric oxide (NO) molecule that plays a central role in endothelial function.

The formation of oxidized compounds such as aldehydes, and oxidized LDL-C (ox-LDL) during the heating of cooking oils can directly impair endothelial function and activate inflammatory pathways. Another study established that exposing rats to repeatedly heated palm and soyabean oil (15% w/w) caused detrimental effects on the vascular structure of the aorta through impaired endothelium-dependent vasorelaxation and heightened contractile response (Leong et al., 2009, Leong et al., 2010). The intake of reheated oils, leading to elevated levels of ox-LDL, activates inflammatory pathways in the endothelium, predominantly through the NLRP3 (NOD, LPR- and pyrin domain-containing protein 3) inflammasome, resulting in heightened oxidative stress and inflammation (Leong, 2021). Hypercholesterolemia associated with reheated oil intake is linked to the upregulation of ox-LDL receptors and NADPH oxidase in the endothelium further impairing function (Jiang et al., 2022). Conversely, Hamsi et al., (2014) found that repeatedly heated virgin coconut oil did not significantly alter inflammatory markers like ICAM-1, VCAM-1, CRP, PGI2, and TXB_2 in rats compared

to fresh oil. However, a study by Ng et al., (2012) on rats who received 15% of repeatedly heated palm oil for six months showed increased expression of adhesion molecules in the aorta, indicating heightened inflammation and endothelial dysfunction. This was linked to elevated blood pressure, suggesting a direct impact on vascular health.

2.5.4 Atherosclerosis

The build-up of fatty deposits or plaques such as cholesterol and ox-LDL on the arterial walls is an essential characteristic of atherosclerosis. The prolonged consumption of thermally oxidized oils, which contain high levels of lipid oxidation products such as malonaldehyde (MDA), and 4- hydroxy-2 -nonenal (4-HNE) has been linked to the commencement and advancement of atherosclerosis, thereby increasing the risk of cardiovascular events. Research by Staprãns et al., (1996) showed that a diet consisting of a 5% oxidized corn oil fed to rabbits over 14 weeks resulted in increased lipid peroxidation in serum and an increased accumulation of fatty deposits in the aorta. Similarly, Idris et al., (2018) found that rabbits who received a diet containing 15% heated palm oil and corn oil for 12 weeks exhibited significant development of atherosclerotic lesions. The groups that were fed thermally oxidized oil had considerably higher atherosclerotic lesion scores than the groups that were fed unoxidized oil. This indicates that heated oil may accelerate the progression of atherosclerosis.

In most of the studies mentioned above, the heated oils have been supplemented directly, with food or other sources for a short duration to evaluate the pharmacological influences. However, the direct influence of heated oil at normal dietary levels over a longer duration is necessary to assess its influence on metabolism and associated pathologies. Also, it is essential to assess the risk groups that can be at higher risk of deleterious effects from the consumption of these oils are still unanswered

Chapter 3

Materials and Methods

3.1 MATERIALS

3.1.1 Chemicals

Name of Chemicals	Manufacturer
Acetic acid	Merck (New Jersey, United States)
Acetone	-do-
Ascorbic acid	-do-
Chloroform	-do-
Copper sulfate pentahydrate	-do-
Ethylene diamine tetra acetic acid	-do-
Ferrous ammonium sulfate	-do-
Folin Ciocalteau Phenol reagent	Sisco Research Laboratories (Mumbai, India)
Formaldehyde	-do-
Hydrochloric acid	Merck (New Jersey, United States)
Hydrogen peroxide	-do-
Isoamyl alcohol	-do-
Isooctane	-do-
Isopropanol	-do-
Methanol	Spectrochem (Mumbai, India)
Nicotinamide adenine dinucleotide Phosphate oxidized (NADP)	Sisco Research Laboratories (Mumbai, India)
Nitrobluetetrazolium (NBT)	-do-
p- Anisidine	-do-
Phenolphthalein indicator solution	Merck (New Jersey, United States)

Potassium chloride (KCl)	-do-
Potassium dihydrogen phosphate	-do-
Potassium Hydroxide (KOH)	Sisco Research Laboratories (Mumbai, India)
Sodium acetate	Merck (New Jersey, United States)
Sodium Azide	-do-
Sodium bicarbonate	-do-
Sodium dihydrogen phosphate dehydrate	-do-
Sodium hydroxide	-do-
Sodium potassium tartrate	-do-
Sulfuric acid	-do-
Thiobarbituric acid (TBA)	Hi-Media (Mumbai, India)
Tris Buffer	Merck (New Jersey, United States)
Tris-HCl	-do-

3.1.2 Diagnostic Kits and Reagents

Name of the Diagnostic Kit	**Manufacturer**
Alanine transaminase kit	Euro Diagnostic Systems Pvt Ltd (Tamil Nadu, India)
Alkaline phosphatase kit	-do-
Aspartate transaminase kit	-do-
Glucose kit	Agape Diagnostics Ltd. (Kerala, India)
Haemoglobin kit	-do-
High-density lipoprotein kit	Euro Diagnostic Systems Pvt Ltd (Tamil Nadu, India)

C-Reactive Protein	Alpha Diagnostic International, (Texas, USA)
Rat Insulin	Origin Diagnostic and Research, India
Rat Interleukin -6 (IL-6)	Peprotech, (New Jersey, USA)
Rat Interleukin- 10 (IL-10)	-do-
Total bilirubin kit	Euro Diagnostic Systems Pvt Ltd (Tamil Nadu, India)
Total cholesterol	-do-
Total protein	-do-
Triglycerides kit	- do-

3.1.3 Instruments

Name of the Instrument	**Manufacturer**
Deep freezer (-20ºC)	Remi Laboratory Instruments, India
Double distillation Unit (Quartz)	Borosil, India
Electronic balance	Schimadzu, Genzo Shimadzu
FTIR spectrophotometer	PerkinElmer, Massachusetts, USA
High-speed cooling centrifuge	Eppendorf, Hamburg, Germany
Hot air oven	Rotex Instruments Pvt Ltd, India
Incubator	Beston Instruments, India
Inverted microscope	Magnus INVI, Bangalore, India
LC-MS/MS	Agilent biotech, California, USA
Micro Plate Reader	Epoch, Biotek, Germany
Microcentrifuge	Tarsons Products Private Limited
Multi dispenser	Eppendorf, Hamburg, Germany
pH meter	Eutech, Waltham, USA
Phase contrast microscope	Magnus INVI, Bangalore, India

Tissue homogenizer	Yorco Scientific, Chennai, India
Ultra-Centrifuge	Thermo Scientific, Waltham, USA
Ultra-low Deep freezer (-80ºC)	New Brunswick, Eppendorf, Germany
UV/Visible Spectrophotometer	PG Instruments Ltd; Systronics India
Vacuum concentrator	Eppendorf, Hamburg, Germany

3.1.4 Software

Name of the Software	**Purpose**
Adobe Acrobat DC Pro	Preparation and edit PDF files
Adobe Illustrator	Image processing
Adobe Photoshop 2022,	Image processing
Endnote X20	Reference Manager
GraphPad prim 9	Statistics & Graph Plotting
Image J 1.48	Biological image processing
IS Capture 3.6.6	Microscope image capture
MS Excel 2010	Calculation
MS Powerpoint 2010	Presentation and artworks
MS Word 2010	Text preparation
Origin 2019 b	Statistics & Graph preparation
UV Win spectrophotometer software	Spectroscopic measurement

3.1.5 Edible Oils and Thermal Oxidation

Four edible oils, Coconut oil (KLF), Mustard oil (V.V.V and Sons' enterprise), Sunflower oil (Gold winner), and Palm oil (Ruchi Gold), were bought from the local market of Thrissur, Kerala, India and subsequently stored under -20ºC conditions to avoid auto-oxidation. A portion of these oils were subjected to thermal oxidation as per the method outlined by Narayanankutty et al., (2018). Briefly, 500 mL of each of the edible oils was heated at a high temperature (160-180ºC) in a stainless-steel frying pan

with the span of 5 hours of heating and cooling and the process was repeated for 72 hours. To avoid further oxidative changes, oils that underwent thermal oxidation were stored at -20°C.

3.1.6 Animals

Male Wistar rats (*Rattus norvegicus*) weighing 160-180g were acquired from the Small Animal Breeding Station, College of Veterinary and Animal Sciences, Mannuthy, Thrissur. They were housed in polypropylene cages with proper bedding in the animal house facility of Amala Cancer Research Centre, Thrissur. The rats were nourished with water and pure rat chow (Sai Durga Feeds, Bangalore, India) over two weeks of acclimatization. Environmental conditions were controlled and monitored with a 12h light and dark cycle, temperature of 22±2°C, and humidity of 60±10%. The institutional animal ethical committee of Amala Cancer Research Centre [Approval No. ACRC/IAEC/17 (1) – P (02)] granted prior permission for all the experimental procedures, were humanly adhering to the stringent guidelines of the Committee for the Control and Supervision of Experiments on Animals (CCSEA), Ministry of Environment, Forest, and Climate Change, Govt. of India.

3.2 METHODOLOGY

3.2.1 Modified Diets

In accordance with the guidelines established by the American Institute of Nutrition (AIN), the diets were meticulously formulated within our laboratory by blending powdered substances, as outlined by Reeves et al., (1993). The diet was comprised of approximately 62 % potato starch as a carbohydrate source, 18 % soya bean powder as a protein source, and the fat source was 5% either unoxidized or thermally oxidized coconut oil, palm oil, mustard oil, and sunflower oil. The respective diet composition is shown in Table 3.1.

Table 3.1: Composition of the Modified Rat Diets**.**

Nutrients (g/ 100g)	**Unoxidized oil Diet**	**Thermally oxidized oil Diet**
Protein	18.0	18.0
Potato Starch	62	62
Fresh, unoxidized oil	5	-
Thermally oxidized oil	–	5
***Mineral Mix**	3.5	3.5
#Vitamin Mix	1.5	1.5
Fibre	5.0	5.0
Moisture	5.0	5.0
Total (g)	**100**	**100**
Energy (kcal/kg)	**3500**	**3500**

*The mineral mixture contained the following (mg/1000g): calcium phosphate dibasic, 7000; sodium chloride, 2000; potassium 9000; magnesium oxide, 2000; manganese carbonate, 95.06; zinc, 9600; iron 1500 mg; copper 12.81; cobalt 0.34; iodine 1.66; sulphur 1141.22 phosphorus 5000; Aluminium 79.37; Chlorine 2.377; Selenium 0.25; Zinc 95.18

#The vitamin mixture contained the following (mg): Vitamin A 4000IU; Vitamin D_3: 1000IU; Vitamin E – 75 IU; Nicotinamide – 30; thiamine hydrochloride, 5; riboflavin, 6; pyridoxine hydrochloride, 6; nicotinic acid, 3000; calcium pantothenate; 15; D-biotin, 0.2; cyanocobalamin, 0.025; retinyl palmitate, 160; DL-tocopherol acetate, 20000; cholecalciferol, 25; DL-Methionine – 1000; Folic acid 2; Vitamin K 0.075.

3.2.2 Experimental design

Eight groups of male Wistar rats were sorted, each consisting of six rats. The groups were fed with respective unoxidized or thermally oxidized diets for six months. Mortality and morbidity were monitored throughout the experimental period. During the dietary intervention period, all animals had unrestricted access to water and food. Table 3.2 illustrates the grouping of the experimental rats. Throughout the dietary intervention, the body weight of experimental rats was monitored, with initial measurements recorded at baseline, followed by weekly monitoring for six months.

The formula was used to determine the percentage of body weight gain.

$$\textbf{Body weight gain}\ (g\%) = \frac{(\textbf{Final body weight} - \textbf{Initial body weight})}{\textbf{Initial body weight}} \times 100$$

At the beginning and end of the experimental period, bleeding and clotting times were recorded. After the dietary intervention, animals were euthanized with CO_2 anaesthesia at concentrations (30–70%). Blood was collected through cardiac puncture. Furthermore, animals were necropsied to retrieve the liver, heart, kidney and visceral adipose tissue for organ weight evaluation. The relative organ weight (organosomatic index) was determined by dividing the organ weight of animals by their body weight.

$$\textbf{Relative Organ weight}\ (\%) = \frac{\textbf{Organ Weight}\ (g)}{\textbf{Final Body weight}\ (g)} \times 100$$

Tissues were stored at −80°C for further biochemical studies, and a portion of internal organs was preserved in 10% neutral buffered formalin for histopathological examination. After collection of blood was allowed to coagulate for an hour at room temperature. Centrifuging the blood for 15-20 minutes at 2000 rpm to separate the serum, which was then aliquoted and kept at -20°C freezer for biochemical analysis.

Table 3.2: Grouping of Experimental Rat

Groups	Treatment/ Intervention
Group I	A diet with 5% Unoxidized Coconut Oil (CO)
Group II	A diet with 5% Thermally oxidized Coconut Oil (TCO)
Group III	A diet with 5% Unoxidized Palm oil (PO)
Group IV	A diet with 5% Thermally oxidized Palm oil (TPO)
Group V	A diet with 5% Unoxidized Mustard oil (MO)
Group VI	A diet with 5% Thermally oxidized Mustard oil (TMO)
Group VII	A diet with 5% Unoxidized sunflower oil (SO)
Group VIII	A diet with 5% Thermally oxidized Sunflower oil (TSO)

3.3 Thermal Characterization of Edible oils

3.3.1 Differential Scanning Calorimetry (DSC)

The Netzsch DSC 204F1 Phoenix calorimeter was employed to measure the heat flow resulting from chemical reactions or changes in the physical characterization of a sample with respect to temperature in order to evaluate the oxidative stability of oils. The instrument temperature scale was calibrated using reference samples (indium (In) and tin (Sn)), based on the manufacturer's guidelines and instructions (Pardauil et al., 2011). A baseline was recorded by running an empty open aluminium pan. Within the sample chamber, open aluminium pans holding oil samples, each weighing 6.0 ± 0.5 mg were placed. At intervals of 10°C min^{-1}, the samples were heated from 30°C to 250°C. Purified oxygen (99.8%) was continuously bubbled through the sample chamber at a flow rate of 50 mL/min. Each measurement was terminated upon the observation of an endothermic peak indicative of thermal degradation.

3.4 Physico–Chemical Characterization of Edible Oils

3.4.1 Density

Procedure: The densities of oil samples were estimated using a modified Harabarkada method (Habarakada et al., 2021). The relative density of each oil was assessed using a 25 mL density bottle. Initially, the density bottle was cleaned, and dried, and the empty weight was recorded. Then, the mass of the 25 mL density bottle with water was measured. After emptying and thoroughly drying the bottle to remove any trace of water residue. Finally, the density bottle was filled with the oil sample and weighed. The measurement was recorded to estimate the mass of the oil sample. Each experiment was repeated three times. The given below equation was used to estimate the densities of oil samples.

Calculation

$$\textbf{Density, } \boldsymbol{\rho} = \frac{\boldsymbol{M}}{\boldsymbol{V}}$$

Where V = volume of oil sample (mL)

M = mass of oil sample in the density bottle (gram)

3.4.2. Conjugated Dienes and Trienes

Principle: Conjugated dienes and trienes are a good measure of the oxidative states of the oils. Due to the free radical reaction, hydroperoxides are formed from PUFA during oil oxidation, leading to the conjugated structure. This causes absorption of UV radiation at 230-234nm for conjugated dienes. Further oxidation leads to the formation of conjugated trienes, and these results increase in absorbance at 270 nm.

Procedure: Conjugated diene (CD) and conjugated triene (CT) contents were determined according to the method AOCS cd 7-58 (AOCS, 1998), where 0.03g of oil was dissolved in 25 mL of isooctane (2,2, 4 – trimethylpentane). The samples were measured quantitatively by UV-visible spectrophotometric measurement. The absorbance was read at 234 nm and 270 nm for conjugated dienes and trienes against isooctane as a blank. The concentration of CD and CT was calculated according to the following formula and expressed in millimoles of linoleic acid hydroperoxides per kilogram using a molar extinction coefficient.

Calculation

$$[\boldsymbol{CD}] = \frac{\boldsymbol{A}}{\boldsymbol{\varepsilon l}}$$

Where A= absorbance

ε = 172540 $M^{-1}.cm^{-1}$ – Molar absorption coefficient of linoleic acid hydroperoxide,

l = optical path of the quartz cell – 1cm.

3.4.3. Acid Value (AV) and Free Fatty Acid (FFA) Content

Principle: The FFA in oil is estimated by titrating the oil against KOH in the presence of a phenolphthalein indicator.

Procedure: Acid values and free fatty acid contents of oil samples were determined

according to the Ca 5a-40 method (AOCS, 1998) with few modifications. Each oil sample was weighed 1 – 10g and mixed with 50 mL of freshly neutralized hot solvent (a mixture of diethyl ether and ethanol, 1:1) in the 250 mL Erlenmeyer flask. The mixture was heated for 30 minutes in a water bath (75-80°C). After cooling, three drops of phenolphthalein indicator (1%) were added to the mixture. The mixture was titrated with 0.1 N alcoholic potassium hydroxide solution, shaking vigorously during the titration. The endpoint was recorded when the colour changed from colourless to light pink (persisting for 15 minutes). All experimental procedures were performed in triplicate. The acid values of the oil samples were calculated using the equation.

Calculation:

$$\boldsymbol{Acid\ Value} = \frac{\boldsymbol{56.1 \times V \times N}}{\boldsymbol{W}}$$

Where 56.1 = Molecular weight of the KOH (g/mol)

V = Volume of potassium hydroxide solution (mL)

N = Normality of potassium hydroxide solution

W = Weight of oil sample (gram)

The acid value was expressed as mg of KOH required per gram of oil sample

Free fatty acid contents in oil samples were calculated using the equation below. Free fatty acid was expressed as % oleic acid equivalents in the one gram of oil sample.

$$\boldsymbol{Free\ fatty\ acid\ content}\ (\%\ \boldsymbol{Oleic\ acid/g\ \ oil}) = \frac{\boldsymbol{Acid\ Value}}{\boldsymbol{2}}$$

3.4.4. p -Anisidine Value (p-AV)

Principle: p- AV is a parameter used to assess the amount of secondary oxidation product (2-alkenes, 2,4-denials) of oils and fats. A schiff's base is formed when aldehyde reacts with the p-anisidine amine group, and it absorbs at 350 nm.

Procedure: The p- anisidine value was determined by standard method according to AOCS p2.4 (AOCS, 1998). Oil (2g) was dissolved in 25 mL isooctane. Absorbance

(A1) of this solution was measured at 350 nm against blank isooctane. An aliquot (5 mL) of this solution, 5 mL of isooctane (as blank) was transferred to each of the two test tubes, and 1 mL anisidine solution (0.25% g/v in glacial acetic acid) was added to each. After 10 minutes, the absorbance A2 was measured at 350 nm against isooctane containing p-anisidine.

Calculation:

The p-AV is determined as;

$$\boldsymbol{p - AV = 25 \times 1.2 \times \frac{(A2 - A1)}{W}}$$

Where W = weight of the sample.

3.4.5 Thiobarbituric Acid Reactive Substances (TBARS) Assay

Principle: In accordance with the procedure by Zeb and Ullah, (2016), the TBARS assay was carried out using MDA equivalents derived from 1,1,3,3 tetra ethoxy propane (TEP). Lipid carbonyls are produced when oils under thermal oxidation, react with thiobarbituric acid, resulting in the development of a pink-coloured complex. The intensity can be measured as the absorbance level at 532 nm.

Procedure: One gram of oil sample was weighed and dissolved into glacial acetic acid and made up to a standard volumetric flask of 25 mL. An equal volume of oil sample and thiobarbituric acid reagent (TBA, 4 mM, 57.66 mg in 100 mL of glacial acetic acid) was mixed and transferred into a dry test tube. The sample- TBA mixture was then heated for an hour at 95°C in a water bath. At 532 nm, the absorbance of the pink-coloured complex was measured using a blank containing TBA reagent with glacial acetic acid. Concentrations of TBARS were determined from a standard curve of malonaldehyde (MDA). A stock standard TEP solution (3mM) was prepared by adding 75 µL of TEP to 100 mL of 0.1N HCl and then heating it for 10 minutes to hydrolyze the TEP. This hydrolyzed stock solution was subsequently used to prepare a 1mM

MDA standard solution in glacial acetic acid, with the concentration expressed as µg MDA/ mL of oil.

Calculation:

$$\boldsymbol{TBARS}\ (\mu \boldsymbol{g\ MDA/mL\ \ of\ oil}) = \frac{(\boldsymbol{Ac} \times \boldsymbol{V})}{\boldsymbol{W}}$$

Where Ac is the amount of MDA equivalent from the standard graph

V = Dilution Factor

W = Weight of Oil

3.4.6 Fourier Transform Infrared (FT-IR) spectroscopy

FTIR was used to measure the IR spectrum of oils by Perkin Elmer Instrument. Approximately 20 µL of edible oil was placed on the top of the potassium bromide polished baseplate using a micropipette, maintained at a temperature of 30°C during the procedure. All infrared spectra were recorded in absorbance mode in the range of 500–4,000 cm^{-1}. For each measurement, 128 interferograms were co-added at a resolution of 4 cm^{-1} and the overall time for the collection of interferograms was approximately 2 min. All measurements have been repeated three times.

3.4.7 Fatty acid Methyl Ester (FAME) Analysis

The gas chromatograph GC-7890 (Agilent, USA) with an FID detector and capillary column (60 mm × 0.25 mm, with 0.25 µm) was used to quantify the fatty acid composition of oils. FAME was synthesized using a modified method by O'Fallon et al., (2007). The oil sample (40 µL) was mixed with 6 mL of 10 N methanolic KOH and incubated in a 55°C water bath for 1.5 hr with vigorous shaking for 5s within 20 minutes. The mixture was incubated for 1.5 hours after 0.58 mL of 24 N sulphuric acid was added. Following the synthesis of FAME, 3 mL of hexane was added, vortexed thoroughly, and allowed the phases to separate. FAME in the hexane layer was collected by evaporation under nitrogen gas. A volume of 1 µL of FAME and helium

was injected into the GC column. Methyl esters were analyzed by comparing the retention time and peak area of the unknown fatty acid with known FAMEs. The fatty acid composition was expressed as a percentage of total fatty acid content.

3.5 Determination of Glycaemic Response

3.5.1 Fasting Blood Glucose (FBS) level

Principle: Fasting blood glucose was determined according to manufacturer instructions (Euro Diagnostic Systems Pvt Ltd). FBS was estimated using the glucose oxidase peroxidase (GOD POD) method. Hydrogen peroxide (H_2O_2) is a by-product of the oxidation of glucose to gluconate, which is catalyzed by the GOD enzyme. The POD enzyme then catalyzes the reaction between H_2O_2 and a chromogen solution, resulting in a coloured compound, which was measured at a wavelength of 505 nm spectrophotometrically. The intensity of the coloured compound is directly proportional to the concentration of glucose in the sample which can be compared with the known absorbance value of a standard.

$$\boldsymbol{Glucose + O_2 + H_2O_2 \xrightarrow{GOD} Gluconic\ acid + H_2O_2}$$

$$\mathbf{H_2O_2 + Phenol + 4AP \xrightarrow{POD} Red\ Quinonimine + 4H_2O}$$

Where, 4AAP - 4 Amino Antipyrine

Procedure: In each tube, 1 mL of reagent was mixed with10 µL of serum and standard, the tube was then incubated for 15 minutes at 37°C. A spectrophotometer set at 505 nm was used to measure the colour developed against the glucose reagent as blank. The formula was used to estimate the blood glucose concentration.

$$\textbf{Fasting blood glucose}\left(\frac{\textbf{mg}}{\textbf{dL}}\right) = \frac{\textbf{OD of Sample}}{\textbf{OD of Standard}} \times \textbf{Conc. of Standard (100mg/dL)}$$

3.5.2 Oral glucose tolerance test (OGTT)

Procedure: Wistar rats were fasted overnight (about 12 hours), and initial fasting glucose was measured using commercially available strips (One Touch Select, India).

Animals were then orally administered 2 g/Kg glucose via a polyethene gastric tube. At 30, 60, 90, and 120 minutes after glucose administration, blood was drawn from the tail vein and measured by a glucometer. The peak blood glucose was compared at all time points. The graph was plotted against the concentration of glucose (mg/dL) on the Y-axis and time (minutes) on the X-axis. The area under the curve over 120 min (AUC0-120 min) was also determined by using GraphPad Prism 9 Software.

3.5.3 Fasting plasma insulin level

Principle: Rat insulin was determined by the sandwich ELISA method. The insulin in the sample binds to an immobilized antibody on a solid surface followed by the addition of a biotinylated detection antibody that targets different epitopes on the insulin. Then streptavidin-HRP conjugate was added into the well, as an enzyme labelled. Well, turned blue colour upon adding the substrate to each well containing the Rat INS, biotinylated detection antibody and HRP conjugate. The enzyme-substrate reaction was halted by adding a stop solution. Using a microplate reader, the absorbance was then measured at a wavelength of 450 nm.

Procedure: A pre-coated ELISA plate was procured from Origin Diagnostic and Research Centre (Kerala, India). In each well, 40 µL of haemolysis-free plasma samples, 50 µL of streptavidin-HRP conjugate, and 10 µL of anti-INS antibody were dispensed and incubated at 37°C for 60 minutes. Following incubation, all the components that had not been attached to the wells were removed by washing with wash buffer (300 µL). A mixture of totalling 100 µL, of chromogen solutions A and B, was added to each well, then incubated for a further 15 minutes. The reaction was subsequently halted by adding 50 µL of stop solution. The colour that developed was read at 450 nm using a microplate reader within 10 minutes. The concentration of insulin was calculated as follows;

$$Conc.of\ insulin\ \left(\frac{mIU}{L}\right) = \frac{OD\ of\ sample}{Slope\ of\ the\ standard\ curve}$$

3.5.4 Calculation of HOMA indices (Levy et al., 1998)

The Homeostatic Model Assessment (HOMA) indices are used to estimate insulin resistance. The most common HOMA indices are HOMA-IR (insulin resistance) and HOMA S (insulin sensitivity). These indices are calculated using fasting insulin and glucose levels.

$$\textbf{HOMA-IR (Insulin Resistance)} = \frac{\textbf{Fasting plasma glucose (mg/dL)} \times \textbf{Fasting plasma insulin (mIU/L)}}{\textbf{405}}$$

$$\textbf{HOMA-S (Insulin Sensitivity)} = \frac{\textbf{1}}{\textbf{HOMA IR}} \times \textbf{100}$$

$$\textbf{HOMA B\% (β cell function)} = \frac{\textbf{20} \times \textbf{Fasting plasma insulin (mMol/L)}}{\textbf{Fasting plasma glucose (mM)- 3.5}}$$

3.6 Coagulation Factors

3.6.1 Platelet Count

The blood samples were thoroughly mixed for 10 minutes using an automated mixer. After mixing, a complete automated blood count was performed using the Mindrary BC 20s, a 3-part differential haematological analyzer. This machine counts various blood cells, including white blood cells, platelets, and red blood cells. It may provide other important information about the blood sample, like the haemoglobin concentration and haematocrit levels.

3.6.2 Bleeding Time Test

Principle of Bleeding Time test: The bleeding time test assesses how long it takes the time for blood to stop flowing from a small incision in the rat's tail. It assesses primary haemostasis, which includes platelet function and blood vessel integrity.

Procedure: Bleeding time was measured using Duke's method. The rats were fasted for a short period (2-4 hours) before the test to avoid interference from food. The tail

tip was disinfected with an alcohol swab; a small incision was made horizontally across the tail tip using a scalpel blade. Immediately blot the incision site with a pre-weighed filter paper, allowing the blood to soak into the paper. Every 30 sec, a filter paper touched the puncture site without pressing or squeezing the wound. This process was continued as the blood spots gradually became smaller and eventually disappeared completely. The time point at which the bleeding stopped was recorded. The number of blood spots observed on the filter paper was then counted and divided by two. This provided the bleeding time in minutes.

3.6.3 Determination of Clotting Time

Principle of clotting Time: The clotting time test calculates how long the time required for blood to form a stable clot in a test tube. It assesses the effectiveness of the coagulation cascade, including factors like fibrinogen and platelets.

Procedure: It was performed by placing one end of the capillary tube on a drop of blood and tilting it downwards until blood was filled in the tube (about 10 cm). After two minutes, small lengths of the tube were broken at 30-second intervals to observe the clot formation. Initially, the blood column broke cleanly, but eventually, a thick strand or blood clot stretched between the broken ends. The appearance of this strand marked the clotting time. The total time was measured from when blood started to flow to when the fibrin strand appeared.

3.6.4 Prothrombin Time (PT time) Test

Prothrombin time was done using Quick's one-stage method (Quick and Stanley-Brown, 1935). The prothrombin time test assesses the time required for plasma to clot when specific reagents are added. Blood was collected from the animals and was transferred into a vacuum tube containing sodium citrate. After centrifugation, the supernatant plasma was removed, placed in a clean glass tube, and mixed with a reagent containing calcium ions and thromboplastin. A stopwatch was started and observed.

The timer was stopped when the clot formed, and the time was recorded in seconds

3.6.5 Activated Partial Thromboplastin Time (APTT) Test

Activated partial thromboplastin time (APTT) was determined by using Agape Diagnostics Ltd. (Kerala, India) in accordance with the standard and protocol given by the manufacturer. The APTT is the most widely used method for monitoring the ability of blood to form a fibrin clot through the intrinsic haemostatic pathway. This process depends on several coagulation factors I, II, V, VIII, IX, X, XI and XII, along with platelet lipids and calcium. Blood was collected from the animals, transferred to a 3.8% sodium citrate-containing vacutainer, mixed well, and centrifuged at 2500 pm for 15 minutes. Plasma was collected immediately after the centrifugation. APTT reagent (100 µL) was mixed with 100 µL of plasma and incubated for 3 minutes at 37°C. After this incubation, 100 µL of the preincubated calcium chloride (0.02 M) was added and record the clotting time after the clot was formed.

3.7 Analysis of Systemic Toxicity

3.7.1 Liver function parameters

3.7.1.1 Total Bilirubin

Principle: Total bilirubin is measured by coupling bilirubin with 3,5-dichloro phenyl diazonium in a strongly acidic medium in the presence of a solubilizing agent to form a pink-coloured complex. Total bilirubin encompasses two forms: unconjugated (indirect) bilirubin, bound to albumin and water-insoluble. Another form is conjugated (direct) bilirubin is water soluble and excreted in bile.

Procedure: Determination of total bilirubin was done using a kit from Euro Diagnostic Systems Pvt. Ltd. (Tamil Nadu, India), as per standard protocol given by the manufacturer. For the test, 50 µL of serum, 50 µL of diazo A and B reagents, and 1 mL of caffeine were added. For the test blank, 50 µL of serum, 50µL of diazo A and 1 mL of caffeine were added. Measured the absorbance at 546 nm against distilled water after

incubation at room temperature for 2 minutes.

Calculation

$$\boldsymbol{Concentration\ of\ Bilirubin\ (mg/dL)}$$

$$\boldsymbol{= (OD\ of\ the\ test - OD\ of\ Blank) \times 26\ (\ Factor)}$$

3.7.1.2 Total Protein (Biuret reaction)

Principle: This method relies on the reaction of protein with copper ions in an alkaline solution, resulting in a blue-violet colour (Zheng et al., 2017). The intensity of the colour is directly proportional to the total protein content in the sample.

$$\boldsymbol{Protein + Cu^{++} \rightarrow Blue\ Violet\ Colored\ Complex}$$

Procedure: Determination of total protein by using a kit from Euro Diagnostic System Pvt Ltd (Tamil Nadu, India) in accordance with the standard protocol given by the manufacturer. Pipetted 10 µL of serum, distilled water, and standard into the respective test, blank and standard test tube. Each tube received, 1 mL of biuret reagent, which was then thoroughly mixed, and incubated at 37°C for 10 minutes at 555 nm, the absorbance of the test and standard measured within 60 minutes against the blank.

Calculation

$$\boldsymbol{Total\ Protein\ \left(\frac{g}{dL}\right) = \frac{Abs\ of\ Test}{Abs.Of\ Sample} \times 6g/dL}$$

3.7.1.3 Albumin

Principle: Estimation of serum albumin is based on the principle of protein binding with a dye, such as bromocresol green (BCG). In the slightly acidic pH, Serum albumin reacts with BCG and turns a green-blue from a yellow-green colour.

Procedure: Determination of albumin was performed by using a kit from Euro Diagnostic System Pvt Ltd. (Tamil Nadu, India) in accordance with the standard protocol given by the manufacturer. Bromocresol green (BCG) reagent (0.26 mmol/L) with succinate buffer (90 mmol/L) mixed with serum (10 µL) for test and 10 µL of

distilled water for blank and incubated at 5 minutes. Absorbance was read at 623 nm against reagent blank within 60 minutes.

Calculation

$$\boldsymbol{Albumin}\left(\frac{\boldsymbol{g}}{\boldsymbol{dL}}\right) = \frac{\boldsymbol{Ab\ of\ Sample}}{\boldsymbol{Ab\ Std}}\ \boldsymbol{X\ Conc.Std}\ (\frac{\boldsymbol{g}}{\boldsymbol{dL}})$$

3.7.1.4 Serum Glutamate Pyruvate Transferase (SGPT)

Principle: Alanine aminotransferase (ALT/ SGPT) catalyzes the conversion of alanine to α-ketoacid with the concomitant reduction of NAD to NADH. The increase in NADH concentration was monitored spectrophotometrically at a specific wavelength of 340 nm. The rate of change in absorbance is directly proportional to the SGPT activity in the sample.

Procedure: Determination of SGPT was measured by using a kit from Euro Diagnostic System Pvt Ltd. (Tamil Nadu, India) in accordance with the standard protocol given by the manufacturer. The serum sample (50 µL) was mixed with 1mL of working reagent and incubated for 1 minute. Measured the change in absorbance at 340 nm for 3 minutes and recorded the initial and final absorbance values for each sample. Distilled water was used as blank for setting zero.

Calculation:

$$\boldsymbol{SGPT\ activity}\ (\boldsymbol{U/L}) = \boldsymbol{\Delta A/min} \times \mathbf{1746}$$

3.7.1.5 Serum Glutamate Oxaloacetate Transferase (SGOT)

Principle: SGOT (AST/ Aspartate transaminase) facilitates the transfer of an amino group from aspartate to oxaloacetate, which is accompanied by the reduction of NADH and the production of glutamate and α-ketoglutarate. The reaction is monitored kinetically at 340 nm by observing the rate of decrease in NADH concentration. The rate at which the absorbance changes is directly proportional to the SGOT activity in the sample.

Procedure: Determination of the activity of SGOT was measured by using a kit from Euro Diagnostic System Pvt Ltd. (Tamil Nadu, India) in accordance with the standard protocol given by the manufacturer. The spectrophotometer was zeroed using distilled water as a blank. A 50µL serum sample was then mixed with 1mL of working reagent and incubated for 1 minute. The change in absorbance was measured for 3 minutes at 340nm and recorded the initial and final absorbance values for each sample.

Calculation:

$$\boldsymbol{SGOT\ activity\ (U/L)\ =\ \Delta A/min \times 1746}$$

3.7.1.6 Determination of Alkaline Phosphatase Activity

Principle: Alkaline phosphatase (ALP) catalyzes the hydrolysis of p-nitrophenyl phosphate at an alkaline pH of 10.4, resulting in the generation of p-nitrophenol and inorganic phosphate. The activity of ALP was determined by measuring the rate of production of p-nitrophenol spectrophotometrically.

$$\boldsymbol{p-\ Nitrophenylphosphate\ +\ H_2O \xrightarrow{ALP} P-nitrophenol + Phosphate}$$

Procedure: Determination of the ALP activity was performed by using a kit from Euro Diagnostic System Pvt Ltd (Tamil Nadu, India) strictly following the standard protocol given by the manufacturer. The spectrophotometer was adjusted to zero with distilled water as blank. The serum sample (20µL) was mixed with 1mL of working reagent and incubated for 1 minute. The change in absorbance was recorded for 3 minutes at 405 nm.

Calculation:

$$\boldsymbol{ALP\ activity\ (U/L)\ =\ \Delta A/min \times 2746}$$

3.7.2 Renal Function Test

3.7.2.1 Determination of Serum Creatinine (Toora and Rajagopal, 2002)

Principle: The creatinine present in the sample forms a red-orange complex when it

reacts with alkaline picric acid. The colour intensity is directly proportional to the concentration of creatinine. It was measured at 520nm by using UV-Visible spectrophotometer.

$$\textbf{Creatinine} + \textbf{Sodium picrate} \xrightarrow{\textbf{Alkali}} \textbf{Creatinine Picrate Complex}$$

Procedure: The concentration of creatinine was determined by using a kit from Euro Diagnostic System Pvt Ltd (Tamil Nadu, India) in accordance with the standard protocol given by the manufacturer. Standard and test (50 µL) was pipetted into each test tube, and mixed with 1 mL of reagent. Read the absorbance of standard(S) and test (T) against distilled water at 520 nm (505-570nm) after 5 minutes of incubation at room temperature.

Calculation

$$\boldsymbol{Serum\ creatinine(mg/dL)} = \frac{\boldsymbol{A_T}}{\boldsymbol{A_S}} \times \mathbf{2}(\boldsymbol{Standard\ Conc.})$$

Where, A_S – Absorbance of sample

A_T – Absorbance of test

3.7.2.2 Determination of Blood Urea Nitrogen

Principle: Urea is enzymatically hydrolysed to ammonia and carbon dioxide by the action of the enzyme urease. In an adapted procedure, ammonium ions react with α-Ketoglutarate and NADH to yield glutamate and NAD. This reaction is quantified by monitoring the rate of NADH oxidation to NAD and is determined by measuring the reduction in absorbance at 340 nm. The decrease in absorbance is directly proportional to the urea concentration in the sample.

Procedure: Determination of urea was using a kit from Euro Diagnostic System Pvt Ltd (Tamil Nadu, India) following the manufacturer's prescribed protocol; the working reagents were reconstituted in the ratio 4:1. Subsequently, 10µL of serum and standard were added to separate test tubes. Following this, 1mL of the reconstituted working

reagent was added to each test tube. The absorbance of each tube was immediately recorded at 340nm after precisely 30 sec (A1) and 60 sec (A2).

Calculation:

$$\Delta A = A1 - A2$$

$$Serum\ Urea\ \left(\frac{mg}{dL}\right) = \frac{\Delta A(sample)}{\Delta A(Standard)} \times 50\ (Conc.Std)$$

3.8 Lipid Metabolism

3.8.1 Determination of Lipid Profile

3.8.1.1 Determination of Serum Total Cholesterol

Principle: The Cholesterol Oxidase-Peroxidase (CHOD-POD) method is a widely used enzymatic assay for quantifying total cholesterol levels in serum samples. Cholesterol esters in the serum are cleaved by cholesterol esterase (CHE) to form cholesterol and fatty acids. Cholesterol oxidase (COD), subsequently oxidized cholesterol resulting in the formation of cholest 4en- 3-one and hydrogen peroxide (H_2O_2). The generated H_2O_2 reacts with amino antipyrine and phenol in the presence of peroxidase (POD), resulting in a red chromophore.

$$\mathbf{Cholesterol\ ester + H_2O \xrightarrow{CHE} Cholesterol + Fatty\ acid}$$

$$\mathbf{Cholesterol + O_2 \xrightarrow{CHOD} Cholesterol - 3 - one + H_2O_2}$$

$$\mathbf{2H_2O_2 + Phenol + 4 - AP \xrightarrow{POD} Quinoneimine + 4\ H_2\ O}$$

Procedure: Determination of cholesterol by using a kit from Euro Diagnostic System Pvt Ltd (Tamil Nadu, India) in accordance with the standard protocol given by the manufacturer. Approximately, 1 mL of cholesterol oxidase peroxidase reagent was pipetted into the test tubes. Following this, 10µL of serum or cholesterol standard was added and incubated for 10 min at 15 - 25°C. Read the absorbance of the sample and standard at 505nm against a blank containing 1 mL reagent.

Calculation

$$\boldsymbol{Cholesterol}\left(\frac{\boldsymbol{mg}}{\boldsymbol{dL}}\right) = \frac{\boldsymbol{Absorbance\ of\ Sample}}{\boldsymbol{Absorbance\ of\ standard}} \times \boldsymbol{Standard\ Conc.}\left(\mathbf{200}\frac{\boldsymbol{mg}}{\boldsymbol{dL}}\right)$$

3.8.1.2 Determination of Triglycerides

Principle: The GPO-POD method is an enzymatic colourimetric assay that measures the triglyceride levels in serum and tissue samples. Triglycerides in the sample are hydrolyzed to produce glycerol and fatty acids by lipoprotein lipase. Glycerol is converted to glycerol 3 – phosphate (G3P) and adenosine – 5 – diphosphate (ADP) by glycerol kinase and ATP. G3P then oxidized using glycerol oxidase and H_2O_2. H_2O_2 reacts with 4 aminophenazone (4- AP) and p- chlorophenol in the presence of peroxidase (POD) to give a red-coloured dye.

$$\boldsymbol{Triglycerides + H_2O \xrightarrow{LPL} Glycerol + Fatty\ acid}$$

$$\boldsymbol{Glycerol + ATP \xrightarrow{Glycerol\ Kinase} G3P + ADP}$$

$$\boldsymbol{G3P + O_2 \xrightarrow{GPO} DAP + H_2O_2}$$

$$\boldsymbol{H_2O_2 + 4 - AP + p - Chlorophenol \xrightarrow{POD} Quinone + H_2O_2}$$

Procedure: Determination of concentration of triglycerides by using a kit from Euro Diagnostic System Pvt Ltd. (Tamil Nadu, India) in accordance with the standard protocol given by the manufacturer. Pipetted out 10 µL of the serum or standard into a clean test tube. About 1 mL of the GPO-POD reagent was added to the test tube and thoroughly mixed. After allowing the mixture to incubate at room temperature for 10 minutes, the absorbance was measured at 505 nm using a spectrophotometer.

Calculation

$$\boldsymbol{Triglycerides}\left(\frac{\boldsymbol{mg}}{\boldsymbol{dL}}\right) = \frac{\boldsymbol{Absorbance\ of\ Sample}}{\boldsymbol{Absorbance\ of\ Standard}} \times \boldsymbol{Standard\ Conc.}\ (\boldsymbol{200\ mg/dL})$$

3.8.1.3 Determination of HDL – Cholesterol

Principle: The enzymatic method for determining serum and tissue HDL cholesterol is based on the hydrolysis of HDL-cholesterol esters by cholesterol esterase in the presence of other lipoprotein particles. The resulting free cholesterol is oxidized by cholesterol oxidase to produce hydrogen peroxide, which is then detected through a peroxidase reaction and results a blue-coloured complex that can be measured spectrophotometrically.

Procedure: Determination of the concentration of HDL-cholesterol by using a kit from Euro Diagnostic System Pvt Ltd (Tamil Nadu, India) in accordance with the standard protocol given by the manufacturer. Pipetted 10 µL of the serum or standard into clean test tubes, followed by 1 mL of the reagent 1 (R1) and mixed well. The mixture was then incubated for 10 minutes at room temperature. Added 340µL of reagent 2 (R2) and incubated for 5 min at 37°C. The absorbance of the mixture was measured at 550 - 650 nm.

Calculation

$$\boldsymbol{HDL\ cholesterol}\ (\boldsymbol{mg/dL}) = \frac{\boldsymbol{Absorbance\ of\ Sample}}{\boldsymbol{Absorbance\ of\ standard}} \times \boldsymbol{Standard\ Conc.}\ (\boldsymbol{200\ mg/dL})$$

3.8.1.4 Determination of LDL – Cholesterol

Principle: The concentration of LDL cholesterol is determined using the CHOD-PAP method. Cholesteryl esters were hydrolysed and oxidation of the 3-OH group of cholesterol by-cholesterol esterase enzyme, which produces H_2O_2 as a by-product. The

H_2O_2 is quantitatively measured in a peroxidase-catalyzed reaction that has a colour. The intensity of the colour is proportional to the concentration of cholesterol.

Procedure: Determination of the concentration of LDL cholesterol by using a kit from Euro Diagnostic System Pvt Ltd (Tamil Nadu, India) using the standard protocol given by the manufacturer. Pipetted out 20 µL of the serum or standard into a clean test tube. About 900 µL of reagent 1 (R1) was added to the test tube and mixed thoroughly. The mixture was then incubated at room temperature for 10 minutes. Following this, 340µL of reagent 2 (R2) was added and the mixture was incubated for an additional 5 min at 37°C. The absorbance of the mixture was measured at 550- 650 nm using a spectrophotometer.

Calculation

$$\boldsymbol{LDL\ cholesterol\ (mg/dL)} = \frac{\boldsymbol{Absorbance\ of\ Sample}}{\boldsymbol{Absorbance\ of\ standard}} \times \boldsymbol{Standard\ Conc.\ (200\ mg/dL)}$$

3.8.1.5 Determination of VLDL-C (Friedewald et al., 1972)

The Friedewald's formula is used to estimate the concentration of very-low-density lipoprotein cholesterol (VLDL-C) in the blood, a component of the total cholesterol calculation.

Calculate the VLDL-Cholesterol using the Friedewald's formula:

VLDL-C(mg/dL) = (Triglycerides / 5).

3.8.2 Extraction of Hepatic Lipids (Folch et al., 1957)

Principle: Hepatic lipids were extracted using a modified Folch method. This method relies on the differential solubility of lipids in organic solvents (chloroform) and water-miscible solvents (methanol). Lipids are extracted into the organic phase due to their hydrophobic nature when tissue is homogenized in a chloroform and methanol mixture. Subsequent centrifugation after the addition of water creates a phase separation,

allowing the lipids to partition into the lower chloroform phase. The filtered chloroform phase contains the extracted lipids, including triglycerides, phospholipids, and cholesterol.

Procedure: Hepatic tissue (typically 0.5-1g) was homogenized with 20 volumes of 2:1 chloroform/methanol solution. The homogenate was centrifuged at 2500 x g for 10 minutes to remove solid tissue debris. The resulting filtrate was mixed with 10 volumes of distilled water, allowing it to separate into two layers. The lower chloroform phase containing the lipids was collected and evaporated off the solvent under vacuum using a rotary evaporator. The dried lipid residue was weighed, and calculated for the lipid content of the hepatic tissue. The lipid residue was dissolved in 1mL of an alcohol: acetone (1:1) mixture and stored in a -20°C freezer for the subsequent determination of hepatic cholesterol and phospholipid content.

3.8.3 Estimation of Hepatic Cholesterol

Determination of hepatic cholesterol was done by a modified method of Zak's (Jeksy Jos et al., 2015). A lipid extract was dried and mixed with alcohol: acetone (1:1). From this mixture, 0.5 mL of the lipid extract was combined with 2.5 mL of 0.05% $FeCl_3$ - acetic acid reagent and 2.5 mL of sulfuric acid. The test tube was shaken and kept in a water bath at 50°C for 10 minutes. The absorbance was measured at 550 -570 nm against a blank containing reagent solution without lipid extract. The concentration of hepatic cholesterol was calculated in the standard graph of cholesterol.

Calculation

$$\boldsymbol{Cholesterol\ (mg/g\ tissue)} = \frac{\boldsymbol{Conc.of\ cholesterol\ From\ standard\ Graph \times 1000}}{\boldsymbol{Weight\ of\ tissue\ (g) \times Volume\ of\ sample\ (ml)}}$$

3.8.4 Estimation of hepatic Phospholipid (Mosmuller et al., 1992)

The hepatic lipid extract was digested with 1 mL of 5N H_2SO_4 for 3 hours in a warm

water bath. When charring occurred, one drop of 2N HNO_3 was added and digested till it became colourless. Pipetted out different volumes of standard and made up to 1mL with 5N H_2SO_4. A blank containing 1 mL water was also taken. Cooled the digestion tube and added 1 mL of digested extract, 1mL of a 2.5% ammonium molybdate solution (prepared by dissolving 2.5g in 3N H_2SO_4) were added. The mixture was incubated for 10 minutes at room temperature. Subsequently, 0.4mL of ANSA reagent (a mixture of 14.625g sodium metabisulphite, 500 mg sodium sulphite and 250 mg 1-amino-2-naphthol-4-sulfonic acid (ANSA) dissolved in 100 mL distilled water) was added to the digestion tube. The absorbance was read at a wavelength of 640 -660 nm after incubating for an additional 20 minutes at room temperature, using a blank for reference. The phospholipid content of the liver tissue was assessed by comparing the absorbance of the sample to a standard curve created with known concentrations of inorganic phosphorus.

3.8.5 Determination of HMG Co. A reductase Activity (Rao and Ramakrishnan, 1975)

The HMG-Co. A reductase activity was determined by measuring the HMG-Co. A/mevalonate ratio method described by Rao and Ramakrishnan (1975). An equal volume of liver tissue homogenate (10%) was mixed with perchloric acid and centrifuged at 2000 rpm for 10 min. The supernatant (1 mL) was mixed with 0.5 mL of freshly prepared hydroxylamine reagent, pH 5.5 (In the case of HMG-Co. A) and pH 2.1 (in the case of mevalonate). 1.5 mL of ferric chloride reagent was added to both tubes after 5 min, mixed well, and absorbance was taken within 15 min at 540 nm against similarly treated saline arsenate (1g of sodium arsenate/L of saline). The ratio of HMG Co. A to mevalonate was calculated. A higher ratio signifies lower enzyme activity, whereas a lower ratio indicates higher enzyme activity.

3.8.6 Determination of Hepatic Lipase Activity (Shirai and Jackson, 1982)

Principle: p-Nitrophenyl butyrate is hydrolysed by lipase enzyme to form p-nitrophenol and butyric acid. Increased absorbance of p-nitrophenol was detected at 400nm.

$$\boldsymbol{p-Nitrophenyl\ butyrate\ +\ H_2O \xrightarrow{Lipase} p-Nitrophenol + Butyric\ acid}$$

Enzyme solution (0.1 mL) was pipetted out into a 1.0 mL reaction mixture that contained 0.099M sodium phosphate buffer (pH 7.2), 0.5% (v/v) Triton X-100, 0.149M sodium chloride, 0.50 mM p-nitrophenyl butyrate (PNPB), 1% (v/v) acetonitrile in the final volume. The hydrolysis of PNPB was measured by monitoring the increase in absorbance at 400 nm.

Calculation

$$\boldsymbol{Units/mg\ protein}$$

$$= \frac{(\Delta A_{400nm}\ /\ \mathbf{min}\ of\ test - \Delta A_{400nm}\ /\ \mathbf{min}\ of\ Blank) \times 1.01 \times d.f}{0.0148 \times 0.1}$$

df = Dilution Factor

1.01 = Total Volume (in millilitres)

0.1 = Volume (in milliliter) of enzyme

0.0148 = micromolar extinction coefficient of nitrophenol at 400nm

3.9 Carbohydrate Metabolism assays

3.9.1 Isolation of Mitochondria (Clayton and Shadel, 2014)

The liver tissues of experimental rats were harvested immediately and placed on ice to preserve the integrity of the mitochondria. After removal of blood and connective tissue, 1g of liver was minced into 1-2 mm pieces and homogenized in 10 mL of ice-cold mitochondria suspension (MS) homogenizing buffer (comprising 1mM EDTA, 70mM sucrose, 210mM Mannitol, 5mM Tris-HCl pH 7.5). The homogenate underwent centrifugation at 1300g for 10 minutes to pellet the nuclei and other debris, yielding the supernatant (S1). Subsequently, S1 was further centrifuged at 600-1300g for 10

min, to obtain supernatant (S2). S2 was then centrifuged at 7000-17000g for 15 minutes to pellet the mitochondria. The supernatant was discarded, and the mitochondrial pellet was washed twice with MS homogenizing buffer at 7000-17000g for 15 minutes.

3.9.2 Mitochondrial purification

Mitochondrial purification was done by sucrose density gradient centrifugation. A 3 mL gradient was prepared by layering 1.5 mL of 1M sucrose solution over 1.5 mL of 1.5M sucrose solution in ultra-centrifuge tubes. The collected mitochondrial suspension was loaded above the sucrose layers and centrifuged at 60,000g for 20 minutes. Mitochondria were gently collected from the top layer of the 1.5M sucrose solution. The mitochondrial suspension was diluted with 5mM Tris HCl/1mM EDTA, washed with MS homogenizing buffer, and re-suspended with Tris-HCl/EDTA buffer. The resulting mitochondrial suspension was promptly stored at -80°C for subsequent analysis.

3.9.3 Fructose 1,6 bis Phosphatase (Gancedo and Gancedo, 1971)

Principle: Fructose 1,6-bisphosphatase (FBPase) activity was assessed by monitoring the release of inorganic phosphate. The enzyme catalyzes the conversion of fructose 1, 6-bisphosphate to fructose 6-phosphate and inorganic phosphate. The released inorganic phosphate was quantified using colorimetric methods.

Procedure: In this assay, a final volume of 2 mL was prepared with, 1.2mL of 0.1M Tris HCl buffer (pH 7), 0.001M EDTA (0.25 mL), 0.1M Magnesium Chloride (0.25 mL), 0.05M Fructose-1,6-diphosphate (0.1 mL), 0.1M Potassium Chloride (0.1 mL), and 0.1 mL of tissue homogenate (10%). The reaction was incubated at 37°C for 15 minutes and terminated by adding 1mL of 10% trichloroacetic acid (TCA). After centrifugation, the phosphorus content in the supernatant was determined using the Fiske and Subbarow method (1925). Enzyme activity was quantified as nanomoles of inorganic phosphate (Pi) released per minute per milligram of protein.

Calculation

$$\boldsymbol{Unit\ Enzyme\ activity(ip\ liberated/min/mg\ protein)}$$

$$= \frac{\boldsymbol{A_{640\ nm} \times T\ V\ (2ml) \times D\ F}}{\boldsymbol{Slope \times T\ (minutes) \times E\ V \times M\ W \times mg\ protein}}$$

T V = Total volume

D. F = Dilution Factor

Slope = From Standard Graph

E V = Enzyme Volume

M W = molecular weight of inorganic phosphate

3.9.4 Isocitrate dehydrogenase Activity (Kim et al., 2003)

Principle: Isocitrate dehydrogenase activity was monitored by the conversion of isocitrate to alpha-ketoglutarate by following the change in absorbance at 340 nm. The decrease in absorbance corresponds to the reduction of NAD+ to NADH during the reaction.

Procedure: The isocitrate dehydrogenase activity was assessed using Kim's modified method. The assay mixture composed of 0.1 M Tris HCl (0.88 mL) pH 8, 0.01 M NADP (0.04mL), 0.1 M $MnCl_2$ (0.04 mL), and 0.02 mL of 0.025 M isocitrate solution in a final volume of 0.98 mL. Absorption was read at 340nm at 25 °C after the addition of 0.02 mL of tissue homogenate.

Calculation

$$\boldsymbol{Enzyme\ activity}\ \left(\frac{\boldsymbol{U}}{\boldsymbol{L}}\right) = \frac{\boldsymbol{\Delta A_{340nm} \times TV\ (1mL)}}{\boldsymbol{min \times 6.22 \times E\ V\ (0.02mL)}} \times \boldsymbol{DF}$$

T V = Total reaction volume of test

E V = Enzyme Volume

Molar extinction coefficient, εNADPH at 340nm = 6.22×10^3 $M^{-1}cm^{-1}$

3.9.5 Succinate Dehydrogenase Activity (Slater and Borner, 1952)

Principle: Succinate dehydrogenase (SDH) activity was assessed through the reduction of the artificial electron acceptor 2,6-dichlorophenolindophenol (DCPIP). SDH facilitates the oxidation of succinate to fumarate while concurrently reducing ubiquinone to ubiquinol. SDH transfers electrons from succinate to oxidized DCPIP, leading to the reduction of DCPIP. Upon receiving electrons from succinate, the oxidized form of DCPIP (initially blue) is transformed into its reduced form (colourless**).** The reduction of DCPIP was monitored spectrophotometrically at 600 nm.

$$\boldsymbol{Succinate} + \boldsymbol{DCPIP}\ (\boldsymbol{blue\ Colour}) \xrightarrow{\boldsymbol{SDH}} \boldsymbol{Fumarate} + \boldsymbol{Reduced\ DCPIP}\ (\boldsymbol{Colourless})$$

Procedure: The succinate dehydrogenase activity was assessed using the modified method of Slater & Borner (1952). The assay mixture was composed of 0.1M Sodium Succinate (0.1mL), 0.05% BSA (0.1mL), and 0.1mL of 0.03M $NaNO_3$ solution in a final volume of 0.9 mL with 0.2M phosphate buffer (pH 7.6). Mitochondrial solution (0.05mL) was added and incubated at 25°C. The absorption was read at 600 nm at 25°C after the addition of 0.05mL of DCPIP solution.

Calculation

$$\boldsymbol{Enzyme\ activity}\left(\frac{\boldsymbol{U}}{\boldsymbol{L}}\right) = \frac{\Delta \boldsymbol{A_{600nm}} \times \boldsymbol{TV}\ (\boldsymbol{1mL})}{\boldsymbol{min} \times \boldsymbol{20.7} \times \boldsymbol{E\ V}\ (\boldsymbol{0.05mL})} \times \boldsymbol{DF}$$

T V = Total reaction volume of test

E V = Enzyme Volume

Molar extinction coefficient, εDCPIP at 600nm = 20.7×10^3 $M^{-1}cm^{-1}$

3.9.6 Malate Dehydrogenase activity (Mehler et al., 1948)

Principle: Malate dehydrogenase (MDH) activity was determined by the measurement of the rate of oxidation of NADH. It catalyzes the reversible conversion of malate to oxaloacetate using the coenzyme NAD^+ (nicotinamide adenine dinucleotide) as a

cofactor. The reaction involves the reduction of NAD^+ to NADH.

Procedure: The MDH activity was assessed using the modified method of Mehler et al.,(1948). The assay mixture contained 0.895mL of 0.05M Tris HCl buffer, pH 6.8, 0.05mL of 0.2M malate, 0.025mL of 0.1M NAD^+, and 0.01mL of 0.1M $MnCl_2$ in a final volume of 0.98 mL in a cuvette. Mitochondrial solution (0.02mL) was added and incubated at 25°C. Absorption was read at 340 nm at 25°C. The increase in absorbance at wavelength of 340 nm is proportional to the malate dehydrogenase activity.

Calculation

$$\boldsymbol{Enzyme\ activity}\left(\frac{\boldsymbol{U}}{\boldsymbol{L}}\right)=\frac{\boldsymbol{\Delta A_{340nm}\times TV\ (1mL)}}{\boldsymbol{min\times 6.22\times E\ V\ (0.02mL)}}\times\boldsymbol{DF}$$

T V = Total reaction volume of test

E V = Enzyme Volume

Molar extinction coefficient, εNADH at 340nm = 6.22×10^3 $M^{-1}cm^{-1}$

3.9.7 Estimation of Inorganic Phosphate (Fiske and Subbarow, 1925)

Principle: The modified method of Fiske and Subbarow (1925) was used to estimate the inorganic phosphorus present in the protein-free supernatant. This technique relies on the reaction between phosphate ions and molybdic acid, which forms phosphomolybdic acid. The formed phosphomolybdic acid undergoes reduction and develops a dark blue colour, which is directly proportional to the concentration of phosphate ions in the sample.

Procedure: In this experiment, 1mL of ammonium molybdate reagent was added to the sample (1mL) and allowed to stand at room temperature for 10 minutes. Subsequently, 0.4 mL of ANSA (1-amino-2-naphthol-4-sulfonic acid) was added and the mixture was left for a further 20 minutes to allow the blue colour to develop. The optical density was quantified at 640-660 nm with the use of a spectrophotometer. The same procedure was applied to the blank and standard (potassium dihydrogen

phosphate (35.1mg), making up to 100 mL of water). The value obtained was expressed as µg of inorganic phosphate/mL of solution.

3.10 Inflammatory Markers

3.10.1 *Estimation of C- Reactive Protein (CRP)*

Principle: The rat CRP ELISA is a sandwich ELISA used to quantify the concentration of CRP in rat serum. This is based on the principle that CRP binds to a monoclonal antibody that is coated onto the wells of a microplate. A second monoclonal antibody conjugated to horseradish peroxidase (HRP) is then added forming a sandwich if CRP is present. The HRP then converts the substrate, 3,3',5,5'-tetramethylbenzidine (TMB), into a coloured product. The intensity of the colour was measured using a plate reader at 450nm; which is directly proportional to the amount of CRP in the sample.

Procedure: Serum CRP was determined using a commercial kit (Alpha International Diagnostic Kit) per the manufacturer's protocol. Specifically, 100µL of control, standards, and rat serum were added to the wells pre-coated with anti-rat CRP antibodies and allowed for the specific binding of CRP in the sample at room temperature for 1 hour. Wells were washed with 300 µL of wash buffer to remove any unbound components. Followed by the addition of 100 µL of anti-rat CRP-HRP conjugate to each well and incubated at room temperature for 30 minutes to bound with captured CRP. The second washing step was performed after incubation to eliminate unbound conjugate. Followed by, 100 µL of TMB substrate was added to each well, TMB reacted with HRP and turned blue colour. After 15 minutes of incubation, 100 µL of stop solution was added, changing the colour from blue to yellow indicating the reaction was halted. The absorbance of the wells was measured at 450 nm using a microplate reader within 30 minutes after the addition of the stop solution. The result was calculated from the standard curve plotted by the concentration of CRP on the X-axis against the corresponding optical density on the Y-axis.

3.10.2 Determination of Lipoprotein Associated Phospholipase A_2 (Lp-PLA_2) activity (de Araújo and Radvanyi, 1987)

Principle: Lp-PLA_2 is an enzyme that hydrolyses oxidized phospholipids, generating oxidized fatty acids and lysophosphatidylcholine. Hydroxylamine reacts with the product of Lp-PLA_2 activity to form a coloured nitrophenyl hydroxamic acid product. The Lp-PLA_2 activity in the sample is directly proportional to the intensity of the colour, which is measured using a spectrophotometer.

$$\boldsymbol{Lecithin \xrightarrow{Phospholipase\ A2} Lysolecithin + Fatty\ acid}$$

$$\boldsymbol{Lysolecithin + Hydroxilamine \rightarrow Colored\ product}$$

Procedure: Lp-PLA_2 was determined according to the modified method of Arujo et al. In a test tube, pipetted into 0.7mL 0.5mM Tris HCl (pH 8.5), 0.7 mL of Phosphatidylcholine (2% in 10mM $CaCl_2$), 0.7mL of 1.5% of deoxycholate solution, and 0.05mL of serum and incubated for 5 minutes. Then, 0.2mL of the test mixture was put into another test tube, 1.5mL of 25% ether, 0.2mL of 2M hydroxylamine solution, and 0.2mL of 14% NaOH solution were added, and incubated for 20 minutes. After incubation, 0.3mL of 3N HCl and 0.3mL of 10% FeCl3 were added, and the read absorbance was at 570nm. Blank was treated as the sample, and 0.05mL of 10mM $CaCl_2$ was used instead of the sample. Lp-PLA_2 activity was calculated from the standard graph by plotting the concentration of phosphatidyl Choline at the X-axis and absorbance at the Y-axis.

Calculation

$$\boldsymbol{Units/mg\ protein = \frac{A\ 570nm/minutes}{M \times 0.5 \times mgenzyme/ml\ of\ RM}}$$

M = slope of the standard curve

RM = reaction mix

0.5 = conversion factor for the formation of lysolecithin has one-half the absorbance of

lecithin at 570nm.

3.10.3 Determination of IL-6

Procedure: IL 6 levels were measured using an ELISA kit from Peprotech, USA and done in accordance with manufacturer instructions. Elisa plate was coated with 100 µL of capture antibody (1µg/mL) for the standard, sample and blank, then sealed and to allowed the antibody to adhere to the wells overnight at room temperature. After incubation, the liquid was discarded, and the wells were rinsed four times with 300 µL of wash buffer (0.05% Tween 20 in PBS). Each well was then filled with 300 µL of blocking buffer (1% BSA in PBS) and allowed to sit for 1 hour at room temperature. After washing step was performed, 100 µL of either standard solution or hemolysate-free sample was added to each well and incubated at room temperature for 2 hrs. Aspirated and washed plate 4 times. Following this 100 µL of detection antibody (0.25 µg/mL) was added to each well and incubated for 2 hrs at room temperature. Following another wash, 100 µL of avidin HRP conjugate was added per well and incubated for 30 minutes at room temperature. Finally, after aspiration and washing the plate 4 times with wash buffer. 100 µL of ABTS substrate solution was added to each well and incubated at room temperature for colour development. The plate was read at 405 nm with a microplate reader.

$$\boldsymbol{Conc.\,of\ IL\ 6\ (pg/ml)} = \frac{\boldsymbol{OD\ of\ sample}}{\boldsymbol{Slope\ of\ the\ standard\ curve}}$$

3.10.4 Determination of IL-10

Procedure: IL 10 was measured using an ELISA kit from Peprotech, USA following the manufacturer instructions. Each well of the Elisa plate was coated with 100 µL of capture antibody (2µg/mL) for standard, sample and blank, and incubated overnight at room temperature. Following incubation, the wells were emptied and washed four times with 300 µL of wash buffer (0.05% Tween 20 in PBS). Each well was then filled

with 300 µL of blocking buffer (1% BSA in PBS) followed by overnight incubation at room temperature. Following aspiration of the block buffer, the plate underwent four rounds of washing. Standard or hemolysate-free samples (100 µL each) were added to each well and incubated at room temperature for 2 hrs. Once the incubation was complete, the samples were removed and the wells were again washed four times with wash buffer. Thereafter, 100 µL of the detection antibody (0.5 µg / mL) was added to each well, followed by a two-hour incubation at room temperature. After removing the detection antibody and washing the plate four times, 100 µL of Avidin- HRP conjugate was added per well and incubated for 30 minutes at room temperature. Following this, the wells were washed four times with wash buffer. Finaly, each well received 100 µL of ABTS substrate solution, and color development proceeded at room temperature. A microplate plate reader was then used to read the Elisa plate at 405 nm.

$$\textbf{Concentration of IL 10 } (pg/mL) = \frac{\textbf{OD of sample}}{\textbf{Slope of the standard curve}}$$

3.11 Estimation of Total Protein (Lowry et al., 1951)

Principle: The Lowry method is a widely used colourimetric assay for the estimation of total protein in a sample. It is based on the reaction between the amino acids in proteins and the Folin-Ciocalteu phenol (FCP) reagent under alkaline conditions. When the amino acids reduce the FCP reagent, it forms a blue complex with the proteins present in the sample. This resulting colour change is quantified spectrophotometrically at a wavelength of around 660- 750 nm.

Procedure: Varying concentrations of standard BSA (10 to 100 µg)) and 0.2mL of the diluted samples were added to the test tubes and mixed with 1 mL of Lowry reagent (Reagent A: 0.5% $CuSO_4$ and 1% sodium potassium tartrate and Reagent B: 2% Na_2CO_3 in 0.1N NaOH, in 1:50 ratio). A 0.1mL of FCP reagent (1N) was added to this solution, mixed thoroughly, and incubated for 30 minutes. Following incubation, a

spectrophotometer was used to measure the developed colour at a wavelength of 660 nm against a reagent blank (without the addition of sample and standard). The concentration of unknown samples was determined from the graph by plotting a standard curve with the absorbance value against known concentration of the BSA.

Calculation

$$\boldsymbol{Concentration\ of\ protein\ (mg/mL)} = \frac{\boldsymbol{A_{660}\ nm}}{\boldsymbol{Slope} \times \boldsymbol{0.2mL}} \times \boldsymbol{DF}$$

Where, DF – Dilution Factor

3.12 Histological Analysis

The tissue (liver, heart, and kidney) was first washed with PBS and then preserved in 10% formalin. Following fixation, a series of graded alcohols (70, 95, and 100%) were used to dehydrate the tissues. This was followed by tissue being immersed in xylene for clearing and embedded in paraffin wax. A microtome was used to slice thin sections that were 4-5 μm in size, which were then spread on glass slides. Then immersed in xylene to remove the paraffin, and rehydrated through a series of graded alcohols (100%, 95%, 70% ethanol). The sections were then subjected to a 5-10-minute immersion in Harris haematoxylin solution, and then washed under running tap water to intensify the blue hue of the nuclei. Counterstaining the sections with eosin Y solution for 1-3 minutes was performed. Post-staining, the sections underwent dehydration via graded alcohols (70%, 95%, 100% ethanol). The finalized slides were mounted using a permanent mounting medium, covered with coverslips, and allowed to dry. The H&E-stained sections were analyzed by Magnus octopus microscope with IS image capturing software for microscopic visualization of the sections, and digital photographs of histological sections were obtained.

3.13 Statistical Analysis

Statistical analysis was conducted using GraphPad Prism software (Version 9) with

results were reported as mean ± SD of six animals per group. A Two–way Analysis of variance (ANOVA) was conducted to evaluate the main effects of oils, oxidation, and their interaction. Significant differences between groups were identified using Tukey's post hoc multiple comparison test, with significant threshold set at $p < 0.05$. The notations used for statistical significance included: ns non- significant, * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

Chapter 4

Physicochemical Characterization of Oils

4.1 Introduction

Edible oils are widely utilized in food preparations to enhance the flavour, texture, and nutritional quality of food products (Zhao et al., 2021). Heating or deep frying of edible oil (Thermal oxidation) is a common procedure in cooking. When oils are exposed to high temperatures, ranging from 100 to 200°C, such as frying, baking, or other cooking methods, they undergo thermal oxidation, which can lead to alterations in their physical properties and chemical composition. Chemical analysis of fats and oils assumes significance regarding their health impact. Especially it is always important to determine the degree of thermal oxidation that occurs during the repeated heating of oils.

Physicochemical characterisation is an essential approach for evaluating the physical and chemical properties of oils. This helps to determining their composition and evaluate the degree of oxidation after heating or frying. Physicochemical properties include density, acid value, percentage of free fatty acid content, conjugated diene (CD) and conjugated triene (CT) values, p-anisidine value, and TBARS, which are vital for analysing the oxidative deterioration of edible oils. Analytical techniques like gas chromatography-mass spectrometry (GC-MS) and Fourier-transform infrared spectroscopy (FTIR) are commonly utilized to detect and measure these chemical entities in the oil (Zahir et al., 2017). Physicochemical characterisation of edible oils varies significantly depending on the oil type. Understanding these properties is crucial for monitoring oil quality and stability, particularly during cooking processes, where oils undergo various chemical transformations due to heat and exposure to air. This information aids in ensuring that oils maintain their nutritional value and are safe to ingest after being heated or fried.

Oils obtained from coconut, palm, sunflower, and mustard seeds are more widely used

vegetable oils in India for cooking purposes, and each is represented as MCFA, LCFA, MUFA and PUFA-rich oils. Many of these oils possess pharmacologically active phytoconstituents. The primary objective of this these is to evaluate the health impact of unoxidized and thermally oxidized oils in the diet, at its physiological concentrations. A detailed physical and chemical characterisation of oils under study is thus very important. Prior to conducting animal experiments, a comprehensive analysis of the physicochemical characteristics of the test oils was undertaken. This chapter describes, in detail, the physical and chemical characterisation of dietary oils coconut (CO), palm (PO), mustard (MO) and sunflower oils (SO) in their unoxidized as well as thermally oxidized forms to ensure-oxidative stability and nutritional values.

4.2 Materials and Methods

4.2.1 Edible oils

In this study, CO, which is rich in medium-chain saturated fatty acids (MCFA); PO, rich in long-chain saturated fatty acids (LCFA); MO, rich in monounsaturated fatty acids (MUFA); and SO, rich in polyunsaturated fatty acids (PUFA) were selected based on their fatty acid composition. The thermal oxidation procedure carried out for these oils is described in Chapter 3.1.5.

4.2.2 Thermal Stability Assessment by Differential Scanning Calorimeter (DSC)

DSC analysed the thermal stability of the oil. The procedure is described in Chapter 3.3.1.

4.2.3 Density measurements (Habarakada et al., 2021)

The density of edible oils was analysed using the modified method of Harabarkada (2021). The procedure is described in Chapter 3.4.1.

4.2.4 Conjugate Dienes (CD) and Trienes (CT) estimation (AOCS, 1998)

CD and CT are good measures of the oxidative states of the oils. The procedure is described in Chapter 3.4.2.

4.2.5 Free fatty acid content analysis (AOCS, 1998)

The FFA in oil was estimated by titrating oils against KOH. The method is outlined in Chapter 3.4.3

4.2.6 Determination of p- anisidine value (p-AV) (AOCS, 1998)

The p-AV was determined by measuring the absorbance of a solution containing p-anisidine and the oil being tested, which has been described in Chapter 3.4.4.

4.2.7 Thiobarbituric acid reacting substances (TBARS) Assay

Lipid peroxidation status is commonly measured using the TBARS assay. It measures the amount of malondialdehyde (MDA) and other carbonyl compounds produced during the breakdown of PUFA. Details of the assay have been given in the section of Chapter 3.4.5.

4.2.8 Fourier – Transform Infrared (FT-IR) Spectroscopy Analysis

FT-IR spectroscopy provides insights into the functional groups present in oil molecules based on their unique infrared absorption spectra. Detailed methodology is given in the section of Chapter 3.4.6.

4.2.9 Fatty Acid Methyl Ester (FAME) Analysis

FAMEs commonly used for the analysis of the composition of fatty acids. It was performed using gas chromatography coupled with mass spectrometry (GC-MS). Details have been described in Chapter 3.4.7.

4.3 Statistical Analysis

Thermal oxidation of oil samples was performed in two different batches, with each sample analysed in triplicate across all biochemical assays. The values were expressed as mean ± SD. ANOVA was conducted using Prism 9 (Graph Pad Software, Inc., CA). Tukey's multiple comparison test was applied to determine the significance level at $p < 0.01$, $p < 0.05$, and $p < 0.001$, denoted as *, **, and ***, respectively.

4.4 Results

4.4.1 Thermal Stability of Edible Oils.

The DSC thermal analysis was done to get a deep insight into oxidation and thermal stability. The DSC analysis results for CO (Figure 4.1a), PO (Figure 4.1b), MO (Figure 4.1c), and SO (Figure 4.1d) showed four endothermic peaks corresponding to melting, oxidation and degradation as depicted in. Heating of CO, PO, and SO from room temperature to 250°C exhibited only the offset melting temperature at 59, 52, and 60°C respectively, followed by oxidation with onset temperature, at 60, 53, and 61°C and offset temperatures 187, 184, and 178°C respectively. Hence, CO and PO, which contain more saturated fats, were observed to be oxidatively stable up to 185°C, and SO with a higher amount of unsaturated fats was found to be oxidatively stable up to 175°C. The degradation of palm oil occurred with an onset temperature of 185°C. Meanwhile, in coconut oil and sunflower oil, the degradation occurred in two stages. The first decomposition of CO and SO occurred around 188°C and 178°C respectively, due to unsaturated fatty acids, and the second event of decomposition occurred about 210°C and 240°C for saturated fatty acids and oxidative compounds. However, the DSC analysis of MO exhibited only the offset melting temperature at 114°C, followed by oxidation with an onset temperature of 115°C and an offset temperature of 234°C; the oxidation events occurred in two stages.

4.4.2 Change in Color and Density

Colour is an excellent visual indicator of the quality of the oils. Unoxidized oils typically exhibit a range of colours, from pale yellow to golden or greenish hues, depending on the type of oil. Different oils have varying natural pigments, such as chlorophyll in olive oil or carotenoids in palm oil, which contribute to their respective colour. Thermal oxidation of oils can cause noticeable colour changes. Initially, the oil may darken, becoming more yellowish or amber in appearance. As oxidation

progresses, the oil can develop a darker brown or reddish colour. These colour changes are primarily due to the formation of oxidation products and the polymerisation of unsaturated fatty acids. The photograph of the comparison of changes in the colour of unoxidized and thermally oxidized oils is depicted in Figure (4.2). Initially, the colour of CO was clear, while the TCO showed a yellow hue (Figure 4.2 a). The PO sample presented itself in a light brown hue, contrasting with the TPO sample, which displayed a deeper, dark brown colouration (Figure 4.2 b). The MO revealed a slight brown colour, which darkened after thermal oxidation (Figure 4.2 c). The SO sample displayed a slight cloudiness coupled with a light yellow. Conversely, the TSO sample changed to a dark brown colour (Figure 4.2 d).

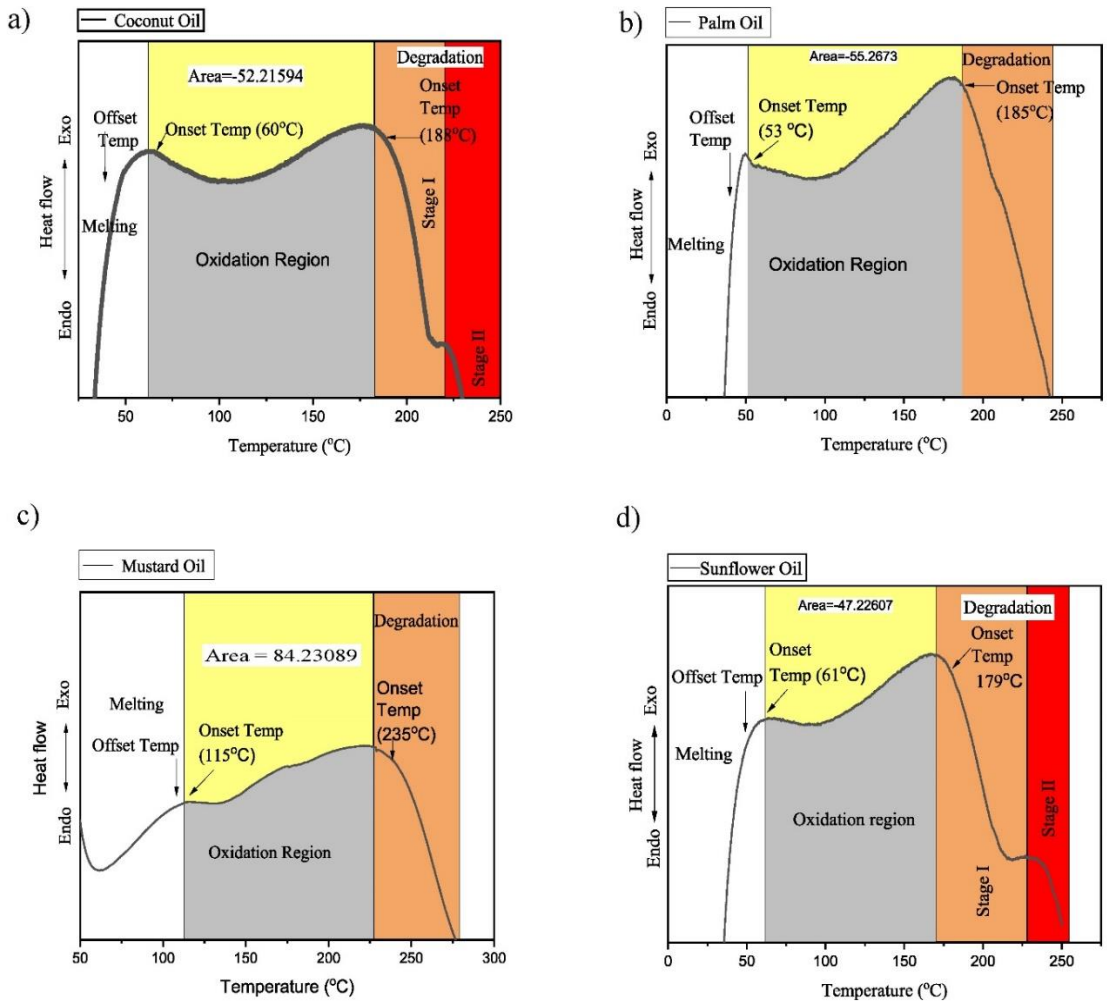

Figure 4.1: The Differential Scanning Calorimetric (DSC) Analysis of a) Coconut oil (CO), b) Palm oil (PO), c) Mustard oil (MO), and d) Sunflower oil (SO).

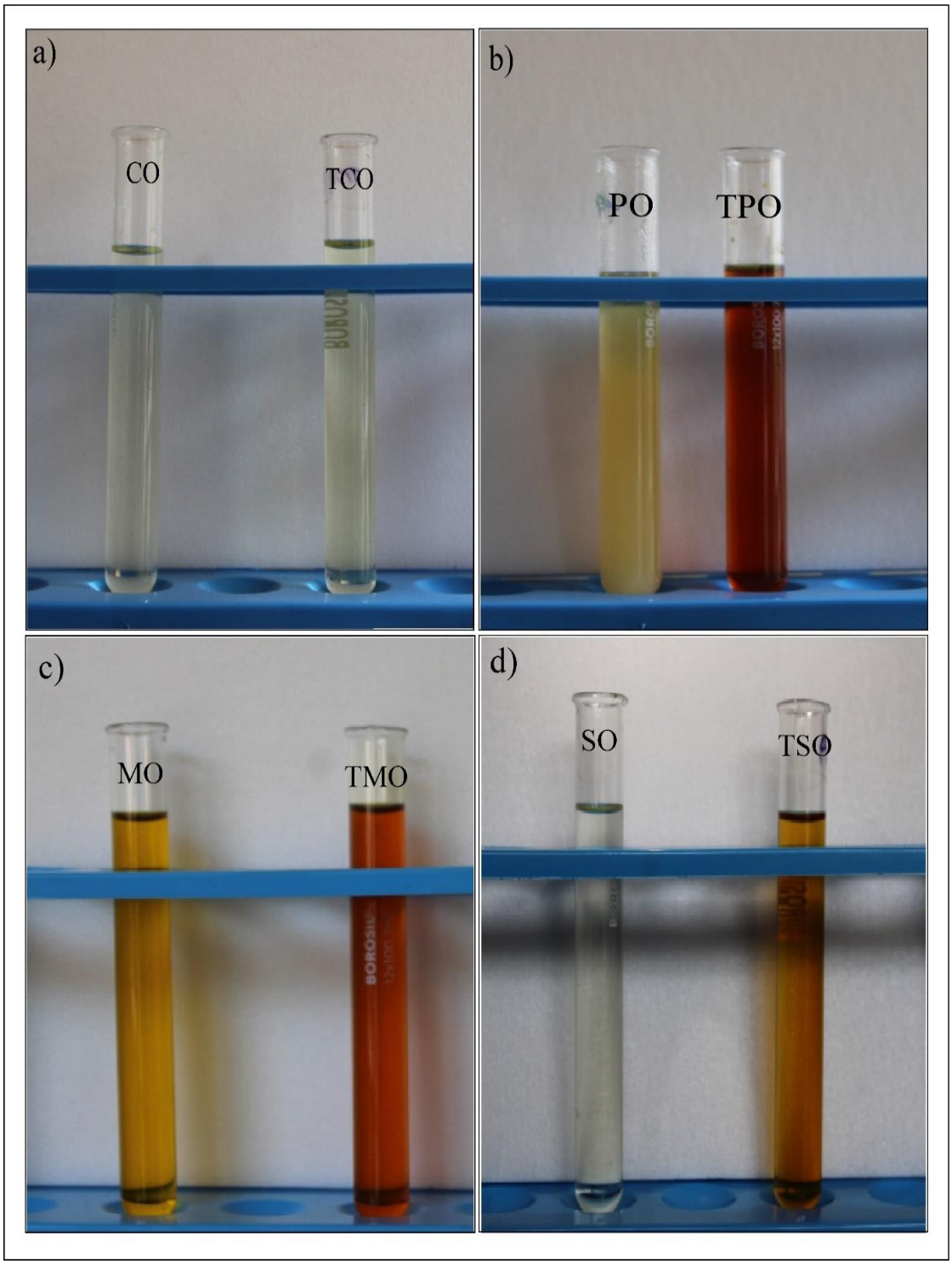

Figure 4.2: Color of Unoxidized and Thermally Oxidized Oils. a) coconut oil turning yellow upon oxidation, b) light brown palm oil becoming dark brown aftet oxidation, c) mustard oil darkening from slight brown to a deeper brown upon oxidation and d) sunflower oil changing from a light yellow to a dark brown. CO – unoxidized coconut oil, TCO – Thermally oxidized coconut oil, PO – Unoxidized palm oil, TPO – Thermally oxidized palm oil, MO – Unoxidized mustard oil, TMO – Thermally oxidized mustard oil, SO -Unoxidized sunflower oil, TSO- Thermally oxidized sunflower oil.

4.4.3 Conjugate Dienes and Trienes

UV spectrophotometric analysis is an effective method for assessing the extent of fatty acid oxidation in oils. Absorbance at 232nm is used to measure the presence of primary oxidation products such as hydroperoxides and conjugated dienes, while absorbance at 270 nm measures the secondary oxidation products including ketones, aldehydes, and other compounds that arise from the breakdown of primary oxidation products. Hydroperoxides formed during the early-stage oxidation of polyunsaturated fatty acids, which subsequently transformed to form conjugated dienes. The obtained data shown in Table 4.1 illustrates the differences in absorption at these wavelengths. The highest concentrations of CD and CT were noticed in SO (18.1 and 11.37 mmol/kg Oil), followed by PO (7.77 and 3.18 mmol/kg Oil), MO (7.38 and 2.17 mmol/ kg Oil), and CO (3.82 and 0.01 mmol/kg oil). However, when oil was heated, the thermal oxidation resulted an increase in these values and the highest value obtained with thermally oxidized form TSO (77.66 and 27.43 mmol/ kg Oil), followed by TMO (74.48 and 21.58 mmol/kg. Oil), TPO (30.75 and 6.83 mmol/kg Oil), and the lowest value was found in TCO (12.54 and 3.35mmols/kg. Oil).

4.4.4 Acid Value (AV) and Free Fatty Acid Content (% FFA)

AV and percentage of FFA content are important indicators of the quality and characterisation of edible oils. FFAs are released when triglycerides in the oil break down, either due to natural processes or degradation caused by factors such as oxidation or hydrolysis. An increase in AV and percentage FFA content indicates the lower quality of oils. Table 4.1 represents the percentage of FFA content and AV in experimental oils and was found to be significantly varied among unoxidized oils, with the following CO at 0.16 ±0.03, PO at 0.65±0.09, MO at 0.83±0.02, and SO at 0.13±0.03. Following thermal oxidation, the percentage change in FFA content increased among the group. The lowest increases were observed in TPO (2.02±0.44)

and TMO (2.52±0.0) both showing a 67% increase. This was followed by TCO (1.09±0.06) at 85% increases, while TSO (1.40±0.28) exhibited the highest increase at 91%. Similarly, AV was significantly different in unoxidized oils (0.32±0.06, 1.31±0.17, 1.66±0.03, 0.26±0.06 for CO, PO, MO, and SO, respectively). Following thermal oxidation, the AV lowered among the group. The lowest increases were observed in TPO at 4.04±0.88 and TMO at 5.05±0.0 both showing a 67% increase. This was followed by TCO at 2.19±0.13 of 85% increases, while TSO (2.81±0.56) exhibited the highest increase at 91%. A two-way ANOVA was conducted to evaluate the effects of oil type and thermal oxidation on the AV and percentage of FFA of CO, PO, MO and SO. The interaction between oil and oxidation was statistically significant ($F(3, 16) = 7.68$) $p<0.01$), indicating that the effect of oxidation on AV and the percentage of FFA differed depending on the oil type. The main effects of both oil type ($F(3, 16) = 72.7$) $p<0.01$) and thermal oxidation ($F(1, 16) = 1198$) were also highly significant $p<0.0001$.

4.4.5. p- Anisidine Value in the oils (p-AV)

The p-AV determines the quantity of secondary oxidation products in edible oils. Table 4.1 illustrates the p-AV across different oils. It primarily represents the levels of ketones, aldehydes, peroxides and other oxidation products, which are formed during the oxidation process. These compounds are responsible for the rancid or off-flavours and odours in oils. The highest p-AV was observed in TSO (42.62 ±0.1) compared to its unoxidized SO (4.22 ±0.7); this substantial increase in p-AV may be attributed to the higher concentration of PUFA. The lowest value was in thermally oxidized CO (17.54 ±1) compared to unoxidized CO (2.36 ±1.5). CO is naturally reported to contain 4-6% unsaturated fats. The changes of p-AV of palm oil were from 3.97 ± 0.4 to 36.77 ±1.9, and moderate changes were found in MO (from 3.25 ± 0.5 to 21.28 ±0.7in TMO) with thermal oxidation. A higher p-AV indicates a higher degree of oxidation and the

potential for sensory quality deterioration in the oil. Fresh, high-quality oils typically have low p-AV, often below 10. Values between 10 and 20 suggest mild to moderate oxidation, which may result in slight flavour changes. Values above 20 indicate significant oxidation and potential development of strong off-flavours and odours. Oils high in PUFAs, are more prone to oxidation and typically have higher p-AV than oils with a higher content of saturated fatty acid, such as coconut oil. The two-way ANOVA revealed a significant effect of oxidation ($F(1, 16) = 2763$, $p < 0.0001$) indicating that oxidation level significantly affects p-AV, similarly, the effect of oil was also significant on p-AV ($F(3, 16) = 405$, $p < 0.0001$). The interaction between oxidation and oil was found to be significant, ($F(3, 16) = 158$, $p < 0.0001$). This interaction effect indicates that the effect of oxidation on p-AV depends on the type of oil, and vice versa.

4.4.6 TBARS content

TBARS values are shown in Table 4.1. TBARS analysis is the most widely used assay to evaluate the progress of lipid oxidation. Higher TBARS values indicate increased lipid peroxidation levels, suggesting a lower quality or freshness of the oil. Lower TBARS values generally indicate lower oxidative deterioration, better stability and hence higher quality of the oil. In this study, the lowest TBARS value was found in oxidized CO (75.8 ± 8.6nmols/ml of oil), whereas the highest was found in unoxidized MO (733 ± 44 nmol/mL of oil) compared to other oils, palm oil (392.7 ± 42 nmol/mL of oil), and sunflower oil (558.1 ± 53.3 nmol/mL of oils). After the thermal oxidation of oils, the TBARS of sunflower oil was found to be increased by 32%, followed by thermally oxidized mustard oil (14.5%) and thermally oxidized palm oil (26.3%). Coconut oil showed a minimum oxidation level during thermal oxidation, 0.8% was increased. The two-way ANOVA revealed a significant effect of oxidation ($F(1, 16) = 65.7$, $p < 0.0001$) indicating that oxidation level significantly affected TBARS, similarly, the effect of oil was also significant on TBARS ($F(3, 16) = 378$, $p < 0.0001$).

The interaction between oxidation and oil was found to be significant, ($F(3, 16) = 11$, $p < 0.0001$).

Table 4.1: Physicochemical Parameters of Unoxidized and Thermally Oxidized Oils.

Parameters	Coconut oil	Palm oil	Mustard Oil	Sunflower Oil	Thermally Oxidized Coconut oil	Thermally Oxidized Palm Oil	Thermally Oxidized Mustard Oil	Thermally Oxidized Sunflower Oil
Density	918 ± 1.01	901 ±2.12	881 ±2.23	846 ± 8.49	926 ± 1.58^{ns}	912 ± 9.19^{ns}	942 ±2.12**	890 ±6.97**
CD (mmol/ Kg Oil)	3.82 ±0.02	7.77±0.0	7.38 ± 0.0	18.11 ± 3.9	12.54 ± 0.2*	30.75 ± 0.0*	74.48 ±1**	77.66 ± 0.0**
CT (mmol/ Kg Oil)	0.01 ±0	3.18 ± 0.2	2.17 ± 0.2	11.37 ±2.4	3.35 ± 0.0^{ns}	13.66 ± 1.0*	21.58 ± 0.2*	27.43 ±5.2*
Acid Value (mg of KOH Required/g oil)	0.32 ±0.06	1.31±0.17	1.66±0.03	0.26±0.06	2.19±0.13**	4.04±0.88*	5.05±0.0*	2.81±0.56**
%FFA (% Oleic acid / g of oil)	0.16 ±0.03	0.65±0.09	0.83±0.02	0.13±0.03	1.09±0.06**	2.02 ±0.44*	2.52±0.0*	1.40±0.28**
p-AV	2.36 ±1.5	3.97 ± 0.4	3.25 ± 0.5	4.22 ±0.7	17.54 ±1**	36.77 ±1.9**	21.28 ±0.7**	42.62 ±0.1**
TBARS (nmol/mL)	75.8±8.6	392.7 ±42	733±44	558.1±53.3	76.4± 8.1^{ns}	532.5±49.6**	857.6±41.1**	824.9±45.7**

The data were expressed as mean ± SD. Two-Way ANOVA was done by using GraphPad Prism Version 9 (Graph Pad Software, Inc., CA). Tukey's multiple comparison test was used for the significant level at $p < 0.05$, $p < 0.01$, and $p < 0.001$, denoted as *, **, and ***, respectively.

4.4.7 Fourier – Transform Infrared (FT-IR) Spectroscopy

FT-IR spectroscopy is an excellent tool for examining the functional group and the intensities of the band in the spectrum. Figure 4.4-4.7 illustrates the plot of transmittance (%) versus wavenumber (cm^{-1}) obtained from the FT-IR study. The peaks are proportional to the concentration of their respective functional group in the edible oils. Unoxidized edible oils possess relatively identical appearances on their FTIR spectra. However, there are some discrepancies in both frequencies and absorbance of the band and varying presence or absence of distinct bands due to their different fatty acid composition. The composition of oil affects the exact positions of the IR spectroscopic bands, leading to shifts as the proportion varies in fatty acids. The notable bands are the following: the band at 3007.44–3008.44 cm^{-1} is the C-H stretching vibration of the *cis*-double bond (=C-H), while the bands at 2852.10–2922.99 cm^{-1} shows -C-H asymmetric stretching vibrations of the aliphatic functional group -CH_2. A double bond stretching region representing the C=O ester carbonyl of triglycerides is observed at 1741 -1743 cm^{-1} (Table 4.2). Additionally, deformations and bending vibrations were identified at 1462–1464 cm^{-1} for the -C-H bending of the CH_2 and CH_3 aliphatic groups and at 1377 cm^{-1} for the -C-H bending of the $-CH_2$ group. The peak spectra 1159- 1160 cm^{-1} showed alcohol compounds that contain the hydroxyl (−OH) group.

Table 4.2: Description of FT IR Spectra of Edible Oils**.**

Wave Number (cm^{-1})	**Types of vibration**	**Functional Group**	**Intensity**
3007 -3008	Asymmetrical Stretching	=C-H of alkenes	-
2921-2922	Asymmetrical stretching	C-H of alkanes	-
2852-2853	Asymmetrical stretching	C-H of methylene	Medium
1741-1743	Stretching	C=O ester bond	-
1462 – 1464	Bending	=C-H (cis)	Variable
1377	Bending in plane	-C-H (CH_3)	Variable
1159-1160	Stretching, Bending	-C-O, -CH_2	-
1097-1118	Stretching	-C-O	Weak
721	Bending	=C-H	-

The table presents the wave numbers (cm^{-1}) corresponding to specific infrared vibrations, categorized by type, associated functional groups, and their intensity. The wavenumber indicates the frequency of molecular vibrations, with higher values representing higher energy. The types of vibrations include stretching (changes in bond length, eg: - symmetrical, or asymmetrical) and bending (changes in bond angles, eg: - in plane or out of plane). Functional group such as =C-H of alkenes, C-H of alkanes ester bonds are responsible for the observed vibrations. Intensity denotes the strength of the absorption band, classified as week, medium, and variable.

The Infrared spectra of unoxidized and thermally oxidized CO (Figure 4.3), PO (Figure 4.4), MO (Figure 4.5), and SO (Figure 4.6) are similar; some differences are found, which are related to the width and intensity, and the presence or absence of IR spectral bands. Thermal oxidation leads to various modifications in the spectral regions of oils. A weak band appeared at 3007-3008 cm^{-1}, indicating the stretching vibration of the *cis* bond in the unoxidized palm oil, mustard oil, and sunflower oil, whereas in CO, there was no peak observed at region 3007- 3008 cm^{-1}. The loss of the *cis* double bond during thermal oxidation decreased peak intensity at 3007cm^{-1} in TPO, TMO, and TSO. Decreased peak intensity at 721cm^{-1} was also confirmed by the loss of the *cis* double bond. In the meantime, there was an observed rise in peak intensity within the spectral region at 967 cm^{-1} caused by an increasing concentration of *trans* double bond**s**. Two peaks at 2921 and 2852 cm^{-1} remained unaltered. Another trend was an increase in the

area of the characteristic bands at 1743 cm^{-1}. This indicates the existence of saturated aldehyde functional groups or secondary oxidation products, likely results from the decomposition of hydroperoxide. This leads to an absorption occurring at 1728 cm^{-1} that overlaps with the triglyceride's ester carbonyl functional group's stretching vibration at 1746 cm^{-1}. The 1464 cm^{-1} methylene (C-H) stretch band showed an upward trend in intensity in the thermally oxidized oils. This would have pertained to the reduction of the UFAs and the fractionation of the carbon chain into smaller molecules by the oxidative process. The rise in the extent of the identifiable band at 1377 cm^{-1} affirmed this pattern. This suggests that the oxidative process fractionated the carbon chain of the fatty acids into smaller molecules. When undergoing lipid oxidation reactions, fatty acids break down into small and volatile molecules that generate unpleasant smells referred to as oxidative rancidity. Two other bands present in all oil samples were near 1236 and 1159 cm^{-1} and are assumed to be related to the proportion of saturated acyl groups in the sample. The frequencies of both bands suffer similar changes during the oxidation process and increase their intensity, although the differences are sharper in the band near 1159 cm^{-1}. The intensity of these bands increased during oxidation because of the decomposition of hydroperoxides through the β-scission mechanism.

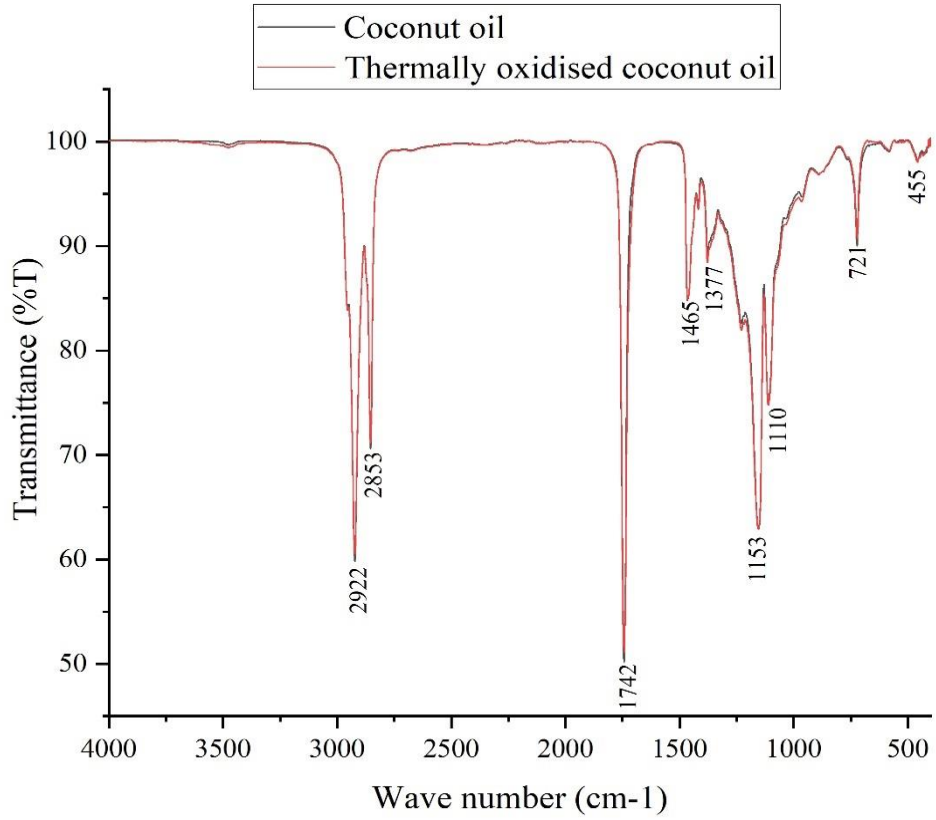

Figure 4.3: FT IR Spectra of Unoxidized and Thermally Oxidized Coconut Oil

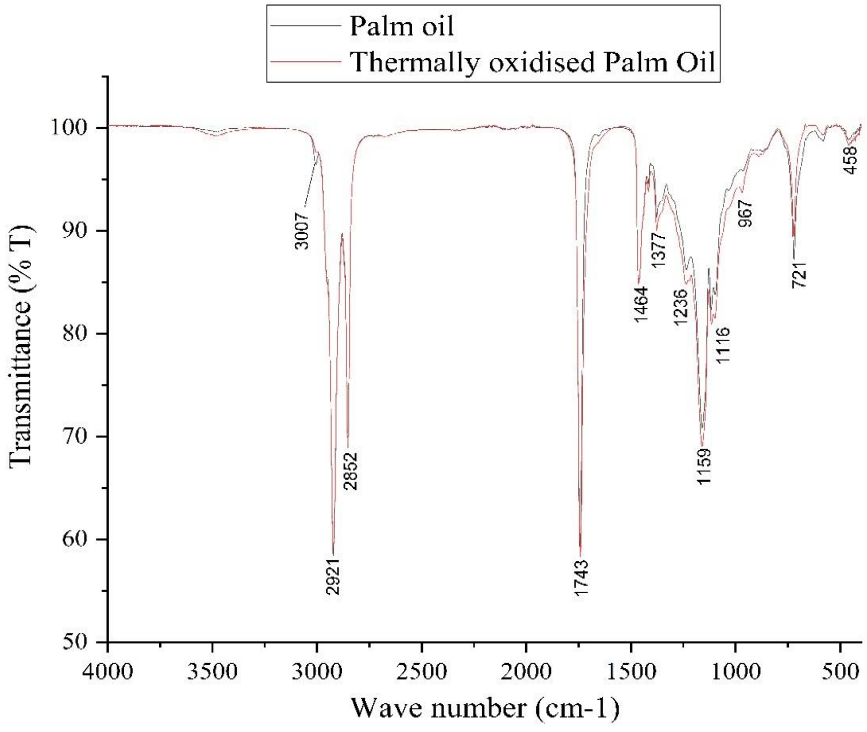

Figure 4.4: FT IR Spectra of Unoxidized and Thermally Oxidized Palm Oil

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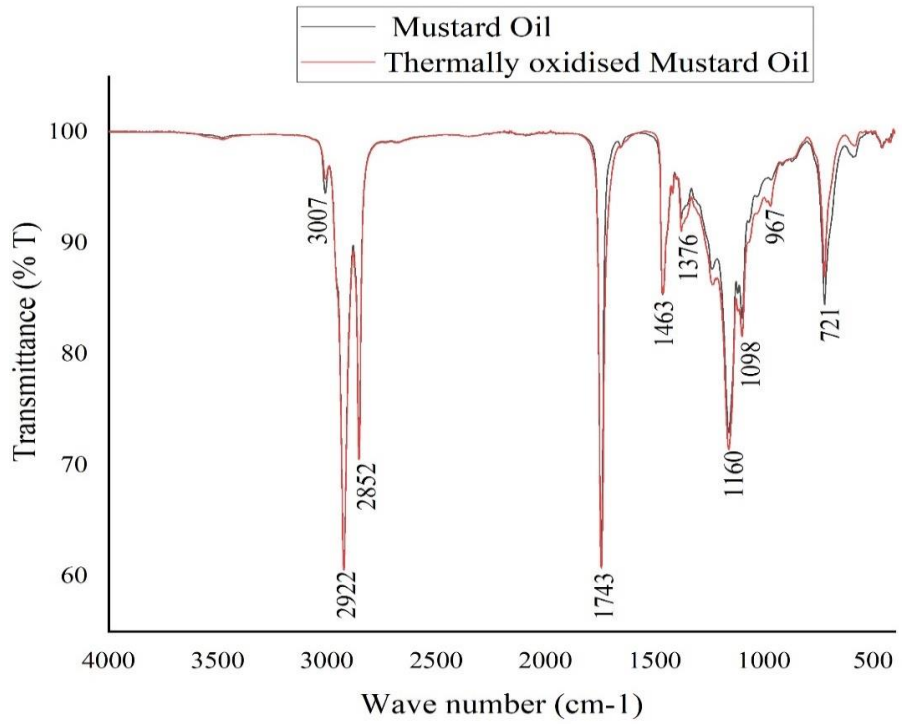

Figure 4.5: FT IR Spectra of Unoxidized and Thermally Oxidized Mustard Oil.

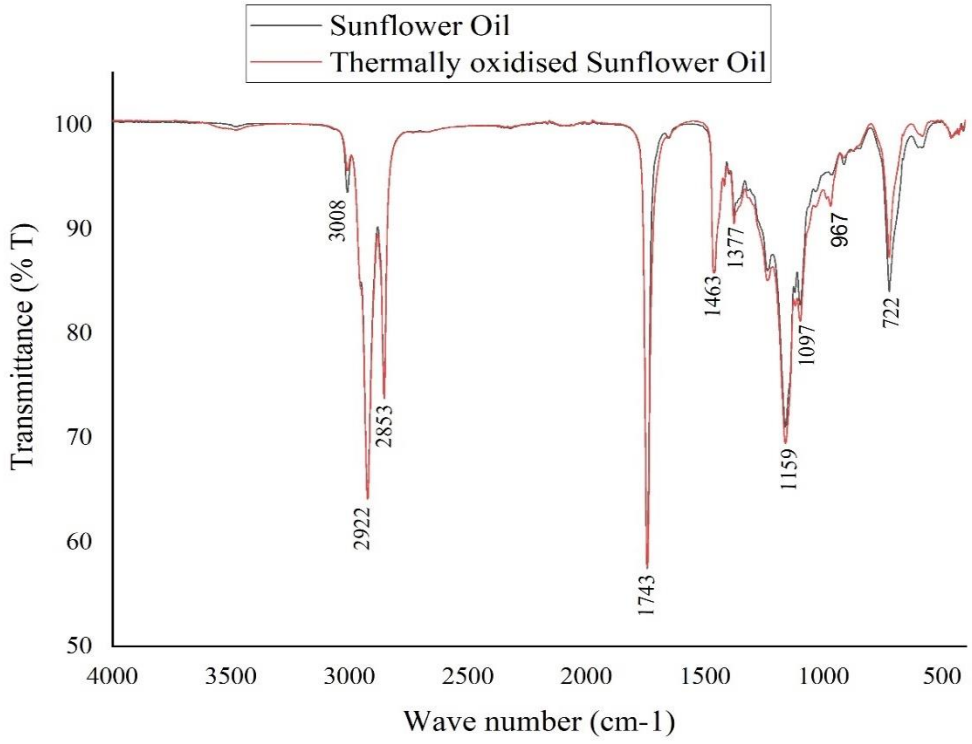

Figure 4.6: FT IR Spectra of Unoxidized and Thermally Oxidized Sunflower Oil.

4.4.8 FAME Analysis

The fatty acid composition of four types of unoxidized and thermally oxidized edible oils was quantified by GC-MS, as shown in Table 4.3. Coconut oil contained 89.92% saturated fats, including caprylic (7.68%), capric (6.11%), lauric (39.21%), myristic (21.01%), as well as long-chain fatty acid palmitic acid (11.65%). Additionally, it has unsaturated fatty acids like linoleic acid (1.60%) and oleic acid (8.48%). Following thermal oxidation, linoleic acid was completely absent, and there was a substantial reduction in oleic acid with only 2.19% remaining, while, saturated fats content in the CO increased, with lauric acid raised to 42.80%, and myristic acid to 22.39%, followed by caprylic acid to 8.91% and capric acid to 6.73%. The GC-MS chromatogram is shown in Figure 4.7

The fatty acid composition of unoxidized PO predominately contained palmitic acid (29.67%), oleic acid (37.54%), linoleic acid (16.12%), and stearic acid (10.38%). After thermal oxidation, the palmitic acid level was found to be slightly increased to 35.97%, while oleic acid reduced to 36.73%, linoleic acid to 2.67%, and stearic acid to 10.19%. The GC- MS chromatogram is shown in Figure 4.8

Mustard oil was found rich in MUFA erucic acid (45.28%), followed by linoleic acid (15.59%), oleic acid (11.50%), and linolenic acid (10.68%). Following thermal oxidation, linoleic acid decreased to 10.82% and linolenic acid to 8.75%, while erucic acid and oleic acid experienced an increase to 48.79% and 13.34% respectively. The GC-MS chromatogram is shown in Figure 4.9

In SO, linoleic acid (56.55%), oleic acid (31.23%), palmitic acid (6.36%), and stearic acid (3.54%) were the predominantly detected fatty acids. Here also a substantial reduction in linoleic acid to 41.29% was observed, along with an increase in oleic acid to 39.30%, palmitic acid to 9.0% and stearic acid to 5.96% following thermal oxidation. The GC-MS chromatogram is shown in Figure 4.10

Linoleic acid (C18:2) is the most abundant PUFA in oils and is more prone to oxidation during heating. While oleic acid (C18:1) was less reactive because it contains only one double bond. After thermal oxidation, there was a decrease in unsaturated fatty acid and increased saturated-rich acid content. Heptanoic acid and caprylic acid are not naturally reported in fresh, unoxidized PO and SO, but these molecules were increasingly documented with thermally oxidized forms of these oils. These could be the products from 9-hydroperoxides formed from linoleic/oleic acid content in the PO and SO. Azelaic acid, detected in thermally oxidized oils is a dicarboxylic acid and an oxidized product form of linoleic acid. During thermal oxidation, keto, and conjugated derivatives were thus found to be formed from PUFA. The FAME composition of SFA, PUFA and MUFA are tabulated in Table 4.4.

Table 4.3: Fatty acid methyl esters of CO, TCO, PO, TPO, MO, TMO, SO, and TSO

Fatty Acids	Coconut Oil		Palm Oil		Mustard Oil		Sunflower Oil	
	Unoxidized	Thermally Oxidized	Unoxidized	Thermally Oxidized	Unoxidized	Thermally Oxidized	Unoxidized	Thermally Oxidized
Heptanoic acid (C7:0)	-	-	-	0.37%	-	-	-	0.11%
Caprylic acid (C8:0)	7.68%	8.91%	-	0.91%	-	-	-	1.01%
Azelaic acid (C9:0)	-	-	-	0.13%	-	-	-	0.10%
Capric acid (C10:0)	6.11%	6.73%	-	-	-	-		
Lauric acid (C12:0)	39.21%	42.80%	0.39%	0.41%	-	-	-	-
Myristic acid (C14:0)	21.01%	22.39%	2.25%	2.26%	-	-	-	0.13%
Pentadecanoic acid (C15:0)	-	-	0.11%	0.12%	-	-	-	-
Palmitic acid (C16:0)	11.65%	11.85%	29.67%	35.97%	2.64%	3.47%	6.36%	9.0%
Cis -Palmitoleic acid (C16:1)	-	-	0.41%	0.32%	-	-	-	0.16%
cis -Hypogeic acid (C16:1)	-	-	0.12%	-	-	-	-	-
Margaric acid (C17:0)	-	-	0.26%	0.24%	-	-	-	-
9-Heptadecenoic acid (C17:1)	-	-	-	0.48%	-	-	-	-
Stearic acid (C18:0)	4.25%	4.10%	10.38%	10.19%	1.29%	1.67%	3.54%	5.96%
cis -Oleic acid (C18:1)	8.48%	2.19%	37.54%	36.73%	10.22%	13.34%	31.23%	39.30%

trans - Oleic acid (C18:1)	-	-	-	-	1.28%	-	0.08%	-
Cis- Linoleic acid (C18: 2)	1.60%	-	16.12%	2.67%	15.59%	10.82%	56.38%	39.95%
trans Linoleic acid (C18:2)	-	-	0.32%	-	-	-	0.17%	1.34%
Linolenic acid (C18:3)	-	-	0.40%	-	10.68%	8.75%	-	-
Arachidic acid (C20:0)	-	-	1%	0.85%	1.08%	1.36%	0.26%	0.44%
Gadoleic acid (C20:1)	-	-	0.47%	-	8.13%	9.31%	-	-
Behenic acid (C22:0)	-	-	0.17%	-	1.30%	1.38%	0.75%	1.27%
Erucic acid (C22:1)	-	-	-	-	45.28%	48.79%	-	-
Cerotic acid (C26:0)	-	-	0.10%	-	-	-	-	-
Methyl -11-eicosenoate	-	-	-	-	-	-	0.15%	-
11,14-Eicosadienoic acid Methyl ester	-	-	-	-	2.50%	1.10%	-	-
Cyclopropaneoctanoic acid 2 – hexyl - methyl ester	-	-	0.07%	-	-	-	-	0.39%
Glycidyl Myristate	-	-	0.11%	-	-	-	-	
Methyl 9- keto stearate	-	-	-	0.77%	-	-	-	0.13%
Methyl 10- keto stearate	-	-	0.09%	0.86%	-	-	-	-
Cis-9,10- Epoxystearic acid	-	0.55%	-	6.70%	-	-	-	0.71%
2-cyclopentene– tridecanoate	-	-	-	-	-	-	1.09%	-

Table 4.4: The proportions of saturated (SFA), monounsaturated (MUFA), and polyunsaturated (PUFA) Fatty acids.

	Coconut Oil		**Palm Oil**		**Mustard Oil**		**Sunflower Oil**	
	Unoxidized	Thermally Oxidized oil	Unoxidized	Thermally Oxidized oil	Unoxidized	Thermally Oxidized oil	Unoxidized	Thermally Oxidized oil
SFA	89.92%	97.37%	44.62%	59.78%	6.31%	7.88%	12%	19.25%
MFA	8.48%	2.63%	38.54%	37.53%	64.91%	76.44%	31.46%	39.46%
PUFA	1.60%	-	16.84%	2.69%	28.77%	15.67%	56.54%	41.29%

Table 4.5: Retention Time of Major Fatty Acid Methyl Esters of Oils in Minutes

Fatty Acids	Coconut Oil		Palm Oil		Mustard Oil		Sunflower Oil	
	Unoxidized	Thermally Oxidized	Unoxidized	Thermally Oxidized	Unoxidized	Thermally Oxidized	Unoxidized	Thermally Oxidized
Caprylic acid (C8:0)	5.071	5.067		5.053	-	-	-	5.063
Capric acid (C10:0)	8.043	8.039	-	-	-	-	-	-
Lauric acid (C12:0)	11.003	11.00	-	-	-	-	-	-
Myristic acid (C14:0)	13.681	13.679	13.671	13.663	-	-	-	-
Palmitic acid (C16:0)	16.150	16.148	16.270	16.208	16.144	16.141	16.199	16.151
Stearic acid (C18:0)	18.525	18.521	18.645	18.572	18.532	18.527	18.540	18.698
cis -Oleic acid (C18:1)	18.786	18.775	18.945	18.855	18.883	18.87	18.890	18.975
Cis- Linoleic acid (C18: 2)	19.384	-	19.489	19.416	19.434	19.408	19.414	19.602
Linolenic acid (C18:3)	-	-	-	-	20.307	20.277	-	-
Gadoleic acid (C20:1)	-	-	-	-	21.529	21.518	-	-
Erucic acid (C22:1)	-	-	-	-	24.661	24.631	-	-

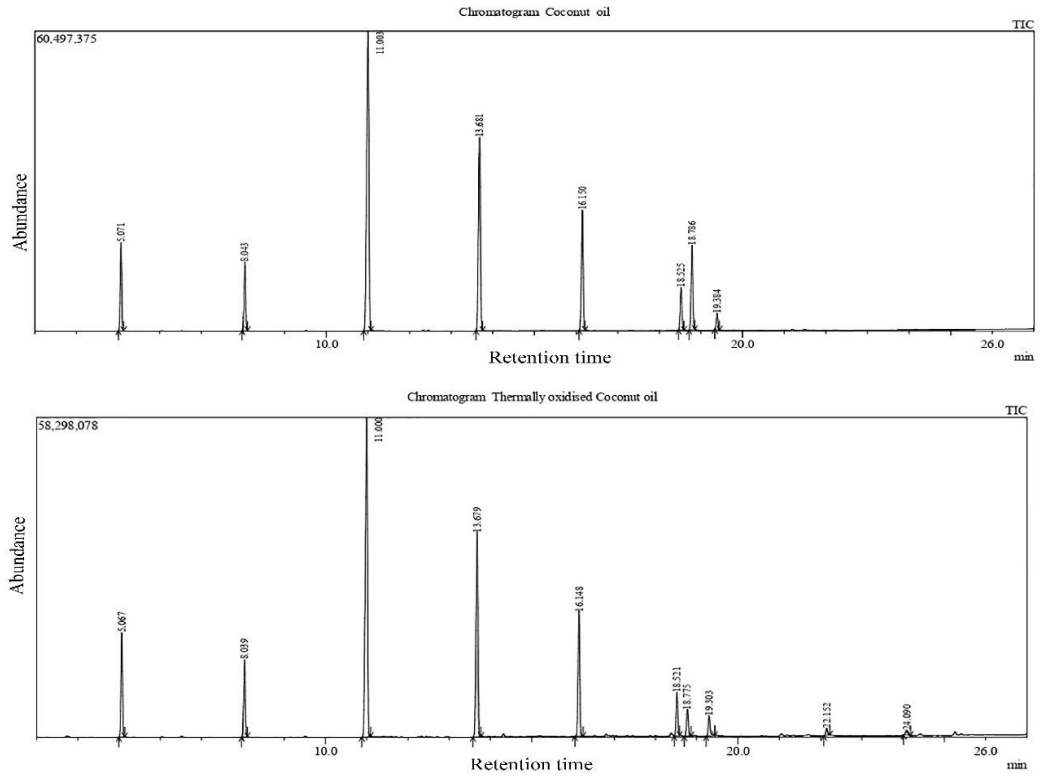

Figure 4.7: GS-MS Chromatogram of Unoxidized and Thermally Oxidized Coconut Oil. Chromatogram with Retention Time on the X-Axis and Abundance on the Y-Axis.

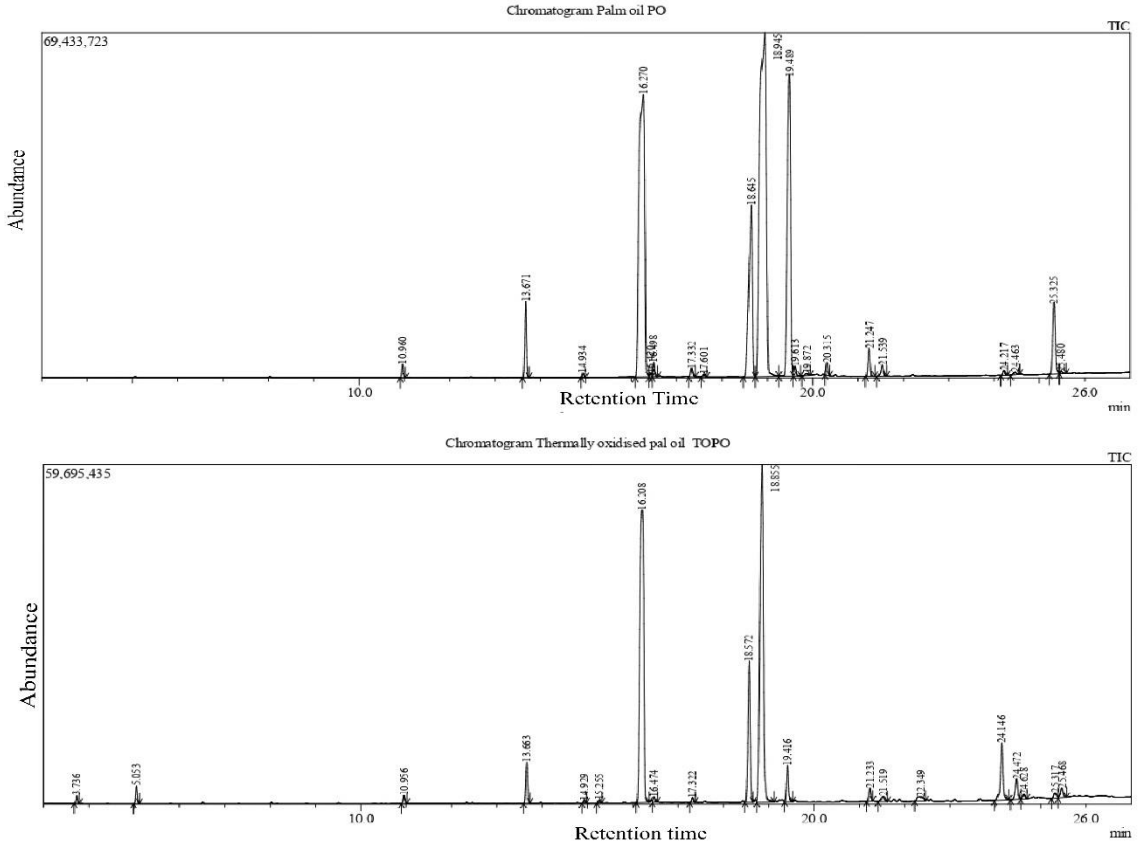

Figure 4.8: GS MS Chromatogram of Unoxidized and Thermally Oxidized Palm Oil. Chromatogram with Retention Time on the X-Axis and Abundance on the Y-Axis.

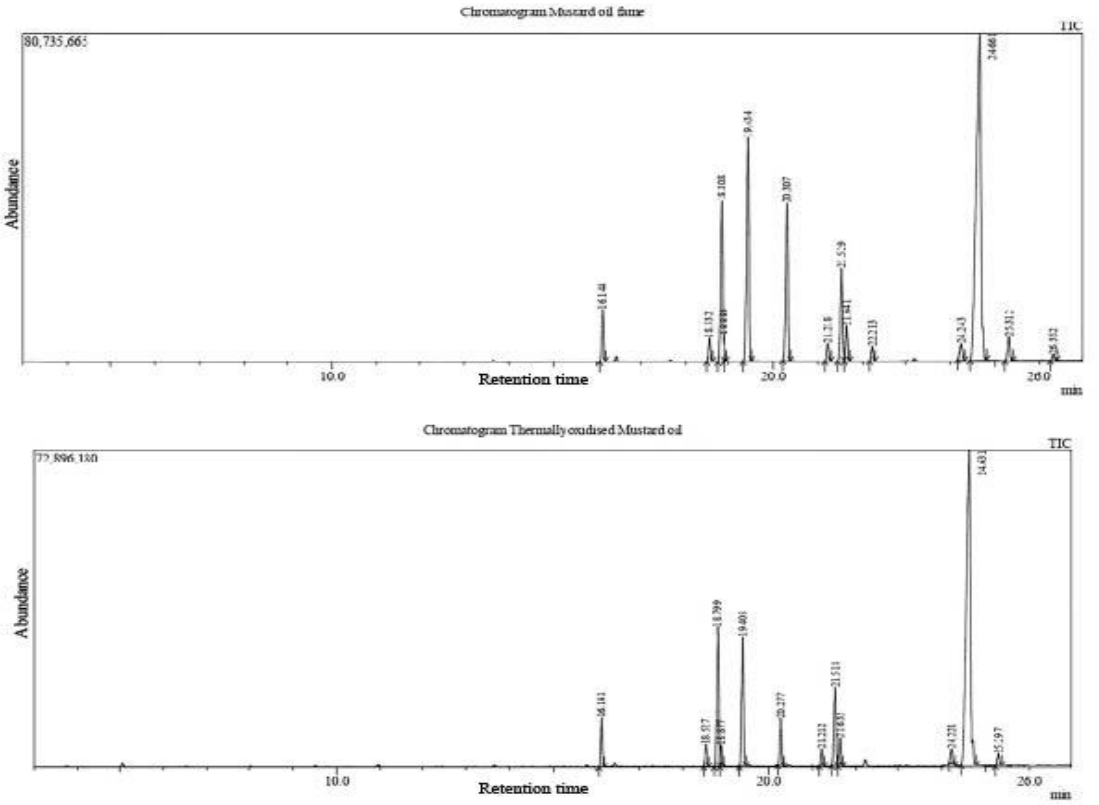

Figure 4.9: GS MS Chromatogram of Unoxidized and Thermally Oxidized Mustard Oil. Chromatogram with Retention Time on the X-Axis and Abundance on the Y-Axis.

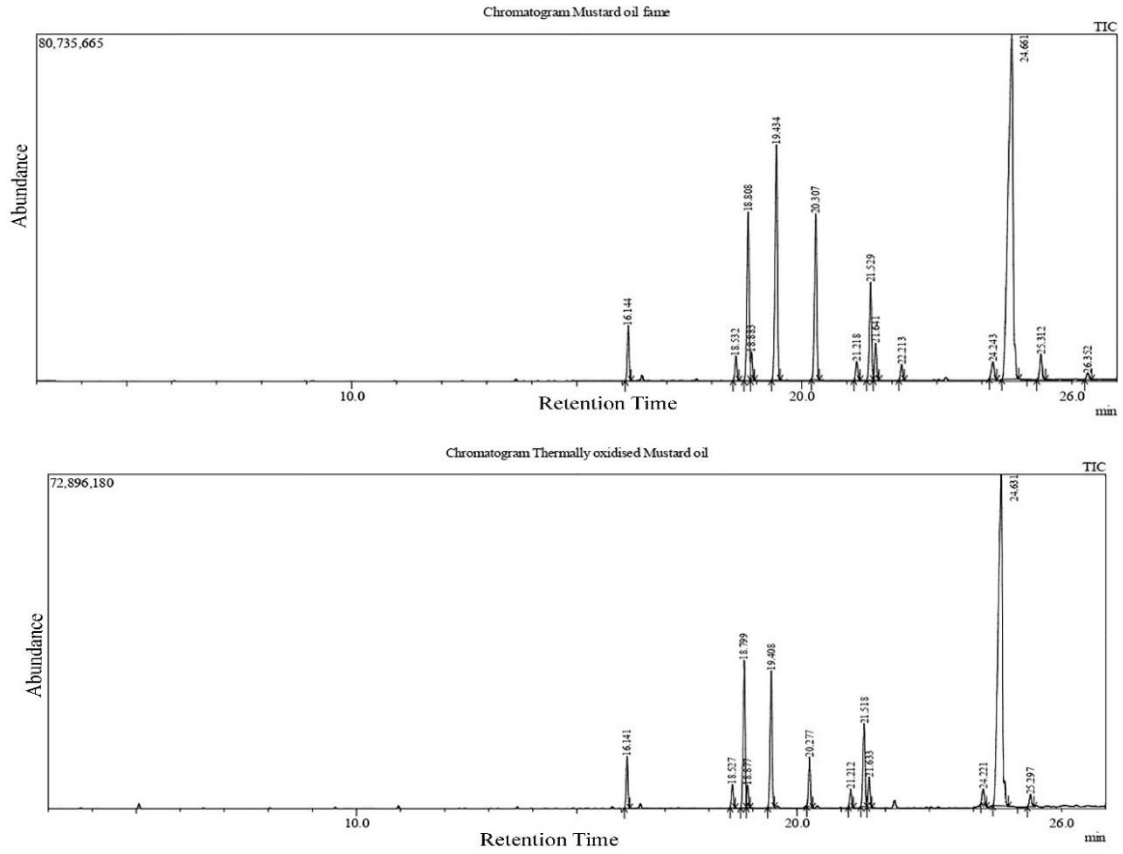

Figure 4.10: GS MS Chromatogram of Unoxidized and Thermally Oxidized Sunflower Oil. Chromatogram with Retention Time on the X-Axis and Abundance on the Y-Axis

4.5 Discussion

The physicochemical analysis of oils has elucidated a distinct amount of oxidation occurred in the thermal oxidation in contrast to their fresh counterparts. The colour and density of oils are important parameters for assessing the deterioration of oil in commercial and household situations. The colour of unoxidized oil was changed to darken under thermal treatment due to the formation of various carbonyl compounds which are the visual indicators of the chemical changes taking place within the oil. The rate of colour change depends on the composition of fatty acid, duration of heating and natural pigments present. More unsaturated fats are typically darkened with a high content of lipid oxidative products. On the other hand, there were the least colour changes in TCO with respect to their unoxidized oil. Different types of edible oils have varying densities due to their unique compositions and fatty acid profiles. Compared to unoxidized oils, saturated oils have shown more densities than oils with unsaturated fats. At the same time, saturated fatty acids containing CO and PO did not show any significant changes in density after the thermal oxidation of these oils. The oxidation process of edible oils can trigger secondary reactions, such as polymerisation and condensation reactions. These reactions can generate larger molecules with higher molecular weights, which may contribute to an increase in density. Another study indicates that heating edible oils to higher temperatures can increase trans fatty acid content, which could also contribute to an increase in density (Bhat et al., 2022).

According to DSC analysis of edible oils, SO and PO are thermally stable up to 225°C but lose their nutritive quality due to the thermal oxidation of polyunsaturated fatty acids (PUFA), specifically linoleic acid (Islam et al., 2022, Tamási and Marossy, 2022). While MO contains a significant amount of monounsaturated and polyunsaturated fats, it also has some saturated fats. However, it is less thermally stable for high-heat culinary practices than SO, PO and CO. Coconut oil (CO) has a higher proportion of

saturated fats, which are less susceptible to heat-induced alterations. FTIR analysis can help to identify functional groups associated with oxidation, including carbonyl groups, which can be indicative of the presence of FFAs resulting from oxidation. The composition of fatty acids in oils significantly influences the formation of new compounds during thermal oxidation. Modifications/changes in the composition of the fatty acid in the oils that underwent thermal oxidation are an important parameter to assess that has been done by FAME analysis. This analytical approach quantifies the extent to which oils are prone to oxidative reactions, offering valuable information regarding their stability and potential for degradation. It also revealed a significant reduction in unsaturated fatty acids, accompanied by a concomitant increase in the proportion of saturated fatty acids. The percentage of unsaturated fatty acids present in the unoxidized CO, PO, MO, and SO were 10.08%, 55.38%, 93.68%, and 88%, respectively, which decreased to 2.63%, 40.22%, 92.11% and 80.75% respectively when thermally oxidation. The saturated fatty acids of unoxidized CO, PO, MO, and SO were 89.92%, 44.62%, 6.31%, and 12%, respectively, which increased to 97.37%, 59.78%, 7.88%, and 19.25% in the respective in thermally oxidized oils. Several studies have shown that the thermal oxidation of fats leads to a decrease in UFAs due to peroxidative reactions, scission, cyclisation, and other side reactions (Szabo et al., 2022, Liu et al., 2021). The rise in saturated fat content in thermally oxidized oils is mainly caused by the degradation of UFAs during thermal oxidation processes. Studies showed that linoleic acid (LA) content decreases significantly during thermal treatment, while oleic acid, and palmitic acid increases. The formation of CDs and other oxidative products indicate the degradation of UFAs which contributes to the rise in SFA levels (Sadoudi et al., 2014). Increased temperature and prolonged exposure to air enhance the oxidation rate and generate LOPs including hydroperoxides and aldehydes resulting in increases in the concentration of saturated fats in the thermally oxidized

oils (Winkler-Moser et al., 2020).

Thermal oxidation of oil can increase the TBARS, CT and CD values, indicating higher oxidative degradation and potentially harmful biological effects due to the presence of oxidative products. p-AV and CD and CT values and TBARS content were found to be higher in TSO, than TMO, followed by TPO compared to TCO. Increased lipid peroxidation can result in the breakdown of fatty acids, which would lower the percentage of free fatty acids. However, a rise in saturated/ unsaturated fatty acids can be formed from polyunsaturated fats. Several studies have reported that heating sunflower oil at high temperatures leads to an increase in the concentration of heptanoic acid, caprylic and azelaic acid, which are LOPs (Zhuang et al., 2022). Palm oil and mustard oil both contain roughly 16% linoleic acid. However, the CT, CD, TBARS content and increase in % free fatty acids of their thermally oxidized form were found to be different.

The AV of edible oil is a critical indicator of the presence of FFAs within the oils. Several reports have demonstrated that the AV of oils increases when oils are heated (e.g., deep frying). An increase in AV indicates increased hydrolysis of TAG and the presence of dicarboxylic acid formation during the thermal oxidation of oils. In this study, TPO and TSO exhibited higher AV values with increased azelaic acid content. In comparison with TPO, TSO have higher azelaic acid content. Azelaic acid is a 9-carbon dicarboxylic acid known to be formed from the oxidation of linoleic acid. Therefore, it is quite natural that SO with a higher amount of linoleic acid generates azelaic acid after thermal oxidation. Even though azelaic acid is reported to be non-toxic and used for the treatment of acne (Skin disorder) and is a proinflammatory molecule. Formation of dicarboxylic acid (azelaic acid) by thermal oxidation of TSO and TPO has also been found to cause an increase in AV, the presence of which was confirmed by FAME analysis.

Thermal oxidation of oils, especially those rich in PUFAs, significantly increased LOPs such as FFAs, peroxides, and aldehydes caused oxidative stress. ROS damages cells and is linked to diseases like CVD and cancer and oxidized lipids deplete antioxidants like vitamin E, weakening cellular defence against oxidative stress. Specific compounds such as acrolein and 4-HNE are noted for their reactivity and potential toxicity (Vieira et al., 2017). In summary, thermal oxidation of edible oils led to deterioration in their quality and stability, reduction in PUFA, and an increase in SFA. The result showed that the thermal oxidation of edible oil changed their physicochemical properties, particularly oils rich in unsaturated fats. The results showed that unsaturated fatty acid-rich oils were more prone to peroxidative degradation due to higher unsaturation in the carbon chain of fatty acids. Whereas, saturated fatty acid-rich oils exhibited enhanced peroxidation resistance, even at high temperatures.

In this study, LOPs formed during the thermal oxidation of oils include peroxides, aldehydes and dicarboxylic acids such as azelaic acid. Oils rich in PUFA such as SO, MO, and PO were particularly susceptible to lipid peroxidation when exposed to high temperatures. SO was found to have the highest levels of lipid oxidation products, including TBARS, CD, and CT values after thermal oxidation. While MO contains both MUFA and PUFAs, it still exhibited considerable oxidation, though to a lesser extent than SO. In contrast, oils such as CO and PO, which are rich in saturated fats, and low in unsaturated fats, were likely prone to form harmful oxidation products. So, these oils are safe for high heat cooking.

Chapter 5

Metabolic Modulation by Thermally Oxidized Coconut and Mustard Oils Promotes Cardiovascular Disease

5.1 Introduction

Coconut and mustard oils are widely accepted cooking oils in India, with CO being particularly popular in southern regions and MO in northern regions. These oils, while integral to traditional culinary practices, have been subjects of significant scientific scrutiny due to their complex fatty acid profiles and potential health impacts. Among these, coconut oil which contains medium-chain saturated fats, has been the subject of controversy due to concern about its potential risk for CVDs. While some pre-clinical studies suggest that CO may enhance HDL-C levels (Maiti et al., 2024), concerns remain about its high saturated fat content and its effects on LDL-C levels, which are critical risk factors for CVDs (Hewlings, 2020). Despite its saturated fat content, CO exhibits anti-inflammatory property and reduces lipid accumulation in liver and adipose tissues (Machado et al., 2023). In contrast, MO is known to have antioxidant and anti-inflammatory properties, largely attributed to a monounsaturated fatty acid, erucic acid. Despite these benefits, erucic acid has been criticized for its toxicity, with pre-clinical rodent models demonstrating its cardiotoxic effects. The heating of these oils at higher temperatures is a concern because it can lead to the formation of harmful oxidation and cyclisation products.

The preceding chapter has clearly shown that edible oils undergo oxidation and chemical alterations. Among the oils tested, unsaturated oils have undergone cis/trans changes, fatty acid alterations and significant peroxidative changes. While saturated fats have undergone addition and cyclic products. According to the current literature, these reactions are quite common to all the classes of fats but the products may vary. The observation is that nearly 1-2% of PUFA of unoxidized CO has been lost completely while the loss in MUFAs is only 68%. The resulting primary or secondary products documented in the TCO might have been attributed to the 7.45% observed

increase in saturated fats. Moreover, a rise in caprylic acid (1.3%), and cis-9,10-epoxy stearic acid (0.55%) is documented in TCO making it a unique combination of biological molecules that may influence metabolic reactions. MO is a monounsaturated fat rich oil characterized by high content of erucic acid, gadoleic acid, behenic acid, oleic acid and ω-3 fatty acids. In TMO, thermal oxidation results in a reduction of unsaturated fats, including beneficial ω-3 fatty acids, and a concomitant increase in saturated fats due to hydrogenation and free radical reactions. Additionally, TMO contains high-molecular-weight polymers and cyclic fatty acid monomers, which are absent in fresh MO. These changes not only reduce the oil's nutritional value but also introduce potentially harmful compounds, making it a distinct molecular entity from fresh MO.

Various degenerative disorders such as diabetes, atherosclerosis, dyslipidaemia, cancer, as well as cardiovascular diseases are thought to be significantly influenced by chronic oxidative stress and associated inflammation (Sharifi-Rad et al., 2020, Chaudhary et al., 2023). Many of the thermally oxidized species of both CO and MO have been found to be toxic and pro-inflammatory. These observations underscore the potential health risks associated with the consumption of thermally oxidized CO and MO. It is important to consider these factors in the context of long-term health outcomes and the development of preventive strategies to mitigate oxidative damage and its associated complications. In recent decades, numerous pathological conditions have underscored the critical role of metabolic functions in the context of various diseases. Even though CO and MO have been traditionally used in culinary practices and assume various health benefits, the metabolic influence of their thermally oxidized counterpart in relation to cardiovascular pathology is of utmost importance and is addressed herein in this chapter.

Based on the importance of metabolic functions in disease conditions, highlighted in

recent decades, the objectives of this study are to examine the effects of the consumption of normolipidic levels of oxidized and thermally oxidized coconut and mustard oil on lipid and carbohydrate metabolism as well as inflammatory markers. The study also focused on determining the impact of thermally oxidized oils varying in fatty acid composition on factors related to cardiovascular disorders, such as serum and tissue lipids, lipoproteins and, coagulation factors.

5.2 Materials and Methods

5.2.1 Preparation of Thermally Oxidized Oil

Coconut oil (KLF) and Mustard oil (VVV and Sons' enterprise) were procured from the local market in Thrissur, Kerala, and kept at -80º C to avoid auto-oxidation. The detailed procedure for the preparation and storage of thermally oxidized oils is outlined in Chapter 3.1.5.

5.2.2 Animals

In this study, male Wistar rats with body weights ranging from 160-180 g were selected. Detailed procedures for managing the animals are described in Chapter 3.1.6

5.2.3 Diets and Experimental Design

The experimental design, the preparation and composition of the oil diet were described in Chapter 2.2.1. Four groups of twenty-four male Wistar rats, each consisting of six animals. Over six months, the rats received a diet containing 5% either unoxidized or thermally oxidized coconut and mustard oils as a fat source.

Groups	Treatment/ Intervention
Group I (CO)	A diet containing 5% Unoxidized Coconut oil
Group II (TCO)	A diet containing 5% Thermally Oxidized Coconut oil
Group III (MO)	A diet containing 5% Unoxidized Mustard oil
Group IV (TMO)	A diet containing 5% Thermally Oxidized Mustard oil

5.2.4 Fasting blood glucose

Fasting blood glucose (FBS) was determined according to the manufacturer's instructions (Euro Diagnostic Systems Pvt Ltd, Tamil Nadu, India). The detailed procedure is described in Chapter 3.5.1.

5.2.5 Oral Glucose Tolerance Test

OGTT was performed to measure the response of glucose after the consumption of 2g/kg Bd. Wt of glucose. The detailed procedure for OGTT is described in Chapter 3.5.2.

5.2.6 Fasting Plasma Insulin

The fasting insulin level was determined using an ELISA kit from Origin Diagnostic and Research, Kerala, India, strictly following the manufacturer's instructions as described in Chapter 3.5.3.

5.2.7 HOMA Indices

HOMA indices are used to estimate IR and insulin sensitivity. The detailed procedure was described in Chapter 3.5.4

5.2.8 Assessment of the Coagulation System

Various parameters of coagulation factors such as bleeding time, clotting time, platelet count, prothrombin time, and Activated Partial Thromboplastin time (APTT) were determined and used to assess the primary homeostasis. The detailed procedure was described in Chapter 3.6

5.2.9 Systemic Toxicity

Liver and renal function markers were analysed in serum samples to assess the impact of the consumption of unoxidized and thermally oxidized oils on the liver and renal performance. The parameters, include ALT, AST, ALP, albumin, total protein, bilirubin, urea, and creatinine. These tests were conducted using a kit from Euro Diagnostic Systems Pvt Ltd, Tamil Nadu, India. The comprehensive description of the

methodology is provided in Chapter 3.7

5.2.10 Lipid Metabolism

Serum total cholesterol, HDL-cholesterol, and both serum and tissue triglycerides were quantified by kits from Euro Diagnostic Systems Pvt. Ltd, Tamil Nadu, India, following the manufacturer's standard protocol. The detailed procedure is described in Chapter 3.8. Tissue Cholesterol was measured spectrophotometrically, the detailed procedure is in Chapter 3.8.3. Lipid metabolic enzymes including HMG Co. A reductase activity was assessed indirectly by determining the HMG Co. A/mevalonate ratio, which is described in Chapter 3.8.5, and hepatic lipase activity was measured spectrophotometrically with the procedure described in Chapter 3.8.6.

5.2.11 Carbohydrate Metabolism

Isolation and purification of mitochondria of hepatic tissue were carried out by density gradient method and activities of Fructose 1,6, bisphosphatse (FBPase), ICDH, MDH, and SDH were measured spectrophotometrically to evaluate the impact of oil on carbohydrate metabolism. The detailed procedure is in Chapter 3.9.

5.2.12 Inflammatory markers

Inflammatory markers such as CRP, LP-PLA_2, IL6, and IL10 were quantitatively using Biochemical and ELISA methods. The detailed procedures are given in Chapter 3.10. specifically, the method for determining Lp-PLA_2 activity is described in Chapter 3.10.2.

5.3 Histopathological analysis

The 10% formalin-fixed hepatic tissues were processed by paraffin embedding technique to visualize the hepatic tissue, heart, and kidney tissue architecture; sections of 4 μm thickness were prepared using a rotary microtome and stained with haematoxylin and Eosin (H and E) dye. The tissues were analyzed under 100× magnification using an inverted microscope (Magnus, INVI). The detailed procedure

is described in Chapter 3.12.

5.4 Statistical analysis

Data was expressed as mean ± SD (6 animals/ group). Statistical analysis was done by GraphPad Prism (prism 9) software. Two-way ANOVA was conducted to assess the effect, which showed significant differences between dietary groups. The detailed procedure is described in Chapter 3.13.

5.5 Results

5.5.1 Changes in Body Weight and Relative Organ Weights of Experimental Animals

After acclimatization, Wistar rats were allocated to access an unoxidized and thermally oxidized coconut (CO, TCO) and mustard oil (MO, TMO) incorporated diet for six months. All these individual animals gained body weight continuously during the experimental period (Figure 5.1a). The mean body weight gain percentage in CO, TCO, MO, and TMO diet-fed group rats were 80.16 ± 8.22, 78.38 ± 9.79, 79.21 ± 9.82, and 78.27±9.73g% respectively, indicating no significant difference among the groups (Figure 5.1b). The organosomatic index of the liver tissues was measured as 3.06 ± 0.13% for CO, 3.31± 0.22% for TCO, 3.04 ± 0.26% for MO, and 3.13±0.22% for TMO, with no significant variation observed across the dietary groups. Additionally, there were no significant changes in the relative weight of kidney, heart and adipose tissue. The relative kidney weight was 0.65 ± 0.06% for CO, 0.65 ± 0.05% for TCO, 0.61 ± 0.06% for MO and 0.64 ± 0.05 % for TMO diet-fed groups, respectively. The relative organ weight of the heart was found to be 0.3 ± 0.03% for CO, 0.31±0.02% for TCO, 0.27±0.02% for MO, and 0.28 ± 0.03% for TMO diet-fed groups. The relative organ weight of the adipose tissue was found to be 2.13±0.37% (CO), 1.91 ±0.25% (TCO), 1.98 ±0.47% (MO), and 1.62 ±0.2% for the TMO diet-fed group. (Fig. 5.1c).

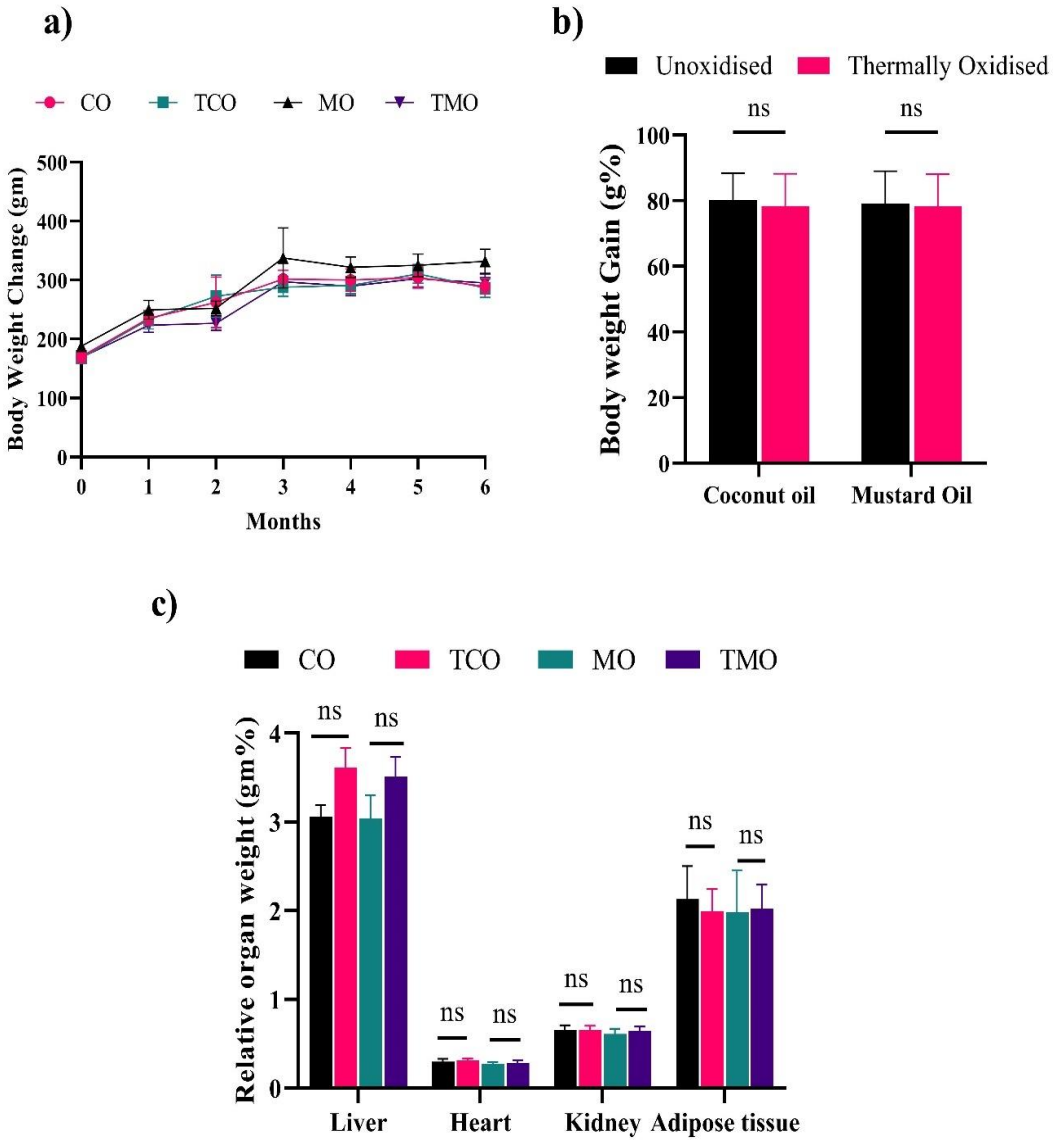

Figure 5.1: a) Body weight change, b) weight gain (g%), and c) relative organ weight of experimental rats assigned to different dietary interventions of unoxidized and thermally oxidized coconut oil and mustard oil for 6 months. Values are expressed as the Mean±SD of 6 animals. Statistical analysis was done by GraphPad Prism software (Prism 9). A two-way ANOVA was conducted to assess the effect of oils and oxidation. Pot hoc test was performed using Tukey's multiple comparison test to determine significant differences between groups. $p<0.05$ is significant, and ns indicates non-significant. CO unoxidized coconut oil, TCO thermally oxidized coconut oil, MO unoxidized Mustard oil, TMO thermally oxidized mustard oil.

5.5.2 Glycaemic Response

Before and after the study period, an oral glucose tolerance test (OGTT) was conducted to evaluate the effects of the coconut and mustard oil diet on the glycaemic response of rats (Figure 5.2a and 5.2b). The total area under the curve (AUC) of the OGTT was calculated for each experimental group. In the initial GTT, all groups displayed a glucose spike at 30 minutes post-administration, followed by a decline in glucose levels over the next 120 minutes with similar glucose clearance in all groups. In the final GTT conducted after the experimental period, specifically, rats fed unoxidized and thermally oxidized coconut oil displayed significantly ($p<0.05$) better glucose tolerance regardless of their oxidation (Figure 5.2c). Conversely, mustard oil did not show a significant difference in AUC between unoxidized and thermally oxidized forms (Figure 5.2d).

As shown in Figure 5.3a, the average insulin level for the CO diet group was 2.17±0.32 IU/L, which was 25% higher than the average insulin level of the TCO diet-fed group (1.74 ±0.20 IU/L). Similarly, the average insulin level for the MO diet group was 3.55 ± 0.30 IU/L, which was 50% higher than the average insulin level for the TMO (1.49±0.24 IU/L) diet group. There was a significant change ($p<0.01$) observed among between both unoxidized and thermally oxidised mustard oil fed diet groups. Insulin resistance was determined by HOMA-IR indices. A higher HOMA- IR index indicates greater insulin resistance. Insulin resistance remained unchanged in CO (0.49 ± 0.04) and TCO (0.40 ± 0.03) diet-fed animals; the HOMA-IR was significantly ($p<0.01$) change in between MO and TMO diet fed groups. The higher in HOMA-IR in MO (0.68 ± 0.03) diet-fed group was found and it was 0.29 ± 0.03 for TMO diet-fed group. HOMA-IR is represented in Figure 5.3b. HOMA-S indicates insulin sensitivity. There were no significant changes in HOMA-S between CO (219.13±26.3) and TCO (239.74±9). The TMO group (325.17±35.2) showed significantly improved insulin

sensitivity ($p<0.01$) compared to the MO group (151.57±9.8) (Figure 5.3c). There is no significant difference (ns) in β-cell function (HOMA-B) between CO (42.51± 7.23) and TCO (46.61±5.09) indicating that consumption of TCO does not adversely affect the pancreatic β-cells ability to produce insulin. MO (69.34±7.02) showed significantly ($p<0.01$) higher HOMA-B compared to TMO (43.24±5.4) (Figure 5.3d).

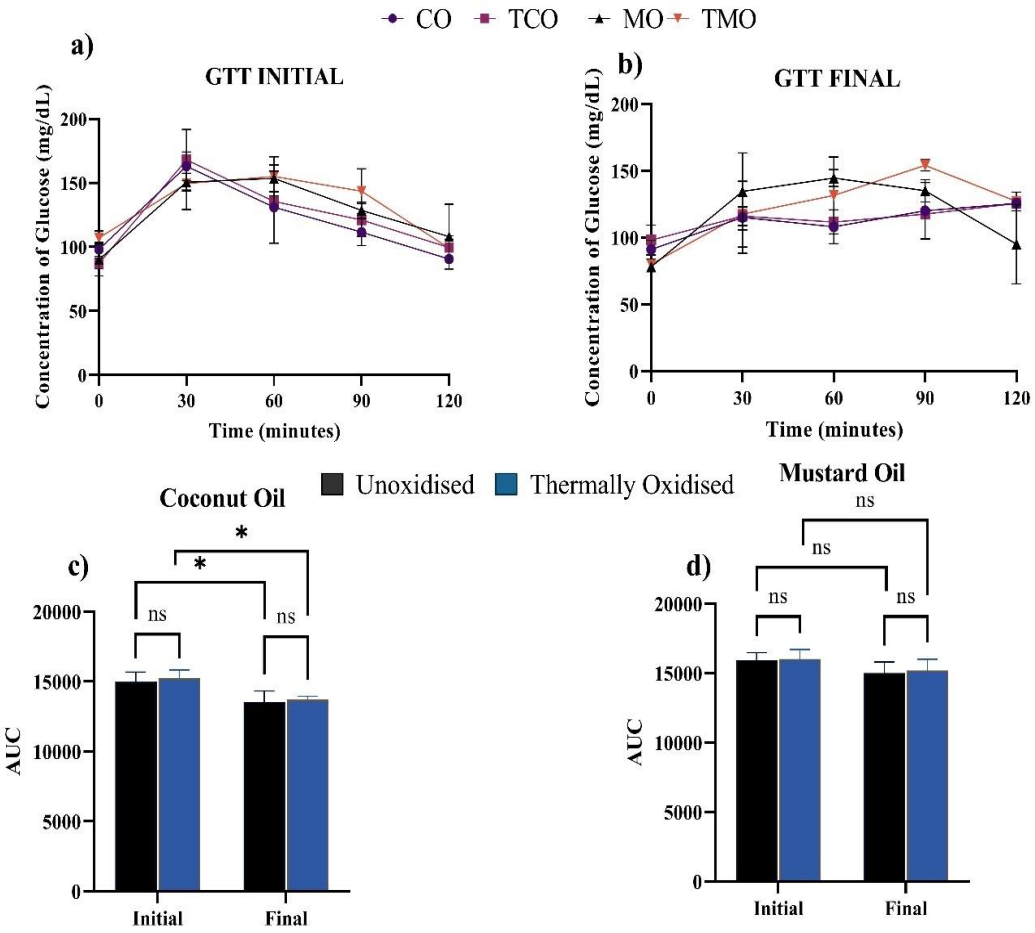

Figure 5.2: Glucose tolerance test of animals consumed 5% unoxidized or thermally oxidized coconut and mustard oil for 6 months. Initial and final oral glucose tolerance test (a and b), the total area under the curve (AUC) of initial and final OGTT (c and d) of Wistar rats. Data are presented as mean ± SD (n=6 rats per group). Results indicated the difference among the group was significant, with $p<0.05$ considered, statistical analysis was performed using Two–way ANOVA followed by Tukey's multiple comparison test, where ns, *, denotes non-significant $p<0.05$, respectively.

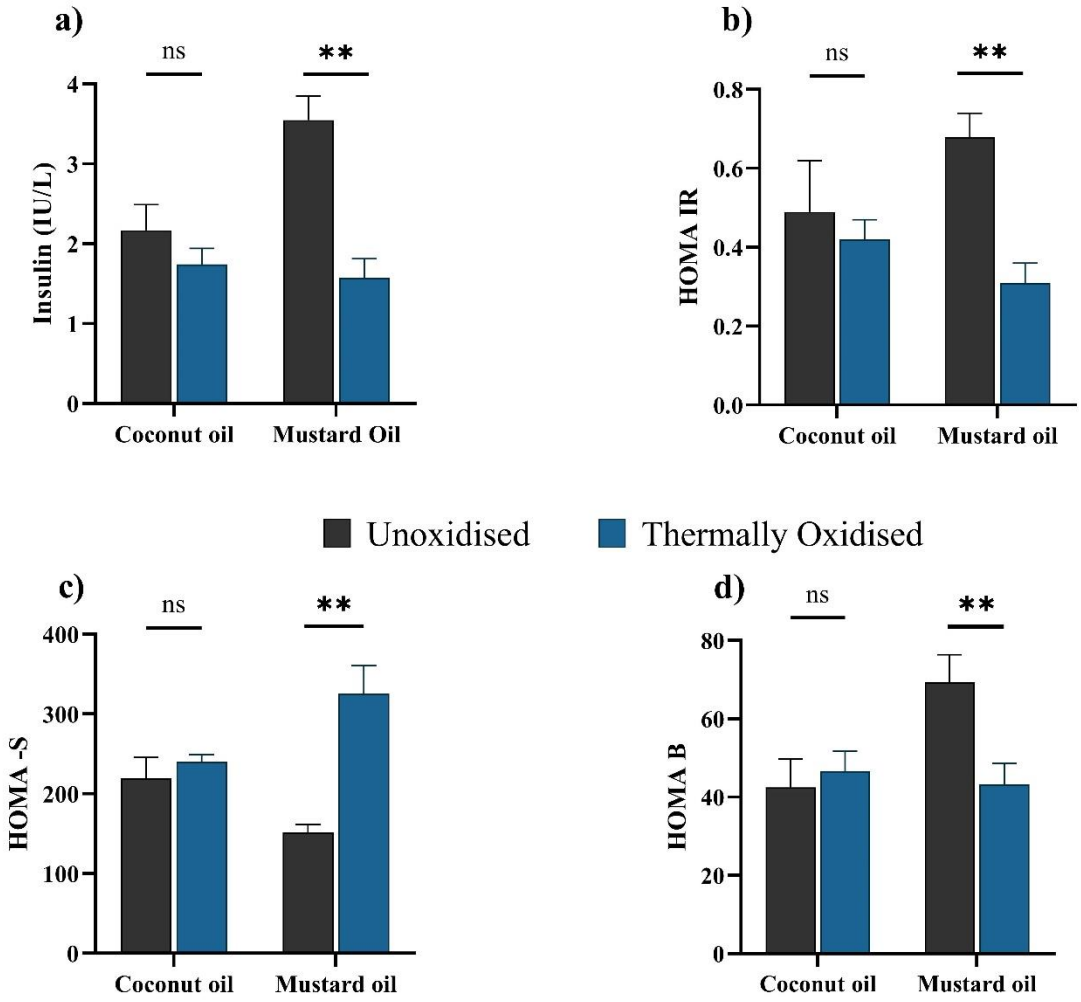

Figure 5.3: Serum insulin and HOMA indices of animals consumed 5% unoxidized or thermally oxidized coconut and mustard oil for 6 months. a) Serum insulin b) HOMA- IR c) HOMA-S d) HOMA -B. Data are presented as mean ± SD (n=6 rats per group). The result indicated the differences among the group were significant, with $p<0.05$ considered, statistical analysis was performed using Two–way ANOVA followed by Tukey's multiple comparison test, where ns, **, denotes non-significant $p<0.01$, respectively.

5.5.3 Coagulation system

Table 5.1 shows the effect of unoxidized and thermally oxidized coconut and mustard oil on various blood clotting parameters. Platelet counts in the CO, TCO, MO, and TMO fed groups were 7.55 ± 0.26, 7.7 ± 0.41, 7.65±0.71, and 7.5±0.80 lakhs/mm^3, respectively with no significant variation found among the diet-fed groups. No significant changes were found in the initial and final bleeding time and clotting time among the groups. The values of the initial and final bleeding time were expressed in

Table 5.1. Prothrombin time for CO, TCO, MO, and TMO groups was 45.33± 5.4, 49.4 ± 3.8, 47.17± 5.1, and 47.67± 4.8 seconds, respectively. There were no significant changes found among the groups. Activated Partial thromboplastin time (APTT) for CO, TCO, MO, and TMO groups was 66.2±2.6, 70.6±1.4, 68±1.5, and 68.7±2.5 seconds, respectively. APTT for TCO groups was significantly ($p<0.05$) higher than CO diet-fed groups.

Table 5.1: Effect of Oil Diet on Blood Clotting Parameters of Experimental Rats**.**

Parameters		**Coconut Oil**		**Mustard Oil**	
		Unoxidized	Thermally Oxidized	Unoxidized	Thermally Oxidized
Platelet Count (lakhs/mm^3)		7.55±0.26	7.7±0.41^{ns}	7.65±0.71	7.5±0.80^{ns}
Bleeding Time (Minutes)	**Initial**	2 ± 0	1.3 ± 0.58^{ns}	2 ± 0.5	1.5 ± 0.41^{ns}
	Final	1.38±0.5	1.2 ± 0.45^{ns}	1.5 ± 0	1.25 ± 0.29^{ns}
Clotting Time (Minutes)	**Initial**	1 ± 0.35	1 ± 0.35^{ns}	1 ± 0.35	1.25 ± 0.5^{ns}
	Final	1 ± 0	1 ± 0^{ns}	1 ± 0	1 ± 0^{ns}
Prothrombin Time (Sec)		45.33± 5.4	49.4 ± 3.8^{ns}	47.17± 5.1	47.67± 4.8^{ns}
APTT (Sec)		66.2±2.6	70.6±1.4*	68±1.5	68.7±2.5^{ns}

Data are expressed as Mean ±SD (n =6 per group). Comparisons among the oil diet groups were conducted using two–way ANOVA followed by Tukey's multiple comparison test to assess the differences. $p<0.05$ is considered statistically significant. ns, non-significant, * $p<0.05$.

5.5.4 Systemic toxicity

Table 5.2 shows the values for various liver function and renal function markers. There was no significant change in total bilirubin, total protein, albumin, globulin, and SGPT among the experimental groups. The total bilirubin levels were unaltered among the groups, with values of 0.33 ±0.05mg/dL for CO, 0.36 ±0.05mg/dL for TCO, 0.34±0.05 mg/dL for MO, and 0.33±0.05 mg/dL for TMO diet-fed groups. The total protein levels showed no significant variation in 7.38 ±0.48mg/dL for CO, 7.47 ± 0.31mg/dL for

TCO, 7.5 ±0.21 mg/dL for MO, and 7.48±0.36 mg/dL for TMO diet-fed group. Additionally, the albumin level was similar among the groups. The values of CO, TCO, MO, and TMO given group animals were 3.59 ± 0.15, 3.54 ± 0.15, 3.56 ± 0.21, and 3.59 ± 0.12 mg/dL respectively. The globulin level was unaltered and the values of CO, TCO, MO, and TMO diet group animal were 3.97 ± 0.41, 3.98 ± 0.17, 4.01±0.10, and 4.1 ±0.3 mg/dL, respectively with no significant variation. The SGPT levels of CO, TCO, MO, and TMO diet group animals were 62.7 ± 6.9, 67 ± 6.3, 70 ± 7 and 69 ± 7.2 U/L respectively, indicating no significant differences. The SGOT levels were lower in the CO (58.5 ± 1.2 U/L) group compared to the TCO (69 ± 3.34 group and both the MO (75± 4.71 U/L) and the TMO (67 ± 2.2 U/L) groups. The ALP levels were elevated in the TCO group (107.7 ± 12.8 U/L) compared to the CO group (85.8 ± 10.6 U/L), MO (94 ±9.5 U/L) and TMO (97.81±9.5U/L) diet-fed groups. Elevated ALP levels can indicate liver or bone tissue diseases. The AST/ALT ratio did not show any significant variation among the groups, with the values of CO, TCO, MO, and TMO diet group rats 0.9 ± 0.2, 1 ± 0.1, 1.1±0.1, and 1.0±0.2 was respectively.

Creatinine is a waste product of muscle metabolism and excreted by the kidneys. It is used as a marker of kidney function. There was no significant difference in creatinine levels among the experimental groups. The creatinine levels of the CO, TCO, MO, and TMO diet group were 1.26 ± 0.28, 1.24± 0.2, 1.14 ± 0.15 and 1.28 ± 0.26 mg/dL, respectively, with no significant variation among the groups. Urea is a waste product produced when the body breaks down proteins and can be used as an indicator of kidney function. There was no significant difference among the experimental groups. The urea levels of the CO, TCO, MO, and TMO diet ingested animals were 29.3± 1.8, 29.2 ± 2.4, 23.48 ± 2.78, and 25 ± 2 mg/dL, respectively (Table 5.2).

Table 5.2. The Influence of Oil Diet on Liver and Renal Functions of Experimental Rats.

Parameters	Coconut Oil		Mustard Oil	
	Unoxidized	Thermally Oxidized	Unoxidized	Thermally Oxidized
Total Bilirubin(mg/dL)	0.33 ±0.05	0.36 ±0.05^{ns}	0.34±0.05	0.33±0.05^{ns}
Total protein (mg/dL)	7.38 ±0.48	7.47 ± 0.31^{ns}	7.5 ±0.21	7.48±0.36^{ns}
Albumin (mg/dL)	3.59 ± 0.15	3.54 ±0.15^{ns}	3.56±0.21	3.59 ±0.12^{ns}
Globulin (mg/dL)	3.97±0.41	3.98±0.17^{ns}	4.01 ±0.10	4.1±0.3^{ns}
SGPT (U/L)	62.7± 6.9	67± 6.3^{ns}	70 ± 7	69 ± 7.2^{ns}
SGOT (U/L)	58.5 ± 1.2	69± 3.34*	75± 4.71	67 ± 2.2^{ns}
ALP (U/L)	85.8±10.6	107.7±12.8*	94 ±9.5	97.81±9.5^{ns}
AST/ALT (U/L)	0.9±0.2	1±0.1^{ns}	1.1±0.1	1.0±0.2^{ns}
Creatinine (mg/dL)	1.26 ± 0.28	1.24 ± 0.2^{ns}	1.14 ± 0.15	1.28 ± 0.26^{ns}
Urea (mg/dL)	29.3 ± 1.8	29.2 ± 2.4^{ns}	23.48 ± 2.78	25 ± 2^{ns}

Values are expressed as Mean ± SD of 6 animals. A two-way ANOVA was conducted to assess the effect of oils and oxidation. Post hoc tests were performed using Tukey's multiple comparison test to determine significant differences between groups. $p<0.05$ is considered significant. * $p<0.05$, ns indicates non-significant.

5.5.5 Lipid Metabolism

Table 5.3 shows the values of serum lipid profile under four different dietary conditions i.e., unoxidized and thermally oxidized coconut (CO, TCO) and mustard oil (MO, TMO) diet-fed group animals. Serum triglyceride levels did not show significant change in both the CO (40.9 ± 7.2 mg/dL) and TCO (41.8 ± 4.5 mg/dL) incorporated diet-fed groups. While there observed a significant change ($p<0.05$) between MO (33.7 ± 8.2 mg/dL) and TMO (46.8 ±8.9 mg/dL) diet-fed groups. Similarly, there was a slight decrease in serum cholesterol levels with TCO (57.95 ± 4.6 mg/dL) compared to CO (65.8 ± 6.2 mg/dL). There was a moderate increase in cholesterol levels in the TMO (72.66±7.6 mg/dL) diet-fed group animals compared to the MO (65.9 ±6.3 mg/dL) diet

groups. Serum HDL-C levels were significantly ($p<0.05$) lower in the TMO (26.4 ±4.3 mg/dL) diet group than in MO (32.3 ± 4.4 mg/dL) diet fed group rats. There were no significant variations among CO (36.8 ± 4.0 mg/dL) and TCO (34.6 ± 4.4 mg/dL) diet fed animals. Serum LDL-C levels were significantly higher in both mustard oil diet fed group rats compared to both coconut oil diet fed groups. TMO-fed animals had significantly ($p<0.05$) increased LDL-C levels than MO-fed ones. The LDL-C levels of MO group was 28.5 ± 3.3 mg/dL and TMO group was 37.8 ± 5.3 mg / dL. However, no significant variation in LDL-C levels was observed between CO (23.1 ± 3.7 mg/dL) and TCO (21.78 ± 4.1 mg/dL) fed group animals. There were no statistically significant differences in VLDL-C levels were observed among the groups. The values of the CO, TCO, MO, and TMO diet groups were 7.3 ± 2.5, 6.7 ± 1.8, 10.5 ±1.1, and 8.5 ± 3.4 mg/dL, respectively. However, TMO-fed animals documented marginally higher VLDL-C levels.

Hepatic TG level was significantly ($p<0.05$) increased in the TMO (748.04 ± 52.22mg/100g tissue) fed group compared to CO, TCO, and MO diet-fed groups (624.11 ± 57.86, 663.83 ± 73.76, and 658.16 ± 65.41 mg/g tissue) respectively. There were statistically ($p<0.01$) significant variations in hepatic cholesterol levels between the coconut and mustard oil diet groups. The values of the CO, TCO, MO, and TMO diet group animals were 99.49 ± 15.53, 112.73 ± 12.30, 167.33 ± 24.08, and 160.12 ± 19.39mg/100g tissue, respectively. Compared to both CO-fed animals, higher level of hepatic TC was observed in both MO diet-fed group animals. The TMO group was also observed to have significantly ($p<0.01$) lower phospholipid levels (428.42 ± 53.3 mg/100g tissue) when compared to the MO group (766.32±55.6 mg/100g tissue). TCO group which exhibited significantly ($p<0.01$) lowest phospholipid levels (412.28 ±66.0 mg/100g tissue) when compared to CO group (504.09 ± 25.6 mg/100 g tissue).

Table 5.3. Serum and Hepatic Lipid Profile of Oil-Diet-Fed Rats

	Parameters	Coconut Oil		Mustard Oil	
		Unoxidized	Thermally Oxidized	Unoxidized	Thermally Oxidized
Serum Lipid Profile (mg/dL)	Triglycerides	40.9 ±7.2	41.8 ±4.5^{ns}	33.7 ±8.2	46.8 ±8.9**
	Cholesterol	65.8 ±6.2	57.95 ±4.6^{ns}	65.9 ±6.3	72.66±7.6^{ns}
	HDL- C	36.8 ±4.0	34.6 ±4.4^{ns}	32.3 ±4.4	26.4 ±4.3*
	LDL-C	23.1±3.7	21.78 ±4.1^{ns}	28.5 ±3.3	37.8 ±5.3*
	VLDL -C	7.3 ±2.5	6.7 ±1.8^{ns}	10.5 ±1.1	8.5 ±3.4^{ns}
Hepatic Lipids (mg/100g)	Triglycerides	624.11±57.86	663.83±73.76^{ns}	658.16±65.41	748.04±52.22*
	Cholesterol	99.49±15.53	112.73±12.30^{ns}	167.33±24.08	160.12±19.39^{ns}
	Phospholipids	504.09 ±25.6	412.28 ±66.0**	766.32± 55.6	468.42±53.3**

Values are expressed as the Mean±SD of 6 animals. A two-way ANOVA was conducted to assess the effect of oils and oxidation. Pot hoc test was performed using Tukey's multiple comparison test to determine significant differences between groups. $p<0.05$ is considered significant, and * $p<0.05$, ** $p<0.01$, ns indicates non-significant.

Figure 5.4 depicts the hepatic lipase and HMG Co. A activities across different dietary groups. The lipase activity was significantly ($p<0.01$) higher in the TMO (61.10±9.13 U/mg protein) fed than in the MO (27.34±3.59 U/mg protein) fed group animals. Conversely, no significant variation was found between the CO (32.15±8.01 U/mg protein) and TCO (38.3±9.82 U/mg protein) fed groups (Figure 5.4a). Figure 5.4b shows the hepatic HMG Co. A reductase activity based on an indirect assessment of the HMG Co. A/ mevalonate ratio for the CO, TCO, MO, and TMO diet-fed group. In the CO diet fed group, the ratio was 1.51±0.31, while in the TCO diet fed, it was 1.93±0.12, indicating a significant reduction ($p<0.05$) in HMG Co. A reductase activity in the TCO fed animals compared to the CO. However, there were no significant variations in the HMG Co. A/mevalonate ratio between the MO (1.44±0.33) and TMO (1.41±0.10) oil diet-fed groups.

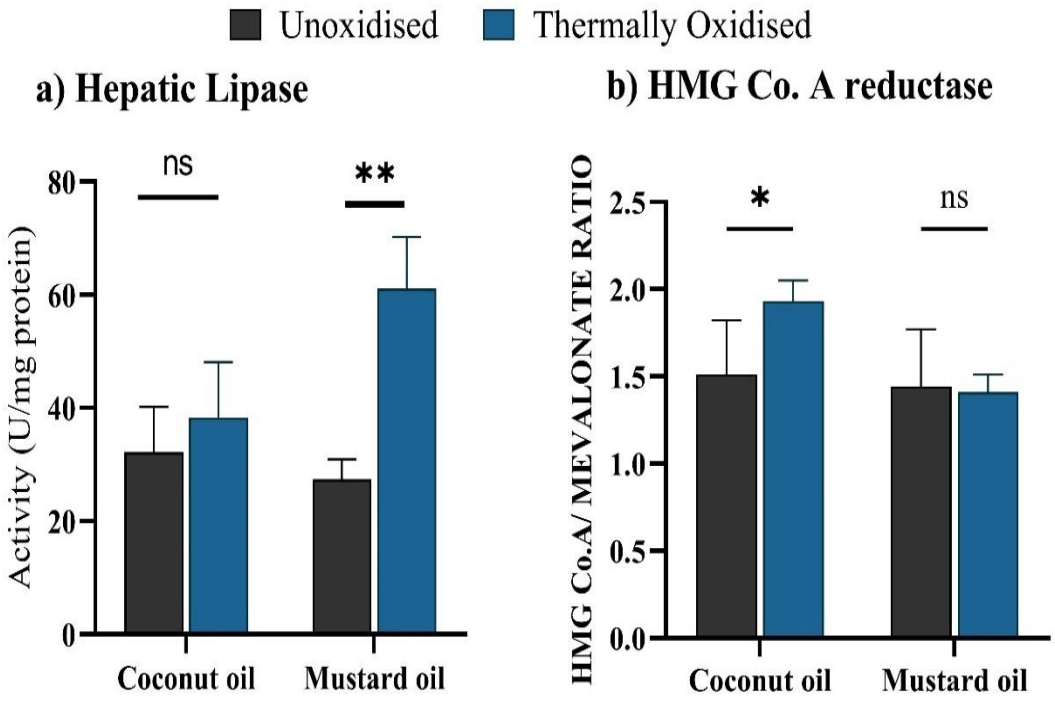

Figure 5.4 Hepatic lipase and HMG Co. A enzyme activities of experimental animals a) hepatic lipase and **b**) HMG Co. A reductase activity. Data represented as Mean ± SD of 6 animals per group. Statistical analysis was conducted using GraphPad Prism employed with Two – way ANOVA followed by Tukey's multiple comparison test. * $p<0.05$, **$p< 0.01$, ns non-significant between oil diet groups.

5.5.6 Carbohydrate Metabolism

Activities of carbohydrate metabolising enzymes were measured after the consumption of an unoxidized and thermally oxidized coconut (CO, TCO) and mustard oil (MO, TMO) diet. The data in Figure 5.5a illustrates that the mean FBPase activity for the coconut oil group was 1.55 ± 0.24 μg of Pi formed/min/mg protein, and for the thermally oxidized coconut oil group was 1.92 ± 0.29 μg of Pi formed/min/mg protein, with no significant changes between CO and TCO oil diet-fed groups. In contrast, there was no statistically significant elevation in FBPase activity observed in rats fed with MO (3.25±0.24 μg of Pi formed/min/mg protein) and TMO (3.25 ± 0.33 μg of Pi formed/min/mg protein) diet-fed groups.

The given Figure 5.5 depicted the activity levels of three mitochondrial enzymes: SDH, MDH, and ICDH activities. SDH activity (Figure 5.5b) was significantly ($p< 0.05$) lower in TMO (26 ± 5.5 U/mg protein) than in MO (34 ± 6 U/mg protein) oil diet-fed groups. There was no statistical significance found in rats fed with a TCO diet, activity level of 30 ± 5 U/mg protein than the group that received the CO diet (28 ± 2 U/mg protein). Figure 5.5c shows MDH activity. MDH activity was significantly ($p<0.05$) increased in TMO diet (70.84 ± 9.7 U/mg protein) fed rats compared to those fed MO diet (55.76 ± 7.6 U/mg protein). No significant change was found between CO and TCO-diet-fed group rats. The MDH activities of the CO and TCO-fed animals were 60.49 ± 9.9, and 55.39 ± 9.8 U/mg protein respectively. The ICDH activities of the CO, TCO, MO, and TMO diet group were 116.21 ± 44.76, 103.4 ± 35.23 U/mg protein, 90.53 ± 10.85 U/mg protein, and 86.96 ± 31.51 U/mg protein, respectively (Figure 5.5d). There was no statistical significance among the group.

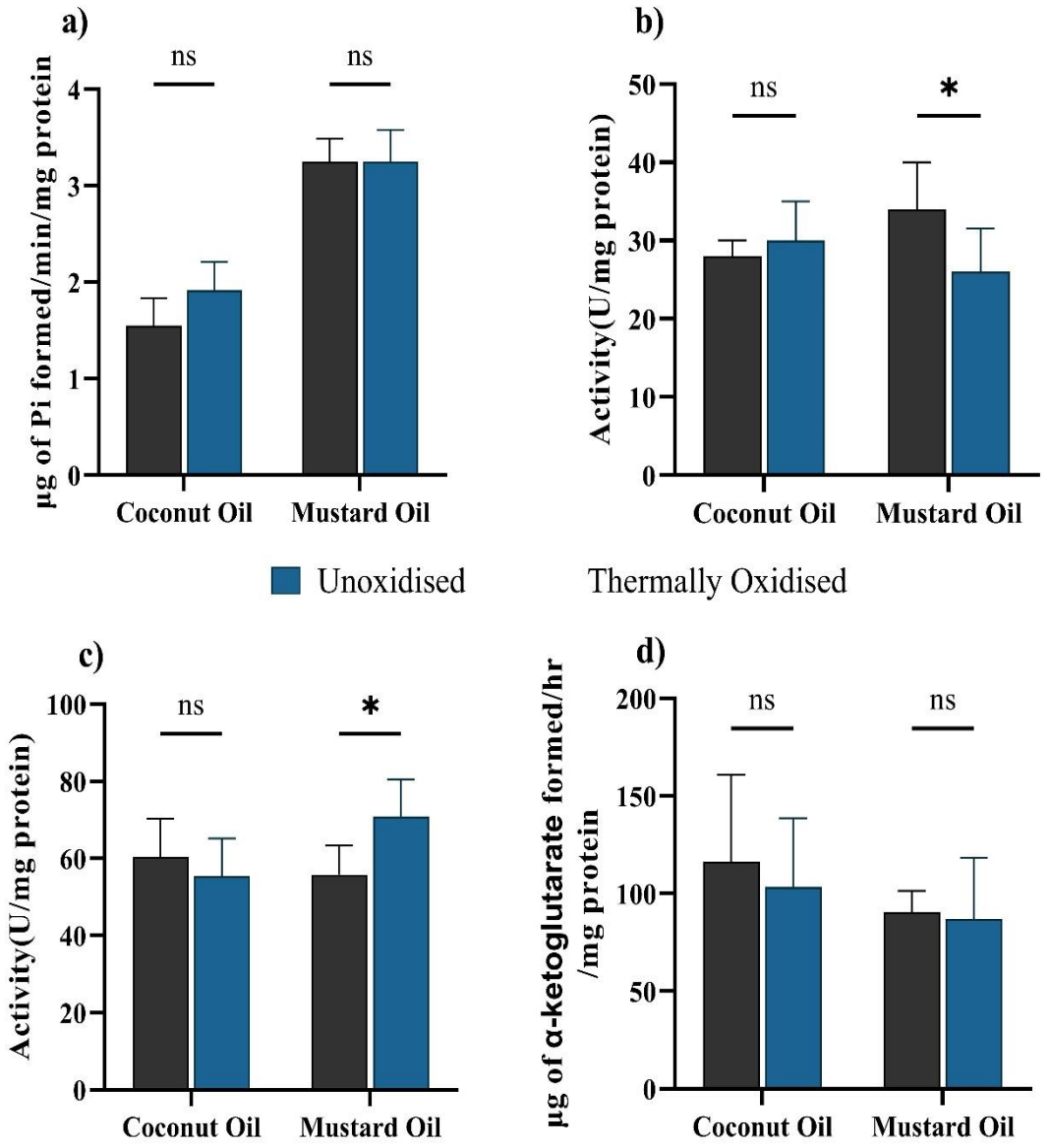

Figure 5.5. Mitochondrial enzyme activities in experimental groups: CO, TCO, MO and TMO were incorporated in the experimental diet at normolipidemic level over six months. a) hepatic FBPase, b) mitochondrial SDH, c) MDH and d) ICDH. Data represented as Mean ± SD of 6 animals per group. Statistical analysis was conducted using GraphPad Prism software (Prism 9), employed with Two – way ANOVA followed by Tukey's multiple comparison test *$p<0.05$, **$p<0.01$, ns non-significant when comparison between oil diet groups.

5.5.7 Inflammatory Markers

Figure 5.6 presents values of LP-PLA_2, CRP, IL-6, and IL-10 for diet-fed unoxidized and thermally oxidized coconut (CO, TCO) and mustard (MO, TMO) oils at normolipidemic levels over six months. Figure 5.6a represents LP-PLA_2 activity. The activity of LP-PLA_2 was significantly ($p<0.01$) elevated in the TMO rats (30.97±2.4 U/mg protein) compared to the MO group rats (24.53±3.2 U/mg protein). The LP-PLA_2 level of CO and TCO diet-fed groups were 19.97 ± 2.7 and 15.51 ± 1.2 U/mg protein. A significant decrease ($p<0.05$) was observed between unoxidized and thermally oxidized CO groups. Figure 5.6b represents the CRP level. The CRP level was significantly ($p < 0.01$) lower in both coconut oil diet-fed animals compared to both mustard oil diet fed ones. However, the CRP level did not show significant differences between CO and TCO-fed groups. The CRP levels were 0.55 ± 0.2 mg/dL for CO and 0.54 ± 0.1 mg/dL for the TCO group. Whereas the CRP level in MO incorporated diet fed group was 0.9±0.3 mg/dL the level of which was found to increase in TMO diet group animals (1.15±0.2 mg/dL). The IL-6 levels (Figure 5.6c) were significantly ($p<0.01$) higher in the TCO diet-fed group (1126 ± 132 pg/mL) compared to CO diet groups (400 ± 20 pg/mL). The IL-6 levels in the MO diet-fed group were 533 ± 176 pg/mL, which was slightly reduced in TMO diet-fed group (408 ±72 pg/m) but not statistically significant. The IL-10 levels (Figure 5.6d) were significantly ($p<0.01$) lower in the TCO diet-fed group (81.23 ± 13.44 pg/mL) compared to the TCO diet groups (178.40 ± 35.35 pg/mL). There was no statistically significant change was found in the IL-10 levels between the MO (303.33 ±27.40 pg/mL) and TMO (326.85 ± 95.80 pg/mL) fed diet groups.

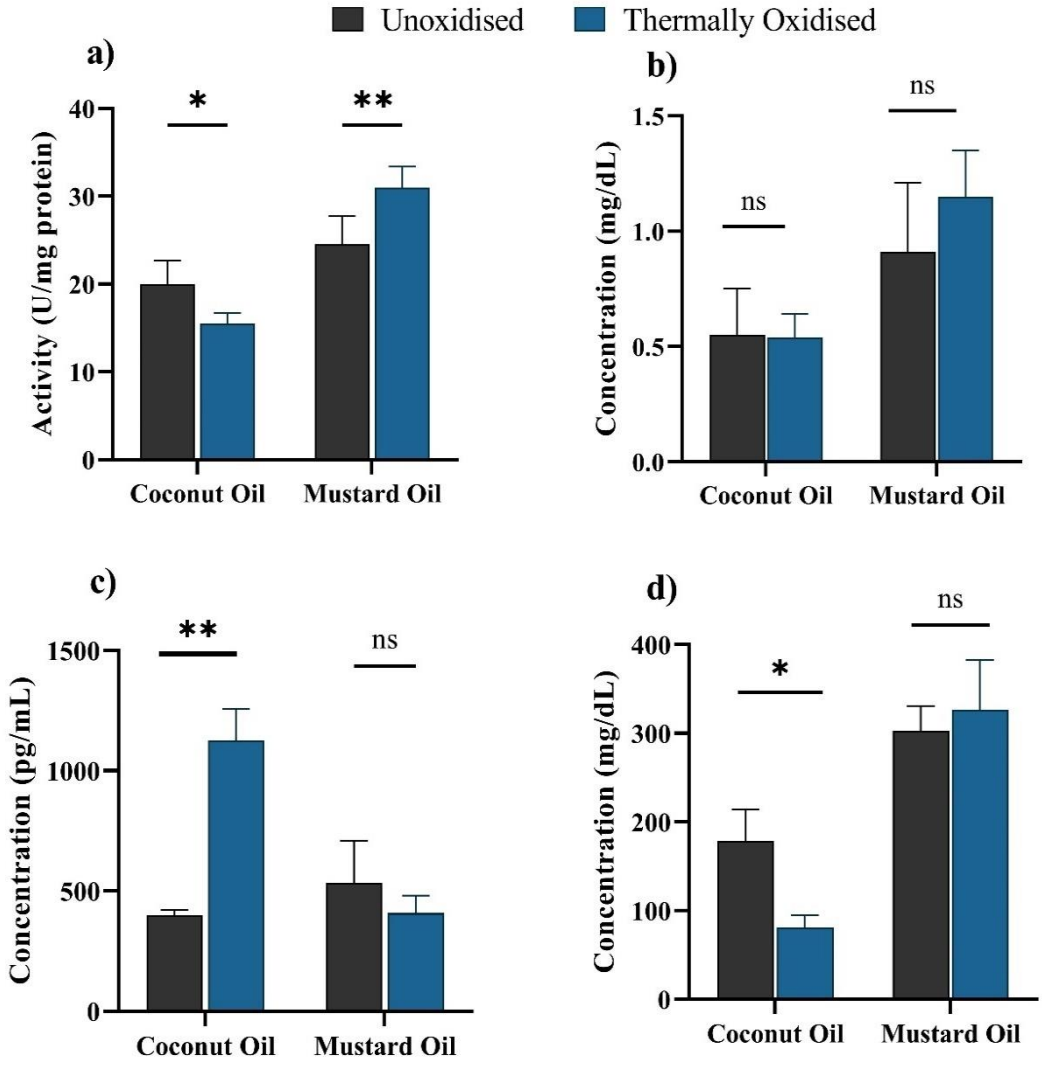

Figure 5.6 Serum inflammatory markers in different experimental groups: CO, TCO, MO and TMO incorporated diet were fed to animals in different groups for six months. The serum levels of a) LP-PLA_2 activity, b) CRP, c) IL-6 and d) IL-10 were measured. Data represented as Mean ±SD of 6 animals per group. Statistical analysis was conducted using GraphPad Prism software, employed with two – way ANOVA followed by Tukey's multiple comparison test. * $p<0.05$, ** $p<0.01$, ns non-significant when comparison between oil diet groups.

5.6. Histopathological analysis

In this study, liver histopathology revealed varying degrees of hepatic damage across different diet groups. CO-incorporated diet-fed rats exhibited a normal radial arrangement of hepatocytes with a normal central vein resulting in a hepatic score of '0' (Figure 5.7 a). In the TCO group animals, hepatic tissue with mild damage

characterized by minimal inflammatory cells diffused microvesicular steatosis of peripheral hepatocytes with intracellular spaces affecting less than 25% of liver tissue. The hepatic score was '1' indicating early-stage fat accumulation (Figure 5.7 b). The MO group showed more pronounced liver damage with moderate infiltration of inflammatory cells found in the portal space with interstitial space and 25-50% steatosis in the liver, with a score of '2.0'. Additionally, mild ballooning of hepatocytes and glycogenated nuclei with scoring of '1', yielding a total score of 3.0 for this group (Figure5.7 c). Similarly, in the TMO group, histopathology of the liver showed mild hepatic damage with a scoring of '1' characterized by glycogenated nuclei, portal tract inflammation, and hepatocyte ballooning. Moderate microvesicular steatosis affected 25-50% area of the liver in TMO group rats, with a score of '2' leading to a combined hepatic score of 3.0. (Fig 5.7 d). Overall, the CO group had no signs of damage, the TCO group had mild liver damage, while the MO and TMO groups experienced more severe liver damage. The scoring of histopathology is shown in the Table 5.4

Table 5.4: Scoring of histopathology**.**

Characteristics	CO	TCO	MO	TMO
Glycogenated nuclei	**0**	**0**	**1**	**1**
Hepatocellular ballooning disarray	**0**	**1**	**1**	**1**
Micro vesicular steatosis	**0**	**1**	**2**	**2**
Portal Tract inflammation	**0**	**1**	**1**	**1**
Sinusoidal dilation	**0**	**1**	**1**	**1**

Scoring indicates 0–2 based on the extent of damage in the hepatocytes, where 0 is normal; 1 marginal (mild); 2 moderate;

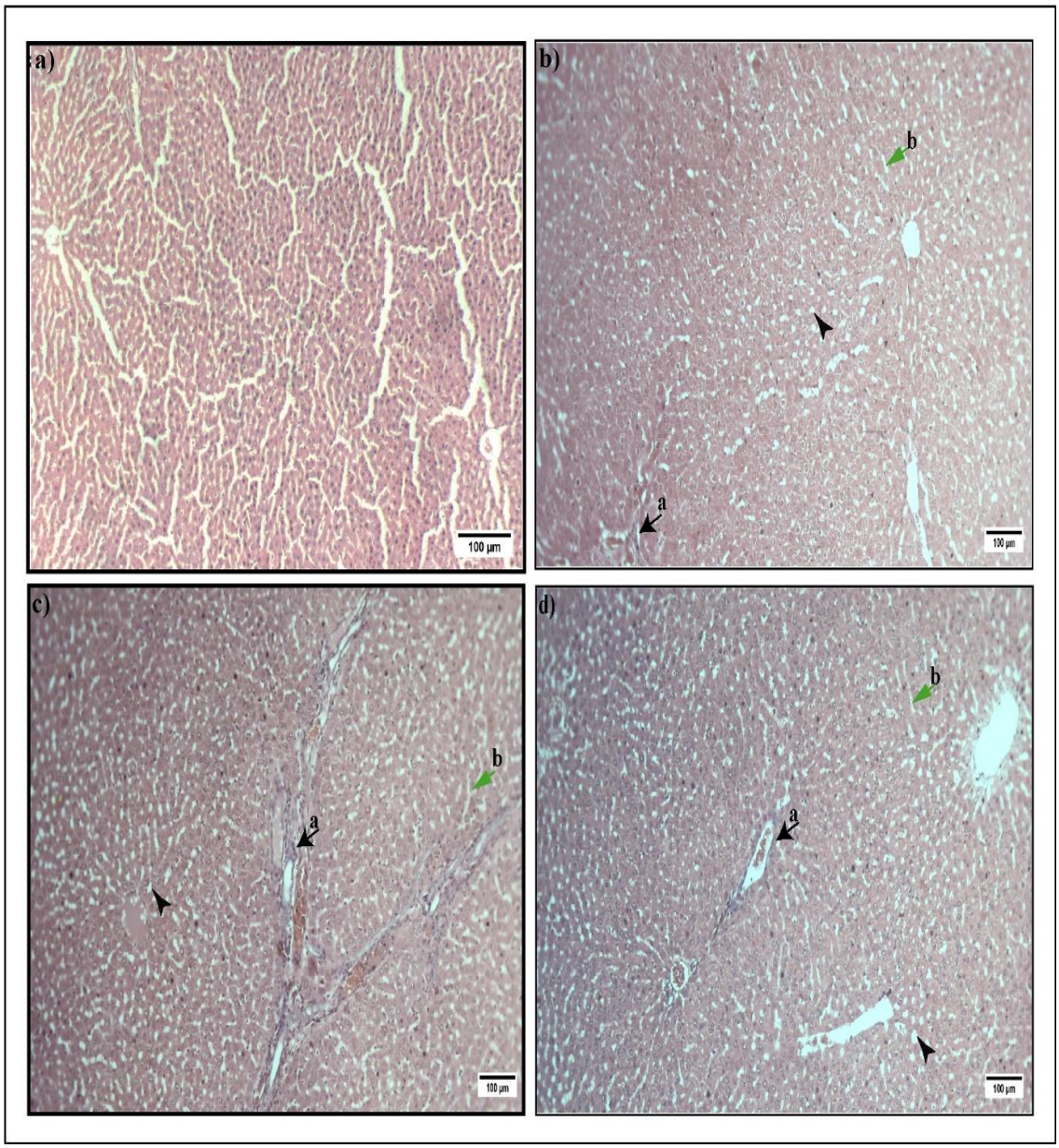

Figure 5.7 Photomicrograph of Rat Liver Tissue Stained with Haematoxylin and Eosin (100x). a) CO group, b) TCO group, c) MO group, and d) TMO group. Arrows a, b, and arrowhead indicate inflammatory cells, intra-sinusoidal space, and microvesicles, respectively.

Figure 5.8 illustrates histological variations in kidney tissues exposed to unoxidized and thermally oxidized coconut oil (CO) and mustard oil (MO). Rats under CO (Figure 5.8a) and thermally oxidized CO (TCO) diets (Figure 5.8b) exhibited kidney sections characterized by normal renal corpuscles, intact glomeruli, and unremarkable proximal convoluted tubes (PCT) and distal convoluted tubes (DCT). In contrast, the group fed with the MO diet-fed animals displayed (Figure 5.8c) kidney tissues featuring normal glomeruli and PCT, but DCT exhibited unstained cytoplasm, indicative of hydropic degeneration. Inflammatory cell infiltration was present, indicating a marginal severity with a grade of "1" damage in the renal cortex. The TMO diet group demonstrated (Figure 5.8d) intact renal corpuscles with normal glomeruli and PCT. However, DCT exhibited hydropic degeneration with the presence of inflammatory cells, showcasing a moderate severity with a grade of "2".

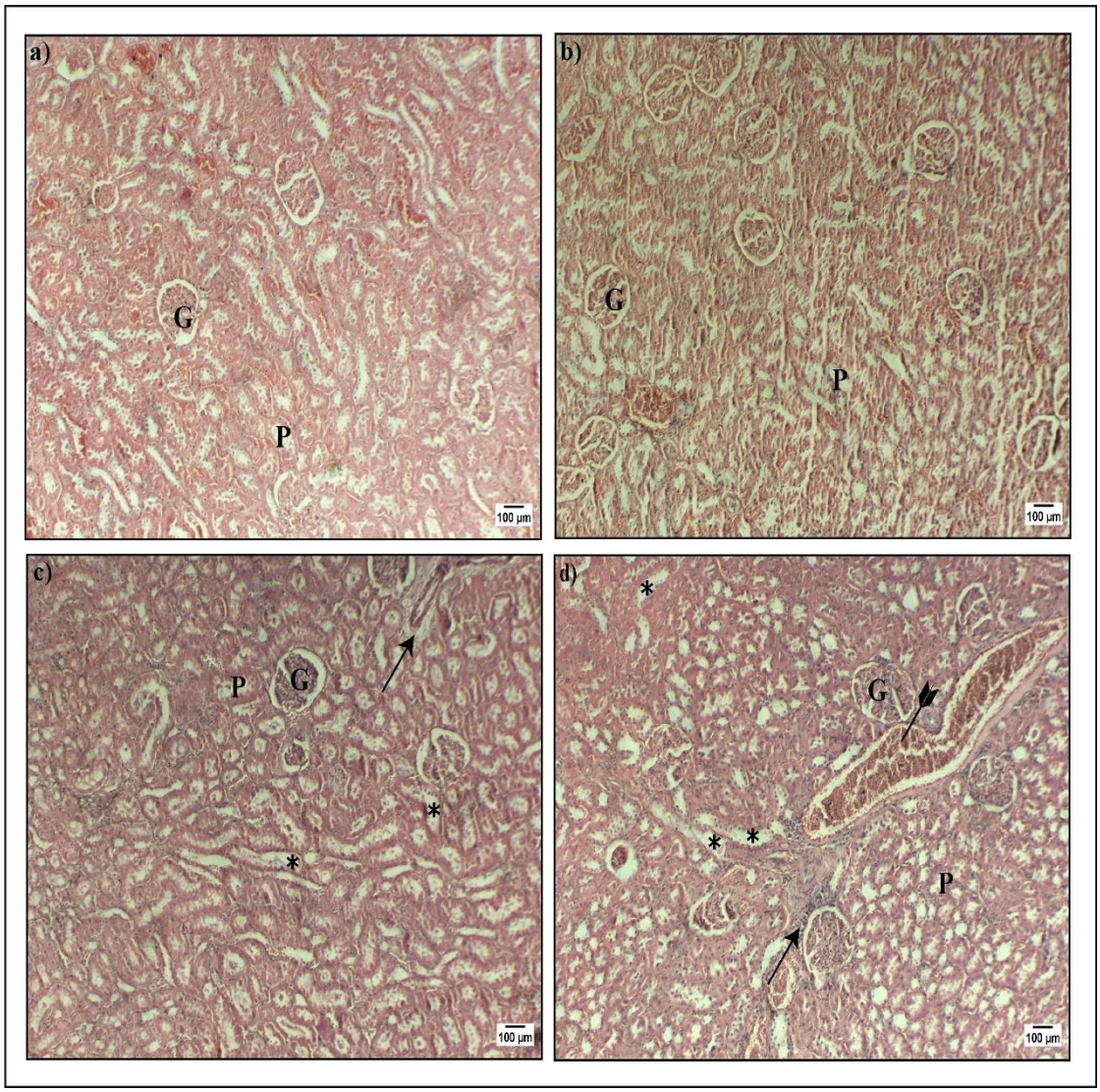

Figure 5.8 Photomicrograph of Rat Kidney Tissue Stained with Haematoxylin and Eosin (100x). a) CO group, b) TCO group, c) MO group, and d) TMO group. Normal renal corpuscles with glomerular capillary loops (G) and proximal convoluted loop (P). The distal convoluted tubules show unstained cytoplasm (hydropic degeneration) and wide lumina (stars), Inflammatory cells (black arrow), and interstitial haemorrhage (black arrow tail).

The histological analysis of the heart in different experimental groups revealed no pathological alteration, indicating that the morphology of the heart tissue remained preserved. Histopathological analysis of the heart is shown in Figure 5.9.

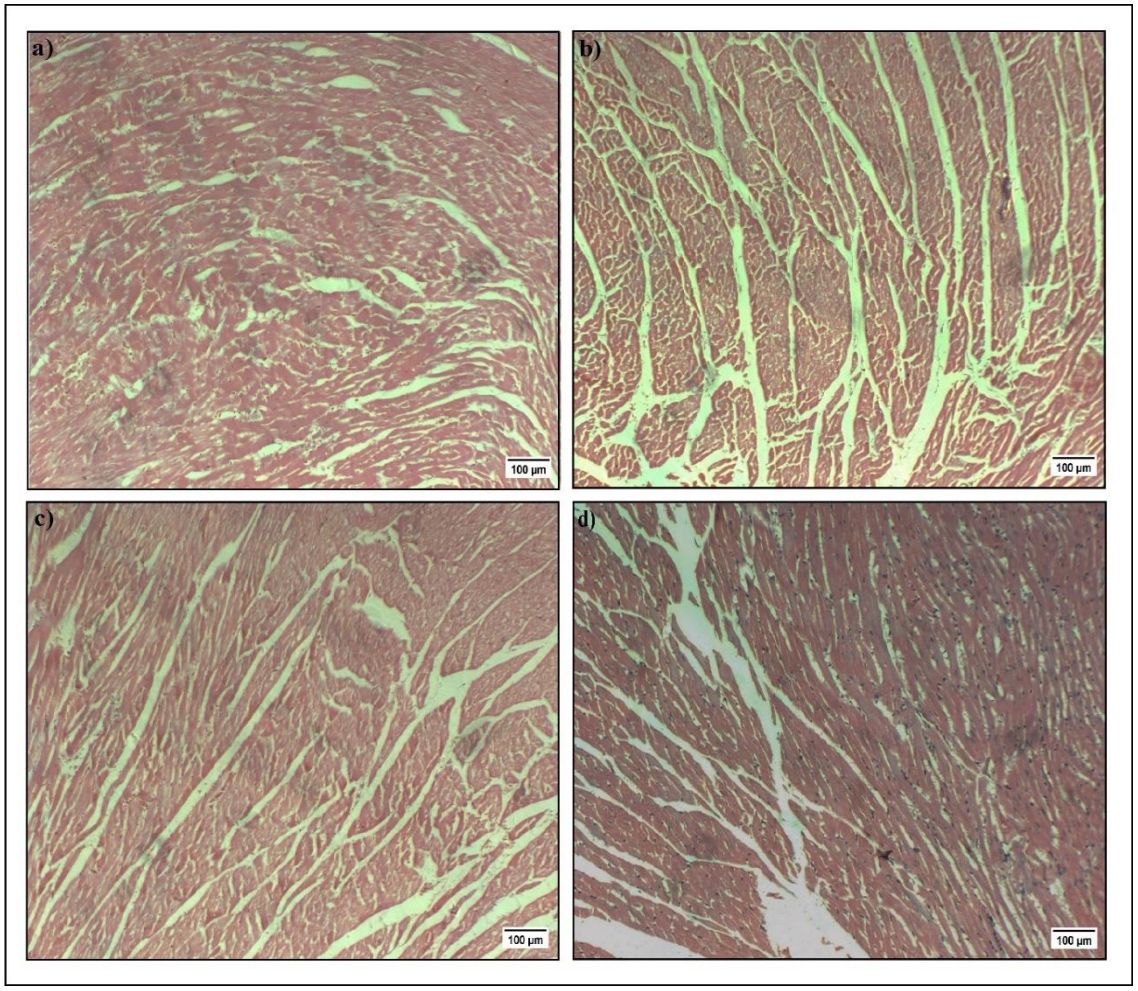

Figure 5.9 Photomicrograph of Rat Heart Tissue Stained with Haematoxylin Eosin and (100x). a) CO group, b) TCO group, c) MO group, and d) TMO group.

5.7 Discussion

The present study investigated the effects of dietary consumption of 5% unoxidized and thermally oxidized coconut oil and mustard oil in experimental animals for six months. The comparative analysis of these oils is crucial for understanding their roles in nutrition and disease prevention, particularly CVD and metabolic disorders. Rats in all experimental groups exhibited an increase in body weight throughout the study period. However, average body weight gain was unchanged irrespective of the composition of fatty acids, individual chain length, saturation and extent of oxidation of fatty acids. Thus, it is clear that the administration of normo-dietary levels of edible oil containing MCTs, MUFAs and its oxidized products does not influence body weight. Contrary to this observation, Islam et al., (2020) reported that oral administration of raw and repeated fried mustard oil with a dose of 5g/kg.bd wt up to 5 weeks showed a reduction in body weight gain compared to their non-oil control diet group. Similarly, Wang et al., (2018), and Swarnamali et al., (2023) have reported that consumption of coconut oil containing MCT results in significant weight loss compared to other oil diets and improved metabolic parameters associated with obesity by increasing energy expenditure and fat degradation. Possibly, a weight loss that could complement obesity/exercise by MCT can be obtained while as per the present observation, CO does not alter the weight of the animals fed.

Even though negligible difference was observed in the organosomatic index for liver, kidney, heart, and adipose tissue in rats fed with unoxidized and thermally oxidized coconut oil and mustard oil. The observed increase in liver weight of rats fed a TCO diet might be due to mild lipid accumulation in the liver, potentially with impaired hepatic metabolism. The presence of oxidized lipid products in the TCO may have a detrimental effect on liver function, contributing to the observed lipid accumulation. On the other hand, in both MO and TMO-fed rats had increased in the organosomatic index

of the liver. The fatty accumulation in the hepatic tissue was evident. This supports that the unoxidized MCT fat (CO) is safe for dietary use.

The effect of unoxidized and thermally oxidized coconut and mustard oils on glucose tolerance was studied and no significant alterations in the initial and final OGTT and area under curve (AUC) of both unoxidized and thermally oxidized mustard oil diet group rats. In contrast, the final OGTT demonstrated that rats fed both unoxidized and thermally oxidized coconut oil exhibited significantly improved glucose tolerance compared to the other dietary groups. This improvement could be attributed to the unique composition of CO, which is rich in MCTs that may enhance insulin sensitivity and promote better glucose metabolism. Previous studies have indicated that CO can improve glucose homeostasis and reduce fat accumulation in metabolic syndrome, suggesting its potential as a beneficial dietary fat for managing blood sugar levels (Mautone Gomes et al., 2023).

The insulin level and HOMA-IR in MO diet fed group were found to be higher which was significantly reduced in TMO-fed animals. The reduction in insulin to HOMA-IR however is proportionate in these groups. The serum insulin concentration and HOMA-IR of CO and TCO-fed rats are found unaltered. Higher insulin levels generally correspond to a higher HOMA-IR, indicating insulin resistance and also indicates reduced insulin sensitivity with lower HOMA-S. Higher insulin levels can be associated with reduced beta cell function with higher HOMA-B. MO diet-fed group showed a tendency to develop insulin resistance with high insulin levels, high HOMA-IR, along with low HOMA-S, and high HOMA-B. CO, TCO and TMO showed low insulin, low HOMA-IR, and high HOMA-S with similar HOMA-B indicating normal insulin sensitivity. These findings suggest that the consumption of a modest quantity of coconut oil irrespective of its oxidation at a normal dietary level may enhance insulin sensitivity and induce a hypoglycaemic effect in rodent models. Various studies have indicated

that coconut oil enhances insulin sensitivity, reduces insulin resistance and exhibits a hypoglycaemic effect. It could be attributed to its high concentration of MCTs, probably lauric acid and polyphenols (Narayanankutty et al., 2016b, Đurašević et al., 2020, Sheela et al., 2017, Malaeb and Spoke, 2020). Furthermore, a small study in healthy men showed that consuming coconut oil for eight weeks enhanced insulin sensitivity (Malaeb and Spoke, 2020). These findings well support this present data. Contrary to this, a systematic review and meta-analysis of clinical studies suggest that long-term consumption of coconut fat may increase insulin resistance and does not appear to be beneficial for long-term glycaemic control (Dhanasekara et al., 2022). Mustard oil plays a role in glycaemic response by not contributing to blood sugar spikes. Erucic acid-rich yellow mustard oil has been shown to decrease PPARγ transcriptional activity and inhibit insulin resistance in obese/diabetic KK-Ay mice (Takahashi et al., 2021). This suggests that mustard oil may help improve insulin sensitivity, which can positively affect glycaemic response. In contrast, in the present study, the MO diet group exhibited a hyperinsulinemia condition.

PT and APTT are important tests to evaluate blood coagulation. PT asses the time it takes for plasma to clot in the presence of thromboplastin and reflects the efficiency of the extrinsic coagulation pathways. On the other hand, APTT measures how long it takes for blood to form a clot and is used to assess the intrinsic pathway and final pathway of blood clotting. In the present study, no changes in coagulation factors were found among the CO and MO oil diet groups. However, APTT was found significantly increased in the TCO group in comparison to the respective unoxidized oil diet group. The impact of oxidized coconut and mustard oils on coagulation parameters such as PT and APTT is not well-established. Previous research on virgin coconut oil indicated that repeated heating of coconut oil resulted in alterations in platelet functions, particularly in cholesterol-fed Sprague-Dawley rats. The study by Nevin and Rajamohan, (2008b)

concluded that the deleterious effects of heated coconut oil on platelet functions were lower than those of heated mustard and sunflower oil.

Liver toxicity and its consequences can be assessed through various parameters such as hepatic enzyme levels, hepatic steatosis. The liver enzymatic activity did not show a significant difference among the CO, MO, and TMO diet-fed groups. This suggests that the composition of these diets did not induce discernible alterations in liver function. However, AST and ALP levels were raised in those animals fed a TCO-based diet. Variations in the concentration of serum albumin and protein indicate an alteration in the normal liver function. In this study, there was no change in other liver parameters. CO was found to have hepatoprotective effects, especially to reverse hepatic steatosis by restoring redox homeostasis and improving lipid metabolism (Narayanankutty et al., 2017b). Another study found that deep-fried edible oils, including coconut and mustard oils, can disrupt hepatic redox equilibrium and lead to increased lipotoxicity and hepatosteatosis (Narayanankutty et al., 2016a). In another study, where female Wistar rats were fed a high-fat diet encompassing either CO or thermally oxidized coconut oil (TCO) for two months, non-alcoholic steatohepatitis (NASH) was established (Gopinath et al., 2021). In all these cases the oils used at higher than the dietary percentage and also along with a high fructose/ fat diet. This suggests that the oxidation process of coconut oil can lead to liver toxicity and fibrosis, whereas unoxidized coconut oil may have protective effects on liver function. Here in this study thermally oxidized coconut oil (TCO) shown to induce minimal hepatotoxicity mediated by redox imbalance in the long run. However, no notable variations were observed in renal function parameters among the groups, suggesting that 5% of unoxidized and thermally oxidized CO and MO consumption impart no detrimental effect on renal function in normal rats.

Animals fed a TMO diet have shown significant changes in serum and tissue lipid levels

compared to their unoxidized oil diet and coconut oil diet. Studies have demonstrated that the inclusion of oxidized oils, characterized by elevated amounts of lipid peroxidation products, in animal diets can impede growth, induce oxidative stress, and impact lipid metabolism (Narasimhamurthy and Raina, 1999, Jaarin et al., 2018, Siddiq et al., 2019). In the current study, rats fed with a 5% TMO diet have shown increased serum TC, TG, and LDL-C and decreased HDL-C compared to those fed with respective unoxidized oil diets. The study investigated the effects of repeatedly frying mustard oil at 180°C for 10 minutes, three times on Wistar rats led to an increased serum LDL-C and triglycerides, while HDL-C levels were decreased, which contributed to the development of NAFLD in rats (Islam et al., 2020). Another study found that thermally oxidized MO contributes to the formation of oxidized components in lipoproteins, which plays a significant role in the development of atherosclerosis in animal models (Khan-Merchant et al., 2002). In contrast, a study on the dietary impacts of diacylglycerol (DAG) rich mustard oil found that this type of MO reduced TC, TG, and LDL-C while simultaneously raised HDL-C (Dhara et al., 2013). The repeated heating of cooking oils has been shown to increase the peroxide values, and lipid peroxidation by-product content, all of which can be harmful to health and increase the risk of many diseases. In this study, compared to heated CO-fed animals, those fed heated MO showed higher lipid levels. Lipoprotein particles that carry low-density lipoprotein (LDL) are recognized for their atherogenic characteristics, and increased concentrations of these particles are associated with hyperlipidaemia and homeostatic changes linked to atherosclerosis. On the contrary, HDL-cholesterol serves a protective function by assisting in the removal of triglyceride-rich lipoproteins, thereby safeguarding the arterial wall from the onset of atherosclerosis. The concentration of liver cholesterol was shown significantly elevated in mustard oil diet-fed animals compared to coconut oil diets, indicating that the consumption of mustard oil may lead to elevated cholesterol

in the liver.

Phospholipids are the matrix of biological membranes, which are the primary targets of peroxidation. ROS interacts with PUFA chain present in phospholipids, leading to the formation of lipid peroxides in response to oxidative stress (Catalá, 2009). The phospholipid level of the liver was significantly reduced in thermally oxidized oil diet-fed rats in comparison with those which fed a fresh unoxidized oil diet. Compared to the TCO group, those fed the TMO group showed a 38% reduction of liver phospholipids. The liver is the primary site for cholesterol synthesis. Cholesterol biosynthesis is regulated by the rate-limiting enzyme HMG Co. A reductase and dietary factors, including the consumption of different types of oils. Among oil-diet-fed animals, the mustard oil diet group showed higher HMG Co. A reductase activity when compared to coconut oil-fed diet animals. Higher activity of HMG Co. A reductase correlates with increased hepatic cholesterol in both unoxidized and thermally oxidized mustard oil diet-fed rats. The consumption of oxidized oils showed to increase HMG Co. A reductase activity in the liver. The increased activity may be responsible for increased serum cholesterol. Hepatic lipase is an enzyme primarily produced in the liver that plays a crucial role in lipid metabolism, specifically in the hydrolysis and remodelling of lipoproteins. Hepatic lipase activity is inversely related to HDL cholesterol levels. This is because hepatic lipase facilitates the removal of lipids from HDL particles, leading to their catabolism and reduced circulating levels. The activity of hepatic lipase in the liver significantly increased in rats, fed the TMO diet when compared to other oil-fed rats. This, in turn, reduced the serum HDL-C and increased serum TG in TMO diet-fed rats. In contrast, other oil diet-fed group rats showed increased concentrations of HDL-C in their serum.

High dietary fat intake has been associated with elevated levels of liver fructose-1,6-bisphosphatase (FBPase), a regulatory enzyme in gluconeogenesis. The present study

revealed that the activity of FBPase was reduced in the group that consumed a coconut oil-based diet compared to those on a mustard oil diet. Furthermore, this decrease in FBPase activity was found to be correlated with a lower concentration of blood glucose in the coconut oil diet group. The TCA cycle is a series of biochemical reactions essential for the breakdown of glucose, fatty acids, and some amino acids. It is the major source of ATP generation in biological systems and is located in the mitochondrial matrix. This study investigated the activities of key enzymes—succinate dehydrogenase, malate dehydrogenase, and isocitrate dehydrogenase in liver mitochondria. The activities of succinate dehydrogenase were significantly lower in animals fed TMO oil diet group compared to those fed both unoxidized MO diet fed group and both unoxidized and thermally oxidized coconut oil diet fed groups. Administration of heated oils resulted in significantly higher lipid peroxidation in the mitochondria. The TCA cycle enzymes affected by the free radicals generated by repeated heating of oils, which can damage cellular membranes, inactivate the enzymes of the TCA cycle, and cause fatty acid oxidation. Individuals with NAFLD, have a high rate of hepatic mitochondrial oxidative and TCA cycle activities indicating increased energy demand during NAFLD (Satapati et al., 2012, Satapati et al., 2015). Activities of lipogenic enzymes, namely, malic enzyme and isocitrate dehydrogenase, were elevated in thermally oxidized oils-fed animals in comparison to those fed unoxidized oils. Malic enzyme and ICDH provide NADPH for fatty acid synthesis, and increased activities of these enzymes in the liver correlate with the increased triglyceride concentration. Compared to TCO oil diet-fed animals, those fed TMO diet showed higher lipogenic enzyme activities.

Lipoprotein-associated phospholipase A_2 (Lp-PLA_2) is an enzyme that significantly contributes to the pathophysiology of atherosclerosis and cardiovascular diseases. It is primarily associated with the breakdown of oxidized phospholipids on the surface of

LDL particles, generating pro-inflammatory and pro-atherogenic by-products such as lysophosphatidylcholine and non- esterified fatty acids (English et al., 2022, Cai et al., 2013). Rats fed a diet rich in coconut oil exhibited decreased Lp-PLA_2 activity compared to those fed a mustard oil diet. Coconut oil is predominantly composed of medium-chain saturated fats and may have minimal direct impact on Lp-PLA_2 activity. In this study, the consumption of CO diet groups was shown to increase the activity of Lp-PLA_2 compared to their thermally oxidized diet might be due to the presence of a small amount of PUFA. Both MO oil diet groups showed increased Lp-PLA_2 activity, primarily MUFAs and PUFAs in MO. Meanwhile, the consumption of TMO could potentially increase Lp-PLA_2 activity due to the presence of oxidized lipids produced from unsaturated fatty acids during thermal oxidation. CRP is a marker of systemic inflammation and has been associated with atherosclerosis and cardiovascular diseases. Some studies have shown that CRP play a role in the progression of atherosclerosis by exerting pro-inflammatory effects, modulating the immune response, and contributing to endothelial dysfunction (Badimon et al., 2018). In rats subjected to a mustard oil diet, CRP levels were notably increased, particularly in the group-fed TMO diet. Conversely, there were no apparent changes observed among the groups fed a coconut diet.

IL-6 and IL-10 are both cytokines involved in immune response and inflammation. IL-6 is a pro-inflammatory cytokine. Elevated levels of IL-6 have been associated with chronic inflammatory conditions, and autoimmune diseases are linked to the development and progression of cardiovascular diseases, including atherosclerosis and coronary artery disease. Research shows that elevated IL-6 levels are associated with increased risk of CVD (Ridker et al., 2000). IL-10, in contrast to IL-6, is an anti-inflammatory cytokine. Consumption of thermally oxidized coconut oil was showed an increased levels of pro-inflammatory cytokines like IL-6, while unoxidized CO exhibits an anti-inflammatory effect. Thermal oxidative products, free radicals and other LOPs

in the CO trigger oxidation-induced inflammation. In contrast to IL-6, IL-10 help to suppress inflammation by inhibiting the production of proinflammatory cytokines. CO induces IL-10 secretion, with the help of anti-inflammatory compounds present in the coconut oil, such as lauric acid and polyphenols. IL-10 has been shown to have protective effects on the cardiovascular system by reducing inflammation and inhibiting the production of pro-inflammatory cytokines. The present data is supported by other studies on the anti-inflammatory potential of coconut oil (Illam et al., 2017, Narayanankutty et al., 2022). MO consumption has been associated with modulating inflammatory responses, including the regulation of IL-10, the bioactive components of mustard oil, such as monounsaturated fats (oleic acid and erucic acid) and ω-3 fatty acids (like linoleic acid) can enhance IL-10 production, thereby exerting anti-inflammatory effects. Interestingly, this modulation appears to occur irrespective of the oxidation state of mustard oil. Chronic inflammation is a known risk factor for CVD. The presence of omega-3 fatty acids in MO could be the contributing factor, as these are well documented for their cardiovascular benefits, including anti-inflammatory and lipid-lowering effects.

Further, the histopathological findings suggest a spectrum of liver disease, with the CO diet-fed animals exhibited early-stage fatty changes, the MO group animals exhibited more advanced damage with inflammation and fibrosis, and the TMO group presenting features of both fat accumulation and abnormal carbohydrate metabolism along with inflammation. These findings indicated that alterations in serum lipids and inflammation, particularly atherogenic index, have not resulted in any pathological changes in the heart and blood vessels. Rats received the unoxidized and thermally oxidized CO diets exhibited normal kidney sections, while the group fed with the MO diet displayed kidney tissues with hydropic degeneration in the distal convoluted tubes (DCT) and inflammatory cells. The group fed with a thermally oxidized MO diet

demonstrated hydropic degeneration in DCT with the presence of inflammatory cells with no elevation renal function marker.

The present study demonstrated the harmful effects of the intake of repeatedly heated dietary oils on lipid parameters as well as enzymes involved in lipid metabolism and carbohydrate metabolism. Results indicated that animals fed thermally oxidized oils had significantly higher concentrations of lipids in serum and tissues, lower serum HDL-C levels, lower phospholipid concentration, and alterations in enzyme activities involved in lipid metabolism compared to those fed fresh oils.

The schematic illustration (Figure 5.10) indicates the modulation of metabolic pathways and the development of cardiovascular diseases. Liver is crucial for regulating glucose and carbohydrate metabolism, and an imbalance of these metabolisms can lead to the development of metabolic diseases such as NFLD and DM, both of which are associated with an increased risk of developing CVDs.

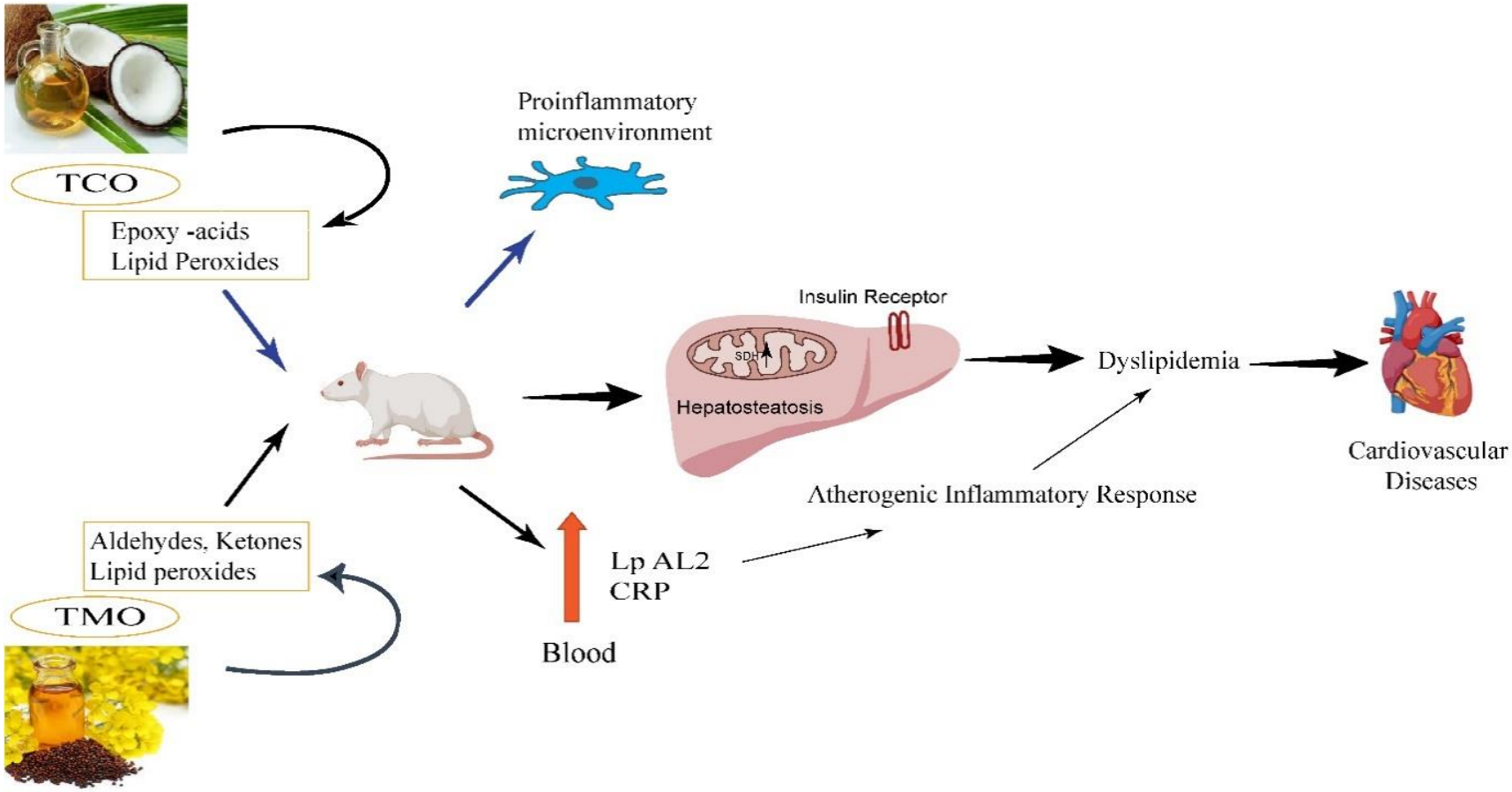

Figure 5. 10: Illustration of Modulation of Development of Cardiovascular Diseases**.**

Chapter 06

Metabolic Impacts of Thermally Oxidized Palm and Sunflower Oils Impart Cardiovascular Risk

6.1 Introduction

Palm oil (PO) and sunflower oil (SO) are abundant sources of long-chain fatty acids, both saturated and unsaturated. These oils are extensively used for cooking purposes in Asian and Indian states. The chemical composition of these oils predominantly consists of long-chain fatty acids. Oxidized products generated following the thermal oxidation of these oils are distinct and have nutritional and health impacts. The variations in the chemical structure of the long-chain fatty acids present in these oils contribute to their different behaviour during cooking and their effects on health when consumed. Thermal oxidation of PO and SO leads to notable changes in their composition. The characterization results described in Chapter 4 indicate that there is a 67% increase in FFA content in TPO and a 90% increase in TSO compared to their unoxidized counterparts due to enhanced triglyceride hydrolysis. Furthermore, essential fatty acids (EFA) like linoleic acid are significantly diminished in both TSO and TPO due to oxidation. In TSO, an increase in trans-fatty acid content has been observed, primarily due to the high levels of PUFA. Behenic acid, cycloperoxides, stearic acid, palmitic acid, cis 9,10 epoxy stearic acid are found in TSO and heptanoic acid, caprylic acid and azelaic acids are the oxidized products of unsaturated fatty acids, which were found in both TSO and TPO. Cis 9,10 epoxy stearic acid, methyl 10- keto stearate, methyl l9-keto stearate, and heptadecenoic acid were also found in TPO.

The assessment of the effect of thermally oxidized CO and MO representing MCSFA and MUFA described in the previous chapter, has found that thermally oxidized products influence carbohydrate and lipid metabolism and promote the inflammatory status of experimental animals and damage liver tissue. Since TSO and TPO have generated more oxidized compounds than CO and MO, the influence of these oils has to be studied in detail. Despite the importance of these findings, dietary intervention studies focusing on the effects of thermally oxidized long-chain saturated, and

polyunsaturated rich oils at normal levels to influence metabolic dysregulation in rats are rarely addressed. Therefore, the present study is designed to investigate the effects of consumption of normolipidic diet containing PO and SO representing LCSFA and PUFA edible oils along with their thermally oxidized products. This focus is on lipid, and carbohydrate metabolism as well as inflammatory status in rats and their contributory role in the development of CVDs.

6.2 Materials and Methods

6.2.1 Preparation of the thermally oxidized oil

Palm oil (Ruchi Gold) and Sunflower oil (Gold Winner) were bought from the local market of Thrissur, Kerala, and kept under refrigerated conditions to avoid auto-oxidation. The preparation and storage of thermally oxidized oils, as described in Chapter 3.1.5

6.2.2 Animals

Male Wistar rats weighing 160-180g were used in this study. The acquisition and maintenance were described in chapter 3.1.6.

6.2.3 Diets and Experimental Design

Male Wistar rats were randomly grouped into four with six animals each. The animals were fed a diet with 5% either unoxidized or thermally oxidized palm and sunflower as fat sources for 6 months. The experimental design and the preparation and composition of the oil diet were described in Chapter 3.2.1. The grouping of experimental rats is given below.

Groups	Treatment/ Intervention
Group I	A diet with 5% Unoxidized Palm oil (PO)
Group II	A diet with 5% Thermally oxidized Palm oil (TPO)
Group III	A diet with 5% Unoxidized sunflower oil (SO)
Group IV	A diet with 5% Thermally oxidized Palm oil (TSO)

6.2.4 Fasting blood glucose (FBS)

Fasting blood glucose levels were assessed by manufacturer guidelines provided by the kit from Euro Diagnostic Systems Pvt Ltd, TN, India). FBS was measured using the glucose oxidase peroxidase (GOD POD) method, described in Chapter 3.5.1.

6.2.5 Oral Glucose Tolerance Test (OGTT)

OGTT was used to measure the response of metabolism to sugar after the consumption of 2g/kg Bd. Wt of glucose. The protocol for OGTT was described in chapter 3.5.2

6.2.6 Fasting Plasma Insulin

The fasting insulin was determined using the ELISA method. The pre-coated ELISA kit was procured from Origin Diagnostic and Research, (Kerala, India). The procedure for insulin assay is described in Chapter 3.5.3

6.2.7 HOMA Indices

HOMA indices are used to estimate IR and insulin sensitivity. The detailed procedure was described in Chapter 3.5.4

6.2.8 Coagulation system

Various parameters of coagulation factors such as platelet count, BT and CT, prothrombin time (PT), and Activated Partial thromboplastin time (APTT) were used to assess the primary homeostasis. The procedure was described in Chapter 3.6

6.2.9 Systemic Toxicity

Liver and renal function tests were used to assess the liver and renal functional assessment. The parameters, including ALT, AST, ALP, albumin, total protein, bilirubin, urea, and creatinine, were determined using the kits from Euro Diagnostic System Pvt. Ltd, TN, India. The procedure is described in Chapter 3.7.1 and Chapter 3.7.2

6.2.10 Lipid Metabolism

Serum total cholesterol, HDL-cholesterol, and both serum and tissue triglycerides were

measured using kits from Euro Diagnostic System Pvt. Ltd, TN, India in accordance with the standard protocol given by the manufacturer and detailed description in Chapter 3.8.1. Hepatic tissue cholesterol was measured spectrophotometrically, the detailed procedure is given in Chapter 3.8.3. Estimation of hepatic phospholipid is described in Chapter 3.8.4. Lipid metabolic enzymes include HMG Co. A reductase activity was assessed by the indirect method of HMG Co. A/mevalonate ratio, described in Chapter 3.8.5. Hepatic lipase activity was measured by spectrophotometrically, as described in Chapter 3.8.6.

6.2.11 Carbohydrate Metabolism

Carbohydrate metabolic enzymes include FBPase and mitochondrial TCA enzymes include ICDH, MDH, and SDH, which were measured spectrophotometrically. The detailed procedure is given in Chapter 3.9.

6.2.12 Inflammatory Markers

Inflammatory markers such as CRP, LP-PLA_2, IL6, and IL10 were measured using ELISA kits. The detailed procedure is given in Chapter 3.10.

6.3 Histopathological analysis

A 10% formalin-fixed tissues were processed by paraffin embedding technique. The sections were stained with H/E stain to visualize the architecture of liver, heart, and kidney tissues. The detailed procedure is described in 3.12.

6.4. Statistical analysis

Data was reported as mean ± SD of 6 animals per group. Statistical analysis was done by using GraphPad Prism (prism 9) software. A Two-Way ANOVA was applied, followed by post–hoc test using Tukey's multiple comparison tests to identify significant differences between groups. A $p<0.05$ was considered statistically significant. ns non-significant, * $p<0.05$, ** $p<0.01$, *** $p< 0.001$.

6.5 Results

6.5.1 Body Weight Changes and Relative Organ Weight.

After acclimatization, Wistar rats were allocated to PO, TPO, SO, and TSO incorporated diet. The rats in all groups experienced continuous weight gain throughout the six-month experimental period (Figure 6.1a). The body weight change was an important parameter to assess the impact of consumption of oxidized products formed from thermal oxidation of oils. The mean body weight gain was significantly ($p<0.01$) lower in TPO diet-fed group rats (64.05 ± 7.6g) compared to PO (81.85 ± 3.8g) diet-fed rats. Similarly, the rats fed with the TSO diet-fed group rats (60.25 ± 5.0g) had lower body weight gain compared to SO diet fed group (75.67 ± 7.2g), with statistical significance ($p<0.01$), as shown in Figure 6.1b. Compared to PO the body weight gain was comparatively lesser in the SO diet-fed group. The relative organ weight analysis revealed a significant ($p<0.05$) increased liver weight in the TPO diet-fed rats (3.01±0.26 g%) compared to the PO diet-fed rats (2.65±0.29g%). Similar to this, the relative organ weight of the liver tissue in TSO diet-fed group rats (3.08 ± 0.29g %) was observed to be higher than SO diet-fed rats (2.85±0.29 %). However, no significant changes were observed in kidney and heart weight. The relative heart weights of the PO, TPO, SO, and TSO diet groups were 0.27 ± 0.03, 0.27 ± 0.05, 0.28 ± 0.03, and 0.37 ± 0.05g % respectively. The relative kidney weight of the PO, TPO, SO, and TSO diet groups was 0.57±0.06, 0.62±0.07, 0.57±0.07, and 0.65±0.07% respectively. There was a significant ($p<0.05$) increased adipose tissue weight found in PO-fed rats (2.28±0.35g%) compared to TPO (1.90±0.27g%) diet-fed rats ($p<0.05$). There was also a significant ($p<0.05$) reduction observed in the weight of adipose tissue between unoxidized and thermally oxidized SO diet groups (1.47±0.21 and 1.21±0.23g% respectively). The relative organ weight is shown in Figure 6.1c.

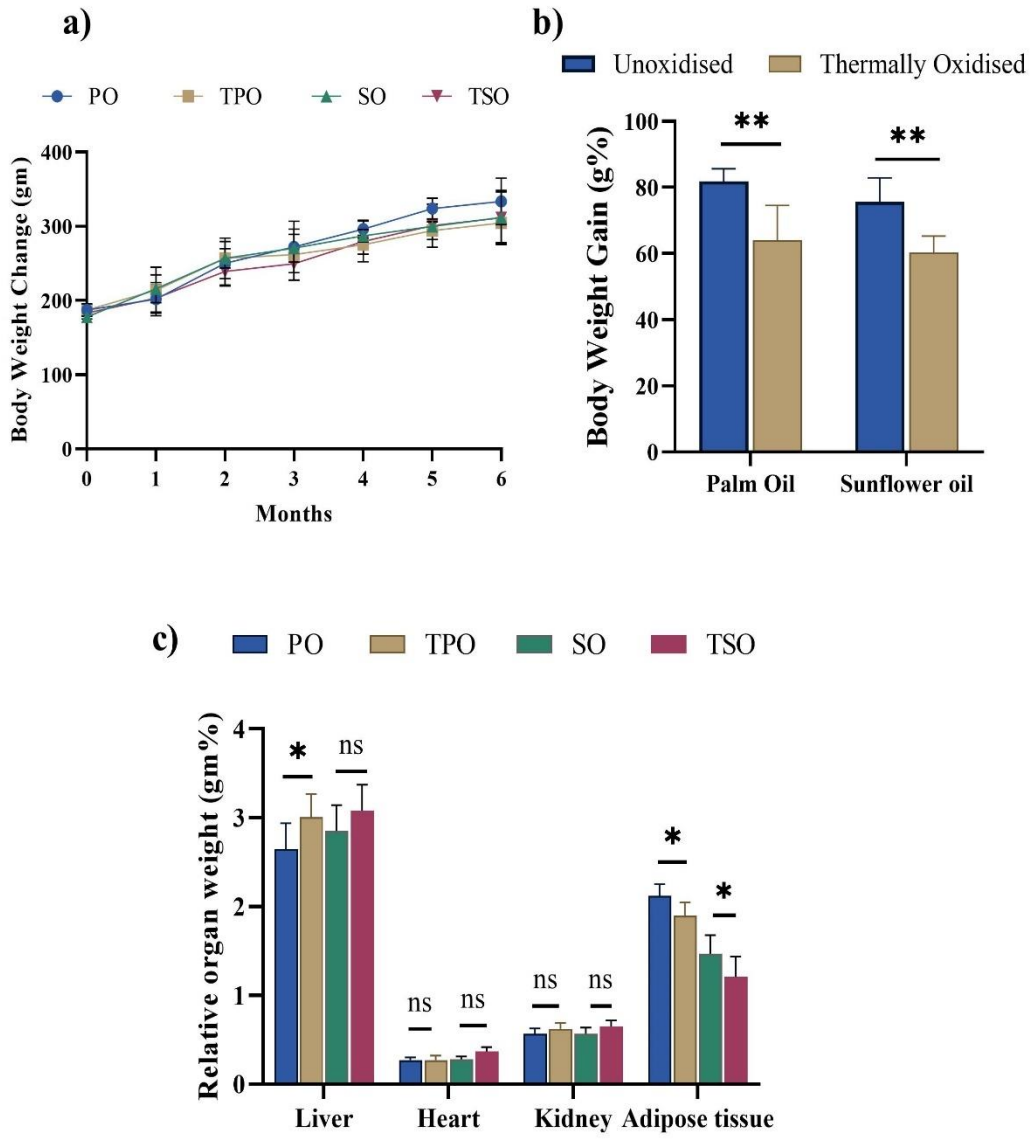

Figure 6.1 Body weight change, weight gain (g%), and relative organ weight of Experimental rats: (PO) unoxidized Palm oil, (TPO) thermally oxidized Palm oil, (SO) unoxidized sunflower oil, (TSO) thermally oxidized sunflower oil incorporated rat chow was given to Wistar rats over 6-month periods. Change in body weight (a) % weight gain (b) and relative organ weight (c) were documented. Data represented as Mean ±SD (n= six animals per group); Statistical analysis was done using GraphPad Prism software (Prism 9). Significant levels were indicated as follows ns non-significant, *p< 0.05, between oil diet groups, as determined by two-way ANOVA followed by Tukey's multiple comparison test.

6.5.2 Glycaemic Response

The impacts of consumption of PO, SO, TPO and TSO-containing diet on the glycaemic response of Wistar rats were assessed through OGTTs conducted at the beginning and end of the experimental period. Following oral administration of glucose (2g/kg bw), blood glucose levels were observed to be increased within 1hr and thereafter reduced over time (Figures 6.2a and 6.2b) to reach the initial level. The total area under the curve (AUC) for each dietary group of animals was calculated. AUC of the initial glucose tolerance test were as follows PO 14944±303, TPO 15484±293.2, SO 15560±334.6, and TSO 15315±322.7 with no significant changes among the groups. However, by the end of the study, the AUC of final OGTT was significantly ($P < 0.01$) reduced in the group fed with the TPO diet (12865±394.5) compared to the group fed with the PO diet (14220±454.1) (Figure 6.2c). Whereas, the SO (12630±450.3) and TSO (12240±452.4) diet-fed animals exhibited decreased AUC of final OGTT compared to other groups (Figure 6.2d).

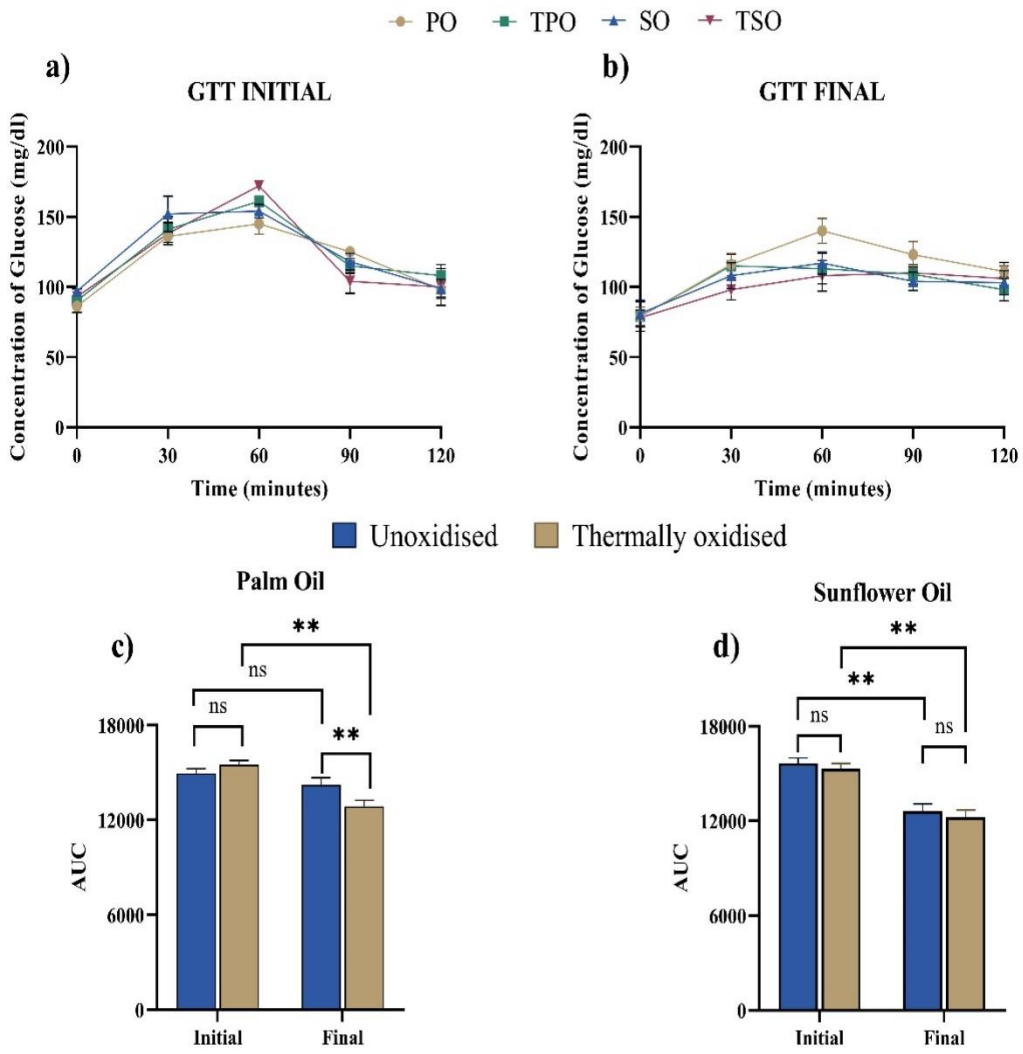

Figure 6.2. Glycaemic response of consumption of 5% of unoxidized and thermally oxidized palm oil and sunflower oil diet in Wistar rats over six-month dietary intervention. Initial and final oral glucose tolerance test (OGTT) (a and b), the total area under the curve (AUC) of initial and final OGTT of palm oil and sunflower oil (c and d). Results were presented as mean ± SD (n =6 rats per group); Statistical analysis was conducted using GraphPad Prism software (Prism 9), employed with Two – way ANOVA followed by Tukey's multiple comparison test. * $p<0.05$, ** $p< 0.01$, ns non-significant between oil diet groups. Unoxidized palm oil (PO) and thermally oxidized palm oil diet (TPO), unoxidized sunflower oil (SO) and thermally oxidized sunflower oil diet (TSO).

Fasting serum insulin level was significantly increased in rats fed with the TPO diet (3.35±0.65mIU/L) compared to those group fed the PO diet (2.3 ± 0.58mIU/L) during six months of dietary intervention ($p<0.01$). Similarly, the fasting serum insulin level was elevated in rats fed with the TSO diet (2.42 ±0.55 mIU/L) than SO diet (1.99±0.67), with no significant changes ($p< 0.45$), as shown in Figure 6.3a. Homeostasis model assessment of insulin resistance (HOMA-IR) further indicated that TPO (0.67±0.1) diet-fed animals had increased HOM-IR ($p<0.01$) than in PO diet-fed (0.32± 0.03) rats. There was a significant ($p<0.05$) change in HOM-IR between SO (0.48±0.05) and TSO (0.58±0.04) diet-fed group rats, as shown in Figure 6.3b. HOMA-S indicates insulin sensitivity. HOMA-S significantly ($p<0.01$) reduced for the TPO (343.98±42.46) diet-fed group compared to the PO group (172.19±52.03). There were no significant changes in HOMA-S between SO (225.36 ± 34.05) and TSO (201.43±45.94) groups (Figure 6.3c). There is no significant difference (ns) in β-cell function (HOMA-B) between SO (21.29± 4.39) and TSO (24.95±7.67) indicating that thermal oxidation does not adversely affect the pancreatic β-cells ability to produce insulin. PO (26.43±7.07) showed significantly ($p<0.05$) reduced HOMA-B compared to TPO (37.75±7.34) (Figure 6.3d).

6.4.3 Coagulation system

Table 6.1 shows the impact of unoxidized and thermally oxidized palm oil and sunflower oil on various blood clotting parameters. There were no significant differences in the initial and final bleeding times among the experimental groups. This suggests that bleeding time remained consistent across all groups throughout the study. Similar to bleeding time, there were no significant changes observed in clotting time between the initial and final measurements among these animals. This implies that clotting time remained stable across all groups. Prothrombin time for the PO diet group was 64 ± 6 seconds; for the TPO diet group, it was 54.67 ± 6.7 seconds; for the SO diet

group, it was 48.33 ± 4 seconds; and for the TSO diet group, it was 47.67 ± 7 seconds. The results indicated no significant differences in prothrombin time among the four groups, as the differences are not statistically significant. APTT determined for the TPO diet group was significantly ($p<0.05$) reduced; it was 63 ± 6.2 seconds when compared to PO diet fed group (96 ± 4.5 seconds). There was no significant change was found in between SO and TSO diet fed groups, 51.3 ± 5.7 and 67.7 ± 2.5seconds, respectively.

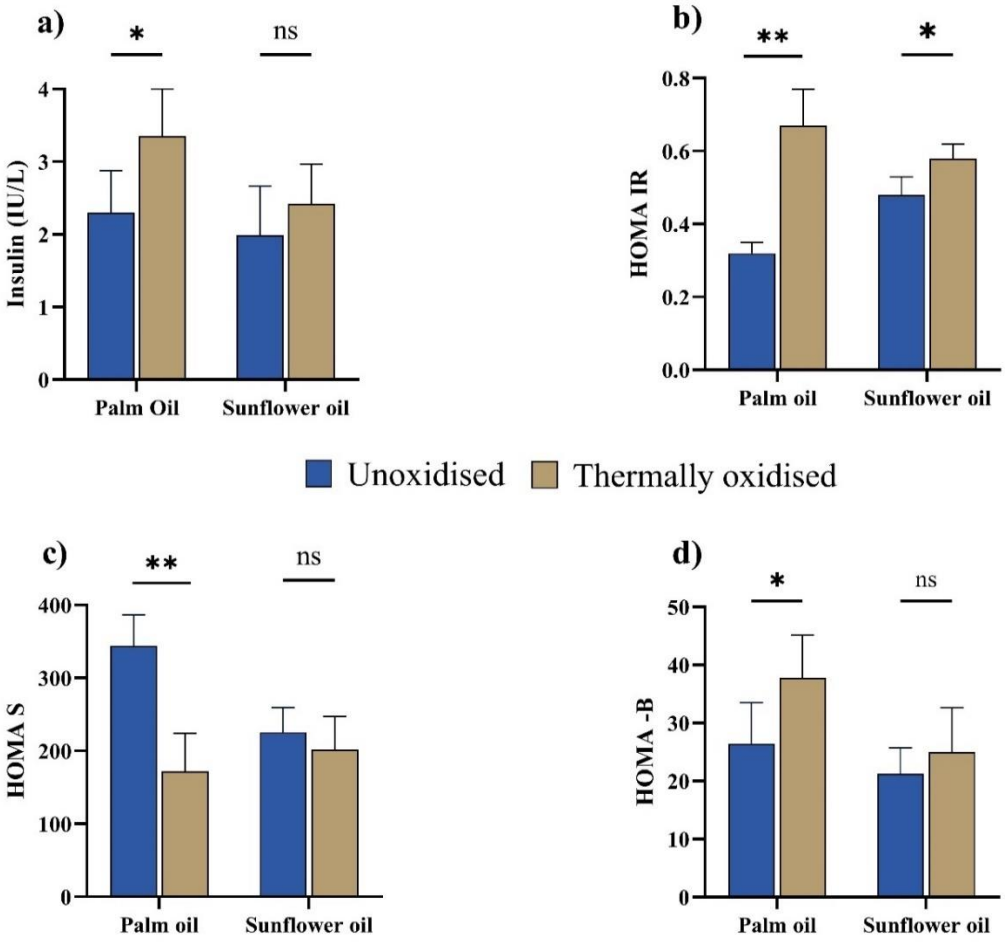

Figure 6.3. Serum insulin and HOMA indices of animals consumed 5% unoxidized or thermally oxidized palm oil and Sunflower oil for 6 months. a) Serum insulin, b) HOMA-IR) c) HOMA-S d) HOMA-B. Results were presented as mean ± SD (n =6 rats per group); Statistical analysis was conducted using GraphPad Prism software (Prism 9), employed with Two – way ANOVA followed by Tukey's multiple comparison test. * $p<0.05$, **$p< 0.01$, ns non-significant between oil diet groups.

Table 6.1: The Effect of Consumption of 5% Of Unoxidized and Thermally Oxidized Palm Oil and Sunflower Oil Diet on Coagulation System

Parameters		Palm Oil		Sunflower oil	
		Unoxidized	Thermally Oxidized	Unoxidized	Thermally Oxidized
Platelet (lakh/mm^3)		9.8±0.67	9.62± 1.06^{ns}	9.4± 1.35	8.5±0.54^{ns}
Bleeding Time	**Initial**	2.1±0.3	1±0.0^{ns}	2.1 ±0.3	1.9±0.3^{ns}
(Minutes)	**Final**	2.1±0.3	1.7± 0^{ns}	1.6 ±0.2	1.5 ±0.2^{ns}
Clotting Time	**Initial**	1.3 ±0.0	1 ±0.0^{ns}	0.8±0.3	0.8 ±0.3^{n}
(Minutes)	**Final**	1.3 ±0.3	1 ±0^{ns}	1.1±0.1	0.8 ±0.3^{ns}
Prothrombin Time (sec)		64± 6	54.67± 6.7^{ns}	48.33± 4	47.67 ± 7^{ns}
APTT (Sec)		96 ± 4.5	63± 6.2**	51.3±5.7	67.7± 2.5^{ns}

The result was expressed as mean ± SD (n= 6 animals per group). Statistical analysis was done by using GraphPad Prism software (Prism 9) * $p<0.05$, ns non-significant between oil diet groups with Two–way ANOVA followed by Tukey's multiple comparison test.

6.5.4 Systemic Toxicity

Table 6.2 illustrates the liver function and renal function markers in rats after the consumption of oxidized and unoxidized palm oil and sunflower oil diet for six months. The concentration of total bilirubin, total protein, and albumin showed no significant difference among the groups. The PO, TPO, SO, and TSO oil diet-fed rats having values of 0.36 ± 0.07, 0.45 ± 0.11, 0.50 ± 0.1, and 0.45 ± 0.05 mg/dL, respectively for total bilirubin. The values of total protein were 7.68±0.23, 7.4±0.52, 7.67 ± 0.5, and 7.38 ± 0.39 mg/dL, respectively. The values were 3.54±0.15, 3.49±0.12, 3.45 ± 0.08, and 3.61 ± 0.19 mg/dL, respectively for albumin concentration. The globulin levels of PO and TPO oil diet group was 4.03±0.43and 3.95±0.4mg/dL respectively with no significant changes. There was a significant change was found in between SO and TSO oil diet fed groups. The values were 4.27 ± 0.28, and 3.75 ± 0.31 mg/dL respectively. SGPT levels were significantly ($p<0.01$) elevated in the TPO (75.63±8.96 U/L) and TSO (116.75 ± 10.61 U/L) diet-fed rats compared to PO (50.38±8.14 U/L) and SO

(57.71 ± 7.68 U/L) diet-fed groups respectively. This is indicative of liver damage or inflammation. SGOT levels were significantly ($p< 0.01$) elevated in PO (104.47±5.4 U/L) compared to that of TPO (82.21±4.6 U/L). In SO diet-fed rats, the level was 74.15 ± 1.9 U/L, and for TSO it was 89.51 ± 2.7 U/L indicating only a marginal increase. This also shows the PO-fed rats had significantly higher SGOT levels than SO or TSO-fed group animals. ALP levels were significantly ($p<0.01$) elevated in the TPO (111.38±6.4 U/L) diet-fed group compared to their PO (77.2±8.4 U/L) diet-fed group. Similarly, a significant ($p<0.01$) increase in ALP activity was found in the TSO (84.92 ± 4.6 U/L) diet-fed group compared to the SO (71.41 ± 7.6 U/L) diet-fed group. However, the increased ALP level in TSO fed animals was observed to be lower than the level in TPO fed animals. The AST/ALT ratio was significantly ($p < 0.05$) higher in TSO (2.67 ±0.5) compared to SO (1.66 ±0.5), PO (1.73±0.32) and TPO (1.56±0.21) diet-fed group rats. The result suggested that the TPO and TSO diet-fed rats were associated with abnormalities in several liver-related parameters, such as total bilirubin, SGPT, SGOT, ALP, and AST/ALT ratio, these findings may indicate liver dysfunction or injury in the TPO and TSO diet- fed group rats. On the other hand, the PO diet-fed rats also showed elevated SGPT, SGOT, and AST/ALT ratio, suggesting potential liver damage, even though to a lesser extent than the TPO-fed groups.

There was no significant difference in the concentration of creatinine among the groups. The creatinine level of the PO, TPO, SO, and TSO group was 1.26 ± 0.28, 1.24 ± 0.2, 1.42±0.08, and 1.34±0.02 mg/dL. Urea is a waste product produced when the body breaks down proteins. The kidneys also excrete it, which can be used as an indicator of kidney function. There was no significant difference among the groups. The urea level of the PO, TPO, SO, and TSO groups was 30 ± 1.5, 27.44± 4.4, 28± 2.3, and 27±1.84 mg/dL.

Table 6.2: The Impact of Consumption of 5% of Unoxidized and Thermally Oxidized Palm Oil and Sunflower Oil Diet on Liver and Renal Function Tests.

Parameters	**Palm Oil**		**Sunflower Oil**	
	Unoxidized	**Thermally Oxidized**	**Unoxidized**	**Thermally oxidized**
Total Bilirubin (mg/dL)	0.36±0.07	0.45±0.11^{ns}	0.50± 0.1	0.45 ± 0.05^{ns}
Total protein (mg/dL)	7.68 ±0.23	7.4±0.52^{ns}	7.67 ± 0.5	7.38 ± 0.39^{ns}
Albumin (mg/dL)	3.54±0.15	3.49 ±0.12^{ns}	3.45 ± 0.08	3.61 ± 0.19^{ns}
Globulin (mg/dL)	4.03 ±0.43	3.95±0.4^{ns}	4.27 ± 0.28	3.75 ± 0.31*
SGPT (U/L)	50.38 ±8.14	75.63 ±8.96**	57.71 ± 7.68	116.75 ± 10.61**
SGOT (U/L)	104.47± 5.4	82.21 ± 4.6**	74.15 ± 1.9	89.51 ± 2.7**
ALP (U/L)	77.2 ±8.4	111.38 ±6.4**	71.41 ± 7.6	84.92 ± 4.6**
AST/ALT	1.73±0.32	1.56±0.21^{ns}	1.66 ±0.5	2.67 ±0.5**
Urea (mg/dL)	30.15± 1.8	27 ± 4.4^{ns}	28± 2.3	27±1.84^{ns}
Creatinine (mg/dL)	1.26 ± 0.28	1.24 ± 0.2^{ns}	1.42±0.08	1.34±0.02^{ns}

Statistical analysis was done using GraphPad Prism software (Prism 9). * $p<0.05$, ** $p< 0.01$, ns non -significant between oil diet groups with Two – way ANOVA followed by Tukey's multiple comparison test.

6.5.5 Lipid Metabolism

Table 6.3 shows the levels of serum as well as hepatic lipid profile of PO, SO, TSO, and TPO oil diet-fed groups. The serum triglycerides were significantly ($p<0.01$) higher in the TPO (142.36 ± 11.1 mg/dL) group animals, followed by PO (118.75 ± 11.3 mg/dL) diet-fed group animals. Statistically, no significant differences were observed between the SO diet group with triglyceride levels at 97 ± 20 mg/dL and the TSO diet group with triglyceride levels at 81.62 ± 13.11 mg/dL. There was no significant interaction between oil and oxidation on serum cholesterol of oil diet-fed

animals. The levels of serum cholesterol were more or less the same for PO, TPO, SO and TSO diet-fed groups (71 ± 7, 66 ± 4, 68±6, and 68±7 mg/dL respectively). There was no significant change among the groups. HDL-C values did not differ significantly among the oil diet groups. The HDL-C levels of PO, TPO, SO and TSO groups were 40.07 ± 5.4, 37.96 ± 3.7, 35.79 ± 2.7, and 40.26 ± 5.3 mg/dL respectively. Similarly, no significant changes were observed in the serum LDL-C between unoxidized and their respective oxidized oils ($p=0.97$). The serum LDL-C levels documented for PO, TPO, SO and TSO were 28.66 ± 6.1, 30±4, 20.39±5.4, and 25.51±6 mg/dL respectively. The VLDL-C levels exhibited a significant change ($p < 0.05$) between the SO diet group (13.4 ± 1.9 mg/dL) and the TSO diet group (19 ± 2.3 mg/dL). There were no significant changes in the serum level of VLDL-C in the PO and TPO groups, (24± 2, and 29± 4 mg/dL).

The levels of hepatic triglyceride contents in the PO, TPO SO, and TSO were 740.32 ± 56.4, 744.65 ± 73.77, 708.32 ± 44.87, and 648.65 ± 42.5 mg/100g tissue respectively. The difference in hepatic cholesterol levels was more pronounced for palm oil than for sunflower oil. Specifically, the hepatic cholesterol levels of rats fed the TPO diet were 187.12 ± 10.26 mg/100g tissue, which was significantly ($p<0.05$) higher than the rats fed the PO diet (163.85 ± 17.66 mg/100g tissue). The hepatic cholesterol levels of rats fed TSO were 101.07 ± 10.76 mg/100g tissue lower than those of rats fed SO diet (112.98 ± 10.37 mg/100g tissue). The consumption of thermally oxidized oil led to a decrease in hepatic phospholipid levels in both palm oil and sunflower oil diets. The decrease was more pronounced in the sunflower oil diet, with a 23.54% decrease compared to a 13.89% decrease in the palm oil diet. The phospholipid content of the PO, TPO, SO, and TSO diet groups was 585.96±54.4 mg/100g tissue, and 504.56±40.85, 575.44±40.83, and 440±58.03mg/100g tissue, respectively.

Figure 6.4 shows the activities of hepatic lipase and HMG CO. A reductase activity of rats fed with PO, TPO, SO, and TSO oil diets. Figure 6.4a shows the indirect assessment of hepatic HMG Co.A activity as measured by the HMG Co.A/ Mevalonate ratio for PO, TPO, SO, and TSO diet-fed group animals. HMG Co.A is a substrate for HMG Co.A reductase, the rate-limiting enzyme in cholesterol biosynthesis. Therefore, an increase in the HMG Co.A/Mevalonate ratio indicates a decrease in HMG Co.A reductase activity. The HMG Co.A/Mevalonate ratio was higher in the PO group (2.27±0.47). However, the HMG Co.A/Mevalonate ratio in the TPO group (1.65 ±0.28) was marginally decreased but not significantly different from the PO diet group. The SO diet-fed group showed a ratio of 1.44 ± 0.47. Interestingly, the TSO diet-fed group exhibited a slightly higher ratio of 1.64 ± 0.30. It's important to note that there was no statistically significant difference in the HMG Co.A/ Mevalonate ratio among the SO diet-fed group and the TSO diet-fed group.

There was no significant difference in hepatic lipase activities observed among PO (26.34±6.9 U/mg protein) and TPO (25.88±4.26 U/mg protein) diet-fed groups. The significant ($p<0.01$) difference was observed in SO and TSO diet fed group animals. Remarkably, the SO diet-fed group exhibited a highest hepatic lipase activity, with a value of 52.58±9.06 U/mg protein, the TSO diet-fed group was shown 30.85±7.64 U/mg protein of lipase activity (Figure 6.4b).

Table 6.3: The Effect of Consumption of 5% of Unoxidized and Thermally Oxidized Palm and Sunflower Oil Diet on Serum and hepatic Lipid Profile.

		Palm Oil		Sunflower oil	
		Unoxidized	Thermally Oxidized	Unoxidized	Thermally Oxidized
Serum Lipid Profile (mg/dL)	**Triglycerides**	118.75 ±11.3	142.36±11.1*	97±20	81.62±13.1^{ns}
	Cholesterol	71 ±7	66 ±4^{ns}	68±6	68±7^{ns}
	HDL-C	40.07±5.4	37.96 ±3.7^{ns}	35.79 ±2.7	40.26 ±5.3^{ns}
	LDL-C	28.66 ±6.1	30 ±4^{ns}	20.39±5.4	25.51±6^{ns}
	VLDL-C	24± 2	29 ±4^{ns}	13.4 ±1.9	19 ±2.3*
Hepatic Lipids (mg/ 100g)	**Triglycerides**	740.32 ± 56.4	744.65 ± 73.77^{ns}	708.32 ± 44.87	648.65 ± 42.5^{ns}
	Cholesterol	163.85 ± 17.66	187.12 ± 10.26**	112.98 ± 10.37	101.07 ± 10.76^{ns}
	Phospholipid	585.96±54.4	504.56±40.85*	575.44±40.83	440±58.03**

The result was expressed as mean ± SD (n= 6 animals per group). Statistical analysis was employed with two – way ANOVA followed by Tukey's multiple comparison test ns non-significant, * $p<0.05$, ** $p<0.01$ between oil diet groups.

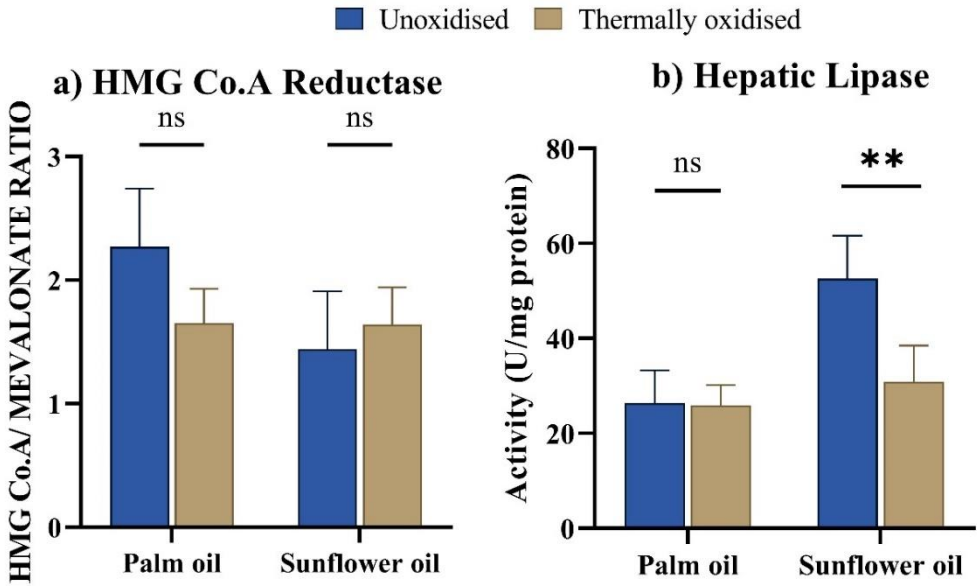

Figure 6.4: The effect of consumption of 5% of unoxidized and thermally oxidized palm and sunflower oil diet for 6 months on a) HMG Co. A reductase activity and b) hepatic lipase. Statistical analysis was done by using GraphPad Prism software (Prism 9) **p< 0.01, ns non - significant between oil diet groups done by two – way ANOVA followed by Tukey's multiple comparison test.

6.4. Carbohydrate metabolism

The activity of FBPase was measured following the consumption of PO, TPO, SO, and TSO oil diet. FBPase plays a key role in gluconeogenesis, the process of making glucose from non-carbohydrate sources. An increase in FBPase activity can lead to an increase in gluconeogenesis. The data illustrated in Figure 6.5a that the consumption of palm oil or sunflower oil diets did not have a significant effect on hepatic FBPase activity. The values of FBPase activity documented in PO, TPO, SO, and TSO oil diet-fed rats were 2.47±0.23, 2.33±0.2, 2.09 ± 0.27, and 2.12 ± 0.26 µg of Pi formed/min/mg protein.

The given Figure 6.5 depicts the activity levels of three mitochondrial enzymes: SDH, MDH, and ICDH activities. Both palm and sunflower oil showed a decrease in succinate dehydrogenase activity after consumption of thermally oxidized oil

compared to their unoxidized oil. SDH activity was significantly reduced ($p< 0.01$) in the TPO-fed group (14±2 U/mg protein) than in the PO (27 ± 3 U/mg protein) diet-fed group. A significant decrease was also found ($p < 0.01$) in the TSO oil diet group (12 ± 4 U/mg protein) compared to the SO oil diet group (29 ± 8 U/mg protein). SDH activity is illustrated in Figure 6.5b.

Figure 6.5c depicts the data of MDH activity. Both TPO and TSO oil diet rats had shown increased hepatic MDH activity after six months of oil diet consumption. However, the increase in hepatic MDH activity was profoundly found in both PO diet groups compared to both SO oil diet groups. Significantly ($p<0.01$) highest MDH activity was exhibited in the TPO (473.77±88.9 U/mg protein) oil diet-fed group than in the PO-fed group (185.37±29.8 U/mg protein). Additionally, the TSO oil diet group showed a significant increase in malate dehydrogenase activity at 236.88 ± 26 U/mg protein relative to the SO oil diet group, which had an activity of 76.49 ± 16.2 U/mg protein ($p < 0.01$).

In contrast, there were no significant differences in hepatic isocitrate dehydrogenase (ICDH) activity among the groups, with activities for the PO, TPO, SO, and TSO diet groups being 164.65 ± 23.3, 142.56 ± 23.78, 133.77 ± 21.14, and 144.4 ± 14.31U/mg protein, respectively. Data is visually represented in Fig. 6.5d.

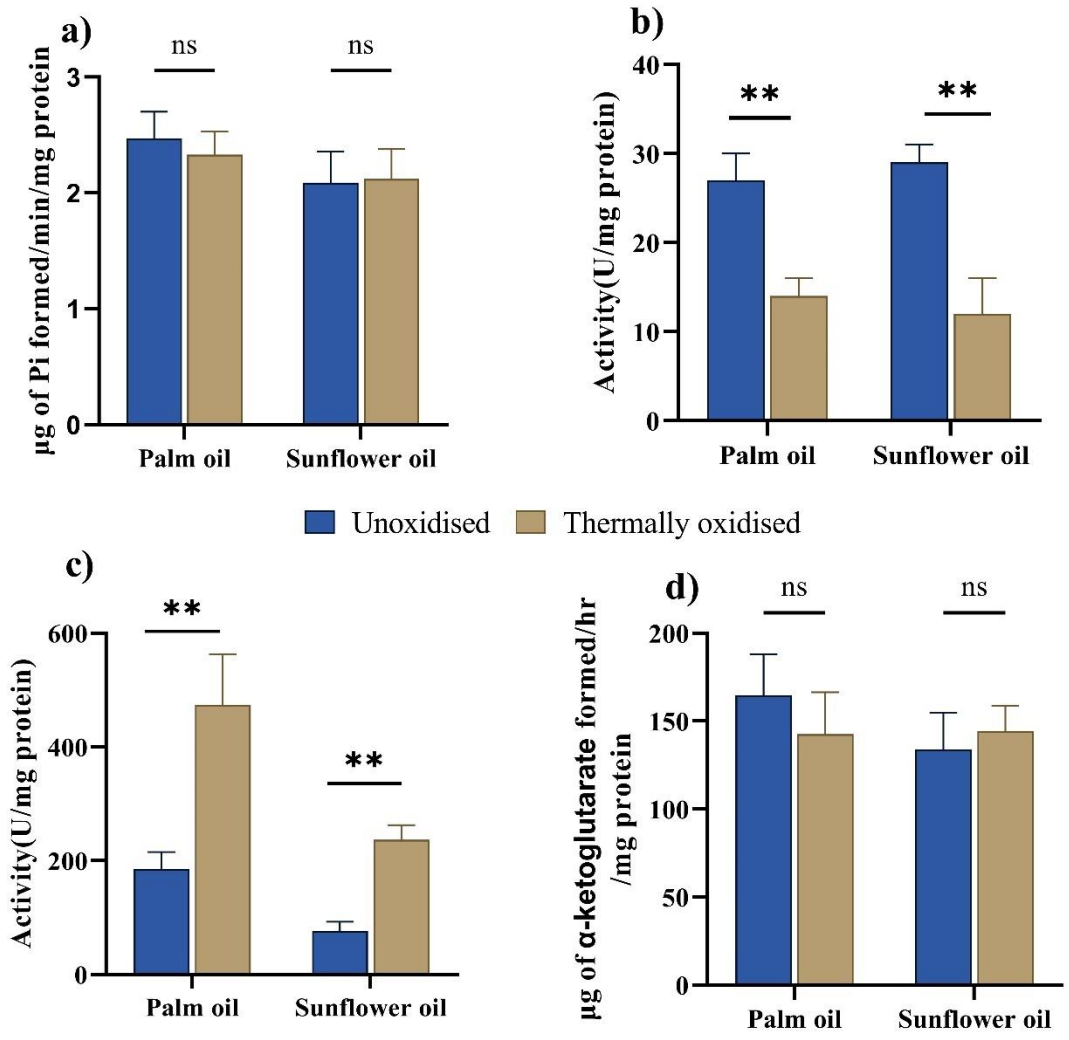

Figure 6.5: The effect of consumption of 5% of unoxidized and thermally oxidized palm oil and sunflower diet for 30 weeks on hepatic a) FBPase, mitochondrial b) SDH c) MDH, and d) ICDH activity. Statistical analysis was done by using GraphPad Prism software (Prism 9) **p< 0.01, ns non -non-significant between oil diet groups done with two – way ANOVA followed by Tukey's multiple comparison test

6.4.7 Inflammatory Markers

Figure 6.6 shows the mean levels of inflammatory markers such as CRP, Lp-PLA_2, IL-6, and IL-10 for PO, TPO, SO, and TSO diet-fed groups. The CRP did not show a significant difference between palm oil diet-fed groups. The CRP level PO and TPO oil diet-fed groups were 0.89 ± 0.1 and 0.82 ± 0.1 mg/dL. There was a slight increase in CRP levels in SO (0.94±0.1mg/dL) and TSO (1.13±0.3mg/ dL) diet-fed animals, but the differences were not statistically significant. The Lp-PLA_2 activity of PO diet fed group was 12.27±3.0 U/mg protein and the TPO group was 11.39 ± 2.7 U / mg protein,

but the differences did not have statistically significance. Both SO diet-fed groups exhibited higher Lp-PLA_2 activity than palm oil diet-fed group animals. Significantly ($p< 0.01$) higher Lp-PLA_2 activity was observed in TSO-fed rats (41.3±5.7 U/mg protein) than the SO (29.88 ±3.3 U/mg protein) diet-fed group.

The TPO diet groups exhibited significantly ($p<0.01$) lower IL-6 levels at 757.5±83.27 pg/mL compared to the PO diet-fed group, which had IL-6 levels of 1315 ± 110 pg/mL. However, in the TSO diet-fed group animals, IL-6 levels were 367.5 ± 125.03 pg/mL, which although was found to be lower than the SO diet-fed group (427.5±52.92pg/mL), with no statistical significance. The data is visually illustrated in Figure 6.6c. The serum IL-10 levels were slightly increased in the TPO (38.15±7.07 pg/mL) fed diet group animals compared to the PO (32.96±8.01 pg/mL) diet-fed animals. On the other hand, the IL-10 levels were found to be decreased in the TSO (51.98±2.36pg/mL) compared to the SO (56.91±9.47pg/mL) diet-fed groups (Figure 6.6d). There were no significant changes found between unoxidized and thermally oxidised palm and sunflower oil.

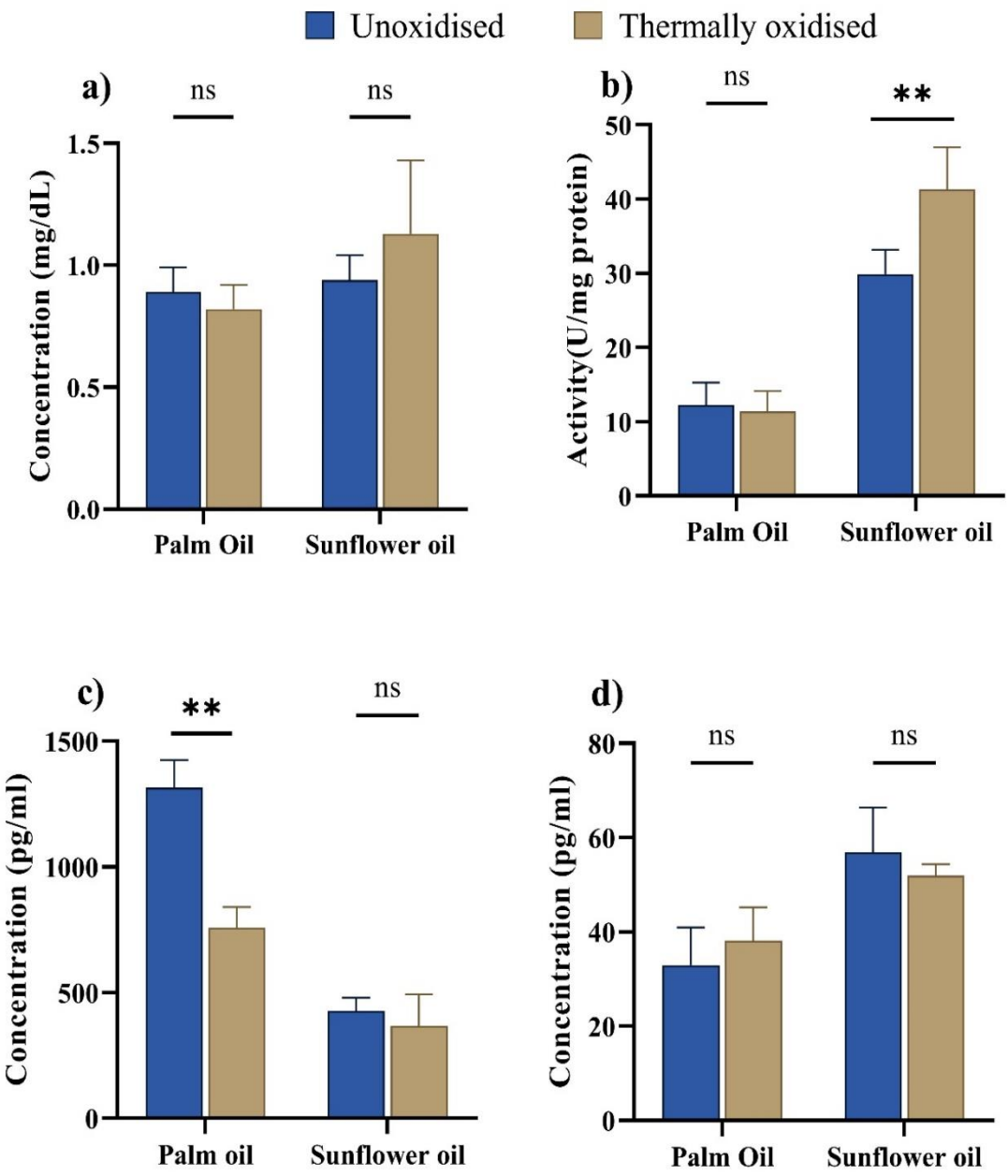

Figure 6.6: The effect of consumption of 5% of unoxidized and thermally oxidized palm oil and sunflower oil diet on serum a) CRP, b) LP-PLA_2 and c) IL-6 and d) IL-10. Statistical analysis was done by using GraphPad Prism software (Prism 9). A $p< 0.05$ was considered statistically significant. Significant levels were indicated as follows, **$p< 0.01$, ns for non-significant between oil diet groups, as determined by two–way ANOVA followed by Tukey's multiple comparison test.

6.5 Histopathological Analysis

The histological analysis of liver tissues of experimental animals is given in Figure 6.6 and the scoring of hepatic tissue damage is depicted in Table 6.4. The impacts of feeding different oil diets on hepatic tissue in rats revealed varying degrees of hepatic

toxicity among the groups fed with unoxidized and thermally oxidized palm and sunflower oil. Rats fed with SO and PO group exhibited marginal liver damage characterized by portal tract inflammation, and macrovesicular steatosis, with scoring of '1'. The palm oil group showed the least liver damage with a total score of '4' (Figure 6.7a). Examination of hepatic tissues from TPO-fed rats, with a total scoring of '7' revealed mild portal tract inflammation and glycogenated nuclei, resulting in a score of 2 and also hepatocellular ballooning, and macrovesicular steatosis with a scoring of '1' (Figure 6.7b). Liver histology of SO rats shown moderate glycogenated nuclei with the scoring of '2'. Hepatocellular ballooning was found in SO-fed rat liver with scoring of "1". In the SO group, moderate liver damage was observed with a total score of '6' (Figure 6.7c). The most severe liver damage was found in TSO oil diet groups, with a total score of '10'. This group demonstrated severe liver toxicity of macrovesicular steatosis, portal tract inflammation and hepatocellular toxicity scoring '2' and also severe glycogenated nuclei scoring '3' (Figure 6.7d).

Table 6.4: Scoring of Liver Histopathology.

Characteristics	PO	TPO	SO	TSO
Glycogenated nuclei	1	2	2	3
Hepatocellular ballooning and disarray	0	1	1	2
Macro vesicular steatosis	1	1	1	2
Portal Tract inflammation	1	2	1	2
Sinusoidal dilation	1	1	1	1

Scoring indicates 0–3 based on the extent of damage in the hepatocytes, where 0 is none; 1 is marginal(mild); 2 moderate; and 3 is severe.

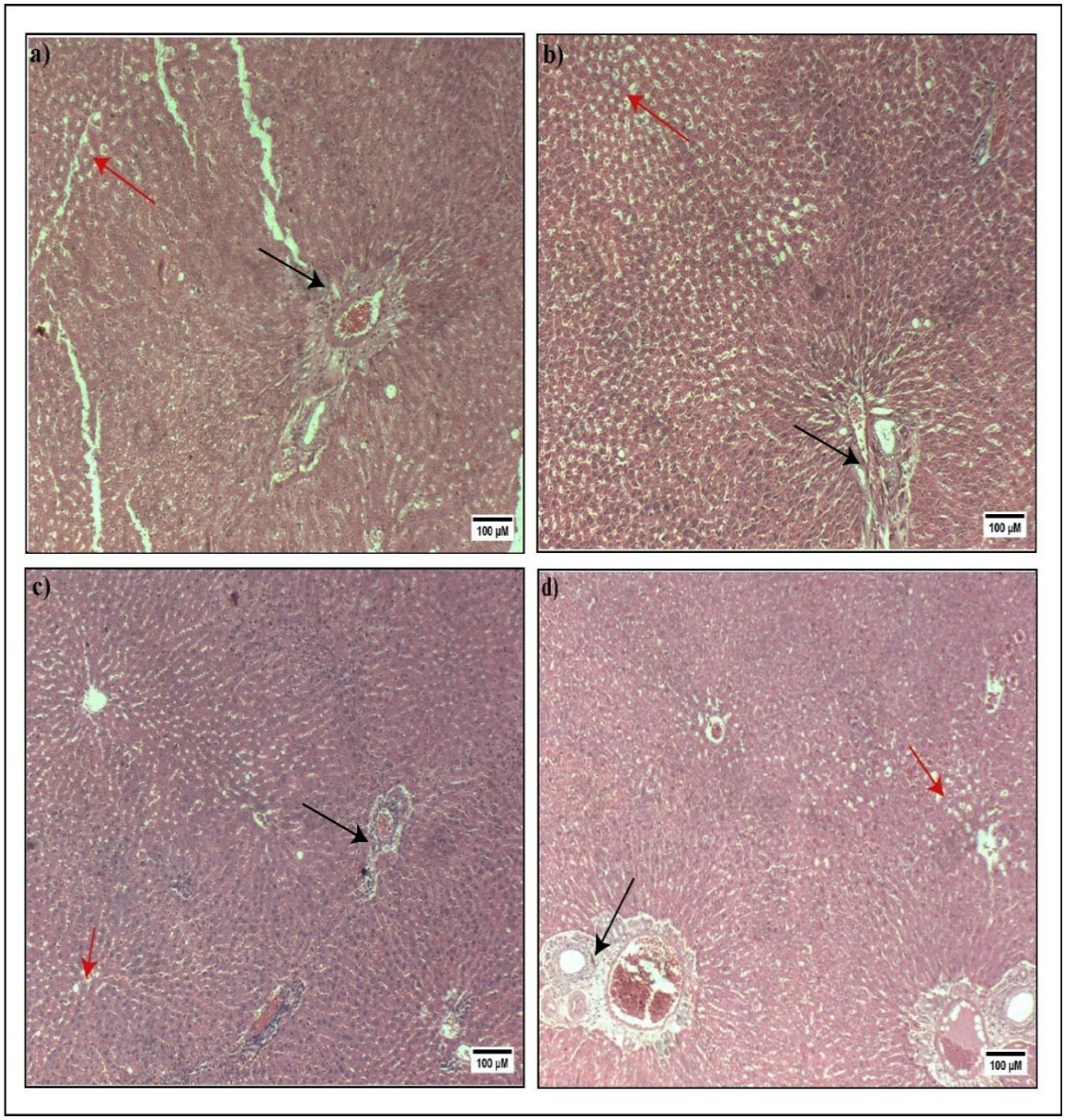

Figure 6.7 Photomicrograph of rat liver tissue stained with hematoxylin and eosin (100x). a) PO group, b) TPO group, c) SO group, d) TSO group. Arrows black and red indicated portal tract inflammation and macrovesicles.

Figure 6.8 illustrates histological variations in renal tissues exposed to unoxidized and thermally oxidized palm oil and sunflower oil. Rats subjected to both unoxidized and thermally oxidized PO and SO exhibited renal tissue characterized by normal renal corpuscles, intact glomeruli, unmarkable proximal convoluted tubes (PCT), and distal convoluted tubes (DCT). No structural variations were thus observed.

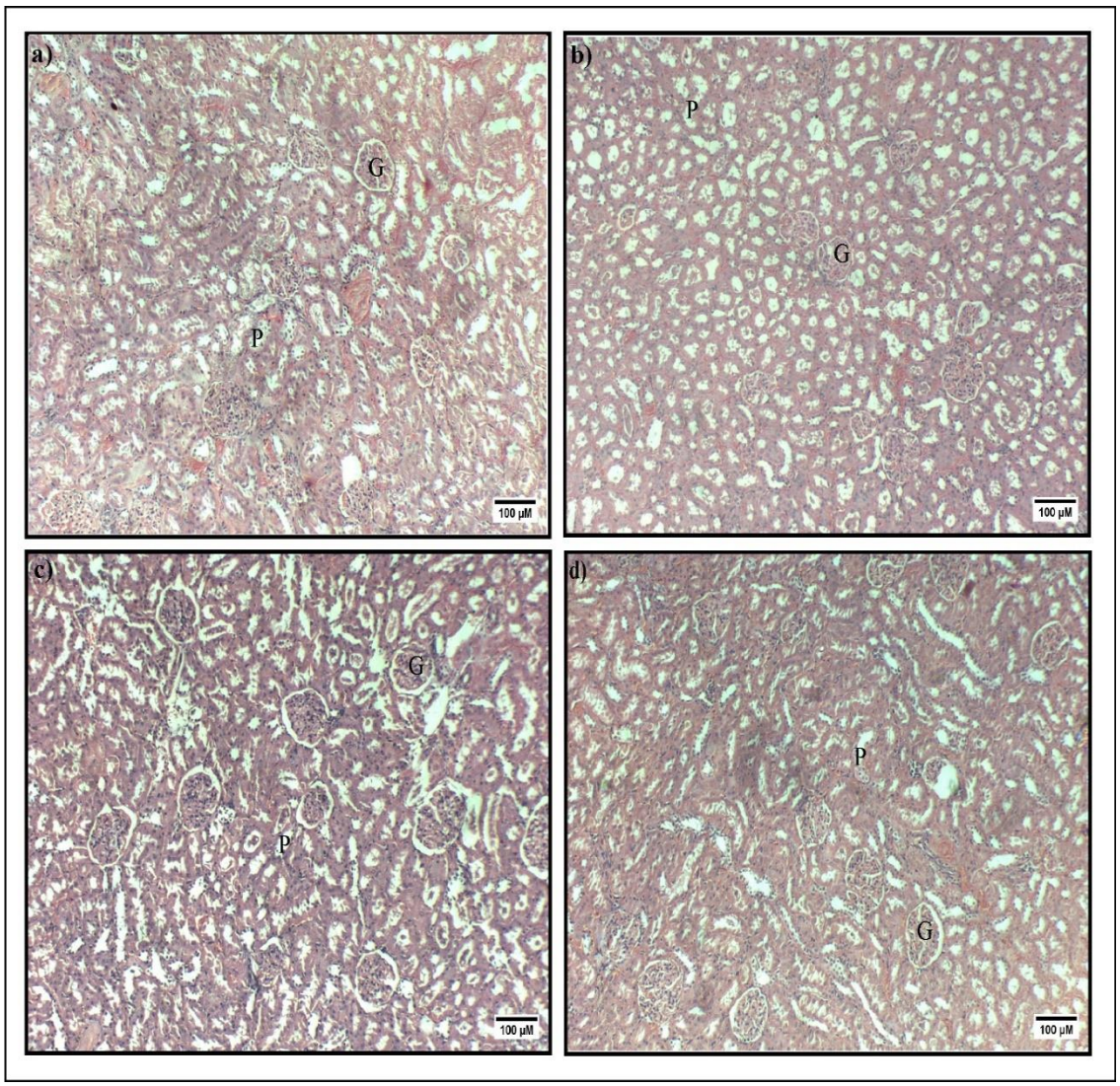

Figure 6.8: Photomicrograph of rat kidney tissue stained with eosin and hematoxylin (100x). a) PO group, b) TPO group, c) SO group, and d) TSO group. Normal renal corpuscles with glomerular capillary loops (G) and proximal convoluted loop (P).

The histological analysis of heart tissue showed normal architecture (Figure 6.8). There was no difference in structural characteristics observed between the experimental group animals**.** Histological details of PO, TPO, SO and TSO exhibited were the same.

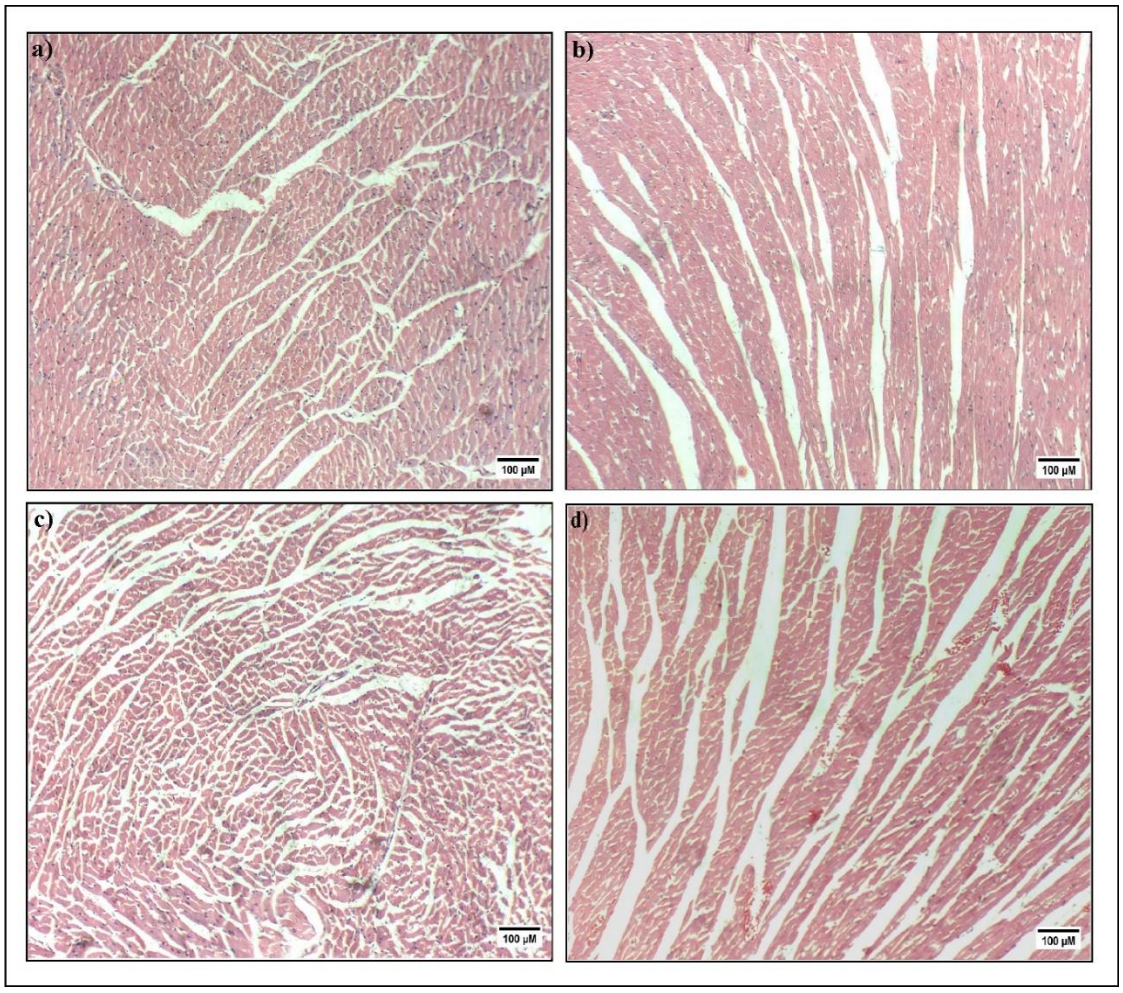

Figure 6.9. Photomicrograph of rat heart tissue stained with hematoxylin and eosin (100x). a) PO group, b) TPO group, c) SO group, and d) TSO group.

Discussion

Thermally oxidized dietary fat is widely recognised as a significant contributor to the development of metabolic disorders, due to its impaired lipid and carbohydrate metabolism at concentrations above normal levels. The current study aims to investigate the impact of a normolipidic (5%) diet incorporating both unoxidized and thermally oxidized PO and SO representing LCFA and PUFA, on carbohydrate and lipid metabolism, and inflammatory markers in rats over a six-month dietary intervention. All groups of rats exhibited body weight gain throughout the experimental period. However, thermally oxidized oil diet-fed group animals failed to attain body weight similar to those fed respective unoxidized oil diet. Although the exact mechanism of reduced body weight gain in thermally oxidized oil groups is not clear but might be due to the depletion of essential fatty acids and bioactive constituents. Increased body weight was found in both palm oil diet-fed groups compared to those fed both sunflower oil diets. A study conducted by Saraswathi et al., (2020) has found that a high-fat diet formulated with palm oil (3% of lard replaced) led to a significant increase in body weight gain as well as hepatic steatosis condition compared to a high-fat diet containing coconut oil (3% of lard replaced). Present results showed that the relative organ weight significantly changes in liver and adipose tissue of thermally oxidized oils-fed animals compared to respective unoxidized oil-fed rats. The relative liver weight was increased in rats fed thermally oxidized oils. The consumption of oxidized palm and sunflower oil can lead to liver weight gain through mechanisms involving oxidative stress, inflammation, fat accumulation, disruption of metabolic pathways such as glucose and lipid metabolism, disruption of cellular energy metabolism and promote hepatic fibrosis. Increased adipose tissue weight was found in both unoxidized and thermally oxidized palm oil-fed diet groups compared to those fed SO and TSO diet fed groups. Both unoxidized and thermally oxidized PO oil diet-

fed rats showed increased serum TG with increased hepatic TG content. This suggests that PO may promote hepatic TG accumulation, which is a risk factor for non-alcoholic fatty liver disease. Some studies have reported that palmitic acid induces obesity through increasing adipogenesis (Murru et al., 2022). Among these oil diet-fed groups, thermally oxidized oil diet group rats showed decreased adiposity.

Compared to the initial and final glucose tolerance in terms of AUC and glucose distribution data of experimental animals, only TPO diet-fed animals experienced a glucose intolerance. Rats fed a TPO diet exhibited an elevated insulin level (hyperinsulinemia), increased HOMA-IR, decreased HOMA-S, and elevated HOMA-B, indicating reduced insulin sensitivity along with heightened β cell activity and developed insulin resistance. PO diet fed rats had improved insulin sensitivity, lower insulin, higher HOMA-S, and unaltered HOMA B. On the other hand, SO and TSO-fed animals were observed to have unaltered insulin, HOMA-IR, HOMA-S, and HOMA-B indicating normal function of pancreatic β cell and insulin sensitivity.

Both PT and APTT were decreased in both thermally oxidized oils-fed groups. Lower prothrombin time and activated partial thromboplastin time are indications of hypercoagulability associated with hyperlipidaemia.

Alanine transaminase (ALT) and aspartate transaminase (AST) are pivotal liver enzymes involved in protein metabolism and their elevated serum levels serve as markers for liver tissue damage due to cellular injury. On the other hand, ALP is a biliary tract enzyme, and an increased level is also associated with liver injury or biliary tract obstruction. Elevated levels of liver enzymes ALP, ALT and AST are significant contributors to the development of fatty liver disease. The ratio between these two enzymes may reflect the nature and severity of liver damage. The ratio of AST/ALT is reported to be less than 1 in non-alcohol fatty liver conditions; however, the ratio rises to greater than 1 in more severe liver damage (Botros and Sikaris, 2013). The results

were suggested that the TPO and TSO diet-fed rats were associated with abnormalities in several liver-related parameters, such as elevated total bilirubin, SGPT, SGOT, ALP, and AST/ALT ratio. These findings indicate liver dysfunction as well as hepatic injury in the TPO and TSO diet-fed group rats. The elevated level of serum ALP was observed in TSO animals possibly in response to biliary duct obstruction. However, SO-fed animals experienced moderate liver damage, On the other hand, the PO diet-fed rats also showed elevated SGPT, SGOT, and AST/ALT ratio, suggesting potential liver damage, even though to a lesser extent than the TPO-fed group rats. These findings are well supported by the observed changes in hepatic tissue histology with TSO-fed animals having a hepatic tissue score of 3 with large macrovesicular foci, inflammatory cell infiltration, hepatic ballooning and enhanced glycogenated nuclei visible in this tissue. It is thus clear that increased liver triglyceride accumulation observed in TSO diet-fed rats contributes to the extent of liver injury observed in these animals.

The study revealed that rats fed with both PO and TPO exhibited significantly higher concentrations of serum TG, LDL-C, VLDL-C, hepatic TG, cholesterol, and phospholipid content compared to both sunflower oil-fed diet groups. These changes in lipid profiles are particularly concerning, as increased serum TG, LDL-C, and VLDL-C levels are well-established risk factors for atherosclerosis and cardiovascular disease (CVD). Additionally, palmitic acid, a major component of palm oil, has been found to increase LDL-C levels, which is associated with elevated blood cholesterol levels. Palm oil, particularly with its high palmitate content, has been associated with potential harm due to lipid oxidation. The study also highlighted the potential mitigating effects of tocotrienols, a minor constituent of PO oil, which is known to suppress cholesterol biosynthesis by downregulating HMG-Co.A reductase activity by post-transcriptional modifications. In this study, increased hepatic HMG Co. A activity was found in the TPO-fed diet group compared to their respective unoxidized oil. This

may cause a mild increase in cholesterol levels in TPO-fed animals. However, it is worth noting that the thermal oxidation of palm oil might have led to the decomposition of tocotrienols, thereby diminishing their protective effects and contributing to the increased cholesterol levels observed in TPO-fed animals. Reduced hepatic lipase activity in turn increased the concentration of VLDL-C in both palm oil-fed diet groups. The findings are also in line with the known association between the cholesterol content of VLDL-C and atherosclerosis. The previous study demonstrated that the association of circulating VLDL-C (VLDL-cholesterol/VLDL-apoB) with 1-year mortality in acute heart failure patients, indicating the prognostic value of VLDL-C content in cardiovascular health (Degoricija et al., 2022). On the other hand, rats fed both sunflower oils had significantly decreased serum TG, LDL-C, and VLDL-C with increased HDL-C and reduced hepatic TG and cholesterol. Slightly increased LDL-C was found in the TSO diet-fed group compared to their unoxidized SO diet-fed group. This is expected as SO was reported to have lipid-lowering activity (Schwingshackl et al., 2018, Wood et al., 1993). Reduced VLDL-C content was found in both sunflower oil diet-fed rats due to their increased hepatic lipase activity. The hepatic phospholipid level was significantly reduced in the thermally oxidized oil diet-fed group in comparison with those who were fed a fresh unoxidized oil diet.

The activities of lipogenic enzymes, namely malic enzyme (MDH) and isocitrate dehydrogenase (ICDH), provide NADPH for fatty acid synthesis, and increased activities of these enzymes in the liver correlate with increased triglyceride concentration (Zhu et al., 2018). The activities of malate dehydrogenase (MDH) were significantly elevated in animals fed thermally oxidized oils compared to those fed fresh oils. In comparison with the group fed a diet of thermally oxidized sunflower oil, increased MDH activity was found in the group fed a diet containing thermally oxidized palm oil. The elevation of MDH activity in both PO oil diet-fed rats correlates with

increased TG in the serum and liver, indicating that enhanced MDH activity contributes to higher lipogenesis by the consumption of PO. In contrast, its activity remains constant across different dietary conditions, indicating that while it contributes to NADPH production, it does not directly correlate with increased triglyceride concentrations.

The incidence between metabolic alterations and inflammatory signalling pathways leads to IR, hepatic steatosis and related diseases. CRP is an acute phase reactant and a marker of inflammation, tissue injury, and infection. Elevated levels are associated with an increased risk of coronary heart disease, ischemic stroke, vascular mortality, and non-vascular mortality (Kaptoge et al., 2010, Shrivastava et al., 2015, Virtanen et al., 2018) and hs-CRP concentration level is associated with the severity of atherosclerosis and are strongly associated with dyslipidaemia. Comparing the CRP level among thermally oxidized oils-fed animals, no significant difference was noticed. Whereas, elevated level was found in TSO diet-fed rats only. CRP selectively binds to oxidized LDL and apoptotic cells through recognition of a common ligand, phosphorylcholine of oxidized phospholipids (Chang et al., 2002) and enhances complement action. CRP and Lp-PLA_2 levels were not significantly altered in both palm oil-fed diet groups. However, CRP and Lp-PLA_2 activity levels have elevated in both sunflower oil diets. TSO diet-fed group rats showed increased levels of CRP and Lp-PLA_2. Previous studies have shown that CRP and Lp-PLA2 are biomarkers significantly increased in metabolic syndrome (Acevedo et al., 2015). Lp-PLA_2 and CRP are characteristics of metabolic syndrome and diabetes, and elevated Lp-PLA2 activity implicates pro-atherosclerotic function (Cojocaru et al., 2010). Therefore, the present results indicated that TSO in the diet promote atherosclerotic CVD. Studies have also reported that consumption of a low palmitic acid/high oleic acid diet exhibits an elevated level of circulatory IL 6 and is a key link between obesity and inflammation

(Dumas et al., 2016; Korbecki and Bajdak-Rusinek, 2019). In this study, GC-MS analysis of palm oil revealed low palmitic acid/high oleic acid content, and the animals that consumed the PO diet showed increasing circulatory levels of IL 6. IL-10 is a cytokine with anti-atherosclerotic and anti-inflammatory potential (Kim et al., 2020, Mallat et al., 1999). Conjugated Linoleic acid is an omega-three fatty acid class of compound and a known anti-inflammatory and anti-atherogenic molecule. In the present study, GC-MS analysis has shown the presence of conjugated linoleic acid in SO. This could be the reason for the increased IL-10 level in the SO diet-fed rats. Thermal oxidation might have converted this conjugated linoleic acid to its oxidized forms, this suggests the reduction of IL-10 in TSO-fed rats.

Histological evaluation of the kidney and heart of rats fed palm oil, and sunflower oil-based diets revealed normal texture of glomerulus and proximal convoluted tubules. The histopathological evaluation of the heart showed normal architecture. The study reveals that edible oil having saturated fats (PO) as well as oxidized form (TPO) induced obesity and associated inflammation and steatosis. On the other hand, SO, and TSO promote inflammation-associated toxicity in hepatic tissues that induced atherogenic development. The schematic diagram represented in Figure 6.10

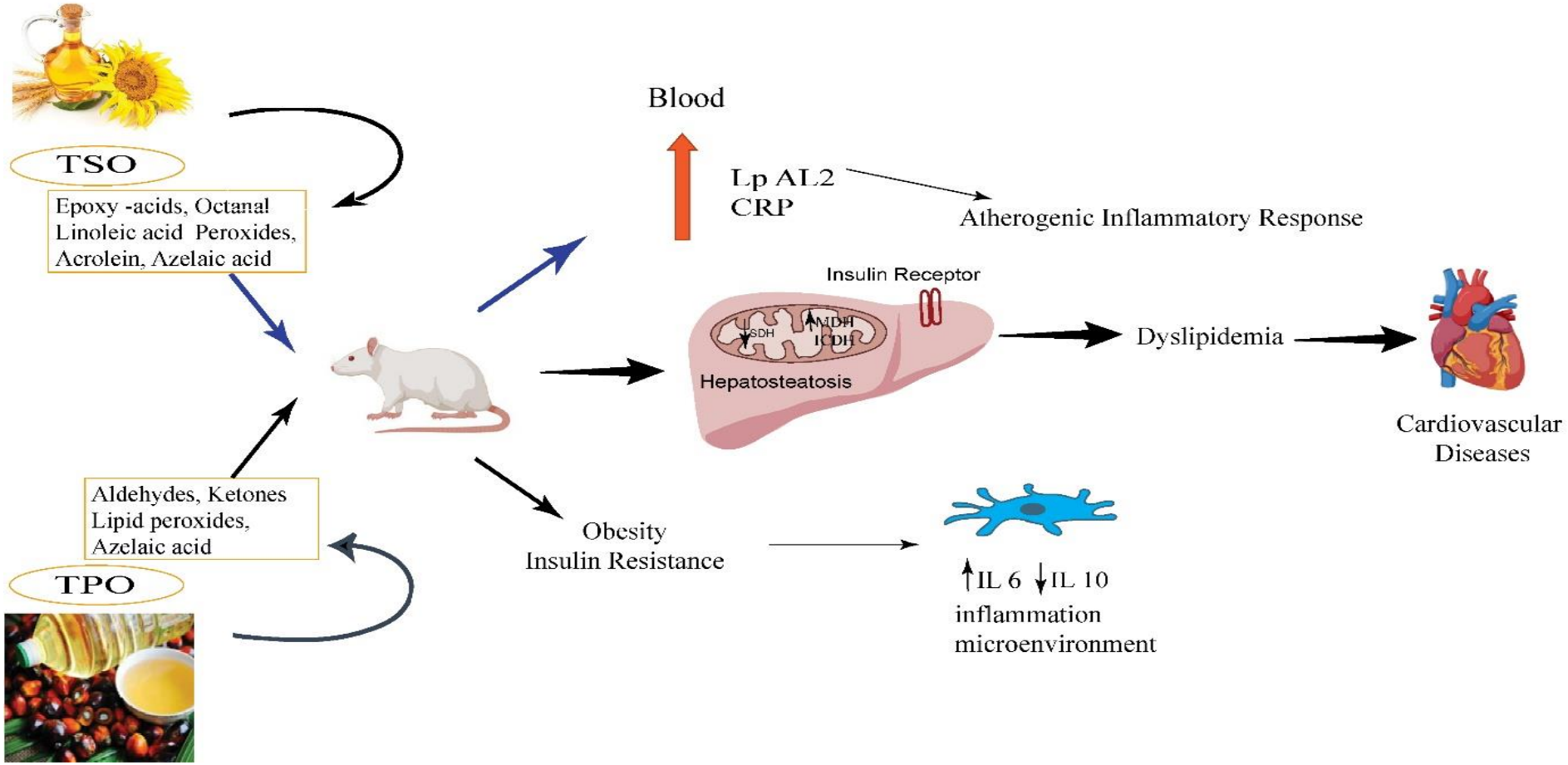

Figure 6.10: The schematic diagram represented in the development of cardiovascular disorder

Chapter 7

Summary and Conclusion

Overall, the thesis documents the impact of thermally oxidized coconut oil, palm oil, mustard oil and sunflower oils at normolipidemic dietary levels on metabolic pathways and cardiovascular disease risk. The initial chapter provided an in-depth review of the chemical processes underlying thermal oxidation in edible oils, highlighting the formation of the various oxidized products such as aldehydes, ketones, and polymerized lipids as well as their potent toxicological effects. Subsequent chapters examined how these oxidized lipids influence key metabolic pathways, particularly those involved in lipid and carbohydrate metabolism and enhanced inflammatory markers. A detailed summary of the significant findings is presented, emphasizing the correlation between the intake of thermally oxidized oils and the development of metabolic dysregulation and cardiovascular risk.

Physicochemical analysis conducted in this study revealed the significant impact of thermal oxidation on the quality, stability and nutritional properties of coconut oil, palm oil, mustard oil and sunflower oil. The findings demonstrated that oils rich in unsaturated fatty acids such as sunflower oil, mustard oil and palm oils were more prone to oxidative degradation when subjected to heat. In contrast, coconut oil, which is predominantly composed of medium-chain saturated fatty acid, exhibited greater resistance to oxidation at elevated temperatures. During thermal oxidation, the susceptibility of unsaturated fatty acids to oxidative breakdown led to noticeable changes in the oil's physical properties, including darkening of colour and an increase in density. These alterations are attributed to the formation of carbonyl compounds and other lipid oxidation products. Furthermore, thermally oxidized sunflower oil, palm oil and mustard oil exhibited significantly higher TBARS values compared to coconut oil. This higher TBARS level in unsaturated oils indicated an increased quantity of secondary lipid peroxidation products, reflecting greater oxidative stress in animals fed with this oil. This study also documented elevated values of CD, CT, and p-AV in

thermally oxidized oils, all of which serve as indicators of a higher degree of oxidative degradation. These metrics highlight the accumulation of primary and secondary oxidation products, signaling a progressive breakdown of fatty acids and other compounds under thermal oxidation. A key observation was the formation of short-chain fatty acids and dicarboxylic acids during the oxidation of oils, especially those oils with high polyunsaturated fatty acid (PUFA) content. Specifically, this study indicates that the oxidative degradation of unsaturated fatty acids leads to the formation of short-chain fatty acids including heptanoic acid (C7:0), caprylic acid (C8:0), as well as dicarboxylic acids such as azelaic acid (C9:0). This formation is closely linked to the increased acid value observed in oils rich in PUFA-containing oils, sunflower oil and palm oil. Thermal oxidation also led to a shift in the fatty acid profile of these oils. The process reduced the proportion of unsaturated fatty acids while simultaneously increased the relative percentage of saturated fatty acids. This shift, which results from the oxidative breakdown of vulnerable unsaturated bonds, was indicative of the overall degradation in oil quality, and nutritional value due to prolonged exposure to heat.

This study also investigated the effect of the consumption of normolipidic (5%) levels of unoxidized and thermally oxidized CO, PO, MO and SO on key metabolic parameters in experimental rats over six months. The primary focus was to explore the effects of different fatty acid contents specifically MCFA, LCFA, MUFA and PUFA-containing oils on body weight, lipid, and carbohydrate metabolism, and inflammatory markers and the development of cardiovascular diseases. Throughout the experimental period, all the rats exhibited body weight gain except those who received diets enriched with TSO and TPO oil. These groups showed reduced body weight gain compared to their unoxidized oils as well as those fed with both unoxidized and thermally oxidized coconut and mustard oil diets. Interestingly, coconut oil and mustard oil diets fed rats, whether oxidized or not, displayed similar levels of body weight gain. A key finding

was the significant increase in liver weight of rats fed thermally oxidized oils, suggesting lipid accumulation in hepatic tissues, likely due to the effects of oxidation-induced lipid peroxidation. Coconut oil, regardless of its oxidation status, appeared to enhance insulin sensitivity and induced hypoglycemic effects, likely due to its high concentration of MCTs and polyphenols. This suggests that the metabolic benefits of coconut oil retained even after thermal oxidation. In contrast, rats consuming diet rich in MO and TPO exhibited hyperinsulinemia indicating impaired insulin regulation and a potential risk of metabolic dysfunction. However, this effect was mitigated in rats fed thermally oxidized mustard oil, suggesting that oxidation may have altered the metabolic impact of mustard oil in a way that improved insulin sensitivity. These findings provide insight into how the thermal oxidation of different oils influences metabolic health, with implications for dietary choices related to fat consumption and the risk of metabolic disorders such as diabetes and cardiovascular disorders.

Lipid profile analysis revealed that rats fed palm oil, especially its thermally oxidized form had significantly elevated levels of serum TG, LDL-C, and VLDL-C, which are risk factors for atherosclerosis and CVD. Conversely, rats fed diets rich in sunflower oil (SO) showed reduced serum TG, LDL-C, and VLDL-C, along with a notable increase in HDL-C levels, suggesting the protective effect against dyslipidemia. In contrast, rats fed TMO displayed an unfavorable lipid profile characterized by elevated serum total cholesterol, TG, and LDL-C and reduced HDL-C. These changes are strongly associated with an increased risk of atherosclerosis and CVD. The decrease in HDL-C, coupled with the rise in LDL-C and TG, indicated impaired cholesterol metabolism and heightened lipid accumulation in the arterial walls, further contributing to atherogenic processes. These findings suggest that thermal oxidation of mustard oil may diminish its cardioprotective properties and enhance its potential to promote lipid dysregulation and cardiovascular risk.

Rats fed thermally oxidized oils exhibited a significant increase in liver weight, alongside elevated levels of liver enzymes indicating liver dysfunction and potential liver damage. Histopathological evaluations further confirmed these findings and revealed the signs of hepatosteatosis and inflammation in rats who consumed thermally oxidized oils. In particular, TCO-fed animals display early-stage fatty changes in the liver, while MO-fed rats shown more severe liver damage, including inflammatory infiltrates and fibrosis. These effects are even more pronounced in rats fed TMO. The progression from steatosis to inflammation and fibrosis marks a key transition in the development of nonalcoholic steatohepatitis (NASH), a more severe form of NAFLD that can lead to cirrhosis and liver failure. Thermally oxidized oils of PO, SO, and MO increased the activity of hepatic lipase which correlated with increased hepatic TG accumulation, and reduced serum HDL-C contributing to the development of NAFLD, CVDs and other metabolic disorders.

Inflammatory markers, including CRP, Lp-AL_2, IL-6, and IL-10 elevated in thermally oxidized oil-fed groups. These markers play critical roles in the body's immune response, and their elevated levels were often associated with tissue damage, metabolic disturbances, and the development of cardiovascular diseases. Rats consuming a diet rich in TSO show elevated levels of CRP, and $LpLA_2$, both of which are risk markers of atherosclerosis. The palm oil diet-fed group showed elevated levels of IL-6, while those on sunflower oil diets had increased IL-10. This suggested that while palm oil promotes proinflammatory pathways, sunflower oil might have anti-inflammatory effects, although thermal oxidation diminishes these effects.

In conclusion, the study underscored the harmful effects of prolonged consumption of thermally oxidized oils, even at normal dietary levels, including coconut oil, palm oil, mustard oil, and sunflower oils, on metabolic health, particularly, lipid, carbohydrate metabolism, and inflammatory response. Among the oils studied, TSO generated the

highest levels of LOPs, which significantly contributed to obesity, IR, dyslipidemia, hepatosteatosis, and an atherogenic inflammatory response, thereby elevating the risk of development of CVDs. TPO also promotes obesity, IR, hepatosteatosis and inflammation, but poses a slightly lower health risk compared to TSO. The consumption of TMO triggered atherogenic inflammatory responses, and hepatosteatosis and posed a moderate cardiovascular health risk. In contrast, TCO produces fewer toxic compounds than other oxidized oils, though it promotes mild inflammation.

Chapter 8

Recommendations

Future Recommendations

Extension of the present study can be directed in the following three aspects.

1. **Gut Microbiota and Systemic Health**: Examine how thermally oxidized oils impact gut microbiota composition and function. Changes in gut health due to oxidized oil may play a significant role in promoting systemic inflammation and metabolic dysregulation, potentially impacting overall health.
2. **Fatty acid-specific toxicity under thermal stress**: Investigate the effect of thermal stress on the individual fatty acid component within oils, including, medium-chain, long-chain, monounsaturated, and polyunsaturated results. This analysis could help pinpoint specific fatty acids that contribute most significantly to toxicity, providing a deeper understanding of oil stability and safety.
3. **Nutrient signalling pathways:** Investigating the molecular mechanisms by which oxidized oils activate carbohydrate and lipid metabolic pathways. This could involve exploring how these oils influence gene expression, enzymatic activity, and biomarkers associated with cardiovascular diseases.
    - Nutrient signalling pathways such as m-TOR (mechanistic target of Rapamycin), AMPK (Adrenaline monophosphate-activated protein Kinase), SIRT1 ((Sitrulin 1) integrated signals from both lipid and carbohydrate metabolism.

Chapter 9

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Publications and Presentations

Peer reviewed publications

1. **Sruthi Panniyan Kandiyil**, Anit Jose, Chanjana Mohanan, Soorya Parathodi Illam, Achuthan C. Raghavamenon.,2024. Virgin coconut oil mitigates ageing-associated oxidative stress and dyslipidaemia in male Wistar rats, Nutrition, Metabolism & Cardiovascular Diseases, 10.1016/j.numecd.2024.08.012.

2. Sruthi Panniyan Kandiyil, Soorya Parathodi Illam, Achuthan C. Raghavamenon., 2023. Normolipidic diet containing deep-fried saturated and unsaturated fatty acids rich edible oils promotes metabolic dysregulation and inflammatory microenvironment in Wistar rats, Food and Chemical Toxicology, , 180 (114029),10.1016/j.fct.2023.114029.

3.Illam, S.P., **Kandiyil, S.P.,** Narayanankutty, A., Veetil, S.V., Babu, T.D., Uppu, R.M. and Raghavamenon, A.C., 2022. Virgin coconut oil complements with its polyphenol components mitigate sodium fluoride toxicity in vitro and in vivo. Drug and Chemical Toxicology, *45*(6), pp.2528-2534.

4. Illam, S. P., Narayanankutty, A., **Kandiyil, S. P**., & Raghavamenon, A. C., 2021.. Variations in natural polyphenols determine the anti-inflammatory potential of virgin coconut oils. Journal of food science, *86*(5), 1620–1628. 10.1111/1750-3841.15705

5. Narayanankutty, A., Anil, A., Illam, S.P., **Kandiyil, S.P**. and Raghavamenon, A.C., 2018. Non-polar lipid carbonyls of thermally oxidized coconut oil induce hepatotoxicity mediated by redox imbalance. Prostaglandins, Leukotrienes and Essential Fatty Acids, *138*, pp.45-51

Non-peer-reviewed publications

1. Panniyan Kndiyil Sruthi, Anit Jose, Chanchana Mohan, Soorya Parathodi illam, Achuthan C. Raghavamenon. “Virgin Coconut oil improves antioxidant and

dyslipidemia in aged rats." 2nd National Conference on Current Trends in Biological Sciences, St. Xavier's College, Palayamkottai, 6-7 Feb, 2020

2. S. P. Kandiyil, Soorya P I, A. Narayanankutty R. M. Uppu, and A. C. Raghavamenon. Long-term dietary consumption of thermally oxidized oils modulates carbohydrate and lipid metabolism and promotes an inflammatory environment in Wistar rats., Society of Toxicology Annual Meeting and Tox Expo, Virtual event, 14-18 March 2021.