SUB-ACUTE ORAL TOXICITY OF TRANSFORMER MINERAL OIL IN WISTAR RATS

BY

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DECLARATION

Declaration by the Student

This thesis is my original work and has not been submitted for any academic award in any institution; and shall not be reproduced in part or full, or in any format without prior written permission from the author and/or University of Eldoret.

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DEDICATION

I would like to dedicate my work to my husband Moses Walela, my children Elaine and Clarence, and my parents Mr. and Mrs. Peter Otunga.

ABSTRACT

There is an increasing trend in Kenya where local deep frying food-vendors use cooking oil blended with transformer mineral oil (TMO) in preparing edible products despite the scant knowledge on possible toxic effects of TMO. This study used a rat model to investigate possible sub-acute oral toxicity of TMO. Forty albino male and female Wistar rats of 6-8 weeks old were randomly distributed into 4 groups of ten animals (five of either sex): Control group were fed on corn oil only (COC, 200µl), low dose heated TMO (HLD-TMO, 50mg/kg bwt), high dose heated TMO (HHD-TMO, 500mg/kg bwt), and high dose unheated TMO (UHD-TMO, 500mg/kg bwt) groups in corn oil. The oral exposure was done by oral gavage once daily for 28 days. Physical observations of rats were done daily while changes in body weight were determined weekly. A full hemogram was conducted to determine haematological changes. Serum levels of Alanine transaminase (ALT), total protein (TP), globulin (GLOB), and albumin (ALB) were indicators of liver toxicity while creatinine (CRE) and urea levels were indicators of kidney toxicity. Malondialdehyde (MDA) levels, an index of lipid peroxidation, were measured in liver tissues using Thiobarbituric Acid Reactive Substances (TBARS) method. Tissues of liver, kidney, and small intestines were examined for histological changes. Data obtained was analysed statistically using student's t test and Analysis of Variance ($p< 0.05$). Significant increase in red blood cells and haemoglobin levels were observed in HHD-TMO females and UHD-TMO males respectively as compared to the control. The HHD-TMO and UHD-TMO male rats showed significant decrease in ALT levels relative to the control. TP and ALB of the HHD-TMO females showed a significant decrease from the COC. The UHD-TMO female animals showed a significant decline in urea levels in comparison to the control. The HHD-TMO males and UHD-TMO males and females showed a significant increase in MDA levels relative to the control. For histopathology, rats in HHD-TMO group had a liver with bile duct proliferation; the female HLD-TMO and UHD-TMO animals showed liver with focal areas of periportal chronic inflammation; the rats in HHD-TMO group had kidneys with mild chronic inflammation; and the rats in HHD-TMO and UHD-TMO groups showed small intestines with chronic inflammation. In conclusion, sub-acute oral administration of TMO induced oxidative stress and varied degrees of toxicities among the various tissues of male and female rats. However, further studies are required to determine other toxicities.

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LIST OF ABBREVIATIONS

ALB	Albumin
ALT	Alanine transaminase
ALTL	Alanine aminotransferase reagent
ANOVA	Analysis of Variance
BCG	Bromcresol green
Bwt	Body weight
CFA	Continuous flow analyzer
COC	Corn oil control
CRET	Creatinine
DBP	2, 6-ditertiary-butyl-phenol
DBPC	2, 6-ditertiary-butyl-para-cresol
DEB	1, 4-Diethoxybutane
DMH	1, 6-Dimethoxyhexane
GLOB	Globulin
GLU	Glucose
HB	Haemoglobin
HCT	Hematocrit
HDL	High density lipoprotein
HE	Haematoxylin-eosin
HHD-TMO	Heated high dose transformer mineral oil
HLD-TMO	Heated low dose transformer mineral oil
KPLC	Kenya Power and Lighting Company
LDL	Low density lipoprotein
MCFA	Medium chain fatty acid
MCH	Mean corpuscular haemoglobin
MCHC	Mean corpuscular haemoglobin
MCV	Mean corpuscular volume
MDA	Malondialdehyde
MSDS	Material Safety Data Sheet
MTRH	Moi Teaching and Referral Hospital

MUFA	Monounsaturated fatty acids
NAD	Nicotanimide adenine dinucleotide
NADH	Nicotanimide adenine dinucleotide hydrogen
NIST	National Institute of Standards and Technology
NOELs	No observed effect levels
OECD	Organization of Economic Corporation and Development
PAH	Polycyclic aromatic hydrocarbon
PCB	Polychlorinated biphenyl
PE	Pentyl ether
PLT	Platelet
POPs	Persistent organic pollutants
PUFA	Polyunsaturated fatty acids
RBC	Red blood cell
RDW	Red blood cell distribution width
ROS	Reactive oxygen species
SEM	Standard error mean
SFA	Saturated fatty acids
SPSS	Statistical package for social sciences
TBA	Thiobarbituric acid
TBARS	Thiobarbituric acid reactive substance
TMO	Transformer mineral oil
TMP	1, 1, 3, 3-tetramethoxypropane
TP	Total Protein
UHD-TMO	Unheated high dose transformer mineral oil
WBC	White blood cell
WSF	Water soluble fractions

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CHAPTER ONE

INTRODUCTION

1.1 General Background

Transformer oil is an insulating oil that is stable at high temperatures and has excellent electrical insulating properties (Powerlink Oil Refinery Ltd, n.d). It is used in oil-filled transformers, some types of high-voltage capacitors, fluorescent lamp ballasts, and some types of high-voltage switches and circuit breakers (Powerlink Oil Refinery Ltd, n.d). Crude oil or petroleum has always been the primary source of transformer mineral oils (TMOs). However, in the past few decades, siloxane-based synthetic fluids and C7-, C8-based fatty acid ester products, have also been used in various parts of the world (Kaplan *et al.*, 2010). Petroleum-based transformer fluids are normally divided into paraffin-rich oils and naphthenic-rich oils. After the polychlorinated biphenyl (PCB) ban in most countries across the world including Kenya (Ministry of Environment, Water, and Natural Resources, 2014), the choice of crude based oil for insulating fluid became naphthenic rich oils, because of their preferred heat transfer and low-temperature characteristics (Rouse, 1998). Capacitors and transformers containing PCBs have been discouraged in many Kenyan enterprises though there are no administrative or restrictive directives to this effect. PCB was found in electrical transformers and capacitors manufactured before 1985 in Kenya (Saoke, 2005). After 1985, use of PCBs in the manufacture of transformers had been banned. Most of the post-1985 manufactured transformer equipment was found using coolants and lubricants that were supplied by different major multinational oil companies like Shell, Caltex, and Total. The

specifications of these products show that they did not contain PCBs therefore, are more environmentally friendly.

Transformer mineral oil (TMO) is a clear and bright liquid with an odor of petroleum and is used as insulating oil in transformers. TMO is a fraction of purified crude oil, obtained by distillation. The oil has a complex structure of hydrocarbon molecules and contains the following main components: paraffin (10-15 %), naphthyl or cycloparaffins (60-70 %), aromatics (15-20%), asphalt resinous substance (2.1 %), sulphur compounds (<1%), nitrogen compounds (< 0.8 %), naphthenic acid (< 0.02 %), and antioxidant additives/ionol (0.2-0.5%) (GlobeCore, n.d; Módenes *et al.*, 2018). TMO should be handled with care to avoid inhalation, skin or eye contact and ingestion. Protective measures should be put in place while handling such as proper ventilation, respirator use, wearing goggles, gloves and protective clothing. The oil should not be disposed in water supplies and sewers (Goldenwest Lubricants, 2014). Despite the complex mixture of hydrocarbons (Godinho *et al.*, 2014; IEEE, 2018) in TMO, people in Kenya are engaged in transformer vandalism to obtain transformer oil for purposes of using the oil in cooking. Furthermore, there are no long-term toxicity studies done on effects of using this oil in cooking and its consumption.

1.2 Statement of Problem

There is increasing trend regarding the percentage of transformers being vandalised by people to obtain transformer mineral oil for purposes of selling or using the oil for preparation of deep fried foods such as French fries in Kenya. Transformer mineral oil (TMO) is preferred by the vendors versus the edible oils because they seem not to be

easily oxidized and are heat stable and so can be used repeatedly for a longer period (Iraki, 2014). The hydrocarbons in the TMO are stable at high temperatures as compared to the fatty acids found in edible cooking oils which are easily oxidised at high temperatures. Many Kenyans unknowingly ingest transformer mineral oil mainly through consumption of french fries, popularly known as chips, and mandazi which are deep fried in this oil amongst other foods prepared by deep frying. Despite the ingestion of TMO through foods deep fried in it, the knowledge is scant on its toxicological effects. Due to its complex mixtures of hydrocarbons (GlobeCore, n.d; IEEE, 2018), transformer oil has the potential to elicit multiple types of toxic effects. It might cause acute toxicity, sub-lethal chronic toxicity or both depending on the exposure, dosage and the organism exposed (Iwuanyanwu *et al.*, 2011). This study is therefore designed to investigate haematological, biochemical and histological toxicity of sub-acute ingestion of transformer mineral oil in rats.

1.3 Justification

Transformer mineral oil (TMO) is being ingested in Kenya through consumption of foods fried in the oil yet there is scant knowledge on the potential toxic effects of ingesting this oil either for a short term or even on long-term basis. Most Kenyan vendors also re-use the oils for frying foods which may pose toxicological effects upon human consumption (Karimi *et al.*, 2017). Short-term toxicity studies with rodents are generally conducted for 14 or 28 days (OECD, 1994). Results of these studies can help predict appropriate doses of the test substance for future sub-chronic or chronic toxicity studies, and can be used to determine no observed effect levels (NOELs) for some toxicological endpoints, and allow future studies in rodents to be designed with special emphasis on identified target organs.

Rats are convenient: small, easily housed and maintained, and adapt well to new surroundings. Rats are also relatively inexpensive, mild-tempered and docile, making them easy for researchers to handle (Jacqueline *et al.*, 2013). The genetic, biological and behavioural characteristics of rats closely resemble those of humans, and many symptoms of human conditions can be replicated in rats (Iannaccone & Jacob, 2009). Wistar albino rats have been used for long (since 1906) in biomedical research. Theses rats are particularly useful in various fields of biomedical research including toxicology, oncology, teratology, immunology, gerontology among others. Since the whole anatomy of Wistar albino rats have been studied and their anatomy and physiological processes verified fit for research, they have become the most used laboratory animals worldwide (Sengupta, 2013). The albino rats are therefore recognized as the preeminent model of the mammalian system.

1.4 Study Objectives

1.4.1 Overall Objective

The overall objective of this study is to investigate the sub-acute oral toxicity of transformer mineral oil in Wistar albino rats.

1.4.2 Specific Objectives

This study proposed to use Wistar albino rats orally exposed to transformer mineral oils for 28 days to specifically examine the following;

i. To monitor effects of ingesting transformer mineral oils used in fried foods on body weights of rats.

ii. To monitor the physical changes in rats after ingestion of transformer mineral oil.

iii. To determine the haematological changes (WBC, RBC, HCT, HB, MCV, MCH, MCHC, RDW, and PLT) in whole blood.

iv. To determine serum biochemical indices of liver (ALT, total protein, albumin, globulin) and kidney (urea, creatinine, and glucose) toxicity.

v. To determine liver malondialdehyde (MDA) levels and histological changes in liver, kidney, and small intestines.

1.5 Hypothesis

1.5.1 Null hypothesis (H_0)

Transformer mineral oil ingestion in rats does not result in changes in body weight, haematological, biochemical indices and organ histology.

1.5.2 Alternative hypothesis (H_1)

Transformer mineral oil ingestion in rat results in changes in body weight, haematological, biochemical indices and organ histology.

1.6 Overall Study Significance

This study has elucidated the potential harmful effects of oral exposure to transformer mineral oil used in fried foods in rats and provided insights on some of the mechanisms of such toxic effects.

CHAPTER TWO

LITERATURE REVIEW

2.1 Transformer oil

Transformer mineral oil is a clear, bright and highly viscous liquid with an odour of petroleum and is used as insulating oil and coolant in transformers (GlobeCore, n.d). Transformer mineral oil (TMO) is a fraction of purified crude oil, obtained by distillation (IEEE, 2018). TMOs have primarily been obtained from petroleum crude oil treatment. However, in the past few decades, synthetic transformer oils are also being produced from vegetable oils. Petroleum-based transformer oil also known as transformer mineral oil is normally divided into paraffin-rich and naphthenic-rich oils (Kaplan *et al.*, 2010; Wang *et al.*, 2018). Naphthenic-rich transformer oil is widely used in most countries across the world. TMO can either be inhibited or uninhibited. Uninhibited oils have antioxidant additives added in their composition as opposed to inhibited oils which lack the additives. Uninhibited transformer oils have a higher concentration of polycyclic aromatic hydrocarbons (PAHs) compounds. TMO has a complex structure of hydrocarbon molecules present in crude oil and other non-hydrocarbon molecules like gases and water (GlobeCore, n.d).

2.2 Sources of transformer oil

Crude oil is a liquid found within the earth comprising of hydrocarbons, organic compounds and small amounts of metals. Majority of the crude oil composition is hydrocarbons whose composition can vary from 50%-97% depending on the type of

crude oil and how it is extracted (OilPrice Editors, 2009). Crude oil is a complex mixture of hydrocarbons from which various petroleum products such as gasoline, kerosene, fuel oil, lubricating oil, wax and asphalt are derived (Azeez *et al.*, 2013; Sunmonu & Oloyede, 2008).Several toxic components of crude oil such as polycyclic aromatic hydrocarbons (PAHs) and water soluble fractions (WSF) have been documented. Organic compounds like nitrogen, oxygen, and sulphur typically make-up between 6%-10% of crude oil while metals such as copper, nickel, vanadium and iron account for less than 1% of the total composition (OilPrice Editors, 2009). Crude oil, also referred to as petroleum, is created through the heating and compression of organic materials (like prehistoric algae and zooplankton remains) over a long period of time. It is mostly extracted from the ground by drilling the exact location where the oil is located. The top five oil producing (Oil extraction from oil reserves) countries are Saudi Arabia, Russia, United States, Iran, and China (OilPrice Editors, 2009). Distillation of crude oil separates a mixture of hydrocarbons into its various components including light gases, petrol, naphtha, kerosene, diesel oil, fuel oil, and residue. The chemical structure and number of carbon atoms in the molecule dictates the boiling point of these hydrocarbons. During distillation process, the temperature is elevated in order to vaporize the fluid (OilPrice Editors, 2009). As the vapours rise through a distillation column they begin to cool and condense back to liquid form when the temperature drops below their boiling point. There are several perforated trays which allow the vapours to rise while collecting condensate at various temperature levels thus separating crude oil into various fluid streams (IEEE, 2018).

Transformer mineral oil is a petroleum by-product, produced by fractional distillation of crude oil. Mineral oils are the most widely used fluids for electrical insulation and heat transfer in equipment such as transformers, capacitors, bushings etc., because of their excellent technical characteristics (good electrical properties, aging behaviour, and low viscosity) and reduced cost of production. The main limitation of using mineral transformer oil is the environmental hazards that occur after leakage due to its poor biodegradability (Bertrand & Hoang, 2004). Mineral oils have cyclic and alkane components and are often the base oil in transformer oil and other lubricants (IEEE, 2018).

Vegetable oils can be an alternative to mineral oils because of its non-fossil origin; it could be an appropriated response to environmental, safety and health problems, and could reduce the exploitation and end-life costs of transformers (Godinho *et al.*, 2014). There exist both synthetic and natural vegetable oils; natural esters can be extracted from palm nuts, soybeans, and groundnuts, while synthetics, are made from petroleum products like pentaerythitol esters which have suitable dielectric properties (Bashi *et al.*, 2006). Some vegetable oils meet the technical requirements of conventional dielectric liquids. Their high biodegradability and non-toxicity are other qualities making these natural oils interesting raw materials for the development of new environmental friendly dielectric fluids. The most promising candidates for electrical transformer application are based on refined rapeseed oil and its derived esters. A study by Bertrand and Hoang demonstrated the ability of these alternative liquids to fulfil the key characteristics for use in transformers (Bertrand & Hoang, 2004). Oxidation and hydrolysis are the main ageing

mechanisms of the oils. Oxidation inhibitors are therefore added to the basic fluid to stabilize these vegetable formulated oils (Bertrand & Hoang, 2004).

2.3 Types of Transformer mineral oil

Generally there are two types of transformer mineral oil (TMO) used in transformers; paraffin based TMO and naphtha based TMO. Naphtha oil is more easily oxidized than paraffin oil but the oxidation product i.e., sludge in the naphtha oil is more soluble than paraffin oil (Kuperman *et al.,* 2011). Thus sludge of naphtha based oil is not precipitated in bottom of the transformer and hence, does not obstruct convection circulation of the oil, meaning it does not disturb the transformer cooling system. But in the case of paraffin oil, although the oxidation rate is lower than that of naphtha oil, the oxidation product or sludge is insoluble and precipitate at bottom of the tank thus obstructs the transformer cooling system (Kuperman *et al.*, 2011).

Over the past three decades, refining technology has improved greatly and has led to the development of both uninhibited- and inhibited-type fluids. The former depends on controlling refining severity to leave the natural organic components of the oil such as non-specific PAH compounds and sulphur compounds (like benzo-thiophenes) to act as oxidation stabilizers by serving as proton donors that stop peroxide degradation (Kaplan *et al.,* 2010). Inhibited type oils are more highly refined in a stepwise fashion. First, the refinery removes natural oxidation inhibitors, and then substitutes with synthetic anti-oxidants. The two synthetic inhibitors approved for use today (and used at least since 1980) are 2, 6-ditertiary-butyl-para-cresol (DBPC or BHT) and 2, 6-ditertiary-butyl-phenol (DBP). Uninhibited oils are only used in distribution transformers that are

considered low stress service. Power transformers and all high stress service use inhibited oil, which is more effective in producing cleaner base oil with better anti-oxidant performance (Kaplan *et al.*, 2010).

2.4 Chemical Composition of Transformer mineral oil

The specific constituents present in TMOs include aromatic, naphthenic, paraffinic fluids, PCBs (currently banned), and other constituents (IEEE, 2018) as described in detail in the subsequent sub-sections.

2.4.1 Aromatics

Aromatic derivatives have a benzene ring type chemical structure. They are also known as polycyclic aromatic hydrocarbons (PAHs) making up about 15-20% of the oil. PAHs comprises up to six benzene rings that are fused together. Aromatics exhibit some of the benzene's chemical behaviours such as higher reactivity and higher solvency when compared to paraffinic and naphthenic products (Kaanagbara *et al.*, 2010). Naphthalene is considered as the simplest form of aromatic hydrocarbon but is often classified in its own group. A study on transformer oils found out that highly branched aliphatic and aromatic hydrocarbons with evidence of proportional balance in both aged and new oils were present (Kaanagbara *et al.*, 2010). PAHs are lipid soluble and therefore can be absorbed through the skin, respiratory tract, and gastrointestinal tract. Metabolism of PAH is complex and occurs primarily in the liver and other tissues. PAH elimination occurs via urine and faeces with urinary metabolites eliminated within few days (Nhanes, 2009).

2.4.2 Naphthenes

Naphthenic fluids have a large proportion of cycloalkane structures with very low wax molecule content (low to no alkanes). Naphthyl or cycloparaffin compounds are found at about 60-70% in the oil (GlobeCore, n.d). Mineral oils are considered naphthenic if they contain less than 55 to 60% alkanes or paraffinic structures. Naphthenic and aromatic fluids have higher solvency than paraffinic fluids. Naphthenic fluids are useful for low pour-point applications because they exhibit low-temperature properties.

2.4.3 Paraffin

Paraffin is an alkane hydrocarbon found at 10-15% in the transformer oil (GlobeCore, n.d). Derivatives of paraffin include paraffin oils and wax. Paraffin wax consists of alkane mixture with straight and branched chain lengths ranging from 20 to 40 (IEEE, 2018). At room temperature, the wax is solid but starts melting or liquifying at approximately 37°C. Just like mineral oil, paraffin oil is also a by-product of petroleum distillation process. Paraffin-based products are relatively non-reactive and have excellent oxidation stability.

2.4.5 PCBs

Polychlorinated biphenyls (PCBs) is present in <1% in the oil, and are persistent organic pollutants (POPs) that are subject to international restrictions on usage and emissions (Saoke, 2005). PCBs are a group of synthetic organic chemicals formed when chlorine atoms replace hydrogen atoms in the biphenyl structure. PCBs have been used in industry as heat exchangers, lubricants, and insulators in electric transformers and capacitors

(Saoke, 2005). PCBs for more than fifty years has been produced on an industrial scale and exported as chemicals to essentially every country in the world. PCBs have taken different trade names such as Asbestos, Askarel, Chlorinol, Chlorphen, Dykanol, and Pyranol in PCB-containing equipment. PCBs' usefulness arises from their chemical stability and heat resistance (Saoke, 2005). As stated above, commercial PCBs consist of a mixture of congeners, the most abundant of which tend to be readily biodegradable. A smaller portion of PCB congeners, however, tends to be "dioxin-like" PCBs, which are very stable and resistant to biodegradation and metabolism (Chemicals, 1999). PCBs bio-accumulate in the fatty tissues of exposed animals and humans (Gioia *et al.*, 2014) and this exposure is shown to be responsible for many health effects (Pedersen *et al.*, 2013). PCBs might have toxic, mutagenic, and/or carcinogenic properties. In Kenya, electric equipment and thermal fluids have depended mostly on imports. Thus, the most feasible avenue of entry of PCBs has been through importation of equipment and synthetic chemicals (Saoke, 2005).

PCB was found in electrical transformers and capacitors manufactured before 1985 in Kenya. After 1985, use of PCBs in the manufacture of transformers had been banned (Saoke, 2005). Most of the transformer equipment manufactured after 1985 was found using coolants and lubricants that were supplied by different major multinational oil companies (Shell, Caltex, and Total). The majority of PCB wastes are destroyed by high-temperature incineration that purportedly gives rise to almost total oxidation of organic products into carbon dioxide and safely disposable oxidation by-products (Saoke, 2005). PCBs can also be decontaminated by chemical sodium process or biological degradation by anaerobic bacteria (Ryoo *et al.*, 2007).

2.4.6 Minor Constituents

Transformer mineral oil also contains sulphur compounds, nitrogen compounds, silicon water content, and antioxidant additives in small percentages (Gray, n.d). Sulphur compounds are kept low at <1% since they may be changed by heat and electrical stress to form corrosive sulphur. Nitrogen compounds are also found at <1% in the oil. Silicon is also an additive in the oil at less than 5-10 parts per million in the oil and has antifoaming properties (Sokolov *et al.*, 2003). Water is usually present in the oil in a soluble, dissolved or bound form (adsorbed by polar aging products) and is increased with heating of the oil. 2, 6-ditertiarybutyl- para-cresol (DBPC/BHT) and 2, 6-ditertiary-butyl-phenol (DBP) found at <0.5% are the commonly used antioxidant additives in uninhibited transformer oils to prevent oxidation and therefore enhance the insulating life (Sokolov *et al.*, 2003).

2.5 Uses of Transformer mineral oil

Transformers and even other electrical equipment generate heat during operation and therefore a coolant is necessary to dissipate this heat. Transformer oil is usually a highly refined mineral oil that is stable at high temperature and has excellent electrical insulating properties (Powerlink Oil Refinery Ltd, n.d). It is used in oil-filled power transformers, some types of high voltage capacitors, high voltage switches and circuit breakers. Its function is to insulate, suppress corona and arcing, and to serve as a coolant (Powerlink Oil Refinery Ltd, n.d).Because it also provides part of the electrical insulation between internal live parts, transformer oil must remain stable at high temperature for an extended

period (IEEE, 2018). To improve cooling of large power transformers, the oil filled tank may have external radiators through which the oil circulates by natural convection.

In Kenya, the Kenya Power and Lighting Company (KPLC) is the leading firm which highly utilizes transformer oil and it distributes electricity throughout the country. There are also a number of other companies or industries in Kenya utilizing transformer mineral oil in their transformers and capacitors as insulating oil and a coolant. On the contrary, In 2015, Al Jazeera featured on an article titled “Thieves fry Kenya's power grid for fast food” with a sub title, “Vandals smash electrical transformers to steal viscous fluid that's later sold as cooking oil for roadside stalls” (Iraki, 2014). Experts said that Kenyans' appetite for fried food and cheap frying oil is stalling the country's urgent efforts to build a modern electrical grid, even as it sows the seeds of a public health crisis. Kenya has even had some success fighting transformer vandalism. In 2013, 535 transformers were vandalised across the country, a stark drop from 898 in 2011, according to Kenya Power (Iraki, 2014). That may be due to a 2013 law that imposes a minimum 10-year jail sentence on transformer vandals. Kenya Power also started mounting transformers in more inaccessible places, such as inside homes and much higher up on poles. Transformer mineral oil is preferred by the vendors versus the edible oils because they seem not to be easily oxidised and are heat stable and so can be used repeatedly for a longer time (Oriedo, 2010). The hydrocarbons in the TMO are stable at high temperatures as compared to the fatty acids found in edible cooking oils which are easily oxidised.

2.6 Safety Assessment of transformer mineral oil

2.6.1 Exposure Scenarios and toxicity

The toxicity of substances can be established by either studying the accidental exposures to an element, in vitro studies using cell lines, or *in vivo* exposure on experimental animals (Azeez *et al.*, 2013). There are currently no studies done on the toxicity of transformer mineral oil both in vivo or in vitro models. Humans and animals can be exposed to transformer oil accidentally or intentionally through ingestion, inhalation and dermal exposure. Some practices that lead to transformer oil exposure include vandalism of the oil for cooking, oil spillage, and improper waste disposal of the oil or equipment containing the oils, and soil contamination with the oil. There are however many documented studies on exposure of fuel oils and other petroleum products to animal models for toxicity studies (Azeez *et al.*, 2013; Njoroge *et al.*, 2015; Poon *et al.*, 2004; Sunmonu & Oloyede, 2008).

Crude oil and some of its products have been shown to cause harmful health effects. One study showed that breathing in high levels of gasoline by petrol attendants for a short period or swallowing large amounts of gasoline may cause harmful effects on the nervous system (Emelike *et al.*, 2015). Another study showed detrimental effects of oxidative stress and induction of pro-oncogenes in workers exposed to gasoline (Ekpenyong & Asuquo, 2017). Bonny light crude oil caused severe pathological changes in the liver in a sub-acute study of the oil in albino rats (Ikanone *et al.*, 2017). A study on short-term oral toxicity in male rats of pentyl ether (PE), 1, 4-Diethoxybutane (DEB), and 1, 6-Dimethoxyhexane (DMH) which are additives of diesel fuel showed many results (Poon

et al., 2004). For instance, there was increased GSH levels in the group administered high DMH, histological changes in the liver for all chemical groups and decreased platelet count. Another study documented the carcinogenic risk of certain PAHs to man (Nadon *et al.*, 1995). The toxic potency of PAH is indicated by Benzo[a]pyrene (BaP) presence which makes PAH considered a Class 1 carcinogen, though other PAH compounds have also been associated with cancer and other adverse health outcomes (Lam *et al.*, 2012). Aromatic hydrocarbons target the lipid model of tissue membranes resulting in impaired mechanisms and histopathological changes (Renegar *et al.*, 2017). The carcinogenic risk of PCBs to humans and animals through ingestion of food contaminated with PCBs, inhalation and skin exposure have been documented (Lauby-Secretan *et al.*, 2013). Gioia *et al.* (2014) also discussed on how PCBs pose a threat to the environment and public health in Africa after the spillage of oils containing PCBs. Another study showed no observed effect levels (NOELs) upon exposure to 3-methylpentane (Chung *et al.*, 2016). From the above studies, it can be concluded that some components of transformer mineral oil and other petroleum products can cause toxic effects to both humans and animals.

In contrast to TMO, edible vegetable oils have a different composition of fatty acids. For instance, soy oil contains 60% polyunsaturated fatty acids (PUFA), 24% monounsaturated fatty acids (MUFA) and 16% saturated fatty acids (SFA). In contrast, palm oil contains 50% MUFA, and 50% SFA. Soy oil is rich in tocopherol, whilst palm oil is rich in tocotrienols. Both oils possess antioxidant properties that act as free radical scavengers. Tocotrienols have greater antioxidant properties compared to the tocopherols (Hamsi *et al.*, 2015). Coconut oil contains mainly medium-chain saturated fatty acid

(MCFA) (50%); lauric acid, short chain saturated fatty acids (SCFA) such as capric, caproic and caprylic acid and unsaturated fatty acids (8%). Corn oil also contains PUFAs, MUFAs and tocopherols as the antioxidant (Kuperman *et al.*, 2011). Palm oil also contains saturated fatty acids; Palmitic (C16) 44.3%, Stearic (C18) 4.6%, Myristic (C14) 1.0%, Monounsaturated fatty acids; Oleic (C18) 38.7%, Polyunsaturated fatty acids-Linoleic (C18) 10.5% and tocotrienols (Mukherjee & Mitra, 2009). Olive oil has higher monounsaturated fatty acids (MUFA) in its composition, being therefore less prone to oxidation than those with higher polyunsaturated fatty acid (PUFA) content (Santos *et al.*, 2013). The above vegetable oils are used in cooking; deep-frying, pan-frying, roasting, microwave cooking, etc. Vegetable oils account for a significant source of calories and also are rich in linoleic acid, an essential nutrient that can help lower risk of heart disease. Vegetable oils are also easy to digest and help the body preserve important vitamins (Santos *et al.*, 2013). They aid in lowering low-density lipoprotein (LDL) and raising high-density lipoprotein (HDL).

2.6.2 Other safety concerns of transformer mineral oil

The Material Safety Data Sheet for Shell Diala insulating oils highlights several health hazards after either short-term or long-term exposure to transformer mineral oil. Some of the health hazards include irritation (eye, respiratory tract, and skin), aspiration hazard, and cancer. Acute ingestion of transformer mineral oil may cause abdominal pain, nausea, or vomiting (Goldenwest Lubricants, 2014). Little amounts of oil aspirated during ingestion or vomiting may cause lung damage; no information is available for long-term exposure to transformer mineral oil.

Transformer mineral oil should, therefore, be handled with care to avoid inhalation, skin or eye contact and ingestion. Protective measures should be put in place while handling: proper ventilation, respirator use, wearing goggles, gloves and protective clothing. The oil should not be disposed in water supplies and sewers (Goldenwest Lubricants, 2014). First Aid measures to be put in place in case of exposure include: If inhaled, move the person to fresh air/ uncontaminated area immediately; If not breathing, give artificial respiration or oxygen by qualified personnel and seek immediate medical attention. In case of skin contact, rinse exposed skin with soap and water for at least 15 minutes and seek medical attention if needed. In case of eye contact, quickly wash eyes, including under the eye lids with sufficient amounts of water for at least 15 minutes and seek immediate medical attention (Goldenwest Lubricants, 2014). When ingestion/aspirated, do not induce vomiting; If vomiting occurs, keep head lower than hips to prevent aspiration. If not breathing, give artificial respiration by qualified personnel and seek immediate medical attention. Furthermore, transformer oil is slightly flammable. Suitable extinguishing media in case of fire are regular dry chemical, carbon dioxide, and regular foam.

2.7 Toxicity assays

2.7.1 Weight monitoring

The assessment of the effects of xenobiotics to body weights (Bailey *et al.*, 2004) and/or organ is important in toxicological experiments. The differences in body weights are often evident between the treatment groups and the control group. Body weight changes occurs due to many reasons including altered growth where the agents modify secretions

of growth hormones, changes in neurotransmitters which affect the consumption of food, or through nonspecific systemic toxicity (Bailey *et al.*, 2004). Some studies have shown a proportional relationship between body weight and organ weight though it is not always the case in many experiments. For instance, organs that do not change even during normal growth like the brain or adrenals are not affected by body weight changes after exposing animals to treatments (Bailey *et al.*, 2004).

2.7.2 Clinical observations of experimental animals

Physical observation of rats for mortality and morbidity are crucial elements in toxicity studies. Clinical abnormalities are also observed in animals receiving treatment which are signs of toxicity (Shirodkar *et al.*, 2015). In rats, the behaviour, skin or fur are usually the main targets for observation. Aggressiveness in rats is defined by burrowing i.e. mechanical removal or moving of bedding materials within cages. It can also be defined by fighting i.e. animals chasing each other, voluntarily attacking other animals, or biting one another (Njoroge *et al.*, 2015). Physical observation is therefore the initial non-invasive indicator in toxicity studies.

2.7.3 Haematological toxicity

Blood is always the primary target of most toxic substances. It is a medium of intercellular and intracellular transport, which comes in direct contact with various organs and tissues of the blood (Dahunsi *et al.*, 2011). The physiological state of an animal at a particular time is reflected in its blood. After a chemical enters an organism, several physiological, haematological and biochemical reactions occur; the organism may adapt to such reactions or they may lead to a toxic state (Montanha *et al.*, 2014).

Haematology acts as a frontline sensitive indicator of vital physiological and biochemical functions as well as status of nutrition, health, diseases and stress responses of the organism subjected to changes in environmental conditions (Montanha *et al.*, 2014). Haematological parameters serve as a focus of this study because of their relationship with energy (blood glucose), respiration (RBC, HCT, and HB) and defence (WBC). The hematopoietic system is a susceptible target of toxic substances. Therefore monitoring haematological parameters like WBC, RBC, HCT, MCV, MCHC, MCH, HB, RDW and PLT in whole blood is important in assessing the physiological and pathological status of animals and humans. The toxicants cause dysfunction of hematopoietic stem cells and abnormalities in bone marrow environment resulting in pathogenesis of blood related disorders. Toxicants also induce oxidative damage that interfere with critical enzymes in hematopoietic biosynthetic pathway (Ibeh *et al.*, 2016). Consequently, the production of various blood cells are interfered with and it is observed in the varying levels of blood cells after exposure to toxicants. However, WBC increase can be associated with the toxicants inducing stress or inflammation.

2.7.4 Biochemical toxicity

Biochemical biomarkers like glucose, certain proteins and enzymes are frequently used as indicators of the general state of health and early warning of toxicity (Montanha *et al.*, 2014).For instance, the liver is the detoxification site for toxic substances. Toxins damage the liver through inflammation, mitochondrial dysfunction, oxidative stress, and dysfunction of cytochrome P450. Increased marker enzymes like ALT which is more specific to the liver indicate liver damage (Seif, 2016). Necrosis or membrane damage enhances the release of enzymes into the circulation. Reactive oxygen species may also

disrupt the liver by oxidising the polyunsaturated fatty acids. The liver synthesizes and secretes albumin, globulin and total protein. Elevated or decreased levels of serum proteins will signify the interference of ROS on the liver's secretory ability (Seif, 2016).

Toxicants damage the proximal tubule in the kidney thus interfering with the glomerular filtration rate. Urea and creatinine levels are indices of renal functions (Azeez *et al.*, 2013). Low urinary concentrations of urea and creatinine result in low urinary clearance. Consequently there will be a rise in serum urea/ creatinine concentrations due to presence of gaps in the tubule that cause a back leak of the filtrate. High urea and creatinine levels in serum therefore indicate an impaired renal function.

Environmental pollutants disrupt the blood glucose integrity (Isehunwa *et al.*, 2016). Products of crude oil like transformer mineral oil contribute to the environmental contaminants that affect glucose metabolism in animals. The pollutants increase stress hormone catecholamines which mobilize glucose from glycogen stores in liver and muscles to enable the animal cope with stress (Isehunwa *et al.*, 2016). The toxicants could also affect the enzyme activities of the carbohydrate metabolism. The toxicants can also disrupt insulin signalling pathways in target tissues like muscles, liver, and adipose tissue (Chamorro *et al.*, 2018). The pollutants stimulate the production of reactive oxygen species which cause oxidative stress that interferes with glucose homeostasis (Chamorro *et al.*, 2018).

Antioxidants play a vital role in the body in terms of protecting the body against free radicals. Different stimuli like environmental toxins are sources of in vivo reactive oxygen species (ROS). Petroleum products like transformer mineral oil are sources of

environmental toxins that induce the generation of ROS (Aksoy, 2015). ROS are free radicals that have unpaired electrons that cause lipid peroxidation. Lipid peroxidation occurs as a result of ROS oxidising the polyunsaturated fatty acids in the membrane structure (Aksoy, 2015). The primary products of lipid peroxidation like lipid hydroperoxides decompose into carbonyl and aldehyde compounds resulting in formation of MDA as the end product.ROS also directly impairs the respiratory chain of liver hepatocytes or indirectly causes oxidative damage to mitochondria genome (Noeman *et al.*, 2011). Under the ideal circumstances, a balance exists between free radicals and the antioxidant defence system. Antioxidant molecules like glutathione protect the structural and functional integrity of cells, tissues, or organs from free radicals. Oxidative damage occurs when this balance is disturbed in favour of free radicals (Aksoy, 2015). High MDA levels therefore indicate tissue damage caused by production of free radicals on biologic membranes.

2.7.5 Histological assays

The histological procedure used to investigate the various body organs/ tissues involves fixing tissues in formalin (10% formaldehyde solution), embedding in paraffin, and sectioning at 5.0μm thickness for routine haematoxylin–eosin (HE) staining (Ramos *et al.*, 2015).Oil-based fuels have been reported to cause histological changes in animals. Petroleum products produce chemical pollutants that are metabolised in the body into reactive metabolites (Ramos *et al.*, 2015). These metabolites interact with excreting and metabolising tissues like liver and kidney to elicit toxic effects (Uhegbu *et al.*, 2015). Also, the hydrocarbons and other constituents of petroleum products are metabolised and excreted in liver and kidney. Tissue damage occurs due to cellular injury by the

metabolites. Toxicants from petroleum products can interfere with gastrointestinal absorption and cause gastric erosion. The small intestine is therefore a target for toxicant-induced ulceration or mucositis (Omotoso *et al.*, 2012). Toxicants also produce ROS that destroy the tissues resulting in tissue damage (Aksoy, 2015). Transformer mineral oil (TMO), just like other crude petroleum products, contains hydrocarbons and other toxic constituents that can cause histological changes in rats.

CHAPTER THREE

MATERIALS AND METHODS

3.1 Materials

Shell Diala Öl G (Trafoöl) was the transformer mineral oil (TMO) used in this study and was obtained from Philips Medical Systems Limited, Nairobi-Kenya. Corn oil (Elianto) used for the control group was locally purchased from Tuskys supermarket, Eldoret-Kenya. 1, 1, 3, 3- tetramethoxypropane (TMP) and Thiobarbituric acid (TBA) were obtained from Sigma Aldrich, UK through Kobian Scientific distributors, Nairobi, Kenya. All the other chemicals used were of analytical grade.

3.2 Ethical Statement

Ethical clearance for this study was granted by the Research Ethics Committee of University of Eastern Africa, Baraton (Reference- REC: UEAB/7/3/2017 in Appendix III). The 28 day sub-acute oral toxicity study was performed according to the Organization of Economic Cooperation and Development (OECD) guideline 407 for toxicity studies (OECD, 2008).

3.3 Study Animals

Forty albino Wistar rats (20 males and 20 females) of approximately the same age (6-8 weeks) and comparable weights (154-187g) were obtained from the Department of Zoology's animal facility of Chiromo campus, University of Nairobi. The rats were housed in cages (males separate from females) at the Department of Biological Science's

animal house, University of Eldoret. Ambient temperature of around 25°C (±5ºC) were maintained in the room. The rats were exposed to 12h of light and 12h of darkness (Njoroge *et al.*, 2015). The rats were acclimatized for a period of 10 days prior to initiation of the experimental treatment. Animals were allowed free access to water and standard rodent chow diet (Diet was obtained from Maraba Agrovet, Eldoret-Kenya) throughout this acclimatization period.

3.4 Experimental Animal Grouping and Design

After acclimatization, 40 rats were randomly grouped into four groups with five rats of either sex per group. Male and female rats were caged separately to avoid mating. Corn oil was administered to the control groups since it is edible oil and it is easily digested and absorbed and has been used for a long time as a control/vehicle in several research studies for toxicity.TMO was given to the treatment groups with varying doses and forms of the oil (Refer to Table 3.1). The rats ingested both the corn oil and TMO delivered by oral gavage using micro pipettes once daily for a period of 28 days. The treatment groups ingesting the test substance (transformer mineral oil) were compared with the control group for possible toxic effects.

3.5 Animal Experimentation

3.5.1 Preparation of Heated Transformer Mineral Oil

TMO was heated separately by deep frying potato slices weighing around 1000g in about 500ml of the oil at temperatures of around 180°C (in order to mimic the usage the oil for preparation of deep fried foods such as French fries in Kenya). The heating was done for

3 consecutive hours per day for five days (Morita *et al.*, 2008) and the oil was stored in a sterile container. There was no addition of fresh oil during the entire heating period.

Table 3.1: Animal grouping and treatment design

Experimental Group	**Sex**	**Treatment and dosage**
1-Corn Oil Control (COC)	Male/Female	Control on vehicle (200µl corn oil)
2-Heated Low Dose Transformer Mineral Oil (HLD-TMO)	Male/Female	Low dose of heated transformer oil (50mg/kg body weight) in corn oil
3-Heated High Dose Transformer Mineral Oil (HHD-TMO)	Male/Female	High dose of heated transformer oil (500mg/kg body weight) in corn oil
4-Unheated High Dose Transformer Mineral Oil (UHD-TMO)	Male/Female	High dose of unheated transformer oil (500mg/kg body weight) in corn oil

3.5.2 Treatment Doses and Administration

All animals were maintained on regular rodent chow diet and had free access to water throughout the study. Animal treatments were conducted according to Poon *et al.* (2009) with minor modifications. Specifically, control animals were orally administered corn oil only (200µl). Low dose heated transformer oil-treated rats were administered 50mg/kg bwt of heated TMO dissolved in corn oil. High dose heated transformer oil-treated rats were administered 500mg/kg bwt of heated TMO dissolved in corn oil while the last group were treated with unheated high dose of 500mg/kg bwt of TMO dissolved in corn oil. Oral administration was performed once daily at 10:00am by oral gavage for 28 days according to OECD Guidelines for the Testing of Chemicals (407) on Repeated Dose 28-Day Oral Toxicity Study in Rodents (OECD, 2008). The animal dosages were adjusted according to the weekly fasting weights (Appendix 1).

3.5.3 Physical Observation and Determination of Body Weights

The behaviour and physical appearance of the rats were observed on a daily basis for aggressiveness and skin/fur texture respectively. The fasting body animal weights were determined at the beginning of the study and on weekly basis (day 0, 7, 14, 21, and 28). The rats were fasted overnight prior to weight measurement. The rats were weighed using an analytical balance (Uni-bloc model UW420H).

3.5.4 Animal Sacrifice and Collection of Blood Samples and Tissues

At the end of the study (day 28), blood samples from the rats were collected via cardiac puncture under chloroform anaesthesia. The rats were fasted overnight prior to collection

of the blood. Blood collection tubes were well labelled before the start of this procedure. Rats were anaesthetized by being placed in a dessicator with chloroform soaked in cotton wool. They were tested for reaction by a toe pinch after removal from the dessicator. Animals were then placed on their back on a dissecting board with their legs held apart with pins. The abdominal skin was sterilized with methylated spirit, a V-cut made through the skin using a pair of scissors, and internal organs moved down. A 21 gauge needle fixed to a 5ml syringe was gently inserted into the heart and negative pressure gently applied on the syringe plunger to withdraw blood. The needle was then withdrawn and blood collected was placed in appropriate tubes.

Blood samples for haematological assay were collected about half full in 5mL EDTA (purple top tubes) vacutainers and promptly mixed by gentle inversion several times to avoid clotting. Blood samples for biochemical assay were collected about half full in 5mL plain tubes without anticoagulant (red top tubes) and tubes gently inverted about 5 times to allow mixing of the blood and then allowed to stand for 1 hour to allow clotting before centrifugation. Centrifugation was done at 3000xg for 10 minutes and then serum was aliquoted into labelled plastic screw-cap vials using clean pipettes. The serum vials were immediately refrigerated at -20ºC awaiting biochemical analysis. For MDA assay, liver tissues were obtained from all the rats after dissection while for histopathology, two rats per cage were randomly selected and dissected to obtain the liver, kidney, and small intestines. The rats were placed in dorsal recumbency on a clean dissection board and the skin incised using a pair of scissors down to the anal region. The tissues were removed from the carcass using forceps and scissors and unnecessary connective tissue or fat was removed by carefully trimming the tissue before placing them in normal saline to clean

from blood. For MDA assay, liver tissues obtained were placed in well labelled simport containers and immediately frozen at -20^{o}C for later analysis. The tissues for histopathology were then placed into well labelled containers with 10% neutral buffer formalin (containers were capped to avoid leakage) for fixation.

3.6 Toxicity Assays

3.6.1 Haematological Assay

The blood samples for haematology obtained and processed as described in section 3.5.4 were immediately taken to Moi Teaching and Referral Hospital (MTRH) haematology laboratory, Eldoret, Kenya for analysis. A complete blood count was done on the samples by a haematology autoanalyzer (Model: Advia 2120i). The parameters assessed included: WBC, RBC, HGB, HCT, MCV, MCH, MCHC, RDW and PLT. The ADVIA 2120i hematology system consists of the following: an analytical module that aspirates, dilutes, and analyzes whole blood samples; an auto sampler that automatically mixes, identifies, and presents the samples for processing; a computer workstation that controls the instrument, provides primary user interface with the instrument and manages the data produced by the instrument; a printer that optionally generates reports based on the instrument results and an auto slide module. The blood sample is mixed with reagents that lyse the cells to differentiate them from each other. Different cells are differentiated by volume or recognition patterns that make it possible for blood cell count.

The haematology auto analyzer utilizes the aperture impedance principle. Blood is aspirated into a chamber and diluted with an isotonic saline solution. The analyzer prepares two dilutions: one lysed and the other unlysed. After the first dilution is

measured, the WBC and HB values are displayed on the screen of the instrument; meanwhile, the analyzer processes the second dilution, which measures RBC, HCT, MCV and PLT. A small portion of the diluted fluid in each bath is allowed to flow through a small aperture (50μm). An electrical current is produced in each aperture by two electrodes, one on the inside and the other on the outside of the aperture. The saline solution conducts current between the electrodes. The cell moves through the aperture, then displaces a volume of electrolyte equal to its size (Chandra *et al.*, 2010; Khanna *et al.*, 2013). The cell acts as an electrical resistor and impedes the flow of the current which produces a voltage pulse proportional to the size of the cell.

3.6.2 Serum Biochemical Assay

As earlier described in section 3.5.4, serum samples were obtained from animal blood after centrifugation at 3000xg for 10 minutes and the analysis of the biochemical parameters (ALT, albumin, total protein, urea, creatinine and glucose) was then performed at MTRH biochemistry laboratory by an auto-chemistry analyzer (COBAS INTEGRA 400 PLUS). The COBAS INTEGRA allows mixing of the samples and reagents for an enzymatic reaction to occur. Optical density is measured at the end of the reaction and the difference from the standard is recorded on a monitor. The machine utilizes the colorimeter to measure the color density of the reactions thus giving the concentration of different substances in a sample.

The autoanalyzer carries out the ordered tests automatically. The machine is equipped with a fluorescence photometer, absorbance photometer, and an ion-selective electrode (MTRH, 2018). The samples are automatically transferred from the sample tube where

optical measurements are made. The Integra cassettes hold all the necessary reagents and are automatically handled to reduce possibilities of errors and save on time. The sample racks are arranged in a way that it allows for continuous access of tests. The machine utilizes the cassette containing Alanine Aminotransferase reagent (ALTL) for quantitative measurement of ALT catalytic activity. ALT catalyzes the reaction between L-alanine and 2-oxoglutarate where pyruvate is formed in the process by oxidation of NADH. The ALT catalytic activity is determined by measuring the decrease in absorbance at 340nm.

The divalent copper reacts with the peptide bonds in proteins under alkaline conditions forming a characteristic pink to purple biuret complex. The color intensity is directly proportional to the protein concentration measured by the increase in absorbance at 552nm (MTRH, 2018). Albumin binds to the anionic dye bromcresol green (BCG) at pH 4.1 forming a blue-green colored complex. The color intensity of this complex is directly proportional to albumin concentration and is measured photometrically by monitoring the increase in absorbance at 583 nm. The difference between total protein and albumin gives the concentration of globulin in serum.

The enzymatic conversion of creatinine liberates hydrogen peroxide which reacts with 4-aminophenazone and HTIB forming a quinine imine chromogen. The color intensity of the chromogen is directly proportional to creatinine concentration measured by increase in absorbance at 552nm. The cassette COBAS Integra 400 Plus Urea/BUN (UREAL) contains a reagent that hydrolyzes urea into ammonia and carbonate (MTRH, 2018). Ammonia then reacts with 2-oxoglutarate in presence of NADH to form L-glutamate. The rate of NADH decrease is directly proportional to urea concentration determined by

measuring the absorbance at 340nm. For glucose determination glucose is phosphorylated to glucose-6-phosphate by hexokinase enzyme. The second reaction involving oxidation of glucose-6-phosphate utilizes NAD^+ forming NADH. The concentration of NADH formed is directly proportional to glucose concentration measured by increase in absorbance at 340nm.

3.6.3 Lipid Peroxidation Assay in Liver Tissues

Malondialdehyde (MDA) levels in liver tissues, an index of lipid peroxidation, was assayed according to the Okhawa's method as described by Alam *et al.* (2013) with few modifications. The frozen liver tissues that were earlier obtained as described in section 3.5.4 were removed from the freezer and allowed to stand for one hour at room temperature. One gram (1g) of the liver tissue to 9mL of 1.15% of cold KCl was homogenized and centrifuged at 2000 rpm for 10 minutes. 0.1mL of the resultant supernatant was mixed with 0.2mL of 8.1% sodium dodecyl sulfate (SDS), 1.5mL of 20% acetic acid and pH adjusted to 3.5 with 10M NaOHbefore adding 1.5mL of 8% thiobarbituric acid (TBA). This mixture was made up to 4mL with distilled water and then heated at 95^oC on a water bath (with boiling chips to reduce bumping) for 60 minutes. The tubes were cooled using tap water to room temperature after incubation. 1mL of distilled water was added to the mixture to make a final volume of 5mL in each tube. 5mL of butanol: pyridine mixture in a ratio of 15:1(v/v) was added and the contents mixed thoroughly for 2 minutes. The total volume of the mixture was 10mL for each sample. The flocculent precipitate (pink organic layer) was pipetted after centrifugation at 4000 rpm for 10 minutes and the absorbance measured at 532 nm using a spectrophotometer (Spectronic 21D from Milton Roy Limited). The MDA levels were

obtained from a standard calibration curve generated by hydrolyzing 1,1,3,3-tetramethoxypropane (TMP) as follows; The MDA standard was prepared by diluting the stock solution of 6.03M of TMP to make 100mM of the solution (1666µl of TMP diluted to 100mL with distilled water). The 100µM stock solution was prepared by taking 50µl of the 100 mM solution and diluting to 50mL with distilled water. The resultant µM stock solution was used to make concentrations of 10µM, 7.5µM, 5µM, 4µM, 3µM, 2µM, 1µM, and 0.5 µM. 0.2mL of 8.1% SDS was added to 0.1mL of each concentration and mixed. 1.5mL 20 % Acetic Acid was then added, mixed and the pH adjusted to 3.5 with 10 M NaOH. 1.5mL of 0.8% TBA was added to the mixture, volume made to 4mL with distilled water before placing in a water bath at 95^0Cfor one hour. 1mL of distilled water and 5mL of Butanol: Pyridine was added to the cooled mixture, mixed, and centrifuged at 4000 rpm for 10 minutes. The upper organic layer was measured at 532nm in a spectrophotometer. The new concentrations for the 0.1mL of the µM solutions were obtained; for instance, the new concentration for the 0.1mL of the 10µM solution gave 100nM and therefore the final working standard concentrations for standard curve plot was in the range of 5nM-100nM (Appendix II). The reference blank was prepared by taking 0.1mL of 1.15% KCl and addition of the same reagents as the samples and standard. The thiobarbituric acid reactive substance (TBARS) method depends on the formation of MDA as an end product of lipid peroxidation. MDA reacts with TBA producing a pink chromogen (TBARS) that is measured spectrophotometrically at 532 nm. The levels of MDA in the liver tissues were expressed in nM.

3.6.4 Histopathological Assay

The fixed tissues obtained and processed as described in section 3.5.4 were taken to MTRH histopathology laboratory for processing. After fixation, the specimens were trimmed (should not be too big or thick to touch the edges) using a scalpel to enable them fit into appropriate labelled tissue cassettes. The filled cassettes were then stored in 10% formalin until further processing. The tissues were dehydrated by passing them in increasing concentrations of alcohol (70% to 95%) to remove water and formalin. A clearing agent (xylene) was used to remove the alcohol and was preferred because it allows infiltration with paraffin wax. The specimens were then infiltrated with an embedding agent (paraffin wax) and allowed to solidify to form blocks. These blocks provide a support matrix that allows thin sectioning. Wax was removed from the surface of the block to expose the tissue. The blocks were chilled on a refrigerated plate for about 10 minutes before sectioning. A microtome was used to slice extremely thin tissue sections off the block in the form of a ribbon. The microtome was pre-set to cut the tissues at thicknesses of 5 µm. The tissue ribbons were thereafter floated on a warm water bath to remove wrinkles. The tissue ribbons were then picked up on a glass microscopic slide. The glass slides were then placed in a warm oven (at 37ºC) for about 15 minutes to help the section adhere to the slide. Before staining, the slides were "deparaffinized" by running them through xylenes to alcohols to water. Haematoxylin and eosin stains were used to provide contrast to tissue sections, making tissue structures more visible and easier to evaluate. Haematoxylin is a nuclear stain and stains the nucleus blue/black/purple, depending on the haematoxylin used. Eosin is a cytoplasmic counter stain, with at least 3 different shades of pink depending on the cell part or tissue type. The

morphological evaluations were then carried out using a light microscope. Light microscopic examination of multiple tissue sections from various organs in all groups were performed and images representative of the typical histological profile examined.

After staining, cover slips were mounted over the tissue specimens on the slides using optical grade glue, to help protect the specimen. The stained slides were finally examined for histological changes under a light microscope (Olympus CX21FS1) for evidence of toxicity. The photomicrographs of the tissues were taken using a Samsung smart phone and labelled.

3.7 Data Management

Data entry and management was done using Microsoft Office Excel 2007 and the Statistical Package for the Social Sciences (SPSS) version 16.0.The results were expressed as mean ± SEM. Data for changes in fasting body weights were analyzed using two-way analysis of variance (ANOVA) with the Statistical Package for the Social Sciences (SPSS) version 16. All the other data for hematology, biochemical, and lipid peroxidation were analyzed using pair wise Student's t-test comparisons with Microsoft Office Excel 2007. The results were considered significant at $p < 0.05$.

CHAPTER FOUR

RESULTS

4.1 Effects of TMO on body weights of rats

4.1.1 Effects of TMO on body weights of male rats

Rats in both the control and TMO treated groups showed an increase in their body weights from day 0 to 14 (Figure 4.1A) but was not statistically significant. Interestingly, there was little decrease in body weights as the animals progressed to day 21 followed by rapid decrease in weights to day 28 in animals treated with TMO. Analysis using t-test indicated significant decrease (3%) in body weights at day 28 of HHD-TMO rats as compared to the control ($p=0.0143$). The UHD-TMO animals also showed a significant increase (7%) in weight at day 28 as compared to the HHD-TMO animals; $p=0.0114$ (Figure 4.1A). Rats in the control group showed increase in body weight throughout the study period (day 0 to day 28). ANOVA analysis indicated that the treatment period and treatments administered did not significantly affect the overall body weight of animals in treatment groups compared to the control and within treatment groups.

4.1.2 Effects of TMO on body weights of female rats

Rats in the control group increased in body weight throughout the entire period (Figure 4.1B). Rats treated with TMO showed body weight gain from day 0 to day 14, followed by decrease in weight from day 14 to day 28. Analysis using t-test indicated a significant decrease (12%) in body weight at day 28 of animals in HHD-TMO group as compared to the control ($p=0.0077$). The HHD-TMO animals also showed a significant decrease in

weights at day 7 (10%), 14 (9.5%), 21 (11%), and 28 (9%) as compared to the HLD-TMO group; p=0.0056, 0.0108, 0.0062, 0.0052 respectively (Figure 4.1B). ANOVA analysis indicates that the treatment period and treatments did not significantly affect the overall body weight of animals in treatment groups compared to the control.

4.2 Physical appearance and mortality of rats following 28 day oral administration of TMO

All the rats in the control and treatment groups appeared alert and with normal behaviour (fighting and burrowing) during the entire period of experimentation. The fur of rats in the control group appeared normal and smooth during the entire study period while fur of rats administered TMO were initially normal and smooth but the changes started appearing at day 14; their fur became rough and started peeling. There was however no mortality of the animals during the study.

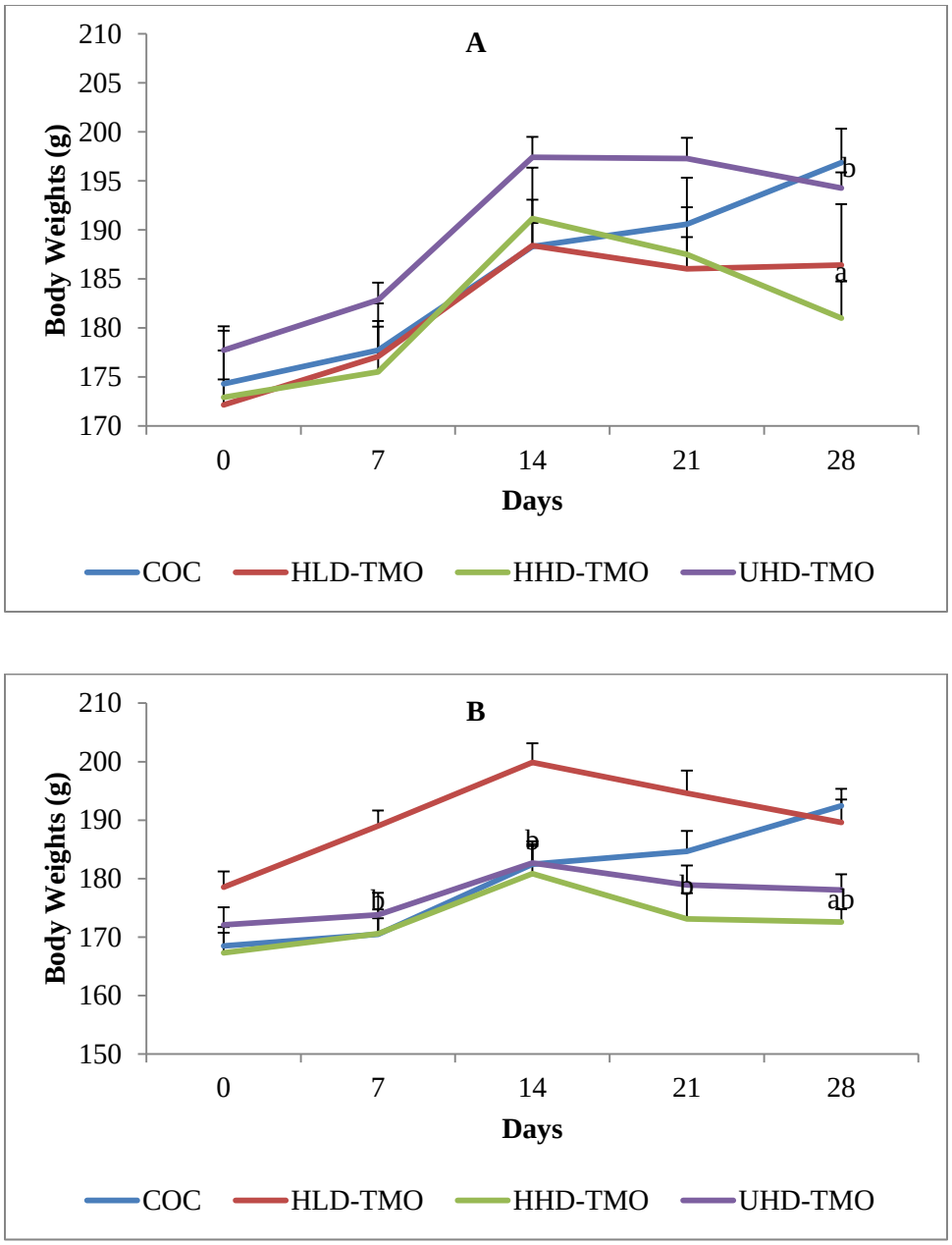


Figure 4.1: Changes in fasting body weight of male rats following 28 days oral administration of TMO

Values are mean ± SEM of five animals per group. COC-Corn oil control; HLD-TMO- Heated low dose transformer mineral oil (50mg/kg bwt); HHD-TMO- Heated high dose transformer mineral oil (500mg/kg bwt); UHD-TMO- Unheated high dose transformer mineral oil (500mg/kg

bwt). 'a' Significantly different from control at p < 0.05; 'b' Unheated high dose significantly different from heated high dose at p< 0.05.

4.3 Effects of TMO on haematological parameters in rats

4.3.1 Haematological changes in male rats

Animals in the HLD-TMO group showed a significant decrease of 11% in RBC as compared to the COC group (Table 4.1). Also, there was a significant increase in PLTs (30%) among HHD-TMO animals as compared to the COC group; p=0.0284. UHD-TMO animals showed significant increase in HGB (7%) and PLTs (44%) when compared to COC group; p=0.0147 and 0.0155 respectively. There was a significant decrease in WBC (38%) and increase in RBC (11%) and HGB (6%) among HHD-TMO animals compared to HLD-TMO animals; p=0.0424, 0.0074, and 0.0375 respectively (Table 4.1). Animals of UHD-TMO group showed no significant differences in any of the parameters as compared to the HHD-TMO group.

4.3.2 Haematological changes in female rats

The haematological parameters of animals in HLD-TMO group did not show any significant changes when compared to the COC animals (Table 4.1). However, animals in HHD-TMO group showed a significant increase in RBC (10%) and a significant decrease (10%) in RDW in comparison to the COC group; p=0.0368 and 0.0421 respectively. The UHD-TMO animals showed significant decrease (10%) in RDW as compared to the COC group (p=0.0355) and a significant decrease in RBC (14%) as compared to animals in the HHD-TMO group (p=0.0379).

Table 4.1: Changes in haematological parameters in rats following 28 days oral administration of TMO

Haematology Parameters	Treatment Groups				
	Sex	**COC**	**HLD-TMO** *(50mg/kg bwt)*	**HHD-TMO** *(500mg/kg bwt)*	**UHD-TMO** *500mg/kg bwt)*
WBC ($x10^3$/µL)	**M**	5.78±1.30	6.03±0.59	3.74±0.68[b]	7.02±2.55
	F	4.24±0.37	4.72±1.16	5.70±0.82	5.15±0.33
RBC ($x10^6$/µL)	**M**	8.20±0.24	7.34±0.23[a]	8.15±0.07[b]	8.37±0.10
	F	7.73±0.23	8.20±0.80	8.52±0.17[a]	7.33±0.42[c]
HGB (g/dL)	**M**	14.88±0.23	14.4±0.36	15.3±0.14[b]	15.86±0.22[a]
	F	14.74±0.38	14.4±1.04	15.6±0.46	14.45±0.46
HCT (%)	**M**	48.84±2.57	45.75±1.08	47.86±0.78	49.66±0.79
	F	45.8±1.07	50.38±3.31	49.45±1.44	44.43±1.96
MCV (fL)	**M**	59.5±2.44	62.48±1.49	58.72±0.84	59.36±0.83
	F	59.26±0.49	65.42±3.36	58.03±0.59	60.78±1.11
MCH (pg)	**M**	18.16±0.42	19.68±0.63	18.8±0.30	18.96±0.25
	F	19.06±0.25	18.58±0.41	18.3±0.2	19.83±0.60
MCHC (g/dL)	**M**	30.72±1.42	31.48±0.38	31.98±0.45	31.96±0.10
	F	32.18±0.30	28.68±1.63	31.58±0.17	32.55±0.43
RDW (%)	**M**	14.06±0.23	14.88±0.62	13.64±0.25	14.12±0.27
	F	13.94±0.28	14.48±0.68	12.9±0.30[a]	12.9±0.27[a]
PLT ($x10^3$/µL)	**M**	547.6±30.79	582.0±43.45	706.4±50.92[a]	787.8±72.06[a]
	F	646.8±75.05	508.0±77.67	710.75±33.57	656.25±55.03

COC-Corn oil control; HLD-TMO- Heated low dose transformer mineral oil; HHD-TMO- Heated high dose transformer mineral oil; UHD-TMO- Unheated high dose transformer mineral oil; M-Male; F-Female; WBC-White blood cells; RBC-Red blood cells; HGB-Haemoglobin; HCT-Hematocrit; MCV-Mean corpuscular volume; MCH-Mean corpuscular haemoglobin; MCHC-Mean corpuscular haemoglobin concentration; RDW-Red cell distribution width; PLT-Platelets. The results are mean ± SEM of five animals per group. 'a' Treatment groups significantly different from control at p < 0.05. 'b' Heated high dose significantly different from heated low dose at p< 0.05. 'c' Unheated high dose significantly different from heated high dose at p< 0.05.

4.4 Effects of TMO on serum indices of liver function in rats

4.4.1 Effects of TMO on serum indices of liver function in male rats

The HLD-TMO animals showed no significant changes in ALT, TP, GLOB, and ALB from the COC group (Figure 4.2A).The HHD-TMO animals and UHD-TMO animals showed significant decrease in ALT by 44% and 34% respectively as compared to the control; p=0.0033 and 0.0130 respectively. However, TP, ALB, and GLOB levels in HHD-TMO and UHD-TMO groups showed no significant changes compared to COC group. There was a significant decrease (44%) in ALT in HHD-TMO animals when compared to the HLD-TMO animals; p=0.0105 (Figure 4.2A). The UHD-TMO animals showed no significant changes in ALT, TP, GLOB, and ALB from the HHD-TMO animals.

4.4.2 Effects of TMO on serum indices of liver function in female rats

There were no significant changes in the ALT and GLOB among the various TMO treated groups (HLD-TMO, HHD-TMO, and UHD-TMO) when compared to the COC group except TP and ALB of the HHD-TMO animals which showed a significant increase by 10% and 15% respectively from the COC animals; p=0.0014 and 0.0428 respectively (Figure 4.2B).

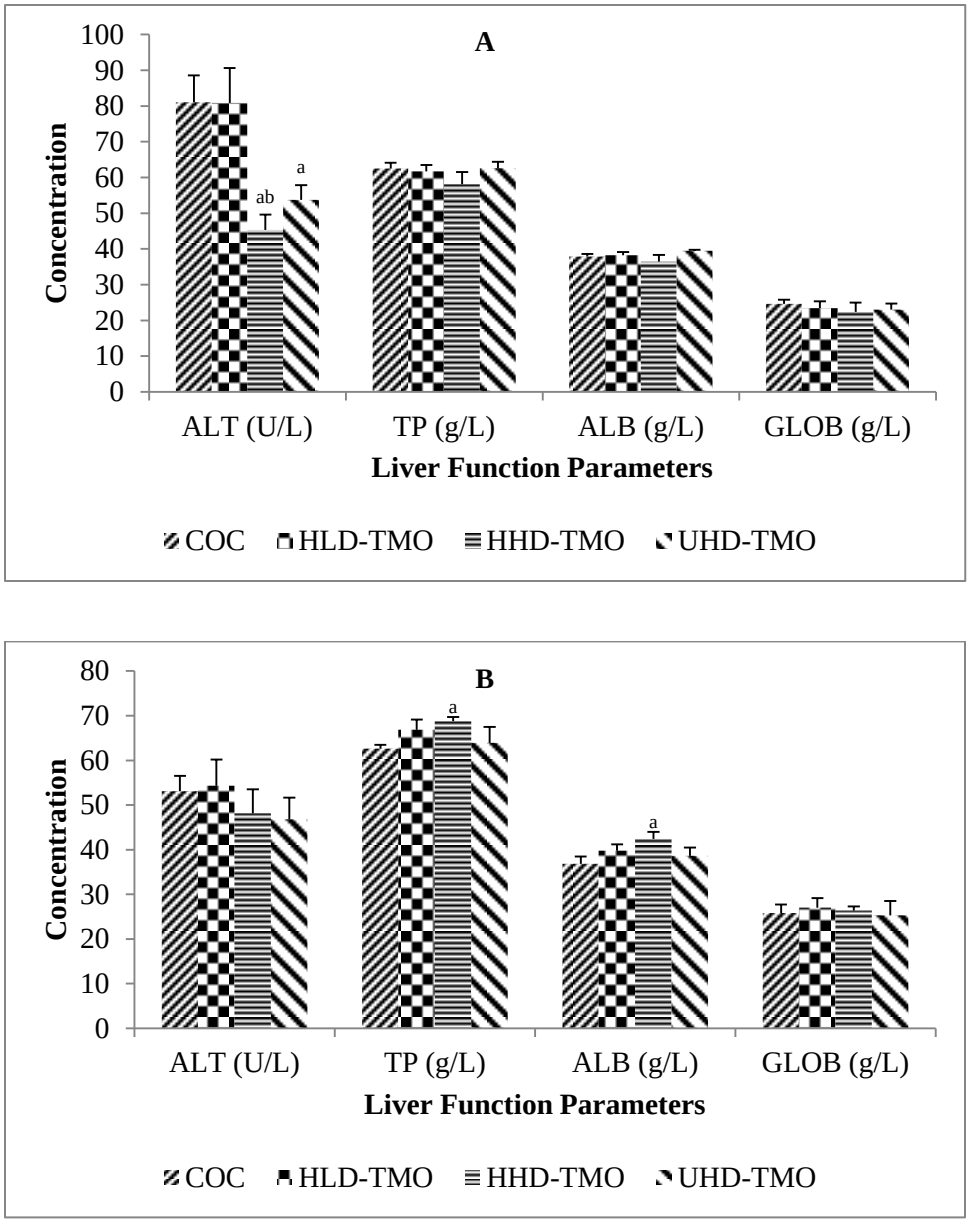


Figure 4.2: Levels of serum indices of liver function in male rats following 28 days oral administration of TMO

COC-Corn oil control; HLD-TMO- Heated low dose transformer mineral oil (50mg/kg bwt); HHD-TMO- Heated high dose transformer mineral oil (500mg/kg bwt); UHD-TMO- Unheated high dose transformer mineral oil (500mg/kg bwt); ALT-Alanine transaminase; TP-Total Protein; ALB-Albumin; GLOB-Globulin. Values are mean ± SEM of five animals per group. 'a' Significantly different from control at $p < 0.05$. 'b' Heated high dose significantly different from heated low dose at $p < 0.05$.

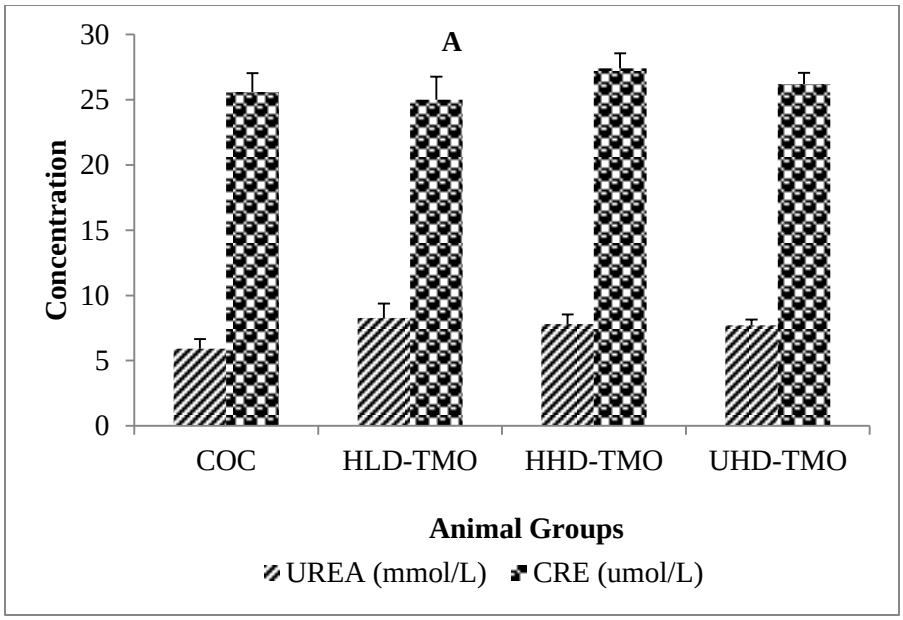
4.5 Effects of TMO on serum indices of kidney function in rats

4.5.1 Effects of TMO on serum indices of kidney function in male rats

The kidney function indices assessed were creatinine and urea levels which showed no significant differences among the various TMO treated groups as compared to the control (COC) and within TMO treated groups (Figure 4.3A).

4.5.2 Effects on serum indices of kidney function in female rats

No significant changes in urea levels were observed among the HLD-TMO animals and HHD-TMO animals when compared to the COC group as shown in figure 4.3B. However, the UHD-TMO animals showed a significant decline in urea levels (22%) in comparison to the control; $p=0.0145$. Changes in creatinine levels among all the TMO treated groups were not significantly different as compared to the control except for the HHD-TMO animals that showed a significant increase of 26% when compared to the HLD-TMO animals; $p=0.0069$ (Figure 4.3B).



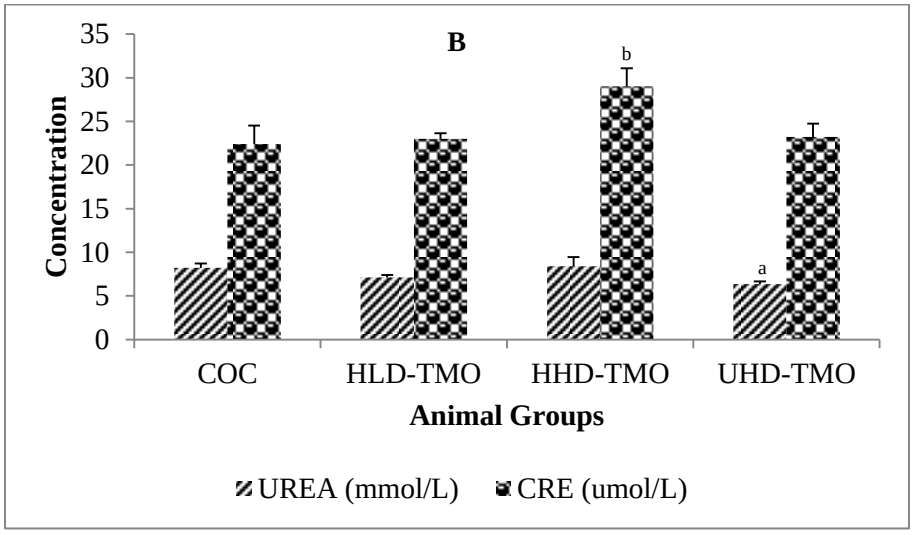


Figure 4.3: Serum urea and creatinine (CRE) levels in male rats following 28 days oral administration of TMO

COC-Corn oil control; HLD-TMO- Heated low dose transformer mineral oil (50mg/kg bwt); HHD-TMO- Heated high dose transformer mineral oil (500mg/kg bwt); UHD-TMO- Unheated high dose transformer mineral oil (500mg/kg bwt); CRE-Creatinine; Values are mean ± SEM of five animals per group. "a" Significantly different from control at p < 0.05. "b" Heated high dose significantly different from heated low dose at p< 0.05.

4.6 Effects of TMO on MDA levels in liver tissues

Both the male and female rats showed an increase in MDA levels with increase in TMO doses though the changes in HLD-TMO males and females and HHD-TMO females were not significant as compared to COC group (Figure 4.4A & 4.4B). Significant changes in MDA levels were observed in liver tissues of HHD-TMO males (22% increase) and UHD-TMO males (24% increase) when compared to the control; p=0.0124 and 0.0117 respectively (Figure 4.4A). The female UHD-TMO animals showed significant increase (34%) in MDA levels as compared to the control; p=0.0434 (Figure 4.4B). No significant changes in MDA levels were observed in HHD-TMO and UHD-TMO male and female animals as compared to HLD-TMO and HHD-TMO groups respectively (Figure 4.4A & 4.4B).

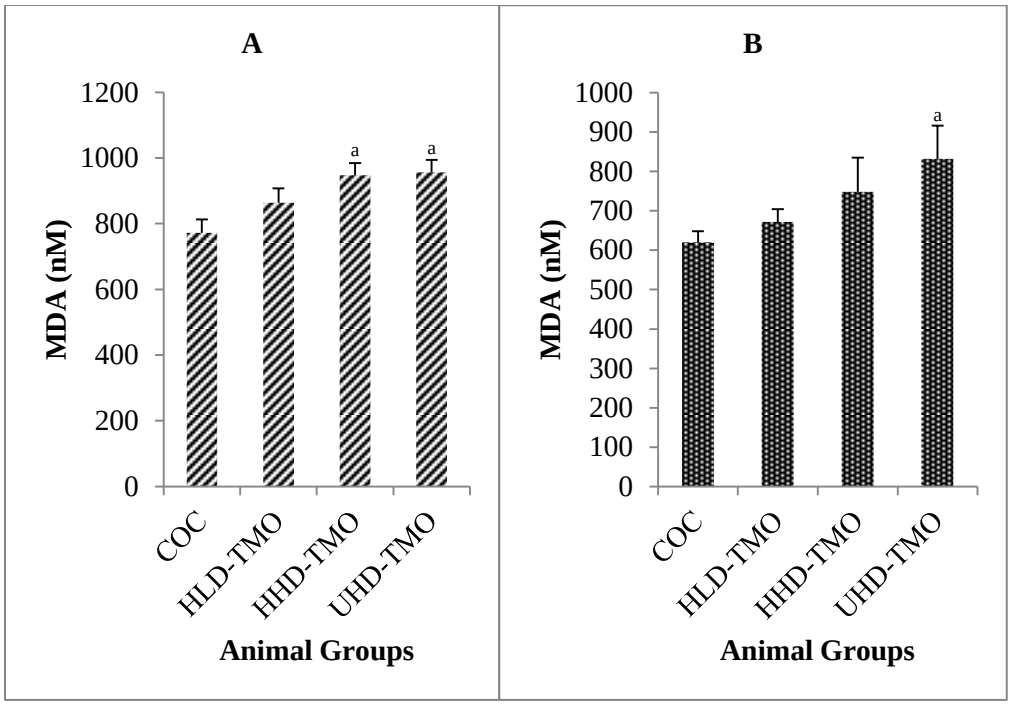


Figure 4.4: MDA levels in liver tissues in male (A) and female (B) rats following 28 days oral administration of TMO

COC-Corn oil control; HLD-TMO- Heated low dose transformer mineral oil (50mg/kg bwt); HHD-TMO- Heated high dose transformer mineral oil (500mg/kg bwt); UHD-TMO- Unheated high dose transformer mineral oil (500mg/kg bwt); MDA- Malondialdehyde. Values are mean ± SEM of five animals per group. "a" Significantly different from control at p < 0.05.

4.7 Effects of TMO on fasting blood glucose (FBS) levels of rats

There were no significant changes in fasting blood glucose levels between the treatments and the control groups among the male rats (Figure 4.5A). However, the female rats showed a significant increase in glucose levels among the HLD-TMO rats (36%), HHD-TMO rats (43%), and UHD-TMO rats (57%) as compared to the control; p=0.0088, 0.0069, 0.0009 respectively (Figure 4.5B). Both the male and female rats showed no significant differences between HHD-TMO compared to HLD-TMO animals and UHD-TMO compared to HHD-TMO rats.

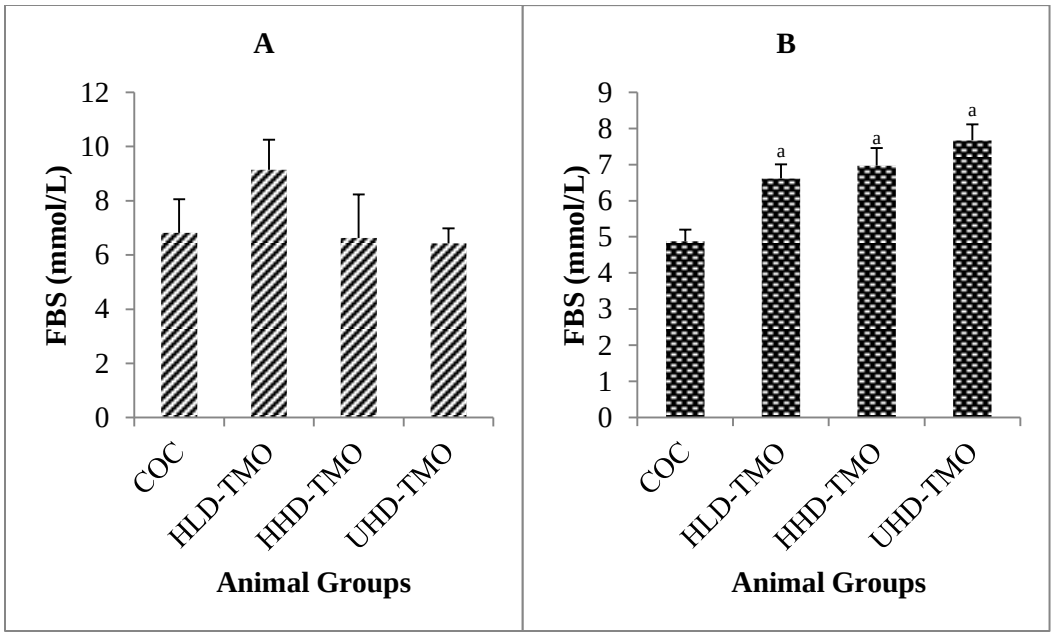


Figure 4.5: Fasting blood glucose (FBS) levels in male (A) and female (B) rats following 28 days oral administration of TMO

COC-Corn oil control; HLD-TMO- Heated low dose transformer mineral oil (50mg/kg bwt); HHD-TMO- Heated high dose transformer mineral oil (500mg/kg bwt); UHD-TMO- Unheated high dose transformer mineral oil (500mg/kg bwt); Values are mean ± SEM of five animals per group. "a" Significantly different from control at p < 0.05.

4.8 Effect of TMO on tissue histology of rats

4.8.1 Histological examination of liver

Animals in the COC group of both the male and female animals had a normal liver (Figure 4.6A). The male (Figure 4.6D, 4.6E) and female (Figure 4.6C) animals in the HHD-TMO showed a liver with bile duct proliferation. The female HLD-TMO animals (Figure 4.6B) and UHD-TMO animals (Figure 4.6F) showed focal areas of periportal chronic inflammation.

4.8.2 Histological examination of kidney

Both the male and female rats in COC, HLD-TMO, and UHD-TMO groups showed a normal kidney (Figure 4.7 A-C). The male and female rats of HHD-TMO group showed focal areas of mild chronic inflammation (Figure 4.7 D-E).

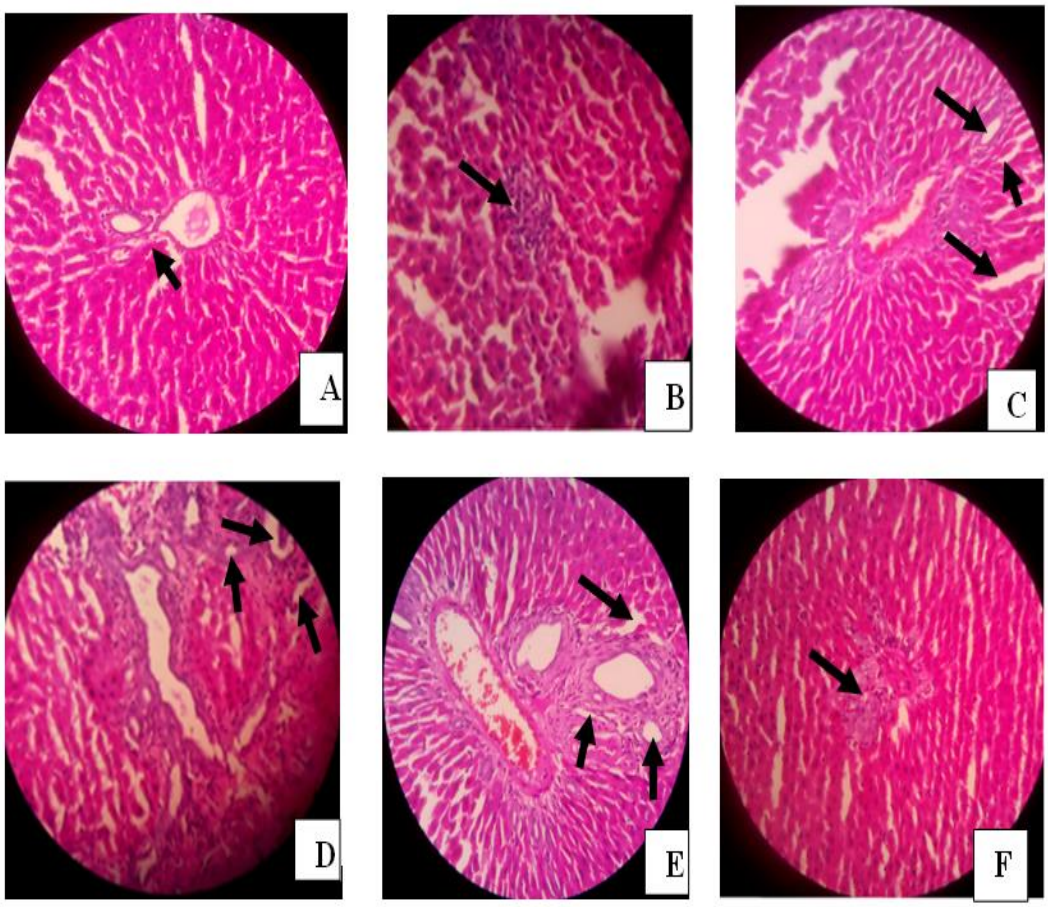


Figure 4.6: Photomicrographs of histological changes in liver tissues of rats after 28 days oral administration of TMO (X40)

A-Representative image of liver tissue of rats in the COC group depicting normal portal triad (arrow);B-Liver tissue of HLD-TMO female rats showing several focci of mild periportal chronic inflammation (arrow):C- Liver tissue of HHD-TMO female rats with bile duct proliferation (arrows); D-Liver tissue of male HHD-TMO rats with mild bile duct proliferation (arrows); E-Liver tissue of female rats with bile duct proliferation (arrow); F-Liver tissue of UHD-TMO female rats with mild periportal chronic inflammation (arrow). COC-Corn oil control (fed on 200µl corn oil); HLD-TMO- Heated low dose transformer mineral oil (50mg/kg bwt); HHD-TMO- Heated high dose transformer mineral oil (500mg/kg bwt); UHD-TMO- Unheated high dose transformer mineral oil (500mg/kg bwt).

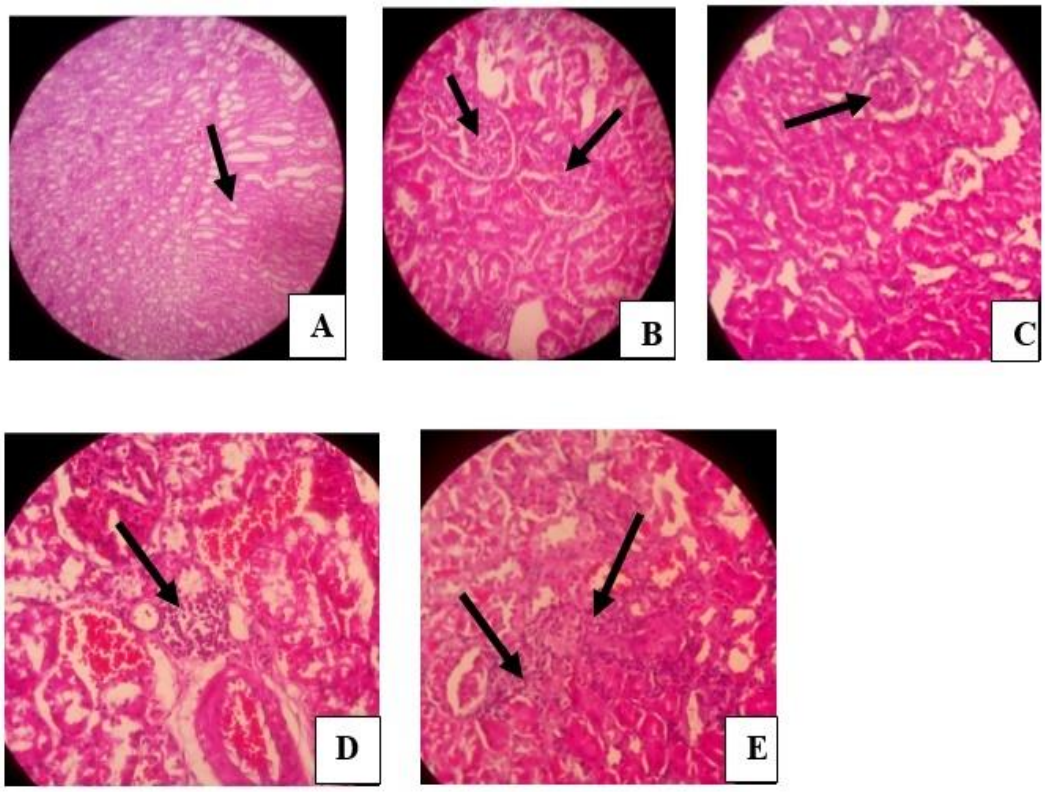


Figure 4.7: Photomicrographs of histological changes in the kidney of rats after 28 days oral administration of TMO

A-Normal kidney of male rats in COC group with arrow depicting normal tubules (X10); B-Normal kidney of male rats in HLD-TMO group with arrows showing normal glomeruli (X40); C-Normal kidney of female rats in UHD-TMO group with arrow showing normal glomeruli (X40); D-Kidney of male rats in HHD-TMO group with mild chronic inflammation at X40 (arrow); E-Kidney of female rats in HHD-TMO group with arrows depicting mild chronic inflammation (X40). COC-Corn oil control (fed on 200µl corn oil); HLD-TMO- Heated low dose transformer mineral oil (50mg/kg bwt); HHD-TMO- Heated high dose transformer mineral oil (500mg/kg bwt); UHD-TMO- Unheated high dose transformer mineral oil (500mg/kg bwt).

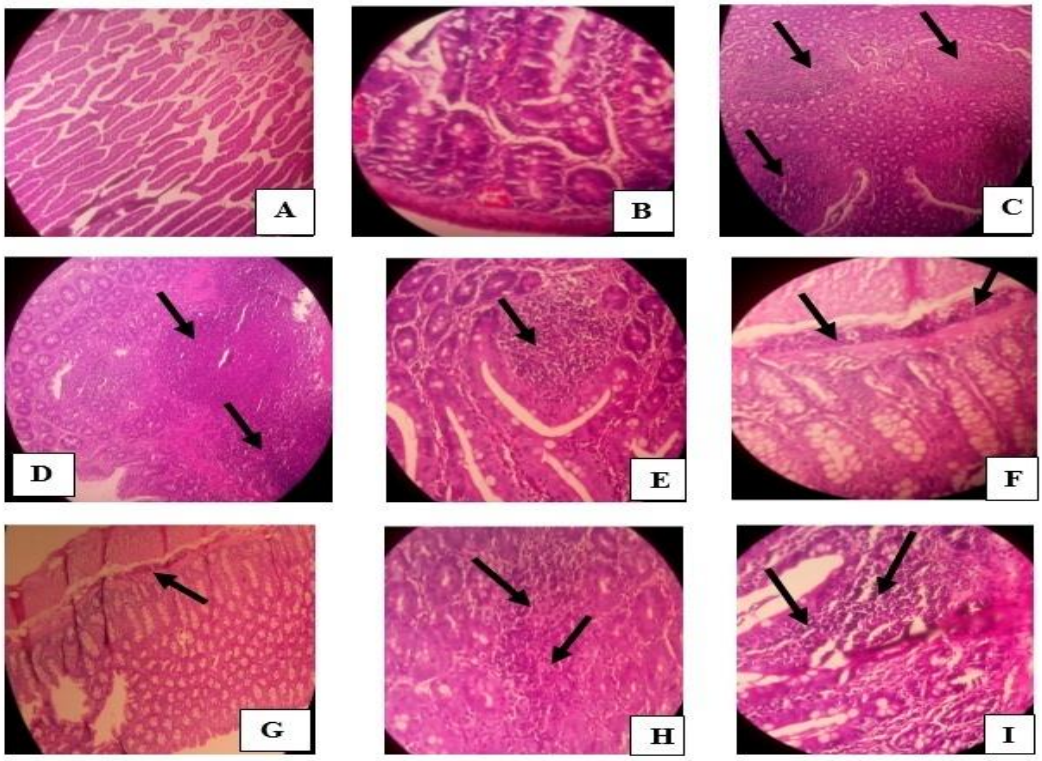


Figure 4.8: Photomicrographs of histological changes in the small intestines of rats after 28 days oral administration of TMO

A-Normal small intestine tissue of female rats in COC group (X10); B- Normal small intestine tissue of male rats in COC group (X40); C-Small intestine tissue of male rats in COC group (X40) indicating prominent lymphoid follicles (arrows); D-Small intestine tissue of female rats in HLD-TMO group (X10) with lymphoid follicles (arrows); E-Small intestine tissue of male rats in HHD-TMO group (X40) with mild chronic inflammation (arrow); F-Small intestine tissue of female rats in HHD-TMO group (X40) with moderate chronic inflammation (arrows);G-Small intestine tissue of male rats in HHD-TMO group (X10) with moderate chronic inflammation (arrow); H-Small intestine tissue of male rats in UHD-TMO group (X40) with moderate chronic inflammation (arrows); I-Small intestine tissue of female rats in UHD-TMO group (X40) with moderate chronic inflammation (arrows); HLD-TMO- Heated low dose transformer mineral oil (50mg/kg bwt); HHD-TMO- Heated high dose transformer mineral oil (500mg/kg bwt); UHD-TMO- Unheated high dose transformer mineral oil (500mg/kg bwt).

4.8.3 Histological examination of small intestine

Some of the male and female animals in the COC group had normal small intestines (Figure 4.8A, 4.8B). However, other male and female animals in the COC groups and all treated groups had small intestines tissue with lymphoid follicles (Figure 4.8C, 4.8D). The presence of lymphoid tissue is not a change that resulted due to treatment with TMO. Both male and female animals in HHD-TMO (Figure 4.8 E-G) and UHD-TMO (Figure 4.8 H-I) groups showed focal areas of chronic inflammation which was absent in the COC group.

CHAPTER FIVE

DISCUSSION

Many local vendors in Kenya are using transformer mineral oil (mixed with edible oils) in frying chips and mandazi. Many Kenyans are therefore ingesting varied amounts of TMO through consumption of such fried foods. Knowledge on the toxicological effects of TMO is still scarce. TMO contains complex mixtures of hydrocarbons which can be toxic to humans and other living creatures (IEEE, 2018; Mukherjee & Mitra, 2009). Some constituents present in TMO include PAHs and benzene which are produced by metabolic transformation by cytochrome P450 linked polysubstrate monooxygenase system (Igwe *et al.*, 2016). These metabolites are highly active and known to be carcinogenic. The potential toxic effects of crude oil are exerted on variety of organs of living systems such as the skin, lungs, liver and kidney (Eyong *et al*., 2004; Adedara *et al*., 2012; Ita *et al*., 2014; Okoye *et al*., 2014).

The TMO treated male and female rats in this study showed no overall significant changes in body weights when compared to the control. Rats exposed to petroleum products from previous studies have shown no significant change in weight compared to the control (Njoroge *et al.*, 2015; Poon *et al.*, 2004). However, there were significant changes in body weight at day 28 in HHD-TMO animals as compared to the control. Other studies have also shown significant decrease in body weight of rats ingesting petroleum products (Asara *et al.*, 2013). There was observed decline in the rats' body weights from day 14 to day 28 of this study though this change was not significant. The decreased body weights might indicate the inability of the animals to convert the feed

consumed into useful nutrients that are required by the body (Iwuanyanwu *et al.*, 2011). The reduced body weight might also be an indication of a pathological response to the toxic hydrocarbons present in TMO or may be the adaptive feature of the animals to the petroleum product administered.

The rats in the control and treated groups appeared alert with fighting and burrowing during the entire period of experimentation thus indicative of normal behavior. Fighting among rats is usually a form of social interactions or communication. Rats fight to compete for some advantage over another or as a defensive tactic in attacks (Himmler *et al.*, 2013). Laboratory rats display burrowing as a form of shelter from perceived threats like humans or loud voices (Makowska & Weary, 2016). The rats also shelter from light which is aversive to rats and may lead to retinal atrophy or blindness. Rats use the burrows extensively for sleeping, eating, and storing food. Rats show alertness through standing upright upon any destruction which is an inherent component of their behaviour (Makowska & Weary, 2016). There was no mortality of the rats during the entire study period. The fur of rats in the control group appeared normal and smooth during the entire study period. Fur of rats administered TMO were initially normal and smooth but the changes started appearing at day 14; their fur became rough and started peeling. This observation on fur changes therefore suggest that TMO may have constituents that can cause dermatitis (inflammation of the skin). Animals exposed to PAHs contained in petroleum products are known to exhibit substantial risk of skin cancer (Iyanda, 2016; Virich *et al.*, 2008). A study by Igwe reported significant dose related alterations in the skin DNA and RNA content of adult albino rats exposed to Bonny light crude oil by topical application (Igwe *et al.*, 2016). Histological examination of the exposed skin area

showed extensive fatty infiltration and vacuolar degeneration. These intrinsic cellular changes were suggested to be capable of inducing skin related defects such as dermatitis, neoplasm and skin cancer. Direct dermal exposure to crude oil has been reported to produce such effects as burning sensation, irritation, erythema, followed by the formation of vesicles, blisters and even extensive epidermolysis (Otaigbe & Adesina, 2005). Future studies should therefore consider the potential carcinogenicity of TMO in rats.

In this study, although certain haematological parameters like WBC, HCT, MCV, and MCHC showed no significant changes between the TMO-treated and control groups, there were significant decreases in RDW of HHD-TMO and UHD-TMO among the female rats and may indicate toxicity. RDW levels measure the variation in size of RBCs and increased levels are usually biomarkers for cardiovascular diseases or metabolic inflammatory disorders. Although there was a significant increase in the PLTs levels of HHD-TMO and UHD-TMO male rats, the values remained within the acceptable published normal reference values for rats (He *et al.*, 2017). This may therefore not have resulted in toxic effects with respect to this parameter. Indeed, increase in platelets may be beneficial, excessive increase becomes toxic as it leads to disorders associated with blood clotting like stroke. In the present study, since the values rose but remained within normal reference ranges, the changes may therefore appear to be favourable to the animals. The effect of continued dosing beyond day 28 (chronic effects) remain to be investigated, however, given the increasing trend of PLT, it is probable that the values may rise to surpass the upper reference values. Abnormally increased platelet levels are associated with oxidative stress, inflammation, iron deficiency, spleen disorder, and some types of anemia (Momoh & Oshin, 2015).

The significant increase in RBCs and HGB in HHD-TMO males compared to the HLD-TMO males may also be beneficial since it increases the capacity of blood to carry oxygen in animal body system. The UHD-TMO males also showed a significant increase in HGB compared to the control. Like their male counterparts, the female HHD-TMO animals also showed significant increase in RBCs compared to their control group. Although the HLD-TMO males showed a significant decline in RBCs, these values were within normal ranges (He *et al.*, 2017). Many studies have shown a decrease in RBCs and HGB in animals that ingested petroleum products. A study by Adeyemi *et al.* (2010) observed reduced RBC and HGB levels in albino rats ingesting water contaminated with phenol and benzene (constituents of TMO). Reduction in RBC and HGB levels were also observed in rats following ingestion of crude oil, petrol, kerosene and diesel (Ita & Udofia, 2011). Ita and Udofia observed reduction in RBC levels and suggested that the treatment induced anemic conditions (Ita & Udofia, 2011). TMO might have destroyed RBCs or interfered with normal production of RBC thus may ultimately lead to anaemia. TMO might also cause release of malondialdehyde (MDA) which can cause oxidative damage to the heart thereby destroying RBC and reduce erythrocyte survival. The oxidative stress can be accounted for by the significant increase in liver MDA levels observed in this study. Hydrocarbons in TMO are expected to undergo oxidation upon heating. The oil oxidation results in formation of intermediates and peroxides (Ushakova *et al.*, 2017). However, there were no significant changes between animals treated with heated and unheated forms of the oil in this study except for the UHD-TMO females that showed significantly lower values of RBCs compared to the HHD-TMO females. TMO has aromatic compounds that act as inhibitors of oxidation upon heating. The inhibitors

can be inactivated at high temperatures of 220^{o}C where the aromatic oxidised compounds do not decompose resulting in continued oxidation (Ushakova *et al.,* 2017). The heating in this study (at 180°C) therefore had fewer effects on changes in TMO properties.

It is interesting to note that TMO did not induce anemic conditions as it is expected with many petroleum products. Most petroleum and crude oil constituents are highly toxic to biological membranes and proteins. Naphthalene, a constituent in TMO, has been reported to cause haemoglobin denaturation and when complexed with other compounds, leads to development of haemolytic anaemia in animals (Oguwike *et al.,* 2014). The toxic constituent of petroleum (benzene) has been shown to be activated in the bone marrow (Ibeh *et al.,* 2016) which results in bone marrow depression that is characterized by inadequate production of RBCs. The presence of these toxic constituents may possibly explain the changes in haematological values observed in this study.

The liver is the organ that is involved in metabolism of endogenous and foreign substances (Montanha *et al.,* 2014). Liver enzymes like ALT are usually used as biomarkers of liver injury. ALT is always present in the cytoplasm and mitochondria of liver cells and increases in blood is due to hepatocellular damage. TMO showed liver toxicity in this study although the toxicity did not appear to increase ALT levels as expected. The HHD-TMO and UHD-TMO male rats showed significant decrease in ALT relative to the control. There was a significant decrease in ALT in HHD-TMO animals when compared to the HLD-TMO animals indicating a dose-dependent change. Though low ALT levels indicate a healthy animal with a normal liver, it might not always be the case. The decreased ALT levels may indicate a non-functioning/ low-functioning liver that is unable to release a lot of ALT into the blood when it becomes damaged. Imo et al.

also demonstrated negative effects on liver functions of male rats exposed to petroleum products (Imo *et al.*, 2015). Many studies have shown petroleum by-products being associated with increased liver enzymes indicating toxicity. For instance, increased liver enzymes in wistar albino rats exposed to bonny light crude oil were observed in a study by Iwuanyanwu *et al.* (2013). The increased levels of serum enzymes such as ALT are always an indication of cellular leakage as a result of lack of plasma membrane integrity in the liver (Uboh *et al.*, 2005). Some hydrocarbons present in petroleum products are responsible for the cellular leakage and destruction of cellular membranes (Imo *et al.*, 2015). However, this study did not show increased ALT levels in blood as an indicator of organ dysfunction.

The TP and ALB of the HHD-TMO female animals showed a significant increase from the COC animals. The increase in these proteins in this study indicates inflammation or liver diseases which are supported by the histological changes observed in the liver. However, some studies observed significant decrease in serum ALB in rats ingesting kerosene (Njoroge *et al.*, 2015) which could suggest direct injury to hepatocytes and loss of function since plasma proteins are synthesized in the liver (Krishna Murti 1991). The results observed in effects of TMO on liver functions can be supported by histological results of liver tissue which showed bile duct proliferation and chronic inflammation of the liver in animals that ingested TMO. These results elucidate liver dysfunction. Globulin levels in this study showed no significant changes. However, globulin elevation during hepatotoxicity are usually good indicators for liver damage (Mattes *et al.*, 2014).

The effect of TMO on kidney function was determined by assessing creatinine and urea levels in serum. Urea is a waste product of protein metabolism while creatinine is a by-

product of muscle metabolism that are all needed to be excreted by the kidney. Increased levels of urea and creatinine in serum are usual biomarkers for kidney malfunction. The UHD-TMO female animals showed a significant decline in urea levels in comparison to the control. The decrease in urea levels may be attributed to liver failure. Urea is synthesized in the liver and decreased levels in serum may indicate liver damage that results in inadequate synthesis of urea. Although this may be the case in this study, these findings are not entirely supported by the results from liver functions tests and therefore require further investigations. The HHD-TMO females showed a significant increase in serum creatinine levels when compared to HLD-TMO which may indicate the kidney's inability to excrete creatinine from the body. Unlike urea levels, creatinine is a more specific and sensitive marker of kidney malfunction. Urea levels can be altered by other factors like dehydration and diet but alterations in creatinine levels is only caused by kidney damage (Garba *et al.*, 2007). A change in urea levels therefore does not necessarily suggest renal failure. Renal failure is indicated by increased urea and creatinine levels in serum since it shows inability of kidney to excrete these substances from animal body. This study finding therefore suggests that transformer mineral oil may be toxic to the kidneys.

MDA levels are useful indicators of damaged biological membranes caused by free radicals. The reactive oxygen species (ROS) oxidise polyunsaturated fatty acids in the membrane structure resulting in lipid peroxidation (Aksoy, 2015). MDA, a reactive aldehyde is generated from the decomposition of the formed lipid hydroperoxides. The observed significant increase of liver MDA levels in this study indicates the formation of excess free radicals and lipid peroxidation. The HHD-TMO males and UHD-TMO males

and females showed a significant increase in MDA levels relative to the control group. Aksoy also observed higher MDA levels among the wistar albino male rats that were fed on diesel, indicating the presence of toxic substances in petroleum products (Aksoy, 2015). The reactive oxygen species target the proteins, lipids and DNA resulting in alteration of the structure and function of the cell, tissue, organ, or the entire system (Azeez *et al.,* 2013). All the major biomolecules in cells are targets of ROS but lipids are the most susceptible targets. High MDA levels in humans have been associated with diseases like chronic kidney diseases or renal failure (Satishkumar *et al.,* 2015). Results from this study therefore suggest the presence of toxic substances in TMO that enhance oxidative stress in tissues.

Many studies in rats ingesting products of crude oil have shown decreased glucose levels. Ita *et al.* (2014) observed reduced fasting blood glucose in rats exposed to Nigerian Bonny Light crude oil. Hypoglycemia was also observed by Achuba and Nwokogba after feeding rats with diet contaminated with gasoline and kerosene (Achuba & Nwokogba, 2015). Alonso-Alvarez and his co-workers suggested polycylic aromatic hydrocarbons (PAHs) to be responsible for the decrease in glucose level even though they acknowledged that crude oil contains other toxic compounds that may possibly have contributed to the observed effects (Alonso-Alvarez *et al.,* 2007). TMO contains PAHs and other toxic compounds that are expected to cause hypoglycaemic effects. This was not the case for this study because no significant changes in glucose levels were observed among the male rats. On the other hand, although the females showed a significant increase of FBS levels in all the TMO treated groups when compared to the control, the FBS levels were within normal reference values (He *et al.,* 2017). Some studies observed

an increase in blood glucose in toads exposed to petrol (Isehunwa *et al.*, 2016). Tawwab observed increased glucose levels in Nile tilapia exposed to kerosene, diesel, or gasoline (Tawwab, 2012). Increased blood glucose levels can be attributed to stress-induced mobilization of the energy reserves. TMO might have induced stress that led to the observed hyperglycemia among the female rats.

Petroleum products are known to cause liver damage in animals. Liver is the main organ that chemically alters the foreign substances that enter the body and it is thus prone to injury. In this study animals in the control group of both the male and female animals had a normal liver while the male and female animals in the HHD-TMO group showed a liver with bile duct proliferation. Bile duct proliferation is a histological feature seen in many forms of liver diseases particularly in choleostatic liver injury. Cholangiocytes are cells that form bile ducts and are responsible for secretion, reabsorption, and regulation of the bile components (Vartak *et al.*, 2016). The bile duct proliferation also known as duct reactions is characterized by increased number of bile duct structures that are accompanied by inflammatory cell infiltration. The proliferation is typically caused by hepatotoxic chemicals (Vartak *et al.*, 2016). The female animals treated in the HLD-TMO and UHD-TMO groups also showed focal areas of periportal chronic inflammation with the male counterparts in the same groups being normal. Periportal inflammation is a hallmark sign of chronic hepatitis and fibrosis in humans. The inflammation is characterized predominantly by lymphocytes and minor components like macrophages, neutrophils, and eosinophils (Mannan, 2017). Other previous studies done in animals exposed to petroleum-related products have also shown the liver being one of the major targets for injury (Iyanda, 2016; Owagboriaye *et al.*, 2017; Poon *et al.*, 2004; Poon *et al.*,

2001; Uboh *et al.*, 2005). The low ALT, TP, and ALB observed in some of the treated groups in this study also indicate liver damage with the liver unable to synthesize enough of these proteins. The oxidative damage (attributed to increased MDA levels in this study) which may have been induced by TMO is likely to be one of the underlying mechanisms responsible for hepatotoxic effects observed in this study.

Both the male and female rats in all the COC, HLD-TMO, and UHD-TMO groups in this study showed a normal kidney except for HHD-TMO animals which showed focal areas of mild chronic inflammation. Chronic inflammation plays a critical role in initiation of many forms of chronic kidney diseases (Waters & Vogt, 2018). The serum levels of urea and creatinine in this study also showed significant changes that could indicate kidney damage. Another study also observed no histological changes in kidney of rats exposed to gasohol (Poon *et al.*, 2001). Kidney is prone to damage by free radicals generated after from metabolism of hydrocarbons present in the TMO and other petroleum products (Iwuanyanwu *et al.*, 2011).

Both the control and treated animals in this study had lymphoid follicles in their small intestines. Lymphoid tissues are normally present in the gut mucosa of animals and humans. The lymphoid follicles line the mucosal walls of the small intestines. Histomorphological changes of these tissues may reflect normal functioning or presence of pathological conditions. Foreign particles in the gut contribute to intestinal inflammation characterized by prominent lymphoid follicles (Haley, 2017). Both the controls and treated groups in this study ingested oils that may be the cause of observed lymphoid tissues in both groups. The presence of lymphoid tissue observed in treated groups of this study may not be significant because it was also observed in the control.

Despite this observation, the toxic effects of TMO on small intestine may not be ruled out since TMO has toxic constituents like naphthalene known to cause organ histopathology (Iyanda, 2016). The animals treated with heated high dose and unheated high dose of TMO in this study showed focal areas of chronic inflammation in tissues of small intestine which was absent in the control group. Chronic inflammation in stomach lumen of rats after kerosene ingestion was also observed in another study (Njoroge *et al.*, 2015). The inflammation observed in the small intestine in this study could be attributed to oxidative stress caused by the increased MDA levels. Chronic inflammation of the gut is usually associated with many intestinal inflammatory diseases that affect any part of the gut including the stomach, small intestines, and colon (Rubin *et al.*, 2012). Prolonged exposure to TMO and increased doses could result in further histological changes in animal tissues.

CHAPTER SIX

CONCLUSION AND RECOMMENDATIONS

6.1 Conclusion

Transformer mineral oil was orally administered to rats and its toxic effects were compared to control group in this study. The decline in body weights and fur changes of the TMO treated groups of rats are indicative of toxicity. The liver is a target of toxic constituents in TMO as evident in the alteration of liver biomarkers and histopathological changes observed in this study. Additionally, the increased liver MDA levels in this study elucidate TMO causing increase in free radicals that could lead to oxidative stress. The kidney biomarkers (serum urea and creatinine levels) and histological observations in this study showed kidney toxicity. As opposed to other petroleum products, TMO did not induce anemia, weight loss, and hyperglycemia. However, TMO has toxic constituents that to some extent altered the hematopoietic system resulting in altered haematological responses. This study has provided insights into the possible toxicity of transformer mineral oil on the haematological, biochemical, and histological systems.

6.2 Recommendations

1. It is recommended that additional studies need to be conducted with higher doses of TMO and longer treatment duration to determine other toxicities including carcinogenicity.

2. The pathophysiological effects observed in this study can also be further investigated to determine the mechanism involved or the reversibility/ irreversibility of the toxic effects of TMO.

3. Since the study on sub-acute oral toxicity of TMO in rats has shown toxicity effects, this is suggestive that the use of TMO in deep frying of foods could possibly have negative impacts on consumers and should be avoided.

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APPENDICES

Appendix I: Dose calculation of treatments

Transformer mineral oil being in liquid form, the initial treatment doses had to be converted from mg/kg to ml/kg using online converter. 500mg/kg converted to ml/kg resulted to 0.562ml/kg while 50mg/kg resulted to 0.056ml/kg. The individual weights of each rat were used to calculate the specific doses of transformer oil to be administered to each rat per group as shown below:

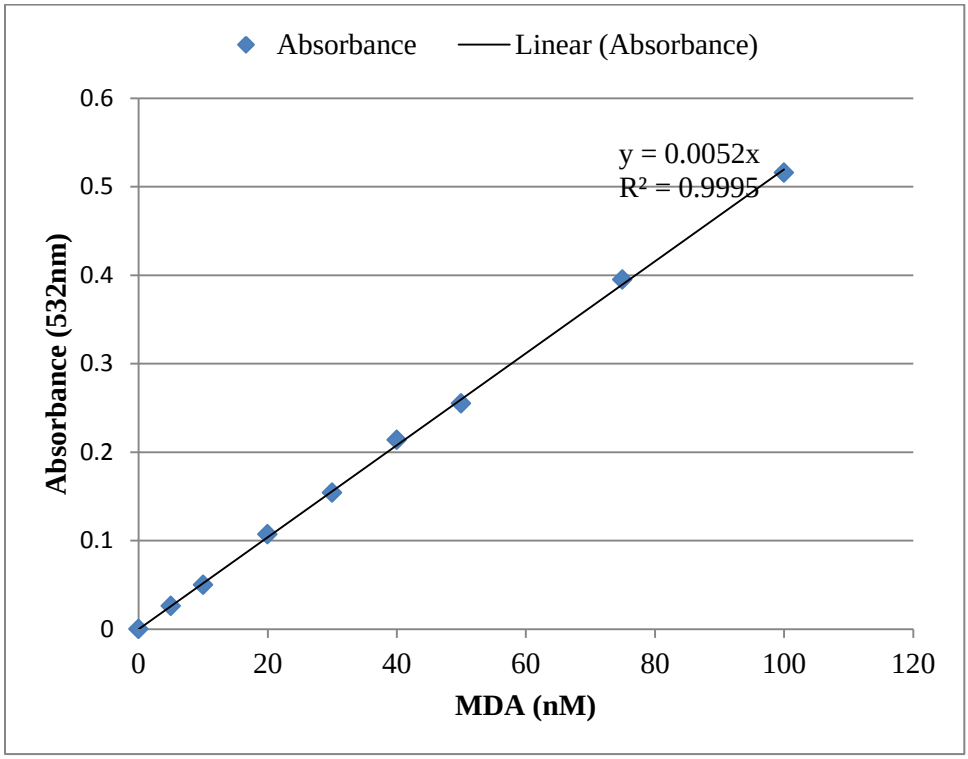
$$\text{Specific doses} = \frac{\text{Weight of rat (g) X Dose per group (ml)}}{1000\ \text{g}}$$

For instance, the dose administered to a rat weighing 180.5g in cage six will be calculated as shown below:

$$\text{Dose administered} = \frac{180.5\text{g X } 0.056\text{ml}}{1000\text{g}} = 0.0101\text{ml}$$

The doses of transformer mineral oil found from the above formula were converted to µl and made up to 200 µl with corn oil. Weekly treatments were prepared for each rat at day 1, 7, 14 and 21. Weekly stock solutions for both corn oil and TMO were prepared using daily doses for each rat multiplied 10 days. The weekly doses were adjusted per the weight changes of the rats. The weekly treatments were stored in sterile and sealed containers at room temperature.

Appendix II: Standard curve for Malondialdehyde (MDA)



Appendix III: Ethical clearance Letters

OFFICE OF THE DIRECTOR OF GRADUATE STUDIES AND RESEARCH

UNIVERSITY OF EASTERN AFRICA, BARATON

P. O. Box 2500-30100, Eldoret, Kenya, East Africa

March 24, 2017

Grace Nelima Otunga
University of Eldoret
School of Science

Dear Grace,

Re: ETHICS CLEARANCE FOR RESEARCH PROPOSAL (REC: UEAB/7/3/2017)

Your research proposal entitled ***"Sub-Acute Oral Toxicity of Transformer Mineral oil in Rats"*** was discussed by the Research Ethics Committee (REC) of the University and your request for ethics clearance was granted approval.

This approval is for one year effective March 24, 2017 until March 24, 2018. For any extension beyond this time period, you will need to apply to this committee one month prior to expiry date. Note that you will need a clearance from the study site before you start gathering your data.

We wish you success in your research.

Sincerely yours,

University of Eastern Africa, Baraton
24 MAR 2017
P. O. Box 2500-30100, ELDORET
Research Ethics Committee

Dr. Jackie K. Obey
Chairperson, Research Ethics Committee

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OFFICE OF THE DIRECTOR OF GRADUATE STUDIES AND RESEARCH

UNIVERSITY OF EASTERN AFRICA, BARATON

P. O. Box 2500-30100, Eldoret, Kenya, East Africa

March 24, 2018

Grace Nelima Otunga
University of Eldoret
School of Science

Dear Grace,

Re: ETHICS CLEARANCE FOR RESEARCH PROPOSAL (REC: UEAB/7/3/2017)

Your research proposal entitled ***"Sub-Acute Oral Toxicity of Transformer Mineral oil in Rats"*** was discussed by the Research Ethics Committee (REC) of the University and your request for ethics clearance was granted approval.

This approval is for one year effective March 24, 2018 until March 23, 2019. For any extension beyond this time period, you will need to apply to this committee one month prior to expiry date.

Note that you will need a research permit from the National Commission for Science, Technology, and Innovation (NACOSTI) and clearance from the study site before you start gathering your data.

We wish you success in your research.

Sincerely yours,

Jackie K. Obey

University of Eastern Africa, Baraton
24 APR 2018
P. O. Box 2500-30100, ELDORET
Research Ethics Committee

Dr. Jackie K. Obey
Chairperson, Research Ethics Committee