UNIVERSITY OF GHANA

COLLEGE OF HEALTH SCIENCES

AMELIORATIVE EFFECT OF NATURAL COCOA ON HEPATIC INJURY CAUSED BY OVERDOSE OF PARACETAMOL IN RATS

BY

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A THESIS SUBMITTED TO THE SCHOOL OF GRADUATE STUDIES IN PARTIAL FULFILMENT OF THE REQUIREMENT FOR THE AWARD OF MASTER OF PHILOSOPHY DEGREE IN ANATOMY

DEPARTMENT OF ANATOMY

JULY, 2019
DECLARATION BY CANDIDATE

I hereby declare that except for references to work of other researchers, which have been duly referenced, this project is the product of my own research carried out under supervision in accordance with regulations of the School of Research and Graduate Studies, University of Ghana. I further declare that this dissertation has neither in whole nor in part been presented for another degree elsewhere, and that I am solely responsible for any residual flaws in this work.

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We declare that the practical work and presentation of this thesis were supervised by us in accordance with guidelines on supervision of thesis laid down by the University of Ghana.

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DEDICATION

This work is dedicated to the Almighty God for his Grace and mercy for making this a reality.
To my mum, Miss Betty Riverson thank you for your guidance, support and relentless pursuit to this great achievement. Also to my beloved husband for your countless contribution and commitment. Finally to my son Kwabena Amoah, it was a tough journey but we sailed through.
May God richly bless you all.
ACKNOWLEDGEMENT

With Christ in the vessel, I smile at the storm! MHB 511 summarizes my expression of love and gratitude for you dear Lord. Indeed we have smiled at the storm!

I am most grateful to my Head of Department, Dr. John Ahenkorah for your patience, encouragement and guidance throughout the entire work.

To Prof. Federick Kwaku Addai. I express my profound gratitude for your constant encouragement and the supervision of this work. I deem it a great honour to be under your mentorship.

Dr. Bismark Hottor, Dr. (Mrs.) Esther Dennis, Dr. Kevin Adutwum-Ofosu, and Dr. Richard Michael Blay are also appreciated for their immense contribution to the success of this project.

I am also grateful to the technical staff of the School of Biomedical and Allied Health Animal Experimentation Unit, especially to Mr Armah and Auntie Augustina, for your expertise and your willingness to help at all times.

To Mr. Kumi and Mr. Jones of the (Department of Immunology, Noguchi Memorial Institute for Medical Research) as well as Mrs Anna Kafintu-Quarshie (Department of clinical virology), I am highly grateful for the assistance rendered during the bench work.

Special thanks to Mr. Hope Azasu, Big Joe, Nana and Mr. Degraft for your directives during the tissue processing and staining techniques.

I’m highly indebted to my colleagues, Mr. Ifeanyi, Miss Akosua Boatemaa Baidoo, Miss Delali Ed- Banson, Mr. Francis Akanyibah and Mr. Bright Dzotef. Words are not enough to express my sincere gratitude for your countless contributions and timely support which pulled me through the completion of this work. May the Almighty God bless you all.
To my dear mother, Betty Riverson, for playing mother to my kids all the times I was away working on my project, Meedase Mama! To Panyin and Kakra and baby Coby for being the most understanding and adorable kids in the world, know that mummy loves you sky high. To my beloved sister Matilda Hammond thanks for the encouragement and constant prayers.

Then to the bone of my bone and flesh of my flesh Dr. Brodrick Yeboah Amoah, I couldn’t have gone this far without you. I love you to Mars.

Now to all who contributed to this work in diverse ways, May heavens open up for your sake!
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LIST OF ABBREVIATIONS

NAPQI: N-Acetyl-P-Quinone Imine
GSH: Reduced Glutathione
ROS: Reactive Oxygen Species
NAC: N-Acetyl Cysteine
NCP: Natural Cocoa Powder
SOD: Superoxide Dismutase
ALT: Alanine Aminotransferase
AST: Aspartate Aminotransferase
SECS: Sinusoidal Endothelial Cells
NPCs: Non-Parenchymal Cells
NAFLD: Non-Alcoholic Fatty Liver Disease
NSAID: Non-Steroidal Anti-Inflammatory Drug
PGHS: Prosta Glandin .H. Synthase
POX: Peroxidase
Cox: Cyclooxygenases
FAAH: Fatty Acid Amide Hydrolase
PMSF: Phenylmethylsulfonyl Fluride
TRPvi: Transient Receptor Ppotentialvi
HMBA: 4-Hydroxy-3-Methoxy Benzyamine
HEK: Human Embryonic Kidney
SULT: Sulprotranserase
DPPH: 2,2-Diphenyl-1-Picryl Hydrazyl
NO: Nitric Oxide
ABSTRACT

Introduction: Paracetamol (acetaminophen) is a widely used over-the-counter analgesic and antipyretic drug which is known to cause liver injuries in both humans and experimental animals when administered in overdose. Supratherapeutic dose of paracetamol results in accumulation of reactive oxygen species (ROS) which impairs the antioxidants defence mechanisms and thus, causes a redox imbalance. The resulting oxidative stress plays a vital role in paracetamol-induced hepatic toxicity. Current well-known intervention for paracetamol toxicity involves the administration of N-acetyl cysteine (NAC) which serves as a glutathione precursor. Cocoa flavanols have antioxidant properties, and hence potential to mitigate the effect of oxidative stress caused by paracetamol overdose and concomitant tissue damage.

Aim: To investigate the ameliorative activity of natural cocoa in paracetamol-induced hepatotoxicity in rats.

Methodology: 20 male Sprague Dawley rats were weighed and randomised into four groups of five (G1, G2, G3, and G4) and given the following daily treatment for 4 weeks: group one (G1) were administered with 350mg/kg body weight paracetamol via oral gavage and tap water for 24 hours; group 2 (G2) received 350mg/kg paracetamol via oral gavage, 2% (w/v) natural cocoa powder for 12 hours, and water for the next 12 hours; group 3 (G3) received 350mg/kg paracetamol via oral gavage, 140mg/kg NAC after 3 hours and water for 24 hours; group 4 (G4), served as control group were given 1ml of distilled water via oral gavage and tap water, for 24 hours. All rats were given standard rat chow daily. After week 4, all rats were weighed and sacrificed; livers were harvested and histologically processed for histomorphometric analyses. Relative volume density of hepatocytes and central veins were assessed using standard stereological procedures.
Blood samples were collected via tail snipping and milking at the commencement and termination of the experiment. The levels of liver transaminases (AST, ALT), and (SOD, GSH) were measured as indicators of hepatocyte damage and oxidative stress, respectively.

**Results:** After 4 weeks of treatment, the mean serum liver enzymes AST increased significantly ($p<0.0001$) in G1 (416.37, SD 53.9 IU/L) when compared to G2 (157.12, SD 13.4 IU/L), G3 (170.50, SD 37.1 IU/L) and G4 (154.22, SD 15 IU/L), respectively. The comparison of mean ALT levels in G1 (160.31, SD 15.1 IU/L) differed significantly from G2 (108.12, SD 35.3 IU/L) G3 (92.50, SD 19.1 IU/L) and G4 (114.00, SD 3.6 IU/L) respectively. The mean SOD activity was significantly high in G2 (from 0.250, SD 0.15 U/ml to 0.3991, SD 0.17 U/ml) in comparison with G1 (from 0.256 ± 0.18 U/ml to 0.018, SD 0.01 U/ml). Post treatment mean GSH activity in the serum of rats was significantly decreased in G1 (2.43.1, SD 0.30 uM) when compared to G4 (5.995, SD 0.46 uM). GSH activity after the experiment was significantly increased among rats in G2 (8.676, SD 0.69 uM) and G3 (8.255, SD 1.18 uM) when compared to G4 (5.995, SD 0.46U/m). Histomorphometric assessment indicated that G1 rats showed significant increase in the volume of damaged hepatocyte (0.13, SD 0.027) in comparison with G2 (0.017, SD 0.005) and G3 (0.010, SD 0.002) groups. However, among the four groups there was a significant increase ($p < 0.0001$) in volume of central vein with G1 group mean volume (4.699, SD 1.297).

**Conclusion:** Natural cocoa powder exerts hepatoprotective action and increased antioxidant activity against paracetamol-induced structural and functional liver damage in male Sprague dawley rats.
CHAPTER ONE

INTRODUCTION

1.1 Background

The human liver is the largest internal organ in the body with a domed superior surface which relates completely to the diaphragm. Its postero-inferior surface however rests against the abdominal oesophagus, stomach, upper duodenum, hepatic flexure of the colon, right kidney and suprarenal gland, in addition to carrying the gall bladder (Ellis, 2011). The human liver is divided into four lobes namely left, right, caudate and quadrate unlike the rats liver with distinct lobes of varying sizes characterised by the right, the medial, the left and caudate (Novak et al., 2015). The liver is actively involved in major functions including synthesis of plasma proteins, blood detoxification and purification, bile production, metabolism of proteins, carbohydrate and fat. The vital role of the liver therefore makes it an important organ which cannot be compensated for when its function is compromised (Saritha et al., 2016). Liver injury may occur from some medicinal agents administered in overdose or even ingested within therapeutic doses. Studies have reported that more than 900 drugs including paracetamol may cause liver damage; other implicated agents of liver damage include some herbs and toxins (Fiedmann, Grendell, Quaid, & Kenneth, 2003).

Paracetamol also known as acetaminophen is a widely used drug because of its analgesic and antipyretic properties. Although the drug is similar to non-steroidal anti-inflammatory drug (NSAID), it does not possess any anti-inflammatory activity. The analgesic activity of paracetamol is essentially by the inhibition of prostaglandin synthesis (Jóźwiak- Bebenista & Nowak, 2014). It is one of the most effective, well tolerated and commonly used over the counter medications, for management of minor to more severe pain (Elkarib, 2014). Administration of paracetamol at the recommended dosage is safe and has minimal side effect
(Ozkaya, Genc, Bek, & Sullu, 2010), however its overdose causes hepatotoxicity and liver injury which accounts for high mortality rate worldwide (Yoon, Choudhary, Kutner, & Pyrsopoulos, 2016). Occasionally, in some individuals a normal dose (500mg-1000mg) can also cause acute liver damage, which can be intensified by alcohol, tobacco, fasting and anorexia nervosa (Malar & Bai, 2012). Other drugs such as phenobarbital, isoniazid, rifampicin, phenytoin and carbamazepin increase paracetamol toxicity (Parameswari, Chetty, & Chandrasekhar, 2012).

A high dose more than 2g/day of paracetamol increases the risk of upper gastrointestinal complications such as stomach bleeding (Malar & Bai, 2012). Administration of paracetamol at a concentration of 300 mg/kg body weight over a period of two weeks induced hepatic damage in experimental rats (Makoshi, Adanyeguh, Nwatu, & Loveth, 2013). Meanwhile, Matić and colleagues reported that a dose of 100 mg/kg per day for 3 consecutive days resulted in increased oxidative stress in rat blood (Matić et al., 2016). Overdose of paracetamol is also associated with inflammation, marked by an increase in the inflammatory cytokines, as well as the upregulation of nitric oxide (NO) from serum, macrophages and hepatocytes (McGill & Jaeschke, 2013). Signs of paracetamol overdose comprise; severe diarrhoea, increased sweating, loss of appetite, nausea and vomiting, stomach cramps or severe pain, swelling, tenderness and pain in the upper abdomen (Koppert et al., 2006). The long-term side effect of administration of paracetamol tablets in children against fever, in their first year increases the prevalence of asthmatic symptoms at 6-7 years (Malar & Bai, 2012). Furthermore, the administration of paracetamol in the first year of life and when children are between the ages of 6-7 years is associated with greater chances of rhino conjunctivitis and eczema (Malar & Bai, 2012).
Paracetamol is first metabolised by the liver and excreted mainly in the urine as glucuronide and sulphate conjugates; with less than 5% being excreted as unmodified Paracetamol. (Canayakin, Bayir, Baygutalp, & Karaoglan, 2016). The metabolism of the drug in the liver is through three pathways: glucuronidation, sulfation, and hepatic cytochrome P450 enzyme system. The cytochrome P450 enzyme is mainly responsible for the poisonous effects of Paracetamol due to reactive metabolite N-acetyl-P-benzo-quinone imine (NAPQI), converting Paracetamol to a toxic metabolite in the liver cells. Glutathione (GSH) then binds to the toxic metabolite and subsequently turns it into a non-hazardous compound. Hepatotoxicity occurs when there is more rapid depletion of glutathione stores than it is regenerated, resulting in accumulation of the toxic metabolite (Lancaster, Hiatt, & Zarrinpar, 2014). Moreover, GSH is necessary to sustain cellular redox balance thereby preventing acute oxidative stress and cellular toxicity (Shanmugam et al., 2016 and Sharoud, 2015). Overdose of paracetamol alters the antioxidant enzyme system and oxidative stress plays an important role in the pathogenesis of paracetamol causing liver injury (Simeonova et al., 2013). The interference of prooxidant-antioxidant balance in tissues increases the levels of reactive oxygen species (ROS) oxidative damage of macromolecules (Nagalekshmi, Menon, Dhanya, & Nair, 2011). This results in several pathological conditions, such as hepatic and renal dysfunction, testicular damage, and cancer in both humans and animals (Hinson, Roberts, & James, 2010).

Several biological compounds with antioxidant properties have been tested to protect against deleterious effect of paracetamol overdose (Wang et al., 2017). Some studies have reported the use of medicinal plants, vitamins, and natural products to protect the cells and tissues from damage caused by reactive oxygen species and free radicals arising from paracetamol overdose (Dadzeasah & Ansah, 2013; Sharma, Makwana, & Rathore, 2008). Current therapeutic practice uses N-acetyl cysteine (NAC) an antioxidant precursor to prevent NAPQI from
binding to hepatic macromolecule during the early phase of paracetamol intoxication (Du, Ramachandran & Jaeschke, 2016).

The beneficial health properties of cocoa have been widely investigated. It is widely accepted that polyphenol compounds in cocoa have antioxidant properties comparable to those found in tea, wine, vegetables, and fruits. Flavanols are antioxidant polyphenols in cocoa including several class of compounds such as epicatechin, catechins and procyanidins (Noori, Nasir, & Mahboob, 2009). In humans, cocoa consumption has been demonstrated to improve antioxidant capacity (Wang et al., 2000). The health benefits of cocoa as well as ability to protect tissues from damage are ascribed to the flavonols which have the potential of scavenging free radicals. Several studies have elucidated the health benefits of cocoa in animal models by demonstrating protection at the tissue level by the ingestion of natural cocoa powder. In the department (Department of Anatomy, College of Health Sciences, University of Ghana, Korle-Bu), several researches on natural cocoa powder have investigated the high antioxidant properties of cocoa. The prolonged intake of dietary cocoa ameliorated haemoglobin glycation associated with pathogenesis of diabetes in rats (Affram et al., 2008). Studies conducted by Aidoo et al., 2012 revealed that ingestion of natural cocoa attenuated liver damage in mice infected with Plasmodium berghei (NK65). Other studies by Toboh, (2009) also indicated that natural cocoa enhanced wound healing in rabbits. Studies have also demonstrated that cocoa ameliorated hepatotoxicity caused by high dose of artemether–lumefantrine in non-malarious guinea pigs (Asiedu-Gyekye et al., 2016). Cocoa flavonols with its powerful antioxidants extenuated hepatic damage in rats following chronic alcohol induced toxicity (Sokpo et al., 2012). The theobromine component of cocoa is known to fight against inflammation, prevent viral ailments and strengthen the immune system (Dillinger et al., 2000 & Addai, 2009). This
study therefore seeks to assess whether natural cocoa reduces liver damage in rats caused by paracetamol ingestion.

1.2 Problem statement

Paracetamol is a widely available antipyretic and analgesic which can be obtained as an over-the-counter drug or as prescribed medication. The World Health Organization has classified paracetamol as a medication for treating all three stages of pain intensity (minor, moderate and severe pains) (Tittarelli, Pellegrini, Scarpellini, & Marinelli, 2017). In Ghana, paracetamol accounts for 52.6% of analgesic prescriptions (Owusu-Ansah, 2009). However, the drug has been listed among medications with the greatest number of mortalities when administered either alone or as a component of a combination therapy (Mowry, Spyker, Brooks, Mcmillan, & Schauben, 2015). Paracetamol has also been reported to be the commonest cause of death due to acute liver failure worldwide. In the United States an overdose of the drug leads to acute liver poison and accounts for about 39% of all cases of acute hepatic injury (Tittarelli et al., 2017). In Africa, although several researchers have extensively reported on the effect of paracetamol overdose, the comparatively weak public health systems have not been effective in addressing adverse reactions of the drug (Dadzeasah & Ansah, 2013).

Presently, the management and treatment options for paracetamol-induced liver damage are limited. Several remedies rely heavily on the use of expensive allopathic agents, notably N-acetyl Cysteine (NAC) whilst others alternatively employ some uncommon medicinal plant extracts. Administration of NAC has common side-effects including nausea, vomiting, and
abdominal pain (Mullins et al., 2004). Moreover, some investigators have reported that the use of NAC in large doses can cause damage to the heart and liver (Palmer et al., 2007). Therefore, it is attractive to search for a safer, affordable, and efficacious substitute of NAC for the treatment of paracetamol-induced hepatic toxicity. For all that is known now, natural cocoa powder offers an efficacious alternative to NAC.

1.3 Justification

Liver injury from overdose of paracetamol resulting in increased mortality rate is alarming worldwide. Nevertheless, due to easy accessibility of paracetamol as an over the counter drug, substantive measures to control and monitor its abuse are lacking. Although generally known for its antipyretic and analgesic properties at therapeutic dose, paracetamol causes an overproduction of NAPQI which leads to the disruption of prooxidant-antioxidant balance in tissues following an overdose administration of the drug (Dröge, 2002). In the liver, excess NAPQI overwhelms the detoxification ability of GSH- a potent antioxidant and thus, increases the reactive oxygen species levels and induces oxidative damage of hepatocytes (Bessems & Vermeulen, 2001). Paracetamol overdose is also associated with marked elevation of pro-inflammatory cytokines which contribute to other pathological conditions including renal injury, testicular damage, respiratory disorders and cancer (Jaeschke et al., 2013; Hinson et al., 2010).

Recent studies have however, reported that paracetamol-mediated oxidative stress or hepatotoxicity could be attenuated by use of naturally-occurring antioxidants and/or free radical scavengers such as vitamins, medicinal plants and natural products (Singh et al., 2011; Jaeschke et al., 2013).
Cocoa flavanols have antioxidant properties which have the inherent ability to scavenge free radicals thereby helping to ease conditions associated with oxidative stress and coincident tissue damage (Cordero-Herrera, Goya, & Ramos, 2015).

Cocoa therefore lends itself as potential alternative antidote without the various side effects of current remedies. In Ghana, cocoa is readily available because it is locally produced. Hence, it is worth investigating whether natural cocoa powder (NCP) ingestion does attenuate liver injury caused by paracetamol intoxication.

1.4 Study hypothesis

Null hypothesis: It is hypothesised that regular ingestion of NCP has no protective effect on liver damage induced by overdose of paracetamol in rats.

1.5 Aim

• To investigate the ameliorative activity of natural cocoa in paracetamol-induced hepatotoxicity in rats.

1.6 Specific objectives

1. To determine histological changes in hepatocytes of rats treated with paracetamol overdose. Tissue damage was assessed by volume composition of perinuclear vacuolations or granulations.

2. To determine the protective effect of natural cocoa powder (NCP) on liver damage caused by paracetamol overdose by comparing percentage of undamaged tissue in animals given the drug overdose with and without NCP ingestion.
3. To determine the biochemical indicators of antioxidant benefit of NCP ingestion in rats given paracetamol overdose. This was done by measuring serum concentration of Superoxide dismutase (SOD) and reduced glutathione (GSH) synthase and compare among treatment groups.

4. To determine the effect of paracetamol overdose on biochemical markers of hepatic parenchymal cell injury by measuring alanine aminotransferase (ALT) and aspartate aminotransferase (AST) in serum of rats in the various experimental groups.

5. To determine relative volume density of hepatocytes and central veins in the various treatment groups as an assessment of altered liver morphology due to paracetamol hepatotoxicity.
CHAPTER TWO

LITERATURE REVIEW

2.1 Anatomy of the liver

The liver is the largest gland in the human body, located in the upper right quadrant of the abdominal cavity. It rests just beneath the diaphragm and is positioned to the right side of the stomach and overlies the gall bladder. The liver is partly divided into lobes and is covered with a thin connective tissue known as the Glisson’s capsule. Grossly, the human liver lobes include the right, left, caudate and quadrate sections with surface peritoneal and ligamentous attachment. The division between right and left lobes of the liver is indicated by the falciform ligament superiorly and inferiorly by the ligamentum venosum (Ellis, 2011). The reddish brown colour of the liver is dependent on the amount of venous blood volume that perfuse the organ (Standring & Gray, 2008). In an adult, the liver constitutes about 2.5 per cent of body mass and serves as site for nutrient storage and bile secretion.

In rats, the liver (Figure 1A) is a firm, smooth and dark red organ located in the cranial abdominal region with half of its volume in the intrathoracic part of the abdominal cavity. The rat liver has a compact appearance and centrally placed with its caudal edges at about the same level on both sides of the abdominal cavity. The caudal edge has the right inferior lobe on the right side and the left lateral lobe by the left side. The diaphragmatic surface is smooth and convex and made up by the medial lobes and the left lateral lobes covered entirely with the peritoneum. In situ, on this surface are the median lobe, the left lateral lobe and the right lobe.
Part of the rat liver is not covered with peritoneum and is found to be directly in contact with the diaphragm. This area is located between the caudate lobe, the caudal vena cava and the two layers of coronary ligament. The visceral surface is deeply concave and related to the stomach, descending duodenum, pancreas, transverse colon and right flexure, spleen, right kidney, and the right suprarenal gland. The right kidney is the only structure that makes a renal imprint on the liver lobe. On the visceral surface, all the four lobes are recognised and completely covered by the peritoneum (Stan, 2018). Further, the rats liver do not have a gall bladder (Martins & Neuhaus, 2007).

Figure 1: A- Gross anatomy of the rat liver and B- Histological section of normal rat liver (Adapted from: Naiko-Ito et al., 2010).

2.1.1 Structural organization of the liver.

The liver is a heterogeneous tissue with the lobule as a functional unit (MacSween et al., 2002). This feature gives the liver a consistent histologic appearance regardless of the angle from which it is sectioned and is therefore referred to as having an isotropic parenchyma (Mart-nez-Sanchis et al., 2018) and a complex, 3-dimensional architecture. Consequently, the lobule and the acinus are considered the functional, operational unit of the liver (Sadri, Jeschke, & Amini-
Nik, 2015). Histologically, hexagonal lobules are oriented around terminal hepatic venules or central veins (Figure 1B). At the edges of the lobule are the portal triads (or portal tracts), containing a branch of the portal vein, a hepatic arteriole, and a bile duct. Blood enters the portal tract via the portal vein and hepatic artery which then enters the hepatic sinusoids and percolates along the cords of parenchymal cells (hepatocytes). The central veins finally receive the blood, thus permitting it to exit the liver via the hepatic vein (Sadri, Jeschke, & Amini- Nik, 2015).

**2.1.2 Lobes of the liver**

Lobes of the human liver have traditionally been designated as left, right, quadrate, and caudate. However, based on the ductal and vascular branching patterns to the left and right sides, the liver can be subdivided into 9 segments (Kogure et al., 1999; MacSween et al., 2002). In rats, all four liver lobes: left, right, median and caudate are further subdivided into at least 2 parts except for the left lobe (Vdoviaková et al., 2016). Comparatively, the hepatic lobes of the rat appear to have similar fundamental portal and hepatic venous systems and segments, with that of the human liver (Kogure et al., 1990). However, there are distinct variations in human vascular systems to or from lobes, compared to rats (Kogure et al., 1999; MacSween et al., 2002). Lobe variations have equally been reported following acetaminophen hepatotoxicity (Heinloth et al., 2004; Irwin et al., 2004), chemical carcinogenesis (Richardson et al., 1986), cirrhosis (Regev et al., 2002) and regeneration (LaBrecque, 1994).

Within the liver lobes, there is a potential incomplete mixing of blood drained from the spleen and gastrointestinal tract leading to variation in delivery of various nutrients, elements and toxins (Thein et al., 2003; Daniel et al., 2004). The portal vein- one of two components of the main vascular systems that supplies blood to the liver, delivers about 70% of blood flow and
40% of oxygen whereas 30% of blood and 60% of oxygen are respectively supplied by the hepatic artery (Burt & Day, 2002). The right and left sides of the liver receive the portal blood drained from the gastric, mesenteric, splenic and pancreatic veins.

2.2 Cells of the liver

2.2.1 The hepatocytes

Although known to comprise at least 15 different cell types, hepatocytes constitute approximately 60% of total liver cells in a healthy liver (Malik et al., 2002); and account for 80% of the human liver tissue by volume. The remaining biologically important cells consist of Kupffer cells, hepatic stellate cells, sinusoidal endothelial cells, and biliary epithelium. Arranged in plates or 1 cell thick, hepatocytes branch and anastomose in a continuum of labyrinth with limiting plates at the capsule and portal regions (MacSween et al., 2002). The hepatocyte has hexagonal surfaces which either adjoin adjacent parenchymal cells, border bile canaliculi, or are exposed to microvilli-covered perisinusoidal space. Rough and smooth endoplasmic reticula constitute about 15% of its volume, and each hepatocyte also contains about 500 peroxisomes and 30 lysosomes which together perform many vital functions (Jones and Spring-Mills, 1983; MaCSween et al., 2002).

The numerous metabolic activities of the liver are generally categorised into synthetic function, detoxification, and bile secretion averaging at about 15 ml/kg/day in humans (Jones and Spring-Mills, 1983). Each of these essential roles becomes strikingly apparent in patients with defective bile drainage, inadequate synthesis of plasma proteins, faulty detoxification as well as in those with liver failure or liver cirrhosis in all of whom there is evidence of the clinical stigmata of liver disease (Stanger, 2015).
2.2.2 Biliary epithelial cells

Apart from hepatocytes, the other parenchymal epithelial cell type which participates in hepatic important functions is the biliary epithelial cells (BECs), also known as cholangiocytes (Stanger, 2015). Although less metabolically active than hepatocytes, BECs have some important metabolic functions including bicarbonate synthesis. The biliary epithelia serve as effective communicators with adjoining cells in the production of mediators involved in response to injury and cell growth. Primarily, BECs act as essential lining for conducting bile flow, modification of canalicular bile and for the concentration of bile in gall bladder (Stanger, 2015).

2.2.3 Sinusoidal endothelial cells

The function of separation of solutes, fluids and particles between blood and space of Disse is carried out by sinusoidal endothelial cells (SECs) which represent approximately 20% of all liver cells; it thus serves as a primary barrier between blood and hepatocytes (Braet and Wisse, 2002; Smedsrod, 2004). Sinusoidal endothelial cells constitute a special endothelium on the basis presence of fenestrae, transfer of particles and molecules by endocytosis, and the lack of a basal lamina (Braet & Wisse, 2002).

As proof of its functional importance, studies have shown that defenestration in sinusoidal endothelial tissue plays a role in hepatoxin-induced cirrhosis. In rabbits for instance, their smaller fenestrae in comparison with rats increase their risk for developing atherosclerosis, as less cholesterol is removed from circulation and consequently accumulates in blood (Braet and Wisse, 2002; Smedsrod, 2004). Fenestrae alterations in SECs therefore play a vital role in conditions such as atherosclerosis, tumour metastases and liver cirrhosis.
2.2.4 Kupffer cells

Stellate cells, endothelial cells, fibroblasts, and Kupffer cells are collectively known as non-parenchymal cells (NPCs) as they are not derived from hepatocytes nor biliary epithelial cells, but instead arise from non-endodermal origins (Scholten et al., 2010; Chu et al., 2011). Kupffer cells derive from circulating monocytes and constitute 15% of liver cells (Macsween et al., 2002; Bykov et al., 2004) and function as the main producers of cytokines for mediating inflammatory processes and communication with other cells. Further, Kupffer cells have phagocytic activity and capable of proliferating locally.

2.3 Functions of the liver

The functional role of the liver relates to digestion, detoxification, fluid and electrolyte balance and haemostasis. However, they are generally divided into synthetic function, bile secretion and detoxification. The vital implication of the liver functions is mostly evident in patients with liver cirrhosis or liver failure, or having inadequate bile drainage, defective detoxification, or deficiencies in synthesis of plasma proteins. Any one or combination of such dysfunctional liver conditions result in clinically diagnosed liver disease. The parenchymal epithelial cell types (hepatocytes and biliary epithelial cells) are responsible for carrying out the functions of the liver (Stanger, 2015).

2.4 Liver injury

The liver is routinely exposed to a vast number of insults contributed by several agents. Liver injuries can be classified based on toxin induced injury, viral hepatitis, biliary obstruction, and metabolic, vasculature and immune-mediated injury (Stanger, 2015)
Toxin induced liver injury include toxins such as alcohol and many commonly used drugs (example paracetamol), viral infection, build-up of fats (steatosis) resulting in non-alcoholic fatty liver disease (NAFLD), fibrosis, irreversible cell death, cancer, and metabolic dysregulation. Excess liver fat is additionally strongly associated with the development of type 2 diabetes mellitus. (Almeda-Valdés, Cuevas-Ramos, & Aguilar-Salinas, 2009). Other phase two mechanisms of pathogenic or toxic liver injuries include; oxidative stress and lipid peroxidation, inflammatory cells, dysfunction of cytochrome P450, and mitochondrial dysfunction (Chun, Tong, Busuttil, & Hiatt, 2009).

2.5 Mechanisms of paracetamol-induced liver injury

Paracetamol has not been considered as a member of the non-steroidal anti-inflammatory drugs (NSAIDs) family in pharmacological textbooks on account of its lack of an anti-inflammatory activity (Jóźwiak- Bebenista & Nowak, 2014). Interestingly, it has been always discussed together with NSAIDs. The mechanism of action of paracetamol is therefore easier to comprehend from the narrative on NSAIDs’ action.

All conventional NSAIDs prevent transformation of arachidonic acid (AA) into prostaglandin H -PGH2. The conversion stage is catalysed by prostaglandin H synthase (PGHS), which is presently referred to as cyclooxygenase (COX) within which isoenzymes COX-1 (PGHS-1) and COX-2 (PGHS-2) occur (Hinz & Brune, 2002). PGHS is a bifunctional enzyme and has two dissimilar enzymatic activities: cyclooxygenase and peroxidase (POX). The transformation of AA to PGH2 involves two reactions: cyclization of AA to unstable 15-hydroxyperoxide (PGG2) with the involvement of a cyclooxygenase constituent and double oxidation in position 9 and 11. Whereas the reduction of PGG2 molecule to its 15-hydroxy analogue, unstable structure of PGH2, takes place due to peroxidase activity of PGHS (POX) (Jóźwiak- Bebenista & Nowak, 2014).
Several studies have reported that paracetamol is able to inhibit COX, provided that the ambient concentration of peroxides is kept low (Bertolini et al., 2006; Boutaud, Aronoff, Richardson, Marnett, & Oates, 2002). Paracetamol is a weak inhibitor of prostaglandin synthesis in broken cell systems, therapeutic concentrations of paracetamol inhibit prostaglandin synthesis in intact cells in vitro when the arachidonic acid level is low. Under these circumstances, prostaglandins are synthesized mainly by COX-2. A study suggested that the effects of paracetamol may be due to selective inhibition of COX-2 dependent pathways that are working at relatively lower rates (Graham, Scott, & Day, 2005). Others studies reported that paracetamol has no affinity for the active site of COXs, but rather blocks their activity by decreasing the conversion of the active oxidized form of the enzymes to an inactive form; which may be attributed to the fact that paracetamol is more effective under reducing conditions of low peroxide concentration (Boutaud et al., 2002; Lucas et al., 2005). Other studies also suggest that the mechanisms of action of paracetamol includes inhibition of nitric oxide generation and hyperalgesia induced by either N-methyl-D-aspartate or substance P (Bujalska, 2004).

2.6 Molecular and genetic aspects of paracetamol metabolism

Although administered for over a century as a treatment option for moderate fever and pain, the exact mechanisms of paracetamol analgesic action are poorly understood and are therefore contrastingly presented in literature and themes of ongoing scientific debate. Its two main widely accepted mechanisms are established on the inhibition of the cyclooxygenases (COX) and the involvement of the serotonergic system (Mallet et al., 2010). Emerging, new targets that have also been named as part of its action via intricate neuronal and metabolic pathways are the T-type calcium-channel Cav3.2 and the vanilloid and endocannabinoid systems (Ayoub et al., 2011).
Current studies support the phenomenon that subsequent to its hepatic deacetylation to p-aminophenol, paracetamol is metabolized by fatty acid amide hydrolase (FAAH) enzyme into an active metabolite- AM404 (N-arachidonoylaminophenol), in the brain (Högestätt et al., 2005; Muramatsu et al., 2016). Incubation of purified FAAH with arachidonic acid and p-aminophenol yields AM404; in FAAH−/− mice or rats pretreated with phenylmethylsulfonyl fluoride (PMSF) – a broad spectrum esterase, amidase and protease inhibitor, the administration of paracetamol does not produce AM404 and consequently fails to provide any analgesic effect (Mallet et al., 2008). Owing to the ubiquitous nature of FAAH, opinions are varied in respect of its peripheral or central involvement in paracetamol action. In providing proof that supra-spinal FAAH is obligatory for presenting the clinical effect of paracetamol, investigators introduced URB597- a FAAH inhibitor which readily traverses the blood-brain barrier, and URB937- a peripherally restricting agent for FAAH in separate animal studies (Clapper et al., 2010; Moreno-Sanz et al., 2011) (Fig 2). It was observed that paracetamol analgesic effect was still maintained following intra-cerebroventricular administration of URB937 but undetectable after URB597 treatment.
Figure 2. Pharmacological strategies to block central and/or peripheral FAAH. (A) Peripheral FAAH was inhibited by a systemic injection of URB597 or URB937, respectively. (B) URB937 was supraspinally injected to specifically inhibit brain FAAH (available at http://dx.doi.org/10.5772/66649).

2.6.1 - Molecular targets of N-arachidonoylphenolamine (AM404)

2.6.1.1 The COX enzyme

In a Nobel Prize-winning research work, the mechanism of action of NSAIDs including aspirin was attributed to the inhibition of prostaglandin (PG)- the local factor primarily involved in pain, inflammation and fever (Vane, 1971). Notwithstanding its similarity in action to NSAIDs, paracetamol was not suggested to be associated with the metabolism of prostaglandins. Currently, however, it is increasingly becoming evident that paracetamol pharmacological effects are basically exerted via the inhibition of prostaglandin synthesis and is mediated ostensibly by its selectivity for COX-2, a member of the COX enzymes. Under precise *in-vitro* conditions of low peroxide concentration of isolated cells, acetaminophen inhibits PG production from arachidonic acid. In many human tissues, the cytosolic phospholipase A₂ is known to hydrolyse phospholipids leading to arachidonic acid generation whereas the
hydrolysis of 2-arachidonoylglycerol by the monoacylglycerol lipase enzyme yields arachidonic acid in brain, lung and liver (Nomura et al., 2011).

Indeed, both COX-1 and COX-2 enzymes are bifunctional; primarily, each possesses a cyclooxygenase activity, and a peroxidase function. However, the apparent preference of paracetamol for the COX-2 isotype has experimentally been proved using low arachidonic acid concentration to show that not only is the COX-1 pathway not activated under such conditions, but is also compartmentalized from COX-2 (Murakami et al., 2000). Investigators demonstrate that at inhibition concentration (IC$_{50}$) values (usually below 10 M) within the known therapeutic plasma concentration limits (Boutaud et al., 2002; Kis et al., 2004; Graham and Scott 2005), COX-2 is essentially involved in the antipyretic and anti-nociceptive actions of paracetamol (Li et al., 2008).

2.6.1.2 The TRPV1 receptor

The application of pharmacological and genetic methods for the inhibition of the transient receptor potential cation channel subfamily V member 1 (TRPV1), also known as the capsaicin receptor, have shown that the TRPV1 is another important contributor to the action of paracetamol (Zygmunt et al., 2000; Kerckhove et al., 2014). In a genetic inactivation experiment involving an intracerebroventricular injection of AM404 in TRPV1$^{-/-}$ mice, Mallet et al. (2010) showed that in addition to abolishing the antinociceptive effects of acetaminophen, the pharmacological impairment of the TRPV1 receptor by capsazepine in rats also results in diminished expression of the analgesic effects of paracetamol. Other investigations carried out by Barrière et al. (2013) in mice in which the loss of antinociceptive action of $p$-aminophenol was observed following capsazepine pre-injection prove that, TRPV1 is a vital effector of paracetamol action and that its precise location of involvement is the lateral ventricle.
Consequently, novel strategies that have been suggested for the alleviation of pain are specifically targeted at TRPV1 activation via pharmacological vectorisation (Mallet et al., 2017). Further, the synthesis of new FAAH substrates and analogues of p-aminophenol or paracetamol has recently been considered potent TRPV1 activators since they form conjugates with arachindonic acid. In order to validate their initial hypothesis, research investigators observed that in both in-vitro and in-vivo conditions, 4-hydroxy-3-methoxybenzylamine (HMBA)- a primary amine analog of p-aminophenol, produces olvanil and arvanil both of which exert FAAH-dependent analgesic effects (Barrière et al., 2013).

2.6.1.3 The Cav3.2 calcium channel

Recently, electrophysiological and behavioral methodologies have been used to provide evidence that brain TRPV1 activation requires Cav3.2 to exert its analgesic action as the first AM404 target (Kerckhove et al., 2014). In human embryonic kidney (HEK) cells that stably expressed the Cav3.2 genetic sequence, electrophysiological recordings obtained following Cav3.2 current induction by depolarization were not affected by capsaicin bath application. However, Cav3.2 electrical current was found to be minimal when HEK cells were transfected with TRPV and subjected to capsaicin application. In other related investigations, the silencing of Cav3.2 gene using oligonucleotide antisense, knockout mice or pharmacological tools or provides strong evidence of the importance of the calcium channel in nociception (Bourinet et al., 2005; Choi et al., 2007; Francois et al., 2013). Arachidonic-related compounds such as 2-arachidonylglycerol and anandamide are also known to interact T-type calcium channels, particularly Cav3.2, and thus mediate its analgesic property (Barbara et al., 2009).
In order to determine whether Cav3.2 participates in the anti-nociceptive effects of paracetamol, investigators introduced TTA-A2—a Cav3.2 blocker, into the cerebral ventricles of mice prior to acetaminophen administration since AM404 is a known arachidonic-related metabolite of paracetamol (Mallet et al., 2017); it was observed that paracetamol analgesic effect was non-existent. However, following an intrathecal injection of TTA-A2 for the investigation of possible spinal involvement of Cav3.2 receptors, there was no alteration in the analgesic property of paracetamol thus, providing the empirical evidence that the antinociceptive effect of paracetamol is Cav3.2-dependent and organ-specific.

2.6.1.4 The Cannabinoid Receptor (CB1)

The relationship between acetaminophen and the cannabinoid receptor CB1 remains poorly understood, as per recent experiments involving rats or mice. It is suggested that the action of CB1 receptor does not depend on AM404 inhibition of cannabinoid re-uptake. This being because the administration of p-aminophenol or paracetamol does not affect the brain content of endocannabinoids namely, anandamide, palmitoylethanolamide or 2-arachidonoylglycerol (Mallet et al., 2010; Barrière et al., 2013). Moreover, paracetamol is not known to bind directly to CB1 receptors (Mallet et al., 2008).

Through the inhibition of anandamide degradation and its re-uptake, AM404 is capable of indirectly activating the CB1 cannabinoid receptor (Beltramo et al., 1997; Giuffrida, Beltramo & Piomelli, 2001; Glaser et al., 2003). In rats pretreated with AM251 (a specific CB1 antagonist) and CB1 knockout mice, the CB1 receptor was shown to be insensitive to paracetamol (Ottani et al., 2006; Mallet et al., 2008). Similar findings from a pain model study conducted by Hama and Sagen (2010) in a neuropathic rat indicate that the additive or
synergistic antinociception of paracetamol with memantine, tramadol or gabapentin is attenuated following pretreatment with AM251.

2.7 Paracetamol metabolism- involvement of the serotonergic system

Studies have pointed out the participation of the serotonergic system in exerting paracetamol action (Tjolsen et al., 1991; Pini et al., 1996). Acetaminophen analgesic effect was reduced subsequent to the absolute depletion of central serotonin [5-hydroxytryptamine (5-HT)] synthesis by p-chlorophenylalanine or following serotonergic bulbospinal pathway lesion caused by 5,6-dihydroxytryptamine or 5,7-dihydroxytryptamine (Dogrul et al., 2011).

Realizing that paracetamol failed to bind serotonin receptors, Pini et al. (1996) and other researchers carried out further investigations into the mobilization of 5-HT neurotransmitter and found out that in a dose-dependent manner, paracetamol increased the tissue concentrations of serotonin in the cortex, hypothalamus, striatum, hippocampus and brainstem (Pelissier et al., 1996; Courade et al., 2001). Later studies indicated that some specific serotonin subtypes namely, 5-HT1A, 5-HT2A, 5HT2C and 5HT7 are involved in the action of acetaminophen (Alloui et al., 2002; Bonnefront et al., 2005; Bonnefront et al., 2007; Liu et al., 2013).

With regards to the 5-HT3 receptor however, recent clinical investigations (Jokela et al., 2010; Tiippana et al., 2013; Ramirez et al., 2015) and animal studies with regards to its involvement in paracetamol action have yielded several conflicting results (Alloui et al., 2002; Libert et al., 2004; Girard et al., 2009; Minville et al., 2011). Some studies showed that the non-specific 5-HT3 receptor antagonist known as tropisetron, blocks the analgesic effect of acetaminophen. Based on the observations that other 5-HT3 antagonists namely, ondansetron and granisetron, or antisense oligodeoxynucleotides targeted at 5-HT3 receptors failed to reverse paracetamol-induced antinociceptive effects (Libert et al., 2004; Högestätt et al., 2005) these reports give
contrasting reasons for the non-involvement of 5-HT3 in the inhibitory effect of tropisetron. There is thus the need to identify the unknown specific spinal receptor since the 5HT-3 does not appear to be mediate spinal tropisetron sensitivity. Overall, the established pathways essential for paracetamol metabolism as a prodrug with significant analgesic effect is pivoted on the final activation of the bulbospinal serotonergic pathways (Fig 3).

![Diagram](image)

**Figure 3.** Proposed sequential mechanisms for the antinociceptive effect of paracetamol. (1) Deacetylation of paracetamol in the liver. (2) FAAH-dependent metabolism of \( p \)-aminophenol into AM404 in the brain. (3) Direct and/or indirect involvement of supra-spinal CB1 receptors by metabolite. (4) Reinforcement of the serotonergic bulbospinal pathways (5) Involvement of spinal pain-suppressing serotonergic receptors (adapted from: Frédérique Koukoff/Inserm from Mallet/UMR 1107/Neuro-Dol Inserm).

**2.8 Pathophysiology of paracetamol toxicity.**

The major toxicity of paracetamol is the result of drug metabolism in both the liver and extra hepatic tissues, with 1% of the drug excreted unchanged in the urine (Gu et al., 2005). In
adults, the therapeutic dosing of paracetamol metabolized is about 50-70% by glucoronidation and 25-35% by sulfation, as well as sulfotranserase (SULT, SULT1A1, 1A3/4 and possibly 1E1 and UGT (UGT1A1 and 1A6) enzymes respectively (McGill & Jaeschke, 2013). These phase II reactions occur mainly in the liver and results in water soluble metabolites which are excreted by the kidney. After the ingestion of therapeutic dose of paracetamol, approximately 5-15% is excreted as mercapturate (Mer) paracetamol or cysteine (Cys) paracetamol conjugates in the urine. After the phase 1 metabolism of paracetamol, the production of the (Mer) and (Cys) conjugates are predominantly mediated by CYP2E1, although other CYP (CYP1A2,2D6 and 3D4) enzymes have been shown to activate paracetamol in vitro (McGill & Jaeschke, 2013).

At the therapeutic dose, about 5% of paracetamol is oxidized by microsomal P-450 enzyme system to N-acetyl-p-benzoquinone imine (NAPQI) reactive metabolite. At the therapeutic dosing, glutathione then reduces NAPQI which is excreted as mercapturic acid which is a benign compound. This leads to the deletion of excess paracetamol sulphate and glutathione stores which shunts more of the paracetamol to CYP-450 mixed function oxidase system, producing more NAPQI reactive metabolites. Although, microsomal enzyme CYP-450 is present in both the liver and the kidney, they differ in each organ.

Ingestion of large doses of paracetamol results in much severe depletion of glutathione (< 30% of the normal) and massive production of the metabolites which results in hepatotoxicity (Ibrahim, Agnihotri, & Agnihotri, 2013) causing large amounts of reactive species unbound. Reactive oxygen species (ROS) production result from metabolic activation of paracetamol and NAPQI then readily reacts with nucleophilic sulfhydryl groups and depletes GSH (Jaeschke, McGill, Williams, & Ramachandran, 2011). NAPQI metabolite can bind to
sulfhydryl groups and hepatic proteins, with the latter spontaneously reacting with glutathione. The binding of the metabolite to the hepatic proteins results in the critical initiating event in cell death during paracetamol induced liver injury and depletion of glutathione. Agents that prevent and scavenge mitochondrial ROS and peroxynitrite are used for paracetamol hepatotoxicity (Du, Ramachandra, & Jaeschke, 2016), however GSH defence against NAPQI led to the use of NAC as an effective antidote for paracetamol hepatotoxicity in clinical practice (McGill & Jaeschke, 2013). Furthermore, research has reported that ingestion of 10-15 g of paracetamol can lead to foetal hepatotoxicity and a single dose ingestion of 150 mg/kg (5-10 g) can also cause severe hepatocellular damage and renal necrosis (Benette & Brown, 2009; Radosavljevic et al., 2010).

2.9 Clinical presentation of paracetamol induced hepatotoxicity

Clinical signs do not show following an acute overdose ingestion of paracetamol within the first 24-48 hours, however liver failure may occur between 2-7 days of ingestion (Rang et al., 2008). The clinical manifestation of paracetamol toxicity is generally divided into 4 phases (Farrell, 2013), as follows.

In the first phase (0-24 hours), the individual appears asymptomatic or may present with features including; anorexia, nausea, vomiting and malaise. Liver function test in this phase indicates a mild increase in serum transaminase level (elevation occurs approximately 12 hours following an acute ingestion). The second phase occurs between 18 and 72 the hours, on which the individual present’s clinical signs including nausea, vomiting, and abdominal pain (right hypochondrium). Upon examination there appears to be tenderness on the right hypochondrium; tachycardia, and hypotension, as well as continuous increase in serum transaminase level. The third phase (72-96 hours) is mainly the critical phase when the patient
becomes severely ill. The symptoms include; jaundice, coagulopathy with tendency of bleeding, hypoglycaemia, hepatic flap, hepatic encephalopathy which arises from hepatic necrosis and dysfunction. Acute renal failure may occur due to hepato-renal syndrome. Ultimately death may occur as a result of multi organ failure. The fourth phase occurs between 3 to 4 weeks which is also the recovery phase, in which individuals who survive the critical illness of phase 3 may likely recover with symptoms as well as resolution of organ failure.

2.10 Management and treatment of paracetamol hepatotoxicity

Prior to treatment a thorough history of all the details of ingestion must be acquired from all individuals who present with acute acetaminophen toxicity. The details must include; all over the counter drug and prescription products, dosages, quantity, illicit substances used and the time course of the overdose. Additionally, laboratory data must include test for liver function, creatinine, and blood urea nitrogen.

The general focus of management of paracetamol toxicity is mainly on the prevention of hepatotoxicity by engaging appropriate line of treatment to limit absorption of the drug and reduce the toxic effect of NAPQI intermediate by replenishing of glutathione stores. Understanding of the general damage caused by paracetamol-induced toxicity suggests that it can only be reversed within the first phase of acute ingestion. Christopherson et al., (2002) reported that the combination treatment of gastric lavage followed by drinking of activated charcoal solution can be replaced by charcoal alone and can resolve the overdose ingestion of paracetamol in patients who arrive within 1-hour post ingestion. N-Acetyl Cysteine (NAC) is the antidote used for specific treatment of paracetamol intoxication and it gives the best hepatoprotective effect when administered within the first 8 hours of exposure to an acute overdose. NAC was listed as an antidote for the treatment of paracetamol overdose by WHO in 2006. NAC serves as a glutathione precursor increasing the amount of GSH (thus decreases
paracetamol toxicity) and subsequently NAPQI induced oxidative stress, whilst increasing the production of GSH (McGill and Jaeschke, 2013) (Fig 4). The administration of NAC reduces the incidence of hepatic necrosis by paracetamol, although it has not established a benefit in preventing nephropathy. Additionally, this supports the theory that there is distinct mechanism of toxicity that exists between the liver and the kidney. This has been evident in analysis which has not yielded any difference in peak creatinine levels between NAC treated patients and those who are not on treatment (Mazer & Perrone, 2008). Administration of NAC is not harmful to the kidney, however its role in patients without hepatotoxicity but isolated renal function is uncertain. This was evidenced in studies showing that the administration of NAC in paracetamol-poisoned mice either orally or intraperitoneally did not protect against nephrotoxicity (Slitt et al., 2004). Therefore the treatment of NAC is solely based on liver necrosis but not on renal dysfunction (Mazer & Perrone, 2008).
Figure 4. Phases of hepatotoxicity after paracetamol overdose. NAC- N-acetylcysteine; NAPQ1-N-acetyl-p-benzoquinone imine; ROS- Reactive oxygen species (adapted from: Du et al., 2016).
2.11 N-acetyl cysteine (NAC)

2.11.1 Background information

N-acetylcysteine (NAC) is a sulphur containing amino acid cysteine metabolite with a molecular formula of HSCH₂CH(NHCOCH₃)CO₂H and a formula weight of 163.19. The administration of NAC can be orally or intravenous infusion and can also be used as an inhaler using a nebulizer. It serves as both an antioxidant and a mucolytic agent (Xu, 2017). Although the therapeutic potential of NAC has been examined, there is ongoing investigation on its efficacy on range of illness including; its antidote for specific toxins, bioprotective agent against oxidative stress and ischemic injury and as a treatment for certain mental and physical illness (Dodd, Dean, Copolov, Malhi, & Berk, 2008).

NAC occasionally serves as a dietary supplement for athletes (Rhodes and Braakhuis, 2017; Dodd et al., 2008). It is currently the antidote for paracetamol overdose and a second line agent for the treatment of acrylonitrile and methacrylonitrile poisoning. Oral administration of NAC is generally tolerated and its use as a nutraceutical increases the demand by patients (Dodd et al., 2008).

2.11.2 NAC as an antioxidant and glutathione precursor

NAC is a free radical scavenger and its major role as a therapeutic antioxidant is due to the precursor of cysteine which is the rate limiting step in glutathione synthesis. The depletion of glutathione due to oxidative stress is reversed by NAC supplementation. Cellular defence mechanism against oxidative stress can include the reduction of hydroperoxides by glutathione peroxidases and conjugations reactions that are catalysed by glutathione S-transferases. Glutathione is vital in maintaining the body’s antioxidant balance. NAC is known for its ability to replenish glutathione stores by forming cysteine and mercapturic acid conjugates to mop up hepatotoxic metabolites in the event of paracetamol toxicity (Heard, 2008).
2.12 The health benefits of natural cocoa

2.12.1 Background information

Cocoa is derived from the bean of cocoa tree (*Theobroma cacao*) and serves as an important agricultural commodity in the production of chocolate and other cocoa products (Hurst et al., 2002). In the crop year 2011/2012, the total population of cocoa beans exceeded 4 million tons worldwide (Krähmer *et. al.*, 2015). The production of cocoa is greatest in West Africa (70%), followed by Asia and Oceania (15.6%) and Latin America (14.1%) (Giacometti, Joli, & Josi, 2015). The world leaders in cocoa production include Ivory Coast, Ghana, Indonesia, Nigeria, Cameroon, Brazil, Ecuador, the Dominican Republic, and Malaysia, contributing 90% of the world production (Fowler, 2009). Cocoa has a significant genetic diversity with over 1400 distinct varieties recognised. (McShea *et al.*, 2008). The four main commercially exploited varieties are Forastero, Criollo, Trinitario, and Nacional (Giacometti *et al.*, 2015). These are distinguished by morphological features of the fruit, geographic origin, and the characteristic flavour (Biehl & Ziegleder 2003a). The Forastero (*Theobroma cacao* L. ssp. *Shaerocarpum* Cuat) has been more voluminously produced and has been cultivated since historic times (Rusconi & Conti 2010; Giacometti *et al.*, 2015).

In West Africa (except Cameroon) the Amelonado which is a subvariety of the Forastero type is extensively cultivated and has a large genetic variability which is used for breeding in the main countries that produce cocoa (Jahurul *et al.*, 2013). The Forastero type exhibits a strong basic chocolate flavour which is categorised as bulk, basic, or ordinary cocoa grade. Bulk cocoa indicate above 90% of cocoa production world production and are used to manufacture cocoa mass, cocoa powder, cocoa butter, and dark chocolate (Fowler, 2009).

Cocoa has attracted increased attention from nutritional and pharmacological viewpoints due to the high level of polyphenols. Research has shown several health benefits of cocoa including
antioxidant capacity, cardiovascular protection, neuroprotective and chemoprotective properties (Andújar et al., 2012; Bernaert, et al., 2012).

2.12.2 Composition of cocoa

Cocoa bean is loaded with high polyphenol compounds constituting about 15% of dry bean weight of cocoa nibs. (Forsyth, 1955; Krahmer et. al., 2015). Cocoa contains 3 major groups of polyphenols: catechins (flavan-3-ols), anthocyanins, and proanthocyanidins. Monomers in total cocoa polyphenol accounts for 5% to 10% and the polymers accounts for 90% (Khan & Nicod 2012).

Catechins (about 29%-38%) contained in total cocoa polyphenol are represented by epicatechin (approximately 35%), catechin, gallocatechin and epigallocatechin. The anthocyanin (about 4% of total polyphenol) is formed by leucoanthocyanins L1, L2, L3 and L4, cyaniding-3-α-Larabinoside and cyanidin- 3- β-D-galactoside. Proanthocyanidins of about 58%-65% of total polyphenol is in cocoa seeds, however the most important proanthocyanidins include; B1, B2, B3, B4, B5, C1 and D (Andújar et al., 2012)

Cocoa also contain flavonols glycosides, quercetin-3- O-arabinoside and quercetin-3-O-glucoside (isoquercitrin), and quercetin aglycone. Research has shown that cocoa also contains quercetin-3-O-galactoside (hyperoside) and quercetin-3-O-glucuronide and the flavones, apigenin, apigenin-8-C-glucoside (vitexin), apigenin-6-C-glucoside (isovitexin), luteolin, and luteolin-7-O-glucoside. (Sánchez-Rabaneda et al., 2003).

Additionally, cocoa also contains minor polyphenols known as flavones (apigenin, luteolin, haempeferol and their glycosides) (Rusconi & Conti, 2010). The amount and composition of
polyphenol greatly depends on factors such as: genotype, origin, the degree of ripeness and bean processing (Kothe et al., 2013).

2.12.3 Antioxidant potential of cocoa

Cocoa beans have a higher antioxidant capacity as compared to red wine or black or green tea. (Jolic et al., 2011). Flavonols in cocoa are noted for their potent antioxidant properties primarily based on their ability to scavenge free radicals, thereby off-setting oxidative stress and consequently liver damage (Cordero-Herrera et al., 2015). Cocoa flavonoids with antioxidant activity include; catechin, epicatechin and procyandinins (Lamuela-Raventós et al., 2005; Crozier et al., 2011). The flavonol quercetin has been shown to prevent hepatic and renal damage caused by paracetamol induced oxidative stress in rats (Yousef, Omar, El-guendi, & Abdelmegid, 2010).

The total polyphenol and flavonoids in cocoa is approximately 611 mg gallic acid equivalents and 564 mg epicatechin equivalents respectively (Johurul et al., 2013). Andújar et al., 2012, in their in vitro experimental studies established that cocoa polyphenols scavenge reactive oxygen species including; 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2’-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid; ABTS), and superoxide radicals, hypochlorite and peroxynitrite anions, inhibit lipid peroxidation, and chelate free pro-oxidant metal ions (Fe2+, Cu+). A chronic supplementation of rats’ diet with 2% cocoa powder was shown to reduce DNA and glutathione oxidation. (Keen et al., 2005).
CHAPTER THREE

MATERIALS AND METHODS

Materials purchased for the experiment comprised; N-Acetyl L- Cystein (NAC) (Vitabay, Germany, VB1281), Diethyl ether Code: 16500 - (Auro Avenida PVT limited India), Superoxide Dismutase Assay kit (Cayman Chemical, item number: 706002, USA), Glutathione Assay Kit (Cayman Chemical, Item number: 703002, USA), Paracetamol (Ayrton Drug, Ghana), Growers mash (GAPFA, Ghana), Good Food® Natural Cocoa Powder (Kakawa Enterprise Limited, Accra, Ghana Batch number KK1802A).

3.1 Pilot study

A four-week study was conducted to determine the dose of paracetamol that could induce liver injury in rats with distinctly observable histological alterations and as well determine the appropriate treatment protocol for the main study. All animals were kept in the Animal Experimentation Unit at the School of Biomedical and Allied Health Sciences (SBAHS), College of Health Sciences, University of Ghana, Korle-Bu campus. Eight rats of ages 12-18 weeks weighing between 230-300 g were used for the study. After a week of acclimatization, the rats were divided into four groups of two (G1, G2, G3 and G4). G1 rats were given 650 mg/kg b.wt paracetamol (Yousef, Omar, El-Guendi, & Abdelmegid, 2010), G2 rats were given 500 mg/kg b.wt, G3 rats were given 350 mg/kg b.wt; and G4 rats were given 200 mg/kg b.wt (Kyong-Shil et al., 2012) at 8.00am each morning via oral gavage.

All eight rats survived at the end of the experimental treatment (4 weeks). At the termination of the experiment the rats were sacrificed by perfusion fixation and their livers were harvested for histological analysis. The results obtained indicated that 350 mg/kg paracetamol dose
caused liver damage with observable morphological alterations under the light microscope compared to the other doses (650 mg/kg, 500 mg/kg and 200 mg/kg body weights).

3.2 Experimental Protocol for Main Study

3.2.1 Study design

The study design was experimental animal study.

3.2.2 Study site

The research was conducted at the Animal Experimentation Unit of the School of Biomedical and Allied Health Sciences, Korle Bu. The unit has a laboratory with ambient temperature of 28 ± 2º C and humidity of 70 ± 2%. The unit has 120 cages (28.7 cm length × 20.3 cm width × 17.3 cm height) each of which can accommodate two rats. The laboratory also has tap water supply, and it is run on an alternating 12-hour period of light and 12-hour period of darkness.

3.2.3 Study population

The study was conducted with twenty (20) male Sprague Dawley rats.

3.2.4 Acquisition and acclimatization of animals

Twenty (20) male Sprague Dawley rats of aged 12-14 weeks old and weighing between 190 – 210 g were purchased from the Centre for Plant Medicine Research (CPMR), Mampong-Akuapem, Ghana. Animals were transported to the Animal Experimentation Unit of the School of Biomedical and Allied Health Sciences Korle Bu, and acclimatised for one week. The conditions of the laboratory were as stated above under study conditions. The rats were fed with nutritionally standardized rat chow (GAPFA, Ghana) and were also given tap water. After
the period of acclimatization, the animals were weighed and grouped into four. The research was approved by the Ethical and Protocol Review Committee of the University of Ghana College of Health Sciences (Protocol identification number: CHS-Et/M.6-5.10/2018-2019). All procedures involving animals conformed to the institutional guidelines, and in accordance with the National and International regulations and guidelines for the use of animals in biomedical research.

### 3.2.5 Preparation of 2% (w/v) unsweetened natural cocoa suspension

Natural Cocoa Powder (GoodFood® brand, Ghana) was prepared at a concentration of 2% w/v of natural cocoa powder. The suspension was freshly prepared daily by weighing 4g of cocoa powder using a chemical weighing balance (Mettler Toledo P1200, Switzerland) and dissolved in 200 ml of freshly boiled tap water in a beaker; the cocoa was stirred until it began to froth, thus ensuring thorough suspension. The cocoa suspension was left to cool and poured into a graduated feeding bottle which was placed at an accessible point of the rat cage (Fig. 5). The cocoa suspension bottle was shaken every 3 hours to prevent sedimentation of particles; this was done to avoid blockage of the inverted bottle teat which would otherwise have made the suspension inaccessible to the rats. Only rats in group two (were given cocoa after paracetamol administration.
3.2.6 Preparation of paracetamol dose

Paracetamol tablets (250000mg) were crushed in a crucible into fine powder using a pestle. 350mg/kg b.wt that was able to cause histomorphological change in the liver obtained from the pilot study was used as a standard dose for administration. However, depending on the body weight of the animal the right dose was measured and given based on the standard dose and weighed in an electronic chemical balance (Lab-kits Analytical Balance, WB12021432). The amount of paracetamol powder calculated was then suspended in 1.0 ml distilled water and administered daily to rats in Groups 1, 2 and 3 (G1, G2 and G3) via oral gavage for four weeks.

3.2.7 Preparation of NAC dose

NAC capsule (72000 mg) was opened and poured into a sample container. 140 mg/kg b.w of the drug was used as a standard dose based on research by Canayakin et al (2016). Therefore,
per the animal’s body weight the appropriate dose was measured based on the standard dose. The dose was then weighed in a weighing balance and dissolved in 1.0ml distilled water and administered to rats in G3 group three (3) hours after paracetamol administration daily via oral gavage for four weeks. The timing between the paracetamol and NAC administration was congruent with work by Içer et al (2015).

3.2.8 Animal grouping and administration of treatments

After one week of acclimatization, the rats were put into four (4) groups of five (5), ensuring that their mean body weights were not significantly different. Each group of rats were kept in a single cage that was adequately spacious. After grouping the animals on the first day of the experiment, their weights were recorded as baseline for each group.

Group 1 (G1) rats were given 350 mg/kg b.wt paracetamol only via oral gavage, and had 24-hour access to 500 ml of filtered tap water. Group 2 (G2) rats were given 350 mg/kg b.wt dose of paracetamol, unrestricted access to 2% (w/v) natural cocoa suspension for 12 hours from (7:00 am- 7:00 pm) and 500 ml of filtered tap water for 12 hours (7:00 pm – 7:00 am). Rats in Group 3 (G3) were given 350 mg/kg b.wt dose of paracetamol, followed by 140 mg/kg bw of NAC three (3) hours later (Içer et al., 2015) in addition to 24-hour access to 500 ml of clean water. Group 4 (G4) rats were the control, and were not given paracetamol, cocoa, or NAC but were given 1.0ml of distilled water via oral gavage daily. Rats in G4 also had free access to 500 ml filtered tap water for 24 hours. All animals had unrestricted 24 hour access to rat chow during the experiment. The daily amounts of water and cocoa consumed were measured and recorded. The experimental treatment lasted for four (4) weeks during which the rats were weighed weekly (Appendix V).
3.3 Blood sampling for biochemical analysis

Blood samples were collected from each rat at start and termination of experiment via tail snipping and milking. The animals were anaesthetised by placing them in a glass chamber with a cotton dubbed with adequate amount of diethyl ether. The rats were properly anaesthetized by showing no movement in their limbs and not responding to pin prick. The tail was then snipped and allowed to hang to allow blood flow into a serum separating gel tube and EDTA tubes. Blood in the serum separating gel was allowed to stand at room temperature to speed up clotting and serum separation. The samples were appropriately coded and centrifuged at 3,500 rpm for 7 minutes using high speed refrigerated centrifuge (TGL-16MC), at the Medical Biochemistry department, SBAHS, CHS, Korle-Bu. The serum obtained was then transferred into 2 ml Eppendorf tube with a pipette and stored in a -20°C freezer. The sera were used later for Superoxide Dismutase and reduced Glutathione assays for assessing oxidative stress, AST and ALT liver enzymes and haematological parameters. The blood sample collected after the four-week experiment in the EDTA sample tube were appropriately identified with labels and put on a roller to ensure evenly distribution of the cells in the sample tube and immediately analysed with a haematological analyser (ABX Micros-ES16, HORIBA).

3.4 Antioxidant assays

3.4.1 Reduced Glutathione (GSH) and Superoxide Dismutase (SOD)

The assays were prepared according to manufacturer’s protocols per the Glutathione Assay Kit and Superoxide Dismutase kit from Cayman Chemical, USA. The assays were performed at Noguchi Memorial Institute for Medical Research (NMIMR). A Spectrophotometer was used to read the end point absorbance (optical densities) at 405 nm after twenty-five (25) minutes of incubation. Optical densities measured were then converted to GSH and SOD concentrations.
by the formula provided by the Kit’s protocol. A Graphpad Prism statistical software (version 5) was used to analyse the final GSH and SOD values.

3.5 Determination of AST and ALT liver enzymes

Blood samples collected into tubes without an anticoagulant were used for the liver enzyme tests. After clotting, the serum was separated by centrifugation and stored at -80°C until assay. Using a Cobas 8000 autoanalyzer (Roche Diagnostic GmbH, Mannheim, Germany) with commercially available kits (Roche Diagnostics), serum aspartate aminotransferase (AST) and alanine transaminase (ALT) activities were measured spectrophotometrically.

3.6 Haematological analysis

Blood samples collected into tubes with anticoagulant (EDTA) were immediately analysed for haematological parameters using an automatic haematological assay analyser. The parameters determined were White blood cells (WBC), red blood cells (RBC), haemoglobin (HB), haematocrit (HCT) percentage, mean corpuscular volume (MCV), mean corpuscular haemoglobin concentration (MCHC), platelets (PLT) and mean cell haemoglobin (MCH).

3.7 Assessment of variables

3.7.1 Weight

The body weight of the experimental rats was measured weekly. A weighing scale was used to determine the weights of the rats. The rats laid in the middle of the scale to ensure no movement before recording the weight. The weighing scale was tared prior to weight measurement.
3.7.2 Determination of fluid consumed by rats

The volume of NCP suspension and water consumed by each animal was measured daily. The volume of the NCP suspension consumed (NCPs) in 12 hours was calculated by subtracting the volume left (NCPf) in the graduated drinking bottles at the end of the 12 hours from the initial volume administered (NCPi). The volume of water consumed (Wc) was also calculated by subtracting the volume of water remaining (Wf) in the bottles from the initial volume of water administered (Wi). A summary of the calculation is provided below:

Volume of NCP consumed

\[ \text{NCPs (12hours)} = \text{NCPi} - \text{NCPf} \]

Volume of Water consumed

\[ \text{Wc (12 hours)} = \text{Wi} - \text{Wf} \]

The total volume of fluid intake within 24 hours per each group was recorded and computed by adding the volume of NCP suspension consumed to the volume of water consumed as;

Total fluid intake per day = NCPs + Wc
Figure 6. Diagram summarizing the daily treatment of rats in this study.
3.8 Harvesting of tissues and histological procedures

3.8.1 Harvesting of tissues

At the termination of the experiment at week 4, rats were transported from the animal experimentation unit to the histology laboratory at Anatomy Department, SBAHS, where they were perfusion-fixed after being euthanized. A standard perfusion set up was mounted and a desiccator without a desiccant was improvised as a rat gas chamber. The rats from each group was anesthetised with diethyl ether (BDH Chemicals Limited, Poole, England) one at a time. The diethyl ether was soaked in cotton wool and placed in the desiccator in which a rat was also placed, and covered. After a rat ceased movement, its total state of anaesthesia was determined by lack of response to a lancet prick on the foot. The animal was quickly removed and pinned unto a dissecting board in a supine position (Fig 7). The furs on the thorax and abdominal wall were rubbed with 70% ethanol. Incisions were then made at the anterior thoracic wall and extended to the abdominal cavity. The rats were dissected to expose the thoracic viscera.

The cardiovascular system of the rat was washed with 0.9% of normal saline running under constant flux of gravity. This was done by the use of a hypodermic needle of 21Gx1 inserted into the left ventricle of the rat. The right atrium was then nicked with a pair of scissors to allow an exit for the blood and normal saline. Adequate clearing was established by visual examination of the liver paleness. After clearing, and fixing with formalin, the liver was dissected out and weighed on a balance.

The four lobes (right, left, median and caudate lobes) of the harvested liver were dissected and stored in differently labelled sample containers with 10% buffered formalin (pH 7.4) for 7 days before further processing.
Figure 6. Picture of a rat being perfused. A = dissection board, B = a rat, (C and G) = a pair of scissors, D = surgical blade in a holder, E = forceps for securing hypodermic needle in position, F = fluid delivery tube, H = toothed forceps, I = hypodermic needle in heart.

3.8.2 Slicing and processing of liver tissue

The right and left rat liver lobes were cut into three longitudinal slices using a scalpel blade and one slice was randomly selected for histological processing. The middle and caudate lobes were each cut into two longitudinal slices and one was randomly selected for processing. Therefore, each rat in a group had four liver slices which were processed separately. The liver slices were further fixed in 10% buffered formalin at a pH of 7.26. The tissues were processed by routine histological protocol. In brief, they were dehydrated through graded ethanol (70% to absolute) and cleared with xylene. The tissues were infiltrated with molten wax and then embedded to form blocks for sectioning with a microtome.
3.8.3 Sectioning of tissues

Each tissue block was trimmed at a thickness of 10 micrometres (μm) to expose the whole profile of the tissue using Leica microtome (Leica RM 2125, Germany). The liver tissues were then sectioned at 5 μm, with three sections systematically selected from each of the four blocks (per rat) for Haematoxylin and Eosin (H&E) staining. The three sections were selected at every 10th, 50th and 100th section. A total of twelve (12) liver sections were obtained for each rat, but these were mounted on six (6) glass slides (76 x 26 mm x1mm). The left and right liver lobes were placed on the same glass slide and the middle and caudate were also placed on the same glass slide. Photomicrographs were then taken from the 6 slides for stereological studies.

3.9 Stereological assessment

3.9.1 Sampling of photomicrographs of liver sections.

A bright field binocular light microscope (Leica Galen III, catalogue no. 317506, serial no ZG6JA4) was used to examine the liver slides with x40 and x10 objective lens. The microscope field was directed to one end of the slide and an eyepiece lens of the microscope was removed and replaced with a digital microscope eyepiece (Lenovo Q350 USB PC Camera) connected to a desktop computer (HP Compaq dx2300 Microtower).

Four graduations x four graduations of the Leica Galen III microscope stage unit intervals on the X and Y axes respectively. The microscope stage was moved from one plane on the X-axis to a different plane on the Y-axis. Photographs of fields of view under the microscope as determined by the X and Y axis movements were captured using X40 digital eye piece (hepatocytes) and X10 digital eyepiece (central vein) (Lenovo Q350 USB PC Camera) attached to a computer (HP Compaq dx 2300 Microtower). The digital eyepiece replaced ocular lens.
on a Leica Galen III Binocular light microscope (catalogue no. 317506, serial no. 1207XU). This procedure was done until the whole area of each section was covered.

![Photograph showing microscope stage graticule superimposed on stereological lattice for calibration using adobe Photoshop. One graticule unit = 0.01 mm.](image)

**Figure 7.** Photograph showing microscope stage graticule superimposed on stereological lattice for calibration using adobe Photoshop. One graticule unit = 0.01 mm.

### 3.9.2 Morphometric analysis of liver sections

A design based stereological method was employed to measure relative volume density and thickness of the various structural variables of the liver tissue.

The relative volume density of the damaged hepatocytes, undamaged hepatocytes and the central veins were determined using Cavalieri’s principle with point counting. A stereological grid (figure 8) of dimension 1cm × 1cm in Adobe Photoshop CS6 Extended (trial version 13.0.1) software was superimposed on each photomicrograph of the liver tissue sampled as described above. Desired parameters, namely, volume density of undamaged hepatocytes,
damaged hepatocytes and the central vein on the micrograph were counted at the point of intersection of the grid lines. Values from the point counting were entered into the formula (cavalieri estimator of volume) below for calculation of relative volume density.

\[ V_v = \frac{\Sigma P \times (\frac{a}{p}) \times t}{M^2} \]

Where \( V_v \) indicates relative volume density, \( \Sigma P \) is the sum of all test points encountered, \( (a/p) \) is the area per point of the stereological grid, \( t \) is the thickness of the section and \( M \) is the linear magnification.

3.10 Statistical analysis

Graph pad Prism version 5.0 was used for the statistical analysis of all data. All data was tested for normal distribution using Shapiro-Wilk normality test. Data sets with p-value less than 0.05 was concluded to be not normally distributed. Data were presented as mean and standard deviation (SD), as well as 95% confidence interval for means was used. One way analysis of variance (ANOVA) and Paired T-test was used to compare the means between and within the various groups. P-value < 0.05 was considered statistically significant.
CHAPTER FOUR

RESULTS

4.1 Assessed experimental variables

4.1.1 Rat body weight

Table 1 and Figure 9 represent one-way analysis of variance (ANOVA) for mean weight between the groups for the four-week experimental period. The baseline mean weight (prior to treatment) of rats per group at week one were 210 (SD 18.7) g, 194.0 (SD 15.1) g, 222.0 (SD 20.5) g, 202.0 (SD 16.4) g for the G1, G2, G3 and G4 respectively (Table 1). One-way ANOVA between the groups at week one indicated no significant difference (p=0.1222) between the mean weight of rats. At week two the mean weight of the rats in G1, G2, and G3 compared to G4 was not statistically different (p=0.4845). In week 3, ANOVA yielded significance at p=0.0231 for mean weight of rats in G1, G2, and G3 compared to G4; but Tukey’s post hoc analysis indicated that only G3 rats had a significant mean weight difference (p < 0.05). Table 1 shows that there was a significant ANOVA difference among rats at week 4 (p=0.009), with post-hoc comparison indicating significant increases in the mean weights of rats in G2 and G3 (p<0.05); whilst weight reduction in G1 rats was not significant in comparison with G4 rats.

One-way ANOVA within the groups recorded no significant difference for mean weight in G1 rats even though the weights of animals reduced in the course of the experimental duration (Table 1). Time course analysis (Fig. 9) shows that, even though there were changes in the mean weights of rats in G3 and G4 the variations were not statistically significant when compared to the baseline weight in week one. As shown in Table 1, significant increases were recorded in the mean weights of rats in G2 at week 3 and week 4 when compared to week one weights (p<0.01 and p<0.001 respectively).
### Table 1: Weekly mean weight (grams) of rats during the 4 weeks experimental period

<table>
<thead>
<tr>
<th>Weeks</th>
<th>Paracetamol (G1)</th>
<th>Paracetamol &amp; Cocoa (G2)</th>
<th>Paracetamol &amp; NAC (G3)</th>
<th>Control (G4)</th>
<th>P-value 1</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean rat body weight in grams</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.</td>
<td>210.0 (18.7)</td>
<td>194.0 (15.1)</td>
<td>222.0 (20.5)</td>
<td>202.0 (16.4)</td>
<td>0.1222</td>
</tr>
<tr>
<td>2.</td>
<td>202.0 (10.8)</td>
<td>202.0 (4.0)</td>
<td>212.6 (25.4)</td>
<td>218.0 (24.8)</td>
<td>0.4845</td>
</tr>
<tr>
<td>3.</td>
<td>209.4 (17.8)</td>
<td>226.6 (14.5)(^b)</td>
<td>230.0 (12.5)(^*)</td>
<td>203.8 (12.8)</td>
<td>0.0231</td>
</tr>
<tr>
<td>4.</td>
<td>196.2 (13.2)</td>
<td>249.5 (13.6)(^c)</td>
<td>246.0 (32.9)(^*)</td>
<td>208.4 (17.8)</td>
<td>0.009</td>
</tr>
</tbody>
</table>

P-value 2  
0.4513    <0.0001  0.1985  0.4620

Values are expressed as mean (SD). P-value 1 represents significance level for one-way ANOVA comparison (followed by Tukey’s post hoc test) of the four groups of rats studied with *=P<0.05 indicating level of significance from Control (G4) value. P value 2 indicate significance level for one-way ANOVA comparison (followed by Tukey’s post hoc test) of weekly values for each group of rats with alphabets indicating significant difference from week 1. c=P<0.001, b=P<0.01 and a=P<0.05.

![Graph showing weight changes over weeks](image_url)
Figure 8. Line plot of weekly weight of rats during the experiment. Each point represents Mean weight of rats in the group for the specific week, error bars are S.E.M. Probability values are for Tukey’s post hoc comparisons with respective controls (G4). *P < 0.05, **P < 0.01, ***P < 0.001. G1 is Paracetamol group; G2 is the Paracetamol and Cocoa group; G3 is the Paracetamol & NAC group and G4 is the control group (without paracetamol, cocoa or NAC).

4.1.2 Fluid consumption

4.1.2.1 Water and cocoa consumption

Fluid consumption by the experimental rats summarized in Table 2 and graphically in Figure 10. Time course analysis as revealed by the line graph in (Fig. 10) shows a statistically significant increase in fluid consumption within the groups during the 4 weeks experimental period. A significant variation was observed for all groups (p < 0.0001). The control group (G4) (Fig. 10) recorded significant increase in fluid consumption from week two to four when compared to the baseline fluid consumed at week one by one-way ANOVA. A similar trend was also observed in G3 rats with significant increase in fluid intake from week 2 at p < 0.001. G1 and G2 rats recorded a significant increase in fluid consumption from week 2 to week 4. Overall, there was a significant reduction in the fluid intake by G1 rats in week three compared to G4 but no such significant difference was observed in fluid consumed between week 1 and week 3. One-way ANOVA (Table 2) indicated a significant difference in the fluid consumption of G3 rats during the four-week treatment duration. In comparison with G4 (control group) fluid intake in all groups differed significantly within each week. At the end of week one, the mean fluid intake recorded for the groups were 218.5 ml, 310.0 ml, 244.3 ml and 257.1 ml for G1, G2, G3 and G4 respectively (Table 2). The table (2) also shows that fluid intake by G2 rats was significantly higher when compared to the G4 group at week 2; no significant difference
was observed in the fluid consumption in G1 and G3. At week 3, fluid consumption significantly decreased \((p=0.001)\) in the G1 group. The increase observed in the mean fluid consumption between G2 and G3 was not statistically significant. Similarly at week 4, no significant increase in fluid intake was recorded for G2 and G3 when compared to G4 by one way ANOVA; the increase in fluid intake group in (G1) was however statistically increased \((p<0.01)\).

### Table 2. Weekly mean fluid intake in millilitres

<table>
<thead>
<tr>
<th>WEEKS</th>
<th>Paracetamol G1</th>
<th>Paracetamol + NCP (G2)</th>
<th>Paracetamol +NAC (G3)</th>
<th>Control (G4)</th>
<th>P-value 1</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>218.5 (17.7)</td>
<td>310.0 (44.7)</td>
<td>244.3 (18.1)</td>
<td>257.1 (13.8)</td>
<td>0.1553</td>
</tr>
<tr>
<td>2</td>
<td>330.0 (23.0)c</td>
<td>374.3 (15.1)**b</td>
<td>360.0 (52.5)c</td>
<td>347.2 (41.1)c</td>
<td>(&lt;0.0001)</td>
</tr>
<tr>
<td>3</td>
<td>352.9 (28.1)**c</td>
<td>485.7 (28.7)c</td>
<td>488.5 (21.9)c</td>
<td>457.2 (26.9)c</td>
<td>(&lt;0.0001)</td>
</tr>
<tr>
<td>4</td>
<td>451.5 (40.1)**c</td>
<td>501.4 (21.1)c</td>
<td>517.1 (14.9)c</td>
<td>512.9 (22.1)c</td>
<td>0.0003</td>
</tr>
</tbody>
</table>

| P-value 2 | \(<0.0001\) | \(<0.0001\) | \(<0.0001\) | \(<0.0001\) |

Values are expressed as mean (SD). P value (1) represent significance level for one-way ANOVA (followed by Tukey’s post hoc) for between group comparison with *=P<0.05, **=P<0.01 and ***=P<0.001. P value 2 indicate significance level for one-way ANOVA (followed by Tukey’s post hoc) for time course assessment within the groups with Alphabets indicate significant difference from week 1. c=P<0.001, b=P<0.01 and a=P<0.05. G1 is Paracetamol only group; G2 is the Paracetamol and Cocoa group; G3 is the Paracetamol and NAC group and G4 is the control group (without treatment).
**Figure 9.** Line plot of weekly volume of fluid consumed by groups of five rats during the experiment. Each point represents Mean volume of fluid consumed by the group for the specific week. Probability values are for Tukey’s post hoc comparisons with respective controls (G4). *P < 0.05, **P < 0.01, ***P < 0.001. G1 represent Paracetamol only group; G2 is the Paracetamol and Cocoa group; G3 is the Paracetamol and NAC group and G4 is the control group (without treatment).

**4.2 Post Treatment Variables Assessed**

**4.2.1 Liver weight**

The average gross liver weight in the four groups of rats studied is presented in Figure 11. A one way ANOVA comparison produced no significant differences in the mean liver weights of the rats in the groups when compared to the control group (G4). P-value recorded was 0.495
4.2.2 Biochemical makers of liver function

4.2.2.1 Serum AST and ALT levels

Biochemical analysis for liver damage was determined by the concentration of liver enzymes AST and ALT before and after the treatment. Time course assessment presented as the bar graph (Figure 12) by paired t-test analysis revealed no significant difference in the AST and ALT levels after the experiment when compared to the baseline AST and ALT levels within each group in G2, G3 and G4 nonetheless, a significant increase was observed in both enzymes after the experiment in the G1 rats when compared to the baseline enzyme levels (Table 3). In G1 rats AST increased significantly at $P<0.00047$ whiles ALT increased significantly at $P=0.001$ after the treatment.

One-way ANOVA for mean AST and ALT levels indicated no significant differences ($P=0.9447$ and $P=0.9695$ respectively for AST and ALT) between the groups before the commencement of the treatment. However, AST and ALT levels in G1 were significantly

![Figure 10. Bar chart of rat liver weight measured at the end of the experiment. Each bar is mean weight in grams with error bars as SD. G1 Paracetamol only group; G2 is the Paracetamol and Cocoa; G3 is the Paracetamol and NAC and G4 is the control group (without treatment).]
higher when compared to that of G4 after experiment. P-values recorded for AST and ALT enzymes were less than 0.001 and 0.05 respectively. However no such significant differences were observed in the enzyme levels in the cocoa fed (G2) and NAC-treated (G3) when compared to the control group (G4) after the treatment.

**Table 3.** Synopsis of Statistical Analysis of mean serum AST/ALT in (IU/L)

<table>
<thead>
<tr>
<th></th>
<th>G1</th>
<th>G2</th>
<th>G3</th>
<th>G4</th>
<th>P-value 1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pre-treatment AST</td>
<td>122.9</td>
<td>123.62</td>
<td>124.31</td>
<td>120.00</td>
<td>0.9447</td>
</tr>
<tr>
<td>Post-treatment AST</td>
<td>416.37</td>
<td>157.12</td>
<td>170.50</td>
<td>154.22</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>P value 2</td>
<td>0.00047</td>
<td>0.0553</td>
<td>0.0513</td>
<td>0.1240</td>
<td></td>
</tr>
<tr>
<td>Pre-treatment ALT</td>
<td>92.37</td>
<td>90.56</td>
<td>91.00</td>
<td>93.77</td>
<td>0.9695</td>
</tr>
<tr>
<td>Post-treatment ALT</td>
<td>160.31</td>
<td>108.12</td>
<td>92.50</td>
<td>114.00</td>
<td>0.0077</td>
</tr>
<tr>
<td>P-value 2</td>
<td>0.001</td>
<td>0.1236</td>
<td>0.4551</td>
<td>0.1241</td>
<td></td>
</tr>
</tbody>
</table>

Values are expressed as mean (SD). P value 1 represents significance level for one-way ANOVA (followed by Tukey’s post hoc) for between group comparison. P value 2 indicates significance level for Paired T-Test for within group data comparisons. G1 Paracetamol only group; G2 is the Paracetamol and Cocoa group; G3 is the Paracetamol and NAC group, and G4 is the control group (without treatment).
Figure 11. Bar chart of serum AST and ALT concentrations before (Pre-) and after (Post-) treatment in the four rat groups studied. Bars are mean values and error bars are SD. G1 Paracetamol only group; G2 is the Paracetamol and Cocoa; G3 is the Paracetamol and NAC group, and G4 is the control group (without treatment).

4.2.2.2 Serum SOD levels

One way ANOVA comparison of mean serum SOD activity presented in Table 4 and Figure 13, indicated that there was no significant difference between groups before the experiment. However, the post-treatment SOD values between the groups revealed a significant difference (p<0.003). Post hoc test revealed significant reduction (p<0.05) in serum SOD activity in G1 rats when compared with the control group (G4). G2 rats also showed a significant increase (P<0.05) in SOD activity when compared to G4. SOD activity within G1 rats was very low as revealed by the bar graph (Figure 13) after the experiment. From the graph no significant difference was observed in the SOD activity for groups 3 and 4. Also, there was no significant increase in the mean SOD activity of G2 rats after the experiment when compared to the pre-
treatment SOD activity. However, a significant reduction (p=0.049) in the mean SOD concentration was observed in G1. The P values recorded for paired T-test for activity of SOD were 0.6580, 0.1548 and 0.9856 for G2, G3 and G4 respectively. (Table 4).

Table 4. Serum Superoxide dismutase (SOD) with summarized statistical analyses (U/ml.)

<table>
<thead>
<tr>
<th></th>
<th>G1</th>
<th>G2</th>
<th>G3</th>
<th>G4</th>
<th>P-value 1</th>
<th>P-value 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>PRE TREATMENT</td>
<td>0.256 (0.18)</td>
<td>0.250 (0.15)</td>
<td>0.318 (0.12)</td>
<td>0.286 (0.08)</td>
<td>0.9105</td>
<td>0.049</td>
</tr>
<tr>
<td>POST TREATMENT</td>
<td>0.018 (0.01)*</td>
<td>0.399 (0.17) *</td>
<td>0.195 (0.14)</td>
<td>0.288 (0.16)</td>
<td>0.003</td>
<td>0.055</td>
</tr>
</tbody>
</table>

Values are expressed as mean (SD). P value 1 represents significance level for one-way ANOVA (followed by Tukey’s post hoc test) for between group comparison with *P < 0.05, **P < 0.01, ***P < 0.001. P value 2 indicates significance level for Paired T-Test for within group comparisons. G1 Paracetamol only group; G2 is Paracetamol and Cocoa group; G3 is Paracetamol and NAC group, and G4 is the control group (without treatment).
Figure 12. Bar chart of serum SOD activity in studied rat groups before and after treatment. Each column represents mean value with SD as error bars. G1 Paracetamol only group; G2 is Paracetamol and Cocoa group; G3 is Paracetamol and NAC group, and G4 is the control group (without treatment).

4.2.2.3 Serum GSH levels

Results of serum GSH assays in this study are presented in Figure 14, and Table 5 with a summary of statistical comparisons performed on them. One-way ANOVA showed that before treatment, the activity of GSH was not significant (p=0.3599) among the four experimental groups. However, after treatment GSH activity was significantly increased (p<0.0001) among the four experimental groups. Unpaired t-test showed that post-treatment GSH activities in G1 (2.431, SD 0.30 uM) was significantly reduced when compared with G4 (5.995, SD 0.46 uM).
From the graph (Figure 14), both G2 and G3 showed significant increases in GSH activity after four weeks of treatment.

**Table 5.** Serum reduced Glutathione (GSH) concentration in uM and summarized statistical analyses.

<table>
<thead>
<tr>
<th></th>
<th>G1</th>
<th>G2</th>
<th>G3</th>
<th>G4</th>
<th>P value 1</th>
</tr>
</thead>
<tbody>
<tr>
<td>PRE TREATMENT</td>
<td>6.185 (0.32)</td>
<td>6.293 (0.27)</td>
<td>6.233 (0.22)</td>
<td>5.793 (0.64)</td>
<td>0.3599</td>
</tr>
<tr>
<td>POST TREATMENT</td>
<td>2.431 (0.30) ***</td>
<td>8.676 (0.69) **</td>
<td>8.255 (1.18) *</td>
<td>5.995 (0.46)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>P Value 2</td>
<td>0.003</td>
<td>0.0032</td>
<td>0.0339</td>
<td>0.6832</td>
<td></td>
</tr>
</tbody>
</table>

Values are expressed as mean (SD). P value 1 represents significance level for one-way ANOVA (followed by Tukey’s post hoc test) for between group comparison with *P < 0.05, **P < 0.01, ***P < 0.001. P value 2 indicates significance level for Paired T-Test for time course assessment within the groups. G1 Paracetamol only group; G2 Paracetamol and Cocoa group; G3 Paracetamol and NAC group, and G4 control group (without treatment).
Figure 13. Bar chart of serum GSH concentrations before and after treatment in the four rat groups studied. Columns represent mean serum GSH concentrations and error bars are SD. G1 Paracetamol only group; G2 is Paracetamol and Cocoa group; G3 is Paracetamol and NAC, and G4 is the control group (without treatment).
4.2.2.4 Haematological assessment

Haematological parameters; White blood cells (WBC), red blood cells (RBC) haemoglobin (HB), haematocrit (HCT) percentage, mean corpuscular volume (MCV), mean corpuscular haemoglobin concentration (MCHC), platelets (PLT) and mean cell haemoglobin (MCH), assessed are presented in Table 6, which also shows summarized statistical comparisons. Data were analysed by one-way ANOVA followed by Tukey’s post hoc test. There was a significant difference in the WBC values among the groups, but only G1 group had significant increase when compared to G4 at 99.9 confidence level (Table 6). However, no significant differences were obtained in the G2 and G3 when compared to G4 group. There was also a significant \( p<0.01 \) reduction in the mean MCV in G1 when compared to G4, however, G2 group also recorded a significant elevation in the mean MCV at a confidence level of 95%. The mean MCV in G3 did not differ significantly from that of G4. No significant differences were obtained in RBC, HGB, HCT, MCHC, PLT and MCH in the blood of rats after the treatment period.
**Table 6.** Haematological parameters

<table>
<thead>
<tr>
<th>Parameters</th>
<th>G1</th>
<th>G2</th>
<th>G3</th>
<th>G4</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>WBC (10^3/mm³)</td>
<td>34.98 (9.7)****</td>
<td>9.52 (11.2)</td>
<td>2.35 (0.4)</td>
<td>0.900 (0.2)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>RBC (10^6/mm³)</td>
<td>6.86 (1.5)</td>
<td>6.75 (1.01)</td>
<td>7.65 (0.5)</td>
<td>7.56 (0.6)</td>
<td>0.4526</td>
</tr>
<tr>
<td>HGB (g/dl)</td>
<td>13.80 (3.0)</td>
<td>13.10 (1.3)</td>
<td>13.63 (0.9)</td>
<td>14.16 (0.8)</td>
<td>0.8358</td>
</tr>
<tr>
<td>HCT (%)</td>
<td>42.04 (13.7)</td>
<td>44.62 (9.7)</td>
<td>46.25 (3.9)</td>
<td>41.10 (4.3)</td>
<td>0.8251</td>
</tr>
<tr>
<td>MCV (µm³)</td>
<td>54.00 (1.4) *</td>
<td>65.60 (6.9)*</td>
<td>60.25 (1.2)</td>
<td>60.00 (5.1)</td>
<td>0.0104</td>
</tr>
<tr>
<td>MCHC (g/dl)</td>
<td>33.60 (3.3)</td>
<td>30.14 (5.1)</td>
<td>29.53 (0.6)</td>
<td>34.84 (5.5)</td>
<td>0.2029</td>
</tr>
<tr>
<td>PLT (10^3/mm³)</td>
<td>492.80 (273.4)</td>
<td>948.40 (332.0)</td>
<td>852.30 (146.5)</td>
<td>1374 (1172.2)</td>
<td>0.2407</td>
</tr>
<tr>
<td>MCH (%)</td>
<td>20.20 (1.2)</td>
<td>19.48 (1.4)</td>
<td>17.83 (0.1)</td>
<td>18.88 (2.7)</td>
<td>0.2523</td>
</tr>
</tbody>
</table>

Values are expressed as mean (SD). P value represents significance level for one-way ANOVA followed by Tukey’s post hoc analysis for between group comparisons with. *P<0.05, **p<0.01 and ***p<0.001
4.3 Histomorphometrically assessed structural changes in the liver

Table 7: Summary of statistics on mean volume densities of hepatocytes and central veins of studied rats.

<table>
<thead>
<tr>
<th>Variable</th>
<th>G1 (Paracetamol-only)</th>
<th>G2 (Paracetamol-cocoa)</th>
<th>G3 (Paracetamol-NAC)</th>
<th>G4 (Controls)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Damaged Hepatocyte</td>
<td>0.136 (SD 0.027)***</td>
<td>0.017 (SD 0.005)</td>
<td>0.010 (SD 0.002)</td>
<td>0.007 (SD 0.003)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>(×10⁴µm³)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Undamaged Hepatocyte</td>
<td>0.003 (SD 0.002)***</td>
<td>0.075 (SD 0.010)</td>
<td>0.066 (SD 0.011)</td>
<td>0.072 (SD 0.010)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>(×10⁴µm³)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Central Vein</td>
<td>4.699 (SD 1.297)***</td>
<td>1.000 (SD 0.200)</td>
<td>1.311 (SD 0.181)</td>
<td>0.886 (SD 0.095)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>(×10⁴µm³)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Values are expressed as mean (SD). P value denotes significance level for One-way ANOVA (followed by Tukey’s post-hoc test) for between–group comparison with *** = p < 0.001, ** = p < 0.01 and * = p < 0.05
4.3.1 Damaged Hepatocytes

The mean volume density of damaged hepatocytes for the various groups of rats is shown in Table 7 and Figure 15. The difference between the mean volume density of damaged hepatocytes was significant when the four groups were compared by ANOVA (p < 0.0001); Tukey’s post hoc multiple comparison indicated significant increase of mean volume density in G1 (0.136, SD 0.027 µm$^3$) and control (G4) group (p < 0.001). However, comparison of mean volume density of G2 (0.017, SD 0.005 µm$^3$), G3 (0.010, SD 0.002 µm$^3$) and G4 (0.007, SD 0.003 µm$^3$) did not yield significant statistics (Figure 15).

![Figure 14](http://ugspace.ug.edu.gh)

**Figure 14.** A bar chart showing mean volume density of damaged hepatocytes for rats in four treatment groups, Error bars represent SD. * = p < 0.05, ** = p < 0.01 and *** = p < 0.0001 compared to control group (G4).
4.3.2 Undamaged Hepatocytes

A comparison of mean volume densities for undamaged hepatocytes was statistically significant among the four groups (p < 0.0001). The analysis of difference in mean volume densities of G1 (0.003, SD 0.002 µm³) and G4 (0.072, SD 0.010 µm³) by Tukey’s post hoc comparison was statistically significant (p < 0.001). However, the analysis of the mean volume densities of G2 (0.075, SD 0.010 µm³) and G3 (0.066, SD 0.011 µm³) did not differ significantly from G4 (Figure 16).

Figure 15. A bar chart showing mean volume density of undamaged hepatocytes for rats in the four treatment groups of rats. Error bars represent SD. * = p < 0.05, ** = p < 0.01 and *** = p < 0.0001 compared to control group (G4).
4.3.3 Central Vein

The mean volume density of central veins for the various groups of rats is shown in Table 7 and Figure 17. ANOVA comparison of the mean volume density of central vein was significant among the four groups (p < 0.0001); further inferential analysis by Tukey’s multiple comparison showed the significant observation was attributable to increased mean volume density of G1 (4.699, SD 1.297 µm$^3$) compared with the control group (0.886, SD 0.095 µm$^3$) (p < 0.001). However, comparison between G2 (1.0, SD 0.200 µm$^3$) and G3 (1.311, SD 0.181 µm$^3$) was not significantly different from G4 value (Figure 17).

![Bar chart showing mean volume density of central vein for rats in Groups 1, 2, 3, and 4. Error bars represent SD. * = p < 0.05, ** = p < 0.01 and *** = p < 0.0001 compared to control group.]

**Figure 16.** A bar chart showing mean volume density of central vein for rats in Groups 1, 2, 3, and 4. Error bars represent SD. * = p < 0.05, ** = p < 0.01 and *** = p < 0.0001 compared to control group.
Morphology of Liver Tissue

**Figure 17.** Photomicrographs showing H & E liver sections from all four experimental groups (G1, G2, G3 and G4) at low power (X10). A from G1 rat showing dilated central vein indicated with black star. B from G2 and C from G3 rat showing preserved central vein which is comparable with D from G4.
Figure 18. Photomicrographs showing H & E liver sections from all four experimental groups (G1, G2, G3 and G4) at high power (X40). A from G1 rat showing distorted arrangements of hepatocytes with disintegrated nuclei. B from G2 rat showing preserved hepatocytes with reduced number of damaged hepatocytes. C from G3 rat shows preserved hepatocytes (hh) with minimal damages. D from G4 rat showing the typical well preserved hepatocytes.

Yellow arrows are pointing to damaged hepatocytes with laterally displaced and disintegrated nuclei. Oval shape indicating distorted arrangement of hepatocytes.
Figure 19. Photomicrographs showing H & E liver sections from Paracetamol only group (G1) at high power (X40). A shows dilated sinusoids (dsn), yellow arrows indicating laterally displaced hepatocyte with fatty accumulation and black arrows indicating steatosis. B shows pycnotic hepatocyte indicated with blue arrow and oval shape indicating disrupted trabecular arrangement of hepatocytes. C shows vascular infiltration indicated with white arrows.
CHAPTER FIVE

DISCUSSION

5.1 General

The focus of this research was to assess the ameliorative activity of prandial natural cocoa powder (NCP) on paracetamol-induced hepatotoxicity in rats. Previous work has demonstrated that ingested 2% NCP ameliorated putative oxidative damage associated with murine falciparum infection (Aidoo et al., 2012), rat alcoholic liver toxicity (Sokpor et al., 2012), and atherogenesis in rabbit pups born to hypercholesterolaemic mothers (Blay et al., 2019). Saleem et al., (2010) and Oyedeji et al., (2013) have reported the harmful effects of paracetamol overdose on the liver. The conventional allopathic remedy for paracetamol induced hepatotoxicity is NAC which replenishes glutathione stores (McGill & Jaeschke, 2013). Therefore, this study compared NCP intake and NAC in attenuating experimental hepatotoxicity induced by paracetamol overdose.

Meanwhile, several plants with natural antioxidants have been used for paracetamol hepatic injury on account of effective ability to scavenge free radicals (Singh et al., 2011; Jaeschke et al., 2013, Dadzeasah & Ansah, 2013). Recent, studies have been conducted on plants containing phenolic compounds to elucidate their antioxidant capacity in the prevention or control of disease such as acute liver failure, cancer and cardiovascular diseases (Khalafalla et al., 2010 and Saleh, Allam, El-rabeaie, & El-sabbagh, 2018).
5.2 Fluid consumption

In their research, Speth et al. (2001), reported that rats administered with acetaminophen had decreased water intake on the day of exposure but significantly increased water intake a day after drug exposure. In the present study it was observed that the mean fluid intake within all four groups significantly varied from week two to week four, however the means of fluid intake between the four groups were not significant at the end of the first week. Additionally, it was observed that the paracetamol only group G1 recorded a significant increase in fluid consumption in the subsequent weeks.

At the end of the fourth week, the mean fluid consumption in G1 was comparatively lower than the control (G4), paracetamol/ cocoa group (G2) and paracetamol/ NAC (G3). The results observed is consistent with study conducted by Bauer et al. (2003) who investigated the post-surgical anaesthetic effect of paracetamol in rats.

The G2 and G3 groups did not differ in their mean fluid intake compared to G4 at the end of the fourth week. This finding may be due to the fact that the theobromine component of cocoa exerted a diuretic effect by causing systemic dehydration which stimulated increased fluid consumption (Sokpor et al., 2012).

5.3 Body weight

Rats (G2) treated with paracetamol and cocoa had significant increase (p<0.0001) in body weight from the first to the fourth week. By contrast G1 rats treated with only paracetamol recorded a decrease in weight from week one to week 4. Weight loss in G1 rats may have resulted from underfeeding since Bauer et al. (2003) showed that the amount of food consumed by the rats reduces, following paracetamol exposure. Weight gain within the G2 group could therefore be explained as suppression of the paracetamol-induced underfeeding attributable to
the hedonic effect of cocoa which is known to contribute to the weight gain by enhancing increased up-take of food in rats (Coccurello & Maccarrone, 2018). The palmitic acid contained in cocoa may also possibly induce weight gain as seen in G2 rats (Olieveira & Genovese, 2013).

5.4 Hepatic injury biomarkers

5.4.1 AST and ALT

Aspartate aminotransferase (AST) and alanine aminotransferase (ALT) are enzymes which are predominantly present in the liver. These enzymes are also found in the red blood cells, heart cells, muscle tissue and organs such as the kidney and pancreas (Huang et al., 2006). In the diagnosis of liver disease, AST and ALT enzymes are the primary serum biomarkers for the identification of liver lesion and are also useful for monitoring the course of many liver disorders (Senior, 2012; Freitag et al., 2015). The above liver enzymes are not specific for liver damage, however, it can be used in combination with other enzymes to monitor the course of many liver disorders. During liver damage plasma membrane disruption leads to excess AST and ALT being released into the bloodstream causing levels of the enzymes to increase. The extent of tissue damage is therefore directly related to the concentrations of AST and ALT in the blood (Huang et al., 2006). Additionally, after treatment with inducers of hepatocyte apoptosis, the initial level of serum ALT is low, however it is elevated in the course of injury (McGill, 2016).

In the present study, four weeks treatment with paracetamol (350 mg/kg b. wt) caused significant increase in serum AST and ALT in G1 rats compared with controls (G4), whereas increases in serum concentration of AST and ALT in G2 and G3 rats were not statistically different from G4. Taken together, these results suggest that NCP ingestion was comparable to
NAC in attenuating liver injury arising from paracetamol overdose. Increase in cellular enzymes in serum is invariably due to leakage of cellular enzymes as a result of disturbance in the transport functions of hepatocytes and hence loss of functional integrity of cell membrane in liver (Zimmerman & Seeff, 1970; Moore et al., 1985). This study and other investigations (Alkiyumi et al., 2012) have demonstrated the loss of liver cell membrane integrity following paracetamol overdose and is evident in the presentation of elevated concentrations of serum AST and ALT. A recent finding by Zengin et al. (2015) shows that the use of montelukast sodium in comparison with NAC following acetaminophen intoxication caused AST and ALT serum enzymes elevation among rats treated with paracetamol only. Several studies have also demonstrated similar findings; (Yousef, Omar, Guendi and Abdelmegid, 2010; Imo et al., 2013; Matić et al., 2016; Tzankova et al., 2017; and Uchida et al., 2017)

5.5 Antioxidant Assays

5.5.1 SOD

Superoxide dismutase (SOD) is a redox biomarker which converts superoxide radical into hydrogen peroxide and molecular oxygen (O₂) (Weydert & Cullen, 2012). SOD is one of the primary antioxidant enzymes which serve as a first line indicator of the antioxidant balance through oxidation or reduction process (Yang & Lee, 2015). The activity of SOD is however reinforced by catalase in metabolising hydrogen peroxide and hydrogen radicals to H₂O (Genestra, 2007; Pacher, Beckman, & Liaudet, 2007)

In this study reduction in supeoxide dismutase measured in the blood serum in G1 post treated rat group suggested a high pro-oxidant biological activity compared to control. However G2
rats recorded a significant increase (p<0.05) in SOD activity compared to control. Although, the comparison of G3 to G4 indicated a decrease in SOD activity it was not significant. The decrease in the antioxidant activity of SOD in the paracetamol treated group may be due to ROS detoxification as a result of depleted enzymes (Mladenovic et al., 2009). The decrease in SOD activity may also be attributed to the intensification of lipid peroxidation which consequently controls liver damage and the inability of the antioxidant defence mechanism to inhibit the formation of excess free radicals (Madkour & Abdel-Daim, 2014). Several studies have reported that oxidative stress and lipid peroxidation influence the development of paracetamol related hepatotoxicity (Yousef et al., 2010; Mahesh et al., 2014 and Matić et al., 2016).

The antioxidant effect of cocoa shown in this study may be attributed its polyphenols and flavanol content, thus rendering the ability to scavenge active oxygen and free radicals and consequently repair liver damages caused by paracetamol intoxication. Several studies have established the antioxidant protective effect of natural cocoa on various liver related injuries (Allgrove & Davison, 2014; Sokpor et al., 2012; Aidoo et al., 2012 and Jolic et al., 2011).

In this study, the reduction in SOD levels among rats administered with paracetamol in combination with NAC (G3) group may be due to the extent of damage caused by paracetamol. Though a well-recognised glutathione precursor which mops up free radicals, the duration of the study may have accounted for the decrease in SOD levels observed in the paracetamol/NAC group in this study.

5.5.2 GSH

Reduced glutathione (GSH) is a tripeptide which is present in all cell types including the liver. GSH is vital in maintaining the intracellular redox balance by reducing reactive oxygen species (ROS) and peroxides produced in the organism, while detoxifying xenobiotics (Myhrstad et
Glutathione is however a non-enzymatic compound which interrupts the radical chain reactions (Balasaheb & Pal, 2015; Mao et al., 2011). Several studies have reported that paracetamol overdose causes fatal hepatic necrosis and hepatic failure due to oxidative stress including lipid, DNA and peroxidation, as well as a decrease in hepatic GSH levels (Jothy et al., 2012, Simeonova et al., 2013 and Hohmann et al., 2015).

In the present study post treatment GSH level was significantly decreased in the paracetamol only treatment group (G1). A decrease in GSH suggest that the metabolic activation of paracetamol led to hepatotoxicity in the liver (Jaeschke et al., 2003). Furthermore the mechanism of paracetamol toxicity in the liver is associated with the covalent binding of the toxic metabolite (NAPQI) to sulphhydryl groups of GSH and other proteins and subsequently leading to oxidation (Gumbrevicius, Sveikata, Sveikatienė and Stankevičius, 2012). The reduction in GSH levels in G1 rats in this study is consistent with previous studies (Abdel-Zaher et al., 2008; Yousef et al., 2010 and Hamid et al., 2012).

The increase in GSH levels in G2 may be due to cocoa polyphenols which were able to counteract oxidative damage induced by paracetamol overdose. Several studies have established the antioxidant activity of cocoa ascribed to its polyphenols (Blay et al., 2019; Sokpor et al., 2012; Aidoo et al., 2012 and Noori, Nasir & Mahboob, 2009).

G3 rats showed a significant increase in GSH activity and this may suggest the ability of NAC to restore depleted glutathione stores as a result of the accumulation of the active metabolite NAPQI due to reactive oxygen species induced by paracetamol overdose. (North et al., 2010, McGill & Jaeschke, 2011; McGill & Jaeschke, 2013 and Saleh et al., 2018).
5.6 Haematological variables

Haematological parameters are important in the assessment of extent of liver damage following paracetamol toxicity. In this study the haematological parameters including; White blood cells (WBC), red blood cells (RBC) haemoglobin (HB), hematocrit (HCT) percentage, mean cell haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC), mean corpuscular volume (MCV), and platelets (PLT) were measured. The study indicated that apart from MCV and WBC which were significantly increased in paracetamol-treated rats, all the other parameters measured did not differ significantly in all the four experimental groups.

There was a significant increase in WBC counts in G1 group compared to G4 group, this observation may be due to oxidative stress that precedes inflammatory changes in the tissue as a result of the toxic effect of paracetamol (Matić et al., 2016). Additionally, innate immune responses under such conditions involve the induction of neutrophil adherence to vascular endothelium, leading to oxidative burst (Cover et al., 2006). The observation of significant increase in total leucocyte counts is therefore consistent with several animal studies conducted recently (Matić et al., 2016; Shanmugam et al., 2016; Imo et al., 2013; & Yousef et al., 2010).

The protective effect of cocoa in G2 suggests a probable combative action of cocoa polyphenols against paracetamol toxicity to ensure physiological total leucocyte levels. Cocoa may also have boosted the immune system by decreasing WBCs. This hypothesis is supported by Addai (2010) who postulated that cocoa has several health benefits including the potential to strengthen the immune system.

The Mean corpuscular volume (MCV) is a measure of the average size of RBCs, the decrease in MCV therefore implies that RBCs are smaller than normal (microcytic) (Cascio & DeLoughery, 2016). In this study, MCV levels in the rat group with paracetamol and cocoa combination (G2) was highly significant compared to G1, G3 and G4. The group (G1) showed
thrombocytopenia compared to increase in platelets count in the paracetamol and cocoa combination (G2) group. The increase in platelets may be attributed to the appreciable amounts of epicatechin and oligomeric procyanidins in cocoa (Rein, 2000).

5.7 Histomorphometric liver variables

The histologic al changes observed in the present study may be due to direct paracetamol toxicity on mitochondria and the oxidative process, as well as the accumulation of triglycerides arising from defective lipoprotein metabolism and plasma membrane damage which increased lipid delivery to hepatocytes. These findings are consistent with several researches (Yousef et al., 2010; Iyanda & Adeyini, 2011; Madkour & Abdel-Daimi, 2014; İçer et al., 2015 and Saleh et al., 2018).

The histological deterioration observed in G1 rats were qualitatively diminished in the paracetamol and cocoa group G2. Quantitative evidence in support of the protective benefit of ingested cocoa in paracetamol overdose was provided by the histomorphometric assessments. The structural variables of undamaged and damaged hepatocytes, as well as central vein relative volume density were significantly better in G2 than G1, and comparable to G3 and G4. It is inferred that cocoa ingestion did significantly ameliorate structural liver damage resulting from paracetamol overdose, and was as effective as the allopathic therapy NAC. The reduced liver damage may be explained by activity of quercetin content of cocoa. As a cocoa flavonoid, quercetin is able to diffuse into cell membranes and thus are able to maintain the integrity of hepatocytes following injury (Lamuela-Raventós et al., 2005; Sokpor et al., 2012). Further, quercetin scavenges free radicals through the lipid bilayer (Miller, 1996), impedes xanthine oxidase, NADPH oxidase, lipoxygenase, and inhibits lipid peroxidation (Change et al., 1993; Saija et al., 1995 & Miller, 1996). The inhibition of these potent pro-oxidants may be responsible for the attenuation of oxidative stress induced by cellular damage (Day et al.,
2000). In addition, it has been reported that quercetin metabolites can inhibit peroxynitrite-mediated oxidation, similar to free quercetin (Klotz and Sies, 2003). This study has therefore demonstrated the antioxidant activity of cocoa in relation to the preservation of liver structure despite exposure to paracetamol overdose.

5.8 Summary of key findings

After four weeks of treatment with paracetamol overdose, serum AST and ALT levels were strikingly increased in Group 1 (paracetamol only) rats. However, AST and ALT levels in Group 2 (paracetamol plus natural cocoa) and Group 3 (Paracetamol and NAC) rats reduced the extent of damage induced by paracetamol and hence the levels of these liver enzymes did not differ from G4 (control) rats.

The activity of SOD and GSH concentrations were also decreased in the serum of rats that were administered with paracetamol only (Group 1). The elevated levels of these antioxidants in the natural cocoa and paracetamol (Group 2) rats suggest that natural cocoa has the potential to scavenge free radicals and maintain antioxidant balance in rats exposed to paracetamol injury. Additionally, the increase in GSH activity in the paracetamol plus NAC (Group 3) rats may also be due to the ability of NAC to restore glutathione stores which were depleted following paracetamol injury.

The weight of the rats after four weeks of treatment also showed that natural cocoa is able to prevent excessive weight loss caused by paracetamol toxicity. Although, there was reduction in weight in (paracetamol only) Group 1 rats at week four of treatment, perhaps if the duration of the study was extended, weight loss would have been significant following long exposure to the drug.
In the study cocoa was able to reduce the white blood cell (WBC) counts in paracetamol plus cocoa (Group 2) rats, this suggest that natural cocoa has the ability to protect and sustain the immune system following paracetamol exposure.

Platelets counts in paracetamol only) G1 at the end of the study were markedly reduced to half the levels in G2 (paracetamol plus cocoa group) suggesting a potential hypercoagulability effect of paracetamol overdose.

The administration of 350 mg/kg b.wt of paracetamol caused striking alterations in liver microstructure after 4 weeks of treatment. The pathology observed in this rat model of paracetamol toxicity was largely associated with disintegration of the hepatocytes, disruption of the sinusoids, vascular infiltrations and enlargement of the central veins.

Design based random and systematic stereology employed in this study to quantify the volume densities of the damaged hepatocytes, undamaged hepatocytes and central veins suggest that natural cocoa significantly mitigated paracetamol-induced alterations in the liver microstructure.

5.9 Conclusion

This study has confirmed that the administration of 350 mg/kg b. wt of paracetamol had toxic effect on the liver. This study, showed for the first time that natural cocoa consumption ameliorated liver damage following paracetamol overdose. The consumption of natural cocoa may successively restore liver function and architecture and inhibit oxidative injury induced by paracetamol. Additionally, natural cocoa consumption may also blunt haematotoxicity and oxidative stress in comparable with conventional allopathic N-acetylcysteine (NAC).
5.10 Limitations of the study

1. The difficulty of paracetamol to dissolve in distilled water contributed to the difficulty in administration via gavage to the animals. This challenge in methodology could have been surmounted by using ethanol as the solvent. However, the resultant solution would have introduced further histological damage.

2. Stereological analysis could not cover biliary epithelial cells and Kupffer cells due to lack of time and logistics.

5.11 Recommendation

1. Further studies should include the determination of systemic and tissue specific inflammatory markers such as high sensitive CRP (hs-CRP) and tumour necrosis factor (TNF).

2. Immunohistochemical and immunofluorescence techniques should be employed in further studies to determine liver tissue damage involving the expression of peroxisome proliferator activated receptor α (PPARα) and fatty acid amide hydrolase (FAAH).
REFERENCES


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APPENDIX I

COLLECTION OF BLOOD FOR BIOCHEMICAL ANALYSIS (TAIL SNIPPING AND MILKING PROCESS)

Materials

Rodent handling gloves, towel, cotton, collection tube and glass chamber, scalpel holder and surgical blade.

- The animal was placed in a glass chamber with cotton dubbed with diethyl ether to make them unconscious
- Local aesthetic was applied to the surface of the tail 30 minutes before the experiment
- A cut of 0.5 cm was cut from the tip of the tail with a scalpel blade.
- The tail was then allowed to hung and placed in collection tube to collect blood. The tail was hung to allow gravity facillitate blood flow.
- After collection, pressure was applied to the tail to stop blood flow.
APPENDIX II

PROTOCOL FOR TISSUE PROCESSING

- Place tissue in tissue cassettes and into 50% alcohol overnight.
- Remove tissue and place into 70% alcohol for 2 hours.
- Transfer tissue into 95% alcohol; for 1 hour.
- Remove tissue and place in absolute alcohol (100%) I, II and III for 2 hours, 2 hours and 1 hour respectively.
- Transfer tissue into xylene I and II for 30 minutes each.
- Remove and place tissue into xylene III for 45 minutes.
- Infiltration.
- Place tissue in cassettes in molten wax I for 1 hour in an oven.
- Remove and place into molten wax II and III for 30 minutes each.
- Tissue in cassettes are embedded in wax and allowed to harden on ice.
APPENDIX III

PREPARATION OF 10% BUFFERED FORMALDEHYDE PH 7.3

- 10% buffered formaldehyde pH 7.26 (IL)
  - Formalin (37 - 40% w/v - BDH, England) ..................100 mL
  - Distilled water.................................................. 900 mL
  - Sodium hydrogen orthophosphate (NaH$_2$PO$_4$) .................4g
  - Disodium hydrogen orthophosphate (Na$_2$HPO$_4$) ............6.5g

- Apparatus and equipment
  - Electronic balance (Mettler CH – 8606)
  - 1000 mL flask
  - Magnetic stirrer
  - pH meter (Philips, PW9418)
  - Conical flasks and beakers
  - Plastic weighing container
  - Measuring cylinder
APPENDIX IV

PROTOCOL FOR HAEMATOXYLIN AND EOSIN (H &E) STAINING

With the aid of Leica Auto Sectioner XL with the following programmed methods, the paraffin embedded pancreatic blocks were sections at 5 micrometre prior to H&E staining for histomorphometry.

Staining Technique

- De-wax sections in xylene for 1 minute.
- Take sections to water (rehydrate) by passing them through graded series of alcohol in the order 100%, 95% and 70%
- Stain in Haematoxylin for 15 minutes.
- Wash in water for 2-3 minutes
- Differentiate in 1% hydrochloric acid in 70% alcohol for 1 minutes
- Wash in water for 10 minutes
- Stain in 1% aqueous eosin for 5 minutes
- Rinse gently in a bowl under running tap water to wash off surplus stain.
- Dehydrate in graded series of alcohol (75%, 95%, 100% and 100%) and keep in xylene for subsequent mounting with DPX.
APPENDIX V

FLOW CHART FOR STEREOLGY

Rat liver

- **Right lobe**: 3 Slices
- Every 2nd slice picked
- 1 liver block
- Microtomy
- 6 sections each from one liver block for H&E
- 6 sections (3 sections each for hepatocytes and central vein; every 10th, 50th, 100th)
- 10 micrographs each per slide for each lobe
- 6x10
- 120 micrographs per rat
- 120x4
- 480 micrographs per group
- 480x4
- 1,920 micrographs

- **Left lobe**: 3 Slices
- Every 2nd slice picked
- 1 liver block
- Microtomy
- 6 sections each from one liver block for H&E
- 6 sections (3 sections each for hepatocytes and central vein; every 10th, 50th, 100th)
- 10 micrographs each per slide for each lobe
- 6x10
- 120 micrographs per rat
- 120x4
- 480 micrographs per group
- 480x4
- 1,920 micrographs

- **Median lobe**: 2 Slices

- **Caudate lobe**: 2 Slices

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