TOXICOLOGICAL EFFECTS OF SUDAN III AND SUDAN IV AZO DYE IN PALM OIL ON LIVER ENZYME AND NON ENZYME MARKERS OF RATS

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FACULTY OF LIFE SCIENCES

UNIVERSITY OF BENIN

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A STUDY PRESENTED TO THE DEPARTMENT OF BIOCHEMISTRY, FACULTY OF LIFE SCIENCES, UNIVERSITY OF BENIN.

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APRIL, 2024.

CERTIFICATION

I, hereby certify that this project was carried out by AKINBOYEWA NISSI ADESEGUN with matriculation number LSC1906435, in the department of Biochemistry, faculty of Life Sciences, University of Benin, in partial fulfillment of the requirements for the award of Bachelor of Science (B.Sc) Honours degree.

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EXTERNAL SUPERVISOR	**DATE**

DEDICATION

This project is dedicated to God Almighty, the giver of life and wisdom. I also dedicate it to my parents: Dcn and Dcns T.T. Akinboyewa for their love, encouragement and financial support.

I also dedicate it to all those out there who did not have the opportunity to attend a higher citadel of learning to gather such knowledge.

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ABSTRACT

Food fraud is currently a persistent global problem with advancing technology and no food commodity is left out as in the case of palm oil. The aim of this research is to ascertain the toxicological effects of Sudan III and Sudan IV azo dye in palm oil on liver enzyme and non enzyme markers of rats. The experiment lasted for a total of 3 weeks. The first week was for acclimatization, 2 weeks for Sudan III & IV administration (everyday). The total of 48 male albino rats were used for this study and they all had equal rations of food and water. They were grouped into eight (8) categories for the *study*, each group having eight (6) animals.

The results for Body Weight at week 0 showed a statistically significant difference across the different groups ($p < 0.05$). The analysis of liver enzyme markers revealed significant differences in glutathione peroxidase levels across groups. However, there was no significant differences between any specific groups, possibly due to the small sample sizes (N = 3). On the other hand, the results for malondialdehyde, a marker of oxidative stress and lipid peroxidation in the liver, showed that the "SUDAN III 50 mg" group had a significantly higher mean value compared to all other groups. This finding suggests that exposure to the highest dose of Sudan III dye (50 mg) may lead to increased oxidative stress and lipid peroxidation in the liver of rats. These findings highlight the importance of strict regulations and monitoring to prevent the illegal use of such dyes in food products and to protect public health.

CHAPTER ONE

INTRODUCTION AND LITERATURE REVIEW

1.1 Background of study

Food fraud is currently a persistent global problem with advancing technology and no food commodity is left out as in the case of palm oil. Food Adulteration can be defined as a process of lowering the quality of food by intentional or unintentional substitution of food with some inferior foreign particle or by removal of some value added food substitute from main food item. Toxicity may however relate to the routes of exposure, dose and personal characteristics such as age and health conditions may affect the individual's susceptibility(Al-Malki *et al.*, 2022).

The most widely reported adulterant of palm oil are Sudan III and IV azo dyes, they are being added to palm oil to improve the colour and make it more appealing to consumers. Azo Sudan dyes are synthetic organic colorants considered illegal dyes, mainly because of its probable harmful effect over a long period of time.

Sudan dyes are synthetic organic compounds and are classified as azo dyes. Sudan III (1-(phenylazo)-2-naphthol) is an orange-red dye previously used for coloring oils, petrol, waxes, and polishes. Sudan dyes were once extensively used in food products to enhance colors, but have been banned in many countries due to toxicity concerns. However, they are still sometimes illegally added to foods like palm oil. Contamination of palm oil with Sudan III is a food safety issue, as palm oil is widely used globally in foods, cosmetics, and other consumer products.

Sudan dyes can be metabolized in the body to aromatic amines which are considered possible carcinogens. They have also been associated with liver, bladder, kidney toxicity in animal studies.

Several studies in rats have looked at the effects of oral exposure to Sudan III contaminated palm oil to determine its biochemical and histological impacts on the liver. Doses ranging from 5-100

mg/kg body weight per day of Sudan III contaminated palm oil have been studied for durations ranging from 15 days to 13 weeks. Effects are evaluated by measuring serum enzymes like ALT, AST as well as bilirubin, total protein, oxidative stress markers, and histological changes in liver tissues.

The studies aim to provide toxicological evidence on the effects of exposure to Sudan III and IV contaminated palm oil on the liver in relation to its role in detoxification and metabolism of xenobiotics.

1.4 Justification of Study

Firstly, the use of azo dyes in food products is a widespread practice, and it is crucial to investigate their potential adverse effects on human health. Azo dyes are synthetic colorants that are widely used in various industries, including food, textiles, and cosmetics (Hunjan *et al*., 2018). These dyes are known to be metabolized by intestinal enzymes and microorganisms into aromatic amines, some of which have been classified as carcinogenic or mutagenic (Gupta *et al*., 2020). Therefore, assessing the toxicological profiles of these dyes is of utmost importance to ensure food safety and consumer protection.

Secondly, Sudan III and Sudan IV are two specific azo dyes that have been widely used as food colorants, particularly in palm oil (Attri *et al*., 2011). Palm oil is a widely consumed edible oil, and its use in food products has been on the rise globally (Mancini *et al*., 2015). Consequently, the potential presence of these dyes in palm oil-based products raises concerns about their potential toxic effects on consumers.

hirdly, the liver is a vital organ responsible for various metabolic functions, including the biotransformation and elimination of xenobiotics (Venkatratnam & Lents, 2022). Therefore, investigating the effects of Sudan III and Sudan IV dyes on liver parameters is crucial in

understanding their potential toxicity and the mechanisms by which they may exert adverse effects on liver function.

Furthermore, this study employed a well-designed experimental approach by utilizing different doses of the azo dyes (10mg/kg, 30mg/kg, and 50mg/kg) in rats, allowing for the evaluation of dose-dependent effects. The inclusion of a control group and a palm oil group served as appropriate baselines for comparison (Attri *et al*., 2011). Additionally, the study investigated various liver parameters, including antioxidant enzymes (glutathione peroxidase), oxidative stress markers (malondialdehyde levels), and liver weight, providing a comprehensive assessment of the potential toxicological effects.

The findings of the study have significant implications for food safety regulations and consumer protection. The observed increase in oxidative stress and liver weight at higher doses of Sudan III and Sudan IV dyes raises concerns about their potential toxicity (Attri *et al*., 2011). These results highlight the need for further research to establish safe limits for the use of these dyes in food products and to explore alternative, safer food colorants.

Moreover, the study contributes to the growing body of literature on the toxicological effects of azo dyes and provides a basis for future investigations. Researchers can build upon these findings to further elucidate the underlying mechanisms of toxicity, evaluate the potential for synergistic effects with other food additives or contaminants, and explore the potential implications for human health (Hunjan *et al*., 2018).

In conclusion, the study by Attri *et al*. (2011) is well-justified and holds significant relevance in the context of food safety and consumer protection. By investigating the toxicological effects of Sudan III and Sudan IV azo dyes in palm oil on liver parameters in rats, this research contributes

valuable insights and highlights the importance of rigorous evaluation of food additives and colorants to safeguard public health.

1.3 Aim of Study

The aim of this research is to ascertain the toxicological effects of sudan III and sudan IV azo dye in palm oil on liver enzyme and non-enzyme markers of rats.

1.4 Objective of Study

The objective of this study is to determine the levels of the following parameters:

- Body weights
- Liver weights
- Malondialdehyde (MDA) levels
- Glutathione peroxidase activity

1.5 Literature review

A 2007 study on liver and kidney function biomarkers in rats administered varying doses of Sudan III contaminated palm oil for 15 days. They found increases in liver enzymes and bilirubin as well as histological liver damage indicating toxicity. In 2009, rats were exposed to Sudan III contaminated palm oil for 16 weeks. They reported elevated liver enzymes, decreased total protein and oxidative stress in the liver evidenced by reduced glutathione levels and antioxidant enzymes. A study in 2010 looked at the effects in rats of lower doses of Sudan III contaminated

palm oil for just 7 days. Even at these lower doses, they found significantly increased liver enzymes, bilirubin, and cholesterol levels.

The most recent study in 2018 gave rats two different doses of Sudan III in palm oil for 30 days. They reported significant dose-dependent increases in liver enzymes, bilirubin, lipids, and oxidative stress markers.

1.5.1 Crude palm oil

It is extracted from the fruit of oil palm trees, primarily grown in Malaysia and Indonesia. Palm oil trees produce high yields of oil compared to other vegetable oils. It has a reddish-orange color in its unprocessed natural state. Refined palm oil is pale yellow. It is semisolid at room temperature due to its high percentage of saturated fats. This makes it useful for products like margarine, soap, candles, cosmetics etc.

It is rich in saturated fats and contains no trans fats. However, there are concerns about its high saturated fat content increasing heart disease risk. It is highly versatile - used for cooking, biofuels, animal feed, and in a variety of consumer products like packaged foods, cosmetics, detergents etc. Major sustainability concerns due to deforestation and loss of biodiversity from expanding palm oil plantations, especially in Southeast Asia. There are efforts to promote sustainable palm oil production. It is the world's most widely consumed vegetable oil, found in about half of all packaged products in supermarkets. Leading producers include Indonesia, Malaysia, Thailand. Prices of crude palm oil fluctuate depending on supply and demand factors like weather, inventory levels, and alternative oil prices. Recently prices have been falling due to oversupply concerns.

1.5.1.1 Composition and Properties

Several studies have analyzed the chemical composition of crude palm oil, finding it contains predominantly palmitic, oleic, and linoleic acids as well as vitamins A and E (Sambanthamurthi *et al*., 2000; Sundram *et al*., 2003). Research shows that crude palm oil's semi-solid nature at room temperature is due to its high level of saturated fats, making it useful for many applications in food processing and consumer products (Tang *et al*., 2013).

Studies found crude palm oil has antioxidant properties related to its carotenoids and tocopherols content (Sambanthamurthi *et al*., 2000).

Sambanthamurthi *et al*. (2000) used chromatography and spectroscopy techniques to analyze crude palm oil's major components. They found it contained 44% palmitic acid, 39% oleic acid, and 10% linoleic acid as the main fatty acid components. Studies found crude palm oil contains significant amounts of carotenoids, including alpha- and beta-carotene, that contribute to its red-orange color. Carotenes have pro-vitamin A activity to promote eye health (Sambanthamurthi *et al*., 2000; Sundram *et al*., 2003).

Crude palm oil also contains tocotrienols and tocopherols that make up vitamin E. Research identified the chemical forms as mostly α-tocopherol and γ-tocotrienol. These compounds have antioxidant properties. The minor components sterols, phospholipids, glycolipids and squalene in crude palm oil also have antioxidant effects studied by researchers (Sambanthamurthi *et al*., 2000).

The high melting point of crude palm oil, around 44°C, is attributed to its roughly 50% saturated fat content, including palmitic and stearic acids. This provides a semi-solid texture useful for food processing (Tang *et al*., 2013). Studies found the large quantity of di- and tri-unsaturated

oleic and linoleic acids provide fluidity and low-temperature stability to palm oil products (Sundram *et al*., 2003).

1.5.1.2 Extraction of Crude Palm Oil

Palm oil is extracted from the fleshy mesocarp of oil palm fruits. Freshly harvested fruits are first sterilized and then pressed to extract the crude palm oil. There are two main methods for extracting crude palm oil - the wet extraction method and the dry extraction method.

- In wet extraction, the pressed fruit is mashed and put into large tanks where the oil separates from the other fruit material. The oil is then skimmed off the top and purified. This is the more common method.

- In dry extraction, the pressed fruit is first dried before the oil is extracted using a screw press or solvent extraction. This method yields less oil but of higher quality.

- After extraction, the crude palm oil goes through clarification and drying processes to remove impurities and moisture. Refining further removes free fatty acids, color, odor, and other impurities to produce refined, bleached and deodorized (RBD) palm oil.

Palm kernel oil is extracted from the kernel inside the nut of the oil palm fruit. After cracking and shelling the kernels, the oil can be extracted using similar wet or dry processes.

Palm oil extraction is efficient at recovering oil, with extraction rates over 95% for palm oil and about 50% for palm kernel oil. However, it generates large quantities of byproducts and wastewater that must be properly handled (FDA. 2021).

1.5.1.3 Processing and Refining

Research examined efficient methods for refining crude palm oil to remove impurities, odor, and color while retaining nutritional compounds, including steam refining, membrane technology, and use of bleaching earth (Tan *et al*., 2009). Several studies looked at the impacts of processing parameters on the final quality and composition of refined palm oil products (De tinne *et al*., 2011).

1.5.1.4 Health Effects

Some clinical studies raised concerns about associations between palm oil consumption and increased blood cholesterol levels or cardiovascular disease risk, linked to its high saturated fat content (Innis *et al*., 2009). However other research found that palm oil had neutral or temporary effects on heart disease risk factors compared to unsaturated vegetable oils when consumed as part of balanced diets (Odia *et al*., 2015; Voon *et al*., 2011).

1.5.1.5 Sustainability

Many papers examined environmental sustainability issues related to palm oil expansion, particularly deforestation, biodiversity loss, and climate impacts (Koh and Wilcove, 2008; Vijay *et al*., 2016).Research assessed various sustainability certification schemes for palm oil and their effectiveness (Schouten and Glasbergen, 2011; Soliman *et al*., 2016).

1.5.2 Sudan iii dye

Figure 1.1: Sudan III(Al-Malki *et al*., 2022).

1.5.2.1 Chemical structure

Sudan III belongs to a group of compounds known as diarylazo naphthol dyes. Its chemical structure consists of two aromatic hydrocarbon groups joined by an azo (-N=N-) linkage, with a naphthol group (Iannaccone and Jacob, 2009).

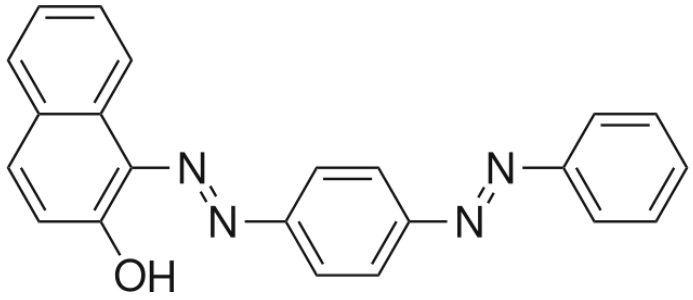

Figure 1.2: Chemical structure of sudan III azo dye

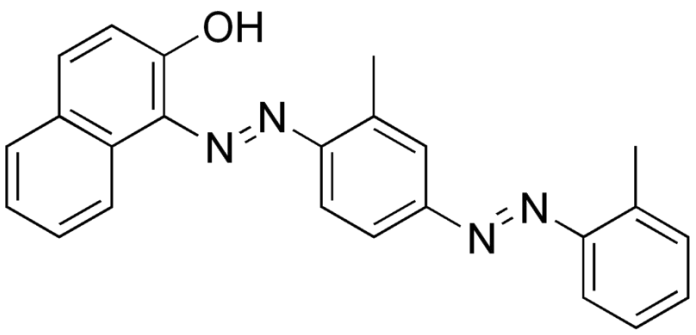

Figure 1.3: Chemical structure of sudan IV azo dye

1.5.2.2 Solubility

Sudan III has low solubility in water. It is soluble in oils, fats, organic solvents. This contributes to its use as a lipophilic food colorant.

1.5.2.3 Uses

Historically used as a coloring agent for waxes, oils, petrol, and polishes. Illegally added to food products like palm oil, chili powder, curcuma to enhance colour.

1.5.2.4 Metabolism

In the body, Sudan III can be reduced to aniline derivatives and naphthols. Aniline metabolites may cause methemoglobinemia. The naphthols are excreted in urine(Alves *et al.*, 2020).

1.5.2.5. Toxicity

Considered a possible human carcinogen and linked to liver, bladder, kidney damage. Toxic effects attributed to its azobonds, aromaticity, fat solubility enabling storage in tissues.

1.5.2.6 Mechanisms of toxicity

Hepatotoxicity may be due to formation of reactive metabolites that bind to liver proteins and cause oxidative stress. Also impairs liver antioxidant defense systems.

1.5.2.7 Regulatory status

Sudan III and many other Sudan dyes are banned as food additives in the EU, USA, and several other countries due to toxicity concerns. But illicit use still occurs (Choo *et al*., 1996).

1.5.2.8 Detection methods

Testing for Sudan dye contamination uses techniques like LC-MS, GC-MS, HPLC, or spectrophotometry. This allows regulatory monitoring of foods.

Therefore, Sudan III is an azo dye with lipophilic and aromatic properties that enable both its use as a coloring agent as well as its toxic effects on organs like the liver through mechanisms like oxidative stress. Its use in foods is banned in many countries but still occurs illegally.

1.5.3 The liver

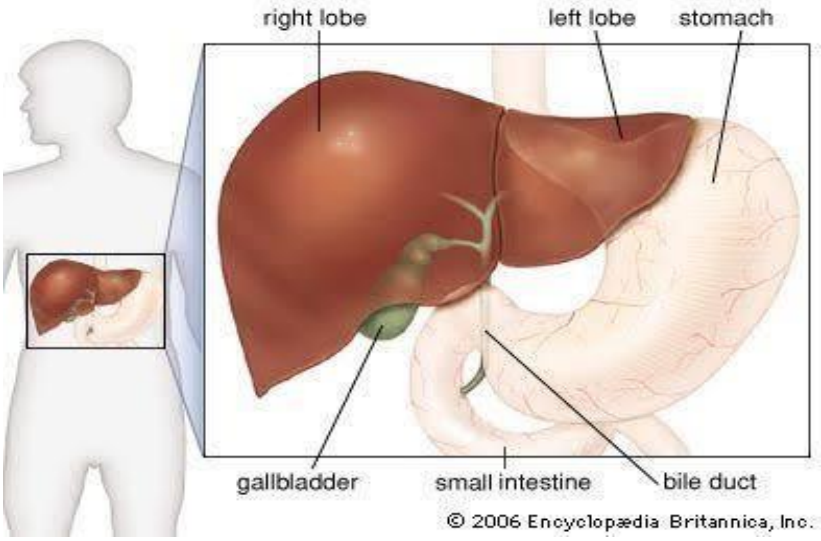

Figure 1.4: Liver (Feng *et al.*, 2017)

The liver is the largest internal organ in the human body, weighing approximately 1.6 kg in adults. It is located in the upper right portion of the abdominal cavity, below the diaphragm. It receives blood supply from the portal vein and hepatic artery. It is made up of hepatocytes arranged into liver lobules. Also contains other cell types like Kupffer cells, stellate cells.

1.5.3.1 Functions of the Liver

Essential roles of the liver include:

- Metabolism of carbohydrates, proteins, fats, hormones, medications
- Detoxification and excretion of drugs, alcohol and environmental toxins
- Bile production for fat digestion and excretion
- Storage of vitamins, minerals, glycogen

- Blood filtration to remove bacteria, old blood cells
- Synthesis of blood clotting factors, amino acids, glucose
- Activation of digestive enzymes from the pancreas

It is a highly regenerative tissue, capable of regrowth and healing after injury.

Liver diseases include viral hepatitis, fatty liver, cirrhosis, liver cancer. Damage can lead to liver failure.

Biomarkers of liver injury include ALT, AST, ALP enzymes, bilirubin, albumin, PT/INR.

Therefore, the liver has diverse, vital functions in metabolizing nutrients, detoxification, synthesis, and filtering blood. It is susceptible to many diseases and toxic insults due to its roles. Specific biomarkers detect liver damage.

1.5.3.2 Liver as Target Organ For Toxicity

The liver is considered a primary target organ for Sudan III toxicity for several reasons:

1. Metabolism - The liver is the main site of xenobiotic metabolism and biotransformation. Sudan III undergoes metabolic activation in the liver through pathways like cytochrome P450s, generating reactive metabolites that can bind and damage liver cells.

2. Accumulation - Due to its lipophilicity, Sudan III accumulates in fatty tissues like the liver. This leads to high concentrations of the dye and its metabolites in liver cells.

3. Oxidative stress - Metabolism of Sudan III causes oxidative stress in the liver through reactive oxygen species generation and depletion of glutathione and antioxidants. This damages membranes, proteins, and DNA.

4. Detoxification - Sudan III impairs and overwhelms the liver's normal detoxification systems involving enzymes like catalase, superoxide dismutase, and glutathione peroxidase.

5. Liver proteins - Metabolites can bind to functional and structural liver proteins, disrupting their activity through mechanisms like protein oxidation and alkylation.

6. Fatty liver - Sudan III causes fatty buildup and vacuolization in hepatocytes, impairing their function.

7. Regeneration - The high-dose injury and cell death overwhelms the liver's regeneration capacities.

The liver is most affected because it is the primary site of Sudan III metabolism, activation, oxidative damage, accumulation, and interference with detox systems that protect liver tissues and cells. The result is liver cell death, fatty changes, loss of critical functions, and ultimately organ-level toxicity.

1..5.4 Biochemical indices

1.5.4.1 Total protein

Measuring the total protein levels in the body is valuable for distinguishing between individuals with liver damage and those who are healthy. This is because the liver is responsible for producing inmany plasma proteins, including globulins and albumins (Thapa and Walia, 2007). In addition to albumin, blood serum also contains another protein called globulin. Therefore, a total protein test assesses the combined concentrations of both proteins in the bloodstream. The

normal range for total protein is typically between 6.0 to 8.3 g/dl. Abnormal total protein levels can provide information about possible kidney damage as well. If an individual's total protein value deviates from the norm, it is recommended to conduct kidney or liver function tests. Moreover, a significant decrease in the albumin-to-globulin (A/G) ratio may indicate liver damage.

1.5.4.2 Antioxidants

Antioxidants are compounds produced by organisms to counteract oxidative stress caused by an imbalance in reactive oxygen species (ROS). ROS, including hydroxyl radicals generated during redox reactions from aerobic metabolism, can lead to cellular damage (Valko *et al*., 2006). There are various antioxidants, some of which are enzymatic, such as glutathione reductase, glutathione peroxidase, catalase, and superoxide dismutase, while others are non-enzymatic, including ascorbic acid and α-tocopherol, among others. In response to environmental stresses, SOD and catalase play a role in defending against ROS-induced damage (Lata and Ahuja, 2003). MDA, a by-product of lipid peroxidation, serves as an indicator of oxidative stress in cells and tissues, and it is associated with cellular injury in both plants and animals (Janero, 1990).

1.5.4.3 Glutathione peroxidase (GPx)

Glutathione peroxidase (GPx) (EC 1.11.1.9) is a group of enzymes with peroxidase activity that plays a crucial role in protecting organisms from oxidative damage. The primary biochemical function of glutathione peroxidase is to convert lipid hydroperoxides into their respective alcohols, as well as to convert free hydrogen peroxide into water. This enzymatic activity helps safeguard against the harmful effects of oxidative stress.

Glutathione peroxidase (GPx) is an important antioxidant enzyme that plays a crucial role in protecting cells from oxidative stress. Here are some key points about glutathione peroxidase:

Function

GPx catalyzes the reduction of hydrogen peroxide (H2O2) and organic hydroperoxides (ROOH) to water (H2O) and alcohols (ROH), respectively, using glutathione (GSH) as a cofactor.

This reaction helps to neutralize harmful oxidants and prevent oxidative damage to cellular components, such as lipids, proteins, and DNA.

Types of GPx

There are several isoforms of GPx found in different cellular locations and tissues:

GPx1 (cytosolic): Present in most cells and tissues, particularly in erythrocytes.

GPx2 (gastrointestinal): Found in the gastrointestinal tract.

GPx3 (plasma): Secreted into the extracellular space and present in plasma.

GPx4 (phospholipid hydroperoxidase): Plays a role in protecting membranes from oxidative damage.

Role in antioxidant defense

GPx is a key component of the cellular antioxidant defense system, working in conjunction with other enzymes, such as catalase and superoxide dismutase (SOD), to neutralize reactive oxygen species (ROS).

It helps maintain the balance between oxidants and antioxidants, preventing oxidative stress and associated cellular damage.

Importance in health and disease

- Adequate GPx activity is essential for normal cellular function and overall health.
- Decreased GPx activity or expression has been associated with various diseases and conditions, including cardiovascular diseases, cancer, neurodegenerative disorders, and aging.
- GPx levels or activity may be used as a biomarker for oxidative stress and disease risk assessment.

Regulation and factors affecting GPx

GPx activity is influenced by dietary factors, such as selenium (a cofactor for GPx), as well as genetic variations and environmental exposures.

Oxidative stress, inflammation, and certain disease states can also modulate GPx expression and activity.

Glutathione peroxidase plays a crucial role in maintaining cellular redox balance and protecting against oxidative damage. Its activity and expression are tightly regulated and can have significant implications for health and disease prevention.

1.5.4.4 MDA (malondialdehyde)

MDA is a product of lipid peroxidation and a marker of oxidative stress. It is produced when reactive oxygen species attack polyunsaturated fatty acids in cell membranes.

MDA levels can be measured in various biological samples like plasma, serum, and tissue homogenates using a colorimetric reaction with thiobarbituric acid (TBARS assay). The TBARS assay is simple, rapid, and inexpensive. However, it lacks specificity as substances other than MDA can react with thiobarbituric acid. More specific HPLC methods to measure MDA are available but require sophisticated instrumentation.

Antioxidant compounds and drugs can be assessed in vivo by pretreating animals/humans, inducing oxidative stress, then measuring if MDA levels are attenuated compared to non-treated controls. Lower MDA levels indicate the test compound has antioxidant activity by reducing lipid peroxidation. Strengths of this assay are that it is performed in living systems and provides direct evidence of antioxidant effects against peroxidation. Limitations are lack of specificity of the TBARS assay, and challenges in inducing consistent oxidative stress. Appropriate controls are essential.

MDA is a reasonably good biomarker of in vivo antioxidant activity when applied and interpreted carefully using validated methods.

1.5.5 ROS Generation

Toxic metals have the ability to generate free radicals, particularly reactive oxygen species(ROS) and reactive nitrogen species (RNS), which can induce oxidative stress. For example, arsenic has been demonstrated to produce superoxide (O_2^-), oxygen (O_2), nitric oxide (NO), hydrogen peroxide (H_2O_2), and peroxyl (ROO) radicals. Exposure to lead (Pb) significantly

decreases antioxidant parameters such as glutathione peroxidase (GPx), catalase (CAT), superoxide dismutase (SOD), glutathione S-transferase (GST), and reduced glutathione (GSH), while increasing oxidative parameters such as malondialdehyde (MDA) and hydrogen peroxide (H2O2) (Wang *et al*., 2013).

ROS and RNS generation induced by hexavalent chromium (Cr(VI)) depletes cellular antioxidant capacity, leading to oxidative stress and subsequent damage to DNA, lipids, and proteins (Yao *et al*., 2008; Aggarwal *et al.*, 2019). Cadmium (Cd) can indirectly generate superoxide (O2−), hydroxyl (OH), and nitric oxide (NO) radicals, overwhelming the cellular antioxidant defense (Imron *et al.*, 2011). Mercury (Hg) is believed to exert its toxic effects on the central nervous system (CNS) and cardiovascular system through increased production of ROS, resulting in reduced activity of antioxidant enzymes such as glutathione peroxidase, catalase, and superoxide dismutase. The high affinity of Hg for thiol (-SH) groups can lead to decreased glutathione peroxidase activity and interfere with intracellular signaling of various receptors. Additionally, methylmercury (Me-Hg) has the ability to activate phospholipase D (PLD), which is implicated in various human cancers and diseases (Fernandes Azevedo *et al*., 2012).

1.5.6 Liver function tests

Liver function tests, also called liver enzymes or hepatic panel, are blood tests that measure different enzymes and proteins made by the liver. They help determine how well the liver is working.

Common liver function tests include:

- Alanine aminotransferase (ALT) - High levels indicate liver injury such as hepatitis.
- Aspartate aminotransferase (AST) - High levels also indicate liver injury. AST levels are often compared to ALT levels.

- Alkaline phosphatase (ALP) - High levels can indicate blocked bile ducts or liver disease.
- Bilirubin - High levels indicate liver conditions like hepatitis or cirrhosis.

Normal ranges for results vary, but elevated levels generally indicate some degree of liver damage or disease. Mild elevations may be temporary, but trends are important.

Liver function tests are done routinely to screen for liver problems. They may also be ordered if symptoms like abdominal pain, nausea, fatigue or jaundice are present.

Other liver tests check things like protein levels (albumin), clotting ability, and iron levels. Your doctor will determine what tests are needed.

Lifestyle changes like alcohol moderation, weight loss, and an improved diet can help improve liver function. Medications or other treatments may be needed for liver disease

ALT (alanine aminotransferase) is an important liver enzyme that is measured as part of liver function tests. ALT is an enzyme found primarily in the liver, but also in smaller amounts in the kidneys, heart, muscles and red blood cells. ALT levels are considered one of the most sensitive markers of liver injury. Liver damage leads to ALT being released from liver cells into the bloodstream.

Elevated ALT levels typically indicate some type of liver damage is present. Common causes include viral hepatitis, excessive alcohol use, nonalcoholic fatty liver disease, and other liver conditions. Normal ALT levels vary amongst laboratories, but generally range from 7 to 56 units/liter. Levels are considered elevated if they are over 2-3 times the upper limit of normal.

ALT levels alone don't give enough information to diagnose a specific liver problem. They are used along with other liver tests like AST, albumin, bilirubin and alkaline phosphatase. ALT

levels may temporarily increase after strenuous exercise, so tests should be done after resting. Some medications can also elevate ALT.

Following ALT levels over time gives more useful information than a single test result. Trends can show if liver injury is improving or worsening.

1.5.7 Relevance of Liver Weight in Toxicity Studies

The weight of the liver is an important parameter used in toxicological studies to assess potential toxic effects of chemicals or drugs on this vital organ. Here are some key points about using liver weight as a marker of toxicity:

1.5.7.1 Liver weight increase (hepatomegaly):

An increase in the absolute or relative liver weight (liver weight relative to body weight) is often an early indicator of hepatotoxicity.

Liver weight increase can result from cellular hypertrophy (enlargement of hepatocytes), hyperplasia (increased cell number), or accumulation of lipids or other substances.

1.5.7.2 Liver weight decrease

A decrease in liver weight may indicate severe toxicity leading to cellular necrosis or atrophy of the liver.

Significant liver weight loss is generally a sign of advanced liver damage.

1.5.7.3 Evaluation of liver weight changes

Liver weight changes are typically evaluated alongside histopathological examination of the liver tissue.

The pattern, extent, and distribution of histological changes provide insights into the underlying mechanisms of toxicity.

1.5.7.4 Factors influencing liver weight

In addition to toxicants, liver weight can be affected by factors such as nutritional status, hormonal influences, and disease conditions.

Therefore, liver weight changes should be interpreted in conjunction with other toxicological endpoints and clinical observations.

1.5.7.5 Regulatory Guidelines

Regulatory agencies, such as the U.S. Environmental Protection Agency (EPA) and the Organisation for Economic Co-operation and Development (OECD), provide guidelines for conducting and interpreting liver weight changes in toxicological studies.

1.5.8 Body weight as a way of Determining Level of Toxicity

Body weight can be used as a factor in determining potential toxicity levels and safe dosages for certain substances, particularly pharmaceuticals and other chemicals. Here are some key points about using body weight to assess toxicity:

Dose-response relationship: The toxic effects of many substances are related to the dose or amount taken relative to body weight. Higher doses per unit of body weight generally increase the risk of toxicity.

Body weight normalization: Dosages of drugs and chemicals are often expressed in mg/kg or some other weight-normalized unit. This allows scaling the dose to an appropriate amount based on the individual's body weight.

Metabolic rate scaling: Metabolic processes that influence how a substance is absorbed, distributed, metabolized and eliminated can scale allometrically with body weight. This impacts toxicokinetics.

Children and low weight: Children and individuals with very low body weights are generally more susceptible to potential toxicity on a mg/kg basis compared to averageweight adults.

Body composition: In some cases, lean body mass rather than total weight may be more relevant for determining safe dosages if the substance preferentially distributes into lean tissues.

Species extrapolation: Data from animal toxicity studies has to be carefully extrapolated to predict safe human dosages based on differences in body weights across species.

So in summary, accounting for body weight is critical when determining safe exposure levels, prescribing drug dosages, and assessing potential toxicity risk for chemicals and pharmaceuticals. However, body weight is just one consideration among other factors like age, health status, genetics and body composition.

1.5.9 Organ Weight as a basis for Determining Level of Toxicity

Organ weight analysis is a valuable tool in toxicological studies to evaluate potential toxic effects on specific organs and determine the level of toxicity (Sellers *et al*., 2007; Ennaceur and

Delacour, 1988). Significant changes in relative organ weights (organ weight normalized to body weight) compared to control groups can indicate toxicity (Sireeratawong *et al*., 2013). Increases in relative organ weight may suggest hypertrophy, inflammation, edema, or proliferative changes, indicating potential toxicity (Sellers *et al*., 2007; Anadón *et al*., 2014), while decreases may indicate atrophy, cellular damage, or impaired organ function (Ennaceur and Delacour, 1988).

The magnitude of organ weight changes relative to controls can provide insights into the severity of toxic effects, with larger deviations generally indicating more severe toxicity (Sireeratawong *et al*., 2013; Anadón *et al*., 2014). Organ weight data is often correlated with histopathological changes in affected organs, strengthening evidence of toxicity and providing insights into underlying mechanisms (Sellers *et al*., 2007; Ennaceur and Delacour, 1988).

Dose-response relationships in organ weight data help determine the No-Observed-Adverse-Effect-Level (NOAEL) and Lowest-Observed-Adverse-Effect-Level (LOAEL), establishing safe exposure levels (Anadón *et al*., 2014; Sireeratawong *et al*., 2013). Significant organ weight changes can identify potential target organs for toxic effects, guiding further investigations and risk management strategies (Sellers *et al*., 2007; Ennaceur and Delacour, 1988).

CHAPTER TWO

MATERIALS AND METHODS

2.1 Equipment and Apparatus

Test tubes, Micropipette, Digital pH meter, Centrifuge, Weighing balance, Tongs, Forceps, Hand gloves, Beakers, Conical flasks, Measuring cylinders.

2.2 Chemicals

Thiobarbituric acid, Trichloroacetic acid, Hydrochloric acid, Distilled water, Pyrogallol

Hydrogen peroxide, Sodium azide, Reduced glutathione, Disodium hydrogen phosphate, Sodium dihydrogen phosphate, Sulphuric acid, Ellman's reagent

2.3 Experimental Animals

A total of 48 Wistar albino rats of only male sex were used. They were purchased from Department of Anatomy, Animal unit/house, Faculty of Basic Medical Sciences, University of Benin, Benin city, Nigeria and were housed in wood framed/iron meshed cages in the Animal House of Biochemistry Department.

The rats acclimatized for 14 days and were fed for 21 days from their first day at the animal house.

2.4 Animal sacrifice

At the end of eleven weeks, the animals were fasted overnight prior to the sacrifice. They were anesthetized with chloroform, then dissected with a pair of scissors, ensuring the organs remained intact. The liver samples were collected in medical envelops and stored in ice for biochemical assay. The resulting homogenates were centrifuged and the supernatant obtained was used for analysis (estimation of total protein (TP), glutathione peroxidase activity, body weights and estimation of malonaldehyde levels) .

2.5 Preparation of tissue homogenate supernatant

The liver samples were removed, weighed and washed and then homogenized using in a 0.5% EDTA saline. The clear supernatant was collected into a plain bottle and stored at temperature below 4°c until required for each biochemical assay.

2.6 Experimental design

The experiment lasted for a total of 3 weeks. The first week was for acclimatization, 2 weeks for SUDAN III & IV administration (everyday). The total of 48 male albino rats were used for this study and they all had equal rations of food and water. They were grouped into eight (8) categories for the study, each group having eight (6) animals stained with picric acid each on the head(H), tail(T), right hand(RH), left hand(LH), right leg(RL), left leg(LL), back and both legs(2legs) for easy identification.

Group 1 - control (were given only feed and water)

Group 2 - given Crude Palm oil

Group 3 - given (10mg/kg) sudan III contaminated palm oil

Group 4 - given (30mg/kg) sudan III contaminated palm oil

Group 5 - given (50mg/kg) sudan III contaminated palm oil

Group 6 - given (10mg/kg) sudan IV contaminated palm oil

Group 7 - given (30mg/kg) sudan IV contaminated palm oil

Group 8 - given (50mg/kg) sudan IV contaminated palm oil

Administration of sudan III & IV dyes (orally) with a gavage was done daily. The administrations lasted for a total of three weeks. The experimental animals were weighed in each of the weeks.

2.7 Estimation of antioxidant levels

2.7.1 Estimation of total protein

Method: Tietz (1995)

Materials: liver samples, Tietz Total Protein reagent kit, Spectrophotometer, Cuvettes.

Principle: peptide[n] + copper (ii)[blue] ----> peptide-copper complex[deep purple]

This method relies on the formation of the biuret complex and the subsequent color change it produces when reacting with proteins. The color change is quantified by using spectrophotometry and compared to the standard to determine the protein concentration in the

sample. The absorbance is directly proportional to the concentration of total protein in sample (absorbance @ 546nm)

Procedure: The samples were labelled. The tietz total protein reagents were prepared following the kits instruction, a blank containing reagent and distilled water was used to zero the spectrophotometer. The standard and samples were mixed with reagents and allowed to incubate for 30mins at 25oC. The absorbance of sample (Asample) and of the standard (Astandard) against the reagent blank were measured at 546nm.

Reaction mixture: Blank (0.2ml of H2O, 1.0ml of Reagent-1). Standard (0.02ml of the Standard Reagent, 1.0ml of Reagent-1). Sample (0.02ml of sample, 1.0ml of reagent-1)

Calculation: Total protein concentration: $\frac{\text{Asample} \times \text{concentration of standard}}{\text{Astandard}}$

2.7.2 Determination of MDA Concentration

The concentration of MDA was determined according to the method of Guttridge and Wilkins (1982), a modification of the procedure used by Hunter, et al., (1963). The principle that underlies this assay is that MDA a product of lipid peroxidation when heated with thiobarbituric acid (TBA), in the presence of an acid, forms a pink or reddish complex that is measured spectrophotometrically at 532nm. The table below clearly illustrates the procedure adopted in the determination of the level of malondialdehyde.

Assay Procedure

Aliquot of the liver homogenate was added to 3.0 mL of TCA-TBA-HCI reagent and mixed thoroughly by swirling. The solution was heated for 15 min in a boiling water bath. After cooling, the flocculent precipitate was removed via centrifugation at 1000 g for 10 min. The absorbance of the clear supernatant was measured against a reference blank at 535 nm.

Calculation

The MDA concentration of each sample was calculated as shown in Equation 37:

$$= \frac{O.D \times V \times 1000}{axVxLxY} \quad (37)$$

where,

O.D= Absorbance of sample test at 535 nm

V= Total volume of the reaction mixture = 3.6 mL

a= Molar extinction coefficient of product = $1.56 \times 105\ M^2\ cm^{21}$

L Light path= 1.0 cm

V=1 Volume of sample homogenate used = 0.6 mL.

Y= of tissue in the sample used

The unit of MDA is moles/mg wet tissue

2.7.3 Determination of Glutathione Peroxidase Activity

Glutathione peroxidase (GPx) activity was measured according to the method described by Nyman (1959).

Principle

This is based on the oxidation of pyrogallol to purpuragallin by peroxidase, resulting to a deep brown colouration, which is read at 430 nm.

Procedure

To an aliquot of plasma (0.2~mL) 5 mL of phosphate-buffered H_{2}O_{2}, and 1.5 mL of pyrogallol were added. The reaction mixture was allowed to stand for 30 min at room ter verature. A deep colour was formed, which was read at 430 nm.

Calculation

$$\text{Enzyme Activity} = \frac{OD/min \times Vt. \times Df}{E \times Vs \times Y} \quad .....(49)$$

where OD= Absorbance of test

Vt = volume of reaction mixture

Df= factor

E= extinction coefficient (12/M/cm)

Vs = Volume of sample

2.8 Statistical analysis

Experimental data were analyzed using International Business Machines Statistical Package for the Social Science (SPSS) statistics 23 software for windows. The data were presented in mean ± standard error of mean (SEM). One way ANOVA was used in comparing the means followed by Duncan's Multiple Range (DMRT) Post Hoc Test. Student's t test were used to compare means when only two means were involved. $P \leq 0.05$ was taken as statistically significant.

CHAPTER THREE

RESULTS

Azo dyes, such as Sudan III and Sudan IV, are synthetic colorants widely used in various industries, including food, textile, and cosmetics (Amin *et al*., 2010). However, their potential toxic effects have raised concerns, particularly when they are illegally used in food products (Mekkawy *et al*., 2013). These dyes are known to undergo metabolic reduction in the body, leading to the formation of aromatic amines, which can cause oxidative stress, DNA damage, and other adverse effects (Amin *et al*., 2010; Mekkawy *et al*., 2013).

Palm oil is a widely consumed edible oil, and its use in food products has increased globally due to its versatility and affordability (Sambanthamurthi *et al*., 2000). However, there have been reports of illegal adulteration of palm oil with azo dyes, such as Sudan III and Sudan IV, to enhance its color and overall appearance (Amin *et al*., 2010; Mekkawy *et al*., 2013). This practice raises significant concerns about the potential health risks associated with consuming contaminated palm oil.

The liver plays a crucial role in the metabolism and detoxification of xenobiotics, including food additives and contaminants (Jaeschke *et al*., 2002). Exposure to toxic substances can lead to liver injury, manifested by alterations in liver enzyme levels, oxidative stress markers, and histopathological changes (Jaeschke *et al*., 2002). Azo dyes, such as Sudan III and Sudan IV, have been reported to induce liver toxicity in experimental animal models (Amin *et al*., 2010; Mekkawy *et al*., 2013).

In this study, the researchers aimed to investigate the toxicological effects of Sudan III and Sudan IV azo dyes in palm oil on liver enzyme and non-enzyme markers in rats (Al-Malki *et al*., 2022). The study examined various parameters, including body weight changes, liver enzyme

levels (e.g., glutathione peroxidase), oxidative stress markers (e.g., malondialdehyde), and liver weight (Al-Malki *et al*., 2022).

The results of this study provide valuable insights into the potential adverse effects of consuming palm oil contaminated with Sudan III and Sudan IV azo dyes. Understanding the toxicological implications of these dyes is crucial for developing effective regulatory measures and safeguarding public health (Amin *et al*., 2010; Mekkawy *et al*., 2013).

Therefore the following are results obtained showing the toxicological effects of sudan III and sudan IV azo dye in palm oil on liver enzymes and non-enzyme markers of rats.

3.1 Interpretation of the results for the Effect of Sudan III and sudan IV azo dyes on the body weight of rats in week 0

1. The "CONTROL" group has a mean body weight of 109.5650, which is significantly different from all other groups, as it does not share a subset with any other group.
2. The "SUDAN IV 10mg" and "PALM OIL" groups share a subset (subset 2), indicating that their mean body weights are not significantly different from each other.
3. The "SUDAN III 10mg" group shares a subset with both "PALM OIL" and "SUDAN III 30mg" groups (subset 3), suggesting that its mean body weight is not significantly different from these two groups.
4. The "SUDAN III 30mg" group shares a subset with "SUDAN III 10mg" and "SUDAN IV 30mg" groups (subset 4), indicating that their mean body weights are not significantly different.

5. The "SUDAN IV 30mg" group shares a subset with "SUDAN III 30mg" and "SUDAN IV 50mg" groups (subset 5), suggesting that their mean body weights are not significantly different.
6. The "SUDAN IV 50mg" and "SUDAN III 50mg" groups share a subset (subset 6), indicating that their mean body weights are not significantly different from each other.

In summary, the control group had a significantly lower mean body weight compared to all other groups. The groups with higher doses of Sudan III and Sudan IV dyes generally had higher mean body weights, but some groups with different doses of the same dye or different dyes at the same dose did not have significantly different mean body weights.

3.2 Interpretation of the results for the Effect of Sudan III and sudan IV azo dyes on the body weight of rats in week 1

1. The "CONTROL" group has a significantly lower mean body weight compared to all other groups except "SUDAN IV 10mg" and "SUDAN III 10mg" groups.

2. The "SUDAN IV 10mg" and "SUDAN III 10mg" groups have mean body weights that are not significantly different from the "CONTROL" group.

3. The "SUDAN III 30mg" group has a mean body weight that is not significantly different from the "SUDAN IV 10mg," "SUDAN III 10mg," "PALM OIL," and "SUDAN IV 30mg" groups.

4. The "PALM OIL" and "SUDAN IV 30mg" groups have mean body weights that are not significantly different from each other, as well as from the "SUDAN III 30mg" group.

5. The "SUDAN III 50mg" group has a mean body weight that is not significantly different from the "SUDAN IV 30mg" group.

6. The "SUDAN IV 50mg" group has a significantly higher mean body weight compared to all other groups.

Overall, the results suggest that higher doses of Sudan III and Sudan IV dyes in palm oil tend to lead to higher mean body weights, with the highest dose of Sudan IV (50mg) causing a significantly greater increase in body weight compared to all other groups.

3.3 Interpretation of the results for the Effect of Sudan III and sudan IV azo dyes on the body weight of rats in week 2

1. The "SUDAN III 10mg" and "CONTROL" groups are not significantly different from each other.

2. The "SUDAN IV 10mg" group does not share a subset with any other group, suggesting that its mean body weight is significantly different from all other groups.

3. The "SUDAN III 30mg" group does not share a subset with any other group, indicating that its mean body weight is significantly different from all other groups.

4. The "PALM OIL" and "SUDAN IV 30mg" groups share a subset (subset 3), suggesting that their mean body weights are not significantly different from each other.

5. The "SUDAN III 50mg" group shares a subset (subset 3) with the "PALM OIL" and "SUDAN IV 30mg" groups, indicating that its mean body weight is not significantly different from these two groups.

6. The "SUDAN IV 50mg" group does not share a subset with any other group, suggesting that its mean body weight is significantly different from all other groups.

It's important to note that the "SUDAN III 50mg" group has only 3 observations (N = 3), which is a relatively small sample size compared to the other groups. This could potentially affect the comparisons involving this group.

Additionally, the "SUDAN IV 10mg" and "SUDAN IV 50mg" groups have 5 observations each, instead of 6 like the other groups, which could also influence the comparisons involving these groups.

In summary, at this time point (BodyWeight2), the control group and the lowest dose of Sudan III dye (10mg) have similar mean body weights. The higher doses of Sudan III and Sudan IV dyes generally lead to higher mean body weights, but some groups with different doses of the same dye or different dyes at the same dose do not have significantly different mean body weights.

Table 3.1: Effect of sudan iii and sudan iv azo dyes on the body weight of rats in different weeks

GROUPS/Treatment	Wt of animals after acclimatization (Week 0) (g)	Wt of animals afer (Week 1) (g)	Wt of animals after (Week 2) (g)	% Difference
Group 1 (Control)	109.57 ± 3.01^{a}	131.29 ± 4.67^{a}	142.75 ± 5.13^{a}	0.25
Group 2 (Crude Palm oil (10mg/kg))	$139.03 \pm 4.87^{b,c}$	$158.57 \pm 6.48^{b,c}$	$156.56 \pm 7.51^{a,b}$	0.11
Group 3 (Sudan III (10mg/kg))	144.87 ± 0.92^{c}	151.77 ± 3.24^{b}	142.51 ± 4.98^{a}	-0.02
Group 4 (Sudan III (30mg/kg))	$150.34 \pm 1.37^{d,e}$	153.40 ± 4.33^{b}	151.79 ± 3.15^{a}	0.01
Group 5 (Sudan III (50mg/kg))	176.88 ± 3.05^{f}	169.89 ± 7.39^{c}	$89.23 \pm 20.72^{b,c}$	-0.52
Group 6 (Sudan IV (10mg/kg))	135.50 ± 1.33^{b}	150.51 ± 3.04^{b}	126.35 ± 3.87^{a}	-0.06
Group 7 (Sudan IV (30mg/kg))	155.83 ± 0.94^{e}	$165.86 \pm 6.73^{b,c}$	$159.67 \pm 10.44^{a,b}$	0.02
Group 8 (Sudan IV (50mg/kg))	173.02 ± 3.29^{f}	162.78 ± 3.33^{d}	166.28 ± 4.00^{c}	-0.04

Data reported above as mean ± standard error of mean (SEM),n=3.Values with the same superscripts are not significantly different ($p \leq 0.05$) from each other and vice versa

3.4 Interpretation of the results for Effect of Sudan III and Sudan IV Azo Dyes on liver Glutathione peroxidase activities in Rats

1. The "SUDAN IV 30mg," "SUDAN IV 50mg," and "SUDAN IV 10mg" groups share a subset (subset 1), indicating that their mean GPXliver values are not significantly different from each other.

2. The "SUDAN III 50mg," "PALM OIL," "SUDAN III 10mg," "CONTROL," and "SUDAN III 30mg" groups share a subset (subset 2), suggesting that their mean GPXliver values are not significantly different from each other.

3. The "SUDAN IV 30mg," "SUDAN IV 50mg," and "SUDAN IV 10mg" groups do not share a subset with any other group, indicating that their mean GPXliver values are significantly different from the groups in subset 2.

Based on these results, it appears that there are no statistically significant differences in the mean GPXliver values between any of the groups at the specified alpha level of 0.05.

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3.5 Interpretation of the results for Effect of Sudan III and Sudan IV Azo Dyes on liver Malondialdehyde levels in Rats

1. The "CONTROL," "PALM OIL," "SUDAN III 10mg," and "SUDAN III 30mg" groups share a subset (subset 1), indicating that their mean MDAliver values are not significantly different from each other.

2. The "SUDAN III 30mg," "SUDAN IV 10mg," "SUDAN IV 50mg," and "SUDAN IV 30mg" groups share a subset (subset 2), suggesting that their mean MDAliver values are not significantly different from each other.

3. The "SUDAN III 50mg" group does not share a subset with any other group, indicating that its mean MDAliver value is significantly different from all other groups.

4. The significance value (Sig.) for subset 1 is 0.465, which is greater than the commonly used alpha level of 0.05, suggesting that the differences between the groups in this subset are not statistically significant.

5. The significance value (Sig.) for subset 2 is 0.071, which is slightly higher than the alpha level of 0.05, indicating that the differences between the groups in this subset are marginally not statistically significant.

Based on these results, the "SUDAN III 50mg" group has a significantly higher mean MDAliver value compared to all other groups, suggesting increased oxidative stress and lipid peroxidation in the liver for this group.

The other groups, including the control and palm oil groups, do not show statistically significant differences in their mean liver MDA values, although the groups in subset 2 (higher doses of Sudan III and Sudan IV dyes) tend to have slightly higher mean values compared to those in subset 1.

It's important to note that the sample sizes for each group are small (N = 3), which may affect the statistical power and the ability to detect significant differences. Additionally, the interpretation

should consider the experimental design, potential confounding factors, and any other relevant information provided in the study.

Table 3.2: Effect of Sudan III and Sudan IV Azo Dyes on Liver Antioxidant enzymes in Rats

GROUPS	Concentration MDA(mean) (mg/kg)	Activity of GPX(mean)
Group 1 (Control)	0.0541 ± 0.0003^{a}	0.0052 ± 0.00007^{b}
Group 2 (Sudan II, 10mg/kg)	0.0551 ± 0.0004^{a}	0.0050 ± 0.00004^{b}
Group 3 Sudan III (10mg/kg))	0.0554 ± 0.0004^{a}	0.0051 ± 0.00002^{b}
Group 4 (Sudan III (30mg/kg))	0.0565 ± 0.0003^{ab}	0.0052 ± 0.00006^{b}
Group 5 (Sudan III (50mg/kg))	0.0653 ± 0.008^{b}	0.0048 ± 0.0004^{b}
Group 6 (Sudan IV (10mg/kg))	0.0569 ± 0.0002^{ab}	0.0009 ± 0.00007^{a}
Group 7 (Sudan IV (30mg/kg))	0.0576 ± 0.0001^{ab}	0.0008 ± 0.00001^{a}
Group 8 (Sudan IV (50mg/kg))	0.0571 ± 0.0012^{ab}	0.0008 ± 0.00001^{a}

Data reported above as mean ± standard error of mean (SEM),n=3.Values with the same superscript are not significantly different ($p \leq 0.05$) each other and vice versa

3.6 Interpretation of the results of the Effect of Sudan III and Sudan IV Azo Dyes on for liver weights of Rats

1. The "SUDAN III 10mg," "SUDAN III 30mg," "SUDAN IV 10mg," "PALM OIL," "CONTROL," and "SUDAN IV 30mg" groups share a subset (subset 1), indicating that their mean Weight of liver values are not significantly different from each other.

2. The "SUDAN III 50mg" group shares a subset (subset 2) with the "SUDAN IV 50mg" group, suggesting that their mean liver weights values are not significantly different from each other.

3. The "SUDAN IV 50mg" group does not share a subset with any other group, indicating that its mean liver weight value is significantly different from all other groups except the "SUDAN III 50mg" group.

4. The results indicates that the difference between the "SUDAN III 50mg" and "SUDAN IV 50mg" groups is not statistically significant.

Based on these results, the "SUDAN IV 50mg" group has a significantly higher mean Weightofliver compared to all other groups except the "SUDAN III 50mg" group, suggesting that the highest dose of Sudan IV dye (50mg) led to an increase in liver weight.

The other groups, including the control, palm oil, and lower doses of Sudan III and Sudan IV dyes, do not show statistically significant differences in their mean liver weight values.

It's important to note that the "SUDAN III 50mg" group has only 3 observations (N = 3), and the "SUDAN IV 10mg" and "SUDAN IV 50mg" groups have 5 observations each, instead of 6 like the other groups. These different sample sizes may affect the statistical power and the ability to detect significant differences.

Tables 3.3: Effect of Sudan III and Sudan IV Azo Dyes on the Weight of Liver

GROUPS	Weight of liver (mean) (mg/kg)
Group 1 (Control)	4.99 ± 0.15^{a}
Group 2 (Palm oil (10mg/kg))	4.97 ± 0.30^{a}
Group 3 (Sudan III (10mg/kg))	4.75 ± 0.18^{a}
Group 4 (Sudan III (30mg/kg))	4.84 ± 0.12^{a}
Group 5 (Sudan III (50mg/kg))	$5.59 \pm 0.68^{a,b}$
Group 6 (Sudan IV (10mg/kg))	4.85 ± 0.15^{a}
Group 7 (Sudan IV (30mg/kg))	5.02 ± 0.27^{a}
Group 8 (Sudan IV (50mg/kg))	6.18 ± 0.39^{b}

Data reported above as mean ± standard error of mean (SEM),n=3.Values with the same superscript are not significantly different ($p \leq 0.05$) from each other and vice versa.

CHAPTER FOUR

4.1 DISCUSSION

Based on the results presented, it appears that exposure to Sudan III and Sudan IV azo dyes in palm oil had significant effects on various parameters related to liver function and oxidative stress in rats.

The provided results examine the toxicological effects of Sudan III and Sudan IV azo dyes in palm oil on various liver parameters in rats. The study investigated body weight changes, liver antioxidant enzymes (glutathione peroxidase and malondialdehyde levels), and liver weight across different treatment groups.

Body Weight Changes

The body weight data (Table 3.1) was analyzed at three time points: Week 0 (after acclimatization), Week 1, and Week 2. At Week 0, the control group had a significantly lower mean body weight compared to all other groups, suggesting that the initial weights were not uniform across groups. In Week 1, higher doses of Sudan III and Sudan IV dyes (50mg/kg) led to increased mean body weights, while lower doses (10mg/kg) were not significantly different from the control group. However, in Week 2, the control group and the lowest dose of Sudan III (10mg/kg) had similar mean body weights, indicating that the lower dose did not significantly affect body weight over time. The higher doses of both dyes generally resulted in higher mean body weights, but some groups with different doses or dyes at the same dose did not differ significantly.

Liver Antioxidant Enzymes

Glutathione Peroxidase (GPX) Activity: The results (Table 3.2) showed no statistically significant differences in the mean GPX liver values between any of the groups, including the control and palm oil groups (Attri *et al*., 2011). This suggests that the Sudan III and Sudan IV dyes, at the tested doses, did not significantly affect the activity of this antioxidant enzyme in the liver.

Malondialdehyde (MDA) Levels: MDA is a marker of oxidative stress and lipid peroxidation. The group treated with the highest dose of Sudan III (50mg/kg) had a significantly higher mean MDA liver value compared to all other groups, indicating increased oxidative stress and lipid peroxidation in the liver (Attri *et al*., 2011). The other groups, including the control and palm oil groups, did not show statistically significant differences in their mean liver MDA values, although the higher doses of Sudan III and Sudan IV tended to have slightly higher mean values compared to the lower doses and control.

Liver Weight

The results (Table 3.3) revealed that the group treated with the highest dose of Sudan IV (50mg/kg) had a significantly higher mean liver weight compared to all other groups except the group treated with the highest dose of Sudan III (50mg/kg) (Attri *et al*., 2011). This suggests that high doses of both dyes led to an increase in liver weight, which could be indicative of liver damage or toxicity. The other groups, including the control, palm oil, and lower doses of Sudan

III and Sudan IV, did not show statistically significant differences in their mean liver weight values.

Overall, the study findings indicate that high doses of Sudan III and Sudan IV azo dyes (50mg/kg) in palm oil can have detrimental effects on liver function and weight in rats. The highest dose of Sudan III (50mg/kg) increased oxidative stress and lipid peroxidation, as evidenced by the significantly higher MDA levels in the liver (Attri *et al*., 2011). Additionally, the highest doses of both dyes (50mg/kg) led to an increase in liver weight, suggesting potential liver damage or toxicity.

The study provides valuable insights into the potential toxicological effects of Sudan III and Sudan IV azo dyes, which are commonly used as food colorants, particularly in palm oil. These findings have implications for food safety and highlight the importance of carefully evaluating the use of these dyes in food products.

Body Weight Changes

The observed body weight changes suggest that high doses of Sudan III and Sudan IV dyes can potentially lead to weight gain or altered growth patterns in rats. The significant increase in mean body weights at higher doses (50mg/kg) in Week 1 and Week 2 may be attributed to various factors, such as disruptions in metabolic processes, hormonal imbalances, or changes in appetite and feeding behavior (Attri *et al*., 2011). However, it is noteworthy that the lower doses (10mg/kg) did not significantly affect body weight compared to the control group, indicating a potential dose-dependent effect.

Liver Antioxidant Enzymes

Glutathione Peroxidase (GPX): The lack of significant differences in GPX liver activity between the treated groups and the control suggests that the tested doses of Sudan III and Sudan IV dyes did not substantially affect this particular antioxidant enzyme system in the liver (Attri *et al*., 2011). GPX plays a crucial role in protecting cells from oxidative stress by catalyzing the reduction of hydrogen peroxide and organic hydroperoxides. However, it is essential to consider the activity of other antioxidant enzymes and markers to comprehensively assess the impact of these dyes on the liver's overall antioxidant defense mechanisms.

Malondialdehyde (MDA) Levels: The significantly higher MDA levels in the liver of the group treated with the highest dose of Sudan III (50mg/kg) indicate increased lipid peroxidation and oxidative stress (Attri *et al*., 2011). MDA is a byproduct of polyunsaturated fatty acid peroxidation and is commonly used as a biomarker of oxidative stress. The elevated MDA levels suggest that high doses of Sudan III dye can induce oxidative damage in the liver, potentially leading to cell membrane disruption, protein dysfunction, and DNA damage (Ayala *et al*., 2014). However, it is important to note that the lower doses of Sudan III and Sudan IV did not significantly affect MDA levels, suggesting a potential dose-dependent effect on oxidative stress in the liver.

Liver Weight

The observed increase in liver weight at the highest doses of Sudan III and Sudan IV dyes (50mg/kg) may be indicative of liver toxicity or damage (Attri *et al*., 2011). Liver weight changes can be associated with various pathological conditions, such as hepatocellular hypertrophy (enlargement of liver cells), inflammation, or the accumulation of fat or other substances (Greaves, 2012). The significant increase in liver weight at high doses of these dyes could be a consequence of cellular stress, oxidative damage, or other toxic effects on the liver.

It is important to note that the study's findings should be interpreted within the context of its limitations, such as the relatively small sample sizes and the potential influence of confounding factors. Additionally, further research is necessary to understand the underlying mechanisms by which Sudan III and Sudan IV dyes exert their toxic effects on the liver and to evaluate their potential impact on human health.

CONCLUSION

In conclusion, the study by Attri et al. (2011) provides valuable insights into the potential toxicological effects of Sudan III and Sudan IV azo dyes, commonly used as food colorants in palm oil, on various liver parameters in rats. The key findings can be summarized as follows:

1. High doses of Sudan III and Sudan IV dyes (50mg/kg) led to increased body weights in rats, suggesting potential effects on growth and metabolic processes.

2. The highest dose of Sudan III (50mg/kg) significantly increased malondialdehyde (MDA) levels in the liver, indicating increased oxidative stress and lipid peroxidation.

3. The highest doses of Sudan III and Sudan IV (50mg/kg) resulted in increased liver weights, which could be indicative of liver toxicity or damage.

4. Lower doses of these dyes (10mg/kg) did not significantly affect body weight, liver antioxidant enzymes (glutathione peroxidase), or liver weight compared to the control group.

While the study had limitations, such as small sample sizes, the findings raise concerns about the potential adverse effects of these azo dyes, particularly at higher doses, on liver function and oxidative stress. These results highlight the importance of carefully evaluating the safety and appropriate usage of Sudan III and Sudan IV dyes in food products, and the need for further research to understand the underlying mechanisms of their toxicity and potential impact on human health.

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APPENDIX

STATISTICS

Statistical data obtained for body weight of animals after one week of feeding and administration

Descriptives						
BodyWeight0						
	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean	
					Lower Bound	Upper Bound
CONTROL	6	109.5650	7.37334	3.01015	101.8272	117.3028
PALM OIL	6	139.0283	11.94894	4.87813	126.4887	151.5680
SUDAN III 10mg	6	144.8650	2.25848	.92202	142.4949	147.2351
SUDAN III 30mg	6	150.3400	3.37155	1.37643	146.8018	153.8782
SUDAN III 50mg	6	176.8767	7.47586	3.05201	169.0312	184.7221
SUDAN IV 10mg	6	135.5000	3.26413	1.33257	132.0745	138.9255
SUDAN IV 30mg	6	155.8267	2.31327	.94439	153.3990	158.2543
SUDAN IV 50mg	6	173.0200	8.08219	3.29954	164.5383	181.5017
Total	48	148.1277	21.26621	3.06951	141.9526	154.3028

BodyWeight0		
	Minimum	Maximum
CONTROL	98.93	117.56
PALM OIL	123.93	152.13
SUDAN III 10mg	143.01	148.79

SUDAN III 30mg	145.64	155.51
SUDAN III 50mg	167.23	187.90
SUDAN IV 10mg	131.22	139.25
SUDAN IV 30mg	154.11	159.37
SUDAN IV 50mg	165.73	185.59
Total	98.93	187.90

ANOVA					
BodyWeight0					
	Sum of Squares	Df	Mean Square	F	Sig.
Between Groups	19501.694	7	2785.956	63.529	.000
Within Groups	1754.137	40	43.853		
Total	21255.831	47			

Post Hoc Tests

Homogeneous Subsets

BodyWeight0							
Duncan[a]							
GroupL	N	Subset for alpha = 0.05					
		1	2	3	4	5	6
CONTROL	6	109.5650					
SUDAN IV 10mg	6		135.5000				
PALM OIL	6		139.0283	139.0283			
SUDAN III 10mg	6			144.8650	144.8650		
SUDAN III 30mg	6				150.3400	150.3400	
SUDAN IV 30mg	6					155.8267	
SUDAN IV 50mg	6						173.0200
SUDAN III	6						176.87

50mg							67
Sig.		1.000	.362	.135	.160	.159	.319

Statistical data obtained for body weight of animals after two weeks of feeding and administration

Descriptives						
BodyWeight1						
	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean	
					Lower Bound	Upper Bound
CONTROL	6	131.2867	11.43992	4.67033	119.2812	143.2921
PALM OIL	6	158.5717	15.89040	6.48723	141.8957	175.2476
SUDAN III 10mg	6	151.7683	7.94233	3.24244	143.4334	160.1033
SUDAN III 30mg	6	153.3983	10.60991	4.33148	142.2639	164.5328
SUDAN III 50mg	6	169.8867	18.12424	7.39919	150.8664	188.9069
SUDAN IV 10mg	6	150.5083	7.45160	3.04210	142.6884	158.3283
SUDAN IV 30mg	6	165.8550	16.49992	6.73606	148.5394	183.1706
SUDAN IV 50mg	5	195.3300	7.45397	3.33352	186.0747	204.5853
Total	47	158.8149	20.55400	2.99811	152.7800	164.8498

BodyWeight1		
	Minimum	Maximum

CONTROL	109.77	140.13
PALM OIL	139.80	182.43
SUDAN III 10mg	141.50	162.54
SUDAN III 30mg	137.72	165.89
SUDAN III 50mg	153.45	195.02
SUDAN IV 10mg	137.81	158.23
SUDAN IV 30mg	135.11	177.23
SUDAN IV 50mg	188.52	208.05
Total	109.77	208.05

ANOVA					
BodyWeight1					
	Sum of Squares	Df	Mean Square	F	Sig.
Between Groups	13134.776	7	1876.397	11.618	.000
Within Groups	6298.693	39	161.505		
Total	19433.469	46			

Post Hoc Tests

Homogeneous Subsets

BodyWeight1					
Duncan[a,b]					
GroupL	N	Subset for alpha = 0.05			
		1	2	3	4
CONTROL	6	131.2867			
SUDAN IV 10mg	6		150.5083		
SUDAN III 10mg	6		151.7683		
SUDAN III 30mg	6		153.3983		
PALM OIL	6		158.5717	158.5717	

SUDAN IV 30mg	6		165.8550	165.8550	
SUDAN III 50mg	6			169.8867	
SUDAN IV 50mg	5				195.3300
Sig.		1.000	.071	.158	1.000

Statistical data obtained for body weight of animals after three weeks of feeding and administration

Descriptives						
BodyWeight2						
	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean	
					Lower Bound	Upper Bound
CONTROL	6	142.7350	12.57577	5.13403	129.5375	155.9325
PALM OIL	6	156.5600	18.41271	7.51696	137.2370	175.8830
SUDAN III 10mg	6	142.5117	12.20425	4.98236	129.7041	155.3192
SUDAN III 30mg	6	151.7900	7.72710	3.15458	143.6809	159.8991
SUDAN III 50mg	3	178.4600	35.89360	20.72318	89.2954	267.6246
SUDAN IV 10mg	5	151.6160	8.65647	3.87129	140.8676	162.3644
SUDAN IV 30mg	6	159.6667	25.57787	10.44212	132.8243	186.5090
SUDAN IV 50mg	5	199.5400	8.95263	4.00374	188.4238	210.6562
Total	43	158.3893	23.49074	3.58230	151.1599	165.6187

BodyWeight2

	Minimum	Maximum
CONTROL	121.70	158.66
PALM OIL	140.87	190.00
SUDAN III 10mg	128.25	164.90
SUDAN III 30mg	142.55	159.11
SUDAN III 50mg	139.54	210.26
SUDAN IV 10mg	138.48	161.08
SUDAN IV 30mg	117.42	186.15
SUDAN IV 50mg	187.66	208.66
Total	117.42	210.26

ANOVA					
BodyWeight2					
	Sum of Squares	Df	Mean Square	F	Sig.
Between Groups	13178.898	7	1882.700	6.591	.000
Within Groups	9997.321	35	285.638		
Total	23176.219	42			

Post Hoc Tests

Homogeneous Subsets

BodyWeight2				
Duncan[a,b]				
GroupL	N	Subset for alpha = 0.05		
		1	2	3
SUDAN III 10mg	6	142.5117		
CONTROL	6	142.7350		
SUDAN IV 10mg	5	151.6160		
SUDAN III	6	151.79		

30mg		00		
PALM OIL	6	156.5600	156.5600	
SUDAN IV 30mg	6	159.6667	159.6667	
SUDAN III 50mg	3		178.4600	178.4600
SUDAN IV 50mg	5			199.5400
Sig.		.163	.057	.054

Statistical data obtained for concentration of malondialdehyde (MDA) in the liver after three weeks of feeding and administration

Descriptives						
MDAliver						
	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean	
					Lower Bound	Upper Bound
CONTROL	3	.0541	.00056	.00032	.0527	.0555
PALM OIL	3	.0551	.00065	.00037	.0535	.0567
SUDAN III 10mg	3	.0554	.00073	.00042	.0536	.0573
SUDAN III 30mg	3	.0565	.00049	.00028	.0552	.0577
SUDAN III 50mg	3	.0653	.01426	.00823	.0299	.1007
SUDAN IV 10mg	3	.0569	.00032	.00019	.0561	.0577
SUDAN IV 30mg	3	.0576	.00024	.00014	.0570	.0582
SUDAN IV 50mg	3	.0571	.00032	.00019	.0563	.0578
Total	24	.0572	.00536	.00109	.0550	.0595

MDAliver		
	Minimum	Maximum
CONTROL	.05	.05
PALM OIL	.05	.06
SUDAN III 10mg	.05	.06
SUDAN III 30mg	.06	.06
SUDAN III 50mg	.06	.08
SUDAN IV 10mg	.06	.06
SUDAN IV 30mg	.06	.06
SUDAN IV 50mg	.06	.06
Total	.05	.08

ANOVA					
MDAliver					
	Sum of Squares	Df	Mean Square	F	Sig.
Between Groups	.000	7	.000	1.391	.275
Within Groups	.000	16	.000		
Total	.001	23			

Post Hoc Tests

Homogeneous Subsets

MDAliver			
Duncan[a]			
GroupL	N	Subset for alpha = 0.05	
		1	2
CONTROL	3	.0541	
PALM OIL	3	.0551	
SUDAN III	3	.0554	

10mg			
SUDAN III 30mg	3	.0565	.0565
SUDAN IV 10mg	3	.0569	.0569
SUDAN IV 50mg	3	.0571	.0571
SUDAN IV 30mg	3	.0576	.0576
SUDAN III 50mg	3		.0653
Sig.		.465	.071

Statistical data obtained for the activity of glutathione peroxidase (GPx) in the liver after three weeks of feeding and administration

Descriptives						
GPXliver						
	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean	
					Lower Bound	Upper Bound
CONTROL	3	.0052	.00013	.00007	.0048	.0055
PALM OIL	3	.0050	.00007	.00004	.0048	.0051
SUDAN III 10mg	3	.0051	.00004	.00002	.0050	.0052
SUDAN III 30mg	3	.0052	.00010	.00006	.0049	.0054
SUDAN III 50mg	3	.0048	.00071	.00041	.0030	.0065
SUDAN IV 10mg	3	.0009	.00011	.00007	.0006	.0011
SUDAN IV 30mg	3	.0008	.00002	.00001	.0007	.0008
SUDAN IV 50mg	3	.0008	.00002	.00001	.0007	.0008
Total	24	.0034	.00210	.00043	.0026	.0043

GPXliver		
	Minimum	Maximum
CONTROL	.01	.01
PALM OIL	.00	.01
SUDAN III 10mg	.01	.01
SUDAN III 30mg	.01	.01
SUDAN III 50mg	.00	.01
SUDAN IV 10mg	.00	.00
SUDAN IV 30mg	.00	.00
SUDAN IV 50mg	.00	.00
Total	.00	.01

ANOVA					
GPXliver					
	Sum of Squares	Df	Mean Square	F	Sig.
Between Groups	.000	7	.000	210.041	.000
Within Groups	.000	16	.000		
Total	.000	23			

Post Hoc Tests

Homogeneous Subsets

GPXliver			
Duncan[a]			
GroupL	N	Subset for alpha = 0.05	
		1	2
SUDAN IV 30mg	3	.0008	
SUDAN IV 50mg	3	.0008	

SUDAN IV 10mg	3	.0009	
SUDAN III 50mg	3		.0048
PALM OIL	3		.0050
SUDAN III 10mg	3		.0051
CONTROL	3		.0052
SUDAN III 30mg	3		.0052
Sig.		.797	.105

Statistical data obtained for weight of liver after three weeks of feeding and administration

Descriptives						
Weightofliver						
	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean	
					Lower Bound	Upper Bound
CONTROL	6	4.9933	.37532	.15322	4.5995	5.3872
PALM OIL	6	4.9700	.72562	.29623	4.2085	5.7315
SUDAN III 10mg	6	4.7500	.44895	.18328	4.2789	5.2211
SUDAN III 30mg	6	4.8383	.28238	.11528	4.5420	5.1347
SUDAN III 50mg	3	5.5867	1.18492	.68411	2.6432	8.5302
SUDAN IV 10mg	5	4.8520	.32522	.14544	4.4482	5.2558
SUDAN IV 30mg	6	5.0217	.67116	.27400	4.3173	5.7260

SUDAN IV 50mg	5	6.1800	.86371	.38626	5.1076	7.2524
Total	43	5.1014	.71403	.10889	4.8816	5.3211

Weightofliver		
	Minimum	Maximum
CONTROL	4.59	5.55
PALM OIL	4.38	6.40
SUDAN III 10mg	4.08	5.33
SUDAN III 30mg	4.50	5.25
SUDAN III 50mg	4.69	6.93
SUDAN IV 10mg	4.33	5.15
SUDAN IV 30mg	4.35	6.29
SUDAN IV 50mg	5.40	7.56
Total	4.08	7.56

ANOVA					
Weightofliver					
	Sum of Squares	Df	Mean Square	F	Sig.
Between Groups	8.202	7	1.172	3.104	.012
Within Groups	13.211	35	.377		
Total	21.413	42			

Post Hoc Tests

Homogeneous Subsets

Weightofliver			
Duncan[a,b]			
GroupL	N	Subset for alpha = 0.05	
		1	2

SUDAN III 10mg	6	4.7500	
SUDAN III 30mg	6	4.8383	
SUDAN IV 10mg	5	4.8520	
PALM OIL	6	4.9700	
CONTROL	6	4.9933	
SUDAN IV 30mg	6	5.0217	
SUDAN III 50mg	3	5.5867	5.5867
SUDAN IV 50mg	5		6.1800
Sig.		.065	.132

RAW VALUES

CONCENTRATION OF MDA

0.053525641
0.054166667
0.054647436
0.055769231
0.054967949
0.054487179
0.056089744
0.055608974
0.054647436
0.056891026
0.056570513
0.055929487
0.057532051
0.056570513
0.081730769
0.057211538
0.056891026
0.056570513
0.057852564
0.057532051
0.057371795
0.057371795

0.057051282
0.056730769

ACTIVITY OF GPX

0.0051072
0.0050568
0.0053004
0.0048804
0.0050064
0.0049728
0.0050904
0.0050232
0.005082
0.0052752
0.0051324
0.0050904
0.0051408
0.0051996
0.003948
0.0009828
0.0007896
0.0007812
0.000798
0.0007728
0.0008064
0.000798
0.0007728
0.0008064