(RESEARCH ARTICLE)

investigation into the effect of acute exposure to chlorogenic acid in male wistar mice

Adekanye Joseph-Peace Oluwabukunmi [1, *], Oluwatoyin A. Adeyemo-Salami [1], Opeyimi Opeoluwa Latinwo [1], Uchenna Collins Ojukwu [2], Oluwasanmi Victor Falade [3], Kenneth Uyi Iyere [4] and Olutomiwa Ayoola Omokore [5]

[1] Department of Biochemistry, Faculty of Basic Medical Sciences, University of Ibadan, Oyo State, Nigeria.
[2] Department of Medicine, Gullas College of Medicine, University of Visayas, Cebu, Philippines.
[3] Department of Biochemistry, Ladoke Akintola University of Technology, Oyo State, Nigeria.
[4] Department of Chemistry, University of Benin, Benin City, Edo State, Nigeria.
[5] Department of Surgery, Babcock University, Ilishan-Remo, Ogun State, Nigeria.

Abstract

Chlorogenic acid (CGA) is a natural polyphenolic compound widely present in various fruits, vegetables, and plant-based foods. It has gained considerable attention due to its potential health benefits including antioxidant, hepatoprotective, antibacterial, anti-inflammatory, anti-obesity, cardioprotective, neuroprotective, central nervous system stimulator, and anti-hypertensive amongst others. There is a dearth of information about acute exposure to chlorogenic acid which is also used as a food supplement. Ninety-six male Wistar mice were weight-matched into 8 groups (n=12). Group 1 was treated with distilled water, Group 2 received 1% ethanol, while Groups 3-8 were treated with 30, 60, 120, 240, 480 and 1,000 mg/kg body weight doses of CGA, respectively. Six animals were bled and sacrificed per group 24 hours post-treatment, and the liver and kidney were excised. Sections of the organs were fixed in 10% formalin for histopathological examination. Various antioxidant parameters were assayed in the liver and kidney samples using standard methods. A liver function test was also carried out on the liver samples. Haematological analysis was carried out as well as histopathology analysis of the liver and kidney samples. The remainder of the treated mice in each group were observed for mortality 24 hours post-treatment and for two weeks. Statistical analysis was performed using one-way analysis of variance (ANOVA) with p-values less than 0.05 considered to be significant. The results of this study show that chlorogenic acid led to dose-dependent increased levels of hydrogen peroxide and albumin, increased activity of alkaline phosphatase with decreased activities and levels of alanine aminotransferase, aspartate aminotransferase and bilirubin in the liver function while there were increased hydrogen peroxide levels in the kidney. The haematological parameters were not affected and the histopathological examination complemented the results with mild to moderate lesions in the liver and none in the kidney. Although, the lethal dose (LD 50-i.e., the dose at 50% mortality) was not established in this study. Therefore, chlorogenic acid should be ingested with caution.

1. Introduction

Natural and herbal remedies are receiving more attention daily as a result of growing worries about the unfavourable effects of utilizing chemical preservatives in the food industry. Due to their numerous beneficial effects, including their anti-inflammatory, anticancer, and antioxidant capabilities, phenolic acids have recently attracted a lot of interest. One of the most readily available phenolic acid components in plant-based foods such as coffee and tea, fruits, and vegetables is chlorogenic acid (CGA). Coffee contains a variety of polyphenols, particularly CGAs, which are well-known

* Corresponding author: Adekanye Joseph-Peace Oluwabukunmi

antioxidants. However, the word "CGA" refers to the entire group of hydroxycinnamic esters (Youyou *et al.*, 2011; Muhammad *et al.*, 2017).

According to Boerjan *et al.* (2003) and Jiali *et al.* (2018), chlorogenic acid (CGA) is the most abundant dietary polyphenol formed from the esterification of caffeic acid and quinic acid. Apart from being a phytonutrient, CGA is also taken as a food supplement. However, there exist scanty reports on the effect in light of an overdose or acute exposure to CGA.

This study aims at investigating the effect of acute exposure of male Wistar mice to CGA which is also taken as a food supplement.

1.1. Rationale

While CGAs are generally assumed to be safe, it is imperative to understand the effect in light of acute exposure. The rationale behind this project lies in the need to comprehensively evaluate the safety of CGA (a phytonutrient and food supplement).

Aim

This study aims to investigate the acute toxicity of chlorogenic acids (CGAs) in male Wistar mice.

Objectives

- To assess the effect of acute exposure to CGA on the function of the liver in male Wistar mice.
- To investigate the effect of acute exposure to GCA on antioxidant parameters in the liver.
- To determine the effect of acute exposure to CGA on antioxidant parameters in the kidney.
- To investigate the effect of acute exposure to CGA on haematological parameters.

2. Literature review

2.1. History of Chlorogenic Acid

Chlorogenic acid is a compound that is found in many plants. The history of chlorogenic acid can be traced back to the mid-19th century. In 1837, Robiquet and Bourton discovered physiologically active components in coffee and isolated acidic substances with green pigments, including ferulic chloride, from green coffee beans. In 1846, Payen coined the phrase "chlorogenic acid" (CQA). In 1854, Ludwig and Kromeyer discovered the chemical in sunflower seeds. In 1908, more than 50 years later, Gorter discovered that this component is widely distributed in the leaves and seeds of several different plants. From a crystalline complex of potassium caffeine chlorogenate, which he isolated, he made the pure compound. He also discovered that the action of alkalis breaks down chlorogenic acid, resulting in the production of caffeine and quinic acids. In 1910, two years later, Charaux, one of the pioneers to attempt measuring the CQA content in plants, described a potential extraction procedure. He confirmed that CQA is widely present in the plant kingdom and claimed that it is present in plants in amounts roughly two times greater than those of caffeic acid. In 1920, Chlorogenic acid, the tannic component of coffee, was first reported by Freudenberg, as a derivative of quinic acid and caffeic acid. In 1932, Fischer and Dangschat determined the structure of the compound, by isolating 3-O-caffeoylquinic acid from green coffee beans, which is now known as 5-O-caffeoylquinic acid (5-CQA) in modern nomenclature. In 1950, a paper from Barnes *et al.* suggested a compound with a trivial name, isochlorogenic acid. This compound was isolated from coffee beans and reported to have similar properties to chlorogenic acid but does not form a complex analogue to the crystalline potassium chlorogenate like chlorogenic acid (Clifford, 1999; Daniel *et al.*, 2015). The research on chlorogenic acid (CGA) can offer valuable insights for understanding nanoparticle-based cancer treatments, particularly in terms of how bioactive compounds interact with biological systems. Like CGA, nanoparticles used for cancer therapies also exhibit dose-dependent effects and have the potential to cause oxidative stress, impact organ function, and induce toxicity. The CGA study's focus on histopathology, antioxidant parameters, and liver and kidney function provides a framework for evaluating the safety and effectiveness of nanoparticles by monitoring their impact on vital organs and oxidative stress levels. Both studies emphasize the importance of understanding dose-response relationships and the biological effects of introducing foreign substances, which is crucial in determining the therapeutic window and toxicity levels for cancer treatment using nanoparticles (Onivefu, et al, 2024, a & b)

2.2. Green Coffee bean

Green coffee beans are the unroasted seeds of the Coffea plant. They are a very rich source of chlorogenic acid, a type of polyphenol that has been associated with various health benefits.

Green coffee beans are composed of both volatile and non-volatile compounds. The volatile compounds are responsible for the aroma of the coffee, while the non-volatile compounds (e.g., Chlorogenic acid) contribute to the basic taste sensation of sourness, bitterness, and astringency. There are approximately 100 different volatile compounds that have been identified in green coffee beans according to research. (Buffo and Cardelli-Freire, 2004; Farah, 2012).

Figure 1 Green coffee bean (Rena, 2017)

2.2.1. Availability of Chlorogenic Acid from the green coffee bean

Green coffee beans have between 6 and 10% CGA by dry matter, depending on the species. In the course of roasting, CGA gradually degrades and gets transformed. For every 1% loss of dry matter in the production of soluble coffee powders and home brews, approximately 8-10% of the dry matter is lost. However, it is important to note that significant amounts of dry matter are still present and can be utilized to make commercially available soluble coffee powders and home brews. A 200 ml cup of roasted and ground coffee can contain anywhere between 20 mg of chlorogenic acid (CGA) for a weak brew with a very dark roast, to 675 mg of CGA for a strong brew with a very mild roast *robusta* (Cruz *et al.*, 2012). However, recent investigations suggest that a more representative range is 70-200 mg of CGA per 200 ml cup of *arabica* coffee, and 70-300 mg (or even 350 mg) per 200 ml cup of robusta coffee (Cruz *et al.*, 2012). These amounts are within the lower range reported for roasted coffees after beverage preparation (Cruz *et al.*, 2012). The content of CGAs in coffee beans can vary depending on the species, with *Coffea arabica* containing 3.4-4.8% CGAs and *Coffea canephora* (robusta) containing 7.88-14.4% CGAs (Li *et al.*, 2020). The extraction method and roast level can also affect the concentration of CGAs in coffee (Fuller and Rao, 2017; Lu *et al.*, 2020). From 1983 to 1984, soluble coffee powder in the UK contained approximately 70 to 220 mg of chlorogenic acid (CGA) per cup, with the composition consisting of 50 to 150 mg of caffeoylquinic acid (CQA), 4 to 21 mg of feruloylquinic acid (FQA), and 15 to 38 mg of other components (Zhang *et al.*, 2018). The Temperature-Programmed Desorption (TPD) approach, used to study the behavior of materials under changing environmental conditions, can offer insights into chlorogenic acid (CGA) exposure by helping analyze how CGA interacts with biological tissues at different concentrations. TPD could theoretically be adapted to model CGA's adsorption, desorption, and reaction kinetics in biological systems, providing insight into its stability, metabolic breakdown, or accumulation in organs such as the liver and kidney. This method could also help identify the thresholds at which CGA induces toxic effects, allowing researchers to better predict safe dosage levels, much like TPD helps assess material performance under stress (Onivefu et al, 2023, 2024).

2.3. Nomenclature

The term "Chlorogenic acid" (CGA) is thought to be the general term that describes the family of esters formed between certain trans-cinnamic acid and quinic acid(1L-1(OH),3,4/5tetrahydroxy cyclohexane carboxylic acid) which has axial hydroxyls on carbons 1 and 3, and equatorial hydroxyls on carbons4 and 5 (Clifford, 1999).

According to Daniel *et al.* (2015), five main classes of chlorogenic acid can be found in coffee beans, they include caffeoylquinic acid (CQA), dicaffeoylquinic acid (diCQA), feruloylquinic acid (FQA), p-coumaroylquinic acid (CoQA) and caffeoyl feruloyl quinic acid (CFQA). There are other known esters of quinic acids that could be from natural or synthetic sources. They include; cinnamoylquinic acid (CiQA), galloylquinic acid (GQA), isoferuloylquinic acid (iFQA), o-camumaroylquinic acid (oCoQA) and sinapolyquinic acid (Clifford, 1999). Other isomers of CGA include 3-CQA, 4-CQA (cryptochlorogenic acid), and 5-CQA (neochlorogenic acid).

According to Muhammad *et al.* (2018), the International Union of Pure and Applied Chemistry (IUPAC) published a new system for numbering cyclitols which changed the numbering of the atoms in the quinic acid ring and caused the name change of CGA from 3-caffeoylquinic acid (3-CQA) to 5-caffeoylquinic acid (5-CQA).

2.4. Sources and chemical formula

CGA is a predominant class of phenolic acids found in a wide variety of foods and beverages, including fruits, olive oil, vegetables, wines, spices, and coffee (Emilija *et al.*, 2018). It has a chemical formula of $C_{12}H_{18}O_9$ and an average molecular weight of 354.31.
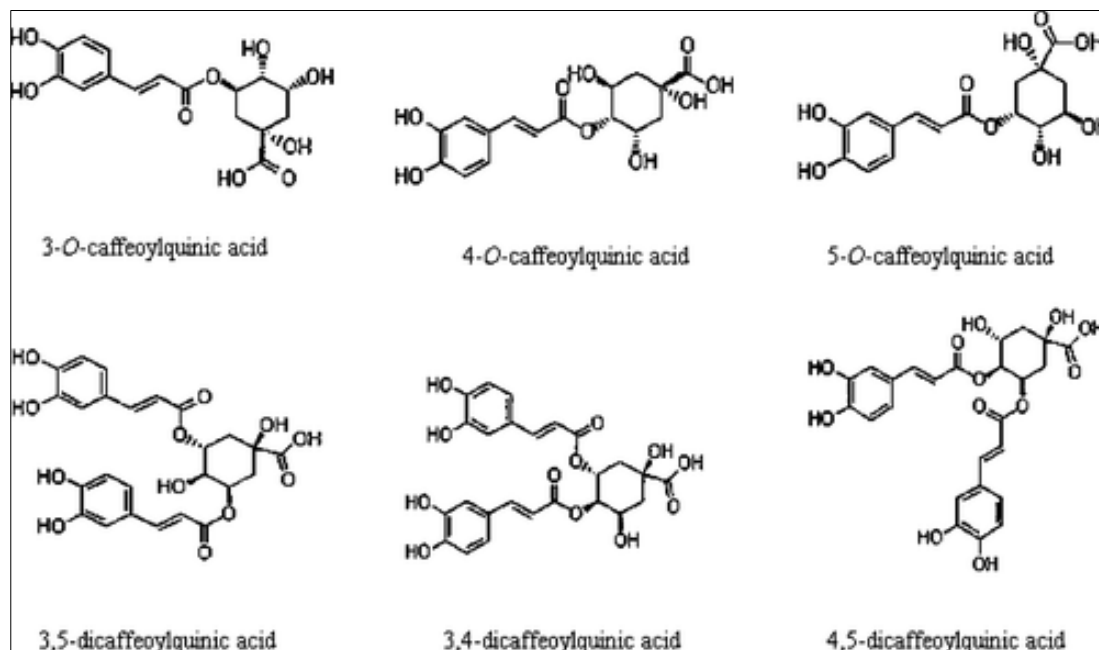

Figure 2 Structure of chlorogenic acid isomers (Xu et al., 2012)

2.5. Physical and chemical properties of chlorogenic acid

- Molecular Formula: Chlorogenic acid has the molecular formular C16H18O9.
- Solubility: Chlorogenic acid is soluble in hot water but insoluble in chloroform, benzene, and ether, because of its polarity. It is also soluble in lower alcohols (e.g., ethanol, methanol, etc.) or alcohol-water mixtures (Clifford, 1985).

Table 1 Data for the Solubility of Chlorogenic Acid

Data for the solubility of chlorogenic acids in water (mg/ml) at room temperature		
Compound	Solubility	Reference
1-CQA	at least 30	Scarpati et al.[34]
4-CQA	max. 20	Scarpati and Esposito[18] Corse et al.[40]
5-CQA	at least 20	Panizzi et al.[41]
1-GQA	at least 7·7	Haslam et al.[29]
3-GQA	at least 15	
5-GQA	at least 7·3	
1,3-diCQA	max. 0·6	Scarpati et al.[30]
3,4,5-triGQA	at least 18	
1,3,4,5-tetraGQA	at least 7·7	

- Stability: Chlorogenic acid is relatively stable under normal conditions but can undergo degradation under certain factors such as heat, light, and enzymatic activity. High temperatures, acidic or alkaline conditions, and prolonged exposure to UV light can lead to the degradation of CGA (Clifford, 1999; Farah, 2012).
- pH Dependence: The stability and solubility of Chlorogenic acid can be influenced by pH. It is more soluble in acidic conditions and less soluble in alkaline conditions. The presence of acid or base can also affect the stability and chemical reactivity of Chlorogenic acid (Clifford, 1999; Farah, 2012).
- Chirality: Chlorogenic acid is a chiral molecule, meaning it exists in two enantiomeric forms (D and L). The naturally occurring form found in plants is the L-enantiomer (Clifford, 1999; Farah, 2012).
- Melting point: The melting points of chlorogenic acids can be as low as 146°c and as high as 210°c depending on their crystalline form and purity. (Clifford, 1985).
- Boiling point: The boiling point of chlorogenic acid is about 407.55°c
- Density: The density of chlorogenic acid is around 1.28 g/cm3.
- Ultraviolet–visible absorption: Chlorogenic exhibit absorption in the Ultraviolet (UV) and visible (Vis) regions of the electromagnetic spectrum. It has characteristics of absorption maxima around 325 nm and 325-330, which can be used for its detection and quantification in analytical methods.
- Storage temperature: 2-8°C
- Predicted acidity potential (pKa): The pKa of chlorogenic acid is 3.3 (Liu and Renard, 2020)
- Colour: white to almost white
- Forms: Powder, crystals, needles, and/or chunks
- Molecular weight: The molecular weight of CGA is approximately 354.31 grams per mole.

2.6. Biosynthesis of Chlorogenic Acid

Chlorogenic acid is synthesized through the phenylpropanoid pathway, which involves the conversion of phenylalanine to cinnamic acid by phenylalanine ammonia-lyase (PAL) (Yan *et al*., 2016). Cinnamic acid is then converted to p-coumaric acid by cinnamate 4-hydroxylase (C4H) and then to caffeic acid by 4-coumarate: CoA ligase (4CL) (Walker *et al*., 2013). Finally, caffeic acid is converted to chlorogenic acid by hydroxycinnamoyl-CoA: quinate hydroxycinnamoyl transferase (HQT) (Ferro *et al*., 2017; Kaushik, 2020).

The synthesis of chlorogenic acid can be affected by various factors. For example, salinity stress has been shown to stimulate the synthesis of chlorogenic acid in honeysuckle leaves (Yan *et al*., 2016). In potatoes, the accumulation of chlorogenic acid isomers requires aromatic amino acids, which are produced from sugars (Torres-Contreras *et al*., 2014). Additionally, trans-cinnamic acid can be converted to caffeic and ferulic acids, while chlorogenic acid can be degraded to caffeic acid (Kulik *et al,* 2017).

Several genes and enzymes are involved in the synthesis of chlorogenic acid. For instance, hydroxycinnamoyl-CoA: quinate hydroxycinnamoyl transferase (HQT) and *p*-coumaroyl ester 3′-hydroxylase (C3′H) are involved in the synthesis of chlorogenic acid (Ferro *et al,* 2017). The hydroxycinnamoyl transferase (HCT) enzyme is also involved in the synthesis of chlorogenic acid (Walker *et al*., 2013). The expression of these genes has been detected in various plant species, including *Coffea arabica*, *Coffea canephora*, *Lonicera japonica*, *Erigeron breviscapus*, and eggplant (Koshiro *et al*., 2007; Yuan *et al*., 2014; Jiang *et al*., 2014; Gramazio *et al*.,2014). Moreover, the overexpression of the hydroxycinnamoyl CoA-quinate transferase (SmHQT) gene has been shown to increase the production of chlorogenic acid in eggplant (Kaushik, 2020). The regulation of chlorogenic acid accumulation during floral organ development in *Lonicera confusa* is mainly controlled by PAL and HQT, which are the rate-limiting enzymes in the biosynthesis of chlorogenic acid (Jiang *et al*., 2014).
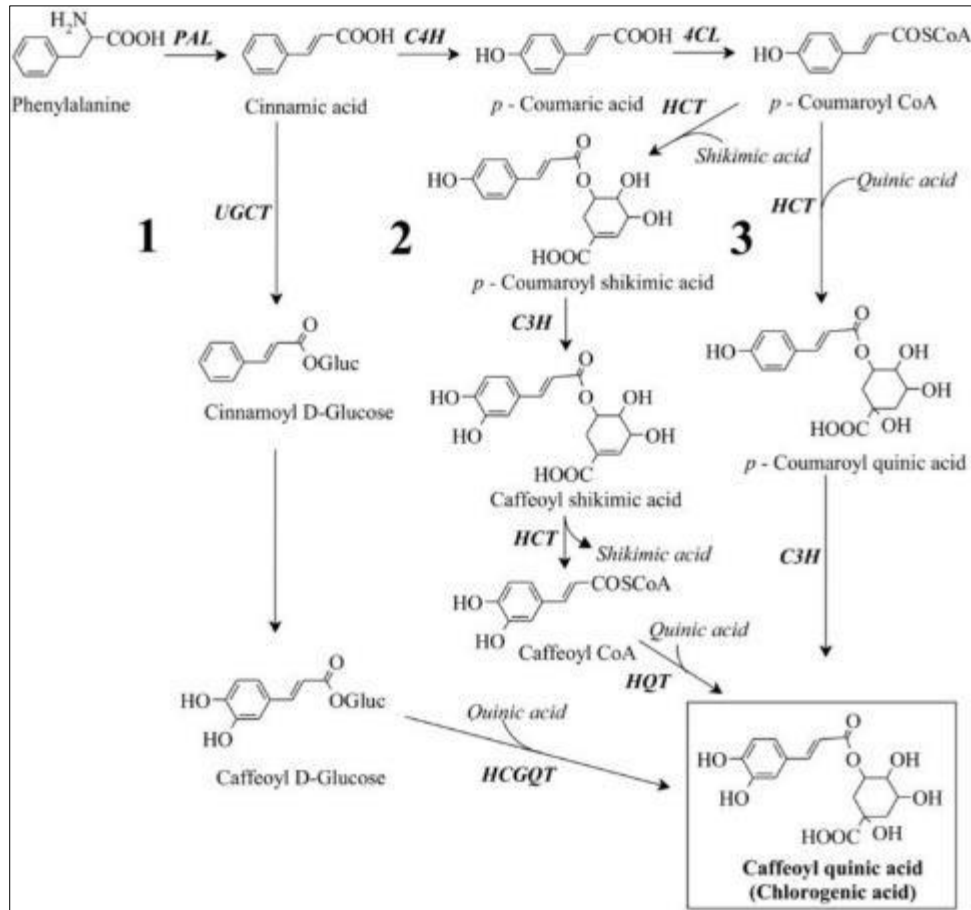
Figure 3 Pathways for Chlorogenic acid synthesis

Three proposed pathways for chlorogenic acid synthesis in plants (labelled 1, 2, and 3). PAL, phenylalanine ammonia-lyase; C4H, cinnamate 4-hydroxylase; 4CL, 4-coumarate CoA ligase; HCT, hydroxycinnamoyl CoA shikimate/quinate hydroxycinnamoyl transferase; UGCT, UDP glucose: cinnamate glucosyl transferase; HCGQT, hydroxycinnamoyl d-glucose: quinate hydroxycinnamoyl transferase. (Tuan *et al.*, 2014)

2.7. Why chlorogenic acid?

2.7.1. Uses and benefits

Phenolic acids are gaining more attention because of their several, practical, biological, and pharmaceutical benefits (Muhammad *et al.*, 2018). Chlorogenic acids are the most abundant and important class of naturally occurring polyphenols from plant sources in the human diet, exhibiting antioxidant, anti-inflammatory and antihypertensive functions (Muhammad *et al.,* 2018; Jilia *et al.*, 2018). CGAs have been found to have many other health benefits including reducing the risk of type 2 diabetes, obesity, Alzheimer's disease, eclampsia, and stroke. Other beneficial effects of CGA include antibacterial, hepatoprotective, neuroprotective, antiviral, antipyretic, cardioprotective, antimicrobial, free radical scavenger, and central nervous system stimulator. In addition, it has been found in some animal studies, to have insulin and lipid modulating functions.

2.7.2. Chlorogenic Acid as an Antioxidant

As an antioxidant, chlorogenic acid works by neutralizing free radicals in the body. Free radicals are unstable molecules that can damage cells and contribute to the development of chronic diseases such as cancer, heart disease, and Alzheimer's disease. By neutralizing these free radicals, chlorogenic acid helps to protect cells from damage and reduce the risk of these diseases (Farah, 2012; Oboh, and Ademiluyi, 2012). Several studies have investigated the antioxidant properties of chlorogenic acid. Wang *et al.* (2017), found that chlorogenic was effective at reducing oxidative stress in rats with liver damage. The study showed that chlorogenic acid was able to decrease levels of oxidative stress markers and improve liver function in rats. Another study found that it had a protective effect on human liver cells exposed to

oxidative stress. The study showed that chlorogenic acid was able to reduce oxidative stress and inflammation in the liver cells, which may help protect against liver damage and disease (Meng *et al.*, 2013).

2.7.3. Anti-inflammatory effects of Chlorogenic acid

In addition to its antioxidant properties, chlorogenic acid has also been shown to have anti-inflammatory effects. Chronic inflammation is a contributing factor to many chronic diseases, including cardiovascular disease, diabetes, and cancer (Farah, 2012; Tajik *et al.*, 2017). A study on mice with ulcerative colitis found that chlorogenic acid reduced inflammation, oxidative stress, and apoptosis in the colon. The study showed that chlorogenic acid was able to alleviate DSS-induced ulcerative colitis in mice, which significantly reduced tissue inflammation and apoptosis, and its mechanism was related to the MAPK/ERK/JNK signalling pathway. This suggests that chlorogenic acid may have potential therapeutic effects on ulcerative colitis (Gao *et al.*, 2019).

A study published in the Journal of Agricultural and Food Chemistry found that chlorogenic acid was able to reduce the inflammatory response in intestinal cells exposed to lipopolysaccharides. The study showed that chlorogenic acid was able to inhibit the production of pro-inflammatory cytokines and chemokines in the intestinal cells, which may help reduce inflammation and improve gut health (Li *et al.*, 2016). Another study was conducted to assess the antioxidant and anti-inflammatory properties of chlorogenic acid-mediated silver nanoparticles. The study found that the chlorogenic acid-mediated silver nanoparticles had significant anti-inflammatory and antioxidant activity. This suggests that chlorogenic acid may enhance the properties of silver nanoparticles and increase their potential health benefits. (Anu and Lakshminarayanan, 2020).

Chlorogenic acid, along with other phytochemicals, has been suggested as a main contributor against inflammation and oxidative stress. The study reviewed the potential health benefits of chlorogenic acid and other phytochemicals and found that they may help protect against metabolic syndrome, which is characterized by inflammation and oxidative stress.

2.7.4. Anti-microbial effect of chlorogenic acid

There is evidence to suggest that chlorogenic acid can disrupt bacterial cell membranes, leading to cell death. A study by Zaixiang *et al.* (2011) investigated the antibacterial activity of chlorogenic acid against *Staphylococcus aureus* and *Escherichia coli*. The results showed that chlorogenic acid caused damage to the bacterial cell membrane, leading to cell death. Chlorogenic acid has shown antimicrobial activity against a wide range of organisms, including bacteria, yeasts, moulds, viruses, and amoebas (Jesús *et al.*, 2011). The study by Arunkumar *et al.* (2013) supports the notion that chlorogenic acid has promise *in vitro* antibacterial and antibiofilm activities against *S. maltophilia.* Chlorogenic acid was found to be one of the main components of the water extract of green coffee beans under high pressure and demonstrated antibacterial activity against both Gram-positive (*Staphylococcus aureus and Listeria innocua*) and Gram-negative (*Escherichia coli* and *Salmonella enterica*) (Keisuke *et al.*, 2015). The mechanism by which chlorogenic acid exerts its antibacterial, antiviral, and antimicrobial effects is not fully understood, but it is thought to involve several different pathways. For example, chlorogenic acid has been shown to disrupt bacterial cell membranes, which can lead to cell death. It has also been suggested that chlorogenic acid may inhibit viral replication by interfering with viral entry into host cells (Zaixiang *et al.*, 2011; Tajik *et al.*, 2017).

2.7.5. Chlorogenic acid as a central nervous system stimulator and neuroprotector

Chlorogenic acid has been shown to have a potential role as a central nervous system stimulator in several studies. For example, Jun-Ming (2018), on the protective effects of chlorogenic acid against triptolide-induced hepatotoxicity found that it had a central nervous system stimulator effect. Chlorogenic acid has been shown to have a protective effect on nigral dopaminergic neurons in an experimental model of Parkinson's disease (Dehnad *et al.*, 2019). In a study by Adeyemo-Salami *et al.* (2021), the ameliorative potential of chlorogenic acid on rotenone-induced neurotoxicity in *Drosophila melanogaster* was evaluated. The results showed that CGA significantly improved the negative geotaxis assay and increased the survival rate of the flies. It has also been suggested that chlorogenic acid may regulate the ubiquitin-proteasome system proteins, which can protect cortical neurons from neuronal damage (Shah *et al.*, 2022). The mechanism by which chlorogenic acid exerts its central nervous system stimulator effects is not fully understood, but it is thought to involve several different pathways. For example, chlorogenic acid has been shown to have antioxidant and anti-inflammatory effects, which may contribute to its neuroprotective effects (Navabi *et al.*, 2017).
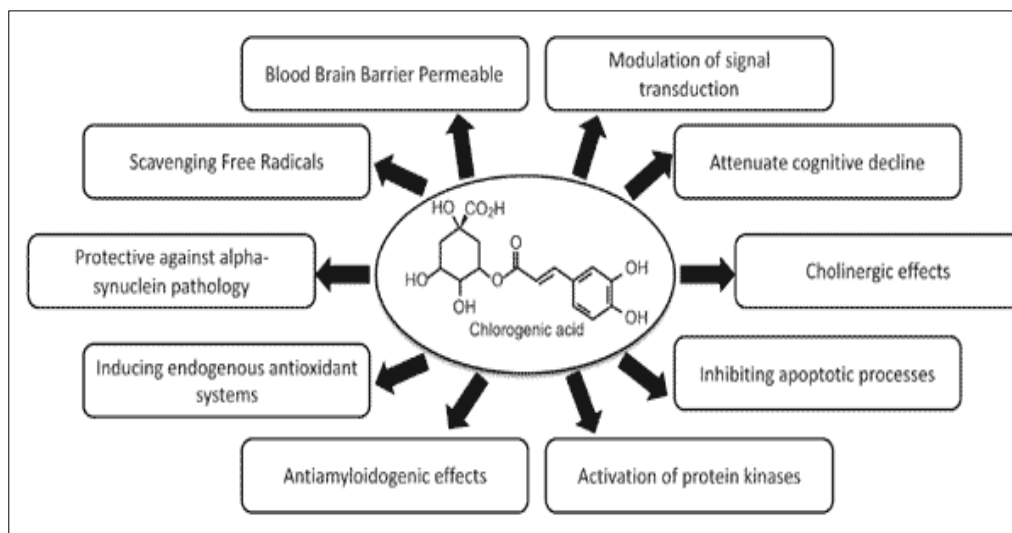
Figure 4 Protective effect of chlorogenic acid in the CNS (Navabi et al., 2017)

2.7.6. Antihypertensive effect of chlorogenic acid

Chlorogenic acid has been shown to have antihypertensive effects in several studies. For example, a recent study found that chlorogenic acid improved high-fructose-induced salt-sensitive hypertension in mice by moderating gut microbiota and bile acid metabolism. (Zhu *et al.*, 2022).

Another study found that chlorogenic acid increased nitric oxide-mediated vasodilation and improved endothelial function, leading to its antihypertensive effects (Kim and Je 2018).

2.7.7. Chlorogenic acid and obesity

Chlorogenic acid has been found to have potential benefits in reducing obesity. Wang *et al.* (2019), reported that chlorogenic acid can alleviate obesity and modulate gut microbiota in high-fat-fed mice. Shimoda *et al.* (2006), also suggested that green coffee bean extract, which contains chlorogenic acid, has an inhibitory effect on fat accumulation and body weight gain in mice. He *et al.* (2021), suggest that supplementation with chlorogenic acid can improve high-fat diet-induced obesity and associated glucose intolerance mainly by regulating food intake and energy expenditure. Zhong *et al.* (2020), suggested that chlorogenic acid can be developed as a lowering blood lipid and obesity treatment drug.

2.7.8. Chlorogenic acid and cosmetics

Chlorogenic acid has been found to have potential uses in cosmetics due to its biomedical activity and antioxidant properties (Dawidowicz and Typek, 2010; Rodrigues *et al.*, 2023). Specifically, it has been used as an exfoliant in cosmetic preparations (Handayani and Muchlis, 2021). Chlorogenic acid has also been found to exhibit anti-ageing and photoprotective abilities, making it a promising ingredient in skincare products (Rodrigues *et al.*, 2023). However, it is important to note that more research is needed to fully understand the potential benefits and limitations of using chlorogenic acid in cosmetics.

2.7.9. Hepatoprotective effect of chlorogenic acid

Chlorogenic acid has been found to have hepatoprotective effects in various studies. It is postulated that the hypo-cholesterolemic effect of chlorogenic acid is the primary beneficial effect that leads to other secondary beneficial effects such as atherosclero-protective, cardioprotective, and hepatoprotective functions. Chlorogenic acid has been shown to up-regulate the gene expression of PPAR-α, which is associated with the attenuation of fatty liver in hyper-cholesterolemic rats (Wan *et al.*, 2013).

According to Zhang *et al.* (2022), chlorogenic acid has been identified as one of the effective constituents that exert hepatoprotective effects, along with hyperoxide and astragalin. Long-term consumption of food-derived chlorogenic acid has been found to protect mice against acetaminophen-induced hepatotoxicity by promoting PINK1-dependent mitophagy and inhibiting apoptosis (Bang-yan *et al.*, 2022).

Studies have suggested that the polyphenolic fraction of coffee, which includes chlorogenic acids, may be responsible for the hepatoprotective effects of coffee (Friedrich *et al.*, 2016; Di Mauro *et al.*, 2021). Chlorogenic acid has been found to possess synergistic hepatoprotective effects with other compounds such as silymarin and melatonin (Al-Rasheed *et al.*, 2016).

2.7.10. Cardioprotective effect of Chlorogenic Acid

Chlorogenic acid has been found to have cardioprotective effects. It has been shown to lower cholesterol levels and attenuate fatty liver by up-regulating the gene expression of PPAR-α in hypercholesterolemic rats (Wan *et al.*, 2013). Chlorogenic acid has also been found to lower blood pressure acutely, which could benefit cardiovascular health if sustained (Mubarak *et al.*, 2012). Additionally, chlorogenic acid has antioxidant and anti-inflammatory effects, which could aid in the prevention of cardiovascular diseases (Ogbonna *et al.*, 2020).

2.7.11. Insulin and lipid modulating effect of chlorogenic acid

Chlorogenic acid has been found to have insulin and lipid-modulating effects in various studies. Chlorogenic acid has been shown to reduce lipid levels in plasma and alter mRNA expression of lipogenesis and lipolysis-related genes in adipose tissue (Wang *et al.*, 2019). Chlorogenic acid has also been found to have a glucose absorption inhibitory effect and modulate oxidative stress, while *p*-Coumaric acid has a glucose-lowering effect in streptozotocin (STZ)-induced diabetic rats (Eyenya *et al.*, 2020). Furthermore, chlorogenic acid has been found to decrease fasting plasma glucose and HbA1c by modulating the adiponectin receptor signalling pathways (Lee *et al.*, 2016).

Chlorogenic acid administration in patients with impaired glucose tolerance has been found to decrease fasting plasma glucose and insulin secretion while increasing insulin sensitivity and improving both anthropometric evaluations and the lipid profile (Zuñiga *et al.*, 2018). Chlorogenic acid has also been found to modulate glucose uptake and gastrointestinal hormones and insulin secretion in humans (Martina *et al.*, 2021). Finally, chlorogenic acid has been found to prevent high-fat diet-induced hepatic steatosis and inflammation by modulating gut microbiota and increasing glucagon-like peptide-1 secretion-related anti-inflammatory effects (Wang *et al.*, 2023; Latinwo et al, 2024).

3. Material and methods

3.1. Chemicals, Reagents, and Kits

All chemicals and reagents used during this project were procured from Sigma Aldrich (U.S.A), and SureChem (U.K.). Chemicals and reagents used during this experiment include the following: Ammonium ferrous Sulphate, Ascorbic acid, Ellman's Reagent (5,5'-dithiobis-(2-nitrobenzoic acid) or DTNB), Epinephrine (Adrenaline), Ethanol, Hydrochloric Acid, Hydrogen peroxide, Potassium Chloride, Tris Buffer, Phosphate buffer, Sodium hydroxide, Sodium carbonate, Sodium-Potassium tartrate, Copper sulfate pentahydrate, Folin-ciocalteu reagent, Dipotassium hydrogen phosphate trihydrate, Potassium dihydrogen phosphate, 1-Chloro-2,4-dinitrobenzene (CDNB), Reduced Glutathione (GSH), Sulphosalicylic acid, Sulphuric acid, Trichloroacetic acid (TCA), Sodium azide, Dipotassium hydrogen orthophosphate, Thiobarbituric acid, Xylenol orange. Aspartate Aminotransferase (AST), Alanine Aminotransferase (ALT), Alkaline phosphate (ALP), Bilirubin, Albumin, and Gamma-glutamyl transferase (GGT) kits were obtained from Fortress Laboratories, UK.

3.1.1. Apparatus/Instrument

The following apparatus and instruments were used during this experiment; Filter papers, conical flasks, beakers, measuring cylinders, glass stirrer, Eppendorf tubes, test tube racks, sample bottles, pipette tips, automatic micropipette, oral cannulas, syringes and needles, dissecting set, dissecting board, plane bottles, capillary tubes, cotton wool, tissue paper, hand gloves, face masks, masking tapes, markers, cuvettes, tabletop centrifuge, cold ultracentrifuge, UV-Visible spectrophotometer, pH meter, water bath, weighing balance, incubator, refrigerator, freezer, and homogenizer. The blood samples were assayed using the Mindray BC 3000 Auto-haematology analyser (China).

3.1.2. Test Phytonutrient/ Food supplement

Chlorogenic acid (AK Scientific, U.S.A) was used.

3.1.3. Experimental Animals

Ethical approval was granted by the Animal Care and Use Research Ethics Committee of the University of Ibadan and the number UI-ACUREC 19/095 was assigned.

Ninety-six male Wistar rats weighing between 17 g to 26 g were purchased. The animals were housed in suitable specially crafted cages in a well-ventilated animal house of the Faculty of Basic Medical Sciences, University of Ibadan where they were provided with rat feed and water. The animals were housed in 8 cages (12 per cage) and subjected to natural photoperiod of 12 hours of light and 12 hours of darkness daily.

3.2. Experimental Design and Treatments

The experimental animals were divided into eight groups of 12 animals per group based on their weights. Before treatment, the experimental animals were allowed two weeks of acclimatization where they were only provided with rat feed and sufficient water. After an overnight fast with free access to water, the animals were then treated by oral gavage with the following protocol once;

- Group 1 (Control): received distilled water alone
- Group 2 (vehicle): received 1% ethanol alone.
- Group 3: received chlorogenic acid at a dose of 30mg/kg
- Group 4: received chlorogenic acid at a dose of 60mg/kg
- Group 5: received chlorogenic acid at a dose of 120mg/kg.
- Group 6: received chlorogenic acid at a dose of 240 mg/kg.
- Group 7: received chlorogenic acid at a dose of 480 mg/kg.
- Group 8: received chlorogenic acid at a dose of 1000mg/kg.

The animals were observed for mortality 24 hours post-treatment and for 14 days post-treatment for delayed toxicity. The weight was also monitored weekly throughout the study

3.3. Sacrifice of Experimental Animals

Twenty-four hours after the treatment, six (6) of the animals were sacrificed. The animals' blood was first collected via ocular puncture into K_2-EDTA sample bottles for haematological analysis and then sacrificed by cervical dislocation. Thereafter, the liver and kidneys of the animals were immediately excised, weighed, and processed for biochemical, and histological analyses.

3.4. Homogenization of the Organs

Harvested liver samples were rinsed in ice-cold 1.15% KCl solution (washing buffer), blotted with filter paper, and weighed to determine their initial weights. Thereafter, the liver samples were sectioned for histological examination and submerged in 10 %formalin. The remaining portions of the harvested liver were homogenized with homogenizing buffer (pH 7.4) using a Teflon homogenizer. The homogenates gotten were then centrifuged at 10,000 revolutions per minute for 15 minutes in an ultracentrifuge (4ºC) to obtain the supernatant. After centrifugation, the supernatants were collected and used for biochemical and histopathology analyses.

3.5. Preparation of reagents for sacrifice

- Washing Buffer (1.15%Potassium chloride)

11.5 g of potassium chloride (SureChem, U.K.) was dissolved in and made up to 1000 ml with distilled water. This buffer was stored at 4°C.

- Formalin (10%)

10 ml of formaldehyde was dissolved in distilled water and made up to 100 ml with distilled water.

- Homogenizing buffer (50mM Tris-HCL, 1.15% KCL, pH 7.4)

7.80g of Tris (hydroxyl methyl) amino ethane (Sigma Chemicals, St. Louis, U.S.A.) and 11.5g of potassium chloride were dissolved in 900ml of distilled water, the pH adjusted to 7.4 and then made up to 1 litre with distilled water. The buffer was stored at 4°C.

3.6. Determination of total body and relative organ weights

The total body weight of each rat was determined using a digital balance before and at weekly intervals throughout the experimental period. Changes in weights weekly were also expressed as percentage weight increases.

Percentage weight increase was calculated from the formula:

$$\frac{W_y - W_x}{W_x} \times 100$$

Where W_x = Initial mean total body weight

W_y = Final mean total body weight

The weights of harvested organs of respective rats were measured with a digital balance and presented as a percentage of liver weight per total body weight. This was calculated from the formula:

$$\frac{Liver\,Weight}{W_y} \times 100$$

3.7. Biochemical assays

- Liver Function Test
    - Aspartate Aminotransferase (AST)
    - Alanine Aminotransferase (ALT)
    - Alkaline Phosphatase (ALP)
    - Gamma-glutamyltransferase (GGT)
    - Bilirubin
    - Albumin
- Antioxidant assays
    - Determination of Catalase activity
    - Determination of Superoxide dismutase (SOD) activity
    - Assay for the reduced Glutathione (GSH) level
    - Estimation of Glutathione –S- Transferase (GST) activity
    - Determination of Ascorbic Acid level
    - Assay for Glutathione Peroxidase (GPx) activity
- Oxidative Stress Indices
    - Estimation of Lipid peroxidation
    - Determination of Hydrogen Peroxide generation
- Histological Examination of Organs

3.7.1. Liver Function Test

Measurement of Aspartate Aminotransferase (AST) Activity

Principle
In the reaction, the AST also known as serum glutamic oxaloacetic transaminase (SGOT) catalyses the reversible transamination of L-aspartate and α-ketoglutarate to oxaloacetate and L-glutamate. The oxaloacetate is then reduced to malate in the presence of malate dehydrogenase with the concurrent oxidation of NADH to NAD. AST measurements are used in the diagnosis and treatment of certain types of liver and heart diseases.

$$\alpha\text{-oxoglutarate} + \text{L-aspartate.} \xrightarrow{\text{AST}} \text{L-glutamate} + \text{Oxaloacetate}$$

Fortress diagnostics kit was used to determine AST activity in the samples.

3.7.2. Reagents and their concentration:

- Reagent 1 (Buffer)
    - 100 mmol/l of phosphate buffer
    - 200 mmol/l of L-aspartate

- o 2.0 mmol/l α- oxoglutarate.
- Reagent 2 Dye Reagent
  - o 2.0 mmol/l of 2,4 dinitrophenylhydrazine
- Reagent 4 Pyruvate Standard
  - o 2.0 mmol/l of Pyruvate
- Reagent 6 Sodium Hydroxide (4.0 mol/l)
  - o 1 bottle of Sodium Hydroxide made up to 1000ml

**Table 2** Procedure for determination of AST levels

	Reagent blank	Sample
Sample (ml)	-	0.1
(R1) AST Buffer (ml)	0.5	0.5
Distilled water (ml)	0.1	
Thoroughly mix and incubate for 30mins at 37°C		
(R2) Dye Reagent (ml)	0.5	0.5
Mix, allow to stand for 20mins at 20 - 25°C		
Diluted NaOH (ml)	5.0	5.0

Samples were mixed and absorbance was read against blank after 5 minutes at 546 nm with a UV- visible spectrophotometer

Measurement of Alanine Aminotransferase (ALT) Activity

Principle

In the reaction, ALT catalyses the reversible transamination of L-alanine and α-ketoglutarate to pyruvate and L-glutamate. The pyruvate is then reduced to lactate in the presence of lactate dehydrogenase (LDH) with the concurrent oxidation of NADH to NAD.

$$α\text{-oxoglutarate} + \text{Alanine} \rightarrow \text{L-glutamate} + \text{Pyruvate}$$
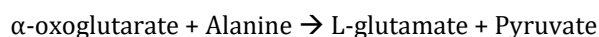
Fortress diagnostics kit was used to determine ALT activity in the samples.

3.8. Reagents and their concentration

- Reagent 1 (Buffer)
  - o 100mmol/L of phosphate buffer
  - o 200 mmol/L of L-alanine
  - o 2 mmol/L of α-oxoglutarate
- Reagent 2 (Dye Reagent)
  - o 2 mmol/L of 2, 4-dinitrophenylhydrazine
- Reagent 4 Pyruvate Standard
  - o 2.0 mmol/l of Pyruvate
- Reagent 6 Sodium Hydroxide (4.0 mol/l)
  - o 1 bottle of Sodium Hydroxide made up to 1000ml

Table 3 Procedure for determination of ALT level

	Sample blank	Sample
Sample (µl)	-	100
(R1) Buffer (µl)	500	500
Mix and incubate for 30mins at 37°C in a water bath		
(R2) Dye Reagent (µl)	500	500
Sample (µl)	100	-
Samples were mixed and allowed to stand for 20mins at 25 - 25°C		
Diluted NaOH (ml)	5.0	5.0

Samples were mixed and absorbance was read against blank after 5 minutes at 546 nm with a UV- visible spectrophotometer.

Measurement of Alkaline phosphatase (ALP) Activity

Principle

The alkaline Phosphatase acts on the AMP-buffered sodium thymolphthalein monophosphate. The addition of an alkaline reagent stops enzyme activity and simultaneously develops a blue chromogen, which is measured at 590nm. It can be a useful indicator for various bone diseases, renal diseases, and some liver disease conditions.

Fortress diagnostics kit was used to determine ALP activity in the samples.

3.8.1. Reagents and their concentration:

- R1: Substrate
  - 3.6mM of Sodium Thymolphthalein Monophosphate
  - 0.2M of 2- Amino-2-Methyl-1-Propanol Buffer pH10.2
  - 1.0mM of Magnesium Chloride
- R2: Colour Reagent
  - 0.09M of Sodium Hydroxide
  - 0.1m of Sodium carbonate
- R4: Standard of 50U/L

Table 4 Procedure for determination of ALP level

	Reagent blank(µl)	Standard/Sample(µl)
R1 Substrate	500	500
Equilibrate at 37ºC for 3 minutes		
DDH$_2$O	50	-
Standard/ Sample	-	50
Incubate for exactly 10 minutes at 37ºC		
R2 Colour Reagent	2500	2500

The resultant solutions were mixed and absorbance was read against a reagent blank at 590 nm with a UV- visible spectrophotometer.

Calculation

Abs of unknown/Abs of standard × Value of Std (IU/L) = Unknown (IU/L)

Measurement of Gamma Glutamyl Transferase (GGT) Activity

Principle
The substrate L- y-glutamyl-3-carboxy-4-nitroanilide, in the presence of glycylglycine, is converted to 5-amino-2-nitrobenzoate by y-GT measured at 405nm. The increase in absorbance is proportional to y-GT activity.

L- y-glutamyl-3-carboxy-4-nitroanilide + glycylglycine → L- y- glutamylglycylglcine + 5-amino-2-nitrobenzoate
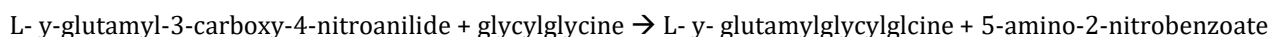
Elevated levels of y-GT are found in many forms of liver disease including primary and secondary liver cancer. Increased levels are also found in cases of alcohol abuse and liver cirrhosis. y-GT is the most sensitive enzymatic marker of hepatobiliary disease.

Fortress diagnostics kit was used to determine GGT activity in the samples

3.8.2. Reagents and their concentration

- R1: Buffer /Glycylglycine
  - 100mmol/l of Tris buffer pH 8.25
- R2: Substrate
  - 2.9 mmol/l of L-γ-glutamyl-3-carboxy-4-nitroanilide

Table 5 Procedure for determination of GGT activity

	Blank (µl)	Sample (µl)
Sample	-	100
Working Reagent	1000	1000

The resultant solutions were mixed well and allowed to stand for 1 minute at 25ºC. Measurement of the absorbance of the samples was taken at 405 nm per minute for 3 minutes.

Calculations
GGT activity (U/l) =1158 x ΔA 405 nm/min

Measurement of Albumin Level

Principle
This test is used for the quantitative determination of albumin in serum. Serum Albumin binds with the bromocresol green indicator in an acid medium to form a green BCG complex, the amount of which produced is directly proportional to the albumin concentration present in the sample. Albumin has two main functions: to maintain water balance in serum and plasma and to transport a variety of ligands, for example, calcium, bilirubin, and hormones.

Fortress diagnostics kit was used to determine the Albumin level in the samples

3.8.3. Reagents and their concentration

- BCG Reagent
  - 0.15 mmol/l of Bromocresol Green
  - Succinate Buffer
- 45 g/l (4.5 g/dl) of Standard

Table 6 Procedure for determination of Albumin level

	Blank	Standard/Sample
DDH$_2$O(µl)	5	-
Standard/Sample(µl)	-	5

| BCG reagent (ml) | 1 | 1 |

The resultant solutions were mixed well and allowed to stand for 5 minutes at 25ºC. Measurement of the absorbance of the calibrator and samples against the reagent blank was taken at 578 nm.

Calculations

$$\text{Albumin Concentration in g/l} = \frac{\text{Sample absorbance}}{\text{Standard absorbance}} \times \text{Standard concentration}$$

Measurement of Total Bilirubin Level

Test Principle
The colorimetric method is based on that described by Jendrassik and Grof (1938). Direct (conjugated) bilirubin reacts with diazotized sulphanilic acid in an alkaline medium to form a blue-coloured complex. Total bilirubin is determined in the presence of caffeine, which releases albumin-bound bilirubin, by the reaction with diazotized sulphanilic acid.

Randox kit was used to determine the Bilirubin level in the samples

Reagents and their concentration

- R1:   29 mmol/l of Sulphanilic acid
  - 0.17 N of Hydrochloric acid
- R2:   38.5mmol/l of Sodium Nitrite
- R3:   0.26 mol/l of Caffeine
  - 0.52 mol/l of Sodium benzoate
- R4:   0.93 mol/l of Tartrate
  - 1.9 N of Sodium Hydroxide

Table 7 Procedure for determination of Bilirubin level

	Sample blank	Sample
Reagent 1	200 µl	200 µl
Reagent 2	-	50 µl
Reagent 3	1000 µl	1000 µl
Sample	200 µl	200 µl
Mix, and incubate for 10 min at 20-25ºC		
Reagent 4	1000 µl	1000 µl

The resultant solutions were mixed, and incubated for further 5-30 minutes at 25ºC and the measurement of the absorbance was taken at 578 nm.

Calculation
Total Bilirubin (mg/dl) = 10.8 × A_{TB} (578 nm)

3.8.4. Determination of Total Protein Concentration

The protein concentration of the Liver and kidney homogenate supernatants was determined using the method of Lowry *et al.* (1951).

Principle

The Reagent Folin-ciocalteau used in this method contains phosphomolybdic acid and tungstate. The aromatic amino acids, tyrosine and tryptophan present in the protein react with Folin's reagent to produce a dark blue colour

Reagents

- Alkaline copper reagent
  - Reagent A: 2 g of sodium carbonate was dissolved in and made up to 100 ml with 0.1N Sodium hydroxide solution.
  - Reagent B: 10 mg of copper sulfate was dissolved in 2.0 ml of 4% sodium Potassium tartrate solution.
  - 50 ml of reagent A and 1 ml of reagent B were mixed fresh at the time of the assay.
- Sodium hydroxide (0.1 N)
  - 0.8 g of NaOH was dissolved in and made up to 200 ml with distilled water and labelled as reagent A
- Sodium Potassium Tartrate (4%)
  - 0.4 g of Na-K Tartrate was dissolved in and made up to 10 ml with distilled water and labelled as reagent B.
- 1% $CuSO_4.5H2O$
  - g of $CuSO_4.5H_2O$ was dissolved in 10 ml of distilled H_2O and labelled as reagent C.
- Folin-Ciocalteau Reagent (1 N)
  - A solution of 1 ml/5 ml of Folin-C was prepared. This was done by adding 40 ml of water to 8 ml of Folin-C, to give 48 ml of Folin-C solution.

3.9. Standard protein (BSA) curve

A 6 ml stock solution of Bovine Serum Albumin was prepared. Serial dilutions of the solutions were placed in two groups of 5 test tubes each and numbered 1-5. 5 ml of the alkaline solution was added into the protein standard solution. The mixture was incubated at room temperature for 20 minutes after which 0.5 ml of Folin's reagent was added and incubated at room temperature for 30 minutes, after which the absorbance was read in a spectrophotometer using a glass cuvette at 650 nm against a blank containing no BSA. A curve of absorbance against protein concentration was plotted.

Table 8 Protocol for protein estimation

Test tube No.	1	2	3	4	5
Stock BSA (ml)	0.20	0.40	0.60	0.80	1.00
Distilled water (ml)	0.80	0.60	0.40	0.20	-
Alkaline $CuSO_4$ (ml)	5.00	5.00	5.00	5.00	5.00
Incubation for 20 minutes at room temperature					
Folin's reagent (ml)	0.50	0.50	0.50	0.50	0.50

The mixture incubated for 30 minutes at room temperature

3.10. Procedure for the determination of protein in samples

The liver and kidney homogenates were diluted 10 times with distilled water. To 1.0 ml of the diluted supernatant, 5 ml of alkaline copper reagent was added, vortexed, and allowed to stand for 20 minutes. 500 µl of Folin's phenol reagent was added and incubated for 30 minutes at room temperature. The blue colour formed is measured at 650 nm in the spectrophotometer. A blank without the protein was used. The protein content of the samples was extrapolated from the standard curve and multiplied by 10 to get the actual amount of supernatant.

3.10.1. Antioxidant Assays

Determination of Catalase Activity

Catalase activity was determined according to the method of Claiborne (1985).

Principle
The method is based on the loss of absorbance observed at 240 nm as catalase splits hydrogen peroxide. Although hydrogen peroxide has no absorbance maximum at this wavelength, its absorbance correlates well enough with concentration to allow its use for a quantitative assay. An extinction coefficient of 0.0394 $mM^{-1}cm^{-1}$ (Noble and Gibson, 1970) was used.

Reagents
- Phosphate buffer (0.05 M, pH 7.4)
  - 16.044 g of K2HPO4 and 7.88 g of KH2PO4 were dissolved and made up to 300 ml distilled water.
- Hydrogen peroxide (19 mM)

430 μl of 30% H_2O_2 was mixed in 100 ml of 0.05 M phosphate buffer, pH 7.

Procedure
And 50 μl of the sample was added to a 2.95 ml Hydrogen peroxide/phosphate buffer mixture in a quartz cuvette and mixed by inversion. The absorbance was read at 240 nm at 0 and 1-minute intervals for 5 minutes.

Calculation

$$\textbf{Catalase activity} = \frac{(\Delta A240/\min)\ x\ volume\ of\ the\ mixture}{0.0394\ x\ volume\ of\ sample\ x\ mg\ protein}$$

Where 0.0394 is the millimolar extinction coefficient of H_2O_2 at 240 nm

Volume of mixture = 3 ml

Volume of sample = 50 μl

Change in absorbance / min = (Abs$_0$ – abs$_5$) / 5

Unit = mmole H_2O_2 consumed/min/mg protein.

Determination of Superoxide Dismutase (SOD) Activity

The activity of SOD was determined by the method of Misra and Fridovich (1972).

Principle
The ability of SOD to inhibit the autoxidation of epinephrine at pH 10.2 makes this reaction a basis for a simple assay for this dismutase. Superoxide radical causes the oxidation of epinephrine to adrenochrome and the yield of adrenochrome produced per superoxide radical introduced increases with increasing pH and concentration of epinephrine.

Reagents

- 0.05 M Carbonate buffer (pH 10.2)
  - 3.58 g of Na2CO3.10H2O and 1.05 g of NaHCO3 were dissolved in 200 ml of distilled water. The pH was adjusted to 10.2 and then made up to 250 ml with distilled water.
- 0.3 mM Epinephrine
  - 0.0125 g of epinephrine was dissolved in 50 ml of distilled water containing 0.5 ml of concentrated HCl.

Procedure
10 μl of the sample was added to 2.5 ml of 0.05 M carbonate buffer (pH 10.2) and 0.3 ml of epinephrine in a cuvette, mixed by inversion, and change in absorbance was monitored every 30 sec for 2 minutes at 480 nm. The reference cuvette was the same as for the samples with water replacing the samples.

Calculation
Increase in absorbance per minute = $\frac{Abs3 - Abs0}{2.5}$

Where A$_0$ is the absorbance at 0 seconds

Abs$_3$ is the absorbance at 150 seconds

% inhibition = 100 – (100 x $\frac{Increase\ in\ absorbance\ per\ min\ for\ substrate}{Increase\ in\ absorbance\ per\ \min\ for\ blank}$)

1 unit of SOD activity was given as the amount of SOD necessary to cause 50% inhibition of the autooxidation of epinephrine.

Assay for the Reduced Glutathione (GSH) Level

The method of Beutler *et al.* (1963) was appropriated/employed.

Principle
The reduced form of glutathione comprises in most instances the bulk of cellular non-protein sulfhydryl groups. This method is therefore based upon the development of arelatively stable yellow colour when 5',5'-dithiobis-(2-nitrobenzoic acid) (Ellman's reagent) is added to sulfhydryl compounds. The chromophoric product resulting from the reaction of Ellman reagent with the reduced glutathione, 2-nitro-5-thiobenzoic acid possesses a molar absorption at 412 nm. Reduced GSH is proportional to the absorbance at 412 nm.

- This method has the following advantages over the earlier modification of the nitroprusside method:
- The precipitation process is carried out with a single easily prepared reagent. It does not require the addition of solid sodium chloride or prolonged shaking.
- The determination may be carried out at any temperature likely to be encountered in the laboratory.
- The colour formed is relatively stable.
- The reagent for colour development is stable for many weeks
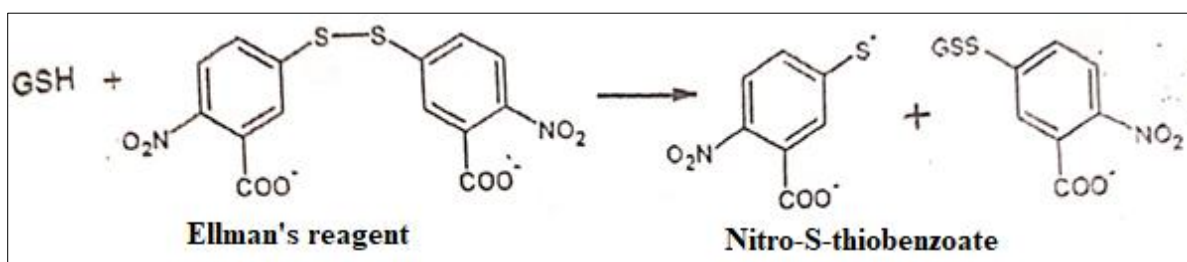- The sensitivity of the method is so great that it may readily be adapted to a micro-procedure

Figure 5 Reaction of reduced GSH with Ellman's reagent

Reagents
GSH working standard (stock)

4 mg GSH (Mol. Wt.307.3 g) was dissolved in 10 ml of 0.1 M phosphate buffer, pH 7.4, and then stored in the refrigerator.

Phosphate buffer (0.1 M, pH 7.4)

17.90 g of $NaHPO_4.12H_2O$ (Mol. Wt. 358.22) was dissolved in 500 ml of distilled water.

3.90 g of $NaH_2PO_4.2H_2O$ (Mol. Wt. 156.03) was dissolved in 250 ml of distilled water. Finally, solutions (a) and (b) were added together and the pH was adjusted to 7.4

Ellman Reagent [5',5'-Dithiobis-(2-nitrobenzoate)-DTNB]

240 mg of DTNB was dissolved in 0.1 M phosphate buffer and made up to 600 ml

Precipitating agent

4% Sulphosalicyclic acid ($C_7H_6S.2H_2O$; Mol. Wt. 254.22) was prepared by dissolving 4 g of sulfosalicylic acid in 100 ml of distilled water.

Procedure

Serial dilutions of the GSH working standard were prepared as shown in the table below

Table 9 Preparation of GSH standard curve

GSH working Standard(ml)	Phosphate buffer(ml)	DTNB (ml)	Absorbance (412mm)	GSH Conc. (µg/ml)
0.02	0.48	4.50	0.065	8
0.05	0.45	4.50	0.177	20
0.10	0.40	4.50	0.383	40
0.20	0.30	4.50	0.695	80
0.30	0.20	4.50	1.109	120
0.40	0.10	4.50	1.514	160

3.10.2. Determination of GSH concentration in samples

800 ul of the Supernatant (since the assay would be done in duplicates) was deproteinated by the addition of an equal volume of 4 % sulfosalicylic acid. This was centrifuged at 4,000 x g for 5 minutes. Thereafter, 0.5 ml of the supernatant was added to 4.5 ml of Ellman reagent. A blank was prepared with 0.5 ml of the diluted precipitating reagent (1 ml of distilled water: 1 ml of precipitating reagent) and 4.5 ml of Ellman reagent.

Reduced glutathione (GSH) level is proportional to the absorbance at 412 nm.

Determination of Glutathione S-Transferase Activity

Glutathione S-transferase activity was determined according to Habig *et al.*, (1974).

Principle
The assay is based on the principle that all known glutathione S-transferase isotypes demonstrate a relatively high activity with 1-chloro-2,4-dinitrobenzene (CDNB) as the second substrate. When CDNB is conjugated to reduced glutathione, its absorption maximum shifts to a longer wavelength and the absorption increase at the new wavelength of 340 nm provides a direct measurement of the enzymatic reaction.

Reagents
- **1-Chloro-2,4-dinitrobenzene (20 mM)**

16.85 mg of 1-chloro-2,4-dinitrobenzene (CDNB) was dissolved in 5 ml of absolute ethanol.

- **Reduced Glutathione (0.1 M)**

30.73 mg of glutathione (GSH) was dissolved in 1 ml of 0.1 M phosphate buffer (pH 6.5).

- **Phosphate buffer (0.1 M, pH 6.5)**

Dipotassium hydrogen phosphate trihydrate (0.381 g) and potassium dihydrogen phosphate (1.134 g) were dissolved in 90 ml of distilled water, the pH was adjusted to 6.5 and the volume made up to 100 ml with distilled water.

Procedure
The medium for the estimation was prepared as shown in the table below and the reaction was allowed to run for 3 min with readings taken every 60 seconds against the blank at 340 nm.

Table 10 Glutathione S-Transferase Assay Medium

Reagent	Blank	Test
Reduced glutathione (GSH) (µl)	30	30
CDNB (20 mM) (µl)	150	150
Phosphate buffer, pH 6.5 (ml)	2.8	2.8
Distilled water (µl)	30	-
Sample (µl)	-	30

Calculations

ΔABS, Change in absorbance = $Abs_{180} - Abs_0$

Where Abs_0 is the absorbance at 0 seconds

Abs_{180} is the absorbance at 180 seconds (3 minutes)

GST activity = $\Delta ABS \times 3.47$ / mg protein

= µmole/min/mg protein

Determination of Glutathione Peroxidase Activity

Glutathione peroxidase (GPx) activity was measured according to the procedure of Rotruck *et al*. (1973) with some modifications.

Principle

Glutathione peroxidase is allowed to conjugate hydrogen peroxide to glutathione for a fixed period after which the reaction is quenched. The remaining glutathione is reacted with Ellman's reagent and the GSH consumed is then used as a measure of enzyme activity.

Reagents

- **Sodium azide (10 mM)**

1.20 mg of sodium azide was dissolved in 20 ml of distilled water.

- **Reduced glutathione (4 mM)**

1 mg of GSH was dissolved in 100 ml of 0.1 M phosphate buffer, pH 7.4.

- **Hydrogen peroxide (2.5 mM)**

28 µl of 30% hydrogen peroxide was dissolved in 100 ml of distilled water.

- **Trichloroacetic acid (10%)**

10 g of TCA was dissolved in distilled water and the volume made up to 100 ml with the same.

- **Dipotassium hydrogen orthophosphate (0.3 M)**

13.08 g of $K_2HPO_4.3H_2O$ was dissolved in distilled water and the volume made up to 250 ml with the same.

- **5'-5'-dithiobis-(2-dinitrobenzoic acid) (DTNB)**

0.06 mg of DTNB was dissolved in 150 ml of 0.1 M phosphate buffer, pH 7.4.

- **Phosphate buffer (0.1 M, pH 7.4)**

Dipotassium hydrogen phosphate trihydrate (1.736 g) and potassium dihydrogen phosphate (3.406 g) were dissolved in 350 ml of distilled water, the pH adjusted to 7.4 and the volume made up to 100 ml with distilled water.

Procedure
To 50 μl of phosphate buffer in a test tube was added 10 μl of NaN_3, 20 μl of GSH, 10 μl of H_2O_2, and 50 μl of the sample (added last). The reaction mixture was incubated for 3 minutes at 37°C after which 50 μl of TCA was added and the final mixture was centrifuged at 3000 rpm for 5 minutes. To 50 μl of the supernatants, 100 μl of K_2HPO_4 and 50 μl of DTNB were added and the absorbance read against a reagent blank of 50 μl distilled water, 100 μl of K_2HPO_4 and 50 μl of DTNB at 412 nm.

Calculation
GSH consumed = initial GSH amount (254.34) – GSH remaining

GPx activity = GSH consumed/mg protein

= μg GSH/mg protein

Determination of Ascorbic Acid Level

Ascorbic acid (Vitamin C) concentration in the samples was determined according to the method of Jakota and Dani (1982).

Principle
Ascorbic acid present in biological samples reacts with Folin's reagent, an oxidizing agent to give a blue colour which has maximum spectrophotometric absorption at 760 nm. The complete absence of the interference of even those substances which interfere in protein estimation by the Folin's reagent may be due to either dilution of the Folin's reagent or too acidic pH. The dissociation of phosphate from molybdate leads to the disappearance of the yellow colour of phosphomolybdate which decreases the reactivity of the Folin's reagent. Only strong reductants like ascorbic acid can react with Folin's reagent under these conditions and any interference by other possible substances is eliminated.

Reagents
- **10% Trichloroacetic acid (TCA)**

10 g of TCA was dissolved in distilled water and made up to 100 ml.

- **Folin- Ciocalteu reagent.**

A commercially prepared Folin's reagent of 2.0 M was diluted 10-fold with double distilled water.

- **Ascorbic acid standard solution (stock)**

0.1 g of ascorbic acid was dissolved in distilled water and made up to one litre to give a final concentration of 100 μg ascorbic acid/ ml.

- **Preparation of Ascorbic acid standard curve**

A standard curve was prepared by taking varying concentrations of standard solutions of ascorbic acid in water. To this, 0.8 ml of 10% TCA was added and vigorously shaken, incubated in an ice bath for 5 minutes, and centrifuged at 3000 xg for 5 minutes. The supernatant of the range was withdrawn and diluted to 2.0 ml using double distilled water. 0.2 ml of diluted Folin's reagent was added and the tubes were vigorously shaken and incubated in an ice bath for 10 minutes. The absorbance of the blue colour developed was measured at 760 nm.

Table 11 Procedure for ascorbic acid (vitamin C) standard curve

Test-tube	1	2	3	4	5	6	7
Ascorbic acid(ml)	-	0.05	0.1	0.2	0.3	0.4	0.5
TCA (ml)	0.8	0.8	0.8	0.8	0.8	0.8	0.8
Vortex, incubate in ice for 5 minutes, and centrifuge at 3000 x g for 5 minutes							
The supernatant (ml)	-	0.05	0.1	0.2	0.3	0.4	0.5
Distilled water (ml)	2.0	1.95	1.9	1.8	1.7	1.6	1.5
Folin's reagent (ml)	0.2	0.2	0.2	0.2	0.2	0.2	0.2
Ascorbic acid conc. (μg/ml)	-	5	10	20	30	40	50
Absorbance (760 nm)	-	0.002	0.008	0.026	0.069	0.092	0.13

3.10.3. Estimation of the ascorbic acid level in test samples

The same procedure employed for the standard curve was used in the determination of ascorbic acid concentration in the test samples. 500 μl of the homogenates was used in place of the ascorbic acid standard.

Estimation of Lipid Peroxidation

Lipid peroxidation was determined by measuring the levels of malondialdehyde produced during lipid peroxidation according to the method described by Varshney and Kale (1990).

Principle
This method is based on the reaction between 2-thiobarbituric acid (TBA) and malondialdehyde: an end product of lipid peroxide during peroxidation. On heating in acidic pH, the product is a pink complex that absorbs maximally at 532 nm and which is extractable into organic solvents such as butanol. Malondialdehyde (MDA) is often used to calibrate this test and thus the results are expressed as the amount of free MDA produced.
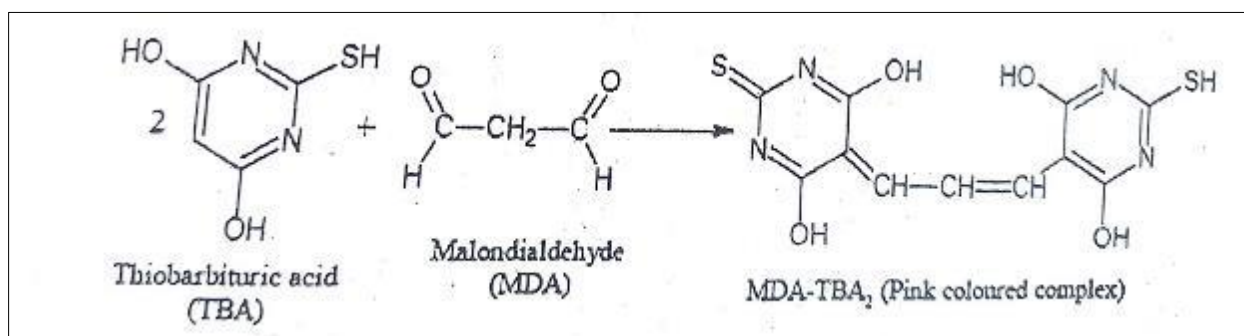

Figure 6 MDA reaction in lipid peroxidation assay

Reagents
- **30% Trichloroacetic acid (TCA)**

9 g of TCA was dissolved in distilled water and made up to 30 ml with the same

Caution: Weigh TCA into a glass beaker and not on aluminium foil and always use gloves when weighing.

- **0.75% Thiobarbituric acid (TBA)**

This was prepared by dissolving 0.225 g of TBA in 0.1 M HCl and made up to 30 ml with the same.

Note: Warm the solution in a water bath at any temperature from 50°C and keep stirring so that the TBA can dissolve.

- **0.15 M Tris-KCL buffer (pH 7.4)**

1.12 g of KCl and 2.36 g of Tris base were dissolved in 100 ml of distilled water and the pH was then adjusted to 7.4.

Procedure
400 μl of the sample was mixed with 1.6 ml of Tris-KCl buffer to which 500 μl of 30 % TCA was added. Then 500 μl of 0.75 % TBA was added and placed in a water-bath for 45 minutes at 80°C. This was then cooled in ice and centrifuged at 3000 g for 5 minutes. The clear supernatant was collected and absorbance was measured against a reference blank of distilled water at 532 nm. Lipid peroxidation expressed as MDA formed/mg protein or gram tissue was computed with a molar extinction coefficient of 1.56×10^5 $M^{-1}cm^{-1}$.

$$\text{LPO (MDA formed/mg protein)} = \frac{Absorbance \; x \; volume \; of \; mixture}{E532 \; nm \; x \; volume \; of \; sample \; x \; mg \; protein}$$

Where: volume of mixture=3 ml

Volume of sample = 400 μl

E_{532nm}, molar extinction coefficient = 1.56×10^5 $M^{-1}cm^{-1}$.

3.10.4. Determination of Hydrogen Peroxide Generation

Hydrogen peroxide generated was evaluated using the method of Wolff, 1994.

Principle
Hydroperoxides oxidize ferrous to ferric ions selectively in dilute acid and the resultant ferric ions can be determined using ferric-sensitive dyes as an indirect measure of hydroperoxide concentration. Xylenol orange (o-cresolsulfonephthalein 3',3"-bis(methylimino) diacetic acid, sodium salt) binds ferric ion with high selectivity to produce a coloured (blue-purple) complex with an extinction coefficient of 1.5×10^4 $M^{-}cm^{-1}$ at 560 nm, the absorbance maximum. In the presence of sorbitol, the yield of ferric ions per mole hydroperoxide increases enormously relative to a system containing only ferrous ions and xylenol orange. Therefore, the apparent extinction coefficient obtained for H_2O_2 in an optimized reaction mixture comprising 100 μM xylenol orange, 250 μM ammonium ferrous sulfate, and 100 mM sorbitol in 25 mM H_2SO_4 is $2.24 \pm 0.067 \times 10^5$ $M^{-1}cm^{-1}$ at 560 nm (the absorbance maximum).

Reagents
- **100 μM H_2O_2**

5.7 μl of 30 % H_2O_2 was made up to 10 ml with distilled water 1 ml of the resulting solution was taken and made up to 50 ml with distilled water.

- **100 μM xylenol orange tetrasodium salt (760.58 g/mol)**

19.025 mg of xylenol orange was dissolved and made up to 25 ml with distilled water.

- **100 mM sorbitol**

4.55 g of sorbitol was dissolved and made up to 25 ml with distilled water.

- **25 mM H_2SO_4**

340 μl of conc. H_2SO_4 was made up to 125 ml distilled water

- **250 μM ammonium ferrous sulfate (392.14 g/mol)**

24.5 mg of ammonium ferrous sulfate was dissolved in 50 ml of 25 mM H_2SO_4.

- **Fox 1 reagent**

10 ml of xylenol orange + 10 ml of sorbitol + 50 ml of ferrous sulfate, made up to 100 ml with distilled water.

Procedure

The procedure for the determination of the standard curve for hydrogen peroxide within the range of 0-5 µM is shown in Table 3.11 below;

Table 12 Procedure for hydrogen peroxide standard curve

	Blank	1	2	3	4	5
H_2O_2 solution (µl)	-	20	40	60	80	100
Distilled water (µl)	100	80	60	40	20	-
Fox 1 reagent (ml)	1.9	1.9	1.9	1.9	1.9	1.9
H_2O_2 concentration (µM)	0	1	2	3	4	5

Vortex, incubate for 30 minutes at room temperature, and centrifuge for 5 minutes at 3000 x g

Absorbance is then read at 560 nm

3.10.5. Procedure for the determination of hydrogen peroxide generation in samples

100 µl of the test sample was added to 1.9 ml of Fox 1 reagent. The mixture was vortexed, incubated for 30 minutes at room temperature, and then centrifuged at 3000x g for 5 minutes to remove any flocculated material. The absorbance is read at 560 nm against the hydrogen peroxide standard curve.

3.11. Histological examination of liver and kidney samples

The liver and kidney from each animal were excised, blotted, and then perfused with 1.15% potassium chloride to remove all traces of haemoglobin that may contaminate the tissues. Sections of liver and kidney samples were obtained and fixed in 10% formalin. These tissues were processed for histopathology examination using a routine paraffin-wax embedded method. Sections of 5-micrometre thickness were stained with haematoxylin and eosin. The slides were then examined using a light microscope for lesions and were evaluated by a pathologist at the Department of Veterinary Anatomy, University of Ibadan.

3.12. Statistical analysis of results

Graphpad prism was used to assess the differences between the groups. Data were expressed as mean ± standard error of the mean. Statistical analysis was carried out using one-way analysis of variance (ANOVA). Values of *$p < 0.05$* were considered to be significant and posthoc tests were carried out using the least significant difference (LSD).

4. Results

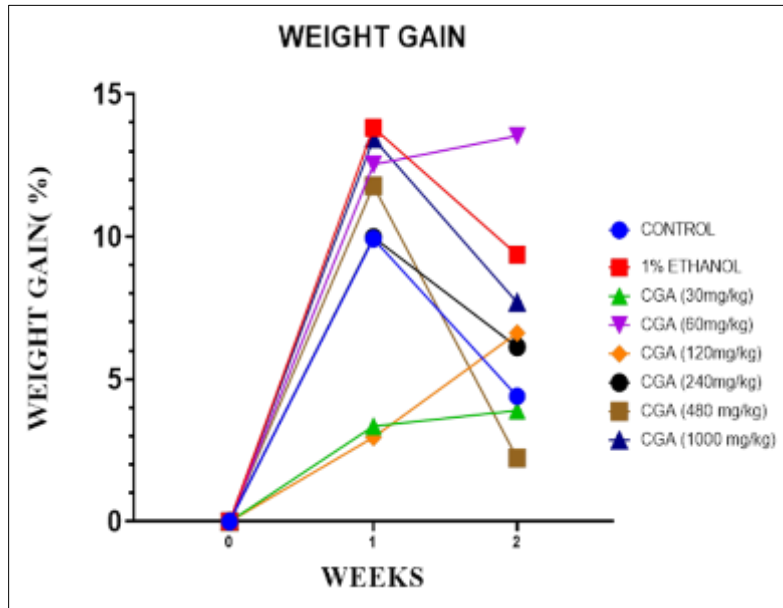### 4.1. Effect of chlorogenic acid on the percentage weight gain of male wistar mice

Figure 7 Effect of chlorogenic acid on percentage weight gain in male Wistar mice Note: n=6

4.2. Effect of chlorogenic acid on the relative organ weight of male wistar mice

Table 13 Effect of CGA on the relative organ weight of male Wistar mice

Relative Organ Weight (%)		
	Kidney	**Liver**
CONTROL	1.06±0.08	5.18±0.55
1% ETHANOL	1.04±0.06	5.42±0.30
CGA (30 mg/kg)	1.25±0.12	5.59±0.36
CGA (60 mg/kg)	1.15±0.10	5.22±0.22
CGA (120 mg/kg)	0.89±0.03	4.88±0.13
CGA (240 mg/kg)	1.11±0.16	5.77±0.47
CGA (480 mg/kg)	1.41±0.19	5.81±0.14
CGA (1000 mg/kg)	1.01±0.17	5.45±0.12

Note: n=6; values are expressed as mean ± standard error of mean

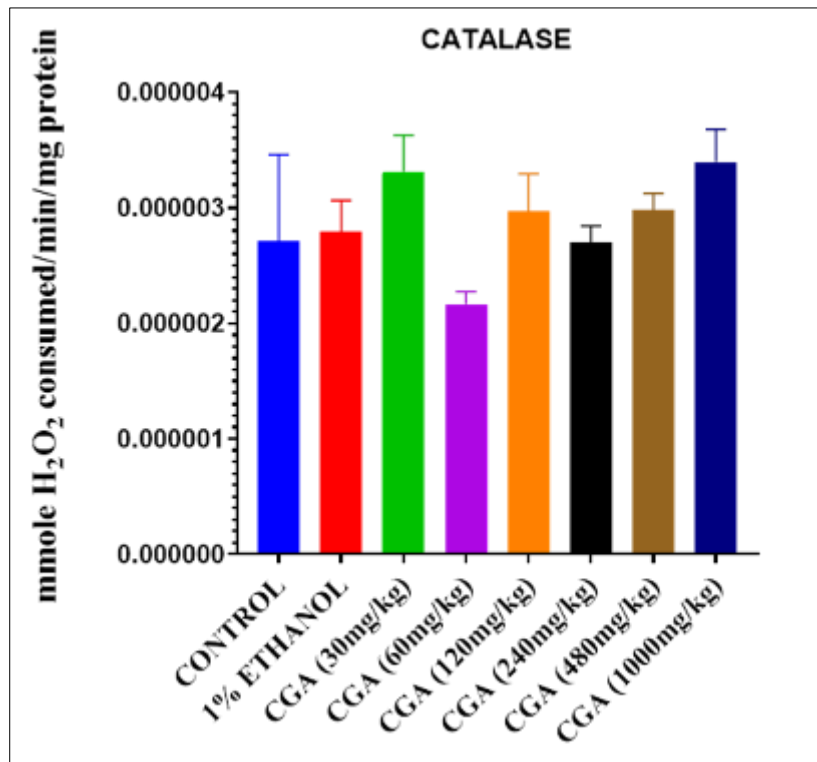**4.3. Effect of chlorogenic acid on antioxidant enzymes activities in the liver of male wistar mice**

Figure 8 Effect of chlorogenic acid on catalase activity in the liver of miceNote: n=6
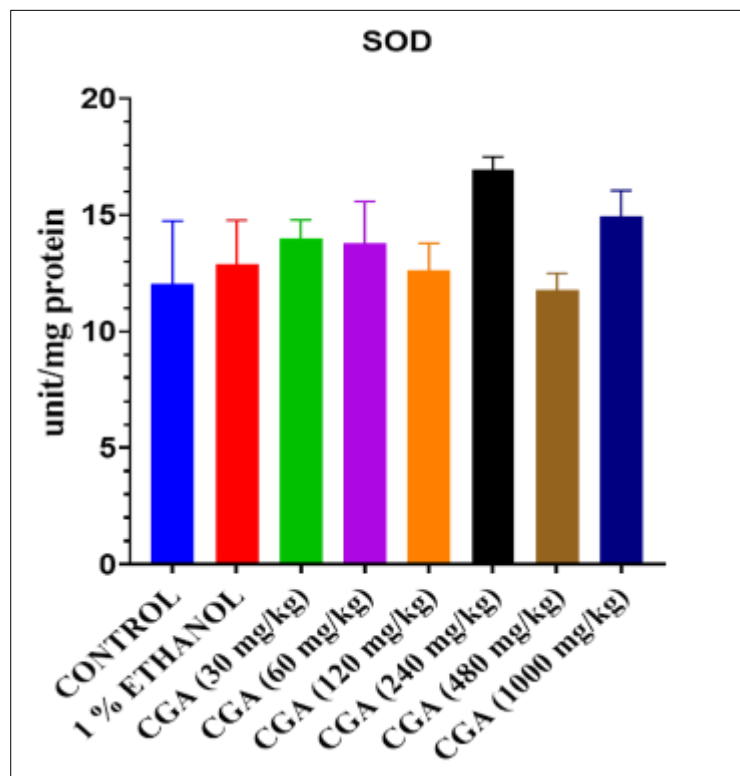

Figure 9 Effect of chlorogenic Acid on Superoxide Dismutase Activity in the Liver of MiceNote: n=6
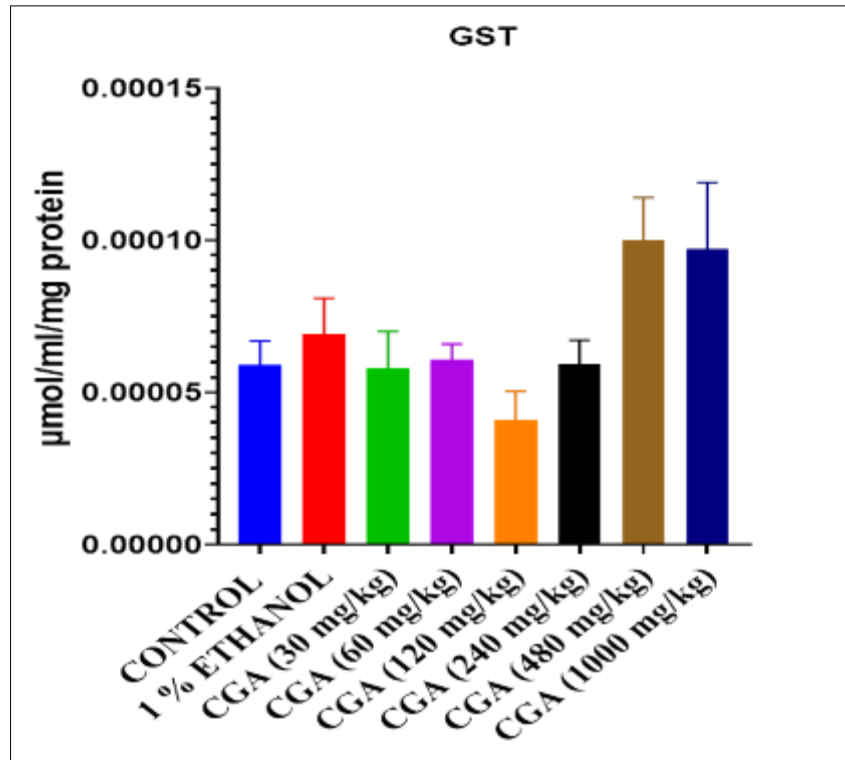
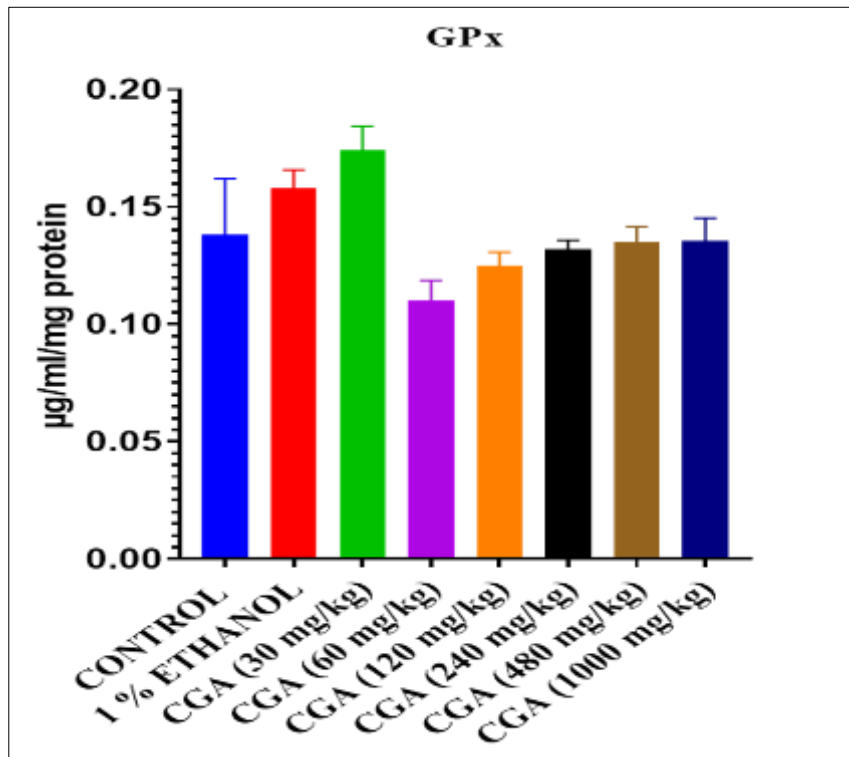**Figure 10** Influence of chlorogenic acid on glutathione-s-transferase activity in the liver of mice Note: n=6

Figure 11 Influence of chlorogenic acid on glutathione peroxidase activity in the liver of mice Note: n=6

4.4. Effect of chlorogenic acid on non-enzymatic antioxidant parameters in the liver of male wistar mice

Table 14 The effect of chlorogenic acid on the concentrations of reduced glutathione and ascorbic acid in the liver of male Wistar mice

	GSH (µmol/g tissue)	Ascorbic acid (µg/ml)
CONTROL	59.13±0.84	3.19±0.88
1% ETHANOL	58.84±0.74	4.93±0.71
CGA (30 mg/kg)	60.07±0.80	4.20±0.69
CGA (60 mg/kg)	59.57±0.50	3.99±0.24
CGA (120 mg/kg)	58.41±0.78	2.83±0.62
CGA (240 mg/kg)	58.04±0.25	3.91±0.53
CGA (480 mg/kg)	61.01±0.60	2.61±0.25
CGA (1000 mg/kg)	59.17±0.41	2.90±0.56

Note: n=6; values are expressed as mean ± standard error of mean

4.5. Effect of chlorogenic acid on the oxidative stress indicator in the liver of male wistar mice

Table 15 The effect of chlorogenic acid on the oxidative stress indicator in the liver of male Wistar mice

	Lipid Peroxidation	Hydrogen Peroxide
CONTROL	5.90E-09±8.40E-10	2.62±0.05
1% ETHANOL	4.90E-09±8.80E-10	2.99±0.06
CGA (30 mg/kg)	4.70E-09±6.20E-10	2.97±0.20
CGA (60 mg/kg)	4.00E-09±5.80E-10	2.60±0.16
CGA (120 mg/kg)	4.60E-09±5.10E-10	2.85±0.09
CGA (240 mg/kg)	5.10E-09±1.10E-09	4.85±0.37*
CGA (480 mg/kg)	5.10E-09±3.60E-10	7.03±0.36*
CGA (1000 mg/kg)	5.30E-09±6.30E-10	6.26±0.13*

Note: n=6; values are expressed as mean ± standard error of mean. The unit for lipid peroxidation is µmol MDA formed/mg protein, unit for hydrogen peroxide is mg protein/ml; *- significant difference when compared to the control group at *p<0.05*; MDA- Malondialdehyde

4.6. Effect of chlorogenic acid on the liver function parameters of male wistar mice

Table 16 The effect of chlorogenic acid on hepatic levels of Albumin, Bilirubin and activities of ALP, ALT, AST, and GGT in male Wistar mice

GROUPS	TOTAL PROTEIN (g/dL)	ALBUMIN (IU/L)	ALP (IU/L)	ALT (IU/L)	AST (IU/L)	BILIRUBIN (IU/L)	GGT (IU/L)
CONTROL	1102.75±175.18	4.11±1.12	41.75±0.70	121.64±2.68	292.75±5.25	0.15±0.03	1.42±0.24
1% ETHANOL	958.96±41.56	3.92±0.70	42.76±1.26	101.19±3.65*	302.46±3.26	0.09±0.01*	1.03±0.19
CGA (30 mg/kg)	863.54± 54.58	4.21±1.03	43.98±0.87	97.02±1.73*	297.39±4.41	0.05±0.01*	1.03±0.08
CGA (60 mg/kg)	1191.88±51.45	4.98±0.36	44.26±2.79	97.08±2.11*	287.17±7.48	0.09±0.01*	0.84±0.06
CGA (120 mg/kg)	1165.00±39.72	5.32±1.77	49.80±1.00*	95.51±1.14*	281.30±1.95	0.12±0.01*	0.90±0.08
CGA (240 mg/kg)	1114.79±32.30	12.48±1.56*	49.04±1.30*	87.89±2.13*	278.91±2.67	0.07±0.01*	1.48±0.12

CGA (480 mg/kg)	1115.21±52.29	12.73±1.15*	51.90±1.75*	93.04±4.87*	275.80±2.40*	0.11±0.01*	1.29±0.24
CGA (1000 mg/kg)	1098.3±75.44	21.34±1.21*	51.23±1.36*	90.18±5.19*	274.42±4.21*	0.07±0.01*	1.09±0.12

Note: n=6; values are expressed as mean ± standard error of mean; *- significant difference when compared to the control group at $p<0.05$; ALP- Alkaline Phosphatase, ALT- Alanine aminotransferase, AST- Aspartate aminotransferase, GGT- Gamma Glutamyltransferase.

4.7. Effect of chlorogenic acid on antioxidant enzymes activities in the kidney of male wistar mice
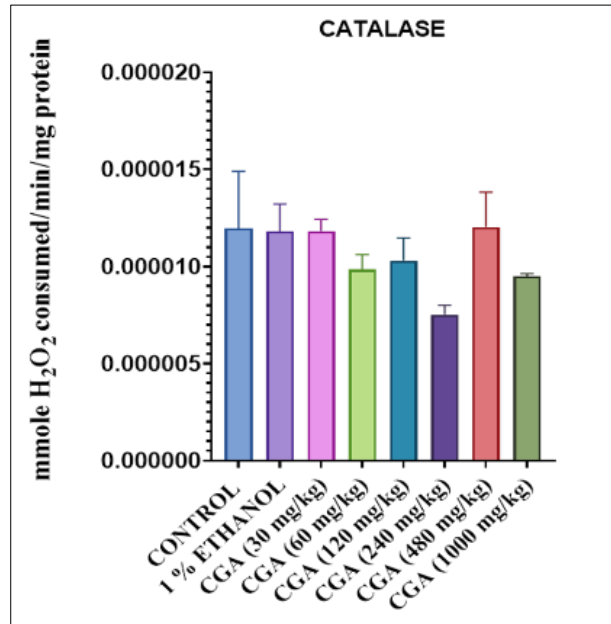

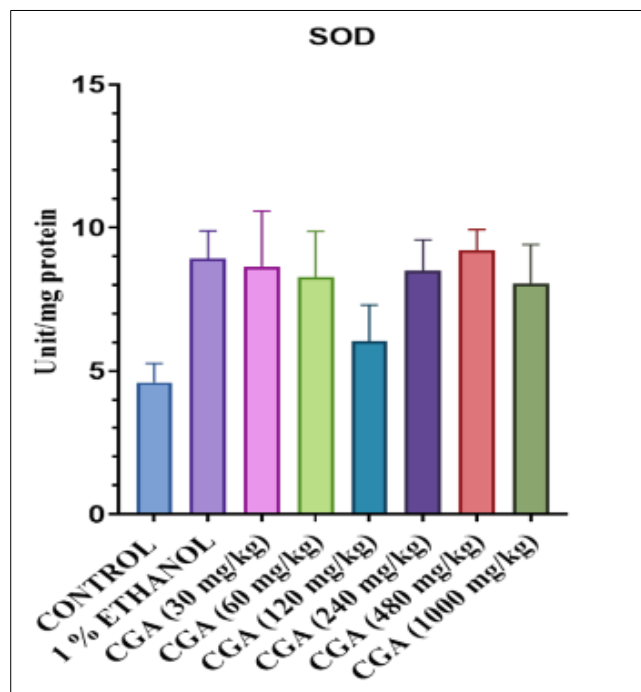**Figure 12** Effect of chlorogenic acid on catalase activity in the kidney of mice Note: n=6

Figure 13 Effect of chlorogenic acid on superoxide dismutase activity in the kidney of mice Note: n=6
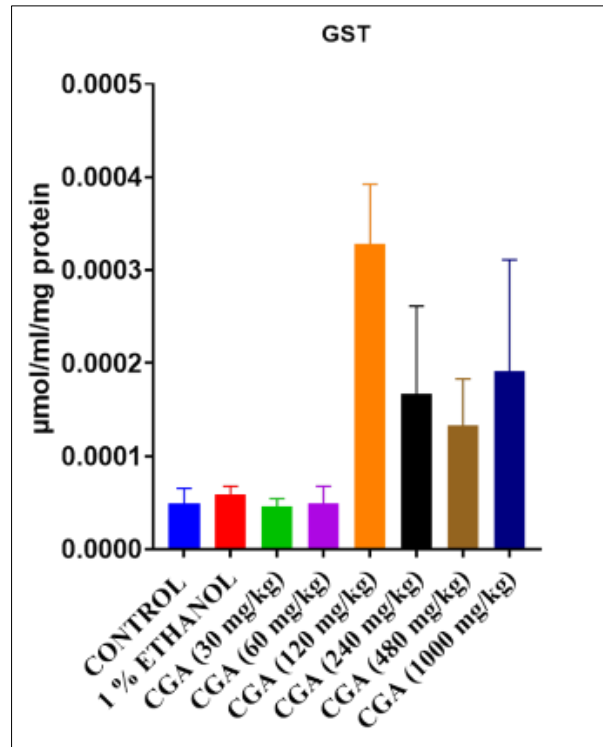
Figure 14 Influence of chlorogenic acid on glutathione-s-transferase activity in the kidney of mice Note: n=6
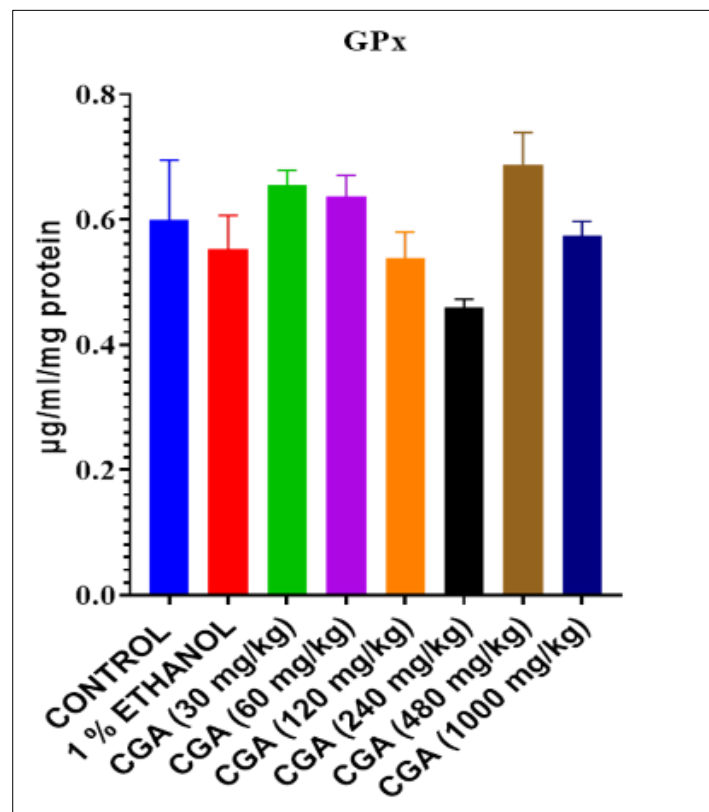

Figure 15 Influence of chlorogenic acid on Glutathione Peroxidase activity in the kidney of mice Note: n=6

4.8. Effect of chlorogenic acid on non-enzymatic antioxidant parameters in the kidney of male wistar mice

Table 17 Effect of chlorogenic acid on the concentrations of reduced glutathione and ascorbic acid in the kidney of male Wistar mice

	GSH (µmol/g tissue)	Ascorbic acid (µg/ml)
CONTROL	61.49±0.46	4.26±0.88
1% ETHANOL	61.96±0.33	5.00±0.27
CGA (30 mg/kg)	62.66±0.26	4.86±0.34
CGA (60 mg/kg)	62.03±0.28	4.06±1.02
CGA (120 mg/kg)	62.50±0.14	4.42±1.09
CGA (240 mg/kg)	62.14±0.50	4.71±0.76
CGA (480 mg/kg)	62.07±0.13	3.84±0.78
CGA (1000 mg/kg)	62.39±0.40	7.04±0.42

Note: n=6; values are expressed as mean ± standard error of mean

4.9. Effect of chlorogenic acid on renal oxidative stress indicator in male wistar mice

Table 18 The effect of chlorogenic acid on the levels of lipid peroxidation and hydrogen peroxide in the kidney

	Lipid Peroxidation	Hydrogen Peroxide
CONTROL	1.60E-08±2.02E-9	2.93±0.17
1% ETHANOL	1.62E-08±2.71E-9	4.22±0.07
CGA (30 mg/kg)	1.36E-08±2.35E-9	9.89±0.34*
CGA (60 mg/kg)	1.18E-08±5.30E-10	8.20±0.44*
CGA (120 mg/kg)	2.53E-06±2.51E-06	13.69±0.65*
CGA (240 mg/kg)	1.04E-08±1.25E-09	8.81±0.49*
CGA (480 mg/kg)	1.78E-08±2.11E-9	5.44±0.38*
CGA (1000 mg/kg)	1.44E-08±1.94E-9	5.14±0.38*

Note: n=6; values are expressed as mean ± standard error of mean; The unit for lipid peroxidation is µmol MDA formed/mg protein, unit for hydrogen peroxide is mg protein/ml; *- significant difference when compared to the control group at *p<0.05*; MDA- Malondialdehyde

4.10. Hematological analysis

Table 19 The effect of Chlorogenic Acid on the Hematological Parameters of Male Wistar Mice

GROUPS	WBC (x10³)	RBC (x10⁶)	HB (g/dL)	PCV (%)	PLATELETS (x10⁵)
CONTROL	6.17±0.36	7.77±0.45	14.42±0.95	41.67±3.24	4.93±0.36
1% ETHANOL	7.65±1.54	7.68±0.45	13.60±0.83	38.83±2.24	6.91±0.27
CGA (30 mg/kg)	6.85±0.69	8.17±0.23	14.07±0.33	40.67±0.92	5.82±0.13
CGA (60 mg/kg)	5.77±0.85	8.17±0.15	15.13±0.29	44.17±1.42	6.00±0.35
CGA (120 mg/kg)	5.97±0.47	8.42±0.27	14.55±0.82	41.67±2.44	6.22±0.65
CGA (240 mg/kg)	5.38±1.33	7.77±0.51	13.65±0.98	39.00±2.97	6.18±0.61
CGA (480 mg/kg)	7.62±0.80	8.72±0.10	15.98±0.67	44.17±0.95	4.69±0.40

CGA (1000 mg/kg)	7.28±0.65	9.00±0.22	16.17±0.43	46.17±1.58	5.27±0.26

Note: n=6; values are expressed as mean ± standard error of mean; WBC- white blood cell, RBC- red blood cell, HB- haemoglobin, PCV- packed cell volume.

Table 20 The influence of chlorogenic acid on differential leukocyte count in Male Wistar Mice

GROUPS	NEUT (%)	LYMP (%)	MONO (%)	BAS (%)	EOS(%)
CONTROL	38.67±6.88	59.33±6.84	0.00±0.00	0.00±0.00	1.33±0.61
1% ETHANOL	48.67±10.39	48.33±10.67	0.00±0.00	1.33±0.21	2.17±0.31
CGA (30 mg/kg)	57.50±5.83	38.83±5.92	0.50±0.22	1.67±0.33	2.50±0.34
CGA (60 mg/kg)	42.50±6.71	55.00±6.65	0.50±0.02	0.00±0.00	2.33±0.33
CGA (120 mg/kg)	39.17±4.66	57.00±3.61	0.33±0.02	0.00±0.00	2.40±0.80
CGA (240 mg/kg)	41.67±5.97	52.17±6.22	0.50±0.02	0.40±0.02	3.00±0.73
CGA (480 mg/kg)	35.67±6.64	57.67±7.02	0.80±0.34	0.17±0.02	3.50±0.67
CGA (1000 mg/kg)	46.00±7.54	49.67±8.30	0.33±0.02	1.00±0.45	3.00±0.86

Note: n=6; values are expressed as mean ± standard error of mean; NEUT- neutrophils, LYMP- lymphocytes, MONO- monocytes, BAS- basophils, EOS- eosinophils.

Table 21 The influence of chlorogenic acid on blood indices in male Wistar mice

GROUPS	MCH (Pg.)	MCHC (g/dL)	MCV (FL)
CONTROL	18.17±0.17	35.50±0.34	51.67±0.61
1% ETHANOL	18.17±0.17	35.00±0.26	52.00±0.68
CGA (30 mg/kg)	17.00±0.26	34.50±0.22	49.83±0.60
CGA (60 mg/kg)	18.60±0.37	35.50±0.43	52.33±1.15
CGA (120 mg/kg)	17.00±0.45	35.00±0.26	48.83±1.28
CGA (240 mg/kg)	17.67±0.33	35.17±0.48	50.00±1.37
CGA (480 mg/kg)	18.17±0.48	34.67±0.21	51.33±1.02
CGA (1000 mg/kg)	18.33±0.33	35.83±0.31	51.00±0.37

Note: n=6; values are expressed as mean ± standard error of mean; MCH- mean corpuscular haemoglobin, MCHC- mean corpuscular haemoglobin concentration, MCV- mean corpuscular volume

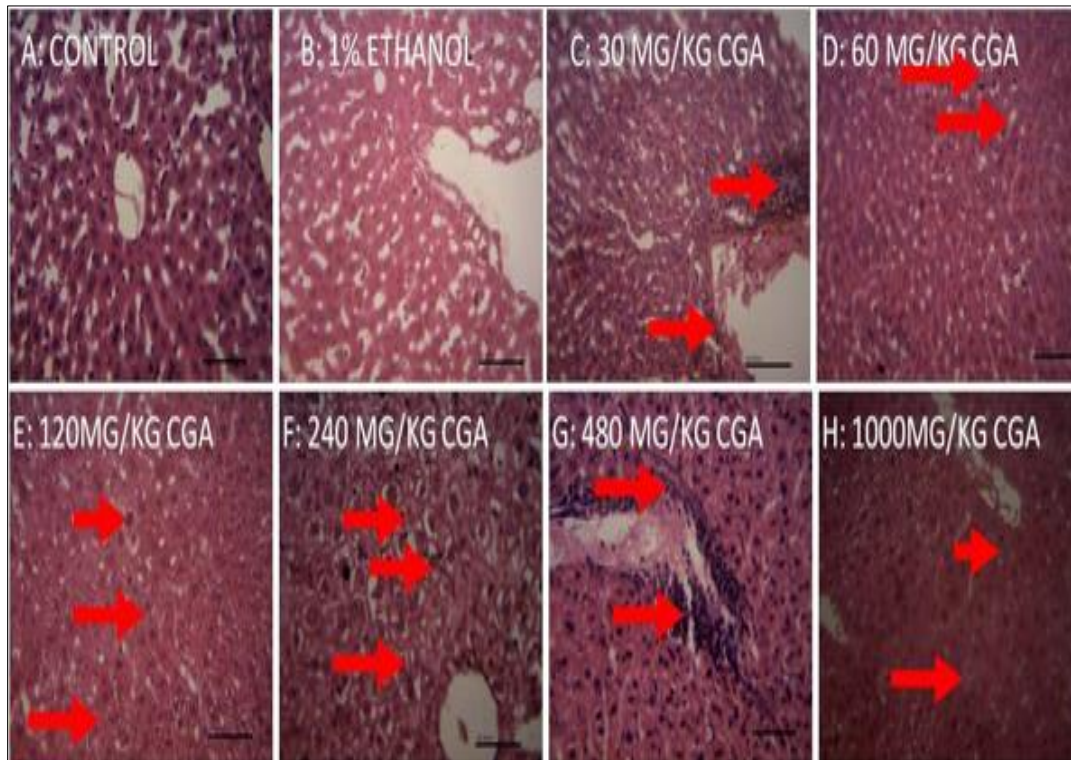## 4.11. Histopathological assessment of the liver

Figure 16 Photomicrographs of the Liver of Male Wistar Mice

A and B: No lesions; C: There is a very mild periportal cellular infiltration; D: Very mild diffuse hydropic degeneration of hepatocytes; E: There is a moderate diffuse vacuolar degeneration of hepatocytes; F: There is a moderate periportal cellular infiltration; G: There is a very mild vacuolar degeneration of the hepatocytes.

5. Discussion

Chlorogenic acid administration led to a reduction in body weight in male Wistar mice in this study. Figure 4.0 revealed that for two weeks of observation, the body weight of mice given doses of 30 mg/kg CGA, and 60 mg/kg CGA, increased in a dose-dependent manner, and mice given 120 mg/kg CGA showed an exponential increase in body weight which could suggest that low doses of chlorogenic acid may help gain weight. However, for mice given 1% ethanol, CGA at doses of 240 mg/kg, 480 mg/kg, and 1000 mg/kg, a gain in body weight was seen in the first week, followed by a drop in their weight in the following week. This implies that large dosages of chlorogenic acid may aid weight loss. According to a study by Santana-Gálvez *et al.* (2017), where male Sprague-Dawley rats were fed a high-fat diet with different doses of chlorogenic acid, it was found that chlorogenic acid significantly reduced body weight in the rats. This was also supported in a study by Rahman *et al.* (2017) where chlorogenic acid was shown to reduce body weight and visceral fat mass in obese mice. However, Shimoda *et al.* (2006) stated that the effect of chlorogenic acid, particularly on body weight gain, has not been established. This study shows that the relative organ weight of male Wistar mice treated with varying doses of chlorogenic acid was not affected (Table 4.1).

Catalase is an enzyme found in the cells of living organisms. It plays a crucial role in the breakdown of hydrogen peroxide (H_2O_2), a harmful by-product of cellular metabolism. Catalase catalyses the hydrolysis of hydrogen peroxide into water (H_2O) and oxygen (O_2), preventing the accumulation of toxic levels of hydrogen peroxide within cells. This is vital for maintaining cellular homeostasis and protecting cells from oxidative damage caused by reactive oxygen species (ROS) (Li *et al.*, 2011; Feng *et al.*, 2012; Jia *et al.*, 2016). This study shows that catalase activity was not affected in the liver and the kidneys (Figure 4.1 and Figure 4.5).

Superoxide dismutase (SOD) is an enzyme responsible for catalysing the conversion of superoxide radicals (O_2-) into less harmful compounds, namely molecular oxygen (O_2) and hydrogen peroxide (H_2O_2), thereby, protecting cells from the damaging effects of reactive oxygen species (ROS) (McCord, and Fridovich, 1969; Valko *et al.*, 2007). SOD works in conjunction with other antioxidant enzymes, such as catalase and glutathione peroxidase, to maintain the balance of

reactive oxygen species within cells and tissues. In this study (Figure 4.2 and Figure 4.6), SOD activity was not affected by acute exposure to chlorogenic acid in the liver and kidneys.

Glutathione S-transferases (GSTs) are a family of enzymes that play a crucial role in the detoxification and elimination of harmful substances, including drugs, environmental pollutants, and carcinogens. They catalyse the conjugation of the antioxidant molecule glutathione (GSH) to various electrophilic compounds, making them more water-soluble and facilitating their excretion from the body (Hayakawa *et al*., 2020). According to this study (Figure 4.3 and Figure 4.7), was not affected in the liver and kidneys upon acute exposure to chlorogenic acid.

Glutathione peroxidase (GPx) is an essential antioxidant enzyme that helps protect cells from oxidative damage. It is involved in the reduction of hydrogen peroxide (H_2O_2) and lipid hydroperoxides, which are reactive oxygen species (ROS) generated during normal cellular metabolism and in response to external stressors. GPx utilizes glutathione (GSH) as a cofactor to carry out its antioxidant function (Brigelius-Flohé, and Maiorino, 2013). This study (Figure 4.4 and Figure 4.8) reveals that the activity of GPx was not altered in the liver and kidneys.

Some non-enzymatic biochemical assays were carried out on the liver and kidneys of male albino Wistar mice. This includes reduced glutathione (GSH) a tripeptide molecule that plays a crucial role in antioxidant defence and cellular protection, and ascorbic acid (vitamin C) which also play a crucial antioxidant function. The concentrations of reduced glutathione and ascorbic acid were not affected upon acute exposure to chlorogenic acid.

This study also tried to prove the effect of chlorogenic acid on the levels of some common oxidative stress indicators, namely lipid peroxides (LPO), and hydrogen peroxide (H_2O_2). Lipid peroxidation is a process that involves the oxidative degradation of polyunsaturated fatty acids (PUFAs) in cell membranes and lipoproteins. It occurs when reactive oxygen species (ROS), such as free radicals, attack the double bonds present in PUFAs, initiating a chain reaction of lipid oxidation. This process can result in the formation of reactive lipid species and lipid hydroperoxides (Sies, 2015). On the other hand, H_2O_2 is a reactive oxygen species that is produced during various cellular processes, including normal metabolism and as a by-product of enzymatic reactions. At low concentrations, H_2O_2 can act as a signalling molecule and participate in cellular processes such as cell signalling and immune responses, regulating various cellular processes like cell proliferation, differentiation, migration, and apoptosis (Bienert *et al*., 2006; Sofo *et al*., 2015; Niu, and Liao, 2016; Černý *et al*., 2018; Lismont *et al*., 2019). However, when H_2O_2 levels become excessive or when the cellular antioxidant defence system is overwhelmed, it can lead to oxidative stress and damage to biomolecules such as proteins, lipids, and DNA.(Semba *et a*l., 2007; Faizal *et al*., 2012; Thanan *et al*., 2014; Mesfer *et al*., 2017; Lan *et al*., 2019; Juan *et al.,* 2021).For the level of lipid peroxidation in the liver and kidney supernatants from the treated mice in this study, there was not much statistical difference from the liver and kidney supernatants of the control mice group, thus showing that toxic exposure to chlorogenic acid treatment had no appreciable impact on lipid peroxidation in the liver and kidneys. However, there was a dose-dependent increase in the level of hydrogen peroxide in the liver at 240, 480 and 1,000 mg/kg doses of chlorogenic acid (Table 4.5, and Table 4.8). Cui *et al*. (2010) indicate that osmotic stress leads to an increase in hydrogen peroxide levels. Additionally, Zhang *et al*. (2020) suggest that isochlorogenic acid B, a derivative of chlorogenic acid, has an inhibitory effect on hydrogen peroxide-induced oxidative damage. These studies highlight the diverse effects of chlorogenic acid on hydrogen peroxide levels, which may be influenced by different experimental conditions and systems.

Total protein refers to the measurement of all the proteins present in a biological sample, such as blood, urine, or tissue/ organs such as the liver. It is an important parameter to assess overall protein status. Abnormalities in total protein levels can indicate underlying health issues, such as malnutrition, liver disease, kidney disorders, or immune system disorders (Doumas *et al. 1981*). In this study, Total protein levels were not affected by acute exposure to chlorogenic acid (Table 4.5).

Albumin is a protein that is made by the liver. It is the most abundant protein in the blood, and it makes up about 60% of the total protein in the blood. Albumin has many important functions, including transporting nutrients and hormones throughout the body, maintaining blood pressure, and protecting tissues from damage, amongst others (Peter, 1996; Fanali *et al*., 2012; Roche and Rondeau 2019). This study shows a dose-dependent increase in the levels of albumin in liver samples administered with 240mg/kg, 480mg/kg, and 1000mg/kg suggesting that at these doses chlorogenic may have inflicted some damage in the liver (Table 4.5).

Alkaline phosphatase (ALP) is an enzyme that is found in various tissues throughout the body, including the liver, bones, kidneys, intestines, and placenta. The main function of alkaline phosphatase is to catalyse the hydrolysis of phosphate esters at an alkaline pH. It removes phosphate groups from various molecules, including nucleotides, proteins, and alkaloids. This enzymatic activity is essential for several biological processes, such as bone mineralization, metabolism

of lipids, and detoxification of certain substances (Millán, 2006). ALP in the liver samples used in this study increased in a dose-dependent manner at the doses of 120 mg/kg, 240 mg/kg, 480 mg/kg, and 1000 mg/kg. This further suggests that the function of the liver is compromised. Kaplan *et al.* (2007) and R *et al*. (1982) reported an increase in ALP synthesis and activity in rat liver following bile duct ligation, which suggests that ALP is associated with liver pathologies.

Alanine aminotransferase (ALT), also known as serum glutamic-pyruvic transaminase (SGPT), is an enzyme primarily found in liver cells. ALT is released into the bloodstream when liver cells are damaged or injured. ALT plays a crucial role in the metabolism of amino acids. Specifically, it catalyses the transfer of an amino group from alanine to alpha-ketoglutarate, resulting in the formation of pyruvate and glutamate. This enzymatic activity is essential for energy production and amino acid metabolism (Pratt and Kaplan, 2000; Giannini *et al*., 2005; Lazo and Clark, 2008). According to this study, there was a dose-dependent decrease in the activity of ALT in all the liver samples treated with CGA (Table 4.5). This supports the fact that the function of the liver may be affected. Moreover, Merzouk *et al*., (2017) conclude that chlorogenic acid displays corrective and preventive effects on hepatocytes exposed to oxidative stress, which could be beneficial for liver protection. This observation may be at lower doses.

Aspartate transaminase (AST), also known as serum glutamic-oxaloacetic transaminase (SGOT), is an enzyme that is found in various tissues throughout the body, with significant concentrations in the liver, heart, skeletal muscles, and kidneys. AST is released into the bloodstream when these tissues are damaged or injured. AST is an enzyme involved in amino acid metabolism. It catalyses the reversible transfer of an amino group from aspartate to alpha-ketoglutarate, resulting in the formation of oxaloacetate and glutamate. This enzymatic activity is essential for energy production and amino acid metabolism (Pratt and Kaplan, 2000; Berg *et al*., 2015; Nelson *et al*., 2017). The Aspartate aminotransaminase activity in the liver samples in this study (Table 4.5) decreased in a dose-dependent manner in the treatment groups and was significant at 480 and 1,000 mg/kg doses.

Bilirubin is a yellowish pigment produced during the breakdown of haem; a component of haemoglobin found in red blood cells. It is formed in the liver as a result of the normal turnover of red blood cells and is excreted in bile (Fevery, 2008; Ishaq *et al*., 2023). There was a reduction in the bilirubin levels in all the treatment groups with it being significant at 30, 60, 240 and 1,000 mg/kg doses. Reduced bilirubin level is an indication of a defect in liver function. This further supports the other observations.

Gamma-glutamyl transferase (GGT), also known as gamma-glutamyl transpeptidase (GGTP), is an enzyme found in various tissues, with high concentrations in the liver, bile ducts, and kidney cells. GGT is primarily involved in the metabolism of glutathione, a molecule that plays a role in antioxidant defence and detoxification processes. GGT catalyses the transfer of the gamma-glutamyl group from molecules such as glutathione to other amino acids or peptides. This enzymatic activity is involved in the metabolism of various compounds, including glutathione, amino acids, and certain drugs (Dringen *et al*., 1999; Sierra-Johnson *et al*., 2008; Li *et al*., 2016; Jalili *et al*., 2022; Kim *et al*., 2022). In this study (Table 4.5), GGT activity was not affected by acute exposure to chlorogenic acid.

Haematological tests are conducted on parameters that measure the number and function of blood cells. These tests can be used to diagnose a variety of conditions, including anaemia, infection, and cancer. The haematological parameters, blood indices and differential leukocyte count were not affected by acute exposure to chlorogenic acid. This suggests that chlorogenic acid does not have an adverse effect on the various aspects of blood function (Table 4.9.1, Table 4.9.2 and Table 4.9.3).

In Figure 4.10, mild to moderate lesions in the liver were observed in all the groups treated with chlorogenic acid and none was observed in the control group. There were no lesions in the kidney samples. These observations complemented the biochemical assays.

6. Conclusion

In this study, the lethal dose (LD50), was not established even at the highest dose of 1000 mg/kg body weight. However, liver function was compromised, especially at higher doses. These show that CGA is safe but caution should be exercised when ingested in large amounts.

Compliance with ethical standards

Disclosure of conflict of interest

No conflict of interest to be disclosed.

Statement of ethical approval

All experimental procedures involving Wistar rats were conducted in accordance with the ethical standards and guidelines set forth by the University of Ibadan and were approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Ibadan, Ibadan, Oyo State, Nigeria. The approval number for this study is 8466879009. All efforts were made to minimize the number of animals used and to reduce suffering

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Appendices