CHAPTER ONE

INTRODUCTION

1.1. Background of the Study

The rate of use of herbal medicines are increasing rapidly worldwide, although the reasons for this development may vary for different people in different environments. The observed safety of herbal medicines and their affordability is a major consideration. Herbal medicines derived from locally available medicinal plants, have been used to manage many medical problems such as hypertension, malaria, arthritis, fatty liver disease, cancer etc. in different parts of Nigeria. The evaluation of the hepatoprotective activity of a Nigerian tri-herbal formulation (GOV) consisting of 50 % ethanolic extract of *Gongronema latifolia* Benth, *Ocimum gratissimum Linn.* and *Vernonia amygdalina Del.* by investigating its effect on the biochemical, antioxidant, anti-inflammatory, anti-proliferative indices in normal albino rats and HepG2 cell line, is the subject of this study.

The liver is the primary site for drug metabolism and hepatotoxicity is one of the most frequently reported human adverse drug reactions. Adverse drug reactions (ADRs) present serious human health problems and cause reactions similar to those of acute viral hepatitis (Mumoli, *et al*., 2006). Potential hepatotoxicity of some of the first-line anti -tubercular and anti-retroviral drugs remains a problem (Shakya, *et al*., 2006). It has been observed that liver diseases present a leading cause of medical emergency in Nigeria resulting from poorly treated or untreated infective hepatitis, late presentation at hospitals for treatment, alcoholism and drug misuse (Adesunkanmi *et al*., 2002). Hepatoprotective drugs are frequently prescribed as part of treatment for tuberculosis in China (Ho, 2006) because the first line anti-tuberculosis drugs isoniazid (INH), rifampicin (RIF) and pyrazinamide (PZA) are also associated with toxic reactions in tissues, particularly in the liver, leading to hepatitis (Liu, *et al*., 2008). Drug-induced liver injury has become a leading cause of severe liver

disease in Western countries and therefore poses a major clinical and regulatory challenge (Russmann, *et al.,* 2009). In England, there is a marked increase in liver diseases with reports of rising morbidity and mortality, particularly in younger age groups (Kaner, *et al.*, 2007).

Acetaminophen (APAP) hepatotoxicity is the most common cause of death due to acute liver failure in the developed world and is increasingly recognized as a significant public health concern (Lee, 2007). Alcohol liver disease (ALD) is the foremost health risk in developing countries and ranks third in developed countries (WHO, 2005). Alcohol abuse and alcoholism represents one of the major health, social and economic issues facing the world and the liver is among the organs most susceptible to the toxic effects of ethanol (Lieber, *et al*., 2008).

Hepatitis and other liver diseases constitute a major cause of mortality and morbidity in Nigeria, and these can be prevented with proper treatments and as well as prophylactic measures (Adesunkanmi *et al*., 2002). Halim and Ajayi (2000) reported that the prevalence of hepatitis C antibody from blood donors in Nigeria varied between 5.8 % – 12.3 %. However, treatment and prophylaxis options for these conditions are limited (Hwang and Chen, 2006).

There has been a rapid expansion of allopathic health care in Nigeria over the last three decades, including an increase in the number of allopathic health care providers. At the same time, because the majority of Nigerians use traditional medicine, the Government of Nigeria has shown appreciation for the importance of traditional medicine in health care delivery (WHO, 2001a). According to Sule (2000), though informal intercommunication between the Government and traditional medicine practitioners can be traced back to the 19th century, formal legislation promoting traditional medicine dates back to 1966 when the Ministry of Health authorized the University of Ibadan to conduct research into the medicinal properties of local herbs (WHO, 2001b).

Despite the widespread use of complementary and alternative medicine (CAM), there is lack of scientific evidence on the efficacy and safety of some of these herbal drugs. Research has been carried out to evaluate the scientific basis for the claimed hepatoprotective activity of herbal agents as a single agent or in formulation. It is therefore very important to make continuous effort to develop more effective therapeutic strategies or prophylactic modalities to eradicate or stem the scourge of liver disease.

1.2. Statement of Problem

The need for organized and continuous improvement of the practice and development of traditional medicine is of great importance in order to protect the population from quacks, fraud, and incompetence. Certain herbal supplements can have adverse effects ranging from nausea and vomiting to fatal conditions like liver or kidney dysfunction. For example, in 2002, the U.S. Food and Drug Administration (FDA) released a warning about potential liver damage from kava root, and it was then, one of the 10 most popular herbal supplements sold in the United States of America (FDA, 2002: Moulds and Malani, 2003) . And in 2004 the FDA banned ephedra, a Chinese weight-loss herb, after it was linked to more than 100 deaths (Haller, *et al*., 2005).

There is a false belief that herbal remedies are safer than conventional medicines, when in fact many of these substances may cause serious illnesses, aggravate pre-existing health problems or result in death, particularly if taken in excess or injected rather than ingested. Some herbal supplements and medications, may be adulterated and thus could be contaminated with pathogenic microbes or dangerous heavy metals, including lead and mercury. Perhaps the greatest potential risk, however, lies in possible interaction with pharmaceutical drugs the patients are already taking. Saint-John's-wort,

which has been shown to help in treating mild to moderate depression, is also known to reduce the effectiveness of some HIV medications and heart drugs such as digoxin and warfarin, and cause intermenstrual bleeding in women taking the oral contraceptive pill, while Gingko and garlic also increase the risk of bleeding with anticoagulants. Access to herbal products is generally unrestricted and many people do not tell their doctor they are taking herbal medicines, their contribution to death may not be fully appreciated during a standard autopsy.

Herbal medicine has become a common form of healthcare, even though several differences exist between herbal and conventional pharmacological treatments, herbal medicine needs to be tested for efficacy using conventional trial methodology. Clinical proof i.e. a randomized, controlled trial is the main standard for establishing a drug's usefulness and safety. There is a clear need for better public and physician understanding of herbal products through health education, early detection and management of herbal toxicities, scientific scrutiny of their uses, and research on their safety and effectiveness. It is therefore important to expand the knowledge base of traditional and complementary/alternative medicine, and to promote its inclusion into health improvement policies especially where they are proven safe and effective.

1.3. Purpose of Study

The aim of this study is to investigate the hepatoprotective, antiproliferative and antioxidant effects of a tri-herbal formulation (GOV) on acetaminophen, alcohol, carbon tetrachloride and D-galactosamine induced toxicity in Wistar albino rats and HepG2 cell line. This investigation will be carried out using both *in vitro* and *in vivo* assay models. The anti-proliferative activity of GOV in

different solvent systems toward human hepatocellular liver carcinoma cell line (HepG2 cells) would be monitored by *in vitro* model.

1.4. Objectives of the Study

The specific objectives of this study include:

1. To determine the hepatoprotective activity of the triherbal formulation (GOV) against acetaminophen (APAP), alcohol (ethanol), d- galactosamine (D-GaIN) and carbon tetrachloride (CCl_4) induced hepatotoxicity in rats by,

i. Ascertaining the effect of GOV on haematologic indices and serum liver marker enzymes.

ii. Determining the antioxidant potential of GOV using serum, liver and kidney homogenates.

iii. Profiling the anti-apoptotic activities of GOV using leukocytes from whole blood to determine the fold-increase in caspase-2, 3 and 9 activities.

iv. Profiling the histopathologic indices of the liver and kidney of pre-treated rats using morphologic observations.

2. To evaluate the antiproliferative activity of GOV using human hepatocellular liver carcinoma cell line (Hep G2 cells) and nasopharyngeal cancer cells (CNE2 and SUME –α- nasopharyngeal cells).

3. To determine the *in vitro* antioxidant potential of GOV using brain homogenates of Wistar albino rats and erythrocyte of Swiss mice.

4. To determine the phytochemical constituents of the triherbal formulation (GOV) consisting of 50 % ethanolic extract of *Gongronema latifolia* Benth, *Ocimum gratissimum Linn.* and *Vernonia amygdalina Del.* using preliminary phytochemical analysis such as, thin layer chromatography (TLC), high performance thin layer chromatography (HPTLC), high performance liquid chromatography **(**HPLC) and the saturation-transfer difference **–** nuclear magnetic resonance spectroscopy (STD-NMR) based screening.

5. To ascertain the safe dose of GOV orally and intraperitoneally using animal models.

6. To investigate the analgesic and anti-inflammatory activity of GOV using appropriate pharmacologic assays.

1.5. Research Questions and / or Hypotheses.

1. Which solvent best extracts the bioactive constituents of GOV?
2. What phytochemicals can be identified in the triherbal formulation?
3. Is GOV safe?
4. Does GOV demonstrate anti-inflammatory and analgesic activities in Swiss mice and Wistar albino rats?
5. What effect does GOV exhibit on haematologic parameters in Wistar albino rats intoxicated with different hepatotoxicants (acetaminophen, alcohol, carbon tetrachloride and D-galactosamine)?

6. What effect does GOV exert on serum liver enzymes of Wistar albino rats intoxicated with these hepatotoxicants?

7. Is there any significant difference ($p \leq 0.05$) in the biochemical and antioxidant indices of serum, liver and kidney homogenates of GOV treated rats compared to the toxin control group?

8. Does GOV have any effect on the concentrations of chemical analytes in Wistar albino rats intoxicated with these hepatotoxicants?

9. Does GOV exhibit anti-apoptotic effect on rats intoxicated with these hepatotoxicants?

10. Is there any morphologic change in internal organs of rats administered GOV before tissue damage with the different hepatotoxicants compared to the toxin control rats?

11. Does GOV demonstrate antiproliferative activity?

1.6. Significance of Study

Many of the herbs and spices used by humans to season food, yield useful medicinal compounds. Similarly to prescription drugs, a number of herbs are thought to cause adverse effects. Adulteration, inappropriate formulation, or lack of understanding of plant interactions and formulations have led to adverse reactions that are sometimes life threatening or lethal such as *Ricinus communis* (castor oil plant). Therefore studies on the bioactive components in these herbs are imperative so as to promote or discourage its use. This will aid the botanists, natural-products chemists, microbiologists and pharmacologists to develop phytochemicals that could be used for treatment of various diseases.

According to Brower (2008), the Botanic Gardens Conservation International report said that "five billion people still rely on traditional plant-based medicine as their primary form of health care".

The World Health Organization (WHO) estimates that 80 % of the world's population presently uses herbal medicine for some aspect of primary health care (WHO, 2008). This can be attributed to the fact that pharmaceuticals are prohibitively expensive for most of the world's population (2.8 billion people live on less than US$ 2 per day) (DaSilva, *et al*., 2002), in comparison to herbal medicines that can be cultivated or gathered from nature for little or no cost. Even in this present technological era, traditional medicine is still the predominant means in the third world for the preservation of health of the rural majority who constitute over 70 % of the total population (Okoli, *et al*., 2007). Based on scientific evidence(s) from this study, government will be encouraged to acknowledge, support and integrate traditional medicine into national health systems as part of primary health care in combination with national policy and regulation to ensure safe, effective and quality products and practices. This will boost access to care for the rural majority and increase general awareness on the efficacy of some indigenous medicinal plants.

This study may help suggest the chemopreventive potentials and antioxidant profiles of GOV as a traditional herbal medicine and to highlight the importance of scientific research on miscellaneous plants with various medicinal properties. It is expected to awaken environmental consciousness on the need to avoid unconscious exposure to different potential hepatotoxicants. It will also help promote awareness on the safety, efficacy and quality of medicinal plants by expanding its knowledge base, promoting therapeutic use of medicinal plants by increasing the quality, quantity and accessibility of clinical evidence to support claims for their effectiveness. This will encourage studies on medicinal plants so as to provide more affordable chemopreventive drugs that are highly accessible especially in poor and marginalized populations thereby reducing excess mortality, morbidity and disability.

These investigations will help to document the uses of indigenous plants for posterity, since knowledge of indigenous cultures are lost because cultures themselves are in danger of extinction and

prior to their possible elimination through urbanization, social development and deforestation. It is also hoped that it will stimulate interest for further research in medicinal plant(s) and the ailments for which they are used with the purpose of developing potential drugs for some common diseases. It will provide scientific evidence to evaluate the safety and effectiveness of traditional medicine products and practices.

Scientific proof of the efficacy of herbs makes herbal medicines a lucrative form of traditional medicine, so it could be used as export commodities, which generates considerable income.

1.7. Operational Definition of Terms

Allopathic medicine - Allopathy is a biologically based approach to healing and it is the type of medicine most familiar to westerners today.

Antiproliferative - used to inhibit cell growth or retard the spread of cells especially malignant cells.

Apoptosis - the programmed death of some of an organism's cells as part of its natural growth and development.

Chemopreventive - The use of a drug or compound to interfere with a disease process.

Cholestasis - interruption in the excretion or flow of bile.

Cirrhosis - a chronic degenerative disease in which normal liver cells are damaged and are then replaced by scar tissue.

Complementary and alternative medicine (CAM) - CAM as a group of diverse medical and health care systems, practices, and products that are not generally considered part of conventional medicine.

Confluence - a coming or flowing together, meeting, or gathering at one point

Conventional medicine - Medicine as practiced by holders of M.D. (doctor of medicine) or D.O. (doctor of osteopathy) degrees and by their allied health professionals such as physical therapists, psychologists, and registered nurses.

Cytosolic extract - the liquid medium of the cytoplasm, i.e., cytoplasm minus organelles and nonmembranous insoluble components.

Dewax - To remove wax from a material or from a surface

Efficacy - The power or capacity to produce a desired effect.

Formulation – combination of different chemical substances to produce a final medicinal product.

Fractionation - To divide or separate into parts; break up Separation of a mixture in successive stages, each stage removing from the mixture some proportion of one of the substances, as by differential solubility in water

Haemolysis - The breakdown of red blood cells.

Hepatitis - inflammation of the liver.

Hepatoprotective drugs - the drugs that prevent liver disease.

Hepatotoxic - The ability of a substance to have damaging effects on the liver.

Hyperglobulinaemia - the presence of excess globulins in the blood.

IC_{50} value - the concentration that brings about 50 percent inhibition or the effective dose required to inhibit the proliferative response by 50 percent

Intraperitoneal - administered by entry into the peritoneum.

Metabolism - The chemical processes occurring within a living cell or organism that are necessary for the maintenance of life.

Necrosis – the death of body tissue when there is not enough blood flowing to the tissue.

Oxidative stress - an imbalance between the production and manifestation of reactive oxygen species and a biological system's ability to readily detoxify the reactive intermediates or to repair the resulting damage

Pharmacovigilance - the detection, evaluation, understanding and prevention of adverse effects, particularly long and short term side effects of medicines.

Prophylaxis - Prevention of or protective treatment for disease.

Steatosis – the abnormal retention of lipids within a cell. It reflects an impairment of the normal processes of synthesis and elimination.

Writhing - To twist as in pain or struggle.

CHAPTER TWO

LITERATURE REVIEW

2.1. Medicinal Plants

Medicinal plants are rich source of novel drugs that forms the ingredients in traditional systems of medicine, modern medicines, nutraceuticals, food supplements, folk medicines, pharmaceutical intermediates, bioactive principles and lead compounds in synthetic drugs (Ncube, 2008). Plants have long served as a useful and natural source of therapeutic agents. Almost all plants have medicinal values and their uses differ from place to place. Medicinal plants are worldwide in distribution and abound in the tropics. Tropical plant species are claimed to contain three to four times the number of active chemical constituents than their temperate counter-parts (Rodríguez and West, 1995). Herbs and vegetables have emphatically contributed to the improvement of human health, in terms of prevention, and or cure of different disorders (Dhiman and Chalwa, 2005; Negi, *et al.,* 2010). Use of medicinal plants has been there even before the discovery of conventional orthodox medicine. Many herbalists and naturopathic doctors use the bark, flowers, fruit, leaves, roots, seeds and/ or stems of plants in the preparation of herbal drugs because of their acclaimed folk medicinal usage. Practice of herbal medicine is dominated by some traditions e.g. Ayurvedic, Siddha and Unani (India) (Mohamed-Saleem, *et al.*, 2010), Kampoo (Japanese), Chinese herbology (China), Traditional African medicine (South Africa –Sangoma and Inyanga; West Africa –Ifa), Shamanic herbalism (South America) and Native American medicine.

Studies are going on throughout the world for the search of protective molecules that would provide maximum protection of the liver, kidney as well as other organs and practically very little or no side effects would be exerted during their function in the body (Montilla *et al.,* 2005; Mansour *et al.,* 2006). Liver diseases pose an enormous health problem in spite of tremendous strides in modern

medicine (WHO, 2006; 2008; 2010). There are hardly any drugs that can effectively control inflammation, protect the liver from the damaging effects of hydrophobic bile acids which are retained in cholestatic disorders, promote protein synthesis, manifest antioxidative, anti-lipid peroxidative and antifibrotic properties, prevent fat from infiltrating the liver, enhance glucuronidation, decreases intestinal absorption and suppresses hepatic synthesis and storage of cholesterol, stabilize hepatocyte membranes, help the liver to replace damaged tissue and regenerate itself, promotes effective metabolism of drugs by maintaining levels of CYP or Cytochrome P450, protect the liver from damage and also regulate the liver enzymes (Akah and Odo, 2010). Because of the affordability, availability and accessibility of dependable hepatoprotective drugs in scientific/ conventional medicine, plants play an important role in the management of various liver disorders and in meeting the demands of primary health care in many developing countries (WHO, 2009). Herbal drugs have gained importance and popularity in recent years because of their safety, efficacy and cost effectiveness (Mohamed-Saleem, *et al*., 2010).

Herbal drugs contain diverse chemical constituents such as Alkaloids, carotinoids, coumarins, essential oils, flavonoids, glycosides, lignans, lipids, organic acids, phenolics, terpenoids and xanthines. Countless number of medicinal plants and polyherbal formulations are proposed to possess hepatoprotective properties though some of them are supported by scientific evidence such as Himoliv (Bhattacharya, *et al.,* 2003), *Trianthema portulacastrum* L (Kumar, *et al.,* 2004), Liv 52 (Dandagi, *et al*., 2008), *Vitex negundo* (Tandon, *et al.,* 2008), *Leucas lavendulaefolia* (Kotoky, *et al.,* 2008), *Clausena dentate* (Rajesh, *et al.,* 2009), *Rubus aleaefolius* Poir (Hong, *et al.,* 2010), *Murraya koenigii* (Sathaye, *et al.,* 2011). Hepatoprotective activity of plants can be attributed to their phytochemical constituent(s) such as alkaloids (Vijyan, *et al*., 2003), Flavonoids (Okwu, 2003), Saponinns (Aka and Odo, 2010), terpenoids (Grabmann, 2005) and phenolics (Zeashan, *et al* 2009).

The triherbal formulation in this study was prepared from leaves of *Gongronema latifolia* Benth Benth, *Ocimum gratissimum Linn.* and V*ernonia amygdalina. Gongronema latifolia* Benth Benth, *Ocimum gratissimum Linn.* and *Vernonia amygdalina* are medicinal plants used for centuries in the African traditional medicine. These plants are commonly used in foods as vegetables and spices for flavouring and in medicine they are used for the treatment of various ailments such as diarrhea, headache, fever, hepatitis, dysentery, malaria, nausea, diabetes among others in the South Eastern part of Nigeria. They have been used singly and in combination with other plants for treatment of different disorders with subsequent scientific supports to these claims (Nwanjo and Alumanah, 2005; Erasto, *et al*., 2007; Adaramoye, *et al.,* 2008; Iwalokun, 2008; Nweze and Eze, 2009). The hepatoprotective potentials of a triherbal formulation containing these three plants are evaluated using different hepatotoxins.

2.1.1. *Gongronema latifolia* Benth

Gongronema latifolia Benth (Asclepiadaceae) commonly called Utazi in the south eastern parts or Arokeke in the western parts of Nigeria, aborode by the Asante and Akans of Ghana, dondo-polole by the Kissis of Sierra Leone or gasub by the Serers of Senegal is a globorous perennial edible climber. The leaves are stalked, alternate and simple while the stalks are pliable and soft. It is commonly used as vegetable and spice in food and in medicine, for the treatment of malaria, diabetes, hepatits and various gastrointestinal ailments in the Eastern part of Nigeria. In the traditional Igbo society, it is used in soups in combination with other herbs and spiced to improve to stimulate milk production in lactating mothers, improve appetite and normalize the menstrual flow. According to Iweala and Obidoa (2009), *G. latifolia* is not toxic to the liver and because it has the ability to reduce the level of

liver enzymes in the blood, its role is rather protective and not destructive to the liver. Flavonoids content in *G. latifolia* have been implicated as the major constituent that gives *G. latifolia* its hepa-protective properties (Perrissoud 1986). Perrissoud (1986) reported that flavonoids exert a membrane-stabilizing action that protects the liver cells from injury. The leaves are known to improve the general conditions of a patient, kill or expel intestinal worms and also stimulate appetite. (Irvine, 1961) and most of these claims have been supported with scientific claims (Ugochukwu and Badaby, 2003; Nwanjo and Alumanah, 2005; Nwanjo, 2005; Nwanjo and Alumanah, 2005; Okafor, 2005).

Phytochemical analysis of the plant extract showed that it contains polyphenols, glycosides, reducing sugars, alkaloids, essential oils, saponins and pregnanes among others (Morebise *et al*., 2002; Eleyinmi, 2007; Antai, *et al*., 2009). The forest vegetable is a good source of iron, vitamins, minerals and proteins (Okafor, 2005). The plants have high potentials as antioxidants, owing to high amount of flavonoids and phenolic compounds (Omale and Okafor, 2009). The active ingredients in *Gongronema* are Gonioanthelma and Gonolobus (Manach, *et al*., 2004).

Teas containing *G. latifolia, Vernonia amygdalina Del.* or *Cryptolepsis sanguinolenta* are also used throughout West Africa for the management of diabetes and other metabolic disease associated with the liver (Seeff, *et al* 2001). The antimicrobial activities of aqueous extracts of *V. amygdalina*, *Garcinia kola*, and *G. latifolia*, and their blends were evaluated against several test organisms, *Staphylococcus aureus, Bacillus subtilis, Escherichia coli*, and *Streptococcus salivarus* and it was observed that the blends have potential for use in the control of beer-spoilage organisms (Oshodi, *et al* 2004). Ugochukwu and Babady, (2003) and Ugochukwu, *et al*., (2003) reported that aqueous and ethanolic *G. latifolia* extracts had hypoglycemic, hypolipidemic and antioxidative properties.

G. latifolia leaves was used locally for the Treatment of Fowl Cough in Nigeria because of its antitussive and antioxidant properties (Essien, *et al*., 2007). Ethanolic leaf extract of *G. latifolia* was shown to have *in- vivo* Schizonticidal activity on *Plasmodium berghei* in Mice (Akuodor, *et al*., 2010). The aqueous extract of the dried leaves of *G. latifolia* significantly inhibited carrageen-induced rat paw oedema, carrageen-induced leucocyte migration in rats and dye leakage induced by intraperitoneal injection of acetic acid in mice indicating its antiinflammatory activity (Morebise, *et al* 2002). Onwuka, (2005) subjected raw beef to chemical and microbial analysis, then treated it with either extracts or powders of four local vegetable leaves namely *Gongronema latifolia* Benth, *Piper guineesis*, *Vernonia amygdalina Del.* and *Ocimum gratissimum Linn.* at three different concentrations. Samples treated with *Vernonia amygdalina Del.* extract and powdered leaves showed the lowest microbial counts followed by *Ocimum gratissimum Linn.*, *Gongronema latifolia* Benth and *Piper guineesis* in that order. Ugochukwu and Cobourne, (2003) and Nwanjo, *et al*., (2006), reported the protective effect of aqueous and ethanolic extracts from *G. latifolia* leaves on biomarkers of oxidative stress in streptozotocin-induced diabetic and non-diabetic rats.

Figure 1: *Gongronema latifolia* Benth**.**

2.1.2. *Ocimum gratissimum Linn.*

Ocimum gratissimum Linn. (African Basil) belongs to the family Lamiaceae commonly called Dai'doya' by the Hausas, 'efinrin' by the Yoruba, 'nchanwu' by the Igbos, *is* a shrub with many branches having simple oblong leaves. The plant is found throughout the tropics and subtropics and its greatest variability occurs in tropical Africa and India (Aruna and Sivaramakrishina, 1990) and has an aromatic smell when crushed. It is planted as a food flavour and for medicinal use. It is used as spices and condiments in salads, soups, pastas, vinegars and in traditional medicine for the treatment of several ailments such as upper respiratory tract infections, diarrhea, headache, fever, ophthalmic and skin diseases, pneumonia, sore throats and tonsillitis, urinary tract, wound, skin gastrointestinal infections and as an insect repellant. Most of these properties of *O. gratissimum* have been supported with scientific claims. (Elujoba, 2000; Pessoa, *et al***.,** 2002; Silva, *et al*., 2005; Travisan, *et al*., 2006; Nweze and Eze, 2009)

It is used as a febrifuge and as an ingredient in many malaria medicines, for rheumatic pains and lumbago and catarrh remedies (Ainslie, 1937). The oils are antiseptic and are used in dressing of wounds, as a mouth gargle, mosquito repellant (Sofowora, 2000), or mixed with alcohol and used topically for skin infections or taken orally for bronchitis. It is used as an expectorant, germicide for toothpastes and mouth washes (Holets, *et al*., 2003; Pessoa, *et al*., 200) and antihelminth (Chitwood, 2003). The essential oils are a potential candidate as a phytotherapeutic agent in some fungal diseases and for the control of fungi in the environment (Nakamura, *et al.*, 2004). They play an important role in preservation of pharmaceutical products (Travisan, *et al*., 2006) and also inhibit *Staphylococcus aureus* at a concentration of 0.75 mg\ml (Nakamura, *et al*., 2004). Nweze and Eze, (2009) reported the antimicrobial effect of *O. gratissimum* suggesting that it may not really threaten the efficacy of some conventional antibiotics that may have been taken concomitantly with it.

Phytochemical screening revealed the presence of tannins, Cardiac glucoside, phenolic compounds, saponin, alkaloids, steroids, flavonoids and terpenoids (Gill, 1992; Okwu, 2003). Eugenol, thymol, citral, geraniol and linalool have been extracted from the oil (Vierra and Simon, 2000). The essential oils of *O. gratissimum* are active against several species of bacteria (*Escherichia coli, Shigella, Salmonella* and *Proteus*), fungi (*Trichophyton rubrum* and *T. mentagrophytes*) and antihelmintic activity (Lemos, *et al*., 2005; Silva, *et al*., 2005; Pessoa, *et al*., 2002; Nweze and Eze, 2009). It can be applied as an insecticidal fumigant to control *Callosobruchus maculates* (Keita, *et al.,* 2001). Rabelo, *et al*., 2003 has reported the antinociceptive property of its essential oil.

A dose-dependent sedative effect of Ocimum oil was observed during the acute toxicity study in mice and rats and in the sub-chronic test in rats (Orafidiya, *et al.,* 2004). Fractions isolated from *O. gratissimum* leaves have been shown to contain components which contract guinea pig ileum, rat colon and raise rat mean arterial blood pressure (Onajobi, 1986). A study by Madeira, *et al.,* (2005) showed that the essential oil extracted from the leaves of *O. gratissimum* collected at different time periods, exerts significant relaxant effects on isolated guinea-pig ileum which may underlie the therapeutic action of the plant. The wound healing effect of leaf extracts of *O. gratissimum* was investigated in adult male Wistar rats. The results suggested that the methanolic extracts of *O. gratissimum* could be a potential wound healing agent due to its ability to enhance wound contraction (Osuagwu, *et al.,* 2004). Studies on *O. gratissimum* proved the plant extract can be a source of medication for people living with Human Immunodeficiency Virus, (HIV) and Acquired Immune Deficiency Virus, AIDS (Elujoba, 2000). The hypoglycemic effect of the methanolic extract of *Ocimum gratissimum Linn.* leaves was evaluated in normal and alloxan-induced diabetic rats. Intraperitoneal injection of the extract (400 mg/kg) significantly reduced plasma levels both in normal and diabetic rats by 56 and 68 %, respectively (Aguiyi, *et al.,* 2000).

Figure 2: *Ocimum gratissimum Linn.*

2.1.3. *Vernonia amygdalina Del.*

Vernonia amygdalina Del. (Asteraceae) have obovate, simple and entire leaves. The flowers occur in copious corymbose panicles white fragrant and are usually bee infested (Irvine, 1961). It is commonly called Bitter leaf because of its bitter taste, Onugbu (Igbo), Ewuro (Ibadan,) Etidot (Cross River) and grows predominantly in tropical Africa. The bitter taste is attributed to anti-nutritional factors such as alkaloids, saponins, tannins and glycosides (Butler and Bailey, 1973). It is commonly used as local iodine for wounds, in treatment of diabetes, cough, feverish condition, constipation, hypertension, emesis, nausea, diabetes, sexually transmitted diseases, loss of appetite-induced abrosia, dysentery and other gastrointestinal tract problems and some have scientific supports to these claims (Gyang, *et al*., 2004; Nwanjo, 2005; Amira and Okubadejo, 2007; Erasto, *et al*., 2007; Adaramoye, *et al.*, 2008; Iwalokun, 2008). Its leaf extract has been demonstrated to function satisfactorily as an alternative to hops in beer brewing (Babalola and Okoh, 1996).

Phytochemical analysis of the leaves yielded 2 known sesquesterpene lactones – Vernolide and Vernodalol, vernodalin, hydroxyvernolide, Vernonioiside B, Myricetin saponins, steroid glycosides, tannins, alkaloids, tannins, flavonoids, steroid glucosides (vernonioside A1-A4; for bitter tasting constituents and vernonioside B1-B3; for nonbitter related constituents) (Babalola, *et al*., 2001; Erasto, *et al.*, 2006). The sesquesterpene lactones have an *in vitro* cytotoxic action against KB tumour cells and Wilme's myeloma (Toubiana, 1975; Izevbigie, 2003; Izevbigie and Ernest, 2005). It is used to stimulate the digestive system as well as reduce fever. They are used as local medicine against leeches. The root and stem are used as chewing stick and the bitter taste has antimicrobial activity against the oral microflora. In Nigeria, it is used in place of hops in beer making because of the bitter taste which is due to antinutritional factors such as alkaloids, tannins, glycosides and saponins (Buttler and Bailey, 1973). In *V. amygdalina*, ascorbic acid decreases with storage time (Faboya, 1990). Elemental analysis

of the leaves revealed that they have adequate concentration of some elements e.g. Iron (1500 + 111ppm) and Calcium (10100 + 895 ppm) that are believed to be essential for normal growth (Ibrahim *et al.*, 2001). Oshodi, (1992) found that the dried leaves of *V. amygdalina* were rich in minerals, especially in phosphorus, and that the content of ascorbic acid was temperature dependent. The antioxidant activities of compounds (luteolin, luteolin 7-O- β -glucuronoside and luteolin 7-O- β -glucoside flavonoid) isolated from the leaves of *V. amygdalina* has been reported using coupled oxidation of β-carotene linoleic acid (Igile *et al*., 1994). The sesquiterpene lactone extract from the leaves has antihepatotoxic activity in hepatotoxic damage in rats (Babalola, *et al*, 2001; Arhoghro, *et al*., 2009).

V. amygdalina extracts may help suppress, delay, or kill cancerous cell in many ways, such as: Induction of apoptosis as determined in cell culture and animal studies. (Sweeney, *et al*., 2005; Song, *et al*., 2005), inhibition of the growth or growth signals of cancerous cells (Kupchan, *et al*., 1969; Jisaka, *et al*., 1993; Izevbigie, *et al*., 2004; Opata and Izevbigie, 2006), suppression of metastasis of cancerous cells in the body by the inhibition of NFκB is an anti-apoptotic transcription factors as demonstrated in animal studies (Song, *et al*., 2005), antioxidants - *V. amygdalina* may provide anti-oxidant benefits (Erasto, *et al*., 2007), enhanced chemotherapy sensitivity - *V. amygdalina* extracts may render cancerous cells to be more sensitive to chemotherapy (Sweeney, *et al.*, 2005), enhancement of the immune system - Many studies have shown that *V. amygdalina* extracts may strengthen the immune system through many cytokines (including NFκB, pro inflammatory molecule) regulation (Sweeney, *et al*., 2005). http//:en.wikipedia.org/wiki/vernonia_amygdalina_–_cite note-sweeny-1

Squeeze-washing and rinsing seem to be the best treatment to preserve both vitamins A and C. Drying at 45 °C is best recommended while freezing for less than ten days is most appropriate for the

conservation of vitamin A and C (Ejoh, 2005). Several stigmastane-type saponins such as vernoniosides A1, B1, A2, A3, B2, D3, A4 and C have been identified in the leaves (Ohigashi, *et al.,* 1991; Jisaka, *et al.,* 1992). The antioxidant activities of luteolin, luteolin 7-0, β-glucuronoside and luteolin 7-O-β-glucoside flavonoid compounds isolated from the leaves of *V. amygdalina* have been reported using coupled oxidation of β-carotene linoleic acid (Igile, *et al.,* 1994). Subsequently Jisaka, *et al.* (1993) and Khalafalla, *et al*., (2009) demonstrated that vernodaline and vernolide elicited antitumoral activities in leukemia cells P-388 and C-1210 with IC_{50} values of 0.11 and 0.17 µg/ml for vernodaline and 0.13 and 0.11 µg/ml for vernolide respectively. *V. amygdalina* extracts have also been suggested to have cell growth inhibitory effects in prostate cancer cell line (PC-3) and no effect on normal human peripheral blood mononuclear cells (PBMC) (Izevbigie, 2003). It significantly attenuated the hepatic triglyceride and LDL cholesterol levels of streptozotocin diabetic rats and has hypoglycemic effect (Nwanjo, 2005; Adaramoye, *et al*., 2008)

The antiplasmodial (Abosi and Raseroka, 2003; Tona, *et al*., 2004) antihelmintic (Abosi and Raseoka, 2005) properties of some sesquiterpene and steroidal constituents of *V. amygdalina in vitro* were studied. It dose dependently restored the efficacy of chloroquine against chloroquine resistance *P. berghei* malaria in mice (Iwalokun, 2008). *V. amygdalina* administration decreased blood glucose by 50 % compared to untreated diabetic animals in studies conducted using streptozotocin-induced diabetic laboratory animals (Uhegbu and Ogbechi, 2004; Nwanjo, 2005). The Fungitoxic and phytotoxic effect of *V. amygdalina* on Cowpea and Cowpea seedling pathogens was reported by Alabi, *et al*., (2005).

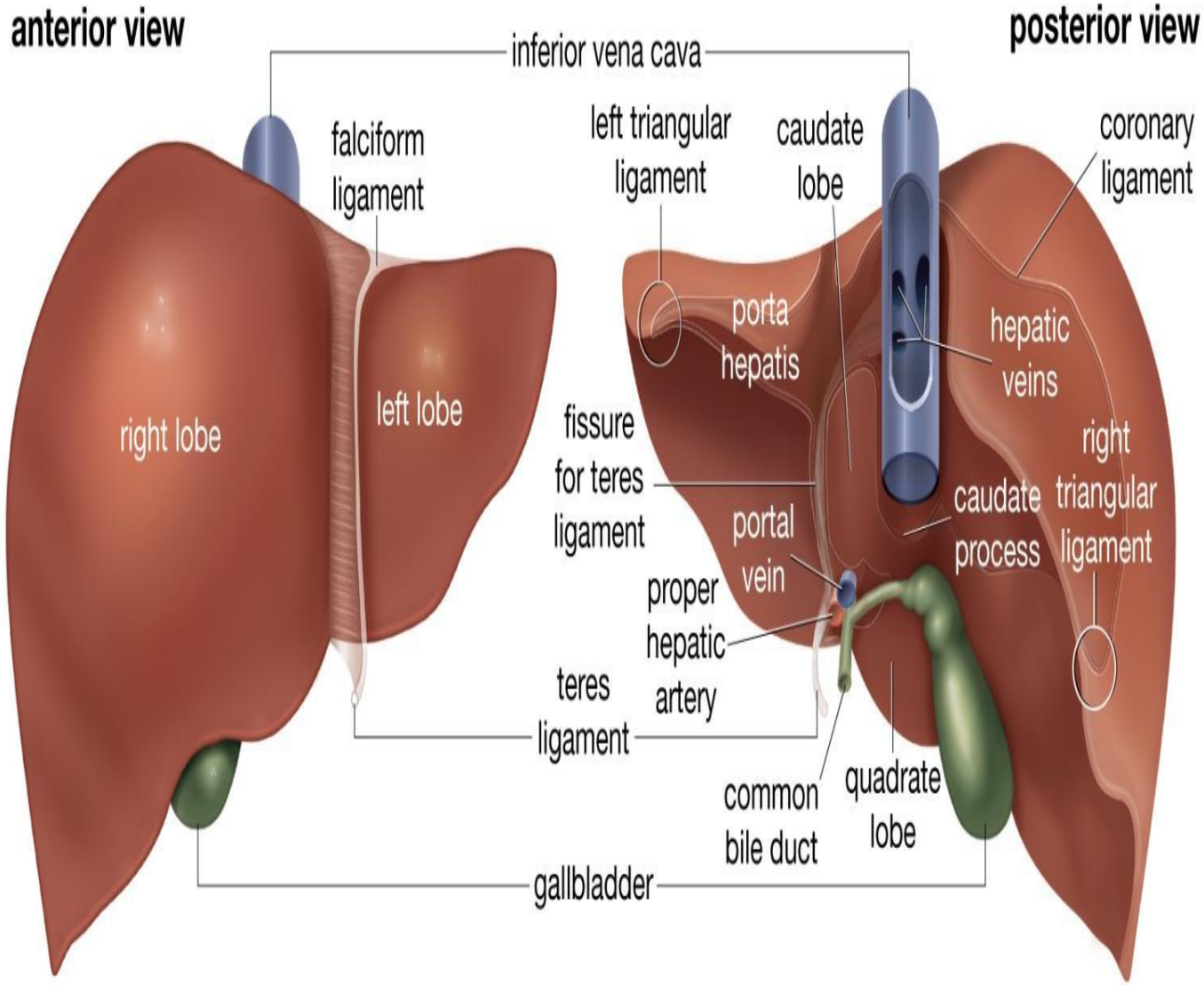
Figure 3: *Vernonia amygdalina Del.*

2.2. The Mammalian Liver

The liver is the largest glandular organ of the body and weighs approximately 1200-1500 g or, on average, one fiftieth of the total adult body weight. It measures about 8 inches (20 cm) horizontally (across) and 6.5 inches (17 cm) vertically (down) and is 4.5 inches (12 cm) thick (Cotran, *et al*., 2005). It is a soft, pinkish-brown or reddish brown, triangular organ located on the right side of the abdomen beneath the diaphragm and protected by the lower rib cage. The liver is covered entirely by visceral peritoneum, a thin, double-layered membrane that reduces friction against other organs. This strategic localization between nutrient-laden capillary beds and the general circulation is associated with hepatic regulation of metabolite levels in the blood through storage and mobilization mechanisms controlled by liver enzymes. It is divided into four lobes of unequal size and shape and contains as many as 100,000 lobules that are served by two distinct blood supplies. Blood is carried to the liver via two large vessels: the hepatic artery carries oxygen-rich blood from the aorta, and the portal vein carries blood containing digested food from the small intestine and the descending colon (Shneider and Sherman 2008). These blood vessels subdivide in the liver repeatedly, terminating in minute capillaries. Each capillary leads to a lobule which is made up of hepatic cells, the basic metabolic cells of the liver. On the surface of the lobules there are ducts, veins and arteries that carry fluids to and from them.

The liver has important and complex functions and plays a major role in metabolism (Das, 2011). Some of which are manufacture and secretion of bile (an alkaline compound which aids in digestion), which is stored in the gall bladder and released in the small intestine, conversion of glucose to its stored form, glycogen, which is reconverted into glucose as the body requires it for energy (Gao, *et al*., 2008), it maintains the proper level of glucose in the blood (glucose buffer function), converts excess carbohydrates and protein into fat, it further breaks down digested proteins in the form of

amino acids (Deamination), synthesis, storage, and processing of fats, including fatty acids (used for energy) and cholesterol, decomposition of red blood cells (Fanne, *et al.*, 2010), formation and secretion of bile that contains bile acids to aid in the intestinal absorption (taking in) of fats and the fat-soluble vitamins A, D, E, and K, synthesis of plasma protein, it metabolizes and stores carbohydrates (Gluconeogenesis - synthesis of glucose from certain amino acids, lactate or glycerol (Das, 2011; Fanne, *et al.*, 2010), Glycogenolysis - breakdown of glycogen into glucose and Glycogenesis - formation of glycogen from glucose), which are used as the source for the sugar (glucose) in blood that red blood cells and the brain use, synthesizes certain amino acids (the so-called nonessential amino acids) from other amino acids (transamination) (Zakim and Boyer, 2003; Das, 2011), it filters harmful substances from the blood (Phagocytic cells in the liver, called Kupffer cells, remove large amounts of debris and bacteria) (Seki, *et al*., 2000) and detoxifies by metabolizing and/or secreting, drugs, alcohol, and environmental toxins (Zakim and Boyer, 2003). The liver eliminates, by metabolizing and/or secreting, the potentially harmful biochemical products produced by the body, such as bilirubin from the breakdown of old red blood cells and ammonia from the breakdown of proteins (Seki, *et al*., 2000) , manufactures about 95% of the plasma proteins (antithrombin, protein C and S) and blood clotting agents (prothrombin, fibrinogen and other coagulation factors e.g. V, VII, IX, X and XI) (Palmer, 2004), converts ammonia to urea, it also plays an important role in thermoregulation; changes in metabolism of its cells varies the heat produced by the body and synthesizes angiotensinogen, a hormone that is responsible for raising the blood pressure when activated by renin, an enzyme that is released when the kidney senses low blood pressure and also disposes of ammonia, an extremely toxic byproduct of protein metabolism using the amino acids arginine and orthinine to control the ammonia levels (Zakim and Boyer, 2003; Das, 2011).



(Snell, 2004)

Figure 4: Diagram of the Mammalian Liver

In addition, the liver's ability to regenerate lost tissues helps maintain these functions, even in the face of moderate damage (Murray *et al.,* 2003; Suzuki, *et al*. 2008). The liver is among the few internal human organs capable of natural regeneration of lost tissue (Out, *et al*., 2007). As little as 25 % of remaining liver can regenerate into a whole liver again. This is attributed to the hepatocytes acting as unipotential stem cells (Sherlock and Dooley, 2002; Suzuki, *et al*., 2008). The liver is necessary for survival and there is currently no way to compensate for the absence of liver function as no artificial organ or device capable of emulating all the functions of the liver. Medical terms related to the liver often start in *hepato-* or *hepatic* from the Greek word for liver.

2.2.1. Diseases of the Liver

Because of its strategic location and multidimensional functions, the liver is also prone to many diseases (Sherlock and Dooley, 2002; Runyon, 2010). Liver disease (also called hepatic disease) is a broad term describing any single number of diseases affecting the liver. Some of the known liver diseases are hepatitis A, B, C, E, cancer of the liver, fatty liver, Gilbert's syndrome i.e. a genetic disorder of bilirubin metabolism, Budd-Chiari syndrome i.e. an obstruction of the hepatic vein, tumours, vascular obstruction, Wilson's disease i.e. a hereditary disease which causes the body to retain copper an autoimmune inflammatory disease of the bile duct, formation of fibrous tissue in the liver instead of replacing dead liver cells leading to cirrhosis, Alagille syndrome, biliary atresia, Hemochromatosis, alcohol damage, drug damage and Glycogen storage disease type II among others Sherlock and Dooley, 2002; Schiff *et al.,* 2003; Schiff *et al.,* 2007).

Jaundice is a common symptom of most liver diseases and this is caused by increase in the bilirubin level in the system since the liver cannot remove it from the blood so it can be excreted

through bile (Zakim and Boyer, 2003). This bilirubin results from the breakup of the hemoglobin of dead red blood cells. Other symptoms might include dark urine (when urine mixes with bilirubin), fatigue, abdominal pain, loss of appetite, abdominal swelling i.e. due to ascites and / or failure of the liver to produce albumin, itching of the skin (starts when bilirubin is deposited on the skin), nausea and vomiting, vomiting blood, bloody/ black stools, light-colored stools (this occurs when stercobilin, a brown pigment, is absent from the stool), and loss of libido. Some diets are believed to protect the liver such as vitamin E, thiamine because it lowers the iron load on the liver which is present in foods like pork, oatmeal, corn, nuts and cauliflower, higher coffee intake because it reduces the risk of cirrhosis and supplements of omega-3 fatty acids (Yurdakok, and Kanra, 1986; Hikino and Kiso, 1988; Wallace and Weeks, 2001).

2.2.2. Hepatitis

Hepatitis is an inflammation of the liver that can be caused by inherited diseases, viruses, drugs, chemicals, alcohol, environmental toxins or the patient's own immune system causing injury or destruction. In severe cases inflammation may interfere with the normal functions of the liver and allow potentially toxic substances to accumulate. Hepatitis varies in severity from a self-limited condition with total recovery to a life-threatening or life-long disease (Worman, 2001).

About two million patients infected with Hepatitis B die yearly and this makes it the ninth leading cause of death worldwide. The risk of cirrhosis and liver cancer increases with coinfection with hepatitis C or D. Diet influences the risk of developing liver cancer. Chronic carriers should be strongly encouraged to avoid consuming alcohol as it increases their risk for cirrhosis and hepatocellular carcinoma.

2.2.3. Causes of Hepatitis

Hepatitis is commonly caused by a virus such as hepatitis A or infectious jaundice caused by hepatitis A virus, a picornavirus transmitted by the fecal-oral route, hepatitis B caused by hepatitis B virus, a hepadnavirus transmissible through contact with blood or bodily fluids, tattoos, sexually or via mother to child by breast feeding , Hepatitis *C* caused by hepatitis C virus an RNA virus that is a member of the Flaviviridae family which is transmitted through the placenta and contact with blood. Others are hepatitis D and E virus. Cytomegalovirus, Epstein-Barr virus and yellow fever may also cause hepatitis.

Hepatitis can also be caused by an overactive immune system leading to chronic liver disease of unknown etiology characterized by circulating autoantibodies, hyperglobulinaemia and interface hepatitis and accounts for about 20 % of all chronic hepatitis cases (Parker and Picut, 2005), The Epstein – Barr virus which causes mononucleosis, measles virus, drug or toxin reaction or a hepatitis virus, can trigger off this reaction (Pieters, *et al*., 2002).

One of the most common causes of chronic hepatitis is fatty liver and this is mainly due to excessive alcohol consumption. It is usually reversible and resolved by abstaining from alcohol. The more severe form of hepatitis which is another cause of fatty liver is nonalcoholic steatohepatitis (NASH), the most common chronic hepatitis not caused by viruses (Perrez-Carreras, *et al*., 2003). While symptoms are usually fairly mild, it may cause cirrhosis. It is seen most commonly in people with hypertension, type 2 diabetes, high triglyceride levels, metabolic syndrome and overweight individuals (Videla, *et al.*, 2004).

Other causes of hepatitis are drugs, alcoholism, chemicals, and environmental toxins. Several inherited diseases can exhibit symptoms of acute or chronic hepatitis. Among these are deficiency of alpha-1-antitrypsin, hemochromatosis and Wilson's disease.

2.2.3.1. Viral Hepatitis

The most common cause of hepatitis is an infection with one of several viruses called Hepatitis Virus. The viruses primarily associated with hepatitis are named in the order of their discovery by the letters A through G. Viral hepatitis is a mostly enterically transmitted liver disease caused by viral infection. The major transmission path of the disease is through ingestion; viral hepatitis is also transmitted through blood transfusion of virus-contaminated blood or blood products such as blood plasma.

Hepatitis A or Infectious Hepatitis is excreted through feces and is contacted from infected food and water. Eating shellfish taken from sewage-contaminated water is a common means of contracting hepatitis A. Hepatitis A is contagious. It is the least serious of the common hepatitis viruses. Symptoms are mild such as Diarrhea, nausea, fatigue and jaundice although most patients recover fully within six months. Radioimmunoassay is generally used to identify IgM antibodies, first produced to fight hepatitis A.

Hepatitis C formerly called non-A non-B hepatitis is spread mainly by exposure to contaminated blood, through sexual activity that results in tissue tears or from mother to baby during childbirth. It is the most common cause of chronic hepatitis and is contagious. Most patients with hepatitis C do not experience symptoms but if they later appear, it develops about a month or two after infection. Patients with chronic hepatitis C may be susceptible to non-liver disorders e.g. : Hodgkin's

lymphomas, Cryoglobulinemia (a disorder in which protein clumps form in the blood), Certain autoimmune disorders, particularly hypothyroidism and rheumatoid arthritis, Porphyria cutanea tarda (a disorder which causes skin color and texture changes and sensitivity to light.) etc. specific tests include enzyme-linked immunosorbent assay (ELISA), They use a polymerase chain reaction (PCR) to detect the Ribonucleic acid (RNA) of the virus and blood tests showing elevated liver enzymes, particularly alanine aminotransferase (ALT).

Hepatitis D only causes an infection when hepatitis B is present and is spread by contact with infected blood while hepatitis E is spread through contaminated food and water. It is not serious except in pregnant women, when it can be life threatening. Hepatitis G is always chronic with probably the same modes of transmission as hepatitis C. To date much research indicates it does not cause disease or even increase the severity of any accompanying virus, including HIV or other hepatitis viruses.

Hepatitis B is a serious disease that causes scarring of the liver, liver failure, liver cancer and even death. The hepatitis B virus is the second most prevalent cause of cancer in humans after Tobacco smoke. Symptoms include jaundice, loss of libido, fatigue, itching, nausea, vomiting, dark urine, light coloured stool, abdominal pain and loss of appetite (Ryan and Ray 2004). Hepatitis B is spread through infected blood, other body fluids such as semen and vaginal secretions, use of contaminated sharp objects such as needles, razor and from mother to child during child birth. Hepatitis B is recognized as endemic in China and various other parts of Asia (Alberts, *et al* 2002).

Viral hepatitis is widespread around the world. China is estimated to have approximately thirty million viral hepatitis patients including an estimated number of nine million new patients each year and about one hundred million hepatitis B virus (HBV) carriers. Ten percent of the pregnant women in

China are estimated to be HBV carriers. About one hundred thousand people in China die each year of liver cancer originated from liver diseases.

2.2.3.2. Autoimmune Chronic Hepatitis

Autoimmune hepatitis is a chronic liver disease of unknown etiology characterized by circulating autoantibodies, hyperglobulinaemia and interface hepatitis and accounts for about 20 % of all chronic hepatitis cases (Parker and Picut, 2005). Their development is as a result of genetic defective immune system which attacks the liver after being triggered by an environmental agent, probably a virus. Autoimmune chronic hepatitis typically occurs in women between the ages of 20 and 40 who have other autoimmune diseases, including glomerulonephritis, rheumatoid arthritis, inflammatory bowel disease systemic lupus erythematosus, and hemolytic anemia (Pieters, *et al*., 2002). Some research shows that during the postmenopausal period, there may be a rise in incidence of autoimmune hepatitis among women (O´Connor, *et al*., 2009; van der Sluijs, *et al*., 2009). In general, no major risk factors have been discovered for this condition. The Epstein - Barr virus which causes mononucleosis, measles virus, drug or toxin reaction or a hepatitis virus, can trigger off this reaction. It is treated with prednisone and azathioprine.

2.2.3.3. Nonalcoholic Fatty Liver Disease (NAFLD)

One of the most common causes of chronic hepatitis is fatty liver which causes liver enlargement, tenderness, and abnormal liver function, and this is mainly due to excessive alcohol consumption. It is usually a reversible, resolved by abstaining from alcohol. The more severe form of hepatitis which is another cause of fatty liver is nonalcoholic steatohepatitis (NASH), the most

common chronic hepatitis not caused by viruses (Perrez-Carreras, *et al*., 2003). While symptoms are usually fairly mild, it may cause cirrhosis. It is seen most commonly in people with hypertension, type 2 diabetes, high triglyceride levels, metabolic syndrome and overweight individuals.

NAFLD has features similar to alcohol-induced hepatitis, but it occurs in individuals who do not drink significant quantity of alcohol. NAFLD is usually very slowly progressive and benign. In certain patients, however, it can lead to cirrhosis, liver failure, or liver cancer. It can be controlled by reducing Weight and managing any accompanying medical condition. Drugs, used to lower triglycerides or those that increase insulin levels may help protect against liver damage. Vitamin E may help reduce liver injury (Videla, *et al* 2004).

2.2.3.4. Inherited forms of Hepatitis

Several inherited diseases can exhibit symptoms of acute or chronic hepatitis. Among these are Deficiency of alpha-1-antitrypsin which is both acute and chronic in children and can lead to cancer and cirrhosis. Hemochromatosis is associated with accumulation of too much iron in the body and it affects mainly the liver and it can be chronic. An accumulation of excess copper in the liver, brain, and in other tissues commonly known as Wilson's disease and can be worse even fatal if not treated.

2.3. Hepatotoxicity

Approved drugs are sometimes withdrawn from the market after a post-marketing discovery of toxicity that was not detected in the extensive preclinical and clinical testing (WHO, 2010). During the last decade liver toxicity has been one of the most frequent reasons for pharmacovigilance safety reports and the withdrawal from the market of an approved medicinal product (EMEA, 2008) e.g. of

such potentially hepatotoxic drugs are Didanosine (WHO, 2010), Olanzapine (FDA, 2009), Bufexamac (EMA, 2010), Rosiglitazone (SFDAA, 2010), Ketoconazole tablets (MHRA, 2008a), Moxifloxacin (MHRA, 2008b; EMEA, 2008). The occurrence of drug-induced liver toxicity is the single most common reason for the regulatory actions concerning drugs (Boelsterli and Lim, 2007; Kodell and Chen, 2010). The liver is the primary site for drug metabolism and hepatotoxicity is one of the most frequently reported human adverse drug reactions and cause reactions that are similar to those of acute viral hepatitis (Mumoli, *et al*., 2006). Symptoms can be observed two weeks to six months after commencing drug treatment although most disappear when the drug is withdrawn; but, in rare circumstances, they may progress to serious liver disease. Specifically, the existence of relatively small, unidentified, hypersensitive subpopulations could explain the occurrence of perculiar liver injury only after approved drugs have been prescribed for large numbers of patients (Shenton, *et al*., 2004; Kaplowitz, 2005).

In some countries, hepatoprotective drugs are frequently prescribed as part of treatment for tuberculosis e.g. China. (Nolan, *et al*., 1999; Ho, 2006). To treat tuberculosis (TB), traditional Chinese medicine practitioners typically advocate a combination of biomedical treatment to eliminate bacteria, and traditional medicine to strengthen qi (Ho, 2006) and herbs to protect the liver (Zhang, *et al*., 1995).

Toxic liver injury produced by drugs and chemicals may virtually mimic any form of naturally-occuring liver disease (Luyendyk, *et al*., 2003; Kaplowitz, 2005). Drug-induced hepatotoxicity is a major issue for drug development, and toxicogenomics has the potential to predict toxicity during early toxicity screening (Maddox, *et al*., 2006; WHO, 2010). Hepatoprotective effect have been studies against chemicals and drugs induced hepatotoxicity in laboratory animals like thioacetamide, alcohol, rifampicin, CCl_4, galactosamine, paracetamol, isoniazid, Aflatoxin B etc (Dhuley, *et al.*, 2002;

Srivastava and Shivanandappa . 2006; Lal *et al.,* 2007; Naaz, *et al.,* 2007; Tandon, *et al.,* 2008; Shaarawy, *et al.,* 2009; Khatri, *et al.,* 2009; Upur, *et al.,* 2009; Somasundaram, *et al.,* 2010; Roy and Das , 2010).

Hepatotoxic signals including reversibility may be identified and evaluated from standard non-clinical studies. Important and easily accessible signals may be obtained from clinical chemistry, histopathology and ultrastructural pathology among others (Dambach, *et al*., 2005).Clinically, the most relevant reactions include liver necrosis, hepatitis, cholestasis, vascular changes and steatosis (Zimmerman, 1978). There are 3 main types of liver injuries biochemically. These are Cholestatic, Hepatocellular and mixed hepatotoxicity (Green and Flamm, 2002; Lee and Senior, 2005; Temple, 2006)

Cholestatic hepatotoxicity is characterized by jaundice and development of pruritus. It is often accompanied by marked elevation of serum alkaline phosphatase and total bilirubin levels. Amoxicillin/clavulanate, Anabolic steroids, Oral contraceptives, Erythromycins and Estrogens are classified to be a potential Cholestatic hepatotoxin. It rarely leads to chronic liver disease and vanishing bile duct syndrome i.e. progressive destruction of intrahepatic bile ducts (Evans and McLeod, 2003).

Hepatocellular hepatotoxicity manifests as malaise and right upper quadrant abdominal pain, associated with marked elevation in aminotransferase levels especially ALT. In severe cases, this may be accompanied by hyperbilirubinemia (hepatocellular jaundice). According to Hy's law, hyperbilirubinemia is associated with mortality rates as high as 50% if hepatocellular liver injury is accompanied by jaundice, impaired hepatic synthesis, and encephalopathy, chance of spontaneous recovery is low, and liver transplantation should be considered (Lewis, 2006; Senior, 2006). This type

of injury can result from drugs such as Acetaminophen, Tetracyclines, Highly Active Antiretroviral Therapy (HAART) drugs, Isoniazid, Ketoconazole, Lisinopril, NSAIDs and Rifampin so they are classified to be a potential Hepatocellular hepatotoxic drugs (Borne, 1995; Goldkind and Laine, 2006).

In mixed hepatotoxicity, neither aminotransferase nor alkaline phosphatase elevations are clearly predominant and symptoms may also be mixed. Drugs such as Azathioprine, Clindamycin, Nitrofurantoin, Phenobarbital, Sulfonamides and Trimethoprim/sulfamethoxazole can cause this type of injury. Liver histopathology should serve as the most important tool for identifying and characterizing liver injury whether or not clinical chemistry changes are also identified. The presence of significant apoptosis/necrosis should be addressed (the pattern of cellular damage, the presence of cellular infiltrates, and the presence of necrotic and/or apoptotic cells) (Jaeschke and Lemasters, 2003; Foster, 2005). Ultrastructural pathology can provide evidence for enzyme induction, mitochondrial changes, drug accumulation, and early indications of cholestasis, necrosis, steatosis etc (Maddox, *et al*., 2006).

2.4. Pharmacological Models for Inducing Hepatitis

Many drugs such as Acetaminophen (Chitturi and George, 2002), statins (Jacobson, 2006) can cause asymptomatic elevation of hepatic enzymes even when taken at therapeutic doses (Setty, *et al*., 2007). However, clinically significant liver injury (eg, with jaundice, abdominal pain, or pruritus) or impaired liver functions that can result in deficient protein synthesis with prolonged Prothrombin Time (PT) or hypoalbuminemia can occur (Boone, *et al*., 2005). The pathophysiology of drug induced liver injury varies depending on the drug or hepatotoxin (Kaplowitz, 2005). Drug-induced

injury mechanisms include covalent binding of the drug to cellular proteins resulting in immune injury, inhibition of cell metabolic pathways, blockage of cellular transport pumps, induction of apoptosis, and interference with mitochondrial function (Lee, 2003; Maddox, *et al*., 2006).

2.4.1. Alcohol

Alcohol, the only legal and socially acceptable recreational drug is a toxin that is especially harmful to the liver. Alcoholic liver diseases (ALD), particularly cirrhosis, is one of the leading causes of alcohol related death. Liver cirrhosis is a major cause of death in the United States (Yoon, *et al*., 2002; Minino, *et al*. 2002). In 2000, it was the 12th leading cause of death (Mann, *et al*., 2003). In an assessment by the WHO in 2005, 4 % of the burden of disease and 3.2 % of all deaths globally were attributable to alcohol. ALD is the foremost health risk in developing countries and ranks third in developed countries. (WHO, 2005)

Alcohol abuse and alcoholism represents one of the major health, social and economic issues facing the world and the liver is among the organs most susceptible to the toxic effects of ethanol (Lieber, 2008). Alcohol (ethanol) is readily absorbed from the stomach, but most is absorbed from the small intestine and cannot be stored. Chronic alcohol intake is known to produce hypercholesterolemia (Polychronopoulos, *et al*., 2005), hyperlipidemia (Kim, *et al*., 2004) and hypertriglyceridemia (Bessembinders, *et al*., 2011). A small amount is degraded in transit through the gastric mucosa. The liver is the major site of ethanol metabolism and thus sustains the most injury from chronic alcohol consumption (Srivastava and Shivanandappa, 2006; Sathaye *et al.,* 2010).

It is metabolized primarily by alcohol dehydrogenase (ADH) but also by, by catalase in a H_2O_2-dependent reaction, the microsomal enzyme oxidation system (MEOS), cytochrome P-450 2E1

(CYP2E1) particularly the ethanol-inducible CYP2E1, which is largely responsible for the increased rate of alcohol metabolism associated with chronic exposure and, to a lesser extent, cytosolic aldehyde dehydrogenases (ALDH2 and ALDH1, respectively). (Srivastava and Shivanandappa, 2006; You *et al.*, 2008; Sathaye *et al.*, 2010). ADH is a cytoplasmic enzyme that oxidizes alcohol into acetaldehyde (Ishak, *et al*., 1991). Acetaldehyde is a highly reactive intermediate which is further metabolized in mitochondria to acetate by acetaldehyde dehydrogenase (ALDH), Ramchandani, *et al.*, 2001). Chronic alcoholism induces the MEOS (mainly in endoplasmic reticulum), increasing its activity. The main enzyme involved is CYP2E1. When induced, the MEOS pathway can account for 20 % of alcohol metabolism. This pathway generates harmful reactive O_2 species, increasing oxidative stress and formation of oxygen-free radicals. CYP 2E1-mediated ethanol metabolism not only leads to the formation of acetaldehyde, but also to the formation of oxygen and hydroxyethyl radicals that in turn promote the formation of other highly reactive intermediates (Tuma and Casey, 2003). Acetaldehyde binds covalently to proteins forming adducts that are antigenic. Humans and animals exposed to long term alcohol abuse develop persistent circulating antibodies that recognize acetaldehyde protein adducts (Wehr *et al.*, 1993; Brooks, 1997). Acetaldehyde modified self proteins may serve as neoantigens, initiating harmful humoral and/or cellular immune responses, which lead to tissue injury (Kannarkat *et al*., 2006; Lieber *et al.*, 2008).

These oxidative reactions generate hydrogen, which converts nicotinamide-adenine dinucleotide (NAD) to its reduced form (NADH), increasing the redox potential (NADH/NAD) in the liver (Bondy, 1992). The increased redox potential inhibits fatty acid oxidation and gluconeogenesis, promoting fat accumulation in the liver which may predispose to subsequent oxidative damage (Baraona, *et al.*, 1983; Xu, *et al.*, 1998).

Alcohol consumption causes oxidative damage which is increased by liver hypermetabolism, reduction in protective antioxidants (eg, glutathione, vitamins A and E), binding of alcohol oxidation products, such as acetaldehyde, to liver cell proteins, forming neoantigens and resulting in inflammation, inflammatory cytokines secreted by WBCs, free radical–induced lipid peroxidative damage and accumulation of neutrophils and other WBCs, which are attracted by lipid peroxidative damage (Fernandez–Checa, *et al* 1997; McCord, 1998). Removal of toxic reactive oxygen species is achieved by three major antioxidant enzymes. These are catalase, superoxide dismutase and glutathione peroxidase (Polavarapu, *et al.*, 1998). The generation of high concentrations of free radicals during the metabolism of alcohol may exceed the capacity of the antioxidant defence mechanisms and contribute to the development of alcohol induced liver injury (Polavarapu *et al*, 1998; Fattaccioli *et al*, 1999).

There are three kinds of liver disease related to alcohol consumption. Fatty liver (Steatosis), alcoholic hepatitis (steatohepatitis) and cirrhosis *(*Sørensen, 1990) are often considered separate, progressive manifestations of alcoholic liver disease. Steatosis is the initial and most common consequence of alcohol–induced liver disorder and is potentially reversible with abstinence. If excess alcohol intake persists, it may progress to cirrhosis (Teli, *et al*, 1996). It is marked by a build-up of fat cells in the liver since a large proportion of the cytoplasm of affected hepatocytes is occupied by a single large triglyceride occlusion (Nanji, *et al*., 1996). This causes the liver to enlarge. Usually there are no symptoms though discomfort in the upper abdomen may be experienced.

Steatohepatitis is a combination of fatty liver, diffuse liver inflammation, and liver necrosis (often focal)—all in various degrees of severity. Severely damaged hepatocytes become necrotic.

Sinusoids and terminal hepatic venules are narrowed. Symptoms may include loss of appetite, nausea, vomiting, abdominal pain and tenderness, fever and jaundice. In its mild form, alcoholic hepatitis can last for years and will cause progressive liver damage while the disease may occur suddenly, after binge drinking, and it can quickly lead to life-threatening complications in its severe form. Cirrhosis may also be present.

Cirrhosis refers to the replacement of normal liver tissue with scar tissue. Alcoholic cirrhosis is advanced liver disease characterized by extensive fibrosis that disrupts the normal liver architecture (Iredale, 2007; Weiler-Normann, *et al*., 2007). It is the most severe form of alcoholic liver injury and is usually of the micronodular type. The feeble compensatory attempt at hepatic regeneration produces relatively small nodules (micronodular cirrhosis). As a result, the liver usually shrinks. In time, even with abstinence, fibrosis forms broad bands, separating liver tissue into large nodules. Many heavy drinkers will progress from fatty liver to alcoholic hepatitis and finally to alcoholic cirrhosis, though the progression may vary from patient to patient. At least 80% of heavy drinkers develop steatosis, 10%–35% develop alcoholic hepatitis, and approximately 10% will develop cirrhosis (Grant, *et al*, 1988). The risk of developing cirrhosis is particularly high for people who drink heavily and have another chronic liver disease such as viral hepatitis C.

Alcoholic liver disease does not affect everyone who drinks alcohol (Frezza, *et al*., 1990). Though it is dependent on the amount of alcohol consumed over time, some people seem to be more susceptible to the adverse effects of alcoholic beverages (Jones–Webb, 1998). Liver damage can be very severe after years of drinking, although genetic factors, high-fat diets and sex may play a role in increasing or decreasing a person's risk for alcoholic hepatitis (Corrao, *et al.*, 1994; Nanji, *et al*., 2001). Poor dietary habits may also contribute to the progression of alcohol liver disease (Lieber,

2003). In fact, malnutrition defined by the lack of calories and protein consumed is noted in almost all people with advanced alcohol liver disease (Stump 2002).

Diagnosis typically relies on laboratory tests of three liver enzymes: gamma–glutamyltransferase (GGT), aspartate aminotransferase (AST), and alanine aminotransferase (ALT) (Marsano, *et al*., 2003). Liver disease is the most likely diagnosis if the AST level is more than twice that of ALT (Marsano, *et al*., 2003), a ratio some studies have found in more than 80 percent of alcoholic liver disease patients.

2.4.2. Acetaminophen (APAP)

Acetaminophen (APAP), also known as paracetamol is a commonly used analgesic and antipyretic drug (Girish, *et al.,* 2009). It is quite safe and well tolerated in therapeutic doses, but an acute APAP overdose can cause potentially fatal hepatic and renal necrosis or acute liver failure characterized by centrilobular necrosis in both man and experimental animals (Rajesh, *et al.,* 2009; Sreedevi, *et al.,* 2009; Roy and Das, 2010). Acetaminophen overdose contributes significantly to cases of drug induced hepatitis (WHO, 2004) and causes hepato-renal oxidative stress (Ghosh and Sil, 2007).

Reactive oxygen (ROS) and nitrogen (RNS) species play an important part in the development of hepatotoxicity caused by APAP (Michael *et al.,* 1999; Knight *et al.,* 2001; Knight and Jaeschke, 2002). Formation of an electrophilic metabolite by the cytochrome P450 system, presumably *N*-acetyl-*p*-benzoquinone imine (NAPQI) in the liver especially CYP2E1, is a prerequisite for the injury (Nelson and Bruschi, 2003; Hinson, *et al*, 2004). NAPQI is effectively detoxified by glutathione but at large doses of acetaminophen, it conjugates with GSH leading to its depletion in the liver causing

oxidative stress (Mitchell *et al.*, 1973; Van de Straat, *et al.*, 1987). This is because at very high doses, the drug can no longer be metabolized by the liver, and the excess is oxidized into NAPQI leading to a rapid depletion of the internal antioxidants glutathione and S-adenosyl-L-methionine in the liver (Gardner, *et al*., 2002). NAPQI also binds to vital cellular and mitochondrial proteins and activate cells of the immune system. When the cellular glutathione stores are depleted, cellular necrosis (Cohen, *et al.*, 1997), release of pro-inflammatory cytokines (Pumford, *et al*, 1997; Cheung, *et al*, 2002; Manov, *et al*, 2008) and cytochrome *c* from the mitochondria (Adams, *et al.*, 2001; Knight and Jaeschke, 2002), impaired mitochondrial respiration (Meyers, *et al.*, 1988; Ramsay, *et al.*, 1989), depletion of hepatocellular ATP levels (Jaeschke, 1990; Tirmenstein and Nelson, 1990), and opening of the mitochondrial membrane permeability transition pore (Kon *et al.*, 2003; Hinson *et al.*, 2004) are observed so liver cell death begins to occur. Necrosis is recognized as the mode of cell death and apoptosis has been ruled out (Lawson, *et al.*, 1999; Gujral, *et al.*, 2002).

Within 24 hours of a toxic dose of acetaminophen, nausea, vomiting, and abdominal tenderness may be present. Elevation of liver enzymes can occur from an acute dose as soon as 36 hours after ingestion (Ankeer, 2001). Within days, liver damage can result, followed by kidney damage. If liver failure occurs, mortality rates are relatively high (Oz, *et al.*, 2004). Kidney function tests and liver enzyme measurements help assess adverse effects from acetaminophen (Wu, 1994; Brestel, 1994). Combinations of drugs such as acetaminophen and alcohol have the potential to cause life-threatening acute liver failure.

2.4.3. Carbon Tetrachloride

Carbon tetrachloride (CCl_4) also called tetrachloroethane, or benziform, is a clear, colorless, nonflammable, volatile liquid that has a high vapor density and characteristic sweetish, aromatic odour (CPHF, 1988). Carbon tetrachloride was originally synthesised by the French chemist Henri Victor Regnault in 1839 by the reaction of chloroform with chlorine, (Regnault, 1839) but now it is mainly produced from methane. It is used as a grain fumigant, dry-cleaning and fabric spotting agent, used to reduce fire hazard in combinations with either carbon disulfide or ethylene dichloride (fire-extinguisher fluid solvent), for the recovery of tin in tin-plating waste, Industrial solvent for nylon, cable and semiconductor manufacture (Channabasavaraj, *et al.,* 2008; Akindele *et al.,* 2010). It is used as solvent for oils, fats, lacquers, varnishes, rubber waxes, resins and as a starting material in manufacture of organic compounds (IARC, 1999).

Exposure to carbon tetrachloride results mostly from breathing contaminated air or drinking contaminated water near manufacturing plants or waste sites, or coming in skin contact with water while showering or cooking with contaminated water, swimming or bathing in contaminated water, contact with or ingesting contaminated soil at waste site. Exposure to very high amounts of carbon tetrachloride can damage the liver, kidneys, and nervous system and can also cause cancer (Channabasavaraj, *et al.,* 2008; Pradeep *et al.,* 2009; Hong, *et al.,* 2010; Akindele *et al.,* 2010). Carbon tetrachloride moves readily through soil and adsorbs only slightly to sediment. Releases or spills of carbon tetrachloride on soil should result in rapid evaporation and leaching to groundwater causing contamination, as it does not adsorb to soil well (U.S. EPA, 1998). The major sources of carbon tetrachloride in drinking water are discharge from chemical plants and other industrial activities. Most carbon tetrachloride in food was residual contamination from fumigation as a pesticide (U.S. EPA, 1984).

CCl_4-induced reactive free radicals initiate cell damage through two different mechanisms of covalent binding to the membrane proteins and cause lipid peroxidation (Kanter, *et al.,* 2003; Kanter, *et al.,* 2005). CCl_4 is rapidly metabolized by mixed function cytochrome P450 oxygenases of the endoplasmic reticulum (ER) resulting in the generation of CCl_3^- (Plaa, 2000; Weber, *et al.,* 2003). This is followed by chloromethylation, saturation, peroxidation and progressive destruction of the unsaturated fatty acid of the endoplasmic reticulum membrane phospholipids (Reynolds and Moslen, 1979). Adduct formation between CCl_3^- and DNA is believed to initiate hepatic cancer (Weber, *et al.,* 2003). CCl_3^- can also react with oxygen to form CCl_3OO^- (Plaa, 2000; Weber, *et al.,* 2003). CCl_3^- initiates a chain reaction leading to lipid peroxidation, changes in membrane permeability, and loss of calcium homeostasis. In addition, tissue lipid levels increase.

Nearly half of the absorbed carbon tetrachloride is excreted unchanged, but the remainder is metabolized by reductive dehalogenation to the trichloromethyl free radical via the cytochrome P-450 enzyme system (McCay, Lai *et al.,* 1984). This radical may undergo both oxidative and reductive biotransformation and the isoenzymes implicated in this process are CYP2EI and CYP2B1/2B2 (Zhang, *et al.,* 2003; Rajesh, 2004), though the major human enzyme responsible for carbon tetrachloride bioactivation at lower, "environmentally relevant" levels is P450-2E1 (Zangar *et al.,* 2000). Known metabolites include phosgene, carbon monoxide, carbon dioxide, chloroform, and hexachloroethane (Fleming and Hodgson, 1992; Pohanish, 2002). This radical quickly adds molecular oxygen to form trichloromethylperoxy radical (Letteron, Labbe *et al.,* 1990). Removal of hydrogen atoms from unsaturated fatty acids by such radical creates carbon-centered lipid radicals (McCay, Lai *et al.,* 1984). These latter radicals quickly add molecular oxygen to form lipid peroxyl radicals, thereby, initiating the process of lipid peroxidation (Letteron, Labbe *et al.,* 1990). Unless scavenged by radical scavengers, these lipid peroxyl radicals in turn abstract hydrogen atoms from other lipids

molecules, thereby propagating the process of lipid peroxidation (Recknagel, 1983). In addition to the binding of carbon tetrachloride metabolites to protein and lipid macromolecules, there is also evidence of binding to DNA (DHS, 1987).

Carbon tetrachloride is a selective hepatotoxic chemical agent (Hong, *et al.,* 2010; Somasundaram, *et al.,* 2010) and one of the most widely used toxicant for experimental induction of liver fibrosis in laboratory animals (Weiler-Normann, *et al*., 2007; Akindele *et al.,* 2010). Carbon tetrachloride is hepatotoxic in humans and the effects appear rapidly. The kidney is also a major target of carbon tetrachloride toxicity. Characteristic renal injuries from carbon tetrachloride exposure are nephritis, nephrosis, and renal failure. The initial cellular injury subsequently takes the form of inflammation (Plaa, 2000; Weber, *et al.,* 2003), and the chronic effects of CCl_4 exposure include fatty degeneration, fibrosis, hepatocellular death, and carcinogenicity (Weber, *et al.,* 2003).

In liver fibrosis induced in rats by carbon tetrachloride (CCl_4) and in human patients with an acute liver disease, Mirza, *et al*., (1997) and Johnson, *et al.,* (1997) found a dramatic rise in tissue transglutaminases activity. After oral administration, CCl_4 concentrates in the liver. CCl_4 decreases secretion of triglycerides as part of very low density lipoproteins and increases hepatic triglyceride by 195% within 3 h in rats (Boll, *et al.,* 2001). A distinctive feature of CCl_4 toxicity is the rapidity of triglyceride accumulation in the liver due to a failure in their secretory mechanisms (Plaa, 2000; Weber, *et al.,* 2003). This failure has been largely attributed to unspecified functional impairment in the Golgi apparatus (Weber, *et al.,* 2003).

Acid and alkaline Phosphatase was a clear indication of cellular leakage and loss of functional integrity of cell membrane (Joel and Lee, 2001) because they are cytoplasmic in location and released into circulation after cellular damages (Rajesh, 2004; Omar *et al.,* 2006). In humans, alterations in

lipid metabolism in the liver may be observed 30 minutes following administration. Histological changes may be observed within one hour. Centrilobular necrosis and hepatic steatosis (fatty degeneration) are characteristic toxic lesions. In mice, CCl_4 decreased plasma triglyceride-rich lipoproteins, increased cellular lipids, and reduced microsomal triglyceride transfer protein (MTP) without diminishing mRNA levels (Pan, *et al.,* 2007). The capacity of liver microsomes to incorporate amino acids is depressed, causing a generalized inhibition of protein synthesis. These changes result in rapid loss of the ability of liver to synthesize albumin (Rothschild, 1972) and other proteins causing an overall significant decrease in the protein and serum albumin content. According to Jadon, *et al*. (2007), uncoupling of oxidative phosphorylation by CCl_4 causes a fall in the activity of ATPase while reduction in SDH activity may be due to the structural and functional disorganization of the mitochondrial assembly. Thus, when mitochondria are damaged, energy generation in them is inevitably inhibited which contributes to the overall loss in the energy production (Gao, *et al.,* 2004; Chattopadhyay, *et al.,* 2006).

Excessive exposure to carbon tetrachloride may affect the brain, digestive system, eye, nervous system kidney, liver and skin, and may cause cancer (Takahashi, *et al*. 2002). Greater toxicity of carbon tetrachloride has been reported in heavy drinkers, possibly due to induction of additional CYP2E1 enzyme by chronic ethanol consumption (Manno and Rezzadore, 1994). Compounds that induce cytochrome or delay tissue repair enhance CCl_4 toxicity (Plaa, 2000; Weber, *et al*., 2003).

2.4.4. D- Galactosamine (D-GaIN)

D-Galactosamine (D-GaIN) is a hexosamine derived from galactose. It is a constituent of some glycoprotein hormones such as follicle-stimulating hormone (FSH) and luteinizing hormone (LH). Galactosamine (GalN) is an established experimental hepatotoxin (Upur, *et al.,* 2009; Jaishree and

Badami, 2010). Liver injury induced by *D*-galactosamine is of clinical importance because the multifocal necrosis produced, and the metabolic and morphological aberrations in the liver of experimental animals closely resembles the lesions caused by acute human viral hepatitis (Kucera, *et al.,* 2006) and also that induced by the H-1 strain virus, one of two serologic types of rat virus, in its symptoms (Keppler *et al*., 1968).

D-GalN hepatitis is induced by a multiple step mechanism (Black *et al.,* 1983). The cholestasis caused by galactosamine may be from its damaging effects on bile ducts or ductless or canalicular membrane of hepatocytes (Saraswat, *et al.,* 1996). It mainly causes liver injury by inhibiting the synthesis of RNA and protein via the generation of free radicals and depletion of cellular UTP nucleotides (a substrate for RNA polymerase) (Wills and Asha, 2006; Han, *et al.,* 2006) followed by the formation of UDP hexosamines (Kmiec, *et al.,* 2000). According to Mangeney-Andreani *et al.* (1982), GaIN inhibits the energy metabolism of hepatocytes, it also injures the enzymes involved in the transport of substrates to the mitochondria and modifies the phospholipid composition of membranes (Sire, *et al.* 1983). These changes affect cell membranes and organelles, the synthesis of proteins and nucleic acids (Keppler and Decker 1969) and the location of proteoglycans are changed in the rat liver (Sasaki *et al.* 1996). This leads to the inhibition of transcription and consequently the translation processes (Keppler, *et al.,* 1974) which finally leads to the necrosis of liver cells (Decker and Keppler, 1974; Kmiec, *et al.,* 2000).

D-GaIN has been proposed to be hepatotoxic due to its ability to destroy liver cells possibly by a free radical mechanism, so hepatic apoptosis and necrosis induced by GaIN intoxication are involved in a free radical dependent fashion in hepatocytes (Quintero, *et al.,* 2002; Ohta, *et al.,* 2007). Oxidative degeneration of polyunsaturated fatty acids, has been linked with altered membrane structure and

enzyme inactivation (Comporti, 1985). The peroxidation of endogeneous lipids is a major factor in the cytotoxic action of GaIN (Hu and Chen, 1992: Sakaguchi and Yokota, 1995). Several reports have suggested that oxidative stress induced by GaIN suppressed hepatic GSH levels in animal treatment (Itokazu, *et al.,* 1999; Daizo, *et al.,* 2005). GaIN-induced hypoglycaemia-related glucose auto-oxidation (Rozance and Jr, 2010) may also be responsible for the increased generation of free radicals in GaIN hepatitis.

GaIN intoxication also induces renal dysfunction in connection with hepatic disorders (Javle, *et al.,* 1998). Renal failure appeared with acute liver damage where the histological status of the kidney was normal but the renal blood flow, sodium excretion, glomerular filtration rate and creatinine clearance was decreased while there was an increase in plasma endothelin-1 (ET-1) concentration and also up-regulation of ET-1 receptor in renal cortex (Anand, *et al.,* 2006). Moore, *et al.,* (1992) reported that levels of plasma ET-1 concentration are elevated in patients with severe liver disease and hepato-renal syndrome.

Daizo, *et al.,* (2005) observed that liver weight decreases 24 hr after GaIN injection as a result of atrophy followed by necrosis. Liver damage induced by GaIN commonly reflects turbulence of liver cell metabolism which leads to characteristic changes in the serum marker enzyme activities (Mitra, *et al* 1998). Administration of GaIN caused an increase in the level of serum transaminases, hepatic necrosis and coma in rats. (Lim, *et al.,* 2000; Muntane, *et al.,* 2000). GaIN induced liver injury can be suppressed by natural compounds and dietary supplementation with plant extracts and their constituents, such as catechin, glycoside, oligosaccharide and soluble dietary fibers (Lim, *et al.,* 2000).

2.5. The Reference Drugs

Some of the herbal hepatoprotective agents used to manage and treat hepatic diseases globally include Liv. 52® (Livercare), Himoliv®, silymarin, Livex®, HD-03®, Hepatomed® and Hepatoguard®.

2.5.1. Liv 52®

Liv 52 was introduced in 1955 as a specially formulated proprietary ayurvedic herbal formula available as tablets and syrup for healthy liver support by the Himalaya Drug Company, Bangalore, India. It is a non-toxic multiherbal hepatotonic composed of *Capparis spinosa, Cichorium intybus, Solanum nigrum, Cassia occidentalis, Terminalia arjuna, Achillea millefolium, Mandur basma, Phyllanthus amarus* and *Tamarix gallica* and is reported to be clinically active in hepatotoxicity and a wide range of hepatic disorders (Girish, *et al*., 2009), and has been used traditionally in India in the treatment of various liver disorders (De Silva *et al*., 2003).

This hepatotonic has also been reported to help regulate levels of enzymes, optimizes assimilation (Bhattacharya, *et al*., 2008), increases serum albumin and helps maintain healthy levels of serum cholesterol, lipoproteins, phospholipids and triglycerides (Baijal, *et al*., 2004), restores the functional efficiency of the liver by protecting the hepatic parenchyma and promoting hepatocellular regeneration (Kolhapure and Mitra, 2004), normalizes biochemical markers indicating protective effect on liver cells, increases synthesis of proteins revealed by increased levels of serum proteins and normalizes gamma-globulin and gamma-glutamic transaminase (Marginean, *et al*., 2002). The antiperoxidative activity of Liv.52 prevents the loss of functional integrity of the cell membrane, maintains cytochrome P-450, hastens the recovery period and ensures early restoration of hepatic

functions in infective hepatitis (Kohapure and Mitra, 2004). Liv.52 facilitates rapid elimination of acetaldehyde, the toxic intermediate metabolite of alcohol metabolism, and ensures protection from alcohol-induced hepatic damage (Singh and Dhawan, 2000) and also diminishes the lipotropic activity in chronic alcoholism thereby prevents fatty infiltration of the liver (De Silva, *et al*., 2003).

2.5.2. Silymarin

Silybum marianum, commonly known as milk thistle also called wild artichoke, holy thistle and Mary thistle is an annual or biannual plant of the Asteraceae family and is found all over the world. The active constituents of the plant are obtained from the dried seeds and consist of four flavonolignans which are collectively known as silymarin (Pradhan and Girish, 2006). Silymarin is a complex mixture of polyphenolic molecules and consists of four flavonolignan isomers namely- silybin, isosilybin, silydianin and silychristin (Laekeman, *et al*., 2003).

Silymarin, the flavonoid extracted from milk thistle a member of the Daisy family, has been studied for treating all types of liver disease. Milk Thistle has been reported to protect liver cells from a number of toxins such as Hepatitis Virus (Mayer, *et al*., 2005). Silymarin has been shown to have positive effects in treating liver diseases of various kinds, including cirrhosis, chronic hepatitis, fatty infiltration of the liver (chemical and alcohol induced fatty liver) and inflammation of the bile duct In human studies (Saller, *et al*., 2001; Mayer, *et al* 2005; Pradhan and Girish, 2006). It has been shown to reverse liver cell damage and decrease elevated liver enzymes and it can also improve the symptoms of abdominal discomfort, fatigue and decreased appetite (Magliulo, *et al* 1978; Crocenzi and Roma, 2006).

Studies on the hepatoprotection provided by silymarin indicate that it acts as an antioxidant - exhibits activity against lipid peroxidation as a result of free radical scavenging and the ability to increase and regulate the intracellular content of glutathione (Kosina, *et al*., 2002; Hassan and EL-Gendy, 2003), as cell membrane stabilizers and permeability regulators - its ability to regulate membrane permeability and to increase membrane stability in the presence of xenobiotic damage (Pradhan and Girish, 2006), as promoters of ribosomal RNA synthesis - its capacity to regulate nuclear expression by means of a steroid-like effect thereby stimulating liver regeneration (Mayer, *et al*., 2005; Saller, *et al*., 2007), as inhibitors of the transformation of stellate hepatocytes into myofibroblasts which are responsible for the deposition of collagen fibres leading to cirrhosis (Gazak *et al*., 2007). It demonstrates anti-inflammatory activity (Saller, *et al*., 2001) and protects the liver against drug and toxin related liver damage (Tasduq, *et al*., 2005). The antioxidant property and cell-regenerating functions as a result of increased protein synthesis are considered as most important (Kosina, *et al*., 2002).

It has also been proposed to block fibrosis (Schuppan, *et al* 1998). Silymarin's effect in preventing liver destruction and enhancing liver function relates largely to its ability to inhibit the factors that are responsible for hepatic damage, i.e., free radicals and leukotrienes, coupled with an ability to stimulate liver protein synthesis (Wagner, 1985; Saller, *et al*., 2001; Kosina, *et al*., 2002). In animals, experimental liver damage can be produced by diverse toxic chemicals as amanita toxin, carbon tetrachloride, galactosamine and praseodymium nitrate (Wang, *et al*., 2002). Silymarin has been shown to protect against liver damage by all of these agents (Gebhardt, 2002; Johnson, *et al*., 2002). The liver can be damaged by the action of leukotrienes which are produced by the transfer of oxygen to a polyunsaturated fatty acid, a reaction catalysed by the enzyme lipoxygenase. Crocenzi and Roma (2006) described the impact of silymarin on normal bile secretion and its anticholestatic

properties in experimental models of cholestasis, with particular emphasis on the cellular/molecular mechanisms involved, including modulation of bile salt synthesis, biotransformation/depuration of cholestatic compounds, changes in transporter expression/activity, and evocation of signaling pathways.

CHAPTER THREE

MATERIALS AND METHODS

3.1. Collection and Identification of Plant Materials

Fresh leaves of *Gongronema latifolia* Benth, *Ocimum gratissimum Linn.* and *Vernonia amygdalina Del.* were purchased from Oyingbo market in Lagos metropolis during the rainy season (May 2007). They were subsequently identified and authenticated by Dr A.B. Kadiri of Botany Department, University of Lagos, Nigeria where voucher specimen (PCGH 444, PCGH 443 and PCGH 432 respectively) were deposited. The identities of the leaves were also authenticated at the Forest Research Institute of Nigeria (FRIN) Ibadan, Nigeria by Mr. A.O. Samuel and also by comparing with herbarium collections. The voucher numbers of *Gongronema latifolia* Benth, *Ocimum gratissimum Linn.* and *Vernonia amygdalina Del.* are FHI 106508, FHI 106507 and FHI 106509 respectively. The leaves were air dried at room temperature and finely ground using Corona® hand grinder.

3.2. Extraction and Fractionation of Plant Materials

3.2.1. Preparation of the 50 % Ethanolic Extract of *Gongronema latifolia* Benth, *Ocimum gratissimum Linn.* and *Vernonia amygdalina Del.* (GOV).

For the animal studies, 3 kg of each of the powdered leaves of *G. latifolia*, *O. gratissimum* and *V. amygdalina* (1:1:1) was mixed and soaked in thirty litres (30 L) of 50 % ethanol (v/v) for 24 hrs. It was filtered using three layers of cheese cloth. The solvent was removed by rotary evaporation under reduced pressure at temperatures below 45 °C while the water was removed by freeze-drying. This extract is known as the triherbal formulation (GOV).

The percentage extract yield was estimated as:

$$\frac{\text{Dry weight}}{\text{Dry material weight}} \times 100$$ (Parekh and Chanda, 2007).

3.2.2. Preparation of the Ethanolic Extract of *Gongronema latifolia* Benth*, Ocimum gratissimum Linn.* and *Vernonia amygdalina Del.*.

One hundred grams of each of the powdered leaves of *G. latifolia*, *O. gratissimum* and *V. amygdalina* (1:1:1) was mixed and soaked in thirty litres (3 L) of absolute ethanol (v/v) for 24 hrs. It was filtered using three layers of cheese cloth. The solvent was removed by rotary evaporation under reduced pressure at temperatures below 45 °C while the water was removed by freeze-drying. This extract is known as the ethanolic extract. The percentage extract yield was estimated.

3.2.3. Preparation of the Aqueous Extract of *Gongronema latifolia* Benth*, Ocimum gratissimum Linn.* and *Vernonia amygdalina Del.*.

One hundred grams of each of the powdered leaves of *G. latifolia*, *O. gratissimum* and *V. amygdalina* (1:1:1) was mixed and soaked in thirty litres (3 L) of water (v/v) for 24 hrs. It was filtered using three layers of cheese cloth. The solvent was removed by rotary evaporation under reduced pressure at temperatures below 45 °C while the water was removed by freeze-drying. This extract is known as the aqueous extract. The percentage extract yield was estimated.

3.2.4. Fractionation of 50 % Ethanolic Extract of *Gongronema latifolia* Benth, *Ocimum gratissimum Linn.* and *Vernonia amygdalina Del.* (GOV)

Thirty grams of GOV was dissolved in 300 ml of distilled water in a separatory funnel, shaken with n-hexane (150 ml) for 2 mins and allowed to settle at room temperature until a separated and clear hexane and aqueous layers were obtained. The hexane fraction was then collected and the aqueous layer was extracted 2 more times with hexane. The three hexane fractions were pooled and evaporated to dryness using a rotary evaporator. The combined hexane extract was evaporated under reduced pressure to yield the hexane fraction. The aqueous layer was then further fractionated by successive solvent extraction with chloroform (3 x 150 ml), ethyl acetate (3 x 150 ml) and n-butanol (3 x 150 ml) using the same procedures as was performed for the hexane extraction, yielding the chloroform, ethyl acetate, butanol and water fractions respectively .

3.3. Preliminary Phytochemical Assays

Preliminary phytochemical analysis was performed as follows.

3.3.1. Test for Tannins

The method of Trease and Evans (1989) was used to test for the presence of tannins. 5 g of extract was stirred with 10 ml distilled water, filter and add ferric chloride to the filtrate. Presence of blue-black, green, or blue green precipitate indicates that the extract contains Tannin.

To confirm the presence of tannins, each portion of alcohol and water extracts (5 g) were stirred with 10 ml of alcohol and distilled water respectively, filter and add ferric chloride reagent to the filtrate and 1ml portion of the extract was treated with bromine water. Presence of blue-black, green, or blue green precipitate indicates that the extract contains tannin.

3.3.2. Test for Alkaloids

This assay was carried out using the method of Farnsworth and Euler (1962). 0.5 g of extract was stirred with 5 ml of 1 % aqueous hydrochloric acid on a steam bath. Add a few drops of Dragendorffs reagent to 1 ml of the extract-aqueous hydrochloric acid solution. To another 1 ml of the solution, add a few drops of Mayer's reagent. Turbidity or precipitation indicates presence of alkaloids in the extract(s).

To confirm and eliminate false results, the method of Trease and Evans (1989) was used. 1 g of extract was treated with 40 % calcium hydroxide solution till it is clearly alkaline on litmus paper and extracted twice with 10 ml portions of chloroform. The extract was combined and concentrated *in vacuo* to about 5 ml. The chloroform extract was spotted on thin layer plates. Four (4) different solvent systems that have wide varying polarity were used to develop the plant extract. The developed chromatograms were sprayed with freshly prepared Dragendorff's spray reagent. An orange or a darker coloured spot against a pale yellow background confirmed the presence of alkaloids. This is a modified form of the thin layer chromatography.

3.3.3. Test for Phlobatannins

The plant extract was boiled with 1 % of aqueous hydrochloric acid. A red precipitate indicates the presence of phlobatannins.

3.3.4. Test for Saponins

The ability of saponins to produce frosting in aqueous solution and to haemolyse red blood cells was used as screening test for these compounds. The methods of Wall *et al.*, (1954) and Southeeswaran and Kenchington (1989) were used.

Frosting Test: 0.5 g of extract was shaken with water in a test tube. If frosting persists on warming, it shows evidence of presence of saponins.

Haemolysis Test: The extract (0.2 g) was dissolved in 10 ml of warm water and filtered. 2 ml of 0.8 % sodium chloride (NaCl) solution was added and it was put into two test tubes. To one of these, 2 ml distilled water was added and to the other 2 ml of the extract was added. The concentration of sodium chloride in each test tube was isotonic with blood serum. 5 drops of blood was added to each test tube and the tubes were gently inverted to mix the content. Haemolysis of the red blood cells shows that the extract contains saponins.

To eliminate all false results, this blood haemolysis test was also performed on all samples that frosted in water. 0.5 g of positive extract(s) was boiled with 50 ml phosphate buffer of pH 7.4, cool and filter. Pass 5 ml of extract through asbestos disc that had been previously soaked with 2 – 3 drops of 1 % cholesterol in ether and dried for 3 hrs. The disc was washed with 0.5 ml distilled water, dried and boiled in 20 ml oxylol for 2 hrs so as to destroy any complex formed between the cholesterol and any saponin in the extract. Ether was used to wash the disc and it was dried and placed on a 7 % nutrient blood agar. If after 6 hrs, there is complete haemolysis of the red blood cells around the disc then it further proves that the extract contains saponins.

3.3.5. Test for Anthraquinones

Tests for the presence of free hydroxyanthraquinones and combined anthraquinones were carried out using the method of Trease and Evans (1989).

Free hydroxyanthraquinones: The Borntrager's test is used. The extract (1 g) was shaken with 2 ml benzene, filtered, and 1 ml of 10 % ammonia solution was added to the filtrate and shaken. A pink, red or violet colour in the lower phase indicates the presence of free hydroxyanthraquinones.

Anthraquinone derivatives: 1 g of extract was boiled with 2 ml aqueous sulphuric acid and filtered while hot. The filtrate was shaken with 1 ml benzene. The benzene layer separated. 10 % ammonia solution was added to half its own volume. A violet, pink or red colour on the lower phase indicates the presence of anthraquinone derivatives in the extract.

Free anthraquinone: The extract (0.1 g) was dissolved with 10 ml of hot water for the water extract and 10 ml of alcohol for the alcohol extracts. All were put in a water bath to steam for 5 mins, the solution was filtered while hot and.

Bound (Glycosides): Another set of mixtures were prepared with 0.1 g sample both of water and alcohol extract with 10 ml each of ferric chloride solution and 5 ml hydrochloric acid. They were hydrolysed by heating on a water bath for 10 mins filtered hot and the filtrates were extracted with chloroform. The chloroform layer was removed and this layer was washed with 5 ml water and was shaken with 5 ml dilute ammonia solution.

3.3.6. Tests for Cardiac Glycosides

The tests for the presence of cardiac glycosides were carried out according to the methods of Trease and Evans (1989) and Harbone (1996)

Salkowski test: 0.5 g of extract was dissolved in 2 ml chloroform and sulphuric acid was carefully added so as to form a lower layer. Formation of a reddish brown colour at the interface indicates the presence of the aglycone portion of the cardiac glycoside i.e. steroidal ring.

Kedde test: 1ml of an 8 % solution of the extract was mixed in methanol with a 2 % solution of 3, 5-dinitrobenzoic acid in methanol and 1ml of a 5.7 % aqueous sodium hydroxide. The appearance of an immediate violet colour indicates the presence of cardenolides. The colour gradually fades through reddish brown to brownish yellow with the precipitation of a whitish crystalline solid. This indicates the presence of a lactone ring in the cardenolide.

Legal test: The extract was dissolved in pyridine, a few drops of 2 % sodium nitroprusside was added and also a few drops of 20 % sodium hydroxide. Appearance of a deep red colour that fades to brownish yellow indicates the presence of cardenolides.

Liebermann-Burchard's test: 0.5 g of extract was dissolved in 2 ml acetic anhydride, cool on ice and sulphuric acid was carefully added. A colour change from violet to blue to green indicates the presence of a steroidal nucleus.

Keller-Kelian's Test: The extract (0.5 g) was dissolved in 2 ml of glacial acetic acid containing one drop of ferric chloride solution. This was then underplayed with 1 ml of concentrated sulphuric acid.

3.3.7. Tests for Cyanogenetic Glycosides

This was performed according to the method of Sofowora (1994). The extract (0.2 g) was put into 2 test tubes A and B. 3 ml of distilled water was added into each test tube. Sodium picrate papers were placed at the tips of the test tubes and plugged with cotton wool in such a way that the picrate papers did not touch the solution inside the test tubes. Test tube A was left at room temperature and test tube B was put in hot water bath for 30 mins.

3.3.8. Identification of Flavonosides

To test for presence of flavonosides, the methods of Sofowora (1994) and Harbone, (1996) were used.

Lead Acetate Test: To 0.2 ml of the extract, 0.2 ml of 10 % lead acetate was added and the mixture was shaken to avoid emulsion.

Ferric Chloride Test: 0.2 ml of the 10 % ferric chloride ($FeCl_3$) was added to the extract. The mixture was shaken together to observe the colour.

Sodium Hydroxide Test: 0.2 ml of dilute sodium hydroxide was added to 0.2 ml of the extract and shaken gently.

3.3.9. Tests for Reducing Compounds

The extracts (0.5 g) were distilled with 1 ml of water and 1 ml of Fehling's solution (A and B) was added and then put in the hot water bath to warm. The reducing sugar reduces copper (II) ions in these test solutions to copper (I), which then forms a brick red copper (I) oxide precipitate. Red copper (I) oxide then precipitates out of the reaction mixture, which indicates a positive result i.e. that redox has taken place (Trease and Evans, 1989).

3.4. Thin Layer Chromatography (TLC)

For TLC analyses, PET foils and Glass TLC plates with dimensions 10 x 10 and 10 x 20 coated with Silica gel 60 F_{254} (Merck) were used. Preliminary experiments were performed in order to choose the best system so as to accomplish good separation of the extracts. The TLC plates were prewashed with methanol before use. The standard reference compounds and GOV were dissolved in methanol (1 g in 100 mL i.e. 1 %). Pencil lines were drawn 1 cm from the bottom of the plate and 1 cm from the top. The plates were spotted with GOV and the standard reference compounds using 1 µl microcaps and labeled using a pencil. The plates were examined under the UV light to be sure that enough of each extract and standard were applied, if not more was added. Glass twin trough development chambers with lid for TLC (10 x 10 and 20 x 10 by CAMAG) were used. The plates were then developed using different solvent systems such as butanol: formic acid: water: methanol (8: 2: 3: 5), heptane: methanol (1:1), toluene: ethyl acetate (1:1) and hexane: ethyl acetate (1:1). The plates were removed the solvent has risen to the 1cm mark on the top. The plates were left in the hood so that the solvent will evaporate from the plates. The plates were scanned with CAMAG Scanner III® at 270 nm using the deuterium source and examined under UV light and the Retention (R_f) values were measured. The plates were visualized by spraying with ninhydrin and natural product reagent (prepared by mixing 5 % aqueous aluminum chloride ($AlCl_3$) solution and 0.05 % diphenylboric acid – β ethylaminoester in 10 % methanol as described by Heinrich *et al*. (2002)). Some plates were heated or placed in an enclosed container with a watch glass along with a few iodine crystals. Pictures were taken using a Kodak EasyShare C433 digital camera.

The TLC was also used to confirm and eliminate false results especially the test for alkaloids. To confirm the presence of alkaloids, 0.1 g of extract was treated with 40 % Calcium hydroxide solution till it was clearly alkaline on litmus paper and extracted twice with 10 ml portions of chloroform. The extract was combined and concentrated *in vacuo* to about 5 ml. The chloroform extract was spotted on TLC plates (10 x 10 or 10 x 20 coated with Silica gel 60 F254 (Merck)). Different solvent systems that have wide varying polarity were used to develop spotted TLC plates. The developed chromatograms were sprayed with freshly prepared Dragendorffs spray reagent. An orange or a darker colored spot against a pale yellow background confirmed the presence of alkaloids. This is a modified form of the thin layer chromatography (Farnsworth and Euler 1962).

The Retention (Rf) Values was calculated as follows

$$\text{Rf value} = \frac{\text{Distance from Baseline travelled by Solute}}{\text{Distance from Baseline travelled by Solvent (Solvent Front)}}$$

3.5. Flash Column Chromatography

The weight of the dry, solvent-free extract to be separated was determined. The ratio of the silica gel used to the extract is 30:1. An appropriate column was chosen and this is determined by the quantity of silica gel to be used and the ability to have 2-3 empty column volumes. The total volume of the column was recorded. The stopcock end of the column was plugged with a small piece of cotton or glass wool so that the silica will not drain out and the column was mount in the hood in a perfectly vertical position. The stopcock was closed and 50 ml of the eluent was added. Slurry of the silica gel was made using the eluent at a ratio of 1:5. The slurry was vigorously stirred so as to eliminate all the air from the silica. The slurry was slowly poured into the column. A pipette containing the eluent was

used to remove any silica on the side of the column. The stopcock was opened and the column was packed using compressed air while the sides of the column was been tapped with a rubber stopper so as aid in packing the silica gel particles. The final height of the packed silica gel will be about half the original height. A protective layer was made using glass wool and then cotton wool so as not to disturb the packed column. Compressed air was used to reduce the volume of the eluent to where the protective plug was. The stopcock was then closed.

The extract was dissolved in a round bottomed flask using chloroform. Silica gel of known weight was added to the dissolved extract and evaporated to dryness using a rotary vapour at 120 rpm till is was completely dry. A powder funnel was used to gently add the extract \ silica gel mixture to the packed column. Sterile cotton wool was used to plug the column so as to protect the extract silica gel mixture layer from being disturbed whenever a solvent system is added. Collection bottles were properly labeled for easy identification of the fractions. A collecting bottle was put under the column outlet. A known volume of the first solvent system was used to rinse the flask and the content was gently poured into the column from the side of the flask using a pipette. The column was gently filled with the solvent system and the source of compressed air was connected. The stopcock was opened and the column was run. The air pressure was adjusted so as have a flow rate of 100 ml\min. the collecting bottles were changed as the required volume was collected. Once the solvent system got to 20 – 30 mls from the extract silica gel mixture layer, the compressed air was turned off and more solvent was added. When it was time to use another solvent system, the previous solvent system was allowed to get to the extract silica gel mixture layer before a new solvent system was introduced. After collection, the column was washed using methanol and compressed air.

The different fractions were evaporated to dryness using a rotary evaporator at 120 rpm at 40 °C and the separation was monitored by TLC and HPLC so that similar fractions can be pulled together.

Fractions that were not pure were further purified using smaller columns. The polarity of the solvent systems was increased gradually while the air pressure was decreased.

3.6. Reverse Phase Chromatography

The aqueous extract of all the plant leaves were subjected to reverse phase chromatography. A Septech – Semia Preparative HPLC system with variable wavelength monitor at 210 nm, a flow rate of 105 ml\min, and a Lichrosphere column (12 µRP C18) with an empty column volume of 680 ml and a GP 901 controller was used. The column was washed using 2000 ml of 50 % MeOH/H_2O and then equilibrated using MeOH: H_2O (10:90). 1 g of the extract was dissolved in 10 ml of water and loaded onto the column using a Q- Pump (FMI Lab Pump) at 140 ml\min. Two empty column volumes of each solvent system were used in eluting and the fractions were collected and evaporated to dryness.

3.7. High Performance Liquid Chromatography (HPLC)

Dionex Summit Series (using 170 S multiple UV detector with an ASI- 100 automated sample injector) and Luna 5 µ C18 (2) 100 A (150 x 4.6 mm) column was used for analysis.

3.8. High Performance Thin Layer Chromatography (HPTLC) Analysis

A HPTLC system comprising a CAMAG automatic TLC sampler 4 software, Optiquest monitor Q7 and CAMAG scanner III was used for this study. Ethyl acetate: formic acid: acetic acid: water (10:1.1:1.1: 2.6 v/v/v/v) was employed as mobile phase while anise aldehyde/ H_2SO_4 and natural

products reagents were used as reagent sprays. They were prepared and used fresh. Chromatography was performed on 10 x 10 and 20 x 10 cm Bioluminex HP-TLC silica gel 60 F_{254} plates. The HPTLC plates were marked using a light pencil at 70 mm showing the developing distance from the lower edge, it was prewashed with methanol in a prewashing chamber by developing methanol off the top of the HPTLC plate. It was dried at 100 ± 15 ^{o}C for 20 minutes.

The extracts used for HPTLC were prepared by weighing approximately 0.5 g of dried powdered plant leaves into a 25 ml scintillation vial and 5 ml of methanol was added to it. Each vial was sonicated for 10 min in a hot water temperature bath at 40 °C using a Telsonic Ultrasonic Bath (TPC25). It was allowed to cool, filtered using a 0.45 µm PTFE syringe filter into a 5 ml volumetric flask and made up to 5 ml using methanol. Using a transfer pipette (SAMCO), it was transferred into a clean scintillation vial and mixed very well. An aliquot (1.2 – 1.8 ml) was transferred into an amber HPLC vial with Teflon cap for analysis.

Using a CAMAG automatic TLC sampler 4® methanolic solutions of samples and standard compounds of known concentrations were spotted on the plates at a temperature of 21°C and relative humidity of 28 %. After spotting, the plate was placed in an oven at 100 ^{o}C for 2 minutes. The HPTLC plate was developed and dried in an oven at 40 ^{o}C for 20 minutes. The Optiquest monitor Q7, Alpha Innotech Optiplex GX 260 by Dell and Alpha Innotech Camera chamber was used to analyze and take pictures of the plates at different wavelengths (365 nm and 245 nm) using the Alpha Innotech Fluorchem® 8900 for UV detection.

The plate was sprayed with natural products reagent and the resulting bands were detected using CAMAG® movie files at 365 nm and the 365 picture was taken. It was then sprayed with anise aldehyde reagent (0.5 ml anisealdehyde - Fluka, 10 ml 99 % acetic acid, 85 ml methanol and 10ml

sulphuric acid mixed in chronological order) and placed in an oven at 115 -135 ^{o}C for 5 seconds. The bands were detected using CAMAG® movie files at 365 nm and derivatized 365 pictures were taken. It was put back into the oven at 125 ^{o}C for 1 minute and the white light picture was taken. Care was taken so that the plates will not stay more than 1 min because the whole plate will turn red. The identification of standard compounds was confirmed by matching the UV spectra of samples and standards within the same R_f window.

3.9. The Saturation-Transfer Difference – Nuclear Magnetic Resonance Spectroscopy (STD-NMR) Based Screening.

Proton NMRs: 0.0035 g samples of GOV and its fractions were dissolved in a 50 mM D_2O/phosphate buffer (pH 7.4). This assumed that GOV had an average molecular weight of 700 g/mol which approximates, a 10 mM aggregate concentration. A 600 MHz Varian NMR Spectrometer, equipped with a cryoprobe, was used to obtain these spectra at 298 K. Proton NMR (also Hydrogen-1 NMR, or ^{1}H NMR) spectra were acquired with 1024 scans.

STD Based Screening: Ten millimoles (10 Mm) of the triherbal formulation was prepared in 0.50 ml of 50 Mm D_2O/phosphate buffer with a PH of 7.4, with 10 M Dihydrodipicolinate reductase (DHPR) protein. To increase solubility of GOV that did not dissolve easily, 5 % of d_6-dimethyl sulfoxide (DMSO) was added to all samples. A 600 MHz varian NMR Spectrometer, equipped with a cryoprobe, was used to obtain these spectra at 298 K, and the protein was irradiated at -0.5 ppm for the difference in spectrum. Each STD experiment consisted of 512 scans for a total run time of 45 minutes.

3.10. Animal Care

Studies were carried out using Swiss mice (20-30 g) and Wistar albino rats (150-200 g) of both sexes, obtained from the Laboratory Animal Centre of the College of Medicine, University of Lagos Nigeria. The animals were grouped and maintained under standard laboratory conditions with dark and light cycle (12/12 hr.). The animals were fed with standard rat chow supplied by Ladokun feeds, Ibadan, and left for 10 days to acclimatize before commencement of experiments.

3.11. Toxicity studies:

3.11.1. Acute Toxicity Test:

This was performed according to the Organization of Economic Co-operation and Development (OECD) guidelines for testing Chemical, TG425 (OECD, 2001). 120 mice were randomly divided into 12 groups of 10 animals each. The animals were fasted overnight but allowed access to water only. Groups 1-5 were administered orally with GOV at different doses (1, 2, 4, 8 and 16 g kg^{-1} respectively) by gastric intubation, while groups 7-11 were administered with different doses of GOV (0.5, 1, 1.5, 2 and 2.5 g kg^{-1} respectively) intraperitoneally. Group 6 and 12 which are the control groups received the dosing vehicle i.e. distilled water (10 ml kg^{-1}). Signs of toxicity and mortality were observed after the administration of the extract at the first, second, fourth, sixth and twenty fourth hrs. Mortality in each group within 24 hrs was recorded. Principle of the limit test (The limit test is primarily used in situations where the experimenter has information indicating that the test material is likely to be nontoxic, i.e., having toxicity only above regulatory limit doses) (OECD, 2000a; 2000b) was used to evaluate safety of GOV.

3.11.2. Subchronic Toxicity Test:

Based on the outcome of the acute toxicity studies, three doses of the GOV (2, 4 and 8 g kg^{-1}) were chosen for further studies. Twenty eight (28) rats were randomly divided into 4 groups of 7 animals each. They were allowed standard rat chow and clean tap water *ad libitum*. Groups 2-4 were administered orally with GOV at different doses (2, 4 and 8 g kg^{-1}) by gastric intubation for 14 days, while animals in group 1 received the dosing vehicle i.e. 10 ml kg^{-1} distilled water (p.o.) and served as the control group. After the experimental period the animals were anaesthetized mildly with ether and blood was collected from the retro-orbital plexus. They were sacrificed and more blood samples were collected by cardiac puncture. The blood samples were used for haematologic, biochemical, antioxidant and apoptotic studies. The liver and kidneys were also dissected out for assay of antioxidants and histopathologic studies. These organs were fixed in 10 % formal saline prior to the histologic studies.

3.12. Preliminary Pharmacological Assays

3.12.1. Analgesic Activity:

3.12.1.1. Hot Plate Test:

To evaluate the analgesic activity of the triherbal preparation the hot plate method described by Woolfe *et al.,* (1944) was used. Twenty five mice were used for this experiment and the animals were divided into 5 groups comprising 5 animals per group. Groups 1, 2 and 3 received GOV (2, 4 and 8 g kg^{-1} p.o.), group 4 morphine (2 mg kg^{-1} sc.) and group 5 normal saline (10 ml kg^{-1} p.o). These were

administered to the animals 30 minutes before the tests. The animals were dropped on a hot plate maintained at a temperature of 56 ± 1 °C. The interval between the time the animal reached the hot plate and the moment it licked its forepaws or jumped up or out of the hot plate was recorded as the reaction time. The percentage increase in reaction time at each time interval was calculated (Vogel, 2002)

3.12.1.2. Mouse Writhing Assay:

This experiment is based on the method described by Koster *et al* (1959). Swiss albino mice of either sex were selected and divided into five groups of five animals each. Groups 1, 2, and 3 were administered with GOV (2, 4 and 8 g kg^{-1} p.o), groups 4 received acetylsalicylic acid (100 mg kg^{-1} sc.) while groups 5 received normal saline (10 mg kg^{-1} p.o.) thirty minutes before intraperitoneal injection of 0.6 % v/v acetic acid solution. Thereafter, the mice were gently dropped inside a transparent glass cage and the number of writhing was counted for 15 minutes. A significant reduction in the number of writhes by acetylsalicylic acid and GOV treated animals as compared to normal saline treated animals was considered as a positive analgesic response. The percentage inhibition of writhes was calculated as the reduction in the number of writhes compared to the control group. Percentage inhibition of writhes was obtained using the formula as given by Vongtau *et al* (2000)

$$\text{Inhibition (\%)} = \frac{\text{Mean number of writhing (control)} - \text{Mean number of writhing (test)}}{\text{Mean number of writhing (control)}} \times 100$$

3.12.1.3. Formalin Test:

The method adopted is similar to that described for the mouse writhing assay. The test animals were administered with GOV (2, 4 and 8 g kg^{-1} p.o.), acetylsalicylic acid (100 mg kg^{-1} sc.) and normal saline (10 ml kg^{-1} p.o.) thirty minutes before the injection of formalin. Twenty microliters of 1 %

formalin was injected subcutaneously into the right hind paw of the mice. The time spent in licking and or biting of the injected paw was recorded as an indicator of pain response. The responses were measured for 5 minutes after the injection of formalin (Turner 1965).

3.12.2. Anti-inflammatory Activity

3.12.2.1. Xylene Induced Ear Oedema:

The animals were divided into 5 groups of 5 animals each. Groups 1, 2 and 3 were administered with GOV (2, 4 and 8 g kg^{-1} p.o.), group 4 received Dexamethasone (4 mg kg^{-1} p.o.) and group 5 normal saline (10 ml kg^{-1}) 30 minutes before inflammation was induced. Xylene was used to induce inflammation in mice by topical application of the xylene (2 drops) on the inner surface of the right ear and was left to act for 15 minutes. The animals were anaesthetized using ether and the left and right ears cut off. The difference between the 2 ears was recorded as the oedema induced by the xylene (Junping, *et al*., 2005)

3.12.2.2. Carrageenan Induced Rat Paw Oedema:

The method described by Winter *et al* (1962) was used. Wistar rats were divided into 5 groups with each group containing 5 test animals. Groups 1, 2 and 3 were administered with GOV (2, 4 and 8 g kg^{-1}, p.o.), group 4 received indomethacin (10 mg kg^{-1}, p.o.) while group 5 which served as control received normal saline (10 ml kg^{-1}, p.o.). Carrageenan was used to induce oedema 1 hour after the administration of the different doses of GOV, indomethacin and normal saline by injecting 0.1 ml of freshly prepared 1 % carrageenan diluted in normal saline (1 % w\v in 0.9 % normal saline) into the sub plantar region of the right hind paw. The paw size was measured before and immediately after the

administration of the carrageenan using a cotton thread at 1, 2, 3, 4, 5 and 6 hrs. intervals. Increases in the linear diameter of the right hind paws were taken as an indication of paw oedema. Any significant reduction in the volume of the injected hind paw of the test group(s) compared to that of the control group was considered as an anti-inflammatory response (Satyanarayana *et al.,* 2004). The Mean increase in paw swelling was measured and the percentage inhibition calculated. The anti-inflammatory effect of the extract was calculated by the following equation:

Anti-inflammatory activity (%) = (1-D/C) X 100

Where D represents the percentage difference in paw volume after the extract was administered to the rats and C represented the percentage difference of volume in the control groups.

The percentage inhibition of the inflammation was calculated using the Newbould formula (Moody *et al*., 2006).

3.13. Pharmacological Models of Inducing Hepatotoxicity

Hepatoprotective effect of many herbal products have been studies against chemical and drug induced hepatotoxicity in laboratory animals using different hepatotoxicants e.g. alcohol, carbon tetrachloride (CCl_4), D-galactosamine (D-GaIN), paracetamol (APAP), etc (Khatri, *et al.,* 2009; Upur, *et al.,* 2009; Somasundaram, *et al.,* 2010; Roy and Das , 2010).

3.13.1. Drug Treatment Protocol for Alcohol Induced Hepatotoxicity

The rats were randomized and divided into seven groups of seven rats each.

Group 1: Served as normal control and the rats received 10 ml distilled water kg^{-1} body weight orally (p.o.) for fourteen days.

Group 2: This is the toxin control group where the rats received distilled water (10 ml kg^{-1} body weight p.o.) for fourteen days and 50 % ethanol (8.5 g kg^{-1} body weight p.o.) on the thirteenth day and four hrs before sacrificing on the fourteenth day.

Group 3: Rats in this group were treated with 2 g kg^{-1} body weight (p.o.) of GOV for fourteen days. The extract was administered 1hr before 50 % ethanol (8.5 g kg^{-1} body weight p.o.) was administered on the thirteenth day and four hrs before sacrificing on the fourteenth day.

Group 4: These rats recieved 4 g kg^{-1} body weight (p.o.) of GOV for fourteen days. The extract was administered 1hr before 50 % ethanol (8.5 g kg^{-1} body weight p.o.) was administered on the thirteenth day and four hrs before sacrificing on the fourteenth day.

Group 5: This group was treated with 8 g kg^{-1} body weight (p.o) of GOV for fourteen days. The extract was administered 1hr before 50 % ethanol (8.5 g kg^{-1} body weight p.o.) was administered on the thirteenth day and four hrs before sacrificing on the fourteenth day.

Group 6: Received LIV 52 syrup (0.3 g kg^{-1} body weight p.o.) for fourteen days. The drug was administered 1hr before 50 % ethanol (8.5 g kg^{-1} body weight p.o.) was administered on the thirteenth day and four hrs before sacrificing on the fourteenth day.

Group 7: Received Silymarin (0.3 g kg^{-1} body weight in distilled water p.o.) for fourteen days. The drug was administered 1hr before 50 % ethanol (8.5 g kg^{-1} body weight p.o.) was administered on the thirteenth day and four hrs before sacrificing on the fourteenth day.

3.13.2. The Effect of GOV on D-galactosamine Induced Hepatotoxicity

Forty nine rats were again randomized and divided into seven groups of seven rats each.

Group 1: These rats served as normal control group and received distilled water (10 ml kg^{-1} body weight p.o.) for fourteen days.

Group 2: This group served as toxin control group and received distilled water (10 ml kg^{-1} body weight p.o.) for fourteen days. D-GaIN (Sigma) in distilled water was administered to the rats in a single dose of 0.5 g kg^{-1} body weight intraperitoneally (i.p.) twenty four hrs before sacrificing on the fourteenth day

Group 3: The rats in this group were treated with 2 g kg^{-1} body weight (p.o) of GOV for fourteen days. The rats were administered the extract 1 hr before treatment with D-GaIN (Sigma) in distilled water in a single dose of 0.5 g kg^{-1} body weight (i.p.) twenty four hrs before sacrificing on the fourteenth day.

Group 4: These rats were treated with 4 g kg^{-1} body weight (p.o.) of GOV for fourteen days. The rats were administered the extract 1 hr before treatment with D-GaIN (Sigma) in distilled water in a single dose of 0.5 g kg^{-1} body weight (i.p.) twenty four hrs before sacrificing on the fourteenth day.

Group 5: Were treated with 8 g kg^{-1} body weight (p.o.) of GOV for fourteen days. The rats were administered the extract 1 hr before treatment with D-GaIN (Sigma) in distilled water in a single dose of 0.5 g kg^{-1} body weight (i.p.) twenty four hrs before sacrificing on the fourteenth day.

Group 6: This group of rats were administered LIV 52 syrup (0.3 g kg^{-1} body weight p.o.) for fourteen days. The rats were administered LIV 52 syrup 1 hr before treatment with D-GaIN (Sigma) in distilled water in a single dose of 0.5 g kg^{-1} body weight (i.p.) twenty four hrs before sacrificing on the fourteenth day.

Group 7: This group received silymarin (0.3 g kg^{-1} body weight in distilled water p.o.) for fourteen days. The rats were administered silymarin 1 hr before treatment with D-GaIN (Sigma) in distilled water in a single dose of 0.5 g kg^{-1} body weight (i.p.) twenty four hrs before sacrificing on the fourteenth day.

3.13.3. The Effect of GOV on Acetaminophen Induced Hepatotoxicity

Animals were randomized and divided into seven groups of seven animals each.

Group 1: served as normal control received distilled water (10 ml kg^{-1} body weight p.o.) for fourteen days.

Group 2: served as toxin control received distilled water (10 ml kg^{-1} body weight p.o.) for fourteen days and received APAP 3 g kg^{-1} body weight in distilled water forty eight hours before sacrificing on the fourteenth day

Group 3: were treated with 2 g kg^{-1} body weight of triherbal extract (GOV) for fourteen days. On the twelfth day, the extract was administered 1hr before treatment (p.o.) with APAP 3 g kg^{-1} body weight in distilled water.

Group 4: were treated with 4 g/kg body weight of triherbal extract (GOV) for fourteen days. On the twelfth day, the extract was administered 1hr before treatment (p.o.) with APAP 3 g kg^{-1} body weight in distilled water.

Group 5: were treated with 8 g kg^{-1} body weight of triherbal extract (GOV) for fourteen days. On the twelfth day, the extract was administered 1hr before treatment (p.o.) with APAP 3 g kg^{-1} body weight in distilled water.

Group 6: received LIV 52 syrup (300 mg kg^{-1} body weight) for fourteen days. On the twelfth day, the drug was administered 1hr before treatment (p.o.) with APAP 3 g kg^{-1} body weight in distilled water.

Group 7: received Silymarin (300 mg kg^{-1} body weight in distilled water p.o.) for fourteen days. On the twelfth day, the drug was administered 1hr before treatment (p.o.) with APAP 3 g kg^{-1} body weight in distilled water.

3.13.4. Drug Treatment Protocol for Carbon Tetrachloride Induced Hepatotoxicity

Animals were randomized and divided into seven groups of seven animals each.

Group 1: served as normal control received distilled water (10 ml kg^{-1} body weight p.o.) for fourteen days and received a single dose liquid paraffin (1 ml kg^{-1} b. wt) 6 hr after the last dose administration. Animals were sacrificed 24 hrs after the last dose and blood was collected.

Group 2: served as toxin control received distilled water (10 ml kg^{-1} body weight p.o.) for fourteen days and received single intraperitoneal injection of CCl_4 in a dose of 1 ml kg^{-1} of a 50 %

(v/v) solution in liquid paraffin after 6 hr of last dose administration. Animals were sacrificed 24 hrs after the last dose and blood was collected.

Group 3: were treated with 2 g kg^{-1} body weight of triherbal extract (GOV) for fourteen days. On the fourteenth day, the animals received a single intraperitoneal injection of CCl_4 in a dose of 1 ml kg^{-1} of a 50 % (v/v) solution in liquid paraffin after 6 hr of last dose administration. Animals were sacrificed 24 hrs after the last dose and blood was collected.

Group 4: were treated with 4 g/kg body weight of triherbal extract (GOV) for fourteen days. On the fourteenth day, the animals received a single intraperitoneal injection of CCl_4 in a dose of 1 ml kg^{-1} of a 50 % (v/v) solution in liquid paraffin after 6 hr of last dose administration. Animals were sacrificed 24 hrs after the last dose and blood was collected.

Group 5: were treated with 8 g kg^{-1} body weight of triherbal extract (GOV) for fourteen days. On the fourteenth day, the animals received a single intraperitoneal injection of CCl_4 in a dose of 1 ml kg^{-1} of a 50 % (v/v) solution in liquid paraffin after 6 hr of last dose administration. Animals were sacrificed 24 hrs after the last dose and blood was collected.

Group 6: received LIV 52 syrup (300 mg kg^{-1} body weight) for fourteen days. On the fourteenth day, the animals received a single intraperitoneal injection of CCl_4 in a dose of 1 ml kg^{-1} of a 50 % (v/v) solution in liquid paraffin after 6 hr of last dose administration. Animals were sacrificed 24 hrs after the last dose and blood was collected

Group 7: received Silymarin (300 mg kg^{-1} body weight in distilled water p.o.) for fourteen days. On the fourteenth day, the animals received a single intraperitoneal injection of CCl_4 in a dose of

1 ml kg^{-1} of a 50 % (v/v) solution in liquid paraffin after 6 hr of last dose administration. Animals were sacrificed 24 hrs after the last dose and blood was collected.

After the experimental period the animals were anaesthetized mildly with ether and blood was collected from the retro-orbital plexus. They were sacrificed and more blood samples were collected by cardiac puncture. The blood samples were used for haematologic, biochemical, antioxidant and caspases studies. The liver and kidneys were also dissected out for assay of oxidative stress indicators or enzymes and histopathology.

One gram (1 g) of the liver and kidneys were weighed, washed with ice-cold normal saline and homogenates (10 % w/v) were prepared in 0.4 M PBS using a Polytron® Ergonomic homogenizer. The homogenates were centrifuged at 5000 × g for 10 minutes at 4 ^{o} C, after removal of the cell debris, supernatant was used for the estimation of lipid peroxidative indices and enzymic and non-enzymic antioxidants.

3.14. Screening for Hepatoprotective Activity of GOV

3.14.1. Haematologic indices

Blood samples were collected by cardiac puncture under mild diethyl ether anesthesia, using 21 gauge (21G) needles mounted on a 5 ml syringe into Ethylene Diamine Tetra-acetic Acid (EDTA) - coated sample bottles for analysis of haematologic indices.

3.14.1.1. Packed Cell Volume (PCV)

Packed cell volume (PCV) is the fraction of whole blood volume that consists of red blood cells. The packed cell volume was determined using the method of Baker (1976). A low haematocrit

may indicate anaemia, blood loss, bone marrow failure, leukaemia, multiple myeloma, nutritional deficiency, over-hydration or rheumatoid arthritis, while a high haematocrit may indicate dehydration.

Procedure:

A well labelled plain capillary about three quarters filled with well mixed EDTA anticoagulated blood sample is sealed using a sealant at the unfilled end. It was spurn in a microhaematocrit centrifuge for 3 minutes (RCF 15,000 x g) and the PCV was read immediately by using a transparent ruler. The length of the total column of blood and the length of the red cell column i.e. from the base to directly below the buffy coat layer were measured. The PCV was calculated as follows

$$PCV = \frac{\text{length of red cell column (mm)}}{\text{length of total column (mm)}}.$$

3.14.1.2. Haemoglobin, (HB)

The principle of the method Cyanmethemoglobin method **(**Bain and Bates, 2001**)** is that when blood is mixed with a solution containing potassium ferricyanide and potassium cyanide, the potassium ferricyanide oxidizes iron in the haemoglobin to form methemoglobin. The potassium cyanide then combines with methemoglobin to form cyanmethemoglobin, which is a stable colour pigment read photometrically at a wave length of 540 nm. The colour relates to the concentration of haemoglobin in the blood. The reading obtained corresponds to 15 g dl^{-1}, haemoglobin

Procedure:

Preparation of reagent blank: 5 ml of Drabkin's neutral diluting fluid is pipetted into a test tube.

Preparation of test sample: 4 ml of Drabkin's neutral diluting fluid is pipetted into a test tube containing 20 µl of EDTA anti-coagulated blood sample. The tube is allowed to stand for 4 – 5 minutes.

The absorbance of the sample is read in the spectrophotometer at 540 nm while the blank reagent is used to zero the spectrophotometer.

3.14.1.3. Red Cell Count (RBC)

This measurement was made with a microscope, mechanical hand tally counter, counting chamber coverslips, pipettes, and an improved Neubauer ruled counting chamber (hemacytometer). Gower's solution is an isotonic solution (so that erythrocytes are not hemolyzed or crenated) that contains a fixative which acts as a preservative and prevents agglutination of cells if counting cannot be done within an hour.

Procedure:

Preparation of blood sample: EDTA anticoagulated blood (10 µl) was mixed with 1990 µl of Gower's solution i.e. the resulting dilution is 1:200 because the normal dilution factor for a red cell count is 200 (McPherson and Pincus, 2007).

The counting chamber and cover slip were cleaned with water and dried. The cover glass was slide into position over the grid area and pressed down on each side until rainbow colours (Newton rings) are seen. It was then mount on the mechanical stage of the microscope. The 20 ml pipette tip was placed on the haemocytometer at the edge of the coverslip. The plunger of the adjustable pipette was slowly pressed so that the sample flowed between the haemocytometer's raised shiny surface and the coverslip i.e. the haemocytometer is charged. It was stopped when the sample touched the three

sides of the chamber (about 10 ml). Care was taken not to let it overflow into the moat which surrounds the chambers. The counting chamber was undisturbed for 2-3 minutes, on the stage so that the cells can settle down on the ruled area. The chamber was examined at 100x magnification for evenness of the red blood cell distribution. It was turned to the 40x objective, focused and the cells in the designated squares were counted. A chart of the squares was drawn and the number of cells in each square was entered while counting.

Calculation:

RBC's Counted x Dilution x Depth x Area = RBC's cmm^{-1}

Dilution = 200 Depth = l0 Area counted = 5

Or

Number of RBC's counted in 5 squares x RBC Factor = $\frac{\text{number RBC}}{mm^3 \text{ of blood}}$

e.g. 500 x 10,000 = 5,000,000 RBC/mm^3 of blood

3.14.1.4. Mean Cell Volume or Mean Corpuscular Volume (MCV)

Mean cell volume is an estimate of the volume of red blood cells (size). It is useful for determining the type of anaemia a person might have. A low MCV are found in microcytic anaemias and may indicate iron deficiency, chronic disease, pregnancy, a haemoglobin disorder such as thalassaemia, anaemia due to blood cell destruction or bone marrow disorders while a high MCV are found in macrocytic anaemias and may indicate anaemia due to nutritional deficiencies, bone marrow abnormalities, liver disease, alcoholism, chronic lung disease, or therapy with certain medications. It is expressed in femtoliters (fl i.e. 10^{-15} litres) and is calculated

$$\text{MCV fl} = \frac{\text{PCV (L/L)}}{\text{RBC X } 10^{12}/\text{ L}}$$

3.14.1.5. Mean Cell Haemoglobin or Mean Corpuscular Haemoglobin (MCH)

The MCH is the haemoglobin content of the average red cell in pictograms (pg). It tells the haematologist how many oxygen carrying proteins are in each red blood cell. It is a further guide to the investigation of anaemia. The MCH may be low in types of anaemia where the red blood cells are abnormally small and also when RBC's are microcytic and normochromic, or high in other types of anaemia where the red blood cells are enlarged (for example, as a result of folic acid or vitamin B12 deficiency) and in macrocytic normochromic anaemias.

The index is calculated as follows:

$$\text{MCH (picograms)} = \frac{\text{Hemoglobin (in g /L)}}{\text{RBC count x } 10^{12} \text{ /L}}$$

3.14.1.6. Mean Cell Haemoglobin Concentration or Mean Corpuscular Haemoglobin Concentration (MCHC)

The MCHC is the average haemoglobin concentration in a given volume of packed red cells and is expressed as g/L in 1 liter of packed red cells. The MCHC is low in iron deficiency, blood loss, pregnancy and anaemias caused by chronic disease while an increased MCHC can occur in marked spherocytosis.

The index is calculated as follows:

$$\text{MCHC (\%)} = \frac{\text{Haemoglobin (in gm/dl) x 100}}{\text{Hematocrit (in \%)}}$$

Or

$$\text{MCHC (g/L)} = \frac{\text{Hb (g/L)}}{\text{PCV (L/L)}}$$

3.14.1.7. White Cell (Leucocyte) Count

A Haemocytometer (imporved Neubauer ruled chamber), a hand tally counter, diluting fluid (Turk's solution), compound microscope and watch glass were used for this assay.

Preparation of test sample: 20 µl of well mixed EDTA anticoagulated blood was pipette into a test tube containing 0.38 ml of Turk's solution and mixed.

The counting chamber and cover slip were cleaned with water and dried. The cover glass was slide into position over the grid area and pressed down on each side until rainbow colours (Newton rings) are seen i.e. the haemocytometer is charged. It was then mount on the mechanical stage of the microscope. A small drop of the diluted blood was used to fill one of the grids of the chamber by just touching the edge of the cover slip with the tip of the pipette (containing the diluted blood sample) on the chamber. Care was taken not to overfill the area. The counting chamber was undisturbed for 2-3 minutes, on the stage so that the cells can settle down on the ruled area.

White cells were counted in the four large outside squares. These squares each have an area of 1 mm^2 so that the total area counted is 4 mm^2. The cells were then counted using 100x magnification. A chart of the squares was drawn and the number of cells in each square was entered while counting.

Calculation:

The total number of cells counted were divided by 2

The number obtained was divided by 10.

The number multiplied by 10^9 is the white cell count.

E.g. if the number of cell counted in 4 squares = 42

42 ÷ 2 = 21.

21 ÷ 10 = 2.1 therefore, the WBC count = 2.1 cells X 10^{9} $^{-}$L

3.14.1.8. Leucocyte (White Cell) Differential Count

White blood cells are classified into granulocytes, lymphocytes, and monocytes. Granulocytes owe their name to the presence of distinct cytoplasmic granulation and three varieties are recognized. These are neutrophils (or polymorphonuclear granulocytes), eosinophils, and basophils.

To perform a differential count, the following materials were used: plain glass microscope slides, Wright stain solution, distilled water, microscope with light source, immersion oil and blood cell counter.

Generally, white cells are identified by their affinity to the dye they prefer such as, cells that prefer the acid dye (eosin) are called eosinophils. Other cells that prefer the basic dye are called basophils. The white blood cell count was determined using the method of Houwen (2001).

Procedure**:**

Making the blood smear: A capillary tube was used to collect EDTA anticoagulated blood sample and a drop of blood was deposited from the capillary tube onto a clean, grease-free slide. Using a second microscope slide as a spreader held at 30-40 degrees, the edge of the blood drop was touched its end and pushed toward the opposite end of the slide (the blood will be drawn into the acute intersection of the slides and will be "pulled" across the slide). The smear was allowed to air dry for about 5 minutes and it was properly labelled.

Staining the cells: the smears were placed in Wright's stain for 15 to 30 seconds, transferred to phosphate buffer (pH 6.2) for 5 to 15 seconds and then briefly rinsed with water. The slide were placed in a vertical support and allowed to air dry.

Results:

Red blood cells - red to pink.

Neutrophils - dark purple nuclei, pale pink cytoplasm, reddish-lilac small granules.

Eosinophils - blue nuclei, pale pink cytoplasm, red to orange-red large granules.

Basophils - purple to dark blue nucleus, dark purple, almost black large granules.

Lymphocytes - dark purple to deep bluish purple nuclei, sky blue cytoplasm.

Platelets - violet to purple granules

Counting the cells: a drop of immersion oil was placed on the blood film and it was covered with a clean coverslip. The stained blood smear was mount on the microscope stage and examined using the 10 x objective. A region in the "feather" end of the smear was located where the RBCs are neither too diffuse nor overlapping then the 40 x objective was used to examine the film. The first 100 WBC encountered were counted and were classified by cell type and maturation. The tally was kept on a multichannel counter specifically designed for WBC differential counting.

Reporting the count: the total number of each type of white cell is recorded.

For example, if 20 lymphocytes were counted among the 100 cells, the differential count for lymphocytes is 20 %. This process was continued until the count totals 100 %. This differential count is referred to as a relative count.

3.14.1.9. **Platelet Count (**Thrombocyte Count**)**

Platelet count is an estimation of the number of platelets per litre of blood. A platelet count is used to investigate abnormal bleeding that can occur when the platelet count is very low or when the patient is being treated with cytotoxic drugs or other drugs that can cause thrombocytopenia.

An improved Neubauer ruled bright-line counting chamber, counting chamber cover slips, pipettes, mechanical hand tally counter and ammonium oxalate 10 g L^{-1} (1 % w/v) were used for this assay.

Procedure:

20 µl of well mixed anti-coagulated blood was mixed with 0.38 ml of filtered ammonium oxalate diluting fluid. The counting chamber and cover slip were cleaned with water and dried. The cover glass was slide into position over the grid area and pressed down on each side until rainbow colours (Newton rings) are seen i.e. the haemocytometer is charged. A small drop of the diluted blood was used to fill one of the grids of the chamber by just touching the edge of the cover slip with the tip of the pipette (containing the diluted blood sample) on the chamber. Care was taken not to overfill the area. The counting chamber was undisturbed for 20 minutes so that the cells can settle down on the ruled area (it was placed on dampened paper in a petri dish and closed so as to prevent it from drying). It was then mount on the mechanical stage of the microscope.

The 10 x objective was used to focus on the rulings of the grid so as to bring the central square of the chamber into view. It was changed to 40 x objective and focused on the platelets (seen as small bright fragments).

The cells were counted and reported as the number of platelets in 1 L of blood. E.g.

$$\text{platelet count (per litre)} = \frac{\gamma \times \alpha \times 10^6}{\beta \times \delta} = \frac{\text{cells counted x 20 x } 10^6}{0.2 \text{ x } 0.1}$$

Where

γ = cells counted α = 1 in 20 dilution of blood

β = 0.2 mm^2 area counted δ = 0.1 mm dept of chamber

3.14.2. Biochemical Assays

Serum alkaline phosphatase (ALP), alanine aminotransferase (ALT), aspartate aminotransferase (AST), L-γ-glutamyltransferase (GGT) and lactate dehydrogenase (LDH) activities were determined on serum using the standard procedures.

3.14.2.1. Alkaline Phosphatase (ALP)

This is an optimized standard method according to the Deutsche Gesellschaft fur Klinische Chemie. Randox kit was used for this assay. Alkaline phosphatase (ALP EC 3.1.3.1: orthophosphoricmonoester phosphohydrolase) enzymes in serum sample, converted a clear *p*-nitrophenyl phosphate reagent to a yellow *p*-nitrophenol in a timed reaction. Colour change, measured by light absorption, was considered a reliable indication of enzyme activity.

Procedures**:**

A vial of the substrate R1b (containing 10 mmol/L of *p*-nitrophenyl phosphate) was reconstituted with the 10 ml of the buffer R1a (containing 1 mol/L diethanolamine buffer at pH 9.8 and 0.5 mmol/L magnesium chloride). This is the working reagent.

Twenty microliters (20 µl) of the serum sample was mixed with 1 ml of working reagent and the initial absorbance was read at 405 nm. The timer was started simultaneously and the absorbance was read after 1, 2 and 3 min.

Calculation: U/L = 2760 x Δ A 405 nm/ min

3.14.2.2. Alanine Aminotransferase (ALT)

Alanine Aminotransferase activity was measured by monitoring the concentration of pyruvate hydrazone formed with 2,4-dinitrophenylhydrazine (Bergmeyer and Horder, 1980). Randox kit was used for this assay. Two solutions labelled R1 (containing 100 mmol/L Phosphate buffer (pH 7.4), 200 mmol/L L-alanine and 2.0 mmol/L α-oxoglutarate) and R2 (containing 2.0 mmol/L 2,4-dinitrophenylhydrazine) was used.

Procedure:

Preparation of reagent blank: 0.5 ml of solution R1 was mixed with 0.1 ml of distilled water and incubated at 37 ^{o}C for 30 minutes. 0.5 ml of solution R2 was added to it, mixed and allowed to stand for 20 minutes at 25 ^{o}C before adding 5 ml of 0.4 mol/L sodium hydroxide. After 5 minutes, the absorbance of the test sample was measured against the reagent blank by using the reagent blank to set the instrument to zero at 546 nm.

Preparation of test sample: 0.5 ml of solution R1 was mixed with 0.1 ml of serum sample and incubated at 37 ^{o}C for 30 minutes. 0.5 ml of solution R2 was added to it, mixed and allowed to stand for 20 minutes at 25 ^{o}C before adding 5 ml of 0.4 mol/L sodium hydroxide. After 5 minutes, the

absorbance of the test sample was measured against the reagent blank by using the reagent blank to set the instrument to zero at 546 nm.

3.14.2.3. Aspartate Aminotransferase (AST)

Serum AST activity was measured by monitoring the concentration of oxaloacetate hydrazone formed with 2,4-dinitrophenylhydrazine (Reitman and Frankel, 1957). Randox kit was used for this assay. Two solutions labelled R1 (containing 100 mmol/L Phosphate buffer (pH 7.4), 100 mmol/L L-aspartate and 2.0 mmol/L α-oxoglutarate) and R2 (containing 2.0 mmol/L 2,4-dinitrophenylhydrazine) was used.

Procedure:

Preparation of reagent blank: 0.5 ml of solution R1 was mixed with 0.1 ml of distilled water and incubated at 37 ^{o}C for 30 minutes. 0.5 ml of solution R2 was added to it, mixed and allowed to stand for 20 minutes at 25 ^{o}C before adding 5 ml of 0.4mol/L sodium hydroxide. After 5 minutes, the absorbance of the test sample was measured against the reagent blank by using the reagent blank to set the instrument to zero at 546 nm.

Preparation of test sample: 0.5 ml of solution R1 was mixed with 0.1 ml of serum sample and incubated at 37 ^{o}C for 30 minutes. 0.5 ml of solution R2 was added to it, mixed and allowed to stand for 20 minutes at 25 ^{o}C before adding 5 ml of 0.4 mol/L sodium hydroxide. After 5 minutes, the absorbance of the test sample was measured against the reagent blank by using the reagent blank to set the instrument to zero at 546 nm.

3.14.2.4. Lactate Dehydrogenase (LDH)

This is an optimized standard method according to the Deutsche Gesellschaft fur Klinische Chemie (Rec. GSCC 1972). Randox kit was used for this assay. A vial of NADH (0.18 mmol/L) (R1b) was reconstituted with 3 ml of buffer / subtrate containing 50 mmol/L phosphate buffer at pH 7.5 and 0.6 mmol/L of pyruvate (R1a). This is the working reagent.

Procedure:

Twenty microliters (20 µl) of serum sample was pipette into cuvette containing 1 ml of the working reagent. It was mixed and the initial absorbance was read at 340 nm after 30 sec and the the timer was started simultaneously. The absorbance was read again after 1, 2 and 3 mins.

Calculation:

U/L = 8095 x ΔA 340 nm / min

3.14.2.5. L-γ-glutamyltransferase (GGT)

Serum L-γ-glutamyltransferase activity was determined using the method of Szasz **(**1969). The substrate L-γ-glutamyl-3-carboxy-4-nitroanilide, in the presence of glycylglycine is converted by γ-GT in the sample to 5-amino-2-nitrobenzoate which can be measured at 405 nm. Randox kit was used for this assay. 3 ml of a solution containing Tris buffer (100 mmol/L at pH 8.25) and Glycylglycine (100 mmol/L) was used to reconstitute 2.9 mmol/L of L- γ -glutamyl-3-carboxy-4-nitroanilide substrate. This is the working reagent.

Procedure:

One milliliter (1 ml) of the working reagent was mixed with 100 µl of serum sample. The initial absorbance was read at 405 nm and a timer started simultaneously. The absorbance is read again after 1, 2 and 3 minutes.

3.14.3. Antioxidant assays

Catalase (CAT), glutathione peroxidase (GPx), reduced glutathione (GSH), glutathione-s-transferase (GST), thiobarbituric acid reactive substances (TBARS), superoxide dismutase (SOD) and total protein (TP) activities were determined in liver and kidney homogenates and serum samples.

3.14.3.1. **Estimation of Lipid Peroxidation Product** (TBARS)

Lipid peroxidation in terms of thiobarbituric acid reactive substances (TBARS) formation was measured using the method of Niehaus and Samuelsson (1968) with a little modification. Malondialdehyde (MDA) is a secondary product of lipid peroxidation and is used as an indicator of tissue damage. The MDA reacts with TBA (Thiobarbituric Acid) reagent to from a pink coloured product which has absorption maxima at 535 nm.

Procedure:

100 µl of test sample was treated with 2ml of TCA–TBA–HCl (TBA 0.37%, 0.25N HCl and 15 % TCA (Trichloroacetic acid)) reagent (1: 1: 1) and incubated in boiling water bath for 15 minutes.

After cooling, the precipitate was removed by centrifugation. The absorbance of the sample was measured at 535 nm using a blank containing all the reagents except the sample. The blank was used to zero the spectrophotometer. TBARS concentration of the plasma, liver and kidney samples were calculated using the extinction coefficient of MDA which is $1.56 \times 10{-}5$ $mmol^{-1}$ cm^{-1} since 99 % of TBARS exists as MDA using this formula.

$$\frac{OD \times V \times 10^6}{\Sigma \times v} = nmol/ml$$

Σ = molar extinction coefficient ($1.56 \times 10{-}5$ $mmol^{-1}$ cm^{-1})

V = final volume of the reaction

v = volume of the sample

10^6 = optimal dilution factor

3.14.3.2. Estimation of Reduced Glutathione (GSH)

Glutathione, an antioxidant, helps protect cells from reactive oxygen species such as free radicals and peroxides (Pompella, *et al*., 2003). It is the most abundant intracellular thiol compound present in virtually all mammalian cells. GSH reacts with DTNB (5,5'-dithiobis-(2-nitrobenzoic acid) and gets reduced to a yellow coloured complex which has an absorption maximum at 412 nm. Reduced glutathione in plasma, kidney and liver were measured by the method of Ellman (1959).

Procedure:

Preparation of Ellmans reagents: 19.8 mg of DTNB was mixed with 100 ml of 0.1 % sodium nitrate

0.5 ml of the test sample were deproteinated with 0.5 ml of 10 % TCA by centrifugation to release GSH into the supernatant. 0.5 ml of the supernatant was derivatized with 0.25 ml of Ellmans reagent and mixed with 0.2 M phosphate buffer (1.5 ml). Color was developed by adding the Ellmans reagent and it was read at 412 nm.

Calculation:

$$\frac{OD \times V \times 10^3}{\Sigma \times v} = nmol/ml$$

Σ = molar extinction coefficient (13600) V = final volume of the reaction

v = volume of the sample 10^3 = optimal dilution factor

3.14.3.3. Glutathione Peroxidase (GPx)

Glutathione peroxidase is the general name of an enzyme family with peroxidase activity, whose main role is to protect the organism from oxidative damage. The biochemical function of GPx is to reduce lipid hydro peroxides to their corresponding alcohols and to reduce free hydrogen peroxide to water. Glutathione peroxidase (GPx) activity in the test samples were determined by the method of Ellman, (1959) based on the ability of GPx to catalyze the reduction of hydroperoxides, including hydrogen peorxide, by reduced glutathione and functions to protect the cell from oxidative damage.

Procedure:

A mixture containing 0.2 ml of 0.4 M phosphate buffer (pH 7), 0.1 ml of 10 mM sodium azide, 0.2 ml of the test sample, 0.2 ml of glutathione solution and 0.1 ml of 0.2 mM hydrogen peroxide was

incubated for 10 minutes at 37 ^{o}C. 0.4 ml of 10 % TCA was added and it was centrifuged for 10 minutes at 3,500 rpm. The supernatant was used to assay for glutathione content using 0.25 ml of Ellmans reagent and 1.5 ml of 0.2 M phosphate buffer. It was read at 412 nm.

Calculation:

$$\frac{OD \times V \times 10^3}{\Sigma \times v} = nmol/ml$$

Σ = molar extinction coefficient (13600) V = final volume of the reaction

v = volume of the sample 10^3 = optimal dilution factor

3.14.3.4. Glutathione S-Transferases (GST)

Glutathione-S-transferases catalyzes the conjugation reaction with glutathione in the first step of mercapturic acid synthesis. The activity of the enzyme was measured according to the method of Habig and Jakoby (1974) with a little modification.

Procedure:

A mixture containing 0.5 ml of phosphate buffer (0.2 M), 50 µl of 20 mM cDNB (1-chloro-2,4-dinitrobenzene) and distilled water was incubated at 37 ^{o}C for 5 minutes. 50 µl of the sample was added to 50 µl of GSH reagent and it was read at 340 nm at the 1st -3rd minutes. A blank was run in the absence of the sample. One unit of GST activity is defined as 1 µmol product formation per minute.

Calculation:

$$\frac{\Delta OD/min \times V \times 10^3}{\Sigma \times v} = \mu mol/ml$$

Σ = molar extinction coefficient (9.6 $mmol^{-1}$ cm^{-1}) v = volume of the sample

V = final volume of the reaction 10^3 = optimal dilution factor

3.14.3.5. Catalase (CAT)

Hydrogen peroxide (H_2O_2) is formed in cells by controlled pathways and elicits a broad spectrum of cellular response ranging from mitogenic growth stimulation (Petros and Lambeth, 2001) to apoptosis (Zhuang, *et al*., 2007) to necrosis (McKeague, *et al*., 2003) at different concentration levels. Removal of H_2O_2 from cells is therefore necessary for protection against oxidative damage. The most common definition of one catalase unit is the amount of enzyme decomposing 1.0 μmole of hydrogen peroxide per minute at pH 7.0 and 25 ºC, with initial H_2O_2 concentration of 10.3 mM. catalase level was measured according to the method of Sinha (1972).

Procedure:

The reaction mixture (1.5ml) contained 1.0 ml of 0.01M pH 7.0 phosphate buffer, 0.1 ml of test sample and 0.4 ml of 2M H_2O_2 . The reaction was stopped by the addition of 2.0 ml of dichromate-acetic acid reagent (5% potassium dichromate and glacial acetic acid were mixed in 1:3 ratio). A decrease in absorbance was observed after the addition of H_2O_2 to the reaction mixture containing the test sample, which is used as the source of catalase.

Catalase was assayed colourimetrically at 620 nm and expressed as mmoles of H_2O_2 consumed/min/mg protein as described by Sinha (1972).

Calculation:

$$\frac{\Delta OD/\ min \times V \times 10^3}{\Sigma \times v} \div \text{total protein content} = \mu mol/min/mg\ protein$$

Σ = molar extinction coefficient (40 $mmol^{-1}$ cm^{-1}) v = volume of the sample

V = final volume of the reaction 10^3 = optimal dilution factor

3.14.3.6. Determination of Superoxide Dismutase (SOD) Activity.

SOD is a metalloenzyme with its active center occupied by copper or zinc, sometimes manganese (or) iron. SOD plays an important role in protection of all aerobic life systems, against oxygen toxicity. The activity of SOD was measured following the method originally developed by Nishikimi *et al.,* (1972) and then modified by Kakkar *et al.,* (1984). One unit of SOD activity is defined, as the enzyme concentration required inhibiting chromogen production by 50% in one minute under the assay condition.

Procedure:

A mixture containing 30 µl of epinephrine (3×10^4 M), 3 ml of 50 mM $NaCO_3$ (Sodium Carbonate) buffer (pH 10.2) and 20 µl of the test sample was read at 485 nm for the first 3 minutes i.e. at 1 minute interval.

Calculation:

$$\frac{\Delta OD/min \times V \times 10^3}{\Sigma \times v} = nmol/ml$$

Σ = molar extinction coefficient ($4.5 \times 10^3 M^{-1} cm^{-1}$) v = volume of the sample

V = final volume of the reaction 10^3 = optimal dilution factor

3.14.4. Chemical Analytes Assay

Albumin (ALB), cholesterol (CHO)**,** creatinine (CREA)**,** total protein, triglycerides (TG) and blood urea nitrogen (BUN) concentrations were determined on serum using these standard procedures.

3.14.4.1. **Total Protein** (TP)

This method, commonly referred to as the Biuret assay (Tietz, 1995), is based on the interaction of cupric (Cu^{2+}) ion with protein in an alkaline solution and the resultant development of absorbance at 545 nm.

Procedure:

Preparation of reagent blank: To 0.02 ml of distilled water, 1 ml of the Biuret reagent was added.

Preparation of sample: To 0.02 ml of test sample, 1 ml of the Biuret reagent was added.

Preparation of standard: To 0.02 ml of standard protein (bovine serum albumin), 1 ml of the Biuret reagent was added.

They were mixed and incubated for 30 min at +20 to +25°C. Within 90 minutes after the addition of the Biuret Reagent, the absorbance of the standard solution, reagent blank and the test samples were determine at the wavelength of maximum absorbance at 545 nm. The absorbance of the test sample and standard were measured against the reagent blank by using the blank to set the instrument to zero.

3.14.4.2. Albumin (ALB)

The measurement of serum albumin is based on its quantitative binding to the indicator 3,3',5,5'-tetrabromo-m cresol sulphonephthalein (bromocresol green, BCG). The BCG albumin procedure is based on the dye-binding properties of serum albumin with bromcresol green. The absorbance of BCG at 630 nm increases with binding to albumin and is proportional to the albumin concentration present (Spencer and Price, 1977).

Procedure:

Preparation of Reagent Blank: 10 µl of deionized water was mixed with 1.0 ml BCG albumin reagent

Preparation of standard: 10 µl of albumin standard was mixed with 1.0 ml BCG albumin reagent

Preparation of test samples: 10 µl of test sample (serum) was mixed with 1.0 ml BCG albumin reagent.

They were incubated for 5 minutes at +20 to +25°C and the absorbance of the test sample and standard were measured against the reagent blank at 630 nm. The absorbance of the test sample and standard were measured against the reagent blank by using the reagent blank to set the instrument to zero.

3.14.4.3. Creatinine (CREA)

Creatinine was measured according to the method of Bartels, *et al.,* **(**1972**).** Creatinine in alkaline solution reacts with picric acid to form a coloured complex. The amount of the complex formed is directly proportional to the creatinine concentration.

Reagents: equal quantity of Picric acid **(**35 mmol/L) and Sodium hydroxide **(**0.32 mol/L) were mixed and used for this assay as the working reagent.

Procedure:

Preparation of reagent blank: 1 ml of the working reagent was mixed with 100 µl of double distilled water.

Preparation of sample: 1 ml of the working reagent was mixed with 100 µl of serum sample.

After 30 seconds, the absorbance (A_1) of the reagent blank was measured against the sample. Exactly 2 minutes later, the absorbance (A_2) of the reagent blank was measured against the sample at 492 nm.

3.14.4.4. Triglycerides (TG)

Triglyceride assay is based on enzymatic reactions (GPO method) including lipoprotein lipase (LPL), glycerol kinase (GK) and glycerol phosphate dehydrogenase (GPO). Triglycerides are hydrolyzed by LPL to liberate glycerol and free fatty acids. Glycerol is converted to glycerol-3-phosphate (G3P) and ADP (Adenosine diphosphate) by GK and ATP. G3P is then converted by GPO to dihydroxyacetone phosphate (DAP) and hydrogen peroxide (H_2O_2). H_2O_2 is quantified by Trinder reaction using 4-aminoantipyrine, 4-chlorophenol and hydrogen peroxidase (Trinder, 1969). Triglycerides are determined after enzymatic hydrolysis with lipases. The indicator a quinoneimine, is

formed from hydrogen-peroxide, 4-aminophenazone and 4-chlorophenol under the catalytic influence of peroxidase. The reaction is monitored at 500 nm.

Randox kit was used for this assay. Three reagents were used. A buffer (reagent 1) containing Pipes buffer (40 mmol/L at pH 7.4), 4-chlorophenol (5.4 mmol/L), magnesium ions (5 mmol/L), ATP (1 mmol/L), peroxidase (EC 1.11.1.7; horseradish at 25°C. 0.5 U/ml.), glycerol kinase (EC 2.7.1.30; microbial at 25°C. 0.4 U/ml.), glycerol-3-phosphate oxidase (EC 1.1.3.21; microbial at 25°C. 1.5 U/ml.) and sodium azide (0.05 %), an enzyme reagent (reagent 2) containing 4-aminoantipyrine (0.4 mmol/L), lipases (EC 3.1.1.3; microbial, 25°C. 150 U/ml) and sodium azide (0.05 %) and a standard (reagent 3) were used. A mixture of the enzyme reagent (250 µl**)** and the buffer (15 ml) was used as the working reagent.

Procedure:

Preparation of reagent blank: 1 ml of the working reagent was mixed with 10 µl of working reagent.

Preparation of sample: 1 ml of the working reagent was mixed with 10 µl of serum sample.

Preparation of standard: 1 ml of the working reagent was mixed with 10 µl of standard.

It was incubated for 10 minutes at 25 °C. The absorbance of the sample and standard were measured against the reagent blank within 60 minutes.

3.14.4.5. Blood Urea Nitrogen (BUN)

Blood urea nitrogen was determined spectrophotometrically from serum samples using the method of Kassirer (1971). Urea is hydrolyzed in the presence of water and urease to produce

ammonia and carbon dioxide. The ammonia produced in the first reaction combines with α-oxoglutarate and NADH in the presence of glutamate dehydrogenase to yield glutamate and NAD^+.

In the presence of glutamate dehydrogenase (GLDH) and reduced nicotinamide-adenine dinucleotide (NADH), ammonia (as ammonium ions; $NH4^+$) reacts with 2-oxoglutarate to form L-glutamic acid and NAD^+.

The amount of NAD+ formed is stoichiometric with the amount of ammonia or with twice the amount of urea. It is NADH consumption which is measured by the decrease in absorbance at 340 nm.

Randox kit was used for this assay. 50 ml of tris-buffer (150 mmol/L at pH 7.6) was reconstituted with 50 ml of an enzyme reagent containing urease (15 U/ml), GLDH (1 U/ml), NADH (0.28 mmol/L), adenosine-5-diphosphate (2.45 mmol/L) and α-oxoglutarate (11.7 mmol/L). This is the working reagent.

Procedure:

Preparation of reagent blank: 1 ml of the working reagent was mixed with 10 µl of distilled water.

Preparation of sample: 1 ml of the working reagent was mixed with 10 µl of serum sample

Preparation of standard: 1 ml of the working reagent was mixed with 10 µl of standard.

The initial absorbance of the serum sample and standard were measured against the reagent blank after 30 sec and a timer was started simultaneously. It was read again after 1 minute.

3.14.5. Caspase Activity Assay

3.14.5.1. Isolation of Leukocytes from Whole Blood

For each 2 ml of EDTA-treated blood (from the rats in the pharmacologically induced hepatotoxicity groups) , the blood sample was spun at 5,000 rmp and the white blood cells i.e. the buffy coat was transferred to a test tube containing 10 ml of 0.17 M ammonium chloride solution. It was incubated for 5 minutes at 4 ^{o}C and then centrifuged for 5 minutes at 2000 rpm and the procedure was repeated 4 times. The supernatant was aspirated using a Pasture pipette and the pellets were suspended in 2 ml of 0.5 % saponin. Phosphate buffered saline was laid over the leukocytes, and tubes were then centrifuged at 1500 rpm for 5 minutes. The supernatant was aspirated.

The concentration of the leukocyte suspension was determined using a Thoma counting chamber. Polymorphonuclear leukocytes and mononuclear leukocytes were discerned using Giemsa staining.

Leukocyte viability was checked using the trypan blue exclusion test since viable cells with intact membrane integrity do not take in (exclude) certain chemical dyes. 200 µl of leucocyte suspension being tested for viability was centrifuged for 5 min at 1500 rpm and the supernatant was discarded. The cell pellet was resuspended in 200 µl PBS (Phosphate buffered saline) and mixed with 200 µl of 0.4 % trypan blue (GIBCO/BRL) in an Eppendorf tube. The mixture was allowed to incubate for 3 min at room temperature (Cells should be counted within 3 to 5 min of mixing with trypan blue, as longer incubation periods will lead to cell death and reduced viability counts). A drop of the trypan blue/cell mixture was applied to a haemacytometer and viewed on a binocular microscope. Nuclei of non-viable cells stained blue. The white blood cells and pellets were resuspended in 10 ml cold 1x phosphate buffered saline (pH = 7.2), aliquot into Eppendorf tubes and stored at -20 ^{o}C.

3.14.5.2. Caspase-2 Assay Procedure:

The increase in caspase-2 activity was determined using the method of Hoglund, *et al.,* (2009). The white blood cells and pellets isolated from the rats in the pharmacologically induced hepatotoxicity groups were used for this assay. White blood cells and pellet (2-5 x 10^6 cells) were counted and resuspended in 50 µl of chilled cell lysis buffer. It was incubated on ice for 10 min and centrifuged for 1 min in a microcentrifuge at 10,000 rpm. The protein concentration of the supernatant was assayed (Biuret method) and 100-200 µg cytosolic extract was diluted in 50 µl cell lysis buffer (BioVision). 50 µl of 2x reaction buffer (Gentaur) (to which dithiothreitol (DTT) (Sigma) had been added giving a final concentration of 10 mM i.e. 10 µl of 1.0 M DTT stock was added to 1 ml of 2X reaction buffer) was added to each sample. 5 µl of 4 mM substrate comprising the amino acids L-valyl- L-aspartyl- L-valyl- L-alanyl- L-aspartic acid- p-nitroanilide (VDVAD-pNA or Val-Asp-Val-Ala-Asp- pNA substrate) (BioVision) was added giving a final concentration of 200 µM. It was incubated at 37 ^oC for 1-2 hr. The absorbance was read using a BIO-RAD microplate reader at 405nm. The increase in caspase-2 activity was determined by comparing the results of treated samples (groups 2-7) with the level of the untreated control (group 1).

3.14.5.3. Caspase-3 Assay Protocol:

The method of Chen, *et al*., (2008) was used to determine the increase in caspase-3 activity. The white blood cells and pellets isolated from the rats in the pharmacologically induced hepatotoxicity groups were used for this assay. White blood cells and pellet 1-5 x 10^6 cells was counted and resuspended in 50 µl of chilled cell lysis buffer. It was incubated on ice for 10 min and centrifuged at 10,000 rpm for 1 min. The supernatant (cytosolic extract) was put on ice and the protein concentration was assayed according to Biurets method. 50-200 µg cytosolic extract was diluted in 50 µl cell lysis buffer and 50 µl of 2x reaction buffer to which DTT had been added giving a final

concentration of 10 mM final concentration i.e. 10 µl of 1.0 M DTT stock was added to 1 ml of 2X reaction buffer was added. 5 µl of the 4 mM DEVD (Ac-Asp-Glu-Val-Asp) p-nitroanilide substrate (Acetyl- L-aspartyl- L-glutamyl- L-valyl- L-aspartic acid- p-nitroanilide substrate) (BioVision) was added giving a final concentration of 200 µM and it was incubated at 37 ^{o}C for 1-2 hr. The absorbance was read using a BIO-RAD microplate reader at 405 nm. The fold-increase in caspase-3 activity was determined by comparing the results of hepatotoxicity induced rats (groups 2-7) with the level of the uninduced control rats (group 1).

3.14.5.4. Caspase -9 Assay Protocol:

Fold-increase in caspase-9 activity was determined using the method of Chen, *et al*., (2008). White blood cells and pellets 2-5 x 10^6 cells were counted and resuspended in 50 µl of chilled cell lysis buffer. It was incubated on ice for 10 min and centrifuged at 10,000 rpm for 1 min. The supernatant (cytosolic extract) was put on ice and the protein concentration was assayed using Biurets method. 100-200 µg of the cytosolic extract was diluted in 50 µl cell lysis buffer and 50 µl of 2x reaction buffer (comprising of 10 µl of 1.0 M DTT stock diluted in 1 ml of 2x reaction buffer). 5 µl of the 4 mM LEHD (Ac-Leu-Glu-His-Asp) p-nitroanilide substrate (Acetyl- L-leucyl- L-glutamyl- L-histidyl- L-aspartic acid- p-nitroanilide substrate) (BioVision) was added giving a final concentration of 200 µM and it was incubated at 37^{o}C for 1-2 hr. The absorbance was read using a BIO-RAD microplate reader at 405-nm.

Fold-increase in caspase-9 activity was determined by comparing the results of hepatotoxicity induced rats (groups 2-7) with the level of the uninduced control rats (group 1).

3.14.6. Histopathology.

The method of Mallory (1961) was used with a little modification. A small chunk of liver and kidney were taken from the sacrificed experimental rats used for hepatotoxicity studies and were

preserved in 10 % formal saline for histologic studies. The tissues were processed and sectioned in paraffin. The paraffin sections of buffered formalin- fixed tissue samples (3 µm thick) were dewaxed and rinsed in alcohol and also water. They were stained with Harris' haematoxylin (Sigma) for 10 minutes, washed in running tap water for 1 minute, differentiated in acid alcohol for 10 seconds and washed again in running tap water for 5 minutes. The tissues were stained with eosin for 4 minutes and washed in running tap water for 10 seconds. It was dehydrated and mounted for photomicroscopic observations of the histologic architecture. The general structure of the liver and kidneys of the normal control group (group 1) was compared with those of the treated groups (groups 2-7).

3.15. *in vitro* Antioxidant Assays

3.15.1. Assay of Lipid Peroxidation using Brain Homogenates

The thiobarbituric acid (TBA) assay was used to assess lipid peroxidation using the method of Chowdhury and Soulsby (2002). For the *in vitro* studies, the brains of normal rats were dissected and homogenized with a Polytron® ergonomic homogenizer (speed setting 7-8) in ice-cold Tris-HCl buffer (20 mM, pH 7 4) to produce a 1/10 homogenate. The homogenate was centrifuged at 14,000 rpm for 15 min. One ml aliquots of the supernatant were incubated with the different fractions of GOV, the aqueous and ethanolic extracts of GOV in the presence of 10 µM FeS04 (Ferrous Sulphate) and 0.1 mM ascorbic acid at 37 °C for 1 hr. The reaction was stopped by addition of 1 ml trichloroacetic acid (TCA, 28 %, w/v) and 1.5 ml thiobarbituric acid (TBA, l %, w/v) in succession, and the solution was then heated at 100°C for 15 mins. After centrifugation to remove precipitated protein, the colour of the malondialdehyde (MDA)-TBA complex was detected at OD 532 nm using a Milton Roy spectronic 3000 spectrophotometer. Butylated hydroxyanisole (BHA) was used as a positive control. The inhibition ratio (%) was calculated using the following formula.

Inhibition ratio (%) = $\frac{(A - A_1)}{A} \times 100\ \%$ where

A = the absorbance of the control, and A_1 = the absorbance of the test sample

3.15.2. Assay of Inhibition of Erythrocyte Haemolysis

The method of Ramchoun, *et al*., (2009) was used to assay the inhibition of erythrocyte haemolysis. Blood was obtained from mice by extirpating their eyeballs and collected in tubes containing 0.15 M NaCl. Erythrocytes were separated from the plasma and the buffy coat, and washed three times with 10 volumes of 0.15 M NaCl. During the last wash, the erythrocytes were centrifuged at 2,500 rpm for 10 min to obtain a constantly packed cell preparation. Erythrocyte haemolysis was mediated by peroxyl radicals in the assay system. A 10% suspension of erythrocytes in pH 7.4 phosphate buffered saline (PBS) was added to the same volume of 200 mM 2,2'-azo-bis (2-amidinopropane) dihydrochloride (AAPH) (Sigma) solution (in PBS) containing different concentrations of the samples to be tested. The reaction mixture was shaken gently while being incubated at 37 ^{o}C for I hr. The reaction mixture was removed, diluted with 8 volumes of PBS, and centrifuged at 2,500 rpm for 10 min.

The absorbance A of the supernatant was read at 540 nm. Similarly, the reaction mixture was treated with 8 volumes of distilled water to achieve complete haemolysis, and the absorbance B of the supernatant obtained after centrifugation was measured at 540 nm. L-ascorbic acid (Sigma) was used as a positive control.

The percentage of haemolysis was calculated by using the equation

$$(1\text{-}A/B) \times 100\ \%.$$

3.16. Antiproliferative Activity toward Human Hepatocellular Liver Carcinoma Cell Line (HepG2 cells) and Nasopharyngeal Cancer Cells (CNE2 and SUME –α- Nasopharyngeal Cells)

The human nasopharyngeal carcinoma cell lines CNE-2 (Human Nasopharyngeal Carcinoma Cell Lines) and SUME –α- nasopharyngeal cells were bought from Sun Yat-sen University of Medical Sciences, Guangzhou, China. Hep G2 (Human hepatocellular liver carcinoma cell line) cell lines were purchased from The American Type Culture Collection (ATCC). All others reagents used were from Sigma (USA) or as mentioned.

Assay of antiproliferative activity on tumor cell lines: Growth was assessed by using the methylthiazolyldiphenyl-tetrazolium (MTT) bromide cellular proliferation assay. (Sieuwerts, *et al.*, 1995; Zhang, *et al*., 2004). Briefly, HepG 2, SUME –α- and CNE-2 cells were maintained in RPMI (Roswell Park Memorial Institute) 1640 medium containing 10 % fetal bovine serum, 1 % penicillin and streptomycin at 37 ^{o}C, an atmosphere of 95 % air and 5 % carbon dioxide. After the cells had grown to 90 % confluence, the cells were trypsinized (with 0.25% (w/v) Trypsin- 0.53 mM EDTA solution to remove all traces of serum that contains trypsin inhibitor) and seeded to a 96-well plate (each well containing approximately 5000 cells per 100 µl) respectively. After incubation for 8 hr, the cells were treated with a serial dilution of the purified trypsin inhibitor at concentrations from 1.6 to 100 µM for 24 and 48 hrs. Two hours (2 hrs.) before the end of each incubation interval, 50 µl of a solution containing 5 mg ml^{-1} of MTT was spiked into each well. After careful removal of the medium, 150 µl dimethyl sulfoxide (Sigma) was added to each well and then agitated on an orbital shaker for 15 minutes. The absorbance at 595 nm was measured using a BIO-RAD microplate reader.

3.17. Statistical analysis

The results were expressed as Mean $\pm$ SEM for seven rats. Statistical analysis of the data was performed using ANOVA statistical SPSS package (15.0) version. The significance of differences among all groups was determined by the Tukey HSD test. P – values less than 0.05 ($p \leq 0.05$) were considered to be statistically significant.

CHAPTER FOUR

RESULTS

4.1. Extraction of Plant Materials

The percentage yields of the aqueous and ethanolic extracts of *Gongronema latifolia* Benth, *Ocimum gratissimum Linn.* and *Vernonia amygdalina Del.* (1: 1: 1) were 12.6 % and 14.1 % respectively. The yield of the triherbal formulation i.e. 50 % ethanol extract (GOV) obtained was 1.42 kg (15.69 %).

4.2. Fractionation of Plant Materials

The 50 % ethanol extract (GOV) was sequentially solvent partitioned into soluble extracts of GOV giving a % yield of Hexane (5.92 %), Chloroform (10.38 %), Ethyl acetate (20.89 %), Butanol (24.68 %) and Water (36.63 %).

4.3. Phytochemical analysis

The results of the phytochemical screening of 50 % ethanolic extract of the triherbal formulation (GOV) is shown in able 1. The phytochemicals alkaloids, flavonoids, tannins, free and bound anthraquinones, saponins and reducing sugars were present in the 50 % ethanolic extract while anthocyanides were absent.

Table 1: Preliminary phytochemical analysis of 50% ethanolic extract of *Gongronema latifolia* Benth*, Ocimum gratissimum Linn.* and *Vernonia amygdalina Del.* (GOV)

Bioactive componenets	**Phytochemical test**	**GOV (50 % ethanolic extract)**
Alkaloids	Dragendorf	+++
Flavonoids	Ferric chloride	++
Tannins (Catechol)	Ferric chloride	+++
Condensed tannins	Bromine water	++
Phlobatanins	Aqueous HCl	+++
Cardiac glycoside (Steroidal ring)	Salkowski	+++
Deoxysugar (characteristic of cardenolide)	Keller-Killian	+++
Anthraquinones	(free and bound)	++
Saponin	Frothing on sample	+++
	Haemolysis on RBC	+++
Reducing sugar	Fehling's solution	+++
Anthocyanides		--

+++ = high concentration; ++ = medium concentration; + = low concentration; -- = not detected

4.4. Thin Layer Chromatogram of GOV

The phytochemical analysis of GOV revealed that it is as a source of some significant secondary metabolites such as rutin, stigmasterol, β sitosterol, hyperoside borneol, ascorbic acid, eugenol, luteolin using the thin layer chromatography. The chromatograms of GOV using different mobile phases and sprayed with different reagents are shown in plates 1- 5. The best separation and highest numbers of bioactive components were detected in the hexane: ethyl acetate (1:1) solvent system while the natural product reagent spray demonstrated the best colour separation compared with the other sprays. Table 2 shows the Rf values of the test samples and standard reference compounds.

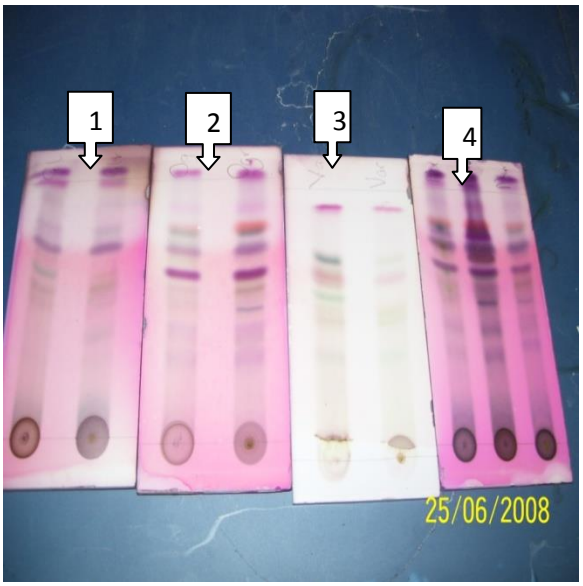


Plate 1: Chromatogram of GOV using (1) ethyl acetate:methanol:water (100:13.5:10) (2) ethyl acetate:glacial acetic acid (99:1) (3) hexane: chloroform (1:1) (4) ethyl acetate: Hexane (1:1) sprayed with a solution of ninhydrin.

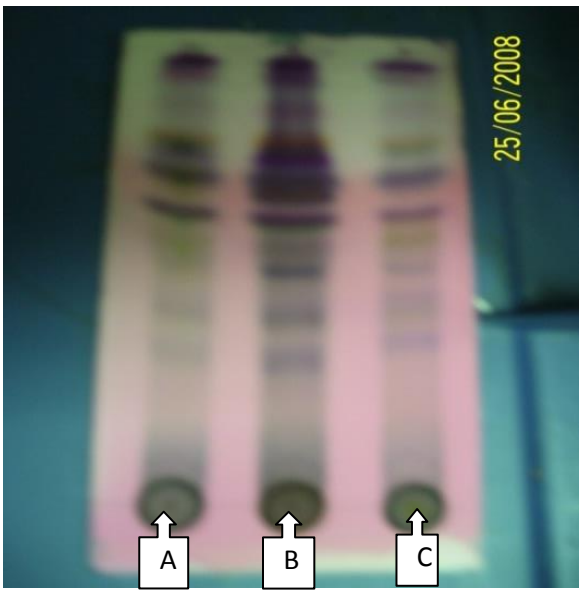


Plate 2: Chromatogram of GOV using ethyl acetate: Hexane (1:1) sprayed with a solution of ninhydrin. Aqueous (A) 50% ethanolic (B) and ethanolic (C) extracts of *Gongronema latifolia* Benth, *Ocimum gratissimum Linn.* and *Vernonia amygdalina Del.*

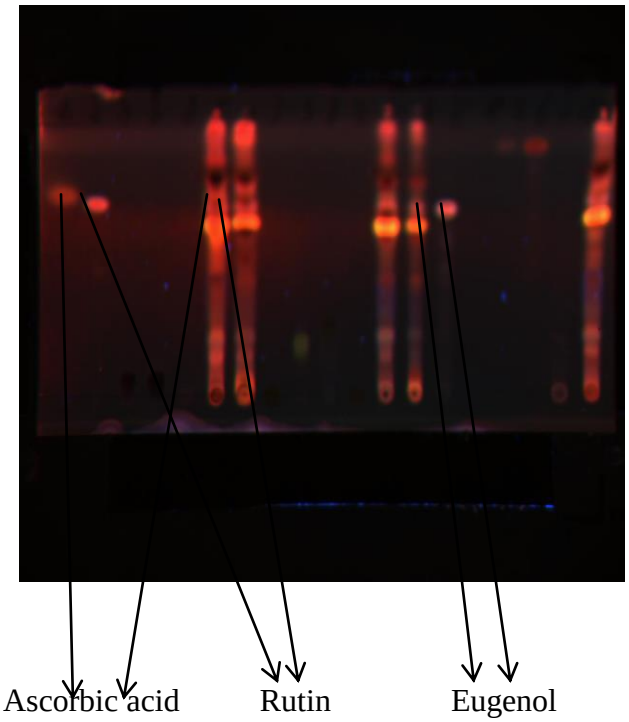


Plate 3: Chromatogram of aqueous and 50% ethanolic extract of GOV sprayed with anisaldehyde / H_2SO_4 solution and scanned with a Scanner III at 365 nm indicating the presence of ascorbic acid, rutin and eugenol

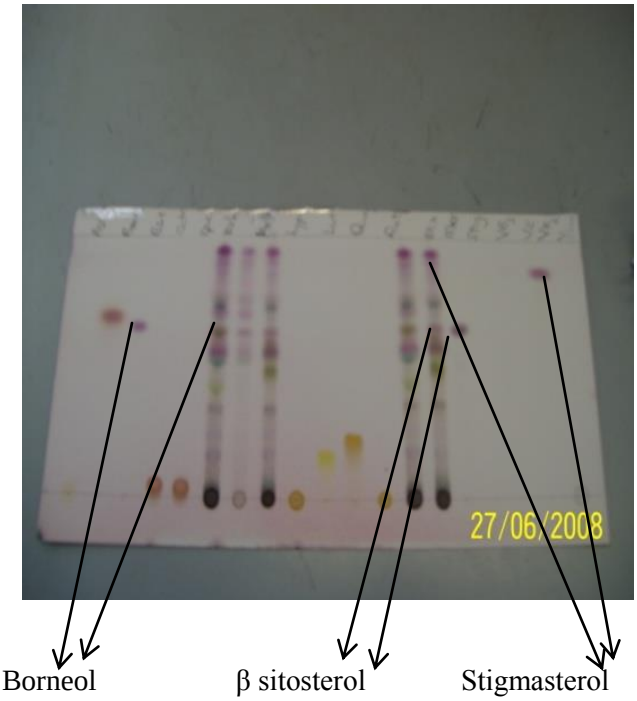


Plate 4: Chromatogram of aqueous and 50% ethanolic extract of GOV sprayed with natural product reagent showing presence of borneol, β sitosterol and stigmasterol.

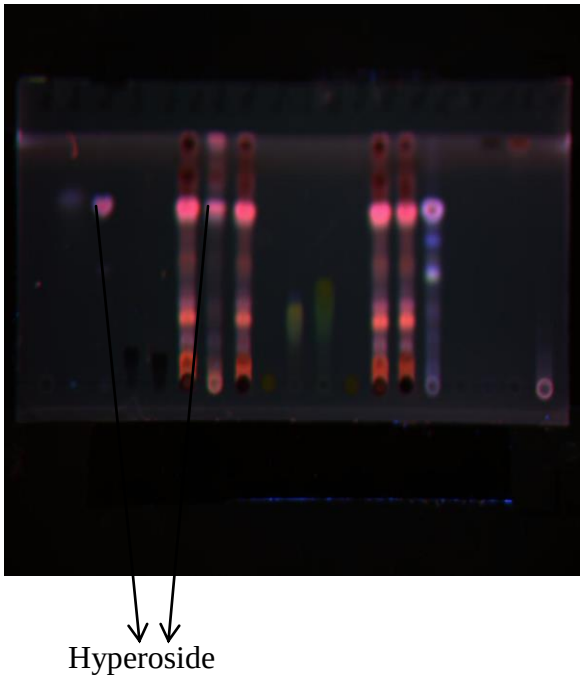


Plate 5: Chromatogram of GOV at 365 nm indicating the presence of borneol and hyperoside.

Thin Layer Chromatogram of the triherbal formulation (GOV)

Table 2: The Rf values of the test samples and standard reference compounds

Plate number	Compound	Rf value		Colour	
		Standard	Sample	Standard	Sample
Plate 3	Ascorbic acid	0.67	0.67	Deep orange	Deep orange
	Rutin	0.73	0.73	Pink	Pink
	Eugenol	0.7	0.7	Rose	Rose
Plate 4	Borneol	0.71	0.71	Purple	Purple
	β sitosterol	0.68	0.68	Dark purple	Dark purple
	Stigmasterol	0.89	0.89	Deep blue	Deep blue
Plate 5	Hyperoside	0.71	0.71	Pink	Pink

Plate 3: Chromatogram of aqueous and 50 % ethanolic extract of GOV sprayed with anisaldehyde / H_2SO_4 solution and scanned with a Scanner III at 365 nm.
Plate 4: Chromatogram of aqueous and 50 % ethanolic extract of GOV sprayed with natural product reagent
Plate 5: Chromatogram of GOV at 365 nm

4.5. High Performance Thin Layer Chromatograms (HPTLC) of GOV Extract

Further separation was achieved using HPTLC. The HPTLC chromatograms of GOV are shown in plates 6 - 7. The migration or Rf values of GOV were compared with reference compounds, sprayed with different reagents and then viewed at different wavelengths.

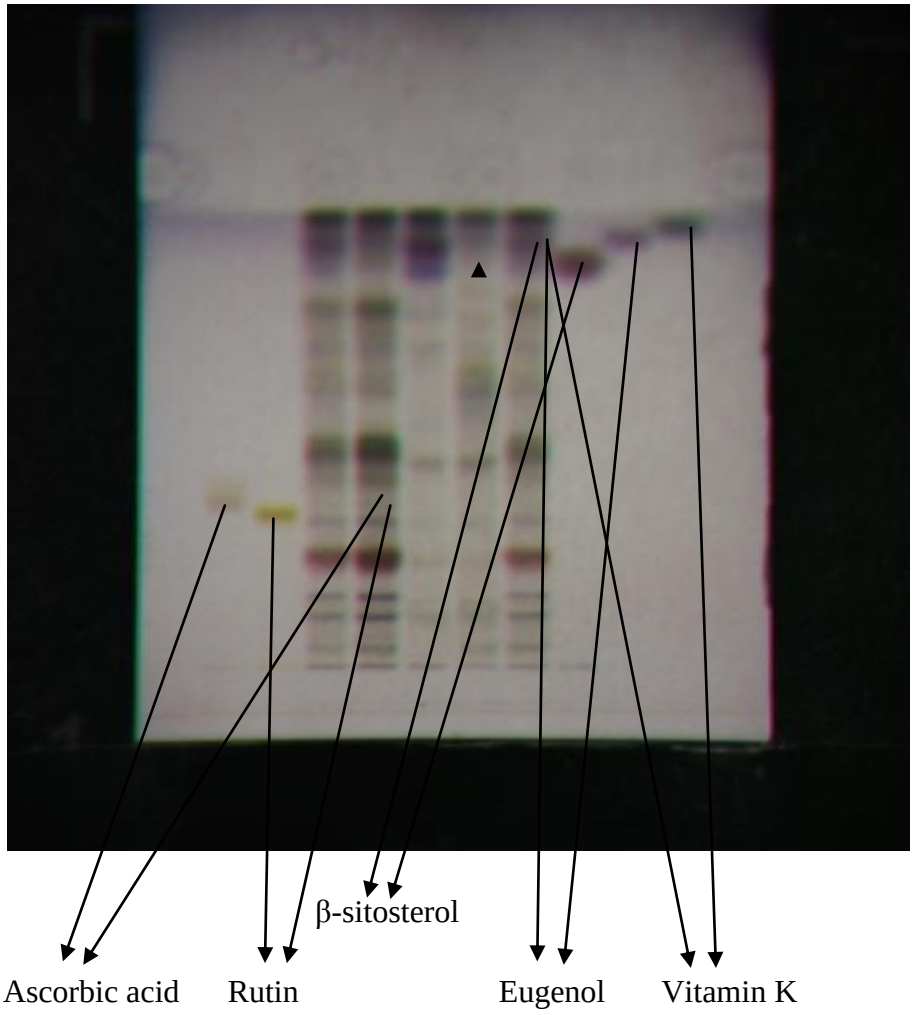


Plate 6: White light chromatogram of GOV sprayed with natural product.

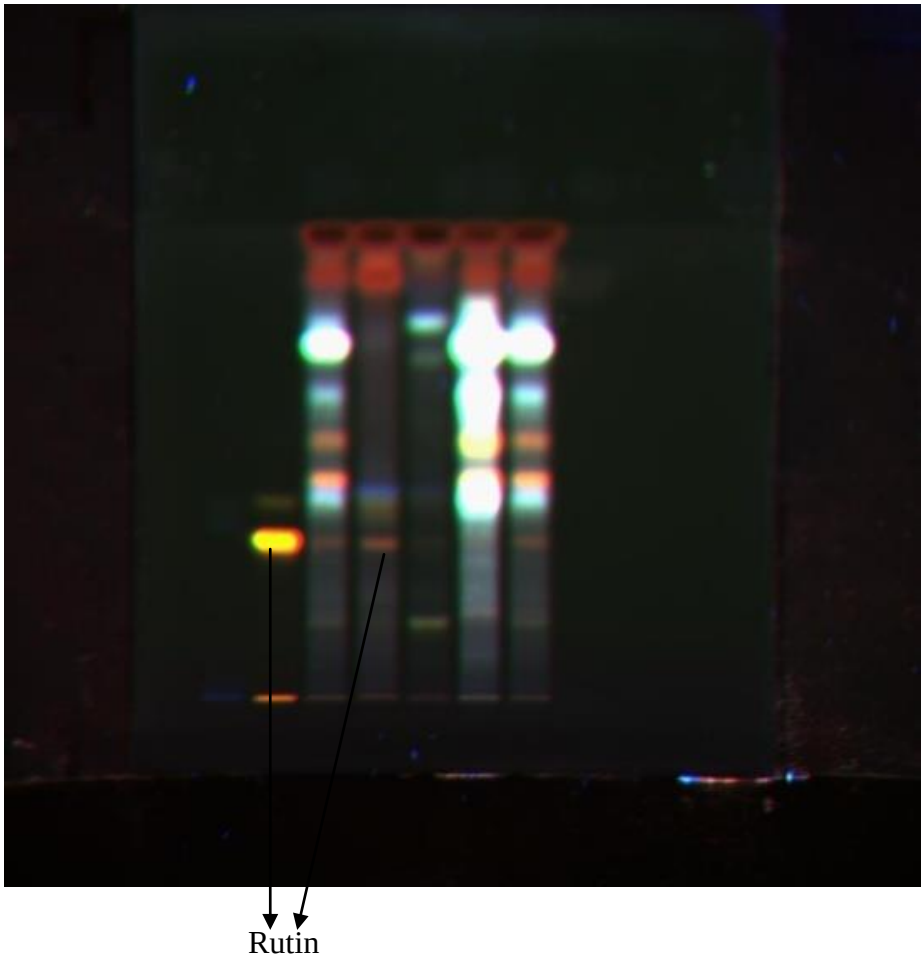


Plate 7: 365nm chromatogram of aqueous and 50% ethanolic extract of GOV sprayed with anisaldehyde/ H_2SO_4

4.6. NMR Spectra for the Binding Study of GOV and its Fractions with Dihydrodipicolinate Reductase (DHPR)

The signals in these areas could indicate compounds that are binding to DHPR. For example, peaks at 0.6, 1.2, and 1.3 ppm in the butanol fraction (Figure 2(A)) seemed to indicate a compound that bound to DHPR. A follow up experiment with a proton NMR of the same sample (Figure 2 (B)) shows that the STD signals maybe due to the excitation of the protein at -0.5 ppm. This phenomenon is due to the irradiation of the protein being near proton NMR signals of the ligands due to methyl groups. Previous STD studies led to familiarity in irradiating DHPR at -0.5 ppm to excite the protein. Another possible signal in the STD at 5.6 ppm was found upon close inspection of the spectrum, Figure 5(A).

Key:

A + on a spectrum indicates that area is of interest and further experimentation is necessary to investigate the nature of the signal.

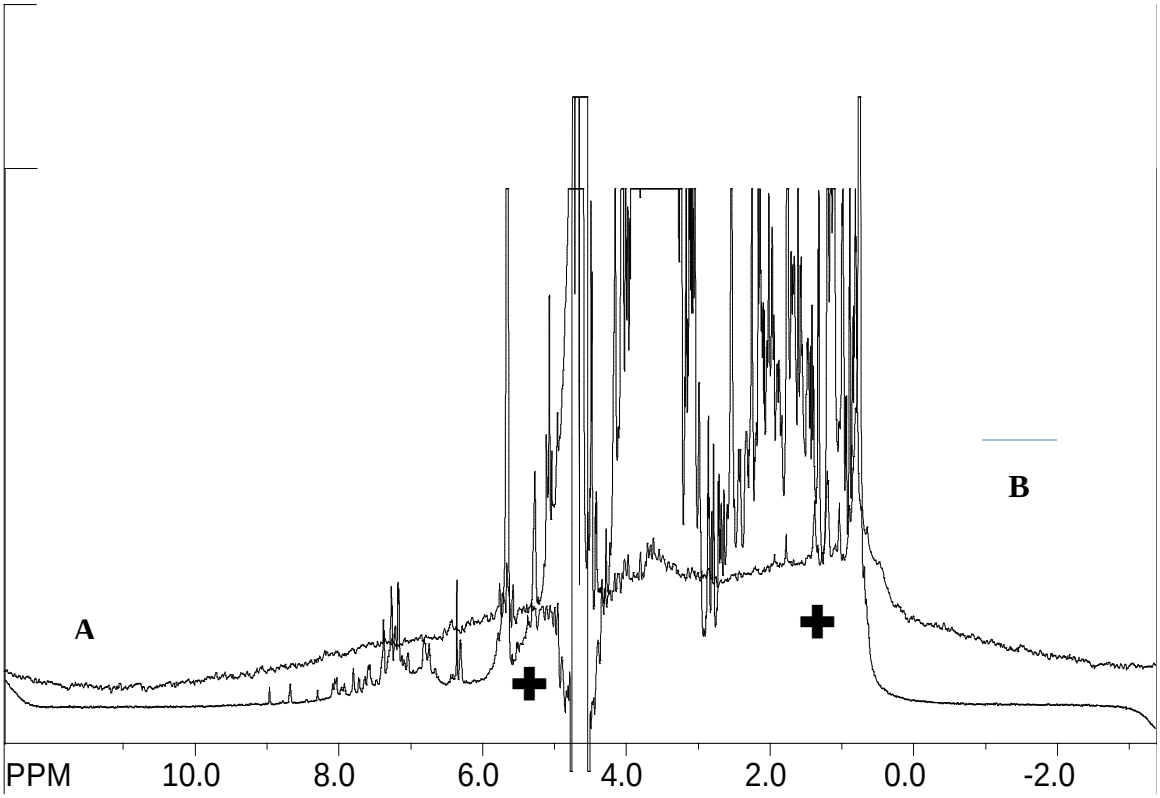


Figure 5: A) STD difference spectrum of GOV with DHPR. B) Proton NMR spectrum of GOV in the presence of DHPR.

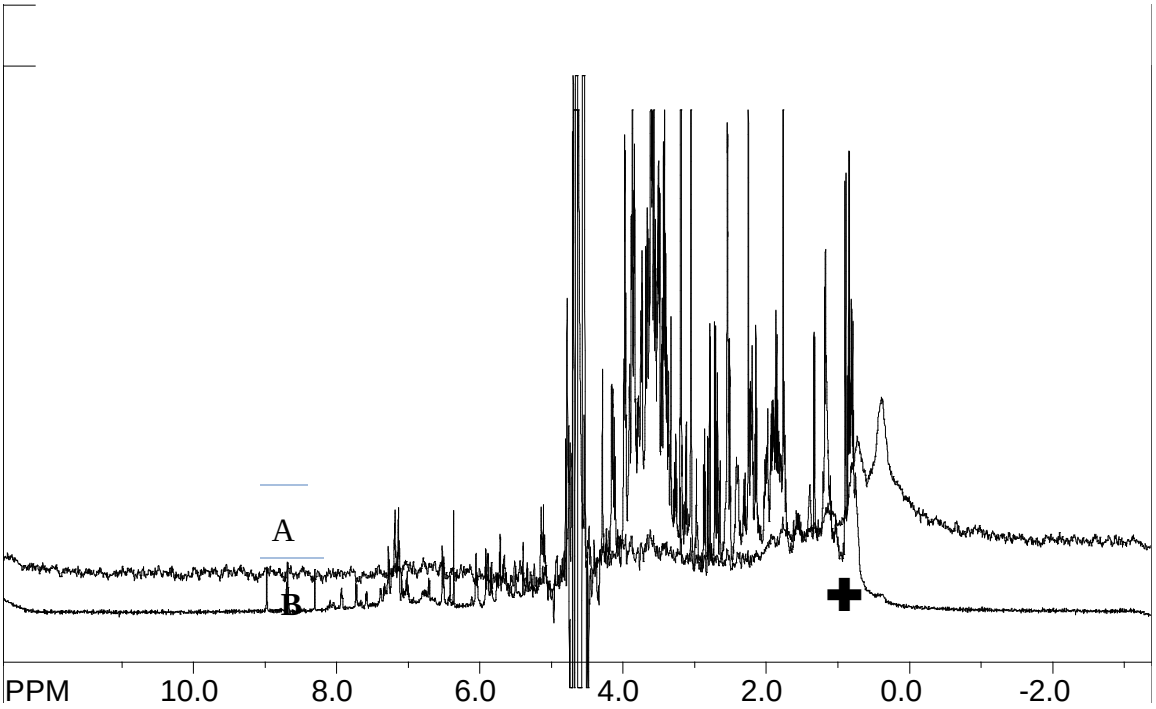


Figure 6: A) STD difference spectrum of the ethyl acetate fraction of GOV with DHPR. B) Proton NMR spectrum of the ethyl acetate fraction of GOV in the presence of DHPR.

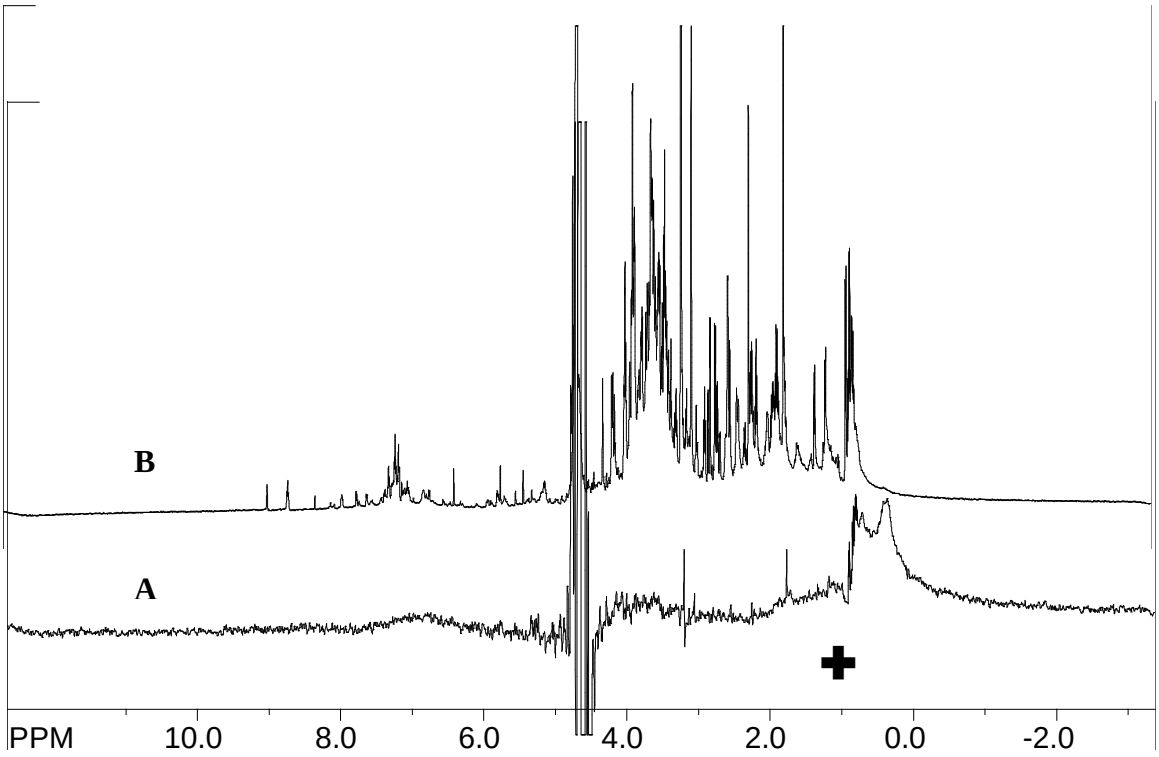


Figure 7: A) STD difference spectrum of the ethanol extract of a mixture of *Gongronema latifolia* Benth, *Ocimum gratissimum Linn.* and *Vernonia amygdalina Del.* with DHPR. B) Proton NMR spectrum of the ethanol extract of a mixture of *G. latifolia, O. gratissimum* and *V. amygdalina* in the presence of DHPR.

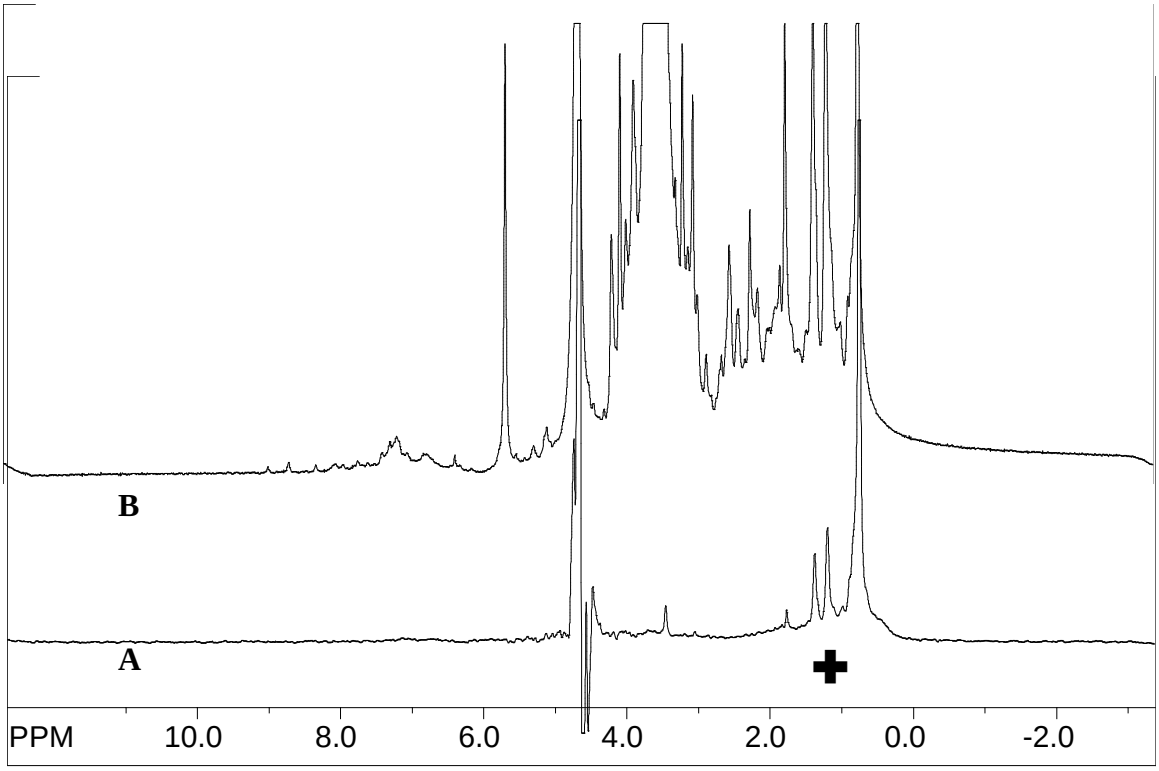


Figure 8: A) STD difference spectrum of the butanol fraction of GOV with DHPR. B) Proton NMR spectrum of the butanol fraction GOV in the presence of DHPR.

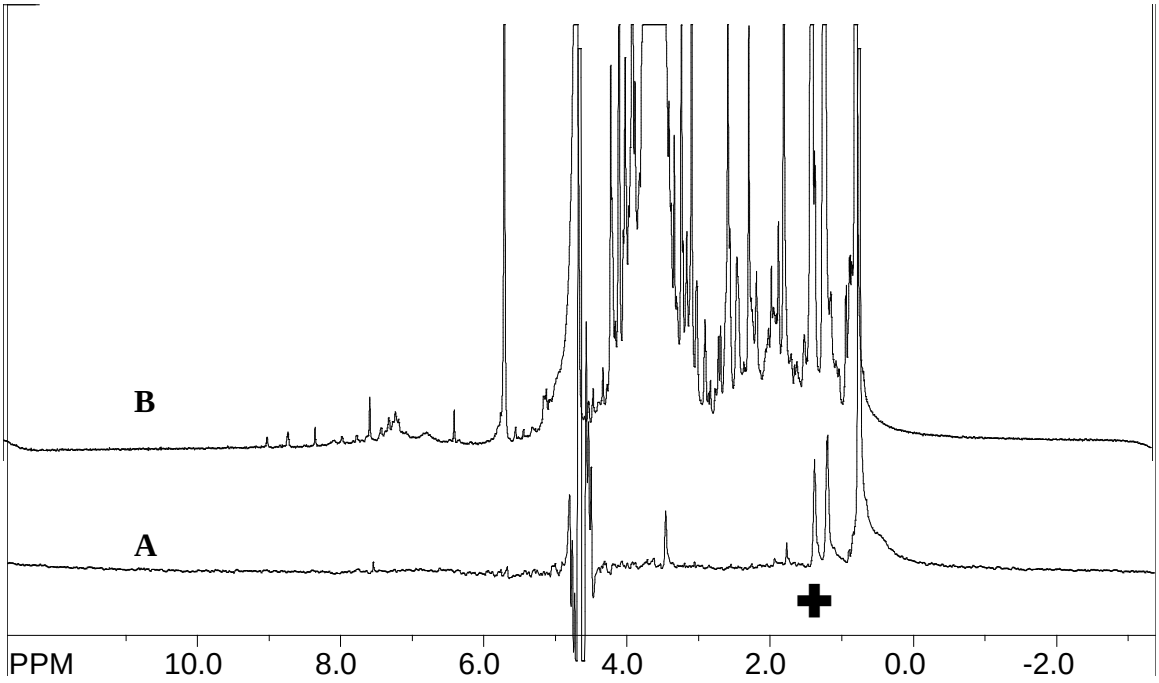


Figure 9: A) STD difference spectrum of the chloroform fraction of GOV with DHPR. B) Proton NMR spectrum of the chloroform fraction of GOV in the presence of DHPR.

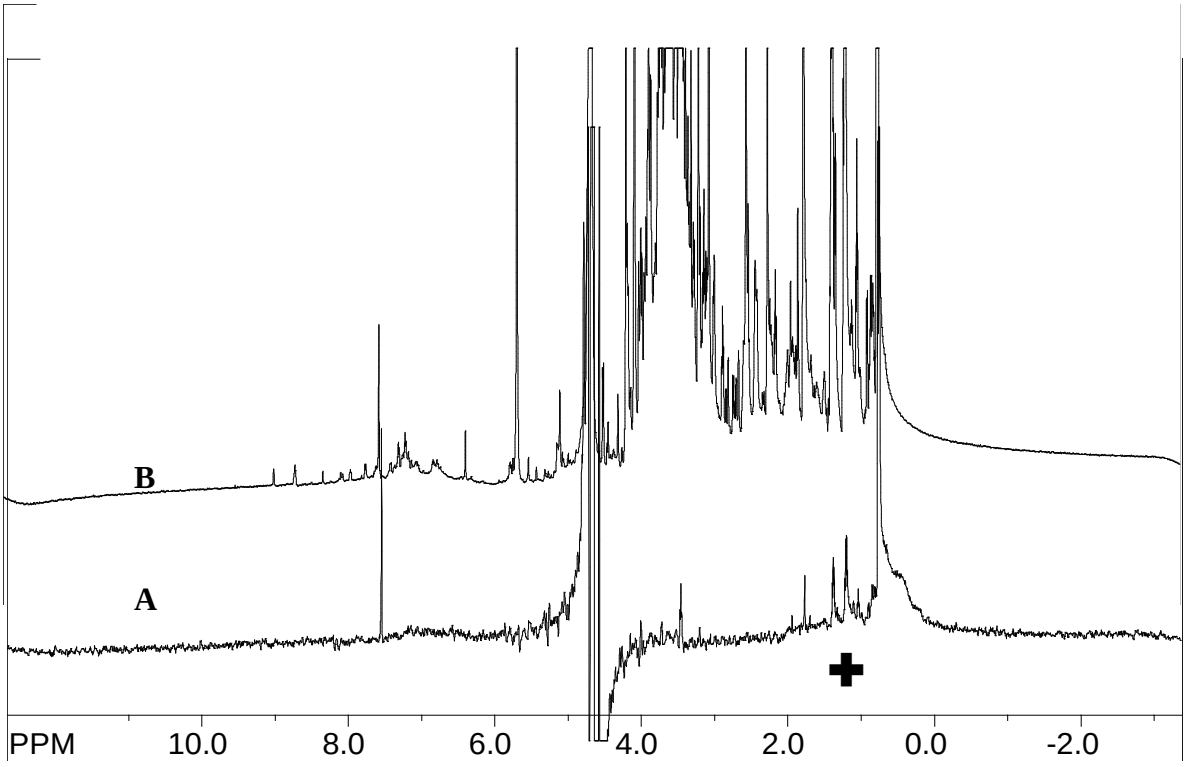


Figure 10: A) STD difference spectrum of the water fraction of GOV with DHPR. B) Proton NMR spectrum of the water fraction of GOV in the presence of DHPR.

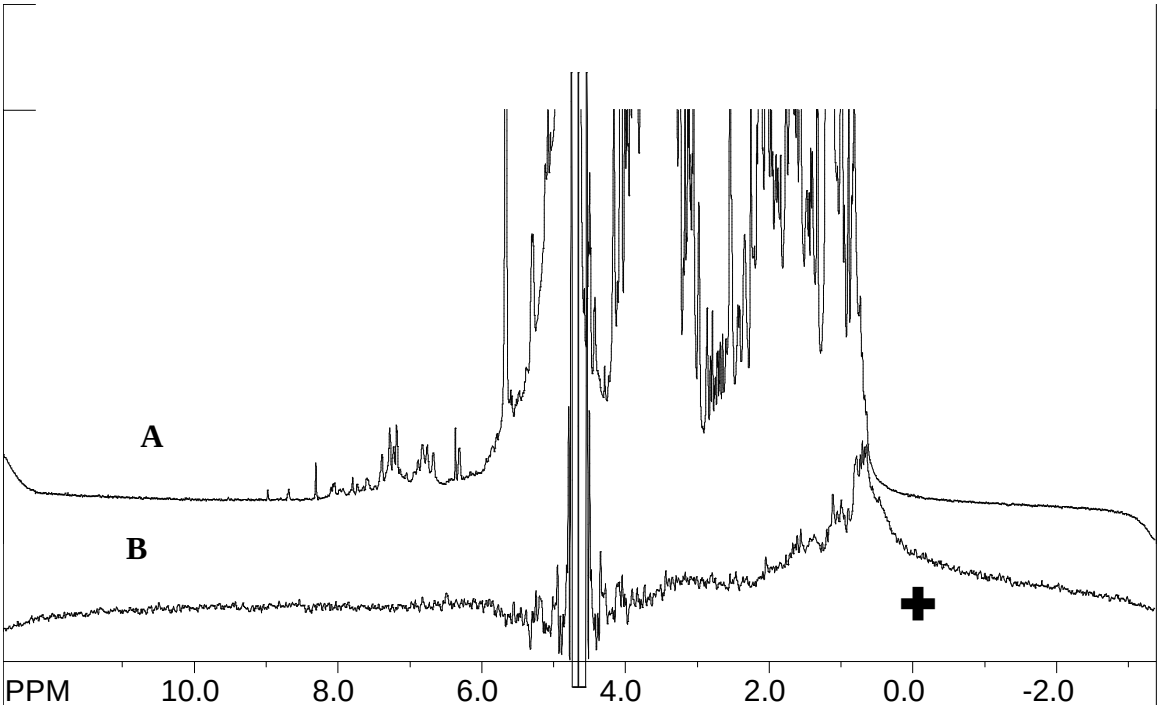


Figure 11: A) STD difference spectrum of the hexane fraction of GOV with DHPR. B) Proton NMR spectrum of the hexane fraction of GOV in the presence of DHPR.

4.7. Toxicity Tests

4.7.1. Acute Toxicity

The acute toxicity studies showed that GOV at 16 g kg^{-1} (p.o.) and 2.5 g kg^{-1} (i.p.) did not produce any mortality nor was there any significant change in the general behaviour of the mice.

4.7.2. Sub-chronic toxicity

There were no visible signs of deleterious effects on the rats both in behaviour and morphology. No deaths were recorded.

4.7.2.1. Sub-chronic Effect on Haematologic Indices.

The effects of pretreatment of normal albino rats with GOV on the haematologic indices are shown in tables 3a and 3b. At 2 g kg^{-1}, the haemoglobin (Hb), platelets, Mean corpuscular haemoglobin concentration (MCHC), Mean corpuscular haemoglobin (MCH) and granulocytes were increased while the Mean corpuscular volume (MCV) had the lowest value compared to the other groups while at 8 g kg^{-1}, the packed cell volume (PCV), red blood cell (RBC), white blood cell (WBC) and lymphocytes were the highest.

There was no significant ($p \leq 0.05$) difference between the GOV treated groups and the control group. GOV dose dependently increased the PCV, Hb, RBC, WBC, platelet, MCHC, MCH, granulocytes and lymphocytes compared to the control group. There was no significant ($p \leq 0.05$) difference between the groups.

Table 3a: Effect of GOV on the hematologic indices in rats

Group	Dose ($g\ kg^{-1}$)	PCV (%)	RBC ($10^6/\mu l$)	Hb (g/dl)	WBC ($10^3/\mu l$)	Platelet ($10^3/\mu l$)
Control		44.46 ± 1.9	6.66 ± 0.14	12.26 ± 0.5	3.1 ± 0.33	50.56 ± 2.92
GOV	2	46.99 ± 1.67	7.16 ± 0.33	14.16 ± 0.34	3.54 ± 0.56	53.21 ± 2.82
	4	46.11 ± 0.76	6.92 ± 0.36	12.6 ± 0.67	3.51 ± 0.3	49.06 ± 2.53
	8	48.09 ± 2.44	7.39 ± 0.23	13.89 ± 0.8	3.81 ± 0.4	53.14 ± 3.01

Values are expressed as Mean ± SEM of seven rats.

Table 3b: Effect of GOV on the hematologic indices in rats

Group	Dose ($g\ kg^{-1}$)	MCHC (%)	MCV (fl)	MCH (pg)	Granulocytes (%)	Lymphocyte (%)	Monocyte (%)
Control		27.75 ± 1.17	66.95 ± 2.98	18.43 ± 0.67	9.27 ± 0.53	85.93 ± 0.67	4.8 ± 0.59
GOV	2	30.35 ± 1.28	62.31 ± 1.65	18.97 ± 1.14	10.17 ± 0.65	85.73 ± 0.74	4.1 ± 0.4
	4	27.35 ± 1.43	67.41 ± 2.55	18.3 ± 0.8	9.63 ± 0.63	86.44 ± 0.56	3.93 ± 0.43
	8	29.67 ± 3.14	65.82 ± 4.76	18.87 ± 1.11	8.99 ± 0.78	87.11 ± 0.84	4.04 ± 0.12

Values are expressed as Mean ± SEM of seven rats.

PCV =Packed Cell Volume, HB = Haemoglobin, RBC = Red Cell Count, MCV = Mean Cell Volume, MCH = Mean Cell Haemoglobin, MCHC = Mean Cell Haemoglobin Concentration, WBC = White Blood Cell.

4.7.2.2. Sub-chronic effect of GOV on serum marker enzymes and chemical analytes

Table 4a and 4b shows how GOV affects serum marker enzymes. GOV at all concentrations significantly ($p \leq 0.05$) attenuated the ALP level compared to the control group while there was no significant difference in GGT and LDH levels. At 2 g kg^{-1}, GOV significantly ($p \leq 0.05$) decreased the ALT level compared to control and GOV at 4 g kg^{-1}. GOV at 2 and 4 g kg^{-1} significantly ($p \leq 0.05$) lowered the AST level compared to the control group

The triglyceride and BUN concentrations were significantly ($p \leq 0.05$) attenuated by GOV at 4 and 8 g kg^{-1} compared to the control group. At 4 g kg^{-1}, GOV significantly ($p \leq 0.05$) decreased the concentration of cholesterol compared to control and 2 g kg^{-1} GOV groups. However, GOV at a concentration of 4 g kg^{-1} presented the lowest cholesterol, creatinine, total protein, triglyceride and BUN concentrations and highest albumin concentration compared to all the groups.

Table 4a: Serum activities of ALT, AST, ALP, LDH and GGT of rats treated with GOV

Group	Dose ($g\ kg^{-1}$)	LIVER FUNCTION ENZYMES				
		ALP (UL)	**ALT (UL)**	**AST (UL)**	**GGT (UL)**	**LDH (UL)**
Control		$139.59 \pm 4.84^{(b, c, d)}$	$14.06 \pm 0.34^{(b)}$	$17.55 \pm 0.41^{(b, c)}$	1637.61 ± 44.99	14.04 ± 1.37
GOV	2	$121.6 \pm 4.65^{(a)}$	$11.33 \pm 0.62^{(a, c)}$	$14.44 \pm 0.72^{(a)}$	1432.3 ± 41.81	13.62 ± 1.49
	4	$119.14 \pm 3.88^{(a)}$	$13.42 \pm 0.26^{(b)}$	$14.25 \pm 0.68^{(a)}$	1597.47 ± 41.69	13.59 ± 1.47
	8	$114.9 \pm 3.72^{(a)}$	12.7 ± 0.64	16.19 ± 0.51	1531.87 ± 83.72	11.99 ± 0.87

Values are expressed as Mean ± SEM of seven rats and triplicate determination. The Mean difference is significant at the 0.05 level. (a) = $p \leq 0.05$ as compared with the normal control group. (b) = $p \leq 0.05$ as compared with the 2 g kg^{-1} GOV group. (c) = $p \leq 0.05$ as compared with the 4 g kg^{-1} GOV group. (d) = $p \leq 0.05$ as compared with the 8 g kg^{-1} GOV group. The significance of differences among all groups was determined by the Tukey HSD test

ALP = Alkaline Phosphatase, ALT = Alanine Aminotransferase, AST=Aspartate Aminotransferase, LDH = Lactate Dehydrogenase, GGT = L-γ-glutamyltransferase

Table 4b: Serum levels of albumin, cholesterol, creatinine, total protein, triglyceride and BUN of rats treated with GOV.

Group	Dose ($g\ kg^{1}$)	BIOCHEMICAL PARAMETERS					
		TP ($g\ L^{-1}$)	ALB ($g\ L^{-1}$)	BUN ($mmol\ L^{-1}$)	CREA ($mmol\ L^{-1}$)	TG ($mmol\ L^{-1}$)	CHO ($mmol\ L^{-1}$)
Control		77.08 ± 3.11	36.62 ± 1.7	7.5 ± 0.4$^{(c, d)}$	82.06 ± 2.75	1.43 ± 0.13$^{(c, d)}$	1.73$^{(c, d)}$
GOV	2	77.47± 2.63	38.69 ± 1.7	6.53 ± 0.65	76.21 ± 3.42	1.15 ± 0.05	1.56 ± 0.15$^{(c)}$
	4	77.06 ± 2.93	43.55 ± 2.3	5.18 ± 0.36$^{(a)}$	71.75 ± 3.25	1.04 ± 0.04$^{(a)}$	1.16 ±.08$^{(a, b)}$
	8	80.96 ± 1.33	40.22 ± 2.2	5.6 ± 0.44$^{(a)}$	74.94 ± 2.26	1.13 ± 0.06$^{(a)}$	1.34 ± 0.07$^{(a)}$

Values are expressed as Mean ± SEM of seven rats and triplicate determination. The Mean difference is significant at the 0.05 level. (a) = $p \leq 0.05$ as compared with the normal control group. (b) = $p \leq 0.05$ as compared with the 2 g kg^{-1} GOV group. (c) = $p \leq 0.05$ as compared with the 4 g kg^{-1} GOV group. (d) = $p \leq 0.05$ as compared with the 8 g kg^{-1} GOV group. The significance of differences among all groups was determined by the Tukey HSD test

TP = Total Protein, ALB = Albumin, BUN = Blood Urea Nitrogen, CREA = Creatinine, TG = Triglyceride, CHO = Cholesterol

4.7.2.3. Sub-chronic Effect on Serum Antioxidant Enzymes

The effect of GOV on serum antioxidant enzymes in rats are shown in table 5 below. GOV increased the CAT and decreased the MDA level compared to control. At the 3 doses, GOV exhibited similar SOD levels. At 2 g kg^{-1}, the total protein concentration was higher than control while the GST levels were almost equal. The GSH levels of GOV at 4 and 8 g kg^{-1} are high compared to control. There was a significant difference in GSH level of GOV at 2 g kg^{-1} compared to control and GOV at 4 and 8 g kg^{-1}.

Table 5: The effect of GOV on serum antioxidant enzymes in rats

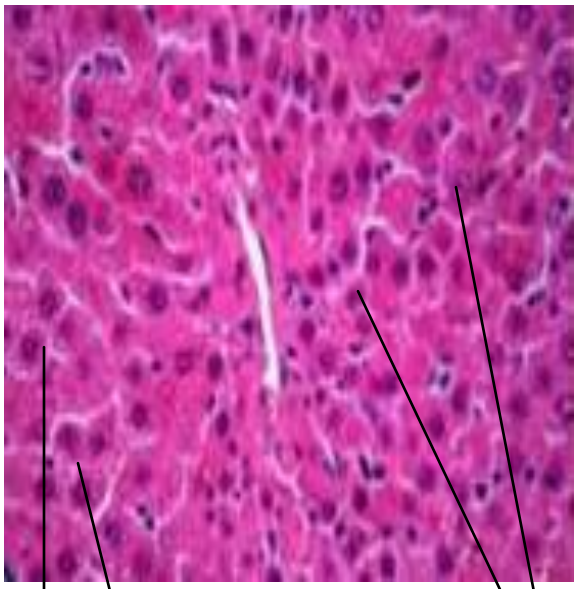
Group	Dose (g kg^{1})	CAT (µmol/min/mg protein)	GPx (µmol/ml)	GSH (µmol/ml)	GST (µmol/ml)	MDA (nmol/ml)	SOD (µmol/ml)	TOTAL PROTEIN (g/L)
Control		112.78 ± 2.74	8.53 ± 0.06	0.56 ± 0.003$^{(b)}$	40.36 ± 2.13	8.74 ± 0.26	69.15 ± 1.02	77.08 ± 3.11
GOV	**2**	121.46 ± 3.2	8.49 ± 0.07	0.55 ± 0.005$^{(a, b, d)}$	39.14 ± 1.09	8.71 ± 0.88	65.52 ± 1.49	77.47 ± 2.63
	4	113.84 ± 1.85	8.42 ± 0.08	0.56 ± 0.003$^{(b)}$	36.86 ± 0.83	8.58 ± 0.59	65.3 ± 2.73	77.06 ± 2.93
	8	118.31 ±1.36	8.24 ± 0.11	0.56 ± 0.002$^{(b)}$	38.37 ± 2.45	8.69 ± 0.77	65.53 ± 2.75	76.02 ± 1.55

Values are expressed as Mean ± SEM of seven rats. The Mean difference is significant at the 0.05 level. (a) = $p \leq 0.05$ as compared with the normal control group. (b) = $p \leq 0.05$ as compared with the 2 g kg^{-1} GOV group. (c) = $p \leq 0.05$ as compared with the 4 g kg^{-1} GOV group. (d) = $p \leq 0.05$ as compared with the 8 g kg^{-1} GOV group. The significance of differences among all groups was determined by the Tukey HSD test.

CAT = Catalase, GPx = Glutathione Peroxidase, GSH = Reduced Glutathione, GST = Glutathione-S-Transferase, MDA = Malondialdehyde, SOD = Superoxide Dismutase

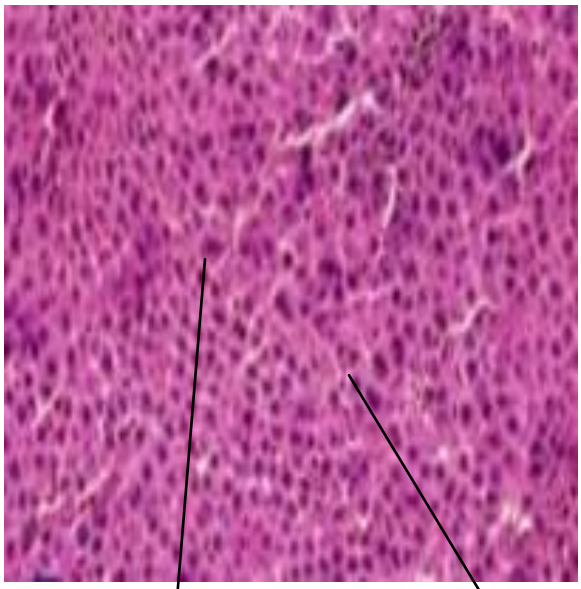
4.7.2.4. The Histopathology of Normal Liver of Rats Treated with GOV

Plates 1- 2 shows that the liver architecture of rats treated with GOV at different doses and control were characterized by polyhedral shaped hepatocytes and granulated cytoplasm. This indicates that GOV is not toxic.



Polyhedral shaped hepatocytes granulated cytoplasm

Plate 1: Control (X100)



Polyhedral shaped hepatocytes granulated cytoplasm

Plate 2: GOV 2 g kg^{-1} (X40)

Liver architecture of rats treated with GOV

4.8. Preliminary Pharmacological Assays

4.8.1. Antinociceptive Activities of GOV

The experimental results of antinociceptive activities of the GOV are depicted in Tables 6 - 8. The number of abdominal constrictions resulting from injection of mice with 100 mg kg $^{-1}$ of 0.6 % acetic acid intraperitoneally presented 28.4 ± 3.4 writhes which is 68.9 % in 15 minutes while treatment with GOV at doses of 2 – 8 g kg $^{-1}$ significantly ($P \leq 0.05$) and dose dependently reduced the number of writhes from 34.6 ± 3.2 to 30.4 ± 2.0 (Table 6) in 15 minutes. In control mice however, the number of abdominal constrictions was 91 ± 12.00.

Table 6: Effect of the GOV on acetic acid induced writhing in mice

Group	Dose ($g\ kg^{-1}$)	No of writhes (Mean ± SEM)	% inhibition
Control		91 ± 1.2	
GOV	2	34.6 ± 3.2	61.9
	4	31.2 ± 1.7	65.7
	8	30.4 ± 2.0	66.6
Acetyl salicylic acid	0.1	28.4 ± 3.4	68.9

Values are expressed as Mean ± SEM of seven rats.

Table 7 indicates that in the formalin induced pain assay, the highest dose of the GOV exhibited a significant inhibition of 46 % (42 ± 5.4) while acetylsalicylic acid produced a significant pain inhibition of 49 % (39.6 ± 2.9).

Table 7: Effect of GOV on formalin induced pain

Group	Dose (g kg $^{-1}$)	0–5min (Mean ± SEM)	% Inhibition
Acetylsalicylic acid	0.1	$39.6 \pm 2.88^{(b, c)}$	49
GOV	2	$55.4 \pm 5.88^{(a,c,d)}$	28.7
	4	$48.8 \pm 8.18^{(a,b)}$	37.2
	8	$42 \pm 5.4^{(b)}$	46

Values are expressed as Mean ± SEM of seven rats. The Mean difference is significant at the 0.05 level. (a) = $p \leq 0.05$ as compared with the Acetylsalicylic acid group. (b) = $p \leq 0.05$ as compared to GOV (2 g kg^{-1}) group. (c) = $p \leq 0.05$ as compared with the GOV (4 g kg^{-1}) group. (d) = $p \leq 0.05$ as compared with the GOV (8 g kg^{-1}) group. The significance of differences among all groups was determined by the Tukey HSD test.

Morphine, a potent opioid agonist inhibited paw licking (180.8 %) inhibition, compared to GOV at 8 g kg^{-1} (138.5 %) (Table 8) in the hot plate assay.

Table 8: Effect of GOV on hot plate test

Group	Dose (g kg $^{-1}$)	Reaction time (sec)	% Inhibition
Control		$5.2 \pm 0.63^{(c,d,e)}$	
GOV I	2	$6.6 \pm 0.32^{(d,e)}$	26.9
GOV II	4	$7.8 \pm 0.48^{(a,d,e)}$	50
GOV III	8	$12.4 \pm 1.89^{(a,b,c)}$	138.5
Morphine	0.02	$14.6 \pm 1.2^{(a,b,c)}$	180.8

Values are expressed as Mean ± SEM of seven rats. The Mean difference is significant at the .05 level. (a) = $p \leq 0.05$ as compared with the control group. (b) = $p \leq 0.05$ as compared to GOV (2 g kg^{-1}) group. (c) = $p \leq 0.05$ as compared with the GOV (4 g kg^{-1}) group. (d) = $p \leq 0.05$ as compared with the GOV (8 g kg^{-1}) group. (e) = $p \leq 0.05$ as compared with the Morphine group. The significance of differences among all groups was determined by the Tukey HSD test.

4.8.2. Anti-Inflammatory Activities of GOV

The anti-inflammatory studies revealed a dose dependent relationship in the activity of the GOV and this is comparable to Indomethacin (Table 9). It showed statistically significant inhibition at doses of 4 and 8 g kg^{-1}, exhibiting 114.71 % and 132.35 % inhibition respectively post 6 hr induction of inflammation. No inhibition was observed in the control group. The results obtained in this study indicated that GOV may possess more lasting or prolonged anti-inflammatory effects compared to the standard steroidal drugs as GOV (8 g kg^{-1}) inhibited the paw oedema at 2 hr more than indomethacin.

Table 9: Effect of GOV on carrageenan-induced rat paw oedema

Group	Dose ($g\ kg^{-1}$)	RB	Difference in oedema circumference (cm)						
			0hr	1hr	2hr	3hr	4hr	5hr	6hr
Control		21±0.58	24 ± 0.91	0.28 ± 0.75	0.68 ± 0.63	0.58±1.03	0.52 ±0.95	0.4 ±1.08	0.34 ±1.11
GOV	**2**	20.4 ± 0.66	23.6 ± 0.88	0.16 ± 0.48	0.36 ±1.89	0.26 ±1.89	0.22 ±1.80	0.14± 1.53	0.1 ±1.56
				(42.86)	(47.06)	(55.2)	(57.69)	(65)	(70.59)
	4	21.4 ± 0.78	24.8 ±1.11	0.2 ±0.63	0.4 ±1.98	0.34 ±1.8	0.18 ±1.05	0.06 ±1.05	-0.05 ±0.95
				(28.54)	(41.18)	(41.38)	(65.39)	(85)	(114.71)
	8	21.2±0.26	24.8 ±0.48	0.08 ±0.32	0.16 ±1.85	0.18 ±1.85	0.08 ±1.71	0 ±1.78	-.11 ±1.71
				(71.43)	(76.47)	(68.97)	(84.62)	(100)	(132.35)
Indomethacin	**0.01**	21±1.23	25.2± 1.49	0.08 ±1.08	0.19 ±1.03	0.16± 1.70	-0.2±1.68	-0.1±1.44	-.21 ±1.16
				(71.43)	(72.06)	(74.14)	(103.85)	(125)	(161.77)

MEAN ± SEM, n= 7, figures in parenthesis indicate the percentage inhibition (%)

RB= circumference of rat paw before start of experiment 0hr= circumference of paw immediately after injection of carrageenan

Differences in oedema circumference is the difference in circumference between the time (1hr – 6hr) and the paw circumference immediately after injection of carrageenan

In this test, the GOV was found to significantly and dose dependently exhibit strong anti-inflammatory effect on xylene-induced ear oedema when compared to test drug dexamethasone as shown in table 10. At 8 g kg^{-1}, GOV had a percentage inhibition of 66 % while dexamethasone was 69%.

Table 10: Effect of the GOV on xylene induced ear oedema in mice

Group	Dose ($g\ kg^{-1}$)	WLE (g)	WRE (g)	IEW (g)	% inhibition
Control		0.26 ±0.01	0.61 ± 0.013	0.35 ± 0.004 (57 %)	
GOV	**2**	0.4 ± 0.011	0.62 ± 0.011	0.22 ± 0.005 (36 %)	37
	4	0.38 ± 0.005	0.54 ± 0.01	0.16 ± 0.008 (30 %)	54
	8	0.37 ± 0.007	0.5 ± 0.012	0.12 ± 0.008 (24 %)	66
Dexamethasone	**0.004**	0.36 ± 0.003	0.49 ± 0.003	0.11 ± 0.003 (22 %)	69

Values are in Mean ± SEM (n =7). P ≤0.05 compared to control (students t-test). Figures in parenthesis indicates % increase in ear weight

(n = 7) – number of mice in each group is = 7

WLE – weight of left ear

WRE – weight of right ear

IEW – increase in ear weight

4.9. Alcohol Induced Hepatotoxicity

4.9.1. Effect of GOV on the Haematologic Indices on Alcohol Induced Hepatotoxicity on Rats.

The administration of GOV dose dependently caused a significant ($p \leq 0.05$) increase in PCV, RBC, Hb, WBC, platelet, MCHC, granulocytes and lymphocytes and decrease in MCV and monocytes compared to the ethanol induced toxin control group (tables 11a and 11b) resulting in GOV associated protection of the haematopoietic system. There was an increase in levels of Hb, platelet count and MCHC and granulocytes on administration of GOV at 2 g kg^{-1} compared to Liv 52 group, all the other groups and control and silymarin groups respectively. However a decrease in MCV was observed compared to control and silymarin group. There was an increase in PCV at the administration of GOV at 4 g kg^{-1} compared to Liv 52 and silymarin groups and it showed an increase in RBC when compared to silymarin treated rats but was almost equal to Liv 52 treated rats. The numbers of monocytes observed in the 4 g kg^{-1} group were lowered compared to control and Liv 52 groups and it exhibited the lowest and highest value of MCH and lymphocytes respectively compared to all the groups.

On administration of GOV (8 g kg^{-1}), the PCV and WBC levels were elevated compared to Liv 52 and silymarin also, its RBC was high compared to silymarin and almost equal to Liv 52 treated rats. The Hb and platelet counts of GOV (8 g kg^{-1}) were elevated compared to Liv 52 though it attenuated the MCH level compared to control and silymarin. It boosted and abated the lymphocyte and granulocytes levels respectively compared to control and Liv 52 experimental groups. There was a significant increase in the PCV and WBC values at administration of GOV at a dose of 8 g kg^{-1}, compared to 2 g kg^{-1}.

Table 11a: Effect of pretreatment with GOV on the hematologic indices in rats with alcohol induced hepatotoxicity.

Group	Dose ($g\ kg^{-1}$)	PCV (%)	RBC ($10^6/\mu l$)	Hb (g/dl)	WBC ($10^3/\mu l$)	Platelet ($10^3/\mu l$)
Control (Grp 1)		42.86 ± 0.91$^{(b, c)}$	7.09 ± 0.27$^{(b, c)}$	12.67 ± 0.41$^{(b, c, d, e)}$	8.2 ± 0.32$^{(b, c, d)}$	46.79 ± 3.18$^{(b)}$
Toxin Control (Grp 2)	**8.5**	25.96 ± 1.46$^{(a, c, d, e)}$	3.09 ± 0.11$^{(a, c, d, e)}$	6.13 ± 0.31$^{(a, c, d, e)}$	1.95 ± 0.28$^{(a, c, d, e)}$	28.46 ± 1.48$^{(a, c, d, e)}$
GOV + Alcohol (Grp 3)	**2**	34.76 ± 1.52$^{(a, b, e)}$	5.71 ± 0.17$^{(a, b)}$	10.87 ± 0.42$^{(a, b)}$	4.74 ± 0.3 3$^{(a, b, e)}$	49.56± 1.3$^{(b)}$
(Grp 4)	**4**	39.7 ± 1.2$^{(b)}$	6.29 ± 0.19$^{(b)}$	10.03 ± 0.25$^{(a, b)}$	5.44 ± 0.51$^{(a, b)}$	43.34 ± 5.95$^{(b)}$
(Grp 5)	**8**	42.21 ± 1.12$^{(b, c)}$	6.29 ± 0.15$^{(b)}$	10.67 ± 0.54$^{(a, b)}$	7.46 ± 0.7$^{(b, c)}$	43.72 ± 1.78$^{(b)}$
LIV 52 + Alcohol (Grp 6)	**0.3**	37.21 ± 2.14$^{(b)}$	6.52 ± 0.6$^{(b)}$	10.33 ± 0.41$^{(a, b)}$	6.57 ± 0.81$^{(b)}$	40.07 ± 2.64
Silymarin + Alcohol (Grp 7)	**0.3**	39.16 ± 1.71$^{(b)}$	6.14 ± 0.22$^{(b)}$	11 ± 0.37$^{(b)}$	6.2 ± 0.27$^{(b)}$	43.75 ± 2.64$^{(b)}$

Values are expressed as Mean ± SEM for seven rats. The Mean difference is significant at the .05 level. (a) = $p \leq 0.05$ as compared with the normal control group. (b) = $p \leq 0.05$ as compared to Alcohol control group. (c) = $p \leq 0.05$ as compared with the GOV + Alcohol ($2\ g\ kg^{-1}$) group. (d) = $p \leq 0.05$ as compared with the GOV + Alcohol ($4\ g\ kg^{-1}$) group. (e) = $p \leq 0.05$ as compared with the GOV + Alcohol ($8\ g\ kg^{-1}$) group. The significance of differences among all groups was determined by the Tukey HSD test.

PCV =Packed Cell Volume, Hb = Haemoglobin, RBC = Red Cell Count, WBC = White Blood Cell.

Table 11b: Effect of pretreatment with GOV on the hematologic indices in rats with alcohol induced hepatotoxicity.

Group	Dose ($g\ kg^{-1}$)	MCHC (%)	MCV (fl)	MCH (pg)	Granulocytes (%)	Lymphocyte (%)	Monocyte (%)
Control (Grp 1)		29.71 ± 1.22	60.91 ± 2.03$^{(b)}$	18.1 ± 0.99	10.01 ± 0.84	83.61 ± 1.28$^{(b)}$	6.37 ± 0.8$^{(b)}$
Toxin Control (Grp 2)	**8.5**	24.34 ± 2.28$^{(c)}$	83.97 ± 3.89$^{(a, c, d, e)}$	19.97 ± 1.25	5.67 ± 0.26$^{(c)}$	65.01 ± 2.11$^{(a, c, d, e)}$	29.31 ± 2.05$^{(a, c, d, e)}$
GOV + Alcohol (Grp 3)	**2**	31.33 ± 0.44$^{(b)}$	60.9 ± 2.04$^{(b)}$	19.04 ± 0.51	11.36 ± 1.46$^{(b)}$	81.47 ± 2.15$^{(b)}$	7.17 ± 0.82$^{(b)}$
(Grp 4)	**4**	25.39 ± 0.95	63.54 ± 3.14$^{(b)}$	15.96 ± 0.77	8.24 ± 0.59	86.66 ± 0.85$^{(b)}$	5.1 ± 0.5$^{(b)}$
(Grp 5)	**8**	25.45 ± 1.64	67.39 ± 2.47$^{(b)}$	17.05 ± 1.01	9.86 ± 0.67	84.36 ± 0.78$^{(b)}$	5.79 ± 0.34$^{(b)}$
LIV 52 + Alcohol (Grp 6)	**0.3**	28.18 ± 1.53	58.53 ± 3.17$^{(b)}$	16.73 ± 1.72	11.9 ± 1.14$^{(b)}$	81.43 ± 1.69$^{(b)}$	6.67 ± 0.69$^{(b)}$
Silymarin + Alcohol (Grp 7)	**0.3**	28.3 ± 1.06	64.52 ± 4.16$^{(b)}$	18.09 ± 0.97	10.64 ± 1.39$^{(b)}$	84.41 ± 1.74$^{(b)}$	4.94 ± 0.62$^{(b)}$

Values are expressed as Mean ± SEM for seven rats. The Mean difference is significant at the 0.05 level. (a) = $p \leq 0.05$ as compared with the normal control group. (b) = $p \leq 0.05$ as compared to Alcohol control group. (c) = $p \leq 0.05$ as compared with the GOV + Alcohol ($2g\ kg^{-1}$) group. (d) = $p \leq 0.05$ as compared with the GOV + Alcohol ($4g\ kg^{-1}$) group. (e) = $p \leq 0.05$ as compared with the GOV + Alcohol ($8g\ kg^{-1}$) group. The significance of differences among all groups was determined by the Tukey HSD test.

MCV = Mean Cell Volume, MCH = Mean Cell Haemoglobin, MCHC = Mean Cell Haemoglobin Concentration,

4.9.2. Effect of GOV on Serum Hepatic Enzymes and Chemical Analytes on Alcohol intoxicated rats

The effects of the triherbal formulation on the serum hepatic enzymes and chemical analytes on alcohol induced toxicity in all the groups are given in tables 12a and 12b. There was an increase in the levels of serum ALP, AST, GGT, ALT, and LDH in the toxin control group compared to the normal control group. Pretreatment of rats with GOV, dose dependently decreased the levels of serum hepatic enzymes activities significantly ($p \leq 0.05$) in experimental rats as compared to the toxin control group. In the 8 g kg^{-1} group, ALP activity was lower than that of silymarin and was significantly ($p \leq 0.05$) increased when compared to Liv 52. In 8 g kg^{-1} group, LDH and ALP activities were decreased when compared to the rats that received silymarin, while its ALT activities were lower than that of Liv 52 group. In the 2 g kg^{-1} group, GOV lowered the AST activity compared to Liv 52 and Silymarin. There was a 76 %, 67.39 %, 66.44 %, 52.5 % and 63.67 % increase in ALP, ALT, AST, GGT and LDH activities respectively in group 2 as compared to group 1 with a 36.8 % decrease in total protein level.

At 4 g kg^{-1}, GOV attenuated the concentrations of cholesterol and creatinine more than Liv 52 and silymarin, lowered the triglyceride concentration more than that of Liv 52 and had almost the same BUN concentration as Liv 52. The protein concentration of GOV at 8 g kg^{-1} was almost equal to that of Liv 52 and silymarin. Silymarin significantly ($p \leq 0.05$) increased the albumin concentration compared to GOV at 2 and 4 g kg^{-1}. The levels of serum albumin and total protein concentrations in alcohol induced toxin control group were significantly ($p \leq 0.05$) decreased while increase in cholesterol, creatinine, triglyceride and BUN concentrations were observed compared to the three doses of GOV.

Table 12a: Serum levels of ALT, AST, ALP, LDH and GGT in rats pretreated with GOV before alcohol damage.

Group	Dose ($g\ kg^{-1}$)	LIVER FUNCTION ENZYMES				
		ALP (U/L)	AST (U/L)	ALT (U/L)	GGT (U/L)	LDH (U/L)
Control (Grp 1)		73.93 ± 1.34$^{(b, c, d, e)}$	56.51 ± 2.32$^{(b, c, d, e)}$	29.43 ± 1.29$^{(b)}$	1917.56 ± 137.87$^{(b)}$	12.18 ± 0.91$^{(b)}$
Toxin Control (Grp 2)	**8.5**	308 ± 24.57$^{(a, c, d, e)}$	168.36 ± 5.43$^{(a, c, d, e)}$	90.24 ± 5.37$^{(a, c, d, e)}$	4037.15 ± 171.62$^{(a, c, d, e)}$	33.53 ± 0.29$^{(a, c, d, e)}$
GOV + Alcohol (Grp 3)	**2**	185.97 ± 4.64$^{(a, b)}$	74.17 ± 2.25$^{(a, b)}$	37.67 ± 6.05$^{(b)}$	2217.04 ± 210.17$^{(b)}$	15.02 ± 1.89$^{(b)}$
(Grp 4)	**4**	188.8 ± 2.9$^{(a, b)}$	79.2 ± 1.52$^{(a, b)}$	39.37 ± 2.27$^{(b)}$	2308.59 ± 118.8$^{(b)}$	15.43 ± 0.93$^{(b)}$
(Grp 5)	**8**	153.6 ± 3.43$^{(a, b)}$	76.84 ± 1.86$^{(a, b)}$	35.6 ± 2.55$^{(b)}$	2268.9 ± 19.08$^{(b)}$	13.56 ± 1.29$^{(b)}$
LIV 52 + Alcohol (Grp 6)	**0.3**	143.68 ± 5.67$^{(a, b, d)}$	76.82 ± 4.87$^{(a, b)}$	34.97 ± 2.92$^{(b)}$	2196.03 ± 111.8$^{(b)}$	12.66 ± 1.55$^{(b)}$
Silymarin + Alcohol (Grp 7)	**0.3**	157.25 ± 4.6$^{(a, b)}$	76.26 ± 3.17$^{(a, b)}$	32.9 ± 1.56$^{(b)}$	2127.78 ± 125.21$^{(b)}$	13.84 ± 0.71$^{(b)}$

Values are expressed as Mean ± SEM for seven rats. The Mean difference is significant at the .05 level. (a) = $p \leq 0.05$ as compared with the normal control group. (b) = $p \leq 0.05$ as compared to D-GaIN control group. (c) = $p \leq 0.05$ as compared with the GOV + D-GaIN (2 g kg^{-1}) group. (d) = $p \leq 0.05$ as compared with the GOV + D-GaIN (4 g kg^{-1}) group. (e) = $p \leq 0.05$ as compared with the GOV + D-GaIN (8 g kg^{-1}) group. The significance of differences among all groups was determined by the Tukey HSD test.

ALP = Alkaline phosphatase, ALT = Alanine aminotransferase, AST = aspartate aminotransferase, GGT = L-γ-glutamyltransferase and LDH = Lactate dehydrogenase

Table 12b: Serum levels of albumin, cholesterol, creatinine, total protein, triglyceride and BUN in rats pretreated with GOV before alcohol damage.

Group	Dose ($g\ kg^{-1}$)	BIOCHEMICAL PARAMETERS					
		TP (g/L)	ALB (g/L)	BUN (mmol/L)	CREA (mmol/L)	TG (mmol/L)	CHO (mmol/L)
Control (Grp 1)		86.07 ± 2.76$^{(b)}$	47.05 ± 1.76$^{(b, c, d)}$	5.02 ± 0.34$^{(b)}$	61.11 ± 3.61$^{(b, c)}$	1 ± 0.03$^{(b)}$	1.78 ± 0.11$^{(b, c, e)}$
Toxin Control (Grp 2)	**8.5**	54.39 ± 2.64$^{(a, c, d, e)}$	25.28± 1.15$^{(a, c, d, e)}$	10.53 ± 1.45$^{(a, c, d, e)}$	93.07 ± 4.38$^{(a, c, d, e)}$	3.28 ± 0.16$^{(a, c, d, e)}$	4 ± 0.18$^{(a, c, d, e)}$
GOV + Alcohol (Grp 3)	**2**	77.42 ± 2.69$^{(b)}$	37.82 ± 0.85$^{(a, b)}$	5.87 ± 0.34$^{(b)}$	76.51 ± 0.78$^{(a, b)}$	1.37 ± 0.16$^{(b)}$	2.7 ± 0.12$^{(a, b)}$
(Grp 4)	**4**	78.34 ± 3.83$^{(b)}$	37.45 ± 0.8$^{(a, b)}$	5.58 ± 0.32$^{(b)}$	63.44 ± 2.57$^{(b)}$	1.24 ± 0.18$^{(b)}$	2.19 ± 0.16$^{(b)}$
(Grp 5)	**8**	80.3 ± 2.2$^{(b)}$	43.29 ± 1.81$^{(b)}$	6.19 ± 0.48$^{(b)}$	72.77 ± 2.38$^{(b)}$	1.45 ± 0.16$^{(b)}$	2.51 ± 0.12$^{(a, b)}$
LIV 52 + Alcohol (Grp 6)	**0.3**	81.19 ± 2.99$^{(b)}$	42.31 ± 1.52$^{(b)}$	5.5 ± 0.25$^{(b)}$	69.01 ± 2.65$^{(b)}$	1.3 ± 0.16$^{(b)}$	2.28 ± 0.14$^{(b)}$
Silymarin + Alcohol (Grp 7)	**0.3**	81.41 ± 1.12$^{(b)}$	43.95 ± 1.28$^{(b, c, d)}$	5.15 ±.35$^{(b)}$	64.89 ± 3.31$^{(b)}$	1.12 ± 0.06$^{(b)}$	2.24 ± 0.05$^{(b)}$

Values are expressed as Mean ± SEM for seven rats. The Mean difference is significant at the .05 level. (a) = $p \leq 0.05$ as compared with the normal control group. (b) = $p \leq 0.05$ as compared to Alcohol control group. (c) = $p \leq 0.05$ as compared with the GOV + Alcohol (2 g kg^{-1}) group. (d) = $p \leq 0.05$ as compared with the GOV + Alcohol (4 g kg^{-1}) group. (e) = $p \leq 0.05$ as compared with the GOV + Alcohol (8 g kg^{-1}) group. The significance of differences among all groups was determined by the Tukey HSD test.

TP = Total Protein, ALB = Albumin, BUN = Blood Urea Nitrogen, CREA = Creatinine, TG = Triglyceride, CHO = Cholesterol

4.9.3. The Effects of Alcohol on Antioxidant Defence Enzymes of Albino Rats

The activity of antioxidant defence enzymes such as CAT, GPx, GSH, GST and SOD were significantly ($p \leq 0.05$) decreased in the serum, liver and kidney tissues of animals in group 3 -5 as compared to that of animals in group 2 respectively in a dose dependent manner as shown in tables 13, 14 and 15.

Table 13 shows that there was significant ($p \leq 0.05$) difference in serum, CAT, GPx, GSH, GST, SOD and total protein levels after ethyl alcohol administration to rats in group 2 compared to group 1 rats. This was significantly ($p \leq 0.05$) reversed by administration of GOV in a dose dependent manner. The SOD value of GOV at 2 g kg^{-1} was elevated more than that of Liv 52 and silymarin and almost equal to that of the control group. At 4 g kg^{-1}, the MDA level of GOV was lower than that of silymarin. At 8 g kg^{-1}, GOV augmented the levels of GPx, GSH, and GST more than Liv 52, the CAT and GST levels more than silymarin and had almost the same GPx and GSH values as silymarin.

Table 13: The effect of alcohol damage on serum antioxidant enzymes in rats pretreated with GOV

Group	Dose ($g\ kg^{-1}$)	CAT (µmol/min/mg protein)	GPx (µmol/ml)	GSH (µmol/ml)	GST (µmol/ml)	MDA (nmol/ml)	SOD (µmol/ml)	Total protein (g/L)
Control (Grp 1)		11.55 ±.48$^{(b, c, d, e)}$	0.65 ±.03$^{(b, c, d, e)}$	0.21 ±.01$^{(b, d)}$	1139.07 ± 56.84 (b, c, d, e))	4.66 ± 0.25$^{(b, c, d, e)}$	69.32 ± 2.56$^{(b)}$	86.07 ± 2.76$^{(b)}$
Toxin Control (Grp 2)	**8.5**	5.7 ±.24$^{(a, c, d, e)}$	0.19±.02$^{(a, c, d, e)}$	0.1 ± 0.01$^{(a, c, e)}$	454.99 ± 10.47$^{(a, c, d, e)}$	14.4 ± 0.42$^{(a, c, d, e)}$	31.65 ± 2.72 $^{(a, c, d, e)}$	54.39 ± 2.64$^{(a, c, d, e)}$
GOV + Alcohol (Grp 3)	**2**	9.02 ± 0.22$^{(a, b)}$	0.48 ±.01 (a, b)	0.15 ± 0.01 $^{(b)}$	810.76 ± 24. 1$^{(a, b)}$	8.14 ± 0.26$^{(a, b)}$	62.13 ± 1.12$^{(b)}$	77.42 ± 2.69$^{(b)}$
(Grp 4)	**4**	9.43 ±.42$^{(a, b)}$	0.5 ±.01$^{(a, b)}$	0.15 ± 0.01$^{(a)}$	850.06 ± 28.18 $^{(a, b)}$	7.5 ± 0.3$^{(a, b)}$	58.15 ± 2.66$^{(b)}$	78.34 ± 3.83$^{(b)}$
(Grp 5)	**8**	9.68 ±.53$^{(a, b)}$	0.52 ± 0.01$^{(a, b)}$	0.17 ± 0.01$^{(b)}$	899.55 ± 9.87$^{(a, b)}$	7.71 ± 0.32$^{(a, b)}$	59.56 ± 3.92$^{(b)}$	80.3 ± 2.2$^{(b)}$
LIV 52 + Alcohol (Grp 6)	**0.3**	9.73 ±.64 $^{(b)}$	0.51 ± 0.01$^{(a, b)}$	0.16 ±.01 (b)	877.02 ± 40.47$^{(a, b)}$	7.38 ± 0.37$^{(a, b)}$	58.05 ± 2.53 $^{(b)}$	81.19 ± 2.99$^{(b)}$
Silymarin + Alcohol (Grp 7)	**0.3**	9.58 ±.27$^{(a, b)}$	0.54 ± 0.03$^{(a, b)}$	0.19 ±.03$^{(b)}$	860.18 ± 57.79$^{(a, b)}$	7.56 ± 0.35$^{(a, b)}$	58.27 ± 2.27$^{(b)}$	81.41 ± 1.12$^{(b)}$

Values are expressed as Mean ± SEM for seven rats. The Mean difference is significant at the .05 level. (a) = $p \leq 0.05$ as compared with the normal control group. (b) = $p \leq 0.05$ as compared to Alcohol control group. (c) = $p \leq 0.05$ as compared with the GOV + Alcohol (2 g kg^{-1}) group. (d) = $p \leq 0.05$ as compared with the GOV + Alcohol (4 g kg^{-1}) group. (e) = $p \leq 0.05$ as compared with the GOV + Alcohol (8 g kg^{-1}) group. The significance of differences among all groups was determined by the Tukey HSD test.

CAT = Catalase, Gpx = Glutathione Peroxidase, GSH = Reduced Glutathione, GST = Glutathione-S-Transferase, MDA = Malondialdehyde, SOD = Superoxide Dismutase

In Table 14, the levels of CAT, GPx, GSH, GST, SOD and total protein in the liver were markedly significantly ($p \leq 0.05$) reduced and that of MDA levels were significantly ($p \leq 0.05$) increased in the ethyl alcohol treated toxin control group. The thiobarbituric acid reaction showed a significant ($p \leq 0.05$) increase in MDA of the ethyl alcohol treated animals in both hepatic tissues and serum. Treatment with GOV at 2, 4 and 8 g kg^{-1}) significantly ($p \leq 0.05$) prevented the increase in MDA level which was almost brought to near normal in both hepatic tissues. There was a sign GOV dose dependently resulted in a significant ($p \leq 0.05$) increase of CAT, GSH, GPx, GST, SOD and total protein when compared to the toxin control group hepatic tissues. At 2 g kg^{-1} and 4 g kg^{-1}, GOV increased the SOD and CAT level more than Liv 52 respectively. The GSH, GST and total protein levels of Liv 52 group was low compared to that of GOV at 8 g kg^{-1} while the MDA level was higher.

Table 14: The effect of alcohol damage on liver antioxidant enzymes in rats pretreated with GOV.

Group	Dose (g kg^{-1})	CAT (µmol/min/mg protein)	GPx (µmol/ml)	GSH (µmol/ml)	GST (µmol/ml)	MDA (nmol/ml)	SOD (µmol/ml)	Total protein (g/L)
Control (Grp 1)		206.68 ± 5.56$^{(b, c)}$	8.82 ± 0.24$^{(b, c, d, e)}$	26.64 ± 1.13$^{(b, c)}$	424.58 ± 17.46$^{(b, c, d, e)}$	10.77 ± 0.56$^{(b)}$	115.81 ± 10.46$^{(b, c, d, e)}$	84.2 ± 2.24$^{(b, c, d)}$
Toxin Control (Grp 2)	**8.5**	55.93 ± 2.83$^{(a, c, d, e)}$	3.94 ± 0.18$^{(a, d, e)}$	10.76 ± 0.8$^{(a, c, d, e)}$	90.2 ± 5.96$^{(a, c, d, e)}$	35.65 ± 2.76$^{(a, c, d, e)}$	42.7 ± 2.49$^{(a, c, d, e)}$	53.97 ± 1.94$^{(a, d, e)}$
GOV + Alcohol (Grp 3)	**2**	157.16 ± 11.48$^{(a, b)}$	4.43 ± 0.1$^{(a, e)}$	18.75 ± 1.34$^{(a, b)}$	265.38 ± 12.24$^{(a, b)}$	14.46 ± 0.46$^{(b)}$	85.49 ± 4.18$^{(a, b)}$	63.13 ± 1.72$^{(a, e)}$
(Grp 4)	**4**	191.27 ± 4.49$^{(b)}$	5.16 ± 0.43 $^{(a, b, e)}$	21.07 ± 1.36$^{(b)}$	214.23 ± 4.24$^{(a, b, e)}$	15.02 ± 0.89$^{(b)}$	71.45 ± 4.2$^{(a, b)}$	67.05 ± 2.23$^{(a, b, e)}$
(Grp 5)	**8**	176.63 ± 10.2$^{(b)}$	6.66 ± 0.28$^{(a, b, c, d)}$	24.22 ± 2.57$^{(b)}$	292.14 ± 34.04$^{(a, b, d)}$	12.66 ± 1.29$^{(b)}$	82.52 ± 6.49$^{(a, b)}$	77.87 ± 3.32$^{(b, c, d)}$
LIV 52 + Alcohol (Grp 6)	**0.3**	185.08 ± 5.38$^{(b)}$	6.72 ± 0.13$^{(a, b, c, d)}$	22.19 ± 1.44$^{(b)}$	275.33 ± 4.54$^{(a, b)}$	12.95 ± 0.61$^{(b)}$	85.37 ± 3.91$^{(a, b)}$	76.9 ± 1.85$^{(b, c, d)}$
Silymarin + Alcohol (Grp 7)	**0.3**	192.71 ± 12.32$^{(b)}$	7.03 ± 0.4 $^{(a, b, c, d)}$	21.01 ± 1.29$^{(b)}$	293.93 ± 9.31$^{(a, b, d)}$	12.31 ± 0.44$^{(b)}$	93.14 ± 4.15$^{(b)}$	78.27 ± 1.67$^{(b, c, d)}$

Values are expressed as Mean ± SEM for seven rats. The Mean difference is significant at the .05 level. (a) = $p \leq 0.05$ as compared with the normal control group. (b) = $p \leq 0.05$ as compared to Alcohol control group. (c) = $p \leq 0.05$ as compared with the GOV + Alcohol (2g kg^{-}1) group. (d) = $p \leq 0.05$ as compared with the GOV + Alcohol (4g kg^{-}1) group. (e) = $p \leq 0.05$ as compared with the GOV + Alcohol (8g kg^{-}1) group. The significance of differences among all groups was determined by the Tukey HSD test.

CAT = Catalase, GPx = Glutathione Peroxidase, GSH = Reduced Glutathione, GST = Glutathione-S-Transferase, MDA = Malondialdehyde, SOD = Superoxide Dismutase

Table 15 shows the effect of alcohol induced toxicity on rat kidney. At 2 g kg^{-1}, GOV exhibited higher concentration of total protein compared to Liv 52 while the GST was almost equal to that of Liv 52 and silymarin. The CAT, GPx and SOD levels of GOV at 4 g kg^{-1} were increased while the MDA was lowered compared to Liv 52 and silymarin. When compared to Liv 52, the GSH and GPx activities of GOV at 4 and 8 g kg^{-1} were increased respectively. The CAT activity of GOV at 4 g kg^{-1} was significantly ($p \leq 0.05$) higher than that of 2 and 8 g kg^{-1}. GOV significantly reversed the effects of alcohol on the rats in a dose dependent manner.

Table 15: The effect of alcohol damage on kidney antioxidant enzymes in rats pretreated with GOV

Group	Dose ($g\ kg^{-1}$)	CAT (µmol/min/mg protein)	GPx (µmol/ml)	GSH (µmol/ml)	GST (µmol/ml)	MDA (nmol/ml	SOD (µmol/ml)	Total protein (g/L)
Control (Grp 1)		263.15 ± 5.01$^{(b, c, d, e)}$	3.91 ± 0.2$^{(b, c, d, e)}$	36.53 ± 1.36$^{(b, c, e)}$	481.79 ± 22.81$^{(b, c, d, e)}$	9.57 ± 0.73$^{(b, e)}$	129.53 ± 3.24$^{(b, c, d, e)}$	84.21 ± 3.59$^{(b, e)}$
Toxin Control (Grp 2)	**8.5**	74.09 ± 5.15$^{(a, c, d, e)}$	1.45 ± 0.15$^{(a, c, d, e)}$	13.71 ± 0.22$^{(a, c, d, e)}$	77.76 ± 5.1$^{(a, c, d, e)}$	38.04 ±. 5$^{(a, c, d, e)}$	52.08 ± 3.09$^{(a, c, d, e)}$	47.19 ± 1.87$^{(a, c, d, e)}$
GOV + Alcohol (Grp 3)	**2**	131.68 ± 12$^{(a, b, d)}$	2.71 ± 0.11$^{(a, b)}$	25.29 ± 0.93$^{(a, b)}$	301.27 ± 17.03$^{(a, b)}$	12.98 ± 1.63$^{(b)}$	73.51 ± 3.11$^{(a, b)}$	77.68 ± 3.41$^{(b)}$
(Grp 4)	**4**	193.71 ± 17.73 $^{(a, b, c, e)}$	3.27 ± 0.15$^{(a, b)}$	28.36 ± 1.43$^{(b)}$	292.4 ± 12.24$^{(a, b)}$	12.48 ± 0.57$^{(b)}$	80.57 ± 3.85$^{(a, b)}$	79.72 ± 3.07$^{(b)}$
(Grp 5)	**8**	136.77 ± 5.47$^{(a, b, d)}$	2.97 ± 0.2$^{(a, b)}$	27.15 ± 4.17$^{(a, b)}$	263.64 ± 16.23$^{(a, b)}$	13.22 ± 0.58$^{(a, b)}$	70.51 ± 1.4$^{(a, b)}$	71.49 ± 2.63$^{(a, b)}$
LIV 52 + Alcohol (Grp 6)	**0.3**	163.03 ± 9.03$^{(a, b)}$	2.92 ± 0.14$^{(a, b)}$	28.01 ± 1.41$^{(a, b)}$	309.16 ± 9.96$^{(a, b)}$	12.65 ± 0.63 $^{(b)}$	79.18 ± 1.29$^{(a, b)}$	76.3 ± 2.31$^{(b)}$
Silymarin + Alcohol (Grp 7)	**0.3**	157.46 ± 2.37$^{(a, b)}$	3.1 ± 0.14$^{(a, b)}$	29.59 ± 1.19$^{(b)}$	310.05 ± 13.82$^{(a, b)}$	12.54 ± 0.53$^{(b)}$	77.43 ± 1.77$^{(a, b)}$	79.81 ± 1.42$^{(b)}$

Values are expressed as Mean ± SEM for seven rats. The Mean difference is significant at the .05 level. (a) = $p \leq 0.05$ as compared with the normal control group. (b) = $p \leq 0.05$ as compared to Alcohol control group. (c) = $p \leq 0.05$ as compared with the GOV + Alcohol ($2g\ kg^{-1}$) group. (d) = $p \leq 0.05$ as compared with the GOV + Alcohol ($4g\ kg^{-1}$) group. (e) = $p \leq 0.05$ as compared with the GOV + Alcohol ($8g\ kg^{-1}$) group. The significance of differences among all groups was determined by the Tukey HSD test.

CAT = Catalase, Gpx = Glutathione Peroxidase, GSH = Reduced Glutathione, GST = Glutathione-S-Transferase, MDA = Malondialdehyde, SOD = Superoxide Dismutase

4.9.4. Effect of Alcohol on Caspase Activities

Table 16 shows the fold increase in caspase 2, 3 and 9 activities in the white blood cell of rats pretreated with GOV before ethanol damage. At 2 g kg^{-1}, GOV significantly ($p \leq 0.05$) increased the caspase 9 activities compared to control, GOV (4 and 8 g kg^{-1}), Liv 52 and silymarin groups though there was a significant ($p \leq 0.05$) decrease compared to the toxin control group.

Table 16: Caspase - 2, 3 and 9 activities in the white blood cell of rats treated with ethanol.

Group	Dose (g kg^{-1})	Caspase 2 (Units/mg of total protein).	Caspase 3 (Units/mg of total protein)	Caspase 9 (Units/mg of total protein)
Control (Grp 1)		0.5 ± 0.06$^{(b, c, d, e)}$	0.95 ±.03$^{(b, c, d, e)}$	1.20 ± .09$^{(b, c, d, e)}$
Toxin Control (Grp 2)	**8.5**	2.96 ± 0.31$^{(a, c, d, e)}$	5.85 ± 0.12$^{(a, c, d, e)}$	7.3 ± 0.45$^{(a, c, d, e)}$
		(4.92)	(5.16)	(5.08)
GOV + Alcohol (Grp 3)	**2**	0.91 ± 0.05$^{(a, b)}$	1.42 ± 0.03$^{(a, b)}$	2.05 ± 0.02$^{(a, b, d, e)}$
		(0.81)	(0.5)	(0.71)
(Grp 4)	**4**	0.98 ± 0.12$^{(a, b)}$	1.39 ± 0.03$^{(a, b)}$	1.69 ± 0.03$^{(a, b, c)}$
		(0.95)	(0.46)	(0.41)
(Grp 5)	**8**	0.88 ± 0.04$^{(a, b)}$	1.34 ± 0.04$^{(a, b)}$	1.56 ± 0.1$^{(a, b, c)}$
		(0.76)	(0.41)	(0.3)
LIV 52 + Alcohol (Grp 6)	**0.3**	0.942 ± 0.05$^{(a, b)}$	1.38 ± 0.04$^{(a, b)}$	1.57 ± 0.07$^{(a, b, c)}$
		(0.84)	(0.45)	(0.31)
Silymarin + Alcohol (Grp 7)	**0.3**	0.82± 0.09$^{(b)}$	1.37 ± 0.03$^{(a, b)}$	1.48 ± 0.02$^{(b, c)}$
		(0.65)	(0.45)	(0.23)

Values are expressed as Mean ± SEM for seven rats. The Mean difference is significant at the .05 level. (a) = $p \leq 0.05$ as compared with the normal control group. (b) = $p \leq 0.05$ as compared to Alcohol control group. (c) = $p \leq 0.05$ as compared with the GOV + Alcohol (2g kg^{-}1) group. (d) = $p \leq 0.05$ as compared with the GOV + Alcohol (4g kg^{-}1) group. (e) = $p \leq 0.05$ as compared with the GOV + Alcohol (8g kg^{-}1) group. The significance of differences among all groups was determined by the Tukey HSD test. The figures in parenthesis = fold-increase in caspase activity compared to the uninduced control.

4.9.5. Histopathology of Liver of Rats Intoxicated with Alcohol

The photomicrograph of the liver of animals in the control group showed normal histology. Pretreatment with GOV at 8 g kg^{-1} before administration of alcohol showed almost normal liver architecture while at 4 g kg^{-1}, it showed almost normal liver with unremarkable central vein and hepatic lobule, with a focus of inflammatory infiltrate but no necrosis. The toxin control group showed loss of architecture, fibrosis, micro- and macrovesicular steatosis and fatty infiltration with extensive necrosis.

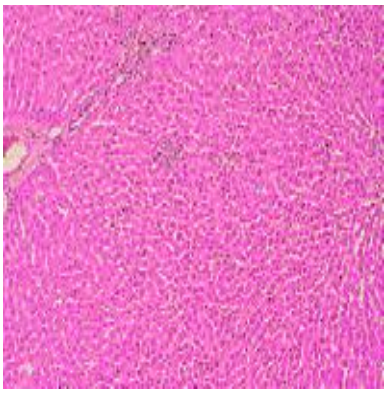


Plate 3 : Control Normal liver (X40)

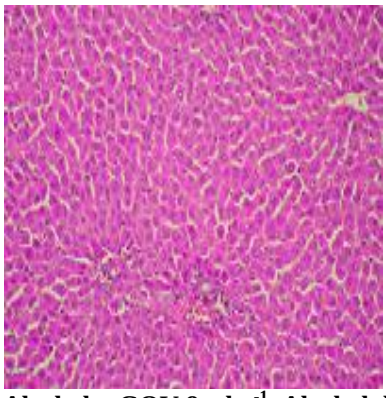


Plate 4: Alcohol + GOV 8 g kg^{-1} Alcohol damaged liver (X40)

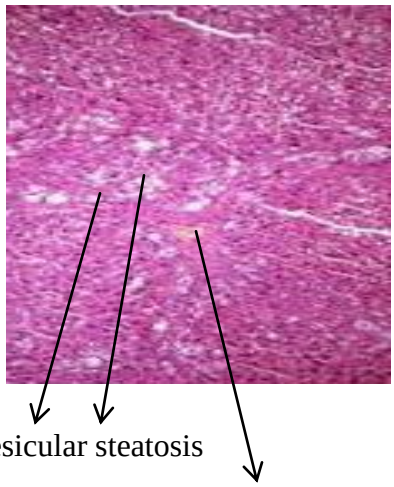


Plate 5: Toxin control Alcohol damaged liver (X40)

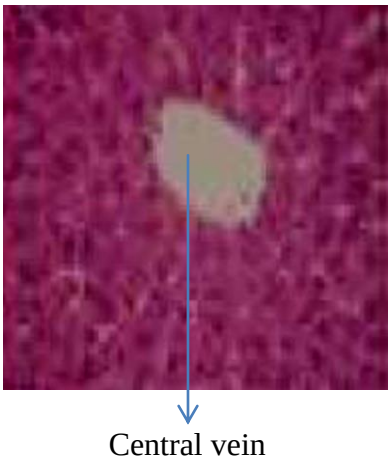


Plate 6: Alcohol + GOV 4 g kg^{-1} Alcohol damaged liver (X100)

Histopathology of Liver of Rats Intoxicated with Alcohol

4.10. D- galactosamine (D-GaIN) Induced Hepatotoxicity

Administration D-GalN on normal rats induced liver injury, as indicated by an increase of serum liver marker enzymes, decrease of albumin and total serum, liver and kidney proteins, decreased serum, liver and kidney antioxidant activities, increased serum and tissue lipid peroxidation, increase in caspase activities and distorted liver and kidney histopathology

4.10.1. Effect of GOV on the Haematologic Indices on D-GaIN Induced Hepatotoxicity on Rats.

Tables 17a and 17b shows the effect of GOV on the hematologic indices of rats with D-GaIN induced hepatotoxicity. There was a significantly ($p \leq 0.05$) increase in PCV, RBC, Hb, WBC, platelet count, MCHC, granulocytes and lymphocytes while the MCV and monocytes significantly ($p \leq 0.05$) depreciated in a dose dependent fashion compared to the toxin control group.

At 2 g kg^{-1}, GOV did not demonstrate any significant ($p \leq 0.05$) difference in PCV, MCHC, MCH and granulocyte compared to the toxin control group. The administration of GOV at 4 g kg^{-1} and 8 g kg^{-1} increased the MCHC and MCH values and RBC and WBC values compared to all the groups respectively. Rats that received GOV at a dose of 4 g kg^{-1} exhibited increments in PCV, RBC, Hb, WBC and lymphocyte but the platelet numbers, MCV, granulocyte and monocytes depreciated compared to Liv 52 group. Compared to rats in the silymarin group, the rats in GOV (8 g kg^{-1}) group manifested an increment in PCV, RBC, WBC, MCV and monocytes while the Hb, platelet count, MCHC, MCH, granulocytes and lymphocytes were decreased. Groups 5 and 7 demonstrated significant ($p \leq 0.05$) increase in RBC compared to group 3.

Table 17a: Effect of pretreatment with GOV on the hematologic indices in rats with D-GaIN induced hepatotoxicity.

Group	Dose ($g\ kg^{-1}$)	PCV (%)	RBC ($10^6/\mu l$)	Hb (g/dl)	WBC ($10^3/\mu l$)	Platelet ($10^3/\mu l$)
Control (Grp 1)		43.77 ± 2.27[(b)]	6.36 ± 0.16[(b, c)]	11.86 ± 0.67[(b)]	12.84 ± 0.44[(b)]	44.99 ± 4.45[(b)]
Toxin Control (Grp 2)	**0.5**	31.29 ± 0.94[(a, d, e)]	3.84 ± 0.2[(a, c, d, e)]	6.31 ± 0.66[(a, c, d, e)]	2.28 ± 0.35[(a, c, d, e)]	21.3 ± 0.82[(a, c, d, e)]
GOV + D-GaIN (Grp 3)	**2**	37.07 ± 1.9	5.38 ± 0.3[(a, b, e)]	9.68 ± 0.48[(b)]	12.39 ± 0.47[(b)]	40.73 ± 4[(b)]
(Grp 4)	**4**	41.17 ± 1[(b)]	5.9 ± 0.07[(b)]	11.9 ± 0.32[(b)]	13.51 ± 0.18[(b)]	41.86 ± 3.33[(b)]
(Grp 5)	**8**	43.1 ± 1.05[(b)]	6.42 ± 0.17[(b, c)]	11.73 ± 0.52[(b)]	13.56 ± 0.99[(b)]	41.14 ± 4.41[(b)]
LIV 52 + D-GaIN (Grp 6)	**0.3**	40.39 ± 1.03[(b)]	5.88 ± 0.2[(b)]	11.47 ± 0.41[(b)]	11.8 ± 0.11[(b)]	49.43 ± 5.52[(b)]
Silymarin + D-GaIN (Grp 7)	**0.3**	42.6 ± 2.11[(b)]	6.36 ± 0.25[(b, c)]	12.14 ± 0.56[(b, c)]	12.48 ± 0.43[(b)]	48.7 ± 6[(b)]

Values are expressed as Mean ± SEM for seven rats. The Mean difference is significant at the .05 level. (a) = $p \leq 0.05$ as compared with the normal control group. (b) = $p \leq 0.05$ as compared to D-GaIN control group. (c) = $p \leq 0.05$ as compared with the GOV + D-GaIN (2 g kg^{-1}) group. (d) = $p \leq 0.05$ as compared with the GOV + D-GaIN (4 g kg^{-1}) group. (e) = $p \leq 0.05$ as compared with the GOV + D-GaIN (8 g kg^{-1}) group. The significance of differences among all groups was determined by the Tukey HSD test.

PCV =Packed Cell Volume, Hb = Haemoglobin, RBC = Red Cell Count, WBC = White Blood Cell.

Table 17b: Effect of pretreatment with GOV on the hematologic indices in rats with D-GaIN induced hepatotoxicity.

Groups	Dose ($g\ kg^{-1}$)	MCHC (%)	MCV (fl)	MCH (pg)	Granulocytes (%)	Lymphocyte (%)	Monocyte (%)
Control (Grp 1)		25.61 ± 2.05	68.72 ± 2.65[(b)]	18.6 ± 0.8	8.4 ± 0.6	86.01 ± 1.18[(b)]	5.59 ± 0.83[(b)]
Toxin Control (Grp 2)	**0.5**	20.25 ± 2.12[(d)]	82.48 ± 4.03[(a, c, d, e)]	16.59 ± 1.82	4.63 ± 0.5[(d)]	66.33 ± 1.92[(a, c, d, e)]	29.04 ± 2.07[(a, c, d, e)]
GOV + D-GaIN (Grp 3)	**2**	26.22 ± 0. 8	69.37 ± 3.07[(b)]	18.23 ± 1.06	9.04 ± 0.8	84.8 ± 1.47[(b)]	6.16 ± 1.05[(b)]
(Grp 4)	**4**	28.98 ± 0.76[(b)]	69.92 ± 2.06[(b)]	20.23 ± 0.68	10.53 ± 1.43[(b)]	83.91 ± 2.14[(b)]	5.41 ± 0.98[(b)]
(Grp 5)	**8**	23.71 ± 2.84	67.63 ± 2.68[(b)]	18.32 ± 0.82	9.64 ± 1.13	84.79 ± 1.62[(b)]	5.57 ± 0.73[(b)]
LIV 52 + D-GaIN (Grp 6)	**0.3**	28.43 ± 0.7[(b)]	69.08 ± 2.87[(b)]	19.58 ± 0.67	10.87 ± 0.89[(b)]	83.34 ± 0.84[(b)]	5.79 ± 0.57[(b)]
Silymarin + D-GaIN (Grp 7)	**0.3**	28.65 ± 0.58[(b)]	66.9 ± 2.17[(b)]	19.1 ± 0.37	10.43 ± 2.1[(b)]	85.66 ± 2.54[(b)]	4.49 ± 1[(b)]

Values are expressed as Mean ± SEM for seven rats. The Mean difference is significant at the .05 level. (a) = $p \leq 0.05$ as compared with the normal control group. (b) = $p \leq 0.05$ as compared to D-GaIN control group. (c) = $p \leq 0.05$ as compared with the GOV + D-GaIN (2 g kg^{-1}) group. (d) = $p \leq 0.05$ as compared with the GOV + D-GaIN (4 g kg^{-1}) group. (e) = $p \leq 0.05$ as compared with the GOV + D-GaIN (8 g kg^{-1}) group. The significance of differences among all groups was determined by the Tukey HSD test.

MCV = Mean Cell Volume, MCH = Mean Cell Haemoglobin, MCHC = Mean Cell Haemoglobin Concentration,

4.10.2. Effect of GOV on Serum Hepatic Enzymes and Chemical Analytes on D-GaIN intoxicated rats

Tables 18a and 18b shows serum marker enzyme activities and biochemical analytes in rats fed GOV before administration of D-GaIN. The increases in these enzyme activities were significantly ($p \leq 0.05$) declined at all doses of GOV tested, although the magnitude of the effect varied. At 2 g kg^{-1}, GOV attenuated the ALP activity compared to all the other experimental groups, had an ALT value lower than Liv 52 and almost equal to the control group and exhibited almost the same level of LDH activity compared to Liv 52 and silymarin.

Pretreatment with GOV significantly ($p \leq 0.05$) attenuated the increased cholesterol, creatinine, triglyceride and BUN concentrations and elevated the reduced albumin and total protein concentrations induced by D-GaIN. At 2 g kg^{-1}, GOV increased albumin and total protein concentrations compared to 2 and 8 g kg^{-1} while the cholesterol concentration is low compared to Liv 52. In the 4 g kg^{-1} group, GOV reduced creatinine more than Liv 52 and silymarin, exhibited a lower triglyceride activity than Liv 52 but it is the same as silymarin. At 4 g kg^{-1}, BUN was reduced compared to 2 g kg^{-1} and 8 g kg^{-1}. At 2 and 8 g kg^{-1}, GOV has the same cholesterol value as Liv 52.

Table 18a: Serum activities of ALP, ALT, AST, GGT and LDH in rats pretreated with GOV before D-GaIN damage.

Group	Dose (g kg^{-1})	LIVER FUNCTION ENZYMES				
		ALP (U/L)	ALT (U/L)	AST (U/L)	GGT (U/L)	LDH (U/L)
Control (Grp 1)		239.59 ± 4.84$^{(b)}$	14.06 ± 0.34$^{(b)}$	15.15 ± 0.73$^{(b)}$	1151.96 ± 62.01$^{(b, e)}$	14.04 ± 1.37$^{(b, e)}$
Toxin Control (Grp 2)	0.5	474.19 ± 45.63$^{(a, c, d, e)}$	68.18 ± 6.72$^{(a, c, d, e)}$	75.75 ± 7.51$^{(a, c, d, e)}$	3722.26 ± 80.33$^{(a, c, d, e)}$	67.99 ± 1.89$^{(a, c, d, e)}$
GOV + D-GaIN (Grp 3)	2	212.45 ± 18.62$^{(b)}$	14.49 ± 0.53$^{(b)}$	16.79 ± 2.6$^{(b)}$	1317.06 ± 72.5$^{(b)}$	15.78 ± 0.94$^{(b)}$
(Grp 4)	4	254.75 ± 10.76$^{(b)}$	16.45 ± 0.87$^{(b)}$	19.79 ± 0.9$^{(b)}$	1465.86 ± 29.33$^{(b)}$	17.59 ± 0.78$^{(b)}$
(Grp 5)	8	267.07 ± 8.47$^{(b)}$	17.83 ± 0.58$^{(b)}$	18.9 ± 0.72$^{(b)}$	1498.38 ± 131.08$^{(a, b)}$	20.47 ± 1.62$^{(a, b)}$
LIV 52 + D-GaIN (Grp 6)	0.3	241.23 ± 8.23$^{(b)}$	15.68± 0.63$^{(b)}$	15.61± 0.73$^{(b)}$	1285.52± 82.92	15.54± 0.95
Silymarin + D-GaIN (Grp 7)	0.3	240.03 ± 15.31$^{(b)}$	13.13 ± 0.38$^{(b)}$	15.02 ± 0.71$^{(b)}$	1184.02 ± 56.78$^{(b)}$	15.58 ± 1.51$^{(b)}$

Values are expressed as Mean ± SEM for seven rats. The Mean difference is significant at the .05 level. (a) = p ≤ 0.05 as compared with the normal control group. (b) = p ≤ 0.05 as compared to D-GaIN control group. (c) = p ≤ 0.05 as compared with the GOV + D-GaIN (2 g kg^{-}1) group. (d) = p ≤ 0.05 as compared with the GOV + D-GaIN (4 g kg^{-}1) group. (e) = p ≤ 0.05 as compared with the GOV + D-GaIN (8 g kg^{-}1) group. The significance of differences among all groups was determined by the Tukey HSD test.

ALP = Alkaline phosphatase, ALT = Alanine aminotransferase, AST = Aspartate aminotransferase, GGT = L-γ-glutamyltransferase and LDH = Lactate dehydrogenase

Table 18b: Serum levels of albumin, cholesterol, creatinine, total protein, triglyceride and BUN in rats pretreated with GOV before D-GaIN damage.

Group	Dose (g kg^{-1})	BIOCHEMICAL PARAMETERS					
		TP (g/L)	ALB (g/L)	BUN (mmol/L)	CREA (mmol/L)	TG (mmol/L)	CHO (mmol/L)
Control (Grp 1)		91.33 ± 3.61$^{(b, d, e)}$	50.05 ± 3.45$^{(b, c, d, e)}$	7.08 ± 0.24$^{(b, e)}$	58.2 ± 3.3$^{(b)}$	0.99 ± 0.03$^{(b)}$	1.21 ± 0.08$^{(b)}$
Toxin Control (Grp 2)	**0.5**	50.34 ± 2.49$^{(a, c, d, e)}$	22.3 ± 0.97$^{(a, c)}$	14.89 ± 0.75$^{(a, c, d, e)}$	100.12 ± 1.68$^{(a, c, d, e)}$	3.01 ± 0.11$^{(a, c, d, e)}$	2.81± 0.29$^{(a, c, d, e)}$
GOV + D-GaIN (Grp 3)	**2**	78.6 ± 4.31$^{(b)}$	37.06 ± 0.76$^{(a, b)}$	9.22 ± 0.69$^{(b)}$	64.82 ± 4.89 $^{(b)}$	1.13 ± 0.07$^{(b)}$	1.74 ± 0.15$^{(b)}$
(Grp 4)	**4**	76.25 ± 2.49$^{(a, b)}$	34.15 ± 0.85$^{(a)}$	8.47± 0.55$^{(b)}$	60.92 ± 4.25$^{(b)}$	1.08 ± 0.02$^{(b)}$	1.8 ± 0.11$^{(b)}$
(Grp 5)	**8**	74.05 ± 3.34$^{(a, b)}$	32.68 ± 1.5$^{(a)}$	10.61± 1.33 $^{(a, b)}$	66.8 ± 2.65$^{(b)}$	1.13 ± 0.13$^{(b)}$	1.75 ± 0.18$^{(b)}$
LIV 52 + D-GaIN (Grp 6)	**0.3**	79.11 ± 2.58$^{(b)}$	40.62 ± 5.76$^{(b)}$	7.68 ± 0.29$^{(b)}$	63.79 ± 2.34$^{(b)}$	1.11± 0.06$^{(b)}$	1.75 ± 0.12$^{(b)}$
Silymarin + D-GaIN (Grp 7)	**0.3**	80.73 ± 2.7$^{(b)}$	40.29 ± 1.51$^{(b)}$	7.57 ± 0.34$^{(b, e)}$	63.1± 4.63$^{(b)}$	1.08 ± 0.1$^{(b)}$	1.41± 0.16$^{(b)}$

Values are expressed as Mean ± SEM for seven rats. The Mean difference is significant at the .05 level. (a) = $p \leq 0.05$ as compared with the normal control group. (b) = $p \leq 0.05$ as compared to D-GaIN control group. (c) = $p \leq 0.05$ as compared with the GOV + D-GaIN (2 g kg^{-1}) group. (d) = $p \leq 0.05$ as compared with the GOV + D-GaIN (4 g kg^{-1}) group. (e) = $p \leq 0.05$ as compared with the GOV + D-GaIN (8 g kg^{-1}) group. The significance of differences among all groups was determined by the Tukey HSD test.

TP = Total Protein, ALB = Albumin, BUN = Blood Urea Nitrogen, CREA = Creatinine, TG = Triglyceride, CHO = Cholesterol

4.10.3. Effect of D-GaIN on Antioxidant Enzymes

Table 19 shows the effect of D-GaIN damage on serum antioxidant enzymes in rats pretreated with GOV. At 2 g kg^{-1}, GOV increased the CAT, GSH and GST compared to Liv 52 and silymarin while its GPx and SOD were increased compared to Liv 52. There was a significant ($p \leq 0.05$) increase in GSH at a dose of 2 g kg^{-1}compared to the 4 and 8 g kg^{-1} doses. The 2 g kg^{-1} attenuated the lipid peroxidation compared to Liv 52. GOV dose dependently increased the SOD activity compared to Liv 52.

Table 19: The effect of D-GaIN damage on serum antioxidant enzymes in rats pretreated with GOV

Group	Dose ($g\ kg^{-1}$)	CAT (µmol/min/ mg protein)	GPx (µmol/ml)	GSH (µmol/ml)	GST (µmol/ml)	MDA (nmol/ml)	SOD (µmol/ml)	Total protein (g/L)
Control (Grp 1)		92.21 ± 5.16$^{(b, d)}$	6.28 ± 0.1$^{(b, c, d, e)}$	37.57 ± 1.15$^{(b, d, e)}$	80.73 ± 2.33$^{(b, c, d, e)}$	36.65 ± 0.58$^{(b, d, e)}$	74.61 ± 1.63$^{(b, c, d, e)}$	91.33 ± 3.61
Toxin Control (Grp 2)	**0.5**	46.19 ± 1.93$^{(a, c, d, e)}$	2.86 ± 0.1$^{(a, c, d, e)}$	19.66 ± 0.28$^{(a, c, d, e)}$	38.60 ± 0.82$^{(a, c, d, e)}$	81.69 ± 0.27$^{(a, c, d, e)}$	22.43 ± 1.6$^{(a, c, d, e)}$	50.34 ± 2.49$^{(a, c, d, e)}$
GOV + D-GaIN (Grp 3)	**2**	86.40 ± 1.8$^{(b)}$	5.18 ± 0.07$^{(a, b)}$	35.77 ± 1.19$^{(b, d, e)}$	70.10 ± 2.24$^{(a, b)}$	40.96 ± 1.1$^{(b)}$	48.66 ± 2.9$^{(a, b)}$	78.6 ± 4.31$^{(b)}$
(Grp 4)	**4**	79.45 ± 1.7$^{(a, b)}$	5.11 ± 0.08$^{(a, b)}$	31.25 ± 0.5$^{(a, b, c)}$	65.52 ± 1.42$^{(a, b)}$	45.55 ± 0.93$^{(a, b)}$	49.09 ± 3.06$^{(a, b)}$	76.25 ± 2.49$^{(a, b)}$
(Grp 5)	**8**	82.82 ± 1.94$^{(b)}$	4.93 ± 0.11$^{(a, b)}$	29.77 ± 0.53$^{(a, b, c)}$	68.60 ± 0.82$^{(a, b)}$	46.82 ± 0.9$^{(a, b)}$	48.83 ± 3.59$^{(a, b)}$	74.05 ± 3.34$^{(a, b)}$
LIV 52 + D-GaIN (Grp 6)	**0.3**	81.04 ± 2.73$^{(b)}$	5.09 ±0.05$^{(a, b)}$	31.58 ± 0.38$^{(a, b, c)}$	68.37 ± 1.08$^{(a, b)}$	42.39 ± 3.01$^{(b)}$	45.63 ± 2.18$^{(a, b)}$	79.11 ± 2.58$^{(b)}$
Silymarin + D-GaIN (Grp 7)	**0.3**	80.52 ± 2.52$^{(b)}$	5.22 ± 0.06$^{(a, b)}$	35.5 ± 0.85$^{(b, d, e)}$	67.75 ± 0.96$^{(a, b)}$	39.83 ± 1.9$^{(b, e)}$	55.36 ± 1.18$^{(a, b)}$	80.73 ± 2.7$^{(b)}$

Values are expressed as Mean ± SEM for seven rats. The Mean difference is significant at the .05 level. (a) = $p \leq 0.05$ as compared with the normal control group. (b) = $p \leq 0.05$ as compared to D-GaIN control group. (c) = $p \leq 0.05$ as compared with the GOV + D-GaIN (2g kg^{-1}) group. (d) = $p \leq 0.05$ as compared with the GOV + D-GaIN (4g kg^{-1}) group. (e) = $p \leq 0.05$ as compared with the GOV + D-GaIN (8g kg^{-1}) group. The significance of differences among all groups was determined by the Tukey HSD test.

CAT = Catalase, Gpx = Glutathione Peroxidase, GSH = Reduced Glutathione, GST = Glutathione-S-Transferase, MDA = Malondialdehyde, SOD = Superoxide Dismutase

Table 20 shows hepatic CAT, GPx, GSH, GST, MDA, SOD and total protein levels in rats fed GOV by intragastral gavage before administration of D-GaIN. The CAT, GPx, GSH, GST, SOD and total protein levels of liver homogenate in the toxin control group were significantly ($p \leq 0.05$) attenuated while the MDA level was significantly ($p \leq 0.05$) high compared to all the other experimental groups. The GPx, GSH, GST and SOD levels of experimental animals on pretreatment with GOV at 2 g kg^{-1} were higher compared to those of Liv 52 group while the CAT was higher than that of Liv 52 and silymarin. Silymarin group had the same GPx and GST values as 2 g kg^{-1} group while the 4 g kg^{-1} group has the same SOD value as Liv 52 group. Control group was dose dependently and significantly ($p \leq 0.05$) different compared to GOV at the different doses.

Table 20: The effect of D-GaIN damage on liver antioxidant enzymes in rats pretreated with GOV

Group	Dose (g kg^{-1})	CAT (µmol/min/mg protein)	GPx (µmol/ml)	GSH (µmol/ml)	GST (µmol/ml)	MDA (nmol/ml)	SOD (µmol/ml)	Total protein (g/L)
Control (Grp 1)		393.52 ± 24.16$^{(b, c, d, e)}$	34.88 ± 4$^{(b, d, e)}$	75.97 ± 2.66$^{(b, c, d, e)}$	408.58 ± 17.46$^{(b, c, d, e)}$	7.6 ± 0.35$^{(b, c, d, e)}$	313.95 ± 17.95$^{(b, c, d, e)}$	102.85 ± 4.24$^{(b, d, e)}$
Toxin Control (Grp 2)	**0.5**	89.34 ± 3.37$^{(a, c, d, e)}$	9.9 ± 0.64 $^{(a, c, d, e)}$	35.07 ± 1.85$^{(a, c, d, e)}$	94.38 ± 6.08$^{(a, c, d, e)}$	22.54 ±1.04$^{(a, c, d, e)}$	77.74 ± 5.07$^{(a, c, d, e)}$	57.14 ± 1.92$^{(a, c, d, e)}$
GOV + D-GaIN (Grp 3)	**2**	308.23 ± 32.8$^{(a, b)}$	29.46 ± 2.24 $^{(b)}$	64.52 ± 1.45$^{(a, b)}$	302.1 ± 28.88$^{(a, b)}$	11.46 ± 0.79$^{(a, b)}$	178.22 ± 13.08$^{(a, b)}$	92.48 ± 2.24$^{(b)}$
(Grp 4)	**4**	287.04 ± 14.43$^{(a, b)}$	24.76 ± 1.37 $^{(a, b)}$	63.03 ± 0.49$^{(a, b)}$	230.95 ± 32.98$^{(a, b)}$	13.45 ± 0.63$^{(a, b)}$	171.52 ±15.52$^{(a, b)}$	83.22 ± 1.93$^{(a, b)}$
(Grp 5)	**8**	267.59 ± 15. 8$^{(a, b)}$	22.75 ± 1.31 $^{(a, b)}$	59.71 ± 1.65$^{(a, b)}$	288.26 ± 8.16$^{(a, b)}$	12.95 ± 0.44$^{(a, b)}$	174.89 ± 11.44$^{(a, b)}$	83.46 ±4.09$^{(a, b)}$
LIV 52 + D-GaIN (Grp 6)	**0.3**	289.16 ± 8.7$^{(a, b)}$	28.35 ± 0.94 $^{(b)}$	60.89 ± 0.58$^{(a, b)}$	282.69 ± 13.2$^{(a, b)}$	11.45 ± 0.58$^{(a, b)}$	171.4 ± 13.92$^{(a, b)}$	96.72 ± 3.36
Silymarin + D-GaIN (Grp 7)	**0.3**	298.93 ± 7.79$^{(a, b)}$	29.85 ± 0.63 $^{(b)}$	67.26 ± 3.38$^{(a, b)}$	302.24 ± 26.46$^{(a, b)}$	10.99 ± 0.77$^{(a, b)}$	190.34 ± 4.77$^{(a, b)}$	97.35 ± 3.89$^{(b)}$

Values are expressed as Mean ± SEM for seven rats. The Mean difference is significant at the .05 level. (a) = $p \leq 0.05$ as compared with the normal control group. (b) = $p \leq 0.05$ as compared to D-GaIN control group. (c) = $p \leq 0.05$ as compared with the GOV + D-GaIN (2g kg^{-1}) group. (d) = $p \leq 0.05$ as compared with the GOV + D-GaIN (4g kg^{-1}) group. (e) = $p \leq 0.05$ as compared with the GOV + D-GaIN (8g kg^{-1}) group. The significance of differences among all groups was determined by the Tukey HSD test.

CAT = Catalase, Gpx = Glutathione Peroxidase, GSH = Reduced Glutathione, GST = Glutathione-S-Transferase, MDA = Malondialdehyde, SOD = Superoxide Dismutase

Table 21 shows the activities and levels of CAT, GPx, GSH, GST, MDA and total protein in kidney tissue homogenates obtained from all the experimental mice. MDA level (34.64 ± 4.79 nmol/g tissue) in kidney tissue homogenates of D-GaIN treated rats was found to be significantly ($p \leq 0.05$) higher than that level compared to normal control rats (7.43 ± 0.2 nmol/g tissue). Pre-treatment with GOV followed by toxin administration decreased the level significantly ($p \leq 0.05$). GOV significantly ($p \leq 0.05$) attenuated TBARS formation and elevated the activities of CAT, GPx, GSH, GST, SOD and total protein compared to D-GaIN intoxicated control group. GOV at 4 g kg^{-1} had higher CAT activity than Liv 52, increased GSH and GST compared to 2 and 8 g kg^{-1}, attenuated TBARS formation in D-GaIN induced kidney damaged rats compared to toxin control and GOV at 2 and 8 g kg^{-1}. The total protein concentration in GOV at 2 g kg^{-1} was almost equal to that of Liv 52. The GPx activity in the 8 g kg^{-1} group is significantly ($p \leq 0.05$) low compared to 4 g kg^{-1}, Liv 52 and silymarin groups while the total protein concentration is significantly ($p \leq 0.05$) low compared to silymarin.

Table 21: The effect of D-GaIN damage on kidney antioxidant enzymes in rats pretreated with GOV

Group	Dose (g kg^{-1})	CAT (µmol/min/mg protein)	GPx (µmol/ml)	GSH (µmol/ml)	GST (µmol/ml)	MDA (nmol/ml)	SOD (µmol/ml)	Total protein (g/L)
Control (Grp 1)		457.5 ± 15.9$^{(b)}$	6.28 ± 0.1$^{(b,c,e)}$	4.19 ± 0.25$^{(b,c,e)}$	345.71 ± 35.69$^{(b,e)}$	7.43 ± 0.2$^{(b)}$	236.73 ± 14.95$^{(b,c,e)}$	71.75 ± 1.35$^{(b,c,d,e)}$
Toxin Control (Grp 2)	**0.5**	112.93 ± 14.08$^{(a)}$	2.7 ± 0.1$^{(acde)}$	1.43 ± 0.11$^{(acde)}$	108.52 ± 4.48$^{(acde)}$	34.64 ± 4.79$^{(acde)}$	63.08 ± 2.55$^{(acde)}$	45.79 ± 2.63$^{(acd)}$
GOV + D-GaIN (Grp 3)	**2**	277.14 ± 11.09$^{(a, b)}$	5.25 ± 0.23$^{(a, b)}$	2.82 ± 0.3$^{(a, b)}$	264.17 ± 33.05$^{(b)}$	9.79 ± 0.87$^{(b)}$	165.38 ± 9.27$^{(a, b)}$	60.62 ± 2$^{(a, b)}$
(Grp 4)	**4**	286.52 ± 11.63$^{(a, b)}$	5.32 ± 0.28$^{(b,e)}$	3.37 ± 0.18$^{(a, b)}$	272.19 ± 16.23$^{(b)}$	9.33 ± 0.52$^{(b)}$	191.11 ± 23.02$^{(b)}$	59.9 ± 2.36$^{(a, b)}$
(Grp 5)	**8**	241.07 ± 7.52$^{(a, b)}$	4.35 ± 0.25$^{(a, b,d)}$	2.83 ± 0.28$^{(b)}$	222.19 ± 13.92$^{(a, b)}$	11.3 ± 0.87$^{(b)}$	153.88 ± 9.33$^{(a, b)}$	54.54 ± 1.69$^{(a)}$
LIV 52 + D-GaIN (Grp 6)	**0.3**	285.18 ± 26.7$^{(a, b)}$	5.34 ± 0.33$^{(b,e)}$	3.44 ± 0.16$^{(b)}$	285.95 ± 7.71$^{(b)}$	8.87 ± 0.29$^{(b)}$	191.76 ± 9.44$^{(b)}$	61.62 ± 2.8$^{(a, b)}$
Silymarin + D-GaIN (Grp 7)	**0.3**	294.77 ± 29.88$^{(a, b)}$	6.07 ± 0.15$^{(b,e)}$	3.48 ± 0.12$^{(b)}$	285.38 ± 32.64$^{(b)}$	8.51 ± 0.54$^{(b)}$	196.9 ± 7.42$^{(b)}$	65.39 ± 1.4$^{(b,e)}$

Values are expressed as Mean ± SEM for seven rats. The Mean difference is significant at the .05 level. (a) = $p \leq 0.05$ as compared with the normal control group. (b) = $p \leq 0.05$ as compared to D-GaIN control group. (c) = $p \leq 0.05$ as compared with the GOV + D-GaIN (2g kg^{-}1) group. (d) = $p \leq 0.05$ as compared with the GOV + D-GaIN (4g kg^{-}1) group. (e) = $p \leq 0.05$ as compared with the GOV + D-GaIN (8g kg^{-}1) group. The significance of differences among all groups was determined by the Tukey HSD test.

CAT = Catalase, Gpx = Glutathione Peroxidase, GSH = Reduced Glutathione, GST = Glutathione-S-Transferase, MDA = Malondialdehyde, SOD = Superoxide Dismutase

4.10.4. Effect of D-GaIN on Caspase Activities

Table 22 shows the fold increase in caspase 2, 3 and 9 activities in the white blood cell of rats pretreated with GOV before D-GaIN damage. At 2 and 4 g kg^{-1}, GOV lowered the fold increase in the caspase 3 activities compared to toxin control, GOV (8 g kg^{-1}), Liv 52 and silymarin groups. GOV significantly ($p \leq 0.05$) decrease the fold increase in caspase 2, 3 and 9 activities compared to the toxin control group.

Table 22: Caspase - 2, 3 and 9 activities in the white blood cell of rats treated with D-GaIN

Group	Dose ($g\ kg^{-1}$)	Caspase 2 (Units/mg of total protein).	Caspase 3 (Units/mg of total protein)	Caspase 9 (Units/mg of total protein)
Control (Grp 1)		$0.97 \pm 0.07^{(b)}$	$0.72 \pm 0.07^{(b)}$	$0.95 \pm 0.02^{(b)}$
Toxin control (Grp 2)	**0.5**	$2.27 \pm 0.15^{(a, c, d, e)}$ (1.34)	$6.28 \pm 0.33^{(a, c, d, e)}$ (7.72)	$4.66 \pm 0.72^{(a, c, d, e)}$ (3.91)
GOV + D-GaIN (Grp 3)	**2**	$1.09 \pm 0.03^{(b)}$ (0.12)	$1.01 \pm 0.1^{(b)}$ (0.4)	$1.14 \pm 0.07^{(b)}$ (0.2)
(Grp 4)	**4**	$1.2 \pm 0.07^{(b)}$ (0.24)	$1 \pm 0.07^{(b)}$ (0.39)	$1.22 \pm 0.04^{(b)}$ (0.28)
(Grp 5)	**8**	$1.15 \pm 0.04^{(b)}$ (0.19)	$1.12 \pm 0.05^{(b)}$ (0.56)	$1.14 \pm 0.02^{(b)}$ (0.2)
Liv 52 + D-GaIN (Grp 6)	**0.3**	$1.01 \pm 0.05^{(b)}$ (0.04)	$1.06 \pm 0.09^{(b)}$ (0.47)	$1.13 \pm 0.08^{(b)}$ (0.19)
Silymarin + D-GaIN (Grp 7)	**0.3**	$1.00 \pm 0.06^{(b)}$ (0.03)	$1.03 \pm 0.01^{(b)}$ (0.43)	$1.11 \pm 0.06^{(b)}$ (0.17)

Values are expressed as Mean ± SEM for seven rats. The Mean difference is significant at the .05 level. (a) = $p \leq 0.05$ as compared with the normal control group. (b) = $p \leq 0.05$ as compared to Alcohol control group. (c) = $p \leq 0.05$ as compared with the GOV + Alcohol ($2g\ kg^{-1}$) group. (d) = $p \leq 0.05$ as compared with the GOV + Alcohol ($4g\ kg^{-1}$) group. (e) = $p \leq 0.05$ as compared with the GOV + Alcohol ($8g\ kg^{-1}$) group. The significance of differences among all groups was determined by the Tukey HSD test. The figures in parenthesis indicate the fold-increase in caspase activity compared to the uninduced control.

4.10.5. Histopathology of rats pretreated with GOV before D-GaIN damage

The plates of the histopathologic studies on rat liver damaged with D-GaIN are shown in plates 7-10. The control group showed normal hepatocytes arranged in roughly rod or pillar-shaped pattern while the Toxin control group showed extensive hepatocellular necrosis, leukocyte infiltration, steatosis, centrizonal necrosis, hepatocellular swelling and vacuolation. Pretreatment with GOV 2 g kg^{-1} before intoxication using D-GaIN showed no remarkable changes while at 4 g kg^{-1} it showed mild steatosis. Pretreatment with Liv 52 and silymarin before damage with D-GaIN showed no remarkable changes.

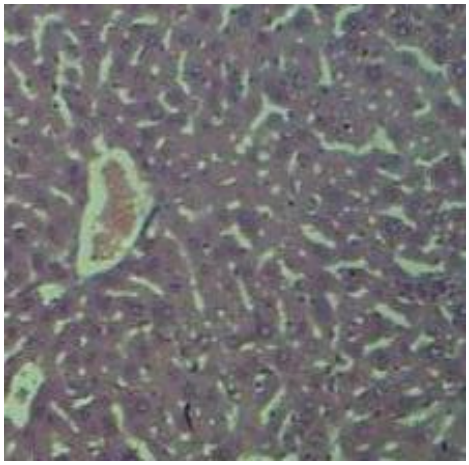


Plate 7: Control group (X100)

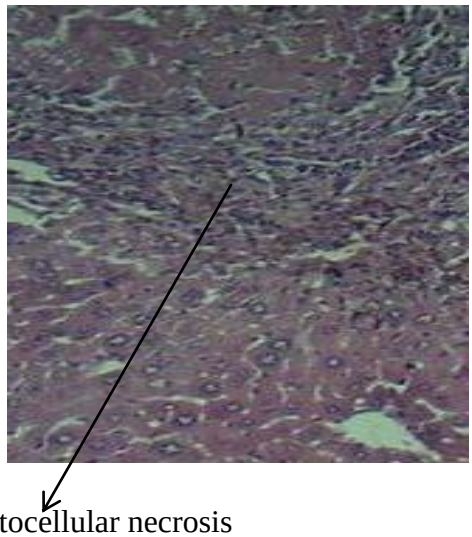


Plate 8: Toxin control group (X100)

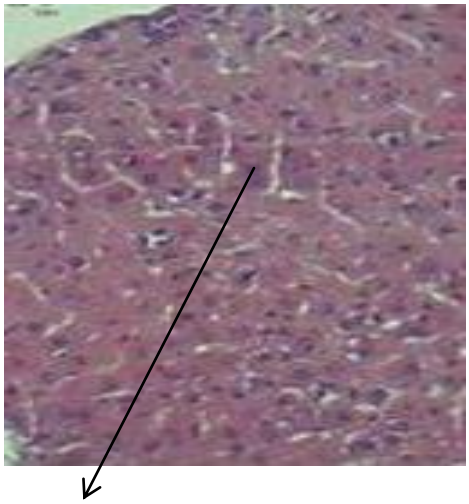


Plate 9: GOV 4 g kg^{-1} + D-GaIN (X100)

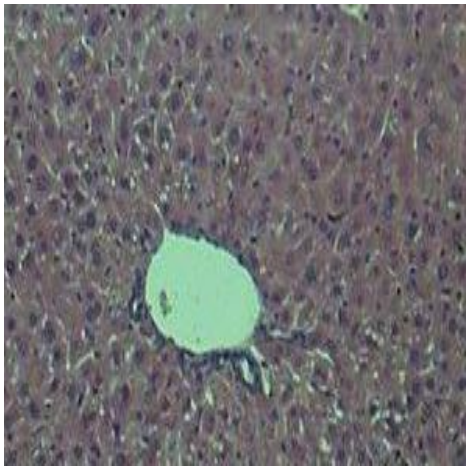


Plate 10: silymarin + D-GaIN (X100)

Histopathology of rats pretreated with GOV before D-GaIN damage

4.11. Acetaminophen Induced Hepatotoxicity

4.11.1. Effect of GOV on Hematological Parameter Acetaminophen-Induced Hepatotoxic Rats

The triherbal formulation (GOV) dose dependently caused a significant ($P \leq 0.05$) increase in the levels of Hb, PCV, RBC, WBC, Platelet count, MCHC, granulocytes and lymphocytes and also significantly ($P \leq 0.05$) reduced the levels of MCV and monocytes when compared to the APAP induced toxin control group (Table 23a and 23b). At 2 g kg^{-1}, GOV increased the Hb, MCHC and neutrophil level while the PCV was almost equal compared to Liv 52 and Silymarin groups. At 4 g kg^{-1}, GOV increased the platelet count compared to Liv 52 group while it was restored to normal with reference to the control group. When compared to the Liv 52 and silymarin groups, GOV at 8 g kg^{-1} increased the Hb and MCHC levels while the RBC, platelet counts and granulocytes levels were increased compared to Liv 52. Administration of GOV at 8 g kg^{-1} reduced the MCV level compared to all the groups.

Table 23a: Effect of pretreatment with GOV on the hematological parameter in rats with APAP induced hepatotoxicity

Group	Dose ($g\ kg^{-1}$)	PCV (%)	RBC ($10^6/\mu l$)	Hb (g/dl)	WBC ($10^3/\mu l$)	Platelet ($10^3/\mu l$)
Control (Grp 1)		42.86 ± 0.91(b, d)	6.53 ± 0.17(b, d)	12.67 ± 0.41(b)	4.33 ± 0.27(b, d)	36.03 ± 2.08(b)
Toxin Control (Grp 2)	3	29.74 ±1.04(a, c, d, e)	3.84 ± 0.146(a, c, d, e)	7.77 ± .25(a, c, d, e)	1.89 ± 0.13(a, c, d, e)	10.63 ± 0.79(a, c, d, e)
GOV + APAP (grp 3)	2	40.07 ± 1.56(b)	6.14 ± 0.08(b)	12.51± 0.37(b)	3.41 ± 0.25(b)	32.19 ± 1.72(a, b)
(Grp 4)	4	36.3 ± 1.36(a, b)	5.27 ± 0.17(a, b, e)	11.26 ± 0.45(b)	3.19 ± 0.32(a, b)	35.04 ± 2.24(b)
(Grp 5)	8	37.97 ± 0.56(b)	6.62 ± 0.31(b, d)	12.07 ± 0.63(b)	3.57 ± 0.33(b)	34.27 ± 1.73(b)
LIV 52 + APAP (Grp 6)	0.3	40.64 ± 1.56(b)	6.43 ± 0.28(b, d)	11.06 ± 0.32(b)	4.26 ± 0.17(b)	34.74 ± 2.36(b)
Silymarin + APAP (Grp 7)	0.3	40.93(b)	6.9 ± 1.32.32(b, d)	11.59 ±. 7(b)	4.19 ± 0.27(b)	38.8 ± 1.11(b)

Values are expressed as Mean ± SEM for seven rats. The Mean difference is significant at the .05 level. (a) = $p \leq 0.05$ as compared with the normal control group. (b) = $p \leq 0.05$ as compared to APAP control group. (c) = $p \leq 0.05$ as compared with the GOV + APAP (2g kg^{-1}) group. (d) = $p \leq 0.05$ as compared with the GOV + APAP (4g kg^{-1}) group. (e) = $p \leq 0.05$ as compared with the GOV + APAP (8g kg^{-1}) group. The significance of differences among all groups was determined by the Tukey HSD test.

PCV =Packed Cell Volume, Hb = Haemoglobin, RBC = Red Cell Count, WBC = White Blood Cell.

Table 23b: Effect of pretreatment with GOV on the hematological parameter in rats with APAP induced hepatotoxicity

Group	Dose (g kg^{-1})	MCHC (%)	MCV (fl)	MCH (pg)	Granulocytes (%)	Lymphocyte (%)	Monocyte (%)
Control (Grp 1)		29.72 ± 1.22(b)	66.01 ± 2.6(b)	19.43 ± 0.54	9.16 ± 0.85(c)	84.04 ± 1.53(b)	6.8 ± 0.92(b)
Toxin Control (Grp 2)	3	21.04 ± 1.35(a, c, d, e)	78.3 ± 2.35(a, c, e)	20.55 ± 1.43	6.89 ± 0.61(c, d, e)	66.46 ± 1.47(a, c, d, e)	26.66 ± 1.29(a, c, d, e)
GOV + APAP (Grp 3)	2	31.2 ± 0.82(b)	65.23 ± 3.54(b)	20.34 ± 1.32	14.43 ± 0.73(a, b)	78.17 ± 1.86(b)	7.4 ± 1.25(b)
(Grp 4)	4	30.96 ± 1.54(b)	68.9 ± 2.45(e)	21.57 ± 0.59	11.94 ± 0.99(b)	80.43 ± 1.86(b)	7.59 ± 1.08(b)
(Grp 5)	8	33.87 ± 1.3(b)	56.53 ± 1.14(b, d)	18.97 ± 0.99	12.23 ± 0.76(b)	80.04 ± 1.56(b)	7.73 ± 1.04(b)
LIV 52 + APAP (Grp 6)	0.3	27.73 ± 1.08	60.25 ± 2.46(b)	17.35 ± 0.83(d)	11.11 ± 0.71(b)	80.57 ± 1.42(b)	8.31 ± 0.9(b)
Silymarin + APAP (Grp 7)	0.3	29.95 ± 3.97(b)	59.64 ± 2.54(b)	18.07 ± 0.59	12.31 ± 0.98(b)	79.76 ± 1.46(b)	7.93 ± 0.81(b)

Values are expressed as Mean ± SEM for seven rats. The Mean difference is significant at the .05 level. (a) = $p \leq 0.05$ as compared with the normal control group. (b) = $p \leq 0.05$ as compared to APAP control group. (c) = $p \leq 0.05$ as compared with the GOV + APAP (2g kg^{-}1) group. (d) = $p \leq 0.05$ as compared with the GOV + APAP (4g kg^{-}1) group. (e) = $p \leq 0.05$ as compared with the GOV + APAP (8g kg^{-}1) group. The significance of differences among all groups was determined by the Tukey HSD test.

4.11.2. Effect of GOV on Serum Hepatic Enzymes and Chemical Analytes on Acetaminophen intoxicated rats

Table 24a shows serum ALP, ALT, AST, LDH and GGT activities in rats fed GOV by intragastral gavage before administration of acetaminophen. The levels of serum marker enzymes were significantly ($p \leq 0.05$) elevated in the toxin control group compared to the control group indicating induction of severe liver damage. Administration of GOV at the doses of 2, 4 and 8 g kg^{-1} significantly ($p \leq 0.05$) repressed hepatotoxicity induced by APAP by reducing the levels of the serum marker enzymes in a dose dependent manner. At 4 and 8 g kg^{-1}, GOV lowered the level of LDH to normal indicating its hepatoprotective potentials.

Serum cholesterol, creatinine, triglyceride and BUN concentrations were significantly ($p \leq 0.05$) increased in the APAP treated toxin control group of animals compared to the normal control group of animals while total protein concentrations were decreased significantly ($p \leq 0.05$) indicating the induction of severe nephro-hepatotoxicity (Table 24b). At 4 g kg^{-1}, GOV significantly increased albumin concentration and also decreased cholesterol, creatinine, triglyceride and BUN concentrations compared to the toxin control group. Treatment with different doses of GOV dose dependently decreased the concentrations of serum cholesterol, creatinine, triglyceride and BUN and that of albumin and total protein were increased compared to the toxin control group indicating its nephro-hepatoprotective potentials.

Table 24a: The activities of ALT, AST, ALP, LDH and GGT in rats treated with a triherbal formulation (GOV) and a single dose of acetaminophen (APAP).

Group	Dose ($g\ kg^{-1}$)	LIVER FUNCTION ENZYMES				
		ALT (U/L)	AST (U/L)	ALP (U/L)	LDH (U/L)	GGT (U/L)
Control (Grp 1)		24.2±1.2 (b, c, d)	10.3±1.0 (b, c, e)	70.0± 6.5 (b, c)	14.0±1.4 (b)	1254.7±84.6 (b)
Toxin Control (Grp 2)	3	95.9±2.2 (a, c, d, e)	77.8±2.1 (a, c, e)	447.0±11.5 (a, c)	56.5±3.5 (a)	3920.0±421.1 (a)
GOV + APAP (Grp 3)	2	45.4±4.4 (a, b, d, e)	18.8±1.5 (a, b)	165.6±16.1 (a, b)	19.7±3.6 (b)	1584.0±57.5 (b)
(Grp 4)	4	65.1±4.7 (a, b, c)	16.8±1.0 (a, b)	116.9±23.1 (b)	13.5±0.6 (b)	2611.9±95.8 (a, b)
(Grp 5)	8	75.7±5.1 (a, b, c)	21.2±0.8 (a, b)	131.1±8.4 (a, b)	10.7±0.4 (b)	2518.3±81.6 (a, b)
LIV 52 + APAP (Grp 6)	0.3	47.8±3.8 (a, b, d, e)	16.55±1.67 (a, b)	93.2±7.3 (b, c)	18.36±2.6 (b)	1719.9±214.2 (b)
Silymarin + APAP (Grp 7)	0.3	28.3±3.7 (b, c, d, e)	11.1±0.4 (b, c, e)	110.1±5.3 (b, c)	10.1±1.8 (b)	1420.6±65.5 (b)

Values are expressed as Mean ± SEM for seven rats. The Mean difference is significant at the .05 level. (a) = $p \leq 0.05$ as compared with the normal control group. (b) = $p \leq 0.05$ as compared to APAP control group. (c) = $p \leq 0.05$ as compared with the GOV+APAP (2 $g\ kg^{-1}$) group. (d) = $p \leq 0.05$ as compared with the GOV+APAP (4 $g\ kg^{-1}$) group. (e) = $p \leq 0.05$ as compared with the GOV+APAP (8 $g\ kg^{-1}$) group. The significance of differences among all groups was determined by the Tukey HSD test

Table 24b: Effect of GOV on serum ALB, CHO, CREA, TP, TG and BUN concentrations in rats treated with a polyherbal formulation (GOV) and a single dose of APAP.

Group	Dose ($g\ kg^{-1}$)	BIOCHEMICAL PARAMETERS					
		TP (g/L)	ALB (g/L)	BUN (mmol/L)	CREA (mmol/L)	TG (mmol/L)	CHO (mmol/L)
Control (Grp 1)		112.8 ± 12.5[b]	34.3±1.9	6.4±0.4[b]	63.9±1.5[b]	1.5±0.1[b]	1.7±0.1[b]
Toxin Control (Grp 2)	3	56.2±1.9[a]	28.9±1.9	13.6±0.7[a]	89.8±3.5[a]	2.9±0.2[a]	3.0±0.1[a, d]
GOV + APAP (Grp 3)	2	71.7±6.1[a]	35.1±0.8	7.5±0.3[b]	70.0±3.1[b]	1.9±0.2 [b]	2.3±0.1
(Grp 4)	4	74.9±3.3[a]	42.4±2.2[b]	5.9±0.1[b]	70.5±2.9[b]	1.5±0.1[b]	1.9±0.2[b]
(Grp 5)	8	73.0±2.9[a]	34.7±2.9	6.1±0.5[b]	67.2±3.1[b]	1.5±0.3[b]	2.3±0.2
LIV 52 + APAP (Grp 6)	0.3	79.6±5[a, b]	38.2±1.9[b]	6.3±0.3[b]	60.7±4.0 [b]	2.0±0.2[b]	2.0±0.1[b]
Silymarin + APAP (Grp 7)	0.3	79.9±3.9[a, b]	38.8±1.7[b]	6.2±0.1[b]	62.2±2.6[b]	1.4 ± 0.09[b]	2.2±0.1[b]

Values are expressed as Mean ± SEM for seven rats. The Mean difference is significant at the .05 level. (a) = $p \leq 0.05$ as compared with the normal control group. (b) = $p \leq 0.05$ as compared to APAP control group. (c) = $p \leq 0.05$ as compared with the GOV+APAP (2 $g\ kg^{-1}$) group. (d) = $p \leq 0.05$ as compared with the GOV+APAP (4 $g\ kg^{-1}$) group. (e) = $p \leq 0.05$ as compared with the GOV+APAP (8 $g\ kg^{-1}$) group. The significance of differences among all groups was determined by the Tukey HSD test

ALB = Albumin, CREA = Creatinine, CHO = Cholesterol, TP = Total Protein, TG = Triglyceride, BUN = Blood Urea Nitrogen

4.11.3. The Effects of APAP on Antioxidant Defence Enzymes of Albino Rats.

Serum CAT, GPx, GSH, GST, MDA and SOD activities and levels in rats fed GOV by intragastral gavage before administration of APAP are shown in table 25. The CAT, GPx, GSH, GST and SOD activity in the APAP treated toxin control group were significantly ($p \leq 0.05$) decreased while the MDA levels were significantly ($p \leq 0.05$) increased when compared to the groups administered the different doses (2, 4 and 8 g kg^{-1}) of GOV. The GST level was reversed to normal by GOV at a dose of 2 g kg^{-1} compared to the normal control and toxin control groups while the GPx levels of the different doses of GOV were dose dependently reversed compared to Liv 52 and Silymarin groups. At 8 g kg^{-1}, GOV reduced the serum MDA level to normal. The GSH activity of GOV at 4 g kg^{-1} was almost the same as that of silymarin while Liv 52 was significantly higher than GOV at 2 and 8 g kg^{-1}.

Table 25: The effect of APAP damage on serum antioxidant enzymes in rats pretreated with GOV

Group	Dose ($g\ kg^{-1}$)	CAT (µmol/min/mg protein)	GPx (µmol/ml)	GSH (µmol/ml)	GST (µmol/ml)	MDA (nmol/ml)	SOD (µmol/ml)	TP (g/l)
Control (Grp 1)		68.73 ± 2.9[(b)]	5.38 ± 0.03[(b)]	19.29 ± 0.53[(b)]	65.01 ± 3.13[(b, e)]	27.25 ± 1.68[(b)]	77.43± 2.63[(b, c, d, e)]	148.50 ± 12.48[(b)]
Toxin Control (Grp 2)	3	26.57± 1.18[(a, c, d, e)]	2.2 ± 0.002[(a, c, d, e)]	4.18 ± 0.26[(a, c, d, e)]	30.58 ± 1.78[(a, c, d, e)]	88.81± .74[(a, c, d, e)]	32.21± 1.51[(a, c, d, e)]	57.67 ± 2.58[(a)]
GOV + APAP (Grp 3)	2	57.75 ± 1.45[(b)]	4.96 ± 0.26[(b)]	12.91 ± 0.78[(a, b)]	67.48 ± 2.21[(b, d, e)]	35.08 ± 1.64[(b)]	54.36 ± 0.81[(a, b)]	71.67± 6.14[(a)]
(Grp 4)	4	68.3 ± 1.53[(b)]	5.16 ± 0.72[(b)]	13.81 ± 0.47[(a, b)]	53.71 ± 2.03[(b, c)]	32.87 ± 1.53[(b)]	59.29 ± 1.84[(a, b)]	73.44 ± 3.7[(a)]
(Grp 5)	8	55.97± 2.77[(b)]	4.75 ±0.48[(b)]	13.2 ± 0.26[(a, b)]	48.12 ± 3.37[(a, b, c)]	29 ± 3.1[(b)]	59.83± 7.08[(a, b)]	68.74± 3.35[(a)]
LIV 52 + APAP (Grp 6)	0.3	82.07± 5.64[(b, c, e)]	4.9 ±0.003[(a, c, d, e)]	15.77 ± 0.6[(a, b, c, e)]	53.36 ± 2.75[(b, c)]	26.16 ± 2.69[(b)]	63.79 ± 3.79[(b)]	63.91 ± 4.16[(a)]
Silymarin + APAP (Grp 7)	0.3	84.76 ± 4.4[(a, c, d, e)]	5.2 ± 0.02[(a, c, d, e)]	13.88 ± 0.64[(a, b)]	56.63 ± 2.98[(b)]	26.3± 3.4[(b)]	73.85 ± 4.45[(b, c)]	71.32 ± 0.8[(a)]

Values are expressed as Mean ± SEM for seven rats. The Mean difference is significant at the .05 level. (a) = $p \leq 0.05$ as compared with the normal control group. (b) = $p \leq 0.05$ as compared to APAP control group. (c) = $p \leq 0.05$ as compared with the GOV+APAP (2 g kg^{-1}) group. (d) = $p \leq 0.05$ as compared with the GOV+APAP (4 g kg^{-1}) group. (e) = $p \leq 0.05$ as compared with the GOV+APAP (8 g kg^{-1}) group. The significance of differences among all groups was determined by the Tukey HSD test.

CAT = Catalase, GPx = Glutathione Peroxidase, GSH = Reduced Glutathione, GST = Glutathione-S-Transferase, MDA = Malondialdehyde, SOD = Superoxide Dismutase

The effect of GOV on CAT, GPx, GSH, GST, SOD and total protein activity were significantly ($p \leq 0.05$) and dose dependently increased compared to the toxin control group, while the MDA activities were significantly ($p \leq 0.05$) reduced as shown in table 26. At 8 g kg^{-1}, GOV significantly ($p \leq 0.05$) increased the activity of GSH compared to all the groups while at 4 g kg^{-1}, GOV increased the levels of GSH, GST and total protein compared to Liv 52 and Silymarin groups. The GPx levels of the different doses of GOV were restored to normal. The triherbal formulation dose dependently reversed the nephro-toxic activity of APAP on rats compared to the control and toxin control groups.

Table 26: The effect of APAP damage on kidney antioxidant enzymes in rats pretreated with GOV

GROUP	Dose ($g\ kg^{-1}$)	CAT (µmol/min/mg protein)	GPx (µmol/ml)	GSH (µmol/ml)	GST (µmol/ml)	MDA (nmol/ml)	SOD (µmol/ml)	TOTAL PROTEIN (g/l)
Control (Grp 1)		238.42 ± 18.04$^{(b)}$	0.46 ± 0.01 $^{(b)}$	0.91 ± 0.08$^{(b, c, e)}$	81.71 ± 1.79$^{(b, c, e)}$	30.48 ± 0.71$^{(b, c)}$	49.1± 0.88 $^{(b, d)}$	17.95 ± 0.32$^{(b, c, d, e)}$
Toxin Control (Grp 2)	3	88.81 ± 3.37$^{(a ,c, d, e)}$	0.3 ± 0.01$^{(a, c, d, e)}$	0.43 ± 0.03 $^{(a, d, e)}$	43.81 ± 2.55$^{(a, d, e)}$	56.64 ± 2. 8$^{(a, c, d, e)}$	28.72 ± 1.18 $^{(a, c, e)}$	7.86 ± 0.43$^{(a, c, d, e)}$
GOV + APAP (Grp 3)	2	193.38 ± 20.49$^{(b)}$	0.49 ± 0.03$^{(b)}$	0.59 ± 0.03$^{(a, e)}$	58.57± 0.78 $^{(a, b)}$	41.4 ± 1.5$^{(a, b, d, e)}$	42.16 ± 1.88$^{(b)}$	11.69 ±0 .76 $^{(a, b)}$
(Grp 4)	4	208.92 ± 4.12$^{(b)}$	0.46± 0.02$^{(b)}$	0.83 ± 0.04$^{(b, e)}$	70.19 ± 1.62$^{(b)}$	32.33± 0.92$^{(b, c)}$	36.8 ± 2.22$^{(a)}$	12.57 ± 0.29$^{(a, b)}$
(Grp 5)	8	204.61 ± 3.17$^{(b)}$	0.47 ± 0.01$^{(b)}$	1.41 ± 0.01$^{(a, b, c, d)}$	67.87± 1.31$^{(a, b)}$	31.48±0.89$^{(b, c)}$	39.98± 1.59$^{(b)}$	12.32±0.48$^{(a, b)}$
LIV 52 + APAP (Grp 6)	0.3	247.8 ± 19.03 $^{(b)}$	0.6 ± 0.02 $^{(a, b, c, d, e)}$	1.12 ± 0.1$^{(b, c, d, e)}$	56.19 ± 0.77 $^{(a, b, d)}$	29.67 ± 0.63$^{(a, b, c, d, e)}$	40.1 ± 4.12$^{(b)}$	13.71 ± 0.21$^{(a, b, c)}$
Silymarin + APAP (Grp 7)	0.3	280.05 ± 27.85 $^{(b, c, e)}$	0.62 ± 0.03$^{(a, b, c, d, e)}$	.76 ± 0.04 $^{(a, b, e)}$	70.33 ± 5.91$^{(b)}$	29.87 ± 2.06$^{(b, c)}$	44.11 ± 3.39 $^{(b)}$	12.85 ± 0.47$^{(a, b)}$

Values are expressed as Mean ± SEM for seven rats. The Mean difference is significant at the .05 level. (a) = $p \leq 0.05$ as compared with the normal control group. (b) = $p \leq 0.05$ as compared to APAP control group. (c) = $p \leq 0.05$ as compared with the GOV+APAP (2 $g\ kg^{-1}$) group. (d) = $p \leq 0.05$ as compared with the GOV+APAP (4 $g\ kg^{-1}$) group. (e) = $p \leq 0.05$ as compared with the GOV+APAP (8 $g\ kg^{-1}$) group. The significance of differences among all groups was determined by the Tukey HSD test.

CAT = Catalase, GPx = Glutathione Peroxidase, GSH = Reduced Glutathione, GST = Glutathione-S-Transferase, MDA = Malondialdehyde, SOD = Superoxide Dismutase

The triherbal formulation significantly ($p \leq 0.05$) and dose dependently increased the activities CAT, GPx, GSH, GST, SOD and total protein compared to toxin control group as shown in table 27. At 4 g kg^{-1}, GOV increased the GPx, GSH, SOD and total protein levels compared to Liv 52 and Silymarin groups, while the catalase activity was restored to normal compared to the control group. The liver of Liv 52 and silymarin treated animals also showed a significant ($p \leq 0.05$) increase in antioxidant enzymes levels compared to acetaminophen treated rats. On treatment with different doses of GOV, MDA contents were significantly ($p \leq 0.05$) reduced compared to the toxin induced control group.

Table 27: The effect of APAP damage on liver antioxidant enzymes in rats pretreated with GOV.

GROUP	Dose (g kg^{-1})	CAT (µmol/min/mg protein)	GPx (µmol/ml)	GSH (µmol/ml)	GST (µmol/ml)	MDA (nmol/ml)	SOD (µmol/ml)	TP (g/l)
Control (Grp 1)	54.22 ± 4.07 [b]	0.6 ± 0.02 [b, c, d, e]	1.31 ± 0.11 [b, c, d, e]	112.57 ± 4.02 [b, d, e]	32.21 ± 0.91 [b, c, d]	62.05 ± 3.16 [b, c, d, e]	22.65 ± 0.72 [b, c, d, e]	
Toxin Control (Grp 2)	3	27.64 ± 19.59 [a, c, d]	0.29 ± 0.01 [a, c, d, e]	0.6 ± 0.01 [a, c, d, e]	65.48 ± 1.29 [a, c, d]	51.42 ± 3.14 [a, c, d, e]	34.85 ± 2.72 [a, c]	9.7 ± 0.53 [a, c, d, e]
GOV + APAP (Grp 3)	2	44 ± 4.48 [b]	0.49 ± 0.01 [a, b, e]	0.89 ± 0.04 [a, b]	123.1 ± 3.13 [b, d, e]	41.95 ± 1.73 [a, b]	39.78 ± 2.69 [a]	14.65 ± 0.2 [a, b]
(Grp 4)	4	54.04 ± 1.88 [b]	0.51 ± 0.02 [a, b, e]	1.05 ± 0.02 [a, b]	91.33 ± 2.11 [a, b, c, e]	39.62 ± 1.31 [a, b]	48.4 ± 3.93 [a, b]	16.29 ± 0.3 [a, b]
(Grp 5)	8	42.47 ± 4.15	0.4 ± 0.02 [a, b, c, d]	0.94 ± 0.01 [a, b]	76.38 ± 2.22 [a, c, d]	36.1 ± 0.6 [b]	46.82 ± 1.38 [a]	15.02 ± 0.68 [a, b]
LIV 52 + APAP (Grp 6)	0.3	61.94 ± 3.9 [b, c, e]	0.45 ± 0.03 [a, b]	1.11 ± 0.08 [b]	96.88 ± 4.48 [a, b, c, e]	35.34 ± 1.16 [b]	50.29 ± 4.28 [b]	16.95 ± 0.42 [a, b, c]
Silymarin + APAP (Grp 7)	0.3	63.27 ± 3.58 [b, c, e]	0.53 ± 0.03 [b, e]	1.17 ± 0.03 [b, c]	103.33 ± 2.51 [b, c, e]	30.63 ± 0.6 [b, c, d]	51.52 ± 0.89 [b]	17.06 ± 0.5 [a, b, c]

Values are expressed as Mean ± SEM for seven rats. The Mean difference is significant at the .05 level. (a) = p ≤ 0.05 as compared with the normal control group. (b) = p ≤ 0.05 as compared to APAP control group. (c) = p ≤ 0.05 as compared with the GOV+APAP (2g kg^{-}1) group. (d) = p ≤ 0.05 as compared with the GOV+APAP (4g kg^{-}1) group. (e) = p ≤ 0.05 as compared with the GOV+APAP (8g kg^{-}1) group. The significance of differences among all groups was determined by the Tukey HSD test.

CAT = Catalase, GPx = Glutathione Peroxidase, GSH = Reduced Glutathione, GST = Glutathione-S-Transferase, MDA = Malondialdehyde, SOD = Superoxide Dismutase

4.11.4. Caspase - 2, 3 and 9 activities in the white blood cell of rats treated with Acetaminophen

Table 28 shows the fold increase in caspase 2, 3 and 9 activities in the white blood cell of rats pretreated with GOV before acetaminophen intoxication. GOV significantly ($p \leq 0.05$) decreased the caspase activities compared to toxin control, though there was a significance ($p \leq 0.05$) compared to the control group. GOV did not demonstrate up to one fold-increase in caspase activity compared to the uniduced control group.

Table 28: Caspase - 2, 3 and 9 activities in the white blood cell of rats treated with APAP

Group	Dose ($g\ kg^{-1}$)	Caspase 2 (Units/mg of total protein).	Caspase 3 (Units/mg of total protein)	Caspase 9 (Units/mg of total protein)
Control (Grp 1)		9.66 ±0.69[(b)]	9.54 ± 0.18[(b)]	6.4 ± 0.36[(b)]
Toxin control (Grp 2)	3	19.91 ± 1.01[(a, c, d, e)]	22.62 ± 1.08[(a, c, d, e)]	19.34 ±0.65[(a, c, d, e)]
		(1.06)	(1.37)	(2.02)
GOV + APAP (Grp 3)	2	10.95 ± 0.3[(b)]	11.66± 0.66[(b)]	6.8 ± 0.48[(b)]
		(0.13)	(0.22)	(0.06)
(Grp 4)	4	12 ±0.85[(b)]	12.08 ± 0.42[(b)]	7.2 ± 0.38[(b)]
		(0.24)	(0.27)	(0.13)
(Grp 5)	8	11.74 ± 0.34[(b)]	11.54 ± 0.16[(b)]	6.84 ± 0.84[(b)]
		(0.22)	(0.21)	(0.07)
LIV 52 + APAP (Grp 6)	0.3	10.92 ± 1.1[(b)]	11.37 ± 0.75[(b)]	6.72 ± 0.48[(b)]
		(0.13)	(0.19)	(0.05)
Silymarin + APAP (Grp 7)	0.3	10.26 ± 0.53[(b)]	11 ± 0.54[(b)]	6.6 ± 0.46[(b)]
		(0.06)	(0.15)	(0.03)

Values are expressed as Mean ± SEM for seven rats. The Mean difference is significant at the .05 level. (a) = $p \leq 0.05$ as compared with the normal control group. (b) = $p \leq 0.05$ as compared to APAP control group. (c) = $p \leq 0.05$ as compared with the GOV + APAP ($2g\ kg^{-1}$) group. (d) = $p \leq 0.05$ as compared with the GOV + APAP ($4g\ kg^{-1}$) group. (e) = $p \leq 0.05$ as compared with the GOV + APAP ($8g\ kg^{-1}$) group. The significance of differences among all groups was determined by the Tukey HSD test. The figures in parenthesis = fold-increase in caspase activity compared to the uninduced control.

4.11.5. Histopathology of Liver of Rats Intoxicated with APAP

The liver of rats in the toxin group showed vacuolization of hepatocytes, sinusoidal dilation and centrilobullar hepatic necrosis compared to control. Additionally, it showed severe steatohepatitis with marked neutrophillic activity and periportal mixed necro-inflammatory cells, infiltrated with lymphocytes and Kupffer cells.

Administration of the different doses of GOV did not show diffuse microvesicular steatosis (plates 11 - 12). However, there was minimal periportal lymphocytic infiltration with necrosis at different concentrations of the extract compared to animals in the toxin group. The morphology of liver of rats in Liv 52 and silymarin groups were almost normal.

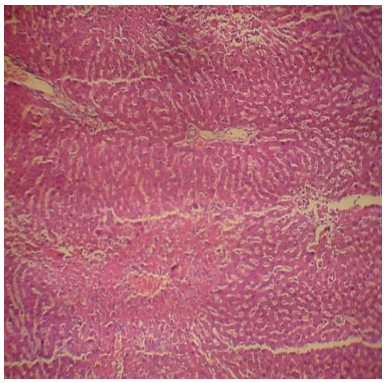


Plate 9: Control (Liver)

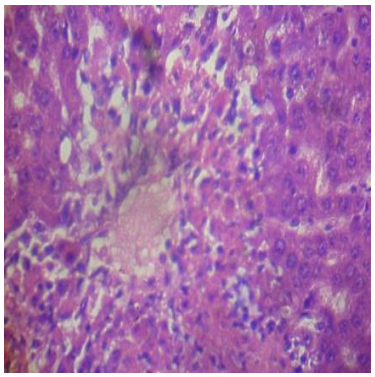


Plate 10: toxin control (liver)

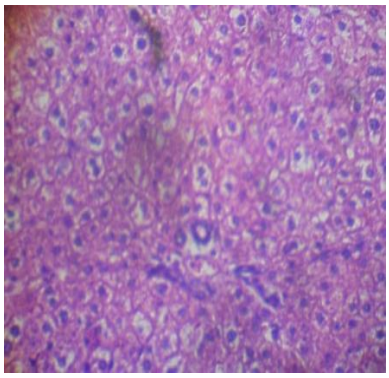


Plate 11: GOV 2 g kg^{-1} + APAP (liver)

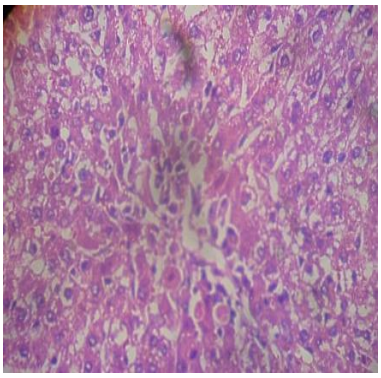


Plate 12: GOV 4 g kg^{-1} + APAP (liver)

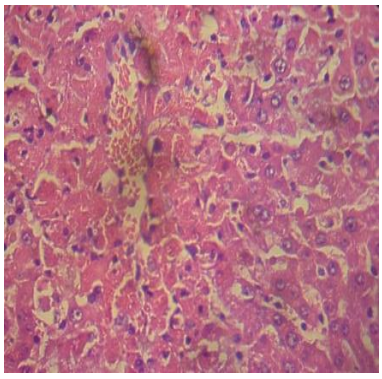


Plate 13: Liv. 52 + APAP (liver)

Histopathological examination of liver and kidney sections of the rats fed with GOV and intoxicated with APAP

4.12. Carbon Tetrachloride Induced Hepatotoxicity (CCl_4)

4.12.1. Effect of pretreatment with GOV on the hematological parameter in rats with CCl_4 induced hepatotoxicity

The administration of GOV dose dependently caused a increase in PCV, RBC, Hb, WBC, platelet, MCHC, granulocytes and lymphocytes and decrease in MCV and monocytes compared to the CCl_4 induced toxin control group as shown in tables 29a and 29b. There was an increase in levels of Hb, PCV, platelet count, MCV, MCH and MCHC and monocytes on administration of GOV at 2 g kg^{-1} compared to Liv 52 group. At 4 g kg^{-1}, GOV lowered the WBC count while at 8 g kg^{-1}, GOV increased the WBC count comparable to Liv 52 and silymarin. GOV increased platelet count and MCHC compared to Liv 52. At 8 g kg^{-1}, GOV increased the number of granulocytes and monocytes compared to control, Liv 52 and silymarin groups. The PCV level of the control group was significantly ($p \leq 0.05$) increased comparable to the toxin control and GOV at 4 and 8 g kg^{-1}. At 8 g kg^{-1}, GOV significantly ($p \leq 0.05$) increased WBC count comparable to the control group. There was no significant ($p \leq 0.05$) difference in MCV, MCHC and MCH in this experiment.

Table 29a: Effect of pre-treatment with GOV on the hematologic indices in rats with CCl_4 induced hepatotoxicity

Group	Dose ($g\ kg^{-1}$)	PCV (%)	RBC ($10^6/\mu l$)	Hb (g/dl)	WBC ($10^3/\mu l$)	Platelet ($10^3/\mu l$)
Control (grp 1)		49.29 ± 0.97$^{(b, d, e)}$	7 ± 0.11$^{(b)}$	12.94 ± 0.46$^{(b)}$	2.24 ± 0.25$^{(b, e)}$	74.14 ± 3.74$^{(b)}$
Toxin Control (Grp 2)		28.71 ± 1.32$^{(a, c, d, e)}$	4.3 ± 0.16$^{(a, c, d, e)}$	7.31 ± 0.38$^{(a, c, d, e)}$	6.13 ± 0.81$^{(a, c, d)}$	28.14 ± 2.01$^{(a, c, d, e)}$
GOV + CCl_4 (Grp 3)	**2**	44.04 ± 1.49$^{(b)}$	6.44 ± 0.31$^{(b)}$	11.83 ±0.64$^{(b)}$	3.4 ±0.31$^{(b)}$	71.54 ±3.03$^{(b)}$
(Grp 4)	4	40.57 ± 3.84$^{(a, b)}$	6.18 ±0.32$^{(b)}$	11.14 ±0.44$^{(b)}$	2.49 ±0.17$^{(b)}$	66.14 ±7.02$^{(b)}$
(Grp 5)	8	39.86 ±1.1$^{(a, b)}$	6.53 ± 0.22$^{(b)}$	10.93 ± 0.53$^{(b)}$	4.71 ±0.87$^{(a)}$	67.64 ±3.41$^{(b)}$
LIV 52 + CCl_4 (Grp 6)	0.3	43.29 ±1.54$^{(b)}$	6.67 ±0.29$^{(b)}$	11.36 ± 0.52$^{(b)}$	4.13 ± 0.26	62 ± 10.04$^{(b)}$
Silymarin + CCl_4 (Grp 7)	0.3	46.91 ± 1.1$^{(b)}$	6.75 ± 0.24$^{(b, c, d, e)}$	14.02 ± 0.47$^{(b)}$	4.13 ± 0.39	69.86 ± 6.17$^{(b)}$

Values are expressed as Mean ± SEM for seven rats. The Mean difference is significant at the .05 level. (a) = $p \leq 0.05$ as compared with the normal control group. (b) = $p \leq 0.05$ as compared to CCl_4 control group. (c) = $p \leq 0.05$ as compared with the GOV + CCl_4 (2000mg kg^{-1}) group. (d) = $p \leq 0.05$ as compared with the GOV + CCl_4 (4000mg kg^{-1}) group. (e) = $p \leq 0.05$ as compared with the GOV + CCl_4 (8000mg kg^{-1}) group. The significance of differences among all groups was determined by the Tukey HSD test.

PCV =Packed Cell Volume, Hb = Haemoglobin, RBC = Red Cell Count, WBC = White Blood Cell.

Table 29b: Effect of pre-treatment with GOV on the hematologic indices in rats with CCl_4 induced hepatotoxicity

Group	Dose (g kg^{-1})	MCHC (%)	MCV (fl)	MCH (pg)	Granulocytes (%)	Lymphocyte (%)	Monocyte (%)
Control (grp 1)		26.38 ± 0.78	70.53 ±1.67	18.52 ± 0.72	10.57 ± 0.87$^{(b)}$	83.29 ± 1.48$^{(b)}$	6.14 ± 0.94$^{(b)}$
Toxin Control (Grp 2)		25.67 ± 1.55	67.33 ± 3.89	14.72 ± 2.41	5.57 ± 0.75$^{(a, d, e)}$	75 ± 1.69$^{(a, c, d)}$	19.43 ± 1.09$^{(a, c, d, e)}$
GOV + CCl_4 (Grp 3)	2	27.06 ± 1.81	69.22 ± 3.94	18.7 ± 1.54	9.43 ± 0.65	83.57 ± 0.65$^{(b)}$	7 ± 0.44$^{(b)}$
(Grp 4)	4	28.93 ± 3.05	65.7 ± 4.65	18.25 ± 0.87	10.86 ± 1.37$^{(b)}$	82.57 ± 2.48$^{(b)}$	5.14 ± 0.86$^{(b)}$
(Grp 5)	8	27.49 ± 1.37	61.65 ± 3.3	16.92 ± 1.1	11.43 ± 0.92$^{(b)}$	81.43 ± 1.29	7.14 ± 0.6$^{(b)}$
LIV 52 + CCl_4 (Grp 6)	0.3	26.46 ± 1.57	65.8 ± 4.17	17.18 ± 0.98	9.71 ±1.11	84.14 ± 1.22$^{(b)}$	6.14 ± 0.63$^{(b)}$
Silymarin + CCl_4 (Grp 7)	0.3	30.08 ± 1.59	67.27 ± 3.12	20.94 ±1.09$^{(b)}$	9.21 ± 1.35	84.96 ± 1.87$^{(b)}$	5.83 ± 0.66$^{(b)}$

Values are expressed as Mean ± SEM for seven rats. The Mean difference is significant at the .05 level. (a) = $p \leq 0.05$ as compared with the normal control group. (b) = $p \leq 0.05$ as compared to CCl_4 control group. (c) = $p \leq 0.05$ as compared with the GOV + CCl_4 (2000mg kg^{-1}) group. (d) = $p \leq 0.05$ as compared with the GOV + CCl_4 (4000mg kg^{-1}) group. (e) = $p \leq 0.05$ as compared with the GOV + CCl_4 (8000mg kg^{-1}) group. The significance of differences among all groups was determined by the Tukey HSD test.

MCV = Mean Cell Volume, MCH = Mean Cell Haemoglobin, MCHC = Mean Cell Haemoglobin Concentration

4.12.2. Effect of GOV on Serum Hepatic Enzymes and Chemical Analytes on CCl_4 intoxicated rats

The effect of GOV on serum hepatic enzymes and chemical analytes on CCl_4 intoxicated rats are shown in Tables 30a and 30b. Pretreatment with GOV at a dose of 8 g kg^{-1} decreased the activity of ALP compared to Liv 52 group. At a dose of 4 g kg^{-1}, GOV significantly ($p \leq 0.05$) decreased serum ALT activity compared to the 8 g kg^{-1} group and also its activity compared to Liv 52 and silymarin groups. There was a significant increase in AST activity of GOV at a dose of 8 g kg^{-1} compared to silymarin and control groups. GOV at a dose of 2 g kg^{-1} decreased the level of total bilirubin compared to all the other experimental groups and it was significant ($p \leq 0.05$) compared to the 8 g kg^{-1} and toxin control groups. At 2 and 8 g kg^{-1}, GOV exhibited a decrease in GGT activities compared to Liv 52 group. The LDH activity of the Liv 52 group was increased compared to GOV at 4 and 8 g kg^{-1}.

Administration of GOV at a dose of 4 g kg^{-1} exhibited the lowest concentration of cholesterol and BUN compared to all the other groups. It showed a decreased concentration in triglyceride level and an increased concentration in total protein compared to Liv 52 group. It also demonstrated almost the same level of albumin concentration as the Liv 52 group. The level of creatinine concentration exhibited by GOV at a dose of 8 g kg^{-1} is comparable to those of Liv 52 and silymarin groups while its triglyceride concentration is decreased compared to Liv 52 group.

Table 30a: Serum activities of ALP, ALT, AST, GGT and LDH in rats pretreated GOV before CCl_4 damage.

Group	Dose ($g\ kg^{-1}$)	LIVER FUNCTION ENZYMES				
		ALP (U/L)	**ALT (U/L)**	**AST (U/L)**	**GGT (U/L)**	**LDH (U/L)**
Control (grp 1)		$30.5 \pm 0.88^{(b, c)}$	$16.4 \pm 0.8^{(b, c, e)}$	$11.83 \pm 1^{(b, c, d, e)}$	$122.38 \pm 6.37^{(b)}$	$8.29 \pm 0.49^{(b)}$
Toxin Control (Grp 2)		$92.93 \pm 2.94^{(a, c, d, e)}$	$74.23 \pm 3.22^{(a, c, d, e)}$	$76.69 \pm 5.29^{(a, c, d, e)}$	$236.5 \pm 10.91^{(a, c, d, e)}$	$34.18 \pm 2.19^{(a, c, d, e)}$
GOV + CCl_4 (Grp 3)	2	$49.81 \pm 6.49^{(b)}$	$34.16 \pm 2.78^{(a, b)}$	$28.46 \pm 0.85^{(a, b)}$	$149.59 \pm 10.45^{(b)}$	$12.47 \pm 1^{(b)}$
(Grp 4)	4	$43.32 \pm 1.45^{(b)}$	$26.76 \pm 0.25^{(b, e)}$	$27.53 \pm 1.17^{(a, b)}$	$161.54 \pm 13.05^{(b)}$	$11.64 \pm 0.88^{(b)}$
(Grp 5)	8	$40.43 \pm 4.79^{(b)}$	$40.48 \pm 4.13^{(a, b, d)}$	$37.67 \pm 5.79^{(a, b)}$	$146.57 \pm 11.05^{(b)}$	$10.59 \pm 1.69^{(b)}$
LIV 52 + CCl_4 (Grp 6)	0.3	$42.48 \pm 1.55^{(b)}$	$34.98 \pm 3.39^{(a, b)}$	$25.85 \pm 2.4^{(b)}$	$153.99 \pm 10.52^{(b)}$	$11.69 \pm 1.27^{(b)}$
Silymarin + CCl_4 (Grp 7)	0.3	$36.09 \pm 2.5^{(b)}$	$30.27 \pm 2.43^{(a, b)}$	$22.23 \pm 1.45^{(b, e)}$	$132.79 \pm 14.68^{(b)}$	$9.35 \pm 0.93^{(b)}$

Values are expressed as Mean ± SEM for seven rats. The Mean difference is significant at the .05 level. (a) = $p \leq 0.05$ as compared with the normal control group. (b) = $p \leq 0.05$ as compared to CCl_4 control group. (c) = $p \leq 0.05$ as compared with the GOV + CCl_4 (2000mg kg^{-1}) group. (d) = $p \leq 0.05$ as compared with the GOV + CCl_4 (4000mg kg^{-1}) group. (e) = $p \leq 0.05$ as compared with the GOV + CCl_4 (8000mg kg^{-1}) group. The significance of differences among all groups was determined by the Tukey HSD test.

ALP = Alkaline phosphatase, ALT = Alanine aminotransferase, AST = aspartate aminotransferase, GGT = L-γ-glutamyltransferase and LDH = Lactate dehydrogenase

Table 30b: Serum levels of albumin, cholesterol, creatinine, total protein, triglyceride and BUN in rats pretreated with GOV before CCl_4 damage.

		BIOCHEMICAL PARAMETERS					
Group	**Dose (g kg^{-1})**	**TP** (g/L)	**ALB** (g/L)	**BUN** (mmol/L)	**CREA** (mmol/L)	**TG** (mmol/L)	**CHO** (mmol/L)
Control (grp 1)		98.85 ± 5.03$^{(b)}$	52.17 ± 1.5$^{(b, c, d, e)}$	6.08 ± 0.65	71.91 ± 4.5$^{(b)}$	1.09 ± 0.07$^{(b)}$	2.65 ± 0.21$^{(b)}$
Toxin Control (Grp 2)		53.84 ± 3.07$^{(a, c, d, e)}$	23.47 ± 1.05$^{(a, c, d, e)}$	8.07 ± 0.33$^{(d)}$	105 ± 3.98$^{(a, c, d, e)}$	2 ± 0.19$^{(a, c, d, e)}$	3.6 ± 0.21$^{(a, d, e)}$
GOV + CCl_4 (Grp 3)	2	83.38 ± 3.62$^{(b)}$	40.14 ± 2.24$^{(a, b)}$	6.02± 0.31	74.28 ± 2.92$^{(b)}$	1.21 ± 0.09$^{(b)}$	2.8 ± 0.14
(Grp 4)	4	85.21 ± 2.88$^{(b)}$	41.88 ± 1.38$^{(a, b)}$	5.96± 0.44$^{(b)}$	75.24 ± 1.81$^{(b)}$	0.92 ± 0.05$^{(b)}$	2.5 ± 0.14$^{(b)}$
(Grp 5)	8	83.25 ± 3.76$^{(b)}$	40.14 ± 3.27$^{(a, b)}$	6.73± 0.46	75.56 ± 3.26$^{(b)}$	1.07± 0.15$^{(b)}$	1.98 ± 0.28$^{(b)}$
LIV 52 + CCl_4 (Grp 6)	0.3	84.97 ± 3.64$^{(b)}$	42.97± 1.23$^{(a, b)}$	6.49 ± 0.34	76.49 ± 2.03$^{(b)}$	1.17± 0.13$^{(b)}$	2.25 ± 0.31$^{(b)}$
Silymarin + CCl_4 (Grp 7)	0.3	85.76 ± 3.32$^{(b)}$	44.4 ± 2.14	6.09 ± 0.65	76.84 ± 1.6$^{(b)}$	0.9 ± 0.09$^{(b)}$	2.35 ± 0.13$^{(b)}$

Values are expressed as Mean ± SEM for seven rats. The Mean difference is significant at the 0.05 level. (a) = $p \leq 0.05$ as compared with the normal control group. (b) = $p \leq 0.05$ as compared to CCl_4 control group. (c) = $p \leq 0.05$ as compared with the GOV + CCl_4 (2 g kg^{-1}) group. (d) = $p \leq 0.05$ as compared with the GOV + CCl_4 (4 g kg^{-1}) group. (e) = $p \leq 0.05$ as compared with the GOV + CCl_4 (8 g kg^{-1}) group. The significance of differences among all groups was determined by the Tukey HSD test.

TP = Total protein, ALB = Albumin, BUN = Blood Urea Nitrogen, CREA = Creatinine, TG = Triglyceride, CHO = Cholesterol

4.12.3. The effect of CCl_4 damage on serum antioxidant enzymes in rats pretreated with GOV

The activities of antioxidant enzymes CAT, GPx, GSH, GST, MDA, SOD and total protein after intr aperitoneal administration of CCl_4 are depicted in tables 31. It is observed that, GOV administration resulted in a significant ($p \leq 0.05$) elevation in the levels of CAT, GPx, GSH, GST, SOD and total protein and significant ($p \leq 0.05$) decrease in lipid peroxidation compared to the toxin control group. Compared to Liv 52 and silymarin, administration of GOV at 4 g kg^{-1} exhibited almost the same CAT and SOD activities while the GSH activity was higher. GOV at a dose of 8 g kg^{-1} and Liv 52 group has the same GSH activities. Pretreatment with GOV at 4 g kg^{-1}exhibited higher GSH activity compared to Liv 52 and silymarin groups while the MDA activity was lower than that exhibited by Liv 52. It also showed almost the same SOD activity compared to Liv 52 and silymarin. GOV at a dose of 2 g kg^{-1} showed significantly ($p \leq 0.05$) attenuated CAT, GSH and SOD activities compared to control, Liv 52, silymarin and GOV at 4 g kg^{-1} groups.

Table 31: The effect of CCl_4 damage on serum antioxidant enzymes in rats pretreated with GOV

Group	Dose (g kg^{-1})	CAT (µmol/min/mg protein)	GPx (µmol/ml)	GSH (µmol/ml)	GST (µmol/ml)	MDA (nmol/ml)	SOD (µmol/ml)	TP (g/l)
Control (grp 1)		87.41 ± 3.36$^{(b, c, d, e)}$	14.96 ± 1.11$^{(b, c, d, e)}$	13.38 ± 0.67$^{(b, c, d, e)}$	126.46 ± 1.66$^{(b, c, d, e)}$	4.92 ± 0.1$^{(b, c, d, e)}$	92.13 ± 3.4$^{(b, c, d, e)}$	98.85 ± 5.03$^{(b, c, d, e)}$
Toxin Control (Grp 2)		22.22 ± .93$^{(a, c, d, e)}$	2.46 ± 0.01$^{(a, c, d, e)}$	4.27 ± .2 $^{(a, c, d, e)}$	39.23 ± 1.76 $^{(a, c, d, e)}$	16.29 ± 0.64 $^{(a, c, d, e)}$	21.21 ± 1.27$^{(a, c, d, e)}$	53.84 ± 3.07 $^{(a, c, d, e)}$
GOV + CCl_4 (Grp 3)	2	52.01 ± 2.75$^{(a, b, d)}$	8.65 ± 0.39$^{(a, b)}$	7.14 ± 0.4$^{(a, b, d, e)}$	89.86 ± 8.54$^{(a, b)}$	9.2 ± 0.47$^{(a, b)}$	55.89 ± 2.81 $^{(a, b, d)}$	74.8 ± 1.3$^{(a, b)}$
(Grp 4)	4	67.13 ± 1.95$^{(a, b, c)}$	9.26 ± 0.24$^{(a, b)}$	10.25 ± 0.24$^{(a, b, c)}$	97.38 ± 3.04$^{(a, b)}$	7.55 ± 0.32$^{(a, b)}$	77.6 ± 1.85$^{(a, b, c)}$	79.49 ± 1.77$^{(a, b)}$
(Grp 5)	8	61.14 ± 1.88$^{(a, b)}$	9.38 ± 0.55$^{(a, b)}$	9.8 ± 0.34$^{(a, b, c)}$	92.12 ± 4.96$^{(a, b)}$	8.68 ± 0.15 $^{(a, b)}$	66.61 ± 3.31 $^{(a, b)}$	77.71 ± 1.21$^{(a, b)}$
LIV 52 + CCl_4 (Grp 6)	0.3	68.23 ± 3.53$^{(a, b, c)}$	9.49 ±. 5$^{(a, b)}$	9.8 ± 0.34$^{(a, b, c)}$	102.84 ± 3.64$^{(a, b)}$	7.71 ± 0.46 $^{(a, b)}$	78.62 ± 1.42$^{(a, b, c, e)}$	82.11 ± 2.74$^{(a, b)}$
Silymarin + CCl_4 (Grp 7)	0.3	69.87 ± 2.19 $^{(a, b, c)}$	10.58 ± 0.51$^{(a, b)}$	10.04 ± 0.33$^{(a, b, c)}$	111.02 ± 7.46$^{(b)}$	6.86 ± 0.44$^{(a, b, c, e)}$	79.88 ± 2.89$^{(a, b, c, e)}$	81.24 ± 2.08$^{(a, b)}$

Values are expressed as Mean ± SEM for seven rats. The Mean difference is significant at the .05 level. (a) = $p \leq 0.05$ as compared with the normal control group. (b) = $p \leq 0.05$ as compared to CCl4 control group. (c) = $p \leq 0.05$ as compared with the GOV + CCl4 (2 g kg^{-1}) group. (d) = $p \leq 0.05$ as compared with the GOV + CCl4 (4 g kg^{-1}) group. (e) = $p \leq 0.05$ as compared with the GOV + CCl4 (8g kg^{-1}) group. The significance of differences among all groups was determined by the Tukey HSD test.

CAT = Catalase, GPx = Glutathione Peroxidase, GSH = Reduced Glutathione, GST = Glutathione-S-Transferase, MDA = Malondialdehyde, SOD = Superoxide Dismutase, TP = Total Protein

Results presented in Table 32 indicates that the elevated level of MDA due to CCl_4 intoxication were significantly ($p \leq 0.05$) attenuated in rats, after treatment with GOV. GOV exhibited lower MDA activity compared the standard reference drug groups. The GOV pretreated rats in the 4 g kg^{-1} group, showed increased GPx activity compared to the standard reference drugs. It also showed significantly ($p \leq 0.05$) decreased SOD activity compared to the control group. At 2 g kg^{-1} and 8 g kg^{-1}, GOV showed significant ($p \leq 0.05$) decrease in the concentration of total protein compared to the standard reference drugs. The catalase activity of GOV was significantly ($p \leq 0.05$) decreased compared to control and standard reference drug groups.

Table 32: The effect of CCl_4 damage on liver antioxidant enzymes in rats pretreated with GOV

Group	Dose (g kg^{-1})	CAT (µmol/min/mg protein)	GPx (µmol/ml)	GSH (µmol/ml)	GST (µmol/ml)	MDA (nmol/ml)	SOD (µmol/ml)	TP (g/l)
Control (grp 1)		210.73 ± 10.96$^{(b, c, d, e)}$	55.12 ± 4.49$^{(b)}$	137.53 ± 14. 2$^{(b, d, e)}$	296.91 ± 31.32$^{(b)}$	13.64 ± 1.27$^{(b)}$	106.65 ± 4.04$^{(b, c, d, e)}$	114.79 ± 5.06$^{(b, c, d, e)}$
Toxin Control (Grp 2)		69.8 ± 5.89 $^{(a, c, d, e)}$	28.57 ± 0.99$^{(a, c, d, e)}$	58.94 ± 4.77$^{(a, c, e)}$	67.86 ± 5.12$^{(a, c, d, e)}$	36.35 ± 3.37 $^{(a, c, d, e)}$	52.76 ± 4.45$^{(a, c, d, e)}$	56.07 ± 2.73$^{(a, c, d, e)}$
GOV + CCl_4 (Grp 3)	2	148.03 ± 1.6$^{(a, b)}$	43.37 ± 1.31$^{(b)}$	91.06 ± 2.28$^{(a, b)}$	222.45 ± 15.86 $^{(b)}$	15.59 ± 0.89$^{(b)}$	85.68 ± 2.1$^{(a, b)}$	80.15 ± 2.23$^{(a, b)}$
(Grp 4)	4	151.98 ± 6.86$^{(a, b)}$	46.81 ± 4.01 $^{(b)}$	103.52 ± 12.21$^{(b)}$	250.43 ± 16.89$^{(b)}$	15.6 ± 1.71$^{(b)}$	80.7 ± 2.65$^{(a, b)}$	84.25 ± 3.15$^{(a, b)}$
(Grp 5)	8	151.76 ± 2.07$^{(a, b)}$	44.78 ± 2.31 $^{(b)}$	100.57 ± 4.35$^{(a, b)}$	262.41 ± 8.09$^{(b)}$	15.14 ± 1.2$^{(b)}$	84.63 ± 4.78$^{(a, b)}$	77.93 ± 1.03$^{(a, b)}$
LIV 52 + CCl_4 (Grp 6)	0.3	187.98 ± 7.57$^{(b, c, d, e)}$	45.33 ± 2.65$^{(b)}$	115.6 ± 5.01$^{(b)}$	296.33 ± 10.67 $^{(b)}$	16.39 ± 0.8$^{(b)}$	93.89 ± 3.73$^{(b)}$	98.31 ± 3.94$^{(a, b, c, e)}$
Silymarin + CCl_4 (Grp 7)	0.3	190.45 ± 5.81$^{(b, c, d, e)}$	45.71 ± 2.21$^{(b)}$	117.54 ± 4.52$^{(b)}$	287.64 ± 35.15 $^{(b)}$	16.56 ± 0.6 $^{(b)}$	98.28 ± 3.19$^{(b, c)}$	95.03 ± 1.96 $^{(a, b, c, e)}$

Values are expressed as Mean ± SEM for seven rats. The Mean difference is significant at the .05 level. (a) = $p \leq 0.05$ as compared with the normal control group. (b) = $p \leq 0.05$ as compared to CCl4 control group. (c) = $p \leq 0.05$ as compared with the GOV + CCl4 (2 g kg^{-1}) group. (d) = $p \leq 0.05$ as compared with the GOV + CCl4 (4 g kg^{-1}) group. (e) = $p \leq 0.05$ as compared with the GOV + CCl4 (8g kg^{-1}) group. The significance of differences among all groups was determined by the Tukey HSD test.

CAT = Catalase, GPx = Glutathione Peroxidase, GSH = Reduced Glutathione, GST = Glutathione-S-Transferase, MDA = Malondialdehyde, SOD = Superoxide Dismutase, TP = Total Protein

In Table 33, pretreatment of rats with different doses of GOV 1 hr before CCl_4 injection, showed that there was a significant ($p \leq 0.05$) increase in CAT, GPx, GSH, GST, SOD and total protein and also decreased MDA activity compared to the CCl_4 intoxicated toxin group. GOV at a dose of 8 g kg^{-1} showed increased CAT, GPx and SOD activities compared to 2 g kg^{-1} and 4 g kg^{-1}. At a dose of 2 g kg^{-1}, GOV demonstrated a significant ($p \leq 0.05$) decrease in CAT activity compared to toxin control and Liv 52 groups. There was a significant ($p \leq 0.05$) increase in GPx activity in the control group compared to all other groups. At 4 g kg^{-1}, GOV exhibited increased GSH activity and decreased MDA activity compared to Liv 52 group. It was also significantly ($p \leq 0.05$) elevated compared to the 2 g kg^{-1} group. At a dose of 8 g kg^{-1}, GOV showed almost the same level of SOD activity and total protein concentration as the Liv 52 and silymarin groups and the Liv 52 group respectively.

Table 33: The effect of CCl_4 damage on kidney antioxidant enzymes in rats pretreated with GOV

Group	Dose (g kg^{-1})	CAT (µmol/min/mg protein)	GPx (µmol/ml)	GSH (µmol/ml)	GST (µmol/ml)	MDA (nmol/ml)	SOD (µmol/ml)	TP (g/l)
Control (grp 1)		170.1 ± 9.28$^{(b, c)}$	72.27 ± 2.35$^{(b, c, d, e)}$	211.67 ± 13.16$^{(b, c, d, e)}$	258.33 ± 11.51$^{(b, c, d, e)}$	8.41 ± 0.49$^{(b)}$	88.34 ± 5.63$^{(b)}$	88.76 ± 5.07$^{(b, c, d, e)}$
Toxin Control (Grp 2)		60.43 ± 3.68$^{(a, c, d, e)}$	26.07 ± 2.83$^{(a, c, d, e)}$	51.93 ± 3.67$^{(a, c, d, e)}$	57.79 ± 5.18$^{(a, d, e)}$	25.73 ± 2.14$^{(a, c, d, e)}$	43.28 ± 5.38$^{(a, c, d, e)}$	44.56 ± 2.91$^{(a, c, d, e)}$
GOV + CCl_4 (Grp 3)	2	114.42 ± 10.65$^{(a, b)}$	47.98 ± 3.08$^{(a, b)}$	79.89 ± 2.23$^{(a, b, d)}$	95.38 ± 3.89$^{(a)}$	10.94 ± 0.42$^{(b)}$	72.37 ± 2.69$^{(b)}$	66.09 ± 2.12$^{(a, b)}$
(Grp 4)	4	158.89 ± 21.15$^{(b)}$	52.8 ± 1.96$^{(a, b)}$	145.4 ± 18.26$^{(a, b, c)}$	156.19 ± 20.78$^{(a, b)}$	9.9 ± 0.44$^{(b)}$	70.45 ± 2.31$^{(b)}$	69.71 ± 2.78$^{(a, b)}$
(Grp 5)	8	159.9 ± 7.8$^{(b)}$	54.31 ± 1.74$^{(a, b)}$	117.7 ± 5.57$^{(a, b)}$	145 ± 21.14$^{(a, b)}$	11.03 ± 0.43$^{(b)}$	74.68 ± 2.56$^{(b)}$	69.79 ± 2.1$^{(a, b)}$
LIV 52 + CCl_4 (Grp 6)	0.3	167.79 ± 12.24$^{(b, c)}$	57.36 ± 3.41$^{(a, b)}$	137.36 ± 13.76$^{(a, b, c)}$	183.86 ± 26.87$^{(a, b, c)}$	10.25 ± 0.55$^{(b)}$	76.57 ± 4.82$^{(b)}$	71.98 ± 3.55$^{(a, b)}$
Silymarin + CCl_4 (Grp 7)	0.3	163.43 ± 7.99$^{(b)}$	57.2 ± 1.48$^{(a, b)}$	188.41 ± 2.68$^{(b, c, e)}$	189.76 ± 11.06$^{(b, c)}$	9.66 ± 0.65$^{(b)}$	75.99 ± 4.59$^{(b)}$	77.92 ± 4.11$^{(b)}$

Values are expressed as Mean ± SEM for seven rats. The Mean difference is significant at the .05 level. (a) = $p \leq 0.05$ as compared with the normal control group. (b) = $p \leq 0.05$ as compared to CCl4 control group. (c) = $p \leq 0.05$ as compared with the GOV + CCl4 (2 g kg^{-1}) group. (d) = $p \leq 0.05$ as compared with the GOV + CCl4 (4 g kg^{-1}) group. (e) = $p \leq 0.05$ as compared with the GOV + CCl4 (8g kg^{-1}) group. The significance of differences among all groups was determined by the Tukey HSD test.

CAT = Catalase, GPx = Glutathione Peroxidase, GSH = Reduced Glutathione, GST = Glutathione-S-Transferase, MDA = Malondialdehyde, SOD = Superoxide Dismutase, TP = Total Protein

4.12.4. Caspase - 2, 3 and 9 activities in the white blood cell of rats treated with CCl_4

Table 34 shows the results of the colorimetric assay of caspase - 2, 3 and 9 activities in the white blood cell of rats pretreated with CCl_4. GOV significantly attenuated the fold increase resulting from CCl_4 induced hepatotoxicity compared to the toxin control. At 4 g kg^{-1}, GOV demonstrated lower caspase 2 and 9 activities compared to Liv 52.

Table 34: Caspase - 2, 3 and 9 activities in the white blood cell of rats treated with CCl_4

Group	Dose ($g\ kg^{-1}$)	Caspase 2 (Units/mg of total protein).	Caspase 3 (Units/mg of total protein)	Caspase 9 (Units/mg of total protein)
Control (Grp 1)		$7.1 \pm 0.39^{(b, c, d, e)}$	$5.95 \pm 0.43^{(a, c, d, e)}$	$6 \pm 0.44^{(a, c, d, e)}$
Toxin control (Grp 2)		$29.36 \pm 1.18^{(a, c, d, e)}$ (3.14)	$29.85 \pm 1.57^{(a, c, d, e)}$ (4.02)	$26.8 \pm 0.96^{(a, c, d, e)}$ (3.47)
GOV + CCl_4 (Grp 3)	2	$11.11 \pm 0.7^{(a, b)}$ (0.57)	$11.22 \pm 0.57^{(a, b)}$ (0.89)	$11.25 \pm 0.37^{(a, b)}$ (0.88)
(Grp 4)	4	$10.58 \pm 0.41^{(a, b)}$ (0.49)	$10.87 \pm 0.35^{(a, b, e)}$ (0.83)	$9.89 \pm 0.36^{(a, b)}$ (0.65)
(Grp 5)	8	$11.88 \pm 0.62^{(a, b)}$ (0.67)	$11.04 \pm 0.95^{(a, b, d)}$ (0.86)	$11.16 \pm 0.6^{(a, b)}$ (0.86)
LIV 52 + CCl_4 (Grp 6)	0.3	$10.92 \pm 0.53^{(a, b)}$ (0.54)	$10. 78 \pm 0.54^{(a, b, e)}$ (0.81)	$9.97 \pm 0.49^{(a, b)}$ (0.66)
Silymarin + CCl_4 (Grp 7)	0.3	$10.02 \pm 0.45^{(a, b)}$ (0.41)	$9.88 \pm 0.47^{(a, b, c, e)}$ (0.66)	$7.88 \pm 0.4^{(b, c, d, e)}$ (0.31)

Values are expressed as Mean ± SEM for five rats. The Mean difference is significant at the .05 level. (a) = $p \leq 0.05$ as compared with the normal control group. (b) = $p \leq 0.05$ as compared to CCl_4 control group. (c) = $p \leq 0.05$ as compared with the GOV + CCl_4 ($2\ g\ kg^{-1}$) group. (d) = $p \leq 0.05$ as compared with the GOV + CCl_4 ($4\ g\ kg^{-1}$) group. (e) = $p \leq 0.05$ as compared with the GOV + CCl_4 ($8\ g\ kg^{-1}$) group. The significance of differences among all groups was determined by the Tukey HSD test. The figures in parenthesis = fold-increase in caspase activity compared to the uninduced control.

4.12.5. Histopathology of rats pretreated with GOV before CCl_4 damage

The photomicrographs of liver sections of the experimental rat from the different groups stained with haemotoxylin and eosin (x100) are shown below. The Control shows the normal architecture while the carbon tetrachloride treated control rats showed steatosis and necrosis. At 2 g kg^{-1} GOV showed moderate restoration of liver architecture while at 4 g kg^{-1} it demonstrated almost normal of liver architecture. The Liv 52 and Silymarin groups exhibited significant restoration of liver architecture comparable with control rat group.

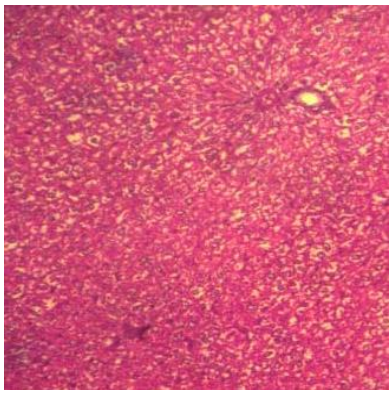


Plate 19:Control rats (Group 1) showing normal architecture

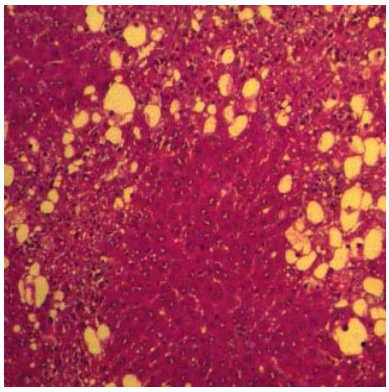


Plate 20: Carbon tetrachloride-treated rats (Group 2) showing steatosis and necrosis

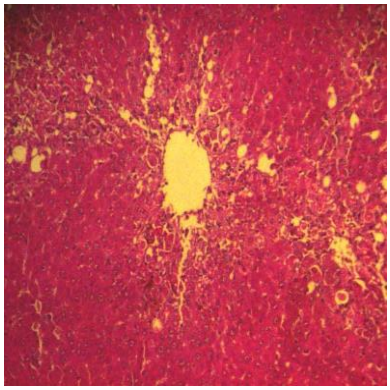


Plate 21: Carbon tetrachloride plus GOV (2 g kg^{-1})-treated rats (Group 3) showing moderate restoration of liver architecture

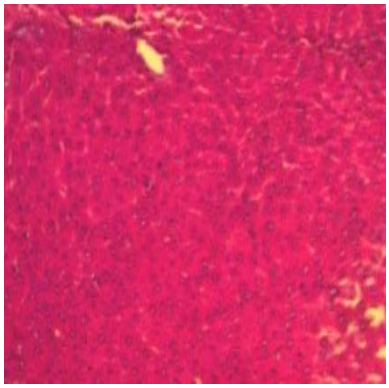


Plate 22: Carbon tetrachloride plus GOV (4 g kg^{-1})-treated rats (Group 4) showing almost normal of liver architecture

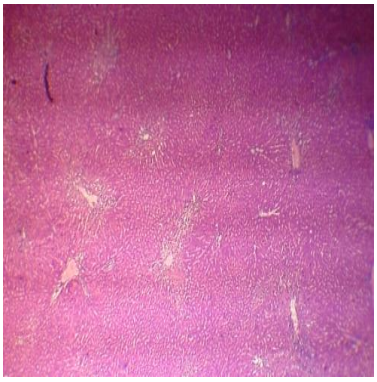


Plate 24: Silymarin-treated rats (Group 7) showing significant restoration of liver architecture comparable with normal rat

<u>Liver sections of rat stained with haemotoxylin and eosin (x100).</u>

4.13. **Antiproliferative activity of GOV towards HepG2 hepatoma, CNE2 and SUME-α nasopharyngeal cells (*in vitro* studies)**

The 50 % ethanolic extract and fractions (butanol, chloroform, ethyl acetate, hexane and water) of GOV were tested for antiproliferative activity toward hepatoma HepG2 cells, CNE2 and SUME-α nasopharyngeal cancer cells. The inhibition of proliferation by the different fractions of GOV followed a time-dependent manner. The antiproliferative activity of GOV towards HepG2 hepatoma, CNE2 and SUME-α nasopharyngeal cells at 24 and 48 hours (hr) are shown in table 35.

It was observed that GOV exerted anti-proliferative activity in HepG2, CNE2 and SUME cells with an IC_{50} value of 41, 1455 and 342 μg ml^{-1} respectively at 48 hr. The following fractions were highly potent toward HepG2 cells, with an IC_{50} value below 200 μg: M2, M3, M5 and M6. M1 and M4 were less potent (500 μg>IC_{50}>200 μg).

As regards the antiproliferative activity toward CNE2 cells, M1, M2, M3, M4, M5, M6 and Me were not potent (500 μg>IC_{50}>200 μg) at 24 and 48 hr. The antiproliferative activity of GOV and its fractions toward SUME-α nasopharyngeal cells, showed that M3 was potent (500 μg>IC_{50}>200 μg) while M1, M2, M4 and M6 were less potent (1000 μg>IC_{50}>500 μg).

Onset of action and the percentage survival of cells have been taken into consideration in order to categories the potency of GOV. The inhibition followed a time-dependent manner and there was a difference in the ranking of antiproliferative potencies of the various fractions when the data collected after 24 hr of incubation were compared with those collected after 48 hr of incubation. Since it took more than 2500 μg for many fractions to achieve 50 % inhibition after 24 hr of incubation, the ranking of IC_{50} values after 48 hr of incubation was selected for the purpose of potency comparison. Within the three cancer cell lines used in this experiment, GOV was more effective against HepG2 cells with IC_{50} 41 μg/ml compared to CNE1 and SUME-α-nasopharyngeal cells.

Table 35: Antiproliferative activity of GOV towards HepG2 hepatoma cells, CNE2 and SUME-α nasopharyngeal cells

	Antiproliferative activity toward HepG2 hepatoma cells		Antiproliferative activity toward CNE2 nasopharyngeal cells		Antiproliferative activity toward SUME-α-nasopharyngeal cells	
	IC_{50} at 24hr (µg)	**IC_{50} at 48hr (µg)**	**IC_{50} at 24hr (µg)**	**IC_{50} at 48hr (µg)**	**IC_{50} at 24hr (µg)**	**IC_{50} at 48hr (µg)**
Me	>2500	1975	>2500	>2500	>2500	1000
M1	>2500	251	>2500	1455	>2500	611
M2	>2500	73	>2500	1831	>2500	928
M3	2000	130	>2500	2206	2000	342
M4	>2500	448	>2500	>2500	>2500	799
M5	>2500	60	>2500	>2500	>2500	1021
M6	>2500	41	>2500	>2500	>2500	533

M1 – butanol fraction of GOV **M2 – chloroform fraction of GOV**
M3 – ethyl acetate fraction of GOV **M4 – hexane fraction of GOV**

M5 – water fraction of GOV **M6 – 50% ethanolic extract of GOV**

Me – ethanolic extract of a mixture of *G. latifolia, O. gratissimum* and *V. amygdalina*.

Antiproliferative activity of GOV toward HepG2 hepatoma cells:

Ranking of potencies at 24h: M3> the rest.

Ranking of potencies at 48h: M6>M5>M2>M3>M1>M4 >Me

Antiproliferative activity of GOV toward CNE2 nasopharyngeal cells:

Ranking of potencies at 24h: all are equal

Ranking of potencies at 48h: M1>M2>M3

Antiproliferative activity of GOV toward SUME-α nasopharyngeal cell:

Ranking of potencies at 24h: M3> the rest

Ranking of potencies at 48h: M3>M6>M1>M4 >Me>M5

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4.14. *in vitro* antioxidant assays

4.14.1. Inhibition of erythrocyte haemolysis using Swiss mice

Table 36 shows anti-oxidant activity of GOV and its fraction using red blood cell. In the screening of various fractions of GOV and ethanolic extracts of a mixture of *G. latifolia, O. gratissimum* and *V. amygdalina* for antioxidant activity using the assay of inhibition of haemolysis of mice erythrocytes, it was found that M1, M2, M3, M5, M6 and Me exhibited antioxidant activity. A 40% inhibition of erythrocyte haemolysis or more was taken as the cut-off point for possession of antioxidant activity.

Table 36: Anti-oxidant activity of GOV and its fraction using Red Blood Cell.

	Burst	Protected	Burst %	Protected %
M1	0.157 ± 0.012	0.917 ± 0.115	17.12	82.88
M2	0.295 ± 0.005	0.86 ± 0.016	34.3	65.7
M3	0.225 ± 0.016	0.889 ± 0.013	25.31	74.69
M4	0.397 ± 0.011	0.467 ± 0.014	85.01	14.99
M5	0.034 ± 0.002	0.87 ± 0.068	3.91	96.09
M6	0.03 ± 0.012	0.89 ± 0.068	3.39	96.61
Me	0.44 ± 0.076	0.917 ± 0.082	47.98	52.02
Positive	0.058 ± 0.004	0.648 ± 0.022	8.95	91.05
Negative	0.442 ± 0.023	0.491 ± 0.011	90.02	9.98

Values are expressed as Mean ± SEM for seven mice.

M1 – butanol fraction of GOV **M2 – chloroform fraction of GOV**

M3 – ethyl acetate fraction of GOV **M4 – hexane fraction of GOV**

M5 – water fraction of GOV **M6 – 50% ethanolic extract of GOV**

Me – ethanolic extract of a mixture of *G. latifolia, O. gratissimum* and *V. amygdalina*.

4.14.2. Lipid Peroxidative Activity of GOV using Brain Homogenates of Wistar Albino Rats

Lipid peroxidative activity of GOV using brain homogenates of Wistar albino rats are shown in Table 37. When the extracts and fractions of GOV were tested for antioxidant activity using rat brain homogenate, it was found that only fractions all the extracts and fractions manifested sufficiently high antioxidant activity to allow estimation of the IC_{50} value (i.e. the concentration that brings about 50 % inhibition). All other fractions had very low inhibition activity on oxidation in rat brain homogenate.

Table 37: Lipid Peroxidative Activity of GOV using Brain Homogenates of Wistar Albino Rats

Sample	10 mg/ml	% Inhibition
M1	0.055	93.86
M2	0.055	93.86
M3	0.059	93.41
M4	0.055	93.86
M5	0.056	93.74
M6	0.056	93.74
Me	0.055	93.85
Negative control	0.895	0
Positive control	0.041	95.42

Values are expressed as Mean ± SEM for seven mice.

CHAPTER FIVE
DISCUSSION

Preliminary phytochemical screening of GOV revealed the presence of different phytoconstituents like flavonoids, triterpenoids, saponins, terpenoids and phenolics and alkaloids and these compounds are known to possess antioxidant and hepatoprotective activity.

Thin layer chromatographic (TLC) analysis of GOV, the ethanolic and water extracts of *G. latifolia, O. gratissimum* and *V. amygdalina* showed that water is not the best solvent for extracting polyphenolic compounds. This is in concordance with the findings reported by Marwah *et al*. (2007) that aqueous alcohols are the best solvents for extracting polyphenolic compounds from plant materials. Stigmasterol which is one of the phytoconstituents suspected has been reported to possess anti-osteoarthritic (Gabay, *et al*., 2010), antioxidant, hypoglycemic and thyroid inhibiting properties (Panda *et al*., 2009) while β-sitosterol another compound suspected, exhibits anti-inflammatory activity in human aortic endothelial cells (Loizou *et al.*, 2010) and is used in Europe for the treatment of breast cancer (Awad, *et al* 2000). Rutin is an antioxidant as reported by Jaganath *et al*., (2009), shows antinociceptive (Lapa *et al* 2009) and anti-inflammatory activity in some animal and *in vitro* models (Chan, *et al*., 2007). Hyperoside could have a protective antioxidant effect on cultured PC12 cells (a cell line derived from a pheochromocytoma of the rat adrenal medulla) (Zhiyong *et al*., 2005) and also in lung fibroblast cells (Piao, *et al*., 2008). Eugenol shows protective activity against oxidized LDL-induced cytotoxicity and adhesion molecule expression in endothelial cells (Ou, *et al* 2006) and exhibited antioxidant, anti-inflammatory and DNA-protective properties in thioacetamide-induced liver injury in rats (Yogalakshmi, *et al*., 2010). Ascorbic acid selectively kills cancer cells and acts as a pro-drug to deliver hydrogen peroxide to tissues (Chen, *et al*., 2005) while linoleic acid demonstrates antiapoptotic activities (Miner, *et al*., 2001). Borneol exhibits antihypertensive and antioxidant activities (Kumar, *et al*., 2010).

The antioxidant, hepatoprotective, antiproliferative, anti-inflammatory, antinociceptive and antiapoptotic activities of GOV could be adduced to the presence of some of these compounds identified from our phytoscreening.

Saturation-transfer difference NMR spectroscopy (STD NMR) studies of the various fractions showed many areas of interest. Initial proton NMR experiments showed the presence of various aromatic compounds in each fraction, due to proton NMR signals between 6-8 ppm. Representative proton NMR spectra of the butanol fraction of GOV are shown in Figures 4. Proton NMR spectra of the other fractions also showed many aromatic protons in the 6-8 ppm range. Other areas of the spectra showing signals, the carbohydrate region, 3-4.5 ppm and the methyl region, 0.5-1 ppm, showed numerous peaks. Previous STD studies have led to familiarity in irradiating DHPR at -0.5 ppm to excite the protein (Ge and Sem, 2007). Because of the anisotropic effect in proteins, some signals can show up in the negative scale of the spectrum. Often these are methylene signals and can be characteristic of lipids. Knowing that these plants produce medicinally relevant chemicals and that aromatic type substructures must be present, it was reasonable to test these fractions for binding against a drug target of interest- initially, we are screening against DHPR. These regions of interest require further experimentation to examine the nature of the origin of the signal (ie. if it indicates binding or if it comes from irradiation of the protein).

The models (hot-plate, tail-flick, mouse writhing test, formalin, capsaicin, carrageenan, turpentine, UV-irradiation or Freund's complete adjuvant) of inflammatory or neuropathic pain could be used to identify effective and potent drugs that may be successful in the clinic while leaving the animals in good health and without any visible changes e.g. excessive weight loss, distress or any behavioural changes.

The analgesic activity assessed by hot plate test, formalin induced pain test and acetic acid-induced writhing test to ascertain if GOV possesses peripheral and/or central action. Aspirin interferes with synthesis of prostaglandins and thromboxanes by irreversibly inhibiting cyclo-oxygenase 1 (COX-1) and modifies the enzymatic activity of COX-2 (Wu, 2003). Aspirin-modified COX-2 produces lipoxins, most of which are anti-inflammatory. Morphine acts directly on the central nervous system (CNS) to relieve pain. Commonly used non-steroidal anti-inflammatory drugs (NSAID) such as aspirin and indomethacin are widely used to reduce swelling associated with pain and inflammation through inhibition of prostaglandin synthesis by direct effect on cyclo-oxygenase (COX) in the arachidonic acid metabolism (Nwafor and Okwuasaba, 2003).

Acute pain is modeled by the hot-plate test while persistent pain by the formalin tests. The hot plate method is very effective for evaluating drugs possessing analgesic property, which act centrally (Al-Naggar *et al*., 2003). Prolongation of reaction time in hot plate test inferred possible central analgesic effects of GOV. The acetic acid induced mouse writhing test is used for rapid evaluation of peripheral type of analgesic action in drugs (Viana *et al*., 2000). The abdominal constriction is related to the sensitization of nociceptive receptors to prostaglandins. Therefore, GOV may have exhibited analgesic effect in mice by inhibiting the acetic acid induced writhes, which is a model of visceral pain, as much as that of the standard drug used-acetylsalicyclic acid. According to Rang *et al*., (1999), acetic acid has been reported to cause hyperalgesia by liberating endogenous substances such as prostaglandins, leukotrieines, 5-HT, histamine, kinins, H^+ and K^+, etc. which have been implicated in the mediation of pain perception (Oyemitran, *et al.,* 2008). Consequently it is suggested that GOV may be exhibiting its analgesic effects in a similar manner to opioids or opiates. Therefore, it is likely that one of the bioactive components of GOV may be useful as an analgesic.

It had been reported that nociceptive activity of the first phase of the formalin test is mediated by the effect on peripheral nociceptors activating primary afferent fibers to transmit the pain signals while the second phase occurs through the activation of ventral horn neurons at the spinal cord levels (Ridtitid *et al*., 2008). Opioid analgesics have been reported to possess antinociceptive effects in both phases having more effect at the 2nd phase (Le Bars *et al*., 2001). GOV has analgesic effects at both phases of the formalin induced paw-licking episodes in mice (Iroanya *et al.,* 2010), pointing to the central mediation of the anti-inflammatory activity of GOV due to its effects on the 2nd phase of the reaction. The results further demonstrated that GOV has comparable effects with the standard drugs used. GOV dose dependently showed significant antinociceptive activity, indicating that it possessed a significant anti-oedematogenic effect that might be interfering with the prostaglandin pathways.

Inflammation is a pathophysiologic response of living tissue to injuries that leads to the local accumulation of plasmatic fluid and blood cells or the reaction of the body to tissue injury or infection. Carrageenan induced rat paw oedema is a multimediated phenomenon that liberates diversity of mediators and is taken as a prototype of exudative phase of inflammation. It is believed to be biphasic; the first phase (1 h) involves the release of serotonin and histamine while the second phase (over 1 h) is caused by the release of bradykinin, protease, prostaglandin, and lysosome (Perianayagam *et al*., 2004). Xylene-induced mouse ear oedema reflects the oedematization during the early stages of acute inflammation, which was probably related with the release and inhibition of the inflammation factors.

Xylene and carrageenan-induced inflammation model are significant predictive tests for anti-inflammatory agents acting by the mediators of acute inflammation (Perianayagam *et al*., 2004). The inhibitory effect of GOV on carrageenan-induced inflammation and xylene induced ear oedema is likely to be due to inhibition of the enzyme cyclooxygenase, leading to the inhibition of prostaglandin synthesis.

The antinociceptive and anti-inflammatory effects of GOV may be attributed to the presence of phytochemicals such as flavonoids, tannins, alkaloids and saponins. These bioactive constituents also present in GOV have been reported to possess antioxidant, analgesic and/or anti-inflammatory properties on different *in vivo* and *in vitro* models.

It has been shown that ethanol intake may lead to oxidative damage in several tissues such as liver, erythrocyte or brain (Hernandez-Munoz, *et al*., 2000). When the amount of alcohol is high in the body, imbalances are created which can lead to hypoglycemia, hyperuricemia, fatty liver, and or hyperlipemia. Blood is a liquid tissue and suspended in the watery plasma are seven types of cells and cell fragments viz red blood cells (RBCs) or erythrocytes, platelets or thrombocytes , five kinds of white blood cells (WBCs) or leukocytes (three kinds of granulocytes - neutrophils, eosinophils, basophils and two kinds of leukocytes without granules in their cytoplasm-lymphocytes and monocytes. The vital function that blood cells perform (such as transport and defence) increases its susceptibility to intoxication by xenobiotics and makes the hematopoeitic system a unique target organ. It ranks with liver and kidney as one of the most important considerations in the risk assessment of potential environmental toxicants or xenobiotics (Adeneye, *et al.,* 2008). Hematological parameters namely PCV, WBC and differentials were monitored in this study because of their diagnostic significance and role in providing information concerning hematological changes caused by drug and toxin-induced toxicity (Patrick-Iwuanyanwu *et al*., 2007).

The significant increase in serum Alkaline Phosphatase (ALP), Alanine Aminotransferase (ALT), Aspartate Aminotransferase (AST), L-γ-Glutamyltransferase (GGT) and Lactate dehydrogenase (LDH) activity levels shows that there is leakage of cellular enzymes into the serum indicating hepatic injury or damage. The observed decrease in the ALP, ALT, AST, GGT and LDH levels and increased total protein level may be attributed to the reduced leakage of these marker

enzymes in serum. It is likely that GOV could be acting by modulating the repair of hepatic injury and or restore cellular permeability thereby lowering the toxicity of ethyl alcohol.

It is possible that GOV dose dependently prevented glutathione depletion by up-regulating the biosynthesis of glutathione in the liver. Many phytochemical constituents such as steroidal saponins could play vital roles as anti-inflammatory agents, in the induction of protein synthesis, and in tissue regeneration and repair (Matsuura, 2001) while flavonoids are the most common and widely distributed group of plant phenolic compounds and are usually very effective antioxidants (Urquiaga and Leighton, 2000). Silymarin has been reported to help stabilize mast cells and inhibit neutrophil migration, Kupffer cells, and the formation of inflammatory prostaglandins and leukotrienes (Luper, 1998). Therefore GOV is likely to produce the same effects since its activities are similar and in some cases better than silymarin. Pretreatment with GOV, Liv 52 and silymarin also showed increased synthesis of protein which may suggest the regeneration of liver.

Inhibition of the generation of free radicals or antioxidation activity is important in the protection against alcohol induced liver damage because it increases free radical induced lipid peroxidative damage, accumulation of granulocytes and other WBCs, which are attracted by lipid peroxidative damage and neoantigens and Inflammatory cytokines secreted by WBCs. The ability of GOV to significantly ($P \leq 0.05$) increase the CAT, GPx, GSH, GST, SOD and total protein activity and significantly ($P \leq 0.05$) deplete the levels of TBARS in both serum and liver tissue after intoxication with ethyl alcohol may be ascribed to decreased lipid peroxidation and improvement of the serum and tissue antioxidant defence enzymes activity levels. GOV may have attenuated the generation of free radicals and also increased the ability to scavenge free radicals thereby stabilizing the structure of the cell membrane by significantly ($P \leq 0.05$) reducing the level of the protective enzymes CAT, GPx, GSH, GST and SOD, previously elevated by ethyl alcohol damage in the serum and liver homogenate.

It is also possible that the mechanism of hepatoprotective action of GOV might be due to its anti-oxidant properties. The significant increase in the level of total protein of rats in GOV, Liv 52 and silymarin groups suggests that there may have been a repair of damaged hepatocytes and restoration of normal functions of liver after alcohol induced hepatotoxicity.

The hepatoprotective formulation may have a role in the process of regeneration, thus, our data indicates that treatment with GOV offers protection against free radical-mediated oxidative stress in serum, liver and kidney of animals subjected to ethanol-induced injury. The antioxidant activity may be by inhibiting the formation of the free radicals or scavenging of the formed radical assisted by the presence of the phenolic compounds.

D-GaIN produces diffuse type of liver injury simulating viral hepatitis (Srinath, *et al*., 2010). Its toxicity increases cell membrane permeability leading to enzyme leakage which eventually causes cell death. Pretreatment with GOV reduced the increased enzyme activities of liver marker enzymes and malondialdehyde induced by D-GaIN damage. D-GaIN causes cholestasis and this can be attributed to its damaging effects on bile ducts. The bile duct obstruction caused pronounced elevation in ALP activity as ALP is mainly produced in the bile duct and its release is enhanced by cholestasis. The significant reduction in ALP on pretreatment with GOV can be attributed to its attenuation of the damaging effect on the bile ducts thereby preventing cholestasis. Elevated levels of serum enzymes are indicative of cellular leakage and loss of functional integrity of cell membrane in liver (Rajkapoor, *et al* 2008). Serum ALP, bilirubin and total protein levels on other hand are related to the function of hepatic cell.

It has been shown that the lowering of serum albumin level is attributed to the reduction of albumin mRNA expression. The reduction in serum albumin concentration in D-GaIN treated rats after GOV treatment could be attributed to its ability to increase the blood flow and irrigation to liver

hence contribute to liver vitality. Serum cholesterol and triglyceride are elevated in cholestasis because metabolic degradation and excretion are impaired. Pretreatment with GOV significantly decreased serum cholesterol and triglyceride concentrations indicating its ability to enhance metabolic degradation and excretion. As D-GalN induced renal failure seems to occur at the end stage of liver cirrhosis, the protective role of GOV against D-GalN induced renal damages is likely to be an indirect effect probably coming into play via the protection against hepatic disorders. Decline in protein synthesis may have arisen due to disruption in synthesis of essential uridylate nucleotides which causes organelle injury and finally cell death, since depletion of essential uridylate nucleotides impede the normal synthesis of RNA. GOV attenuated the decrease in the total serum, kidney and liver proteins demonstrating that it participates in improving the conditions of liver and kidney. It shows that GOV may support optimum metabolic conditions for the high rate of energy dependent recovery processes required for repairing the tissues damaged by D-GaIN intoxication.

The restoration by GOV of the decreased serum, hepatic and renal levels of glutathione as well as decreased activities of glutathione S-transferase and glutathione peroxidase by GaIN towards normalization is suggestive of its hepatoprotective activity. This hepatoprotectivity may consist of maintaining adequate levels of hepatic glutathione for xenobiotics removal and increased blood flow to liver thus increasing its antioxidant capacity.

The protective role of GOV against D-GaIN induced renal damages is likely to be an indirect effect, since GOV possesses hepatoprotective activity, it may first ameliorate liver damage and subsequently the renal disorders are reduced.

Caspases are known to mediate the apoptotic pathway (Kikuchi, *et al*., 2010). The results obtained from this experiment indicated that D-GaIN induced cell death occurs through activation of caspases-2, 3 and 9. Zhivotovsky and Orrenius (2005), reported that genotoxic stress causes activation

of caspase-2 upstream of mitochondria and that this caspase is the apical caspase which is required for apoptosis. In rats, it has been shown that D-GaIN causes apoptosis in the liver by activating caspase-3, which is released to the plasma by secondary necrosis, as indicated by the concomitant AST increase (Sun *et al*., 2003). The reduction in caspase 3 activity by GOV supports the attenuation of AST level earlier observed. Chan *et al*., (2006) and Kang and Reynolds, (2009) reported that cytotoxic stress either from DNA damage or mitochondrial impairment leads to apoptosis via the intrinsic pathway. The intrinsic pathway involves the release of proapoptotic proteins including cytochrome c from the inner membrane of mitochondria to the cytosol leading to activation of caspase-9 (Riedl and Salvesen, 2007). It is likely that GOV may have decreased the extent of cytotoxic stress induced by these toxins by lowering the extent of release of proapoptotic proteins including cytochrome c and subsequent decrease in caspase activity. Thus it is suggested that GOV may inhibit apoptosis by down-regulating caspase-2, 3 and 9 activities.

In this toxicity study, treatment of rats with a toxic dose of acetaminophen caused significant ($p<0.05$) alterations in most of the measured hematological parameters, except for the MCH which were not significantly ($p<0.05$) different (table 23a and 23b) compared to the normal control group. Oral treatment with graded doses of GOV reversed the significant ($p<0.05$) decrease in the Hb, PCV, RBC, WBC, platelet count, MCHC, granulocytes and lymphocytes value recorded for APAP hematotoxicity and also caused a significant ($p<0.05$) dose related decrease in the MCV, MCH and monocytes value compared to the toxin control group. This result shows that APAP induced hematotoxicity can cause leukopenia, granulocytosis and neutropenia, thrombocytopenia and pancytopenia in rats. The recorded hematotoxicity could be secondary to the deleterious effect of APAP on organs of hematopoeisis in the body which include liver and kidneys. Results of this study showed that the extract could contain active biological principle(s) annulling the hematotoxic effect of

APAP, with ensuing improvement of hematopoiesis. The biological principle(s) could also be arbitrating hematopoietin-like effect or augmenting the release of hematopoietin from hematopoetic organs such as the kidneys or liver.

The laboratory feature of hepatotoxicity induced by APAP resembles other kinds of acute inflammatory liver disease with prominent increase of AST, ALT, and ALP levels (Davidson and Eastham, 1966). The effects of GOV on serum marker enzymes are presented in table 24a. In the present study, the serum level of hepatic enzymes AST, ALT, ALP, GGT and LDH of the APAP induced toxin control group were significantly ($p< 0.05$) increased compared to the control group. This reflected the hepatocellular damage in the APAP-induced hepatotoxicity animal model and is indicative of cellular leakage and loss of functional integrity of cell membrane in liver (Drotman and Lawhan, 1978). Increase in serum level of ALP is due to increased synthesis, in presence of increasing biliary pressure (Muriel and Garcipiana, 1992). Administration of GOV at the doses of 2, 4 and 8 g kg^{-1} significantly ($p<0.05$) prevented acetaminophen-induced hepatotoxicity in rats. The hepatoprotective effect of the different doses of GOV was shown by the decreases in the activities of ALT, ALP, AST, LDH and GGT. This is an indication of stabilization of plasma membrane as well as repair of hepatic tissue damage caused by acetaminophen. The reversal of increased serum enzymes in acetaminophen-induced liver damage by GOV may be due to the prevention of the leakage of intracellular enzymes by its membrane stabilizing activity. Acting as a stabilizing agent, GOV increased the stability of membrane and simultaneously prevented the intracellular leakage of enzymes. This is in agreement with the commonly accepted view that serum levels of transaminases return to normal with the healing of hepatic parenchyma and the regeneration of hepatocytes (Thabrew and Joice, 1987). The effects of the different doses of GOV were comparable with that of the standard drugs Liv 52 and silymarin.

In Table 24b, APAP induced toxicity showed a significant ($P<0.05$) increase in the serum cholesterol, triglyceride, urea and creatinine concentrations in the APAP induced toxin control group when compared to the normal control group. Elevation of urea and creatinine levels in the serum was taken as the index of nephrotoxicity (Ali, *et al.*, 2001). The reduction in albumin and protein concentrations and increase in triglyceride concentration is attributed to the initial damage produced and localized in the endoplasmic reticulum which results in the loss of P450 leading to its functional failure with a decrease in protein synthesis and accumulation of triglycerides leading to fatty liver. Oral administration of GOV dose dependently and significantly ($P<0.05$) attenuated serum cholesterol, triglyceride, urea and creatinine concentrations and increased the albumin and total protein concentrations when compared to the toxin control group. At 4 g kg^{-1}, GOV increased the albumin concentration, lowered cholesterol and urea concentrations more than the standard drugs. The rise in protein and albumin concentrations and decrease in triglyceride concentration suggests the stabilization of endoplasmic reticulum leading to protein synthesis and the anti-hyperlipidemic effect of GOV. Silymarin, Liv 52 and the different doses of GOV decreased acetaminophen induced elevated enzyme levels in tested groups thereby, indicating the protection of structural integrity of serum, kidney and liver cell membrane or regeneration of damaged liver cells.

SOD, CAT and GPx enzymes are important scavengers of superoxide ion and hydrogen peroxide. The non-enzymic antioxidant, glutathione is one of the most abundant tripeptides present in the liver and its functions are mainly concerned with the removal of free radical species such as hydrogen peroxide, superoxide radicals and alkoxy radicals, maintenance of membrane protein thiols and as a substrate for glutathione peroxidase and GST (Prakash *et al.*, 2001). These enzymes prevent generation of hydroxyl radical and protect the cellular constituents from oxidative damage (Scott, *et*

al., 1991). Recent studies have demonstrated that oxidative stress is a major mechanism in the development of APAP-induced hepatotoxicity (Rajkapoor, *et al*., 2008: Hanczko, *et al.,* 2009).

In the present study, the elevations in the levels of end products of lipid peroxidation in the serum, kidney and liver of rats treated with paracetamol were observed (tables 25 -27). The increase in malondialdehyde (MDA) levels in serum, kidney and liver of the APAP induced toxin control groups suggests enhanced lipid peroxidation leading to tissue damage and failure of antioxidant defense mechanisms to prevent formation of excessive free radicals. The result in table 25 - 27 indicates the anti-lipid peroxidation and/or adaptive nature of the systems as brought about by different doses of the polyherbal formulation against the damaging effects of free radical produced by APAP.

It was observed that the different doses of GOV significantly ($p< 0.05$) and dose dependently increased the serum, kidney and hepatic CAT, GPx, GSH, GST, SOD and total protein activity in APAP induced damage in rats compared to the toxin control groups. Administration of GOV significantly ($p<0.05$) increased the level of GPx and GST in a dose dependent manner. It also caused a significant ($p< 0.05$) increase in SOD, CAT and GSH activity and thus reduces reactive free radical induced oxidative damage, protected the tissues from highly reactive hydroxyl radicals and attenuated lipid peroxidation in the liver, kidney and blood APAP treated rat. This shows that GOV can dose dependently reduce reactive free radicals that might lessen oxidative damage to the tissues and improve the activities of the hepatic antioxidant enzyme.

The biochemical results obtained agrees with the histological studies of the liver and kidney sections. The hepatoprotective property of this polyherbal mixture can be attributed to the presence of these active principles which alone or in combination may be responsible for the hepatoprotection

demonstrated in this study. This indicates that GOV has hepato- and nephro-protective effects against APAP induced toxicity.

The experimentally induced cirrhotic response by carbon tetrachloride (CCl_4) in rats and mice has been shown to be similar to human cirrhosis of the liver (Weiler-Normann *et al*., 2007). CCl4 can induce liver damage through the formation of reactive free radicals that can bind covalently to cellular macromolecules forming nucleic acid, protein and lipid adducts, through the induction of hypomethylated ribosomal RNA, resulting in inhibition of protein synthesis and it can also affect hepatocellular calcium homeostasis (Weber, *et al*., 2003: Manibusan *et al*., 2007). CCl4 treatment can result in centrilobular steatosis, inflammation, apoptosis and necrosis (Shi *et al*., 1998: Weber, *et al*., 2003: Manibusan *et al*., 2007). The liver will progress to fibrosis and cirrhosis if the damage exceeds the ability of the liver to repair (Manibusan *et al*., 2007).

Mansour (2000) reported that a single dose of CCl_4 induced hepatotoxicity manifested biochemically by significant elevation of activities of liver functions, such as ALT and AST. The results of the present study shows that GOV at different doses (2, 4 and 8 $g\,kg^{-1}$) significantly ($P<0.05$) protected the rats against CCl_4-induced hepatotoxicity as demonstrated by its inhibition of the elevation of serum AST ALT, ALP, GGT, and LDH activities (Tables 30a and 30b). CCl_4-induced acute liver damage may be initiated by the trichloromethyl free radical, which is formed by cytochrome P450 and could induce peroxidation of the unsaturated fatty acids of cell membrane. This leads to membrane injury and leakage of enzymes e.g. AST and ALT (Lee *et al*., 2002). Aminotransferase levels are sensitive indicators of liver-cell injury and are helpful in recognizing hepatocellular diseases while gamma glutamyl transferase a sensitive indicator of cholestasis is useful to help determine if elevations of alkaline phosphatase are due to bone or liver disorder.

The main mechanism by which CCl_4 is known to mediate its toxic effects is through oxidative stress and oxidative damage due to an increased production of Reactive Oxygen Species (ROS) (Arteel, 2003). A variety of enzymatic and non-enzymatic mechanisms have evolved to protect cells against ROS, including the superoxide dismutases, which remove O_2; catalase and the glutathione (GSH) peroxidase system which remove H_2O_2; glutathione transferases which can remove reactive intermediates and lipid aldehydes (Yu, 1994: Halliwell, 1999). GOV significantly ($P<0.05$) increased the activities of catalase, GSH, GPx, GST and superoxide dismutase but decreased the lipid peroxidation level compared to the toxin control rats (Tables 31 – 33). Malondialdehyde (MDA) is a cytotoxic reactive aldehyde (Manibusan, *et al* 2007) formed as a byproduct of lipid peroxidation. The ability of GOV to attenuate the elevated levels of MDA indicates it hepatoprotective potentials. Depletion of GSH has been shown to enhance CYP2E1 resulting in CYP2E1-derived ROS leading to toxicity (Zhuge and Cederbaum, 2007). Since CYP2E1 is a key contributor to injury produced by CCl_4, one possible mechanism involved in the prevention of this toxicity by GOV could have been an inhibition of CYP2E1 catalytic activity. The mechanism of protection can be attributed to decreased production of ROS and lipid peroxidation when the CYP2E1 mediated oxidative stress was produced in hepatocytes with pro-oxidant as CCl_4.

Rat hepatocytes undergo apoptosis after injection of CCl4 (Shi, *et al*., 1998). It increased accumulation of apoptotic cells and is thought to play an important role in the progression of liver injury. A great deal of evidence indicates that cell exposure to ROS, in addition to producing direct chemical injury to cell components, also induces apoptosis, which leads to cell disruption and tissue necrosis (Ozben, 2007). Caspase 9 initiates apoptosis following mitochondrial dysfunction while caspase 2 has been implicated in cell death by endoplasmic reticulum stress (the unfolded protein response) and following DNA damage (Kumar, 2009). GOV significantly ($P<0.05$) inhibited the fold

increase attributed to CCl_4 induced hepatotoxicity compared to the toxin control group as shown in table 34, thereby inhibiting death receptor-mediated apoptosis (Witek, *et al*., 2009). GOV can be suggested to attenuate hepatic injury and fibrosis in preclinical models of cholestasis and nonalcoholic liver disease because of its potential as a caspase inhibitor.

GOV administration led to amelioration of the effects of CCl4 on liver function tests including serum levels of albumin, lipids, liver enzymes, antioxidant enzymes and histology of the liver and kidneys. These benefits were seen with the three doses used. These reports, shows that GOV can protect against CCl_4-induced toxicity and oxidative stress.

There is an increasing realization that chemotherapeutic agents act primarily by inducing cancer cell death through the mechanism of apoptosis, though there are many cancers that are intrinsically resistant to apoptosis (Muscella, *et al.*, 2008), thereby making it vital to develop novel drugs for combination chemotherapy.

The ranking of antiproliferative activity of the various fractions toward hepatoma cells is grossly similar to that toward nasopharyngeal cancer cells although not completely identical. It is known that ribosome inactivating proteins have different antiproliferative activities toward different tumor cell lines (Chu and Ng, 2006). The ability of GOV to inhibit cancer cells proliferation further purports it as a potential anticancer agent in liver and nasopharyngeal carcinomas, thus making it a promising agent for chemotherapy, which merits further study. This finding may be a prelude to the development of new drugs as an alternative in the treatment liver diseases.

CONCLUSION

This study complements the on-going activities of evaluation of different uses of medicinal plants and the development of new improved traditional medicine in Nigeria. Phytochemical, pharmacological and toxicologic studies were carried on this triherbal formulation in order to ascertain its effectiveness as a hepatoprotective agent. The traditional knowledge of plants with medicinal properties supported by experimental studies could serve as an innovative and powerful discovery engine for newer, safer and affordable medicines. The development of these traditional systems of medicines with the perspectives of safety, efficacy and quality will help not only to preserve this traditional heritage but also to rationalize the use of natural products in the health care. Thus, we can easily identify rare and extinct plants for conservation and preserve the traditional heritage of the traditional practitioners.

The ability of a hepatoprotective agent to reduce the injurious effects, or to preserve the normal hepatic physiologic mechanisms which have been disturbed by a hepatotoxin, is an index of its protective effects. Although serum enzyme levels are not a direct measure of hepatic injury, they show the status of the liver. The lowering of enzyme levels is an indication of the hepatoprotective action of this triherbal formulation. The *in vitro* and histopathologic studies are also direct evidence of the efficacy of this formulation as a hepatoprotectant. The results of the analgesic and antiinflammatory properties of GOV further justify the use of the plant in ethnomedicine for alleviating pain and treating inflammatory disorders. Thus, the presence of rutin, borneol, stigmasterol, beta sitosterol, eugenol, hyperoside, ascorbic acid and other antioxidants in GOV may be the contributing factor towards its hepatoprotective activity and justifies the folkloric use of the plant in treatment of liver diseases. The

hepatoprotective properties of GOV may be attributed to the individual or combined action of these bioactive constituents.

The data presented here indicate that GOV exhibits antioxidant, liver and kidney protective properties both in ethyl alcohol and D-galactosamine treated rats and antiproliferative properties in Hep G2 cells. These findings show the prophylactic efficacy of GOV in maintaining the integrity and functional status of hepatocytes in rats and its antiproliferative property on Hep G2 cells. In addition, the *in vivo* studies carried out using GOV also proved to be highly efficient in terms of dosage, tolerability, and restoring the liver.

It can be said that this triherbal formulation (GOV) has demonstrated liver protective effect against ethyl alcohol and D- galactosamine-induced hepatotoxicity. It exhibited antioxidant activities in a dose dependent manner and demonstrated significant protection to the liver thus justifying its use as a hepatoprotective agent. The present findings provide scientific evidence to the ethnomedicinal use of this trihebal formulation by the tribal group of Eastern Nigeria in treating liver diseases.

Contributions to Knowledge

1. The hepatoprotective potentials of GOV were established using different models of inducing hepatotoxicity in Wistar albino rats.
2. This study has demonstrated that GOV modulates liver cancer proliferation using HepG2 hepatoma cells.
3. This study has demonstrated the reduced release of proapoptotic proteins and subsequent decrease in caspase activity on administration of GOV to Wistar albino rats.
4. GOV exhibited antinociceptive and anti-oedematogenic activity comparable to some standard reference drugs, such as Liv 50, Silymarin, Acetaminophen.
5. This study has shown that GOV offered protection to the liver in cases of alcohol or toxin induced damage as reflected from histopathologic studies and also against free radical mediated oxidative stress.

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