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EFFECT OF METHANOL EXTRACT OF *Prosopis africana* FERMENTED SEED ON PARACETAMOL-INDUCED LIVER DAMAGE IN WISTAR ALBINO RATS

BY

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UNIVERSITY OF NIGERIA
NSUKKA

SEPTEMBER, 2014
CHAPTER ONE
INTRODUCTION

1.0 Background of the study

The study of herbal medicines and the use of plant leaves, stems, roots, seeds and the latex, for human benefits, is an age long event for human benefits (Okafor et al., 1994). Herbal medicine is fast emerging as an alternative treatment to available synthetic drugs for the treatment of diseases possibly due to lower cost, availability, fewer adverse effects and perceived effectiveness (Ubaka et al., 2010). The exploitation of cheap agricultural materials to manufacture industrial products will enhance the development of rural agro-based economy (Kronbergs, 2000; Sain and Panthapulakkal, 2006). The historic role of medicinal plants in the treatment and prevention of diseases and their role as catalysts in the development of pharmacology do not however, assure their safety for uncontrolled use by an uninformed public (Matthews et al., 1999). The use of plants in the management and treatment of diseases started with life. In more recent years, with considerable research, it has been found that many plants do indeed have medicinal values. Some medicinal plants used in Nigeria include *Garcina kola*, used in the treatment of asthma, *Carica papaya*, used as a remedy for hypertension, *Ocimum basilicum*, a cure for typhoid fever, and *Cola nitida*, for treatment of pile (FAO, 1996). In Nigeria, fermented *Prosopis africana* seeds are popularly used as food seasoning. It is evident that fermented food condiments are good sources of nutrients and could be used to produce complementary food supplements (Achi, 2005). The food flavouring condiments are prepared by traditional methods of uncontrolled solid substrate fermentation resulting in extensive hydrolysis of the protein and carbohydrate components (Fetuga et al., 1973; Eka, 1980). Apart from increasing the shelf life, and a reduction in the anti-nutritional factors (Odunfa, 1985; Barimalaa et al., 1989; Achi and Okereka, 1999), fermentation markedly improves the digestibility, nutritive value, and flavours of the raw seeds. Fermented products remain of interest since they do not require refrigeration during distribution and storage. The traditional condiments have not attained commercial status due to the very short shelf life, objectionable packaging materials, stickiness and the characteristic putrid odour (Arogba et al., 1995). Fermented condiments often have a stigma attached to them; they are often considered as food for the poor.
Liver damage due to ingestion or inhalation of hepatotoxins such as drugs is increasing worldwide, and conventional drugs used in the management of drug induced liver damage are mostly inadequate and have serious adverse effects (Ozugwu, 2011). In spite of the tremendous strides in modern medicine, there are grossly few drugs that stimulate liver function, offer protection to the liver from damage or help regeneration of hepatic cells. Chronic hepatic diseases is one of the foremost health problems worldwide, with liver cirrhosis and drug induced liver injury accounting for the ninth leading cause of death amongst the western and developing countries population (Mohamed Saleem et al., 2010). About 20,000 deaths are reported every year due to liver disorders (Gupta and Misra, 2006). As said earlier conventional drugs used in the management of drug induced liver damage are mostly inadequate and have serious adverse effects (Ozugwu, 2011). It is, therefore, necessary to explore the herbal options in the management of drug induced liver damage to replace currently used drugs of low efficacy and safety.

1.1 *Prosopis africana*

1.1.1 Ecological and some pharmacological importance of *Prosopis africana* seed

*Prosopis africana* is a leguminous plant of the Fabaceae family. It is a flowering plant that is locally called “kiriya” in Hausa, “okpehe” in Ibo and Idoma and “gbaaye” in Tiv languages of Nigeria. The leaves, branches, bark and roots are used for several purposes in traditional medicines (Kalinganire et al., 2007). *Prosopis africana* plant is a tropical leguminous tree that is readily distinguished by its dark, pale drooping foliage with small pointed leaflets. The tree is about 12m to 18m high and up to 2.2m in girth. The dry pods which are between 10cm and 15cm long and about 2cm thick contain numerous ellipsoid seeds of about 15 to 18 (Ogunshe et al., 2007). The only known usage of these seeds, presently in Nigeria, is as food seasoning, which is particularly common among the Idomas of Benue State. The seeds are processed in the same way as locust bean seeds. The seeds have protein content of between 39 and 40 per cent (Balogun, 1982). It is traditionally used for formulation of animal feeds and preparation of local condiments through boiling and fermentation processes (Aremu et al., 2006).

The tree is of great economic value to man and animal, it fixes nitrogen to enrich the soil, generates hardy timbers, produces protein rich leaves and sugary pods used as feed stuffs for ruminants (Annongu et al., 2004). However, the disadvantage of *Prosopis* is the high content of anti-nutritive factor such as tannins, haemagglutinins, prosopine and toxic amino acids
which are capable of inducing adverse effect on simple stomached animals when consumed without adequate processing (Cheeke and Shull, 1985). The seeds could be used as a protein supplement for low-protein foods and seeds such as cereal grains for animals (Maragoni and Alli, 1987). The seeds could also serve as a good source of carbohydrate concentrate for all classes of livestock.

1.1.2 Pharmacological Properties of *Prosopis africana*.

The methanol stem bark extract of *Prosopis africana* is used for anti-inflammatory and pain relief medicine in humans. Likewise, the tannins and dye in the bark is utilized in the leather industry (Ayanwuyi *et al*., 2010). The leaves and stem are used for treating toothache. The fruits (pods) are used as fodder for ruminant animals (Amusa *et al*., 2010). In the middle belt states of Nigeria, fermented *Prosopis africana* seeds are popularly used as food seasoning. It is a source of low cost protein. Gels that could be used for pharmaceutical tablet formulation is obtained from *Prosopis africana* gum. The endocarp gum of *Prosopis africana* seed contains high content of galactose and mannose. Galactose is a special type of natural sugar that gives sustained energy for a longer time compared to other sugar. Mannose is important for treatment of urinary tract infections (Achi and Okolo, 2004). Likewise, the seeds have been reported to have 4445 kcal/kg of food energy which is higher than the 2500 to 3000 kcal/kg daily requirement by humans (Barminas *et al*., 1998).

According to (Kolapo *et al*., 2009) the stem and root of *P. africana* indicates a potential candidate plant parts in dentrifice production. The bark of *Prosopis africana* in this study is used to treat pile (Lawal *et al*., 2010).

Anticonvulsant properties of *Prosopis africana* were seen on strychnine (STR) and pentylenetetrazol (PTZ) induced convulsion. Plant extract were effective against PTZ and STR induced convulsions (Ngo-Bum *et al*., 2009).

*Prosopis africana* plant extract was pre-screened and evaluated as anti-trypanosomal agents. The result suggests that the plant with promising bioactivity may possess component that may provide the chemical lead towards the discovery of new generation trypanocides that are more potent and less toxic than the currently available and marketed trypanocidal drugs (Osho and Lajide, 2012).

*Prosopis africana* belonging to the family Fabaceae is being used traditionally as medicine in many African homes. This includes the leaves used in treatment of headache and toothache;
leaves and bark are combined in the treatment of rheumatism, skin disease and eyewashes; the roots are used as diuretic, and in the treatment of dysentery, bronchitis and stomach cramps (Gilbert and Neil, 1986; Arbonnier, 2002). The prosopis gum has been used in the present day research as bio-adhesive agent in delivery of metformin, this show a synergistic effect (Adikwu and Nnamani, 2005).

Prosopis gum can be used to treat infection, skin irritation and in the management of wound. These studies suggest that a mixture of bovine mucin, cicatrin and prosopis gum has a better healing effect than cicatrin powder alone (Momoh et al., 2008). According to Ojo et al. (2006), *Prosopis africana* leaf extract was observed to have hepatoprotective potentials, this could be related to the high concentration of tannin in the leaf.

### 1.1.3 Scientific Classification of *Prosopis africana* Seed

<table>
<thead>
<tr>
<th>Kingdom</th>
<th>Plantae</th>
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<tr>
<td>Subkingdom</td>
<td>Tracheobionta</td>
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<tr>
<td>Superdivision</td>
<td>Spermatophyta</td>
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<tr>
<td>Division</td>
<td>Magnoliophyta</td>
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<tr>
<td>Class</td>
<td>Magnoliopsida</td>
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<tr>
<td>Subclass</td>
<td>Rosidae</td>
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<tr>
<td>Order</td>
<td>Fabales</td>
</tr>
<tr>
<td>Family</td>
<td>Fabaceae</td>
</tr>
<tr>
<td>Genus</td>
<td>Prosopis</td>
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<tr>
<td>Species</td>
<td>africana</td>
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**Source:** (Achi and Okereka, 1999)
Liver is a self regenerating organ that plays important roles in the body. It functions not only in metabolism and removal of exogenous toxins and therapeutic agents responsible for metabolic derangement but also in the biochemical regulation of fats, carbohydrates, amino acids, protein, blood coagulation and immunomodulation function (Ram and Goel, 1999). Due to its ability to regenerate, even a moderate cell injury is not reflected by measurable change in its metabolic function. However, damage caused by lipid peroxidation on the membrane of the hepatocytes allows the leakage of some cytosolic enzymes of the liver into the blood stream (Plaa and Hewitt, 1982).

1.2.1 Epidemiology and Statistics of Drug Induced Liver Injury.
Drug induced liver damage is a health problem worldwide and is expected to increase as the number of drugs being consumed increases. It is a major health issue that challenges not only health care professionals but also the pharmaceutical industry and drug regulatory agencies (Saleem et al., 2008). Drug induced liver injury is the most commonly cited reason for withdrawal of already approved drugs from the market (Butura, 2008). According to the United States Acute Liver Failure Study Group, drug-induced liver injury accounts for more than 50% of acute liver failure, with hepatotoxicity caused by overdose of paracetamol accounting for 39% and idiosyncratic liver injury triggered by other drugs accounting for about 13% (Holt and Ju, 2006). Drug-induced liver toxicity accounts for approximately half
of the cases of acute liver failure and mimics all forms of acute and chronic liver disease (Kaplowitz, 2001). The reported incidence of anti-tuberculosis drugs induced hepatotoxicity indicated that the developing countries are having difficulties in systematic steps for prevention and management of tuberculosis drugs induced hepatotoxicity. Despite the frequency of drug induced liver injury being relatively low, data from the centers for disease control and prevention in the U.S reported approximately 1600 new acute cases of liver failure annually, of which paracetamol hepatotoxicity accounts for approximately 41% (Norris, and Lewis, 2008). The most commonly implicated drugs involved in acute liver injury are summarised in Table 1.

Table 1: Commonly reported drugs associated with drug induced liver injuries.

<table>
<thead>
<tr>
<th>Paracetamol</th>
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<tr>
<td>Non-Steroidal Anti-Inflammatory Drugs</td>
</tr>
<tr>
<td>Diclofenac</td>
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<tr>
<td>Ibuprofen</td>
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<tr>
<td>Naproxen</td>
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<tr>
<td>Antibiotics</td>
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<tr>
<td>Amoxicillin/Clavulanate (Augmentin)</td>
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<td>Flucloxacillin</td>
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<td>Erythromycin</td>
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<td>Ciprofloxacin</td>
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<tr>
<td>Anti-Tuberculosis Drugs</td>
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<td>(Isoniazid, Rifampicin, Pyrazinamide)</td>
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<tr>
<td>Anti-Retroviral Drugs (E.g Ritonavir)</td>
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<td>Immunosuppressant</td>
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<td>Azathioprine</td>
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<td>Cyclophosphamide</td>
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<td>Anti-Arrhythmia Drugs</td>
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<td>Amiodarone</td>
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<tr>
<td>Anti-Epileptics</td>
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<tr>
<td>Phenytoin</td>
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<tr>
<td>Carbamazepine</td>
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<tr>
<td>Valproic Acid</td>
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<tr>
<td>Psychiatric Drugs</td>
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<tr>
<td>Chlorpromazine</td>
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<td>Paroxetine</td>
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Source: (Chau, 2008)

1.3 Acetaminophen (Paracetamol)
Acetaminophen is an effective antipyretic and analgesic, but its anti-inflammatory properties are minimal, especially compared with non-steroidal anti-inflammatory drugs (NSAIDs). Nevertheless, acetaminophen is preferred over NSAIDs in some patients because it carries a lower risk of gastrointestinal toxicity (e.g., ulceration, bleeding) and so may be better tolerated (Burke et al., 2006). It is one of the most important drugs used for the treatment of mild to moderate pain when an anti-inflammatory effect is not necessary (Nwachukwu, 2006). Acetaminophen structure is shown below in Fig. 3.

![Acetaminophen structure](image)

**Fig. 3:** N-acetyl-p-aminophenol (Acetaminophen)

### 1.3.1 History of paracetamol

Its history says that when Cinchona tree became scarce in the 1880s, people began to look for alternatives. Two alternative antipyretic agents were developed in 1880s; Acetanilide in 1886 and Phenacetin in 1887. Harmon Northrop Morse first synthesized paracetamol via the reduction of p-nitrophenol with Tin in glacial acetic acid in 1878; however, paracetamol was not used in medical treatment for another 15 years. In 1893, Paracetamol was discovered in the urine of individuals that had taken Phenacetin and was concentrated into white crystalline compound with a bitter taste. In 1899, paracetamol was found to be a metabolite of acetenilide. This discovery was largely ignored at that time. In 1948, Brodie and Axelrod determined that the analgesic effect of acetanilide was due to its active metabolite paracetamol. The product was then first sold in 1955 by McNeil laboratories as a pain and fever reliever for children, under the brand name Tylenol children’s elixir (Vidhya and Metillda, 2012).

### 1.3.2 Pharmacokinetics of Paracetamol

Paracetamol is administered orally. Absorption is related to the rate of gastric emptying and peak blood concentrations are usually reached in 30-60 minutes. Acetaminophen is slightly bound to plasma proteins and is primarily metabolized by hepatic microsomal enzymes and converted to acetaminophen sulphate and glucoronide, which are pharmacologically inactive. Less than 5% is excreted unchanged. A minor but highly active metabolite (N-acetyl-p-
benzoquinone) is important in large doses because of its toxicity to both liver and kidney (Prescott et al., 2006). The half life of acetaminophen is 2-3 hours and is relatively unaffected by renal function.

1.3.3 Intravenous and Oral Administration of Paracetamol

Paracetamol has previously been available for intravenous use in the form of its pro-drug, propacetamol. Used in France since 1985, propacetamol, provided as a powder for reconstitution, is water soluble and rapidly hydrolysed by plasma esterases to form paracetamol and diethylglycine; a dose of 1 g propacetamol provides 0.5 g paracetamol after hydrolysis. In a study of patients undergoing dental extraction, propacetamol was significantly better than placebo for all measured parameters; pain relief, pain intensity, patient’s global evaluation and duration of analgesia (Moller et al., 2005). Advantages of intravenous paracetamol over not associated with pain on injection or contact dermatitis. Paracetamol is bioequivalent to propacetamol (Flouvat et al., 2004).

In a study of 35 patients undergoing day-surgery, intravenous propacetamol (the IV prodrug of Paracetamol) reached therapeutic plasma concentrations more quickly and predictably than oral Paracetamol (Holmer-Pettersson et al., 2004). Paracetamol plasma concentrations were observed for the first 80 minutes after administration of either 1 g or 2 g oral Paracetamol or 2 g intravenous propacetamol. Intravenous paracetamol provided an average concentration within the therapeutic range after 20 minutes. There was a large and unpredictable variability with oral administration; some patients who received 1 g orally did not achieve detectable plasma levels within the 80 minute study period, and the average plasma concentration after receiving this dose was sub therapeutic throughout. 2 g oral paracetamol achieved a median plasma concentration within the therapeutic range after 40 minutes, suggesting that when paracetamol is given orally, a loading dose can reduce the time needed to achieve therapeutic levels.

1.3.4 Metabolism of Paracetamol

Paracetamol is metabolised in the liver via three pathways – glucuronidation, sulphation (both account for 95% of metabolism) and cytochrome P450 system (5%). Acetaminophen is metabolically activated by cytochrome P450 to form a reactive metabolite that covalently binds to protein (Mitchell et al., 1973). The reactive metabolite was found to be N-acetyl-p-benzoquinone imine (NAPQI), which is formed by a direct two-electron oxidation (Dahlin et al,
NAPQI is detoxified by glutathione (GSH) to form an acetaminophen-GSH conjugate. After a toxic dose of acetaminophen, total hepatic GSH is depleted by as much as 90%, and as a result, the metabolite covalently binds to cysteine groups on protein, forming acetaminophen-protein adducts (Mitchell et al., 1973). This mechanism is shown in Fig. 4.

Paracetamol is rapidly absorbed from the small intestine. Peak serum concentrations occur within 1-2 hours for standard tablet or capsule formulations and within 30 minutes for liquid preparations. Peak serum concentrations after therapeutic doses do not usually exceed 130nmol/l (20gm/l) (Leshna et al., 1976).

Twenty percent (20%) of the ingested dose undergoes first-pass metabolism in the gut wall (sulphation). Distribution is usually within 4 hours of ingestion for standard preparations and 2 hours for liquid preparation. Volume of distribution is 0.91/kg. Further elimination occurs by hepatic biotransformation. After therapeutic doses, the elimination half-life is 1.5-3 hours.

Fig 4: Schematic representation depicting the role of metabolism of acetaminophen toxicity

Source: (James et al., 2003)
(Nahid et al., 2005). Over 90.6% is metabolised to inactive sulphate and glucuronide conjugates that are excreted in the urine. Metabolism of the remainder is via cytochrome P450 and results in the highly reactive intermediary compound N-acetyl-p-benzoquinone imine (NAPQI). In normal conditions, NAPQI is immediately bound by intracellular glutathione and eliminated in the urine as mercapturic adducts (Daly et al., 2008). With increased paracetamol doses, greater production of NAPQI may deplete glutathione stores. When glutathione depletion reaches a critical level (about 30% of normal stores), NAPQI binds to other proteins, causing damage to the hepatocyte. Glutathione depletion itself may be injurious (Kupeli et al., 2006).

The commonest target organ in paracetamol poisoning is the liver and the primary lesion is acute centrilobular hepatic threshold. In adults, the single acute threshold dose for severe liver damage is 150-250mg/kg though there is marked individuals variation in susceptibility (Prescott et al., 2006).

1.3.5 Pharmacodynamics of Paracetamol

The major active metabolites of paracetamol are sulphates and glucuronide conjugates. Its main mode of action is to inhibit the activity of the enzyme cyclooxygenase (COX) (James et al., 2003). COX enzymes are necessary for the production of prostaglandins. Prostaglandins are a form of hormone (although rarely classified as such) that are indicated to be mediators of pain, fever and inflammation. The half-life of paracetamol may be measured either by salivary or by plasma counts. Both measurements give varying half-life between 1 and 4 hours (Lee et al., 1996). Peak levels are reached 40-60 minutes after ingestion. It has been proposed that paracetamol aids in the reduction of pain by increasing serotonergic neurotransmissions (Garrone et al., 2007).

1.3.6 Paracetamol Hepatotoxicity

Overdose of paracetamol leads to ‘paracetamol hepatotoxicity,’ which mainly results into liver injury but is also one of the most common causes of poisoning all over the world (Norris, and Lewis, 2008). Many people who develop paracetamol toxicity may feel no symptoms at all in the first 24 hours that follow overdose of paracetamol. Others may initially experience nonspecific complaints like vague abdominal pain and nausea. As the paracetamol toxicity increases, signs of liver failure like low blood sugar; low blood pH, easy bleeding, and hepatic encephalopathy may develop. Timely treatment can cure the condition
of the patient but untreated cases may result in death. Often a liver transplant is needed if damage to the liver gets severe. The risk of paracetamol toxicity increases with excessive alcohol intake, fasting or anorexia nervosa, and also with the use of certain drugs like isoniazid (Vidhya and Mettilda, 2012).

Events that produce hepatocellular death following the formation of acetaminophen protein adducts are poorly understood. One possible mechanism of cell death is that covalent binding to critical cellular proteins results in subsequent loss of activity or function and eventual cell death and lysis. Primary cellular targets have been postulated to be mitochondrial proteins, with resulting loss of energy production, as well as proteins involved in cellular ion control (Nelson, 1990). Tirmenstein and Nelson (1989) and Tsokos-Kuhn et al. (1988) reported alterations of plasma membrane ATPase activity following toxic doses of acetaminophen.

In addition to hepatotoxicity, NAPQI inhibits mitochondrial respiration by blocking electron transport between the cytochrome b/c complex and the cytochrome oxidase complex within the electron transport chain (Porter and Dawson, 1979). Fasting is a risk factor, possibly because of depletion of hepatic glutathione reserves (Nolan et al., 1994). Chronic alcoholism which also induces CYP2E1 is also well known to increase the risk of paracetamol induced hepatotoxicity (Nwodo et al., 2010).

In normal doses, paracetamol does not irritate the lining of the stomach or affect blood coagulation, the kidney or the fetal ductus arteriosus (as NSAIDs can) like NSAIDs and unlike opioid analgesics paracetamol has not been found to cause euphoria or alter mood in any way. Since this molecule is achiral, it does not have a specific rotation (Prescott et al., 2006). The words acetaminophen and paracetamol are both derived from the chemical names for the compounds N acetyl-para-aminophenol and para-acetyl amino-phenol respectively.

1.3.6.1 Paracetamol Hepatotoxicity and Alcohol Consumption

It is claimed that chronic alcoholics are at increased risk of paracetamol (acetaminophen) hepatotoxicity not only following over dosage but also with its therapeutic use. Increased susceptibility is supposed to be due to induction of liver microsomal enzymes by ethanol with increased formation of the toxic metabolite of paracetamol. However, the clinical evidence in support of these claims is anecdotal and the same liver damage after overdosage occurs in patients who are not chronic alcoholics. Many alcoholic patients reported to have liver damage after taking paracetamol with ‘therapeutic intent’ had clearly taken substantial overdoses (Vidhya and Mettilda, 2012).
The paracetamol-alcohol interaction is complex; acute and chronic ethanol has opposite effects (Garry et al., 2004). In animals, chronic ethanol causes induction of hepatic microsomal enzymes and increases paracetamol hepatotoxicity as expected (ethanol primarily induces CYP2E1 and this isoform is important in the oxidative metabolism of paracetamol). However, in man, chronic alcohol ingestion causes only modest (about two fold) and short-lived induction of CYP2E1, and there is no corresponding increase (as claimed) in the toxic metabolic activation of paracetamol. Acute ethanol inhibits the microsomal oxidation of paracetamol both in animals and man. This protects against liver damage in animals and there is evidence that it also does so in man. The protective effect disappears when ethanol is eliminated and the relative timing of ethanol and paracetamol intake is critical (Vidhya and Mettilda, 2012).

Hepatotoxicity from therapeutic doses of paracetamol is unlikely in patients who consume moderate to large amounts of alcohol daily. However, patients with severe alcoholism should be instructed or supervised about the correct dosage of paracetamol. The depression often associated with alcoholism may make them more likely to take an overdose of paracetamol (Garry et al., 2004).

In many of the reports where it is alleged that paracetamol hepatotoxicity was enhanced in chronic alcoholics, the reverse should have been the case because alcohol was actually taken at the same time as the paracetamol. Chronic alcoholics are likely to be most vulnerable to the toxic effects of paracetamol during the first few days of withdrawal but maximum therapeutic doses given at this time have no adverse effect on liver function tests. Although the possibility remains that chronic consumption of alcohol does increase the risk of paracetamol hepatotoxicity in man (perhaps by impairing glutathione synthesis), there is insufficient evidence to support the alleged major toxic interaction (Prescott, 2000). Chronic consumption of alcohol for three consecutive days may cause inflammation and scarring of the liver cells depending on diet, sex, immune status, gut flora and the capacity of the metabolising enzymes (Nwodo, 2012).

### 1.3.6.2 Diagnosis of Paracetamol Toxicity

The most effective way to diagnose poisoning is by obtaining a blood paracetamol level. A drug nomogram developed in 1975, called the Rumack-Matthew nomogram, estimates the risk of toxicity based on the serum concentration of paracetamol at a given number of hours
after ingestion (Rumack and Matthew, 1975). To determine the risk of potential hepatotoxicity, the paracetamol level is traced along the nomogram. Use of a timed serum paracetamol level plotted on the nomogram appears to be the best marker indicating the potential for liver injury (Daly et al., 2008). A paracetamol level drawn in the first four hours after ingestion may underestimate the amount in the system because paracetamol may still be in the process of being absorbed from the gastrointestinal tract. Therefore a serum level taken before 4 hours is not recommended (Dart et al., 2006).

Clinical or biochemical evidence of liver toxicity may develop in one to four days, although, in severe cases, it may be evident in 12 hours. Right-upper-quadrant tenderness may be present and can aid in diagnosis. Laboratory studies may show evidence of hepatic necrosis with elevated AST, ALT, bilirubin, and prolonged coagulation times, particularly an elevated prothrombin time (Bartlett, 2004). After paracetamol overdose, when AST and ALT exceed 1000 IU/L, paracetamol-induced hepatotoxicity can be diagnosed. In some cases, the AST and ALT levels can exceed 10,000 IU/L (Jones, 2000).

1.4 Phytochemistry

Phytochemicals are a large group of plant-derived compounds hypothesized to be responsible for much of the disease protection provided by diets high in fruits, vegetables, beans, cereals, and plant-based beverages such as tea and wine (Arts and Hollman, 2005). Based on their chemical structure, phytochemicals are classified into the following ten categories as shown in Fig. 5 below.

Fig. 5: Classes of Phytochemicals
Source: (Arts and Hollman, 2005).
The most important of these bioactive constituents of plants are alkaloids, tannins, flavonoids, and phenolic compounds (Hill, 1952). Some of these naturally occurring phytochemicals are anti-ulcerative agents, anti-hepatic agents, anti-carcinogenic and some possess other beneficial properties, while some prevent oxidation by free radicals and therefore known as chemo-preventers. Antioxidant activity is one of the predominant mechanisms of their protective action which is due to their capacity to scavenge free radicals (Del Rio, 1997).

1.4.1 Alkaloids

These are the largest group of secondary chemical constituents made largely of ammonia compounds comprising basically of nitrogen bases synthesized from amino acid building blocks with various radicals replacing one or more of the hydrogen atoms in the peptide ring, most containing oxygen. The compounds have basic properties and are alkaline in reaction, turning red litmus paper blue. In fact, one or more nitrogen atoms that are present in an alkaloid, typically as 1°, 2° or 3° amines, contribute to the basicity of the alkaloid. The degree of basicity varies considerably, depending on the structure of the molecule, and presence and location of the functional groups (Sarker and Nahar, 2007). They react with acids to form crystalline salts without the production of water (Firn, 2010). Alkaloids have pharmacological applications as anesthetics and CNS stimulants. Other important alkaloids of plant origin include the addictive stimulants caffeine, nicotine, codeine, atropine, morphine, ergotamine, cocaine and ephedrine (Madziga et al., 2010).

1.4.2 Flavonoids

Flavonoids are important group of polyphenols widely distributed among the plant flora (Kar, 2007). Structurally, they are made of more than one benzene ring in its structure (a range of C15 aromatic compounds) and numerous reports support their use as antioxidants or free radical scavengers (Kar, 2007). Flavonoids are widely distributed throughout plant kingdom and they give the flowers and fruits of plants their vibrant colours. More importantly, the consumption of foods containing flavonoids has been linked to numerous health benefits. Though research shows flavonoids alone provide minimal antioxidant benefit due to their absorption by the body, it has been indicated that they biologically trigger the production of natural enzymes that fight pathogens and diseases. Indeed, once consumed and absorbed, flavonoids act favourably in the body through actions such as inhibiting xanthine oxidase and arachidonic acid metabolism (Nijveldt et al., 2001).
1.4.3 Tannins
These are widely distributed in plant flora. They are phenolic compounds of high molecular weight. Tannins are soluble in water and alcohol and are found in the root, bark, stem and outer layers of plant tissue. Tannins have a characteristic feature to tan, i.e. to convert things into leather. They are acidic in reaction and the acidic reaction is attributed to the presence of phenolics or carboxylic group (Kar, 2007). Tannins are astringent, bitter plant polyphenols that both binds and precipitate proteins and various other organic compounds including amino acids and alkaloid (Petridis, 2010). The astringency from tannins is what causes the dry and pucker feeling in the mouth following the consumption of unripened fruit or red wine (Serafini et al., 1994). Many human physiological activities, such as stimulation of phagocytic cells, host-mediated tumour activity, and a wide range of anti-infective actions, have been assigned to tannins (Haslam, 1996).

1.4.4 Sterols
Sterols in plant are generally described as phytosterols with three known types occurring in higher plants: sitosterol, stigmasterol and campest erol while some are confined to lower plants; one of which is ergosterol, found is yeast and many fungi and others in both plants e.g. fucosterol. Steroids are triterpenes with cyclopentane hydrophenanthrene ring system (Harborne, 1998). In all eukaryotes, sterols form integral components of the membrane lipid bilayer where they play an important role in the regulation of membrane fluidity and permeability (Corey et al., 1993).

1.4.5 Saponins
Saponins therefore possess ‘soaplike’ behaviour in water, i.e. they produce foam. On hydrolysis, an aglycone is produced, which is called sapogenin. Saponins are also necessary for activity of cardiac glycosides (Kar, 2007). They possess a bitter and acrid taste, besides causing irritation to mucous membranes. Saponins are also important therapeutically as they are shown to have hypolipidemid and anticancer activity (Sarker & Nahar, 2007). It has also been shown to possess beneficial effects such as cholesterol lowering properties and exhibits structure dependent biological activity (Harborne, 1998).

1.4.6 Terpenoids
Terpenes are among the most widespread and chemically diverse groups of natural products. They are flammable unsaturated hydrocarbons, existing in liquid form commonly found in essential oils, resins or oleoresins (Firm, 2010). The sesquiterpene acts as irritants when applied externally and when consumed internally their action resembles that of gastrointestinal tract irritant. A number of sesquiterpene lactones have been isolated and broadly they have antimicrobial (particularly antiprotozoal) and neurotoxic action (Singh, 2009).

1.5 Haematological Indices

Haematology offers a wide spectrum of interest and interaction in medicine and offers the unique opportunity to combine laboratory and clinical data in a rapidly changing science (Fincher and Page, 2003). Haematological Indices are the parameters that are used in the assessment of the status of blood in the body. These parameters include red blood cells, total white blood cell, differential white blood cell, packed cell volume, erythrocyte sedimentation rate and haemoglobin (Fincher and Page, 2003).

1.5.1 Packed Cell Volume (PCV)

PCV measures the percentage by volume of packed RBC in a whole blood sample after centrifugation (Wynne and Edwards, 2003). It is a reasonable index of the red cell population. It can equally be expressed as the fraction of the volume occupied by erythrocytes when a sample of whole blood in a capillary tube is centrifuged.

1.5.2 Haemoglobin (Hb)

Hb test measures the amount of Hb in grams in 1dl of whole blood and provides an estimate of oxygen carrying capacity of the RBCs (Wynne and Edwards, 2003). Haemoglobin is a specialized protein that is contained in red cells of the blood. The main function of the red cells is to carry oxygen (O₂) from the lungs to the tissues and to return carbon dioxide (CO₂) from the tissues to the lungs (Hoffbrand et al., 2006). Haemoglobin consists of four polypeptide chains, a₂B₂, with each containing individual haem group. Haem synthesis occurs largely in the mitochondria through a series of biochemical reactions.

1.5.3 White Blood cells (WBCs)

White blood cells (leukocytes) are the cells of the immune system. They defend the body against pathogens, infections and foreign materials (Stock and Hoffman, 2000). White blood
cells are of two broad groups: the phagocytes and the immunocytes. The phagocytes include the three types of cell-neutrophils (polymorphs), eosinophils and basophils and also the monocytes. The immunocytes include the lymphocytes, their precursor cell and plasma cells.

### 1.5.4 Red Blood cells (RBCs)

Red blood cell count is a blood test that measures the number of red blood cells (RBCs). The red blood cell (erythrocytes) count is almost always part of the complete blood count test (Brose, 2003). The major function of red blood cells is to carry oxygen to all tissues from the lungs as well as transport carbon (iv) oxide from the cells to the lungs (Nelson and Cox, 2000). The cells are flexible biconcave disc that are involved in the transport of oxygen. The red blood cells carry haemoglobin into close contact with the tissues and successful gaseous exchange, the red cell, 8um in diameter, must be able: to pass repeatedly through the microcirculation whose minimum diameter is 3.5um, to maintain haemoglobin in a reduced (ferrous) state (Hoffbrand et al., 2006).

### 1.6 Liver Function Tests (Enzyme Markers)

When the integrity of the membrane of the hepatocytes is compromised, certain enzymes located in the cytosol are released into the blood. Their estimation in the serum is useful quantitative marker for the evaluation of liver damage (Ram and Goel, 1999). Glutamate dehydrogenase activity is not found in normal serum but moderate elevation is found in most cases of acute hepatitis indicating cellular damage. Another demonstrable type of membrane damage involves injury to lysosomes which leads to the release of acid ribonuclease and acid phosphatases, and other liver enzymes such alanine transaminase, aspartate transaminase and alkaline phosphatase, into the blood stream. These enzymes are elevated to distinguish and assess the extent and type of hepatocellular injury (Ram and Goel, 1999).

#### 1.6.1 Classification of Liver Function Tests

- Test of the liver’s capacity to transport organic anions and to metabolise drugs (serum bilirubin, urine bilirubin and urobilirubin).
- Test that detect injury to hepatocytes (serum enzyme tests) – Aminotransferases, alkaline phosphatase, glutamyl transpeptidase, 5-nucleotidase, leucine aminopeptidase.
• Test of the liver’s biosynthetic capacity – serum protein, albumin, prealbumin, serum ceruloplasmin, procollagen III peptide, a lantitrypsin, a fetoprotein, prothrombin time (Thapa and Anuj, 2007).

1.6.2 Aspartate Aminotransferase (AST)

AST is more widely distributed than ALT. It is present in the liver, heart, kidneys, skeletal muscle and red blood cells. AST levels are raised in shock and it is less specific for liver disease. AST levels are also raised in pregnancy and after exercise. Ratios between ALT and AST are useful to physicians in addressing the etiology of liver enzyme abnormalities and also useful in differentiating between causes of liver damage (Mason, 2004).

AST: Aspartate + α-Ketoglutarate \[\text{AST}\] Oxaloacetate + Glutamate.

1.6.3 Alanine Aminotransferase (ALT)

The enzyme ALT is present in high concentration in the liver. It is also found in cardiac and skeletal muscle. However, ALT is considered as a specific marker of hepatocellular damage because levels are generally only significantly raised in liver damage. ALT is present in the heart and muscles in much lower concentrations – only marginal elevations occur in acute myocardial infarction. People with acute liver damage have particularly high ALT levels and those with chronic liver disease and obstructive jaundice have more modestly raised levels. Low ALT (and AST) levels suggest vitamin B6 deficiency. The level of ALT abnormality is increased in conditions where cells of the liver have been inflamed or undergone cell death. As the cells are damaged, the ALT leaks into the blood stream leading to a rise in the serum level (Mason, 2004).

ALT: Alanine + α-Ketoglutarate \[\text{ALT}\] Pyruvate + Glutamate.

1.6.4 Alkaline Phosphatase (ALP)

ALPs are a family of zinc metaloenzymes, with a serine residue at the active centre; they release inorganic phosphate from various organic orthophosphates and are present in nearly all tissues. ALP is produced in the lower bile duct, bone and gut and is widely distributed in the body. In liver, alkaline phosphatase is found histochemically in the microvilli of bile
canaliculi and on the sinusoidal surface of hepatocytes (Thapa and Anuj, 2007). ALP levels in plasma will rise with large bile ducts obstruction, intrahepatic cholestasis or infiltrative diseases of the liver. It is present in the bone and planceta. So it is higher in growing children (as their bones are being remodelled) and elderly patients with paget’s disease (Mason, 2004). Elevations occur as a result of both intrahepatic and extrahepatic obstruction to bile flow.

1.7 Lipid Profile

Lipid profile or lipid panel is a panel of blood tests that serves as an initial broad medical screening tool for abnormalities in lipids, such as cholesterol and triglycerides. The results of this test can identify certain genetic diseases and can determine approximate risks for cardiovascular disease, certain forms of pancreatitis, and other diseases. Lipid panels are commonly ordered as part of a physical exam, along with other panels such as the complete blood count (CBC) and basic metabolic panel (BMP) (Murray et al., 2003).

Liver plays an essential role in lipid metabolism, several stages of lipid synthesis and transportation. It has been well documented that chronic liver dysfunction might interfere with lipid metabolism in vivo and could change plasma lipid and lipoprotein patterns (Miller, 1990).

The liver carries out the following major functions in lipid metabolism:
(1) It facilitates the digestion and absorption of lipids by the production of bile, which contains cholesterol and bile salts synthesized within the liver de novo or from uptake of lipoprotein cholesterol.
(2) The liver has active enzyme systems for synthesizing and oxidizing fatty acids and for synthesizing triacylglycerols and phospholipids.
(3) It converts fatty acids to ketone bodies (ketogenesis).
(4) It plays an integral part in the synthesis and metabolism of plasma lipoproteins (Murray et al., 2003).

1.7.1 Cholesterol

Cholesterol is a waxy substance that is present in the blood plasma and in all animal tissues. Chemically, cholesterol is an organic compound belonging to the steroid family; its molecular formula is C_{27}H_{46}O (Abell et al., 1952). Cholesterol is essential to life; it is a primary component of the membrane that surrounds each cell, and it is the starting material
or an intermediate compound from which the body synthesizes bile acids, steroid hormones, and vitamin D. Cholesterol circulates in the bloodstream and is synthesized by the liver and several other organs. Human beings also ingest considerable amounts of cholesterol in the course of a normal diet. Cholesterol is insoluble in the blood; it must be attached to certain protein complexes called lipoproteins in order to be transported through the bloodstream. A compensatory system regulates the amount of cholesterol synthesized by the liver, with the increased dietary intake of cholesterol resulting in the liver's decreased synthesis of the compound (Murray et al., 2003).

1.7.2 Triacylglycerol (TAG)

The triacylglycerols play an important role in furnishing energy in animals. They have the highest energy content over 9kcal/mole. They provide more than half the energy need of some organs like the brain, liver, heart and resting skeletal muscle. Lipid mainly as triacylglycerol can accumulate in the liver. Extensive accumulation is regarded as a pathologic condition. When accumulation of lipid in the liver becomes chronic, fibrotic changes occur in the cells that progress to cirrhosis and impaired liver function (Murray et al., 2003).

1.7.3 High Density Lipoprotein (HDL)

HDL is the smallest of the lipoprotein particles. It is the densest because it contains the highest proportion of protein to cholesterol. Its most abundant apolipoproteins are apo A-I and apo A-II (Desprès, 2009). The liver synthesizes these lipoproteins as complexes of apolipoproteins and phospholipid.

HDL cholesterol is cholesterol that is packaged for delivery to the liver, where the cholesterol is removed from the body. HDL and its protein and lipid constituents help to inhibit oxidation, inflammation, activation of the endothelium, coagulation, and platelet aggregation. Pharmacologic (1 to 3 gram/day) niacin doses increase HDL levels by 10–30% (Rader, 2004) making it the most powerful agent to increase HDL-cholesterol (Chapman et al., 2004). In contrast, while the use of statins is effective against high levels of LDL cholesterol, it has little or no effect in raising HDL cholesterol (Chapman et al., 2004).

1.7.4 Low Density Lipoprotein (LDL)

Lipoprotein molecules enable the transportation of lipids (fats), such as cholesterol, phospholipids, and triglycerides, within the water around cells (extracellular
fluid), including the bloodstream. Studies have shown that higher levels of type-B LDL particles (as opposed to type-A LDL particles) are associated with health problems, including cardiovascular disease. Although the nickname is overly simplistic and thus misleading, LDL cholesterol molecules are often informally called bad cholesterol because they can transport their content of many fat molecules into artery walls, attract macrophages, and thus drive atherosclerosis. In contrast, HDL cholesterol molecules are frequently referred to as good cholesterol or healthy cholesterol, because they can remove fat molecules from macrophages in the wall of arteries (Murray et al., 2003).

Niacin (B3), lowers LDL by selectively inhibiting hepatic diacylglycerol acyltransferase 2, reducing triglyceride synthesis and VLDL secretion through a receptor HM74 (Meyers et al., 2004) and HM74A or GPR109A (Soudijn et al., 2007). LDL particles appear harmless until they are within the blood vessel walls and oxidized by free radicals (Teissedre et al., 1996), it is postulated that ingesting antioxidants and minimizing free radical exposure may reduce LDL's contribution to atherosclerosis, though results are not conclusive.

1.8 Membrane Stabilisation on Hypotonicity-Induced Haemolysis of Human Red Blood Cells (HRBCs)

Membrane stabilization is a process of maintaining the integrity of biological membranes such as erythrocyte and lysosomal membranes against osmotic and heat-induced lyses (Oyedapo and Famurewa, 1995; Sadique et al., 1989). Biological membranes forming closed boundaries between compartments of varying composition consist mainly of proteins and lipids. They are asymmetric, fluid structures that are thermodynamically stable and metabolically active. The erythrocyte membrane resembles that of lysosomal membrane and as such, the effect of drugs on the stabilization of erythrocyte could be extrapolated to the stabilization of lysosomal membrane (Omale and Okafor, 2008). Temperature, ionic environments and fatty acid compositions of phospholipids and glycolipids and presence or absence of cholesterol can affect the general physical state of biological membrane. Presently many membrane stabilizers (enfenamic acid, phenyl butazone etc.,) and destabilizers (Vitamin A, bile salts etc.,) have been identified. Several agents capable of releasing hydrolytic enzymes from lysosomes are also known to interfere with the stability of erythrocyte membrane. It has been shown that non-steroidal anti-inflammatory drugs protected erythrocyte membranes from hypotonic haemolysis (Brown and Mackey, 1968).

1.9 Aim of Study
The aim of this study is to determine the possible hepato-protective and ameliorative effects of methanol extract of fermented seeds of *Prosopis africana* on paracetamol-induced liver damage in rats.

### 1.10 Research Objectives

The aim of this study was achieved through the following specific objectives:

- To determine the qualitative and quantitative phytochemical constituents of *Prosopis africana* methanol seed extract.
- To determine the median lethal dose toxicity (LD$_{50}$) of the extract.
- To determine the effect of the methanol extract of fermented seed on the activities of some liver function enzymes (ALT, AST and ALP) in paracetamol-induced liver damage in rats.
- To determine the effect of the methanol extract of fermented seed on serum lipid profile (total cholesterol, LDL cholesterol, HDL cholesterol, TAG) in paracetamol-induced liver damage in rats.
- To determine the effect of treatment with different doses of the methanol extract of fermented seed on the levels of some haematological indices (haemoglobin, red blood cell, packed cell volume, white blood cell) in paracetamol-induced liver damage in rats.
- To determine the effect of the extract on hypotonicity-induced haemolysis of human red blood cells.
CHAPTER TWO

MATERIALS AND METHODS

2.1 Materials

2.1.1 Plant material

The fermented seeds of *Prosopis africana* were used for this study. The fermented seeds of *Prosopis africana* were bought from Ogige Market in Nsukka, LGA of Enugu State, Nigeria.

2.1.2 Animals

Thirty two (32) male adult Wistar albino rats and eighteen (18) adult albino mice were used for this study. All the animals used were obtained from the animal house of the Department of Zoology, University of Nigeria Nsukka. The animals were acclimatized for 7 days under standard environmental conditions, with a 12 hour light and dark cycle maintained on a regular feed and water *ad libitum*.

2.1.3 Equipment/Instruments

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<tr>
<th>Equipment</th>
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<tr>
<td>Centrifuge</td>
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<tr>
<td>Colorimeter</td>
<td>EI Scientific Co. India</td>
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<tr>
<td>Electron microscope</td>
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<td>Haematocrit Centrifuge</td>
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<td>Haemoglobin graph</td>
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<td>Oven</td>
<td>Gallenkamp, England</td>
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<td>Refrigerator</td>
<td>Thermocool, England</td>
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<td>Spectrophotometer (E312 Model)</td>
<td>Jenway, UK</td>
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<tr>
<td>Water bath</td>
<td>Gallenkamp, England</td>
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<tr>
<td>Weighing Balance</td>
<td>Vickas Ltd, England</td>
</tr>
</tbody>
</table>

2.1.4 Chemicals and Reagents
The chemicals and reagents used were of analytical grade and include:

1% Thiobarturic acid      BDH England
Acetone                  Sigma Aldrich, Germany
Aluminium chloride      BDH, England
Ammonia                   BDH, England
Anticoagulant (EDTA, heparin)      Randox USA
Ascorbic acid                  May and Baker, England
Bismuth carbonate        BDH, England
Butanol                   Sigma, England
Chloroform                Sigma, England
Potassium Dichloromate   BDH England
Glacial acetic acid      BDH England
Drangendorff’s reagent   May and Baker, England
Ferric chloride          Merck Darmstadt, Germany
Hydrochloric acid        May and Baker, England
Hydrogen peroxide        BDH, England
Lead acetate solution     Merck Darmstadt, Germany
Mayer’s reagent           BDH, England
Methanol                 Sigma, England
Olive oil                Solive oil, Nigeria
Picric acid              Merck Darmstadt, Germany
Potassium hydroxide      Sigma Aldrich, Germany
Sodium chloride          BDH, England
Sodium chloride          BDH, England
Sodium dodecyl sulphate  BDH, England
Sodium hydroxide         May and Beakers, England
Trichloroacetic acid (TCA)  Merck Darmstadt, Germany
Trichloroacetic acid     Sigma Aldrich, Germany
Tungstic acid            Merck Darmstadt, Germany
Turk’s solution (20% glacial acetic acid)  Merck Darmstadt, Germany
Wagner’s reagent          BDH, England

2.2 Methods

2.2.1 Collection of plant material
The fermented seeds of *Prosopis africana* was bought from Ogige Market in Nsukka, LGA of Enugu State, Nigeria.

**2.2.2. Preparation of Paracetamol (Acetaminophen)**

Paracetamol (Emzor) was suspended in normal saline and administered orally at a dose of 2500mg/kg b.w. This dosage is known to cause liver damage in rats (Mitchell *et al.*, 1973).

**2.2.3 Extraction of plant material**

Folch’s method for lipid extraction was used (Folch *et al.*, 1957). The extraction was done by soaking the fermented seeds with chloroform-methanol in the ratio of 2:1 for 24 hours at room temperature (26-28°C). The macerate was filtered using Whatman No. 1 filter paper. The filtrate was shaken with 20% of distilled water to obtain two (2) layers. The lower layer (chloroform layer) was separated from the upper layer (methanol layer) using separating funnel. The methanol filtrate was concentrated using rotary evaporator 40-60°C. The extract was stored in an air-tight plastic container in the refrigerator (4°C) and used for the study.

**2.2.4 Preparation of reagents**

**2.2.4.1 Ferric chloride solution (5% (w/v))**

A quantity, 5.0 g ferric chloride was dissolved in 100 ml of distilled water.

**2.2.4.2 Ammonium solution**

Stock concentrated ammonium solution (187.5 ml) was diluted in 31.25 ml of distilled water and then made up to 500 ml with distilled water.

**2.2.4.3 Ethanol (45% (v/v))**

A quantity, 45 ml of absolute ethanol was mixed with 55 ml of distilled water.

**2.2.4.4 Aluminium chloride solution**

Aluminium chloride (0.5 g) was dissolved in 100 ml of distilled water.

**2.2.4.5 Dilute sulphuric acid**

A 10.9 ml aliquot of concentrated sulphuric acid was mixed with 5 ml of distilled water and made up to 100 ml.
2.2.4.6  Lead sub acetate solution
A quantity, 45 ml of 15% lead acetate (i.e. 15.0 g of lead acetate in 100 ml of distilled water) was dissolved in 20 ml of absolute ethanol and made up to 100 ml with distilled water.

2.2.4.7  Wagner’s reagent
A known quantity, 2.0 g, iodine crystals and 3.0 g potassium iodide were dissolved in minimum amount of water and then made up to 100 ml with distilled water.

2.2.4.8  Mayer’s reagent
A known weight, 13.5 g, of mercuric chloride was dissolved in 50 ml of distilled water. Also, 5.0 g of potassium iodide was dissolved in 20 ml of distilled water. The two solutions were mixed and the volume was made up to 100 ml with distilled water.

2.2.4.9  Dragendorff’s reagent
Bismuth carbonate (0.85 g) was dissolved in 100 ml of glacial acetic acid and 40 ml of distilled water to give solution A. Another solution designated solution B was prepared by dissolving 8.0 g of potassium iodide in 20 ml of distilled water. Both solutions were mixed to give a stock solution.

2.2.4.10  Hydrochloric acid (2% (v/v))
Measured volume, 2.0 ml concentrated hydrochloric acid was diluted with some distilled water and made up to 100 ml.

2.2.4.11  Picric acid (1% (w/v))
Picric acid (1.0 g) was dissolved in 100 ml of distilled water.

2.2.4.12  Preparation of normal saline
Normal saline was prepared by dissolving 0.9 g sodium chloride in distilled water and made up to 100 ml.

2.2.4.13  Preparation of ranitidine
A known quantity, 150 mg of ranitidine was dissolved in 10 ml of distilled water to give 15 mg/ml.

2.2.4.14  Preparation of 25% trichloroacetic acid (TCA)
A known weight, 25 g trichloroacetic acid was dissolved in distilled water and made up to the 100 ml mark with distilled water in a measuring cylinder.

2.2.4.15  Preparation of 1% thiobarbituric acid (TBA)
One (1 g) thiobarbituric acid was dissolved in distilled water and made up to the 100 ml mark with distilled water in a measuring cylinder.

2.2.4.16 Preparation of 0.3% sodium hydroxide (NaOH)
Sodium hydroxide (0.3 g) was dissolved in a little amount of water and made up to 100 ml mark with distilled water in a measuring cylinder.

2.2.4.17 Preparation of 20% Sodium dodecyl sulphate (SDS)
A quantity, (20 g) sodium dodecyl sulphate was dissolved in some quantity of distilled water and made up to the 100 ml mark with distilled water in a measuring cylinder with distilled water.

2.2.4.18 Preparation of 2% Glacial acetic acid
A known quantity, 2 g of glacial acetic acid was dissolved in distilled water and made up to 100 ml with distilled water in a measuring cylinder.

2.2.4.19 Preparation of phosphate buffer pH 7.4
Phosphate buffer was prepared by dissolving (5.67 g) anhydrous monopotassium phosphate in 1 litre of distilled water and the pH adjusted.

2.2.5 Qualitative phytochemical analysis of the methanol extract of *Prosopis africana* seed.
The phytochemical analysis of the seed of *Prosopis africana* was carried out according to the methods of Harborne (1998) and Trease and Evans (2002) to identify its active constituents.

2.2.5.1 Test for alkaloids
A quantity, 0.2 g of the sample was boiled with 5 ml of 2% HCl on a steam bath. The mixture was filtered and 1ml aliquots of the filtrate were treated with 2 drops of the following reagents
(i) Dragendorff’s reagent: An orange precipitate indicates the presence of alkaloids.
(ii) Mayer’s reagent: A creamy-white precipitate indicates the presence of alkaloids.
(iii) Wagner’s reagent: A reddish-brown precipitate indicates the presence of alkaloids.
(iv) Picric acid (1%): A yellow precipitate indicates the presence of alkaloids.

2.2.5.2 Test for flavonoids
A quantity, 0.2 g of the sample was heated with 10 ml ethyl acetate in boiling water bath for 3 minutes. The mixture was filtered, and the filtrate was used for the following tests.

(i) Ammonium test: 4 ml of the filtrate was shaken with 1ml of dilute ammonium solution to obtain two layers. The layers were allowed to separate. A yellow precipitate observed in the ammonium layer indicates the presence of flavonoids.

(ii) Aluminium chloride test: 4ml of the filtrate was shaken with 1 ml of 1% aluminium chloride solution and observed for light yellow colouration that indicates the presence of flavonoids.

2.2.5.3 Test for saponins

A quantity, (0.1g) of the sample was boiled with 5ml of distilled water for 5 minutes. The mixture was filtered while still hot. The filtrate was used for the following tests.

(i) Emulsion test: A quantity, 1 ml of the filtrate was added to two drops of olive oil. The mixture was shaken and observed for the formation of emulsion.

(ii) Frothing test: A quantity, 1 ml of the filtrate was diluted with 4 ml of distilled water. The mixture was shaken vigorously and then observed on standing for a stable froth.

2.2.5.4 Test for glycosides

A quantity, (2.0 g) of the sample was mixed with 30 ml of distilled water and 15 ml of dilute sulphuric acid respectively and heated in a boiling water bath for 5 minutes. The mixtures was filtered and the filtrates used for the following test.

(i) To 5 ml of each of the filtrate 0.3 ml Fehling’s solutions mixtures of A and B was added until it turned alkaline (tested with litmus paper) and heated on a boiling water bath for 2 minutes. A brick-red precipitate indicates the presence of glycosides.

2.2.5.5 Test for tannins

A quantity, 2 g of the sample was boiled with 5 ml of 45% ethanol for 5 minutes. The mixture was cooled and then filtered and the filtrate was treated with the following solutions.

(i) Lead sub acetate solution: To 1ml of the filtrate, 3 drops of lead sub acetate solution was added. A gelatinous precipitate indicates the presence of tannins.

(ii) Bromine water: To 1 ml of the filtrate was added 0.5 ml of bromine water and then observed for a pale brown precipitate.

(iii) Ferric chloride solution: a quantity, 1 ml of the filtrate was diluted with distilled water and then 2 drops of ferric chloride solution was added. A transient greenish to black colour indicates the presence of tannins.
2.2.5.6 Test for terpenoids and steroids
A known volume, 9 ml of ethanol was added to 1g of the sample and refluxed for a few minutes and filtered. The filtrate was concentrated to 2.5 ml on a boiling water bath, and 5 ml of hot water was added. The mixture was allowed to stand for 1 hour, and the waxy matter filtered off. The filtrate was extracted with 2.5 ml of chloroform using a separating funnel. To 0.5 ml of the chloroform extract in a test tube was carefully added 1 ml of concentrated sulphuric acid to form a lower layer. A reddish-brown interface shows the presence of steroids.

Another 0.5 ml aliquot of the chloroform extract was evaporated to dryness on a water bath and heated with 3 ml of concentrated sulphuric acid for 10 minutes on water. A grey colour indicates the presence of terpenoids.

2.2.5.7 Test for reducing sugars
A quantity, 0.1 g of the sample was shaken vigorously with 5 ml of distilled water and filtered. To the filtrate was added equal volumes of Fehling’s solutions A and B and shaken vigorously. A brick-red precipitate indicates the presence of reducing sugars.

2.2.6 Quantitative phytochemical analysis of methanol extract of *Prosopis africana* seed.
2.2.6.1 Quantitative determination of tannins
The method of Swain (1979) was used for the determination of the tannin content of *P. africana*. A quantity, 0.2 g of finely ground sample was measured into a 50 ml beaker. About 20 ml of 50% methanol was added and covered with paraffin and placed in a water bath at 77-80°C for 1 hr and stirred with a glass rod to prevent bumping. The extract was filtered using a double layer of Whitman No. 1 filter paper into a 50 ml volumetric flask then 20 ml distilled water, 2.5 ml Folin-Denis reagent and 10 ml of 17% Na₂CO₃ were added and mixed properly. The mixture was made up to mark with distilled water and allowed to stand for 20 mins when a Mish-green colouration developed. Standard tannic acid solutions of range 0.10 mg were treated similarly as 1 ml of sample above. The absorbances of the tannic acid standard solutions as well as samples were read after colour development at 760 nm. The tannin content was calculated using the formula:

\[
\text{Tannin (mg/100g)} = \frac{\text{Absorbance of sample} \times \text{Average gradient} \times \text{Dilution factor}}{\text{Weight of sample} \times 1000}
\]
2.2.6.2 Quantitative determination of cyanogenic glycoside
The extraction was according to Wang and Filled method as described by Onwuka (2005). A portion, 5 g of sample was made into paste and dissolved in 50 ml distilled water. The extract was filtered and the filtrate was used for cyanide determination. To 1ml of the sample filtrate, 4 ml of alkaline picrate was added and absorbance was read at 550 nm and cyanide content was extrapolated from a cyanide standard curve.

\[
\text{Cyanide (mg/g)} = \frac{\text{Absorbance} \times \text{GF} \times \text{DF}}{\text{Sample weight}}
\]

Where: GF = gradient factor and DF = dilution factor.

2.2.6.3 Quantitative determination of flavonoids
This was determined according to the method of Harborne (1998). A quantity, 5 g of the sample was boiled in 50 ml of 2 MHCl solution for 30 min under reflux. It was allowed to cool and then filtered through Whatman No. 1 filter paper. A measured volume of the filtrate was treated with equal volume of ethyl acetate starting with drops. The solution was filtered into a weighed crucible and heated to dryness in an oven at 60°C. The dried crucible was weighed again and the difference in the weight gave the quantity of flavonoid present in the sample.

2.2.6.4 Quantitative determination of alkaloids
The quantitative determination of alkaloid was described by Harborne (1998). A known quantity, 5 g of the sample was weighed into a 250 ml beaker and 200 ml of 10% acetic acid in ethanol was added, covered and allowed to stand for 2 hours. This was filtered and the filtrate was concentrated down in a water bath for 4 h with continuous stirring at about 55°C. Concentrated ammonia was added drop-wise to the filtrate till a precipitate was formed. The precipitate was collected and washed with dilute ammonium hydroxide and then filtered. The residue, the alkaloid, was dried and weighed.

2.2.6.5 Quantitative determination of Saponins
The method used was that of Obadoni and Ochuko (2001). The samples were ground and 20 g of each were put into a conical flask and 100 cm3 of 20% aqueous ethanol were added. The samples were heated over a hot water bath for 4 h with continuous stirring at about 55°C. The mixture was filtered and the residue re-extracted with another 200 ml of 20% ethanol. The combined extracts were reduced to 40 ml over water bath at about 90°C. The concentrate was transferred into a 250 ml separating funnel and 20 ml of diethyl ether was added and
shaken vigorously. The aqueous layer was recovered while the ether layer was discarded. The purification process was repeated. 60 ml of n-butanol was added. The combined n-butanol extracts were washed twice with 10 ml of 5% aqueous sodium chloride. The remaining solution was heated in a water bath. After evaporation the samples were dried in the oven to a constant weight; the saponin content was calculated as mg/g.

2.2.6.6 Quantitative determination of steroids

This was determined by the method described by Edeoga et al. (2005). A known weight of each sample was dispersed in 100 ml freshly distilled water and homogenized in a laboratory blender. The homogenate was filtered and the filtrate was eluted with 0.1 normal ammonium hydroxide solution (pH 9). The eluent (2 ml) was put in test tube and mixed with 2 ml of chloroform. Ice-cold acetic anhydride (3 ml) was added to the mixture in the flask and 2 drops of conc. H$_2$SO$_4$ were cautiously added. Standard sterol solution was prepared and treated as described above. The absorbances of standard and prepared sample were measured using spectrophotometer at 420 nm.

2.2.7 Acute toxicity test of methanol seed extract of *Prosopis africana* seed.

The method of Lorke (1983) was used for the acute toxicity test of the methanol seed extract of *Prosopis africana*. Eighteen (18) albino mice were utilized in this study. The test involved two phases. In phase one, the animals were grouped into three (3) groups of three mice each and were given 10, 100 and 1000 mg/kg body weight of the extract respectively and in the second phase, 1600, 2900 and 5000 mg/kg body weight of the extract were administered to the groups respectively. The administration of the extract was done orally using intubation tube. The animals were then observed for 24hrs for nervousness, dullness, in-coordination or death.

2.2.8 Induction of Liver Damage

The induction of liver damage was according to (Mitchell *et al*., 1973). Paracetamol was suspended in normal saline and administered orally at a dose of 2500mg/kg b.w.. The animals were starved for 24 hours before the commencement of the experiment but had free access to drinking water. Group II, the paracetamol control group received a daily dose of normal saline and feed for 6 days and paracetamol (2500mg/kg) suspension on day 7 and on day 14.

2.2.9 Experimental Design
Thirty-two (32) male albino rats weighing 120-180 g were used for the study. The animals were maintained under hygienic conditions, with feed and water available ad libitum for seven (7) days before the onset of the experiment. After acclimatization, the animals were randomly divided into eight (8) groups of four (4) rats each. The route of administration was via oral route with the aid of an oral intubation tube. The groups and doses administered are summarised below.

Group 1: received 5ml/kg of normal saline for 14days (Normal control).
Group 2: received paracetamol (2500mg/kg) suspension on day 7 and day 14 (Positive control).
Group 3: received low dose of extract (100mg/kg bw) for 14days.
Group 4: received high dose of extract (400mg/kg bw) for 14days.
Group 5: received low dose of extract (100mg/kg bw) + paracetamol (2500mg/kg). They received the extract for 7days. After 30mins on the 7th day, paracetamol was administered. This group continued receiving the extract for the next 7 days.
Group 6: received high dose of extract (400mg/kg bw) + paracetamol (2500mg/kg). (The same procedure as group 5)
Group 7: received paracetamol (2500mg/kg) + low dose of extract (100mg/kg bw).
Paracetamol was administered on the 1st day, treatment with the extract commenced after 24hours for 14days.
Group 8: received paracetamol (2500mg/kg) + high dose of extract (400mg/kg bw). (The same procedure as group 7)
Groups 5 and 6 represent the hepatoprotective effects of the extract while groups 7 and 8 represent the curative effects of the extract. Blood samples were collected and analyzed on the 7th and on the 14th day of the experiment. Blood was collected into sample bottles through rectobulba plexus in the eye and carefully mixed with the anticoagulant, EDTA -10% w/v in distilled water to prevent clotting for the determination of haematological parameters and into non-heparinised sample bottles to obtain serum for the determination of some biochemical parameters.

2.2.10 Haematological parameters of rats treated with methanol extract of Prosopis africana seed.
Haematological parameters were determined using the method described by Dacie and Lewis (1991).
2.2.10.1 Determination of packed cell volume (PCV).

**Principle:** When whole blood sample is subjected to a centrifugal force for maximum RBC packing, the space occupied by the RBCs is measured and expressed as percentage of the whole blood volume.

**Method:** Using microhaematocrit method, a well-mixed anticoagulated whole blood was allowed to enter capillary haematocrit tubes until they are approximately 2/3 filled with blood. Blood filling was done for each tube. One end of each tube was sealed with plastic seal and placed in the medial groves of the centrifuge, head exactly opposite each other, with the sealed end away from the centre of the centrifuge. All tubes were spun for five minutes at 1000 rpm. The tubes were removed as soon as the centrifuge had stopped spinning.

**Calculation:** PCV was obtained for each tube using microhaematocrit-reader by measuring the height of the RBC column and expressing this as a ratio of the height of the total blood column.

\[
PCV(\%) = \left( \frac{\text{Height of cell column}}{\text{Height of total blood column}} \right) \times 100
\]

2.2.10.2 Determination of haemoglobin (Hb) concentration

**Principle:** Whole blood is added to Drabkin’s regent: a solution containing KCN and (CN)$_6$ KCN converts Hb-Fe$^{2+}$ (ferrous) to Hb-Fe$^{3+}$ (ferric) state to form methaemoglobin which then combines with KCN to form a stable pigment, cyanmethaemoglobin complex. The colour intensity of this mixture is measured in a spectrophotometer at a wavelength of 540 nm or using a yellow-green filter. The optical density (OD) of the solution is proportional to the haemoglobin concentration. All forms of Hb (Hb-C, Hb-O, etc) except Hb-S are measured with this cyanmet-method.

**Method:** Exactly 5.0 ml of Drabkin’s reagent was pipetted into two test tubes 1 and 2 and a well-mixed sample of EDTA treated blood (0.02 ml) was pipetted into the tubes, rinsing the pipette five times with the reagent, until all the blood had been removed from the pipette. The solutions were well mixed and allowed to stand at 25°C for 10 minute in order to allow the formation of cyano-methaemoglobin. The mixtures were read in a spectrophotometer at a wavelength of 540 nm. The Drabkin’s reagent in tube I was used to blank the machine (setting the percentage transmittance at 100%). The readings were recorded using a pre-calibrated chart and the actual Hb values in g/dl were determined.

2.2.10.3 Determination of white blood cells (WBCs) counts
**Principle:** When whole blood is mixed with weak acid solution, it dilutes the blood and haemolyses the RBCs, enabling the WBCs to be counted.

**Method:** Manual WBC counting method was used as follows:

**Dilution of Blood:**
(a) The blood specimen was mixed approximately for one minute; using the aspirator and WBC pipette, blood was drawn to the 0.5 mark in the pipette.
(b) Blood was removed from the outside of the pipette with clean gauze.
(c) Holding the pipette almost vertically, the tip was placed into the counting diluting fluid to draw it slowly. While gently rotating the pipette, to ensure proper mixing, the diluting fluid was aspirated until it reached the 11 mark.
(d) The pipette was placed in a horizontal position and firmly holding the index finger of either hand over the opening in the tip of the pipette, aspirator was detached from the other end of the pipette. This is 1:20 dilution
(e) Having now completed the dilution of blood, the counting chamber and cover glass were cleaned with a lint-free cloth.

**Filling the counting chamber:** Approximately 0.02 ml of well mixed EDTA-anticoagulated blood sample was added to 0.38 ml of dilute fluid, dispensed into a small container. One of the grids of the counting chamber was filled with re-mix of the diluted blood sample using a Pasteur pipette, taking care not to overfill the area. The filled area was left undisturbed for two minutes to allow time for the white blood cells to settle, after which the underside of the chamber was dried and placed on the microscope stage.

**Counting the white blood cells:** Using the x10 objective with the condenser iris closed sufficiently to give good contrast, the ruling of the chamber and white cells were focused until the cells appeared as small black dots. The cells in the four large squares of the chamber were then squarely counted.

**Calculation:**
- The number of white cells per litre of blood was calculated as follows:
- The total number of cells counted was divided by 2
- The number obtained was then divided by 10
- The result was then multiplied by $10^9$ to give the white cell count.

**2.2.10.4 Determination of red blood cell (RBCs) counts**
**Principle:** When whole blood is diluted with an isotonic fluid, it prevents lysis and facilitates counting of the red cells. Some isotonic solutions in use include Hayem’s solution, Gower’s solution or 0.85% sodium chloride (NaCl) solutions.

**Method:** Using the Thoma (manual counting) method, anti-coagulated blood was drawn up to the 0.5 ml mark in the RBC count pipette and diluted to a 101 mark with RBC diluting fluid (1:200 dilution). Dilution was repeated with the replicate tube. The counting chamber was cleaned; both pipettes were shaken three times; counting chamber filled (first expelling the first 4 drops of the mixture), allowing approximately three minutes for the RBCs to settle, Red cells were counted using the counting steps as follows:

1. The filled counting chamber was carefully placed on the microscope stage.
2. Using low power (x10 objective) the large centre square was placed in the middle of the field of vision and the entire large square was carefully examined for even distribution of RBCs.
3. The high-dry objective was carefully changed, moving the counting chamber so that the small upper left corner square (this square is further sub-divided into 16 even smaller squares) is completely in the field of vision.
4. All the RBC were counted in the squares, also counting the cells on the two of the margins but excluding those lying on the other two sides

**Calculation** The RBCs (in mm$^3$) = cells counted $\times$ correction for volume $\times$ correction for dilution$=$ RBCs counted in 5 small squares $\times$ 200 $\times$ 10$^4$. 

2.2.11 Biochemical Parameters

2.2.11.0 Liver function test of rats treated with methanol extract of fermented *Prosopis africana* seed

Liver Function Test (ALT, AST and ALP) was determined using the method of Reitman and Frankel (1957) and Klein *et al.* (1960)

**2.2.11.1 Determination of aspartate aminotransferase (AST)**

**Principle:** AST or SGOT is measured by monitoring the concentration of oxaloacetate hydrazone formed with 2, 4-dinitrophenylhydrazine. The colour intensity is measured against the blank at 546 nm.
**Method:** The blank and sample test tubes were set up in duplicates. A volume, 0.1ml of serum was pipetted into the sample tubes and 0.5ml of reagent 1 was pipette into both sample and blank tubes. The solutions were thoroughly mixed and incubated for exactly 30 minutes at 37 °C ml and pH 7.4. 0.5ml of Reagent 2 containing 2, 4-dinitrophenylhydrazine was added into all the test tubes followed by 0.1ml of sample into the blank tubes. The tubes were mixed thoroughly and incubated for exactly 20 minutes at 25 °C and 5.0ml of sodium hydroxide solution was then added to each tube and mixed. The absorbance was read against the blank after 5 minutes at 546 nm.

**Calculation:** the activity of AST was read up from Table 1b from the appendix.

### 2.2.11.2 Determination of alanine aminotransferase (ALT)

**Principle:** ALT is measured by monitoring the concentration of pyruvate hydrazone formed with 2, 4-dinitrophenylhydrazine. The colour intensity is measured against the blank at 546nm.

**Method:** The blank and sample test tubes were set up in duplicates. 0.1ml of serum was pipetted into the sample tubes. To these were added 0.5ml buffer solution containing phosphate buffer, L-alanine and α-oxoglutarate. The mixtures were thoroughly mixed and incubated for exactly 30 minutes at 37 °C and pH 7.4. A volume, 0.5ml of reagent containing 2, 4-dinitrophenylhydrazine was later added to both tubes while 0.1ml of sample was added to sample blank tube. The tubes were mixed thoroughly and incubated for exactly 20 minutes at 25 °C. Five milistres of sodium hydroxide solution was then added to each tube and mixed. The absorbance was read against the blank after 5 minutes at 546 nm.

**Calculation:** The activity of ALT was read up from Table 1a (see appendix).

### 2.2.11.3 Determination of alkaline phosphatase (ALP)

**Principle:** The principle of this method is based on the reaction involving serum alkaline phosphatase and a colourless substrate of phenolphthalein monophosphate, giving rise to phosphoric acid and phenolphthalein which at alkaline pH values, turn pink that can be determined spectrophotometrically.

\[ \text{P-nitrophenylphosphate} + \text{H}_2\text{O} \rightarrow \text{ALP} \rightarrow \text{PO}_4^{2-} + \text{P-nitrophenol (pink at pH=9.8)} \]
**Method:** The blank and sample test tubes were set up in duplicates and 0.05ml of sample was pipette into the sample test tubes. 0.05ml of distilled water was pipetted into the blank tube. Three millilitres (3.0ml) of substrate was pipetted into each tube respectively, which was then mixed and the initial absorbance taken at 405nm. The stop watch was started and the absorbance of the sample and the blank read again three more times at one minute intervals.

**Calculation:** alkaline phosphatase activity was calculated as follows:

\[
\text{Activity of ALP (in U/L)} = \frac{\text{Absorbance of sample}}{\text{Absorbance of standard}} \times 3300
\]

### 2.2.12.0 Lipid profile test of rats treated with methanol extract of fermented *Prosopis africana* seed

#### 2.2.12.1 Cholesterol determination

The method of Abell *et al.*, (1952) was followed.

**Principle**

Cholesterol was determined after enzymatic hydrolysis and oxidation. The indicator quinoneimine is formed from hydrogen peroxidase and 4-aminoantipyrine in the presence of phenol and peroxidase.

**Test procedure**

Three (3) test tubes were set up in a test tube rack and labelled blank, standard and sample respectively. To the blank, was added (10µl) distilled H₂O, 10µl standard specimen to the standard test tube and 10µl sample (serum) to the sample test tube. To each of these test tubes was added 1000µl of the cholesterol reagent. It was thoroughly mixed and incubated for 10minutes at room temperature (20-25°C). The absorbance of the sample \(A_{\text{sample}}\) against the blank was taken within 60 minutes at 500nm.

#### 2.2.12.2 Triacylglycerol

The concentration of triacylglycerol (TAG) was determined according to the method of Otvos (1999).

**Clinical significance:**

Triacylglycerols measurements are used in the diagnosis and treatment of diseases involving lipid metabolism and various endocrine disorders e.g diabetes mellitus, nephrosis and liver obstruction.
**Principle:**

The triacylglycerols are determined after enzymatic hydrolysis with lipases. The indicator is a quinoneimine formed from hydrogen peroxide, 4-aminophenazone and 4-chlorophenol under the catalytic influence of peroxidase.

\[
\text{Triacylglycerol} + H_2O_{\text{lipases}} \rightarrow \text{Glycerol + fatty acids}
\]

\[
\text{Glycerol} + \text{ATP} \xrightarrow{\text{GK}} \text{Glycerol-3-phosphate} + \text{ADP}
\]

\[
\text{Glycerol-3-phosphate} + O_2 \xrightarrow{\text{GPO}} \text{Dihydroxyacetone phosphate} + H_2O_2
\]

\[
2H_2O_2 + 4\text{-aminophenazone} + 4\text{ chlorophenol} \xrightarrow{\text{POD}} \text{Quinoneimine} + \text{HCl} + 4H_2O
\]

**Method:** A quantity, 0.1 ml of the sample was pipetted into a clean labelled tube and 1.0 ml of trichloroacetic acid (TCA) was added, mixed and then centrifuged at 300 rpm for 10 minutes. The supernatant was decanted and reserved for use. The assay procedure was carried out as follows:

<table>
<thead>
<tr>
<th>Reagents (ml)</th>
<th>Blank</th>
<th>Standard</th>
<th>Sample</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Distilled water</td>
<td>0.5</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>2. Standard solution</td>
<td>-</td>
<td>0.5</td>
<td>-</td>
</tr>
<tr>
<td>3. TCA</td>
<td>0.5</td>
<td>0.5</td>
<td>-</td>
</tr>
<tr>
<td>4. Supernatant</td>
<td>-</td>
<td>-</td>
<td>1.0</td>
</tr>
<tr>
<td>5. Reagent mixture</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
</tr>
</tbody>
</table>

The mixtures were allowed to stand for 20 minutes at 25 °C and the absorbance of the sample and standard read against the blank at 540 nm.

**Calculation:** The concentration of triacylglycerol in serum was calculated as follows:

\[
\text{Absorbance of sample} \times \text{Standard concentration (mmol/l)} = \text{Absorbance of standard}
\]

2.2.12.3 High density lipoprotein (HDL) Cholesterol

The concentration of high density lipoprotein (HDL) was determined according to the method of Kameswara et al. (1999).
Principle:

LDL and VLDL (low and very low density lipoproteins) are precipitated from serum by the action of a polysaccharide in the presence of divalent cations. Then, high density lipoproteins (HDL) present in the supernatant is determined.

Procedure:

The precipitant solution 0.1ml was added to 0.3ml of the sample and mixed thoroughly and allowed to stand for 15 min. This was centrifuged at 2,000 x g for 15 min. The cholesterol concentration in the supernatant was determined.

2.2.12.4 Low density lipoprotein (LDL) Cholesterol

The concentration of low density lipoprotein (LDL) cholesterol was determined according to the method of Kameswara et al. (1999).

Principle:

LDL-C can be determined as the difference between total cholesterol and the cholesterol content of the supernatant after precipitation of the LDL fraction by polyvinyl sulphate (PVS) in the presence of polyethyleneglycol monomethyl ether.

Procedure:

The serum samples were kept at 2-8°C. The precipitant solution (0.1ml) was added to 0.2ml of the serum sample and mixed thoroughly and allowed to stand for 15 min. This was centrifuged at 2,000 x g for 15 min. The cholesterol concentration in the supernatant was determined. The concentration of the serum total cholesterol as described by Kameswara et al. (1999) was used.

Calculation:

\[
LDL-C (\text{mmol/L}) = \text{Total Cholesterol (mmol/L)} - 1.5 \times \text{Supernatant Cholesterol (mmol/L)}.
\]

2.2.13 Determination of membrane stabilization effect of fermented Prosopis africana seed extract on hypotonicity induced haemolysis of human red blood cells.

The membrane stabilization effect of the extract was evaluated using hypotonicity-induced haemolysis of human red blood cells. The modified method of Shinde et al. (1999) was used. Exactly 5 ml of blood was collected in a tube containing 0.1 vol. of 3.8% trisodium citrate
and was used within 8 hours. The blood sample was centrifuged at 3000rpm for 10mins. The supernatant (plasma) was discarded. The pellet was washed twice by resuspending exact amount of normal saline equal to the volume of the supernatant discarded. The washing was done by centrifuging at 3000rpm for 10mins. The pellet (0.1ml) was resuspended in 2.5ml of normal saline and incubated at 37°C for 1 hr. After incubation, the mixture was centrifuged at 3,000rpm for 10 min to terminate the reaction and the absorption of the supernatant measured at 418 nm to assess the extent of lysis of the red cells. The incubation was repeated in the presence of the extract (0.1, 0.2, 0.4 and 0.5 mg/ml). 1.2ml normal saline and 0.8ml distilled water was used as blank. % inhibition of haemolysis was calculated using this formula:

\[
\% \text{ inhibition of haemolysis} = 100 \times \left( \frac{\text{OD}_1 - \text{OD}_2}{\text{OD}_1} \right)
\]

Where \( \text{OD}_1 \) = Optical density of hypotonic solution (control)

\( \text{OD}_2 \) = Optical density of test sample in hypotonic solution.

2.3 Statistical Analysis

The data obtained were analysed using Statistical Package for Social Sciences (SPSS) version 16.0 and the results expressed as mean ± standard error of mean. Significant differences of the result were established, T-Test and one-way ANOVA and the acceptance level of significance was \( p<0.05 \) for all the results.
CHAPTER THREE

RESULTS

3.1 Qualitative phytochemical composition of methanol extract of fermented seeds of *Prosopis africana*

Table 2 shows the phytochemical composition of the methanol extract of *P. africana* seeds. A bioactive compound such as cyanogenic glycosides was slightly present. On the other hand, alkaloids, steroids, saponins and tannins were found to be moderately present. While flavonoids was found to be highly present, terpenoids and reducing sugar were not detected in the methanol extract of the fermented seeds of *P. africana*.

3.2 Quantitative phytochemical composition of methanol extract of fermented seeds of *Prosopis africana*

Table 3 shows quantitative phytochemical composition of the methanol extract of *P. africana* seeds. Bioactive compounds such as tanins were found to be highest (5.445 ± 0.005 mg/100g) relatively compared to flavonoids (0.077±0.006 mg/100mg), alkaloids (3.341±0.004 mg/100g), saponins (1.137±0.002 mg/100g) and steroids (1.645±0.002 mg/100g). Cyanogenic glycosides were found to be lowest (0.077 ± 0.006 mg/100g).
Table 2: Qualitative phytochemical composition of methanol extract of fermented seeds of *Prosopis africana*.

<table>
<thead>
<tr>
<th>Phytochemicals</th>
<th>Relative Presence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alkaloids</td>
<td>++</td>
</tr>
<tr>
<td>Flavonoids</td>
<td>+++</td>
</tr>
<tr>
<td>Steroids</td>
<td>++</td>
</tr>
<tr>
<td>Saponins</td>
<td>++</td>
</tr>
<tr>
<td>Cyanogenic glycosides</td>
<td>+</td>
</tr>
<tr>
<td>Tannins</td>
<td>++</td>
</tr>
<tr>
<td>Terpenoids</td>
<td>ND</td>
</tr>
<tr>
<td>Reducing Sugars</td>
<td>ND</td>
</tr>
</tbody>
</table>

**Key:**
- + Slightly present
- ++ Moderately present
- +++ Highly present
- ND Not Detected

Table 3: Quantitative phytochemical composition of methanol extract of fermented seeds of *Prosopis africana*

<table>
<thead>
<tr>
<th>Phytochemical composition</th>
<th>Quantity/Amount (mg/100g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tannins</td>
<td>5.445±0.005</td>
</tr>
<tr>
<td>Cyanogenic glycosides</td>
<td>0.077±0.006</td>
</tr>
<tr>
<td>Flavonoids</td>
<td>4.394±0.003</td>
</tr>
</tbody>
</table>
### 3.3 Acute toxicity (LD₅₀) test of methanol extract of fermented seeds of *Prosopis africana*

The acute toxicity test of the methanol extract of *P. africana* seeds showed no death up to 5000 mg/kg body weight within 24 hours of administration as shown in table 4. This result shows that the extract is safe at dose below 5000 mg/kg body weight.
Table 4: The median lethal dose of methanol extract of fermented seeds of \textit{P. africana}

<table>
<thead>
<tr>
<th>Phase 1</th>
<th>Dose mg/kg body weight</th>
<th>Mortality</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group 1</td>
<td>10</td>
<td>0/3</td>
</tr>
<tr>
<td>Group 2</td>
<td>100</td>
<td>0/3</td>
</tr>
<tr>
<td>Group 3</td>
<td>1000</td>
<td>0/3</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Phase II</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Group 1</td>
<td>1600</td>
<td>0/3</td>
</tr>
<tr>
<td>Group 2</td>
<td>2900</td>
<td>0/3</td>
</tr>
<tr>
<td>Group 3</td>
<td>5000</td>
<td>0/3</td>
</tr>
</tbody>
</table>
3.4 Effect of methanol seed extract of *Prosopis africana* on packed cell volume in paracetamol-induced liver damage in rats

Results obtained showed that on day 7, significant (p<0.05) increase were observed in the packed cell volume (PCV) of groups 5, 6, 7 rats administered 100 mg/kg b.w. of the extract before paracetamol induction, 400 mg/kg b.w. of the extract before paracetamol induction and paracetamol-induced and treated with 100 mg/kg b.w. of the extract respectively compared with PCV of group 2 rats which represented the paracetamol-induced group as shown in Fig. 6. Significant (p<0.05) decrease was observed in PCV of group 4 rats administered 400 mg/kg b.w. of the extract compared with PCV of normal rats in group 1. There was no significant (p>0.05) difference in the PCV of group 4 rats compared with PCV of paracetamol-induced rats in group 2. Also, non-significant (p>0.05) change was observed in PCV of group 5 rats administered 100 mg/kg b.w. of the extract before paracetamol induction of liver damage compared with PCV of group 1 (normal control). On day 14, significant (p<0.05) increases were observed in the PCV of groups 4, 5, 6, 7, 8 rats administered 400 mg/kg b.w. of the extract only, 100 mg/kg b.w. of the extract before paracetamol induction, 400 mg/kg b.w. of the extract before paracetamol induction, paracetamol-induced and treated with 100 mg/kg b.w. of the extract and paracetamol-induced and treated with 400 mg/kg b.w. of the extract respectively when compared with PCV of paracetamol-induced rats in group 2. However, a relative increase was observed in PCV of group 3 rats administered 100 mg/kg b.w. of extract compared with PCV of group 2 rats treated with paracetamol only.
Group 1
Group 2
Group 3
Group 4
Group 5
Group 6
Group 7
Group 8

Treatment Groups

Mean PCV (%)

Day 7
Day 14

Group 1
Group 2
Group 3
Group 4
Group 5
Group 6
Group 7
Group 8
Fig. 6: Effect of methanol seed extract of *Prosopis africana* on packed cell volume in paracetamol-induced liver damage in rats

<table>
<thead>
<tr>
<th>Group 1=Normal Control</th>
<th>Group 2=Paracetamol (2500mg/kg bw)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group 3=100mg/kg bw of Extract</td>
<td>Group 4=400mg/kg bw of Extract</td>
</tr>
<tr>
<td>Group 5=100mg/kg bw of Extract + Paracetamol</td>
<td>Group 6=400mg/kg bw of Extract + Paracetamol</td>
</tr>
<tr>
<td>Group 7=Paracetamol + 100mg/kg bw of Extract</td>
<td>Group 8=Paracetamol + 400mg/kg bw of Extract</td>
</tr>
</tbody>
</table>

3.5 Effect of methanol seed extract of *Prosopis africana* on haemoglobin concentration in paracetamol-induced liver damage in rats

Fig. 7 shows that on day 7, a significant (p<0.05) increase in the mean haemoglobin (Hb) level of groups 5, 6, 7 rats administered 100 mg/kg b.w. of the extract before paracetamol induction, 400 mg/kg b.w. of the extract before paracetamol induction and paracetamol-induced and treated with 100 mg/kg b.w. of the extract respectively when compared with the Hb concentration of rats in group 2 which represented the paracetamol-induced group. Significant (p<0.05) reduction was observed in group 4 rats administered 400 mg/kg b.w. of the extract only compared with group 1 normal control rats. A relative increase was observed in the mean Hb level of groups 3 and 8 administered 100 mg/kg of the extract only and paracetamol-induced and treated with 400 mg/kg b.w. of the extract compared with Hb concentration of paracetamol-induced liver damage in rats in group 2. On day 14, the mean Hb concentration of rats in groups 4, 5, 6, 7 and 8 rats administered 400 mg/kg b.w. of the extract only, 100 mg/kg b.w. of the extract before paracetamol induction, 400 mg/kg b.w. of the extract before paracetamol induction, paracetamol-induced and treated with 100 mg/kg b.w. of the extract and paracetamol-induced and treated with 400 mg/kg b.w. of the extract respectively increased significantly (p<0.05) when compared to the mean Hb concentration of group 2 rats treated with paracetamol only. An increase was observed in the mean Hb concentration of group 3 rats though not significant (p>0.05) when compared with the Hb concentration of paracetamol-induced group 2 rats.
Fig. 7: Effect of methanol seed extract of *Prosopis africana* on haemoglobin concentration in paracetamol-induced liver damage in rats
3.6 Effect of methanol seed extract of *Prosopis africana* on total white blood cell count in paracetamol-induced liver damage in rats

As shown in Fig. 8, significant (p<0.05) increase was observed in the total white blood cell (WBC) count of groups 3, 4, 5 and 6 rats administered 100 mg/kg b.w. of the extract only, 400 mg/kg b.w. of the extract only, 100 mg/kg b.w. of the extract before paracetamol induction and 400 mg/kg b.w. of the extract before paracetamol induction when compared with the WBC count of group 2 which represented the paracetamol-induced group on day 7. In all the test groups, a significant (p<0.05) decrease was observed in total WBC count when compared with the total WBC count of normal control rats represented by group 1. As shown on day 14, significant (p<0.05) increase was observed in the total WBC count of groups 4 and 5 rats administered 400 mg/kg b.w. of the extract only and 100 mg/kg b.w. of the extract before paracetamol induction when compared with the total WBC count of paracetamol-induced rats in group 2. Groups 3, 6, 7, 8 rats administered 100 mg/kg b.w. of the extract only, 400 mg/kg b.w. of the extract before paracetamol induction, paracetamol-induced and treated with 100 mg/kg b.w. of the extract and paracetamol-induced and treated with 400 mg/kg b.w. of the extract shows a significant (p<0.05) reduction compared with the total WBC count of normal rats in group 1.
Fig. 8: Effect of methanol seed extract of *Prosopis africana* on total white blood cell count in paracetamol-induced liver damage in rats

Group 1=Normal Control  
Group 2=Paracetamol (2500 mg/kg bw)  
Group 3=100mg/kg bw of Extract  
Group 4=400mg/kg bw of Extract  
Group 5=100mg/kg bw of Extract + Paracetamol  
Group 6=400mg/kg bw of Extract + Paracetamol
3.7 Effect of methanol seed extract of *Prosopis africana* on red blood cell count in paracetamol-induced liver damage in rats

Fig. 9 shows relative increase in red blood cell (RBC) count in all the test groups on day 7, though such increase was not significant (p>0.05) when compared with the RBC count of paracetamol-induced rats in group 2. Day 14 showed a non-significant (p>0.05) reduction in the RBC count of groups 4, 5, 6, 7, and 8 rats administered 400 mg/kg b.w. of the extract only, 100 mg/kg b.w. of the extract before paracetamol induction, 400 mg/kg b.w. of the extract before paracetamol induction, paracetamol-induced and treated with 100 mg/kg b.w. of the extract and paracetamol-induced and treated with 400 mg/kg b.w. of the extract respectively when compared with the RBC count of control groups 1 and 2 which represented normal and standard controls. A significant (p<0.05) increase was observed in the RBC count group 3 rats administered 100 mg/kg b.w of the extract when compared with RBC count of the control groups.
Fig. 9: Effect of methanol seed extract of *Prosopis africana* on red blood cell count in paracetamol-induced liver damage in rats

Group 1=Normal Control  
Group 2=Paracetamol (2500mg/kg bw)  
Group 3=100mg/kg bw of Extract  
Group 4=400mg/kg bw of Extract  
Group 5=100mg/kg bw of Extract + Paracetamol  
Group 6=400mg/kg bw of Extract + Paracetamol  
Group 7=Paracetamol + 100mg/kg bw of Extract  
Group 8=Paracetamol + 400mg/kg bw of Extract
3.8 Effect of methanol seed extract of *Prosopis africana* on aspartate aminotransferase activity in paracetamol-induced liver damage in rats

As shown in Fig. 10 on day 7, aspartate aminotransferase (AST) activity indicated a significant (p<0.05) reduction in groups 6 and 7 rats administered 400 mg/kg b.w. of extract before paracetamol induction and paracetamol-induced and treated with 100 mg/kg b.w. of extract respectively when compared with the AST activity of group 2 rats treated with paracetamol only. Day 14 in Fig. 10 shows a significant (p<0.05) reduction in the AST activity of groups 6 and 8 rats when administered 400 mg/kg b.w. of extract before paracetamol induction and paracetamol-induced and treated with 400 mg/kg b.w. of extract compared with the AST activity of group 2 rats. Although a relative decrease was observed on both days 7 and 14 in groups 3, 4 and 5 rats administered 100 mg/kg b.w. of the extract, 400 mg/kg b.w. of the extract and 100 mg/kg b.w. of extract before paracetamol induction when compared to the AST activity of group 2 rats.
Fig. 10: Effect of methanol seed extract of *Prosopis africana* on aspartate aminotransferase activity in paracetamol-induced liver damage in rats

Group 1=Normal Control
Group 2=Paracetamol (2500mg/kg bw)
Group 3=100mg/kg bw of Extract
Group 4=400mg/kg bw of Extract
Group 5=100mg/kg bw of Extract + Paracetamol
Group 6=400mg/kg bw of Extract + Paracetamol
Group 7=Paracetamol + 100mg/kg bw of Extract
Group 8=Paracetamol + 400mg/kg bw of Extract
3.9 Effect of methanol seed extract of *Prosopis africana* on alanine aminotransferase activity in paracetamol-induced liver damage in rats

Fig. 11 shows a significant (p<0.05) reduction in the alanine aminotransferase (ALT) activity of all the test groups on day 7 when compared to the ALT activity of paracetamol-induced rats in group 2. A significant (p<0.05) increase was observed in the ALT activity of group 2 rats treated with paracetamol only when compared to the ALT activity of normal control rats in group 1. However, a non-significant (p>0.05) decrease was observed in the ALT activity of all test groups compared to the ALT activity of group 2 rats on day 14.
Fig. 11: Effect of methanol seed extract of *Prosopis africana* on alanine aminotransferase activity in paracetamol-induced liver damage in rats

Group 1=Normal Control
Group 2=Paracetamol (2500mg/kg bw)
Group 3=100mg/kg bw of Extract
Group 4=400mg/kg bw of Extract
Group 5=100mg/kg bw of Extract + Paracetamol
Group 6=400mg/kg bw of Extract + Paracetamol
Group 7=Paracetamol + 100mg/kg bw of Extract
Group 8=Paracetamol + 400mg/kg bw of Extract
3.10 Effect of methanol seed extract of *Prosopis africana* on alkaline phosphatase activity in paracetamol-induced liver damage in rats

Fig. 12 shows a non-significant (p>0.05) increase in alkaline phosphatase (ALP) activity in all the test groups on day 7 when compared with the ALP activity of normal and paracetamol groups (groups 1 and 2 respectively). Day 14 also showed a non-significant (p>0.05) increase in the ALP activity of group 3 rats administered 100 mg/kg b.w. of the extract only when compared with the ALP activity of groups 1 and 2. A non-significant (p>0.05) decrease was observed in the ALP activity of groups 4, 5, 6, 7 and 8 rats administered 400 mg/kg b.w. of the extract only, 100 mg/kg b.w. of the extract before paracetamol induction, 400 mg/kg b.w. of the extract before paracetamol induction, paracetamol-induced and treated with 100 mg/kg b.w. of the extract and paracetamol-induced and treated with 400 mg/kg b.w. of the extract when compared with the ALP activity of groups 1 and 2.
Fig. 12: Effect of methanol seed extract of *Prosopis africana* on alkaline phosphatase activity in paracetamol-induced liver damage in rats

Group 1=Normal Control  
Group 2=Paracetamol (2500mg/kg bw)  
Group 3=100mg/kg bw of Extract  
Group 4=400mg/kg bw of Extract  
Group 5=100mg/kg bw of Extract + Paracetamol  
Group 6=400mg/kg bw of Extract + Paracetamol  
Group 7=Paracetamol + 100mg/kg bw of Extract  
Group 8=Paracetamol + 400mg/kg bw of Extract
3.11 Effect of methanol seed extract of *Prosopis africana* on total cholesterol levels in paracetamol-induced liver damage in rats

As shown in Fig. 13, total cholesterol level decreased significantly (p<0.05) in groups 4, 6 and 8 rats administered 400 mg/kg b.w. of the extract only, 400 mg/kg b.w. of the extract before paracetamol induction and 400 mg/kg b.w. of the extract after paracetamol induction when compared to the total cholesterol concentration of normal rats in group 1 on day 7. A non-significant (p<0.05) increase was observed in the total cholesterol concentration of groups 3, 5 and 7 rats administered 100 mg/kg b.w. of the extract only, 100 mg/kg b.w. of the extract before paracetamol induction and 100 mg/kg b.w. of the extract after paracetamol induction when compared with the total cholesterol concentration of group 2 rats induced with paracetamol. Day 14 shows a significant (p<0.05) increase in total cholesterol concentration of group 7 rats when compared with the control groups. Also, a significant (p<0.05) increase was seen in total cholesterol level of group 3 rats when compared with rats in group 1. A non-significant (p>0.05) decrease was observed in total cholesterol concentration of groups 4, 5, 6 and 8 rats when compared with total cholesterol concentration of rats in group 2.
Fig. 13: Effect of methanol seed extract of *Prosopis africana* on total cholesterol levels in paracetamol-induced liver damage in rats

Group 1=Normal Control  
Group 2=Paracetamol (2500mg/kg bw)  
Group 3=100mg/kg bw of Extract  
Group 4=400mg/kg bw of Extract  
Group 5=100mg/kg bw of Extract + Paracetamol  
Group 6=400mg/kg bw of Extract + Paracetamol  
Group 7=Paracetamol + 100mg/kg bw of Extract  
Group 8=Paracetamol + 400mg/kg bw of Extract
3.12 Effect of methanol seed extract of *Prosopis africana* on triacylglycerol levels in paracetamol-induced liver damage in rats

As shown in Fig. 14, the triacylglycerol (TAG) concentration was observed to have increased significantly (p<0.05) in group 7 rats induced with paracetamol before the treatment with 100mg/kg of extract when compared with the TAG concentration of normal control rats in group 1 on day 7. A significant (p<0.05) decrease was observed in the TAG concentration of group 4 rats administered 400 mg/kg of extract when compared with the TAG concentration of paracetamol-induced rats in group 2. Day 14 shows a significant (p<0.05) increase in the TAG concentration of group 4, 7 and 8 rats administered with 400 mg/kg b.w. of extract only, 100 mg/kg b.w. of extract after induction of paracetamol and 400 mg/kg b.w. of extract after induction of paracetamol when compared with the TAG concentration of rats in group 1. Non-significant (p>0.05) increase was observed in the TAG concentration of groups 7 and 8 rats when compared with the TAG concentration of paracetamol-induced rats in group 2.
Fig. 14: Effect of methanol seed extract of *Prosopis africana* on triacylglycerol levels in paracetamol-induced liver damage in rats

<table>
<thead>
<tr>
<th>Treatment Groups</th>
<th>Group 1 Normal Control</th>
<th>Group 2 Paracetamol (2500mg/kg bw)</th>
<th>Group 3 100mg/kg bw of Extract</th>
<th>Group 4 400mg/kg bw of Extract</th>
<th>Group 5 100mg/kg bw of Extract + Paracetamol</th>
<th>Group 6 400mg/kg bw of Extract + Paracetamol</th>
<th>Group 7 Paracetamol + 100mg/kg bw of Extract</th>
<th>Group 8 Paracetamol + 400mg/kg bw of Extract</th>
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<tr>
<td><strong>Day 7</strong></td>
<td>80</td>
<td>100</td>
<td>120</td>
<td>140</td>
<td>160</td>
<td>180</td>
<td>200</td>
<td>220</td>
</tr>
<tr>
<td><strong>Day 14</strong></td>
<td>100</td>
<td>120</td>
<td>140</td>
<td>160</td>
<td>180</td>
<td>200</td>
<td>220</td>
<td>240</td>
</tr>
</tbody>
</table>
3.13 Effect of methanol seed extract of *Prosopis africana* on high density lipoprotein levels in paracetamol-induced liver damage in rats

Fig. 15 shows a significant ($p<0.05$) decrease in high density lipoprotein (HDL) cholesterol levels of group 3 rats administered 100 mg/kg of extract only compared with group 1 rats (normal control) on day 7. A non-significant ($p>0.05$) increase was observed in HDL cholesterol levels of groups 4, 5, 6, 7 and 8 rats administered 400 mg/kg b.w. of the extract only, 100 mg/kg b.w. of the extract before paracetamol induction, 400 mg/kg b.w. of the extract before paracetamol induction, paracetamol-induced and treated with 100 mg/kg b.w. of the extract and paracetamol-induced and treated with 400 mg/kg b.w. of the extract respectively when compared with HDL cholesterol level of paracetamol-induced group 2 rats. On day 14 it was observed that HDL cholesterol level in group 7 rats induced with paracetamol and treated with 100 mg/kg b.w. of extract increased significantly ($p<0.05$) when compared with HDL cholesterol level of normal rats in group 1. Significant ($p<0.05$) decrease was observed in the HDL cholesterol level of group 5 rats when compared with the HDL cholesterol level of rats in group 2 treated with paracetamol only.
Fig. 15: Effect of methanol seed extract of *Prosopis africana* on high density lipoprotein levels in paracetamol-induced liver damage in rats

Group 1=Normal Control  
Group 2=Paracetamol (2500mg/kg bw)  
Group 3=100mg/kg bw of Extract  
Group 4=400mg/kg bw of Extract  
Group 5=100mg/kg bw of Extract + Paracetamol  
Group 6=400mg/kg bw of Extract + Paracetamol  
Group 7=Paracetamol + 100mg/kg bw of Extract  
Group 8=Paracetamol + 400mg/kg bw of Extract
3.14 Effect of methanol seed extract of *Prosopis africana* on low density lipoprotein levels in paracetamol-induced liver damage in rats

Fig. 16 shows a non-significant (p>0.05) increase in the low density lipoprotein (LDL) cholesterol level of groups 3 and 5 rats administered 100 mg/kg b.w. of extract only and 100 mg/kg b.w. of extract before paracetamol induction respectively when compared with the LDL cholesterol level of rats in groups 1 and 2 on day 7. Non-significant (p>0.05) decrease was observed in the LDL concentration of groups 4, 6, 7 and 8 rats administered 400 mg/kg b.w. of extract, 400 mg/kg of extract before paracetamol induction, paracetamol-induced and treated with 100 mg/kg b.w. of extract and paracetamol-induced and treated with 400 mg/kg b.w. of extract when compared with the LDL cholesterol level of control rats in group 1 and 2. On day 14, a significant (p<0.05) increase was seen in LDL cholesterol level of groups 3 and 7 rats administered 100 mg/kg b.w. of extract only and paracetamol-induced and treated with 100 mg/kg b.w. of extract when compared with the LDL cholesterol level of control groups.
Fig. 16: Effect of methanol seed extract of *Prosopis africana* on low density lipoprotein levels in paracetamol-induced liver damage in rats

Group 1=Normal Control
Group 2=Paracetamol (2500mg/kg bw)
Group 3=100mg/kg bw of Extract
Group 4=400mg/kg bw of Extract
Group 5=100mg/kg bw of Extract + Paracetamol
Group 6=400mg/kg bw of Extract + Paracetamol
Group 7=Paracetamol + 100mg/kg bw of Extract
Group 8=Paracetamol + 400mg/kg bw of Extract
3.15  Effect of *Prosopis africana* seed extract on hypotonicity-induced haemolysis of red blood cells. (membrane stabilization effect of the extract)

Table 5 shows that *Prosopis africana* extract at concentration of 0.5, 0.4, 0.2 and 0.1mg/ml inhibited haemolysis by 97.4, 83.0, 79.2 and 63.2% respectively. The erythrocytes were fairly stable in isotonic solution; the absorbance at 418nm was 0.051. The value 0.701 was the maximum lysis of red cells when it was incubated in water, a low osmotic medium. This caused the absorption of the supernatant solution at 418nm to increase to 0.701. The extent of reduction of haemolysis was found to be dose-dependent.
Table 5: Effect of *Prosopis africana* seed extract on hypotonicity induced haemolysis of normal red blood cells (membrane stabilization effect of the extract)

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Extract concentration (mg/ml)</th>
<th>Normal saline (ml)</th>
<th>Distilled water (ml)</th>
<th>RBC (blood) (ml)</th>
<th>Absorbance % inhibition of haemolysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Isotonic solution</td>
<td>-</td>
<td>1.8</td>
<td>-</td>
<td>0.2</td>
<td>0.051</td>
</tr>
<tr>
<td>Hypotonic solution</td>
<td>-</td>
<td>0.6</td>
<td>1.2</td>
<td>0.2</td>
<td>0.701</td>
</tr>
<tr>
<td>Test sample</td>
<td>0.1</td>
<td>0.5</td>
<td>1.2</td>
<td>0.2</td>
<td>0.258 63.2</td>
</tr>
<tr>
<td>Test sample</td>
<td>0.2</td>
<td>0.4</td>
<td>1.2</td>
<td>0.2</td>
<td>0.146 79.2</td>
</tr>
<tr>
<td>Test sample</td>
<td>0.4</td>
<td>0.2</td>
<td>1.2</td>
<td>0.2</td>
<td>0.119 83.0</td>
</tr>
<tr>
<td>Test sample</td>
<td>0.5</td>
<td>0.1</td>
<td>1.2</td>
<td>0.2</td>
<td>0.018 97.4</td>
</tr>
</tbody>
</table>
CHAPTER FOUR

DISCUSSION

The liver plays a central role in transforming and clearing chemicals and is susceptible to the toxicity from these agents (Friedman et al., 2003). The liver is the vital organ of paramount importance involved in the maintenance of metabolic function and detoxification from the exogenous and endogenous challenges such as xenobiotics, drugs, viral infection and chronic alcoholism. If during all such exposures to the above mentioned challenges the natural protective mechanisms of the liver are overpowered or compromised, the result is hepatic injury.

Paracetamol is a known antipyretic, analgesic drug which produces hepatic necrosis at high doses and normally eliminated as sulfate and glucuronide conjugate (Mitchell et al., 1973). Administration of toxic doses of paracetamol makes the sulfation and glucuronidation routes become saturated; hence, higher percentages of paracetamol molecules are oxidized to highly reactive N-acetyl-p-benzoquinemine by cytochrome-P450 enzymes. The inhibitors of CYPs are known to curtail the toxicity of paracetamol (Tanaka and Misawa, 2000). The semiquinone radicals, obtained by one electron reduction of N-acetyl-p-benzoquineimine, can covalently bind to macromolecules of cellular membrane which increases the lipid peroxidation resulting in tissue damage. Higher doses of paracetamol and N-acetyl-p-benzoquineimine can alkylate, oxidise intracellular GSH which could result to the depletion of liver GSH pool with subsequent step to increased lipid peroxidation; thereby potentiating liver damage (Arnaiz et al., 1995) as observed in the result. The choice of Prosopis africana used in this research was based on their numerous pharmacological properties as reported by Arbonnier (2002).

In this study, the potential effect of Prosopis africana seeds on some haematological parameters and serum biochemical parameters that are indicators of liver damage was investigated. The phytochemical active compounds of the methanol seed extract of Prosopis africana were qualitatively analyzed and the results were presented in Table 2. In the screening process, the presence of alkaloids, flavonoids, steroids, saponins, cyanogenic glycosides and tannins were detected. Flavonoids were found to be highly present while cyanogenic glycosides were found to be slightly present. Excessive ingestion of cyanogenic glycosides can be fatal. Some foodstuffs containing cyanogenic glycosides can cause poisoning (severe gastric irritations and damage) if not properly handled. The presence of flavanoids suggests the possibility of the hepatoprotective principle of Prosopis africana.
Table 3 shows the quantitative phytochemical analysis of fermented methanol seed extract with the following composition: tannins (5.445±0.005mg/g), flavonoids (4.394±0.003mg/g), alkaloids (3.341±0.004mg/g), saponins (1.645±0.002mg/g), steroids (1.137±0.002mg/g) and cyanogenic glycosides (0.077±0.006mg/g). This indicates that the extract possesses some biologically active compounds which could serve as potential sources of drugs. This probably suggests that the extract may have the ability to scavenge free radicals due to the presence of alkaloids and flavonoids which serve as the chief sources of antioxidant in plants. Tannins have been shown to possess some medicinal properties and protective property (Ekeanyanwu et al., 2010); although, they could be considered as antinutrients (Doss et al., 2011). Alkaloids also have pharmacological applications as anesthetics and CNS stimulants (Madziga et al., 2010). This result is in line with the findings of Liener (1994) who stated that lower concentrations of tannins in plants are found to be desirable for human and animal consumption. It could be due to the reduced concentration of tannins in the methanol fermented seed extract that enhanced the protective property.

The acute toxicity (LD$_{50}$) test of the methanol fermented seed extract of $P.$ africana did not show any toxic or deleterious effects by oral route up to 5000 mg/kg b.w. indicating that the seed extract is safe for use even at high doses. The high degree of safety is also consistent with the popular use of the fermented seed as food condiment to enhance the flavors of foods (Achi, 2005).

The packed cell volume (PCV) and the haemoglobin concentration of the animals in the experimental groups 5, 6 and 7 rats administered 100 mg/kg b.w. of the extract before paracetamol induction, 400 mg/kg b.w. of the extract before paracetamol induction, paracetamol-induced and treated with 100 mg/kg b.w. of the extract showed significant (P<0.05) increase when compared with that of group 2 (paracetamol group) on days 7 and 14. PCV measures the percentage by volume of packed red blood cells (RBC) in a whole blood sample after centrifugation (Wynne and Edwards, 2003) while haemoglobin (Hb) test measures the amount of Hb in grams in 1 dl of whole blood and provides an estimate of oxygen carrying capacity of the RBCs. Increases in PCV and haemoglobin concentration show that the rats were not anaemic and the oxygen carrying capacity of the animals was not compromised. A significant (p<0.05) decrease in the PCV of group 2 (paracetamol group) compared with the group 1 (control group) rats suggests that the animals were anaemic resulting from the damage to the liver caused by paracetamol. This result is in line with Nwodo et al. (2010) who reported that coconut water could have the potential of boosting the levels of RBC, total WBC and PCV in both normal and pathological conditions.
Significant (p<0.05) increase was observed in the total white blood cell of test groups 3, 4, 5 and 6 rats when compared with group 2 (paracetamol group). WBC count is the number of WBC in a cubic millimeter of whole blood; and is usually important in fighting against infections (Schalm et al., 1975). The increase in WBC counts could be as a result of the responses to the induced liver damage which could lead to the mobilisation of the white cells. It has been known that WBC counts increase rapidly following foreign attack by exogenous factors and the system’s normal physiologic response will be to boost the body defence mechanism (Eyong et al., 2004). The interpretation above explains the increase in the WBC count of the rats. The significant (p<0.05) decrease observed in group 2 (paracetamol group) when compared to the control group suggests the suppression of the production of the WBC resulting from toxic reactions seen in paracetamol metabolism.

Increase in RBC counts of all the test groups were observed; though, the increase was found to be non-significant (p>0.05) when compared with group 2 rats (paracetamol group), except for group 3 rats administered 100 mg/kg b.w. of extract only which showed significant (p<0.05) increase compared to the control groups. The major function of red blood cells is to carry oxygen to all tissues from the lungs as well as transport carbon (iv) oxide from the cells to the lungs (Nelson and Cox, 2000). The extract may contain some mineral such as iron which helps to bind and transport oxygen to the cells. A decrease observed in group 2 (paracetamol group) compared to normal control (group 1) could be attributed to the RBCs destruction in an enlarged spleen (hypersplenism) and acquired alterations in the red cell membrane of group 2 rats caused by paracetamol.

In the assessment of liver damage by paracetamol or any other hepatotoxin, the assay of enzyme activities such as ALT and AST activities are largely used (Dobbs et al., 2003). Necrosis or membrane damage releases the enzyme into circulation; therefore, it can be measured in serum. AST is predominantly found in mitochondria of hepatocytes. ALT is more specific to liver; thus it is a better parameter for detecting liver injury. Serum ALP is also associated with liver cell damage. Increase in the activity of above enzymes would indicate liver toxicity. Administration of paracetamol caused a significant elevation of AST, ALT and ALP activities has been attributed to the damage of the structural integrity of the liver; this is due to the fact that they are cytoplasmic in location and released into circulation after cellular damages indicating development of hepatotoxicity (Sallie et al., 1991). This explains the significant (p<0.05) increase found in AST and ALT activities of group 2 rats when compared with rats in normal control group.
The result of this study also shows a significant (p<0.05) reduction in the AST activity of groups 6, 7 and 8 rats administered 400 mg/kg bw of extract after paracetamol induction, 100 mg/kg bw and 400 mg/kg bw of extract before paracetamol induction respectively when compared with group 2 (paracetamol group). From the result above we can deduce that at a higher dose (400 mg/kg bw) of the extract, the injurious effects of paracetamol were overcome. Furthermore, the 100 and 400 mg/kg bw of the extract administered after paracetamol were able to ameliorate the damage to an extent.

Significant (p<0.05) reduction was observed in the ALT activity of all the test groups when compared with group 2 (paracetamol group). This result actually indicates the protective and ameliorative effect of the extract since ALT is more specific to liver; thus it is a better parameter for estimating liver injury. Its protective activity indicates that hepatic tissue damage was repaired and plasma membrane stabilized. This could be attributed to the presence of flavonoids and alkaloids in the extract. Lower than normal ALT (and AST) activities suggest vitamin B6 deficiency. The AST and ALT results are in line with the earlier work of Ojo et al. (2006) and Ramadoss et al. (2011).

Alkaline Phosphatase (ALP) is excreted normally via bile by the liver. The liver injury due to toxins can result in defective excretion of bile by hepatocytes which are reflected as their increased levels in serum (Rajesh and Latha, 2004). ALP elevation in group 2 rats compared with the normal group could be associated with cholestasis due to biliary obstruction or hepatic infiltration of rats in group 2. Day 7 shows a non-significant (p>0.05) increase of ALP activity in all the test groups when compared with the control groups (group 1 and 2) while day 14 shows a non-significant (p>0.05) decrease in groups 4, 5, 6, 7 and 8 rats when compared with groups 1 and 2. This result suggests that the extract was able to regenerate the damaged hepatic cells with time. Furthermore, ALP activity of group 3 rats administered100 mg/kg bw of extract only was found to be elevated compared with the control groups throughout the experiment. This result implies that the extract was not able to effectively control the activity of ALP since effective control of alkaline phosphatase activity points towards an early improvement in the secretory mechanism of the hepatic cell (Mason, 2004).

The test extract appears to be effective in ameliorating the effect of paracetamol, observed in the study. There was an indication of stabilization of plasma membrane, as well as repair of hepatic tissue damage caused by paracetamol. The results are in agreement with the commonly accepted view that serum activities of transaminase, return to normal with
healing of hepatic parenchyma and the regeneration of hepatocytes (Thabrew et al., 1987; Shukla et al., 1992).

Derangement of serum lipid profile is a common observation in cirrhotics. Therefore, examination of plasma lipid and lipoprotein levels will be helpful to evaluate the extent of the hepatic damage. It is well known that plasma levels of cytokines (Nanji et al., 1999), lipid peroxides (Wang et al., 2008) and anti-oxidant status (Peterhans, 1997) could be changed under acute or chronic hepatitis which may also interfere with the lipid metabolism in vivo.

The cholesterol necessary for normal metabolism is manufactured by the liver. It can build up inside the arteries when it is present in greater than normal amounts. High levels are a major risk factor for heart and blood vessel disease. Significant (p<0.05) increase was observed on day 14 in groups 3 and 7 rats administered 100 mg/kg bw of extract only and 100 mg/kg bw of extract before paracetamol induction when compared with group 1. This implies that with time and at the particular dose mentioned above, the animals might be at high risk of developing coronary heart disease. Total cholesterol concentration decreased significantly (p<0.05) in groups 4, 6 and 8 rats when compared with group 1 (normal control) on day 7. At a higher dose (400 mg/kg bw), the extract could be said to possess cholesterol lowering property thereby could prevent cardiovascular disorders. This result is in line with earlier work of Sanjay et al. (2013) who reported and concluded that dyslipidemia exists in patients with liver cirrhosis.

Determination of triacylglycerols concentrations are used in the diagnosis and treatment of diseases involving lipid metabolism and various endocrine disorders, example, diabetes mellitus, nephrosis and liver obstruction. Triacylglycerols are storage form of fat released in bloodstream. High levels are a major risk factor for heart and blood vessel disease. Lipid mainly as triacylglycerol can accumulate in the liver. Extensive accumulation is regarded as a pathologic condition during liver damage. This may explain their significant (p<0.05) increase in group 2 (paracetamol group) rats when compared with normal group. Group 2 rats could be at risk of heart and blood vessel disease. TAG concentration was observed to have increased significantly (p<0.05) in group 7 rats pre-treated with paracetamol before the administration of 100 mg/kg bw of extract when compared with control groups. Also, animals in group 7 could be at risk of coronary heart disease. Triacylglycerols play an important role in furnishing energy in animals and fuel for muscle contraction (Murray et al., 2003). A decrease was found in groups 3, 4, 5 and 6 rats when compared with group 2 rats. The extract contain niacin that reduces TAG (Meyers et al., 2004) in both pathological and
non-pathological condition. Luo et al. (2010) also reported that serum levels of TAG and LDL-C were obviously higher in the patients with acute hepatitis than in normal subjects.

HDL particles remove fats and cholesterol, from cells, including atheroma within artery wall and transport it back to the liver for excretion or re-utilization, the reason why the cholesterol carried within HDL particles (HDL-C) is sometimes called "good cholesterol". However, HDL carries many lipid and protein species, several of which have very low concentrations but are biologically very active. For example, HDL and its protein and lipid constituents help to inhibit oxidation, inflammation, activation of the endothelium, coagulation, and platelet aggregation. All these properties may contribute to the ability of HDL to protect from atherogenesis, and it is not yet known which are the most important. Those with higher levels of HDL-C tend to have fewer problems with cardiovascular diseases, while those with low HDL-C levels have increased rates for heart disease (Toth, 2005). Fig. 15 shows a non-significant (p>0.05) increase in HDL-C in groups 4, 5, 6, 7 and 8 rats when compared with group 2 (paracetamol group) on day 7. The extract which contains niacin (Meyers et al., 2004) and magnesium (Rosanoff and Seelig, 2004) has the ability to increase HDL levels.

LDL molecules are often informally called bad cholesterol because they can transport their content of many fat molecules into artery walls, attract macrophages, and thus drive atherogenesis. LDL particles pose a risk for cardiovascular disease when they invade the endothelium and become oxidized, since the oxidized forms are more easily retained by the proteoglycans. A complex set of biochemical reactions regulates the oxidation of LDL particles, chiefly stimulated by presence of necrotic cell debries (Otvos, 1999) and free radicals in the endothelium. As shown in Fig. 16, it was observed that a higher concentration of 400 mg/kg b.w. of the extract was able to reduce LDL-C levels. This could be attributed to the fact that the extract contain phytosterols which are widely recognized as having a proven LDL cholesterol lowering efficacy (Demonty et al., 2009) and niacin (B3) which lowers LDL by selectively inhibiting hepatic diacyglycerol acyltransferase 2, reducing triglyceride synthesis and VLDL secretion through a receptor HM74 (Meyers et al., 2004).

Table 5 shows that Prosopis africana seed extract exhibited high membrane stabilization effect against hypotonicity induced haemolysis of the red cells as is shown by the percent inhibition of haemolysis. Hypotonicity-induced haemolysis may arise from shrinkage of the cells due to osmotic loss of intracellular electrolyte and fluid components. The extract may inhibit processes which stimulate or enhance the efflux of these intracellular...
components. Hypotonicity-induced haemolysis of red blood cells occurs due to water uptake by the cells and leads to the release of haemoglobin which absorbs maximally at 418 nm. Inhibition of haemolysis was found to be dose-dependent, increasing with increased concentration of the extract in the medium. Dose-dependent decrease in the optical density shows decreased amount of haemoglobin released into the medium. Any toxicity usually results in distortion of hepatocytes membrane integrity due to hepatocellular injury and plasma levels rise, as a consequence of high toxin levels present within hepatocytes. The stabilizing effect of this extract on lysosomal membranes (because erythocyte membrane is analogous to the lysosomal membrane) as seen in this study suggests a possible mechanism of action for anti-inflammation. This result is in line with the findings of Anosike and Obidoa (2010) and Iwueke et al. (2006) that reported high membrane stabilization effect of coconut extract and Vitex doniana leaves respectively.

4.2 Conclusion

From the results, it could be deduced that the methanol seed extract of Prosopis africana has significant action on paracetamol-induced hepatotoxicity. Also, it shows that incorporation of the fermented seed of Prosopis africana in the human diet may protect against liver damage, enhances the synthesis of HDL and TAG and also improve on the haematological parameters of the consumer. The extract could also say to have potential therapeutic value in the treatment of some liver disorders.

4.3 Suggestions for Further Studies

- The fermented seed and the unfermented seed of Prosopis africana should be compared to know the one that gives a better protection of the liver against paracetamol-induced hepatotoxicity.
- Antioxidant properties of the fermented seed should be investigated since antioxidants have a very important role to play in metabolism of paracetamol.
- Isolation and characterization of the active agent(s) responsible for the protective mechanism need to be done.
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