AMELIORATIVE POTENTIALS OF *Alstonia boonei* ON FORMALIN-INDUCED HEPATIC NECROSIS IN MALE WISTAR RATS

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JULY, 2021

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A PROJECT WORK SUBMITTED TO THE DEPARTMENT OF MEDICAL LABORATORY SCIENCE, SCHOOL OF BASIC MEDICAL SCIENCES, UNIVERSITY OF BENIN, BENIN CITY, EDO STATE IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE AWARD OF BACHELOR OF MEDICAL LABORATORY SCIENCE DEGREE (BMLS) IN MEDICAL LABORATORY SCIENCE.

JULY, 2021

CERTIFICATION

This is to certify that this work was carried out by **EDEMA, PRECIOUS OCHUKO** with matriculation number **BMS1501885** of the Department of Medical Laboratory Science, School of Basic Medical Sciences, College of Medical Sciences, University of Benin, Benin City, under the supervision of Mr. Frank O. Amegor, in partial fulfilment of the requirement for the award of Bachelor of Medical Laboratory Science degree.

_____ _____

MR. FRANK O. AMEGOR **DATE**
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Head of Department

_____ _____

EXTERNAL EXAMINER **DATE**

DEDICATION

This study is dedicated to the Almighty God for His grace, guidance, wisdom and knowledge; to my parents, Mr & Mrs EDEMA and to my sibling Mr. Moses Edema for family and moral support in the actualization of this programme.

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To my dearest aunty, Blessing Adjejevwe , I appreciate all of your support. To my colleagues and friends, Judith Oyiza, Mary Egbon and others too numerous to mention, I say a big thank you to you all. You are simply the best.

I am immensely grateful to my family; especially my Parents, Mr. and Mrs. Edema, for financial and moral support. May God bless you.

Finally, but most importantly, I thank God for His blessings. The sentiment of John Newton's classic hymn summarizes my life: through many dangers, toils and snares, I have already come. God has kept me safe this far and grace will lead me home.

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ABSTRACT

Inflammation is a reaction of a living vascularised tissue to an injury. Conventional or synthetic drugs used in the treatment of inflammatory diseases are available. However, there is paucity of information on the use of herbal medicine in the treatment of inflammation. The present study is aimed at evaluating the effects of aqueous extract of *Alstonia boonei* on formalin-induced hepatic necrosis in male Wistar rats, using liver enzyme parameters. This study was done using 90 male Wistar rats. The animals were divided into 6 groups and 12 subgroups each comprising of 5 animals for the various plant extracts; leaf, stem bark and root bark, and treated accordingly: Normal control, formalin with *A. boonei* extract (150mg/kg bw), formalin with *A. boonei* extract (300mg/kg bw), formalin with *A. boonei* extract (600mg/kg bw); group 5 (test group) received 5mg/kg, *p.o* of indomethacin and 300mg/kg bw extract only. After the experimental period of 10 days, the animals were sacrificed and the blood samples were collected and the biochemical analyses were carried out. The activity of alanine aminotransferase, aspartate aminotransferase, alkaline phosphatase and lactate dehydrogenase-5 were measured using standard colorimetric methods. Data were analysed using ANOVA and a P-value of <0.05 was considered significant. It was observed that there was a significant increase ($p<0.05$) in serum aspartate aminotransferase, alanine aminotransferase, alkaline phosphatase and lactate dehydrogenase 5 in the leaf, stem bark and root bark extract compared with the control group. Serum alkaline

phosphatase had no significant increase in the root bark extract compared to the control. In this study, it was observed that the aqueous extract of *A. boonei* is effective against chronic inflammation in a dose-related manner. However, the present study suggests that the extract is toxic in high doses and longer dosage regimen. To minimise the toxicity, more toxicity studies should be carried out to understand more about the toxic effects of the plant on the liver.

CHAPTER ONE

1.0 INTRODUCTION

1.1 Background of Study

Natural products from plants, animals and minerals are the basis for treating human diseases (Singh, 2002). Medicinal plants are currently in demand and their acceptance is increasing progressively. No doubt, plants play an essential role by providing important services in the ecosystems. Without plants, humans and other living organisms cannot strive freely. Medicinal herbs have constantly acted as an overall indicator of the ecosystem health (Firenzuoli and Gori, 2007). Humans have considered medicinal plants since ancient times. Medicinal plants have been transformed into one of the oldest sciences in countries such as China, Egypt, Greece and India. Plants were commonly used as drugs, disinfectant and aromatic agents in ancient Persia (Hamilton, 2004). The use of medicinal plants for disease treatment dates back to the history of human life, that is, since human beings have sought a tool in their environment to recover from a disease, plants use was a major choice of treatment (Halberstein, 2005). Over a tenth of plant species are used in pharmaceutical and cosmetics products (Fatemeh *et al.,* 2018).

Alstonia boonei de Wild (Apocynaceae) is a medicinal plant widely used across Africa for various ailments. It is a large tropical deciduous medicinal plant originating from West Africa.

A. boonei is named after Professor Dr C. Alston (1685-1760), at department of Botany in University of Edinburgh (Akinloye *et al.,* 2013). More than 40 species are known. *A. boonie* is commonly known as Ahun in Yoruba, Egbu-ora in Igbo, Ukhu in Edo and Ukpukunu in Urhobo. Preparations of the stem bark is traditionally used for snakebites, venereal diseases, malaria,

measles, boils, wounds, arterial hypertension, arthritis, cataracts, placenta retention, worm infestation, rheumatic pains and for muscle relaxation (Emelia *et al.,* 2020).

Therapeutically, the stem bark was shown to have antipyretic, antirheumatic, anti-inflammatory, analgesic/pain-killing, antimalarial/antipyretic, antidiabetic (slightly hypoglycemic), anthelmintic, aphrodisiac, antimicrobial and antibiotic properties (Adotey *et al.,* 2012).

Adversely, high doses of this plant extract are known to cause Steven-Johnson's syndrome. Various species of *A. boonei* are rich in alkaloids, steroids, triterpenoids and phenolic compounds, these compounds are known to contribute to their toxicity (Akbar *et al.,* 2014).

Inflammation is the immune system's response to infection and injury and has been implicated in the pathogenesis of arthritis, cancer, and stroke, as well as in neurodegenerative and cardiovascular disease (Das, 2011). It is a local response (reaction) of the living vascularized tissues to endogenous and exogenous stimuli. It is derived from the Latin "inflammare" meaning to burn. Inflammation is fundamentally destined to localize and eliminate the causative agent and to limit tissue injury (Atyaf and Zainab, 2019).

Inflammation is a protective response of organism to injury (caused by physical stimuli, chemical agents, etc.) or infection (caused by microorganisms), whose role is to eliminate injury-induced agents, prevent tissue damage and/or initiate repair processes and restore physiological functions of the tissue or organ affected by the inflammatory process (Das, 2011).

Arthritis is a form of joint disorder that involves inflammation of one or more joints. The term arthritis includes more than 100 different rheumatic diseases and conditions, the most common of which is osteoarthritis. Rheumatoid arthritis, lupus, fibromyalgia and gout are other forms of

arthritis that occurs often. Pain, aching, stiffness and swelling in or around the joints are some of the symptoms (Emery *et al.,* 2014).

The liver is the largest internal organ in the body. It weighs about 1.5 kg in adult human and 0.9kg in rabbits. It is also the most active and most complex organ. Suffice it to say that, the liver plays a major role in metabolism by independently handling virtually all the biochemical transformations. Consequently, the replenishing power of the liver is not in doubt because it carries out inter alia metabolic, secretory, excretory, storage, and detoxification functions (Edet *et al.,* 2011). The liver can thus be said to be the sine qua non for life.

Despite its many interesting functions, the liver is prone to suffer a lot of damage. These damages give rise to various forms of liver diseases. The end result is that the liver malfunctions and its efficacy in carrying out its function is hampered. These defects in liver function could have implications on other organs. For instance, acute liver failure correlates with acute renal failure with an approximately 55% frequency of occurrence, although the relationship between cirrhosis and renal dysfunction has not been fully established. The defects in liver function could be as a result of alcohol, drugs, chemical compounds etc (Jaramillo-Juarez *et al.,* 2008).

Formaldehyde is a water soluble, colourless gas whose pure form is irritant and fetor. Its solid form is called paraformaldehyde and it can turn into its gas state in room temperature. Formalin and urea-formaldehyde are the most commonly used forms. In epidemiological and experimental animal studies, formalin-induced a variety of toxic effects, especially in the liver tissue, after inhalation. These effects include dose-related focal hepatic necrosis, hepatic enlargement, decreased weight, and hepatocellular fatty degeneration (Selman *et al.,* 2010).

1.2 Justification Of Study

There have been reports on chemical responses that occur due to inflammation in cells, tissues and organs . However, there is paucity of information as regards the use of *A. boonei* plant extracts. In view of this, this study is designed to determine the alkaline phosphates, alanine amino transferase, aspartate amino transferase and lactate dehydrogenase 5 levels in formalin-induced arthritic male Wistar rats treated with aqueous stem bark, leaf and root bark extracts of *A. boonei* with the aim to establishing a connection between the effects of aqueous extracts of *A. boonei* on liver enzymes in formalin-induced arthritic male Wistar rats.

1.3 Aim of Study

This study is done to evaluate the effects of aqueous extract of *A. boonie* on formalin-induced hepatic necrosis in male Wistar rats, using liver enzyme parameters.

1.4 Specific Objective of the Study

1. To investigate the effects of aqueous extract of *A. boonei* on Alanine Aminotransferase (ALT).
2. To investigate the effects of aqueous extract of *A. boonei* on Aspartate Aminotransferase (AST).
3. To investigate the effects of aqueous extract of *A. boonei* on Alkaline Phosphatase (ALP).
4. To investigate the effects of aqueous extract of *A. boonei* on Lactate Dehydrogenase 5 (LDH-5).

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 The Liver

The liver is a reddish brown organ with four lobes of unequal size and shape. A human liver normally weighs 1.44 to 1.66 Kg (3.2 to 3.7 Ib), (Cotran *et al.,* 2005) and is a soft pinkish-brown, triangular organ. It is both the largest internal organ and the largest gland in the human body. It is located in the left upper quadrant of the abdominal cavity, resting just below the diaphragm. The liver lies to the right of the stomach and overlies the gallbladder. It is connected to two large blood vessels, one called the hepatic artery and other called the portal vein (Ozougwu, 2017).The hepatic artery is supplied by the aorta, while the portal vein carries blood rich in digested nutrients from the entire gastrointestinal tract and also from the spleen and pancreas. The functional units of the liver are called hepatocytes. Two major types of cells populate the liver lobes karat parenchymal and non-parenchymal cells. The parenchymal cells refered to as hepatocytes occupy about 80% of the liver volume. Non-parenchymal cells constitute 40% of the total number of liver cells but only 6.5% of its volume. Some non-parenchymal cells includes Kupffer cells, sinusoidal cells and hepatic stellate cells (Ozougwu, 2017).

2.1.1 Functions of The Liver

The liver has numerous functions, grouped into secretion of bile, metabolism of bilirubin, vascular and hematologic functions, metabolism of nutrients, metabolic detoxification and storage of minerals and vitamins (Ozougwu, 2017).

Fig. 1 : Showing *A. boonei* leaves (Abel and Busia, 2005).

Fig. 2 : Showing *A. boonei* plant (Abel and Busia, 2005).

Fig. 3 : Showing *A. boonei* bole (Palla, 2005).

Fig. 4 : Showing *A. boonei* bark and slash (Abel and Busia, 2005).

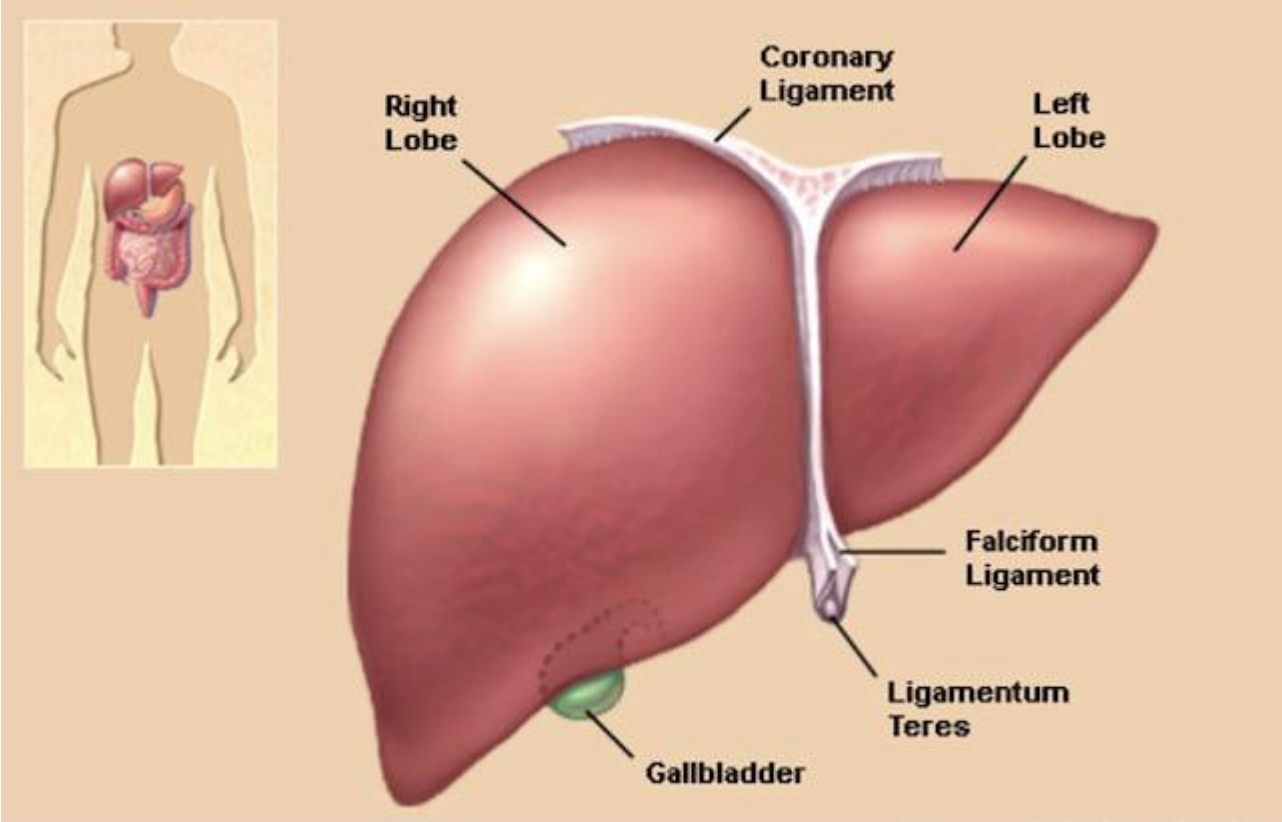

Fig. 5 : Diagram of the liver (Ozougwu, 2017)

2.1.2 Liver Enzymes

The liver releases several enzymes when it is injured. Liver damage is a serious disease characterized by disturbances in normal functions of the liver. It is clinically diagnosed by determining the serum concentrations of liver enzymes (ALT, AST and ALP). These enzymes are non-plasma specific enzymes and were reported to reach higher than normal levels in the blood when there is necrosis of the parenchymal cells of the liver as in viral or toxic hepatitis, with ALT being the most specific liver injury marker and a more selective liver parenchymal enzyme (Muhammad et al., 2015). Alkaline phosphatase (ALP) test is also used to detect bone disorders. In conditions affecting the liver, damaged liver cells release increased amounts of ALP into the blood. This test is often used to detect blocked bile ducts because ALP is especially high in the edges of cells that join to form bile ducts. If one or more of them are obstructed, then blood levels of ALP will often be high (Sherlock and Dooley, 2002).

2.1.2.1 Alanine aminotransferase

Alanine aminotransferase (ALT) is an enzyme mainly aggregated in the cytosol of the hepatocyte. It consist of 496 amino acids, with an half-life of approximately 47 hours and is coded by the ALT gene, which is on the long arm of chromosome 8. The ALT enzyme physically catalyzes the transfer of amino groups from L-alanine to alpha ketoglutarate, and the converted products are L-glutamate and pyruvate in the liver, which is a critical process of the tricarboxylic acid (TCA) cycle. ALT activity in hepatic cells is approximately 3000 times higher than serum ALT activity. When the liver is injured, there is an increase in serum ALT activity. ALT also exits in muscles, adipose tissues, intestines, colon, prostate, and brain; however, the concentration of ALT in these organs is much lower than the liver (Kim *et al.,* 2008). ALT measurement is not only widely used in detecting the incidence, development, and prognosis of

liver disease with obvious clinical symptoms, but also provides reference on screening the overall health status during health check-ups (Zhengtao *et al.,* 2014). ALT has diurnal variation; the nadir value is at 4:00 hours, and the peak value at 16:00 hours. It is higher in males compared to females. This gender-based differences in ALT levels are possibly due to hormonal differences between males and females.

2.1.2.2 Aspartate aminotransferase

Aspartate aminotransferase (AST) is an enzyme found mainly in the liver, but also found in red blood cells, muscle tissue and other organs, such as pancreas and kidneys. It was formerly called serum glutamic oxaloacetic transaminase (GOT). AST is a valuable aid for diagnosis of liver disease. Although not specific for liver disease, it can be used in combination with other enzymes to monitor the course of various liver disorders (Wang, 2002). AST is involved in the transfer of an amino group between aspartate and alpha-keto acids. It is commonly referred to as transaminase as it is an enzyme that belongs to the transferase group. Aspartate aminotransferase is widely distributed in human tissue with the highest concentrations found in cardiac tissue, liver and skeletal muscle. Smaller amounts can also be found in the kidney, pancreas and erythrocytes. It exists as two isoenzyme fractions found in the cell cytoplasm and mitochondria. The intracellular concentration of AST may be 7000 times higher than the extracellular concentration. Although raised AST levels can be found in a wide variety of diseases, the clinical use of AST is limited mainly to the evaluation of cardiac, hepatocellular disorders and skeletal muscle involvement. It plays a role as a general purpose enzyme for the evaluation of myocardial infarction and liver disorders (Bishop *et al.,* 2006).

2.1.2.3 Alkaline Phosphatase

Alkaline phosphatase (ALP) enyzme hydrolyzes the phosphomonoesters from number of organic molecules like ribonucleotides, deoxyribonucleotides, proteins, alkaloids, phosphate esters and anhydrides of phosphoric acid. It is a metallodependent enzyme which shows it's catalytic activity optima at alkaline pH (Rani *et al.,* 2012). Human alkaline phosphatase exist in multiple forms and the forms found in different organs possess distinct properties by which they may be recognized. Identification has been based on immunochemical and electrophoretic properties, thermal stability etc. Alkaline phosphatase (ALP; E.C.3.1.3.1.) is an ubiquitous membrane-bound glycoprotein that catalyzes the hydrolysis of Phosphatase monoesters at basic pH values. It is divided into four isoenzymes depending on the site of tissue expression that are intestinal ALP, placental ALP, Germ cell ALP and tissue nonspecific alkaline phosphatase or liver/bone/kidney (L/B/K) ALP. The intestinal and placental ALP loci are located near the end of the long arm of chromosome 2 and L/B/K ALP is located near the end of the short arm of chromosome 1. The heat-liabile isoenzyme represent the liver/bone/kidney or tissue nonspecific (TNSALP) form. It is expressed in many tissues throughout the body and is especially abundant in hepatic, skeletal, and renal tissue. The normal serum range of ALP is 20 to 140U/L. The enzyme alkaline phosphatase is an important serum analyte and it's elevation in serum is correlated with the presence of bone, liver, and other diseases (Sharma *et al.,* 2013).

2.1.2.4 Lactate dehydrogenase 5

Lactate dehydrogenase 5 (LDH5) is one of the five LDH isoenzymes and, apparently, the most important for promoting anaerobic glycolysis. LDH5 is transcriptionally regulated by the hypoxia inducible factors (HIF) 1alpha and 2alpha. LDH5 is found in the liver and muscles of skeleton. It has the highest efficiency to catalyze pyruvate transformation to lactate (Kayser *et al.,*

2010). LDH is produced in human body in little amounts with low monitory value. LDH5 can be used as a good biomarker for diagnosis of liver, muscle and even cancer diseases (Chaudhary and Chauhan, 2015). Another consideration regarding serum LDH in liver disease is its more rapid decline than ALT, because of its shorter half-life in serum. Concerning liver diseases, it is well known that dominant elevation of serum LDH is observed in hypoxic hepatitis caused by shock or heart failure. Although the elevation of LDH activity in acute liver injury has been simply supposed to be enyzme leakage through damaged hepatocyte membranes (Kotoh *et al.,* 2008).

2.2 Effects of *A. boonei* Extract On Serum ALT, AST, and ALP In Malaria Parasite Infection

The result from previous study showed a significant ($p<0.05$) increase in mean serum of ALT, AST and ALP in negative control compared to normal control, This could be due to infection by the malaria parasite that undergo developmental stage in the liver cell. Upon administration of the extracts, a steady fall in marker enzymes was observed in a dose dependent pattern with aqueous extract possessing the highest hepato-curative ability against plasmodium berghei induced liver damage. On the other hand, an increase in mean serum of ALT, AST and ALP was seen in Chloroquine administered group compared to negative control. The observed increase may be as a result of infection of the liver by the malaria parasite during the exo-erythrocytic cycle stage (Alhassan *et al.,*2017). The significant increase ($P<0.05$) in serum total protein and the non-significant increase in albumin and conjugated bilirubin (Direct bilirubin) of negative control compared to normal control could be due to onset the liver damage which trigger the released of newly synthesized albumin and conjugated bilirubin in addition to challenging

immune system. These indices (total protein, albumin and direct bilirubin) are good indicators of chronic liver damage rather than acute and their values are expected to be lower (Hou *et al.,* 2011).

Earlier findings of in vitro and in vivo antimalarial studies of the extracts previously revealed elevations in parasitaemia levels of malaria parasite in infected but untreated mice and converse reductions in parasitaemia levels upon treatment of infected mice with the extracts. In this study, malaria infected but untreated animals (Negative Control) and positive control (malaria infected and treated with Chloroquine) showed pronounced elevations in serum levels of ALT, AST, ALP, and total bilirubin when compared with the normal control group (uninfected mice) indicating negative impacts of malarial and Chloroquine on the liver. Similarly when compared to the negative control group (infected but untreated), a significant decrease ($P<0.05$) in the mean serum levels of ALT, AST, Total protein and direct bilirubin was observed suggesting that the extracts have hepatocurative effects in addition to the observed anti-malarial activity. This observation which is similar to that reported by Onwusonye *et al.,* (2014) serve as more evidence in support of the continued use of the herb in malaria treatment. One possible mechanism for the hepatocuration may be due to their antioxidant properties, which could counteract the toxic effect of malaria parasite. The bark was said to contain flavonoids, phenolic and triterpenoids which may bind to and scavenge the free radicals by the parasite, thus preventing microsomal lipid and protein peroxidation which is thought to be the cause of liver damage by plasmodium berghei (Chime *et al.,* 2015).

2.3 Paracetamol and Ethanol Induced Liver Damage Effects on ALT, AST and ALT IN Male Wistar Rats

The liver participates in a variety of enzymatic metabolic activities. Administration of ethanol causes elevation of serum ASP, ALT, ALP and total bilirubin levels in rats, indicating that ethanol may induce hepatocellular damages which in turn alters the structure and function of liver cells (Tsukamoto *et al.,* 2001; Pradham *et al.,*2006; Zhou *et al.,* 2003; Wills *et al.,* 2006). The study on the ethanol induced hepatic damage are in accordance with previous reports (Ige *et al.,* 2011; Abdel Salam *et al.,* 2007). It was reported that lipid peroxidation and oxidative stress were implicated in paracetamol (PCM) induced hepatic damage. Due to malignant infiltration and circhosis of liver, serum ALT and AST levels were significantly increased due to the leakage of these enzymes from the cytosine of the liver (Zimmerman *et al.,* 1978). The increase in ALP levels are due to damage of cell membrane of tissue where the enzymes are firmly attached. Hepatic necrosis or membrane damage release these enzymes into circulations and can be measured in serum. ALT (SGPT) is a more specific enzyme for assessing liver damage and also a better parameter than AST (SGOT) which is a non specific enzyme to the liver (Donald, 1994).

Previous study demonstrated that Alstonia scholaris aqueous bark extract had reduced levels of AST, ALT and ALP which were elevated by paracetamol and ethanol administration. The results were in accordance with the findings of other investigators (Obioha *et al.,* 2009; Deepti *et al.,* 2011). Our results are consistent with earlier studies, which strongly suggest that Alstonia scholaris may protect the structural integrity of hepatocytes and prevent the release of cytosolic enzymes into bloodstream.

2.4 Toxicity and Novelty-Induced Mama Decotion In Wistar Rats

In a severe liver damage, the level of this enzyme reduction can lead to an increase in the concentration of cholesterol in the plasma. The implication of this is the accumulation of these lipids in the arterial walls leading to atherosclerosis due to obstruction and distortion of the arteries with serious implications related to cardiac disorders such as angina, myocardial infarction, strokes, peripheral vascular disease and hypertension (Brunzell et al., 2008). Conversely, abnormally low levels of cholesterol, known as hypocholesterolemia, have been reported to be associated with depression, cancer and cerebral hemorrhage (Lewington et al., 2007). From the results obtained from previous study, the low levels of both triglyceride and cholesterol obtained are still within the normal range. Thus, MD can not cause any cardiovascular disease that may be attributed to lipid concentrations. The ALT, AST and ALP results showed that all these biochemical parameters were significantly increased dose-dependently in serum while in the liver only ALT was significantly decreased dose-dependently. There was no significant effect on ALP but AST was significantly increased in the liver. The significant increase in ALT, AST and ALP levels in plasma, following the administration of MD clearly suggested the possibility of liver or bone damage since these are diagnostic markers for the detection of possible liver or bone damage as a diagnostic tool (Attiah et al., 2013). However, further biochemical analysis of the liver did not show this pattern completely except for the increase in AST similarly observed in the liver. Slight-to-moderate elevations of ALT (usually with higher increases in AST levels) may appear in any condition that produces acute hepatocellular injury. Confirmatory procedures to ascertain the liver status were performed and it was observed that there were no significant histopathological effects on the liver. In fact, it was very clear that most of the organs were essentially normal.

2.5 Serum Alkaline Phosphatase In Bone and Liver Disease

Pathological causes of increased serum ALP concentration is observed in subjects with bone diseases and liver diseases. Several clinical and epidemiological investigation attest that elevated ALP concentration increases the risk of coronary artery calcification and coronary artery disease, as well as mortality of chronic kidney disease (Palmer *et al.,* 2011; Adeney *et al.,* 2009). It has been demonstrated in previous trials that serum ALP concentration is associated with the risk of cardiovascular disease (CVD) in subjects with subclinical atherosclerosis (Toneilli *et al.,* 2005; Grandi *et al.,* 2012).

Accumulating data have demonstrated that the concentration of increased ALP is associated with C-reactive protein (CRP) concentration, which indicates serum ALP level may be a marker to reflect the low-grade chronic inflammation (Kerner *et al.,* 2005).

In fact, the association of serum ALP concentration and coronary heart disease (CHD) has been reported in previous studies, and serum ALP concentration is associated with the outcomes of various adverse events such as reduced lung function, inflammation, endothelial dysfunction and solidification (Abramowitz *et al.,* 2010). It is then noteworthy that the relationship between serum ALP concentration and CVD should be interpreted by inflammation since atherosclerosis is associated with inflammatory processes (Xie *et al.,*2014) and the high expression of ALP is observed in the atherosclerotic plaque (Webber *et al.,* 2010) whereas vascular inflammation is a promoter for the initiation and development of atherosclerosis (Kerner *et al.,* 2005). Thus, inflammatory processes may contribute to increase serum ALP concentration in the presence of vessel inflammation.

2.6 Toxicology Of Solvent Extract and Fractions Of *Alstonia boonei* (DC.) Wild Stem Bark in Rats

Study by Oludele and Olufunro (2018) showed that the basal level of AST and ALT found in the plasma may increase when there is damage to tissues such as liver, kidney and heart. Increase in the serum level of ALP indicates liver injury or hepatitis (Godwa *et al.,* 2009). The reference values for AST (0 to 40 U/L) and ALT (0 to 45 IU/L) in human (Melissa, 2004) showed that all values obtained for these enzymes from all the drug candidates at both doses and at 7 and 21 days were significantly higher than normal and it was evidently clear that damage was done to the liver. The increase in the activities of these enzymes after 7 days is indicative of the toxic effects of the extract and fractions of the stem bark extract of A. boonei. Subsequent decrease in the serum activities of these enzymes after 21 days did not reveal complete recovery. Microscopic examination of the liver and kidney sections after the recovery period of 21 days showed various lesions such as vacuolar degeneration, portal cellular infiltration by mononuclear cells and severe, diffuse hepatic vacuolation with central venous congestion, diffuse hepatic vacuolation and severe portal congestion with cellular infiltration by mononuclear cells and mild fibroplasias, renal cortical congestion and hemorrhage. The congestion observed in the liver is evidence that the liver is involved in the biotransformation of xenobiotics.

2.7 The Effects of Ethanolic Extract of *P. guajava Leaf* On AST, ALT, ALP

The effects of administration of ethanolic extract of P. guajava leaf to rats on the activities of AST, ALT, and ALP, respectively, from previous studies showed that there was a significant increase ($p < 0.05$) in the activities of aspartate aminotransaminase, alanine aminotransaminase, ALP and ACP in the serum of all animals treated with ethanolic leaf extract throughout the course of study relative to control (Adeyemi and Akanji., 2010).

ALP is a marker enzyme for the plasma membrane and endoplasmic reticulum. It is often employed to assess the integrity of plasma membrane (Akanji *et al.,*1993), since it is exclusive to the microvilli of the bile canaliculi, located in the plasma membrane. Both aspartate aminotransaminase (AST) and alanine aminotransaminase (ALT) are excellent markers of liver damage caused by exposure to toxic sub- stances (Roy *et al.,* 2006). AST is not specific for the liver only but is also located in other organs like the heart, brain, kidney and skeletal muscle. ALT is the more liver- specific enzyme for diagnostic use when the integrity of the hepatocellular membrane is compromised, there is extrusion of the enzyme into the plasma (Muruganandan *et al.,* 2001). In previous studies (Awobode *et al.,* 2006; Wurochekke *et al.,*2008). it has been reported that changes in enzyme levels are a good marker of soft tissue damage. Damage to body cells may result in the alteration of membrane permeability and consequent release of enzymes into the extracellular fluid. The ethanolic extract caused significant increase in serum concentration of AST, ALT and ALP probably as a result of extrusion of these enzymes from tissues that may have been damaged. Elevation of serum ALP activity has been implicated in hepatobiliary diseases (Qian and Nihorimbere, 2004; Guyton and Hall, 2000), while increases in serum ALT and AST activities have been indicated in conditions involving necrosis of hepatocytes, myocardial cells, erythrocyte and skeletal muscle cells (Mactarlane *et al.,* 2000). The initial increase ($p < 0.05$) in activities of AST and ALT observed from previous studies may be as a result of stress likely imposed on the liver and kidney as a consequence of the metabolism and excretion of the extract by the liver and kidney, respectively (Moss and Henderson, 1996). The tissues in a bid to offset the stress introduced by exposure to the ethanolic extract could have increased de novo synthesis of the enzymes.

2.8 Clearance Of Liver Enzymes

Enzymes are rapidly cleared by reticulo-endothelial system such as bone marrow, spleen and liver cells. The mechanism appears to be by receptor mediated endocytosis. The process include recognition, specific accumulation and uptake of protein by specific cell surface receptor followed by fusion with the lysosome, digestion of the ingested protein and recycling of the receptor back to cell membrane (Ozougwu, 2017).

2.9 Inflammation

Inflammation is a biological reaction to a disrupted tissue homeostasis, it is a tissue-destroying process that involves blood derived products recruitment such as plasma proteins, fluid, and leukocytes, into perturbed tissue. This migration is facilitated by alterations in the local vasculature that lead to vasodilation, increased vascular permeability, and increased blood flow (Noah *et al.,* 2012). Inflammation is part of the body's defense mechanism. It is the process by which the immune system recognizes and removes harmful and foreign stimuli and begins the healing process (Pahwa *et al.,* 2020).

Parham, (2000) described inflammation as a localized protective reaction of cells/tissues of the body to allergic or chemical irritation, injury and/or infections. Pain, heat, redness, swelling and loss of function that result from dilation of the blood vessels resulting in an increased blood supply and from increased intercellular spaces in leukocytes movement, protein and fluids into the inflammed regions are the symptoms of inflammation. Inflammation is complex and is initiated by several factors ranging from bacterial infection and chemical injury to environmental pollution that result in cell injury or death (Paterson *et al.,* 2003). There are several components to an inflammatory reaction injury such as oedema formation, leukocyte infiltration and granuloma formation (Mitchell and Cotton, 2010). On an evolutionary level, Inflammation is a

highly conversed phenomenon and appears to be an important first line of defense for both invertebrates and vertebrates. Chemotaxis and phagocytosis amongst the many components associated with inflammatory cascade are readily employed by unicellular organisms (Noah *et al.,* 2012).

Inflammation is an active process that lasts from a few minutes to a few years. Depending on: the extent of the injury, the type of injury and the vascularity of the tissue. The primary functions of Inflammation are to rapidly destroy or isolate the underlying source of disturbance, remove damaged tissue, and then restore tissue homeostasis (Medzhitov, 2008; Soehnlein and Lindbon, 2010).

2.9.1 Causes of Inflammation

Inflammation is caused by etiologic agents (such as viruses, bacteria, fungi and parasites), hypersensitivity (body reacts against itself, there are four types of reactions), physical and chemical agents (such as sunburn, acid, trauma etc.).

Inflammation can be grouped into two basic phases: acute Inflammation and chronic Inflammation.

2.9.2 Acute Inflammation

This is relatively of short duration, lasting from a few minutes up to a few days and it is characterized by fluid and plasma exudation and neutrophilic leukocyte accumulation

predominantly (Cohran *et al.,* 2002). Rapid influx of blood granulocytes, typically neutrophils, followed swiftly by monocytes which mature into inflammatory macrophages and subsequently proliferate affecting functions of resident tissue macrophages are the acute phase characteristics.

Tissue damage due to trauma, microbial invasion, or noxious compounds can induce acute phase inflammation. It starts rapidly, becomes severe in a short time and may remain for a few days for example cellulitis or acute pneumonia. Subacute inflammation is the period between acute and chronic Inflammation and may last 2 to 6 weeks (Pahwa *et al.,* 2020).

The usual outcome of acute Inflammation is successful resolution and repair of tissue damage. It may be anticipated , therefore that failure of acute Inflammation to resolve may predispose to autoimmunity, chronic dysplastic Inflammation, and excessive tissue damage (Nathan, 2002).

2.9.3 Chronic Inflammation

It's is manifested histologically by influx of lymphocytes and macrophages and by tissue destruction and repair. Chronic Inflammation is of longer duration (days to years) and is associated with vascular proliferation and fibrosis (Cohran *et al.,* 2002). An initial inflammatory stimulus triggers chemical mediators release from plasma or cells, which subsequently regulate the vascular and cellular responses.

Chronic Inflammation is also referred to as slow, long-term inflammation lasting for prolonged periods. Generally the extent and effects of chronic Inflammation vary with the cause of the injury and the body's ability to repair and overcome the damage (Pahwa *et al.,* 2020). Examples of chronic Inflammation includes: impaired foreign body in a wound, tuberculosis, rheumatoid arthritis, and inflammatory bowel disease (Crohn's disease and ulcerative colitis).

2.9.4 Rheumatoid Arthritis

Rheumatoid arthritis (RA) is a chronic, autoimmune, inflammatory disease that may cause progressive joint damage and deformity, leading to functional disability and reduced quality of life (Smolen *et al.,* 2016). The chronic inflammatory state of RA is also associated with extra-articular problems and increased mortality. Over the last one to two decades, modern therapeutic strategies involving the early diagnosis of RA, the treatment to-target principle, and disease-modifying antirheumatic drugs (DMARDs), which can prevent or reduce progression of structural joint damage and reduce mortality risk, have greatly improved the management of rheumatoid arthritis (Carmen *et al.,* 2019). Clinical features of RA can be divided into articular and extra-articular manifestions. Extra-articular features may involve multiple organs including the skin, eyes, lungs and blood vessels. The key presenting symptoms of joint Inflammation are: joint pain and swelling and early morning stiffness lasting for more than 60 minutes (Chang *et al.,* 2016).

Rheumatoid arthritis is therefore a disease which combines chronic Inflammation and bone loss; growing evidence shows that Inflammation per se is a major precipitator for skeletal destruction. Stimulation of monocytes, macrophages, and synovial fibroblasts, by T-cells, leads to the secretion of a number of mediators including enzymes which are involved in the erosion and degradation of bone and cartilage (Schett, 2008).

2.9.5 Mechanisms of Inflammation

Inflammation comprise of a tightly regulated cascade of immunological, physiological, and behavioral processes that are orchestrated by soluble immune signaling molecules called cytokines. Recognition of infection or damage is the first step of the inflammatory cascade. This is achieved by pathogen-associated molecular patterns (PAMPs) detection specifically directed towards general motifs of molecules expressed by pathogens which is necessary for pathogen survival. Endogenous molecules such as alarmins, or damage-associated molecular patterns (DAMPs) signal damage or necrosis and are also recognized by the innate immune system. Unlike adaptive immunity, the innate immunity lacks the capability to differentiate among several strains of pathogens and to determine if these strains are harmful to the host (virulent) (Janeway *et al.,* 2005). Germ-line encoded receptors (transmembrane Toll-like receptors (TLRs) and intracellular nucleotide binding domain and leucine-rich-repeat containing receptors (NOD-like receptors or NLRs) recognises many signal damage (Proell *et al.,* 2008). TLRs activation of common signaling pathways that culminate in the activation of nuclear factor kappa-light-chain-enhancer of activated B cells takes place once ligand recognition occurs. All cell types contain this transcription factor and remains in an inactivated state bound to an inhibitor protein (Friedman and Hughes, 2002). The last phase of Inflammation is its resolution, which is critical for limiting collateral damage to the host (Serhan and Savill, 2005). After the first few hours of Inflammation, a coordinated program of resolution is set into motion by tissue resident and recruited macrophages.

2.9.6 Inflammatory Markers

Inflammatory markers, including C-reactive protein (CRP), erythrocyte sedimentation rate (ESR), and plasma viscosity (PV) are commonly used in primary care for diagnosis and monitoring of inflammatory conditions, including infections, autommune conditions , and cancers (O'Sullivan *et al.,* 2018). Generally, inflammatory markers or inflammatory parameters refers to proteins or other substances that are produced in the liver or other organs that increases in the presence of Inflammation.

Detection of acute Inflammation that might indicate specific diseases or providing a marker of treatment response are the two main functions of measurement of inflammatory markers. Inflammatory markers measurement can also be used as a general, but non-specific test for serious underlying disease. Diseases with prominent inflammatory response activation fall into three main groups: infections, autommune diseases, and some haematological malignancies (Jessica *et al.,* 2012).

2.9.7 Osteoarthritis

Osteoarthritis is a chronic inflammatory joint disease in the world. It is a complex disease whose pathogenesis, changes the tissue homeostasis of articular cartilage and subchondral bone, determine the predominance of destructive process. Pain, joint stiffness, muscle weakness, bone enlargement and swelling are some of the signs and symptoms of osteoarthritis. Systemic risk factors for osteoarthritis include age, gender, genetics hormones and diet. Local risk factors include joint injury and trauma, obesity, occupation and physical activity/sports (Zahra *et al.,* 2013). It is the most common joint disorder worldwide, which affects approximately 10% men and 18% of women over 60 years old (Mandl, 2018). Inflammation can occur in osteoarthritis

but is more like a wear process, where the cartilage in the joint can not tolerate the load which has been placed on it (Weiland *et al.,* 2005).

2.10 Nonsteroidal Anti-Inflammatory Drugs (NSAIDs)

Nonsteroidal anti-inflammatory drugs (NSAIDs) is a drug class that provide analgesic (pain-killing) and antipyretic (fever-reducing) effects, and, in higher doses, anti-inflammatory effects. The analgesic and anti-inflammatory properties acts by inhibiting two recognized isoenzymes of postaglandin G/H synthase also known as cyl-oxygenase (COX), which are COX 1 and COX 2. Inhibition of COX 2 is the pharmacodynamic action of these drugs while the adverse reactions are largely due to COX 1 inhibition (Fokunang *et al.,* 2018). The Nonsteroidal anti-inflammatory drugs are prescribed widely for the treatment of pain and Inflammation in many conditions, including osteoarthritis and rheumatoid arthritis (Fosbol *et al.,* 2010). NSAIDs are typically divided into groups based on their chemical structure and selectively : acetylated salicylates (Aspirin), non-acetylated salicylates (diflunisal, salsalate), propionic acids (naproxen, ibuprofen, acetic acid (diclofenac, indomethacin), enolic acids (meloxicam, peroxicam), anthranilic acids (mefanemic acid), napthylalanine (nabumetone), and selective COX 2 inhibitors (celecoxib) (Oyler *et al.,* 2015).

2.11 Indomethacin

Indomethacin is a potent nonselective COX inhibitor and may also inhibit phospholipase A and C, reduce neutrophil migration, and decrease T cell and B cell proliferation. Indomethacin's half life is prolonged by probenecid by inhibiting both renal and billiary clearance. Indomethacin is indicated for use in gout, juvenile rheumatoid arthritis and ankylosing spondylitis, postepisiotomy pain, etc. A high incidence (up to 50%) of gastrointestinal tract and central nervous system

side effects is produced : diarrhea, GI bleeding, frontal headache, mental confusion, etc. (Fokunang *et al.,* 2018). It is a non steroidal anti-inflammatory drug whose mode of action like that of other anti-inflammatory drugs, is not known. It is a potent inhibitor of prostaglandin synthesis in vitro. Prostaglandins sensitize afferent nerves and potentiate the action of bradykinin in inducing pain in animal models. Postaglandins are known to be among the mediators of Inflammation. Indomethacin is an inhibitor of prostaglandin synthesis, its mode of action may be due to a decrease of prostaglandins in peripheral tissues. Indomethacin has be shown to be an effective anti-inflammatory agent, appropriate for long-term use in rheumatoid arthritis, ankylosing spondylitis and osteoarthritis. Following single oral doses of indomethacin capsules 25mg or 50mg, indomethacin is readily absorbed, attaining peak plasma concentration of about 1 and 2 mcg/mL, respectively at about 2 hours. Biological effects of this drug is seen on tight junctions, neutrophil adhesion and transmigration, and cell apoptosis and proliferation (Takada and Aggarwal*,* 2004).

2.12 Biochemical and Histological Evaluation Of Indomethacin-induced Hepatotoxicity in Wistar Rats.

Over 50 years ago, inflammation is treated by non steroidal anti-inflammatory drugs (NSAIDs) such as salicylates, naproxen, ibuprofen, and indomethacin that represent one of the most common classes of medications used world-wide, with an estimated usage of >30 million per day , which are differ in their structure but all have similar anti-inflammatory, analgesic and anti-pyretic effects through the suppression of prostaglandin (PG) synthesis, by inhibiting the enzyme cyclooxygenase (COX) (Hörl, 2010). Indomethacin is 1-(p-chlorobenzoyl)-5-methoxy-2-methyllindole-3-acetic acid that emerged as one of the most extremely potent -

antipyretic ,analgesic, and anti-inflammatory properties through non-selective potent inhibitor of the cyclooxygenases (COX) 1 and 2 enzyme which responsible for the conversion of arachidonic acid to prostaglandins(Scholz *et al.,* 2012, Katary, 2017). Liver as a major organ plays an astonishing array of vital functions in the maintenance, performance and regulation of homeostasis of the body, its major functions are, carbohydrate, protein and fat metabolism, immunity, exogenous (drug) and endogenous substances detoxification, secretion of bile and storage of vitamin (Omar and Dass, 2018). Indomethacin is considered to be safe at therapeutic doses and is a widely used antipyretic and analgesic drug in clinical practice. However, overdose of indomethacin in both humans and animals causes severe hepatotoxicity and necrosis. Liver is responsible for the metabolism and excretion of indomethacin. Most of the toxic compounds in the body are metabolized in liver (Olatosin, 2013) .The mechanism of indomethacin overdose induced hepatic injury have not been fully illustrated, but Chougule *et al.,* 2018 suggests that, inhibition of protective prostaglandins PGE1, PGE2 and prostacyclin (PG12) may be one of the mechanism by which indomethacin induces injury(Chougule *et al.,* 2018). Previous study showed significant increase in serum concentrations of AST, ALT , GGT and ALP in the groups administered indomethacin because liver marker enzymes (ALT,AST and GGT) might reflect cell rupture ,a major permeability, cellular leakage , loss of functional integrity of the cell membrane and the release of these enzymes from the damaged liver parenchymal cells. In addition, significant increase in the ALP which may be attributing to elevated biliary pressure, and acute cell necrosis, releasing of ALP from its membranes bound site and entry into blood is facilitated due to amphillic nature of bile salts (Hanaa, 2015, Nawal and Entedhar, 2018).

2.13 Formaldehyde

Formaldehyde is a noxious, colourless, flammable, highly water soluble gas discovered in 1867 by the British chemist, August Wilheld Von Hofman. It has a pungent, distinct odour and may cause a burning sensation to the eyes, nose, and lungs at high concentrations. Formaldehyde with its very short half-life of about 1.5minutes is metabolised into formic acid in the liver and erythrocytes. Formaldehyde dehydrogenase requires glutathione as a co-factor during this metabolic reaction (Egwurugwu *et al.,* 2018). It is mostly available commercially as a 30-50% (by weight) aqueous solution, commonly referred to as 'formalin' . In dilute aqueous solution, the predominant form of formaldehyde is its monomeric hydrate, methylene glycol. Methanol (slightly 15%) and other substances (e.g. various amine derivatives) are usually added to the solutions as stabilizers, in order to reduce intrinsic polymerization (Reuss *et al.,* 2003).

2.13.1 Chemical and Physical Properties

a) *Description:* Colourless gas with a pungent odour (Reuss *et al.,* 2003).

b) *Boiling-point:* -19.1°C

c) *Melting-point:* -92 °C

d) *Density:* 0.815 at -20 °C

e) *Spectroscopy data:* Infrared [prism, 2538], ultraviolet [3.1] and mass spectral data have been reported (Sadtler, 1991).

f) *Solubility:* Soluble in water, ethanol and chloroform; miscible with acetone, benzene and diethyl ether.

g) *Stability:* Commercial formaldehyde-alcohol solution are stable; the gas is stable in the absence of water; incompatible with oxidizers, alkalis, acids, phenols and urea (Gerberich and Seaman, 2004).

h) *Reactivity:* Reacts explosively with peroxide, nitrogen oxide and performing acid; can react with hydrogen chloride or other inorganic chlorides to form bis (chloromethyl) ether (Reuss *et al.,* 2003).

i) *Conversion factor:* mg/m3 = 1.23 x ppml.

2.13.2 Mechanisms Of Action Of Formalin in Inducing Chronic Inflammation

The formaldehyde-induced model of chronic Inflammation, aids in the assessment of potential arthritic and anti-inflammatory effects of a substance. Formaldehyde induction causes chronic Inflammation in the site of induction, which involves the subsequent proliferation phase of Inflammation elicited by COX mediators. At low dose, formalin induced an oedema which mainly results from a neurogenic Inflammation mediated by neuropeptides such as substance P. At higher doses, formalin induced an oedema which mainly depends on the release of substance P, prostanoids, 5-hydroxytryptamine and histamine. Formalin has been used as a classic model to produce experimental Inflammation. The effect of the formalin is expressed by the hindlimb licking and shaking that occurs principally in two phases (Amina *et al.,* 2016).

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Study Area

This work was conducted within the premises of University of Benin, Benin City, Edo state, South-South Nigeria.

3.2 Plant Specimen Collection and Authentication

The stem bark, root and leaves of *A. boonei* was collected from the growing tree inside University of Benin, Benin city, in March 2021 and was authenticated by Dr Akinibosun A. O. (Plant Taxonomist), Department of Plant Biology and Biotechnology (PBB, University of Benin, Benin City. The sample was washed, air-dried and pulverized using milling machine (Kenwood UK LTD). It was stored in air tight containers respectively prior to extraction.

3.3 Aqueous Extract Preparation

The aqueous extract was obtained by cold maceration method.The pulverised stem bark, root bark and leaves weighed respectively was soaked in 10L, 7L, and 10L of distilled water respectively. The different soaked plant extracts were stirred with a stirrer at one hour interval and left overnight for 24 hours. The extract was filtered and then concentrated under pressure in a rotary evaporator at 68 °C and dried in an oven, at 50 °C for 48 h (yield 5.5%). The dried extracts 2g each was dissolved in 5mL of Tween 80 solution and 15mL of distilled water. It was mixed thoroughly and each of the

preparation was stored in an air tight clean container and preserved in the refrigerator at 4 °C until use.

3.4 Control Drug Preparation

Indomethacin (5mg) was used as the control drug for this experiment. 100mg of Indomethacin was weighed and dissolved in 4mL of 2% sodium bicarbonate and 16mL of normal saline. It was thoroughly mixed and the preparation was stored in an air tight container in the refrigerator at 4°C.

3.5 Animal Stabilization and Feeding

Seventy Male Wistar rats with average weight of 148-250g were obtained from the animal house of the Department of Pharmacology and Toxicology, University of Benin, Benin City. The animals were stabilized for 11 days in the animal house of the Department of Pharmacology and Toxicology, University of Benin, Benin City. The animals were fed with the standard rodent feed and had free access to feed and water *ad libitum.* All animals were fasted overnight before the beginning of each experiment. Animals were exposed to natural lighting condition and were handled according to standard experimental protocols approved by the Faculty of Pharmacy Animal Ethics Committee, University of Benin.

3.5.1 Determination Of Body Weight

Individual rat was weighed using a weighing balance before the commencement of the experiment. The animals were weighed after, before sacrificing from each group.

3.6 Experimental Design

Formalin induced chronic Inflammation.

The animals were divided into 6 groups and 12 sub-groups, each comprising of 5 animals each for the various plants extracts; stem, leaf and roots respectively. They were fully described, weight identified and marked, prior to the experiment.

Inflammation was induced by subaponeurotic injection of 0.1mL of 2% w/v formalin in distilled water in the right hind paw of the rats on the first and third day in groups II, III, IV, and V. The plant extracts were administered to groups II, III, IV and VI daily using an oral gastric tube and a syringe.

Group 1: This is the control group. This group was fed with the standard rodent feed. No inflammation was induced and 3mL/kg *p.o* of distilled water was administered to the animals for ten days. No plant extract was administered to this group.

Group 2: This group was administered 150mg/kg, *p.o* of the various extract; leaf, stem bark and root bark respectively once a day for ten days as follows:

- **Sub group I:** 150mg/kg, *p.o* of leaf extract once a day for ten days.
- **Sub group II:** 150mg/kg, *p.o* of stem bark extract once a day for ten days.
- **Sub group III:** 150mg/kg, *p.o* of root extract once a day for tens days.

Group 3: This group was administered 300mg/kg, *p.o* of the various extract; leaf, stem bark and root bark respectively once a day for ten day as follows:

- **Sub group I:** 300mg/kg, *p.o* of leaf extract once a day for ten days.
- **Sub group II:** 300mg/kg, *p.o* of stem bark extract once a day for ten days.
- **Sub group III:** 300mg/kg, *p.o* of root bark extract once a day for ten days.

Group 4: This group was administered 600mg/kg, *p.o* of the various extract; leaf, stem bark and root bark respectively once a day for ten days as follows:

- **Sub group I:** 600mg/kg, *p.o* of leaf extract once a day for ten days.
- **Sub group II:** 600mg/kg, *p.o* of stem bark extract once a day for ten days.
- **Sub group III:** 600mg/kg, *p.o* of root bark extract once a day for ten days.

Group 5: This control drug group was administered 5mg/kg, *p.o* of indomethacin once a day for ten days.

Group 6: This negative control group was administered 300mg/kg, *p.o* only of the various extract; leaf, stem bark and root respectively once a day for ten days without formalin induction as follows:

- **Sub group I:** 300mg/kg, *p.o* of leaf extract once a day for ten days.
- **Sub group II:** 300mg/kg, *p.o* of stem bark extract once a day for ten days.
- **Sub group III:** 300mg/kg, *p.o* of root extract once a day for ten days.

The right paw thickness was measured daily for ten days using Vernier calliper. The percentage inhibition of the mean increase in the paw edema of each group was calculated on the 10th day and compared with the control.

At the end of the experiment, all the animals were anaesthetised using cotton wool soaked in chloroform placed in a container. They were dissected using a dissecting set . Blood was collected form the abdominal aorta and directly from the heart using a 5mL syringe into a plain container and then allowed to clot. It was then centrifuged at 4000rpm for 10 minutes. The serum was collected into plain sterile bottles and used for alanine amino transferase, aspartate amino transferase, alkaline phosphates and lactate dehydrogenase 5 analyses.

3.7 Determination of Alkaline Phosphatase (Kochmar and Moss, 1986).

3.7.1 Principle:

The alkaline phosphatase acts on the AMP-buffered sodium thymolphthalein monophosphate. The addition of an alkaline reagent (sodium hydroxide) stops the enzyme activity and simultaneously develops a blue chromogen, which is measured photometrically at 630nm.

3.7.2 Sample: Unhaemolysed serum

3.8 Determination of Aspartate Aminotransferase (AST) (Reitman and Frankel, 1957).

3.8.1 Principle:

Aspartate aminotransferase aids the conversion of alpha-keto-glutarate and aspartate to glutamate and oxaloacetate respectively via the amino group transfer. The oxaloacetate thus formed is coupled with 2,4-dinitrophenylhydrazine to produce a coloured complex in which the absorbance in alkaline solution is then measured spectrophotometrically at 540nm. Aspartate aminotransferase (AST) is measured by monitoring the concentration of oxaloacetate of hydrazone formed with 2-4-dinitrophenylllhydrazine.

$$\alpha\text{- oxoglutarate} + \text{L- aspartate} \xrightarrow{\text{GOT}} \text{L- gluatamate} + \text{Oxaloacetate}$$

3.8.2 Sample: Unhaemolysed serum

3.9 Determination of Alanine Aminotransferase (ALT) (Reitman and Frankel, 1957).

3.9.1 Principle:

Alanine aminotransferase catalysis the conversion of alpha-keto-glutamate and alanine sulfinate to pyruvate and glutamate. The pyruvate thus formed is coupled with 2,4-dinitrophenylhydrazine to produce a coloured complex in which the absorbance in the alkaline solution is measured spectrophotometrically at 540nm. Alanine aminotransferase (ALT) is measured by monitoring the concentration of pyruvate hydrazine formed with 2-4-dinitrophenylllhydrazine.

$$\alpha\text{- oxoglutarate + L- alanine} \xrightarrow{\text{ALT}} \text{L- gluatamate + Pyruvate}$$

3.9.2 Sample: Unhaemolysed serum

3.10 Determination Of Lactate Dehydrogenase (UV method) (Wroblewski and Ladue,1995).

3.10.1 Principle:

The lactate dehydrogenase catalyzes the reversible oxidation of L-Lactate to Pyruvate with the concurrent reduction of ß-Nicotinamide Adenine Dinucleotide (NAD) to ß-Nicotinamide Adenine Dinucleotide (reduced form) (NADH). The absorbance is read at 340nm

$$\text{L-Lactate + NAD+} \xrightarrow{\text{LD.}} \text{Pyruvate + NADH + } H^+$$

3.10.2 Sample : Unhaemolysed serum

3.11 Quality Control

3.12 Statistical Analysis

The statistical analyses of the results were done and the various results obtained from the study were expressed as Mean ± Standard Error in Mean (S.E.M). The differences among the groups were determined by one-way ANOVA. The data were statistically analysed using SPSS Software (2007) version 16.0 by SPSS Ink. The Turkey-Kramer Multiple comparison Test was used as the post test for the determination of significant difference between means. A P-value (<0.05) was considered to be statistically significant and N represent the number of samples analysed.

CHAPTER FOUR

4.0 RESULTS

The results obtained are shown below in tables:

Table 4.1; Mean Comparison (Leaf extract of *A. boonei*)

		3 mL/kg	150 mg/kg	300 mg/kg	600 mg/kg	Indomethacin	300 mg/kg only	P
Leaves	AST	33.40±0.93^{a}	198.60±10.36^{bcd}	222.60±28.62^{cd}	142.20±7.22^{b}	154.40±17.26^{bc}	228.40±13.88^{d}	0.000
	ALT	34.00±1.00^{a}	107.20±11.77^{bc}	98.20±4.36^{bc}	78.80±2.91^{b}	81.40±7.10^{b}	113.20±6.12^{c}	0.000
	ALP	31.60±0.54^{a}	80.24±6.20^{b}	54.12±2.53^{b}	59.86±7.21^{bc}	44.44±5.30^{ac}	60.66±5.06^{bc}	0.000
	LDH5	171.80±7.50^{a}	1671.00±220.00^{bc}	2088.40±434.57^{c}	1527.20±348.52^{bc}	916.60±130.42^{ab}	1746.80±174.80^{bc}	0.000

Values are expressed as mean±SEM. Means with different superscripts are statistically significant at $p<0.05$

Table 4.1 shows a comparison between liver enzymes (AST, ALT, ALP and LDH5) in Group 1 (3mL/kg control group), Group II (150mg/kg bw leaf extracts and formalin), Group III (300mg/kg bw leaf extract and formalin), Group IV (600mg/kg bw leaf extract and formalin), Group V (5mg/kg Indomethacin and formalin) and Group VI (300mg/kg bw leaf extract only).

Table 4.2: Mean Comparison (Stem bark of *A. boonei*)

		3 ml/kg	150 mg/kg	300 mg/kg	600 mg/kg	Indomethacin	300 mg/kg only	P
Stem	AST	33.40±0.93^{a}	195.00±14.87^{b}	163.00±13.66^{b}	166.75±19.07^{b}	154.40±17.26^{b}	145.20±11.06^{b}	0.000
	ALT	34.00±1.00^{a}	122.20±16.52^{b}	93.20±7.79^{b}	108.75±18.72^{b}	81.40±7.10^{b}	86.00±2.51^{b}	0.000
	ALP	31.60±0.54^{a}	59.24±5.72^{ab}	55.34±6.37^{ab}	62.50±12.57^{b}	44.44±5.30^{ab}	60.86±4.79^{b}	0.013
	LDH5	171.80±7.50^{a}	1687.80±208.12^{b}	1294.60±267.63^{b}	1091.25±245.58^{b}	916.60±130.42^{ab}	1238.80±261.63^{b}	0.001

Values are expressed as mean±SEM. Means with different superscripts are statistically significant at $p<0.05$

Table 4.2 shows a comparison between liver enzymes (AST, ALT, ALP and LDH5) in Group 1 (3mL/kg control group), Group II (150mg/kg bw stem bark extracts and formalin), Group III (300mg/kg bw stem bark extract and formalin), Group IV (600mg/kg bw stem bark extract and formalin), Group V (5mg/kg Indomethacin and formalin) and Group VI (300mg/kg bw stem bark extract only).

Table 4.3: Mean Comparison (Root bark of *A. boonei*)

		3 mL/kg	150 mg/kg	300 mg/kg	600 mg/kg	Indomethacin	300 mg/kg only	p
Root	AST	33.40±0.93[a]	164.00±15.57[b]	134.00±7.57[b]	131.80±4.50[b]	154.40±17.26[b]	164.40±9.03[b]	0.000
	ALT	34.00±1.00[a]	90.80±13.31[b]	72.33±3.71[b]	78.60±7.10[b]	81.40±7.10[b]	84.60±4.25[b]	0.000
	ALP	31.60±0.54[a]	41.68±1.29[a]	34.23±1.37[a]	56.08±20.48[a]	44.44±5.30[a]	41.56±2.81[a]	0.537
	LDH5	171.80±7.50[a]	1127.00±180.53[b]	966.67±64.79[ab]	968.80±280.14[ab]	916.60±130.42[ab]	1085.80±198.43[b]	0.010

Values are expressed as mean±SEM. Means with different superscripts are statistically significant at $p<0.05$

Table 4.3 shows a comparison between liver enzymes (AST, ALT, ALP and LDH5) in Group 1 (3mL/kg control group), Group II (150mg/kg bw root bark extracts and formalin), Group III (300mg/kg bw root bark extract and formalin), Group IV (600mg/kg bw root bark extract and formalin), Group V (5mg/kg Indomethacin and formalin) and Group VI (300mg/kg bw root bark extract only).

Table 4.4: Mean Comparison of Weight (kg**) of Animals**

	3mL/kg	150mg/kg	300mg/kg	600mg/kg	Indomethacin	p
Leaves	191.72±6.96^{a}	189.38±9.91^{a}	175.78±6.45^{a}	199.60±6.30^{a}	197.46±5.90^{a}	0.197
Stems	191.72±6.96^{ab}	198.16±10.44^{ab}	174.62±6.61^{a}	210.88±2.98^{b}	197.46±5.90^{ab}	0.039
Roots	191.72±6.96^{a}	180.44±5.95^{a}	185.50±7.16^{a}	211.28±11.89^{a}	197.46±5.90^{a}	0.108

Values are expressed as mean±SEM. Means with different superscripts are statistically significant at $p<0.05$

Table 4.4 shows a comparison between mean weights for the leaf, stem and root extract in Group 1 (3mL/kg control group), Group II (150mg/kg bw root extracts and formalin), Group III (300mg/kg bw root extract and formalin), Group IV (600mg/kg bw root extract and formalin), Group V (5mg/kg indomethacin and formalin) and Group VI (300mg/kg bw root extract only).

Table 4.5: Mean comparison of paw thickness (mm**) of Animals**

	3mL/kg	150mg/kg	300mg/kg	600mg/kg	Indomethacin	p
Leaves	0.43±0.02^{a}	0.68±0.03^{bc}	0.63±0.04^{b}	0.85±0.03^{d}	0.80±0.02^{cd}	0.000
Stems	0.43±0.02^{a}	0.65±0.04^{b}	0.66±0.05^{b}	0.86±0.02^{c}	0.80±0.02^{bc}	0.000
Roots	0.43±0.02^{a}	0.87±0.03^{b}	0.85±0.03^{b}	0.86±0.03^{b}	0.80±0.02^{b}	0.000

Values are expressed as mean±SEM. Means with different superscripts are statistically significant at $p<0.05$

Table 4.5 shows a comparison between paw thickness for the leaf, stem and root extract in Group 1 (3mL/kg control group), Group II (150mg/kg bw extracts and formalin), Group III (300mg/kg bw extract and formalin), Group IV (600mg/kg bw extract and formalin), Group V (5mg/kg indomethacin and formalin) and Group VI (300mg/kg bw extract only).

Table 4.6: %Inhibition of The Leaf, Stem bark and Root bark extract of *A. boonei*

	Leaves	Stems	Roots
3mL	-	-	-
150mg/kg	56.77	50.44	99.87
300mg/kg	45.62	51.15	96.39
600mg/kg	95.14	98.10	97.97
Indomethacin	83.28	83.28	83.28

Table 4.6 shows the percentage inhibition of the leaf, stem bark and root bark extract of *A. boonei* in Group 1 (3mL/kg control group), Group II (150mg/kg bw extract and formalin), Group III (300mg/kg bw extract and formalin), Group IV (600mg/kg bw extract and formalin), Group V (5mg/kg indomethacin and formalin) and Group VI (300mg/kg bw extract only).

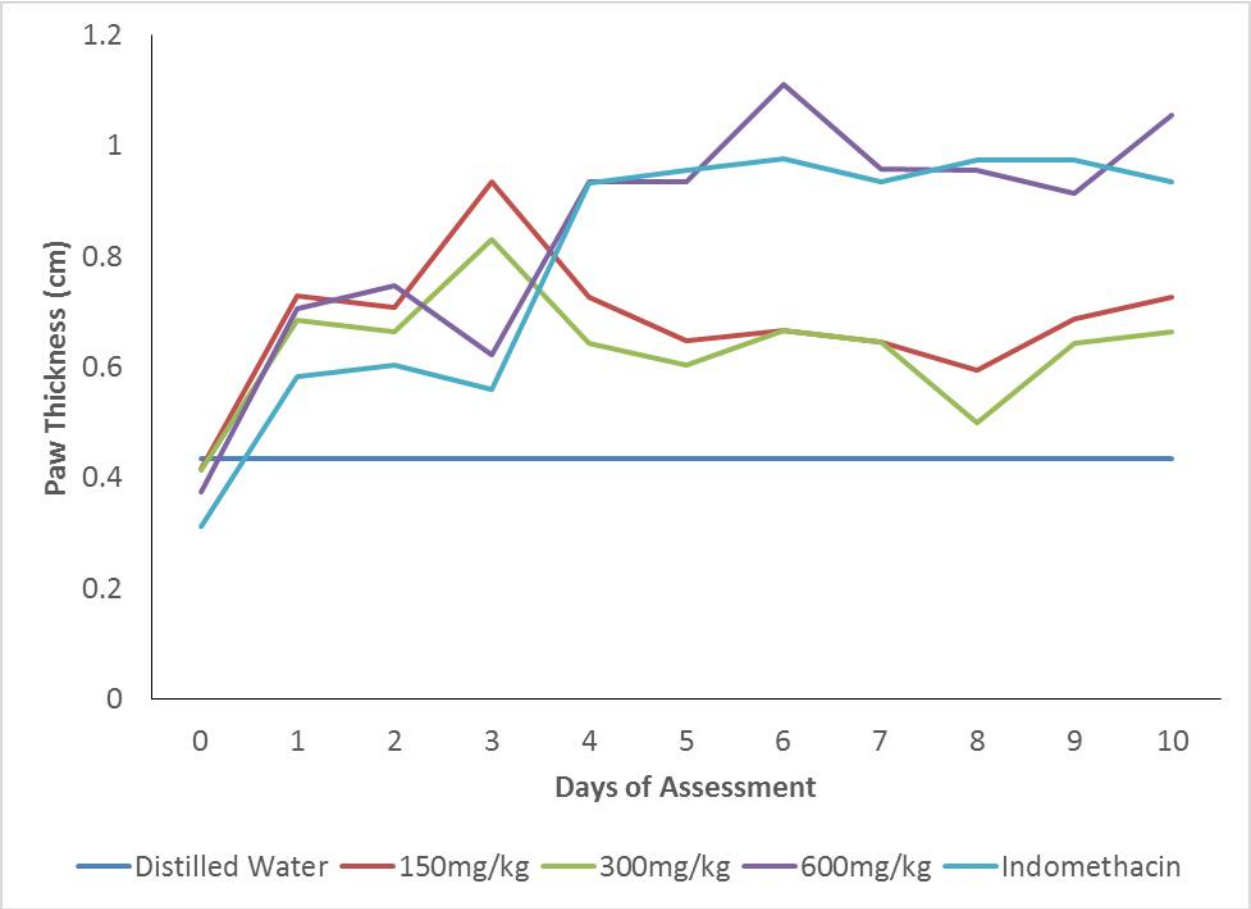

Figure 4.1: shows a graphical comparison of the Paw thickness measured from day1 to day 10 between Group I (3mL/kg bw), Group II (150mg/kg bw leaf extract of *A. boonei* + Formalin), Group III (300mg/kg bw leaf extract of *A. boonei* + Formalin), Group IV (600mg/kg bw leaf extract of *A. boonei* + Formalin) and Group V (Indomethacin + Formalin).

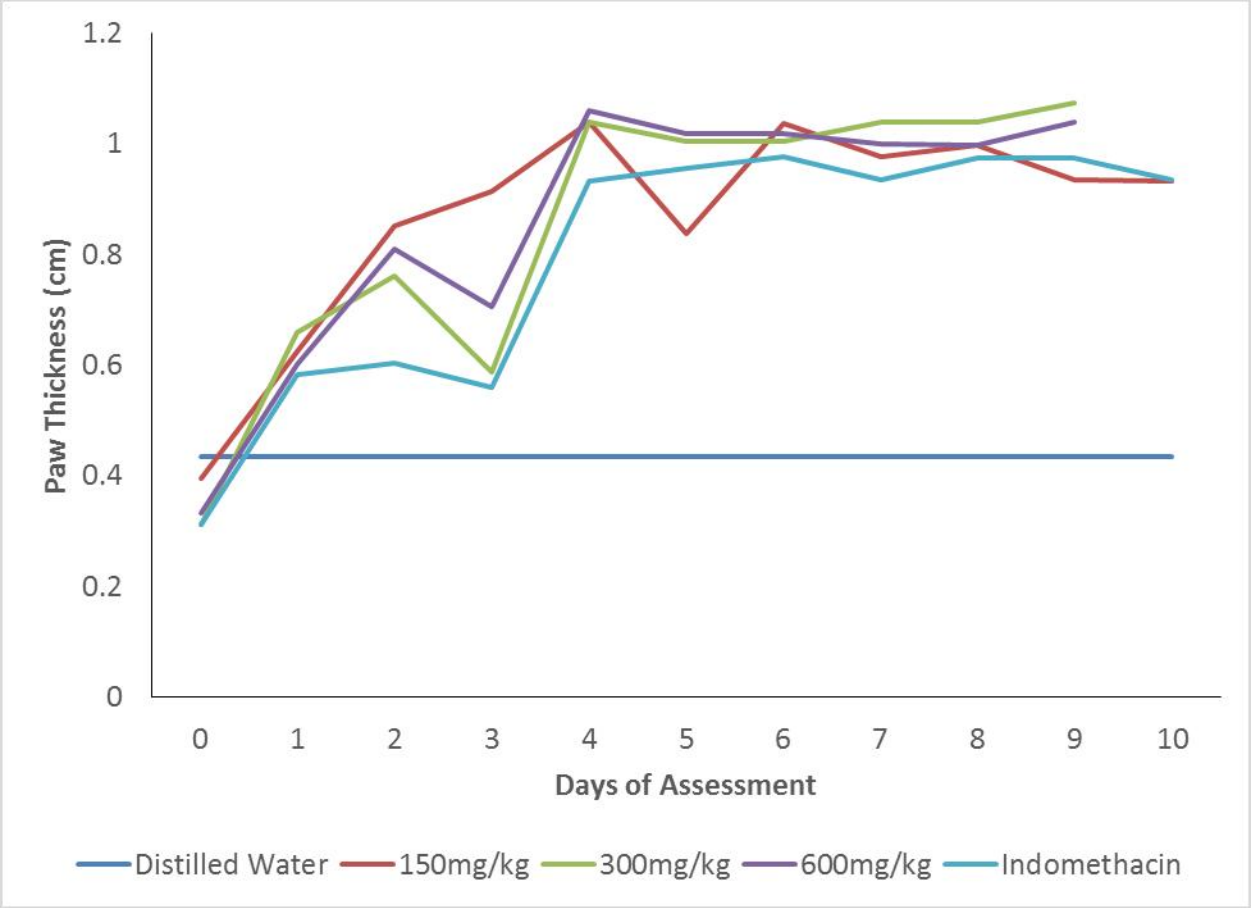

Figure 4.2: shows a graphical comparison of the Paw thickness measured from day1 to day 10 between Group I (3mL/kg bw), Group II (150mg/kg bw stem bark extract of *A. boonei* + Formalin), Group III (300mg/kg bw stem bark extract of *A. boonei* + Formalin), Group IV (600mg/kg bw stem bark extract of *A. boonei* + Formalin) and Group V (Indomethacin + Formalin).

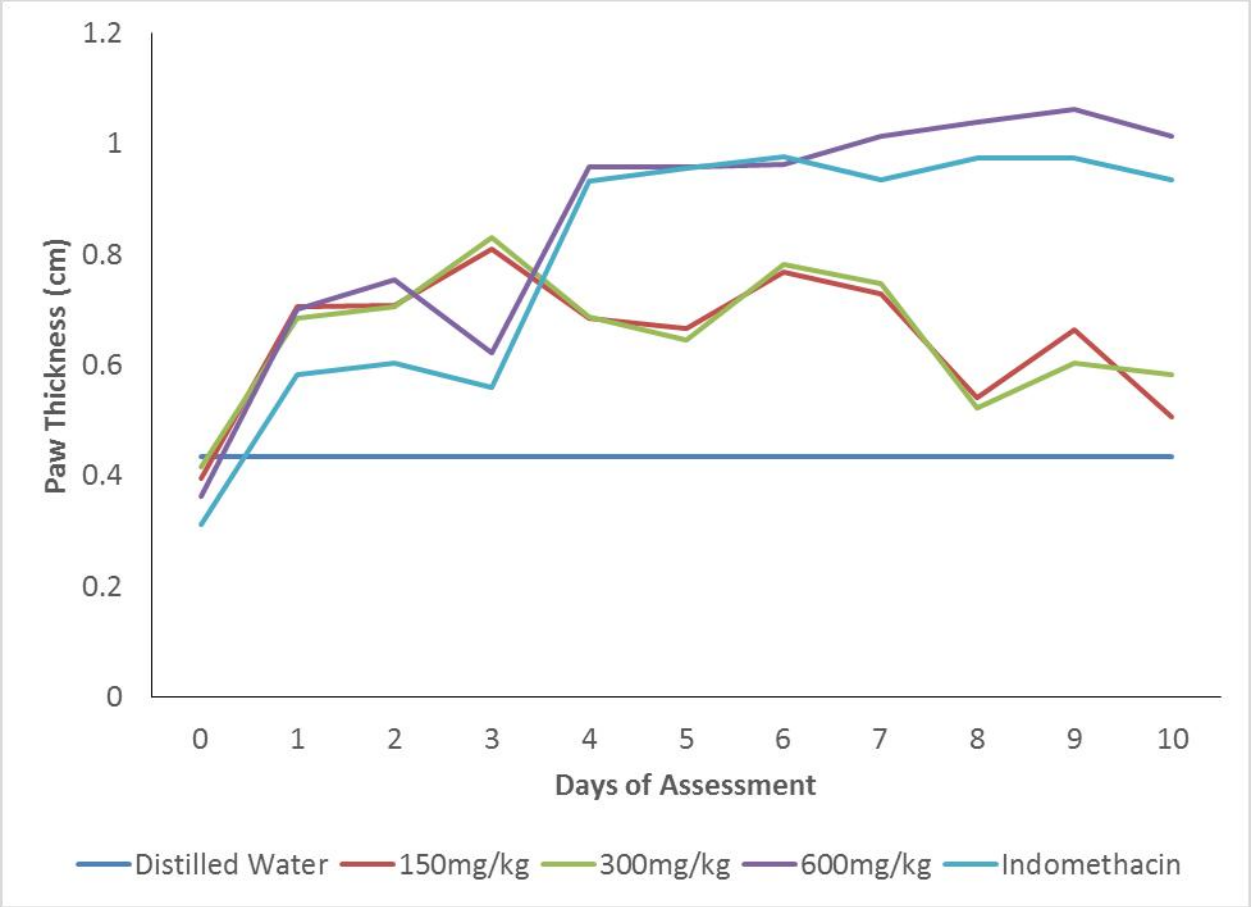

Figure 4.3: shows a graphical comparison of the Paw thickness measured from day1 to day 10 between Group I (3mL/kg bw), Group II (150mg/kg bw root bark extract of *A. boonei* + Formalin), Group III (300mg/kg bw root bark extract of *A. boonei* + Formalin), Group IV (600mg/kg bw root bark extract of *A. boonei* + Formalin) and Group V (Indomethacin + Formalin).

CHAPTER FIVE

5.1 Discussion

In the present study, the anti-inflammatory activity of the aqueous extract of *A. boonei* has been evaluated in chronic inflammatory models. Formalin induced paw oedema in rats is one of the most suitable test procedure to screen for chronic inflammation and it is believed to be a biphasic event. The inhibition of formalin induced inflammation in rats is an established model for evaluating anti-inflammatory drugs, which has been used to assess anti oedematous effect of natural products. The first phase occurs within one hour of formalin inflammation and is attributed to the release of cytoplasmic enzymes, histamine and serotonin, from the mast cells. The second phase is mediated by an increased release of prostaglandin in the inflammatory area and continuity between the two phases is provided by kinins. Since the extract significantly inhibited paw edema induced by formalin in the second phase, this finding suggests a possible inhibition of cyclooxygenase synthesis by the extract (Di Rosa *et al.,* 1974). This effect is similar to that produced by non-steroidal anti-inflammatory drugs such as indomethacin, whose mechanism of action is inhibition of the cylooxygenase enzyme, which catalysed the synthesis of cyclic endoperoxides important in the formation of prostaglandins.

The liver which is the largest mammalian internal organ plays a vital role in metabolism, detoxification and inflammatory response (Tacke *et al.,* 2009). The organ is primarily charged with the responsibility of regulating the internal chemical environment. In attempt to perform this function the liver is at most times faced with toxic challenges which may have great negative consequences. Hepatotoxic agents reduce the functional integrity of liver hepatocytes.

Impairment of functionality of liver cells results to accumulation of waste products of metabolism such ammonia in the blood (Mao *et al.,*2014), Ravichandran *et al*.,(2014). ALT is the important liver enzyme that catalyses transamination reaction. The occurrence of conditions that can cause liver damage such as cancer, injury and hepatitis will result in higher levels of this enzyme (Kodavanti *et al.,* 1989). AST and ALP, the biomarkers of liver damage, are cytotoxic and mitochondrial enzymes whose levels are usually increased in cases of chronic illness and necrosis due to loss of hepatocellular integrity. These enzymes are involved in the transfer of alpha-amino groups from alanine and aspartate to the alpha-keto group of ketoglutarate to form pyruvate and oxaloacetate respectively (Schiff *et al.,* 2007). Paracetamol induced hepatotoxicity has been reported to occur by covalent binding of its metabolite n-acetyl-p-benzoquinoneimine to the sulfhydryl group of protein leading to lipid peroxidation and cell necrosis (Kapur et al., 1994), Ravichandran *et al*.,(2014). When the cells of the liver are damaged, biochemical marker enzymes like AST, ALT and Alkaline phosphatase find their ways as a result of leakage through compromised plasma membranes. This results to increased activities of the enzymes in the serum.

Findings from the leaf extract of *A. boonei* showed significant increase in AST, ALT and ALP at all doses; 150mg/kg, 300mg/kg, 600mg/kg, indomethacin group and the 300mg/kg extract group only respectively when compared to the control group. This is in line with previous study on *Psidium gaujava* ethanolic leaf extract administration over a period of 7 days by Adeyemi and Akanji, 2010 which also showed significant increase in AST, ALT and ALP. The initial increase in activities of AST, ALT and ALP as observed in all groups when compared to the control group may be as a result of stress likely imposed on the liver as a consequence of the metabolism and excretion of the extract by the liver. It is also possible that the leaf extract of *A. boonei* caused membrane damage which allowed the enzymes to leak through the cell membrane into

the extracellular compartment (Adeyemi and Akanji, 2010). Cellular damage likely to arise from the plant extract administration can result in leakage of the liver enzymes to the extracellular fluid (Vieira *et al.,* 2001).

Lactate dehydrogenase 5, is a sensitive indicator of liver injury (Zilva and Pannal, 1984). There was a significant increase for LDH-5 in the leaf, stem bark, and root bark of *A. boonei* at all doses; 150mg/kg, 300mg/kg, 600mg/kg, Indomethacin group and 300mg/kg extract only compared to the control group. This significant increase may be as a result of liver injury by the extract of *A. boonei* as Zilva and Pannal, 1984 showed that increased lactate dehydrogenase 5 is a sensitive indicator of liver injury. Also, previous studies showed that rats induced with CCl4 had marked elevation in liver enzymes: alanine transaminase, aspartate transaminase, alkaline phosphatase, lactate dehydrogenase and other biochemical parameters: bilirubin, creatinine and urea, thus indicating liver injury (Taj *et al.,* 2014).

Indomethacin is considered to be safe at therapeutic doses and is a widely used antipyretic and analgesic drug in clinical practice. Indomethacin group showed a significant increase in serum ALT, AST, ALP and LDH-5 compared to the control group. This may be due of increase dosage as seen from previous studies (Entedhar, 2018). However, overdose of indomethacin in both humans and animals causes severe hepatotoxicity and necrosis. Liver is responsible for the metabolism and excretion of indomethacin. Most of the toxic compounds in the body are metabolized in liver (Olatosin, 2013). Previous study showed significant increase in serum concentrations of AST, ALT , GGT and ALP in the groups administered with indomethacin because liver marker enzymes (ALT,AST and GGT) might reflect cell rupture ,a major permeability, cellular leakage , loss of functional integrity of the cell membrane and the release

of these enzymes from the damaged liver parenchymal cells. In addition, significant increase in the ALP which may be attributing to elevated biliary pressure, and acute cell necrosis, releasing of ALP from its membranes bound site and entry into blood is facilitated due to amphillic nature of bile salts (Hanaa, 2015, Nawal and Entedhar, 2018).

Findings from the stem extract of *A. boonei* showed a significant increase in AST, ALT and ALP in all groups compared to the control group. From previous studies, the stem bark has been shown to contain flavonoids, phenolic and triterpenoids which may bind to and scavenge the free radicals by the parasite, thus preventing microsomal lipid and protein peroxidation which is thought to be the cause of liver damage by plasmodium berghei (Chime *et al.,* 2015). These observed high levels of AST, ALT, and ALP compared to the control group is line with previous study by Oludele and Olufunro (2018). Despite its many interesting functions, the liver is prone to suffer a lot of damage. These damages give rise to various forms of liver diseases. The end result is that the liver malfunctions and its efficacy in carrying out its function is hampered. Study by Oludele and Olufunro (2018) showed that the basal level of AST, ALT and ALP found in the plasma may increase when there is damage to tissues such as liver, kidney and heart. Increase in the serum level of ALP indicates liver injury or hepatitis (Godwa *et al.,* 2009). The reference values for AST (0 to 40 U/L) and ALT (0 to 45 IU/L) in human (Melissa, 2004) showed that all values obtained for these enzymes from all the subjects at both doses and at 7 and 21 days were significantly higher than normal and it was evidently clear that damage was done to the liver. The increase in the activities of these enzymes after 7 days is indicative of the toxic effects of the extract and fractions of the stem bark extract of *A. boonei* in a dose-related manner.

The significant increase in ALT, AST and ALP levels in plasma, following the administration of *A. boonei* may clearly suggest the possibility of liver or bone damage since these are diagnostic markers for the detection of possible liver or bone damage (Attiah *et al.,* 2013).

Findings from the root extract of *A. boonei* showed a significant increase for AST and ALT in all doses compared to the control group. From previous studies these enzymes were reported to be higher than normal in serum when there is liver necrosis (Keith and Robert, 2001; Price and Stevens, 2003). These values are significantly higher in group II compared to Group I and it indicates possible induction of liver damage (Obi and Uneh, 2003; Alhassan *et al.,*2009). The concurrent elevations of serum AST together with ALT may likely be due to the toxicity induced by formaldehyde. However in comparison to other plant parts, the root extract have some role in decreasing serum AST and ALT . This is in line with previous study of ashwagandha (*Withania somnifera*) root extract, which may have some role in decreasing serum AST, ALT towards normal levels in gentamicin intoxicated rats due to its free radical scavenging activity which reflects its hepatoprotective effects (Nayman *et al.,* 2012). The root extract showed no significant increase for ALP in all doses 150mg/kg, 300mg/kg, 600mg/kg, Indomethacin group and 300mg/kg extract only compared to the control group.

Previous studies have demonstrated that exposure to formaldehyde could cause hepatic necrosis and hepatocellular fatty degeneration in the liver of mice exposed to FA (Cikmaj *et al.,* 2010), resulting in increased liver enzymes in the rats exposed to FA. Fisher (1905) also revealed that acute exposure to formalin caused inflammation and cloudy swellings in the liver. This was as a result of vacuolated protoplasm, destruction of nuclei and focal hepatic necrosis.

The stem bark extract caused a significant increase in the weight of rats across all doses (150mg/kg, 300mg/kg, 600mg/kg, indomethacin group and 300mg/kg extract only group) when compared to the control group and the other plant parts as shown from previous studies (Kucera *et al.,* 1972). This significant increase is in a dose-dependent manner and the aqueous extract of *A. boonei* stem bark significantly increased the percentage of the body weight gain in male rats vs. control. Decrease in body weight of the rats may be due to the activities of echitamine and echitamidine, potent constituents of *A. boonei* which have earlier been reported to have diuretic and hypotensive properties (Kucera *et al.,* 1972, Maurica, 1993).

Findings from the graphical comparison of the paw thickness measured from day 1 to day 10 revealed that the paw size of the control remained constant from day 1 to day 10 because no inflammation was induced, however, the leaf and root bark extract of *A. boonei* showed significant inhibition of inflammation compared to the stem bark extract of *A. boonei.* 300mg/kg leaf extract appeared more effective compared to 600mg/kg leaf extract of *A. boonei* and indomethacin. This is in line with previous study by Omowumi *et al., (2*017) which showed that 50, 150 and 250mg/kg b.w aqueous fraction of *A. boonei* leaf suppressed carrageenan-induced rat paw oedema thickness in a dose-dependent manner which indicates that the anti-inflammatory mechanism of the action of *A. boonei* leaf may be related to prostaglandin inhibition. This could explain the folkloric use of *A. boonei* leaf in the treatment of rheumatoid arthritis. The stem extract showed no significant reduction of the inflammation compared with the control group. The root bark extract showed a good inhibition of inflammation from day 4 to day 10, reducing the paw thickness. However, 300mg/kg root bark extract appeared more effective compared to 600mg/kg root bark extract of *A. boonei* and indomethacin. This is in line with previous study by Adotey *et al.,* (2012) which showed that histological examinations of the

proximal interphalengeal foot joints showed reduced synovial proliferation in the paw thickness by the root extract. Similar work showed that the extract of *A. boonei* root bark had anti-inflammatory effects in CFA-induced arthritic rats (Kweifo-okai and Caroll, 1993).

5.2 Conclusion

The aqueous extract of *A. boonei* has been shown to be effective against chronic inflammation (formalin induced paw oedama) in a dose related manner. This present study supports the claim in the use of the extract of *A. boonei* in the traditional medicine for the treatment of inflammatory conditions. However, The present study suggests that the aqueous extract of *A. boonei* De Wild is toxic in high doses and longer dosage regimen. The extract and fractions of *A. boonei* are not only toxic, but also recovery from the toxic effects is not within a short-term limit. Although, administration of herbal medicines may be useful in the treatment of diseases, the secondary adverse effects must not be overlooked because in some cases, these side effects are more deleterious than the diseases these phytochemicals are originally used to treat. The safety of the administration of herbs must be considered when using them for therapeutic and prophylactic purpose. Reduction in the given doses could prevent the toxic effects produced. Therefore, aqueous extract of *A. boonei* stem bark appears to have no order of toxicity when ingested in therapeutic amounts. Finally, to minimize the toxicity, more toxicity studies should be carried out to understand more about the toxic effects of the plant on the liver.

5.3 Recommendations

Further studies should be carried out with the view of identifying and isolating the active components involved in the expression of toxicity.

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APPENDIX I

The reagents used for this research were of high analytical grade. They include:

- Double distilled water

- Indomethacin (St Louis, MO, U.S.A).

- Formaldehyde (Vardhman Exports India)

- Randox AST reagent

- Randox ALT reagent

- Randox ALP reagent

- Randox LDH reagent

Alkaline Phosphatase Analysis

Reagent Composition

1. Alkaline Phosphatase Substrate:

3.6 mM, Sodium Thymolphthalein Monophosphate in 0.2 M 2-Amino-2-Methyl-1-Propanol buffer. Magnesium Chloride 1.0mM, wetting agent, inactive ingredients, preservatives; pH 10.2 ±0.1.

2. Alkaline Phosphatase Color Developer: 0.1 M Sodium Hydroxide, 0.1 M Sodium Carbonate.

3. Alkaline Phosphatase Standard: Thymolphthalein in n-Propanol 0.5mM/L. Equivalent to 50U/L enzyme activity when used according to the Alkaline Phosphatase Procedure.

Procedure:

0.5mL of Alkaline Phosphatase Substrate was dispensed into labelled test tubes and it was equilibrated to 37°C for 3 minutes. 0.05mL of standard and test sample was added to each tubes respectively. Deionised water was used as sample for Reagent Blank. It was incubated for exactly 10 minutes at 37°C. 2.5mL Alkaline Phosphatase Color Developer was added to each

tubes respectively and mixed well. The absorbance of the reaction was read after 5 minutes at 630nm against reagent blank using a spectrophotometer.

Aspartate Aminotransferase Analysis

Reagent Composition

R1. Buffer

Phosphate buffer	100mmol/L, pH 7.4
L-aspartate	100mmol/L
Alpha- oxoglutarate	2.0mmol/L
R2. 2,4 dinitrophenylhydrazone	2.0mmol/L

Procedure:

Exactly 50µl of test sample was placed in a test tube and 50µl of distilled water was placed in blank test tube. Thereafter, 0.25ml of Reagent 1 was added to each. The solution was mixed and incubated at 37°C for 30minutes. Also, 0.25ml of Reagent 2 was added. The solution was mixed and allowed to stand for 20 minutes at 25°C. 2.5ml of Sodium hydroxide was then added. The absorbance of the reaction mixture was read after 5minutes at 540nm against a reagent blank using a spectrophotometer.

Calculation:

The activity of AST was extrapolated from the graph previously plotted against absorbance

Normal Range: ≤ 40 IU/L

Alanine Aminotransferase Analysis

Reagent Composition

R1. Buffer

Phosphate buffer	100mmol/L, pH 7.4
L-alanine	200mmol/L
Alpha- oxoglutarate	2.0mmol/L
R2. 2,4 dinitrophenylhydrazone	2.0mmol/L
R3. Sodium Hydroxide	4.0mmol/L

Procedure:

Exactly 50µl of test sample was placed in a test tube. Thereafter, 0.25ml of Reagent 1 was added. The solution was mixed and incubated at 37°C for 30 minutes. Also, 0.25ml of Reagent 2 was added. The solution was mixed and allowed to stand for 20minutes at 25°c. 2.5ml of Sodium hydroxide was then added. The absorbance of the reaction mixture was read after 5minutes at 540nm against a reagent blank using a spectrophotometer.

Calculation: The ALT activity was extrapolated from the table previously plotted against absorbance.

Normal Range: ≤ 40 IU/L

Lactate Dehydrogenase Analysis

Reagent Composition

R1a. Buffer/Substrate

Phosphate buffer	50mmol/L, pH 7.5
Pyruvate	0.6mmol/L
R1b. NADH	0.18mmol/L

Procedure:

0.1mL of test sample was added to 3.0mL of reagent at 25°C . It was mixed and the initial absorbance was read at 30 seconds at 340nm. It was simultaneously read again after 1, 2 and 3 minutes.

Calculation:

U/I= 4921 × Δ A

Normal Range: 125 - 220 U/L

APENDIX II

APPARATUS

- Spectrophotometer

- Centrifuge

- Digital weighing balance (METLER MT-5000D)

- Pestle and Mortar

- Glass pipettes

- Beakers

- Measuring Cylinders (Pyrex, England)

- Syringes

- Micropipettes

- Cuvette

-Pasteur pipette

-Test tubes

- Test tube rack

- Rat cages

- Feeding troughs

- Drinking troughs

- Refrigerator

- Dissecting sets

- Cotton wool

- Water bath

- Gloves

- Vernier calliper

Fig. 6 : Showing induction of inflammation through subaponeurotic injection of 0.1mL of 2% w/v formalin in the right hind paw of the rat from this study.

Fig. 7 : showing Inflammed right hind paw of the male Wistar rat from this study.

Fig. 8 : Showing normal hind paw of the control group from this study