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Haematological Profile and Hepatoprotective Property of Stem bark Extracts of *Anogeissus leiocarpus* against Formalin - Induced Pain and Inflammation in Wistar Rats

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Medicinal plants are rich in phytochemical constituents that have been used by man as therapeutic agents in the treatment of several inflammatory-related diseases. *Anogeissus leiocarpus* DC. (Guill & Perr.) (African birch) belongs to the family of Combretaceae, it has been used in African traditional medicine particularly in South-western part of Nigeria to treat many illnesses including gastro-intestinal disorders, wounds, arthritis, malaria, diabetes, ulcer amongst others. Therefore, the present study is to evaluate the haematological profile and hepatoprotective potential of hexane and ethanolic stem bark extracts of *Anogeissus leiocarpus* in formalin induced pain and inflammation in wistar albino rats in order to validate folkloric claim of the study plant in the treatment of inflammation. Formalin-induced paw oedema model was used to investigate anti-inflammatory effects of hexane and ethanolic stem bark extract of *Anogeissus leiocarpus* in experimental rats. Liver tissue used to assay for biochemical parameters including aspartate aminotransferase (AST), alanine transaminase (ALT), superoxide dismutase (SOD), glutathione peroxidase (GP_X), malondialdehyde (MDA) and nitric oxide (NO). Haematological parameters including total leucocytes count, Total Red Blood Corpuscles (RBC), Total White Blood Corpuscles (WBC) and Haemoglobin (Hb) were also determined using an automatic hematology analyzer. There is a dearth of information on hepatoprotective potential of this study plant in pain and inflammation models induced by formalin in rats. The results of the study showed that formalin-induced pain in rats model produced reduction in the levels of total RBC and Hb and increase in the levels of total leucocytes and total WBC. However, daily oral administration of stem bark extracts of *Anogeissus leiocarpus* for fourteen days significantly increased the levels of total RBC and Hb and caused reduction in levels of total leucocytes and total WBC. The formalin-induced pain and inflammation in the animal model also resulted in a significant ($p < 0.05$) increase in AST, ALT, NO, MDA and significant ($p < 0.05$) reduction in GPx, SOD and GSH tissue activities. However, the administration of hexane and ethanolic stem bark extracts of *Anogeissus leiocarpus* resulted in significant ($p < 0.05$) reduction in tissue levels of ALP, AST, ALT, NO MDA and significant ($p < 0.05$) increase in tissue activities of GPx, SOD and GSH in a dose dependent manner. Results obtained from this study revealed that hexane and ethanolic stem bark extracts of *Anogeissus leiocarpus* could be explored as a natural product in preventing oxidative stress mediated diseases and liver damage.

Keywords: *Anogeissus leiocarpus,* inflammation, haematology, hepatoprotective.

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INTRODUCTION

Liver is a vital organ that regulates several biochemical reactions and functions. It plays a pivotal role in the detoxification of toxins, drugs and metabolites (Schiff, 2007; Wang *et al*., 2016). Liver has also many functions in hormone synthesis, plasma protein synthesis, digestion and lipid and glucose homeostasis (Wang *et al*., 2016). Liver is a sine qua non for life, and there is no way to compensate for the absence of liver functions. Hence, there is need for hepatoprotective agents to guarantee its functions.

Formalin has over the years been linked to oxidative stress and has the ability to increase and over a long period of time elevate liver enzymes which would result in hepatotoxicity. Formalin is toxic for the liver, its injection, ingestion, or inhalation may lead to hepatotoxicity and acts on cellular compounds such as proteins, nucleic acids and lipids (Schiff, 2007). It forms cross linkages between protein and single stranded DNA by linking of primary amino groups in proteins with other nearby nitrogen atoms in DNA or protein through a $-CH_2-$ linkage (Samuel *et al*., 2020). The toxic effect of formalin destroys the cell structures and cause cellular dysfunction (Samuel *et al*., 2020). After ingestion, formaldehyde rapidly diffuses into numerous tissues like the brain, liver and lung. Hence, it is regarded as hepatotoxic and carcinogenic (Schiff, 2007; Samuel *et al*., 2020).

Inflammation and pain are common non-specific manifestations of many diseases (Hossain *et al*., 2011). It is a defence mechanism targeted to remove the injurious stimuli and initiate the tissue healing process (Maldini *et al*., 2009; Hossain *et al*., 2011). Prolonged inflammation can result into numerous diseases including rheumatoid arthritis, psoriasis and inflammatory bowel disease (Wang *et al*., 2013). Rheumatoid arthritis is a debilitating autoimmune disease. Rheumatoid arthritis can lead to the destruction of synovial fluids causing swelling, pain and stiffness in polyarticular joints leading to disability and premature mortality (Aletaha *et al*., 2010).

Rheumatoid arthritis is a chronic inflammatory disease and it is also one of the leading causes of chronic morbidity in the developed world, mostly affecting the work force population throughout the world (Mody, 2009). Formalin-induced inflammatory reaction is similar to that reported during arthritis and this animal model, is a standard for the evaluation of therapeutic agents with suspected anti-arthritic activity (Okoli *et al*., 2008).

In developing countries, especially in Nigeria, the use of traditional means in the treatment of diseases is still in practice (Balekar *et al*., 2013). Extracts of plants of natural origin are rich in phyto constituents that have been used by man as therapeutic agent in the treatment of numerous inflammatory-related diseases (Balekar *et al*., 2013).Therefore, there is need for an alternative source of anti-inflammatory drugs to combat inflammatory disorders because the use of either synthetic steroidal or non-steroidal anti-inflammatory drugs produce undesirable side effects such as ulcer, kidney problems and hypertension (Musa *et al*., 2012). In addition, the World Health Organization (WHO) had reported that these synthetic drugs are often associated with drug induced toxic effects or secondary adverse effects on long term use (Sarwar *et al*., 2011).

Anogeissus leiocarpus DC. (Guill & Perr.) occurs as a shrub or tree about 21 meters high, often with pendulous branches. It is commonly known as 'African Birch' (Lemmens *et al*., 2012).Different parts such as leaves, stem bark and roots are used by traditional healers in Africa especially in the South-Western part of Nigeria for the treatment of variety of diseases including malaria, acute respiratory tract infections, general body pain, asthma, common cold, cough, diabetes and wound healing (Ademola and Eloff, 2011; Barku and Grace, 2013).

In addition, the interaction with some traditional healers in South-western part of Nigeria revealed that stem bark of *Anogeissus leiocarpus* is a good source of traditional medicine for wound healing, pneumonia, arthritis, aches, microbial infection and treatment of malaria. Therefore, the present study seeks to evaluate the haematological profile and hepato protective potential of hexane and ethanolic stem bark extracts of *Anogeissus leiocarpus* in formalin induced pain and inflammation in wistar albino rats in order to validate folkloric claim of the study plant in the treatment of inflammation.

MATERIALS AND METHODS

Plant Collection and Extraction

One (1) kg of stem bark of *Anogeissus leiocarpus* was purchased from herbal practitioners at Akure main market, Akure, Ondo State, Nigeria. Identification and authentication of the study plant was carried out by the Crop, Soil and Pest Management (CSP) Department at the Federal University of Technology, Akure (FUTA), Nigeria. The voucher sample was deposited at Federal University of Technology, Akure, Nigeria's herbarium with reference number FUTA/HB/0259 allotted and deposited in the Department of Neurofarba, Pharmaceutical and Neuroceutical section, Research Unit of Phytolab, University of Florence, Italy. The air-dried stem bark was coarsely powdered and subjected to cold extraction using hexane and 70 % ethanol solvents separately for 72 hours, the filtrate was concentrated using rotary evaporator and freeze dried to obtain crude extract.

Drugs and Chemicals

Ibuprofen (Ranbaxy), formalin and other chemicals were of analytical grade.

Animals

Albino wistar rats weighing approximately (160–250 g body weight) were used. Rats were housed and used at least one week after their arrival in plastic cages with filter tops at the Animal House Lab, Department of Biochemistry, Federal University of Technology Akure, Ondo State, Nigeria. Six rats were housed per cage; animals were fed a standard laboratory diet and tap water ad libitum and kept at under standard environmental conditions (23–25°C, 12-h light/12-h dark cycle) (OECD, 2010).

Induction of inflammation using rat model of formalin-induced paw oedema

(Experimental Protocol)

The anti-inflammatory activity of hexane and ethanolic stem bark of *Anogeissus leiocarpus* (Al) was measured in rat model of formalin-induced paw oedema. Albino rats fasted overnight were divided into 9 groups of six animals each, the dosage of the drugs administered to the different groups was as follows:
Group I- control (normal saline)
Group II- Formalin induced (0.1ml/kg bw)
Group III-V -Formalin + (hexane extract of Al; 75, 150 and 300 mg/kg b.w) for 14 days
Group VI-VIII -Formalin + (ethanolic) extract of Al; 75,150 and 300 mg/kg b.w) for 14 days
Group IX - Standard drug (Ibuprofen) for 14 days

Formalin-induced paw oedema

Thirty minutes pre oral treatment with extract/drug, following injection of formalin (0.1ml of 10% v/v) into the right hind paw of the tested rats. No injection of formalin into the control group animals. The paw thickness was measured before and after induction of inflammation by using vernier calliper. The increase in paw oedema was measured by vernier calliper according to method described by Taylor *et al*. (2000) and Joseph *et al.* (2005) with some modifications.

The difference in paw thickness after and before induction of inflammation was calculated and presented as mean increase in paw thickness (cm). The ability of hexane and ethanolic stem bark extract of *Anogeissus leiocarpus* and the standard (ibuprofen) used as anti-inflammatory drugs to suppress paw inflammation was expressed as a percentage of inhibition of paw oedema (Taylor *et al*., 2000; Joseph *et al.,*2005).

Twenty-four hours after the end of treatment periods of induction of inflammation using rat model, the rats were euthanized using cervical dislocation and subsequently sacrificed. Blood samples were collected through cardiac puncture using 5mL syringes into ethylenediamine tetraacetic acid (EDTA) and heparinized bottles to prevent the blood samples from clotting.

Haematological Analysis

Blood samples obtained using EDTA bottles were subjected to haematological analysis to determine total white blood corpuscles (WBC), total red blood corpuscles (RBC), haemoglobin (HB), lymphocytes, neutrophils and eosinophils counts using an automatic hematology analyzer (Swelab Alfa 3-Part Haematology Analyzer, Boule Medicals, Spanga, Sweden) at The Federal University of Technology, Akure, Ondo-State, Nigeria).

Assessment of Liver Function,

Liver function biomarkers including alanine aminotransferase (ALT), aspartate aminotransferase (AST) and alkaline phosphatase (ALP) were performed according to the manufacturer's procedure (Randox Laboratories, Crumlin, UK).

Oxidative Stress and Pro-inflammatory Biomarkers

The excised liver samples were homogenized accordingly using a 50mMTris–KCl buffer at pH 7.4 consisting of 1.15% KCl, then further centrifuged at 12,000 X g for 15 min at 4^{o}C to obtain the post mitochondrial fraction, which was used for

the assessment of oxidative stress and inflammatory biomarkers. Misra and Fridovich (1972) method was used to determine the activities of Superoxide dismutase (SOD). Reduced glutathione (GSH) level was determined according to Habig *et al*. (1974) and Rotruck *et al*. (1973) whereas Glutathione peroxidase (GPx**)** activity was assessed according to the established method of Granell *et al*. (2003). The assay of pro-inflammatory biomarkers including NO and MDA were assessed according to established protocol as described by Green *et al*. (1982) and Varshney and Kale (1990) respectively.

Statistical analysis

Results were expressed as mean ± standard deviation (SD). Statistical analyses were performed using Microsoft Excel and Graph Pad prism 8 software. Data were analyzed using student t-test and analysis of variance (One-way ANOVA) followed by Turkey's test. Values of $p < 0.05$ were considered as statistically significant.

RESULTS

Table 1. Level of Leucocytes in Experimental Rats

Groups	Lymphocytes (%)	Eosinophils (%)	Neutrophils (%)
Control	40.5 ± 0.71^{a}	$4.0 \pm 0.00^{a, b}$	47.0 ± 0.03^{a}
Induced	72.5 ± 0.76^{i}	8.5 ± 0.71^{k}	74.5 ± 0.78^{j}
I + Hex (75 mg/kg)	50.5 ± 0.82^{e}	$6.1 \pm 0.14^{d, e}$	$59.0 \pm 0.01^{e, f}$
I + Hex (150 mg/kg)	47.5 ± 0.53^{d}	5.8 ± 0.01^{d}	$57.5 \pm 4.95^{d, e}$
I + Hex (300 mg/kg)	45.5 ± 0.72^{c}	5.3 ± 0.05^{c}	$55.0 \pm 0.02^{c, d}$
I + EtOH (75 mg/kg)	64.5 ± 0.55^{f}	$8.1 \pm 0.14^{g, h}$	68.5 ± 0.71^{i}
I + EtOH (150 mg/kg)	61.5 ± 0.75^{g}	$7.8 \pm 0.28^{f, g}$	$67.5 \pm 2.12^{g, h}$
I + EtOH (300 mg/kg)	60.0 ± 0.02^{h}	7.4 ± 0.15^{f}	$66.5 \pm 0.74^{g, h}$
I + Ibuprofen (400 mg/kg)	$42.0 \pm 0.04^{b, c}$	3.7 ± 0.02^{a}	$49.0 \pm 0.05^{a, b}$

Key: I-Induced; Hex-Hexane; EtOH- Ethanol. Values are expressed as mean ± standard deviation (n=6). Values with different superscript(s) in a column are significantly different ($p < 0.05$).

Table 2. Levels of Haemoglobin, Total RBC and WBC in Experimental Rats

Groups	HB (g/l)	RBC (10^{12}g/l)	WBC (10^{9}g/l)
Control	13.5 ± 0.71^{a}	5.8 ± 0.28^{a}	$4.3 \pm 0.14^{a, b}$
Induced	8.0 ± 0.04^{i}	2.8 ± 0.14^{i}	8.9 ± 0.10^{i}
I + Hex (75 mg/kg)	10.0 ± 0.02^{e}	4.7 ± 0.12^{e}	6.0 ± 0.02^{e}
I + Hex (150 mg/kg)	10.5 ± 0.05^{d}	$4.8 \pm 0.00^{c, d}$	5.8 ± 0.14^{d}
I + Hex (300 mg/kg)	11.0 ± 0.01^{c}	$5.0 \pm 0.03^{c, d}$	5.4 ± 0.10^{c}
I + EtOH (75 mg/kg)	8.8 ± 0.02^{h}	3.2 ± 0.14^{g}	7.7 ± 0.05^{h}
I + EtOH (150 mg/kg)	8.9 ± 0.14^{g}	3.3 ± 0.23^{g}	7.5 ± 0.03^{g}
I + EtOH (300 mg/kg)	9.0 ± 0.02^{f}	$3.5 \pm 0.11^{f, g}$	7.1 ± 0.12^{f}
I + Ibuprofen (400 mg/kg)	12.0 ± 0.01^{b}	$5.7 \pm 0.16^{a, b}$	4.4 ± 0.14^{b}

Key: I-Induced; Hex-Hexane; EtOH- Ethanol; WBC-total white blood corpuscles; RBC-total red blood corpuscles; haemoglobin (HB). Values are expressed as mean ± standard deviation (n=6). Values with different superscript(s) in a column are significantly different ($p < 0.05$).

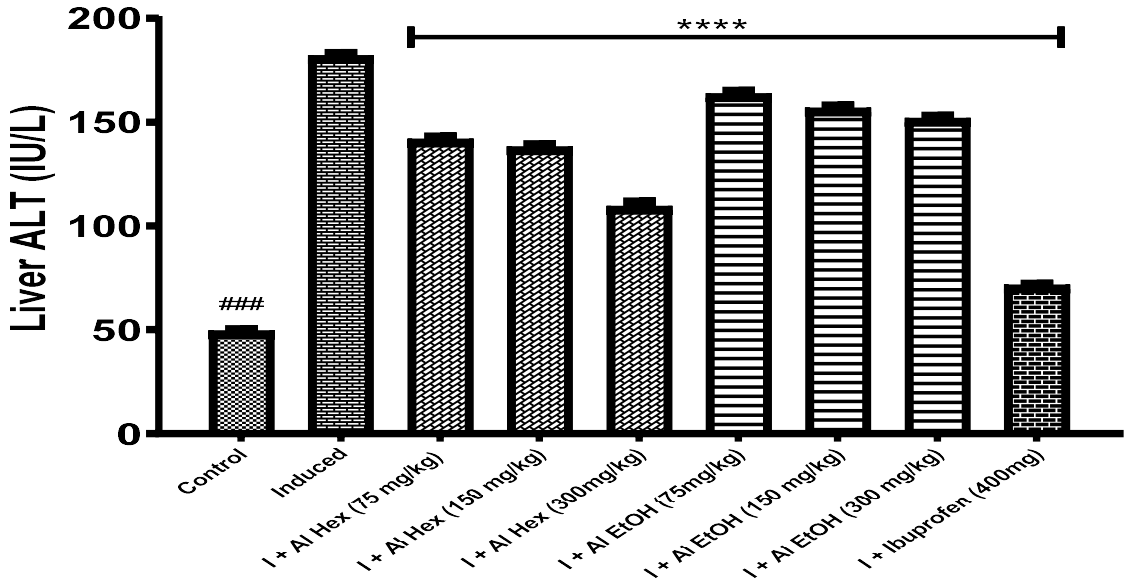

Figure 1. Effect of Hexane and Ethanolic Stem Bark Extracts of *Anogeissus leiocarpus* on Liver ALT level in Formalin-Induced Inflammation in Rats. Data are expressed as mean ± standard deviation (n=6). Values with different superscript(s) in a column are significantly different ($p < 0.05$); # denotes that Induced with Control and ∗ denotes treatment groups were compared with Induced. I-induced; EtOH- Ethanol; Hex- Hexane.

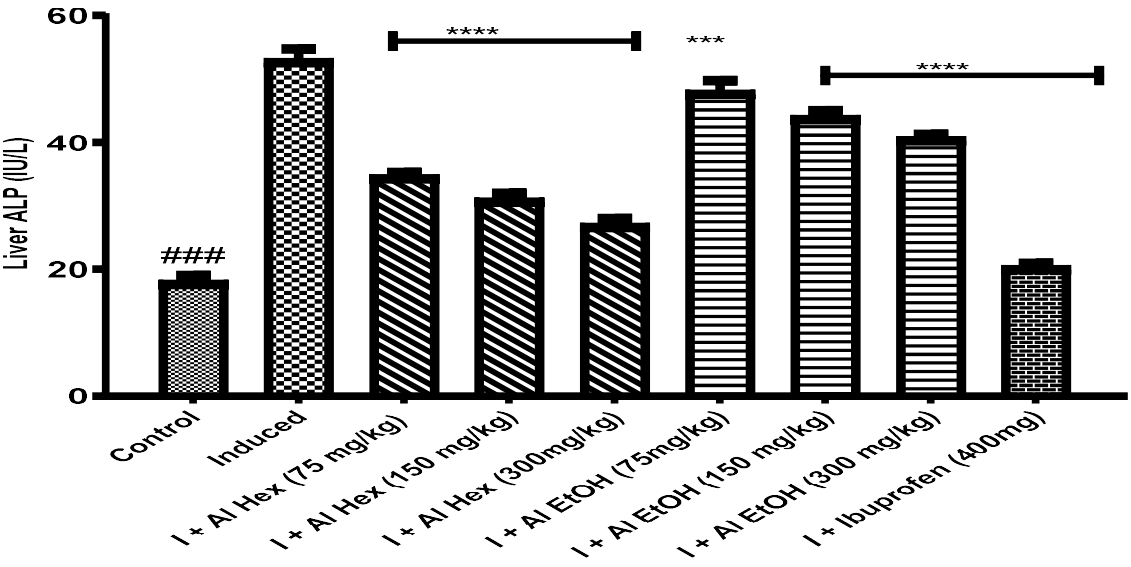

Figure 2. Effect of Hexane and Ethanolic Stem Bark Extracts of *Anogeissus leiocarpus* on Liver ALP level in Formalin-Induced Inflammation in Rats. Data are expressed as mean ± standard deviation (n=6). Values with different superscript(s) in a column are significantly different ($p < 0.05$); # denotes that Induced with Control and ∗ denotes treatment groups were compared with Induced. I-induced; EtOH- Ethanol; Hex- Hexane.

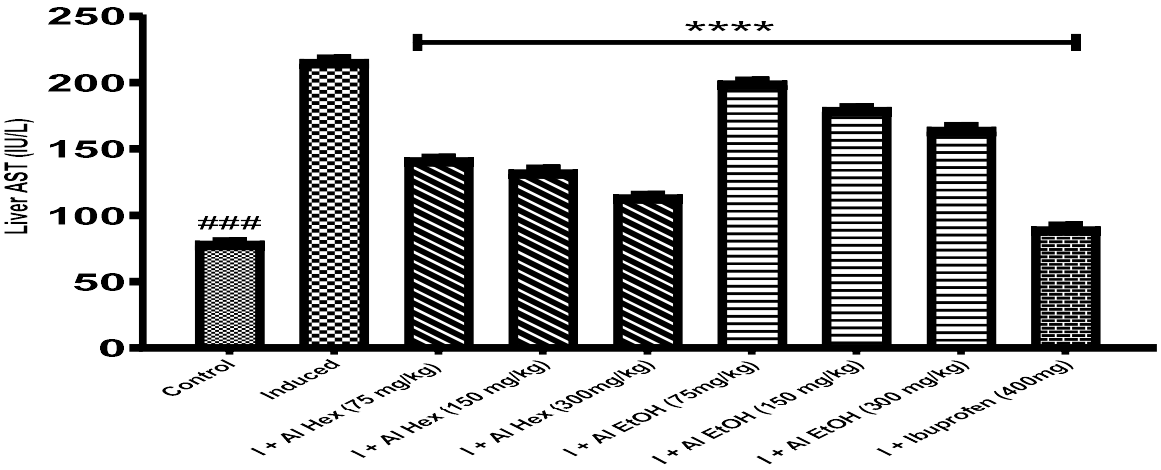

Figure 3. Effect of Hexane and Ethanolic Stem Bark Extracts of *Anogeissus leiocarpus* on Liver AST level in Formalin-Induced Inflammation in Rats. Data are expressed as mean ± standard deviation (n=6). Values with different superscript(s) in a column are significantly different ($p < 0.05$); # denotes that Induced with Control and ∗ denotes treatment groups were compared with Induced. I-induced; EtOH- Ethanol; Hex- Hexane.

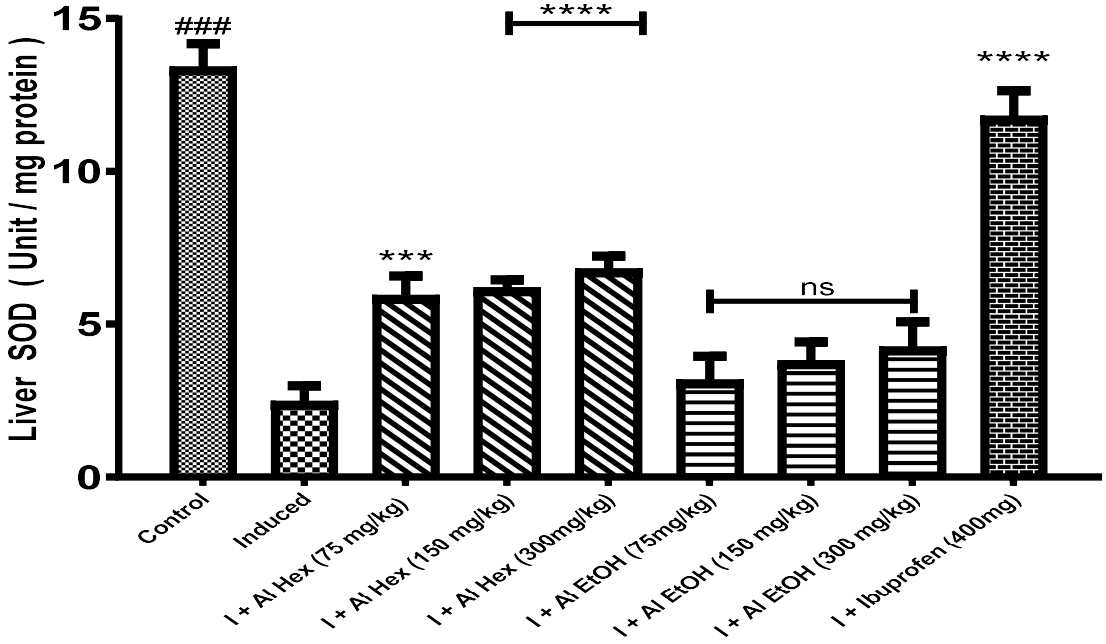

Figure 4. Effect of Hexane and Ethanolic Stem Bark Extracts of *Anogeissus leiocarpus* on Liver SOD activity in Formalin-Induced Inflammation in Rats. Data are expressed as mean ± standard deviation (n=6). Values with different superscript(s) in a column are significantly different ($p < 0.05$); # denotes that Induced with Control and ∗ denotes treatment groups were compared with Induced. I-induced; EtOH- Ethanol; Hex- Hexane.

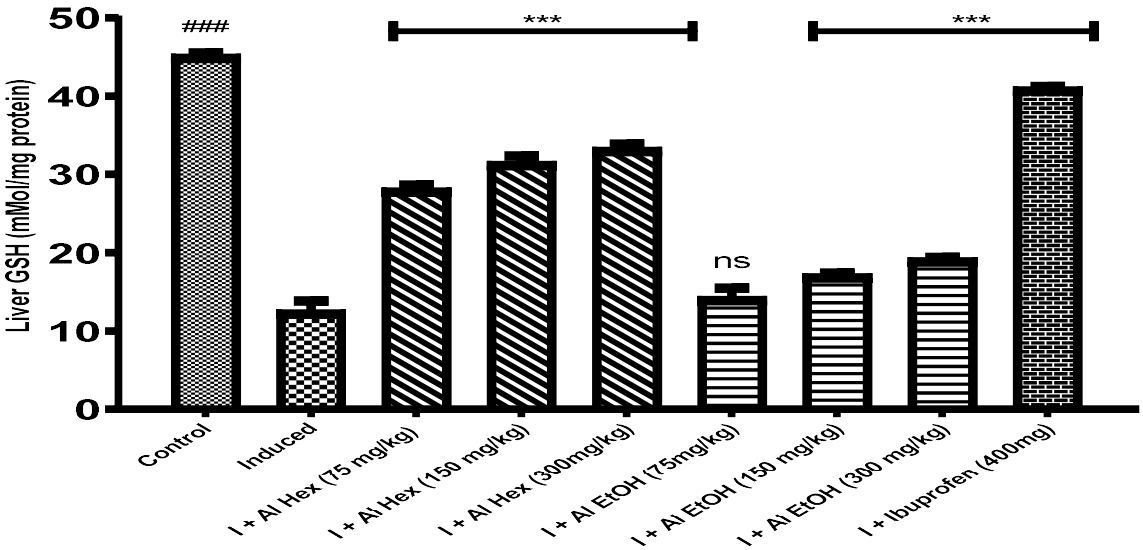

Figure 5. Effect of Hexane and Ethanolic Stem Bark Extracts of *Anogeissus leiocarpus* on Liver GSH produced in Formalin-Induced Inflammation in Rats. Data are expressed as mean ± standard deviation (n=6). Values with different superscript(s) in a column are significantly different ($p < 0.05$); # denotes that Induced with Control and ∗ denotes treatment groups were compared with Induced. I-induced; EtOH- Ethanol; Hex- Hexane.

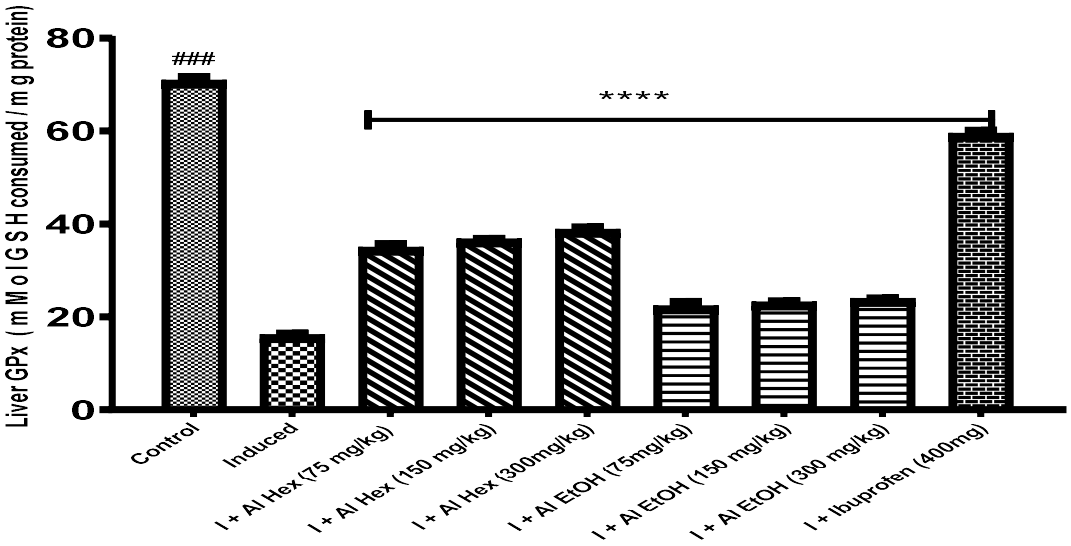

Figure 6. Effect of Hexane and Ethanolic Stem Bark Extracts of *Anogeissus leiocarpus* on Liver GPx activity in Formalin-Induced Inflammation in Rats. Data are expressed as mean ± standard deviation (n=6). Values with different superscript(s) in a column are significantly different ($p < 0.05$); # denotes that Induced with Control and ∗ denotes treatment groups were compared with Induced. I-induced; EtOH- Ethanol; Hex- Hexane

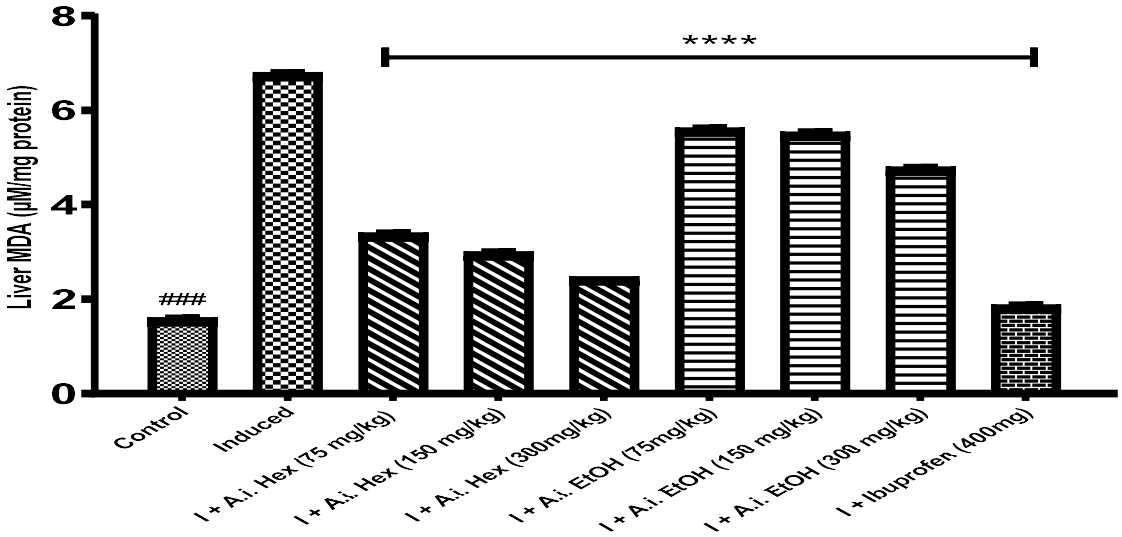

Figure 7. Effect of Hexane and Ethanolic Stem Bark Extracts of *Anogeissus leiocarpus* on Liver MDA level in Formalin-Induced Inflammation in Rats. Data are expressed as mean ± standard deviation (n=6). Values with different superscript(s) in a column are significantly different ($p < 0.05$); # denotes that Induced with Control and ∗ denotes treatment groups were compared with Induced. I-induced; EtOH- Ethanol; Hex- Hexane

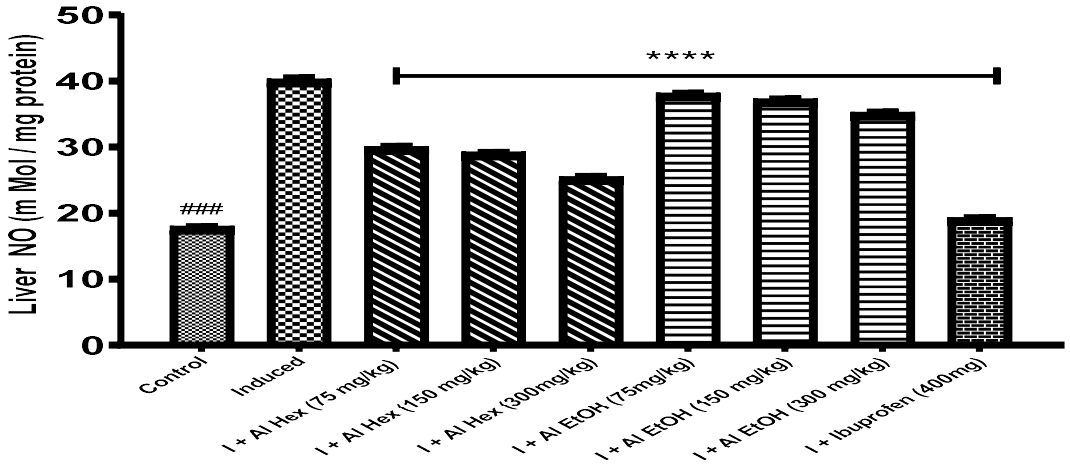

Figure 8. Effect of Hexane and Ethanolic Stem Bark Extracts of *Anogeissus leiocarpus* on Liver NO produced in Formalin-Induced Inflammation in Rats. Data are expressed as mean ± standard deviation (n=6). Values with different superscript(s) in a column are significantly different ($p < 0.05$); # denotes that Induced with Control and ∗ denotes treatment groups were compared with Induced. I-induced; EtOH- Ethanol; Hex- Hexane

DISCUSSION

From the time immemorial, the use of medicinal plants in the prevention and management of various ailments has been in existence (Oyebode *et al*., 2016). Leucocytes play a vital role in the development and manifestation of inflammation. Neutrophils play a crucial role in the development and propagation of inflammation and they are the main source of free radicals at the site of inflammation. In the present study a reduction in the levels of neutrophils (Table 1)

after the pre-treatment and daily oral administration of hexane and ethanolic stem bark extracts of *Anogeissus leoicarpus* was observed thus establishing its involvement in suppressing inflammation (Goel *et al*., 2001).

Eosinophils are granule having leucocytes, they differentiate from stem cell precursors. It is used to synthesize and release lipid derived mediators that stimulate responses in tissues**.** In chronic inflammation, lymphocytes are the predominant cells that caused permanent distortion of the tissue thus interfering its function. The reduction in the population of lymphocytes and eosinophils in formalin induced pain and inflammation in rats treated with hexane and ethanolic stem bark extracts of *Anogeissus leiocarpus* as shown in Table 1 revealed its anti-inflammatory property (Goel *et al*., 2001).

Total white blood cells (WBC) plays a vital role in body defense mechanism. There is an increase in total white blood cells count during inflammation, this may be due to the release of interleukins, responsible for the production of both granulocytes and macrophage colony stimulating factor (Eric and Lawrence, 1996). The present study reveals high level of white blood cell count (Table 2) in formalin induced pain and inflammation. Pre-treatment and daily oral administration of hexane and ethanolic stem bark extracts of *Anogeissus leiocarpus* at the dose levels of 75, 150 and 300 mg/kg body weight significantly decrease the total white blood cell count (Table 2) in a dose dependent manner which shows the significant recovery from the inflammatory process.

Haemoglobin (Hb) and RBC play a crucial role in the oxygen transport. Formalin induction causes the significant decrease in the RBC and Hb which leads to anaemia (Table 2). Pre-treatment and daily oral administration of hexane and ethanolic stem bark extracts of *Anogeissus leiocarpus* at the dose levels of 75, 150 and 300 mg/kg body weight reversed these levels to normal in a dose dependent manner. The low concentration of haemoglobin is noted in chronic inflammatory disease such as rheumatoid arthritis which is typically associated with the anorexia and weight loss. Such decline in Hb level has been reported earlier by Goel *et al*. (2001).

Formalin has over the years has been linked to oxidative stress and has the ability to increase and over a long period of time elevate liver enzymes which would result in hepatotoxicity. The hepato protective potential of hexane and ethanolic stem bark extracts of *Anogeissus leiocarpus* against formalin induced pain and inflammation in rat paw was investigated by determining ALT, ALP and AST. ALT is an important liver damage enzyme which catalyzes transamination reactions. The incidence of conditions that can lead to liver damage such as injury, cancer and hepatitis will result in increased levels of this enzyme (Uthman *et al*., 2021). AST, which is biomarker of liver damage, are cytosolic and mitochondrial enzymes of which levels are usually increased as a result of chronic illness and necrosis due to loss of hepatocellular integrity.

Liver marker enzymes are involved in the transfer of α-amino groups from alanine and aspartate to the α-keto group of ketoglutarates to form pyruvate and oxaloacetate, respectively (Schiff, 2007). As shown in Figures 1, 2 and 3, there is a significant increase ($p < 0.05$) in the levels of these enzymes in the group administered formalin when compared to the control. However, pre-treatment and daily oral administration of varying concentrations of hexane and ethanolic stem bark extracts of *Anogeissus leiocarpus* significantly reduced the elevated levels in a dose dependent manner as indicated in Figure 1, 2 and 3.

When there is impairment in body metabolism, an increase in the production of toxic molecules such as free radicals will be observed. Antioxidants, known as free radical scavengers, will be needed to reduce or neutralize the free radical formation (Wang *et al*., 2016). The hepatic destruction caused by formalin causes oxidative stress and produces reactive oxygen species (ROS), as shown in the significant increase in MDA and NO, that are known to be oxidative stress markers, and also a decrease in GSH, GPx and SOD, which are antioxidant markers. These observations are in accordance to Payani *et al.* (2019) and Mesole *et al*. (2020).The two authors reported that formalin exposure significantly reduced the levels of enzymatic and non-enzymatic antioxidants.

However, pre-treatment and daily oral administration of varying concentrations of hexane and ethanolic stem bark extracts of *Anogeissus leiocarpus* significantly increases the activities and levels of these endogenous antioxidant enzymes in dose dependent manner, which is in agreement with the report of Mesole *et al*. (2020). This study indicated that rats treated with hexane and ethanolic stem bark extracts of *Anogeissus leiocarpus* caused a significant increase in the levels of antioxidant enzymes.

This research established the hepato protective potential of hexane and ethanolic stem bark extracts of *Anogeissus leiocarpus* in a dose dependent manner with hexane extract at 300 mg/kg body weight showing the best hepato protective ability. In addition, the research showed the ameliorative potential of the haematological parameters in formalin-induced pain and inflammation. The hexane extract was more effective in ameliorating the negative alterations to liver biomarkers and haematological parameters than the ethanolic extract.

CONCLUSION

Subcutaneous injection of formalin in rat paw resulted in elevated levels of liver biomarkers and production of free radicals; hence elevated levels of MDA, NO and reduced activity of important free radical scavenging enzymes such as GSH, GPx and SOD within the liver. The administration of hexane and ethanolic stem bark extracts of *Anogeissus leiocarpus* resulted in a reduced level of liver biomarkers and oxidative stress mediated diseases (MDA and NO) and increased in the tissue activity of GSH, GPx and SOD in a dose dependent manner. Results obtained from this present study revealed that hexane and ethanolic stem bark extracts of *Anogeissus leiocarpus* are efficacious at preventing oxidative stress mediated diseases and liver damage in formalin induced pain and inflammation.

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Conflict of interest: The authors declare that there are no conflicts of interest.

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