CHAPTER ONE

INTRODUCTION

1.1 Background to the study

Blood is the liquid that moves through the vessels and includes plasma (the liquid portion, which contains water, proteins, salts, lipids, and glucose) and the cells (red and white cells) and cell fragments called platelets (Charles,2012).

Blood is a necessary component of the human body, and the loss of the blood may be life-threatening.Hematopoiesis is the process through which blood is produced and then used to transport oxygen to tissues and cells. The clotting system in the human body guards against blood loss.Theprocess through which blood coagulates is known as **Blood coagulation**.It is a method of preventing hemorrhage by stopping the flow of blood and retaining it within the injured artery walls (Periayah et al. 2017). The second stage in the process of stopping blood loss from a damaged vessel, the creation of a clot is commonly referred to as secondary hemostasis.The first stage, primary hemostasis, is characterized by blood vessel constriction (vasoconstriction) and platelet aggregationat the site of the vessel's damage.Clots can form in a vessel that has not been breached under unusual circumstances, and these clots can cause occlusion (blockage) of the vessel.

When a vascular tissue injury occurs, blood coagulation is a complex chain mechanism involving a sequence of stimulus responses in combination with coagulation factors and enzymes, with the aim of staunching blood flow (Ngo et al. 2012).Blood coagulation is a biochemical event that occurs on the cell surface and is designed to not only stop blood loss after a vascular injury (hemostasis), but also to provide the required genetic, cellular, and protein constituents for growth and repair.Furthermore, coagulation of arteries and veins of a medium sizemay have serious consequences, ranging from organ injury to death.Blood coagulation is a complicated but well-coordinated sequence of events involving tissue factor-bearing cells and platelets. The mechanism by which cells and soluble protein elements combine to form an intravascular blood clot is known as blood coagulation (Hoffman et al. 2006). This is an effective protective

mechanism that acts to seal vascular bleeds and avoid excessive hemorrhage when it occurs in response to vessel injury.

Inflammation is a developed protective mechanism that occurs in higher species to defend them from harmful insults such as microbial infection, tissue damage, and other unhealthy conditions. It is a crucial host immune response that enables harmful stimuli to be removed and damaged tissues to be repaired (Ahmed, 2011*)*.The body's early response to damage or infection is inflammationand it is a defensive response by the body's self-defense system (Shiel 2020). Inflammation is a tissue support mechanism that stimulates the immune system to repair damaged tissues and defend against disease caused by foreign organisms (Siraj 2020). The immune response to damage or disease, as well as harmful stimuli such as bacteria, contaminated cells, toxic contaminants, or irradiation, works by removing harmful stimuli and initiating the healing process (Medzhitov, 2010).

Inflammation and hemostasis are closely linked pathophysiologic mechanisms that have a significant impact on one another. Inflammation activates the hemostatic system, which has a significant impact on inflammatory activities in thisbidirectionalinteractions(Verhammep,2009). Inflammation causes blood to clot, reduces the effectiveness of natural anticoagulant pathways, and the fibrinolytic system is affected (Verhammep,2009*)*.The main mediators in the activation of coagulationare inflammatory cytokines.When blood clots are formed, these clots have the potential to migrate to other parts of the body and cause damage.

Phylantusamarus commonly calledinYoruba culture's Eyinolobe; Ngwu among the Igbo tribe; and Mache dogooyo in Hausa tribe, belongs to the family of Euphorbiceae with approximately 800 species spread over Australian, American, African and Asia continent (Joseph and Raj,2011;Iranloye et al.2010). *P. amarus*has been reported to be a medicinal plant; the ayurvedic literature has recorded many medicinal uses of the plant such as antiviral, antioxidant, anti-diabetic, (Gupta and Vaghela 2019), anti-carcinogenic, anti-coagulant, and anti-inflammatory properties (Aldred, 2009*;* Matoset al. 2013). The plant contains various phytochemicals, nutritional and mineral compounds. *P. amarus* has been used in folklore and traditional medicine to treat different health complications such as wounds, hypertension, urinary tract infections, and diabetes (*Patel* et al. 2011). Bearing in mind the above uses of *P. amarus*, this study aims at

exploring the anti-coagulation property of *Phyllantusamarus* plant and its ultimate effects in carrageenan induced inflammation mice models.

1.2 Statement of the problem

The relevance of Inflammation and hemostasis are closely linked and have a significant impact on one another. Inflammation activates the hemostatic system (Verhammep,2009*).* inflammation causes blood to clot, reduces the effectiveness of natural anticoagulant pathways, andthe fibrinolytic system is affected.(Bonart et al.*2010*) The main mediators involved in coagulation activation are inflammatory cytokines (Levi et al.*2010*) When blood clots are formed, these clots have the potential to migrate toother areas of the body and cause damage. There are clinical circumstances in which in which the near interdependence of inflammation and hemostasis plays a significant role in pathogenesis, which can lead to bothersome blood clots, as well as other severe conditions. (Levi and Vander poll 2010).

Inflammation and hemostasis have a close relationship and it explains the prothrombin tendencies in some of these clinical conditions, thereby inflammation shifts the hemostatic activity towards activation of coagulation by proinflammatory mediators which then inhibitsfibrinolytic and anticoagulant properties.(Vander pollet al.2010) Examples of clinical conditions include: Systemic inflammatory response to inflection also called Sepsis and Acute arterial thrombosis, inflammatory bowel disease. (Esmon and Levi 2010). Some of orthodox anticoagulant agents are expensive and reportedly accompanied with undesirable side effects. Therefore, there is increased research focus towards the discovery of novel natural anticoagulant and anti-inflammatory agents. The pivot of the present research is therefore to explore the possible anti-coagulant and anti-inflammatory activity of *P. amarus* ethanol extract and fractions in induced inflammation mice models.

1.3 Aim and Objectives of the study

This study aims to investigate the effect of hydroethanol extract and fractions of *Phyllantusamarus* plants on blood coagulation and some biochemical parameters in carrageenan induced active inflammation mice models.

The specific objectives are to:

- determine the effects hydroethanol extract and fractions of *P. amarus*on clotting (CT) and bleeding (BT) times of active inflammatory mice.
- determine the effects of hydroethanol extract and fractions of *P. amarus* on the protrombin (PT) and activated partial thromboplastin (aPPT) times of the experimental mice.
- determine the effects of hydroethanol extract and fractions of *P. amarus*on some biochemical parameters; Total Protein (TP), Aspartate aminotransferase (AST) and Alanine transferase (ALT) in inflammatory mice.
- characterize the phytochemical components of the crude extract and fractions of *P. amarus* using Gas chromatography-mass spectrometry (GC-MS).

1.4 Scope of the study

This study will investigate and determine the effects of the *P. amarus*plant on blood coagulation parameters in active inflammatory mice, as well as to see if the plant has anti-inflammatory properties. The phytochemical components of the plant extract and fraction will be characterized by GC-MS in order to identify some bioactive compounds present in *P. amarus* that may be responsible for any observed activity.

1.5 Significance of the study

There may be possible discovery of novel anti-coagulant and anti-inflammatory properties of *P. amarus* plant. The mechanism of the interaction between inflammation and coagulation will be discussed using active inflammatory mice models by determining the bleeding and clotting times, prothrombin and active partial thromboplastin times, and other relevant biochemical parameters. Some important phytochemicals in the hydroethanol extract of *P. amarus* may be identified. The possible mechanism of the plant in reducing the complications in disease state via the downplay of the interaction between inflammation and blood coagulation may be elucidated.

1.6 Definition of terms

Activated partial thromboplastin time (aPTT): this is a routinely used coagulation test that is simple to run, inexpensive, and widely utilized in clinical and research coagulation laboratories around the world. The aPTT is founded on the idea that addinga platelet replacement, factor XII activator, and $CaCl_2$ to citrated plasma allows for the development of a stable clot. The amount of time it takes for a stable clot to develop is recorded in seconds and represents the actual aPTT result**.**

Bleeding time: is a diagnostic examination that evaluates a person's platelet function. It entails causing a patient to bleed and then keep track of how long the bleeding lasts. When the test is carried out according to specified guidelines, the term template bleeding time is used.

Clotting time: this measures the time necessary to produce a clot. In most studies, an activator is utilized to induce coagulation and assess the response of a component or portions of the cascade model of coagulation. A reduction in the number or function of the coagulation components involved might cause abnormal clotting times.

Fibrinogen: Fibrinogen (factor I) is a glycoprotein complex produced in the liver and circulated in all vertebrates' blood. It is transformed enzymatically by thrombin to fibrin and then to a fibrin-based blood clot during tissue and vascular injury. Fibrin clots are mainly used to avoid bleeding by occluding blood vessels.

Prothrombin time: The extrinsic and common pathways of coagulation are assessed using the prothrombin time. Protime INR and PT/INR are two other names for this blood test. They are used to assess the clotting propensity of blood, in the measure of warfarin dosage, liver damage, and vitamin K status. I (fibrinogen), II (prothrombin), V (proaccelerin), VII (proconvertin), and X (Stuart–Prothrombin) are the coagulation factors measured by PT.

CHAPTER TWO

LITERATURE REVIEW

The shift in the attention to the use of medicinal plants to treat diseases and infections in the world today is due to the minimum side effects and their improved safety, and reliability when compared to synthetic drugs (Joseph and Raj 2011). Medicinal herbs are vital store houses of bioactive compounds and nutrients, including minerals and vitamins. (Aldred et al.2010).

2.1 GENERAL OVERVIEW OF *PHYLLANTUS AMARUS* PLANT

Phyllantusamarus is small erect, tropical annual medicinal herb plant which is widely distributed. It grows 30-40cm in height, with small leaves and flowers which comes in various colors such as yellow, green, or white. It has five white sepals and an apical anther (Danladiet al.2018*;* Vernaet al.2014).

*P. amarus*is a plant from the family of Euphorbiaceae. Phyllantus; its generic name has approximately 800 species over Austrialia, African, America and Asian continent (Joseph and Raj, 2011; Iranloye et. al., 2010). In Africa, particularly Nigeria, it has various names in different tribes. In Yoruba tribe it is called "dobisowo or eyinolobe", in Igbo tribe it is called "ngwu" and Hausa it is called "machedagooyo" (Joseph and Raj 2011).

Other species of phyllantus include:

- Phyllantusemblica
- Phyllantusniruri
- Phyllantusurinaria
- Phyllantusdebillis

TAXONOMY OF *P. amarus*

Kingdom: Plantae

Division: Tracheophyta

Class: Magnoliopsida

Order: Malpighiales

Family: Phyllanthaceae

Genus: Phyllantus.L

Species: *P. amarus*

(Article6 ; International journal of pharmaceutical sciences., volume 4, 2018)

HABITAT: It is widely spread throughout the tropics and sub-tropics, sandy regions, and as a weed in cultivated and waste lands.

Figure 2.1: *Phyllantusamarus* (Jenna et al. 2020)

P. amarus has been utilized traditionally as Ayurvedic medicine before being hijacked by medical experts. The plant contains bitter, astringent, cooling, diuretic, stomachic, antiseptic, antidiabetic, hypotensive, and febrifuge properties (Patelet al.*2011)* which is traditionally used in treatment of jaundice, diarrhea, dysentery, diabetes, fevers, ulcers and wounds (*Patel* et al.*2011*).

2.2 REPORTED PHYTOCHEMICAL CONSTITUENTS OF *P. amarus*

Phytochemicals are bioactive compounds found naturally in plants. They account for unique color, flavor, and aroma of plant. These functions protect plants against invasion, diseases and infection (Adnan et al. 2010; Achi et al. 2017).

*P. amarus*has been discovered to havevarious phytochemicals that are associated to the leaves, roots, and stem. However, more phytochemicals are found in its leaves which

have been reported to possess anticarcinogenic, antitumour, antioxidant, antibacterial, antifungal, antiviral and antidiabetic activities (Gupta and Vaghela 2019). These phytochemicals include: Alkaloids, Flavonoids, Tannins, Lignans, phyllanthin, hypophyllantin, phylletralin, niranthin) (Patel et al. 2011), Coumarins, Saponins, Steroids, Reducing sugar, Cardiac glycosides, Phenolic compounds, Volatile oils (Aldred, 2009; Achi et. al.2017; Ashok and Upadhayaya; 2012).

The cardiac glycosides are found in the leaves of the plants exert positive effects on the heart during cardiac failures by increasing the capacity of the heart muscles to pump blood (Aldred, 2009). Due to these bioactive compounds, the plant has been used over the years has an essential herb in treating heart failures and cardiac rhythm disorders (Aldred, 2009). Coumarins another important phytochemical present in the leaves of the *P. amarus* has demonstrated anticoagulant, antifungal and antibacterial activities (Gupta and Vaghela, 2019). Tannins have astringent properties and is used in the treatment of stomach ulcers and diarrhoea. They form a protective layer over wounds and prevent infections (Ashok and Upadhyaya, 2012).

The presence of alkaloids and terpenoids in the plant has been used in treatment of malaria, hypertension, and cancer (Achi et al. 2017). Flavonoids are associated with the prevention of diseases that are associated with oxidative stress (Huang et al. 2010). Saponins are natural antibiotics used to fight infections and microbial invasions. They also have hypocholesterolemia properties, which assist in some chemo protection against heart diseases (Okwu and Emenike, 2006). Table 2.1 showed some reported phytochemicals constituents in *P. amarus*

Table 2.1: Phytochemicals in *Pyllantusamarus* and their bioactive constituents

S/NO	PHYTOCHEMICALS	BIOACTIVE CONSTITUENTS	REFERENCES
1.	TANNINS	Geraniin, amariin, amarulone	(Guha et al.,2010).
2.	LIGNANS	Phyllanthin, hypophyllanthin, phyltetralin, niranthin	(Harikumar et al. 2009).
3.	ALKALOIDS	Securinine,nor-securinine, dihydrosecurinine.	(Etta 2008).
4.	TRITERPENES	Phenazine, and phenazine derivatives	(Achi et al.2017).
5.	VIOLATILE OILS	Linalool	(Luo et al.2016).

2.3 NUTRITIONAL COMPOSITION OF *Phyllantusamarus*

P. amarus plant has shown to possess nutritional components in its leaves such as: crude fats, protein, carbohydrate, fiber and anti- nutrient components such as oxalate and phytate, they were determined. It also shown to possess moisture and ash which was also determined alongside the nutritional components using the standard methods of the Association of Official Analytical Chemists (AOAC, 2000). According to the results gotten after using the AOAC method, different percentage of nutritional composition present in the *P. amarus*leaves indicate that the plant has high level of fibre (36.3%), with moderate levels of fat (19.01%), carbohydrate (13.15%), protein (1.13%), ash (13.5%), and moisture content (16.91%). The anti-nutritional components were found to be relatively low: oxalate (0.95%), and phylate (0.21%) (AOAC, 2000).

Moisture content is a major determinant in safe guarding and sustenance of plant. The presence of high moisture value in the *P. amarus* shows that it will promote the activities of spoilage microorganism and result to reduction of shelf life of the plant (Umofiaet al.*2017*). The presence of ash in that contents reveal that the *P. amarus* is rich in ash, making it a good source of plant minerals by man for normal metabolic activity of body tissues as well as proper assimilation of vitamins (Umohet al.*2013).* It is also rich in fiber, which helps to prevents constipation, supports the proper functioning of the digestive tracts and avert colon cancer, it helps to lowers cholesterol levels and maintains blood sugar (Dhingraet al.*2012*).

2.4 PHARMACOLOGICAL ACTIVITIES OF *P. amarus*

ANTIDIABETIC ACTIVITY

Diabetes is a metabolic disorder of carbohydrate, lipid and protein. (Seriramaet al.*2010*). In vitro, methanol extract of *P. amarus* inhibited lipid peroxidation and scavenged hydroxyl and superoxide radicals. The extract was found to reduce the blood sugar in alloxan- induced diabetic rats(Sreejayanet al. *2010*).

WOUND HEALING ACTIVITY

The anti-oxidant activity contributes to wound healing activity (Ali et al.2006). The extracts were reported to enhance the wound healing process, by reducing the size of the scar formed and enhances the recovery of periphery nerves after injury. The plant was also reported to inhibits microbial and fungal growth at the site of injury. It also boosts angiogenesis resulting in the formation of fibroblasts and leads to improved wound healing time (Servasta et al.2008).

ANTIFERTILITY ACTIVITY

P. amarus possess antifertility activity. After an experimental study carried out by some scientists, it was reported that alcoholic extracts of *P. amarus* brought changes in 3- beta cells and 17- beta hydroxyl steroid dehydrogenase (HSDS) levels, thereby, effecting hormonal conversions in the female mice used for the experiments (Aliet al. *2*006).

ANTIVIRAL ACTIVITY

P. amarus possess antiviral and anticancerous properties. Evaluation of the antiviral activity of the plant were evident from an experimental study where aqueous extract of *P.amarus* were evaluated against Herpes Simplex Virus type 1 and type 2 in Vera cells by quantitative polymerase chain reaction (Umohet al.2013).

IMMUNODATORY ACTIVITY

P. amarus is found to have a very strong oxidative burst of polymorph nuclear leukocytes with luminal- based chemiluminescence (Panakpapornet al.2012).

ANTI ATHEROSCLEROTIC ACTIVITY

P. amarus has been found to act as hypotensive agents. The extract of the plant is known to lower the cholesterol level in the body and also enhances lipid metabolism. The effect of the plant extract in lowering blood pressure was studied with the use of rabbit (Mannikkothet al.2011).

ANTI-INFLAMMATORY ACTIVITY

It has shown that the ethanol extract of *P. amarus*possess pronounced anti-inflammatory properties. Lignin which is an important phytochemical of *P. amarus* possesses variety of effects acting as anti-inflammatory agents.

2.5 BLOOD COAGULATION /HEMEOSTASIS

Homeostasis is a natural process that preventsblood loss when an injury occurs. It is derived from a Greek phrase that means "blood stopping." The process consists of a series of cellular and biochemical activities that work together to preserve blood in a liquid state within veins and arteries and to limit blood loss after an injury by forming a blood clot (Rodak, 2002).During an injury, blood flows and clots form to limit blood loss, and blood changes from a liquid to a gelatinous condition. Platelets, plasma coagulation cascades, fibrinolytic proteins, blood vessels, and cytokine mediators all work together to maintain hemostasis(Periayah, 2017). Blood clotting, also known as coagulation, plays an important function in the hemostatic system, which is a highly conserved machinery(Henri, 2013). The systems work together when the blood vessel endothelial lining is disrupted by mechanical trauma, physical agents, or chemical trauma to produce clots. The clots stop bleeding and are eventually dissolved through the fibrinolytic process.As a result, during the hemostatic process, there is a delicate balance between clot creation and disintegration.A disruption of this balance may precipitate thrombosis or hemorrhage as a consequence of hyper coagulation or hypo coagulation, respectively (Rodak, 2002).

2.5.1 MECHANISM OF HEMOSTASIS

The mechanism of hemostasis falls under three categories namely:

Primary hemostasis, secondary hemostasis and tertiary hemostasis. Table 2.2 shows the mechanism of each category of hemostasis.

Table 2.2: Types of Hemostasis and corresponding mechanism/pathway

Type of hemostasis	Mechanism
Primary hemostasis	Blood vessel contraction /vasoconstriction
	Platelet plug formation upon platelet adhesion and aggregation
Secondary hemostasis	Activation of the coagulation cascade
	Deposition and stabilization of fibrin
Tertiary hemostasis	Dissolution of fibrin clot
	Dependent on plasminogen activation

(Periayah, 2017).

Primary hemostasis involves the response of the vascular system and platelets to vessel injury. It takes place when there are injuries on small vessels during which the affected vessels contract to seal off the wound and platelets are mobilized, aggregate, and adhere to components of the sub endothelium of the vasculature (Rodak, 2002).

2.5.2 Vasoconstriction

This is the first step in halting blood flow (Kumar et al., 2009). When the blood vessels are injured or damaged, vascular spasm occurs. This will cause a vasoconstriction, which may result in the blood flow being cut off.Within 30 minutes, this reaction develops, and it is limited to the wounded area. Exposed collagen fibers will produce ATP and other inflammatory mediators to

attract macrophages at this point. Furthermore, the ECM increases its thrombogenicity, which promotes platelet adhesion and aggregation (Periayah, 2017).

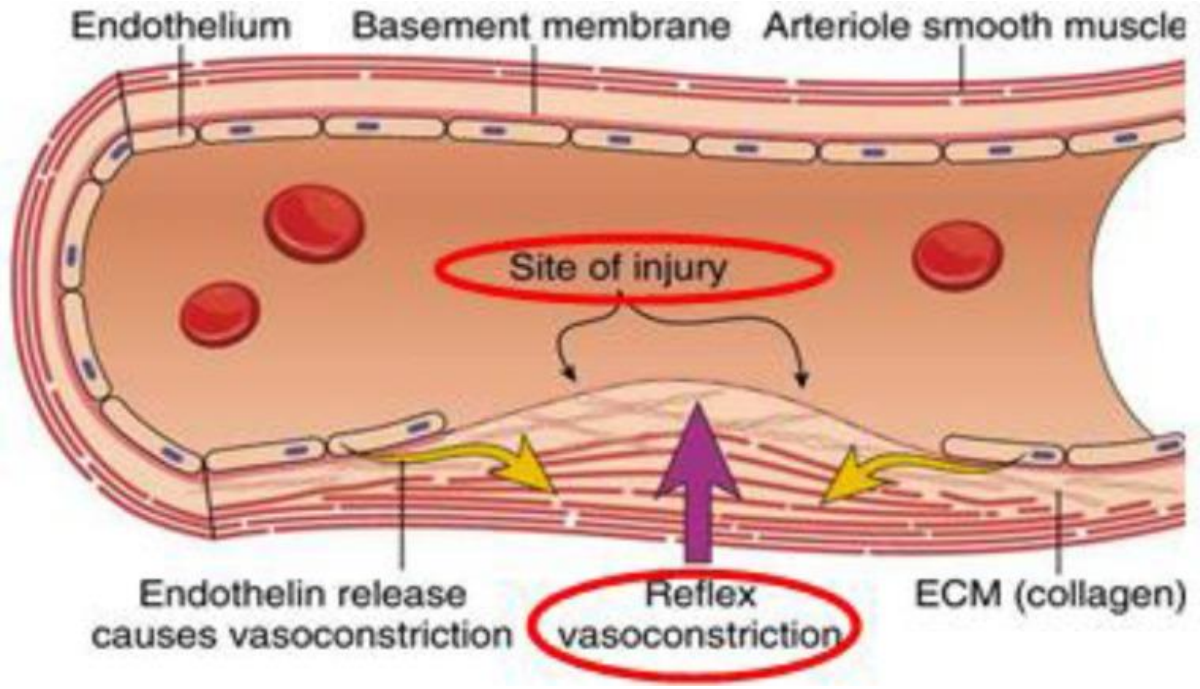

Figure 2.2: Vasoconstriction phase (kumar et al. 2009)

2.5.3 Platelet Plug Formation

Platelets clump together at the site of vascular injury to form a plug, which is the first step in the creation of a blood clot. Coagulation begins within twenty seconds of a damage to the blood vessel's epithelial wall being disrupted. After a few minutes, fibrin strands grow among the wound, and the platelet plug is totally created by fibrin. Following vasoconstriction,to produce a platelet plug, platelets will connect, activate, and aggregate (the three phases in platelet creation), enclosingthe injured area. (Periayah, 2017) Endothelial cell injuries expose platelets to thrombogenic, sub endothelial ECM, which facilitates platelet adhesion and activation.

platelet activation triggers platelet shape changes by releasing secretory granules. Primary hemostasis is formed when released secretary granules recruit more platelets to create a platelet plug.(Kumar et al. 2009).

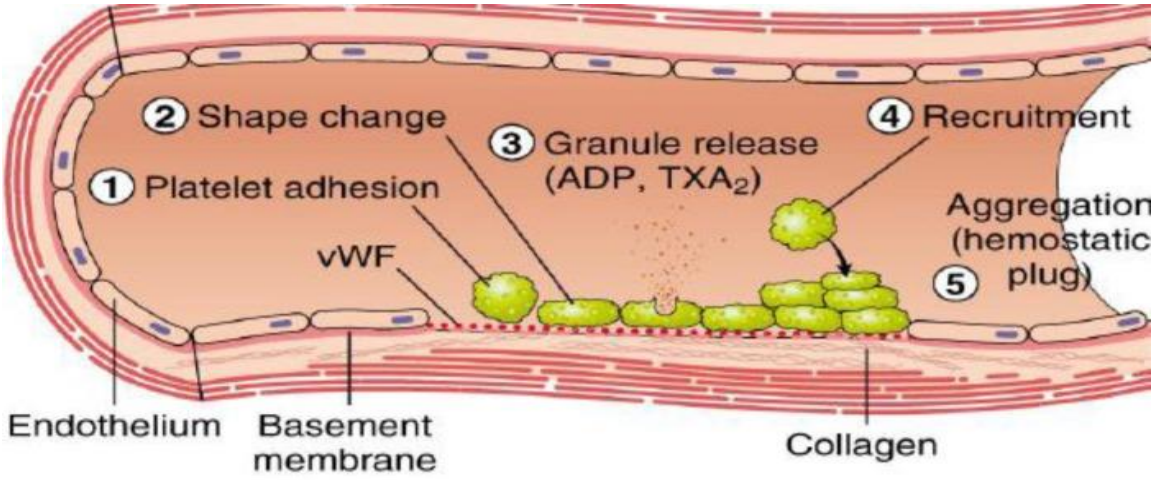

Figure 2.3: Platelet Plug Formation (Kumar et al. 2009)

The platelet plug formation is divided into three phases which are: Platelet adhesion, Platelet activation and Platelet aggregation.

Platelet Adhesion

While platelets flow through the blood arteries, endothelial cells normally express chemicals that prevent platelet adhesion and activation. Nitric oxide, prostacyclin (PGI_2), and endothelial ATPase are among these compounds.

As normal epithelial cells, sub endothelial collagen from the extracellular matrix beneath the endothelium cells is exposed on the epithelium during an injury are harmed and eliminated, resulting in the release of the von Willebrand Factor (VWF). VWF causes platelets to change shape and develop adhesive filaments (extensions) that bind to the endothelium wall's sub endothelial collagen (Lumen, 2014).

Platelet Activation

Platelets can be activated by a variety of triggers. Biomaterial surface stimulation can also activate platelet cells. Degranulation occurs when adhered platelets release cytoplasmic granules containing serotonin, platelet activation factors, and ADP. ADP is a physiological agonist that platelets store in their dense bodiesand is necessary for optimal hemostasis and thrombosis. (Gupta, 2014).

Platelet Aggregation

Aggregation of platelets into a barrier-like plug is the final step in platelet plug creation (Lumen, 2014). Platelet aggregation begins when platelets become activated, prompting the GpIIbIIIa receptor (50-100/platelet), which attaches to vWF or Fib and causes platelets to clump together. Each active platelet grows pseudopods, which clump together and agglomerate. The activations are amplified even more by the production of primary platelets plug. (Kumar et al. 2009; Offermanns , 2006).

2.5.4COAGULATION CASCADE

The coagulation cascade, which forms a fibrin mesh to strengthen the platelet plug, is referred to as secondary hemostasis. Secondary hemostasis can happen at the same time as primary hemostasis, but it usually happens after it. Coagulation factors circulate as inactive enzyme precursors that, once activated, participate in the coagulation cascade's chain of events. Serine proteases are the most common coagulation factors (enzymes)(Lumen, 2014).

Approximately fifty chemicals have a substantial impact on blood coagulation processes. The blood coagulation cascade of secondary hemostasis mainly consists of two primary pathways:

(i) intrinsic (contact activation pathway)
(ii) extrinsic (TF pathway).

The following are three crucial stable phases in the blood clotting process::

(i) The involvement of a complicated cascade, which triggers chemical events mediated by coagulation factors to generate fibrin strands for the consolidation of platelet plugs;

(ii) the conversion of prothrombin (PT) into thrombin, which is catalyzed by the PT activator; and

(iii) Conversion of Fib into fibrin, which eventually enmeshes the plasma, platelets and blood cells to build a firmer clot (Lefkowitz , 2006; Guyton and Hall, 2011).

The three pathways that makeup the classical blood coagulation pathway

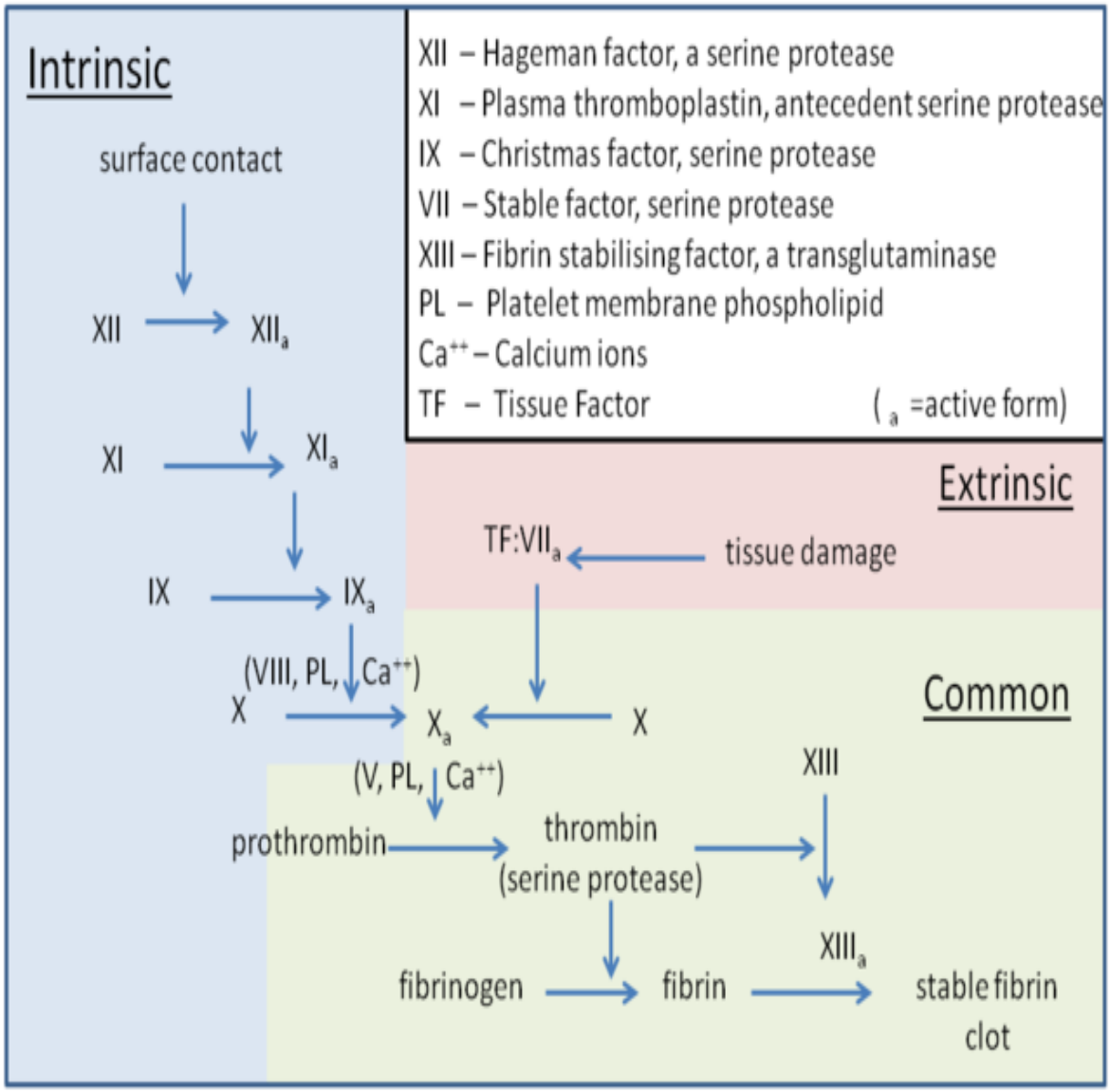

Figure 2.4: Coagulation Cascade Pathway

The third stage of hemostasis is the formation of blood clot. If the platelet plug is insufficient to halt the bleeding, a blood clot will form.Platelets have secretory granules that adhere to the protein in artery walls, degranulate, and release ADP (adenosine diphosphate), serotonin, and thromboxane A2. During this phase, blood begins to gel, and at least 12 chemicals known as blood clotting factors participate in a series of chemical events that result in the formation of a fibrin mesh inside the blood. Each clotting factor has a distinct purpose. The primary factors involved in the coagulation cascade are prothrombin, thrombin, and fibrinogen. The liver produces prothrombin and fibrinogen, which are proteins that are deposited in the blood.

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MECHANISM

When blood arteries are injured, the vessels and adjacent platelets release a chemical called prothrombin activator, which activates the conversion of prothrombin, a plasma protein, into the enzyme thrombin. Calcium ions are required for this process. Thrombin aids in the conversion of fibrinogen, a soluble plasma protein, into lengthy, insoluble fibrin fibers or threads. Fibrin threads form an interlocking network of fibers and clot framework around the platelet plug at the injured portion of the blood artery.The initial clot is formed by a net of fibers that captures and helps to hold platelets, blood cells, and other molecules tight to the site of injury. Before platelets connect, this temporary fibrin inhibits blood flow. The platelets in the clot then begin to shrink, tightening the clot and bringing the vessel walls together to begin the wound healing process.The whole process of blood clotting takes less than an hour.

2.5.5 BLOOD COAGULATION FACTORS

Table 2.3: Shows Blood Coagulation factors (researchgate.net)

Factor	Name	Source	Pathway	Description	Function
I	Fib	Liver	Common	Plasma glycoprotein; Molecular Weight (MW)= 340 kilodaltons (kDa)	Adhesive protein which aids in fibrin clot formation.
II	Prothrombin	Liver	Common	Vitamin K-dependent serine protease; MW= 72 kDa	Presence in the activated form and the main enzyme of coagulation
III	Tissue factor	Secrete by the damaged cells and platelets	Extrinsic and Intrinsic	Known as thromboplastin; MW= 37 kDa	Lipoprotein initiator of the extrinsic pathway
IV	Calcium ions	Bone and gut	Entire process	Required for coagulation factors to bind to phospholipid (formerly known as factor IV)	Metal cation which is important in coagulation mechanisms
V	Proaccererin / Labile factor	Liver and platelets	Intrinsic and extrinsic	MW = 330 kDa	Cofactor for the activation of prothrombin to thrombin (prothrombinase complex)
VII	Proconvertin (stable factor)	Liver	Extrinsic	MW = 50 kDa; vitamin K-dependent serine protease	With tissue factor, initiates extrinsic pathway (Factor IX and X)
VIII	Antihemophilic factor A (cofactor)	Platelets and endothelium	Intrinsic	MW = 330 kDa	Cofactor for intrinsic activation of factor X (which it forms tenase complex)
IX	Christmas factor / Antihemophilic factor B (plasma thromboplastin component)	Liver	Intrinsic	MW = 50 kDa; vitamin K-dependent serine protease	Activated form is enzyme for intrinsic activation of factor X (forms tenase complex with factorVIII)
X	Stuart-Prower factor (enzyme)	Liver	Intrinsic and extrinsic	MW = 58.9 kDa; vitamin K-dependent serine protease	Activated form is the enzyme for final the common pathway activation of prothrombin (forms prothrombinase complex with factor V)
XI	Plasma thromboplastin antecedent	Liver	Intrinsic	MW = 160 kDa; serine protease	Activates intrinsic activator of factor IX
XII	Hageman factor	Liver	Intrinsic; (activates plasmin)	MW = 80 kDa; serine protease	Initiates activated partial thromboplastin time (aPTT) based intrinsic pathway; Activates factor XI, VII and prekallikrein
XIII	Fibrin stabilizing factor	Liver	Retards fibrinolysis	MW = 320 kDa; Crosslinks fibrin	Transamidase which cross-links fibrin clot

2.5.6 BLOOD COAGULATION DISORDERS

Bleeding disorders are a group of disorders that share the inability to form a proper blood clot**.** Blood is transformed from a liquid to a solid during the clotting process, also known as coagulation.The body normally begins clotting process on an account of injury or broken blood vessel in other to prevent excess loss of blood in a process called hemostasis. Some certain conditions inhibit this normal physiological process from occurring and this can result in prolonged or heavy bleeding (April, 2018).

Causes of bleeding disorder

Defects in blood components such as platelets and/or clotting proteins, also known as clotting factors, and blood cell fragments known as platelets can cause improper clotting.The body synthesizes 13 clotting factors.Blood clotting is impaired if any of them are defective or inadequate, which can result in a mild, moderate, or severe bleeding condition (April, 2018). Platelets clump together to form a plug at the site of a damaged or injured blood artery in normal circumstances. After that, the clotting components combine to form a fibrin clot. This holds platelets in place and inhibits blood from leaking out of the blood artery (April, 2018). Clotting factors or platelets, on the other hand, do not perform properly or are in insufficient quantity in persons with bleeding problems. Excessive or persistent bleeding can occur when the blood fails to clot.It can also cause spontaneous or unexpected bleeding in muscles, joints, or other bodily components (April, 2018).

Types of Bleeding Disorders

Inherited or acquired bleeding problems are also possible. Following an accident or injury, some bleeding diseases can cause significant bleeding. Heavy bleeding can occur quickly and without warning in different illnesses (April, 2018).It can also lead to spontaneous or sudden bleeding in the muscles, joints, or other parts of the body (April, 2018).

Most of bleeding disorders are inherited; the disorders are passed from a parent to child. However, some disorders may develop as a result of other medical conditions, such as liver disease. The drugs that have the ability to interfere with the clotting of blood are classified as anticoagulants (April., 2018).

These bleeding disorder includes:

Hemophilia is a rare, genetic bleeding illness that can range in severity based on the amount of clotting factor in the blood. Hemophilia is divided into two types: type A and type B, depending on which type of clotting factor is missing (factor VIII in type A and factor IX in type B). Hemophilia is caused by a genetic flaw on the X chromosome. Hemophilia A and B are blood clotting disorders caused by low levels of clotting factors in the blood. It produces excessive or unusual joint bleeding. Hemophilia is an uncommon blood disorder with life-threatening implications.

Factor II, V, VII, X, or XII deficiencies are bleeding disorders related to blood clotting problems or abnormal bleeding problems.

The most prevalent inherited bleeding problem is Von Willebrand's disease. It occurs when the blood lacks von Willebrand factor, which aids in clotting and also transports factor VIII, another clotting protein. It is usually less severe than hemophilia and affects both men and women (April, 2018).Based on the levels of von Willebrand factor and factor VIII activity in the blood, Von Willebrand disease is divided into three categories (Types 1, 2, and 3). Type 1 is the mildest and most common kind, whereas Type 3 is the most severe and rarest.

2.6 INFLAMMATION

Inflammation is an early response to injury or infection by the body which is a is a defensive reaction by the body’s system of self-defense (Shiel, 2020). It is also a support system to tissues which stimulate the immune system to repair injured tissues and defense against disease from foreign organism (Siraj, 2020). It is the immune response to injury or illness or harmful stimuli such as bacteria, infected cells, radioactive chemicals or irradiation, which works by eliminating harmful stimuli and starting the healing process (Medzhitov, 2010).This is a procedure that occurs after hemostasis and occurs during damage. When vasoconstriction comes to an end and vasodilation commences.During injury, tissues are damaged and inflammation occurs as a result of inflammatory mediator release from the immune system cells (such as mast cells or NK cells) that receives cell stress cytokines from damaged endothelial cells or vasoactive amine (serotonin) which are secreted by activated platelets. During inflammation, vasodilation occurs along with

increased vascular permeability and leukocyte chemotaxis, ending the spasm of vasoconstriction and hemostasis as wound healing begins (Siraj, 2020).

Inflammation reaction is triggered by living tissue damage. When something unwelcome is introduced into the body, for example, when one is sick or injured, the immune system signals an inflammatory response thereby releasing hormone which causes tiny blood vessels to expand around the source of the affected area shifting more blood, fluid and proteins towards it to contain and heal the damage (Siraj, 2020). The chemical release increases blood flow to the affected area, this is why the site of the inflammation becomes red, hot and swollen. After this, the offending irritant will be terminated and the affected tissues begin to repair., The cells may regenerate, if possible, scar tissues would be formed (Barhum, 2020).

The inflammatory response is a defense mechanism that arose in higher organisms to protect tissues from infection and injury.When there is presence of inflammation in the body, high levels of substance known as biomarkers will be present such as C-reactive protein. (Punchard et al. 2005) Without inflammation, wounds would fester and infections may become more lethal as a physiological reaction. However, it may become problematic if the inflammatory phase goes on for too long or if the inflammatory response happens in areas where it is not needed *(*Szala, 2018). Inflammation can also cause redness, heat and pain (Gilroy, 2010).

2.6.1 Types of inflammation

1. Acute inflammation: This is the body's immune system's rapid and early reaction to an injurious agent; it is a defensive reaction after an injury or infection. Redness, swelling, a rise in body temperature, and pain are all symptoms of this rapid reaction (Siraj, 2020). It begins quickly and quickly escalates into a serious situation. The symptoms can only be present for a short time (Pahwa et al. 2020).Trauma, infectious substances, or microbial invasion may all induce acute inflammation, and the body responds by releasing cytokines (proteins that stimulate inflammation), which kicks off the healing process (Barhum, 2020). Acute inflammation is a well-coordinated series of events involving a wide range of genetic, cellular, and physiological changes. Symptoms of acute inflammation last a few days. Subacute inflammation lasts 2–6 weeks (Adam, 2020).

2. Chronic inflammation: This is a slow long-term inflammation, which last for a long period of time. The effects of chronic inflammation differ according to the cause of the injury and the body's ability to heal the damage and resolve it. In certain situations, chronic inflammation may cause the body to overreact and attack itself (Pahwa et al .2020),which will eventually damage the cells, tissues and organs that are healthy. So many autoimmune diseases can lead to chronic inflammation thereby the body turn it on by itself (Barhum, 2020). Chronic inflammation is linked to various diseases, such as: diabetes, cardiovascular disease (CVD), arthritis and other joint diseases, allergies, chronic obstructive pulmonary disease (COPD), psoriasis, rheumatoid arthritis (Adam, 2020). The disease's symptoms will vary, but they may include discomfort and exhaustion (Adam, 2020).

Table 2.4: Differences between acute and chronic inflammation

	Acute	**Chronic**
Cause	Harmful pathogens or tissue injury.	Pathogens that the body cannot break down, including some types of viruses, foreign bodies that remain in the system.
Onset	Rapid	Slow
Duration	A few days.	From months to years.
Outcomes	Inflammation improves, or an abscess develops or becomes chronic	Tissue death, thickening, and scarring of connective tissue.

2.6.2 DISEASES MEDIATED BY INFLAMMATION

Several diseases that can result from inflammation are:

1) Fatty liver disease: This is a disease whereby it signals an inflammatory response when an individual is not eating rightly. This disease can lead to cirrhosis, liver failure, liver cancer which can therefore result to death (Kaspers, 2015).
2) Cystitis: This is a disease where the bladder is being inflamed (Kaspers, 2015*).*
3) Asthma: it is an inflammatory disease which causes the breathing difficult thereby making the airways to swell and become narrow. It also causes the airways to produce more mucus and makes them more sensitive to asthma triggers (*Zelman., 2020*).
4) Dermatitis: This is a disease where the skin is inflamed.
5) Cancer: This is an inflammatory disease caused by chronic inflammation.
6) Persistent acute inflammation**:** In some cases, a person may not fully recover from acute inflammation. Sometimes, this can lead to chronic inflammation.

2.6.3 Anti-inflammatory Agent

Infection causes inflammation in both the innate and adaptive immune systems, which is a normal response. Inflammation, on the other hand, can lead to autoimmune or auto inflammatory illnesses, neurological disease, or cancer if left untreated. Aspirin and other nonsteroidal anti-inflammatory drugs (NSAIDs), among others, are among the safe and effective anti-inflammatory medications available. In particular, "biologicals" such as anti-cytokine treatments and small compounds that block kinase activity are part of the new era of anti-inflammatory drugs.Other anti-inflammatories in use or in development include statins, histone deacetylase inhibitors, PPAR agonists, and short RNAs. Examples of anti-inflammatory agent include:

Aspirin: Aspirin inhibits the cyclooxygenase (COX) enzymes COX-1 and COX-2, which produce prostaglandins and thromboxane, inflammatory mediators. It is the most often utilized therapeutic drug because of its ability to inhibit the formation of prostaglandins and thromboxane.

Nonsteroidal anti-inflammatory medicines (NSAIDS), which target COX-2 and thus prostaglandin synthesis, particularly PGE2, come in second to aspirin. Many inflammatory

illnesses are treated using synthetic analogues of natural cortisol (called glucocorticoids). Examples of NSAIDS arediclofenac, ibuprofen, etodolac etc. (David et al., 2013).

Diclofenac is an analgesic, anti-inflammatory, and antipyretic nonsteroidal anti-inflammatory medication (NSAID) that has been demonstrated to be useful in the treatment of a number of acute and chronic pain and inflammatory diseases. Diclofenac, like all NSAIDs, works by inhibiting prostaglandin synthesis by inhibiting both cyclooxygenase-1 (COX-1) and cyclooxygenase-2 (COX-2) with equal efficacy. However, substantial research demonstrates that diclofenac's pharmacologic activity comprises multimodal and, in some cases, new modes of action in addition to COX inhibition (MOA)

GCs are anti-inflammatory drugs that work primarily by altering nuclear DNA activity, a genomic impact that activates or suppresses the expression of target genes. Inflammatory and anti-inflammatory enzymes, receptors, cytokines, chemokines, and adhesion molecules are all produced as part of a complicated and coordinated process(David et al., 2013).

2.7 Interrelationship between blood coagulation and inflammation

Inflammation and hemostasis are closely linked pathophysiologic processes that have a significant impact on one another. Inflammation activates the hemostatic system, which has a significant impact on inflammatory activity in this bidirectional interaction. (Hoylaerts et al.2010).Inflammation shifts the hemostatic activity towards procoagulant state by the ability of proinflammatory mediators to activate coagulation system and to inhibit anticoagulant and fibrinolytic activities. Once the activation of hemostatic system occurs in inflammatory states, amplification of the hemostatic disorder can result in thrombosis and organ damage. In turn, uncontrolled activation of the hemostatic system can also amplify the initial inflammatory response thus causing additional organ injury (Bonart et al.2010).

The hemostatic system works in tandem with the inflammatory cascade to form an inflammation-hemostasis cycle in which each active process stimulates the other and the two systems feed back on each other. Inflammation's purpose is to restore the integrity of tissues that have been threatened or harmed by injury or infectious microorganisms. Hemostasis, on the

other hand, is a physiological defensive system that prevents bleeding caused by vessel wall injury (Arnout et al.2006). The hemostatic system is a complicated network of separate components that is activated by vessel wall. Primary hemostasis is made up of vascular endothelial cells (ECs) and platelets, which results in the creation of platelets clots.

Following this, the plasma coagulation cascade is activated, resulting in secondary hemostasis and the production of a fibrin clot. The hemostatic activation results in a stable platelet-fibrin clot. These processes are tightly controlled by physiologic anticoagulant mechanisms that keep the coagulation system under control, as well as the fibrinolytic system, which is in charge of degrading the platelet-fibrin clot after it has served its purpose (Levi, 2010).

All components of the hemostatic system, including vascular ECs, platelets, the plasma coagulation cascade, physiologic anticoagulant pathways, and fibrinolytic action, are involved in extensive crosstalk between immune and hemostatic systems. Inflammatory mediators, especially proinflammatory cytokines, play a key role in the effects on hemostatic function during an inflammatory response. (Levi et al. *2010*). The present study thus aims at investigating the effects of *P. amarus* ethanol extract and its fractions on blood coagulation parameters in inflammatory mice model.

2.8 Liver marker enzymes

liver function tests are routinely used to screen for liver disease, monitor the development of a known condition, and assess the effects of potentially hepatotoxic medicines.The most common liver function tests include the aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP) (Salmela et al. 2004).

Alanine aminotransferase and aspartate aminotransferase measures the concentration of intracellular hepatic enzymes that have leaked into the circulation and serve as a marker of hepatocyte injury (Samela et al. 2004).

2.8.1 Aspartate aminotransferase

The enzyme aspartate aminotransferase (AST) is mostly present in the liver, but it can also be found in red blood cells, heart cells, and kidneys (Yoon et al. 2016). AST levels are mainly used to diagnose liver diseases, but they can also be used in conjunction with other enzymes to monitor the progression of different liver disorders. When body tissue or an organ, such as the liver or heart, is sick or damaged, AST is released into the bloodstream, causing levels of the enzyme to rise. As a result, the level of AST in the blood is proportional to the degree of tissue injury(Saikrishna et al. 2020).

2.8.2 Alanine aminotransferase

Serum glutamate pyruvate transaminase is a transaminase enzyme (SGPT) also known as ALT. In the alanine cycle, alanine aminotransferase catalyzes the transition of an amino group from alanine to alpha-ketoglutarate, forming pyruvate and glutamate (York, 2017). Pyridoxal phosphate, a coenzyme that acts as an amino carrier, is needed for ALT. It is important for gluconeogenesis and amino acid metabolism. Serum activation is proportional to the amount of infected hepatocytes, with large increases indicating permanent cell loss and necrosis and small increases indicating membrane blubbing and reversible cell damage. (Dzoyem et al. 2014). The ALT enzyme is found in serum and organ tissues, particularly liver.

2.9 Total Protein Concentration

The total serum protein (TP) concentration is the total protein of specific proteins in plasma, excluding those utilized in the production of clots, such as fibrinogen and clotting factors. The difference between serum and plasma protein is roughly 3-5 g/L. (Wanda, 2010). When analyzing protein changes, the animal's hydration condition should be taken into account.

Principle of action

The biuret reaction, in which an alkaline copper (II) solution reacts with peptide linkage to generate a complex that absorbs light at 546nm, is the most widely used method for measuring total protein (Williams, 2012).

CHAPTER THREE

METHODOLOGY

3.1 MATERIALS AND REAGENTS

Volumetric flask, weighing balance, beakers, glass rod, spatula, jute bag, filter paper, rotary evaporator, separating funnel, retort stand, petri dish, aluminum foil, measuring cylinder, centrifuge, funnel, sample bottles, Eppendorf tubes, hand gloves, nose mask, rack stand, ethanol, distilled water, butanol, ethyl acetate, hexane, 0.9% normal saline, phosphate buffer saline(PBS), 3.2% trisodium citrate, sodium hydroxide.

3.1.1 Collection and preparation of plant materials

Phyllantusamarus plant was collected from Mountain Top University surroundings, Ibafo, Ogun state, Nigeria in the month of March 2021 and was identified by Dr. Nodza George Department of Botany, University of Lagos, Nigeria. Voucher specimen was deposited in the Herbarium of the same Department having reference number 8786 on the 23rd June 2021.

The root was separated from the stem and dried in the oven at 40 ℃ to remove moisture content present in them. After the plant was thoroughly dried, they were pulverized by means of a mechanical blender into coarse powder. The powdered *P. amarus* plant was weighed and stored in an air- tight containers and kept in a refrigerator.

3.1.2 Plant Extraction and Concentration

Two hundred grams (200 g) of powdered plant sample was soaked in 70% ethanol and 30% water in the ratio (8:1 w/v) which was shaken intermittently at room temperature for 72 hours. The solution was then filtered using sterile muslin cloth. The filtrate was concentrated in vacuum at 45^{o}C using a rotary evaporator. The concentrate was later spread out into petri dishes in the oven at 40℃ for rapid drying and then the dried residue extracts were stored in a refrigerator at 4℃.

The hydroethanol extract was subjected to solvent-solvent extraction in a separating funnel using butanol, ethyl acetate, and hexane in order of increasing polarity. This yields distinct fractions

that were then placed in the rotary evaporator where the solvents evaporate at a low, regulated temperature of 45℃before being dried in the oven and stored in the refrigerator until needed.

3.2 Experimental animals

A total of 45 Wistar albino mice of both sexes weighing (21-38 g) was used for this study. The animals were obtained from the Animal facility of Mountain Top University's Department of Biological Sciences in Nigeria. Animals were housed in well-ventilated cages in a controlled laboratory environment at room temperature with a 12 hours light/dark cycle and free access to standard food and water.

The mice were divided into nine (9) groups, each with five (5) mice, and given two weeks to acclimatized, provided with food and water *ad libitum*. The Institutional Animal Ethics Committee's ethical requirements for the care and use of laboratory animals (IAEC) were strictly followed during all mouse experiments. After two weeks of acclimatization, the animals were treated for one week according to their grouping as shown in the experimental design below.

3.3 Experimental design

Group I- Normal Control (given distilled water)
Group II- Negative control (mice administered with normal saline)
Group III- Positive control (mice administered with standard drug called diclofenac)
Group IV- administered with 100mg/kg crude ethanol extract of *P. amarus*
Group V- administered with 200mg/kg crude ethanol extract of *P. amarus*
Group VI- administered with 100mg/kg ethyl acetate fraction
Group VII- administered with 200mg/kg ethyl acetate fraction
Group VIII- administered with 100mg/kg aqueous fraction
Group IX - administered with 200mg/kg aqueous fraction

3.4 Experimental Induction of inflammation

Experimental inflammation was induced in the mice with carrageenan. 500mL of 0.9% saline was poured into a beaker and 2.5g of carrageenan powder was added slowly with continuous stirring, the solution was heated to 90℃ and not allowed to boil. The carrageenan was freshly prepared. The mice were pretreated with the standard drugs and plant extracts according to their groups 1 hour prior to carrageenan injection. They were then induced by intradermal injection on the right hind paw and the thickness of the paw was measured in mm using a Vernier caliper at the 1, 2, 3, and 4 h after the carrageenan injection.

Oral administration of plant extract and standard drug was continued once daily for 7 days using a cannula. At the end of administration, the rats were sacrificed under light anesthesia (by placing the animal in chloroform fume chamber for 10 seconds), then blood samples were collected by ocular puncture into Lithium heparin bottles to be used for biochemical assays.

3.5 Blood sample Preparation

Blood samples for the assay of PT and aPTT were collected into Eppendorf tubes containing 3.2% trisodium citrate in ratio (1; 9) and centrifuge at 2500 g for 15 minutes. The plasma was separated and stored in the refrigerator at 4℃ until use. Blood samples for other biochemical assays were collected into lithium heparin bottles and centrifuged at 2500 g for 15 minutes.

3.6 Tissue Preparation

The rats were dissected to remove two major organs which are the liver and kidney. A portion of the liver was rinsed in ice-cold 0.1 M PBS. The tissues were then homogenized using mortar and pestle, and centrifuged at 12000 g for 10 minutes to obtain the supernatant, which was then separated and refrigerated at 4℃ for biochemical assays.

3.7 Determination of Bleeding and clotting times

Materials used: scalpel, glass slides, stop watch, Filter paper.

Principle: A standardizedincision is made on the tail. The time the incision bleeds was measured. Cessation of bleeding indicate the formation of haemostatic plug which depends on the adequate no of platelets and on the ability of the platelets to adhere to the subendothelium.

Clotting Time is the time required for blood to form a clot in vitro**.**The test is based on the fact that when entire blood is exposed to a foreign surface, such as a glass tube, it forms a solid clot. In the absence of tissue factors, it is an approximate estimate of all intrinsic clotting factors.

The bleeding time was determined using Ivy's method (1vy, 1941). It was carried out on the first day of treatment and last day of treatment before sacrifice. The mice's tail was cut with a scalpel 1-2cm proximal from the end. A stop watch was started immediately to observe, till the bleeding stops. Spots were made with the bleeding tail on a filter paper every 10 seconds until the bleeding stops. The time was recorded as the bleeding time of the mice.

The clotting time was determined using the method by Shrivastava (1987) as reported by *Raoof*et al. *2013.* After the tail of the mice was cut with a scalpel, a drop of blood from the mice's tail was placed on a clean glass slide and a stop watch is started at the same time. A pin was passed across the drop of blood once every 10seconds. When a thread of fibrin is noticed, the stopwatch was stopped and the time was recorded as the clotting time for the mice.

3.8 Determination of Prothrombine Time (PT)

Materials used**:** test tubes, test tube racks, water bath, stop watch, micropipette

Principle:In the presence of ca++, tissue thromboplastin initiates the extrinsic pathway of the human blood coagulation cascade. The concentration of individual clotting components involved in the coagulation cascade determines the activation time. This aids in determining the source and scope of the hemorrhagic illness.

Procedure: This assay was carried out using Agappe's kit for PT reagent following manufacturer's instruction. The PT reagent (0.2 mL) was placed in a test tube and placed into a water bath at 37℃and incubated for 1-2 minutes. Then plasma of the mice (0.1 mL) was added into the test tube after incubation then still placed in the water bath, a stop watch was immediately started upon adding the plasma to the reagent. The tube was slightly tilted at regular intervals (returning to the water bath between tilting) until the formation of a clot was observed. The stop watch was stopped immediately a clot was observed and time was recorded.

3.8.1 Determination of Activated partial thromboplastin time

Principle:The Activated Partial Thromboplastin Time (APTT) is based on the idea that adding a platelet substitute, factor XII activator, and CaCl2 to citrated plasma allows for the development of a stable clot. The time it takes for a stable clot to develop is measured in seconds and represents the real APTT result.

Procedure:aPTT was determined using Agappe's kit for aPTT reagent following the manufacturer's instruction. The reagent was first reconstituted with 4mL distilled water according to the manufacturer's instruction, 0.2 mL of the reagent was measured into a test tube using micropipette and place into water bath at 37℃ for 1-2 minutes. After which 0.1 mL of test plasma was added, the tube was gently titled at intervals for exactly two minutes. Then 0.1 mL of 0.02M calcium chloride was added while simultaneously starting a stop watch, the tubes were titled at regular intervals until clot formation was observed. The stopwatch was instantly stopped when a clot was observed and the time was recorded.

3.8.2 Determination of Total Protein in the Plasma and Liver

Principle:Cupric ions, in an alkaline medium, interact with protein peptide bond resulting in the formation of a colored complex.

Procedure: This assay was carried out using Randox kit for Total protein. It was carried out in both plasma and liver. Reagent blank was first prepared by adding 0.02 mL of distilled water into a test tube, then 0.1 mL of reagent 1(Biuret reagent) was added. Simultaneously, standard was also prepared by adding 0.02 mL of the standard reagent and 1.0 mL of reagent1(Biuret reagent) in a test tube. Sample was also prepared by adding 0.02 mL of the plasma and 1.0 mL of reagent1(Biuret reagent). After adding all these, it was mixed together and incubated for 30 minutes at room temperature. Using a spectrophotometer, reagent blank prepared was used to blank, absorbance of standard and each sample was measured at 546 nm, cuvette size: 1 cm light path. This assay was also carried out for liver samples using the same procedures.

3.8.3 Determination of Alanine Aminotransferase

The method described by Reitman and Frankel (1957) was used for assaying the activity of alanine aminotransferase. Alanine aminotransferase activity was determined by monitoring the concentration of pyruvate hydrazine formed with 2,4- dinitrophenyl hydrazine.

ȧ-oxoglutarate + L-alanine ——> L-glutamate + pyruvate

Procedure: This assay was carried out using Randox kit for Alanine Aminotransferase. It was carried out on the liver. Reagent blank was first prepared by adding 0.5mL of buffer (Phosphate buffer, L-aspartate and α-oxoglutarate) and 0.1 mL of distilled water in a test tube. Then sample was prepared by adding 0.1 mL of each liver sample in different test tubes and 0.5 mL of buffer into the same test tube, it was then mixed together and incubated for exactly 30 minutes at 37℃. After this, 0.5 mL of 2,4-dinitrophenylhydrazine was both added to the reagent blank and sample, then it was mixed and allowed to stand for exactly for 20 minutes at 25℃. 1 vial of sodium hydroxide was reconstituted up to 1000 mL with distilled water in a volumetric flask. 5.0 mL of the sodium hydroxide was added to both the reagent blank and sample and mixed. The absorbance of the sample was read at wavelength 546 nm, cuvette size: 1 cm light path.

3.8.4 Determination of Aspartate transferase

Principle:The enzyme catalyzes the reversible process that produces L-glutamate and oxaloacetate from -ketoglutarate and L-aspartate. The concentration of oxaloacetate hydrazone produced with 2,4-dinitrophenyl hydrazine at 546 nm was used to evaluate the activity of aspartate aminotransferase.

Procedure: The concentrations of the liver aspartate transferase of the experimental animals were determined using Randox kit for Aspartate transferase. Reagent blank was first prepared by adding 0.5 mL of buffer (R1) and 0.1 mL of distilled water in a test tube. Then, the test samples were prepared by adding 0.1 mL of each liver sample in different test tubes and 0.5 mL of buffer (R1) into the same test tubes. The contents were mixed together and incubated for exactly 30 minutes at 37℃. After this, 0.5 mL of 2,4-DNP (R2) was added to both the reagent and the sample, it was mixed and allowed to stand for exactly 20 minutes at 20℃ to 25℃. Then 0.4 M of

sodium hydroxide was prepared. And 5.0 mL of the prepared sodium hydroxide was added to both sample and reagent blank and mixed together. The absorbance was read at 546 nm.

3.8.5 Phytochemical characterization of extracts and fractions of *P.amarus* using GC-MS

The GC-MS study was carried out using a Hewlett Packard Gas Chromatograph (Model 6890 series) fitted with a flame ionization detector and a Hewlett Packard 7683 series 250 °C MS transfer line temperature injector. The GC was fitted with an HP-5MS (30 x 0.25 mm) fused silica capillary column, 1.0 μm film thickness. The oven temperature was kept at 50 ° C for 5 min of held time and increased at a rate of 2 ° C / min from 50 to 250 ° C, using helium gas (99.999 percent) as a carrier gas at a steady flow rate of 22 cm / s. A 1.0micron extract (1 mg diluted in 1 ml of absolute alcohol) was injected at a 1:30 split ratio. Agilent Science Network Mass Spectrometer (Model 5973 series) coupled with Hewlett Packard Gas Chromatograph (Model 6890 series) with NIST08 Library software database was studied by MS. Mass spectra were taken at 70 eV/200 °C, 1 scan/s scanning rate. Compound recognition was carried out using the NIST08 Library database. The mass spectrum of each unknown compound was compared with the known compounds contained in the library software database.

3.9 Statistical analysis

The statistical analysis was done using Graph Pad prism 9.0. The results were reported as mean ± SEM (standard error of mean). The data were subjected to one-way analysis of variance (ANOVA) to test for variations of the different parameters observed in the study. Test of significance was at 0.05% probability ($p < 0.5$).

3.10 Waste Disposal

The experiment wastes were incinerated and the experiment animals were buried.

CHAPTER FOUR

RESULTS

4.1 PERCENTAGE YIELD

$$\text{Percentage Yield} = \frac{\text{Weight after extraction}}{\text{Weight before extraction}} \times 100$$

The ethanol extract gave a yield of 62.25%; while the aqueous and ethyl acetate fractions yielded 33.58 % respectively.

4.2 Effects of Oral administration of aqueous, ethyl acetate and ethanol extract of *P. amarus* on the paw edema of carrageenan induced inflammation in the mice

From figure 4.2, the injection of carrageenan, triggered inflammation in the hind limb for the first hour upon administration of standard drug (Diclofenac) and various fractions of *P.amarus*extracts, it was observed that positive control(diclofenac) reduced significantly in % inhibition of paw edema, and for other fractions of *P. amarus* extracts increases the % inhibition, these report is significant in the first three hours. However, for the fourth hour there is a reduction in % inhibition in groups administered with Ethylacetate (200 mg/kg) and Aqueous (200 mg/kg).

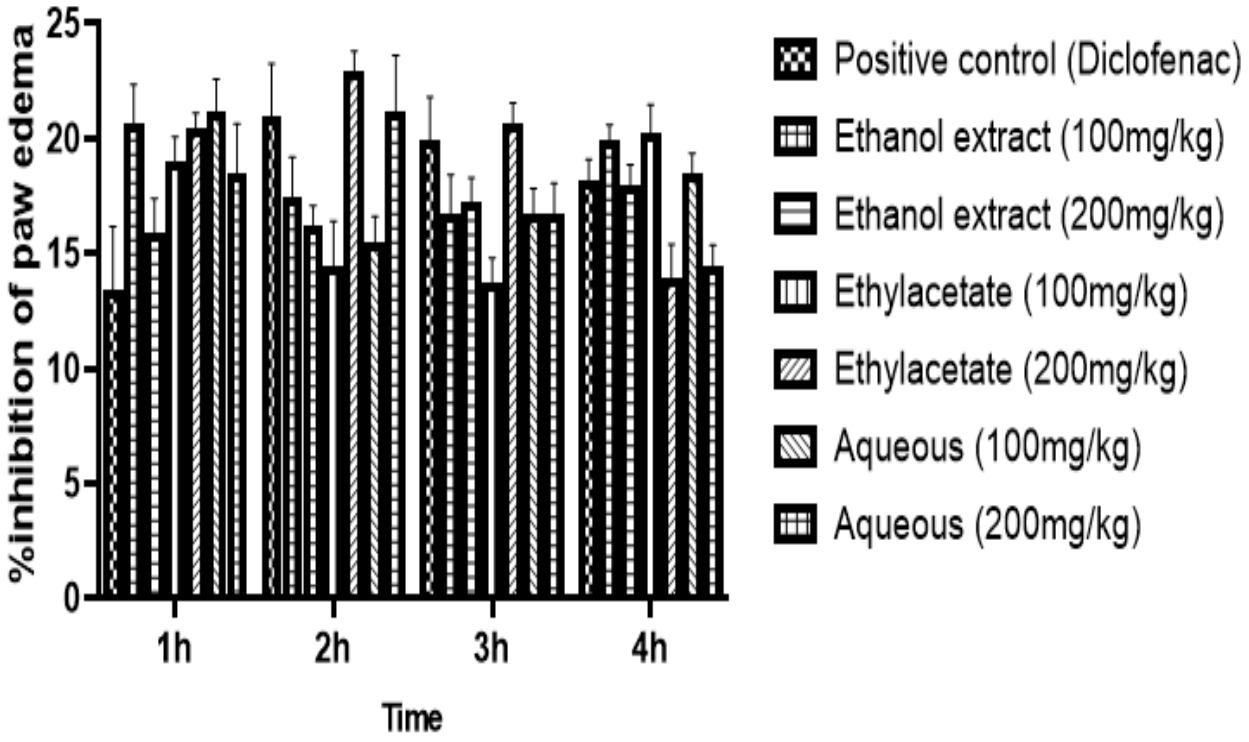

Figure 4.1: Percentage inhibition of paw edema in inflamed mice treated with graded concentrations of ethanol extract and fractions of *P. amarus*

Data are presented as mean ± SD of replicate determinations; n =4

4.3 Effects of Oral administration of aqueous, ethyl acetate and ethanol extract of *P. amarus* on the bleeding and clotting times of the experimental mice

From figure 4.2, the result shows there was a significant($p< 0.05$) decrease of bleeding time upon administration ethanol extract (100 mg/kg) and ethylacetate (100 mg/kg) for the first day of administration when compared to the normal control.While on the eight day, it was observed still showed a decrease in bleeding time upon administration of ethanol fraction (200 mg/kg) when compared to the negative control which have a similar effect in standard drug (Diclofenac) group.

From figure 4.3, the result shows there was a significant ($p< 0.05$) decrease) in clotting time on the first day upon administration aqueous extract (100 mg/kg and 200 mg/kg) when compared to the normal control. On the eight day, there was a significant decrease in the groups treated with Ethyl acetate fraction (200mg/kg) and Aqueous extract (100 mg/kg and 200 mg/kg) when compared to the negative control.

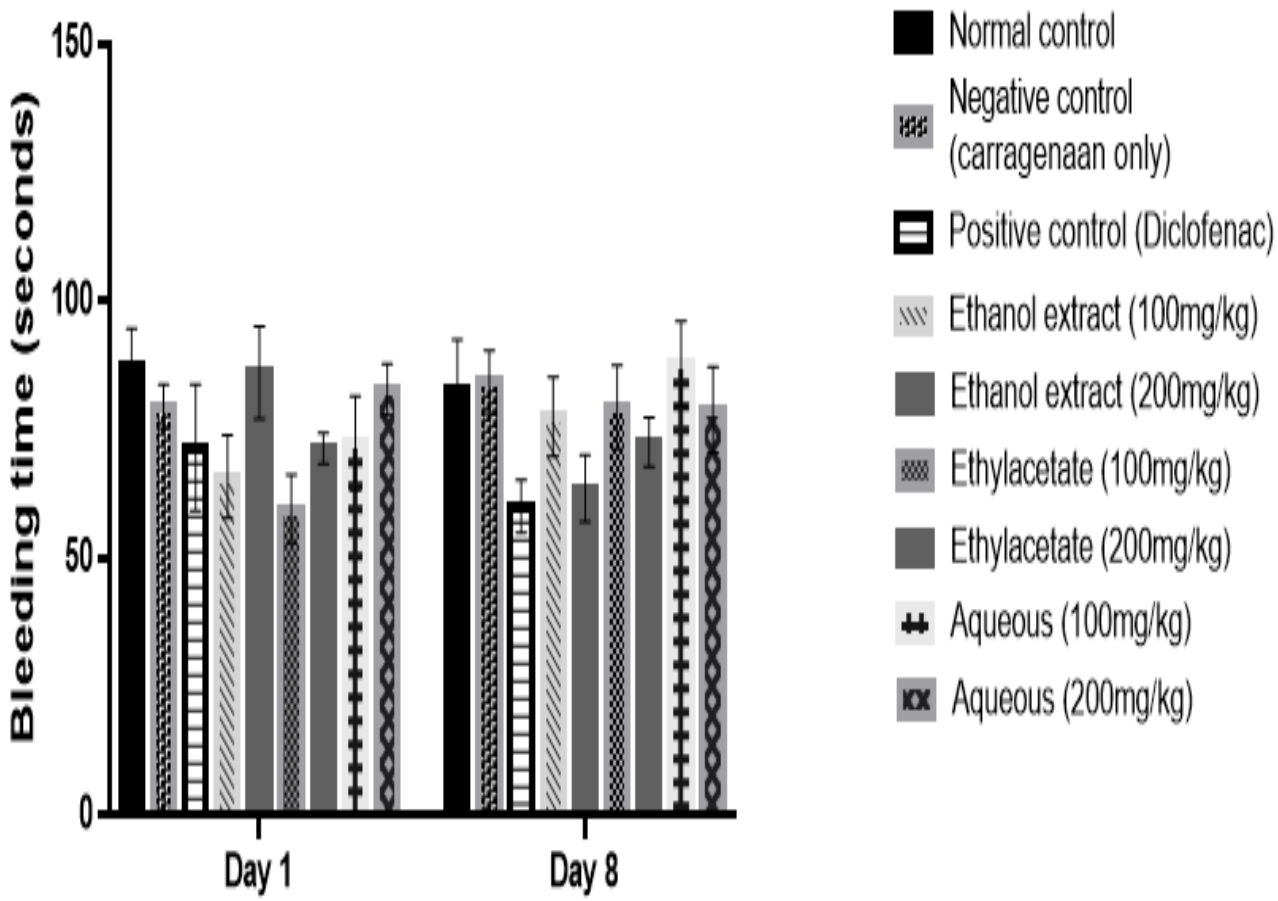

Figure 4.2: Bleeding time of control and experimental mice treated with graded concentrations of ethanol extract and fractions of *P. amarus*

Data are mean ± SD of replicate determinations; n =4

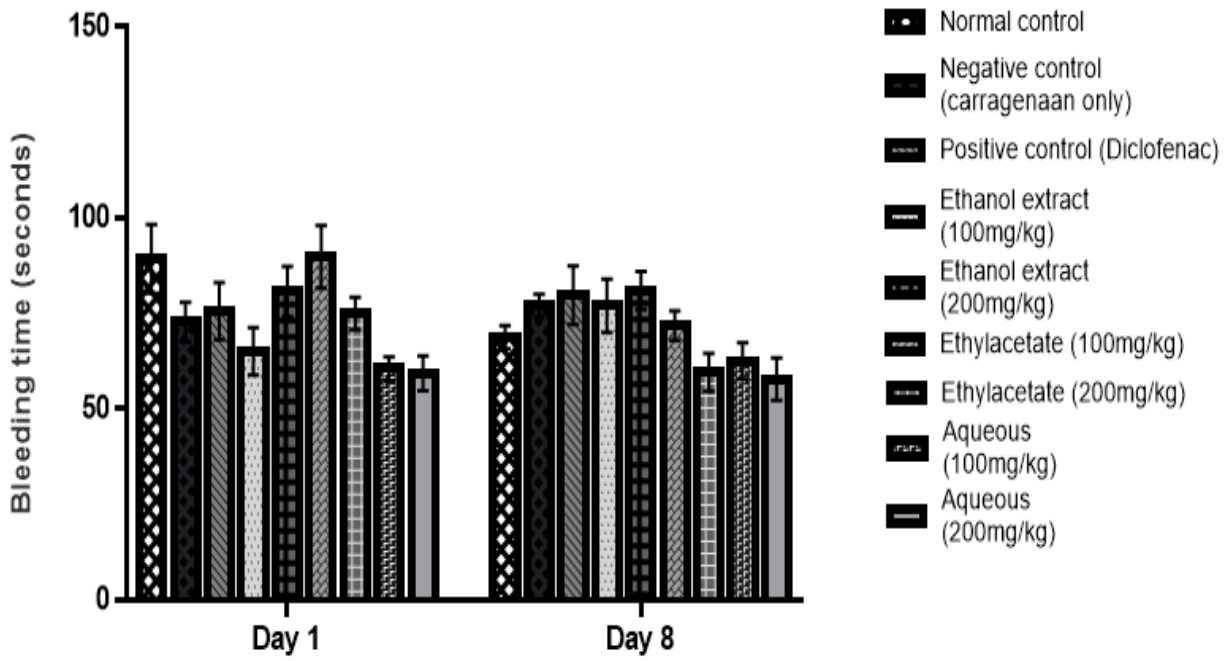

Figure 4.3: Clotting time of control and experimental mice treated with graded concentrations of ethanol extract and fractions of *P. amarus*

Data are mean ± SD of replicate determinations; n =4

4.4 Effects of Oral administration of aqueous, ethyl acetate and ethanol extract of *P. amarus* on the Prothrombin time and Activated partial thromboplastin time of the experimental mice

From figure 4.4, the result showed a significant ($p< 0.05$) increase of prothrombin time upon administration of Aqueous extract (100mg/kg) when compared to normal control mice, whereas there was significant decrease upon administration of all other fractions of *P. amarus*extract which is also similar in the group treated with standard drug(diclofenac) when compared to normal control.

From figure 4.5, this result shows that there is no significant difference across the group treated with plant extracts. However, there was slight increase in Activated partial thromboplastin time in group administered with Ethyl acetate fraction (100 mg/kg and 200 mg/kg).

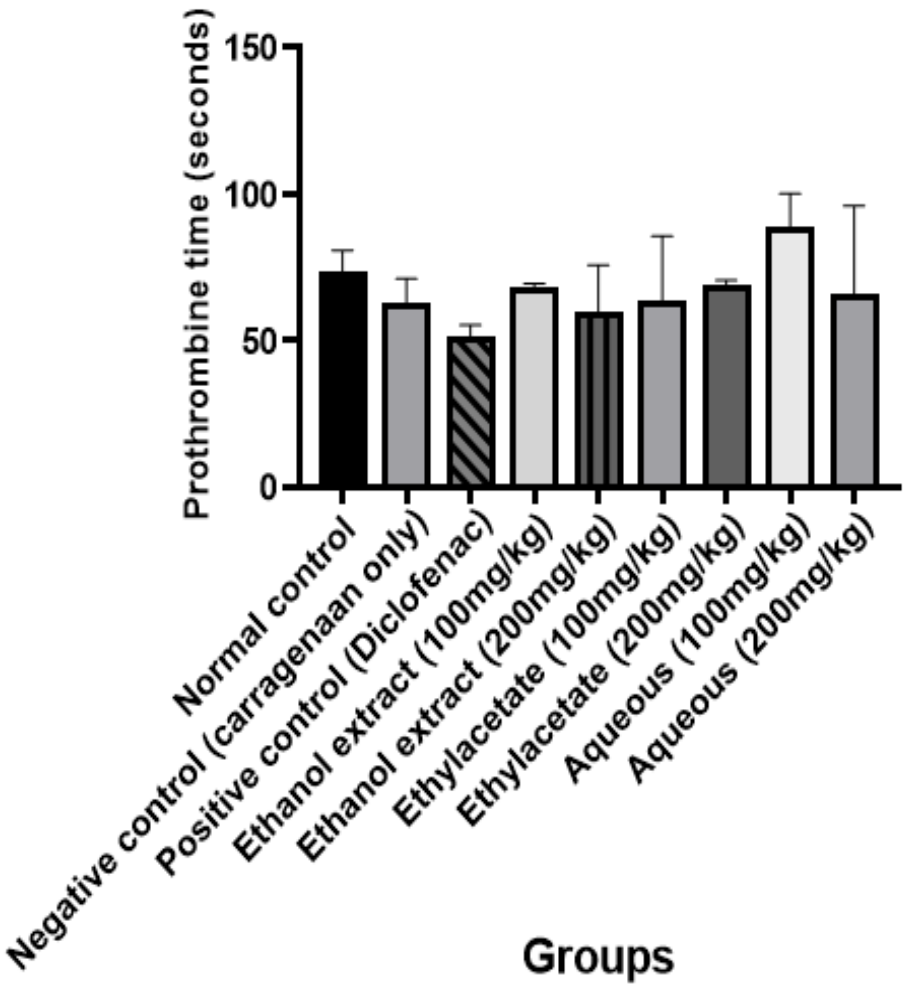

Figure 4.4: Prothrombin time of control and experimental mice treated with graded concentrations of ethanol extract and fractions of *P. amarus*

Data are mean ± SD of replicate determinations; n =4

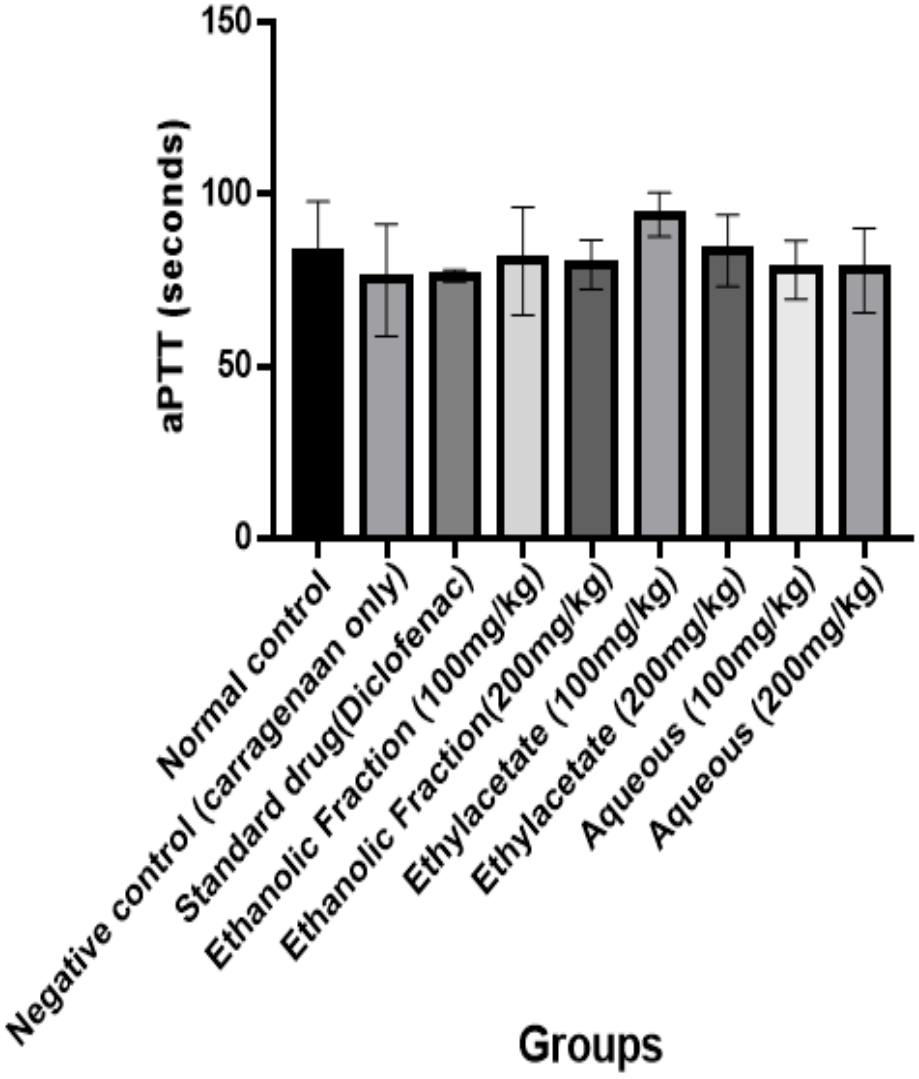

Figure 4.5: Activated partial thromboplastin time of control and experimental mice treated with graded concentrations of ethanol extract and fractions of *P. amarus*

Data are mean ± SD of replicate determinations; n =4

4.5 Effects of Oral administration of aqueous, ethyl acetate and ethanol extract on the plasma and liver total protein concentrations of the experimental mice

Figure 4.6, showed that there was a significant increase *(p*<0.05) in the plasma total protein levels upon administration of various plant extracts when compared with normal control. It was observed that the 100mg/kg ethylacetate fraction of *P. amarus*, increased the total protein concentration most significantly.

From figure 4.7, The result shows that there is no significant difference in Total protein(liver) concentration upon administration of various fractions of plant extract with exception to group treated with ethyl acetate and ethanol extract (200mg/kg) which decrease slightly when compared to the normal control.

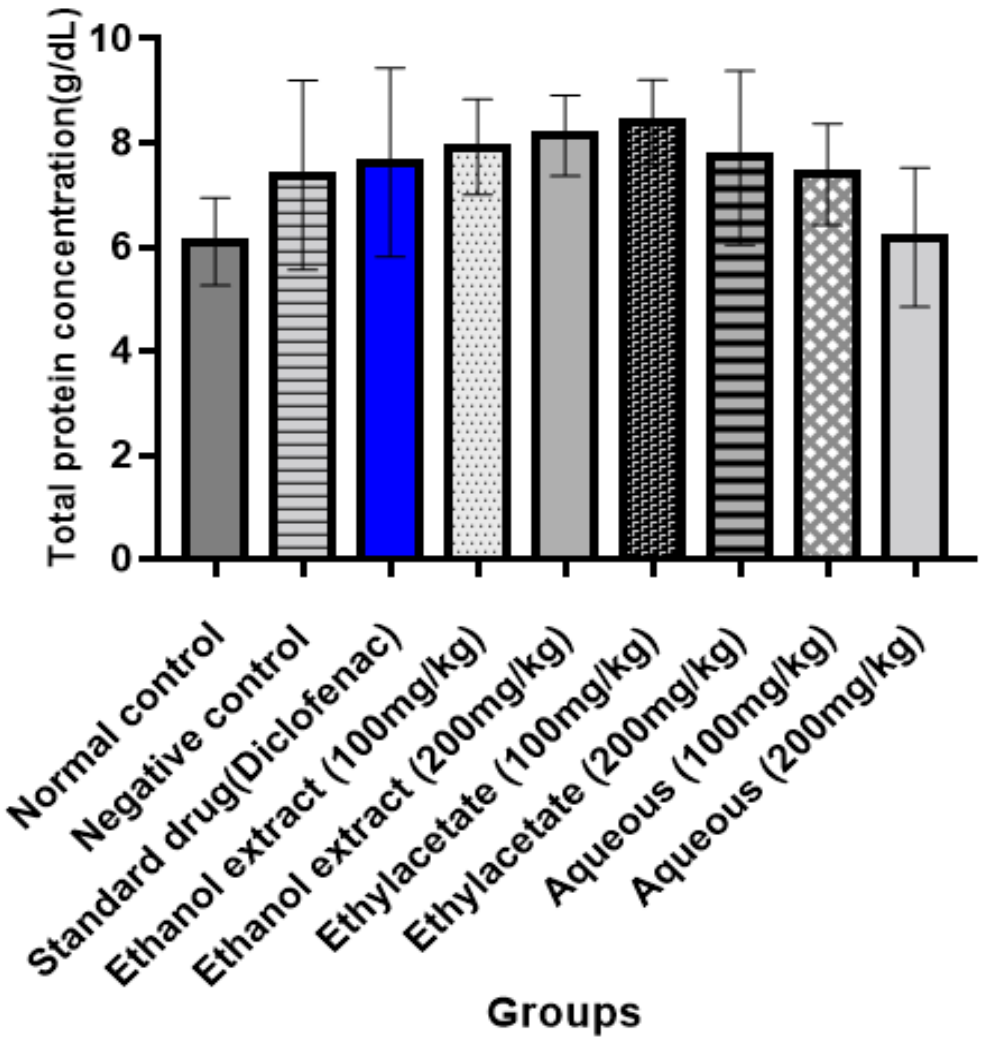

Figure 4.6: Plasma total protein concentrations of control and experimental mice treated with graded concentrations of ethanol extract and fractions of *P. amarus*

Data are mean ± SD of replicate determinations; n =4

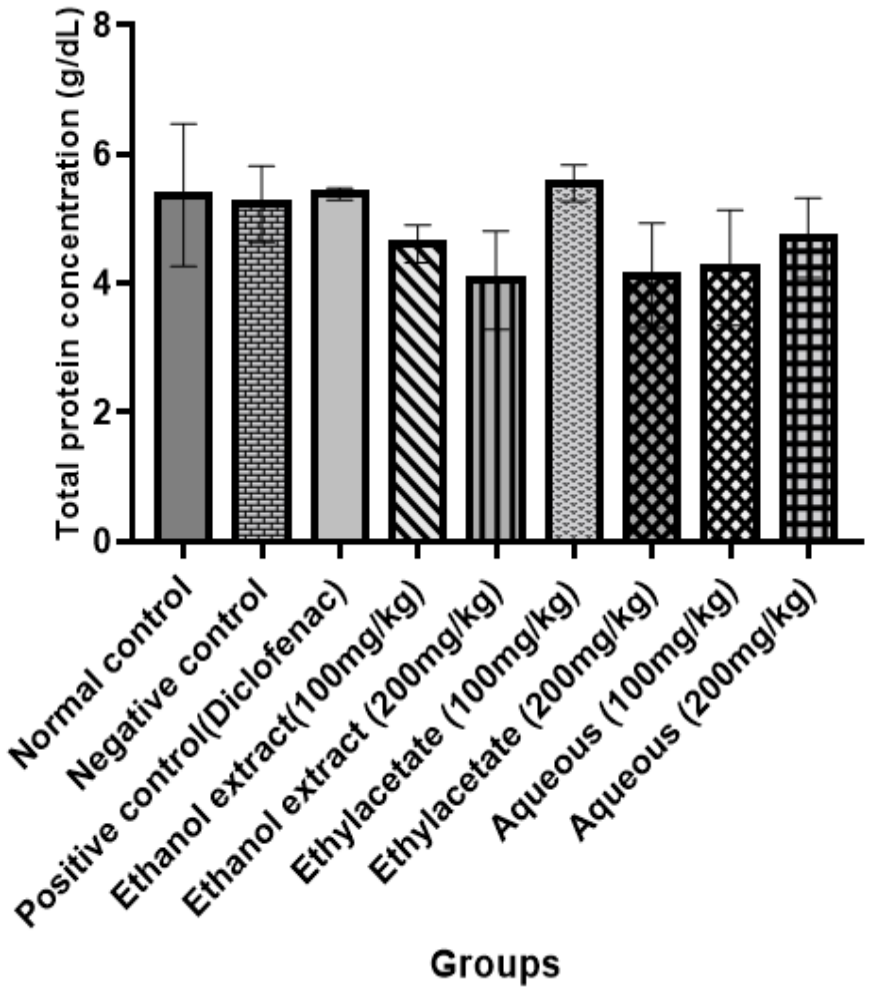

Figure 4.7: Liver total protein concentrations of control and experimental mice treated with graded concentrations of ethanol extract and fractions of *P. amarus*

Data are mean ± SD of replicate determinations; n =4

4.6 Effects of Oral administration of aqueous, ethyl acetate and ethanol extract on the liver Aspartate aminotransferase (AST) concentrations of the experimental mice

From figure 4.8, the result showed that upon administration, comparing result with the normal control there was a significant decrease ($p < 0.05$) in Aspartate transferase concentration in group treated with various fractions of the plant extract when compared to normal control. However, it was observed that 200mg/kg Ethanol fraction increase significantly in Aspartate transferase concentration level.

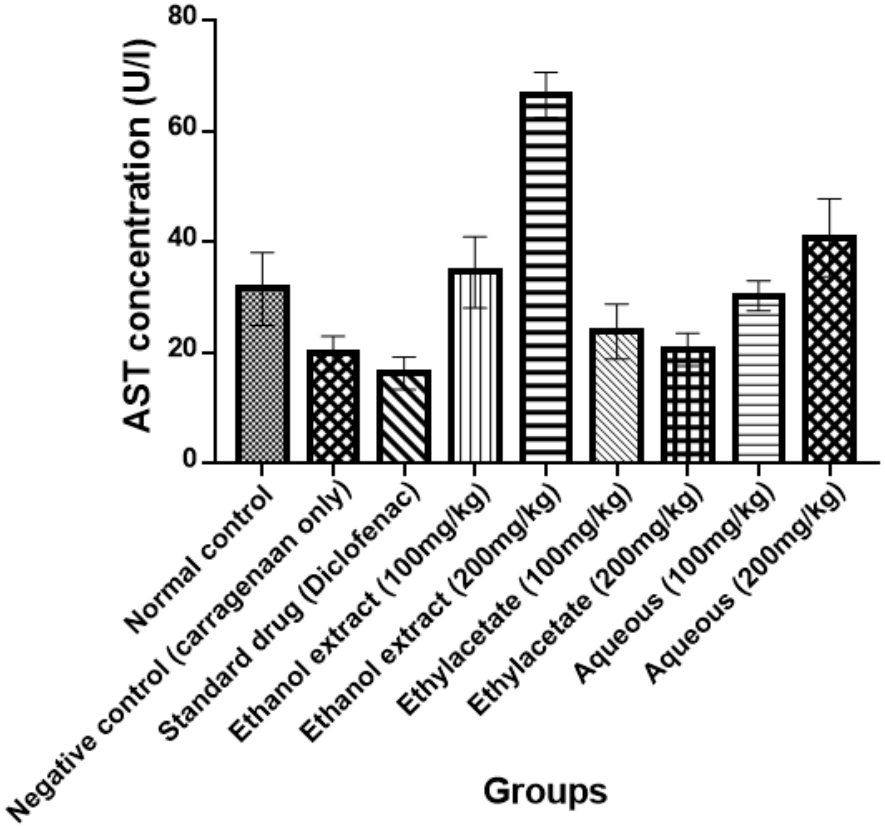

Figure 4.8: Liver AST concentrations of control and experimental mice treated with graded concentrations of ethanol extract and fractions of *P. amarus*

Data are mean ± SD of replicate determinations; n =4

4.7 Effects of Oral administration of aqueous, ethyl acetate and ethanol extract on the liver Alanine aminotransferase (ALT) concentrations of the experimental mice

From figure 4.9, This result shows that there was a significant decrease ($p < 0.05$) in concentration of ALT upon administration of Aqueous extract (100mg/kg) when compared to normal control. whereby no significant difference in other groups, when they are all compared to the normal control.

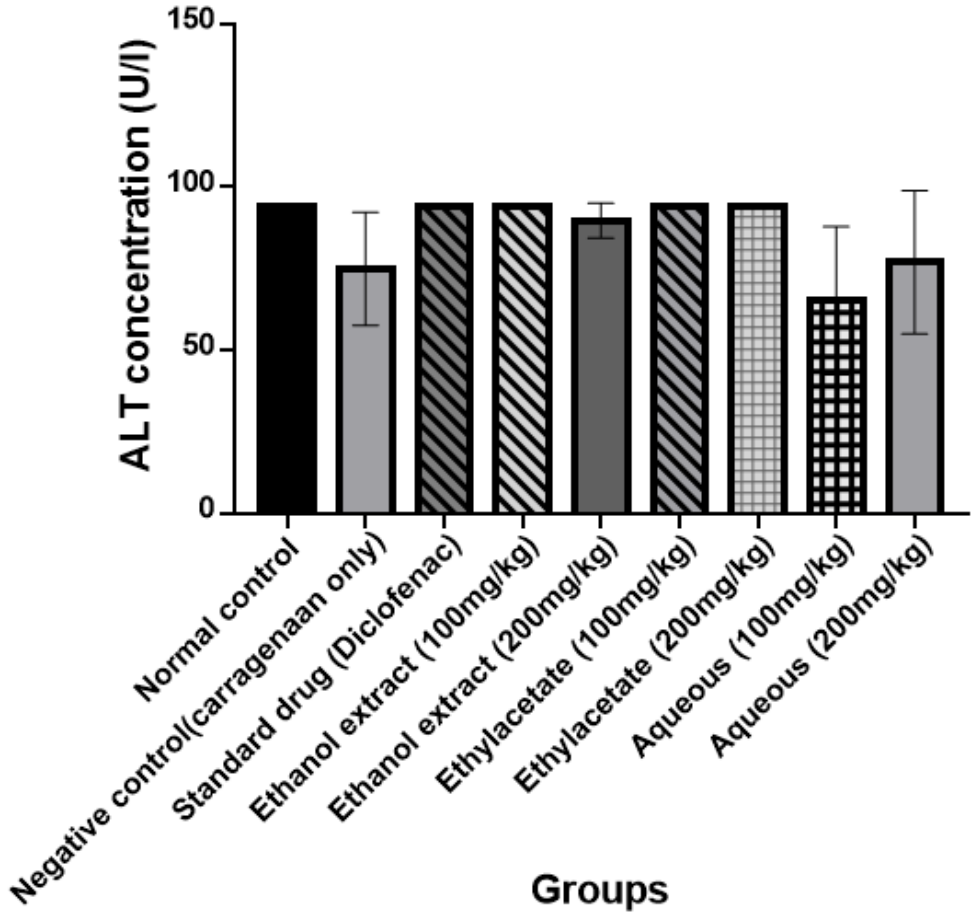

Figure 4.9: Liver ALT concentrations of control and experimental mice treated with graded concentrations of ethanol extract and fractions of *P. amarus*

Data are mean ± SD of replicate determinations; n =4

4.8 Results of Phytochemical Characterization of the Ethanol extract of *P. amarus* by GCMS

Table 4.1: GCMS result of Ethanol extract of P.amarus

Peak	Name of Compound	% Area	Retention Time	Chemical Compound Formula
1	Tetraethyleneglycolmonomethylethe	0.03	2.625	$C_9H_{20}O_5$
2	Allyl fluoride	1.01	3.257	C_3H_5F
3	Trimethylsilylmethanol	1.77	3.469	$C_4H_{12}OSi$
4	Aziridine, 2-methyl-	2.45	3.613	$C_4H_9NO_2S$
5	Formamide, N-methylthio	1.93	3.851	C_2H_5NS
6	Cyclopropane, 1-methyl-2-(3-methyl	5.00	4.502	C_9H_{18}
7	3,6,9-Trioxa-2-silaundecane	0.18	5.315	$C_9H_{22}O_3Si$
8	1-Butanol, 3-methyl-, acetate	0.07	5.509	$C_7H_{14}O_2$
9	Urethane	0.35	5.746	$C_3H_7NO_2$
10	Propanenitrile, 2-hydroxy-	1.77	6.028	C_3H_5NO
11	Pentane, 1-(1-ethoxyethoxy)-	0.15	6.753	$C_9H_{20}O_2$
12	Hexanoic acid, ethyl ester	0.06	7.241	$C_8H_{16}O_2$
13	1-Methyl-1-silabenzocyclobutene	0.13	7.879	C_8H_9Si
14	Hexanoic acid	0.13	8.355	$C_6H_{12}O_2$
15	Thiocyanic acid, 2,4,6-trinitrophe	0.02	8.580	$C_7H_3N_3O_4S$
16	Cyclopentasiloxane, decamethyl-	0.02	8.743	$C_{10}H_{30}O_5Si_5$
17	Nonanal	0.03	8.905	$C_9H_{18}O$
18	Diglycerol	1.15	9.656	$C_6H_{14}O_5$
19	Quinoline, 2 nitro-	0.11	10.37	$C_9H_6N_2O_2$
20	Undecane 3,9-dimethyl-	0.13	10.54	$C_{13}H_{28}$
21	Methenamine	0.16	10.73	$C_6H_{12}N_4$
22	Succinic acid, heptyl tetrahydrofurfuryl ester	0.03	10.94	$C_{17}H_{30}O_5$
23	2-Hexen-4-yn-1-ol, (E)	0.03	11.06	C_6H_8O
24	Cyclohexene, 1-chloro-	0.09	11.34	$C_8H_{10}C_{l2}$
25	Nonane, 4,5-dimethyl-	0.10	11.61	$C_{11}H_{24}$
26	5,9-Docosadienoic acid, DMOX derivative	0.08	11.71	$C_{26}H_{47}NO$
27	Caryophyllene	0.26	12.06	$C_{15}H_{24}$
28	Hexadecane	0.14	12.24	$C_{16}H_{34}$
29	Precocene I	0.50	12.52	$C_{12}H_{14}O_2$
30	6-Octadecenoic acid	2.45	13.65	$C_{18}H_{34}O_2$
31	Methyl tetradecanoate	0.97	13.87	$\mathbf{C_{15}H_{30}O_2}$
32	1H-Indene, 2-butyl-3-hexyl-	0.57	14.01	$C_{19}H_{28}$
33	Methyl 18-methylicosanoate	0.72	14.24	$C_{22}H_{44}O_2$
34	Pentadecanoic acid, methyl ester	1.63	14.46	$C_{17}H_{34}O_2$

35	Hexadecanoic acid, methyl ester	3.38	15.10	**$C_{17}H_{34}O_2$**
36	6-Octadecenoic acid	22.80	16.21	$C_{18}H_{34}O_2$
37	10,13-Octadecadienoic acid, methyl ester	14.80	16.48	$C_{19}H_{34}O_2$
38	n-Hexadecanoic acid	34.80	17.41	**$C_{16}H_{32}O_2$**

4.9 Discussion of results

Inflammation is the body's early response to damage or infection, and it is a protective response by the body's self-defense mechanism (Shiel, 2020). *P. amarus* is a promising herbal resource with therapeutic potential against a variety of ailments. The effect of *P.amarus* plant extracts and fractions on blood coagulation parameters and liver function tests was investigated in this study.

The paw oedema of mice inflamed with carrageenan on the right hind limb was greatly reduced by *P.amarus* plant extracts, particularly the ethanol extracts, as observed in this study. It was discovered that within the first four hours of induction, the paw size reduces. This demonstrates the anti-inflammatory activity of the regular medicine (diclofenac) and ethanol extract of *P. amaus* similar to the regular drug (diclofenac). Diclofenac is a non-steroidal anti-inflammatory drug that works by suppressing the production of prostaglandins by cyclooxygenase-2.

The anti-inflammatory activity of the *P.amarus* plant is related to the presence of secondary metabolites like flavonoids, alkaloids, and tannins which has the ability to inhibit the production of prostaglandins by cyclooxygenase 2 (COX 2) (Agnel and Shobana, 2012). COX is an enzyme that aids in the conversion of arachidonic acid to prostaglandins. It comes in two isomers: COX-1 and COX-2. COX-2 causes an overabundance of Prostanglandin E2 (PGE2) to be released in the inflammatory area (Aluwi et al. 2017). Several studies have shown that anti-inflammatory drugs significant inhibitory effects on COX-2 expression are useful in preventing and curing various illnesses (Baraf, 2007). *P. amarus*extract anti-inflammatory action may likely be via the inhibition of PGE2 synthesis in LPS-stimulated macrophages by downregulating the COX-2 protein (Baraf, 2007).

The present study when compared to normal mice, the standard drug-treated group and part of the groups treated with P. amarus extracts demonstrated a reduction in bleeding and clotting times. On the eighth day of administration, *P. amarus*plant aqueous and ethyl acetate extracts reduced clotting and bleeding times even more. The ability of *P. amarus*plant extracts to reduce bleeding and clotting times revealed the plant's procoagulant tendency, which might be related to *P.amarus's* anti-coagulant action. Thus, the plant may be useful in the treatment of hemophilia, and related blood coagulation disorder.

The coagulation factors Prothrombin time and Activated Partial Thromboplastin time were measured in this study after administration of the standard drug and plant extracts. It was discovered upon administration, there was decrease in PT concentration in ethanol extract(200 mg/kg)when compared to the normal control, implying that ethanol extract(100mg/kg) could be used as a procoagulant. When comparing Appt to normal control, there was no significant change.

In comparison to normal rats, plasma total protein levels were shown to be considerably higher in this investigation. In treated carrageenan-induced inflamed mice, administration of P.amarus fractions and extracts significantly enhanced($p< 0.05$) Plasma Total Protein concentrations. This indicates that the plant has the ability to protect and strengthen liver cells (Rajeshkumar and kutan. 2000). Cells are still protected even when total protein concentrations exceed normal.

This study also shows that, there was a significant decrease in Aspartate transferase concentration in group treated with the standard drug(diclofenac) and other groups treated with various fractions of the plant extract in comparison with the normal control. However, the Aspartate transferase concentration level increased drastically in the 200mg/kg Ethanol fraction. There were decreases in alanine aminotransferase levels in the group treated with diclofenac and Aqueous extract (100mg/kg). ALT and AST are two enzymes found in the liver. The functions of these enzymes are evaluated using their activity. These enzymes' activities are utilized to measure the liver's functional status and as biochemical indicators of liver disease (Moss and Ralph Handerson, 1999). The decrease in liver enzyme activity levels indicates that the extracts do not induce liver harm and that the enzyme activity is adequately managed, preventing it from leaking into the bloodstream.

The result of the GCMS analysis showed that the ethanol extract of *P. amarus* contains a great proportion of compounds. Some of which are responsible for the procoagulant and anti-inflammatory effects in carrageenan induced mice. Examples of such compounds are:6-octadecanoic acid (procoagulant activity), urethane (anti-inflammatory activity), hexanoic acid (procoagulant activity), formamide (anti-inflammatory activity).

CHAPTER FIVE
CONCLUSION AND RECOMMENDATION

5.1 CONCLUSION

The anti-inflammatory effects of the aqueous and ethanol extracts of *P. amarus* were demonstrated in this study corroborating the traditional use of the plant for treatment of inflammation. *P. amarus* demonstrated procoagulant activity, and thus may be a source of natural procoagulant agent in the pharmaceutical drug development for the treatment of blood coagulation disorders.

5.2 RECOMMENDATION

Further toxicology studies are recommended on *P. amarus*to check appropriate dosage be given which will not affect the tissues. Also, isolation of procoagulant agent present in *P. amarus* that will be of great advantage in pharmaceutical company to produce drugs in treatment of hemophilia, and related blood coagulation disorder is recommended.

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APPENDIX

TABLE FOR CLOTTING AND BLEEDING TIME OF MICE

GROUPS OF MICE	DAY ONE BLEEDING TIME (secs)	DAY EIGHT BLEEDING TIME (sec)	DAY ONE CLOTTING TIME (sec)	DAY EIGHT CLOTTING TIME (sec)
GROUP 1				
TAIL	87	82	98	72
HEAD	96	70	96	70
BLUE BACK	78	86	83	65
GREEN BACK	88	93	80	68
GROUP 2				
UNMARK	80	78	76	80
TAIL	78	80	78	73
BACK	85	90	70	77
	75	89	67	78
GROUP 3				
UNMARK	64	70	85	72
HEAD	89	90	70	75
BACK	62	67	78	83
	71	84	69	89
GROUP 4				
UNMARK	80	74	66	72
RIGHT LEG	75	88	72	84
LEFT HAND	70	78	78	82

	77	80	77	70
GROUP 5				
UNMARK	74	72	80	79
HEAD	90	68	75	75
LEFTLEG	85	75	78	84
	95	80	90	86
GROUP 6				
TAIL	79	70	90	70
RIGHT LEG	80	89	100	68
HEAD	85	82	89	72
	78	76	80	77

GROUP 7				
UNMARK	88	70	85	72
HEAD	73	77	87	70
LEFTARM	75	67	79	89
	86	76	78	76
GROUP 8				
UNMARK	90	90	38	86
HEAD	72	97	18	90
LEFT LEG	89	87	39	75
	75	77	32	73
GROUP 9				
RIGHT LEG	80	88	70	69

HEAD	82	78	75	76
BACK	79	81	80	88
	70	68	87	74

TABLE FOR PROTHROMBINE TIME AND ACTIVATED PARTIAL PRO THROMBINE TME

APPT TABLE

GROUPS	IDENTITY	INTIAL TIME (sec)	FINAL TIME (sec)
	HEAD	40	92
1	TAIL	77	95
	UNMARK	58	62
	BACK	59	83
2	UNMARK	44	70
	BACK	65	97
	NECK/HEAD	38	58
	TAIL	49	75
3	UNMARK	64	77
	TAIL	65	78
	HEAD	75	74
	BACK	68	76
4	UNMARK	77	109
	TAIL	27	66
	RIGHT LEG	41	69
	HEAD	26	108
5	LEFT LEG	57	82

	BACK	60	87
	HEAD	58	70
	TAIL	58	79
6	TAIL	46	107
	HEAD	54	95
	UNMARK	29	85
	RIGHT LEG	43	96
7	RIGHT LEG	70	79
	LEFT ARM	72	130
	TAIL	33	71
	HEAD	58	93
8	RIGHT LEG	20	66
	UNMARK	58	84
	LEFT ARM	16	84
	HEAD	31	78
9	BACK	44	64
	UNMARK	64	125
	HEAD	19	71
	TAIL	42	86

TABLE FOR PROTHROMBINE TIME

GROUPS	IDENTITY	INTIAL(sec)	FINAL(sec)
1	HEAD	50	60
	TAIL	71	77
	UNMARK	77	79
	BACK	66	72
2	NECK/HEAD	48	49
	UNMARK	52	60
	HEAD	48	72
	BACK	45	64
3	UNMARK	35	48
	TAIL	40	45
	HEAD	50	57
	BACK	41	50
4	UNMARK	34	65
	HEAD	59	70
	RIGHTLEG	37	66
	TAIL	38	67
5	HEAD	19	35
	BACK	29	68

	RIGHT LEG	22	73
	TAIL	23	59
6	TAIL	30	54
	HEAD	61	94
	UNMARK	20	40
	RIGHT LEG	37	62
7	HEAD	28	69
	RIGHT ARM	30	70
	TAIL	17	63
	LEFT ARM	25	67
8	UNMARK	78	104
	RIGHT LEG	32	74
	HEAD	49	87
	LEFTARM	37	85
9	HEAD	106	108
	TAIL	46	66
	UNMARK	25	40
	BACK	35	45

RESULT OF TOTAL PROTEIN ASSAY

LIVER

STANDARD: 0.839

GROUPS	IDENTITY	ABSORBANCE 1	TOTAL PROTEIN CONCENTRATION 1 (g/dl)	ABSORBANCE 2	TOTAL PROTEIN CONCENTRATION 2 (g/dl)
1	TAIL	0.753	5.340	0.762	5.403
	HEAD	0.953	6.737	0.853	6.070
	UNMARK	0.568	4.028	0.807	5.723
	BACK	0.758	5.375	0.807	5.723
2	UNMARK	0.627	4.446	0.504	3.574
	HEAD	0.758	5.375	0.444	3.148
	HEAD/NECK	0.827	5.864	0.677	4.801
	BACK	0.737	5.226	0.542	3.844
3	UNMARK	0.776	5.503	0.746	5.290
	TAIL	0.746	5.290	0.567	4.021
	BACK	0.761	5.397	0.657	4.659
	HEAD	0.754	5.347	0.612	4.340
4	TAIL	0.604	4.383	0.734	3.645
	UNMARK	0.709	5.028	0.514	5.765
	HEAD	0.627	4.446	0.813	4.297
	RIGHTLEG	0.647	4.588	0.687	4.872

5	HEAD	0.455	3.226	0.784	5.559
	LEFTLEG	0.539	3.822	0.814	5.772
	BACK	0.712	5.049	0.774	5.489
	RIGHTLEG	0.578	4.099	0.790	5.603
6	HEAD	0.975	6.914	0.838	5.942
	TAIL	0.568	4.028	0.756	5.361
	UNMARK	0.518	3.673	0.757	5.368
	RIGHTLEG	0.687	4.872	0.784	5.559
7	HEAD	0.443	3.141	0.466	3.163
	LEFTARM	0.436	3.092	0.726	5.143
	RIGHTLEG	0.785	5.567	0.566	4.014
	TAIL	0.555	3.935	0.586	4.156
8	LEFTARM	0.469	3.326	0.602	4.269
	UNMARK	0.654	4.638	0.442	3.135
	HEAD	0.747	5.297	0.751	5.325
	LEFTARM	0.623	4.418	0.598	4.240
9	HEAD	0.592	4.198	0.497	3.524
	BACK	0.785	5.567	0.780	5.531
	TAIL	0.612	4.340	0.607	4.304
	UNMARK	0.663	4.701	0.628	4.454

RESULT TABLE FOR TOTAL PROTEIN

PLASMA

STANDARD: 0.269

GROUPS	IDENTITY	ABSORBANCE 1	CONCENTRATION 1	ABSORBANCE 2	CONCENTRATION 2
1	HEAD	0.188	4.158	0.104	2.300
	TAIL	0.323	7.144	0.151	3.339
	UNMARK	0.255	5.640	0.128	2.831
	BACK	0.289	6.392	0.139	3.076
2	HEAD	0.135	2.986	0.095	2.101
	NECK	0.237	5.242	0.438	9.688
	UNMARK	0.553	12.231	0.233	5.153
	BACK	0.609	13.470	0.495	10.948
	NECK/HEAD	0.725	16.036	0.717	15.859
3	UNMARK	0.446	9.865	0.370	8.184
	TAIL	0.553	7.564	0.550	12.165
	HEAD	0.245	5.419	0.230	5.087
	BACK	0.346	7.653	0.340	7.520
4	RIGHTLEG	0.151	3.339	0.332	7.343
	TAIL	0.342	7.564	0.520	11.501
	HEAD	0.520	11.502	0.330	7.299
	UNMARK	0.338	7.476	0.450	9.954

	TAIL	0.420	9.289	0.500	11.059
5	UNMARK	0.341	7.542	0.193	4.268
	BACK	0.681	15.063	0.415	9.179
	HEAD	0.126	2.786	0.154	3.406
	TAIL	0.760	16.810	0.387	8.272
6	TAIL	0.510	11.280	0.407	9.002
	HEAD	0.666	14.731	0.565	12.497
	UNMARK	0.520	11.501	0.714	15.792
	RIGHTLEG	0.565	12.497	0.562	12.430
7	LEFTARM	0.897	19.840	0.811	17.938
	RIGHTLEG	0.731	16.168	0.558	12.342
	UNMARK	0.265	5.861	0.245	5.419
	TAIL	0.350	7.741	0.538	11.900
	LEFTARM	0.448	9.909	0.540	11.944
8	RIGHTLEG	0.897	19.840	0.863	19.088
	LEFTARM	0.731	16.168	0.382	8.449
	HEAD	0.280	6.193	0.355	7.852
	UNMARK	0.320	7.078	0.688	15.217
9	BACK	0.614	13.581	0.613	13.558
	TAIL	0.323	7.144	0.236	5.220
	HEAD	0.220	4.866	0.775	17.142
	UNMARK	0.340	7.520y	0.541	11.966

RESULT TABLE FOR ALT ASSAY

GROUPS	IDENTITY	ABSORBANCE1	CONCENTRATION	ABSORBANCE2	CONCENTRATION	
1	TAIL	0.512	94	0.515	94	
	HEAD	0.612	94	0.620	94	
	UNMARK	0.562	94	0.567	94	
	BACK	0.587	94	0.593	94	
2	UNMARK	0.432	77	0.437	77	
	HEAD	0.306	52	0.309	52	
	BACK	0.567	94	0.577	94	
	TAIL	0.435	77	0.441	77	
3	TAIL	0.672	94	0.677	94	
	UNMARK	0.767	94	0.777	94	
	HEAD	0.719	94	0.727	94	
	BACK	0.743	94	0.752	94	
4	TAIL	0.702	94	0.701	94	
	RIGHT LEG	0.534	94	0.535	94	
	HEAD	0.510	94	0.514	94	
	UNMARK	0.582	94	0.583	94	
5	HEAD	0.463	83	0.461	83	
	LEFT LEG	0.584	94	0.597	94	
	BACK	0.487	88	0.494	88	
	UNMARK	0.511	94	0.517	94	
6	HEAD	0.608	94	0.613	94	

	TAIL	0.605	94	0.604	94	
	UNMARK	0.765	94	0.766	94	
	BACK	0.659	94	0.661	94	
7	HEAD	0.635	94	0.636	94	
	LEFT ARM	0.617	94	0.623	94	
	RIGHT LEG	0.557	94	0.568	94	
	UNMARK	0.603	94	0.609	94	
8	LEFT ARM	0.414	77	0.418	72	
	UNMARK	0.517	94	0.517	94	
	HEAD	0.262	48	0.255	43	
	RIGHT LEG	0.303	52	0.302	52	
9	HEAD	0.296	48	0.296	48	
	BACK	0.500	94	0.505	94	
	TAIL	0.416	72	0.416	72	
	UNMARK	0.755	94	0.755	94	

RESULT FOR AST ASSAY

GROUPS	IDENTITY	ABSORBANCE	CONCENTRATION
1	HEAD	0.161	76
	TAIL	0.148	59
	UNMARK	0.156	76
	BACK	0.153	67
2	UNMARK	0.160	76
	HEAD	0.135	52
	BACK	0.155	67

	TAIL	0.170	89
3	UNMARK	0.145	59
	TAIL	0.164	76
	BACK	0.166	76
	HEAD	0.170	89
4	TAIL	0.150	67
	UNMARK	0.168	76
	RIGHTLEG	0.158	67
	HEAD	0.162	76
5	HEAD	0.140	59
	LEFTLEG	0.161	76
	BACK	0.150	67
	UNMARK	0.153	67
6	HEAD	0.139	52
	TAIL	0.145	59
	UNMARK	0.158	67
	BACK	0.160	76
7	HEAD	0.170	76
	UNMARK	0.172	76
	LEFTARM	0.162	76
	RIGHTLEG	0.166	76
8	LEFTARM	0.148	59
	UNMARK	0.158	67
	HEAD	0.153	67

	RIGHTLEG	0.163	76
9	HEAD BACK TAIL UNMARK	0.140 0.148 0.162 0.153	59 59 76 67

Paw inhibition calculation

Groups	Identity	1h	2h	3h	4h
3	Unmark Head Tail back	10 22 11 10	24 20 25 14	15 25 19 20	20 19 15 18
4	Unmark Head Tail Back	23 16 24 19	15 21 13 20	21 18 12 15	20 19 22 18
5	Unmark Head Tail Back	16 12 15 20	14 19 16 15	16 14 20 18	20 19 15 17
6	Unmark Head Tail Back	20 21 19 15	20 15 10 12	13 15 16 10	24 20 19 17

7	Unmark	20	23	20	13
	Head	22	25	23	10
	Tail	21	23	21	14
	Back	18	20	18	18
8	Unmark	17	17	16	17
	Head	20	18	18	16
	Tail	23	14	19	19
	Back	24	12	13	21
9	Unmark	25	16	13	17
	Head	18	25	15	15
	Tail	14	26	18	13
	Back	16	17	20	12