University of Zululand

Antithrombotic and anti-inflammatory activity of ethyl acetate extract of *Protorhus longifolia* stem bark

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DECLARATION

I, Londiwe Cynthia Majola, declare that the experimental work described in this dissertation was conducted in the Department of Biochemistry and Microbiology, Faculty of Science and Agriculture, University of Zululand under the supervision of Dr. R.A. Mosa and Prof. A.R Opoku. This study represents the original work by the author. Where use was made of the work of others, it has been duly acknowledged in the text.

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DEDICATION

This work is dedicated to all those who did not give up on me through this journey including my family and supervisors.

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To the Lord Jesus for his grace and mercy that are new every morning, would not have made it thus far without him.

A profound thank you to the following people whom without their contributions and guidance the success of this project would not have been possible:

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“I know that you can do all things no purpose of yours can be restrained “

-Job 42:2

ABSTRACT

Blood clotting related disorders are the major underlying cause of cardiovascular diseases, which are the major cause of premature deaths globally. Despite great efforts made in the discovery of antithrombotic agents, ischemic events continue to claim more lives. Thus, there is a need to discover and develop new and more effective antithrombotic drugs, preferably from natural sources. The study is aimed at investigating the anti-thrombotic and anti-inflammatory activity of the ethyl acetate extract of *Protorhus longifolia* stem bark.

The *in vitro* antioxidant activity of the crude ethyl acetate was evaluated against DPPH, ABTS and nitric oxide radicals. Its reducing power and metal ion chelating potential were also determined. A chromogenic substrate was used to determine the antithrombin activity of the extract. While the thrombolytic activity of the extract was determined in rat whole blood clot, the antiplatelet aggregation activity was evaluated on rat platelet rich plasma. The carrageenan induced rat paw edema and rat tail bleeding time models were used to investigate the anti-acute inflammatory and antithrombotic activity of the extract, respectively. The experimental rats were orally pre-treated with the extract at 100 and 350 mg/kg for 8 consecutive days. While the water displacement method was used to measure the rat paw edema, the time it took for the rat tail to stop bleeding following a 5 mm amputation was taken as the bleeding time of the rats. At the end of the experiments, all the rats were euthanized and blood and the hind paws of the rats were collected for biochemical analysis of some inflammation and oxidative stress biomarkers.

The extract showed, to a varying degree of efficacy, concentration dependent scavenging activity on DPPH, ABTS and nitric oxide radicals. While the plant extract showed a strong reducing power, only a moderate metal ion (Fe^{2+}) chelating activity was observed. The extract also exhibited concentration dependent antithrombin and antiplatelet aggregation activities. At the highest concentration of 10 mg/ml, the extract exhibited up to 35.7% thrombolytic activity. While a marked decrease in tail bleeding time (3.18 min) was observed in the untreated inflammation induced rats when compared to the normal control group, prior oral administration of the extract (at 100 and 350 mg/kg) to the rats seemed to prolong the bleeding time up to 8.8 min. The animals pre-treated with the extract also displayed anti-inflammatory activity as they effectively reduced the rat paw swelling. Even though no significant

changes were observed in the tissue levels of IL-6 and TNF-α on the extract treated groups, a marked decrease in COX-2 and TGF- β1 was observed. Increased tissue levels of superoxide dismutase and total antioxidant status were also observed in the inflammation induced rats pre-treated with the extract at both 100 and 350 mg/kg in comparison to the untreated control group. Even though lower serum levels of some kidney and liver function biomarkers (urea, creatinine, AST, ALT and ALP) were observed in the group treated with the extract at 100 mg/kg, relatively higher serum levels of BUN and AST were observed in the rat group administered with the extract at a higher concentration of 350 mg/kg.

The results obtained suggest that the ethyl acetate extract of *P. longifolia* stem bark possess antithrombotic and anti-inflammatory activities. The observed increases in serum levels of BUN and AST at the higher dosage of the extract indicate its potential nephro-and hepatotoxicity and that it be used as a treatment with caution.

LIST OF ABBREVIATIONS USED

AA	Ascorbic acid
ABTS	2, 2′-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid)
ADP	Adenosine diphosphate
AST	Aspartate transaminase
ALP	Alanine transaminase
ALP	Alkaline phosphatase
BHA	Butylated hydroxyl-anisole
BUN	Blood urea nitrogen
CA	Citric acid
COX	Cyclooxygenase
DMSO	Dimethyl sulfoxide
DPPH	2, 2-Diphenyl-1-picryl-hydrazyl
EA	Ethyl acetate extract
EDTA	Ethylenediaminetetra-acetic acid
GSH	Glutathione reduced
IC_{50}	Inhibitory concentration at 50%
MDA	Malondialdehyde
NADPH	Nicotinamide adenine dinucleotide phosphate oxidase
NO	Nitric oxide
ROS	Reactive oxygen species
SOD	Superoxide dismutase
PAR	Protease activated receptor
TF	Tissue factor

TM	Thrombomodulin
Tpa	tissue plasminogen activator
uPA	urokinase plasminogen activator
vWF	von Willebrand factor
WHO	World Health Organization

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Chapter 4

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Discussion of results obtained in this study

Chapter 6

This chapter consists of the conclusion made from the obtained results, limitation(s) of the current study and future recommendations.

CHAPTER 1

1.0 Introduction

Blood clotting is an important and necessary physiological process responsible for preventing excessive bleeding during vascular injury. Unnecessary formation of a blood clot (thrombus) within a blood vessel disrupts and blocks blood flow, leading to nutrient and oxygen deprivation in the affected tissues (Verhamme and Hoylaerts, 2009, Thiruvenkatarajan *et al*., 2014;). Excessive clot formation is the underlying causes of ischemic events such as stroke and myocardial infarction, which are among the major contributors to the high rate of morbidity and premature deaths globally (Raskob *et al.,* 2014). The risk factors that pre-dispose people to clot formation include diabetes mellitus, cardiovascular diseases, oxidative stress, obesity, high blood cholesterol levels, hypertension and inflammation (Cerquozzi *et al.,* 2016). Inflammation is known to suppress the anticoagulant system and promote expression of blood clotting factors (Verhamme and Hoylaerts, 2009).

Current treatment for thrombosis consists of anti-platelet therapy, anticoagulants, thrombolytic agents, inhibitors of fibrin formation and direct thrombin inhibitors (Fintel, 2012). Despite great efforts made in the discovery of these therapeutic agents, ischemic events continue to claim more lives. Moreover, risks of excessive bleeding and other various adverse effects with long term use of these therapeutic agents (Fintel, 2012; Lamberts *et al.,* 2014; Daley *et al.,* 2015) also limit their clinical use. Thus, there is a need to discover and develop new therapeutic drugs, preferably from natural sources, that are more effective with less side effects.

Over the years, medicinal plants have been used as primary healthcare sources, and have substantially contributed to the discovery and development of pharmacologically active drugs in clinical use today. Various stem bark extracts of *Protorhus longifolia* (Benrh.) Engl (Anacardiaceae), a plant commonly used by Zulu traditional healers to manage blood clotting related diseases, have been reported to possess antiplatelet aggregation activity (Mosa *et al.,* 2011a). Anticoagulant and anti-inflammatory activities of a lanosteryl triterpene isolated from the chloroform extract of *P. longifolia* stem bark have also been reported (Mosa *et al*., 2011b; Mosa *et al.,* 2015). Since medicinal plants possess an unmatched diversity of bioactive

compounds with different properties, other extracts of the plant could exhibit even more exciting and improved efficacy against blood clotting related disorders. Thus, this study evaluated the antithrombotic and anti-inflammatory activity of the ethyl acetate extract of *P. longifolia* stem bark.

CHAPTER 2

2.0 Literature Review

Normal blood flow through blood vessels is vital in supporting life as blood delivers oxygen and nutrients to the tissue, as well as removing metabolic waste, from the tissues. Under physiological conditions, equally important is the process of blood clotting that prevents excessive loss of blood from only minor tissue injuries (Thiruvenkatarajan *et al*., 2014). Blood clotting and dissolution are important components of hemostasis. However, under pathophysiological conditions, the balance between blood clot formation and dissolution is disturbed, resulting in life threatening blood clot formation, a condition referred to as thrombosis (Verhamme and Hoylaerts, 2009, Thiruvenkatarajan *et al*., 2014;). Unwanted and unnecessary internal blood clot formation is the underlying cause of various ischemic events including stroke and heart attacks.

2.1 Coagulation system and platelet aggregation in haemostasis

Hemostasis is an important process that regulates and maintains vascular integrity and blood flow upon vascular injury (Furie and Furie, 2008; Chen *et al.,* 2015). The mechanisms that ensure hemostasis upon vascular injury include platelet activation and aggregation, coagulation and thrombolytic or fibrinolytic systems. Platelet activation and aggregation is responsible for the platelet plug formation at the site of injury. Platelets also provide the surface for the coagulation cascade occurrence and generate thrombin and fibrin, respectively (Furie, 2009). Thrombin is a proteolytic enzyme that produces fibrin from fibrinogen which is deposited to stabilize the clot. Clot formation and stabilization is maintained during hemostasis by the anticoagulant system that regulates coagulation. The fibrinolytic system is responsible for dissolving the formed clot (Cesarman-Maus and Hajjar, 2005).

2.1.1 Mechanism of blood coagulation

Blood coagulation and platelet activation are important biological process responsible for preventing excessive bleeding during injury. The coagulation cascade involves two pathways, extrinsic and intrinsic pathways (Figure 2.1). The intrinsic pathway is triggered by exposure of blood to collagen during vascular injury. This pathway is initiated by the activation of factor XII on the exposed collagen,

followed by the activation of coagulation factors including factor XII, HMW kininogen, perkallekerin and factor XI. Active factor XI, in the presence of calcium ions, activates factor IX which forms a complex with activated factor VIII, responsible for activating factor X (Palta *et al.,* 2014). The extrinsic pathway is triggered upon tissue injury on the ruptured endothelial wall. The tissue factor (TF) expressed on the sub-endothelial binds to factor VIIa. In the presence of calcium ions, TF-VIIa activates factor X to factor Xa (Lasne *et al.,* 2006, Owens and Mackman, 2010, Palta *et al.,* 2014).

The activation of factor X, from both the intrinsic and extrinsic pathway, forms the prothrombinase complex in the presence of calcium ions, activating factor V, vitamin K and the phospholipid surface of the platelets. This complex promotes the generation of thrombin from prothrombin (Hall, 2010, Palta *et al.,* 2014). Thrombin is responsible for the conversion of fibrinogen to fibrin polymers. Generated thrombin activates factor V and VIII, causing a burst in the generation of thrombin until the clot is stabilized (Furie and Furie, 2008; Butenas *et al.,* 2009; Geddings and Mackma, 2014). Since platelets provide the surface for coagulation to occur, platelets aggregate and form stable clots with cross-linked fibrin chains (Versteeg *et al*., 2013; Yau *et al.,* 2015).

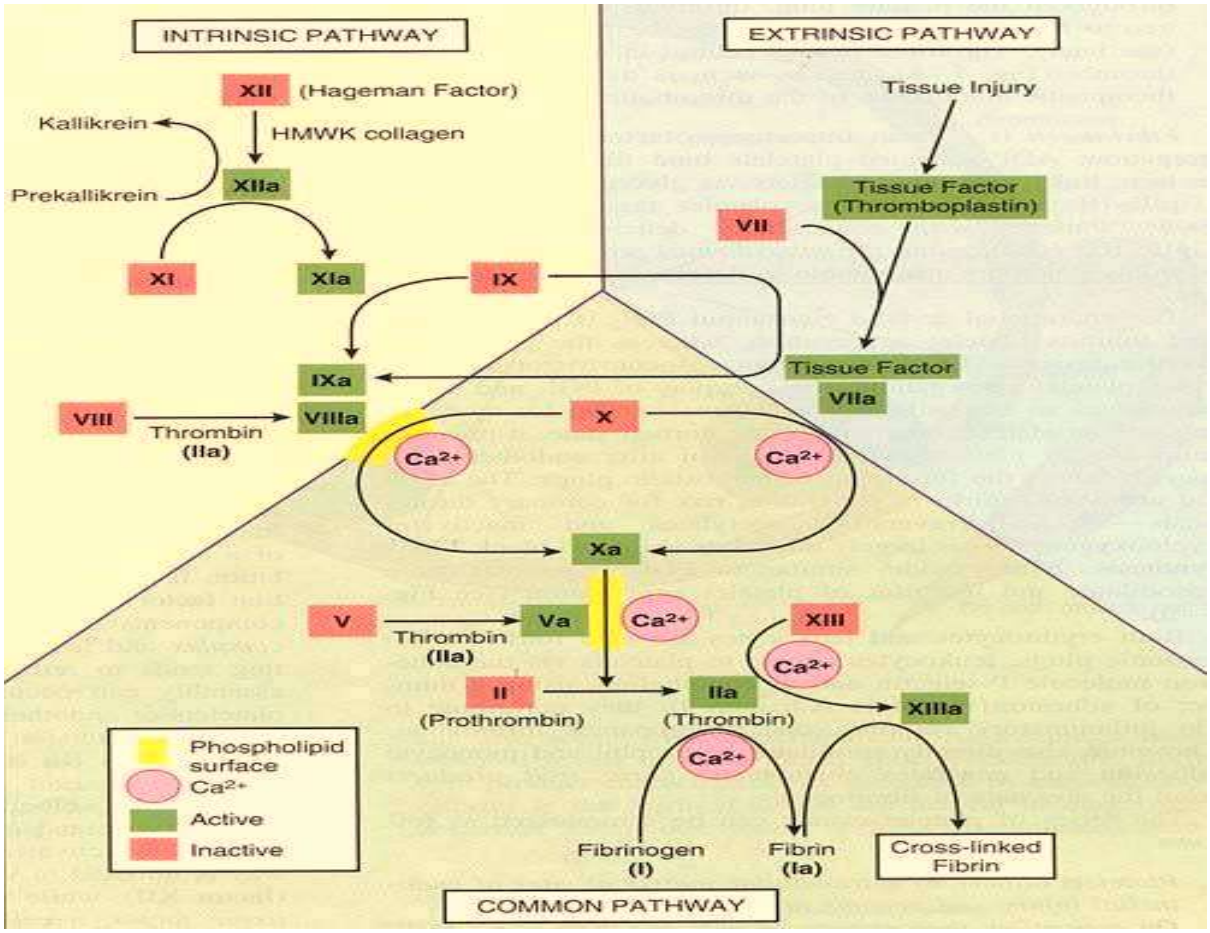

Figure 2.1: The coagulation cascade. The extrinsic and intrinsic pathways converge at the common pathway leading to the production of thrombin from prothrombin (Palta *et al.,* 2014).

The coagulation cascade is normally regulated by physiological anticoagulants such as protein C activation, antithrombin and tissue factor plasminogen inhibitor. These physiological anticoagulants are responsible for reducing the effects of thrombin (Ezihe-Ejiofor and Hutchinson, 2013).

2.1.2 Platelets activation and aggregation

Platelet activation and coagulation usually occur simultaneously during hemostasis (Palta *et al.,* 2014). During vascular injury and disrupted endothelium, circulating inactivated platelets migrate towards the site of injury and bind to exposed collagen via the von Willebrand factor (vWF) and the platelet membrane glycoprotein receptors Ib-IX-V complex (Heemskerk *et al.,* 2002). Platelet agonists, including thromboxane A_2 and ADP, recruit circulating platelets to the site of injury for the formation of the platelet plug. This process amplifies the response of platelets. ADP

activates platelets by binding to the $P2Y_{12}$ glycoprotein receptors present on the platelet surface (Offermanns, 2006). Thromboxane A2 is not a product of platelet activation but is the product of inflammation. Thromboxane is produced due to the conversion of arachidonic acid by the cyclooxygenase-1 to prostaglandin $H_{2;}$ that is further metabolised to thromboxane A2 (Karlíčková, *et al.,* 2016). This agonist activates more platelets to the site of injury by binding to the thromboxane A2 receptor on the inactivated platelets (Offermanns, 2006). Thrombin, produced during coagulation on the surface of activated platelets, strongly activates circulating platelets by binding to protease activated receptors 1 and 4 (PAR-1 and 4) present on the surface of the platelets and also stabilizes the developing clot by generating fibrin polymers from fibrinogen (Heemskerk *et al.,* 2002).

Dense and alpha granules, released by activated platelets, mediate various biological functions that influence hemostasis. These mediators elevate inflammation, cell and platelet adhesion, proliferation, chemotaxis, coagulation and proteolysis. Pro-inflammatory cytokines include interleukin 1 beta (IL-1β), β-thromboglobin, CD40 ligand, growth factors including transformation growth factor beta 1 (TGF-β1), platelet-derived growth factor (PDGF), epidermal growth factor (EGF), adhesion proteins, glycoprotein IIb/IIa, vWF, fibrinogen and coagulation factors (Kaplanski *et al*., 1994; Gawaz *et al.,* 2005).

Platelet aggregation occurs when glycoprotein receptor IIb -IIa present on the surface of platelets is converted to its highest affinity integrin $\alpha II_b\beta_3$ state. This state allows activated platelets to expand and aggregate at the site of injury. This expansion occurs when fibrinogen and vWF bind to the high affinity integrin $\alpha II_b\beta_3$ (Holinstat and Bray, 2016). During the hemostatic state normal endothelial cells on the vascular lining of the arteries release nitric oxide and prostaglandin I_2 to prevent platelet activation (Thomas and Storey, 2015).

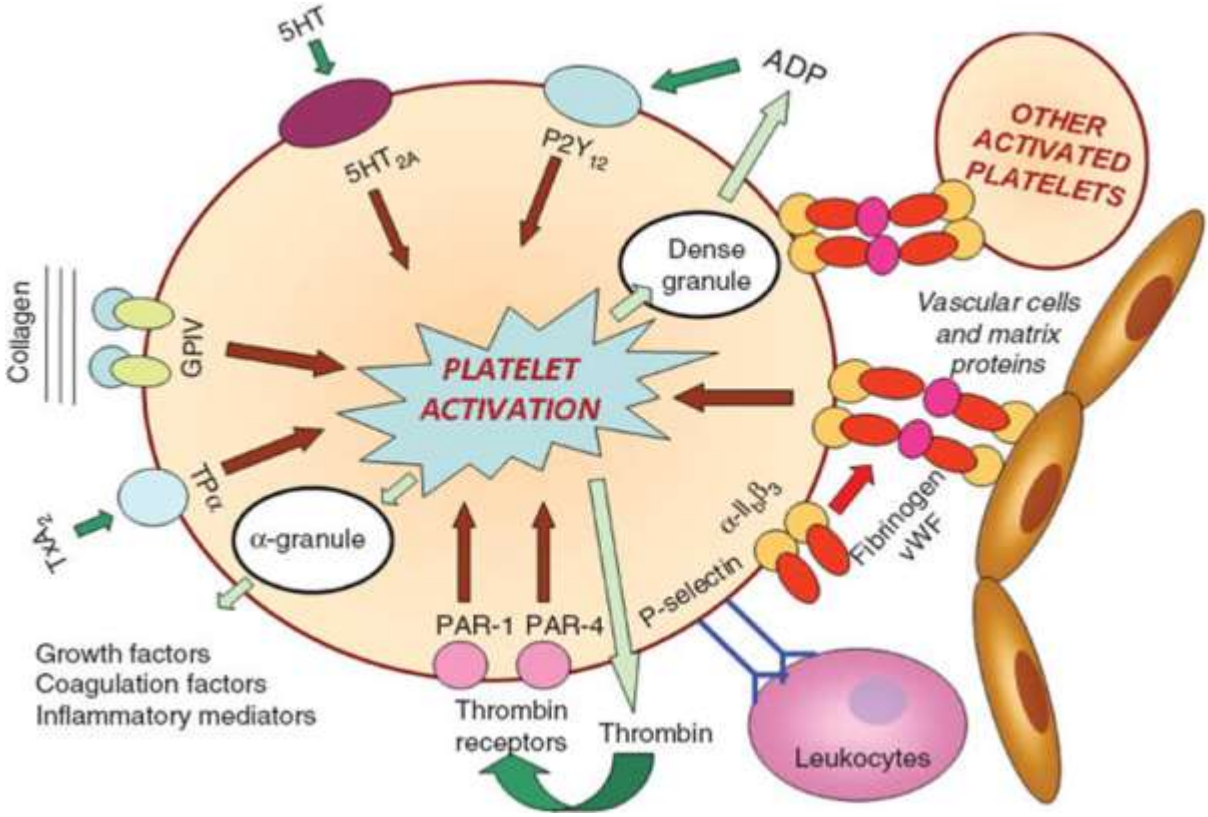

Figure 2.2: Platelet activation. Binding of platelet agonists such as collagen, thrombin, ADP and thromboxane A_2, on their respective receptors, activates and causes morphological changes in the platelets. The activated platelet releases ADP, coagulation factors, inflammatory mediators and growth factors (Farré *et al*., 2014).

2.1.3 Fibrinolytic system

Similar to the coagulation cascade that is tightly regulated by the anti-coagulant system, the fibrinolytic system is closely regulated by inhibitors, cofactors and receptors (Chapin and Hajjar, 2015). During healing of the injured vessel, the formed fibrin clot is lysed as a result of plasmin action into fibrin degradation products. Plasminogen is activated into plasmin by either urokinase plasminogen activator (uPA) or the tissue plasminogen activator (tPA) (Chapin and Hajjar, 2015). Plasmin can increase the activator activity by converting the uPA and tPA single chains into doubled chain counterparts, thus exerting a feedback mechanism that allows its own activation from plasminogen (Hoylaerts *et al.,* 1982; Cesarman-Maus and Hajjar, 2005; Chapin and Hajjar, 2015). These two plasminogen activators are synthesized by macrophages, monocytes and endothelial cells, respectively (Sprengers and Kluft, 1987; Cesarman-Maus and Hajjar, 2005).

The fibrinolytic system is tightly regulated by the plasmin inhibitors that prevent the enhanced plasminogen activator and plasmin activity. These inhibitors include PAI-1 plasminogen inhibitor, PAI-2 and α-2antiplasmin, which all co-exist in the circulatory system (Schneider and Nesheim, 2004; Chapin and Hajjar, 2015). PAI-1 is produced by activated platelets, endothelial cells and other cells, and is released into the circulatory system where it rapidly and irreversibly inhibits the activity of tPA and uPA. An elevated concentration of PAI-1 inhibitor also reduces the life span of the plasminogen activators tPA and uPA in the circulatory system. α-2antiplasmin regulates the fibrinolytic system by binding to plasmin (Plow and Collen, 1981; Kimura and Aoki, 1986). Normally plasmin binds to fibrin for dissolution to occur, hence preventing the regulatory action of α-2antiplasmin (Schneider and Nesheim, 2004). PAI- 2 is normally expressed during pregnancy and also inhibits the activity of tPA and uPA. The TAFI is another potent fibrinolytic proenzyme activated by thrombin. TAFI reduces the affinity of plasminogen to fibrin (Cesarman-Maus and Hajjar, 2005; Palta *et al.,* 2014.). The main role of TAFI in hemostasis is to mediate a common understanding between coagulation and fibrinolysis (Mosnier *et al*., 2001). The fibrinolytic (thrombolytic) system is disturbed when plasminogens are insufficiently activated, excessive enzyme inhibitors are present or when there is a deficiency in their production (Collen *et al.,* 1986; Gonzalez-Gronow *et al.,* 2017). During the pathophysiological state, the normal haemostatic state of the vessel is disturbed by the increased pro-coagulant and reduced anticoagulant activity, leading to thrombosis.

2.2 Thrombosis

Thrombosis is the development of a blood clot (thrombus) in the vessel instead of the vascular wall, thus reducing blood flow in the circulatory system (Thiruvenkatarajan *et al.,* 2014). The thrombus blocks the transportation of oxygen and nutrients to the brain and heart which leads to myocardial infarction and ischemic stroke. These are all major causes of morbidity and mortality world-wide (Misra and Dikshit, 2014). Thrombosis occurs in two forms, which are arterial and venous thrombosis. Arterial thrombosis is known to be rich in platelets and venous thrombosis is known to be rich in red blood cells (Koupenova *et al*., 2016).

Thrombosis is the underlying cause of various cardiovascular diseases including venous thromboembolism, ischemic heart disease (acute coronary syndrome) and cerebral stroke (Raskob *et al.,* 2014). In 2016 cardiovascular diseases including thrombotic disorders, had been reported to account for over 17.9 million deaths globally and 85% of these deaths were due to strokes and ischemic heart disease (Figure 2.3) (WHO, 2018). These figures are expected to increase to over 23.6 million (41%) by 2030 (Mozaffarian *et al*., 2016). The World Health Organisation (WHO) has reported that cardiovascular disease in both low and middle-income countries accounts for 37% death rate globally (WHO,2018). STATS SA (2017) has also indicated that, in South Africa, ischemic event related health cases are the prominent cause of death, more than HIV and cancer cases combined. Stroke and myocardial infarction have been reported to account for 77.9% and 77.3% of mortality cases, respectively, when compared to other ischemic events (STATS SA, 2017).

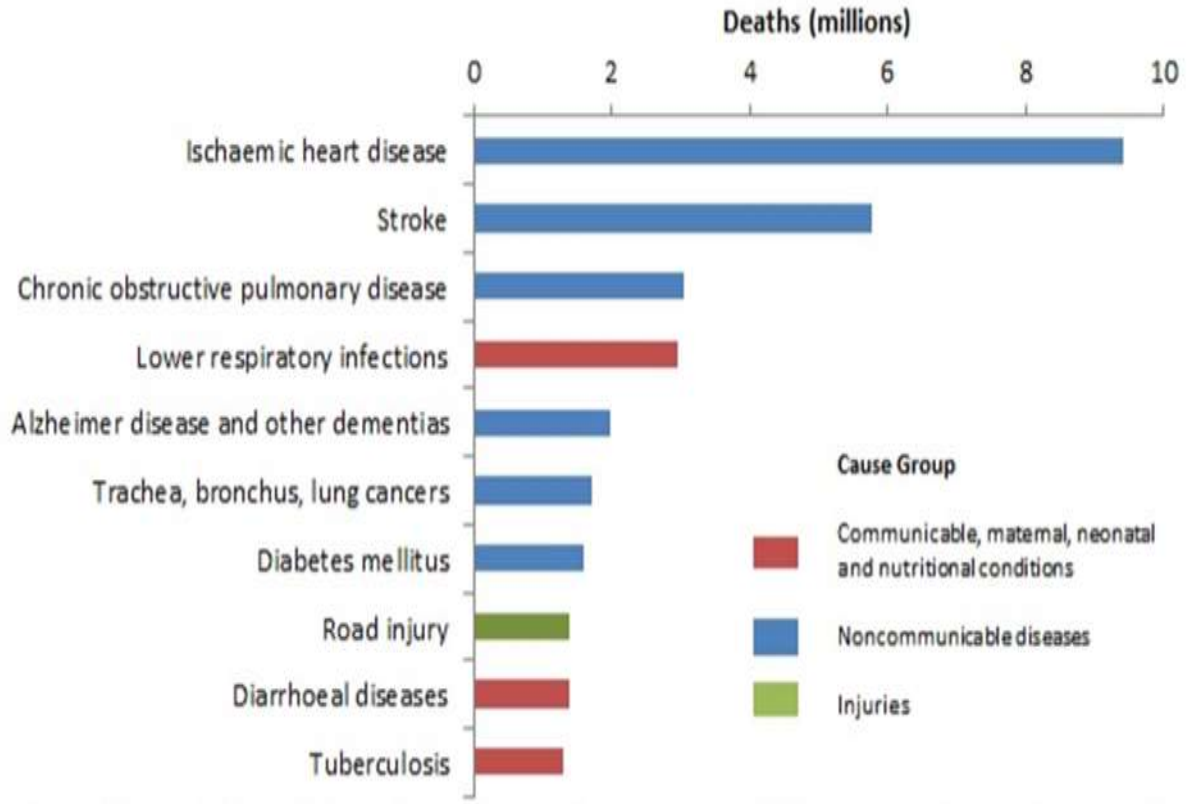

Figure 2.3: Top 10 global causes of deaths, 2016 (Source: Global Health Estimates 2016: Deaths by Cause, Age, Sex, by Country and by Region, 2002-2016. Geneva, World Health Organisation; 2018).

The risk factors that predisposes people to thrombosis and related disorders include inflammation, diabetes mellitus, cardiovascular diseases, oxidative stress, obesity, high blood cholesterol levels and hypertension (Cerquozzi *et al.,* 2016). During pathophysiological conditions of thrombosis, inflammation supresses anticoagulation and promotes procoagulant events including platelet activation, aggregation and coagulation, which promotes excessive clot formation (Esom, 2005; Verhamme and Hoylaerts, 2009; Yau *et al.,* 2015).

Upon vascular injury, due to plaque rupture, the exposed collagen from the subendothelial wall allows circulating platelets to adhere to the injury site via vWF. ADP, thromboxane A_2 and serotonin are released by activated platelets. This action recruits more activated platelets at the site of injury (Shattil *et al*., 2010). Activated platelets release mediators that cause inflammatory response, interactions of various cells at the site of injury and coagulation factors that amplify inflammatory response and coagulation during thrombosis (Esmon, 2005). The protein disulphide isomerases released by activated endothelial cells and platelets have been reported to be key players in the pathophysiology of thrombosis (Furie and Furie, 2008).

2.3 Inflammation

Inflammation is the body's instant response to tissue injury caused by noxious stimuli such as physical injury or chemical, thermal and antigen-antigen reaction. It can also be caused by foreign pathogens (Weiss, 2008). Inflammation is characterised by pain, heat, swelling and loss of function (Gautamand Jachak, 2009; Bellik *et al.,* 2012). The expression of inflammatory mediators, including tumour necrosis factor (TNF), interleukins (IL-6, IL-12, IL-1), nitric oxide, inducible cyclooxygenase-2 (COX--2), inducible nitric oxide synthase (iNOS) and interferon (INF-γ), responds to inflammation (Mueller *et al*., 2010). These inflammatory mediators initiate and amplify inflammation (Calixto *et al*., 2004; Hussein *et al*., 2012). Inflammation exists in two forms, acute inflammation and chronic inflammation. Acute inflammation is the initial stage of inflammatory response to injurious stimuli mediated by the activation of the immune system cells (Anderson *et al.,* 2013). During acute inflammation, leukocytes infiltrate the injured region to repair the injury by removing the stimulus.

Chronic inflammation refers to long term inflammation that lasts for long period of time due to an autoimmune disorder on normal tissue and failure to eradicate acute inflammation causing agents (Nordqvist, 2017). This prolonged inflammation is linked with disrupted tissue, active inflammation mediators and attempted tissue repair (Weiss, 2008; Elmajdoub *et al.,* 2017).

The development and progression of thrombus formation is directly linked to inflammation by tissue injury alongside platelet activation and aggregation (Jenne *et al*., 2013). The central role played by platelets links the coagulation and inflammation processes. Activated platelets release mediators of inflammation, such as pro-inflammatory cytokines, including IL-1β, β-thromboglobin, CD40 ligand and growth factors, including TGF-β1, PDGF and EGF, adhesion proteins, fibrinogen and coagulation factors (Kaplanski *et al*., 1994; Gawaz *et al.,* 2005). Some of the inflammatory mediators and growth factors released during platelet activation, enhance inflammation during thrombosis. TGF-β1 is a chemotactic cytokine that initiates inflammation by stimulating the migration of lymphocytes, monocytes, neutrophils and fibroblasts to the site of inflammation (McCartney-Francis and Wahle *et al*., 2012).

Elevated levels of IL-1 and TNF-α promote the expression of tissue factor on monocytes and macrophages (Edgington *et al*., 1991; Esmon, 2003). This occurs as a result of a hemostatic shift, caused by enhanced inflammation that favours coagulation during thrombosis (Drake *et al.,* 1989; Esmon, 2003). The expression of various coagulation factors and adhesion molecules including vWF, fibrinogen factor VII and tissue factor are induced by the IL-6 (Kerr, 2001). The concentrations of protein S and antithrombin are also reduced when IL-6 levels are increased (Amrani, 1990; Burstein *et al.,* 1996; Neumann *et al.,* 1997; Kerr *et al.,* 2001).

Coagulation is also directly linked to inflammation through thrombin. Thrombin is an important serine protease with multifunctional roles in both hemostasis and thrombosis (Crawley *et al.,* 2007; Ebrahimi *et al.,* 2017). Thrombin binds to the specific protease activated receptors (PARs) 1-4 and directly influences inflammation (Cheng *et al*., 2011; D'Angelo, 2015). The PAR receptors on the platelet cells express inflammatory responses when thrombin binds to them. Protease activated

receptors induce various expressions of inflammatory molecules including adhesion molecules MCP-1, VCAM-1 and MCP-1, tissue necrosis factor (TNF-α), interleukins1, 6 and 8 and growth factors, including PDGF and VEGF (Szaba and Smiley, 2002; Chu, 2011). The downregulation of the anticoagulant system and suppression of fibrinolysis during thrombosis is an action of inflammation (Verhamme and Hoylaerts, 2009). The expression of cytokines TNF-α and lL-1β inhibits the fibrinolytic system, increasing the release of tissue plasminogen and urokinase-type activator of plasminogen from vascular endothelial cells (Aksu *et al*., 2012). The expression of TNF-α and lL-1 also supresses thrombomodulin (TM) (Levi and van der Poll, 2010; Aksu *et al*., 2012).

Biosynthesis of prostaglandins is important for inflammatory response and generation. Prostaglandin levels are increased in inflamed tissue and are also responsible for the development of the cardinal signs of acute inflammation (Ricciotti *et al.,* 2011). Prostaglandins are products of the arachidonic acid (AA) metabolism. Cyclooxygenases, COX-1 and COX-2, are isoenzymes that catalyse the synthesis of prostaglandins from arachidonic acid during inflammation . While COX-1 is constitutively expressed in the majority of cells, the expression of COX-2 is induced during inflammation (Oesch-Bartlomowicz and Oesch, 2007; Koontongkaew and Leelahavanichkul, 2012). COX-1 expression is important in the generation of prostaglandins, regulating vascular hemostasis, renal function and gastrointestinal cytoprotection (Nakano *et al.,* 2007). During inflammation, the arachidonic acid is converted to prostaglandin H_2 by COX (Figure 2.4). Prostaglandin H_2 is further metabolised by tissue specific isomerases to thromboxane A_2, PDG_2, PGF_2, PGE_2, and PGI_2 (Nakano *et al.,* 2007; Ricciotti *et al.*, 2011). Prostaglandins mediate inflammatory response and tissue injury (Vane *et al.,* 1998; Charlier and Michaux, 2003).

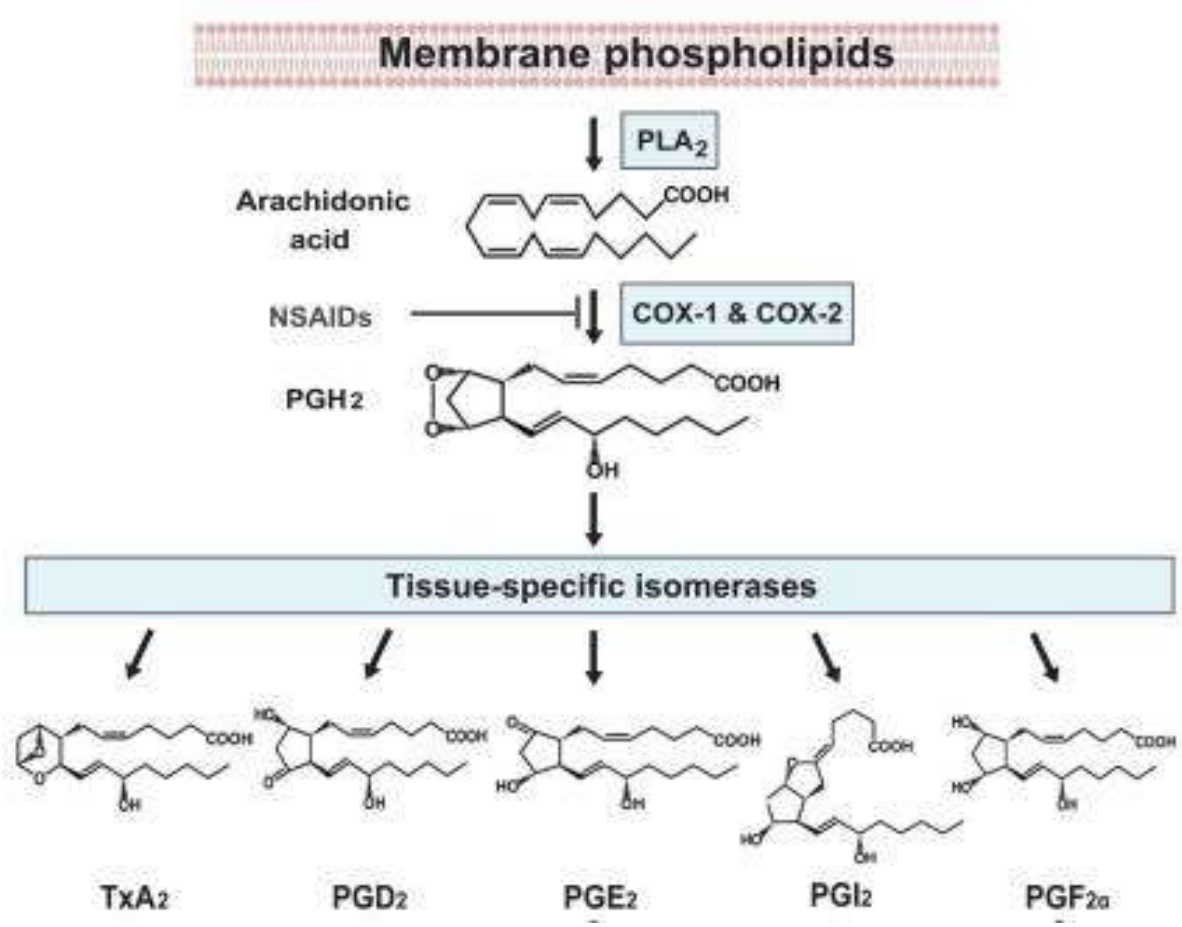

Figure 2.4: Prostaglandin is responsible for mediating inflammatory responses during inflammation and tissue injury. Arachidonic acid metabolism for prostaglandins is dependent on the cyclooxygenase enzymes (Ricciotti *et al*., 2011).

2.4 Oxidative stress

Oxidative stress results from the imbalance of the intracellular antioxidant capacity and the production of the reactive oxygen species (ROS) under pathophysiological conditions (Ray *et a*l., 2012). The accumulation of ROS disturbs the normal functioning of cells and causes damage to the cells and tissues, thus increasing the inflammatory state (Bishop, 2008; Aitken *et al.,* 2009; Shaikh *et al.,* 2016). Cells, including platelets, neutrophils and endothelial cells, produce ROS, and are all implicated during thrombosis (Ide *et al*., 1999; Bartimoccia *et al*., 2014).

The enzymes NADP oxidase, uncouple nitric oxide synthase (NOS), and the enzyme myeloperoxidase triggers the production of ROS from platelets (Violi and Pignatelli, 2014). NADPH oxidase generates the unstable superoxide anion($O_2^{\bullet -}$) during thrombosis (Ghasabeh *et al*., 2016; Ghasemzadeh and Hosseini, 2017). The

upregulation and expression of the tissue factor in activated platelets yields the activation of NADPH oxidase, promoting the generation of the ROS during thrombus formation (Görlach *et al*., 2000, Herkert *et al*., 2002, Madamanchi *et al*., 2005). The interaction and aggregation between platelets and leukocytes occur due to the release of a superoxide anion produced by leukocytes (Madamanchi *et al*., 2005). The superoxide radical is a major source of oxidative stress that directly affects thrombosis and also limits the biological function of nitric oxide produced by the normal endothelial cells during thrombosis (Ohara *et al*., 1993; Pignatelli *et al*., 1998; Bartimoccia *et al*., 2014).

The natural occurring antioxidants, including glutathione peroxidase, superoxide dismutase and catalase, inhibit the platelet aggregatory activity by scavenging ROS, thus altering platelet function during thrombus formation (Violi *et al*., 2010). Pharmacological agents that possess antioxidant properties could also be crucial in the treatment of thrombotic disorders.

2.5 Current management strategies against thrombosis related disorders

The current treatment for thrombosis consists of anti-platelet therapy, anticoagulants, thrombolytic agents, inhibitors of fibrin formation and direct thrombin inhibitors (Fintel, 2012). The current therapeutic drugs used against thrombosis are effective, but their prolonged use is linked with undesirable side-effects. These include cardiovascular problems, renal failure, gastric lesions and bleeding (Fintel, 2012). The use of thrombolytic agents such as streptokinase, alteplase, urokinase, antistreplase and tissue plasmogamy activator are associated with bleeding risk, anaphylactic reaction and lack specificity (Donnan *et al.,* 1996; Furolan *et al.,* 1999; Bivard *et al.,* 2013; Daley *et al.,* 2015). Reports also suggest that thrombotic events and cardiovascular disease recur when taking anti-inflammatory drugs, coupled with either anticoagulant or antiplatelet treatment (Fintel, 2012; Lamberts et al., 2014). Meanwhile prolonged use of current NSAID and cyclooxygenase -2 inhibitors is associated with increased risk of acquiring cardiovascular disease and renal failure (Evans *et al.,* 1995, Rodríguez and Hernández-Díaz, 2003; Bhala *et al*., 2013; Patrono, 2016)

There is a need to find alternative treatment preferably from natural sources that will be more effective, with less side effects. These natural products could effectively complement or completely replace the current treatment used for thrombosis related disorders. Due to dissatisfaction with the use of current conventional drugs, researchers continue to focus their attention on discovering new therapeutic drugs, preferably from medicinal plants.

2.6 Medicinal plants and their derivatives as potential therapeutic targets

Medicinal plants use in the management of various diseases have a long history. Medicinal plants are those plants that possess bioactive compounds (phytochemicals) responsible for therapeutic properties (Negi *et al.,* 2011, Chikezi *et al.,* 2015). The bioactive ingredients are found in various parts of the plants including stems, roots, flowers, leaves and seeds (Cragg and Newman, 2001; Dougharie, 2012). The bioactive compounds include glycosides, saponins, flavonoids, phenolics and tannins and alkaloids (Saxena *et al.,* 2013). Phenolics have been reported to be the most abundant and diverse phytochemicals possessing potent bioactivities (Dai and Mumper, 2010; Saxena *et al.,* 2013).

The continuous health threat posed by venous and arterial thromboembolic disorders has increased interest in the search of new antithrombotic molecules from medicinal plants. Literature produced evidence of various medicinal plants that exhbit antithrombotic activity. Plants such as *Pinus densiflora* (Park *et al.,* 2016), *Termininalia belerica* fruit extracts (Ansari *et al.,* 2012), *Crataegus orientalis (*Arslan *et al.,* 2011) and *Melastoma malabathricum* (Manicam *et al.,* 2013) have been reported to possess antithrombotic activity. Medicinal plants and isolated compounds have been reported to exhibit antithrombotic activity by inhibiting thrombin and thrombin induced platelet aggregation *in vitro*. These include bioactive compounds such as *oleanolic* acid*, betulinic* acid and *Calamintha offinalis extract* (Zoheir *et al.,* 2013; Lee *et al.,* 2012; Osunsanmi *et al.,* 2015; Osusanmi *et al.,* 2018).

Some extracts of medicinal plants such as *Aphanmixs polystachya*, *Enhydra fluctuans* Lour, *Averrho abclimb*, *Clerodendeum viscosum, Cutmina* and *Carsia berbesi* have been reported to dissolve whole blood clots, thus exhibiting

thrombolytic activity (Apu *et al.,* 2013; Kuri *et al.,* 2014; Fatima *et al.,* 2015; Ramjan *et al.,* 2014). Upon vascular endothelial injury, an autocatalytic response occurs between blood coagulation and inflammation, thus attention has been paid to the natural products with both anti-inflammatory and anticoagulant activities. The methanolic extract of *Ajuga bracteosa* has been reported to possess both anticoagulant and anti-inflammatory activity (Kayani *et al.,* 2016). Medicinal plants such as *Vitex negundo* and *Pinus roxburghili* have been reported to possess anti-inflammatory activity by inhibiting COX-2 activity (Chattopadhyay *et al.,* 2012; Kaushik et al., 2012). *Bulbine natalensis, Rapanea melanophloeos* and *Protohus longifolia* are some plants the Zulu traditional healers from KwaZulu-Natal use to treat blood clotting related disorders (Mosa *et al.,* 2011a).

2.6.1 *Protorhus longifolia (*Benrh.) Eng

Figure 2.5: The leaves, fruits and steam bark of *Protorhus longifolia*.

Protorhus longifolia (Benrh.) Engl (Figure 2.5) commonly known as Reed beech (English), rooimelkhout (Afikaans) and uhlangothi (Isizulu) belongs to the Anacardiaceae family. *P. longifolia* is a South African indigenous tree that is predominantly found in the forests and on the river sides of Kwa-Zulu Natal, Limpopo and the Eastern Cape. The stem bark of *P. longifolia* is used by Zulu traditional healers to treat blood clotting related disorders. Various reports have been given regarding the medicinal properties of the plant stem bark, including its use to treat diarrhoea and heartwater in cows (Dold and Cocks, 2001). Mosa *et al.* (2011a) reported that crude extracts, including water, hexane, chloroform and ethyl acetate, of *P. longifolia* stem bark possessed antioxidant and anti-platelet aggregation activity by inhibiting platelet aggregation induced by ADP, thrombin and epinephrine.

Several triterpenoids such as 3-oxo-5α-lanosta-8-24 diene-21oic acid, 3β-hydroxylanosta-9, 24-dien-oic acid methyl-3β-hydroxylanosta-9 and 24-dien-21-oate (Figure 2.6) have been isolated and characterized from the chloroform extract of *P. longifolia* (Mosa *et al.,* 2011b; Mosa *et al.,* 2014).

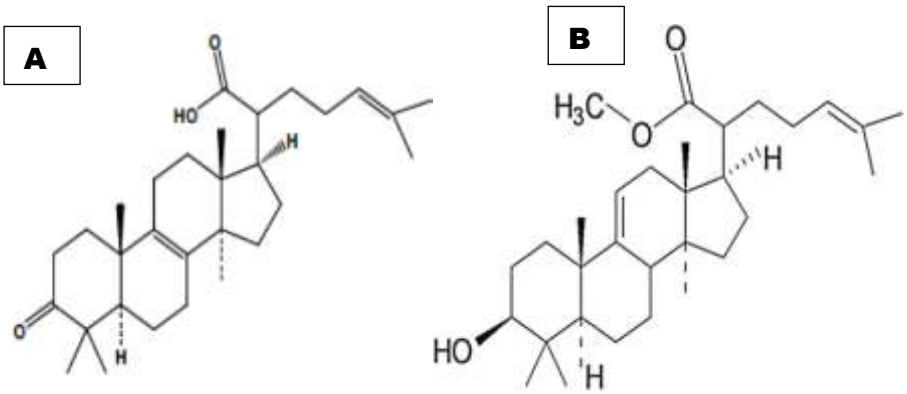

Figure 2.6: The chemical structures of acid methyl-3β-hydroxylanosta-9, 24-dien-21-oate (A) and 3β-hydroxylanosta-9, 24-dien-oic acid (B)

The lanosteryl triterpenes from *P. longifolia* stem bark have been reported to possess various bioactivities such as antiplatelet aggregation, anticoagulant, anti-inflammation (Mosa *et al*., 2011b), cardioprotective (Mosa *et al*., 2016), antihyperlipidemic (Machaba *et al*., 2014) and antihyperglycemic (Mosa *et al*., 2015; Mabhida *et al*., 2017) activities.

Antiplatelet aggregatory, anti-inflammatory, anticoagulant and antioxidant activities of the extracts and the isolated triterpenes from *P. longifolia* have been evaluated extensively over the years (Mosa *et al.*,2011a; Mosa *et al*., 2011b; Mosa *et al*., 2015; Mabhida *et al*., 2017). Medicinal plants are known to possess thousands of bioactive compounds distributed according to their varying polarities. The bioactive constituents present in the chloroform extract could be different from those present in the ethyl acetate extract. This project aimed to evaluate the antithrombotic and anti-inflammatory activities that could bring forth the isolation of new bioactive compounds from the ethyl acetate extract of the *P. longifolia*.

2.7 Common experimental models in anticoagulant and anti-inflammatory drug discovery

Various *in vivo* models have been utilized to evaluate the anticoagulant and anti-inflammation activities of potential pharmacological agents. Some of the anticoagulant methods used to evaluate antithrombotic activity include the tail bleeding time test, ferric chloride ($FeCl_3$) electrical and laser induced thrombosis in rat models. The $FeCl_3$ induced thrombosis involves a fluorescent dye that is used to observe platelets and leukocytes adhering to the site of injury using intravital microscopy attached on the mesentery of the rat. (Bonnard and Hagemeyer, 2015). In the laser induced thrombosis method, laser is used to target the vessel in order to initiate vascular injury and automatically triggering thrombus formation (Atkinson *et al*., 2010). The bleeding time test is a method widely utilized to evaluate the hemostatic parameters including platelet response and coagulation in *vivo* (Day *et al*., 2004; Liu *et al*., 2012). This test is simple and quick to perform, using no specific and sophisticated equipment. (Øvlisen *et al*., 2008; Greene *et al*., 2010; Molina *et al*., 2014). In this test, a 5 mm amputation is made on the rat tail and blotted on a filter paper per time intervals until the bleeding stops (Nieswandt *et al*., 2001; Liu *et al*., 2012). The interaction of injured vessels and platelet action leads to platelet plug formation which largely influences the bleeding time (Liu *et al*., 2012).

Histamine and serotonin are some *in vivo* inflammatory models used to evaluate acute inflammation. These increase vascular permeability and vasodilatation (Gupta *et al*., 2005). The carrageen induced paw edema model is used to determine the potential of pharmacological agents to withstand the biochemical changes linked with inflammation (Begum *et al.,* 2015). Carrageenan induced acute inflammation is known to be biphasic. The 1^{st} phase is linked with the release of serotonin and histamine. The 2^{nd} phase of carrageenan induced inflammation is associated with the elevated prostaglandin release and begins after the 1^{st} hour of inflammation (Vinegar *et al.,* 1969). The link between these phases involves the release of Bradykinins after 1hr 30min. The cotton pellet granuloma test (Nair *et al*., 2012) is normally used to evaluate the effect of novel agents that could be used against the proliferative phase, involved during chronic inflammation. In this study, considering the link between the coagulation system and inflammation, the carrageenan induced

rat paw edema and tail bleeding time test have been employed to investigate the antithrombotic and anti-inflammatory activity of the plant extract.

2.8 Aim and Objectives of the study

2.8.1 Aim

The aim of the study was to investigate the anti-thrombotic and anti-inflammatory activity of the ethyl acetate extract of *Protorhus longifolia*.

2.8.2 Objectives

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

i. To extract the plant material with ethyl acetate
ii. To investigate the anti-oxidant activity of the plant extract in vitro
iii. To determine the anticoagulant, antiplatelet aggregation and thrombolytic activities of the plant extract *in vitro.*
iv. To investigate the antithrombotic and anti-inflammatory activity of the extract in rats.

CHAPTER 3

3.0 Materials and Methods

This chapter describes the methods used to prepare and obtain the crude extract from the fresh plant material. It also describes the methods used to investigate the antioxidant, anti-thrombotic, antiplatelet aggregation, thrombolytic, anticoagulant and anti-inflammatory activities of the extract. Details the preparations of some reagents are presented in Appendix A.

3.1 Materials

3.1.1 Chemical and Reagents

The listed Chemicals were all purchased from Sigma Aldrich Co. Ltd (Steinheim, Germany):

2,2'-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid) (ABTS), ascorbic acid (AA), butylated hydroxyl-asinole (BHA), Carrageenan, collagen, citric acid, dextrose, dimethyl sulfoxide (DMSO), 2,2-diphenyl-1picryl-hydrazyl (DPPH), ethylenediaminetetra-acetic acid, ferrozine, gallic acid, heparin, indomethacin, potassium ferricyanide, tris-HCL, trichloro acetic acid (TCA), thrombin, streptokinase, quercetin, glutathione assay kit (Catalog number: CSO260), superoxide dismutase assay kit (Catalog number: 19160), antioxidant assay kit (Catalog number: CSO790), interleukin-6 ELISA kit (Catalog number: RABO333), Malondialdehyde assay kit (catalog number: MAK085)

The following ELISA kits were purchased from Elabscience (Houston,Texas, U.S.A): Rat Tumor Necrosis Factor (Catalog number: E-EL-R1037), Transforming Growth Factor Beta 1 (catalog number E-EL-R0084) and Prostaglandin Endoperoxide Synthase 2 (catalog number E-EL-R0792)

3.1.2 Equipment

Digital Plethysmometer LE 7500 (Panlab, Spain), Eppendorf centrifuge 5804 R (Merck), platform shaker (Labcon, Polychem supplies), rotary evaporator (Heidolph

Instruments), microplate reader (Synergy HT, BioTek Instruments), homogeniser (Ultra-Turrax), light microscope (Carl Zeiss)

3.2 Methodology

3.2.1 Plant collection and extraction

Fresh stem barks of *Protorhus longifolia* (Benrh) Engl were collected from Kwa-Hlabisa Kwa-Zulu Natal. The identity of the plant (voucher specimen: RAUZ01) was confirmed a Botanist in the Department of Botany, University of Zululand. The plant material was washed, cut in to small pieces and air dried. The dried plant material was sequentially extracted with n-hexane, chloroform and ethyl acetate, each for 24 hours at room temperature with constant shaking on platform shaker at 150 rpm. The extracts were separately filtered, and the filtrates concentrated *in vacuo* (using rotary evaporator at 40 ± 2 °C) to obtain crude extracts. The crude ethyl acetate extract (EA) was used in the study.

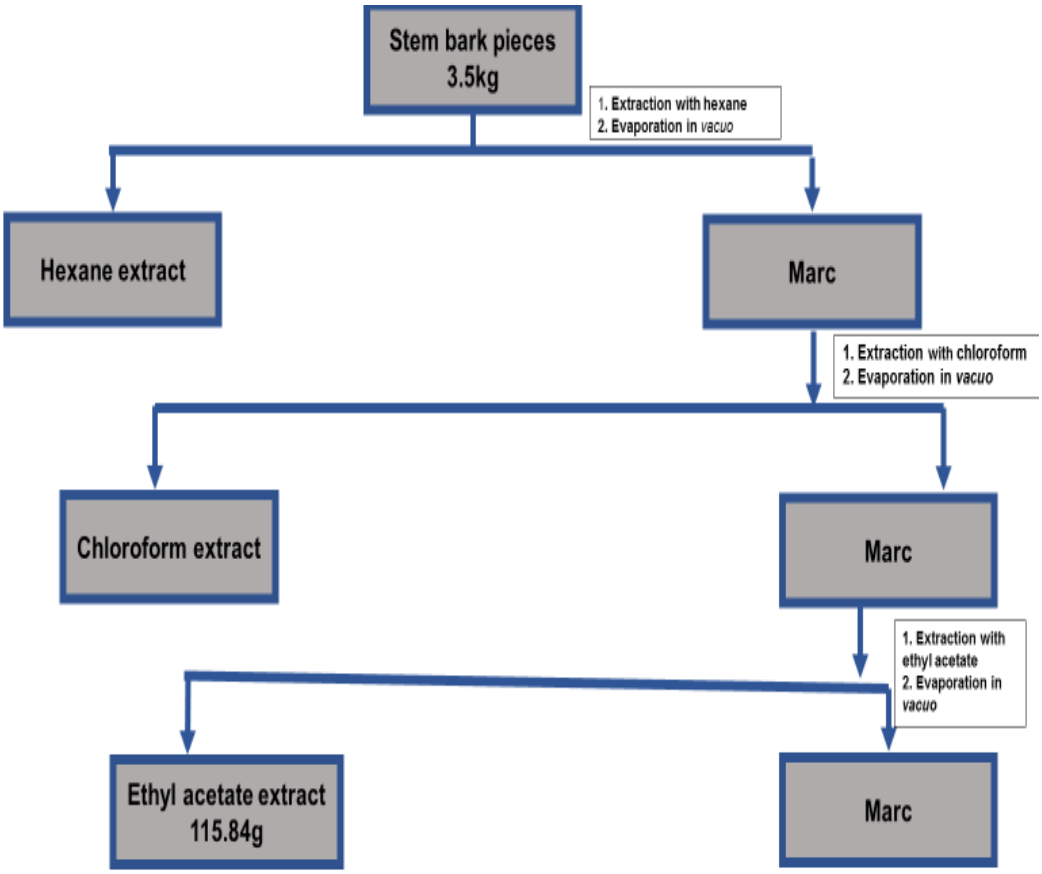

Figure 3.1: Experimental design for extraction of the plant material to obtain the crude ethyl acetate extract.

3.2.2 Quantitative phytochemical screening

3.2.2.1Total flavonoid content

The alcoholic aluminium chloride colorimetric assay described by Ordonez *et al.,* (2006) was used to determine the total flavonoid content of the extract. The extract (0.5 mg/ml) was mixed with 0.5 ml of 2% aluminium chloride solution. The reaction mixture was incubated at room temperature for 1 hour. Quercetin, a known flavonoid, was used as the standard and the blank consisted of 2% alcoholic aluminium chloride solution. Absorbance was read at 420 nm and total flavonoid content of the extract was determined from the calibration curve of quercetin. The results were expressed as mg/g dry weight of the plant material.

3.2.2.2Total phenolic content

The total phenolic content of the extract was determined using the Folin-Ciocalteu method as described by Kim *et al*. (2003). The crude extract (0.5 mg/ml) was mixed with 1.5 ml Folin-Ciocalteu (1:10 v/v) reagent and 1.2 ml sodium carbonate (7.5%). The reaction mixture was incubated in the dark for 30 min at room temperature. The total phenolic content was determined from the calibration curve of gallic acid. The results were expressed as mg/g dry weight of the plant material.

3.2.3 *In vitro* antioxidant activity

The antioxidant activity of the crude ethyl acetate was evaluated against DPPH, ABTS and nitric oxide radicals. Its reducing power and metal ion chelating potential were also determined. Unless otherwise stated, all the experiments were replicated three times and mean values of the results reported. Ascorbic acid (AA) and butylated hydroxyl-anisole (BHA) were used as standards. Biotek micro plate reader (Synergy HT, Biotek Instruments) was used to measure absorbance of the samples. Percentage activity of the extracts was calculated using the formula

$$\% \text{ scavenging activity} = [1-A_E/A_C] \times 100$$

Where, A_E =absorbance in the presence of the extract, A_C = absorbance of the control sample.

3.2.3.1 1, 1-diphenyl-2-picryl hydrazyl (DPPH) scavenging activity

DPPH radical scavenging activity of the plant extract was determined using the method of Brad-Williams (1995). DPPH (0.02 mg/ml) was mixed (1:1) with a different concentration (0-20 µl/ml) of the extract. The mixture was allowed to stand at room temperature for 30-60 min with intermittent shaking. Absorbance was read at 517 nm against methanol as blank.

3.2.3.2 ABTS radical scavenging activity

The ABTS assay (Re *et al*., 1999) was used to determine the free radical scavenging activity of the extract. The ABTS stock solution (in 3 ml of distilled water) was prepared by mixing 7mM ABTS and 2.45 mM potassium persulfate. The mixture was incubated in the dark for 16 hours The ABTS radical working solution was prepared by diluting 1 ml of the stock solution with 60 ml of methanol. The extract, at different concentrations (0-20 µl/ml), was mixed (1:1) with the ABTS radical. The mixture was then incubated at room temperature for 6 min. The absorbance was read at 734 nm.

3.2.3.3 Nitric oxide ($NO^{\bullet}$) radical scavenging activity

The nitric oxide radical assay described by Badami *et al*. (2013) was followed to evaluate the nitric oxide radical scavenging activity of the extract. The reaction mixture contained 2 ml of 10 mM sodium nitroprusside, 0.5 ml of phosphate buffer saline (pH 7.4, 0.01 M) and 0.5 ml of different concentrations (0 – 5 mg/ ml) of the plant extract. The mixture was incubated at 25 °C for 150 min and then 1.0 ml of sulphanilic acid reagent (0.33% in 20% glacial acetic acid) was added. The reaction mixture was allowed to stand at room temperature for 5 min and this was followed by the addition of 0.1 ml of 0.1% naphthylethylenediamine dihydrochloride. The mixture was allowed to stand for a further 30 min in the dark. Absorbance was read at 540 nm.

3.2.3.4 Reducing power

The reducing power of the plant extract was evaluated following the method described by Ferreira *et al*. (2007). The reaction mixture consisted of 100 µl of 0.2 M phosphate buffer (pH 6.6), 100 µl of 1% potassium ferricyanide and 100 µl of plant

extract at different concentrations (0-4 mg/ml). The reaction mixture was incubated at 50ºC for 20 min. After incubation, 500 µl of 10% trichloroacetic acid was added to the reaction mixture and mixed well. The mixture was centrifuged at 1000 rpm for 10 min. Thereafter, 500 µl of the supernatant was collected and diluted with 500 µl of distilled water and 100 µl of 0.1% $FeCl_3$ was added to the reaction mixture. Ascorbic acid was used as standard and absorbance was read at 700 nm.

3.2.3.5 Metal ion (Fe^{2+}) chelating activity

The metal ion chelating activity of the extract was evaluated using the method of Decker and Welch (1990). The reaction mixture contained 0.5 ml of the extract at different concentrations (0- 5 mg/ml), 0.05 ml of 2 mM $FeCl_2$ and 1.6 ml of deionised water. The reaction was initiated by adding 0.1 ml of 5 mM ferrozine. The mixture was then mixed and incubated for 10 min at room temperature. Citric acid and EDTA were used as standards. Absorbance was read at 562 nm spectrophotometrically.

3.2.4 Antithrombin activity *in vitro*

The antithrombin activity of the ethyl acetate extract of *P. longifolia* was evaluated using an artificial chromogenic substrate (H-D-phenylalanyl-L-pipecolyl-p-nitroandile dihydrochloride) (Rob *et al.,* 1997). The plant extract (50 µl) at different concentrations (0.04, 0.2 and 1 mg/ml of 1% DMSO) were mixed with 10 µl of thrombin (5 U/ml). The mixture was allowed to stand at room temperature for 10 min and the reaction was initiated by adding 190 µl of the chromogenic substrate. Absorbance was read at 412 nm for 8 min at 1 min intervals.

3.2.5 Animals

The approval (UZREC 171110 – 030 PGM 2017/438) for laboratory animal use and protocols in the study was obtained from the University of Zululand Ethics Committee (UZREC). Adult Sprague Dawley rats of either sex were obtained from the Department of Biochemistry and the Microbiology Animal Unit. The rats were kept under standard living conditions (12 h light- dark cycle, temperature 23 ± 2ºC). The animals had free access to food (pelleted rat feed) and drinking water.

3.2.6 Thrombolytic activity

The thrombolytic activity of the extract was evaluated following the method of Prasad *et al*. (2006). Blood (0.5 ml) was collected from the Sprague Dawley rats and put into separate pre-weighed non-citrated tubes. The tubes were incubated at 37ºC for 45 min to develop a firm clot. Excess serum was removed completely without disturbing the formed clot and weight of the clot was determined. To these tubes, 100 µl of the extract at different concentrations (1, 5, and 10 mg/ml) was added. Equal volumes of streptokinase (10 KU) and 1% DMSO solution were added into the positive and negative control tubes, respectively. All the tubes were incubated at 37ºC for 90 min and thereafter, any fluid formed in the tubes was completely removed. The weight of the remaining clot in each tube was again determined. The experiment was replicated three times. The thrombolytic activity of the extract was calculated using the formula.

Clot weight difference= Original $_{\text{clot weight}}$ – lysed $_{\text{clot weight}}$

Clot dissolution % = (clot weight difference / Original clot weight) x 100

3.2.7 Antiplatelet aggregation activity

3.2.7.1 Blood platelet preparation

The method described by Tomita *et al.* (1983) was followed to collect and prepare blood platelets. A rat was euthanized under anaesthesia and blood was immediately collected by cardiac puncture. The blood was immediately mixed (5:1 v/v) with an anticoagulant [containing 0.65 M citric acid, 2% dextrose and 0.085 M tri-sodium citrate]. The blood was first centrifuged at 1200 rpm for 15 min and then at 220 rpm for 3 min consecutively. The supernatant was collected and re-centrifuged at 3200 rpm for 15 min. The supernatant was discarded and the remaining sediment containing platelets was washed with 5 ml of wash buffer (pH 6.5). The platelets and wash buffer solution were centrifuged at 3000 rpm for 15 min and the supernatant was discarded. The sediment (platelets) was dissolved in 1 ml of resuspending buffer (pH 7.4) to serve as stock solution. The working solution was prepared by diluting (1:10 v/v) the platelets with the resuspending buffer. This was supplemented with 10 % $CaCl_2$ (1: 0.04 v/v).

3.2.7.2 Antiplatelet aggregation activity

The antiplatelet aggregation activity of the extract was evaluated using the method of Osunsanmi *et al*. (2018). The reaction mixture consisted of 200 µl of the prepared platelet rich plasma and 20 µl of the extract at different concentrations (0.2, 1 and 5 mg/ml). The reaction mixture was then incubated at 37ºC for 5 min. DMSO solution (1%) and aspirin (0.2, 1 and 5 mg/ml) were used as negative and positive controls, respectively. Platelet aggregation was stimulated by the addition of 20 µl of thrombin (5 Units/ml) into the reaction mixture. After 3 min, 5 µl of the reaction mixture was placed on a slide and the prepared slides were view under a light microscope (magnification X400) for any platelet aggregation.

3.2.8 Carrageenan-induced acute inflammation

The anti-acute inflammatory activity of the plant extract was evaluated using the carrageenan-induced hind rat paw oedema model (Winter *et al.,* 1962). Sprague-Dawley rats of either sex (180-200 g) were randomly divided into six groups of five rats per group. The animals in the different groups were treated as shown in Figure 3.2. Seven consecutive days prior to induction of inflammation, the rats in their respective groups received a single oral dose of the extract (at 100 and 350 mg/kg), indomethacin (10 mg/kg, positive control 1), aspirin (30 mg/kg, positive control 2) and 2% Tween 20 (carrier solvent, negative control). The normal group received an equivalent oral dose of distilled water.

On the 8^{th} day, 1 hour after oral administration of the drugs, the experimental rats were subcutaneously injected with a freshly prepared 1% Carrageenan solution (0.1 ml) on the right hind paw to induce acute inflammation. The left hind paw was injected with 0.1 ml of normal saline to serve as a control. The water displacement method, using a digital plethysmometer (EL 7500), was used to measure the swelling volume of the paws. The swelling volumes were taken at hourly intervals for four hours. The difference between the right and left hind paws in the respective groups was used to determine the level of inflammation. The swelling volumes were compared between the different groups to determine the anti-acute inflammatory activity of the plant extract.

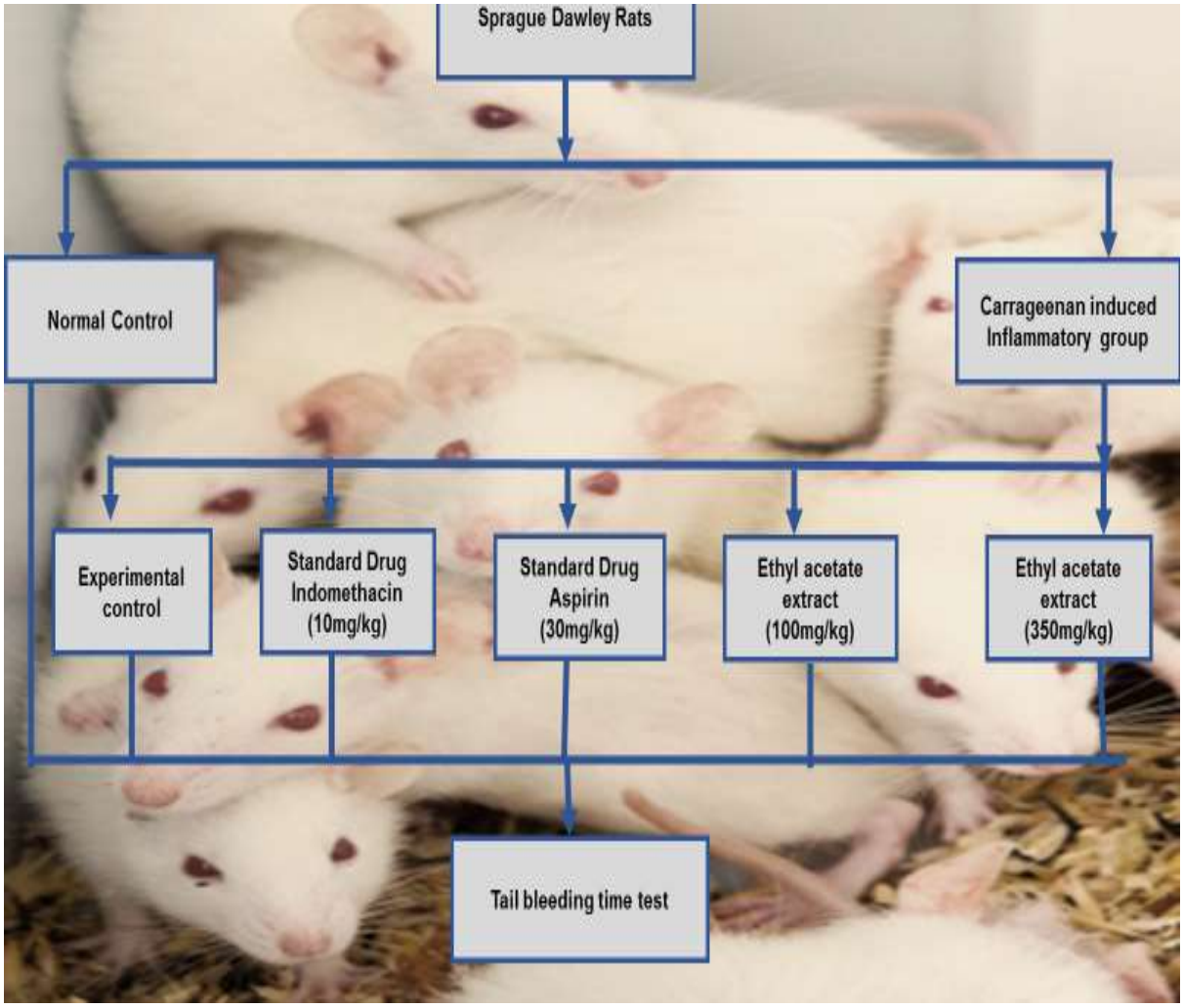

Figure 3.2: The experimental design of the anti-inflammatory effect of the extract in carrageenan induced inflammation in rat model.

3.2.8.1 Tail bleeding time test

Considering the link between inflammation and the blood coagulant system, the anticoagulant property of the extract was also evaluated at the end of the four hours of the anti-inflammation evaluation. The tail bleeding time test was used to evaluate the anticoagulant properties of the extract (Klafke *et al.,* 2011). The rats were slightly anaesthetized, and 5 mm of the rat's tail tip was amputated. The tail was then blotted on the filter paper at 30s intervals until the bleeding of the tail stopped. The time it took for the bleeding to stop was taken as the bleeding time. The bleeding times were then compared between the groups. At the end of the experiment, all the rats were then euthanized under anaesthesia, blood and the hind paws of the rats were collected for biochemical analysis of some inflammatory and oxidative stress biomarkers.

3.2.8.2 Blood serum and paw sample preparation

The collected blood samples were allowed to clot and then centrifuged at 1200 rpm for 15 min to obtain serum. The rat paw homogenate was prepared by separately homogenizing the paws (1:10) in Tris homogenization buffer (0.1 M, pH 7.4). The homogenate was centrifuged at 5000 rpm for 30 min and the supernatant collected. The serum and paw homogenate (supernatant) were kept at -80 ºC until required for use.

3.2.9.3 Biochemical analysis

Standard pathology procedures (Global Labs, Richards Bay, KZN) were followed to determine the serum levels of liver function enzymes (AST, ALT, ALP) and kidney function biomarkers (creatinine, urea). The homogenate supernatant was used to estimate the tissue (paw) levels of some inflammation biomarkers (transforming growth factor beta 1 (TGF-β1), cyclooxygenase-2 (COX-2), interleukin -6 (IL-6) and tissue nuclear factor-α (TNF-α), as well as the antioxidant markers superoxide dismutase (SOD) and reduced glutathione (GSH) content). Commercial assay kits were used following the respective manufacturers' instructions to estimate the tissue levels of the listed biochemical parameters.

3.2.10 Data analysis

The experiments were at least triplicated, and the results obtained were presented as mean ± SEM or mean ± SD. The results were statistically analysed by one-way analysis of variances (ANOVA) and turkey using Graph Pad Prism v6. The statistical difference between the groups was considered significant where $p < 0.05$.

CHAPTER 4

4.0 Results

4.1 Total phenolic and flavonoid contents

The total flavonoids and phenols present in the ethyl acetate extract of *P. longifolia* stem bark were quantified as quercetin and gallic acid equivalents, respectively. The flavonoids content (0.08 mg/g) of the extract was detected to be higher than the total phenolic content (0.05 mg/g) (Figure 4.1).

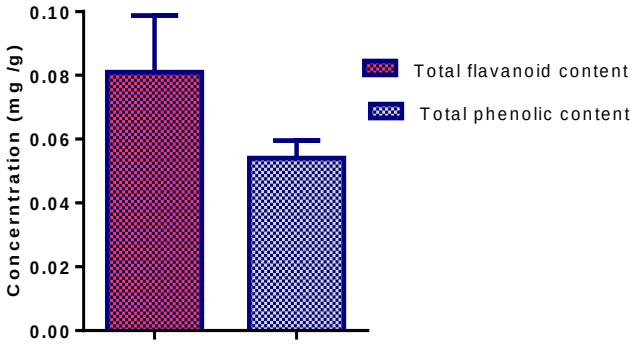

Figure 4.1: Total flavonoid and phenolic contents present in ethyl acetate extract of *P. longifolia* stem bark. Data are expressed as mean ± S.D, n=3.

4.2 Antioxidant activity

The free radical scavenging activity of the ethyl acetate extract of *P. longifolia* was evaluated against DPPH, ABTS and nitric oxide radicals. The extract showed, to a varying degree of efficacy, a concentration dependant free radical scavenging activity (Table 4.1). The highest activity of the extract (with IC_{50} values ranging from 4.8 µg/ml to 5.78 µg/ml) was observed against DPPH and ABTS when compared to the nitric oxide radical with the IC_{50} value < 5 mg/ml.

The reducing power and metal ion chelating activity of the extract was also evaluated and the results are presented in Figure 4.2. While the plant extract showed a strong reducing power, only a moderate metal ion (Fe^{2+}) chelating activity was observed.

Table 4.1: Free radical scavenging activity of ethyl acetate extract on DPPH, ABTS and nitric oxide radicals.

Concentration (µg/ml)	DPPH	ABTS	Concentration (mg/ml)	NO•
1.25	29.8 ± 0.01	28.0 ± 0.02	**0.2**	10.5 ± 0.08
2.5	35.3 ± 0.02	36.2 ± 0.03	**0.6**	10.5 ± 0.09
5	46.0 ± 0.04	52.0 ± 0.03	**1.0**	14.5 ± 0.06
10	56.0 ± 0.02	41.8 ± 0.21	**3.0**	12.3 ± 0.05
20	73.8 ± 0.04	83.6 ± 0.02	**5.0**	5.4 ± 0.13
IC_{50}	5.75 µg/ml	4.8 µg/ml	**IC_{50}**	< 5
BHA (IC_{50})	1.13 µg/ml	10.8 µg/ml	**BHA (IC_{50})**	0.467
AA (IC_{50})	**1.92 µg/ml**	**4.25 µg/ml**	**AA (IC_{50})**	**0.183**

Data are expressed as mean ± S.D, (n=3).

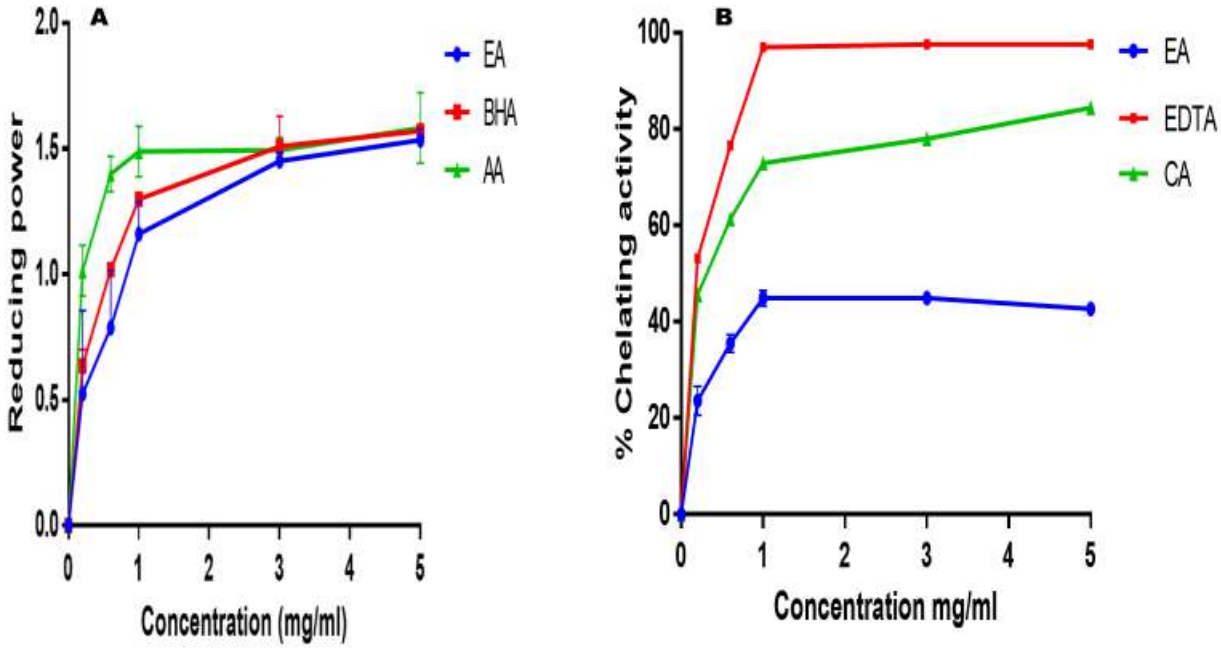

Figure 4.2: Reducing power (A) and Fe^{2+} chelating activity (B) of the ethyl acetate extract (EA) of *P. longifolia.* Data are expressed as mean ± S.D, n=3.

4.3 Antithrombin activity

Figure 4.3 presents the *in vitro* effect of the plant extract on thrombin activity. The extract showed thrombin inhibitory activity in a concentration dependent manner. The extract at 1 mg/ml showed the most noticeable thrombin inhibitory activity.

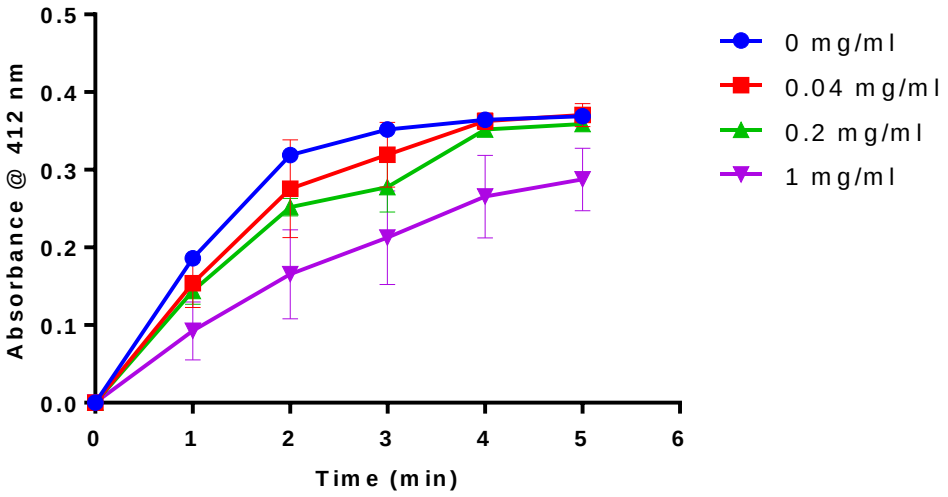

Figure 4.3: The inhibitory effect of the ethyl acetate extract on thrombin activity. Data are expresses as mean ± S.D, n=3.

4.4 Thrombolytic activity

The thrombolytic activity (clot dissolution) activity of the extract was evaluated on the whole blood clot. The extract also showed a concentration dependent thrombolytic activity (Table 4.2). Even though not comparable to that of streptokinase, the extract showed a significantly higher percentage dissolution activity at 5 mg/ml and 10 mg/ml when compared to the effect of 1% DMSO solution which was used as solvent control.

Table 4.2: The results of the thrombolytic activity of the plant extract

Concentration (mg/ml)	Initial Clot weight (g)	Lysed clot weight (g)	% Clot Dissolution
1	0.15 ± 0.09	0.13 ± 0.03	11.4 ± 2.99
5	0.23 ± 0.05	0.17 ± 0.04*	23.5 ± 7.86*
10	0.22 ± 0.05	0.13 ± 0.04	35.7 ± 7.00***
Streptokinase (10 KU)	0.19 ± 0.05	0.05 ± 0.05	95.6 ± 6.16
1% DMSO	0.41 ± 0.05	0.37 ± 0.67	8.4 ± 4.81

Data are expressed as mean ± S.D, n=3. *p ≤ 0.05, **p ≤ 0.01, ****p ≤ 0.0001 vs experimental control

4.5 Antiplatelet aggregation activity

The antiplatelet aggregation activity of the extract was evaluated on platelet rich plasma and the obtained results are shown in Figure 4.4. The results revealed that the inactivated platelets (normal platelets without stimulus) are free from each other and red blood cells (RBC), but upon activation with thrombin, aggregates of platelets on RBC were observed. The presence of the extract seemed to inhibit platelet aggregation in a concentration dependent manner, as seen by an increasing number of platelets dissociating from RBC as the concentration of the extract increases. Much better dissociation of platelet aggregates were observed upon treatment of the platelets with aspirin.

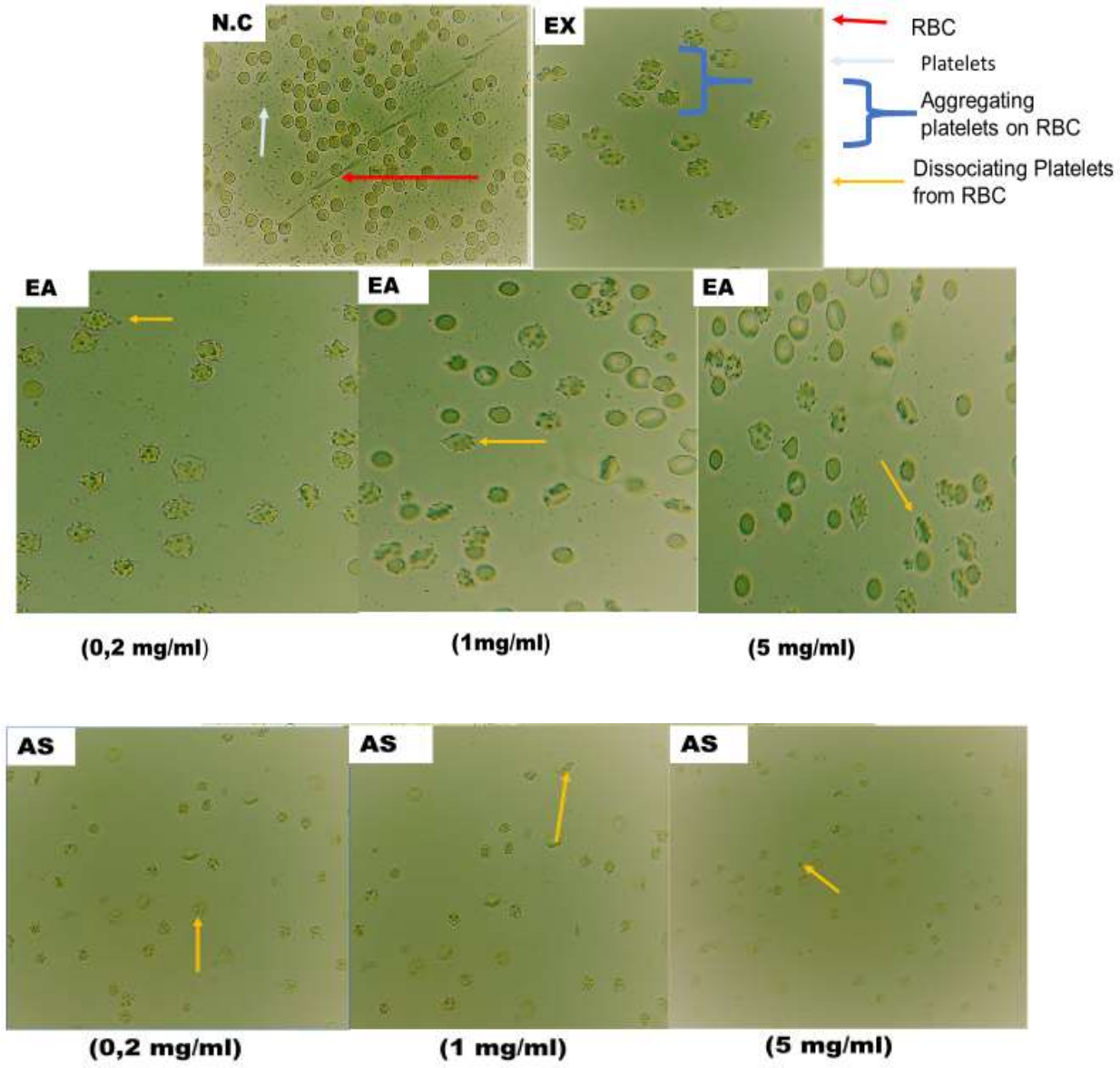

Figure 4.4: Microphotographs showing the extract's effect on thrombin induced platelet aggregation (magnification 400X. NC- normal control, normal platelets; EX- experimental control, platelets activated with thrombin; EA– ethyl acetate extract at different concentrations (0.2, 1 and 5 mg/ml), platelets activated with thrombin in the presence of the extract. AS-Aspirin at different concentrations (0.2, 1, 5 mg/ml), platelets activated with thrombin in the presence of aspirin.

4.6 The effect of the extract on inflammation and coagulation *in vivo*

4.6.1 Serum levels of some kidney function biomarkers and liver function enzymes

The potential toxicity of the extract on the rat's kidney and liver was determined by estimating the serum levels of some kidney function biomarkers and liver function enzymes. Table 4.3 shows elevated serum levels of kidney and liver function biomarkers in the untreated group when compared to the normal control group. The group, orally administered with the extract at 100 mg/kg, showed low serum levels of the liver and kidney function biomarkers. However, relatively higher serum levels of BUN and AST were observed in the rats group administered with the extract at a higher concentration of 350 mg/kg.

Table 4.3: Effect of *P. longifolia* ethyl acetate extract on the serum levels of BUN, creatinine, ALP, AST and ALT

Group	Urea (mmol/L)	Creatinine (umol/L)	AST (IU/L)	ALT (IU/L)	ALP (IU/L)
Normal group	4.75 ± 0.56	27.7 ± 3.30	359 ± 81.7	74 ± 3.6	108.3 ± 14.7
Untreated group	6.65 ± 0.64	40.3 ± 4.40	430 ± 71.4	145 ± 29	123.3 ± 8.6
EA (100 mg/kg)	5.00 ± 0.85	20.0 ± 8.00*	437 ± 102.2	74.3 ± 12	23.3 ± 3.8***
EA (350 mg/kg)	7.63 ± 0.59**	43.3 ± 1.85	707 ± 39.6*	163.5 ± 19	122 ± 11.4
Indomethacin (10 mg/kg)	7.67 ± 0.49**	43.0 ± 2.08	701 ± 217	180 ± 67*	135 ± 16.5
Aspirin (30 mg/kg)	6.27 ± 0.23	34.0 ± 2.08	308 ± 51.7	110.7 ± 6	110 ± 18.1

Data are expressed as mean ± S.E.M, n=5. *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.0001 vs experimental control

4.6.2 The anticoagulant activity of the extract *ex vivo*

Figure 4.5 shows the results obtained from evaluation of the *ex vivo* anticoagulant activity (tail bleeding time test) of the plant extract. The normal control group showed a normal bleeding time of about 5.5 min. However, an obvious decrease in bleeding time was observed in the inflamed and untreated group with a bleeding time of 3.18 min. Oral administration of the rats with the extract seemed to prolong the bleeding

time of the rats in a concentration dependent manner. The highest concentration of the extract (350 mg/kg) prolonged the bleeding time up to 8.8 min. A similar effect was observed in the aspirin treated group.

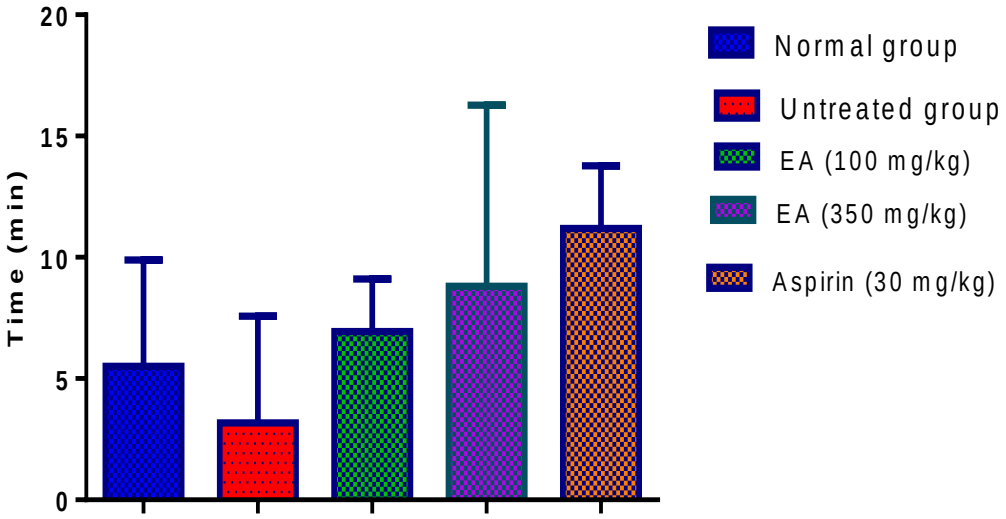

Figure 4.5: The effect of ethyl acetate extract on tail bleeding time assay. Data are expressed as mean ± S.E.M, n=5.

4.6.3 The anti-inflammatory activity of the extract on carrageenan induced rat paw edema

The carrageenan induced hind rat paw oedema model is commonly used to investigate acute anti-inflammatory activity of plant extracts. In this study, an increase in the paw volume was observed in the untreated group for a period of 3 hours, followed by a drop after the 4th hour (Figure 4.6). The extract pre-treated groups showed decreased paw volumes when compared to the untreated group. The rats pre-treated with the extract at 100 mg/kg exhibited a marked decrease in the paw swelling size after 3 hours of the experiment when compared to the indomethacin treated group that showed the marked decrease in the rat paw swelling after only 2 hours.

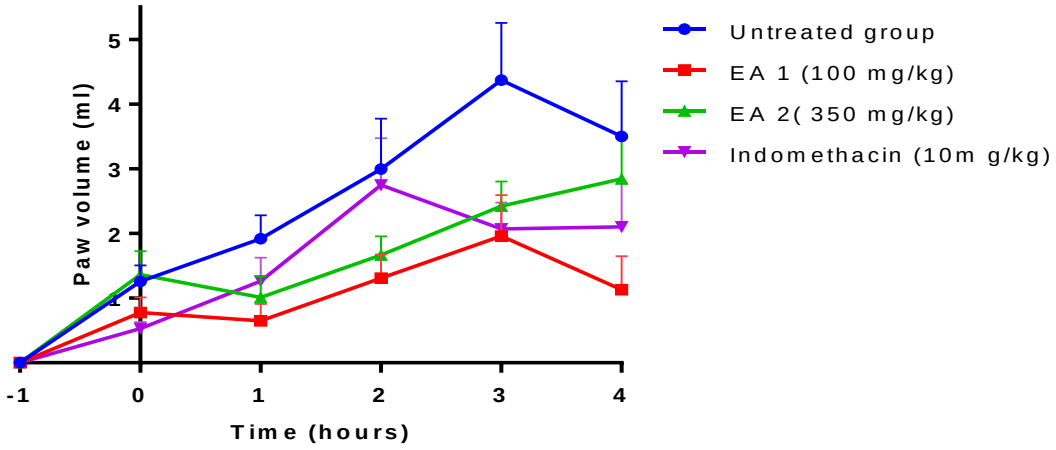

Figure 4.6: The effect of the ethyl acetate extract on carrageenan induced inflammation. The swelling volume was measured at hourly intervals for four hours following inflammation induction. (-1 represents the reading before inducing inflammation). Data are expressed as mean ± S.E.M, n= 5

4.6.3.1 Tissue levels of some inflammation biomarkers

Figure 4.7 shows the results of the levels of some tissue inflammation biomarkers such as IL-6, TNF-α, COX-2 and TGF-β1. An increase in the levels of IL-6, TNF-α, COX-2 and TGF-β1 were observed in the untreated group when compared to the levels observed in the normal group. However, even though no significant changes in the levels of IL-6 and TNF-α were observed in the groups administered with the extract, a marked decrease in the levels of COX-2 and TGF- β1 were observed. A similar pattern was observed in the indomethacin treated group.

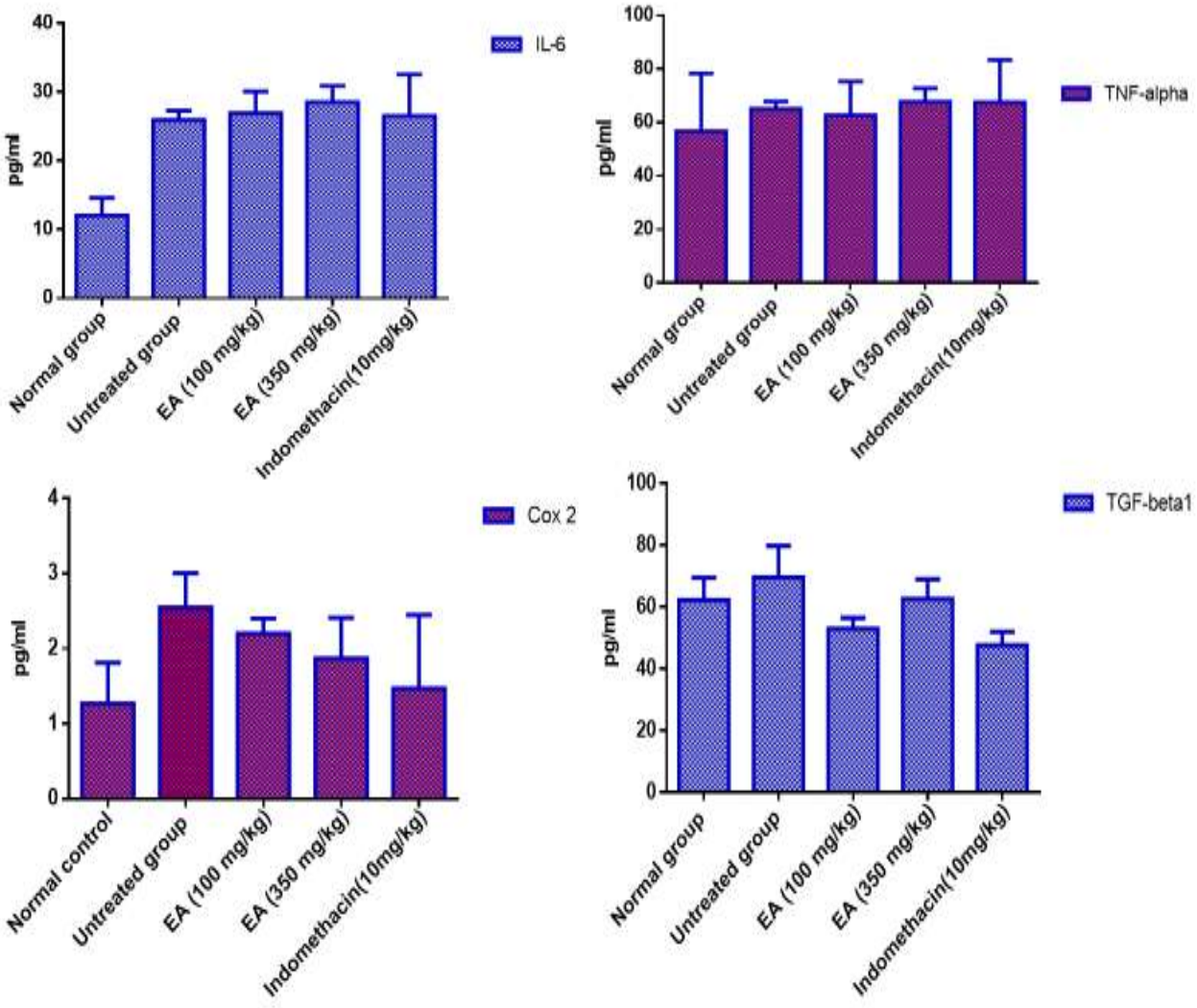

Figure 4.7: Effect of the ethyl acetate extract (EA) on some inflammation biomarkers (IL-6, TNF-α, COX-2 and TGF-β1). Data are expressed as mean ± S.E.M, n=5.

4.6.3.2 Effects of the *P. longifolia* ethyl acetate extract on the antioxidant levels in carrageenan induced acute inflammation in rats.

Table 4.4 shows the results of the antioxidant status on the inflamed paws. The obtained results showed a noticeable decrease in the antioxidant status of the untreated group when compared with the normal group. Increased levels of total antioxidant capacity and SOD were observed in the rats pre-administered with the plant extract at both concentrations. At 350 mg/kg a significant increase of the total antioxidant capacity was observed when compared to the untreated group. Even though noticeable changes in antioxidant status and SOD activity were observed in

all the groups, no significant changes where observed in the MDA and GSH levels. Indomethacin also increased the antioxidant status of the animals.

Table 4.4: Effect of the extract on total antioxidant capacity, SOD, GSH and MDA levels in inflamed rat paws.

Groups	**TAC (mM)**	**SOD (% inhibition)**	**GSH (nmol/ml)**	**MDA (nmol/ml)**
Normal group	1.58 ± 0.12	26.4 ± 5.40	$4 \times 10^{-3} \pm 2 \times 10^{-3}$	$6 \times 10^{-4} \pm 4 \times 10^{-4}$
Untreated group	0.65 ± 0.23	19.1 ± 0.65	$6 \times 10^{-4} \pm 6 \times 10^{-5}$	$7 \times 10^{-4} \pm 3 \times 10^{-4}$
EA (100 mg /kg)	1.08 ± 0.50	24.3 ± 5.80	$6 \times 10^{-4} \pm 2 \times 10^{-5}$	$7 \times 10^{-4} \pm 2 \times 10^{-4}$
EA (350 mg/ kg	$2.22 \pm 0.05^{**}$	32.9 ± 1.00	$6 \times 10^{-4} \pm 2 \times 10^{-4}$	$7 \times 10^{-4} \pm 2 \times 10^{-4}$
Indomethacin (10 mg/kg)	1.10 ± 0.17	33.9 ± 3.10	$7 \times 10^{-4} \pm 3 \times 10^{-4}$	$7 \times 10^{-4} \pm 3 \times 10^{-4}$

Data are expressed as mean ± S.E.M, n=5, **p ≤ 0.01, vs experimental control, TAC- Total antioxidant capacity

CHAPTER 5

5.0 Discussion

Thrombosis is the main underlying cause of cardiovascular diseases which are the major cause of premature deaths globally. Despite great efforts made in the discovery of antithrombotic agents, ischemic events continue to claim more lives. In a search for new therapeutic agents, the antithrombotic activity of various medicinal plants, as crude extracts or pure isolated compounds, have been explored (Ansari *et al.,* 2012; Park *et al.,* 2016; Ku *et al*., 2015; Osunsanmi *et al.,* 2015; Bukhari *et al.,* 2016). Anticoagulant and anti-inflammatory activities of a lanosteryl triterpene, isolated from the chloroform extract of *P. longifolia* stem bark, have also been reported (Mosa *et al.,* 2011b; Mosa *et al.,* 2015). Since medicinal plants possess an unmatched diversity of bioactive compounds with different properties, the current study investigated the antithrombotic and anti-inflammatory activity of the ethyl acetate extract of *P. longifolia* stem bark.

The *in vitro* antithrombin, antiplatelet aggregation and thrombolytic assays were used to investigate the antithombotic activity of the ethyl acetate extract of *P. longifolia* stem bark. Internal blood clot (thrombus) formation requires platelet activation and coagulation reactions. Thrombin is one of the major platelet agonists that stimulates platelet aggregation. Thrombin is also an important enzyme in the coagulation cascade as it converts soluble fibrinogen molecules into insoluble fibrin strands that form a meshwork with blood cells to form a clot. The results obtained indicate the antithrombotic potential of the ethyl acetate extract of *P. longifolia*. This is indicated by the ability of the extract to inhibit thrombin activity (Figure 4.3) and platelet aggregation (Figure 4.4). The inhibition of thrombin induced platelet aggregation was observed by the disintegration of platelets from the red blood cells in the presence of the extract. Some plant-derived bioactive compounds and crude extracts of some medicinal plants have been reported to inhibit thrombin and thrombin induced platelet aggregation (Lee *et al.,* 2012; Rao *et al.,* 2014; Ku *et al.,* 2015; Osunsanmi *et al.,* 2018). The obtained results are also consistent with those reported by Mosa *et*

al. 2011a and Mosa *et al*. 2011b in which both the crude extracts and a pure compound (triterpene) from *P. longifolia* stem bark showed antiplatelet aggregation activity.

Inhibition of platelet aggregation and coagulation factors prolongs the bleeding time. The tail bleeding time assay is commonly used to assess the *in vivo* anticoagulant activity of plant extracts (de Caterina *et al.,* 1994; Chen *et al.,* 2014; Mosa *et al.,* 2015; ul Ain *at al*., 2018). The noticeable decrease in tail bleeding time of the rats in the untreated experimental group (Figure 4.5) was an indication of a possible suppression of the anticoagulant and fibrinolytic system as a result of the induced inflammatory state in the rats. The observed prolonged tail bleeding time, following the pre-treatment of the rats with the extract (at both concentrations), suggested the anticoagulant effect of the extract, which thus supported the observed *in vitro* antithrombin and antiplatelet aggregation activity of the extract. Literature reports have demonstrated the influence on antiplatelet aggregation of several medicinal plants, such as *Juglans regia* (Amir ou *et al.,* 2018), *Ramulus mori* (Lee *et al.,* 2016) and *Dioscorea zingiberensis* (Gong *et al.,* 2011) extracts; and on prolonged tail bleeding times exhibited by various medicinal plants (Amirou *et al.,* 2018). The anticoagulant activity exhibited by the extract *in vivo* could be attributed to its ability to directly inhibit thrombin activity as well as inhibition of platelet aggregation.

The thrombolytic activity displayed by the extract (Table 4.2) further indicated the potential of the extract to dissolve the already formed blood clot. Unnecessary thrombus formation in a blood vessel, if not dissolved, causes ischemic stroke or myocardial infraction (Jin *et al.,* 2004; Lee *et al.,* 2016). Some extracts of other medicinal plants, such as *Aphanmixs polystachya* (Apu *et al.,* 2013), *Enhydra fluctuans* Lour (Kuri *et al.,* 2014) and *Clerodendeum viscosum* (Ramjan *et al.,* 2014), have also been reported to possess thrombolytic activity. Phytochemicals such as tannins, saponins, alkaloids, steroids and cardiac glycosides have been reported to influence thrombolytic activity of some medicinal plants (Mahmud *et al.,* 2015; Islam *et al.,* 2018).

There is also a strong link between blood coagulation and inflammation (Verhamme and Hoylaerts, 2009; Chu, 2011). Under pathophysiological conditions, the natural anticoagulant system is suppressed by inflammation which favours expression of

prothrombogenic molecules (Verhamme and Hoylaerts, 2009). The link between the coagulation system and inflammation is via thrombin and activated platelets. Thrombin binding to PARs induces the expressions of pro-inflammatory molecules (Szaba and Smiley, 2002; Chu, 2011). Activated platelets, in addition to pro-inflammatory cytokines, release chemokines that directly affect vascular injury (Aggrey *et al.,* 2013; Morrell *et al.,* 2014). The obtained results further indicate that in addition to its anticoagulant effect, the extract can also inhibit inflammation and its related complications including thrombosis and tissue damage. Thus, therapeutic agents with both anticoagulant and anti-inflammatory properties are important in the management of thrombotic disorders (Hirsh *et al.,* 2007).

The anti-inflammatory activity of the extract was investigated using the carrageenan induced paw edema rat model. The mechanism of the Carrageenan induced acute inflammation is known to be biphasic. The 1st phase is linked with the release of serotonin and histamine. The 2nd phase of carrageenan induced inflammation is associated with elevated prostaglandin levels after the 1st hour. (Vinegar *et al*., 1969). In this study, the observed increase in the paw volume for a period of 3 hours, followed by a drop on the 4th hour in the untreated group (Figure 4.6), indicated the induction of inflammation. The marked decrease in paw swelling (especially in the third hour for 100 mg/kg) in the extract pre-treated groups indicated its anti-acute inflammatory properties. Results obtained in this study concur with the results reported by Begum *et al. (*2015) on the anti-inflammatory activity of the methanolic extract of *Careya arborea.* The anti-inflammatory activity of the methanolic extract of *Careya arborea* was linked to inhibition of synthesis and the release of prostaglandins, a mechanism similar to that of non-steroidal anti-inflammatory drugs.

Carrageenan induced acute inflammation is known to be mediated by clyclooxygenases (COX) resulting in stimulation of prostaglandin biosynthesis (Saxena *et al*., 1984; Mossa *et al*., 1995). The observed lower tissue levels of COX-2 in the extract treated groups (Figure 4.7) indicated COX-2 inhibitory activity of the extract and consequent suppression of prostaglandin biosynthesis. Similar to the results obtained in this study, the anti-inflammatory activity of the *Vitex negundo* oil has also been linked to its ability to reduce the expression and activity of COX-2 thus decreasing swelling in the Carrageenan induced inflammation in rats

(Chattopadhyay *et al*, 2012). Even though the effect of the extract on COX-1 activity, which is constitutively expressed in most tissues, was not determined in this study, the observed potential COX-2 inhibitory effect of the extract was interesting, for this isozyme is only expressed under an inflammatory state. Owing to the physiological importance of COX-1 (Nakano *et al.,* 2007), selective COX-2 inhibitors are currently the most sought-after anti-inflammatory agents.

The observed reduced tissue levels of TGF-β1 on the extract pre-treated groups further supported the anti-inflammatory properties of the plant extract. TGF-β1 is a chemotactic cytokine that initiates inflammation by stimulating the migration of lymphocytes, monocytes, neutrophils and fibroblasts to the site of inflammation (McCartney-Francis and Wahle, 2012; Han *et al*., 2012). Even though there was no noticeable effect on the tissue levels of IL-6 and TNF-α, the ability of the extract to reduce TGF-β1 (Figure 4.7) suggested that the extract was able to decrease the initial inflammatory responses in the carrageenan induced rats' paw edema. The ethyl acetate extract of *Salvadora persica. L*. also reduced the levels of TGF- β1 in the carrageenan induced inflammation in rats (Ibrahim *et al.,* 2011). The reduction of the TGF- β1 levels by the extract was linked to reduce vascular permeability that promotes edema resolution.

Inflammation is also linked with oxidative stress and the two are commonly implicated in various pathophysiologies including thrombotic disorders (Levonen *et al.,* 2008; Kumar *et al.,* 2015). The anti-inflammatory activity of various medicinal plants has been attributed to their antioxidant potential (Ben Khedir *et al.,* 2016; Zouari- boussida *et al.,* 2018). The relatively higher tissue SOD activity and total antioxidant status in the extract treated groups, compared to the untreated group, indicated the potential of the extract to improve tissue antioxidant capacity during inflammation. Elevation of the total antioxidant capacity is the cumulative action of all non-enzymatic and enzymatic antioxidants present, providing an integrated potential of antioxidant potential (Bahrami *et al.,* 2016). The observed effective *in vitro* free radical scavenging activity and reducing potential of the extract support the antioxidant activity of the extract. The antioxidant properties of the extract could be attributed to its flavonoid and phenolic contents as these are well known of their antioxidant activity (Kumar *et al.,* 2013; Kumar and Pandey, 2013). The antiplatelet

aggregation activity of some plants extracts has been influenced by their ability to suppress the generation of ROS and thus reduce oxidative stress (Bartimoccia *et al.,* 2014; Meshkini and Tahmasbi, 2017). The antioxidant activity exhibited by the extract in this study could also be important in the prevention of oxidative stress-induced platelet activation and aggregation.

Even though medicinal plants have been used by traditional healers for ages, the majority of these plants have not been evaluated for their toxicity and they also have non- standardized dosage (Musila *et al.,* 2017). In addition to efficacy, it is also equally important to have a non-toxic therapeutic agent. Elevated serum levels of liver and kidney function biomarkers following drug administration serve as a quick indication of potential hepato- and nephrotoxicity of the drug. Significantly higher serum levels of both urea and AST following administration of the extract, at the highest concentration of 350 mg/kg, indicated the potential hepato- and nephrotoxicity of the extract. Thus, the medicinal use of the ethyl acetate extract of *P. longifolia* stem bark should be with caution.

CHAPTER 6

6.0 Conclusion

The results obtained in this study indicate that the ethyl acetate extract of *P. longifolia* exhibits antithrombotic and anti-inflammatory activities. The potential antithrombotic effect of the *P. longifolia* extract was related to the extract's thrombolytic activity on whole blood clots and its ability to inhibit the activity of thrombin and the thrombin induced platelet aggregation. More so, the extracts anti-inflammatory potential was linked to its ability to reduce the levels of TGF-β1 and COX-2 activity. Since inflammation is also linked with oxidative stress, and the two are commonly implicated in thrombotic disorders and other pathophysiologies (Levonen *et al.,* 2008; Kumar *et al.,* 2015), the exhibited antithrombotic and anti-inflammatory activities of the extract could also be associated with its antioxidant properties. The obtained results suggest that the ethyl acetate extract of *P. longifolia* could serve as a potent therapy in the prevention of thrombosis and inflammation. Nevertheless, it would also be important to document a safe therapeutic dosage of the extract since the results from this study indicated that high concentrations could potentially lead to hepatic and/or renal toxicity.

6.1 Limitation

The isolation of the bioactive compounds from the ethyl acetate extract of *P. longifolia* could not be achieved due to time constraints.

6.2 Recommendation for future study

The bio-guided isolation of the bioactive constituents present in the ethyl acetate extract, and confirmation of their antithrombotic and anti-inflammatory activities are recommended for future study

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APPENDIX A

A0. Details of Reagents Preparation

A.1 2, 2'-azinobis (3-ethylbenzothiazoline-6-sulfonate)

The mixture containing 2.45 mM potassium persulfate, 7mM of ABTS and ionized water was incubated for 16 hours at room temperature in the dark which resulted in the production of radical cation ABTS. The ABTS* was diluted (1:60 methanol, v/v).

A.2 Sulphanilic acid reagent

Sulphanilic acid (0.33 %) was dissolved in 20 % glacial acetic acid.

A3 Tris-HCl buffer (pH 7.4)

Tris-HCl (0.302 g), 1.095 g of EDTA and 0.051 g of NaCl was dissolved in water up to 50 ml

A.4 Anticoagulant solution

Citric acid (30.89 g), 8.377g of dextrose and 2.679g of NaCl was dissolved in 500 ml deionized water

A.4 Wash buffer (pH 6.4)

K_2HPO_4 (14.98 g),4.95 g of glucose ,3.30 g of NaCl and 0.61g of Na_2HPO_4 was dissolved in 500 ml distilled water.

A.5 Resuspension buffer (pH 7.4)

Tris HCl (9.09 g), 4.09 g of NaCl and 4.54 g of glucose were dissolved in 500 ml distilled water

A.6 Homogenizing buffer (pH 7.4)

Tris-HCl (1.214 g), 0.24g of $MgCl_2$ and 0.292 g of NaCl were dissolved in 1 litre and 1ml protease inhibitor was added into the buffer solution.

APPENDIX B

B0. Details of some Methods

B1 Extraction of the plant material

Fresh stem barks of *Protorhus longifolia* (Benrh) Engl were collected from Kwa-Hlabisa Kwa-Zulu Natal. The identity of the plant (voucher specimen: RAUZ01) was confirmed a Botanist at the Department of Botany, University of Zululand. The plant material was washed, cut in to small pieces and air dried. The dried plant material was sequentially extracted with n-hexane, chloroform and ethyl acetate, each for 24 hours at room temperature with constant shaking on platform shaker at 150 rpm. The extracts were separately filtered, and the filtrates concentrated *in vacuo* (using rotary evaporator at 40 ± 2 °C) to obtain crude extracts. The crude ethyl acetate extract (EA) was used in the study.

B2Total phenolic content

The total phenolic content of the plant extract was evaluated using the Folin-Ciocalteu reagent method (Kujala *et al*., 2000). Crude extract (0.5 mg/ml) was mixed with 1.5 ml dilute (1:10 v/v). Different concentrations of gallic acid were prepared in diethyl ether (0.01,0.02,0.04,0.08,0.1). These were evaporated and Folin-Ciocalteu reagent and 1.2 ml of 7.5% sodium carbonate were added into the solution. The mixture was allowed to stand for 30 min in the dark at room temperature. The blue coloured mixture was measured at 765nm. The total phenolic content of the plant extract was calculated as garlic acid equivalent to a calibration curve of gallic acid (Figure B1) and expressed as mg/g dry plant material.

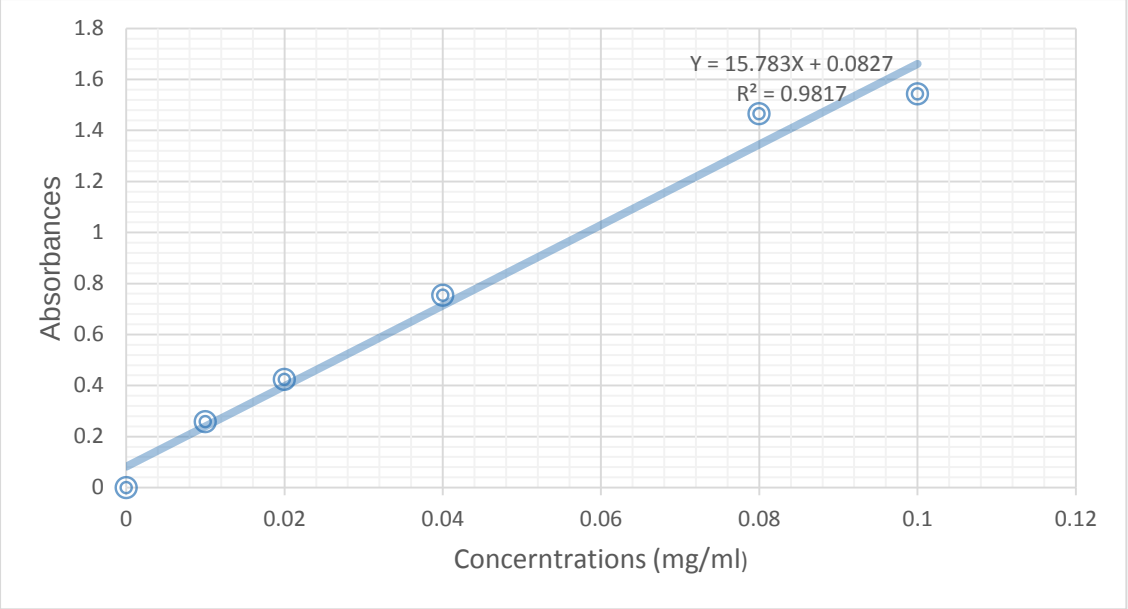

Figure B1: Calibration curve for gallic acid concentration against absorbance (nm vs mg/ml).

B.2 Total flavonoid content

The total flavonoid content of the crude plant extract was estimated using the colourimetric method described by Ordonez et al. (2006). The extract (0.5 mg/ml), different concentrations (0.01,0.02,0.04,0.08,0.1). of quercetin were prepared in diethyl ether. The standard was allowed to evaporate and 0.5 ml of 2% alcoholic aluminium chloride added into the solution. The mixture was incubated for 1 hour at room temperature. Quercetin was used as a standard and absorbance of the yellow coloured mixture was measured at 420 nm against the blank containing alcoholic aluminium chloride. The total flavonoid content of the extract was calculated from a calibration curve of quercetin (Figure B2) and expressed as mg/g dry plant material.

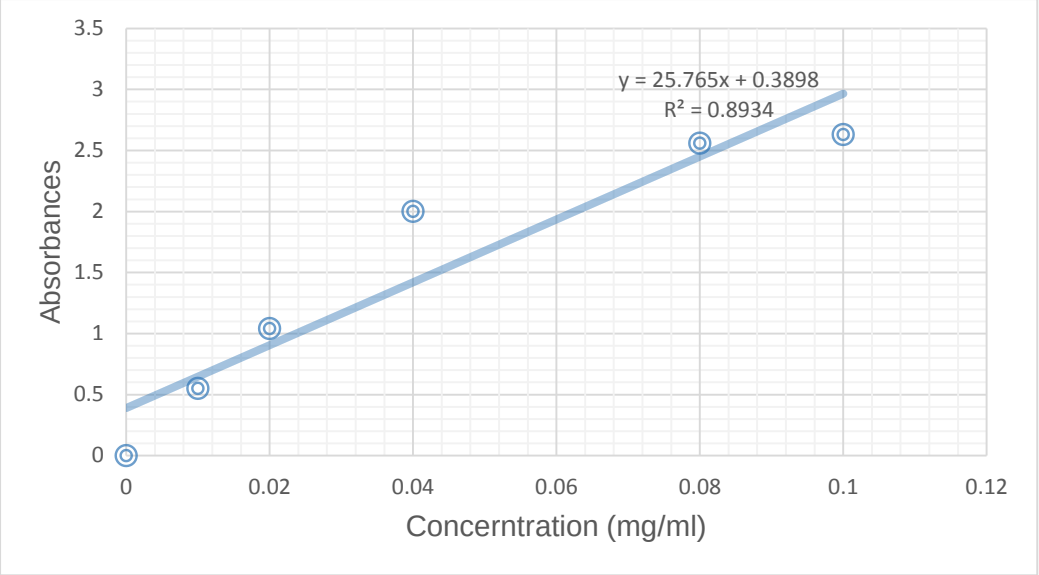

Figure B2: Standard curve for quercetin equivalent concentration against absorbance (nm vs mg/ml).

APPENDIX C

C 0. Additional data

C1. Free radical scavenging activity of the ethyl acetate extract

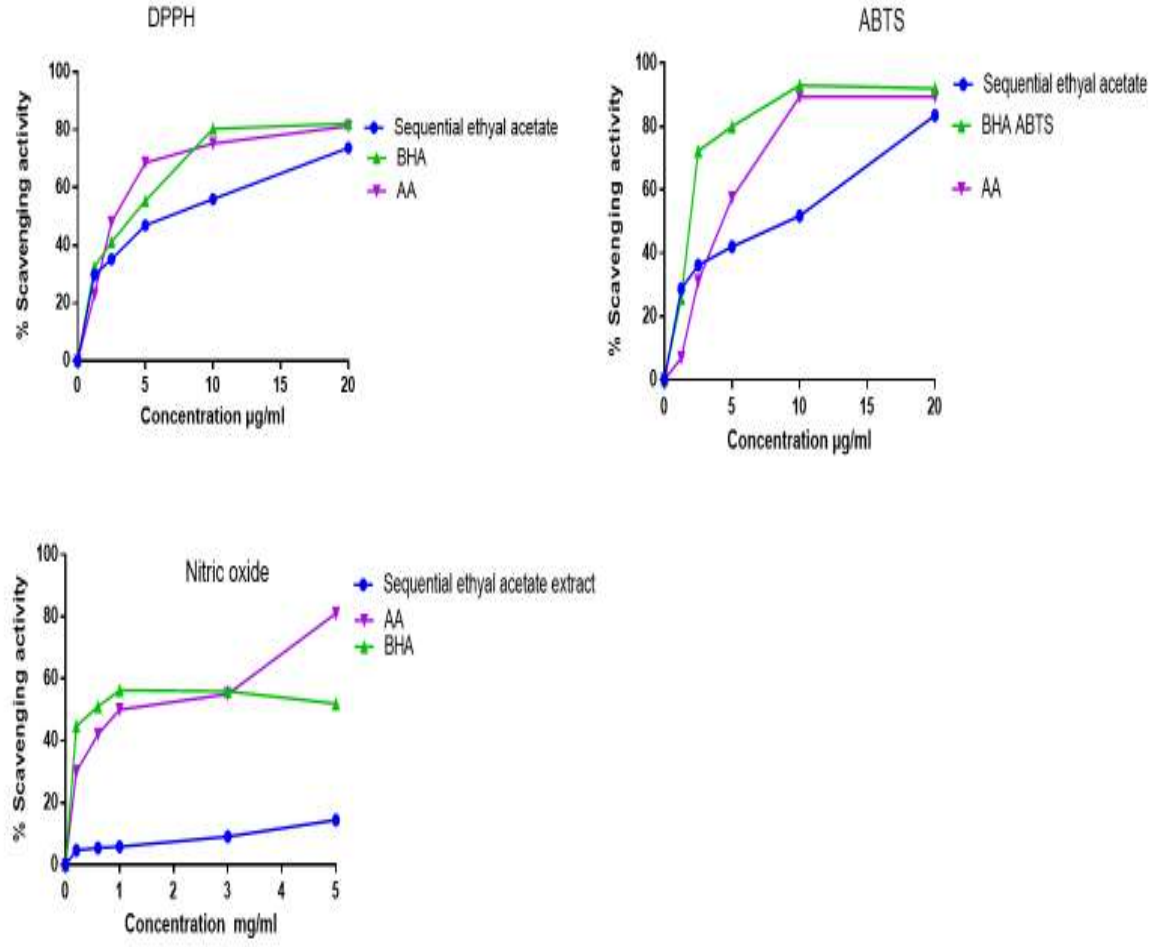

Figure C1.1: DPPH, ABTS and Nitric oxide scavenging activity of the ethyl acetate extract (EA).

C2. Thrombolytic activity of the ethyl acetate extract

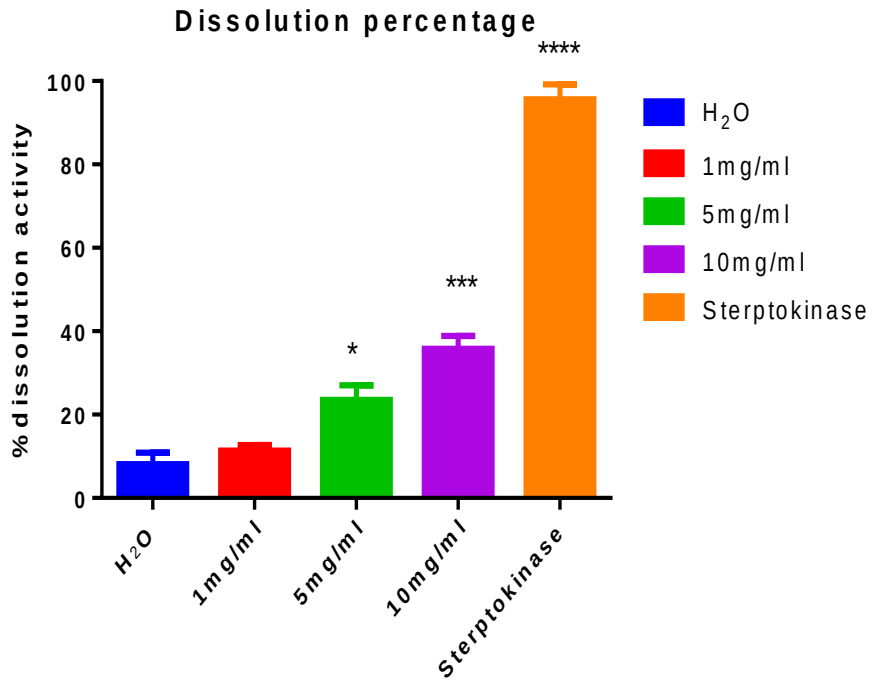

Figure C2.1: Thrombolytic activity of the extract

APPENDIX D

UNIVERSITY OF ZULULAND
RESEARCH ETHICS COMMITTEE
(Reg No: UZREC 171110-030)

RESEARCH & INNOVATION
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ETHICAL CLEARANCE CERTIFICATE

Certificate Number	UZREC 171110-030 PGM 2017/438							
Project Title	Antithrombotic and anti-inflammatory activity of ethyl acetate extract of Protorhus longifolia stem bark							
Principal Researcher/ Investigator	Majola LC							
Supervisor and Co-supervisor	Dr R Mosa		Prof A Opoku					
Department	Biochemistry and Microbiology							
Faculty	Science & Agriculture							
Type of Risk	Low risk – Desktop research							
Nature of Project	Honours/4th Year		Master's	x	Doctoral		Departmental	

The University of Zululand's Research Ethics Committee (UZREC) hereby gives ethical approval in respect of the undertakings contained in the above-mentioned project. The Researcher may therefore commence with data collection as from the date of this Certificate, using the certificate number indicated above.

Special conditions:

(1) This certificate is valid for 2 years from the date of issue.
(2) Principal researcher must provide an annual report to the UZREC in the prescribed format [due date-01 July 2018]
(3) Principal researcher must submit a report at the end of project in respect of ethical compliance.
(4) The UZREC must be informed immediately of any material change in the conditions or undertakings mentioned in the documents that were presented to the meeting.

The UZREC wishes the researcher well in conducting research.

Professor Gideon De Wet
Chairperson: University Research Ethics Committee
Deputy Vice-Chancellor: Research & Innovation
16 November 2017

CHAIRPERSON
UNIVERSITY OF ZULULAND RESEARCH ETHICS COMMITTEE (UZREC)
REG NO: UZREC 171110-30
29-11-2017
RESEARCH & INNOVATION OFFICE