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Evaluation of extracts of South African plant species for the treatment of venous thromboembolism

Submitted in partial fulfilment of the degree:

MSc Medicinal Plant Science

Department of Plant and Soil Sciences

Faculty of Natural and Agricultural Sciences

University of Pretoria

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Abstract

Venous thromboembolism (VTE) is a collective term used to describe deep vein thrombosis (DVT) and pulmonary embolism (PE). Cardiovascular diseases are globally responsible for the majority of cases of mortality. A large portion of the world's population who live in developing countries rely on herbal medicinal products as a primary source of healthcare; thus, exploring the medicinal properties of plants in relation to their potential use for VTE is necessary.

The main pathologic mechanism underlying venous thromboembolism is thrombosis, which is regulated by coagulation. Therefore, the aim of this research was to evaluate the antithrombotic properties of extracts of selected South African medicinal plants. A total of 11 plants were selected based on traditional use and pharmacological properties. The selected plants were collected and extracted with ethanol, dichloromethane and water. A total of 33 plant extracts were tested.

The antiproliferative activity of all the plant extracts was tested using an antiproliferative assay against the human hepatocellular carcinoma (HepG2) cell line. The most potent HepG2 antiproliferative activity was exhibited by the ethanolic extract of *Hypoestes forskaolii* at a IC_{50} (50% inhibitory concentration) lower than 3.13µg/mL. The antiproliferative activity of *Sideroxylon inerme* and *Barleria obtusa* leaf extracts against the HepG2 cell line are documented for the first time in this study. Ten extracts had IC_{50} values higher than the highest tested concentration of 400µg/mL. These extracts were then tested further for antioxidant activity using the DPPH assay.

The antioxidant activity of *Pelargonium graveolens*, *Carpobrotus dimidiatus* and *B. obtusa* is reported for the first time in this study. The aqueous extract of *Heteropyxis natalensis* showed the most potent antioxidant activity, with a IC_{50} value of 2.71µg/mL. Four plant extracts had IC_{50} values less than 20µg/mL and these extracts were then tested for the ability to inhibit activated coagulation factor X (FXa) using the FXa inhibition assay. The assay revealed that the aqueous extract of *H. natalensis* was a potent inhibitor of FXa (IC_{50} = 2.64µg/mL) and the mechanism of inhibition was found to be competitive inhibition. This study reports the FXa inhibitory activity of *H. natalensis* for the first time.

The aqueous extract of *H. natalensis* was then tested for cyclooxygenase-1 (COX-1) inhibition using the COX-1 Inhibitor Screening Assay Kit (ab240698, Abcam), where it inhibited COX-1 (IC_{50} = 25.32µg/mL). The ability of *H. natalensis* to inhibit COX-1 enzyme is also reported for the first-time in this study. The phytochemistry of *H. natalensis* aqueous extract was determined using ultra

performance liquid chromatography mass spectrometry (UPLC-MS) which confirmed the presence of β-carotene in the extract.

The study concluded that amongst the 33 extracts tested, the aqueous extract of *H. natalensis* was found to be the best candidate for future antithrombotic studies in relation to VTE. The strong antioxidant activity of the extract would prevent the up regulatory effects of reactive oxygen species (ROS) on coagulation. Additionally, the competitive inhibition of FXa at low concentrations, would prevent progression of both pathways of the coagulation cascade. The ability of the extract to also inhibit COX-1 in the present study, indicated that this extract could prevent platelet aggregation. In addition to these findings, the present study revealed that the extract is not likely to cause any antiproliferative effects in the liver. Future studies should focus on the mechanisms of action on FXa and COX-1 as well as bioactive compound identification and isolation from this lead plant.

Research Outputs

- Denga, L.L., Kok, A., Lall, N., *Barleria obtusa*, in: Underexplored Medicinal Plants from Sub-Saharan Africa. 2nd Edition (In Progress)
- Denga, L.L., Kok, A., Lall, N., *Carpobrotus dimidiatus*, in: Underexplored Medicinal Plants from Sub-Saharan Africa. 2nd Edition (In Progress)
- Denga, L.L., Kok, A., Lall, N., *Heteropyxis natalensis*, in: Underexplored Medicinal Plants from Sub-Saharan Africa. 2nd Edition (In Progress)
- Denga, L.L., Kok, A., Lall, N., *Pelargonium graveolens*, in: Underexplored Medicinal Plants from Sub-Saharan Africa. 2nd Edition (In Progress)
- Denga, L.L., Lall, N., 2021. Chapter 17: The potential of plants as treatments for venous thromboembolism: A review of traditional use, biological activity and chemical constituents, in: Medicinal Plants in Cosmetics, Health and Disease. Taylor & Francis (Final accepted draft submitted)
- Denga, L.L., Lall, N., Evaluation of the antithrombotic potential of selected South African plant extracts for venous thromboembolism. Blood Cells, Molecules and Diseases, Impact Factor 3.039 (Submitted)

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List of abbreviations

ANOVA: Analysis of variance
APC: Activated protein C
AT: Antithrombin
COX: Cyclooxygenase
COX-1: Cyclooxygenase-1
COX-2: Cyclooxygenase-2
DCM: Dichloromethane
DMEM: Dulbecco's Modified Eagles Medium
DMSO: dimethylsulfoxide

DPPH: 2,2-diphenyl-1-picrylhydrazyl

DVT: Deep vein thrombosis

EDTA: Ethylenediaminetetracetic acid

EI: Electron ionisation

FII: Prothrombin

FIIa: Thrombin

FIX: Coagulation factor IX

FIXa: Activated coagulation factor IX

FV: Coagulation factor V

FVa: Activated coagulation factor V

FVIIa: Activated coagulation factor VII

FVIII: Coagulation factor VIII

FVIIIa: Activated coagulation factor VIII

FX: Coagulation factor X

FXa: Activated coagulation factor X

FXIa: Activated coagulation factor XI

GGACK: 1,5-Dansyl-Glu-Gly-Arg Chloromethyl Ketone

GPP8: GraphPad Prism 8

HepG2: Hepatocellular carcinoma

IC_{50}: 50 percent inhibitory concentration

IL-1: Interleukin-1

IL-1β: Interleukin 1-beta

IL-6: Interleukin-6

K_m: Michaelis constant

MIC: Minimum inhibitory concentration

MPs: Microparticles

NETs: Neutrophil extracellular traps

PBS: Phosphate buffered saline

PE: Pulmonary embolism

PEG-6000: Polyethylene glycol 6000

ROS: Reactive oxygen species

TF: Tissue factor

TF-FVIIa: Tissue factor and activated coagulation factor VII complex

TFPI: Tissue factor pathway inhibitor

TLRs: Toll-like receptors

TM: Thrombomodulin

TNF: Tumour necrosis factor

TNFα: Tumor necrosis factor alpha

tPA: Tissue plasminogen activator

Tris: tris(hydroxymethyl)aminomethane

TXA_2: Thromboxane A_2

uPA: Urokinase plasminogen activator

UPLC-MS: Ultra-performance liquid chromatography mass spectrometry

V_{max}: Maximum rate of reaction

VTE: Venous thromboembolism

VWF: von Willebrand factor

ZPI: Protein Z dependant protease inhibitor

Structure of thesis

Chapter 1: General Introduction

The chapter provides a brief introduction and motivation for the study. The main objectives of the study are highlighted, and the methods employed are briefly mentioned.

Chapter 2: Literature Review

The chapter focuses on the role of the coagulation cascade and platelets in the progression of VTE as well as the role of different risk factors in the development of VTE. The mechanisms of action of current treatments are also discussed. This chapter provides comprehensive information on the use of plants for VTE, highlighting traditional use and biological activity of various plants against the research targets.

Chapter 3: Plant Selection

The chapter provides information on the 11 selected South African plant species in the form of monographs. The ethnobotanical use and phytochemical constituents of the plants are provided. Additionally, the *in vitro* and *in vivo* results of the pharmacological activities of plant extracts and plant-derived compounds are provided where available.

Chapter 4: Evaluation of the Antithrombotic Potential of Selected South African Plant Extracts for Venous Thromboembolism

The chapter provides the aims, materials and methods, results and discussions of the various experiments conducted in the study.

Chapter 5: Conclusion and Future Research

In this chapter, the conclusion and main findings of the study have been discussed, and recommendations for future research have been suggested.

Chapter 1: General Introduction

Chapter 1: General Introduction

1.1. Background and motivation of the study

The purpose of this study was to evaluate the bioactivity of South African plant species in relation to the treatment of venous thromboembolism (VTE). This research is not solely applicable to South Africa, but also holds great relevance worldwide. Globally, cardiovascular diseases are the major cause of deaths. It has been reported that more than 75% of these deaths occur in low- and middle-income countries (World Health Organisation, 2017). Many conventional synthetic medicines are available and used for cardiovascular diseases, but it is also important to note that about 80% of those who live in developing countries, rely on herbal medicinal products as a primary source of healthcare (Ekor, 2014).

In many parts of Africa, medicinal plants are the most easily accessible health resource available to the community (Mahomoodally, 2013). Medicinal plants are a valuable source of healthcare in South Africa; due to the abundant biodiversity of plants in terms of species richness with almost 3 689 species of plants used as traditional medicines (Cherry, 2005). Moreover, South Africa houses 20 500 vascular plant taxa, which make up nearly 6% of the world's plant diversity (Klopper et al., 2010). Therefore, investigating plants for their potential use for VTE, is applicable to South Africa.

Venous thromboembolism is a collective term consisting of two cardiovascular diseases, one being deep vein thrombosis (DVT) and the other, pulmonary embolism (PE) (Anderson and Spencer, 2003). Deep vein thrombosis is the development of a blood clot, also known as a thrombus, in the large veins of the leg (Stone et al., 2017). The thrombus is not tightly adherent to the endothelium and may easily dislodge (Koupenova et al., 2017).

When a part of this thrombus breaks away and travels through the circulatory system, it is known as an embolus (Tarbox and Swaroop, 2013). The blood flow will carry the embolus to the pulmonary circulation in the lungs, where the embolus has the potential to block an artery (Tarbox and Swaroop, 2013). This results in a condition known as pulmonary embolism, with PE occurring in up to one third of DVT cases and is the main contributor to mortality (Mackman, 2008).

A thrombus forms when coagulation is upregulated in the coagulation cascade (Jin and Gopinath, 2016). The coagulation cascade consists of a series of reactions, which involves the activation of serine proteases known as coagulation factors or procoagulants (Schenone et al., 2004). Coagulation factors exist in an inactivated state and become more active when they form a complex with another

specific coagulation factor (Smith et al., 2015). Upregulation of the coagulation cascade results in enhanced activation of platelets, which aggregate and contribute to the formation of a thrombus (Nurden et al., 2008). Conditions such as obesity, pregnancy and long periods of hospitalisation are risk factors for thrombus formation (Mukhopadhyay et al., 2019).

The Global Burden of Diseases, Injuries, and Risk Factors (GBD) study, conducted in 2010, revealed that ischemic heart disease and stroke collectively caused one in four deaths globally (Horton, 2012). Like VTE, this is relevant because the key pathologic mechanism underlying most of these cardiovascular disease cases, is thrombosis (Raskob et al., 2014). This indicates that thrombosis is a major contributor to the pathology of cardiovascular diseases with a high rate of fatalities. Therefore, targeting specific factors that lead to thrombosis is an effective way to treat VTE. Drugs typically used for the treatment of VTE display anti-coagulant and anti-platelet activity.

1.2. Objectives of the study

The main objective of this research project was to evaluate the efficacy of selected plant extracts against specific factors that contribute to the development of VTE. The study findings provided information on the potential anti-coagulant and anti-platelet properties of the selected South African plant species. The specific objectives of the study follow.

1.2.1. To prepare extracts of the selected plant species

Ethanolic, dichloromethanoic and aqueous extracts of the leaves and non-woody stems of selected plants were prepared using the method described by Mativandlela et al. (2006) albeit with slight modifications. The use of diverse solvents ensured that compounds with dissimilar polarities were extracted from the selected plants.

1.2.2. To investigate the antiproliferative effects of the selected plant extracts on the liver

In this undertaking, it was important that the plant extracts were not harmful to the liver as this organ is responsible for detoxification of chemicals and metabolising drugs. All plant extracts were therefore tested for antiproliferative activity against the hepatocellular carcinoma (HepG2) cell line to ensure that the plant samples did not cause any damage to the liver. The HepG2 cell line is regularly used as an alternative to primary human hepatocytes (Vinken and Rogiers, 2015). The method described by Steenkamp and Gouws, (2006) was applied with slight modifications. This served as a screening method to remove any samples which may cause adverse hepatic effects.

1.2.3. To evaluate the antioxidant activity of selected plant extracts

Studies have shown that reactive oxygen species play a role in the activation of platelets, while *in vitro* studies have demonstrated that potent antioxidants inhibit activation of platelets. Those plant extracts which did not display hepatotoxicity when tested at the highest concentration, were subsequently tested for the ability to scavenge free radicals using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay described by du Toit et al. (2001). The DPPH assay is considered a valid and accurate method to evaluate radical scavenging activity of antioxidants (Kedare and Singh, 2011).

1.2.4. To investigate the effect of selected plant extracts on FXa

Inhibition of activated coagulation factor X (FXa) is an effective mechanism to treat VTE as both the intrinsic and extrinsic pathway of the coagulation cascade converge at this point. The plant extracts with significant antioxidant activity were tested for inhibitory potential against FXa using the assay described by Chu et al., (2000) with slight modifications. The method is based on the ability of FXa to cleave a chromogenic substrate and cause a colour change in solution.

1.2.5. To investigate the effect of selected plant extracts on cyclooxygenase-1 (COX-1)

Inhibition of cyclooxygenase-1 (COX-1) prevents the aggregation of platelets. The plant extract, which displayed significant anti-FXa activity, was tested for the ability to inhibit COX-1 using the COX-1 inhibitor screening assay kit (ab240698, Abcam). The assay is based on the fluorometric detection of Prostaglandin G2, the intermediate product generated by the COX enzyme.

1.2.6. To determine the phytochemical constituents of selected plant extracts

Knowing what the phytochemical constituents of certain plant extracts are, can further assist in understanding the biological activity of these plant extracts. To this end, ultra performance liquid chromatography mass spectrometry (UPLC-MS) was used to analyse the plant extract which displayed significant activity in order to determine its phytochemical constituents.

1.3. References

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Chapter 2: Literature Review

Chapter 2: Literature Review

Statement

This chapter forms part of "Chapter 17: The potential of plants as treatments for venous thromboembolism: A review of traditional use, biological activity and chemical constituents", in the future book publication titled: "Medicinal Plants in Cosmetics, Health and Disease".

2.1. Introduction

Venous thromboembolism (VTE) is a collective term for two cardiovascular diseases: deep vein thrombosis (DVT) and pulmonary embolism (PE) (Anderson and Spencer, 2003). Understanding the biological mechanism that leads to the development of VTE is critical when investigating potential treatments as it provides key factors and conditions that contribute to disease development (Cann, 2016). These key factors and conditions can be targeted when evaluating potential therapies for VTE.

The key biological mechanism that causes VTE is thrombosis (Raskob et al., 2014). The process of thrombosis is the formation of a coagulated blood mass, known as a venous thrombus, within a blood vessel (Schafer et al., 2003). In the case of DVT, the venous thrombus forms in the large veins of the legs (Stone et al., 2017). The venous thrombus has the potential to dislodge from the endothelium of the veins and travel further through the bloodstream (Koupenova et al., 2017). The dislodged venous thrombus is now known as an embolus and the embolus can travel to the pulmonary circulation, resulting in PE (Tarbox and Swaroop, 2013). Thrombosis is regulated in the coagulation cascade by coagulation factors and platelets (Schenone et al., 2004; Nurden et al., 2008). Current treatments for VTE target coagulation factors and platelets to prevent progression of the disease.

This chapter focuses on the role of the coagulation cascade and platelets in the progression of VTE as well as the role of different risk factors in the development of VTE. The mechanisms of action of current treatments are discussed and the research project targets will be elucidated. Furthermore, this chapter evaluates plants as potential treatments for VTE, highlighting biological activity against the research project targets.

2.2. Coagulation

Haemostasis is a natural physiological process in the body that prevents excessive bleeding (Pilli, 2018). Coagulation is a haemostatic mechanism that prevents excessive bleeding by converting blood from a liquid state to a gel state (Jin and Gopinath, 2016). VTE is a condition where coagulation is

upregulated to a greater degree than is necessary, resulting in thrombosis and the development of a venous thrombus (Pilli, 2018).

2.2.1. Structure of a venous thrombus

A venous thrombus is composed of red blood cells and immune cells encased in an interlaced structure of platelets and fibrin (Ariens, 2015) (Figure 2.1). Fibrin is the final product of the coagulation cascade and it is an essential protein that forms a fibrous meshwork with platelets (Kattula et al., 2017). The fibrin fibres contribute to the elasticity of the thrombus and provide a proteinaceous structural backbone which stabilises the thrombus (Mukhopadhyay et al., 2019).

Platelets are small disc-shaped anucleate blood cells that function as procoagulant surfaces which recruit additional platelets and leukocytes such as macrophages and neutrophils (Davì and Patrono, 2007; Wohner, 2008). During inflammation, neutrophils release extracellular DNA fibres, known as neutrophil extracellular traps (NETs), which stimulate thrombosis (Mukhopadhyay et al., 2019). Furthermore, macrophages express procoagulant factors and release inflammatory cytokines that stimulate thrombus formation and recruit additional immune cells (Bokarewa et al., 2002).

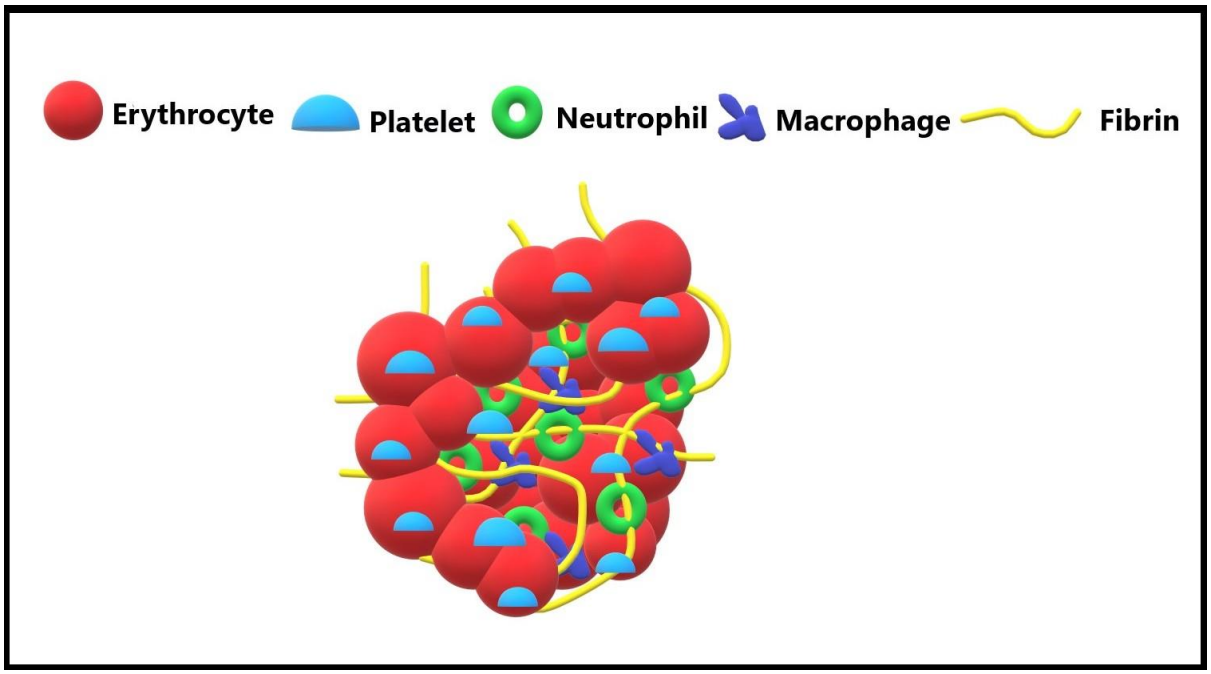

Figure 2.1: Structure of a venous thrombus (Paint 3D)

2.2.2. The coagulation cascade

The coagulation cascade is a series of reactions that lead to haemostasis (Pilli, 2018). The plasma portion of blood carries a set of serine proteases that exist in an inactivated state. These serine proteases are known as coagulation factors or procoagulants. They are normally activated upon injury

to promote the formation of a plug at the site of an injury; however, they can be activated in the absence of gross vessel wall injury (Pilli, 2018; Schenone et al., 2004). Inactive coagulation factors are represented by Roman numerals and are appended with a lower case "a" once proteolytically converted to the active form. The activity of the coagulation factors can increase by as much as five orders of magnitude when activated (Smith et al., 2015). The coagulation cascade consists of two pathways, i.e., the intrinsic and extrinsic pathway (Mackman, 2012).

2.2.2.1. The extrinsic pathway

The extrinsic pathway is the initiation step of coagulation and it requires an integral membrane protein known as tissue factor (TF) (Mackman et al., 2007). This membrane protein (TF) is expressed on endothelial cells surrounding blood vessels as well as on monocytes (Schecter et al., 2000; Chu, 2006). Expression of TF is normally induced when a blood vessel wall is damaged; however, active TF can be expressed by smooth muscle and adventitial cells in the absence of gross vessel wall injury (Koupenova et al., 2017; Manly et al., 2011).

According to Owens and Mackman, (2010), patients with VTE tend to have increased levels of circulating TF expressed on microparticles (MPs), which may trigger venous thrombus formation. The MPs are membrane vesicles released from activated or apoptotic cells and can be generated from monocytes, vascular smooth muscle cells and possibly from platelets (Manly et al., 2011). Furthermore, under pathological conditions such as inflammation, expression of TF by endothelial cells and monocytes can be induced by inflammatory cytokines such as tumor necrosis factor (TNF) and interleukin 1 (IL-1) (Bokarewa et al., 2002; Chu, 2006).

The coagulation cascade is initiated when TF forms a complex with the serine protease, activated coagulation factor VII (FVIIa) (Mackman et al., 2007). The tissue factor and activated coagulation factor VII complex (TF-FVIIa) is a potent activator of coagulation because it activates the downstream substrates, coagulation factors IX (FIX) and X (FX), which become activated coagulation factors IX (FIXa) and X (FXa) respectively (Smith et al., 2015). The catalytic site of FVIIa is stabilised by TF on a plasma membrane to facilitate optimal interaction with its substrates FIX and FX (Grover and Mackman, 2018).

During the initiation phase of coagulation, TF-FVIIa proteolytically creates small amounts of FXa, which then converts initial amounts of prothrombin (FII) to thrombin (FIIa) (Dobesh and Stacy, 2019). Thrombin is a serine protease that is responsible for the amplification of the coagulation cascade and conversion of fibrinogen to fibrin in the intrinsic pathway (Monroe et al., 2002; Brass, 2003).

2.2.2.2. The intrinsic pathway

The intrinsic pathway is the amplification and propagation step of the coagulation cascade as it creates numerous positive feedback loops which accelerate the coagulation cascade (Palta et al., 2014). The initial creation of thrombin promotes the production of additional thrombin by activating coagulation factors V(FV) and VIII(FVIII), which become activated coagulation factors V(FVa) and VIII(FVIIIa) respectively (Dobesh and Stacy, 2019). Activated coagulation factor VIII exists in complex with the glycoprotein von Willebrand factor (VWF), which stabilises FVIII in the circulation (Peyvandi et al., 2011). Thrombin activates FVIII by cleaving it from VWF (Monroe et al., 2002) and FVIIIa forms a complex with FIXa known as the tenase complex (Autin et al., 2004).

The tenase complex proteolytically creates more FXa by activating additional FX (Smith et al., 2015). Activated factor X then forms a complex with FVa, known as the prothrombinase complex (Palta et al., 2014). The prothrombinase complex rapidly converts prothrombin to thrombin, which further amplifies the cascade and converts soluble fibrinogen to insoluble fibrin (Weisel, 2005). In addition to cascade amplification and fibrinogen conversion, thrombin is also a potent activator of platelets (Brass, 2003). Figure 2.2 illustrates the initiation and amplification of the coagulation cascade in a schematic representation below.

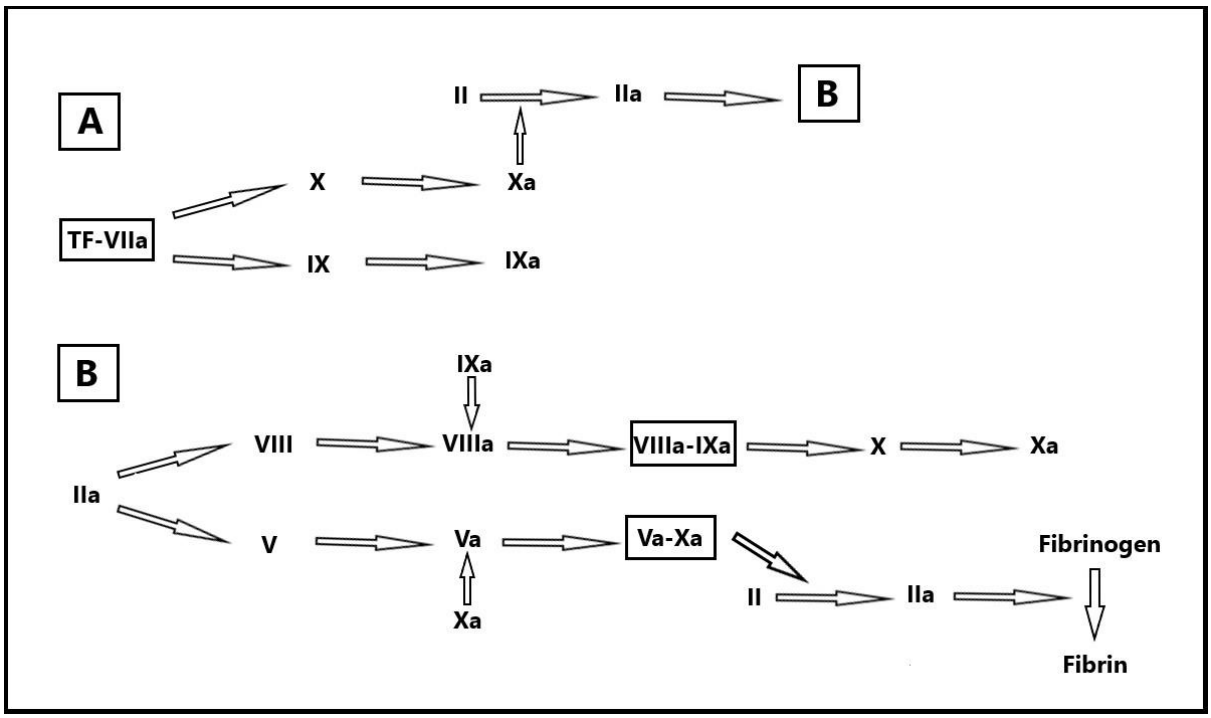

Figure 2.2: The coagulation cascade (Paint 3D). (A) Initiation of the coagulation cascade: Tissue factor (TF) forms complex with activated coagulation factor VII(VIIa). Complex activates coagulation factors X(X) and IX(IX) resulting in activated coagulation factors X(Xa) and IX(IXa). Xa converts prothrombin (II) to thrombin (IIa). **(B) Propagation of the coagulation cascade:** IIa activates coagulation factors VIII(VIII) and V(V) resulting in activated coagulation factors VIII(VIIIa) and V(Va). VIIIa and IXa form tenase complex (VIIIa-IXa) resulting in additional Xa. Va and Xa form prothrombinase complex (Va-Xa). Va-Xa converts II to IIa, which converts fibrinogen to fibrin.

2.2.2.3. The role of platelets in the coagulation cascade

Coagulation reactions do not occur physiologically in solution but are localised to a surface. Studies have shown that the protein components of platelets provide virtually all the specificity required for procoagulant activity and subsequent thrombin generation (Monroe et al., 2002). Under normal physiological conditions, damage to the endothelial cell layer of a blood vessel exposes collagen and VWF binds to collagen (Peyvandi et al., 2011). Platelets then adhere to collagen via VWF through their activated glycoproteins (Nurden et al., 2008; Koupenova et al., 2017). Under some pathological conditions, TF may be expressed on smooth muscle and adventitial cells in the absence of gross vessel wall injury (Manly et al., 2011; Koupenova et al., 2017).

Exposed TF leads to the generation of thrombin, which activates platelets and localizes them near the site of exposed TF (Monroe et al., 2002). Thrombin activation of platelets results in the release of partially active FV, FVIII and FIX. Thrombin then cleaves the partially activated coagulation factors to fully active forms, which rapidly bind to the activated platelet (Monroe et al., 2002).

The initial FIXa formed by TF-VIIa, diffuses to the platelet surface (Monroe et al., 2002; Mackman et al., 2007). Additionally, activated coagulation factor XI (FXIa) on the platelet surface activates FIX and provides additional FIXa, which forms the tenase complex with FVIIIa (Monroe et al., 2002; Autin et al., 2004; Smith et al., 2015). Coagulation factor X is then activated by the tenase complex and forms the prothrombinase complex with FVa on the surface of the platelet, resulting in a burst of thrombin (Palta et al., 2014; Monroe et al., 2002). Fibrinogen binds to activated platelets via activated fibrinogen receptors and is converted to fibrin by thrombin (Dorsam and Kunapuli, 2004).

Activated platelets also facilitate the infiltration of leukocytes into the thrombus. The first leukocytes to be recruited are neutrophils, followed by monocytes that differentiate into macrophages (Mukhopadhyay et al., 2019). The binding of coagulation factors and fibrinogen to the surface of activated platelets is illustrated in Figure 2.3 below.

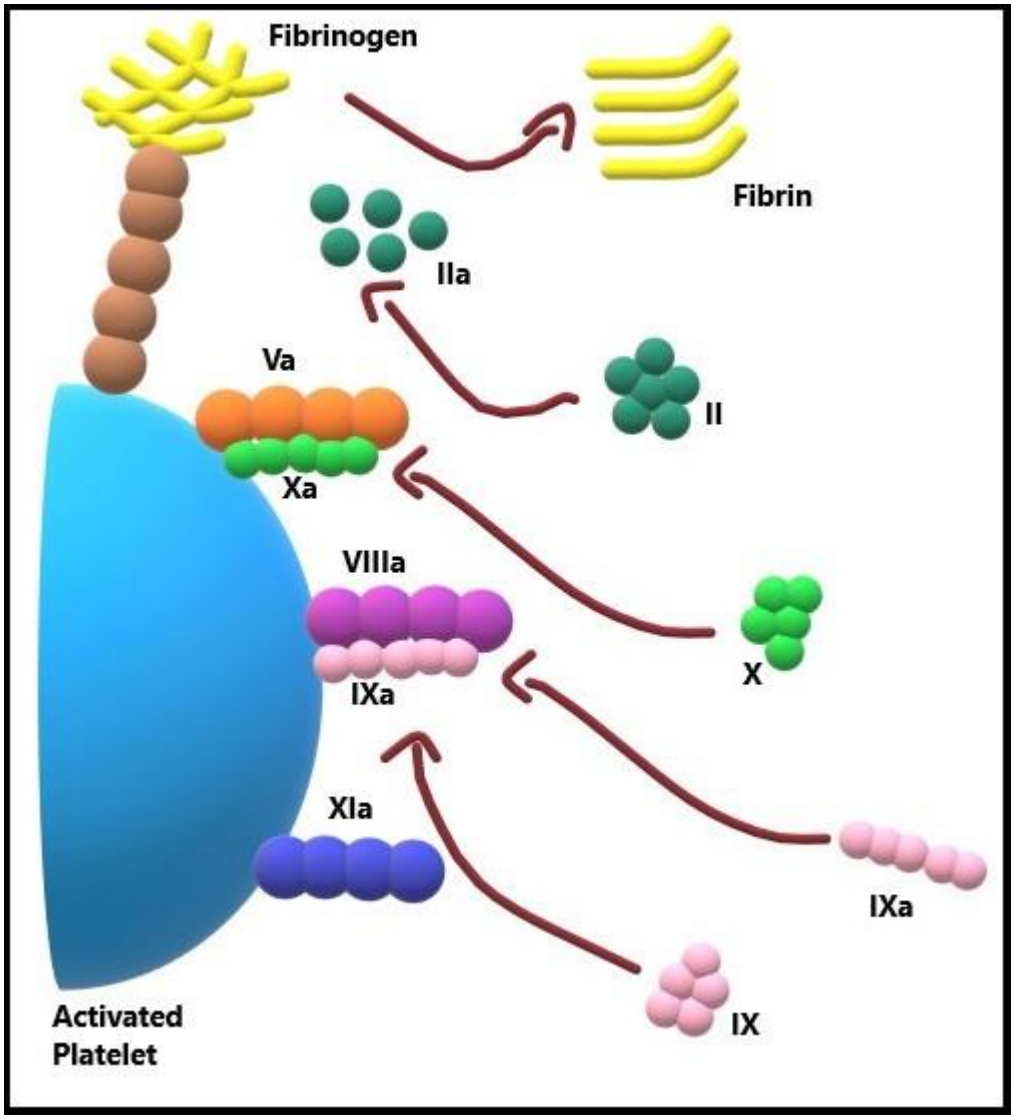

Figure 2.3: Activated platelet (Paint 3D). Activated platelet rapidly binds activated coagulation factors V(Va), VIII(VIIIa) and XI(XIa). Activated coagulation factor IX(IXa) diffuses to the platelet surface and activated coagulation factor XI (XIa) on the platelet surface provides more IXa by activating coagulation factor IX(IX). IXa forms the tenase complex with VIIIa which activates coagulation factor X(X). Activated coagulation factor X(Xa) converts prothrombin (II) to thrombin (IIa), which converts fibrinogen to fibrin.

2.3. Anticoagulant mechanisms

Physiological anticoagulant mechanisms exist to regulate coagulation in the bloodstream (Ezihe-ejiofor and Hutchinson, 2013). Regulation of coagulation occurs at multiple stages in the coagulation cascade, by enzyme inhibition or modification of the activity of coagulation factors (De-Caterina et al., 2013). People with a deficiency in any of these anticlotting mechanisms tend to develop VTE. Understanding the natural anticoagulation mechanisms aids in the development of treatments for VTE. Anticoagulant mechanisms can be divided into two systems being the fibrinolytic system and the anticoagulant system (De-Caterina et al., 2013).

2.3.1. Anticoagulant system

The main purpose of the anticoagulant system is to reduce the formation of fibrin by reducing the production and effects of thrombin (Ezihe-ejiofor and Hutchinson, 2013). The different proteins involved in the anticoagulant system as well as their mechanisms of action are summarised in Table 2.1.

Table 2.1: Proteins involved in the anticoagulant system and mechanisms of action

Anticoagulant Protein	Mechanism of action	Source
Antithrombin (AT)	Binds to thrombin and irreversibly inhibits action on fibrinogen, platelets, FV and FVIII.	Ezihe-ejiofor and Hutchinson, 2013
	Directly inhibits FXa, FIXa and TF-VIIa complex.	Thomas, 2001
Tissue Factor Pathway Inhibitor (TFPI)	Forms a complex with FXa, which subsequently forms a quaternary complex with TF-FVIIa, leading to inhibition of FXa and FVIIa	Dahm et al., 2008
Activated Protein C (APC)	Proteolytically degrades FVa and FVIIIa once activated by thrombin	Maqbool et al., 2013
Thrombomodulin (TM)	Forms a complex with thrombin that accelerates the activation of Protein C	Conway, 2012
Protein S	Functions as a cofactor for APC to enhance the degradation of FVa and FVIIIa as well as direct inhibition of the prothrombinase complex	Van Cott et al., 2016
Protein Z dependant protease inhibitor (ZPI)	Inhibits FXa in the presence of protein Z as a cofactor	Ezihe-ejiofor and Hutchinson, 2013
	Inhibits FIXa and FXIa in the absence of Protein Z	

FIXa: Activated coagulation factor IX; **FV:** Coagulation factor V; **FVa:** Activated coagulation factor V; **FVIIa**: Activated coagulation factor VII; **FVIII:** Coagulation factor VIII; **FVIIIa:** Activated coagulation factor VIII; **FXa:** Activated coagulation factor X; **FXIa:** Activated coagulation factor XI; **TF-VIIa:** Tissue factor and activated coagulation factor VII complex

2.3.2. Fibrinolytic system

The main purpose of the fibrinolytic system is to resolve thrombi that have already formed. The thrombi are resolved by cleaving fibrin to soluble degradation products (Lijnen and Rijken, 2013). The enzyme responsible for fibrin degradation is plasmin and the inactive precursor of plasmin is plasminogen (Heit, 2013). Plasmin is created when plasminogen is released into the bloodstream and activated by tissue plasminogen activator (tPA) or urokinase plasminogen activator (uPA) (Del Priore et al., 2008). Tissue plasminogen activator binds to clot-bound plasminogen and converts it to plasmin

(Ezihe-ejiofor and Hutchinson, 2013). Urokinase plasminogen activator binds to the urokinase plasminogen activator receptor on cell surfaces and converts cell-bound plasminogen to plasmin (Ezihe-ejiofor and Hutchinson, 2013).

2.4. Hypercoagulability

Hypercoagulability or thrombophilia is a state of increased risk for thrombus formation (Schafer et al., 2003). A hypercoagulable state describes a pathologic state where coagulation is exaggerated in the bloodstream due to the hyperactivity of pro-coagulant factors or a deficiency in anti-coagulants (Sun and Kroll, 2018). Hypercoagulability can occur as a result of inherited genetic factors or, it can be acquired through physiological responses to stress or trauma (Shackford, 2018). In many VTE cases, a hypercoagulable state develops due to an interaction between inherited and acquired factors.

2.4.1. Inherited hypercoagulability

Inherited hypercoagulability or thrombophilia occurs when an inherited DNA mutation results in the production of lower concentrations of anticoagulant proteins or production of mutated anticoagulant proteins (Khan and Dickerman, 2006). The most common inherited hypercoagulability disorders that pose as a risk factor for VTE are summarised in Table 2.2.

Table 2.2: Inherited hypercoagulability disorders that pose as a risk for VTE

Disorder	Description	Risk	Source
AT deficiency	Low concentration or lower activity of AT	Heterozygous individuals critically pre- disposed to DVT and 10-fold increased risk of thrombosis	Thomas, 2001
Protein C deficiency	Low concentration of Protein C	Homozygous individuals develop thrombotic disorders and heterozygous individuals are at increased risk for VTE	Maqbool et al., 2013
Factor V Leiden	Mutation in FV	Causes APC resistance resulting in failure of APC-mediated degradation of FVa and FVIIIa, found in 15–25% of patients with DVT	Van Cott et al., 2016
Protein S deficiency	Low concentration of Protein S	2 to 11-fold increased risk for developing VTE in comparison with those without a deficiency	Lipe and Ornstein, 2011

Prothrombin G20210A	Higher levels of prothrombin	Prothrombin levels of more than 110% increases risk of VTE	Jadaon, 2011

APC: Activated Protein C; **AT:** Antithrombin; **DVT:** Deep vein thrombosis; **FV:** Coagulation factor V; **FVa:** Activated coagulation factor V; **FVIIIa**: Activated coagulation factor FVIII**; VTE:** Venous thromboembolism

2.4.2. Acquired hypercoagulability

Acquired hypercoagulability refers to medical conditions or physiological responses that create an environment that favours thrombus formation (Thomas, 2001). Medical conditions such as obesity, as well as pregnancy and prolonged hospitalisation after injury increase the risk of VTE. Furthermore, physiological responses such as inflammation, oxidative stress and response to bacterial invasion can increase the risk of VTE.

2.4.2.1. The role of inflammation in VTE

Inflammation is an essential response by the immune system to stresses such as bacterial infections and injury (Ahmed, 2011). Inflammation is mediated by inflammatory cytokines released by immune cells, which include macrophages and neutrophils. Macrophages act by phagocytising bacteria and damaged tissue (Rambhia and Ma, 2019) while neutrophils act by releasing NETs to trap foreign invaders (Shah et al., 2017). Acute and chronic inflammatory conditions promote thrombosis by upregulating procoagulant factors and downregulating natural anticoagulants (Esmon, 2003).

The coagulation cascade is upregulated during inflammation when an expression of TF on endothelial cells and monocytes is induced by inflammatory cytokines (Bokarewa et al., 2002)**;** such as tumor necrosis factor alpha (TNFα), Interleukin-6 (IL-6) and IL-1 (Chu, 2006). Furthermore, TNFα and IL-1 downregulate thrombomodulin (TM), resulting in less activation of Protein C (Esmon, 2003). The elastase enzymes and NETs released from activated neutrophils during chronic inflammation, degrade antithrombin (AT) (Keser, 2012; Mukhopadhyay et al., 2019). Furthermore, chronic inflammation impairs the synthesis of AT (Mukhopadhyay et al., 2019).

2.4.2.2. The role of reactive oxygen species (ROS) in VTE

Oxidative stress plays a central role in the pathogenesis of many disorders, including thrombotic disorders (Santhakumar, 2015). Reactive oxygen species stimulate coagulation by increasing the expression of TF in endothelial cells, monocytes and vascular smooth muscle cells (Cadroy et al., 2000; Herkert et al., 2002). Furthermore, ROS induce coagulation by inhibiting anticoagulant proteins through oxidative modifications (Gutmann et al., 2020). These proteins include tissue factor pathway inhibitor (Ohkura et al., 2004), thrombomodulin (Glaser et al., 1992), activated protein C (Nalian and

Iakhiaev, 2008) and protein Z dependant protease inhibitor (Huang et al., 2017). Reactive oxygen species can further promote coagulation by oxidising fibrinogen, which is more readily converted to fibrin upon oxidation (Upchurch et al., 1998). Inhibition and activation of platelets is also, in part, regulated by ROS (Gutmann et al., 2020).

2.4.2.3. Obesity as a risk factor

Obesity is a risk factor for the development of VTE due to the lack of mobility of heavier obese individuals and increased inflammation in obese individuals (Previtali et al., 2011; Blokhin and Lentz, 2013). In obese individuals, there is a high accumulation of adipose tissue, which is made up mainly of adipocytes which secrete pro-inflammatory cytokines such as IL-6, interleukin 1-beta (IL-1β) and TNFα (Surmi and Hasty, 2008). The release of inflammatory cytokines activates toll-like receptors (TLRs) on macrophages (Hirayama et al., 2018) and promote increased generation of ROS by macrophages and monocytes. Furthermore, the inflammatory cytokines create an inflammatory state in the body and stimulate the activation of coagulation factors in the coagulation cascade (Blokhin and Lentz, 2013).

2.4.2.4. Pregnancy as a risk factor

Major changes in many aspects of haemostasis occur during a normal pregnancy in order to maintain placental function and protect a woman from haemorrhage during delivery (Prisco et al., 2005). However, these changes increase the risk of developing VTE as it creates a hypercoagulable state. In pregnant women, the concentrations of FVII, FVIII, FIX, and FX increases by more than 100% when compared to the non-pregnant state (Katz and Beilin, 2015). Furthermore, pregnant women have an increased concentration of plasma fibrinogen (Prisco et al., 2005) and the growing uterus causes mechanical obstruction which compromises venous outflow (Alsheef et al., 2020).

2.4.2.5. Injury, surgery and hospitalisation as risk factors

Individuals with decreased mobility due to bedrest or, blood vessel trauma due to surgery or serious injury, have an increased risk of developing VTE (Engbers et al., 2014). Venous thromboembolism is recognised as a major complication facing patients who undergo surgery in the lower parts of the body (Motohashi et al., 2012). Decreased mobility results in reduced blood flow, which causes an accumulation of procoagulant factors and induces thrombosis (Mackman, 2012). Blood vessel trauma induces thrombosis by exposing collagen and TF to procoagulant factors in the bloodstream (Schenone et al., 2004). Furthermore, blood vessel trauma induces an inflammatory response, which further upregulates thrombosis (Rambhia and Ma, 2019).

2.5. Current treatments for VTE

Antithrombotic drugs display anticoagulant, antiplatelet, or thrombolytic activity. This study focused on the anticoagulant and anti-platelet activity of selected plant extracts. Drugs with anticoagulant activity prevent further development of blood clots and allow an individual's natural fibrinolytic system to lyse an existing clot (Bauer, 2008). Drugs with anti-platelet activity decrease platelet aggregation by interfering with platelet receptors and signalling molecules (Hovens et al., 2006).

2.5.1. Anticoagulants

The main types of anticoagulants are direct thrombin inhibitors (Haqqani et al., 2013), direct FXa inhibitors (Alexander and Singh, 2005), Vitamin K antagonists (Dobesh and Stacy, 2019) and unfractionated and low molecular weight heparins (Acquisto, 2014). The intrinsic and extrinsic pathway of the coagulation cascade converge at the activation of FX. Therefore, this study focused on FXa as a target for anticoagulant activity.

2.5.1.1. FXa Inhibitors

Direct FXa inhibitors selectively bind to the active site of FXa and prevent it from interacting with substrates, therefore resulting in less generation of thrombin (Eriksson et al., 2009). Direct FXa inhibitors target FXa within a clot and free FXa in solution and have no direct effect on platelet aggregation (Cabral and Ansell, 2015). Inhibition of FXa is an effective mechanism to prevent the development of thrombosis as it is a more targeted approach that will interrupt the progression of both the intrinsic and extrinsic pathways (Kubitza et al., 2014).

Inhibiting thrombin generation earlier at FXa may require less drug dosage when compared to the dosage needed to directly inhibit thrombin (Alexander and Singh, 2005). Furthermore, FXa functions directly in coagulation; therefore, negative effects outside of coagulation will be minimal (Bauer, 2008). Rivaroxaban, apixaban, and edoxaban are known FXa inhibitors that have been studied in Phase III clinical trials for the treatment of VTE (Cabral and Ansell, 2015). Side effects of these drugs include headache, nausea and bleeding (Abdalwahab et al., 2015).

2.5.2. Antiplatelets

Antiplatelet drugs have different mechanisms of action, including inhibition of P_2Y_{12} receptors (Damman et al., 2012), inhibition of glycoprotein IIb/IIIa (Seiffert and Billheimer, 2007), inhibition of phosphodiesterase enzymes (Schulman and Hirsh, 2012) and inhibition of cyclooxygenase-1 enzyme (COX-1) (Armstrong et al., 2011). It was noted that findings from previous studies revealed

that plants traditionally used for pain and inflammatory disorders have displayed COX-1 inhibitory activity. Therefore, this study focused on COX-1 as a target for antiplatelet activity.

2.5.2.1. Cyclooxygenase-1 inhibitors

Cyclooxygenases are enzymes which are involved in the synthesis of thromboxanes and prostaglandins. Prostaglandins are active lipid compounds that have an important role in the production of pain, inflammation and fever (Bushra and Aslam, 2010). Thromboxanes are lipid compounds involved in vasoconstriction and platelet aggregation (Hanson and Maddison, 2008). Two distinct cyclooxygenase isoforms have been characterised: COX-1 and cyclooxygenase-2 (COX-2). Cyclooxygenase-1 is primarily responsible for homeostatic prostaglandin synthesis, while COX-2 is mainly induced in response to inflammatory stimuli (Süleyman et al., 2007).

Once activated, platelets proximal to a site of injury or exposed TF, recruit additional platelets by releasing aggregatory mediators, which recruit other circulating platelets (Koupenova et al., 2017). The main aggregatory mediator is thromboxane A_2 (TXA_2) which is synthesised by COX-1 (Gabrielsen et al., 2010). Inhibition of COX-1 prevents the production of TXA_2 in platelets and therefore, prevents platelet aggregation (Armstrong et al., 2011).

The most common COX-1 inhibitor used by physicians is acetylsalicylic acid (Konduru and Vanita, 2014). This drug inhibits COX-1 by acetylation of serine residue 529, located on the enzyme, thereby preventing substrate access to the catalytic site of COX-1 (Eikelboom et al., 2012). Cyclooxygenase-1 is constitutively expressed in almost all cell types and can result in side effects such as gastrointestinal erosions as well as renal and hepatic insufficiency (Süleyman et al., 2007).

2.6. Research targets and methods

As previously mentioned, this study focused on FXa and COX-1 as targets for selected plant extracts to determine the anticoagulant and antiplatelet activity. Additionally, the antiproliferative activity against the human hepatocellular carcinoma (HepG2) cell line as well as the antioxidant activity of the plant extracts were investigated. Furthermore, the phytochemistry of the most active plant was determined.

2.6.1. Phytochemistry

In this research study, plant extracts were prepared using dichloromethane (DCM), ethanol, and water. The different extraction solvents allowed for the extraction of secondary metabolites with varying polarities (Mohd et al., 2012). Water is the most polar solvent while DCM is the least polar solvent.

DCM and ethanol are effective in extracting secondary metabolites such as sterols, phenolics and flavonoids while water is effective in extracting alkaloids and glycoside compounds (Widyawati et al., 2014). The composition of the most active plant extract was determined using ultra performance liquid chromatography mass spectrometry (UPLC-MS).

2.6.2. Antiproliferative effects on the liver

The liver is a large organ in the human body responsible for metabolism and detoxification of endogenous and exogenous compounds (Ahsan et al., 2009). The liver is involved in almost all biochemical pathways in the human body, therefore, any damage to the liver will have negative health implications (Subramaniam et al., 2015). Several medicines can cause damage to the liver when taken in overdose or when introduced into therapeutic ranges (Abou Seif, 2016). About 5% of hospital admissions and 50% of all acute liver failures are associated with drug induced hepatotoxicity (Dey et al., 2013). Furthermore, hepatotoxicity is one of the main reasons of drug withdrawal from the market (Dey et al., 2013).

Hence, the antiproliferative assay was used in this study to test all selected plant extracts for antiproliferative activity against the human hepatocellular carcinoma (HepG2) cell line. The HepG2 cell line is an adherent cell line isolated from a human liver hepatocellular carcinoma (Tham et al., 2019). The cell line serves as an appropriate model for normal hepatocytes as it is a non-tumorigenic cell line, and it performs many hepatic functions (Vinken and Rogiers, 2015). The antiproliferative assay is a cell culture based *in vitro* test which determines the antiproliferative effect of a selected plant extract on a cell line. Actinomycin D is an antitumor agent that is used as the positive control in the antiproliferative assay (Lu et al., 2015). It works as a transcription blocker and induces apoptosis in cells (Kleeff et al., 2000). The PrestoBlue™ reagent is used as a cell viability indicator in the antiproliferative assay. The reagent is resazurin based and changes from a blue to pink colour when viable cells reduce resazurin to resorufin (Lall et al., 2013).

2.6.3. Antioxidant activity

There is a need for the development of novel treatments, which prevent thrombosis and/or promote thrombus resolution and have less side effects. An emerging target for such a treatment is ROS, which appear to be important inducers of coagulation. Research in the last decade has provided evidence that plant extracts with significant and well-known antioxidant properties also have anticoagulant effects (Bijak et al., 2016).

By decreasing the level of ROS in the system, the expression of TF in endothelial cells, monocytes and vascular smooth muscle cells will decrease (Herkert et al., 2002). Furthermore, the inhibition of specific anticoagulant proteins by ROS and the activation of platelets by ROS will decrease (Gutmann et al., 2020). This is a relatively underexplored mechanism of action in thrombosis research. It will provide a foundation for future research and an improved understanding of the regulatory role of ROS in thrombosis. The antioxidant activity of the plant extracts that had no activity in the antiproliferative assay was evaluated using the 2,2-diphenyl-1-picryl-hydrazyl-hydrate (DPPH) assay.

The DPPH assay is a test which determines the antioxidant activity of a selected plant extract by measuring its ability to scavenge DPPH free radical (Sharma and Bhat, 2009). The DPPH free radical is a stable free radical with an unpaired electron at the nitrogen bridge (Eklund et al., 2005). When added to DPPH solution, antioxidants reduce DPPH free radical by donating a hydrogen atom which causes a loss of the solutions violet colour (Kedare and Singh, 2011). Vitamin C is a potent antioxidant used as the positive control in the DPPH assay. It rapidly scavenges free radicals by donating a hydrogen atom and forming a stable ascorbate molecule (Nimse and Pal, 2015). The results of the DPPH assay are highly reproducible and comparable to other free radical scavenging methods (Kedare and Singh, 2011).

2.6.4. FXa inhibition

Many medicinal chemists have made the development of direct, potent and orally bioavailable FXa inhibitors their focus (Patel et al., 2016). An active molecule of FXa can generate more than 1000 thrombin molecules (Toschi and Lettino, 2011); therefore, inhibiting FXa will effectively block coagulation. Those plant extracts which displayed significant antioxidant activity were tested for the ability to inhibit FXa using the FXa inhibition assay.

The FXa inhibition assay is an enzyme assay, which determines the ability of a plant extract to inhibit the activity of FXa enzymes. The method is based on the ability of FXa to cleave a chromogenic substrate and cause a colour change in solution (Luan et al., 2017). When the chromogenic substrate is cleaved, it produces a yellow colour. The intensity of the yellow colour is directly proportional to the percentage activity of the FXa enzyme. The positive control in the FXa inhibition assay is GGACK (1,5-Dansyl-Glu-Gly-Arg Chloromethyl Ketone) dihydrochloride, which is a potent, irreversible inhibitor of FXa (Chu, 2006).

2.6.5. COX-1 inhibition

Cyclooxygenase-1 synthesises the aggregatory mediator TXA_2 (Gabrielsen et al., 2010) which recruits additional platelets to a site of exposed TF (Koupenova et al., 2017). By inhibiting COX-1, the production of TXA_2 will decrease and result in the prevention of platelet aggregation (Armstrong et al., 2011). The plant extract which displayed significant inhibition of FXa was tested for its ability to inhibit COX-1 using the COX-1 inhibitor screening assay kit (ab204698, Abcam). The COX-1 inhibitor screening assay kit is based on the fluorometric detection of Prostaglandin G2, the intermediate product generated by the COX enzyme. The positive control for the kit is SC560.

2.7. Plants for the treatment of VTE

Medicinal plants have the potential to advance drug discovery in the global market (Frenzel and Teschke, 2016). As mentioned previously, a large portion of the population living in developing countries, rely on plants as a source of medicine (Ekor, 2014). This is mainly due to the easy accessibility and availability of medicinal plants as a health resource in communities (Mahomoodally, 2013). Almost 3 689 plant species are used as traditional medicines in South Africa (Cherry, 2005).

Medicinal plants produce secondary metabolites with medicinal properties, and therefore have the potential to be used for VTE. Plant secondary metabolites are compounds involved in a plant's interaction with the surrounding environment. These compounds are mainly produced in response to abiotic and biotic stresses (Pagare et al., 2015). Secondary metabolites from plants include chemical groups such as alkaloids, flavonoids and glycosides. These compounds display medicinal properties and they have been used extensively in the pharmaceutical industry (Jain et al., 2019). Plant extracts have shown anticoagulant, antioxidant and antiplatelet activity in *in vitro* studies.

2.7.1. Natural FXa inhibitors

Various anticoagulant studies in the literature survey indicated that plant extracts and isolated compounds have been found to display inhibitory activity of FXa. A study by Bijak et al., (2014) found that the flavonoids: procyanidin B2, cyanidin, quercetin and silybin, had inhibitory effects on FXa activity at 50 percent inhibitory concentrations (IC_{50}) of 1.2µM, 3µM, 5.5µM and 35µM, respectively. Furthermore, a study by Ku et al., (2013) discovered that two flavonoids, persicarin and isorhamnetin, inhibited the activity of FXa in a dose-dependent manner without potentiation by antithrombin.

In a different study, the ethanolic extracts of plants traditionally used for pain and blood flow were tested for their effects on FXa activity (Ibrahim et al., 2020). The IC_{50} values were 0.363mg/mL,

0.729mg/mL and 0.866mg/mL for *Glycyrrhiza glabra* L. (Liquorice), *Trifolium alexandrinum* L. (Egyptian clover) and *Olea europaea* L. (Olive), respectively. An earlier study by (Correia-da-Silva et al., (2011), found that polysulfated flavonoids could directly inhibit FXa. These studies revealed that plants with a high concentration of flavonoids may be considered as potential FXa inhibitors. The literature makes it evident that South African plants have not been explored as potential FXa inhibitors.

2.7.2. Natural antioxidants for thrombosis

Research in the last decade has provided evidence that plant extracts with significant and well-known antioxidant properties also have anticoagulant effects. A study by Bijak et al. (2016) revealed that the grape seed extract of *Vitis vinifera* L. displayed dual antiplatelet and anticoagulant activity. Another *in vitro* study found that resveratrol, a potent antioxidant, inhibited platelet aggregation by scavenging free radicles produced after collagen induced activation of platelets (Sobotková et al., 2009). Another study found that resveratrol downregulated the expression of TF in human umbilical vein endothelial cells and mononuclear cells by removal of highly reactive free radicals such as superoxide (Santo et al., 2003). Based on this finding, plants with high concentrations of polyphenolic compounds, such as resveratrol can be considered for VTE.

2.7.3. Natural COX-1 inhibitors

Plants traditionally used for pain and inflammatory disorders frequently have the ability to inhibit cyclooxygenase enzyme. This is due to the role that cyclooxygenase plays in the production of pain and inflammation (Bushra and Aslam, 2010). Plant extracts with these properties can be effective treatments for VTE as inflammation promotes thrombosis (Esmon, 2003) and COX-1 is directly involved in platelet aggregation (Armstrong et al., 2011). Table 2.3 summarises some South African plants traditionally used for pain and inflammatory disorders which displayed COX-1 inhibition during *in vitro* studies.

Table 2.3: Plants traditionally used for pain and inflammatory disorders with reported COX-1 activity

Plant	Traditional Use	Reported COX-1 Activity	Phytochemicals responsible for activity	Source
***Merwilla plumbea* (Lindl.) Speta** **(Blue hyacinth)**	Sprains and fractures, menstrual pains, support woman when giving birth, boils,	DCM extract demonstrated 80% inhibition of COX-1 and hexane extract displayed 71% inhibition of	Saponins and Bufadienolides	Street and Prinsloo, 2013 Sparg et al.,

	veld sores	COX-1 at a concentration of 250 µg/mL		2002
***Siphonochilus aethiopicus* (Schweinf.) B.L.Burtt.** **(Wild ginger)**	Rhizomes are chewed fresh for pain relief and dysmenorrhea	Ethanolic extract of leaves demonstrated 80% inhibition of COX-1 at a concentration of 250 µg/mL	Sesquiterpenes	van Wyk et al., 1997 Light et al., 2002 Igoli and Obanu, 2011
***Eucomis autumnalis* (Mill.) Chitt.** **(Pineapple lily)**	Leaves and bulbs administered for backache, post-operative recovery, fevers and fractures	Ethanolic extract of leaves, bulbs and roots displayed IC_{50} of 15 µg/mL, 72 µg/mL and 27 µg/mL respectively against COX-1	Flavonoids	Masondo et al., 2014 Taylor and van Staden, 2002
***Aloe ferox* Mill.** **(Bitter aloe)**	Bruises, burns, inflamed skin	Methanolic extract displayed 80% inhibition of COX-1	Cinnamic acid	van Wyk et al., 1997 Lindsey et al., 2002 Lawrence et al., 2009
***Ocotea bullata* (Burch.) Baill.** **(Black Stinkwood)**	Bark is taken as a snuff for headaches	Methanolic extracts of dried leaves displayed 70% inhibition of COX-1 at a concentration of 100µg/mL	Sesquiterpenes	Zschocke et al., 2000
***Harpagophytum procumbens* (Burch.) DC ex Meisn.** **(Devil's claw)**	Analgesic and remedy for fever and allergies	A fraction decreased the activity of COX-1 by 37.2% at a concentration of 30 µg/mL	Harpagoside	Mncwangi et al., 2012 Anauate et al., 2010

DCM: dichloromethane; **IC50**: 50 percent inhibitory concentration

2.8. Conclusion

The key target for the treatment of venous thromboembolism is thrombosis. Antithrombotic drugs display antiplatelet and anticoagulant properties. There are many scientific studies that support the use of medicinal plants for VTE. In this research study's focus on antiproliferative effects on the liver, antioxidant activity, FXa inhibition, COX-1 inhibition and the phytochemistry of selected plant

extracts, it became evident that the antithrombotic potential of South African plant species is underexplored. Hence, the results of this study provide new information and consideration of the selected South African plant species for further studies for VTE.

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Chapter 3: Plant Selection

Chapter 3: Plant Selection

Statement

The plants chosen for this study are part of a collaborative project contributed to by different students. The monographs have been prepared for a future book publication titled: "Underexplored Medicinal Plants from Sub-Saharan Africa, 2nd Edition". The monographs for the following plants can be found under Appendix A: Additional Plant Monographs; *Cotyledon orbiculata* (Author: Jacqueline Maphutha), *Cussonia spicata* (Author: Jacqueline Maphutha), *Hypoestes aristata* (Author: Jacqueline Maphutha), *Hypoestes forskaolii* (Author: Jacqueline Maphutha), *Pelargonium citronellum* (Author: Lydia Gibango), *Portulacaria afra* (Author: Lydia Gibango), *Sideroxylon inerme* (Author: Lydia Gibango)

3.1. Introduction

A total of 11 plant species were selected for the current study. The plants were chosen based on traditional use and pharmacological properties. Plants traditionally used for pain, bleeding disorders and wound healing as well as plants with anti-inflammatory and antioxidant pharmacological properties were selected.

3.2. *Heteropyxis natalensis*

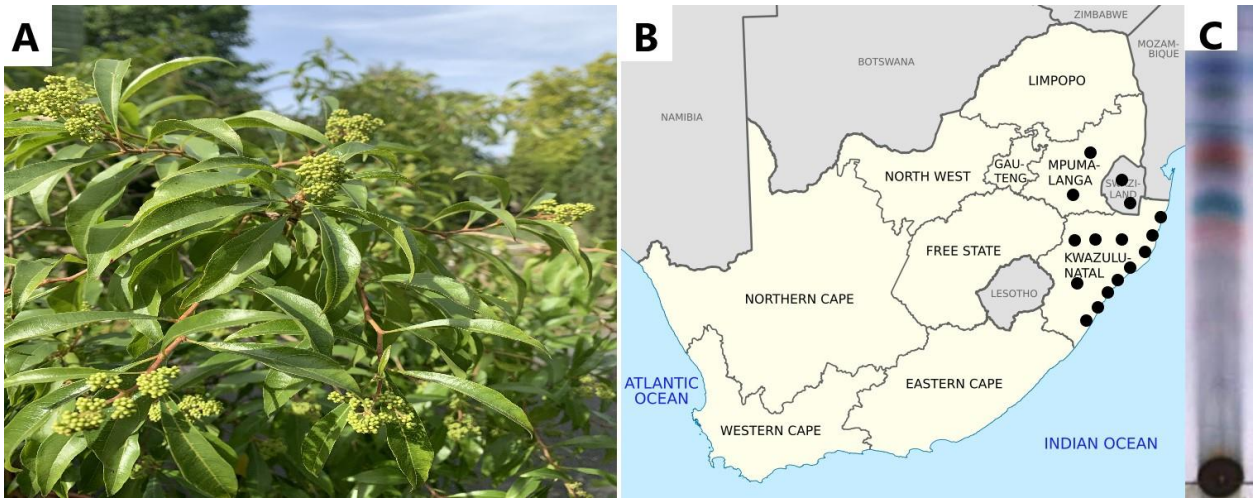

Figure 3.1: (A) *Heteropyxis natalensis* aerial plant parts (Wolf, 2020). **(B)** Distribution of *H. natalensis* (Htonl, 2010). **(C)** TLC chromatogram of *H. natalensis* ethanolic extract

3.2.1. General description

3.2.1.1. Botanical nomenclature

Heteropyxis natalensis Harvey

3.2.1.2. Botanical family

Heteropyxidaceae

3.2.1.3. Vernacular names

Lavender tree (English)

Laventelboom (Afrikaans)

Inkunzi (Zulu)

3.2.2. Botanical description

Heteroropyxis natalensis is a relatively small (15m) deciduous tree with dense leaves and aromatic foliage with a similar smell to lavender (Coates-Palgrave, 2002). The leaves are elliptic, spirally arranged, and droop from the stem with a glossy green colour above and a lighter green colour below (van Wyk and van Wyk, 2013). The flowers are small, light yellow to green in colour and mature into oval shaped capsules which split and shed small dark seeds (Palmer and Pitman, 1972).

3.2.3. Distribution

Heteroropyxis natalensis occurs in the coastal regions of KwaZulu Natal as well as inland regions, mainly along streams, through Mpumalanga to Swaziland (Palmer and Pitman, 1972; Coates-Palgrave, 2002).

3.2.4. Ethnobotanical usage

The leaves of *H. natalensis* are used for colds, weaning, toothaches and gum infections (Braithwaite et al., 2008; van Wyk, 2011; Henley-Smith et al., 2018; van Wyk and Gericke, 2018;). The leaves are also used as a blood purifier in humans and as a dewormer for animals (van Wyk et al., 2009). The bark of *H. natalensis* is used for wounds, impotence and used as an aphrodisiac (Hutchings and Scott, 1996; Long, 2005). The roots of *H. natalensis* are used for bleeding gums (Palmer and Pitman, 1972). The bark, roots and leaves of *H. natalensis* are used for anti-infection, bleeding disorders, nose-bleeding, menorrhagia, respiratory disorders and as a decongestant (Arnold and Gulumian, 1984; van Wyk et al., 2009; van Wyk, 2011).

3.2.5. Phytochemical constituents

More than 190 compounds have been isolated from the essential oil of *H. natalensis* (Maroyi, 2019). A study by Weyerstahl et al. (1992), found that the essential oil of *H. natalensis* was rich in monoterpenes which varied seasonally in quantity. The main monoterpenes identified were (E)-β-

ocimene, linalool, myrcene, l,8-cineole and p-mentha-1(7),8-diene. In a study by Henley-Smith et al. (2018), aurentiacin A, cardamomin, 5-hydroxy-7-methoxy-6- methylflavanone, quercetin and 3,5,7-trihydroxyflava were isolated from the dried ethanolic leaf extract of *H. natalensis*. The chalcone, (E)-1-(2', 4'-dihyroxy, 5'-methoxy, 3'-methylphenyl)-3-phenylprop-2-en-1-one, was isolated from the leaves of *H. natalensis* in a study on non-volatile compounds present in the plant (Adesanwo et al., 2009). In a study of the geographical variation of the essential oil of *H. natalensis*, it was found that (Z)-3-hexenyl nonanoate was a major essential oil component of *H. natalensis* from Lagalametse, South Africa (van Vuuren et al., 2007).

3.2.6. Pharmacological Properties

Heteroropyxis natalensis has been investigated for antibacterial, antifungal, antioxidant, anti-inflammatory, pro-inflammatory and anticancer activities.

3.2.6.1. Antibacterial activity

The essential oil of *H. natalensis* as well as crude extracts and isolated compounds have been tested against several bacterial pathogens using the broth microdilution assay (Table 3.1). In a study by Gundidza et al. (1993), the essential oil of *H. natalensis* was tested against several bacterial pathogens using the disc diffusion method (Table 3.2).

Table 3.1: Minimum inhibitory concentration (MIC) of the essential oil, extracts and compounds isolated from *Heteropyxis natalensis* against various bacterial pathogens

Pathogen	Sample	MIC (mg/mL)	Reference
Actinomyces israelii **ATCC 10049**	Aurentiacin A*****	0.06	Henley-Smith et al., 2018
	Leaf EtOH	0.88	
Aeromonas hydrophilia **ATCC 7965**	Leaf $MetOH_a$	3.08	Cock and van Vuuren, 2015
Alicaligenes faecalis **ATCC 8750**	Leaf $MetOH_a$	0.53	
Bacillus cereus **ATCC 11778**	Essential Oil	4.50	van Vuuren and Viljoen, 2006
	APP Smoke ex	0.35	Braithwaite et al., 2008
	APP Ace	0.25	
	APP $MetOH_a$	0.25	
	Essential Oil	4.00	

	(Z)-3-hexenyl nonanoate*	1.560	van Vuuren et al., 2007
	(E)-3-hexenyl nonanoate*	1.560	
	hexyl nonanoate*	1.560	
	(Z)-2-hexenyl nonanoate*	1.560	
Bacillus subtilis **ATCC 6051**	Essential Oil	32.000	van Vuuren et al., 2007
Entorococcus faecalis **ATCC 29212**	Essential oil	18.000	van Vuuren and Viljoen, 2006
	(Z)-3-hexenyl nonanoate*	3.100	van Vuuren et al., 2007
	(E)-3-hexenyl nonanoate*	1.210	
	hexyl nonanoate*	3.500	
	(Z)-2-hexenyl nonanoate*	1.060	
Escherichia coli **ATCC 11775**	Essential oil	4.500	van Vuuren and Viljoen, 2006
Escherichia coli **ATCC 25922**	(Z)-3-hexenyl nonanoate*	1.560	van Vuuren et al., 2007
	(E)-3-hexenyl nonanoate*	1.560	
	hexyl nonanoate*	1.560	
	(Z)-2-hexenyl nonanoate*	1.560	
Fusobacterium nucleatum **ATCC 25586**	Leaf (1:1) DCM:MetOH ex	2.000	Akhalwaya et al., 2018
	Stem (1:1) DCM:MetOH ex	1.330	
Klebsiella pneumoniae **ATCC 9633**	APP Smoke	0.700	Braithwaite et al., 2008
	APP Ace	2.000	
	APP $MetOH_a$	1.000	
	Essential Oil	8.000	
Klebsiella pneumoniae **NCTC 9633**	Essential Oil	10.10	van Vuuren and Viljoen, 2006
Lactobacillus acidophilus **ATCC 4356**	Leaf (1:1) DCM:MetOH ex	0.250	
	Stem (1:1) DCM:MetOH ex	1.330	

	Stem Aqua	4.000	Akhalwaya et al., 2018
Lactobacillus casei **ATCC 344**	Leaf (1:1) DCM:MetOH ex	0.250	Akhalwaya et al., 2018
	Stem (1:1) DCM:MetOH ex	1.000	
	Leaf Aqua	8.000	
Lactobacillus paracasei **Oral clinical strain A54**	Leaf EtOH	9.380	Henley-Smith et al., 2018
Moraxella catarrhalis **ATCC 23246**	(Z)-3-hexenyl nonanoate*	1.560	van Vuuren et al., 2007
	(E)-3-hexenyl nonanoate*	1.560	
	hexyl nonanoate*	2.610	
	(Z)-2-hexenyl nonanoate*	2.080	
Mycobacterium aurum **NCTC 10437**	Leaf Ace	0.080	Dzoyem et al., 2016
Mycobacterium fortuitum **ATCC 6841**	Leaf Ace	0.625	
Mycobacterium smegmatis **ATCC 1441**	Leaf Ace	0.156	
Mycobacterium tuberculosis **TB 8104**	Leaf Ace	0.312	
Porphyromonas gingivalis **ATCC 33277**	Leaf (1:1) DCM:MetOH ex	1.000	Akhalwaya et al., 2018
	Stem (1:1) DCM:MetOH ex	0.500	
	Leaf Aqua	2.000	
	Stem Aqua	1.000	
Prevotella intermedia **ATCC 25611**	Leaf EtOH	12.50	Henley-Smith et al., 2018
Propionibacterium acnes **ATCC 11827**	Leaf EtOH	0.250	Sharma and Lall, 2014
Proteus mirabilis **ATCC 43071**	Leaf $MetOH_a$	6.150	Cock and van Vuuren, 2015
Proteus vulgaris **ATCC 33420**	Leaf $MetOH_a$	3.065	Cock and van Vuuren, 2015

***Pseudomonas aeruginosa* ATCC 27858**	(Z)-3-hexenyl nonanoate*	0.850	van Vuuren et al., 2007
	(E)-3-hexenyl nonanoate*	0.450	
	hexyl nonanoate*	0.490	
	(Z)-2-hexenyl nonanoate*	1.190	
***Pseudomonas aeruginosa* ATCC 9027**	Essential Oil	8.600	van Vuuren and Viljoen, 2006
***Staphylococcus aureus* ATCC 12600**	Essential Oil	8.600	van Vuuren and Viljoen, 2006
***Staphylococcus aureus* ATCC 25923**	(Z)-3-hexenyl nonanoate*	3.130	van Vuuren et al., 2007
	(E)-3-hexenyl nonanoate*	1.560	
	hexyl nonanoate*	1.560	
	(Z)-2-hexenyl nonanoate*	2.350	
	APP Smoke ex	1.860	Braithwaite et al., 2008
	APP Ace	0.380	
	APP $MetOH_a$	0.380	
	Essential Oil	32.00	
***Staphylococcus epidermidis* ATCC 2223**	Essential Oil	5.700	van Vuuren and Viljoen, 2006
***Streptococcus mutans* ATCC 25175**	Leaf (1:1) DCM:MetOH ex	1.330	Akhalwaya et al., 2018
	Stem (1:1) DCM:MetOH ex	2.000	
	Stem Aqua	4.000	
	Leaf EtOH	2.600	Henley-Smith et al., 2018
***Streptococcus sanguis* ATCC 25175**	Leaf (1:1) DCM:MetOH ex	2.000	Akhalwaya et al., 2018
	Stem (1:1) DCM:MetOH ex	0.210	
	Leaf Aqua	8.000	
	Stem Aqua	6.000	

*****Isolated compound; **Ace:** Acetone extract**; APP:** Aerial plant parts; **Aqua:** Aqueous extract **DCM:** Dichloromethane; **EtOH:** Ethanolic extract; **ex:** Extract; **MetOHa:** Methanolic extract; **MetOH:** Methanol; **MIC:** Minimum Inhibitory Concentration

Table 3.2: Zone of inhibition of *Heteropyxis natalensis* essential oil at a concentration of 1000mL/L against bacterial pathogens

Pathogen	Zone of inhibition at 1000mL/L (mm)
Brevibacterium linen **NCIB 8456**	18.5
Clostridium perfringens **NCIB 10696**	31.5
Escherichia coli **NCIB 8879**	12
Flavobacterium suaveolens **NCIB 8992**	15.4
Klebsiella pneumoniae **NCIB 418**	16.4
Staphylococcus aureus **NCIB 6571**	12.6
Streptococcus faecalis **NCTC 775**	14.6

3.2.6.2. Antifungal activity

The essential oil of *H. natalensis* as well as crude extracts and isolated compounds have been tested against *Candida* and *Cryptococcus* species, using the broth microdilution assay (Table 3.3). In a study by Gundidza et al. (1993), the essential oil of *H. natalensis* was tested against *Aspergillus* species using the disc diffusion method (

*****Isolated compound; **Ace:** Acetone extract**; APP:** Aerial plant parts; **Aqua:** Aqueous extract; **DCM:** Dichloromethane; **EtOH:** Ethanolic extract; **ex:** Extract; **MetOHa:** Methanolic extract; **MetOH:** Methanol; **MIC:** Minimum Inhibitory Concentration

Table 3.4).

Table 3.3: Minimum inhibitory concentration (MIC) of different extracts and compounds isolated from *Heteropyxis natalensis* against *Candida* and *Cryptococcus* species

Pathogen	Sample	MIC (mg/mL)	Reference
Candida albicans **ATCC 10231**	Essential Oil	4.5	van Vuuren and Viljoen, 2006
	Leaf EtOH	8.33	Henley-Smith et al., 2018
	(Z)-3-hexenyl nonanoate*	1.53	van Vuuren et al., 2007
	(E)-3-hexenyl nonanoate*	1.09	

	hexyl nonanoate*	1.09	
	(Z)-2-hexenyl nonanoate*	1.50	
***Candida albicans* ATCC 102331**	Leaf (1:1) DCM:MetOH ex	1.00	
	Stem (1:1) DCM:MetOH ex	4.00	Akhalwaya et al., 2018
	Leaf Aqua	0.25	
	Stem Aqua	4.00	
***Candida albicans* 1051604**	Leaf EtOH	12.5	Henley-Smith et al., 2018
***Candida albicans* ATCC 90030**	Leaf (1:1) DCM:MetOH ex	1.00	
	Stem (1:1) DCM:MetOH ex	0.50	Akhalwaya et al., 2018
	Stem Aqua	8.00	
***Candida krusei* ATCC 14243**	Leaf (1:1) DCM:MetOH ex	3.33	
	Stem (1:1) DCM:MetOH ex	1.00	Akhalwaya et al., 2018
***Cryptococcus neoformans* ATCC 90112**	APP Smoke	0.93	
	APP Ace	1.50	Braithwaite et al., 2008
	APP $MetOH_a$	0.83	
	Essential Oil	8.00	
	(Z)-3-hexenyl nonanoate*	0.50	
	(E)-3-hexenyl nonanoate*	0.89	van Vuuren et al., 2007
	hexyl nonanoate*	0.89	
	(Z)-2-hexenyl nonanoate*	0.89	
***Cryptococcus neoformans* ATCC 9012**	Essential Oil	5.70	van Vuuren and Viljoen, 2006

*****Isolated compound; **Ace:** Acetone extract**; APP:** Aerial plant parts; **Aqua:** Aqueous extract; **DCM:** Dichloromethane; **EtOH:** Ethanolic extract; **$_{ex}$:** Extract; **MetOHa:** Methanolic extract; **MetOH:** Methanol; **MIC:** Minimum Inhibitory Concentration

Table 3.4: Zone of inhibition of *Heteropyxis natalensis* essential oil at a concentration of 1000mL/L against *Aspergillus* species

Pathogen	Zone of inhibition (mm)
Aspergillus flacus **IMI 89717**	19.5
Aspergillus niger **IMI 17454**	39.3
Aspergillus ochraceus **IMI 132424**	19.0

3.2.6.3. Antioxidant, anti-inflammatory, anticancer and pro-inflammatory activity

The antioxidant activity of the ethanolic extract of the leaves and stems of *H. natalensis* was tested using the 2,2-diphenyl-1-picrylhydrazyl hydrate (DPPH) radical scavenging assay (Muchuweti et al., 2006). The percentage inhibition was determined to be 29.7% at a concentration of 1mg/mL. The anti-inflammatory activity of the essential oil of *H. natalensis* was investigated using the 5-lipoxygenase inhibitory assay (Frum and Viljoen, 2006). The 50 percent inhibitory concentration (IC_{50}) of 5-lipoxygenase by the extract was determined to be 46.64ppm. In another study by Henley-Smith et al., (2018), the leaf ethanolic extract of *H. natalensis* exhibited pro-inflammatory activity by reducing the production of interleukin-8 by macrophages. In the same study, the extract inhibited proliferation of human laryngeal epidermoid carcinoma cells at an IC_{50} of 35.56µg/ml in a PrestoBlue™ based cell viability assay.

3.2.7. TLC Fingerprinting of plant extract

The ethanolic extract of *H. natalensis* (2mg) was dissolved in 200µL of ethanol. The separation of the compounds present in the extract were observed on a Silica gel 60 F254 TLC plate. The extract was spotted on the plate and left to dry. The plates were developed with a Hexane: Ethyl acetate (7:3) solvent system. Once developed, the plate was observed under long and short wavelength ultraviolet light and sprayed with vanillin solution to detect bands.

3.2.8. Additional information

3.2.8.1. Safety data

A single-dose acute toxicity study was conducted to determine the acute toxicity of *H. natalensis* ethanolic extract (Naidoo, 2014). Mice were dosed with *H. natalensis* ethanolic extract at 300mg/kg and 2000mg/kg. The mice were euthanised after 14 days from exposure to the single dose. Signs of

mild hepatotoxicity were evident in the mice and there was an accumulation of blood in the thoracic cavity of all mice with a more severe accumulation in the 2000mg/kg group. In another study by Henley-Smith et al. (2018), the cytotoxicity of the leaf ethanolic extract of *H. natalensis* was tested against human monocyte (U937) and African green monkey kidney epithelium (Vero) using a PrestoBlue™ based cell viability assay. The IC_{50} values for human monocyte (U937) and African green monkey kidney epithelium (Vero) were found to be 147µg/ml and 33.66µg/ml respectively. In another study which utilised the MTT assay, Dzoyem et al. (2016), concluded that the Selectivity index (SI) value for *H. natalensis* acetone extract was 3.3. This, therefore, indicated a large safety margin between the mycobactericidal concentration and the Vero cytotoxicity concentration.

3.2.8.2. Therapeutic (proposed) usage

Antibacterial, antifungal, anticancer

3.2.8.3. Trade information

Not threatened. Least concern on South African National Biodiversity Institute (SANBI) Red List

3.2.8.4. Dosage

Not available

3.3. *Pelargonium graveolens*

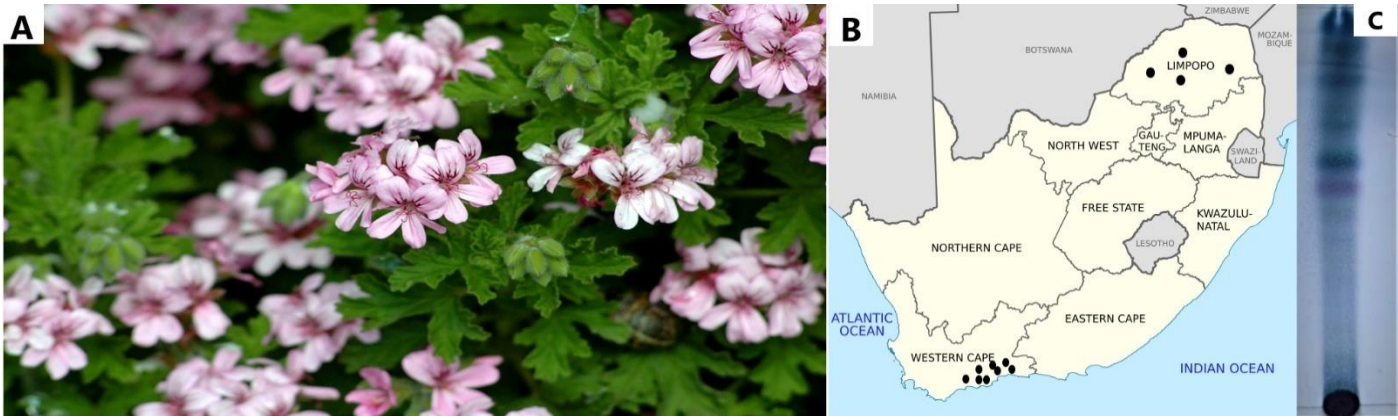

Figure 3.2: (A) *Pelargonium graveolens leaves and flowers* (Hunt, 2005). **(B)** Distribution of *Pelargonium graveolens* (Htonl, 2010). **(C)** TLC chromatogram of *P. graveolens* ethanolic extract

3.3.1. General description

3.3.1.1. Botanical nomenclature

Pelargonium graveolens L'Heritier

3.3.1.2. Botanical family

Geraniaceae

3.3.1.3. Vernacular names

Rose-scented pelargonium (English)

Wildemalva (Afrikaans)

3.3.2. Botanical description

Pelargonium graveolens is an aromatic perennial, shrub that can reach 1.3m in height and 1m in spread (Hutchings and Scott, 1996; Boukhris et al., 2013) The leaves have multiple glandular hairs with a strong rose scent (Verma et al., 2010; Boukhris et al., 2013;). The stem of the plant is hairy and herbaceous and becomes woody as the plant ages (Boukhris et al., 2013). The flowers are small, pink and arranged in an umbel inflorescence (Lawrence, 2002; Asgarpanah and Ramezanloo, 2015;). The fruit of *Pelargonium* species resemble a stork beak, hence the genus name *Pelargonium,* which originates from the Greek word "pelargos", which means stork in English (Lawrence, 2002).

3.3.3. Distribution

Pelargonium graveolens occurs in the Limpopo province and the south-east region of the Western Cape province on dry rocky slopes (Lawrence, 2002; Rana et al., 2002). While the plant is endemic to South Africa, however, it was introduced as an ornamental plant in Northern Africa (Broomhead et al., 2020).

3.3.4. Ethnobotanical usage

The roots and aerial plant parts of *Pelargonium* species are used in traditional remedies for wounds, fever, sore throats and abscesses (Hutchings and Scott, 1996; Lalli et al., 2008)

3.3.5. Phytochemical constituents

Pelargonium graveolens is cultivated worldwide for its essential oil, which is used in many industries (Ennaifer et al., 2018). A study by Rana et al. (2002) identified the chemical constituents of the essential oil of *P. graveolens*. The main compounds identified were citronellol (33.6%), geraniol (26.8%), linalool (10.5%), citronellyl formate (9.7%) and ρ-menthone (6.0%). In another study by Boukhris et al. (2013), the flavonoids myrisetin, quercetin, kaempferol and isorhamnetin aglycone, were identified in the non-polar extracts of the leaves and flowers of *P. graveolens.*

3.3.6. Pharmacological Properties

Pelargonium graveolens has been investigated for antibacterial, antifungal, antioxidant, and anticancer activity. Furthermore, the plant has been tested for enzyme inhibition and photoprotective properties.

3.3.6.1. Antibacterial activity

The essential oil of *P. graveolens* as well as crude extracts have been tested against several bacterial pathogens using the broth microdilution assay (Table 3.5).

Table 3.5: Minimum inhibitory concentration (MIC) of the essential oil and crude extracts of *Pelargonium graveolens* against various bacterial pathogens

Pathogen	Sample	MIC	Reference
Atopobium vaginae*	Essential Oil	1.00[a]	Schwiertz et al., 2006
Bacillus cereus **ATCC 11778**	Leaf Essential Oil	2.00[a]	Ali et al., 2020
	Leaf MetOH	10.00[a]	
	Leaf EtOH	1.00[a]	
	Leaf Aqua	5.00[a]	
	Flower Essential Oil	0.50[a]	
	Flower MetOH	2.00[a]	
	Flower EtOH	1.00[a]	
	Flower Aqua	2.00[a]	
Bacillus subtilis **DSM 6633**	MetOH	0.47[b]	El Aanachi et al., 2020
	DCM	1.87[b]	
	Hex	7.50[b]	
Bacteroides vulgatus*	Essential Oil	1.00[a]	Schwiertz et al., 2006
Enterobacter cloacae **Clinical strain**	Leaf MetOH	5.00[b]	Ali et al., 2020
	Leaf EtOH	1.00[b]	
	Leaf Aqua	5.00[b]	
	Flower Essential Oil	1.00[b]	
	Flower MetOH	2.00[b]	

	Flower EtOH	0.50[b]	
	Flower Aqua	5.00[b]	
Escherichia coli **ATCC 25922**	Leaf Essential Oil	2.00[a]	
	Leaf MetOH	5.00[a]	
	Leaf EtOH	1.00[a]	
	Leaf Aqua	5.00[a]	Ali et al., 2020
	Flower Essential Oil	1.00[a]	
	Flower MetOH	2.00[a]	
	Flower EtOH	1.00[a]	
	Flower Aqua	2.00[a]	
Escherichia coli **ATCC 25922**	MetOH	0.94[b]	
	DCM	1.87[b]	El Aanachi et al., 2020
	Hex	3.75[b]	
Escherichia coli **K12 CECT 433**	MetOH	1.87[b]	
	DCM	3.75[b]	El Aanachi et al., 2020
	Hex	3.75[b]	
Gardnerella vaginalis*	Essential Oil	1.00[a]	Schwiertz et al., 2006
Klebsiella oxytoca **Clinical strain**	Leaf Essential Oil	0.50[a]	
	Leaf EtOH	2.00[a]	
	Leaf Aqua	1.00[a]	
	Flower Essential Oil	0.50[a]	Ali et al., 2020
	Flower MetOH	5.00[a]	
	Flower EtOH	2.00[a]	
	Flower Aqua	1.00[a]	
Klebsiella pneumoniae **ATCC 10031**	Leaf Essential Oil	0.50[a]	
	Leaf MetOH	10.00[a]	
	Leaf EtOH	2.00[a]	
	Leaf Aqua	5.00[a]	Ali et al., 2020

	Flower Essential Oil	0.50[a]	
	Flower MetOH	2.00[a]	
	Flower EtOH	2.00[a]	
	Flower Aqua	5.00[a]	
Lactobacillus acidophilus*	Essential Oil	10.00[a]	Schwiertz et al., 2006
Lactobacillus casei*	Essential Oil	10.00[a]	Schwiertz et al., 2006
Listeria innocua	MetOH	0.94[b]	
CECT 433	DCM	3.75[b]	El Aanachi et al., 2020
	Hex	3.75[b]	
Morganella morgani	Leaf Essential Oil	5.00[b]	
Clinical strain	Leaf MetOH	10.00[b]	
	Leaf EtOH	1.00[b]	
	Leaf Aqua	5.00[b]	Ali et al., 2020
	Flower Essential Oil	5.00[b]	
	Flower MetOH	2.00[b]	
	Flower EtOH	2.00[b]	
	Flower Aqua	2.00[b]	
Proteus mirabilis	Leaf Essential Oil	10.00[b]	
Clinical strain	Leaf MetOH	10.00[b]	
	Leaf EtOH	1.00[b]	
	Leaf Aqua	2.00[b]	Ali et al., 2020
	Flower Essential Oil	1.00[b]	
	Flower MetOH	5.00[b]	
	Flower EtOH	1.00[b]	
	Flower Aqua	5.00[b]	
Pseudomonas aeruginosa **CECT 118**	MetOH	0.47[b]	
	DCM	3.75[b]	El Aanachi et al., 2020
	Hex	3.75[b]	

***Pseudomonas aeruginosa* ATCC 27853**	Leaf Essential Oil	0.50[a]	Ali et al., 2020
	Leaf MetOH	10.00[a]	
	Leaf EtOH	1.00[a]	
	Leaf Aqua	1.00 [a]	
	Flower Essential Oil	1.00[a]	
	Flower MetOH	2.00[a]	
	Flower EtOH	1.00[a]	
	Flower Aqua	2.00[a]	
***Salmonella typhimurium* NCTC 6017**	Leaf Essential Oil	10.00 [b]	Ali et al., 2020
	Leaf EtOH	2.00 [b]	
	Leaf Aqua	2.00[b]	
	Flower Essential Oil	2.00 [b]	
	Flower MetOH	10.00 [b]	
	Flower EtOH	0.50 [b]	
	Flower Aqua	5.00 [b]	
***Staphylococcus aureus* ATCC 25923**	MetOH	0.47[b]	El Aanachi et al., 2020
	DCM	1.87[b]	
	Hex	3.75[b]	
***Staphylococcus aureus* ATCC 6538**	Leaf Essential Oil	5.00 [a]	Ali et al., 2020
	Leaf MetOH	10.00 [a]	
	Leaf EtOH	1.00 [a]	
	Leaf Aqua	5.00 [a]	
	Flower Essential Oil	0.50 [a]	
	Flower MetOH	2.00 [a]	
	Flower EtOH	1.00 [a]	
	Flower Aqua	2.00 [a]	
***Staphylococcus epidermis* ATCC 12228**	Leaf Essential Oil	2.00 [a]	
	Leaf MetOH	10.00 [a]	

	Leaf EtOH	1.00 [a]	
	Leaf Aqua	2.00 [a]	Ali et al., 2020
	Flower Essential Oil	0.50 [a]	
	Flower MetOH	2.00 [a]	
	Flower EtOH	1.00 [a]	
	Flower Aqua	2.00 [a]	
Streptococcus agalactiae*	Essential Oil	1.00[a]	Schwiertz et al., 2006

*: Isolated from patients diagnosed with bacterial vaginosis; **a:** MIC(µL/mL); **Aqua:** Aqueous extract; **b:** MIC (mg/mL); **DCM:** Dichloromethane extract; **EtOH:** Ethanolic extract; **Hex:** Hexane extract; **MetOH:** Methanolic extract; **MIC:** Minimum Inhibitory Concentration,

3.2.6.2. Antifungal activity

In a study by El Aanachi et al. (2020), the methanolic, ethanolic and hexane extracts of *P. graveolens* were tested against *Candida albicans* and *Cryptcoccus neoformans* using the broth microdilution assay (Table 3.6).

Table 3.6: Minimum inhibitory concentration (MIC) of the essential oil of *Pelargonium graveolens* against *Candida albicans* and *Cryptcoccus neoformans*

Pathogen	Extract	MIC (mg/mL)
***Candida albicans* ATCC 10231**	MetOH	0.47
	DCM	1.87
	Hex	3.75
***Cryptcoccus neoformans* Clinical Strain**	MetOH	1.87
	DCM	3.75
	Hex	3.75

MetOH: Methanolic extract, **DCM:** Dichloromethane extract, **Hex:** Hexane extract

3.2.6.3. Antioxidant activity

The antioxidant activity of *Pelargonium graveolens* crude extracts and essential oils have been tested in various studies using the DPPH radical scavenging assay. In a study by, El Aanachi et al. (2020), the methanolic and hexane extracts of the aerial plant parts exhibited IC_{50} values of 12.96µg/mL and 37.60µg/mL respectively. In another study by Ali et al. (2020), the methanolic extracts of the leaves and flowers of *P. graveolens* displayed IC_{50} values of 12.24µg/mL and 16.03µg/mL respectively. In

the same study, the ethanolic extracts of the leaves and flowers exhibited an IC_{50} of 14.68µg/mL and 19.31µg/mL respectively. Boukhris et al. (2015), determined the antioxidant activity of *P. graveolens* essential oil at different phenological stages. The IC_{50} was 1.5µg/mL, 1.00µg/mL, 2.00µg/mL and 1.2µg/mL at the floral budding, full flowering, post flowering and vegetative stages, respectively. In another study of the essential oil of *P. graveolens*, 76.80% of DPPH free radical was scavenged at a concentration of 200µg/mL (Fayed, 2009).

3.2.6.2. Anticancer activity

Fayed, (2009) tested the anticancer activity of the essential oil of *P. graveolens* against the human promyelocytic leukemia cell lines, HL-60 and NB4. A tryphan blue cell viability assay was used, and it revealed that the essential oil decreased the number of viable HL-60 and NB4 cells by 79.27% and 79.8% respectively at a concentration of 200µg/mL. In another study, Wiseman et al., (2007), concluded that the compound geraniol caused G_0/G_1 phase arrest in human pancreatic adenocarcinoma cell lines, MIA PaCa-2 and BxPC-3, while Duncan et al., (2004) revealed that geraniol inhibited the growth of human breast adenocarcinoma cells (MCF-7) by G_0/G_1 arrest and a slowing of G_2/M phase at higher concentrations.

3.2.6.2. Enzyme inhibition, photoprotection

A study by Ali et al. (2020), revealed that the essential oils from the flowers and leaves of *P. graveolens* were able to inhibit acetylcholinesterase at IC_{50} values of 294µg/mL and 272 µg/mL respectively. El Aanachi et al. (2020), tested the methanolic extract of *P. graveolens* for its potential to inhibit tyrosinase enzyme using an L-DOPA based assay. The extract inhibited tyrosinase at an IC_{50} value of 21.11µg/mL. In the same study, the methanolic extract of *P. graveolens* was tested for its potential to inhibit urease enzyme using the indophenol method. The study concluded that the IC_{50} of the extract was 31.05µg/mL. Furthermore, the same extract displayed photoprotective activity in this study with a sun protection factor (SPF) of 31.91.

3.3.7. TLC Fingerprinting of plant extract

The ethanolic extract of *P. graveolens* (2mg) was dissolved in 200µL of ethanol. The separation of the compounds present in the extract were observed on a Silica gel 60 F254 TLC plate. The extract was spotted on the plate and left to dry. The plates were developed with a Hexane: Ethyl acetate (7:3) solvent system. Once developed, the plate was observed under long and short wavelength ultraviolet light and sprayed with vanillin solution to detect bands.

3.3.8. Additional information

3.3.8.1. Safety data

Geraniol can cause contact dermatitis and it is recognised as a fragrance allergen in the European Union (Buckley, 2007)

3.3.8.2. Therapeutic (proposed) usage

Antibacterial, antifungal, anticancer, UV protection, Pigmentation

3.3.8.3. Trade information

Not threatened. Least concern on SANBI Red List

3.3.8.4. Dosage

Not available

3.4. *Barleria obtusa*

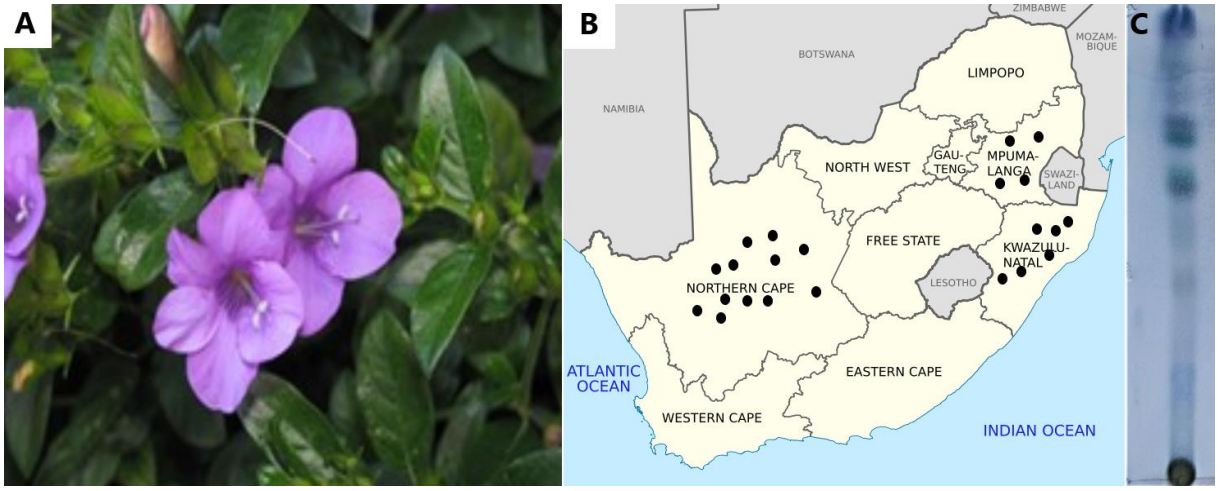

Figure 3.3: (A) *Barleria obtusa* leaves and flowers. **(B)** Distribution of *Barleria obtusa* (Htonl, 2010). **(C)** TLC chromatogram of *B. obtusa* ethanolic extract

3.4.1. General description

3.4.1.1. Botanical nomenclature

Barleria obtusa Nees

3.4.1.2. Botanical family

Acanthaceae

3.4.1.3. Vernacular names

Bush violet (English)

Bosviooltjie (Afrikaans)

Idololenkonyane (Zulu)

3.4.2. Botanical description

Barleria obtusa is a climbing shrub with multiple stems and can grow up to 2m under shady conditions (Isaacs, 2001). The flowers are a deep purple colour with a protruding style and two protruding stamens from the corolla (House and Balkwill, 2019). The leaves are arranged oppositely and are without stipules. The stems of the plant are swollen to a small degree above nodes and the fruit is 2-valved and obovate (van Wyk and van Wyk, 2013).

3.4.3. Distribution

Barleria obtusa occurs in the Northern Cape, Mpumalanga and Kwa-Zulu Natal provinces, growing on hills and along the margins of subtropical regions (Isaacs, 2001).

3.4.4. Ethnobotanical usage

The Xhosa communities of the Amathole District in Eastern Cape South Africa use the leaves of *B. obtusa* for burns (Afolayan et al., 2014).

3.4.5. Phytochemical constituents

Barleria obtusa has not been extensively researched for its phytochemical constituents. A study on the effects of elevated ultraviolet-B radiation on Southern African plants, revealed that *B. obtusa* had a high concentration of carotenoids and flavonoids (Musil et al., 2002).

3.4.6. Pharmacological Properties

The pharmacological properties of *B. obtusa* have not been comprehensively investigated extensively. However, carotenoids and flavonoids, which are present in this plant, are known to have strong antioxidant activity (Pietta, 2000; Fiedor and Květoslava, 2014;).

3.4.7. TLC Fingerprinting of plant extract

The ethanolic extract of *B. obtusa* (2mg) was dissolved in 200µL of ethanol. The separation of the compounds present in the extract were observed on a Silica gel 60 F254 TLC plate. The extract was spotted on the plate and left to dry. The plates were developed with a Hexane: Ethyl acetate (7:3)

solvent system. Once developed, the plate was observed under long and short wavelength ultraviolet light and sprayed with vanillin solution to detect bands.

3.4.8. Additional information

3.4.8.1. Safety data

Not available

3.4.8.2. Therapeutic (Proposed) usage

Not available

3.4.8.3. Trade information

Not threatened. Least concern on SANBI Red List

3.4.8.4. Dosage

Not available

3.5. *Carpobrotus dimidiatus*

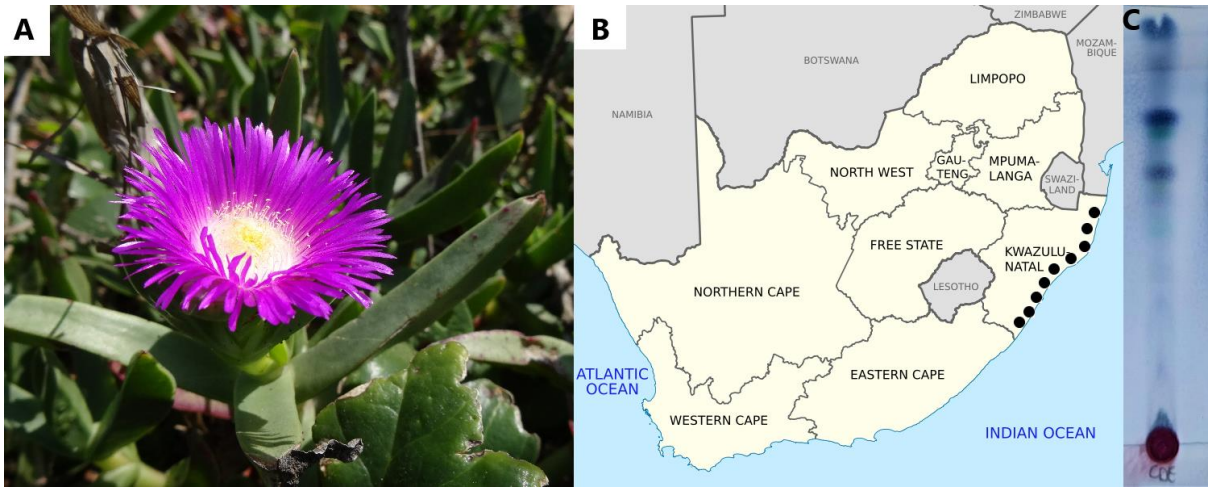

Figure 3.4: (A) *Carpobrotus dimidiatus* leaves and flower (Rulkens, 2014). **(B)** Distribution of *C. dimidiatus* (Htonl, 2010). **(C)** TLC chromatogram of *C. dimidiatus* ethanolic extract

3.5.1. General description

3.5.1.1. Botanical nomenclature

Carpobrotus dimidiatus (Haw.) L. Bolus

3.5.1.2. Botanical family

Mesembryanthemaceae

3.5.1.3. Vernacular names

Natal sour fig (English)

Strandvy (Afrikaans)

Ikhambi lamabulawo (Zulu)

3.5.2. Botanical description

Carpobrotus dimidiatus is a trailing plant with succulent, three-sided leaves (Nonjinge, 2007). The genus name *Carpobrotus* refers to the edible fruit of the plant. The name is derived from the Greek words *karpos* and *brotas*, which mean fruit and edible, respectively (Broomhead et al., 2020). The flower of the plant is large and ranges from mauve to dark pink in colour (Nonjinge, 2007).

3.5.3. Distribution

Carpobrotus dimidiatus mainly occurs in the coastal regions of Kwa-Zulu Natal province (Broomhead et al., 2020)

3.5.4. Ethnobotanical usage

The leaf juice of *C. dimidiatus* is used for wound healing, burns and toothache (van Wyk and Gericke, 2000)

3.5.5. Phytochemical constituents

The phytochemical constituents of *C. dimidiatus* have not been studied in depth. A study by Mulaudzi et al. (2019) found that the leaves of *C. dimidiatus* contained flavonoids and condensed tannins.

3.5.6. Pharmacological properties

The pharmacological properties of *C. dimidiatus* have not been studied extensively. The plant has shown antidiabetic, anti-inflammatory, antioxidant and anticancer activities.

3.5.6.1. Antidiabetic, anti-inflammatory, antioxidant and anticancer activity

In a study by Mulaudzi et al., (2019), the 70% acetone leaf extract of *C. dimidiatus*, inhibited 15-lipoxygenase (LOX) enzyme at a IC_{50} of 528.7µg/mL. Additionally, 50% of DPPH free radical was scavenged by the same extract at a concentration of 9.2µg/mL. In the same study, the aqueous leaf

extract of *C. dimidiatus* inhibited alpha-glucosidase and 15- LOX at IC_{50} values of 652µg/mL and 700µg/mL, respectively. The same extract showed cytotoxicity at an IC_{50} of 69.58µg/mL, against the human hepatocellular carcinoma (HepG2) cell line.

3.5.7. TLC Fingerprinting of plant extract

The ethanolic extract of *C. dimidiatus* (2mg) was dissolved in 200µL of ethanol. The separation of the compounds present in the extract were observed on a Silica gel 60 F254 TLC plate. The extract was spotted on the plate and left to dry. The plates were developed with a Hexane: Ethyl acetate (7:3) solvent system. Once developed, the plate was observed under long and short wavelength ultraviolet light and sprayed with vanillin solution to detect bands.

3.5.8. Additional information

3.5.8.1. Safety data

Carpobrotus dimidiatus aqueous leaf extract demonstrated an IC_{50} of more than 1000µg/mL against the African green monkey kidney (Vero) cell line.

3.5.8.2. Therapeutic (proposed) usage

Antidiabetic, anticancer

3.5.8.3. Trade information

Not threatened. Least concern on SANBI Red List

3.5.8.4. Dosage

Not available

3.6. References

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Chapter 4: Evaluation of the Antithrombotic Potential of Selected South African Plant Extracts for Venous Thromboembolism

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Statement

This chapter forms part of the manuscript titled "Evaluation of the antithrombotic potential of selected South African plant extracts for venous thromboembolism", prepared for the *Blood Cells, Molecules and Diseases* journal.

4.1. Introduction

This chapter highlights the research methodology and findings of the current study. A total of 33 plant extracts were prepared and tested against the research targets. All extracts were tested in an antiproliferative assay against the hepatocellular carcinoma (HepG2) cell line to determine the antiproliferative effects of the extracts on the liver. The plant extracts which did not exhibit antiproliferative activity, were tested for antioxidant activity using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay. The extracts with significant antioxidant activity were then tested for activated coagulation factor X (FXa) inhibition. The extract with significant FXa inhibitory activity was tested for cyclooxygenase-1 (COX-1) inhibition and the phytochemistry of the extract was determined with ultra-performance liquid chromatography mass spectrometry (UPLC-MS).

4.2. Materials and methods

4.2.1. Collection of plant material

The plant species tested (Table 4.1) were collected from the University of Pretoria's Hatfield and Hillcrest campus and purchased from Random Harvest Nursery (Krugersdorp, South Africa). The species identity of the plants was confirmed by Mr Jason Sampson, the curator of the Manie van der Schijff Botanical Garden. Voucher specimen numbers were allocated at the H.G.W.J. Schweickerdt Herbarium.

Table 4.1: Plant species collected

Plant Species	Herbarium Number
***Barleria obtusa* Nees**[a]	PRU 125926
***Carpobrotus dimidiatus* (Haw.) L.Bolus**[a]	PRU 125927

***Cotyledon orbiculate* L.[b]**	N/A
***Cussonia spicata* Thunb.[a]**	N/A
***Heteropyxis natalensis* Harvey[c]**	N/A
***Hypoestes aristata* (Vahl) Sol. Ex Roem & Schult[a]**	PRU 125925
***Hypoestes forsakolii* (Vahl) Roem. & Schult.[a]**	PRU 127863
***Pelargonium citronellum* J.J.A. Van der Walt[a]**	PRU 127869
***Pelargonium graveolens* L'Heritier[a]**	N/A
***Portulacaria afra* Jacq.[a]**	N/A
***Sideroxylon inerme* L.[b]**	N/A

a: Collected from University of Pretoria Hatfield Campus; **b:** Purchased from Random Harvest Nursery (Krugersdorp, South Africa)**; c:** Collected from University of Pretoria Hillcrest Campus; **N/A:** Not available

4.2.2. Preparation of extracts

Ethanolic, dichloromethane (DCM) and aqueous extracts of the leaves and non-woody stems of each plant species were prepared using the methods employed by Mativandlela et al. (2006) with slight modifications (Table 4.2). Non-succulent plants were shade dried and macerated to powder form with a grinding mill. By contrast, succulent plant species were not shade dried, but were macerated with solvent in a blender, while fresh.

4.2.2.1. Ethanolic and DCM extract preparation

Ethanolic and DCM extracts of non-succulent plants were prepared by adding 99.9% ethanol and dichloromethane, respectively, to dried powdered plant material. Ethanolic extracts of succulent plants were prepared by macerating fresh plant material with 99.9% ethanol in a blender**,** whereas DCM extracts of succulent plants were prepared by macerating fresh plant material with dichloromethane using a mortar and pestle. Ethanol and dichloromethane were added at a specific plant mass to solvent volume ratio (Table 4.2). The plant material in the solvent was left for 3 days with continuous shaking and then it was filtered through a Whatmann® filter system (11µm pore size). The filtrate was concentrated in a rotary evaporator (Buchi, RII) under reduced pressure at 36℃ and then frozen at -80°C. The frozen extracts were then freeze dried in a freeze drier (Christ, Alpha 1-2 LDplus) until the solvent evaporated.

4.2.2.2. Aqueous extract preparation

Aqueous extracts of non-succulent plants were prepared by adding hot distilled water to dried powdered plant material**,** whereas aqueous extracts of succulent plants were prepared by macerating fresh plant material with hot distilled water in a blender. Hot distilled water was added at a specific

plant mass to solvent volume ratio (Table 4.2). The plant material in solvent was refrigerated at 8°C for 24 hours and then left to shake continuously for 24 hours. The plant material in solvent was then filtered through a Whatmann® filter system (11 µm pore size) and the filtrate was frozen at -80°C. The frozen extracts were then freeze dried (Christ, Alpha 1-2 LDplus) until the solvent evaporated.

4.2.2.3. Extract percentage yield calculation

The extract percentage yield was calculated using the following equations:

(i) Non-succulent plants

$$Extract\ percentage\ yield\ (\%) = \frac{Final\ extract\ mass\ (g)}{Dried\ plant\ material\ mass(g)}$$

(ii) Succulent Plants

$$Extract\ percentage\ yield\ (\%) = \frac{Final\ extract\ mass\ (g)}{Fresh\ plant\ material\ mass(g)}$$

Table 4.2: Plant extraction data

Plant species	Fresh plant material collected (g)	Mass after drying (g)	Extraction Type	Plant material used (g)	Plant mass(g): Solvent (mL)
Barleria obtusa[a]	590.00	95.33	Ethanolic	29.76[c]	1:20
			DCM	35.81[c]	1:20
			Aqueous	29.76[c]	1:20
Carpobrotus dimidiatus[b]	395.00	N/A	Ethanolic	130.00[d]	1:5
			DCM	130.00[d]	1:5
			Aqueous	130.00[d]	1:5
Cotyledon orbiculata[b]	725.00	N/A	Ethanolic	80.00[d]	1:10
			DCM	215.00[d]	1:5
			Aqueous	215.00[d]	1:5
			Ethanolic	45[c]	1:20

***Cussonia spicata*[a]**	295.00	53.18	DCM	8.18[c]	1:20
			Aqueous	8.18[c]	1:20
			Ethanolic	91.00[c]	1:20
***Heteropyxis natalensis*[a]**	785.00	272.92	DCM	91.00[c]	1:20
			Aqueous	91.00[c]	1:20
			Ethanolic	23.36[c]	1:20
***Hypoestes aristata*[a]**	480.00	64.72	DCM	18.00[c]	1:20
			Aqueous	23.36[c]	1:20
			Ethanolic	26.56[c]	1:20
***Hypoestes forsakolii*[a]**	207.08	59.46	DCM	6.34[c]	1:20
			Aqueous	26.56[c]	1:20
			Ethanolic	27.71[c]	1:20
***Pelargonium citronellum*[a]**	500.00	100.43	DCM	27.71[c]	1:20
			Aqueous	27.00[c]	1:20
			Ethanolic	8.75[c]	1:20
***Pelargonium graveolens*[a]**	500.00	24	DCM	8.75[c]	1:20
			Aqueous	6.50[c]	1:40
			Ethanolic	145.00[d]	1:5
***Portulacaria afra*[b]**	445.00	N/A	DCM	200.00[d]	1:5
			Aqueous	100.00[d]	1:5
			Ethanolic	43.50[c]	1:20
***Sideroxylon inerme*[a]**	517.50	130.55	DCM	43.50[c]	1:20
			Aqueous	43.50[c]	1:20

a: non-succulent plant species; **b:** succulent plant species; **c:** dried plant material; **d**: fresh plant material; **N/A:** Not applicable

4.2.3. Antiproliferative assay

The antiproliferative activity of the 33 plant extracts was tested against the HepG2 cell line using the method employed by Steenkamp and Gouws (2006)**,** with slight modifications. The HepG2 cell line was donated by Prof Lyn-Marie Birkholtz from the University of Pretoria. Dulbecco's Modified Eagles Medium (DMEM), Phosphate buffered saline (PBS), actinomycin D, penicillin, streptomycin,

fungizone, trypsin ethylenediaminetetracetic acid (EDTA), foetal bovine serum, PrestoBlue™ cell viability reagent and tryphan blue were purchased from Thermo Fisher Scientific (Johannesburg, Gauteng, South Africa).

The cells were grown in a flat-sided tissue culture flask in DMEM supplemented with 1% antibiotics [100 U/mL penicillin, 100 µg/mL streptomycin, 250 µg/L fungizone] and 10% foetal bovine serum. The cells were incubated in a humidified incubator (Thermo Forma™ CO_2 incubator) at 37°C and 5% CO_2. The cells were washed with PBS and detached from the surface of the flask with 0.25% trypsin EDTA (Ethylenediaminetetraacetic acid) solution after a monolayer of cells formed on the surface of the flask. The cells were then centrifuged (BOECO U-32) at 980 rpm for 5 minutes and the pellet was suspended in 1mL of DMEM.

A portion of the cell suspension was stained with tryphan blue, and the number of viable cells were counted using an automated cell counter (Countess II FL). The cell suspension was then diluted in DMEM to a final stock concentration of 1×10^4 cells/mL. The cells were plated in 96-well plates to a final concentration of 1000 cells/well and the plates were incubated overnight at 37°C and 5% CO_2 to allow adherence to the wells. The plant extracts were dissolved in dimethylsulfoxide (DMSO) and then tested in triplicate at concentrations ranging from 1.56 - 400 µg/mL and 3.91×10^{-4} - 0.05 µg/mL for actinomycin D.

The plates were incubated for 72 hours, after which PrestoBlue™ cell viability reagent was added to all wells of the plates. The plates were incubated for 2 hours followed by the measurement of fluorescence (Ex/Em = 560/590nm) using the Victor® Nivo™ multimode plate reader (PerkinElmer, Waltham, Massachusetts).

Actinomycin D served as the positive control, 2% DMSO served as the solvent control, DMEM served as the negative control and PrestoBlue™ with DMEM and no cells served as the PrestoBlue™ control. The cell viability was determined using the equation below and the 50 percent inhibitory concentration (IC_{50}) was determined using GraphPad Prism© 8 (hereafter, GPP8) software (GraphPad Software, San Diego, California, USA).

$$Percentage\ viability\ (\%) = \frac{Fluorescence\ Extract - Fluorescence\ PrestoBlue™ control}{Fluorescence\ solvent\ control - \ Fluorescence\ PrestoBlue™\ control} \times 100$$

4.2.4. DPPH antioxidant assay

The plant extracts which displayed an IC_{50} higher than 400µg/mL in the antiproliferative assay were tested for antioxidant activity. The antioxidant activity of the plant extracts were tested in a 96-well

plate using the method described by du Toit et al. (2001) with modifications. Vitamin C and DPPH were purchased from Sigma Aldrich (Modderfontein, Gauteng, South Africa).

Aqueous extracts were dissolved in distilled water, DCM extracts were dissolved in dichloromethane and ethanolic extracts were dissolved in 99.9% ethanol. Vitamin C was dissolved in 99.9% ethanol and served as the positive control. Ethanol (99.9%) served as the blank and diluted plant extract served as the negative control. The plant extracts were tested in triplicate at concentrations ranging from 0.16 - 20µg/mL for *H. natalensis* aqueous extract, 0.41 - 100µg/mL for vitamin C and 3.91 - 500µg/mL for all other extracts.

The reaction was initiated by the addition of DPPH followed by incubation for 30 minutes at room temperature in the dark. The DPPH was omitted from the negative control reactions. Absorbance was read at 515nm using a BIO-TEK®PowerWave™ XS multi-well plate reader (A.D.P., Weltevreden Park, South Africa) and KC Junior software (BIO-TEK®, Winooski, Vermount, USA). The percentage antioxidant activity was determined using the formula below and the IC_{50} values were determined using GPP8 software.

$$Percentage\ antioxidant\ activity\ (\%) = \frac{Absorbance\ Blank - (Absorbance\ Extract - Absorbance\ Negative\ Control)}{Absorbance\ Blank} \times 100$$

4.2.5. FXa inhibition assay

The plant extracts which displayed an IC_{50} lower than 20µg/ml in the DPPH assay, were tested for FXa inhibition. The inhibitory activity of the plant extracts was tested using the method described by Chu et al. (2000) with slight modifications. Tris(hydroxymethyl)aminomethane (Tris), NaCl, Polyethylene glycol 6000 (PEG-6000), FXa from bovine plasma and FXa chromogenic substrate, were purchased from Sigma-Aldrich. The positive control, 1,5-Dansyl-Glu-Gly-Arg Chloromethyl Ketone (GGACK) dihydrochloride, was purchased from Biocom Africa (Pty) Ltd. (Centurion, South Africa). The enzyme assays were conducted in a buffer containing 0.15 M NaCl, 0.05M Tris and 0.1% PEG-6000, pH 7.5. The buffer and DMSO (0.5%) served as the solvent controls

Plant extracts were dissolved and serially diluted in DMSO (10%). The GGACK dihydrochloride was dissolved and serially diluted in buffer. The FXa chromogenic substrate was dissolved in buffer. Serially diluted samples were preincubated with FXa from bovine plasma for 5 minutes at 37°C in a 96-well plate. The final concentration of FXa from bovine plasma was 0.425µM. The plant extracts were tested in triplicate at final concentrations of 1.56 - 100µg/mL for *H. natalensis* aqueous extract and 7.8 - 500µg/mL for all other extracts. GGACK dihydrochloride was tested in triplicate at final concentrations of 0.008 - 0.5µg/mL.

The reaction was initiated by adding FXa chromogenic substrate to each well at a final concentration of 161µM. The absorbance of the reaction was read at 405nm for 15 minutes at 37°C using a BIO-TEK® PowerWave™ XS multi-well plate reader and KC Junior software. The IC_{50} values were determined using GPP8 software.

4.2.6. FXa enzyme kinetics

The plant extract which displayed significant FXa inhibitory activity was subjected to enzyme kinetic studies to determine the type of inhibition. The plant extract and GGACK dihydrochloride were tested in triplicate at the IC_{50} values determined in the FXa inhibition assay. The plant extract was dissolved in DMSO (10%) while GGACK dihydrochloride was dissolved in buffer. The buffer and DMSO (0.5%) served as solvent controls. The FXa chromogenic substrate was dissolved and serially diluted in buffer.

Diluted samples were preincubated with FXa from bovine plasma for 5 minutes at 37°C in a 96-well plate. The final concentration of FXa from bovine plasma in each well was 0.425µM. The reaction was initiated by adding FXa chromogenic substrate to each well at a final concentration range of 1.26-161µM. The absorbance of the reaction was read at 405nm for 15 minutes at 37°C using a BIO-TEK®PowerWave™ XS multi-well plate reader and KC Junior software. The Michaelis constant (K_m) and maximum rate of reaction (V_{max}) were determined using Michaelis-Menten analysis in GPP8 software. Furthermore, kinetic graphs were developed using GPP8 software.

4.2.7. COX-1 inhibition

The COX-1 inhibitory potential of the plant extract which displayed significant inhibition of FXa was tested using the COX-1 Inhibitor Screening Assay Kit (ab204698, Abcam). The assay was conducted according to the kit instructions provided. The COX assay buffer served as the enzyme control, SC560 served as the inhibitor control and DMSO (0.5%) served as the solvent control.

The COX (Cyclooxygenase) reaction mixture consisting of COX assay buffer, COX-1, COX cofactor and COX probe was prepared and added to a 96-well plate at room temperature. The plant extract was dissolved and serially diluted in DMSO (10%) and added to the 96-well plate. The plant extract was tested in triplicate at concentrations ranging from 1.56 - 100µg/mL. The inhibitor control was prepared by adding 2µL of SC560 to the COX assay buffer.

The reaction was initiated by adding Arachidonic acid and NaOH (50:50) solution. The fluorescence of the reaction was read in a kinetic mode for 15 minutes at 25°C using the Victor® Nivo™ multimode plate reader (Ex/Em = 535/587). The maximum slopes and the IC_{50} values were determined using GPP8 software.

4.2.8. Ultra-performance liquid chromatography

UPLC analyses was performed by Ms Madelien Wooding, University of Pretoria, Department of Chemistry (Appendix B: UPLC-MS Protocol). The plant extracts were dissolved and diluted ×1000 in 50% acetonitrile. Analysis was performed at positive and negative ionisation and mass spectra were generated. The positive and negative ion mass spectra were analysed using Mnova© 14.1 chemistry software (Mestrelab Research, Santiago de Compostela, Spain). The identity of the compounds was determined by comparing the mass spectra to the NIST main electron ionisation (EI) mass spectral library (mainlib).

4.2.9. Statistical analysis

The IC_{50} values were determined using a 4-parameter logistic equation in GPP8 with constraints on the top (100) and bottom (0) parameters. A one-way ANOVA (Analysis of variance) followed by a Tukey's multiple comparison test in GPP8 was used to determine whether the differences between the positive controls and the treatments were significant in the hepatotoxicity and antioxidant assays. The unpaired parametric t-test with Welch's correction in GPP8 was used to determine whether the difference between the positive control and the treatment in the FXa inhibition and COX-1 inhibition assay was significant.

4.3. Results

4.3.1. Extract Yield

The extract yield is summarized in Table 4.3

Table 4.3: Final mass and percentage yield of extracts

Plant species	Extract Type	Final Extract Mass (g)	Percentage Yield (%)
Barleria obtusa	Ethanolic	0.71	2.39
	DCM	2.24	6.26
	Aqueous	4.76	16.00
Carpobrotus dimidiatus	Ethanolic	2.95	2.27
	DCM	1.2	0.92
	Aqueous	4.99	3.84
Cotyledon orbiculata	Ethanolic	0.68	0.85
	DCM	0.58	0.23
	Aqueous	2.75	1.23

Cussonia spicata	Ethanolic	0.77	1.71
	DCM	0.52	6.36
	Aqueous	1.46	17.85
Heteropyxis natalensis	Ethanolic	12.94	14.22
	DCM	6.74	7.41
	Aqueous	2.23	2.45
Hypoestes aristata	Ethanolic	1.50	6.42
	DCM	3.43	19.06
	Aqueous	3.94	16.87
Hypoestes forsakolii	Ethanolic	3.58	13.48
	DCM	1.83	28.86
	Aqueous	2.19	8.25
Pelargonium citronellum	Ethanolic	2.89	10.43
	DCM	2.05	7.40
	Aqueous	3.26	12.07
Pelargonium graveolens	Ethanolic	0.69	7.89
	DCM	0.32	3.66
	Aqueous	1.64	25.23
Portulacaria afra	Ethanolic	5.18	3.57
	DCM	1.06	0.53
	Aqueous	1.70	1.70
Sideroxylon inerme	Ethanolic	4.10	9.43
	DCM	1.39	3.20
	Aqueous	10.37	23.84

4.3.2. Antiproliferative activity

The antiproliferative activity of the selected plants extracts is summarized in **Error! Reference source not found.** A third of the plant extracts tested displayed an IC_{50} higher than the highest tested concentration. The only plant extract which displayed an IC_{50} lower than the lowest tested

concentration was *H. forskaolii* ethanolic extract. Additionally, 10 of the plant extracts had IC_{50} values lower than 100µg/mL, and 6 plant extracts had IC_{50} values between 100µg/mL and 300µg/ml. Furthermore, 4 plant extracts displayed an IC_{50} between 300µg/mL and 400µg/mL.

According to the one-way ANOVA test, there was a significant difference among the mean IC_{50} values of the extracts that displayed activity at concentrations lower than 400µg/mL ($p < 0.05$). Tukey's multiple comparisons test revealed that the same extracts displayed significantly higher mean IC_{50} values than the positive control, actinomycin D. However, there was no significant difference between the mean IC_{50} values of the ethanolic extracts of *H. aristata* and *P. citronellum* ($p = 0.2654$).

Table 4.4: Antiproliferative activity of selected plant extracts

Plant	Extract Type	IC_{50} (µg/mL) ± SD
Actinomycin D[a]	-	0.0006 ± 0.07
Barleria obtusa	Ethanolic	146.3 ± 0.12*
	DCM	297.2 ± 0.06*
	Aqueous	NI
Carpobrotus dimidiatus	Ethanolic	NI
	DCM	205.1 ± 0.04*
	Aqueous	396.7 ± 0.04*
Cotyledon orbiculata	Ethanolic	NI
	DCM	61.96 ± 0.18*
	Aqueous	NI
Cussonia spicata	Ethanolic	99.40 ± 0.04*
	DCM	232.4 ± 0.04*
	Aqueous	NI
Heteropyxis natalensis	Ethanolic	72.76 ± 0.11*
	DCM	132.7 ± 0.53*
	Aqueous	NI
Hypoestes aristata	Ethanolic	26.10 ± 0.02*
	DCM	14.76 ± 0.03*
	Aqueous	294.60 ± 0.24*

Hypoestes forskaolii	Ethanolic	IL
	DCM	38.61 ± 0.04*
	Aqueous	7.66 ± 0.18*
Pelargonium citronellum	Ethanolic	26.77 ± 0.05*
	DCM	34.14 ± 0.04*
	Aqueous	212.6 ± 0.11*
Pelargonium graveolens	Ethanolic	94.05 ± 0.12*
	DCM	306.5 ± 0.62*
	Aqueous	NI
Portulacaria afra	Ethanolic	NI
	DCM	327.3 ± 0.14*
	Aqueous	NI
Sideroxylon inerme	Ethanolic	151.4 ± 0.06*
	DCM	NI
	Aqueous	342.2 ± 0.65*

*: p-value < 0.05 when compared to positive control (one-way ANOVA p < 0.05, Tukey's multiple comparison test, n=3); **a:** positive control; **IL:** Inhibition lower than lowest tested concentration of 3.13µg/mL; **NI:** No inhibition at highest tested concentration of 400µg/mL; **SD:** Standard deviation

4.3.3. Antioxidant activity

The antioxidant activity of the selected plant extracts is summarised in Table 4.5. The lowest IC_{50} was displayed by *H. natalensis* aqueous extract with an IC_{50} of 2.71µg/mL. The ethanolic extract of *C. dimidiatus* and the aqueous extract of *P. graveolens* and *B. obtusa* displayed IC_{50} values lower than 20 µg/mL. In addition, 5 of the plant extracts had IC_{50} values between 20µg/mL and 65µg/mL. The DCM extract of *S. inerme* is the only sample which had no activity at the highest tested concentration.

According to the one-way ANOVA test, there is a significant difference amongst the means of all samples which exhibited activity at concentrations lower than 500µg/mL ($p < 0.05$). The same extracts had mean IC_{50} values which were significantly higher than the positive control, vitamin C, according to Tukey's multiple comparisons test ($p < 0.05$)

Table 4.5: Antioxidant activity of selected plant extracts

Sample	Extract Type	IC_{50} (µg/mL) ± SD
Vitamin C[a]	-	2.50 ± 0.04
Barleria obtusa	Aqueous	14.93 ± 0.04*
Carpobrotus dimidiatus	Ethanol	11.92 ± 0.02*
	Ethanol	41.04 ± 0.04*
Cotyledon orbiculata	Aqueous	64.86 ± 0.03*
Cussonia spicata	Aqueous	27.94 ± 0.03*
Heteropyxis natalensis	Aqueous	2.71 ± 0.03*
Pelargonium graveolens	Aqueous	15.67 ± 0.03*
	Ethanol	28.92 ± 0.03*
Portulacaria afra	Aqueous	35.51 ± 0.04*
Sideroxylon inerme	DCM	NI

*: p-value < 0.05 when compared to positive control (one-way ANOVA p < 0.05, Tukey's multiple comparison test, n=3); **a:** positive control; **NI:** No inhibition at highest tested concentration of 500µg/mL

4.3.4. FXa inhibition

The FXa inhibitory potential of the selected plant extracts is summarised in Table 4.6. The only plant extract which displayed activity was *H. natalensis* aqueous extract with an IC_{50} of 2.64µg/ml. The positive control displayed an IC_{50} of 0.23µg/mL. According to the unpaired t-test with Welch's correction, there is no significant difference between the mean IC_{50} values of the positive control, GGACK dihydrochloride, and *H. natalensis* aqueous extract (p= 0.0940).

Table 4.6: FXa inhibition of selected plant extracts

Sample	Extract Type	IC_{50} (µg/mL) ± SD
GGACK Dihydrochloride [a]	-	0.23 ± 0.07
Heteropyxis natalensis	Aqueous	2.64 ± 1.71*
Carpobrotus dimidiatus	Ethanolic	NI
Barleria obtusa	Aqueous	NI
Pelargonium graveolens	Aqueous	NI

***** p-value = 0.0940 when compared to positive control (Unpaired t test with Welch's correction, n=3); **a:** positive control; **NI:** No inhibition at highest tested concentration of 500µg/ml

4.3.5. FXa enzyme kinetics

The Km and Vmax values are summarised in Table 4.7. The Vmax and Km values of *H. natalensis* were lower than the Vmax and Km values of the DMSO [0.5%] control, indicating competitive inhibition (Figure 4.1). The Vmax and Km values of GGACK dihydrochloride were lower than the Vmax and Km values of the buffer control, indicating competitive inhibition (Figure 4.2).

Table 4.7: Vmax and Km values of selected samples

Sample	Km	Vmax
DMSO [0.5%]	185.0	24.08
***H. natalensis* aqueous extract [2.64µg/ml]**	102.0	12.09
Buffer	236.3	29.49
GGACK Dihydrochloride [0.23µg/ml]	247,9	11,63

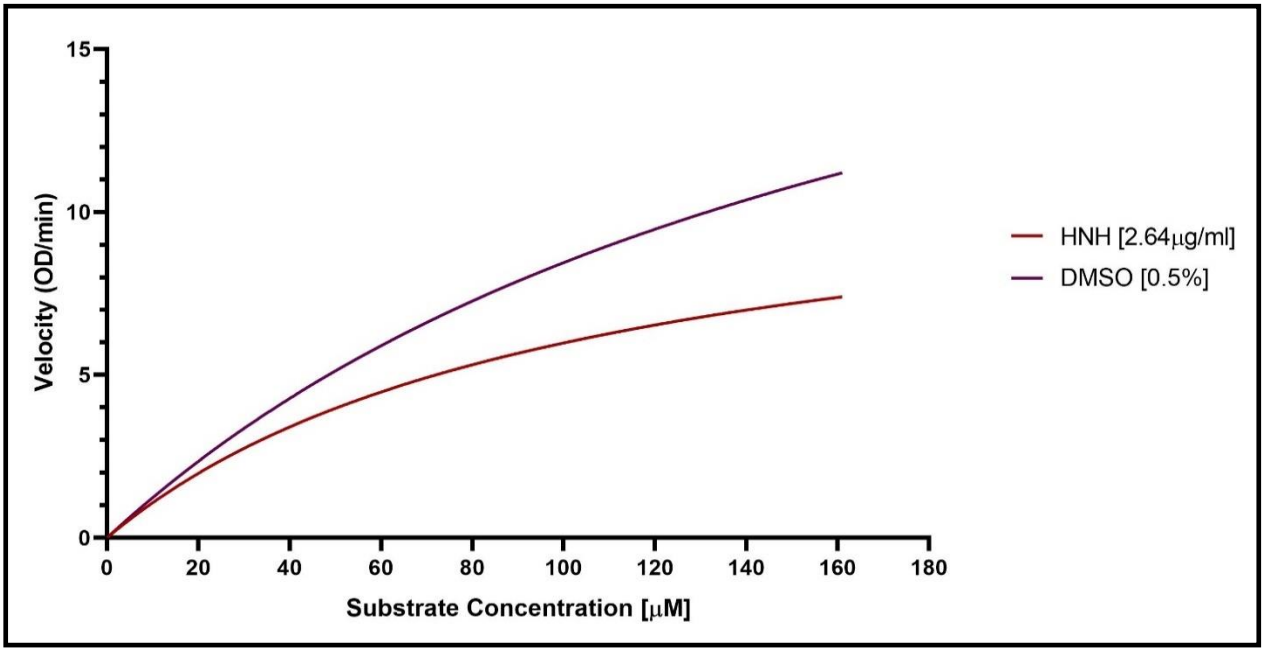

Figure 4.1: Competitive inhibition of FXa by *H. natalensis* aqueous extract (HNH). DMSO [0.5%] curve represents enzyme at 100% activity

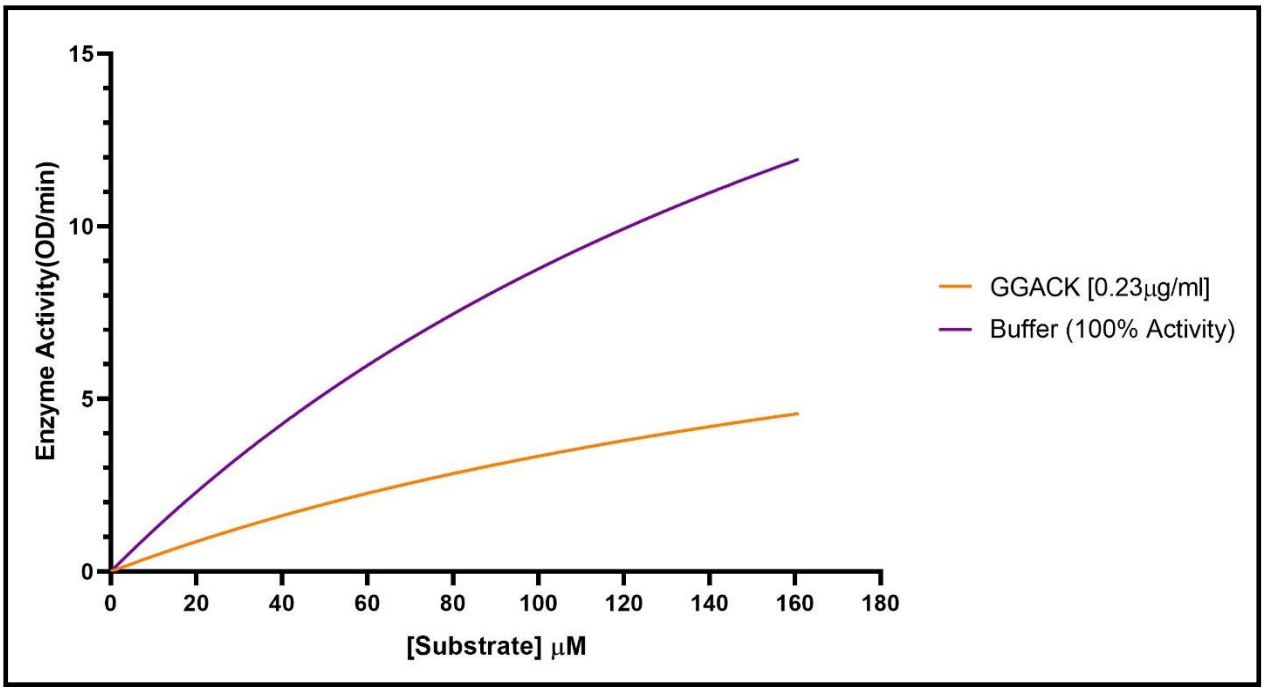

Figure 4.2: Competitive inhibition of FXa by GGACK dihydrochloride (GGACK). Buffer curve represents enzyme at 100% activity

4.3.6. COX-1 inhibition

The IC_{50} of *H. natalensis* aqueous extract was determined to be 25.32µg/mL. According to the product protocol, SC560 has an IC_{50} of 6.45nM which translates to 0.002µg/mL. According to the unpaired t-test with Welch's correction, there is a significant difference between the mean IC_{50} values of SC560 and *H. natalensis* aqueous extract ($p < 0.05$).

4.3.7. Ultra-performance liquid chromatography

The NIST main EI mass spectral library search produced a 100% probability match for the compound β-carotene (Figure 4.3) at a retention time of 13.99 at positive ionisation in the aqueous extract of *H. natalensis.*

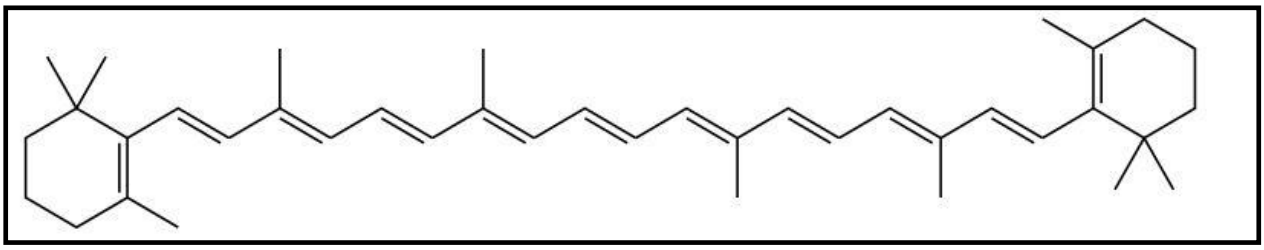

Figure 4.3: Structure of beta-carotene (NEUROtiker, 2007)

4.4. Discussion

4.4.1. Antiproliferative assay

In the current study, the ethanolic, DCM and aqueous extracts of *H. forskaolii* displayed IC_{50} values lower than 30µg/mL. In a previous study of the methanolic extract of *H. forskaolii* against the HepG2 cell line, the extract displayed an IC_{50} value of 29.9µg/mL. In the same study, the subsequent petroleum ether, chloroform and η-Butanol fractions displayed IC_{50} values of 13.3µg/mL, 4.57µg/mL and 4.98µg/mL respectively. These studies further emphasise that extracts of *H. forskaolii* have strong antiproliferative effects on the HepG2 cell line. This antiproliferative effect may be due to alkaloids which have previously been isolated from the plant (Abdel-sattar et al., 2020).

The ethanolic and DCM extracts of *H. aristata,* as well as the ethanolic extract of *P. citronellum,* also displayed IC_{50} values lower than 30µg/ml in the current study. The activity of *H. aristata* may be due to the bioactive lignan, hinokinin, which was previously isolated (Ramabulana et al., 2020) and displayed G2/M cell cycle arrest in another study (Cunha et al., 2016). Lignans have also shown strong cytotoxicity against the HepG2 cell line in another study (Suthiwonga et al., 2018). The presence of essential oils in *P. citronellum* may account for the antiproliferative activity observed in the current study. The oil of *Pelargonium citronellum* contains the monoterpene geranic acid (Lalli et al., 2006). Monoterpenes are the major compounds found in plant essential oils and many studies have confirmed that monoterpenes exhibit antiproliferative activity in *in vitro* experimental settings (Zárybnický et al., 2018).

In previous studies, the monoterpene linalool was identified in the essential oil of *H. natalensis* (Weyerstahl et al., 1992) and *P. graveolens* (Rana et al., 2002). A study by Rodenak-Kladniew et al. (2018), revealed that linalool induces apoptosis in HepG2 cells. This may account for the low IC_{50} values observed in the current study for the ethanolic and DCM extracts of *H. natalensis* as well as the ethanolic and DCM extracts of *P. graveolens*. Additionally, the essential oil of *P. graveolens* contains the monoterpene geraniol (Rana et al., 2002) which decreased the cell viability of HepG2 cells by 38.3% at a concentration of 800µM in a study by Crespo et al. (2020). Furthermore, the compound cardomomin was isolated from the ethanolic extract of *H. natalensis* leaves (Henley-Smith et al., 2018) and it displayed strong antiproliferative activity against the HepG2 cell line in another study, with an IC_{50} value of 22.63µM (Li et al., 2008).

In the current study, the DCM extract of *C. orbiculata* displayed antiproliferative activity at an IC_{50} value lower than 100µg/mL. This may be due to the presence of bufadienolides, which are known to cause livestock poisoning (Botha and Penrith, 2008). Additionally, the aqueous extract of *C. dimidiatus* displayed low antiproliferative activity at an IC_{50} close to 400µg/mL. However, in a previous study by Mulaudzi et al. (2019), the aqueous extract had higher antiproliferative activity with

an IC_{50} value of less than 100µg/mL. The activity of *C. dimidiatus* may be due to the presence of condensed tannins (Mulaudzi et al., 2019) which are known to cause liver damage (Thompson et al., 2017). The activity of the ethanolic extract of *C. spicata* (IC_{50} < 100µg/mL) may likewise be due to the presence of condensed tannins (Naumann et al., 2013).

In the current study, the DCM extracts of *P. afra* had low antiproliferative activity with an IC_{50} value of 327µg/mL. In a previous study of the methanolic extract of *P. afra* against the HepG2 cell line, the extract displayed an IC_{50} value of 4mg/mL (Khanyile et al., 2021). Furthermore, the study revealed the presence of tannins and alkaloids (Khanyile et al., 2021) which may account for the observed activity in the current study (Thompson et al., 2017). This study documents the antiproliferative activity of *S. inerme* and *B. obtusa* leaf extracts against the HepG2 cell line for the first time.

In general, the ethanolic and DCM extracts of all the plants had higher antiproliferative activity than their respective aqueous extracts. This trend could be due to the presence of toxic compounds with high volatility not being present in the aqueous extracts (Mamede and Pastore, 2006). Plant extracts, which do not cause adverse hepatic effects are good candidates for further VTE therapy studies. Therefore, the extracts which did not show antiproliferative activity at the highest tested concentration, were investigated further.

4.4.2. Antioxidant activity

The aqueous extract of *H. natalensis* had the best antioxidant activity in the current study. In a previous study by Muchuweti et al. (2006), the ethanolic leaf extract of *H. natalensis* scavenged 29.7% of DPPH free radicles at a concentration of 1mg/mL. Therefore, the aqueous extract in the current study appears to be more potent. Studies have determined that *H. natalensis* is rich in polyphenolic compounds (Muchuweti et al., 2006) and quercetin has been isolated from the plant (Henley-Smith et al., 2018). This may account for the activity observed in the current study as quercetin is known to have strong antioxidant properties (Xu et al., 2019).

Quercetin has also been isolated from *P. graveolens* (Boukhris et al., 2015) which displayed strong antioxidant activity in the current study. The aqueous extracts of *B. obtusa* also displayed strong antioxidant activity and this may be due to the presence of carotenoids and flavonoids (Musil et al., 2002), which are known to have good antioxidant activity (Fiedor and Květoslava, 2014). Flavonoids are also present in *C. dimidiatus* (Mulaudzi et al., 2019) which also demonstrated strong antioxidant activity in the current study.

The IC_{50} values of the *P. afra* and *C. orbiculata* extracts were slightly higher; however, they displayed note-worthy antioxidant activity. In a previous study, the acetone extract of *P. afra* had an IC_{50} of 32.05µg/mL in the DPPH assay (Olaokun et al., 2017), which falls within the range of the IC_{50} values of the aqueous and ethanolic extracts of the current study. The antioxidant activity of *P. afra* may be due to the presence of alkaloids (Khanyile et al., 2021), which are major antioxidants (Gan et al., 2017).

The ethanolic and aqueous extracts of *C. orbiculata* were previously tested using the DPPH assay (Ondua et al., 2019). The IC_{50} value for the ethanolic extract was 16.2µg/mL, which is lower than the IC_{50} observed in the current study. The IC_{50} for the aqueous extract was more than 100µg/mL which is higher than the IC_{50} observed in the current study. The antioxidant activity of *C. orbiculata* may be due to the presence of flavonoids (Maroyi, 2019; Fiedor and Květoslava, 2014). The plant extracts with strong antioxidant activity will prevent the oxidative modifications of anticoagulant proteins and decrease the activation of platelets (Gutmann et al., 2020) as well as the expression of TF in endothelial cells, monocytes and vascular smooth muscle cells (Herkert et al., 2002). Therefore, the plant extracts which displayed the best antioxidant activity (IC_{50} < 20µg/mL) were investigated further.

4.4.3. FXa Inhibition and enzyme kinetics

The aqueous extract of *H. natalensis* was the only extract which inhibited FXa in the current study. Furthermore, the mechanism of FXa inhibition was determined to be competitive inhibition. This mechanism of inhibition reveals that the inhibitory effect of the extract can be overcome with an increase of substrate (Ouertani et al., 2019). The FXa inhibitory activity of the extract may be due to the presence of quercetin (Henley-Smith et al., 2018), which inhibited FXa in a previous study at a concentration of 5.5µM (Bijak et al., 2014).

In a single dose cytotoxicity study on mouse models by Naidoo, (2014), the ethanolic extract of *H. natalensis* caused bleeding in the thoracic cavity of mice. Since the aqueous extract was able to inhibit FXa in the current study, the bleeding observed by Naidoo, (2014) may have been caused by the inhibition of FXa, which resulted in blood thinning and bleeding. However, *H. natalensis* is traditionally used to prevent bleeding, which contradicts the findings of the current study. In another study, the ethanolic extracts of *Glycyrrhiza glabra* L. (Liquorice), *Trifolium alexandrinum* L. (Egyptian clover) and *Olea europaea* L. (Olive) inhibited FXa with IC_{50} values of 363µg/mL, 729µg/mL and 866 µg/mL for *G. glabra*, *T. alexandrinum* and *O. europaea* respectively (Ibrahim et al., 2020). It is evident that the aqueous extract of *H. natalensis* is a more potent FXa inhibitor when compared to the plant extracts in the study by Ibrahim et al. (2020).

According to studies by Bijak et al. (2014) and Correia-da-Silva et al. (2011), flavonoids may be the compounds responsible for FXa inhibition by plant extracts. However, this trend was not observed in the current study where plants with recorded presence of flavonoids did not inhibit FXa at the highest concentration tested.

4.4.4. COX-1 Inhibition

The aqueous extract of *H. natalensis* exhibited note-worthy inhibition of COX-1 in the current study. In a study undertaken by Adesanwo et al., (2009), the chalcone (E)-1-(2',4'-dihyroxy,5'-methoxy,3'-methylphenyl)-3-phenylprop-2-en-1-one, was isolated from *H. natalensis*. In another study, chalcone derivatives inhibited 68.47-79.95% of COX-1 activity (Bandgar et al., 2012). Therefore, the presence of chalcones may account for the COX-1 inhibitory activity observed in the current study.

4.4.5. Ultra-Performance Liquid Chromatography

The presence of β-carotene in the aqueous extract of *H. natalensis* may further contribute to the potent antioxidant activity of the extract (Fiedor and Květoslava, 2014). However, to the best of our knowledge, there are no reports of inhibition of FXa and COX-1 by β-carotene.

4.5. Conclusion

The results of the study revealed that amongst the 33 extracts tested, the aqueous extract of *H. natalensis* was the best candidate for future antithrombotic studies in relation to VTE. The extract exhibited strong antioxidant activity; hence it will prevent the up regulatory effects of ROS on coagulation. Additionally, the extract competitively inhibited FXa at a low concentration, indicating that the extract will prevent progression of both pathways of the coagulation cascade. The extract also inhibited COX-1 and will therefore be able to prevent the aggregation of platelets. Furthermore, it was determined that the extract is not likely to cause any adverse hepatic effects. Future studies should focus on the mechanisms of action of the aqueous extract of *H. natalensis* on FXa and COX-1 as well as further identification and isolation of compounds.

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Chapter 5: Conclusion and Future Research

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In general, most of the plant extracts displayed some degree of antiproliferative activity against the HepG2 cell line. The antiproliferative assay revealed that the plant extracts of *Hypoestes forskaolii, Hypoestes aristata* and *Pelargonium citronellum* had significant antiproliferative activity. Furthermore, these were the only plants in the study where all 3 extracts exhibited antiproliferative activity. Considering that the liver metabolises drugs, these plants may cause adverse hepatic effects when administered. Therefore, the findings of this study can be of added advantage when evaluating the medicinal properties of these plants in relation to the treatment of any disease.

The majority of those plant extracts which did not evidence antiproliferative activity, exhibited good antioxidant activity in the DPPH assay. Reactive oxygen species play a central role in the progression of many diseases; therefore, these plants can also be considered for future research of such diseases. The most potent antioxidant in this study was the aqueous extract of *Heteropyxis natalensis*, which also inhibited FXa and COX-1. The ability of *H. natalensis* to inhibit FXa and COX-1 supports the trend that plants with antioxidant activity also have anticoagulant and antiplatelet effects. Furthermore, the COX-1 inhibitory activity observed supports the trend of plants traditionally used for pain and inflammation, having the ability to inhibit cyclooxygenase enzyme. This knowledge could be used as a potential screening method for antithrombotic agents in future thrombosis research.

The inhibition of FXa and COX-1 by the aqueous extract of *H. natalensis* is reported for the first time in this study. From the literature, it is evident that crude plant extracts have not been extensively studied for their ability to inhibit FXa. Furthermore, the aqueous extract of *H. natalensis* displayed better FXa inhibitory activity when compared to the extracts in previous FXa reports. Studies on direct FXa inhibitors have focused mainly on isolated compounds. Therefore, future studies on the aqueous extract of *H. natalensis* can focus on compound isolation and the effects of the compounds on FXa. The current study revealed that the aqueous extract of *H. natalensis* competitively inhibited FXa, indicating that the extract competes with substrate for the active site of FXa. Future studies can focus on the interactions of *H. natalensis* aqueous extract and isolated compounds with the active site of FXa. Furthermore, future studies can focus on the mechanism of inhibition of COX-1 by *H. natalensis* aqueous extract.

The current study revealed that the aqueous extract of *H. natalensis* has antithrombotic properties that may be explored further for VTE. The findings of this research are valuable as cardiovascular diseases such as VTE cause the majority of deaths, globally. The use of an antithrombotic agent from a plant source will be especially beneficial for developing countries as plants are utilised by majority of the population as a healthcare source and the plants are easily accessible. Furthermore, *Heteropyxis*

natalensis is part of the richness of South Africa's extensive range of plant species and the findings of this research will hopefully encourage further exploration of the said South African plant species.

Appendix A: Additional Plant Monographs

Appendix A: Additional Plant Monographs

Sideroxylon inerme

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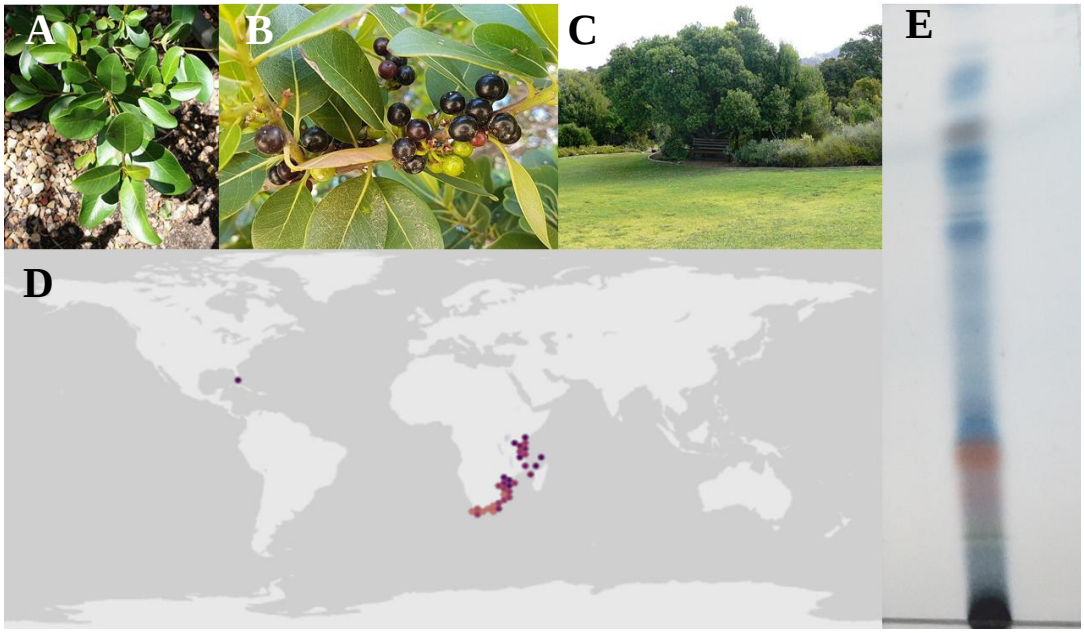

Figure A.1: (A) *Sideroxylon inerme* leaves (Shawka, 2010a). **(B)** Fruits of *S. inerme* (Shawka, 2010b)**. (C)** Aerial parts of *S. inerme* (Venter, 2017). **(D)** Distribution of *S. inerme* (GBIF, 2021) **(E)** TLC Chromatogram of *S. inerme* ethanolic extract

1. General description

1.1. Botanical nomenclature

Sideroxylon inerme L.

1.2. Botanical family

Sapotaceae

1.3. Vernacular names

White milkwood (English)

Witmelkhout (Afrikaans)

Melkhoutboom (Afrikaans)

Melkbessie (Afrikaans)

aMasethole (Xhosa)

umQwashu (Xhosa)
aMasethole-amhlope (Zulu)
uMakhwela-fingqane (Zulu)

2. Botanical description

Sideroxylon inerme is a medium evergreen tree that can reach heights of 10 - 15m (Figure A.1C). The bark appears to be grey brown to black. Its young branches are covered with very fine hairs. The leaves are spirally arranged, dark green and leathery and have fine hairs (Figure A.1A). The flowers are greenish white with a strong pungent smell and flowers during summer and autumn. The fruits are purplish black, round, small and fleshy (**Error! Reference source not found.**B). Both the leaves and fruits contain milky latex (Bosman, 2006).

3. Distribution

This plant species usually appears in dune forests, coastal woodlands and littoral forests. It appears inland as well as in Zimbabwe and Gauteng (Bosman, 2006).

4. Ethnobotanical usage

The root and bark of the tree can be administered as an enema (Okatch et al., 2012). It is reported to be used for treatment of gall sickness in stock, treatment of fevers and as a skin lightener (Sharma, 2014). The roots have been used to treat conjunctivitis, coughs, paralysis and hernias (Chhabra et al., 1993).

5. Phytochemical constituents

Sideroxylon inerme has been reported to have triterpenes, sterols and alkaloids present (Shelembe et al., 2016). Preliminary screening of crude extracts of *S. inerme* has revealed the presence of phytocompounds, procyanidin B and epigallocatechin gallate (Momtaz et al., 2008). The bark contains compounds such as cinnamic acid, leucanthocyanins, and kaemperfol (Grace et al., 2003; Hutchings et al., 2006).

6. TLC fingerprinting of plant extract

The ethanolic extract of *S. inerme* was weighed at 2mg and dissolved in 200µL ethanol. The plant extract was spotted on a Silica gel 60 F254 TLC plate. The mobile phase was prepared at a ratio of 7:2:1 of hexane, dichloromethane, and ethanol respectively at a total volume of 10mL. The TLC plate

was run in a chromatographic chamber with the respective mobile phase and covered with a glass plate for the prevention of evaporation of the solvent. The developed TLC plate was observed under long (365nm) and short (254nm) wavelength and sprayed with vanillin solution for detection of the bands.

7. Pharmacological properties

The crude methanolic extracts of the stem bark of *S. inerme* have been tested for their antioxidant activity using DPPH assays and results reported was 150µg/mL. The fruits of *S. inerme* have exhibited free radical scavenging activity with an IC_{50} value of 800µg/mL and therefore suggested to be potentially beneficial to human health in terms of abating oxidative stress (Shelembe et al., 2016).

7.1. Antifungal activity

In a study conducted by Shikwambana & Mahlo (2020), acetone and aqueous extracts of *S. inerme* have been reported to show antifungal activity with the lowest MIC value of 0.02mg/mL. The antifungal activity of the extracts was tested using the microdilution method (Table A.1).

Table A.1: *Sideroxylon inerme* antifungal activity

Pathogen	Sample/Extract	MIC (mg/mL)	Plant part
***Candida albicans* (ATCC 10231)**	Acetone	0.63	Root
	Aqueous	0.02	
***Trichophyton rubrum* (MTCC 2961)**	Acetone	0.02	Root
	Aqueous	0.08	
***Microsporum canis* (ATCC 36299)**	Acetone	1.25	Root
	Aqueous	0.04	
***Candida albicans* (ATCC 10231)**	Acetone	0.31	Stem
	Aqueous	0.02	
***Trichophyton rubrum* (MTCC 2961)**	Acetone	0.02	Stem
	Aqueous	1.25	
***Microsporum canis* (ATCC 36299)**	Acetone	0.16	Stem
	Aqueous	0.02	

***Candida albicans* (ATCC 10231)**	Acetone	0.63	Bark
	Aqueous	0.02	
***Trichophyton rubrum* (MTCC 2961)**	Acetone	0.31	Bark
	Aqueous	0.31	
***Microsporum canis* (ATCC 36299)**	Acetone	0.16	Bark
	Aqueous	0.02	

7.2. Cytotoxicity and antioxidant activity

As stated by a study conducted by Rademan et al., (2019), *S. inerme* extract displayed promising activity on the A431 celline with IC_{50} values of 41.8µg/mL and 46.7µg/mL, respectively. The plant extract was found to have a relatively good safety margin due to its high IC_{50} value of 119.2µg/mL against the HaCat cell line when comparing its activity with the A431 cell line. The antioxidant activity of the extract was determined by the evaluation of its capacity to scavenge DPPH free radicals, the nitric superoxide reactive oxygen species and the nitric oxide reactive species. Results are summarized in the table below (Table A.2).

Table A.2: Cytotoxicity and antioxidant activity of ethanolic extract of aerial plant parts of *S. inerme*

Activity	**Cell line/ Reagent/ Enzyme**	**IC_{50} (µg/mL)**
Cytotoxicity	A431	46.7
	HaCat	119.2
	HCT-116	137.2
	HeLa	> 400
	MCF-7	93.1
	UCT-Mel 1	90.1
Antioxidant	DPPH	11.5
	Nitric oxide	131.5
	Superoxide	115.6

7.3. Antityrosinase activity

Tyrosinase is a multicopper monooxygenase enzyme that has a wide distribution in nature. It is responsible for pigmentation of skin, hair and eyes in mammals. Its function includes monophenolase (cresolase) and diphenolase (catecholase) activity and is involved in melanin biosynthesis (Likhitwitayawuid, 2008). Momtaz et al., (2008) tested acetone, dichloromethane, and methanol *S. inerme* bark extracts for antityrosinase activity (Table A.3). The methanol and acetone extracts showed significant inhibition of tyrosinase (Momtaz et al., 2008).

Table A.3: Monophenolase and diphenolase inhibitory activity of bark extracts of *S. inerme*

Enzyme	Sample/Extract	IC_{50} (µg/mL)
Monophenolase	Acetone	63
	Methanol	82
	Dichloromethane	> 400
Diphenolase	Acetone	> 400
	Methanol	> 400
	Dichloromethane	-

8. Additional information

8.1. Therapeutic (proposed) usage

Antifungal, antioxidant, and antityrosinase.

8.2. Safety Data

As stated previously (Table A.2), the plant seems to have a good safety margin against HaCat cell lines (Rademan et al., 2019). More studies need to be done on other cell lines involved in the metabolism of herbal drugs in order to provide more valuable information on the plant.

8.3. Trade information

Least concern according to the SANBI Red List

8.4. Dosage

Not available.

9. References

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Shawka, A., 2010b. Sideroxylon inerme or White Milkwood tree. Cape Town. This image was

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Hypoestes forskaolii

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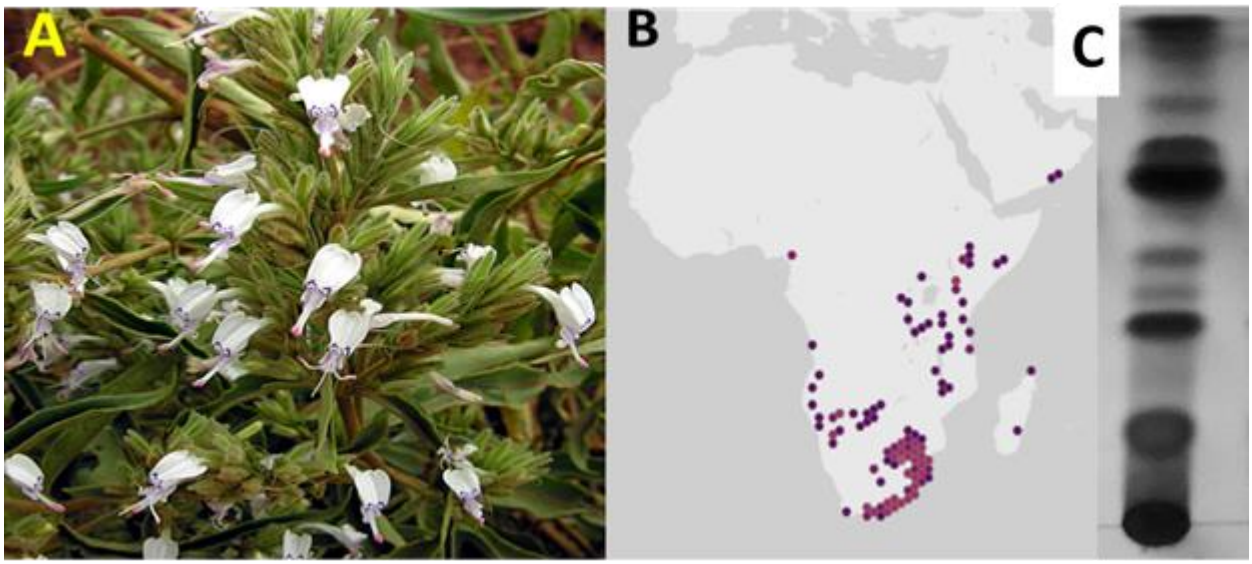


Figure A.2: (A) Depiction of *Hypoestes forskaolii* leaves and flowers. **(B)** Distribution map of *Hypoestes forskaolii*. **(C)** TLC chromatogram of ethanol extract

1. General description

1.1. Botanical nomenclature

Hypoestes forskaolii (Vahl) Roem. & Schult. subsp. forskaolii

1.2. Botanical family

Acanthaceae

2. Botanical description

Hypoestes forskaolii is an annual herb that grows up to 1 m with its stems and leaves almost globous. It gives rise to pale pink or white flowers (Hyde, 2002).

3. Distribution

Hypoestes forskaolii is widely distributed in Southern Africa, Saharan highlands, Arabia and Madagascar (Hyde, 2002).

4. Ethnobotanical usage

The fresh leaves of *H. forskaolii* are applied onto wounds to halt bleeding and dried leaves are mixed with water to form a solution that can be taken orally for diabetes (Beyi, 2018). The juice obtained from the crushed leaves of *H. forskaolii* is taken orally for jaundice (Araya et al., 2015). According to East African folk medicine, the whole *H. forskaolii* plant is used to treat vomiting, headaches, nausea and heartburn (Haidari, 2018). In Cameroon, the poultice of the whole plant is applied topically for the treatment of skin infections (Fongod et al., 2013).

5. Phytochemical constituents

Several C-fusicoccane type diterpenoids have been isolated from the methanol extract of *H. forskaolii* (Figure A.3). Table A.4 details the compound, source, molecular formula and biological activity:

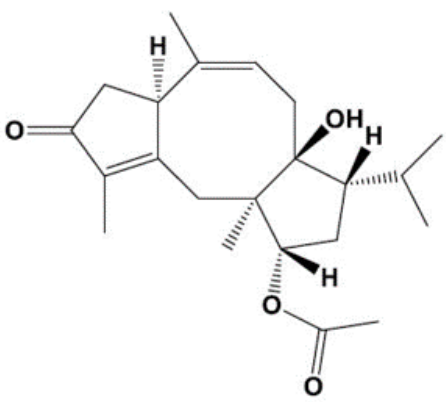
Table A.4: Compounds isolated from *Hypoestes forskaolii*

No.	Compound	Source	Molecular formula	Biological activity	Reference
1	Hypoestenonol A	Aerial parts	$C_{22}H_{32}O_4$	Moderate anti-plasmodial activity (IC_{50}	Mohamed et al., 2014

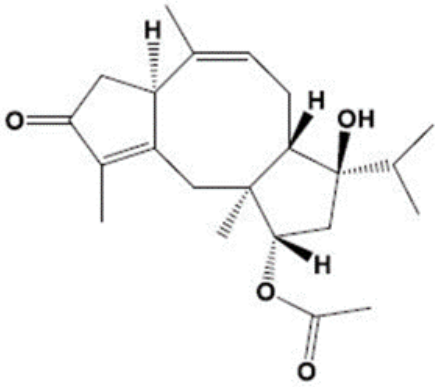
				of 18.9 µM) Not cytotoxic to MRC5-SV2 cells with an IC_{50} > 64.0 µM	
2	Hypoestenonol B	Aerial parts	$C_{22}H_{32}O_4$	No reported activity	Mohamed et al., 2014
3	Verticillarone	Aerial parts	$C_{20}H_{26}O_5$	Moderate antiplasmodial activity (IC_{50} of 25.1 µM)	Mohamed et al., 2014
4	Hypoestenone	Aerial parts	$C_{20}H_{28}O_2$	Moderate antiplasmodial activity (IC_{50} of 16.7 µM)	Mohamed et al., 2014
5	Deoxyhypoestenone	Aerial parts	$C_{20}H_{30}O$	No reported activity	Muhammad et al., 1998
6	Dehydrohypoestenone	Aerial parts	$C_{20}H_{26}O_2$	No reposted activity	Muhammad et al., 1998
7	Hypoestene	Aerial parts	$C_{21}H_{28}O_4$	No reported activity	Muhammad et al., 1998
8	Fusicoplagin D	Aerial parts	$C_{22}H_{32}O_6$	No reported activity	Muhammad et al., 1998
9	8-α epoxydeoxyhypoestenone	Aerial parts	$C_{21}H_{30}O_2$	No reported activity	Muhammad et al., 1998
10	Sesamin	Aerial parts	$C_{20}H_{18}O_6$	Anti-inflammatory activity through the inhibition of delta 5-desaturase leading to a reduction in pro-inflammatory mediators. Significantly halts the production of excess nitric oxide (NO) in the murine microglial cell line, BV2 and rat primary microglia cells. Lastly, sesamin has also been found to inhibit angiogenesis	Chavali et al., 1998 Chung et al., 2010 Shimizu et al., 1991 Hou et al., 2003

IC50: 50% minimum inhibitory concentration. **MRC5-SV2**: Human foetal lung fibroblast cells transformed with the SV40 virus.

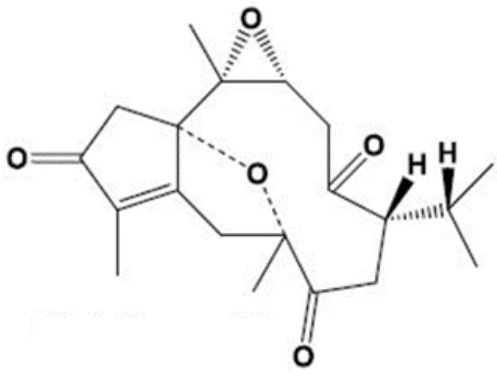
Figure A.3: Chemical Structures of compounds 1-10 (Al Haidari, 2018)



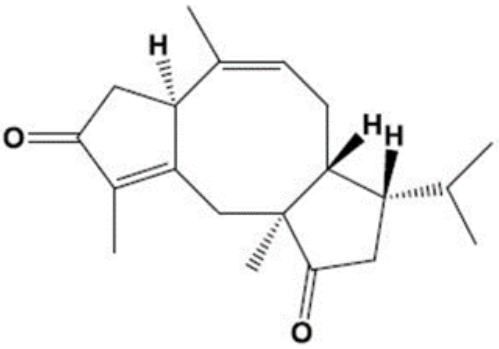
Hypoestenonol A (1)



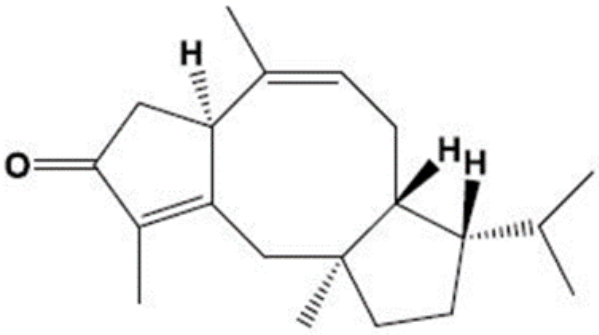
Hypoestenonol B (2)



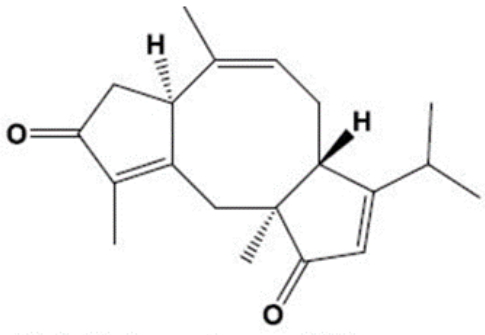
Verticillarone (3)



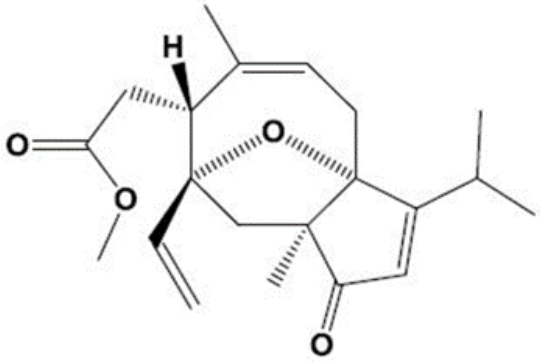
Hypoestenone (4)



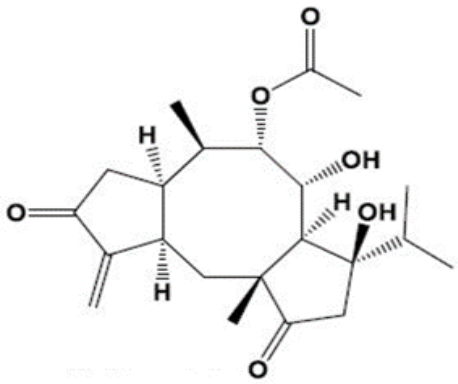
Deoxyhypoestenone (5)



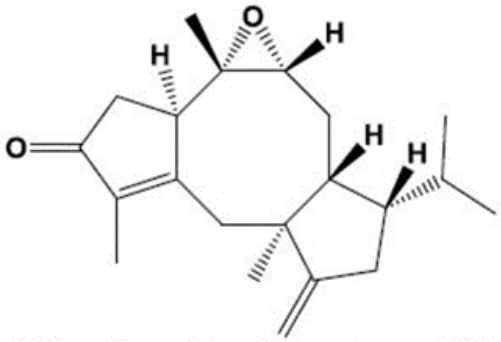
Dehydrohypoestenone (6)



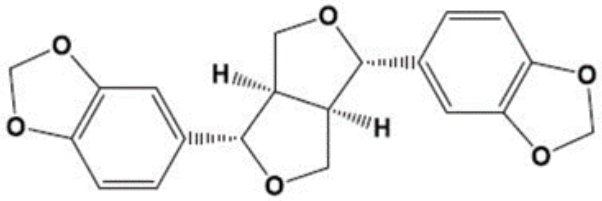
Hypoestene (7)



Fusicoplagin D (8)



8(9)-α-epoxydeoxyhypoestenone (9)



Sesamin (10)

6. TLC fingerprinting of plant extract

For the detection of the chemical profile of *H. forskaolii* through thin layer chromatography (TLC), 2mg of the ethanol extract was diluted in 100 µL of ethanol. The solution was spotted on silica gel 60 F254 TLC plates using glass Pasteur pipettes. The spotted silica gel 60 F254 TLC plates were placed in a TLC tank containing a solvent system that comprised of 7mL of hexane, 2mL of dichloromethane and 1mL of ethyl acetate. The solvent was allowed to rise until the solvent front was reached (1cm from the top of the TLC plate) then the plate was airdried, placed under an ultraviolet lamp (short and long wavelength) then sprayed with vanillin in order to see the chemical profile.

7. Pharmacological properties

Hypoestes forskaolii has not undergone extensive *in vitro* and *in vivo* testing but significant antiplasmodial activity, anti-angiogenic activity and antiproliferative/cytotoxic activity has been identified from the methanol extract and compounds isolated and identified from the methanol extract.

7.1. Antimalarial activity

The *in vitro* antimalarial activity of a methanolic extract prepared using *H. forskaolii* was assessed against *Plasmodium falciparum* KI (a chloroquine resistant strain). Moderate antimalarial activity was observed with a IC_{50} of 5.5µg/mL as plant extracts with IC_{50}'s greater than 12.5µg/mL are considered to be inactive. Furthermore, a novel phenanthrol-quinolizidine alkaloid (15-β-hydroxycryptopleurine-N-oxide) isolated from *H. forskaolii* through bio-guided fractionation displayed significant antimalarial activity with an IC_{50} of 6.11nm against the chloroquine resistant KI strain of *Plasmodium falciparum* and an IC_{50} of 5.13nm against the chloroquine sensitive (FCR) strain of *Plasmodium falciparum* (Abdel-Sattar et al., 2020).

7.2. Anti-angiogenic activity

The *in vitro* anti-angiogenic activity of a compound isolated from the methanol extract of *H. forskaolii* (sesamin) was observed on breast cancer cell lines (MCF-7 and MDA-MB-231) treated with conditioned media from macrophages (THP-1 cells differentiated with 12-o-tetradecanoyl-13-phorbol acetate, TPA). The levels of interleukin-6 (IL-6), interleukin-8 (IL-8) and tumour necrosis factor alpha (TNF-α) were substantially enhanced after treatment with the conditioned media from the macrophages in MCF-7 cells. The treatment of MCF-7 cells (treated with conditioned media from the macrophages) with sesamin inhibited the production of IL-6, IL-8 and TNF-α thus, this also led to an inhibition of vascular endothelial growth factor (VEGF) and matrix metalloproteinase-9 (MMP-9). Vascular endothelial growth factor and matrix metalloproteinase-9 are major precursors of angiogenesis (Lee et al., 2011).

7.3. Antiproliferative/cytotoxic activity

The methanol extract prepared using the aerial parts of *H. forskaolii* displayed cytotoxic activity against Hepatocellular carcinoma (HepG2) cells and Human epithelial carcinoma cells (Hela). The IC_{50} values obtained for both cell lines were 29.9µg/mL (HepG2) and 16.3µg/mL (Hela) respectively (Almehdar et al., 2012).

8. Additional information

8.1. Therapeutic (proposed) usage

Antimalarial/antiplasmodial, anti-angiogenic and anticancer.

8.2. Safety data

The fusiccocane diterpenoid isolated from the *H. forskaolii* methanolic extract displayed low cytotoxicity against MRC5-SV2 cells with an IC_{50} greater than 64.0µM (Mohamed et al., 2014).

8.3. Trade information

Not threatened or endangered. Status of least concern on the SANBI red data list.

8.4. Dosage

Not available.

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Hypoestes aristata

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Figure A.4: (A) Depiction of *Hypoestes aristata* flower. **(B)** Depiction of *Hypoestes aristata* leaves and flowers **(C)** Distribution map of *Hypoestes aristata* (KENPEI, 2008, Rotational, 2009, GBIF Secretariat, 2019). **(D)** TLC Chromatogram of *Hypoestes aristata* ethanolic extract

1. General description

1.1. Botanical nomenclature

Hypoestes aristata (Vahl) Sol. Ex Roem & Schult var aristata

1.2. Botanical family

Acanthaceae

1.3. Vernacular names

Ribbon bush (English)

Seeroogblommetjie (Afrikaans)

uHladlwana olukhulu, uHlonyane, uHlalwane (Zulu)

uHlololwane (IsiXhosa)

2. Botanical description

Hypoestes aristata grows to approximately 1.5m, the leaves are oval shaped with fine hairs and the flowers are typically purple and white (Joffe, 2001, Pooley, 1998).

3. Distribution

Hypoestes aristata is native to Sub-Saharan Africa but in South Africa it is found in the Western Cape, Eastern Cape, Kwa-Zulu Natal, Mpumalanga and Limpopo (Joffe, 2001; Pooley, 1998).

4. Ethnobotanical usage

The AmaZulu (Tribe in South Africa) reportedly used crushed *H. aristata* leaves for the treatment of sore eyes and decoctions prepared using *H. aristata* were used for breast diseases (Hulme, 1954, Kokwaro, 1976). The root bark was used for the treatment of Malaria and the roots were often chewed for influenza, cough, colds and sore throats in East Africa (Iwu, 1993, Kokwaro, 1976). Lastly, the Xhosa tribe (Tribe in South Africa) uses the plant for the treatment of tuberculosis, arthritis, bone fractures and cancer (Bhat, 2014).

5. Phytochemical constituents

The *Hypoestes* genus gives rise to different phytochemicals such as diterpenoids, alkaloids, lignans and pentacyclic triterpenes (Haidari, 2018). Known lignans (hinokinin, savinin, medioresinol, cubebins) and three new benzylbutyrolactone type lignans were identified in the aerial parts of *H. aristata* (Ramabulana et al., 2020).

6. TLC fingerprinting of plant extract

For the detection of the chemical profile of *Hypoestes aristata* through thin layer chromatography (TLC), 2mg of the ethanol extract was diluted in 100µL of ethanol. The solution was spotted on silica gel 60 F254 TLC plates using glass Pasteur pipettes. The spotted silica gel 60 F254 TLC plates were placed in a TLC tank containing a solvent system that comprised of 7mL of hexane, 2mL of dichloromethane and 1mL of ethyl acetate. The solvent was allowed to rise until the solvent front was reached (1cm from the top of the TLC plate) then the plate was airdried, placed under an ultraviolet lamp (short and long wavelength) then sprayed with vanillin in order to see the chemical profile.

7. Pharmacological properties

Hypoestes aristata has not undergone extensive *in vitro* and *in vivo* testing but moderate anti-HIV activity was exhibited by lignans isolated from the chloroform extract of the stems of *H. aristata*. Furthermore, moderate cytotoxic activity against two breast cancer cell lines (MCF-7 and MDA-MB231) as well as significant cytotoxic activity was displayed against human drug sensitive T-lymphoblastoid (CCRF-CEM) and multidrug resistant T-lymphoblastoid (CEM/ADR5000) cell lines.

7.1. Anti-HIV activity

Lignans isolated from the stems of *H. aristata* through chromatographic purification of the chloroform (CH_2Cl_2) extract (hinokinin and a benzylbutyrolactone-type lignan, (7S,8S,7′S,8′R)-7,7′ diacetoxyhinokinin), exhibited moderate inhibitory activity against the HIV-1 protease enzyme at concentrations lower than 60µM (Ramabulana et al., 2020).

7.2. Antiproliferative/cytotoxic activity

Compound 4, (7S,8S,7′S,8′R)-7′-acetoxy-7-hydroxyhinokinin, compound 6, (7S,8S,7′S,8′R)-7′-acetoxy-7-hydroxyhinokinin and compound 7a, (7S,8S,7′S,8′R)-7′-acetoxy-7-hydroxyhinokinin) were tested against two breast cancer cell lines (MCF-7 and MDA-MB231) to determine the antiproliferative/cytotoxic activity. The compounds (4,6 and 7a) did not exhibit any antiproliferative/cytotoxic activity at concentrations below 90µM (Ramabulana et al., 2020). Furthermore, the antiproliferative/cytotoxic activity of *H. aristata* was also investigated against human drug sensitive T-lymphoblastoid (CCRF-CEM) and multi-drug resistant T-lymphoblastoid (CEM/ADR5000) cell lines. *H. aristata* inhibited the growth of both cell lines by 60% (Saeed et al., 2016).

8. Additional information

8.1. Therapeutic (proposed) usage

Anti-HIV and anticancer.

8.2. Safety data

No data.

8.3. Trade information

Not threatened or endangered. Status of least concern on the SANBI red data list.

8.4. Dosage

Not available.

9. References

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Cussonia spicata

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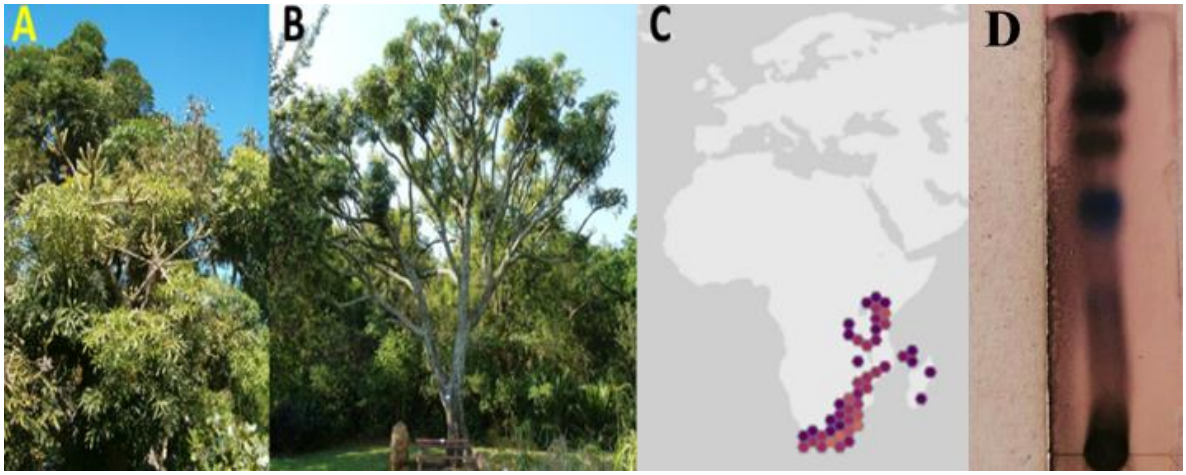


Figure A.5: (A) Depiction of *Cussonia spicata*. **(B)** Clear depiction of *Cussonia spicata* canopy. **(C)** Distribution map of *Cussonia spicata* (GBIF Secretariat, 2019, User:BotBln, 2010a, User:BotBln, 2010b). **(D)** TLC chromatogram of *Cussonia spicata* ethanolic extract

1. General description

1.1. Botanical nomenclature

Cussonia spicata Thunb.

1.2. Botanical family

Araliaceae

1.3. Vernacular names

Cabbage tree (English)

Kiepersol (Afrikaans)

Musenzke (Venda)

uMsenge (Xhosa)

uMsenge (Zulu)

Motshetshe (Northern Sotho)

2. Botanical description

Cussonia spicata is an ever-green tree that grows up to 17 m (Palgrave et al., 1997). The roots are large and fleshy, the colour of the leaves vary between green, blue-green or gray-green. The flowers of *C. spicata* are small and typically green (Palmer and Pitman, 1972).

3. Distribution

Cussonia spicata is found in Botswana, Comoros, The Democratic republic of Congo, Kenya, Malawi, Mozambique, South Africa, South Sudan, Swaziland, Tanzania, Uganda, Zambia and Zimbabwe (Palmer and Pitman, 1972).

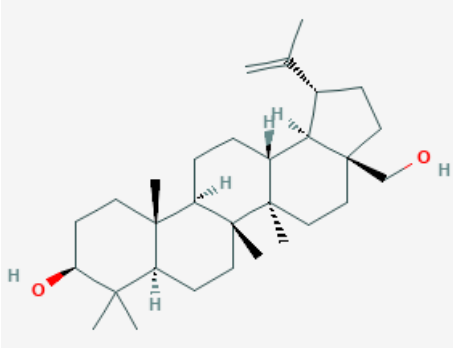
4. Ethnobotanical usage

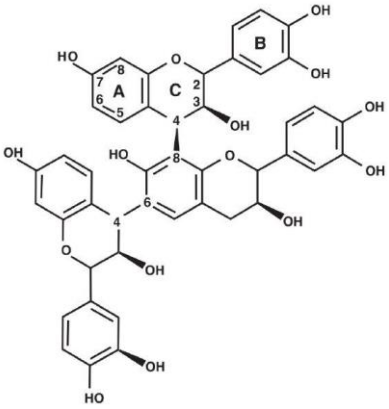
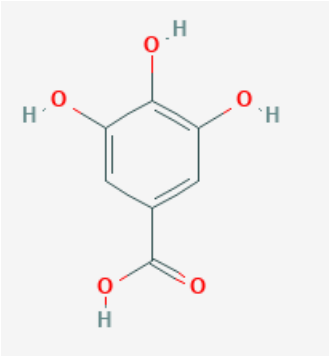
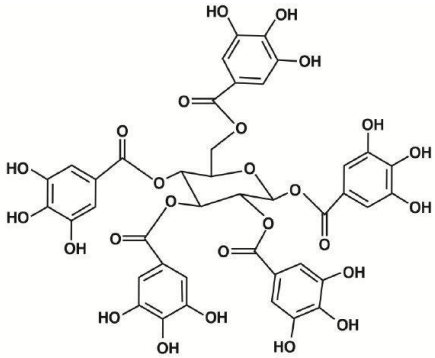
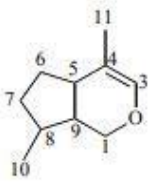
The boiled bark of *C. spicata* is used for the treatment of abdominal pain, however, the bark can also be chewed for the same effect (Kigen et al., 2017). The roots of *C. spicata* were reportedly used for the treatment of venereal diseases and the bark was used for malaria (Chhabra et al., 1984). In addition to the various uses of *C. spicata*, it was also used for the treatment of diabetes by Bapedi healers (Semenya et al., 2012).

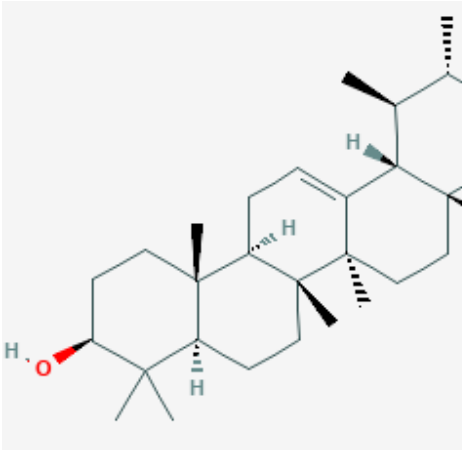
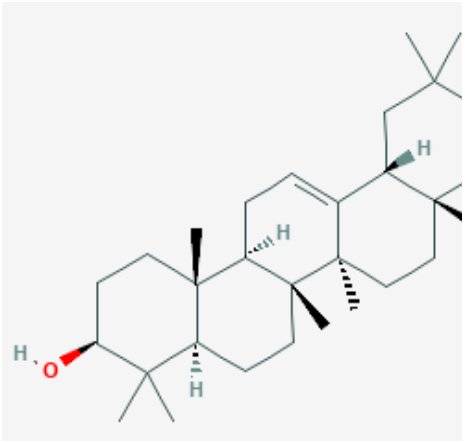
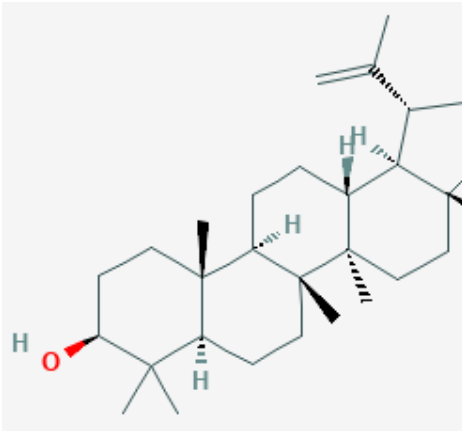
5. Phytochemical constituents

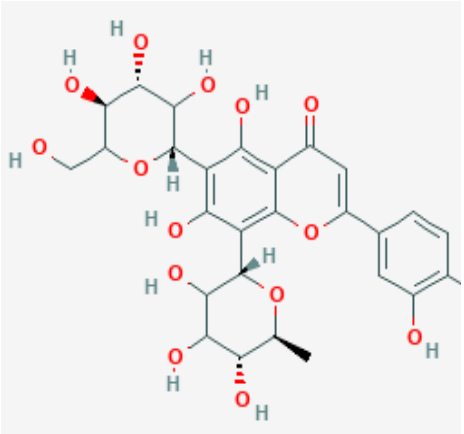
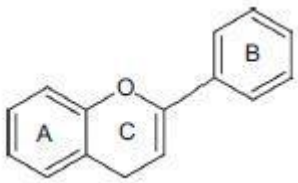
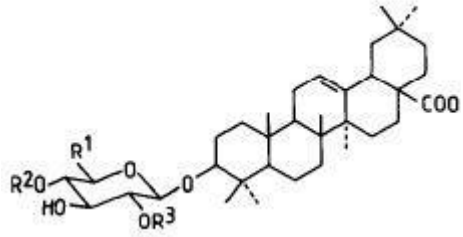
Several phytochemicals have been identified in the aerial parts of *C. spicata*. Table A.5 depicts the phytochemical, source and structure:

Table A.5: Phytochemicals identified in *Cussonia spicata*

Phytochemical	Source	Structure	Reference
Betulin	Leaves and stems		Wollenweber et al., 1999

Condensed tannins	Leaves		Amoo et al., 2012 Naumann et al., 2013
Free gallic acid	Leaves		Amoo et al., 2012
Gallotannins	Leaves		Amoo et al., 2012 Naumann et al., 2013
Iridoids	Leaves		Amoo et al., 2012 Viljoen et al., 2012

α-amyrin	Leaves and stems		(Wollenweber et al., 1999)
β-amyrin	Leaves and stems		(Wollenweber et al., 1999)
Lupeol	Leaves and stems		(Wollenweber et al., 1999)

Total flavonoids	Leaves		(Amoo et al., 2012)
Total phenolics	Leaves		(Amoo et al., 2012, Vuolo et al., 2019)
Saponins	Stem bark		(Gunzinger et al., 1986)

6. TLC fingerprinting of plant extract

For the detection of the chemical profile of *C. spicata* through thin layer chromatography (TLC), 2mg of the ethanol extract was diluted in 100µL of ethanol. The solution was spotted on silica gel 60 F254 TLC plates using glass Pasteur pipettes. The spotted silica gel 60 F254 TLC plates were placed in a TLC tank containing a solvent system that comprised of 5mL of chloroform, 2mL methanol and 3mL of ethyl acetate. The solvent was allowed to rise until the solvent front was reached (1cm from the top of the TLC plate) then the plate was airdried, placed under an ultraviolet lamp (short and long wavelength) then sprayed with vanillin in order to see the chemical profile.

7. Pharmacological properties

Cussonia spicata exhibited various pharmacological properties ranging from anti-bacterial, anti-malarial and antiproliferative/cytotoxic activity.

7.1. Antibacterial activity

Cussonia spicata's efficacy against *Bacillius subtilis*, *Escherichia coli*, *Klebsiella pneumoniae* and *Staphylococcus aureus* was determined through the disc diffusion assay with neomycin as a positive control. The ethanol and water extracts prepared from *C. spicata* were active against various pathogens with minimum inhibitory concentration (MIC) values ranging from 3.1mg/mL to 12.5mg/mL (McGaw et al., 2000). Furthermore, the aqueous and methanol extracts of *C. spicata* were evaluated against *Enterococcus faecalis*, *Escherichia coli*, *Neisseria gonorrhoeae*, *Staphylococcus aureus* and *Pseudomonas aeruginosa* using the microdilution assay with ciprofloxacin as the positive control. The extracts displayed MIC values ranging from 0.3mg/mL to 16mg/mL (De Villiers et al., 2010).

7.2. Antimalarial activity

Methanol and water extracts prepared using the leaves of *C. spicata* were also evaluated for their anti-malarial activity. The IC_{50} of the methanol extract against *Plasmodium falciparum* was 28.20 ± 1.23µg/mL and the IC_{50} of the water extract was > 50µg/mL compared to the positive control, quinine, which had an IC_{50} of 0.04 ± 0.00052 µg/mL (De Villiers et al., 2010).

7.3. Antiproliferative/cytotoxic activity

The antiproliferative/cytotoxic activity of *C. spicata* was evaluated against the T-cell leukemia (jurkat) cell line with camptothecin as a postive control. The extracts (methanol and water) exhibited moderate cytotoxicity with IC_{50}'s ranging from 23.9µg/mL (methanol) to > 50µg/mL compared to the IC_{50} of the positive control, camptothecin, 0.07 ± 0.02µg/mL (De Villiers et al., 2010).

8. Additional information

8.1. Therapeutic (proposed) usage

Antibacterial, antimalarial/antiplasmodial and anticancer.

8.2. Safety data

No data

8.3. Trade information

Not threatened or endangered. Status of least concern on the SANBI red data list.

8.4. Dosage

Not available.

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User:BotBln, 2010a. At Kirstenbosch National Botanical Gardens Species Cussonia spicata. This image was obtained from wikimedia under the Creative Commons Attribution 3.0 Unported Licence. Online. Available at: https://commons.wikimedia.org/wiki/File:Cussonia_spicata_KirstenboshBotGard09292010L.jpg (Accessed 3.26.20).

User:BotBln, 2010b. At Kirstenbosch National Botanical Gardens Species Cussonia spicata. This image was obtained from wikimedia under the Creative Commons Attribution 3.0 Unported Licence. Online. Available at: https://commons.wikimedia.org/wiki/File:Cussonia_spicata_KirstenboshBotGard09292010K.JPG (Accessed 3.26.20).

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Cotyledon orbiculata

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Figure A.6: (A) Depiction of the leaves of *Cotyledon orbiculate*. **(B)** Flowers of *Cotyledon orbiculata*. **(C)** Distribution map of *Cotyledon orbiculata* (Dinkum, 2011; Shawka, 2010; GBIF Secretariat, 2019). **(D)** TLC Chromatogram of *Cotyledon orbiculata* ethanolic extract

1. General description

1.1 Botanical nomenclature

Cotyledon orbiculata L. var. oblonga (Haw.) DC.

1.2 Botanical family

Crassulaceae

1.3 Vernacular names

Pig's ear (English)

Plakkie, platjies, varkoorblare, varkoor, kouterie (Afrikaans)

imPhewula (isiXhosa)

iPewula (isiZulu)

2. Description

The leaves of *C. orbiculata* are fleshy with a red border and waxy layer. The flowers are bell-shaped and may be yellow or orange (Harris, 2004, Van Wyk et al., 2009).

3. Distribution

Cotyledon orbiculata is found in Angola, Lesotho, Mozambique, Namibia, South Africa and Swaziland (Maroyi, 2019).

4. Ethnobotanical usage

A hot poultice prepared using the leaves of *C. orbiculata* was used for boils and the leaf was applied for the treatment of corns and warts. The leaf sap of *C. orbiculata* was used for earache and toothache (Moteetee and Wyk, 2011, Watt and Breyer-Brandwijk, 1962). *Cotyledon orbiculata* was also reportedly used for inflammation and epilepsy (Maroyi, 2019, Watt, 1968).

5. Phytochemical constituents

Several phytochemicals have been identified in *C. orbiculata* such as cardiac glycosides, saponins, tannins, reducing sugars and triterpene steroids (Kabatende, 2005). Characteristic compounds isolated from *C. orbiculata* were bufadienolide-type cardiac glycosides: Orbicuside A-, B-, C- and tyldeoside C (Anderson et al., 1985, Steyn et al., 1986). Bufadienolides are extremely toxic and were responsible for the death of several small livestock in the little Karoo and southern regions of the Great Karoo (Botha, 2016). Lastly, it was found that if humans (Bushmen and Hottentot people) fed on the meat of these livestock, secondary intoxication occurred with neurotoxic effects such as paralysis (Botha, 2016). The structures of the bufadienolides are depicted below in Figure A.7 and Figure A.8 (Botha, 2016).

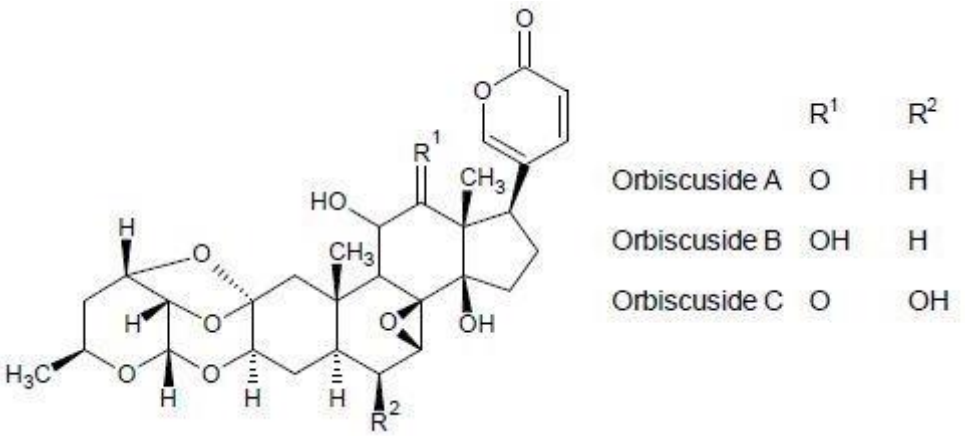

Figure A.7: Orbicusides A-, B- and C-

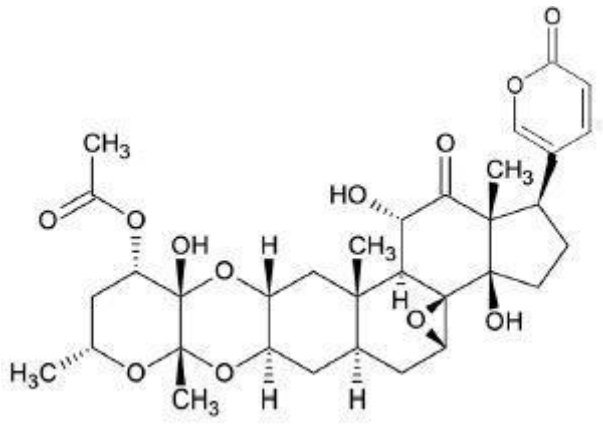

Figure A.8: Tyledoside C

6. TLC fingerprinting of plant extract

For the detection of the chemical profile of *C. orbiculata* through thin layer chromatography (TLC), 2mg of the ethanol extract was diluted in 100µL of ethanol. The solution was spotted on silica gel 60 F254 TLC plates using glass Pasteur pipettes. The spotted silica gel 60 F254 TLC plates were placed in a TLC tank containing a solvent system that comprised of 5mL of chloroform, 4mL hexane and 1mL of methanol. The solvent was allowed to rise until the solvent front was reached (1cm from the top of the TLC plate) then the plate was airdried, placed under an ultraviolet lamp (short and long wavelength) then sprayed with vanillin in order to see the chemical profile.

7. Pharmacological properties

Cotyledon orbiculata has not been studied extensively *in vitro* and *in vivo* but it has exhibited anti-inflammatory activity and anti-convulsant properties.

7.1. Anti-inflammatory activity

The anti-inflammatory activity of *C. orbiculata* was evaluated on lipopolysaccharide stimulated murine macrophages (Raw 264.7 cells) based on the inhibition of nitric oxide (NO). Nitric oxide is a major precursor of inflammation. It was found that the acetone extract of *C. orbiculata* exhibited good inhibitory activity with 99.37% inhibition of NO (Maroyi, 2019).

7.2. Anti-convulsant activity

The water and methanol extracts reportedly displayed anti-convulsant properties *in vivo* but the ethanol extract did not exhibit similar activity *in vitro* as $GABA_A$ -benzodiazepine binding activity was absent (Amabeoku et al., 2007, Stafford et al., 2005).

8. Additional information

8.1. Therapeutic (proposed) usage

Anti-inflammatory and anticonvulsant

8.2. Safety data

No data

8.3. Trade information

Not threatened or endangered. Status of least concern on the SANBI red data list.

8.4. Dosage

Not available

9. References

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Portulacaria afra

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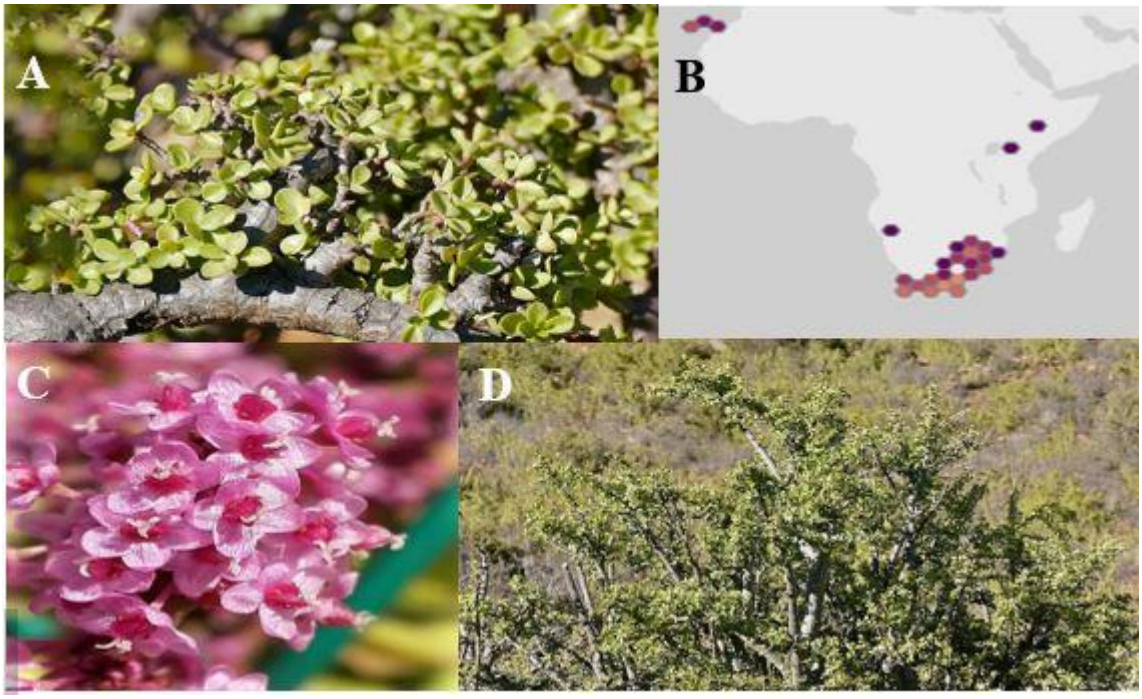

Figure A.9: (A) Leaves of *Portulacaria afra* (Du Pont, 2016). **(B)** Distribution of *P. afra* in Africa (GBIF, 2021). **(C)** Flowers of *P. afra* (Marchant, 2017). **(D)** Aerial parts of *P. afra* (Du Pont, 2016)

1. General description

1.1 Botanical nomenclature

Portulacaria afra Jacq.

1.2. Botanical family

Didiereaceae

1.3. Vernacular names

Elephant bush (English)

Porkbush (English)

Spekboom (Afrikaans)

iGqwanitsha (Xhosa)

isiCocco (Zulu)

2. Botanical description

Portulacaria afra is a small evergreen succulent shrub or small tree that can grow up to 2 – 5 m in height and about 1.5 – 2 m in gardens (Figure A.9D). Its succulent leaves are round and small and red in colour at the stems (Figure A.9A). Its flowers are small star-shaped and pink and in cultivation flowering can occur erractically (Figure A.9C) (Hankey, 2002).

3. Distribution

The succulent is found on rocky slope areas with warm conditions in the succulent karoo scrub, bushveld, thicket, and the dry river valleys in the eastern regions of South Africa in the Eastern Cape (Figure A.9B) (Hankey, 2002).

4. Ethnobotanical usage

Portulacaria afra is used for several ailments such as chronic sores and rashes (Nciki et al., 2016), and included in treatment for diabetes, ear aches, ear infections, otitits, tonsilitis (Hulley and Van Wyk, 2019)

5. Phytochemical constituents

The plant has been reported to contain anthroquinones, tannins, saponins and flavonoids (Nciki et al., 2016).

6. Pharmacological properties

Some antimicrobial activity has been noted in the leaf extract of *P.afra* (Table A.6) (Nciki et al., 2016). In a study by Salaheldin et al. (2019), *P. afra* showed little to no inhibitory potential against a few microbes such as *Bacillus subtilis*, *Staphylococcus aureus*, *Escherichia coli*, *Nesseria gonorrhoea*, *Pseudomonas aeruginosa*, and *Streptococcus faecalis*. The antibacterial potential of the *P. afra* extract increased slightly with biosynthesized silver nanoparticles ($AgNO_3$), and more significantly with gold nanoparticles (AgNPs).

6.1. Antimicorbial activity

The antimicrobial activity of the dichloromethane: methanol (1:1) leaf extract of *P.afra* (Nciki et al., 2016) is summarised in the table below (Table A.6)

Table A.6: *P. afra* antimicrobial activity

Pathogen	MIC (mg/mL)
Staphylococcus aureus **(ATCC 25925)**	1000
Staphylococcus aureus **Methicillin-resistant (ATCC 43300)**	380
Staphylococcus aureus **Gentamycin and methicillin resistant (ATCC 33592)**	1000
Staphylococcus epidermis **(ATCC 2223)**	3000
Propionibacterium acnes **(ATCC 11827)**	8000
Brevibacterium agri **(ATCC 51663)**	3000
Brevibacterium linens **(DSM 20425)**	> 8000
Pseudomonoas aeruginosa **(ATCC 27853)**	500
Escherichia coli **(ATCC 25922)**	2000
Trichophyton mentagrophytes **(ATCC 9533)**	250
Microsporum canis **(ATCC 36299)**	1940
Candida albicans **(ATCC 10231)**	2000

6.2. Cytotoxicity and antioxidant activity

The cytotoxicity of the *P. afra* extract was assessed against HepG2 cells at different concentrations. It was determined that the extract was only safe for use up to 2500µg/mL (Khanyile et al., 2021). Similar observations were observed in other studies where plant extracts exhibited high toxic thresholds at higher concentrations (Mensah et al., 2019; Maliehe et al., 2017).

7. Additional information

7.1. Therapeutic (proposed) usage

It has the potential to be an antimicrobial agent.

7.2. Safety Data

As stated previously in the study by Khanyile et al., (2021), the plant extract is safe for use up to 2500µg/mL

7.3. Trade information

Least concern according to the SANBI Red List

7.4. Dosage

Not available.

8. References

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Pelargonium citronellum

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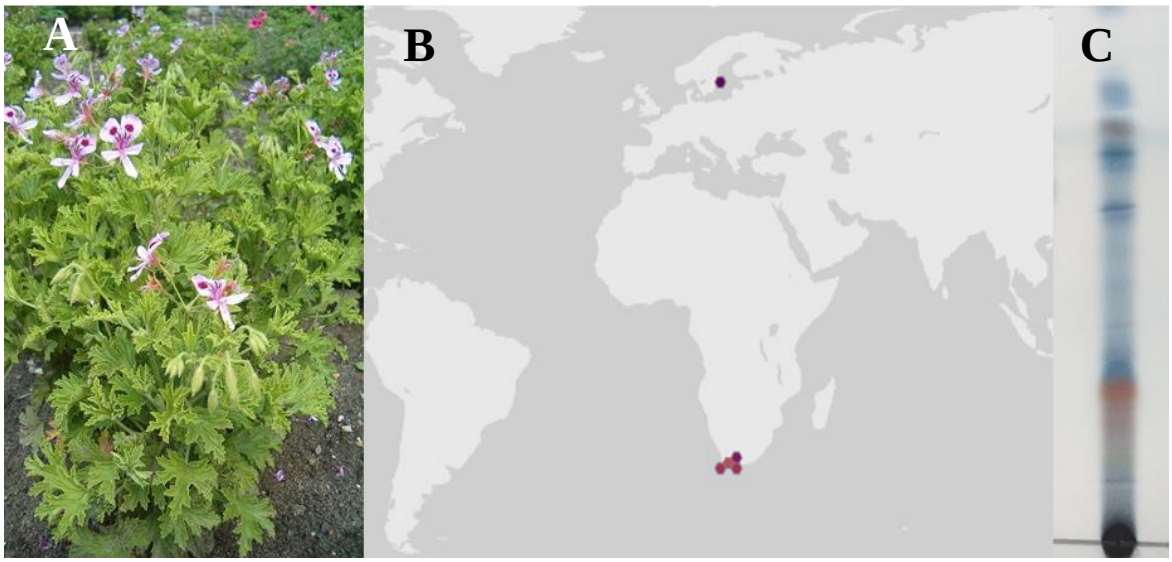

Figure A.10: (A) Aerial plant parts of *P. citronellum* (Botbln, 2011). **(B)** Distribution of *P. citronellum* (GBIF, 2019). **(C)** TLC Chromatogram of *P. citronellum* DCM extract

1. General description

1.1. Botanical nomenclature

Pelargonium citronellum J.J.A. Van der Walt

1.2. Botanical family

Geraniaceae

1.3. Vernacular names

Lemon-scented pelargonium (English)

Malva (Afrikaans)

2. Botanical description

Pelargonium citronellum is a bushy, woody, much-branched and strongly lemon-scented shrub that reaches heights up to 2m and spreads up to 1m in diameter. It has herbaceous stems when young and is woody at the base (Demarne and Van der Walt, 1993). The leaves and stems are covered in

numerous small glandular hairs. The leaves are conspicuously veined abaxially, palmately shaped with sharp pointed lobes, and simple and alternately arranged. Inflorescence is a branched system of peduncles that are 20 – 50mm long, each ending in a pseudo umbel of 5,6, or 8 flowers. The flowers are pink purple with dark markings on the two larger upper petals only. Flowering occurs during spring and summer and is at its best in early summer (Van der Walt, 1977, Vorster & Van der Walt, 1982).

3. Distribution

The plant grows near Ladysmith, a village in the south-eastern Western Cape region but is also commonly found on the northern foothills of Langeberg Mountains located from Miskraal to Herbertsdale. It also grows near streams and well-drained soils (Van der Walt, 1977, Vorster & Van der Walt, 1982, Mjuleni, 2007).

4. Ethnobotanical usage

The strong lemon scent is reported to have natural insecticidal properties. The fragrant and attractive foliage makes this species an interesting addition to the garden and can be used a culinary herb (Mjuleni, 2007).

5. Phytochemical constituents

Comprehensive studies have been conducted to present the chemical composition of the essential oil produced by *P. citronellum.* GC and GC/MS analysis reveal that the essential oil mainly contains aldehyde mixtures with a lemon scent usually encountered in lemongrass. More than 80 of the constituents could be separated by GC but most of them were trace constituents. It was noted that in the *Pelargonium* genus, only *P. citronellum* and *P. crispum* synthesize large quantities of the monoterpenoid, nerol (Figure A.11) and geraniol (Figure A.12) (Demarne and Van der Walt, 1993).

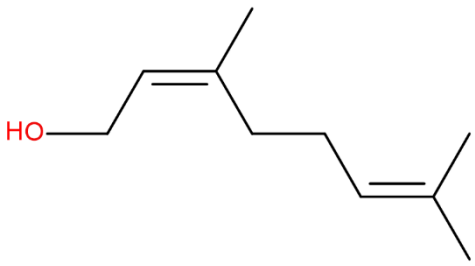

Figure A.11: Nerol

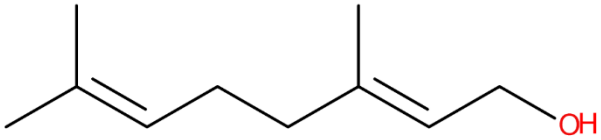

Figure A.12: Geraniol

6. TLC fingerprinting of plant extract

The DCM extract of *P. citronellum* was weighed at 2mg and dissolved in 200µL of DCM. The plant extract was spotted on a Silica gel 60 F254 TLC plate. The mobile phase was prepared at a ratio of 7:2:1 of hexane, dichloromethane, and ethanol respectively at a total volume of 10 mL. The TLC plate was run in a chromatographic chamber with the respective mobile phase and covered with a glass plate for the prevention of evaporation of the solvent. The developed TLC plate was observed under long and short wavelength and sprayed with vanillin solution for detection of the bands (Figure A.10C).

7. Pharmacological properties

There is a limited number of studies that have been conducted to determine the biological properties of *P. citronellum.* The biological activity of *P. citronellum* aerial plant parts according to a study by Lalli et al. (2008) is summarized in the table below (Table A.7):

Table A.7: Biological activity of *P. citronellum*

Activity	Pathogen/Cell line/Reagent/Enzyme	Sample/Extract	MIC (mg/mL)	IC_{50} (µg/mL)
Antifungal	*Candida albicans* (ATCC 10231)	Acetone	500	59.94
Antioxidant	DPPH	Acetone	-	84.01
Antibacterial	*Klebsilla pneumonia* (NCTC 1633)	Acetone	3000	-
	Bacillus cereus (ATCC 11778)	Acetone	410	-
	Staphylococcus aureus (ATCC 12600)	Acetone	160	-
Cytotoxicity	Human epithelial kidney cells	Acetone	-	19.14

8. Additional information

8.1. Safety data

Based on the study conducted by Lalli et al., (2008), the acetone extract of *P. citronellum* exhibited high toxicity with an IC_{50} of 19.14 µg/mL on human epithelial kidney cells.

8.2. Therapeutic (proposed) usage

Potential antifungal, antibacterial, and anticancer.

8.3. Trade information

According to the SANBI Red List, this is a rare species and not threatened

8.4. Dosage

Not available

9. References

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Appendix B: UPLC-MS Protocol

Appendix B: UPLC-MS Protocol

Compound separation and detection were performed using a Waters® Synapt G2 high-definition mass spectrometry (HDMS) system (Waters Inc., Milford, Massachusetts, USA). The system comprises of a Waters Acquity Ultra Performance Liquid Chromatography (UPLC®) system hyphenated to a quadrupole-time-of-flight (QTOF) instrument. The system was operated with MassLynx™ (version 4.1) software (Waters Inc., Milford, Massachusetts, USA) for data acquisition and processing. An internal lock mass control standard, 2 ng/µL solution leucine enkephalin (m/z 555.2693), was directly infused into the source through a secondary orthogonal electrospray ionisation (ESI) probe allowing intermittent sampling. The internal control was used to compensate for instrumental drift, ensuring good mass accuracy, throughout the duration of the runs. The instrument was calibrated using sodium formate clusters and Intellistart functionality (mass range 112.936 – 1 132.688 Da). Resolution of 20 000 at m/z 200 (full width at half maximum (FWHM)) and mass error within 0.4 mDa were obtained.

The source conditions were as follows: the capillary voltage for ESI was 2.6 kV and 2.0 kV for positive and negative mode ionisation. The source temperature was set at 120 ºC, the sampling cone voltage at 25 V, extraction cone voltage at 4.0 V and cone gas (nitrogen) flow at 10.0 L/Hr. The desolvation temperature was set at 350 ºC with a gas (nitrogen) flow of 600.0 L/Hr.

Quantitative data-independent acquisition (DIA) was done using two simultaneous acquisition functions with low and high collision energy (MSE approach) with a QTOF instrument. The high energy MS scan can be time aligned with the low energy scan in order to predict which fragment ions belong to which precursor ions, consequently the full mass spectrum is acquired. Fragmentation patterns can thus be used for qualitative confirmation. Fragmentation was performed using high energy collision induced dissociation (CID). The fragmentation energy was set at 2 V and 3 V for the trap and collision energy, respectively. The ramping was set from 3 to 4 V and 20 to 40 V for the trap and transfer collision energy, respectively.

Mass spectral scans were collected every 0.3 seconds. The raw data was collected in the form of a continuous profile. Mass to charge ratios (m/z) between 50 and 1 200 Da were recorded.

Separation was completed using a reverse phase step gradient elution scheme from 95% H2O (0.1% formic acid) to 100% acetonitrile (0.1% formic acid). Formic acid was added to the solution as buffer (pH correction), preservative and proton source for ionisation. The gradient started with an isocratic flow (hold 0.1 min) followed by a linear increase to 100% ACN; subsequently the column was washed for 1 min followed by conditioning and re-establishing of initial conditions to allow for

equilibration before the start of the next run for the complete elution scheme. The column temperature was kept constant at 40 ºC and the flow rate was set at 0.4 mL/min for the entire run giving a total run time of 20 min. Injection volumes were set at 5 µL. A Kinetex® 1.7 µm EVO C18 100 Å (2.1 mm ID x 100 mm length) column was used. The positive and negative ion mass spectra were collected in separate chromatographic runs (employing the same separation conditions). Solvents used: ultra-purity water and acetonitrile (Romil-UpS™,Microsep, South Africa), 99+% purity Formic acid (Thermo Scientific, South Africa)

Appendix C: Antiproliferative and Antioxidant Assay Pictures

Appendix C: Antiproliferative and Antioxidant Assay Pictures

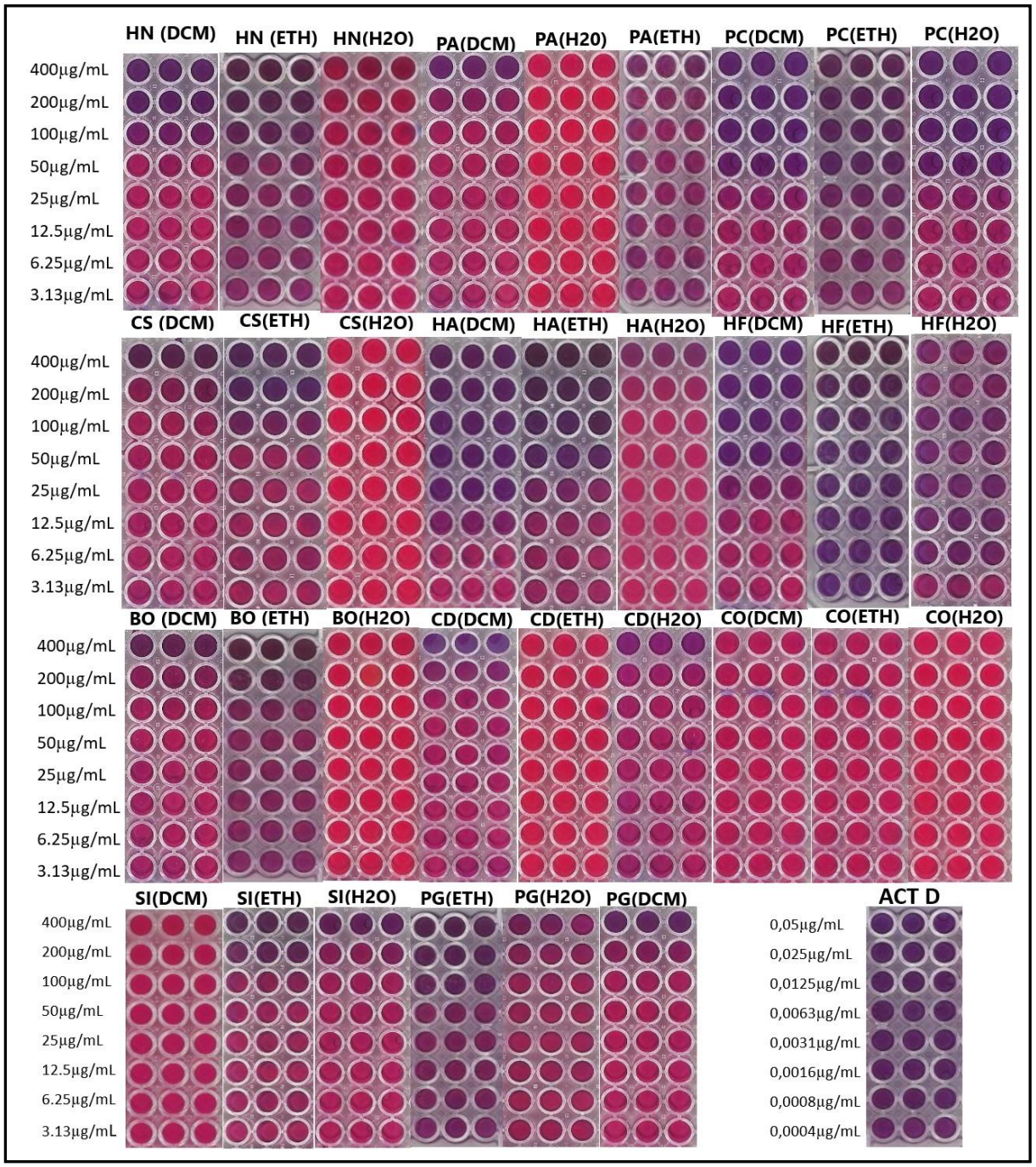

Figure C.1: Antiproliferative assay results. (DCM) Dichloromethanoic. **(ETH)** Ethanolic. **(H2O)** Aqueous**. (HN)** *Heteropyxis natalensis*. **(PA)** *Portulacaria afra*. **(PC)** *Pelargonium citronellum.* **(CS)** *Cussonia spicata*. (**HA)** *Hypoestes aristata*. **(HF)** *Hypoestes forskaolii***. (BO)** *Barleria obtusa*. **(CD)** *Carpobrotus dimidiatus*. **(CO)** *Cotyledon orbiculata*. **(SI)** *Sideroxylon inerme***. (PG)** *Pelargonium graveolens*. **(ACT D)** Actinomycin D

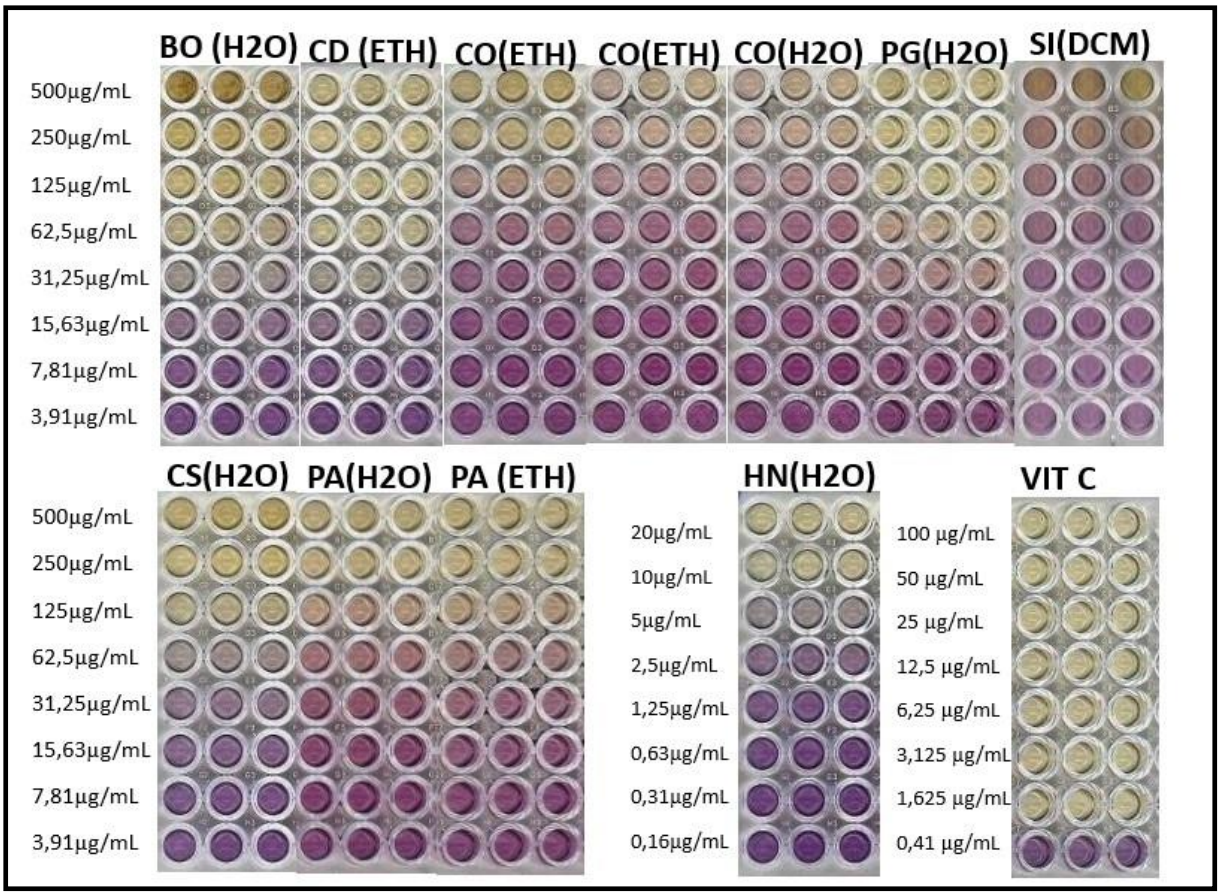

Figure C.2: DPPH Assay results. (DCM) Dichloromethanoic. **(ETH)** Ethanolic**. (H2O)** Aqueous. **(HN)** *Heteropyxis natalensis*. **(PA)** *Portulacaria afra*. **(PC)** *Pelargonium citronellum*. **(CS)** *Cussonia spicata*. **(BO)** *Barleria obtusa*. **(CD)** *Carpobrotus dimidiatus*. **(CO)** *Cotyledon orbiculata*. **(SI)** *Sideroxylon inerme*. **(PG)** *Pelargonium graveolens*. **(VIT C)** Vitamin C.