

EVALUATION OF EFFECTS OF SELECTED MEDICINAL PLANTS ON ANGIOGENESIS BY DETERMINING THEIR INHIBITORY EFFECTS ON COX-2, NOS AND LOX ENZYMES

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DECLARATION OF INDEPENDENT WORK

DECLARATION WITH REGARD TO INDEPENDENT WORK

I, Paballo Irvin Direko, identity number _____ and student number _____, do hereby declare that this research project submitted to the Central University of Technology, Free State for the Degree MASTER OF HEALTH SCIENCE IN BIOMEDICAL TECHNOLOGY, is my own independent work; and complies with the Code of Academic Integrity, as well as other relevant policies, procedures, rules and regulations of the Central University of Technology, Free State; and has not been submitted before to any institution by myself or any other person in fulfilment (or partial fulfilment) of the requirements for the attainment of any qualification.



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Summary

Introduction: Angiogenesis is the process of forming new capillaries from existing ones. This process is important for tumor growth and metastasis. It is also important during wound healing. During tumor metastasis this process is amplified, as new capillaries are needed for cancer to spread, but in ulcerations, when this process does not occur, ulceration does not heal. Cyclooxygenase-2 (COX-2), nitric oxide synthase (NOS) and lipoxygenase (LOX) are some of the enzymes that are highly expressed during angiogenesis and therefore provide a useful way to detect angiogenesis. Some medicinal plants have been shown to possess angiogenic modulating properties, which has led to the development of anti-angiogenic drugs. *Euphorbia tirucalli*, *Spirostachys Africana* sond, and *Synadenium cupulare* (Boiss.) L.C. have traditionally been used for treatment of open wounds, ulcers and cancer. Their mechanism(s) of action is, however, still not clear. These plants have therefore been selected to determine their effect on angiogenesis, as angiogenesis is a denominator for cancer and tissue regeneration, which is critical for wound healing and healing of ulcers.

Aim: The study aims to evaluate the effects of selected medicinal plants for their angiogenesis modulating effects by determining their inhibitory effects on COX-2, LOX and NOS enzymes, as well as their growth inhibitory effects on MCF-7 cells.

Methods: The plants were collected from their natural habitat in Pretoria and the Kruger National Park, Limpopo. Plants were dried and extractions were made with water and sequentially with hexane, dichloromethane, methanol and ethyl acetate. Phytochemical analysis was performed to determine the classes of compounds present in the plants. The MCF-7 cells were cultured in DMEM and 10% fetal bovine serum under normal culture conditions. The cells were treated with different concentrations of the plant extracts. After exposure, the cell viability was measured using MTT assay. The cell lysate was then evaluated for enzymatic activity of COX-2 and LOX. Pure enzymes obtained commercially were used as positive controls, while

commercial substrates were used for standard curves using enzyme. The culture media were tested for the amount of nitric oxide produced by cells after incubation with extracts.

Results and discussion: The plant ingredients were extracted successfully and the yields were recorded. Phytochemical analysis revealed the presence of tannins, saponins and glycosides on all plants. *Euphorbia tirucalli* was one of the plants that had the lowest number of phytochemicals, namely saponins, glycosides, tannins and triterpenoids. This plant also had the lowest cell growth inhibitory activity around 10 $\mu\text{g/ml}$ or higher and the lowest enzyme inhibitory activity. *Spirostachys africana* had most phytochemicals and the highest growth inhibition of MCF-7 cells with IC_{50} around 1 $\mu\text{g/ml}$ and selectivity for cancer cells over MCF-10A and CHO cells. *E. cupularis* had the second highest number of phytochemicals and also exhibited good cell growth inhibition, ranging around 5 to 1 $\mu\text{g/ml}$, and good selectivity for cancer cells, especially the nonpolar extracts. The extracts induced NOS, which is indicative of apoptosis or which could imply induction of apoptosis by activating inducible NOS. Selective extracts of *E. cupularis* and *S. africana* might inhibit angiogenesis through cyclooxygenase inhibition. LOX inhibitory activity was concentration-dependent. The highest extract concentration had the highest inhibitory activity.

Conclusion: From our findings, we suggest that non-polar extracts of *S. africana* and *E. cupularis* have anti-cancer activity and anti-angiogenic activity as well. Our results were also contradictory to the traditional use of the plants.

Keywords: Angiogenesis, cyclooxygenase, lipoxygenase, nitric oxide synthase, cell growth inhibition, selectivity, enzyme inhibition, MCF-7 cell culture, phytochemical analysis, *Euphorbia tirucalli*, *Spirostachys africana*, *Euphorbia cupularis*.

TABLE OF CONTENT

Contents

Summary	4
LIST OF TABLES.....	8
CHAPTER 1	10
INTRODUCTION.....	10
1.1 Problem statement	12
1.2 Hypothesis.....	12
1.3 Aim	13
1.4 Objectives.....	13
CHAPTER 2	14
2. LITERATURE REVIEW	14
2.1 Angiogenesis	14
2.2 Tumor angiogenesis	15
2.3 Angiogenesis and ulcerations	18
2.4 Cyclooxygenase 2.....	19
2.5 Lipoxygenases	21
2.6 Nitric oxide synthase.....	22
CHAPTER 3	23
3. MEDICINAL PLANTS.....	23
3.1. <i>Spirostachys Africana Sond</i>	23
3.2. <i>Synadenium cupulare (Boiss.) L.C.</i>	24
3.3 <i>Euphorbia tirucalli</i>	25
CHAPTER 4	27
4. MATERIALS AND METHODS	27
4.1. Plant collection and extraction	27
4.2 Phytochemical analysis	27
4.3 Cell culturing	30
4.3.1 Cell culture conditions	30

4.3.2	Cell trypsinization and sub-culturing	30
4.3.3	Cell viability count.....	31
4.3.4	Cell plating.....	31
4.3.5	Assay.....	31
4.4	MTT assay.....	32
4.4.1	Measuring cell viability.....	32
4.5	Nitric oxide synthase activity	33
4.6	LOX activity assay.....	33
4.7	Cyclooxygenase-2 activity assay	34
CHAPTER 5	35
RESULTS	35
5.1	Plant extraction.....	35
5.2	Phytochemical analysis	36
5.3	Cell culture	38
5.3.1	Cell growth inhibition of MCF-7 breast cancer cells	38
5.3.1.1	Cell growth inhibition of MCF-7 cells by extracts of <i>S. africana</i>	39
5.3.1.2	Cell growth inhibition of MCF-7 cells by <i>E. tirucalli</i>	42
5.3.1.3	Cell growth inhibition of MCF-7 cells by <i>E. cupularis</i>	43
5.3.2	Selectivity of extracts for breast cancer cells.....	45
5.4	Enzyme activity assays	49
5.4.1	NOS activity.....	49
5.4.2	Cyclooxygenase-2.....	50
5.4.3	Lipoxygenase activity	56
CHAPTER 6	62
6.1	DISCUSSION.....	62
6.2	CONCLUSION.....	68
REFERENCES	70
Appendix	82

LIST OF TABLES

Table 1. A summary of the names and labels of samples that were evaluated for MCF-7 cell growth inhibition.....	33
Table 2. Yield percentage of plant extracts after extraction with various solvents.	35
Table 3. Phytochemicals present in <i>E. tirucalli</i> , <i>E. cupularis</i> , and <i>S. africana</i>	36
Table 4. Cell growth inhibitory activity of extracts of different plant parts of the three plants	39
Table 5. Selectivity index of selected extracts	48
Table 6. The percentage yield of extracts from powdered material	86
Table 7. Phytochemicals tested in <i>S. Africana</i>	87

LIST OF FIGURES

Figure 1. The process of angiogenesis.....	15
Figure 2. Schematic presentation that shows the multi-stage development of a tumor.....	16
Figure 3. Signaling pathways of tumor angiogenesis	17
Figure 4 Schematic presentation of the role of COX-2 in controlling proliferation, invasion, apoptosis and tumor angiogenesis.....	20
Figure 5. Picture of <i>Spirostachys Africana Sond</i>	24
Figure 6. <i>Euphorbia cupularis</i>	25
Figure 7. Image of <i>Euphorbia tirucalli</i>	26
Figure 8. Images showing color changes observed during phytochemical testing <i>E. cupularis</i>	37
Figure 9. Images showing color changes observed during phytochemical testing of <i>S. africana</i>	37
Figure 10. Images showing color changes observed during phytochemical testing of <i>E. tirucalli</i>	38
Figure 11. Cell growth inhibition of MCF-7 cells by <i>S. africana</i> (tamboti) plant extracts.....	40
Figure 12. Cell growth inhibitory of MCF-7 cells by <i>S. africana</i> leaf extracts tested at concentration range of 100 -0.001 µg/ml (full 10-fold dilutions).	41
Figure 13. Cell growth inhibition of MCF-7 cells by <i>S. africana</i> stem and bark extracts tested at concentration range of 100 -0.001 µg/ml (full 10-fold dilutions).....	41
Figure 14. Cell growth inhibition of MCF-7 by <i>Euphorbia tirucalli</i> extracts.....	42
Figure 15. Cell growth inhibition of MCF-7 cells by <i>E. tirucalli</i> extracts tested at concentration range of 100 -0.001 µg/ml (full 10-fold dilutions).....	43
Figure 16. Growth inhibition of MCF-7 cell line by <i>Euphorbia cupularis</i> extracts	44
Figure 17. Cell growth inhibition of MCF-7 cells by <i>E. cupularis</i> extracts tested at concentration range of 100 -0.001 µg/ml (full 10-fold dilutions).....	45
Figure 18. Cell growth inhibition of MCF-7 cells by extract of tamboti leaves and <i>E. cupularis</i> stems.	46
Figure 19. Cell growth inhibition of CHO cells by extracts of <i>E. tirucalli</i>	47
Figure 20. Nitrite standard curve	49
Figure 21. Effects of selected extracts of <i>S. africana</i> , <i>E. tirucalli</i> , and <i>E. cupularis</i> on NOS activity	50
Figure 22. COX-2 standard curve	51

Figure 23. Enzyme kinetic activity of COX enzyme with (Enz + inh) and without a commercial inhibitor (enzyme).	51
Figure 24. The graphs show the effect of <i>S. africana</i> extracts on activity of COX-2 over time..	52
Figure 25. The graphs show the effect of <i>S. africana</i> hexane bark extract on activity of COX-2 over time.	53
Figure 26. The graphs show the effect of <i>E. cupularis</i> leaf extracts on activity of COX-2 over time.....	53
Figure 27. The graphs show the effect of <i>E. cupularis</i> stem extracts on activity of COX-2 over time.....	54
Figure 28. Activity of COX-2 against selected extracts.	55
Figure 29. LOX standard curve	56
Figure 30. Kinetic activity of LOX enzyme.....	57
Figure 31. The graphs show the effect of <i>S. africana</i> leaf extracts on activity of LOX over time.....	58
Figure 32. The graphs show the effect of <i>S. africana</i> bark extracts on activity of LOX over time.	58
Figure 33. The graphs show the effect of <i>E. cupularis</i> stem extracts on activity of LOX over time.....	59
Figure 34. The graphs show the effect of <i>E. cupularis</i> stem extracts on activity of LOX over time.....	60
Figure 35. . The graphs show the effect of selected <i>E. tirucalli</i> extracts on activity of LOX over time.	
Figure A shows the effect of DCM:MeOH extract and B shows the methanol extract.	60
Figure 36. Activity of LOX against selected extracts.	61
Figure 37. Effects of <i>S. africana</i> extracts on MCF-7 cell growth inhibition	87
Figure 38. Effects of <i>S. africana</i> bark and leaf extracts on normal human breast MCF-10A cell growth inhibition	88
Figure 39. The effect of <i>S. africana</i> extracts on nitric oxide synthase	89
Figure 40. Activity of COX-2 against <i>S. africana</i> extracts.....	90
Figure 41. Effects of selected extracts on the activity of LOX enzyme..	91

CHAPTER 1

INTRODUCTION

Angiogenesis is the processes of forming new capillary vessels from existing ones. It is a normal biological process that occurs during the female reproductive cycle, wound healing and treatment of ulcers (Nishida et al, 2006). Studies have shown that abnormal angiogenesis is related to tumor growth and progression to a metastatic phenotype (Folkman, 1971). Metastasis refers to the spreading of neoplasm to other tissues or organs and for this to occur, new vessels are required to supply the tumor with nutrients and oxygen (Nishida et al, 2006). Angiogenesis is a complex process that involves growth factors, cytokines and enzymes such as cyclooxygenases (COX), nitric oxide synthase (NOS) and lipoxygenase (LOX). These three enzymes, in particular, have been selected as the focus of the study, because of their significant role in angiogenesis and their implication in tumor initiation and growth.

Cyclooxygenases are the enzymes that catalyze the oxidation of a wide variety of xenobiotics, including prostaglandin (Smith et al, 1991). There are two forms of COX: COX1- and COX-2. COX-1 is constitutive, is found in most tissues and maintains homeostasis, while COX-2 is inducible and functions in inflammation and tumor angiogenesis (Sano et al, 1995; Kujubu et al, 1993).

Lipoxygenases are a group of enzymes with a non-heme iron atom that catalyze the oxidation of poly-unsaturated fatty acids such as arachidonic acid (Wisastra & Dekker, 2014). They are classified as 5-, 8-, 12- and 15-LOX according to their preferred

oxidation position on the fatty acids (Brash, 1999). Expression of 12-LOX in prostate cancer induced tumor angiogenesis (Nie et al, 1998).

Nitric oxide (NO) was initially described as an endothelium-derived relaxing factor with vasomotor regulatory effect by Furchgott and Zawadzki in 1980. Further investigation of this molecule led to the discovery of more regulatory effects, such as smooth muscle proliferation, inhibition of platelet activation and inhibition of leukocyte function (Schmidt & Walter, 1994). NOS forms NO from L-arginine. Higher levels of inducible NOS were detected in metastatic tumors than in benign tumors (Cianchi et al, 2003).

Medicinal plants are widely used in South Africa, especially in rural areas where there is a lack of good health care services. Most rural people choose medicinal plants over western medicine because medicinal plants are affordable and accessible. *Spirostachys Africana* Sond, *Synadenium cupulare* (Boiss.) L.C. and *Euphorbia tirucalli* are some of the medicinal plants that have traditionally been used for the treatment of ulcers and open wounds, which are the processes that require angiogenesis. Gastrointestinal ulcers are caused by a shearing of the stomach lining. Wounds and ulceration require angiogenesis to be healed, so that new vesicles are produced at the healing site, to supply oxygen and nutrients (DiPietro, 2016). These plants have also been used traditionally for the treatment of various cancers. These two disease conditions share the process of angiogenesis, where a shift in balance of angiogenesis can result in either tumor initiation or wound healing.

1.1 Problem statement

Angiogenesis is important for tumor metastasis and wound healing, as new capillaries are required for delivery of nutrients and oxygen as well as removal of metabolic waste. Drugs used in the treatment of cancer have adverse side effects such as hair loss, ulcers and damage to the reproductive system (Redd et al, 2001). Therefore there is a need for better and efficient drugs that target and eradicate cancer cells only. Medicinal plants have been used for generations, proving that they are safe to consume. Moreover, there is still much scientific knowledge to be discovered from these plants. Angiogenesis is a complex process with multiple pathways, which makes it difficult to inhibit this process in tumor metastasis. Medicinal plants may provide different pathways through which they exert inhibition on this process.

1.2 Hypothesis

One of the ways to inhibit tumor angiogenesis is to inhibit the enzymes that regulate these pathways; these include COX-2, LOX and NOS. The three enzymes stimulate angiogenesis in different pathways. The effects of selected plants on different biological targets would give an indication of their mode of action. MCF-7 cells are derived from metastatic breast cancer cells, have ideal tumor characteristics and have been chosen as the *in vitro* model to study anti-proliferation and inhibition of enzymes involved in tumor angiogenesis.

1.3 Aim

The aim of the study is to evaluate the effects of the selected medicinal plants for their angiogenesis modulating effects on the growth of MCF-7 cells and by determining their inhibitory effects on COX-2, LOX and NOS enzymes, which are highly implicated in the process of angiogenesis.

1.4 Objectives

- To collect selected plants, extract substances from the plants, and screen the extracts for their proliferative or anti-proliferative effect on breast cancer MCF-7 cells
- To determine the selectivity effects of the extracts on normal breast MCF-10A cells and CHO cells
- To determine the effects of the extracts on the enzymatic activity of COX-2, NOS and LOX in MCF-7 cells
- To perform phytochemical analysis of the plants to identify the classes of secondary metabolites present.

CHAPTER 2

2. LITERATURE REVIEW

2.1 Angiogenesis

Angiogenesis has been the focus of study ever since Judah Folkman (1971) found out that it promotes tumor progression to a metastatic phenotype. Therefore much research has been done on angiogenesis since the 1980s and many regulatory factors have been identified (Otrock et al, 2007). Angiogenesis occurs from early embryonic development to adulthood and if it is not properly regulated, especially in adults, it results in the development of tumors. There are a number of diseases that result from abnormal angiogenesis, which include cancer, diabetic retinopathy, rheumatoid arthritis and cardiovascular disorders (Pandya et al, 2006).

Angiogenesis is controlled by a balance between angiogenic and angiostatic growth factors (Toomey et al, 2009). When more angiogenic growth factors are activated, the balance tips off to the side of angiogenesis; this is called the 'angiogenic switch' and it occurs owing to inflammation, neoplasia or hypoxia (McMahon, 2000). When the 'angiogenic switch' is turned on, growth factors bind to specific receptors on endothelial cells, causing the endothelial cells to proliferate and migrate out of existing vessels. Adhesion molecules or integrins help pull the new vessels outwards, while matrix metalloproteinases dissolve the tissue in front of the sprouting vessels. Tissues then form around new vessels and the blood begins to flow (figure 1) (Pandya et al, 2006).

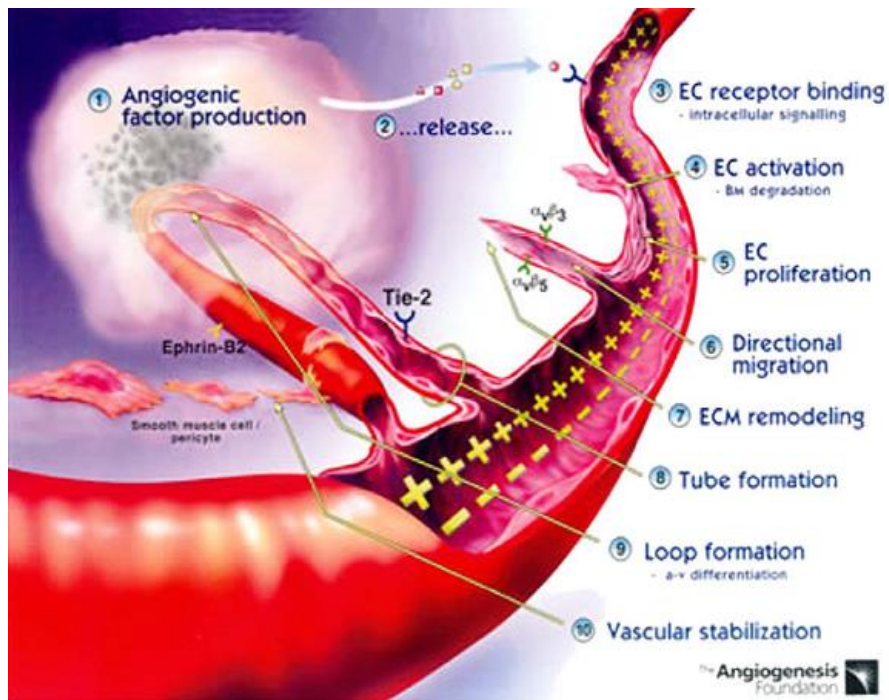


Figure 1. The process of angiogenesis (Pandya et al, 2006).

2.2 Tumor angiogenesis

Angiogenesis is important for the growth of tumors and metastasis (Pandya et al, 2006). This has been proven by a study conducted by Muthukkaruppan et al (1982) when they compared the behavior of cancer in different regions of the same cells. It was found that cancer cells placed in a region where angiogenesis was possible grew more than the cells placed where angiogenesis did not occur (Muthukkaruppan et al, 1982). The relationship between angiogenesis and tumor metastasis was first described by Folkman and coworkers in the 1971. It was showed that tumor development is dependent on neovascularization and suggested that this involves growth factors released during angiogenesis.

The development of a tumor is a multi-stage process (Parangi et al, 1996). Tumorigenesis occurs in two stages; the first step is a switch from dormancy to hyperproliferation of the oncogene expressing pancreatic f3 cells associated with activation of the expression of insulin-like growth factor II (IGF-II) (Christofori et al, 1994). The second step is the activation of angiogenesis, where new capillaries are formed from existing ones (figure 2).

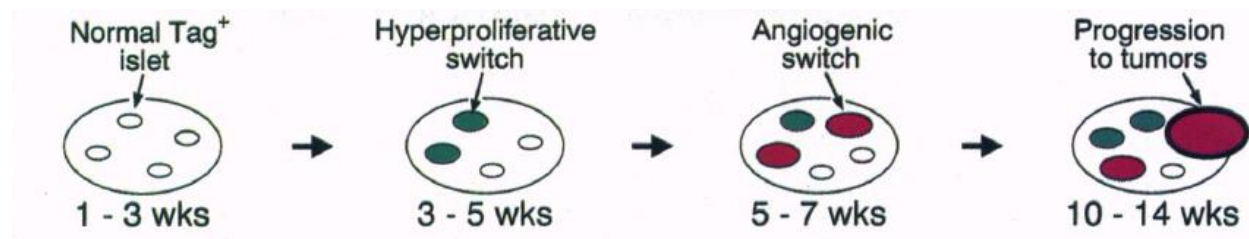


Figure 2. Schematic presentation that shows the multi-stage development of a tumor (Christofori et al, 1994)

Evidence that tumor metastasis is dependent on angiogenesis has been shown in many research projects. This hypothesis, however, does not include the early development of solid tumors when the cells are about 10^{16} (Folkman, 1990). Tannock (1970) estimated the kinetic parameters for endothelial cells of capillary walls, fibroblasts and carcinoma cells of transplanted C3H mouse mammary cells. He found that the labeling index, mitotic index and mean grain count per labeled cells decreased with increased distance of the carcinoma cells from the capillaries (Tannock, 1970). Folkman and coworkers (1966) compared tumors grown in isolated perfused organs and found that tumors grown where blood vessels did not proliferate were growth-limited but grew rapidly after vascularization when they were implanted into mice (Folkman et al, 1966). When

Gimbrone et al (1972) implanted anaplastic Brown Pearce carcinoma into the iris of rabbits, the cells always vascularized, and when they were implanted far from the iris, they did not vascularize and remained small and mitotically active. However, when the same cells were placed in the iris, they grew and became invasive. This suggests that if tumor angiogenesis is blocked, tumors will be growth-restricted, non-invasive and non-lethal.

Tumor angiogenesis is a complicated process in that it has a number of signaling pathways (figure 3). One of the ways to inhibit tumor angiogenesis is to inhibit the enzymes that regulate these pathways; these include COX-2, LOX and NOS. These enzymes are discussed in detail below.

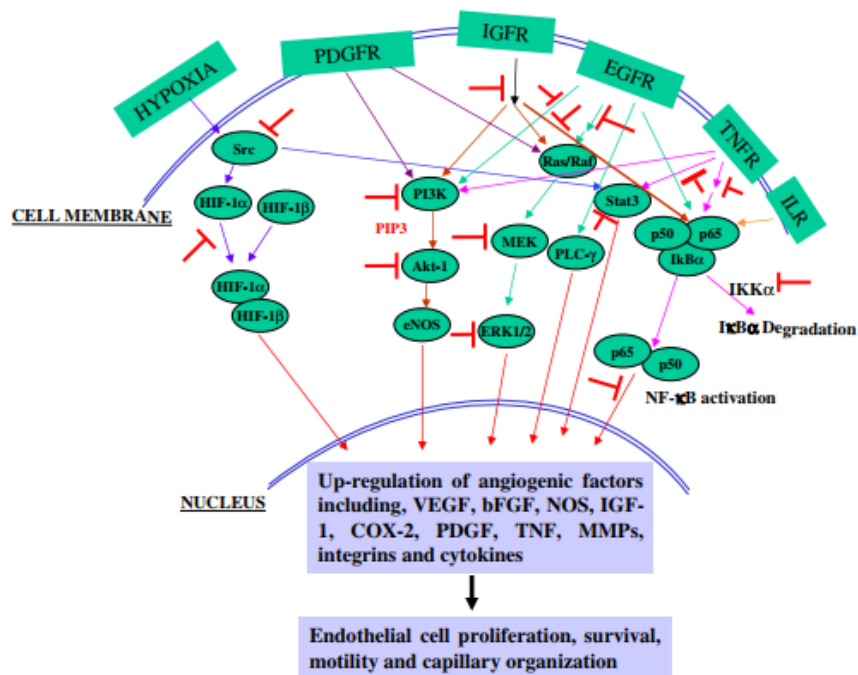


Figure 3. Signaling pathways of tumor angiogenesis (Bhat and Singh, 2008)

2.3 Angiogenesis and ulcerations

Ulcerations are open wounds that fail to heal and like regular wounds, they contain inflammatory cells, granulation tissue, exposed collagen and necrotic debris (Folkman et al, 1991). They also lack an epithelial covering and the granulation tissue is made up of capillary blood vessels, monocytes and fibroblasts. Folkman et al (1991) administered basic fibroblast growth factor (bFGF-C523) to rats with duodenal ulcers for 21 days and this stimulated an over nine-fold increase in angiogenesis and accelerated healing.

Non-steroidal anti-inflammatory drugs (NSAIDs) have been used to treat cancer, but the continued use of these drugs increases the occurrence and delayed healing of gastroduodenal ulcers (Hawkey, 1990). These drugs function by non-selectively inhibiting angiogenesis, a process that plays a key role in healing ulcers by stimulating granulation tissue maturation (Tarnawski et al, 1991; McGrath, 1985).

Gangrene is a skin ulcer that is resistant to common treatments (Asai et al, 2006). It occurs mostly in people with diabetes mellitus, a disease that causes microvascular disorders and an immunocompromised state that leads to gangrene (Asai et al, 2006). A study on genetically diabetic mice with gangrene showed that the healing site had reduced infiltration of monocytes and macrophages, as well as keratinocyte growth factor expression and vascular endothelial factor, which are increased in normal wound healing (Werner et al, 1994). In another study, Asai et al (2006) treated a 65-year-old man who had aggressive diabetic gangrene on his left foot with peripheral blood mononuclear cells and bFGF. The ulcer closed completely and no new ulcerations were observed thereafter.

NSAIDS cause ulcers because they block angiogenesis completely, therefore there is a need for selective inhibitors that will not block regulatory angiogenesis that maintains homeostasis ulcers (Hawkey, 1990). In order for gastroduodenal ulcers and gangrene to heal, angiogenesis must take place at the healing site. Gangrene is normally treated by excising the affected site; however, this does not always stop the disease (Asai et al, 2006). Therefore there is a need for better treatment of gangrene in the form of angiogenesis activators at the healing site.

2.4 Cyclooxygenase 2

COXs catalyse the conversion of arachidonic acid to prostaglandins (Menczer, 2009). The enzyme has two catalytic sites: the COX site, which converts arachidonic acid to endoperoxide PGG₂, and the peroxidase site, which converts PGG₂ to PGH₂ (Botting, 2006). There are two forms of COX: COX-1 is constitutive, is found in most tissues and maintains homeostatis, while COX-2 is inducible and functions in inflammation and tumor angiogenesis (Sano et al, 1995; Kujubu et al, 1993).

COXs produce prostaglandin PGG₂, which has been found to be higher in human colon tumor than in normal cells (Bennet, 1987). Furthermore, Sano et al (1995), showed that immunoreactive COX-2 was expressed in colorectal cancer cells, inflammatory mononuclear cells, vascular endothelial cells and fibroblasts, but immunoreactive COX-1 was expressed in extremely low amounts when histochemical analysis was performed. This indicates that COX-2, but not COX-1, is involved in abnormal angiogenesis, which leads to the progression of metastatic tumors.

Prostaglandins produced by COX-2 bind receptors and activate signaling pathways that control proliferation, invasion and angiogenesis of tumors (Wang & DuBois, 2006). Chang et al (2004) found that endomethacin, a COX-2 inhibitor, inhibited up-regulation of angiogenic regulatory genes, suggesting that prostaglandins release by COX-2 induce angiogenesis.

Gallo et al (2001) found elevated levels of prostaglandin E2 in tumor front zone compared to normal mucosa and a significant correlation between COX-2 expression and tumor vascularization, microvessel density and VEGF expression in head and neck cancer. Huang et al (2005) found the same results in gastric carcinoma, which means that COX-2 and VEGF are both involved in the same pathway to activate tumor angiogenesis.

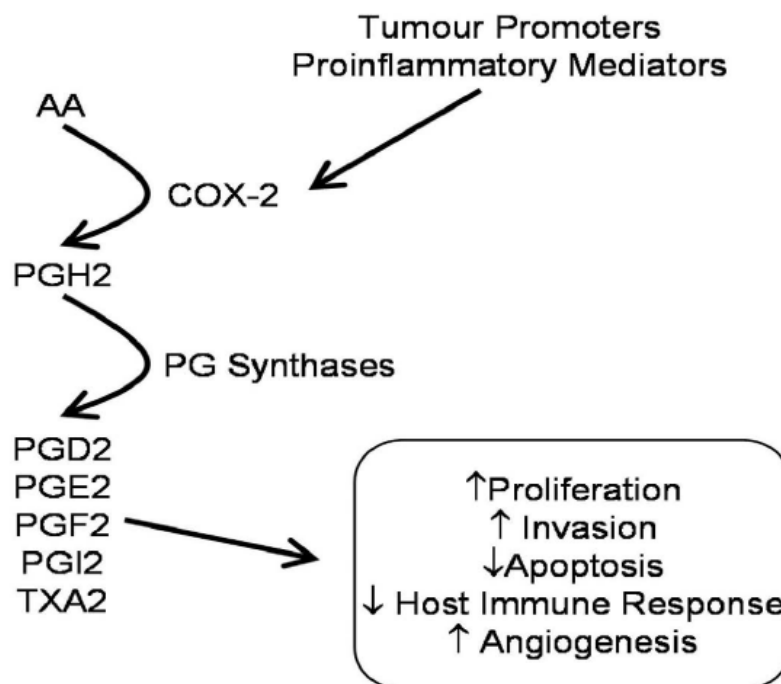


Figure 4. A Schematic presentation of the role of COX-2 in controlling proliferation, invasion, apoptosis and tumor angiogenesis (Toomey et al 2009).

2.5 Lipoxygenases

LOXs catalyze the oxidation of arachidonic acid (Wisastra & Dekker, 2014). LOXs are classified as 5-, 8-, 12- and 15-LOX, according to their preferred oxidation position on the fatty acids (Brash, 1999), and of these, the platelet type 12-LOX and 5-LOX are involved in tumor angiogenesis. Gao et al (1995) found elevated levels of platelet-type 12-LOX messenger ribonucleic acid (mRNA) in the metastatic prostate tumor cells. This led Nie et al (1998) to investigate the effects of this LOX further. They found that it stimulates tumor angiogenesis in prostate adenocarcinoma cells.

In 2000, Nie et al found that N-benzyl-N-hydroxy-5-phenylpentanamide (BHPP), a 12-LOX inhibitor, reduced endothelial cell proliferation, migration and the formation of tube-like structures in the Matrigel. These processes were stimulated by vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF); overexpression of 12-LOX stimulates endothelial cell migration and tube differentiation (Nie et al, 2000). VEGF and bFGF induce tumor angiogenesis. This therefore proves that 12-LOX is involved in the same pathways as these growth factors to induce angiogenesis.

Another LOX has been implicated in the development of tumors. Boado et al (1992) found more abundant 5-LOX transcripts in brain tumors than in normal bovine brain. Avis et al (2001) exposed breast cancer cells to 5(S)-hydroxyeicosa-6E,8C,11Z,14Z-tetraenoic acid (5-HETE), a product of 5-LOX, and found that this process stimulated growth, but when they added a 5-LOX selective inhibitor, growth was reduced and apoptosis increased. Gupta et al (2001) found higher levels of 5-LOX mRNA, up-

regulation of 5-LOX and higher levels of 5-HETE in prostate malignant tissue than in normal prostate tissue.

2.6 Nitric oxide synthase

NO is synthesized by NOS isoenzymes. These isoenzymes form NO from L-arginine with L-citrulline as the coproduct (Iyengar et al, 1987), and of these isoenzymes, endothelial NOS is involved in angiogenesis (Papapetropoulos et al, 1997). Gallo et al (1998) assessed the role of NO in angiogenesis and found that the levels of NOS were higher in tumor than in normal mucosa and that blocking NO production decreases tumor angiogenesis.

Cianchi et al (2003) found elevated levels of inducible nitric oxide synthase (iNOS) and VEGF in colorectal cancer, and higher activity of iNOS in metastatic tumors than in non-metastatic ones. Jadeski and Lala (1999) treated metastatic murine mammary adenocarcinoma cells with an iNOS inhibitor called L-NAME (N-nitro-L-arginine methyl ester) and it reduced tumor-induced neovascularization. Ziche et al (1997) found that L-NAME blocked VEGF-induced angiogenesis but not bFGF-induced angiogenesis in rabbits bearing corneal implants transfected with MCF-7 breast cancer cells. Furthermore, NOS and guanylate cyclase inhibitors blocked post-capillary endothelial cell migration and VEGF-induced growth, suggesting that VEGF and bFGF are involved in different pathways to induce tumor angiogenesis and that iNOS is only involved in VEGF pathways.

CHAPTER 3

3. MEDICINAL PLANTS

Long before the birth of western medicine, plants were the only source of medicine. Plants were used to treat many kinds of diseases. The most common use was to apply a decoction to open wounds to prevent microbial infection (Rios & Recio, 2005). Most of the pharmaceutical drugs used today are also derived from medicinal plants and over 80% of the world population still use medicinal plants (Kamboj, 2000) to treat various ailments. Medicinal plants are in demand in both developing and developed countries because of their safety, efficiency and lower side effects, in addition to the fact that they have been tried and tested for generations.

Spirostachys Africana Sond, *Synadenium cupulare* (Boiss.) L.C. and *Euphorbia tirucalli* are some of the medicinal plants that have traditionally been used for the treatment of ulcers, open wounds and cancer.

3.1. *Spirostachys Africana* Sond

Spirostachys Africana Sond, which is also known as tamboti, is a member of the family Euphorbiaceae. This plant species is found all over Southern Africa and Central Africa (Palgrave, 1990). In South Africa, the stem and bark is used to treat stomach pain, stomach ulcers, kidney complaints and cough and eye complaints (Mabogo, 1990; Cocks & Dolds, 2000; McGaw et al, 2000; Verschaeve et al, 2004; Mathabe et al, 2006). Crude extracts from the bark have antibacterial activity against bacteria that

cause diarrhea (Mathabe et al, 2006). However no studies have been done on its ethnomedicinal usage to treat ulcers. The processes of healing ulcers require angiogenesis.

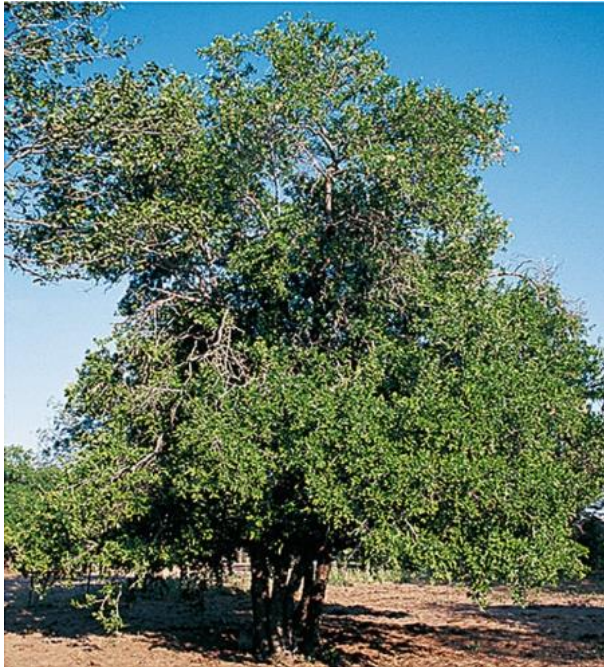


Figure 5. Picture of *Spirostachys Africana* Sond (<https://za.pinterest.com/mackboshoff/indigenous-sa-trees>)

3.2. *Synadenium cupulare* (Boiss.) L.C.

Synadenium cupulare (Boiss.) L.C., also known as *Euphorbia cupularis*, belongs to the Euphorbiaceae as well. It is found in South Africa and Mozambique. In South Africa, the dried leaves are used to treat headaches and flu. The latex is used to treat toothaches by placing it on a tooth cavity, and to treat infected wounds. The ethanolic leaf extract has been found to inhibit prostaglandin synthesis by inhibiting COX (Jager et al, 1996). Prostaglandins are mediated in inflammation pain sensation.



Figure 6. *Euphorbia cupularis* (http://www.ethnopharmacologia.org/recherche-dans-prelude/?plant_id=5738)

3.3 *Euphorbia tirucalli*

Euphorbia tirucalli, known as the pencil tree because of its pencil-like branches, is an evergreen succulent plant that grows up to 10 meters (Van Damme, 2001). This plant is so widely spread throughout the tropical regions of the world that its origin is unknown; however, some speculate that it originated from East Africa. It is endemic in Angola, Senegal, Ethiopia, Malawi, Zanzibar, Eritrea, Sudan, Rwanda, Uganda and Tanzania (Van Damme, 1989; Schmelzer & Gurib-Fakim, 2008). The plant has many medicinal uses in different parts of the world, of which some are the same.

In East Africa, the plant's latex is used to cure sexually transmitted infections, epilepsy, toothache, snake bites and hemorrhoids (Van Damme, 1989). In Malaysia, a poultice of the roots and stem is applied to nose ulcerations, hemorrhoids and swelling, while in Brazil, it is used against cancer, sarcomas, chancroids, tumors and warts (Duke, 1983).



Figure 7. Image of *Euphorbia tirucalli* (<https://kumbulanursery.co.za/plants/euphorbia-tirucalli-cultivar-rosea>)

The latex protects the plant from herbivores, as it is an irritant. It contains compounds such as phorbol esters, triterpenes, polyphenols and steroids (Mwine & Van Damme, 2011). The acetone extract of this plant's latex contains tumor-promoting compounds (Roe & Peirce, 1961). These findings conflict with the anti-cancer use of this plant by the Brazilians; however, the *E. tirucalli* used in the particular research project was not from Brazil, and Kinghorn (1979) found a compound in the latex of a Colombian *E. tirucalli* that was not present in the South African *E. tirucalli*. Fürstenberger and Hecker (1985) isolated the irritant and tumor-promoting compounds in the latex of a South African *E. tirucalli* and identified them as ingenane and tiglane type diterpene esters, as well as 4-deoxyphorbol, phorbol and ingenol.

Lin et al (2012) isolated euphol from the methanol extract of *E. tirucalli* found in Taiwan and found that this compound inhibits human gastric cancer cell growth by inducing ERK1/2-mediated apoptosis. This introduces the question whether the plant is carcinogenic or cures cancer. The literature points to both, and that similar species of *E. tirucalli* growing in different places have different latex compositions.

CHAPTER 4

4. MATERIALS AND METHODS

4.1. Plant collection and extraction

Plants were collected from their natural habitat in Pretoria and the Kruger National Park, Limpopo Province, South Africa during late summer and early autumn. The plants were washed with tap water, dried at room temperature and ground to a fine powder. A portion of the ground plant material was extracted with water (40 g/L) twice for 24 hours and another portion was extracted (40 g/L) sequentially with hexane, dichloromethane (DCM), DCM:MeOH (1:1 ratio), Methanol (MeOH) and ethyl acetate. The organic solvent filtrate was concentrated with a rotary evaporator (Buchi rotavapor R-100), while the water extract was dried using freeze dryer (Dura-Dry microprocessor) Percentage yield was calculated using this formula:

$$\% \text{yield} = \text{mass of extract} / \text{mass of powder}$$

4.2 Phytochemical analysis

Phytochemicals studies of the powdered plant parts were carried out using colorimetric assays described by Yadav & Agarwala, 2011.

Determination of phytosterols

The powdered plant material was weighed to yield 0.05 g. To the powdered material, 10 ml of chloroform was added, then 1 ml of concentrated H₂SO₄ was added with caution down the side of the test tube to 0.5 ml of chloroform extract. The appearance

of a reddish brown color in the chloroform layer was indicative of the presence of phytosterols.

Determination of pentose

Forty milliliters (40 ml) of distilled water was added to 2 g of powdered plant material. The mixture was then filtered. To the 2 ml filtrate, 2 ml of hydrochloric acid containing a little phloroglucinol was added. The mixture was then heated for five minutes. The formation of a red color was indicative of the presence of pentose.

Determination of tannins

Twenty milliliters (20 ml) of distilled water was added to 0.5 g of powdered plant material. The mixture was then boiled and filtered while it was still hot. The filtrate was treated with three drops of 0.1% of ferric chloride. A blue-black precipitate was indicative of the presence of tannins.

Determination of glycosides

Two milliliters (2 ml) of acetic acid was added to 0.5 g of powdered plant material. The mixture was then treated with one drop of 0.1% of ferric chloride, after which 1 ml of concentrated sulfuric acid was added to the mixture with caution. The appearance of a brown ring was indicative of the presence of deoxy sugars.

Determination of triterpenoids

One milliliter (1 ml) of chloroform was added to 2 mg of powdered plant material, then 3 ml of concentrated sulfuric acid was added to the mixture with caution. The formation of

an interface with reddish brown coloration was indicative of the presence of triterpenoids.

Determination of anthroquines

Twelve milliliters (12 ml) of 10% HCl solution was added to 1 g of powdered plant material and boiled for five minutes. The mixture was filtered and left to cool before 10 ml of chloroform was added to the filtrate. The chloroform layer was then placed in a clean test tube and 10 ml of 10% ammonia solution was added to this mixture. The mixture was shaken and the formation of a rose pink color in the top layer was indicative of anthroquines.

Determination of saponins

Five milliliters (5 ml) of distilled water was added to 0.5 g of powdered plant material, boiled and filtered, then 3 ml of distilled water was added to the filtrate. The mixture was shaken vigorously for five minutes. Frothing indicated the presence of saponins.

Determination of flavonoids

Ten milliliters (10 ml) of ethyl acetate was added to 0.5 g of powdered plant material and heated for three minutes, allowed to cool and then filtered, before 1 ml of dilute ammonia solution was added to 5 ml of the filtrate. The mixture was shaken. A yellow precipitate was indicative of the presence of flavonoids.

Determination of alkaloids

Two milliliters (2 ml) of 1% HCl was added to 0.2 g of powdered plant material and 1 ml of Meyer's reagent was added to the mixture, followed by 1 ml of Drangendorff reagent. The appearance of an organic precipitate indicated the presence of alkaloids.

4.3 Cell culturing

4.3.1 Cell culture conditions

The culture environment was maintained at 37°C in a humidified, concentrated CO₂ (5%) atmosphere. The cells were grown in DMEM media (Sigma, South Africa) supplemented with 10% FBS until 80% confluent.

4.3.2 Cell trypsinization and sub-culturing

Once MCF-7 cells had reached approximately 80% confluency in the flask, which was confirmed by microscopic analysis, they were trypsinized. Cells were rinsed with 10 ml of sodium phosphate buffer (PBS). Aliquots of 2 ml of warm (37°C) trypsin-EDTA solution were added for two minutes to detach the cells. After two minutes, the cells were checked for detachment under a microscope, then trypsin-EDTA was neutralized by adding equal amounts of complete medium. The cell suspension was centrifuged at 700 rpm for five minutes to obtain a cell pellet. The cell pellet (MCF-7 cells) was re-suspended in 4 ml fresh growth medium. An aliquot of 20 µl was removed for cell counting, to determine cell viability. The amount of cell suspension to be added to 10 ml

of complete medium to obtain a cell concentration of 1×10^5 cells/ml was calculated and added; thereafter the cells were plated in a 96-well plate and incubated at 37°C in a humidified 5% CO_2 atmosphere.

4.3.3 Cell viability count

Cell viability was determined using trypan blue staining solution: 20 μl of cell suspension was mixed with 20 μl of trypan blue staining solution in an Eppendorf, then 20 μl of the cell suspension was pipetted into a sealed hemocytometer and viable and dead cell concentrations were obtained using an automated cell counter (Invitrogen Countess II automated cell counter).

4.3.4 Cell plating

Following the concentration achieved above, the cells were diluted to the desired concentration (1×10^5 cell/ml) with supplemented media. Aliquots of 100 μl of the cell suspension containing 1×10^5 cells/ml were introduced into each well of a 96-well plate, after which 100 μl of growth medium was added. Next, the plate was incubated for 24 hours at 37°C and 5% CO_2 incubator to enable the cells to attach.

4.3.5 Assay

After a 24-hour incubation period, the medium in the plates was aspirated and the cells were treated with 100 μl of the crude extracts (in triplicate) according to the dilutions (100, 10 and 1 $\mu\text{g}/\text{ml}$) and 100 μl of media was added to make a final volume of 200 μl . These cells were then incubated for 48 hours at 37°C and 5 % CO_2 .

For test samples that showed inhibitory activity at 10 µg/ml and less, a series of five 10-fold dilutions of plant extracts ranging from 100 µg/ml to 0.01 µg/ml was used to determine the IC₅₀.

4.4 MTT assay

Cell growth was measured using the 3-[4, 5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay as described by Mosmann (1983). The MTT assay is a non-radioactive quantitative colorimetric assay used for *in vitro* measurement of the cytotoxicity and metabolic activity of cell cultures subjected to different culture conditions. It is based on the ability of viable cells to reduce the yellow water-soluble tetrazolium salt to a water-insoluble purple formazan product. Since this activity requires functional mitochondria, only viable cells can cleave the tetrazolium ring. The metabolic activity in the cells and the number of viable cells are directly proportional to the amount of formazan crystals formed.

4.4.1 Measuring cell viability

Once the 48-hour incubation was complete, 25 µl of sterile MTT (5 mg/ml in PBS) was added to each well and the plates were incubated for four hours at 37°C. The supernatant was aspirated from the wells and 100 µl of 100% dimethylsulfoxide (DMSO₄) was added to each well to dissolve the formazan crystals. The plates were gently shaken on a microtiter plate shaker for five minutes and then read immediately on a microtiter plate reader at 540 nm. For cell viability analysis, dose-response curves were plotted on bar charts with Microsoft Excel.

Table 1. A summary of the names and labels of samples that were evaluated for MCF-7 cell growth inhibition. *ND=Not done

Extract	<i>Euphorbia tirucalli</i>		<i>Spirostachys africana</i>			<i>Euphorbia cupularis</i>	
	Dried plant material	Fresh plant material	Barks	Stems	Leaves	Stem	Leaves
Hexane	ETD1	ETF1	TB1	TS1	TL1	ECB1	ECL1
DCM	ETD2	ETF2	TB2	TS2	TL2	ECB2	ECL2
DCM: MeOH	ETD3	ETF3	ND	ND	ND	ND	ND
MeOH	ETD4	ETF4	TB3	ND	TL3	ECB3	ECL3
Ethyl acetate	ND	ND	TB4	ND	TL4	ECB4	ECL4
Water	ETD5	ETF5	ND	ND	ND	ND	ND

4.5 Nitric oxide synthase activity

The Griess reagent system was used to measure the amount of NO produced by the cells after exposure to the plant extracts. The assay relies on a diazotization reaction that was first described by Griess. It detects nitrite (NO_2^-), a stable form of NO, in the culture media, which reacts with sulfanilamide and N-1-naphthylethylenediamine dihydrochloride (NED) under acidic (phosphoric acid) conditions. In this assay, the cell culture supernatant (100 μl) taken after 48-hour incubation of the cells with extracts was mixed with sulfanilamide (100 μl), incubated for 10 minutes at room temperature and then NED solution (100 μl) was added and incubated for 10 minutes. Thereafter, the absorbance was measured at 550 nm. The nitrite concentration was calculated from the standard curve using nitrite as a standard.

4.6 LOX activity assay

LOX activity was determined using the LOX activity assay kit from BioVision. In this assay kit, LOX converts its substrate into an intermediate that reacts with the probe, and

in doing so, generates a fluorescent product, which is measured on a spectrophotometer that can read fluorescence.

The cells (4×10^5) were homogenized with 100 μ l ice-cold LOX buffer and kept on ice for 10 minutes, followed by centrifugation at 10 000xg for 15 minutes. The cell lysate (10 μ l) was then mixed with 10 μ l plant extract (100, 10 and 1 μ g/ml), 20 μ l LOX substrate, 2 μ l LOX probe and 78 μ l of LOX buffer. The mixture was immediately placed on the fluorescence plate reader at an excitation wavelength of 500 nm and emission wavelength of 536 nm and the fluorescence was measured at 30-second intervals for 30 minutes. A standard curve was generated and a LOX inhibitor that came with the kit was used as a positive control. The experiment was performed in duplicate and repeated twice.

4.7 Cyclooxygenase-2 activity assay

Inhibition of COX-2 in MCF-7 cells was determined using the COX activity kit from Biovision. The cells (4×10^5) were homogenized with 100 μ l ice-cold LOX buffer and kept on ice for 10 minutes, followed by centrifugation at 10 000 Xg for 15 minutes. In each well, cell lysate (10 μ l) was mixed with 10 μ l plant extract (100, 10 and 1 μ g/ml), 2 μ l COX probe, 4 μ l diluted COX cofactor and 74 μ l COX assay buffer. The mixture was immediately placed on the fluorescence plate reader at an excitation wavelength of 536 nm and emission wavelength of 587 nm and the fluorescence was measured at 30-second intervals for 30 minutes. A standard curve was generated and a supplied COX-2 inhibitor was used as a positive control. The experiment was performed in duplicate and repeated twice. All the data was analyzed with Microsoft excel.

CHAPTER 5

RESULTS

5.1 Plant extraction

The powdered plant materials were extracted and the percentage yields were calculated. The percentage yields are shown in table 2 below. The methanol extract of *S. africana* bark had the highest yield of 39.9% and the lowest was the DCM extract of fresh *E. tirucalli* stems with a 0.12% yield. The dried plant material of *E. tirucalli* yielded more extracts than the fresh.

Table 2. Yield percentage of plant extracts after extraction with various solvents. [DCM= dichloromethane, DCM:MeOH= dichloromethane:methanol 1:1 ratio] *ND=Not done

	Hexane	DCM	DCM:MeOH	MeOH	Ethyl acetate	Water
<i>E. cupularis leaves</i>	10.72%	3.4%	ND	3.6%	0.66%	17.2%
<i>E. cupularis stem</i>	0.12%	7.6%	ND	11.9%	0.3%	4.5%
<i>S. africana leaves</i>	0.5%	0.7%	ND	21.1%	0.2%	ND
<i>S. africana bark</i>	5.9%	5.1%	ND	39.9%	5.9%	ND
<i>S. africana stem</i>	1.9%	0,02%	ND	ND	ND	ND
<i>E. tirucalli aerial parts (fresh)</i>	0.27%	0.12%	0.87%	0.21%	ND	0.53%
<i>E. tirucalli aerial parts (dry)</i>	1.9%	2.3%	6.3%	1.6%	ND	5.2%

5.2 Phytochemical analysis

The three plants, *Euphorbia tirucalli*, *Spirostachys Africana* and *Euphorbia cupularis*, were analyzed to determine the types of phytochemicals present in them. All three plants tested positive for the presence of tannins, glycosides and saponins. The bark of *S. africana* tested positive for all the phytochemicals except flavonoids and it was the only plant part that contained anthroquines.

Table 3. Phytochemicals present in *E. tirucalli*, *E. cupularis*, and *S. africana*, (+) means present and (-) means absent.

	<i>E. tirucali</i>	<i>E. cupularis</i>		<i>S. africana</i>		
Plant part	aerial parts	stems	leaves	stems	leaves	barks
Phytosterols	-	+	-	+	-	+
Pentose	-	+	-	-	-	+
Tannins	+	+	+	+	+	+
Glycosides	+	+	+	+	+	+
Triterpenoids	+	+	-	+	-	+
Anthroquines	-	-	-	-	-	+
Saponins	+	+	+	+	+	+
Flavonoids	-	+	+	-	-	-
Alkaloids	-	-	-	+	+	+

Secondary metabolites such as tannins, glycosides, saponins and flavonoids were detected in the powdered plant material of *Euphorbia Cupularis Boiss* leaves and in the stem phytosterols, pentose, tannins, glycosides, triterpenoids, saponins and flavonoids.

In the powdered plant material of *S. africana*, three parts of the plant were analyzed: leaves, stem and bark. In the leaf parts, triterpenoids, tannins, glycosides, saponins and

alkaloids were detected. In the stem tannins, phytosterols, glycosides, alkaloids, saponins and triterpenoids were detected.

Spyrostachys africana



A) Branches, (+) saponins **B)** Barks, (+) saponins **C)** Leaves, (+) saponins

Figure 9. Images showing color changes observed during phytochemical testing of *S. africana*. Frosting indicates the presence of saponins, A represents *S. africana* stems, B= barks and C= leaves.

Euphorbia cupularis



A) Stems, tannins **B)** Leaves, saponins **C)** Stems, pentose

Figure 8. Images showing color changes observed during phytochemical testing *E. cupularis*. Figure A shows the presence of tannins, B shows the presence of saponins in powdered leaves and C shows the presence of pentose. A blue-black precipitate indicates tannins, a reddish color showed the presence of pentose.

Euphorbia tirucalli



A) saponins

B) tannins

C) triterpenoids

Figure 10. Images showing color changes observed during phytochemical testing of *E. tirucalli*. Figure A shows the presence Saponins, B shows the presence of tannins and C shows the presence of triterpenoids. Triterpenoids are indicated by the presence of a brown interphase.

5.3.1 Cell growth inhibition of MCF-7 breast cancer cells

Extracts of the three plants, *S africana* (tamboti), *E tirucali* and *E cupularis*, were tested for their cell growth inhibitory effect on MCF-7 cells using MTT. Extracts were tested in triplicate in concentrations of 100, 10 and 1 $\mu\text{g/ml}$. Most extracts exhibited dose-dependent cell anti-proliferative activity. Most of the extracts showed 100% inhibition at 100 $\mu\text{g/ml}$, while some showed inhibition higher than 50%. The concentration at which a plant extract inhibits 50% cell growth is known as IC_{50} . A summary of the results is shown in table 4 below. An IC_{50} that is equal to or less than 10 $\mu\text{g/ml}$ was considered good activity, whereas higher than 10 $\mu\text{g/ml}$ was considered poor.

The national cancer guidelines state that an IC_{50} less than 30 $\mu\text{g/ml}$ is considered active (Suffness and Pezzuto, 1990), hence we decided on extracts with an IC_{50} of 10

µg/ml or lower. Extracts that showed IC₅₀ of 10 or lower were then tested in six serial 10-fold dilutions.

Table 4. Cell growth inhibitory activity of extracts of different plant parts of the three plants; *S. africana*, *E. tirucali*, and *E. cupularis* on MCF-7 cells. X= IC₅₀ higher than 10 µg/ml and ND= not done

Plant	Plant part	Extract and Inhibitory activity				
		Hexane	DCM	DCM:MeOH	Methanol	Ethyl Acetate
<i>Spirostachys africana</i>	Stems	x	IC ₅₀ <10 µg/ml	x	x	x
	Leaves	IC ₅₀ <10 µg/ml	IC ₅₀ <10 µg/ml	ND	IC ₅₀ <10 µg/ml	IC ₅₀ <1 µg/ml
	Bark	IC ₅₀ <10 µg/ml	IC ₅₀ <1 µg/ml	ND	x	x
<i>Euphorbia tirucali</i>	Stems (dried)	IC ₅₀ <10 µg/ml	IC ₅₀ <10 µg/ml	Proliferative	Proliferative	ND
	Stems (wet)	x	x	x	x	ND
<i>Euphorbia cupularis</i>	Leaves	IC ₅₀ ≤10 µg/ml	IC ₅₀ <10µg/ml	ND	x	x
	Stems	IC ₅₀ ≤10 µg/ml	IC ₅₀ ≤10µg/ml	IC ₅₀ ≤10 µg/ml	x	IC ₅₀ ≤10 µg/ml

5.3.1.1 Cell growth inhibition of MCF-7 cells by extracts of *S. africana*

The extracts of *S. africana* obtained from the leaves, stems and bark were tested against MCF-7. Of the four extracts of the bark (hexane, dichloromethane, methanol and ethyl acetate), hexane and dichloromethane extracts exhibited anti-proliferative activity at IC₅₀ values of 10 µg/ml or lower. All leaf extracts showed good activity with

IC₅₀ of around 10 µg/ml or lower. The DCM extract of the stem showed activity higher than 40% at 1 µg/ml, the results are shown in figure 11.

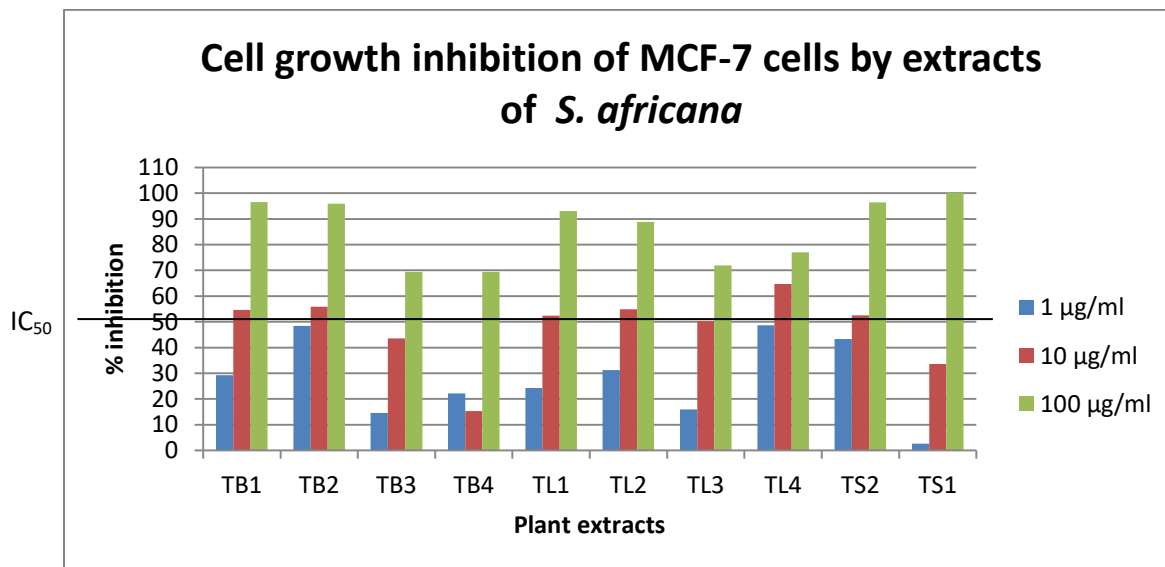


Figure 11. Cell growth inhibition of MCF-7 cells by *S. africana* plant extracts. TB= bark extracts, TL= leaf extracts, TS= stem extracts, 1= hexane, 2= DCM, 3=MeOH, and 4= ethyl acetate extract

A series of five 10-fold dilutions of plant extracts ranging from 100 µg/ml to 0.01 µg/ml was used to determine the IC₅₀ of the extracts better. The graph of *S. africana* leaf extract revealed that DCM extract (TL2) had the lowest IC₅₀ of **0.09 µg/ml**. The extract inhibited nearly 40% of cell growth even at the lowest concentration of 0.001 µg/ml. The hexane and ethyl acetate (TL1 and TL4) achieved IC₅₀ values of 4 and 6 µg/ml respectively, while the highest IC₅₀ was exhibited by methanol (TL3), with an IC₅₀ of 8 µg/ml (figure 12). The leaf extract were considered highly active.

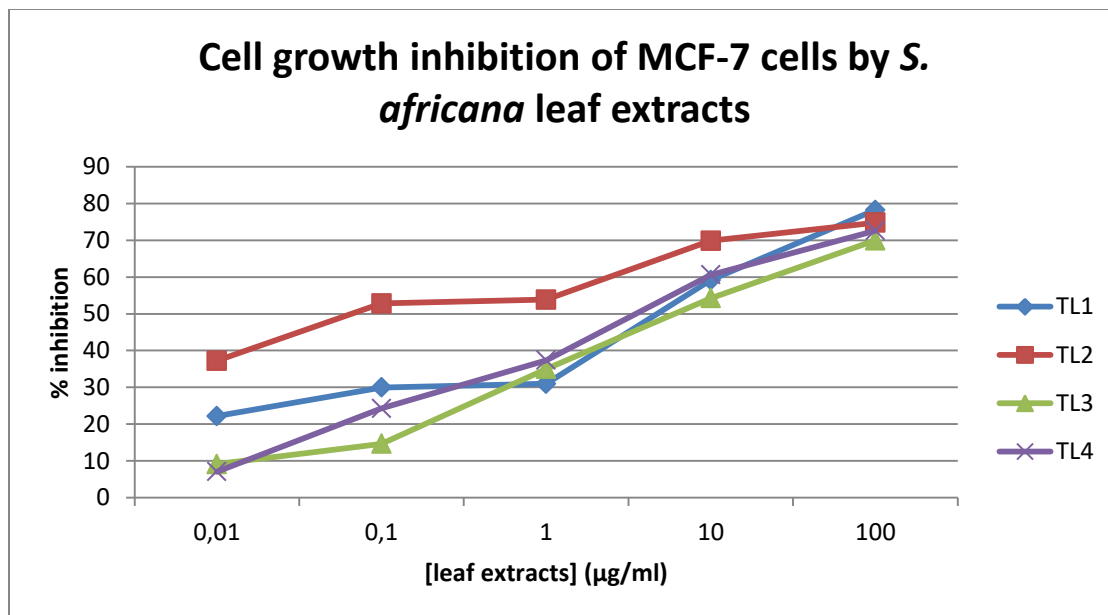


Figure 12. Cell growth inhibitory of MCF-7 cells by *S. africana* leaf extracts tested at concentration range of 100 -0.001 µg/ml (full 10-fold dilutions).

The three extracts of the stem and bark (TS2, TB1 and TB2) were selected for full dilution based on activity observed from the 3-dilution results.

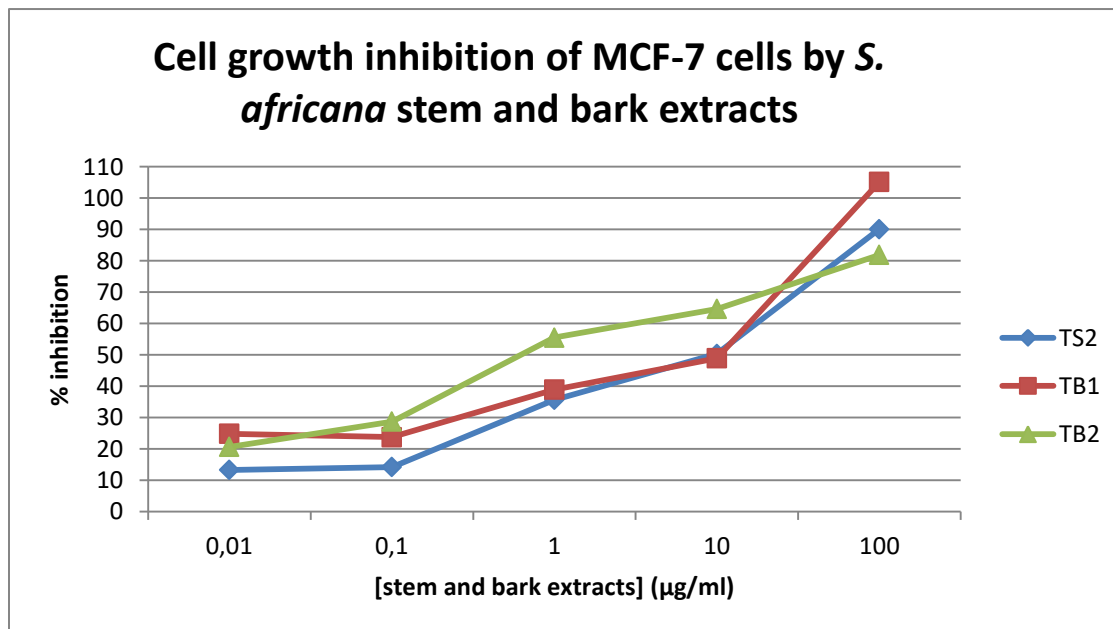


Figure 13. Cell growth inhibition of MCF-7 cells by *S. africana* stem and bark extracts tested at concentration range of 100 - 0.001 µg/ml (full 10-fold dilutions). TS2 = DCM stem extract,, TB1 = hexane bark and TB2= DCM bark extract.

Figure 13 shows the growth inhibition activity and IC₅₀ values of selected stem and bark extracts. The DCM extract of the bark had the lowest IC₅₀ of **0.8 µg/ml**, while the hexane bark (TB1) and DCM stem (TS2) extracts both had an IC₅₀ of 11 µg/ml.

5.3.1.2 Cell growth inhibition of MCF-7 cells by *E. tirucalli*

The four extracts of *Euphorbia tirucalli*; hexane, DCM, DCM:methanol (1:1) and methanol, were tested against MCF-7 cells. The fresh (wet) and dried plant material were extracted. Only the dried extracts of hexane and DCM had an IC₅₀ < 10 µg/ml. These extracts, exhibited decreased activity at a concentration of 1 µg/ml. The DCM:MeOH and MeOH extracts of the stems showed proliferation of MF-7 cells. Interestingly, the highest proliferative activity was observed at the lowest concentration. All these activities were only observed in dried plant material, the results are shown in figure 14 below.

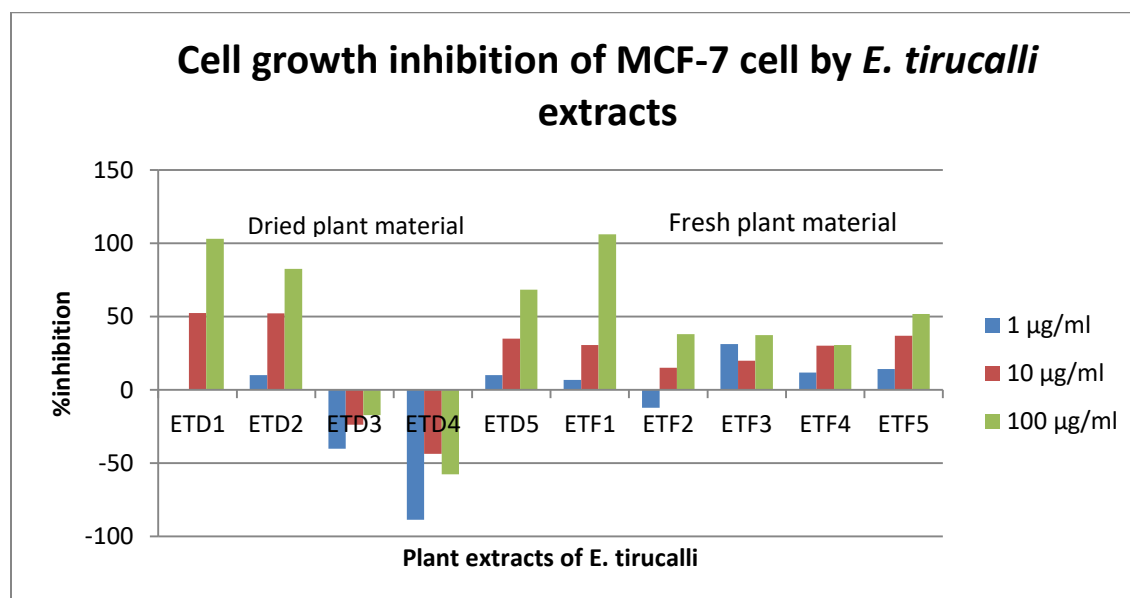


Figure 14. Cell growth inhibition of MCF-7 by *Euphorbia tirucalli* extracts. ETD= dried plant material, ETF= fresh plant material. 1= hexane, 2= DCM, 3= DCM:MeOH, 4= MeOH, 5= water extracts.

The plant *E. tirucalli* yielded the lowest number of extracts that met the criteria. The hexane and DCM of the dried parts had a similar IC₅₀ of 9.5 µg/ml. These were selected for full range dilutions to obtain the exact IC₅₀ values. Results are shown in Figure 15.

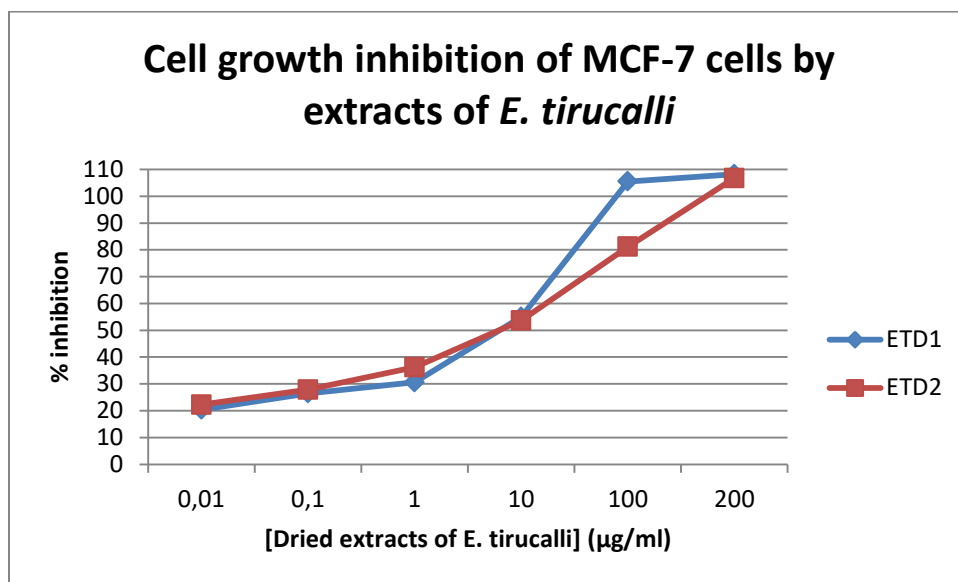


Figure 15. Cell growth inhibition of MCF-7 cells by *E. tirucalli* extracts tested at concentration range of 100 -0.001 µg/ml (full 10-fold dilutions)

5.3.1.3 Cell growth inhibition of MCF-7 cells by *E. cupularis*

The leaf and stem extracts of *Euphorbia cupularis* were analyzed for their growth inhibitory activity. The DCM leaf extract as well as hexane and DCM extracts of the stems had the highest activity, with an IC₅₀ below 1 µg/ml.

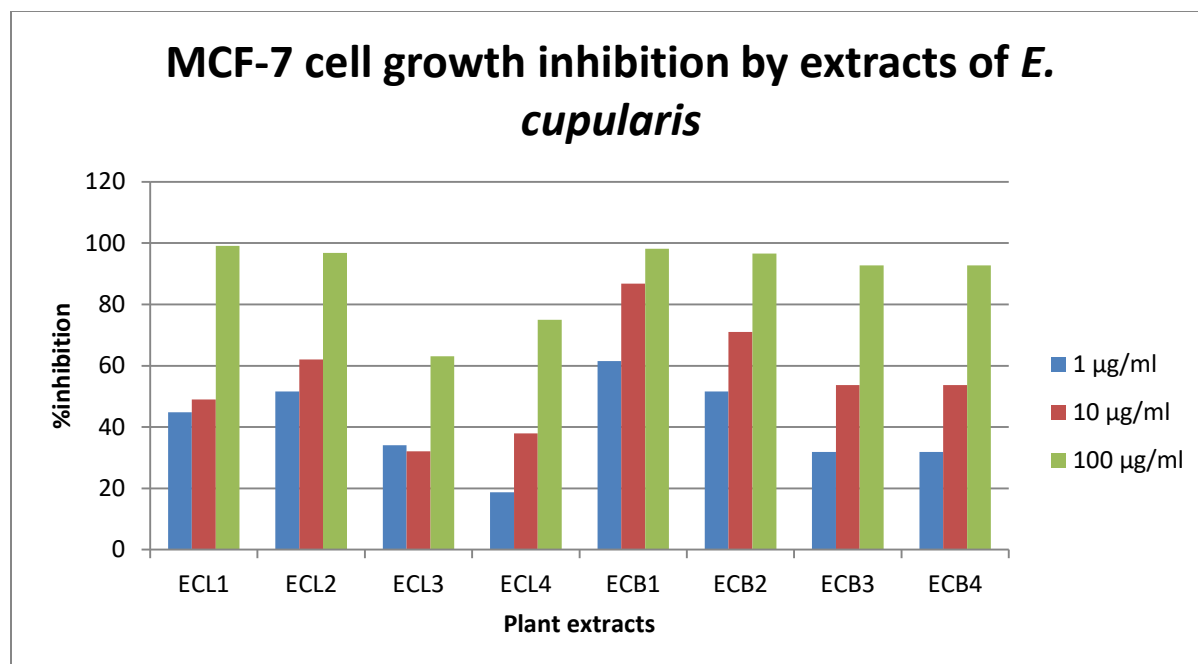


Figure 16. Growth inhibition of MCF-7 cell line by *Euphorbia cupularis* extracts; ECL represents the leaf extract while ECB represents the stems. 1=hexane, 2=DCM, 3=methanol and 4= ethyl acetate

The extracts that had an IC_{50} below or equal to 10 µg/ml and those that showed proliferative activity were then taken further in the studies to determine selectivity and enzyme inhibition studies.

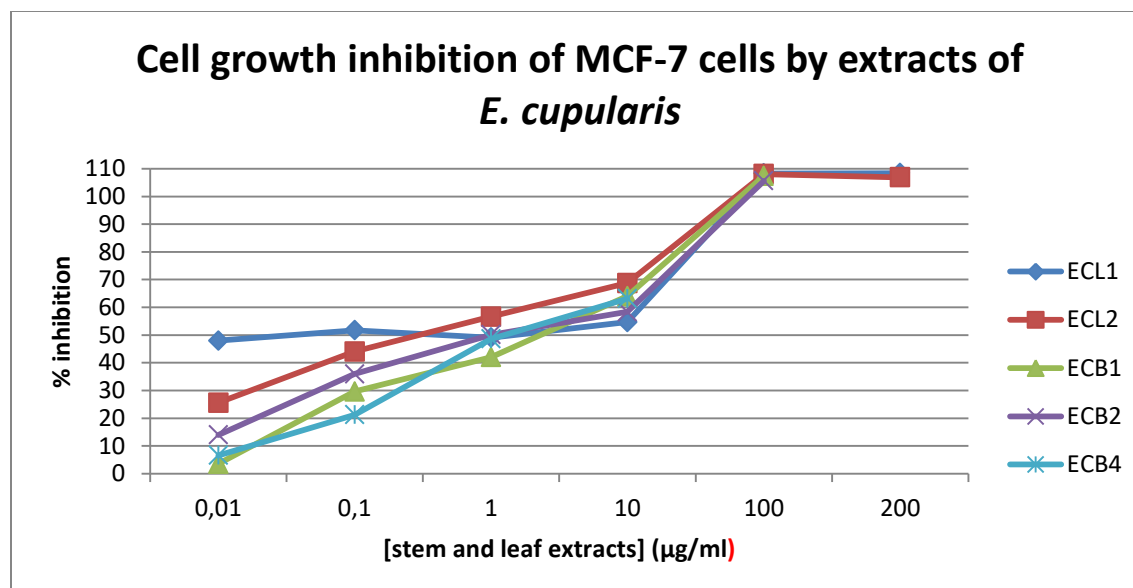


Figure 17. Cell growth inhibition of MCF-7 cells by *E. cupularis* extracts tested at concentration range of 100 -0.001 µg/ml (full 10-fold dilutions). ECL= hexane leaf extract, ECL2= DCM leaf, ECB1= hexane stem, ECB2= DCM stem, and ECB4= ethyl acetate stem extract.

Figure 17 shows the growth inhibitory activity of the *E. cupularis* MCF-7 cell line. The selected leaf extracts had the lowest activity and the hexane (ECL1) extract showed cytostatic behavior from a concentration of **0.01 µg/ml** to 1 µg/ml with about 50% growth inhibition maintained in that range of concentration. The IC_{50} was determined as **0.6 µg/ml**, which is the same as that of DCM (ECL2). The ethyl acetate (ECB4) and DCM (ECB2) stem extract had a slightly similar IC_{50} of 1 µg/ml and 1.2 µg/ml respectively. The hexane stems extract exhibited the highest IC_{50} of 5 µg/ml.

5.3.2 Selectivity of extracts for breast cancer cells

The extracts that had an IC_{50} below or equal to 10 µg/ml and those that showed proliferative activity were studied further to determine their selectivity for the breast

cancer cell line. The selected extracts were screened for inhibitory activity against the breast normal cell line MCF-10A, and CHO cell line.

Spirostachys africana leaf extracts showed low anti-proliferation against normal breast cells compared to breast cancer cells. The hexane extract (TL1) that had the highest activity against cancer cells showed the least activity against normal breast cells. Ethyl acetate extract (TL4) also had good selectivity, meaning that *S. africana* leaves selectively inhibited breast cancer cell growth.

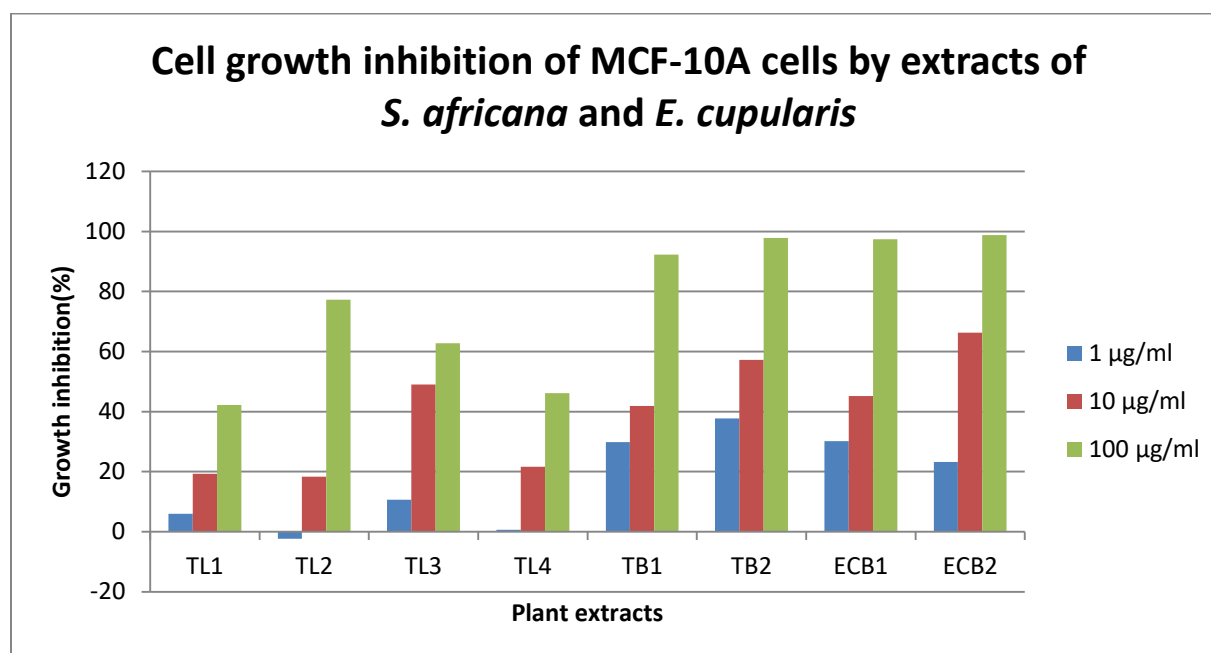


Figure 18. Cell growth inhibition of MCF-7 cells by extract of tamboti leaves and *E. cupularis* stems. TL= *S. africana* leaf extracts, TB= *S. africana* bark extracts and ECB= *E. cupularis* stem extracts. 1=hexane, 2=DCM, 3=methanol and 4= ethyl acetate

Other extracts were tested on the CHO cells instead of the normal breast cells (as the normal breast cells grew slowly and required costly growth supplements). The *E. cupularis* DCM leaf extract (ECL2) showed most selectivity, with the highest

concentration at only 11%. This was followed by the stem extracts of the same plant, with the ethyl acetate extract (ECB4) showing 49% growth inhibition at 100 µg/ml.

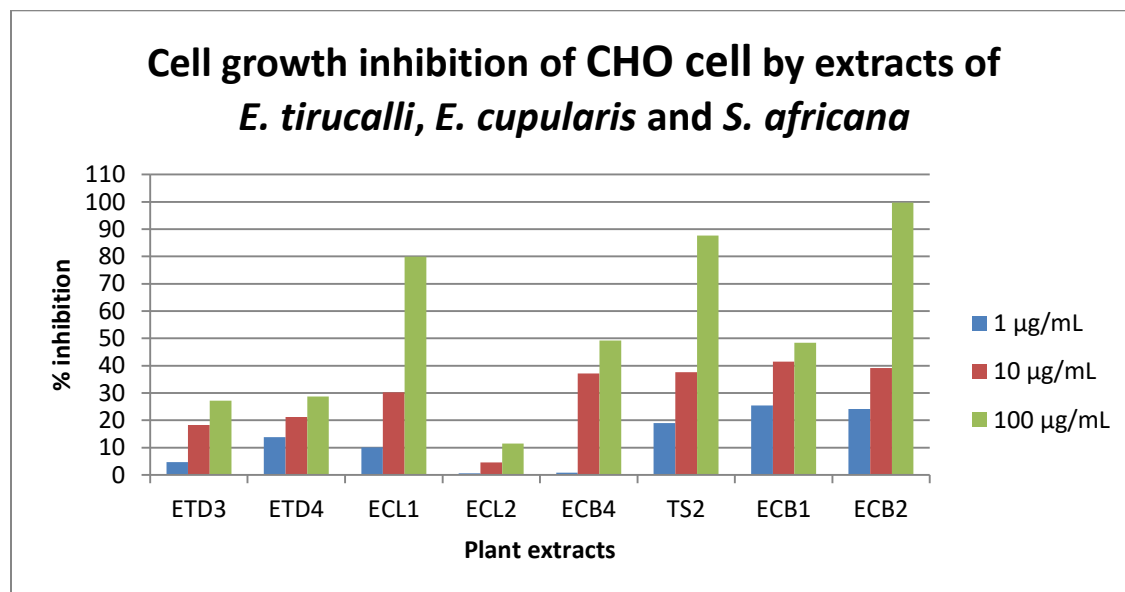


Figure 19. Cell growth inhibition of CHO cells by extracts of *E. tirucalli* (ETD3= DCM:MeOH and ETD4= MeOH), *E. cupularis* leaves (ECL1= hexane, ECL2= DCM extracts) and stems (ECB1= hexane, ECB2 DCM, ECB4 ethyl acetate extract), and *S. africana* stems (TS2= DCM extract).

The selectivity index (SI) was then calculated using a method described by Badisa et al (2009). An SI of less than 2 is regarded as not selective, and in the extracts tested only TL3 had a selective index of 1.8, which makes it unselective. The highest SI was 688 from TL2.

Table 5. Selectivity index of selected extracts

Extract	IC₅₀ in normal breast cells or CHO cells	IC₅₀ in cancer cells	Selectivity Index (SI)
TL1	122	4	30,50
TL2	62	0,09	688,88
TL3	15	8	1,88
TL4	110	6	18,33
TS2	20	11	1,82
TB1	49	11	4,45
TB2	7	0,8	8,75
ECL1	30	0,6	50,00
ECL2	150	0,6	250,00
ECB1	20	5	4,00
ECB2	7	1,2	5,83
ECB4	105	1	105,00

The extracts that showed considerable anti-proliferative activity and those that showed proliferative behavior were taken further to understand their mode of action through determining their effects on LOX, COS and NOS enzymes.

5.4 Enzyme activity assays

5.4.1 NOS activity

NOS activity was determined by measuring the amount of NO present in the culture media after incubating the cells with the plant extract for 24 hours. A standard curve (figure 20) was created and used to determine the concentration of NO, by determining the levels of nitrite, a stable form of NO.

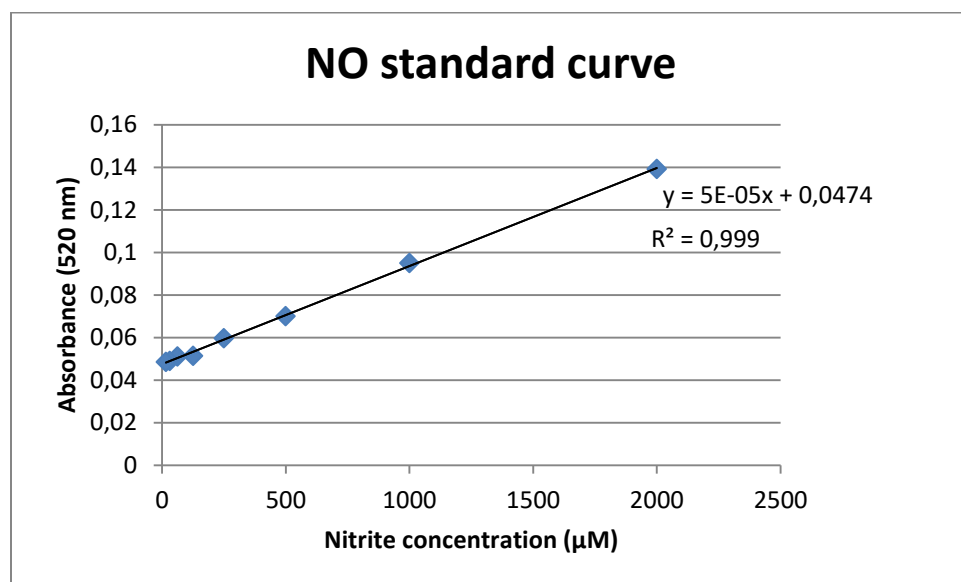


Figure 20. Nitrite standard curve

Figure 21 below shows the activity of NOS against selected extracts. These results show that the levels of NO increase with the concentration of extracts.

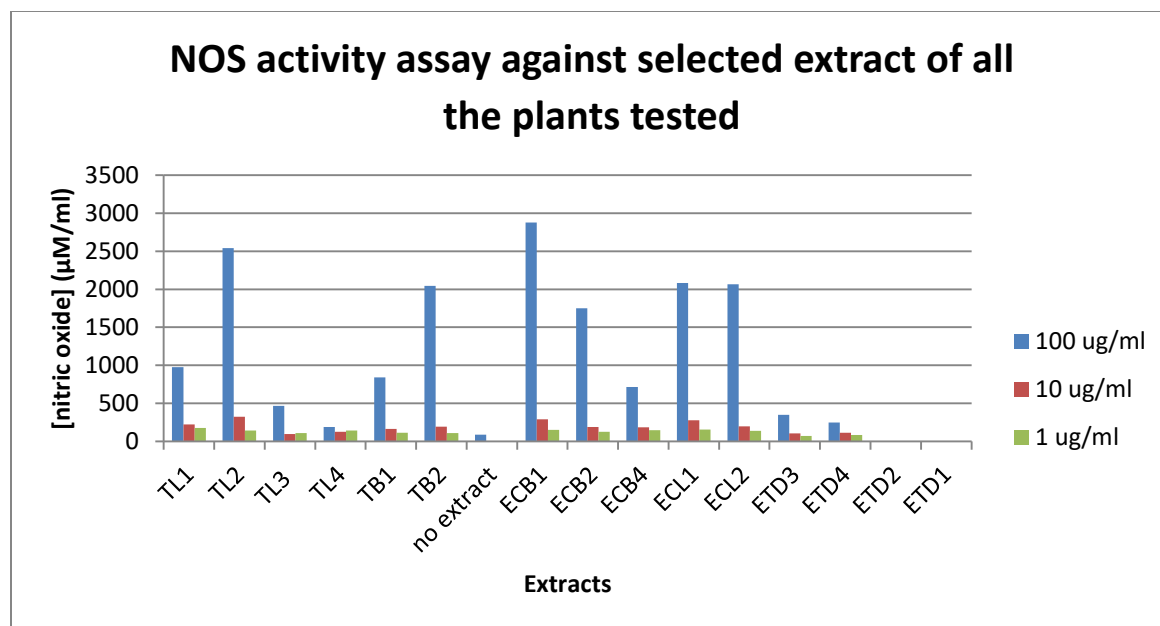


Figure 21. Effects of selected extracts of *S. africana*, *E. tirucalli*, and *E. cupularis* on NOS activity. TL= *S. africana* leaf extracts (1= hexane,2= DCM, 3= MeOH, and 4= ethyl acetate), TB= *S. africana* bark extracts (1= hexane, 2= DCM), ECB= *E. cupularis* stem extracts (1=hexane, 2= DCM, 4= ethyl acetate), ECL= *E. cupularis* leaf extracts(1= hexane, 2= DCM), ETD= *E. tirucalli* dried extracts(1= hexane, 2= DCM, 3= DCM:MeOH, 4= MeOH) and no extract= untreated cells.

5.4.2 Cyclooxygenase-2

COX-2 is the inducible isoform of COX; when induced it produces prostaglandins that induce inflammation and tumor angiogenesis (Chang et al, 2004). In this study, we determined the effects of the selected extracts on COX-2 using the COX activity assay kit from Biovision. The inhibitory effects of the extracts were determined in a kinetic study, as shown with the relative fluorescence units (RFU) per time graphs below. From the kinetic graphs, enzyme activity was calculated by deducing the gradient and determining the concentration of the product using the standard curve.

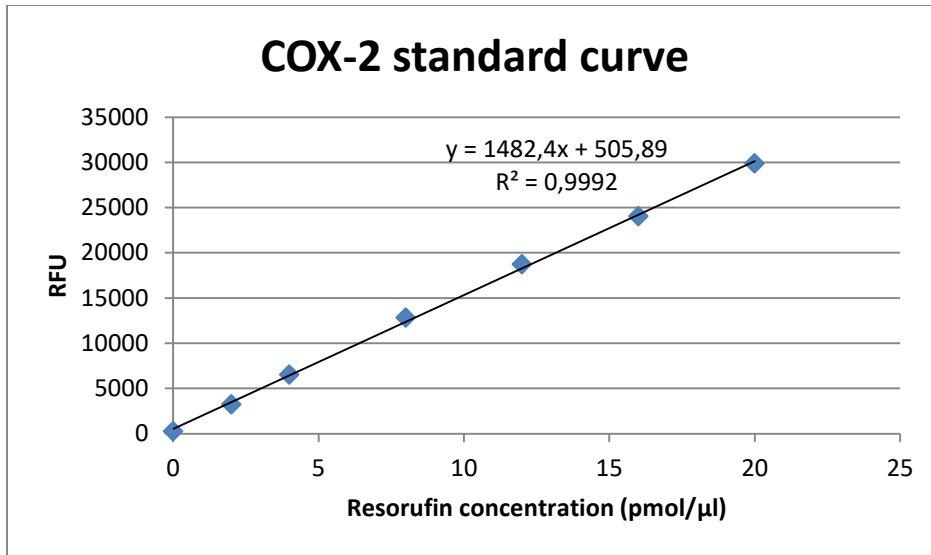


Figure 22. COX-2 standard curve

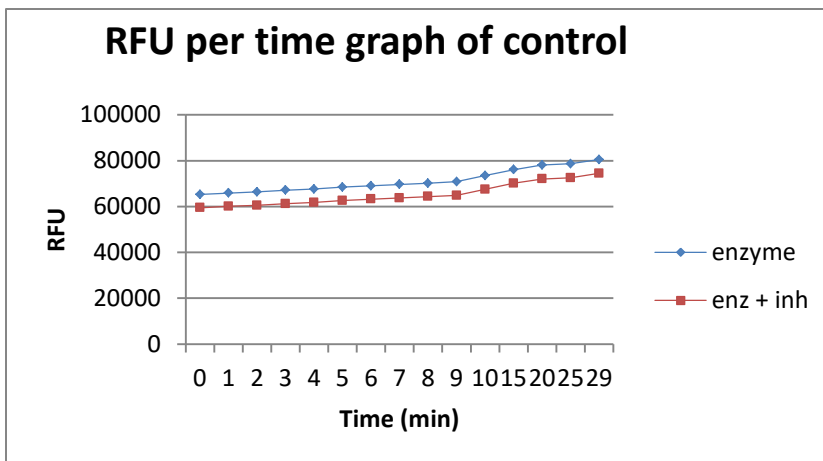


Figure 23. Enzyme kinetic activity of COX enzyme with (Enz + inh) and without a commercial inhibitor (enzyme).

Figure 23 shows the kinetic activity of COX control enzyme with and without a positive control inhibitor. The enzyme has higher activity than the enzyme inhibitor and both systems follow a similar trend over time. The behavior of the enzyme does not seem to be altered during inhibition; only the amount of product formed is altered. Thus, in the presence of an inhibitor, less product formation is observed.

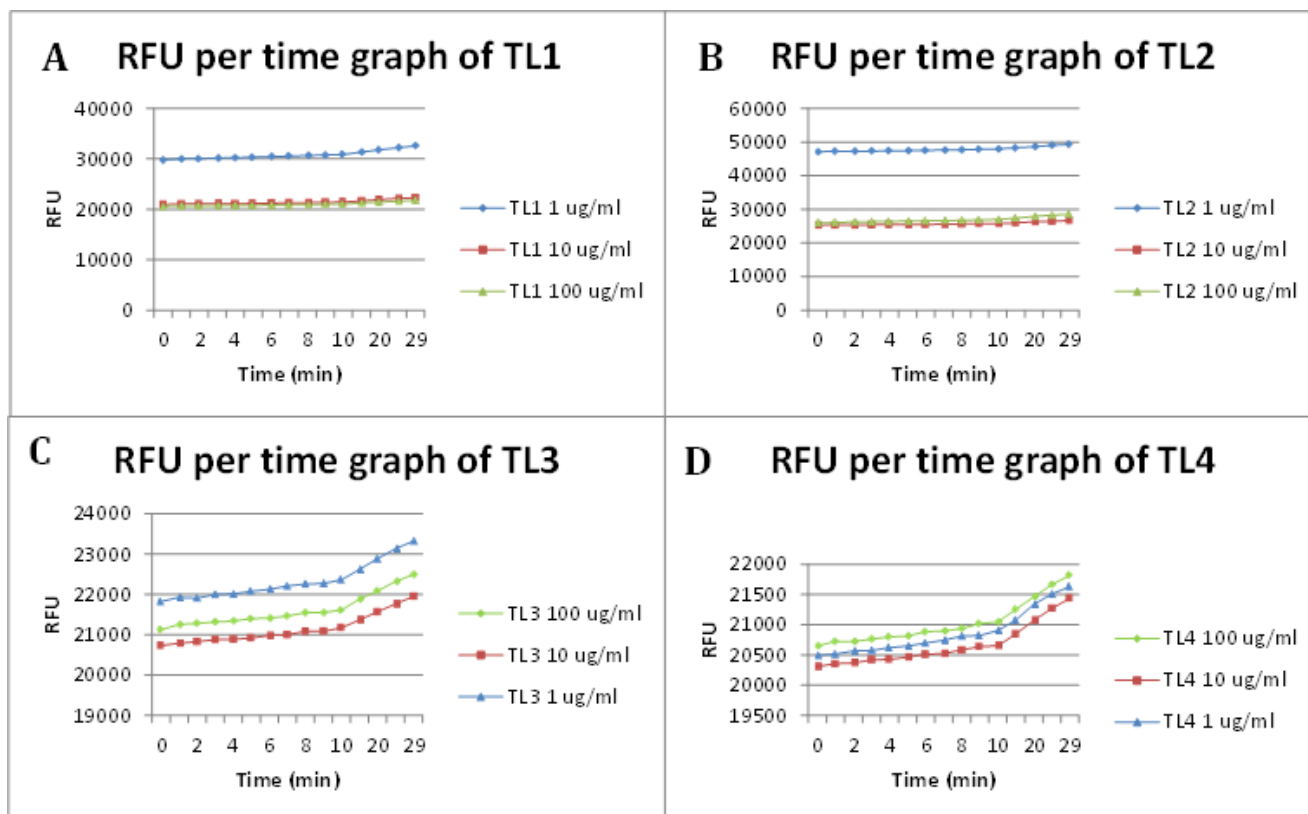


Figure 24. The graphs show the effect of leaf extracts of *S. africana* on activity of COX-2 over time. Figure A shows the effect of hexane leaf extract, B is DCM leaf extract, C is the methanol leaf extract, D is the ethyl acetate leaf extract and E shows the hexane bark extract.

The same behavior was noticed with the enzyme isolated from MCF-7 cells, where concentration-dependent inhibition was observed. Figure 24 shows the kinetic behavior of COX against the leaf extracts from *S. Africana*. The non-polar extracts, (TL1 and TL2) have similar activity at concentrations 10 and 100 μ g/ml.

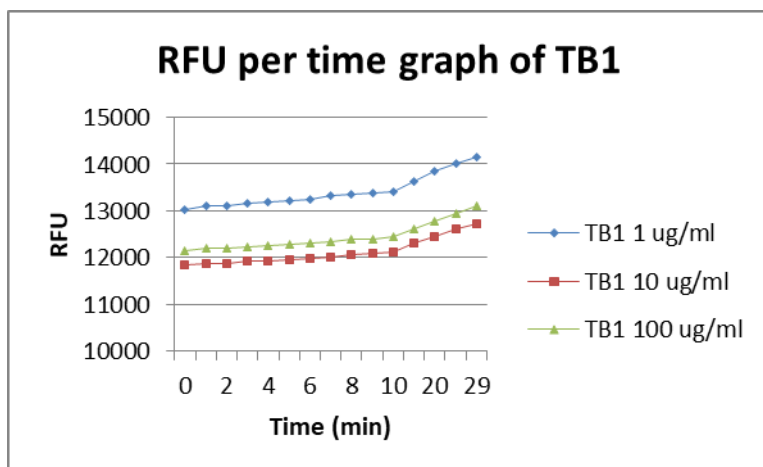


Figure 25. The graphs show the effect of *S. africana* hexane bark extract on activity of COX-2 over time.

Hexane bark extract of *S. africana* induced a slightly similar activity at 100 and 10 $\mu\text{g/ml}$, with the highest inhibitory activity at 10 $\mu\text{g/ml}$.

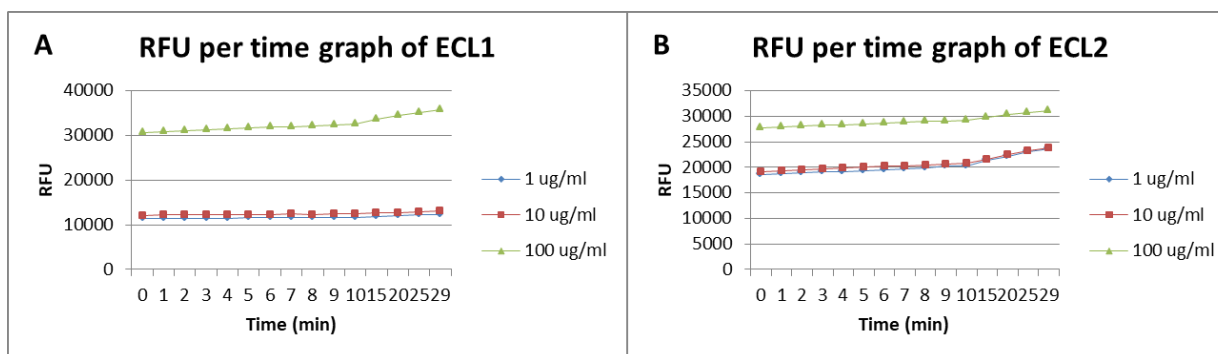


Figure 26. The graphs show the effect of *E. cupularis* leaf extracts on activity of COX-2 over time. Figure A shows the effect of DCM extract and B shows that of hexane extract.

However, other extracts showed a different trend; for example, the hexane and DCM Extracts of *E. cupularis* leaves (ECL1 and 2) showed an increase in activity with increasing extract concentration, but there was no distinguishable activity between concentrations 1 and 10 $\mu\text{g/ml}$.

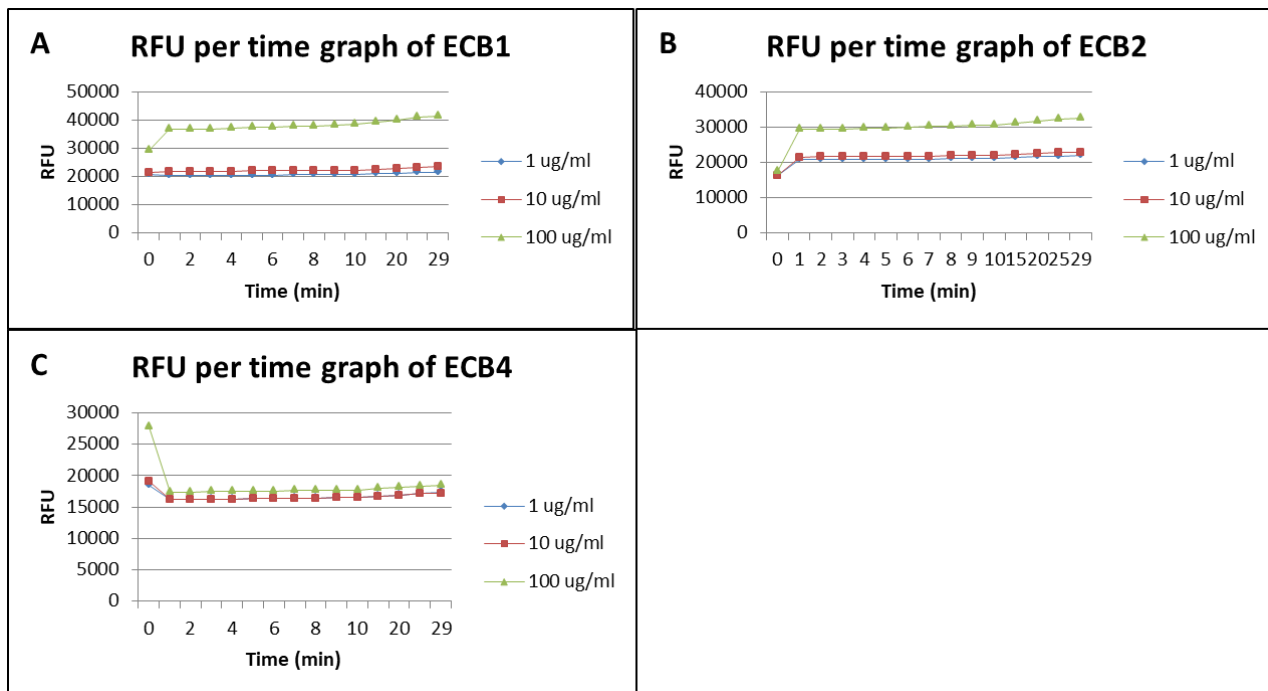


Figure 27. The graphs show the effect of *E. cupularis* stem extracts on activity of COX-2 over time. Figure A shows the effect of hexane extract, B shows the DCM extract and C shows ethyl acetate extract.

Another interesting trend was observed with ethyl acetate extract of *E. cupularis* stems (ECB4): the activity decreased rapidly in the first minute and then increased steadily for 20 minutes, the results are shown in figure 27.

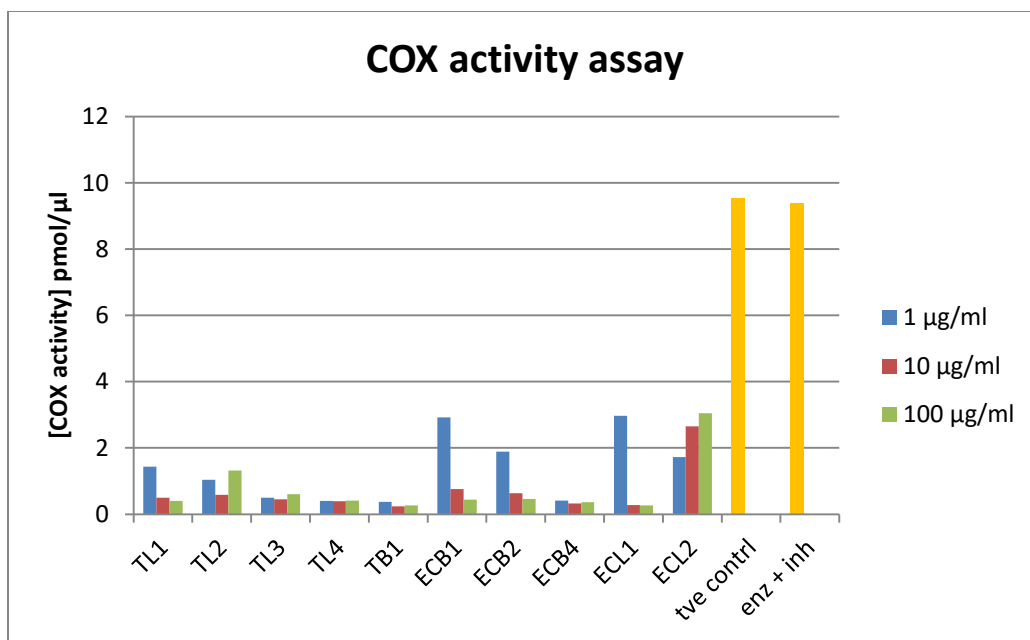


Figure 28. Activity of COX-2 against selected extracts, where the tve control represents positive control, a pure enzyme supplied, and enz + inh is the pure enzyme with celecoxib (COX-2 inhibitor) added.

Figure 28 shows activity of COX-2 against the selected extracts. This activity was determined from the kinetic studies shown above. The results show that COX-2 was inhibited in a concentration-dependent manner and TB1 was most active. The DCM extract, however, showed increasing enzyme activity with increasing extract concentration, while the ethyl acetate of *S. africana* and *E. cupularies* showed no distinguishable concentration activity. Compared to celecoxib, a known inhibitor of COX-2, the extracts were most active.

5.4.3 Lipoxygenase activity

The effect of the plant extracts on LOX was determined using a kinetic study. The RFU time graphs are shown below. The results show concentration-dependent inhibition of LOX by all the plant extracts. LOX activity was determined in the same manner as COX activity. The standard curve is shown below.

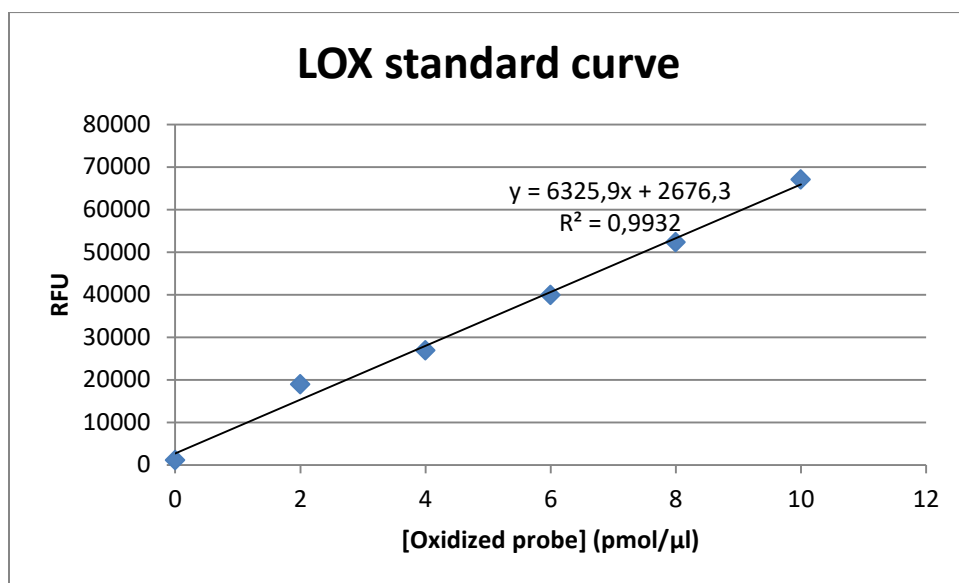


Figure 29. LOX standard curve

The kinetic characteristics of the controls are shown in figure 29 below. The graph shows that the activity of the LOX from MCF-7 lysate is more than that of the pure enzyme (PC) supplied; however, when a known inhibitor is added the LOX lysate becomes inactive.

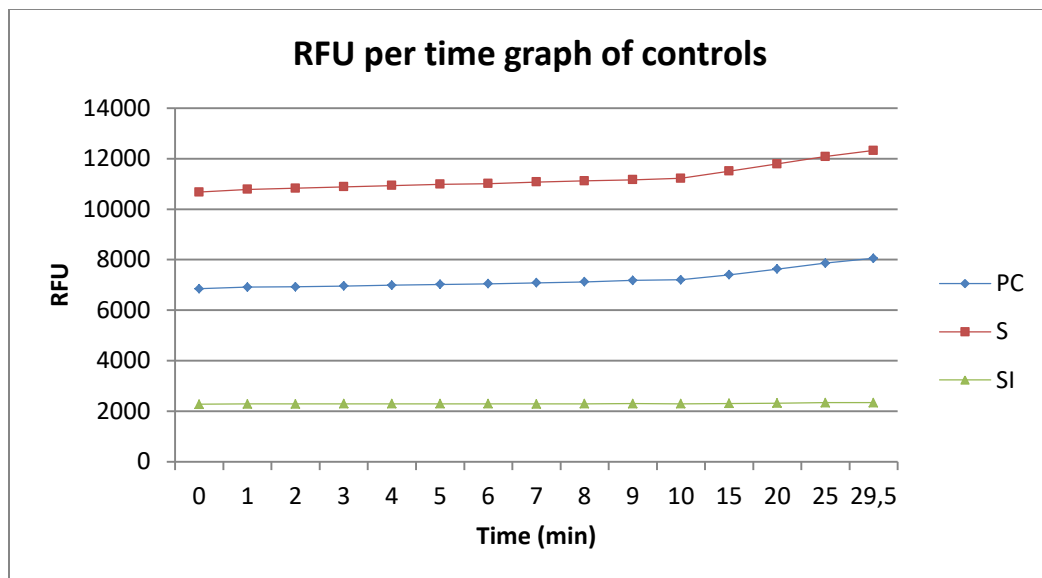


Figure 30. Kinetic activity of LOX enzyme. PC= Positive control (Pure enzyme obtained commercially), S= MCF7 untreated cells, SI=Inhibitor added to MCF-7 cells (inhibitor obtained commercially). High RFU is indicative of high LOX activity.

As shown in figure 30, MFC-7 cells showed highest LOX activity overtime, followed by the Positive control, which was a pure LOX enzyme obtained commercially. The results showed that the commercial inhibitor did inhibit the activity of LOX. The result proof that the assay worked and the experiments with extract can be regarded as credible.

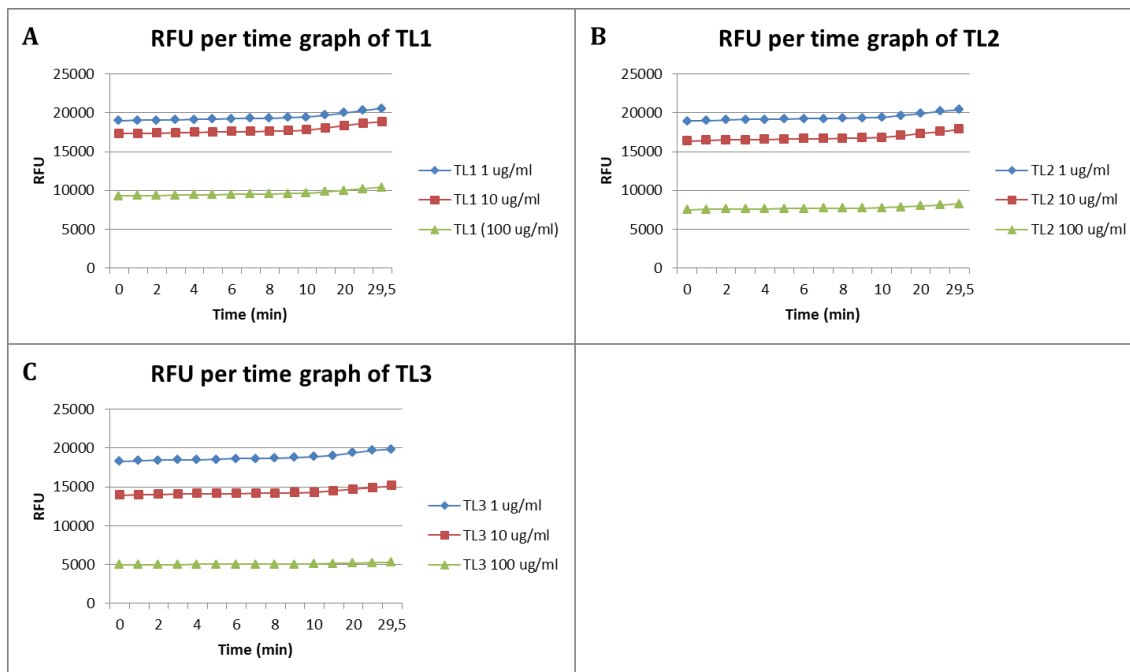


Figure 31. The graphs show the effect of *S. africana* leaf extracts on activity of LOX over time. Figure A shows the effect of hexane leaf extract, B is DCM leaf extract, C is the methanol leaf extract.

The effect of *S. africana* leaf extracts on LOX kinetic activity are shown in figure 31. Hexane extract shows slightly similar activity at 10 and 1 $\mu\text{g/ml}$, thus no significant difference was observed.

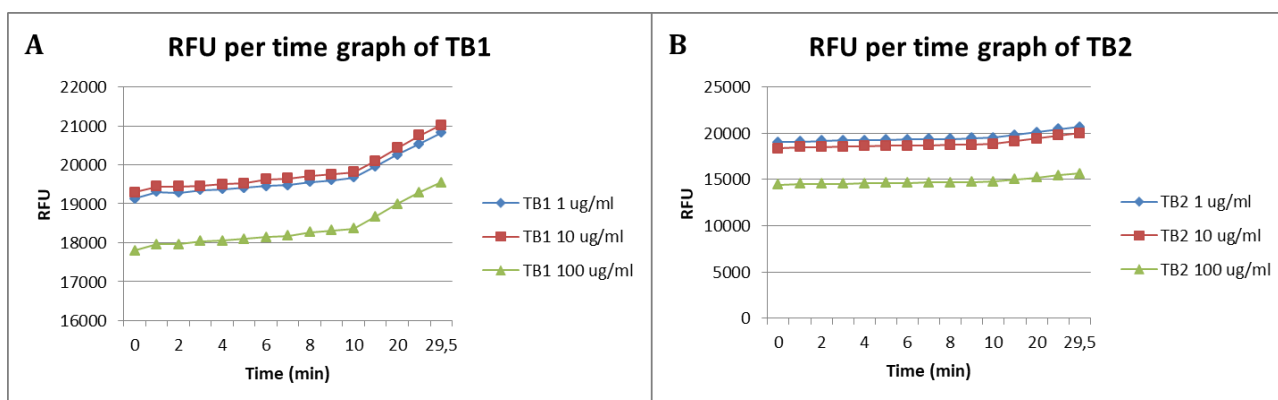


Figure 32. The graphs show the effect of *S. africana* bark extracts on activity of LOX over time. Figure A shows hexane bark extract and figure B shows the DCM bark extract. TB1= hexane extract and TB2= DCM extract.

The effect of *S. africana* bark extracts on LOX kinetic activity are shown in figure 32. Both TB1 and TB2 have a similar activity at concentration of 10 and 1 µg/ml. TB1 also shows an exponential increase in LOX activity from the 10th minute.

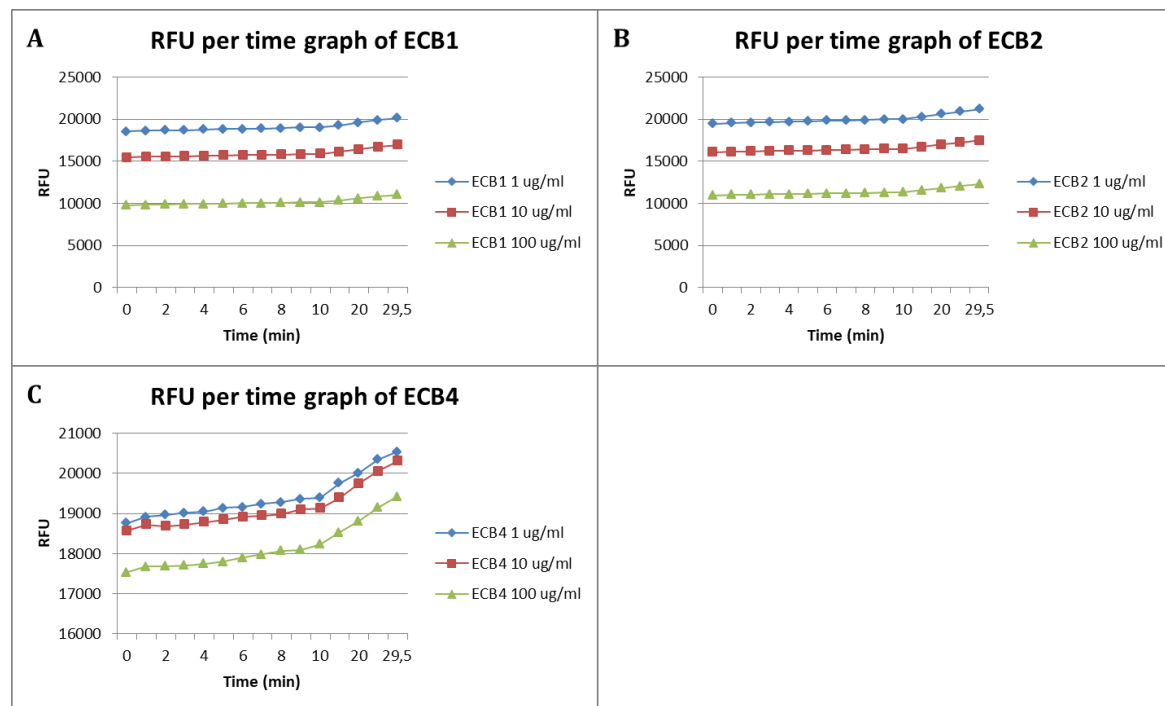


Figure 33. The graphs show the effect of *E. cupularis* stem extracts on activity of LOX over time. Figure A shows the effect of hexane extract, B shows the DCM extract, and C shows ethyl acetate stem extract. D shows hexane leaf extract and E shows DCM leaf extract.

Euphorbia cupularis was tested for its effect on LOX over time in figure 33. Ethyl acetate extract of the stems shows a steady increase in LOX activity from 0 minutes to 10 minutes and from 10 minutes onwards that activity increases exponentially.

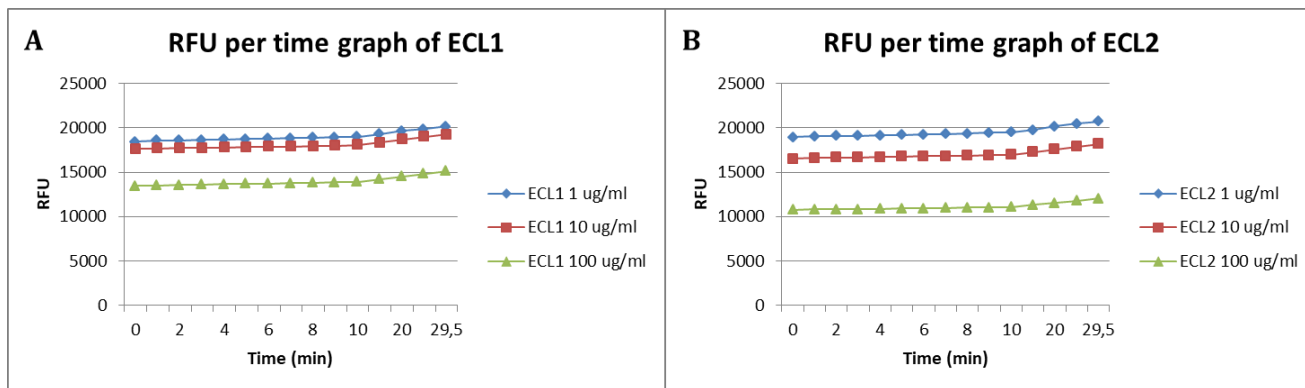


Figure 34. The graphs show the effect of *E. cupularis* stem extracts on activity of LOX over time. Figure A shows the effect of hexane extract, B shows the DCM extract

The leaf extracts of *E. cupularis* also showed a concentration dependent inhibition and no distinguishable activity at 10 and 1 µg/ml as well. The results are shown in figure 34.

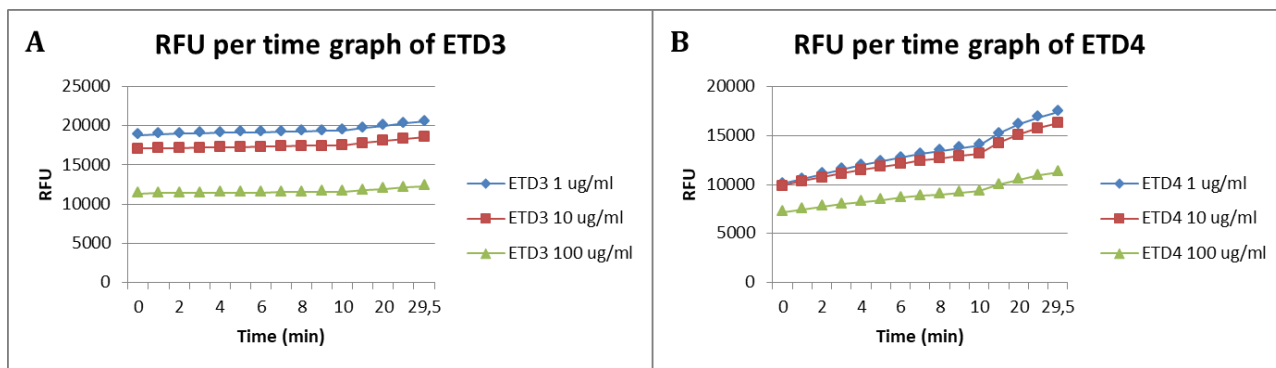


Figure 35. . The graphs show the effect of selected *E. tirucalli* extracts on activity of LOX over time. Figure A shows the effect of DCM:MeOH extract and B shows the methanol extract.

Euphorbia tirucalli was the least active of the plants tested. The DCM:MeOH and methanol extracts were the only extracts tested. The methanol extract showed a higher increase in activity over time, while the ETD3 was more static over time (figure 35).

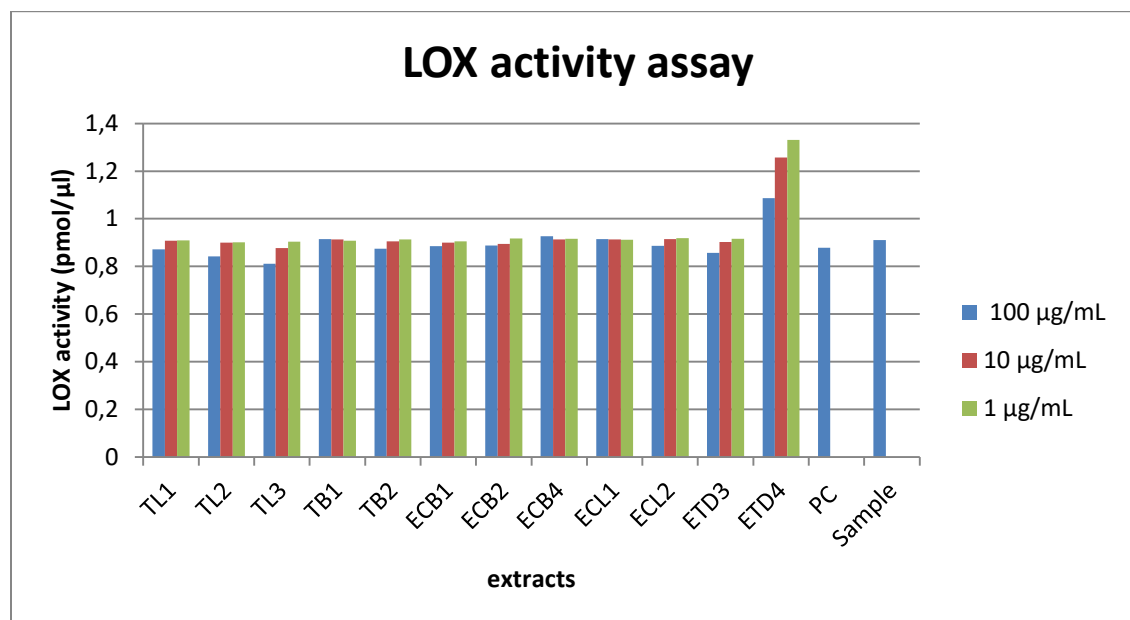


Figure 36. Activity of LOX following exposure to selected extracts. PC= positive control (pure enzyme obtained commercially) and sample = untreated MCF-7 cells .

The kinetic activity results were used to determine LOX activity based on the concentration of the product formed, figure 36 shows the results. The results show that inhibition was concentration-dependent, with ETD4 having the least inhibitory activity; in fact the LOX activity of ETD4 was higher than that of the positive control. This may suggest that ETD4 induces LOX activity rather than inhibiting activity. However, when comparing the activity of other extracts with the sample that had no extract, the activity was similar, which may suggest that the other extracts did not affect LOX activity; only ETD4 showed distinguishable activity.

CHAPTER 6

6.1 DISCUSSION

Plants produce secondary metabolites as defense against pathogens and harsh environmental conditions. These metabolites, which are known as phytochemicals (Bennett & Wallsgrove, 1994), are important in cancer treatment owing to their antioxidant, anti-inflammatory and anti-mutagenic activities (Kaur et al, 2018).

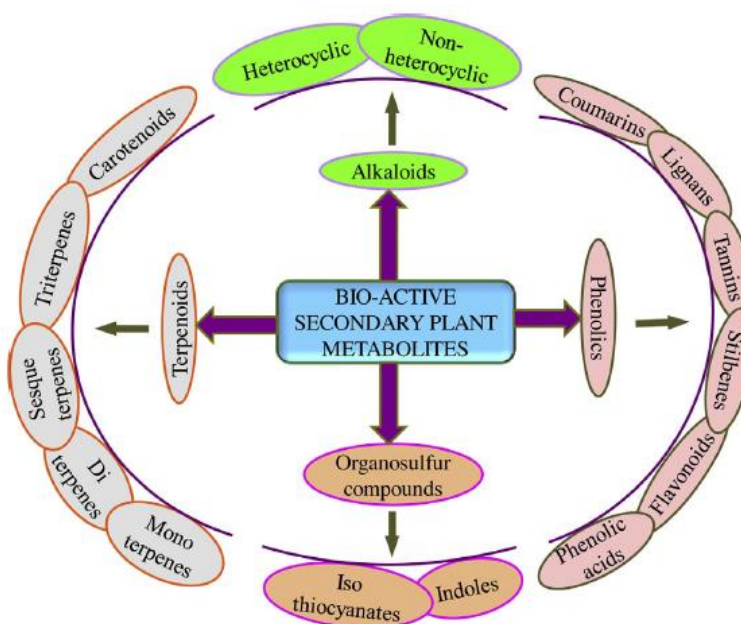


Figure 34: Bioactive secondary metabolites from medicinal plants (Kaur et al, 2018).

In the stems of *Euphorbia tirucalli*, tannins, glycosides, triterpenoids and saponins were detected. In phytochemical studies of *E. tirucalli* from Jaipur city in India, researchers detected the presence of alkaloids, coumarins, triterpens, polyphenols and tannins (Upadhyah et al, 2010). However, we did not detect alkaloids. This proves that the

same plant species growing in different geographical locations produce different secondary metabolites, since they face different environmental conditions.

In previous studies, the stem bark of *S. africana* ethanolic extract was analyzed for phytochemicals using silica gel column chromatography. It was found to contain diterpenoids and triterpenoids (Mathebe et al, 2008). This confirms our analysis of the bark of *S. africana* in which we found triterpenoids in the powdered plant part.

Tannins, flavonoids and glycosides have been shown to have anti-inflammatory and anti-oxidant activity (Nascimento et al, 2017). Inflammation is related to tumor metastasis and the enzyme responsible for inflammation is COX-2, an enzyme that is known to promote tumor angiogenesis. Antioxidant activity removes reactive oxidation species in the body that are known to cause cancer (Rice-Evans et al, 1997). Tannins also display anti-cancer and anti-allergic activity, specifically relating to hypersensitivity to food (Li & Wang, 2003; Akiyama et al, 2005). Alkaloids have been reported to show analgesic, anti-angiogenic anti-spasmodic and antibacterial activity (Yadav & Agarwala, 2011; Khalid et al, 2016). Glycosides are known to reduce hypertension and saponins have anti-inflammatory activity (Nyarko & Addy, 1990; Just et al, 1997). Tannins, saponins and glycoside were present in all the powdered plants, whereas alkaloids were only present in *S. africana* and flavonoids in *E. cupularis*. The presence of these phytochemicals could be the result of anti-cancer activity observed in these plants. However, it has been shown that plant phytochemicals usually work in synergy to exert activity (Liu, 2004), it can therefore be a combination of these phytochemicals that resulted in the anti-cancer activity observed in the plants tested. Further study is required to determine this.

To determine anti-cancer activity, the MCF-7 breast cancer cells were exposed to the extracts. The extracts that had an IC_{50} of 10 $\mu\text{g/ml}$ or lower were considered active and were selected for further studies. The national cancer institute regards an IC_{50} of less than 30 $\mu\text{g/ml}$ as active (Suffness and Pezzuto, 1990). The extracts that also showed proliferation were selected. *E. tirucalli* was the only plant that exhibited this trend. Both dried and fresh plant materials were extracted. The yield was found higher in dried material than in fresh. Furthermore, cell growth inhibition activity was also found in dried material. The hexane and DCM extracts of the dried plant material exhibited considerable activity in the range of 10 $\mu\text{g/ml}$. The latex of this plant, which is known to be poisonous, did not show activity either. It therefore seems that drying concentrated and/or preserved the phytochemicals present in the plant. However, in another study it was found that drying reduced the total phenolic content and anti-oxidant activity of that plant (Barimah et al, 2017), but that study focused on solar and oven drying, while we placed our plant away from direct sunlight.

The non-polar extracts of *S. africana* showed the highest activity in the range of 1 $\mu\text{g/ml}$, while the more polar extract (methanol) had reduced activity, which implies that the bioactive secondary metabolites in the plant are non-polar. The leaf extracts showed higher activity than the roots, while the bark extract showed comparative activity of 9 $\mu\text{g/ml}$ to 15 $\mu\text{g/ml}$. The non-polar extracts of *E. cupularis* stem and leaves also showed high cell growth inhibition.

The extracts were then tested for selectivity using the epithelial breast cell MCF10A and Chinese hamster ovarian cell lines. The SI was determined for all extracts that were

tested against cancer and normal cells. Of all the extracts tested, only TL3 was nonselective and the most selective was TL2.

All *S. africana* leaf extracts, except methanol, exhibited selectivity against MCF-7 breast cancer cells. This therefore implies that this plant part is a good candidate for targeted anti-cancer treatment drug development. Other extracts were tested on CHO cells. The *E. tirucalli* extracts were also nontoxic to these cells; instead they had a proliferative effect on CHO cells. The DCM extract of *E. cupularis* was least toxic of the extracts tested on CHO cells. The powdered material of all the extracts that showed selectivity had the lowest number of phytochemical constituents tested. All these extracts had tannins, saponins and glycosides. *E. tirucalli* had triterpenoids, *E. cupularis* had flavonoids and *S. africana* leaves had alkaloids as the distinguishing phytochemicals. The DCM extract of *E. cupularis* stem was most toxic to both cell types and had phytosterols, pentose, triterpenoids and flavonoids in addition to the common three phytochemicals. We can therefore assume that there is a synergic relationship with these absent phytochemicals in all the extracts that are present, which makes *E. cupularis* DCM extract lethal. We can also assume that the presence of alkaloids in *S. africana* leaves and the presence of flavonoids in *E. cupularis* leaves result in selectivity.

The selected extracts were then analyzed for NO activity in MCF-7 cells. The results showed that the extracts that had low cell growth inhibition also induced very low NO activity. These were *E. tirucalli* extracts. *E. tirucalli* plant extracts exhibited very little NO

production and also induced proliferation of CHO cells, which might suggest that they induce tumor angiogenesis. The results also suggested a direct proportional increase in NO concentration to growth inhibition. Therefore the extracts that induced growth inhibition also induced NO activity. Since these extracts induce cell growth inhibition of the cancer cells used, this also shows that high levels of NO in the cells result in cell death (Kumar & Kashyap, 2015). NO has been known to induce angiogenesis and tumor metastasis; however, high levels of NO promote apoptosis (Tschugguel et al, 1999). Therefore NO has cancer-promoting activity at low levels and anti-cancer activity at high levels. In our research, we observed that the levels of NO produced by the effect of the selected plant extracts showed anti-cancer activity, and perhaps the low levels seen in cells treated with *E. tirucalli* extracts, which resulted in proliferation, could be an indication of induction of tumor angiogenesis.

Inhibitory effects of selected extract on COX was studied to determine whether the extract affected angiogenesis. Relative to the control, all the extracts tested inhibited COX activity. *S. africana* hexane bark extracts inhibited COX-2 more than other extracts. Inhibitory activity at 10 and 100 µg/ml was not distinguishable, which could mean that saturation was reached at 10 µg/ml. Extracts that showed dose-dependent inhibition of COX included TL1, ECB1, ECB2, and ECL1. This indicates that COX-2 inhibitors are non-polar, since the extracts that showed inhibitory activity were hexane and DCM of *S. africana* leaves and *E. cupularis* stems and leaves. To the best of our knowledge no inhibition studies of COX-2 isolated from MCF-7 cell have been reported yet. However, in another study, more COX inhibitory activity was found in ethanol, a polar solvent extract, than in hexane, a non-polar extract (Shaikh et al, 2016). This is

why medicinal plants are a field of interest; they offer new and attractive sources of medicine and lead compounds.

To investigate the effects of plant extracts on angiogenesis further, LOX inhibition studies were conducted. LOXs convert arachidonic acid to hydroperoxyeicosa-6E, 8C, 11Z, 14Ztetraenoic acid (HETE) (Avis et al, 2001). Both LOX and COX have an arachidonic acid as their substrate to form eicosanoids (Chi et al, 2001). The plant extracts exhibited dose-dependent inhibition of LOX; however, the difference in activity between the concentrations is very low compared to the control. The methanol extract of *E. tirucalli* is the only extract with distinguishable activity; however, instead of inhibitory activity, the extract showed inducible activity. This activity decreased with increasing extract concentration. The same extract was found to induce proliferation in MCF-7 cells, therefore at a low concentration, this extract may induce angiogenesis by inducing LOX activity.

In another study, inhibition of 5-LOX was shown to induce apoptosis in MCF-7 cells (Avis et al, 2001). This may also suggest that our extracts induce apoptosis through a different mechanism that does not involve LOX. Moreover, MK886, a LOX inhibitor, is known to be effective *in vivo*, but in the cell lysate it has no effect (Rouzer et al, 1990). Therefore there is a possibility that our extract may inhibit LOX *in vivo*. Some compounds need to be metabolized to be active. They behave like pro-drugs, hence they are non-active *in vitro* but become active *in vivo*.

6.2 CONCLUSION

The results showed that *E. tirucalli* had low cytotoxicity against MCF-7 and CHO cells, and only three classes of phytochemicals tested positive. They also showed that DCM and methanol extracts of *E. tirucalli* might possess proliferative activity, while the methanol extracts may also induce angiogenesis through induction of LOX activity. The extract had low NOS activity, which might also explain the proliferative activity observed.

The stems and leaf parts of *Euphorbia capularis* were investigated. *E. capularis* is the only plant that had flavonoid content. Hexane and DCM extracts of both the stem and leaves, as well as the ethyl acetate extract of the stem, showed good anti-proliferative activity. This was confirmed by relatively good NOS activity. The extracts also had good selectivity based on the SI. ECB1, ECB2 and ECL1 at 10 to 100 ug/ml, as well as ECB4 at all concentrations, induced high COX-2 inhibition, which may suggest that *E. capularis* might inhibit angiogenesis through COX-2.

Spirostachys africana had the highest phytochemical composition and activity. All the leaf extracts had anti-proliferative activity and were also selective towards MCF-7 cells. The leaf extracts induced NOS activity as well. The extracts further exhibited inhibitory activity against COX-2, suggesting that they may inhibit angiogenesis by inhibiting COX-2 activity. The hexane and DCM extracts from the bark also had good anti-proliferation and NOS activity; however, they did not have a considerable effect on LOX and COX-2 enzymes. This suggests that they only induce apoptosis through the activation of NOS, as perhaps one of the mechanisms.

To the best of our knowledge, no studies have described the effect of the medicinal plants used in this study on angiogenesis. More research is under way to characterize the active constituents contained in these plants further. Some of the results are contradictory to the traditional use of the plants. *S. africana* and *E. cupularis* are traditionally used for wound healing and we found anti-cancer activity in the plants. *E. tirucalli* is traditionally used to cure cancer, but we found no (strong) anti-cancer activity *in vitro*. *E. tirucalli* is also used traditionally for wound healing, and the results agree with this. The plant showed growth inhibition at a concentration of 100 ug/ml, which we do not (according to NCI guidelines) consider good activity.

It is difficult to make concrete conclusions based only on *in vitro* studies. More studies *in vivo* are needed to draw proper conclusions.

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Appendix

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Investigation of angiogenic modulating properties of *Spirostachys Africana* by determining inhibitory activity against COX-2, LOX and NOS enzymes in MCF-7 breast cancer cell line

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Abstract

Angiogenesis is implicated in tumor growth, metastasis and wound healing. During tumor metastasis, this process is amplified as new capillaries are needed for cancer to spread, but in ulcerations, when this process does not occur, ulceration does not heal. Cyclooxygenase-2 (COX-2), Nitric oxide synthase (NOS) and lipoxygenase (LOX) are some of the enzymes that are highly expressed during angiogenesis, and therefore provide a useful way to detect angiogenesis. Some medicinal plants have shown to possess angiogenetic-modulating properties, which has led to development of anti-angiogenic drugs. *Spirostachys Africana* Sond has traditionally been used for treatment of open wounds, ulcers and cancer. Its mechanism(s) of action is however still not clear. The aim of the study is to elucidate mechanisms of anti-cancer and wound healing activities of *Spirostachys Africana* by evaluating its angiogenesis modulating effects, through determination of its growth inhibitory effects on MCF-7 cells as well as its inhibitory effects on COX-2, LOX and NOS enzymes. The dried plant materials were extracted with water and sequentially with organic solvents in order of increasing polarity. Extracts were screened for cell growth inhibitory activity against breast cancer MCF7 cells, and for selectivity against normal breast MCF-10A cells, and CHO cell line. Extracts that showed growth inhibitory activity with IC₅₀ values $\leq 10\mu\text{g/ml}$ were further evaluated for effects on COX-2, LOX, and NOS enzymatic activity. The nonpolar extracts of all plant parts had anti-proliferation activity with IC₅₀ around 10 $\mu\text{g/ml}$ or lower. All the leaf extracts showed selectivity for MCF-7 breast cancer. The selected extracts also induced NOS activity and inhibited LOX and COX activity in a concentration dependent manner. It was concluded that the selected extract may inhibit angiogenesis by inhibiting COX and LOX, and induce apoptosis by increasing NOS activity. *S. Africana* was found to contain tannins, glycosides, saponins and alkaloids which could be responsible for the biological activity observed.

Keywords: Angiogenesis, *Spirostachys Africana*, cyclooxygenase-2, lipoxygenase, MCF-7 breast cancer, nitric oxide synthase

Introduction

Angiogenesis is the processes of forming new capillary vessels from existing ones. It is a normal biological process that occurs during the female reproductive cycle, wound- healing, and

treatment of ulcers (Nishida et al, 2006). Studies have shown that abnormal angiogenesis is related to tumor growth and progression to a metastatic phenotype (Folkman, 1971). For metastasis to occur, new vessels are required to supply the tumor with nutrients and oxygen (Nishida et al, 2006). Furthermore, angiogenesis is complex process that involves growth factors, cytokines and enzymes such as cyclooxygenases, nitric oxide synthase and lipoxygenase.

Cyclooxygenases (COX) are the enzymes that catalyze the oxidation of a wide variety of xenobiotics, including prostaglandins (Smith et al, 1991). COX-2 is inducible and functions in inflammation and tumor angiogenesis (Sano et al, 1995; Kujubu et al, 1993).

Lipoxygenases (LOX) are group of enzymes with a non-heme iron atom that catalyze the oxidation of poly-unsaturated fatty acids like arachidonic acid (Wisastra & Dekker, 2014). Expression of 12-LOX in prostate cancer was found to induce tumor angiogenesis (Nie et al, 1998). More 5-LOX transcripts were found in brain tumors than in normal bovine brain (Boado et al, 1992), and 5(S)-hydroxyeicosa-6E,8C,11Z,14Z-tetraenoic acid (5-HETE), a product of 5-LOX, stimulated growth whereas a 5-LOX selective inhibitor reduced growth in breast cancer cells (Avis et al, 2001).

Nitric oxide synthase (NOS) form nitric oxide from L-arginine. Cianchi et al (2003) found elevated levels of iNOS and VEGF in colorectal cancer, and a high activity of iNOS in metastatic tumors than in non-metastatic ones. Thus, these three enzymes in particular, COX-2, LOX and NOS, have been selected as the focus of the study due to their significant role in angiogenesis and their implication in tumor initiation and growth.

Spirostachys Africana Sond is a member of the family *Euphorbiaceae* and is found all over the Southern Africa and also in the Central Africa (Palgrave, 1990). In South Africa, the stem and bark are used to treat stomach pains, stomach ulcers, kidney complaints and cough and eye complaints (Mabogo, 1990; Cocks & Dolds, 2000; McGaw et al, 2000; Verschaeve et al, 2004; Mathabe et al, 2006). Crude extracts from the bark of this plant have shown antibacterial activity against bacteria that cause diarrhea (Mathabe et al, 2006). The aim of the study is to investigate *Spirostachys africana* for its effect on growth of MCF-7 breast cancer cell line, and its angiogenesis modulating properties, by determining its inhibitory effects on COX-2, LOX and NOS enzymes, which are involved in tumour angiogenesis. MCF-7 cells were selected for the study as it has characteristics of a tumor, such as enzyme expression (Soule et al, 1973; Kumar & Kashyap, 2015).

Materials and Methods

Plant collection and extraction

The plants were collected from Pretoria and Kruger National Park, Limpopo Province, South Africa during the summer season. The dried plant material were then ground, weighed, then extracted with water or organic solvents in order of increasing polarity (hexane, DCM, methanol) and left to shake them for 48h before filtering. Filtrates were concentrated by freeze drying (for

water extracts) and by rotatory evaporation for organic extracts. Percentage yields were calculated.

Phytochemical analysis

The powdered plant material was subjected to different methods for determination of phytosterols, pentose, tannins, glycosides, triterpenoids, anthroquines, saponins, flavonoids, and alkaloids (Yadav & Agarwala, 2011).

Cell culturing

MCF-7 cells were grown in DMEM supplemented with 10% FBS and 0.6% streptomycin. The culture environment was maintained at 37°C in humidified, concentrated CO₂ (5%) atmosphere. Cells were sub-cultured once they reached ~80% confluency following methods described by (Huang et al, 2010). Cell viability was determined by trypan blue staining, and automated cell counter was used to obtain a cell concentration of 1x10⁵ cells/ml that was used in all experiments. The cells were plated in 96-well plate and incubated for 24 hours to adhere. After a 24 hour incubation period, the medium was aspirated and the cells were treated with 100 µl of the crude extracts (in triplicate) according to the dilutions (100, 10 and 1 µg/ml) and 100 µl of media was added to make a final volume of 200 µl. Cell proliferation was measured by MTT assay (Mosmann 1983) following a 48 hour incubation. Absorbance was read at 540 nm wavelength.

Selectivity was assessed on non-cancerous normal MCF-10A and CHO cells. Cells were grown in EMEM supplemented with epidermal growth factors, and in DMEM: HAMS F12 (1:1) respectively. Both media were supplemented with 10% FBS.

Nitric oxide synthase activity

The Griess reagent system was used to measure the amount of nitric oxide (NO) produced by the cells after exposure to the plant extracts. The assay relies on a diazotization reaction that was first described by Griess. It detects nitrite (NO₂⁻), a stable form of NO, in the culture media which reacts with sulfanilamide and N-1-naphthylethylenediamine dihydrochloride (NED) under acidic (phosphoric acid) conditions. In this assay, cells (1x10⁵ cells/ml) were incubated with the extracts at different concentration for 48hrs. The cell culture supernatant (100 µl) was obtained from culture and mixed with sulfanilamide (100 µl). The mixture was incubated for 10 minutes at room temperature and then NED solution (100 µl) was added and incubated for 10 minutes. Thereafter, the absorbance was measured at 550nm. The nitrite concentration was calculated from nitrite standard curve. Cells without extract were used as negative control.

LOX activity assay

Lipoxygenase activity was determined using the lipoxygenase activity assay kit from BioVision. In this assay, Lipoxygenase converts its substrate into an intermediate that reacts with the probe, and in doing so, generates a fluorescent product which is measured with a spectrophotometer.

The cells (4×10^5 cells/ml) were homogenized with 100 μ l ice-cold LOX buffer and kept on ice for 10 minutes, followed by centrifugation at 10 000 Xg for 15 minutes. The cell lysate (10 μ l) was then mixed with 10 μ l plant extract (100, 10 and 1 μ g/ml), 20 μ l LOX substrate, 2 μ l LOX probe and 78 μ l of LOX buffer. The mixture was immediately placed on the fluorescence plate reader at an excitation wavelength of 500 nm and emission wavelength of 536 nm and the fluorescence was measured at 30 seconds interval for 30 minutes. Standard curve was generated from the LOX substrate and pure LOX enzyme supplied. LOX inhibitor was used a positive control. The experiment was performed in duplicates and repeated twice.

Cyclooxygenase-2 activity assay

Inhibition of COX-2 in MCF-7 cells was determined using cyclooxygenase activity kit from Biovision. The cells (4×10^5 cells/ml) were homogenized with 100 μ l ice-cold LOX buffer and kept on ice for 10 minutes, followed by centrifugation at 10 000 Xg for 15 minutes. In each well, cell lysate (10 μ l) was mixed with 10 μ l plant extract (100, 10 and 1 μ g/ml), 2 μ l COX probe, 4 μ l diluted COX cofactor and 74 μ l COX assay buffer. The mixture was immediately placed on the fluorescence plate reader at an excitation wavelength of 536 nm and emission wavelength of 587 nm and the fluorescence was measured at 30 seconds interval for 30 minutes. A standard curve was generated and a supplied COX-2 inhibitor was used a positive control. The experiment was performed in duplicates and repeated twice.

Results

The ground plant material was extracted and the yields are shown in table 1 below. Methanol extracts had the highest yield for both leaves and barks while the lowest yield was observed in DCM stem extract.

Table 6. The percentage yield of extracts from powdered material

	hexane	DCM	MeOH	ethyl acetate
<i>S. africana leaves</i>	0.5%	0.7%	21.1%	0.2%
<i>S. africana barks</i>	5.9%	5.1%	39.9%	5.9%
<i>S. africana stem</i>	1.9%	0,02%		

Phytochemical analysis

In the powdered plant material of *S. africana*, three parts of the plant were analysed; leaves, stem and barks. In the leaf parts, triterpenoids, tannins, glycosides, saponins and alkaloids were detected. In the stem tannins, phytosterols, glycosides, alkaloids, saponins and triterpenoids were detected. All the phytochemicals were present in the barks except for flavonoids, which were absent in all plant parts.

Table 7. Phytochemicals tested in *S. Africana*, + indicates present and – present absent

	stems	leaves	bucks
Phytosterols	+	-	+
Pentose	-	-	+
Tannins	+	+	+
Glycosides	+	+	+
Triterpenoids	+	-	+
Anthroquines	-	-	+
Saponins	+	+	+
Flavonoids	-	-	-
Alkaloids	+	+	+

Cell growth inhibition studies

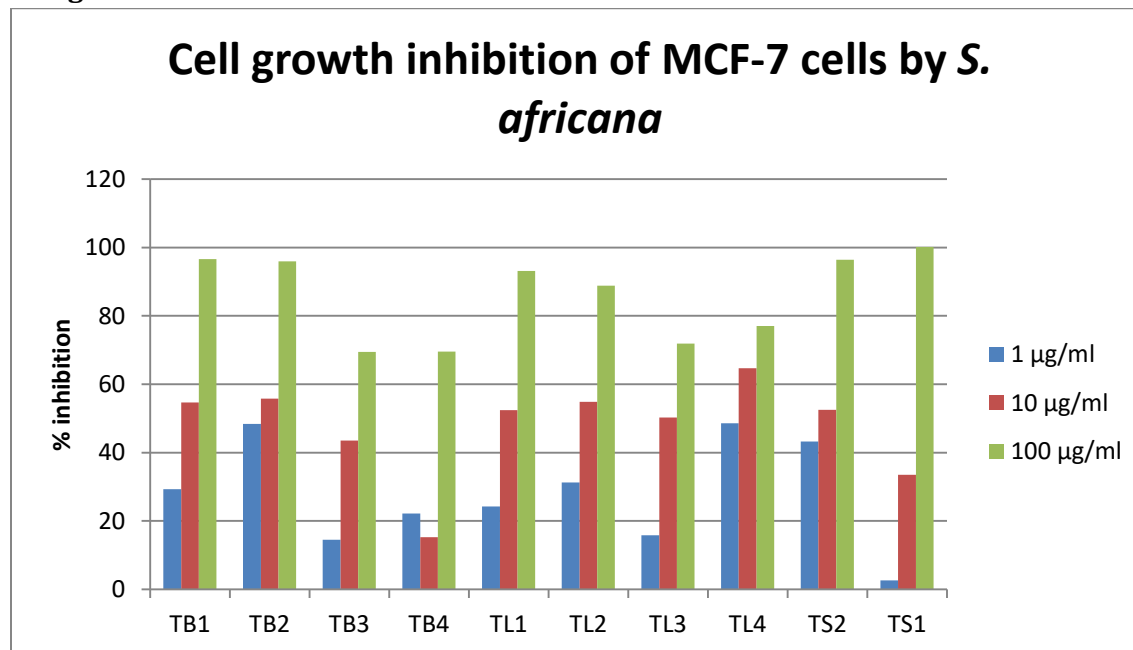


Figure 37. Effects of *S. africana* extracts on MCF-7 cell growth inhibition . TB represents the bark extract, TL the leaf and TS represents the stem extract. 1= hexane, 2= DCM, 3= methanol and 4= ethyl acetate.

Each plant part: stem, leaf and bark had 2 to 4 extracts. The bark had 4 extracts: hexane (TB1), dichloromethane (TB2), methanol (TB3) and ethyl acetate (TB4). From the 4 extracts, hexane

and dichloromethane had the anti-proliferative activity with IC_{50} of 10 $\mu\text{g/ml}$ or lower. All the leaf extracts showed considerable activity with IC_{50} ranging from 1 $\mu\text{g/ml}$ to 10 $\mu\text{g/ml}$. For the stem extracts, only the DCM extract (TS2) showed anti-proliferation activity.

Selectivity

Selectivity of the extracts was studied by determining their anti-proliferative activity against the normal human breast MCF-10A cells. All the leaf extracts showed low anti-proliferation against normal breast cells compared to activity against breast cancer cells. The hexane extract (TL1), which had the highest cytotoxic activity against the cancer cells, showed the least activity of all the extracts, indicating that *S. Africana* leaves selectively inhibited breast cancer cell growth. Extracts that showed IC_{50} of 10 $\mu\text{g/ml}$ and below showed reduced inhibition (20% inhibition and less) at the same concentration against the normal breast cell line. The leaf extracts showed negligible activity (<5 $\mu\text{g/ml}$) against the normal breast cells at Concentration of 1 $\mu\text{g/ml}$.

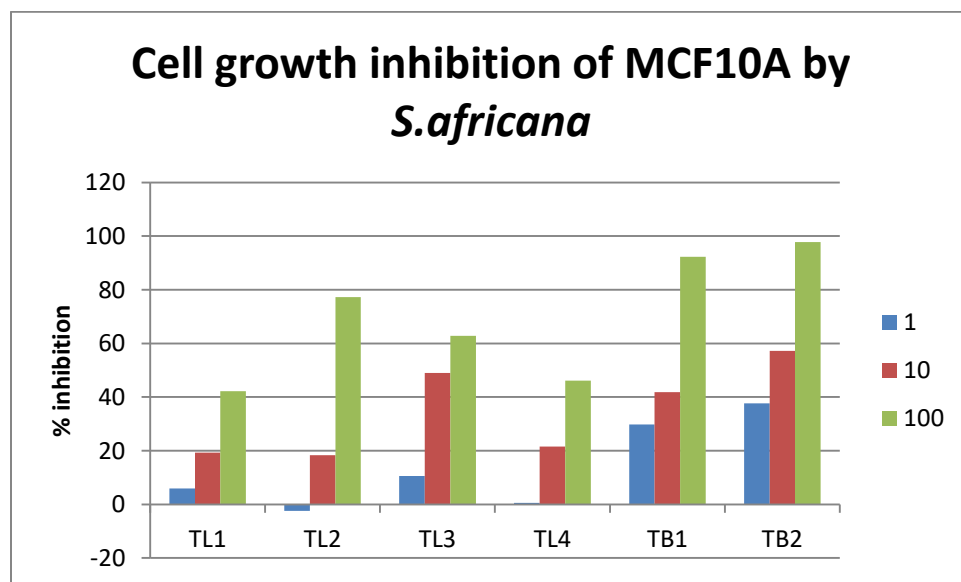


Figure 38. Effects of *S. africana* bark and leaf extracts on normal human breast MCF-10A cell growth inhibition

NOS inhibition studies

Figure 3 shows the results of nitric oxide synthase activity against plant extracts. The results show that the levels of nitric oxide increased with the concentration of extracts. The hexane and DCM extracts of the both the leaves and barks exhibited high levels of NO (over 1000 and 2000 $\mu\text{M/ml}$ respectively) compared to the negative control, which were cells that were not exposed to

the extract. The two nonpolar extracts (TL1, TL2, TB1 and TB2) exhibited highest inhibition against MCF-7 cells, suggesting a strong association between the level of NO and induction of cell death.

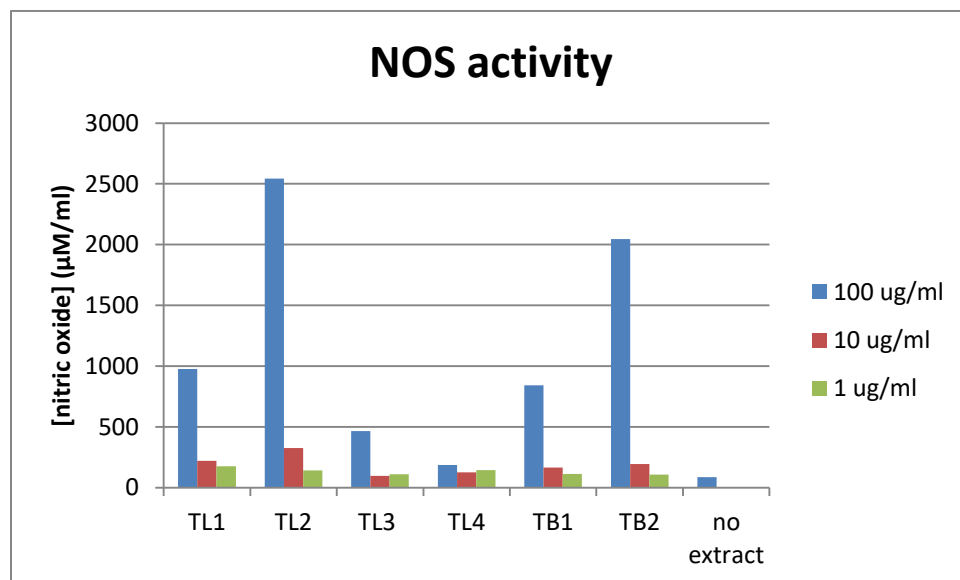


Figure 39. The effect of *S. africana* extracts on nitric oxide synthase

COX-2 inhibition studies

The results in figure 4 show that COX-2 was inhibited in a concentration dependent manner. The hexane extract of the bark-(TB1) exhibited the highest inhibition with enzyme activity of ~0.26 pmol/µl at 100 µg/ml, compared to celecoxib, a known inhibitor of COX-2 which had activity of 9.3 pmol/µl).

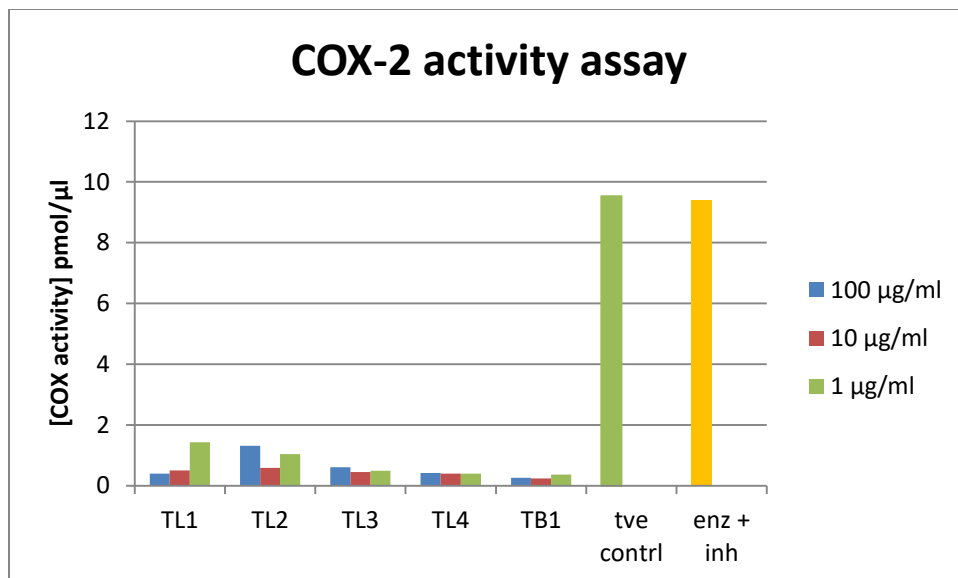


Figure 40. Activity of COX-2 against *S. africana* extracts, where tve contrl represents positive control (pure enzyme supplied) while enz + inh is the pure enzyme with celecoxib (COX-2 inhibitor). N=2

2

LOX inhibition studies

LOX activity was determined by measuring RFU and deducing concentration from the LOX standard probe. The results are shown in figure 5, and they show a concentration dependant inhibition with methanol leaf extract (TL3) showing the lowest inhibition (0.81 pmol/μl) at 100 μg/ml. The negative control had activity of 0.91 pmol/μl, which is similar that of TB1 at 1 μg/ml and TB2 at 100 μg/ml.

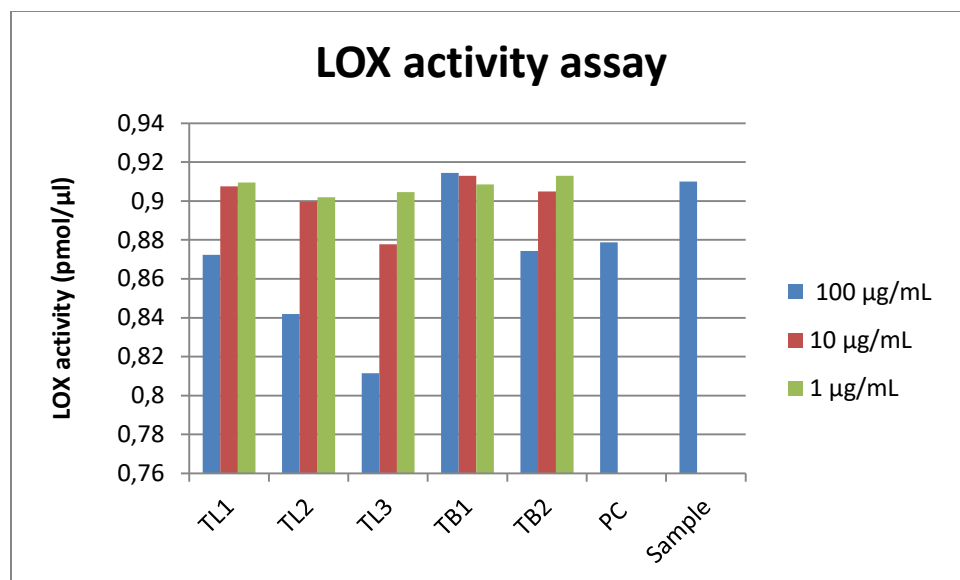


Figure 41. Effects of selected extracts on the activity of LOX enzyme. PC= positive control and sample = cells without extract.

Discussion

Spirostachys Africana tested positive for many of the secondary metabolites, including tannins, glycosides, saponins and alkaloids. In previous studies, the stem and bark of *S. africana* ethanolic extracts were found to contain diterpenoids and triterpenoids (Mathebe et al, 2008). This corroborates with our findings.

Tannins, saponins, flavonoids and glycosides have been shown to have anti-inflammatory and anti-oxidant activity (Nascimento et al, 2017). Inflammation is related to tumour metastasis and cyclooxygenase-2 is implicated in both. Tannins also have anticancer activity (Li & Wang, 2003; Akiyama et al, 2005). Glycosides are known for several biological activities including toxicity and anti-inflammatory activity (Nyarko & Addi, 1990; Just et al, 1997). We also observed that all the non-polar solvents had the highest activity, suggesting that the active phytochemicals are non-polar. The non-polar and semi-polar phytochemicals that could be present in the plant include alkaloids, phytosterols and triterpenoids (Widyawati et al, 2014). The leaf extracts selectively inhibited MCF-7 breast cancer cells over MCF-10A normal breast epithelial cells. The leaves only tested positive for alkaloids while the stem and barks tested positive for all three, which may suggested that the compound(s) with selectivity could be an alkaloid. However, this would only be verified after our future study has been completed, which is on the isolation and identification of pure compounds from this plant species, especially alkaloids.

Angiogenesis is the key process in wound healing and, tumor growth and metastasis. Complete inhibition of angiogenesis result in gastric ulcers (Wallace, 2000), hence there is a need for

selective inhibition of angiogenesis only in cancer cells. The plant extract have demonstrated selective inhibition of MCF-7 breast cancer cells over MCF-10A breast epithelial cells.

Literature shows that Lipoxygenases and cyclooxygenase-2 are highly expressed in tumor angiogenesis (Ara &Teicher, 1996) and that successful inhibition of both these enzymes does inhibits tumor angiogenesis (Ye et al.2005). We have shown that *S.africana* leaf extracts and DCM bark extracts inhibit angiogenesis through inhibition of Lipoxygenase activity in MCF-7 cells.

Nitric oxide has been known to induce angiogenesis and tumor metastasis, however high levels of nitric oxide promote apoptosis (Tschugguel et al, 1999). Therefore, nitric oxide has cancer promoting activity at low levels and anti-cancer activity at high levels. In our research, we observed that the levels of NO produced by the effect of the selected plant extracts have anti-cancer activity. Moreover, this might be due to the ability of *S. africana* plant extracts in inducing the iNOS (inducible-nitric oxide synthase) enzyme to produce more nitric oxide (Kumar & Kashyap, 2015), thus promoting apoptosis in return.

Conclusion

S. africana leaves had a few but essential phytochemicals that are known to have anti-inflammatory and anti-cancer activity. The leaf extracts selectively inhibited cancer cell over normal cells and induced nitric oxide production in lethal concentrations. The extracts also inhibited COX and LOX activity in concentration dependent manner, suggesting that *S. africana* leaf extract inhibit angiogenesis in MCF-7 cells. From the bark extracts, only the DCM and hexane extract had the desired cytotoxic activity at an IC₅₀ of 10 µg/ml, inducing high NO production and inhibited LOX activity. According to the author's knowledge, no studies of this manner have been performed on this plant.

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