ANTIOXIDATIVE, ANALGESIC AND ANTI-INFLAMMATORY ACTIVITIES OF *ACOKANTHERA OPPOSITIFOLIA, PLANTAGO LANCEOLATA, CONYZA CANADENSIS,* AND *ARTEMISIA VULGARIS*

By

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Submitted in accordance with the requirements

for the degree of

MASTER OF SCIENCE

in the subject

LIFE SCIENCES

at the

UNIVERSITY OF SOUTH AFRICA

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FEBRUARY 2015

DECLARATION

I declare that the study entitled: Antioxidative, analgesic and anti-inflammatory activities of *Acokanthera oppositifolia, Plantago lanceolata, Conyza canadensis*, and *Artemisia vulgaris,* is my own work and that all the sources that I have used or quoted have been indicated and acknowledged by means of complete references. It has been submitted and shall not be submitted in any form to any institution of higher learning for the award of any degree.

Signature of student Date 2015/02/17

DEDICATION

This work is dedicated to my dear father Elada Faustin and my darling mother Bekono Paulette.

‘But after you have suffered a little while, the God of all undeserved kindness, who called you to his everlasting glory in union with Christ, will himself finish your training. He will make you firm, He will make you strong, He will firmly ground you. To Him be the might forever. Amen’. 1 Peter 5:10, 11.

ACKNOWLEDGEMENTS

I would like to express my deepest gratitude to my supervisor and co-supervisors, Dr S L Lebelo and Dr L J Shai for their training, support, and patience throughout this project.

I would like to thank:

- Prof J E Eloff, University of Pretoria, Phytomedicine Programme, for the lipoxygenase test.
- Mr Salmon Alani Adebayo, Tshwane University of Technology, for his assistance.
- National Student Financial Aid Scheme (NSFAS) and the University of South Africa for funding this project.
- Friends and colleagues for their support.
- I would like to thank my wife, Stephanie Tiogang Djopmegne for her love, encouragement, patience, and believing in me; my twin brother Etoundi Jephte Godefroy for his encouragement and support.

ABSTRACT

The anti-inflammatory properties of four medicinal plants were investigated. These plant extracts were subjected to screening for their possible effects as antioxidative, analgesic, and anti-inflammatory agents. In the antioxidant activity, the *Plantago lancelota* extracts resulted in an IC_{50} value of 0.4 mg/mL compared to the positive control quecertin with IC_{50} 0.04 mg/mL *Plantago lanceolata* inhibited COX-2 activity with IC_{50} values of 0.41 mg/mL. However, the COX-1 inhibition indicated an IC_{50} of 68.99 mg/mL. The lipoxygenase assay indicated that *Plantago lanceolata* was the most active plant species with an IC_{50} value of 4.86 mg/mL compared to the positive control (quecertin) with an IC_{50}<2mg/mL. The nitric oxide assay of the plant extracts indicates a dose-dependent activity of our plant extracts. Likewise the cell viability result indicated a good activity at dose 100 mg/mL.

Key words: *Plantago lanceolata*, *Conyza canadensis*, *Acokantera oppositifolia*, *Artemisia vulgaris,* antioxidant, anti-inflammatory, nitric oxide, cell viability, Raw 264.7 macrophages, lypoxygenase, cyclooxygenase-1 and cyclooxygenase-2.

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LIST OF NON-STANDARD ABBREVIATIONS

AA	Arachidonic acid
ACE^{TM}	Acetylcholinesterase tracer
Ache	Acethycholinesterase
BK	Bradykinin
BLK	Blank
bNOS	Brain nitric oxide synthase
Bo	Maximum binding
CGRP	Calcitonin gene-related peptide
CHF	Congestive heart failure
CML	Chronic myeloid leukaemia
COX-1	Cyclooxygenase-1
COX-2	Cyclooxygenase-2
DMEM	Dulbecco's modified eagle medium
DMSO	Dimethyl sulfoxide
DPPH	2, 2'-diphenyl-1-picrylhydrazyl
EDTA	Ethylenediaminetetra-acetic acid
EIA	Enzyme immunoassay

EL	Endothelium
eNOS	Endothelium nitric oxide synthase
FCS	Foetal calf serum
HPLC	High performance liquid chromatography
5-HETE	5-hydroxy eicosatetraenoic acid
5-HPETE	5-hydroperoxy eicosatetraenoic acid
5-HT	5-hydroxytryptamine
IBD	Inflammation bowel disease
IC_{50}	Inhibition concentration with 50% inhibition
IL	Interleukin
iNOS	Inducible nitric oxide synthase
KOH	Potassium hydroxide
LOX	Lipoxygenase
LPS	Lipopolysaccharide
LT	Leukotriene
MS	Multiple sclerosis
MTT	3(4,5-imethylthiazol-2-yl) 2,5-diphenyltetrazolium bromide
NGF	Nerve growth factor
NO	Nitric oxide
NOS	Nitric oxide synthase
NSAIDs	Non-steroidal anti-inflammatory drugs
NSB	Non-specific binding
PAF	Platelet-activating factor

PBS	Phosphate buffer saline
PGI_2	Prostacyclin
PLA_2	Phospholipase A_2
POMC	Pro-opomelanocortin
PSF	Penicillin streptomycin fungizone
PUFA	Polyunsatured fatty acid
RNOS	Reactive nitrogen oxide species
ROS	Reactive oxygen species
SP	Substance P
SRS-A	Slow-reaction substance
TA	Total activity
TLRs	Toll-like receptors
TNF-α	Tumour necrosis factor alpha
TX	Thromboxane

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CHAPTER ONE

1. Introduction

1.1 Overview of pain

Pain is a common health problem with substantial socioeconomic impact because of its high incidence. It is a symptom of many diseases. It is estimated that 80-100% of the world's population experience back pain at least once in their life time (Jain *et al*., 2002). The treatment of pain requires analgesics, including anti-inflammatory products. Most of the so-called non-steroidal anti-inflammatory agents also have analgesic activity. Although many analgesics and anti-inflammatory agents are present in the market, modern drug therapy is associated with some adverse effects, like gastrointestinal irritation, fluid retention, bronchospasm and prolonged bleeding time (Osadebe and Okoye, 2003; Jain *et al*., 2002).

The World Health Organisation (WHO) estimates that up to 80% of the people in developing countries still depend on local medicinal plants to fulfil their primary healthcare needs (WHO 2002). In certain African countries, up to 90% of the population still relies exclusively on plants as sources of medicines (Hostettman *et al*., 2000). Furthermore, an estimated 25% of prescription drugs and 11% of drugs considered essential by WHO are derived from plants. A large number of synthetic drugs are obtained from precursor compounds originating from plants (Hostettman *et al*., 2000). According to Konno (2004), easy accessibility, efficacy and affordability drive the massive preference traditional medicine over modern medication in developing and third world countire. In Sub-Saharan Africa, the ratio of traditional healers to the population is approximately 1:500, while medical doctors have a 1:40000 ratios to the rest of the population (Konno, 2004).

1.2 Inflammation

Inflammation is the response of the organism to invasion by a foreign body, such as bacteria, parasite, or viruses. In this context, the inflammatory response is a critical protective reaction to irritation, injury, or infection, characterized by redness, heat swelling, loss of function, and pain (Levine and Reichling, 1999). Redness and pain result from an increase in blood flow, swelling is associated with increased vascular permeability, and pain is a consequence of activation and sensitisation of primary afferent nerve fibres. Under normal conditions, these changes in inflamed tissue serve to isolate the effects of the tissue damage or infection and thereby limit the threat to the affected site (Levine and Reichling, 1999).

Inflammation is a process involving localised increases in the number of leukocytes and a variety of complex mediator molecules. Prostaglandins are ubiquitous substances that modulate cellular and tissue responses involved in inflammation (Lipsky, 1999). Their biosynthesis has also been implicated in the pathophysiology of cardiovascular diseases, cancer, colonic adenomas and Alzheimer's disease (Smith, 1995; Lipsky, 1999). Inflammation is designed to limit invasions and damage after injury, an essential process for the survival of mankind in the absence of medication such as antibiotics. The onset to conclusion of an inflammation is a self-limiting and controlled process of the immune system (Margarethe *et al*., 2012).

General understanding of the molecular and cellular mechanisms involved in the inflammatory process has increased dramatically in recent decades, and has permitted the discovery of many promising targets for the development of new drugs to treat chronic diseases such as rheumatoid arthritis, allergy, asthma, inflammatory bowel disease and others. A great number of inflammatory mediators including kinins, platelet-activating factors, prostaglandins, leukotrienes, amines purines, cytokines, chemokine, and adhesion molecules act on specific targets, leading to the local release of other mediators from leukocytes (e.g. mast cells and basophiles) and

the further attraction of leukocytes, such as neutrophils, to the site of inflammation (Levine and Reichling, 1999).

To appreciate the inflammatory process, it is important to understand the role of chemical mediators. These mediators are the substances released as plasma proteins, or secreted by cells like mast cells, platelets, neutrophils and monocytes/macrophages following stimulation by allergic or chemical irritation, injury and infections (Shailasre *et al*., 2012). These mediators, depending on the duration of injury, or trigger determine the severity of inflammation and are termed pro-inflammatory fundamental factors. These substances bind to specific target receptors on the cells and may increase vascular permeability, promote neutrophil chemotaxis, stimulate smooth muscle contraction, increase direct enzymatic activity, induce pain and/or mediate oxidative damage (Coleman, 2002). Examples of these mediators include: nitric oxide (NO), prostaglandins (PG), leukotriene (LK), vasoactive amines (histamine, serotonin), and cytokines (tumour necrosis factor and interleukins–1, 12). Although some of the cytokines (IL-3 -4,-5,-6,-10,-13) released are beneficial by acting as anti-inflammatory mediator within the cells (Esch and Stefano, 2002), these are pro-inflammatory. Even though the innate cascade process of inflammation is complex, it is mainly divided into two parts i.e. acute and chronic which could either be beneficial or detrimental. Under specific circumstance, it could turn into a chronic state and subsequently become a causative factor in the pathogenesis. Inflammation is a self-defence reaction in its first phase, hence regarded as the main therapeutic target and often, the best choice to treat the disease and alleviate the associated symptoms (Shailasre *et al*., 2012).

1.2.1 Acute inflammation

Acute inflammation is short-term response characterised by rapid onset. It is characterised by the exudation of fluids and plasma proteins; and the migration of leukocytes, most notably neutrophils into the injured area. This acute inflammatory response is believed to be a defence mechanism aimed at the killing of bacteria,

viruses and parasites with simultaneous facilitations of wound repairs (O'Byrne and Dalgleish, 2001; Dalgleish and O'Byrne, 2002).

The inflammatory response stimulates release of Tumour Necrosis Factor alpha (TNF-α) from stimulated mast cells. Other cells involved in inflammation have receptors for TNF-α. They are activated by the binding of TNF-α. Activation of these recruited cells produces their own mediators of inflammation. This positive feedback quickly amplifies the response. The phagocytotic cells, macrophages and neutrophils, produce reactive oxygen species (ROS). Macrophages and activated platelets release interleukin (IL)-1, a cytokine. IL-1 causes fever by stimulating the release of prostaglandins (PGs), which act on the temperature control centre of the hypothalamus (Duff and Durum, 1982). IL-1 is synthesised from a larger precursor that is cleaved by caspase-1. Caspase-1 is part of two (or more) multi- protein complexes in the cytosol of macrophages and neutrophils that are called inflammasomes (Franchi *et al*., 2009). Inflammasomes are activated by several different products produced by invading bacteria that interact with toll-like receptors (TLRs), thus providing a link between the innate immune system and inflammation. Chemical mediators such as histamine and bradykinin induce the production of PGs and leukotrienes with a role to potentiate the plasma exudation (Oh-Ishi, 1997). These potent mediators of inflammation are derivatives of arachidonic acid (AA), a 20-carbon unsaturated fatty acid produced from membrane phospholipids.

Arachidonic acid, esterified at the second carbon in the glycerol backbone of membrane phospholipids is released in a hydrolytic reaction catalysed by phospholipase A_2 (PLA_2). It is subsequently metabolized by cyclooxygenase (COX) and lipoxygenase (LOX) to prostaglandins. The COX-1 is constitutively expressed and produces PGs in a basic housekeeping manner for normal functioning of the body. The expression of COX-2 is inducible, upregulated in response to cytokines, mitogens and endotoxins (Shailasre *et al*., 2012).

1.2.2 Chronic inflammation

Chronic inflammation is of a more prolonged duration and manifests histologically by the presence of lymphocytes and macrophages, resulting in fibrosis and tissue necrosis. The persistent chronic inflammation increases the development of the degenerative diseases such as rheumatoid arthritis, atherosclerosis, heart disease, Alzheimer, asthma, acquired immunodeficiency disorder (AIDS), cancer, congestive heart failure (CHF), multiple sclerosis (MS), diabetes, infections (bacteria, fungi, parasites), gout, IBD-inflammatory bowel disease, aging and other neurodegenerative CNS depression, all of which are associated with immunopathology that appears to play a key role in the onset of the condition (O'Byrne and Dalgleish., 2001; Dalgleish and O'Byrne., 2002).

1.3 Aim of the study

The aim of the study is to investigate antioxidative, analgesic and anti-inflammatory effects of *Acokanthera oppositifolia, Plantago lanceolata, Artemisia vulgaris* and *Conyza canadensis*.

1.4 Objectives

- Evaluate and establish some of the basic ethnopharmaceutical properties of *Acokanthera oppositifolia*, *Plantago lanceolata*, *Conyza canadensis* and *Artemisia vulgaris.*
- Investigate a preliminary phytochemical screening of the plants to rationalise their therapeutic usefulness in African traditional medicine.
- Determine their analgesic, antioxidative and anti-inflammatory effects and compare *Plantago lanceolata* to three other extract plants (*Acokanthera oppositifolia, Conyza canadensis* and *Artemisia vulgaris*.
- Evaluate plants toxicity effects in cell cultures by using greiss reagent.

CHAPTER 2 LITERATURE REVIEW

2.1 Introduction

Pain is a disabling accompaniment of many medical conditions. Pain control is one of the most important therapeutic priorities today. Many analgesic drugs, notably opioids and non-steroidal anti-inflammatory drugs (NSAIDs) have their origin in natural products that have been used for centuries (Rang *et al*., 2012).

Inflammation is a normal, protective response to tissue injury caused by physical trauma, noxious chemicals or microbiological agents (Kumar *et al*., 2013). There are many inflammatory pathways and the more important are cyclooxygenase pathway and lipoxygenase pathway. The use of herbal medicines is fast becoming more popular due to toxicity and side-effects of allopathic medicines. Medicinal plants play an important role in the development of potent therapeutic agents. There are over 1.5 million practitioners in the traditional medicine system using medicinal plants in preventive, promotional and curative applications (Kumar *et al*., 2013).

2.2 Traditional medicine

2.2.1Herbal medicines

Herbal medicines are also referred to as herbal remedies, herbal products, herbal medicinal products, phytomedicine, phytotherapeutic agents and phytopharmaceuticals. The use of herbal medicine in evidence or science-based approach to treatment and prevention of diseases is known as phytotherapy. Herbal medicines have been used since time immemorial. Ancient Chinese tests and Indian records contain detailed description of the use of a variety of plant-derived medications. Even in present days, plants remain the main source of medicines for a large proportion of a world's population, particularly in the developing countries. Over the years, synthetic drugs have been plagued by unwanted side-effects, toxicity and inefficiency, among other problems (Vadde *et al*., 2012). In addition, the search for new drugs to treat and manage a variety of illnesses through chemical synthesis has

not been encouraging. These factors, as well as the emergence of new diseases, the proliferation of disorders such as cancers and growing multidrug resistance in pathogenic microorganisms have prompted renewed interest in the discovery of potential drug molecules from medicinal plants (Vadde *et al*., 2012). Herbal medicine is now globally accepted as a valid alternative system of therapy in the form of phytochemicals, and functional foods, a trend recognised and advocated by WHO. Though ancient medicinal treatments have been documented, a large number of medicinal plants remain undocumented, uncharacterized, with minimal knowledge of their use being orally passed from generation to generation (Khare, 2007; Barnes *et al*., 2007; Yang, 2010).

The documentation of medicinal uses of African plants is becoming increasingly urgent because of the accelerated loss of the natural habitat of some of these plants due to anthropogenic activities. The continent is estimated to have 216,634,000 hectares of closed forest areas with a calculated annual loss of about 1% due to deforestation. Many of the medicinal plants become extinct before they are even documented. Africa has one of the highest deforestation rates in the world, for example, Cote d'Ivoire and Nigeria have 6.5 and 5% deforestation per year, respectively. The rates in these countries are much higher than the global average rate of 0.6%. Lack of habitat conservation and protection threatens not only the loss of plant resources but also traditional community life, cultural diversity as well as the accompanying knowledge of the medicinal value of several endemic plant species (Iwu, 2014).

South Africa is blessed with a rich cultural diversity, reflected in the formal and informal systems of medicines presently practised in different parts of the country. The informal oral-tradition medical systems of the Khoisan people, Nguni and Sotho-speaking people has not yet been systematised, and are passed on by word of mouth from one generation to the next (Van Wyk, 2009). The formal system of medicine, which is well documented and systematised, was introduced to the country over a period of more than 300 years (since 1652) by Europeans and other settlers, and is exemplified by today's modern Western medicine. Ayurvedic medicine from

India, Traditional Chinese medicine and Homeopathic medicine are also commonly practised in certain parts of South Africa (Van Wyk, 2009).

2.2.2 Medicinal plants of South Africa

Medicinal plants are an important aspect of the daily lives of many people and an important part of the South African cultural heritage. Southern Africa has well over 30 000 species of higher plants. The Cape Floral Kingdom alone has nearly 9 000 species and is the most diverse temperate flora on earth, rivalling the tropical rain forest in terms of forest richness. With South Africa's remarkable biodiversity and cultural diversity, it is not surprising to find that approximately 3 000 species of plants are used as medicines, with some 350 species are traded in medicinal plants markets (Van Wyk, 2009).

Plants were once a primary source of all the medicines in the world and they will continue to provide mankind with new remedies. Well-known examples of plants-derived medicines include quinine, morphine, codeine, aspirin, Atropine reserpine and cocaine. Recently, important anti-cancer drugs such as taxol and vincristine have been developed from medicinal plants. South African contribution to world medicine includes Cape Aloes (*Aloes ferox*), Buchu (*Agathosma betulina*) and Devil's Claw (*Harpagophytum procumbens*). Local equivalents also exist for many of the famous remedies used elsewhere. There is a growing interest in natural and traditional medicines as a source of new commercial products (Van Wyk, 2009). It is clear that traditional healers play an influential role in the lives of African people and have the potential to serve as crucial role players in a comprehensive health care strategy (Kambizi and Ofolayan, 2001). According to Clarke (1998), traditional healers are deeply interwoven into the fabric of cultural and spiritual life, are the first health practitioners to be consulted in up to 80% of cases (especially in rural areas), and are present in almost every community due to their accessibility in remote areas characterised by under developed healthcare facilities (Clarke, 1998).

2.2.3 Traditional healing in South Africa

There are an estimated 200 000 indigenous traditional healers in South Africa, and up to 60% of the South African population consults these healers, usually in addition to using modern biomedical services (Van Wyk, 2009). Traditional healers in South Africa are most commonly known as ‘isangoma’ (Zulu), ‘ixwele’ (Xhosa), ‘ngaka’ (Sotho), ‘bossiedokter’ (Western and Northern Cape). The term ‘inyanga’ and ‘sangoma’ are used to refer exclusively to the herbalist and diviner, respectively. In modern time the distinction has become blurred, with the healers practicing both arts. In addition to herbalists and diviners who are believed to be spiritually empowered, there are traditional birth attendants, prophets, spiritual healers, spirit mediums, intuitives and dreamers (Van Wyk, 2009).

2.2.4 Other aspects of traditional medicine in South Africa

Of the nearly 4 000 ethnomedicinal plant taxa used in South African traditional healthcare (Arnold *et al*., 2000), relatively few are considered likely to give rise to serious toxicity. Poisoning from traditional medicines is usually a consequence of misidentification, incorrect preparation or inappropriate administration and toxic dosage (Stewart and Steenkamp, 2000), frequently due to self-administration rather than innate risks of using traditional healthcare (Popat *et al*., 2001). Traditional healers possess considerable knowledge of medicinal plants and how to avoid acute poisoning (Savage and Hutchings, 1987). Several plants used in South African traditional medicine can cause damage to genetic material and, therefore, have the potential to cause long-term damage in patients when administered as medical preparations. The use of the abovementioned plant material for treatment of ailments should be treated with caution and rigorous. Toxicological and clinical studies are necessary before prescribed in traditional medicine (Stewart and Steenkamp, 2000).

2.3 Some medicinal plants used against pain and inflammation

About 18% of the world depends on herbal-based alternative systems of medicine. An estimated 70 000 plants (including lower plants) are used in medicine.

Herbal medicine is now expanding at an astonishing pace due to the great inputs from ethnomedicinal practice being pooled from all over the world (Daniel M, 2006). African traditional medicine is the oldest and perhaps the most diverse of all medicine systems (Daniel M, 2006). The biological and cultural diversity that constitute the cradle of mankind (there are more than 2 000 languages in Sub-Saharan Africa is reflected in the market regional differences in healing practices. Unfortunately, the various systems were poorly recorded and remain so to this day (Van Wyk and Wink, 2004).

2.3.1 *Boophone disticha*

Boophone disticha: Common names, Century Plant, Poison Bulb, Sore-eye Flower, Kxutsana-yanaha Motlasisa (Sesotho) Inkwadi (Xhosa, Zulu); Ibhade (Zulu) is an attractive, deciduous bulbous plant with a thick covering of dry scales above the ground. The large, round heads are sometimes on such sort terms that they appear to grow directly from the bulbs, almost at ground level. The colour of flowers are from shades of pink to red and are sweetly scented (July to October). The pedicels (flowers stalks) elongate after flowering to form a large seed head. This breaks off at the top of the scape (stalks) and tumbles across the veld dispersing the seed (Van Der Spuy, 1971).

The greyish green leaves are erect arranged in a conspicuous fan and are usually produced after flowering in spring. The bulb is very poisonous*. Boophone disticha* contains extremely toxic alkaloids and several human deaths have been recorded (Du Plooy *et al*., 2001). *Boophone disticha* has many medicinal uses. The Bushmen once used the poison for their arrows, and traditional healers use it to treat pain and wounds. Parts of the plant are used by certain African tribes and also by some Europeans to cure specific ailments. The outer covering of the bulb is applied to boils and abscesses. Fresh leaves are used to stop bleeding and wounds. The name ‘sore-eye’ flower refers to the fact that if a person is exposed to the open flowers in a confined place, it may lead to sore eyes and even to a headache (Van Vyk, 1998; Jackson, 1990).

2.3.2 *Lippia javanica*

Lippia javanica commonly called fever tea lemon bush (English), Koorsbossie Benkesbossie (Afrikaans), inZinziniba (Xhosa), unSuzwane (Zulu) is 1-2 m high woody shrub that stands erect. The multistems have a square appearance when looked at in cross-section. The leaves are hairy with noticeable veins, giving off a lemon-like odour when crushed. It is said to be one of the most aromatic plant of South African's indigenous shrubs. The small cream flowers can be found on the shrub from summer to autumn in some areas. These flowers are arranged in dense rounded flower heads. The fruits are rather inconspicuous, small and dry (Wan Wyk, 1998; Van Wyk, 2000). These plants are widespread throughout a large part of South Africa, with the exception of the Western Cape. *Lippia javanica* grows from the Eastern Cape Northwards extending into Tropical Africa including Botswana, Swaziland, Malawi, Zambia, Tanzania and Kenya. This herb is said to be an effective treatment of fever, especially in case of malaria influenza, measles and as prophylactic against lung infections. The smoke from the herb has proven to be effective, when inhaled against asthma, chronic cough and pleurisy. Skin disorders such as heat rash and other rashes, as well as scratches, stings and bites can also be treated (Van Wyk, 1997; Fox, 1983). *Lippia javanica* is rich in volatile oil and numerous monoterpenoids have been identified, including myrcene, caryophyllene, linalool, *p*-cymene and ipsdienone. The volatile oil of *Lippia javanica* has decongestant, strong anti-septic effects and anti-inflammatory activity (Van Wyk, 2009).

2.3.3 *Anemone vesicatoria*

2.3.3.1 Plant description

Anemone vesicatoria (previously known as *Knowltonia vesicatoria*) is a perennial herb with a short rhizome and fleshy roots, usually growing on shady slopes and in forests. The leaves are firm-textured, dark green and divided into three leaflets. The leaf stalks are purple-red towards their bases. White or yellowish flowers are produced in the winter and early spring, followed by small black fleshy fruits. Several other known species of *Anemone* (formerly know Tonia), include

Anemone anemonoides (Zulu: "uxaphusa"), *Anemone brateata* (Zulu: "umvuthaza") and *Anemone knowltonia* (Anemone *capensis*; Afrikaans: “katjedrieblaar"). The fresh leaves are mainly used in medicine, sometimes also the roots. *Anemone vesicatoria* is widely distributed in the western and southern parts of South Africa, mainly in the Western Cape (Watt and Breyer-Brandwijk, 1962; Hutchings *et al*, 1996).

2.3.3.2 Medicinal uses of *Anemone vesicatoria*

The plant is an old Cape remedy for lumbago and rheumatism (Forbes, 1986; Smith, 1966). Decoctions of the roots, mixed with *Pelargonium* roots, have also been used to treat colds and influenza (Watt and Breyer-Brandwijk, 1962). Fresh leaves and roots are bruised and applied to the skin, producing blisters (hence the names "vesicatoria" and "brandblaar") (Watt and Breyer-Brandwijk, 1962; Smith, 1966). Infusions may be used for the same purpose. Smoke from burning leaves (Hutchings *et al*., 1996) or the fumes from crushed leaves (Watt and Breyer-Brandwijk, 1962) may be inhaled for headaches.

2.3.3.3 Active ingredients

Many species of the Ranunculaceae family are known to contain irritant, toxic yellow oil known as protoanemonin (Bruneton, 1995). When the leaves or roots are dried, protoanemonin is rapidely dimerised to form non-toxic anemonin (Bruneton, 1995). A structurally related glycoside, ranunculin, is found in several members of the family. Protoanemonin produces a strong allergic reaction on the skin, resulting in blistering. This has an effect similar to mustard oil, which is a powerful irritant and has been used as a counter-irritant and rubefacient (Bruneton, 1995).

2.4 Plants used in this study

2.4.1 *Plantago lanceolata*

A rosette-forming perennial herb bearing narrowly oblong, sparsely hairy and distinctly parallel-veined leaves distinctly ridged flowering stalks and numerous inconspicuous white or pale pink flowers in a dense solitary clusters (Van Wyk, 2004). Plantain is used mainly against catarrhs of the respiratory tract and inflammation of the mouth and throat. It is externally used to treat wounds and inflammation of the skin. Greater plantain herbs are traditionally used against cystitis, haematuria and for relief from irritating and bleeding haemorrhoids. It is used to treat colds and flu in Chinese modern herbalism (Van Wyk, 2004).

Plantain draws toxicity from the body. It has a long history of use as a remedy for blood poisoning and is considered an ‘alternative blood purifier in the old sense of the word. Its rich nutriments stimulate the liver and enrich or ‘cleanse the blood. It is used to treat liver problems, poor digestion, hepatitis, jaundice, skin eruption and eruptive personality (too much heat in the body). Plantain is the poultice herb supreme. The leaves can be chopped, mashed, and placed directly over the problem area (Figure 2.1). They can be made into a strong tea, and a cloth soaked in the tea is placed directly over the area. As a poultice, plantain is a highly effective remedy for bites and insect stings, boils and other eruptive skin disorders and any deep-seated infection. Plantain has such excellent drawing properties that it can be used to remove slivers that are too deep to pull out (Rosemary, 2012).

Figure 2.1: *Plantago lanceolata* (Lam).

Plantain also has haemostatic properties, meaning that it can help check bleeding. As a tea or tincture, plantain can also be used to stanch heavy menstrual bleeding. Though it can be used alone to stop bleeding, it's more effective when blended with yarrow and nettle for this purpose. It's also an excellent wound healing agent shortening recovery time. All parts of plantain are used for medicinal purpose mainly seeds, roots, and leaves (Rosemary, 2012).

Plantain is nutrient dense, containing proteins, starch, and a host of vitamins and can thus serve as an excellent emergency food. Though it can be bitter and stringy as it gets older, it's a tasty ingredient in many wild-food dishes. The plantain seeds, which grow at the top of a long slender stalk, are rich in mucilage and mildly laxative. A cultivated variety of plantain, *Plantago phylum*, is grown for its large, abundant seed. *Psyllium* seeds are the main ingredients in metamucil. Plantain is safe; no known reactions or harmful side effects (Rosemary, 2012). *Plantago lanceolata* contain irridoid glycosides up to 2.5% of dry weight. The main compound is aucubin, accompanied by catalpol and asperuloside, furthermore, 2% mucilage, tannins, phenolic acids (chlorogenic and caffeic acid), unidentified saponins and flavonoids (Van-Wyk, 2004).

2.4.2 *Acokanthera oppositifolia*

Common names: Bushman's Poison bush (English); Gewone Gifboom, Boesmansgif (Afrikaans); Uhlunguyembe (Zulu); Nthunguyembe (Xhosa).

2.4.2.1 Botanical description

Acokanthera oppositifolia Lam is an evergreen shrub or small tree (Kupicha, 1982; Codd, 1963), usually about 2-5 m in height. The leaves are thick and leathery, glossy dark green above the paler below, often tinged red, without any hairs but with conspicuous veins. The fragrant white flowers are about 10 mm long and in dense clusters almost throughout the year (Figure 2.2). The plum-like berries are 10-15 mm long turning dark purple at maturity. Leaves and roots are used for medicinal purpose (Van Wyk *et al*., 2009).

Figure 2.2: *Acokanthera oppositifolia (*Lam) Codd (JMK, 2014).

2.4.2.2 Medicinal uses

The dried leaves (or roots) are used to treat headaches or as a treatment for snake bite. Leaf infusions are taken for abdominal pain. Colds, anthrax, and tapeworm infestation are also treated with extracts of this plant. *Acokanthera* species are best known as sources of extremely toxic arrows poison (Forbes, 1986).

2.4.2.3 Preparations and dosage

The leaves are dried, powdered and sniffed, or may be soaked in water and the extract used as a nasal spray. As a snake bite treatment, the leaves or roots are powdered and applied either directly to the bite or as a paste (Van Wyk *et al*., 2009).

2.4.2.4 Active ingredients

The plant is extremely toxic due to a high content of heart glycosides such as acovenoside A, with smaller amounts of acolongifloroside K and several other minor

constituents. Among these is ouabain, the famous arrow poison from the East Africa *Acokanthera schimperi* (Van Wyk *et al*., 2009).

2.4.2.5 Pharmacological effects

Toxic, analgesic and decongestant properties have been ascribed to *Acokanthera*. Ouabain (Strophantin-G) has been used as an injection in congestive heart failure (Van Wyk *et al*., 2009).

2.4.2.6 Distribution

This species is widely distributed along the western and northern parts of South Africa where it occurs in various habitats, from dense forest to open savannah, often in rocky places. It is also distributed in Mozambique, Zimbabwe, Zambia, DRC, Malawi, Tanzania and Kenya. (Van Wyk *et al*., 2009).

2.4.3 *Artemisia vulgaris*

Artemisia vulgaris is distributed in Europe, in Africa, in Asia, and in America. This plant may have been transported throughout the world by early humans who needed it for its medicinal and food value. It can be easily transported as seeds. The meaning of Mugwort may be marsh root since it grows near permanent sources of water. The scientific name *Artemisia* comes from Artemis, Greek Goddess of the hunt, wild animals, wilderness, childbirth and virginity. Artemis is capable of bringing or relieving disease in women (James *et al*., 2012). The three species of *Artemisia* differ somewhat in appearance, perhaps the result of growing in different habitats for thousands of years. *Artemisia* is easy to grow from seeds and likes shade. The perennial plant grows to 2.5 m high and has variably lobed oblanceolate leaves to 1 to 7 lobes. The leaves can be up to 15 cm long, are green on top and white, tomentose on the underside. The stem and roots are woody (Figure 2.3). The flowers grow in panicles as small disciform heads, less than 5 mm in diameter, contain 5-9 pistillate flowers and 6-25 disk flowers (James *et al.*, 2012).

Figure 2.3: *Artemisia vulgaris* (Lam).

Since ancient times, *Artemisia vulgaris* has been used for medicinal and magical purposes. The main modern use is as an aromatic bitter to treat dyspepsia and lack of appetite. It is traditionally considered to be useful medicine for the treatment of neuroses, depression, restlessness, insomnia, anxiety and irregular or painful menstruation (Van Wyk, 2004). It is also used to treat flatulence, distension, colic, diarrhoea, constipation, cramps, worm infestation, hysteria, epilepsy, vomiting, menstrual problems, irregular periods and to promote circulation and as a sedative. The root has different uses, as a tonic, for psychoneurons, neurasthenia, depression, autonomic neuroses, irritability, restlessness, insomnia and anxiety. *Artemisia vulgaris* is described as an abortifacient without discussion of the preparation method (Bisset, 2000).

The plant contains many active compounds including the monoterpenes, eucalyptol, camphor, linalool, thujone, 4-terpineol, borneol, spathulenol and 21 others (Bisset, 2000). These monoterpenes are present in the essential oils that make up to 0.03%-0.3% of the plant. The plant also contains sesquiterpenes and

sesquiterpene lactones such as eudesmane, vulgarin, psilostachyin and psilostachlyin C (Bisset, 2000). The plant also contains flavonol glycosides, quarcetin 3-O-glucoside, rutin and isorhamnetin 3-*O*-glucoside (Bisset, 2000), coumarins such as easculin, umbelliferone, scopoletin, coumarin and 6-methoxy-7-8-methylene-dioxycoumarin (Bisset, 2000). Polyacetylenes, carotenoids, and pentacyclic triterpenes such as sitosterol and stigmasterol have been isolated or reported (Bisset, 2000).

2.4.4 *Conyza canadensis*

The genus *Conyza* belongs to a well-known family called Asteraceae which consists of about around 50 species. Some species of this genus are traditionally used for a variety of pharmacological applications such as the treatment of smallpox, chicken pox, sore throat, ringworm, skin diseases, toothache and bleeding (Shinwari *et al*., 2000). *Conyza canadensis* is used for rheumatism, antidiarrheal and as antihaemorrhoidal (Shahkirullah *et a*l., 2011). *Conyza canadensis* also have antiviral activity (Hakizamungu *et al*., 1992; Mathiu *et al*., 2007; Titanji *et al*., 2008), and antibacterial activity (Nisar *et al*., 2013). Unidentified compounds in chloroform and ethylacetate extracts of *Conyza canadensis* had antifungal activity (Nisar *et al*., 2013). Secondary compounds possessing anti-inflammatory activity were isolated from the genus (Figure 2.4) (Manguro *et al*., 2011; Mohammad *et al*., 2009).

Figure 2.4: *Conyza canadensis (*Lam) Cronquist.

2.5 Phytochemical analysis of plants

The active ingredients in medicinal plants are chemical compounds that act directly or indirectly to prevent of treat disease, and maintain health. The active compound may be extracted from the plant in a pure form, after which it is identified and tested. Natural, plant-based products are becoming increasingly popular nowadays. These phytomedicines are sold as extracts or powders in which the concentration of the active ingredient are standardised to ensure safety and efficacy (Van Wyk *et al*., 2009).

Medicinal plants contain some organic compounds which produce definite physiological action on the human body and these bioactive substances include tannins, alkaloids, carbohydrates, terpenoids, steroids and flavonoids. They are of great importance to the health of individuals and communities. Many of these indigenous medicinal plants are used as spices and food plants. Phenolics have been known to possess a capacity to scavenge free radicals. The antioxidant activity

of phenolic is principally due to their redox properties, which allow them to act as reducing agents, hydrogen donors. Phenolics are especially common in leaves, flowering tissues and woody parts, such as stems and barks. Studies have shown that they play an important preventive role in the development of cancer, heart diseases and ageing related diseases (Anjali and Sheetal, 2013).

2.6 Antioxidative activity

Antioxidative act as a defence mechanism to protect against oxidative damage, and include compounds to remove or repair damage molecules. They can prevent or retard the oxidation caused by free radicals, and sufficient intake of antioxidants is supposed to protect against certain diseases (Celiktar et *al*., 2007). Free radicals are not only produced naturally in the cell following stress or respiration, but can also emerge as a result of radiation, bacterial and viral toxins, smoking, alcohol or physiological or emotional stress. The body produces many antioxidant enzymes such as superoxide dismutase, catalase and glutathione peroxidase, which neutralize many types of free radicals (Gamiotea-Turro et al., 2004).

Oxidative stress is a factor for many human diseases, as either a cause or an effect. Plants are the source of medication for preventive, curative, protective or promotive purpose (Sidhu *et al*., 2007). However, the natural antioxidant compounds become important (Malpure *et al*., 2006). Several groups of constituents in plants have been identified as potentially health promoting in animal studies, including cholesterol lowering factors, antioxidants, enzyme inducers, and others (Dragster *et al*., 2006). Many of these herbal medicines are finding their way into the world market as alternatives to prescribed allopathic drugs currently available to treat various disorders and ailments (Huang *et al*., 2005). The rapid increase in the consumption of traditional herbal remedies worldwide has been stimulated by several observations which have shown their use as alternative medicine. In particular, cancer patients are reported to benefit from treatment with herbal medicine and survivability in many cases is significantly increased. Plants that are

eaten as foods in southern Africa were shown also to provide important health benefits in the form of antioxidative activity (Lindsey *et al*., 2002).

The medicinal value of plants have assumed important dimensions in the past few decades owing mainly to the discovery that extracts from plants contain not only minerals and primary metabolites but also a diverse array of secondary metabolites with antioxidative potential (Akinmoladun *et al*., 2007). Plant-based antioxidants are now preferred over synthetic because of safety concerns (Akinmoladun *et al*., 2007). This factor has inspired the widespread screening of plants for possible medicinal, antimicrobial and antioxidative properties (Jayaprakasha *et al*., 2001). Antioxidative substances block the action of free radicals implicated in the pathogenesis of many diseases including atherosclerosis, ischemic heart disease, cancer, Alzheimer's disease and in the aging process (Aruoma, 1998).

2.7 Pathways of inflammation

2.7.1 Cyclooxygenase pathway

Cyclooxygenase-1 and cyclooxygenase-2 (COX-1 and COX-2) are prostaglandin synthase that catalyse sequential synthesis of prostaglandins G_2 (PGG_2) and PGH_2 from arachidonic acid through intrinsic COX and peroxidase activities (Agarwal *et al*., 2009). This group includes the traditional (in the historical sense) non-steroidal anti-inflammatory drugs as well as newer coxibs that are more selective for COX-2 than COX-1. Non-steroidal anti-inflammatory drugs (NSAIDs), sometimes called the aspirin-like drugs or antipyretic analgesics are among the more widely used of all agents. There are now more than 50 different NSAIDs on the global market. These drugs provide symptomatic relief from pain and swelling in chronic joint diseases such as occurs in osteo and rheumatoid arthritis, as well as in more acute inflammatory conditions such as fractures, sprains, sports and other soft tissue injuries (Rang *et al*., 2012). They find application in the treatment of postoperative, dental and menstrual pain. Several NSAIDs available over the counter are widely used for other types of minor aches and pains. Virtually all these drugs, particularly the traditional NSAIDs, can have significant unwanted effects, especially in the elderly. Newer agents have fewer adverse effects (Rang *et al*., 2012).

Prolonged use of NSAIDs is also associated with severe side effects such as gastro-intestinal haemorrhage due to COX-1 inhibition (Lee *et al*., 2003).

While there are differences between individual NSAIDs, their primary pharmacology is attributed to the inhibition of COX enzymes, to decrease the production of prostaglandins and thromboxane. There are two common isoforms in the enzyme, COX-1 and COX-2. While COX-1 and COX-2 are closely related and catalyse the same reaction, there are differences in the expression and role of these two isoforms. Cyclooxygenase-1 is constitutively expressed in most tissues, including blood platelets. It has a 'housekeeping' role in the body, involved in tissue homeostasis, the production of prostaglandin (Rang *et al.,* 2012). In contrast, COX-2 is induced in inflammation activated by the inflammatory cytokines-interleukins (IL)-1 and tumour necrosis factor (TNF)-1. Then, the COX-2 isoform is mainly responsible for the production of the prostanoid mediators of inflammation (Vane and Botting, 2001). There is also a considerable pool of constitutive COX-2 present in the central nervous system (CNS) and some other tissues, although its function at these sites is yet to be completely understood (Rang *et al*., 2012). The new COX-2 selective drugs do not seem to be free of risk either since several COX-2 inhibitors cause cardiovascular problems (Muklerjee *et al*., 2001). Steroids have an obvious role in treatment of inflammatory diseases but, due to their potential dependency and toxicity, there is a strong need for natural products with minimum side effects (Figure 2.5).

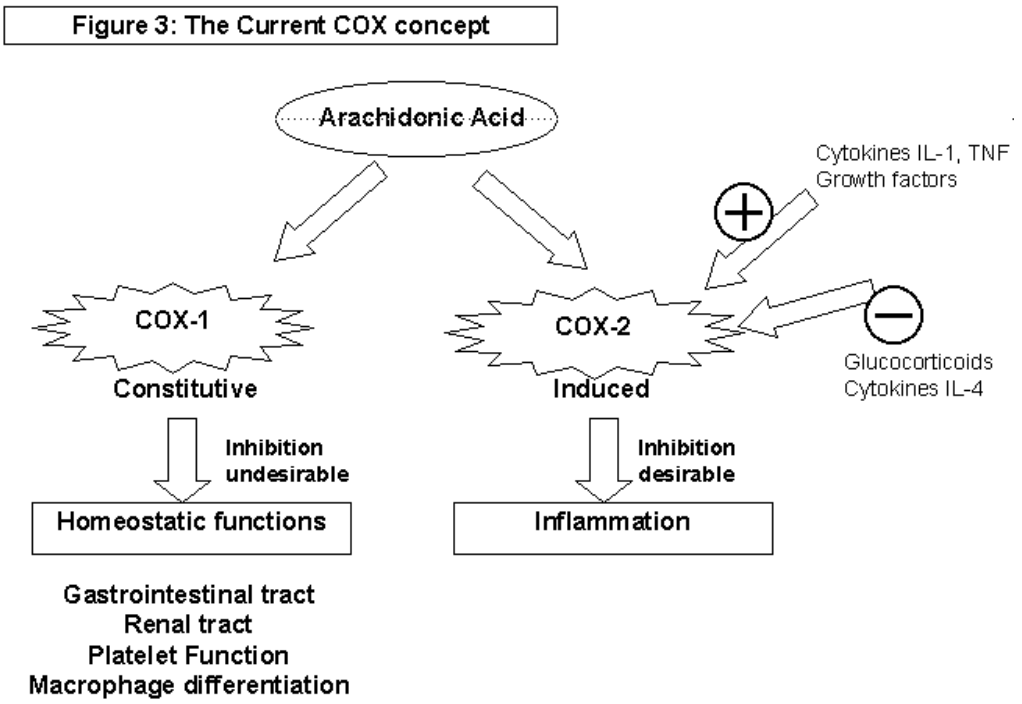

Figure 2.5: Cyclooxygenase-1 and cyclooxygenase-2 (Portanova *et al*., 1996).

Most traditional NSAIDs inhibit both COX-1 and COX-2, although they vary in the degree to which they inhibit each isoform. It is believed that the anti-inflammatory probably most analgesic and antipyretic actions of the NSAIDs are related to inhibition of COX-2, while their unwanted effects, particularly those affecting the gastrointestinal tract are largely a result of their inhibition of COX-1 (Rang *et al*., 2012). Compounds with a selective inhibitor action on COX-2 are employed clinically. COX-2 seems to be an important target in healing and resolution. There is also a concern about the cardiovascular effects of all NSAIDs when these are taken over a long time (Rang *et al*., 2012).

While the pharmacological actions of NSAIDs are broadly similar, there are exceptions. Aspirin has other quantitatively different pharmacological actions, and Paracetamol is an interesting exception to the general NSAIDs ‘stereotype’. While it

is an excellent analgesic, and antipyretic, anti-inflammatory activity is slight and seems to be restricted to a few specials cases such as inflammation following dental extraction (Skjelbred *et al*., 1984). Paracetamol inhibits prostaglandin biosynthesis in some experimental settings (during fever) but not in others.

2.7.1.1 Mechanism of action

The main actions of non-steroidal anti-inflammatory drugs (NSAIDs) emanate from the inhibition of arachidonic acid (AA) oxidation by the fatty acid COXs. These are bi functional enzymes, having two distinct catalytic activities. The first, dioxygenase step incorporates two molecules of oxygen into the arachidonic (or other fatty acid substrate) chain at C11 and C15, giving rise to the highly unstable endoperoxide intermediate PGG_2 with a hydroperoxy group at C15. A second peroxidase function of the enzyme converts PGG_2 to PGH_2 with a hydroxyl group at C15, which can then be transformed in a cell-specific manner by separate isomerase, reductase or synthase enzymes into other prostanoids. Both COX-1 and COX-2 are heme containing homodimeric oligomeric enzymes linked to intracellular membranes. Structurally, the isoforms are similar, both containing a hydrophobic channel into which the arachidonic or other substrate fatty acids dock for catalyses (Rang *et al*., 2012).

Most NSAIDs are competitive inhibitors interfering with the initial deoxygenation reaction through competition with the normal substrate for the active site. They inhibit COX-1 more rapidly, and COX-2 in a more time-dependant manner and is often irreversible. To block the enzyme, NSAIDs enter the hydrophobic channel, forming hydrogen bonds with an arginine residue in position 120, preventing substrate fatty acids from entering into a catalytic domain. However, a single amino acid change in the structure of the entrance of this channel in COX-2 results in a bulky side pocket that is not found in COX-1. This is important in understanding why some drugs, especially those with large sulphur-containing side groups, are most selective for the COX-2 (Rang *et al*., 2012).

Other actions, besides inhibition of cyclooxygenase they may contribute to the anti-inflammatory effects of some NSAIDs. Reactive oxygen radicals produced by neutrophils and macrophages are implicated in tissue damage in some degenerative conditions. Some NSAIDs have oxygen radical-scavenging effects as well as COX inhibitory activity, and thus may decrease tissue damage caused by reactive oxygen radicals. Aspirin also inhibits expression of the transcription factor, which has a key role in the transcription of the genes for inflammatory mediators (Rang, *et al*., 2012).

2.7.2 The lipoxygenase pathway

Lipoxygenases (LOXs) belong to the groups of dioxygenases involved in the insertion of one molecule of oxygen at different sites in arachidonic acid (AA). The particular site of incorporation of oxygen is tissue and enzyme-specific. Lipoxygenases catalyse the oxygenation of poly-unsaturated fatty acid (PUFA) containing a 1,4-*Cis*, *cis*-pentadiene system, producing a 1-hydroperoxy-2,4-*trans*, *cis*-pentadine product. Four main types of LOXs with positional specificities occur in animal tissues: 5-lypoxygenase, 8-LOX, 12-LOX, and 15-LOX. 5-LOX is found in mast cells, macrophages and neutrophils, where if functions in the synthesis of 5-hydroperoxy eicosatetraenoic acid (5-HPETE) and leukotrienes B4 (LTB4), C4 (LTC4) and D4 (LTD4). 5-hydroxy eicosatetraenoic acid (5-HETE), a reduced product of (5-HPETE) has potent chemotactic activity towards neutrophils, eisinophils monocytes and macrophages. Leukotriene C4 (LTC4) and leukotriene D4 (LTD4) are derived from leukotriene A4 (LTA4) and are bronchoconstrictor components of slow-reacting substance of anaphylaxis (SRS-A) (Poeckel and Punk, 2010). In comparison to histamine, LTC4 and LTD4 are 1000-fold more potent bronchoconstrictors and probably extremely important mediators of asthma allergic reactions (Chung, 1995).

Three lipoxygenases, 5-LOX, 8-LOX, and 12-LOX have procarcinogenic potential, whereas 12-LOX has anticarcinogenic effects. Products of 12R-LOX are associated with various skin diseases (Argawal *et al*., 2009) and 15-LOX products are associated with atherosclerosis. The roles of various LOX metabolites in

regulating carcinogenesis have been studied. 15-lypoxygenase (15-LOX) is a critical regulator of leukaemia cancer stem cells in chronic myeloid leukaemia (CML). Treatments of CML with a 5-LOX inhibitor also impair the functions of leukaemia cancer stem cells. Inhibition of 5-LOX by vitamin E and benzyl propargyl ether has been reported (Poeckel and Punk, 2010).

There are three LOX isozymes, 5-, 12-, and 15-LOX, that have been identified in humans with the ability to convert AA into pro-inflammatory hydroperoxy eicosatetraenoic acids (HPETEs), 5-, 12-, and 15-HPETE (Kuhn *et al*., 2000). In contrast to other LOX enzymes, 15-LOX also initiates the synthesis of lipoxins (LXs) involved in the resolution phase of inflammation (Serhan C.N *et al*., 1984). This will focus on the role of 5-LOX and synthesis of LTs as targets for permeability leading to oedema by contracting endothelial cells (EC) in the microvasculature (Figure 2.6). Leukotrienes also play an important role in GI mucus protection (Rioux *et al*., 1994; Vaananen *et al*., 1992). In addition, they can interact with sensory nerve fibres, leading to changes in their excitability and enhanced release of tachykinins. Therefore, LTs are important mediators of various inflammatory diseases and allergic disorders including asthma, rheumatoid arthritis, inflammatory bowel disease, ulcerative colitis, psoriasis and allergic rhinitis (Lewis et al., 1990).

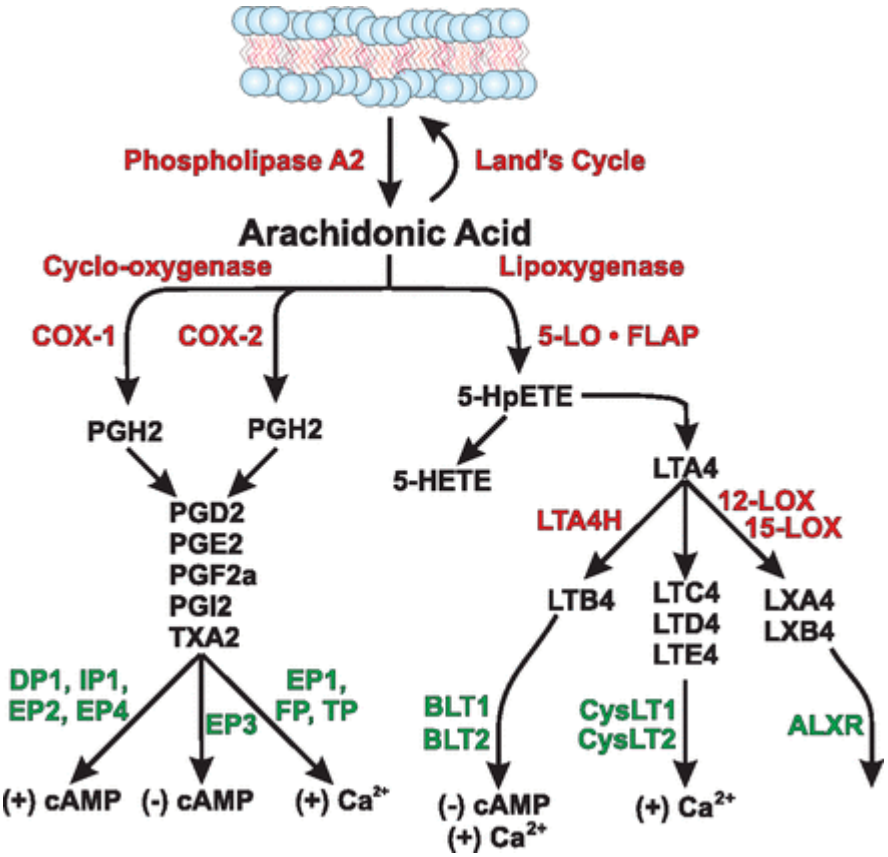

Figure 2.6: Arachidonic acid metabolism from phospholipase A2 indicating cyclooxygenase and lypoxygenase pathways (Su and O'Connor, 2013).

2.7.3 Nitric oxide

2.7.3.1 Nitric oxide chemistry

There are direct chemical reactions that are defined as those in which nitric oxide (NO) interacts with biological targets. The most common reaction is between NO and haeme-containing proteins. Such reactions are generally rapid, requiring low concentrations of NO and most likely account for the majority of the physiological effects of NO. The inductive form of nitric oxide synthase (NOS) is the most important pro-inflammatory enzyme responsible for increasing the level of NO (Eun-Jin *et al*., 2012). Nitric oxide, synthesised from L-arginine through NOS is a short-lived free radical and a potentially toxic molecule. It functions as an intracellular

messenger mediating a wide range of biological functions. To date, three isoforms of NOS have been identified. Conversely, indirect effects involve reactive nitrogen oxide species.

2.7.3.2 Nitric oxide and inflammation

There is a large body of evidence that nitric oxide (NO) is involved in several inflammatory disorders. Indeed, virtually every cell and many immunological parameters are modulated by NO. It has been shown that NO can be pro-inflammatory (immunostimulatory, anti-apoptotic) or anti-inflammatory (immunosuppressive, pro-apoptotic), host-protective or host damaging during infections (Giuseppe *et al*., 2006).

2.8 Mechanism of pain

2.8.1 Inflammation related pain

Pain is a subjective experience, hard to define exactly, even though we all know what we mean by it. Typically, it is a direct response to an untoward event associated with tissue damage or stress, such as injury, inflammation or cancer, but severe pain can arise independently of any obvious predisposing cause (e.g. trigeminal neuralgia), or persist long after the precipitation injury has healed (e.g. phantom limb pain). It can also occur as a consequence of brain or nerve injury (e.g. following a stroke or herpes infection). Painful conditions of the latter kind, not directly linked to tissue injury, are often described as 'neuropathic pains'. They are very common and a major cause of disability and distress, and in general they respond less well to conventional drugs than do conditions where the immediate cause is clear. In these cases, we need to think of pain in term of disordered neurone neural function rather than simply as a normal response to tissue injury (Rang *et al*., 2012).

2.8.2 Nociceptive Afferent Neurons

Under normal conditions, pain is associated with impulse activity in small-diameter (C and Aδ) primary afferent fibres of peripheral nerves. These nerves have sensory endings in peripheral tissues and are activated by stimuli of various kinds (mechanical, thermal, chemical) (Julius and Basbaum, 2001; Julius and McCleskey, 2006). The majority of unmyelinated (C) fibres are associated with polymodal nociceptive endings and convey a dull, diffuse, burning pain, whereas myelinated (Aδ) fibres convey a sensation of sharp, well-localised pain. C and Aδ fibres convey nociceptive information from muscle and viscera as well as from the skin. With many pathological conditions, tissue injury is the immediate cause of the pain and results in the local release of a variety of chemicals that act on the nerve terminals, either activating them directly or enhancing their sensitivity to other forms of stimulation (Julius and Basbaum. 2001).

2.8.3 Modulation in the nociceptive pathway

Acute pain is generally well accounted for in term of nociception: An excessive noxious stimulus giving rise to an intense and unpleasant sensation. In contrast, most chronic pain states are associated aberrations of the normal physiological pathway, giving rise to hyperalgesia (an increased amount of pain associated with a mild noxious stimulus), allodynia (pain evoked by a non-noxious stimulus) or spontaneous pain without any precipitating stimulus (Tracey, 2008).

2.8.4 Transmission of Pain to Higher Centres

From the dorsal horn, ascending nerve axons travel in the contralateral spinothalamic tracts, and synapse on neurons in the ventral and medial parts of the thalamus, from which there are further projections to the somatosensory cortex. In the medial thalamus in particular, many cells respond specifically to noxious stimuli in the periphery, and lesions in this area cause analgesia. Functional brain imaging studies in conscious subjects have been performed to localise regions involved in pain processing. These include sensory, discriminatory areas such as primary and secondary somatosensory cortex, thalamus and posterior parts of insula as well as

affective, cognitive areas such as the anterior parts of insula, anterior cingulate cortex and prefrontal cortex (Tracey, 2008).

2.8.5 Neuropathic Pain

Neurological disease affecting the sensory pathway can produce severe chronic pain termed neuropathic pain unrelated to any peripheral tissue injury. This occurs with central nervous system disorders such as stroke and multiple sclerosis or with conditions associated with peripheral nerve damage, such as mechanical injury, diabetic neuropathy or herpes zoster infection (shingles). The pathophysiological mechanisms underlying this kind of pain are poorly understood, although spontaneous activity in damaged sensory neurons, due to overexpression or redistribution of voltage-gated sodium channels, is thought to be a factor (Lai *et al*., 2004; Chahine *et al*., 2005).

The sympathetic nervous system also plays a part, because damaged sensory neurons can express α-adrenoceptors and develop sensitivity to noradrenaline that they do not possess under normal conditions. Thus, physiological stimuli that evoke sympathetic responses can produce severe pain, a phenomenon described clinically as sympathetically mediated pain. Neuropathic pain, which appears to be a component of many types of clinical pain (including common conditions such as back pain and cancer pain, as well as amputation pain), responds poorly to conventional analgesic drugs but can be relieved by some antidepressant and antiepileptic agents . Potential new targets are discussed at the end of this chapter (Chahine *et al*., 2005).

2.9 Types of analgesics

Analgesics are divided into the opioids (with similar properties to drugs derived from opium, such as Morphine) and non-opioids. Non-opioids include all the other analgesics, including paracetamol, nefopam, and also the non-steroidal anti-inflammatory drugs (NSAIDS), the best known in which is aspirin. The non-opioids

are all less powerful as painkillers than the opioids (O'Shaughnessy, 2011). Opioid drugs and paracetamol act directly on the brain and spinal cord to alter the perception of pain. Opioids act like the endorphins; hormones naturally produced in the brain and stop the cell-to-cell transmission of pain sensation (O'Shaughnessy, 2011).

2.9.1 Opioids analgesics

Opioids are defined as compounds with effects that are antagonized by naloxone. There are three families of opioid peptides, which are derived from large precursor molecules, encoded for by separate genes. Pro-opomelanocortin (POMC) gives rise to the opioid peptide β-endorphin and a number of other non-opioid peptides, including leu-enkephalin and met-enkephalin. Prodynorphin gives rise to a number of opioid peptides, which contain leu-enkephalin and their amino terminal (e.g. dynorhin A). The peptides derived from each of these three precursor molecules have a distinct anatomical distribution in the central nervous system and have varying affinity to the different types of opioid receptors. The precise function of these opioid peptides in the brain and elsewhere is still unclear (Neal, 2005).

Opioid analgesics are drugs that mimic endogenous opioids peptides by causing a prolonged activation of opioid peptides receptors (usually μ-receptors). This produces analgesia, respiratory depression, euphoria and sedation. Pain acts as an antagonist of respiratory repression that may, however, become a problem if the pain is removed, e.g. with a local anaesthetic. Opioids often cause nausea and vomiting and antiemetic may be required. Effects of the nerves plexuses in the gut, which also possess opioids peptides and receptors, cause constipation, and laxatives, are usually required. Continuous treatment with opioids analgesic results in tolerance and dependence in addicts. However, in terminally ill patients, a steady increase in morphine dosage is not automatic and, if it does occur, is more likely to result from progressively increasing pain rather than tolerance. Similarly, the clinical concept of dependence is unimportant. Unfortunately, over caution in the use of opioid analgesic frequently results in unnecessarily poor pain control in patients (Neal, 2005).

Some analgesics, such as codeine and dihydrocodeine are less potent than morphine and cannot be given in equianalgesic doses because of the onset of adverse effects. As a result of this restriction in dosage, they are less likely, in practice, to produce respiratory depression and dependence. They are useful in controlling mild to moderate pain (Neal, 2005).

2.9.2 Morphine analogues

Morphine is a phenanthrene derivative with two planar rings and two aliphatic ring structures, which occupy a plane roughly at right angles to the rest of the molecule. The most important parts of the molecule for the opioid activity are the free hydroxyl on the benzene ring that is linked by two carbon atoms to a nitrogen atom. Variants of the morphine molecule have been produced by substitution at one or both of the hydroxyls (e.g. diamorphine 3,6-diacethylmorphine, codeine 3-methoxymorphine, and oxycodone). Substitution of a bulky substituent on the nitrogen atom introduces antagonist activity to the molecule (e.g. naloxone) (Rang *et al*., 2012).

2.9.3 Non-steroidal anti-inflammatory drugs (NSAIDS)

Non-steroidal anti-inflammatory drugs (NSAIDS) and opioids are used in management of mild to moderate and severe pain respectively. These drugs have serious limitations due to the side effects. Opioids cause respiratory depression, euphoria, tolerance and dependence while non-steroidal anti-inflammatory drugs produce gastrointestinal irritation and renal damage (Howland and Micek, 2006). The reign of morphine as the king of narcotic analgesics, is associated with addictive properties and numerous side effects which include respiratory depression, drowsiness, decreased gastrointestinal mobility, nauseas and several alterations of endocrine and autonomic nervous system (Almeida et al., 2001).

2.9.3.1 Aspirin

Used for many years to relieve pain and reduce fever, aspirin also acts to reduce inflammation by blocking the production of prostaglandin, which contributes to the swelling and pain in inflamed tissue. Aspirin is useful for headaches, toothache, mild rheumatic pain, sore throat, and discomfort caused by feverish illnesses. Administered regularly, aspirin may also relieve the pain and inflammation associated with chronic rheumatoid arthritis (O'Shaughnessy, 2011). Aspirin is a weak organic acid that is unique among the NSAIDS in irreversibility acetylating cyclooxygenase. The other NSAIDs, including salicylates, are all reversible inhibitors of cyclooxygenase. The NSAIDs, including aspirin have three major therapeutic actions, namely, they reduce inflammation (anti-inflammation), pain (analgesia), and fever (antipyrexia) (Mycek *et al*., 2000). Aspirin is found in combination with other substances in a variety of medicines. It is also used in the treatment of some blood disorders, since aspirin helps to prevent abnormal clotting of blood by preventing platelets from sticking together. Aspirin, in the form of tablets, dissolved in water before being taken, is absorbed into the bloodstream more quickly, thereby relieving pain faster than tablets. Soluble aspirin is not, however, less irritant to the stomach lining (O'Shaughnessy, 2011).

2.9.3.2 Paracetamol

This analgesic is believed to act by reducing the production of prostaglandins in the brain. However, paracetamol does not affect production of prostaglandin in the rest of the body, so it does not reduce inflammation, although it can reduce fever. Paracetamol can be used for everyday aches and pain such as headache, toothache, and joint pains (O'Shaughnessy, 2011). As well as being the most widely used analgesic, it is one of the safest when taken correctly. It seldom irritates the stomach and allergic reactions are rare. However, an overdose can cause severe and possibly fatal liver or kidney damage. Its toxic potential may be increased in heavy drinkers. The degradative metabolites of paracetamol cause liver toxicity (Graham, 2005).

CHAPTER 3 MATERIALS AND METHODS

3.1 Introduction

The research project was conducted as follows: plants were collected then extracted and kept in dose 10 mg/mL in dimethyl sulfoxide (DMSO) at room temperature for further experimentations. A couple of week later, qualitative phytochemical analysis was done to determine plants chemical constituents of *Plantago lanceolata.* Thereafter, antioxidant activity was done in four plants before anti-inflammatory activity (cyclooxygenase-1 and cyclooxygenase-2, and lipoxygenase). Nitric oxide analysis was then done before cytotoxicity (in four plant materials).

3.2 Plant collection

Leaves of the four plant species: *Acokanthera oppositifolia*, *Plantago lanceolata*, *Conyza canadensis* and *Artemisia vulgaris* were used in this study. *Acokanthera oppositifolia* was collected in June 2012 from the Lowveld Botanical Garden, in Nelspruit. A voucher specimen (PRU/120583/1/adebayoSA) was deposited in the Herbarium of University of Pretoria. *Plantago lanceolata*, *Artemisia vulgaris* and *Conyza canadensis* were collected in the vicinity of Midrand, Johannesburg. A voucher specimen (MosesTUT1, MosesTUT2, and MosesTUT3) were deposited in the Herbarium of Tshwane University of Technology. The leaves were subsequently dried at room temperature in a ventilated room, milled to a fine powder in an atomy mill Polymax (PC-MFC 90 D) and stored in closed containers until used.

3.3 Plant extraction

Plant material (5 g) from each species investigated was separately extracted with 20 mL of acetone SMM instruments), ethyl acetate (SMM instruments), chloroform (SMM instruments), hexane (Merck), and water in polyester centrifuge tubes. The solvents were selected on their solubility. The tubes were vigorously

shaken for 30 minutes in an orbital shaker (Velp Scientifica). Tubes were centrifuged at 2 000 rpm for 10 minutes and the supernatant was filtered using Whatman No.1 filter paper before being transferred into pre-weighed glass containers. The solvent was removed by evaporation under a stream of air in a fume hood at room temperature to produce the dried extract (Eloff, 2004). The extract was reconstituted in DMSO (Merck Schuchardt OHG) at the dose of 10 mg/mL and tested in the assays.

3.4 Extraction of plant materials for phytochemical analysis

Plantago lanceolata leaves were air-dried at room temperature 27^{o}C for two weeks, after they were ground to a uniform powder. The *n*-hexane extract was prepared by soaking 100 g of the dry powdered ground material in 1 L of *n*-hexane at room temperature for 48 h. The extract was filtered after 48 h through a Whatmann filter paper number 42 (125 mm). The extract was then concentrated using a rotary evaporator with the water bath set at 40 ℃. The extract was kept in the DMSO at a dose 10 mg/mL.

3.4.1 Test for anthraquinones

0.5 g of the extract was boiled with 10 mL of sulphuric acid and filtered while hot. The filtrate was shaken with 5 mL of chloroform. The chloroform layer was pipetted into another test tube and 1 mL of dilute ammonia was added. The resulting solution was observed for colour changes.

3.4.2 Test for terpenoids

To 0.5 g of the extract was added to 2 mL of chloroform. Concentrated sulphuric acid, 3 mL was carefully added to form a layer. A reddish brown colouration for the interface indicated the presence of terpenoids.

3.4.3 Test for flavonoids

Three methods were used to test for flavonoids. First dilute ammonia (5 mL) was added to a portion of an aqueous filtrate of the extract. Concentrated sulphuric acid (1 mL) was added. A yellow colouration that disappears on standing indicates the presence of flavonoids. Second, a few drops of 1% of aluminium solution were added to a portion of the filtrate. A yellow colouration indicates the presence of flavonoids. Third, a portion of the extract was heated with 10 mL of ethyl acetate over a steam bath for three minutes. The mixture was filtered and 4 mL of the filtrate was shaken with 1mL of dilute ammonia solution. A yellow colouration indicates the presence of flavonoids.

3.4.4 Test for saponins

To 0.5 g of extract was added 5 mL of distilled water in a test tube. The solution was shaken vigorously and observed for a stable persistent froth. The frothing was mixed with 3 drops of olive oil and shaken vigorously after which it was observed for the formation of an emulsion.

3.4.5 Test for tannins

About 0.5 g of the extract was boiled in 10 mL of water in a test tube and then filtered. A few drops of 0.1 g ferric chloride was added and observed for brownish green or a blue-black colouration.

3.4.6 Test for cardiac glycosides

To 0.5 g of extract diluted to 5 mL in water 2 mL of glacial acetic acid containing one drop of ferric chloride solution was added. This was underplayed with 1 ml of concentrated sulphuric acid. A brown ring at the interface indicated the presence of a deoxysugar characteristic of cardenolides. A violet ring may appear below the brown ring, while in the acetic acid layer a greenish ring may form just above the brown ring and gradually spread throughout this layer.

3.4.7 Test for steroids

2 mL of acetic anhydride was added to 0.5 mL crude extract of plant sample with 2 mL of sulphuric acid. The colour changed from violet o blue or green in sample which indicates the presence of steroids.

3.4.8 Test for quinones

Dilute NaOH was added to 1 mL of crude extract. Blue-green or red colouration indicates the presence of quinones.

3.4.9 Test for coumarin

10% of NaOH was added to the extract and chloroform was added for observation of yellow colour, which shows the presence of coumarin.

3.5 1, 1-diphenyl-2-picryl-hydrazyl assay

The free radical scavenging activity of these plants were measured by 1,1-diphenyl-2-picryl-hydrazyl (DPPH.) method, modified for working with 96 well plate. With this method it was possible to determine the antiradical power of an antioxidant by measuring the decrease in the absorbance of DPPH at 517 nm. As a result of the colour changing from purple to yellow the absorbance is decreased when the DPPH radical is scavenged by an antioxidant through donation of hydrogen to form a stable DPPH-H molecule (Matthäus, B., 2002). This method was applied to ethanolic, methanolic and aqueous extracts of plants (acetone, ethyl acetate, chloroform and hexane).

3.5.1 Plate layout for DPPH assay

Three plate wells number 1-3 were used for this test. Plate number one, contained blank (DMSO A1 to A3), negative control (B1 to B3), vitamin C (C1-3 to

F1-4) in four serial dilutions, positive control (quecertin G1-3), plants extracts (18 samples, acetone, ethyl acetate, chloroform, hexane, and water in triplicate, four serial dilutions). Then 50 µL of extract sample, negative control, positive control, and vitamin C were added to each well. The plate was incubated at room temperature in dark for 30 minutes then read on the plate reader at 570 nm. Then, 50 µL of this solution was added to 150 µL of extracts of plants. The mixture was shaken vigorously and allowed to stand at room temperature for 30 min. Lower absorbance of the reaction mixture indicated higher free radical scavenging activity (Oktay et al., 2003).

The DPPH scavenging effect calculated using following equation: %DPPH scavenging activity = 100- $\frac{sample-blank}{negative-blank}$x100

Where blank is the absorbance of the control reaction (containing all reagents except the test compound) and sample is the absorbance of the test compound. Extract concentration providing 50% inhibition IC_{50} was calculated using the graph by plotting inhibition percentage against extract concentration. Ascorbic acid (Vit C) and quecertin were used as positive control.

3.6 Cyclooxygenase-1 and cyclooxygenase-2 assay

3.6.1 Material used

Water was used to prepare all enzyme immunoassay (IEA) reagents and buffers were deionized and free of trace organic contaminants (‘Ultra-Pure’ available for purchase from Cayman, item No 400000), PG screening EIA antiserum (one vial, Cayman, item No 414016), PG screening AchE tracer (one vial, Cayman, item No 414006), PG screening IEA standard (one vial, Cayman item No 414026), EIA buffer concentrate 10x (2 vial, Cayman item No 400060), wash buffer concentrate 400x (1 vial, Cayman item No 400062), polysorbate 20 (1 vial, Cayman item No 400035), mouse anti-rabbit IgG coated plate (1 plate, Cayman item No 400004), 96-well cover sheet (one cover, Cayman item No 400012), Elman’s reagent (three vial, Cayman item No 400050), reaction buffer 10x (one vial, Cayman item No 460104), Cox-1 ovine (one vial, Cayman item No 460100), Cox-2 human recombinant (one vial, Cayman item No 460121), Heme (one vial, Cayman item No 460102),

arachidonic acid substrate (one vial Cayman item No 460103), potassium hydroxide (one vial, Cayman item No 460105), hydrochloric acid (one vial, Cayman item No 460106), and stannous chloride (one vial, Cayman item No 460107).

3.6.2 Description of Acetylcholinesterase Tracer (ACE^{TM}) competitive Enzyme Immunoassays (EIA$_S$)

This assay was based on the competition between PGs and PG-acetyl cholinesterase (Ache) conjugate (PG tracer) for a limited agent of PG antiserum. Because the concentration of PG tracer is held constant while the concentration of PG varies, the amount of PG tracer that was able to bind to the PG antiserum was inversely proportional to the concentration of PG in the well. This rabbit-antiserum-PG (either free or tracer) complex binds to a mouse monoclonal anti-rabbit antibody that has been previously attached to the well. The plate was washed to remove any unbound reagents and then Elman's reagent (containing the substrate) was added to the well. The product of this enzymatic reaction was a distinct yellow colour and absorbed strongly at 412 nm. The intensity of this colour, determined spectrophotometric ally, is proportional to the amount of PG tracer bound to the well, which is inversely proportional to the amount of free PG present in the well during the incubation (Macleod *et al*., 1987; *Parcells et al*., 1985).

3.6.3 Performing cyclooxygenase reactions

The following reagents were added to two test tubes: 970 µL of Reaction Buffer, 10 µL of Hemet, and 10 µL of inactive COX-1 or inactive COX-2, 950 µL of Reaction Buffer, 10µL of Hemet, and 10 µL of COX-1 to two test tubes, 950 µl of Reaction Buffer, 10 µL of Hemet, and 10 µL of COX-1 to six test tubes, 950 µL of Reaction Buffer, 10 µL of Hemet, and 10 µL of COX-2 to two test tubes, 950 µL of Reaction Buffer, 10 µL of Hemet, and 10 µL of COX-2 to six test tubes, 20 µL of inhibitor to the COX-1 and COX-2 inhibitor tubes and 20 µL of Reaction Buffer or solvent to the 100% initial activity tubes and vortex. 50 µL of 1 M HCL was added to each test tube to stop enzyme catalysis. The test tubes were then removed from the water bath and 100 µL of the saturated Stannous Chloride Solution was added to

each test tube and vortex. The incubation took five minutes at room temperature. Cyclooxygenase-1 and Cyclooxygenase-2 were inactivated by transferring 20 µL of each enzyme to a 500 µL microfuge tube and placing the tube in boiling water for three minutes. The inactivated enzymes were used to generate the background values.

3.6.4 Preparation of assay-specific reagents

3.6.4.1 Prostaglandin screening standard

After dissolving the lyophilized PG Screening EIA Standard (Item No 414026) in 1mL of EIA buffer, the concentration of this solution (the bulk standard) was 10ng/ml. then we stored this solution. The standard was prepared for use in EIA: first eight clean test tubes were numbered 1 through 8. 800 µL EIA buffer was aliquot to tube 1 and 500 µL EIA Buffer to tubes two to eight. 200 µL of the bulk standard (10 µg/mL) was transferred to tube one and mixt thoroughly. Serial dilutions were made. The standard was diluted by removing 500 µL from tube one and placing in tube two; mixed thoroughly. 500 µL was removed from tube two and placed into tube three; mixed thoroughly. This process was repeated for tubes four to eight and stored for 24 h.

3.6.4.2 Cyclooxygenase reaction dilutions

Two clean test tubes were obtained and labelled BC1 and BC2; they were then aliquot 990 µL of EIA Buffer on each test tube. 10 µL of Background COX-1 was added to the tube labelled BC1, 10 µL of Background COX-2 to the dilute labelled BC2, and mixed thoroughly. Each test tube contains 1: 100 dilution of the original sample.

3.6.5 Ninety-six well plate set up for cyclooxygenase reaction dilutions.

A total of 18 samples were used in triplicate. Each plate contained two blanks (BLK), two Non-Specific Binding wells (NSB), two Maximum Binding wells (Bo), one

Total Activity well (TA), and an eight point standard curve run in duplicate. Each COX reaction sample was assayed at two dilutions, and each dilution was assayed in duplicate. A minimum of one 100% initial activity sample was assayed for both COX-1 and COX-2.

3.6.6 Performing of enzyme immunoassay

100 µL of enzyme immunoassay (EIA) Buffer was added to Non-Specific Binding (NSB) wells, 50 µL of EIA Buffer to Maximum Binding (Bo) wells, 50 µL from tube eight to both of the lower standard wells (S8), and 50 µL from tube seven to all of the next standard wells (S7). This procedure was done until all the standards were aliquot. The sample pipette tip was used to aliquot all the standards. 50 µL of background sample was added (BC1 and BC2) per well (Artemisia *vulgaris* in four samples, *Acokanthera oppositifolia* in five samples, *Plantago lanceolata* in four samples and *Conyza canadensis* in five samples). Each sample was assayed in duplicate. 50 µL of COX 100% Initial Activity Samples was added in two dilutions with each dilution assayed in duplicate. 50 µL of COX inhibitors samples were added per well, each sample at two dilutions with each dilution assayed in duplicate, then 50 µL of PG screening acetylcholinesterase (AChE) tracer was added to each well except the Total Activity (TA) and the Blank (BLK) wells and 50 µL of PG Screening EIA Antiserum to each well except the Total Activity (TA), the Non-Specific Binding (NSB), and the Blank (BLK) wells. Each plate was covered with plastic film (Item No 400012) and incubated for 18 h at room temperature on an orbital shaker.

3.6.7 Development and reading of the plate

When ready to develop the plate, one DTN vial of Elman's reagent was reconstituted (Item No 400050) with 20 mL of Ultra-Pure water. This Reagent was sufficient to develop 100 wells. Empty the wells and rinse five times with Wash Buffer. 200 µL of Elman's reagent was added to each well, and 5 µL of Tracer to the Total Activity wells. The plate was covered with plastic film. Optimum development was obtained by using an orbital shaker equipped with a large, flat cover to allow the plate to develop in the dark in 60-90 minutes. The bottom of the plate was wiped

with a clean tissue to remove fingerprints, dirt, etc. The plate cover was removed being careful to keep Elman's reagent from plashing on the cover. The plate was read at the wavelength between 405 and 420 nm. The absorbance was checked periodically until the Bo wells had reached a maximum of 0.3 A. U. (Blank subtracted). The plate was read when the absorbance of the wells exceeded 1.5, wash the plate, add fresh Elman's Reagent and let it develop again.

3.6.8 Preparation and data calculation

The following procedure was done for preparation of the data prior the graphical analysis.

Average the absorbance readings from the NSB wells.

Average the absorbance readings from the Bo wells

Subtract the NSB average from the Bo average. This was the corrected Bo or corrected Maximum Binding.

Calculate the %B/Bo (%sample or standard Bound/ maximum bound) for the remaining wells. To do this, the average NSB absorbance was subtracted from the S1 absorbance and divided by the corrected Bo, then multiplied by 100 to obtain %B/Bo. The calculations were repeated for S2-S8 and all sample wells.

3.7 Lipoxygenase assay

3.7.1 Kit material supplied

Lipoxygenase inhibitor screening assay buffer (10X) (1 vial), developing reagent 1 (1 vial), developing reagent 2 (1 vial), 15-Lipoxygenase Standard (1 vial), arachidonic acid (Substrate)(1vial), linoleic acid (Substrate) (1 vial), potassium hydroxide (1 vial) , 96-well solid plate (colorimetric assay) 1 plate , 96-well cover sheet 1 cover.

3.7.2 Material needed but not supplied by cyclooxygenase-1 and cyclooxygenase-2 kit material

A plate reader capable of measuring absorbance between 490-500 nm , adjustable pipettor and a repeat pipettor, a source of pure water. Glass distilled water or HPLC-grade water is acceptable hydrogen peroxide (420 µM).

3.7.3 Reagent preparation

Lipoxygenase Inhibitor Screening Assay Buffer (10X) was Diluted 3 mL of Assay Buffer concentrate with 27 mL of HPLC-grade water. This final Assay Buffer (0.1 M Tris-HCl, pH 7.4) was used for dilution of samples and the 15-LOX standard prior to assaying. When stored at 4 °C, this diluted Assay Buffer was stable for at least two months. Chromogen was prepared prior to use by mixing equal volumes of Developing Reagent 1 and Developing Reagent 2 in a test tube and vortexing. The volume of Chromogen to be prepared was dependent on the number of wells assayed, and then we calculated 100 µL for each well. Chromogen was used within one hour. 15-Lipoxygenase Standard, a solution of 15-LOX (soybean) was supplied as a positive control. Then 10 µL of the supplied enzyme were transferred to another vial and diluted with 990 µL of diluted Assay Buffer prior to use, stored on ice, and used within one hour. A 90 µL aliquot of the enzyme per well caused a final absorbance of approximately 0.19 nm under the standard assay conditions.

3.7.4 Arachidonic Acid (Substrate)

This vial contained a solution of arachidonic acid in ethanol and was stored at -20 °C when not being used. After Transferring 25 µL of the supplied substrate to another vial, 25 µl of Potassium was added, Hydroxide, vortex, and diluted with 950 µL of HPLC-grade water achieved a final concentration of 1 mM. The prepared arachidonic acid solution was used within 30 minutes. A 10 µL aliquot yielded a final concentration of 100 µM in the wells. NOTE: linoleic acid was used as substrate in the assay. The vial Linoleic Acid (Substrate) contained a solution of linoleic acid in ethanol and was stored at -20 °C when not being used. 25 µL of the supplied substrate was transferred to another vial, 25 µL of Potassium Hydroxide was added, vortex, and diluted with 950 µL of HPLC-grade water to achieve a final concentration of 1 mM. The prepared linoleic acid solution was used within 30 minutes. A 10 µL aliquot yielded a final concentration of 100 µM in the wells. NOTE: linoleic acid was used as subtract in the assay. This vial contained 0.1 M potassium hydroxide (KOH). The reagent was ready to use as supplied.

3.7.5 Plate setup for lipoxygenase assay

There was no specific pattern for using the wells on the plate. However, it was necessary to have some wells (at least two) designated as non-enzymatic controls (blanks). The absorbance of these wells was subtracted from the absorbance measured in the sample wells. In this experiment, two wells were designated as positive controls

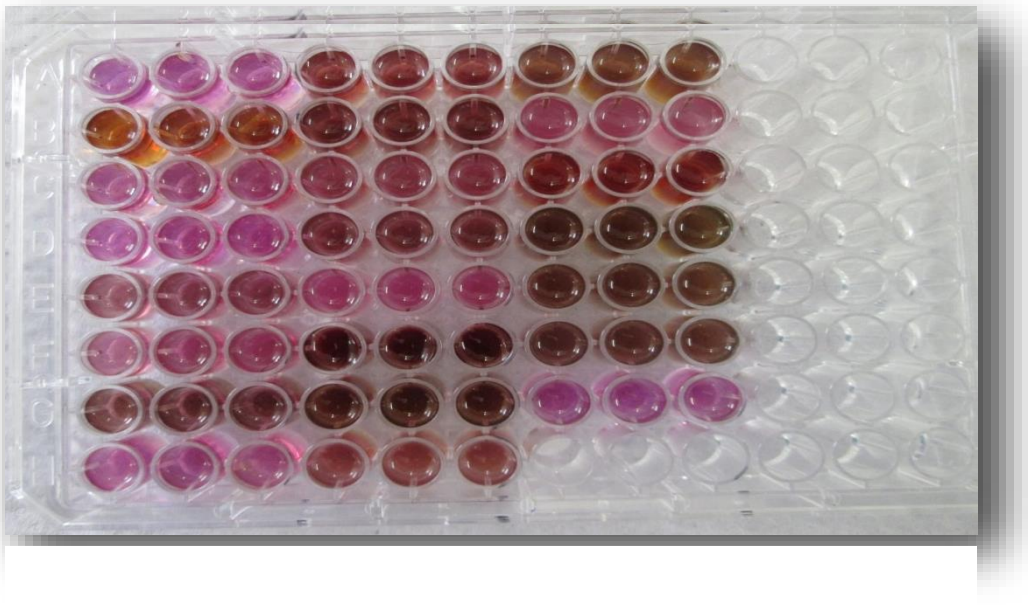

Figure 3.1: Ninety-six plates ready for reading after performing anti-lipoxygenase activity of eighteen plant extracts. The lipoxygenase test was done at the University of Pretoria, Onderstepoort, Faculty of Veterinary Medicine, Department of Paraclinical Sciences, Phytomedicine Programme.

3.7.6 Assay procedure

To achieve the most accurate results, the purified lipoxygenases (LOX) (5-, 12- or 15- LOX) was used for this assay. The sample was free of particulates to avoid interference in the absorbance measurement. In the blank well portion, 100 µL of assay buffer was added to at least two wells. To positive control wells (15-LOX standard), 90 µL of 15-LOX standard and 10 µL of assay buffer were added to at least two wells. In 100% initial activity wells 90µL of lipoxygenase enzyme and 10µl of solvent (the same solvent used to dissolve the inhibitor) were added to two wells. The 100% initial activity resulted in approximately 10 nmol/min/mL of activity. In inhibitor wells, 90 µL of lipoxygenase enzyme and 10 µL of inhibitor were added to two wells. After this the reaction was initiated by adding 10 µl of subtract (linoleic acid) to all the wells. Thereafter, the 96-wells plate was put on a shaker for at least five minutes. After adding 100 µL of chromogen to all wells to stop enzyme catalysis, and develop the reaction, a plate was covered and we placed the 96-wells plate on a

shaker for five minutes, then the cover was removed and read in a plate reader (see Fig 8).

3.7.7 Data analysis

The first step was to determine the average absorbance of the blank, 100% initial activity (IA), and inhibitor wells, then subtracted the average absorbance of the Blank from the average absorbance of the 100% IA and inhibitor wells, and determine the percentage inhibition or percentage IA for each inhibitor using one of the following equations.

% inhibition =100- $\frac{sample-blank}{OD\ negative}$ x100

Then the percentage inhibition or the percentage initial activity was plot as a function of the inhibitor concentration to determine IC_{50} value (concentration at which there was 50% inhibition).

3.8 Nitric oxide assay

3.8.1 Material used for Nitric Oxide assay

Biological safety cabinet (Vivid Air, air filtration and ventilation supplied), DMEM medium (Highveld), trypsin (MERCK), foetal calf serum, mouse leukaemic macrophages cell line RAW 264.7, sodium nitrite, LPS solution (*Escherichia coli)*, penicillin (MERCK), CO_2 incubator (Thermo Electron Corporation), Water bath (Memmert), ground extract apparatus (Polymix PC-MFC 90 D), microscope (Olympus CKX 31), vortex apparatus (Velp Scientifica), pipettes and multi pipettors (Thermo Scientific), hot plate (FMH instruments), DMSO (MERCK Schuchardt OHG), hemocytometer, cover glass (Marienfeld, Germany), centrifuge (Thermo Electron Corporation), tissue culture flask (Greiner Bio-one), disposable serological pipettes (5 mL, 10 mL and 25 mL) (Biocom), 25 cm cell scraper (Biofil), centrifuge tubes (gamma irradiation).

3.8.2 Preparation of growing cells

Tissue culture is a general term used to cover methodologies for the removal of cells, cultures, or organs from an animal or plant and their successive placement into an artificial environment favourable for growth. Bacterial, yeast, plants and animals cells are often grown *in vitro* to study how they grow, divide or differentiate, how they interact with each other and how they respond to various types of mechanical or pharmacological stimuli. Cell culture provides a controlled environment which cells can be incubated under defined conditions and treated with growth factors, drugs, toxins or mechanical stimuli in a reproducible fashion (Bonner and Hargreaves, 2011).

Upon receiving cells the first day, cells were thawed before use, medium was added before centrifuge, then medium supernatant was removed before fresh medium was added, cells were suspended once again and seeded into a culture flask. Two days later, the medium was removed, cells had attached, new media was added, and this was repeated until cells were attached. From one million cells in concentration millilitres medium, 10 mL was added, viewed and incubated. The medium was changed the day after and allowed growing Vero cells once again by decanting added medium and washing cells carefully by shacking slightly, then new medium was added to the top side so that cells were not disturbed, 10 mL medium was added, cells incubated till they reached 70-80% (ready for sub culturing in bigger 75 cm^2 flask.

3.8.3 Sub culturing cells

The lamina floor (Vivid Air) was cleaned thoroughly with 70% ethanol before experiments, then everything needed (medium, FBS, and trypsin) was prepared. Five minutes later, Vero cells were removed from incubator. Then cells were washed twice gently in PBS, 2 mL of trypsin was added then cells were allowed to incubate at 37 + 5% CO_2 incubator for five minutes. After cells were suspended they were transferred into a centrifuge tube to spin at 200 rpm for 10 minutes, thereafter, trypsin cell suspended in 2 mL of medium was first removed, and then cells were

transferred in a bigger flask (75 cm^2) containing 30 mL in medium. Cells culture was allowed to incubate till next use.

3.8.4 Cells counting

After removing cells from the incubator and aspirating off the media, they were scraped from the walls of the flask, and then put into a sterile centrifuge tube. They were spin in 10000 rpm for 10 minutes, and then the supernatant was removed. Medium + PBS were prepared (after warming them). Then 19 ml of medium plus 1 mL of PBS were prepared. The total volume obtained was 20 mL (5% PBS). 153 µL were removed from the prepared medium in which 153 µL was added to stock cells then vacuum. That small volume of cells was transferred to an eppendorf. A new eppendorf was prepared with 20 µL typan blue. A 1:1 dilution was prepared by mixing 20 µL of cells with 20 µL of typan blue (dilution factor is 2). Then 10 µL typan blue cells was transferred and mixed. The haemocytometer was cleaned, and the cover slip was added. 10 mL of mixture was added to both sides of the Haemocytometer. After all the final preparation was observed and counted with haemocytometer into microscope.

3.8.5 Nitric oxide performing

3.8.5.1 Nitric oxide inhibitory assay in lipopolysaccharide-activated mouse leukaemic monocyte macrophage cell line (RAW 264.7 Macrophages)

Macrophage culture (the murine MF cell line RAW 264.7) was grown in DMEM supplemented with 10% heated-inactivated FCS (foetal calf serum) and 5.5 mL of PSF (Penicillin/Streptomycin/Fungizone) to confluence at 37 ºC in a humidified incubator of 5 % CO2. For some experiments, cells were starved, which means that cells were washed with phosphate-buffered saline (PBS) and incubated in DMEM supplemented with FCS and PBS. Experimental design Cells were plated in 96 well plates (for nitrite [NO_2^-] or urea measurements) at 5×10^5 cells/well. When the cells fully adhered after starvation for 12 hours, they were exposed to our eighteen samples as follow: *Acokanthera oppositifolia* (acetone, chloroform, ethylacetate, hexane and water), *Plantago lanceolata* (acetone, chloroform, ethyl acetate, hexane,

and water), *Artemisia vulgaris* (acetone, chloroform, ethyl acetate and hexane), and *Conyza canadensis* (acetone, chloroform, ethyl acetate, and hexane) respectively (50 µL each). 50 µL of LPS was added at a final concentration of 40 µg/mL in DMEM. Then the 96 well plates were incubated for 24h. At the scheduled time points, the supernatant from the cells stimulated by extract plants samples was collected for NO2- measurement using the colorimetric Griess reaction (Green *et al*., 1982). The best incubation time was determined by the preceding time points. After incubation, measurement of NO2- was done for our 18 plants samples and measurement of urea was done for plants extract samples to determine the best concentration for stimulus. Optimal concentrations plant extracts samples, as determined by the earlier experiments, were used to determine the best times. The production of NO was measured by determining NO^{2-} in the culture supernatants using the colorimetric griess reaction (Corraliza *et al*., 1994). Aliquots (60 µL) of cell supernatant were combined with an equal volume of griess reagent [1% sulphanilamide (Alfa Aesar)/0.1% N-(1-napthyl) ethylenediamine (International Laboratory USA) – each in 2.5% H3PO4] in a 96-well plate at room temperature for 10 minutes, and the absorbance at 550 nm was measured with a Multiscan plate reader (Genios, Tencan). Absorbance measurements were averaged and converted to µmol/L of NO_2- per well using a standard curve of sodium nitrite.

3.9 Cell viability (toxicity)

The traditional use of plants to treat various illnesses is widespread. Herbs have been used for medicinal throughout history. Today herbal and food remedies, recommended for the treatment of a variety of diseases, are experiencing increased popularity. Hundreds of natural products are available to the consumer although some could be potentially toxic when ingested in overdoses or in combination with other medications (Santa Maria *et al*., 1997).

3.9.1 Cytotoxicity activity by 3-(4, 5-dimethyl-2-thiazolyl)-2, 5-diphenyl-4H tetrazolium bromide assay

Mouse leukemic macrophage cell line (RAW264.7) was cultured in DMEM supplement with 10% FCS and 1% PSF. The cells were harvested with trypsin-EDTA and diluted to a suspension in fresh DMEM medium. The cells were seeded in 96 wells plate with 1 x 10^5 cells per well and allowed adhering for 1 hour at 37 ℃ in a humidified atmosphere containing 5% CO_2. The medium was then replaced with fresh medium to test our plant samples at various concentrations (2, 10, 30, 100 µg/L) and then incubated for 48 h. Then we used another 96 well plate and added the supernatant for the toxicity test. Cytotoxicity was determined by using the 3-(4, 5-dimethyl-2-thiazolyl)-2, 5-diphenyl-4H tetrazolium bromide (MTT) calorimetric method.

After 48h incubation with test samples, MTT solution (10 µL, 5 mg/mL in PBS) were added to all the wells. After two hours incubation, the medium was removed. 96 wells plate was filled as follows: blank (DMEM medium + 10 µL MTT + 100 µL cells + 50 µL LPS), control (medium +10 µL MTT + sample + 100 µL cells + 50 µL LPS), sample (medium + 100 µL cells +10 µL sample + 50 µL LPS), standard (medium + 100 µL cells + 50 µL quecertin + 50 µL LPS). After two hours incubation, the medium was removed and Isopropanol was added containing 0.04 M HCL to dissolve the formazan production in the cells. To measure optical density of formazan solution with a microplate reader at 570 nm, quecertin was used as positive control. The stock solution of each sample was dissolved in DMSO and the solution was added to the DMEM medium.

CHAPTER 4 RESULTS AND DISCUSSION

4.1 Introduction

Previous studies demonstrated that *Plantago lanceolata* has many medicinal effects. The leaves of the plant are used as astringent, anti-inflammatory, expectorant, topical anodyne, antibacterial, styptic, haemoptysis, haematuria, sore throats, coughs, diarrhoea, dysentery, haemorrhoids, cervicitis, rectal fissures, insect bites, snake bites, cuts, bruises and abscess (Miser-Salihoglu *et al*., 2013). Previous studies were done in ethanol or methanol extracts (Miser-Salihoglu *et al*., 2013). This study was designed not only to confirm antioxidant, analgesic and anti-inflammatory properties of *Plantago lanceolata*, but also to compare *Plantago lanceolata* to *Acokantera oppositifolia, Conyza canadensis,* and *Artemisia vulgaris* by using hexane, ethyl acetate, chloroform, ethanol and water extracts.

4.2 Phytochemical analysis

Phytochemical screening revealed the presence of anthraquinones, phlobatannins, flavonoids, alkaloids, cardiac glycosides, terpenoids, tannins and saponins (Table 4.1). The flavonoids, as an anti-oxidant in this plant may contribute to the effects of this plant as hepatoprotective and nephroprotective, antimicrobial, anti-inflammatory, and anti-carcinogenic effect. Table 1 indicates the phytochemical analysis of *Plantago lanceolata* hexane extract.

Table 4.1: Preliminary phytochemical analysis of *Plantago lanceolata* hexane extracts + means positive. – means negative.

Phytochemicals	*Plantago lanceolata* hexane extract
Anthraquinones	+
Terpenoids	+
Flavonoids	+
Saponins	+
Tannins	+
Quinones	-
Coumarins	-
Cardiac glycosides	+
Phlobatannins	+
Phenols	+
Alkaloids	+

4.3 Antioxidative test

The antioxidative activity of *Acokanthera oppositifolia, Plantago lanceolata*, *Conyza canadensis* and *Artemisia vulgaris* in acetone, chloroform, ethyl acetate , hexane and water, a total of eighteen samples were examined by comparing them one another and comparing them to the activity of known antioxidants such as ascorbic acid and quecertin with inhibition of DPPH radicals. Substances capable of donating electrons/hydrogen atoms are to convert DPPH to their non-radical form 1, 1'-diphenyl-2-picrylhydrazine, a reaction which can be followed spectrophotometrically. On the other hand, the cell walls contain unsaturated fatty acids such as linoleic and arachidonic acid. The cell permeability is changed after the oxidation of these fatty acids then chronic diseases may occur. The extension of self-life and control of deterioration of fatty foods can be achieved via the protection of these acids. Ascorbic acid and quecertin were used as positive control in this test. Free radicals scavenging of those four southern Africa medicinal plants were investigated. *Plantago lanceolata* hexane extract indicates the lowest IC_{50} (0.41 mg/mL), the same with *Plantago lanceolata* chloroform extract (0.41 mg/mL). It is

important to note that the lower IC_{50} value reflects better protective action. Table 4.2 and Figure 4.1 indicate that among the 19 samples, *Plantago lanceolata* hexane extract and chloroform extract indicate the lowest IC_{50} and closer to the positive control ascorbic acid and quacertin than the other samples. The antioxidative activity of the hexane and chloroform extract could be attributed to content of flavonoids. Besides, phenolic compounds and flavonoids are also widely distributed in plants which have been reported to exert many biological effect, including antioxidative, free radical scavenging abilities, anti-inflammatory, and anticarcinogenic (Miller, 1996).

Table 4.2: Antioxidative test results of plant extracts. Data represent the percentage of inhibition and IC_{50} (µg/mL) in concentrations mg/ml.

Plant species	H_20	Acetone	Ethyl acetate	Chloroform	Hexane
Artemisia vulgaris	ND	2.10	2.20	1.36	3.51
Acokanthera oppositifolia	2.23	0.54	1.81	0.87	2.42
Plantago lanceolata	1.81	2.02	0.56	0.41	0.41
Conyza Canadensis	0.56	0.87	2.42	2.23	2.02

Vitamin C IC_{50} = 0.04 mg/ml; Quecertin IC_{50} = 0.06 mg/ml; ND, not done.

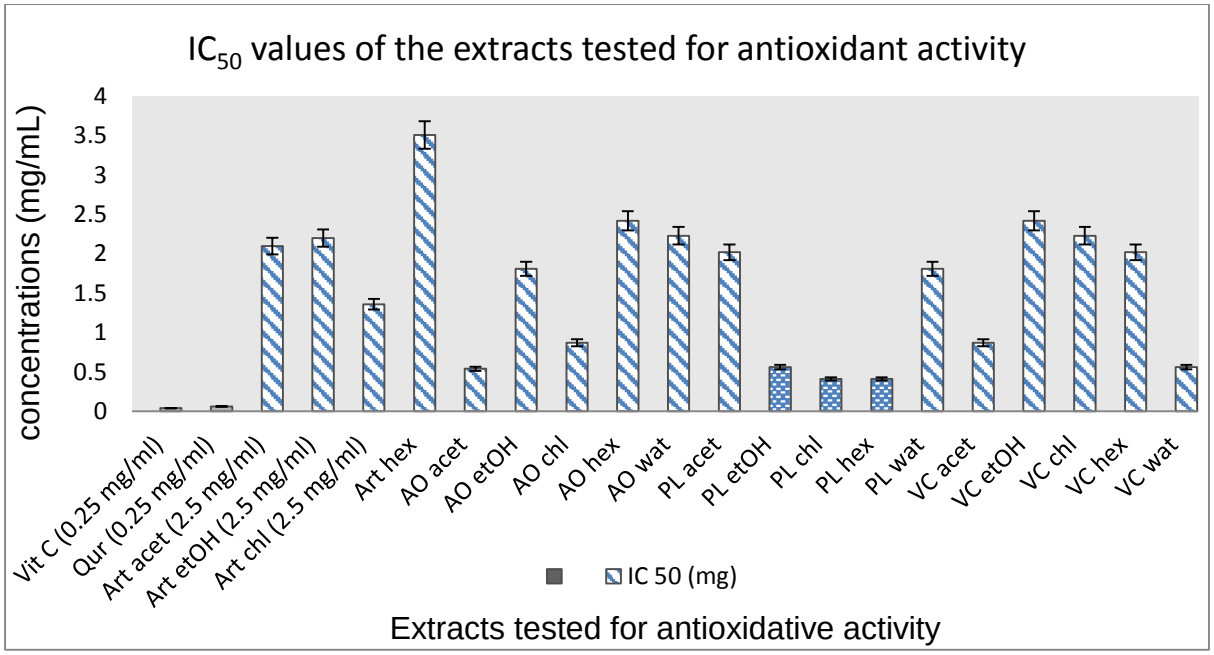

Figure 4.1: Antioxidative activity of *Acokanthera oppositifolia, Plantago lanceolata, Conyza canadensis*, and *Artemisia vulgaris.*

4.4 Anti-inflammatory activity

4.4.1 Cyclooxygenase-1 and cyclooxygenase-2 test

Cyclooxygenase enzymes (COXs, prostaglandin endoperoxide synthases) catalyse two reactions, the first being a cyclooxygenase function consisting of the addition of molecular oxygen to arachidonic acid (AA) to form prostaglandin G_2 (PGG_2). The second is the conversion of PGG_2 to PGH_2 by a peroxidase function. Hence, this COX enzyme performs the critical initial reaction in the AA metabolic cascade leading to the formation of pro inflammatory prostaglandins, thromboxane and prostacyclin. Prostaglandins regulate smooth muscle contractility, blood pressure and platelet aggregation and mediate pain and fever. Inhibition of cyclooxygenase activity is the mechanism by which non-steroidal anti-inflammatory drugs (NSAIDs) exert their analgesic, antipyretic, anti-inflammatory, and antithrombotic effects (Lee *et al*., 2003). The constitutive form COX-1 is responsible for the maintenance of physiological prostanoid biosynthesis. In contrast, COX-2 is an inducible isoform linked to inflammatory cell types and tissues. Prolonged use of NSAIDs is also associated with severe side effects such as gastro- intestinal

haemorrhage due to COX-1 inhibition (Lee *et al*., 2003). The new COX-2 selective drugs do not seem to be free of risk either since several COX-2 inhibitors has been found to cause cardiovascular problems (Mukherjee *et al*., 2001). Steroids have an obvious role in the treatment of inflammatory diseases but, due to their toxicity, they can only be used over short periods of time except in very serious cases for which the risks are acceptable. Consequently, there is a strong need for natural products with minimum side effects. The percentage inhibitory activity against COX-1 and COX-2 by all the plant extracts was reported as percentage inhibition of prostaglandin biosynthesis in Fig 8 and Fig 9. Among our four medicinal plants (Acokanthera *oppositifolia, Plantago lanceolata, Artemisia vulgaris and Conyza canadensis), Plantago lanceola* hexane extract is the more active fraction and the IC_{50} value (0.2 mg/mL) concentration is even less than the positive control IC_{50} = 0.42 mg/mL (quecertin). On the other hand, Figure 4.2 and Figure 4.3 indicate the COX-1 and COX-2 activity of our four medicinal plants in percentage inhibition and IC_{50}. *Plantago lanceolata* hexane extract indicates a percentage of inhibition of 7.25 and IC_{50} of 68.99 mg/mL. The same extract has an IC_{50} of 1.96 mg/mL in COX-2 (Table 4.4) compared to IC_{50} 68.99 mg/mL in COX-1 (Table 4.3). On the other hand, the positive control quecertin indicate an IC_{50} of 4.6 mg/mL (COX-1) and 8.39 mg/mL (COX-2) It may be concluded that *Plantago lanceolata* is a selective COX-2 inhibitor. A therapeutic advantage, in relation to selective COX-2 inhibitors, is the low ulcer toxicity. Vane and Botting have suggested a parallel relationship between COX-2 selectivity and gastrointestinal side effects with NSAID treatment, such that COX-2 selective compounds cause fewer ulcers (Vane and Botting, 2001). Masferrer (1996) demonstrated that administration of COX-2 selective inhibitors did not produce stomach lesions, in contrast to administration of nonselective NSAIDs (Masferrer *et al*., 1996). This indicates its interest in inflammation.

Table 4.3: Anti-cyclooxygenase-1 inhibitory of *Acokanthera oppositifolia*, *Plantago lanceolata*, *Conyza canadensis*, and *Artemisia vulgaris* indicating their percentage of inhibition and IC_{50}.

Plant species	H_20	Acetone	Ethyl acetate	Chloroform	Hexane
Artemisia vulgaris	ND	46.00	76.67	ND	86.25
Acokanthera	ND	62.73	57.50	ND	46.00
oppositifolia	ND	53.08	40.59	ND	69.00
Plantago lanceolata					
Conyza canadensis	ND	40.59	ND	ND	40.59

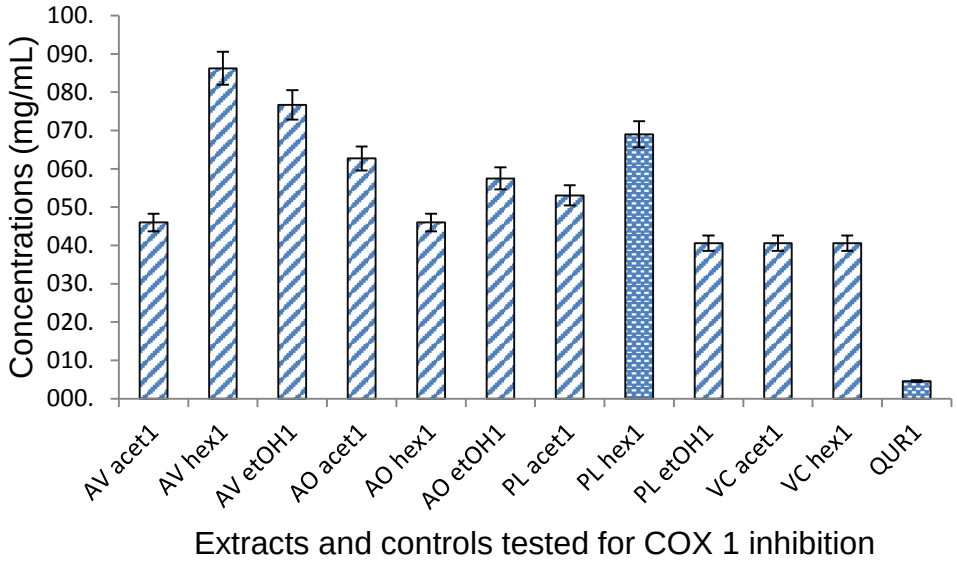

Figure 4.2: IC_{50} values of *Acokanthera oppositifolia*, *Plantago lanceolata*, *Conyza canadensis*, and *Artemisia vulgaris* tested in COX-1 inhibition.

Table 4.4: Anti-cyclooxygenase-2 activity of *Acokanthera oppositifolia*, *Plantago lanceolata*, *Conyza canadensis*, and *Artemisia vulgaris* indicating their percentage inhibition and IC_{50}.

Plant species	H_20	Acetone	Ethylacetate	Chloroform	Hexane
Artemisia vulgaris	ND	50.33	83.89	50.33	53.93
Acokanthera oppositifolia	ND	53.93	83.89	83.89	> 100
Plantago lanceolata	ND	1.98	> 100	> 100	1.96
Conyza canadensis	ND	ND	> 100	53.93	ND

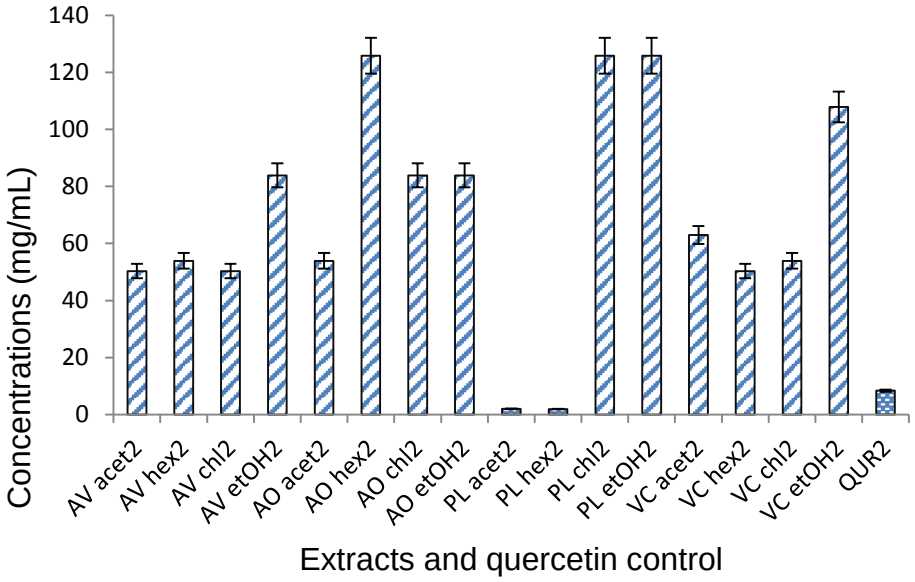

Figure 4.3: IC_{50} values of *Acokanthera oppositifolia*, *Plantago lanceolata*, *Conyza canadensis canadensis*, and *Artemisia vulgaris* tested in COX-2 inhibition.

4.4.2 Lipoxygenase assay

The lipoxygenase (LOX) activity was performed in four medicinal plants. Inhibition of 15-lipoxygenase enzymes by methanol was analysed to evaluate anti-inflammatory activity. LOX catalyzes dioxygenation of polyunsaturated fatty acids to yield cis, trans-conjugated diene hydroperoxides. Results for LOX inhibitory activity (IC_{50}) are shown in Figure 4.4. A total of 15 samples were tested for their anti lipoxygenase activity three times in triplicate. The first test showed among our 15 samples, *Artemisia vulgaris* hexane extract, *Plantago lanceolata* hexane extract and *Acokanthera oppositifolia* acetone extract were the most active samples with the IC_{50} of 4.86, 6.32, and 6.44 mg/mL respectively. The second test was performed with 11 samples. The third test was performed and 11 samples were used. The three tests were summarised in Table 4.4. The highest inhibitory effect was observed for *Acokanthera oppositifolia* acetone extract and *Plantago lanceolata* hexane extract with an IC_{50} of 4.75 mg/mL and 7.73 mg/mL respectively compared to the positive control (quecertin). The results reported here suggest that *Acokanthera oppositifolia* hexane extract and *Plantago lanceolata* hexane extract has potentially high anti-LOX effect, which might be related to the polyphenolic content and antioxidant property of the extracts. Lipoxygenase plays an important role in the pathophysiology of several inflammatory diseases (Wedi and Kapp, 2001).

Plant derived chemical constituents such as flavonoids, coumarins, quinones, pentacyclic triterpenes, sesquiterpenes, alkaloids and polyacetylates have been reported to inhibit 15- lipoxygenase (Werz, 2007). Table 4.1 of the primary phytochemical analysis of *Plantago lanceolata* acetone extract indicates the presence of flavonoids, coumarins, quinones, and terpenoids. The presence of these constituents confirms the potential anti-lipoxygenase activity of *Plantago lanceolata*. Lipoxygenases are sensitive to antioxidants and the most of their action may consist of inhibition of lipid hydroperoxide formation due to scavenging of lipidoxy or lipid peroxy- radical formed in course of enzyme peroxidation. This can limit the availability of lipid hydroperoxide substrate necessary for the catalytic cycle of LOX (Govindappa *et al*., 2011). The results obtained from our studies on these four medicinal plants have shown as potential anti-lipoxygenase activity.

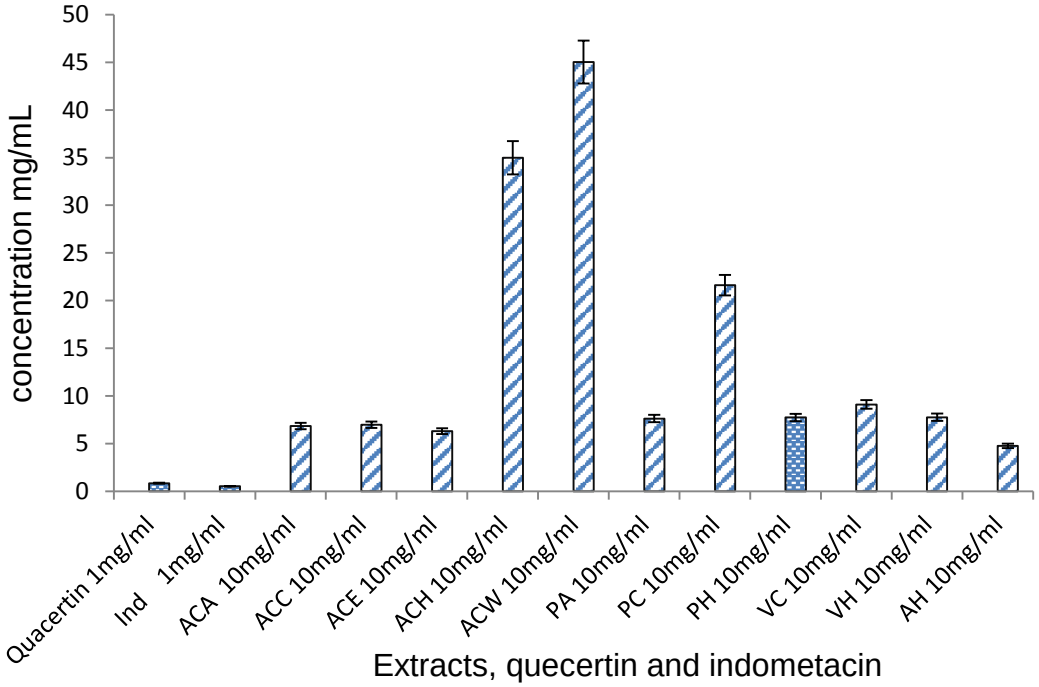

Figure 4.4: IC_{50} values of test 1, test 2, and test 3.

4.5 Nitric oxide test

Nitric oxide (NO) is an important chemical mediator generated by endothelial cells, macrophages, neurons, etc. and is involved in the regulation of various physiological processes. Excess concentration of NO is associated with several diseases. NO is generated in biological tissues by specific nitric oxide synthesis (NOSs), which metabolizes arginine to citralline with the formation of NO via a five electron oxidative reaction (Ross, 1993). These compounds are responsible for altering the structural and functional behaviour of many cellular components. Incubation of sodium nitroprusside solution in PBS at 25 $°C$ for 2 h resulted in linear time dependent nitrite production, which is reduced by the tested extracts of the plants. NO scavenging capacity is determined by the decrease in the absorbance at 550 nm, induced by antioxidants. In order to evaluate the antioxidant potency through NO scavenging by the test samples, the change of optical density of NO was monitored. Table 4.5 indicates the inhibition of NO production in LPS-activated RAW 234.7 by our four extracts in different fractions.

The results of NO scavenging activity of the selected plant extracts are shown as amount of NO (µM) and as percentage of NO inhibition Nitric oxide or reactive nitrogen species, formed during their reaction with oxygen or with superoxide, are very reactive. These compounds are responsible for altering the structural and functional behaviour of many cellular components. *Conyza canadensis* acetone extract and *Conyza canadensis* ethylacetate extract have greater inhibition comparative to other plant. The percentage of inhibition of *Conyza canadensis* ethylacetate extract is 94.31 in concentration 10 mg/mL and 96.33 in 100 µg/mL with IC_{50} <2mg/mL. *Conyza canadensis* acetone extract also indicates a percentage of inhibition of 97.31 at concentration 10 mg/mL and an IC_{50} <2mg/mL.

The cell viability indicates in Table 4.5 that *Conyza canadensis* extract kills most of cells at the concentrations of 100 and 30 mg/mL (the ethyl acetate extract) and in concentrations of 100 and 30 mL/mg (acetone extract). On the other hand, *Plantago lanceolata* indicates an IC_{50} of 47.66 mg/mL but with good cell

viability (91.90 and 100%) at the doses 100 and 30 mg/mL respectively (Table 4.5). The primary phytochemical analysis of *Plantago lanceolata* hexane extract indicates the presence of flavonoids and phenols. Phenolic compounds and flavonoids have been reported to be associated with antioxidative action in biological systems, acting as scavengers of singlet oxygen and free radicals (Rice-Evans *et al*., 1997; Jorgensen *et al*., 1999). The nitric oxide scavenging activity of flavonoids and phenolic compounds are known (Madson *et al*., 2000; Jagethia *et al*., 2004).

Table 4.5: Inhibition of NO production in LPS-activated macrophages RAW 234.7 by *Acokanthera oppositifolia, Plantago lanceolata, Conyza canadensis* and *Artemisia vulgaris.*

Plant species	[Extract] (µg/mL)	IC_{50} (µg/mL)	% Inhibition of NO				
			H_20	Acetone	Ethyl acetate	Chloroform	Hexane
Acokanthera oppositifolia	100		ND	86.72±2.25	98.04±1.06	93.27±4.47	86.97±1.97
	30	32.57	ND	37.09±2.44	83.97±4.79	50.62±7.76	39.48±1.52
	10		ND	24.31±2.29	37.22±9.25	21.67±1.83	26.88±1.96
	2		ND	16.47 ±0.94	16.96±5.67	10.35±00	23.33±0.68
Artemisia vulgaris	100		68.24±2.6	100±1.95	97.19±2.82	98.29±0.96	49.15±2.56
	30		38.32±1.5	96.21±1.21	85.80±2.69	87.21±2.25	29.02±1.20
	10	41.13	24.9±4.68	68.18±3.39	51.84±1.36	53.13±4.50	25.47±1.87

	2		17.64±9.9	27.79±2.40	23.5±3.461	18.55±7.12	16.35±2.29
Plantago lanceolata	100		84.4 ± 3.7	93.76±1.17	87.76±7.71	85.74±2.48	84.46±1.26
	30		37.89 ±1.7	51.78±1.73	50.68±5.56	44.13±1.34	38.81±2.19
	10	44.05	21.43 ± 3.6	29.87±2.99	28.10±4.17	26.88±0.48	25.83±1.04
	2		10.66±3.33	21.67±2.01	18.25±1.5	21.67±1.72	18.68±0.78
Conyza canadensis	100		95.9 ± 2.88	96.88±1.98	96.33±1.17	98.53±0.00	99.20±0.38
	30		53.9 ± 7.04	98.29±1.46	96.94±1.31	97.67±0.57	98.71±0.40
	10		31.71 ±2.26	97.31±1.67	94.31±4.43	80.11±2.64	71.61±0.77
	2	17.69	15.5±6	51.60±1.65	53.80±4.08	48.17±0.69	37.58±1.59

Plant species	[Extract] (µg/mL)	IC_{50} µg/mL	% of cell viability				
			H_20	Acetone	Ethyl acetate	Chloroform	Hexane
Acokanthera oppositifolia	100		ND	67.98±1.53	48.53±2.60	88.10±4.09	93.33±10.60
	30	32.57	ND	76.94±4.37	88.06±7.27	96.55±1.37	86.82±8.84
	10		ND	86.32±3.28	99.92±8.76	74.30±3.99	83.72±7.39
	2		ND	95.16±2.85	85.78±6.64	80.47±6.80	ND
Artemisia vulgaris	100		84.46 ±8.17	10.50±1.96	25.00±2.33	40.35±33	59.53±4.16
	30	41.13	81.09 ±5.52	54.96±3.55	73.29±4.17	71.78±0.97	71.63±5.00
	10		70.62 ±6.87	99.34±10.9	91.01±7.83	79.61±3.82	92.09±6.27
	2		74.96 ±4.61	99.76±6.38	94.88±3.70	88.45±1.99	94.92±10.30
Plantago lanceolata	100		96.36 ±19.86	88.80±8.12	94.84±8.60	75.35±2.44	91.90±7.43
	30		94.92 ±7.20	99.6±10.98	99.16±6.72	91.05±8.87	99.05±10
	10	44.05	99.64 ±11.38	99.57±0.86	99.9±10.81	98.91±4.67	97.93±6.62

	2		99.37	±1.42	100±10.10	100±4.98	99.31±3.02	83.99±10.53
Conyza canadensis	100	17.6	85.97	±9.83	6.20±0.11	5.89±0.05	5.31±0.15	5.70±0.19
	30		99.65	±11.29	6.01±0.05	6.67±0.71	54.22±15.80	98.76±2.41
	10		99.25	±16.16	89.57±7.91	89.96±8.62	77.21±0.33	98.18±0.46
	2		100	±1.06	78.84±8.53	94.2±11.07	99.72±7.78	81.20±0.56

Key: W, water; A, acetone; E, ethylacetate; C, chloroform, H, hexane. Positive control quecertin $IC_{50} < 2$. Percentage of NO inhibition at dose 20µg/mL, 96.71±0.31. Percentage of cell viability 6.82 ± 0.24

4.6 Cytotoxicity test

Mouse Leukemic macrophage cell line (RAW264.7) was used for the cytotoxicity activity by using MTT calorimetric method. The results were combined with the nitric oxide table results (Table 4.5). Four plants (A*cokanthera oppositifolia*, *Plantago lanceolata*, *Artemisia vulgaris* and *Conyza canadensis*) were tested; each plants in four or five different solvents (acetone, ethylacetate, chloroform, hexane, and water). *Conyza canadensis* ethyl acetate extract kills cells at the dose of 100 mg/mL and 30 mg/mL with a percentage of cell viability of 5.89 ± 0.05 and 6.67 ± 0.71 respectively. *Conyza canedensis* chloroform extract is also toxic at the dose of 100 mg/mL with a percentage of cell viability 5.31 ± 0.15. *Conyza canadensis* acetone extract indicates a percentage of cell inhibition of 6.20 ± 0.11 and 6.01 ± 0.05 at doses 100 mg/mL and 30 mg/mL respectively. Table 4.5 also indicates that *Conyza canadensis* is toxic at dose 100 mg/mL. On the other hand, *Plantago lanceolata* indicated a good cell viability with the percentages between 75.35 ± 2.44 and 100% cell viability.

CHAPTER 5 DISCUSSION AND CONCLUSION

The objectives of this study were to investigate analgesic and anti-inflammatory activity of *Acokanthera oppositifolia, Plantago lanceolata, Conyza canadensis* and *Artemisia vulgaris*. The extracts were tested for their antioxidative, analgesic, anti-inflammatory activity by determination of inhibitory activity on lipopolysaccharides (LPS) induced nitric oxide production in RAW 264.7 cell lines using griess reagent, lipoxygenase, COX-1 and COX-2, and cell viability. The plants were extracted in five solvents each (acetone, chloroform, ethyl acetate hexane and water). A total of ten to twenty samples were used in this study.

For antioxidative activity, the IC_{50} coefficient was calculated to compare the antioxidant activity of individual extracts (Table 4.2). *Plantago lanceolata* hexane extract indicated a higher IC_{50} (0.41 mg/mL) compared to the other plant extracts and to the positive control. On the other hand, phytochemical analysis of the hexane *Plantago lanceolata* hexane extract shows the presence of flavonoids tannins and phenols. Flavonoids and Tannins are a major group of compounds that act as primary antioxidants or free radical scavengers (Polterait, 1997). Free radical(s) are oxygen-centered with at least one unpaired electron and are end products of several physiological and biochemical processes. They attack and damage cell membrane, cell organelles and DNA resulting in faulty translation of genetic information. This is implicated in contributing to cancer, atherosclerosis, aging, immunosuppression, inflammation, diabetes, neurodegenerative disorder etc. (Shailasree *et al*., 2013). Phenols are very important plant constituents because of their free radical scavenging ability due to their hydroxyl groups (Hatano *et al*., 1999). The phenolic compounds may contribute directly to antioxidant action. It is suggested that polyphenolic compounds have inhibitory effects on mutagenesis and carcinogenesis in human beings (Tsao and Akhtar, 2005). The antioxidant capacities of the plant extracts largely depend on the composition of the extracts. DPPH, scavenging gave reliable information concerning the antioxidant ability of the tested compounds (Shailasree, *et al*., 2013). The antioxidative characteristics might be attributed to the presence of polyphenolic compounds. Based on the result in the study, it was concluded that *Plantago lanceolata* hexane extract was found to probably have anti-

oxidant effect. Further studies are required to identify specific active principles of these plants for the significant antioxidative effect.

Inflammation is a pathological condition mediated through production of PGE2 from arachidonic acid (AA) generated by enzyme system PG synthetase, a complex enzyme including COX-2. The enzymatic oxygenation of arachidonic acid via the COX and LOX pathways play a key role in the mediation of inflammation. As a result, the key enzymes of these pathways COX-1, COX-2, and LOX have become the target for the development of anti-inflammatory drugs (Kumar, 2011) Another group of compounds eliciting inflammatory conditions are leukotrienes which are derived directly from AA by enzymatic action of lipoxygenase (LOX) (Shailasree *et al*., 2012). For the anti-inflammatory activity, the plant extracts were tested for their anti COX-1 and COX-2 activity, anti lipoxygenase activity, and inhibition of LPS (Lipopolysaccharides). The enzyme cyclooxygenase-1 (COX-1, prostaglandin endoperoxide H synthase-1, PGHS-1), a catalyst of prostaglandin synthesis, has been extensively used as a tool for studying the anti-inflammatory effects of plant extracts and plant-derived compounds (Bauer, 1996). With the discovery of the isoenzyme COX-2, whose production is induced by inflammation mediators, interest in cyclooxygenase inhibitors has grown. The adverse effects observed with traditional nonsteroidal anti-inflammatory drugs (NSAIDs), such as aspirin and indomethacin, are believed to stem from an inhibition of constitutive COX-1 activity, and it is hypothesized that selective COX-2 inhibitors exhibit an improved safety profile. The constitutive form COX-1 is responsible for the maintenance of physiological prostanoid biosynthesis. In contrast, COX-2 is an inducible isoform linked to inflammatory cell types and tissues. Prolonged use of NSAIDs is also associated with severe side effects such as gastro- intestinal haemorrhage due to COX-1 inhibition (Lee *et al*., 2003). The new COX-2 selective drugs do not seem to be free of risk either since several COX-2 inhibitors has been found to cause cardiovascular problems (Mukherjee *et al*., 2001). The Figure 4.2 and Figure 4.3 indicate the IC_{50} of the sample extract used in this study. *Plantago lanceolata* hexane extract indicates the lower IC_{50} in COX-2 inhibition. The IC_{50} value is close to the control (quecertin) compared to the other extract plants. *Plantago lanceolata* hexane extract indicates an IC_{50} 68.99 mg/mL in COX-1 inhibition Table 4.2. This indicates

the great interest of the *Plantago lanceolata* hexane extract by the fact that the extract inhibits only the COX-2 enzyme and is without inhibition in COX-1.

The anti lipoxygenase activity was also investigated for the anti-lipoxygenase activity. Table 5 indicates that among the four plants, *Acokanthera oppositifolia* hexane extract and *Plantago lanceolata* hexane extract with an IC_{50} of 4.75 mg/mL and 7.73 mg/mL respectively are the most active extract plants because of their lower IC_{50}. Lipoxygenases are sensitive to antioxidants as antioxidants are involved in inhibition of lipid hydroperoxide formation due to scavenging of lipidoxy or lipidperoxy radicals. This could lead to less availability of lipid hydroperoxide substrate required for LOX catalysis (Rackova *et al*., 2007). Lipoxygenase are the family of the key enzyme in the biosynthesis of leukotrienes which plays an important role in the pathophysiology of several inflammatory diseases. Lipoxygenases are sensitive to antioxidants and the most of their action may consist of inhibition of lipid hydroperoxide formation due to scavenging of lipidoxy or lipidperoxy radicals formed in course of enzymatic peroxidation (Kumaraswamy and Satish, 2008). *Plantago lanceolata* inhibited the lipoxygenase enzyme. This indicates that *Plantago lanceolata* may have an interest in anti-inflammation studies.

Nitric oxide (NO) is a potent pleiotropic inhibitor of physiological processes such as smooth muscle relaxation, neuronal signalling, inhibition of platelet aggregation and regulation of cell mediated toxicity. It is a diffusible free radical that plays many roles as an effector molecules in diverse biological systems including neuronal messenger, vasodilatation and antimicrobial and antitumor activities (Hagerman *et al*., 1998). Table 4.5 indicates the percentage of inhibition of NO production is dose dependent for all the plant extracts as we used an increasingly dose of 2, 10, 30 and 100 mg/mL for the nitric oxide test. On the other hand, *Plantago lanceolata* may not be toxic to the cells by the fact that Table 4.5 indicates the percentage of cell viability is very high in all the *Plantago* tests, even in our maximum dose used (100 mg/mL). The observed NO scavenging activity of *Plantago lanceolata* hexane extract might be useful for the development of newer and more potent natural antioxidants. Observed NO scavenging activity of *Plantago*

lanceolata extract might be useful for the development of newer and more potent natural antioxidants.

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APPENDIX: RAW DATAS

A- Antioxidant analysis

Table: A1 Antioxidant values of samples, controls, blank, and negative

Conc	Sample	OD	S-BLANK	% Inhb	IC 50
	BLK	0,304	0,000		
	NEG	0,427	0,123		
1 mg/ml	**VIT C 1**	0,133	-0,171	239,16	0,21
0,5	**VIT C 2**	0,074	-0,230	286,79	0,17
0,25	**VIT C 3**	0,070	-0,234	290,31	0,04
0,125	**VIT C 4**	0,077	-0,227	284,57	0,02
1	**QUR 1**	0,155	-0,149	220,92	0,23
0,5	**OUR 2**	0,167	-0,138	211,69	0,12
0,25	**OUR 3**	0,164	-0,141	214,15	0,06
0,125	**QUR 4**	0,216	-0,088	171,45	0,04
10	Art acet 1	0,825	0,520	-322,41	-1,55
5	Art acet 2	0,456	0,151	-22,90	-10,92
2,5	Art acet 3	0,354	0,050	59,59	2,10
1,25	Art acet 4	0,338	0,034	72,50	0,86
10	Art etOH 1	1,031	0,727	-489,93	-1,02

5	Art etOH 2	0,488	0,184	-49,04	-5,10
2,5	Art etOH 3	0,355	0,051	58,94	2,12
1,25	Art etOH 4	0,369	0,064	47,71	1,31
10	Art chl 1	0,990	0,685	-456,45	-1,10
5	Art chl 2	0,563	0,259	-110,31	-2,27
2,5	Art chl 3	0,315	0,010	91,61	1,36
1,25	Art chl 4	0,380	0,076	38,59	1,62

Table A1 continue

Conc	**Sample**	**OD**	**S-BLANK**	**% Inhb**	**IC 50**
10 mg/ml	Art hex 1	0,383	0,079	35,70	14,01
5 mg/ml	Art hex 2	0,435	0,130	-5,79	-43,17
2,5 mg/ml	Art hex 3	0,384	0,079	35,59	3,51
1,25	Art hex 4	0,469	0,164	-33,37	-1,87
10	AO acet 1	0,094	-0,210	270,37	1,85
5	AO acet 2	0,138	-0,167	235,18	1,06
2,5	AO	0,145	-0,160	229,55	0,54

	acet 3				
1,25	AOacet 4	0,241	-0,063	151,45	0,41
10	AO etOH 1	0,792	0,487	-295,64	-1,69
5	AO etOH 2	0,308	0,003	97,35	2,57
2,5	AO etOH 3	0,342	0,038	68,96	1,81
1,25	AO etOH 4	0,204	-0,101	181,79	0,34
10	AO chl 1	0,621	0,317	-157,43	-3,18
5	AO chl 2	0,310	0,006	95,16	2,63
2,5	AO chl 3	0,250	-0,055	144,36	0,87
1,25	AO chl 4	0,217	-0,087	170,61	0,37
10	AO hex 1	0,543	0,238	-93,48	-5,35
5	AO hex 2	0,286	-0,018	114,70	2,18
2,5	AO hex 3	0,364	0,060	51,56	2,42
1,25	AO hex 4	0,338	0,034	72,21	0,87
10	AO wat 1	0,438	0,134	-8,66	-57,73
5	AO wat 2	0,435	0,131	-6,22	-40,16

Table A1 continue

Conc	Sample	OD	S-BLANK	% Inhb	IC 50
2,5	AO wat 3	0,358	0,054	56,04871	2,23
1,25	AO wat 4	0,360	0,056	54,91204	1,14
10	PL acet 1	0,270	-0,034	127,5778	3,92
5	PL acet 2	0,315	0,011	91,4479	2,73
2,5	PL acet 3	0,351	0,047	61,94858	2,02
1,25	PL acet 4	0,381	0,077	37,42896	1,67
10	PL etOH 1	0,514	0,210	-70,5007	-7,09
5	PL etOH 2	0,250	-0,054	144,2219	1,73
2,5	PL etOH 3	0,153	-0,151	222,977	0,56
1,25	PL etOH 4	0,128	-0,176	242,8958	0,26
10	PL chl 1	0,051	-0,253	305,2233	1,64
5	PL chl 2	0,053	-0,251	303,6806	0,82
2,5	PL chl 3	0,053	-0,251	303,8972	0,41
1,25	PL chl 4	0,050	-0,254	306,2788	0,20
10	PL hex 1	0,052	-0,252	304,7632	1,64

5	PL 2	hex	0,051	-0,253	305,4127	0,82
2,5	PL 3	hex	0,052	-0,252	304,8173	0,41
1,25	PL 4	hex	0,051	-0,253	305,521	0,20
10	PL 1	wat	0,053	-0,252	304,3572	1,64
5	PL 2	wat	0,052	-0,253	305,0609	0,82
2,5	PL 3	wat	0,342	0,038	68,95805	1,81
1,25	PL 4	wat	0,204	-0,101	181,7862	0,34

Table A1continue

Conc	**Sample**	**OD**	**S-BLANK**	**% Inhb**	**IC 50**
10	VC acet 1	0,621	0,317	-157,429	-3,18
5	**VC acet 2**	0,310	0,006	95,15562	2,63
2,5	**VC acet 3**	0,250	-0,055	144,3572	0,87
1,25	**VC acet 4**	0,217	-0,087	170,6089	0,37
10	**VC etOH 1**	0,543	0,238	-93,4777	-5,35
5	**VC etOH 2**	0,286	-0,018	114,6955	2,18
2,5	**VC**	**0,364**	0,060	51,55616	2,42

	etOH 3				
1,25	**VC etOH 4**	**0,338**	0,034	72,20568	0,87
10	**VC chl 1**	**0,438**	0,134	-8,66035	-57,73
5	**VC chl 2**	**0,435**	0,131	-6,22463	-40,16
2,5	**VC chl 3**	**0,358**	0,054	56,04871	2,23
1,25	**VC chl 4**	**0,360**	0,056	54,91204	1,14
10	**VC hex 1**	**0,270**	-0,034	127,5778	3,92
5	**VC hex 2**	**0,315**	0,011	91,4479	2,73
2,5	**VC hex 3**	**0,3511**	0,0469	61,94858	2,02
1,25	**VC hex 4**	**0,3813**	0,0771	37,42896	1,67
10	**VC wat 1**	**0,5142**	0,2100	-70,5007	-7,09
5	**VC wat 2**	**0,2498**	-0,0545	144,2219	1,73
2,5	**VC wat 3**	**0,1528**	-0,1515	222,977	0,56
1,25	**VC wat 4**	**0,1282**	-0,1760	242,8958	0,26

A2 Cyclooxygenase-1 and cyclooxygenase-2

Table A2$_1$ Cyclooxygenase-1 and cyclooxygenase-2 values for standard plot

std	Conc	std	Conc
0,097	**2000**	0,097	
0,149	**1000**	0,149	
0,245	**500**	0,245	500
0,308	**250**	0,308	250
0,37	**125**	0,37	125
0,423	**62,5**	0,423	62,5
0,416	**31,3**	0,416	31,3
0,4215	**15,6**	0,4215	15,6

Fig A2$_1$ standard curve

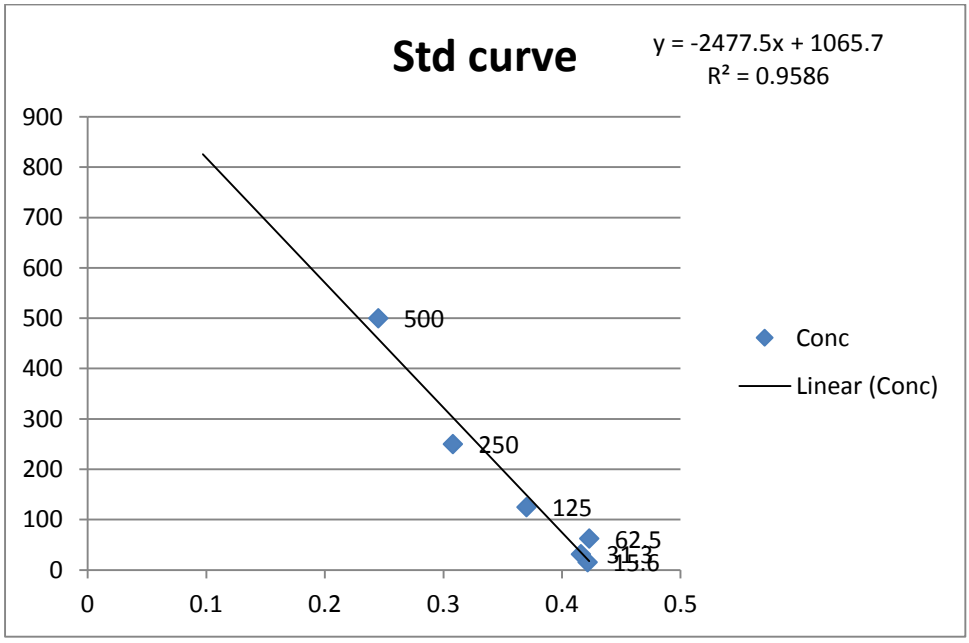

Table A2$_2$ Cyclooxygenase-1 values

COX 1	OD-BLK	%B/B0	x dil factor	% INHB	IC50
AV acet1	0,016	1,539	3077,977	10,8695663	46
AV hex1	0,012	0,821	1641,588	5,79710203	86,24999
AV etOH1	0,013	0,923	1846,786	6,52173978	76,66666
AO acet1	0,014	1,129	2257,183	7,97101529	62,72727
AO hex1	0,016	1,539	3077,977	10,8695663	46
AO etOH1	0,014	1,231	2462,382	8,69565304	57,49999
PL acet1	0,015	1,334	2667,580	9,4202908	53,07692
PL hex1	0,013	1,026	2051,985	7,24637754	68,99999
PL etOH1	0,017	1,744	3488,374	12,3188418	40,58823
VC acet1	0,017	1,744	3488,374	12,3188418	40,58823
VC hex1	0,017	1,744	3488,374	12,3188418	40,58823
QUR1	0,016	1,539	3077,977	10,8695663	4,6

Table A2$_3$ Cyclooxygenase-2 values

Samples Cox2			**pg/ml**		
	OD	**%B/B0**	X Dil Factor	**% Inhb**	IC 50
AV acet2	0,016	1,539	6155,955	9,93377429	50,33
AV hex2	0,015	1,436	5745,558	9,27152267	53,93
AV chl2	0,016	1,539	6155,955	9,93377429	50,33
AV etOH2	0,013	0,923	3693,573	5,96026458	83,89
AO acet2	0,015	1,436	5745,558	9,27152267	53,93
AO hex2	0,011	0,616	2462,382	3,97350972	125,83
AO chl2	0,013	0,923	3693,573	5,96026458	83,89
AO etOH2	0,013	0,923	3693,573	5,96026458	83,89
PL acet2	0,199	39,090	156361,3	252,317867	1,98
PL hex2	0,201	39,603	158413,2	255,629125	1,96
PL chl2	0,011	0,616	2462,382	3,97350972	125,83
PL etOH2	0,011	0,616	2462,382	3,97350972	125,83
VC acet2	0,014	1,231	4924,764	7,94701944	62,92
VC hex2	0,016	1,539	6155,955	9,93377429	50,33

VC chl2	0,015	1,436	5745,558	9,27152267	53,93
VC etOH2	0,012	0,718	2872,779	4,63576134	107,86
QUR2	0,013	0,923	3693,573	5,96026458	8,39

A3 Lypoxygenase test

BLK= BLANK(DMSO)
QU= POSITIVE CONTROL QUACERTINE
IND= INDOMETACIN
NEG= NEGATIVE CONTROL BORATE BUFFER ONLY
ACA= ACOKANTHERA OPP(ACETONE)
ACC= ACOKANTHERA OPP(CHLOROFORM)
ACE= AKOKANTHERA OPP(ETHYLACETATE)
ACH= ACOKANTHERA OPP(HEXANE
ACW= ACOKANTHERA OPP(WATER)
PA= PLANTAGO (ACETONE)
PC= PLANTAGO(CHLOROFORM)
PE= PLANTAGO(ETHYLACETATE)
PH= PLANTAGO (HEXANE)
PW= PLANTAGO(WATER)
VA= VERGERETTE(ACETONE)
VC= VERGERETTE(CHLOROFORM)

VE= VERGERETTE(ETHYLACETATE)
VH= VERGERETTE(HEXANE)
VW= VERGERETTE(WATER)
AA= ARTEMISIA (ACETONE)
AC= Artemisia chloroform
AE= ARTEMISIA ETHYLACETATE
AH= ARTEMISIA HEXANE

Table A3$_1$ Lypoxygenase test value from test 1

Sample	OD	S-BLANK	%inhib	Ic50
blanck	0,872667	0		
Quac	0,921333	0,048667	93,96694	0,532102
Ind	0,796	-0,07667	109,5041	0,456604
Neg	0,806667	-0,066		
ACA	1,053333	0,180667	77,60331	6,443024
ACC	1,116667	0,244	69,75207	7,168246
ACE	0,947667	0,075	90,70248	5,512528
ACH	1,098	0,225333	72,06612	6,938073
ACW	1,254667	0,382	52,64463	9,497645
PA	1,398667	0,526	34,79339	14,37055
PC	1,126333	0,253667	68,55372	7,29355
PE	1,336667	0,464	42,47934	11,77043
PH	1,041333	0,168667	79,09091	6,321839

PW	2,267333	1,394667	-72,8926	-6,85941
VA	2,089333	1,216667	-50,8264	-9,8374
VC	1,191	0,318333	60,53719	8,259386
VE	1,69	0,817333	-1,32231	-378,125
VH	1,092333	0,219667	72,7686	6,871096
VW	1,546	0,673333	16,52893	30,25
AA	1,682667	0,81	-0,41322	-1210
AC	1,652	0,779333	3,38843	147,561
AE	1,858667	0,986	-22,2314	-22,4907
AH	0,849	-0,02367	102,9339	4,857487

BLK= BLANK(DMSO)
QU= POSITIVE CONTROL QUACERTINE
IND= INDOMETACIN
NEG= NEGATIVE CONTROL BORATE BUFFER ONLY
ACA= ACOKANTHERA OPP(ACETONE)
ACC= ACOKANTHERA OPP(CHLOROFORM)
ACE= AKOKANTHERA OPP(ETHYLACETATE)
ACH= ACOKANTHERA OPP(HEXANE
ACW= ACOKANTHERA OPP(WATER)
PA= PLANTAGO (ACETONE)
PC= PLANTAGO(CHLOROFORM)

PE= PLANTAGO(ETHYLACETATE)
PH= PLANTAGO (HEXANE)
PW= PLANTAGO(WATER)
VA= VERGERETTE(ACETONE)
VC= VERGERETTE(CHLOROFORM)
VE= VERGERETTE(ETHYLACETATE)
VH= VERGERETTE(HEXANE)
VW= VERGERETTE(WATER)
AA= ARTEMISA (ACETONE)
AC= Artemisa chloroform
AE= ARTEMISA ETHYLACETATE
AH= ARTEMISA HEXANE

Table A3$_2$ Lypoxygenase value from test number 2

Sample	OD	S-BLANK	%inhib	Ic50
blanck	0,578667			
Quac	0,878333	0,299667	53,89744	0,927688

Ind	0,636333	0,057667	91,12821	0,548678
Neg	0,65	0,071333		
ACA	0,777333	0,198667	69,4359	7,200886
ACC	0,892333	0,313667	51,74359	9,663033
ACE	0,944333	0,365667	43,74359	11,43025
ACH	0,708	0,129333	80,10256	6,241997
ACW	1,093667	0,515	20,76923	24,07407
PC	0,815	0,236333	63,64103	7,856567
PE	1,08	0,501333	22,87179	21,86099
PH	0,859	0,280333	56,87179	8,791704
VC	0,853333	0,274667	57,74359	8,65897
VH	0,920667	0,342	47,38462	10,55195

Table A3$_3$ Lypoxygenase value from test number 3

Sample	**OD**	**S-BLANK**	**%inhib**	**Ic50**
blanck	0,603			
Quac	0,851	0,248	59,89218	0,834833
Ind	0,611	0,008	98,7062	0,506554
Neg	0,618333	0,015333		
ACA	0,726	0,123	80,10782	6,241588
ACC	0,769	0,166	73,15364	6,83493
ACE	1,147667	0,544667	11,91375	41,96833
ACH	0,730333	0,127333	79,40701	6,296673
ACW	1,133	0,53	14,28571	35
PA	1,152667	0,549667	11,10512	45,02427
PC	0,815333	0,212333	65,66038	7,614943
PE	1,078333	0,475333	23,12668	21,62005
PH	0,821333	0,218333	64,69003	7,729167
VC	0,881667	0,278667	54,93261	9,102061

VH	0,822667	0,219667	64,47439	7,755017
AH	0,571	-0,032	105,1752	4,753972

A4 Nitric oxide test

Table A4$_1$ Nitric oxide values for standard curve

	400	200	100	50	25	12,5	6,25	3,12	1,56	0,78	0,39	0,19
NaNO2	3,507	2,297	1,408	0,762	0,444	0,263	0,164	0,112	0,081	0,07	0,059	0,053
NaNO2	3,519	2,304	1,459	0,853	0,488	0,285	0,175	0,115	0,085	0,067	0,059	0,055
NaNO2	3,833	2,357	1,278	0,694	0,374	0,232	0,153	0,144	0,088	0,064	0,61	0,056
NaNO2	3,645	2,398	1,339	0,731	1,171	0,227	0,39	0,097	0,257	0,062	0,092	0,387
	46,14	23,34378	11,80411	6,138222	3,289	1,732111	0,821556	0,429	0,259333	0,144667	0,159111	0,114444

400	**46,14**
200	23,3438
100	11,8041
50	6,13822
25	3,289
12,5	1,73211
6,25	0,82156
3,12	0,429
1,56	0,25933
0,78	0,14467
0,39	0,15911
0,19	0,1444

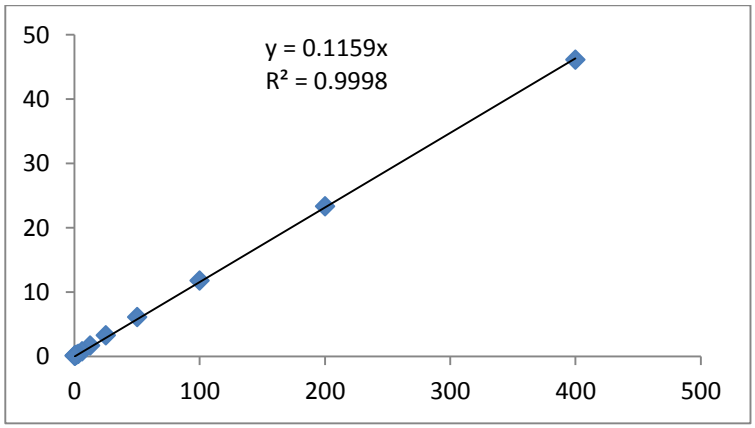

Fig A4 standard curve

Table A4$_2$ Nitric oxide value from plate reading

NITRIC OXIDE ASSAY

Conc.	VE	VE	VE	VC	VC	VC	VA	VA	VA	bkVE	bkVC	bkVA
100	0,082	0,083	0,096	0,073	0,073	0,073	0,097	0,077	0,102	0,067	0,065	0,075
30	0,082	0,087	0,07	0,082	0,083	0,089	0,092	0,079	0,073	0,063	0,072	0,072
10	0,131	0,089	0,074	0,202	0,167	0,181	0,098	0,094	0,077	0,067	0,075	0,075
2	0,351	0,306	0,302	0,35	0,342	0,35	0,34	0,318	0,328	0,068	0,065	0,065
Conc.	VW	VW	VW	VH	VH	VH	PH	PH	PH	bkVW	bkVH	bkPH
100	0,133	0,109	0,095	0,071	0,068	0,066	0,165	0,166	0,151	0,09	0,064	0,076
30	0,368	0,321	0,274	0,08	0,085	0,081	0,41	0,428	0,399	0,07	0,075	0,079
10	0,463	0,445	0,433	0,223	0,216	0,226	0,476	0,488	0,476	0,075	0,067	0,076
2	0,586	0,515	0,511	0,424	0,42	0,404	0,52	0,529	0,529	0,077	0,076	0,083

Conc.	PE	PE	PE	PC	PC	PC	PA	PA	PA	bkPE	bkPC	bkPA
100	0,202	0,115	0,111	0,174	0,155	0,141	0,133	0,132	0,146	0,076	0,079	0,103
30	0,381	0,333	0,308	0,376	0,361	0,377	0,339	0,339	0,359	0,072	0,067	0,083
10	0,488	0,447	0,435	0,474	0,469	0,468	0,485	0,446	0,458	0,065	0,072	0,081
2	0,521	0,5	0,513	0,499	0,511	0,522	0,528	0,503	0,507	0,066	0,084	0,086
Conc.	PW	PW	PW	AA	AA	AA	AH	AH	AH	bkPW	bkAA	bkAH
100	0,202	0,17	0,153	0,066	0,065	0,088	0,339	0,372	0,363	0,09	0,079	0,081
30	0,429	0,413	0,407	0,098	0,084	0,084	0,457	0,464	0,473	0,078	0,068	0,078
10	0,526	0,496	0,478	0,269	0,243	0,224	0,499	0,475	0,481	0,072	0,072	0,079
2	0,538	0,577	0,576	0,487	0,469	0,455	0,559	0,558	0,532	0,077	0,077	0,094

Conc.	AE	AE	AE	AC	AC	AC	ACW	ACW	ACW	bk	bk	bk
100	0,128	0,094	0,097	0,097	0,087	0,099	0,274	0,274	0,244	0,091	0,085	0,091
30	0,169	0,139	0,137	0,156	0,13	0,13	0,43	0,417	0,41	0,071	0,069	0,083
10	0,332	0,335	0,318	0,308	0,36	0,308	0,522	0,466	0,47	0,066	0,07	0,077
2	0,492	0,494	0,453	0,492	0,58	0,505	0,618	0,489	0,53	0,063	0,082	0,097
Conc.	ACE	ACE	ACE	ACA	ACA	ACA	ACH	ACH	ACH	bk	bk	bk
100	0,081	0,076	0,067	0,164	0,135	0,143	0,154	0,17	0,144	0,064	0,075	0,085
30	0,189	0,126	0,148	0,425	0,394	0,401	0,413	0,396	0,414	0,067	0,064	0,078
10	0,479	0,367	0,378	0,5	0,47	0,48	0,469	0,475	0,494	0,066	0,071	0,081
2	0,576	0,511	0,51	0,532	0,523	0,535	0,505	0,514	0,51	0,08	0,075	0,092

Conc.	ACC	ACC	ACC	FH2O	FH2O	FH2O	A1	A1	A1	bk	bk	bk
100	0,154	0,1	0,105	0,262	0,198	0,238	0,285	0,296	0,277	0,083	0,087	0,11
30	0,397	0,296	0,327	0,412	0,388	0,457	0,408	0,414	0,406	0,071	0,08	0,085
10	0,51	0,494	0,486	0,473	0,452	0,458	0,488	0,474	0,453	0,07	0,077	0,09
2	0,58	0,55	0,542	0,547	0,595	0,585	0,532	0,597	0,55	0,069	0,07	0,089
Conc.	A16	A16	A16	A20	A20	A20				bk	bk	bk
100	0,092	0,09	0,079	0,073	0,075	0,064				0,089	0,072	0,085
30	0,242	0,119	0,117	0,216	0,124	0,107				0,1	0,076	0,081
10	0,575	0,341	0,343	0,335	0,353	0,346				0,074	0,071	0,079
2	0,586	0,58	0,551	0,525	0,561	0,551				0,085	0,091	0,161

Conc.	Quer	Quer	Quer	CTRL	CTRL	CTRL	bkCTR	bkCTR	bkCTR	bkQuer	bkCTR	Media
20	0,086	0,083	0,082	0,515	0,506	0,5	0,06	0,064	0,062	0,066	0,053	0,075
5	0,084	0,087	0,084	0,541	0,521	0,516	0,066	0,062	0,064	0,066	0,055	0,077
2	0,2	0,194	0,187	0,553	0,531	0,526	0,065	0,074	0,065	0,065	0,063	0,06
0,5	0,386	0,374	0,4	0,595	0,566	0,54	0,071	0,073	0,072	0,062	0,051	0,077

Table A4$_3$ Amount of nitric oxide produced, average and standard deviation

	Amount of NO produced														
Conc.	VE	VE	VE	AVG	SD	VC	VC	VC	AVG	SD	VA	VA	VA	AVG	SD
100	0,129422	0,13805	0,250216	0,17	0,06	0,069025	0,069025	0,069025	0,07	0,00	0,189819	0,017256	0,232959	0,15	0,09
30	0,163934	0,207075	0,060397	0,14	0,06	0,086281	0,094909	0,146678	0,11	0,03	0,172563	0,060397	0,008628	0,08	0,07
10	0,5522	0,189819	0,060397	0,27	0,21	1,095772	0,793788	0,914582	0,93	0,12	0,198447	0,163934	0,017256	0,13	0,08
2	2,44176	2,053494	2,018982	2,17	0,19	2,459016	2,389991	2,459016	2,44	0,03	2,372735	2,182916	2,269198	2,27	0,08
Conc.	VW	VW	VW			VH	VH	VH			PH	PH	PH		
100	0,371009	0,163934	0,043141	0,19	0,14	0,060397	0,034513	0,017256	0,04	0,02	0,767903	0,776531	0,64711	0,73	0,06
30	2,571182	2,16566	1,760138	2,17	0,33	0,043141	0,086281	0,051769	0,06	0,02	2,85591	3,011217	2,761001	2,88	0,10
10	3,347714	3,192407	3,08887	3,21	0,11	1,345988	1,285591	1,371872	1,33	0,04	3,451251	3,554789	3,451251	3,49	0,05
2	4,391717	3,77912	3,744607	3,97	0,30	3,002588	2,968076	2,830026	2,93	0,07	3,770492	3,848145	3,848145	3,82	0,04
Conc.	PE	PE	PE			PC	PC	PC			PA	PA	PA		
100	1,087144	0,336497	0,301984	0,58	0,36	0,819672	0,655738	0,534944	0,67	0,12	0,258844	0,250216	0,371009	0,29	0,06
30	2,666091	2,251941	2,036238	2,32	0,26	2,666091	2,53667	2,67472	2,63	0,06	2,208801	2,208801	2,381363	2,27	0,08
10	3,649698	3,295945	3,192407	3,38	0,20	3,468507	3,425367	3,416739	3,44	0,02	3,485764	3,149267	3,252804	3,30	0,14
2	3,925798	3,744607	3,856773	3,84	0,07	3,580673	3,684211	3,77912	3,68	0,08	3,813632	3,597929	3,632442	3,68	0,09
Conc.	PW	PW	PW			AA	AA	AA			AH	AH	AH		
100	0,96635	0,69025	0,543572	0,73	0,18	-0,11217	-0,12079	0,077653	-0,05	0,09	2,226057	2,510785	2,433132	2,39	0,12
30	3,028473	2,890423	2,838654	2,92	0,08	0,258844	0,13805	0,13805	0,18	0,06	3,27006	3,330457	3,40811	3,34	0,06
10	3,91717	3,658326	3,50302	3,69	0,17	1,699741	1,47541	1,311475	1,50	0,16	3,623814	3,416739	3,468507	3,50	0,09
2	3,977567	4,314064	4,305436	4,20	0,16	3,537532	3,382226	3,261432	3,39	0,11	4,012079	4,003451	3,77912	3,93	0,11
Conc.	AE	AE	AE			AC	AC	AC			ACW	ACW	ACW		
100	0,319241	0,025884	0,051769	0,13	0,13	0,103538	0,017256	0,120794	0,08	0,05	1,578947	1,578947	1,320104	1,49	0,12
30	0,845557	0,586713	0,569456	0,67	0,13	0,750647	0,526316	0,526316	0,60	0,11	2,99396	2,881795	2,821398	2,90	0,07
10	2,295082	2,320966	2,174288	2,26	0,06	2,053494	2,502157	2,053494	2,20	0,21	3,839517	3,356342	3,390854	3,53	0,22
2	3,701467	3,718723	3,36497	3,60	0,16	3,537532	4,296808	3,649698	3,83	0,33	4,495255	3,382226	3,735979	3,87	0,46
Conc.	ACE	ACE	ACE			ACA	ACA	ACA			ACH	ACH	ACH		
100	0,146678	0,103538	0,025884	0,09	0,05	0,767903	0,517688	0,586713	0,62	0,11	0,595341	0,733391	0,50906	0,61	0,09
30	1,052632	0,50906	0,698878	0,75	0,23	3,114754	2,847282	2,907679	2,96	0,11	2,890423	2,743745	2,899051	2,84	0,07
10	3,563417	2,597066	2,691976	2,95	0,43	3,701467	3,442623	3,528904	3,56	0,11	3,347714	3,399482	3,563417	3,44	0,09
2	4,279551	3,718723	3,710095	3,90	0,27	3,943054	3,865401	3,968939	3,93	0,04	3,563417	3,64107	3,606557	3,60	0,03
Conc.	ACC	ACC	ACC			FH2O	FH2O	FH2O			A1	A1	A1		
100	0,612597	0,146678	0,189819	0,32	0,21	1,509922	0,957722	1,302847	1,26	0,23	1,509922	1,604832	1,440897	1,52	0,07
30	2,81277	1,941329	2,208801	2,32	0,36	2,864538	2,657463	3,252804	2,92	0,25	2,786885	2,838654	2,769629	2,80	0,03
10	3,796376	3,658326	3,589301	3,68	0,09	3,416739	3,235548	3,287317	3,31	0,08	3,433995	3,313201	3,13201	3,29	0,12
2	4,408973	4,150129	4,081104	4,21	0,14	4,115617	4,529767	4,443486	4,36	0,18	3,822261	4,383089	3,977567	4,06	0,24
Conc.	A16	A16	A16			A20	A20	A20							
100	0,025884	0,008628	-0,08628	-0,02	0,05	0,008628	0,025884	-0,06903	-0,01	0,04					
30	1,225194	0,163934	0,146678	0,51	0,50	1,207938	0,41415	0,267472	0,63	0,41					
10	4,322692	2,30371	2,320966	2,98	0,95	2,277826	2,433132	2,372735	2,36	0,06					
2	4,322692	4,270923	4,020708	4,20	0,13	3,744607	4,05522	3,968939	3,92	0,13					
Conc.	Quer	Quer	Quer			CTRL	CTRL	CTRL							
20	0,172563	0,146678	0,13805	0,15	0,01	3,986195	3,908542	3,856773	3,92	0,05					
5	0,155306	0,181191	0,155306	0,16	0,01	4,19327	4,020708	3,977567	4,06	0,09					
2	1,164797	1,113028	1,052632	1,11	0,05	4,227783	4,037964	3,994823	4,09	0,10					
0,5	2,795513	2,691976	2,916307	2,80	0,09	4,693701	4,443486	4,219154	4,45	0,19					
								4,129997							

Table A4₄ percentage of nitric oxide inhibition

	% of NO inhibition														
Conc.	VE	VE	VE	AVG	SD	VC	VC	VC	AVG	SD	VA	VA	VA	AVG	SD
100	97,25	97,06	94,68	96,33	1,17	98,53	98,53	98,53	98,53	0,00	95,96	99,63	95,04	96,88	1,98
30	96,51	95,59	98,71	96,94	1,31	98,16	97,98	96,88	97,67	0,57	96,33	98,71	99,82	98,29	1,46
10	88,25	95,96	98,71	94,31	4,43	76,69	83,11	80,54	80,11	2,64	95,78	96,51	99,63	97,31	1,67
2	48,05	56,31	57,04	53,80	4,08	47,68	49,15	47,68	48,17	0,69	49,52	53,55	51,72	51,60	1,65
Conc.	VW	VW	VW			VH	VH	VH			PH	PH	PH		
100	92,11	96,51	99,08	95,90	2,88	98,71	99,27	99,63	99,20	0,38	83,66	83,48	86,23	84,46	1,26
30	45,29	53,92	62,55	53,92	7,04	99,08	98,16	98,90	98,71	0,40	39,24	35,93	41,26	38,81	2,19
10	28,77	32,08	34,28	31,71	2,26	71,36	72,65	70,81	71,61	0,77	26,57	24,37	26,57	25,83	1,04
2	6,56	19,59	20,33	15,49	6,32	36,12	36,85	39,79	37,58	1,59	19,78	18,12	18,12	18,68	0,78
Conc.	PE	PE	PE			PC	PC	PC			PA	PA	PA		
100	76,87	92,84	93,57	87,76	7,71	82,56	86,05	88,62	85,74	2,48	94,49	94,68	92,11	93,76	1,17
30	43,27	52,09	56,68	50,68	5,56	43,27	46,03	43,09	44,13	1,34	53,00	53,00	49,33	51,78	1,73
10	22,35	29,87	32,08	28,10	4,17	26,20	27,12	27,30	26,88	0,48	25,83	32,99	30,79	29,87	2,99
2	16,47	20,33	17,94	18,25	1,59	23,82	21,61	19,59	21,67	1,72	18,86	23,45	22,71	21,67	2,01
Conc.	PW	PW	PW			AA	AA	AA			AH	AH	AH		
100	79,44	85,31	88,43	84,40	3,73	102,39	102,57	98,35	101,10	1,95	52,64	46,58	48,23	49,15	2,56
30	35,56	38,50	39,60	37,89	1,70	94,49	97,06	97,06	96,21	1,21	30,42	29,14	27,49	29,02	1,20
10	16,66	22,16	25,47	21,43	3,63	63,84	68,61	72,10	68,18	3,39	22,90	27,30	26,20	25,47	1,87
2	15,37	8,21	8,39	10,66	3,33	24,73	28,04	30,61	27,79	2,40	14,64	14,82	19,59	16,35	2,29
Conc.	AE	AE	AE			AC	AC	AC			ACW	ACW	ACW		
100	93,21	99,45	98,90	97,19	2,82	97,80	99,63	97,43	98,29	0,96	66,41	66,41	71,91	68,24	2,60
30	82,01	87,52	87,88	85,80	2,69	84,03	88,80	88,80	87,21	2,25	36,30	38,69	39,97	38,32	1,52
10	51,17	50,62	53,74	51,84	1,36	56,31	46,76	56,31	53,13	4,50	18,31	28,59	27,85	24,92	4,68
2	21,25	20,88	28,40	23,51	3,46	24,73	8,58	22,35	18,55	7,12	4,36	28,04	20,51	17,64	9,88
Conc.	ACE	ACE	ACE			ACA	ACA	ACA			ACH	ACH	ACH		
100	96,88	97,80	99,45	98,04	1,06	83,66	88,99	87,52	86,72	2,25	87,33	84,40	89,17	86,97	1,97
30	77,60	89,17	85,13	83,97	4,79	33,73	39,42	38,13	37,09	2,44	38,50	41,62	38,32	39,48	1,52
10	24,18	44,74	42,72	37,22	9,25	21,25	26,75	24,92	24,31	2,29	28,77	27,67	24,18	26,88	1,96
2	8,95	20,88	21,06	16,96	5,67	16,11	17,76	15,55	16,47	0,94	24,18	22,53	23,26	23,33	0,68
Conc.	ACC	ACC	ACC			FH2O	FH2O	FH2O			A1	A1	A1		
100	86,97	96,88	95,96	93,27	4,47	67,87	79,62	72,28	73,26	4,85	67,87	65,85	69,34	67,69	1,43
30	40,15	58,70	53,00	50,62	7,76	39,05	43,46	30,79	37,77	5,25	40,70	39,60	41,07	40,46	0,62
10	19,23	22,16	23,63	21,67	1,83	27,30	31,16	30,06	29,51	1,62	26,94	29,51	33,36	29,93	2,64
2	6,19	11,70	13,17	10,35	3,00	12,43	3,62	5,46	7,17	3,80	18,68	6,74	15,37	13,60	5,03
Conc.	A16	A16	A16			A20	A20	A20							
100	99,45	99,82	101,84	100,37	1,05	99,82	99,45	101,47							
30	73,93	96,51	96,88	89,11	10,73	74,30	91,19	94,31							
10	8,03	50,98	50,62	36,54	20,16	51,54	48,23	49,52							
2	8,03	9,13	14,45	10,54	2,81	20,33	13,72	15,55							
Conc.	Quer	Quer	Quer			CTRL	CTRL	CTRL							
20	96,33	96,88	97,06	96,76	0,31	15,19	16,84	17,94							
5	96,70	96,14	96,70	96,51	0,26	10,78	14,45	15,37							
2	75,22	76,32	77,60	76,38	0,98	10,05	14,09	15,00							
0,5	40,52	42,72	37,95	40,40	1,95	0,13	5,46	10,23							

A5 Cytotoxicity test

Table A5$_1$ MTT test values from plate reading

MTT ASSAY

Conc.	VE	VE	VE	VC	VC	VC	VA	VA	VA	bkVE	bkVC	bkVA
100	0,05	0,051	0,051	0,047	0,044	0,046	0,054	0,054	0,052	0,048	0,046	0,05
30	0,065	0,057	0,05	0,612	0,285	0,502	0,052	0,051	0,052	0,052	0,673	0,054
10	0,868	0,766	0,687	0,666	0,666	0,66	0,865	0,738	0,708	0,624	1,299	0,975
2	0,941	0,773	0,717	0,93	0,948	0,798	0,596	0,664	0,774	1,352	1,315	1,321
Conc.	VW	VW	VW	VH	VH	VH	PH	PH	PH	bkVW	bkVH	bkPH
100	0,842	0,741	0,635	0,049	0,051	0,047	0,778	0,719	0,874	0,923	0,049	1,177
30	0,85	1,077	0,902	1,037	0,992	1,035	0,867	0,815	1,209	1,294	1,006	1,579
10	0,985	0,97	0,683	0,98	0,86	1,08	0,863	0,879	0,991	1,76	1,352	1,486
2	0,876	0,862	0,854	0,694	0,696	0,705	0,627	0,696	0,844	1,492	1,537	1,542
Conc.	PE	PE	PE	PC	PC	PC	PA	PA	PA	bkPE	bkPC	bkPA
100	0,77	0,92	0,757	0,657	0,668	0,619	0,689	0,745	0,857	1,288	1,292	1,269
30	0,884	0,918	0,782	0,798	0,868	0,683	0,76	0,934	0,979	1,338	1,344	1,305
10	0,916	1,109	0,908	0,809	0,905	0,838	0,882	0,9	0,89	1,177	1,306	1,217
2	0,938	1,043	0,991	0,912	0,968	0,966	1,055	1,234	1,244	1,104	1,195	1,072
Conc.	PW	PW	PW	AA	AA	AA	AH	AH	AH	bkPW	bkAA	bkAH
100	0,916	0,98	0,59	0,112	0,088	0,071	0,494	0,48	0,562	0,761	0,121	1,078
30	0,824	0,888	0,737	0,515	0,459	0,444	0,606	0,569	0,673	1,039	0,752	1,212
10	0,839	1,043	1,05	0,979	0,833	0,751	0,857	0,725	0,794	1,212	1,16	1,255
2	0,904	0,889	0,874	0,925	0,881	0,793	0,854	0,694	0,901	1,236	1,391	1,374

Conc.	AE	AE	AE	AC	AC	AC	ACW	ACW	ACW	bk	bk	bk
100	0,187	0,225	0,233	0,368	0,364	0,309	0,627	0,775	0,777	0,231	0,281	1,006
30	0,661	0,65	0,58	0,62	0,626	0,606	0,642	0,692	0,758	0,833	0,899	1,193
10	0,857	0,797	0,694	0,696	0,718	0,64	0,548	0,586	0,688	0,958	1,023	1,26
2	0,826	0,849	0,773	0,782	0,76	0,74	0,598	0,641	0,695	1,007	1,217	1,221
Conc.	ACE	ACE	ACE	ACA	ACA	ACA	ACH	ACH	ACH	bk	bk	bk
100	0,437	0,386	0,429	0,602	0,57	0,582	0,728	0,749	0,931	0,254	1,072	0,983
30	0,817	0,784	0,671	0,711	0,654	0,62	0,64	0,812	0,788	1,076	1,208	1,174
10	0,836	0,961	0,781	0,782	0,726	0,719	0,657	0,807	0,696	1,172	1,322	1,328
2	0,776	0,78	0,657	0,848	0,788	0,819	0,814	0,949	0,902	1,354	1,452	1,339

Conc.	ACC	ACC	ACC	FH2O	FH2O	FH2O	A1	A1	A1	bk	bk	bk
100	0,784	0,781	0,708	0,642	0,646	0,772	0,659	0,688	0,704	0,689	0,949	0,994
30	0,823	0,821	0,847	0,855	0,857	0,861	0,744	0,79	0,853	1,355	1,339	1,371
10	0,598	0,637	0,682	0,684	0,64	0,741	0,619	0,678	0,618	1,018	1,392	1,361
2	0,687	0,623	0,766	0,599	0,639	0,636	0,51	0,58	0,55	0,953	1,129	1,131
Conc.	A16	A16	A16	A20	A20	A20	DMSO	DMSO	DMSO	bk	bk	bk
100	0,737	0,272	0,304	0,06	0,056	0,047	0,51	0,572	0,517	0,206	0,062	0,977
30	0,86	0,86	0,777	0,635	0,642	0,734	0,557	0,517	0,514	1,04	1,161	1,07
10	0,972	0,957	0,837	0,807	0,698	0,876	0,598	0,69	0,639	1,264	1,452	1,295
2	0,933	0,98	0,9	0,836	0,756	0,957	0,716	0,653	0,719	1,506	1,67	1,3

Conc.	Quer	Quer	Quer	CTRL	CTRL	CTRL	bkCTR	bkCTR	bkCTR	bkQuer	Media	Media
20	0,059	0,056	0,061	0,76	0,815	0,702	1,325	1,275	1,343	0,052	0,075	0,075
5	0,225	0,249	0,241	0,953	0,859	0,747	1,389	1,39	1,165	0,134	0,075	0,075
2	0,73	0,689	0,776	0,864	1,047	0,962	1,506	1,467	1,524	1,014	0,084	0,084
0,5	0,753	0,723	0,733	0,906	0,85	0,888	1,269	1,582	1,212	0,992	0,086	0,086
						0,86			1,37			

Table A5$_2$ percentage of cell viability

Conc.				AVG	SD				AVG	SD				AVG	SD
100	5,81	5,93	5,93	5,89	0,05	5,47	5,12	5,35	5,31	0,15	6,28	6,28	6,05	6,20	0,11
30	7,56	6,63	5,81	6,67	0,71	71,16	33,14	58,37	54,22	15,80	6,05	5,93	6,05	6,01	0,05
10	100,93	89,07	79,88	89,96	8,62	77,44	77,44	76,74	77,21	0,33	100,58	85,81	82,33	89,57	7,91
2	109,42	89,88	83,37	94,22	11,07	108,14	110,23	92,79	103,72	7,78	69,30	77,21	90,00	78,84	8,53
Conc.															
100	97,91	86,16	73,84	85,97	9,83	5,70	5,93	5,47	5,70	0,19	90,47	83,60	101,63	91,90	7,43
30	98,84	125,23	104,88	109,65	11,29	120,58	115,35	120,35	118,76	2,41	100,81	94,77	140,58	112,05	20,32
10	114,53	112,79	79,42	102,25	16,16	113,95	100,00	125,58	113,18	10,46	100,35	102,21	115,23	105,93	6,62
2	101,86	100,23	99,30	100,47	1,06	80,70	80,93	81,98	81,20	0,56	72,91	80,93	98,14	83,99	10,53
Conc.															
100	89,53	106,98	88,02	94,84	8,60	76,40	77,67	71,98	75,35	2,44	80,12	86,63	99,65	88,80	8,12
30	102,79	106,74	90,93	100,16	6,72	92,79	100,93	79,42	91,05	8,87	88,37	108,60	113,84	103,60	10,98
10	106,51	128,95	105,58	113,68	10,81	94,07	105,23	97,44	98,91	4,67	102,56	104,65	103,49	103,57	0,86
2	109,07	121,28	115,23	115,19	4,98	106,05	112,56	112,33	110,31	3,02	122,67	143,49	144,65	136,94	10,10
Conc.															
100	106,51	113,95	68,60	96,36	19,86	13,02	10,23	8,26	10,50	1,96	57,44	55,81	65,35	59,53	4,16
30	95,81	103,26	85,70	94,92	7,20	59,88	53,37	51,63	54,96	3,55	70,47	66,16	78,26	71,63	5,00
10	97,56	121,28	122,09	113,64	11,38	113,84	96,86	87,33	99,34	10,96	99,65	84,30	92,33	92,09	6,27
2	105,12	103,37	101,63	103,37	1,42	107,56	102,44	92,21	100,74	6,38	99,30	80,70	104,77	94,92	10,30
Conc.															
100	21,74	26,16	27,09	25,00	2,33	42,79	42,33	35,93	40,35	3,13	72,91	90,12	90,35	84,46	8,17
30	76,86	75,58	67,44	73,29	4,17	72,09	72,79	70,47	71,78	0,97	74,65	80,47	88,14	81,09	5,52
10	99,65	92,67	80,70	91,01	7,83	80,93	83,49	74,42	79,61	3,82	63,72	68,14	80,00	70,62	6,87
2	96,05	98,72	89,88	94,88	3,70	90,93	88,37	86,05	88,45	1,99	69,53	74,53	80,81	74,96	4,61
Conc.															
100	50,81	44,88	49,88	48,53	2,60	70,00	66,28	67,67	67,98	1,53	84,65	87,09	108,26	93,33	10,60
30	95,00	91,16	78,02	88,06	7,27	82,67	76,05	72,09	76,94	4,37	74,42	94,42	91,63	86,82	8,84
10	97,21	111,74	90,81	99,92	8,76	90,93	84,42	83,60	86,32	3,28	76,40	93,84	80,93	83,72	7,39
2	90,23	90,70	76,40	85,78	6,64	98,60	91,63	95,23	95,16	2,85	94,65	110,35	104,88	103,29	6,51
Conc.															
100	91,16	90,81	82,33	88,10	4,09	74,65	75,12	89,77	79,84	7,02	76,63	80,00	81,86	79,50	2,17
30	95,70	95,47	98,49	96,55	1,37	99,42	99,65	100,12	99,73	0,29	86,51	91,86	99,19	92,52	5,20
10	69,53	74,07	79,30	74,30	3,99	79,53	74,42	86,16	80,04	4,81	71,98	78,84	71,86	74,22	3,26
2	79,88	72,44	89,07	80,47	6,80	69,65	74,30	73,95	72,64	2,12	59,30	67,44	63,95	63,57	3,33
Conc.															
100	85,70	31,63	35,35	50,89	24,66	6,98	6,51	5,47	6,32	0,63	59,30	66,51	60,12	61,98	3,22
30	100,00	100,00	90,35	96,78	4,55	73,84	74,65	85,35	77,95	5,25	64,77	60,12	59,77	61,55	2,28
10	113,02	111,28	97,33	107,21	7,03	93,84	81,16	101,86	92,29	8,52	69,53	80,23	74,30	74,69	4,38
2	108,49	113,95	104,65	109,03	3,82	97,21	87,91	111,28	98,80	9,61	83,26	75,93	83,60	80,93	3,54
Conc.															
20	6,86	6,51	7,09	6,82	0,24	88,37	94,77	81,63	88,26	5,36	154,07	148,26	156,16	152,83	3,35
5	26,16	28,95	28,02	27,71	1,16	110,81	99,88	86,86	99,19	9,79	161,51	161,63	135,47	152,87	12,31
2	84,88	80,12	90,23	85,08	4,13	100,47	121,74	111,86	111,36	8,69	175,12	170,58	177,21	174,30	2,77
0,5	87,56	84,07	85,23	85,62	1,45	105,35	98,84	103,26	102,48	2,71	147,56	183,95	140,93	157,48	18,91