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Antiasthmatic Properties of *Lippia javanica* and *Myrothamnus flabellifolius* using an *in Vitro Model*

by

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DECLARATION

I, **Ayiman Alnamni**, declare that thesis on ***"*Antiasthmatic properties of *Lippia. javanica* and *Myrothamnus flabellifolius* using an *in vitro* model"** is my own work, that it has not been submitted for any degree or examination at any other university, that it is free of plagiarism and that all the sources used have been indicated and acknowledged by complete references.

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Date Signed : 12 May 2022

DEDICATION

This thesis is dedicated to my husband, Dr Emad, who is the best husband in the world and to my dear wonderful mother, and to my friend, PhD student Dr Mariam Abobaker Rado, for their support, encouragement, and motivation which have played a tremendous role in helping me to achieve all my dreams and goals, not only academically, but personally as well. Thank you for always pushing me to uncover my true potential and bestowing a great philosophy of never to quit to succeed in the ideals you want most in life. Your zest for life has been a true inspiration to me to be the best version of myself and you've taught me that even the darkest times can be overcome through perseverance.

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I would also like to dedicate this thesis to anyone who has fallen victim to the grave disease of asthma and to survivors who were fortunate to overcome this battle. Your bravery and will to fight is truly admirable. You are forever in our thoughts and prayers.

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"Verily, with every hardship comes ease."

Quran 94:5-6

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ABSTRACT

Background and objectives: Asthma is a reversible, chronic inflammatory disease that causes obstruction to the airways that causes an increase in airway hyper-responsiveness that leads to recurrent episodes of chest tightness and coughing. *Lippia javanica* and *Myrothamnus flabellifolius* are among the most widely used medicinal plants in Southern Africa for asthma management, control, and treatment. The aim of this study was to investigate the potential antiasthmatic properties of *L. javanica* and *M. flabellifolius* in a cell culture model.

Method: Human bronchial smooth muscle cells (HBSMCs) were pre-treated with methanolic extracts of *L. javanica* and *M. flabellifolius,* at increasing concentrations (3.125, 6.25, 12.5, 25, 50 and100 µg/ml) in quadruplicate, and the cells were incubated for 1 hour, then cells treated with IL-1β (10 U/ml) with change medium every day followed by pre-treatments with treatments for 4 days. Cells were then exposed to XTT assay for 3hr. Morphological changes of cells were visualized by light microscopy. Dexamethasone and quercetin served as positive controls in the study.

Results: *L. javanica* did not significantly inhibit the proliferation of cells stimulated by IL-1β (p=0.3184). However, there was a general trend towards inhibition of cell proliferation with increasing concentrations of *L. javanica*. The IC_{50} for *L. javanica* was 24.27 µg/ml. *M. flabellifolius* significantly inhibited the proliferation of cells stimulated by IL-1β (p=0.0159). The IC_{50} for *M. flabellifolius* was 41.85 µg/ml. The extracts of both plants at the above concentrations caused sparse cell distribution and decreased cell size when compared to controls.

Conclusion: The methanolic extracts of *L. javanica* and *M. flabellifolius* inhibited the proliferation human airway smooth muscle cells stimulated with IL-1β. The antiproliferative effects observed in the study suggests that both plants may reverse

airway remodelling in asthma. However, the methanolic extract of *M. flabellifolius* inhibited the proliferation human airway smooth muscle cells stimulated with IL-1β more than *the L. javanica*.

Keywords: human bronchial smooth muscle cells (HBSMCs), methanolic extract of *L. javanica and M. flabellifolius,* dexamethasone, quercetin, cell proliferation, interleukin-1β.

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LIST OF ABBREVIATIONS

AHR	Airway Hyper-Responsiveness
ANOVA	Analysis of Variance
AoSMC	Human Aortic Smooth Muscle Cell Line
ASM | BSMCs	Airway Smooth Muscle Cells | Bronchial Smooth Muscle Cells
BALF	Bronchoalveolar Lavage Fluid
BFGF	Basic Fibroblast Growth Factor
BHR	Bronchial Hyper-Responsiveness
BSM	Bronchial Smooth Muscle
BSMCs | ASM	Bronchial Smooth Muscle Cells | Airway Smooth Muscle Cells
BSS	Buffered Saline Solution
C2H5OH	Ethanol
C4H8O2	Ethyl Acetic Acid
C15H10O7	Quercetin
C22H29FO5	Dexamethasone
cAMP	Cyclic Adenosine Monophosphate
CCR	G-Protein-Coupled Chemokine Receptor(s)
CCR3	G-Protein-Coupled Chemokine Receptor-3
CH3OH	Methanol
COX	Cyclooxygenase
C. serratum	*Clerodendrum serratum*
CXC (α-Type)	Chemokine Receptor α-Type
CXC (β-Type)	Chemokine Receptor β-Type
DC	Dendritic (Antigen-Presenting) Cells
DMEM/F-12	Dulbecco's Modified Eagles Medium: Nutrient Mixture F-12
DMSO | (CH3)2SO	Dimethyl Sulfoxide
DPPH	2,2-Diphenyl-1-Picrylhydrazyl
ECM	Extracellular Matrix

EDTA	Ethylenediaminetetraacetic Acid
EIB	Exercise-Induced Bronchospasm
FBS	Foetal Bovine Serum
GINA	The Global Initiative for Asthma
GM-CSF	Granulocyte-Macrophage Colony-Stimulating Factor
HBSMCs	Human Bronchial Smooth Muscle Cells
HEPES	Hydroxyethyl Piperazineethanesulfonic Acid
HICs	High-Income Countries
HL-60	Human Leukemia Cell Line
ICS	Inhaled Corticosteroids
IgE	Immunoglobulin E
IL-1β	Interleukin-1-Beta
IL-4	Interleukin-4
IL-5	Interleukin-5
IL-6	Interleukin-6
IL-10	Interleukin-10
IL-13	Interleukin-13
KCl	Potassium Chloride
LMICs	Low- and Middle-Income Countries
LOX	Lipoxygenase
MALME-3M	Human Malignant Melanoma Cell Line
MCF-7	Human Breast Cancer Cells, Metastatic Breast Cancer (Adenocarcinoma)
MCP-3	Monocyte-Chemotactic Protein-3
MCP-4	Monocyte-Chemotactic Protein-4
MDA-MB-435	Highly Metastatic Breast Cancer Derived Cell Line—Actually of Melanocytic Origin
MDA-N	Methylenedioxyamphetamine-N
MLCK	Myosin Light Chain Kinase
PBS	Phosphate-Buffered Saline

PGE2	Prostaglandin E2
PDGF	Platelet-Derived Growth Factor
TNF-α	Tumour Necrosis Factor Alpha
S.D.	Standard Deviation
S.E.M.	Standard Error of the Mean
SM-α-Actin	Smooth Muscle α-Actin
SMBM	Smooth Muscle Basal Medium
SMMHC	Smooth Muscle Myosin Heavy Chain
T-Cells	T Lymphocytes
TGF-β1	Transforming Growth Factor Beta 1
Th2 Cells	T-Lymphocyte Helper 2 Cells
TNF-α	Tumour Necrosis Factor-α
TK-6	Thymidine Kinase-6
TNBC	Triple Negative Breast Cancer
WHO	World Health Organization
XTT	2,3-Bis-(2-Methoxy-4-Nitro-5-Sulfophenyl)-2*H*-Tetrazolium-5-Carboxanilide

CHAPTER 1

INTRODUCTION

1.1 Introduction

Asthma is a reversible inflammatory obstructive disorder of the airways that presents with recurrent wheezing, breathlessness, and coughing [1]. Asthma is ranked among the top 20 chronic diseases for disability-adjusted life years in children aged 5-14 years and young adults [2,3]. Mortality rates in children vary from 0 to 0.7 per 100,000 people [4,5]. According to the 2018 Global Asthma Report, in excess of 339 million people worldwide suffer from the disease and, by 2025, another 100 million people will be affected [6,7]. Notwithstanding the stark global and regional variations in the prevalence of asthma symptoms, the disease is escalating steadily in both low- and middle-income countries (LMICs) and high-income countries (HICs), suggesting that the global prevalence, morbidity, mortality, and economic burden associated with asthma over the last few decades have persisted [5,8].

In Africa, the prevalence of asthma increases by 50% every decade [8]. The pervasive effects of childhood asthma in African countries is observed more frequently than in HICs [9]. According to estimates, there are 3.9 million people living with asthma in South Africa as reported recently by the Global Initiative for Asthma [8]. Asthma is also the eighth leading contributor to the burden of disease in South Africa and the second most important chronic disease after HIV/AIDS [10]. Despite the availability of medications, asthma remains poorly controlled in many patients worldwide.

A wide array of antiasthmatic drugs currently being used to treat asthma lack efficacy, are associated with side effects, and are costly, which hinder treatment compliance [11]. In recent years, traditional herbal medicines have come into sharper focus as alternative therapeutics for respiratory disorders, including asthma [12,13]. Many of these herbs are used to relieve convulsive bronchitis and bronchial asthma. Since asthma is a chronic inflammatory disease of the respiratory tract implicating several immune cells, including eosinophils, neutrophils, macrophages, T-lymphocytes, mast cells and epithelial cells, traditional phytochemicals with antiasthmatic potential may be exploited for their diverse mechanisms of action to counteract the proinflammatory effects mediated by these immune cells [14,15].

In Southern Africa, *Lippia javanica* and *Myrothamnus flabellifolius* are among the most commonly used medicinal plants for asthma control, management, and treatment [16,17]. *L. javanica* is widely available throughout South Africa. It belongs to the Verbenaceae family, which includes approximately 200 herbs [18]. *L. Javanica* is commonly known as 'fever tea' [19]. *M. flabellifolius* generally grows in rocky areas throughout Namibia, Botswana, South Africa, and Zimbabwe [17]. *Myrothamnus* is the only member of the family Myrothamnaceae. The herb *M. flabellifolius* is usually taken orally as an infusion or decoction to treat colds and respiratory problems [20]. Several reports indicate that both *L. javanica* and *M. flabellifolius* possess anti-inflammatory, antidiabetic, antifungal, antiviral, and antioxidant properties [126]. This beneficial effect is likely attributed to polyphenols, including phenolic acids, tannins, and flavonoids [22]. Recent studies suggest that flavonoids can inhibit the production and release of IL-4, IL-13, and transcription factors, and directly stimulate cells that initiate allergies [23]. Additionally, these compounds may reduce airway hyper-responsiveness and inhibit free radicals and mast cell degranulation, which can lead to histamine release [23].

In Namibia, the Nama people use the leaf extract of *M. flabellifolius* to treat asthma and other chest diseases [17,24]. In *M. flabellifolius* leaves, 3,4,5-tri-O-galloylquinic acid is the principal polyphenolic compound with significant activity to attenuate bronchial hyperreactivity and allergic reactions [17,24]. The smoke released by combustion of *M. flabellifolius* leaves is hailed in traditional African medicine as beneficial to health, particularly chest conditions [24]. Numerous members of the *L. javanica* family (e.g., *Clerodendrum indicum* (L.) Kuntze, *Clerodendrum viscosum* Ven, *Lippia dulcis* Trev, and *Clerodendrum serratum*) are rich in trans-pinocarveol, an essential oil component used in pharmaceutical preparations to treat respiratory tract disorders, including asthma [19].

The traditional uses of the pharmacologically bioactive constituents of Verbenaceae family of plants are being applied efficaciously in Bangladesh for numerous health conditions such as cold, asthma, respiratory problems, bloating, tuberculosis and bronchitis [25]. The most prevalent chemical constituents in *Lippia dulcis* Trev and *Clerodendrum serratum* (Verbenaceae) are carbohydrates and phenolics that have biological effects such as antioxidant and antiproliferative properties, as well as essential oils and flavonoids that possess antiasthmatic and antitumour properties [26].

No studies have reported on the evaluation of the antiasthmatic effects of *L. javanica and M. flabellifolius.* As such, the study aimed to investigate the claim that *L javanica* and *M. flabellifolius* have antiasthmatic effects using an *in vitro* cell culture model.

1.2 Research Hypothesis

- *L. javanica* and *M. flabellifolius* extracts have have anti-asthmatic (antiproliferative activities) on human bronchial smooth muscle cells.

1.3 Aims and Objective

The aim of this study was to:

- Evaluate and investigate the antiasthmatic properties of *L. javanica and M. flabellifolius* in an *in vitro* cell culture model.

The specific objectives of the study were to:

- Prepare and characterize the antiproliferative activity of crude extracts of *L. javanica and Myrothamnus flabellifolus* on human bronchial smooth muscle cells.

- Evaluate the effects of the extracts of *L. javanica and Myrothamnus flabellifolus* on the morphology of human bronchial smooth muscle cells.

CHAPTER 2

LITERATURE REVIEW

2.1 Introduction

Asthma is a chronic inflammatory disease of the respiratory tract involving many cells and cellular elements triggered by genetic or environmental and lifestyle factors, and is characterized by airway hyper-responsiveness (AHR), inflammation, and variable airflow obstruction [27]. Asthma often presents as a heterogeneous disease clinically which may impact on its prognosis. Accordingly, the International Classification of Diseases diagnostic system, generally categorizes asthma patients based on disease severity and manifestation of complications [28], whereas novel methodologies afford identification of subtypes of asthma symptoms such as atopic status, wheeze, cough, breathlessness, chest tightness, coughing, particularly at night or in the early morning [29]. Recurrent episodes of wheezing are commonly associated with airflow obstruction that is often reversible, either spontaneously or with treatment [1]. Asthma leads to disability, increased health care costs, and a poor quality of life for those who suffer from it. It is a chronic disease that is most common among children and young adults, especially due to early-onset (one in four in the general population develops asthma before the age of 40) [4]. The early identification of such conditions and the appropriate treatment is crucial because children's behaviour can predict adult disease development due to the patterns they develop during childhood [30].This increases the risk of developing additional morbidities such as obesity or cardiac disease due to the restrictions (such as exercising) placed on an individual with improperly managed asthma [31].

2.2 Pathophysiology of Asthma

Asthma is a common chronic disorder of the airways that involves a complex interaction of airflow obstruction, airway hyper-responsiveness (AHR) and underlying inflammation (Figure 2.1). This section presents an overview of asthma, a description of the processes on which asthma is based, i.e., asthma pathophysiology and pathogenesis [32]. Many cells and cellular elements contribute to the chronic inflammation of the airways in asthma—among them are mast cells, eosinophils, T lymphocytes, macrophages, neutrophils, and epithelial cells. This inflammation commonly causes wheezing, breathlessness, chest tightness, and coughing in individuals who are susceptible [33].

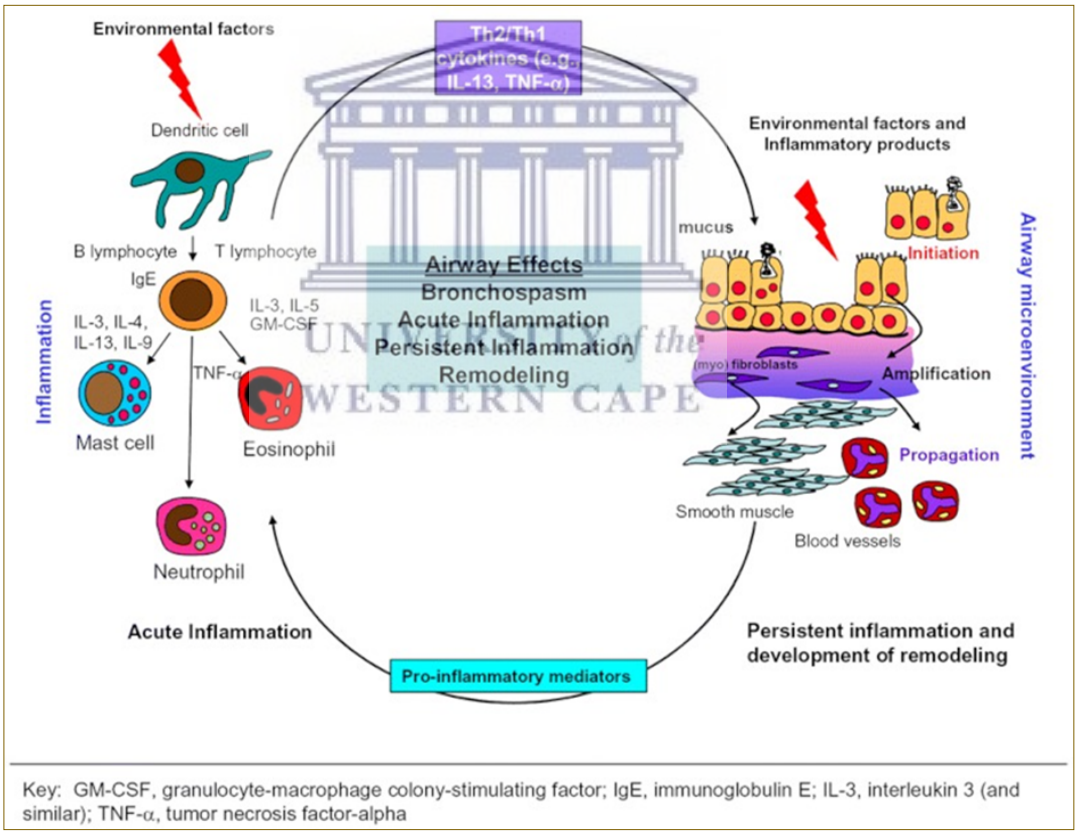


Figure 2.1: Pathophysiology and pathogenesis of asthma [34]

2.2.1 Pathophysiologic Factors in Asthma

2.2.1.1 Bronchoconstriction

Bronchoconstriction is the constriction of the airways in the lungs due to a dominant physiological response triggering clinical symptoms in airway narrowing and ensuing obstruction of airflow (Figures 2.2 and 2.3).

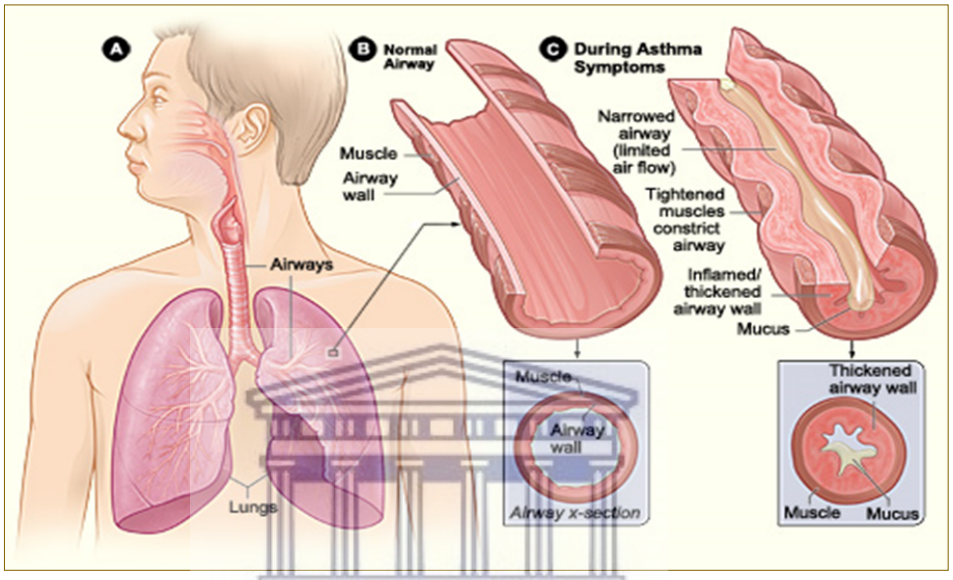


Figure 2.2: Location of the lungs and airways in the body (A) and cross-sections of a normal airway (B) and an airway during asthma symptoms (C)

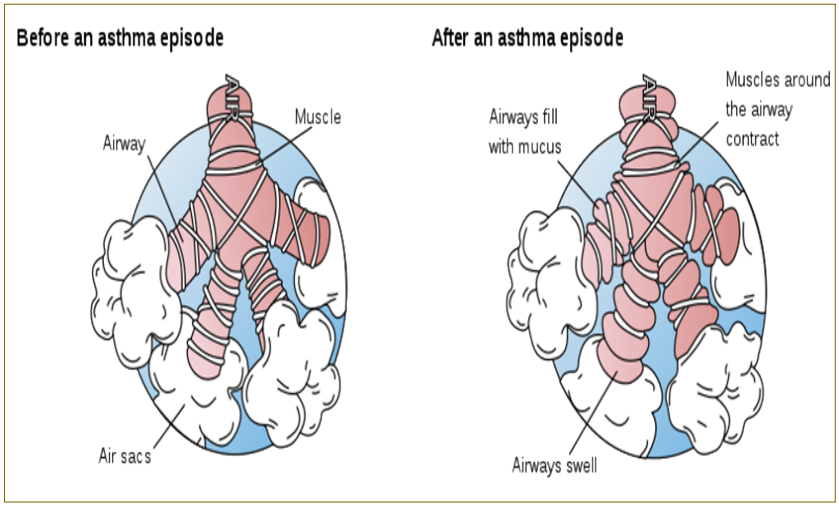


Figure 2.3: Pathophysiology of asthma before and after an asthma episode

[35]

In acute exacerbations of asthma, bronchial smooth muscle contraction (bronchoconstriction) occurs rapidly to narrow the airways in response to exposure to an array of stimuli, including allergens or irritants [33]. Allergen-induced acute bronchoconstriction results from an IgE-dependent release of mediators from mast cells that includes histamine, tryptase, leukotrienes, and prostaglandins that directly contract airway smooth muscle [36].

Aspirin and other nonsteroidal anti-inflammatory drugs can also cause acute airflow obstruction in some patients and evidence indicates that the non-IgE-dependent response also involves mediator release from airway cells [37,38]. Besides, other stimuli such as exercise, cold air, and irritants can also cause acute airflow interruption. Stress may as well play a role in precipitating asthma exacerbations. The mechanisms involved have yet to be elucidated and may comprise enhanced production and release of proinflammatory cytokines [33].

2.2.1.2 Airway Oedema

As asthma becomes more persistent and the inflammation more progressive, additional immune-related components may promote airflow constraints even further. These include oedema, inflammation, mucus hypersecretion and the formation of inspissated mucus plugs, as well as structural changes, including hypertrophy and hyperplasia of the airway smooth muscle. These latter changes may confer a clinical refractory state or resistance to conventional treatment regimens [33].

2.2.1.3 Airway Hyper-Responsiveness (AHR)

Airway hyper-responsiveness (AHR) is a response to stimuli that causes the

bronchospasm to be exaggerated. Asthma severity is correlated with the extent to which AHR may be determined by contractile responses to methacholine challenges. Several factors contribute to AHR, including inflammation, dysfunctional neuroregulation, and structural changes. Inflammation is a discernible determinant of the degree of AHR and, therefore, treatment targeted towards reducing inflammation can lessen AHR and improve asthma control [33,39].

2.2.1.4 Airway Remodeling

Some asthmatics may only have partial reversibility of airflow limitation. Airways can undergo changes that are permanent and associated with a progressive loss of lung function that cannot be prevented or fully reversed with current therapies [33]. Remodelling of the airways is caused by activation of many structural cells, leading to permanent changes that increase airway obstruction, decrease responsiveness, and make the patient more resistant to therapy [34].

2.3 Bronchial Smooth Muscle Cells

Airway smooth muscle consists of spindle-shaped, single-nuclear, non-striated cells that cannot be voluntarily controlled. Smooth muscle is distributed throughout the body in the walls of hollow organs, such as airways, blood vessels and intestines, where its primary function is to regulate the flow of air, circulation of blood or peristaltic movement via contraction and relaxation [40]. Airway and vascular smooth muscle are organized as multi-unit (visceral) smooth muscles in which each smooth muscle cell is separately innervated.

By contrast, single-unit muscles, such as intestinal and detrusor smooth muscle, contain more gap-junctions and fewer neuronal innervations, that allow fluctuations in membrane potentials and myogenic activity [41]. Furthermore, variations in excitation-

contraction coupling as well as heterogeneity in the distribution of ion channels and receptors, cause the response to a certain hormone, neurotransmitter or pharmacological agent to differ between smooth muscles [42]. In humans, the entire respiratory tree from the trachea to the smaller airways is surrounded by airway smooth muscle. Historically, the airway smooth muscle has solely been considered an effector cell-type in asthma, which causes airway narrowing because of spasmogen release from mast cells and nerves, and an impaired release of epithelium-derived relaxing factors [43].

2.4 The Proliferation of Bronchial Smooth Muscle Cells

Airway inflammation promotes the proliferation of airway smooth muscle (ASM) cells and plays a central role in the hyper-responsiveness and remodelling of the asthmatic airway, which further trigger hyperplasia and hypertrophy of smooth muscle cell. The proliferation of ASM cells is induced by a plethora of mitogens and upregulation of proinflammatory mediators in the airway (e.g., growth factors, cytokines, inflammatory mediators and allergens), and has been proposed as a primary mechanism underlying increase in ASM mass [44,45].

Furthermore, ASM cell proliferation as a feature of the inflammatory response in asthma, is regulated by the proinflammatory cytokines and chemokines, including IL-1β and tumour necrosis factor-α (TNF-α). These proinflammatory cytokines have been shown to promote human ASM cell proliferation *in vitro,* which is due to cyclooxygenase-2 expression, production of prostaglandin E2, and increased cAMP levels. It has been shown that ASM cells cultured from asthmatic patients *in vitro* grow more rapidly than cells derived from non-asthmatic patients [45].

In the airway wall of healthy subjects, the smooth muscle layer consists mainly of differentiated ASM cells, which are characterized by low proliferation rates [46], low

fractions of biosynthetic organelles, and relatively high expression levels of contractile proteins, including smooth muscle α-actin (SM-α-actin), calponin, and smooth muscle myosin heavy chain (smMHC) [47]. In contrast to skeletal myocytes and cardiomyocytes, ASM cells maintain their capacity to re-enter the cell cycle [48].

Thus, exposure to mitogenic stimuli (e.g., PDGF) activates the induction of a more proliferative/synthetic ASM cell phenotype [49], which correlates with a loss of contractile responsiveness, most likely the corollary of downregulated contractile protein expression [46].

Long-term serum deprivation results in the reinduction of a contractile phenotype, accentuating the reversibility of the ASM phenotype [50]. This phenotypic plasticity might be indispensable in growth and repair processes of inflamed airways and may contribute to airway remodelling in chronic asthma [46,51]. Extracellular matrix (ECM) proteins or macromolecules that envelop the tissue cells modulate many facets of cellular behaviour, including migration, differentiation, survival, and proliferation of cells originating from a variety of tissues, including ASM [52,53].

Compositional analysis of patient biopsies has demonstrated that the quantity of ECM components are altered in the airways of chronic asthmatics which, together with increased ASM mass, are major contributors to airway remodelling in asthma and chronic obstructive pulmonary disease [46,54]. Deposition of collagen IV and elastin is decreased in the airway wall of asthmatic patients, whereas collagen types I, III, and V, and fibronectin, tenascin, hyaluronan, versican, and laminin α2/β2-chains are increased compared with healthy subjects [46].

Further, in studies using ASM cells in culture, it has been observed that ECM proteins may differentially affect growth factor-induced phenotypic modulation. Thus, in human

ASM cells cultured on fibronectin or collagen I matrixes, progression towards a proliferative phenotype, induced by either PDGF or α-thrombin, was promoted, whereas culturing on a laminin or matrigel matrix inhibited phenotype switching by these mitogens [49]. Enhancement of PDGF-dependent proliferation of human ASM cells on a fibronectin or collagen I matrix is dependent on activation of α2β1 - α4β1 and α5β1-integrins [55]. These findings indicate a high clinical significance of ECM remodelling in asthma. It has been shown that in ASMs the presence of the soluble forms of fibronectin and collagen I can decrease the contractile, but increase the proliferative capacity of ASMs [46].

The airways are thought to contribute to the persistent airway obstruction and AHR of the airways of asthmatic patients by intensifying airway wall thickness and stiffness of the airways, thus modifying the contractility/relaxation of the airway [56]. In histologic studies of asthmatic airways, the normal profile of ECM proteins is altered such that collagen I, III, and V, fibronectin, tenascin, hyaluronan, versican, and laminin a2/b2 are upregulated, while collagen IV and elastin are downregulated [57]. Specifically, around the ASM of asthmatic patients, collagen I, hyaluronans, and versican are overexpressed. The complex network of macromolecules constituting the ECM not only provides mechanical support that plays an fundamental role in the maintenance of airway structure and function, but presumably also regulates an assortment of cellular functions, including cell proliferation [58].

In addition to its contractile function, the ASM is recognized for its active involvement in the progression of asthma [54]. Consequently, the ASM can produce and secrete a number of growth factors, chemokines and cytokines [59] that can promote the recruitment and survival of inflammatory cells, such as mast cells [60]. Additionally, ASM cells from asthmatics display amplified synthetic machinery [61], because of an altered ECM and the diversity of mediators that are released during inflammation [62].

These increased synthetic functions may contribute to hyperplasia and hypertrophy, and the increased ASM mass that is observed in patients with asthma. The ASM has, because of the increased sensitivity of the airways to direct constrictors of the airway smooth muscle, also logically been linked to AHR [63-65]. These structural changes include thickening of the sub-basement membrane, subepithelial fibrosis, ASM hypertrophy and hyperplasia, blood vessel proliferation and dilation, and mucous gland hyperplasia and hypersecretion [33].

Even though the causes and types of airway remodelling that occur in asthma are heterogeneous, they share commonalities with uncontrolled migration, proliferation and differentiation of resident cells playing key roles. These include ASM hypertrophy and hyperplasia, subepithelial fibrosis, myofibroblast accumulation, mucus gland and goblet cell hyperplasia and epithelial disruption [52,66]. In addition to the airways, remodelling may also be detected in the vasculature. This is a key pathological feature of pulmonary arterial hypertension where increased stiffness and muscularization of proximal pulmonary arteries occurs due to hyperproliferation and transdifferentiation of pulmonary artery smooth muscle cells [67].

However, it remains an open question whether vascular remodelling also occurs in asthma where a degree of neovascularization is observed leading to enhanced tissue perfusion, which may affect normal gas exchange and airway dynamics [68,69]. However, these investigations focused primarily on human bronchial smooth muscle cell cultures and airways to quantify the hyper proliferative response of the ASM that leads to thickening of the airways in asthma. This ASM hypertrophy/hyperplasia can be monitored using cell proliferation assays and image analysis [69].

The key pathological trait of chronic asthma is an increase in the mass of ASM that stems from increased cell number (hyperplasia) combined with an increase in cell size

[70]. Maintenance of ASM contractile properties requires an increased ASM mass in asthma for exacerbated airway constriction. Thus, it is critical to elucidate the factors that may modify the proliferation of human ASM cells in culture [71,72]. In several animal species, an increase in ASM proliferation has been associated with leukotriene D4, histamine, insulin-like growth factor-I, interleukin (IL)-1β, IL-6, and epidermal growth factor [73]. Since human ASM cells in asthmatic lungs are exposed to several inflammatory mediators, it is imperative to differentiate the influences of these mediators on muscle growth and division. Proliferation occurs in response to histamine, thrombin, epidermal growth factor, basic fibroblast growth factor (bFGF), and PDGF [74], whereas dexamethasone, salbutamol [75], TNF-α, heparin, PGE2, and vasoactive intestinal peptide suppress mitogen-induced proliferation of human ASM cells in culture [72,76].

Furthermore, two patterns of ASM proliferation have been identified in necropsy studies. In type 1, increased muscle mass correlates with hyperplasia that is limited to the large central airways, while in type 2 mild hyperplasia occurs in the large airways, but hypertrophy is extended throughout the bronchial tree, specifically in small peripheral airways [70]. Increases in ASM are induced by several mechanisms, including proliferation induced by inflammatory mediators, cytokines and growth factors [52]. ASM hypertrophy may be due to repeated episodes of bronchospasm, downregulated inhibitory control, and build-up of enriched plasma in the ASM milieu.

Modifications in ASM morphology occur in parallel with alterations in the functional characteristics of ASM in asthma. Previous studies have shown that asthmatic ASM has an increased maximal velocity of muscle shortening due to an increase in the activity of myosin light chain kinase [77], which may account for the reduced bronchodilatory effect of inspiration in asthmatics. Undoubtedly, the insufficiency of this protective mechanism may be regarded as one of the leading activators of AHR

[78].

Exposure of ASM cells derived from nonasthmatic patients to proinflammatory mediators such as interleukins revealed increased cell proliferation in the presence of bronchoalveolar lavage fluid (BALF) from asthmatic patients, an effect which was further corroborated when the BALF was obtained after allergen challenge [79]. Such observations increase the possibility for the development of new therapeutic agents that specifically target the ASM, thus affording an effective method for reversing or preventing the airway remodelling that is associated with chronic severe asthma.

2.5 Mechanisms in the Development of Airway Inflammation

2.5.1 Inflammatory Cells

Mast cells release bronchoconstrictor mediators (histamine, cysteinyl-leukotrienes, prostaglandin D2) [80-82]. Even though allergen activation is mediated through high-affinity IgE receptors, sensitized mast cells may also be activated by osmotic stimuli to account for exercise-induced bronchospasm (EIB). Increased numbers of mast cells in ASM may correlate clinically with AHR [82,83]. Mast cells also release abundant quantities of cytokines to alter the airway microenvironment and promote inflammation even though exposure to allergens is insufficient [33].

Eosinophils exist in increased numbers in the airways of most people with asthma [33,84-86]. These cells contain inflammatory enzymes, generate leukotrienes, and express a wide variety of proinflammatory cytokines. Increases in eosinophils often correlate with greater asthma severity. Several lines of evidence suggest that treating asthma with corticosteroids reduces circulating and airway eosinophils, thus improving clinical outcome. However, the current understanding of the complex pivotal involvement of eosinophils in asthma has been confounded further by studies

that showed significantly reduced eosinophils upon anti-IL-5 treatment, but this did not affect asthma control [87]. Therefore, although the eosinophil may not be the only primary effector cell in asthma, it likely has a distinct role in different phases of the disease [33].

Neutrophils are increased in the airways and sputum of persons who have severe asthma, during acute exacerbations, and in the presence of smoking. Neutrophils have been implicated in a lack of response to corticosteroid treatment, but pathophysiological role requires further elucidation [88]. The mechanisms underlying the regulation of neutrophil recruitment, activation, and alteration in lung function are keenly being investigated, but leukotriene B4 may contribute to these processes [89-91].

Macrophages are the most numerous cells in the airways and are activated by allergens through low-affinity IgE receptors to release inflammatory mediators and cytokines that amplify the inflammatory response [92]. In asthma, macrophages are derived from circulating monocytes that migrate to the ASM microenvironment to facilitate inflammation through the production and release of a variety of cytokines that either increase or decrease inflammation, depending on the inducing stimulus.

Asthma is characterized by impaired lymphocyte function caused by altered alveolar macrophage function after exposure to allergens. Macrophages secrete IL-10, which is known to be anti-inflammatory in its actions. In patients with asthma, alveolar macrophages secrete low levels of IL-10. It is therefore likely that macrophages play an important role in the prevention of allergic inflammation by acting as anti-inflammatory agents [93].

Epithelial cells in asthmatic persons may be injured upon generation of inflammatory

mediators, recruitment, and activation of inflammatory cells. Infection by respiratory viruses can cause epithelial cells to overproduce inflammatory mediators to injure the epithelium further [94]. The repair process, following an injury to the epithelium, may be abnormal in asthma, thus promoting the obstructive lesions that occur in asthma [33].

T-Lymphocyte Helper 2 (Th2) cells are critical proinflammatory cells in asthma, promoting both bronchospasm and inflammation by stimulation of mast cells, eosinophils and B-lymphocytes. The Th2 cell is nonspecific in its response and relies on stimulation by IL-4 and IL-13 from dendritic antigen-presenting cells (DC) both for its generation from naive T-cells and its subsequent activation to produce its proinflammatory cytokines [95]. The airway epithelium contains many DCs which have toll-like receptors, as do the epithelial cells [96]. Once activated by their specific antigen, the DCs migrate to lymphoid tissue in the lungs to control the division of naive lymphocytes into their various subtypes. Upon Th2 stimulation, DCs respond to a wide range of pathogens that enter the lungs, and trigger the immune pathway encompassing normal pulmonary defences against infection [97].

Lymphocytes play a central role in coordinating the inflammatory response in asthma through the release of specific categories of cytokines, initiating in the recruitment and proliferation of eosinophils and the accumulation of mast cells in the airways. T-lymphocytes are encoded to express a distinctive pattern of cytokines, including IL-3, IL-4 and IL5 [98]. It has been suggested that antigen-presenting cells such as DCs are involved in this programmed function of T-lymphocytes, since they migrate from the epithelium to lymph nodes or interact with lymphocytes in the microenvironment of the airway mucosa [99].

2.5.2 Inflammatory Mediators

Cytokines modulate the inflammatory response in asthma and likely determine its severity [100]. Th2-derived cytokines include IL-5, which is needed for eosinophil differentiation and survival, and IL-4 which is important for Th2 cell differentiation and IL-13 is important for IgE formation. Key cytokines include IL-1β and tumour necrosis factor-α (TNF-α), which amplify the inflammatory response, and granulocyte-macrophage colony-stimulating factor (GM-CSF), which prolongs eosinophil survival in airways. In recent studies, treatments targeting one cytokine (e.g., monoclonal antibodies against IL-5 or soluble IL-4 receptor) did not improve asthma outcomes [33].

Immunoglobulin E (IgE) a special type of antibody, responsible for the activation of allergic reactions, plays a critical role in the development and persistence of allergies. The high-affinity receptors on the surface of cells allow IgE to bind to them. The number of IgE receptors on mast cell surfaces is amplified when activated by interaction with antigen, and the ligand-receptor complexes so formed trigger the release a wide variety of mediators to initiate acute bronchospasm and extended release of proinflammatory cytokines to perpetuate underlying airway inflammation [80,101]. Basophils, DCs, and lymphocytes also have high-affinity IgE receptors. The development and use of monoclonal antibodies against IgE provide proof of concept that the reduction of IgE is efficacious in asthma treatment [102,103]. These clinical observations further support the importance of IgE in asthma management [33,93].

Cysteinyl-leukotrienes are potent bronchoconstrictors derived mainly from mast cells. They are the only mediators whose inhibition is associated with an improvement in lung function and asthma symptoms [36,104,105]. Recent studies have also shown leukotriene B4 can contribute to the inflammatory process by recruitment of

neutrophils [106].

Chemokines are mainly produced by airway epithelial cells, and they play an important role in recruiting inflammatory cells into the airways [107]. There are two kinds of chemokines, (a) CXC (*-type) and CXC (*-type), which act via G-protein-coupled receptors (CCR). The synthesis and release of chemokines are induced by asthma exacerbation. Activated eotaxin, eotaxin-2, MCP-3, MCP-4, and CCR3 have been shown to be associated with increased AHR in asthmatic patients [93]. Our understanding of the pathophysiology of asthma and other respiratory illnesses have been greatly enhanced by the elucidated roles these mediators play in orchestrating injury, repair, and many other processes [33].

Nitric oxide, an endogenous vasodilator ,mainly produced by airway epithelial cells, acts as an inflammatory agent and vasodilator [108,109].

2.6 Management of Asthma

The guidelines for the treatment of asthma are described in a five-step protocol by the Global Initiative for Asthma consortium and focus on the control of symptoms, the prevention of exacerbations and decline in lung function, as well as minimizing the risk of side-effects. Asthma management is achieved using controller medications that affect airway inflammation, and reliever therapies that reverse airway obstruction and can be used when symptoms of asthma worsen [110].

2.6.1 Significance of Medicinal Plants and Traditional Medicines in the Management of Asthma

Despite recent recommendations made by the Global Initiative for Asthma (GINA), on the use of as-required inhaled corticosteroid (ICS)–long-acting β2-agonists in symptomatic mild or moderate asthma, indicated also for current or former smokers,

evidence is largely lacking for its effectiveness in all cases of asthma [111,112]. Therefore, more pragmatic experimental designs, including stratification of patients according to their clinical phenotype and molecular features [29], and broader populations to increase the generalizability of evidence to asthma in clinical practice are clearly desirable [112-114]. Furthermore, due to the lack of organized health care systems in developing countries, people with chronic diseases like asthma are among the worst affected. Compared to high-income countries (HICs), many populations in low- and middle-income countries (LMICs) do not have access to modern medications, especially those living in remote areas [5]. Instead, they turn to traditional medications to alleviate a variety of health issues. Many plant species have been isolated for use as drugs, lead compounds, or pharmacological agents [115-117]. As a result, many South Africans use traditional medicines to treat illnesses, including respiratory infections and asthma [118-120]. Moreover, the WHO (World Health Organization) recommends developing countries to incorporate traditional herbal preparations into their health programmes, as long as they are proven non-toxic [119].

In many countries, traditional medicines have become popular alternatives to conventional medicine [116], including South Africa, because of their affordability, accessibility, and cultural significance [1,9,10,16,118-121]. In addition, South Africa has a rich culture and biological diversity that has spurred successful and sustained ethnopharmacology research [119]. Several hundred plants are used in traditional medicine of Southern Africa, which has one of the richest plant diversities in the world [16,118-120,122].

Over the years, medicinal plants have successfully served as essential components of traditional medicine and have demonstrated their use as potential remedies for respiratory tract infections [121]. *In vitro* screening is important for the validation of the

traditional use of medicinal plants and provides leads in the search for new active principles [116,117,121]. The noteworthy antimicrobial and immunological activities from *in vitro* testing may not directly confirm that the tested plant extracts are effective medicines, but affords a straightforward understanding of the efficacy of these medicinal plants in traditional medicine and their potential use as a source of antiasthmatic therapy [123]. Asthma sufferers may greatly benefit from traditional medicine as it has over the years been thought to treat conditions better than Western medicine [119,123].

Research conducted to evaluate the immunomodulatory/anti-inflammatory effects of medicinal plant extracts revealed that *in vivo* and *in vitro*, medicinal plant extracts attenuated asthmatic reactions [121]. The findings further support the hypothesis that herbal medicines may help patients with asthma as adjuvant therapies in the future, highlighting the potential for herbal medicine to be used in immunotherapy [119]. A number of herbal constituents have been used to cure asthma, but their pharmacological/therapeutic basis remains unknown [118]. Furthermore, the mechanisms of their action are not completely understood. For this reason, herbal principles used for asthma should be supported by scientifically validated evidence, including preclinical and clinical assessments, which have become the standard of evaluation for herbal health claims [13,124]. The efficacy of plants used in African traditional medicine against asthma and chest infections has recently been reported [119].

Many medicinal plants have been identified as having the ability to treat respiratory disorders [118]. These herbs are primarily used in asthma therapy to treat convulsive bronchitis and bronchial asthma, which is caused by a continuous contraction of the smooth muscle of the bronchial passages and is associated with mucous oedema and increased secretions [120]. Since asthma is a chronic inflammatory disorder rather

than a bronchospastic disease, medicinal plants that can regulate the underlying airway inflammation as well are required [13]. Various medicinal plants have been used to treat asthma. In natural asthma treatment, vitamins, minerals, and herbs are used to prevent attacks and relieve symptoms [125].

2.6.2 Important Medicinal Plants with Antiasthmatic Potential

In African and the Indian subcontinent, *L. javanica* [18,126-129] and *M. flabellifolius* [17,24,130] are amongst the most widely used medicinal plants as a source of natural anti-inflammatory agents in the management, control and/or treatment of asthma.

2.6.2.1 *Lippia javanica*

L. javanica is widely distributed throughout South Africa, where it is used extensively in traditional herbal remedies. About 200 herbs are included in the family Verbenaceae, which includes *Lippia* (Houst.) [18]. *Javanica* was named by Nicolaas Laurens Burman (1734–1793), who mistakenly believed that the type of specimen had been collected in Java, Indonesia in 1768 [126]. *L. javanica* is commonly known as *"fever tea".* The name comes from its use as a weak infusion to treat fever symptoms [19]. As a medicinal tea, *L. javanica* is used to treat specific conditions such as asthma in Zimbabwe for a limited number of days [126]. *L. javanica* is one of the four species of *Lippia* indigenous to South Africa, where it grows as an erect shrub to about 2 meters in height. The plant has hairy leaves with compound veins and is highly aromatic with a strong lemon scent, and produces dense rounded heads of small yellowish-white flowers during the flowering season [18].

Figure 2.4: The *L. javanica* in its habitat, November and December 2019, Kirstenbosch Garden Centre in South Africa; photographs taken by the researcher.

L. javanica may be developed commercially as an international product as soon as it becomes available beyond some limited local use. According to research, this plant is one of only a few species that could be integrated with the domestication process in sub-Saharan farming systems to support health, income security, and nutritional security for local communities [126]. The different parts of *L. javanica*, such as the leaves and twigs, are used in Bangladesh, Botswana, Ethiopia, Kenya, South Africa, and Zimbabwe to treat asthma, colds, chronic coughs, bronchitis, and tuberculosis. The preferred method to treat asthma and coughs in Botswana, South Africa, and Zimbabwe involves burning the leaves, twigs, and inhaling the smoke [131-133]. The medicinal plant "*muti*" markets in South Africa also sell the stems and leaves of *L. javanica* as herbal medicine [126].

L. javanica extracts provide an alternative therapy for asthmatic patients because a large proportion of patients are not responsive to corticosteroids (40%) [125]. *L. javanica* grows naturally in central, eastern, and southern Africa and has also been discovered in the tropical Indian subcontinent [133,134]. *L. javanica* is reviewed for its potential as a tea and medicinal plant and for its phytochemistry and biological properties [125]. In a thorough literature review, it was discovered that *L javanica* is used as a herbal tea and is used in ethnomedicine for asthma, colds, coughs, fevers,

chest pains, and bronchitis [131,135].

Compositional analyses of extracts derived from *L. javanica*, identified numerous classes of phytochemicals, including volatile and non-volatile compounds such as alkaloids, amino acids, flavonoids, triterpenes, iridoids and minerals. Studies have shown that *L. javanica* has antimicrobial and antioxidant properties, as well as anticancer, antidiabetic, and antimalarial activities [136-138]. *L. javanica* has been reported to be able to treat allergic asthma [125].

It has been demonstrated that flavonoids can be beneficial for allergic disease [23]. By inhibiting the production and release of IL-4 and IL-13, and transcription factors, flavonoids can directly affect the cells that trigger allergic reactions [139,140]. In addition to inhibiting free radical formation and their antitumour action, flavonoids are also known to inhibit mast cell degranulation and subsequent histamine release [23]. In addition to being a flavonoid, quercetin has been found to be antiproliferative in many cell types, such as in cancer cell lines [141]. The exact mechanisms involve in the antitumour effects flavonoids remain largely unknown, but they have been reported to predominantly inhibit cell proliferation. Researchers have observed quercetin's antiproliferative properties in cultured prostate tumour cells and human MCF-7 breast cancer cells [142]. Additionally, quercetin has been shown to inhibit bronchial obstruction and AHR in guinea pigs [143] and perturb non-cytotoxic histamine release from rat peritoneal mast cells *in vitro* [144]. Moreover, quercetin was observed to induce bronchodilation of smooth muscle of the trachea *in vitro* using tracheal rings [145].

L. Javanica has many different local names belonging to the family Verbenaceae. It is one of the four indigenous *Lippia* species in South Africa (Figure 2.4). The species grows to a height of approximately 2 m as an erect woody shrub. This plant has hairy

leaves with compound veins and is highly fragrant with a strong lemon scent. Yellowish-white flowers appear in dense round heads during the blooming season [126]. This plant is distributed in central, eastern, and southern Africa and has also been recorded in the tropical Indian subcontinent [134].

2.6.2.2 *Myrothamnus flabellifolius*

M. flabellifolius is found in the rocky regions of Namibia, Botswana, South Africa (the Northwest Province and Limpopo) and Zimbabwe [17]. It is found mostly in the north of South Africa, on exposed rocky slopes and dry mountainous terrain [146]. Among the flowering plants, *Myrothamnus* belongs to the family Myrothamnaceae, the only genus in this family. Currently, there are two species of *Myrothamnus*: *M. flabellifolius* and *M. moschatus* [17]. Generally regarded as a medicinal plant, *M. flabellifolius* carries a name independently attributed to it by local tribes.

The names refer to the plant's resurrection ability, regarded as a symbol of hope in African cultures [22]. In southern Africa, there are many different resurrection plants of which *M. flabellifolius* is one. It is a woody shrub of about 0.4 m in height, with stiff, strong branches. Its leaves and twigs have strong aromatic properties, and these components are used in many medicinal preparations. *M. flabellifolius* leaves are traditionally burned and the smoke inhaled for treating asthma and chest pains [17].

Traditionally, the leaves and twigs of this plant have been utilized for a number of purposes. This plant is usually consumed in the form of an infusion or decoction, or the leaves are burned and inhaled as smoke. The smoke from burning leaves has been shown to relieve chest pains and asthma [135,147]. Besides, it is used orally for the treatment of respiratory ailments and colds [20]. Traditional African medicine utilizes the upper aerial parts and leaf tips of *M. flabellifolius* for treating respiratory

and urinary tract infections [148].

M. flabellifolius is only one of the plant species that have not been commercialized yet. It has been used traditionally as an herbal tea and in the treatment of various illnesses, such as asthma, epilepsy, etc. [17,20]. *M. flabellifolius* may be studied from a chemical perspective in order to isolate some natural products that may be used as precursors in the development of new drugs [17]. It has been reported that *M. flabellifolius* leaves contain high levels of polyphenol W(tannins) [22]. Of these, 3,4,5-tri-O-galloylquinic acid was detected as the most prevalent polyphenol [17,149]. Galloylquinic acid has been identified as a compound that has a high level of activity against AHR and allergic reactions [22]. Other essential oil components of *M. flabellifolius*, such as trans-pinocarveol, are useful for treating respiratory problems, including asthma [19]. Interestingly, this plant has antibacterial, antifungal, antiviral, anti-inflammatory, anti-diabetic and antiasthmatic and antioxidant properties. These beneficial activities probably result from phenolic acids, tannins and flavonoids found in numerous polyphenols, including phenolic acids and flavonoids [23]. *M. flabellifolius* is the only genus belonging to the Myrothamnaceae family, which includes flowering plants (Figure 2.5) [17].


Figure 2.5: *M. flabellifolius* in is habitat; collected in February 2020, Limpopo Province, South Africa

Based on the literature surveyed, no studies have evaluated the antiasthmatic effects of *L. javanica* and *M. flabellifolius.* Therefore, this study evaluated the potential antiasthmatic effects of *L. javanica* and *M. flabellifolius* using an *in vitro* cell culture model. By improving lung function through bronchodilation, current asthma therapeutic approaches aim to treat asthma bronchodilation [119]. By reducing side effects and increasing the efficacy of asthma treatments, new and improved treatments become essential to provide better therapeutic value. The medicinal properties of *L javanica* and *M flabellifolius* are due to the presence of bioactive compounds.

2.6.3 Review of Studies That Have Evaluated Antiasthmatic Properties of Medicinal Plants

The study of herbal medicines can open new vistas to drug discovery [7]. Critical to such investigations are *in vitro* proliferation assays that need to be adhered to when working with cultured cells. A proliferation assay *in vitro* can be used to determine whether cells are triggered to divide after exposure to a specific stimulus or to assess differences between cell populations in their ability to divide in response to the same

stimulus.

When using *in vitro* assays, a given procedure is performed outside of the body of a living organism, in a controlled environment. This involves the use of a solid lung transplant in a simulated physiological environment. In the present study, we used *in vitro* models (isolated cells), which provide a simple, rapid and sensitive method for screening drug candidates for biological activity with a moderate to high throughput. In natural product research, using the assay method for screening for antiproliferation activity is a very useful system because of its rapidity, accuracy, and simplicity. As a screening parameter, antiproliferation activity has been increasingly used in the search for new antiasthmatic agents derived from herbs [125].

This study uses an *in vitro* assay method to determine the activity of the antiproliferative agent by measuring cell proliferation using the XTT (2,3-bis [2-methyl-4-nitro-5-sulfophenyl]-2H-tetrazolium 5-carboxanilide) assay. Despite the relatively high costs for all the reagents and substrates, the procedure has the advantage of using less time and reagent.

CHAPTER 3

MATERIALS AND METHODS

3.1 Materials and Equipment

The materials and equipment used in the study are summarized in Tables 3.1 and 3.2, respectively.

Table 3.1: List of chemicals

Chemical	Supplier	Batch number
Methanol (CH3OH)	Sigma -Aldrich R & D System, USA	(SHBC1673V)
Ethanol (C2H5OH)	Sigma -Aldrich R & D System, USA	(V001229)
Ethyl acetic acid (C4H8O2)	Sigma -Aldrich R & D System, USA	(6128K25/0419)
Distilled water	Lab Chem	
Dimethyl sulfoxide (DMSO) | (CH3)2SO	Lab Chem, Sigma-Aldrich R & D System, USA	(SHBH9944)
Quercetin (C15H10O7)	Sigma-Aldrich R & D System, USA	(SLCC9071)
Dexamethasone (C22H29FO5)	Sigma-Aldrich R & D System, USA	(B00064136)
Smooth muscle basal medium (SMBM)	Lonza,MD USA	(0000867704)
Dulbecco's Modified Eagles Medium: Nutrient Mixture F-12 (DMEM/F-12)	Gibco (Thermo Scientific)	(2115826)
Supplements and growth factors (hFGF, insulin, hFGF-B, FBS, and gentamicin/amphotericin-B)	Lonza,MD USA	(2090407)
Trypsin/EDTA, HEPES buffered saline solution and trypsin neutralizing solution	Lonza,MD USA	(0000859859)

Phosphate-buffered saline (PBS)pH7.4	Gibco, Island in New York	(1858680)
Interleukin-1β (10 U/ml)	Sigma -Aldrich R & D System, USA	(MKCK0057)
XXT assay (sodium3-[1-(phenylaminocarbonyl)-3,4-tetrazolium]-bis(4-Methoxy6-nitro) benzene sulfonic acid hydrate	Sigma -Aldrich R & D System, USA	(2115281)
Human bronchial smooth muscle cells	Lonza, MD USA	(0000581076)

Table 3.2: List of equipment

Equipment	Model and Manufacturer
Hammer mill or mortar and pestle	Lab Chem, Germany Industrial Corp, Taiwan
Laboratory rotator, rotary vacuum evaporator	Lab Chem, Germany Industrial Corp, Taiwan
Analytical balance	Model PA413, Ohaus Corporation, USA
Vortex mixer	Model VM-300, Germany Industrial Corp, Taiwan
Freeze drier	Vertis TM mobile freeze-dryer, model 125L
Whatman No. 1 paper filter, nylon syringe filters	Lab Chem, Germany Industrial Corp, Taiwan
Oven	Model LDO-080F, Daihan, Labotech Co. Ltd, Korea
Micro pipette, Eppendorf® Research® plus pipette, variable volume, pipette, tips pipette	Sigma-Aldrich R & D System, USA
96-well microplate (TPP®)	Whitehead Scientific – Service and Support
Culture flask 25-cm^2, 75-cm^2	Whitehead Scientific – Service and Support
Syringe	Whitehead Scientific – Service and Support
NEST standard centrifuge tubes 15-ml, 50-ml	Whitehead Scientific – Service and Support
Advanced multi-purpose clinical centrifuge machine	Masiye Labs in South Africa
UV/VIS Spectrophotometer	Lab Chem, Germany Industrial Corp, Taiwan
Light microscope	Lab Chem, Germany Industrial Corp, Taiwan

3.2 Methods

3.2.1 Collection and Preparation of Plant Material

L. javanica was collected in the summer (November and December 2019) from Kirstenbosch Garden Centre in Rhodes Dr, Newlands, Cape Town, 7735, South Africa (Coordinates: 33°59′15″S 18°25′57″E.) while *M. flabellifolius* was collected on the 22nd of February 2020 in Rakgoadi Village (also called Ditholong) in Sekhukhune District, Limpopo Province, South Africa (Coordinates: 24.5028°S 30.6069°E.). Drying was performed immediately after plant collection at ambient temperature in ventilated ovens with the flow of warm air or air drying at room temperature for three weeks. *M. flabellifolius* was further dried in an oven at 30°C for four days.

3.2.2 Preparation and Extraction of Crude Plant Extracts

The next operation in preparing raw plant material for analysis was proper size reduction or homogenization as extraction is governed by the golden rule “deceased particle size, better surface area, improved extraction” so proper size reduction is a very critical pre-extraction step. The dried leaves and stems of *L. javanic*a and *M. flabellifolius* were disintegrated by crushing in a mortar and pestle or a blender (Figure 3.1). The extraction process entailed maceration of 100 g of coarsely powdered raw plant material, in 1000 ml of methanol inside a flask. The flask was closed and kept for at least three days in a dark place away from light in a temperature-controlled (20°C) laboratory room.

The content was stirred and shaken periodically to ensure complete extraction. At the end of extraction, the resulting methanol extract was filtered through Whatman No. 1 filter paper. The filtrate collected was concentrated under low pressure using a Rotovac–Flash evaporator (Figure 3.2). The solvent was removed using a rotary vacuum evaporator with a water bath temperature of 50°C. Finally, the residue (crude

extract) has collected and used for the experiments.

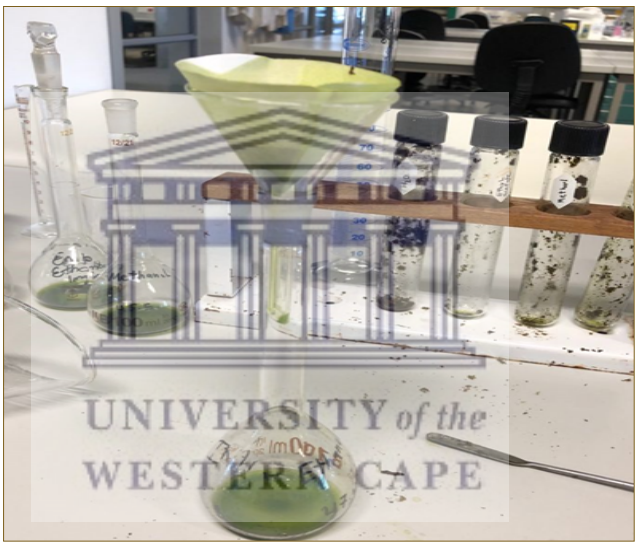


Figure 3.1: The filtration process of *L. javanica* and *M. flabellifolius*

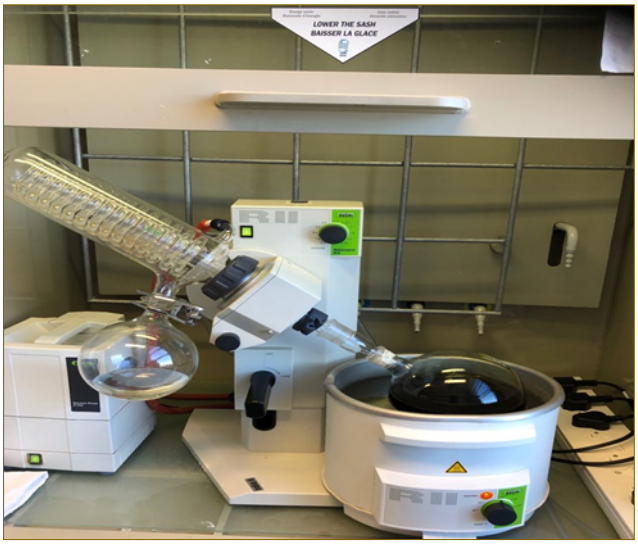


Figure 3.2: Photograph of the Rotovac-Flash evaporator used to evaporate the solvent to yield the crude extracts

3.2.3 Preparation of Primary Cell Cultures

Human bronchial smooth muscle cells (HBSMCs) Lonza, MD USA were isolated from human bronchial smooth muscle received from solid lung transplant benefactors after abrupt demise. The donor was tested and found non –reactive by an FDA approved method for the presence of HIV-l, hepatitis C virus. Where donor testing was not possible, cells has tested for the presence of viral nucleic acid from HIV, hepatitis B virus, and hepatitis C virus. Testing cannot offer complete assurance that HIV-1, hepatitis B virus, and hepatitis C virus are absent. HBSMCs were purchased from LONZA (Pharma & Biotech).

The human bronchial smooth muscle cells were cultured in a smooth muscle basal medium (SMCBM) Lonza, MD USA, supplemented with 5% foetal bovine serum (FBS), 1% penicillin (10 U/ml), 1% streptomycin (10,000 microgram/ml), amino acids

(1:100), l-glutamine (2 mM), and insulin (4 mg/ml), then transferred 6ml of BSMCs suspension into culture flask (25 -cm^2) and incubated at 37°C in 5% CO_2/95% air as described in appendix 1.

The culture medium was replaced after 4–5 days with 6 ml of SMCBM 5%FBS. After 10–14 days, the cells reached confluence. The medium was aspirated and the cells rinsed with (HEPES) Lonza, MD USA at room temperature by using a pipette and the flask was agitated carefully for 15 seconds. The HEPES was discarded. Then cells subcultured with 3.5ml (Trypsin/EDTA) Lonza, MD USA for detaching the adherent cells and were incubated at 37°C in 5% CO_2/95% air., for 2 min, adding the trypsin neutralizing solution to neutralize the trypsin activity in the flask.

The resultant cell suspension was carefully transferred to a centrifugation tube. After centrifugation of the tube for 5 minutes at 220 x g (Advanced multi-purpose clinical centrifuge machine, Masiye Labs in South Africa), pelleted cells were retained by removing most of the supernatant and cells resuspended in 12 ml supplemented SMCBM containing 5%FBS and mechanically dispersing the cells by repeated gentle pipetting and plated in a 75 -cm^2 culture flask. Cells were usually confluent in 10 -12 days, after which further subculture allowed half the cells to be used for experimental work and other half the cells to be maintained in a 75 -cm^2 flasks to continue each cell line. Placed the flask in an incubator at 37°C in 5% CO_2/95% air and medium was changed every 2-3 days. All experiments were performed with passages 2-4 cells. The presence of cells was confirmed by the light microscopy(Lab Chem, Germany Industrial Corp, Taiwan).

HBSMCs were seeded at a density of 7000 per well in 96-well microplate (TPP®) in quadruplicate. When cells reached 70% confluence, before the cell seeding process, the cells growth were arrested by suspended in insulin-free SMCBM for 24h, then the

medium was removed, and the cells washed with HEPES. Proteins were extracted in BSS (buffered saline solution).

3.2.4 Treatment and Stimulation of Human Bronchial Smooth Muscle Cells

HBSMCs were pre-treated with extracts of *L. javanica, M. flabellifolius*, dexamethasone and quercetin at several concentrations (3.125, 6.25, 12.5, 25, 50 and100 µg/ml) in quadruplicate, and the plates were incubated for 1hr. The cells were then treated with IL-1β (10 U/ml, Sigma-Aldrich R & D System, USA) for 4 days with a medium change every day.

3.2.5 Proliferation and Cell viability Assay

In a separate series of experiments, the test procedure involved was cultivation, trypsinized and seeded of cells in 96-well plates to assess the antiproliferative effects of the methanolic extracts of *L. javanica* and *M. flabellifolius* as described in appendix 2. Experimental wells contained 50µl of plant extracts, 50µl of cell suspensions containing 7000 cells and 50µl of XTT reagents ,each well contained a final volume of 150µl. Cells were incubated for 3 hours in standard incubator conditions (in most cases incubation for 2-5 hours is sufficient).After 3 hours, antiproliferative activity was assessed and determined by a colorimetric XTT assay system. Absorbance of each well was read at 490nm with a microplate reader (UV/VIS Spectrophotometer, Sigma-Aldrich, Germany Industrial Corp, Taiwan). All experiments were performed at a period of 7 weeks. The proliferation studies were performed in quadruplicate and results are expressed as a percentage of proliferation compared with controls. Proliferation curves were drawn according to the results. The method is based on the ability of metabolically active cells to reduce the tetrazolium salt, XTT, to an orange-coloured compound of formazan. The dye formed is water-soluble. The intensity of the dye is proportional to the number of metabolically active cells, during which an orange colour was formed. The greater the

number of metabolically active cells in the well, the greater the activity of mitochondrial enzymes and the higher the concentration of the dye formed, which was then be measured using optical intensity of the dye. The morphologic examination of human bronchial smooth muscle cells were examined using ECLIPSE Ti2 inverted microscope equipped with auto correction collar, and external phase contrast system. The base of choice for live-cell imaging, content applications, confocal and super-resolution, and images were captured using a PC-driven digital camera (Leica DC300F;Heerbrugg,Switzerland).

3.3 Statistical Analysis

Results are expressed as a percentage of proliferation compared with controls and are presented on graphs as mean ± standard error of the mean (SEM). Data were analyzed by One-Way ANOVA and Kruskal-Wallis test, as appropriate, and post-hoc comparisons (Dunn's multiple comparisons test and Dunnett's test) when comparing more than two variables and unpaired t-test (two-tailed), at a 0.05 level of significance. IC_{50} calculations were determined from no-linear regression analysis using GraphPad Prism 9 and expressed as mean ± standard deviation (SD), based on the concentration-dependent curves performed four times in triplicate) [141].

3.4 Ethical and Safety Considerations

There are simple precautions that can be taken to reduce the risk of misidentification and contamination of cell lines used for *in vitro* assays. These are common obstacles in human bronchial smooth muscle cell cultures (HBMCs). Despite frequent mistakes in cell line identification and contamination by mycoplasma, as well as genotypic and phenotypic instability, researchers often do not take these issues seriously. Scientists must regularly recant or modify scientific data when cell lines are misidentified. Often, occult contamination by microorganisms (especially mycoplasma) and phenotypic drift

are encountered due to serial transfers between laboratories. Additionally, human cell lines may carry pathogens, including viruses, which could create a health risk for laboratory workers. As well as becoming contaminated with bacteria, fungi, mycoplasma, and viruses, they may also spread to other cell lines. Some of these contaminants may be pathogenic. So, it is important that some precautions are taken to minimize the risk of misidentification and contamination. Therefore, all culture vessels should be labelled correctly (including the full name of the cell line, passage number, and date of transfer), as well as storage containers, and bottles of medium should be dedicated to use with a single cell line. In general, it is advisable to return to frozen stocks periodically (except where essential, no cell line should be grown continuously for more than 3 months or 10 passages, whichever is shorter), and to minimize the contamination, laminar flow cabinets and the work surfaces must be sterilized with 70% alcohol before any procedure. Therefore, good aseptic technique requires that any of the surfaces with sterile instruments, pipettes, and so on are not touched without hand sanitizing. Quick movements inside the cabinet might disrupt the airflow. Using more than one cell line in the cabinet at a time is prohibited. At the bench, there are several considerations in the context of ethics, such as keeping good documentation, practicing proper data collection and management, and being transparent with data sharing.

CHAPTER 4

RESULTS

4.1 Introduction

This chapter presents the results and analysis of the antiproliferative activity of methanolic extracts of *L. javanica* and *Myrothamnus flabellifolus* on human bronchial smooth muscle cells (HBSMCs).

4.2 Plant Extraction Yield

The methanolic extraction of 100 g of the ground powder of *L. javanica* yielded 12 g of the crude extract (i.e., 12% yield). Extraction of 100g of the ground powder of *M. flabellifolus* yielded 16 g of crude extract (i.e., 16% yield).

4.3 Induction of Cell Proliferation with IL-1β

The treatment of HBSMCs with IL-1β (10 U/ml) significantly increased cell proliferation (127.5 ± 16.42, p=0.0154). Therefore, it can be concluded that cell proliferation was successfully induced using 10 U/ml IL-1β. Figure 4.1 shows the relative number of HBSMCs treated with IL-1β and untreated cells.

Figure 4.2 shows the morphological changes of cells, which were observed by the light microscopy. The cells reached confluence gradually and retained their shape after treatment with IL-1β. The higher cell density and increased mitosis confirmed the increased cell proliferation observed using the XTT assay.

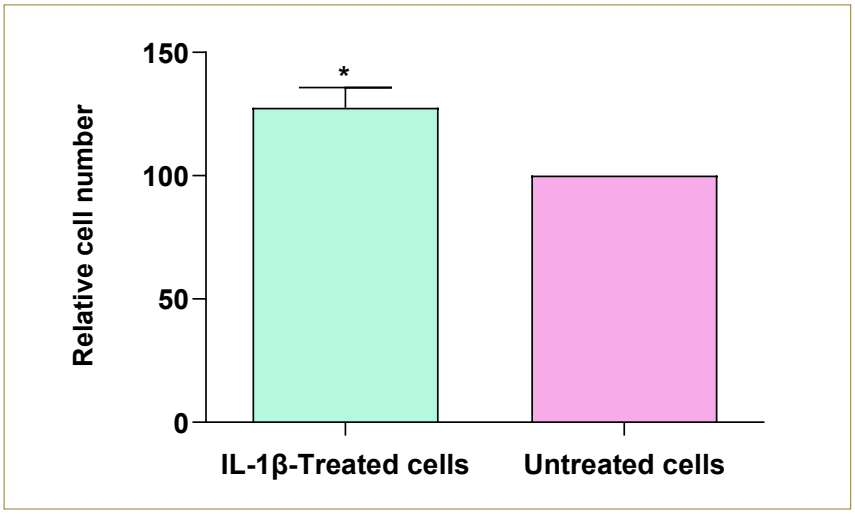


Figure 4.1: Relative number of human bronchial smooth muscle cells stimulated with IL-1β and unstimulated cells

Figure 4.2: Comparison of the morphological appearance of unstimulated cells [A] and cells stimulated with IL-1β [B]. Individual cells displayed a broad, spread shape with nuclei and small clusters of fusiform-shaped. Cells appeared confluent with individual regions of cells aligned in close, parallel orientation with spindle- or triangle-like shape. [B] Cells retained a similar spread shape as in [A] with individual cells making multiple cell-to-cell contacts. Numerous cells undergoing mitosis and increased proliferation as shown by higher cell density. All images are magnified at 20X.

4.4 Effects of Plant Extracts on Cells Stimulated with IL-1β

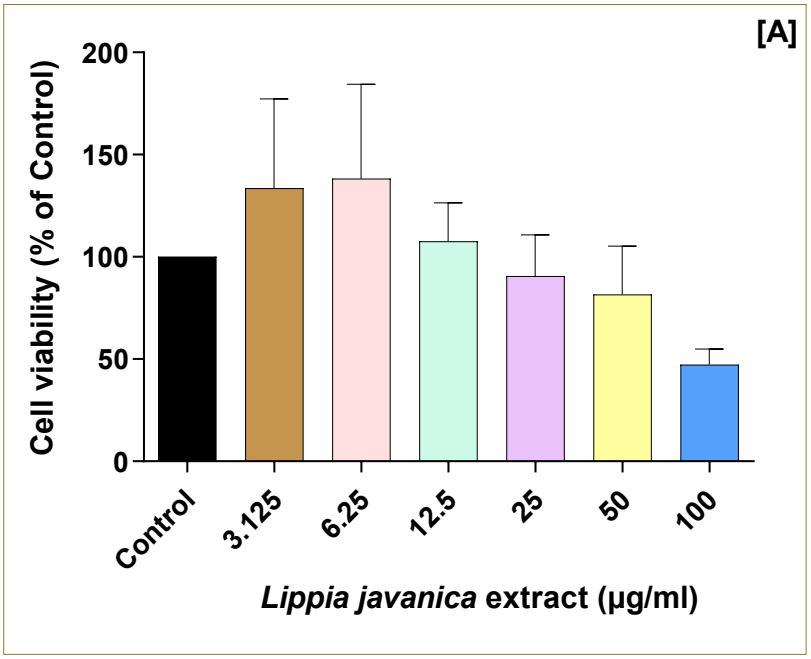
4.4.1 Effects of *L. javanica* Extracts on the Proliferation of Cells Stimulated with IL-1β

Table 4.1 and Figure 4.3 show the viability of cells treated with *L. javanica. L. javanica* did not significantly inhibit the proliferation of cells stimulated by IL-1β (p=0.3184). However, as shown in Figure 4.3, there was a general trend towards inhibition of cell proliferation. This inhibition on cell proliferation increased with higher concentrations of *L. javanica*. The IC_{50} for *L. javanica* was 24.27 µg/ml.

Figure 4.4 shows the morphological changes of cells after treatment with different concentrations of *L. javanica*. Cells treated with 3.12 and 6.25 µg/ml of *L. javanica* had higher proliferation, more elongated spindle-shaped, making multiple cell-to-cell contacts compared to untreated cells (control). However, cells treated with higher concentrations of *L. javanica* had decreased proliferation, increased cell size, with fusiform shape and aggregation into nodules with spindle-like networks compared to control.

Table 4.1: Effects of *L. javanica* extracts on the proliferation of cells stimulated with IL-1β

Concentration (µg/ml)	Experiment 1 (%)	Experiment 2 (%)	Experiment 3 (%)	Experiment 4 (%)	Mean±S.D.
3.75	71	102	98	263	**133.5±87.4**
6.25	66	130	86	271	**138.3±92.4**
12.5	63	155	111	101	**107.5±37.8**
25	57	136	113	56	**90.5±40.3**
50	32	125	119	50	**81.5±47.4**
100	25	50	60	54	**47.2±15.4**



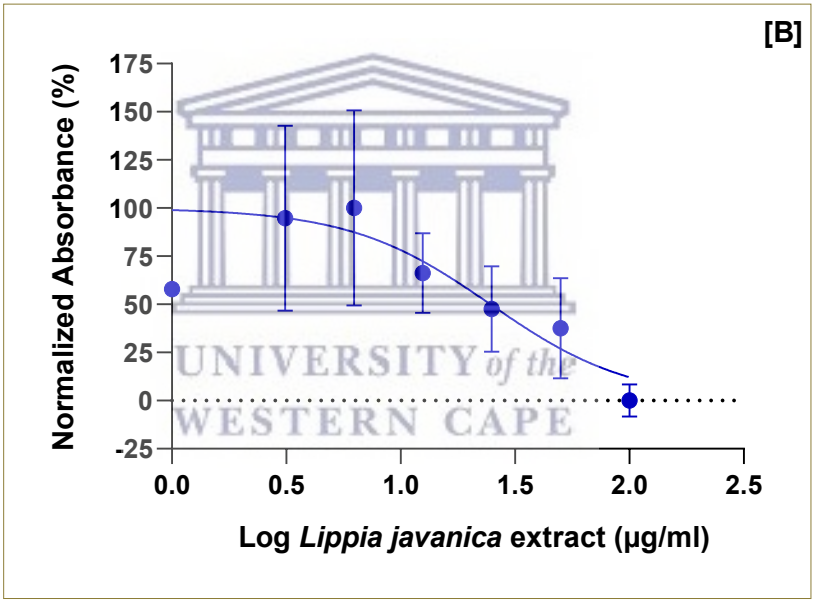


Figure 4.3: Antiproliferative effect of *L. javanica* extracts on cells stimulated with IL-1β, A=Cell viability; B=Dose-response curve. [IC_{50}=24.27 µg/ml; log IC_{50}=1.393; R=0.1843]. Values are Mean ±S.E.M. (n=4)

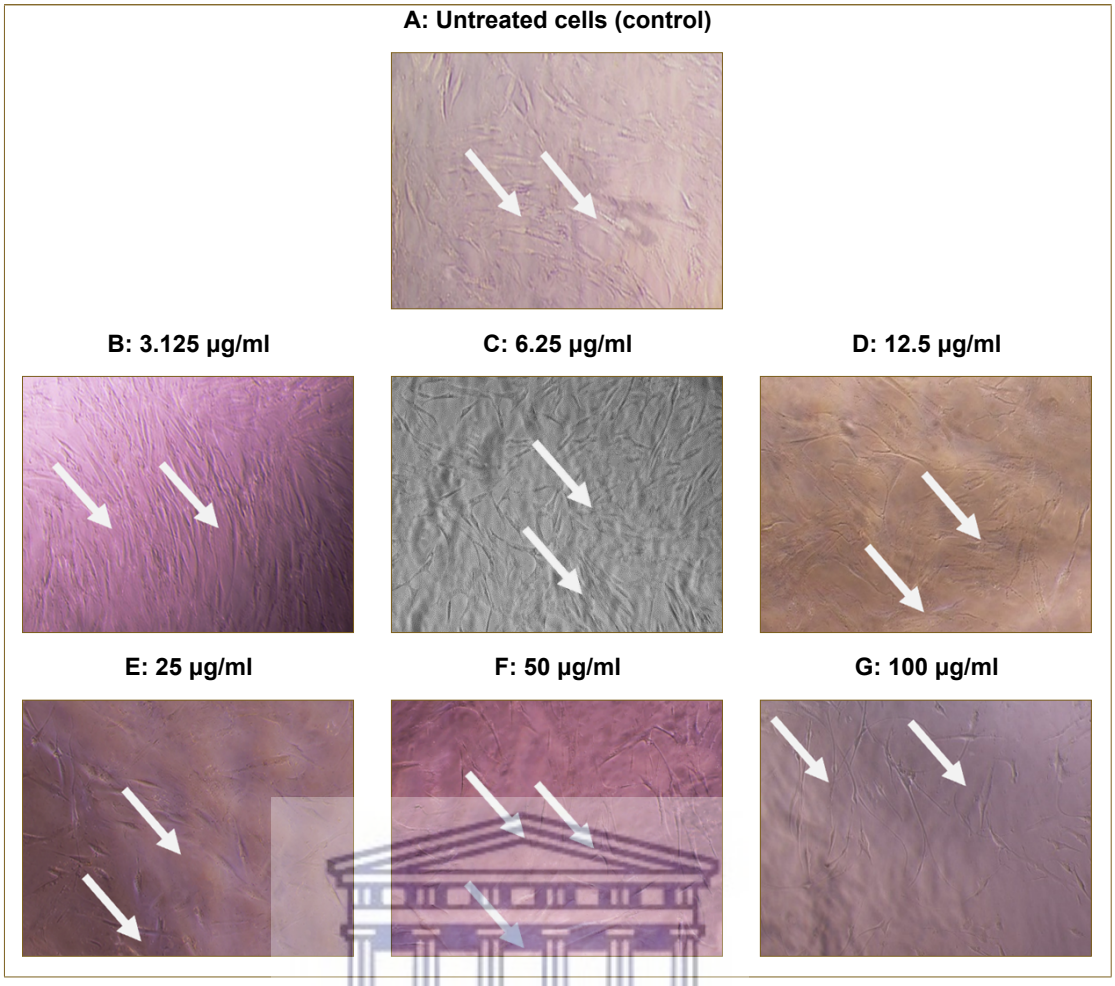


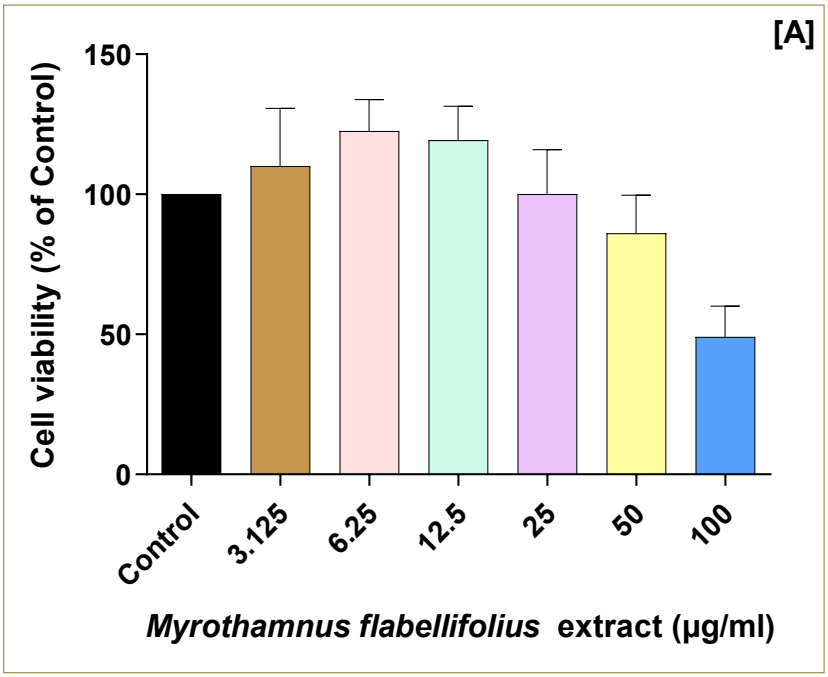
Figure 4.4: Effects of increasing *L. javanica* extract concentrations on the appearance of cells stimulated with IL-1β. [A] Untreated cells stimulated with IL-1β (control); [B], [C], [D] highly increased proliferation of cells, decreased cell size, cells with spindle- or triangle-like shape compared to control. Individual cells making multiple cell-to-cell contacts. [E], [F], [G] decreased proliferation of cells compared to control. The cells show a fusiform shape with an increase in size and hill-and-valley growth. The cells are characterized by aggregation into nodules with spindle-like networks or strands of elongated cells extending to neighbouring nodules. All images are magnified at 20X.

4.4.2 Effects of *M. flabellifolius* Extracts on the Proliferation of Cells Stimulated with IL-1β

Table 4.2 shows the cell viability of cells treated with *M. flabellifolius.* Figure 4.5 shows the effect of *M. flabellifolius* on cells stimulated with IL-1β. *M. flabellifolius* significantly inhibited the proliferation of cells stimulated by IL-1β (p=0.0159). This inhibition of cell proliferation increased with higher concentrations of *M. flabellifolius*. The IC_{50} for *M. flabellifolius* was 41.85 µg/ml. Figure 4.6 shows the morphological changes of cells after treatment with *M. flabellifolius*. Cells treated with 3.125, 6.25 and 12.5 µg/ml of *M. flabellifolius* had higher proliferation rates, elongated cells with spindle-like networks and fusiform cellular shape with regions of cells aligned in parallel compared to untreated cells (control). However, cells treated with higher concentrations of *M. flabellifolius* had decreased proliferation, decreased cell size, with fusiform shape and aggregation into nodules with spindle-like networks compared to control.

Table 4.2: Effects of *M. flabellifolius* extracts on cell proliferation of stimulated with IL-1β

Concentration (µg/ml)	Experiment 1 (%)	Experiment 2 (%)	Experiment 3 (%)	Experiment 4 (%)	Mean±S.D.
3.75	151	69	80	140	**110±41.5**
6.25	156	106	116	112	**122.5±22.7**
12.5	148	98	100	131	**119.3±24.4**
25	130	109	55	106	**100.0±31.8**
50	107	112	59	66	**86.0±27.3**
100	62	70	20	44	**49.0±22.2**



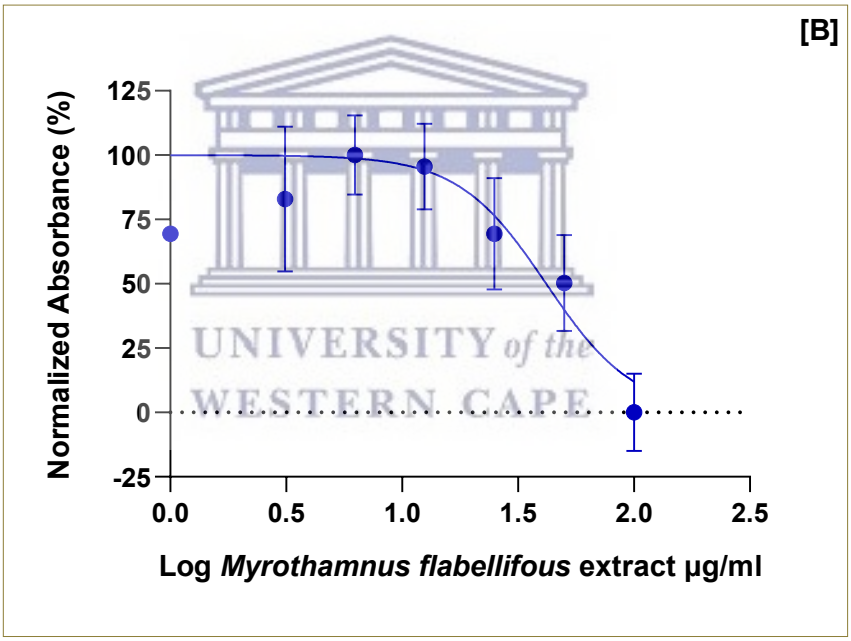


Figure 4.5: Antiproliferative effect of *M. flabellifolius* extracts on cells stimulated with IL-1β, A=Cell viability; B=Dose-response curve. [IC_{50}=41.85 µg/ml; log IC_{50}=1.622; R=0.3869]. Values are Mean±S.E.M. (n=4)

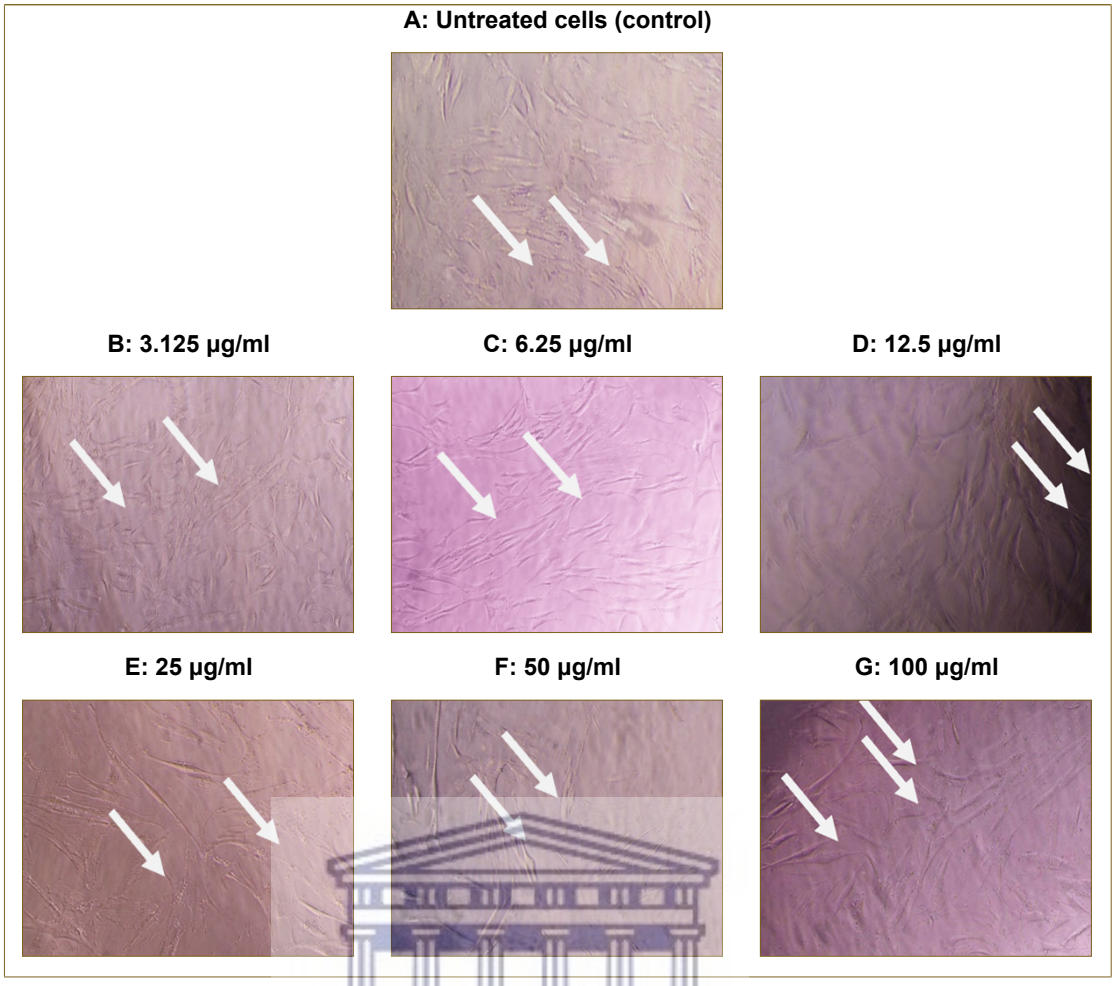


Figure 4.6: Effects of increasing *M. flabellifolius* extract concentrations on the appearance of cells stimulated with IL-1β. [A] Untreated cells stimulated with IL-1β (control); [B]; [C] increased proliferation cells compared to control the cells appeared confluent with individual regions of cells aligned in close, parallel orientation with decreased in size of cells with spindle- or triangle-like shape. [D] increased proliferation of cells compared to control; The cells had a hypertrophic appearance; [E]; [F]; [G] decreased proliferation of cells compared to control. The cells appeared spindle elongated with decreased size; most cells appeared fusiform with centrally located. All images are magnified at 20X.

4.4.3 Effects of Quercetin on the Proliferation of Cells Stimulated with IL-1β

Table 4.3 shows the cell viability of cells treated with of quercetin. Figure 4.7 shows the effect of quercetin on cells stimulated with IL-1β. Quercetin did not significantly inhibit the proliferation of cells stimulated by IL-1β (p=0.0751). However, as shown in Figure 4.7, there was a general trend towards inhibition of cell proliferation. This inhibition on cell proliferation increased with higher concentrations of quercetin. The IC_{50} for quercetin was 7.086 µg/ml. Figure 4.8 shows the morphological changes of cells after treatment with different concentrations of quercetin. There was decreased proliferation and cells were smaller in size and designated as nodules with spindle-like networks or cables of elongated cells extending to neighbouring nodules, compared to untreated cells (control).

Table 4.3: Effects of quercetin on cell proliferation stimulated with IL-1β

Concentration (µg/ml)	Experiment 1 (%)	Experiment 2 (%)	Experiment 3 (%)	Experiment 4 (%)	Mean±S.D.
3.75	57	44	78	148	**81.7±46.3**
6.25	28	55	85	99	**66.7±31.7**
12.5	38	58	72	88	**64.0±21.2**
25	40	77	68	52	**59.2±16.5**
50	22	65	30	50	**41.7±19.5**
100	76	50	46	43	**53.7±15.1**

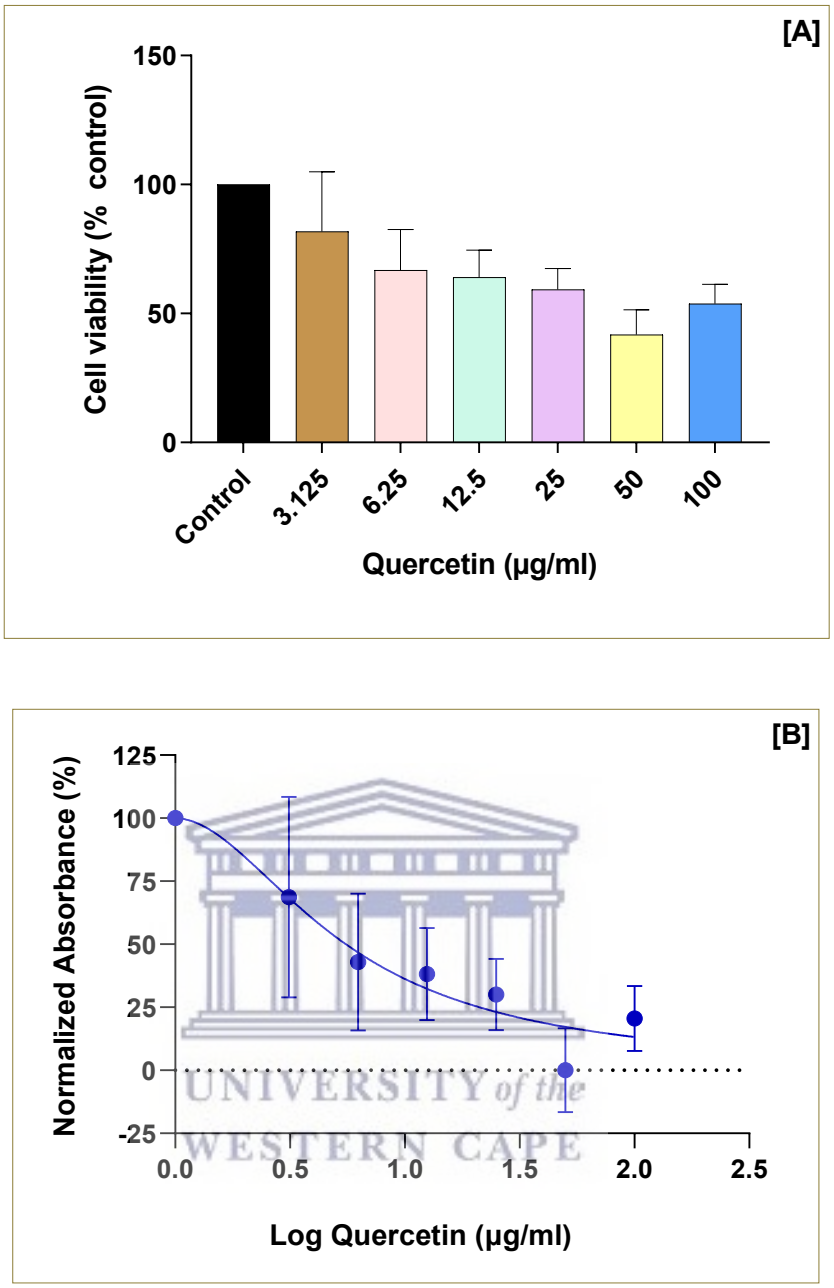


Figure 4.7: Antiproliferative effect of quercetin on cells stimulated with IL-1β, A=Cell viability; B=Dose-response curve. [IC_{50}=7.086 µg/ml; log IC_{50}=0.8504; R=0.3532]. Values are Mean±S.E.M. (n=4)

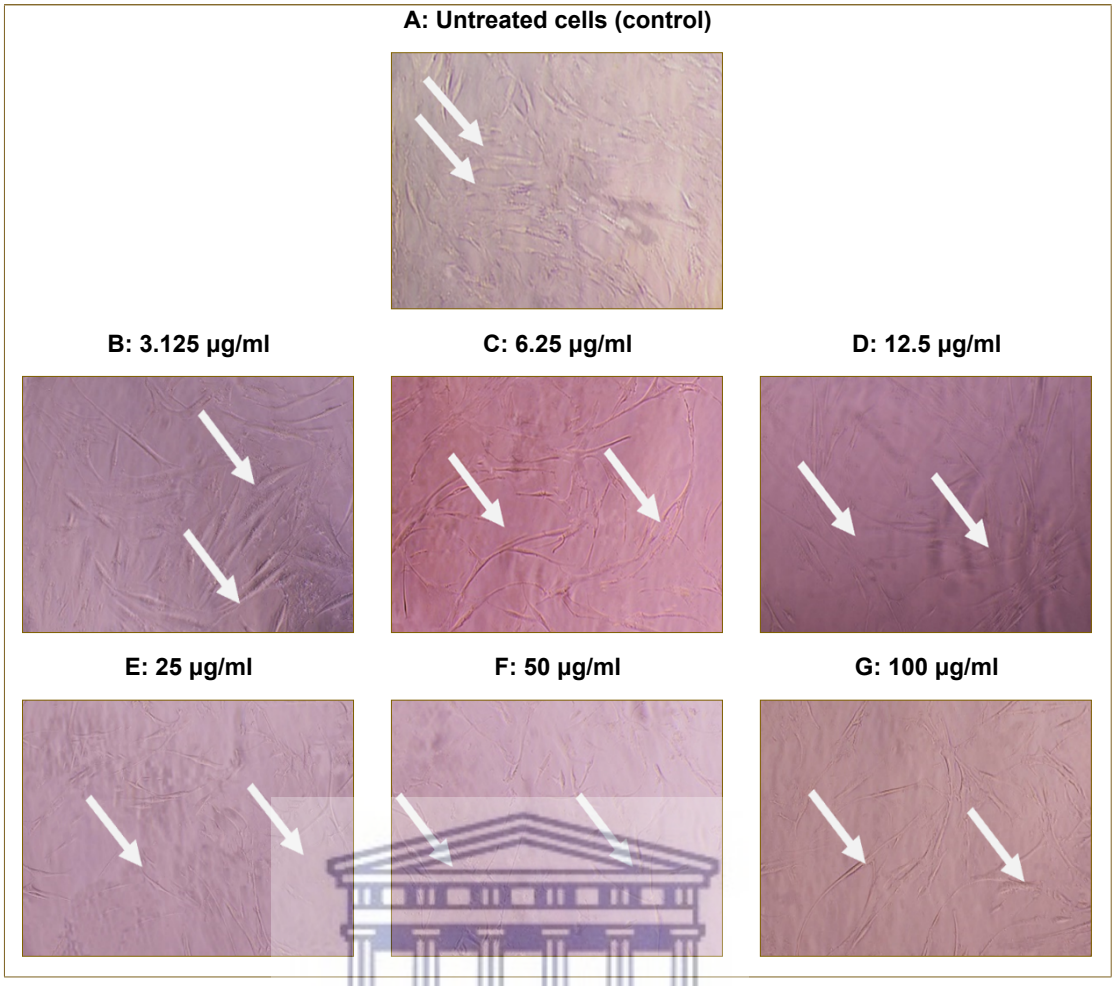


Figure 4.8: Effects of increasing quercetin concentrations on the appearance of cells stimulated with IL-1β. [A] untreated cells stimulated with IL-1β (control); [B]; [C]; [D]; [E]; [F]; [G] decreased cell proliferation compared to control. The cells had decreased size and were characterized by aggregation into nodules with spindle-like networks or cables of elongated cells extending to neighboring nodules. All images are magnified at 20X.

Effects of Dexamethasone on Cell Proliferation Stimulated with IL-1β

Table 4.4 and Figure 4.9 show the cell viability of cells treated with dexamethasone. Dexamethasone did not significantly inhibit the proliferation of cells stimulated by IL-1β ($p=0.1921$). However, as shown in Figure 4.9, there was a general trend towards inhibition of proliferation cells. This inhibition on cell proliferation increased with higher concentrations of dexamethasone. The IC_{50} for dexamethasone was 21.14 µg/ml. Figure 4.10 shows the morphological changes of cells after treatment with different

concentrations of dexamethasone. Cells treated with 3.12 and 6.25 µg/ml of dexamethasone had higher proliferation, more elongated spindle-shaped cells, making multiple cell-to-cell contacts compared to untreated cells (control). However, cells treated with higher concentrations of dexamethasone had decreased proliferation, decreased cell size, with fusiform shape and aggregation into nodules with spindle-like networks compared to control.

Table 4.4: Effects of dexamethasone on cell proliferation stimulated with IL-1β

Concentration (µg/ml)	Experiment 1 (%)	Experiment 2 (%)	Experiment 3 (%)	Experiment 4 (%)	Mean±S.D.
3.75	150	111	99	101	**115.3±23.7**
6.25	99	173	57	109	**109.5±47.9**
12.5	89	152	45	91	**94.2±43.9**
25	80	99	50	60	**72.2±21.8**
50	75	121	37	43	**69.0±38.5**
100	50	99	50	60	**64.7±23.3**

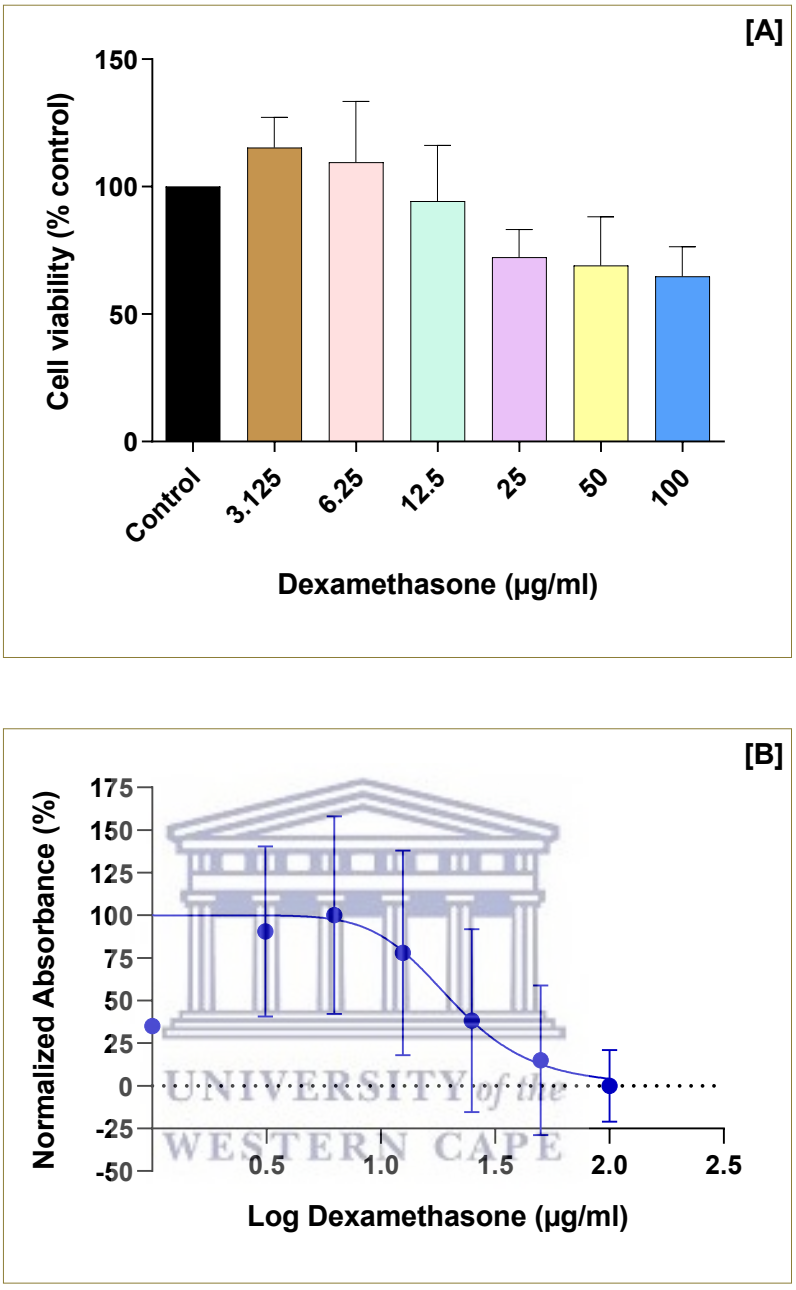


Figure 4.9: Antiproliferative effect of dexamethasone on cells stimulated with IL-1β, A=Cell viability; B=Dose-response curve. [IC_{50}=21.14 µg/ml; log IC_{50}=1.325; R=0.088]. Values are Mean±S.E.M. (n=4)

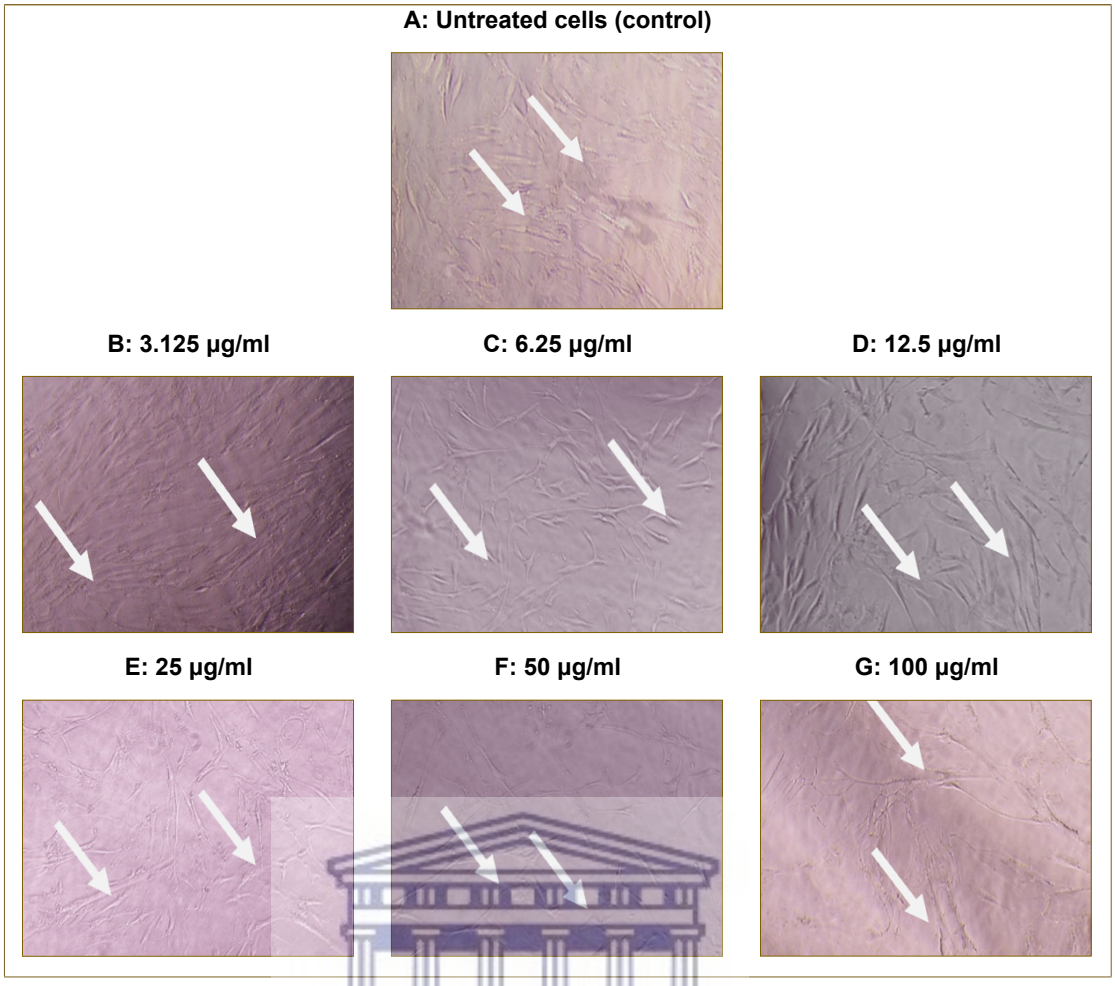


Figure 4.10: Effects of increasing dexamethasone concentrations on the appearance of cells stimulated with IL-1β. [A] cells treated with IL-1β (control); [B]; [C] increased proliferation cells compared to control the cells appeared confluent with individual regions of cells aligned in close, parallel orientation with decreased in size of cells with spindle- or triangle-like shape and with small clusters of fusiform-shaped [D]; [E]; [F]; [G] decreased proliferation cells compared to control. The cells appeared spindle elongated with decreased size and most cells appeared more fusiform cellular shape with regions of cells aligned in parallel that was characterized by elongated cells with spindle-like networks. All images are magnified at 20X.

4.4.5 Comparison of the Antiproliferative Effects of *L. javanica* & *M. flabellifolius* Extracts, Quercetin, and Dexamethasone on Cells Stimulated with IL-1β

Figure 4.11 shows a comparison of the antiproliferative effects of *L. javanica*, *M. flabellifolius,* dexamethasone, and quercetin on cells stimulated with IL-1β. At concentrations up to 50 µg/ml, quercetin exhibited the highest antiproliferative activity, followed by dexamethasone, *L. javanica*, and *M. flabellifolius* extracts. All four test products had similar antiproliferative activity at a concentration 100 µg/ml.

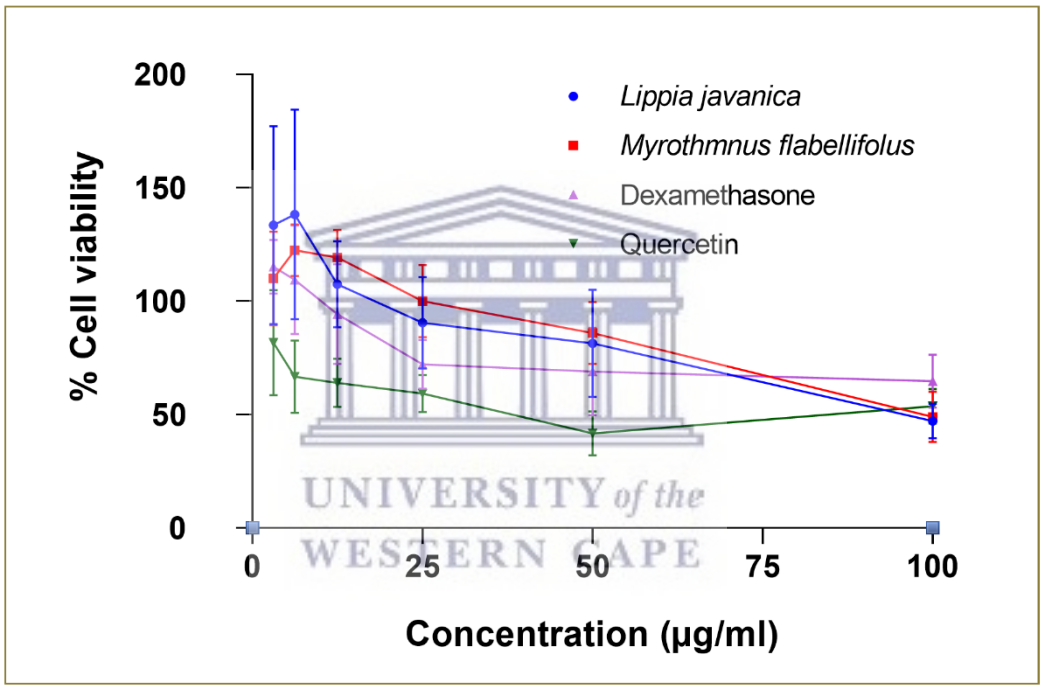


Figure 4.11: The antiproliferative effects of *L. javanica* and *M. flabellifolius* extracts, quercetin, and dexamethasone at various concentrations on cells stimulated with IL-1β. Values are Mean±S.E.M. (n=4)

4.5 Effects of Plant Extracts on Cells Not Stimulated with IL-1β

4.5.1 Effects of *L. javanica* Extracts on the Proliferation of Cells Not Stimulated with IL-1β

Table 4.5 and Figure 4.12 show the cell viability of cells treated with *L. javanica*. There were significant differences in proliferation between cells treated with *L. javanica* with the control ($p<0.0001$). However, at lower concentrations up to 12.5 µg/ml, there was higher cell proliferation in cells treated with *L. javanica* than in untreated cells (control). Post-hoc tests revealed that cells treated with 12.5 µg/ml of *L. javanica* had significantly higher proliferation compared to untreated cells ($p=0.0042$). Although there was no clear dose-response relationship, inhibition of cell proliferation at higher concentrations by *L. javanica* cannot be ruled out. The IC_{50} for *L. javanica* was 13.08 µg/ml. Figure 4.13 shows the morphological changes of cells after treatment with *L. javanica*. Cells treated with 3.125, 6.25 and 12.5 µg/ml of *L. javanica* had higher proliferation rates, more elongated spindle-shaped cells, making multiple cell-to-cell contacts compared to untreated cells (control). However, cells treated with higher concentrations of *L. javanica* had decreased proliferation, with fusiform shape and hill-and-valley growth compared to control.

Table 4.5: Effects of *L. javanica* extracts on proliferation of cells not stimulated with IL-1β

Concentration (µg/ml)	Experiment 1 (%)	Experiment 2 (%)	Experiment 3 (%)	Experiment 4 (%)	Mean±S.D.
3.75	162	128	94	55	**109.8±45.9**
6.25	160	173	118	80	**132.8±42.3**
12.5	163	200	156	228	**186.8±33.6**
25	69	115	87	55	**81.5±25.9**
50	18	40	38	69	**41.2±21.0**
100	39	107	72	79	**74.2±27.9**

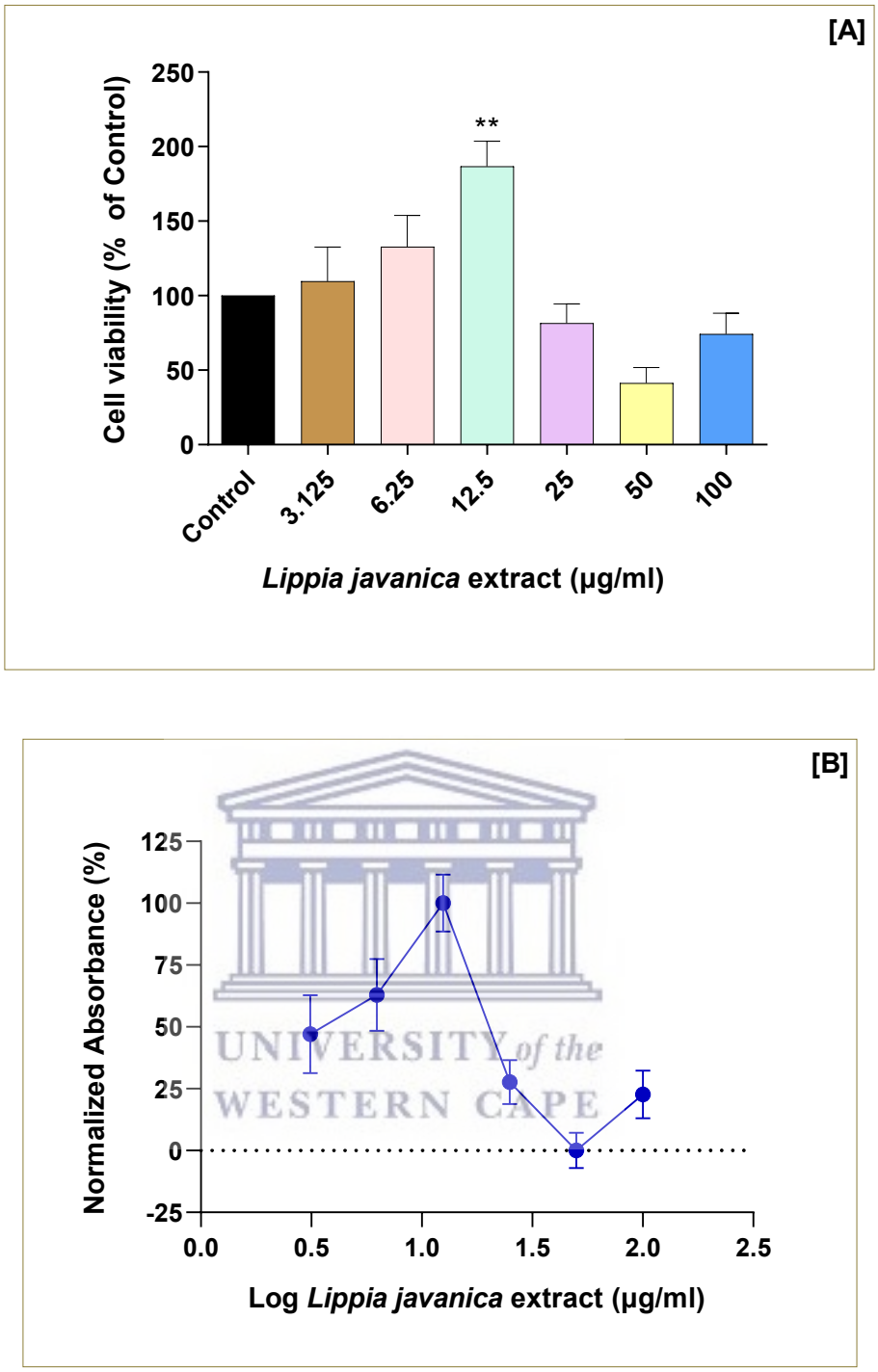


Figure 4.12: Antiproliferative effect of *L. javanica* extracts on cells not stimulated with IL-1β, A=Cell viability; B=Dose-response curve. [IC_{50}=13.08 µg/ml; log IC_{50}=1.117; R=0.2485]. Values are Mean±S.E.M. (n=4)

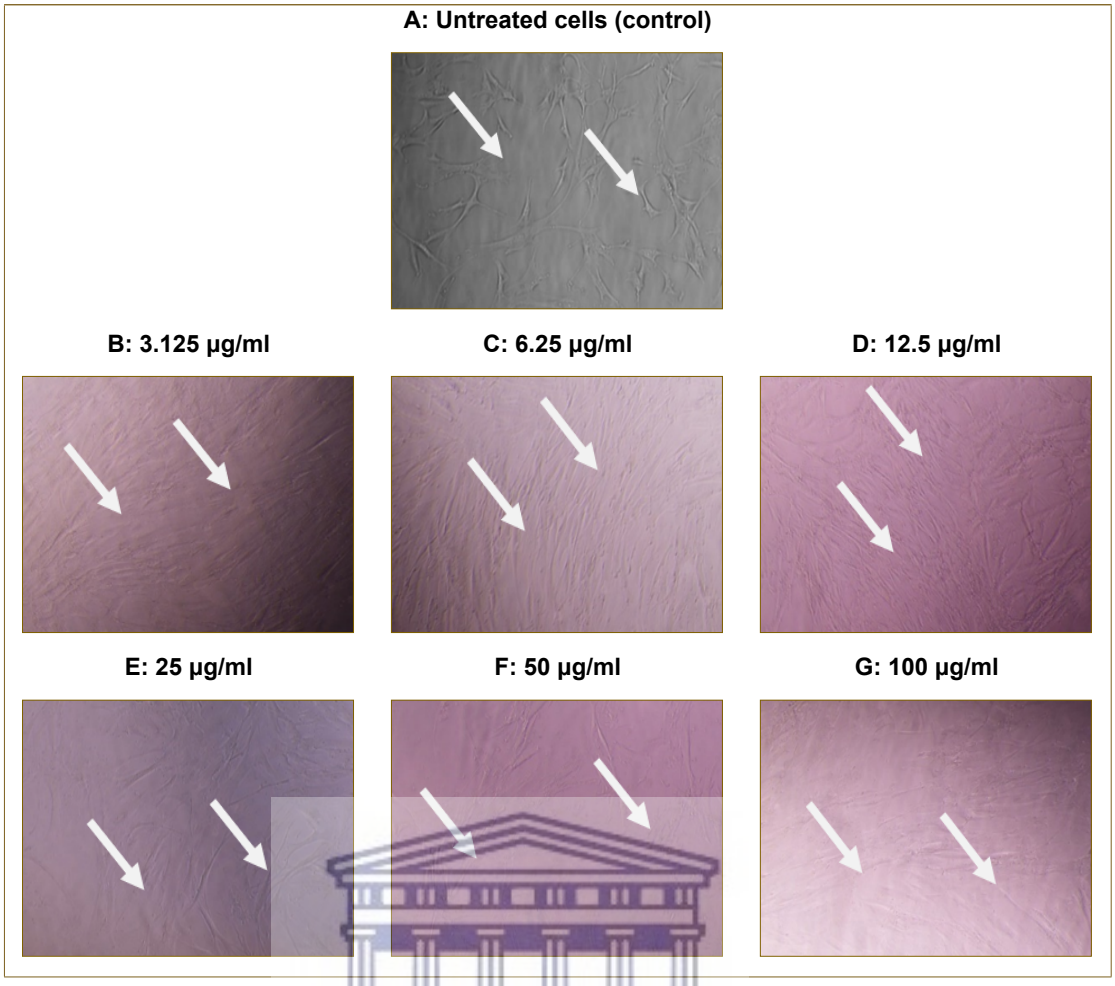


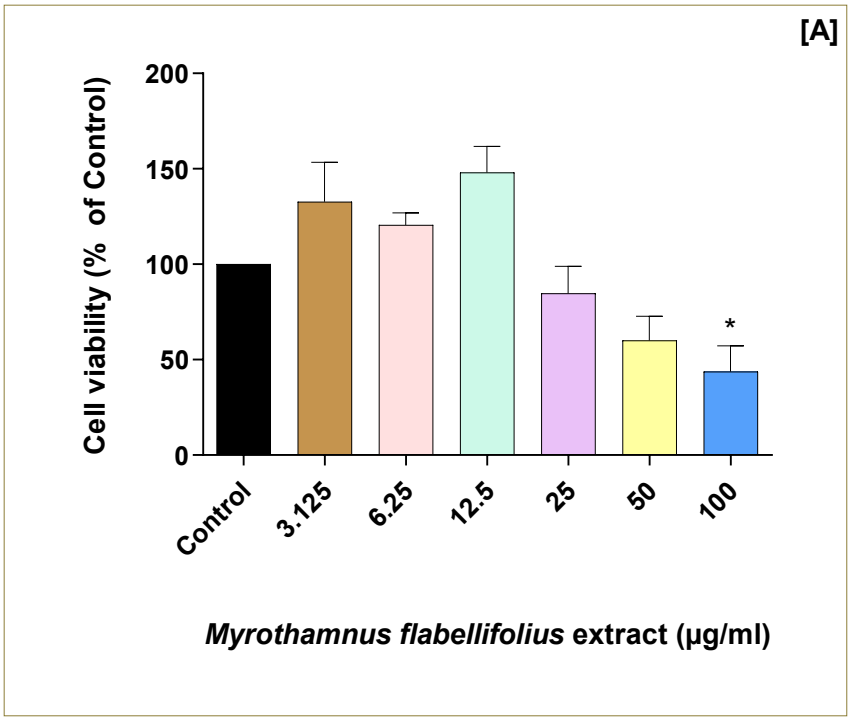
Figure 4.13: Effects of increasing *L. javanica* extract concentrations on the appearance of cells not stimulated with IL-1β. [A] cells not stimulated with interleukin (control); [B]; [C]; [D] highly increased proliferation cells, decreased cell size, cells with spindle- or triangle-like shape compared to control. Individual cells making multiple cell-to-cell contacts. [E]; [F]; [G] decreased proliferation of cells compared to control. The cells show a fusiform shape with slightly increase in size and hill-and-valley growth. The cells appeared spindle elongated, and most cells appeared fusiform with centrally located. All images are magnified 20X.

4.5.2 Effects of *M. flabellifolius* Extracts on the Proliferation of Cells Not Stimulated with IL-1β

Table 4.6 and Figure 4.14 show the cell viability of cells treated with *M. flabellifolius*. There were significant differences in proliferation between cells treated with *M. flabellifolius* with the control ($p<0.0001$). However, at lower concentrations up to 12.5 µg/ml, there was higher cell proliferation in cells treated with *M. flabellifolius* than in untreated cells (control). Post-hoc tests revealed that cells treated with 100µg/ml of *M. flabellifolius* had significantly lower proliferation compared to untreated cells ($p=0.0299$). The IC_{50} for *M. flabellifolius* was 23.06 µg/ml. Figure 4.15 shows the morphological changes of cells after treatment with *M. flabellifolius*. Cells treated with 3.125, 6.25 and 12.5 µg/ml of *M. flabellifolius* exhibited higher proliferation rates, more elongated spindle-shaped, making multiple cell-to-cell contacts compared to untreated cells (control). However, cells treated with higher concentrations of *M. flabellifolius* had decreased proliferation. The cells appeared spindle elongated as nodules with centrally located with fusiform shape and hill-and-valley growth compared to control. Although there was no clear dose-response relationship, inhibition of cell proliferation at higher concentrations by *M. flabellifolius* cannot be ruled out.

Table 4.6: Effects of *M. flabellifolius* extracts on the proliferation of cells not stimulated with IL-1β

Concentration (µg/ml)	Experiment 1 (%)	Experiment 2 (%)	Experiment 3 (%)	Experiment 4 (%)	Mean±S.D.
3.75	99	192	128	112	**132.8±41.2**
6.25	129	130	102	121	**120.5±12.9**
12.5	189	132	140	131	**148.0±27.6**
25	56	124	81	78	**84.7±28.4**
50	32	84	79	45	**60.0±25.5**
100	29	82	21	43	**43.7±27.0**



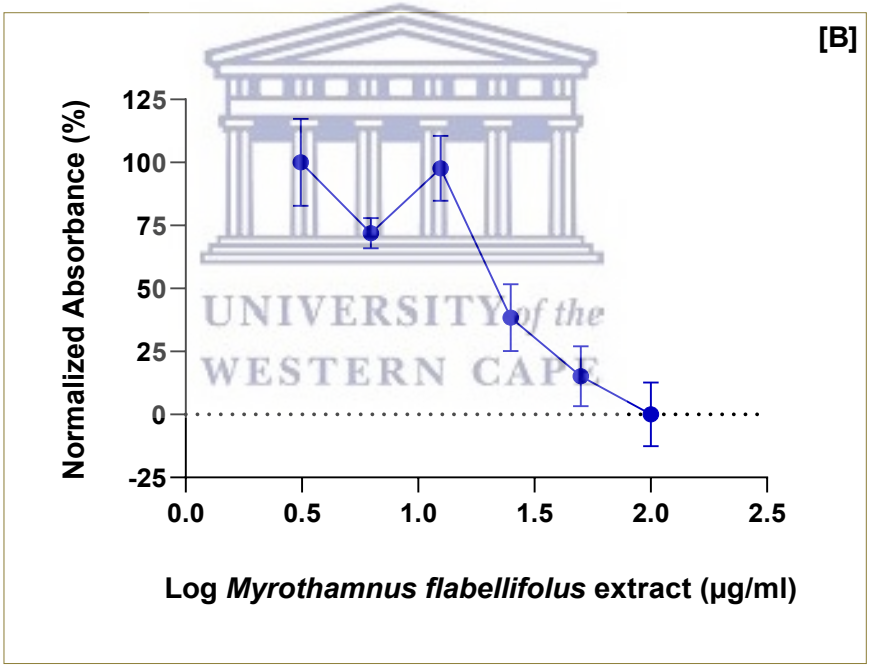


Figure 4.14: Antiproliferative effect of *M. flabellifolius* extracts on cells not stimulated with IL-1β, A=Cell viability; B=Dose-response curve. [IC_{50}=23.06 µg/ml; log IC_{50}=1.3.63; R=0.6792]. Values are Mean±S.E.M. (n=4)

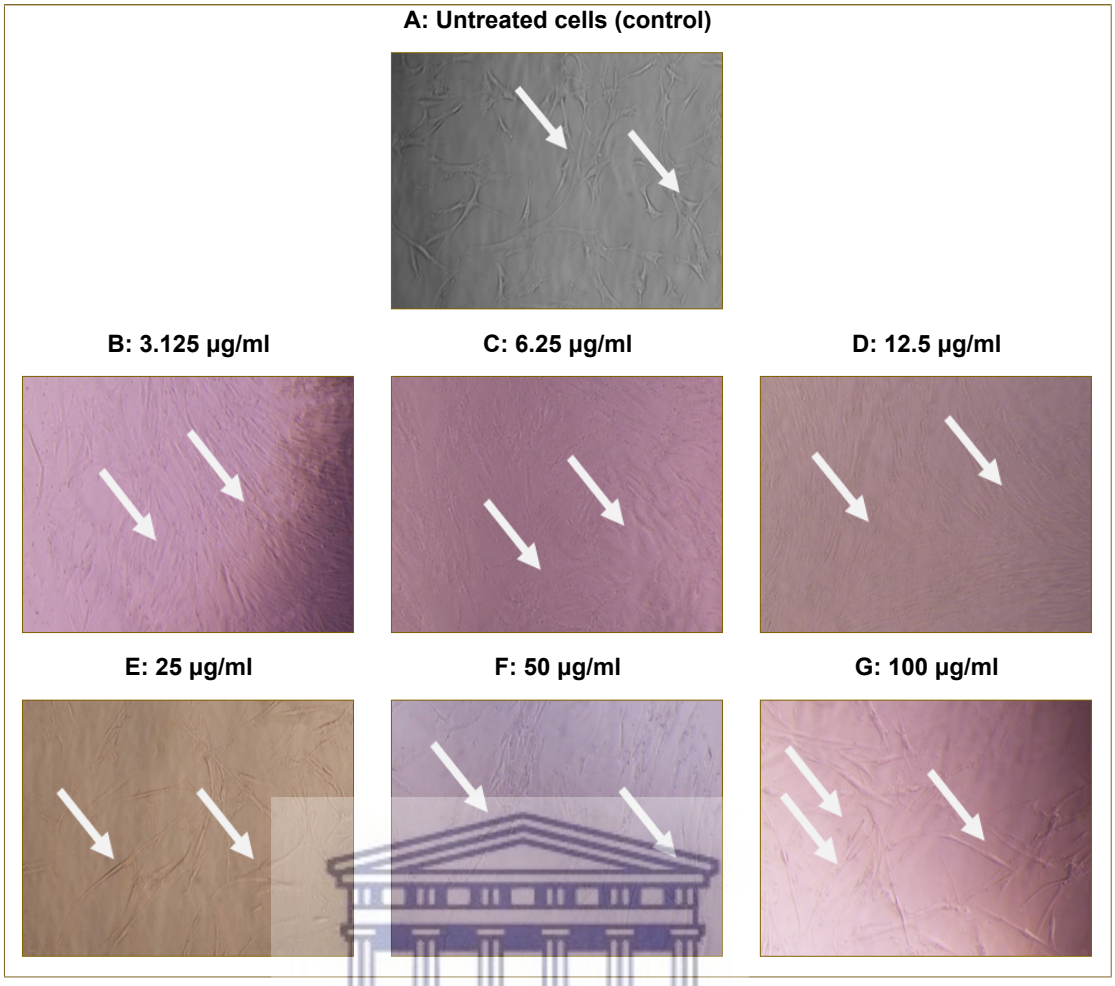


Figure 4.15: Effects of increasing *M. flabellifolius* extract concentrations on the appearance of cells not stimulated with IL-1β. [A] cells untreated with interleukin (control); [B]; [C]; [D] highly increased proliferation cells, decreased cell size, cells with spindle- or triangle-like shape compared to control. Individual cells making multiple cell-to-cell contacts. [E]; [F]; [G] decreased proliferation of cells compared to control. The cells show a fusiform shape with a slight increase in size and hill-and-valley growth. The cells appeared spindle elongated as nodules and most cells appeared fusiform with centrally located. All images are magnified at 20X.

4.5.3 Effects of Quercetin on the Proliferation of Cells Not Stimulated with IL-1β

Table 4.7 and Figure 4.16 show the viability of cells treated with quercetin. Similarly, there were significant differences in proliferation between cells treated with quercetin with untreated cells (p=0.0310). At lower concentrations up to 50 µg/ml, there was lower cell proliferation compared to untreated cells (control). Post-hoc tests revealed that cells treated with 3.125 µg/ml and 12.5 µg/ml of quercetin had significantly lower proliferation compared to untreated cells (p=0.0190 and p=0.0250, respectively). However, cells treated with higher concentrations of quercetin had higher proliferation than cells treated with lower concentrations. There was a clear dose-response relationship in the effect of quercetin on cell proliferation. Quercetin appears to have promoted cell proliferation. The IC_{50} for quercetin was 52.24 µg/ml. Figure 4.17 shows the morphological changes of cells after treatment with different concentrations of quercetin. Cells treated with quercetin had decreased proliferation and designated as nodules with spindle-like networks or cables of elongated cells extending to neighbouring nodules, compared to untreated cells (control).

Table 4.7: Effects of quercetin on the proliferation of cells not stimulated with IL-1β

Concentration (µg/ml)	Experiment 1 (%)	Experiment 2 (%)	Experiment 3 (%)	Experiment 4 (%)	Mean±S.D.
3.75	50	58	61	22	**47.7±17.8**
6.25	24	61	46	93	**56.0±28.9**
12.5	26	50	43	80	**49.7±22.5**
25	25	83	52	78	**59.5±26.7**
50	53	99	18	59	**57.2±33.1**
100	100	70	88	78	**84.0±12.9**

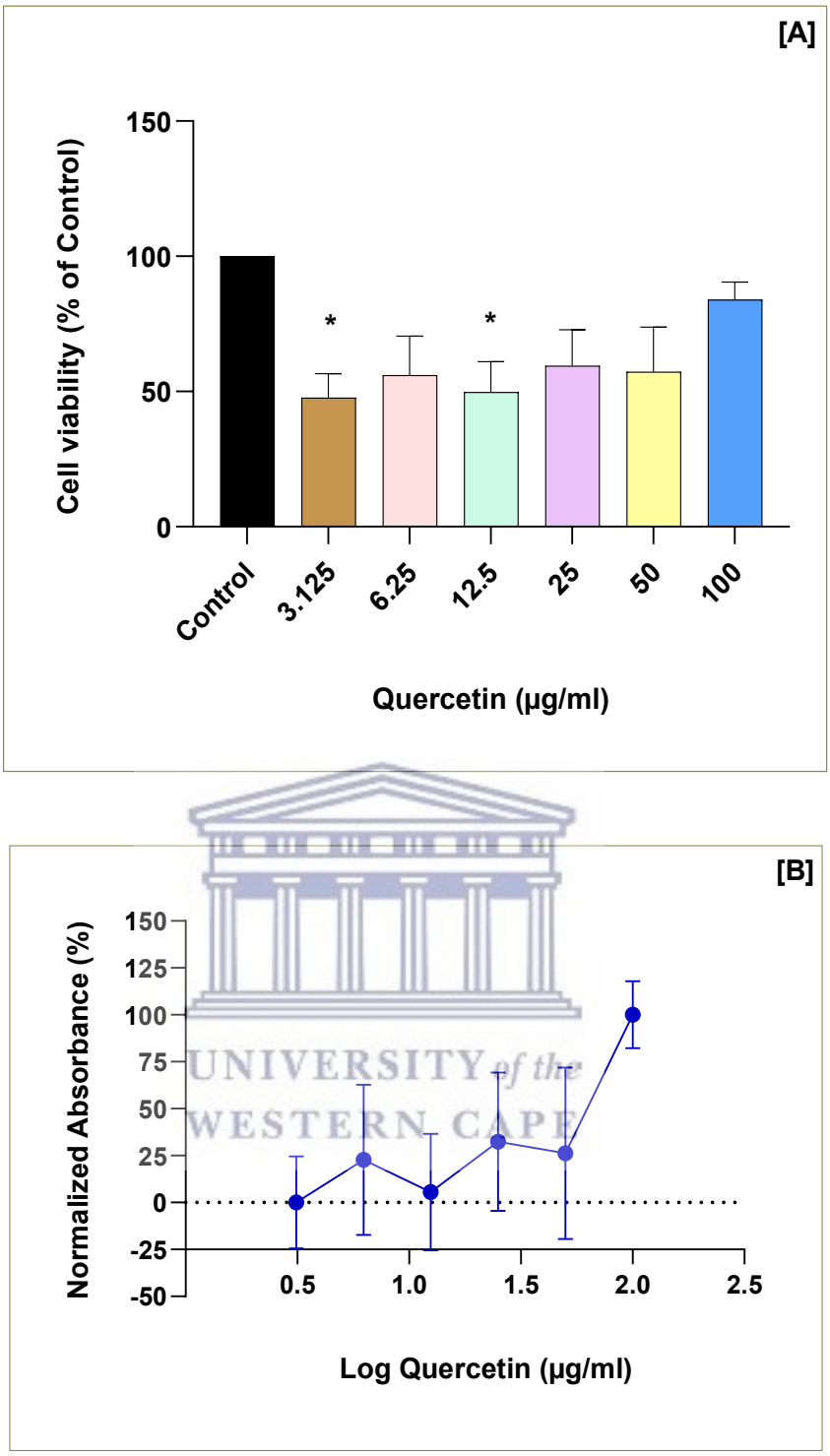


Figure 4.16: Antiproliferative effect of quercetin on cells not stimulated with IL-1β, A=Cell viability; B=Dose-response curve. [IC_{50}=52.24 µg/ml; log IC_{50}=1.718; R=0.1797]. Values are Mean±S.E.M. (n=4)

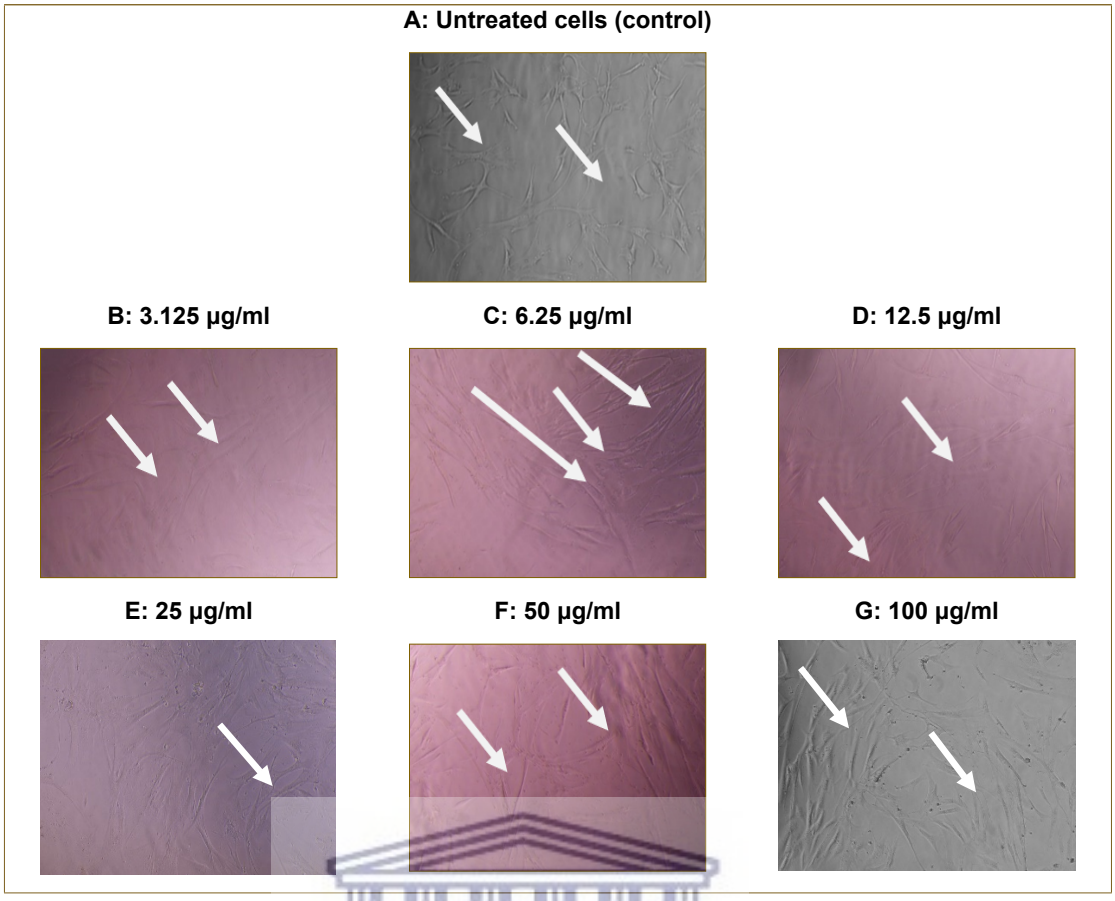


Figure 4.17: Effects of increasing quercetin concentrations on the appearance of cells not stimulated with IL-1β. [A] cells untreated with interleukin (control); [B]; [C]; [D]; [E]; [F]; decreased proliferation cells compared to control. The cells had decreased in size and were characterized by aggregation into nodules with spindle-like networks or cables of elongated cells extending to neighbouring nodules. [G] increased proliferation of cells compared to control; The cells show a fusiform shape with a slight increase in size and hill-and-valley growth and most cells appeared fusiform with centrally located. All images are magnified at 20X.

4.5.4 Effects of Dexamethasone on the Proliferation of Cells Not Stimulated with IL-1β

Table 4.8 and Figure 4.16 show the cell viability of cells treated with dexamethasone. Dexamethasone did not significantly inhibit proliferation (p=0.3022). However, there was a clear trend towards inhibition of cell proliferation with increasing concentrations

of dexamethasone. At lower concentrations up to 12.5 µg/ml, there was higher cell proliferation in cells treated with dexamethasone than in untreated cells (control). Although there was no significant dose-response relationship, it appears at higher concentrations dexamethasone might significantly inhibit cell proliferation. The IC_{50} for dexamethasone was 11.45 µg/ml. Figure 4.19 shows the morphological changes of cells after treatment with different concentrations of dexamethasone. Cells treated with 3.125, 6.25 and 12.5 µg/ml of dexamethasone had higher proliferation, more elongated spindle-shaped, making multiple cell-to-cell contacts compared to untreated cells (control). However, cells treated with higher concentrations of dexamethasone had decreased proliferation. The cells appeared spindle elongated with centrally located with fusiform shape with spindle-like networks compared to control

Table 4.8: Effects of dexamethasone on the proliferation of cells not stimulated with IL-1β

Concentration (µg/ml)	Experiment 1 (%)	Experiment 2 (%)	Experiment 3 (%)	Experiment 4 (%)	Mean±S.D.
3.75	138	165	97	100	**125.0±32.5**
6.25	135	206	150	130	**155.3±34.9**
12.5	125	207	46	77	**113.8±70.1**
25	127	136	42	60	**91.2±47.2**
50	89	146	40	59	**83.5±46.3**
100	136	124	75	75	**102.5±32.1**

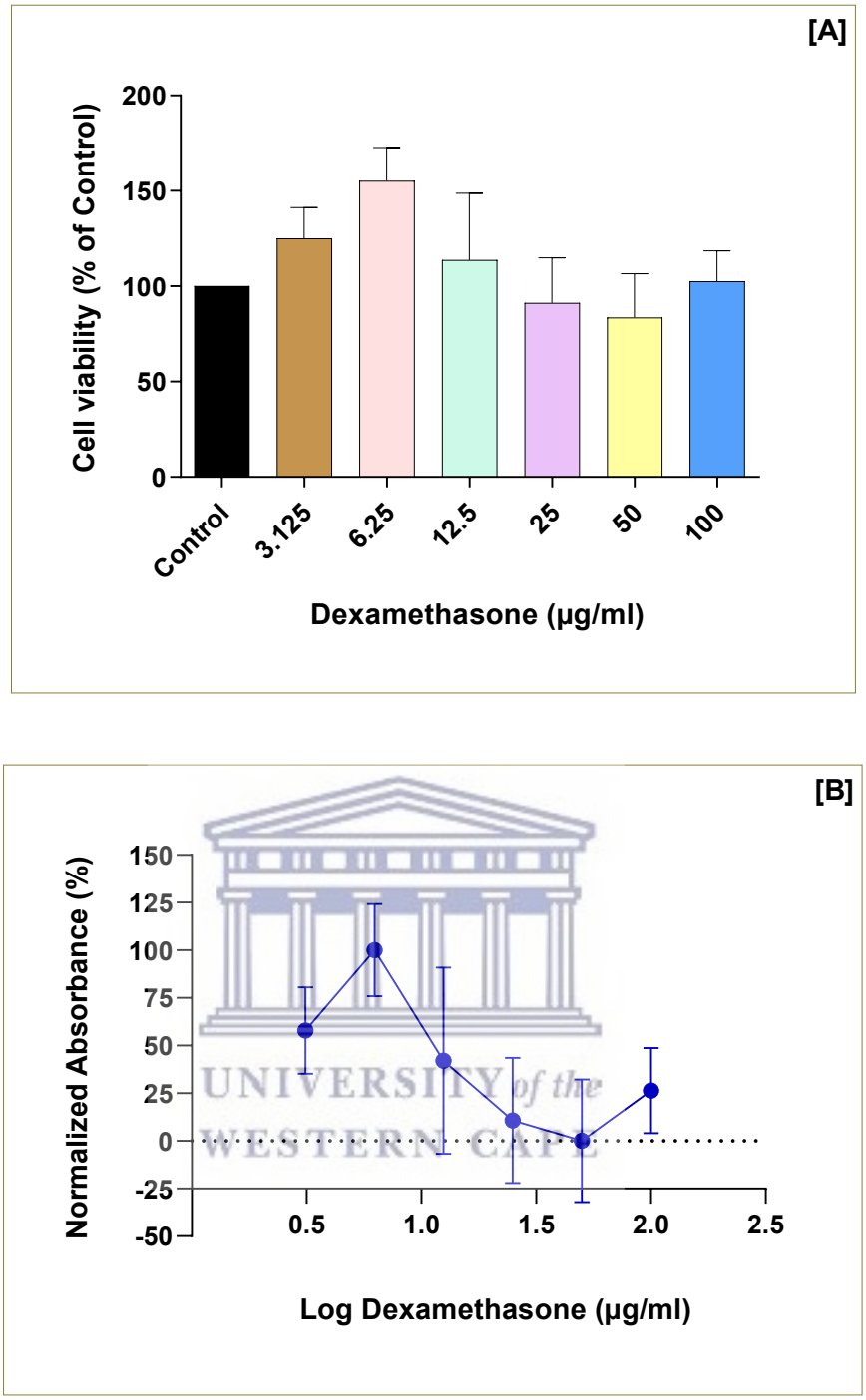


Figure 4.18: Antiproliferative effect of dexamethasone on cells not stimulated with IL-1β, A=Cell viability; B=Dose-response curve. [IC_{50}=11.45 µg/ml; log IC_{50}=1.059; R=0.1636]. Values are Mean±S.E.M. (n=4)

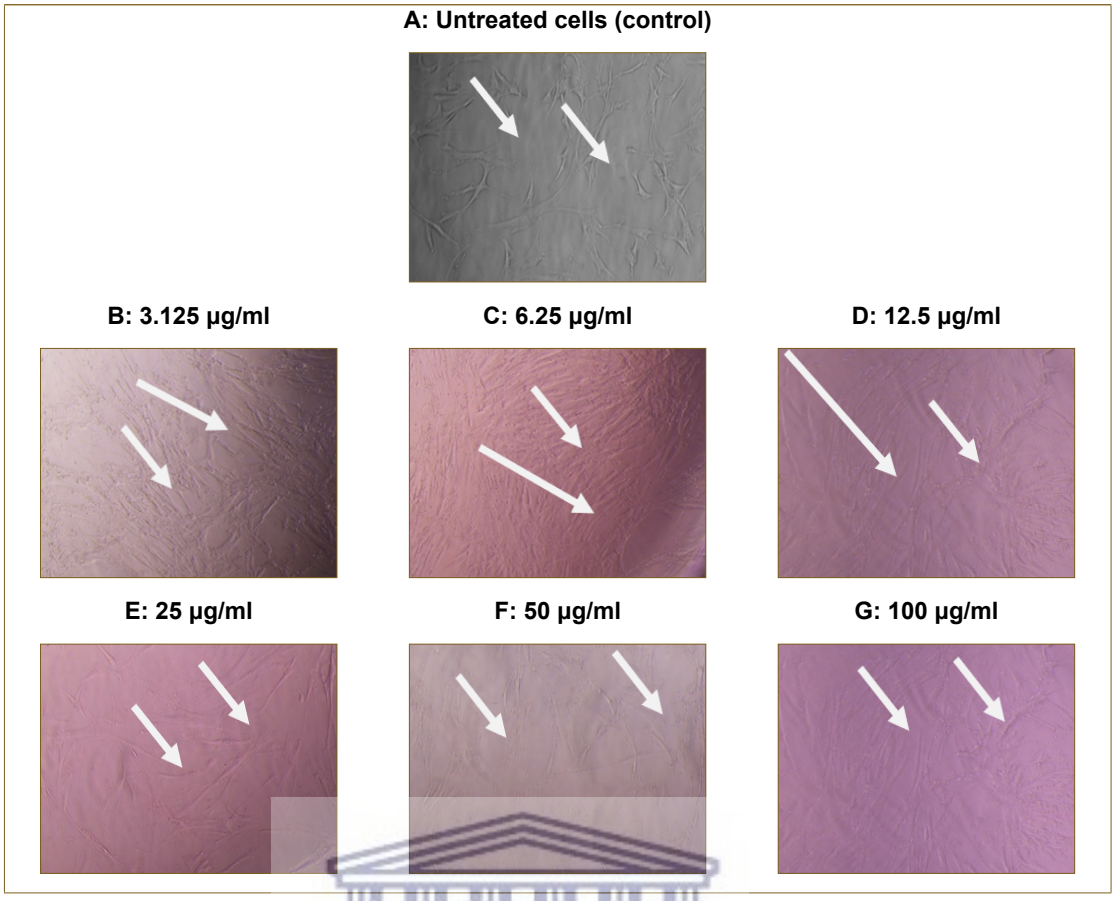


Figure 4.19: Effects of increasing dexamethasone concentrations on the appearance of cells not stimulated with IL-1β. [A] cells untreated with interleukin (control); [B]; [C]; [D] highly increased proliferation cells, decreased cell size, cells with spindle- or triangle-like shape compared to control. Individual cells making multiple cell-to-cell contacts and with small clusters of fusiform shaped [E]; [F]; [G] decreased proliferation of cells compared to control. Cells shows decreased size with spindle- or triangle-like shape and it is more fusiform cellular shape with regions of cells aligned in parallel that was characterized by elongated cells with spindle-like networks. All images are magnified at 20X.

4.5.5 Comparison of the Antiproliferative Effects of *L. javanica, & M. flabellifolius* Extracts, Quercetin, and Dexamethasone on Cells Not Stimulated with IL-1β

Figure 4.20 shows that *L. javanica* and *M. flabellifolus* had the highest antiproliferative activity on cells growing normally. Dexamethasone had lower antiproliferative activity on normally growing cells compared to *M. flabellifolus* and *L. javanica.* There was no clear trend on the effect of quercetin on the proliferation of normally growing cells.

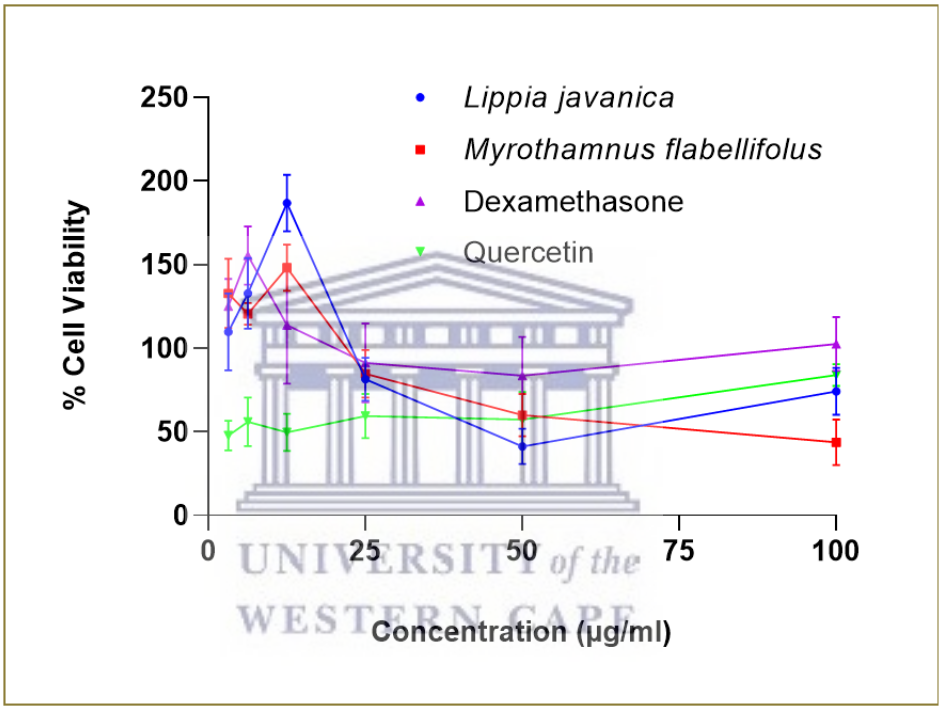


Figure 4.20: The antiproliferative effects of *L. javanica* and *M. flabellifolius* extracts, quercetin, and dexamethasone at various concentrations on cells not stimulated with IL-1β. Values are Mean±S.E.M. (n=4)

CHAPTER 5

DISCUSSION AND CONCLUSION

5.1 Discussion

Asthma often presents as a heterogeneous disease clinically which may impact on its prognosis. Accordingly, the International Classification of Diseases diagnostic system generally categorizes asthma patients based on disease severity, persistence of symptoms and manifestation of complications [28]. Severe therapy resistant asthma [150] and side effects of existing antiasthmatic drugs [116,117,151] are significant impediments to the successful treatment and management of patients. However, several efforts are underway to delineate the innumerable pathophysiological mechanisms and symptoms that characterize the disease and its exacerbations so that more multidimensional, rationalized and personalized treatment options may be developed [152-156].

Despite the use of natural products and traditional medicines in the management of asthma [6,16,116,117,119], there is limited evidence on the antiasthmatic effects of *L. javanica* [118,120,157] and *M. flabellifolius* [17,24,130,158-160]. However, recent research has shown that *L. javanica* (Zumbani) herbal tea infusion decreases allergic airway inflammation via inhibition of Th2 cell activation and suppression of oxidative stress [127]. *L. javanica* has also been reported as one of the most widely used and preferred plant species for the treatment of rhinitis and/or related symptoms by traditional healers in the Limpopo Province of South Africa [157].

Also, *L. javanica* has phytochemical and pharmacological significance, e.g., as remedies for colds, cough, fever, malaria, wounds, diarrhoea, chest pains, bronchitis, and asthma [126]. In this study, methanolic extracts of *L. javanica and M. flabellifolius* inhibited IL-1β-induced proliferation of the human bronchial smooth muscle cell line (HBSMCs) in a dose-dependent manner.

Treatment with *M. flabellifolius* showed decreased cell size while *L. javanica* increased cell size. These results suggest that both plants have potential antiasthmatic properties. Airway smooth muscle (ASM) cells represent a significant proportion of all cells present in the airways and the pathogenesis of asthma [96] and might therefore be expected to be a prominent cellular target for inhaled corticosteroids (ICS)[161,162].

Despite this, little is known of the action of glucocorticoids on ASM. It is becoming increasingly clear that in addition to its contractile properties, ASM can potentially contribute to the pathogenesis of asthma by increased proliferation and by expression and secretion of proinflammatory cytokines and mediators, which in turn may lead to the activation and recruitment of key inflammatory cells in the airways [96].

In this study, dexamethasone was used as a standard antiasthmatic (control) and reduced proliferation and size of HBSMCs. Inhibition of proliferation of cells is a well-known mode of action of glucocorticoids [163]. Glucocorticoids have been demonstrated to inhibit thrombin-stimulated proliferation in ASM cells in culture. [164]. Dexamethasone decreases AHR and inflammatory response to allergens by downregulating eosinophil and mast cell activation [164,165]. Their main action in the airways of subjects with asthma is believed to be the inhibition of the recruitment of inflammatory cells and the inhibition of the release of proinflammatory mediators and cytokines from activated inflammatory and airway epithelial cells [164]. Other works in the literature showed that glucocorticoids such as dexamethasone inhibit the growth

of certain tumor cell lines both *in vitro* and *in vivo* [166,167]. Therefore, extracts derived from *L. javanica* and *M. flabellifolius* may possess similar therapeutic effects as dexamethasone. Although many studies have addressed the effects of glucocorticoids on many aspects of airway inflammation, the impact of glucocorticoids on the remodelling of ASM cell proliferation has not been fully explored [164].

In a 10-yr follow-up study, inhaled steroid treatment did not cause any significant reduction in AHR [168]. Together, one implication of this report is that the remodelling of the airways is poorly reversible and is the result of persistent changes in airway structure. However, glucocorticoids may have several other actions and cellular targets in the airways that contribute to their therapeutic efficacy in asthma management [164].

It has been observed from this study that cells treated with higher concentrations of *L. javanica* extracts showed a decrease in proliferation and increased cell sizes. Therefore, the *L. javanica* extracts are able to decrease airway remodelling by a decrease in proliferation (hyperplasia). In asthmatic patients, there is increased proinflammatory cytokines like IL-1β in ASM cells and in the airway lining fluid. IL-1β can decrease the relaxation response to β-adrenergic agonists in ASM and can contribute to ASM cell proliferation and airway remodelling processes [169].

Bronchial smooth muscle (BSM) remodelling encompasses increased deposition of ECM proteins in and around the BSM bundles, an increased BSM cell size or hypertrophy, and an increased BSM cell number or hyperplasia [170]. Furthermore, these lead to bronchoconstriction and hyperresponsive airways [170-172]. Therefore *L. javanica* may decrease airway remodelling by a decreasing cell proliferation (hyperplasia) induced by inflammatory agents such as IL-1β.

However, *L. javanica* extracts might cause lung remodelling that worsens asthma, since the cells treated with lower concentrations of the extract showed a reduction in cell size and cells treated with higher concentrations revealed an increase in cell size (hypertrophy).

The increase in cell size occurs because of elevated levels of myosin light chain kinase (MLCK) [170]. Furthermore, studies on animal models have demonstrated that *L. javanica* suppresses inflammatory cell infiltration and cytokines, as well as decreasing inflammation-induced oxidative stress in ovalbumin-sensitized rats by reducing Th2-mediated immune response and oxidative stress related to the immune system, likely due to its phenolic compounds [127].

Flavonoids and phenols isolated from *L. javanica* showed antioxidant, anti-inflammatory, bronchodilation, and antiasthmatic effects by inhibiting the synthesis of Th2 type cytokines [127] and releasing chemical mediators, including histamine, acetylcholine, leukotrienes [173], and prostaglandins leading to inflammation [127,128,138]. Traditional use of *L. javanica* as an infusion to treat bronchial infections and respiratory disorders has previously been validated [174].

Generally, flavonoids prevent allergy symptoms [23], and inhibit IL-4, IL-13, transcription factors, and have a direct effect on allergic cells. In addition to their ability to reduce airway hypersensitivity, flavonoids can also inhibit free radical formation, aid in anti-infective activity and even inhibit mast cell degranulation that leads to the release of histamine [23]. Many of the compounds that have been isolated from *L. javanica* such as the flavonoids and phenols possess bronchodilation and antiasthmatic effects due to the presence of xanthine [126,129], which is a demethylated derivative of caffeine that has pharmacological effects such as relaxing smooth muscles (especially BSM) resulting in relaxation of smooth muscle, [126,127].

In this study when the cells were treated with higher concentrations of *M. flabellifolius* extract, decreases in proliferation and cell sizes were observed. Therefore, the *M. flabellifolius* extracts may decrease airway remodelling by decreasing cell size (hypertrophy) and proliferation (hyperplasia) of ASM cells. In concurrence numerous studies have shown that *M. flabellifolius* has anti-inflammatory [175], anti-diabetic and potential antiasthmatic properties [160,176,177].

All these beneficial activities are likely to be due to the presence of numerous polyphenols, including phenolic acids, tannins and flavonoids [23]. Several studies have been conducted to assess anti-inflammatory activity *M. flabellifolius*, both *in vitro* and *in vivo*. These involve inhibition of enzymes such as cyclooxygenase (COX), lipoxygyenase (LOX), which are responsible for the production of the inflammatory mediators such as cytokine (interleukins) [130,178].

This study also provides some new information on quercetin that was the most potent dose-dependent antiproliferative compound among other substituted flavonoids. Quercetin inhibited bronchial obstruction and AHR and bronchial hypertrophy in the guinea pig [143]. According to research conducted in cardiovascular diseases, quercetin suppressed proliferation and migration of Human Aortic Smooth Muscle Cell (AoSMC), concomitant with inhibition of mitogen-activated protein kinase phosphorylation.

Quercetin has also been demonstrated to have *in vitro* relaxant effects on guinea pig trachea pre-contracted with histamine, carbachol or KCl [179-181] and to inhibit the release of histamine *in vitro* from rat peritoneal mast cells [144]. These findings provide new insights and a rationale for the potential use of quercetin in the treatment of asthma [182].

The anti-inflammatory mechanism of quercetin can be attributed to the LOX and PDE4 inhibition and reduction of histamine and leukotriene release, which promote a decrease in the proinflammatory cytokine formation and production of IL-4, respectively. In addition, quercetin also promoted the inhibition of human mast cell activation by Ca^{2+} influx and prostaglandin release inhibition, favouring the therapeutic relief of the asthma symptoms and decreasing the short-acting β-agonist dependence [116].

Therefore, *L. javanica* and *M. flabellifolius* may possess similar therapeutic effects as quercetin due to the presence of numerous polyphenols, including phenolic acids, tannins and flavonoids [17,23,125]. Several studies reported that the flavonoids as a group of compounds can be used in asthma treatment [116].

The present study demonstrates that the antiproliferative activity of the *L. javanica* and *M. flabellifolius* extracts in comparison to dexamethasone and quercetin on cells not stimulated with IL-1β (normally growing cells). It has been observed from this study that cells treated with higher concentrations of *L. javanica* extracts and *M. flabellifolius* extracts showed high decrease in proliferation while dexamethasone had lower antiproliferative activity on normally growing cells compared to *M. flabellifolus* and *L. javanica.*

There was no clear trend on the effect of quercetin on the proliferation of normally growing cells. According to the study, lower concentrations of these plants did not inhibit normal cell proliferation without harming normal cells; thus, herbal remedies have a similar effect as dexamethasone, which does not appear to affect cell proliferation at low concentrations.

5.2 Recommendations and Limitations

5.2.1 Recommendations

From the results presented in the previous chapter, a number of recommendations for future work on *L. javanica and M. flabellifolius* are listed below:

- Further studies should be done to determine isolated phytochemical compounds and the biological activities of the extracts. The flavonoids of *L. javanica* and *M. flabellifolius* will in future also be tested for their antiproliferative activity, because there are no literature reports about the antiproliferative properties of *L. javanica* and *M. flabellifolius.*
- Further studies on animal models of normal or abnormal human function.
- The potential toxicity of the *Lippia* and *Myrothamnus* species still needs to be further investigated. Reproductive toxicity test should be done to evaluate effects of these plants on the growth of human cells, this test uses laboratory animals to check for adverse effects. The reproductive toxicity test is of great importance in pre-clinical testing.

5.2.2 Limitations

Primary HBSMCs have a lower doubling time than most cell lines and have limited growth potential. Even with optimal growth conditions, primary cells eventually senesce and die, thus rendering it challenging to obtain more passages. As these cells are difficult to handle, they are more susceptible to contamination. Primary cells, in contrast to cell lines, are extremely sensitive cells requiring additional nutrients not included in classical media. To optimize survival and growth, primary cells perform best in specialty media customized for each cell type. In addition, whatever results you obtain, you should probably try to replicate them in primary cells, as they are a

superior model of the in vivo situation.

5.2.3 Conclusions

The methanolic extract of *L. javanica and M. flabellifolius* had antiproliferative effects on the human airway smooth muscle cells stimulated with IL-1β. Therefore, *M. flabellifolius* may decrease airway remodelling by a decrease in the size and proliferation of HBSMCs. *L. javanica* may also decrease airway remodelling by inhibiting cell proliferation. However, *L. javanica* increased cell size and this may promote airway remodelling by increasing cell mass.

The potential antiproliferative effects of *L. javanica and M. flabellifolius* was also observed in normally growing cells, suggesting that they may affect cell proliferation through multiple mechanisms. Even though further studies are needed to confirm these properties of *L. javanica and M. flabellifolius in vivo*, the present work corroborates asthma treatment based on ethnopharmacological studies.

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Appendix

Appendix 1

Protocol of cells culture:

Single Quots TM growth factors can be stored at 4°C and added to basal medium within 72 hours of receipt. After Single Quots TM are added to basal medium, use within 1 month. Do not re-freeze.

Reagent Pack TM Thawing Trypsin/EDTA and aliquot it into 5ml sterile centrifuge tubes and refreeze at 20c, we need use 2ml of Trypsin/EDTA

HEPES.BSS (Buffered Saline Solution)

TNS (Trypsin Neutralizing Solution)

We recommend that the HEPES-BSS and the Trypsin Neutralizing Solution be stored at 4°C for no more than one month.

Preparation of media (SMCBM- 5%FBS):

Perform the following steps:

1. Decontaminate the external surfaces of all supplement vials and the medium bottle with ethanol

2. Aseptically open each supplement vial and add the entire amount to the basal medium with a pipette.

3. Rinse each cryovial with the medium. It may not be possible to recover the entire volume listed for each cryovial. Small losses, even up to 10%, should not affect the cell growth characteristics of the supplemented medium.

4. Transfer the label provided with each kit to the basal medium bottle being supplemented. Use it to record the date and amount of each supplement added. We recommend that you place the completed label over the basal medium label (avoid covering the basal medium lot # and expiration date) to avoid confusion or possible double supplementation.

5. Record the new expiration date on the label based on the shelf life.

Setting up smooth muscle cells culture

1. Take aliquot 10ml of Basal media into a 50ml conical tube
2. Add 6ml of media by use micropipette into 25 flask
3. Put the flask in the incubator at 37c at least 30 minutes
4. Wipe cryovial with ethanol before opening and thaw cryovial of cells as fast as possible (using a water bath) at 37c for 2minutes , don't submerge the entire vial
5. Transfer the cells into flask by use micropipette
6. Gently rock the flask to distribute the cells
7. Then return the flask to incubator
8. Observe the flask under microscope to ensure there is no clumping if there is clumping (repeat pipetting up and down in the flask
9. Put the flask in the incubator at 37c, 5% CO_2
10. Replace the medium after 16 – 24hours and every two to three days

Proliferating Cells

1. (Note: immediately put the flask in an incubator (37°C, 5% CO2) for 3 hours to allow the cells to recover from the transportation)
2. Carefully open the flask, rinse the inner side of the cap with 70% ethanol, and let air dry.
3. Aspirate the medium from the flask.
4. Rinse the cells with 5ml Of HEPES Buffered Saline Solution
5. Remove the HEPES Buffered Saline Solution from flask and discard
6. Add 10 ml of media into flask.
7. Observe the flask under microscope and check the cell density. Open the cap half
8. Then return the flask to incubator (37°C, 5% CO2). Change the medium every two to three days.

The cells should be subcultured, according to the subcultivation protocol once they have reached > 70% confluence.

Passaging culture (Sub culturing)

1. Place the reagents at room temperature for at least 30 minutes to adjust the temperature of the reagents
2. .Check cells to ensure growth is optimal (when they are 70 to 80% confluent and contain many mitotic figures throughout the flask).
3. Observe flask under microscope
4. Remove used media from flask and discard
5. Rinse the cells with 5ml Of HEPES Buffered Saline Solution at room temperature by using pipette and agitate the flask carefully for 15 seconds
6. Remove the HEPES Buffered Saline Solution from flask and discard
7. Add 2ml Of Trypsin/EDTA by pipette at a room temperature.
8. Put the flask in the incubator at 37c, for 2min
9. Observe the flask under microscope (we will see detach the cells)
10. Allow the trypsinization to continue until approximately 90% of the cells are rounded up. The entire process takes about 2-6 minutes, depending on the cell type.
11. At this point, rap the flask against the palm of your hand to release the majority of the cells from the culture surface. If only a few cells detach, you may not have let them trypsinize long enough Wait 30 seconds and rap again. If cells still do not detach, wait and rap every 30 seconds thereafter.
12. After cells are released, IMMEDIATELY neutralize the trypsin in the flask with 4 ml of room temperature Trypsin Neutralizing Solution. And gently agitate.
13. Carefully aspirate the cell suspension and transfer it to a centrifugation tube. Spin down the cells for 5 minutes at 220 x g.
14. Aspirate most of the supernatant
15. Flick the cryovial with your finger to loosen the pellet

Dilute the cells in 2-3ml of media and resuspend the cells by carefully pipetting up and down. Plate the cells according to the recommended seeding density in new cell culture vessels containing prewarmed Medium. Place the flask in an incubator (37°C, 5% CO2) and change the media every two to three days.

Seeding stage:

1. Transfer 20 µl of diluted cells on the foil silver paper by micropipette
2. Take 20 µl of trypan blue dye paper by micropipette and then mix up down 4×
3. Transfer the mixture into slide
4. Put the slide in the cells counter

5. Calculate the final volume of cells and volume of media

   $C_1 \times V_1 = C_2 \times V_2$

   C_1= final conc of cells, V1= final volume of cells

   C_2= initial conc of cells, V2= initial volume of cells

6. Calculate the final volume of cells and the media by use the equation and mix it in new tube then take 100 µl of cells by micropipette and add into each well in plate
7. Incubate the plate at 37c for 24hr.

Preparation of 5 ml Stock solution (20mg/ml) each of Lippia Javanica and Myrothamnus Flaelliolius and Dexamethason and Qarcentin:

1. Weigh 0.1 gram of crude extract in a small container and then transfer directly to a volumetric flask. A funnel might be helpful when transferring the solid into the slim neck of the vol flask
2. First add 2.5 ml of DMSO and swirl gently
3. contents until powder is completely dissolved
4. Add more DMSO until the meniscus of the liquid reaches the calibration mark (5ml) on the neck of the vol flask (a process called "diluting to volume"). The vol flask is then capped and inverted several times until the contents are mixed and completely dissolved.
5. Store the stock solution 5 degrees for a maximum of 28 days. Store the stock solution in a refrigerator until the day for bioassays

Dilution of stock solution:

Dilution is the addition of more solvent (DMSO) to produce a solution of reduced concentration. Most often a diluted solution is created from a small volume of a more concentrated stock solution.

1. Take 25 µl of stock solution from drug and add into 2 centrifuge tube (15ml). Then add 4975 µl of media and mix (up and down) by micropippte (these tubes have conc 100 µg/ml)
2. Then take 5 cryovial and add 500 µl of media into each vial
3. Take 500 µl of 100 µg/ml of the first tube and add to next vial which is has conc 50 µg/ml and then mix up and down than transfer 500 µl into next vial which is has conc 25 µg/ml and……..until last vial

Test each concentration in triplicate for biological activity in cell culture:

1. After 24hr incubation, take the plate from incubator and discard the media by micropippte

2. Change it with another media has 0.3% FBS and free insulin (arrested growth) and put the plate in incubator for 3hr then discard the media and wash it with HEPES-BSS (Buffer solution)

3. Add 50 µl of each diluted stock solution (drug) in triplicate in each well plate and incubate plate at 37c for 1hr or 2hr

4. Then add 50 µl of interleukin B1 mixed with media has all components with growth factors then change the media every 24hr for 4days

(Notes: Treatment every day with drugs and interleukin for 4days with change media)

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Protocol for the bioactivity assays:

The crude extract will be assessed for biological activity using the XTT assay on HASMC:

Prepare an aseptic environment:

A. Always use sterile technique when working with cells
B. Close hood sash to proper position to maintain laminar air flow
C. Use only sterile pipette tips
D. Spray all surfaces with 70% ethanol

Steps

1. **Preparation of cell culture:** Human bronchial smooth muscle cells (HASMC) will be cultured from human bronchial smooth muscle obtained from healthy lung transplant donors after sudden death.
2. The smooth muscle cells will be cultured in Dulbecco's modified Eagle's medium (DMEM/F12), supplemented with 10% foetal bovine serum (FBS), penicillin (50 U/mL), streptomycin(50g/mL), amino acids (1:100), l-glutamine (2 mM), and insulin (5 g/mL) in a humidified chamber (37 ◦C, 5% CO2) with the medium changed every other day.
3. Cells will be treated with interleukin-1β (IL-1β) or its solvent for 4 days with medium changed daily (Cells will be pre-treated with the lippia javanica and Myrothamnus flabellifolius ethanolic extract, for 1 h before IL-1 treatment, every day for 4 days)
4. Add 10 µl of the prepared XTT Mixture to each well without introducing air bubbles using a repeating pipette (Preparation of XXT solutions: Thaw XTT labeling reagent and electron-coupling reagent, respectively in a water bath at 37 °C. Mix each vial thoroughly to obtain a clear solution).
5. Mix gently for one minute on an orbital shaker.
6. Incubate the cells at 37 ◦C for0.5- 4 hours in a CO^2 incubator.

7. Before reading the plate mix gently on an orbital shaker for one minute to ensure homogenous distribution of color.
8. Measure the absorbance of each sample using a microplate reader at 450 nm
9. The proliferation studies will be performed three times in triplicate and results are expressed as a percentage of proliferation compared with controls
10. Cell proliferation will be measured by the XTT assay (2,3-bis[2-methoxy-4-nitro-5-sulfophenyl]-2H-tetrazolium5-carboxanilide, "Cell Proliferation kit II XTT"
11. Concentration-response curves to the compounds will be performed in order to calculate its potency expressed as inhibitory concentration 50 (IC50). Cell proliferation will be measured by the XTT assay.

General description of the Cell Proliferation Kit II (XTT)

- Is a colorimetric assay for the nonradioactive quantification of cellular proliferation, viability, and cytotoxicity. Sample material is either adherent or suspension cells cultured in 96-well microplates.
- Colorimetric assays analyze the number of viable cells by the cleavage of tetrazolium salts added to the culture medium. This technique requires neither washing nor harvesting of cells, and the complete assay, from microculture to data analysis by an ELISA reader, is performed in the same microplate.
- More recently, the tetrazolium salt XTT was described. In contrast to MTT, the cleavage product of XTT is soluble in water; therefore, a solubilization step is not required. The tetrazolium salt XTT is cleaved to formazan by a complex cellular mechanism. This bioreduction occurs in viable cells only, and is related to NAD (P) H production through glycolysis. Therefore, the amount of formazan dye formed directly correlates to the number of metabolically active cells in the culture.

Note:

- To obtain reliable results thaw and mix XTT labeling reagent and electron coupling reagent immediately before use.
- The incubation time varies with the individual experimental setup (*e.g.,* cell type and cell concentration, used). Therefore, we recommend to measure the absorption as described at

different time points after addition of XTT labeling mixture (*e.g.,* 4, 6, 8, 12, and 18 hours) using one and the same microplate to determine the optimal incubation period for the particular experimental setup.

- Avoid repeated thawing and freezing.

Storage conditions (working solution): Thaw reagents immediately before use. It is recommended to prepare appropriate aliquots [5 ml XTT labeling reagent and 0.1 ml electron coupling reagent are required for the performance of the assay with one microplate (96 wells)]

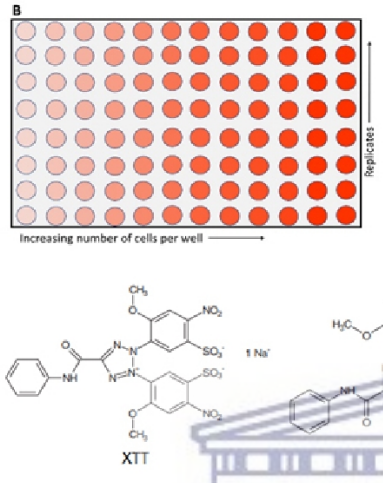

Figure 1: Metabolism of XTT to a water-soluble orange colored formazan salt by viable cells. Schematic of an XTT assay plate.