Antiproliferative, anti-inflammatory and antioxidant activities of leaf extracts, fractions and isolated compounds from *Ptaeroxylon obliquum*

Edward Thato Khunoana
04892471

Supervisor: Prof LJ McGaw
Co-supervisors: Prof JN Eloff
: Dr TE Ramadwa

Thesis submitted in fulfilment of the requirements for the degree of Doctor of Philosophy (PhD)

Phytomedicine Programme

Department of Paraclinical Sciences

Faculty of Veterinary Science

University of Pretoria

NOVEMBER 2022

Declaration

I declare that this thesis titled “Antiproliferative, anti-inflammatory and antioxidant activities of leaf extracts, fractions and isolated compounds from *Ptaeroxylon obliquum*” hereby submitted to the University of Pretoria has not been submitted by me or any other person for a degree at this at any other institution of higher learning. It is my own original work and all material contained herein has been duly acknowledged.

_____

Mr E.T. KHUNOANA

Acknowledgements

I would firstly like to take this opportunity to thank God; this study would not have been feasible without him. He has been the source of my strength throughout the years. I would like to thank him with hymn 166 “E, kajeno ke fodile ke ngoana oa Molimo, ke dumetse, ke thabile ha ke na pelaelo”.

I would like to express my gratitude to my mentor Prof. L.J. McGaw for giving me the chance to join the Phytomedicine family as an intern and then as a PhD candidate. Additionally, I would like to express my gratitude to my co-supervisor, Dr T.E. Ramadwa, who went above and beyond to ensure the success of this study. It was an honour to work under the mentorship of my second co-supervisor, Prof. J.N. Eloff, who has been essential in helping with the project.

I appreciate the help with the lab assays from the Phytomedicine family, particularly Dr Sanah Nkadimeng and Dr Fikile Makhubu. I would like to express my gratitude to the LC-MS Synapt Facility at the Department of Chemistry, University of Pretoria, for the chromatography and/or mass spectrometry services offered by Ms. Madelien Wooding. Sincere thanks to the NRF for providing money for the project.

Lastly, I would like to thank my family, my dad Rev. M.E. Khunoana and my mum B.R. Khunoana and my two brothers A.O. Khunoana and G.O. Khunoana, also my spiritual parents M.M. Khunoana and C. Maema for supporting me throughout my life. I dedicate this PhD to my late great grandfather Mr MORIBOLA JOHANNES KHUNOANA who had great influence in my life and dedicate it also to my late grandparents Rev. L.W. Khunoana, Rev. M.E. Khunoana, S.M. Mabuse and E.T. Mabuse. Thanks to all my support structures and my friends for the support

List of Abbreviations

ANOVA	Analysis of variance
ATCC	American Type Culture Collection
BCL-2	B-cell lymphoma 2
CANSA	Cancer Association of South Africa
CHCL3	Chloroform
COX	Cyclooxygenase
DISC	Death-inducing signaling complex
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
ETOAC	Ethyl acetate
FADD	Fas associated death domain
FASL	Fas ligand
FBS	Foetal bovine serum
H_2O	Water
HELA	Human cervical cancer cells
HepG2	Human liver hepatocarcinoma cells
IBD	Inflammatory bowel disease
IL	Interleukin
LOX	Lipoxygenase
LPS	Lipopolysaccharides
MCF-7	Human breast adenocarcinoma cells
MEM	Minimum Essential Medium
MEOH	Methanol
MTT	3-(4,5-dimethyltetrazolium bromide)
N-BUOH	N-butanol
NF-ϗB	Nuclear factor kappa B
NO	Nitric oxide
INOS	Inducible nitric oxide
NSAID	Non-steroidal anti-inflammatory drugs

LTB4	Leukotriene B4
PBS	Phosphate buffer saline
PGE2	Prostaglandin endoperoxide synthase 2
PGG2	Prostaglandin G2
PSF	Penicillin/streptomycin/fungizone
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
SANBI	South African National Botanical Institute
SI	Selectivity index
TNFR1	Tumour necrosis factor receptor 1
TNF-α	Tumour necrosis factor alpha
TRADD	TNF receptor associated death domain
WHO	World Health Organization

Conference presentations

Khunoana E.T., Eloff J.N., Ramadwa T.E., Nkadimeng S.M., McGaw L.J. 2022. Biological activity of *Ptaeroxylon obliquum* collected in four different geographic regions of South Africa (Oral). 47th South African Association of Botanists (SAAB) Annual Conference, 17-20 January 2022, Mafikeng, North-West, South Africa.

Khunoana E.T., Eloff J.N., Ramadwa T.E., Nkadimeng S.M., McGaw L.J. 2022. Antiproliferative, anti-inflammatory and antioxidant activity of *Ptaeroxylon obliquum* leaf extracts and fractions (Poster). 70th International Congress and Annual Meeting of the Society for Medicinal Plant and Natural Product Research (GA), 28-31 August 2022, Thessaloniki, Greece.

Khunoana E.T., Eloff J.N., Ramadwa T.E., Nkadimeng S.M., Selepe M.A., McGaw L.J. 2022. Anti-cancer, anti-inflammatory and antioxidant activity of extracts, fractions and compounds from *Ptaeroxylon obliquum* leaves. (Oral). Faculty Day, 20 October 2022 held at the University of Pretoria, Faculty of Veterinary Science, Tshwane, Gauteng, South Africa.

Publications

Khunoana, E. T., Eloff, J. N., Ramadwa, T. E., Nkadimeng, S. M., Selepe, M. A. & McGaw, l. J. 2022. *In vitro* antiproliferative activity of *Ptaeroxylon obliquum* leaf extracts, fractions and isolated compounds on several cancer cell lines. Applied Sciences, 12, 11004. https://doi.org/10.3390/app122111004.

Manuscripts under review and in preparation

Khunoana, E. T., Eloff, J. N., Ramadwa, T. E., Nkadimeng, S. M., M. A. & McGaw, l. J. 2022. *Ptaeroxylon obliquum* extracts, fractions and bioactive compounds as potential inhibitors of arachidonic acid metabolic pathways in the inflammation mechanism. Journal of Ethnopharmacology (submitted)

Khunoana E.T., Eloff J.N., Ramadwa T.E., Nkadimeng S.M., Makhubu F.N., McGaw L.J. 2022. *Ptaeroxylon obliquum* acetone leaf extracts and obliquumol as potential apoptosis mediators in HepG2 and HeLa cells by caspase-3 and TNF-α. Plants (to be submitted)

List of Figures

List of Tables

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Faculty of Veterinary Science
Research Ethics Committee

14 October 2022

LETTER OF APPROVAL

Ethics Reference No	**REC235-19**
Protocol Title	**Endophytes from Ptaeroxylon obliquum and Melianthus comosus: Antimicrobial activity and interactions**
Principal Investigator	**Mr ET Khunoana**
Supervisors	**Prof LJ McGaw**

Dear Mr ET Khunoana,

We are pleased to inform you that your submission conforms to the requirements of the Faculty of Veterinary Sciences Research Ethics committee.

Please note the following about your ethics approval:

1. Please use your reference number (REC235-19) on any documents or correspondence with the Research Ethics Committee regarding your research.
2. Please note that the Research Ethics Committee may ask further questions, seek additional information, require further modification, monitor the conduct of your research, or suspend or withdraw ethics approval.
3. Please note that ethical approval is granted for the duration of the research as stipulated in the original application (for Post graduate studies e.g. Honours studies: 1 year, Masters studies: two years, and PhD studies: three years) and should be extended when the approval period lapses.
4. The digital archiving of data is a requirement of the University of Pretoria. The data should be accessible in the event of an enquiry or further analysis of the data.

Ethics approval is subject to the following:

1. The ethics approval is conditional on the research being conducted as stipulated by the details of all documents submitted to the Committee. In the event that a further need arises to change who the investigators are, the methods or any other aspect, such changes must be submitted as an Amendment for approval by the Committee.
2. **Applications using Animals:** FVS ethics recommendation does not imply that AEC approval is granted. The application has been pre-screened and recommended for review by the AEC. Research may not proceed until AEC approval is granted.

Approved

We wish you the best with your research.

Yours sincerely

PROF M. OOSTHUIZEN
Chairperson: Research Ethics Committee

100 YEARS

Room 8-6, Arnold Theiler Building
University of Pretoria, Faculty of Veterinary Science
Private Bag X04, Onderstepoort, 0110, South Africa
Tel +27 (0)12 529 5390
Email marie.watson-kriek@up.ac.za
www.up.ac.za

Abstract

Inflammation is a primordial response to protect the body against infection or trauma from outside or inside the body, such as harmful stimuli, viruses, bacteria and chemical exposures. This response is aimed at restoring damaged tissue to its normal physiological function. In the process of inflammation, macrophages act as the body's first line of defence, coordinating the inflammatory response by releasing a number of mediators. However, excessive or unregulated mediator production can induce chronic inflammation, which is the foundation of many unpleasant human diseases, including rheumatoid arthritis and cancer.

Cancer is a leading cause of death not just in South Africa but also worldwide. The disease is an abnormal cell formation brought on by multiple gene expression alterations that result in an unbalanced ratio of cell proliferation and death. Oncogenes are activated and tumour suppressors are deactivated by DNA mutations that cause cancer to begin. The host metabolism and cell structure must change for cancer to proliferate. It is widely acknowledged that the inflammation microenvironment plays a role in tumorigenesis. Numerous studies have shown a connection between chronic inflammation and cancer formation as well as the influence of tumour-induced inflammation on tumour growth. Significant pathways between inflammation and the growth or metastasis of tumours have been discovered. In the inflammatory microenvironment, elevated levels of ROS, cytokines, prostaglandins, nuclear factor kB (NF-kB), and microRNAs affect angiogenesis, DNA mutation rates, cell death and cellular senescence. Numerous cancers release cytokines like IL-6, which have been associated with metastasis, carcinogenesis and inflammation. CD8+ T cells release anti-inflammatory cytokines including IL-10, which have anti-inflammatory and anti-tumorigenic characteristics as they reduce levels of IL-6 and TNF-α. Chronic inflammation and other factors like oxidative stress are the cause of more than 25% of malignancies. Free radicals, such as reactive oxygen/nitrogen species (ROS/RNS), are created by epithelial and inflammatory cells during chronic inflammation. Oxidative stress occurs when the balance of ROS production to ROS detoxification favours an increase in ROS levels. Unregulated ROS/RNS generation during inflammation damages DNA in organs and promotes the development of cancer. Given that chronic inflammation is connected

to more than one-fifth of cancer incidence, research into chemopreventive drugs or preparations against inflammation-related carcinogenesis is urgently needed.

Ptaeroxylon obliquum (Rutaceae)*,* commonly known as sneezewood, was selected based on its traditional uses against inflammatory disorders such as fevers, arthritis, and rheumatism and work done on this species in the Phytomedicine Programme relating to other applications. Extracts, fractions and purified compounds from the leaves of the tree were evaluated for anti-proliferative, antioxidant and anti-inflammatory activities in this thesis.

Column chromatography was used to fractionate the active extracts based on bioactivity, and NMR and UPLC-MS analyses were used to identify and characterize the isolated compounds. Using a colorimetric tetrazolium bromide test, antiproliferative activity was evaluated against normal Vero cells as well as a number of cancerous human cells, including lung adenocarcinoma (A549), human breast cancer (MCF-7), hepatocarcinoma (HepG2) and human cervical cancer cells (HeLa). The 2, 2'-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS) and 2, 2-diphenyl-1-picrylhydrazyl (DPPH) tests were used to measure the free radical scavenging activity. The nitric oxide (NO) inhibition assay in lipopolysaccharide (LPS)-induced RAW 264.7 mouse macrophages, soybean 15-lipoxygenase (15-LOX) inhibitory assay and cyclooxygenase (COX)-2 enzyme activity utilizing an ELISA method were used to determine the anti-inflammatory activity. Additionally, the effects of the extracts and isolated compounds on the production of the anti-inflammatory cytokine (IL-10) and pro-inflammatory cytokines (IL-1) and TNF-α were evaluated using ELISA kits in this study. Using mouse ELISA kits, the effects of the substances on the levels of caspase-3 and TNF- were determined and quantified in HepG2 and HeLa cancer cells. The alterations in HepG2 and HeLa cell morphology were observed using light microscopy.

The secondary metabolite composition of the four *P. obliquum* acetone leaf extracts showed some geographical variation, as shown by the UPLC-MS chromatograms and the positive mode of the ESI. Using silica gel open column chromatography, obliquumol, O-methylalloptaeroxylin, and a combination of lupeol and β-amyrin were isolated from the chloroform fraction. Acetone extracts were relatively toxic to the HepG2 cells with IC_{50} values from 8 to 200 µg/mL, but they were less toxic to the

other cell lines, with selectivity index values as high as 14. At concentrations evaluated against all cell lines, aqueous extracts and fractions has low toxicity (IC_{50} > 100 µg/mL). Isolated compounds had IC_{50} values against HepG2 and HeLa cells ranging from 52 to 539 µg/mL and 189 to 247 µg/mL, respectively. The cytotoxicity of the acetone extracts was confirmed by light microscopy, which revealed alterations in the morphology of HepG2 and HeLa cells. *P. obliquum* extracts showed selective cytotoxicity against cancer cell lines, rendering them potential sources for development for use in anticancer therapy.

Water extracts scavenged ABTS and DPPH radicals with IC_{50} values as low as 29.06 µg/mL and 43.4 µg/mL, respectively. The 15-LOX inhibitory activity of all acetone extracts was better than that of the positive control and aqueous extracts, with IC_{50} values ranging from 5.7 to 10.4 µg/mL. Obliquumol had the highest level of 15-LOX inhibition with an IC_{50} of 3.66 µg/mL, which was twice as high as quercetin (positive control). The isolated components, fractions and extracts from *P. obliquum* *P. obliquum* all reduced NO production in a dose-dependent manner at the tested concentration. At the lowest measured concentration (1.6 µg/mL), the acetone extracts reduced nitric oxide production with percentages as high as 95% and 102% with the Walter Sisulu and Nelspruit acetone extracts, respectively. The high cell survival of >100% RAW 264.7 macrophage cells showed that the low NO production was not caused by acetone extracts being toxic to the cells. The extracts and the compounds reversed LPS-induced COX-2 production significantly ($p<0.001$) in a dose dependent manner in comparison with the positive control, quercetin.

The levels of LPS-induced pro-cytokines that were assessed in the study were reduced by the extracts and compounds. Obliquumol and the acetone extract of Walter Sisulu leaves significantly reduced the levels of TNF-α induced by LPS ($p<0.027$ and $p<0.008$). *P. obliquum* acetone extracts and the isolated compound obliquumol increased the expression of caspase-3 in HepG2 cells by 2% at 100 µg/mL. Caspase-3 production in HeLa cells was raised by 8% at 100 µg/mL by the isolated compound obliquumol. The Hatfield acetone extract (100 µg/mL) increased TNF-α production by over 10%, which was more than the effect of doxorubicin, a positive control, in HepG2 cells. Walter Sisulu acetone extracts improved TNF-α production in HeLa cells by 11.5% at the lowest tested concentration (50 µg/mL), surpassing the activity of the positive control. The morphology of both cell lines

evaluated in the study showed a concentration-dependent effect against tested samples. HepG2 cells were more sensitive and showed increased morphological changes than HeLa cells.

Anti-inflammatory activity and anti-cancer activity were correlated, with r-values ranging from 0.42 to 0.66. The correlation was significant ($P<0.01$). The anti-inflammatory activity and the antioxidant activity had a strong, perfect negative correlation, with $r = -0.79$ and $r = -0.80$ for ABTS, DPPH, and 15-LOX, respectively. This correlation was highly significant ($P<0.01$). The medicinal usage of *P. obliquum* against inflammation is supported by the strong anti-inflammatory activity of the acetone extracts, fractions, and isolated components of this plant. The acetone extract and isolated compounds from *P. obliquum* may help to induce pro-apoptotic cytokines (caspase-3 and TNF-α). There was significant correlation between antioxidant, anti-inflammatory and antiproliferative properties of crude extracts, fractions and isolated compounds. Considering that *P. obliquum* extracts displayed specific cytotoxicity against cancer cell lines, they may be valuable as sources for anticancer therapy.

Table of Contents

Chapter 1

1.1 Introduction

Background and motivation

Chronic inflammatory disorders are among the leading causes of death globally. For the next 30 years, it is expected that chronic inflammation-related disorders will become more common (Greten and Grivennikov, 2019). Chronic inflammation is thought to be responsible for about 25% of the factors that lead to cancer (Murata, 2018). Steroids and non-steroidal drugs have been successfully used as anti-inflammatory therapeutics to treat various inflammatory diseases for a number of years (Ramadwa et al., 2022).

During inflammation, the body reacts by releasing proinflammatory cytokines, including interleukins (IL-1 and IL-6) and tumour necrosis factor-alpha (TNF-α) (Conforti et al., 2008). Anti-inflammatory cytokines are produced to halt the process by regulating pro-inflammatory cytokine responses, including IL-1 receptor antagonist (IL-1ra), IL-4, IL-6, IL-10, IL-11 and IL-13 (Dinarello, 2000). Following this, cyclooxygenase-2 (COX-2) is induced to produce prostaglandins, which are involved in inflammation, swelling and irritation, and are even connected to certain types of cancer and arthritis (Brand-Williams et al., 1995, Schneider and Pozzi, 2011). The lipoxygenase (LOX) pathway is another inflammatory response route in the human body. COX-2 and 15-LOX-mediated reactions generally produce prostaglandins and leukotrienes that act as pro-inflammatory mediators. The pro-inflammatory mediators are involved in pathogenesis, LOX-12/15 generates protectins and resolvins, which are derived from polyunsaturated fatty acids, as well as lipoxins derived from arachidonic acid (Arita, 2016).

Reactive oxygen species (ROS), which lead to oxidative stress, are produced when macrophages are activated. Nitric oxide (NO) and proinflammatory cytokines are released as a result of oxidative stress, which is an inflammation activator (Nworu and Akah, 2015). ROS also promote the development of tumours through several processes, including inflammation, DNA damage, immune evasion, signaling pathway modulation governing autophagy and apoptosis, angiogenesis and drug resistance (Arfin et al., 2021). Overproduction of ROS is caused by significant

activation of COX-2, LOX, and inducible nitric oxide synthase (iNOS) in inflammatory tissues (Aggarwal, 2014). Nitric oxide inhibits mitochondrial enzymes, stimulates COX enzymes to synthesize prostaglandins, and contributes to the progression of inflammatory disorders (Cuzzocrea et al., 2001). Additionally, NO contributes significantly to the inflammation-induced catabolism of cartilage and the worsening of inflammatory joint disease. Inflammatory mediators like bacteria and pyrogen/lipopolysaccharide (LPS) activate iNOS, which in turn produces NO by converting L-arginine to L-citrulline (Lee, 2015). In order to prevent and treat inflammatory disorders, it is therefore expected that an inhibitor of COX-2, LOX, NO, ROS and certain cytokines will be useful.

There is a wealth of evidence demonstrating the link between chronic inflammation and the development of cancer, as well as the effect that tumour-induced inflammation has on the development of tumours (Lin and Karin, 2007). Some significant pathways that connect inflammation to the development or spread of tumours have been discovered. Increased amounts of ROS, cytokines, prostaglandins, nuclear factor kB (NF-kB), and microRNAs in the inflammatory microenvironment have an impact on angiogenesis, DNA mutation rates, cell death and cellular senescence (Schetter et al., 2010). Numerous malignancies produce cytokines like IL-6, which have been linked to carcinogenesis and metastasis (Taniguchi and Karin, 2014). CD8+ T cells release anti-inflammatory cytokines, like IL-10, which have anti-inflammatory and anti-tumorigenic characteristics as they reduce levels of IL-6 and TNF-α (Schetter et al., 2010, Onuma et al., 2011). Research on antiproliferative medicines against inflammation-related cancer is urgently needed, given that chronic inflammation is linked to more than 20% of cancer incidence (Kanda et al., 2017).

The most commonly prescribed anti-inflammatory medications are either steroidal (e.g., betamethasone, dexamethasone and prednisolone) or nonsteroidal (e.g., aspirin, ibuprofen, indomethacin, diclofenac, nimesulide, naproxen and celecoxib), and they are used to treat both acute and chronic inflammatory conditions, including rheumatoid arthritis (Kawai and Akira, 2011, Van Furth, 2013). As a result of the long-established link between inflammation and cancer, numerous therapies have been developed to either prevent or treat cancer by reducing inflammation. One strategy is to use selective blockers or non-steroidal anti-inflammatory drugs

(NSAIDs) to reduce cyclooxygenase (COX) activity (Munn, 2017). However, some of these medications have negative side effects, and chronic inflammation is frequently treated with long-term dosing. (Ramadwa et al., 2022). Therefore, the search for novel and selective anti-inflammatory, anti-proliferative and antioxidant inhibitors is still necessary.

Since the beginning of time, plants have been considered the backbone of many traditional medical systems around the world, and they continue to offer humanity a source of innovative treatments for a wide range of illnesses. The efficacy of medicinal plants for reducing pain and inflammation is well documented in traditional medicine (Zou et al., 2005, Gacche et al., 2011). Numerous studies have been conducted to assess the anti-inflammatory potential of secondary metabolites from natural products using human and mouse macrophage cell lines as well as activity against inflammatory enzymes like LOX and COX (Dzoyem and Eloff, 2015).

Ptaeroxylon obliquum (Thunb.) Radlk. (Rutaceae), a tree found in southern Africa, is used in South Africa as a traditional medicine to treat a number of ailments, especially those including signs of inflammation like rheumatoid arthritis, fever, chest pain, and headaches (Iwalewa et al., 2007, Moyo and Masika, 2009, Ramadwa et al., 2021, Ramadwa et al., 2022). Previous studies on *P. obliquum* leaf extracts in the Phytomedicine Programme led to the isolation of a newly described compound, obliquumol, which was more active than amphotericin B (the gold standard antifungal drug) against *Candida albicans* (Van Wyk, 2012). Obliquumol was also not toxic to fibroblast cells and this information was patented (Van Wyk et al., 2018). Patents were granted in the USA and in Europe on these activities. Further studies were conducted on obliquumol and other isolated compounds to determine the anthelmintic, larvicidal, antifungal, antimycobacterial, anti-inflammatory and cytotoxic activities of acetone leaf extracts, fractions and isolated compounds from *P. obliquum* (Ramadwa, 2010, Ramadwa et al., 2017, Ramadwa et al., 2019).

Based on the previous results, there was a motivation to determine anti-inflammatory activities of *P. obliquum* via different inflammation mechanisms to ascertain or validate the traditional uses of the plant to treat or prevent inflammation-related ailments and symptoms. Numerous studies have shown a connection between persistent inflammation and the emergence of cancer, and there is a need for

selective anti-inflammatory and/or anti-proliferative inhibitors against cancerous cell lines. This motivated the study reported in this thesis.

1.2 Hypothesis

Based on the above description, it is hypothesized that:

The leaf extracts of the traditionally used *Ptaeroxylon obliquum* tree contain bioactive compounds, that can be isolated and characterized, with potential use in human health, particularly against inflammation related disorders and cancer.

- *P. obliquum,* which is used in traditional medicine to treat inflammatory diseases and their symptoms, contains phytochemicals that have anti-inflammatory, antiproliferative, and antioxidant properties.
- The identity and quantity of secondary metabolites in plants of the same species can vary depending on their geographic location.
- *P. obliquum* aqueous and acetone extracts, fractions and secondary metabolites that affect 15-LOX, COX-2, NO, IL-1β, IL-6, IL-10, and TNF-α have the potential to inhibit or reduce the inflammatory process.
- Extracts, fractions, and bioactive compounds from *P. obliquum* selectively inhibit the proliferation of human breast cancer (MCF-7), hepatocarcinoma (HepG2), lung adenocarcinoma (A549), and cervical cancer (HeLa) cells.

1.3 AIM AND OBJECTIVES

1.3.1 Aim

The aim of the study was to investigate the anti-inflammatory, antiproliferative and antioxidant activities of leaf extracts, fractions and isolated compounds from *Ptaeroxylon obliquum* collected from different locations in South Africa.

1.3.2 Objectives

To achieve the aim of the study, the following objectives were set:

- ❖ To isolate and characterize bioactive compounds from *P. obliquum* leaves.
- ❖ To analyse the chemical composition of *P. obliquum* leaf extracts by Ultra Performance Liquid Chromatography-Mass Spectrometry (UPLC-MS).
- ❖ To determine anti-inflammatory activities of *P. obliquum* aqueous extracts, acetone extracts, fractions and isolated compounds as potential inhibitors of 15-LOX, NO, COX-2, IL-1β, IL-6, IL-10 and TNF-α.

- To determine the radical scavenging activity of the extracts and fractions using 2, 2-diphenyl-1-picrylhydrazyl (DPPH) and 2, 2'-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS) assays.
- To evaluate antiproliferative activity of *P. obliquum* aqueous extracts, acetone extracts, fractions and isolated compounds against Vero cells, hepatocarcinoma (HepG2*)*, lung adenocarcinoma (A549*)*, human breast cancer (MCF-7) and human cervical cancer cells (HeLa) using a colorimetric tetrazolium bromide assay.
- To determine the possible anti-cancer mechanism of action of the most active samples against TNF-α and caspase-3.
- To establish the correlations between antioxidant, anti-inflammatory and antiproliferative activities of crude extracts, fractions and isolated compounds.

Chapter 2

Literature review

2.1 Mediators of inflammation and the inflammatory process

Inflammation is the adaptive response of an innate immune system, which is triggered by the exposure of cells or tissues to chemical irritation, injury and invasion of pathogens (Medzhitov, 2008). When inflammation is unregulated, it may result in various diseases and disorders. Prolonged inflammation is linked to both acute and chronic illnesses, including type 2 diabetes, rheumatoid arthritis, Alzheimer's disease, asthma, cancer, heart disease, atherosclerosis, sclerosis, acquired immunodeficiency syndrome (AIDS) and inflammatory bowel disease (IBD) (O'Byrne and Dalgleish, 2001, Ramadwa et al., 2022). A wide variety of mediators that act together to establish intricate regulatory networks and bind to certain target receptors on cells to control the inflammatory response. Pro-inflammatory mediators, which are molecules secreted as plasma proteins or from cells such as mast cells, platelets, neutrophils, and monocytes or macrophages that promote inflammation, are increased in response to the stimulus (Iwalewa et al., 2007). Examples of pro-inflammatory mediators include nitric oxide (NO), inducible nitric oxide synthase (iNOS), prostaglandins, cytokines (interleukin 1β (IL-1β), interleukin 6 (IL-6) and tumour necrosis factor alpha (TNFα), as well as enzymes such as cyclooxygenase-2 (COX-2) (Muniandy et al., 2018).

Arachidonic acids (AA) are one of the body's important fatty acids, and are well known metabolic precursors involved in various inflammatory pathways. The stimulation of phospholipase within the AA cascade activates the cleavage and release of AA from phospholipid (Nkadimeng et al., 2021). AA is then catalysed by one of three major inflammatory enzymes, namely lipoxygenase (LOX), cytochrome P450 monooxygenase and cyclooxygenase (COX). LOX and COX enzymes are the most commonly investigated enzymes and are responsible for advancing inflammation. There are three cyclooxygenase enzymes: cyclooxygenase-1 (COX-1), cyclooxygenase-2 (COX-2), and the more recently described cyclooxygenase-3 (COX-3). Acetaminophen and related compounds are able to specifically inhibit COX-3 (Botting, 2003, Adebayo et al., 2015). Cyclooxygenase enzymes, also called

prostaglandin endoperoxide synthases, are enzymes with similar structures but their function is totally different. Cyclooxygenase-1 (COX-1) is constitutively expressed in various tissues of the body including the lungs, kidney and small intestine, while COX-2 is a mediator of the inflammatory process. The cyclooxygenase pathway starts with the synthesis of prostaglandin G2 (PGG2) from AA which results in the oxygenation of AA (Tan et al., 2011). The PGG2 is then reduced by peroxidase activity to produce prostaglandin G2 (PGH2) which is a major intermediate for the synthesis of all further substrates, catalysed by various cell-specific isomerases including prostaglandin and thromboxane (SAMUELSSON, 1978, Kawahara et al., 2015, Nkadimeng et al., 2021). In healthy tissue, COX-2 is generally undetectable and is induced by endogenous and exogenous stimuli such as inflammatory cytokines, stress, LPS and hypoxia (Tan et al., 2011). Once activated, COX-2 can induce elevated levels of prostaglandin endoperoxide synthase 2 (PGE2) which, when combined with other prostaglandins, may stimulate pain, increase vascular permeability and increase the likelihood of inflammatory diseases or disorders. Therefore, inhibiting the COX-2 enzyme is critical to reduce inflammatory disorders or diseases (SAMUELSSON, 1978).

Cytokines are small cell-signalling proteins which are specifically responsible for communication and interaction between cells. Macrophages are dominantly associated with the synthesis of pro-inflammatory cytokines which are responsible for the up-regulation of inflammatory reactions (Zhang and An, 2007). According to studies, the development of pathological pain is directly influenced by pro-inflammatory cytokines including IL-1, IL-6 and TNF-α (Nkadimeng et al., 2021). IL-1 may act alone or in synergy with TNF-α to lower the threshold of pain by stimulating the increase in synthesis of prostaglandin endoperoxide synthase 2 (PGE2), and many biological activities of IL-1 are due to increased PGE2 production. When COX inhibitors are administered concurrently with an IL-1 injection, the symptoms of fever, headache, myalgias and arthralgias in humans are all alleviated (Dinarello, 2000). IL-1 was found to be pathogenic in an animal model study of rheumatoid arthritis (Isomäki and Punnonen, 1997). It was also found that TNF-α, IL-1β and IL-6 were present at high levels in the synovial fluid from patients with rheumatoid arthritis and are also produced by the synovial cells *in vitro* (Isomäki and Punnonen, 1997).

TNF-α is a cytokine that promotes inflammation and has a role in a variety of cell signaling pathways that can result in necrosis or apoptosis (Idriss and Naismith, 2000). Many of the actions of TNF-α are caused by binding to either of its two cell membrane receptors, TNFR-1 or TNFR-2. Binding does not only regulate apoptosis but also NF-kB activation of inflammation, and activates stress activated protein kinases (Nkadimeng et al., 2021). The protein is also important for resistance to infection and cancers. The inhibition of cytokine IL-6, which is also known to play a significant role in the neuronal response to nerve injury, has reduced the effects of regeneration (Nkadimeng et al., 2021). In contrast, interleukin 10 (IL-10) is regarded as a potent anti-inflammatory cytokine that is essential for preventing inflammatory and autoimmune diseases by inhibiting the release of pro-inflammatory cytokines by activated macrophages, including TNF- α, IL-6, and IL-1 (Iyer and Cheng, 2012). Additionally, endogenous anti-inflammatory and pro-inflammatory cytokine receptors can be up- and down-regulated by IL-10. As a result, it can influence pro-inflammatory cytokine synthesis and activity at a wide range of levels (Iyer and Cheng, 2012, Porro et al., 2020).

Nitric oxide (NO) is also part of the pro-inflammatory mediators generated by macrophages. Nitric oxide synthase is an enzyme responsible for producing NO, a transient free radical gas (NOS) (Lee, 2015). There are three isoforms of NOS, which include iNOS (found in macrophages, neutrophils, fibroblasts, vascular smooth muscle and fibroblasts), eNOS and nNOS (mostly present in endothelial cells) (Rang et al., 2012). Numerous cells, including macrophages, respond to bacterial lipopolysaccharides (LPS) by expressing inducible nitric oxide synthase (iNOS), which is directly responsible for the creation of high quantities of NO and is crucial for immunoregulation (Ramadwa et al., 2022). Lipopolysaccharide-mediated activation by macrophages initiates the production of some pro-inflammatory cytokines such as interleukins (IL-1β, IL-2, IL-4, IL-5, IL-6) as well as TNF-α and NO (Wang et al., 2011). Lipopolysaccharide-mediated activation of macrophages also leads to the expression of proteins of the nuclear transcription factor kappa-B (NF-κB) and mitogen activated protein kinase (MAPK) pathways. Two molecular targets implicated in the inflammatory process, NF-κB and MAPK pathways, play a key role in inflammation, and regulate inflammatory gene transcription, such as the expression of pro-inflammatory cytokines, COX-2 and iNOS (Arthur and Jobin,

2013). Excessive production of NO may lead to destruction of tissue, as well as immunological and inflammatory diseases (Ramadwa et al., 2017).

Lipoxygenase (LOX) enzymes are iron-containing enzymes which mostly catalyse deoxygenation of polyunsaturated fatty acids such as linoleic acid (Nkadimeng et al., 2021). They are expressed in epithelial, tumour and immune cells displaying a variety of physiological functions, including inflammation, tumorigenesis and skin disorders. LOX enzymes are present in plants, animals, fungi and also in humans where they are associated with the synthesis of leukotrienes and prostaglandins, which are linked with the stimulation of inflammatory activities (Singh and Chauhan, 2009). Arachidonic acid (AA) is a substrate for mammalian lipoxygenases, and these enzymes are classed as 5-, 8-, 12-, and 15-lipoxygenases depending on where AA is oxygenated (Ueda et al., 1998). The lipoxygenase isoform 5-LOX is involved in the synthesis of 5-hydroperoxy eicosatetraenoic acid (5-HPETE) and leukotriene B4 (LTB4). In humans the enzymes are expressed in a tissue-specific form where 15-LOX is mainly expressed in reticulocytes, eosinophils, and microphages (Klil-Drori and Ariel, 2013). The 15-LOX enzyme was found to be implicated in different inflammatory related diseases and in some cases it was found to induce pro-inflammatory cytokines such as IL-6 and IL-12 (Nkadimeng et al., 2021). The 15-LOX enzyme is a pro-inflammatory mediator in synovial tissue of patients suffering from rheumatoid arthritis (Corminboeuf and Leroy, 2015). Leukotrienes play a role as chemo-attractants, recruiting cells from the immune system to bring them to the sites of inflammation. The production of leukotrienes by LOX causes bronchoconstriction leading to bronchospasm. Selective LOX inhibitors are used as a therapeutic strategy in the treatment of asthma (Schneider and Bucar, 2005). To treat inflammatory disorders, it is urgent to find targeted 15-LOX inhibitors. Since soybean lipoxygenase enzymes similarly catalyze the oxidation of linoleic acid, arachidonic acid, and other unsaturated fatty acids, they are frequently utilized in experiments to identify possible compounds that might serve as mammalian lipoxygenase inhibitors (Ondua et al., 2019).

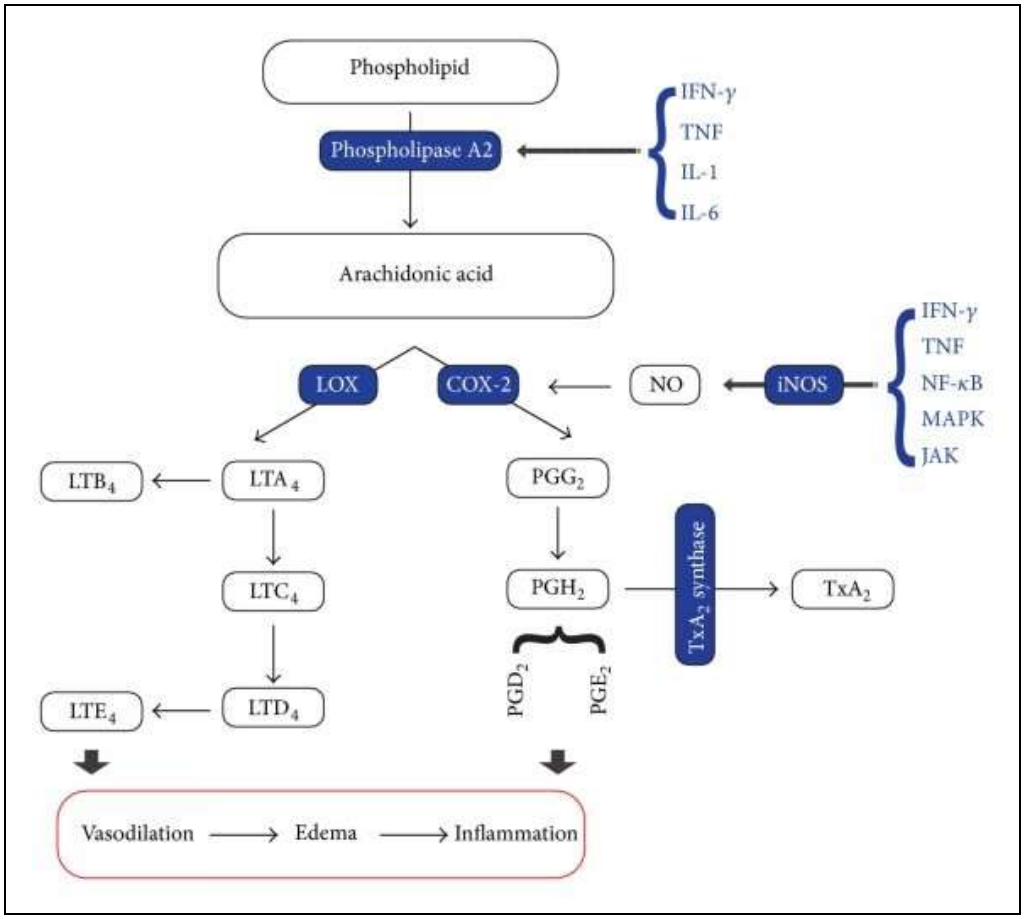


Figure 2.1: Inflammation pathway. COX, cyclooxygenase; LOX, lipoxygenase; PG, prostaglandin; LT, leukotriene; TX, thromboxane; NO, nitric oxide; iNOS, inducible NO synthase; IFN, interferon; TNF, tumour necrosis factor; NF-κB, nuclear factor-κB; MAPK, mitogen activated protein kinase; JAK, janus kinase; IL, interleukin (Ghasemian et al., 2016).

2.2 Link between inflammation, cancer and oxidative stress

Rudolf Virchow's discovery of leukocytes in tumours in the 19th century was the first evidence that inflammation and cancer might be connected. However, it has only been proven conclusively in the last ten years that inflammation is a key factor in carcinogenesis (Karin, 2006). It is now universally acknowledged that the inflammation microenvironment plays a role in carcinogenesis and all tumours, even those for which a clear causative link to inflammation has not yet been shown (Mantovani et al., 2008). Germline mutations are responsible for a small percentage of malignancies, although somatic mutations and environmental factors account for the great majority (90%) of cancer cases. Chronic inflammation seems to have a multitude of associations with cancer risk factors and causes (Aggarwal et al., 2009,

Aggarwal, 2014). In comparison to its unique immune cell makeup, the tumour microenvironment's cytokine and chemokine expression profile may be more important. Regardless of their source, many cytokines can either promote or hinder the growth and spread of tumours (Lin and Karin, 2007).

Pro-inflammatory and anti-inflammatory cytokines are mostly produced by macrophages (Zhang and An, 2007). There are two types of macrophages: M1 and M2 (Sica et al., 2008). M1 macrophages are primarily involved in the production of pro-inflammatory cytokines (TNF-a, IL-1, IL-6, IL-12, or IL-23), which are responsible for enhancing inflammatory responses. M2 is closely associated with the activation of the anti-inflammatory cytokines IL-4, IL-10 and IL-13. The majority of identified tumour-promoting cytokines are "M1 cytokines" and research on colorectal cancer suggests that the M2 cytokine IL-10 may have tumour-suppressive properties (Berg et al., 1996, Lin and Karin, 2007). Other pathways have been suggested, such as inhibiting cell-cycle progression, reducing carcinogen activation, and enhancing immune surveillance. Inducible nitric oxide synthase and complex molecules can kill infections and activate the immune system's anti-tumour response (Greten and Grivennikov, 2019).

The immunological and inflammatory environment is controlled by cytokines through the activation of a variety of downstream effectors, including the transcription factors NF-kB, AP-1, STAT and SMAD as well as caspases, to either promote anti-tumour immunity or suppress it, and have an immediate impact on the survival and proliferation of cancer cells. (Ondua et al., 2019). Caspases are associated with apoptosis which can also be induced by the release of cytochrome c from mitochondria. This directly causes the development of the cytochrome c/Apaf-1/caspase-9-containing apoptosome complex, resulting in caspase-3 activation. (Cain et al., 2002).

TNF-α is a crucial mediator of inflammation and is a pro-inflammatory cytokine. Despite its moniker, TNF-α has a significant impact on the early phases of tumour development by controlling a series of cytokines, chemokines, adhesions, and pro-angiogenic activities. TNF-α may therefore be one mechanism through which inflammation promotes tumour growth (Zhang and An, 2007). There may be uses for

blocking antibodies in cancer therapy that are effective in treating other inflammatory illnesses.

It is now evident that inflammatory cells have a significant impact on tumour growth. These cells are potent tumour promoters early in the neoplastic process, creating a favourable environment for tumour growth, enabling genetic instability, and encouraging angiogenesis (Coussens and Werb, 2002). Inflammatory cells and the chemokines and cytokines they produce control all cell types in the tumour microenvironment, including malignant cells, fibroblasts, and endothelial cells, in order to control their growth, movement, and transformation. The task going forward is to normalize the inflammatory network in order to return the host response to normal. To do this, infiltrating cells' high levels of tumour-promoting properties, like pro-inflammatory cytokines, must be decreased while their levels of tumour-suppressing properties, like anti-inflammatory cytokines, are increased. This will allow us to harness anti-tumour activity later in the course of the tumour while suppressing pro-tumour entities (Coussens and Werb, 2002).

Approximately 20% of cancers are induced by chronic inflammation or other infections (Pinlaor et al., 2004). During chronic inflammation, reactive oxygen and nitrogen species (ROS/RNS) are produced from inflammatory cells and epithelial cells (Pinlaor et al., 2004). ROS/RNS cause DNA damage in organs during inflammation, leading to cancer development. There are strong ties existing between oxidative stress and every aspect of cancer, including tumour development, progression and invasion (Noda and Wakasugi, 2001). Numerous studies have shown that oxidative stress and human pathophysiological disorders may be fundamentally related. Oxidative stress happens when the ratio of ROS production to ROS detoxification favours an increase in ROS levels, impairing cellular function. Therefore, a variety of defensive mechanisms, including several antioxidants and detoxifying enzymes, are required to manage the amounts of ROS (Adwas et al., 2019).

An antioxidant is a chemical that has the power to stop or delay the oxidation of macromolecules. Antioxidants contribute to lowering or halting these chain reactions by neutralizing free radicals or blocking other oxidation events by being oxidized. (Adwas et al., 2019). As a result, the ability of antioxidants to limit oxidative damage

makes them a desirable treatment option for lowering the risk of diseases like cancer and inflammation (Dai et al., 2006). Studying oxidative stress is crucial since it is yet another pathway that might increase the risk of cancer and inflammation. Several medicinal plants have many antioxidant compounds, like polyphenols, that absorb and neutralise free radicals as well as decomposing peroxide and neutralising singlet and triplet oxygen (Charami et al., 2008). There is therefore a need to investigate highly active antioxidants derived from bioactive phytochemicals that are efficient scavengers of free radicals (Iqbal et al., 2015).

2.3 Drugs used in the management of inflammation disorders or related symptoms

Steroids and non-steroidal drugs have been successfully used as anti-inflammatory therapeutics to treat various inflammatory diseases throughout a number of years. However, several of these medications have serious negative side effects, and treating chronic inflammation frequently requires long-term use. Non-steroidal anti-inflammatory drugs (NSAIDs) may be used as COX-2 inhibitors, thus exerting anti-inflammatory effects. The inhibition of COX-1 is associated with the unwanted side effects of such drugs. Therefore, researchers began looking for bioactive molecules that specifically inhibit COX-2 (Zarghi and Arfaei, 2011). Ibuprofen, paracetamol and aspirin are popularly marketed as anti-inflammatory medication, however, they non-selectively inhibit COX enzymes, resulting in a variety of adverse symptoms including (ulceration and gastrointestinal bleeding) brought on by the body's COX-1-regulation processes being inhibited (Park et al., 2018, Ho et al., 2018, Theoduloz et al., 2019). Celecoxib and rofecoxib are examples of selective COX-2 inhibitors that have been developed. These drugs were more effective at suppressing COX-2 than COX-1 and specifically suppressed the biosynthesis of proinflammatory prostaglandins that COX-2 catalyzes (Ho et al., 2018). However, these potent selective COX-2 inhibitors showed serious cardiovascular and cutaneous side effects, including myocardial infarction, which resulted in the partial withdrawal of this class of medications from the market (Ho et al., 2018).

2.4 The use of traditional medicine as an alternative or complementary to conventional medicine

Plants have been used to cure common infectious diseases since antiquity, and some of those ancient remedies are still regularly utilized to treat a variety of

maladies (Rios and Recio, 2005). Traditional plant-based medicine is still widely used in underdeveloped nations, particularly in Africa (Makunga et al., 2008). Due to their effectiveness, cultural preferences and lack of affordable pharmaceutical alternatives, medicinal plants remain the primary form of treatment in many rural areas (Silambarasan and Ayyanar, 2015). Traditional medicinal plants include chemical compounds or combinations of chemical compounds that work singly or in combination to treat or prevent disease (Nielsen et al., 2012). Most traditional plant-based medicines are made using liquids to extract a variety of ingredients, including the active compounds. The most typical liquid for making infusions and decoctions is water. There are primary metabolites like sugars and amino acids, which are present in all plants, as well as secondary metabolites, some of which are only present in a particular genus or species (Heinrich, 2010). It is preferable to use plant leaves rather than the roots, bark, stems or fruit for the sake of sustainability, but this is restricted to cases where the bioactive compounds are present in the leaves (Ramadwa et al., 2017).

Medicinal plants harvested generally vary in consistency as well as quality of the active secondary metabolites due to ecological, environmental and genetic differences (Bopana and Saxena, 2007). The secondary metabolite composition of medicinal plants is known to be influenced by a number of environmental factors, including altitude, soil type, geographic location, and seasonal fluctuations. The life cycle, distribution and phytochemical makeup of the world's vegetation, including medicinal plants, are all being significantly impacted by the changing temperatures brought on by climate change (von Ahlefeld and Gordon-Gray, 2003, Applequist et al., 2020). It is important to gather plant material from various geographic places since all these factors cause variations in pharmacological activity.

2.5 Medicinal plants used in the treatment of inflammation

For centuries, plants or phytochemicals have been used to treat inflammatory diseases and related symptoms. Medicinal plants yield secondary metabolites that may serve as templates for the development of brand-new anti-inflammatory drugs with high pharmacological activity and minimal negative effects (Dzoyem and Eloff, 2015). South African medicinal plants traditionally used to alleviate pain, inflammation and also other symptoms have been widely reported and investigated

(Adebayo et al., 2015, Dzoyem and Eloff, 2015, Elisha et al., 2016, Nabatanzi et al., 2020). Several studies on LPS-stimulated RAW 264.7 cell lines have reported that some phytochemicals extracted from plants (such as p-coumaric acid) and herbal medicines exhibited their anti-inflammatory activity via the inhibition of iNOS, COX-2 and pro-inflammatory cytokines (including IL-1β, TNF-α) (Zhao et al., 2016). Thus, substances like phytochemicals that can inhibit NO, COX, LOX, ROS and pro-inflammatory cytokine release may have therapeutic benefits for inflammatory disorders.

2.6 *Ptaeroxylon obliquum*: A medicinal plant used in South Africa

Ptaeroxylon obliquum (Thunb). Radlk, commonly known as “sneezewood” or “nieshout”, is a member of the Rutaceae family.. *P. obliquum* presented in Figure 2.2 is the only species in the genus *Ptaeroxylon* and only southern Africa contains it; its primary habitats are South Africa, Mozambique, Namibia, Zimbabwe and Zambia (Agostinho et al., 2013). Sneezewood has long been used as a traditional remedy to treat animal and human illnesses. The remedies are traditionally prepared as decoctions, infusions and pastes that are administered both orally or externally . The bark is used to cure fevers, arthritis and rheumatism (Pujol, 1990). The Xhosa people snuff the powdered bark material as a recreational and therapeutic remedy for headache relief (Watt and Breyer-Brandwijk, 1962). The plant's wood is used as a therapy for anthrax, rheumatism, heart conditions, lupus, warts, sinusitis, the treatment of individuals who experience fits, as a tick repellent for cattle, and in ritual sacrifices to ancestor spirits. Wooden pegs made from the tree are frequently used as lightning protection (Hutchings, 1996)..To keep moths and other insects away from cupboards, wood pieces are still used. Its ability to deter insects made it a popular wood for bedsteads (Venter and Venter, 2002).

Figure 2.2: *Ptaeroxylon obliquum* tree at the University of Pretoria, Onderstepoort campus (25,64768°S, 28,18185°E).

The leaves of the trees, which have three to eight asymmetrical, dark green leaflets with a terminal pair of points, can be used to identify them. During the autumn and winter, the leaves may change colour, but the tree does not lose its leaves (Archer and Reynolds, 2001). The trees produce cream-colored buds from August to December, which open to reveal tiny flowers with four white petals and an orange centre. The fruit is made up of oblong capsules with a notched top and winged seeds inside. Young stems have smooth pale bark with blotchy, fissured bark as they age.

Although trees in drier places are little shrubs just 3 metres tall, tree heights range from 20 to 35 metres (Archer and Reynolds, 2001).

A study on *P. obliquum* leaves conducted in the Phytomedicine Programme laboratory at the University of Pretoria led to the isolation and characterization of the novel compound, obliquumol, which had good activity against *Candida albicans* (Van Wyk, 2012). Further studies were done on obliquumol and other compounds isolated from the leaves to determine the antibacterial, antimycobacterial, antifungal, anti-inflammatory and antiparasitic activities (Ramadwa, 2010, Ramadwa et al., 2017, Ramadwa et al., 2019). Since *P. obliquum* is traditionally used in southern Africa to alleviate a variety of ailments, including inflammation disorders and related symptoms such as arthritis, rheumatism, fever, pain and headache, there is a need to determine the anti-inflammatory activity of the crude extracts, fractions and isolated compounds since inflammation can be exerted through various mechanisms. Previous work done on the *P. obliquum* acetone crude extracts and non-polar fractions were toxic to Vero cell lines (Ramadwa et al., 2017). As a consequence, there is a need to isolate the potential cytotoxic compounds and investigate the activity of the bioactive compounds against cancerous cell lines to ascertain potential selective activity. This is particularly relevant as it has been established that there is a link between cancer, inflammation and oxidative stress.

Postscript

In the next chapter, potential bioactive compounds from *Ptaeroxylon obliquum* will be isolated and characterized using methods developed in our laboratory. The antiproliferative activity of the crude extracts, fractions and isolated compounds will be assessed against cancer cells.

Chapter 3

In vitro antiproliferative activity of *Ptaeroxylon obliquum* leaf extracts, fractions, and isolated compounds on several cancer cell lines

Preface

In this chapter the anti-proliferative/cytotoxicity of the crude extracts, fractions and isolated compounds was investigated against cancer and normal cell lines. The following article has been published in the Journal of Applied Science with the following reference:

Khunoana, E.T., Eloff, J.N., Ramadwa, T.E., Nkadimeng, S.M., Selepe, M.A., McGaw, L.J. 2022. *In vitro* antiproliferative activity of *Ptaeroxylon obliquum* leaf extracts, fractions and isolated compounds on several cancer cell lines. Applied Sciences 12, 11004. https://doi.org/ 10.3390/app122111004.

Abstract

Several cancers are induced by microbial infections or chronic inflammation. *Ptaeroxylon obliquum* is traditionally used to treat various infections characterized by inflammation. The *in vitro* antiproliferative and antioxidant activity of *P. obliquum* leaf extracts, fractions and isolated compounds were determined. Antiproliferative activity was assessed against normal Vero cells, and several cancerous human cells, including human breast cancer (MCF-7), hepatocarcinoma (HepG2), lung adenocarcinoma (A549) and human cervical cancer cells (HeLa) using a colorimetric tetrazolium bromide assay. Radical scavenging activity was tested using 2, 2-diphenyl-1-picrylhydrazyl (DPPH) and 2, 2'-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS) assays. Obliquumol, *O*-methylalloptaeroxylin and a mixture of lupeol and β-amyrin were isolated from the chloroform fraction using silica gel open column chromatography. Acetone extracts were toxic to HepG2 cells with IC_{50} values from 8 to 200 µg/mL but were less toxic to other cells with selectivity index as high as 14. Aqueous extracts and fractions were non-toxic at concentrations tested against all the cell lines (IC_{50} >100 µg/mL). Isolated compounds had IC_{50} values ranging from 52 to 539 µg/mL and 189 to 247 µg/mL against HepG2 and HeLa cells respectively. Light microscopy showing changes in HepG2 and HeLa cell morphology supported the cytotoxicity of the acetone extracts. Water extracts scavenged ABTS and DPPH radicals with IC_{50} values as low as 29.06 µg/mL and 43.4 µg/mL. *P. obliquum* extracts may be useful as sources of anticancer therapy as they have selective cytotoxicity against cancer cell lines.

Key words: *Ptaeroxylon obliquum,* cancer, antiproliferative, Vero, HepG2, HeLa

3.1 Introduction

Cancer is an unusual formation of cells caused by various changes in gene expression leading to dysregulated balance of cell proliferation and cell death (Preiser, 2012). This finally leads to a population of cells that invade tissues and metastasize to distant sites, causing significant morbidity, and if not treated, mortality (Ruddon, 2007). Cancer is initiated by mutations in DNA that activate oncogenes and inactivate tumour suppressors; it thrives when changes occur in the host metabolism and cell structure (Metzcar et al., 2019, Katti et al., 2022). The development process of normal, healthy cells turning into cancer cells is termed carcinogenesis and this process takes place in three stages, namely initiation, promotion and progression (Ruddon, 2007).

One of the leading causes of death globally is cancer, which has a 20% mortality rate estimated at 18.1 million under non-communicable diseases, with a 33% increase in newly diagnosed cases estimated at 9.6 million between 2015 and 2018. (Mandal and Basu, 2018, Organization, 2019, Lubuzo et al., 2022). The World Health Organization (WHO) projections indicate that by 2040, these numbers will have increased to 29.5 million of new cancer diagnoses and 16.5 million cancer-related deaths yearly across the globe (Bray et al., 2018, Shah et al., 2019, Organization, 2019). In South Africa, cancer is emerging as a critical public health problem with an estimated 107 467 new reported cancer cases and a total of 57 373 deaths occurring in 2018 (Made et al., 2017, Twilley et al., 2020). Breast cancer has the highest incidence rate (14 097 cases, 13.1%) followed by cervical (12 983 cases, 12.1%) and prostate cancers (12 452 cases, 11.6%) and the incidence of lung cancer was reported at 7.7%, ranking fourth among all the cancers which gives it the highest mortality rate of 13.5%. Lung and prostate cancers are the most predominant types in males while breast and cervical cancers are the predominant types in females (Twilley et al., 2020, Lubuzo et al., 2021).

Treatment options for cancer include chemotherapy, radiation, hormone and gene therapy; however, they all have various negative side effects such as fatigue, weight loss, nausea, vomiting and bleeding (Markham et al., 2020). Chemotherapy is one of the most commonly used anticancer treatments, but some cancers are resistant to

cytotoxic/chemotherapeutic agents which poses a major threat to anticancer therapy. The limitation in the efficacy of the therapeutic agents leads to non-satisfactory treatment outcomes and eventually death (Rahman et al., 2021). Cancer cells may be resistant to apoptosis by avoiding potential apoptotic pathways, such as faulty apoptosis initiation and execution, up-regulated anti-apoptotic markers, up-regulated pro-apoptotic signals, and down-regulated pro-apoptotic signals..

There is a need to seek alternative anticancer therapeutics such as phytochemicals as there is increasing evidence that suggest they could exhibit anticancer effects. Scientific evidence suggests that phytochemicals have substantial anticancer potential that may be considered for drug development (Elmore, 2007, Sak, 2012, Zhang et al., 2020). Modern medicine has embraced plants used in traditional medicine as potential leads for the development of therapeutic drugs. South Africa has a large variety of plant species, which are yet to be explored for their potential against cancer activity. Approximately 60% of currently utilized anticancer agents are natural compounds derived from plants, animals, and microbes, with roughly 25% coming from plants. (Newman et al., 2002; Juárez, 2014).

Approximately 20% of cancers are induced by chronic inflammation or other infections (Pinlaor et al., 2004). During chronic inflammation, reactive oxygen/nitrogen species (ROS/RNS) are produced from inflammatory cells and epithelial cells (Pinlaor et al., 2004). ROS/RNS cause DNA damage in organs during inflammation, leading to cancer development. A biological system is under oxidative stress when there is an imbalance between the synthesis and expression of reactive oxygen species and its ability to quickly detoxify the reactive intermediates or to repair the resultant damage (Preiser, 2012). Every element of cancer, including prevention, tumor formation, and treatment, is directly related to oxidative stress. (Noda and Wakasugi, 2001). Numerous studies have shown that oxidative stress and human pathophysiological disorders may be fundamentally related (Dalle-Donne et al., 2006: Agarwal et al., 2012: Tangvarasittichai, 2015). Specifically, it is well recognized that oxidative stress affects the DNA molecule, changes signaling pathways, and controls the development of a variety of cancers, including those of the breast, lung, liver, colon, prostate, ovary and brain (Saijo et al., 2016: Oh et al., 2016: Wang et al., 2016: Jaroonwitchawan et al., 2017: Lee et al., 2017: Saed et al., 2017: Zhang et al., 2017).

Ptaeroxylon obliquum (Thunb.) Radlk. (Rutaceae) is traditionally used to treat many infections, including inflammatory-related diseases in South Africa (Ramadwa et al., 2021). Therefore, the present study was aimed to investigate the antiproliferative and antioxidant properties of *P. obliquum* extracts, fractions, and isolated compounds against different cancer cell lines. Leaf samples were gathered from a variety of geographic regions since regional diversity may have some effects on the concentration of bioactive compounds in plants of the same species (Abuto et al., 2016).

3.2 Materials and Methods

3.2.1 Plant collection

Ptaeroxylon obliquum (Thunb.) Radlk leaves used in this study were collected during the summer of 2019 from trees growing at the Hatfield Campus (University of Pretoria), National Botanical Gardens of the South African National Biodiversity Institute (SANBI) in Pretoria, the Lowveld Botanic Gardens (Nelspruit, Mpumalanga) and Walter Sisulu National Botanical Garden (Roodepoort). Leaves were collected in open weave nylon bags, dried at room temperature in the shade and powdered using a grinder. The powders were stored in closed containers in the dark until needed. Voucher specimens (PRU130509, PRU130510, PRU130628 and PRU130627) were prepared and kept at the HGWJ Schweickerdt Herbarium of the University of Pretoria. Bulk plant material for isolation could only be collected at SANBI and Nelspruit due to limited availability of leaves.

3.2.2 Preparation of extracts

Exactly 5 g of the powder was extracted using 50 mL acetone and distilled water (hot and hold) separately. The mixture was placed in an airtight container on a shaker and left for 24 h, after which the supernatant was filtered through Whatman No. 1 filter paper and placed into a pre-weighed honey jar. The process was repeated three times for each solvent. Then the supernatants for each solvent were combined in a single pre-weighed honey jar and dried under a stream of cold air.

3.2.3 Fractionation and isolation of bioactive compounds from *P. obliquum*

P. obliquum leaf powder (500 g) from SANBI and Nelspruit were extracted separately with 5L of acetone and vigorously shaken for 8 hours on a Labotec shaking machine. A Büchner funnel was used to filter the supernatant through

Whatman No. 1 filter paper, and a Büchi Rotavapor R-114 (Labotec) was used to evaporate the solvent under vacuum. The concentrated extract was then transferred to a pre-weighed beaker. The same technique was carried out again on the plant material. The extracted mass was then measured after the extract had been allowed to dry at room temperature, yielding 36.6 g (Nelspruit) and 42.17 g (SANBI). The solvent-solvent extraction/fractionation of plant extracts protocol developed by the National Cancer Institute was modified by elimination of the carbon tetrachloride step. Five solvent-solvent fractions containing compounds with different polarities from *P. obliquum* acetone leaf extract were then obtained (Suffness, 1979, Eloff, 1998). To obtain the initial $CHCl_3$ and H_2O fractions, the acetone extract was reconstituted in 500mL of $CHCl_3$: H_2O (1:1) in a separatory funnel and the two layers were partitioned. The H_2O and *n*-BuOH fractions were then obtained by combining the H_2O fraction with an equivalent volume of *n*-BuOH. The *n*-hexane fraction was obtained by extracting the initial $CHCl_3$ fraction with an equal volume of *n*-hexane and a 10% H_2O-methanol mixture after the initial $CHCl_3$ fraction had been dried in a vacuum rotary evaporator. To create the 35%H_2O-MeOH fraction and the $CHCl_3$ fraction, the 10% H_2O-MeOH fraction was first diluted to 35% H_2O-MeOH. Therefore, five fractions were obtained: H_2O, n-BuOH, 35% H_2O-MeOH, $CHCl_3$, and *n*-hexane fractions.

Column chromatography was used to isolate the bioactive chemicals from the SANBI $CHCl_3$ fraction, with silica gel serving as the stationary phase. A uniform slurry made from about 1000 g of silica gel (Merck) and *n*-hexane was then loaded into a glass column with the dimensions of 40 cm in height and 4.5 cm in diameter. The dried $CHCl_3$ fraction (36.47 g) was combined with 50 g of silica gel and dissolved in 100 mL of acetone before drying for approximately 2-3 hours at room temperature. The column bed was then overlaid with the dried $CHCl_3$ fraction. *n*-Hexane with ethyl acetate (EtOAc) was added in increasing percentages (5%) to 100% to yield various fractions that were collected.

After the fractions had dried, white precipitates were observed in some honey jars from 70% to 60% *n*-hexane fractions and were then washed with acetone to remove impurities and yielded 50 mg of pure white crystals of obliquumol.

By constantly purifying the dried fractions collected at *n*-hexane concentrations ranging from 95 to 85% with EtOAc, a 140 mg combination of a mixture of lupeol and β-amyrin was obtained.

Fractions collected from 30-5% hexane were combined since they contained similar compounds based on TLC finger printing. The 1.2 g yield from the combined fractions from the first column was dissolved in acetone, combined with 1 g of silica gel, and allowed to dry at room temperature. The sample was then deposited into a silica gel (0.063-0.200 mm) bed with dimensions of 40 cm in height by 2.5 cm in diameter, and it was eluted using a solution of 70% to 30% EtOAc and *n*-hexane. About 50 mg of *O*-methylalloptaeroxylin was then isolated. Compound 4 was isolated by eluting a solution of 85% to 15% EtOAc and *n*-hexane while compound 5 was obtained by eluting a solution of 90% to 10% EtOAc and *n*-hexane, however the compounds were isolated in small amounts of 10 mg and 15 mg respectively. Nuclear Magnetic Resonance (NMR) spectroscopy and liquid chromatography-mass spectrometry (LC-MS) were used to analyse the samples and the structures of the isolated compounds were elucidated. The 1 dimensional (1D) NMR (^{1}H, ^{13}C, and dept-135) and 2 dimensional (2D) NMR (COSY, HMBC, HSQC, and NOESY) spectra were used. The data was also compared with literature to conclusively interpret the structures.

3.2.4 *In vitro* cytotoxicity assay

3.2.4.1. Cell Cultures

Human liver hepatocarcinoma cells (HepG2*)*, human breast adenocarcinoma cells (MCF-7), lung adenocarcinoma (A549), human cervical cancer cells (HeLa) and African green monkey kidney cells (Vero) were cultured in Dulbecco's Modified Eagle's Medium (DMEM) and Minimum Essential Medium (MEM), supplemented with 10% foetal bovine serum (FBS) and 1% gentamicin solution. The cells were grown in 5% CO_2 at 37ºC in a humidified atmosphere.

3.2.4.2. The 3-(4,5-dimethyltetrazolium bromide) (MTT) reduction assay

Cytotoxicity was evaluated using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) reduction assay described by (Mosmann, 1983) with slight modifications. Cells were seeded at a density of 1 x 10^5 cells/mL (100 µl) in 96-

well microtitre plates and incubated at 37ºC in a 5% CO_2 humidified incubator for 24 h to attach. After the incubation period, 100 µl of each sample were added to the wells containing cells. Doxorubicin was used as a positive control. Negative controls with equivalent concentrations of the solvents were also included, and the plates were further incubated for 48 h in a CO_2 incubator. Thereafter, the medium in each well was aspirated from the cells which were then washed with PBS. Finally, fresh medium (200 µl) was added to all the wells, and 30 µl of MTT (5 mg/mL in PBS) was added to each well and the microtitre plates were incubated at 37ºC for 4 h. Following 4 h incubation, the medium was aspirated from the wells, and 50 µl of DMSO added to solubilize the formed formazan crystals. The absorbance was measured on a BioTek Synergy microplate reader at 570 nm and reference wavelength of 630 nm. The IC_{50} values were calculated as the concentration of the tested samples resulting in a 50% reduction of absorbance compared to untreated cells. The relative safety of each sample was assessed using the selectivity index, which was calculated as follows:

% viability = (absorbance of sample treated cells/absorbance of control cells) x100.

All experiments were performed in triplicate and mean values were calculated. Spectrophotometric determinations were performed using Quant Universal Microplate Spectrophotometer (Bio-Tek, Instruments Inc, USA).

3.2.5 Selectivity Index (SI)

The selectivity index (SI) indicates the degree of cytotoxic selectivity of tested sample against cancer cells versus normal cells (Vero) and was calculated dividing the IC_{50} of the tested sample in normal cells by the IC_{50} of cancer cells.

The SI values were calculated applying the formula: SI = IC_{50} normal cell/IC_{50} cancer cell

3.2.6 Morphological Study

Morphological alteration of HepG2 and HeLa cell lines after exposure to test substances was assessed under the microscope. The cells were seeded at a density of 1 x 10^5 cells/mL in 5 mL medium in a 25 cm^3 flask. After 24 h, the medium was removed and replaced with new medium. Thereafter, HepG2, and HeLa cell lines were treated with 100 µg/mL, 50 µg/mL and 25 µg/mL of the acetone leaf extracts for

24 h. After the treatment, the images were captured at 100x magnification, using a phase contrast inverted microscope (Nikon Eclipse Ti Optical Co., Ltd., Tokyo, Japan). Doxorubicin (12 ug/mL) was used as the positive control while the untreated cells were the negative control. The effect of *P. obliquum* acetone leaf extracts from two different geographic locations on morphological changes of HepG2 and HeLa cells was assessed and photographed.

3.2.7 Antioxidant activity of *P. obliquum* extracts and fractions

3.2.7.1. Quantitative 1,1-diphenyl- 2-picrylhydrazyl (DPPH) free radical-scavenging method.

The effects that the extracts and fractions had on the DPPH radical were determined using a DPPH radical scavenging assay as described by (Mensor et al., 2001) with slight modifications. The extracts and fractions were re-dissolved to a concentration of 10 mg/mL in methanol. First, the DPPH solution's optical density (OD) was calibrated at 517 nm to a range of 0.9 to 1.00. Then, 160 µg/mL of the DPPH solution were added to 40 µg/mL of various crude extracts and fractions at various concentrations (3.125-200 µg/mL). Using a microplate reader, the mixture was incubated in the dark for 30 min to measure the absorbance at 517 nm (BioTech Epoch spectrophotometer). Higher free radical scavenging activity was shown by the solution's lower absorbance. Ascorbic acid and trolox were used as positive controls, with methanol serving as the negative control. The experiment was repeated three times. The percentage inhibition was calculated as:

$$\%\ inhibition = 100 - \left(\left(\frac{\text{Sample} - \text{Control}}{\text{DPPH}}\right) \times 100\right)$$

The IC_{50} is the concentration of the sample that can inhibit 50% of the radicals in the DPPH. The lower the IC_{50} value of the samples, the more effective is the antioxidant activity (Mensor et al., 2001).

3.2.7.2. ABTS free radical-scavenging method

The 2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonate) radical cation (ABTS+) assay was carried out on all the extracts and fractions. The ABTS stock solution was made by combining a 2.45 mM potassium persulfate solution with a 7 mM ABTS in

methanol solution, and letting the combination sit at room temperature in a dark area for 12 to 16 hours (Ozgen et al., 2006). The extracts and fractions were re-suspended in methanol to a concentration of 10 mg/mL. A volume of 40 µl of the samples were diluted to 50% with methanol and then serially diluted in a 96-well microplate. The absorbance of the stock ABTS solution was measured using a spectrophotometer at 734 nm to an absorbance of 0.7-1.0, then 160 µl was added to all the wells of the microplate. The microplates were then incubated in the dark at room temperature for 6 min, and then the absorbance was measured. Methanol was used a negative control. The percentage of inhibition and the IC_{50} was determined following the same methods as described in section 2.6.1.

3.3 Results and discussion

3.3.1 Structures of the isolated compounds

The isolated compounds were identified as obliquumol (12-*O*-acetylptaeroxylinol or ptaeroxylinol acetate) (Agostinho et al., 2013, Malefo et al., 2020), mixture of lupeol and a minor triterpenoid, possibly β-amyrin (Chen et al., 2017), and *O*-methylalloptaeroxylin (Okorie, 1982, Kumar et al., 1982). The structures were assigned on the basis of mass spectrometry and NMR spectroscopy data, which were in agreement with those already reported for the compounds (Ahluwalia et al., 1982, Malefo et al., 2020). Several studies on *O*-methylalloptaeroxylin report ^{1}H NMR data only (Ahluwalia et al., 1982, Okorie, 1982). Therefore, the ^{1}H and ^{13}C NMR data of *O*-methylalloptaeroxylin is provided in Table 1. The assignment was based on 1D and 2D NMR data. The ^{1}H NMR spectrum showed two one-proton singlets at δ_H 5.99 (H-3) and 6.26 (H-6), and two three-proton singlets at δ_H 2.28 (CH_3-2) and 3.91 (OCH_3-5). The signals of the dimethylpyran unit appeared as two doublets at δ_H 6.69 (H-1') and 5.56 (H-2'), and a singlet integrating for six protons at δ_H 1.47 ((CH_3)$_2$-3'). The ^{13}C and dept-135 NMR spectra showed signals of the 5-methoxy and 2-methyl substituents at δ_C 56.3 and 19.7, respectively. Those of the chromone core appeared at δ_C 162.6 (C-2), 111.8 (CH-3), 177.6 (C=O, C-4), 108.4 (C-4a), 160.6 (C-5), 96.3 (CH-6), 157.6 (C-7), 102.3 (C-8), 154.2 (C-8a), and the signals of the dimethylpyran scaffold resonated at δ_C 115.2 (CH-1'), 127.3 (CH-2'), 77.9 (C-3') and 28.2 ((CH_3)$_2$-3'). The molecular formula of the compound was confirmed to be $C_{16}H_{16}O_4$ from the HRESIMS data, which had a protonated molecular ion peak at *m/z* 273.1140 (Cald for $C_{16}H_{17}O_4$, 273.1127).

3.3.2 Cytotoxicity

In categorizing cytotoxicity of plant extracts, the US National Cancer Institute Guidelines consider extracts to have notable *in vitro* anti-proliferative activity against cancer cells if 50% inhibitory concentration (IC_{50}) value is less than 20 µg/mL, extracts with IC_{50} ranging from 20 µg/mL to 50 µg/mL are considered moderately toxic while those from 50 µg/mL to 200 µg/mL are less toxic and IC_{50} above 200 µg/mL are non-toxic (Twilley et al., 2020). The acetone crude extracts were more cytotoxic than water extracts and had substantial antiproliferative activity with IC_{50} values ranging from 8 to 374 µg/mL (Table 1). Chloroform fractions were relatively non-toxic to Vero cells with IC_{50} values as high as 284 µg/mL and also had moderate toxicity with IC_{50} values of 33 µg/mL on HepG2 cells. Acetone extracts had better antiproliferative activity compared to aqueous extracts and fractions. Liver and cervical cancer cells had susceptibility against Hatfield and Walter Sisulu plant acetone extracts while breast cancer cells were susceptible against SANBI acetone extracts. However, SANBI acetone extracts were toxic against normal kidney cells which questions their safety. These results also corroborated previous studies conducted on Vero cells (Ramadwa et al., 2021). The aqueous extracts had lower cytotoxic activity against normal cell lines tested. Similar findings were seen in other studies, which is encouraging because traditional medicine made from plants is typically prepared as decoctions, infusions, and tinctures made primarily from water (Benzie and Wachtel-Galor, 2011). However, it is important to note that all the aqueous extracts tested in the study were also not toxic to all the cancer cell lines tested in the study. The three isolated bioactive compounds were not toxic to the normal cells and the cancer cell lines tested in the study. Therefore, the isolated compounds from the non-polar fraction appear to not be those responsible for the low toxicity observed in both the acetone extracts and the chloroform fraction from which all the compounds were isolated.

The main objective of cancer therapy is to use compounds that can specifically target cancer cells without toxicity against normal cells. Thus the selective toxicity of extracts, fractions or compounds against cancer cells must be considered during discovery of leads for cancer treatment (Wong et al., 2012). We therefore determined if *P. obliquum* acetone extracts, fractions and isolated compounds had selective activity to cancer cells. Tested samples with SI > 2 were considered to

have selective toxicity against tested cancer cell line (Ondo et al., 2012, Artun et al., 2017). Acetone extract results also had selective cytotoxic activity against HepG2 and HeLa cancer cell lines with selective index values as high as 14 (Table 2). The Hatfield acetone extract had the highest cytotoxic activity with IC_{50} of 8.4 µg/mL against HepG2 cells, and had the highest SI value of 14, which means that the extracts was approximately 7 times more toxic to cancer cells than normal Vero cells.

The plant extracts which had significant activity against the tested cancer cells were prepared from organic solvent (acetone) and similar results have been observed in other scientific studies where organic solvent extracts were found to possess more antiproliferative activity than aqueous extracts (Ogbole et al., 2017, Juckmeta et al., 2019). The type of solvent used for extraction clearly plays a crucial role as it determines the class and polarity of compounds which may be isolated. The extractant used influences the biological activity of *P. obliquum* extract, particularly against the normal and cancer cell lines used in the study.

Plants produce a vast range of secondary metabolites, typically as a coping mechanism against attacks from microbes, insects, viruses, herbivores, and other plants (Ramadwa et al., 2021). Due to seasonal shifts and geographic location, a plant's chemical composition can alter over time (Mukeshwar et al., 2011). Geographical location appears to have affected the antiproliferative activity and perhaps phytochemical composition of *P. obliquum*. It was interesting to note that SANBI acetone leaf extracts had some toxicity against normal cell lines tested while other acetone leaf extracts collected from different geographical locations were all less toxic. It is likely that the plant leaf material collected from SANBI had a higher concentration of toxic compounds compared to plant material collected from other different geographical locations. Moreover, two acetone extracts from Hatfield and Walter Sisulu, which had the best anticancer activity against HepG2 and HeLa cells, were less toxic to the normal cell lines. This further indicates that there was a difference in the phytochemical concentrations from this plant species based on the geographical location.

Table 3.1. Cytotoxicity (IC_{50} in µg/mL) and selectivity index (SI) of the extracts, fractions and isolated compounds from *P. obliquum.*

Extracts	IC_{50} (µg/mL)								
	MCF7	**SI**	**HEPG2**	**SI**	**A549**	**SI**	**HELA**	**SI**	**VERO**
WALTER SISULU									
Acetone	197.3 ± 26.5	0.6	**14.5 ± 0.2**	**8.6**	147.4 ± 9.6	0.8	87.2 ± 9.6	1.4	126.1 ± 4.5
H_2O (cold)	487.8 ± 11.9	0.9	832.1 ± 42.1	0.5	353.1 ± 59.5	1.3	946.6 ± 104.9	0.5	449.5 ± 0,8
H_2O (hot)	418.7 ± 175.4	0.5	455.8 ± 24.1	0.5	830 ± 60.9	0.3	911.6 ± 56.6	0.2	214.3 ± 15.1
UP HATFIELD									
Acetone	194.7 ± 27.2	0.6	**8.6 ± 0.8**	**14.2**	64.1 ± 20.4	1.9	**34.8 ± 6.9**	**3.5**	122.1 ± 6.1
H_2O (cold)	>1000	0.3	754.6 ± 22.2	0.7	>1000	0.2	>1000	0.4	535.3 ± 20.5
H_2O (hot)	666.7 ± 109.6	1.5	372.2 ± 8.3	2.74	490.8 ± 117.1	2.1	>1000	1	>1000
SANBI									
Acetone	23.3 ± 6.6	0.7	85.8 ± 6.8	0.2	166.9 ± 20.6	0.0	99.6 ± 4.9	0.2	**16.1 ± 0.7**
H_2O (cold)	764.1 ± 18.9	0.6	>1000	0.5	188.7 ± 12.3	2.6	820.4 ± 169.8	0.6	485.9 ± 121.9
H_2O (hot)	>1000	0.4	607 ± 146.9	0.8	>1000	0.2	694.5 ± 61	0.7	464.4 ± 90.3
$CHCl_3$ fraction	357.6 ± 11.9	0.0	213.3 ± 18.8	0.1	129.4 ± 25.4	0.2	67.2 ± 5.6	0.5	32.6 ± 3.1
Hexane fraction	167.5 ± 34.6	0.2	295.4 ± 18.1	0.1	250.9 ± 34.9	0.1	971.6 ± 81.3	0.0	37.8 ± 4.2
NELSPRUIT									
Acetone	269.8 ± 33.2	0.4	248.4 ± 38.9	0.40	374.7 ± 8.4	0.27	>1000	0.07	100.3 ± 0.8
H_2O (cold)	>1000	0.7	246 ± 4.6	3.91	961.5 ± 137.1	1.00	>1000	0.71	961.5 ± 19.2
H_2O (hot)	658 ± 162.1	0.5	550.9 ± 70.4	0.59	136.6 ± 17.8	2.4	>1000	0.18	322.5 ± 85.9
$CHCl_3$ fraction	284.2 ± 38.4	1.0	**33.5 ± 3**	**8.5**	218.9 ± 9	1.3	824.5 ± 139.1	0.34	284.2 ± 68.1
Hexane fraction	189.2 ± 12.8	1.1	312.4 ± 16.7	0.7	180.4 ± 33.4	1.1	153.1 ± 2	1.3	203.2 ± 2.5
Obliquumol	454.2 ± 57	0.7	52.7 ± 4.8	6	192.7 ± 1.6	1.6	188.5 ± 1.6	1.7	314.8 ± 24.1
Lupeol & β-amyrin	167.8 ± 6.7	0.7	122.6 ± 1.8	1	247.1 ± 49.1	0.5	247.1 ± 2.7	0.5	122.6 ± 5.5
O-methylalloptaeroxylin	248.2 ± 0.1	0.6	364.4 ± 15.7	0.4	279.8 ± 57.6	0.5	212.7 ± 1.8	0.7	151.5 ± 38.7
Doxorubicin	0.18 ± 0.01	55	2.73 ± 0,36	3.6	1,6 ± 0.04	6.3	1,6 ± 0,07	6.3	9,9 ± 1,3

3.3.3. Morphology of HepG2 and HeLa cells

Figures 1 and 2 show modifications in the morphology of HepG2 and HeLa cells caused by *P. obliquum* acetone leaf extracts. Generally, both cancer cells were in a scattered pattern, most of the cells were dead and appeared as floating, rounded cells, as compared to the adherent spindle-shaped live cells. As expected, a concentration-dependent effect was observed in the morphology of both cell lines tested in the study. Significant cell death and morphological alterations were observed more on HepG2 cells as compared to HeLa cells after 48 hours treatment with 10-25 µg/mL Hatfield and Walter Sisulu acetone extracts. As compared to the control, both the HepG2 and HeLa cells lost their typical shape and morphology, became rounded and lost their adherence capacity after the exposure of 10-25 µg/mL of the *P. obliquum* acetone leaf extracts. It is evident that at the tested concentrations, the acetone leaf extracts of *P. obliquum* are effectively cytotoxic and alters the cell morphology of HepG2 and HeLa cells.

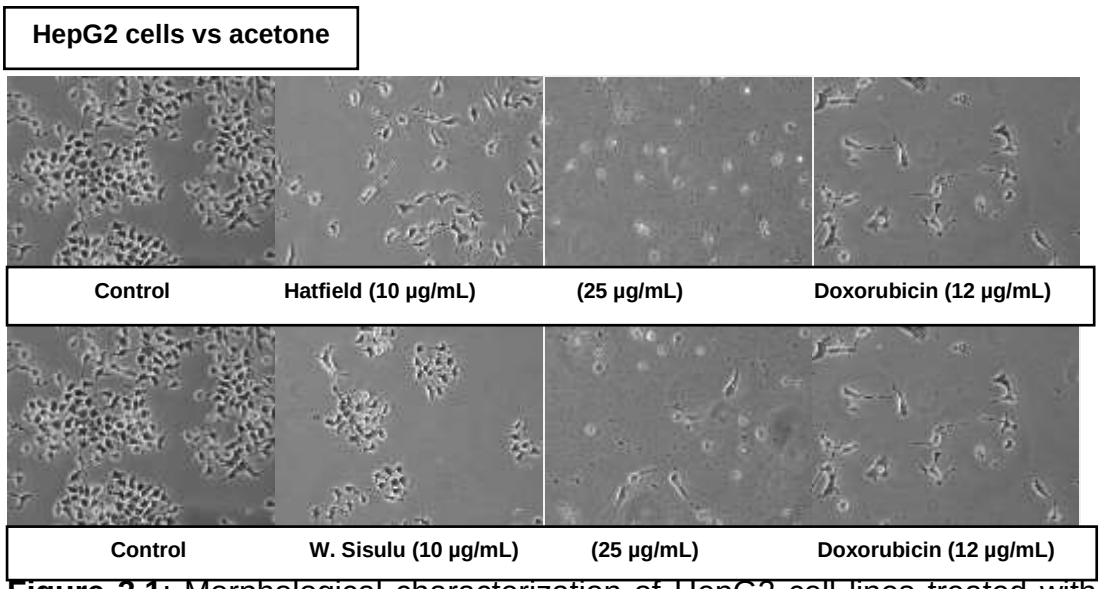


Figure 3.1: Morphological characterization of HepG2 cell lines treated with 10 and 25 µg/mL of Hatfield and Walter Sisulu acetone crude extracts, and 12 µg/mL doxorubicin

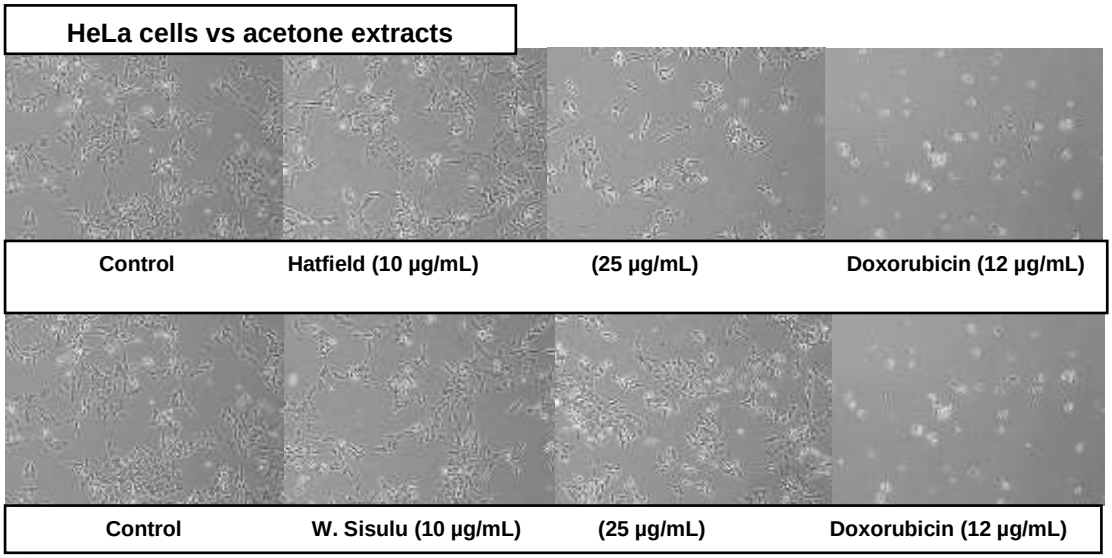


Figure 3.2: Morphological characterization of HeLa cell line treated with 10 and 25 µg/mL of Hatfield and Walter Sisulu acetone crude extracts, and 12 µg/mL doxorubicin for 48 h.

3.3.4. Antioxidant activity of *P. obliquum* extracts and fractions

A molecule or atom that has one or more unpaired electrons and may exist on its own is referred to as a free radical. A few examples of free radicals are the hydroxyl radical, superoxide radical anion, lipid peroxyl, lipid peroxide, and lipid alkoxyl. Radical derivatives like singlet oxygen and hydrogen peroxide are known as reactive

oxygen species (ROS) (Poyton et al., 2009). The initial line of defense against oxidative stress and damage brought on by free radicals is comprised of antioxidant enzymes. There is a possibility that a disease like cancer could emerge when there is an imbalance between oxidative stress and antioxidant enzymes (Waris and Ahsan, 2006). The antioxidant potential of the extracts and fractions from *P obliquum* collected from different geographical locations was determined using DPPH and ABTS assays as shown in Table 3.2 These assays are among the most widely used radical scavenging assays methods because they are rapid, repeatable, and affordable. They are used to evaluate various natural products and functional food products (Nkadimeng et al., 2020). It has been proposed that the radical scavenging activity of extracts with IC_{50} < 100 µg/mL has good antioxidant potential while the ones with IC_{50} < 50 µg/mL are considered potent antioxidant agents (Phongpaichit et al., 2007). The aqueous extract was the most active extract with the lowest IC_{50} of 21.5 µg/mL on ABTS. The aqueous extract had better scavenging activity against ABTS. Generally, the acetone extract and the two non-polar fractions had less scavenging activity in all the antioxidant methods used. Oxidative stress plays a role in various clinical conditions including cancer. However, given that the acetone extracts of *P. obliquum* have extremely little antioxidant activity in the methods utilized in this investigation, it indicates that the antiproliferative activity exerted by these extracts may not have been caused by oxidative stress.

Table 3.2: Antioxidant activities (IC_{50} µg/mL) of the *P. obliquum* acetone extracts, aqueous extracts, and fractions

	DPPH	ABTS
Extracts	IC_{50} µg/mL	
WALTER		
SISULU	269.1 ± 4.6	251.2 ± 50
Acetone		
H_2O (cold)	138.3 ±17.5	**37.5 ± 10**
H_2O (hot)	**43.4 ± 6.1**	**21.5 ± 0.2**
UP HATFIELD		
Acetone	150.6 ± 12	178.4 ± 17
H_2O (cold)	140 ± 9.3	86.1 ± 1.5
H_2O (hot)	85.4 ± 6.6	59 ± 0.2
SANBI		
Acetone	275.5 ± 8.9	318.1 ±19.2
H_2O (cold)	75.7 ± 2.5	**43 ± 1.6**
H_2O (hot)	**46.1 ± 9.5**	**29.1 ± 0**
$CHCl_3$ fraction	423.5 ±54.3	240.4 ±28.8
Hexane fraction	418.5 ± 9.6	143.7 ± 3.3
NELSPRUIT		
Acetone	333.2 ±24.9	268 ± 29.9
H_2O (cold)	62.1 ± 6.1	**36.6 ± 0.6**
H_2O (hot)	83.1 ± 3.2	56.4 ± 4.4
$CHCl_3$ fraction	387.4 ±27.3	214.2 ±13.1
Hexane fraction	236.5 ±42.1	180.2 ± 2.7
Trolox	2.4 ± 0.8	1.6 ± 0.0
Ascorbic Acid	2.6 ± 0.2	1.4± 0.2

3.4 Conclusion

Since there is growing evidence that some phytochemicals may have anticancer properties, plants present a useful source of potential alternative anticancer treatments. Many phytochemicals have significant anticancer potential and could be

used in drug development. Traditional medicinal plants have found favour in modern medicine as possible sources for new therapeutic medications.

Natural products have historically led to the discovery of numerous innovative anticancer medications. Many of these products have demonstrated preliminary anticancer action *in vitro*, as evidenced by their ability to be cytotoxic or antiproliferative, as well as by their impact on mechanisms involved in cancer cell growth. The major goals of studying crude plant extracts are to either isolate bioactive compounds for use directly as medications or to find bioactive compounds that can be utilized as lead ingredients in the development of semi-synthetic pharmaceuticals, thus it is imperative to isolate the cytotoxic compounds in *P. obliquum*. By collaborating with mainstream chemotherapeutic medications, these cytotoxic natural compounds may be able to significantly contribute to the treatment of some malignancies by increasing their efficacy or decreasing their toxicity. Antiproliferative agents such as *P. obliquum* acetone extracts that can induce selective cytotoxicity against cancer cell lines, without causing much harm to normal cells, are highly desirable for therapeutic purposes and may be considered in the development of novel cancer chemotherapeutic drugs.

Postscript

In the next chapter the anti-inflammatory activities of *P. obliquum* leaf extracts, fractions and isolated compounds will be investigated. This is because *P. obliquum* is also used traditionally to treat various inflammatory diseases or inflammatory related symptoms such as arthritis, rheumatism, fever and headaches.

Chapter 4

The activity of *Ptaeroxylon obliquum* extracts, fractions and bioactive compounds as potential inhibitors of arachidonic acid metabolic pathways in the inflammation mechanism.

Preface

Ptaeroxylon obliquum extracts are used traditionally to treat inflammatory diseases and inflammatory related symptoms. Consequently, the anti-inflammatory activities of the crude extracts, fractions and isolated compounds are investigated in this chapter.

This chapter has been submitted as a manuscript to the Journal of Ethnopharmacology and was formatted in accordance with the instructions provided to authors by the journal.

Abstract

Ethnopharmacological relevance: *Ptaeroxylon obliquum* (Thunb.) Radlk is traditionally used to treat inflammation and related symptoms such as arthritis, rheumatism, fever, pain and headache. Extracts and isolated compounds from tree leaves have many positive activities and low cytotoxicity in several indications leading to patents in Europe and the USA. There is no comprehensive study on the anti-inflammatory activity on *P. obliquum* through different inflammatory mechanisms.

Aim of the study: The aim of the study was to determine anti-inflammatory activities by investigating different potential mechanisms of action of the crude extracts, fractions and isolated compounds from *P. obliquum* leaves from different localities in South Africa.

Materials and Methods: The anti-inflammatory activity of *P. obliquum* acetone leaf extracts, aqueous extracts (hot and cold), fractions and isolated compounds

(obliquumol, O-methylalloptaeroxylin and a mixture of lupeol and β-amyrin) was determined by investigating the following: the nitric oxide (NO) inhibition assay in lipopolysaccharide (LPS)-induced RAW 264.7 macrophages, the soybean 15-lipoxygenase (15-LOX) inhibitory assay, as well as cyclooxygenase (COX)-2 enzyme activity using an ELISA method. The effects of the extracts on the pro-inflammatory cytokines interleukin (IL)-1*β* and TNF-*α,* as well as the anti-inflammatory cytokine (IL-10) production, were also determined using ELISA assays.

Results: All acetone extracts demonstrated greater 15-LOX inhibitory activity compared to aqueous extracts and the positive control, with IC_{50} values ranging from 5.7 to 10.4 µg/mL. Obliquumol had the highest level of 15-LOX inhibition with an IC_{50} of 3.66 g/mL, which was twice as high as quercetin (positive control). At the tested concentration, the isolated compounds, fractions, and extracts from *P. obliquum* all inhibited NO production in a dose-dependent manner. The acetone extracts reduced nitric oxide with percentages as high as 95% and 102% on Walter Sisulu and Nelspruit acetone extracts, respectively, at the lowest tested concentration (1.6 µg/mL). The high proportion of NO was not caused by acetone extracts being toxic to the cells, as was demonstrated by cell viability of >100% RAW 264.7 macrophage cells. The extracts and the compounds reversed the LPS-induced COX-2 significantly ($p<0.001$) in a dose dependent manner in comparison with the positive control quercetin treatment. The LPS-induced pro-inflammation cytokines that were assessed in the study were reduced by the extracts and compounds. The Walter Sisulu acetone extract and obliquumol significantly inhibited the LPS-induced TNF- *α* levels ($p< 0.027$ and $p<0.008$, respectively).

Conclusion: The acetone extracts, fractions and isolated compounds of *P. obliquum* had good anti-inflammatory activity thus supporting its medicinal use against inflammation.

Key words: *Ptaeroxylon obliquum,* inflammation, cytokines, Interleukin, 15-Lipoxygenase, Nitric oxide, Cyclogenase 2, Lipopolysaccharides, Tumour necrosis factor

4.1 Introduction

Inflammation is a defence mechanism of the body's immune system against tissue damage, undesired external objects, or pathogens that enter tissue cells (Ribaldone et al., 2018). Prolonged or unregulated inflammation is linked with acute and chronic diseases including rheumatoid arthritis, cancer, heart disease and inflammatory bowel disease (Ramadwa et al., 2022, O'Byrne and Dalgleish, 2001). When foreign pathogens invade host tissue, native tissue cells produce a number of inflammatory mediators that activate different signalling pathways to release and attract leukocytes (Chen et al., 2018). The response is accompanied by an increase in the level of pro-inflammatory mediators including nitric oxide (NO), inducible nitric oxide synthase (iNOS), prostaglandins, cytokines including interleukin 1β (IL-1β), interleukin 6 (IL-6) and tumor necrosis factor alpha (TNF-α) and enzymes such as cyclooxygenase-2 (COX-2) and lipoxygenase (LOX) (Muniandy et al., 2018). Anti-inflammatory cytokines including IL-1 receptor antagonist (IL-1ra), IL-4, IL-6, IL-10, IL-11, and IL-13 are released to prevent the process by regulating pro-inflammatory cytokine responses (Boshtam et al., 2017). As one of the key cytokines that promotes inflammation, TNF-α plays a critical role in the pathophysiology of inflammatory diseases such as rheumatoid arthritis (Li et al., 2017).

COX-2 is an important pro-inflammatory mediator that promotes inflammation via the production of prostaglandins, which are known as molecules that play a key role in the propagation of inflammation (Nanjundaiah et al., 2016). Pro-inflammatory COX-2 is inducible by cytokines (IL-1, IL-6, and TNF-α) expressed in rheumatoid synovial endothelial cells, monocytes, and macrophages as well as the vascular endothelium ((Vane et al., 1998, Brune and Patrignani, 2015).

Lipoxygenases (LOX) are dioxygenases that also produce appropriate hydroperoxides like polyunsaturated fatty acids linoleic acid and arachidonic acid. LOX enzymes are expressed in immunological, epithelial, and tumour cells that exhibit a range of physiological functions, such as inflammation, rashes and carcinogenesis. Depending on where oxygen is inserted into the arachidonic acid, the lipoxygenase enzymes present in mammals are divided into three groups: 5, 12, and 15. (Schneider and Bucar, 2005). Humans express the enzymes in a tissue-

specific manner, with 15-LOX being mostly expressed in reticulocytes, eosinophils and microphages (Klil-Drori and Ariel, 2013). Several disorders associated with inflammation have been linked to 15-LOX's activity. Since 15-LOX's metabolites have been found to have both pro- and anti-inflammatory characteristics, there is growing evidence that 15-LOX plays a role in inflammation (Adebayo et al., 2015).

Numerous inflammatory mediators, such as prostaglandin and nitric oxide (NO), are produced by macrophages. NO is a crucial cellular signalling molecule that plays a role in several physiological processes in mammals, such as vasodilation, smooth muscle relaxation, neurotransmission and the immunological response (Keibel et al., 2009). Overproduction of NO may be a factor in immune-pathology of macrophage-dependent inflammation and degenerative illnesses, such as cancer. NO, a free radical, is created by a group of enzymes called nitric oxide synthases (NOSs). Inhibition of NO production is a promising therapeutic target in the development of potential anti-inflammatory agents (Ramadwa et al., 2022).

Numerous nonsteroidal anti-inflammatory medications have been demonstrated to lessen pain and inflammation by preventing isoforms of the cyclooxygenase enzyme from metabolizing arachidonic acid, which lowers the generation of prostaglandin. The use of nonsteroidal anti-inflammatory medicines, however, is accompanied with a number of negative effects. The African continent is incredibly rich in medicinal plants, many of which have anti-inflammatory properties and are used successfully in traditional medicine to treat inflammatory diseases. In southern Africa, over 500 medicinal plants are utilized in the treatment of inflammatory and pain conditions (Khumalo et al., 2022). Inflammation and the pain it causes, such as toothaches, headaches, backaches, rheumatism, oedema or swellings, generalized body pains, earaches, abdominal pains, arthritis, chest pains, internal body pains, haemorrhoids, labour pains, and rheumatic fever, are among the disorders treated (Khumalo et al., 2022).

Ptaeroxylon obliquum (Thunb.) Radlk (Rutaceae), commonly known as sneezewood, is used traditionally to manage a variety of ailments, including conditions and symptoms linked to inflammation such arthritis, rheumatism, fever, and headaches (Iwalewa et al., 2007, Moyo and Masika, 2009, Ribeiro et al., 2010 Ramadwa et al., 2021). Secondary metabolites produced by medicinal plants may be used as models

to create new anti-inflammatory drugs with high pharmacological activity and fewer adverse effects. The aim of the study was to determine the anti-inflammatory activities in terms of different mechanisms of action of the crude extracts, fractions and isolated compounds from *P. obliquum* leaves from different localities in South Africa.

4.2 Materials and Methods

4.2.1 Plant collection

P. obliquum leaves used in this study were collected during the summer of 2019 from trees growing at the Hatfield Campus (University of Pretoria), National Botanical Gardens of the South African National Biodiversity Institute (SANBI) (Brummeria, Pretoria), the Lowveld Botanic Gardens (Nelspruit, Mpumalanga) and Walter Sisulu National Botanical Garden (Roodepoort, Johannesburg). The leaves were gathered in nylon open weave bags, allowed to dry at room temperature in the shade, and then ground into powder. The powder was kept until needed in sealed containers in the dark. Some of the leaves were prepared as voucher specimens (PRU130509, PRU130510, PRU130511 and PRU130512) and were sent for identification and authentication at the HGWJ Schweickerdt Herbarium of the University of Pretoria.

4.2.2 Fractionation and isolation of bioactive compounds from *P. obliquum*

The plant fractionation procedure and isolation of bioactive compounds were carried out as previously described and led to isolation of obliquumol, lupeol and β-amyrin mixture and O-methylalloptaeroxylin from *P. obliquum* acetone leaf extract (Khunoana et al., 2022). The acetone crude extracts, the two fractions (hexane and chloroform fractions), and the isolated pure compounds were reconstituted in dimethyl sulphoxide (DMSO) for the anti-inflammatory assays.

4.2.3 The 15-lipoxygenase (15-LOX) inhibitory assay

The anti-inflammatory activity of extracts was evaluated *in vitro* via the soybean 15-lipoxygenase (15-LOX) (obtained from Sigma-Aldrich) inhibitory assay according to the method of Ondua et al. (2019). This was done in the presence of linoleic acid based on the formation of the complex Fe3+/xylenol orange (Sigma-Aldrich) with

absorption at 560 nm. Percentage inhibition of 15-lipoxygenase activity was determined and the 50% inhibition concentrations (IC_{50}) were determined using the non-linear regression curve of the 15-LOX percentage inhibition against the logarithm of concentrations tested (Ondua et al., 2019). LOX activity was determined using a spectrophotometric assay according to (Del Carmen Pinto et al., 2007) with slight modifications. The substrate linoleic acid (Sigma-Aldrich, Burlington, MA, United States) (final concentration, 140 µM) was prepared in Tris-HCl buffer (50 mM, pH 7.4). All crude extracts and fractions (10 mg/mL) were prepared in 100% DMSO and further diluted to 2 mg/mL in Tris-HCl buffer, except the water extracts which were directly prepared at 2 mg/mL in the Tris-HCl buffer.

Except for the water extracts, which were prepared directly at 2 mg/mL in the Tris-HCl buffer, all crude extracts and fractions (10 mg/mL) were prepared in 100% DMSO and further diluted to 2 mg/mL in Tris-HCl buffer. Exactly 20 µL of various concentrations (100-0.78 g/mL) of test samples or quercetin (positive control) were combined with 40 µL of 15-LOX, diluted in ice-cold Tris-HCl buffer (final concentration, 0.2 U/mL), at 25 °C for 5 min. Linoleic acid (40 µL) was added to the mixture and was further incubated at 25 °C for 20 min in the dark. In order to terminate the experiment, 100 L of freshly made FOX reagent [iron (II) sulphate (100 M) in methanol/water (9:1), sulphuric acid (30 mM), and xylenol orange (100 M)] were added. The enzyme 15-LOX solution, Tris-HCl buffer, substrate, and FOX reagent made up the negative control, whereas the enzyme 15-LOX and buffer were present in the blanks but the substrate was introduced after the FOX reagent. As shown in formula (2) below, the lipoxygenase inhibitory activity was calculated by calculating the percentage of hydroperoxide generation inhibition from changes in absorbance values at 560 nm after 30 min at 25 °C.

$$15LOX\ inhibition\ (\%) = \frac{Absorbance\ (control) - Absorbance\ (sample)}{Absorbance\ (control)} \times 100$$

The 50% inhibitory concentrations (IC_{50}) were determined using the non-linear regression curve of the percentage (15-LOX) inhibition against the logarithm of concentrations tested

4.2.4 Inhibition of nitric oxide (NO) production

RAW 264.7 macrophages cells obtained from the American Type Culture Collection (ATCC TIB-71™) (Rockville, MD, USA) were used. The cells were cultured in DMEM (Dulbecco's Modified Eagle's Medium) at 37°C with 5% CO_2, 4.5 g/L of glucose, and 4 mM of L-glutamine (HycloneTM), with 10% foetal calf serum (FCS) (Capricorn Scientific Gmbh, South America) and 1% penicillin/streptomycin/fungizone (PSF) added as supplements (PSF). RAW 264.7 cells were seeded in a 96-well microtitre plate using a cell suspension in the amount of 100 µl (2 x 10^6 cells/mL) and were then incubated overnight at 37°C with 5% CO_2 to facilitate adhesion. The RAW 264.7 cells were stimulated by incubation in a medium containing 5 µg/mL of lipopolysaccharide (LPS), treated simultaneously with various doses of the samples dissolved in DMSO, and then further diluted in culture media.

Nitrite oxide concentration in supernatant was measured using the Griess reagent after RAW 264.7 macrophages had been incubated for 24 h at 37°C and 5% CO2. In a new 96-well microtitre plate, 100 µL of cell supernatant from each well was transferred, and the same volume of Griess reagent (Sigma-Aldrich, Burlington, MA, United States) was added (Adebayo et al., 2015). Serial dilutions of sodium nitrite (0-50 µM) were used to calculate the concentrations of the nitrites from the standard curve. Based on how well extracts, fractions or chemicals prevented RAW 264.7 macrophages from producing nitric oxide in comparison to the control (cells treated with LPS without samples), the percentage of NO inhibition was calculated.

4.2.5 Cell viability

The cytotoxicity of crude extracts, fractions and isolated compounds was determined using the 3-(4,5-dimethythiazol- 2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay (Mosmann, 1983).

After removing the culture media from the wells, 200 µL of phosphate buffered saline (PBS) was used to wash the wells; then 200 µL of fresh culture medium, and 30 mL of MTT solution (5 mg/mL in PBS) were added to each well. The plates were then incubated at 37°C with 5% CO_2 for 4 h.

A suction pump (Integra, USA) was used to carefully aspirate the culture media after incubation, and 50 µL of dimethyl sulfoxide (DMSO) was then added to each well. By comparing the absorbance of the samples to the negative control (cells treated

exclusively with LPS were regarded as 100% viable), the percentage of cell viability was estimated.

4.2.6 Treatment of the cells for cyclooxygenase and cytokine detection

The cells were prepared and treated with the extracts and compounds according to (Nkadimeng et al., 2020) to determine their effects on the levels of cytokine and cyclooxygenase-2 activity. Briefly, the RAW 264.7 macrophage cells were plated at 1 × 10^6 cells per 25 cm^2 tissue culture flask (NEST, Whitehead scientific, Johannesburg, South Africa) and incubated for 24 h. Thereafter, the medium was removed and fresh media was added. The cells were stimulated with LPS at 1 µg/mL and treated with 50 and 100 µg/mL concentrations of the extracts and compounds. Quercetin was used as a positive control. The LPS-control cells were cells that were stimulated with LPS but not treated and were referred to as LPS cells in the study. Control cells were cells that were neither stimulated with LPS nor treated with extracts. The cells were exposed to LPS and treated over 24 h. After 24 h, medium was removed and stored at −80°C until the day of analysis.

4.2.7 Treatment of the cells for cyclooxygenase and cytokine detection

The cells were prepared and treated with the extracts and compounds according to (Nkadimeng et al., 2020) to determine their effects on the levels of cytokine and cyclooxygenase-2 activity. Briefly, the RAW 254.7 macrophage cells were plated at 1 × 106 cells per 25 cm2 tissue culture flask (NEST, Whitehead scientific, Johannesburg, South Africa) and incubated for 24 h. Thereafter, the medium was removed and fresh media was added. The cells were stimulated with LPS at 1 µg/mL and treated with 50 and 100 µg/mL concentrations of the extracts and compounds. Quercetin was used as a positive control. The LPS-control cells were cells that were stimulated with LPS but not treated and were referred to as LPS cells in the study. Control cells were cells that were neither stimulated with LPS nor treated with extracts. The cells were exposed to LPS and treated over 24 h. After 24 h, medium was removed and stored at −80°C until the day of analysis of COX-2 activity measurements**.**

The human PTGS2/COX-2 Prostaglandin endoperoxide synthase 2 (PGE2) ELISA kit E-EL-H1846 (Elabscience, Biocom Africa, Johannesburg South Africa) was used to evaluate the effects of extracts on COX-2 levels. The methods were applied in

accordance with the manufacturer's manual protocol. The absorbance correlated closely with the levels of PTGS2/COX-2 in the sample medium. From the standard curve, concentrations of human COX-2 in the cell culture media samples were determined.

4.2.8 Cytokine activity measurements

Using the human ELISA kits E-EL-M0049, E-EL-M0037 and E-EL-M0046 (Elabscience, Biocom Africa), the effects of the extracts, fractions and isolated compounds on TNF-α, IL1 β, IL6 and IL10 concentrations were identified and quantified. The assay was done according to manufacturer's instructions. Standard curves were used to calculate the concentration

4.2.9 Statistical analysis

Results are expressed as mean ± standard deviations, and statistically significant values were compared using one-way ANOVA analysis of variance using an interactive statistical program (Sigmastat, SPSS version 26, San Jose, CA, USA) and pairwise multiple comparison procedures using Holm—Sidak method. The results are presented as mean standard deviations, and statistically significant values were compared using one-way ANOVA analysis of variance performed using an interactive statistical program and pairwise multiple comparison techniques utilizing the Holm-Sidak method. Normality testing was done using Shapiro-Wilk and equal variance test using Brown-Forsythe. The p-value of ≤0.050 was considered statistically significant.

4.3 Results and discussion

4.3.1 Lipoxygenase inhibitory activity of the acetone and aqueous extracts, fractions, and isolated compounds from *P. obliquum*

In this study, the potential inhibition of the 15-LOX activity by *P. obliquum* extracts was investigated. This enzyme is essential for the biosynthesis of leukotrienes, which are involved in the pathophysiology of many inflammatory illnesses. All the acetone extracts had good 15-LOX inhibitory activity compared to aqueous extracts and the positive control with IC_{50} values ranging from 5.7-10.4 µg/mL (Table 4.1). Acetone extracts from SANBI had the best 15-LOX inhibitory activity of the extracts collected from different localities with IC_{50} of 5.4 µg/mL.

Although all the water extracts had poor activity, it was interesting to note that cold water extracts had better anti-inflammatory activity than hot water extracts. Both hot and cold water extraction methods were used since traditional healers use water as an extractant.

The two non-polar fractions tested had IC_{50} values ranging from 10.87-22.72 µg/mL which were comparable with acetone extract activity. Furthermore, the chloroform and hexane fractions indicated that the compounds responsible for the good activity in the acetone extract are the non-polar compounds. Obliquumol had the best 15-LOX inhibitory activity with IC_{50} as low as 3.66 µg/mL which was two times better activity than that of the positive control quercetin. Other tested compounds, O-methylalloptaeroxylin and the mixture of lupeol and β-amyrin had some anti-inflammatory activity against 15-LOX with IC_{50} of 18.30 µg/mL and 11.30 µg/mL, respectively.

Table 4.1. Inhibition of 15-LOX (IC_{50} in µg/mL) by crude extracts, fractions and isolated compounds from *P. obliquum*

Samples	**15-LOX IC_{50} (µg/ml)**
WALTER SISULU	
Acetone	**9.71 ± 0.99**
H_2O Cold	189.78 ± 5.42
H_2O Hot	226.15 ± 9.7
UP HATFIELD	
Acetone	**8.60 ± 0.89**
H_2O Cold	127.61 ± 4.04
H_2O Hot	259.06 ± 10.14
SANBI BRUMMERIA	
Acetone	**5.70 ± 1.12**
H_2O Cold	114.01 ± 6.65
H_2O Hot	238.61 ± 18.74
$CHCl_3$ fraction	**14.35 ± 2.33**
Hexane fraction	**21.57 ± 1.63**
NELSPRUIT	
Acetone	**10.44 ± 1.92**
H_2O Cold	185.74 ± 11.13
H_2O Hot	328.78 ± 12.48
$CHCl_3$ fraction	**10.87 ± 1.35**
Hexane fraction	22.72 ± 0,44
Obliquumol	**3.66 ± 0.15**
Lupeol and β-amyrin	**11.30 ± 3.85**
O- Methylalloptaeroxylin	**18.30 ± 0.66**
Quercetin	**10.08 0,64**

4.3.2 Inhibition of nitric oxide production from LPS-induced RAW 264.7 cells by the crude extracts, fractions and isolated compounds from *P. obliquum*

In the LPS-activated RAW 264.7 macrophage cell line, the ability of *P obliquum* crude extracts, fractions and isolated compounds to suppress NO generation was tested (Table 4.2). To illustrate the efficacy of the assays, untreated cells were used as the negative control, LPS-stimulated cells as the positive control, and additionally, a cell group was utilized as the reduction control group with LPS-stimulated cells, co-incubated with quercetin, which is used as an inhibitor of NO. All *P. obliquum* isolated compounds, fractions and extracts had dose dependent inhibition of NO production at the range of concentrations tested. At the lowest tested concentration (1.6 µg/mL), the crude extracts were able to suppress NO with percentages as high as 95% and 102% for Walter Sisulu and Nelspruit acetone extracts, respectively.

The high proportion of NO was not caused by acetone extracts being toxic to the cells, as was demonstrated by the excellent cell viability of >100% on RAW 264.7 macrophage cells. Aqueous extracts from all the different localities had low NO inhibition with an average of 59% nitric oxide inhibition. Both hexane and chloroform fractions were able to inhibit nitric oxide production at the lowest concentration tested with percentage of inhibition ranging from 64.7-79% and cell viability of >100%. Obliquumol inhibited >80% nitric oxide production at the lowest concentration tested. A mixture of lupeol and β-amyrin had notable NO inhibition of >70% with cell viability of above 100%. Blocking or lowering iNO synthesis is significant in anti-inflammatory research since it is a critical macrophage-derived inflammatory mediator involved in the emergence of several inflammation disorders (Muniandy et al., 2018). By reducing iNO generation, the extracts, fractions, and isolated substances have potential therapeutic benefit in prevention ot treatment of pathological inflammation.

Table 4.2. Inhibitory effects of the acetone and aqueous extracts, fractions and isolated compounds from *P. obliquum* on NO production in LPS-induced RAW 264.7 cells and their viability against macrophage RAW 264.7 cells.

Samples	Conc (µg/mL)	% NO inhibition	Cell viability (%)
WALTER SISULU			
Acetone	1.6	**95.5 ± 3.1**	**135.4 ± 1.9**
	12.5	99.6 ± 3.3	124.4 ± 0.1
	50	108.9 ± 5.6	114.5 ± 1.5
	100	111.1 ± 6.3	105 ± 2.4
H_2O (hot)	1.6	29.6 ± 1.8	139.4 ± 6.2
	12.5	35.3 ± 2.9	130.4 ± 5.6
	50	46.7 ± 5.2	119 ± 5.1
	100	65.4 ± 7.8	108.3 ± 4.4
UP HATFIELD			
Acetone	1.6	**62.7 ± 2.3**	**149.2 ± 6.1**
	12.5	100 ± 5.4	132.5 ± 2.9
	50	107 ± 5.2	112.2 ± 6.6
	100	112.2 ± 11.2	90.2 ± 5
H_2O (hot)	1.6	20.7 ± 2.1	105.2 ± 3.7
	12.5	29.4 ± 5.5	92.9 ± 6.1
	50	36.3 ± 6.4	92.9 ± 13.4
	100	51.5 ± 9.1	83.9 ± 12.7
SANBI			
Acetone	1.6	**78.9 ± 25.1**	**125 ± 5.9**
	12.5	89.5 ± 12	112.2 ± 2.6
	50	109.8 ± 2.2	96 ± 15.1
	100	110 ± 4.0	84 ± 15.6
H_2O (hot)	1.6	23.4 ± 2.9	119.9 ± 8.6
	12.5	44.5 ± 4.0	113.3 ± 1.7
	50	51 ± 4.8	100.7 ± 13
	100	72.1 ± 7.7	86.1 ± 4.6
$CHCL_3$ fraction	1.6	79.1 ± 4.3	121.4 ± 2.22

	12.5	89.7 ± 4.8	108.9 ± 15.2
	50	101.5 ± 0.5	93.9 ± 15.4
	100	104.7 ± 1.9	70.8 ± 8.4
Hexane fraction	1.6	76 ± 3.8	115.2 ± 3.7
	12.5	89.8 ± 5.7	106.1 ± 2.7
	50	98.3 ± 12.9	99.5 ± 13.7
	100	104.7 ± 3	69 ± 4.6
NELSPRUIT			
Acetone	1.6	**102.8 ± 1.5**	**115.2 ± 3.7**
	12.5	109.4 ± 7.4	106.1 ± 2.7
	50	117.3 ± 13.6	99.5 ± 13.7
	100	105.6 ± 3.8	63.3 ± 10.6
H_2O (hot)	1.6	35.1 ± 10.9	117.1 ± 11.7
	12.5	46.2 ± 4.8	108.8 ± 5.5
	50	47.2 ± 6.5	106.1 ± 3.8
	100	51.7 ± 4.8	99.5 ± 0.9
$CHCL_3$ fraction	1.6	77.3 ± 4.4	126.3 ± 2.2
	12.5	80.2 ± 0.4	114.4 ± 0.1
	50	98.3 ± 2.3	101.1 ± 7.1
	100	101.6 ± 3.3	60.5 ± 10.1
Hexane fraction	1.6	64.7 ± 13.1	136.2 ± 9.2
	12.5	87.6 ± 6.7	136.1 ± 3.3
	50	102.8 ± 12.7	121.7 ± 5.9
	100	111.5 ± 3.6	78.9 ± 10.1
Obliquumol	1.6	82.1 ± 7.4	136.6 ± 11.2
	12.5	86.9 ± 1.7	127.9 ± 6.4
	50	93.5 ± 4.8	118.2 ± 1.0
	100	101.4 ± 1.6	98.6 ± 12.8
O- Methylalloptaeroxylin	1.6	18.8 ± 2.6	121.9 ± 4.4
	12.5	50.9 ± 3.2	111 ± 8.3
	50	94.2 ± 4.9	103.2 ± 3.3
	100	97.6 ± 16.8	93.3 ± 4.7
Lupeol and β-amyrin	1.6	70.3 ± 22.5	126.7 ± 12.2

	12.5	92.0 ± 4.7	121.4 ± 11.9
	50	103.7 ± 18.5	116.3 ± 8.7
	100	118.2 ± 36,8	98.4 ± 11,6
Quercetin	1.6	53.84 ± 6.37	99.82 ± 5.42
	12.5	89.18 ± 7.32	83.35 ± 7.66
	50	93.46 ± 1.71	63.97 ± 3.77
	100	95,68 ± 2.89	39.54 ± 4.31

4.3.3 Concentration effects of *P. obliquum* acetone extracts and isolated compounds on LPS-induced IL-1β production in RAW 264.7 macrophages

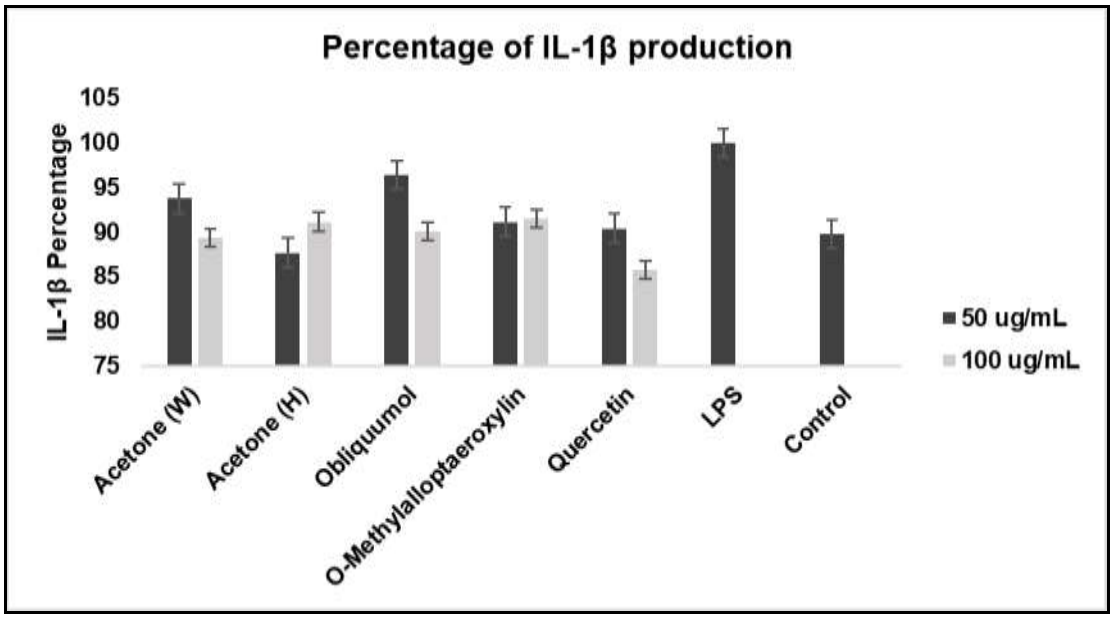


Figure 4.1. Effects of *P. obliquum* acetone extracts from different locations, obliquumol and O-methylalloptaeroxylin on LPS-induced IL-1β production in RAW 264.7 macrophages treated with different concentrations (50 and 100 µg/mL), and positive controls quercetin (50 µg/mL) and Control (No LPS) over 24 h. Key: Acetone (W); Acetone (Walter Sisulu, Roodepoort), Acetone (H); Acetone (University of Pretoria, Hatfield).

Cytokines play a critical role in maintaining our body's optimal performance and protecting it from dangerous agents. IL-1β is one of the cytokines that is regarded as a pro-inflammatory mediator since it helps to initiate an inflammatory cascade (Zhang and An, 2007). However, IL-1β should be controlled and kept at tolerable levels because an accumulation of this proinflammatory cytokine at excessive levels worsens inflammation. TNF and IL-6 are induced by IL-1β (Dinarello, 2000). A

dependent cytokine may therefore be reduced if IL-1β production is suppressed. In this study, exposure of the cells with LPS-induced macrophages increased the amount of IL-1β production in comparison to the non-stimulated controls (Figure 4.1). However, the increase was not significant. Treatment with *P. obliquum* acetone leaf extracts and two tested compounds suppressed the production of the IL-1β levels non-significantly similar to the positive control quercetin (Figure 4.1). Effects of *P. obliquum* acetone extracts and isolated compounds on LPS-induced TNF-α production in RAW 264.7 macrophages.

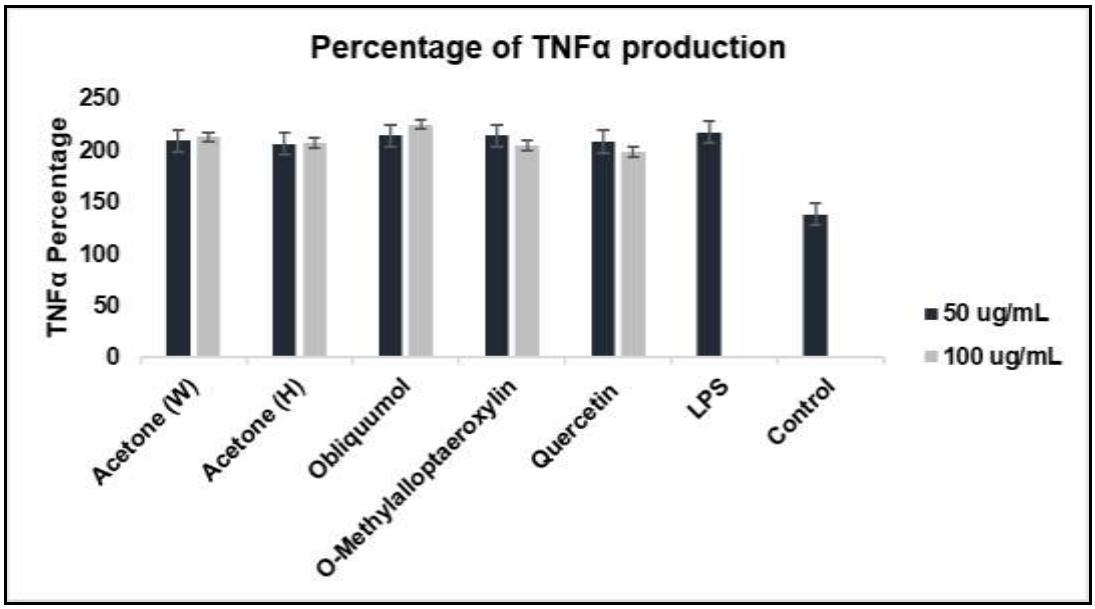


Figure 4.2. Effects of *P. obliquum* acetone extracts from different locations, obliquumol and O-methylalloptaeroxylin on LPS-induced Tumour Necrosis Factor (TNF-α) production in RAW 264.7 macrophages treated with different concentrations (50 and 100 µg/mL), and positive controls quercetin (50 µg/mL) and Control (No LPS) over 24 h. Key: Acetone (W); Acetone (Walter Sisulu), Acetone (H); Acetone (University of Pretoria, Hatfield).

The results of the effects of acetone leaf extracts and the two isolated compounds as potential stimulant of TNF-α production are presented in Figure 4.2. Stimulation with LPS induced a significant increase in TNF-α levels of the cells in comparison to the non-stimulated control cells. Quercetin lowered the LPS-induced TNF-α levels and the decrease was significant ($p < 0.001$) with the highest concentration. Treatment with the extracts and compounds also reduced the LPS-induced TNF-α levels and

the inhibition was significant with the lower 50 ug/mL concentration of the Walter Sisulu acetone ($p < 0.027$) and the highest compound obliquumol concentration ($p < 0.008$), Figure 4.2.

TNF-α is a member of the peptide mediator family. This cytokine functions in inflammatory processes as an intracellular chemical messenger. (Idriss and Naismith, 2000). TNF is primarily produced by monocytes and macrophages, but it can also be released by other cells including smooth and cardiac muscle cells, natural killer cells, mast and endothelial cells, neutrophils, and T and B lymphocytes. (Boshtam et al., 2017).

4.3.5 Effects of *P. obliquum* acetone extracts and isolated compounds on LPS-induced IL-10 production in RAW 264.7 macrophages

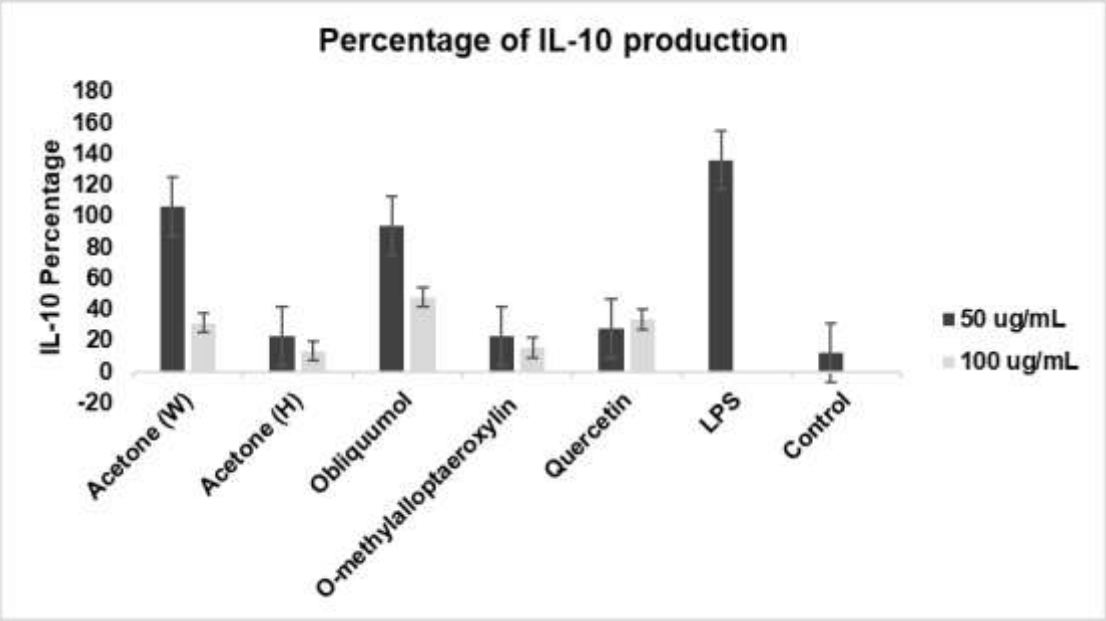


Figure 4.3. Effects of *P. obliquum* acetone extracts from different locations, obliquumol and O-methylalloptaeroxylin on LPS-induced IL-10 production in RAW 264.7 macrophages treated with different concentrations (50 and 100 µg/mL), and positive controls quercetin (50 µg/mL) and Control (No LPS) over 24 h. Key: Acetone (W); Acetone (Walter Sisulu), Acetone (H); Acetone (University of Pretoria, Hatfield).

Interleukin 10 `is an effective anti-inflammatory cytokine that is often essential in preventing inflammatory and autoimmune disorders. The nine-membered IL-10 family of cytokines, which first appeared before the adaptive immune response, is

crucial in the treatment of numerous infectious and inflammatory disorders (Iyer and Cheng, 2012). IL-10 is primarily produced by CD4 + Th2 cells, monocytes, and B cells as a homodimer of two closely packed 160 amino acid proteins (Boshtam et al., 2017). The most potent anti-inflammatory cytokine, IL-10 can suppress pro-inflammatory reactions and reduce tissue changes brought on by inflammation (Iyer and Cheng, 2012). The major way that IL-10 exerts its anti-inflammatory effects is by blocking the production of the inflammatory cytokines TNF-α, IL-1, IL-6, IL-8, IL-12, granulocyte colony-stimulating factor, macrophage inflammatory protein (MIP)-1a, and MIP-2a (Oft, 2014). As demonstrated in Figure 4.3, stimulation with LPS triggered a significant ($p < 0.001$) increase in IL-10 concentrations compared to the non-stimulated control cells. Treatment with the extracts and compounds did not increase the IL10 levels higher than LPS similar to quercetin indicating that they did not have a direct activity on IL10 anti-inflammatory cytokine.

4.3.6 Effects of *P. obliquum* acetone extracts and isolated compounds on LPS-induced COX-2 production in RAW 264.7 macrophages

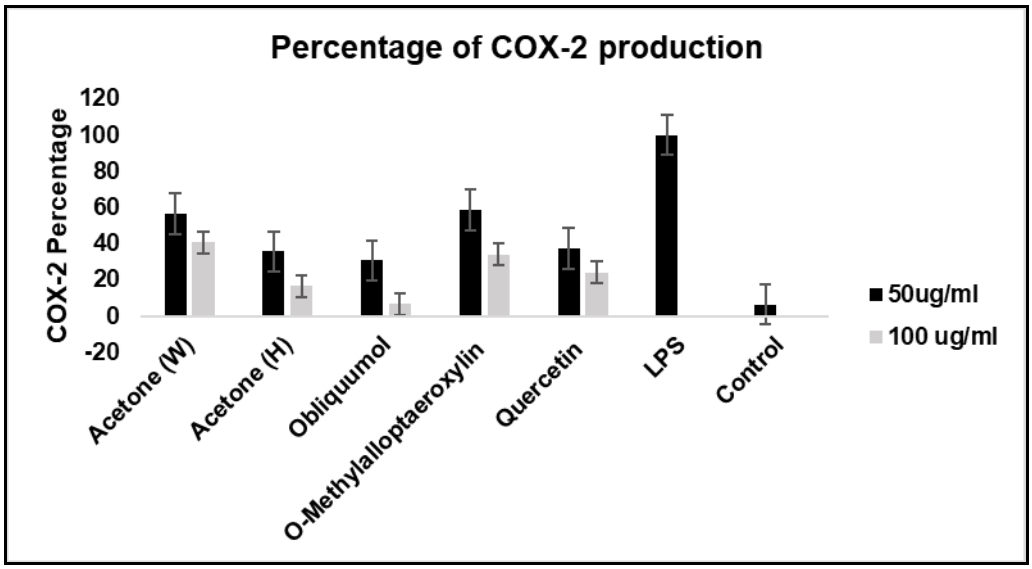


Figure 4.4. Effects of *P. obliquum* acetone extracts from different locations, obliquumol and O-methylalloptaeroxylin on COX-2 production in RAW 264.7 macrophages treated with different concentrations (50 and 100 µg/mL), and positive controls quercetin (50 µg/mL) and Control (NO LPS) over 24 h. Key: Acetone (W); Acetone (Walter Sisulu), Acetone (H); Acetone (University of Pretoria, Hatfield).

Inhibition of inducible COX-2 enzymes is important in pathological inflammation because these enzymes cause tissue damage, discomfort and edema that are related to the illness process. As a result, numerous investigations have identified treatments with potential capacity to suppress COX-2 expression or concentrations as useful tools in the prevention or treatment of pathological inflammatory diseases (Baraf, 2007). As shown in Figure 4, LPS induced a significant increase in COX-2 ($p < 0.001$) compared to non-stimulated control cells. Treatment with quercetin reversed these effects significantly ($p < 0.001$) in a dose dependent manner (Figure 4). The extracts and the compounds also reversed LPS-induced COX-2 significantly ($p < 0.001$) in a dose dependent fashion similar to the quercetin treatment. These inhibition effects were more pronounced with the Walter Sisulu acetone extract and obliquumol treatment with COX-2 levels that were even lower than that of the positive control, quercetin. These results demonstrated the significant inhibitory effects of the plant extracts and compounds on LPS-induced COX-2, which is one of the most potent mediators of inflammation, indicating their potential ability to alleviate inflammation in a pathological inflammation (Baraf, 2007)

4.4 Conclusion

The purpose of the current study was to investigate *in vitro*the potential effectiveness of *P. obliquum* in treating pain and inflammation using *in vitro* studies. Isolated compounds and acetone extracts had anti-inflammatory effects by reducing the expression of the pro-inflammatory cytokine IL-1 in macrophages stimulated by LPS, and also by inhibiting the production of 15-LOX, NO, and COX-2. The acetone extracts and the two tested compounds (O-methylalloptaeroxylin andobliquumol) reduced production of LPS-induced pro-inflammatory cytokines, although not significantly. The LPS-induced TNF-α levels were significantly inhibited after treatment with the acetone extracts and compounds. Similar to quercetin, treatment with the acetone extracts and compounds did not elevate the levels of IL-10 above those induced by LPS, showing that they had no significant impact on the cytokine's ability to inhibit inflammation. These results demonstrated a considerable inhibition of COX-2, one of the most potent inflammatory mediators, by *P. obliquum* extracts and compounds. The acetone extracts, fractions, and obliquumol suppressed NO production and exhibited good cell viability at the lowest concentration tested, indicating that the larger percentage of NO production was not caused by the tested

sample's toxicity to the RAW 24.7 macrophage cells. The anti-inflammatory activity of water extracts in the NO and 15-LOX tests was significantly lower than that of the acetone extract, which was an interesting finding. Most of the time, traditional healers merely employ water as an extractant. Traditional healers use mainly bark and extracting the bark with water from a river or a pond over extended periods may lead to solubilisation of non-polar compounds. *P. obliquum* acetone extract, fractions and isolated compounds may reduce inflammation by inhibiting pro-inflammatory cytokines, activating IL-10, and inhibiting NO and 15-LOX. However, in order to properly understand the mechanism of action, it is necessary to continue investigations on the effect of the extracts and compounds on COX-2 and iNOS. The historic usage of *P. obliquum* to treat inflammatory-related illnesses and symptoms is supported by the *in vitro* anti-inflammatory efficacy in various bioassays by its acetone extracts, fractions and isolated components, providing motivation for additional *in vivo* studies.

The results indicate the anti-inflammatory activity of obliquumol in addition to other uses such as antifungal, antiparacitical and anticancer activities (Ramadwa et al., 2021: Khunoana et al., 2022).

Postscript

In the next chapter the effect of the crude extracts and isolated compounds on pro-apoptotic cytokines will be investigated.

Chapter 5

Ptaeroxylon obliquum **acetone leaf extracts and obliquumol as potential apoptosis mediators in HepG2 and HeLa cells by caspase-3 and TNF-α.**

Abstract

Approximately 20% of cancers are induced by chronic inflammation or other infections. The previous study showed that antiproliferative agents such as *P. obliquum* acetone extracts and obliquumol can induce selective cytotoxicity against cancer cell lines, without causing much harm to normal cells. Therefore, there was a need to investigate the possible mechanism of action against cancer cells. The present study aims at investing the effects of the acetone extracts and obliquumol isolated from *P. obliquum* leaves on pro-apoptotic cytokines (caspase-3 and TNF-α) and morphological alterations from trees growing in different areas in Gauteng.

The effects of the samples on levels of caspase-3 and TNF-α were determined and quantified using ELISA kits. Light microscopy was used to capture the changes in HepG2 and HeLa cell morphology. *P. obliquum* acetone extracts and obliquumol increased caspase-3 production by 2% at 100 µg/mL on HepG2 cells. Obliquumol increased the production of caspase-3 by 8% at 100 µg/mL on HeLa cells. The acetone leaf extract from a tree growing in Hatfield (100 µg/mL) increased TNF-α production by almost 10% which was higher than the activity of the positive control doxorubicin in HepG2 cells. Acetone leaf extracts from a tree growing in Roodepoort increased TNF-α production by 11.5 % at the lowest concentration tested (50 µg/mL) on HeLa cells which was better than the positive control.

A concentration-dependent effect was observed on the morphology of both cell lines tested in the study. Higher cell death and morphological alterations were observed on HepG2 cells than on HeLa cells. The *P. obliquum* acetone extract and isolated compounds may therefore contribute to the induction of pro-apoptotic cytokines (caspase-3 and TNF-α).

Key words: *Ptaeroxylon obliquum,* obliquumol, cancer, antiproliferative, HepG2, HeLa, caspase-3, TNF-α

5.1 Introduction

Over 9.6 million people died from cancer in 2018, making it the second highest cause of death worldwide. Sadly, by 2030, there will likely be an increase in the burden due to 75 million new instances of cancer and 17 million new cancer-related fatalities (Sung et al., 2021). Consequently, cancer is a major global public health

concern with over 200 distinct types of cancer known to harm humans. The prevalence varies by gender, with men having greater rates of certain tumours like the lung, prostate, colorectum, stomach, and liver, while women usually express cases of the breast, colorectum, lung, cervix, and stomach (Khader et al., 2018). The Cancer Association of South Africa (CANSA) estimates that each year, 115 000 South Africans are given cancer diagnoses (Twilley et al., 2020). Men are more likely to develop prostate, colorectal and lung cancer than women, who are more likely to develop breast and cervical cancer (Abudu et al., 2019). Options for cancer treatment include chemotherapy, radiation therapy, hormone replacement therapy, and gene therapy. however, they all have a variety of adverse effects, including fatigue, weight loss, nausea, vomiting, and bleeding (Markham et al., 2020). One of the most common anti-cancer treatments is chemotherapy. However, certain cancer cells are able to become resistant to some of the chemotherapeutic agents, posing a serious threat to anti-cancer therapy. Treatment outcomes are therefore unsatisfactory as a result of limited drug efficacy, which ultimately results in mortality (Rahman et al., 2021).

One of the main methods for preventing the development of cancer or stopping cancer is apoptosis, or programmed cell death (Lopez and Tait, 2015). Given that cancer is characterized by the capacity to evade apoptosis regardless of the kind of cancer, focusing on apoptosis is most beneficial for treating various cancers. Cancer cells can develop resistance by avoiding potential apoptotic mechanisms, such as improper apoptosis initiation and execution, up-regulation of anti-apoptotic signals, and down-regulation of pro-apoptotic signals. The mitochondrial pathway and the death receptor pathway are both extrinsic (also known as the central pathway) and intrinsic (also known as the mitochondrial pathway) processes that are connected to apoptosis (Elmore, 2007).

The intrinsic pathway is started inside the cell and controlled by a series of B-cell lymphoma 2 (Bcl-2) related proteins. Pro-apoptotic and anti-apoptotic proteins from the Bcl-2 family are the two groups that control the process. Cytochrome-C is released from the mitochondria when a protein is pro-apoptotic, whereas an anti-apoptotic protein prevents this from happening. Balance between pro- and antiapoptotic proteins is necessary for the start of apoptosis (Rahman et al., 2021, Wong, 2011). Apoptosome, comprised of cytochrome-C, apoptotic protease

activating factor-1, and caspase-9, is formed as soon as cytochrome protein is released in the cytoplasm and activates caspase-3.

The activation of "death receptors," which are cell surface receptors that deliver apoptotic signals after ligation with particular ligands, mediates the extrinsic pathway. The two most widely recognized death receptors are TNFR1 (Tumour Necrosis Factor Receptor 1)-related protein named Fas and their respective ligands, TNF and Fas ligand (FasL) (Hengartner, 2001). An intracellular death domain found in these death receptors attracts adaptor proteins. The TNF receptor associated death domain (TRADD), the Fas Associated Death Domain (FADD), and cysteine proteases like caspase-8 are among them. It creates a binding site for an adapter protein when the death ligand attaches to its death receptor. The Death-Inducing Signaling Complex is the collective name for the ligand-receptor-adaptor protein complex (DISC) (O'Brien and Kirby, 2008). Procaspase-8 is activated and the assembly is started by the DISC. After processing downstream effector caspases that go on to cleave particular substrates, active caspase-8 causes cell death (Wong, 2011).

The execution pathway that is considered to be the final step of apoptosis is where both the extrinsic and intrinsic pathways connect (Goldar et al., 2015). The last pathway results in cell death and is started by the activation of execution caspases like caspase-3 on certain substrates. (Rahman et al., 2021). In the intrinsic pathway, caspase-9 is the upstream caspase, and in the extrinsic pathway, caspase-8 is the upstream caspase. A converging intrinsic and extrinsic pathway leads to caspase-3. Following that, the caspase-3 cleaves the deoxyribonuclease inhibitor, which oversees nuclear apoptosis (Ghobrial et al., 2005). Additionally, proteins like protein kinases, cytoskeletal proteins, DNA repair proteins, and inhibitory endonuclease components are cleaved by downstream caspases. Together, these result in morphological alterations in apoptosis (Wong, 2011).

Thus, a novel treatment approach for improving chemotherapy effectiveness may involve looking into important apoptotic pathway variables (Rahman et al., 2021). More than 60% of the currently used anticancer drugs are made from natural compounds originating from plants, marine organisms, and microbes, with plants accounting for around 25% of these natural anticancer agents (Newman et al., 2002,

Juárez, 2014). The primary source of medication and treatment for many people in South Africa is traditional medicine, particularly the usage of plants (Mander, 1998). In South Africa, there are many plant species that have not yet been investigated for their possible anti-cancer activity. Consequently, more plants with anticancer potential need to be researched.

Previous studies showed that *P. obliquum* acetone extracts had selective cytotoxic activity against HepG2 and HeLa cancer cell lines (Khunoana et al., 2022). The mechanism of action for antiproliferative plants, like *P. obliquum* acetone leaf extracts which can induce selective cytotoxicity against cancer cell lines without doing significant harm to normal cells, is generally unknown. However, it is thought that the cytotoxicity/anti-proliferation effects may have been brought on by pro-apoptotic processes. Consequently, this chapter will focus on examining the impact of *P. obliquum* extracts on pro-apoptotic cytokines such as caspase-3 and TNF-α.

5.2 Materials and Methods

5.2.1 Plant collection

P. obliquum leaves used in this study were collected during the summer of 2019 from trees growing at the Hatfield Campus (University of Pretoria), and Walter Sisulu National Botanical Garden (Roodepoort). Leaves were collected in open weave nylon bags, dried at room temperature in the shade and powdered using a grinder. The powders were stored in closed containers in the dark until needed. Voucher specimens (PRU130509 and PRU130627) were prepared and kept at the HGWJ Schweickerdt Herbarium of the University of Pretoria.

5.2.2 Preparation of extracts

Exactly 5 g of the powder was extracted using 50 mL acetone. The mixture was placed in an airtight container on a shaker and left for 24 h, after which the supernatant was filtered through Whatman No. 1 filter paper and placed into a pre-weighed honey jar. The process was repeated three times. Then the supernatants for each solvent were combined in a single pre-weighed honey jar and dried under a stream of cold air.

5.2.3 Fractionation and isolation of bioactive compounds from *P. obliquum*

The plant fractionation procedure and isolation of bioactive compounds were carried out as described earlier, and led to isolation of obliquumol, lupeol and β-amyrin mixture and O-methylalloptaeroxylin from *P. obliquum* acetone leaf extract (Khunoana et al., 2022). The acetone crude extracts, the fractions (hexane and chloroform fractions), and the isolated pure compounds were reconstituted in dimethyl sulphoxide (DMSO)

5.2.4 Ultra-performance liquid chromatography qTof mass spectrometry (UPLC- qTof-MS) analysis of P. obliquum acetone extracts.

Samples were sent to the Department of Chemistry at the University of Pretoria (Hatfield campus) for analysis. Compound separation and detection were performed using a Waters® Synapt G2 high definition mass spectrometry (HDMS) system (Waters Inc., Milford, Massachusetts, USA). The system comprises of a Waters Acquity Ultra Performance Liquid Chromatography (UPLC®) system hyphenated to a quadrupole-time-of-flight (QTOF) instrument. The system was operated with MassLynx™ (version 4.1) software (Waters Inc., Milford, Massachusetts, USA) for data acquisition and processing. For mass detection, only the positive mode was used for identification. Ultra-Performance Liquid Chromatography coupled to quadrupole time-of-flight mass spectrometry (UPLC-qTOF-MS) analysis was carried out on the *P. obliquum* acetone leaf extracts from different geographical locations as previously described by Wooding et al. (2022). The acetone extracts were reconstituted, first in 100% acetonitrile followed by water (0.1% formic acid) such that the final concentration was ~1 mg/mL of total crude extract. MS-grade acetonitrile was purchased from Romil. Water with 0.1% formic acid was purchased from Sigma-Aldrich. The extracts were pooled and centrifuged at 10 000 x g for 10 min to remove particulates. Prior to analyses, the instrument was calibrated over a mass range of 50–1200 Da using a sodium formate solution, typically to an absolute mass accuracy of f <0.5 mDa using the Intellistart functionality of the software. The instrument was centrally operated and controlled with MassLynx v4.1 software for data acquisition. A form of data independent analysis termed MSE was used to acquire both low energy (precursor ions) and high energy (product ions) utilising a collision energy ramp from 10 V to 40 V over a scan time of 0.3 s. An internal control (the lockspray), namely

leucine encephalin, was directly infused into the source through a secondary orthogonal electrospray ionisation probe allowing intermittent sampling (every 10 s). The lockspray was used to compensate for instrument drift, thus ensuring good mass accuracy throughout the duration of the runs. Exactly 5 µL of the reconstituted extracts was injected into the UPLC-MS system. All the samples were run in both positive ionization models.

5.2.5 Cytokine activity measurements

The effects of the extracts and isolated compound on TNF-α and caspase-3 concentrations were identified and quantified using the human ELISA kits E-EL-H0109 and E-EL-H0017 (Elabscience, Biocom Africa, Johannesburg South Africa). The assay was done following the instructions on the manufacturer's manual. Standard curves were used to calculate the concentration

5.2.6 Statistical analysis

Results are expressed as mean ± standard deviations, and statistically significant values were compared using one-way ANOVA analysis of variance using an interactive statistical program (Sigmastat, SPSS version 26, San Jose, CA, USA) and pairwise multiple comparison procedures using Holm—Sidak method. The results are presented as mean standard deviations, and statistically significant values were compared using one-way ANOVA analysis of variance performed using an interactive statistical program and pairwise multiple comparison techniques utilizing the Holm—Sidak method. Normality testing was done using Shapiro—Wilk and equal variance test using Brown—Forsythe. The p-value of ≤0.050 was considered statistically significant.

5.2.7 Morphological Study

Morphological alteration of HepG2 and HeLa cell lines after exposure to test substances was assessed under a microscope (Nikon Eclipse Ti Optical Co., Ltd., Tokyo, Japan). The cells were seeded at a density of 1 x 10^5 cells/mL in 5 mL medium in a 25 cm^3 flask. After 24 h, the medium was removed and replaced with new medium. Thereafter, HepG2, and HeLa cell lines were treated with 100 µg/mL and 50 µg/mL of the acetone leaf extracts for 48 h. After the treatment, the images were captured at 100x magnification, using a phase contrast inverted microscope

(Nikon Eclipse Ti Optical Co., Ltd., Tokyo, Japan). Doxorubicin (12 ug/mL) was used as the positive control, while the untreated cells were the negative control. The effect of *P. obliquum* acetone leaf extracts from two different geographic locations on morphological changes of HepG2 and HeLa cells was assessed and photographed.

5.3 Results and discussion

5.3.1 UPLC-MS chromatograms of the four *P. obliquum* acetone extracts from various locations in South Africa.

Analysis of the UPLC-MS data obtained from the electrospray positive mode of the acetone extracts from Hatfield and Walter Sisulu Botanical Garden of *P. obliquum* in comparison to literature led to the tentative identification of two compounds, *O*-methylalloptaeroxylin and obliquumol (Figures 5.1 and 5.2, Table 5.1). *O*-methylalloptaeroxylin molecular ion peak was high in the acetone extract collected in both regions while the one of obliquumol was only high in the Walter Sisulu extract.

Table 5.1. UPLC-MS chromatogram of Hatfield Acetone, ESI positive mode.

Peak	Retention time	Observed[M+H]$^+$ m/z	Exact mass	Molecular Formula	Compound name	Reference to isolated compounds in plants using NMR
1	7.34	273.1149 [M+Na]$^+$	272.1069	$C_{13}H_{16}O_6$	*O*-methylalloptaeroxylin	(Okorie, 1982, Ahluwalia et al., 1982, Khunoana et al., 2022)
2	8.35	317.1105 [M+Na]$^+$	316.1025	$C_{15}H_{18}O_6$	Obliquumol	(Van Wyk et al., 2018, Malefo et al., 2020, Khunoana et al., 2022)

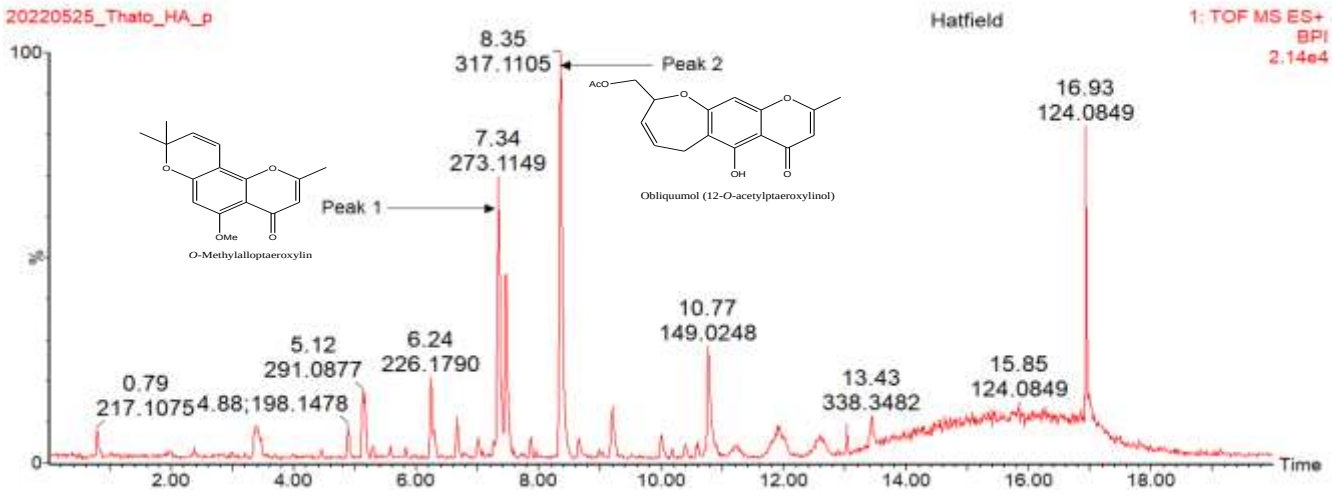


Figure 5.1: ESI positive-mode chromatograms of acetone leaf extracts of *P. obliquum* collected from University of Pretoria Botanical Garden (Hatfield).

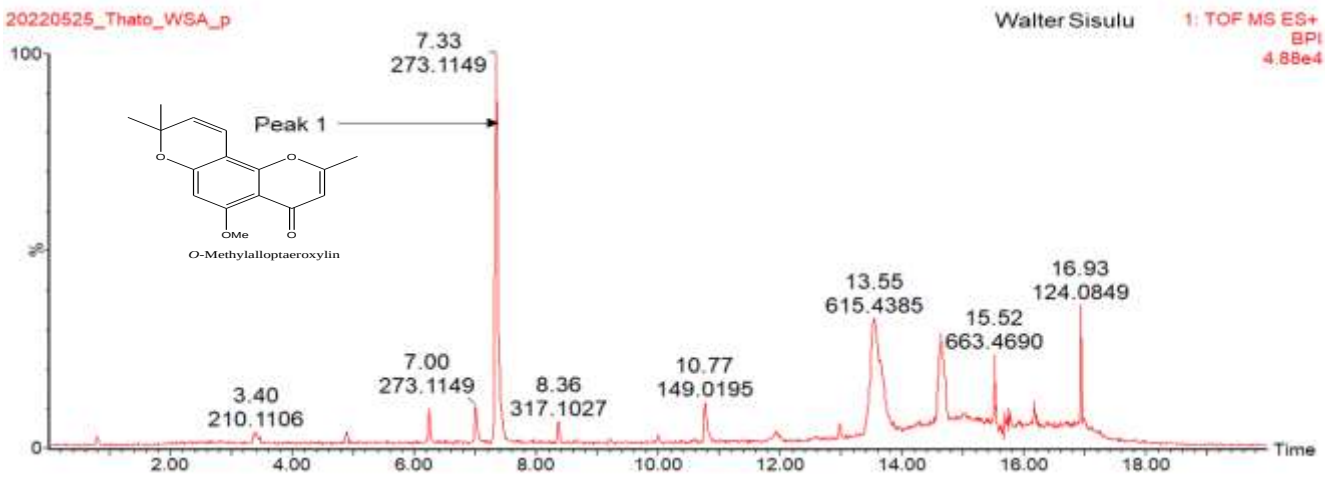


Figure 5.2: ESI positive-mode chromatograms of acetone leaf extracts of *P. obliquum* collected from Walter Sisulu Botanical Garden (Roodepoort).

5.3.2 Effects of *P. obliquum* acetone extracts and isolated compounds on caspase-3 production in HepG2 and HeLa cells.

Caspases play a key role as programmed cell death mediators (apoptosis). Caspase-3 is a commonly active death protease that catalyses the particular cleavage of numerous essential cellular proteins. The release of mitochondrial cytochrome c and the action of caspase-9 may either be required or not for the activation of caspase-3. Caspase-3 is crucial for healthy brain development and plays a vital role in various apoptotic conditions specifically in response to tissue cell or death stimuli. Additionally, caspase-3 is essential for the DNA fragmentation and chromatin condensation that occurs during apoptosis in all cell types analysed. Caspase-3 is therefore necessary for several processes involved in cell death and the production of apoptotic bodies, but it may also act before or during the moment

when the decision to lose cell viability is made (Porter and Jänicke, 1999).

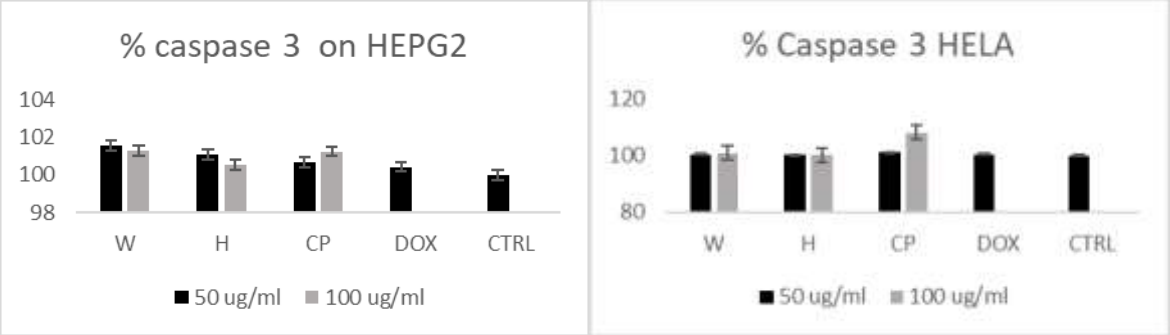


Figure 5.3. Effects of *P. obliquum* acetone extracts from different locations and obliquumol on caspase-3 production in HepG2 and HeLa cancer cells treated with different concentrations (50 and 100 µg/mL), and positive controls doxorubicin (50 µg/mL) and control (no treatment) over 24 h.

P. obliquum acetone extracts and isolated compound obliquumol increased caspase-3 production with 2% at 100 µg/mL on HepG2 cells. The actvity of the extracts and the efficacy of obliquumol was higher than that of the positive control, doxorubicin as illustrated in Figure 5.3. The isolated compound obliquumol increased the production of caspase-3 with 8% at 100 µg/mL on HeLa cells. Although the increase in caspase-3 was not statistically significant (P>0.05), based on the results it is evident that obliquumol and *P. obliquum* acetone appeared to increase caspase-3 production. This is important since caspase-3 mediates apoptosis, which then results in the death of cancer cells

5.3.3 Effects of *P. obliquum* acetone extracts and isolated compounds on human tumour necrosis factor production in HepG2 and HeLa cells.

TNF cytokines are linked to a number of illnesses, including tumorigenesis, rheumatoid arthritis, septic shock, viral replication, diabetes and other inflammatory conditions (Gaur and Aggarwal, 2003). These cytokines regulate either proliferation, survival, or apoptosis. TNF participates in the extrinsic pathway and is frequently referred to as an initiator, activator, or executioner caspase. In order to induce apoptosis in apoptotic cells, the extracellular transmembrane domain of death receptors (DR) is activated by the Fas ligand, tumour necrosis factor (TNF), and TNF-related apoptosis inducing ligand (TRAIL), which triggers the extrinsic pathway. (Guicciardi and Gores, 2009) During apoptosis, they communicate cell death signals to the intracellular signalling pathways.

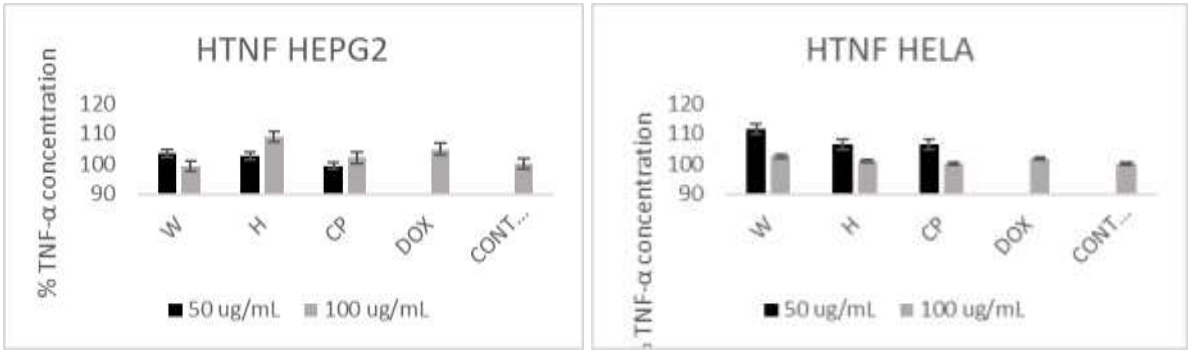


Figure 5.4. Effects of *P. obliquum* acetone extracts from trees growing in different locations and obliquumol on human tumour necrosis factor production in HepG2 and HeLa cancer cells treated with different concentrations (50 and 100 µg/mL), and positive controls, doxorubicin (50 µg/mL) and Control (No treatment) over 24 h.

P. obliquum acetone extracts and obliquumol increased TNF-α production more than did the control (untreated cells) even at the lower tested concentration of 50 µg/mL. The Hatfield acetone extract (100 µg/mL) increased TNF-α production with almost 10% which was higher than the activity of the positive contol, doxorubicin in HepG2 cells. The Walter Sisulu acetone extract also increased TNF-α by 3% followed by the isolated compound obliquumol with 2% in HepG2 cells. Walter Sisulu acetone extracts increased TNF-α production with 11.5% at the lowest concentration tested (50 µg/mL) on HeLa cells which was better than the positive control. Both Hatfield and obliquumol increased TNF-α production by 6%. Based on the results, the increase in TNF-α was not statistically significant ($P>0.05$). It does, however, shows that acetone extracts and the isolated compound from *P. obliquum* may increase TNF-α production. Although the differences observed here were not statistically significant, it may be worthwhile to investigate these observations in more depth.

5.3.4 Morphology of HepG2 and HeLa cells

The effects of *P. obliquum* acetone leaf extracts and the isolated compound obliquumol on the morphology of HepG2 and HeLa cells are depicted in Figures 5.5–5.7.

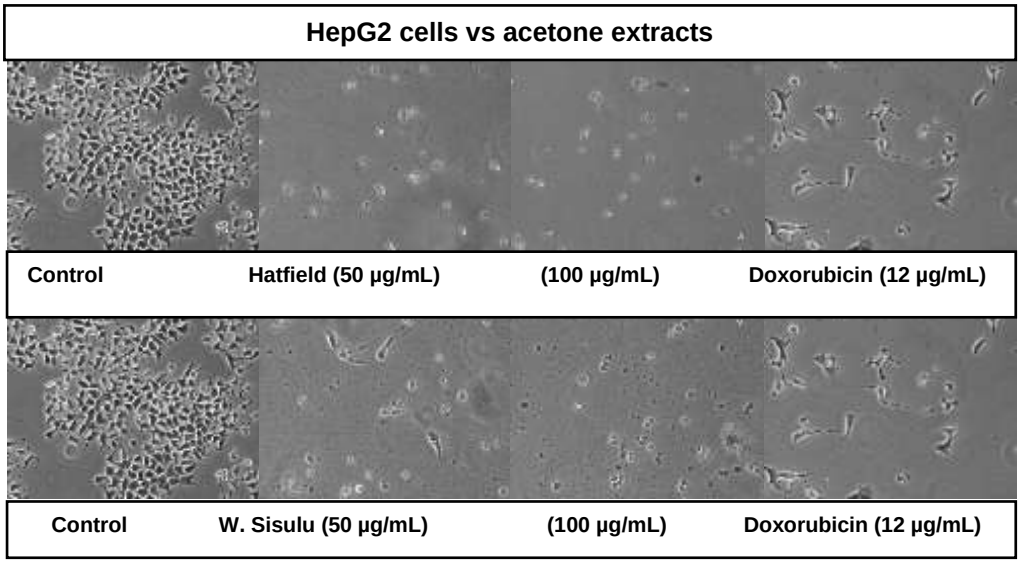


Figure 5.5. Morphological characterization of HepG2 cell lines treated with 50 and 100 µg/mL of Hatfield and Walter Sisulu acetone crude extracts, and 12 µg/mL doxorubicin

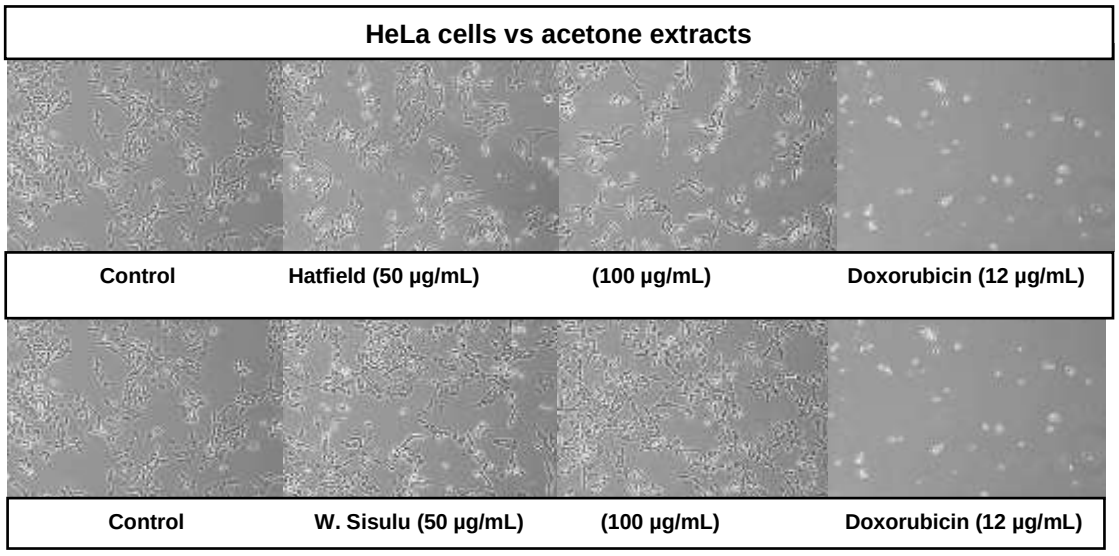


Figure 5.6. Morphological characterization of HeLa cell line treated with 50 and 100 µg/mL of Hatfield and Walter Sisulu acetone crude extracts, and 12 µg/mL doxorubicin for 48 h.

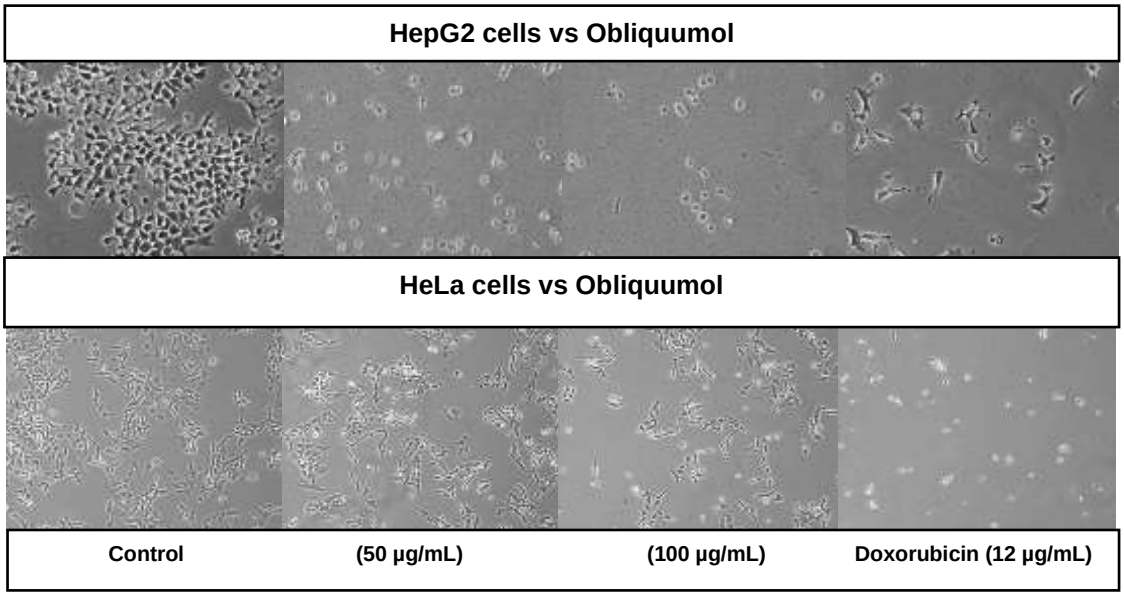


Figure 5.7. Morphological characterization of HepG2 and HeLa cell line treated with 50 and 100 µg/mL of isolated compound obliquumol, and 12 µg/mL doxorubicin for 48 h.

The morphology of HepG2 and HeLa cells changed as a result of exposure to *P. obliquum* acetone leaf extracts and the isolated compound obliquumol, as shown in Figures 5.5-5.7. Generally, both cancer cells were in a scattered pattern, most of the cells were dead and seemed as floating, circular cells, as compared to the adherent spindle-shaped live cells. As expected, a concentration-dependent effect was observed in the morphological changes in both cell lines tested in the study. The HepG2 cells were more sensitive than HeLa cells leading to higher cell death and morphological alterations after 48 hours treatment with acetone extracts and obliquumol. Both the HepG2 and HeLa cells lost their typical shape and morphology compared to the control treatment and became rounded and lost their adherence capacity after the exposure of 50 and 100 µg/mL of the *P. obliquum* samples. It is evident that at the tested concentrations, the acetone leaf extracts and compound obliquumol of *P. obliquum* are effectively cytotoxic and alters the cell morphology of HepG2 and HeLa cells. The cytotoxicity was selective to HepG2 and HeLa as the samples were relatively safe to normal kidney cells (Khunoana et al., 2022).

5.4 Conclusion

The results indicate that treatment with *P. obliquum* extracts and compounds increased (although not statistically significantly) the production of both caspase-3 and TNF-α, which are both key mediators of apoptosis. It also appeared that *P. obliquum* extracts altered the cell morphology of HepG2 and HeLa cells in a dose dependent way. The extracts of *P. obliquum* may be useful in the treatment of selected malignancies. It is also possible to investigate the extracts or compounds' potential synergy with the efficiency of conventional chemotherapy drugs by combining them. Further studies are required to clarify the specific mechanism of action responsible for anti-cancer and apoptotic activity.

Acknowledgment

The LC-MS Synapt Facility (Department of Chemistry, University of Pretoria) is acknowledged for chromatography and mass spectrometry services provided by Dr Madelien Wooding.

Postscript

In the next chapter the possible interaction and correlation between different activities of extracts, fractions and isolated compounds from *P. obliquum* will be examined.

Chapter 6

Correlation between the antioxidant, anti-inflammatory and anti-proliferative activity of crude extracts, fractions and compounds derived from *P. obliquum*

6.1 Introduction

This chapter focuses on the relationship between antioxidant activity, anti-inflammatory activity and cancer. Diseases such as cancer and those associated with inflammation may emerge from the imbalance brought about by oxidative stress. In Chapter 3, the antioxidant potential of the crude extracts, fractions and isolated compounds were screened for their potential to scavenge the free radicals, ABTS and DPPH. Furthermore, cytotoxicity was determined on normal cells (Vero), and cancer cell lines (MCF-7, HepG2, A549 and HeLa). Oxidative stress plays a major role in inflammation, as it is a mediator which induces the release of pro-inflammatory cytokines and nitric oxide (Nworu and Akah, 2015). In Chapter 4, the anti-inflammatory activity was tested against lipoxygenase and inducible nitric oxide synthase.

Correlation analysis measures and interprets the strength of a linear or nonlinear link between two continuous variables. There are numerous ways to evaluate the degree of correlation, and in practice, the correlation may be helpful for pointing out an interesting predictive relationship. Either the Pearson linear correlation coefficient or the Spearman rank correlation coefficient may be used in a correlation analysis to determine the relationship between two random variables. The Pearson correlation coefficient ranging from −1 to 1 was developed by Karl Pearson and founded on Francis Galton's related idea. Pearson's r is defined as the covariance of two variables divided by the product of their standard deviations. It only responds well to linear correlations, whereas other approaches are generally more resistant to non-linear correlations. (Pearson, 1895, Zou et al., 2003, Wang et al., 2015, Schober et al., 2018). In this chapter, the correlations between the antioxidant, anti-inflammatory, and anti-proliferative activity of crude extracts, fractions and compounds derived from *P. obliquum* were investigated.

6.2 Materials and Methods

The obtained data was analysed using the IBM SPSS Statistic 25, software package for Windows. The imported IC_{50} values were obtained using linear regression calculated for all the extracts, fractions and compounds from *P. obliquum* for anti-proliferation, anti-inflammation and the antioxidant activity (as calculated in Chapters 3 and 4). Data were analysed using Pearson correlation statistics.

6.3 Results

Probabilities of 0.01 and 0.05 were used as thresholds for statistical significance, with 0.01 being highly significant. As suggested by Cohen (1988), the interpretation for correlation coefficient values is: weak where r = 0.0 to 0.29; moderate where r = 0.30 to 0.49 and strong correlation with r = 0.50 to 1.0. In the present study (Table 6.10), toxicity to Vero cells had moderate to high correlation with all tested cancer cell line r = 0.39 - 0.71. A strong and significantly high ($p<0.01$) correlation was observed between Vero and MCF-7 (0.71) followed by Vero against HeLa (0.53). A moderate correlation was observed between Vero, A549 and HepG2 cells which were not statistically significant ($P<0.05$). When looking at the relationship between cancer and inflammation, a strong correlation was observed with I5-LOX and cancer cells (HeLa, MCF-7, and HepG2) with r = 0.67, 0.57, and 0.53, respectively. The other inflammation pathway studied, nitric oxide, also had a strong and high ($p<0.01$) correlation with MCF-7 cancer cell. The correlation between cancer and inflammation is not uncommon since the growth of cancer is significantly influenced by the tumour microenvironment. Pro-inflammatory cytokines released by the inflammatory cell within the tumour microenvironment influence NF-kB, STAT3, and HIF1a, activating iNOS and COX-2. The development and spread of cancer is aided by the proteins COX-2 and iNOS (Muniandy et al., 2018).As oxidative stress contributes to both cancer and inflammation, it was found that there was a strong and perfect negative correlation between anti-proliferative and antioxidant activity in the results of the DPPH and ABTS assays against MCF-7 and HepG2 cells. These correlations were significant, with r values ranging from -0.56-0.64. There was a highly significant ($p<0.01$) and strong perfect negative correlation between antioxidant and anti-inflammatory activity, with r = -0.73 to -0.80. Reactive oxygen and nitrogen species (ROS/RNS), which are free radicals, are produced by epithelial and inflammatory cells during chronic inflammation (Pinlaor et al., 2004). When produced in regulated

quantities, ROS/RNS have beneficial effects on the body. However, unregulated production of ROS/RNS leads to oxidative stress, which damages DNA in inflamed tissues and promotes the proliferation of cancer cells.

Table 6.1 Correlation between anti-proliferation, anti-inflammation and antioxidant activity of *P. obliquum* extracts, fractions and isolated compounds.

		MCF	HEPG2	A549	HELA	VERO	LOX 15	NO	DPPH	ABTS
MCF	Pearson Correlation									
	Sig0. (2-tailed)									
	N	19								
HEPG2	Pearson Correlation	0.546*								
	Sig0. (2-tailed)	0.016								
	N	19	19							
A549	Pearson Correlation	0.798**	0.378							
	Sig0. (2-tailed)	<,001	0.11							
	N	19	19	19						
HELA	Pearson Correlation	0.585**	0.44	0.344						
	Sig0. (2-tailed)	0.008	0.059	0.149						
	N	19	19	19	19					
VERO	Pearson Correlation	0.712**	0.42	0.388	0.527*					
	Sig0. (2-tailed)	<,001	0.073	0.101	0.021					
	N	19	19	19	19	19				
LOX 15	Pearson Correlation	0.574*	0.530*	0.426	0.666**	0.662**				
	Sig0. (2-tailed)	0.01	0.019	0.069	0.002	0.002				
	N	19	19	19	19	19	19			
NO	Pearson Correlation	0.520*	0.444	0.472	0.492	0.418	0.631*			
	Sig0. (2-tailed)	0.047	0.098	0.075	0.062	0.121	0.012			
	N	15	15	15	15	15	15	15		
DPPH	Pearson Correlation	-0.587*	-0.555*	-0.432	-0.345	-0.649**	-0.795**	-0.733**		
	Sig0. (2-tailed)	0.017	0.026	0.094	0.19	0.007	<,001	0.007		
	N	16	16	16	16	16	16	12	16	
ABTS	Pearson Correlation	-0.638**	-0.591*	-0.423	-0.388	-0.694**	-0.787**	-0.773**	0.876**	
	Sig0. (2-tailed)	0.008	0.016	0.103	0.138	0.003	<0.001	0.003	<0.001	
	N	16	16	16	16	16	16	12	16	16
*** Correlation is significant at the 00.05 level**										

(2-tailed)0.						
**** Correlation is significant at the 00.01 level (2-tailed)0.**						

6.4 Conclusion

In the previous chapters, it was reported that *P. obliquum* displayed selective cytotoxicity against cancer cell lines and it also significantly prevented inflammation mediators *in vitro*. Thus, the strong correlation between the efficacy of *P. obliquum* in anti-inflammatory (15-LOX and NO) and anti-proliferation assays may be due to the presence of secondary metabolites in the plant such as obliquumol, which acted as anti-inflammatory and anti-proliferative agents. Oxidative stress is closely linked to every aspect of cancer, including tumour development, therapy, and prevention. Thus, the correlation between anti-inflammatory, antioxidant and anti-proliferative activity might be due to various pathways including oxidative stress.

Chapter 7

General Discussion, Conclusions and Recommendations

Numerous malignancies are brought on by microbial infections or chronic inflammation. *Ptaeroxylon obliquum* is traditionally used to treat various infections that could be characterized by inflammation. The aim of the study was to investigate the anti-inflammatory, antiproliferative and antioxidant activities of leaf extracts, fractions and isolated compounds from *Ptaeroxylon obliquum* collected in different locations in South Africa. The overall discussion of the results presented in the thesis will be provided in terms of the initial objectives of the study.

7.1 Discussion

7.1.1 *In vitro* antiproliferative and antioxidant activity of *P. obliquum* leaf extracts, fractions and isolated compounds on several cancer cell lines

Objective: To evaluate antiproliferative activity of *P. obliquum* extracts, acetone extracts, fractions and isolated compounds against cancerous and normal Vero cells using a colorimetric tetrazolium bromide assay, and to determine the radical scavenging activity of the extracts and fractions using 2, 2-diphenyl-1-picrylhydrazyl (DPPH) and 2, 2'-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS) assays.

Many cancers are brought on by various infections or chronic inflammation. Different infections characterized by inflammation are traditionally treated with *Ptaeroxylon obliquum* in traditional medicine. *P. obliquum* leaf extracts, fractions and isolated compounds were tested for their *in vitro* antiproliferative and antioxidant properties. A colorimetric tetrazolium bromide assay was used to evaluate antiproliferative efficacy against normal Vero cells as well as a number of cancerous human cells, including human cervical cancer cells (HeLa), hepatocarcinoma (HepG2), lung adenocarcinoma (A549) and breast cancer (MCF-7) cells. DPPH and ABTS assays were used to evaluate the radical scavenging activity. O-Methylalloptaeroxylin, obliquumol, and a combination of lupeol and β-amyrin were isolated from the chloroform fraction using silica gel open column chromatography. Acetone extracts had IC_{50} values against HepG2 cells ranging from 8 to 200 µg/mL, however, they

were less toxic to other cells, with selectivity index values of up to 14. When tested against all the cell lines, aqueous extracts and fractions were non-toxic at the highest concentration tested (IC_{50} > 100 µg/mL). The IC_{50} for isolated compounds against HepG2 and HeLa cells, respectively, ranged from 52 to 539 µg/mL and 189 to 247 µg/mL. The cytotoxicity of the acetone extracts was confirmed by light microscopy, which showed dose related alterations in the morphology of HepG2 and HeLa cells. The IC_{50} values for water extracts were as low as 29.06 µg/mL and 43.4 µg/mL for scavenged ABTS and DPPH radicals, respectively.

7.1.2 *Ptaeroxylon obliquum* extracts, fractions and bioactive compounds as potential inhibitors of arachidonic acid metabolic pathways in the inflammation mechanism

Objective: To determine anti-inflammatory activities of *P. obliquum* aqueous extracts, acetone extracts, fractions and isolated compounds as potential inhibitors of 15-LOX, COX-2, NO, IL-1β, IL-6, IL-10, and TNF-α.

Ptaeroxylon obliquum extracts have been used for decades to treat inflammation and its related symptoms, including arthritis, rheumatism, fever, pain and headaches. The anti-inflammatory efficacy of *P. obliquum* through various inflammatory systems has not been thoroughly investigated. The purpose of the study was to evaluate the anti-inflammatory activities of crude extracts, fractions and isolated compounds from *P. obliquum* leaves from various locations in South Africa. *P. obliquum* leaf extracts in terms of acetone and aqueous extracts (prepared with hot and cold water), fractions (hexane and chloroform), and isolated chemicals (obliquumol, O-methylalloptaeroxylin, and a combination of lupeol and α-amyrin) were all tested for their ability to alleviate inflammation in different *in vitro* assays. The effects of the test substances on pro-inflammatory cytokines, such as interleukin (IL)-1 and TNF-α, as well as the anti-inflammatory cytokine (IL-10) production using ELISA kits were examined in this study. The methods used included the inhibition of soybean 15-lipoxygenase (15-LOX), nitric oxide (NO) inhibition in lipopolysaccharide (LPS)-induced RAW 264.7 macrophages and cyclooxygenase (COX)-2 enzyme activity using an ELISA method. Ultra-Performance Liquid Chromatography coupled to quadrupole time-of-flight mass spectrometry (UPLC-qTof-MS) analysis was carried out on the *P. obliquum* acetone leaf extracts from different geographical locations to identify compounds present.

The 15-LOX inhibitory activity of all acetone extracts was higher than that of the positive control and aqueous extracts, with IC_{50} values ranging from 5.7 to 10.4 µg/mL. With an IC_{50} of 3.66 µg/mL, obliquumol inhibited 15-LOX at the highest level and twice as effectively as quercetin (positive control). The isolated compounds, fractions, and extracts from *P. obliquum* all reduced NO production in a dose-dependent manner at the measured concentration. At the lowest measured concentration (1.6 µg/mL), the acetone extracts reduced nitric oxide with percentages as high as 95% and 102% on Walter Sisulu and Nelspruit acetone extracts, respectively. The high cell viability of >100% RAW 264.7 macrophage cells confirmed that the high concentration of NO was not caused by acetone extracts being toxic to the cells. When compared to acetone extracts from Walter Sisulu and the positive control quercetin, Hatfield's *P. obliquum* substantially reduced COX-2 production at 100 µg/mL. The most active compound, obliquumol, reduced COX-2 synthesis by as much as 69.3% and 93.4% at 50 µg/mL and 100 µg/mL, respectively. The synthesis of IL-1 was reduced by *P. obliquum* acetone leaf extracts and two tested compounds.

7.1.3 *Ptaeroxylon obliquum* acetone leaf extracts and obliquumol as potential apoptosis mediators in HepG2 and HeLa by modulating caspase-3 and TNF-α

Objective: To determine the possible anti-cancer mechanism of action of the most active samples against TNF-α and caspase-3.

Approximately 20% of cancers are induced by chronic inflammation or other infections. Anti-proliferative agents such as *P. obliquum* acetone extracts and obliquumol can induce selective cytotoxicity against cancer cell lines, without causing much harm to normal cells. Therefore, there was a need to investigate the possible mechanism of action against cancer cells. The purpose of the study was to investigate for the first time how pro-apoptotic cytokines (caspase-3 and TNF-α) and morphological changes are affected by acetone extracts and an isolated compound from *P. obliquum* leaves. Using ELISA kits, the effects of the substances on levels of caspase-3 and TNF-α were identified and quantified. The morphological alterations in HepG2 and HeLa cells were observed using light microscopy. *P. obliquum* acetone extracts and the isolated compound obliquumol stimulated the production of

caspase-3 in HepG2 cells by 2% at 100 μg/mL. Caspase-3 production on HeLa cells was increased by 8% at 100 μg/mL by the isolated compound obliquumol. In comparison to the effect of the positive control drug, doxorubicin, the Hatfield acetone extract (100 μg/mL) increased TNF production in HepG2 cells by almost 10%. Walter Sisulu acetone extracts improved TNF-α production on HeLa cells by 11.5% at the lowest tested concentration (50 μg/mL), outperforming the positive control. However, the differences were not statistically significant so further research is needed. Both of the evaluated cell lines showed a concentration-dependent effect on their morphology. HepG2 cells, the liver carcinoma cells, were more likely than HeLa cervical cancer cells to experience significant cell death and morphological changes.

7.1.4 Correlation between the antioxidant, anti-inflammatory and anti-proliferative activity of crude extracts, fractions and compounds derived from *P. obliquum*

Objective: To establish correlations between antioxidant, anti-inflammatory and anti-proliferative activities of *P. obliquum* crude extracts, fractions and isolated compounds.

P. obliquum extracts were cytotoxic against selected cancer cell lines and non-toxic against normal non-cancerous cells at the same concentrations, thus explaining the correlation between cancer and normal cells. The existence of secondary metabolites in the plant, such as obliquumol, which was active as an anti-inflammatory and anti-proliferative agent, may explain the significant correlation between the efficacy of *P. obliquum* extracts against inflammation and proliferation. The relationship between anti-inflammatory, antioxidant and anti-proliferative activities may therefore be caused by oxidative stress.

7.2 Conclusion and recommendations for future study

Antiproliferative agents such as *P. obliquum* acetone extracts that can induce selective cytotoxicity against cancer cell lines, without causing much harm to normal cells, are highly desirable for therapeutic purposes and may be considered in the development of novel cancer chemotherapeutic drugs. Geographical location didn't seem to have an impact on the anti-inflammatory properties of *P. obliquum* acetone leaf extracts collected from various locations. *P. obliquum* acetone extract, fractions,

and isolated compounds may reduce inflammation by activating anti-inflammatory cytokines, and decreasing NO and 15-LOX as well as pro-inflammatory cytokines. However, it is necessary to investigate the plant's activity on COX-2 and iNOS enzyme expression, as well as cytokines implicated in causing inflammation, using Real-Time (RT) PCR and Western blotting in order to fully comprehend the mechanism of action. It is necessary to conduct further research using *in vivo* models since various components of *P. obliquum* may be responsible for its historical use in treating inflammation-related disorders and symptoms.

According to these findings, *P. obliquum* treatment raised the percentage of caspase-3 and TNF production, two important apoptotic mediators. Additionally, it was clear that *P. obliquum* considerably altered the morphology of HepG2 and HeLa cancerous cells. The current findings thus suggest that *P. obliquum* extracts may be able to substantially contribute to the treatment of some cancers by enhancing the efficacy of standard chemotherapy medicines. Thus, the correlation between anti-proliferation, anti-inflammation, and antioxidant activity is supported by all of these findings. To pinpoint the precise mechanism of action underlying anti-cancer and apoptotic actions, more research is required.

Furthermore, potential synergistic interactions of the extracts, fractions and purified compounds in combination with known anticancer drugs, particularly against cancer cell lines expressing resistant phenotypes, would be useful. Enhancement of the activity of current drugs, or reduction in negative side effects such as damage to liver cells, could be a useful outcome. Additionally, *in vivo* tests to confirm safety and efficacy of selected potential therapeutic preparations derived from *P. obliquum* for use in prevention or treatment of cancer of inflammation-related illnesses would be beneficial.

Chapter 8

References

ABUDU, R. M., CIRA, M. K., PYLE, D. H. & DUNCAN, K. 2019. Landscape of global oncology research and training at national cancer institute–designated cancer centers: results of the 2018 to 2019 global oncology survey. *Journal of Global Oncology,* 5**,** 1-8.

ABUTO, J., MUCHUGI, A., MBURU, D., MACHOCHO, A. & KARAU, G. 2016. Variation in antimicrobial activity of *Warburgia ugandensis* extracts from different populations across the Kenyan Rift Valley. *J Microbiol Res,* 6**,** 55-64.

ADEBAYO, S. A., DZOYEM, J. P., SHAI, L. J. & ELOFF, J. N. 2015. The anti-inflammatory and antioxidant activity of 25 plant species used traditionally to treat pain in southern African. *BMC Complementary and Alternative Medicine,* 15**,** 1-10.

ADWAS, A. A., ELSAYED, A., AZAB, A. & QUWAYDIR, F. 2019. Oxidative stress and antioxidant mechanisms in human body. *J. Appl. Biotechnol. Bioeng,* 6**,** 43-47.

AGGARWAL, C. 2014. Targeted therapy for lung cancer: present and future. *Ann Palliat Med,* 3**,** 229-235.

AGOSTINHO, D., BOUDESOCQUE, L., THERY-KONE, I., DEBIERRE-GROCKIEGO, F., GUEIFFIER, A., ENGUEHARD-GUEIFFIER, C. & ALLOUCHI, H. 2013. A new meroterpenoid isolated from roots of *Ptaeroxylon obliquum* Radlk. *Phytochemistry Letters,* 6**,** 560-566.

AHLUWALIA, V. K., JAIN, A. & GUPTA, R. 1982. A Convenient Synthesis of Linear 2-Methylpyranochromones. *Bulletin of the Chemical Society of Japan,* 55**,** 2649-2652.

APPLEQUIST, W. L., BRINCKMANN, J. A., CUNNINGHAM, A. B., HART, R. E., HEINRICH, M., KATERERE, D. R. & VAN ANDEL, T. 2020. Scientists' warning on climate change and medicinal plants. *Planta Medica,* 86**,** 10-18.

ARCHER, R. & REYNOLDS, Y. 2001. Ptaeroxylon obliquum (Thunb.) Radlk. *South Africa. National Biodiversity institute.(http://www.//bold. cf. ac. uk/Bot. DermFolder/Bot DermR/RUTA. html)*.

ARFIN, S., JHA, N. K., JHA, S. K., KESARI, K. K., RUOKOLAINEN, J., ROYCHOUDHURY, S., RATHI, B. & KUMAR, D. 2021. Oxidative stress in cancer cell metabolism. *Antioxidants,* 10**,** 642.

ARITA, M. 2016. Eosinophil polyunsaturated fatty acid metabolism and its potential control of inflammation and allergy. *Allergology International,* 65**,** 2-5.

ARTHUR, J. C. & JOBIN, C. 2013. The complex interplay between inflammation, the microbiota and colorectal cancer. *Gut Microbes,* 4**,** 253-258.

ARTUN, F. T., KARAGOZ, A., OZCAN, G., MELIKOGLU, G., ANIL, S., KULTUR, S. & SUTLUPINAR, N. In vitro anticancer and cytotoxic activities of some plant extracts on HeLa and Vero cell lines. Proceedings, 2017. 1019.

BARAF, H. S. 2007. Efficacy of the newest COX-2 selective inhibitors in rheumatic disease. *Current Pharmaceutical Design,* 13**,** 2228-2236.

BENZIE, I. F. & WACHTEL-GALOR, S. 2011. *Herbal medicine: biomolecular and clinical aspects,* Bundoora, Victoria, Australia, CRC Press/Taylor & Francis, Boca Raton (FL).

BERG, D. J., DAVIDSON, N., KÜHN, R., MÜLLER, W., MENON, S., HOLLAND, G., THOMPSON-SNIPES, L., LEACH, M. W. & RENNICK, D. 1996. Enterocolitis and colon cancer in interleukin-10-deficient mice are associated with aberrant cytokine production and CD4 (+) TH1-like responses. *The Journal of Clinical Investigation,* 98**,** 1010-1020.

BOPANA, N. & SAXENA, S. 2007. Asparagus racemosus—Ethnopharmacological evaluation and conservation needs. *Journal of Ethnopharmacology,* 110**,** 1-15.

BOSHTAM, M., ASGARY, S., KOUHPAYEH, S., SHARIATI, L. & KHANAHMAD, H. 2017. Aptamers against pro-and anti-inflammatory cytokines: a review. *Inflammation,* 40**,** 340-349.

BOTTING, R. 2003. COX-1 and COX-3 inhibitors. *Thrombosis Research,* 110**,** 269-272.

BRAND-WILLIAMS, W., CUVELIER, M.-E. & BERSET, C. 1995. Use of a free radical method to evaluate antioxidant activity. *LWT-Food science and Technology,* 28**,** 25-30.

BRAY, F., FERLAY, J., SOERJOMATARAM, I., SIEGEL, R. L., TORRE, L. A. & JEMAL, A. 2018. Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA: A Cancer Journal for Clinicians,* 68**,** 394-424.

BRUNE, K. & PATRIGNANI, P. 2015. New insights into the use of currently available non-steroidal anti-inflammatory drugs. *Journal of Pain Research,* 8**,** 105.

CAIN, K., BRATTON, S. B. & COHEN, G. M. 2002. The Apaf-1 apoptosome: a large caspase-activating complex. *Biochimie,* 84**,** 203-214.

CHARAMI, M. T., LAZARI, D., KARIOTI, A., SKALTSA, H., HADJIPAVLOU-LITINA, D. & SOULELES, C. 2008. Antioxidant and antiinflammatory activities of Sideritis perfoliata subsp. perfoliata (Lamiaceae). *Phytotherapy Research: An International Journal Devoted to Pharmacological and Toxicological Evaluation of Natural Product Derivatives,* 22**,** 450-454.

CHEN, D., XU, F., ZHANG, P., DENG, J., SUN, H., WEN, X. & LIU, J. 2017. Practical Synthesis of α-Amyrin, β-Amyrin, and Lupeol: The Potential Natural Inhibitors of Human Oxidosqualene Cyclase. *Arch Pharm (Weinheim),* 350.

CHEN, L., DENG, H., CUI, H., FANG, J., ZUO, Z., DENG, J., LI, Y., WANG, X. & ZHAO, L. 2018. Inflammatory responses and inflammation-associated diseases in organs. *Oncotarget,* 9**,** 7204.

CONFORTI, F., SOSA, S., MARRELLI, M., MENICHINI, F., STATTI, G. A., UZUNOV, D., TUBARO, A., MENICHINI, F. & DELLA LOGGIA, R. 2008. In vivo anti-inflammatory and in vitro antioxidant activities of Mediterranean dietary plants. *Journal of Ethnopharmacology,* 116**,** 144-151.

CORMINBOEUF, O. & LEROY, X. 2015. FPR2/ALXR agonists and the resolution of inflammation. *Journal of medicinal chemistry,* 58**,** 537-559.

COUSSENS, L. M. & WERB, Z. 2002. Inflammation and cancer. *Nature,* 420**,** 860-867.

CUZZOCREA, S., RILEY, D. P., CAPUTI, A. P. & SALVEMINI, D. 2001. Antioxidant therapy: a new pharmacological approach in shock, inflammation, and ischemia/reperfusion injury. *Pharmacological Reviews,* 53**,** 135-159.

DAI, F., MIAO, Q., ZHOU, B., YANG, L. & LIU, Z.-L. 2006. Protective effects of flavonols and their glycosides against free radical-induced oxidative hemolysis of red blood cells. *Life Sciences,* 78**,** 2488-2493.

DEL CARMEN PINTO, M., TEJEDA, A., DUQUE, A. L. & MACÍAS, P. 2007. Determination of lipoxygenase activity in plant extracts using a modified ferrous oxidation− xylenol orange assay. *Journal of Agricultural and Food Chemistry,* 55**,** 5956-5959.

DINARELLO, C. A. 2000. Proinflammatory cytokines. *Chest,* 118**,** 503-508.

DZOYEM, J. P. & ELOFF, J. N. 2015. Anti-inflammatory, anticholinesterase and antioxidant activity of leaf extracts of twelve plants used traditionally to alleviate pain and inflammation in South Africa. *Journal of Ethnopharmacology,* 160**,** 194-201.

ELISHA, I. L., DZOYEM, J.-P., MCGAW, L. J., BOTHA, F. S. & ELOFF, J. N. 2016. The anti-arthritic, anti-inflammatory, antioxidant activity and relationships with total phenolics and total flavonoids of nine South African plants used traditionally to treat arthritis. *BMC Complementary and Alternative medicine,* 16**,** 1-10.

ELMORE, S. 2007. Apoptosis: a review of programmed cell death. *Toxicologic Pathology,* 35**,** 495-516.

ELOFF, J. 1998. Which extractant should be used for the screening and isolation of antimicrobial components from plants? *Journal of Ethnopharmacology,* 60**,** 1-8.

GACCHE, R., SHAIKH, R., PUND, M. & DESHMUKH, R. 2011. Cyclooxygenase inhibitory, cytotoxicity and free radical scavenging activities of selected medicinal plants used in Indian traditional medicine. *Pharmacognosy Journal,* 3**,** 57-64.

GAUR, U. & AGGARWAL, B. B. 2003. Regulation of proliferation, survival and apoptosis by members of the TNF superfamily. *Biochemical Pharmacology,* 66**,** 1403-1408.
GHASEMIAN, M., OWLIA, S. & OWLIA, M. B. 2016. Review of anti-inflammatory herbal medicines. *Advances in pharmacological sciences,* 2016.
GHOBRIAL, I. M., WITZIG, T. E. & ADJEI, A. A. 2005. Targeting apoptosis pathways in cancer therapy. *CA: A Cancer Journal for Clinicians,* 55**,** 178-194.
GOLDAR, S., KHANIANI, M. S., DERAKHSHAN, S. M. & BARADARAN, B. 2015. Molecular mechanisms of apoptosis and roles in cancer development and treatment. *Asian Pacific Journal of Cancer Prevention,* 16**,** 2129-2144.
GRETEN, F. R. & GRIVENNIKOV, S. I. 2019. Inflammation and cancer: triggers, mechanisms, and consequences. *Immunity,* 51**,** 27-41.
GUICCIARDI, M. E. & GORES, G. J. 2009. Life and death by death receptors. *The FASEB Journal,* 23**,** 1625-1637.
HEINRICH, M. 2010. Ethnopharmacology in the 21st century-grand challenges. *Frontiers in Pharmacology,* 1**,** 8.
HENGARTNER, M. O. 2001. Apoptosis: corralling the corpses. *Cell,* 104**,** 325-328.
HO, K. Y., GWEE, K. A., CHENG, Y. K., YOON, K. H., HEE, H. T. & OMAR, A. R. 2018. Nonsteroidal anti-inflammatory drugs in chronic pain: implications of new data for clinical practice. *Journal of Pain Research,* 11**,** 1937.
HUTCHINGS, A. 1996. *Zulu medicinal plants: An inventory,* Scottsville, South Africa, University of Natal press.
IDRISS, H. T. & NAISMITH, J. H. 2000. TNFα and the TNF receptor superfamily: Structure-function relationship (s). *Microscopy Research and Technique,* 50**,** 184-195.
IQBAL, E., SALIM, K. A. & LIM, L. B. 2015. Phytochemical screening, total phenolics and antioxidant activities of bark and leaf extracts of Goniothalamus velutinus (Airy Shaw) from Brunei Darussalam. *Journal of King Saud University-Science,* 27**,** 224-232.
ISOMÄKI, P. & PUNNONEN, J. 1997. Pro-and anti-inflammatory cytokines in rheumatoid arthritis. *Annals of Medicine,* 29**,** 499-507.
IWALEWA, E., MCGAW, L., NAIDOO, V. & ELOFF, J. 2007. Inflammation: the foundation of diseases and disorders. A review of phytomedicines of South African origin used to treat pain and inflammatory conditions. *African Journal of Biotechnology,* 6.
IYER, S. S. & CHENG, G. 2012. Role of interleukin 10 transcriptional regulation in inflammation and autoimmune disease. *Critical Reviews™ in Immunology,* 32.
JUÁREZ, P. 2014. Plant-derived anticancer agents: a promising treatment for bone metastasis. *BoneKEy Reports,* 3.
JUCKMETA, T., PIPATRATTANASEREE, W., JAIDEE, W., DECHAYONT, B., CHUNTHORNG-ORN, J., ANDERSEN, R. J. & ITHARAT, A. 2019. Cytotoxicity to five cancer cell lines of the respiratory tract system and anti-inflammatory activity of Thai traditional remedy. *Natural Product Communications,* 14**,** 1934578X19845815.
KANDA, Y., OSAKI, M. & OKADA, F. 2017. Chemopreventive strategies for inflammation-related carcinogenesis: current status and future direction. *International Journal of Molecular Sciences,* 18**,** 867.
KARIN, M. 2006. Nuclear factor-κB in cancer development and progression. *Nature,* 441**,** 431-436.
KATTI, A., DIAZ, B. J., CARAGINE, C. M., SANJANA, N. E. & DOW, L. E. 2022. CRISPR in cancer biology and therapy. *Nat Rev Cancer,* 22**,** 259-279.
KAWAHARA, K., HOHJOH, H., INAZUMI, T., TSUCHIYA, S. & SUGIMOTO, Y. 2015. Prostaglandin E2-induced inflammation: Relevance of prostaglandin E receptors. *Biochimica et Biophysica Acta (BBA)-Molecular and Cell Biology of Lipids,* 1851**,** 414-421.
KAWAI, T. & AKIRA, S. 2011. Toll-like receptors and their crosstalk with other innate receptors in infection and immunity. *Immunity,* 34**,** 637-650.

KEIBEL, A., SINGH, V. & SHARMA, M. C. 2009. Inflammation, microenvironment, and the immune system in cancer progression. *Current Pharmaceutical Design,* 15**,** 1949-1955.
KHADER, Y. S., SHARKAS, G. F., ARKOUB, K. H., ALFAQIH, M. A., NIMRI, O. F. & KHADER, A. M. 2018. The epidemiology and trend of cancer in Jordan, 2000–2013. *Journal of Cancer Epidemiology,* 2018.
KHUMALO, G. P., VAN WYK, B. E., FENG, Y. & COCK, I. E. 2022. A review of the traditional use of Southern African medicinal plants for the treatment of inflammation and inflammatory pain. *Journal of Ethnopharmacology,* 283**,** 114436.
KHUNOANA, E. T., ELOFF, J. N., RAMADWA, T. E., NKADIMENG, S. M., SELEPE, M. A. & MCGAW, L. J. 2022. In Vitro Antiproliferative Activity of Ptaeroxylon obliquum Leaf Extracts, Fractions and Isolated Compounds on Several Cancer Cell Lines. *Applied Sciences,* 12**,** 11004.
KLIL-DRORI, A. J. & ARIEL, A. 2013. 15-Lipoxygenases in cancer: a double-edged sword? *Prostaglandins & Other Lipid Mediators,* 106**,** 16-22.
KUMAR, A. V., ANJULA, J. & RANJNA, G. 1982. A Convenient Synthesis of Linear 2-Methylpyranochromones. *Bulletin of the Chemical Society of Japan,* 55**,** 2649-2652.
LEE, J.-H. 2015. In-vitro evaluation for antioxidant and anti-inflammatory property of flavanone derivatives. *Food Bioscience,* 11**,** 1-7.
LI, P., ZHENG, Y. & CHEN, X. 2017. Drugs for autoimmune inflammatory diseases: from small molecule compounds to anti-TNF biologics. *Frontiers in Pharmacology,* 8**,** 460.
LIN, W.-W. & KARIN, M. 2007. A cytokine-mediated link between innate immunity, inflammation, and cancer. *The Journal of Clinical Investigation,* 117**,** 1175-1183.
LOPEZ, J. & TAIT, S. 2015. Mitochondrial apoptosis: killing cancer using the enemy within. *British Journal of Cancer,* 112**,** 957-962.
LUBUZO, B., HLONGWANA, K. & GININDZA, T. 2021. Cancer care reform in South Africa: A case for cancer care coordination: A narrative review. *Palliative & Supportive Care***,** 1-9.
LUBUZO, B., HLONGWANA, K. & GININDZA, T. 2022. Cancer care reform in South Africa: A case for cancer care coordination: A narrative review. *Palliative & Supportive Care,* 20**,** 129-137.
MADE, F., WILSON, K., JINA, R., TLOTLENG, N., JACK, S., NTLEBI, V. & KOOTBODIEN, T. 2017. Distribution of cancer mortality rates by province in South Africa. *Cancer Epidemiology,* 51**,** 56-61.
MAKUNGA, N., PHILANDER, L. & SMITH, M. 2008. Current perspectives on an emerging formal natural products sector in South Africa. *Journal of Ethnopharmacology,* 119**,** 365-375.
MALEFO, M. S., RAMADWA, T. E., FAMUYIDE, I. M., MCGAW, L. J., ELOFF, J. N., SONOPO, M. S. & SELEPE, M. A. 2020. Synthesis and Antifungal Activity of Chromones and Benzoxepines from the Leaves of Ptaeroxylon obliquum. *Journal of Natural Products,* 83**,** 2508-2517.
MANDAL, R. & BASU, P. 2018. Cancer screening and early diagnosis in low and middle income countries. *Bundesgesundheitsblatt-Gesundheitsforschung-Gesundheitsschutz,* 61**,** 1505-1512.
MANDER, M. 1998. Marketing of indigenous medicinal plants in South Africa: a case study in KwaZulu-Natal.
MANTOVANI, A., ALLAVENA, P., SICA, A. & BALKWILL, F. 2008. Cancer-related inflammation. *Nature,* 454**,** 436-444.
MARKHAM, M. J., WACHTER, K., AGARWAL, N., BERTAGNOLLI, M. M., CHANG, S. M., DALE, W., DIEFENBACH, C. S., RODRIGUEZ-GALINDO, C., GEORGE, D. J. & GILLIGAN, T. D. 2020. Clinical cancer advances 2020: annual report on progress against cancer from the American Society of Clinical Oncology. *Journal of Clinical Oncology,* 38**,** 1081.
MEDZHITOV, R. 2008. Origin and physiological roles of inflammation. *Nature,* 454**,** 428-435.
MENSOR, L. L., MENEZES, F. S., LEITÃO, G. G., REIS, A. S., SANTOS, T. C. D., COUBE, C. S. & LEITÃO, S. G. 2001. Screening of Brazilian plant extracts for antioxidant activity by the use of DPPH free radical method. *Phytotherapy Research,* 15**,** 127-130.

METZCAR, J., WANG, Y., HEILAND, R. & MACKLIN, P. 2019. A review of cell-based computational modeling in cancer biology. *JCO Clinical Cancer Informatics,* 2**,** 1-13.
MOSMANN, T. 1983. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *Journal of Immunological Methods,* 65**,** 55-63.
MOYO, B. & MASIKA, P. 2009. Tick control methods used by resource-limited farmers and the effect of ticks on cattle in rural areas of the Eastern Cape Province, South Africa. *Tropical Animal Health and Production,* 41**,** 517-523.
MUKESHWAR, P., MOUSUMI, D., SHOBIT, G. & SURENDER, K. C. 2011. Phytomedicine: An ancient approach turning into future potential source of therapeutics. *Journal of Pharmacognosy and Phytotherapy,* 3**,** 27-37.
MUNIANDY, K., GOTHAI, S., BADRAN, K. M., SURESH KUMAR, S., ESA, N. M. & ARULSELVAN, P. 2018. Suppression of proinflammatory cytokines and mediators in LPS-induced RAW 264.7 macrophages by stem extract of Alternanthera sessilis via the inhibition of the NF-κB pathway. *Journal of Immunology Research,* 2018.
MUNN, L. L. 2017. Cancer and inflammation. *Wiley Interdisciplinary Reviews: Systems Biology and Medicine,* 9**,** e1370.
MURATA, M. 2018. Inflammation and cancer. *Environmental Health And Preventive Medicine,* 23**,** 1-8.
NABATANZI, A., NKADIMENG, S. M., LALL, N., KABASA, J. D. & MCGAW, L. J. 2020. Antioxidant and Anti-Inflammatory Activities of Kigelia africana (Lam.) Benth. *Evidence-Based Complementary and Alternative Medicine,* 2020.
NANJUNDAIAH, Y. S., WRIGHT, D. A., BAYDOUN, A. R., O'HARE, W. T., ALI, Z., KHALED, Z. & SARKER, M. H. 2016. Lactobacillus rhamnosus GG conditioned media modulates acute reactive oxygen species and nitric oxide in J774 murine macrophages. *Biochemistry and Biophysics Reports,* 6**,** 68-75.
NEWMAN, D. J., CRAGG, G. M., HOLBECK, S. & SAUSVILLE, E. A. 2002. Natural products and derivatives as leads to cell cycle pathway targets in cancer chemotherapy. *Current Cancer Drug Targets,* 2**,** 279-308.
NIELSEN, T. R., KUETE, V., JÄGER, A. K., MEYER, J. J. M. & LALL, N. 2012. Antimicrobial activity of selected South African medicinal plants. *BMC Complementary and Alternative medicine,* 12**,** 1-6.
NKADIMENG, S. M., NABATANZI, A., STEINMANN, C. M. & ELOFF, J. N. 2020. Phytochemical, cytotoxicity, antioxidant and anti-inflammatory effects of *Psilocybe natalensis* magic mushroom. *Plants,* 9**,** 1127.
NKADIMENG, S. M., STEINMANN, C. M. & ELOFF, J. N. 2021. Anti-Inflammatory Effects of Four Psilocybin-Containing Magic Mushroom Water Extracts in vitro on 15-Lipoxygenase Activity and on Lipopolysaccharide-Induced Cyclooxygenase-2 and Inflammatory Cytokines in Human U937 Macrophage Cells. *Journal of Inflammation Research,* 14**,** 3729.
NODA, N. & WAKASUGI, H. 2001. Cancer and oxidative stress. *Japan Medical Association Journal,* 44**,** 535-539.
NWORU, C. S. & AKAH, P. A. 2015. Anti-inflammatory medicinal plants and the molecular mechanisms underlying their activities. *African Journal of Traditional, Complementary and Alternative Medicines,* 12**,** 52-61.
O'BRIEN, M. A. & KIRBY, R. 2008. Apoptosis: A review of pro-apoptotic and anti-apoptotic pathways and dysregulation in disease. *Journal of Veterinary Emergency and Critical Care,* 18**,** 572-585.
O'BYRNE, K. J. & DALGLEISH, A. G. 2001. Chronic immune activation and inflammation as the cause of malignancy. *British Journal of Cancer,* 85**,** 473-483.
OGBOLE, O. O., SEGUN, P. A. & ADENIJI, A. J. 2017. In vitro cytotoxic activity of medicinal plants from Nigeria ethnomedicine on Rhabdomyosarcoma cancer cell line and HPLC analysis of active extracts. *BMC Complementary and Alternative Medicine,* 17**,** 1-10.

OKORIE, D. A. 1982. Chromones and limonoids from Harrisonia abyssinica. *Phytochemistry,* 21**,** 2424-2426.

ONDO, J. P., LEKANA-DOUKI, J.-B., BONGUI, J.-B., ZANG EDOU, E., ZATRA, R., TOURE-NDOUO, F. S., ELOMRI, A., LEBIBI, J. & SEGUIN, E. 2012. In vitro antiplasmodial activity and cytotoxicity of extracts and fractions of Vitex madiensis, medicinal plant of Gabon. *Tropical Medicine & International Health,* 17**,** 316-321.

ONDUA, M., NJOYA, E. M., ABDALLA, M. A. & MCGAW, L. J. 2019. Anti-inflammatory and antioxidant properties of leaf extracts of eleven South African medicinal plants used traditionally to treat inflammation. *Journal of Ethnopharmacology,* 234**,** 27-35.

ONUMA, K., SUENAGA, Y., SAKAKI, R., YOSHITOME, S., SATO, Y., OGAWARA, S., SUZUKI, S., KURAMITSU, Y., YOKOYAMA, H. & MURAKAMI, A. 2011. Development of a quantitative bioassay to assess preventive compounds against inflammation-based carcinogenesis. *Nitric Oxide,* 25**,** 183-194.

ORGANIZATION, W. H. 2019. International agency for research on cancer.

OZGEN, M., REESE, R. N., TULIO, A. Z., SCHEERENS, J. C. & MILLER, A. R. 2006. Modified 2, 2-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS) method to measure antioxidant capacity of selected small fruits and comparison to ferric reducing antioxidant power (FRAP) and 2, 2 '-diphenyl-1-picrylhydrazyl (DPPH) methods. *Journal of Agricultural and Food Chemistry,* 54**,** 1151-1157.

PARK, E. J., KIM, Y. M., KIM, H. J. & CHANG, K. C. 2018. Luteolin activates ERK1/2-and Ca2+-dependent HO-1 induction that reduces LPS-induced HMGB1, iNOS/NO, and COX-2 expression in RAW264. 7 cells and mitigates acute lung injury of endotoxin mice. *Inflammation Research,* 67**,** 445-453.

PEARSON, K. 1895. VII. Note on regression and inheritance in the case of two parents. *proceedings of the royal society of London,* 58**,** 240-242.

PHONGPAICHIT, S., NIKOM, J., RUNGJINDAMAI, N., SAKAYAROJ, J., HUTADILOK-TOWATANA, N., RUKACHAISIRIKUL, V. & KIRTIKARA, K. 2007. Biological activities of extracts from endophytic fungi isolated from *Garcinia* plants. *FEMS Immunology & Medical Microbiology,* 51**,** 517-525.

PINLAOR, S., MA, N., HIRAKU, Y., YONGVANIT, P., SEMBA, R., OIKAWA, S., MURATA, M., SRIPA, B., SITHITHAWORN, P. & KAWANISHI, S. 2004. Repeated infection with Opisthorchis viverrini induces accumulation of 8-nitroguanine and 8-oxo-7, 8-dihydro-2'-deoxyguanine in the bile duct of hamsters via inducible nitric oxide synthase. *Carcinogenesis,* 25**,** 1535-1542.

PORRO, C., CIANCIULLI, A. & PANARO, M. A. 2020. The regulatory role of IL-10 in neurodegenerative diseases. *Biomolecules,* 10**,** 1017.

PORTER, A. G. & JÄNICKE, R. U. 1999. Emerging roles of caspase-3 in apoptosis. *Cell Death and Differentiation,* 6**,** 99-104.

POYTON, R. O., BALL, K. A. & CASTELLO, P. R. 2009. Mitochondrial generation of free radicals and hypoxic signaling. *Trends in Endocrinology & Metabolism,* 20**,** 332-340.

PREISER, J. C. 2012. Oxidative stress. *Journal of Parenteral and Enteral Nutrition,* 36**,** 147-154.

PUJOL, J. 1990. *The herbalist handbook: African Flora medicinal plants,* Ithaca, New York, United States of America., Natural Healers Foundation.

RAHMAN, M., HANNAN, M. A., DASH, R., RAHMAN, M., ISLAM, R., UDDIN, M. J., SOHAG, A. A. M. & RHIM, H. 2021. Phytochemicals as a complement to cancer chemotherapy: pharmacological modulation of autophagy-apoptosis pathway. *Frontiers in Pharmacology,* 12**,** 718.

RAMADWA, T., DZOYEM, J., ADEBAYO, S. & ELOFF, J. 2022. *Ptaeroxylon obliquum* leaf extracts, fractions and isolated compounds as potential inhibitors of 15-lipoxygenase and lipopolysaccharide-induced nitric oxide production in RAW 264.7 macrophage cells. *South African Journal of Botany,* 147**,** 192-196.

RAMADWA, T., ELGORASHI, E., MCGAW, L., AHMED, A. & ELOFF, J. 2017. Antimicrobial, anti-inflammatory activity and cytotoxicity of Funtumia africana leaf extracts, fractions and the isolated methyl ursolate. *South African Journal of Botany,* 108**,** 126-131.

RAMADWA, T. E. 2010. *Isolation and characterization of antimicrobial compounds from Funtumia africana (Apocynaceae) leaf extracts.* University of Pretoria.
RAMADWA, T. E., AWOUAFACK, M. D., SONOPO, M. S. & ELOFF, J. N. 2019. Antibacterial and antimycobacterial activity of crude extracts, fractions, and isolated compounds from leaves of sneezewood, ptaeroxylon obliquum (rutaceae). *Natural Product Communications,* 14**,** 1934578X19872927.
RAMADWA, T. E., MCGAW, L. J., ADAMU, M., MADIKIZELA, B. & ELOFF, J. N. 2021. Anthelmintic, antimycobacterial, antifungal, larvicidal and cytotoxic activities of acetone leaf extracts, fractions and isolated compounds from *Ptaeroxylon obliquum* (Rutaceae). *Journal of Ethnopharmacology,* 280**,** 114365.
RIBALDONE, D. G., PELLICANO, R. & ACTIS, G. C. 2018. Inflammation: A highly conserved, Janus-like phenomenon—A gastroenterologist'perspective. *Journal of Molecular Medicine,* 96**,** 861-871.
RIBEIRO, A., ROMEIRAS, M. M., TAVARES, J. & FARIA, M. T. 2010. Ethnobotanical survey in Canhane village, district of Massingir, Mozambique: medicinal plants and traditional knowledge. *Journal of Ethnobiology and Ethnomedicine,* 6**,** 1-15.
RIOS, J.-L. & RECIO, M. C. 2005. Medicinal plants and antimicrobial activity. *Journal of Ethnopharmacology,* 100**,** 80-84.
RUDDON, R. W. 2007. *Cancer biology*, Oxford University Press.
SAK, K. 2012. Chemotherapy and dietary phytochemical agents. *Chemotherapy Research and Practice,* 2012.
SAMUELSSON, B. Prostaglandins and thromboxanes. Proceedings of the 1977 Laurentian Hormone Conference, 1978. Elsevier, 239-258.
SCHETTER, A. J., HEEGAARD, N. H. & HARRIS, C. C. 2010. Inflammation and cancer: interweaving microRNA, free radical, cytokine and p53 pathways. *Carcinogenesis,* 31**,** 37-49.
SCHNEIDER, C. & POZZI, A. 2011. Cyclooxygenases and lipoxygenases in cancer. *Cancer and Metastasis Reviews,* 30**,** 277-294.
SCHNEIDER, I. & BUCAR, F. 2005. Lipoxygenase inhibitors from natural plant sources. Part 1: Medicinal plants with inhibitory activity on arachidonate 5-lipoxygenase and 5-lipoxygenase [sol] cyclooxygenase. *Phytotherapy research: An international journal devoted to pharmacological and toxicological evaluation of natural product derivatives,* 19**,** 81-102.
SCHOBER, P., BOER, C. & SCHWARTE, L. A. 2018. Correlation coefficients: appropriate use and interpretation. *Anesthesia & Analgesia,* 126**,** 1763-1768.
SHAH, S. C., KAYAMBA, V., PEEK JR, R. M. & HEIMBURGER, D. 2019. Cancer control in low-and middle-income countries: is it time to consider screening? *Journal of Global Oncology,* 5**,** 1-8.
SICA, A., LARGHI, P., MANCINO, A., RUBINO, L., PORTA, C., TOTARO, M. G., RIMOLDI, M., BISWAS, S. K., ALLAVENA, P. & MANTOVANI, A. Macrophage polarization in tumour progression. Seminars in cancer biology, 2008. Elsevier, 349-355.
SILAMBARASAN, R. & AYYANAR, M. 2015. An ethnobotanical study of medicinal plants in Palamalai region of Eastern Ghats, India. *Journal of Ethnopharmacology,* 172**,** 162-178.
SINGH, P. P. & CHAUHAN, S. 2009. Activity guided isolation of antioxidants from the leaves of Ricinus communis L. *Food Chemistry,* 114**,** 1069-1072.
SUFFNESS, M. 1979. Drugs of plant origin. *Methods Cancer Res.,* 26**,** 73-126.
SUNG, H., FERLAY, J., SIEGEL, R. L., LAVERSANNE, M., SOERJOMATARAM, I., JEMAL, A. & BRAY, F. 2021. Global cancer statistics 2020: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA: A Cancer Journal for Clinicians,* 71**,** 209-249.
TAN, A. C., KONCZAK, I., SZE, D. M.-Y. & RAMZAN, I. 2011. Molecular pathways for cancer chemoprevention by dietary phytochemicals. *Nutrition and Cancer,* 63**,** 495-505.
TANIGUCHI, K. & KARIN, M. IL-6 and related cytokines as the critical lynchpins between inflammation and cancer. Seminars in immunology, 2014. Elsevier, 54-74.

THEODULOZ, C., ALZATE-MORALES, J., JIMÉNEZ-ASPEE, F., ISLA, M. I., ALBERTO, M. R., PERTINO, M. W. & SCHMEDA-HIRSCHMANN, G. 2019. Inhibition of key enzymes in the inflammatory pathway by hybrid molecules of terpenes and synthetic drugs: In vitro and in silico studies. *Chemical Biology & Drug Design,* 93**,** 290-299.

TWILLEY, D., RADEMAN, S. & LALL, N. 2020. A review on traditionally used South African medicinal plants, their secondary metabolites and their potential development into anticancer agents. *Journal of Ethnopharmacology,* 261**,** 113101.

UEDA, N., SUZUKI, H. & YAMAMOTO, S. 1998. Mammalian lipoxygenases: structure, function and evolutionary aspects. *Eicosanoids and Related Compounds in Plants and Animals***,** 47-68.

VAN FURTH, R. 2013. *Mononuclear phagocytes: functional aspects*, Springer Science & Business Media.

VAN WYK, C. 2012. *In vitro biological activity of extracts and compounds from Ptaeroxylon obliquum (Thunb.) Radlk. against oral strains of Candida albicans.* University of Pretoria.

VAN WYK, C., BOTHA, F. S., VLEGGAAR, R. & ELOFF, J. N. 2018. Obliquumol, a novel antifungal and a potential scaffold lead compound, isolated from the leaves of *Ptaeroxylon obliquum* (sneezewood) for treatment of *Candida albicans* infections. *Suid-Afrikaanse Tydskrif vir Natuurwetenskap en Tegnologie,* 37**,** 1-5.

VANE, J., BAKHLE, Y. & BOTTING, R. 1998. CYCLOOXYGENASES 1 AND 2. *Annual Review of Pharmacology and Toxicology,* 38**,** 97-120.

VENTER, F. & VENTER, J. 2002. Making the most of indigenous trees, Briza Publications. *Pretoria, South Africa.[Google Scholar]*.

VON AHLEFELD, D. & GORDON-GRAY, K. 2003. *Medicinal plants traded on South Africa's eastern seaboard*, Ethekwini Parks Department.

WANG, J., MARKOVA, D., ANDERSON, D. G., ZHENG, Z., SHAPIRO, I. M. & RISBUD, M. V. 2011. TNF-α and IL-1β promote a disintegrin-like and metalloprotease with thrombospondin type I motif-5-mediated aggrecan degradation through syndecan-4 in intervertebral disc. *Journal of Biological Chemistry,* 286**,** 39738-39749.

WANG, Y., LI, Y., CAO, H., XIONG, M., SHUGART, Y. Y. & JIN, L. 2015. Efficient test for nonlinear dependence of two continuous variables. *BMC Bioinformatics,* 16**,** 1-8.

WARIS, G. & AHSAN, H. 2006. Reactive oxygen species: role in the development of cancer and various chronic conditions. *Journal of Carcinogenesis,* 5**,** 14.

WATT, J. M. & BREYER-BRANDWIJK, M. G. 1962. *The Medicinal and Poisonous Plants of Southern and Eastern Africa being an Account of their Medicinal and other Uses, Chemical Composition, Pharmacological Effects and Toxicology in Man and Animal,* 16-17, Teviot Place, Edinburgh, United Kingdom, E. & S. Livingstone Ltd.

WONG, R. S. 2011. Apoptosis in cancer: from pathogenesis to treatment. *Journal of Experimental & Clinical Cancer Research,* 30**,** 1-14.

WONG, Y. H., ABDUL KADIR, H. & LING, S. K. 2012. Bioassay-guided isolation of cytotoxic cycloartane triterpenoid glycosides from the traditionally used medicinal plant *Leea indica*. *Evidence-Based Complementary and Alternative Medicine,* 2012.

ZARGHI, A. & ARFAEI, S. 2011. Selective COX-2 inhibitors: a review of their structure-activity relationships. *Iranian Journal of Pharmaceutical Research: IJPR,* 10**,** 655.

ZHANG, J.-M. & AN, J. 2007. Cytokines, inflammation and pain. *International Anesthesiology Clinics,* 45**,** 27.

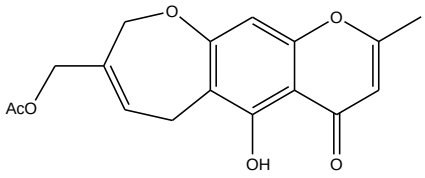
ZHANG, Z., ZHOU, L., XIE, N., NICE, E. C., ZHANG, T., CUI, Y. & HUANG, C. 2020. Overcoming cancer therapeutic bottleneck by drug repurposing. *Signal Transduction and Targeted Therapy,* 5**,** 1-25.

ZHAO, R., LIANG, H., CLARKE, E., JACKSON, C. & XUE, M. 2016. Inflammation in chronic wounds. *International Journal of Molecular Sciences,* 17**,** 2085.

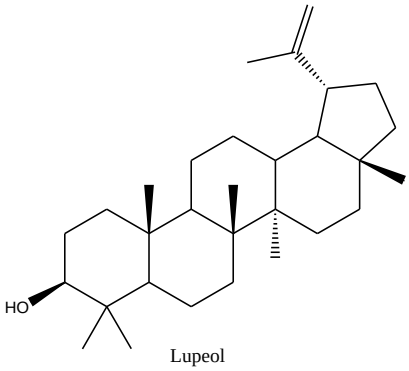
ZOU, J., JIN, D., CHEN, W., WANG, J., LIU, Q., ZHU, X. & ZHAO, W. 2005. Selective Cyclooxygenase-2 Inhibitors from Calophyllum m embranaceum. *Journal of Natural Products,* 68**,** 1514-1518.

ZOU, K. H., TUNCALI, K. & SILVERMAN, S. G. 2003. Correlation and simple linear regression. *Radiology,* 227**,** 617-628.

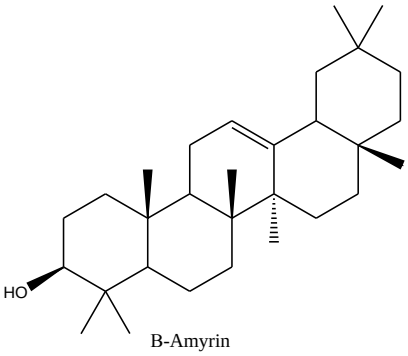
Appendix



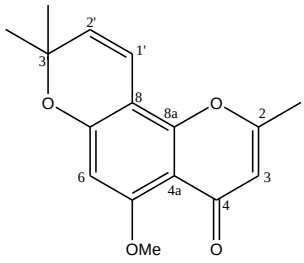
Obliquumol (12-*O*-acetylptaeroxylinol)



Lupeol



B-Amyrin



O-Methylalloptaeroxylin

Figure A1. Structures of isolated compounds from *P. obliquum* leaves.

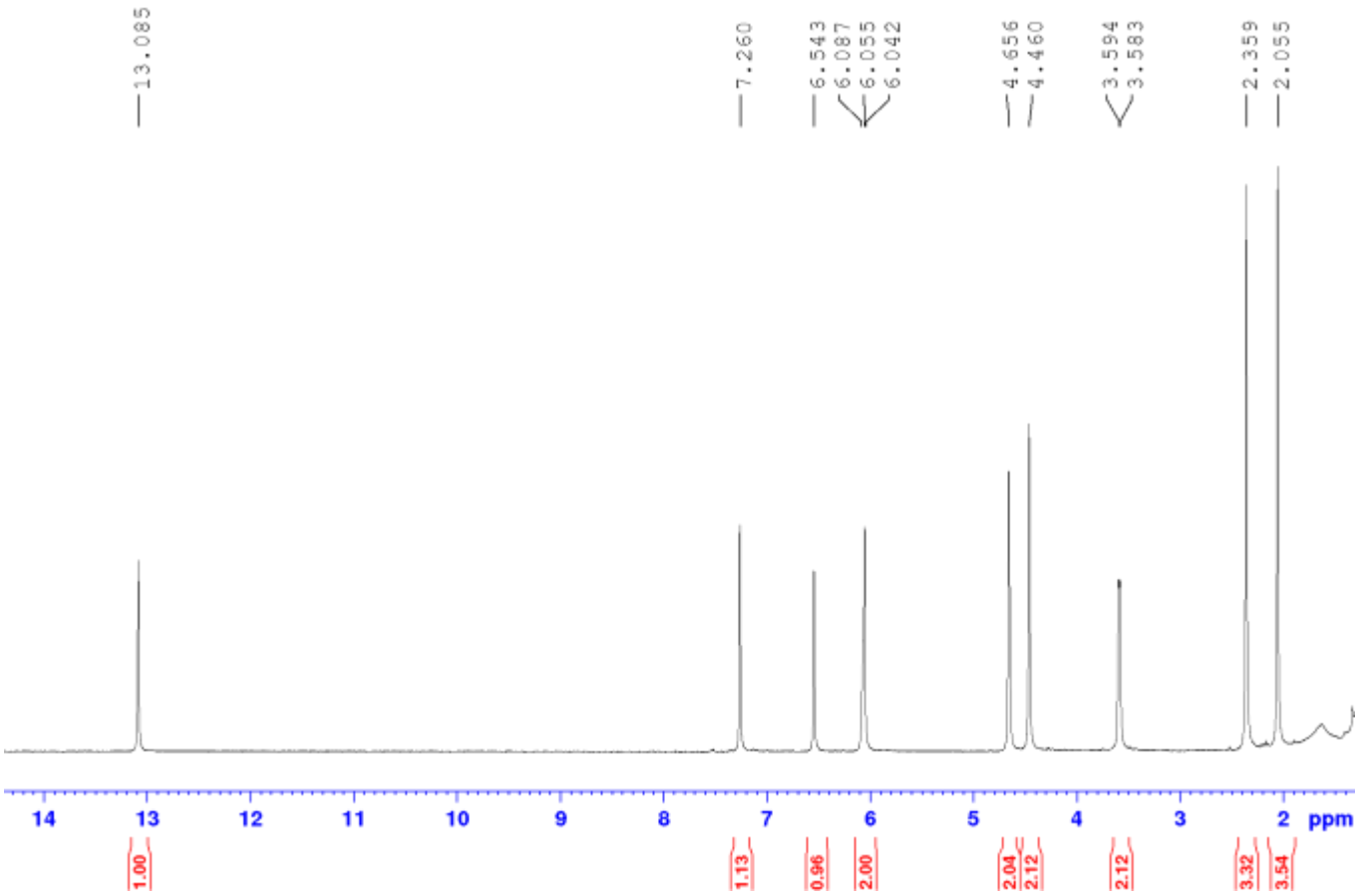


Figure A2. ^{1}H NMR spectrum of obliquumol isolated from *P. obliquum* ($CDCl_3$)

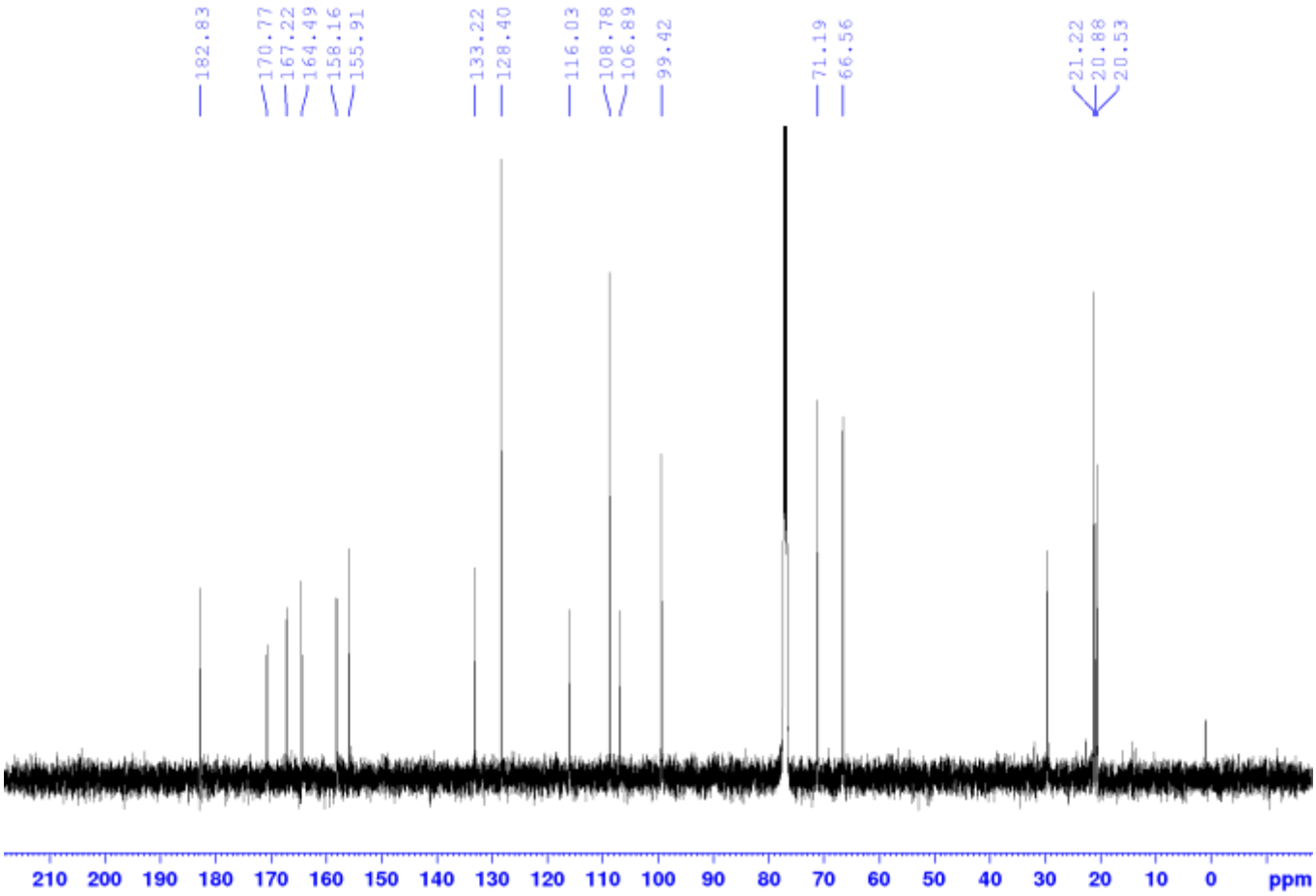


Figure A3. ^{13}C NMR spectrum of obliquumol isolated from *P. obliquum* ($CDCl_3$)

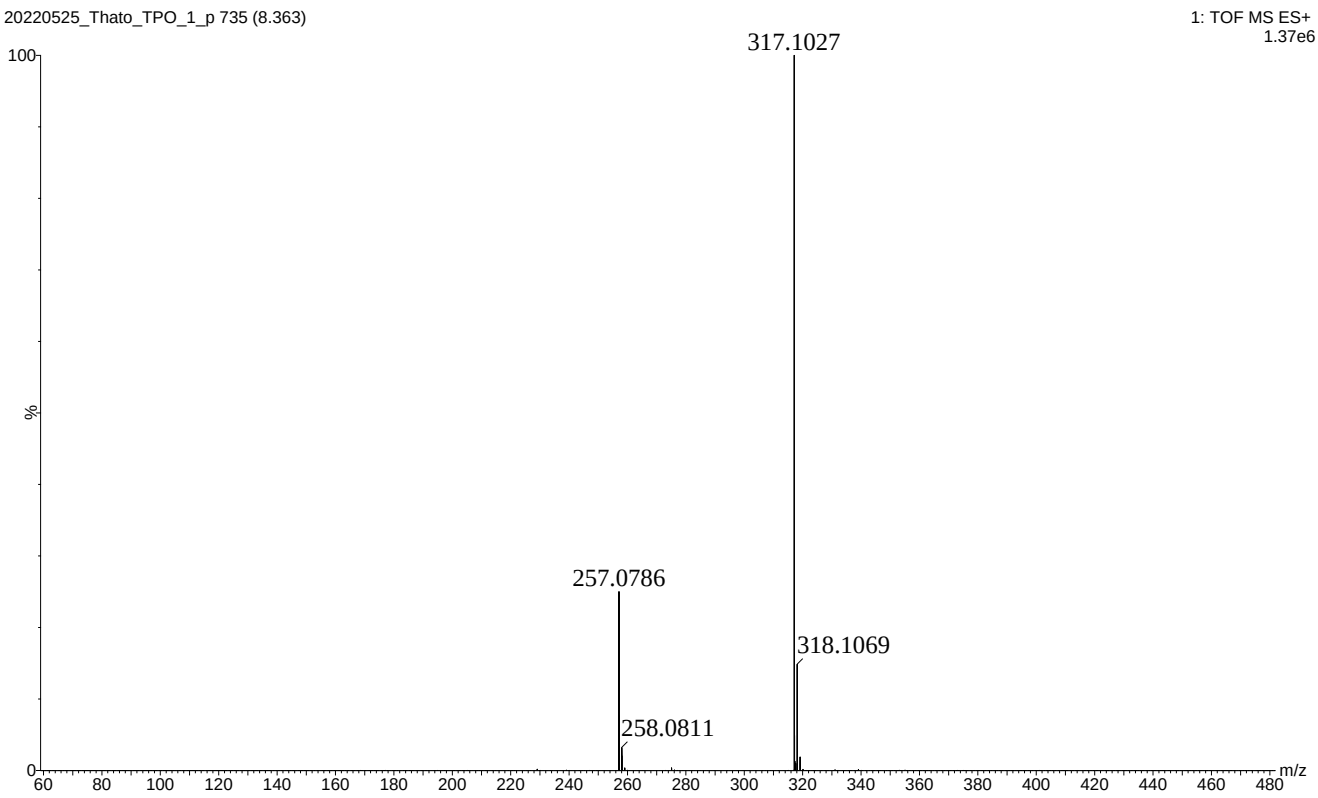


Figure A4. HRESIMS of obliquumol

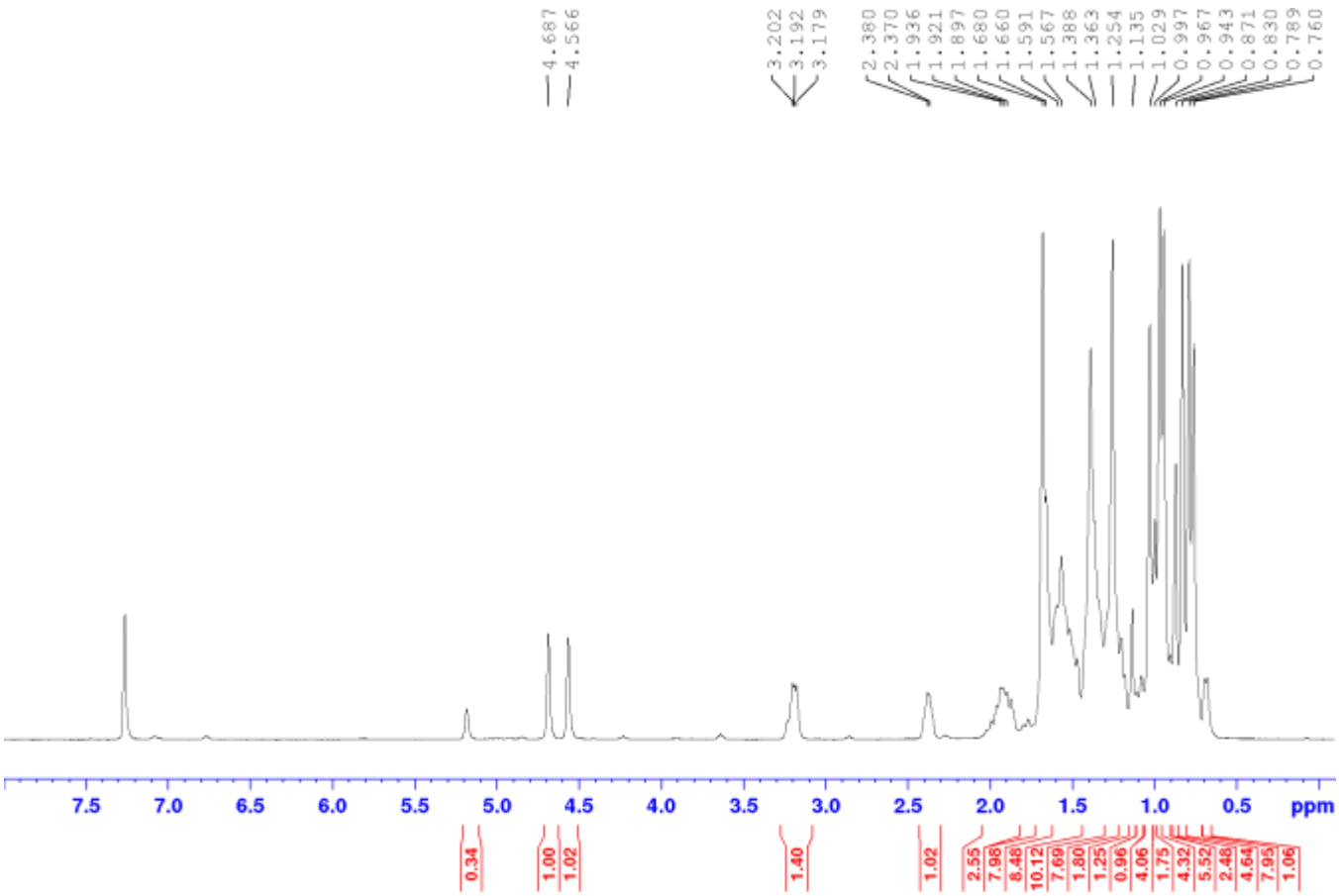


Figure A5. ^{1}H NMR spectrum of lupeol and β-amyrin isolated from *P. obliquum* ($CDCl_3$)

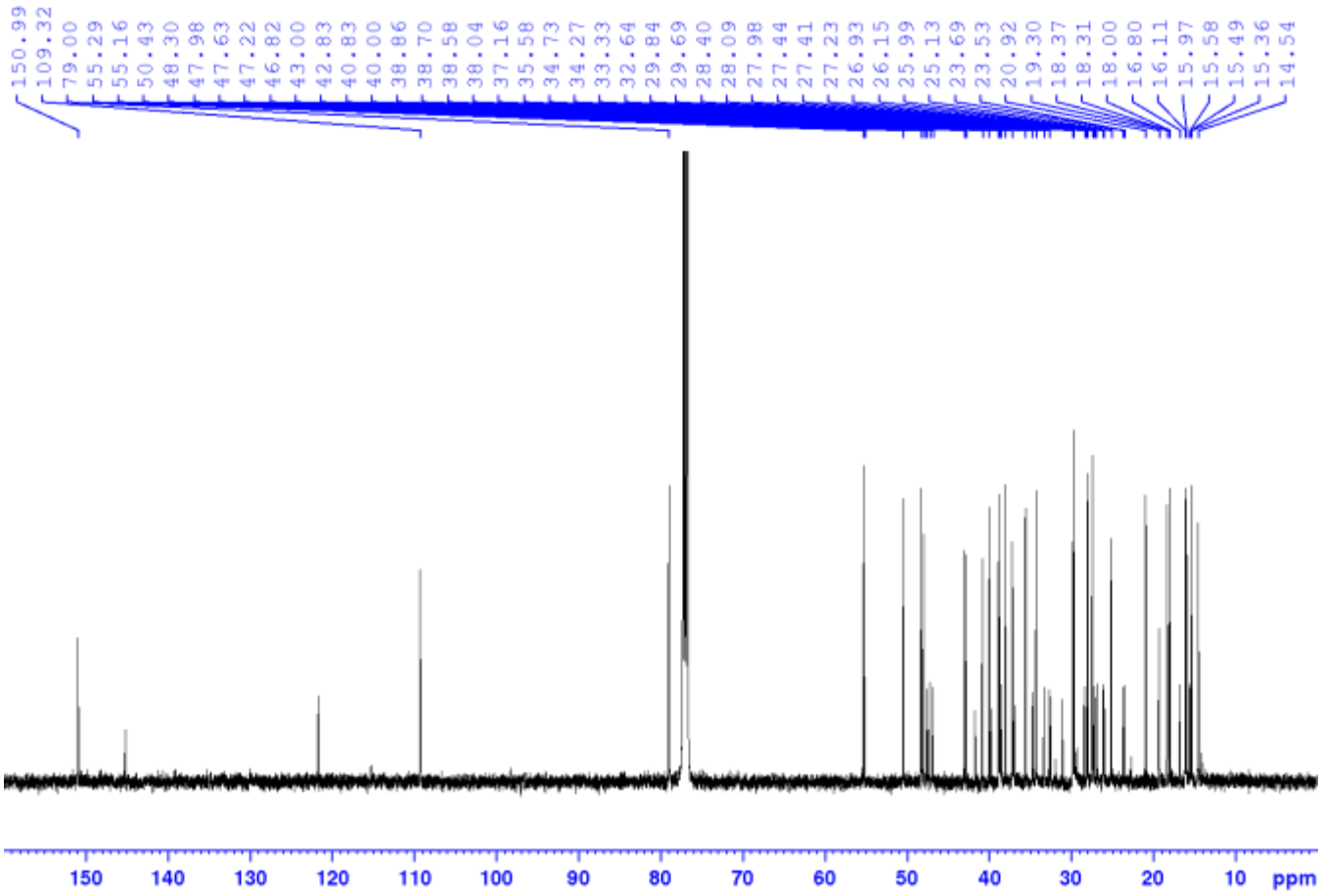


Figure A6. ^{13}C NMR spectrum of lupeol and β-amyrin isolated from *P. obliquum* ($CDCl_3$)

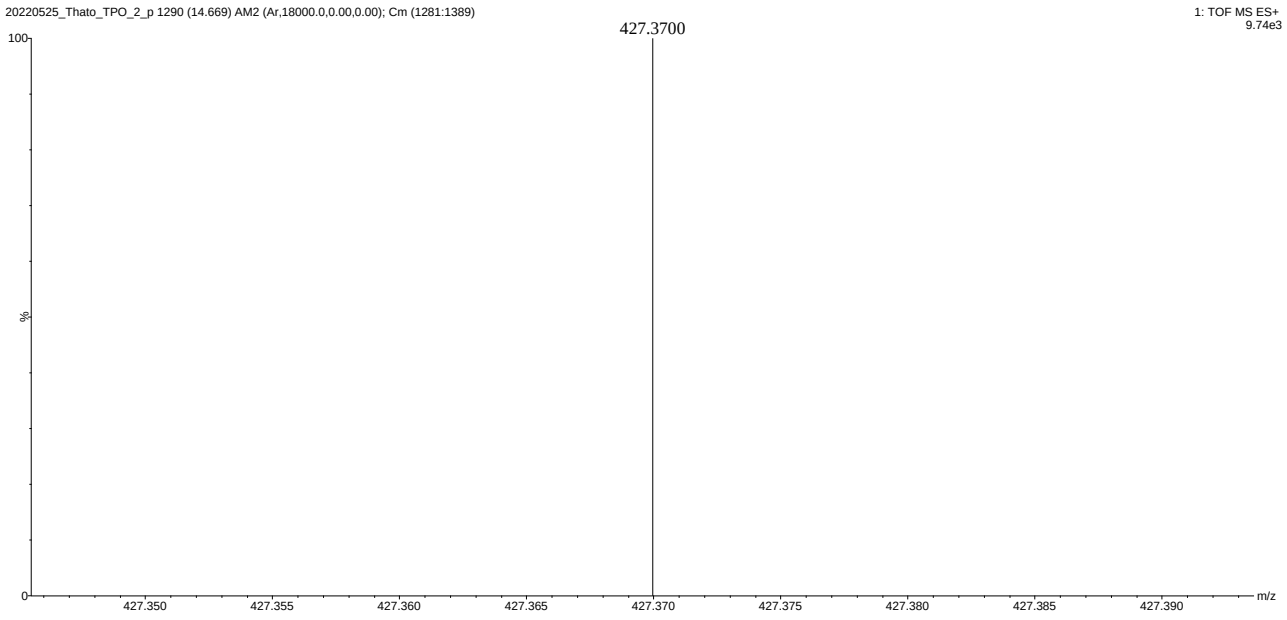


Figure A6. ESIMS of lupeol and β-amyrin isolated from *P. obliquum*

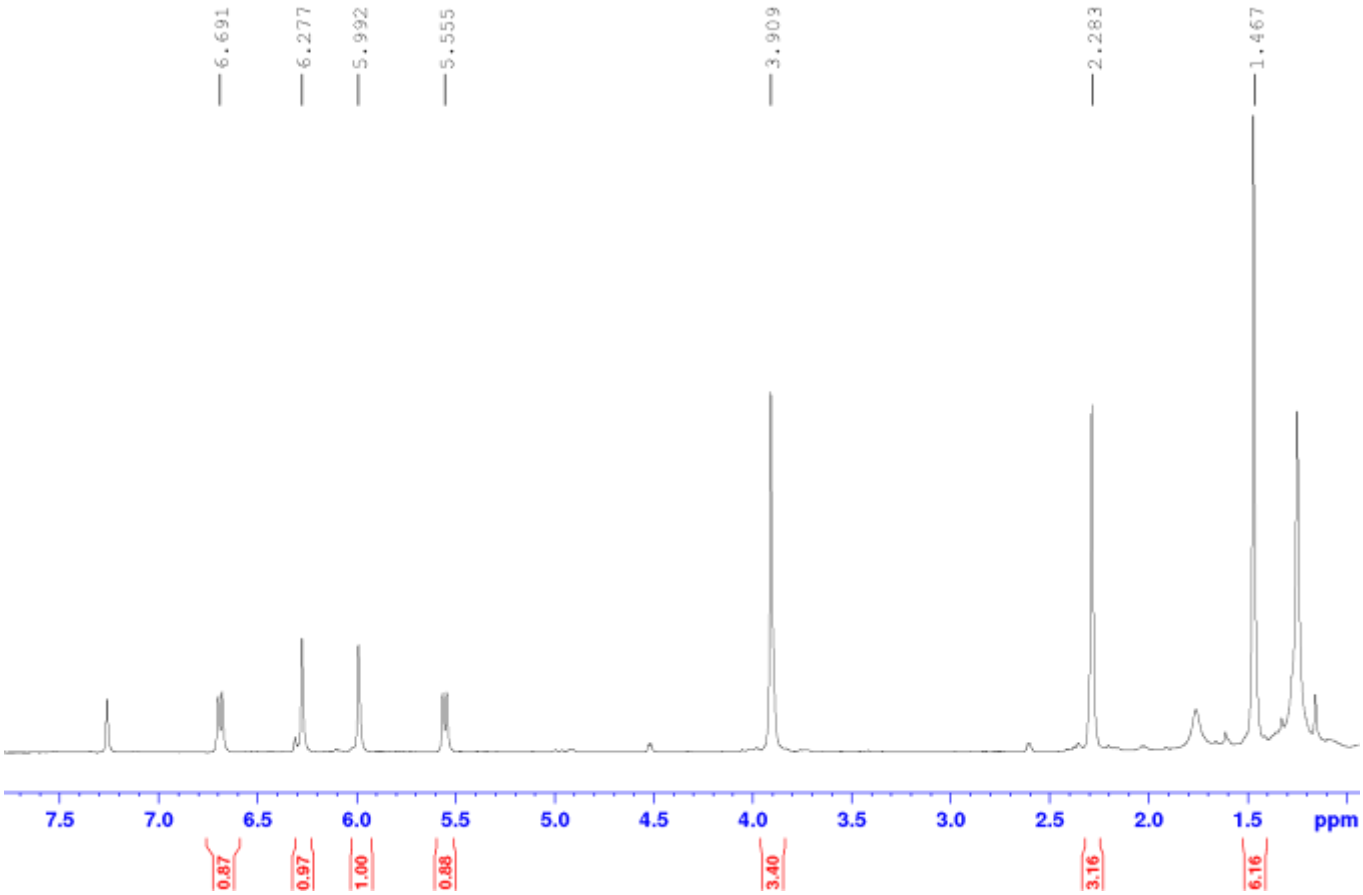


Figure A7. ^{1}H NMR spectrum of O- methylalloptaeroxylin isolated from *P. obliquum* ($CDCl_3$)

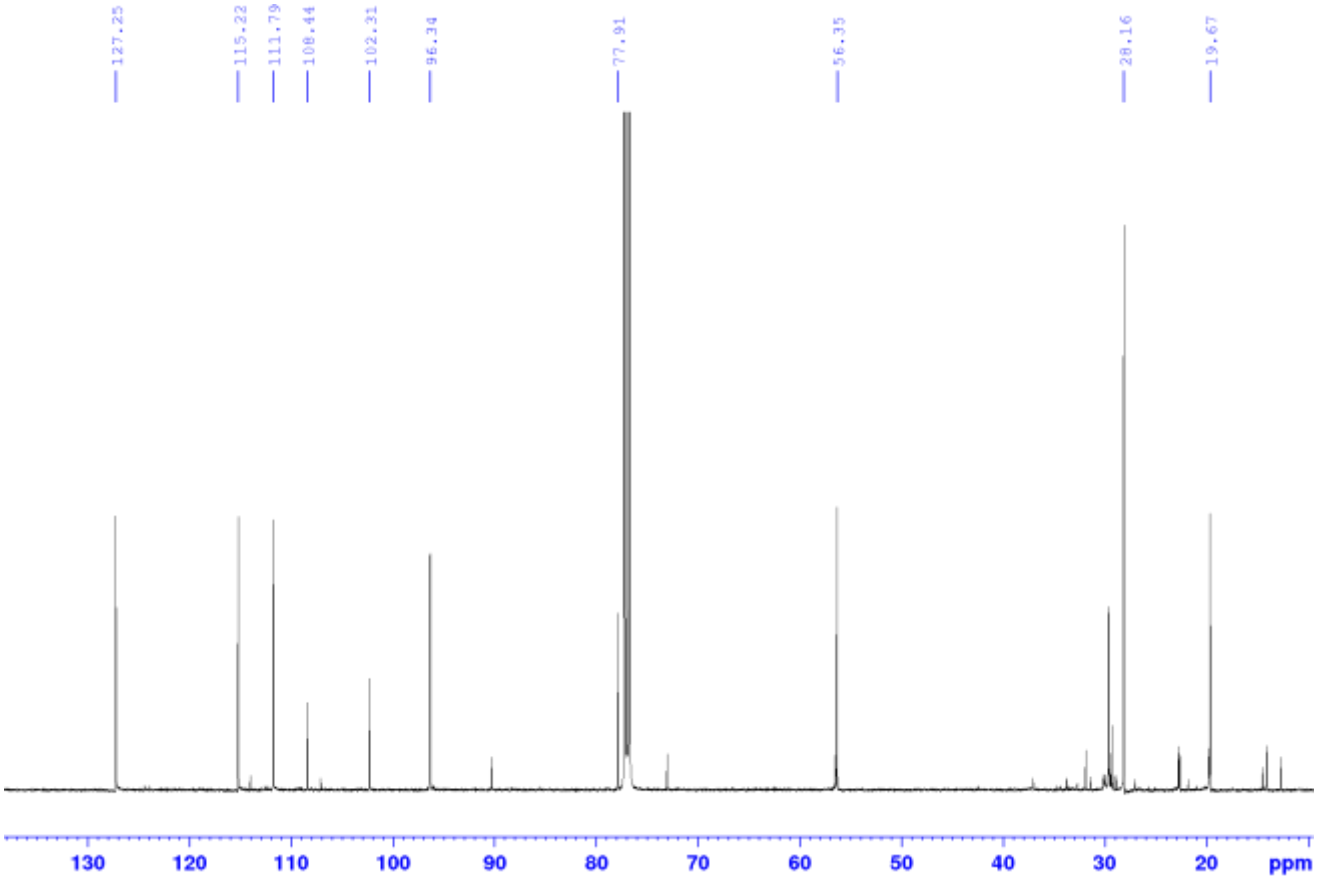


Figure A8. ^{13}C NMR O- methylalloptaeroxylin isolated from *P. obliquum* ($CDCl_3$)

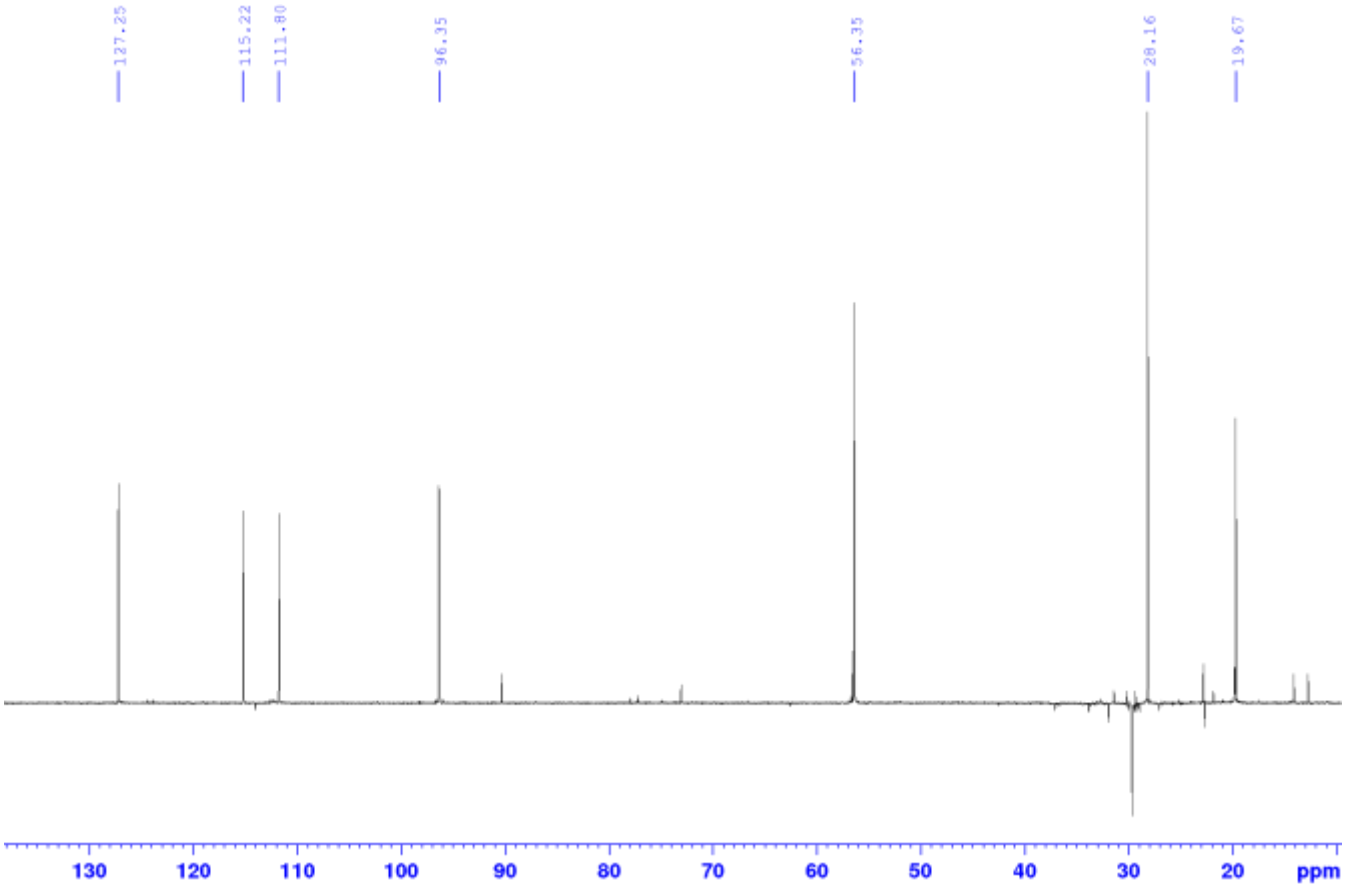


Figure A9. Dept-135 NMR spectrum of O- methylalloptaeroxylin isolated from *P. obliquum* ($CDCl_3$)

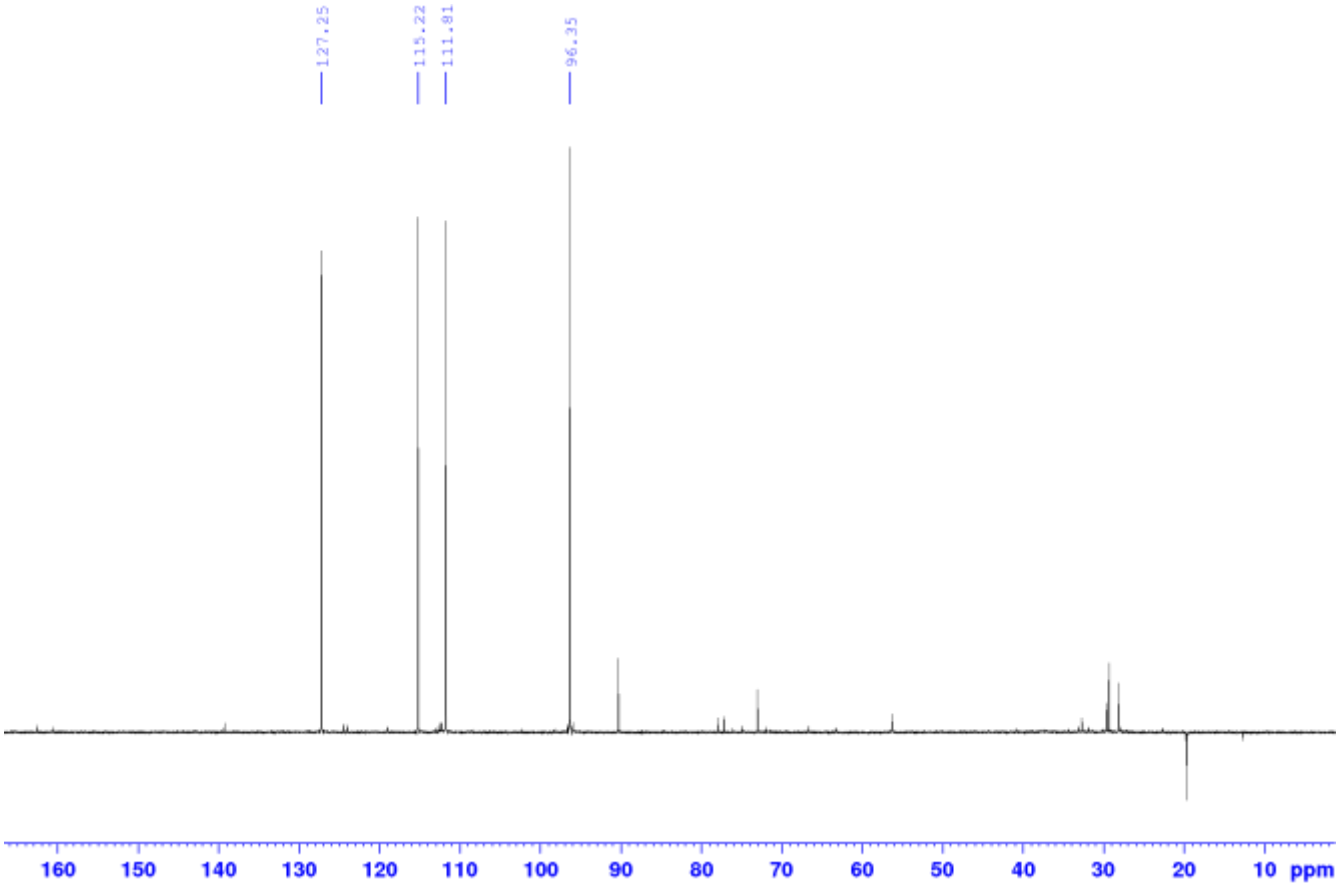


Figure A10. Dept-90 NMR spectrum of O- methylalloptaeroxylin isolated from *P. obliquum* ($CDCl_3$)

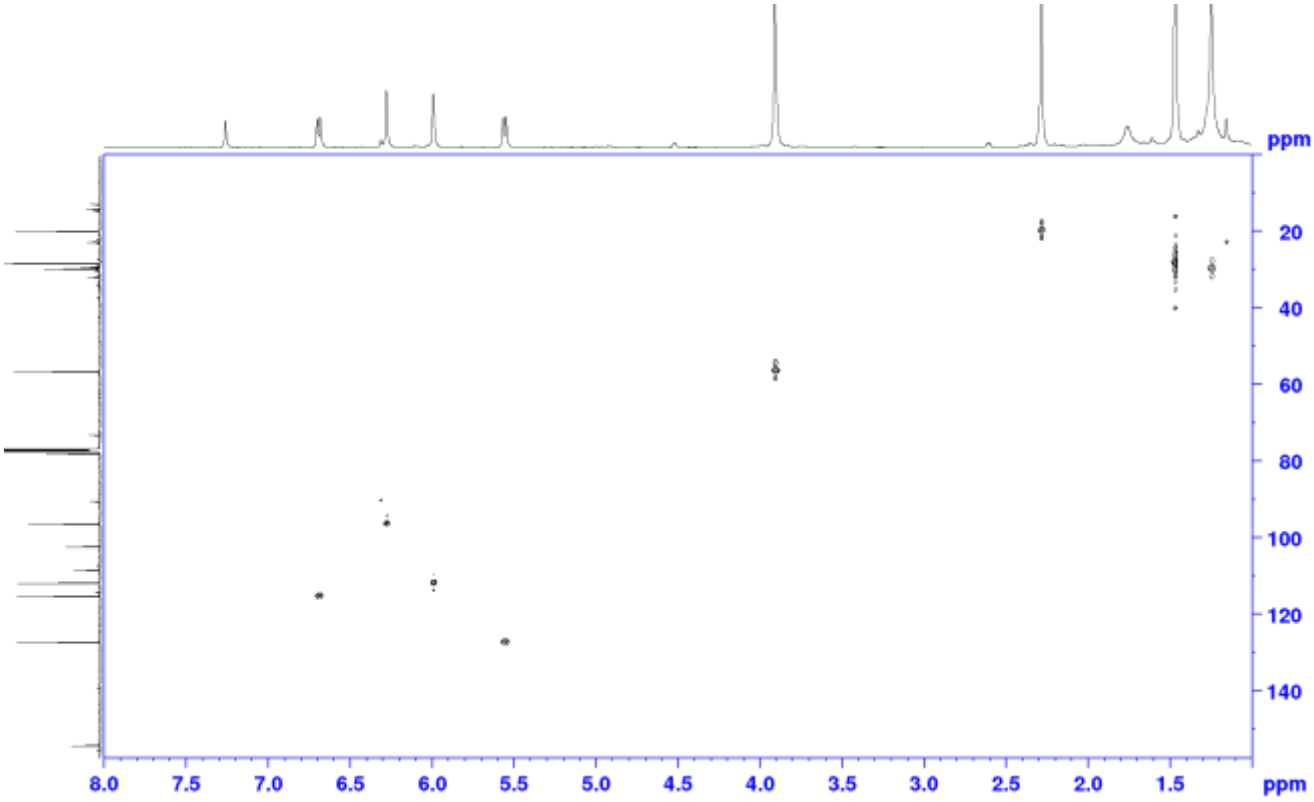


Figure A11. HSQC NMR spectrum of O- methylalloptaeroxylin isolated from *P. obliquum* ($CDCl_3$)

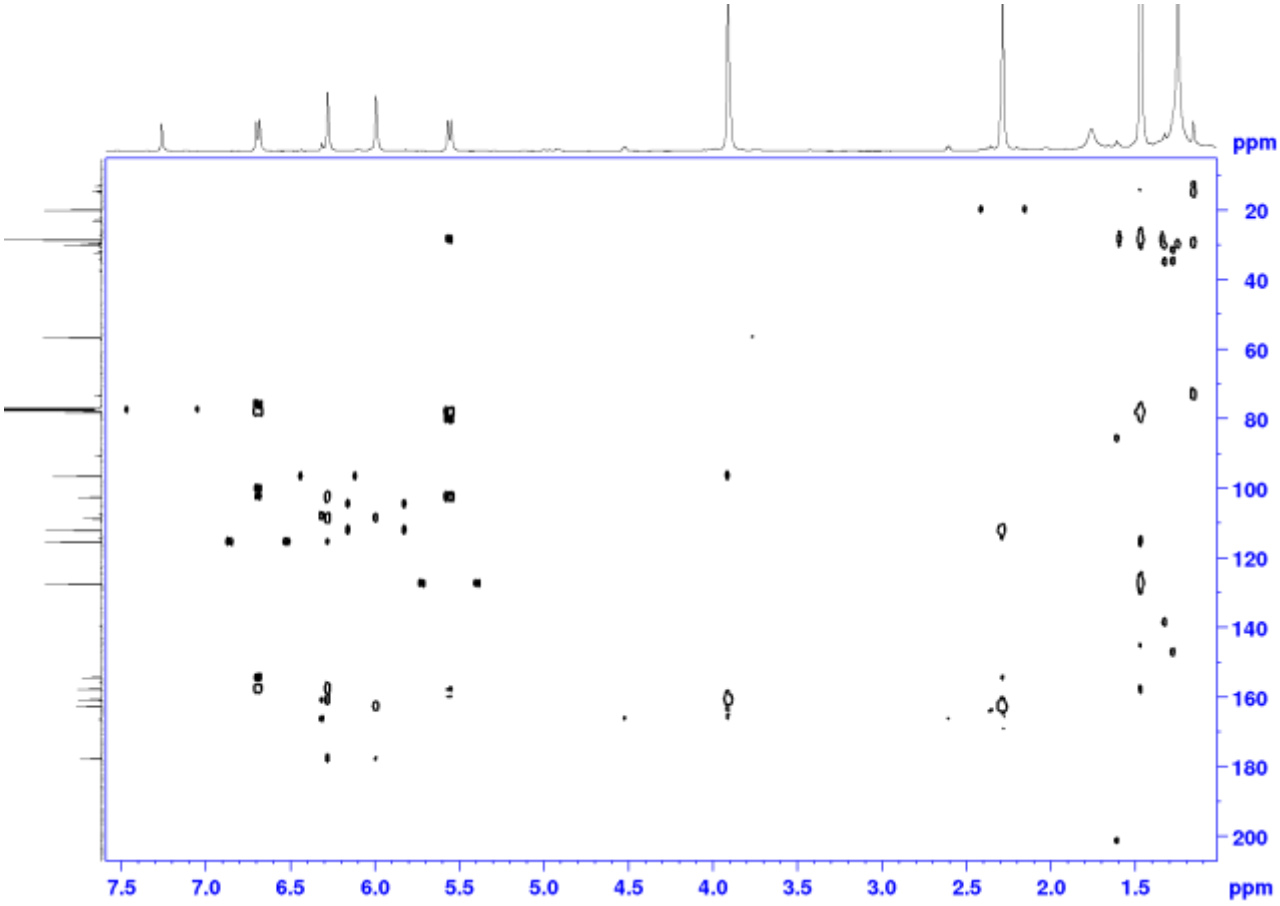


Figure A12. HMBC NMR spectrum of O- methylalloptaeroxylin isolated from *P. obliquum* ($CDCl_3$)

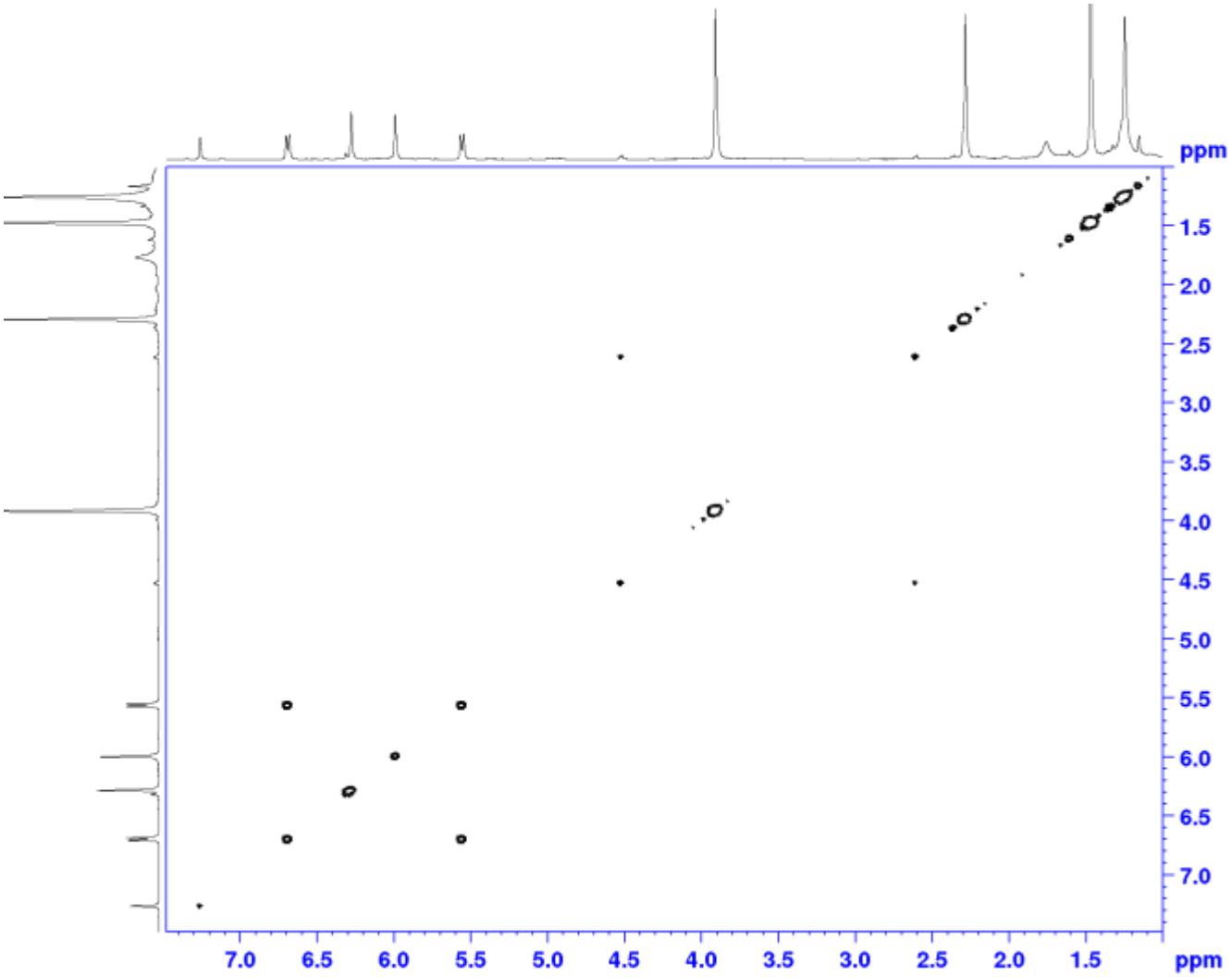


Figure A13. COSY NMR spectrum of O- methylalloptaeroxylin isolated from *P. obliquum* ($CDCl_3$)

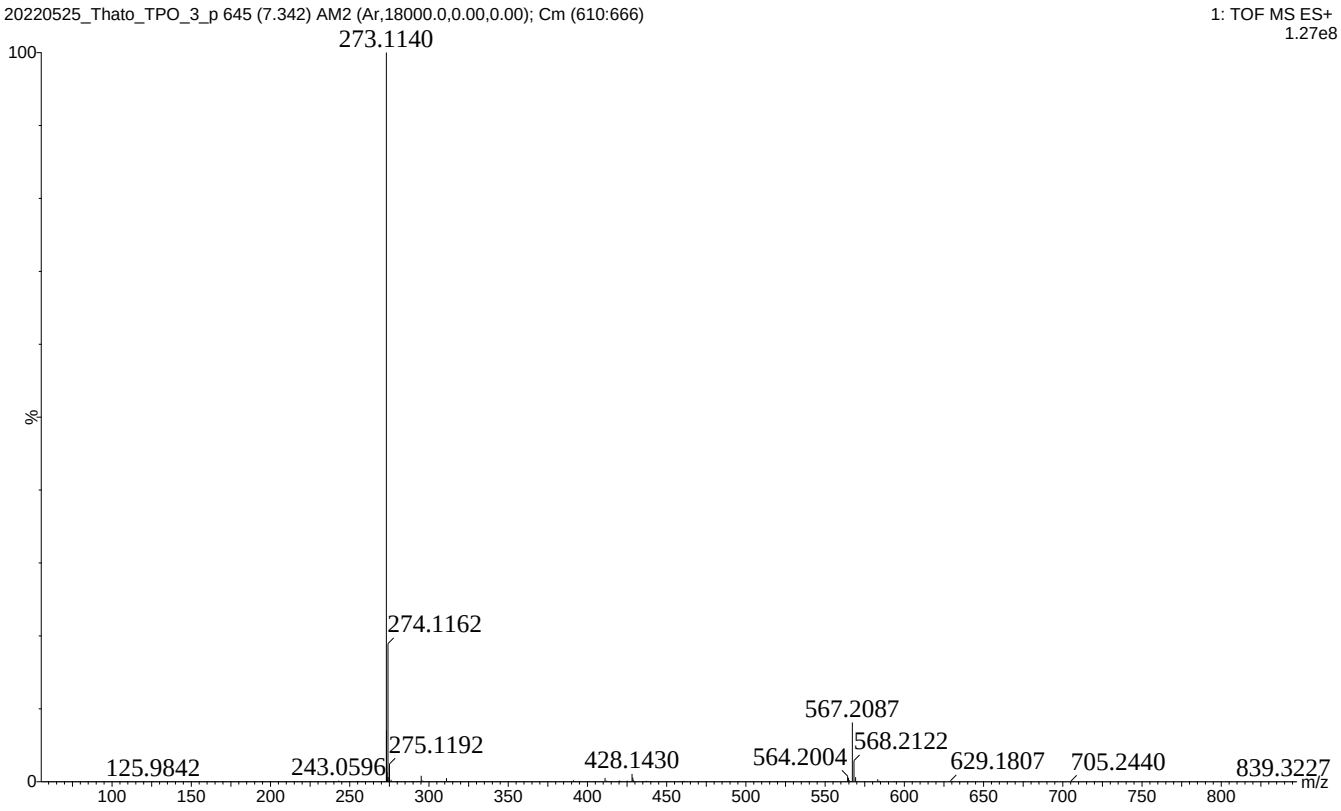


Figure A14. HRESIMS of O- methylalloptaeroxylin