Pharmacokinetics and hepatoprotective effects of *Lippia scaberrima* Sond. and *Aspalathus linearis* (Burm.f.) R. Dahlgren

By

Anna-Mari Kok

Submitted in partial fulfillment of the requirements for the degree

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in the

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of the

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DECLARATION

I, Anna-Mari Kok, declare that this thesis, which I hereby submit for the degree, Doctor of Philosophy in Medicinal Plant Sciences at the University of Pretoria, is my own work and has not previously been submitted by me for a degree at this or any other tertiary institution.

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SUPERVISOR: **Prof. Namrita Lall**

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CO-SUPERVISOR: **Markku Pasanen**

DEDICATION

This thesis is dedicated to my loving husband, for all his love and encouragement, which he has always provided me, especially during my studies. You truly mean the world to me.

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DOCTORAL RESEARCH OUTPUTS

PUBLICATIONS

- **Reid, A.,** Juvonen, R., Huuskonen, P., Lehtonen, M., Pasanen, M., Lall, N. 2019. *In vitro* human metabolism and inhibition potency of verbascoside for CYP enzymes. *Molecules.* 24 (11). (IF: 3.098).
- **Reid, A.,** Oosthuizen, C. B., Lall, N. 2020. *In vitro* antimycobacterial and adjuvant properties of two traditional South African teas, *Aspalathus linearis* (Burm.f.) R. Dahlgren and *Lippia scaberrima* Sond. *South African Journal of Botany.* (Accepted) (IF: 1.504).

PUBLICATIONS UNDER PREPARATION

- **Reid, A.,** Lall, N. 2020. A Polyherbal Combination of South African Plants for the Treatment of TB and its Associated Symptoms. *African Journal of Traditional, Complementary and Alternative Medicines.* (Under preparation) (IF: 0.553).
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- **Reid, A.,** Juvonen, R., Huuskonen, P., Lehtonen, M., Pasanen, M., Lall, N. 2020. Inhibition potency of *Lippia scaberrima* Sond. extract in combination with Aspalathus linearis (Burm.f.) R. Dahlgren extract for human CYP enzymes. Pharmacognosy Magazine. (Under preparation) (IF: 1.525).
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- **Reid, A.,** Oosthuizen, C. B., Fibrich, B. D., Twilley, D., Lambrechts, I, A., de Canha, M. N., Rademan, S., Lall, N. *Traditional Medicine: The Ancient Roots of Modern Practice in Marvel of Medicinal Plants: Uses and Applications.* In: Medicinal Plants for Holistic Health and Wellbeing. ISBN: 9780128124758. 2018 Elsevier. Ch. 1, 1-10.

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AWARDS

- National Research Foundation Innovation Bursary, 2015.
- SABINA Conference Travel Award, 2015.
- IPUF Young Scientist Best Poster Award, 2015.
- National Research Foundation Innovation Bursary, 2016.
- National Research Foundation Innovation Bursary, 2017.

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LIST OF ABBREVIATIONS

ACK: Ammonium Chloride and Potassium

ANOVA: Analysis of variance

ART: Antiretroviral treatment

ARV: Antiretrovirals

ATTC: American Type Tissue Culture

BSA: Bovine Serum Albumin

CO_2: Carbon dioxide

COX-II: Cyclooxygenase II

DMEM: Dulbecco's modified eagle's medium

DMSO: Dimethylsulfoxide

DPPH: 2, 2 diphenyl-1-picrylhydrazyl

EC_{50}: Fifty percent effective concentration

ELISA: Enzyme linked immunosorbent assay

EPMC: Ethyl-p-methoxycinnamate

EPTB: Extrapulmonary tuberculosis

FBS: Foetal Bovine Serum

FTIR: Fourier Transform Infrared Spectroscopy

GC-MS: Gas-Chromatography-Mass-Spectroscopy

$HAuCl_4.3H_2O$: Gold (III) chloride trihydrate

HepG2: Human liver hepatocellular carcinoma cells

HIV/AIDS: Human immunodeficiency virus/ acquired immunodefiency syndrome

IC_{50}: Fifty percent Inhibitory concentration

IFN-γ: Interferon-gamma

IL-4: Interleukin 4

IL-10: Interleukin 10

IL-12: Interleukin 12

IL-13: Interleukin 13

INH: Isoniazid

MABA: Microtitre Alamar Blue Assay

MDR-TB: Multidrug resistant tuberculosis

MHC: Major histocompatibility complex

MIC: Minimum inhibitory concentration

M.tuberculosis*: Mycobacterium tuberculosis*

M. smegmatis*:* Mycobacterium smegmatis

NaCl: Sodium Chloride

NO: Nitric Oxide

NF-κβ: Nuclear factor kappa beta

OADC: Oleic Albumin Dextrose Catalase

RIF: Rifampicin

RNI: Reactive nitrogen intermediates

ROS: Reactive oxygen species

RT: Retention time

SD: Standard deviation

SI: Selectivity index

SOD: Superoxide dismutase

TB: Tuberculosis

TEM: Transmission Electron Microscopy

TGA: Transgravimetric analysis

Th-1: T- helper 1

Th-2: T- helper 2

TLC: Thin layer chromatography

TDR-TB: Totally drug-resistant tuberculosis

U937: Human leukemic monocyte lymphoma cells

UV-Vis: Ultraviolet Visible

WHO: World Health Organization

XDR-TB: Extensively drug resistant

XRD: X-ray Diffraction

XTT: 2, 3 – bi (2 – methoxy-4- nitro-5-sulfophenyl) – 5- [phenylamino- carbonyl] – 2 H- tetrazolium hydroxide

XXDR-TB: Extremely drug resistant tuberculosis

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SUMMARY

Pharmacokinetics and hepatoprotective effects of *Lippia scaberrima* Sond. and *Aspalathus linearis* (Burm.f.) R. Dahlgren.

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In developing countries, the presence and impact created by tuberculosis (TB) is alarming. With the rise of resistant TB, initiatives such as adjunct host-directed therapy (AHDT) that aim to reduce treatment time, lower side-effects and costs involved, should be investigated. The Dutch East Indian Company mainly drank herbal medicinal teas in the Cape region during the settlement of the Cape. Many of the indigenous plants within the immediate area were used as substitutes for the much more expensive and imported tea. *Lippia scaberrima* Sond. (*L. scaberrima*) is an under investigated shrub which is endemic to South Africa. Although *Lippia javanica* may have been an obvious choice for testing in this regard, the fact that not much is known on *L. scaberrima* prompted the investigation into the biological properties of this specific species. *Aspalathus linearis* (Burm.f.) R. Dahlgren (*A. linearis*) a medicinal plant that has a variety of biological properties documented. Both these plants are consumed as teas and in this study an attempt was made to investigate their combined biological and therapeutic activity. The biological investigation in the present study included using a combination of ethanolic extracts of both *L. scaberrima* and *A. linearis*. *L. scaberrima* is known to be rich in volatile compounds and the essential oil was isolated and included in the study. The essential oil of *L. scaberrima* was incorporated as another sample to be tested for its biological activity, as this plant is known to be rich in volatile compounds. This study further included an investigation of the biological activity of verbascoside, a known compound, found in all endemic *Lippia* species. Lastly, the evaluation of synthesized gold nanoparticles from *L. scaberrima* were also included.

Significant antimycobacterial activity was found for *L. scaberrima* against *Mycobacterium tuberculosis* H37Rv. Low to no cellular toxicity was observed against hepatocellular carcinoma cells (HepG2) at the tested concentrations. Hydro-steam distillation

was effective in isolating the essential oils from the aerial parts of *L. scaberrima,* which showed effective hepatoprotective activity against acetaminophen-induced toxicity. The cytochrome P450 (CYP) inhibitory activity of *L. scaberrima*, combinations of *L. scaberrima* and *A. linearis,* and verbascoside has been reported in this study for the first time. Potent CYP inhibitory activity was found for *L. scaberrima* in combination with the green *A. linearis*. Verbascoside had no significant inhibitory activity against the CYP isoforms and therefore, it can be considered that it may have low risk for herb-drug interactions.

This study included the cyclooxygenase-II (COX-II) inflammatory activity of *L. scaberrima*. All the samples, except *L. scaberrima,* that were investigated for their DPPH free radical scavenging potential were found to have significant activity. Moderate nitric oxide scavenging activity was found for verbascoside and green *A. linearis* in combination with *L. scaberrima*. Antioxidant and anti-inflammatory activity play a profound role in assisting with the adverse effects experienced during treatment for TB infection.

This study provided a more comprehensive look into the specific biological properties of *L. scaberrima* relating to TB infection as well as its efficacy when combined with fermented and green *A. linearis* extracts. Synthesized nanoparticles of *L. scaberrima* provided the opportunity to lower the cytotoxicity towards HepG2 cells even more than was initially found.

The investigated samples showed the potential to decrease drug-induced hepatotoxicity, one of the main adverse effects experienced. In addition, all the samples tested, showed potential as an adjuvant for host-directed therapies for TB. These initial results pave the way for further research into specialized avenues with endless possibilities for the discovery of new specialized treatment regimens that are tailored to be host specific and for specific stages of infection. Please note that all experiental studies and results reported on were on the ethanolic extracts of both *L. scaberrima* and *A. linearis* and not of the tea and/or the boiling water extract of *L. scaberrima* and *A. linearis* or otherwise stated.

ABSTRACT

Tuberculosis (TB), as an epidemic, has had devastating consequences across the globe. South Africa is one of the countries with the highest numbers of TB cases. Numerous plant extracts have been investigated for their ability to treat TB and/ or its associated symptoms. As part of their potential as adjunct host-directed therapy (AHDT), the study investigated various biological properties that may assist within the framework of the current treatment regimen for TB. AHDT can be tailored to a specific individual and their needs or to a specific phase during bacterial infection.

Both *L. scaberrima* Sond. and *A. linearis* (Burm.f.) R. Dahlgren, are popular forms of health teas in southern Africa. Many traditional uses have been noted for both these endemic medicinal plants. This study aimed at identifying the potential therapeutic effects of these two herbal teas. This was accomplished by investigating their antimycobacterial, anti-inflammatory and hepatoprotective properties. In addition, the pharmacokinetic characteristics and potential drug- herb interactions were determined by investigating the effect of the plant extracts, and an isolated compound, on human cytochrome P450's. Finally, gold nanoparticles were synthesized, and the properties compared to that of the crude extract.

The biological investigations were conducted on five samples. These included the ethanolic extracts from *L. scaberrima* and *A. linearis* (fermented as well as green), the hydro-steam distilled essential oil from *L. scaberrima*, and verbascoside, a known compound found within *Lippia* spp. In addition, combinations of the above-mentioned samples were included in the study. During the course of the study, differences were found between the fermented and green extracts of *A. linearis*, showing that oxidizing does change the active components found within the plant. Green *A. linearis* showed increased activities when investigating free radical scavenging properties especially, compared to the fermented extracts. The essential oil from the aerial parts of *L. scaberrima* showed differences in the main components when compared to the ethanolic extract, with carvone found in high concentrations in the ethanolic extract and limonene in the essential oil.

The ethanolic extract of *L. scaberrima* showed effective antimycobacterial activity against *M. tuberculosis* with a minimum inhibitory concentration (MIC) value of 125 μg/mL. Combining the ethanolic extracts of *L. scaberrima* and *A. linearis* showed no increase in activity with all combinations showing MIC values of 500 μg/mL.

No effective antimycobacterial activity was found for the essential oil of *L. scaberrima* against *M. tuberculosis* (> 1000 µg/mL). Low to no cellular toxicity at the tested concentrations were found for the essential oil, *L. scaberrima* and combinations of fermented and green *A. linearis* with *L. scaberrima* as well as verbascoside when evaluated against human liver carcinoma (HepG2) cells. For *L. scaberrima*, the combinations and the essential oil, the IC_{50} obtained ranged between 109.20±8.05 µg/mL (Mea+SE) and 244.90±4.97 µg/mL. The essential oil had an IC_{50} value of 244.90 ± 4.97 µg/mL and showed the lowest level of toxicity.

The antioxidant and anti-inflammatory activity of the samples were investigated for their DPPH scavenging activity. Green *A. linearis* alone and in combination with *L. scaberrima*, showed potent DPPH free radical scavenging properties, with IC_{50} values of 2.18±1.75 µg/mL (Mean±SD) and 4.49±0.16 µg/mL, respectively (ascorbic acid IC_{50} =7.70±1.12 µg/mL). The COX-II anti-inflammatory activity of *L. scaberrima* has been reported for the first time and found to be at an IC_{50} value of 36.39±1.62 µg/mL (Mean±SD). Significant hepatoproteciton was observed on drug-induced hepatotoxicity. Where the essential oil and green *A. linearis* extract, showed a protection of 32.02±2.23 % and 33.20±3.91 % at a concentration of 25 µg/mL, respectively.

Many TB-infected individuals make use of medicinal plants as their primary form of healthcare. The potential of these medicinal plants and their herb-drug interactions with the drugs that are taken during TB treatment need to be investigated. The inhibitory properties of verbascoside, *L. scaberrima* alone and in combination with *A. linearis* on human hepatic cytochrome P450 (CYP) enzymes were investigated. *Lippia scaberrima* alone and in combination with fermented and green *A. linearis* showed CYP inhibitory potential. Verbascoside showed low inhibitory activity against CYP1A2 and CYP1B1 with IC_{50} values of 83 µM and 86 µM, respectively. Verbascoside showed an even lower inhibition of CYP1A1, CYP2A6, CYP2C19, CYP2D6 and CYP3A4 with IC_{50} values greater than 90 µM. All the samples tested, possessed low to none CYP interaction risks concerning their potential herb-drug interactions. The study reported on the *in vitro* metabolism of verbascoside in human hepatic microsomes and cytosol incubations. These two different liver fractions consist of their own distinctive components that form part of the human metabolic process. The metabolism and excretion of a compound has a direct relation to its potential toxicity in humans. After hydrolysis, CYP and glucuronide conjugation, no metabolites were found to be formed. However, five different methylated conjugates of verbascoside could be found in the S-

adenosylmethionine incubation, three different sulphate conjugates with the PAPS incubation and very low levels of glucuronide metabolites after incubation with recombinant human UDP-Glucuronosyltransferases (UGT1A7, UGT1A8 and UGT1A10).

Finally, this study investigated the synthesis and characterization of gold nanoparticles from *L. scaberrima* and gum arabic as a stabilizer. An intense peak at a wavelength of 540 nm showed the absorption of the gold nanoparticles formed. Transmission electron microscopy (TEM) images confirmed that most of the formed gold nanoparticles were spherical in shape and the X-Ray diffraction (XRD) pattern confirmed the crystalline nature of the nanoparticles that formed. The possible functional groups were identified by Fourier transform infrared spectroscopy (FTIR) and were, among others, from the alkene functional group. The cytotoxic potential of the gold nanoparticles was reduced by 2-fold when compared to the ethanolic extract.

The current study investigated various biological properties of *L. scaberrima* and *A. linearis*. According to this study, one can conclude that *L. scaberrima*, the essential oil of *L. scaberrima*, *A. linearis*, the 1:1 combinations of *L. scaberrima* with fermented and green *A. linearis* and verbascoside have the potential to be part of AHDT by lessening the adverse effects that are experienced by TB infection and treatment.

Therefore, from all the samples tested, *L. scaberrima*, the essential oil of *L. scaberrima* and the combinations with *A. linearis* (fermented and green *A. linearis*), could be considered for further adjuvant (drug) development due to their biological properties found and the low risk of interactions with other drugs taken concurrently.

In the current study, the novel aspects of the studies conducted are as follows:

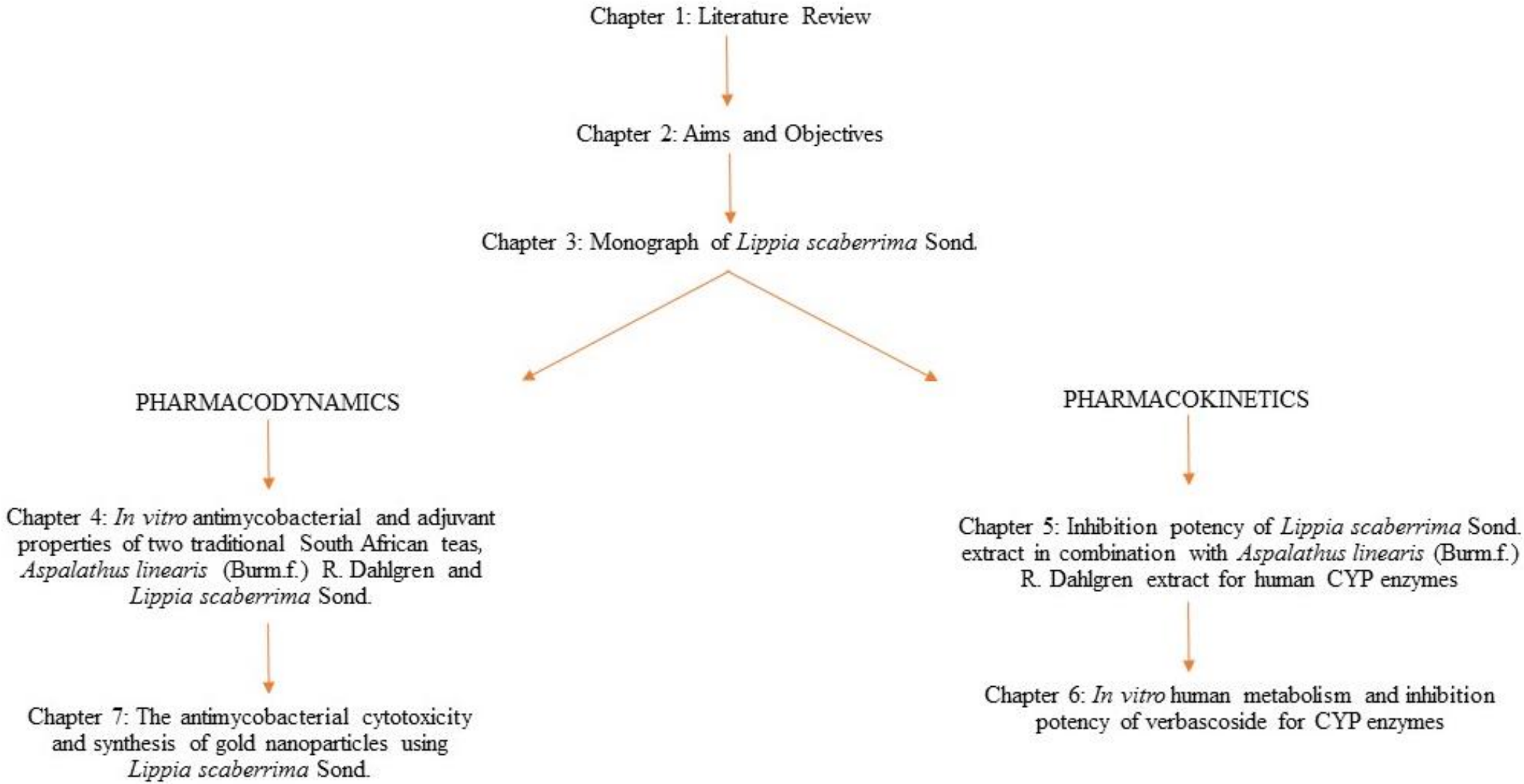
- The first reported testing of the ethanolic extract of *L. scaberrima* against *M. tuberculosis*, *M. smegmatis*, for its anti-inflammatory and antioxidant properties (DPPH, nitric oxide, COX-II), drug induced hepatoprotection and gold nanoparticle synthesis with the ethanolic extract of *L. scaberrima.*
- The first reported studies on the *in vitro* CYP P450 (CYP1A1, CYP1A2, CYP1B1, CYP2A6, CYP2C19, CYP2D6, CYP3A4 and CYP19A1) inhibitory properties of *L. scaberrima* and *A. linearis*.
- The first reported studies on the *in vitro* human metabolism of verbascoside.

In conclusion, this study provides a more comprehensive analysis of the biological potential of not just *L. scaberrima*, which has not yet been investigated in detail, but also added therapeutic value when in combination with *A. linearis*. All initial results indicated that *L. scaberrima* has the potential for adjunct host-directed therapies to lessen the impact of adverse effects experienced during infection such hepatotoxicity. Specifically, verbascoside a constituent found within *Lippia* spp. and endemic to South Africa, showed no potential for any herb-drug interactions following the *in vitro* studies such as hepatoxicity conducted. Please note that all experiental studies and results reported on were on the ethanolic extracts of both *L. scaberrima* and *A. linearis* and not of the tea and/or the boiling water extract of *L. scaberrima* and *A. linearis* or otherwise stated.

Flow diagram of the study and the samples used.

Chapter	*L. scaberrima*	Essential oil of *L. scaberrima*	Fermented *A. linearis*	Green *A. linearis*	*L. scaberrima* & fermented *A. linearis* (1:1)	*L. scaberrima* & green *A. linearis* (1:1)	Verbascoside	Gold nanoparticles of *L. scaberrima*
1	Not applicable							
2	Not applicable							
3	Not applicable							
4	X	X	X	X	X	X		
5	X	X			X	X		
6							X	
7	X							X
8	Not applicable							

Flow diagram 1: Current study and samples used throughout.



Flow diagram 2: Chapter layout and description of thesis. Describing the flow and thought process behind the current study.

1

BACKGROUND AND MOTIVATION

This chapter introduces the tuberculosis epidemic, the history, current treatments and highlights the motivation for the research conducted in the thesis.

Sections of this chapter have been published as three book chapters in the following books:

1) *Natural Products and Drug Discovery: An Integrated Approach and Medicinal Plants for Holistic Heath and Wellbeing.*

2) *Medicinal Plants: From Farm to Pharmacy*

Citation 1: **Reid, A.,** Oosthuizen, C. B., Fibrich, B. D., Twilley, D., Lambrechts, I, A., de Canha, M. N., Rademan, S., Lall, N. *Traditional Medicine: The Ancient Roots of Modern Practice in Marvel of Medicinal Plants: Uses and Applications.* In: Medicinal Plants for Holistic Health and Wellbeing. ISBN: 9780128124758. 2018 Elsevier. Ch. 1, 1-10.

*Citation 2: Oosthuizen, C, B., **Reid, A**., Lall, N. 2018. Can medicinal plants provide an adjuvant for Tuberculosis patients? In: Medicinal Plants for Holistic Health and Wellbeing. ISBN: 9780128124758. 2018 Elsevier. Ch. 7, 213-247.*

*Citation 3: **Reid, A.,** Lall, N. Natural products as possible vaccine adjuvants for infectious diseases and cancer. In: Medicinal Plants: Farm to Pharmacy. ISBN: 9783030312688. 2019 Springer, Life Sciences. Ch. 9.*

1. INTRODUCTION AND PROBLEM STATEMENT

1.1. The Tuberculosis epidemic

Tuberculosis (TB) has had a devastating impact and has caused havoc worldwide with mortality rates reaching 1.3 million for the year 2016. Another infamous epidemic is claiming its fair share with 374 000 of the fatalities, being individuals that are co-infected with human immunodeficiency virus/ acquired immunodeficiency syndrome (HIV/AIDS). It is estimated that one third of patients that have HIV will die of TB (WHO, 2017).

According to the World Health Organization Report (WHO, 2017) on tuberculosis, TB is still the largest burden, geographically, in Africa and Asia. Due to the lack of proper sanitation and poverty that is evident in these countries, it becomes the perfect setting for the undesirable spread of this disease. Figure 1.1 indicates the globally estimated rates for TB incidents for 2016. Although globally, there has been a drop in the number of TB incidents from previous years (1.4%) (Figure 1.2), this drop is still less than what is needed (4-5%) to achieve the milestones set by the End TB strategy by 2020 (WHO, 2017).

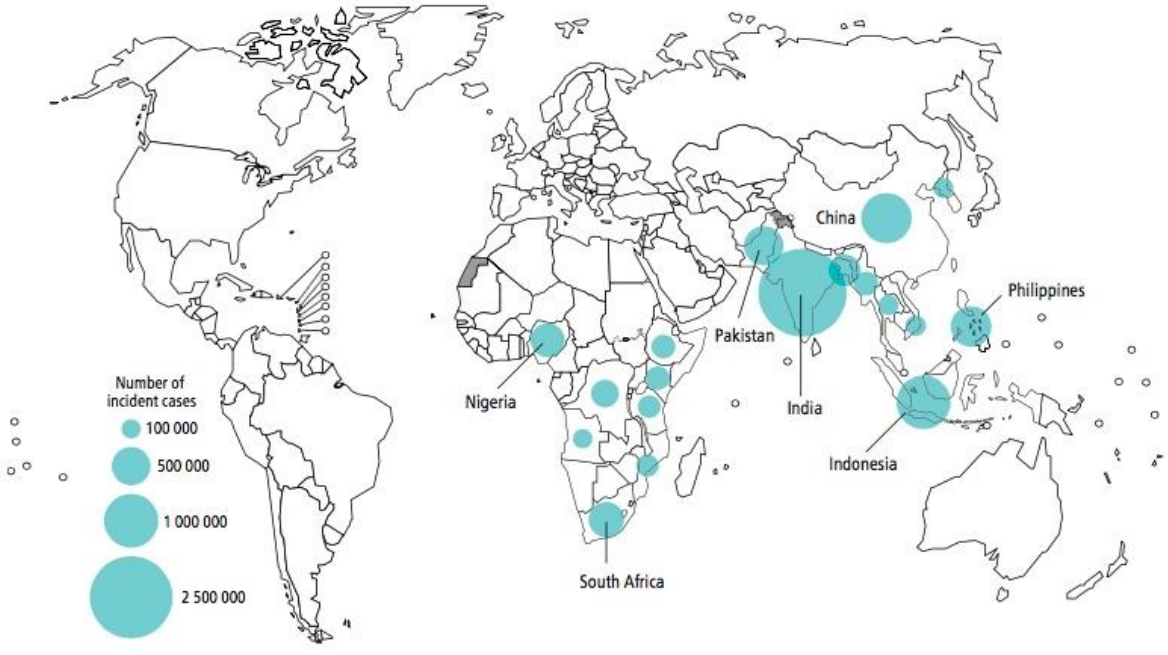


Figure 1.1 Estimated Tb incidences with at least 100 000 cases for 2016 (WHO, 2017).

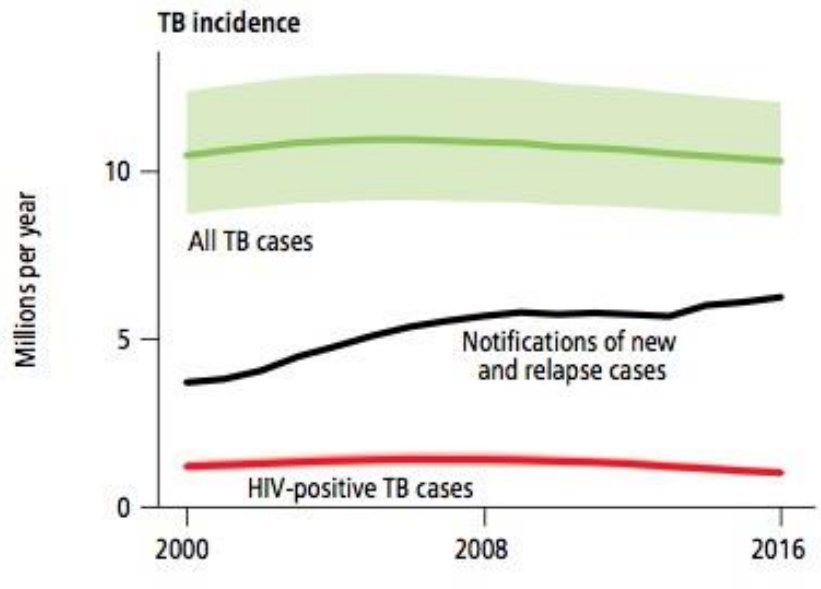


Figure 1.2 Global trends in the estimated TB incidents and deaths (in millions) reported from 2000-2016. Shaded areas represent uncertainty intervals (WHO, 2017).

The causative agent of TB is *M. tuberculosis*, a bacterial pathogen. This bacterium is most often found and readily spread in areas where overcrowding, malnutrition, and poverty are evident (Green et al., 2010). This bacterium is a facultative aerobe and needs oxygen for survival; therefore, in most circumstances, *M. tuberculosis* is found to attack the respiratory system of individuals. This bacterium is, however, not always restricted to the lungs only and has been found to infect different parts of the body, such as the kidneys, spine or brain (CDC, 2014). This type of TB infection is referred to as extrapulmonary TB (EPTB) and is the form of TB that is most often observed in patients who are co-infected with HIV/AIDS (WHO, 2012). Sharma and Mohan, (2004) stated that in HIV-positive individuals, EPTB accounts for more than 50% of all TB cases. It has been observed that when peripheral blood mononuclear cells of HIV infected individuals are exposed to *M. tuberculosis in vitro*, they produce less interferon-gamma (IFN-γ) but equal amounts of Interleukin 4 (IL-4) and IL-10 as compared to HIV negative individuals. This means that in HIV-infected individuals there is a reduced T-helper 1 (Th-1) response. This immunological response is responsible for the resistance against infection with mycobacteria and therefore HIV-positive individuals are more susceptible to TB infections (primary or reactivation of TB as well as exogenous reinfection) (Sharma and Mohan, 2004). Jung and Paauw (1998), showed the relation between CD4 count and HIV-related diseases that as soon as the CD4 count drops below 500/mm, TB usually occurs. It was shown that the *Mycobacterium avium* complex is one of the most common opportunistic infections that are associated with AIDS. This form of Mycobacterium usually occurs at CD4

counts that are below 75/ mm (Baroud et al., 1995; Jung and Paauw, 1998). Tuberculosis can spread through a myriad of ways; one such way is when an infected individual with an active infection of the lungs and throat, sneezes, coughs or spits near an uninfected individual. Droplets expelled from infected individuals are stable for a few hours in the outside environment where they can be inhaled by an unsuspecting new host (O' Garra et al., 2013). Despite all the treatment plans and strategies that are implemented by a wide variety of governmental and non-governmental organizations to combat TB, multi-drug resistant (MDR-TB) and extensively drug-resistant TB (XDR-TB) are still found to be prevalent. In most countries, it was observed that even today, only a small number of the newly and previously infected individuals were tested for MDR-TB in 2010 (WHO, 2011).

1.2. HIV/aids and TB co-infection

The association with HIV/AIDS has a big part to play in the statistics of incidence and mortalities with an additional 374 000 deaths occurring in HIV-positive individuals (WHO, 2017). HIV/AIDS not just raises the mortality rates but also raises the number of incidences by increasing an individual's chances of contracting TB due to a suppressed immune system. Worldwide, TB is the ninth leading cause of death and for five years (2012-2016), the leading cause of death from a single infectious agent, ranking above HIV/AIDS (Figure 1.3)

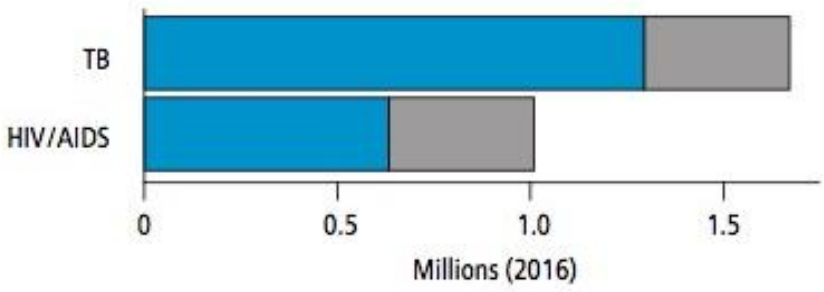


Figure 1.3 The number of estimated deaths from TB versus HIV/AIDS for 2016. The areas in grey are the estimated number of deaths due to HIV-positive individuals co-infected with TB. Deaths among HIV-positive individuals co-infected with TB are officially classified as deaths due to HIV/AIDS (WHO, 2017).

With HIV/AIDS and TB co-infection, the need arises to take drugs for both diseases together. With TB, it is typically a six-month regime that includes taking rifampicin, isoniazid, pyrazinamide, and ethambutol daily or periodically for two months. This is then followed by rifampicin and isoniazid for four months (Lienhardt et al., 2010). In HIV-positive individuals, antiretrovirals (ARVs) form part of the prescribed drugs that need to be taken. These ARVs are mainly prescribed to patients to inhibit the multiplication of the virus and positively control

their CD4+ count. In HIV-infected individuals, the use of ARVs at an early stage is known to decrease the chances of contracting TB, although the optimum time to use ARVs has not yet been noted (WHO, 2010). According to these studies, it has been shown that ARVs are recommended to be given simultaneously with TB treatments regardless of what the CD4+ count may be (Blanc et al., 2011; Havlir et al., 2011; Karim et al., 2011). From these studies, the WHO is now recommending that TB treatment be initiated first; followed by ARVs within 8 weeks of starting the TB treatment. However, those who are found to have a profound suppression of the immune system should start taking ARVs within the first two weeks of the TB treatment (WHO, 2012). In 2012, a meta-analysis was conducted that showed that taking ARVs reduces an individual's risk of contracting TB by 65% (Suthar et al., 2012; WHO, 2012). The difficulty that has arisen during HIV and TB co-infection is the observation of drug interactions between the drugs Rifampicin and selected ARVs such as the protease inhibitors. The presence of the immune reconstitution syndrome (IRIS) which is characterized by the appearance of new TB lesions is also very common (Lienhardt et al., 2010).

1.3. The causative agent

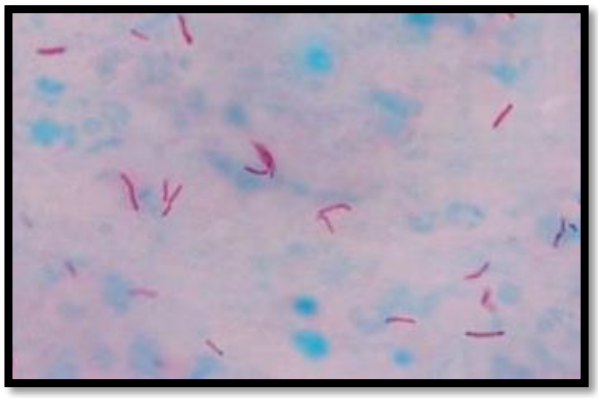


Figure 1.4 Staining of sputum indicating the presence of *Mycobacterium tuberculosis* as red bacilli (adistian.com, 2014).

M. tuberculosis forms part of the *Mycobacterium tuberculosis* complex that consists of *M. tuberculosis, M. bovis, M. microti, M. africanum, M. pinnipedii, M. avium, M. intracellulare, M scrofulaceum* and *M. caprae*. It is a Gram-positive pathogen, which is non-motile and consists of a rod-like structure. It formed part of the Actinomycetes family but has now been placed in a family of its own, the Mycobacteriaceae. It is an intracellular pathogen that proliferates in areas where high oxygen levels can be found, such as the lungs. Due to its intracellular and pathogenic nature, it has been found to infect macrophages. This pathogen has

cell walls that are characteristically lipid rich, with mycolic acids present on the surface. These mycolic acids give them another unique distinguishing factor, which is their acid-fastness, as they can only be stained by acid-fast staining methods. They are impermeable to basic dyes such as Gram-staining but have been detected by the use of the Ziehl-Neelsen staining procedure as shown in (Figure1.4).

In this staining, method organisms can be detected by their red colour between the surrounding blue tissues. Robert Koch first described this pathogen in 1882, for which he received the Nobel Prize in Physiology and Medicine in 1905. Since its discovery, TB as a disease has received several names. Among these names, we may find, "The Great White Plague". This name was given to the infection during the time when a TB epidemic devastated the European population around the 17th century (Microbiologybytes, 2014).

1.4. Pathogenesis of TB

The outcome of infection with *M. tuberculosis* depends on the interactions that occur between the bacteria and the host's innate and acquired immune responses. These interactions can lead to control of infection, advanced disease or the establishment of latent infection (Torrado et al., 2011). After exposure to *M. tuberculosis*, 90% of those who develop cellular immune responses will remain healthy. This is known as latent *M. tuberculosis* infection and reflects a successful immune response and containment of *M. tuberculosis* (Grotzke and Lewinsohn, 2005). *M. tuberculosis* is transmitted through aerosol droplets and when inhaled, in 95% of the cases a primary infection will take place. Cell mediated immunity that consists of a T-cell response will clear this infection or cause containment of the tubercle bacilli within a granulomatous structure where latent *M. tuberculosis* disease is initiated. This form of TB is normally not associated with any visible symptoms as, although the tubercle bacilli are persistent, but are just in a dormant state within the unsuspecting host. This dormant state of this disease may last a lifetime without the individual developing any form of active disease. Yet progression to active TB by reactivation occurs in 5-10% of the individuals infected (Kumar et al., 2011). Co-infection with HIV/AIDS is therefore, an important aspect to consider, as HIV is known to be an immunosuppressant. The failing of the immune system by HIV causes many individuals with a latent infection to progress to active TB infection. Other factors that may compromise the immune system include diabetes, pollution, tobacco smoke as well as a genetic predisposition (Kumar et al., 2013). With reactivated TB, individuals who are poorly treated for this infection, if treated at all, run the risk of developing tuberculosis lesions in the lung.

Cavities develop close to the airway spaces and allow the coughing up of tubercle bacilli through the airway to the outside environment. This process forms part of the transmission stage where many individuals can get infected if they are in close proximity to the infected individual (Kumar et al., 2011). The virulence lifecycle of *M. tuberculosis* and the progression of the disease are illustrated in Figure 1.5.

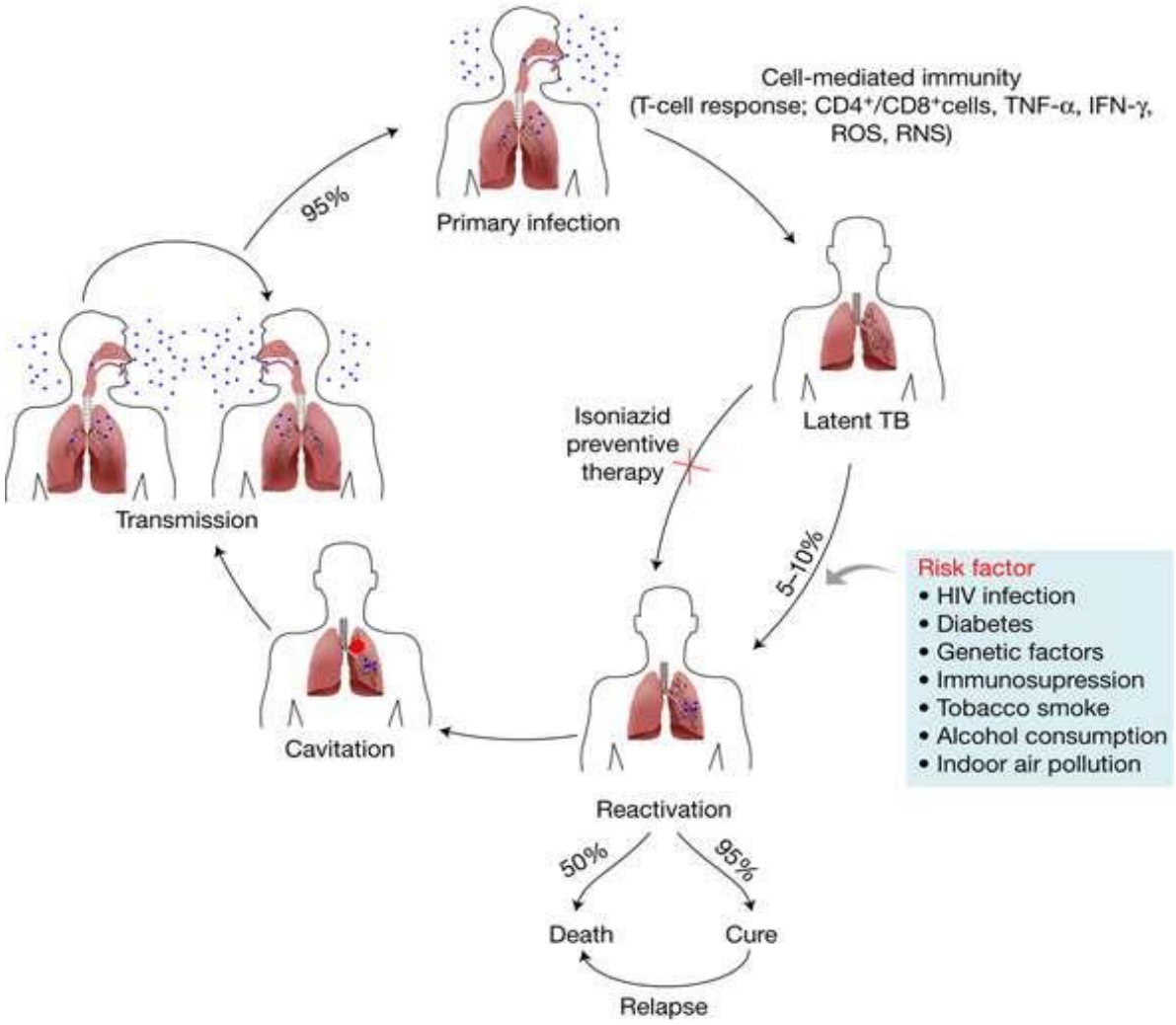


Figure 1.5: The virulence lifecycle of *M. tuberculosis* and the progression of tuberculosis disease (Kumar et al., 2011)

1.5. Adaptive immunity

An important feature of the adaptive immunity is the presence of the CD4+ T-cells, especially when co- infection with HIV/AIDS is seen. HIV co-infection results in depletion of the CD4+ T-cells which gives rise to increased susceptibility to re-infection (Schwander and Dheda,

2011). Adaptive immunity is mainly mediated by these CD4+ T-cells but is also supported by the CD8+ T-cells (Boom et al., 2003).

The local innate immunity which is mediated by the alveolar macrophages may fail to control the replicating bacilli and as a result, the immune system of the infected host is exposed to increasing amounts of mycobacterial antigens which then result in the development of the adaptive immune response. This response is mediated by the T-cells and after this, acute adaptive immunity develops. This is followed by a chronic memory immune phase. This phase is important for the control of the bacilli that are persistent as well as for the surveillance for possible re-infections. Failure of the acute or chronic adaptive immunity phase may then result in clinical TB which makes it possible for the transmission of *M. tuberculosis* to a new host (Boom et al., 2003).

Due to such a high number of the global population that has latent TB infection, it is indicative that *M. tuberculosis* has developed many mechanisms that enable this pathogen to modulate or avoid detection by the host (Flynn and Chan, 2003). Several strategies have been identified that *M. tuberculosis* may implement during latent infection and these can be summarized as follows:

- Preventing the recognition of the infected macrophages by T-cells by inhibiting MHC Class II processing and presentation.
- Evading macrophages killing mechanisms such as those mediated by nitric oxide and the reactive nitrogen intermediates (Flynn and Chan, 2003; Gupta et al., 2012).

1.6. Drug-resistant TB

1.6.1. Multidrug-resistant TB (MDR-TB)

Multi-drug resistant TB involves the strains that are resistant to two of the first line drugs namely isoniazid and rifampicin. With the use of Xpert® MTB/RIF for the simultaneous detection of both TB and the resistance to rifampicin (without further testing for resistance against isoniazid), a number of cases have been reported. The cases of MDR-TB complicate the treatment of TB, especially with its ability to be resistant to the first line drugs and the lack of testing initiatives by TB patients. Figure 1.5 shows the new cases of TB that can be associated with MDR-TB and RR-TB (rifampicin resistant TB) (WHO, 2017). Table 1.1 shows

the presence of MDR-TB and XDR-TB cases widely spread across several provinces within South Africa from 2007 to 2010. (Kanabus, 2017).

1.6.2. Extensively drug-resistant TB (XDR-TB)

Extensively drug resistant TB or XDR-TB as it is better known is an emergent form of TB. This is a form of TB that has also been reported from most parts of the world (Figure 1.6) (WHO, 2012). To confirm XDR-TB one needs to do an acid-fast stain as well as a sputum culture with antibiotic susceptibilities. In countries where funding is limited, solid media are used for *M. tuberculosis* cultures. This can delay the diagnosis for up to a month and therefore, is insufficient in providing timely detection.

Figure 1.6 The estimated incidences of MDR-TB and RR-TB for 2016 (WHO, 2017).

The influx for funding, therefore, also seems to fuel this infection; and large-scale upgrades of treatment centers and clinics especially in developing countries, where poverty is also associated with this disease, needs to be undertaken (Madariaga et al., 2008).

A noticeable outbreak of XDR-TB occurred in 2006 in the province of Gauteng and this made the health institutions, as well as the government of South Africa, focus on the fact that this is a serious disease that just cannot be ignored (Figure 1.7).

Table 1.1 The incidence rate of MDR and XDR-TB (in brackets) in each province of South Africa for the year 2007- 2010 (Dept. Health, 2017).

	2007 MDR TB (XDR-TB)	2008 MDR TB (XDR-TB)	2009 MDR TB (XDR-TB)	2010 MDR TB (XDR-TB)
Eastern Cape	1,092 (108)	1,501 (175)	1,858 (123)	1,782 (320)
Free State	179 (4)	381 (1)	253 (3)	267 (7)
Gauteng	986 (38)	1,028 (30)	1,307 (65)	934 (37)
KZN	2,208 (241)	1,573 (181)	1,773 (254)	2,032 (201)
Limpopo	91 (2)	185 (2)	204 (6)	126 (6)
Mpumalanga	506 (12)	657 (14)	446 (18)	312 (5)
North West	397 (4)	363 (4)	520 (13)	158 (14)
Northern Cape	199 (7)	290 (19)	631 (40)	353 (39)
Western Cape	1,771 (42)	2,220 (60)	2,078 (72)	1,422 (112)
South Africa	**7429 (446)**	**8198 (486)**	**9070 (594)**	**7386 (741)**

Data in Table 1.1 showed a wide array of regions where drug-resistant types of TB infection occurred within a country; these types of TB infections are also observed globally, with reports of these types of infections in regions everywhere across the globe. First line drugs are ineffective against MDR-TB and XDR-TB. The treatment for these types of TB involves taking combinations of the first-line drugs, to which they are resistant, and the second line drugs. The second line drugs include aminoglycosides, cycloserine, terizidone, ethionamide, protioamide, capreomycin, aminosalicylic acid and fluoroquinolones (Ma et al., 2010).

1.6.3. Extremely drug-resistant TB (TDR-TB)

A new form of TB has also made its abysmal mark on the world, the extremely drug-resistant TB (TDR-TB). This form of TB has not yet formally been introduced by the WHO, but notice has since been taken as seen in their Annual Tuberculosis Report of 2012 (WHO, 2012).

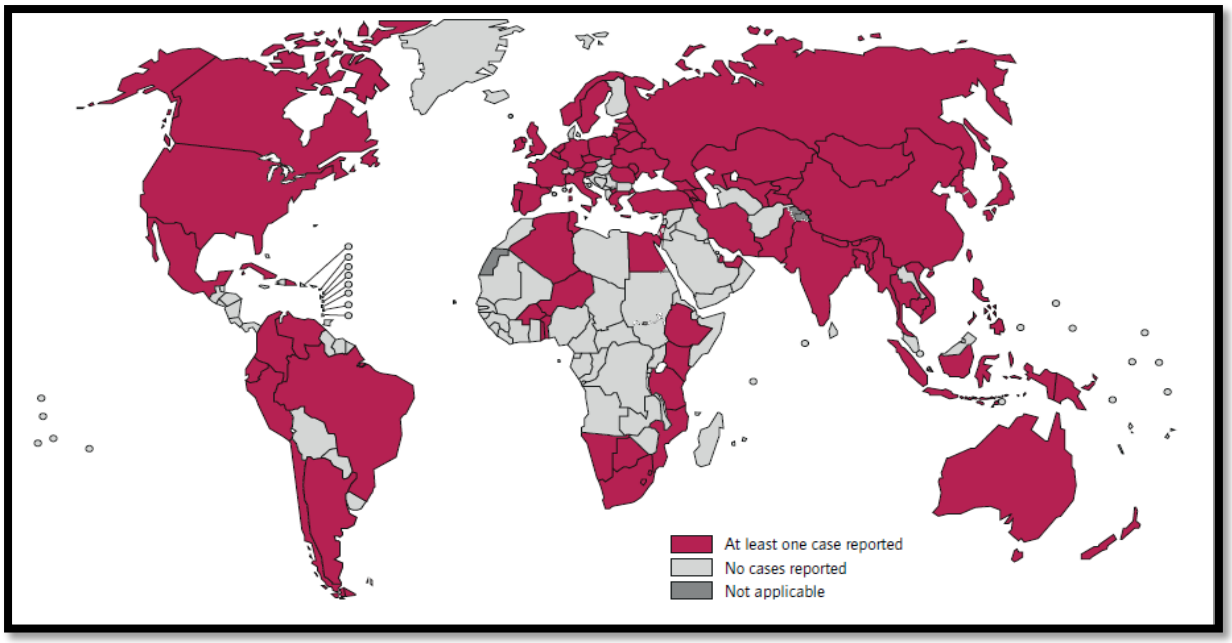


Figure 1.7 Countries that have notified at least one case of XDR-TB by the end of 2011 (WHO, 2012).

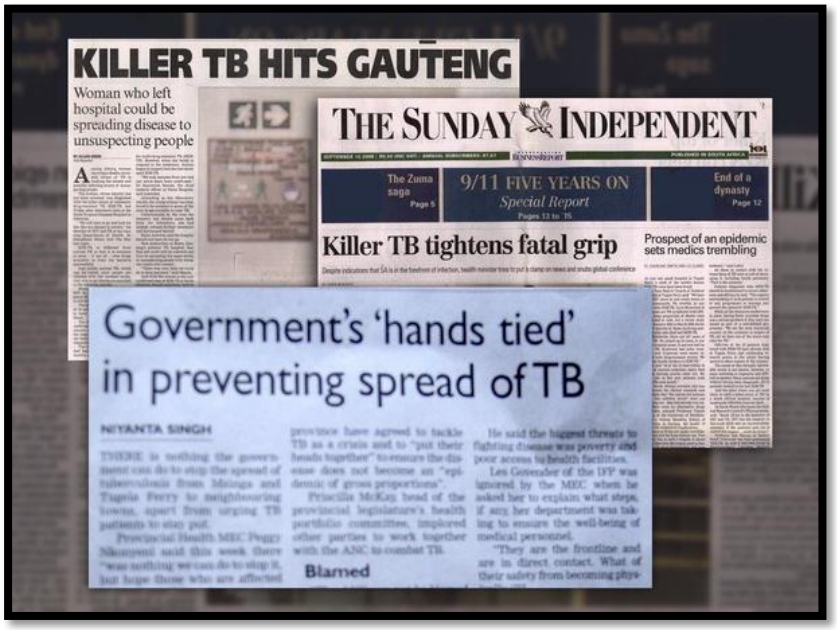


Figure 1.8 Newspaper headlines reporting on the XDR-TB outbreak in Gauteng in 2006 (CSC.com, 2006).

Individuals suffering from TDR-TB show resistance to all first- and second-line drugs. Two cases of TDR-TB were reported from Italy; cases were also reported from India. These cases were found to be resistant to all drugs (Migliori et al., 2007; Udwadia et al., 2012). Mostly

these cases have been found in very impoverished and isolated areas, but experts believe that there could be many undocumented cases as well. Both cases in Italy suggested initial mismanagement and non-compliance to the regimen (Migliori et al., 2007). Tuberculosis is a serious infection that needs to be managed in an optimum way so that it can be ensured that such cases as these and new forms do not emerge due to human error. All these resistant forms of TB may develop due to non-compliancy of the infected individuals, as along with the high number of drugs it involves a long treatment plan (Korbel et al., 2008). It has been observed that in situations where poverty is evident, there are high levels of co-infection with HIV/AIDS and poor access to the high-quality treatment (Ma et al., 2010) and this restricts the efficacy of the control programs. Only 1% of the MDR-TB cases in 2008 received proper treatment that agreed with the standards recommended by WHO (Ma et al., 2010). The drugs that are currently available for treatment are incapable of solving the multiple challenges and problems concerning resistance and effective treatment plans. To prevent the spreading of MDR-TB, XDR-TB, and TDR-TB, shorter treatment plans are needed that are safer, less expensive and more effective as compared with the existing treatment regimes. This only emphasizes the need for new drugs, drug targets and new regimes to be put in place to control the rise in the number of resistant strains.

1.7. Latent tuberculosis

Many individuals throughout their lifetime have direct exposure to *M tb*, may it be as contacts of the individuals that have active TB infection or through random contact with an infected individual with clinical TB. It has been estimated that about 90% of the individuals having evidence of a cellular immune response remain healthy and do not go on to develop active TB (Grotzke and Lewinsohn, 2005). This latent form of TB is prevalent in about one third of the world population (WHO, 2008) and an estimated 10% of these individuals move on to develop active TB. Latent TB is defined as the protective immunity process, which inhibits the growth of the pathogen. It is an ongoing immune-cycle, which consists of immune activation, and suppression, which prevents the bacilli from spreading and replicating (Korbel et al., 2008). After protective immunity sets in, the mycobacterium lowers its metabolism and goes over to a silent state with slow or non-replication and therefore a persistent dormant state (Korbel et al., 2008). This dormant state may be a status for a few individuals for a couple of years or even a lifetime, but for other individuals, the reactivation of the tubercle bacilli may be due to another immune-compromising disease (Korbel et al., 2008). The prevalence of this

reactivation is especially widespread in African countries where co-infection with HIV is evident.

The objective of most of the research in TB has been to reduce the risk of reactivation in individuals who are in high-risk situations, such as those who may be in close contact with individuals having active TB or any other immune compromising diseases. Presently isoniazid monotherapy for 6-9 months seems to be an effective treatment for reducing the risk by as much as 60%, especially for those in contact for over 2 years, with individuals having active TB (Lienhardt et al., 2010). There is also the development of new drug candidates such as PA 824 and TMC-207, of which, TMC-207 is the only successful drug candidate that completed phase III clinical stage development and was projected to move forward to the next stage by the fourth quarter of 2014 (Figure 1.8) (Klein et al., 2014., WGND, 2014).

TMC-207 also known as bedaquiline is a diarylquinoline, which forms part of the synthetically produced antibiotics (Figure 1.9). It belongs to the quinoline class of compounds with a novel mechanism of action, an ATP synthase inhibitor. This compound showed effective activity against non-replicating *M. tuberculosis* for individuals who had latent TB infection as well as showed inhibition against *M. tuberculosis* strains that were sensitive (the MIC value ranged between 0.030-0.120 µg/mL). The compound showed a MIC value of 0.03 µg/mL against the resistant strains, resistant to the first- and second line drugs (Van Daele et al., 2005; Ma et al., 2010; Matteelli et al., 2010; Tasneen et al., 2011). Together with known drugs such as rifapentine (RPT), TMC-207 boasts of an innovative shorter treatment regime (Tasneen et al., 2011).

PA-824 was synthetically produced as a derivative from the known nitroimidazoles antibiotics and forms a part of the group known as the nitroimidazo [2, 1-b] oxazines (Barry et al., 2004). This drug has shown to be effective *in vitro* activity against both resistant and susceptible strains of *M. tuberculosis*, with MIC values that ranged between 0.015 and 0.25 µg/mL. (Ginsberg et al., 2009). PA-824 has had an unknown mechanism of action, but a study conducted by Manjunatha et al., (2009) showed the possible involvement of respiratory poisoning through the release of nitric oxide as the possible mechanism of action.

It is with ongoing research that people are trying to develop drugs that are active against latent *M. tuberculosis* and are safe, short-term compliant and cost-effective. There are currently many drugs in the pipeline moving through the different phases until they are ready for

commercialization. Figure 1.10 shows the different phases and the many drugs that currently form part of the new drug pipeline (WGND, 2014). Table 1.2 in detail describes the origin of the drugs currently in the pipeline, their activity found and what their mechanism of action is

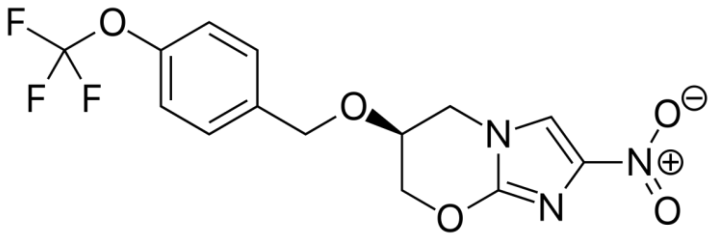


Figure 1.9 The candidate drug PA 824, for TB infection (commons.wikipedia.org, 2014).

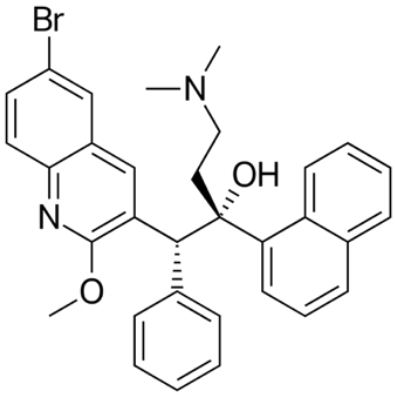


Figure 1.10 Candidate drug, TMC 207, for TB infection (commons.wikipedia.org, 2015).

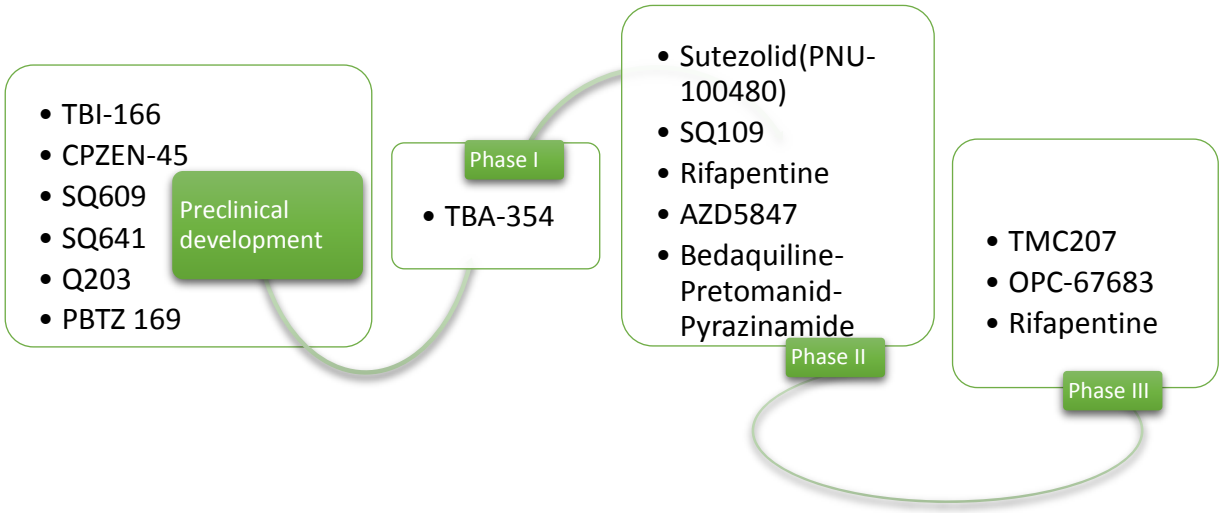


Figure 1.11: Different phases of the drug pipeline. (WGND, 2014)

1.8. The mechanisms of current Tuberculosis drugs

1.8.1. The first line antituberculosis drugs

1.8.1.1. Rifampicin

Rifampicin is said to be active against both growing and non-growing bacilli of mycobacterium, with its method of action resting in binding to the β-subunit of the RNA-polymerase, which will in effect hinder the elongation of the messenger RNA (Blanchard, 1996; Palomino and Martin, 2014).

1.8.1.2. Isoniazid

Isoniazid is known to be active against only metabolically active replicating bacilli, whereas Rifampicin is known to be active against both these bacteria, but also against non-replicating bacteria. The mechanism of action of Isoniazid is by inhibition of the synthesis of the much-needed mycolic acids, a cell wall component of *M. tuberculosis*, through the NADH-dependent-enoyl-acyl carrier protein (ACP) – reductase, which is encoded by the gene, *inhA* (Rawat et al., 2003; Palomino and Martin, 2014).

1.8.1.3. Ethambutol

The mechanism of action of ethambutol, first introduced in 1966, is attributed to the biosynthesis of arabinogalactan in the cell wall of the mycobacterium. Ethambutol is effective against multiplying bacterial bacilli (Takayama and Kilburn, 1989; Palomino and Martin, 2014).

1.8.1.4. Pyrazinamide

This first line drug is effective against semi-dormant bacilli that reside in acidic regions of TB lesions. The mechanism of action is attributed to the conversion of pyrazinamide into pyrazinoic acid which disrupts the bacterial membrane energetic which in turn inhibits membrane transport (Zhang and Mitchison, 2003; Palomino and Martin, 2014).

1.8.1.5. Streptomycin

Once again, Streptomycin is a first line drug that is active against actively growing bacilli and has a mechanism of action that involves the inhibition of the translation in protein synthesis of the bacteria (Moazed and Noller, 1987; Palomino and Martin, 2014).

1.8.2. Second line drugs

1.8.2.1. Fluoroquinolones

These second-line drugs are known for treatment against MDR-TB, these drugs are involved in the inhibition of the topoisomerase II, necessary for the viability of the bacteria (Fabrega et al., 2009; Palomino and Martin, 2014).

1.8.2.2. Kanamycin and Amikacin

Both of these second-line drugs are aminoglycosides and are used in the treatment of MDR-TB. The mechanism of action proposed for these drugs is in the inhibition of protein synthesis by alteration at the level of 16S rRNA (Palomino and Martin, 2014).

1.8.2.3. Capreomycin and Viomycin

These second-line drugs are known to be cyclic peptides that have the same mechanism of action as Kanamycin and Amikacin by binding at the same position in the ribosome (Stanley et al., 2010; Palonimo and Martin, 2014).

1.8.2.4. Ethionamide

This drug is responsible for treating MDR-TB or suspected cases of MDR-TB (Brossier et al., 2011). The drug interferes with mycolic acid synthesis by the formation of an adduct with NAD which then inhibits the enoyl-ACP-reductase enzyme (Palomino and Martin, 2014).

1.8.2.5. Para-amino salicylic acid

This second line drug has a mechanism of action that is not clearly defined against MDR-TB as the other drugs, but it is said that it might involve the competition with para-amino benzoic acid for dihydropteroate synthase, which interferes with the synthetic process of folate (Palomino and Martin, 2014).

1.8.2.6. Cycloserine

Cycloserine is an analog of D-alanine and by blocking the activity of D-Alanine: D-alanine ligase, it inhibits the synthesis of peptidoglycan. It is also an inhibitor of D-alanine racemase, which is needed for the conversion of L-alanine to D-alanine (Zhang, 2005; Palomino and Martin, 2014).

1.8.2.7. Thiacetazone

Although effective against TB by inhibiting the synthesis of mycolic acid, this drug is known to be toxic (Grzegorzewicz et al., 2012; Palonimo and Martin, 2014). Lethal cutaneous adverse drug reactions are common among HIV-positive individuals taking treatment that includes Thiacetazone (Chan and Iseman, 2002). Although Thiacetazone is only used in cases of multi-drug resistant TB for which there is no other treatment, it is currently not included in the list of essential medicines or as reserve treatment as recommended by the WHO (WHO, 2017).

1.8.2.8. Clofazimine

Used for treatment against MDR-TB, this drug also exhibited a few adverse effects such as pigmentation of the skin and therefore, was rather used to treat cases of leprosy (Browne and Hogerzeil, 1962). Clofazimine is currently included in the reserve list of essential medicines for multi-drug resistant TB treatment (WHO, 2017). Recent studies have shown that this drug's mechanism of action involves the outer membrane of the bacteria as the target (Cholo et al., 2012; Palonimo and Martin, 2014).

1.8.2.9. Linezolid

Originally used for the treatment of skin conditions caused by other Gram-positive bacteria, the mode of action is attributed to the inhibition of a step in the early stages of protein synthesis by binding to the 50S ribosomal subunit (Zhang, 2005; Leach et al., 2011; Palonimo and Martin, 2014).

Table 1. 2 Origin, activity found, mechanism of action and references of the drugs currently forming part of the new drug pipeline.

Name of candidate drug	Origin	Activity	Mechanism of action	References
TBI-166	Clofazimine analogues	*In vitro* activity against *M tb* H37Rv (culture and macrophages), drug resistant clinical isolates	Exact mechanism unknown	Quan et al., 2017
CPZEN-45	Caprazamine analogue	*In vitro* activity against *M. tuberculosis* H37Rv	WecA transferase inhibitor involved in arabinogalactan synthesis	Quan et al., 2017
SQ609	Adamantine-containing hydroxydipiperidine	*In vitro* activity against *M. tuberculosis* H37Rv	Believed to target cell wall synthesis	Quan et al., 2017
SQ641	Capuramycin analogue	Activity against both drug resistant and susceptible strains of *M. tuberculosis*	Translocase 1 inhibitior	Quan et al., 2017
Q203	An imidazopyridine compound	Activity against both latent and drug-resistant TB	Targets the cytochrome b subunit (QcrB) of the cytochrome bc_1 complex, depleting the ATP levels intracellularly	Hoagland et al., 2016

Table 1.2 Origin, activity found, mechanism of action and references of the drugs currently forming part of the new drug pipeline (*continued*)

PBTZ 169	A derivative of BT043, a piperidine containing benzothiazinone	Activity in synergy with clofazimine against replicating and non-replicating *M. tuberculosis* H37Rv *in vitro*	Inhibits DprE1, an enzyme involved in arabinogalactan and arabinomannan synthesis pathway	Quan et al., 2017
TBA-354	Pyridine- containing biaryl compound	Activity against both replicating and non-replicating *M. tuberculosis*	Similar to Pretomanid and is hypothesized to involve both respiratory poisoning and cell wall inhibition	Manjunatha et al., 2009; Upton et al., 2015
Sutezolid (PNU-100480)	A Linezolid analog	Based on bactericidal activity	Blocking of microbial protein synthesis	Wallis et al., 2014
SQ109	A 1,2 ethylenediamine	Activity against both drug sensitive and multi-drug resistant TB	Targets a mycolic acid transporter (MmpL3) required for the incorporation of mycolic acid into the mycobacterium cell wall	Sacksteder et al., 2012
AZD5847	A novel oxazolidinone	*In vitro* bactericidal activity against extra-cellular and intracellular mycobacteria	Targets the 50S ribosomal subunit	Balasubramanian et al., 2014

Table 1.2 Origin, activity found, mechanism of action and references of the drugs currently forming part of the new drug pipeline (*continued*)

Bedaquiline-Pretomanid-Pyrazinamide	A mixture of existing drugs	Activity against both drug resistant and drug sensitive mycobacteria	Various mechanisms of action	Peterson et al., 2016
TMC207	A diarylquinoline compound	Activity against both drug-resistant and drug-sensitive TB	Inhibition of bacterial ATP synthase	Matteelli et al., 2010
OPC-67683	A nitro-dihydro-imidazooxazole derivative	Activity against both drug sensitive and drug resistant TB	A mycolic acid biosynthesis inhibitor	Matsumoto et al., 2006
Rifapentine	Rifamycin derivative	Activity within macrophages	Inhibition of DNA-dependent RNA polymerase in susceptible strains of *M. tuberculosis*	Egelund and Peloquin, 2016; Quan et al., 2017,

1.9. Major problems faced with the current Tuberculosis drug treatment

Together with the unhealthy setting in which this disease is prevalent, the termination or interruption of TB treatment is also identified as one of the biggest pitfalls concerning the spread of infection. The progression of the treatment is mainly determined by the adverse effects that are experienced by the individuals undertaking TB drug treatment.

Major and minor adverse effects normally form part of the TB treatment plan. Some major adverse effects that are experienced with the use of the first line drugs include hepato- and ototoxicity (involves toxicity of the ear) as well as hypersensitivity reactions and nausea (Gulbay et al., 2006; Arya, 2011). All the drugs that form part of the treatment plan and their biggest adverse effects are depicted in Table 1.3 (Arya, 2011). The major adverse effects as mentioned in Table 1.3 together with others not mentioned may prove to be fatal if left untreated. These adverse effects cause cessation of the treatment or part of the treatment as the drugs that are attributable to the side-effects are then excluded. This does not just lengthen the treatment period and the risk of termination and relapses, but it also involves financial implications to the individual. The economic feasibility of the government healthcare plans that are in place are also on the line.

The resurgence of MDR-TB and XDR-TB has also complicated the treatment of this epidemic. This is also one of the major factors focusing on the importance of completing the treatment plan. There are various ways to manage the completion of TB treatment. This can be done by administering supplements and/ or drugs which have hepatoprotective effects and which can boost the immunity of the infected individuals.

1.10. Hepatotoxicity due to Tuberculosis drug treatment

For the TB treatment regime to effectively eradicate the invading pathogen and infection it needs to be successfully carried out. This entails to lower the risk of resistance and to enhance the effectiveness. The success of the treatment relies on the compliance of patients and for these many institutions have initiated programs, such as the STOP TB partnership established in Amsterdam in 2000, to ensure compliance as well as good quality anti-TB drugs at a lower cost (WGND, 2014). Countless infected individuals cease treatment due to the adverse effects experienced with anti-TB drugs.

Table 1. 3 Various TB treatment drugs and their associated side-effects (Adapted from Arya, 2011).

Drugs	Side-effects
Isoniazid	Skin rash and hepatitis
Rifampicin	Abdominal pains, nausea, vomiting, hepatitis and thrombocytopenic purpura
Pyrazinamide	Hepatitis and arthralgia
Streptomycin	Vestibular and auditory nerve damage as well as renal damage
Ethambutol	Retrobulbar neuritis and ocular side-effects
Thioacetazone	Skin rash and exfoliative dermatitis
Para-aminosalicylic acid	Anorexia, nausea, vomiting and hypersensitivity reactions
Kanamycin	Vertigo, auditory nerve damage and nephrotoxicity
Ethionamide	Diarrhoea, abdominal pain and hepatotoxicity
Cycloserine	Dizziness, headache, depression, psychosis and convulsions

First line drugs that form part of the treatment regime has been known to cause hepatotoxicity, especially rifampicin (RIF) which is metabolized in the liver and therefore makes it most vulnerable to injury (Tasaduq et al., 2003). Oxidative stress and the subsequent increase in lipid peroxides are mechanisms of pathogenesis that can cause hepatic injury (Kumar et al., 2013). Isoniazid (INH) and RIF in part cause hepatotoxicity through the mechanisms of oxidative stress and lipid peroxidation (Kumar et al., 2013).

Experimental studies have shown that the hepatic injury due to anti-TB drugs can be lowered and eliminated by the use of herbal formulations (Tasaduq et al., 2003). Many plant-derived samples have been reported to show some extent of hepatoprotective abilities against anti-TB drug-induced hepatotoxicities such as the aqueous leaf extracts from the famous Neem tree, *Azadirachta indica* and the polyherbal formulation called Liv.52, is a polyherbal formulation

consisting of several plants such as, *Capparis spinosa*, *Cichorium intybus*, *Mandura bhasma*, *Solanum nigrum*, *Terminalia arjuna*, *Cassia occidentalis*, *Achillea millefolium* and *Tamarix gallica* produced by an Indian company called Himalaya Herbal Healthcare (Figure 1.12) (Kumar et al., 2013; Himalayawellness.com, 2014).

Figure 1.12 The neem tree, *Azadirachta indica* and Liv52, a polyherbal formulation created for liver disease by Himalaya Wellness (www.prota4u.org, 2014; himalyawellness.com, 2014).

Another popular plant with hepatoprotective abilities is Garlic (*Allium sativum*) which significantly lowers important liver transaminases ALT and AST, which are biomarkers for liver injury, as well as the bilirubin levels. Garlic is well known for its therapeutic properties and these can all be ascribed to several known secondary metabolites such as thiosulfanates, terpenoids, and steroids, to name a few (Kumar et al., 2013). A few modern drugs such as tricholine citrate, trithioparamethoxy phenyl propene, phospholipids and a combination of L-ornithine, L-aspartate and pancreatin, silymarin and ursodesoxy cholic acid, are known to exhibit hepatoprotective properties (Kumar et al., 2013). Although there are several drugs on the market that aid in liver difficulties, many have several adverse effects associated with them and no complete solution has yet been found to completely manage these injuries to the liver without any adverse effects. Many people also rely on the herbal formulations as the modern synthetic drugs come at a significant price.

1.11. The role of antioxidants during Tuberculosis infection

Studies have reported that a lack of antioxidants in patients infected with TB, make them prone to hepatotoxicity; therefore, a lot of emphasis has been put on antioxidants and their role in reducing hepatotoxicity through their action against these harmful oxidants (Sodhi et al., 1998; Dhuley, 2002; Kumar et al., 2013). Ascorbic acid or Vitamin C as it is more commonly known

and Quercetin, a major flavonoid in some fruits and vegetables, have been shown to protect cells against the damaging effects of oxidative stress especially neuronal cells in oxidative stress-induced neurotoxicity (Heo and Lee, 2004). It has been found in several cases that untreated individuals with TB infection have very high levels of oxidants (Mohod and Kumar, 2012). High levels of oxidants such as reactive oxygen species (ROS) and other free radicals lead to oxidative stress, the condition which is implicated during the pathogenesis of TB (Mohod and Kumar, 2012). Although the immune response to TB infection is to induce ROS and reactive nitrogen intermediates (RNI), it can also lead to inflammation, injury to tissues and immunosuppression (Beers and Sizer, 1952; Madebo et al., 2003; Mohod and Kumar, 2012; Mokondjimobe et al., 2012).

Antioxidants act as a defense mechanism towards oxidants and with malnutrition, being one of the foremost conditions found in people suffering from TB, there exists an imbalance (Mohod and Kumar, 2012). Increased levels of ROS during TB infection can, therefore, be described as the leading cause of the decrease in antioxidant levels (Mohod and Kumar, 2012). Although Mohod and Kumar, (2012) found that the levels of antioxidants were higher in treated individuals than in the untreated individuals, it was still at levels lower than the positive controls used during the study. This means that the state of oxidative stress still exists even after treatment has been initiated. It was also found in literature that even after a patient has been successfully treated for TB and the treatment has been stopped the high oxidative state persists. There is, therefore, sufficient evidence to indicate that antioxidant treatment should be paired with the ongoing TB treatment regime and even after the treatment has been concluded.

Not just, does the host's immune response implicate an increase in the production of ROS as part of its defense against TB infection but the drugs that are taken during treatment are also the primary cause of ROS and free radical production. Many of the first-line drugs that are taken during treatment produce ROS as part of their metabolism. INH is one of the main drugs taken during treatment, which inhibits the biosynthesis of the mycolic acids that form part of the mycobacterium's cell wall. It also induces ROS generation, which may lead to higher levels of oxidative stress as well as be the cause of oxidative hepatotoxicity (George et al., 1995; Siez and Stabl., 1995; Wang et al., 1998; Betts et al., 2003). As previously mentioned, an increase in ROS production causes oxidative stress and this leads to immune suppression, which in turn

might also predispose the individual to the toxicity, and adverse effects that are experienced whilst taking the anti-TB drugs (Walubo et al., 1995; Reddy et al., 2004).

In a study conducted by Wilson et al., (2015) it was proposed that ingesting botanicals such as *Sutherlandia frutescens*, together with the first line TB drugs may present antagonistic effects. During a double-blind randomized placebo-controlled trial they concluded that the CD4 T-lymphocyte count was not impacted in any participants but that the duration of secondary infections was much longer than observed in placebo patients, partly due to two individuals that developed TB even though they were on Isoniazid therapy for prevention against TB development. The antagonistic interaction was hypothesized to be due to the antioxidative nature of *Sutherlandia frutescens* that blocked the mechanism of action of Isoniazid. Isoniazid is characterized as a prodrug and requires oxidative activation. If botanicals are ingested with INH or any other drugs that requires oxidative activation there will be the possibility of interference as all medicinal plants have some measure of antioxidative activity. This highlighted the susceptibility of drugs when ingested together with botanicals. Adjunct host-directed therapy for the treatment of TB can ensure that for instance in this case, a botanical with a high antioxidant content can be taken at a later stage during treatment (after 6 months in the case of Isoniazid) to ensure that any prodrugs are fully activated. In this manner, the prodrugs are activated but the adverse effects that are experienced due to the treatment can be also be managed. Many drugs require metabolic activation before exerting their mechanisms of action and this emphasizes the need for further research into the mechanisms of action of not just the first line drugs, but also botanicals ingested during treatment.

1.12. Medicinal plants and their role in combatting infectious diseases

Medicinal plants have played a major role in the traditions and lives of many people all over the world and from all walks of life. Plants that were used medicinally by various people and tribes had initiated the development of traditional healthcare systems such as Ayurveda and Unani, which have formed a key part of mankind for thousands of years (Gurib-Fakim., 2006). Today the medicinal importance of higher plants is still recognized with natural products and their derivatives having a 50% share of all drugs in clinical use (Gurib-Fakim., 2006). Some of the first drugs to be discovered were in 1803/04 by Friedrich Serturner, who isolated morphine in a test tube from opium (Klockgether-Radke, 2002) and aspirin, which is based on a natural product called salicin that is isolated form the tree *Salix alba* (Kinghorn, 2001). Pharmacologically natural medicines have shown their importance in their use as starting

material for drug synthesis or directly as therapeutics. Natural medicines can also function as models for pharmacologically active compounds that may possess higher activity and less toxicity than their synthetic counterparts (Verma and Singh, 2008).

Plants have been used in many instances to treat infectious diseases especially when these infectious diseases build up resistance against synthetic antibiotics. In a study conducted by Lakshmanan et al., (2011), plant specimens were chosen from the Ayurvedic treatise Shaasrayogam, to be used in TB treatment. Seven plants were chosen, *Elettaria cardamomum*, *Zingiber officinalis*, *Cyperus rotundus*, *Kaempferia galanga*, *Tinospora cordifolia*, *Asparagus racemosus*, and *Coleus vettiveroides*. From these seven plants, four had inhibition against *M. tuberculosis*. A lead compound known as EPMC (ethyl-p-methoxycinnamate) was isolated from the essential oil of *Kaempferia galanga* and found to inhibit all strains of *M. tuberculosis* (Figure 13).

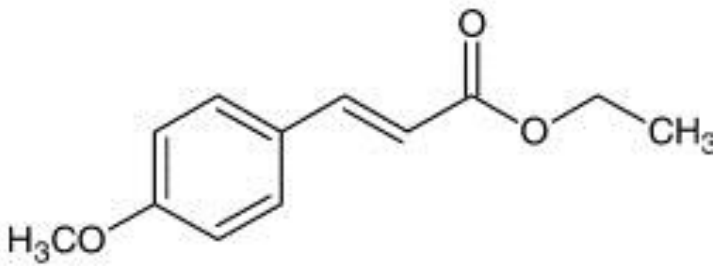


Figure 1.13 Ethyl-p-methoxycinnamate (EPMC) (Lakshmanan et al., 2011).

These strains included avirulent and virulent susceptible laboratory strains of *M. tuberculosis*, also susceptible and multi-drug resistant clinical strains from patients suffering from TB (MIC values ranging from 0.242-0.485 mM). What makes this lead compound so unique is that although it inhibited all the previously mentioned strains of *M. tuberculosis*, it had no effect against the tested Gram-positive or Gram-negative bacteria, including no inhibition against *M. smegmatis*. This may be due to EPMC having a unique target specific to *M. tuberculosis*. It is suggested that this unique target may be the involvement in the mycolic acid biosynthetic pathway (Lakshmanan et al., 2011).

Multi-drug resistant forms of TB are a major threat to the infected individuals and to the overall cure rate for South Africa. In many instances, medicinal plants have been used to help with the treatment of diseases as with the case of Linctagon. *Pelargonium sidoides* forms the major component of this over the counter drug and has been successfully shown to combat respiratory infections associated with colds and flu (Nativa, 2014). As previously discussed, Liv 52 is

known as a potent hepatoprotectant especially against chemically induced hepatotoxicity and is a globally prescribed adjuvant for patients suffering from TB adverse effects including in countries such as Russia (ExpressPharma, 2014). Another polyherbal formulated phytoconcentrate that has played a major part in the possible curing of infectious diseases in the Ukraine is known as Dzherelo-I (Immunoxel). The proposed main properties attributed to this phytoconcentrate are that it functions as an immunomodulator and a hepatoprotectant (Nikolaeva et al., 2009). Several studies have concluded that this phytoconcentrate assists in eradicating infections caused by hyposensitive bacteria such as TB and chlamydia and was approved as an adjunct for TB therapy in 1999 by the health authorities in the Ukraine (Melnik et al., 1999; Zaitzeva et al., 2009). The mode of action is said to be due to an increase in the production of interferon, enhancement of the protection provided by the mucous membranes of the respiratory and digestive systems and also preventing the spread of infection (Ekomed.com, 2010). Dzherelo is commonly used in combination with other immunomodulators on the market in the Ukraine namely Svitanok and Lizorm to treat infectious diseases such as TB and HIV infection (Zaitzeva, 2006, Zaitzeva et al., 2009).

Svitanok is known as a hepatoprotectant and commonly used as therapy for hepatitis, whereas Dzherelo is regularly used to relieve symtpoms due to autoimmune disorders (Bodnar et al., 2002). Dzherelo and Svitanok are particularly recommended as adjuvants for pulmonary TB treatment (Melnik et al., 1999). Studies on the combinations of Dzherelo with Svitanok and Lizorm have indicated that when combined with anti-TB treatment for MDR-TB during a small-scale study that sputum culture conversion was effective at an average of 32 days whereas with successful chemotherapy in a study conducted by Yew et al., (2003), the conversion was 14.5 months. This combination of the immunomodulators with the TB therapy enhances the effectiveness of the drugs taken and shortens treatment time (Zaitzeva, 2006). Further studies have indicated that the combination of these drugs have a drastic impact on the improvement of liver function within the infected individuals (Prihoda et al., 2007). Liver function as previously described is one of the main adverse effects that take place during TB treatment. This has shown that adjuvants play a significant role in combination with Western medicines against infection and diseases. There are many South African plants that are still unexplored and may harbour the potential, as these examples above, as adjuvants or direct treatment for infections and diseases.

Although there have been several studies conducted on natural products and their role as hepatoprotectants, these studies are limited in their scale and evidence presented. More thorough investigation is therefore still needed, with larger scale studies one of the main limitations in the current evidence presented. The products consumed in Ukraine as discussed above have been approved in their country of origin and studies have been conducted in other countries in Europe and recognized by a clinical trial site in the US, but the legislation regarding use of natural products differ between countries. These natural products have also been deemed safe for use in Ukraine as they are described within their local pharmacopeia. This also emphasizes that to really make strides much greater international collaboration is needed especially when indigenous plants are investigated for their biological properties.

Currently, adjuvants are specifically used together with subunit vaccines as there are normally poorly immunogenic and adjuvants are needed to stimulate the needed immune response. Plants have been introduced as a safer, more potent and natural alternative adjuvant. Crude extracts and compounds that have been isolated from plants have always been used as immunostimulators and therefore have the potential to act as adjuvants for their immunostimulatory properties (Harikrishnan et al., 2011). Various classes of compounds from plant sources have been identified over the years as known immunostimulators. These classes are alkaloids, saponins, polysaccharides, triterpenoids, iridoids and organic acids to name a few (Alamgir and Uddin, 2010).

In South Africa, it is estimated that approximately 80% of its population is dependent on medicinal plants for their primary healthcare needs (Street and Prinsloo, 2013). This places a large burden on the indigenous plants of South Africa. The controversial subject of biodiversity concerns is still a very important aspect that needs to be considered by individuals interested in the market that involves healthcare products from natural origin. Most of the South African continent is enveloped in generations of families with rich traditional and knowledgeable information concerning medicines of natural origin. For many individuals, medicinal plants as part of their main healthcare option, is the only choice mainly due to the high cost of primary healthcare as well as the availability of clinics in close proximity. The traditional knowledge is what proves to be an important part of searching for medicinal plants of interest and for the conservation and lessened impact on the surrounding plant community.

South Africa consists of many plant species that were identified to be commercially important (Van Wyk, 2008). Some of these plants include *A. linearis* (Figure 1.14), also known as

Rooibos tea and *Hypoxis hemerocallidea*, the African potato. South Africa is rich in plant species and biodiversity and a reported 25% of all higher plants in the world are concentrated in the southern part of Africa (Van Wyk, 2008). This all concludes to a wide array of chemically and structurally different compounds that can be found in plants and have the potential of being medicinally important. Several South African plants have been reported with good antimycobacterial and antioxidant activities. *Euclea natalensis* a South African plant with rich traditional knowledge linking it to the treatment of chest ailments gave rise to a monomer of diospyrin known as 7-methyljuglone, which had effective activity against *M. tuberculosis* comparable to that of the anti-TB drugs, streptomycin, and ethambutol (Mahapatra et al., 2007).

Figure 1.14 *Aspalathus linearis* (prota4u.org, 2014).

As a developing country, South Africa has many areas that are underdeveloped and poverty-stricken. These areas have thousands of people living in overcrowded and unsanitary conditions. These conditions are, however, very favourable for the pathogen *M. tuberculosis* as is evident in the number of infected individuals seen in these areas. Together with unsanitary conditions, low living standards and inadequate healthcare, the medicinal plants in the area are useful as a remedy against the many ailments that plague these areas. Medicinal plants, found in South Africa, that are identified by the traditional healers in these communities, can become potential remedies for TB and can also aid in the symptomatic relief of this disease.

1.13. Herb-drug interactions

With the high level of users using medicinal plants as their main and only type of healthcare, the risks exist that these are used concomitantly with prescribed medicine without the knowledge of a healthcare professional. Many healthcare professionals do however, in some instances prescribe other herbal drugs to be taken concurrently with prescribed drugs for the management of adverse effects experienced when taking the prescribed medicine as is the case with TB. Herb-drug interactions occur when one drug's metabolism is influenced by the simultaneous intake and interaction of a herb taken. This interaction may include the inhibition or induction of certain key enzymes that form part of the CYP P450 enzymes. These CYP enzymes are found in the liver and transporter proteins (Zhang et al., 2010). CYP1A2, CYP2D6, and CYP3A4 are involved in metabolizing approximately 83% of the pharmaceuticals marketed and initial assessments on the inhibition potential of herbs on these enzymes can give a glance into the herb-drug interaction that may occur by taking these herbs with drugs concomitantly (Rodrigues, 2008). In summary, CYP2D6 is mostly associated with polymorphisms between individuals and metabolizes 25% of marketed pharmaceuticals. CYP3A4 is mostly involved when herb-drug interactions are concerned and is involved in metabolizing 50% of marketed pharmaceuticals. Even though CYP1A2 only metabolizes 8% of marketed pharmaceuticals, this 8% of drugs are most frequently prescribed drugs under patients and therefore, carries a huge risk for herb-drug interactions (Delgoda and Westlake, 2004; Rodrigues, 2008; Strandell and Wahlin, 2011). CYP1 enzymes play an intricate role in cancer and inhibition of these enzymes especially, CYP1B1, CYP1A1, and CYP1A2 *in vitro* may also have chemopreventative potential *in-vivo* (Picking et al., 2018).

With so many TB-infected individuals found in areas where the use of medicinal plants is used as primary healthcare, it is of key importance that herb-drug interactions involving drugs taken during TB infection are investigated. Many of the CYP P450 enzymes have been identified by researchers as being induced or inhibited by drugs taken during TB infection. For instance, rifampicin, a common first-line drug taken during the first six months of treatment is a potent inducer of CYP3A4. Any drugs that use CYP3A4 for metabolism will be severely affected if taken concomitantly with rifampicin. Induction of a CYP P450 enzyme increases the rate at which these enzymes metabolize and excrete drugs and therefore large doses are required to obtain an effective therapeutic effect (Swart and Harris, 2005).

By investigating higher plants, as part of the rich biodiversity in southern Africa, phytomedicines from these plants may be identified, which could have beneficial properties

such as higher biological activity, antioxidant and hepatoprotective effects than their synthetically produced counterparts (Lakshmanan et al., 2011). Selected plants may be identified through sustainable means by making use of plants that have been known through traditional usage in South Africa. These indigenous plants of southern Africa may possess antimycobacterial properties, immunomodulatory and hepatoprotective potential that could make them active forms for treatment against TB disease or as supportive agents during the course of treatment to counter the adverse effects that may occur.

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2

AIMS AND OBJECTIVES

This chapter introduces the aims and objectives of the current study, including the hypothesis of the present study.

2. AIMS AND OBJECTIVES

2.1. Research questions

Tuberculosis (TB) still presents an enigma to scientists as the race to find a drug/treatment that is shortened and without side-effects, yet effective in eradicating the bacterial infection, is still ongoing. The introduction of new treatments as part of the regimen have been slow and costly. Natural products have long since been the first step in finding cures for infectious diseases. Many treatments have found their origins from a single chemical entity found within the plant kingdom. Currently, no South African plant has been reported to be effective for the treatment of TB directly or indirectly for the treatment of the symptoms caused as a result of the current treatment regimen. The research questions with regards to this project are as follows:

- Can the ethanolic extract of *Lippia scaberrima* Sond. (*L. scaberrima*) and *Aspalathus linearis* (Burm.f.) R. Dahlgren (*A. linearis*) and the 1:1 combination thereof, the essential oil of *L. scaberrima* and one of the constituents, verbascoside, directly inhibit the bacterial pathogen, *M. tuberculosis* H37Rv?
- Does the ethanolic extract of *L. scaberrima* and *A. linearis* and the 1:1 combination thereof, the essential oil of *L. scaberrima* and one of the constituents, verbascoside, have any potential for herb-drug interactions?
- Does the ethanolic extract of *L. scaberrima* and *A. linearis* and the 1:1 combination thereof, together with the essential oil of *L. scaberrima* possess any hepatoprotective activity?
- Can the synthesis of gold nanoparticles from the ethanolic extract of *L. scaberrima* with the use of gum arabic, show any lowered toxicity potential against hepatocellular carcinoma (HepG2) cells and increased activity against *M. tuberculosis* H37Rv?
- Does the ethanolic extract of *L. scaberrima* and *A. linearis* and the 1:1 combination thereof, the essential oil of *L. scaberrima* and one of the constituents, verbascoside, show any potential for use as an adjuvant for therapy of TB such as the drugs currently on the market, Dzherelo, Svitanok and Lizorm?

2.2. Hypothesis

The hypothesis of the present study is that the ethanolic extract of *L. scaberrima* and in combination with *A. linearis*, have the ability to lower the mycobacterial load and have the ability to lower the adverse effects experienced during the current TB treatment regimen.

2.3. Aims and objectives

The main aims of this study are as follows:

- What are the possibilities and method for the optimam isolation of an essential oil through hydro-steam distillation of the aerial parts of *L. scaberrima*?
- What is the potential of the ethanolic extract of *L. scaberrima* and *A. linearis* and the 1:1 combination thereof, the essential oil of *L. scaberrima* and one of the constituents, verbascoside to possess antimycobacterial activity?
- Does the ethanolic extract of *L. scaberrima* and *A. linearis* and the 1:1 combination thereof, together as before with the essential oil and one of the constituents, verbascoside, have any cellular toxicity against hepatocellular carcinoma (HepG2) cells?
- What is the potential of the ethanolic extract of *L. scaberrima* and *A. linearis* and the 1:1 combination thereof, together as before with the essential oil and one of the constituents, verbascoside having hepatoprotective activity against acetaminophen-induced toxicity?
- What, if any is the extent of any potential for drug interactions measured by the CYP inhibitory activities of ethanolic extract of *L. scaberrima* and the 1:1 combination with *A. linearis*, the essential oil of *L. scaberrima* and one of the constituents, verbascoside.
- Does the ethanolic extract of *L. scaberrima* and *A. linearis* and the 1:1 combination thereof, the essential oil of *L. scaberrima* and one of the constituents, verbascoside possess and antioxidant and anti-inflammatory?
- Do the synthesis gold nanoparticles of the ethanolic extract of *L. scaberrima* and characterization of these gold nanoparticles, have a lowered cytotoxic potential and increased antimycobacterial activity?

The main objectives of this study are as follows:

- Isolation of an essential oil through hydro-steam distillation of the aerial parts of *L. scaberrima*.
- Determine the antimycobacterial activity of the ethanolic extract of *L. scaberrima* and *A. linearis* and the 1:1 combination thereof, the essential oil of *L. scaberrima* and one of the constituents, verbascoside.
- Determine the cytotoxic potential of the ethanolic extract of *L. scaberrima* and *A. linearis* and the 1:1 combination thereof, together as before with the essential oil and one of the constituents, verbascoside, against hepatocellular carcinoma (HepG2) cells.
- Determine the hepatoprotective activity against acetaminophen-induced toxicity of the ethanolic extract of *L. scaberrima* and *A. linearis* and the 1:1 combination thereof, together as before with the essential oil and one of the constituents, verbascoside.
- Determine the CYP inhibitory activities of ethanolic extract of *L. scaberrima* and the 1:1 combination with *A. linearis*, the essential oil of *L. scaberrima* and one of the constituents, verbascoside.
- Determine the antioxidant and anti-inflammatory potential of the ethanolic extract of *L. scaberrima* and *A. linearis* and the 1:1 combination thereof, the essential oil of *L. scaberrima* and one of the constituents, verbascoside.
- The synthesis gold nanoparticles of the ethanolic extract of *L. scaberrima* and characterization of these gold nanoparticles, its cytotoxic potential and antimycobacterial activity.

Current treatment of TB involves an intense treatment regime. Due to this, compliance to the anti-TB drugs is failing. *L. scaberrima* Sond. and *A. linearis* (Burm.f.) R. Dahlgren may have the potential to mitigate toxicity experienced by the liver, which is the main organ involved in metabolizing many important drugs but also anti-TB drugs. This study will be investigating the antioxidant, anti-inflammatory and hepatoprotective activity of *L. scaberrima* and *A. linearis*. Effective antimycobacterial activity measured by the MABA method means a first line of defense that is actively lowering the colony forming units (CFU) of the bacteria within an infected individual. An increase in COX-II (investigated with a COX-II analysis) also forms part of the first line of defense against the mycobacteria as it prevents the lysing of macrophages. Investigating directly, the antimycobacterial activity and indirectly the

biological properties (antioxidant and anti-inflammatory activities) can identify medicinal plants that can provide relief of the side-effects that are experienced. By providing a herbal formulation or product that has all these abilities, may ensure that compliancy does not dwindle and help stem the surge in multi-drug resistance. By incorporating nanoparticle synthesis by using *L. scaberrima* and gum arabic as a stabilizer there is potential for increasing these desired activities and lowering toxicity levels. The novelty of using *L. scaberrima* is that many analyses with regards to the biological activity have not been thoroughly investigated and may open up new avenues for drug research and development for the current TB regimen.

2.4. Thesis structure and layout

Chapter 1 Background and motivation, this includes a literature review on the current TB epidemic, the statistics as well as the role of medicinal plants in South Africa.

Chapter 2 Aims and objectives, this includes the hypothesis and research questions for the current study.

Chapter 3 Monograph of *L. scaberrima* Sond. which includes the distribution and botanical characteristics as well as the current research on this particular species of *Lippia*.

Chapter 4 *In vitro* antimycobacterial and adjuvant properties of two South African teas, *A. linearis* (Burm.f) R. Dahlgren and *L. scaberrima* Sond.

Chapter 5 Inhibtion potency of *L. scaberrima* Sond. in combination with *A. linearis* (Burm.f.) R. Dahlgren for CYP enzymes.

Chapter 6 *In vitro* human hepatic metabolism and inhibition potency of verbascoside for CYP enzymes.

Chapter 7 The antimycobacterial, cytotoxicity and synthesis of gold nanoparticles using *L. scaberrima* Sond.

Chapter 8 Final conclusion and future recommendations.

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3

MONOGRAPH

This chapter introduces Lippia scaberrima Sond. an indigenous South African plant used during the study. This includes up to date literature and results from various initial studies conducted.

This chapter currently under production as a book chapter in the following book:

1) *Underexplored Medicinal Plants from Sub-Saharan Africa.*

Citation: ***Reid, A.,*** *Lall, N. Lippia scaberrima. In: Underexplored Medicinal Plants from Sub-Saharan Africa. ISBN: 9780128168141. 2019 Elsevier. Ch. 29.*

3. MONOGRAPH: *LIPPIA SCABERRIMA* SOND.

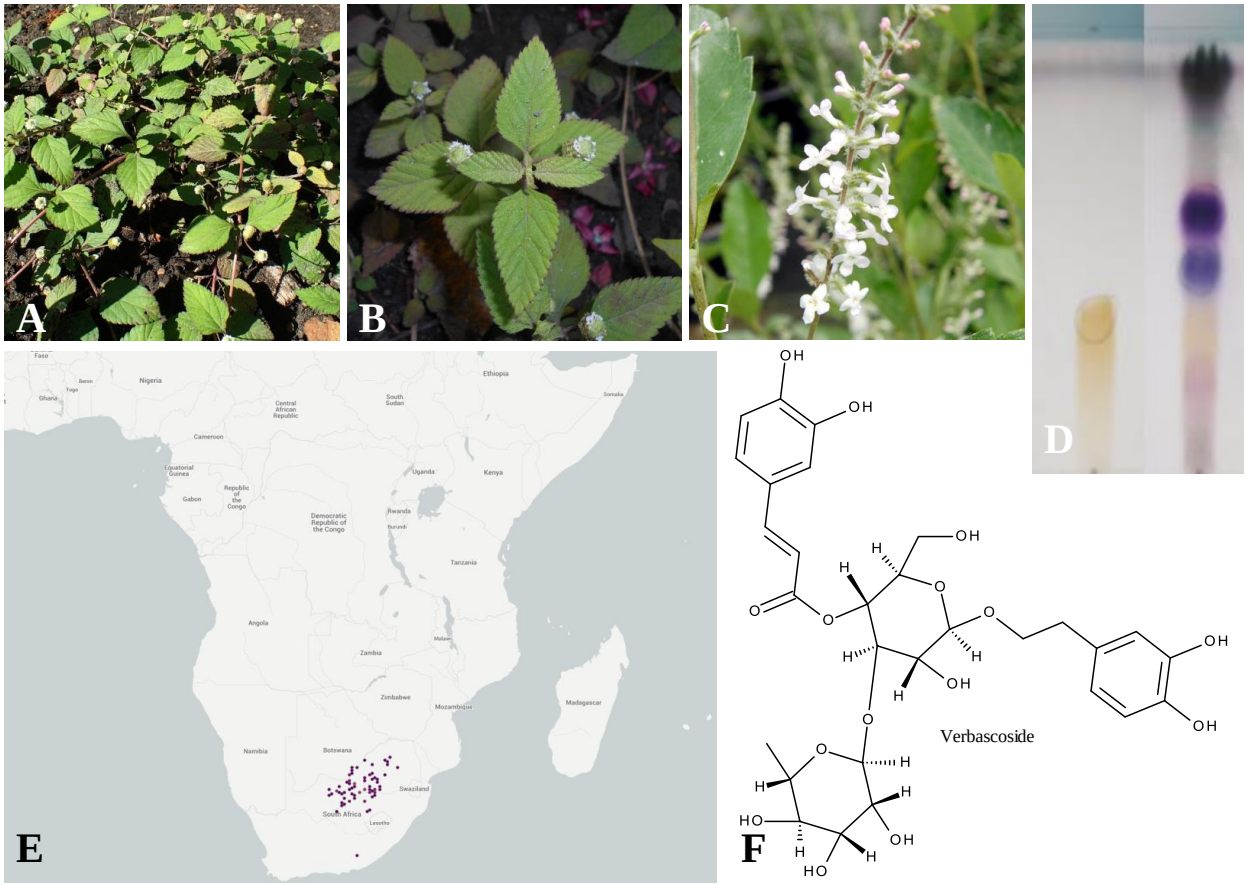

Figure 3.1: Aerial parts of *Lippia scaberrima* (Kenraiz, 2016) (**A**), close-up view of a related *Lippia* species (Salicyna, 2017) (**B**), flowers of *L. scaberrima* (Noarovivaio, 2018) (**C**), TLC chromatogram, Lane 1; verbascoside, Lane 2; *L. scaberrima* ethanolic extract (**D**), distribution map of *L. scaberrima* in sub-Saharan Africa (GBIF, 2018) (**E**), chemical structure of verbascoside (**F**).

3.1. Abstract

Lippia scaberrima Sond. (*L. scaberrima*) is an aromatic and perennial shrub found only in selected provinces throughout South Africa. The scientific research conducted is limited to only research of the essential oil and its constituents. Many compounds and their reported activities have been identified in the essential oil of the plant. The traditional usage of *L. scaberrima* is well-known among Tswana, Pedi, Zulu and Xhosa cultures. Furthermore, *L. scaberrima* is found distributed and sold as a herbal tea in the borders of Botswana. This chapter includes the botanical description, distribution, traditional usage of the plant and its pharmacological activities.

3.2. General description

3.2.1. Botanical nomenclature

Lippia scaberrima Sond.

3.2.2. Botanical family

Verbanaceae

3.2.3. Vernacular names

Beukesbossie (Afrikaans)
Laventelbos (Afrikaans)
Mosukutswane (Tswana)
Umsuzwane (Zulu)

3.3. Botanical description

Lippia scaberrima Sond. is an aromatic, perennial shrub that forms part of the Verbenaceae family. Together with *Lippia javanica*, *Lippia rehmannii*, *Lippia wilmsii*, and *Lippia pretoriensis*, *L. scaberrima* is the only indigenous *Lippia* spp. in South Africa (Regnier et al., 2008; Germishuizen et al., 2006). It reaches an approximate height of 60 cm and will be seen growing in dry climatic conditions with rainfalls during the summer months. This perennial shrub is known to have deciduous leaves with a woody stem (Combrinck et al., 2006; Retief, 2003; Retief and Herman, 1997; Wells et al., 1986).

3.4. Distribution

Lippia scaberrima is found distributed among five provinces within South Africa namely, North West, Northern Cape, Mpumalanga, Gauteng and the Free State (Foden and Potter, 2005).

3.5. Ethnobotanical usage

Lippia scaberrima is well known for its medicinal properties among the Tswana, Pedi, Zulu and Xhosas (Van Wyk et al., 1997; Watt and Breyer-Brandwijk; 1962). It has also been sold as a tea, with health properties namely, “Mosukudu” in Botswana (Shikanga et al., 2010). A tonic created from the leaf infusions is used as a treatment for coughs, fever, colds and bronchitis (Combrinck

et al., 2006; Van Wyk and Gericke, 2000; Smith et al., 1966; Watt and Breyer-Brandwijk, 1962). According to Pascual, et al., (2001) the vast majority of the *Lippia* spp. are used to treat respiratory and gastrointestinal ailments. Some species within the genus *Lippia* have shown antimalarial (Gasquet et al., 1993), antiviral (Abad et al., 1995) and cytostatic properties (Lopez et al., 1979).

3.6. Phytochemical constituents

The essential oil of *L. scaberrima* has been thoroughly investigated for its constituents by various research groups. According to Combrinck et al., (2006), the essential oil (oil extracted from the flowers, leaves and twigs) is rich in R-(-) carvone, (*d)*-limonene and 1, 8-cineole. This was also confirmed in a previous investigation of the essential oil gathered from the flowers and leaves by Terblanche et al., (1998). Some of the other components found in the essential oil of the leaves and flower heads by Combrinck et al., (2006) yielded the following components; α- pinene, β-pinene, camphene, sabinene, myrcene, α-phellandrene, p-cymene, γ-terpinene, linalool, camphor, borneol, α-terpineol, dihydrocarvone, carveol, β-caryophyllene and α-humulene.

3.7. TLC fingerprinting of plant extract

The separation of *L. scaberrima* can be observed by weighing off 2 mg of an ethanolic extract, dissolved in 200 µL ethanol. A standard, verbascoside (acteoside) found within various indigenous species of *Lippia* can be used by weighing off in the same manner as the plant extract but dissolved in 600 µL ethanol. The samples are stored at 4°C until further use as described in the methods section. The thin layer chromatography (TLC) analysis of the ethanolic *L. scaberrima* extract comprised of silica gel 60 F254 TLC plates for the observation of separation of the compounds that may be present within the extract. The plates are marked with a soft pencil and glass capillaries are used to spot the samples. A reference standard (verbascoside), should be spotted to determine its presence within the extract. The spots need to dry completely before placing the TLC plates within a TLC tank which contains a 10 mL eluent of 9:1 chloroform: methanol solvent system. The samples will be allowed to separate until the eluent reached the solvent line, 1 cm from the top of the TLC plate. Freshly prepared vanillin is used to detect the bands present within the TLC plate as well as observation under short and longwave UV light.

3.8. Pharmacological properties

Although *L. scaberrima* has not been extensively investigated for its pharmacological properties many research groups have done initial investigations into the activity of the crude extract. Wide-ranging work has been completed on the activity of the essential oil isolated from *L. scaberrima*, especially as treatment for postharvest pathogens and diseases for economically important fruit.

3.8.1. Antifungal activity

Regnier et al., (2008) studied the antifungal potential of the essential of *L. scaberrima* as well as the major components found within the essential oil, R-(-)-carvone, S-(-)-carvone, limonene and 1, 8-cineole. These major components studied within the essential oil yielded antifungal potential against *C. gloeosporioides* as well as R-(-)-carvone, S-(-)-carvone and 1, 8-cineole showing antifungal activity against *B. parva*. Limonene showed the lowest efficacy against both these pathogens. Regnier et al., (2010) expanded this investigation by including the antifungal properties of the essential oil of *L. scaberrima*. In this study, once again carvone as a major component of the essential was tested and found to have the best activity against three major avocado pathogens, by effectively controlling the growth of these fruit pathogens. Limonene when applied as a vapour to the avocadoes instead, showed the promotion of growth of the fungi under investigation.

3.8.2. Antioxidant activity

A phenylethanoid glycoside, verbascoside, is known to occur in measurable amounts within *L. scaberrima* (Olivier et al., 2010). This glycoside is known for its antioxidant properties. Shikanga et al., (2010) measured the free radical scavenging properties of *L. scaberrima* and that of the pure compound, verbascoside. The EC_{50} values were reported as 1150 μg/mL and 89 μg/mL for *L. scaberrima* and verbascoside respectively.

3.8.3. Antibacterial activity

A previous publication by Pennachio et al., (2005) indicated the antibacterial properties that verbascoside may possess. Following the serial microdilution method by Shikanga et al., (2010), both *L. scaberrima* and verbascoside were tested for their antibacterial properties against four human bacterial pathogens namely *Staphylococcus aureus* (ATCC 29213), *Enterococcus faecalis*

(ATCC 29212), *Pseudomonas aeruginosa* (ATCC 27853) and *Escherichia coli* (ATCC 25922). *Lippia scaberrima* showed the highest minimum inhibitory concentration (MIC) values against these four pathogens with the highest activity against *E. faecalis* (0.63 mg/mL). The MIC values against the other bacteria were all reported to be 1.3 mg/mL (Shikanga et al., 2010). Verbascoside showed the best activity against these four pathogens with the lowest MIC value reported to be 0.06 mg/mL against *S. aureus* and a slightly higher MIC value of 0.1 mg/mL was reported against both *E. faecalis* and *E. coli*. The MIC value against *P. aeruginosa* was reported to be 0.25 mg/mL.

3.9. Additional information

3.9.1. Therapeutic (proposed) usage

Antibacterial, antimycobacterial, immunostimulant and hepatoprotectant

3.9.2. Safety data

Not available

3.9.3. Trade information

Not threatened or listed as an endangered species

3.9.4. Dosage

Not available

3.9.5. Possible products

An immune stimulant and hepatoprotectant. Especially for use during the first line TB regime.

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4

In vitro antimycobacterial and adjuvant properties of two South African teas, *Aspalathus linearis* (Burm.f.) R. Dahlgren and *Lippia scaberrima* Sond.

This chapter introduces the investigation into the in vitro biological activity of Lippia scaberrima Sond. in combination with Aspalathus linearis (Burm. f) R. Dahlgren. The activities tested were antimycobacterial, antioxidant, anti-inflammatory, cytotoxicity and hepatoprotective activity of the samples.

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4. *IN VITRO* ANTIMYCOBACTERIAL AND ADJUVANT PROPERTIES OF TWO TRADITIONAL SOUTH AFRICAN TEAS, *ASPALATHUS LINEARIS* (BURM.F.) R. DAHLGREN AND *LIPPIA SCABERRIMA* SOND.

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Abstract

Many plant extracts have been studied for their ability to treat TB and its associated symptoms. Both *L. scaberrima* Sond. and *A. linearis* (Burm.f.) R. Dahlgren, are popular forms of health teas in South Africa. This study focused on the ability of the ethanolic plant extracts of *L. scaberrima* and *A. linearis*, as well as the essential oil of *L. scaberrima* to act as adjuvants in host-directed therapy against TB. The ethanolic extract of *L. scaberrima* was found to have a minimum inhibitory concentration (MIC) of 125 µg/mL against *M. tuberculosis* H37Rv, whereas the antiproliferative activity on HepG2 hepatocyte cells ranged between 109.20 ± 8.05 µg/mL (Mean±SD) and >400 µg/mL for all the samples tested. The antioxidant properties of the samples exhibited potent activities, with IC_{50} values between 4.49±0.16 µg/mL and 73.00±3.51 µg/mL (Mean±SD) against DPPH. The essential oil of *L. scaberrima* and the ethanolic extract of green *A. linearis* exhibited good hepatoprotective activity, with up to 34% and 40% protection against acetaminophen-induced toxicity, respectively. Due to the high antimycobacterial property of *L. scaberrima*, the sample was further tested for its cyclooxygenase (COX) II inhibitory potential and found to have an IC_{50} value of 36.39±1.62 µg/mL. Additionally, the study investigated the

composition of the ethanolic extract and essential oil of *L. scaberrima* through gas-chromatography mass spectrometry. Carvone and limonene were found to be the main components for the ethanolic extract and essential oil, respectively. The essential oil from *L. scaberrima* showed promising results with noteworthy hepatoprotective activity as well as moderate antimycobacterial activity. These herbal teas showed potential as an adjunct host-directed therapy in TB patients through the demonstration of its biological activities and should be considered for further investigation.

4.1. Introduction

Adjunct host-directed therapy (AHDT) has been proposed many times for the treatment of TB, either through accessing the host immune system to eliminate the pathogenic bacteria efficiently or through limiting the subsequent damages as a result of infection (Tobin, 2015). Adjunct host-directed therapies have the potential of enhancing an existing immune response against TB or have the ability to create new ones (Tobin, 2015). Host-directed therapy has only been laid out theoretically and only in the instance of TB has it been utilized.

Inflammation has been one of the major causes of morbidity in severe cases of TB infection. Glucocorticoids are now included as part of the standard of care regimen to aid in these severe cases (Tobin, 2015). Nonsteroidal anti-inflammatory drugs such as aspirin are one of the host-directed therapies in pre-clinical trials for the limitation of prostaglandin synthesis through inhibition of cyclooxygenase (COX) I and COX II. Through the inhibition of these pathways, the inflammatory response can be limited during TB infection and as such has been tested in a zebrafish model for hyperinflammatory conditions during infection with mycobacteria (Tobin et al., 2012). One clinical trial involving aspirin indicated a beneficial effect as adjunct therapy on the survival rate (Misra et al., 2010; Schoeman et al., 2011). Another study conducted in a murine model of active TB by Vilaplana et al., (2013) showed the anti-inflammatory properties of ibuprofen by decreasing the amount and size of lung lesions found within *M. tuberculosis* infected C3HeB/FeJ mice as well as it was found that Ibuprofen lowered the bacillary loads in the mice lungs. Other host-directed therapies for TB currently in phase 1, 2 and 3, involve the enhancement of host immunity and proper elimination and containment of the bacteria itself (Tiberi et al., 2018). *Aspalathus linearis*, commonly known as Rooibos, can be found growing in the Western Cape region of the Cedarberg mountains in South Africa. Currently, this endemic herbal tea is in high

demand across the world. Many studies have included the health benefits provided by the intake of Rooibos tea, many of which involve its antispasmodic potential (Van Wyk et al., 1997). *Aspalathus linearis* is found commercially available in two forms namely fermented (Rooibos) or unfermented (green Rooibos). It is during a fermentation process that the polyphenols within the rooibos plant are oxidized and it then turns into its characteristic red colour. *L. scaberrima* is an aromatic shrub, which is enjoyed as a herbal tea and goes by the name of 'Musukudu' in southern Africa (Shikanga et al., 2010). Scientific investigation of *L. scaberrima*, and its health benefits are lacking, and most studies have been done on the essential oil and its ability to protect against fungal pathogens such as *Colletotrichum gloeosporioides* and *Botryosphaeria parva* (Regnier et al., 2008).

An investigation into the potential biological properties from the extracts of *L. scaberrima* and *A. linearis* is needed to understand their potential as adjunct host-directed therapy for TB patients. The current study investigated the essential oil and ethanolic extract of *L. scaberrima* and the ethanolic extract of *A. linearis* for their antimycobacterial, hepatoprotective and anti-inflammatory properties. Furthermore, antiproliferative activity was evaluated to facilitate the selectivity.

4.2. Materials and methods

4.2.1. Bacterial strains, cell lines, chemicals and reagents.

Mycobacterium tuberculosis (H37Rv), in MGIT media, and *M. smegmatis* (MC^2155) were kindly donated by the South African Medical Research Council, Pretoria, and Department of Medical Microbiology, University of Pretoria, respectively. The HepG2 cell line (HB-8065) was obtained from the American Type Tissue Culture Collection (ATCC). Dimethyl-sulfoxide (DMSO), Middlebrook 7H9 broth, Middlebrook 7H11 agar base and Middlebrook OADC (Oleic Albumin Dextrose Catalase) growth supplement were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise stated. Cell culture materials and reagents such as Fetal Bovine Serum (FBS), DMEM media and antibiotics were supplied by Highveld Biological. The PANTA Plus antibiotic mixture was purchased from BD Biosciences (San Jose, CA, USA). PrestoBlue was purchased from Thermo Fisher (Carlsbad, CA, USA). All reagents obtained were of analytical grade.

4.2.2. Plant material, collection and extraction.

Lippia scaberrima was collected in autumn from the Kopela community situated nearby Delareyville, North West Province, South Africa. The plant was identified, and a voucher specimen deposited at the H. G. W. J Schweickerdt herbarium, University of Pretoria (PRU) (Table 1). Powdered green and fermented *A. linearis* leaf material were kindly donated by Rooibos Limited, Clanwilliam, Western Cape, South Africa and a voucher specimen deposited. The collected aerial parts of *L. scaberrima* (stem, flowers, and leaves) were mechanically ground to a uniform size of 0.2 mm. The powdered plant material from both plants was extracted with absolute ethanol at a ratio of 1:10 (weight: volume) and macerated for 72 hours. The extracts were filtered, and the plant material was subsequently extracted with fresh ethanol at a ratio of 1:5 for 48 hours, followed by filtration. The filtrates were combined and dried under reduced pressure using a rotary evaporator which resulted in a dark green extract for *L. scaberrima* and reddish extracts for the green and fermented *A. linearis*. The three extracts were stored in airtight containers at 4°C for the duration of the study.

4.2.3. Essential oil extraction by hydro-steam distillation and yield calculation.

Lippia spp. are known for their essential oil content. The aerial parts of *L. scaberrima* were subjected to essential oil extraction using hydro-distillation. Air-dried plant material (stem, flowers, and leaves) (0.415 kg) were subjected to a Clevenger apparatus for hydro-steam distillation for one hour. The extracted essential oil was collected and stored at –20 °C until further use. The yield was determined by the amount of essential oil obtained from 0.415 kg of plant material and was calculated by the following equation:

$$Yield = \frac{VEO \times 100}{M}$$

Eq. 1 Where VEO is the volume of the extracted oil (in mL) and M, the initial biomass of the plant material (in g).

4.2.4. Gas chromatography-mass spectrometry (GC-MS) of *L. scaberrima*

For the identification of compounds within the essential oil, gas chromatography-mass spectroscopy was carried out using an Agilent 7000 C Triple/ Quadrupole GC-MS (Agilent, Santa Clara, United States) with the analysis carried out in scan mode. Mass spectroscopy scan conditions were as follows; an ion source temperature of 200 °C, interface temperature of 280 °C, and ionization energy of 70 eV. The separation of the compounds was achieved using an Agilent J&W DB-5ms Ultra Inert fused silica column 5% phenyl-poly-dimethyl-siloxane (DB- 5ms) 30m x 0.25 mm i.d. with 0.25 µm film thickness. The GC oven temperature and programme were as follows; from 40 °C (5 min hold), raised at 2.5 °C/ min to a final temperature of 300 °C with a 20 °C/ min hold. The injection mode was a split-splitless injection at a split ratio of 1:20 and an injector temperature of 250 °C. The essential oil was diluted with GC-MS grade hexane in a 1: 200 µL ratio and 1 µL was injected. Data acquisition was performed with MassHunter software with mass scan ranges of 30 – 600 u and a scan speed of 380 ms. The compounds were identified with an Agilent MassHunter analysis of the unknown by use of the mass spectra with data collected from a NIST 14 Mass spectral library. The relative percentage of the compounds present in the essential oil was computed from a peak area of the GC-MS analysis.

For the identification of compounds within the ethanolic extract, gas chromatography-mass spectroscopy was carried out using a LECO Pegasus 4D GC-TOFMS (LECO Africa., Kempton Park, South Africa). Mass spectrometry scan conditions were as follows; an ion source temperature of 230 °C, transfer line temperature of 280 °C and ionization energy of 70 eV in the electron ionization mode (EI+). The separation of compounds was achieved using a GC column (Rxi-5Silms) 30m x 0.25 mm with 0.2 µm film thickness. The GC oven temperature and programme were as follows; 40 °C (3 min hold), raised at 10 °C/ min to a final temperature of 300 °C (5 min hold). The injection mode was splitless injection with a time of 30 s. The carrier gas was UHP helium at 1 / min on constant flow mode. The scan ranges for identification of the compounds were 40 – 550 Da and a scan speed of 10 spectra/ s.

4.2.5. Bacterial strain and growth conditions

Both *M. tuberculosis* and *M. smegmatis* were cultured in Middlebrook 7H9 media supplemented with glycerol, OADC and PANTA (1%) and left to incubate for three weeks and 48 hours, respectively, at 37 °C. Both bacteria were adjusted to a 0.5 McFarland standard of (1.5×10^8 colony forming units/mL) [CFUs/mL]). The bacteria were further diluted 50-fold to obtain the final test inoculum (1.5×10^6 CFUs/mL).

4.2.6. Antimycobacterial activity

The minimum inhibitory concentration (MIC) values of all the samples were determined according to the method of Lall et al., (2013). All samples were dissolved in 20% DMSO, in sterile Middlebrook 7H9 media. Sterile, distilled water (200 µL) was added to the outer wells of the plate to compensate for evaporation during the incubation period for *M. tuberculosis*. Serial, two-fold dilutions of each sample were made in sterile Middlebrook 7H9 media to yield a final test concentration range of 31.25 to 1000 µg/mL. The diluted bacterial inoculum (100 µL) was added to each well to yield a final assay volume of 200 µL. Isoniazid (INH), at a final concentration range of 0.03 to 2.5 µg/mL served as the positive drug control for *M. tuberculosis,* whereas ciprofloxacin, at a concentration range of 0.078 to 10µg/mL served as the positive drug control for *M. smegmatis.* An untreated bacterial control, media control, as well as a solvent control (DMSO 5%) were included in triplicate. The plates were incubated at 37 °C for seven days and 24 hours for *M. tuberculosis* and *M. smegmatis*, respectively. PrestoBlue (20 µL) was added to each well, and the plates were incubated for a further 2 to 24 hours. The MIC value was defined as the lowest concentration where no colour change from blue to pink could be observed.

4.2.7. Antiproliferative activity

Cell viability was determined by the method of Berrington and Lall (2012). The liver hepatocellular carcinoma (HepG2) cells (100 µL) were counted and seeded in 96 well plates with a cell density of 10 000 cells/well and left to incubate overnight at 37 °C and 5% CO_2 to allow for attachment. The samples were all prepared to a stock solution of 2000 µg/mL. Serial dilutions of the extracts were made with final test concentrations ranging from 1.53 to 400 µg/mL. Plates were

incubated for 72 hours at 37 °C and 5% CO_2. An untreated cell, solvent control (DMSO 2%) and actinomycin D (positive control) with a final test concentration range of 0.002 to 0.5 µg/mL were included. After incubation, PrestoBlue (20 µL) was added to each well and incubated for 4 hours. The absorbance was read at 490 nm with a reference wavelength of 690 nm using a BIO-TEK Power-Wave XS multi-well reader. The assay was performed in triplicate in three independent assays, and the mean 50% inhibitory concentration (IC_{50}) values calculated.

4.2.8. DPPH inhibitory activity.

For the determination of the radical scavenging activity of all the samples, the method of Berrington and Lall (2012) was followed. Briefly, stock solutions of the samples and the positive control, ascorbic acid (Vitamin C), were prepared at 2000 µg/mL and 1000 µg/mL respectively. The final concentrations of the plant extract in the 96-well plate ranged from 3.9 to 500 µg/mL and for the positive control, from 0.78 to 100 µg/mL. For the blank control ethanol (100%) was used. Finally, 2, 2-diphenyl-1-picrylhydrazyl radical (DPPH) (0.04 M) in an ethanolic solution was added to all the wells, except for the negative control, where distilled water was added. The plate was wrapped in foil and left for 30 min to develop. The absorbency values were measured using a BIO-TEK Power-Wave XS multi-plate reader at a wavelength of 515 nm. The assay was performed in three independent assays, and the mean 50% scavenging concentration (IC_{50}) values calculated.

4.2.9. COX-II inhibitory activity

Due to the high antimycobacterial activity that was found for *L. scaberrima* it was further investigated for its COX-II inhibitory activity according to the method by Reininger and Bauer (2006). Briefly, in a 96-well plate, TRIS buffer at pH 8.0, COX-II (0.5 units/reaction), porcine hematin (5 µM), L-epinephrine (18 mM) and NA_2EDTA (50 µM) was added. Thereafter, the plant extract at a stock solution of 10 mg/mL was serially diluted and added, with final test concentrations ranging from 2.5 to 160 µg/mL. DMSO (1.6 %) as a solvent control was also added. Ibuprofen (10 µM) was used as the positive control. After 5 min of incubation at room temperature, the reaction was initiated by the addition of arachidonic acid (10 µM) to a final assay volume of 200 µL and incubated for 20 min. To stop the reaction, 10 µL of formic acid (10%) was added to

all the wells. For the measurement of the amount of prostaglandin E2 (PGE_2) formed, a PGE_2 ELISA kit was used, following the manufacturer's instructions. The absorbency values were measured using a BIO-TEK Power-Wave XS multi-well reader at a wavelength of 405 nm. The results are presented as the percentage PGE_2 synthesis inhibition when compared with the blank. The IC_{50} values were calculated by the use of Microsoft Excel 2010.

4.2.10. Hepatoprotective activity

The hepatoprotective ability of the samples was evaluated against an acetaminophen-induced toxicity assay according to Lall et al., (2016) with modifications. Briefly, HepG2 cells were seeded at a density of 10 000 cells/well within a 96-well plate and incubated overnight at 37 ºC in a humidified atmosphere of 5% CO_2 to allow for attachment of the cells. All samples were prepared to a stock solution of 2000 µg/mL. The final test concentrations were 25, 100 and 400 µg/mL for all samples. Silymarin at 50 mM was included in the assay as the positive control. The samples and controls were added to the cells in triplicate. Cellular toxicity was induced by the addition of acetaminophen at 25 mM, to a final test volume of 200 µL and incubated for 3 hours at 37 ºC in a humidified 5% CO_2 incubator. After incubation, PrestoBlue (20 µL) was added to each well and incubated for 4 hours. The absorbance was read at 490 nm with a reference wavelength set to 690 nm using a Bio-Tek Power-Wave XS multi-well reader. The percentage protection was calculated by using the acetaminophen-treated cells as the baseline.

4.2.11. Statistical analysis

Statistical analysis was performed using GraphPad Prism (Version 7) using one-way analysis of variance (ANOVA). A Dunnett's multiple comparison test was performed to identify significance as compared to a control value. The data is expressed as the mean ± standard deviation mean.

4.3. Results and discussion

4.3.1. Essential oil isolation by hydro-steam distillation.

The isolated yield of the essential oil from *L. scaberrima* was found to be at 0.60% (w/w) in the current study. The amount of essential oil isolated can be influenced by many factors such as humidity, temperature, the age of the plant parts collected and season (Bueno Da Costa et al., 2014;

Sebei et al., 2015). Typical essential oil yields from *L. scaberrima* as obtained by Combrinck et al., (2006) were found to be 0.25% (w/w). This value was obtained by isolating from air-dried leaves indicating a high amount of essential oil loss. It has been shown that after harvesting, postharvest drying of plant material bearing essential oil may have increased yields due to heat transfer. Oil yields may also be lowered upon mechanical damage of the leaves after drying (Combrinck et al., 2006). The reason for the increased yields of dried and intact leaves in the present study could be due to the continuing metabolic activity which may shift the secondary metabolites from the stems into the leaves (Whish and Williams, 1996). The usage of dried leaves for medicinal treatments will, therefore, still be productive as phenolic compounds tend to stay behind within the tissues of the air-dried plant structures instead of being lost through the drying process as indicated by Combrinck et al., (2007). This supported the fact, why similar compounds are found within the air-dried leaves subjected to hydro-steam distillation and the ethanolic extract prepared from these air-dried leaves.

4.3.2. Gas chromatography-mass spectrometry of the ethanolic extract and its essential oil of *L. scaberrima*

The identified compounds that had a contribution of more than 1% are represented in Table 1 for the essential oil and Table 2 for the ethanolic extract of *L. scaberrima*. Limonene was found to be the major component within the essential oil (35.84%) which correlates with the reports by Regnier et al., (2008) and Combrinck et al., (2006). Carvone was found to be the major component within the ethanolic extract (62.49%) and the second most abundant compound in the essential oil of *L. scaberrima*.

The treatment of various ailments with *L. scaberrima* has shown to be supported by the fact that the major constituents within the plants, which are volatile compounds remain intact albeit in marginal quantities within the dried ethanolic extract. Extraction with ethanol could, however, have targeted the extraction of carvone as it has a ketone within its structure increasing its polarity as seen with the higher amount as compared to limonene which is less polar. Several chemotypes of *L. scaberrima* may also exist due to inter- and intraspecies variation as several different types of chemotypes will present different compositions of compounds due to different metabolic pathways (Viljoen et al., 2005; Maroyi, 2017). Compounds such as phenylethanoid glycosides

which are known to be present within *Lippia* spp. were not identified after GC-MS analysis of the plant extract and the essential oil. This may be due to the fact that GC-MS analysis is mostly used for non-polar, volatile components whereas HPLC-MS analysis is used for more polar, non-volatile compounds such as verbascoside and isoverbascoside. The chromatograms for the essential oil and ethanolic extract of *L. scaberrima* are found in the appendix together with the complete list of identified compounds (Appendix A- Figure 1A and 2A; Table 1A and 2A)

4.3.3. Antimycobacterial activity

All samples were tested against both *M. tuberculosis* and *M. smegmatis* including combinations of *L. scaberrima* with fermented and green extracts of *A. linearis*.The lowest MIC value was observed by the ethanolic extract of *L. scaberrima* which showed an MIC value of 125 µg/mL against Isoniazid (INH) (a positive drug control), with a MIC value of 0.32 µg/mL (Table 3). Due to the known antibacterial properties of *A. linearis*, combinations of the ethanolic extracts of *L. scaberrima* and green and fermented *A. linearis* extracts were also tested. Both combinations exhibited MIC values of 500 µg/mL against *M. tuberculosis*. The essential oil of *L. scaberrima*, showed no activity at the highest tested concentration (1000 µg/mL) against *M. tuberculosis.* However, the essential oil showed the highest activity against *M. smegmatis* with an MIC value of 1000 µg/mL (ciprofloxacin, a positive drug control, MIC of 0.63 µg/mL). All the other samples showed no activity at the highest tested concentration against *M. smegmatis*.

Natural products that show MIC values of 1000 µg/mL and lower are considered to have noteworthy activity according to Gibbons, (2004). A study conducted by Shikanga et al., (2010), indicated the antibacterial potential of the methanolic extract of *L. scaberrima* against other gram-positive bacteria, showing MIC values of 1300 and 630 µg/mL against *S. aureus* and *E. faecalis*, respectively. Many studies have attributed the antibacterial activity to the presence of verbascoside and isoverbascoside in the plant. Although not detected by the GC-MS analysis, verbascoside is known to be a constituent found within the *Lippia* spp. indigenous to South Africa (Shikanga et al., 2010) which was present in the extract after co-TLC analysis. (Appendix A, Figure 3A). In another study, *Lippia javanica* showed much higher activity against *S. aureus* and *E. faecalis* than that of *L. scaberrima*, but much of the activity is attributed to the compounds of verbascoside and apigenin found within this species of *Lippia* (Shikanga et al., 2010, Sandoval-Montemayer et al.,

2012). Sandoval-Montemayer et al., (2012), tested carvone as a constituent found within the hexane extract of *Citrus aurantifolia* and found that carvone had a MIC value of 200 µg/mL against *M. tuberculosis* H37Rv. This further supports the antimycobacterial activity observed by *L. scaberrima*, as carvone was found to be one of the major constituents of the essential oil and ethanolic extract of *L. scaberrima*.

Table 4. 1 Identified compounds with the greatest contribution within the essential oil of *L. scaberrima*, determined by GC-MS analysis (>1%)

RT[a]	Name of the compound	Synonym of compound	Molecular Formula	% Composition	Nature of the compound
11.93	(1R)-2,6,6-Trimethylbicyclo [3.1.1] hept-2-ene	Pinene	$C_{10}H_{16}$	1.74	Terpene
12.71	Camphene	Camphene	$C_{10}H_{16}$	6.53	Bicyclic monoterpenoids
14.25	Bicyclo [3.1.0] hexane, 4-methylene-1-(1-methylethyl)-	Sabinene	$C_{10}H_{16}$	2.16	Terpene
17.31	p-Cymene	Cymene	$C_{10}H_{14}$	5.12	Hydrocarbon-related to monoterpene
17.64	D-Limonene	Limonene	$C_{10}H_{16}$	35.84	Terpene
24.22	(+)-2-Bornanone	Camphor	$C_{10}H_{16}O$	12.72	Terpene
25.50	endo-Borneol	Borneol	$C_{10}H_{18}O$	3.57	Terpene
27.01	L-. alpha -Terpineol	Terpineol	$C_{10}H_{18}O$	2.60	Monoterpene alcohol
28.67	2-Cyclohexen-1-ol, 2-methyl-5-(1-methylethenyl)-, cis-	Carveol	$C_{10}H_{16}O$	1.28	Monoterpene alcohol
30.06	(-)-Carvone	Carvone	$C_{10}H_{14}O$	14.30	Terpene
31.51	2-Cyclohexen-1-one, 3-methyl-6-(1-methylethenyl)-, (S)-	Isopiperitenon	$C_{10}H_{14}O$	3.77	Menthane monoterpenoids

[a]RT: retention time

Table 4. 2 Identified compounds with the greatest contribution within the ethanolic extract of *LS* determined by GC-MS analysis (>1%)

RT[a]	Name of the compound	Synonym of the compound	Molecular Formula	% Composition	Nature of the compound
5.75	Bicyclo [2.2.1] heptan-2-one, 1,7,7-trimethyl-, (1S)-	Camphor	$C_{10}H_{16}O$	3.35	Terpene
6.17	Bicyclo [2.2.1] heptan-2-ol, 1,7,7-trimethyl-, (1S-endo)-	(-)- Borneol	$C_{10}H_{18}O$	1.88	Bicyclic monoterpenoid
6.59	à-Terpineol	Terpineol	$C_{10}H_{18}O$	1.32	Monoterpene alcohol
7.00	trans-p-mentha-1(7),8-dien-2-ol	Terpineol	$C_{10}H_{16}O$	1.40	Monoterpene alcohol
7.27	Octane, 4-methyl-	None	C_9H_{20}	4.24	Octane
7.32	(-)-Carvone	Carvone	$C_{10}H_{14}O$	62.49	Terpene
7.66	2-Cyclohexen-1-one, 3-methyl-6-(1-methylethenyl)-, (S)-	Isopiperitenon	$C_{10}H_{14}O$	1.00	Methane monoterpenoid
7.77	Carvone oxide, cis-	Carvone oxide	$C_{10}H_{14}O_2$	1.21	Oxepane
7.78	Carvone	Carvone	$C_{10}H_{14}O$	1.21	Terpene
8.79	1,2-Cyclohexanediol, 1-methyl-4-(1-methylethenyl)-	Limonene-diol	$C_{10}H_{18}O_2$	1.36	Menthane monoterpenoids
9.95	Humulene	Humulene	$C_{15}H_{24}$	1.17	Sesquiterpene
16.92	Phytol	Phytol	$C_{20}H_{40}O$	1.22	Diterpene
17.40	9,12,15-Octadecatrienal	None	$C_{18}H_{30}O$	1.09	Fatty acid
17.42	E-11-Hexadecenoic acid, ethyl ester	None	$C_{18}H_{34}O_2$	1.09	Fatty acid

[a]RT: retention time

Andrade-Ochoa et al., (2015), hypothesized that the mechanism of action of the essential oils tested for their antimycobacterial activity may lie within the effects that they have on the structure and functioning of the mycobacterial cell wall and membrane, especially for the essential oils that are monoterpic in nature. The monoterpenoids have the ability to interact with the lipid membranes as an impurity within the ordered structure of the bilayer due to their lipophilic nature (Sikkema et al., 1995; Trombetta et al., 2005). Mycobacterial cell walls are known for their lipophilicity due to the presence of mycolic acids. Essential oils interacting with the cell walls of the bacteria have the ability to change the permeability factors and ultimately result in mycobacterial death (Andrade-Ochoa et al., 2015). However, in the present study the MIC of the essential oil of *L. scaberrima* was observed to be rather high i. e. 1000 µg/mL against *M. smegmatis*. The essential oil did not exhibit an inhibitiory activity against *M. tuberculosis* at the highest concentration tested (1000 µg/mL).

4.3.4. Antiproliferative assay

All samples were tested against liver hepatocellular carcinoma (HepG2) cells for their antiproliferative activity and found to have IC_{50} values ranging from between 109.20 ± 8.05 µg/mL (Mean±SD) and >400 µg/mL after 72 hours of incubation (Table 3). Plant extracts with an IC_{50} value of more than 100 µg/mL after 72 hours of incubation are considered as non-cytotoxic to the specific cell line tested. The green *A. linearis* extract, among the plant samples, showed the lowest toxicity against the HepG2 cells with an IC_{50} value greater than 400 µg/mL. *L. scaberrima* essential oil exhibited an IC_{50} value of 244. 90 ± 4.97 µg/mL. The combinations of the ethanolic extract of *L. scaberrima* with fermented and green *A. linearis* extracts showed higher IC_{50} values when compared to *L. scaberrima* on its own with IC_{50} values of 163.30 ± 2.53 µg/mL, 191.20 ± 9.37 µg/mL and 109.20 ± 8.05 µg/mL, respectively.

Lippia javanica is a well-known medicinal plant found within the same genus and is also indigenous to South Africa. Piperitenone, found to be a constituent within the extract and the essential oil has shown to be of low toxicity when tested against human adenocarcinoma (HCT- 8) cells with an IC_{50} value of 265.6 ± 8.53 µg/mL. Similar results were found for limonene, the major constituent within the essential oil extract which had an IC_{50} value of 285 ± 49 µM (38.83 µg/mL) against human lung (CCD-19Lu) cells after 24 hours incubation (Rolseth et al., 2002). A carvone-

rich chemotype of *Lippia alba*, rich in limonene and carvone, showed slight toxicity against the human cervix epithelioid carcinoma (HeLa) cell line with an CC_{50} (50% cytotoxicity concentration) value of 74.5 ± 13.1 µg/mL and a CC_{50} value against African green monkey kidney (Vero) cells of more than 200 µg/mL. L-carvone showed considerable toxicity towards breast cancer cell lines and was explored for its apoptotic ability within these cell lines. The IC_{50} values against Michigan Cancer Foundation-7 (MCF-7), epithelial, human breast cancer cel line (MDA MB 231) and non-tumorigenic epithelial cell line (MCF 10 A) were found to be 1.2 mM (180.26 µg/mL), 1.0 mM (150. 22 µg/mL) and 20 mM (3004, 4 µg/mL), respectively (Patel and and Thakkar, 2014). Cytotoxicity of methanolic and aqueous extracts of fermented *A. linearis* and its major components were tested against immortalized keratinocytes (HaCat), fibroblast-like skin (CRL 7761) cells and basal carcinoma malignant (CRL 7762) cells. All showed IC_{50} values greater than 100 µg/mL on all cell lines (Macgwebeba et al., 2016).

4.3.5. COX-II inhibition and other antioxidant activity

Effective antioxidant scavenging potential was seen for some of the samples tested except for the ethanolic extract and essential oil of *L. scaberrima* (73.00±3.51 µg/mL and >500 µg/mL, respectively). The ethanolic extract of green *A. linearis* and the combination with the ethanolic extract of *L. scaberrima* showed IC_{50}-values of 2.18 ±1.75 and 4.49±0.16 µg/mL (Mean±SD), respectively, even lower than the positive control, ascorbic acid, which exhibited an IC_{50} value of 7.70± 1.12 µg/mL. The combination of the fermented *A. linearis* extract with *L. scaberrima* showed a higher IC_{50} value of 13.98± 3.44 µg/mL when compared to both the green extract alone (2.18±1.75 µg/mL) and the fermented extract alone (9.17±0.73 µg/mL).

The higher antioxidant capacity seen in the current study for the ethanolic extracts of green *A. linearis* is comparable to the results found by Standley et al., (2001), who compared the DPPH scavenging activities of both the fermented and green rooibos. Similar results were also found by Von Gadow et al., (1997a) who found that green rooibos had higher DPPH inhibition potential as the fermented rooibos with a calculated loss of activity of 3,2 % between the two variations of rooibos. The major constituents that were found to be attributed to the antioxidant potential of *A. linearis* was aspalathin, which is known to occur in higher concentrations within green rather than the fermented rooibos although other, now oxidized, constituents within the fermented

rooibos tea still play a considerable part as it still has significant antioxidant capacity as shown in literature and the current study (Von Gadow et al., 1997b).

There are significant changes that occur enzymatically during the process of enzymatic fermentation, and therefore there might be differences in the concentration of flavonoids found between fermented and green rooibos (Standley et al., 2001). According to a study by Marnewick et al., (2001), green rooibos presented significantly higher amounts of polyphenols, flavonoids, and non-flavonoids. Aspalathin is oxidized during the fermentation process to dihydro-iso-orientin, and therefore lower concentrations are to be found within fermented rooibos (Bramati et al., 2003). These are not the only constituents that undergo oxidation, as the oxidation of isoorientin, vitexin and nothofagin were also reported (Joubert, 1996; Joubert and Ferreira, 1996). Low levels of DPPH scavenging potential by *L. scaberrima* was reported by Shikanga et al., (2010). Based on this data the ethanolic extract of green *A. linearis* is an effective radical scavenger which may have the ability to lower the damage that occurs due to free radicals released during severe cases of TB infection as well as tolerates the radicals released as a result of TB drug metabolism by the host. The scavenging ability can be attributed to the significant presence of flavonoids and polyphenols found in the plant and extract.

It has been reported that compounds which are very good antioxidants exert effective anti- inflammatory properties, due to their phenolic content. An inflammatory response against invading bacteria (phagocytosis), such as *M. tuberculosis,* is mostly followed by an oxidative burst that results in the formation of reactive oxygen species (ROS) and reactive nitrogen species (RNS) (Miguel, 2010; Naz et al., 2017). Most of the components found within the ethanolic and essential oil of *L. scaberrima* were found to be flavonoids, and they are known to have high antioxidative potential (Jung et al., 2003; Leyva-Lopez et al., 2016). The phenolic compounds can also create a link between the extent of antimycobacterial and anti-inflammatory properties. Both *Lippia scaberrima* and *Aspalathus linearis* have measurable amounts of phenolic compounds present and this has also been associated with their biological activity. Positive correlations have been found by Naz et al., (2017) between the presence of phenolic compounds and the degree of antimicrobial activity as well as between the phenolic content and the degree of anti-inflammatory activity. Methanolic extracts of *Pereskia bleo* exhibited low MIC values against Gram-positive bacteria such as *Staphylococcus aureus* and *Streptococcus pyogenes* (Johari and Khong, 2019). Similarly,

Ruiz-Ruiz et al., (2017) stated that the presence of phenolic compounds are the major constituents within honey, and they are known to be involved in the stabilization of cell membranes through the reduction of lipid peroxidation. Phenolic compounds inhibit important enzymes during the inflammatory process such as prostaglandins and COX-II enzymes which are responsible for the production of mediators during inflammation.

The activation of nuclear factor kappa-light-chain-enhancer of activated B cells (NF- Kβ) requires nuclear translocation which is ROS-dependent. After activation, NF- Kβ, initiates the activation of inducible nitric oxide synthase (iNOS) and cyclooxygenase II (COX-II). Arachidonic acid metabolism is one of the key processes during an inflammatory response (Miguel, 2010). During inflammation arachidonic acid is released from the cell membranes by phospholipase A_2 and is metabolized through COX or lipoxygenase (LOX) pathways within prostaglandins and leukotrienes. Due to the high antimycobacterial activity found for the ethanolic extract of *L. scaberimma* and the fact that natural products that show good antibacterial activities normally exhibitff good anti-inflammatory activity due to the presence of phenolics (Naz et al., 2017), the COX-II inhibitory activity of the ethanolic extract of *L. scaberrima* was studied. The inhibition was compared to the activity of ibuprofen, a selective COX-II inhibitor (Table 3). The IC_{50} value for the sample was found to be 36.39 ± 1.62 μg/mL, higher than the IC_{50} value found for ibuprofen at 0.61 ± 0.10 μg/mL. *Torreya nucifera* oils that mainly consist of limonene, δ-3-carene, and α- pinene, were found to be selective COX-II inhibitors with effective inhibition activity against prostaglandin E_2 (PGE_2) production (Yoon et al., 2009). The compound; 1, 8-cineole which is a major component of the essential oil and the extract of *L. scaberrima* was reported as an inhibitor of leukotrienes and PGE_2 (Miguel, 2010). Further studies into the inhibitory potential of *L. scaberrima* against NF- Kβ are needed to determine if its high free radical scavenging potential has the ability to inhibit NF- Kβ and therefore, cause downstream inhibition of iNOS, NO and COX-II.

4.3.6. Hepatoprotective activity

Considering the reduced cytotoxic activity observed for the essential oil of *L. scaberrima* and the green extract of *A. linearis,* only these two samples were considered for further studies to evaluate their hepatoprotective effects. The hepatoprotective activity of the samples was calculated after

acetaminophen was introduced to HepG2 liver cells to induce cellular toxicity. To assess the hepatoprotective ability of the samples, the viability of the toxic induced cells (acetaminophen, 0% protection threshold), was compared to the untreated cells (100% protection threshold). Significant protection was established when 20% or more protection against acetaminophen was shown. The ethanolic green *A. linearis* extract showed the highest level of hepatoprotection with 40.79±3.46%, 37.65±5.09%, and 32.02±2.23% (Mean±SD) for the concentrations 400 µg/mL, 100 µg/mL and 25 µg/mL tested, respectively (Table 4). A study done by Olawale et al., (2013) have concluded that the hepatoprotective effects might be due to the inhibition of lipid peroxidation, thus stabilization of the membranes of hepatocytes.

The essential oil of *L. scaberrima* showed high levels of hepatoprotection with 17.79±4.63%, 34.34±3.10% and 33.20±3.91% for the concentrations 400 µg/mL, 100 µg/mL and 25 µg/mL, respectively. Different approaches have been used to explain the mechanism of action of natural products and their ability to exert hepatoprotection against a variety of sources (CCl_4, APAP, H_20_2 etc.). The main mechanism of action is due to the antioxidative capacity, the ability to scavenge free radicals as well as the inhibition of CYP 2E1 enzyme (Jaeschke et al., 2010, Kumar et al., 2013). Kumar et al., (2013), indicated that an increase in the activity of various enzymes such as glutathione peroxidase (GPx), glutathione- S-transferase (GST), superoxide dismutase (SOD) and catalase (CAT) together with the reduction of lipid peroxide influence the integrity of liver cells as well as decrease the levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST) and alkaline phosphatase (ALP). Further research is warranted into the mechanism of action of the essential oil of *L. scaberrima* and its ability to protect the liver against induced toxicity.

4.4. Conclusions

Investigations into the constituents of the ethanolic and essential oil of *L. scaberrima* confirm the presence of terpenoids. These terpenoids are lipophilic in nature and may interfere with the cell wall of bacteria and ultimately lead to the death of the bacteria, especially with mycobacterial spp. which possess mycolic acids. Mycolic acids are known to prevent the penetration of many compounds and therefore inhibit the activity of many of these compounds which need entry within these cells to exert their therapeutic action. During the invasion of the host by bacterial pathogens such as *M. tuberculosis*, nitric oxide is produced in large quantities by the host's macrophages and

neutrophils. The inflammatory process is facilitated by the initiation of several proinflammatory mediators such as reactive oxygen species (ROS) and nitric oxide (NO) (Kumar et al., 2013; Leyva-Lopez et al., 2016). With regards to inflammation, reactive oxygen species (ROS) are a major part of the inflammation process, through the activation of transcription factors such as NF- Kβ, which have the ability to override pro-inflammatory gene expression (Grigore et al., 2013). Treatments that can inhibit ROS production may also restrict the development of inflammation (Leyva-Lopez et al., 2016). The chemical composition of plants or a compound can act as an anti-inflammatory agent by affecting the arachidonic acid pathway, cytokine production or through the modulation of pro-inflammatory gene expression. The ability of *L. scaberrima* and *A. linearis* were found to be effective, at least in an *in vitro* setting, as adjunct host-direct therapy.

The combination of the ethanolic extracts of *L. scaberrima* and green *A. linearis* can be considered as a key candidate for adjunct host-directed therapy in TB patients, as it exhibited both anti-inflammatory properties and has existing antioxidant potential. An in-depth information is, however, needed to show how these samples affect the host's ability to interact with invading bacterial pathogens such as *M. tuberculosis* with regards to which pathways are inhibited or induced. Tea is a beverage that is consumed across the world, and the introduction of teas as additives may have the ability to aid in the treatment of various disorders and diseases. *Lippia scaberrima* is already well known as a health tea in the neighbouring countries of South Africa and the popular Rooibos tea, made from *A. linearis,* a plant endemic to the Cape areas of South Africa is a product exported worldwide.

Table 4. 3 The antimycobacterial, antioxidant, antiproliferative, anti-inflammatory and hepatotoxicity results for *L. scaberrima, A. linearis* and combinations of *L. scaberrima* with both fermented and green *A. linearis* as well as the essential oil of *L. scaberrima*

Sample	PRU number[a]	*M.smegmatis* MIC[b] (µg/mL)	*M.tuberculosis* MIC[b] (µg/mL)	Antiproliferative activity IC_{50}[c] (µg/mL)± SD	DPPH IC_{50}[d] (µg/mL)± SD	COX-II inhibition IC_{50}[e] (µg/mL)± SD
***L. scaberrima* (EtOH) extract**	119010	>1000	125	109.20±8.05	73.00±3.51	36.39±1.62
***L. scaberrima* (Essential oil)**	-[f]	1000	>1000	244.90±4.97	>500	-
***A. linearis* (Fermented)**	122176	>1000	1000	230.6±3.31	9.17±0.73	-
***A. linearis* (Green)**	122176	1000	1000	>400	2.18±1.75	-
***L. scaberrima* and fermented *A. linearis* (1:1)**	-	>1000	500	163.30±2.53	13.98±3.44	-
***L.* scaberrima and green *A. linearis* (1:1)**	-	>1000	500	191.20±9.37	4.49±0.16	-
Ciprofloxacin[g]	-	0.63	-	-	-	-
Isoniazid[h]	-	-	0.31	-	-	-
Actinomycin-D[i]	-	-	-	8.56±8.24	-	-
Ibuprofen[j]	-	-	-	-	7.70±1.12	0.61±0.10

[a]Voucher specimen code for the H. G. W. J. Schweickerdt Herbarium, [b]Minimum inhibitory concentration, [c]fifty percent inhibitory concentration of HepG2 cell viability, [d]Fifty percent inhibitory concentration of 2,2-diphenyl-1-picrylhydrazyl; [e]Fifty percent inhibitory concentration of cyclooxyegnase II, [f]Not applicable or not available, [g]Positive control for *M. smegmatis* inhibitory assay, [h]Positive control for *M. tuberculosis* inhibitory assay, [i]Positive control for antiproliferative activity, [j]Positive control for COX-II inhibition assay.

Table 4. 4 The hepatoprotective activity of the ethanolic extract of green *A. linearis* and the essential oil of *L. scaberrima* on acetaminophen-induced toxic HepG2 hepatocyte cells.

Sample	% Hepatoprotection		
	400 (µg/mL) ± SD	100 (µg/mL) ± SD	25 (µg/mL) ± SD
***A. linearis* (green)**	40.79±3.46*	37.65±5.09*	32.02±2.23*
***L. scaberrima* (Essential oil)**	17.79±4.63*	34.34±3.10*	33.20±3.91*
Acetaminophen[a]		0.00±1.00	
Silymarin[b] 50 mM		91.70±5.35*	
Untreated cells[c]		100±4.65*	

[a]Toxic inducer, used as the 0% protection value, [b]Positive control, [c]Untreated cells, used as the 100% protection value, Data is represented as mean±SD, n=3, ANOVA with Dunnett's multiple comparison, *Statistically significantly different from acetaminophen-induced cells (p-value <0.05).

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5

Inhibition potency of *Lippia scaberrima* Sond. in combination with *Aspalathus linearis* (Burm. f.) R. Dahlgren for CYP enzymes

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This chapter introduces the inhibition potency of Lippia scaberrima Sond. in combination with Aspalathus linearis (Burm. f) R. Dahlgren for several CYP P450 enzymes.

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5. INHIBITION POTENCY OF *LIPPIA SCABERRIMA* SOND. EXTRACT IN COMBINATION WITH *ASPALATHUS LINEARIS* (BURM.F.) R. DAHLGREN EXTRACT FOR HUMAN CYP ENZYMES

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Abstract

Background: The ability of medicinal plants to modulate the activity of metabolizing enzymes is of main importance when considering that medicinal plants are mainly used adjunctly with the current first line TB drug regimen to minimize the impact of side-effects experienced. **Objective:** Indigenous South African medicinal plants, *L. scaberrima* Sond. and *A. linearis* (Burm.f.) R. Dahlgren were studied for their inhibitory properties, alone and in combination on human hepatic CYP enzymes. **Materials and Methods:** Different concentrations of *L. scaberrima* extract alone and in combination with fermented and green *A. linearis* extract were used to determine the inhibitory potential on placental, microsomal and recombinant CYP P450 enzymes. **Results:** *L. scaberrima* extract alone and in combination with fermented and green *A. linearis* extract showed varying levels of CYP inhibitory potential. The most potent inhibition took place for CYP1B1, with an IC_{50}-value of less than 1 µg/L for all combinations and extracts tested. The IC_{50}-values of the extract were between 1 – 10 µg/L against both CYP1A2 and CYP2D6. The IC_{50} values found in the current study were mostly higher than 10 µg/L and therefore were not inhibited. **Conclusion:** Based on our data *L. scaberrima* and in combination with *A. linearis* do not possess any clinically significant interaction potential.

5.1. Introduction

The value and importance of medicinal plants are still essential today, especially in developing countries where access to primary healthcare is limited. Many medicinal products are derived from these plants either as pure compounds or as standardized extracts and are used to alleviate a myriad of symptoms and diseases. *Aspalathus linearis* is extensively investigated, especially with regards to its antioxidant potential as stated by Joubert et al., (2008)[1]. Minimal data exists on any extracts of *L. scaberrima*, and most of the work has been conducted on the essential oil extract. Both of these popular medicinal plants are enjoyed as a herbal "tisane"[2]. The kinetics of a xenobiotic determines its internal dose and concentration in a specific target of the body. No extensive data is available on the pharmacokinetic properties of *L. scaberrima* or *A. linearis* in humans. In addition, to being metabolized, *L. scaberrima* and *A. linearis* may cause herb-drug interactions through induction or inhibition of the metabolic enzymes found in the liver or intestines[3]. The metabolic profile of plant extracts and pure compounds are very important to determine if any future herb-drug metabolic interactions will occur. Due to previous work that was done, the inhibitory potential of *L. scaberrima* and combinations with *A. linearis* were studied against recombinant CYP enzymes and selective marker substrates of hepatic CYP enzymes. This information further sheds light on the potential of these tested plant extracts and their ability to be developed into adjuvants for the current TB drug regimen. This is the first assessment of the effect of the essential oil and ethanolic extracts of *L. scaberrima* and in combination with *A. linearis* against a range of important cytochrome P450 enzymes.

5.2. Materials and methods

5.2.1. Chemicals and reagents

Coumarin and 7-hydroxycoumarin were purchased from Sigma Aldrich (St. Louis, Mo, USA). Synthesis and purity of (TFD024 (3-(3-methoxyphenyl)-6-methoxycoumarin), OCA349 (3-(4-trifluoromethylphenyl)-6-methoxycoumarin), TFD008_1 (3-(4-phenylacetate)-6-chlorocoumarin, coumarin, TFD032 (3-(3-methoxyphenyl)coumarin), TFD023 (3-(4-phenyl)-7-methoxycoumarin) and OCA369 (3-(3-benzyloxo)phenyl-7-methoxycoumarin) are described in published papers[4-6], Tris-HCl, magnesium chloride ($MgCl_2$), $MnCl_2$, isocitric acid, isocitric acid dehydrogenase, Glycin, NaOH and trichloroacetic acid (TCA) were all bought from Sigma-Aldrich (Steineim, Germany). Nicotinamide adenine dinucleotide phosphate ($NADP^+$) were bought from Roche Diagnostics

(Mannheim, Germany). The NADPH regenerative system consisted of 1.12 mM $NADP^+$, 12.5 mM $MgCl_2$, 12.5 $MnCl_2$, 16.8 mM isocitric acid, 0.056 mM KCl and 15 U isocitric acid dehrydogenase in 188 mM Tris-HCl buffer pH 7.4. cDNA expressed human wild-type CYPs (CYP1A1, CYP1A2, CYP2A6, CYP3A4, CYP1B1, CYP2C19 and CYP2D6) were obtained from BD Biosciences Discovery Labware (Bedford, MA). All chemicals were of the highest purity available from the suppliers. The livers used during this study were obtained from University of Oulu Hospital as excess from kidney transplantation donors. The excess tissue collection was approved by the Ethics committee of the Medical Faculty of the University of Oulu. The liver samples were surgically excised and immediately transferred to ice, then cut into pieces and snap frozen in liquid nitrogen and stored at 80 °C until microsomal preparation. The liver microsomes were prepared as described by Lang et al., (1981)[7]. Microsomal protein concentration was determined by using the Bradford method. Placental microsomes were obtained from both non-smoking and smoking mothers of previous studies and prepared according to Huuskonen et al., (2015)[8].

5.2.2. Plant material, collection and extraction

Lippia scaberrima was collected in autumn from the Kopela community situated nearby Delareyville, North West Province, South Africa. The plant was identified, and a voucher specimen deposited at the H. G. W. J Schweickerdt herbarium, University of Pretoria (PRU) (Table 1). Powdered green and fermented *A. linearis* leaf material were kindly donated by Rooibos Limited, Clanwilliam, Western Cape, South Africa and a voucher specimen deposited. The collected aerial parts of *L. scaberrima* (stem, flowers, and leaves) were mechanically ground to a uniform size of 0.2 mm. The powdered plant material from both plants was extracted with absolute ethanol at a ratio of 1:10 (weight: volume) and macerated for 72 hours. The extracts were filtered, and the plant material was subsequently extracted with fresh ethanol at a ratio of 1:5 for 48 hours, followed by filtration. The filtrates were combined and dried under reduced pressure using a rotary evaporator which resulted in a dark green extract for *L. scaberrima* and reddish extracts for the green and fermented *A. linearis*. The three extracts were stored in airtight containers at 4 °C for the duration of the study.

5.2.3. Inhibition of placental CYP1A1, microsomal and recombinant CYP1A2, CYP2A6, CYP3A4, CYP1B1, CYP2C19 and CYP2D6 oxidation by the essential oil and ethanolic extract of *L. scaberrima* and combinations with *A. linearis*.

The IC_{50} determinations of *L. scaberrima* and combinations with *A. linearis* for human recombinant CYPs, human liver microsomes and placental microsomes were determined by the methods described by Juvonen et al. (2018)[6], Huuskonen et al. (2016)[9] and Crespi et al. (1997)[10]. The incubations were carried out in a total volume of 100 µL of 100 mM Tris-HCl buffer (pH 7.4) in all black, flat bottom Costar 96-well plates (Corning Incorporated, Corning, NY). The reaction mixtures contained 20 % NADPH-regenerating system in Tris-HCl buffer (pH7.4), 10 µM coumarin or its derivative substrate ((TFD024 (3-(3-methoxyphenyl)-6-methoxycoumarin), OCA349 (3-(4-trifluoromethylphenyl)-6-methoxycoumarin), TFD008_1 (3-(4-phenylacetate)-6-chlorocoumarin, coumarin, TFD032 (3-(3-methoxyphenyl)coumarin), TFD023 (3-(4-phenyl)-7-methoxycoumarin) or OCA369 (3-(3-benzyloxo)phenyl-7-methoxycoumarin), 0.010 g/L microsomal protein or 5nM recombinant CYP and inhibiting agent of 3 – 500 mg/L essential and ethanolic extracts of *L. scaberrima* or in combination with *A. linearis.* A full reaction (100 %) did not contain any inhibiting agent and blank reactions did not contain any substrate or enzyme. Substances were added from a stock solution containing ethanol so that the final concentration in the incubation mixture was at 1%. The samples were then pre-incubated at 37ºC for 10 minutes. The reactions were initiated by the addition of NADPH. 0 - 10 µM 7-Hydroxycoumarin was used as the standard. The fluorescent signal was measured with a Victor^2 1420 multilabel plate counter (PerkinElmer Lifesciences Wallac, Turku, Finland) with excitation and emission wavelengths of 405 nm and 460 nm, respectively. The fluorescence was monitored every 2 min for a total period of 40 minutes, from where the concentration was calculated at the various time points and from these, the oxidation rates (µM/min) and the relative remaining activity of the sample at different concentrations were calculated. The data were fit to sigmoidal dose-response curves with non-linear regression and IC_{50} values (the concentration at which the sample reduced the metabolism of the CYP substrate by 50%) were determined using GraphPad Prism 5 (San Diego, CA, USA). The calculation was based on the equation $vi/v0= 1/ (1 + i/IC_{50})$, in which (vi) is the rate at the specific concentration of the inhibiting agent, (v0) is the rate without the inhibitor, (IC_{50}) is the sample concentration with 50 % inhibition and (i) is the inhibitor concentration. The degree of inhibition was categorized as potent (IC_{50} < 1 µM), marginal/moderate (1 µM <IC_{50}> 10 µM), weak (IC_{50} > 10 µM), or no inhibition (IC_{50} >100 µM)[11]. Statistical analysis was performed with GraphPad Prism (Version

7) using one-way analysis of variance (ANOVA). The results are expressed as the mean ± standard deviation, n= 3 or more.

5.3. Results

To find out if the essential oil and ethanolic extracts of *L. scaberrima* and combinations with *A. linearis* could inhibit individual human CYP enzymes, concentration dependent inhibitions were determined for eight recombinant CYP enzymes and for selective CYP substrate assays with hepatic and placental microsomes (Table I). The inhibition potency of the extracts varied against the different CYP enzymes tested. The greatest inhibition was for CYP2C19 by *L. scaberrima* essential oil extract with an IC_{50} value of 0.04 mg/L (0.01-0.08) (Fig 1B- supplementary material) as well as CYP1B1 by *L. scaberrima* with an IC_{50} value of 0.07 mg/L (0.06-0.07) (Fig 2B- Suplementary material). The most potent inhibition across all CYP enzymes tested took place against recombinant CYP1B1, with IC_{50} values that were less than 1 mg/L for all extracts tested except for *L. scaberrima* essential oil. The IC_{50} value range of the tested extracts were between 1 – 10 mg/L for both recombinant CYP1A2 and CYP2D6. Other recombinant CYP IC_{50} values were higher than 10 mg/L and therefore did not show any noteworthy inhibition.

Table 5. 1 The IC_{50}-values of *L. scaberrima* and combinations with *A. linearis* against recombinant CYP enzymes and hepatic microsome catalyzed CYP oxidations.

		Recombinant CYP	Microsomal	
CYP enzyme	**Substrates**	**IC_{50}[a] (mg/L) with 95% confidence intervals**	**IC_{50} (mg/L) with 95% confidence intervals**	**Samples**
CYP 1A1	**TFD024**	N/A[b]	0.40 (0.31-0.51)	
CYP 1A2	**OCA349**	4.10 (2.82-5.95)	4.26 (0.32-8.20)	
CYP 1B1	**TFD008_1**	0.07 (0.06-0.07)	No inhibition[c]	
CYP 2A6	**coumarin**	22.9 (15.4-34.69)	18.37 (9.15-27.6)	*L. scaberrima*
CYP 2C19	**TFD032**	4.19 (2.90-5.48)	No inhibition	
CYP 2D6	**TFD023**	7.65 (4.93-12)	44.93 (34.87-58.44)	
CYP 3A4	**OCA369**	0.41 (0.04-16.28)	25.81 (19.36-32.27)	
CYP 1A1	**TFD024**	N/A	0.49 (0.30-0.80)	
CYP 1A2	**OCA349**	1.62 (0.86-2.95)	0.21 (0.09-0.34)	*L. scaberrima*
CYP 1B1	**TFD008_1**	0.21 (0.17-0.24)	Stimulation[d]	with green *A.*
CYP 2A6	**coumarin**	14.08 (9.47-20.47)	7.66 (6.15-9.17)	*linearis*
CYP 2C19	**TFD032**	8.79 (4.75-15.58)	Stimulation	

CYP 2D6	**TFD023**	4.19 (3.10-5.73)	6.17 (3.67-8.67)	
CYP 3A4	**OCA369**	No inhibition	3.04 (0.79-5.28)	
CYP 1A1	**TFD024**	N/A	0.40 (0.39-0.48)	
CYP 1A2	**OCA349**	2.5 (1.57-3.89)	2.22 (-0.62-5.05)	
CYP 1B1	**TFD008_1**	0.25 (0.19-0.32)	No inhibition	*L. scaberrima*
CYP 2A6	**coumarin**	10.16 (6.84-15.15)	12.37 (10.39-14.35)	with fermented
CYP 2C19	**TFD032**	6.96 (3.48-10.44)	2.36 (-1.16-5.83)	*A. linearis*
CYP 2D6	**TFD023**	6.96 (4.34-11.05)	18.98 (12.87-25.09)	
CYP 3A4	**OCA369**	No inhibition	12.96 (10.79-15.13)	
CYP 1A1	**TFD024**	N/A	No inhibition	
CYP 1A2	**OCA349**	3.91 (3.51-4.32)	7.65 (-9.90-25.19)	
CYP 1B1	**TFD008_1**	5.93 (0.97-10.90)	1.17 (0.64-1.70)	
CYP 2A6	**coumarin**	0.71 (0.45-0.97)	1.40 (0.87-1.92)	*L. scaberrima* essential oil
CYP 2C19	**TFD032**	0.04 (0.01-0.08)	No inhibition	
CYP 2D6	**TFD023**	2.66 (0.80-4.46)	6.43 (2.30-10.56)	
CYP 3A4	**OCA369**	No inhibition	15.85 (3.19-28.51)	

[a]50% inhibition concentration (the concentration at which the sample reduced the metabolism of the CYP substrate by 50%) of recombinant CYP enzymes, placental and liver microsomes; [b]No recombinant CYP1A1 available, CYP1A1 is only available within placental microsomes of tobacco smoking mothers; [c]No inhibition of CYP enzyme at the various concentrations tested; [d]Stimulation of CYP enzyme at the various concentrations tested. The IC_{50} values and the 95% confidence intervals (CI) were determined from the appropriate dose response curves.

5.4. Discussion

Metabolism is an important component of the kinetic characteristics of herbal constituents and it often determines internal dose and concentration of effective constituents at the target site. In this study we studied the inhibition of hepatic and placental CYPs by the essential oil and ethanolic extracts of *L. scaberrima*, and combinations with *A. linearis*. *In vitro* analysis involving liver enzymes is a frequently used method to determine the possibility of *in vivo* drug interactions and has yielded dependable results thus far[12]. The CYP family forms an integral part of Phase I metabolizing enzymes and is divided into different families and subfamilies depending on the homology of their nucleotide sequences. They are also highly specific for different substrates which can be used as "diagnostic" targets for their enzymes[3]. The most potent inhibition across all CYP enzymes tested took place against recombinant CYP1B1, with IC_{50} values that were less than 1 mg/L for all extracts tested except

L. scaberrima essential oil. *L. scaberrima* had the highest inhibition of CYP1B1 with an IC_{50} value below 1 mg/L. CYP1B1 is extrahepatic and a key CYP enzyme involved in the metabolism of hormone responsive cancer. CYP1B1 is also present within the liver albeit in low levels and is found to be responsible for the oxidative metabolism of estrogen and the conversion of certain compounds from procarcinogens to carcinogens[13-14]. Due to inhibition taking place throughout for all extracts tested against CYP1B1, this warrants further research into the *in vitro* and *in vivo* inhibition mediated by these samples. Although no significant inhibition is noted for microsomal CYP1A1, these values are slightly lower, in relation to the IC_{50} values obtained for the rest of the recombinant and microsomal CYPs tested. Specificity is key when considering inhibition of CYP1B1 and CYP1A1, as the inhibition of CYP1B1 is a main target for cancer prevention, whereas CYP1A1 plays a key part in metabolically activating dietary compounds that aid in cancer prevention as well as detoxify procarcinogens found in the environment[15].

The extracts tested in this study, especially *A. linearis* are recognized for their high flavonoid content. Flavonoids are known for their interactive potential with CYP1 as they may act as inhibitors and substrates[16]. Limonene which is the main constituent found within the essential oil extract of *L. scaberrima*, has been shown to selectively inhibit CYP1B1 (K_i ~ 2 μM) and not CYP1A1[17]. Although contradictory to this study, the IC_{50} values are lower than for the rest of the values noted for the other CYP enzymes tested. According to the current study, *L. scaberrima* and the combinations with fermented and green *A. linearis* were found to inhibit CYP2C19 indicating targeted specificity of the compounds; as no other activities were affected. CYP2C19 is responsible for the metabolism of many important drugs such as proton pump inhibitors and anti-depressants[18]. A marked decrease in the activity of this enzyme may cause differences in the metabolism of these important drugs and therefore there needs to be a more in detailed investigation done into which constituents might be responsible for the activity found. Most of the potent and moderate enzyme inhibition of the CYP enzymes investigated, results from the combination of *L. scaberrima* and green *A. linearis*. Flavonoids have been implicated several times as the main component exerting inhibition of CYP enzymes[3]. A study conducted by Patel et al., (2016)[19] indicated that Aspalathin, an abundant main component of *A. linearis*, together with fermented and green *A. linearis* showed inhibition of CYP3A4 as compared to erythromycin at set concentrations of 25, 50 and 100 μg/ mL. Structure-based molecular docking studies and computer modelling could identify the binding sites and whether the drugs bind on the active centre or allosteric sites. Levels of enzymes differ within microsomes as compared to recombinant enzymes specific to that enzyme. An effect is more effectively seen within the

recombinant proteins as compared to the microsomes that include a variety of enzymes and somewhat higher level of specific enzyme. Differences in the activity between microsomes may also be due to the fact that the metabolites formed after metabolism may possess greater inhibition properties than the parent compound (Kerns et al., 2007)[20].

5.5. Conclusion

The present study shows that many herb-drug CYP-interactions are capable with *L. scaberrima* and with the combinations of *A. linearis* albeit at significantly lowered levels and thus further supports that the pharmacokinetic profile of a herbal mixture and an active pure compound, isolated from medicinal plants are indeed important and necessary for the prediction of possible herbal-drug interactions. Many medicinal plants are being used adjunctly for instance, with the current first line drugs for treatment against tuberculosis and it is important to determine if there is any herb-drug interaction potential. It can be concluded that *L. scaberrima* and *A. linearis* can be recommended to be used as an adjuvant due to the low levers of interactions with the CYP P450 enzymes.

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6

In vitro human hepatic metabolism and inhibition potency of verbascoside for CYP enzymes

This chapter introduces the hepatic metabolism of verbascoside, one of the main constituents found within Lippia scaberrima Sond. This chapter includes the inhibition potency of verbascoside for several CYP P450 enzymes and aromatase.

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6. *IN VITRO* HUMAN METABOLISM AND INHIBITION POTENCY OF VERBASCOSIDE FOR CYP ENZYMES

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Abstract

Verbascoside is found in many medicinal plant families such as Verbenaceae. Important biological activities have been ascribed to verbascoside. Investigated in this study is the potential of verbascoside as an adjuvant during TB treatment. The present study reports on the *in vitro* metabolism in human hepatic microsomes and cytosol incubations and the presence and quantity of verbascoside within *L. scaberrima* Sond. Additionally, studied are the inhibitory properties on human hepatic CYP enzymes together with antioxidant and cytotoxic properties. The results yielded no metabolites in the hydrolysis or cytochrome P450 (CYP) oxidation incubations. However, five different methylated conjugates of verbascoside could be found in the S-adenosylmethionine incubation, three different sulphate conjugates with the 3′-phosphoadenosine 5′-phosphosulfate (PAPS) incubation with human liver samples and very low levels of glucuronide metabolites after incubation with recombinant human uridine 5'-diphospho-glucuronosyltransferase (UGT) 1A7, UGT1A8 and UGT1A10. Additionally, verbascoside showed weak inhibitory potency against CYP1A2 and CYP1B1 with IC_{50}-values of 83 µM and 86 µM, respectively. Potent antioxidant and low cytotoxic potential were observed. Based on this data, verbascoside does not possess any clinically relevant CYP-mediated interaction potential but effective biological activity. Therefore, verbascoside could be considered as a lead compound for further drug development and as an adjuvant during TB treatment.

6.1. Introduction

Verbascoside, also known as acteoside is a phenylethanoid consisting of a cinnamic acid and hydroxyphenylethyl moieties attached to a β-glucopyranose through a glycosidic bond [1-3]. Verbascoside has extensively been studied for its *in vitro* and *in vivo* biological activities. Many publications have investigated the free radical scavenging effect of verbascoside *in vitro* and found it was comparable to that of α-tocopherol [4]. Verbascoside is also a known constituent to occur within the five *Lippia* species endemic to South Africa [5].

The kinetics of a xenobiotic determines its internal dose and concentration in a specific target of the body. No extensive data is available on the pharmacokinetic properties of verbascoside. However, the pharmacokinetic properties of verbascoside, such as absorption and elimination rate has been previously studied in rats, stating fast absorption and elimination [6]. Verbascoside has the ability to be absorbed into the blood stream and undergo further metabolism [7-8]. A low oral bioavailability of verbascoside has been reported [6]. Cui et al., (2016) [8] investigated the metabolic profile of verbascoside in the presence of human and rat intestinal bacteria as well as with rat intestinal enzyme. This study provided an insight into the metabolic pathway of verbascoside as well as established the fact that phenylethanoid glycosides are metabolized by both intestinal bacteria and enzymes, both in humans and rats. The low oral bioavailability in rats has been described to be due to the multiple routes of hydrolysis by the bacteria of the gastrointestinal ducts with several degradation products as a result thereof. The structure of verbascoside suggests that it is most likely to be metabolized by hydrolyzing and conjugating enzymes, as it contains an ester and several hydroxyl groups.

In addition, to being metabolized, verbascoside, may cause herb-drug interactions through induction or inhibition of metabolic enzymes found in the liver or intestines [9]. The metabolic profile of plant extracts and pure compounds are very important to determine if any future herb-drug metabolic interactions will occur. In the present study, an ultra-high-performance liquid chromatography-and a high-resolution mass spectrometry (UHPLC-QTOF-MS) was used to analyze metabolites of verbascoside after incubation with human liver microsomes or cytosol and in the presence of NADPH, UDP-glucuronic acid, S-adenosylmethionine and PAPS. Also, the presence and quantity of verbascoside within *L. scaberrima*, an indigenous species of *Lippia* in South Africa was investigated through UHPLC-QTOF-MS method. This method served to confirm that the biological activity found for *L. scaberrima* in previous studies (Chapter 4, 5 and 6) conducted may be attributed to the presence of verbascoside. Additionally,

the inhibition of hepatic CYP enzymes by verbascoside were studied with recombinant CYP enzymes and selective marker substrates of hepatic CYP enzymes and placental CYP19A1. Also investigated were the antioxidant properties of verbascoside as well as an investigation into the cytotoxic potential of verbascoside on both first (PBMCs) and secondary (HepG2) cell lines.

6.2. Results

6.2.1. Identification of verbascoside through UHPLC-QTOF-MS

The presence and the calculated mass of verbascoside within *L. scaberrima* was investigated through UHPLC-QTOF-MS in negative ion mode. The identification of verbascoside was based on comparison of retention times of pure standard and samples, together with high accuracy mass and isotopic pattern of analyte and its metabolites. The content of verbascoside was found to be 0.17 mg/mL or 6.8% of the total weight. See supplementary material (Figure 1C and 2C).

6.2.2. In vitro metabolism of verbascoside

In vitro oxidation, hydrolysis, glucuronidation, sulfonation and methylation metabolism of verbascoside was studied in the presence of human liver microsomes or cytosol and specific reactions requiring cofactors. The level of verbascoside decreased when it was incubated with UDP-glucuronic acid, PAPS and S-adenosylmethionine, but no decrease was detected with hydrolysis and NADPH containing oxidation incubations (Table 1). The S-adenosylmethionine incubation produced five different methylated conjugates of verbascoside, whose level increased up to 60 min (Table 1). The PAPS incubation produced three different sulphate conjugates of verbascoside (Table 1), whose level increased up to 20 min. No glucuronide conjugate of verbascoside could be identified from the UDP-glucuronic acid incubations together with human liver microsomes. However, incubations with recombinant human UGT1A7, 1A10, and UGT1A8 produced low levels of glucuronide metabolites of verbascoside (Table 1)

6.2.3. Inhibition of CYP activities by verbascoside

To find out if verbascoside could inhibit clinically relevant individual human CYP enzymes, concentration dependent inhibitions were determined for eight recombinant CYP enzymes and

for selective CYP substrate assays with hepatic and placental microsomes (Table 2). Verbascoside was a weak inhibitor for many CYP enzymes. The IC_{50} value of verbascoside was more than 50 µM for CYP1A2, 1B1, 2D6 and 3A4 and did not inhibit CYP1A1, 2A6, 2C19 and 19A1 (placental aromatase) enzymes.

6.2.4. DPPH and nitric oxide inhibitory activity of verbascoside

The DPPH radical scavenging assay is a method that indicates the free radical scavenging potential of samples or compounds [10]. During this investigation verbascoside showed potent DPPH radical scavenging activity with an IC_{50} value of 2.50±0.02 µM, even lower than for the positive control, ascorbic acid which had an IC_{50} value of 43.72±1.12 µM (Table 3).

According to Table 3, the NO inhibitory activity of verbascoside was moderately effective when compared to that of the positive control, ascorbic acid, with the IC_{50}-values of 382.01±4.15 µM and 143.94±3.30 µM.

6.2.5. Cellular antiproliferative activity of verbascoside

The antiproliferative activity of verbascoside was determined on both primary (PBMC) and secondary (HepG2) cell lines. Verbascoside had a weak antiproliferative effect on both the PBMCs and the HepG2 cell line as both the IC_{50}-values obtained were above 100 µM (Table 3).

6.3. Discussion

Metabolism is an important component of the kinetic characteristics of herbal constituents and it often determines internal dose and concentration of effective constituents at the target site. In this study we studied 1) the *in vitro* human hepatic metabolism of verbascoside, which is found from many medicinally used herbal plants, and 2) inhibition of hepatic and placental CYPs by verbascoside. Verbascoside was metabolized efficiently to methyl and sulphate conjugates, but not hydrolysed or oxidized by human liver subcellular fractions. Three recombinant UGTs had the ability to conjugate verbascoside to glucuronides. Through this *in vitro* study it could be summarized that verbascoside indeed can undergo first pass metabolism in the liver and most probably also in the intestine. Therefore, it could explain the low oral bioavailability as seen in previous studies [11]. It was confirmed through UHPLC-QTOF-MS

that verbascoside is present within the indigenous species of *L. scaberrima* and therefore, may contribute to the biological activity found for the ethanolic plant extract.

The inhibition potency of verbascoside against human CYP enzymes was very weak and therefore, indicate the low potential of verbascoside in clinical herb-drug interactions. However, no nuclear receptor binding studies were carried out to resolve whether any receptor-mediated inductions of metabolizing genes could be detected. Instead, verbascoside had a weak ability to stimulate the CYP2C19 activity, both with the recombinant CYP2C19 and within liver microsomes, which can be due to allosteric binding of the chemical on the metabolizing enzyme. However, all these are *in vitro* results and additional confirmatory clinical trials will be needed to confirm the clinical relevance of this finding. According to the current study, verbascoside, was found to stimulate CYP2C19 indicating targeted specificity of the compounds; no other activities were stimulated.

Table 6. 1 *In vitro* metabolites of verbascoside (VMs are methylation, VS-sulfate and VGs-glucuronide conjugates of verbascoside).

Metabolite	RT (min)	Calculated mass	Structure	*m/z*	Δ mass (ppm)	Score of Isotopic Pattern Matching
Verbascoside	4.76	624.21	$C_{29}H_{36}O_{15}$	623.20	1.17	99.6
Methyl conjugation						
VM1	5.25	638.22	$C_{30}H_{38}O_{15}$	637.21	0.40	96.1
VM2	5.34	638.22	$C_{30}H_{38}O_{15}$	637.21	-0.40	98.7
VM3	5.44	638.22	$C_{30}H_{38}O_{15}$	637.21	-1.39	84.9
VM4	5.60	638.22	$C_{30}H_{38}O_{15}$	637.21	-0.68	95.2
VM5	5.73	638.22	$C_{30}H_{38}O_{15}$	637.21	-0.93	94.2
Sulfonation conjugation						
VS1	4.52	704.16	$C_{29}H_{36}O_{18}S$	703.16	1.08	99.0
VS2	4.78	704.16	$C_{29}H_{36}O_{18}S$	703.16	1.64	66.9

VS3	4.98	704.16	$C_{29}H_{36}O_{18}S$	703.16	0.25	85.2
Glucuronide conjugation						
VG1	3.96	800.24	$C_{35}H_{43}O_{21}$	799.23	0.51	94.5
VG2	4.49	800.24	$C_{35}H_{43}O_{21}$	799.23	1.27	91.5
VG3	4.76	800.24	$C_{35}H_{43}O_{21}$	799.23	1.43	99.0

Table 6. 2 Inhibitory concentrations (μM) of verbascoside against recombinant CYP and hepatic or placental microsome catalyzed CYP oxidations

		Recombinant CYP	**Microsomal**
CYP enzyme	**Substrates**	**IC_{50} (μM) with 95% confidence intervals**	**IC_{50} (μM) with 95% confidence intervals**
CYP 1A1	**TFD024**	N/A[a]	350 (0-870)
CYP 1A2	**OCA349**	83 (21-144)	24 (0-48)
CYP 1B1	**TFD008_1**	86 (71-101)	No inhibition[b]
CYP 2A6	**coumarin**	No inhibition	135 (92-180)
CYP 2C19	**TFD032**	Stimulation[c]	
CYP 2D6	**TFD023**	131 (37-225)	96 (21-171)
CYP 3A4	**OCA369**	314 (0-953)	76 (24-129)
CYP19A1	**Androstendione[d]**	N/A	No inhibition

[a]N/A: No recombinant CYP1A1 available, CYP1A1 is only available within placental microsomes of tobacco smoking mothers; [b]No inhibition of CYP enzyme at the various concentrations tested; [c]Stimulation of CYP enzyme at the various concentrations tested. The calculated IC_{50} values (μM) (the concentration at which the sample reduced the metabolism of the CYP substrate by 50%) of recombinant CYP enzymes, placental and liver microsomes. The IC_{50} values and the 95% confidence intervals (CI) were determined from the appropriate dose response curves. [d]No inhibition of CYP19A1 was observed for verbascoside. As a positive control for the assay finrozole (1 μM and 10 μM) was used within human placental microsomes.

On the other hand, Lee et al., (2004) [12] found that verbascoside showed a protective effect after carbon tetrachloride-induced hepatotoxicity. One explanation could be that verbascoside could act as a "scavenger" against tetrachloride-induced toxicity. Verbascoside showed potent free radical scavenging activity as compared to ascorbic acid,

the positive control used. According to a study by Chen et al., (2012) [13] verbascoside had an IC_{50}-value of 11.4 µM, a higher value as obtained during the current study.

Table 6. 3 The antioxidant (DPPH and NO) and cellular antiproliferative properties of verbascoside

Sample	DPPH IC_{50} [a] (µM)	NO IC_{50} (µM)	PBMC (µM) 48 hours	HepG2 (µM) 48 hours
Verbascoside	2.50±0.02	382.01±4.15	169.55±3.73	>640.42
Ascorbic acid[b]	43.72±1.12	143.94±3.30	-[c]	-

[a]Inhibitory concentration (50%); [b]Positive control for both DPPH and NO inhibitory assays; [c]Not applicable. (Mean±SD, n=3)

This may be due to discrepancies in the two methods used and the lab conditions, as DPPH was dissolved in ethanol and not methanol as indicated in the study done by Chen et al., (2012) [13]. The mechanism of action of the free radical scavenging is believed to be due to a free hydroxyl group in the glucose moiety found within the structure of verbascoside [13]. Another study done by Koo et al., (2006) [14] also indicated the effect of verbascoside on DPPH radical scavenging. The EC_{50} value obtained for verbascoside during this study was 1.28 µM, fairly similar to the results found in the current study. Another study, conducted by Koo et al., (2006) [14], showed that verbascoside had the ability to boost the endogenous antioxidative system.

Nitric oxide inhibition is one of the main mechanisms of down-regulating inflammation during infection, especially on the protein level through iNOS (inducible nitric oxide synthase) inhibition. Verbascoside is known for its anti-inflammatory effects as seen through studies conducted both *in vitro* and *in vivo* [15-17]. During the current study, verbascoside had effective inhibition of NO when compared with the positive control, ascorbic acid which had an IC_{50}-value of 143.94±3.30 µM (Mean±SD). The main mechanism through which verbascoside exerts its inhibitory effect has been shown to be through the inhibition of AP-1 (activator protein-1) [18]. Verbascoside dissolved in PBS showed a much lower IC_{50}-value of 89.66±0.02 µM via the same method, with another study conducted by Koo et al., (2006) [14], yielding an EC_{50}-value of 5.19 µM.

Verbascoside had no noteworthy levels of toxicity observed in the current study on both the cell lines tested. A study conducted by Sipahi et al., (2016) [19] investigated the cellular toxicity of an aqueous extract of verbascoside on PBMCs. The associated IC_{50}-value was found

to be 384 µM. The difference in the values obtained may be due to the difference in the solvents that the extracts were dissolved in. In agreement with Etemad et al., (2015) [17] no cellular toxicity was detected for verbascoside on the HepG2 cell line after 24 and 72 hours with IC_{50}-values higher than the highest concentration tested of 400 µM. These values are comparable to the values obtained in the current study.

6.4. Materials and Methods

6.4.1. Cell lines, chemicals and reagents

Verbascoside with a purity of >99%, coumarin, 7-hydroxycoumarin and finrozol were purchased from Sigma Aldrich (St. Louis, Mo, USA). Synthesis and purity of (TFD024 (3-(3-methoxyphenyl)-6-methoxycoumarin),OCA349(3-(4-trifluoromethylphenyl) 6-methoxycoumarin), TFD008_1 (3-(4-phenylacetate)-6-chlorocoumarin, coumarin, TFD032 (3-(3-methoxyphenyl)coumarin), TFD023 (3-(4-phenyl)-7-methoxycoumarin) and OCA369 (3-(3-benzyloxo)phenyl-7-methoxycoumarin) are described in published papers [20-22], Tris-HCl, magnesium chloride ($MgCl_2$), $MnCl_2$, isocitric acid, isocitric acid dehydrogenase, Glycin, NaOH and trichloroacetic acid (TCA) were all bought from Sigma-Aldrich (Steineim, Germany). Androstenedione and ^{3}H-Androst-4-ene, 3, 17-dione was obtained from PerkinElmer. KCl was from J.T. Baker and nicotinamide adenine dinucleotide phosphate ($NADP^+$) were bought from Roche Diagnostics (Mannheim, Germany). The NADPH regenerative system consisted of 1.12 mM $NADP^+$, 12.5 mM $MgCl_2$, 12.5 $MnCl_2$, 16.8 mM isocitric acid, 0.056 mM KCl and 15 U isocitric acid dehrydogenase in 188 mM Tris-HCl buffer pH 7.4. cDNA expressed human wild-type CYPs (CYP1A1, CYP1A2, CYP2A6, CYP3A4, CYP1B1, CYP2C19, CYP2D6, CYP19A1) were obtained from BD Biosciences Discovery Labware (Bedford, MA). The livers used during this study were obtained from University of Oulu Hospital as excess from kidney transplantation donors. The excess tissue collection was approved by the Ethics committee of the Medical Faculty of the University of Oulu. The liver samples were surgically excised and immediately transferred to ice, then cut into pieces and snap frozen in liquid nitrogen and stored at -80 ^{0} C until microsomal preparation. The liver microsomes were prepared as described by Lang et al., (1981) [23]. Microsomal protein concentration was determined by using the Bradford method. Placental microsomes were obtained from both non-smoking and smoking mothers of previous studies and prepared according to Huuskonen et al., (2015) [24]. The HepG2 cell line (HB-8065) was obtained from the American Type Tissue Culture Collection (ATCC). Cell culture materials and reagents such

as Fetal Bovine Serum (FBS), Dulbecco's Modified Eagles Medium (DMEM) media, trypsin-EDTA and antibiotics were supplied by Highveld Biological (Pty) Ltd. (Johannesburg, RSA). Ficoll-Hypaque was obtained from Pharmacia Biotech (Piscataway, NJ, USA). PrestoBlue was purchased from Thermo Fisher (Carlsbad, CA, USA). All reagents obtained were of analytical grade. The XTT Cell Proliferation Kit II was supplied by Roche Diagnostics (Pty) Ltd. (Johannesburg, RSA). ACK was obtained from Life Technologies (Carlsbad, CA, USA). The Trypan blue solution was supplied from Thermo Fischer Scientific (Waltman, MA, USA). All chemicals were of the highest purity available from the suppliers.

6.4.2. In vitro metabolism and identification of verbascoside through UHPLC-QTOF-MS

To determine *in vitro* metabolism, 10 µM verbascoside was incubated at 37 °C in 1 mL of 100 mM phosphate buffer (pH 7.4) with 400 µg human hepatic microsomal or cytosolic protein with the different cofactors for 1 hour. From the incubation mixture, 150 µL of the samples were added to 450 µL acetonitrile to stop the reaction at 0-, 10-, 20-, 40- and 60 min time points. The CYP incubation mixture contained 20 % NADPH regenerating system, whereas the glucuronidation incubation mixture contained 0.5 mM UDP-glucuronic acid with 5 mM $MgCl_2$. The sulfonation incubation mixture contained 10 µM PAPS, 5 µM $MgCl_2$ and 500 µg cytosolic protein instead of microsomes and the methylation incubation mixture contained 0.5 mM S-adenosylmethionine, 5 mM $MgCl_2$ and 500 µg cytosolic protein instead of microsomes. Blank samples did not contain any of the cofactors. The hydrolysis reaction was studied with an incubation mixture that contained 400 µg microsomal protein, or 500 µg cytosolic proteins, and 5 mM $MgCl_2$. A sample with the absence of any enzyme was considered as the blank sample. After stopping the reactions, the samples were centrifuged for 20 min 10 000 × *g* and stored at –80 °C for analysis by the UHPLC-QTOF-MS system (Agilent Technologies, Waldbronn, Karlsruhe, Germany), which consisted of a 1290 LC system, a Jetstream ESI source, and a 6540 UHD accurate-mass quadrupole-time-of-flight (QTOF) mass spectrometry. The sample tray was kept at 4 °C during these analyses. UHPLC-QTOF-MS was used to both identify the metabolites formed after metabolism of verbascoside and the quantity of verbascoside found within the ethanolic extract of *L. scaberrima* (Figure 1C and 2C- Supplementary material). Two microliters sample was injected onto a column (Zorbax Eclipse XDB-C8, 2.1 × 100mm, 1.8 µm, Agilent Technologies, Palo Alto, CA, USA) that was kept at 50 °C. Mobile phases, delivered at 0.4 mL/min, consisted of water (eluent A) and

methanol (eluent B), both containing 0.1 % (v/v) of formic acid. The gradient employed was as follows: 2% →100% B (0-10 min); 100% B (10-14.5 min); 100%→2% B (14.5-14.51 min); 2% B (14.51-16.50 min).

A Jetstream ESI source, operated in negative ionization mode, used the following conditions: drying gas temperature 325 °C and a flow of 10 L/min, sheath gas temperature 350 °C and a flow of 11 L/min, nebulizer pressure 45 psi, capillary voltage 3500 V, nozzle voltage 1000 V, fragmentary voltage 100 V and skimmer 45 V. Nitrogen was used as the instrument gas. For data acquisition, a 2 GHz extended dynamic range mode was used from *m/z* 20 to *m/z* 1600. Data was collected in the centroid mode at an acquisition rate of 1.67 spectra/s with an abundance threshold of 150. The TOF was calibrated on a daily basis and subsequently operated at high accuracy (<2 ppm). Continuous mass axis calibration was performed by monitoring two reference ions from an infusion solution throughout the runs. The reference ions were *m/z* 112.985587 and *m/z* 966.000725. Identification of the metabolites found in the treatments were based on accurate mass and isotope information; i.e., ratios, abundances, and spacing. The software for the identification of metabolites used was MassHunter Metabolite ID B.04.00 (Agilent Technologies, Santa Clara, CA, USA).

6.4.3. Inhibition of placental CYP1A1 and CYP19A1 oxidation, microsomal CYP oxidation, recombinant CYP1A2, CYP2A6, CYP3A4, CYP1B1, CYP2C19 and CYP2D6 oxidation by verbascoside.

The IC_{50} determinations of verbascoside, for human recombinant CYPs, human liver microsomes and placental microsomes were determined by the methods described by Juvonen et al. (2018) [22], Huuskonen et al. (2016) [25] and Crespi et al. (1997) [26]. The incubations were carried out in a total volume of 100 µL of 100 mM Tris-HCl buffer (pH 7.4) in all black, flat bottom Costar 96-well plates (Corning Incorporated, Corning, NY). The reaction mixtures contained 20 % NADPH-regenerating system in Tris-HCl buffer (pH7.4), 10 µM coumarin or its derivative substrate ((TFD024 (3-(3-methoxyphenyl)-6-methoxycoumarin), OCA349 (3-(4-trifluoromethylphenyl)-6-methoxycoumarin), TFD008_1 (3-(4-phenylacetate)-6-chlorocoumarin,coumarin, TFD032 (3-(3-methoxyphenyl)coumarin), TFD023 (3-(4-phenyl)-7-methoxycoumarin) or OCA369 (3-(3-benzyloxo)phenyl-7-methoxycoumarin), 0.010 g/L microsomal protein or 5 nM recombinant CYP and inhibiting agent of 0.4 µM, 2 µM, 10 µM and 50 µM verbascoside. A full reaction (100 %) did not contain any inhibiting agent and blank reactions did not contain any substrate or enzyme. Substances were added from a stock solution

containing ethanol so that the final concentration in the incubation mixture was at 1%. The samples were then pre-incubated at 37ºC for 10 minutes. The reactions were initiated by the addition of NADPH. 0 - 10 µM 7-Hydroxycoumarin was used as the standard. The fluorescent signal was measured with a Victor^2 1420 multilabel plate counter (PerkinElmer Lifesciences Wallac, Turku, Finland) with excitation and emission wavelengths of 405 nm and 460 nm, respectively. The fluorescence was monitored every 2 min for 40 minutes, from where the concentration was calculated at the various time points and from these, the oxidation rates (µM/min) and the relative remaining activity of the sample at different concentrations were calculated. The data were fit to sigmoidal dose-response curves with non-linear regression and IC_{50}-values (the concentration at which the sample reduced the metabolism of the CYP substrate by 50%) were determined. The calculation was based on the equation vi/v0= 1/ (1 + i/IC_{50}), in which (vi) is the rate at the specific concentration of the inhibiting agent, (v0) is the rate without the inhibitor, (IC_{50}) is the sample concentration with 50 % inhibition and (i) is the inhibitor concentration. To determine whether a sample inhibited CYP 19A1 (aromatase), an aromatase inhibition assay was performed according to the method of Pasanen (1985) [27]. The relative remaining enzyme activity was calculated by measuring the tritiated water (^3H_2O) formed from the aromatization of ^{3}H- Androst- 4-ene, 3, 17-dione. Each of the incubation tubes contained 50 µM ^{3}H- Androst- 4-ene, 3, 17-dione; androstenedione in an acetone-tween solution, distilled water, 100 mM Tris-HCl buffer pH 7.4, placental microsomes, either 4 µM, 20 µM, 100 µM or 500 µM verbascoside, 1 % DMSO and 1 or 10 µM Finrozole as a positive control. Twenty percent NADPH regenerative system was added to initiate the reaction. Reactions were terminated by the addition of 33% trichloroacetic acid. The supernatant was investigated for tritiated water formed in a scintillation mixture (Optiphase Hisafe 2, Perkin Elmer) using a Wallac microbeta 1450 TriLux liquid scintillation and luminescence counter (PerkinElmer). The degree of inhibition was categorized as potent (IC_{50} < 1 µM), marginal/moderate (1 µM <IC_{50}> 10 µM), weak (IC_{50} > 10 µM), or no inhibition (IC_{50} >100 µM) [28].

6.4.4. DPPH and nitric oxide inhibitory activity of verbascoside

The antioxidant properties of verbascoside were determined by the method described by Berrington and Lall (2012) [29]. A stock solution of verbascoside and ascorbic acid (positive control) were prepared at 10 mg/mL and 2 mg/mL in 100% ethanol respectively. Twenty microliters of verbascoside stock solution was added to the top well of a 96-well plate and

serially diluted to concentrations that ranged from 3.90- 500 µg/mL. Ascorbic acid was serially diluted to a concentration range of 0.78- 100 µg/mL. Ethanol (10%) was used as a blank. Ninety microliters of a 0.04 M DPPH (2, 2-diphenyl-1-picrylhydrazyl) in ethanol was added to each well. The plates were incubated for 30 min covered in a layer of foil. Colour controls (negative controls) were prepared in the exact same manner as above but distilled water was used to add to each well instead of DPPH. The absorbencies were measured at a wavelength of 515 nm using a BIO-TEK Power- Wave XS multiplate reader. From the absorbencies an IC_{50}-value was determined. All concentrations of verbascoside and ascorbic acid were tested in triplicate.

The nitric oxide scavenging properties of verbascoside were determined according to the method of Twilley et al., (2017) [30]. Verbascoside and the positive control (ascorbic acid) were all prepared in ethanol to stock concentrations of 10 mg/mL. Verbascoside and ascorbic acid (20 µL) to the top wells of a 96 well plate. Serial dilutions were made and the concentration range for verbascoside and ascorbic acid ranged between 12.50 - 1601.05 µM and 44.35 - 5677.95 µM, respectively. Ethanol (10%) was used as a blank. Nitroprusside (50 µL) at a concentration of 10 mM was added to all the wells. The plates were incubated for 90 min at room temperature. After incubation, Griess reagent (100 µL) was added to all the wells except for the colour controls (negative controls) where instead distilled water was added. The absorbencies were read at a wavelength of 546 nm using a BIO-TEK Power- Wave XS multiplate reader. From the absorbencies an IC_{50}-value was determined. All concentrations of verbascoside and ascorbic acid were tested in triplicate.

6.4.5. Cellular antiproliferative activity of verbascoside

To determine the cellular viability of verbascoside against both first and secondary line cell lines the method of Berrington and Lall, (2012) [29], was performed. Briefly, the peripheral blood mononuclear cells were isolated from blood, freshly donated on the same day at the Student Clinic at the University of Pretoria. Mononuclear cells were isolated by centrifugation over Ficoll-Hypaque, a density gradient solution, with a density gradient of 1.07 g/mL. A layer of fresh heparinized venous blood was layered on the Ficoll- Hypaque in a ratio of 1:1 with supplemented RPMI 1640 media and then subjected to centrifugation at 3000 rpm for 30 min. Through centrifugation, the whole blood sample was separated into its different layers (Figure 1). As observed from the top, the different layers consist of the plasma with its other constituents followed by a white buffy-coated layer of mononuclear cells, the PBMCs. After the PBMC layer, a layer of Ficoll is found and then finally a layer of erythrocytes (red blood

cells) and granulocytes. The white, buffy-coated layer of PBMCs was aspirated out gently and transferred aseptically to a sterile Falcon tube (50 mL).

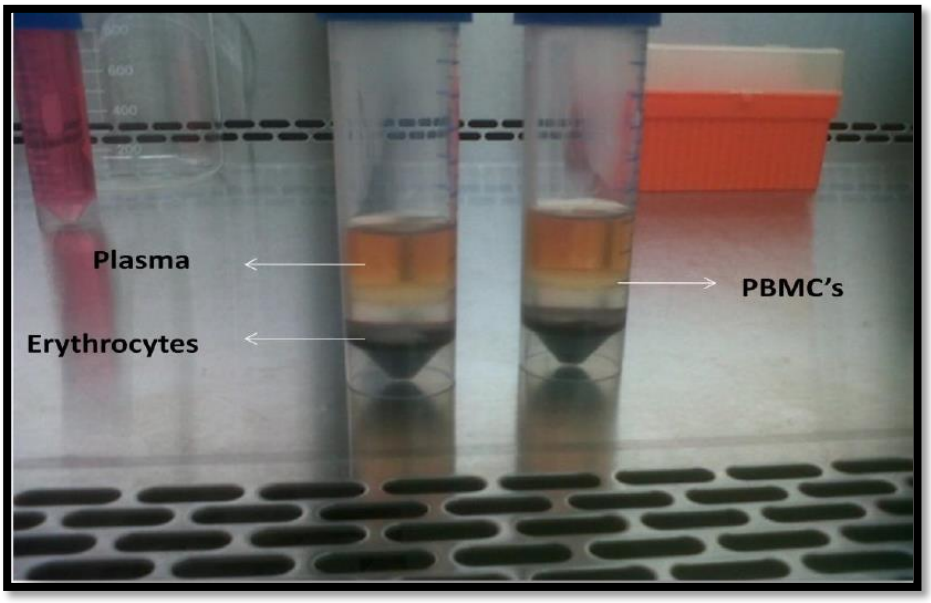

Figure 6.1 Blood layered into its many components (plasma, PBMCs and erythrocytes) through the density gradient centrifugation method.

The resultant suspension of cells was then washed with supplement RPMI 1640 media (30 mL) with an antibiotic (Gentamycin 1%) and subjected to centrifugation at 2200 rpm for 10 min. The supernatant was aspirated from the tube and the resultant pellet of cells washed with ACK (Ammonium, Chloride and Potassium). ACK is a lysis buffer that causes any contaminant red blood cells in the solution to be lysed. After 5 min with the added ACK, supplemented RPMI 1640 media with Gentamycin was added and subjected to centrifugation at 1200 rpm for 10 min. The supernatant was poured off and the resultant pellets of cells were resuspended in approximately 5 mL of supplemented RPMI 1640 media with Gentamycin. The cells were counted and seeded in 96 well plates with a cell density of 10 000 cells/ mL. The following formula was used to adjust the cell concentrations (Equation 1):

$$\text{Concentration of the cells} = \frac{\text{Cells counted}}{\text{number of blocks counted}} \text{ x } 10\,000 \qquad (1)$$

The HepG2 cell line was seeded with a volume of 100 µL with a concentration of 1 x 10^5 cells/well. The plates were incubated for 24 hours at 37 ºC and 5% CO_2 to allow for cellular attachment to the bottom of the wells. Verbascoside was prepared to a stock solution of 2000 µg/mL. Serial dilutions were made with final test concentrations ranging from 400- to 1.53

μg/mL. Plates were incubated for 72 hours (HepG2) and 48 hours (PBMC) at 37 °C and 5% CO_2. DMSO as a solvent control (DMSO 2%) and Actinomycin D as a positive control, with a final test concentration of 0.5- 0.002 μg/mL were added. After incubation, PrestoBlue (20 μL) was added to each well and plates further incubated for 4 hours. The absorbance was read at 490 nm with a reference wavelength of 690 nm using a BIO-TEK Power-Wave XS multi-well plate reader. The assay was performed in triplicate and the mean IC_{50} values calculated.

6.4.6. Statistical analysis

Statistical analysis was performed with GraphPad Prism (Version 7) using one-way analysis of variance (ANOVA). The results are expressed as the mean ± standard deviation, n= 3 or more.

6.5. Conclusions

In the current study verbascoside was found to undergo extensive conjugation metabolism and has no significant inhibitory potential against the most important CYPs found to metabolize many known drugs and compounds. Verbascoside was also found to have noteworthy antioxidant, anti-inflammatory and low levels of cellular toxicity. All these activities indicate the potential of verbascoside as an adjuvant compound that can be used during first line drug treatment of TB as it holds no potential of herb-drug interactions and possesses important additional biological activity.

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7

The antimycobacterial, cytotoxicity and synthesis of gold nanoparticles using *Lippia scaberrima* Sond.

This chapter introduces the synthesis of gold nanoparticles from Lippia scaberrima Sond. together with gum arabic as a stabilizer.

This chapterwill be published as a publication for the following journal:

1) *Natural Product Communication*
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7. THE ANTIMYCOBACTERIAL, CYTOTOXICITY AND SYNTHESIS OF GOLD NANOPARTICLES USING *LIPPIA SCABERRIMA* SOND.

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Abstract

Millions of people die every year due to TB, an infectious disease caused by the pathogen, *Mycobacterium tuberculosis* (*M. tuberculosis*). Many botanical extracts and combinations have been used traditionally to treat infectious diseases. *Lippia scaberrima* Sond. (*L. scaberimma*) is known to be drunk as a health tea in the southern regions of Africa. *L. scaberrima* was studied in its ability to inhibit both *M. tuberculosis* and *Mycobacterium smegmatis* (*M. smegmatis*), a replacement model for *M. tuberculosis*. *L. scaberrima* was found to have a minimum inhibitory concentration (MIC) of 125 µg/mL against *M. tuberculosis*. The antiproliferative activity values obtained on HepG2 cells were found to be 109.20 ± 8.05 µg/mL. Furthermore, the study investigated the synthesis and characterization of gold nanoparticles from *L. scaberrima* (*LSAUNP*). The absorption of the gold nanoparticles that were formed, showed an intense peak at a wavelength of 540 nm. The Transmission Electron Microscopy (TEM) images confirmed that most of the formed gold nanoparticles were spherical in shape. The X-Ray diffraction (XRD) pattern confirmed the crystalline nature of the nanoparticles that formed. The possible functional groups were identified by Fourier Transform Infrared Spectroscopy (FTIR) and were, among others, from the alkene functional group. Finally, the cytotoxic potential of *LSAUNP* was reduced by 2-fold when compared with *L. scaberrima*. *Lippia scaberrima* and *LSAUNP* can be further studied as potential natural products that aid in the treatment of TB.

Keywords

Bioactivity, botanicals, characterization, gold nanoparticles, secondary metabolites, tuberculosis

Despite substantial strides made in the treatment of both resistant and non-resistant strains of *M. tuberculosis*, many individuals still fall prey to this bacterium, with approximately 10.4 million individuals newly infected in 2016[1]. Treatment failures and non-compliance has added further strain on treatment regimens with individuals with drug resistant *M. tuberculosis* strain accounting for 490 000 of the newly infected cases. Clinicians are lacking effective treatment that is short enough and void of any side-effects to curb non-compliance amongst patients[2]. Nanoparticle-based therapeutics have been approved for various treatments and ailments such as cancer and infectious diseases[3-4]. Many applications using silver nanoparticles have been used for drug development and delivery by using the body's own natural mechanisms to fight against infections and disease. The chemical synthesis of nanoparticles may take a short duration but can be toxic and have hazardous by-products. Thus, there is an ever-increasing demand for the synthesis of green nanotechnology which may present less hazardous by-products and lower toxicity[5]. There are several biological approaches of synthesizing nanoparticles using biological methods such as bacteria, fungi and plants[6-7]. Nanoparticles have distinct properties such as larger surface to volume ratios and can introduce new or improved properties based on size, distribution and morphology which ultimately leads to improved therapeutic usage for infections such as TB and cancer[8]. Many different types of nanoparticles exist; copper, zinc and titanium as well as magnesium, silver and gold[9-11]. Gold nanoparticles are reported to be used for the treatment of rheumatoid diseases, nephrotoxicity as well as cancer[12]. Many plant extracts have been utilized as stabilizers and capping agents to produce nanoparticles[13-15]. The benefits of using gold nanoparticles is that they have distinctive attributes such as adjustable size and shapes. Gold nanoparticles have been used in prostate cancer where they will seek out cancer cells and through imaging, the nanoparticles are hit with a laser which causes these nanoparticles to vibrate at a high frequency which in turn kills the cancer cells. *Mycobacterium tuberculosis* is known to have very lipid rich cell walls which makes penetration by drugs and their effectiveness a difficult process. Drug delivery systems can be effective in ensuring that the treatment necessary to kill the bacteria cells directly are delivered to within the bacteria itself within the cell walls. In this instance research is needed into how gold

nanoparticles can be modified to reach within the bacterial cell wall and deliver the treatment necessary to kill the bacteria.

Various plant extracts have shown good antibacterial properties but, in many cases, found to be very toxic and therefore, deemed unsuitable for further use in studies. *Lippia scaberrima* an aromatic plant belonging to the Verbenaceae family is traditionally known for its ability to treat respiratory ailments and for its rich presence of flavonoids such as verbascoside[16]. Many nanoparticles formed from the use of medicinal plant extracts have shown their ability to reduce the toxicity profile of the plant extract in use but also seem to increase their therapeutic potential, be it antibacterial or even anthelmintic as illustrated by Kar et al.,[12]. For the current study, *L. scaberrima* was used for the biosynthesis of gold nanoparticles and the characterization thereof determined through various methods. This study also showcased the ability of the formation of nanoparticles in changing the biological activity attributed to a plant extract.

Both, *L. scaberrima* and *LSAUNP* were tested for antimycobacterial activity against both, *M. smegmatis* and *M. tuberculosis*. The lowest MIC value was observed by *L. scaberrima* which had a MIC value of 125 μg/mL against *M. tuberculosis* as compared to the positive control, Isoniazid (INH), which had a MIC- value of 0.32 μg/mL. Notable antimycobacterial results for natural products are reported to be MIC values that are lower than 1000 μg/mL[16]. Shikanga et al.,[16] reported on the antibacterial properties of the methanol extract of *L. scaberrima*. *Lippia scaberrima* was tested on *Staphylococcus aureus* and *Escherichia faecalis*, both Gram-positive bacteria, similar to *M. smegmatis* and *M. tuberculosis*. Much of the activity found for species of *Lippia* are attributed to the presence of verbascoside and isoverbascoside, which are found in varying levels in all five of the indigenous *Lippia* species. It is shown in literature that due to their cell wall structure Gram positive bacteria are more susceptible to nanoparticles than Gram negative bacteria. However, no noteworthy activity was found for *LSAUNPs* tested against *M. smegmatis*. Gold nanoparticles were used as a control when Mmola et al.,[36] tested for antimicrobial activity against several pathogens as it was expected that gold nanoparticles in general do not show antimicrobial activity. Although several research groups have shown that gold nanoparticles do possess some antimicrobial activity as shown by Shamaila et al.,[37] it might be that the *Mycobacterium* species tested (Gram positive bacteria) were not susceptible to the gold

nanoparticles and due to their cell wall structure, a higher concentration is needed to exert significant antimycobacterial activity.

Both *L. scaberrima* and *LSAUNP* were tested against liver hepatocellular carcinoma (HepG2) cells and found to have IC_{50} values of 109.20±8.05 μg/mL (Mean±SD) and 268.50±1.34 μg/mL after 72 hours of incubation, respectively, according to Table 3. Any extracts of plants and combinations with an IC_{50} value of more than 100 μg/mL after 72 hours of incubation are not considered as cytotoxic to the specific cell line tested. The *LSAUNPs* exerted the highest IC_{50} value (268.50±1.34 μg/mL). This gives an indication of the advantage that synthesis of gold nanoparticles has above an extract alone. The IC_{50} value has increased 2-fold when compared to *L. scaberrima*. This may be due to the different functional groups present on the surface of the nanoparticles as compared to *L. scaberrima*. The toxicity of nanoparticles especially that of gold nanoparticles have been shown to be linked to size, where gold nanoparticles tested of between 8 and 37 nm size has had devastating results on the viability of mice studied[38]. All the other sizes up to 100 nm had no lethal effects. The size of nanoparticles, therefore, are shown to have a dramatic effect on its toxicity potential and should be further investigated.

With the reduction of Au^{3+} to Au^0 in the $HAuCl_2.3H_2O$ solution, the colour of the reaction mixture turned from a yellowish pale green to a dark purple brown colour, which agrees with previous studies conducted, and was considered characteristic with the formation of gold nanoparticles.

Table 7. 1 The hepatotoxicity and antimycobacterial results for *L. scaberrima* and *LSAUNP*

Sample	PRU number	HepG2 IC_{50}[a] (μg/mL)± SD 72 hours	*M. smegmatis*[b]	*M. tuberculosis*[c]
			MIC[d] (μg/mL)	
L. scaberrima	119010	109.20 ± 8.05	>1000	125
LSAUNP[f]		268.50 ±1.34	1000	1000
Actinomycin-D[g]	-	8.56 ± 8.24	-	-
Ciprofloxacin[h]	-	-	0.63	-
INH[i]	-	-	-	0.31

[a]50% inhibitory concentrations, [b]*M. smegmatis*, [c]*M. tuberculosis*, [d]Minimum inhibitory concentration value, [e]Not applicable or not tested, [f]Gold nanoparticles formed with *L. scaberrima* extract and gum arabic [g]Positive control for HepG2 cells, [h]Positive control for *M. smegmatis* assay, [i]Positive control for *M. tuberculosis* assay. (Mean±SD, n=3)

The appearance of the dark purple colour is due to the excitation of the surface plasmon vibrations in the nanoparticles formed[45] as seen in Figure 7.1.

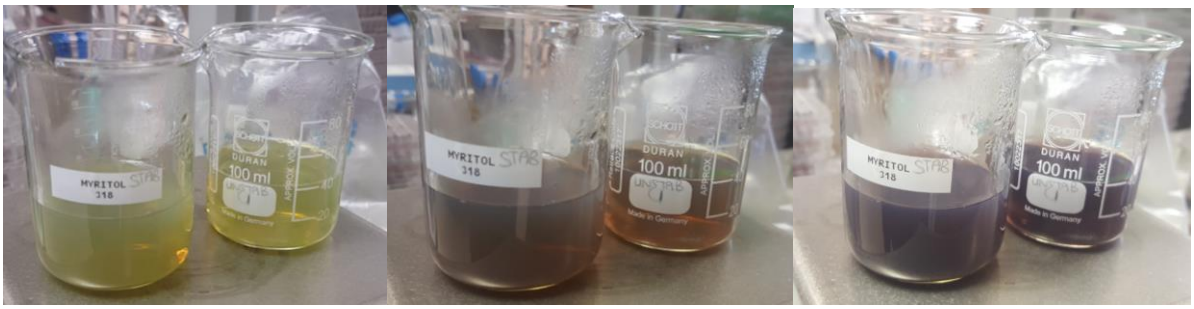

Figure 7.1 Visual inspection of the colour change as the formation of gold nanoparticles with LS and gum arabic progresses. This is the reduction of Au^{3+} to Au^{0} in the $HAuCl_2.3H_2O$ solution turning the solution from yellowish pale green to a dark purple brown colour. The dark purple brown colour is characteristic with the formation of gold nanoparticles within the solution.

To investigate the formation of gold nanoparticles, the absorption spectrum of the mixture was investigated[46]. A strong absorbance band can be found in the visible region of between 500-600 nm. The λ_{max} was found at approximately 540- 555 nm as shown in Figure 7.2-7.7 for Day 0. The results are similar with previous findings where the synthesis of gold nanoparticles using extracts of *Crocus sativus* showed an absorbance value of 549 nm[8, 47]. A similar study by Elia et al.,[48] also used four common household plants and investigated their potential in the synthesis of gold nanoparticles. The stability of the formation of the gold nanoparticles within several different media (NaCl, BSA and DMEM), over a certain time period (0, 1, 4, 5, 7) and pH (pH7 and pH9) were also investigated through their UV/Vis absorption spectra. Figure 3-8 shows the different absorption spectra at various concentrations and two different pH (7 and 9) readings. For all the samples ratios tested (2:3, 1:4 and 1:9), the different pH's and the different media tested, their peak absorbance for the formation of *AUNP* was found to be at 540 nm. Although with the lowered concentration of *AUNPs*, the less significant the peak became but as expected with lowered concentrations of the measured metal particles. Figure 3-8 illustrates the stability of *AUNPs* within

several different media and pH readings. The stability is most probably enhanced due to the use of gum arabic together with *L. scaberrima.*

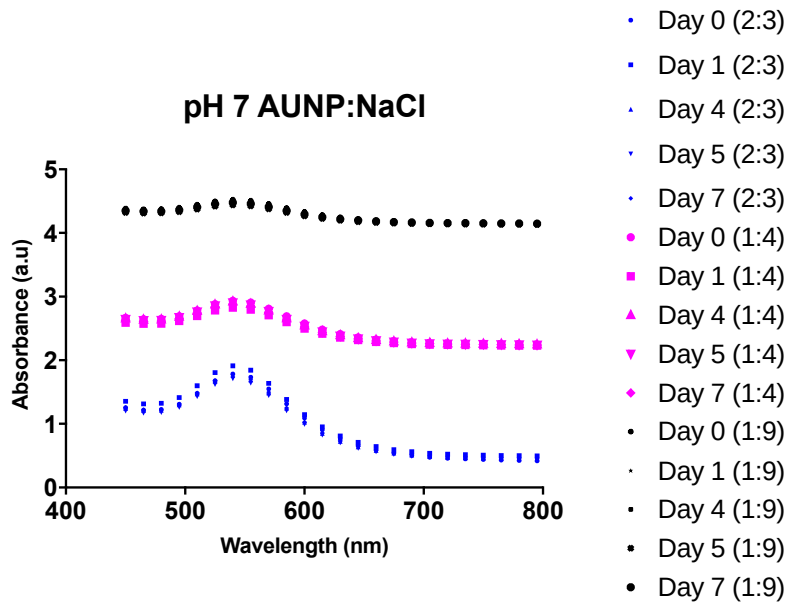

Figure 7.2 The stability of *LSAUNP* in NaCl at pH 7 at various concentrations (2:3, 1:4 and 1:9).

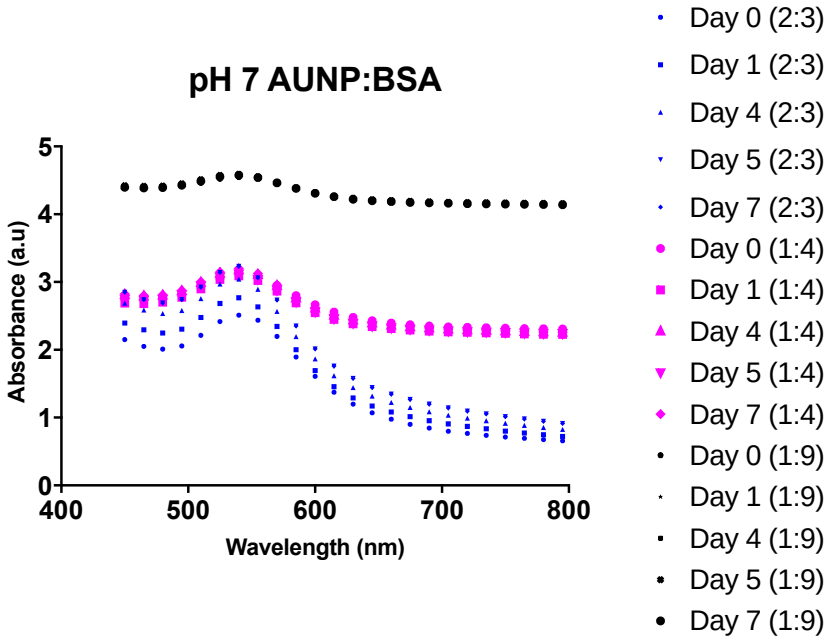

Figure 7.3 The stability of LSAUNP in BSA at pH 7 at various concentrations (2:3, 1:4 and 1:9).

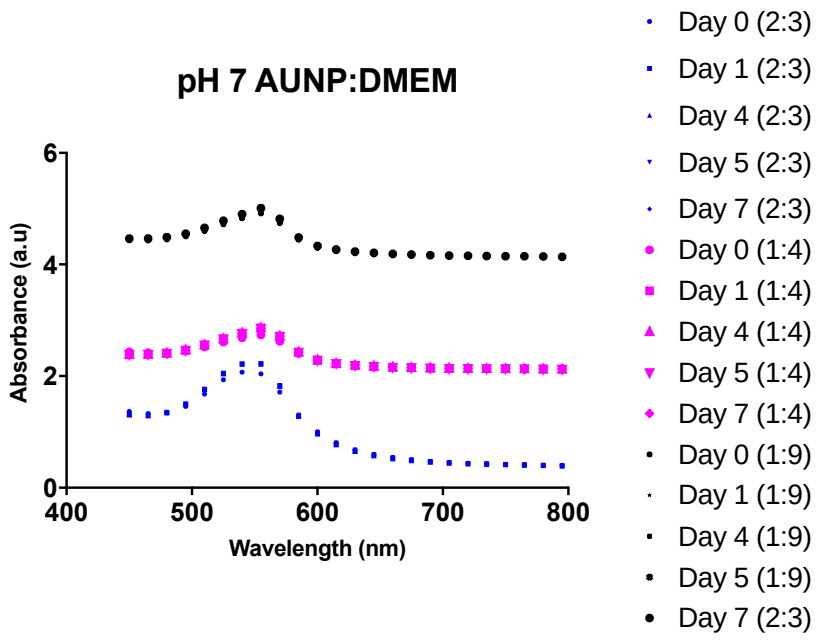

Figure 7.4 The stability of LSAUNP in DMEM at pH 7 at various concentrations (2:3, 1:4 and 1:9).

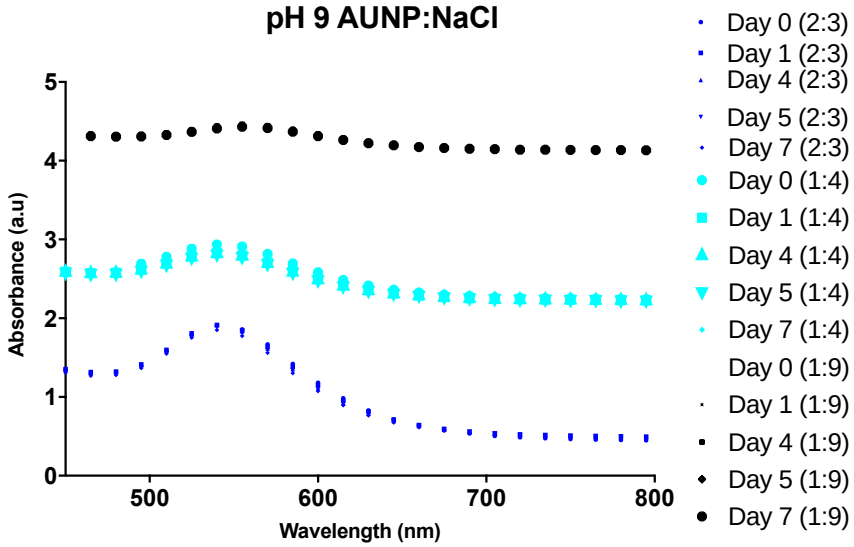

Figure 7.5 The stability of LSAUNP in NaCl at pH 9 at various concentrations (2:3, 1:4 and 1:9).

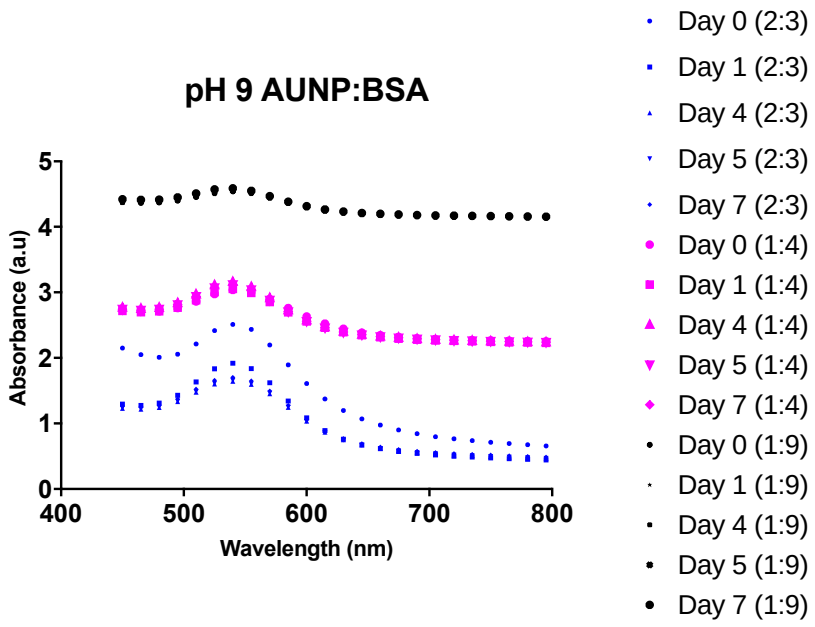

Figure 7.6 The stability of LSAUNP in BSA at pH 9 at various concentrations (2:3, 1:4 and 1:9).

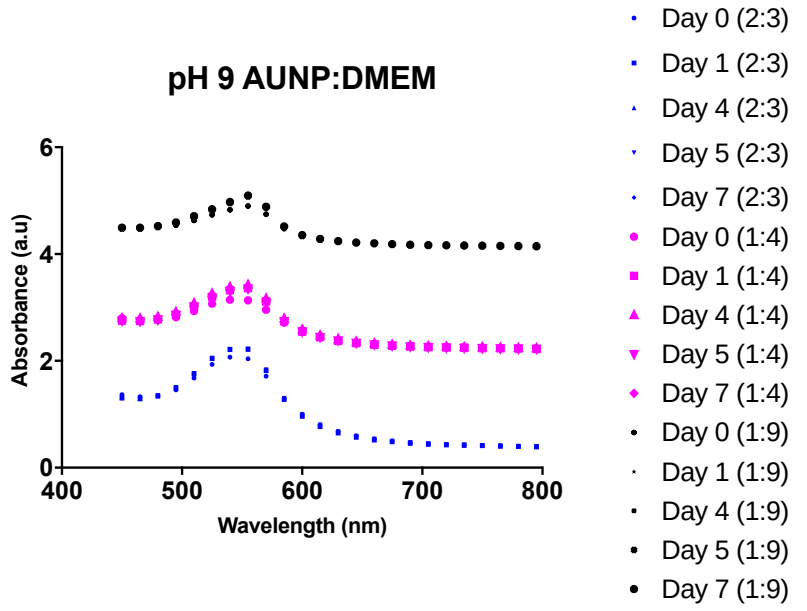

Figure 7.7 The stability of LSAUNP in DMEM at pH 9 at various concentrations (2:3, 1:4 and 1:9).

Gold nanoparticles, mainly spherical in shape were obtained. *AUNP*s were found to be present in several different sizes and predominantly in spherical shape but were also triangular in shape as seen in Figure 7.8 below. This variety of sizes and shapes have been described before within literature and is a common occurrence with gold nanoparticles[48].

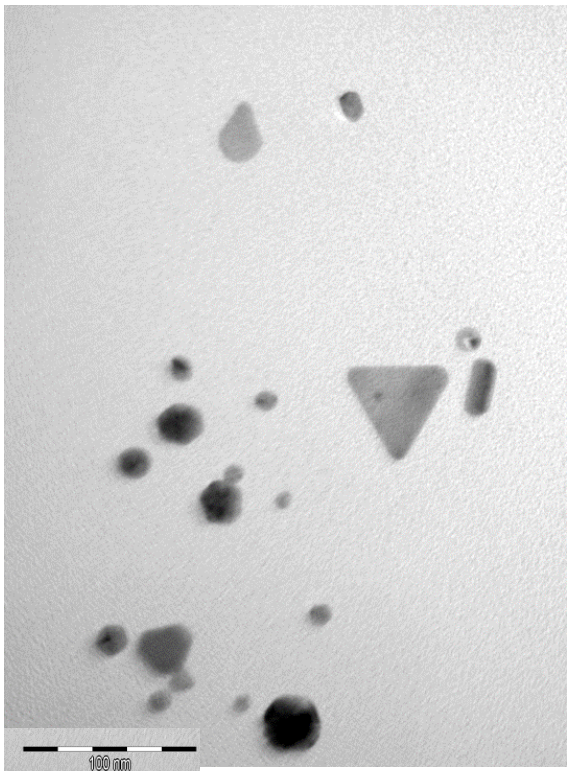

Figure 7.8 Several different shapes and sizes of gold nanoparticles formed in solution with *L. scaberrima* extract and gum arabic investigated using JEOL JEM-2100F Field Emmision Electron microscope.

FTIR measurements were carried out to investigate the possible molecules that are responsible for the capping and stabilization of the gold nanoparticles synthesized when the ethanolic extract of *L. scaberrima* was added as shown in Figure 7.9. The peaks found in the FTIR spectra represent the functional groups that might be present on the surface of *LSAUNP* when synthesized. The FTIR of the ethanolic extract of *L. scaberrima* (blue line) and *LSAUNP* (green line) showed broad peaks at 3288 cm^{-1} and 3293 cm^{-1} characteristics of O-H bonds contributed to by the carboxylic acids. The peaks observed at 2913 cm^{-1} and 2918 cm^{-1} were attributed to by the C-H bonds of the alkenes. The very weak peaks depicted at 2344- and 2343 cm^{-1} are due to C=O groups, whereas the peaks depicted at 2295 cm^{-1} and 2298 cm^{-1} are due to the C=C bands (alkenes). The peaks that occur in

the range of 1750- 1650 cm^{-1} and 1715- and 1650 cm^{-1} are found due to the presence of carbonyls (C=O) while the peaks depicted in the ranges of 1365- 1254 cm^{-1} and 1361-1250 cm^{-1} are due to nitrile compounds (N-O). All the peaks that were found in the spectra of the crude extract corresponded to the peaks found in the gold nanoparticles synthesized. These peaks are all due to the functional groups already present in the ethanolic extract of *L. scaberrima*.

The FTIR analysis of *LSAUNP* featured all the peaks found in the ethanolic extract of *L. scaberrima* confirming the presence of these secondary compounds on the surface of the gold nanoparticles synthesized.

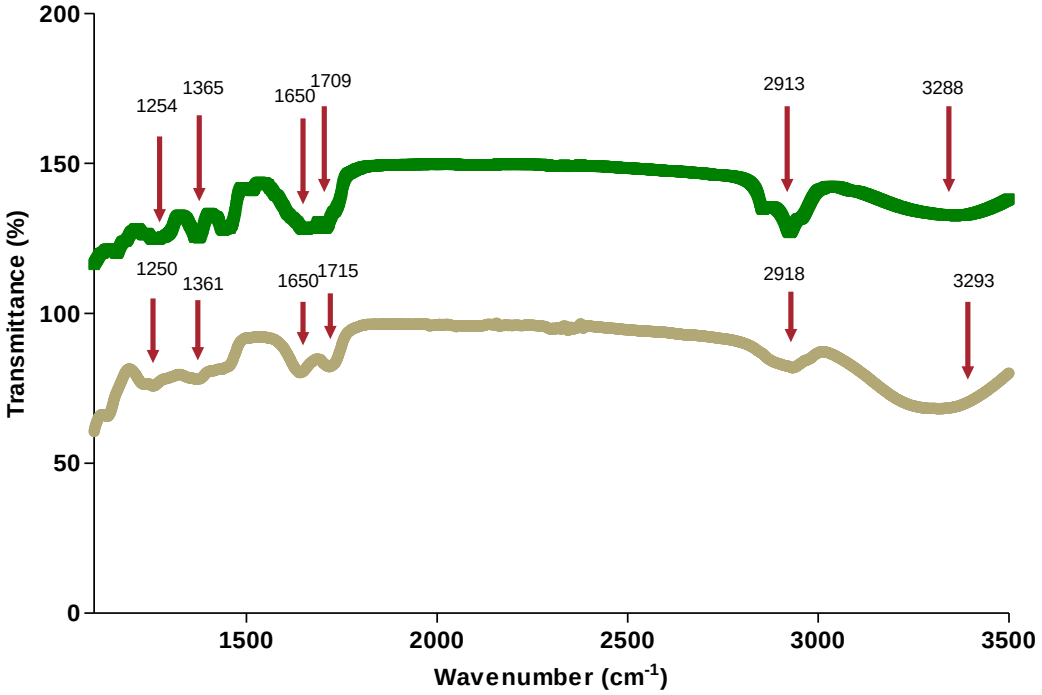

Figure 7.9 FTIR measurement of LSAUNP and *L. scaberrima*, showing several functional groups present at their specific wavenumbers (cm^{-1}) (indicated by the arrows) measured using the PerkinElmer Spectrum FTIR Spectrometer in the wave range extending from 4000 to 400 cm^{-1}.

These secondary compounds act as capping agents on the surface of the gold nanoparticles which assist in their stability but also to prevent agglomeration from occurring. Limonene forms part of the alkene functional group (C=C) and due to its highlighted presence within the ethanolic extract of *L. scaberrima*, it is therefore, not uncommon to find it on the surface of the newly formed,

LSAUNP. The *Lippia* genus is also known for the presence of a wide array of flavonoids and phenolics[16]. Many different compounds including flavonoids and diterpenoids as well as polysaccharides are known for the reduction of gold nanoparticles. These compounds are known to be present within *L. scaberrima*[49]. FTIR analysis confirmed that the reduction of the Au^{3+} to Au^{0} are due to the capping material found within the plant extract. Proteins that are found within the plant extract may also bind to the gold nanoparticles through either free amino or carboxyl groups found within the proteins. Prasad et al[50] also indicated that many of the carboxyl (-C=O), hydroxyl (-O-H) and amine groups (-NH) found within plant extracts may be involved in the creation of the gold nanoparticles.

The gold nanoparticles were subject to XRD analysis to confirm the formation of the gold nanoparticles as well as the confirmation of the crystalline structure of the gold present. Figure 7.10 shows the XRD pattern that was obtained from *LSAUNP* synthesized using *L. scaberrima* extract and gum arabic. The intense peak and planes of the gold nanoparticles found at 38.2° (111), 44.3° (200), 64.5° (220) and 77.7° (311) signifies the presence of gold nanoparticles that are crystalline in nature. The planes are also representative of the face centered cubic (fcc) planes of gold (Au). The fcc of *LSAUNP* synthesized, matched those of the Joint Committee on Powder Diffraction Standards (JCPDS no. 00-004-0784), which confirms the presence of crystalline gold. Sett et al.,[51] also reported that the peaks of gold nanoparticles formed will be in the region as seen in the current study and that this is a typical XRD pattern for the formation of gold nanoparticles[52].The most distinctive peak was at 38.2° and the (111) plane and was used to determine the average diameter for the gold nanoparticles formed as determined by the Debye-Scherrer formula (Equation 3). The average particle diameter for the gold nanoparticles as determined by the Debye-Scherrer formula, using the half widths of the most intense XRD peak (111) was 17.57 nm.

Different parameters may have an effect in the amount of gold nanoparticles formed and have been investigated by several research teams[52-53]. By investigating different factors when synthesizing gold nanoparticles, a variety of nanoparticles can be synthesized by using plant extracts[53].

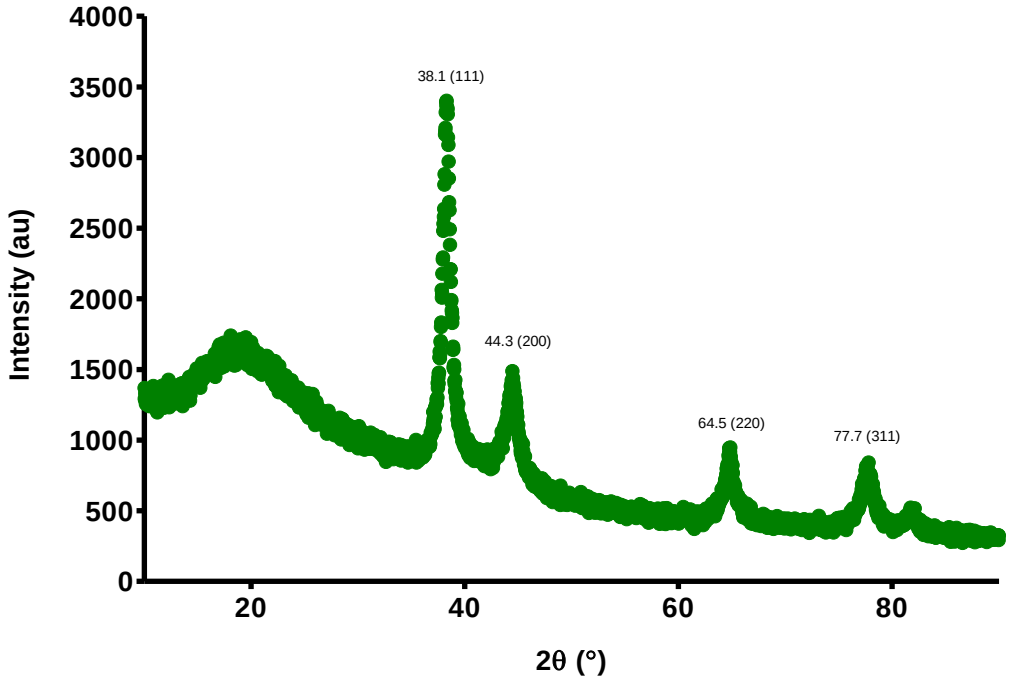

Figure 7.10 The XRD pattern (crystalline structure) of LSAUNP formed with *L. scaberrima* extract and gum Arabic was calculated using the PANalytical X'PERT PRO with an instrumentation setup of 30kV, 40 mA with Cu-K*α* (1.540598) from 5˚ to 90˚ (2θ).

The TGA spectra showed significant weight loss (%) between 46- and 900 ˚C (Figure 7.11), indicating that at higher temperatures, the compounds from *L. scaberrima* that are surrounding the gold nanoparticles, acting as the capping and stabilizing components for the nanoparticles, were completely degraded. Hence, this serves as confirmation that *LSAUNP* synthesized with *L. scaberrima* and gum arabic were capped with organic compounds. This reduction in weight (%) decreased linearly with the increase in temperature. The reduction of the gold by using *L. scaberrima* and gum arabic, forming *LSAUNP* is a reliable and economic method of obtaining gold nanoparticles with enhanced, lowered cytotoxicity potential. Many methods have been proposed for the synthesis of gold nanoparticles and includes the addition of plant extracts that already act as natural stabilizers. The addition of gum arabic to the gold and plant extract solution adds to the stabilizing effect as seen in the current study.

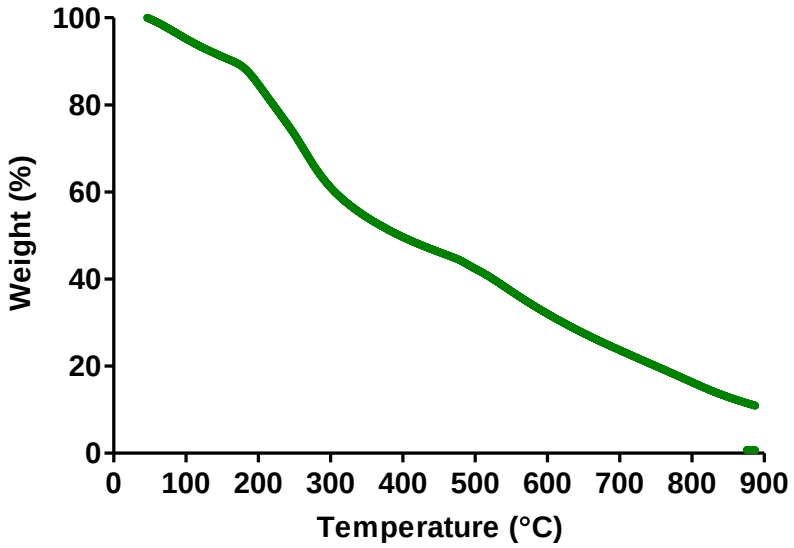

Figure 7.11 Thermogravimetric analysis of LSAUNP formed with *LS* extract and gum arabic recorded between 30 ˚C and 900 ˚C by measuring with a TA Instrument TGA Q500. The flow rate for oxygen and nitrogen was 40 mL/min and 60 mL/min respectively. The heating rate was 30 ˚C.

Stabilizers form a protective layer that prevents the agglomeration of the nanoparticles into larger particles[48]. In the present study most of the compounds found within *L. scaberrima* are known for their antioxidative properties and are thought to be the main reducing agents that led to the swift formation of the gold nanoparticles. Many of these antioxidants are highly water soluble and may act as a better reducing agent for the synthesis of gold nanoparticles[48]. Further investigation is however, required into the size distribution of the nanoparticles as well as the effect of gum arabic on the activity of *LSAUNP*. Ramamurthy et al.,[54] has indicated the possible interference of a stabilizer on the abilities of the synthesized nanoparticles and that they may also have the potential to exert possible toxicity. The use of plant extracts and combinations have been used over several centuries for the treatment of infectious diseases. These plant extracts and combinations consist of many secondary metabolites which are responsible for the activity found. South Africa is home to many different plant species that have not yet been investigated for their unique potential. Many novel compounds may still be found for the development of drugs from natural resources. The successful synthesis of gold nanoparticles is possible with plant extracts, which may have the ability to reduce metal ions at a faster rate as any fungi or bacteria.

Experimental

Bacterial strains, cell lines, chemicals and reagents.

The HepG2 cell line (HB-8065) was obtained from the American Type Tissue Culture Collection (ATCC). *M. tuberculosis* (H37Rv), in MGIT media, and *M. smegmatis* (MC^2155) was kindly donated by the South African Medical Research Council, Pretoria and Department of Medical Microbiology, University of Pretoria, respectively. Dimethyl-sulfoxide (DMSO), Middlebrook 7H9 broth, Middlebrook 7H11 agar base and Middlebrook OADC (Oleic Albumin Dextrose Catalase) growth supplement, Gold (III) chloride trihydrate ($HAuCl_4.3H_2O$), *N*-Acetyl-L-Cysteine, Bovine Serum Albumin (BSA) and Sodium Chloride (NaCl) were all obtained from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise stated. Cell culture materials and reagents such as Fetal Bovine Serum (FBS), DMEM media and antibiotics were supplied by Highveld Biological. The PANTA plus antibiotic mixture was obtained from BD Biosciences (San Jose, CA, USA). PrestoBlue was purchased from Thermo Fisher (Carlsbad, CA, USA). All reagents obtained were of analytical grade.

Plant material, collection, extraction and yield calculation.

Lippia scaberrima was collected in autumn from the Kopela community situated nearby Delareyville, North West Province, South Africa. The plant was identified, and a voucher specimen deposited at the H. G. W. J Schweickerdt herbarium, University of Pretoria (PRU). The collected aerial parts of *L. scaberrima* (stem, flowers, and leaves) were mechanically ground to a uniform size of 0.2 mm. The powdered plant material was extracted with absolute ethanol at a ratio of 1:10 (weight: volume) and macerated for 72 hours. The extract was filtered, and the plant material was subsequently extracted with fresh ethanol at a ratio of 1:5 for 48 hours, followed by filtration. The filtrate was combined and dried under reduced pressure using a rotary evaporator which resulted in a dark green extract. The extract was stored in airtight containers at 4 °C for the duration of the study. The yield was determined through drying 1 mL of the nanoparticles in solution in an eppie and then re-weighing of this same sample after drying. This was the approximate amount (mg) of nanoparticles (per mL) utilized during the current study.

Bacterial strain and growth conditions.

Both *M. tuberculosis* and *M. smegmatis* were cultured in Middlebrook 7H9 media supplemented with glycerol, OADC and PANTA (1%) and left to incubate for three weeks and 48 hours, respectively, at 37 °C. Both bacteria were adjusted to a 0.5 McFarland standard of (1.5×10^8 colony forming units/mL) [CFUs/mL]). The bacteria were further diluted 50-fold to obtain the final test inoculum (1.5×10^6 CFUs/mL).

Antimycobacterial assay.

The minimum inhibitory concentration (MIC) values of *L. scaberrima* and *LSAUNP* were determined according to the method of Lall et al.,[18]. All samples were dissolved in 20% DMSO, in sterile Middlebrook 7H9 media. For *M. tuberculosis*, 200 µl of sterile, distilled water was added to the outer wells of the plate for the compensation of the evaporation during the incubation period. Two-fold, serial dilutions of the samples were made to produce concentrations ranging from 1000- to 31. 25 µg/mL. The bacteria diluted in media (100 µL) was added to each well of the plate. The positive drug control for *M. tuberculosis*, Isoniazid (INH) was used at a concentration of 2.5- to 0.03 µg/mL. For *M. smegmatis*, the positive drug control used was, Ciprofloxacin at a concentration range of 10- to 0.078 µg/mL. Control and solvent control wells were also included, which contained no sample and only DMSO (5%), respectively. The plates were incubated for 7 days and 24 hours for *M. tuberculosis* and *M. smegmatis*, respectively. To determine MIC value, Prestoblue at a volume of 20 µL was added to each well and incubated for a further 24 hours. The MIC value was defined as the lowest concentration where there was no colour change from blue to pink.

Cellular antiproliferative activity.

The cellular antiproliferative activity of *L. scaberrima* and *LSAUNP* were determined by the method of Berrington and Lall[19]. The liver hepatocellular carcinoma (HepG2) cells were counted and seeded (100 µL) in 96 well plates with a cell density of 10 000 cells/mL and left to incubate (37 °C and 5% CO_2) overnight to allow for attachment. The following formula was used to adjust the cell concentrations (Equation 1)

Concentration of the cells = $\frac{cells\ counted}{number\ of\ blocks\ counted}\ x\ 10\ 000$ (1)

Lippia scaberrima and *LSAUNP* were prepared to stock solutions of 2000 μg/mL. Serial dilutions of the samples were made to a final concentration range of 400- to 1.53 μg/mL. All plates were incubated for 72 hours (37 °C and 5% CO_2). Actinomycin D was used a positive control with a final concentration range of 0.5- 0.002 μg/mL. DMSO (2%) was also included as a solvent control. To determine the inhibitory concentrations of the samples, PrestoBlue (20 μL) was added to each well in the plates and incubated for 4 hours. The absorbance values were read at 490 nm with a reference wavelength of 690 nm using a BIO-TEK PowerWave XS multiplate reader. The assay was performed in triplicate and the mean IC_{50}-values were calculated.

Biosynthesis of the gold nanoparticles using L. scaberrima and gum arabic.

A stock solution of 18 mg/mL of the ethanolic extract of the aerial parts of *L. scaberrima* was prepared. One mililitre of the stock solution was added to 17 mL of distilled water to obtain a final concentration of 1 mg/mL. The solution was boiled together for approximately 15 min at 45 °C to which 36 mg of gum arabic was added and stirred until completely dissolved. Once the gum arabic was dissolved, 300 μl of $HAuCl_2.3H_2O$ (0.1 M) solution was added. The formation of the stabilized gold nanoparticles was observed by the deep purple colour change of the solution after a few minutes. The gold nanoparticle solution was stirred continuously for an additional 15 min.

UV/Visible Absorbance Spectroscopy.

UV absorption was used to identify the formation and stability of *LSAUNP* through the observed Surface Plasmon Resonance (SPR). The absorption spectra were measured on a Bio-Tek Power-Wave XS multi-well plate reader. The data was analysed using KC junior with a spectral scan that ranged from 450 and 800 nm. *In vitro* stability of the gold nanoparticles was investigated in the presence of 10% NaCl, 100% DMEM and 0.5% bovine serum albumin (BSA) after 0, 1, 4, 5 and 7 days. A SPR band at 540 nm confirmed the stability of the gold nanoparticles within all of the above media.

Transmission Electron Microscopy (TEM).

TEM analysis was performed on *LSAUNP* to investigate the formation as well as the size and shape. The micrographs were obtained using a JEOL JEM-2100F Field Emission Electron microscope. The nanoparticle solution (5 μL) was added onto a carbon coated copper grid, left to dry overnight and examined using TEM.

Fourier Transform Infrared Spectroscopy (FTIR).

The gold nanoparticle solution was freeze-dried for further analysis. For the investigation into the surface functional groups of the gold nanoparticles synthesized, the freeze-dried nanoparticle was compressed to form a pellet and examined using the PerkinElmer Spectrum FTIR Spectrometer in the wave range extending from 4000 to 400 cm^{-1}.

X-ray diffraction (XRD).

The crystalline structure of *LSAUNP* was calculated using the PANalytical X'PERT PRO with an instrumentation setup of 30 kV, 40 mA with a Cu-Kα (1.540598) from 5° to 90° (2θ). The crystalline structure of the gold nanoparticles was analysed from the width of the XRD peaks, by using the Debye-Scherrer equation (Equation 2):

$$D = \frac{\kappa\lambda}{\beta cos\theta^{\circ}} \qquad (2)$$

Where D = mean crystalline diameter of the gold nanoparticles, λ = wavelength of the X-rays, β = angular full width half maxima of the XRD peak at the diffraction angle (Bragg's angle) 2 θ. Phase identification was carried out by use of the JCPDS database.

Thermogravimetric analysis (TGA).

The thermogravimetric analysis of *LSAUNP* was investigated by using a TA Instrument TGA Q500. The flow rate for oxygen and nitrogen was 40 mL/min and 60 mL/min respectively. An amount of 6.31 mg of the freeze-dried gold nanoparticles were placed on a platinum pan and heated. The temperature range was 30 °C to 900 °C with a heating rate of 30 °C/ min.

Statistical analysis.

Statistical analysis was performed with GraphPad Prism (Version 5) using one-way analysis of variance (ANOVA) with post-Tukey's comparison was used with the difference of $p<0.05$ as statistically significant. The results are expressed as the mean ± standard deviation, n= 3 or more.

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8

FINAL CONCLUSION AND FUTURE RECOMMENDATIONS

This chapter concludes the current study and links with the summary in the first part of the thesis. Future recommendations into further research into natural products as therapy for infectious diseases and especially further research into indigenous South African plants are also mentioned in this chapter.

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8. FINAL CONCLUSION AND FUTURE RECOMMENDATIONS

As our knowledge expands on the bacteria, *M. tuberculosis*, the causative agent for the current TB epidemic, so too does our knowledge expand about the floral kingdom and their provision for the fight against infectious diseases. New chemical entities that are of natural origin have the possibility of providing these answers. The hard work starts by the identification of new natural leads and doing an in-depth study into what this lead comprises of. This includes an investigation into possible herb-drug interactions as well as enhancement of current activities found by utilizing new technologies such as nanoparticles formation. New avenues such as adjunct host-directed therapies have the potential of targeting certain phases of infection and disease and as a result may reduce the morbidity and mortality rates and increase compliance by reducing the side-effects experienced. The main side-effect targeted through the current study is that of hepatotoxicity (toxicity of the liver). As the main organ involved in the metabolism of drugs, and in this case anti-TB drugs, the diminishing of toxicity of the liver through the use of natural products is an important aspect to consider. South Africa has a rich biodiversity of natural flora which may have the possibility for new drug targets or treatment against TB and side-effects experienced.

The current study attempted to evaluate the various biological properties that may be provided by *L. scaberrima* Sond. and if any promising results could be found with regards to treatment for TB. This plant genus provides a rich traditional history as medicinal treatment for various ailments as seen with *Lippia javanica*, especially for treatment of respiratory ailments.

This provided initial steering of the research study and further included *A. linearis* (Burm.f.) R. Dahlgren as both are drunk as health teas or "tisanes" for various health conditions. Both plant species are rich in flavonoids and provide favourable characteristics for the identification of natural new chemical entities that may provide us with an opportunity to fight infectious diseases.

Significant antimycobacterial activity was found for *L. scaberrima* against *Mycobacterium tuberculosis* H37Rv. Low to no cellular toxicity was observed against hepatocellular carcinoma cells (HepG2) at the tested concentrations. Hydro-steam distillation was effective in isolating the essential oils from the aerial parts of *L. scaberrima*, which showed effective hepatoprotective activity against acetaminophen-induced toxicity. The cytochrome P450 (CYP) inhibitory activity of *L. scaberrima*, combinations of *L. scaberrima* and *A. linearis*, and

verbascoside has been reported in this study for the first time. Potent CYP inhibitory activity was found for *L. scaberrima* in combination with the green *A. linearis*. Verbascoside had no significant inhibitory activity against the CYP isoforms and therefore, it can be considered that it may have low risk for herb-drug interactions.

This study included the cyclooxygenase-II (COX-II) inflammatory activity of *L. scaberrima*. All the samples, except *L. scaberrima,* that were investigated for their DPPH free radical scavenging potential were found to have significant activity. Moderate nitric oxide scavenging activity was found for verbascoside and green *A. linearis* in combination with *L. scaberrima*.

This study provided a more comprehensive look into the specific biological properties of *L. scaberrima* relating to TB infection as well as its efficacy when combined with fermented and green *A. linearis* extracts. Synthesized nanoparticles of *L. scaberrima* provided the opportunity to lower the cytotoxicity towards HepG2 cells even more than was initially found, but further research into optimization of the method is needed.

The investigated samples showed the potential to decrease drug-induced hepatotoxicity, one of the main adverse effects experienced. In addition, all the samples tested, showed potential as an adjuvant for host-directed therapies for TB.

Future work includes the following:

- Many new technologies such as the synthesis of gold nanoparticles need to be further investigated for enhanced efficiency, efficacy and yields.
- Isolation of pure compounds need to be investigated especially targeting the terpenoid structures and the highly volatile components that exist within *L. scaberrima*.
- Seasonal variation and chemotypes of *L. scaberrima* should be investigated to determine the most biologically active variant of the species. In this regard genetic selection for a certain chemotype, rich in specific volatile components can be explored.
- Investigating the effect on the immunomodulatory capabilities of either *L. scaberrima* alone or in combination with *A. linearis*.
- The addition of a wider variety of clinically relevant CYP enzymes to be studied such a CYP2B6, CYP2C8 and CYP2C9 which will give a better conclusion on the potential herb-drug interactions.

- Investigation into the time-dependant inhibition of the CYP enzymes by preincubating the inhibitors (medicinal plant samples) with NADP.

In conclusion, this study provides a more comprehensive analysis of the biological potential of not just *L. scaberrima*, which has not yet been investigated in detail, but also added therapeutic value when in combination with *A. linearis*. The results indicated that *L. scaberrima* has the potential for adjunct host-directed therapies to lessen the impact of adverse effects experienced during infection such hepatotoxicity. Specifically, verbascoside a constituent found within *Lippia* spp. and endemic to South Africa, showed no potential for any herb-drug interactions following the *in vitro* studies conducted. Please note that all experiental studies and results reported on were on the ethanolic extracts of both *L. scaberrima* and *A. linearis* and not of the tea and/or the boiling water extract of *L. scaberrima* and *A. linearis* or otherwise stated.

END

Appendix A

In vitro antimycobacterial and adjuvant properties of two traditional South African teas, *Aspalathus linearis* (Burm.f.) R. Dahlgren and *Lippia scaberrima* Sond.

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- The complete list of compounds identified by GC-MS analysis of both the essential oil (Table 1A) and the ethanolic extract of *L. scaberrima* (Table 2A), together with their chromatograms (Figure 1A and 2A)
- TLC chromatogram indicating the presence of verbascoside within the ethanolic extract of *L. scaberrima* (Figure 3A)

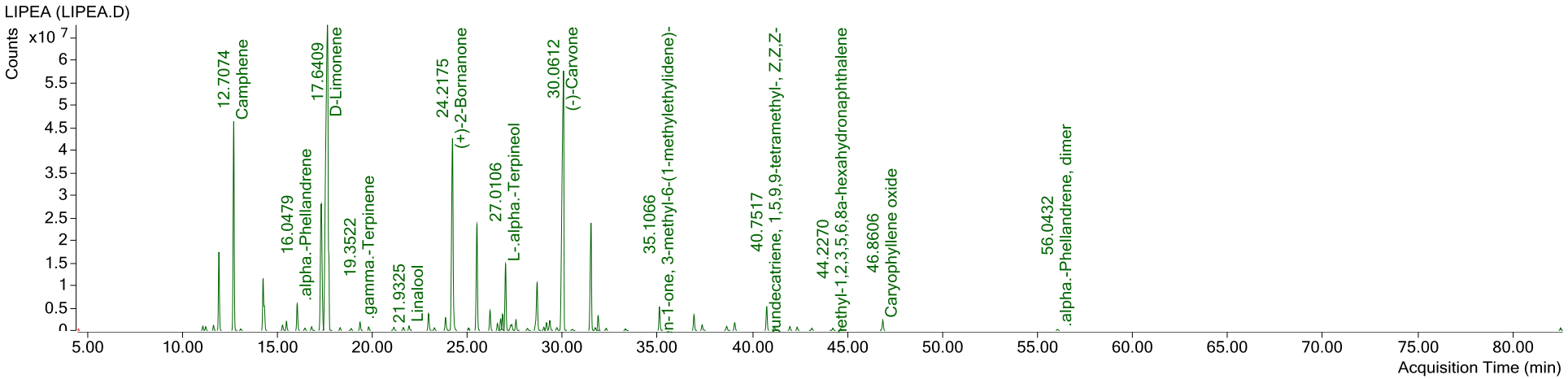

Fig. 1A: TIC chromatogram of the essential oil of *L. scaberrima*

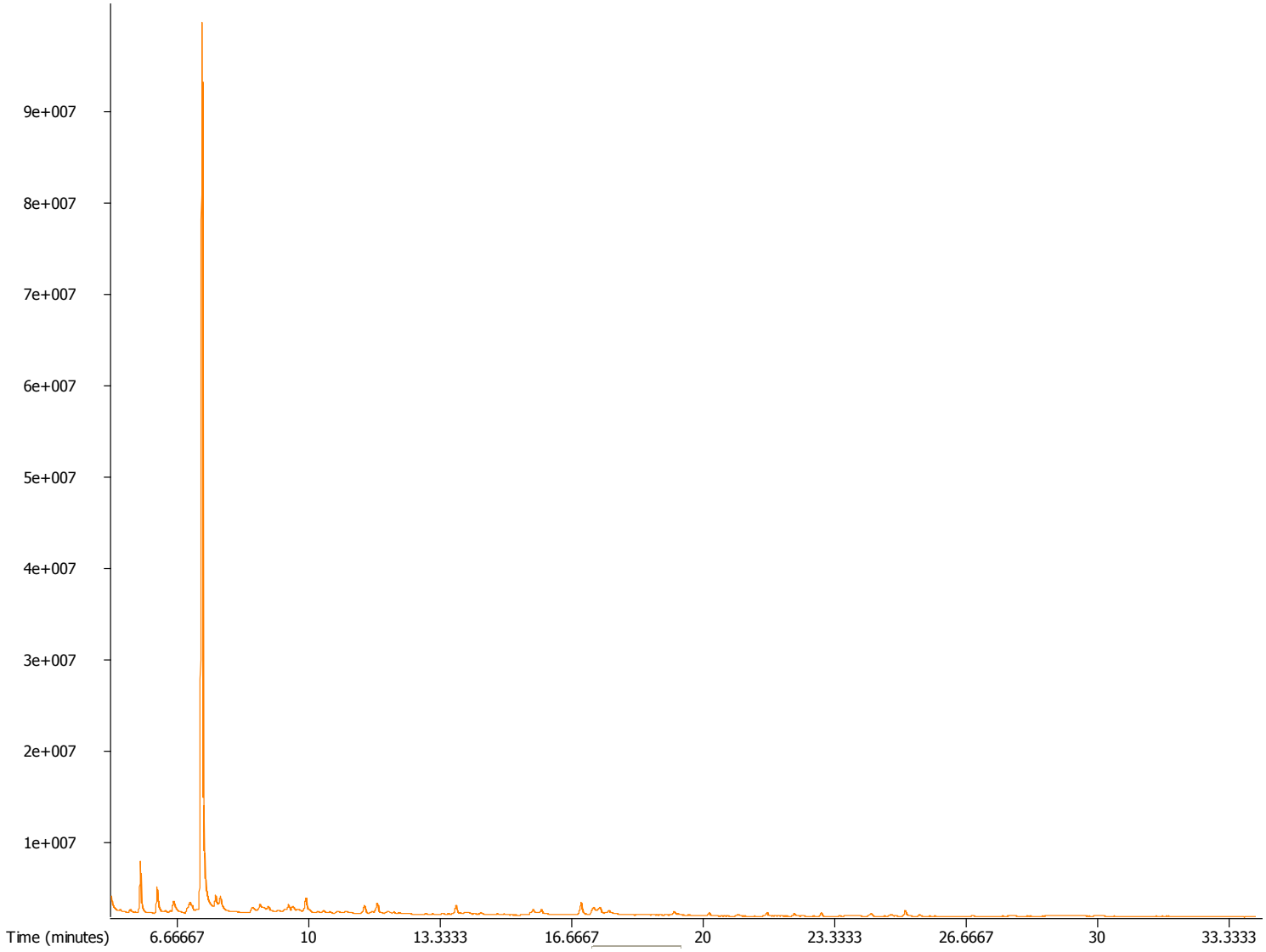

Fig. 2A: Chromatogram of the ethanolic extract of *L. scaberrima*.

Table. 1A: Constituents found within the essential oil extract of *L. scaberrima* with less than 1% contribution

RT[a]	Name of the compound	Molecular Formula	% Composition
5,48833	2-Cyclohexen-1-ol, 1-methyl-4-(1-methylethenyl)-, trans-	$C_{10}H_{16}O$	0,24
6,37167	Butanoic acid, 4-oxopentyl ester	$C_9H_{16}O_3$	0,15
6,52333	Benzenemethanol, à, à,4-trimethyl-	$C_{10}H_{14}O$	0,14
6,62833	1-Dodecene	$C_{12}H_{24}$	0,48
6,94667	Benzothiazole	C_7H_5NS	0,19
7,05833	2-Pentanone, 5-hydroxy-	$C_5H_{10}O2$	0,22
8,58	2-Cyclohexen-1-one, 3-methyl-6-(1-methylethylidene)-	$C_{10}H_{14}O$	0,59
8,99	alfa-Copaene	$C_{15}H_{24}$	0,47
9,07167	(-)-á-Bourbonene	$C_{15}H_{24}$	0,02
9,13	Piperidine, 1,1'-methylenebis-	$C_{11}H_{22}N_2$	0,05
9,225	2,3-Octanedione	$C_8H_{14}O_2$	0,13
9,435	3,5,5,9-Tetramethyl-4a,5,6,7,8,9-hexahydro-2H-benzo [7] annulene	$C_{15}H_{24}$	0,21
9,50167	Caryophyllene	$C_{15}H_{24}$	0,57
9,60167	2-Cyclohexen-1-one, 4-hydroxy-3-methyl-6-(1-methylethyl)-, trans-	$C_{10}H_{16}O_2$	0,67
9,61667	trans-p-mentha-1(7),8-dien-2-ol	$C_{10}H_{16}O$	0,57
10,0467	Spiro(1,3-dioxolane)-2,3'-(5'-androsten-16'-ol)	$C_{21}H_{32}O_3$	0,23
10,26	ç-Muurolene	$C_{15}H_{24}$	0,09
10,395	Benzene, 1-(1,5-dimethyl-4-hexenyl)-4-methyl-	$C_{15}H_{22}$	0,18
10,745	(R)-1-Methyl-4-(6-methylhept-5-en-2-yl) cyclohexa-1,4-diene	$C_{15}H_{24}$	0,26
10,895	trans-Calaenene	$C_{15}H_{22}$	0,11
10,975	2,2-Dimethyl-4-octenal	$C_{10}H_{18}O$	0,21
11,0783	n-Caproic acid vinyl ester	$C_8H_{14}O_2$	0,12
11,4333	Caryophyllene oxide	$C_{15}H_{24}O$	0,76
11,6367	2,2-Dimethyl-4-octenal	$C_{10}H_{18}O$	0,22
11,755	(1R,3E,7E,11R)-1,5,5,8-Tetramethyl-12-oxabicyclo [9.1.0] dodeca-3,7-diene	$C_{15}H_{24}O$	0,98
13,7533	à-Phellandrene	$C_{10}H_{16}$	0,73
13,86	à-Phellandrene, dimer	$C_{20}H_{32}$	0,14
14,0767	à-Phellandrene, dimer	$C_{20}H_{32}$	0,17
14,3783	Neophytadiene	$C_{20}H_{38}$	0,15
14,9567	à-Phellandrene	$C_{10}H_{16}$	0,06
15,63	Oxalic acid, allyl octyl ester	$C_{13}H_{22}O_4$	0,06
15,7017	n-Hexadecanoic acid	$C_{16}H_{32}O_2$	0,79
15,9133	Decanoic acid, ethyl ester	$C_{12}H_{24}O_2$	0,67
17,205	Bicyclo [7.1.0] decane	$C_{10}H_{18}$	0,98
17,235	9,12,15-Octadecatrienoic acid, (Z, Z, Z)-	$C_{18}H_{30}O_2$	0,93
17,3583	8-Dodecenol	$C_{12}H_{24}O$	0,35
17,6217	Hexadecanoic acid, 1,1-dimethylethyl ester	$C_{20}H_{40}O_2$	0,27
17,6983	Octadecanoic acid, ethyl ester	$C_{20}H_{40}O_2$	0,11
19,275	Hexanedioic acid, bis(2-ethylhexyl) ester	$C_{22}H_{42}O_4$	0,33
19,3883	Heptacosane	$C_{27}H_{56}$	0,09
20,1633	Hexatriacontane	$C_{36}H_{74}$	0,23
20,91	Heptacosane	$C_{27}H_{56}$	0,16
21,6317	Heptacosane	$C_{27}H_{56}$	0,33
22,3283	trans-Farnesol	$C_{15}H_{26}O$	0,21
23,0017	Tetracosane	$C_{24}H_{50}$	0,30
24,2433	dl-à-Tocopherol	$C_{29}H_{50}O_2$	0,09
24,286	Hexatriacontane	$C_{36}H_{74}$	0,31
25,1383	á-Sitosterol	$C_{29}H_{50}O$	0,53
25,4917	Cholesterol 3-O-[[2-acetoxy] ethyl]-	$C_{31}H_{52}O_3$	0,16

Table. 2A: Constituents found within the essential oil extract of *L. scaberrima* with less than 1% contribution.

RT[a]	Name of the compound	Molecular Formula	% Composition
4.5376	Toluene	C_7H_8	0,04
11.0846	5,5-Dimethyl-1-vinylbicyclo [2.1.1] hexane	$C_{10}H_{16}$	0,12
11.2357	Tricyclo[2.2.1.0(2,6)]heptane, 1,7,7-trimethyl-	$C_{10}H_{16}$	0,11
11.6444	Bicyclo [3.1.0] hex-2-ene, 2-methyl-5-(1-methylethyl)-	$C_{10}H_{16}$	0,14
13.0790	Bicyclo [3.1.0] hex-2-ene, 4-methylene-1-(1-methylethyl)-	$C_{10}H_{14}$	0,06
15.2743	2,3-Dehydro-1,8-cineole	$C_{10}H_{16}O$	0,20
15.4811	beta. -Myrcene	$C_{10}H_{16}$	0,21
16.0479	alpha. -Phellandrene	$C_{10}H_{16}$	0,91
16.4532	Cyclotetrasiloxane, octamethyl-	$C_8H_{24}O_4Si_4$	0,09
16.8078	1,3-Cyclohexadiene, 1-methyl-4-(1-methylethyl)-	$C_{10}H_{16}$	0,14
18.3104	(1R)-2,6,6-Trimethylbicyclo [3.1.1] hept-2-ene	$C_{10}H_{16}$	0,09
18.8881	1,3,6-Octatriene, 3,7-dimethyl-, (Z)-	$C_{10}H_{16}$	0,09
19.3522	gamma. -Terpinene	$C_{10}H_{16}$	0,27
19.8005	Bicyclo [3.1.0] hexan-2-ol, 2-methyl-5-(1-methylethyl)-, (1. alpha.,2. beta.,5. alpha.)-	$C_{10}H_{18}O$	0,04
21.1319	Benzene, 1-methyl-3-(1-methylethenyl)-	$C_{10}H_{12}$	0,10
21.6322	Bicyclo [3.1.0] hexan-2-ol, 2-methyl-5-(1-methylethyl)-, (1. alpha.,2. alpha.,5. alpha.)-	$C_{10}H_{18}O$	0,05
21.9325	Linalool	$C_{10}H_{18}O$	0,10
22.9576	2-Cyclohexen-1-ol, 1-methyl-4-(1-methylethenyl)-, trans-	$C_{10}H_{16}O$	0,60
23.2700	alpha. -Campholenal	$C_{10}H_{16}O$	0,10
23.8537	2-Cyclohexen-1-ol, 1-methyl-4-(1-methylethenyl)-, trans-	$C_{10}H_{16}O$	0,41
25.0583	Bicyclo [3.1.0] hexan-2-one, 5-(1-methylethyl)-	$C_9H_{14}O$	0,02
26.1939	Terpinen-4-ol	$C_{10}H_{18}O$	0,45
26.5853	Ethanone, 1-(3-methylphenyl)-	$C_9H_{10}O$	0,33
26.7545	Benzene methanol, alpha.,. alpha.,4-trimethyl-	$C_{10}H_{14}O$	0,52
26.8512	p-Mentha-1(7),8-dien-2-ol	$C_{10}H_{16}O$	0,45
27.3435	Cyclohexanone, 2-methyl-5-(1-methylethenyl)-, trans-	$C_{10}H_{16}O$	0,38
27.5552	2-Cyclohexen-1-ol, 3-methyl-6-(1-methylethenyl)-	$C_{10}H16O$	0,28
28.1625	Benzofuran, 4,7-dimethyl-	$C_{10}H_{10}O$	0,06
29.0251	Bicyclo [2.2.1] heptan-2-ol, 1,7,7-trimethyl-, formate, endo-	$C_{11}H_{18}O_2$	0,13
29.1672	cis-p-mentha-1(7),8-dien-2-ol	$C_{10}H_{16}O$	0,13
29.3398	Carveol	$C_{10}H_{16}O$	0,19
29.7004	Propanal, 2-methyl-3-phenyl-	$C_{10}H_{12}O$	0,10
30.5302	2-Cyclohexen-1-one, 3-methyl-6-(1-methylethyl)-	$C_{10}H_{16}O$	0,04
31.7302	4-Hydroxy-3-methylacetophenone	$C_9H_{10}O_2$	0,13
31.8817	1-methyl-4-(prop-1-en-2-yl)-7-oxabicyclo[4.1.0]heptan-2one	$C_{10}H_{14}O_2$	0,39
32.3019	Bornyl acetate	$C_{12}H_{20}O_2$	0,03
33.3199	Thymol	$C_{10}H_{14}O$	0,05
35.1066	2-Cyclohexen-1-one, 3-methyl-6-(1-methylethylidene)-	$C_{10}H_{14}O$	0,46
36.9252	alfa. -Copaene	$C_{15}H_{24}$	0,50
37.3503	(-)-. beta. -Bourbonene	$C_{15}H_{24}$	0,17
38.6408	1H-3a,7-Methanoazulene, 2,3,4,7,8,8ahexahydro-3,6,8,8-tetramethyl-, [3R-(3. alpha.,3a. beta.,7. beta.,8a. alpha.)]-	$C_{15}H_{24}$	0,15

39.0611	Caryophyllene	$C_{15}H_{24}$	0,30
40.7517	1,4,7, -Cycloundecatriene, 1,5,9,9-tetramethyl-, Z, Z, Z-	$C_{15}H_{24}$	0,61
41.1440	(1R,4R,4aS,8aR)-4,7-Dimethyl-1-(prop-1-en-2-yl)-1,2,3,4,4a,5,6,8a-octahydronaphthalene	$C_{15}H_{24}$	0,10
41.9734	Naphthalene, 1,2,3,4,4a,5,6,8a-octahydro-7-methyl-4-methylene-1-(1-methylethyl)-, (1. alpha.,4a. beta.,8a. alpha.)-	$C_{15}H_{24}$	0,12
42.3517	Benzene, 1-(1,5-dimethyl-4-hexenyl)-4-methyl-	$C_{15}H_{22}$	0,09
43.1347	alpha. -Muurolene	$C_{15}H_{24}$	0,06
44.2270	1-Isopropyl-4,7-dimethyl-1,2,3,5,6,8ahexahydronaphthalene	$C_{15}H_{24}$	0,06
44.6406	cis-Calamenene	$C_{15}H_{22}$	0,06
46.8606	Caryophyllene oxide	$C_{15}H_{24}O$	0,14
56.0432	alpha. -Phellandrene, dimer	$C_{20}H_{32}$	0,03
82.5253	Bis(2-ethylhexyl) phthalate	$C_{24}H_{38}O_4$	0,02

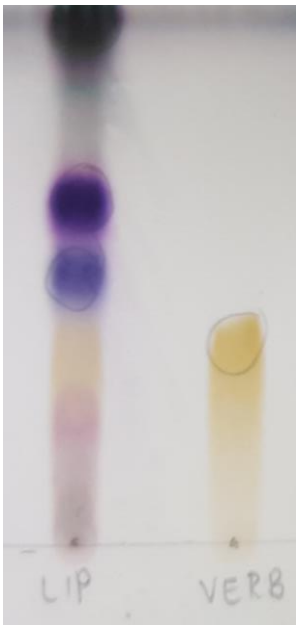

Fig 3A: TLC chromatogram indicating the presence of verbascoside within the ethanolic extract of *L. scaberrima* (MeOH: DCM; 2:8)

Appendix B

Inhibition potency Of *Lippia scaberrima* Sond. extract in combination with *Aspalathus linearis* (Burm.F.) R. Dahlgren extract for human CYP enzymes

Anna-Mari Reid[1*], Risto Juvonen[2], Pasi Huuskonen[2], Marko Lehtonen[3] Markku Pasanen[2], Namrita Lall[1,4,5]

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[2]School of Pharmacy, University of Eastern Finland, Kuopio, Finland
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[5]College of Pharmacy, JSS Academy of Higher Education and Research, Mysuru, Karnataka, India

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- Recombinant CYP1A2, CYP2A6, CYP2C19, CYP2D6 and CYP1B1 inhibition by the essential oil of *L. scaberrima*
- Recombinant CYP1A2, CYP2A6, CYP2C19, CYP2D6 and CYP1B1 inhibition by the ethanolic extract of *L. scaberrima*

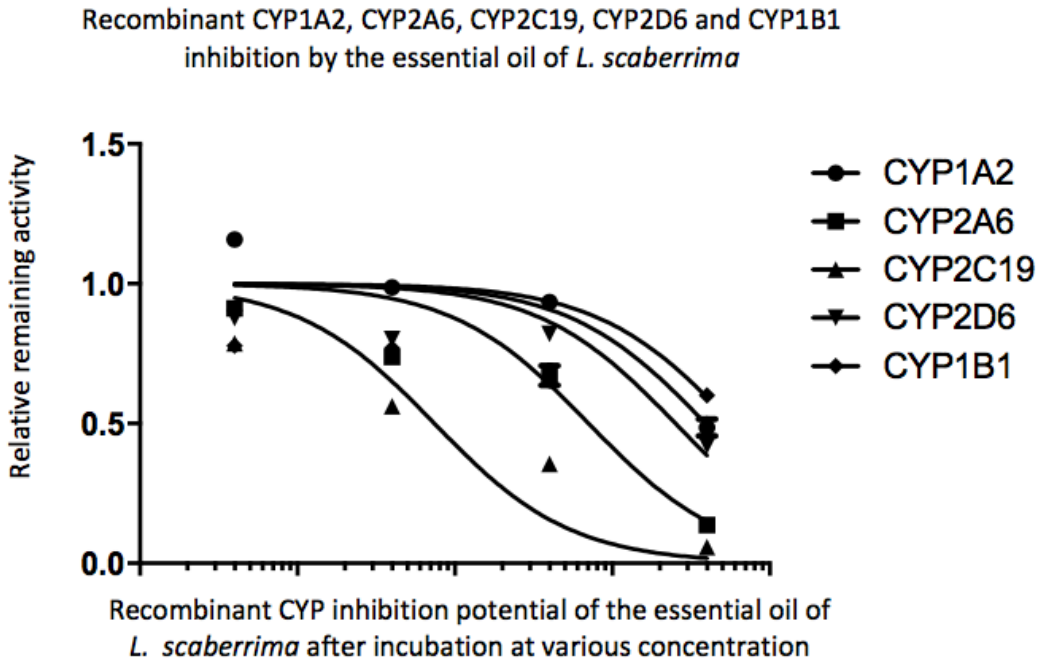

Figure 1B: The CYP inhibition potential of the essential oil of *L. scaberrima* against the recombinant CYP enzymes (CYP1A2, CYP2A6, CYP2C19, CYP2D6 and CYP1B1)

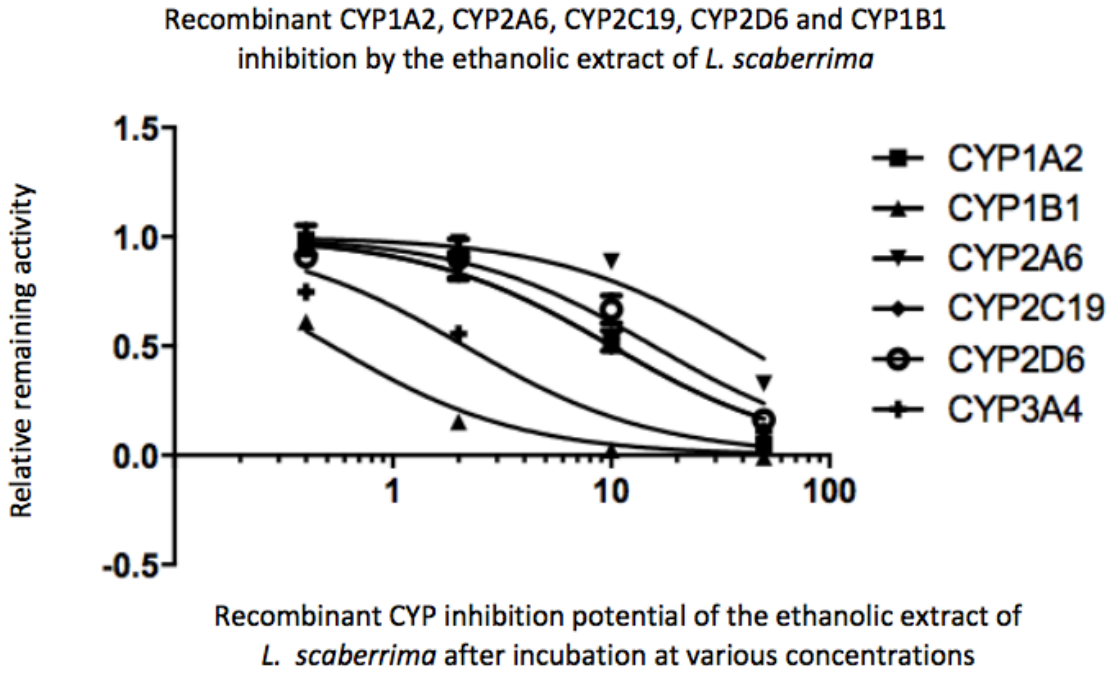

Figure 2B: The CYP inhibition potential of the ethanolic extract of *L. scaberrima* against the recombinant CYP enzymes (CYP1A2, CYP2A6, CYP2C19, CYP2D6 and CYP1B1)

Appendix C

In vitro human metabolism and inhibition potency of verbascoside for CYP enzymes

Anna-Mari Reid [1], Risto Juvonen [2], Pasi Huuskonen [2], Marko Lehtonen [3], Markku Pasanen [2] and Namrita Lall [2,3,5]*

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- The EIC Chromatogram of verbascoside as a standard use to determine its presence and quantity within *L. scaberrima*. The area in red indicates the area of interest (Figure 1C).
- The EIC chromatogram of the ethanolic extract of *L. scaberrima* and indicated in red the presence of verbascoside (Figure 2C).

Supplementary material

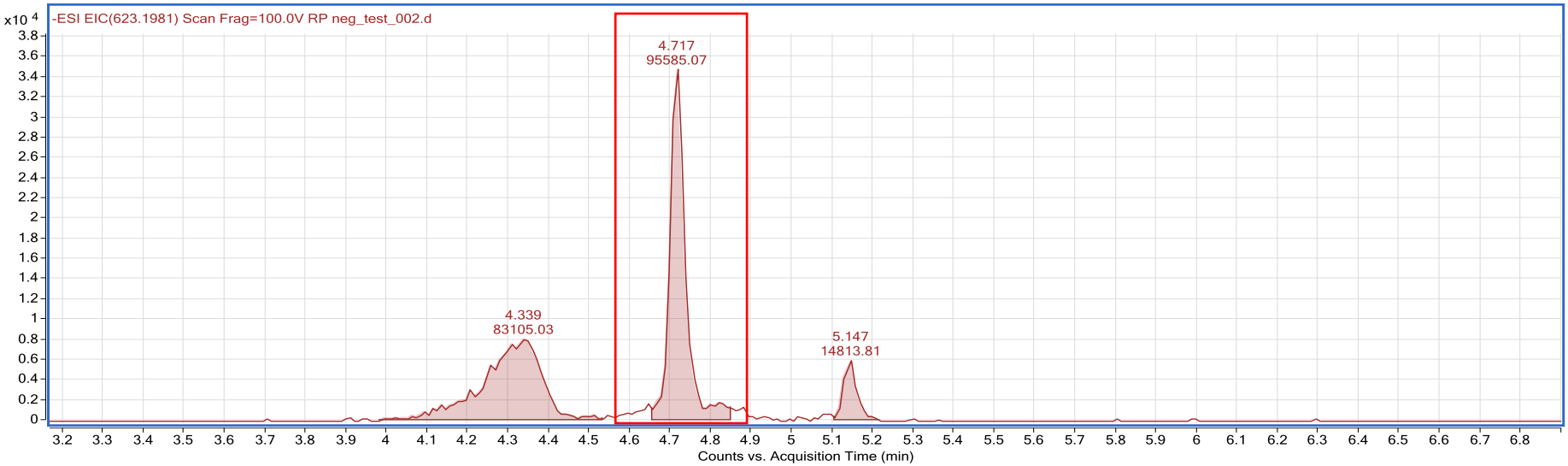

Figure 1C: The EIC Chromatogram of verbascoside as a standard use to determine its presence and quantity within *L. scaberrima*. The area in red indicates the area of interest.

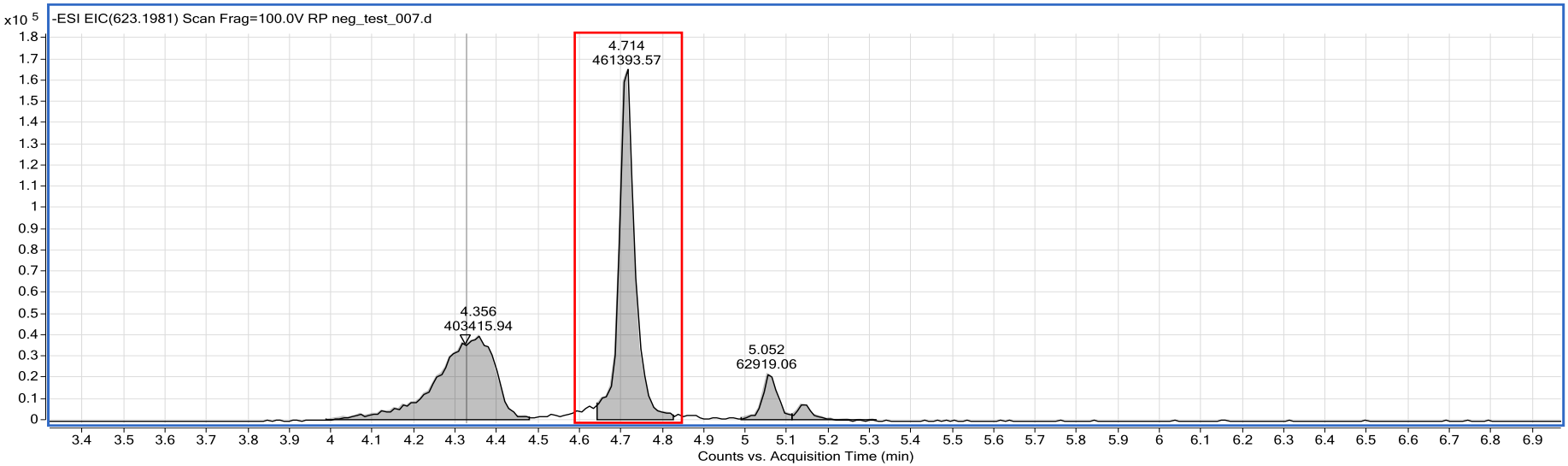

Figure 2C: The EIC chromatogram of the ethanolic extract of *L. scaberrima* and indicated in red the presence of verbascoside