ANTIOXIDATIVE, ANTI-INFLAMMATORY AND ANTIMYCOBACTERIAL ACTIVITIES OF *ARTEMISIA AFRA* SUBFRACTION AGAINST *MYCOBACTERIUM SMEGMATIS*

By

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[redacted]

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DECLARATION

I, **Precious Mabasa Matlala**, declare that the dissertation hereby submitted to the University of Limpopo for the degree of Master of Science in Microbiology has not previously been submitted by me or anyone for the degree at this or any other University, and that this is my own work in design and execution. All the materials contained therein have been duly acknowledged.

…….. ……

Signature Date

DEDICATION

I dedicate this work to:

My grandmother, Salamida Maduane Matlala, thank you Granny, for being my pillar of strength. You encouraged me from early childhood days that “education is the key to success”.

My mother, Joyce Matlala, and siblings: Eddie, Blessing and Lesedi Matlala. Standing together as a family has once again proven to be the best weapon to conquer the world.

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"*Be the change you wish to see in the world"* **~ Mahatma Gandhi**

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LIST OF ABBREVIATIONS

A	Acetone
ABTS	2, 2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid)
AVG	Average
B	Butanol
BEA	Benzene ethanol ammonium hydroxide
C	Chloroform
CEF	Chloroform ethyl acetate
D	Dichloromethane
DMSO	Dimethyl sulfoxide
DPPH	2,2-diphenyl-1-picrylhydrazyl
E	Ethanol
EA	Ethyl acetate
EMW `	Ethyl acetate methanol water
FRAP	Ferric reducing antioxidant power
GAE	Gallic acid equivalence
H	hexane
LC-MS	Liquid chromatography-Mass spectroscopy
M	Methanol
Mtb	*Mycobacterium tuberculosis*
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NSAIDs	Non-steroidal anti-inflammatory drugs
ROS	Reactive Oxygen Species
TB	Tuberculosis

TLC	Thin layer chromatography
UV-VIS	Ultraviolet visible

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2. Matlala MP and Masoko P (2023). Isolation and characterisation of compounds with antimycobacterial, antioxidant and anti-inflammatory effects from the leaves of *Artemisia afra* against *Mycobacterium smegmatis*. 48th Annual South African Association of Botanists Conference (SAAB), Protea Hotel Ranch Resort, Limpopo Province, 17–20 January.
3. Matlala MP, Chokoe KPK and Masoko P (2023). Isolation and characterisation of compounds with antioxidant, anti-inflammatory and antimycobacterial effects against *Mycobacterium smegmatis* from the leaves of *Artemisia afra*. 22nd Biennial Congress of the South African Society for Microbiology (SASM), Stellenbosch University, 17-20 September.
4. Matlala MP, Chokoe KPK and Masoko P (2023). Antioxidative, anti-inflammatory and antimycobacterial activities of *Artemisia afra* against *Mycobacterium smegmatis*. Faculty of Science and Agriculture, 13th Research Day, Polokwane (Bolivia Lodge), 20-22 September.

ABSTRACT

Tuberculosis (TB) is a well-known communicable disease discovered decades back and continues to be a persistent socio-economic burden worldwide with the increasing number of multidrug-resistant and extensive-drug resistant forms. Medicinal plants have been accredited as potential sources of natural pharmaceuticals against TB. The aim of the study was to investigate the efficacy of antioxidative, anti-inflammatory and antimycobacterial activities of *Artemisia afra* extracts and sub-fractions. *Mycobacterium smegmatis* was used as a surrogate for *Mycobacterium tuberculosis* (*Mtb)*. The aerial parts of *A. afra* plant were dried and ground into fine powder. The powdered plant material was extracted using hexane, chloroform, dichloromethane, ethyl acetate, acetone, ethanol, butanol, methanol, and water. All the solvents demonstrated good extraction capacity. The qualitative phytochemical analysis was done using standard chemical tests and thin layer chromatography. Standard chemical tests showed the presence of saponins, steroids, tannins, cardiac glycosides, terpenes, and flavonoids in the extracts. Phytochemical analysis revealed more fluorescing compounds at 365 nm. The methanol extract had the highest amount of total phenolic (190.31±5.81 mg GAE/g), tannin (339.92±11.28 mg GAE/g) and flavonoid contents (1333.07±12.97 mg QE/g). All the tested *A. afra* extracts had low ferric ion reducing antioxidant power. However, the acetone extract showed notable 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical-scavenging potential. Anti-inflammatory activity was investigated using the egg-albumin denaturation assay, where the acetone extract demonstrated the higher activity than diclofenac sodium. Furthermore, the acetone extract exhibited noteworthy antimycobacterial activity against *M. smegmatis* observed on the three chromatograms developed in BEA, CEF and EMW mobile systems with minimum inhibitory concentration of 0.521 mg/mL. Cytotoxicity was tested against the THP-1 cell-line monocytes. The differentiation of THP-1 monocytes into macrophage-like cells was induced by phorbol 12-myristate-13-acetate (PMA). The acetone and methanol extracts had less toxicity at the lowest concentration of 125 µg/mL. Column chromatography was used to fractionate the active acetone extract; and its subfraction of intermediate polarity had the highest inhibitory activity against *M. smegmatis* at MIC value of 0.078mg/mL. Moreover, the subfraction was able to prevent initial cell attachment to form biofilms in a concentration dependent manner and the matured formed biofilm after 24hrs was also

reduced. Growth inhibitory activity monitored in different time intervals and anti-inflammatory activity were also observed. Results obtained from LC-MS analysis revealed several compounds at different retention times from both the acetone crude extract and the sub-fraction. The crude extract contained lesser number of compounds as compared to the sub-fraction. These results suggest that the acetone sub-fraction from the aerial parts of *A. afra* may be a good candidate for further anti-TB drug development.

CHAPTER 1

1.1 GENERAL INTRODUCTION

Tuberculosis (TB) is a well-known communicable disease that has a long history of adding to the high morbidity and mortality rates across the globe with increasing number of drug-resistant strains (Reddy *et al.,* 2021). TB is part of the top ten deadly diseases in South Africa and across the globe (Rahman *et al.,* 2020). The World Health Organization (WHO) estimated that TB, which infected around 10 million people worldwide and resulted in roughly 1.3 million fatalities in just the year 2020, is the second most common infectious agent to contribute to mortality rates (WHO, 2021). Furthermore, WHO has reported South Africa as one of the 30 countries with a high burden of TB (Smeeth *et al.,* 2004; Ayles *et al.,* 2022). TB infections are caused by a group of pathogenic microbes from the Mycobacteriaceae family, namely: *Mtb, Mycobacterium microti, Mycobacterium bovis, Mycobacterium caprae, Mycobacterium pinnipedii, Mycobacterium Canetti, and Mycobacterium africanum. Mtb* is the main cause of the disease with the first site of infection being the lungs. Close contact and the load of bacilli cells inhaled or transferred by aerosols from an infected individual are some of the distinct elements contributing to the severity of the infection in a host (Ahmad, 2011). Phagocytes, such as macrophages and dendritic cells, are released into the infected area after a primary pulmonary TB infection in the lungs and proceed to engulf the pathogenic *Mtb* cells. Pro-inflammatory chemicals are also secreted. *Mtb* can manipulate the host's systems to enhance the conditions that are favourable to it. These bacilli can remain dormant inside the host (Cooper, 2009; Sasindran and Torrelles, 2011). During the dormant stage, there will development of granulomas, which is a site of infection with host immune cells, bacterial cells, and dead cell debris. Granulomas are formed to contain the macrophages with latent *Mtb* and dead host infected cells, as such preventing their spread to other nearby healthy cells. Later, when the latent *Mtb* infection gets reactivated, which could possibly be due to several factors including improvised host's immune system by external factors and/or genetic implications, it results in the rupturing of the granulomas leading to lung cavitation and extra pulmonary infection extending to other organs of the host (Kaplan *et al.,* 2003; Dheda *et al.,* 2005; Ulrichs and Kaufmann, 2006; Russell, 2007). Due to the pathogenicity of *Mtb*, it becomes hard for health care facilities to treat most patients

with TB infections. Therapeutic regimens for TB are prepared from different groups of drugs (first, second and third line of defence). Isoniazid (INH), ethambutol (EMB), pyrazinamide (PZA), and rifampicin (RIF) were the most active antibiotics used against *Mtb* infections for a long time either as single doses or in combinations. The emergence of multidrug-resistant and extensive-resistant TB strains lead to the regimens reduced efficacy; therefore, new therapeutics have to be developed (Verma *et al*., 2020; WHO, 2021; Rossini and Dias, 2023). The well-known vaccine, Bacilli Calmette-Guerin (BCG), has also been reported to have lost efficacy against pulmonary TB, especially in adults (Gong *et al.,* 2018). There are many factors contributing to the TB-drug resistant such as the pro-longed use of antibiotics by patients, incorporation of antibiotics into livestock feed, hydrolysis of antibiotics by microbes, adjustments in the target structure by bacteria, etc. (Zine *et al.,* 2018). Poor patient adherence to the conventional 6-month anti-TB chemotherapy also contributes to *Mtb* -drug resistance (Pienaar *et al.,* 2018). Drug-resistant bacteria pass on the survival genes to their daughter cells enabling them to develop new strategies to change their target site that interacts with therapeutic agents (Javed *et al.,* 2020). For decades, medicinal plants have been acknowledged as the new sources of natural pharmaceuticals. Unlike synthetic medications, medicinal plants are easily accessible, cost effective and have less side effects. People from different communities in developing countries, including South Africa, have been using herbal medicine to treat diseases, infections, and injuries with acquired expertise passed from one generation to another depending on cultural beliefs on the use of the herbs (Amoo *et al.,* 2009; Borokini and Omotayo, 2012; Masoko and Nxumalo, 2013). Some of the medicinal plants with Antimycobacterial activity against *Mtb* include the essential oil of *Ocimum sanctum* (Jayapal *et al.,* 2021), fractions of *Diospyros anisandra* (Uc-Cachón *et al.,* 2014) and *Paederia foetida Linn*, which was tested for its antimycobacterial and antibiofilm activity against *M. smegmatis* (Priyanto *et al.,* 2022).

Artemisia afra, also known as African wormwood (*Umhlonyane*/*Lengana*), is an herbal plant from the family Asteraceae that grows up to 2 m tall and has been reported to be abundant in African countries such as South Africa, Ethiopia, Zimbabwe, Lesotho, Namibia, and Swaziland. The plant herbs are prepared differently to treat various diseases either as inhalants, infusions, or decoctions (Watt and Breyer-Brandwijk, 1962; Liu *et al.,* 2009, Germishuizen *et al.,* 2006). The plant has been reported to treat

several diseases and respiratory infections, including TB related symptoms such as, fever, coughs, convulsions, colds, asthma, headaches, malaria, chills, colic, diabetes, influenza, rheumatism, and inflammation (Watt and Breyer-Brandwijk, 1962; Van Wyk *et al*., 2009; Lawal *et al.,* 2014; Sharifi-Rad *et al.,* 2020). Researchers have established various potent biological activities associated with the herbal medicines of *Artemisia afra*, and these include antimycobacterial activities (Ntutela *et al.,* 2009), antidiabetic (Sunmonu and Afolayan, 2013), antibacterial (Graven *et al*., 1992; Buwa and Afolayan, 2009), antioxidant activity, anti-inflammatory activity (Burits *et al.,* 2001), antifungal activity (Muyima and Nkata, 2005) and antimalarial activity (Clarkson *et al.,* 2004; Liu *et al.,* 2010). The aim of this study was to investigate the efficacy of antioxidative, anti-inflammatory and antimycobacterial activity of *Artemisia afra* extracts and sub-fractions intended to help discover novel phytochemicals with potential as anti-TB drugs influenced by the season and location of plant harvest.

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CHAPTER 2

2. Literature review

2.1 Antibiotic resistance of *Mycobacterium tuberculosis*

In 2019, the World Health Organization (WHO) released new TB treatment regimens and advised the incorporation of RIF and INH with fluoroquinolones, which are broad spectrum bactericidal agents (Jhun and Koh, 2020; Mirzayev *et al.,* 2021). These conventional anti-TB drugs make patients develop severe side effects and have now become less effective. Some of the anti-TB drugs' side effects include skin rashes, oxidative stress; damage to intracellular macromolecules, chronic inflammatory response, peripheral neuritis, ototoxicity, and fever (Mangwani *et al.,* 2020). The antibiotic resistance of many microorganisms is attributed to their ability to modify their cell compartmentalisation, thus helping them to survive in unfavourable environments (Tang *et al.,* 2021). Long overuse of the TB chemotherapy, which can take up to more than the normal period of 6-9 months, also contributes highly to the development of drug-resistant strains (Kumar *et al.,* 2013; Ranjitha *et al.,* 2020). The slow growth rate of *Mtb*, its pathogenic nature, increased virulence, impermeable cell membrane, and hydrophobicity present challenges in the development of TB treatments as well (Smith *et al.,* 2012). During environmental stress or nutrient deficiency, *Mtb* can go through a dormant phase inside the host's cells. This inactivity creates a challenge for treatment and TB drug research (Lelovic *et al.,* 2020). *Mtb* has also been reported to form biofilms in infected patients as a mode of survival, and they help them develop resistant towards drugs (Chakraborty *et al.,* 2021).

Following infection with *Mtb*, an individual's innate immune response will be activated, releasing macrophages and neutrophils to the site of infection, which will then result in the formation of granulomas (Zine *et al.,* 2018). The pathogen *Mtb* will be phagocytosed by macrophages, neutrophils, and dendritic cells as a result of the immune response, releasing cytokines and reactive oxygen species into the alveolar cells. This results in an inflammatory reaction in the lungs, which triggers the programmed cell death of some infected cells (Sasindran and Torrelles, 2011; Blomgran *et al.,* 2012). However, *Mtb* has the ability to escape phagocytosis, thus disrupting the autophagy signalling pathway (Gong *et al.,* 2018). An array of inflammatory response reactions from several *Mtb* infections results in the overproduction of reactive oxygen species that induce cellular and organ damage

(Arulselvan *et al.,* 2016; Racanelli, 2018; Divangahi, 2013). In some studies, integrating metabolomics and transcriptomics, it was discovered that the production of valine, tyrosine, isoleucine, leucine, phenylalanine, and tryptophan was downregulated in *Mtb* during macrophage infection. In addition, it is possible that the harsh intracellular environment in macrophages, which causes *Mtb* to seek out alternate techniques to deal with these stresses, contributes to the downregulation of these pathways, which shows a general slowdown of these amino acid biosynthesis. Therefore, targeting metabolic pathways that are important for stress tolerance mechanisms and also contribute to *Mtb* persistence in hosts may lead to a faster removal of *Mtb* cells and biofilms from the host (Awasthy *et al.,* 2009; Zimmermann *et al.*, 2017).

2.2 *Mycobacterium smegmatis* as a model in anti-TB drug discovery

TB is a disease caused by a group of strains from the Mycobacteriaceae family, including *Mtb*. Microorganisms from this family grow in aerobic environments. *Mtb* is a slow-growing pathogenic microorganism (Ramos, 2018). Due to this pathogenicity, a closely related, non-pathogenic species from the same family is used in TB research. *Mycobacterium smegmatis* is a saprophytic, fast-growing, non-pathogenic organism that is closely related to *Mtb*. It is similar to *Mtb* in structure and biochemical properties, and the two microbes share genes that allow them to adapt in stressful conditions (Gong *et al.,* 2018). A comparative study by Mamadou *et al*. (1989) reported that during the dormancy stage undergone by mycobacterium species induced by environmental stress and host immune responses, transcription rates and metabolic activities gets reduced. However, later after 24 hours, the microbes, including *M. smegmatis* resuscitation, resulting in changes in *denova* transcription and radioactive uracil incorporation. The expression of transcription factor genes and fatty acid synthase systems I and II will then be boosted. Furthermore, central metabolism genes will start to transcribe ribosomal proteins, NADH dehydrogenases, ATP synthases, and cell division after the second resuscitation phase, which lasts for around 4 days.

Both *Mtb and M. smegmatis* have mycolic acids in their cell walls that help in preventing the entry of hydrophobic and hydrophilic solutions, allowing only lipids to pass through, thus contributing significantly to their drug resistance. *Mycobacterium smegmatis* make a good model for *Mtb* because they are 90% genetically comparable and can also generate thiol, which is crucial for the proliferation of *Mycobacterium*

species through mycothiol biosynthesis (Ranjitha *et al.,* 2020). In addition to metabolic and structural similarities between the two microbes, they further contain N-acetylmuramic acid (MurNAc) and N-glycolylmuramic acid (MurNGlyc), which affords them the ability to resist lysozyme hydrolysis during an innate response (Ranjitha *et al.,* 2020).

2.3 The use of medicinal plants by humankind

The indigenous knowledge and usage of plants as a source of medicine stem from age-old cultural practices of people from different parts of the world, especially in developing communities (Wanjohi *et al.,* 2020). Ancient man depended on plants for shelter, medicine, food, and clothes (Botelho *et al.,* 2019). Today, many people from developing countries continue to rely on medicinal plants as primary treatment of diseases and infections. To prepare these herbal medicines, water is used as an extractant for concoctions made using various plant parts; however, neither the measurements of these ingredients nor the dosage of the treatments is known (Matotoka and Masoko, 2017).

Medicinal plants have gained much recognition due to their abundance in bioactive compounds, minerals, and vitamins (Shakya, 2016). Production of secondary metabolites by plants depends on the location as well as the environmental pressures affecting that particular plant (Erb and Kliebenstein, 2020). To ensure the preparation of affordable and safe herbal medicines with improved quality, there has been development of scientific methods to validate the biological activities of the compounds found in plants (Obakiro *et al.,* 2020).

2.4 Medicinal plants as sources of anti-TB drugs

Due to the different negative reactions of patients and strain resistance to the current TB treatment regimens, the search for new anti-TB drugs offering fewer side effects is still ongoing. Medicinal plants such as *Artemisia afra* (Adewumi *et al.,* 2020), *Spondias purpurea L*. (Shakya, 2016), *Asparagus africanus* Lam. (Sharifi-Rad, 2020), *Erythrina abyssinica* and *Allium sativum* (Hannan *et al.,* 2011), with an extensive number of bioactive secondary metabolites, have gained much recognition and are being investigated as leads for the development of new therapeutic agents for TB and other diseases. These bioactive compounds have different biological activities, which are attributed to their structure, number and position of substituent groups and the

topographic origin of the plant (Vaou *et al.,* 2021). Secondary metabolites produced by medicinal plants have been reported to have numerous biological activities such as anti-inflammatory, anticancer, antioxidative and antimicrobial activities (Jamshidi-Kia *et al.,* 2018; Vaou *et al.,* 2021). The nasal cavity, bronchi, and lungs are all affected by respiratory disorders, which are known to affect the airways. However, due to their wide range in severity and dependence to the patient's age, these diseases are frequently challenging to categorise (Villena-Tejada *et al.,* 2021; Khadka *et al.,* 2021). Upper and lower respiratory infections that cause common cold, whooping cough asthma, pneumonia and bronchitis are among many serious respiratory disorders (Khan *et al.,* 2014). The latter mentioned conditions form part of the TB symptoms displayed by most patients.

A great source of phytochemicals, which have numerous advantageous impacts on health, including liver function, are traditional medicines used to treat TB. Phytochemicals inside TB herbs serve as hepatoprotective agents, thus restoring normal liver cell function, histology, and enzymatic activity caused by chemical hepatotoxicity and anti-TB drugs. High levels of protein, serum enzymes, and total bilirubin can be decreased by these herbs. They can also reverse liver damage brought on by anti-TB drugs as well as the improper function of enzymatic antioxidants (Sharma *et al.,* 2004; Madrigal-Santillán and Madrigal-Bujaidar, 2014; Singh *et al.,* 2016). Traditional TB treatments come from plants including *Fumaria indica*, *Apium graveolens*, *A. paniculate*, *Apium indica*, *W. somnifera*, *Ficus religiosa*, *Syzygium aromaticum*, *Glycyrrhiza glabra*, and *Tinospora cordifolia*. These plants have a wealth of compounds that have anti-microbial and liver protective properties, including diterpenoids, flavonoids, alkaloids, lipids, tannins, and sterols (Gupta *et al.,* 2008; Singh *et al.,* 2013; Jadeja *et al.,* 2015). Research is prioritising finding new strategies to develop high efficacy, safe and affordable drugs from natural sources such as plants, animals, and minerals (Süntar, 2020). Incorporating target-specific features of anti-TB medications with the numerous health benefits of medicinal plants may be a successful strategy for treating TB and its accompanying adverse effects (Mangwani *et al.,* 2020).

2.5 Primary and secondary plant metabolites

Metabolites such as antibiotics, amino acids and enzyme inhibitors are produced as intermediates and end products of normal biochemical reactions of plants (Jamshidi-Kia *et al.,* 2018). These plant compounds are classified into three categories: hormones that help in regulating metabolism and other processes of the plant, primary metabolites that play significant roles in the growth of the plant and secondary metabolites (phytochemicals) essential in adaptation to different environmental conditions and for defence against predators (Erb and Kliebenstein, 2020). Examples of primary metabolites include carbohydrates that play a crucial role in the metabolic processes of plants as a source of carbon and energy in a cell (Cao *et al.,* 2018), and polyamines that are key components for plant development and growth, and further play a significant part in higher plants' ability to withstand abiotic stress (Alcázar *et al.,* 2020). Organic phytochemicals produced by plants provide protective mechanisms that help treat diseases like heart disease, TB, cancer, hypertension, and other conditions (Khatiwada *et al.,* 2018). Common established secondary metabolites produced by medicinal plants include phenolic acids, flavonoids, tannins, saponins, alkaloids, cardiac glycosides, steroids, and terpenes (Shakya, 2016; Builders, 2019).

2.5.1 Phenolics

Phenolic compounds (**Figure 2.1**) are widely spread organic compounds used by the plants to protect against diseases and are thus abundant in medicinal plants (Awuchi, 2019). Phenolic compounds contain an aromatic hydrocarbon ring and a single hydroxyl group (Sharma *et al*., 2019). Examples of common phenolic compounds are flavonoids, stilbenes, lignin, tannins, and phenolic acids (Builders, 2019). Phenolic acids are characterised by the carboxylic group in their structure. They can kill or prevent the growth of bacteria by disrupting the cytoplasmic membrane and changing its permeability (Liu *et al.,* 2020). Some of the phenolic compounds derived from the plant *Pinus sylvestris* include the flavonoids kaempferol, taxifolin and quercetin, which have antibacterial activity against certain common disease-causing organisms such as *Escherichia coli* and *Staphylococcus (*Metsämuuronen and Sirén, 2019). These three compounds are also known to inhibit oxidative stress. In addition, quercetin and taxifolin can be detected in the laboratory using ultraviolet (UV) radiation because they naturally shield plants from UV light (Patil *et al.,* 2019). Coumarins are phenolic

compounds that inhibit the protein synthesis and activity of carbonic anhydrases on microorganisms like *Mtb* (Gautam *et al.,* 2023).

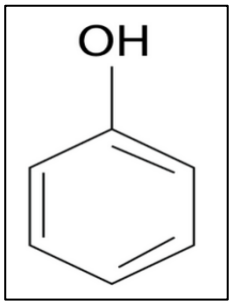

Figure 2.1: Structure of a typical phenolic compound (Builders, 2019)

2.5.1.1 *Flavonoids*

Flavonoids are secondary metabolites forming a large part of phenolics with clinical, biological and nutraceutical uses (Patil *et al*., 2019). As shown in **Figure 2.2**, they contain a three-ring structure in their centre but are differentiated from other phenolics by a heterocyclic ring. Flavonoids are found in fruits, flowers, and leaves with distinct colours. It has been reported that their antibacterial activity stems from the potential to form numerous complexes with bacterial cell walls and extracellular proteins. Flavonoids include flavonols, flavanones, anthocyanidins and flavones (Metsämuuronen and Sirén, 2019), with each of these contributing highly to the antioxidative property of most plants (Hossain *et al.,* 2011). The presence of acyl chain groups on the structure of flavonoid compounds increases their affinity to the lipid bilayers of the membranes, leading to interactions between the hydrophilic heads of flavonoids and polar phospholipids of the cell membranes while the hydrophobic heads of flavonoids are creating a shielding complex inside the lipid bilayers as such, reducing the integrity and fluidity of the cell membrane, therefore resulting in microbial inhibition (Yuan *et al.,* 2021).

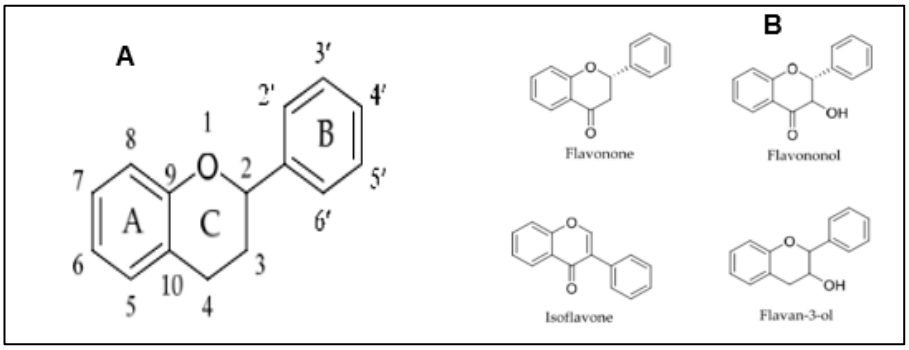

Figure 2.2: Heterocyclic ring structure of flavonoids (**A**) and some common well known isolated flavonoid compounds (**B**) (Shamsudin *et al*., 2022).

2.5.1.2 *Tannins*

Tannins are secondary metabolites that fall under the phenolic compounds. They are found in almost all plant parts such as stems, roots, leaves, seeds, fruit and bark. Tannins defend plants against pathogens, herbivores, and insects because they have the ability to halt substrate availability to microbial cells and precipitate proteins (Das *et al.,* 2020; Javed *et al.,* 2020).

There are two classes of tannins, namely; condensed and hydrolysable tannins, which differ due to the availability of a sugar core in their structure (**Figure 2.3**). Hydrolysable tannins comprise a glucose molecule while condensed tannins are primarily polymerised flavan-3-ols (Girard *et al.,* 2019). As antimicrobial agents, they destabilise the cell wall and membrane of microorganisms, and further interfere with gene expression and oxidative phosphorylation of the pathogen (Vaou *et al.,* 2021). Tannins have iron-chelating properties, therefore preventing bacterial growth due to the limited iron availability (Farha *et al*., 2020), and stopping biofilm formation of certain microbes as well such as *Pseudomonas aeruginosa* by interfering with their quorum sensing (Villanueva *et al*., 2022). Additionally, it was shown that the common hydrolysable tannin (tannic acid) can prevent *Staphylococcus aureus* bacteria from initial adherence to surfaces and from generating polysaccharides, which are intercellular adhesion molecules (Slobodníková *et al.,* 2016).

Amongst other commercial functions, tannins are used in industry as anti-corrosive agents, leather treatment and for mineral absorption (Akhlash and Sunil, 2019; Kaghazchi *et al.,* 2020). Furthermore, tannin extracts contain proanthocyanidins,

which can form complexes with proteins through hydrogen bonds, therefore converting raw skin to leather (Jordaan and Van Der Westhuizen, 2013).

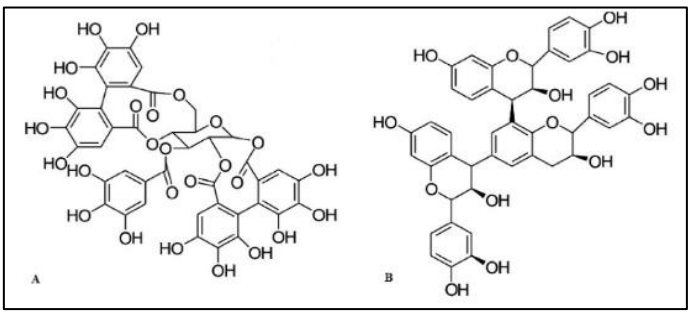

Figure 2.3: Structure of the two classes of tannin compounds, (**A**) hydrolysable tannin with a glucose molecule and (**B**) condensed tannin (Raja *et al.,* 2014).

2.5.2 Saponins

Saponins are a group of compounds that have two sugars in their structure and are mostly present in foods such as lentils, soybeans and legumes (di Gioia and Petropoulos, 2019). These phytochemicals possess a polycyclic aglycone that is connected to a C3 and an ether bond (**Figure 2.4**). Plants produce saponins in abundance during pathogenic attacks and saponins are therefore found in plant tissues that are susceptible to bacterial and fungal assaults. Saponins are non-volatile and can easily dissolve in water. Due to their solubility, saponins are used to increase cellular macromolecule penetrations and are very useful in the production of cosmetic products and as vaccine adjuvants (Ravi and Manasvi, 2016). Saponins are also known to possess notable cytotoxic, antiproliferative and antitumor activities. Furthermore, a class of saponins, known as the triterpenoids, has been reported to have antiviral properties as well (Elekofehinti, 2021).

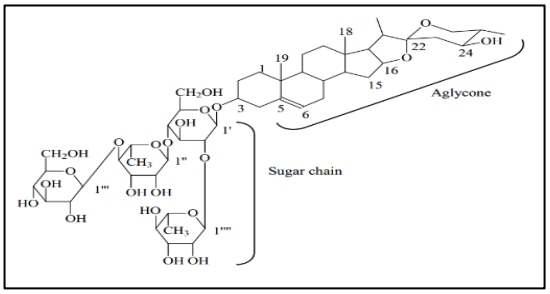

Figure 2.4: Structure of typical saponin compound with a sugar chain and polycyclic aglycone that is connected to a C3 and an ether bond (Moghimipour and Handali, 2015).

2.5.3 Alkaloids

Alkaloids are basic compounds with a nitrogen in their heterocyclic ring, as illustrated in **Figure 2.5**, and are normally obtained from amino acids. The biochemical properties of alkaloids are attributed to their precursors and different final structure (Ncube *et al.,* 2008). They are bitter-tasting phytochemicals that are mostly concentrated in plant stems and are often toxic. Common examples of alkaloids include morphine, caffeine, and nicotine. Galantamine, mostly found in bulbous flowers including *Narcissus spp* and *Galanthus spp,* is another example of alkaloids that are used to treat Alzheimer's diseases (Awuchi, 2019; Santos *et al*., 2020). Approximately 20 percent of plants with flowers produces alkaloids and most of those agents act as insecticides (Croteau *et al.,* 2000). Alkaloids possess several therapeutic activities such as antispasmodic, antifungal, antimalarial and antiviral activities. In addition, they destabilise the microbial cellular membrane and can act as enzyme inhibitors (Builders, 2019; Vaou *et al.,* 2021). Isoquinoline alkaloid is an alkaloid compound isolated from Bark of *Phellodendron amurense Rupr.* with antimycobacterial activity against *Mtb* (Balážová *et al.,* 2022; Gautam *et al,* 2023).

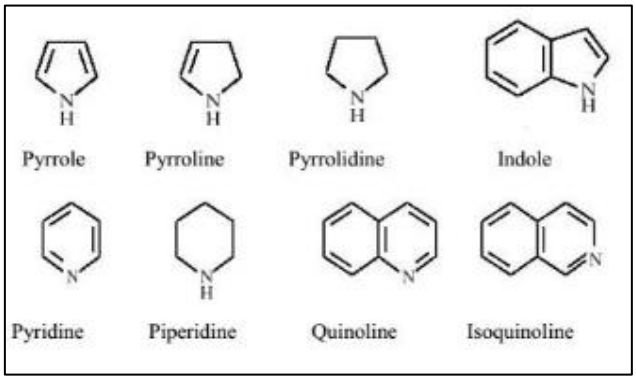

Figure 2.5: Structures of isolated alkaloids from medicinal plants with a nitrogen element (Bribi, 2018).

2.5.4 Steroids

Steroids are a network of lipophilic molecules and their structure consist of four-ringed organic molecules (**Figure 2.6**). There are distinct groups of steroids isolated from plants, which include steroid alkaloids, cardiac glycosides, steroid saponins, stigmastane, androstane, cholestane, pregnane and sterols. Brassinosteroids are also part of the steroids family, and they contribute to plant growth and hormonal functions (Di Gioia and Petropoulos, 2019; Harneti and Supratman, 2021). Pregnane glycosides are yet another group of steroids isolated from various plant families, such as

Asclepiadaceae and Zygophyllaceae, from which researchers have reported numerous biological activities, namely, anti-inflammatory, immune suppressive, antidepressive, antioxidative, anti-tumour and antibacterial activities (Feng *et al.,* 2008; Panda *et al*., 2003; Song *et al*., 2014; Si *et al.,* 2022). *Boswellia serrata* produces steroid compounds such as boswellic acid that aids in the treatment of pain and burn wounds (Morsy *et al.,* 2019).

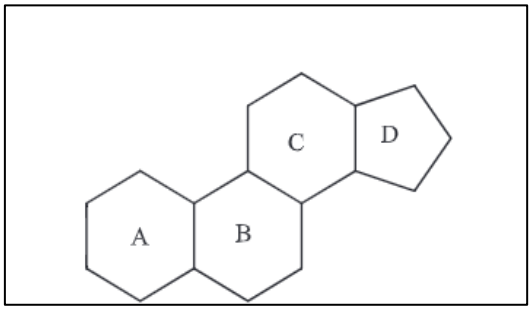

Figure 2.6: Ring structure of organic steroid compounds (Di Gioia and Petropoulos, 2019).

2.5.4.1 Cardiac glycosides

Cardiac glycosides are a group of steroids with similar structures comprised of a sugar group, lactone, and steroid ring (**Figure 2.7**). They have a small structure compared to other steroids. Cardiac glycosides are known to improve the tone of cardiac muscle by elevating systolic contractions and are therefore used to treat atrial fibrillation. Digoxin, a cardiac glycoside isolated from *Digitalis purpurea* (foxglove) that has been approved by the food and drug administration committee as treatment for heart failure (Shakya, 2016). Most isolated cardiac glycosides are not used in drug development because they are often found to be toxic (Bartnik and Facey, 2017). Hunters in the regions of South America, Asia and Africa have taken advantage of the cardiac glycosides' toxicity and use them as poison on their arrows during hunting (Morsy, 2017). Cardiac glycosides have other functions that are unrelated to the heart. Anthraquinones, a group of cardiac glycosides isolated from the medicinal plant *Senna alexandrina,* are the main compounds responsible to relieve constipation and stomach pains (Elansary *et al.,* 2018). Furthermore, this class of secondary metabolites is also well-known for its antifungal and antibacterial properties.

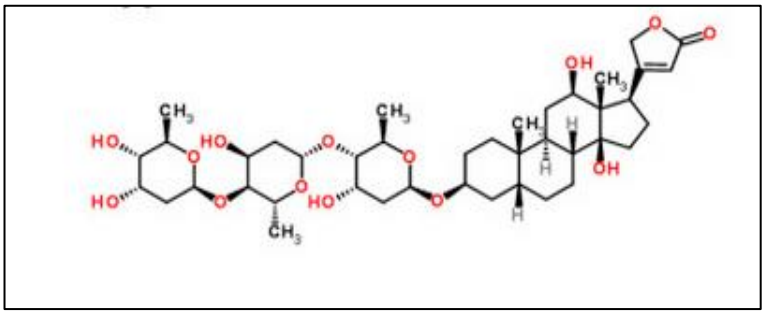

Figure 2.7: Structure of cardiac glycosides containing a sugar group, lactone, and steroid ring (Botelho *et al.,* 2019).

2.5.5 Terpenes and terpenoids

Terpenes are described as low molecular weight, aromatic compounds produced by plants, while terpenoids are modified terpenes (**Figure 2.8**). They both take part in electron transfer reactions and are constituents of cell membranes (Petrović *et al.,* 2019). Terpenoids differ with terpenes by having an extra oxygen molecule on their hydrocarbon structure (Croteau *et al.,* 2000). Oils containing terpenes tend to have a good scent, which gives them an advantage of being used in industry for the production of cosmetic products such as perfumes. The essential oils of *Thymus vulgaris* contains thymol, a terpene compound that serves as an antiseptic, anti-inflammatory and antifungal agent (Awuchi, 2019). Oils extracted from *Cinnamomum osmophloeum* contain terpenes and were found to have antibacterial activity against *E. coli*, *S. aureus* and *Enterococcus spp.* (Ncube *et al.,* 2008). Terpenes serve to regulate the defence mechanism of plants against attacks from pathogen and herbivores (Erb and Kliebenstein, 2020).

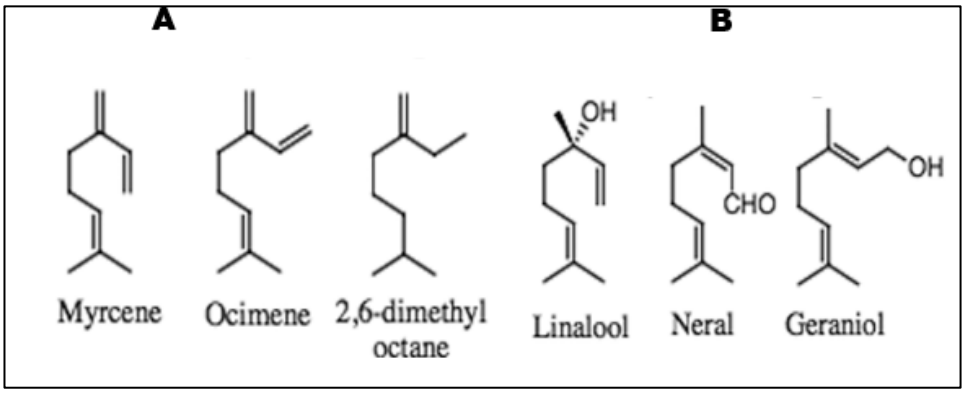

Figure 2.9: Hydrocarbon structures of (**A**) Acyclic monoterpenes and (**B**) Acyclic monoterpenoids with extra oxygen molecule (Masyita *et al.,* 2022).

2.6 Biological activities of compounds derived from medicinal plants

Plants are rich in phytochemicals with a wide range of health benefits. This has fuelled the intense interest in developing novel drugs using plants as natural sources (Jamshidi-Kia *et al.,* 2018). Alongside their antimicrobial activity, these plant secondary compounds also have antioxidant, anti-inflammatory and antifungal activities (Ncube *et al.,* 2008). Phenolic compounds are the ubiquitous largest group of phytochemicals produced by plants, contributing to the numerous biological activities medicinal plants possess. Among other phenolics, flavonoids are present in significant quantities in plant parts and have a number of documented medicinal functions, including, anti-inflammatory, antidiabetic, antioxidative, anti-allergic, antimicrobial, and anticancer properties (Bengal, 2019). It is imperative to find new natural, highly effective, easily accessible, and safe medicinal plants that can help in the development of new anti-TB drugs because medicines developed from synthetic compounds have proven to have severe side effects (Pourmorad *et al.,* 2006; Karimi *et al.,* 2015; Vaou *et al*., 2021).

2.6.1 Antioxidative activity

Antioxidants are molecules with the ability to mitigate oxidation during cellular redox reactions by donating hydrogens and quenching single oxygen molecules. Synthetic antioxidants, such as butylated hydroxyl anisole, propyl gallate, and butylated hydroxyl toluene (Taghvaei and Jafari, 2015), are used as food supplements; however, natural products are still preferred due to safety concerns, such as the development of allergies, neurological anomalies, and asthma. Many plants produce phytochemicals that possess antioxidative properties, with phenolics being most notable (Anand and Sati, 2013; Khatiwada *et al.,* 2018). Antioxidants are divided into 2 classes, primary antioxidants that function to interfere with oxidation reactions as well as secondary antioxidants that react directly with free radicals or pro-oxidants and can scavenge reactive oxygen molecules produced during normal metabolic reactions such as nitric oxide, singlet oxygen, the hydroxyl radical ($^{\cdot}OH$) and hydrogen peroxide (H_2O_2) (Ifeanyi, 2018). In the absence of antioxidants, the accumulation of free radicals and reactive oxygen species causes oxidative stress that leads to the impairment of nucleic acids, proteins, and lipids (Mangwani *et al.,* 2020). Among other key risk factors for the development of TB infection into active TB disease is a low nutritional status (Cegielski and McMurray, 2004; WHO, 2013).

In addition to low levels of antioxidant vitamin concentrations in the blood, oxidative stress has been linked to elevated levels in TB patients, which could be a result of contributing factors like poor diet and uncontrolled inflammation (Vijayamalini and Manoharan, 2004; Madebo *et al.,* 2003). Vitamins and carotenoids, which are antioxidant substances, help prevent possible harm from free radicals (Wintergerst *et al.,* 2007; Aslani and Ghobadi, 2016). N-acetyl cysteine (Oberley-Deegan *et al.,* 2010) and manganese (II) meso-tetrakis-(N-methylpyridinium-2-yl) porphyrin (Oberley-Deegan *et al.,* 2009) are some of the antioxidants that have been reported to prevent the growth of TB bacilli within tubercular abscesses in a host. Therefore, it is important to take antioxidant supplements along with the recommended treatment regimen and/or follow proper diet that will provide one with adequate nutrients, vitamins and minerals when infected with TB to help boost the immune system and speed up the healing process.

2.6.2 Anti-inflammatory activity

One of the multiple defence systems the body employs in response to foreign agents is inflammation (Torres-Moreno *et al.,* 2019, Romeo *et al.,* 2019). This response also helps to repair tissue and organ damage, and it can be acute or chronic. Acute inflammation is a consequence of non-specific immune responses, does not last long and the response is identified by the production of fluid from white blood cells called pus. On the other hand, chronic inflammation is a long-lasting immune response that releases fibrous tissues from fibroblasts and collagen as a response (Arulselvan *et al.,* 2016). The type of inflammatory response depends on the pathogenic infection as well as the mediators produced to regulate and maintain the response. Chronic inflammation can lead to diseases like cancer, diabetes, cardiovascular disease and arthritis, amongst others, and it is, therefore, important to counteract the inflammation with natural agents possessing anti-inflammatory activity (Pahwa *et al.,* 2022). After the activation of an immune response during a TB infection, *Mtb* ligand(s) interact with toll-like receptors (TLRs) on innate immune cells like macrophages and dendritic cells through a Myeloid differentiation primary response protein 88 (MyD88)-dependent pathway, which will ultimately lead to nuclear transcription factor (NF)-B activation and the production of proinflammatory cytokines like tumour necrosis factor (TNF)-, nitric oxide, chemokines, interleukin (IL)-1, and IL-12. However, *Mtb* can develop mechanisms that cause signals to inhibit or regulate the innate immune response in

its favour (Jo, 2008; Harding and Boom, 2010.). Signalling of *Mtb* lipoprotein TLR2 for a long time inhibits major histocompatibility (MHC-II) synthesis and macrophage antigen processing. This then causes some of the infected macrophage to be unable to transmit *Mtb* antigens to CD4+ T cells, leading to reduced effector T cell activation; therefore, enabling *Mtb* to evade immune surveillance and create techniques in which it can survive and thrive (Noss *et al.,* 2001; Fulton *et al.,* 2004; Pai *et al.,* 2004). To reduce the possibility of causing excessive inflammation that could harm host tissues, TLR-induced proinflammatory signals must be limited (Rothlin *et al.,* 2007). Inflammatory response has also been associated with protein denaturation inside a host (Osman *et al.,* 2016).

Substances that can prevent protein denaturation may be of great importance in the development of anti-inflammatory drugs. One of the characteristics of many non-steroidal anti-inflammatory drugs (NSAIDs) has been characterised as their ability to protect or maintain heat-treated albumin at the physiological pH (pH: 6.2-6.5) (Williams *et al.,* 2008; Mendez-Encinas *et al.,* 2023). However, as an alternative to non-steroidal anti-inflammatory medicines (NSAIDs), which have shown to develop side effects, new safe, innovative sources of anti-inflammatory plant extracts are tested for their *in vitro* anti-inflammatory efficacy using the thermal inhibition of egg albumin protein denaturation (Osman *et al.,* 2016; Gunathilake *et al.,* 2018; Dharmadeva *et al.,* 2018). Some exogenous and endogenous substances, including medications, are carried through the blood to different sites/organs by plasma albumin using binding ligands, although bad nutrition status and chronic inflammation contribute to the breakdown of this plasma albumin thus affecting its normal function (Bisaso *et al*., 2014). Therefore, considering the infectious nature of *Mtb* and its ability to manipulate immune response, thus contributing to prolonged inflammatory response, it is deemed paramount to search for alternative drugs as anti-inflammatory agents and it will be more advantageous if it is the same drug with antimicrobial effect against *Mtb.* Several plant species from the *Artemisia* genus, including *A. annua* and *A. abrotanum* (Adewumi *et al.,* 2020)*,* have phytochemicals with documented chronic inflammation inhibition. This anti-inflammatory benefit offered by plants is an attribute of phytochemicals that include saponins, steroids, phenols, terpenoids and steroids (Shakya, 2016; Truong *et al.,* 2019).

2.6.3 Antimycobacterial activity

The discovery of antibiotics was thought to have brought a solution to combat infectious microorganisms. However, these pathogens continue to develop mechanisms to resist the effects of drugs that are designed to slow their growth or kill them (Gill *et al.,* 2015). The inappropriate use of antibiotics in agriculture and health care facilities has contributed greatly to bacterial resistance because the microbes find new ways to survive in their presence (Javed *et al.,* 2020). Despite efforts by pharmaceutical companies to develop new synthetic antibiotics, antibiotic resistance remains a problem to date (da Cunha *et al.,* 2019).

Medicinal plants are loaded with natural compounds that act as antimicrobial agents and can either be used individually or in combination to inhibit the growth of a variety of microorganisms (Shakya, 2016). Phenolics, steroids, alkaloids and triterpenes are classes of phytocompounds with exceptional antimicrobial activity (Anza *et al.,* 2015; Rex *et al.,* 2018; Vaou *et al.,* 2021). Phytocompounds with extensive antimicrobial properties include gallic acid, flavonols, and quercetin (Builders, 2019). Caffeic acid alkyl esters, a group of phenolic acids, have good antibacterial activity against disease-causing bacteria such as *Staphylococcus aureus*, *Escherichia coli* and *Mtb* (Andrade *et al.,* 2015). Caffeic acids are cinnamic acid derivatives and were previously used in combination with other anti-TB drugs like rifampicin to treat TB infections, but their usage was discontinued due to the lack of evidence available about their toxicity (Sova, 2012). There is a huge quantity of phytocompounds with antibacterial activity, yet continuous research for the discovery and development of new drugs is vital because microbes are becoming increasingly resistant to current antibiotics (Vaou *et al.,* 2021).

2.6.4 Antibiofilm activity

Bacteria evolve to adapt and survive nutrient limitations and environmental changes (Nguyen *et al.,* 2021). One mechanism implemented for their survival is the creation of a biofilm, which forms when a mass of bacteria fixes to each other and/or a surface. The ability of bacteria to form these clusters enables them to communicate effectively and thus continuously develop new systems to resist antibiotics (Vestby *et al.,* 2020). In addition to this efficient communication channel, this mass of bacteria also produces various extracellular polymeric substances (EPS) on their biofilm surroundings (matrix) for protection (Toyofuku *et al.,* 2016). The power of biofilms is enhanced by

the gene expression of the distinct multiple cells clustered together. Bacteria adapt in diverse ways due to their range of phenotypes, and they employ quorum sensing as a means of communication to control the expression of survival genes. Additionally, the cells share plasmids with resistant genes through conjugation (Kuchma and O'Toole, 2000; Donlan and Costerton, 2002).

It is practical for researchers to investigate a single microbial strain at a time in the search for alternative antimicrobials. However, it becomes increasingly challenging to investigate a community of microorganisms as each of them evolves differently and assists one another in becoming tolerant to their environmental conditions. Formation of biofilms makes it evidently difficult for researchers to find the minimum inhibitory concentration (MIC) of antimicrobial agents following treatment of the microorganism (Wu *et al.,* 2015). The MIC value is the lowest concentration that prevents the growth of planktonic cultures; however, due to numerous factors, such as the developed EPS matrix, high cell density, resistant mutations, and persistent cells, there is less adsorption and penetration on biofilm cells (Khan, 2021).

Organisms like *Mtb* and *Mycobacterium smegmatis* are known to form biofilms in their natural environment. They contain mycolic acids in their cell walls, affording them the special character of high resistance to antibiotics, antibiofilm agents and disinfectant (Esteban and García-Coca, 2018). Microbial drug resistance and ability to form biofilms qualify the need for development of new drugs from natural sources (Miethke *et al.,* 2021). Current popular reservoirs of natural compounds with several biological activities are medicinal plants. They contain phytochemicals like tannins and flavonoids that have antioxidative, anti-inflammatory, antimicrobial properties and can decrease the formation of biofilms by microbes (Shamsudin *et al.,* 2022).

2.7 *Artemisia afra*

The development of drugs using medicinal plants is attributed to the distinct phytochemicals they produce (Motshudi *et al.,* 2021). *Artemisia afra* Jacq. ex Willd. is a popular plant traditionally used to treat different diseases such as asthma, cough, influenza, and diabetes in Africa (Motshudi *et al*., 2021), particularly in Zimbabwe, South Africa, Ethiopia, Lesotho, Namibia, and Swaziland (Liu *et al.,* 2009, Germishuizen *et al*., 2006). The plant is an evergreen plant with little yellow flowers (**Figure 2.10**) and grows from 0.6 to 2 m tall. The plant is mainly found on the eastern

and southern coasts of Africa (du Toit and van der Kooy, 2019). *Artemisia afra* forms part of the *Asteraceae* family – a family with plants from which many well-known phytochemicals such as flavonoids, terpenes, sterols, terpenes, and acetylenes have been isolated. Plants belonging to this family have a rather astringent taste that is associated with the presence of terpenes and terpenoids (Adewumi *et al*., 2020). The antibacterial activity of *A. afra* has been proven against common diseases-causing organisms such as *S. aureus* and *Mtb* (Haile and Jiru, 2022). *Artemisia afra* and *Artemisia annua* are well-known for producing artemisinin – a drug used to treat malaria; however, *A. afra* does not produce it in large amounts. (Martini *et al.,* 2020). In addition, *A. afra* also has antiplasmodial and anti-inflammatory properties (Shinyuy *et al.,* 2023).

Figure 2.11: *Artemisia afra* plant (**A**) leaves, which are usually used in the treatment of colds, flu, respiratory infections, etc., and (**B**) the yellow flowers that normally appear in the late summer and autumn (van der Walt, 2004; du Toit and van der Kooy, 2019).

2.8 Aims and objectives

2.8.1 Aim:

To investigate the efficacy of antioxidative, anti-inflammatory and antimycobacterial activities of *Artemisia afra* extracts and sub-fractions.

2.8.2 Objectives:

- Evaluate the phytochemical profile of *A. afra* plant extracts;
- investigate the antioxidative activity of *A. afra* extracts;
- evaluate the anti-inflammatory effects of *A. afra* extracts;
- determine the antimycobacterial activities of *A. afra* against *M. smegmatis*;

- to obtain subfraction(s) from *A. afra* extracts with antioxidative, anti-inflammatory and antimycobacterial activities;
- evaluate the effects of *A. afra* acetone extract and subfraction on growth of *M. smegmatis* after treatment.

2.9 References

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CHAPTER 3

3. Extraction, phytochemical analysis, and screening

3.1 Introduction

Medicinal plants are natural sources of key compounds that play an extensive role in the advancement of novel drugs that can help treat complicated diseases like TB (McGaw *et al*., 2019). Secondary metabolites are substances produced by plants as a form of defence against herbivores, microbial invasions, and adverse environmental conditions. Therefore, researchers explored these phytochemicals' effectiveness against disease-causing microorganisms in humans and/or animals because of their notable purpose in plants, and numerous biological activities have been documented regarding them (Shakya, 2016). *Artemisia afra* is an herbal medicinal plant, natively known as *Umhlonyane* and *Lengana*, in South Africa. It is traditionally used in the treatment of sicknesses such as influenza, inflammation, colds, headaches, coughs, and asthma. *A. afra* is a plant of interest in TB treatment research because patients present the above-mentioned illnesses as some of the TB symptoms (Liu *et al.,* 2009).

To obtain the variety of compounds synthesised by medicinal plants, extraction is a process that is used because it helps to maximise the quantity of phytochemicals released from the plant material. The quality and effectiveness of extraction depends on the method that is used, duration, solvents, and the nature of the plant material, either dry or wet. Selection of solvents for extraction is important because different phytochemicals solubilise differently in the solvents due to their various polarities (Chang *et al.,* 2002; Ajanal *et al.,* 2012). Although recently many solvents have been established for effective extraction, methanol, acetone, water, and ethanol are common solvents for most of the successful extractions (Mahdi-Pour *et al*., 2012). The yield and quality of the phytochemicals extracted affect the prospective biological activities of that particular plant (Turkmen *et al*., 2006; McDonald *et al*., 2001). Soxhlet extraction, percolation, maceration, decoction, sonication, and infusion are some of the extraction techniques used (Handa *et al.,* 2008; Azwanida, 2015). Various separation methods, such as thin layer chromatography (TLC), which is conventional, easily applicable, and fast method of phytochemical analysis, are used to separate and isolate pure compounds from medicinal plants. This method is commonly used due to its ability to present the prospective quality of phytochemicals present in the

plant extracts even before one can proceed to other assays or isolation techniques (Bulugahapitiya, 2013). Several phytochemicals, namely, cardiac glycosides, phenolics, flavonoids, tannins and saponins were found in *A. afra* extracts, which are attributed to the plant's antibacterial, antioxidant and anti-inflammatory properties (Haile and Jiru, 2022). The objective of this chapter was to use qualitative and quantitative methods to evaluate the phytochemical profile of the plant extracts.

3.2 Materials and Methods

3.2.1 Plant collection and storage

The aerial parts of *Artemisia afra* plant were collected in *autumn* at Haenertsburg (Limpopo), South Africa. The verification of the plant was done at the Larry Leach Herbarium (UNIN) at the University of Limpopo by Dr E Brownyn. The plant parts were dried at an ambient temperature in the dark. Thereafter, the dried parts were then ground to fine powder using a desc scientific blender (Immelman, model: 722) and preserved in an airtight container in the dark until further use.

3.2.2 Preliminary extraction and phytochemical analysis

3.2.2.1 Extraction

Finely ground plant material (1 g) was extracted with 10 mL of solvents of varying polarity, namely, hexane, chloroform, dichloromethane, ethyl acetate, acetone, ethanol, methanol, butanol, and water. Each mixture was placed on a shaking incubator (New Brunswick scientific co. G-25, USA) for 30 minutes at 200 rpm and the procedure was repeated twice for 20 minutes each to enable exhaustive extraction of the plant material. The crude extracts were filtered through Whattman no. 1 filter paper (Whattman international Ltd., CAT:1004110, Maidstone, England) and poured into pre-weighed glass vials. The solvents were evaporated under a stream of cold air at room temperature, and the mass of each dry extract was recorded. The extracts were then reconstituted to a final concentration of 10 mg/mL in acetone.

3.2.2.2 Qualitative phytochemical analysis

The phytochemical profiling of the extracts was done by loading 10 µL of each extraction aluminium-backed TLC plates (ALUGRAM® SILg/ UV 254-365 Macherey-Nagel, Germany) and developing the TLC plates without delay to minimise photo-oxidative change to the phytocompounds. The plates were developed in 3 mobile phase systems developed by Kotze and Eloff (2002); benzene: ethanol: ammonium

hydroxide: 18:2:0.2 [BEA] (nonpolar and basic), chloroform: ethyl acetate: formic acid: 10:8:2 [CEF] (intermediate polarity and acidic) and ethyl acetate: methanol: water: 40:5.4:5 [EMW] (polar). Upon removal from the TLC tanks, the plates were dried under a stream of air at an ambient temperature to evaporate the solvents. The separated components were visualised under visible and ultraviolet light (254 and 365 nm) (Sigma-Aldrich, Spectroline® E-series Z169625-1EA UV lamp). For the detection of non-fluorescent chemical compounds, the chromatograms were sprayed with vanillin-sulphuric acid reagent [0.1 g vanillin: 28 mL methanol: 1 mL concentrated sulphuric acid (H_2SO_4)] and heated at 110°C for 2 minutes for colour development.

3.2.3 Phytoconstituents screening

3.2.3.1 Saponins

The presence of saponins in the dried plant material was tested using a persistent frothing test described by Odebiyi and Sofowora (1978). Exactly 1 g of the powdered leaf sample was suspended in 30 mL distilled water (dH_2O). The mixture was vigorously shaken for at least 5 minutes by hand, transferred into a glass vial, which was then suspended into a beaker containing water and heated until there was a formation of froth. The sample was observed for the formation of froth to draw an inference.

3.2.3.2 Terpenes/ terpenoids

The presence of terpenoids in the dried plant material was verified using the Salkowski test, as described by Odebiyi and Sofowora (1978). The powdered leaf sample (0.5 g) was mixed with 2 mL of chloroform followed by careful addition of 3 mL H_2SO_4. The mixture was observed for a reddish-brown colour change to draw an inference.

3.2.3.3 Phlobatannins

The presence of phlobatannins in the plant material was verified using the method described by Borokini and Omotayo (2012). The powdered leaf material (0.2 g) was dissolved in 10 mL of distilled water (dH_2O) and put into shaker incubator (New Brunswick scientific co. G-25,USA) at 200 rpm for 20 minutes, and then filtered through Whatman no.1 filter paper (Whatman international Ltd., CAT:1004110,Maidstone,England), and poured into a glass vial. The filtrate was boiled with 2 mL of 1% hydrochloric acid (HCl) in a beaker with water for at least 10 minutes.

The sample was observed for the formation of a red coloured precipitate to draw an inference.

3.2.3.4 Tannins

The method described by Borokini and Omotayo (2012) was used to test for the presence of tannins in the plant material. The powdered leaf sample (0.2 g) was dissolved in 10 mL of distilled water and put into a shaker incubator (New Brunswick scientific co. G-25,USA) at 200 rpm for 20 minutes; filtered through Whatman no. 1 filter paper (Whatman international Ltd., CAT:1004110,Maidstone,England) and poured into a glass vial, and then put inside a beaker with water and boiled gently for at least 10 minutes. Thereafter, it was cooled to a room temperature. One millilitre (1 mL) of the cooled solution was transferred to a clean test tube and 3 drops of 1% ferric chloride ($FeCl_3$) were added. The sample was observed for colour change (blue-black, brown-green, green or blue-green) to draw an inference.

3.2.3.5 Cardiac glycosides

The Keller-Killiani test described by Odebiyi and Sofowora (1978) was used to test for the presence of cardiac glycosides in the dried plant material. Two millilitres (2 mL) of glacial acetic acid was added to 0.5 g of powdered leaf material followed by a single drop of 0.1% $FeCl_3$ solution and 1 mL of H_2SO_4. The sample was observed for colour change (brown colour with a grey ring) to draw an inference.

3.2.3.6 Flavonoids

The method described by Borokini and Omotayo (2012) was used to detect the presence of flavonoids in the dried plant material. Ammonia (5 mL) was added to the whole aqueous filtrate portion of the plant material that was prepared by mixing 0.5 g of dried plant material with 25 mL dH_2O and filtered through Whatman no. 1 filter paper (Whatman international Ltd., CAT:1004110, Maidstone, England) and poured into a different glass vial, followed by the addition of 1 mL H_2SO_4. The sample was observed for colour change (yellow precipitate) to draw an inference.

3.2.3.7 Steroids

The method described by Borokini and Omotayo (2012) was used to detect the presence of steroids in the plant material. Two millilitres (2 mL) of acetic anhydride ($C_4H_6O_3$) were added to 0.5 g of the powdered leaf sample, followed by the addition

of 2 mL of H_2SO_4. The sample was observed for colour change (violet to blue or green) and/or (brown-red) to draw an inference.

3.2.3.8 Alkaloids

Drangendoff's reagent method, described by Odebiyi and Sofowora (1978), was used to test for the presence of alkaloids in the plant material. Finely ground leaf material (0.5 g) was extracted with 95% ethanol (5 mL) in a shaking incubator (New Brunswick scientific co. G-25, USA) at 200 rpm for 10 minutes and filtered through Whatman no. 1 filter paper (Whatman international Ltd., CAT:1004110,Maidstone,England) using a Buchner funnel. The filtered solvent was allowed to dry under a stream of cold air at room temperature. The dried extract was re-dissolved in 5 mL of 1% HCl, followed by the addition of 5 drops of Drangendoff's reagent. A colour change (orange to orange-red) was observed to draw an inference.

3.2.4. Quantification of polyphenols

3.2.4.1 Total phenolic content

The quantity of phenolics present in each plant extract was determined by using the Folin-Ciocalteu reagent method (Velioglu *et al.,* 1998) with minor modifications (Humadi and Istudor, 2008). Each plant extract (10 mg/mL) was reduced to a concentration of 5 mg/mL in a test tube to form a stock. Exactly 100 µL of each stock was transferred to a clean test tube and diluted with 900 µL of dH_2O, followed by the addition of 100 µL of Folin-Ciocalteu reagent. To stop the reaction, 1 mL of 7% sodium carbonate (Na_2CO_3) was added and the mixture was incubated in the dark at room temperature for 30 minutes. A blank sample was prepared similarly, with the plant extract replaced by 100 µL acetone. The absorbance of each sample was measured using an ultraviolet/visible (UV/VIS) spectrophotometer (Thermo Scientific, CAT:840-209800, Genesys 10S UV-VIS) at 550 nm. Gallic acid reference standard solutions (0.08, 0.16, 0.31, 0.63, 1.25, mg/mL) were prepared from a stock of 2.5 mg/mL, followed by serial dilutions throughout the test tubes and their absorbances were measured against the blank. The total phenolic content was expressed as milligram gallic acid equivalents per gram of the extract (mg of GAE/g extract) calculated using the equation obtained from gallic acid standard curve ($y= 2{,}0459x + 0{,}0676$, $R^2 = 0{,}9985$).

3.2.4.2 Total tannin content

The Folin–Ciocalteu method described by Tambe and Bhambar (2014) was used to determine the tannin content in the plant extracts. Briefly, 50 µL of 10 mg/mL plant extract was added to a clean test tube containing 3.8 mL of dH_2O. The Folin–Ciocalteu reagent (0.25 mL) was added to the mixture and vortexed, followed by the addition of 0.5 mL of a 35% solution of Na_2CO_3. The mixture in the tube was made up to 10 mL by the addition of dH_2O. The mixture was vortexed thoroughly and incubated at room temperature for 30 minutes in the dark. A blank was prepared in the same manner as the test solutions with the extract substituted by 50 µL of acetone. The absorbance of each sample was measured using the UV/VIS spectrophotometer (Thermo Scientific,CAT:840-209800, Genesys 10S UV-VIS) at 725 nm. Gallic acid reference standard solutions (1, 0.5, 0.25, 0.125, 0.625 mg/mL) were prepared from a stock of 2 mg/mL, followed by serial dilutions throughout the test tubes and the absorbances for the solutions were measured against the blank. Tannin content was expressed as milligrams of Gallic acid equivalents per gram of extract (mg GAE/g extract) calculated using the equation obtained from the Gallic acid standard curve ($y=0,5085x-0,0059$,$R^2 = 0,9991$).

3.2.4.3 Total flavonoid content

Total flavonoid content was determined by the aluminium chloride colorimetric assay described by Tambe and Bhambar (2014). Briefly, 100 µL of 10 mg/mL of each plant extract was added to 4.9 mL of dH_2O, followed by the addition of 300 µL of 5% sodium nitrite ($NaNO_2$) and incubated at room temperature for 5 minutes. Thereafter, 300 µL of 10% aluminium chloride ($AlCl_3$) was added to the reaction mixture. The reaction was allowed to stand for 5 minutes at room temperature, after which 2 mL of 1M sodium hydroxide (NaOH) was added. The mixture in the test tube was then made up to 10 mL with dH_2O. A blank sample was prepared in the same manner as the experimental samples; however, the plant extracts were substituted with 100 µL dH_2O. Quercetin was used as a standard. Different concentrations (31.25 , 62.5, 125, 250, 500 µg/mL) of the quercetin were prepared using the same method as that used to prepare the extracts. The absorbance of the experimental samples and the standard were measured using a UV/VIS spectrophotometer (Thermo Scientific, CAT:840-209800, Genesys 10S UV-VIS) at a wavelength of 510 nm. The total flavonoid content of the samples was expressed as milligrams of quercetin equivalents per gram of extract (mg

QE/g extract) calculated using the equation obtained from the quercetin standard curve (y=0,1129x+0,0043, R^2 = 0,9993).

3.3 Statistical analysis

Where appropriate, the experiments were conducted in triplicates for each plant extract and the results were expressed as means±standard deviation (SD) of triplicate determinations. Statistical analysis was performed by Microsoft Excel.

3.4 Results

3.4.1 Preliminary extraction

Extraction procedure was carried out with nine organic solvents, namely; Hexane, chloroform, dichloromethane, ethyl acetate, acetone, ethanol, methanol, butanol, and water to extract phytochemicals from *Artemisia afra* plant material. The results depicted in **Figure 3.1** show the different masses extracted, where it was observed that all the selected solvents for extraction had quite a good extraction capacity above 50 mg. Although ethanol and water as polar solvents had the highest masses of 102 mg and 100 mg, respectively. The most non-polar solvent, hexane extracted the lowest mass of 63 mg.

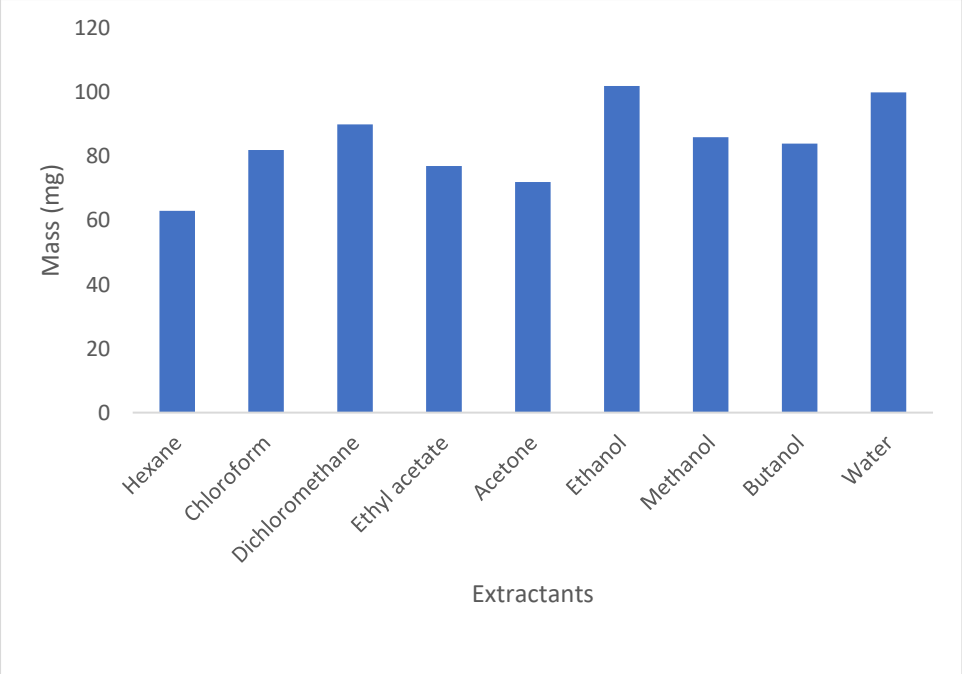

Figure 3.1: Mass of *Artemisia afra* extracts extracted from 1 g of dried plant parts using solvents of varying polarity: Hexane. Chloroform, Dichloromethane, Ethyl acetate, Acetone, Ethanol, Methanol, Butanol, and water.

3.4.2 Phytochemical analysis

3.4.2.1 Qualitative phytochemical analysis

Qualitative and quantitative methods were used to analyse the phytochemicals present in the plant material and extracts. TLC was utilised as a separation technique to analyse the phytochemical profiles of the extracts and **Figure 3.2A** and **Figure 3.2B** are the results of the fluorescing phytochemicals present in the plant extracts, where it was revealed that there were more bands of fluorescing phytocompounds visualised under the UV light at 365 nm (**Figure 3.2B**) after the TLC plates were developed in three mobile systems, BEA, CEF and EMW, as compared to those visualised with 254 nm (**Figure 3.2A**). (Vanillin) sulphuric acid reagent was used as another visualisation method for non-fluorescing phytochemicals, and it was observed that more compounds were able to separate on the chromatogram developed in the non-polar mobile system (BEA) as compared to the plates developed in the other mobile systems (**Figure 3.2C**).

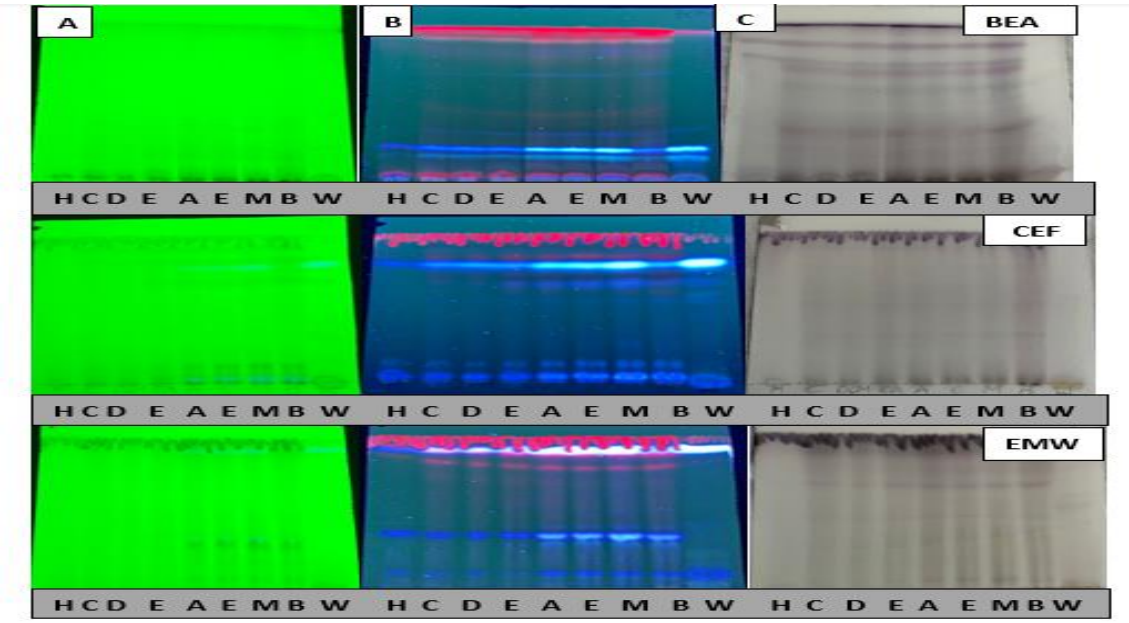

Figure 3.2: Chromatograms of *Artemisia afra* dried plant material extracted with different solvents and developed in BEA, CEF and EMW mobile systems. Thereafter, visualised under UV light at 254 nm (**A**) and 365 nm (**B)** for fluorescing and sprayed with vanillin sulfuric acid for non-fluorescing compounds (**C**).

Key: H:Hexane, C:Chloroform, D:Dichloromethane, E:Ethyl acetate, A:Acetone, E:Ethanol, M:Methanol, B:Butanol and W:Water.

3.4.2.2 Preliminary screening of Phytoconstituents

The diverse number of phytochemicals present in the dried plant material was screened using standard chemical tests. *A. afra* aerial plant parts possess most of the tested common phytochemicals, saponins, flavonoids, tannins, steroids, cardiac glycosides and terpenoids (**Table 3.1**).

Table 3.1: Bioactive phytoconstituents screened from the dried plant material

Phytoconstituents	***Artemisia afra***
Saponins	+
Flavonoids	+
Tannins	+
Steroids	+
Phlobatannins	-
Alkaloids	-
Cardiac glycosides	+
Terpenoids	+

3.4.2.3 Quantification of polyphenols

The quantity of some of the present phytochemicals inside the extracts, particularly, phenolics, flavonoids and tannins standard curves used to calculate the concentrations of phenolics, tannins and flavonoids, are presented on **Figures 3.3, 3.4 and 3.5**, respectively. The polar methanol extract had a high amount of total phenolics, flavonoids and tannin content. The Concentrations of total phenolic content ranged from 92.16±8.41 to 190.31±5.87 mgGAE/g and for tannin content 167.64±6.59 to 339.92±11.28 mg GAE/g. The hexane extract had the lowest quantity of phenolics and tannin. Additionally, flavonoid content ranged between 830,56±53,70 and 1333,07±12,97 mgQE/g and dichloromethane extracts depicted the lowest quantity for flavonoids (**Table 3.2**).

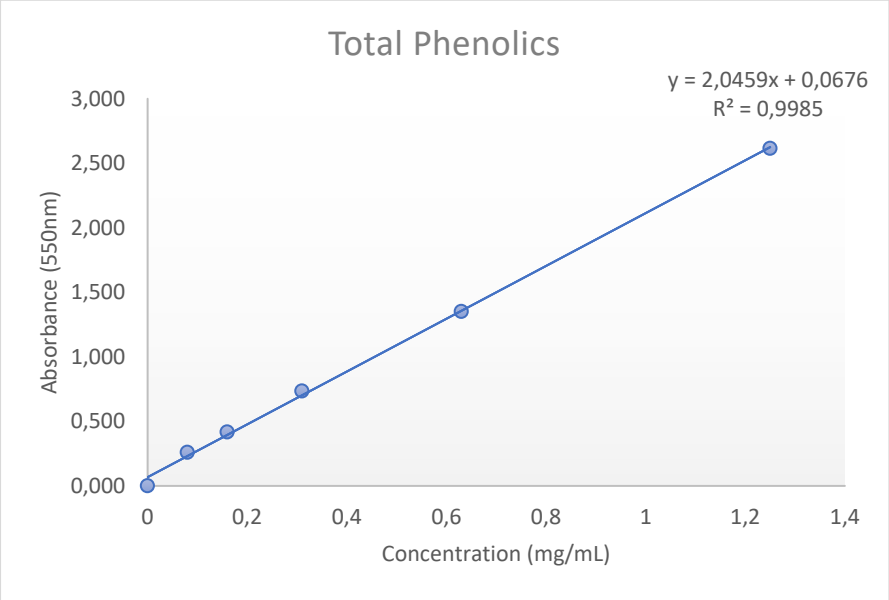

Figure 3.3: Gallic acid standard curve for total phenolic content determination.

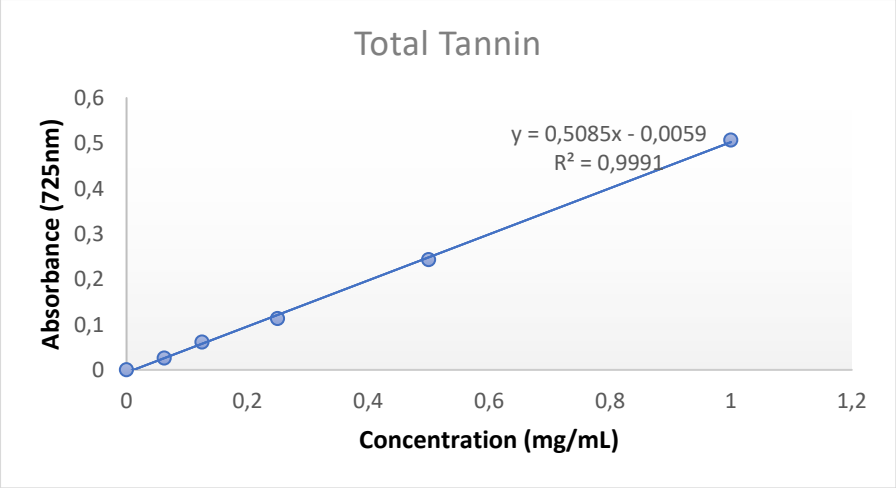

Figure 3.4: Gallic acid standard curve for total tannin content determination.

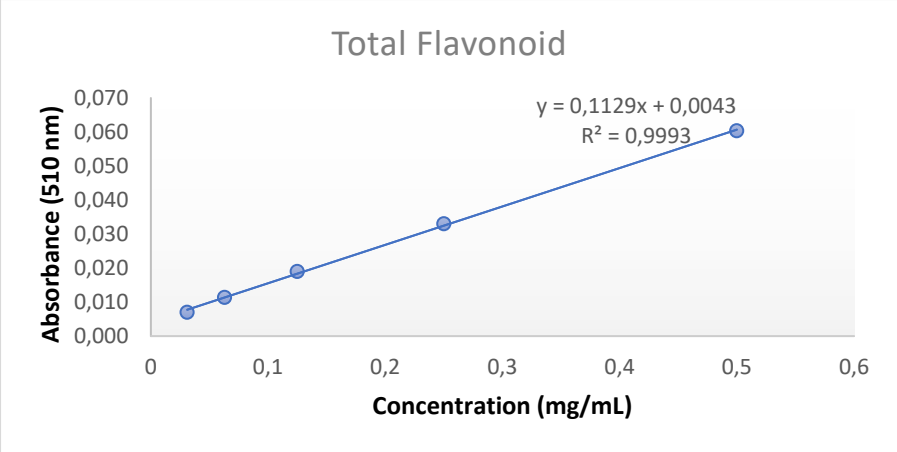

Figure 3.5: Quercetin standard curve for total flavonoid content determination.

Table 3.2: Total phenolics, flavonoids and tannin contents of the plant extracts.

Solvents	Phenolics(mgGAE/g)	Flavonoids (mgQE/g)	Tannins (mgGAE/g)
Hexane	92,16±8,41	1058,78±19,65	167,64±6,59
Chloroform	111,45±4,32	1073,84±44,92	243,87±101,03
Dichloromethane	123,38±5,27	830,56±53,70	185,77±14,68
Ethyl Acetate	109,59±5,87	952,79±144,54	311,52±13,08
Acetone	129,73±8,68	1063,51±56,83	312,04±11,98
Ethanol	112,30±4,56	1185,44±78,61	287,87±10,37
Methanol	190,31±5,87	1333,07±12,97	339,92±11,28
Butanol	154,49±5,04	885,47±23,48	310,42±12,90
Water	133,31±6,40	1183,67±72,01	275,65±6,42

3.5 Discussion

The quality and effectiveness of extraction depends on the method that is used, duration, solvents, and the nature of the plant material, either dry or wet. The selection of solvents for extraction is important because different phytochemicals solubilise differently in the solvents due to their various polarities (Chang *et al*., 2002, Ajanal *et al*., 2012). The aim of this chapter was to evaluate the phytochemical profile of the plant extracts using qualitative and quantitative methods. It was observed that ethanol and water were the best extractants with higher mass contents. Traditionally, people have been using water to prepare their herbal medicines, either as macerations or decoctions depending on their cultural beliefs and knowledge. This is simply because they only had water at their disposal compared to the other organic solvents and it has no toxic effects (Masoko *et al.,* 2008). However, scientific methods have now verified that water is extremely polar and does not solubilise non-polar phytochemicals (Masoko *et al.,* 2008, Seo *et al.*, 2014). In addition, ethanol and water are common solvents usually used in food industries for extraction due to their availability, low cost and for hygiene purposes (Moure *et al.*, 2001).

The simple, fast, and effective method of visualising different phytochemicals present in plant extracts is the TLC method incorporated with other techniques to show available compounds after their separation on the silica plates. In this study, the two methods used were UV-light for fluorescing and vanillin sulphuric acid spray reagent for non-fluorescing compounds. UV-light radiation is frequently used for aromatic phytochemicals applying the spectra wavelengths, 254 nm (short-wave) that results in dark spots against a green colour and 365 nm (long-wave) show different visible colours of compounds against a blue background. For compounds visualised under Ultraviolet light, their fluorescing intensity depends on the chemical structure and functional groups contained in it. Thereafter, vanillin stain method utilises spraying reagents, and the TLC plates are heated to enhance the chemical reaction between the phytochemicals and the staining reagent used. The vanillin stain method has been reported to mostly visualise ketones, aldehydes, alcohols, amines; however, the selection of a staining reagent is more important for one's outcome (Ochwang'I *et al.,* 2016; Holler and Crouch, 2016; Nichols and College, 2022). The results showed several bands in all chromatograms developed in the three mobile systems (BEA,

CEF, EMW) with significant glowing intensity after visualisation with the 365 nm long wavelength. Although, those sprayed with vanillin sulphuric acid only displayed bands in the plate developed in BEA mobile system, which is a non-polar mobile phase. Gitelson *et al.* (2017) reported that flavonoids are some of the secondary metabolites that absorb light at the 365 nm, of which this effect could be due to the fact that naturally flavonoids are produced by plants for protection against external stress that includes radiations from sunlight. Another study by Lorrain *et al.* (2013) indicated that UV-light methods are ideal for the identification of phenolic compounds because of their similar function of protecting plants against UV radiations. The UV-light property of flavonoids and phenolics is attributed to the OH- functional group contained in polyphenols. The red-purple colour depicted on chromatograms sprayed with vanillin sulphuric acid reagent is a result of oxidising chemical reaction between the reagent and saponins and triterpenes (Cheok *et al.,* 2014). This could simply mean that the visible bands with a purple colour on the TLC plate (BEA) depicts either saponins and/or triterpenes/triterpenoids. However, techniques like liquid chromatography-mass spectroscopy (LC-MS) are needed to confirm the specific group of the identified phytochemicals. Standard chemical tests were used to test for various common phytochemicals present in the plant material. The methods apply different colour reagents for exhibition of specific secondary metabolites. The screening technique is very much simple and gives quick insight about the phytochemicals present in the plant extracts and this information can help when doing a further analysis of other biological activities of the plant (Sasidharan *et al*., 2011). The common phytochemicals detected in the plant material after screening were saponins, tannins, flavonoids, terpenoids, steroids, and cardiac glycosides. However, alkaloids and phlobatannins were absent. A study conducted by Masoko and Nxumalo (2013) using a similar plant, *Artemisia afra*, reported the absence of phlobatannins, and Haile and Jiru (2022) also reported the absence of alkaloids. In contrast, Yimam and Desalew (2022) found the presence of alkaloids when using essential oils extracted from *A. afra* fresh leaves. Plants produce phytochemicals in response to biotic and abiotic factors around their environments. The absence or failure to detect phytochemicals in some of the medicinal plants could be due to low concentrations or the plant does not produce them at all.

Seasonal variations, location and age of plant harvest play a huge role on the type and quantity of phytochemicals that plants produce (Street *et al.,* 2008). The plant used in the study was collected during autumn. Thereafter, it was observed after quantification that the methanol extract had the highest content of flavonoids, tannins, and phenolics. This was expected because methanol has been determined to have the best extraction index and efficiency to solubilise both polar and non-polar compounds (Eloff, 1998). In addition, it was noted that overall, all plant extracts had a notable quantity of flavonoids, tannins, and phenolics, respectively. Therefore, it could be confirmed that *Artemisia afra* produce more flavonoids, compared to phenolic acids because tannins are also a member of the flavonoid group, Truong *et al.* (2019) also reported that methanol showed an extensive extraction of phenolics and flavonoids. Medicinal plant species' type plays a paramount role on the selectivity, quantity and efficacy of the phytochemicals they will produce (Taulavuori *et al.*, 2016). The secondary metabolites produced by medicinal plants have been reported to have high efficacy as anti-inflammatory, antioxidative and antimicrobial agents, among other biological activities (Jamshidi-Kia *et al.,* 2018; Vaou *et al.,* 2021). It is paramount to investigate the possible phytocompounds present in plant extracts when searching for new drugs or therapeutic agents for a certain disease or infection (Masoko and Masiphephethu, 2019). This is due to the variety of compounds that medicinal plants contain. Therefore, screening and quantification methods help to develop an idea of what kind of metabolites are present. This, in addition, assists to predict the outcomes of the future bioassays that are still to be conducted, especially those of antimycobacterial activity with reference to the different functional groups that phytocompounds have. Furthermore, flavonoids have antibacterial and antioxidant properties (Al-Harbi *et al.,* 2021) and that the glycosidic residue in tannins also affords, them the potency to act as antimicrobial agents and to elevate pharmacokinetic characteristics must be considered (Ragab *et al*., 2020).

3.6 Conclusion

The study demonstrated that *Artemisia afra* possess saponins, terpenoids, cardiac glycosides, steroids, flavonoids, tannins, and phenolic compounds. Considering that the quantification of polyphenols showed a notable number of flavonoids and tannins, it could be predicted that the plant extracts are abundant with phenolic compounds, which could be advantageous because they have been established to be good

antimicrobial, antioxidants and anti-inflammatory agents and thus can help in the discovery of new drugs for TB infections. Therefore, the documentation of the potential compounds present in a plant material before continuing with other assays is very much imperative because that information gives insight into the possible expected outcomes of future experiments like antimycobacterial activity. Several factors such as a season of harvest, location, and plant age have been shown to play a huge role in the diversity of phytochemicals that medicinal plants produce. In addition, it is important for researchers and pharmaceutical companies conducting research with medicinal plants for the purpose of drug discovery to consider these factors so that there can be progress in the search for new drugs for the treatment of diseases such as TB instead of choosing plants based on traditional uses only. Good extraction solvent plays a huge role as well on the type and quantity of phytocompounds that will be extracted from the plant material. The different solvents used in the study with varying polarity showed notable extraction capacity and efficiency.

3.7 References

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CHAPTER 4

4. Antioxidant and anti-inflammation of crude extracts

4.1 Introduction

The immune system serves as the security of our bodies by protecting them through the prevention of pathogenic infections or the proliferation of acquired diseases. Normal metabolic reactions take place daily for a successful function of the body systems for a healthy life. However, the response against foreign particles and pathogens must be regulated (Rahman, 2007). During normal biochemical processes, free radicals are produced. These are unstable individual molecules with unpaired electrons that are either produced as intermediates or end products. Types of free radicals include reactive oxygen species (ROS), reactive sulphur species (RSS) and reactive nitrogen species (RNS). Common examples are superoxide anion (O_2), hydrogen peroxide (H_2O_2), and nitric oxide (NO) (Lü *et al.,* 2010). Apart from the internal metabolic reactions that produce free radicals, some external factors can contribute to their accumulation too, namely, radiations, industrial solvents, smoking, ozone, and environmental pollutants (Lobo *et al.,* 2010). When free radicals are produced, they look to form bonds with other macromolecules to become stable and their targets are always nucleic acids, sugars, proteins, and lipids (Lü *et al.*, 2010; Craft *et al*., 2012).

Although the presence of these molecules helps regulate cellular homeostasis, their production in high quantities can have detrimental effects that can further result in oxidative stress. Oxidative stress is basically the imbalance between unstable molecules (*i.e*., ROS) production and antioxidants (Wiernsperger, 2003; Huyut *et al.,* 2017). Similar to free radicals and reactive oxygen species, antioxidants, which are substances that delay or prevent the oxidation of unstable molecules, can either be produced during biochemical reactions or be supplemented through diet (Halliwell and Gutteridge, 1995; Halliwel, 2010). BHT (butylated hydroxytoluene) and BHA (butylated hydroxyanisole) are common synthetic antioxidants in the market (EFSA, 2012). These antioxidants are mainly used in food industries to improve the shelf life of food and prevent oxidation during storage, which might affect their quality (Carocho and Ferreira, 2013).

Inflammatory processes induced after pathogenic infections contribute to the accumulation of free radicals as well (Velayutham *et al.,* 2011). Inflammation serves as an array of immune response reactions against internal and external stimuli. The result of inflammatory response can be acute or chronic depending on the severity of the response (Torres-Moreno *et al.,* 2019). Pro-longed inflammatory responds lead to the lysis of lysosomes, thus releasing more molecules that influence further inflammatory response and therefore resulting in immune disorders (Truong *et al.,* 2019). Inflammation has also been associated with protein denaturation inside a host during an immune response (Osman *et al.,* 2016). Some exogenous and endogenous substances, including medications, are carried through the blood to different organs by plasma albumin using binding ligands, although the production of unstable molecules by chronic inflammation contributes to the breakdown of this plasma albumin thus affecting its normal function (McIntyre and Bircher, 1991; Bisaso *et al*., 2014). Chemical and physical agents denature proteins by disrupting their hydrophobic, hydrogen, disulphide bonds and electrostatic forces, thus altering their normal cellular functions. Tissue injury is mostly associated with protein denaturation inside the cells, thus resulting in inflammation (Sangeetha and Vidhya, 2016; Osman *et al.,* 2016). In the study, heat was used as a physical external source to denature egg albumin proteins. Furthermore, the infectious *Mtb* has the ability to manipulate immune response and further contributes to a prolonged inflammatory response and that can lead to more protein damage (Jo, 2008; Harding and Boom, 2010).

The proliferation of reactions promoting the production of ROS and RNS have been reported to induce severe diseases like cancer, auto-immune deficiency diseases, cardiovascular diseases, rheumatoid arthritis, inflammation, adult respiratory distress syndrome, degenerative disorders associated with, Parkinson's and Huntington's diseases, diabetes mellitus, aging, and Alzheimer's (Rahman, 2007; Lobo *et al.,* 2010; Lü *et al.,* 2010; Singh *et al.,* 2010). Rifampicin is a well-known antibiotic used to treat pulmonary TB infections; however, it has been reported to induce the production of toxic metabolites (ROS) in patients after prolonged exposure/use, resulting in robust inflammatory response (Mangwani *et al.,* 2020). Thousands of medicinal plants have been reported to have antioxidative and anti-inflammatory properties. These biological activities are attributed to compounds like phenolic acids and flavonoids. Flavonoids can react as superoxide radical scavengers, reducing agents, singlet oxygen

quenchers, metal chelators and as well as hydrogen donators (Rice-Evans *et al*.,1996; Procházková *et al.,* 2011), while phenolic acids can react as free radical scavengers and metal chelators (Krimmel *et al.,* 2010; Terpinc *et al.,* 2011). The objective of this chapter was to evaluate the antioxidative and anti-inflammatory activity of the plant extracts.

4.2 Materials and Methods

4.2.1 Antioxidant activity

4.2.1.1 Qualitative DPPH free radical scavenging assay

The DPPH assay, as previously used by Braca et al. (2002), was employed using 2, 2-diphenyl-1-picrylhydrazyl (DPPH) (Sigma-Aldrich) serving as a free radical. DPPH is purple in colour and is reduced to 2,2-diphenyl-1-picryl hydrazine (DPPH-H), which depicts a yellow colour when it reacts with a reducing agent. An amount of 10 µL of each extract was loaded on a TLC plate and developed in EMW, CEF, and BEA mobile phase systems. The plates were dried in a stream of air at an ambient temperature to evaporate the solvents and sprayed with 0.2% DPPH in methanol.

4.2.1.2 Quantitative DPPH free radical scavenging assay

The free radical scavenging activity of the plant extracts was quantified using the DPPH method reported (Chigayo *et al.,* 2016). Briefly, 2 mL 0.2 mmol/L DPPH solution dissolved in methanol was added to an equal volume of different concentrations of the plant extract (15.63, 31.25, 62.5, 125, 250, µg/mL). All the prepared mixtures were vortexed thoroughly and incubated in the dark for 30 minutes. The blank was prepared with 2 mL methanol and 1 mL of acetone that substituted the plant extracts. Ascorbic acid served as a reference standard and was prepared at the same concentration range as the plant extracts. A control solution was prepared by adding 2 mL of 0.2 mmol/L DPPH to 1 mL of acetone. After the elapsed time, the solutions were analysed with a UV/VIS spectrophotometer. The absorbance of the solutions was read at 517 nm and the percentage inhibition was calculated using the formula below.

$$\%Inhibition = \frac{Ac-As}{Ac} x100$$

Key: Ac= Absorbance of the control solution

As=Absorbance of the plant extract

a. 4.2.1.3 Ferric reducing antioxidant power assay

The ferric reducing power of the plant extracts was determined using the method of Vijayalakshmi and Ruckmani (2016) and Ahmed *et al.* (2012). This reducing power of the extracts is attributed to their ability to donate electrons to ferric ion (Fe3+) to a ferrous (Fe2+). Five different concentrations of the plant extracts (39.06, 78.13, 156.25, 312.50, 625 µg/mL) were prepared with acetone to 2.5 mL by serially diluting a stock solution of 1250 µg/mL followed by the addition of 2.5 mL of sodium phosphate buffer (0.2M, pH 6.6) and 2.5 mL of 1% potassium ferricyanide to each sample. The samples were briefly vortexed and incubated at 50°C in a water bath for 20 minutes before an addition of 2 mL of 10% trichloroacetic acid to each test tube. The samples were centrifuged at 3000 rpm for 10 min and 5 mL of the resulting supernatant was transferred to a clean test tube. To this supernatant, 5 mL of dH_2O and 1 mL of 0.1% ferric chloride were added consecutively with thorough vortexing after each addition. A blank sample was prepared using the same method; however, an equal volume of acetone substituted the plant extracts. Ascorbic acid (39-625 µg/mL) that was used as a positive control and was prepared similarly to the plant extracts. A UV/VIS spectrophotometer was used to measure the absorbance of samples at 700 nm. The reducing antioxidative power principle is that antioxidant activity should be noted by an increased in absorbance, which is attributed to the change in colour of the reaction.

4.2.2 Anti-inflammatory activity

4.2.2.1 Egg-albumin denaturation assay

The reaction mixture (1250 µL) consisted of 50 µL of egg albumin (from a fresh hen's egg), 700 µL of 0.05 M phosphate buffer (pH 6.4) and 500µL of varying concentrations (2, 1, 0.5, mg/mL) of the plant extracts and standard drug (Diclofenac sodium) (2,1, 0.5 mg/mL). Product (negative) control solution (1250 µL) consisted of 750 µL of 0.05 M phosphate buffer and 500 µL of each extract solution to consider the colour of the extracts when using the UV/Vis spectrophotometer. The egg albumin solution (50µL and 1200µL Tris buffered saline) was used as the test solution (positive) control. The positive control represented 100% protein denaturation. The mixtures were incubated at 37±2°C in a biochemical oxygen demand (BOD) incubator for 15 minutes and then heated at 70°C for 5 minutes. After heating, the solutions were allowed to cool to room temperature for 30 minutes. After cooling, their absorbance was measured at 660 nm by using the phosphate buffer as a blank (Uttra and Alamgeer, 2017; Rahman *et al*.,

2015). The percentage inhibition of protein denaturation was calculated by using the following formula:

% Anti-denaturation activity = $\frac{Absorbance\ of\ control - Absorbance\ of\ test\ sample}{Absorbance\ of\ control} x100$

*Absorbance of test sample = absorbance of test solution – absorbance of negative control.

4.3 Statistical analysis

Where appropriate, results were expressed as means ± standard deviation (SD) of triplicate determinations. Statistical analysis was performed by Microsoft Excel 365 and Graph pad prism v9.5.0 by a two-way analysis of variance (ANOVA), followed by Dunnet multiple comparison test. Significant difference was considered when $p<0.05$ and conversely, non-significance was indicated by $p>0.05$ values.

4.4 Results

4.4.1 Qualitative DPPH assay

Notable antioxidant activity was observed in acetone, ethanol, methanol, and butanol extracts in all the chromatograms developed in the 3 different mobile systems (BEA, CEF, EMW). These results show the potential of active antioxidative compounds of intermediate to polar polarity and it was also observed that the active compounds were able to separate properly in the EMW mobile phase (**Figure 4.1**).

4.4.2 Quantitative antioxidant activity assays

The DPPH free radical scavenging and ferric reducing power assays were used to quantify the antioxidative activity of the extracts. It was then observed that hexane, chloroform, acetone, ethyl acetate, and butanol extracts had more than fifty percent capacity to scavenge the free radical, DPPH, although all the other extracts depicted notable activity in a concentration dependent manner **(Figure 4.2)**. Furthermore, it was then discovered that all the extracts had very low metal chelating properties as compared to the positive control ascorbic acid, in an increasing concentration manner when tested with the ferric reducing power method (**Figure 4.3**). The antioxidant activity tested using the two methods exhibited a concentration dependent pattern, where the activity increased with an increase in concentration.

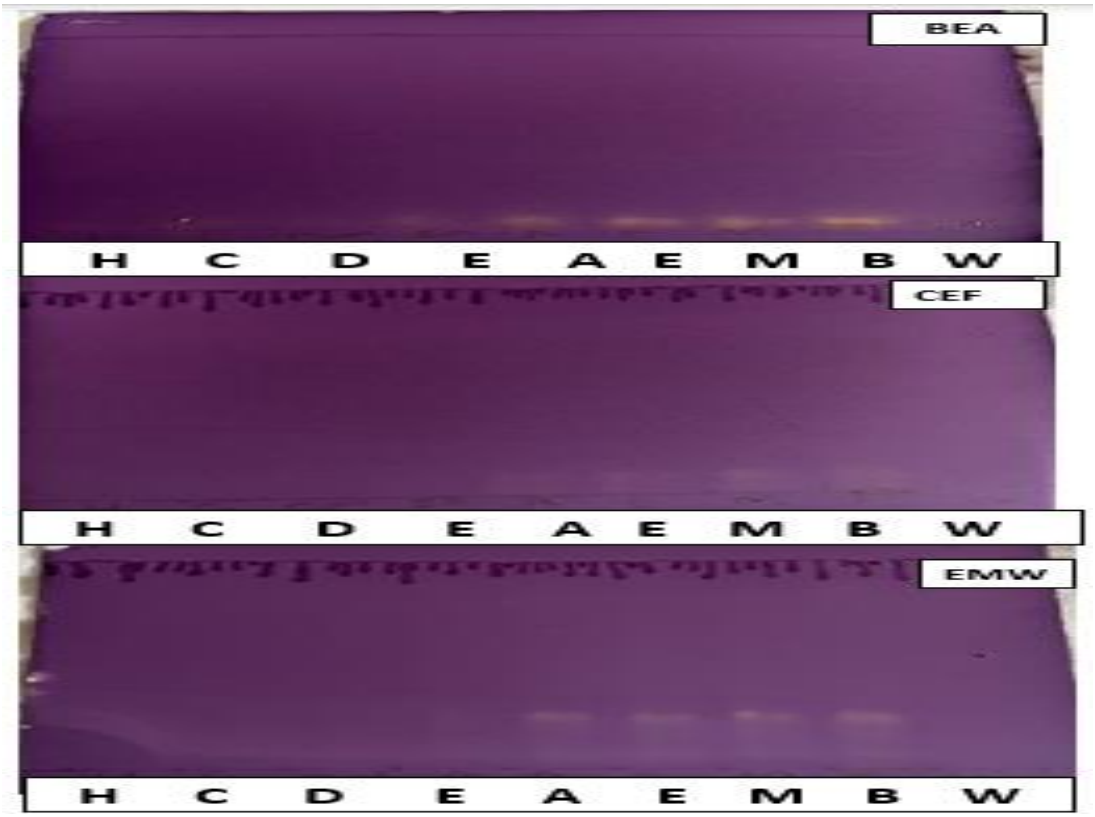

Figure 4.1: Chromatograms of *Artemisia afra* plant extracts, extracted with different solvents and developed in BEA, CEF and EMW mobile systems. Thereafter, sprayed with 0.2% DPPH in methanol. The yellow colour indicates antioxidant activity.

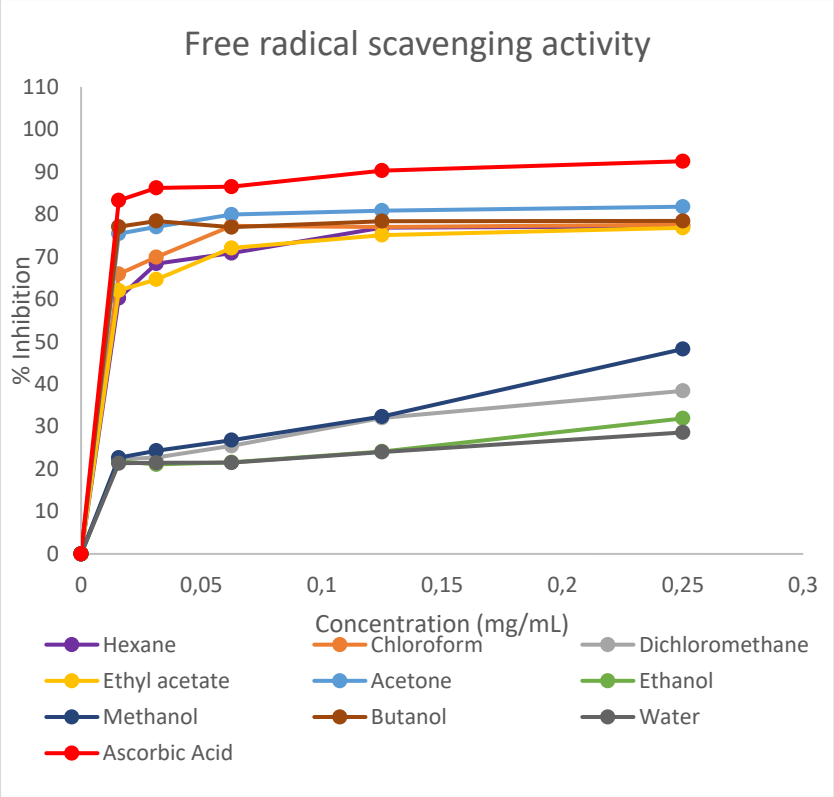

Figure 4.2: Percentage inhibition of free radical (DPPH) scavenging activity of *Artemisia afra* plant extracts measured at 517 nm.

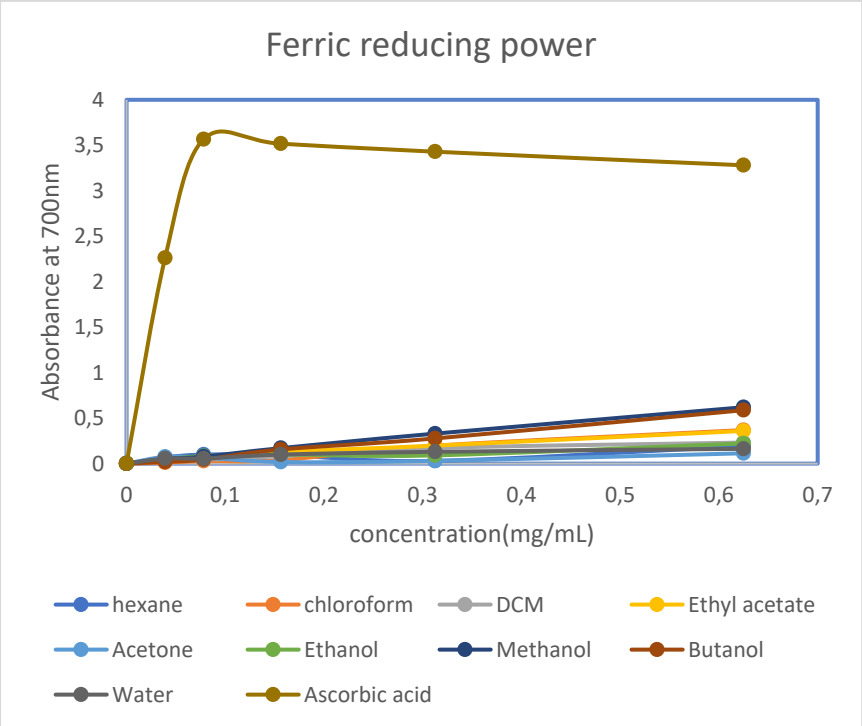

Figure 4.3: Percentage inhibition of ferric reducing antioxidative power of *Artemisia afra* plant extracts measured at 700 nm.

4.4.3 Egg albumin denaturation assay

The egg albumin denaturation assay that uses heat as a source of denaturation to the albumin proteins inside the egg white and was used to evaluate the anti-inflammatory activity of the extracts. The results illustrated in **Figure 4.4** display the anti-inflammatory activity of hexane, dichloromethane, acetone, and methanol extracts. All the extracts, comparable to the positive control diclofenac sodium, depicted a very significant activity ($p<0.0001$), although methanol extracts had the least activity. The results further show that acetone had a very remarkable activity above the positive control at concentration of 0.5 mg/mL, followed by hexane extracts, which had no significance difference when compared to the control. The concentration of the extracts was then increased to 1 mg/mL to assess the effect of higher concentration on the ability to inhibit denaturation of the egg albumin. The results displayed a similar trend and there was not much difference in activity when compared to the lowest concentration tested.

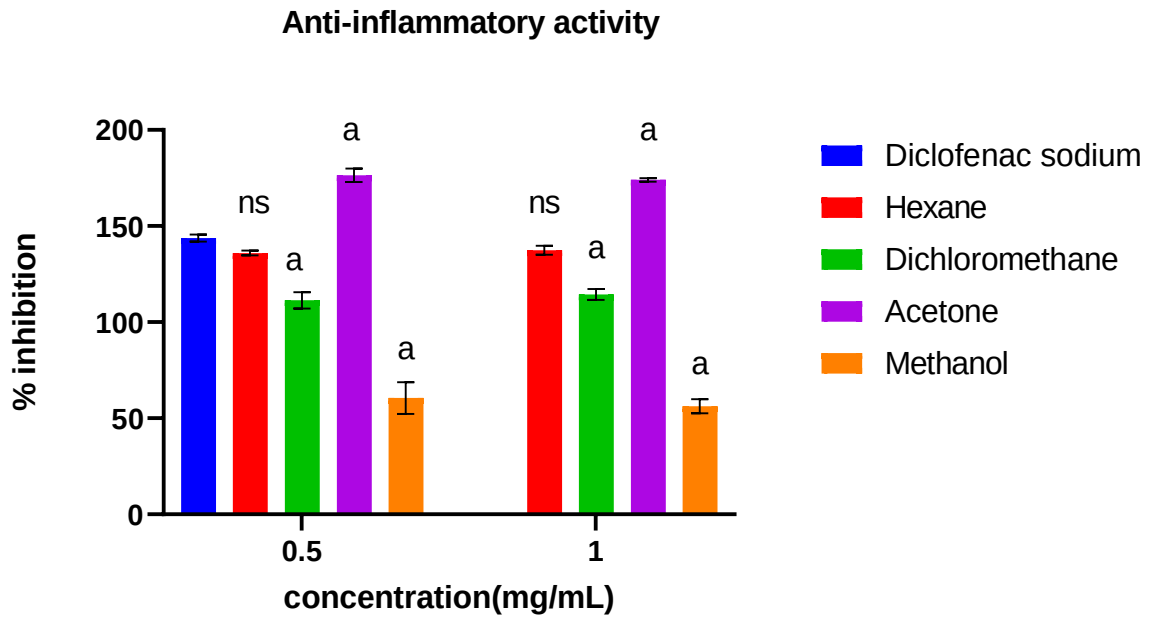

Figure 4.4: Percentage inhibition of egg albumin denaturation by *Artemisia afra* plant extracts and diclofenac which served as a positive control measured at 660 nm.

Key: a=p<0,0001, ns= non-significant

4.5 Discussion

Antioxidants are molecules that are notable for their exceptional property of maintaining the balance of free radicals that are produced during normal metabolic reactions or effects of external stimuli (Rahman, 2007; Poljsa *et al.,* 2011). In this study, it was observed that the extracts, extracted with intermediate and polar organic solvents, acetone, ethanol, methanol, and butanol showed a remarkable antioxidative activity, with exception to water. After the quantification of antioxidants, it was observed that hexane, chloroform, acetone, ethyl acetate, and butanol extracts had more than fifty percentage capacity to scavenge the free radical, DPPH. All the other extracts depicted notable activity in a concentration dependent manner. Additionally, it was noted that even extracts that showed no or less activity (hexane, chloroform, and ethyl acetate) on the TLC chromatograms were able to depict significant activity after quantification. The effect of compounds not separating on the TLC plates and showing less intensity could be due to the concentration, chemical structure of phytochemicals (polarity dependent) and or the effect of separation because some compounds use synergistic properties to showcase their biological activities (Pan *et al*., 2017; Güner *et al.,* 2019). Furthermore, the ability of the plant extracts to chelate metal ions was investigated using the ferric reducing power assay. The method requires low acidic pH to sustain the solubility of the irons (Gulcin, 2012). It was then discovered that all the extracts had a very low metal chelating property. However, although the extracts exhibited poor reducing antioxidative power, Elemike *et al.* (2018) reported that *Artemisia afra* extracts can be used as reducing agents to reduce ferric ions. The quantification of the extracts demonstrated a good quantity of flavonoids, tannins and phenolics, which are secondary metabolites reported daily to be abundant in medicinal plants, including *Artemisia afra* and they have carboxylic and hydroxyl groups that afford them the ability to make bonds with metal ions (Ahmad *et al.,* 2010; Joseph and Mathew, 2015). The overall antioxidant activity was observed to follow a concentration dependent pattern when compared with the positive control ascorbic acid. However, the extracts did show remarkable free radical scavenging property as opposed to metal chelating effects. When there is an imbalance of free radicals and antioxidants, free radicals and reactive oxygen species tend to accumulate leading to the disruption of proteins, lipids, and nucleic acids structures. This will in turn cause unwanted oxidative stress (Mangwani *et al*., 2020). Low levels of antioxidant, vitamin

concentrations in the blood and increased oxidative stress has been observed in most TB patients, which could be due to factors such as poor diet and uncontrolled inflammation since there is no balance between free radicals and antioxidants (Vijayamalini and Manoharan, 2004; Madebo *et al.,* 2003). As a result, it is crucial to maintain a healthy diet full of nutrient-rich foods when suffering from TB to strengthen the immune system and trigger an efficient response to the infection. Additionally, taking vitamins and/or antioxidant supplements that will help to lessen the high potency of oxidative stress in TB patients might be beneficial. Receiving TB therapeutic regimens with both antimycobacterial and antioxidant activity can be very helpful to patients and can further encourage compliance with taking medication because patients frequently, if not always, stop taking their medication due to the large number of medications and supplements they must consume at the same time.

After testing for anti-inflammatory activity using the egg-albumin assay, the results revealed that the extracts, hexane, dichloromethane, acetone, and methanol had a remarkable activity beyond 50%, although methanol extracts were low. The acetone extract had a potent notable activity exceeding the positive control, diclofenac sodium at a lowest tested concentration. Diclofenac sodium is a nonsteroidal anti-inflammatory drug used to regulate arthritis related conditions (Karthik *et al.,* 2013), even though the concentration of the extracts was doubled to assess the effect of higher concentration on the ability to inhibit denaturation of the egg albumin. The results displayed a similar trend and there was not much difference in activity when compared to the lowest concentration tested. The antioxidative and anti-inflammatory activity of *Artemisia afra* extracts could be attributed to the notable phytochemicals that were observed to be present after screening and quantification, namely, saponins, cardiac glycosides, terpenoids, steroids, phenolics, flavonoids and tannins. Phenolics have been reported several times to be good radical scavengers and do present moderate metal chelating properties (Krimmel *et al.,* 2010; Terpinc *et al.,* 2011). In addition, flavonoids as well can inhibit the formation of reactive oxygen species and contain trace elements to chelate metal ions. Furthermore, they are associated with anti-inflammatory, anticarcinogenic and antioxidative effects (Agati *et al.,* 2012; Erlund, 2004).

4.6 Conclusion

Artemisia afra extracts do possess phytochemicals, particularly phenolics, flavonoids and tannins that give them their antioxidative and anti-inflammatory properties. With the maximal antioxidative and anti-inflammatory activities observed from the results, it will be very much beneficial for TB patients because these effects could help boost and strengthen the immune system, thus increasing its response to the infection. In addition, antimycobacterial activity of the extracts should be tested. The anti-inflammatory activity did not show much difference when evaluated with different concentrations. Therefore, it can be recommended that only smaller concentrations be used. Synergistic interactions between the extracts were observed when testing the free radical scavenging activity of the extracts using two qualitative and quantitative methods. This further suggested that *Artemisia afra* phytochemicals would be highly effective as antioxidant supplements, only when administered as extracts and not as pure compounds, as separating the compounds reduces their efficacy. Consuming antioxidants in the form of dietary supplements is also crucial and can help in avoiding to take numerous supplemental drugs on top of the TB treatment because it might lead to the development of side effects.

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CHAPTER 5

5. Antimycobacterial and antibiofilm activity of the crude extracts

5.1 Introduction

In 2014, The World Health Organization (WHO) had to create "*The End TB strategy*" to help prevent additional infections and end TB by the year 2035 because contagious TB infections are still on the rise globally (WHO, 2015). *Mtb,* which was isolated back in 1882 by Robert Koch, is the causative agent of the TB infections among other mycobacterium species. It is a slow growing, acid-fast pathogen with a thick cell wall containing a peptidoglycan layer enclosed by lipids and mycolic acids (Brennan, 2003). Pre-exposure to TB infections occurs through the lungs, but it can also travel to other body parts, including the central nervous system, joints, bones, and lymphatic system. This new condition impacting these additional sites is known as extra-pulmonary TB (Bennett *et al.,* 2019). *M. smegmatis* is non-pathogenic and fast-growing organism from the Mycobacteriaceae family similar to *Mtb* and shares similar structural and biochemical properties with MTB; hence, it is used in laboratories as model to conduct TB research (Reyrat and Kahn, 2001).

Many bacterial species in nature form biofilms as a mode of survival in unfavourable conditions. However, for research purposes, they are mostly utilised as planktonic cells (single cells). Biofilms are a community of microbes packed together and covered by an extracellular matrix produced by multiple species embedded inside. Furthermore, the phenotypic nature of planktonic cells is very much different from those forming biofilms even though there are identical species because the cell morphology and gene regulation patterns are different (Flemming *et al.,* 2016; Chakraborty and Kumar, 2019), as such, biofilms require very high concentrations compared to the single cells, thus making it more difficult to eradicate them (Ceri *et al.,* 1999; Chakraborty and Kumar, 2019). The noteworthy antimicrobial activity is reported to be at MIC values equal or less than 1 mg/mL (van Vuuren and Viljoen, 2011). The strength of a biofilm depends mainly on the extracellular polymeric substances (EPS) produced by the cells to form the matrix because the EPS promotes formation of hydrogen bonds, electrostatic forces and van der Waals interactions to make the matrix stable (Donlan, 2002; Tsuneda *et al.,* 2003; Khan, 2021). EPS has an arrangement of polymers,

including polysaccharides, nucleic acids, proteins, and lipids that provide carbon and energy to the cells (Flemming and Wingender, 2010).

When infected with pulmonary TB, the *Mtb* sessile bacilli is incorporated in a sac like granulomas, which is somehow associated with the similar setting of biofilms (Saunder and Cooper, 2000; Lenaerts *et al.,* 2007; Harper *et al.,* 2012). Stress conditions like low oxygen and nutrient concentrations cause some mycobacteria to go dormant and form structures resembling biofilms (Hett and Rubin, 2008; Bhunu *et al.,* 2017). *Mtb* creates biofilms to withstand the host's immune pressure and antimicrobial drug therapy. Therefore, its capacity to form biofilms in vitro can be used to explain the necessity for prolonged treatment with a range of drugs (Orme, 2014; Trivedi *et al.,* 2016). The over-expression of efflux proteins in a formed biofilm has a considerable impact on antibiotic resistance and the development of the multidrug resistance (MDR) phenotype in Mycobacterium species, including *Mtb* (Viveiros *et al.,* 2003; Rossi *et al*., 2006; Danquah *et al.,* 2021). It has been reported that *Mtb* and *M. smegmatis* biofilms are able survive in ten times MIC values of the anti-TB drugs rifampicin and isoniazid (Ojha *et al.,* 2008; Danquah *et al.,* 2021). High concentrations of samples compared to the MIC values showing bacteriostatic effects towards planktonic cells, are needed to inhibit the biofilm of *M. smegmatis* (Bhunu *et al.,* 2017).

Biofilms naturally are a hassle to treat; however, discovery has been made that even the single cells are developing resistance to available drugs that they were susceptible to. But clinical isolates of *Mtb* have already been found to be resistant to bedaquiline and delamanid, demonstrating the rapid rise of *Mtb* drug resistance (Mokrousov *et al.,* 2019; Polsfuss *et al.,* 2019). Although available TB treatment can reduce patient fatalities, most TB patients nevertheless experience health problems years after the initial disease episode, and research has shown that this group is more likely to experience mortality and long-term disability (Romanowski *et al.,* 2019). Other patients during post-TB, experience pulmonary hypertension, chronic obstructive pulmonary disease (COPD) and bronchiectasis (Amaral *et al.,* 2015; Ravimohan *et al*., 2018; Allwood *et al.,* 2020). For decades, many drugs with potential efficacy have been isolated from plants due to their abundant phytochemicals. Their use from acquired traditional knowledge and modern pharmacological evaluations has brought mild relieve to drug development research because they are natural sources and contain different secondary metabolites, which have been reported to have numerous

biological activities, including antibacterial activity (Sufian *et al.,* 2012). *Artemisia afra* is of the exceptional medicinal plants with several reported biological activities, including the treatment of TB related symptoms (Du Toit and Van der Kooy, 2019). The objective of this chapter was to evaluate the antimycobacterial activity of *Artemisia afra* extracts against *M. smegmatis*.

5.2 Materials and Methods

5.2.1 Bacterial culture

Mycobacterium smegmatis (ATCC 1441) was obtained from Professor Green at the Department of Biotechnology and Food Technology, University of Johannesburg. Work conducted with *M. smegmatis* was conducted in a biosafety level 2 (BSL2) laboratory. The culture was maintained on Middlebrook 7H10 agar plates supplemented with at 4°C. A colony was inoculated in Middlebrook 7H9 (Fluka) broth containing glycerol (Sigma) and Middlebrooks Oleic Albumin Dextrose Catalase (OADC) growth supplement (Sigma) and incubated at 37 °C for 24 hours for bioassays.

5.2.2 Qualitative antibacterial activity assay

5.2.2.1 *Bioautographic assay*

The qualitative analysis of potential antimycobacterial phytocompounds found in the plant extracts was done using the bioautographic method (Begue and Kline, 1972). TLC plates were loaded with 20 µL of each extract (10 mg/mL) and developed in EMW, CEF and BEA as described in Chapter 3 (Section 3.2.2.2). The plates were then dried at room temperature under a stream of air for 5 days to remove the remaining solvents. The developed TLC plates were sprayed with a concentrated suspension of the overnight *M. smegmatis* bacterial culture until completely moist in a laminar flow cabinet (Labaire Pty Ltd., SN:0051). Thereafter, the plates were incubated overnight at 37°C in 100% humidity. The plates were then sprayed with a 2 mg/mL solution of p-iodonitrotetrazolium violet (INT) and incubated for 3 hours at 37°C in the dark. Bacterial growth led to the development of a purple-pink colour resulting from the reduction of INT by the succinate dehydrogenase into the corresponding formazan salt. White bands on the TLC plates indicated the inhibition of *M. smegmatis* growth by the active compounds present in the plant extract.

5.2.3 Quantitative antibacterial activity assay

b. 5.2.3.1 Serial broth microdilution assay

A serial microdilution assay (Eloff, 1998) was used to determine the MIC of the plant extracts against *Mycobacterium smegmatis.* Two-fold serial dilutions of the plant extracts (2.5-0.02 mg/mL) were prepared in 96-well microtiter plates. From working culture was inoculated in supplemented Middlebrook 7H9 broth at a starting OD_{600} 0.1. The culture was incubated at 37 °C at 150 rpm. The culture was allowed to grow to an OD_{600} 0.8-0.9 to maintain the cells at exponential phase and 100 µL was added to each well of the 96 well plate excluding 4 wells representing a negative control (media only). Rifampicin was used as positive control and acetone was used as a negative control. As the indicator of growth, 40 µL of 0.2 mg/mL *p*-iodonitrotetrazolium violet (INT) was added to the microtiter plate wells. The covered microtiter plates were incubated for 30 minutes at 37°C at 100% relative humidity. The growth of *M. smegmatis* was shown by the presence of a pink/purple colour in the wells after incubation with INT while growth inhibition by the plant extracts was displayed by a marked reduction in intensity of the colour. The MIC was recorded as the lowest concentration of the extract that inhibited bacterial growth. The total antimycobacterial activity was calculated by dividing the quantity extracted (in mg) from 1 g of plant material by the MIC (in mg/mL).

5.2.4 Antibiofilm screening

5.2.4.1 Inhibition of biofilm formation – prevention of initial bacterial cell attachment

The potential of the extracts to prevent initial cell attachment was investigated through the crystal violet assay (Sandasi *et al*., 2008). Briefly, a 100 µL of *Mycobacterium smegmatis* culture with OD_{600} = 0.02 (≈1.0×10^6 CFU/mL) was added into individual flat-bottomed 96-well micro-titre plates in three replicates and incubated at 37 °C for 4 hours without shaking. Thereafter, the plates were removed from the incubator and 100 µL (100 mg/mL) of plant extracts with concentrations (4MIC, 2MIC, MIC and 0.5MIC mg/mL) were added and then incubated further at 37 °C for 24 hours without agitation. Untreated microbial cells served as positive control, acetone and sterile media served as negative controls and rifampicin (4MIC, 2MIC, MIC and 0.5MIC

mg/mL) was used as a standard for the assay. The biomass was quantified using the modified crystal violet staining method (Djordjevic *et al*., 2002).

5.2.4.2 Inhibition of development of pre-formed biofilms – assessment of destruction of biofilm mass

The ability of the extracts to prevent irreversible biofilm or destruction of pre-formed biofilms was investigated. A 100 µL of *Mycobacterium smegmatis* cultures with OD600 = 0.02 (1.0 × 106 CFU/ml) was added into individual flat-bottomed 96-well micro-titre plates and incubated at 37 °C for 24hrs (irreversible attachment phase) and 48 hours (mature biofilm) without shaking for the development of a multilayer biofilm. Therefore, after 24 hours, the plate incubated for irreversible attachment phase was removed from the incubator and treated with 100 µL of plant extracts (100 mg/mL), while the plate incubated for mature biofilm remained in the incubator for further 24 hours. The irreversible phase micro-titre plates were then incubated for another 24 hours after treatment with extracts. The same procedure was followed for the micro-titre plates with mature biofilm after 48 hours. Untreated microbial cells served as a positive control while acetone and sterile media served as negative controls. The biofilm biomass was assayed using the modified crystal violet (CV) staining assay (Djordjevic *et al.,* 2002).

5.2.4.3 Crystal violet staining assay

The assay was done as previously described by (Djordjevic *et al.,* 2002) with some modifications (Sandasi *et al.,* 2008). Briefly, the 96-well micro-titre plates were washed three times with sterile distilled water, air dried and then oven-dried at 60 °C for 45 minutes. The wells were then stained with 100 µL of 0.1% crystal violet and incubated at room temperature for 15 minutes after which the plates were washed thrice with sterile distilled water to remove unabsorbed stain. At this point, biofilms were observed as purple rings at the side of the wells. The semi-quantitative assessment of biofilm formation was done by adding 125 µL of methanol to destain the wells. A 100 µL aliquot of the destaining solution was transferred to a new sterile plate and the absorbance was measured at 590 nm using a microplate reader (Thermo Scientific, CAT:1530, Multiskan sky, Singapore). The mean absorbance of the samples was determined, and the percentage inhibition of biofilm was determined using the equation below (Sandasi *et al.*, 2008).

Percentage (%) inhibition = $\frac{\text{OD Positive control} - \text{OD Experimental}}{\text{OD Positive control}} x100$

5.3 Statistical analysis

Where appropriate, results were expressed as means±standard deviation (SD) of triplicate determinations. Statistical analysis was performed by Microsoft Excel 365 and Graph pad prism v9.5.0 by a two-way analysis of variance (ANOVA), followed by the Dunnet multiple comparison test. Significant difference was considered when $p<0.05$ and conversely, non-significance was indicated by $p>0.05$ values.

5.4 Results

5.4.1 Bioautography assay

The chromatograms were developed in three (3) mobile phases (BEA, CEF, EMW) that differ in polarity and the white zones of inhibition against the purple background after contact with INT demonstrated antimycobacterial compounds. Most clear zones observed on the CEF chromatogram, nevertheless, both the BEA and EMW had well separated compounds (**Figure 5.1**). This indicated the *A. afra* had numerous antimycobacterial compounds that differed in polarity in the plant.

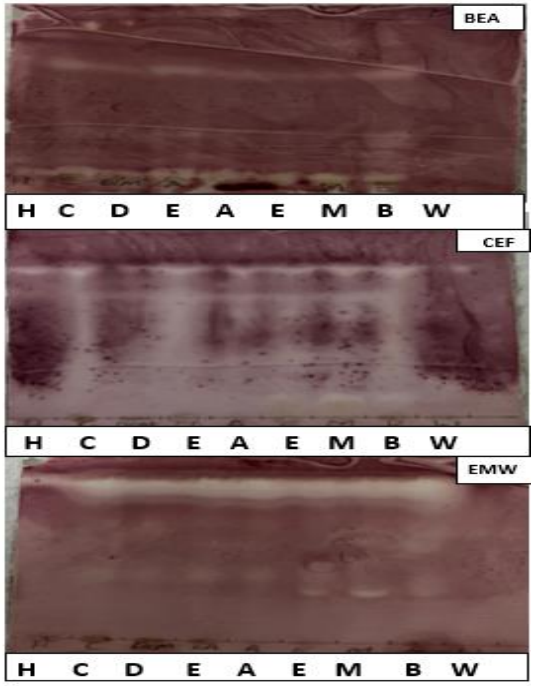

Figure 5.1: Chromatograms of *Artemisia afra* extracts developed in BEA, CEF and EMW mobile systems. Thereafter, sprayed with *Mycobacterium smegmatis.* White areas represent zones of inhibition after incubation with tetrazolium salt.

5.4.2 Serial broth microdilution assay

Micro broth serial dilution assay was also used to evaluate the antimycobacterial activity of the extracts by determining the MIC. The results showed a moderate activity, where the MICs were ranging between 1.25 mg/mL and 2.5 mg/mL, with the lowest concentrations depicted on the chloroform (1.62 mg/mL) and butanol at 1.25 mg/mL. The positive control rifampicin had better activity than the crude extracts where an MIC of 1.56 μL was determined. Hexane and water extracts had no detectable activity (**Table 5.1**).

Table 5.1: MICs (mg/mL) of plant extracts against *Mycobacterium smegmatis.*

Microorganism	Extracts(mg/mL)										
	Hexane	Chloroform	Dichloromethane	Ethyl acetate	Acetone	Ethanol	Methanol	Butanol	Water	Average	Rifampicin
Mycobacterium smegmatis	—	1.62	2.5	2.08	2.08	2.08	2.08	1.25	—	1.96	0.00156

5.4.3 Antibiofilm activity

The hexane, dichloromethane, acetone, and methanol were evaluated for their antibiofilm potential. The crude extracts were not able to prevent the initial cell attachment (**Figure 5.2A**); instead, they induced the biofilm formation of *M. smegmatis*. However, some of the extracts did have the capacity to reduce the irreversible formed biofilm after 24 hours, except for Hexane extracts that had no activity at all (**Figure 5.2B)**. Methanol extracts managed to have remarkable activity after the same 24 hours within all the concentrations. In addition, for the lowest concentrations of the extract's methanol, acetone and dichloromethane showed significant activity that was even better than that of the positive control rifampicin (**Figure 5.2B**). Additionally, all the extracts induced further formation of the matured biofilm (**Figure 5.2C**).

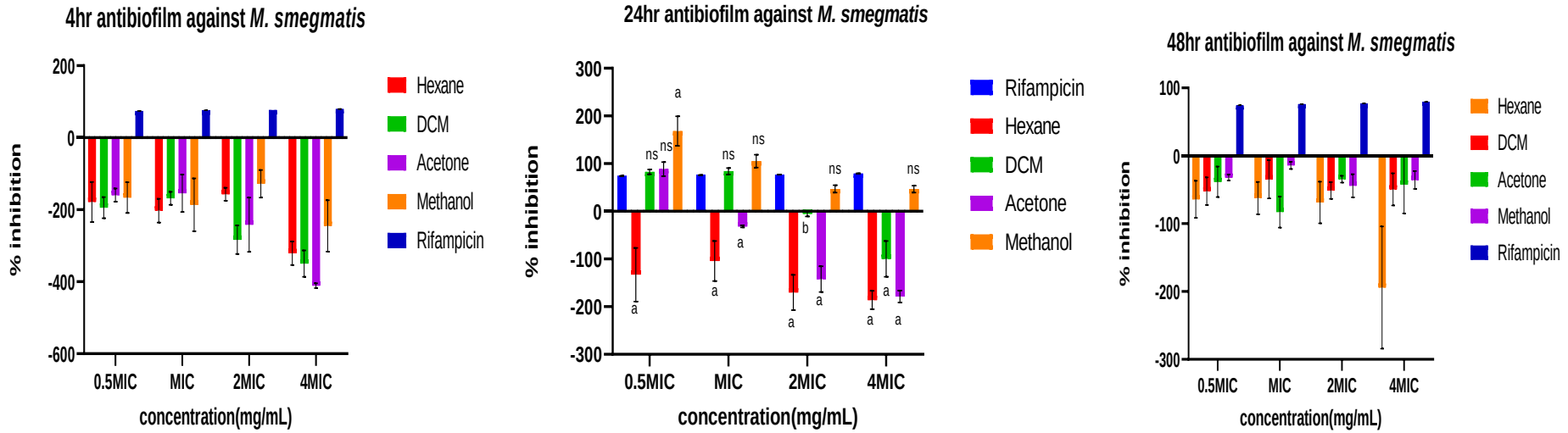

Figure 5.2: Antibiofilm activity of *Artemisia afra* plant extracts measured at 600 nm after at different time intervals (**A**) initial cell attachment, (**B**) Irreversible cell attachment and (**C**) Mature biofilm. Rifampicin served as a positive control.

Key: Biofilm: ≥100%=complete eradication, ≥50%=good inhibition, <50%=poor inhibition and <0%=enhanced biofilm. a=p<0.0001, ns=non- significant.

5.5 Discussion

Although available TB treatment can reduce patient fatalities, most TB patients nevertheless experience health problems years after the initial disease episode (Romanowski *et al.,* 2019). The results revealed moderate activity by zones of inhibition on the two chromatograms developed in the CEF and EMW mobile phases, which separate intermediate and polar compounds, respectively. Antimycobacterial activity observed on chromatograms give insight into what kind of compounds are active inside the extracts looking at the 3 distinct mobile phases used to separate the compounds. The broth microdilution assay displayed average activity of chloroform at MIC 1.62 mg/mL and butanol at MIC of 1.25 mg/mL and noteworthy, the extremely non-polar hexane and extremely polar water extracts had no activity against *M. smegmatis*. The notable antimicrobial activity of extracts at MIC values were equal or less than 1 mg/mL (van Vuuren and Viljoen, 2011). Considering that the other extracts, dichloromethane, acetone, ethyl acetate, ethanol and methanol were able to inhibit the growth of *M. smegmatis* at very high concentrations, it can only be hypothesised looking at the bioautography chromatograms that the possible phytochemicals in *A. afra* extracts responsible for the antimycobacterial activity against *M. smegmatis* are of intermediate and polar polarity. However, identification and characterisation techniques like mass spectroscopy should be applied to help with the identification of the actual compounds. Tuyiringire *et al.,* 2022 reported an opposing activity as compared to the one observed in this study against *M. smegmatis*, where methanolic extracts of *Cryptolepis sanguinolenta* had the lowest MIC value of 0.574 mg/mL, whereas in this study, the *A. afra* methanolic extract was not able to inhibit the growth of *M. smegmatis.* Mycobacteria form biofilms as a virulence factor to enhance their pathogenicity and survival in various environmental conditions. The strength of a biofilm depends mainly on the extracellular polymeric substances (EPS) produced by the cells to form the matrix because the EPS allows communication between the cells, formation of hydrogen bonds, electrostatic forces and van der Waals interactions to make the matrix stable (Donlan, 2002; Tsuneda *et al.,* 2003; Khan, 2021). *Artemisia afra* plant has been reported several times to have potent antibacterial activity against both Gram-negative and Gram-positive bacteria (Jäger, 2003; More *et al.,* 2012; Martini *et al.*, 2020). The antibiofilm activity of the hexane, dichloromethane, acetone, and methanol extracts was determined using the crystal violet assay. The extracts

were investigated for their ability to inhibit initial cell attachment (4hrs), reduce formed biofilm (24 hours), and eradicate matured formed biofilm (48 hours) of *M. smegmatis*. The results exhibited a poor antibiofilm activity of the extracts on cell attachment and eradication of the matured biofilm. The poor inhibition of initial cell attachment by the extracts could be attributed to the inefficiency of the plant extracts to interfere with the formation of hydrogen bonds, electrostatic forces and van der Waals and activity of mycolic acids interactions that are required for attachment to surfaces (Donlan, 2002; Tsuneda *et al.,* 2003; Khan, 2021). The ability of *M. smegmatis* to form these biofilm clusters enables them to communicate effectively and thus continuously develop new systems to resist antibiotics and antimicrobial agents like plant extracts (Vestby *et al.,* 2020). Both *Mtb and M. smegmatis* have mycolic acids in their cell walls that help in preventing the entry of hydrophobic and hydrophilic solutions, allowing only lipids to pass through, thus contributing significantly to their drug resistance. This impermeable membrane acts as a barrier to prevent the crude extracts from penetrating the cells (Ranjitha *et al.,* 2020). In addition, *M. smegmatis* is a fast-growing microorganism when compared to other species in the same genus (Newton and Fahey, 2002). Furthermore, for survival under various growth and stress situations, bacterial populations are known to retain heterogeneous phenotype in cellular length, size, shape, intracellular components, and metabolic status (Hallez *et al.,* 2004; Ackermann, 2015,). On the other hand, when the extracts were tested for their ability to reduce formed biofilm after 24 hours in a concentration dependent manner, it was observed that the hexane extracts had no antibiofilm activity at all. Therefore, considering that the broth microdilution assay also indicated no activity against the planktonic cells when treated with hexane extracts, this could mean that the hexane extracts contain no compounds with antimycobacterial effect against *M. smegmatis*. Methanol extracts, however, had activity against the formed biofilm after 24 hours in all the tested concentrations (0.5MIC, MIC, 2MIC and 4MIC), possibly attributed to its polarity index that allows it to extract both polar and non-polar compounds. In addition, the notable activity of the methanolic extract could be attributed to the synergistic effects among the different compounds present in the extracts, thus enabling potency to penetrate through the formed biofilm, possibly having managed to interfere between the numerous bonds formed on the matrix. Furthermore, there could have been interference with the structure of the cells during their active metabolism when changing morphology from one form to another as a mode of survival. The highest

activity compared to the positive control rifampicin was noted on the smallest concentration, which was a contradicting effect to reports by (Chakraborty and Kumar, 2019), where they stated that the inhibition of biofilms requires concentrations higher than the initial MIC value that had activity against planktonic cells. Although the crystal violet method is being improved daily for investigation of antibiofilm activity, it is a common dye that binds to polysaccharides and negatively charged surface molecules in the extracellular matrix of biofilms. However, it cannot be effectively used as the only method because it stains both living and dead cells (Li *et al.*, 2003; Pitts *et al.*, 2003), which leads to the observation of misleading/incorrect results when measured using spectrophotometry, thus making it hard to distinguish, if the enhanced biofilm is that of actual viable cells or not. Given that the biofilms are treated with crude extracts, it is possible that there may be excess compounds that are inactive interfering with the active phytocompounds inside the extracts at higher concentrations because multiple zones of inhibitions were observed on the antimycobacterial chromatograms, indicating that there are several active phytocompounds present. Therefore, it is important to fractionate the crude extracts and remove them to increase the efficacy of the active ones. *A. afra* plant material has shown to possess flavonoids, tannins and phenolics, which are abundant secondary metabolites that are notorious for diverse biological activities compared to other phytochemicals. Boakye *et al.* (2016) reported that tannins have the capacity to inhibit the growth of both gram positive and gram-negative bacteria. Due to the fact that bacteria need iron to survive, tannins have the ability to bind and solubilise metal ions in a bacterial environment, leading to an iron deficiency and eventual death of the bacterial cells (Slabbert, 1992). The latter effect of tannins is an advantage to the *A. afra* extracts as antimycobacterial agents since they contain tannins.

5.6 Conclusion

A. afra extracts do have the potential to be used as antimycobacterial agents, including treatment against biofilms. Having observed that the chromatograms showed several compounds separated by the intermediate and polar mobile systems, the low efficacy of the extracts in both planktonic and biofilms could have been due to the interference of inactive compounds with the active phytocompounds at higher concentrations. However, considering that some extracts like methanol were able to reduce the irreversible formed biofilm is an advantage to the plant. Further recommendations will

be the test of the cytotoxicity of the extract and fractionation to remove the interfering inactive phytocompounds.

5.7 References

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CHAPTER 6

6. Cytotoxicity of the crude extracts

6.1 Introduction

Flavonoids, anthraquinones, terpenes, tannins, alkaloids, saponins, lactones, steroids, and volatile oils are chemical substances derived from plants that have attracted notable attention recently because of their diverse pharmacological qualities (Saisha *et al*., 2015). Some of the medicine derived from medicinal plants has been linked to serious negative effects. For instance, in South Africa, patients who reported using traditional medicines frequently had dehydration, diarrhoea, vomiting, and changed mental status (Luyckx *et al.,* 2004). Toxicities related to plants intensify effects like gastrointestinal tract irritation, allergic reactions, injury to nervous system, kidneys, liver and other important organs of the body (Nondo *et al.,* 2015; Anand and Lal, 2016; Hewawasam *et al.,* 2016). Cause of toxicity to medicinal plant herbs is from several factors, including contamination by microbes, aflatoxins, combination of herbs with other drugs and toxic compounds present in the very same herbal medicine. Additionally, mislabelling and limited or no knowledge about the verification of that plant is another contributing factor (Jain, 2016; Chugh *et al.,* 2018; Mensah *et al.,* 2019). Macrophages are primary immune system cells that perform several roles such as triggering and controlling the immunological responses to foreign antigens, and they are crucial for maintaining immune homoeostasis (Albrahim *et al.,* 2020). THP-1 cells are human monocyte leukemia cells that behave like native monocyte-derived macrophages after differentiating (Bosshart and Heinzelmann, 2016). When THP-1 cells are at the monocyte stage, they can be differentiated into macrophage-like cells using either macrophage colony-stimulating factor (M-CSF), phorbol-12-myristate-13-acetate (PMA) or 1,25-dihydroxyvitamin D3 (vD3) (Phillips *et al*., 2005; Takahashi, 2001). Determining cytotoxicity effects of medicinal plants is important because it gives insight into their toxicities and how harmful they will be to the human body (Castaño and Gómez-Lechón, 2005; Schultz *et al.,* 2020a).

6.2 Materials and Methods

6.2.1 Assessment of cytotoxicity on THP-1 cells

6.2.1.1 *Cell culture and maintenance*

THP-1 cells, a human monocytic leukemia cell line, were grown in 10 ml complete RPMI (Roswell Park Memorial Institute) medium (RPMI-1640-glutamine (Gibco) medium supplemented with 10% fetal bovine serum (FBS) in T75 flasks. The cells were incubated in a humidified incubator at 37^{o}C and 5% CO_2. Every 2-3 days, the cells were passaged and kept at a density of 2-6 x 10^5 cells/mL.

6.2.1.2 *Differentiation induction*

THP-1 cells (10 ml) were transferred into 50 ml sterile Falcon tubes and centrifuged at 250 x g for 10 minutes at 4^{o}C. The supernatant were discarded, and cell pellets were re-suspended in a complete medium containing 100 ng/ml phorbol 12-myristate 13-acetate (PMA), (Sigma). The cells (200µL) were seeded into 96-well plates at a density of 100,000 cells/well. To allow differentiation, the cells were incubated for 24 hours in a humidified incubator at 37^{o}C and 5% CO_2.The growth medium was removed from the wells, and cells were washed twice with pre-warmed RPMI-1640 medium. Pre-warmed complete RPMI medium was added into the differentiated, adhered naïve macrophages (M0) and incubated for 24 hours.

6.2.1.3 *Assessment of plant extract cytotoxicity*

All plant extracts were dissolved in 100% DMSO to make 100 mg/ml working stock concentrations. Growth medium was removed from the wells of adhered naïve macrophages (M0). Plant extracts (200 µL) that had been diluted in the complete RPMI medium to the relevant concentrations (1000, 500, 250 and 125 µg/ml), were added into the wells containing adhered cells. The control wells contained 200µL of 1% DMSO in complete RPMI. The plates were incubated for 24 hours in a humidified incubator at 37^{o}C and 5% CO_2.The growth medium was removed from the wells and 100 ml TriplE solution (Gibco) was added and incubated for 10 minutes in a humidified incubator at 37^{o}C and 5% CO_2 to detach the cells. Cell suspensions (20 µL) were diluted with equal volumes of trypan blue solution, mixed thoroughly, and placed in a hemo-cytometer and a cover slip. The hemacytometer was placed under a microscope and focused on the cells using a 40x magnification objective lens. The cells were counted using squares of the grid-cells and the squares were counted separately and

averaged. The total number of cells/ml in the cell suspension were determined by multiplying the average number of cells per square by the dilution factor and by the hemacytometer conversion factor.

6.3 Results

Cytotoxicity effects of the extracts were evaluated against differentiated THP-1 monocytes. The results illustrated in (**Figure 6.1**) show that the extracts were toxic to the cells at the highest concentration of 1000 µg/mL. Dichloromethane extracts were more toxic to the cells in all the concentrations tested. However, acetone, and methanol had less toxicity towards the cells at the lowest concentration of 125 µg/mL.

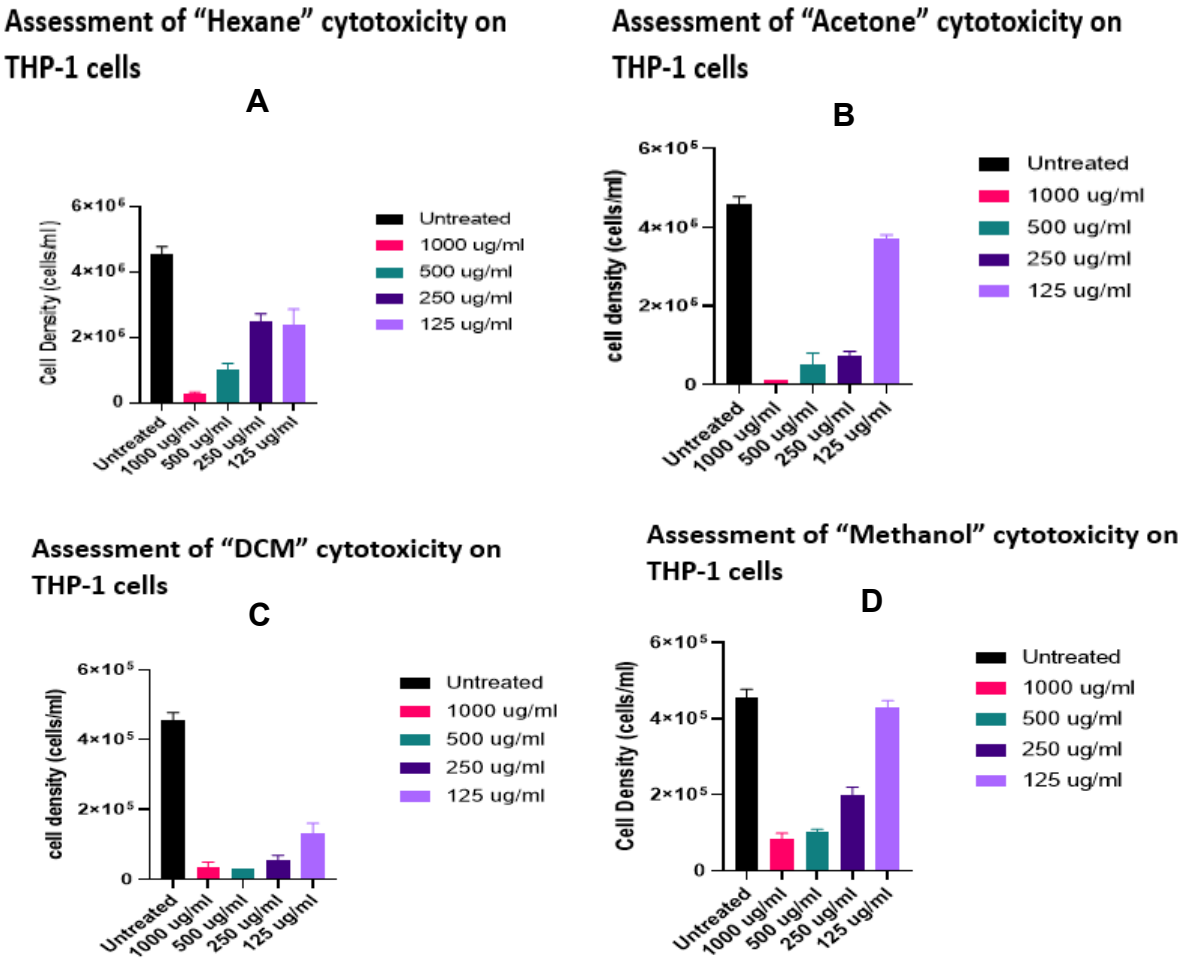

Figure 6.1: Cytotoxicity assessment of four *Artemisia afra* plant extracts, hexane (A), acetone (**B**), dichloromethane (**C**), and methanol (**D**) against THP-1 macrophages.

6.4 Discussion

The aim of this chapter was to investigate the cytotoxicity effects of the *A. afra* extracts against THP-1 macrophages. Bone marrow-derived monocytes transform into macrophages in response to acute infection and inflammation in affected areas and some organs (Geissmann *et al.,* 2010; Hettinger *et al.,* 2013; Wynn *et al*., 2013). THP-1 cells have not been found to contain any harmful substances or contagious viruses, making their use reasonably simple and secure. These transformed cell lines can be maintained *in vitro* for up to three months without experiencing any alterations in sensitivity and activity (Chanput *et al.,* 2014). Phorbol 12-myristate 13-acetate (PMA) is an ester that was confirmed by literature to be the most potent agent for the differentiation of THP-1 monocytes into matured macrophages that are similar to PBMC (Bastiaan-Net *et al*., 2013; Chanput *et al*., 2013; Chanput *et al*., 2012). It was used in this study to induce THP-1 monocyte differentiation into macrophages. The results revealed that acetone and methanol extracts were less toxic at the lowest concentration because there was a significant number of viable cells observed when compared to the untreated cells. More *et al.,* (2012) reported the less toxicity of *A. afra* extracts when texted with McCoy fibroblast cell line. The toxicity observed on the hexane and dichloromethane extract could possibly be due to other phytochemicals present in the extract (Jain, 2016; Chugh e*t al*., 2018; Mensah *et al.,* 2019). Dichloromethane extracts showed notable biofilm activity at lower concentrations; however, when the concentrations were increased, the biofilm was induced. This could then mean that the compounds that exhibited high toxicity effects on the THP-1 cell line overpowered the bioactive compounds inside the crude extracts, thus interfering with their efficacy towards the *M. smegmatis* biofilm. Furthermore, the hexane extracts that were the most toxic to the THP-1 cells, exhibited no antibiofilm and antimycobacterial activity, thus implying that the crude extracts have no bioactive compounds at all, or they are present in small amounts.

Therefore, because *Mtb* initially infects the lungs and further proliferate inside the phagocytic alveolar macrophages and dendritic cells (Zahrt, 2003), it was imperative to investigate the toxicity of the plant extracts prior to confirming their usage as therapeutic agents, to avoid alteration of treatment and weakening of the immune response cells and proteins they produce, thus allowing *Mtb* to thrive more inside the host. Additionally, THP-1 cells were selected as model for macrophages in this study

because of their genetic homogeneity that allows them to have less phenotypic variety. This characteristic is particularly important for studying the biological function of chemicals and plant extracts. Typically, a cell line with little phenotypic variation yields the most accurate results (Rogers *et al.,* 2003; Cousins *et al.,* 2003; Qin, 2012).

6.5 Conclusion

Methanol and Acetone extracts from *A. afra* aerial parts are eligible to be used as source of safe herbal medicine and for the development of therapeutic agents because they contain phytochemicals that showed less toxicity to the THP-1 cell line, and they also showed notable antimycobacterial activity against *M. smegmatis*. In addition, pure compounds that will be isolated from these extracts should be tested for cytotoxicity *in vivo*.

6.6 References

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CHAPTER 7

7. Fractionation of phytocompounds with antioxidative, anti-inflammatory and antimycobacterial activity

7.1 Introduction

Mankind have been using medicinal plants for years to treat different infections, diseases, and wounds. The selection of herbs and their application as therapeutic agents was based on traditional knowledge and cultural beliefs; hence, if research is conducted about a particular plant from several ethnic groups, they will provide different information about the medicinal property of that similar plant; therefore, making it paramount to use modern methodologies to test and provide better understanding about the safety and biological activities of all medicinal herbs. Medicinal plants are used either as crude extracts and/or pure isolated compounds; however, due to their chemical composition diversity, it is important to further ascertain their biological activities as pure isolates, especially for drug development (Cos *et al.,* 2006; Petrovska, 2012). In addition, toxicity effects of the fractions and pure compounds also need to be evaluated because when people are using herbs as a source of medicine to treat ailments without knowledge of their toxicity to the human body, it might result in dire outcomes such as the destruction of red blood cells, allergic reactions, damage to body organs, among others (Nondo *et al.,* 2015; IARC, 2012).

Microbial resistance and adverse effects of synthetic drugs have elevated the need to isolate and purify bioactive phytochemicals for new, safe, and immediate response therapeutics (Sasidharan *et al.,* 2011). To be able to fractionate, isolate, purify and characterise the structure of bioactive compounds, extraction is the first step to go through because it allows one to draw out various phytochemicals from the plant material, depending on which method and solvents one is using for the process. Basic procedures to follow before extraction include drying, pre-washing and grinding, which help to acquire uniform sample for one's extraction and increase the surface of contact between the plant material and the solvent used. Failure to follow the proper procedures of extraction might result in lost and/or destroyed chemical nature of phytochemicals, which will affect their bioactivity (Fabricant and Farnsworth, 2001). Hydrophilic phytocompounds are extracted with polar solvents such as ethyl acetate, ethanol, and methanol, while dichloromethane and methanol are used to extract lipophilic phytochemicals. The most non-polar solvent, hexane, is used in extraction

processes to eliminate chlorophyll from the plant extracts (Cos *et al.,* 2006). Since phytochemicals are different, it is necessary to separate them before evaluating each one's unique medicinal qualities to determine their efficacy. Some of the separation or fractionation procedures entail the use of TLC, High Performance Liquid Chromatography (HPLC), column chromatography, flash chromatography, etc. (Sasidharan *et al*., 2011). TLC is a conventional, inexpensive, and fast method used to identify the different phytochemicals present in a plant extract. Retention factor (RF), the use of spraying reagents for colour development and ultraviolet (UV) light are typical approaches used to visualise and confirm the phytochemicals separated on a TLC plate. It is an ideal procedure to follow before fractionating/isolating, purifying and characterising pure compounds (Shahverdi, 2007). Bioautography is an assay used to evaluate the antibacterial activity of plant extracts against pathogenic organisms. It is a technique that also utilises TLC plates, where the extracts are loaded on the plate and later the microorganism is sprayed over the same plate and the activity of extracts against the organism is observed by white zones of inhibition. It is also a fast and inexpensive procedure to use in search of new antimicrobials (Cos *et al.,* 2006). Therefore, it is important to use a proper extraction method, analyse the present phytochemicals in one extract and confirm their efficacy against the tested organism before proceeding to isolation and purification.

Bioassay-guided fractionation is a technique that involves chromatographically fractionating and refractionating crude extracts until a pure active compound is obtained. Separated fractions or pure compounds are further subjected to bioassays to evaluate their different efficacy in relation to finding new antimicrobials for diseases such as TB (Malviya and Malviya, 2017). One advantage of column chromatography is that little information about the molecules must be known before the purification procedure. In addition, the rate at which the compounds travel/separate inside the column can give an insight about their polarity, depending on what kind of eluent is used and the affinity to the silica gel serving as a stationary phase. Therefore, this could be helpful in drug discovery to predict the type of compounds that will be isolated (Ebere *et al.,* 2019). Secondary metabolites have an advantage of exhibiting synergistic effects as a form of defence mechanism (Harvey *et al.,* 2015). However, others demonstrate high potency as singe agents (Balunas and Kinghorn, 2005). Crude extracts contain a numerous number of distinct phytocompounds with different

properties and biological effects. Moreover, it becomes complicated to work with mixed compounds, especially if they are of different families or functional groups because this affords them the ability to behave differently. This can also make the phytocompounds hinder each other's effectiveness and thus reduce their potency to inhibit or kill pathogens such as *Mtb*. Furthermore, due to the presence of different compounds in the crude extracts, there is also a chance of unintended side effects or herbal toxicity (Ighodaro *et al.,* 2016). This explains why bioassay guided fractionation of crude extracts is needed to remove the other inactive phytocompounds interfering with the active ones and reducing their efficacy. Another analytical technique used to separate compounds in mixed samples is called liquid chromatography-mass spectroscopy (LC-MS). This is a technique that combines sensitive and focused mass spectrum detection with high-resolution chromatographic separation (Parasuraman *et al.,* 2014). In LC-MS, mass spectrometry aids in the identification of a sample's constituent elements and their structure (Pitt, 2009). The LC-MS technique quantitatively and qualitatively measures the isotopic weight of proteins, chemical substances and pharmaceutical agents. One must evaluate gene expressions, determine aflatoxins from fungi and further give structural information of the spectra fragmentations obtained from the analysed masses. Moreover, it provides the pharmaceutical profiles of substances such as drug metabolites and chiral impurities (Prakash *et al.,* 2007; Parasuraman *et al.,* 2014).The objective of this chapter was to fractionate the active acetone extracts of *Artemisia afra* with antioxidative, anti-inflammatory and antimycobacterial properties.

7.2 Materials and Methods

7.2.1 Serial exhaustive extraction

Serial exhaustive extraction was used to extract bioactive compounds from the *A. afra* leaf material. A total mass of 360 g of powdered leaf material was weighed and extracted sequentially with 3.5 L of *n*-hexane, dichloromethane, acetone, and methanol in duplicates, respectively. The mixture was vigorously shaken overnight using a shaker (Thermo Scientific, MaxQ, SHKA3000-1CE) at 200 rpm and filtered using Whatman. Shaking was repeated for 4 hours and lastly for 2 hours with each solvent before beginning the extraction process with the next solvent. The filtrates were concentrated using a rotary evaporator (Lasec, Lauda CAT:RE400/MS) at different boiling points for each of the solvents and transferred into pre-weighed

labelled beakers. The remaining solvents were evaporated from the extracts under a stream of cold air at room temperature.

7.2.2 Qualitative Phytochemical analysis

The chemical profiles of the *A. afra* extracts were analysed on aluminium-backed TLC plates using a method developed by Kotze and Eloff (2002), as described in Chapter 3.

7.2.3 Qualitative DPPH assay on TLC plates

Qualitative DPPH assay was done using TLC, according to the method described by Braca *et al.* (2002) and outlined in Chapter 4 (section 4.2.1.1).

7.2.4 Egg-albumin denaturation assay

Egg albumin denaturation assay was performed according to the method described in chapter 4 (section 4.2.2.1).

7.2.5 Antimycobacterial activity

7.2.5.1 Bioautography assay

Bioautography was done according to the method described by Begue and Kline

(1972), as discussed in Chapter 5 (section 5.2.2.1).

7.2.5.2 Serial broth micro-dilution assay

A serial broth micro-dilution method described by Eloff (1998) was used to determine the MIC values of the extracts against *M. smegmatis*, as outlined in Chapter 5 (section 5.2.3.1).

7.2.5.3 Antibiofilm activity

The crystal violet assay was done as previously described by (Djordjevic *et al.,* 2002) with some modifications (Sandasi *et al.,* 2008), as outlined in chapter 5.

7.2.5.4 Growth curve of M. smegmatis after treatment with fraction

To investigate the effect of *Artemisia afra* plant extracts on the growth of *M. smegmatis* (ATCC1441), the growth curve method was performed with modifications. *M. smegmatis* culture grown overnight (OD600 <1) was diluted to OD_{600} 0.1 at log-phase and inoculated into conical flasks containing 20 mL Middlebrooks 7H9 broth containing glycerol (Sigma) and supplemented with Oleic Albumin Dextrose Catalase (OADC) growth supplement (Sigma). Thereafter, it was treated with the plant extracts (0.5MIC,

MIC, 2MIC, and 4MIC mg/mL) concentrations and incubated in a shaker incubator at 37 °C. The wild type *M. smegmatis* without the extracts was used as a positive control. Growth was followed by monitoring the optical density (OD) at the absorption of 600 nm using a spectrophotometer at the intervals of 3, 6, 9, 18, and 24 hours (Jiang *et al*., 2011).

7.2.6 Fractionation of active phytocompounds

7.2.6.1 Open column chromatography

The acetone extracts (A1—A3) obtained from the serial exhaustive extraction were subjected to open column chromatography, as they had significant antimycobacterial activity. An open column (35 cm height × 3 cm radius) was packed with silica gel 60 (particles size 0.063 —0.200 mm) using 100% *n*-hexane. The extracts (12.789 g) were mixed with silica gel to a paste-like consistency and loaded on the column. The chemical constituents of the extracts were eluted using 1.2 L of the solvent systems illustrated in **Table 7.1**. The fractions were collected until each 1.2 L of the eluent solvents was finished and concentrated using a rotary evaporator to 100 mL. Thereafter, the solvents were completely evaporated under a stream of cold air in pre-weighed beakers at room temperature and the masses of the fractions were determined. Phytochemical analysis of the fractions was then determined using TLC, as described in Chapter 3. The antioxidative DPPH assay was performed, as described in chapter 4, and bioautography and serial broth micro-dilution assays were also done, as outlined in chapter 5.

Table 7.1: Solvent systems used to run the first column.

Numbering	Solvent systems	Percentages (%)
1	Hexane	100
2	Hexane	100
3	Hexane: Ethyl Acetate	90
4		80
5		70
6		50
7		30
8		10
9	Ethyl Acetate	100
10	Ethyl Acetate: Methanol	90
11		80
12		70
13		60
14		50
15		40
16	Methanol	100

7.2.6.2 Determination of solvent system for second column chromatography

Combinations of hexane and ethyl acetate were used as mobile phases (**Table 7.2**), using results from the first open column as guidance. TLC plates were loaded with 20 µL of 20 mg/mL of biologically active fractions (30:10:100), reconstituted in acetone and the plates were developed in the different mobile phases. Phytochemical analysis (described in Chapter 3) and bioautography assay (Chapter 5) were performed to identify the mobile phase that best separates the compounds through antioxidative activity as well as antimycobacterial activity against *M. smegmatis*.

Table 7.2: Solvent systems used as mobile phases to determine the eluent to run second column chromatography.

Numbering	Solvent systems	Percentages (%)
1	Hexane	100
2	Hexane: Ethyl Acetate	80
3		60
5		40
6		50
7		20
8		30
9		40
10		60
11		70
12	Ethyl Acetate	100

7.2.6.3 Second open column chromatography

A mass of 1.963 g of combined fractions (30:10:100) was loaded onto a column packed with silica gel 60 and eluted with 30% hexane and 70% ethyl acetate. The eluents were collected in small test tubes and placed under a stream of air to concentrate. Thereafter, groups of 5 sub-fractions were collectively concentrated and analysed for phytochemical profile and antimycobacterial activity against *M. smegmatis*. The sub-fractions 15-175 were combined and evaluated for antimycobacterial activity using bioautography and serial broth micro-dilution assays.

7.3 Liquid chromatography-Mass spectroscopy

The LC-MS/MS analysis was carried out using a Waters Synapt G2 qTOF mass spectrometer. The Synapt G2 qTOF from Waters (Milford, USA) is a high-resolution quadrupole time-of-flight (qTOF) mass spectrometer capable of data independent analysis (DIA) using Waters ms E technology. When linked to a Waters Acquity UPLC, the system can achieve good chromatographic separation between compounds followed by simultaneous acquisition of both fragmented and unfragmented mass spectra of all compounds within each peak eluting off the column, together with UV spectra produced by the photodiode array (PDA) detector placed upstream of the qTOF. The acetone crude extract and the sub-fraction were centrifuged at 12,000 rpm for 10 minutes before analysis. A waters HSS T3 column, 2.1 × 150 mm was used in obtaining the separation of the phytoconstituents. Two mobile phases (A) and (B) were used, where (A) consisted of 0.1% formic acid in water and (B) had acetonitrile 5 mM ammonium formate. A 5 μL volume of the extracts was injected into the analytical

column for analysis. The sample flow rate was set at 0.4 mL/min. The MS spectra were acquired in the positive ion mode. The mass fragmentations were identified by using a spectrum database for organic compounds.

7.4 Statistical analysis

Where appropriate, results were expressed as means±standard deviation (SD) of triplicate and duplicate determinations. Statistical analysis was performed by Microsoft Excel 365 and Graph pad prism v9.5.0 by a two-way analysis of variance (ANOVA), followed by a Dunnet multiple comparison test. Significant difference was considered when $p<0.05$ and conversely, non-significance was indicated by $p>0.05$ values.

7.4 Results

7.4.1 Serial exhaustive extraction

Serial exhaustive extraction was done in three different time intervals (overnight, 4 hours and 2 hours) using solvents of varying polarity, namely, hexane, dichloromethane, acetone, and methanol. It was found that methanol had extracted a total mass of 56.147 g, followed by dichloromethane with 28.099 g and acetone was the least extractant at 13.384 g (**Table 7.3**).

Table 7.3: Masses (g) extracted from *Artemisia afra*.

Extracts		**Mass residue (g)**	
		Mass	**total**
Hexane	H1	12.374	
	H2	5.181	19.437
	H3	1.882	
Dichloromethane	D1	19.071	
	D2	6.782	28.099
	D3	2.246	
Acetone	A1	7.564	

	A2	4.014	13.384
	A3	1.806	
Methanol	M1	36.252	
	M2	13.211	56.147
	M3	6.684	

7.4.2 Phytochemical analysis

Ultraviolet light and vanillin sulphuric acid reagent were the two methods used to visualise phytochemicals after separating them using TLC. There were few dark spots of bands detected under 254 nm UV-light on the acetone extracts (**Figure 7.1A**). Thereafter, visualisation with a longer wavelength of 365 nm (**Figure 7.1B**), and several colourful bands were observed in all the chromatograms. After spraying with vanillin sulphuric acid, some few bands were also detected in all the plates (**Figure 7.1C**). After serial exhaustive extractions, all the mobile phases, non-polar (BEA), intermediate (CEF) and polar (EMW) were able to separate compounds from the plant extracts, especially in acetone.

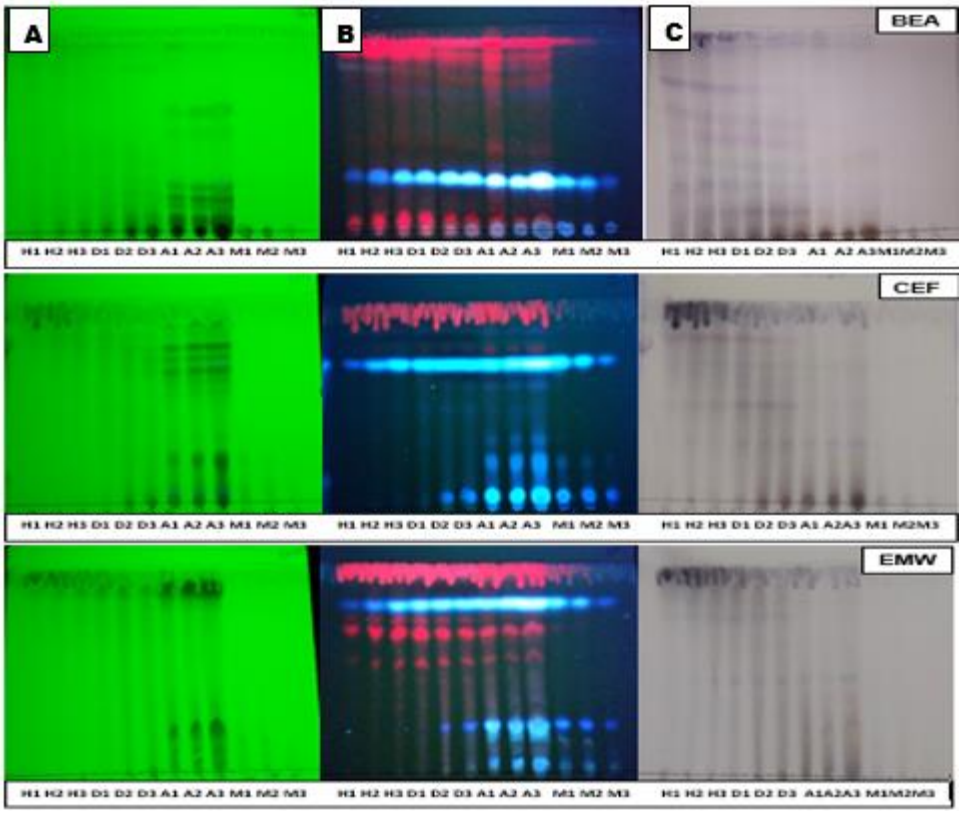


Figure 7.1: Chromatograms of *Artemisia afra* plant extracts extracted with different solvents and developed in BEA, CEF and EMW mobile systems then visualised under UV light at 254 nm (**A**), 365 nm (**B**) and then sprayed with vanillin-sulphuric acid reagent (**C**).

Key: H= Hexane, D= Dichloromethane, A= Acetone, M= Methanol.

7.4.3 TLC-DPPH assay

Antioxidant activity was determined using DPPH as a free radical and the activity was observed by a yellow colour against the purple background. The two mobile phases CEF and EMW were able to separate some active compounds. However, those in BEA were able to show activity but did not migrate from the baseline. Notable antioxidant activity was detected only in acetone extracts (**Figure 7.2**).

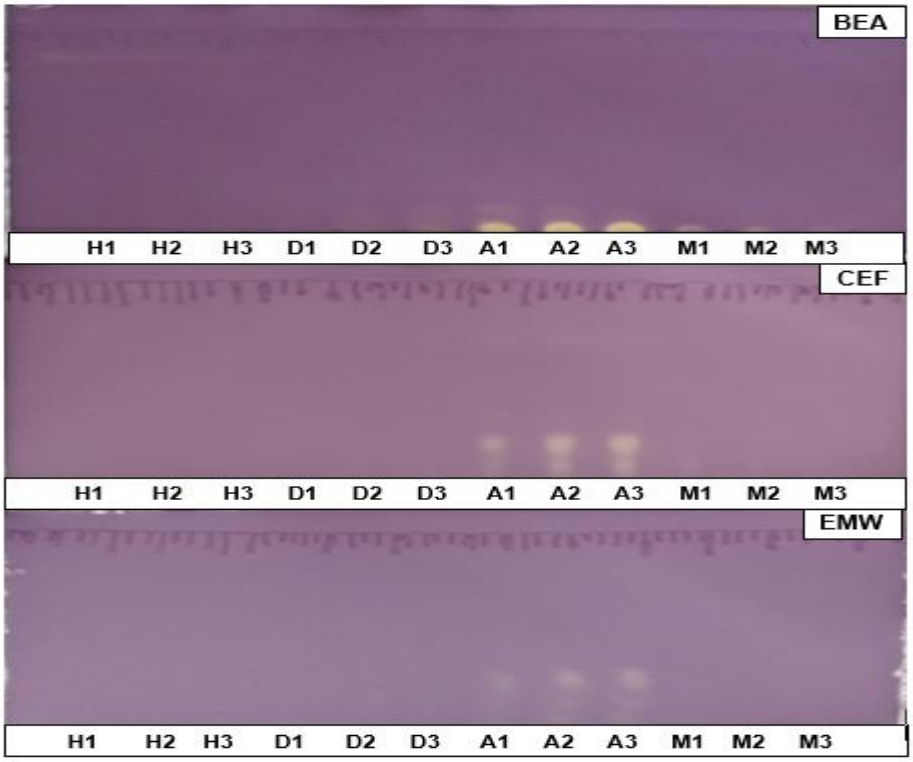


Figure 7.2: Chromatograms of *Artemisia afra* plant extracts, extracted with different organic solvents and developed in BEA, CEF and EMW mobile systems and then sprayed with 0.2% DPPH in methanol. The yellow colour indicates antioxidative activity.

Key: H= Hexane, D= Dichloromethane, A= Acetone, M= Methanol

7.4.4 Bioautography assay

Qualitative antimycobacterial activity was evaluated using bioautographic assay with tetrazolium salt as an indicator, and activity was observed by white areas indicating zones of inhibition. Compounds were able to migrate from the base line after the plates were developed in BEA, CEF and EMW mobile phases. Notable antimycobacterial activity was in the acetone extracts in all chromatograms, although on the plate developed in EMW, it was detected at the solvent front line (**Figure 7.3**).

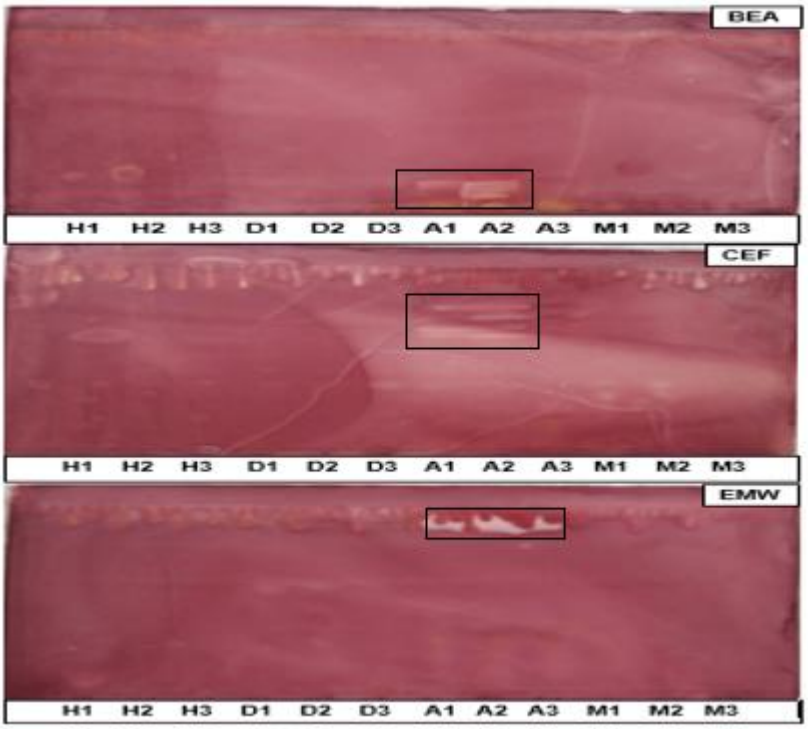


Figure 7.3: Antimycobacterial activity of *Artemisia afra* extracts separated in BEA, CEF and EMW then sprayed with *M. smegmatis*. White areas indicate zones of inhibition.

Key: H= Hexane, D= Dichloromethane, A= Acetone, M= Methanol.

7.4.5 Serial broth microdilution assay

To quantify the MICs of the extracts against *M. smegmatis*, a serial broth microdilution assay was utilised, and tetrazolium salt was used as an indicator. The non-polar and intermediate extracts showed remarkable antimycobacterial activity at MIC values ranging from 0.208 mg/mL to 0.833 mg/mL. Methanol extracts had no activity against *M. smegmatis* at all. In addition, the positive control rifampicin had notable activity at the MIC value of 0.00156 mg/mL (**Table 7.4**).

Table 7.4: MICs (mg/mL) of *Artemisia afra* plant extracts against *Mycobacterium smegmatis.*

Microorganism	Extracts(mg/mL)													
	H1	H2	H3	DCM1	DCM2	DCM3	A1	A2	A3	M1	M2	M3	Average	Rifampicin
Mycobacterium smegmatis	0.625	0.833	0.313	0.208	0.417	0.261	0.417	0.313	0.833	—	—	—	0.469	0.00156

7.4.6 Biological activities of acetone fraction

Figure 7.4 illustrates the process of fractionation to obtain the active acetone subfraction. Four solvents of varying polarity, hexane, DCM, acetone, and methanol were used during the serial exhaustive extraction procedure and the resultant masses are displayed in the diagram. Colum chromatography was further used to fractionate the active acetone extracts obtained from extraction. The masses and active subfractions after both first and second column chromatography are also shown in the diagram. The subfraction obtained after the second column was further subjected to multiple assays, including microbroth dilution, antibiofilm, growth curve and egg-albumin denaturation assay.

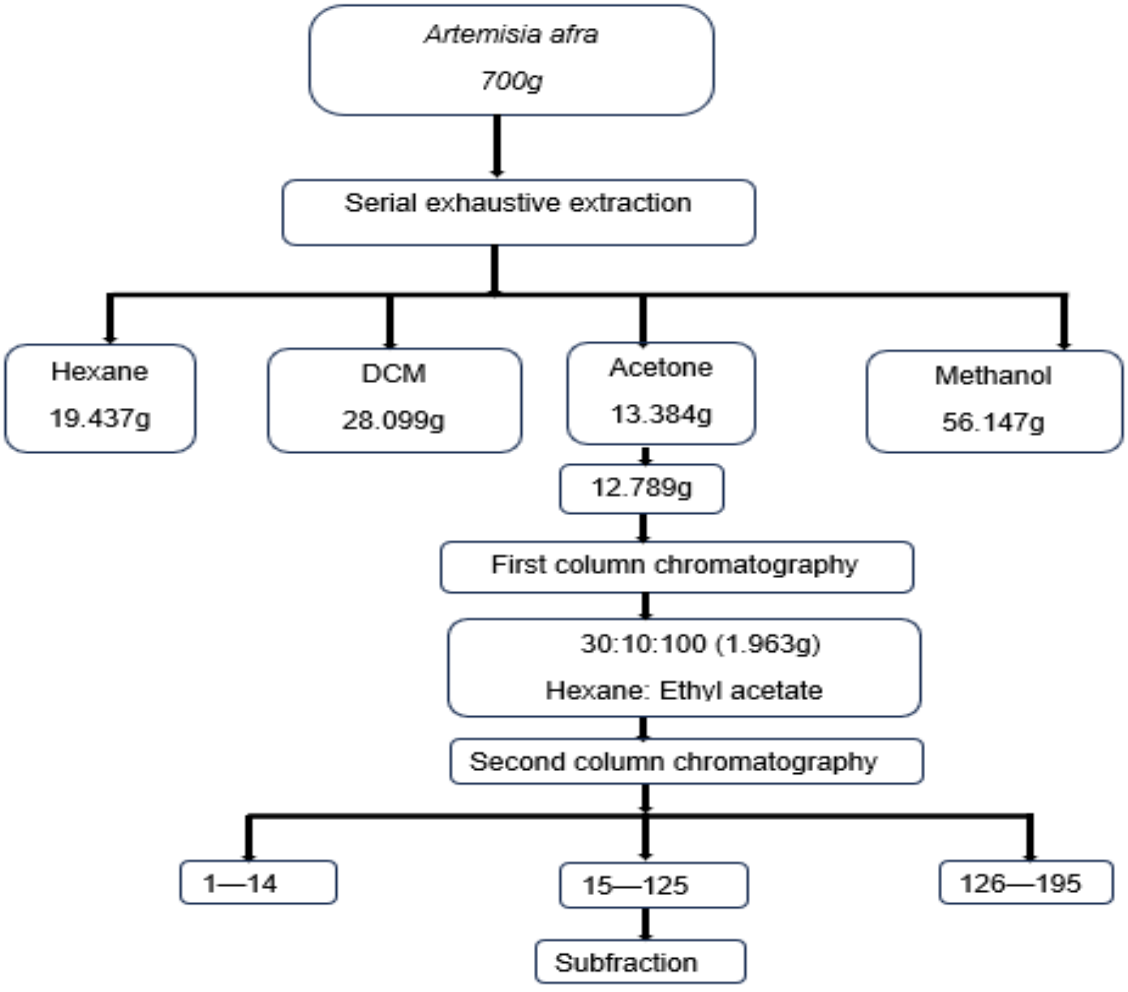


Figure 7.4: Flow diagram of the extraction and fractionation process of the active acetone extracts.

7.4.6.1 Fractionation—first column chromatography

a. Phytochemical analysis

Column chromatography was used to separate the active acetone crude extracts using several ratios of different solvents. After collections of the subfractions, a phytochemicals analysis was done to analyse the different compounds present inside the subfractions. It was therefore, observed that the acetone subfractions contain several compounds with different characteristics. Those that had fluorescing characteristics were visualised with UV-light at 254 nm (**Figure 7.5A**), and some dark spots were detected showing the presence of phytochemicals. The longer wavelength of 365nm was also used to detect other compounds and there were glowing bands detected in all the chromatograms (**Figure 7.5B**). Additionally, non-fluorescing compounds were visualised with vanillin spraying reagent and there were colourful bands observed as well (**Figure 7.5C**). All the mobile phases, BEA, CEF and EMW were able to separate phytochemicals inside the subfractions.

Figure 7.5: Chromatograms of *Artemisia afra* subfractions separated in BEA, CEF and EMW mobile systems then visualised under UV light at 254nm(**A**), 365nm(**B**) and then sprayed with vanillin-sulphuric acid reagent (**C**).

b. TLC-DPPH assay

The antioxidative power of the subfractions after they were separated from other compounds in the crude extracts was determined using DPPH as a free radical. The three mobile phases, BEA, CEF and EMW, were able to separate the compounds and those with antioxidant activity were detected. The activity was noted by a yellow colour against the purple background of DPPH. Overall, in all the chromatograms, activity was spotted from subfractions (30,10.100) hexane: ethyl acetate and (90,80,70, 60 and 50) ethyl acetate: methanol ratios. Although those in the BEA system did not migrate as much (**Figure 7.6**).

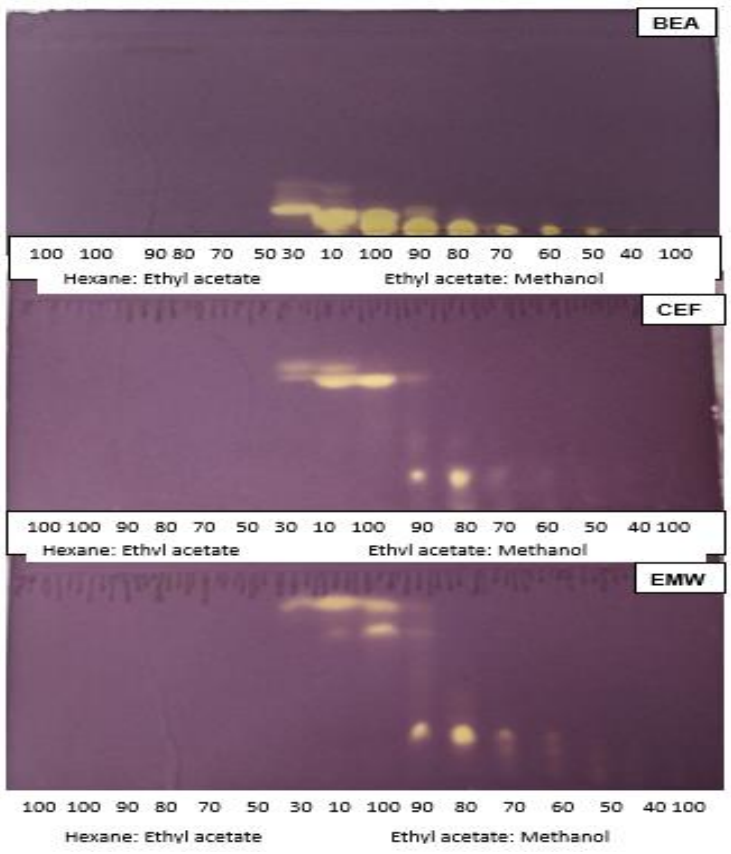


Figure 7.6: Chromatograms of *Artemisia afra* subfractions of hexane: ethyl acetate and ethyl acetate: methanol, developed in BEA, CEF and EMW mobile systems and then sprayed with 0.2% DPPH in methanol. The yellow zones indicate antioxidative activity.

c. Bioautographic assay

Antimycobacterial activity of the acetone subfractions eluted with first column was determined. Activity against *M. smegmatis* was observed on all chromatograms, however, the plate that was developed in EMW mobile phase, depicted activity at the front line while those spotted on BEA and CEF did not separate well. The notable

subfractions with activity were those of hexane: ethyl acetate (30,10,100), which were further subjected to a second column (**Figure7.7**).

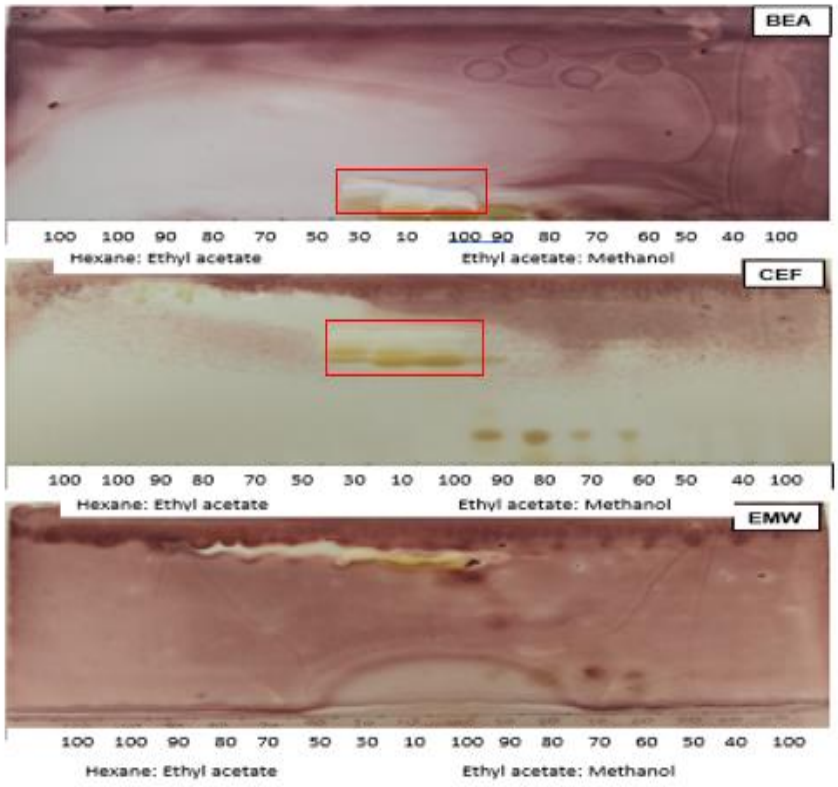


Figure 7.7: Chromatograms of *Artemisia afra* subfractions of hexane: ethyl acetate and ethyl acetate: methanol separated in BEA, CEF and EMW and then sprayed with *M. smegmatis*. White areas indicate zones of inhibition.

d. Serial broth microdilution assay

MIC of the subfractions was evaluated. The MIC values ranged between 0.833 mg/mL and 2.083 mg/mL. Most of the subfractions, especially those with high amount of hexane ratio, had no activity and those that were eluted with ratios of ethyl acetate and methanol did not have any activity against *M. smegmatis* as well. The ratios 30,10 and 100 had MIC values of 0.833 mg/mL,0.833 mg/mL and 1.042 mg/mL , respectively (**Table 7.5**).

Table 7.5: MICs (mg/mL) of subfractions against *Mycobacterium smegmatis*.

Microorganism	Subfractions(mg/mL)																
	100	100	90	80	70	50	30	10	100	90	80	70	60	50	40	100	Average
Mycobacterium smegmatis	—	—	—	1.25	—	1.042	0.833	0.833	1.042	2.083	—	—	—	—	—	—	1.181

7.4.6.2. Fractionation—second column chromatography

For a proper separation of the active subfractions acquired from the first column, several mobile phases had to be prepared and tested to distinguish which one will be able to separate the active compounds efficiently. From the mixture of the 30,10 and 100 subfractions, 10 µl was spotted on the TLC plates and the plates were developed inside the different mobile phases prepared with hexane and ethyl acetate. UV-light and vanillin sulphuric acid were used as visualisation methods and several spots and colourful bands were observed (**Figure7.8**).

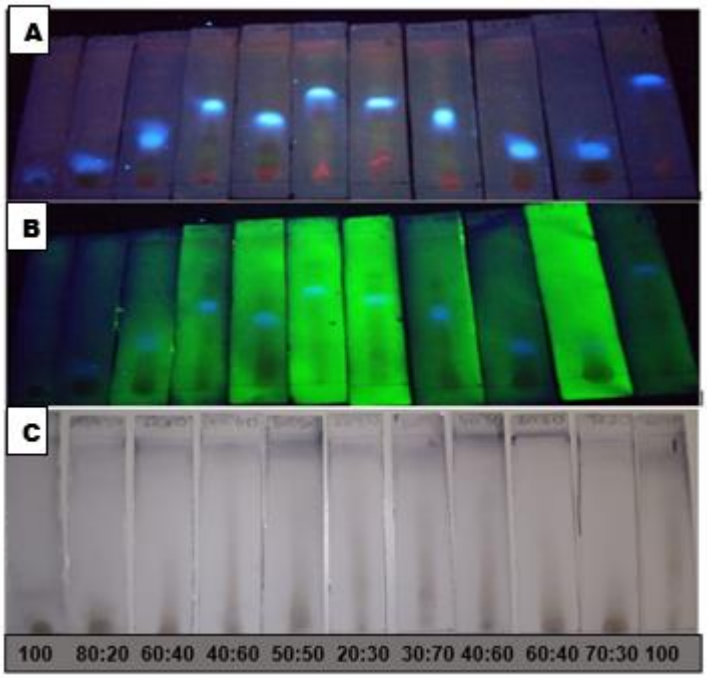


Figure 7.8: Chromatograms of hexane and ethyl acetate fractions used to determine mobile phase to run second column. Then visualised under a UV light at 365 nm (**A**) and 254 nm (**B**), and then sprayed with vanillin sulphuric acid (**C**).

a. Bioautographic assay

To confirm the activity of the subfraction mixtures, bioautography assay was used. Plates displayed on **Figure 7.9A** were loaded with 10 µl of 10 mg/mL subfractions mixture and the observed activity was not significant. Therefore, the concentration of the subfraction had to be increased to 20 mg/mL and the activity was well notable on the plate developed in 70:30 ratio of ethyl acetate and hexane (**Figure 7.9B**).

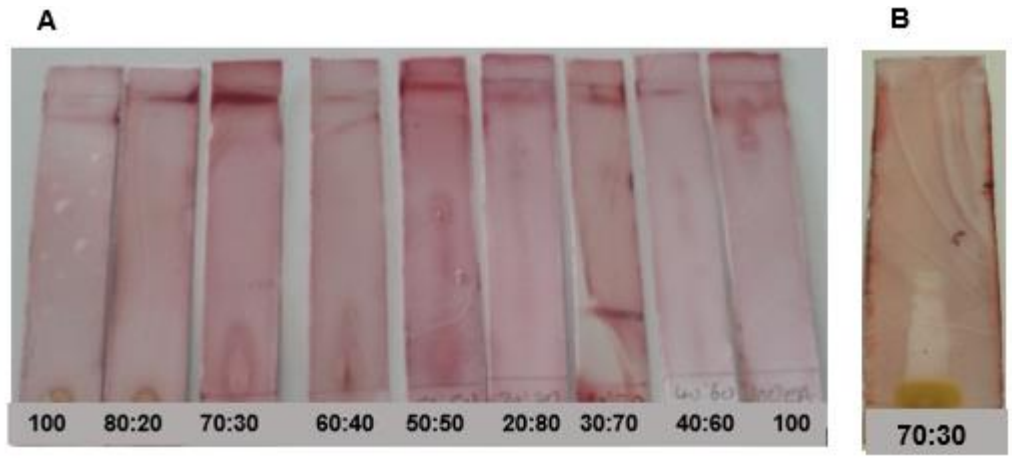


Figure 7.9: Chromatograms of hexane and ethyl acetate fractions used to determine mobile phase to run second column. Then sprayed with *M. smegmatis* (**A**) and (**B**).

7.4.6.2.2. Biological activities of second column subfractions

a. Phytochemical analysis of fractions

Second open column chromatography was loaded with the hexane: ethyl acetate (30,10,100) subfractions mixture with a mass of 1.963 g. Phytochemical analysis using UV-light at 254 nm, 365 nm and vanillin sulphuric acid reagent resulted in observation of compounds on the 3 plates after development in BEA mobile system (**Figure 7.10**).

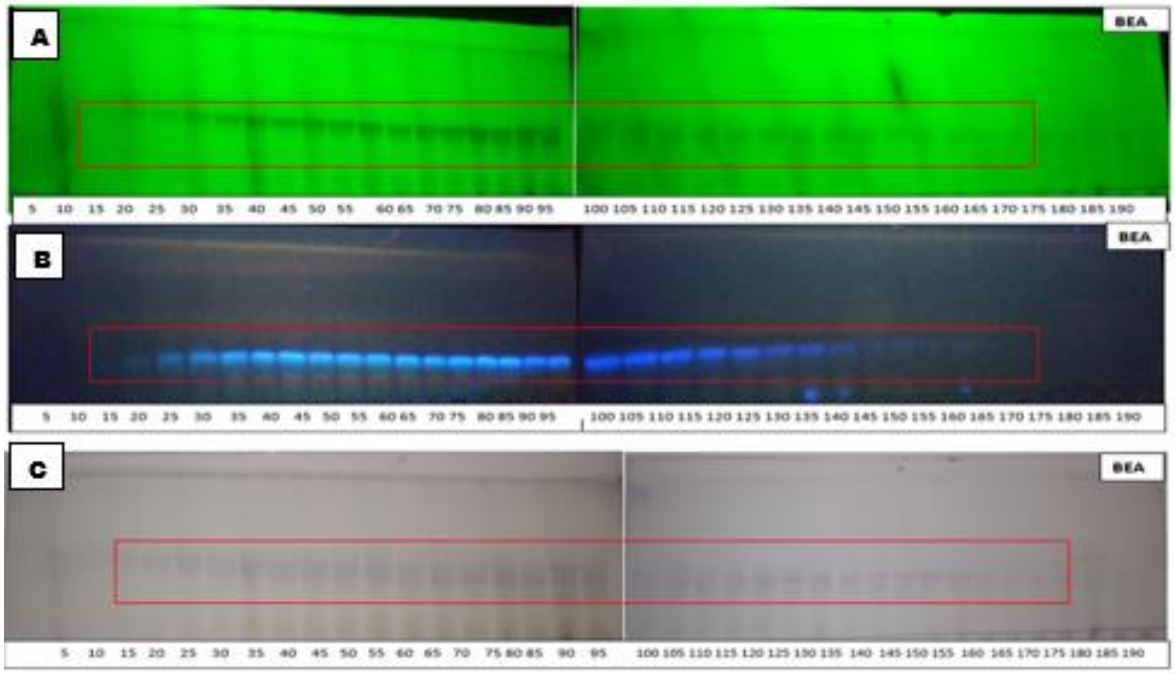


Figure 7.10: Chromatograms of subfractions collected and selected by multiples of 5 (5-190) from second open column eluted by 30%hexane and 70% ethyl acetate and then developed in BEA mobile phase. Further, visualised under a UV light at 254 nm (**A**), 365 nm (**B**) and sprayed with vanallin sulphuric acid (**C**).

b. Bioautographic assay

Antimycobacterial activity of the subfractions collected from second column was evaluated against *M. smegmatis*. It was then observed that subfractions 15 to 125 had notable activity and the compounds showed by white areas seem to be three in number (**Figure 7.11**).

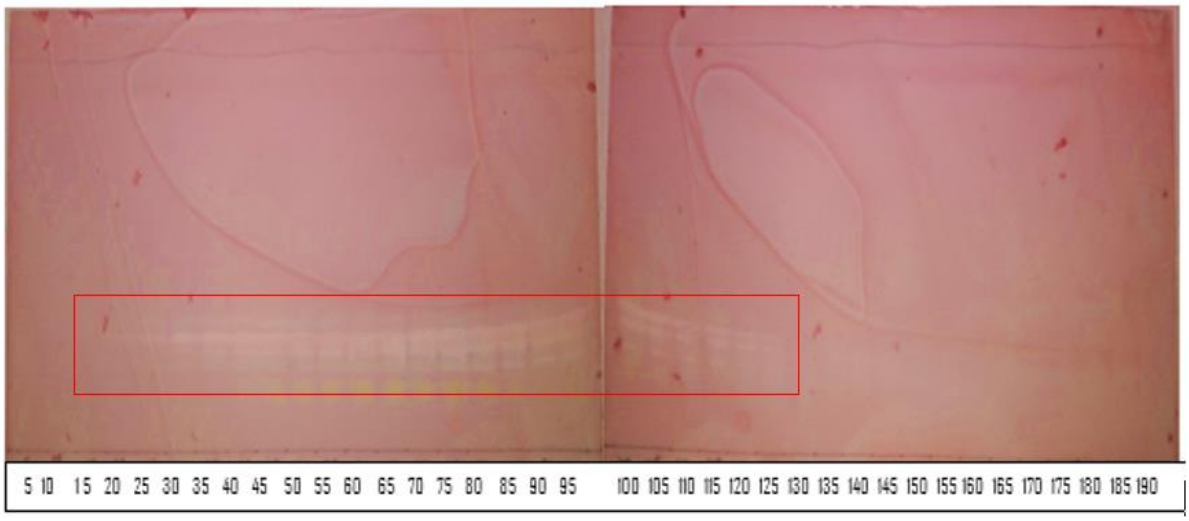


Figure 7.11: Antimycobacterial activity of subfractions collected and selected by multiples of 5 (5-190) from second open column eluted by 30% hexane and 70% ethyl acetate and then developed in BEA mobile phase. Then sprayed with *M. smegmatis.*

c. Serial broth microdilution assay

The subfractions obtained from second column were mixed together to make one subfraction and the MIC of the subfraction was determined. The subfraction, showed remarkable activity at MIC value of 0.078 mg/mL (**Table 7.6**).

Table 7.6: MIC (mg/mL) of subfraction against *Mycobacterium smegmatis*

Microorganisms	Subfraction (mg/mL)
Mycobacterium smegmatis	0.078

d. Antibiofilm activity

The subfraction was tested for its efficiency to prevent initial cell attachment, inhibit biofilm formation, and eradicate matured formed biofilm using the crystal violet assay. After 4 hours of cell attachment, it was observed that the subfraction was able to prevent the cell attachment at the MIC, 2MIC and 4MIC concentrations as compared to the positive control rifampicin (**Figure 7.11A**). There was also remarkable inhibition

of biofilm after 24 hours by the subfraction at all concentrations (**Figure 7.11B**). However, the subfraction was not able to eradicate the matured biofilm after 48 hours; instead, it was enhanced (**Figure 7.11C**).

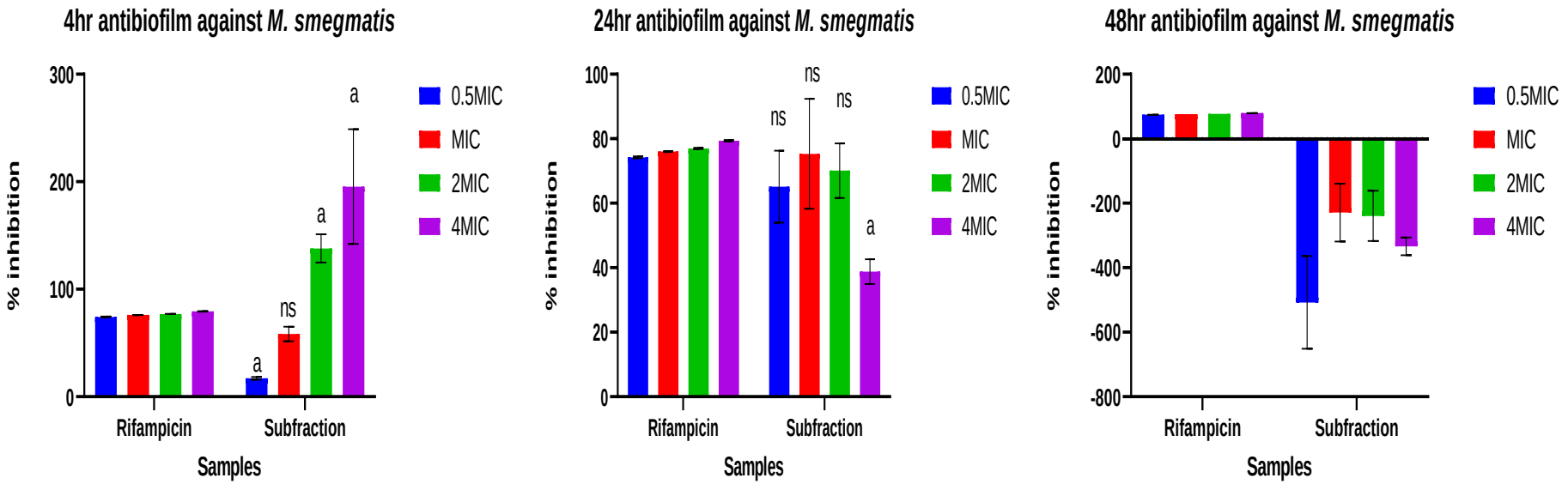


Figure 7.12: Antibiofilm activity of *Artemisia afra* acetone subfraction measured at 590 nm after different time intervals 4 hours, 24 hours and 48 hours.

Key: a=p<0.05, ns=non-significant

e. Growth curve assay

The potency of both the acetone crude extract and sub-fraction to disrupt the growth of *M. smegmatis* at different time intervals 3 hours, 6 hours, 9 hours,18 hours and 24 hours was evaluated using the growth curve assay. After treatment with the acetone crude extract (**Figure 7.13A**), it was noted that the different MIC values were able to disrupt the growth of *M. smegmatis* efficiently when compared to the untreated cells. Additionally, after treatment with the acetone subfraction, it was observed that the higher MIC (4MIC) was able to completely disrupt the growth of the *M. smegmatis* in the first 9 hours; however, after that persistere cells gradually continued to grow, although the growth was still very much lower than that of the untreated cells (**Figure 13B**). Both the crude and sub-fraction were able to reduce the rate of growth of *M. smegmatis* at all the tested concentrations even at sub-MIC.

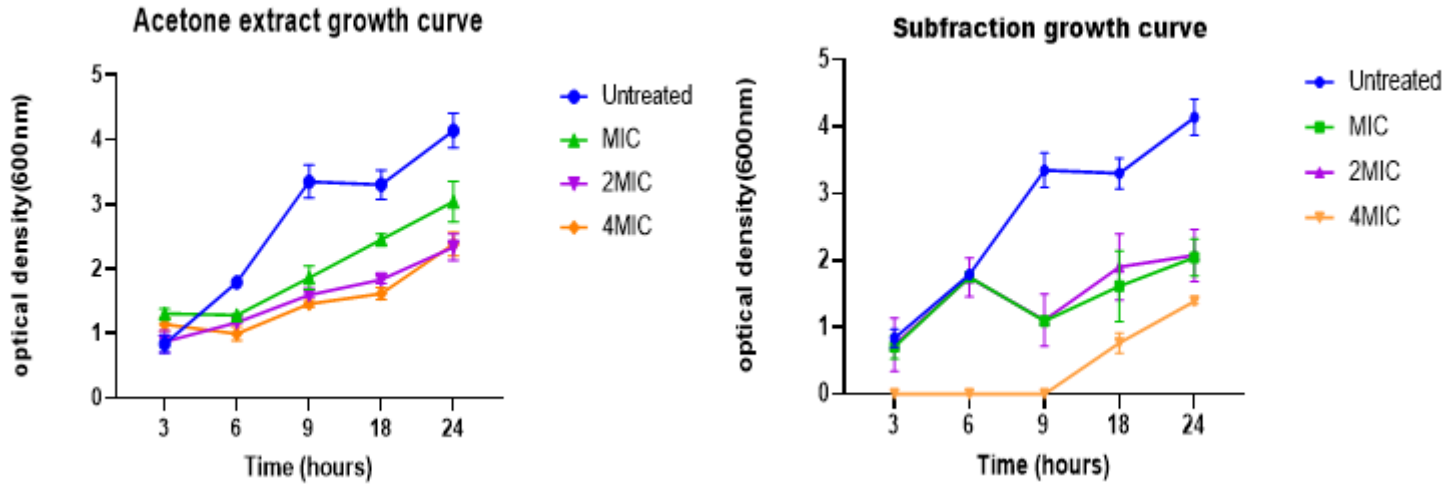


Figure 7.13: Optical density of *M. smegmatis* planktonic cells upon treatment with acetone crude extracts and the subfraction monitored at different time intervals 3 hours, 6 hours, 9 hours, 18 hours and 24 hours measured at 600nm.

f. Egg-albumin denaturation assay

The efficiency of the subfraction to inhibit denaturation of the albumin protein was evaluated. Diclofenac sodium was used as a positive control. The results illustrated in **Figure 7.14** show that the subfraction had comparable activity to the positive control.

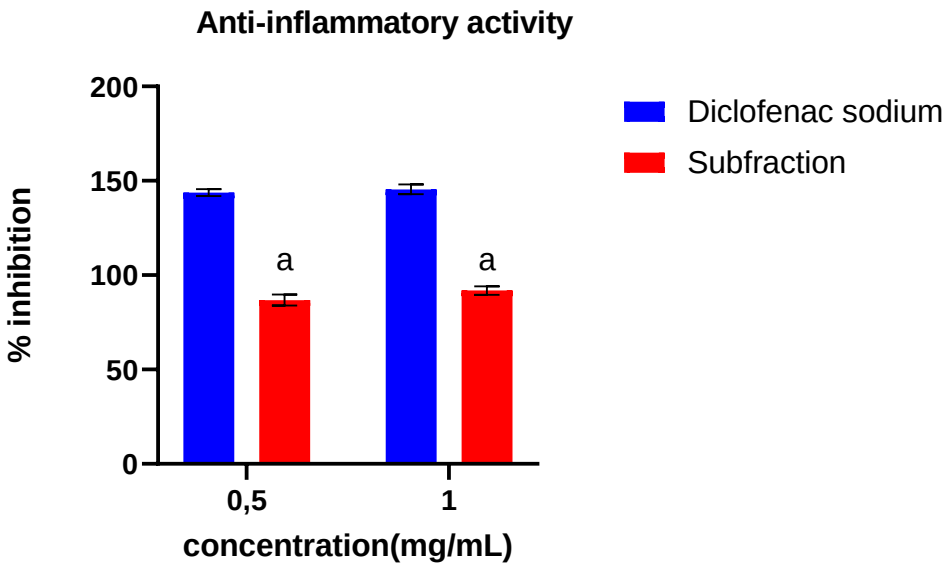


Figure 7.14: Percentage inhibition of egg albumin denaturation by acetone subfraction.

Key: a=p<0.0001

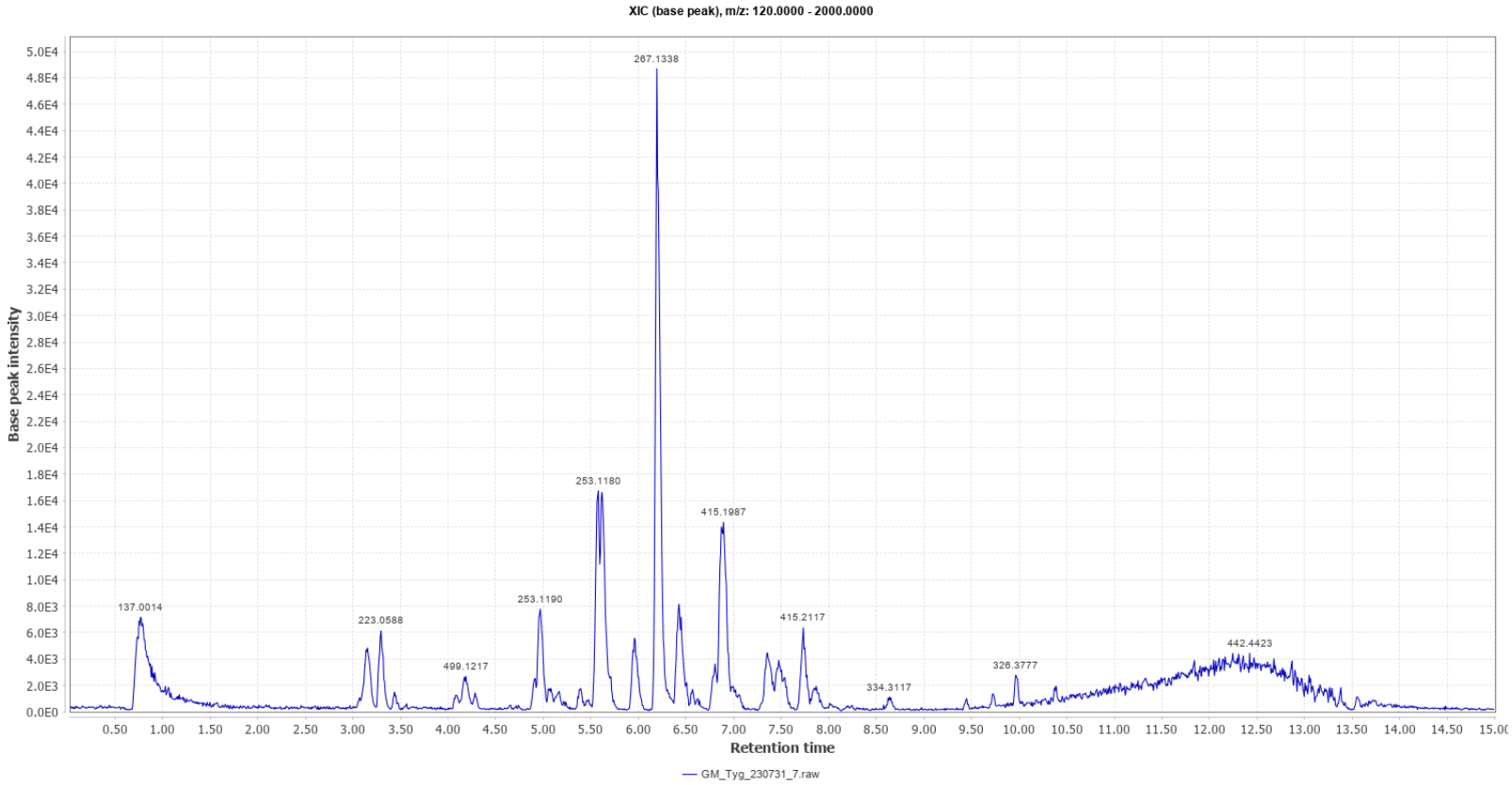


Figure 7.15: LC-MS diagram of compounds in the acetone crude extract**.**

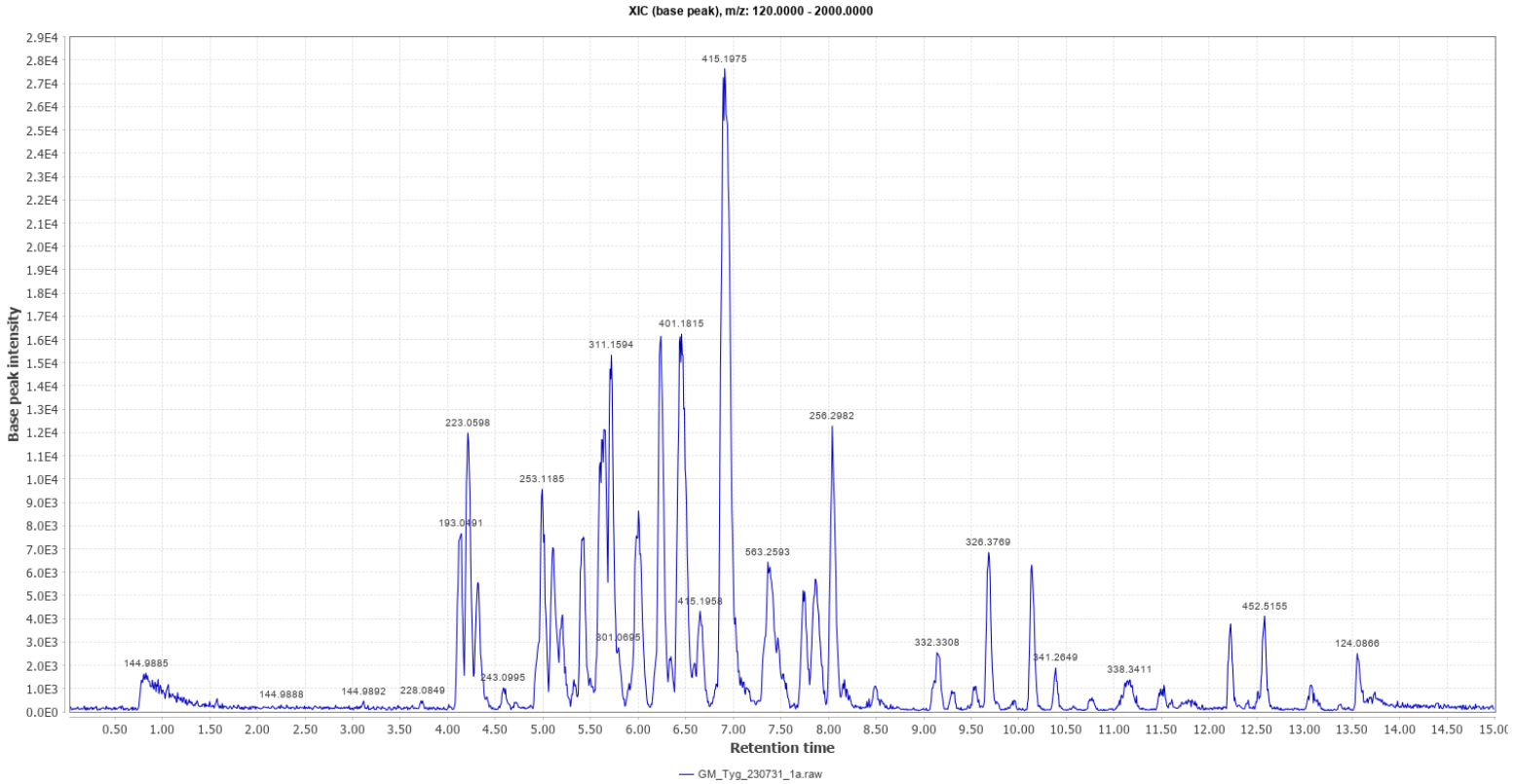
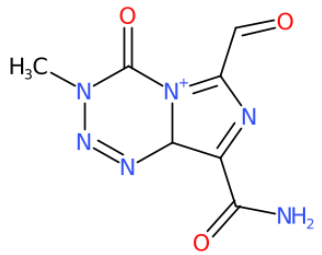
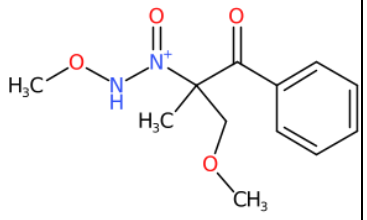
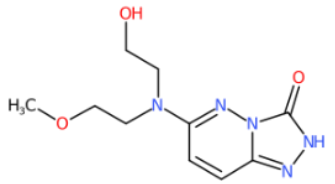
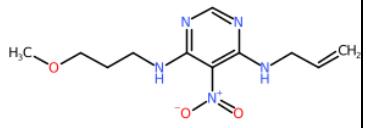
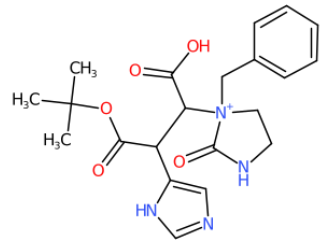
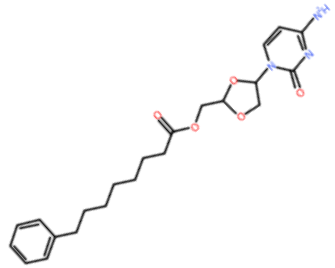
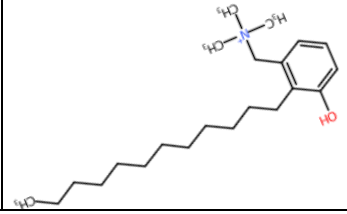


Figure 7.16: LC-MS diagram of compounds in the acetone sub-fraction.

Table 7.7: Possible compounds from Acetone crude extract obtained from PubChem through liquid chromatography-mass spectrometry data guidelines.

Isotopic mass	Formula	Ionization type	Identifier	Retention time	Name	Structure	Source
223.058	$C_7H_7N_6O_3$	[M-H]-	59835578	3.30	6-formyl-3-methyl-4-oxo-8aH-imidazo[5,1-d][1,2,3,5]tetrazin-5-ium-8-carboxamide		PubChem
253.119	$C_{12}H_{17}N_2O_4$	[M-H]-	102096670	4.97	(methoxyamino)-[1-(methoxymethyl)-1-methyl-2-oxo-2-phenyl-ethyl]-oxo-ammonium		PubChem
253.117	$C_{10}H_{15}N_5O_3$	[M-H]-	116051327	5.57	6-[2-hydroxyethyl(2-methoxyethyl)amino]-2H-[1,2,4]triazolo[4,3-b]pyridazin-3-one		PubChem
267.133	$C_{11}H_{17}N_5O_3$	[M-H]-	23487911	6.19	N4-allyl-N6-(3-methoxypropyl)-5-nitro-pyrimidine-4,6-diamine		PubChem

415.198	$C_{21}H_{27}N_4O_5$	[M-H]-	88125337	6.89	(2S)-2-(1-benzyl-2-oxo-imidazolidin-1-ium-1-yl)-4-tert-butoxy-3-(1H-imidazol-5-yl)-4-oxo-butanoic acid		PubChem
415.211	$C_{22}H_{29}N_3O_5$	[M-H]-	59035720 10273309	7.73	[(2S,4S)-4-(4-amino-2-oxo-pyrimidin-1-yl)-1,3-dioxolan-2-yl]methyl 8-phenyloctanoate		PubChem
334.311	$C_{22}H_{40}NO$	[M-H]-	88796125	8.64	(2-dodecyl-3-hydroxy-phenyl)methyl-trimethyl-ammonium		PubChem

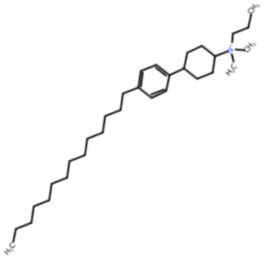
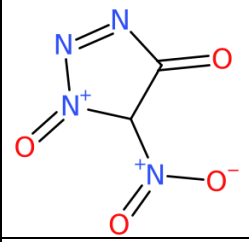
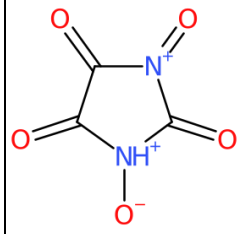
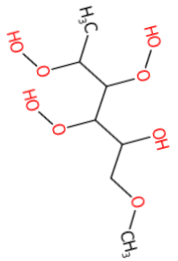
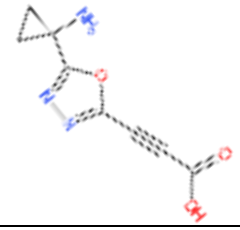
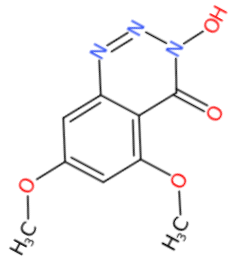
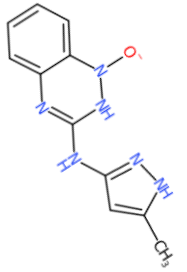
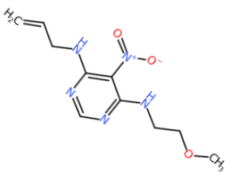
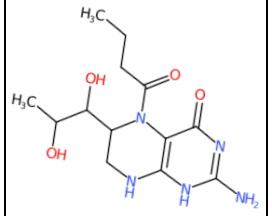
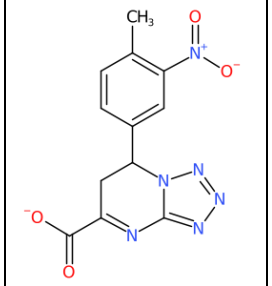
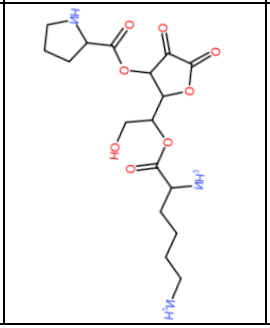
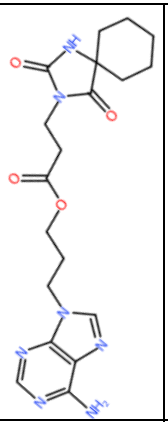
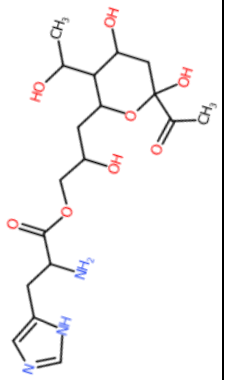
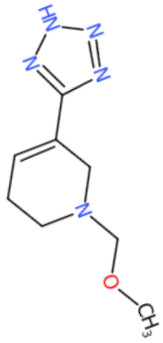
442.441	$C_{31}H_{56}N$	[M-H]-	71458558	12.42	dimethyl-propyl-[4-(4-tetradecylphenyl)cyclohexyl]ammonium		PubChem

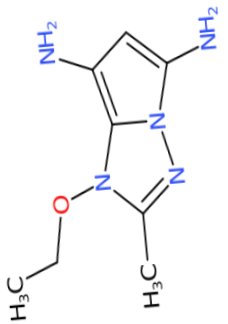
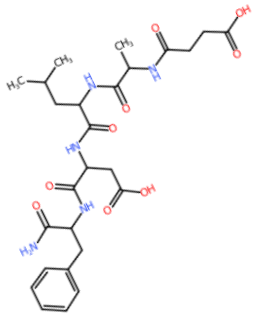
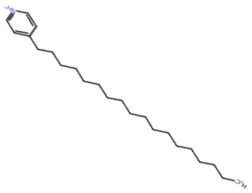
Table 7.8: Possible compounds from Acetone sub-fraction obtained from PubChem through liquid chromatography-mass spectrometry data guidelines.

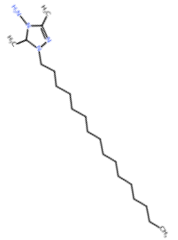
Isotopic mass	Formula	Ionization type	Identifier	Retention time	Name	Structure	Database
145.0	$C_2HN_4O_4$	[M-H]-	87396588	0.83	4-nitro-3-oxo-4H-triazol-3-ium-5-one		PubChem
144.989	$C_3HN_2O_5$	[M-H]-	88813764	3.12	1-oxido-3-oxo-imidazolidine-1,3-diium-2,4,5-trione		PubChem
228.085	$C_7H_{16}O_8$	[M-H]-	89009828	3.73	3,4,5-trihydroperoxy-1-methoxy-hexan-2-ol		PubChem

193.049	$C_8H_7N_3O_3$	[M-H]-	82669819	4.14	3-[5-(1-aminocyclopropyl)-1,3,4-oxadiazol-2-yl]prop-2-ynoic acid		PubChem
223.059	$C_9H_9N_3O_4$	[M-H]-	70341629	4.21	3-hydroxy-5,7-dimethoxy-1,2,3-benzotriazin-4-one		PubChem
243.099	$C_{11}H_{11}N_6O$	[M-H]-	91255347	4.58	N-(5-methyl-1H-pyrazol-3-yl)-1-oxido-2H-1,2,4-benzotriazin-3-amine		PubChem
253.117	$C_{10}H_{15}N_5O_3$	[M-H]-	23487968	4.99	N4-allyl-N6-(2-methoxyethyl)-5-nitro-pyrimidine-4,6-diamine		PubChem

311.159	$C_{13}H_{21}N_5O_4$	[M-H]-	66680682 25028428	5.72	2-amino-5-butanoyl-6-(1,2-dihydroxypropyl)-1,6,7,8-tetrahydropteridin-4-one		PubChem
301.069	$C_{12}H_9N_6O_4$	[M-H]-	26835474 26835472	5.79	(7R)-7-(4-methyl-3-nitro-phenyl)-6,7-dihydrotetrazolo[1,5-a]pyrimidine-5-carboxylate		PubChem
401.18	$C_{17}H_{27}N_3O_8$	[M-H]-	91595513	6.46	[(2R)-2-[(1S)-1-[(2S)-2,6-diaminohexanoyl]oxy-2-hydroxy-ethyl]-4,5-dioxo-tetrahydrofuran-3-yl] (2S)-pyrrolidine-2-carboxylate		PubChem

415.195	$C_{18}H_{29}N_3O_8$	[M-H]-	89564021	6.65	[(2S)-3-[6-acetyl-4,6-dihydroxy-3-[(1R)-1-hydroxyethyl]tetrahydropyran-2-yl]-2-hydroxy-propyl] (2R)-2-amino-3-(1H-imidazol-5-yl)propanoate		PubChem
415.197	$C_{19}H_{25}N_7O_4$	[M-H]-	56284089	6.91	3-(6-aminopurin-9-yl)propyl 3-(2,4-dioxo-1,3-diazaspiro[4.5]decan-3-yl)propanoate		PubChem
195.112	$C_8H_{13}N_5O$	[M-H]-	82365250	6.89	1-(methoxymethyl)-5-(2H-tetrazol-5-yl)-3,6-dihydro-2H-pyridine		PubChem

195.112	$C_8H_{13}N_5O$	[M-H]-	69463083	6.91	1-ethoxy-2-methyl-pyrrolo[1,2-b][1,2,4]triazole-5,7-diamine		PubChem
563.259	$C_{26}H_{37}N_5O_9$	[M-H]-	101614490	7.63	(3S)-4-[[(1S)-2-amino-1-benzyl-2-oxo-ethyl]amino]-3-[[(2S)-2-[[(2S)-2-(3-carboxypropanoylamino)propanoyl]amino]-4-methyl-pentanoyl]amino]-4-oxo-butanoic acid		PubChem
332.332	$C_{23}H_{42}N$	[M-H]-	101940085	9.14	4-octadecylpyridin-1-ium		PubChem

338.341	$C_{20}H_{42}N_4$	[M-H]-	71442591	11.17	2-hexadecyl-3,5-dimethyl-3H-1,2,4-triazol-4-amine		PubChem

7.5 Discussion

Microbial resistance and adverse effects of synthetic drugs have elevated the need to isolate and purify bioactive phytochemicals for new, safe, and immediate response therapeutics (Sasidharan *et al.,* 2011). The aim of this chapter was to fractionate the active acetone extracts of *Artemisia afra* with antioxidant, anti-inflammatory and antimycobacterial properties. Serial exhaustive extraction was done using organic solvents of varying polarity, hexane, dichloromethane, acetone, and methanol. Methanol extract had a high yield of extract of 56.147 g, whereas acetone produced the lowest yield of 13.384 g. Dark spots, glowing and colourful bands of phytochemicals were observed on the chromatograms after phytochemical analysis and visualised with UV- light at 254 nm, 365 nm and vanillin sulphuric acid spraying reagent. The acetone extract with the lowest extracted yield, exhibited a good scavenging activity of DPPH free radical and furthure, demonstrated antimycobacterial activity against *M. smegmatis*. The MICs of the three acetone extracts ranged between 0.208 mg/mL and 0.833 mg/mL. A study conducted by Muleya *et al*., (2014) with *Artemisia afra* collected at KwaZulu Natal, showed different results from those presented in this study. They found that their acetone extract had a higher yield than the methanol extract when using solvent extraction. The yield and quality of the phytochemicals extracted affect the prospective biological activities of that particular plant (Turkmen *et al*., 2006; McDonald *et al*., 2001). Additionally, plant age, harvest site, etc., contribute to the phytochemicals that the plant will produce (Street *et al*., 2008). Column chromatography was done to further fractionate the active acetone extracts. Visible bands and dark spots were observed in all the TLC plates developed in the BEA, CEF and EMW mobile systems thereafter visualised with different methods. The displayed phytochemicals had a similar separating trend on the plates, possibly meaning that they were of the same phytochemical group, although having slight difference in structure; hence, they were visualised by different methods. Fractions 30-50 (hexane: ethyl acetate and ethyl acetate: methanol) ratios showed antioxidant activity after the plates were sprayed with 0.2% DPPH free radical in methanol. However, only fractions 30,10 and 100 (hexane: ethyl acetate) had a good antimycobacterial activity against *M. smegmatis* with MIC values ranging from 0.833 mg/mL and 1.042 mg/mL. These fractions with antimycobacterial activity could

possibly denote that the active compounds are of intermediate polarity because the fractions were eluted with solvent ratios containing high amount of ethyl acetate. Fractions that had high amount of hexane and methanol had no activity when tested with broth microdilution assay. Second column chromatography was packed with the active 30,10 and 100 fractions obtained from first column. The column was eluted with 30% hexane in combination with 70% ethyl acetate. Chromatograms prepared for phytochemical analysis and developed in BEA mobile phase all depicted visible bands and dark spots of present phytochemicals. Subfractions collected and selected by multiples of 5 (15-125) from second open column revealed possible three bands with activity against *M. smegmatis.* The active subfractions were combined together to form one subfraction and quantification of their activity was done using the broth microdilution assay, where the MIC against *M. smegmatis* was found to be 0.078 mg/mL. Fractionation improved the activity of the compounds inside the acetone extracts, because after the second column the MIC value was observed to be very much lower than those of fractions obtained from first column. Muleya *et al.,* (2014) reported that fractionation does improve activity after they conducted a study on *Artemisia afra* and found that methanol and acetone fractions had good activity against *Escherichia coli.*

The growth curve assay was used to monitor the growth of *M. smegmatis* cells after treatment with the acetone crude extract and the acetone subfraction acquired from second column at different concentrations of the MIC. It was then observed that both the extract and subfraction had potency against the bacterial cells because both the acetone crude extract and its subfraction were able to reduce the rate of growth of treated *M. smegmatis* cultures compared to the untreated control. Interestingly, at 4xMIC the subfraction was able to completely arrest the growth of the culture for approximately 9 hours. The growth of the *M. smegmatis* planktonic cells could have been inhibited or prevented by the acetone crude extracts and the sub-fraction applying common mechanisms used by most antimicrobial agents like targeting the cellular ribosome to halt production of essential proteins and also by inhibiting activity of enzymes like DNA gyrase, which is responsible for DNA replication and repair (Walsh, 2003; Vaou *et al.,* 2021). Alkaloids, polyphenols, flavonoids and tannins have been reported to disrupt microbial growth by inhibiting efflux pumps, interfering with cell membrane permeability and integrity, among other functions (Farhadi *et al.,* 2019;

Vaou *et al.,* 2021), which is interesting because *A. afra* plant material does possess this phytochemical. Some phytocompounds like polyphenols have been shown to induce growth of microorganisms after treatment (Milutinović *et al.,* 2021). This could have been a similar effect that caused the residual growth of *M. smegmatis* after 9 hours when treated with the sub-fraction.

Most pathogenic bacteria form biofilms as their mechanism of survival and virulence in their environment (Gellatly and Hancock, 2013). Biofilms are formed in different layers of microbial cells and other components are encompassed inside the matrix, some of the bacterial cells are imbedded deep into the inner layer of the biofilm and they tend to grow slow as compared to those exposed to the outer layer making it difficult for antibiotics to inhibit or kill them (Høiby *et al.,* 2010). The subfraction acquired from the second column was evaluated for its antibiofilm activity, and a great capacity to prevent attachment of *M. smegmatis* planktonic cells after 4 hours compared to the positive control rifampicin at the highest concentration of 2MIC and 4MIC was observed. The activity of the subfraction was increasing with an increasing concentration. With regard to the formed biofilm within 24 hours, the subfraction showed notable signification activity compared to the control. In comparison to the crude extracts, the sub-fraction demonstrated extreme antimycobacterial and anti-biofilm activity, indicating that fractionation did help to remove some of the inactive phytocompounds that were interfering with the efficacy of the active ones. However, both the crude extract and the sub-fraction were not able to eradicate the matured biofilm formed after 48 hours. Therefore, it can be hypothesised that if the *A. afra* *a*cetone crude extract and its subfraction are not effective to *M. smegmatis* matured biofilms, they will have no activity against *Mtb* infections for more than 24 hours in a host, meaning that if they were to serve as TB medication, patients will have to take it after every 24 hours. Most cells contained in biofilm were at a stationary phase and that reduced their susceptibility to antibiotics because most antibiotics are active against metabolically active viable cells (Amato *et al.,* 2014; Maisonneuve and Gerdes, 2014). The sub-fraction demonstrated a remarkable antimycobacterial and antibiofilm activity against *M. smegmatis in vitro* compared to its original acetone crude extracts. After fractionation, there was a huge change in the activity of the and the MICs were reduced to lower amounts, considering that the noteworthy MIC value is equal or less than 1 mg/mL (van Vuuren and Viljoen, 2011). The disruption of tissue proteins

induces inflammation and thus leads to arthritic diseases (Alamgeer *et al.,* 2017; Gupta *et al.,* 2013). Non-steroidal anti-inflammatory drugs (NSAIDs), which block the COX enzymes, are used to treat inflammation, thrombosis, fever, and pain. However, they have potential to badly injure the digestive tract and cause ulcers (Kim *et al.*, 2005). Diclofenac sodium, a type of NSAIDs was used in this study as a positive control to test for inflammatory activity. The subfraction showed significant moderate anti-denaturation activity against the egg albumin ($p<0.0001$) at concentrations 0.5 mg/mL and 1 mg/mL. Study conducted by Khan *et al.,* (2015) reported anti-inflammatory activity of aqueous fraction of *Artemisia scoparia*, which is a medicinal plant from the same family as *A. afra.*

The results obtained from LC-MS analysis revealed several compounds at different retention times from both the acetone crude extract and the sub-fraction. The crude extract contained a lesser number of compounds compared to the sub-fraction. The analysis was run for approximately 15 minutes for both samples and it was observed that more peaks were recorded on the chromatograms after 4 minutes. However, on the sub-fraction chromatogram, it shows that more compound peaks were detected until towards the end of the analysis; therefore, this could mean that most of the compounds in the sub-fraction had high affinity to the stationary phase (Parasuraman *et al*., 2014); hence, the longer retention time, which is defined as the period a solute sample spent inside a column or within the stationary and mobile phase (Hussain, 2018). In addition, both samples possessed several compounds with ammonia and its derivatives as principial functional groups. Kwaśniewska *et al.,* (2020) reviewed several antimicrobial mechanisms of ammonium salts and their derivatives. The crude extracts were, 6-formyl-3-methyl-4-oxo-8aH-imidazo[5,1-d][1,2,3,5]tetrazin-5-ium-8-carboxamide (3.30), (methoxyamino)-[1-(methoxymethyl)-1-methyl-2-oxo-2-phenyl-ethyl]-oxo-ammonium(4.97), N4-allyl-N6-(3-methoxypropyl)-5-nitro-pyrimidine-4,6-diamine (6.19), (2-dodecyl-3-hydroxy-phenyl)methyl-trimethyl-ammonium (8.64) and dimethyl-propyl-[4-(4-tetradecylphenyl)cyclohexyl]ammonium (12.42) . Those obtained from the subfraction are, N-(5-methyl-1H-pyrazol-3-yl)-1-oxido-2H-1,2,4-benzotriazin-3-amine (4.58), N4-allyl-N6-(2-methoxyethyl)-5-nitro-pyrimidine-4,6-diamine (4.99), 1-ethoxy-2-methyl-pyrrolo[1,2-b][1,2,4]triazole-5,7-diamine (6.91), 4-octadecylpyridin-1-ium (9.14) and 2-hexadecyl-3,5-dimethyl-3H-1,2,4-triazol-4-amine (11.17).

Compounds that contain carboxylic acids in their structure have been associated with antimicrobial activity (Jarboe *et al.,* 2013). In the subfraction, two compounds with carboxylic acids were detected, 3-[5-(1-aminocyclopropyl)-1,3,4-oxadiazol-2-yl]prop-2-ynoic acid (4.14) and (3S)-4-[[(1S)-2-amino-1-benzyl-2-oxo-ethyl]amino]-3-[[(2S)-2-[[(2S)-2-(3-carboxypropanoylamino)propanoyl]amino]-4-methyl-pentanoyl]amino]-4-oxo-butanoic acid (7.63). From the crude extract only one was detected, (2S)-2-(1-benzyl-2-oxo-imidazolidin-1-ium-1-yl)-4-tert-butoxy-3-(1H-imidazol-5-yl)-4-oxo butanoic acid (6.89). Furthermore, some of the obtained structures contain imidazole, which is a molecule that has been reported to be used as a precursor to derive bioactive molecules with various antimicrobial activities (Valls *et al.,* 2020). The prediction of the possible compounds present in the crude extract and the sub-fractions using LC-MS has revealed several compounds, which are associated with antimicrobial activity and that further justify that more analysis of the compounds in silico is needed to confirm their mechanisms and binding affinities to pathogen proteins.

7.6 Conclusion

The results demonstrated that the acetone sub-fraction has more potential as an antimycobacterial and antibiofilm agent compared to the acetone crude extract. Therefore, this verified that fractionation does improve the activity of phytochemicals present inside plant extracts by removing any other components that hinder their efficacy. The active compounds in the subfractions were possibly from intermediate polarity because they were able to be separated by intermediate mobile phases. Further isolation and characterisation of the active compounds structure is recommended. The LC-MS results also revealed several compounds associated with antimicrobial activity; therefore, it would be beneficial to evaluate their mechanism of action with pathogen proteins in silico using molecular docking.

7.7 References

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CHAPTER 8

8.1 General discussion

The antibiotic resistance of many microorganisms is attributed to their ability to modify their cell compartmentalisation, thus helping them to survive in unfavourable environments (Tang *et al*., 2021). The aim of the study was to investigate the efficacy of antioxidative, anti-inflammatory and antimycobacterial activities of *Artemisia afra* extracts and sub-fractions. Travel, overcrowding brought on by urbanisation, and inadequate health care systems lead to disease transmissions between humans and animals and highly contributes to the extensive growth of emerging infectious diseases (Alirol *et al*., 2011; Muleya *et al*., 2014). Seasonal variations, location and age of plant harvest also play a huge role on the type and quantity of phytochemicals that plants produce (Street *et al.,* 2008). The study confirmed that *Artemisia afra* possess saponins, terpenoids, cardiac glycosides, steroids, flavonoids, tannins, and phenolic compounds. A phytochemical analysis of the active crude extracts and the sub-fraction revealed that the bioactive compounds of *A. afra* with antioxidant, anti-inflammatory, antimycobacterial and antibiofilm activity against *M. smegmatis* are of intermediate polarity due to the mobile systems that were used to separate them. Although acetone was the best organic solvent to extract active phytochemicals in this study, another study conducted by Motshudi *et al.,* (2021) found that chloroform was the best extractant when working with *Artemisia afra* that were collected from 3 different vendors. Therefore, indeed geographical area plays a significant role in the diversity of phytochemicals produced by medicinal plants (Ramadwa *et al.,* 2019). Microbial invasion is typically the first sign of infection, followed by oxidative stress and severe inflammation, despite the fact that it is widely known that macrophages can eliminate *Mtb* through a number of methods, such as altering cell death programmes, inducing autophagy, and regulating inflammatory responses, during an infection. However, this organism reveals its pathogenicity by avoiding phagocytosis through the production of chemicals or molecules that interfere with the phagosome and as such destructing macrophages from destroying the cells (Liang *et al.,* 2018; Jia *et al.,* 2018), thus further alternating to remain dormant and persistent inside the immune response cells (Behar and Briken, 2019). An array of inflammatory response reactions from several *Mtb* infections results in the overproduction of reactive oxygen species that induce cellular and organ damage (Arulselvan *et al.,* 2016; Racanelli, 2018; Divangahi, 2013).

By delivering and maintaining sufficient quantities of exogenous and endogenous antioxidants, pro-inflammatory cytokine activation and proliferation in respiratory epithelial cells and macrophages are inhibited (Kühn and O'Donnell, 2006; Iwalewa *et al.,* 2007). In this study, it was observed that the plant extracts, extracted with intermediate and polar organic solvents, acetone, ethanol, methanol, and butanol showed a remarkable antioxidative activity by scavenging the DPPH free radical, with exception to water. However, all the extracts depicted low ferric reducing antioxidative power. Muleya *et al.,* (2014) reported the good antioxidant activity of *Artemisia afra* methanol and acetone fractions. TLC separates compounds and gives insight into the type of extraction solvent and polarity of the compounds showing notable antioxidant activity. Furthermore, it helps validate if the extracts have synergistic effects or not when compared to other quantitative methods. When subjected to outside stimuli like a strong acid or base, concentrated inorganic salt, organic solvent, or heat, proteins may get denatured. The inflammatory mediators are produced by plasma proteins or cells, such as mast cells, neutrophils, platelets, and macrophages, which are activated by bacterial products or host proteins. They produce oxidative damage, vascular permeability, neutrophil chemotaxis, smooth muscle contraction, and pain when they bind to specific receptors. Most mediators have short lives but generate detrimental effects (Wan *et al.,* 2013; Yu *et al.,* 2013). *Artemisia afra* extracts exhibited extreme notable anti-inflammatory activity, especially acetone extracts that were greater than the actual positive control diclofenac sodium and the acetone sub-fraction. The remarkable anti-inflammatory activity of the plant will be very much beneficial for TB patients because it can be used as an anti-inflammatory agent that will help reduce free radicals and reactive oxygen species that will be produced in high amounts by the host during immune response and thus protecting proteins and other molecules that could be affected by their overproduction. Furthermore, *Mtb* has demonstrated to manipulate the immune system and interfere with its response, thus elevating inflammation. Therefore, the use of this plant extracts or sub-fractions can highly assist in controlling the results of that effect.

Some herbal medicines used for treatment of infections and diseases, pose dire effects such as liver and kidney dysfunctions, leading to high patient mortality rate (Luyckx *et al*., 2004). The noteworthy low toxicity effect of the acetone extract is a great advantage because the extract is the most active and will further be considered

for isolation of possible compounds with antioxidative, anti-inflammatory and antimycobacterial activities. Fluoroquinolones or injectable drugs, such as capreomycin, amikacin, and kanamycin, which are frequently used in therapy as broad-spectrum antibiotics, have increased toxicity and their efficiency can take up to two years (Mitnick *et al.,* 2009; Grover *et al.,* 2014). Acetone extracts elicited remarkable antimycobacterial activity compared to other extracts after serial exhaustive extraction. Several studies have indicated that the production of biofilms may be the cause of caseous necrosis and cavitation in *Mtb* lung tissue (Basaraba and Ojha, 2017). *Mtb* creates biofilms to withstand host immune pressure and antimicrobial drug therapy. Therefore, its capacity to form biofilms *in vitro* can be used to explain the necessity for prolonged treatment with a range of drugs (Orme, 2014; Trivedi *et al.,* 2016). The formation of biofilms makes it evidently difficult for researchers to find the MIC of antimicrobial agents following the treatment of the microorganisms (Wu *et al.,* 2015). However, in the study, the obtained sub-fraction depicted great capacity to prevent attachment of *M. smegmatis* planktonic cells after 4 hours when compared to the positive control rifampicin, and after 24 hours, there was a notable signification activity against the formed biofilm as well. In addition, the MICs of the acetone crude extract and the subfraction were observed to have a potential to reduce the growth of the planktonic cells of *M. smegmatis* within 24 hours. The components in the biofilm matrix help to anchor the biofilm to the surface as well as trap nutrients, offer structural support, and protect against antimicrobial treatments and host immunological reactions (Flemming *et al.,* 2007). Antibiofilm drugs that inhibit or disperse biofilms have attracted notable research, but because of their inherent lack of antimicrobial action, they must be used in concert with conventional antibiotics. The antibacterial activity of antibiotics against multidrug-resistant TB bacteria could also be increased by the synergistic interactions of natural bioactive substances with conventional antibiotics (Mun *et al.,* 2018; Verderosa *et al.,* 2019.). Jin *et al.,* (2011) and Šimunović *et al.,* (2020) reported that synergistic activity can generate greater effects than some single pure compounds. The antibacterial activity of *A. afra* has been displayed against several microorganisms, not limited to but including, *Staphylococcus aureus*, *Mycobacterium smegmatis*, *Candida albicans* fungi and the protozoa *Plasmodium falciparum* (Van Wyk, 2008). The LC-MS analysis gave an insight into the different compounds that are likely to be present inside both the acetone crude extracts and the sub-fraction. The results revealed that the compound

structures have principal functional groups such as carboxylic acids, ammonia and their derivatives, which have been reported by several studies to be associated with numerous biological activities, including antimicrobial activity. The discovery of these compounds reveals an expected potential of the efficacy of the *A. afra* medicinal plant as a source of drugs for TB treatment, especially the sub-fraction.

8.2 Conclusion and Recommendations

Artemisia afra has shown to possess several phytochemicals associated with distinct biological activities, namely, saponins, flavonoids, tannins, terpenoids, cardiac glycosides, phenolics and steroids. The study confirmed the notorious biological activities of the plant, namely, antioxidative, anti-inflammatory and antimycobacterial activities. From the extraction solvents and mobile phases used, it was observed that the possible active compounds inside the acetone crude extract and sub-fraction are of intermediate polarity. In addition, the acetone extract showed good antioxidative, anti-inflammatory and less toxicity at a lower concentration when tested against THP-1 macrophages. That is an added advantage for a substance that is considered for further TB drug discovery. The sub-fraction, on the other hand, demonstrated remarkable antimycobacterial and antibiofilm activity better than the crude extract. However, it had no efficacy to eradicate matured biofilm after 48 hours. Therefore, if administered as a TB drug or substance, it will have to be administered after every 24 hours to contain its efficacy. The potential of the sub-fraction to depict greater biological potency as compared to the crude extract, validates that fractionation does help to improve efficacy of phytochemicals because it removes unnecessary inactive interfering components inside the extract. For future research, the recommendations could be that the active compounds in the sub-fraction be isolated and characterised for further structure elucidation. Synergistic combinations with conventional antibiotics can be applied to investigate the efficacy of the two against *M. smegmatis,* which will assist to develop new active therapeutic regimen guidelines for TB. Evaluations of the efficacy of the subfraction and or pure compounds on quorum sensing of biofilms can also be considered. Studies with animal models can be conducted as well to substantiate the biological activities of the sub-fraction. Furthermore, the LC-MS analysis revealed numerous compounds with reported antimicrobial activities. Therefore, evaluating their protein binding affinity with *M. smegmatis* proteins using

molecular docking can be beneficial and help provide more insight into the mechanisms of action of the compounds with regards to TB drug discovery.

8.3 References

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