COMPARISON OF *IN VITRO* ANTIMICROBIAL ACTIVITY OF *CANNABIS* EXTRACTS AND MODIFIED ISONIAZID DERIVATIVES AGAINST DRUG RESISTANT *CANDIDA* AND *MYCOBACTERIA* SPECIES

by

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submitted in accordance with the requirements

for the degree

MASTER OF SCIENCE

in the

COLLEGE OF AGRICULTURE AND ENVIRONMENTAL SCIENCES
DEPARTMENT OF LIFE AND CONSUMER SCIENCE

at the

UNIVERSITY OF SOUTH AFRICA
FLORIDA CAMPUS

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YEAR OF FINAL REGISTRATION: 2021

DEDICATION

I dedicate this dissertation to my mother Ms G. N. T Mokoto, who always believes in me and has encouraged me from the get go; to see the vision, believe in it and to strive to achieve it.

DECLARATION

I **Tebogo Matlhogonolo Lorato Mokoto** hereby declare that the dissertation/thesis, with the title: **Comparison of *in vitro* antimicrobial activity of *Cannabis* extracts and modified isoniazid derivatives against drug resistant *Candida* and *Mycobacteria* species** which I hereby submit for the degree of **Master of Science in Life Sciences** at the University of South Africa, is my own work and has not previously been submitted by me for a degree at this or any other institution.

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I declare that during my study I adhered to the Research Ethics Policy of the University of South Africa, received ethics approval for the duration of my study prior to the commencement of data gathering, and have not acted outside the approval conditions.

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ACKNOWLEDGEMENTS

I would like to express my sincere gratitude to the following individuals and organizations for their assistance and support towards the completion of this study:

- I would firstly like to thank my Creator for the success and opportunities granted to me during my academic career.
- My mom for her continuous support and motivation to aspire for more and encouraging me to dream big. This is the beginning of big things; it all starts here – the dreams you have for me are coming to fruition.
- My brother and mentor Mr Tshepo Mokoto for always encouraging me.
- I would also like to extend a warm thank you to my supervisor Mr Garland Kgosi More for his support, encouragement and guidance in regards to my project as well as shaping of the project.
- I would also like to extend my gratitude to my co-supervisor Ms Itumeleng Bridgette Setshedi, for her support and guidance, as well as her contribution to guiding and shaping my scientific development.
- Mr Chris van Lille, for aiding with the growing and taking care of my *Cannabis* strains.
- An extended thank you to Mr Sol Sauna Nety for the assistance in relation the gas-chromatography and mass spectrometry (GC-TOF-MS) analysis of the plant extracts.
- The financial assistance of the National Research Foundation (NRF) towards this research is hereby acknowledged. Opinions expressed and conclusions arrived at, are those of the author and not necessarily to be attributed to the NRF. (Grant UID: 118127-)

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ABBREVIATIONS

Microorganisms	
MAC	*Mycobacterium avium* complex
MOTT	*Mycobacterium* other than tuberculosis
MTB	*Mycobacterium tuberculosis*
NCAC	Non-*Candida albicans Candida*
NTM	Non-tuberculous *mycobacterium*
TB	Tuberculosis
Chemicals, reagents and media	
AMB	Amphotericin B
CO_2	Carbon dioxide
DMEM	Dulbecco's modified eagle's medium
DMSO	Dimethyl sulfoxide
DPPH	2,2-diphenyl-1-picrylhydrazyl
EtOAc	Ethyl acetate
EtOH	Ethanol
FBS	Fetal bovine serum
FLC	Flucazole
H_2O	Water
INH	Isoniazid
INT	*p*-iodonitrotetrazolium chloride
ITR	Itraconazole
MTT	(3- [4,5-dimethylhiazol-2-yl]-2,5-diphenyl tetrazolium bromide)
NO	Nitrogen oxide
Pen/Strep	Penicillin/ Streptomycin
PI	Propidium Iodide
POS	Posaconazole
RMP	Rifampicin
SDA	Sabouraud dextrose agar

SDB	Sabouraud dextrose broth
VRC	Voriconazole
Units of measurement	
ºC	Degrees Celsius
µg/mL	Microgram per milliliter
IC_{50}	Inhibitory concentration at 50%
LC_{50}	Lethal concentration at 50%
mg	Milligram
Instruments	
NMR	Nuclear Magnetic Resonance
GCxGC-TOF-MS	Gas Chromatography–Time-of-Flight Mass Spectrometry
UHPLC/Q-TOF-MS	Ultra-High Performance Liquid Chromatography-Quadrupole Time-of-Flight Mass Spectrometry
Sundry abbreviations	
Δ^9-THC	Delta-9-tetrahydrocannabidiol
AMR	Antimicrobial resistance
CBD	Cannabidiol
CBG	Cannabigerol
COLD	Chronic obstructive lung disease
COX	Cyclooxygenase
DNA	Deoxyribonucleic acid
ESR	Electron spin resonance
FSC-H	Forward scatter – height
IFNγ	Cytokines interferon gamma
IL-2	Interleukin-2

IL-4	Interleukin-4
INH-NAD	Isoniazid-nicotinamide adenine dinucleotide
LOX	Lipoxygenase
LP	Lipopolysaccharide
MIC	Minimum inhibitory concentration
OSI	Oxidative stability index
PCR	Polymerase chain reaction
Q_2	Quadrant 2
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RTCA	Real-time cell analysis
SSC-H	Side scatter – height

Organisations	
FDA	Food and drug administration
WHO	World health organisation

ABSTRACT

Microbial resistance to their respective treatment methods of microorganisms has become a major problem in the public health sector. Both *Candida* and *Mycobacterium* species have been noted as multi-drug resistant species which result in treatment difficulties. Various methods of treatment have been undertaken for both fungal and mycobacterial infections. Resistance is due to overexposure and overuse of drugs as well as shortened treatment courses which evade the drug's mechanism of action. Medicinal plants are viewed as an alternative form of treatment which can be evaluated for the treatment and management of infections caused by these resistant microorganisms. Another form of treatment that has been recently explored is the repurposing of drugs.

This study was set to focus on the antioxidant, cytotoxic and antimicrobial activity of *Cannabis indica/sativa* extracts and modified isoniazid derivatives. Six *Cannabis indica/sativa* extracts derived from vegetative leaves and mature inflorescence parts were evaluated; these plant parts were extracted by means of the Soxhlet extraction method using three solvents namely: *n*-hexane, ethyl acetate and ultrapure water. Modified isoniazid derivatives were received from an ongoing study. However, they also form an important part of the study. The 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging assay was used to evaluate the antioxidant scavenging potential. These were measured using the inhibitory concentration at which 50% (IC_{50}) of the radicals have been scavenged of both extracts and isoniazid derivatives. Of the tested agents, the ethyl acetate extract of the mature inflorescence was the best radical inhibitor with an IC_{50} = 34.8µg/mL and among isoniazid derivatives, *N*'-((4-aminophenyl) phenyl) methylene) isonicotinohydrazide· (salicylic acid) (IBS 010) was the strongest IC_{50} = 105.4µg/mL. The cytotoxicity assay was performed to determine which concentration of the *Cannabis indica/sativa* extracts and modified isoniazid derivatives would kill 50% of the murine macrophages (RAW 264.7) and African green monkey kidney (Vero) cells (LC_{50}). The plant extracts and modified isoniazid derivatives were evaluated in triplicates, the vegetative leaves of *n*-hexane extract showed the best activity against the Vero cells with an LC_{50} = 2 404.6µg/mL and mature inflorescence ethyl acetate extract against RAW264.7 LC_{50} = 1 073.16µg/mL.

Both *Candida* species and *Mycobacterium* species had their minimum inhibitory concentrations (MIC) tested using the minimum inhibitory concentration testing method. In the study *Candida auris, Candida glabrata, Candida tropicalis* and *Candida parapsilosis* were used to evaluate both the use of modified isoniazid derivatives as repurposing agents and the *Cannabis indica/sativa* extracts. The derivatives showed activity that could consider them probable treatment agents against candidiasis caused by non-*Candida albicans Candida* species. Compounds such as *N*'-(bis (2-hydroxyphenyl) methylene) isonicotinohydrazide · (salicylic acid) (IBS 008), *N*'-(propan-2-ylidene) isonicotinohydrazide (IBS 013) and *N*'- (propan-2-ylidene) isonicotinohydrazide · (salicylic acid) (IBS 014), all showed activity against *Candida auris* at an MIC = 31.25µg/mL. *Cannabis indica/sativa* vegetative leaves of ultra-pure water extract also showed noteworthy results against *Candida auris* with an MIC = 62.5µg/mL. These results were confirmed by the performance of a flow cytometry assay where the parent-gated cells showed an apoptotic rate ≥80% for both *Cannabis* extracts and modified isoniazid derivatives. The *Cannabis indica/sativa* extracts were evaluated against the *Mycobacterium* species, the vegetative leaves of ethyl acetate extracts displayed the most noteworthy activity against both *Mycobacterium smegmatis* and *Mycobacterium aurum* with MIC = 31.25µg/mL.

A phytochemical evaluation was also performed using the two dimensional gas chromatography – time of flight – mass spectrometry (GC x GC –TOF-MS). This analysis was able to highlight important compounds found in all six extracts, these compounds also have been found by previous studies to have medicinal properties such as anti-inflammatory, antibiotic and analgesic activity. Some of the compounds analysed include commonly known *Cannabis* compounds such as Δ^9-tetrahydrocannabinol, TMS derivative detected at 19:47,6min and cannabichromene which was found in both leaves and inflorescence *n*-hexane extracts and were detected at the periods of 18:08,6min and 18:52,6min. Other compounds include Oxabenzo (f) cyclobut (cd) inden- 8-ol-1a-à,2,3,3a,8b-à,8c-à- hexahydro- 1,1,3a-trimethyl -6-pentyl- found at 18:35,7min and 4,8,12,16- Tetramethylheptadecan -4-olide detected at 18:39,8min. This study highlighted the importance of medicinal plants used in traditional practices with both vegetative leaves and mature inflorescence of *Cannabis* as sources of phytochemicals that aid in combating microbial growth. Furthermore, a unique discovery of repurposing isoniazid derivatives showed the potential inhibitory activity against *Candida* species and an enhanced activity against *Mycobacterial* species.

Chapter 1

Introduction and Literature Review

1.1 Ethnopharmacology

Ethnopharmacology is a field of study that looks into the use of medicine derived from natural products such as fungi and plants, this often harnesses indigenous knowledge from specific traditional groups (Yeung *et al.*, 2018). The utilization of plants for medicinal use has been a wide practice for the longest time. Many people particularly those in rural areas have relied on medicinal plants for the relief of various ailments for the longest time (Vermani and Garg, 2002). Many contemporary drugs originate from traditional medicine; thus, highlighting the importance of natural products as they remain a significant part of scientific research advancements in the quest to find new drugs for various ailments (Patwardhan, 2005).

Modern science has made it possible to evaluate the effects of ethnopharmacological constituents through the application of model systems (Heinrich and Gibbons, 2001). Often extractions do not yield novel compounds. In most cases, new compounds bear no significant biological properties; however, the ability of known compounds to possess new biological activities is a significant lead in the production of new drugs (Kramer and Cohen, 2004).

Medicinal plants have long been used as agents to for the treatment of human ailments and infections (Tabutia *et al.*, 2010). The products arising and developing from natural sources have always been encouraged as there are approximately 300 000 plant species which exist in the world, of which 15% of these plants have been evaluated for their pharmacological properties (Palhares *et al*., 2015). The importance and efficacy of these medicinal plants has been demonstrated in various studies worldwide (Palhares *et al*., 2015).

1.2 Traditional medicine in South Africa

According to Fokunang *et al*., (2011) traditional medicine is defined as: “the practices of health, approaches, knowledge as well as belief which incorporates the use of animals, plants and mineral-based medicines accompanied by spiritual therapies, exercises and manual techniques”. These techniques are applied in combination or in singularity in order to diagnose, form treatment and prevent illnesses or maintain well-being (Sobiecki, 2014).

Approximately 85% of the world's population use medicinal plants as a primary source of healthcare and source of drug discovery, of this; a considerable 80% of synthetic drugs are derived from medicinal plants (Fitzgerald *et al.*, 2020). The Sub-Saharan African region is one of many places in the world where traditional, complementary and alternative medicine is still widely used. Many people still rely on traditional medicine to maintain health or prevent and treat many communicable as well as non-communicable diseases (James *et al.*, 2018). In South Africa, traditional medicine is viewed as a form of alternative medicine; it is estimated that 72% of the Black African population rely on this form of therapy – this accounts for approximately 26.6 million consumers (Sobiecki, 2014).

South Africa has a large diversity of plants; over 4 000 plant species have been found, many of which are reported to possess medicinal properties (Erasmus *et al.*, 2012). South African medicinal plant practices dates back from ancient times and are still practiced to date. The increased attention received by medicinal plant usage has sparked an interest of many policy-makers, researchers and healthcare professionals to dive deeply into the subject and adjust and transform the area for economic and healthcare purposes (James *et al.*, 2018). Traditional, complementary and alternative medicine plays an enormous role in the country's economy, by generating at least R2.9 million to the South African economy alone by means of trading with international healthcare pharmaceutical companies between the years 1999 and 2003 (James *et al.*, 2018). It is therefore not surprising that approximately 80% of South Africans rely on these plants as a source of primary healthcare (Street and Prinsloo, 2012).

1.3 Cannabis

Cannabis is a single genus of plants belonging to the family Cannabaceae (ElSohly and Slade, 2005). The genus comprises of dioecious flowering plants, meaning that some plants either have male or female flowers while others have both male and female flowers (Gloss, 2015). The leaflets of the plants radiate from the base making them palmate and are serrated (Gloss, 2015). The *Cannabis* plant is indigenous to various parts of central, North-Eastern and some Southern parts of Asia, the plant is currently used worldwide as a recreational drug or for medicinal purposes (ElSohly and Slade, 2005). According to Hillig and Mahlberg (2004), the *Cannabis* plant can be divided into three species namely: *Cannabis ruderalis, Cannabis indica,* and *Cannabis sativa.* Each species can be interbred with other species in order to form hybrids (Gloss, 2015).

For medicinal purposes, the leaves of *Cannabis* are used as tonic, aphrodisiac, astringents and for their analgesic properties. They are used for the treatment and management of convulsions, abdominal disorders, otalgia, malarial fever, diarrhoea, dysentery, hysteria, skin diseases, hydrophobia, colic, insomnia and tetanus (Biosci *et al*., 2014). The resin is smoked to allay bronchitis and hiccough, it is useful in treating and managing headaches, insomnia, neuralgia, whooping cough, dysuria, mania, asthma and in relieving pain associated with menorrhagia and dysmenorrhoea (Biosci *et al.*, 2014). Indian hemp is a strain of *Cannabis sativa* subspecies *indica*, is traditionally used to treat cachexia, depression, glaucoma, eye problems, pain and hypertension (Biosci *et al*., 2014). *Cannabis indica* is recommended for the treatment of insomnia, pain, inflammation, muscle spasms, epilepsy and glaucoma (McPartland, 2018). Dilara and Nath, (2000) suggest that *Cannabis* can also be used for allergies, cuts, burns, wounds, inflammation, leprosy, leuconderma, scabies and smallpox. *Cannabis sativa* is also used to treat headaches, depression, loss of appetite and nausea (Biosci *et al*., 2014). Due to its high cannabidiol content, *Cannabis ruderalis* is commonly used for the treatment of epilepsy and anxiety as well as sclerosis, loss of appetite and cancer (Gloss, 2015).

There is a claim that *Cannabis* has polypharmaceutical properties due to the number of medicinal compounds isolated from the plant, thus this provides two main advantages over single-ingredient synthetic drugs. The first advantage is that other compounds may mitigate the side effects of the primary constituents and the second advantage is that other compounds may be synergized by the therapeutic effects of active primary *Cannabis* constituents (McPartland and Russo, 2001).

Cannabis is not indigenous to South Africa; however, it is the most illicit substance used in the country. *Cannabis* is easy to produce, law prohibition is rarely enforced on those considered dealers and the plant is inexpensive to cultivate. In the year 2012, the annual *Cannabis* prevalence was at 3.65%; in the same year 43.3% of patients needed treatment for *Cannabis* related use (Ramlagan *et al.*, 2017). In the year 2018, the South African Constitutional Court decriminalised the possession and private cultivation of *Cannabis* by adults (van Rensburg *et al.*, 2020). South African *Cannabis* medicinal use is regulated by the Medicines and Related Substances Act (1965), which catagorises the plant as a Schedule 8 medicine, meaning that it is considered a controlled drug (Bulose, 2021). Therefore, there are currently low-level or no convincing evidence for the use of *Cannabis* as a medicinal plant, in many cases the plant is

subject to bias, clinically non-significant and poor-quality studies (van Rensburg *et al.*, 2020). In Africa, *Cannabis* is used for fever, asthma, to facilitate childbirth, treatment of snake bites and blood poisoning. Present-day uses of *Cannabis* include the treatment of high blood pressure and the treatment of indigestion as well as to deworm donkeys and horses (Chandra *et al.*, 2019).

1.3.1 *Cannabis indica*

Cannabis indica is a 2-4 feet tall (~0.6 – 1.2m) shrub with a woody stalk and has bushy budded leaves (Gloss, 2015). *Cannabis indica* is characterized by its strong odour and an acrid "skunky" aroma (McPartland, 2018). The *Cannabis indica* species has been recorded to have substantial quantities of cannabinols which can be used as medicine or for mood alterations (van Wyk and Gericke, 2003). The strains mainly originated from Afghanistan, India, Nepal, and northern Pakistan which lay in central Asia (Turner *et al*., 1973; Hillig and Mahlberg, 2004) indicated in green on the map (**Figure 1.3**).

1.3.2 *Cannabis sativa*

Cannabis sativa is a scanty tree that is distinguished by its soaring fibrous stalks, growing up to 5-18 (1.5 – 5m) feet tall (McPartland, 2018). Flowering of the plant is usually initiated by darkness of over 11 hours per day (Gloss, 2015). *Cannabis sativa* is characterized by its lack of psychoactive effects and is grown mainly for its production of fibre and seed oil, (van Wyk and Gericke 2003). According to McPartland (2018), the plant produces a "herbal" or "sweet" aroma due to its terpenoid profile. The plant species is indigenous to North-Eastern Asia mainly China and Thailand (Florian *et al*., 1991). The areas where the plant species are prevalent are marked in orange on the map (**Figure 1.3**).

Figure 1.3: Origin of *Cannabis indica* and *Cannabis sativa* across Central, South and North-Eastern Asia.

(Source: The map was constructed using MapChart)

1.4 Cannabis constituents

1.4.1 Cannabinoids

Cannabinoids are chemicals found in the *Cannabis* plant. Nearly one hundred and thirteen cannabinoids (Appendix: **Table 10.1**), all exhibiting various effects including medicinal properites have been isolated (Aizpurua-Olaizola *et al*., 2016), these compounds can be found on appendages found on the surface of *Cannabis* inflorescence or flowers called *Cannabis* trichomes (Tanney *et al*., 2021) (**Figure 1.4.1**). They are a potent anti-inflammatory agent, which exert their effects through the induction of apoptosis, suppression of cytokine production, the inhibition of cell proliferation and induction of T-regulatory cells (Nagarkatti *et al*., 2009). The mean Δ^9-tetrahydrocannabinols (THC) levels and B_T frequency in *Cannabis indica* were found to be higher as compared to those of *Cannabis sativa* (Hillig and Mahlberg, 2004). *Cannabis indica* is said to produce more cannabidiol (CBD) than *sativa* with a 1:1 THC-CBD ratio (McPartland, 2018). The essential oils extracted from *Cannabis indica* are found to contain caryophyllene, caryophyllene oxide, linalool, trans-α-bergamotene, cis-β-farnesene, menthol, δ-limonene, eucalyptol and carvone (Naz *et al*., 2017). The resin of the *Cannabis ruderalis* plant has less THC as compared to other *Cannabis* species (Stafford, 1992) and is high in CBD (Clarke, 1981).

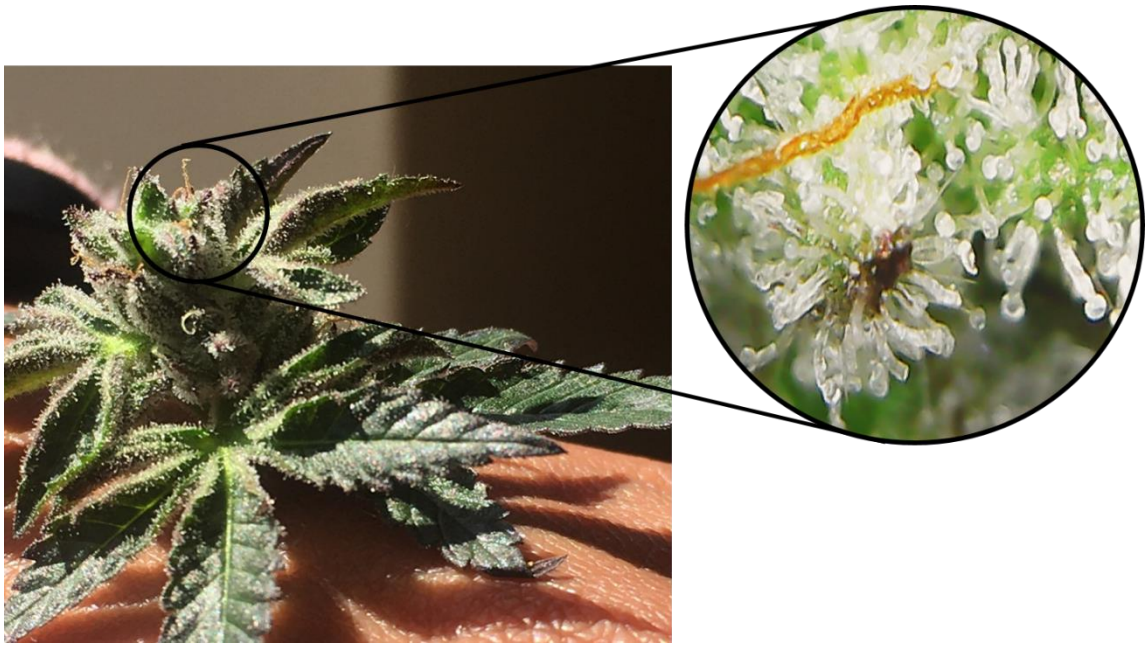

Figure 1.4.1: Trichomes found on the surface of *Cannabis indica/sativa* inflorescence, these adjuncts produce and hold major cannabinoid phytochemicals.
(Source: Pictures were taken during the process of investigation).

1.4.1.1 Cannabigerol

Cannabigerol (CBG), a non-acidic form of cannabigerolic acid is the parent molecule which all cannabinoids are synthesized from, yet it is said to be a minor constituent of the *Cannabis* plant (Morales *et al*., 2017). Cannabigerol is also characterized by its antibacterial activity against Gram-positive bacteria (ElSohly and Slade, 2005), anti-inflammatory and anti-proliferative properties (Iseppi *et al*., 2019). According to Brenneisen (2007), CBG is also well known for its antifungal, antibiotic and analgesic properties.

1.4.1.2 Δ^9-tetrahydrocannabinol

Tetrahydrocannabinols (THC) are the primary constituents responsible for the *Cannabis* plant's psychoactivity, as with pharmacological active secondary metabolites of the plant. Tetrahydrocannabinol is known to be an analgesic, euphoriant, antiemetic, anti-inflammatory and antioxidant medicinal properties, (Brenneisen, 2007). All *Cannabis* species contain varying concentrations of THC (Δ^9-tetrahydrocannabinols), the subjective psycho-effects of euphoria which are seen in oral administration of THC, *Cannabis* preparations such as hashish and smoked THC were compared (Wachtel *et al.,* 2002). It was found that applications produced results and effects which were dose-dependent (Brenneisen, *2007*).

1.4.1.3 Cannabidiol

Cannabidiols (CBD) are major constituents found in the *Cannabis* plant (Aizupura-Olaizola *et al.*, 2016). They have been compounds of interest due to their promising pharmaceutical neuroprotective (Ibeas Bih *et al*., 2015), antiepileptic (Wright *et al*., 2015), hypoxia-ischemia (Mori *et al*., 2017), anti-inflammatory (Ruiz-Valdepeñas *et al.*, 2011), analgesic (Maione *et al*., 2011), anti-asthmatic (Vuolo *et al*., 2015) and antitumor properties (Massi *et al*., 2013). In addition, Brenneisen (2007) also reported that CBD also has antipsychotic, anxiolytic, antispasmodic and antioxidant properties.

1.4.2 Terpenes

A wide range of biological activity is displayed by terpenoids and they are therefore considered to play a major role in many pharmacological effects in various *Cannabis* preparations (El-Alfy *et al.,* 2010). There are approximately 120 identified terpenes in *Cannabis,* attributing to the various aromas which are found in the different strains of *Cannabis* (Andre *et al.*, 2016). Natural terpenes are recognized for their antimicrobial properties and their detrimental effects

are based on their effects on structure and overall function of cell walls and microbial membranes (Andrade-Ochoa *et al*., 2015). Pharmacologically, terpenes have been associated with exerting entourage effects on cannabinoids, thus forming a synergetic medicinal effect (Russo, 2011). Terpenes may be present as either acyclic, monoacyclic or polyacyclic hydrocarbons with substitutions patterns that include alcohols, ethers, aldehydes, ketones and esters. Sabinene, α1,8-cineole, γ-terpinene, bornyl acetate, α-copaene, viridiflorene, pulegone, γ-cardinene, alloaromadendrene, β-bisabolene, *trans*-β-farnesene, -terpinene, *trans*-nerolidol, β-bisabolol and terpineol-4-ol are some of the other terpenoids found to be present in trace amounts (Ross and ElSohly, 1996; Mediavilla and Steinemann, 1997).

1.4.2.1 β-myrcene

Myrcene also known as 7-methyl-3-methylene-octa-1,6-diene is an alkene natural hydrocarbon, classified as a monoterpene and is one of the components in essential oils including *Cannabis* oil (Behr and Johnen, 2009). The anti-inflammatory, antioxidant and anxiolytic activity of β-myrcene has been widely studied and has sparked great interest (Surendran *et al*., 2021). According to Souza *et al*., (2003) β-myrcene is effective in the inhibition of lipopolysaccharide (LPS) induced inflammation, this includes cell migration which is a key feature of pleurisy and of general inflammation response. It was found that β-myrcene possess immune-regulatory activity which inhibits the production of nitrogen oxide (NO), as well as cytokines interferon gamma (IFNγ) and interleukin-4 (IL-4), which would normally be over produced in the lungs during inflammation (Gour and Wills-Karp, 2015).

1.5 Repurposing and remodelling of drugs

Drug repurposing is a technique is used to identify new applications for pioneering drugs, it is used to investigate the use of drugs outside of their intended medical application (Pathak *et al.*, 2020). Due to the slow pace of the discovery and development of new drugs, their high rates of abrasion and substantial costs; the repurposing of many drugs has become a popular go-to for both common and rare diseases (Pushpakom *et al*., 2021). An example of this is Remdesivir, a drug initially used to treat Hepatitis C virus infections, was unsuccessfully tested as a treatment agent for Ebola virus infections (Sahragardjoonegani *et al*., 2021). Other drugs which have been studied for repurposing include amphotericin B which is an antifungal drug for the treatment visceral Leishmaniasis disease (Sundar and Chakravarty, 2010) and azithromycin which is an antibacterial drug that have been tested for the treatment of COVID-19-related (coronavirus infectious disease-2019) coinfections (Rudrapal *et al.*, 2020).

1.6 Isonicotinic acid hydrazide

Isonicotinic acid hydrazide (INH), commonly known as isoniazid is an antibacterial agent and is primarily used as a tuberculostatic drug and remains the first choice in the treatment of latent tuberculosis (TB) (Denholm *et al*., 2014). Isoniazid has a simple structure (**Figure 1.6**) and contains two essential components namely a hydrazide group and a pyridine ring which are required for anti-mycobacterial activity (Jena *et al*., 2014). This drug is commonly used to treat pulmonary and extra pulmonary TB infections and is also utilized as a chemoprophylaxis therapy to prevent or delay the appearance of microbial resistance (Mahmoud *et al*., 2017). Isoniazid is often used in conjunction with pyrazinamide, rifampicin, ethambutol or streptomycin for active tuberculosis; however, it can be used by itself for the treatment of latent tuberculosis (World Health Organization, 2009).

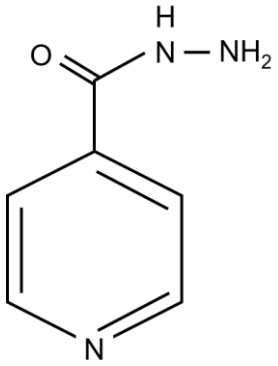

Figure 1.6: Chemical structure of isonicotinic acid hydrazide or isoniazid.
(Source: The diagram was created using EdrawMax Software).

1.7 Side effects

Treatment with isonicotinic acid hydrazide therapy has high toxicity risks which can either manifest as chronic or acute ailments. Acute toxicity usually presents itself as neurological symptoms such as seizures due to the decrease in the levels of the neurotransmitter gamma-aminobutyric acid (GABA) (Hasan *et al.,* 2019). Contrarily, chronic symptoms present as hepatotoxicity (Denholm *et al*., 2014) this is linked to the splintering of deoxyribonucleic acid (DNA) strands and the disruption of the mitochondrial membrane in hepatocytes caused by isoniazid. Isoniazid is able to produce reactive metabolites which are therefore responsible for the damage to liver cells (Metushi *et al.,* 2016). Factors which contribute to the increased probability of isoniazid hepatotoxicity are patient age, use of alcohol, gender (females are at higher risk as compared to males), slow acetylator status, concurrent use of cytochrome-inducing

agents and prior hepatitis infection (Sharma *et al.*, 2016).

Other side effects of the drug include asymptomatic elevation of hepatic enzyme serum; these are common adverse drug reactions. Uncommon adverse drug reactions to isoniazid include hepatitis, peripheral neuropathy and cutaneous hypersensitivity as well as rare reactions including gynaecomastia, lipoid reactions, haemolytic anaemia, arthralgia, mental symptoms, optic neuritis, convulsions, fever and giddiness (Hasan *et al.*, 2019).

1.8 Mechanism of action

Isoniazid is a pro-drug, it diffuses passively through the mycobacterial envelope and is activated by the catalase-peroxidase *katG* and $MnCl_2$ into an isonicotinoyl radical anion. This inhibits the isoniazid target - InhA through the covalent attachment to NADH within the protein active site (Rickman *et al.*, 2013). The catalysis of NADH-dependent reduction of 2-trans-encyl-ACP molecules with sixteen or more carbons has been shown preferably by InhA. The biosynthesis of mycolic acids is also inhibited by isoniazid. The inhibition of InhA and mycolic acid synthesis is provided by the mutation in the *InhA* gene which convenes the resistance of isoniazid and leads to mycolic acid biosynthesis inhibition (**Figure 1.8**) (Jena *et al.*, 2014).

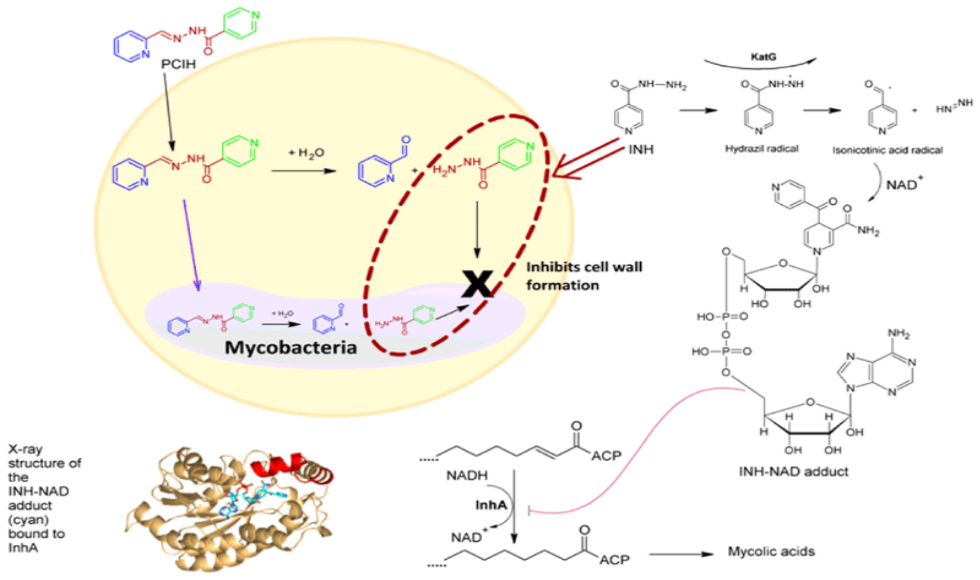

Figure 1.8: The proposed schematic representation of isoniazid's (INH) mechanism of action and hydrazine derivative (2-pyridylcarboxaldehydeisonictinoylhydrazone PCIH). INH-NAD adduct (cyan) bound to InhA (1 zid.pdb) represented as X-ray structure. Representation of substrate binding loop (red) (Angelova *et al.*, 2017).

1.9 Isoniazid resistance

Isoniazid has been the most effective drug for latent tuberculosis since its introduction in 1952 (Ando *et al*., 2011). The activation of isoniazid is reliant on the protein catalase peroxidase enzyme (*KatG*) (Bardal *et al.*, 2011), a mutation of the enzyme is responsible for the resistance of several *Mycobacterial* species to isoniazid (Ando *et al*., 2011). Resistance occurs due to the deletions in the *KatG* gene or *inhA* gene, these genes are responsible for mycolic acid synthesis (Pitso *et al.*, 2019).

1.10 Repurposing of isoniazid

Isoniazid amongst many other drugs is no stranger to this trend, the popular anti-tubercular agent has been repurposed in previous studies for the treatment of prostate cancer by exhibiting the inhibition of monoamine oxidase A (MAOA) levels, this enzyme is responsible for the aggregation prostate cancer (Lv *et al*., 2018). Isoniazid, as well as the anti-tubercular drug rifampicin (RMP) were both trialled in a COVID-19 study as repurposed drugs. In this study, rifampicin showed promising results by presenting good binding energy, however more *in vitro* studies and clinical trials needed to be performed to validate these claims (Pathak *et al.*, 2020).

1.11 Problem Statement

Antimicrobial resistance (AMR) of microorganisms to their respective drugs has advanced into one of the most crucial principle health problems preventing the effective treatment of microbial infections (Prestinaci *et al.*, 2015). Bandow *et al*., (2003) documented that the emergence of clinical resistance of microbes to their respective treatments was due to the continuous deployment of drugs, the microbe's ability to combat the attacks from the drugs and the relation to the rise in immunocompromising conditions (Tanwar *et al*., 2014) this makes treating several infections very difficult and complicated.

Candidiasis is a fungal infection caused by any *Candida* species (Yapar, 2014). A recent estimate stated that the global prevalence of candidiasis stood at 700 000 cases per annum in the year 2017 (Fu *et al.*, 2017). A systematic review and meta-analysis conducted by Omrani and colleagues, (2014), revealed that there was an 18.83% prevalence of *Candida* infections in Africa and the Middle East, respectively. The sub-Saharan region and central Africa had the highest prevalence of 12.34% with the South African region at 4.56% of hospitalized cases (Omrani *et al.*, 2014).

Currently there are three classes of antifungal agents which are clinically used to treat candidiasis. There is an intrinsic variation of drug susceptibility of different *Candida* species which is highlighted by the emerging issue of acquired resistance to its treatment and has inevitably become problematic (Ksiezopolska and Gabaldón, 2018). The resistance rate of flucazoles, which are the most commonly prescribed azole class of anti-*Candida* drug, ranges between 4 – 13% against *Candida parapsilosis*, *Candida glabrata* and *Candida tropicalis* and 90% for *Candida auris* (Tan *et al.,* 2021). Polyene resistance remains quiet unclear; amphotericin B which is a polyene has a 30% resistance rate against *Candida auris* (Carolus *et al.,* 2020). *Candida parapsilosis* and *Candida glabrata* have a 100% efficacy rate against rate and *Candida tropicalis* has now observed an amphotericin B resistance (Carolus *et al.,* 2020). Anidulafungin, caspofungin and micafungin are part of the class echinocandins, which are used as first-line treatment of invasive candidiasis (Sumiyoshi *et al.,* 2020). Caspofungin has an 81% effeciency rate with micafungin at 83.3% and a 72% displayed by anidulafungin, these numbers are conirmed clinical success rates against *Candida* species (Sumiyoshi *et al.,* 2020).

Mycobacterium other than tuberculosis (MOTT) is a causative agent of both pulmonary and non-pulmonary diseases, these bacteria are found in animals, soil, water and humans (Walsh *et al*., 2019). The global prevalence of *Mycobacterium* other than tuberculosis ranged between 4 – 15% amongst suspected tuberculosis cases and 18 – 20% in drug resistant tuberculosis cases in the Middle East and Africa between the years 2004 and 2009 (Stout *et al.,* 2016). According to a systematic review done between 1940 and 2016, Sub-Saharan Africa had a prevalence MOTT pulmonary colonization of 7.5% (Zulu *et al.,* 2021). *Mycobacterium* isolates collected in South Africa between the years 1999 and 2011 indicated that 56% of the isolates were known to be MOTT species, these were isolated from various environmental sources such as animal tissue, soil and water (Gcebe *et al*., 2013).

MOTT pathogens are highly resistant to their antibiotics including anti-tuberculosis treatment agents (Quang and Jang 2021). Consequently, the current therapeutic agents need long remedial periods and their outcomes are not always satisfactory (Quang and Jang 2021). The current treatment for *Mycobacterium* other than tuberculosis infections is a class of antibiotics referred to as macrolide-based antibiotics (Saxena *et al.,* 2021). In spite of the fact that MOTT interspecies are erratic and that they have the ability to be susceptible to drugs used for the treatment of *Mycobacteria*; treatment courses are often lumped together to ensure effectiveness. The various therapeutic routines are often designed in a particular pattern to suit the particular species

targeted; these pathogenic species are often the ones commonly tackled (Wu *et al*., 2018). According to Vilchèze and Jacobs (2019), isoniazid rapidly kills 99% of *Mycobacterium tuberculosis* within the first three days of treatment, the other 1% surviving cells are persisters, these increases the probability of regrowth.

Despite research having been ongoing for numerous years, there are still no licensed vaccines on the market for these infections. Despite the medical management of infections caused by the aforementioned microorganisms, their infections often than never have tendency to reoccur. New drugs have to be designed, explored and if need be repuposed or repositioned in order to combat the resistance of these microorganisms to their respective treatments.

1.12 Purpose of the study

The purpose of this study is to explore the *in vitro* anti-mycobacterial and antifungal potential of *Cannabis indica/sativa* species and to repurpose isoniazid derivatives against nosocomial and communically acquired infections. The study concentrates specifically on the utilization of this plant species as potential therapeutic techniques for these global epidemics due to its reputation as a being gateway drug, it is also concentrates on the use of existing marketed drugs as a therapeutic aid for diseases which were not part of their original design. There has been a dramatic increase in the resistance of various microorganisms to their respective therapeutic agents over the last decade (Tabutia *et al.*, 2010). The resistance of these microorganisms to their respective treatment drugs has shown to be problematic as they threaten the effective prevention and treatment of these microbial infections. Antimicrobial resistance poses as a stern public health threat as they are the root cause of both a social and economic burden on the effective health care of patients. This can be attributed to additional testing to esnure that treatment offered is targeted at the particular organism and the prescription and use of expensive drugs for treatment. It is also a social burden as many people are eschewed away from their communities and have stigmas attached to them.

This study may prove to be significant due to its contribution to the underdeveloped area of research related to antidrug resistant microorganisms. The main significance of this study lies in the fact that, it increases knowledge in the field of phytomedicine and ethnobotany and expantion of drug use outside of the original design’s intention. Highlighting the use of both plants and existing drugs as a new form of commercial medicine ensures that strategies are always put into place once new mutants which are causatives to drug reistsance are discovered.

This research will prove to be significant as it draws interest in the prospect of using plant-based medicine for diseases and shifting the focus of therapeutic drugs intended for one disease to treat another.

1.13 Aim and objectives

Aim

The study aimed to evaluate the anti-mycobacterial, anti-candida, antioxidant activity and cytotoxic effects of *Cannabis indica/sativa* species and isoniazid derivatives.

Objective 1

To investigate the antifungal activity of *Cannabis indica/sativa* extracts and isoniazid derivatives against various *Candida spp.* using the microdilution assay and cell apoptosis using flow cytometry.

Objective 2

To evaluate the anti-mycobacterial activity of *Cannabis indica/sativa* extracts and isoniazid derivatives against *mycobacteria* species other than tuberculosis using minimum inhibitory testing and organism identification.

Objective 3

To evaluate the cytotoxic effects of *Cannabis indica/sativa* extracts and isoniazid derivatives on murine macrophage (RAW 264.7) and African Green Monkey (Vero) cell lines, using the MTT cytotoxicity assay and real-time cell analysis.

Objective 4

To determine the antioxidant activity of *Cannabis indica/sativa* extracts and isoniazid derivatives using 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay.

Objective 5

To profile bioactive compounds found in the *Cannabis indica/sativa* extracts which are responsible for the activity by putative identification of compounds using two dimensional gas chromatography – time of flight – mass spectrometry (GC x GC – TOF – MS).

1.14 Hypothesis

Cannabis indica/sativa extracts and isoniazid derivatives possess anti-mycobacterial, antifungal, antioxidant inhibitory and non-toxic properties.

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Chapter 2

Non-*Candida albicans Candida* and *Mycobacterium* other than tuberculosis species

2.1 Non-*Candida albicans* Candida (NCAC) species

Candidiasis is an invasive fungal infection, this infection is primarily caused by any fungi in the family Candidaceae and genus *Candida* (Hazen and Howell, 2007). *Candida* species usually exist as commensals of the host, however, they also present themselves as opportunistic pathogens and can be found as either hyphae (formed from budding) or pseudohyphae (formed from yeasts) (Silva *et al*., 2012). There are approximately 350 known species of *Candida* and of those, eight have been isolated in humans (Shi En Tan and Yang Hsu, 2018). There are five common species which are associated with candidiasis and these include *Candida albicans* which is the most abundant and common cause of candidiasis, other causative species include non-*Candida albicans Candida* species such as *Candida parapsilosis, Candida krusei, Candida glabrata* and *Candida tropicalis* (Turner and Butler, 2014). Candidiasis encompasses an array of diseases ranging from superficial to systemic manifestations. These could include oesophageal or oropharyngeal candidiasis to those deemed more serious such as nosocomial bloodstream infections (BSI) and disseminated candidiasis (Yapar, 2014), thus referred to as invasive candidiasis (Cortés and Corrales, 2018). Some diseases are known to be encompassed within the broader spectrum of severe candidiasis infections include candidemia, central nervous system infections, endocarditis, endophthalmitis, disseminated infections and osteomyelitis (Pappas, 2006).

2.1.1 *Candida auris*

Candida auris, is a budding yeast and shows characteristics of pseudohyphae when the fungus is treated with genotoxins (Ruiz *et al*., 2021). It forms a smooth shiny, whitish-grey, viscous fungal colonies when cultured on Sabouraud's dextrose agar with microscopically ellipsoid in shape (Jeffery-Smith *et al.*, 2018). It appears beige to pink in colour when cultured on *Candida* selective medium chromogenic media (CHROM-agar) (Kordalewska and Perlin, 2019). *Candida auris* can cause invasive nosocomial candidiasis in immunocompromised patients and is easily confused with other *Candida* species, thus making it a little challenging to diagnose (Osei Sekyere, 2018). This can result in mistreatment, which is more likely a contributing factor towards its multidrug resistance (Chatterjee *et al*., 2015). The pathogen isolates have been

found to be resistant to many azole classes of drugs particularly fluconazoles as well as voriconazole. It also shows resistance towards amphotericin B and to echinocandins which is usually considered the treatment of choice. Triazoles, do however show better activity against *C. auris* (Kuehn, 2020).

2.1.2 *Candida glabrata*

Candida glabrata is a haploid yeast, these strains are asexual yeasts and laboratory attempts to make the species mate have so far failed (Gabaldón and Fairhead, 2019). Both mating types (sexual and asexual) have been found in this species, this is despite having no sexual life cycle (Fairhead *et al*., 2008). The presence of *C. glabrata* does not cause any harm to the human mucosal tissue due to it also being found in the human flora. However, there is a belief that it is the second common cause of candidiasis in immunosuppressed individuals (Fidel *et al.*, 1999). *Candida glabrata* is resistant to many antifungal agents, it has the highest incidence of resistance to azoles amongst clinical isolates and is able to exhibit a decreased susceptibility to them, this includes the newest class addition, isavuconazoles (Whaley *et al*., 2016).

2.1.3 *Candida parapsilosis*

Candida parapsilosis is a non-true form hyphae fungus that can either exist in a yeast phase or in a pseudohyphae form when cultured on cornmeal agar (Trofa *et al.,* 2008). It appears white, creamy and shiny on dextrose agar with an oval, cylindrical or round shape; however, it exhibits wrinkled or smooth, shiny, creamy or white colonies when cultured on Sabouraud dextrose agar (SDA) (Trofa *et al.,* 2008). When in yeast form the phenotype is smooth or cratered as compared to its pseudohyphae phenotype which is wrinkled or concentric. Since *C. parapsilosis* is extensively distributed in nature, it is not just obligated to human infection. Unlike *C. tropicalis* and *C. albicans*, *C. parapsilosis* has been isolated in non-human species as well such as insects, domesticated animals and has also been isolated from soil (Trofa *et al.,* 2008). Robeldo-Leal *et al.,* (2014) consider *C. parapsilosis* a fungal antagonist and killer yeast solely based on its chemical production abilities and its cytotoxic effects on host cells. Azoles, more specifically fluconazoles are used as prophylactic and therapeutic drugs against *C. parapsilosis*. However, the fungus has acquired a rapid and stable resistance against azoles (Lamoth *et al*., 2018).

2.1.4 *Candida tropicalis*

Candida tropicalis, is a diploid yeast whose genome was first sequenced in 2009 (Butler *et al.,*

2009). *Candida tropicalis* is an asexual yeast, there was however a study where a/α tetraploid cells were generated by mating diploid cells a and α (Seervai *et al.*, 2013). It is considered a strong biofilm-producing species which is adherent to endothelial and epithelial cells (Zuza-Alves *et al.*, 2017). Biologically, *C. tropicalis* is known for its ability to ferment galactose, sucrose, maltose, trehalose and other carbohydrates through oxidative pathways (Kurtzman *et al.*, 2011). Zuza-Alves *et al*., (2016), claimed that these *Candida* species are osmotolerant meaning that they can survive in high salt concentrations and this majorly contributes to their various virulence factors. In recent investigations, it was found that *C. tropicalis* is resistant to currently available antifungal drugs such as amphotericin B, azole derivatives and echinocandins (Choi *et al*., 2016).

2.2 Fungal pathogenesis

Candida albicans is the most common *Candida* species with known virulence factors which facilitate the spread of infection in human beings and aid in its pathogenicity. These virulence factors play a role in the pseudohyphae formation which is attached to endothelial cells (blood vessels), epithelial cells (respiratory tract), surface recognition molecules, extracellular hydrolytic enzymes, hyphal and phenotypic switching. The production of proteinase and phospholipase has been suggested to attribute to the virulence of *Candida tropicalis* and *Candida albicans* (Singh and Urhekar, 2013).

Extracellular hydrolytic enzymes play a vital role in the overgrowth of *Candida*. These enzymes help facilitate the adherence and penetration on tissue; thus resulting in host invasions. Tsang *et al*., (2007) suggest that phospholipase and aspartyl proteinases are the two most important hydrolytic enzymes produced by *Candida* species, mainly secreted by *C. tropicalis* and *C. albicans*. All species of *Candida* secrete aspartyl proteinase 9 and 5 (SAP9 and SAP5), which are a class of proteolytic enzymes responsible for virulence in human fungal pathogens (Cassone *et al.*, 2016). This is done through the invasion of the host cell, where the SAP5 and SAP9 create copies of the same fungal genes resulting in genomic replication (Singh and Urhekar, 2013).

Due to their virulence factors, the colonization adhesion property of *Candida* takes place in superficial tissue (local site). It invades deeper into the host tissue when in yeast form and is then transformed into the hyphal form during active infections (Singh and Urhekar, 2013).

Biofilms are defined as microbial community encasing matrices of extracellular polymeric substances. They are associated with most manifestations of candidiasis displaying phenotypic features which differ from their planktonic or free-floating counterparts this is where individual microorganisms in the biofilm are embedded in the matrix which is often an extracellular polymer (Singh and Urhekar, 2013).

2.3 Fungal epidemiology

The following section will focus on the various sysmptoms and methods of diagnosis of candidiasis and invasive candidiasis caused by *non-Candida albicans Candida* species.

2.3.1 Symptoms

Common symptoms of oral candidiasis include white cottage cheese-like patches on the tongue and/or the mouth, throat and oesophageal area. Infections occuring in the mouth are more prominent in people who are immunocompromised (Puebla, 2012). Patients usually complain about burning of the mouth and a cotton-like taste sensation, as well as pain and difficulty swallowing (Patil *et al*., 2018). Clinical manifestations can be localized in the fingers, nails, scalp, lungs, bronchi or gastrointestinal tract (Puebla, 2012). When candidiasis affects the vagina, it is referred to as vulvovaginal candidiasis and is categorized with white, clumpy and thick vaginal discharge as well as itching and inflammation of the vulva (Martin Lopez, 2015). There are no obvious clinical symptoms or signs which indicate invasive candidiasis, patients which are suspected or known to be risk factors would present an unexplained fever which would be unresponsive to antibiotic treatment (Pappas *et al.,* 2018).

2.3.2 Diagnosis

Oral candidiasis is diagnosed by inspecting the oral cavity for white patches and irritation. A sample of the infected area may be taken for tests to determine the organism which is causing the infection (Patil *et al*., 2015). Symptoms of vaginal candidiasis are common in bacterial vaginosis, the diagnosis of yeast infections are done with a culturing or microscopic examination (Chakravarthi and Nagaraja, 2010). An endoscopy is required in order to make a diagnosis for respiratory, gastrointestinal and oesophageal candidiasis (Erdogan and Rao, 2015). A diagnostic laboratory would be notified if a patient is suspected to have invasive candidiasis, this is done to ensure the use of culturing media containing bacterial growth inhibitors. Another diagnostic option for invasive candidiasis is the use of polymerase chain reaction tests (PCR) and antigen detections (Pappas *et al.,* 2018).

2.4 Antifungal therapeutic drugs

2.4.1 Azoles

Azoles possess fungistatic activity against *Candida* species as well as fungicidal activity against *Aspergillus* species (Robbins *et al.*, 2011). Agents of this class include fluconazoles (FLC), itraconazoles (ITR), voriconazoles (VRC) and posaconazoles (POS) (Bondaryk *et al.*, 2013). Cannon and collegues (2009) suggest that azoles are lanosterol demethylase (Erg11p) targets, this is a crytochrome P-450 enzyme and mediates a rate-limiting step in biosynthesis. The accumulation of intermediates of ergosterol biosynthesis, this is a subsequent mechanism of sterol metabolism that is mediated by the C5,6 desaturase enzyme (encoded by ERG 3) and is then activated (Vale-Silva *et al*., 2012). Erg3p aids in the mediation of the conversion of non-toxic 14α-methylfecosterol to 3,6-diol (14α methylergosta-8,24(28)-3β, 6α-diol). Growth inhibition is led by the conversion of the Erg11, an enzyme in the ergosterol synthesis pathway, substrate into toxic methylated sterols (Bondaryk *et al.*, 2013).

Itraconazole, ketoconazole and fluconazole are common treatments for superficial candidiasis. Fluconazole has low activity against *C. krusei* and *C. glabrata.* There is also evidence of colonization and probable increased infection in both species when the drug is routinely used for prophylaxis (Bondaryk *et al.*, 2013).

2.4.2 Polyenes

Polyenes are antifungal agents derived from *Streptomyces* fermentation (Larson *et al.*, 2000). Ergosterol is the primary target of polyenes (Cannon *et al.*, 2009). The three main polyenes used for the treatment of superficial candida infections and vaginal candidiasis include nystatins, natamycin and amphotericin B. Nystatins can be administered orally or topically and may be used for congenital cutaneous candidiasis (Bondaryk *et al.*, 2013). This antifungal agent has no significant toxic side effects as its not absorbed by either the genitourinary tract or the skin (Bondaryk *et al.*, 2013).

Wilson *et al*., (2016) state that amphotericin B (AMB) is an antifungal agent commonly used to treat *Candida* infections. This agent can be used to topically treat superficial candidiasis, it can be used for a variety of *Candida spp.* and other fungal species such as *Cryptococcus spp., Mucor spp., Rhizopus spp.* to name a few (Bondaryk *et al.*, 2013). Amphotericin B has a wide tissue distribution, half-life and significant toxicity, despite its broad spectrum of antifungal

activity (Cannon *et al.,* 2009). The antifungal mechanism of amphotericin B is binding to ergosterol in the fungal membrane, this leads to the formation of pores and increased permeability of the cellular membrane. This in turn causes oxidative damage and leakage of the cellular contents (Bondaryk *et al.,* 2013). Barker and Rogers (2006) claim that resistance to AMB is very uncommon, however other *Candida* species apart from *Candida albicans* naturally are thought to be resistant to AMB.

2.4.3 Echinocandins

Echinocandins are semisynthetic antifungal lipopeptide compounds and are synthetically derived from the natural lipopeptides produced by *Zalerion arboricola, Asperdillus rugulovalvus and Papularia Spheaerosperma* (Vandeputte *et al.,* 2012). There are currently three licensed members of this class: caspofungins, micafungins and anidulafungins (Bondaryk *et al.,* 2013). Caspofungins are as effective as AMB in the treatment of oropharyngeal candidiasis (Bondaryk *et al.,* 2013). The adverse side effects of echinocandins include headaches, fevers, rashes, hepatotoxic effects, phlebitis, the release of histamines and haemolysis (Denning, 2003).

Echinocandins disturb the cell wall biosynthesis, by inhibiting 1,3-β-glucan synthase. This is the enzyme responsible for the synthesis of 1,3-β-glucan. This enzyme is a crucial cell wall strengthening component of C. *albicans*. Lack of the glucan component results in osmotic instability and inevitable cell lysis (Bondaryk *et al.,* 2013). The 1,3-β-glucan synthase complex mutation is linked to echinocandins resistance in fungi. The 1,3-β-glucan synthase consists of two Rho1p units being the regulator and Fksp active site. Mutation of the *FKS* gene results in echinocandins resistance (Cannon *et al.,* 2009).

2.5 Plants with anti-*Candida* activity against non-*Candida albicans Candida* species

In Southern Africa, the plant species *Withania somnifera* is used as a common treatment of Candidiasis, (van Wyk and Gerricke, 2003). Other plant extracts which have been reported activity against *Candida* infections include *Clerodendrum glabrum, Acacia caffra, Osyris lanceolata, Croton gratissimus, Schotia brachypetala, Vangueria infausta subsp. Infausta, Faurea saligna, Richardia brasiliensis, Schkuhria pinnata, Spilanthes acmella, Elaeodendron transvaalense, Strychnos potatorum* and *Hippocratea longipetiolata* (Masevhe *et al.,* 2015). *Origanum vulgare* (Oregano), *Cinnamomum verum* (Cinnamon) (Soares *et al*., 2015). *Lawsania inermis* (*Henna*), *Avicennia marina* (*Qurm*), *Portulaca oleracea* (*Baq'lah*), *Fagonia indica* (*Shoka'a*) *Ziziphus spina- Christi* (*Sidr*) and *Salvadora persica* (*Souwak*) (Soliman *et al*.,

2017).

Plants such as *Allium sativum* (Garlic) (Ebrahimy *et al.*, 2015) have compounds such as allicin which have strong anti-*Candida* activity, this is through the inhibition of thiol-containing proteins and amino acids; this results in the interruption of cell metabolic activities (Soliman *et al*., 2017). *Uncaria tomentosa,* a woody plant native to central America, displays as much effectiveness in terms of anti-*Candida* activity as miconazole on *C. krusei, C. albicans*, and *C. glabrata* and *C. tropicalis*. Other active compounds against *Candida spp.* are anthroquinones isolated from *Heterophylleae pustulata* and they have been found to inhibit the formation of *C. tropicalis* biofilm. Linalool and linalyl acetate are major constituents of *Salvia sclerea* oil and *Heuchera saguinea* which induces apoptosis of the *Candida* cells (Soliman *et al*., 2017). Sphingolipid glucosylceramides interact with the plant *Raphanus sativus* causing an antifungal inhibitory complex in the Candida membrane (Soliman *et al*., 2017). The diterpenoid compound taxodone isolated from *Taxodium distichum* and *Metasequoia glypstroboides* can cause loss of cell integrity and increased permeability in *Candida* species (Kusumoto *et al.,* 2010). Chemical components of *Rosmarinus officinalis* such as cymene, cineole and limonene have showed anti-adherent biological activity against *C. albicans* (Soliman *et al*., 2017).

2.6 *Mycobacterium* other than tuberculosis (MOTT) species

Mycobacterium is a genus of bacteria and belongs to the family Mycobacteriaceae which causes myriad of debilitating infections found in humans (Vilchèze *et al*., 2016), soil and in animals (Walsh *et al*., 2019). These microbes are small and rod-shaped species and can be classified into three main categories; there are those that cause Hansen disease (leprosy) such as *Mycobacterium leprae* (Bhat and Prakash, 2012), *Mycobacterium tuberculosis* complex this complex includes mycobacteria such as *Mycobacterium tuberculosis, Mycobacterium africanum, Mycobacterium microti, Mycobacterium caprae, Mycobacterium canettii, Mycobacterium pinnipedii* and *Mycobacterium bovis* these are causatives of tuberculosis (Forrellad *et al*., 2013; Riojas *et al*., 2018). The final group is *Mycobacteria* other than tuberculosis (MOTT), this group consist of about 200 species collectively classed in this group (Walsh *et al.*, 2019), these are *Mycobacterium* which do not cause leprosy or tuberculosis (TB) and they include *Mycobacterium avium complex* (MAC), *Mycobacterium fortuitum, Mycobacterium kansasii, Mycobacterium smegmatis* and *Mycobacterium abscessus* (Zulu *et al.,* 2021) to name a few. Historically, many *Mycobacterium* infections were due to *Mycobacterium tuberculosis* (MTB), more recently MOTT has been frequently isolated in humans, animals and soil (Johnson and

Odell, 2014).

There are over 150 species of mycobacteria recognized and can vastly be categorized into slow-growing pathogens which are associated with the pathogenicity of the host and fast-growing species which are not associated with host pathogenicity (King *et al*., 2017). Mycobacteria are aerobic, non-motile organisms they appear positive with acid-fast alcohol stains, they are lipid-rich, hydrophobic cell walls and are substantially thicker than most bacteria (Ray and Ryan, 2004). Mycobacteria cell walls are impermeable to hydrophilic nutrients and are resistant to heavy metals, disinfectants and antibiotics due to thickness and cell wall composition (Jarlier and Nikaido, 1994).

2.6.1 *Mycobacterium aurum*

Mycobacterium aurum is an environmental, acid-fast, Gram-positive bacterial species with high cytosine and guanine content belonging to the genus *Mycobacterium* (Phelan *et al*., 2015). Unlike fast-growing *Mycobacterium*, its low pathogenicity enables *Mycobacterium aurum* to survive within the macrophage, this makes it a perfect candidate to use in studies of drug screening throughput, this can be conducted by assessing the permeability of the cell membrane by the drug and their stability within the cell (Phelan *et al*., 2015). *Mycobacterium aurum* is highly susceptible to isoniazid; however, there is a need to conduct more studies on NADH dependant trans enoyl-acyl carrier protein called InhA, a protein responsible for mycolic acid and fatty acid biosynthesis in mycobacteria, to further explore it as a drug target (Gupta *et al*., 2009).

2.6.2 *Mycobacterium avium*

The *Mycobaterium avium* complex (MAC) is a group of non-tuberculous *Mycobacterium*, which are acid-fast and slow-growing (Payeur, 2014), under this group the subspecies *Mycobacterium avium* and *Mycobacterium intracellulare* are found (Daley, 2017). Other MAC subspecies responsible for pulmonary diseases are *Mycobacterium yongonense, Mycobacterium chimaera, Mycobacterium arosiense, Mycobacterium bouchedurhonense, Mycobacterium marseillense, Mycobacterium colombiense*, *Mycobacterium vulneris,* and *Mycobacterium timonense* (Kim *et al.*, 2019). *Mycobacterium avium* and *Mycobacterium intracellulare* are grouped in coalition as they infect humans together to form a *Mycobacterium avium-intracellulare* infection (Daley, 2017). These two species are indistinguishable from one another by

using classic microbiological techniques; however, techniques such as polymerase chain reaction (PCR) aids in providing a clear distinction between these microbial species (Pampaloni *et al*., 2020). Even though the two species tend to co-infect the most prominent one in causing infections is *Mycobacterium avium*, this species is responsible for disseminated disease in immunocompromised patients (Pampaloni *et al*., 2020). The natural niche where MAC is found can be dust, soil and water (Nishiuchi, Iwamoto and Maruyama, 2017). Treatment for MAC includes a cocktail of drugs including rifampicin, ethambutol and macrolide, this combination has a varied outcome (Kim *et al*., 2019).

2.6.3 *Mycobacterium bovis*

Mycobacterium bovis or bovine tuberculosis, is an aerobic, slow-growing, acid-fast bacteria and causative agent of tuberculosis in cattle (Pérez-Lago, Navarro and García-de-Viedma, 2014) and in wildlife (Michel, Müller and van Helden, 2010). The bacteria are of a non-motile, straight or curved filament or rod morphology and fragment into cocci or bacilli once it is distributed into the host environment (Ünüvar, 2018). *Mycobacterium bovis* can be transferred directly to humans through the consumption of contaminated meat and dairy products from infected cattle (Waters *et al*., 2010). *Mycobacterium bovis* is a member of the *Mycobacterium tuberculosis* (*M. tuberculosis* or TB) complex (Lan, Bastos and Menzies, 2016) and acts very similarly to *M. tuberculosis* in terms of its sensitivity to antibiotics (Michel, Müller and van Helden, 2010). Wild-type *M. bovis* is sensitive to all treatment drugs used for TB, these include ethionamide, ofloxacin, streptomycin and ethambutol. Despite its natural resistance to pyrazinamide in human cases, *M. bovis* tuberculosis will heal with standard treatment of ethambutol, pyrazinamide, rifampicin and isoniazid (Michel *et al*., 2010).

2.6.4 *Mycobacterium fortuitum*

Mycobacterium fortuitum is a rapidly-growing bacterium which is non-chromogenic and commonly found in water and soil (Okamori *et al*., 2018). *Mycobacterium fortuitum* is one of the most common rapidly-growing *Mycobacterium* and is causative of extra-pulmonary infections in humans (Winthrop and Roy, 2020). Common infections include soft tissue and skin infections after cardiac or plastic surgery interventions (Gutierrez and Somoskovi, 2014). Treatment of *M. fortuitum* infections include prolonged use of two antibiotics, these include ciprofloxacin, amikacin or cefoxitin (Fabbian *et al*., 2011).

2.6.5 *Mycobacterium smegmatis*

Mycobacterium smegmatis is a fast-growing aerobic bacterium (Hiroyuki *et al.*, 2018) with a long bacillus shape, with its surface morphology appearing to be coarsely collapsed or slightly wrinkled, this microbe has been found to be non-pathogenic (*Sundarsingh et al*., 2020). *Mycobacterium smegmatis* is mainly found in plants, soil and water (Best and Best, 2009*)*. It serves as a useful tool for *Mycobacterium* research and is the most commonly and widely used organism for the genetic investigation of *M. tuberculosis* it is associated with *in vitro* latency (*S*undarsingh *et al*., 2020). This species of *Mycobacterium* is resistant to rifampicin and isoniazid; however, it is susceptible to ethambutol (Best and Best, 2009).

2.6.6 *Mycobacterium tuberculosis*

Mycobacterium tuberculosis a causative agent of tuberculosis and is a non-spore-forming, slow-growing, non-motile, chemo-organotrophic, aerobic bacillus (Gordon and Parish, 2018). *Mycobacterium tuberculosis* is an airborne pathogen that affects both animals and humans alike (LoBue, Enarson and Thoen, 2010) by aerosol droplets which are then deposited into the alveolar surface (Smith, 2003). This bacterium has been found to evolve in mammals and has also been isolated from soil (Smith, 2003). *Mycobacterium tuberculosis* is susceptible to various antibiotic drugs, the first to be introduced includes isoniazid, pyrazinamide and rifampicin; another batch of drugs include ethambutol, streptomycin, capreomycin, kanamycin and ethionamide *(*Sundarsingh *et al*., 2020).

2.7 Mycobacterial pathogenesis

Mycobacterial infections are acquired either intestinally or through the respiratory system (To *et al*., 2020). The most successful strategy used by bacteria for survival is the formation of a biofilm. The assembly of bacterial biofilms is done in various phases, in which are reversible attachment, irreversible attachment formation of mature biofilm and dispersion (Sousa *et al*., 2015). Innate immune response lymphocyte macrophages are important and play a key role in controlling infections (Larsson *et al*., 2017).

In pulmonary infections, once inhaled the *Mycobacterium* species enters the alveolar macrophages through multiple receptor-mediated pathways which are taken through phagosomes. Once processed by the macrophages, the mycobacteria are then presented to the T-lymphocytes and leads to the recruitment of antigen-specific T-lymphocyte clones. Ultimately, there is an

interaction between the lymphocytes and infected macrophages to induce intracellular destruction of the infected macrophages or the mycobacteria itself. In the immune response, cytokines such as IFN-γ, TNF-α and IL-2 play an important role in the regulation and immune response to mycobacteria. The formation of granulomas where infected macrophages are surrounded by epithelioid histiocytes and mononuclear inflammatory cells, serves as a defence mechanism to encapsulate the mycobacteria. However, the granuloma may erupt and disseminate viable mycobacteria throughout the body (Larsson *et al*., 2017).

2.8 Mycobacterial epidemiology

The following section will discuss the symptoms and diagnosis of the non-tuberculous *Mycobacterium* species.

2.8.1 Symptoms

Non-tuberculous *Mycobacterium* (NTM) symptoms generally depend on where the infection takes place in the body and which species causes the infection and what part of the body is affected (Desai and Hurtado, 2021). According to Johnson and Odell, (2014), the most common clinical symptoms of NTM lung disease chronic cough with purulent sputum. Hemoptysis and systemic symptoms such as fatigue, weight loss and malaise may also occur in association with the progression of the NTM lung disease and may reflect other underlying lung diseases such as chronic obstructive lung disease (COLD) or bronchiectasis (Kwon and Koh, 2016). Disseminated bone infections can cause pain in the bones and or joints, where skin infections can produce nodules. Infections which become widespread can cause sweating, fever and weight loss (Desai and Hurtado, 2021).

2.8.2 Diagnosis

Mycobacterium other than tuberculosis, isolated in pulmonary specimens do not equate with any mycobacterial disease. The diagnosis of NTM pulmonary infections require symptoms, radiologic abnormalities and microbiologic cultures which in conjunction are able to exlude other aetiologies (Johnson and Odell, 2014).

2.9 Anti-mycobacterial therapeutic drugs

2.9.1 Amikacin

Amikacin is a semi-synthetic, aminoglycoside antibiotic and is used for a wide range of bacterial infections, it is considered an effective drug against most non-tuberculous mycobacteria

(NTM) species, however daily or intermittent use of systemic amikacin has adverse side effects such as ototoxicity and nephrotoxicity (Egelund *et al*., 2015). The recommended guidelines of aminoglycosides use states that they should be utilized for systemic administration, however it has been suggested that inhalation of amikacin is an effective treatment of refractory NTM lung disease and exhibits less toxicity as compared to the systemic administration of amikacin (Ryu *et al*., 2016).

2.9.2 Linezolid

Linezolid is a synthetic antibiotic and is the first oxazolidinone antibacterial agent approved for clinical use. It has excellent potential against the multidrug-resistant (MDR)-TB and other species of NTM by interrupting the growth of bacteria by inhibition of the initiation stage of protein synthesis (Ryu *et al*., 2016). Clinical use of linezolid for the treatment of NTM lung disease is limited due to lack of long-term safety data and concern over adverse hematologic events as well as costs (Winthrop *et al*., 2015).

2.9.3 Isonicotinic acid hydrazide

Isoniazid or isonicotinic acid hydrazide is an antibacterial agent, primarily used as a tuberculostatic drug and still remains the first line treatment of choice for latent tuberculosis (TB) since 1971 (Denholm *et al*., 2014). It is commonly used in conjunction with ethambutol, rifampicin, streptomycin or pyrazinamide for the treatment of active tuberculosis (WHO *et al*., 2009). Isoniazid is a pro-drug *in vivo* activated by the *katG*-encoded mycobacterial catalase-peroxide in order to generate an INH-NAD (isoniazid-nicotinamide adenine dinucleotide) adduct (Elitas, 2017). Mutations of the *katG*-enzyme in the upstream region of the *fabG1-inhA operon* is the primary cause of isoniazid resistance in *Mycobacterium tuberculosis* (Ando *et al*., 2011). Although isoniazid has shown efficacy for tuberculosis, concerns still remain about its hepatotoxic side effects which include liver failure and/or inflammation (James and Roberts, 2014).

2.9.4 Rifampicin

Rifampicin is most potent first line anti-tuberculosis antibiotic used to treat a wide range of mycobacterial infections including leprosy, *Mycobactrium avium* complex and more commonly those caused by *Mycobactrium tuberculosis* (Siu *et al*., 2011). Rifampicin's mechanism of action lies in the inhibiton of bacteria DNA-dependant RNA polymerase and its appearance is the result of the drug binding in the polymerase subunit deep within the ribonucleic acid (RNA) or deoxyribonucleic acid (DNA) channel. This in turn facilitates the blocking of RNA

elongation, which is thought to inhibit mycobacterial DNA-dependant RNA polymerase (Mtb-RNAP) and appears to occur as a drug binding result of rifampicin in the polymerase subunit deep within the DNA/RNA channel, this facilitates the direct blocking of the elongation of RNA (Beloor *et al*., 2021). Due to the mutation of the primary molecular target *Mycobacterium tuberculosis* RNA polymerase, there has been an increased resistance of mycobacteria tuberculosis to rifampicin (Zhang *et al*., 2019). Rifampicin is known to be fatally toxic at concentrations ranging above 14 – 60g/L and these result in haematological, hepatic and/or renal disorders (Sridhar *et al*., 2012).

2.10 Plants with anti-mycobacterial activity against *Mycobacterium* other than tuberculosis (MOTT) species

Some popular South African plant species used for the treatment of tuberculosis are *Warburgia salutaris* and *Artemisia afra* (van Wyk and Gerricke, 2003; Semenya and Maroyi, 2019). Other plants which have anti-mycobacterial activity include *Bidens pilosa L., Ocimum sanctum, Cirtullus colosnthis, Morinda citrifolia, Peltophorum africanum, Ziziphus mucronata* and *Leonotis leonurus* (Anochie *et al*., 2018). In a study reported by Elisha *et al*., (2017), the extracts from *Securidaca longipedunculata, Maerua edulis, Tabernaemontana elegans* and *Zanthoxylum capense* exhibited high activity against *Mycobacteria spp. (Mycobacterium tuberculosis* H37Ra and *Mycobacterium aurum* A+).

In India, there are a wide variety of plants used for the treatment of tuberculosis including *Alstonia scholaris (L.) R. Br., Mallotus philippensis (Lam.) Müll. Arg., Holorrhena antidysentrica (Roth) Wall. Ex A. DC., Eulophia nuda Lindl., Cocculus hirsutus (L.) Diels, Plumbago zeylanica (L.), Pueraria tuberosa (Willd.) DC., Cyperus rotundusL.,* and *Sphaeranthus indicus (L.) Curcumacaesia Roxb*. (Gupta *et al*., 2018). Native Himalayan plants such as *Aegle marmelos (L.), Lawsoniainermis (L.), Syzygiumaromaticum (L.), Pterolobiumstellatum (Forssk), Pipernigrum (L.), Glycyrrhizaglabra (L.), PerseaamericanaMill L.* and *Otostegiaintegrifolia Benth (L).* which inhibited *Mycobacterium bovis* and *Mycobacterium tuberculosis* and *Piper longum (L.)* strains have also showed activity against *Mycobacterium smegmatis* (Ravindran *et al*., 2020). *Glycyrrhiza glabra (L.)* used by ancient Egyptians, Greek, Chinese, Indians and Romans is also used for various pulmonary diseases including tuberculosis (Ravindran *et al*., 2020).

In synergy with isoniazid, compounds such as 5-(hydroxymethyl) furan-2(5H)-one and 5-(hydroxymethyl) dihydrofuran-2(3H)-one, isolated from the South African plant *Knowltonia vesicatoria (Ranunculaceae)* are used for the treatment of tuberculosis (Anochie *et al*., 2018). Flavonoids found in *Vitex trifolia* such as artemetin, luteolin, casticin, iridoid glycosides and orientin have also exhibited activity against *Mycobacterim smegmatis. Azadirachta indica* A. Juss. also has tannins and flavonoids isolated and are known to have exhibited antibacterial activity against *Mycobacterium aurum* and *Mycobacterium smegmatis* (Anochie *et al*., 2018).

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Chapter 3

Materials and methods

3.1 Plant material

The plant materials were collected based on their traditional medicinal uses around the world and in South Africa. The interest in the plant is based on its antimicrobacterial activity on a wide array of mircoorganisms. The vegetative leaves and the mature aerial inflorescence of the *Cannabis indica/sativa* hybrid plant were grown and harvested by an independent *Cannabis* farmer in Pretoria, Gauteng, South Africa. The leaves and inflorescent parts of the *Cannabis indica/sativa* hybrid species were grown indoors at 25ºC and a humidity of between 50 to 60%.

3.2 Preparation of the extracts

Inflorescence and leaves of the *Cannabis indica/sativa* plants were harvested at various periods, the vegetative leaves were harvested at 9 weeks while the mature inflorescence was harvested at 16 weeks of growth, respectively. During collection, the inflorescence and leaves were placed in a polystyrene box containing ice for transportation to prevent the deterioration of valuable metabolites. Both the leaves and the flowers were treated similarly once the samples arrived at the laboratory, the mature flowers and vegetative leaves of the *Cannabis* plants were immediately processed. Both samples were removed from the ice and rinsed with distled water to remove any debirs, the samples were then air dried and kept at -80ºC until use. The samples were then freeze dried using a Freeze Dryer (Labconco, Vacutec) at a pressure of 0.030 Torr and temperature of -55ºC. The plant material was then ground using a sterile mortar and pestle (**Figure 3.2a**).

Figure 3.2a: Grinding freeze dried vegetative *Cannabis* leaves using a mortar and pestle. (Source: Pictures were taken during the process of investigation).

To obtain the quantity of extract needed for the investigation, the extraction procedure used was followed as described by Redfern *et al*., (2014). Five grams (5g) of the vegetative dried leaf plant material and three grams (3g) of the inflorescence plant material was weighed out and loaded into a Soxhlet extractor. To obtain crude extract, 300mL of each organic solvent; namely: *n*-hexane, ethyl acetate and ultra-pure water, respectively were used. The Soxhlet apparatus was allowed to reflux at 30 – 100°C for ± 6h until each of the respective solvents ran clear, the sample material was extracted with 300mL of *n*-hexane, ethyl acetate and ultra-pure water at various intervals and yielded the weights noted in (**Table 3.2**). The Soxhlet apparatus was allowed to reflux at 30 – 100°C for ± 6h until the respective solvent ran clear. To ensure maximum extraction the entire process was repeated twice. The *n*-hexane and ethyl acetate extracted samples obtained were then concentrated using a Rotary Evaporator RE300 with a Digital Water Bath RE300DB, the ultra-pure water extracts were then divided into six 40mL aliquots and frozen at -80ºC overnight. All extracts were then dried using a Freeze Dryer (Labconco, Vacutec) at a pressure of 0.030 Torr and temperature of -55ºC for four days, the dried extracts were then reconstituted using 10% DMSO and respective solvents (**Figure 3.2b** and **Figure 3.2c**) to yield a stock concentration of 5mg/mL for each extraction.

Table 3.2: Extraction solution and conditions in the Soxhlet extractor for vegetative leaves and mature inflorescence of *Cannabis*.

Extraction solvent	Time taken (hours)	Temperature (°C)	Yield (g)
Vegetative leaves			
n-hexane	6	50	0.258
Ethyl acetate	4	30	1.233
Ultra-pure water	6	100	0.106
Mature inflorescence			
n-hexane	2	50	1.011
Ethyl acetate	4	30	0.335
Ultra-pure water	4	100	16.788

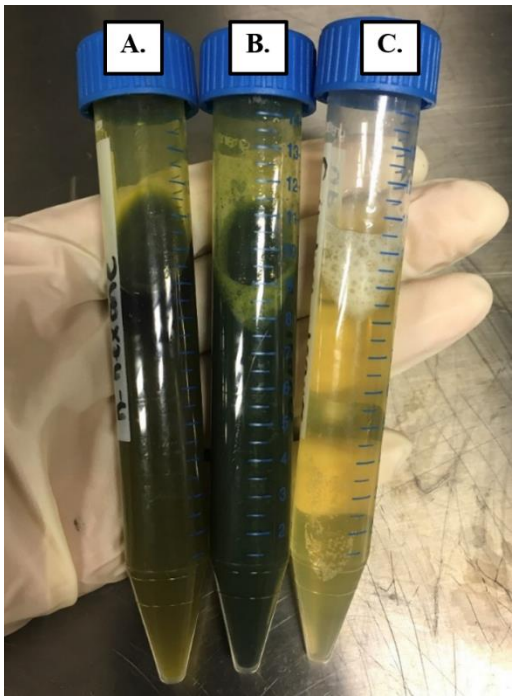


Figure 3.2b: a. n-hexane **b**. ethyl acetate **c**. ultra-pure water 10% DMSO reconstituted vegetative leaf extracts at a concentration of 5mg/mL.
(Source: Pictures were taken during the process of investigation).

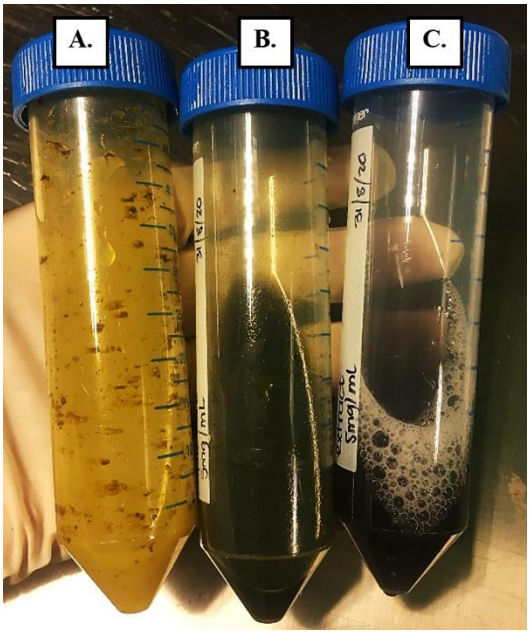


Figure 3.2c: a. n-hexane **b**. ethyl acetate **c**. ultra-pure water 10% DMSO reconstituted mature inflorescence extracts at a concentration of 5mg/mL.
(Source: Pictures were taken during the process of investigation)

3.3 Synthesis of derivatives

The various derivative compounds (**Table 3.3: 1 – 4**) were synthesized in accordance to the methods described by Smith *et al.,* (2015) with modifications. The crystals were synthesized using a one-pot synthesis method which allowed isonicotinic acid hydrazide, salicylic acid and a solvent of preference such as methanol and the addition of a chemical catalyst (used depending on the desired synthetic outcome) to be added to a 10 mL dram-vial or 10ml. The solids were then dissolved with AP-grade methanol, refluxed between 70°C– 110°C for 72 hours with either an open or closed lid and allowed to stand at room temperature until the solvent had crystallized. All reagents used were commercially available and used without further distillation.

The modified isoniazid compounds (**Table 3.3: 5, 6 and 8**) were synthesized using the methods described by Setshedi *et al.,* (2021). All the reagents which used were acquired and utilized without any further distillation. Isonicotinic acid hydrazide and 2-butanone were added into a 25 mL stainless steel jar, this jar contained stainless steel balls (2x7mm). The jar with the contents was tightly closed and shaken at 25Hz for 30 min using the Retsch Mixer Mill MM 200. The mixed contents were transferred into an amber coloured Schott's bottle and left a tad open to enable slow evaporation to take place at room temperature until translucent crystals were

witnessed after three days.

The derivative compound (**Table 3.3: 7**) was synthesized according to the method described by Setshedi and Smith (2021), isonicotinic acid hydrazide, 2-butanone and 2-hydroxy-benzoic acid were placed into a screw-top dram vial. The solution was then stirred at 300 rpm for 10 min at 60 °C. With the vial closed, the solution refluxed in the vial at 60 °C for 24 hours. The solution was then left slightly open to allow slow evaporation at room temperature. This resulted in the formation of colourless crystals which were witnessed after three days.

Table 3.3: Isoniazid derivative compound IUPAC names and their laboratory codes.

	Compound IUPAC name	**Laboratory code**
1.	*N*'-(bis(2-hydroxyphenyl)methylene)isonicotinohydrazide	IBS 007
2.	*N*'-(bis(2-hydroxyphenyl)methylene)isonicotinohydrazide ·(salicylic acid)	IBS 008
3.	*N*'-((4-aminophenyl)phenyl) methylene)isonicotinohydrazide	IBS 009
4.	*N*'-((4-aminophenyl)phenyl) methylene)isonicotinohydrazide·(salicylic acid)	IBS 010
5.	*N*'-(propan-2-ylidene)isonicotinohydrazide	IBS 013
6.	*N*'-(propan-2-ylidene)isonicotinohydrazide· (salicylic acid)	IBS 014
7.	*N*'-(Butan-2-ylidene)isonicotinohydrazide	IBS 017
8	*N*'-(Butan-2-ylidene)isonicotinohydrazide· (salicylic acid)	IBS 018

3.4 Preparation of derivatives

Various modified isoniazid derivative compounds were received from an ongoing study (**Figure 3.4a** and **Figure 3.4b**). These were received in crystals form with an initial stock concentration of 10mg/mL. Each derivative was then weighed out to create a working solution concentration of 1mg/mL by dissolving in ice chilled 99.9% acetone (GPR RECTAPUR, VWR®).

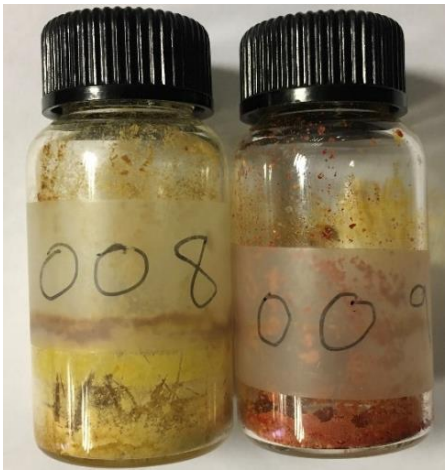
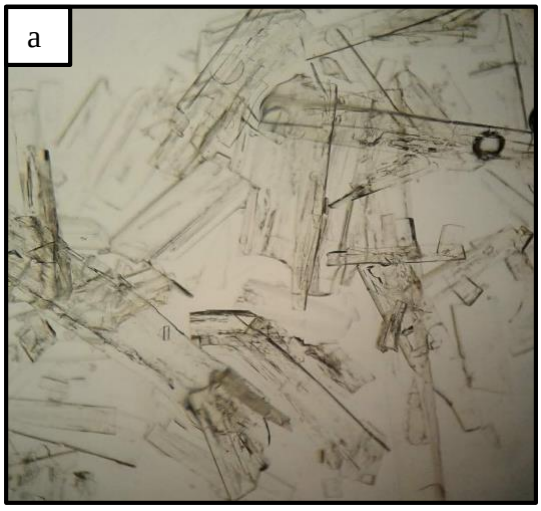
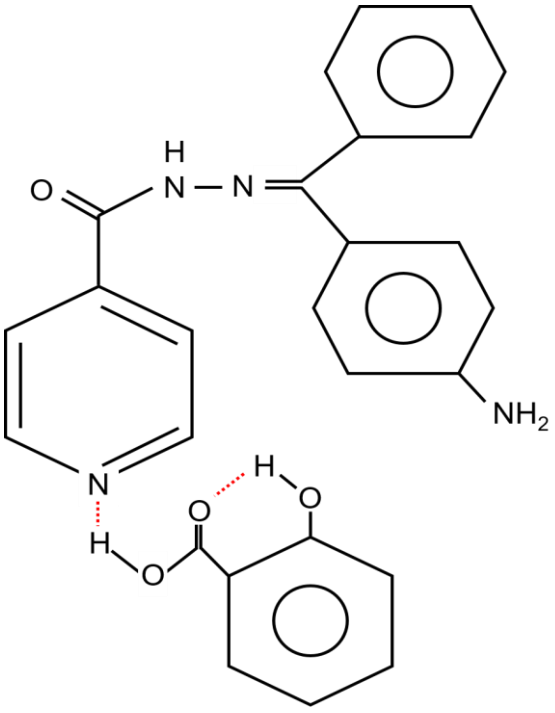


Figure 3.4a: Crystalized modified isoniazid derivatives coded co-crystal *N'*-(*bis*(2-hydroxy-phenyl)methylene)-isonicotinohydrazide)·(salicyclic acid) (IBS 008) and *N'*-((4-aminophe-nyl)(phenyl)methylene)-isonicotinohydrazide (IBS 009).
(Source: pictures were taken during the process of investigation).





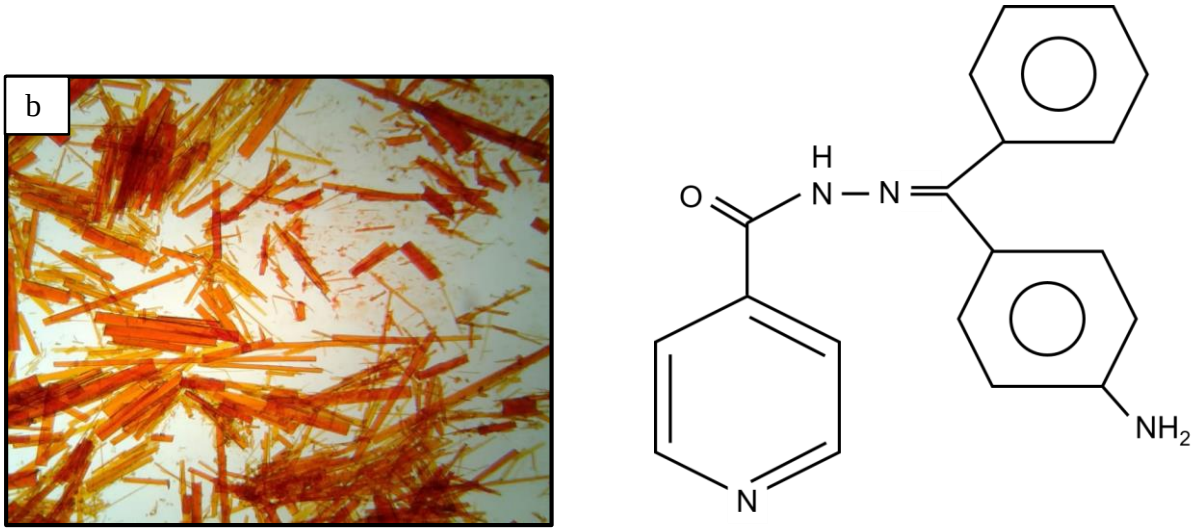


Figure 3.4b: Crystalized modified isoniazid derivatives coded **a.** co-crystal *N'*-(*bis*(2-hydroxyphenyl)methylene)-isonicotinohydrazide)·(salicyclic acid) (IBS 008) and **b.** *N'*-((4-aminophenyl)(phenyl)methylene)-isonicotinohydrazide (IBS 009) microscopic images and chemical structures.
(Source: diagram was created using EdrawMax Software).

3.5 Cell culture

3.5.1 Vero and RAW 264.7 cells maintenance

The cytotoxic evaluation of Vero cells (African green monkey kidney cells) and RAW267.4 (Murine macrophages) cells were evaluated using [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide] (MTT) tetrazolium reduction assay, both cell lines were cultured and maintained in DMEM (Dulbecco's Modified Eagle Media) (Hyclone, United States of America), FBS (Fetal Bovine Serum) (Hyclone South America), and 1% Pen/Strep (Penicillin/ Streptomycin) (Separations Scientific, Honeydew, South Africa). The cells were passaged and left to propagate until approximately 90% confluent (**Figure 3.5.1a**) for Vero cells, (**Figure 3.5.1b)** shows the cells at 40% confluency of RAW264.7 cells (Separation Scientific Cellonex, passage #9, lot: 1, South Africa).

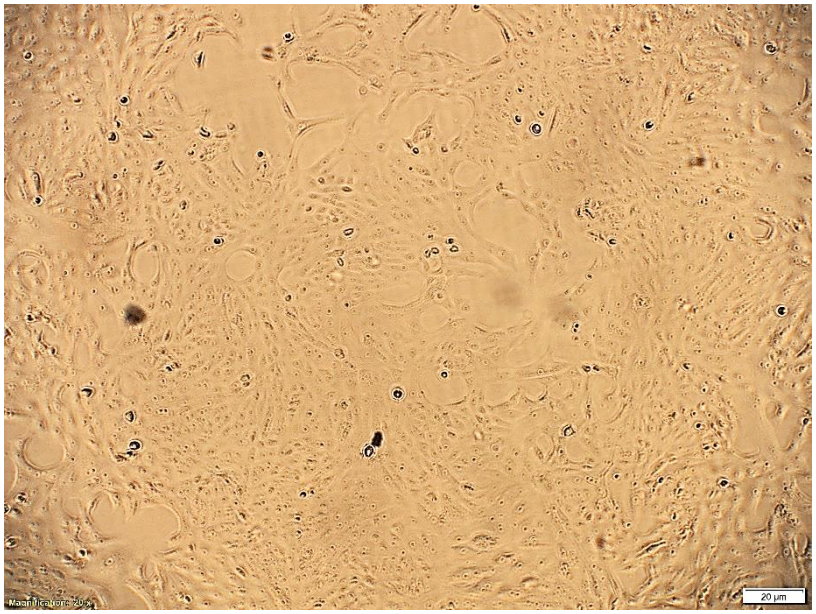


Figure 3.5.1a: Seeded Vero (African green monkey kidney) cells 90% confluent in a microtitire plate observed under 20x magnification (Olympus cellSens Standard 1.17, USA).

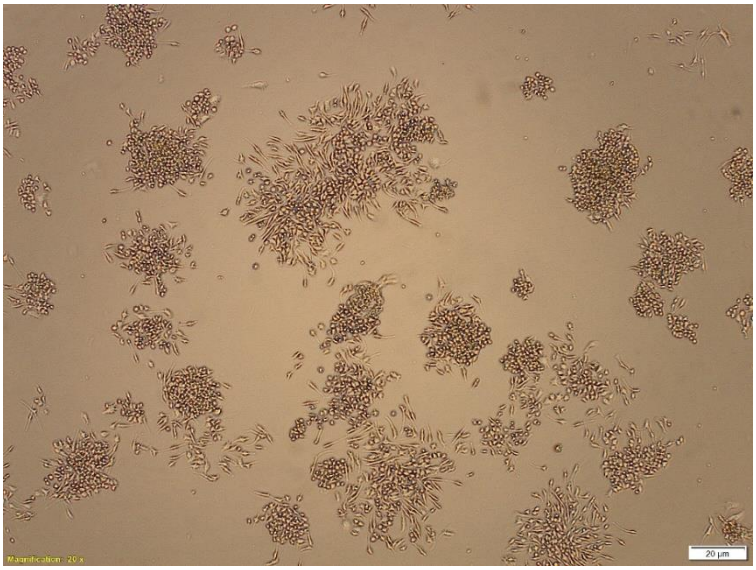


Figure 3.5.1b: RAW264.7 (murine macrophage) cells 40% confluent observed under 20x magnification (Olympus cellSens Standard 1.17, USA).

3.6 Microorganisms

The microorganisms which were used in this study were various *Candida* species namely, *Candida auris* (CDC B11903), *Candida parapsilosis* (ATCC 22019), *Candida tropicalis* (ATCC 1369), *Candida krusei* (ATCC 14243) *and Candida glabrata* (ATCC 15126). The test strains

also included *Mycobacterium* species namely *Mycobacterium fortuitium* (ATCC 6841), *Mycobacterium smegmatis* (ATCC 14468), *Mycobacterium aurum* (NCTC 10437), *Mycobacterium avium* (ATCC 25291), *Mycobacterium bovis* (BCG) (ATCC 27290) and *Mycobacterium tuberculosis* (H37-Ra) (ATCC 25177). The *Cannabis* vegetative leaf and mature inflorescence extracts were dissolved in 10% DMSO (Promark Chemicals, South Africa) to a final concentration of 5mg/mL. The modified isoniazid derivative compounds were weighed out to 1mg and dissolved in 99.9% acetone (GPR RECTAPUR, VWR®) to create a working solution of 1mg/mL. The positive drug controls used were Amphotericin B (*Candida spp*.) at 2mg/mL, Rifampicin and Isoniazid (*Mycobacterium spp*.) at 2mg/mL, respectively.

3.6.1 *Candida spp.* cultures

The *Candida* species were received as Microbiologics ® KWIK STIK™ devices containing a lyophilized pallet of each of the respective *Candida* strain. Each strain was inoculated onto Sabouraud Dextrose Agar (SDA) (Merck, South Africa Pty (Ltd.) Halfway House) prepared petri dish (**Figure 3.6.1**) to form pure, single colonies and were incubated at 37ºC for 24-72 hours. In order to obtain a 0.5 McFarland solution, the colonies were collected into a 50mL tube containing sterile 0.85% sodium chloride (saline) solution and were adjusted using a visible spectrophotometer (Genesys 10S UV-Vis Spectrophotometer) at an OD_{625} to give the microbial absorbance of 0.003nm or set to 0.5 McFarland Standard.

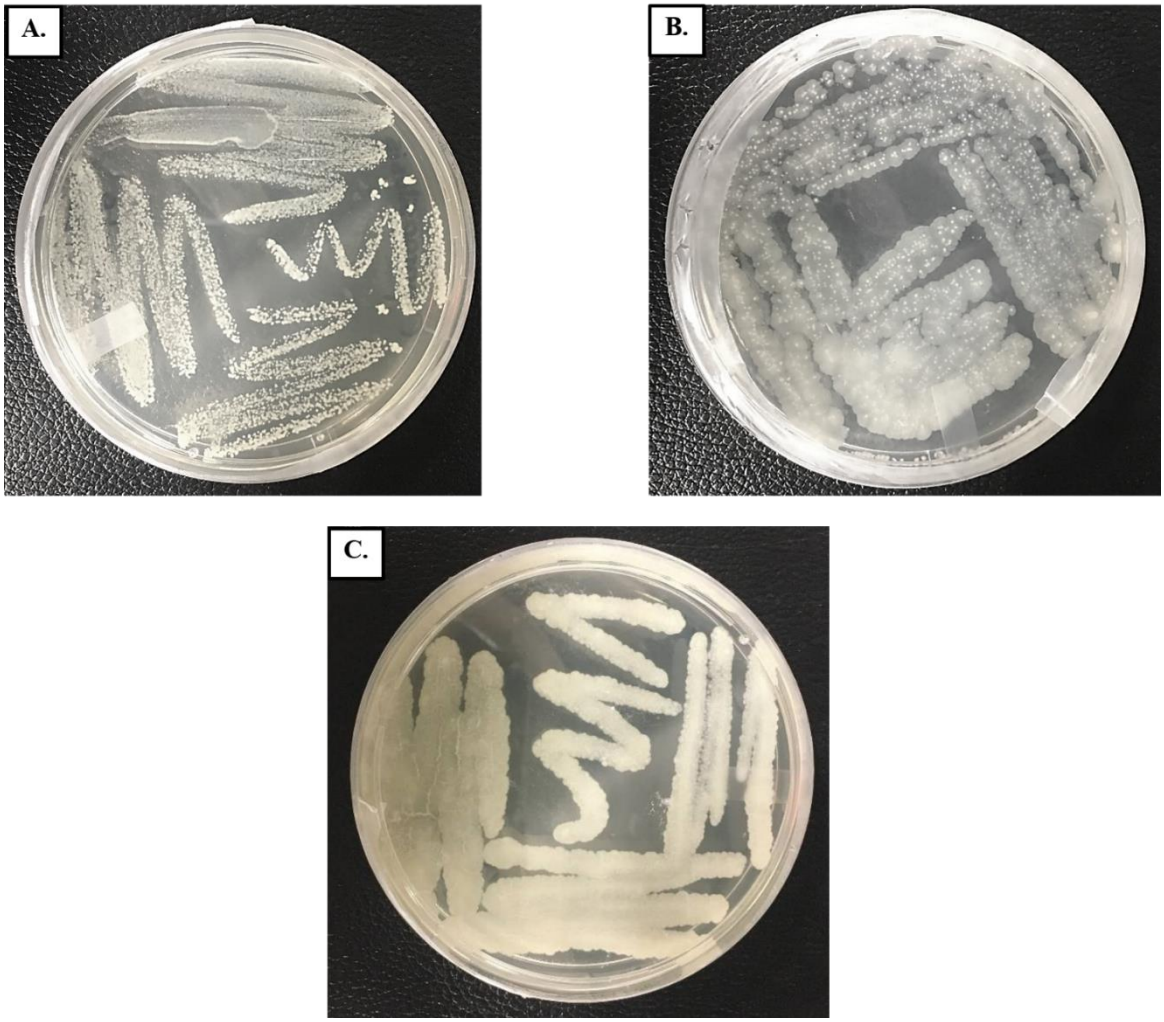


Figure 3.6.1: Cultures of **a.** *Candida parapsilosis* (ATCC 22019) **b.** *Candida tropicalis* (ATCC 1369) **c.** *Candida auris* (CDC B11903) on Sabouraud Dextrose agar (SDA).
(Source: pictures taken during the process of investigation).

3.6.2 *Mycobacterium spp.* cultures

The various *Mycobacterium* cultures were revived from Microbiologics® KWIK STIK™ swabs and culture on Middlebrook 7H11 agar plates. Subcultures were then made in Middlebrook 7H9 broth (Sigma Aldrich, France) that contained 1mL 20% Tween80 (Sigma Aldrich, France) to prevent clumping and 2.5mL Glycerol (AnalaR NORMAPUR, France) to act as a carbon source for the microorganisms. The *Mycobacterium* was then re-cultured on Middlebrook 7H11 agar (Condalab, Spain) and incubated for 1-14 days depending on the species (**Figure 3.6.2**). The *Mycobacterium* species used in this study include *Mycobacterium smegmatis* (ATCC 14468), *Mycobacterium aurum* (NCTC 10437**)**, *Mycobacterium fortuitum*

(ATCC 68841), *Mycobacterium avium* (ATCC 25291), *Mycobacterium bovis* (BCG) (ATCC 27290), *Mycobacterium fortuitum* (ATCC 6841), and *Mycobacterium tuberculosis* (H37Ra) (ATCC 25177).

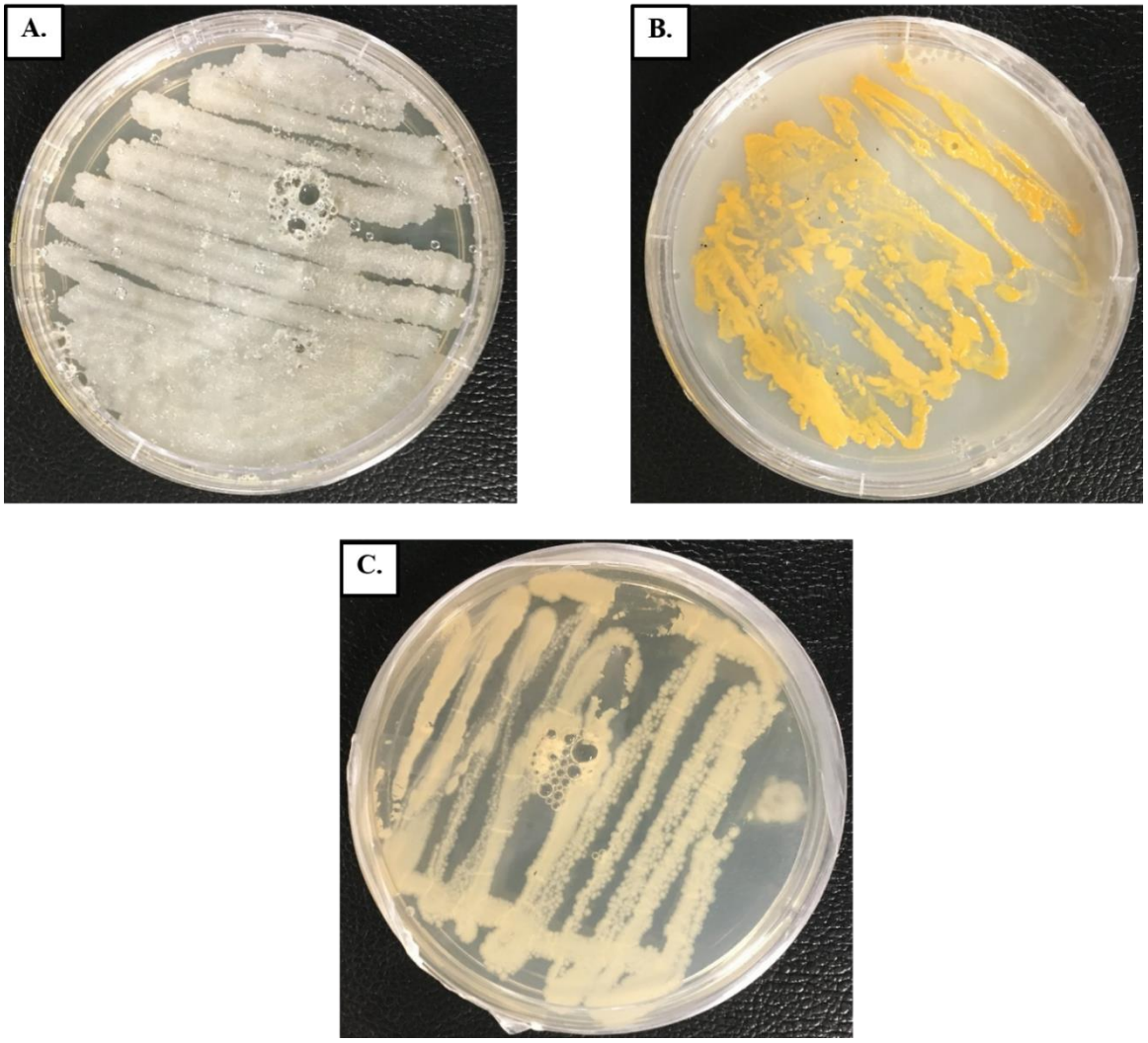


Figure 3.6.2: Petri dish cultures of **a.** *Mycobacterium smegmatis* (ATCC 14468), **b.** *Mycobacterium aurum* (NCTC 10437**)** and **c.** *Mycobacterium fortuitum* (ATCC 6841) on Middlebrook 7H11 agar.
(Source: pictures taken during the process of investigation).

3.6.3 Acid-fast characterization

The determination of fast-acid non-*tuberculosis Mycobacteria* (NTM) was done using Yeware *et al.,* (2019) method. Approximately 20µL of the various NTM cultures at different time points were squarely spread out on a glass slide as a thin smear. The smears were then quickly heat-

fixed by flame and cooled to room temperature before staining. Fluorescent, acid-fast staining dye Auramine-O (Sigma Aldrich, USA) was used to determine the acid-fast bacteria, those that fluoresced yellowish-green were acid-fast and those which were not acid-fast did not fluoresce.

3.7 Reagents and microorganisms

Hexane (AnalaR NORMAPUR VWR®), Ethyl acetate (Sigma-Aldrich, Germany), Ultra-pure water (Sigma Aldrich, Germany), DMSO (dimethyl sulfoxide) (Promark Chemicals), Ascorbic acid (Sigma-Aldrich, Japan), ABTS solution (Calbiochem, USA and Canada), Sabouraud Dextrose Broth (Merck SA Pty (Ltd.) Halfway House), Sabouraud Dextrose Agar (Merck SA Pty (Ltd.) Halfway House), Bacteriological agar (Merck, South Africa), Luria-Bertani (LB) agar (Merck LGaA, Germany), Yeast extract (Sigma-Aldrich, USA), Potassium persulfate solution (Sigma-Aldrich, Germany), Amphotericin B (Sigma-Aldrich, Israel), INT (*p*-iodonitrotetrazolium chloride) (Sigma-Aldrich, Austria), MTT Formazan (Sigma-Aldrich, USA), Thiazolyl Blue Tetrazolium (Sigma-Aldrich, USA), Sodium chloride (Sigma-Aldrich, USA), Acetone (GPR RECTAPUR, VWR®), Formic acid (Sigma-Aldrich, Germany), Benzene (Sigma-Aldrich, United Kingdom), Methanol (AnalaR NORMAPUR VWR®), Ethanol (Promark Chemicals), Chloroform (AnalaR NORMAPUR VWR®), Diethyl ether (AnalaR NORMAPUR VWR®), Ethyl acetate (Merck, USA), Toluene (Merck, USA), Vanillin (Merck, USA), Rifampicin (Sigma-Aldrich, Israel), Isoniazid (Sigma-Aldrich, Germany), Resazurine (Sigma Aldrich, USA), Auramine-O (Sigma Aldrich, USA) Tryptone (Melford, United Kingdom), Tween 80 (Sigma Aldrich, France), OADC growth supplement (Sigma Aldrich, India), Middlebrook 7H9 broth (Sigma Aldrich, France), Middlebrook 7H11 agar (Condalab, Spain). *Candida* species (Microbiologics ® KWIK STIK™) and *Mycobacterium* species (Microbiologics ® KWIK STIK™). Dulbecco's Modified Eagle Medium/ High Glucose (DMEM) (Hyclone, USA), Fetal Bovine Serum (FBS) (Hyclone South America), Penicillin and Streptomycin (Pen/Strep) (Separations Scientific, South Africa), L-glutamine (Lonza, Belgium).

3.8 References

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Chapter 4

Antioxidant activity of *Cannabis* and isoniotinic acid hydrazide derivatives

4.1 Introduction

Antioxidants are defined as bioactive compounds that are categorized as either being natural or synthetic (Altemimi *et al*., 2017) and play an inhibitory or delaying role in the oxidation of free radical molecules (Mahdi-Pour *et al*., 2012). They have the ability to quench free radicals therefore protecting the body from oxidative stress damage (Qasim *et al*., 2017). Free radicals do not only cause cancer or stroke related diseases, but also advance the imbalance between oxidants and antioxidants (Altemimi *et al.*, 2017). Their accumulation in the human body has been associated with several pathophysiological diseases (Rahman *et al*., 2015) such as stroke, diabetes, cancer, rheumatoid arthritis and coronary artery diseases (More and Makola, 2020).

Oxidative stress is a phenomenon triggered by an imbalance between the accumulation and production of reactive oxygen species in tissue and cells and the detoxifying ability of the body (Pizzino *et al*., 2017). Reactive oxygen species (ROS) are highly active chemicals which are formed from oxygen molecules (O_2) (Hayyan *et al.*, 2016). Examples of these oxidising compounds include superoxides ($O_2^{\cdot-}$), hydrogen peroxides (H_2O_2), hydroxyl radicals ($^{\cdot}OH$), peroxides, alpha-oxygen (α-O_2) and singlet-oxygen ($^1O_2$), these compounds are all by-products of various metabolic systems (Pizzino *et al*., 2017). An increase in excess ROS production has effects that can be seen in various metabolic processes which in turn may cause damage either by activating cyclooxygenases (COX) and lipoxygenases (LOX), damaging nucleic acids such as deoxyribonucleic acid (DNA) or stimulating cytokine release by activating kappa B (NF-kB) protein complex which ultimately leads to mitochondrial dysfunctions and cell death (Collin, 2019).

Plants have been known to possess antioxidants which delay or inhibit oxidation caused by ROS (Altemimi *et al.*, 2017). Natural antioxidants are produced by medicinal and dietary plants. These natural antioxidative classes of compounds include phenols, flavonoids, tannins, proanthocyanidins, carotenoids, alkaloids, saponins which might aid in the prevention of free radical oxidative damage (Qasim *et al*., 2017). A healthy diet comprising of fruit and vegetable consumption is associated with low risk of diseases due to the attribution of the antioxidants found in fruit and vegetables (Alvarez-Suarez *et al*., 2012). Polyphenolic compounds are the

most abundant anti-oxidative plant-based compounds possessing powerful antioxidant activity and are commonly found in both edible and inedible plants (Houde *et al.,* 2006). Polyphenols are one of the largest group of phytoconstituents found in various plants and are renowned for their radical scavenging activity and have proven to be effective in *in vitro* studies and thus may significantly contribute to cytoprotective effects *in vivo* (Rice-Evans *et al.*, 1997).

Previous studies reported the antioxidant activity of *Cannabis sativa* extracts and its constituents using the peroxide value, oxidative stability index (OSI), electron spin resonance (ESR) forced oxidation, 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging assay, nitric oxide scavenging activity and the reducing power assay (Tura *et al.,* 2019; Luana Izzo, 2020). The study by Luana Izzo (2020), revealed that *Cannabis* extracts apart from cannabinoids, contained abundant traces of phenolic compounds and flavonoids such as catechin and epicatechin which are responsible for antioxidant activity. A study by Nadeem *et al*., (2012) evaluated the antioxidant and radical scavenging activities using the DPPH and 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonate) radical cation ($ABTS^{\bullet+}$) assays of leaves, stem and inflorescence of *Cannabis sativa* extracted in methanol and subsequently partitioned in n-hexane, chloroform, ethyl acetate and 1-butanol, successively.

Drug repurposing is a growing popular strategy which is cost-effective and involves identifying known approved drugs for treatment of infectious diseases (An *et al*., 2020). Over the past five decades, there has been many suggestions and efforts undertaken to develop anti-mycobacterial drugs (Hu *et al*., 2017) which have effective anti-tubercular properties, good biocompatibility and an improved toxicological profile (Dragostin *et al*., 2019). Many derivatives of the compound isoniazid have been synthesized over the years as a way to counteract resistance of the mycobacterium species to the drug. These include the design of new compound derivatives such as isonicotinoylhydrazone with an intention to improve pharma-toxicological profiles of the drug towards the organism (Dragostin *et al*., 2019) and the *N*'-(bis(2-hydroxyphenyl)methylene)isonicotinohydrazide·(salicylic acid) co-crystals whose addition of salicylic acid is likely to increase bioavailability of the drug (Smith *et al*., 2015).

The aim of this study was to evaluate and compare the antioxidant activities of *Cannabis* (n-hexane, ethyl acetate and ultra-pure water) plant extracts at various stages of their respective growths and to evaluate the comparative effectiveness of isoniazid derivatives using the DPPH method.

4.2 Sample collection and preparation

As described in Chapter 3, the preparation of the *Cannabis indica/sativa* plant extracts is described in section **3.2 Preparation of extracts** and isoniazid derivatives in section **3.3 Synthesis of derivatives** and **3.4 Preparation of derivatives**.

4.3 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging assay

The DPPH free radical scavenging ability of the extracts and derivatives was evaluated using the method described by Swathi, (2016) with modifications. Approximately 120µL of methanol was added to each well of the 96-well plate then topped up with 80µL of each of the six plant extracts with a stock concentration of 5mg/mL to form various concentrations of 2 000 – 15.63µg/mL after being serially diluted in the microtitire plate, the concentration in the first row of the plate was 2 000µg/mL. One hundred microliters (100µL) of DPPH (Sigma-Aldrich, Germany) at a concentration of 0.04mg/mL was added into the microtiter plate, under a dim light. The plates were then incubated at room temperature, in the dark for 1 hour. The resultant absorbance of the mixture was measured at 517nm using a UV-spectrophotometer (Thermo Scientific, Varioskan® Flash UV-spectrophotometer, Finland). Ascorbic acid (Sigma-Aldrich, Japan) was prepared to a concentration of 5mg/mL and was used as a positive control with distilled water being used as a negative control, respectively. The same method was followed for the eight isoniazid derivatives, where 100µL of isoniazid derivatives were added to 100µL of methanol to form concentrations of 100 – 0.781µg/mL. The experiment was conducted in triplicates and the radical scavenging activity (RSA) of each extract, derivative and control was evaluated using the equation below (Kumara *et al.*, 2018):

$$\boldsymbol{RSA = \left(1 - {}^{A_E}/_{A_D}\right) x\, 100}$$

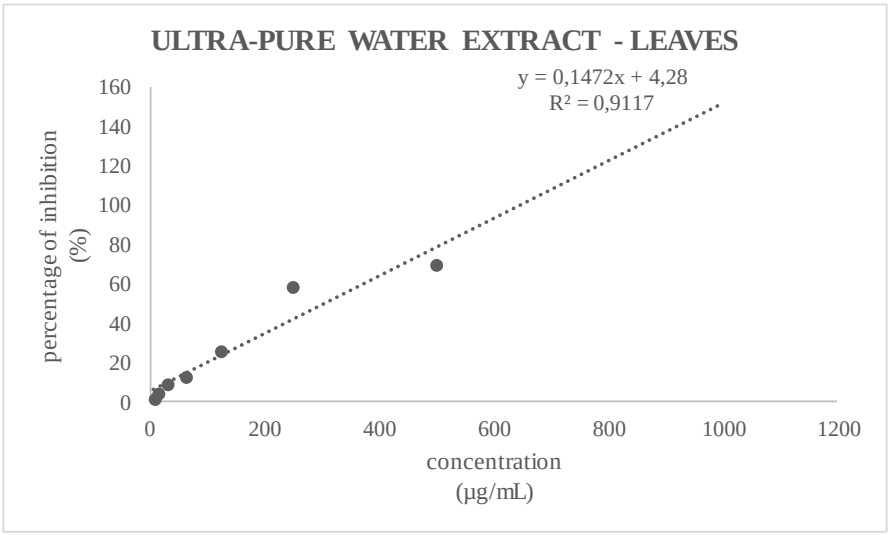
A_E = absorbance of plant extracts/ derivatives/ ascorbic acid mixture minus their colour.
A_D = absorbance of DPPH without extract/ derivatives/ ascorbic acid.

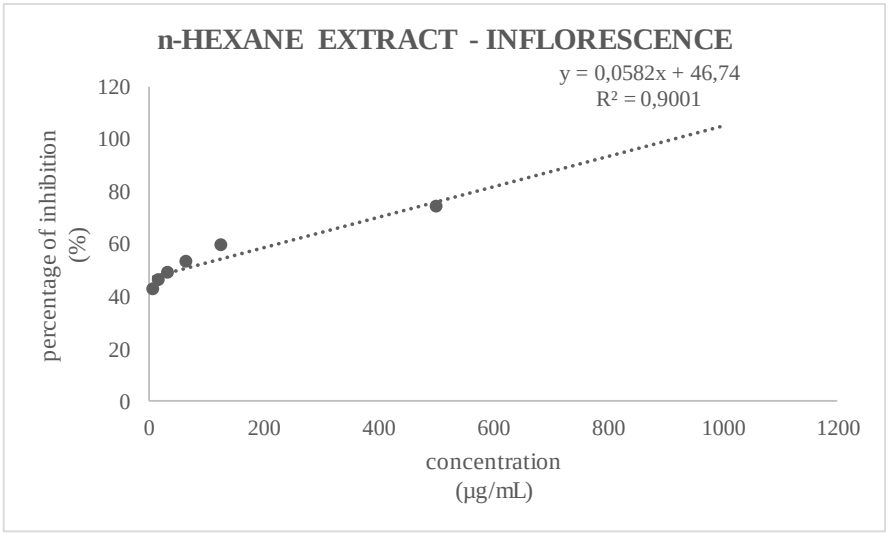
The inhibitory concentration at 50% (IC_{50}), was determined using the radical scavenging assay percentages. A line graph was generated using the percentages and using the line equation y = mx + c and the R^2 value was then used to adjust the line of best fit as seen in (**Graph 4.1.1**).

4.4 Results

4.4.1 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging assay

DPPH free radical scavenging assay is designed to measure the antioxidizing potential of plants and compounds, (Rahman *et al*., 2015). The DPPH free radical solution is a dark violet colour, it gives off a pale-yellow or becomes colourless when neutralized into DPPH-H (**Figure 4.4.1a and 4.4.1b**), this indicates that the tested extract or compound has hydrogen acceptor abilities towards antioxidants (Lalhminghlui and Jagetia, 2018).





Graph 4.4.1: DPPH assay regression graphs for determing the inhibitory concentration at 50% (IC_{50}) **a.** ultrapure water leaves and **b.** n-hexane inflorescence *Cannabis indica/sativa* extracts. (Source: graphs plotted using MicroSoft Excel)

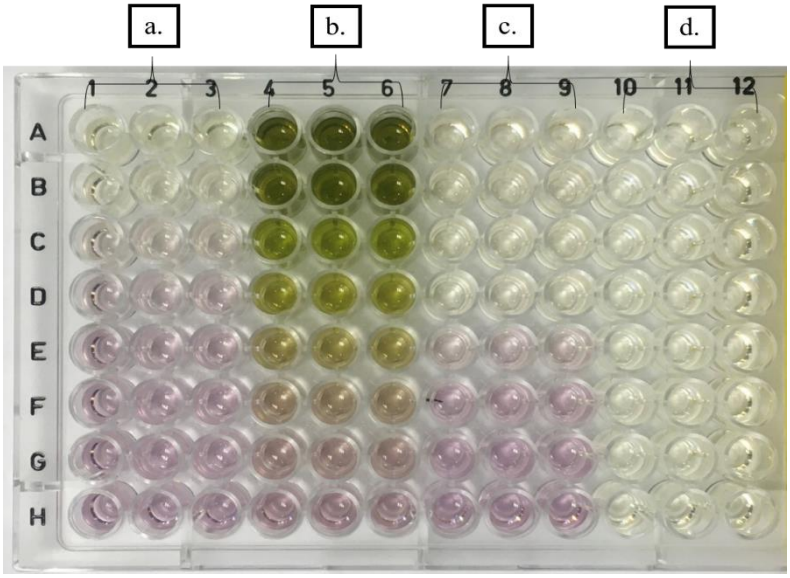


Figure 4.4.1a: DPPH assay results of a. n-hexane **b.** ethyl acetate **c.** ultra-pure water *Cannabis indica/sativa* mature inflorescence extracts and **d.** ascorbic acid.
(Source: Pictures were taken during the process of investigation)

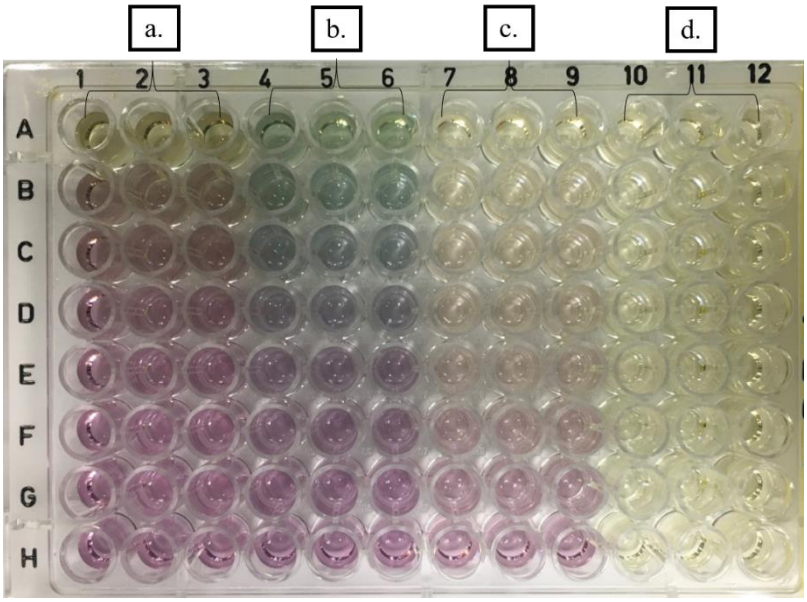


Figure 4.4.1b: DPPH assay of **a**. *N'*-(*bis*(2-hydroxyphenyl)methylene)-isonicotinohydrazide (IBS007) **b**. *N'*-((4-aminophenyl)(phenyl)methylene)-isonicotinohydrazide (IBS 009) **c**. *N'*-((4-aminophenyl)(phenyl)methylene)-isonicotinohydrazide·(salicylic acid) (IB0S10) isoniazid derivatives and **d**. ascorbic acid.

(Source: Pictures were taken during the process of investigation)

The radical scavenging activity of various *Cannabis* extracts were evaluated and compared with the positive control ascorbic acid which had an IC_{50} of 3.71µg/mL. The inhibitory concentration at 50% (IC_{50}) of a compound or plant extracts refers to its ability to scavenge or reduce 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical as compared to the control. Based on the IC_{50} (**Table 4.4.1a**), the *Cannabis* leaf ultra-pure extracts showed the best activity amongst the other tested extracts with a value of 310.60µg/mL, while the ethyl acetate extract showed the least DPPH scavenging activity with a value of 918.28µg/mL. Extracts of mature inflorescence and leaves of *Cannabis* were evaluated for antioxidant activity (**Table 4.4.1b**), of the three extracts the ethyl acetate extract had the best activity with an inhibitory concentration of 34.8µg/mL at 50% (IC_{50}) followed by the *n*-hexane extract at 56.0µg/mL and then the ultrapure extract at 77.7µg/mL, respectively.

Eight modified isoniazid derivatives were evaluated for antioxidant activity. Based on the inhibitory concentration (**Table 4.4.1c**) of the eight drugs, the co-crystal *N'*-((4-aminophenyl)(phenyl)methylene)-isonicotinohydrazide·(salicylic acid) (IBS 010) (Smith *et al.,* 2015) showed the best activity with an IC_{50} of 105.4µg/mL followed by *N'*-((butan-2-ylidene)-isonicotinohydrazide · 0.5 H_2O (IBS 017) (Setshedi *et al.,* 2021) with a concentration of 180.7µg/mL, meaning that this drug is the most potent to scavenge free radicals. The highest inhibitory concentration was found at compound *N'*-(*bis*(2-hydroxyphenyl)methylene)-isonicotinohydrazide (IBS 007) (Smith *et al.,* 2015), this compound showed antioxidant activity at 2 669.8µg/mL and is considered to be the weakest drug of all those which were tested, followed by the co-crystal *N'*-(*bis*(2-hydroxyphenyl)methylene)-isonicotinohydrazide·(salicylic acid) (IBS 008) (Smith *et al.,* 2015) with a concentration of 2333.6µg/mL.

Table 4.4.1a: DPPH assay IC_{50} values for *Cannabis indica/sativa* vegetative leaves extracts and ascorbic acid.

Extracts	**IC_{50} value (µg/mL)**
n-Hexane	356.8 ± 11.5
Ethyl acetate	918.3 ± 20.5
Ultra-pure water	310.6 ± 5.0
Ascorbic acid	3.71 ± 1.4

Table 4.4.1b: DPPH assay IC_{50} values for *Cannabis indica/sativa* mature inflorescence extracts and ascorbic acid.

Extracts	**IC_{50} value (µg/mL)**
n-Hexane	56.0 ± 2.8
Ethyl acetate	34.8 ± 12.3
Ultra-pure water	77.7 ± 1.6
Ascorbic acid	3.71 ± 1.4

Table 4.4.1c: DPPH assay IC_{50} values for modified isoniazid derivatives and ascorbic acid.

Extracts	**IC_{50} value (µg/mL)**
N'-(bis(2-hydroxyphenyl)methylene)isonicotinohydrazide (IBS 007)	2 669.8 ± 1.1
N'-(bis(2-hydroxyphenyl)methylene)isonicotinohydrazide ·(salicylic acid) (IBS 008)	2 333.6 ± 1.3
N'-((4-aminophenyl)phenyl) methylene)isonicotinohydrazide (IBS 009)	275.9 ± 1.0
N'-((4-aminophenyl)phenyl) methylene)isonicotinohydrazide·(salicylic acid) (IBS 010)	105.4 ± 1.1
N'-(propan-2-ylidene)isonicotinohydrazide (IBS 013)	244.3 ± 1.7
N'-(propan-2-ylidene)isonicotinohydrazide· (salicylic acid) (IBS 014)	859.6 ± 1.7
N'-(Butan-2-ylidene)isonicotinohydrazide (IBS 017)	180.7 ± 1.1
N'-(Butan-2-ylidene)isonicotinohydrazide· (salicylic acid) (IBS 018)	672.3 ± 1.1
Ascorbic acid	3.71 ± 1.4

4.5 Discussion

The DPPH free radical scavenging assay was conducted to assess and compare the antioxidant activity of *Cannabis india/sativa* inflorescence and leaf extracts as well as modified isoniazid derivatives. The results of this assay show that the matured *Cannabis* inflorescence parts have

better antioxidant activity as compared to the vegetative leaves. Based on literature and the observed results, it can be concluded that the matured inflorescence extract has more radical scavenging activity and chelate ferrous ions than leaves and seeds of the same plant (Cantele *et al*., 2020). According to a study done by Manosroi *et al*., (2019), both leaves and seeds of *Cannabis* show a higher effective concentration at 50% (EC_{50}), this is the concentration of a drug which induces half of its maximum effect at a stable state; essentially a higher EC_{50} indicates low radical scavenging and ion chelating activity. In a study conducted by Jin *et al*., (2020), evidence was presented that the cannabinoid content in the *Cannabis* plant decreases from the inflorescence to the leaves, roots and stems. Thus, justifying the observed high scavenging activity shown by the inflorescence in this study. According to a study based on the neuroprotective properties of cannabigerols (CBG), tetrahydrocannabinols (THC), cannabidiols (CBD) and other cannabinoids act as powerful antioxidants (Atalay *et al.,* 2019). Cannabidiols are one of the main phytocannabinoid components of hemp, they influence the activity and levels of antioxidants and oxidants through their antioxidant activity (Stasiłowicz *et al*., 2021). There were approximately twenty-six flavonoids found in the *Cannabis* plant after a flavonoid profile was conducted on leaves, stem, inflorescence, roots and bark (Jin *et al*., 2020), *in vitro* experiments have shown that there is antioxidant activity presented by these flavonoids (Alvarez-Suarez *et al*., 2012). Phenolic acids and flavonoids in particular have good health promoting benefits such as anti-cancer, anti-inflammatory, antiviral and antioxidant activity (Pamplona *et al*., 2018; André *et al*., 2020.).

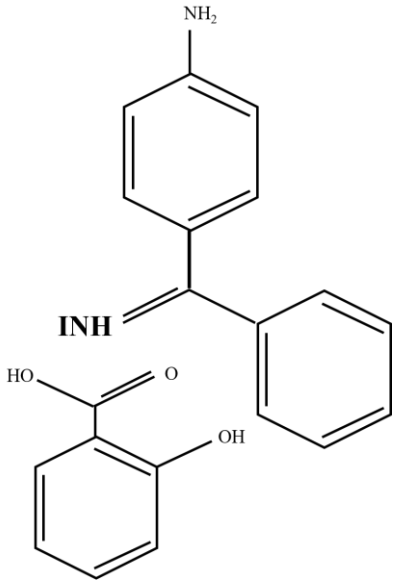
The mechanism of isoniazid to date still remains unclear; however, the assumptions on its labyrinth mechanism is set on its oxidative process. The initial oxidation of isoniazid by the *KatG* enzyme is assumed to produce reactive intermediates which in turn play an integral role in isoniazid's anti-tubercular activity (Rickman *et al*., 2013). According to Rickman *et al*., (2013), studies which have been conducted have detected various isoniazid intermediates, these include nitrogen-, oxygen- and carbon-centred radical intermediates as well as secondary free radical decomposition products which include nitric oxide, superoxide and hydroxyl radicals; these radicals have been detected using an electron paramagnetic resonance (EPR) spectroscopy. Thus, demonstrating some form of antioxidant activity exhibited by isoniazid as a drug.

The modified isoniazid derivatives that have been used in this study have taken various structures with new functional groups attached to the amino functional group (NH_2) on the isoniazid main structure, in addition to form co-crystals the compounds were also reacted with salicylic

acid. These groups which are attached to the isoniazid structure include amino radicals, hydroxides, phenolic groups and carboxylic acids which are attached to the second nitrile. Phenolic groups are compounds which are made up of one or more phenolic aromatic ring with an attached hydroxyl group (Minatel *et al.*, 2016). According to Chen *et al.*, (2020), phenolic acids, are a class derived from the phenolic group which include benzene rings with a carboxylic acid group and contain at least one hydroxyl group. These phenolic acids are considered excellent antioxidants that have the potential to quench free-radicals in the body, the antioxidant ability of these phenolic groups comes from the phenolic hydroxyl group (Chen *et al*., 2020).

Isoniazid derivatives comprising of either one or more phenolic group other than the α-benzene ring are the ones which showed the best activity. A prime example of this is the derivative *N'*-((4-aminophenyl)(phenyl)methylene)-isonicotinohydrazide·(salicylic acid) (IBS010) (**Figure 4.5a**) (Smith *et al.,* 2015) which contains three phenolic groups, one being a phenol aromatic ring, phenolic acid and the other being an aniline – phenolic group with an amine. The other modified derivative containing phenolic groups also exhibited good antioxidant activity was *N'*-(*bis*(2-hydroxyphenyl)methylene)-isonicotinohydrazide (IBS 007) (**Figure 4.5b**) (Smith *et al.,* 2015) exhibited the highest inhibitory concentration. This could be due to the molecule containing two phenolic aromatic rings attached to the main isoniazid compound. It may be assumed that their phenolic group respective positions on the benzene ring could be directly linked with high inhibitory concentration observed during antioxidant testing. This observation can, to a certain extent be justified by the claim made by Chen *et al*., (2020) stating that, the position of phenolic hydroxyls are in direct relation to the phenolic compound's antioxidant ability. In addition to this; Flora, (2009) claims that antioxidant activity is owed to the free hydroxyl group attached to an aromatic ring.

a.



b.

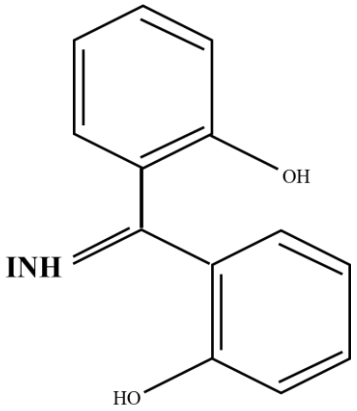


Figure 4.5: Chemical structures of isoniazid with their added functional groups to form **a.** co crystal derivative *N'*-((4-aminophenyl)(phenyl)methylene)-isonicotinohydrazide·(salicylic acid) (IBS 010) and **b.** *N'*-(*bis*(2-hydroxyphenyl)methylene)-isonicotinohydrazide (IBS 007).
(Source: The diagram was created using EdrawMax Software).

4.6 Conclusion

In this study the antioxidant activity of *Cannabis* vegetative leaves, matured inflorescence as well as isoniazid derivatives were examined. The purpose was to evaluate the potential of the extracts and derivatives to scavenge free radicals which are responsible for various diseases in both animals and humans. The matured inflorescence of the *Cannabis* plant had the best activity in comparison to the vegetative leaves and the isoniazid derivatives. Due to understanding that some compounds and functional groups play a vast and important role in antioxidant activity according to literature. An annotated chemical analysis was conducted as seen in Chapter 9, compounds such as Δ^9-tetrahydrocannabinols, β-myrcene, humulene and cannabidiols were identified and are known to have potent antioxidant activity. This will aid in supporting the literature that some compounds have greater scavenging activity than others.

We also have a greater understanding of the modified isoniazid that the newly formed functional groups which make up the individual derivatives have their own potential characteristics which may play a distinctive role in not only tuberculosis but in the repurposing of the drug for other infections. In summary, both *Cannabis indica/sativa* extracts and isoniazid derivatives exhibit antioxidant activity which imparts confidence that they would make respectable drugs

for various infections.

4.7 References

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Chapter 5

Evaluation of the cytotoxic effects of *Cannabis indica/sativa* extracts and isoniazid derivatives on RAW264.7 and Vero cell lines

5.1 Introduction

Cell-based assays are used to screen a collection of drugs in order to test the effects of cell proliferation and exhibit cytotoxicity inevitably leading to cell death (Riss *et al*., 2013). Cell-based assays are also widely used for a variety of signal transduction events involved in genetic reporter expression, organelle function monitoring, the measuring of receptor binding the expression of genetic reporters, trafficking of cellular components, or monitoring organelle function (Nierode *et al*., 2016). It is important to know the amount viable cells that remain during and at the end of an experiment regardless of which assay is being performed. A variety of assays can be used to determine the number of eukaryotic cells which are still viable (Riss *et al*., 2013).

This chapter will provide an overview of the major methods used in multi-well formats where data is recorded using a multi-plate reader. The methods described include: tetrazolium reduction and real-time cytometry assay. Cytotoxicity is defined as the ability of a therapeutic agent to be toxic to living cells (Mukherjee, 2019). Cytotoxicity assays are fast, inexpensive and easy methods used to assess the effects of a compound on mammalian cells (Tonder *et al.*, 2015). MTT assay or 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide reduction assay is a colorimetric technique used to measure metabolic activity within the cell and serves as an indicator for cytotoxicity, cell proliferation and viability. This colorimetric assay is based on the reduction of tetrazolium salts from yellow to purple by metabolically active cells (Riss *et al*., 2013). Cells with an active metabolism are considered viable cells, they will be able to convert the MTT into a purple coloured formazan product when measured at an absorbance of 570nm (Riss *et al*., 2013). Real-time cell analysis uses the xCELLigence system, this system is suitable for the detection of cell morphological changes induced by drugs, these include growth and adhesion (Bernardo *et al*., 2021).

The present study was undertaken to evaluate the cytotoxic effects of *Cannabis* extracts and modified isoniazid derivatives. Furthermore, to investigate the rate of cell proliferation caused by these drugs at lethal concentrations.

5.2 Sample collection and preparation

As described in Chapter 3, the preparation of the *Cannabis indica/sativa* plant extracts is described in section **3.2 Preparation of extracts** and isoniazid derivatives in section **3.3 Synthesis of derivatives** and **3.4 Preparation of derivatives**. The cell culture methods of both Vero and RAW264.7 cells are described in section **3.5 Cell culture**.

5.3 MTT cytotoxicity assay

The reduction of MTT (3- [4,5-dimethylhiazol-2-yl]-2,5-diphenyl tetrazolium bromide) during cell proliferation includes the conversion of MTT into formazan crystals by mitochondrial succinate dehydrogenase enzyme activity of living cells; this, controls the measurement of viable cells and determination of their viability. The cytotoxicity of each extract and isoniazid derivative were determined using the MTT colorimetric assay as described by More and Makola, (2020). The experiment was conducted in a 96-well microtiter plate by seeding each cell line at 100µL in a monolayer. Vero cells and Raw 264.7 cells were seeded at 1.124 $x10^5$ cells/well (**Figure 5.3a**) and 3.308 $x10^5$ cells/well (**Figure 5.3b**) (Sceptor™ 3.0, Handheld Automated Cell Counter, Millipore, Germany), respectively.

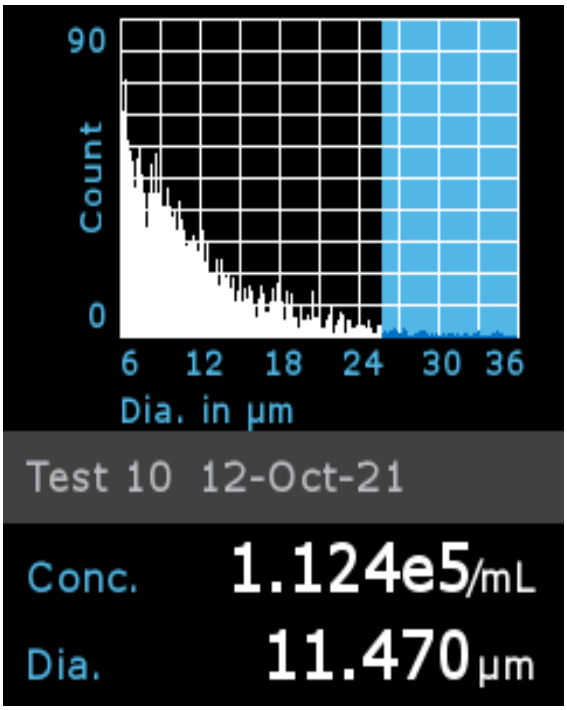

Figure 5.3a: Vero cell count, concentration and diameter from unknown passage number using a Sceptor™ 3.0, handheld Automated Cell Counter, (Millipore, Germany).

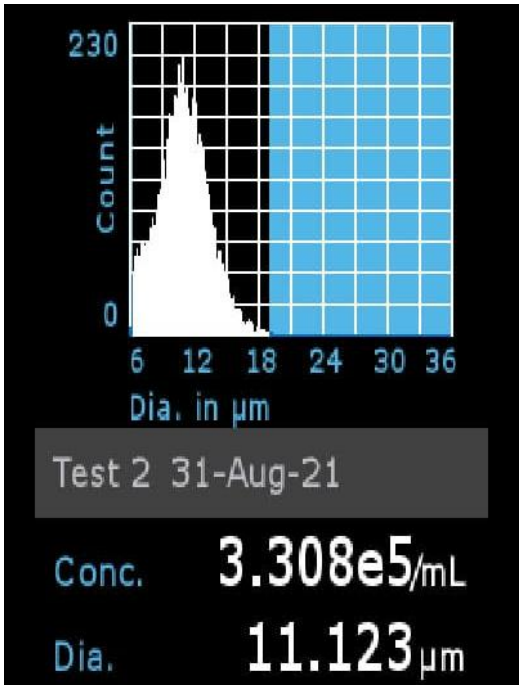

Figure 5.3b: RAW267.4 cell count, concentration and diameter from passage number 9 using a Sceptor^{TM} 3.0, handheld Automated Cell Counter, (Millipore, Germany).

After seeding, the cells were left to incubate at 37°C for 24 hours under 5% CO_2 to enhance attachment. Thereafter the media was aspirated from the wells of the 96-well plate and replaced with 80µL of extract and 120µL media or 40µL derivatives and 160µL media, which were serially diluted in another plate to obtain various concentrations and then added to the 96-well plate that contained the confluent cells. The plates were then further incubated at 37°C in a 5% CO_2 chamber for 48 hours. After incubation, the cells were then treated with 50µL MTT solution (Sigma-Aldrich, Germany) and incubated for 3 hours, 100µL of DMSO (dimethyl sulphoxide) (Promark Chemicals, South Africa) was then added and incubated for a further hour to dissolve any formazan crystals which had formed. The absorbance of the solution was then measured using a UV-Spectrophotometer (Thermo Scientific, Varioskan® Flash UV-spectrophotometer, Finland) at 570nm and a reference wavelength of 630nm. The untreated cells were used as a negative control.

In this experiment, 100µL of amphotericin B (AMB), isoniazid (INH) and rifampicin (RMP) were used as positive controls, respectively. The percentage of the cell viability was then calculated using the formula (More and Makola, 2020):

$$\text{cell viability (\%)} = {}^{AT}/_{AC} \times 100$$

AT: absorbance of extract or derivative treated cells.

AC: absorbance of untreated cells.

5.4 Real-time cell analysis (RTCA) of cytotoxicity

The assessment of the real-time effects of the plant extracts and compounds on macrophages (RAW 264.7) and Vero cells was done in accordance to the method described by More and Makola, (2020) with modifications. The cells were monitored by utilizing the RTCA-DP system (xCELLigence, ACEA Biosciences, Roche Applied Science, United States of America) (xCELLigence, ACEA Biosciences, Roche Applied Science Software, version 2.0.0.1301). The cells were grown and maintained in cell culture flasks until they were between 80-90% confluent, they were then seeded at 3.3 x 10^5 cells/mL for RAW 264.7 and 1.1 x 10^5 cells/mL for Vero cells into E-plates and incubated for a further 24-hours prior to treatment. The cells were treated with the extracts and compounds using the lethal concentration at 50% (LC_{50}), the positive controls used in this experiment were amphotericin B (AMB), isoniazid (INH) and rifampicin (RMP) which were also tested at LC_{50}, untreated cells were considered the negative controls. This experiment was done in duplicates and ran for 24-hours.

5.5 Results

5.5.1 Vero cells

Six *Cannabis indicia/sativa* extracts (**Table 5.5.1a** and **5.51b**) and eight modified isoniazid derivatives (**Table 5.5.1c**) were tested for their cytotoxic effects against African Green Monkey kidney cells with three positive controls amphotericin B (AMB), isoniazid (INH) and rifampicin (RMP) (**Table 5.5.1d**). The concentrations evaluated ranged from 0.98 – 2 000µg/mL for the extracts and 0.098 - 200µg/mL for the modified isoniazid derivatives and positive controls on Vero cells using the MTT reduction assay. Cell toxicity is expressed as lethal concentrations at 50% (LC_{50}), this means that a given concentration of the extract or derivatives is lethal to 50% of the cells (Zhang *et al*., 2007). After evaluation it was determined that the *Cannabis* vegetative leaf n-hexane extract showed lowest toxicity in comparison to all of the tested agents, being lethal to 50% of the cells at 2 404.6µg/mL, this was followed by the mature inflorescence ultra-pure water extract at 1 445.81µg/mL and vegetative leaf extract ultra-pure extract at 1 428.73µg/mL.

Although none of the derivatives showed toxicity, the most positive performing derivative tested *N*'-((4-aminophenyl) phenyl) methylene) isonicotinohydrazide (IBS 009) with an (LC50) of 300.27µg/mL. This was followed by *N*'-(Butan-2-ylidene) isonicotinohydrazide (IBS 017) 260.42µg/mL and *N*'-((4-aminophenyl) phenyl) methylene) isonicotinohydrazide· (salicylic acid) (IBS 010) 205.87µg/mL, respectively. The most toxic extract between the six tested was mature inflorescence ethyl acetate extract 568.51µg/mL and the most toxic derivative in comparison to those tested was the co-crystal *N*'-(bis(2-hydroxyphenyl) methylene) isonicotinohydrazide· (salicylic acid) (IBS 008) with an LC_{50} of 86.85µg/mL. Of the three positive controls tested, rifampicin (RMP) exhibited the lowest toxicity with a lethal concentration of 549.91µg/mL followed by isoniazid (INH) at 476.22µg/mL and amphotericin B (AMB), being the most toxic at 243.98µg/mL.

Table 5.5.1a: MTT assay LC_{50} values for *Cannabis indica/sativa* vegetative leaves extracts.

Extracts	**LC_{50} value (µg/mL)**
n-Hexane	2 404.6 ± 25.3
Ethyl acetate	600.48 ± 10.7
Ultra-pure water	1 428.73 ± 2.3

Table 5.5.1b: MTT assay LC_{50} values for *Cannabis indica/sativa* mature inflorescence extracts.

Extracts	**LC_{50} value (µg/mL)**
n-Hexane	1 119.75 ± 25.6
Ethyl acetate	568.51 ± 10.5
Ultra-pure water	1 445.81 ± 34.6

Table 5.5.1c: MTT assay LC_{50} values for modified isoniazid derivatives.

Compounds	**LC_{50} value (µg/mL)**
N'-(bis(2-hydroxyphenyl)methylene)isonicotinohydrazide (IBS 007)	184.82 ± 22.31
N'-(bis(2-hydroxyphenyl)methylene)isonicotinohydrazide· (salicylic acid) (IBS 008)	86.85 ± 19.64
N'-((4-aminophenyl)phenyl) methylene)isonicotinohydrazide (IBS 009)	300.27 ± 18.03

N'-((4-aminophenyl)phenyl) methylene)isonicotinohydrazide· (salicylic acid) (IBS 010)	205.87 ± 28.44
N'-(propan-2-ylidene)isonicotinohydrazide (IBS 013)	128.70 ± 14.70
N'-(propan-2-ylidene)isonicotinohydrazide· (salicylic acid) (IBS 014)	145.58 ± 27.23
N'-(Butan-2-ylidene)isonicotinohydrazide (IBS 017)	260.42 ± 18.80
N'-(Butan-2-ylidene)isonicotinohydrazide· (salicylic acid) (IBS 018)	194.06 ± 35.35

Table 5.5.1d: MTT assay LC_{50} values for positive controls.

Drug	**LC_{50} value (µg/mL)**
Amphotericin B (AMB)	243.98 ± 4.69
Isoniazid (INH)	476.22 ± 38.81
Rifampicin (RMP)	549.91 ± 39.45

5.5.2. RAW 264.7 cells

Figure 5.5.2 shows the MTT colorimetric assay conversion of yellow tetrazolium salts to purple salts, cells which are still metabolically active will change colour. The vegetative leaves (**Table 5.5.2a**) and mature inflorescence (**Table 5.5.2b**) extracts were tested for their lethal concentrations at 50%, as well as three positive controls namely: amphotericin B, isoniazid and rifampicin (**Table 5.5.2c**). For the *Cannabis indica/sativa* vegetative leaves, the ethyl acetate extract exhibited the lowest toxicity 805.78µg/mL in comparison to the other two extracts, however the ethyl acetate inflorescence extract displayed the best cytotoxic activity, being the least toxic in comparison to all the tested extracts at 1 073.16µg/mL. The most toxic reference drug was amphotericin B 23.05µg/mL at followed by rifampicin 98.40µg/mL at and the least toxic being isoniazid at 186.97µg/mL.

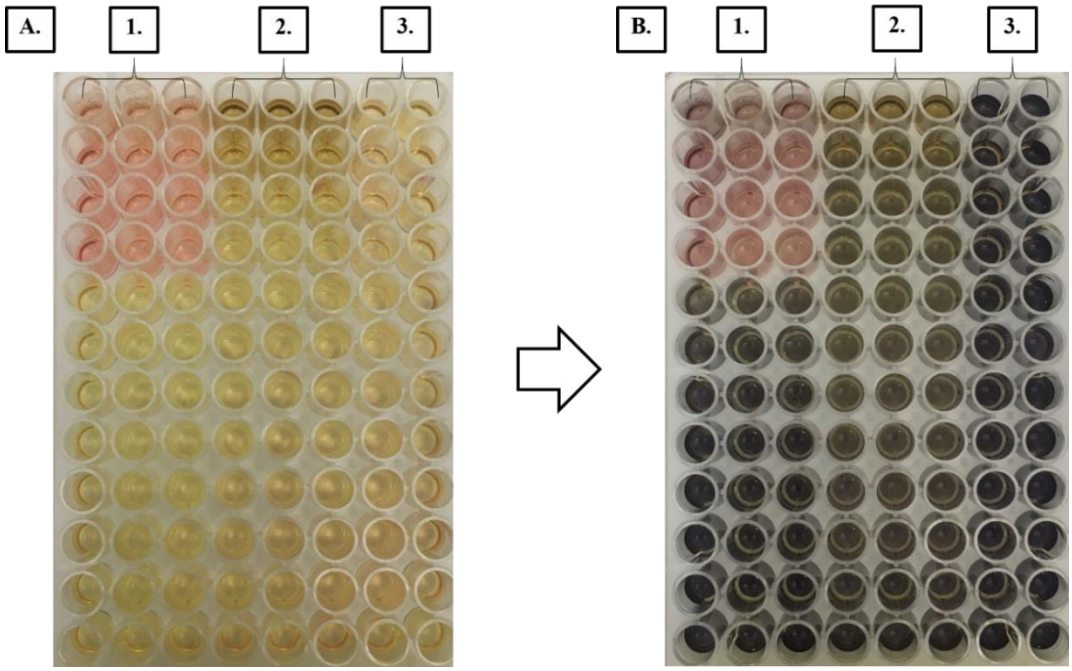

Figure 5.5.2: RAW267.4 cytotoxicity assay for *Cannabis indica/sativa*

1. mature inflorescence and **2.** vegetative leaves ethyl acetate extracts and **3.** blank wells.

a. before MTT staining and incubation **b.** after MTT staining and 3-hour incubation.

(Source: Pictures were taken during the process of investigation).

Table 5.5.2a: MTT assay LC_{50} values for *Cannabis indica/sativa* vegetative leaves extracts.

Extracts	**LC_{50} value (µg/mL)**
n-Hexane	173.32 ± 37.1
Ethyl acetate	805.78 ± 5.7
Ultra-pure water	599.61 ± 10.9

Table 5.5.2b: MTT assay LC_{50} values for *Cannabis indica/sativa* mature inflorescence extracts.

Extracts	**LC_{50} value (µg/mL)**
n-Hexane	783.54 ± 18.6
Ethyl acetate	1 073.16 ± 10.6
Ultra-pure water	562.23 ± 34.2

Table 5.5.2c: MTT assay LC_{50} values for positive controls.

Drug	**LC_{50} value (µg/mL)**
Amphotericin B (AMB)	23.05 ± 10.8
Isoniazid (INH)	186.97 ± 14.0
Rifampicin (RMP)	98.40 ± 17.6

5.5.3 Real-time cell analysis (RTCA) of cytotoxicity

xCELLigence – real time cell analysis (RTCA) using (xCELLigence, ACEA Biosciences, Roche Applied Science) is a system that uses biosensors to monitor the real-time events of cellular viability, adhesion, motility, numerical change and morphology (More and Makola, 2020). Each *Cannabis indica/sativa* extract and modified isoniazid derivative was tested using the LC_{50} obtained from the MTT reduction assay. The measurement of cell adhesion to the respective individual 8 well E-plate well is referred to as the cell index.

The Vero cells were allowed to settle and adhere to the bottom of the plate enabling the bio-sensors to read the cell index coefficient for the first 4-hours, pre-treatment. The biosensors detected that some wells showed significant cell growth over the 4-hour adhesion period with the cell index steadily over 0.00 (**Figure 5.5.3a**). As illustrated on **Figure 5.5.3b** between the treatment point and an hour post treatment, both n-hexane extracts (leaves 2 404.6µg/mL and inflorescence 1 119.75µg/mL), both ultra-pure water extracts (leaves 1 428.73µg/mL and inflorescence 1 445.81µg/mL) as well as the derivative IBS 014 (145.58µg/mL) showed a sharp increase in cell index. The increase in adhesion is attributed to a disturbance of the cells after the respective agent has been added, once this time frame has passed and the cells have settled a pattern of plateau formed. The derivative IBS 014 (145.58µg/mL) showed a steady decline after two hours of treatment where the cell index decreased to 0.00 until the 19-hour mark. From the beginning of the experiment, there was no significant cell growth in most of the wells, at the 14-hour mark post treatment IBS 010 (205.87µg/mL) displayed a negative cell index from the index being steady. Eighteen hours post treatment IBS 008 (86.85µg/mL) showed a negative cell index < -0.10.

At the 30-hour mark, most of the extracts showed positive growth curve spurts on the RAW264.7 cells displaying an increase in cell index >0.40. The most notable of these are the n-hexane (173.32µg/mL) and ethyl acetate (805.78µg/mL) vegetative leaves extracts (**Figure**

5.5.3c) which showed an increased cell index (CI) number from the 30-hour mark which marks 5 hours after treatment in comparison to the untreated cells. The pattern then continues for 30 more hours (at the 35-hour mark) where the growth then showed a steady decline until the experiment was aborted. The mature inflorescence ethyl acetate extract (805.78µg/mL) showed a growth pattern similar to the untreated cells, however the cell index coefficient began to decline after the 45-hour mark. The n-hexane (783.54µg/mL) inflorescence extracts, showed a decrease in the cell index coefficient over a period of 5 hours which showed a steady index to the end of the experiment.

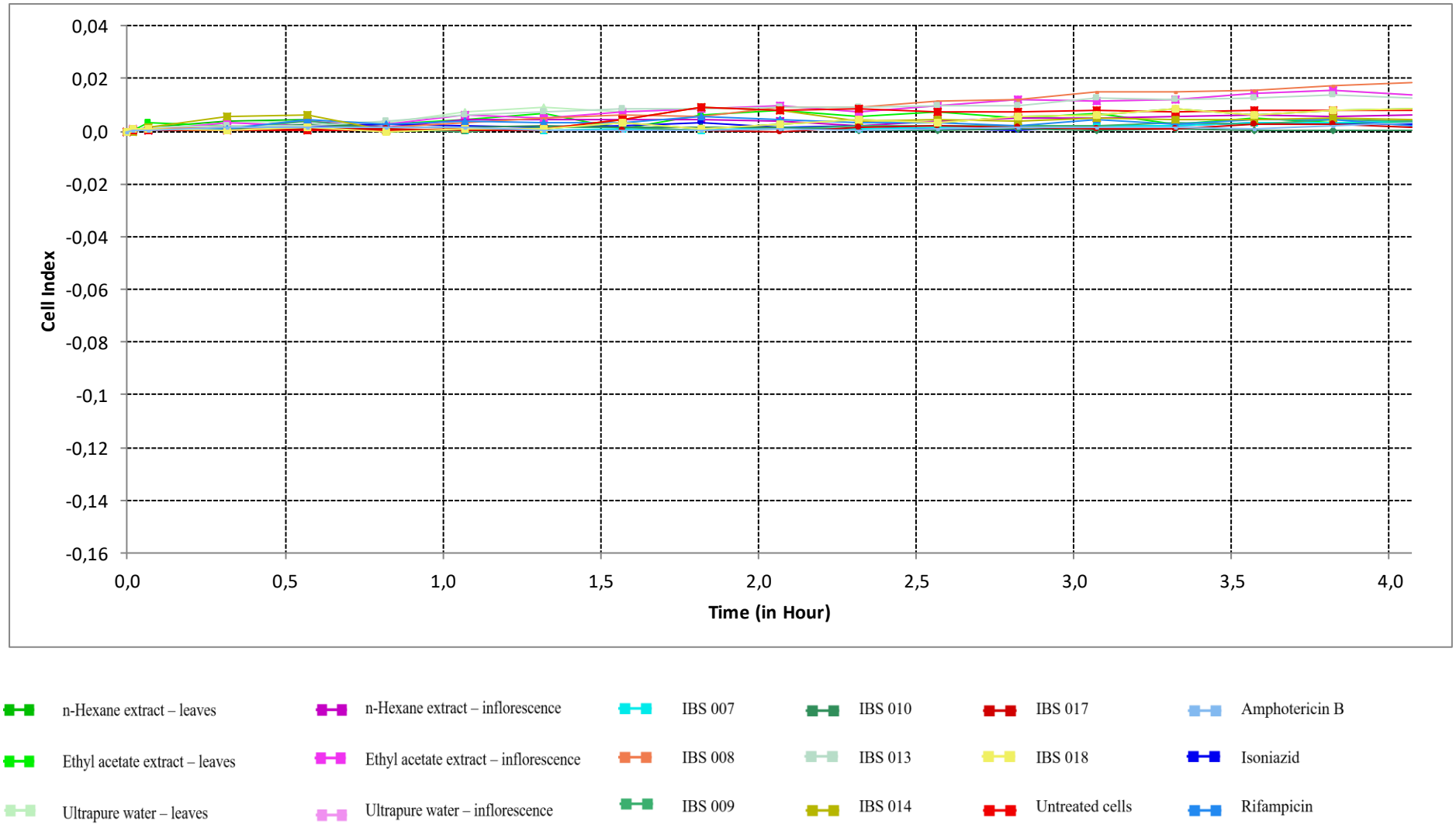

Figure 5.5.3a: Real-time monitoring of the effects of *Cannabis indica/sativa* vegetative leaves, mature inflorescence extracts and modified isoniazid derivatives at their respective LC_{50} on the cell growth and viability of Vero cells exposure for 22 hours.

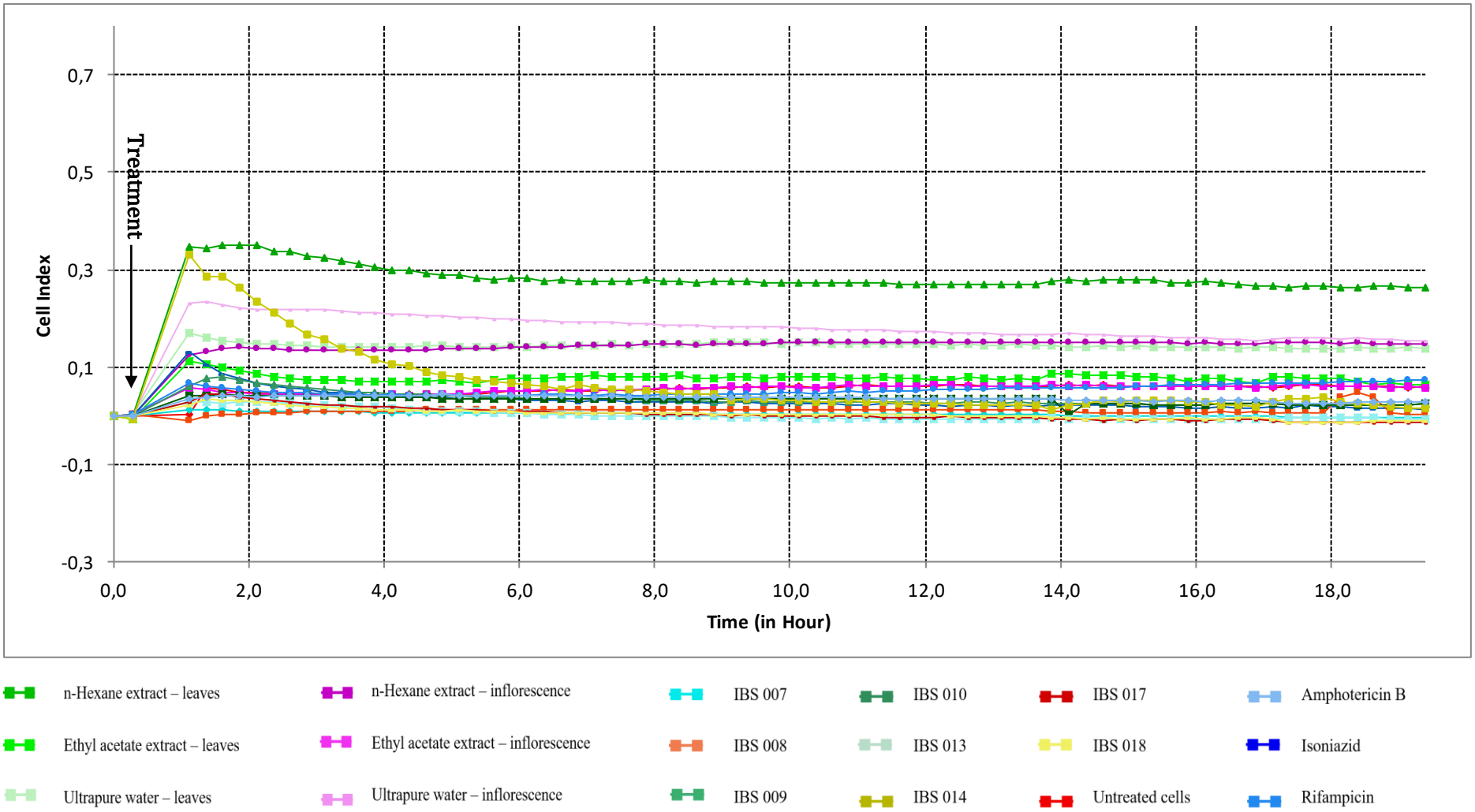

Figure 5.5.3b: Real-time monitoring of the effects of *Cannabis indica/sativa* vegetative leaves and mature inflorescence extracts at their respective LC_{50} on the cell growth and viability of Vero cells exposure for 22 hours.

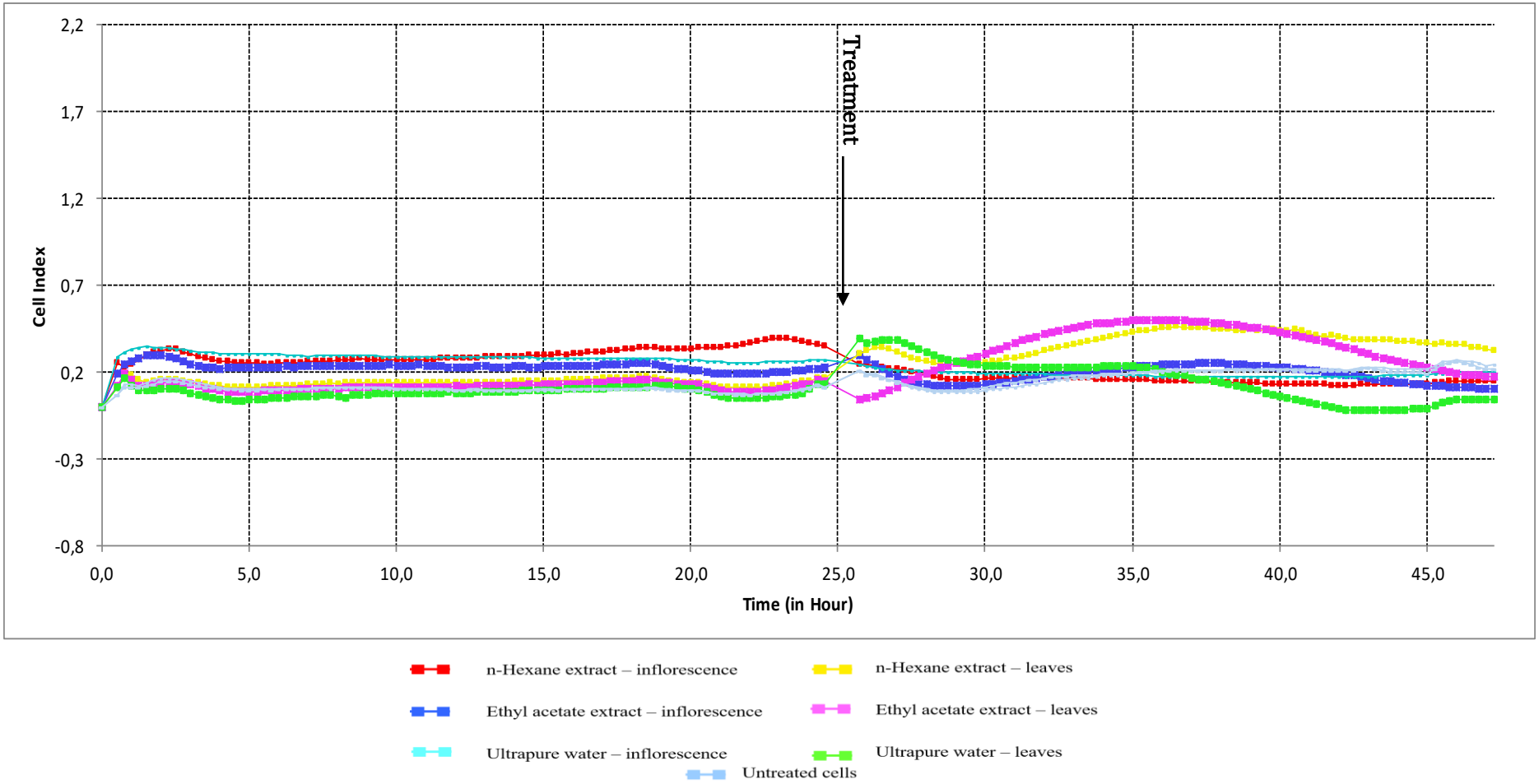

Figure 5.5.3c: Real-time monitoring of the effects of *Cannabis indica/sativa* vegetative leaves and mature inflorescence extracts at their respective LC_{50} on the cell growth and viability of macrophages (RAW 264.7) exposure for 48 hours.

5.6 Discussion

In this study two *Cannabis indica/sativa* extracts and isoniazid derivatives were evaluated for their cytotoxic effects against Vero and RAW264.7 cells. Cytotoxic drugs are described as pharmaceutical drugs which are toxic to cells, preventing growth and replication. It is of great importance that treatment agents used for communicable diseases are non-toxic as they are usually administered over long periods of time (Clumeck and de Wit, 2010). The reason for the evaluation of kidney cells is to determine if the extracts or compounds would be toxic during the excretory process as this forms part of the functioning of the kidneys; these endocrine organs play a major role in excretion. Vero cells derived from the kidney of an adult African green monkey are mammalian cells used for cell biology and microbiological studies (Ammerman *et al*., 2008). Macrophages are specialized, innate immune cells that play a significant role in the immune response by detecting, exhibiting phagocytosis and destroying of pathogens (Viola *et al*., 2019). RAW 264.7 cells monocyte/macrophage-like cells which originate from Abelson leukaemia virus transformed cells from BALB/c mice (Taciak *et al*., 2018).

From the results it can be deduced that *Cannabis indica/sativa* extracts have a higher lethal concentration at 50% (LC_{50}) in comparison to the isoniazid derivatives. Analysis of the cell viability assay state that the higher the lethal concentration the better as a high concentration allows more of the drug agent to be utilized without risking any toxic and/ or poisonous effects, although both the extracts and the drugs do not exhibit any toxic traits in this study on either cell line. All six *Cannabis indica/sativa* extracts which were tested had a lethal concentration >500µg/mL, making each extract less toxic in comparison to other studies which have been conducted on both RAW 264.7 and Vero cell concentrations. Studies which have tested for Vero cell cytotoxicity have exhibited very low lethal concentration, for an example in a study conducted by Vijayarathna and Sasidharan, (2012), an *Elaeis guineensis* methanol extract was evaluated for its effects on Vero cells and a LC_{50} of 22µg/mL was obtained. In a previous study, testing the cytotoxic effects of *Albertisia papuana Becc*. roots against Vero cells, the ethanol extracts of the plant presented an LC_{50} 233µg/mL and ethyl acetate: ethanol (2:3) fractions presented an LC_{50} of 42.5µg/mL (Kristiani *et al*., 2016). The results observed in the *Cannabis indica/sativa* extracts corresponds with previous studies on tests done on RAW264.7 cells. A study done by Joo *et al*., (2014), the crude fractions of *Ulmus pumila* extracts were tested for their cell viability properties against RAW264.7 cells, the LC_{50} values of ethyl acetate fraction was 161µg/mL hot water extract 279.1µg/mL, water fraction 677.5µg/mL and *n*-butanol fraction 645.9µg/mL, respectively. These results correspond with what was obtained in the study

where the LC_{50} values of the *Cannabis* extracts are >170µg/mL, but <1 073µg/mL for the six extracts.

The results exhibited in the MTT reduction assay show that none of the extracts tested had showed any signs of cytotoxicity on both cell lines. This is trend can also be seen in a study conducted by Mooko *et al*., (2021), where bud and flower extracts of *Cannabis sativa* L. which included hexane, water, dichloromethane and methanol were evaluated for their cytotoxic properties and exhibited not toxicity to Vero cells.

In this study the modified isoniazid derivatives displayed non-cytotoxic effects, the LC_{50} of the derivatives against Vero cells was ranged in >80µg/mL and <300µg/mL. During oxidative stress it was shown that isoniazid-protein adduct formation has induced cytoprotective properties on HL-60 cells (human promyelocytic leukemia cells) in a study by Khan *et al*., (2016). Many studies have been conducted on the effects of isoniazid derivatives on mammalian cells, many of which have their apoptotic effects on cancer evaluated. The isoniazid derivative *N*'-(2,3,4-trihydroxybenzylidene) isonicotinohydrazide has been tested for its cytotoxic effects against HCT 116 (human colon cancer) cell line which showed promising cytotoxic effects (Barathan *et al*., 2021). Although the modified isoniazid derivatives and *Cannabis indica/sativa* extracts did not display any toxicity activity, the extracts showed better activity compared to the derivatives and positive controls.

5.7 Conclusion

This study has demonstrated the potential lethal concentrations of *Cannabis* extracts and modified isoniazid derivatives against Vero and RAW264.7 cells. Both extracts and compounds have proved to be less toxic in comparison to the commercial drugs utilized for this study as positive controls. The results suggested that the extracts and the compounds could be used at high concentrations and minimum damage could be seen in kidney cells and the macrophage cells as seen in the *in vitro* study. This could be attributed to the presence of the cannabinoid cannabigerol; this compound was identified in the tentative analysis in Chapter 9. Cannabigerol is known to have antiproliferation properties in T-lymphocytes. The evaluation of the time taken for the cells to come to their demise also serves as a positive response to the low toxic effects of the agents. Further studies would be needed to validate this claim, however from the results obtained in the study, there can be a deduction on a positive response of the cells to the agents tested.

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Chapter 6

Antifungal activity of *Cannabis* and isoniazid derivatives

6.1 Introduction

Candida species are the most common fungal species that cause a wide range of life-threatening invasive and non-life-threatening communicable diseases worldwide (Taei *et al.,* 2019). *Candida albicans* is an opportunistic fungal pathogen that makes up part of the human microbiome, often found as a harmless commensal fungus colonizing certain human parts such as the oral cavity, vagina and the digestive system (Gow and Yadav, 2017). However, *Candida albicans* can cause superficial infections of the skin as well as life threatening infections within bodily systems (Mayer *et al.,* 2013). It has always been recognized as the most common cause of candidiasis; however, recently there has been a shift from the prominence of infections caused by *Candida albicans* to non-*Candida albicans Candida* species (Seyoum *et al.,* 2020). Factors such as the utilization of immunosuppressive drugs, a wide spectrum of antibiotics as well as the use of prophylactic antifungal agents has increased patient's susceptibility to non-*Candida albicans* species induced opportunistic candidiasis (dos Santos Pedroso *et al*., 2019).

The rise in fungal infection incidence has brought the need for next generation drugs as the current available drugs have undesirable side effects or are ineffective against new and re-emerging fungal strains (Weitzman and Summerbell, 1995). The development of non-toxic antifungal drugs for clinical use will greatly benefit patients, particularly those who are immunocompromised (Da *et al.,* 2019). Antifungal drug discovery is becoming increasingly vital particularly the development of drugs which would display low toxicity, possess a broad-spectrum efficacy and possibly have a new mode of action (Da *et al*., 2016).

There are currently four classes of drugs used to treat candidiasis, but the increase in multidrug resistance in *Candida* limits the treatment of the infection (Palande *et al.,* 2015). Several plants have shown effective anti-*Candida* activity observed in secondary plant derived metabolites such as terpenes which have been shown to possess anti-fungal activity (dos Santos Pedroso *et al*., 2019). The repurposing of drugs for antifungal treatment also serves as an alternative intervention strategy, this means that non-fungal drugs could be repositioned as novel antifungal drugs (Kim *et al*., 2019).

The focus of this study was to investigate and compare the potential antifungal efficacy of *Cannabis indica/sativa* extracts and modified isoniazid derivatives towards *Candida spp*.

6.2 Sample collection and preparation

As described in Chapter 3, the preparation of the *Cannabis indica/sativa* plant extracts is described in section **3.2 Preparation of extracts** and isoniazid derivatives in section **3.3 Synthesis of derivatives** and **3.4 Preparation of derivatives**. The *Candida* species culturing method is described in the section **3.6.1 *Candida* spp. cultures**.

6.3 Antifungal activity

6.3.1 Minimum inhibitory concentration testing

The minimum inhibitory concentration of each plant extract and modified derivative was conducted according to dos Santos Pedroso *et al.,* (2019). In each well 120µL of Sabouraud dextrose broth (SDB) (Merck SA Pty (Ltd.) Halfway House) was added, this was followed by 80µL aliquots of plant extracts (5mg/mL), into the first row of the 96-well plate and was two-fold serially diluted into wells containing 100µL of broth. Amphotericin B (Sigma-Aldrich, Israel) reference drug was used as a positive control and was prepared at a concentration of 16mg/mL and was also two-fold serially diluted, untreated cells were used as a negative control. Aliquots of 100µL of the *Candida spp*. namely *Candida auris, Candida glabrata*, *Candida parapsilosis* and *Candida tropicalis,* respectfully were then added to each well of the 96-well plate, the plates were then sealed with parafilm and incubated at 37ºC overnight. Fifty microliters (50µL) of 0.4mg/mL INT (*p*-iodonitrotetrazolium chloride) (Sigma-Aldrich, Austria) was added to the plates and incubated at 37ºC for 24 hours. The plates were then visualized, any well which turned red indicated microbial growth while clear wells indicated microbial growth inhibition. The same method was applied for the modified isoniazid derivatives, 100µL of Sabouraud Dextrose Broth (Merck SA Pty (Ltd.) Halfway House), followed by 100µL of isoniazid derivatives to form a concentration range of 1 000 – 7.81µg/mL atfter serial dilution. This was then added into the first row and then two-fold serially diluted.

6.3.2 Flow cytometry

The determination of cell apoptosis was done according to the method described by Martins *et al*., (2017) with modifications. The minimum inhibitory concentration (MIC) of the various *Candida* species was determined using the minimum inhibitory concentration testing method. Colonies of cells were transfered from the SDA plates into freshly prepared broth, this was

then incubated for 24 hours at 37°C and agitated at 160rpm. During the exponential phase, the *Candida* species were then re-suspended in saline solution and adjusted to 0.5 McFarland standard. The cell-saline solution was incubated for 24 hours at 37°C in SDB with MIC of the extracts and isoniazid derivatives obtained from the minimum inhibitory testing assay. Cells which were treated with 50% ethanol were used as a positive control to determine the degree of cell apoptosis. The cells were then collected by centrifugation, which were re-suspended in 1mL of saline solution and stained in the dark with 10µL (Propidium Iodide) PI/mL then incubated for 30 minutes.

The stained cells were evaluated using the BD FACSAria™ III Cell Sorter (BD Bioscience, San Jose, USA). Standard model with a 70-micron nozzle, side-scatter height (SSC-H) filter for cell surface markers to determine cell death (FL1: 445/15nm and FL2: 488/10nm; no mirrors) and 488nm Argon Laser. The *Candida* cells were analysed by determining the percentage of stained cells using BD FACSDiva Software (Version 8.0.1, BD Bioscience, San Jose, USA).

6.4 Results

6.4.1 Minimum inhibitory concentration testing

Four *Candida* species namely *Candida auris, Candida glabrata Candida parapsilosis* and *Candida tropicalis* were tested against the vegetative leaf extracts of *Cannabis indica/sativa*; the extracts were tested at concentrations 15.626 – 2 000µg/mL (**Table 6.4.1a**). The ethyl acetate extract solvent showed the best activity against all *Candida* species, it inhibited *Candida parapsilosis* at 125µg/mL. Ultra-pure water extract showed the best inhibitory activity against *Candida auris* at 62.5µg/mL. All three extracts showed activity however the lowest activity of the extracts was seen in *Candida tropicalis* which exhibited an inhibitory activity at the concentration 1 000µg/mL for all the extracts which were tested. The results of the minimum inhibitory concentration of *Candida* species which were tested against *Cannabis* mature inflorescence extracts are shown in (**Table 6.4.1b**) from 15.626 – 2 000µg/mL. Of those, the inflorescence water extract showed no activity at the highest tested concentration of 2 000µg/mL. The inflorescence ethyl acetate extract had the best activity of 250µg/mL in comparison to the other extracts against both *Candida glabrata* and *Candida parapsilosis*. The n-hexane extract also had an active concentration of 250µg/mL against *Candida glabrata*, this concentration of 250µg/mL can be measured up to that of the positive control used in this study amphotericin B (AMB) which also had the same active concentration against all the fungal species which were tested.

Eight modified isoniazid derivatives were evaluated against five *Candida* species (**Table 6.4.1c**). The modified isoniazid derivatives were tested at concentrations from 3.90625, 7.8125, 15.625, 31.25, 62.5, 125, 250 and 500µg/mL against *C. auris, C. glabrata, C. parapsilosis* and C. *tropicalis* (**Table 6.4.1c**) with Amphotericin B as a positive control. Of all the modified derivatives IBS 008 showed the best activity against all the *Candida* species with the lowest minimum inhibitory concentration being 31.25µg/mL and the highest inhibiting concentration being 500µg/mL, respectively. The poorest performing modified derivative was IBS 010 with the lowest inhibiting concentration being 250µg/mL and the highest concentration which is able to inhibit being >500µg/mL. All of the modified derivatives had unsurpassable activity against *Candida auris* with the lowest concentration needed to inhibit the microbe being 62.5µg/mL from IBS 008, IBS 013 and IBS 014, respectively and the highest concentration needed was > 500 µg/mL which was seen in IBS 010. Isoniazid and rifampicin were used as reference repurposing drugs; rifampicin gave the best MIC value of 0.49µg/mL against *Candida tropicalis.* Although the modified isoniazid seemed to give positive results isoniazid itself had an MIC of >500µg/mL for each of the five microbes. Amphotericin B (AMB) which was used as the positive control gave a minimum inhibitory concentration value of 250µg/mL.

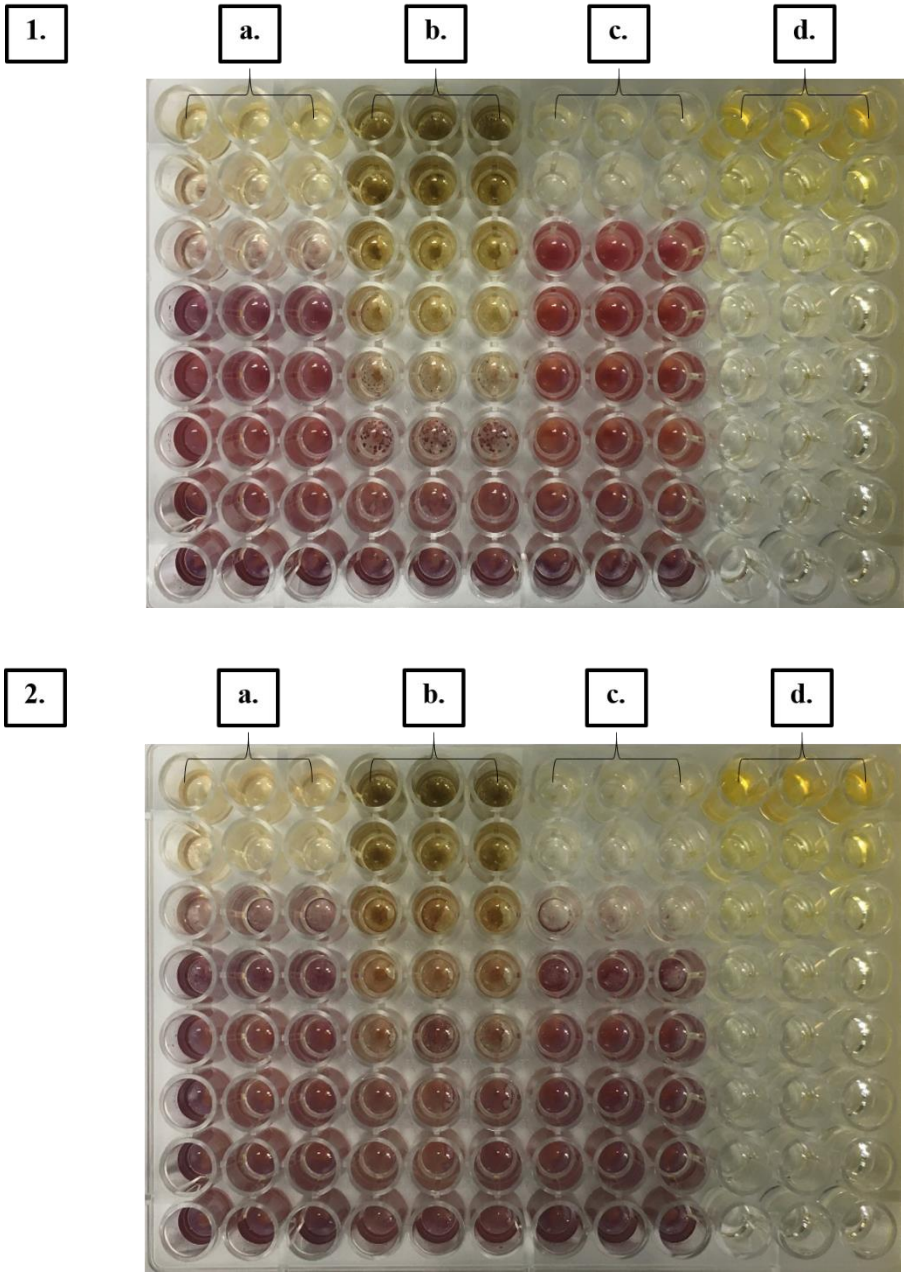

Figure 6.4.1a: Minimum inhibitory concentration testing of *Cannabis indica/sativa* vegetative leaves against **1.** *Candida parapsilosis* and **2.** *Candida glabrata* using extracts **a**. n-hexane **b**. ethyl acetate **c.** ultra-pure water and positive control **d.** amphotericin B.
(Source: Pictures were taken during the process of investigation).

Table 6.4.1a: Minimum inhibitory concentration (µg/mL) of *Cannabis indica/sativa* vegetative leaves against *Candida* species and positive control Amphotericin B.

	Candida auris	***Candida parapsilosis***	***Candida glabrata***	***Candida tropicalis***
n-hexane	1 000	500	1 000	1 000
Ethyl acetate	500	125	250	1 000
Ultra-pure water	62.5	1 000	500	1 000
Amphotericin B (AMB)	250	250	250	250

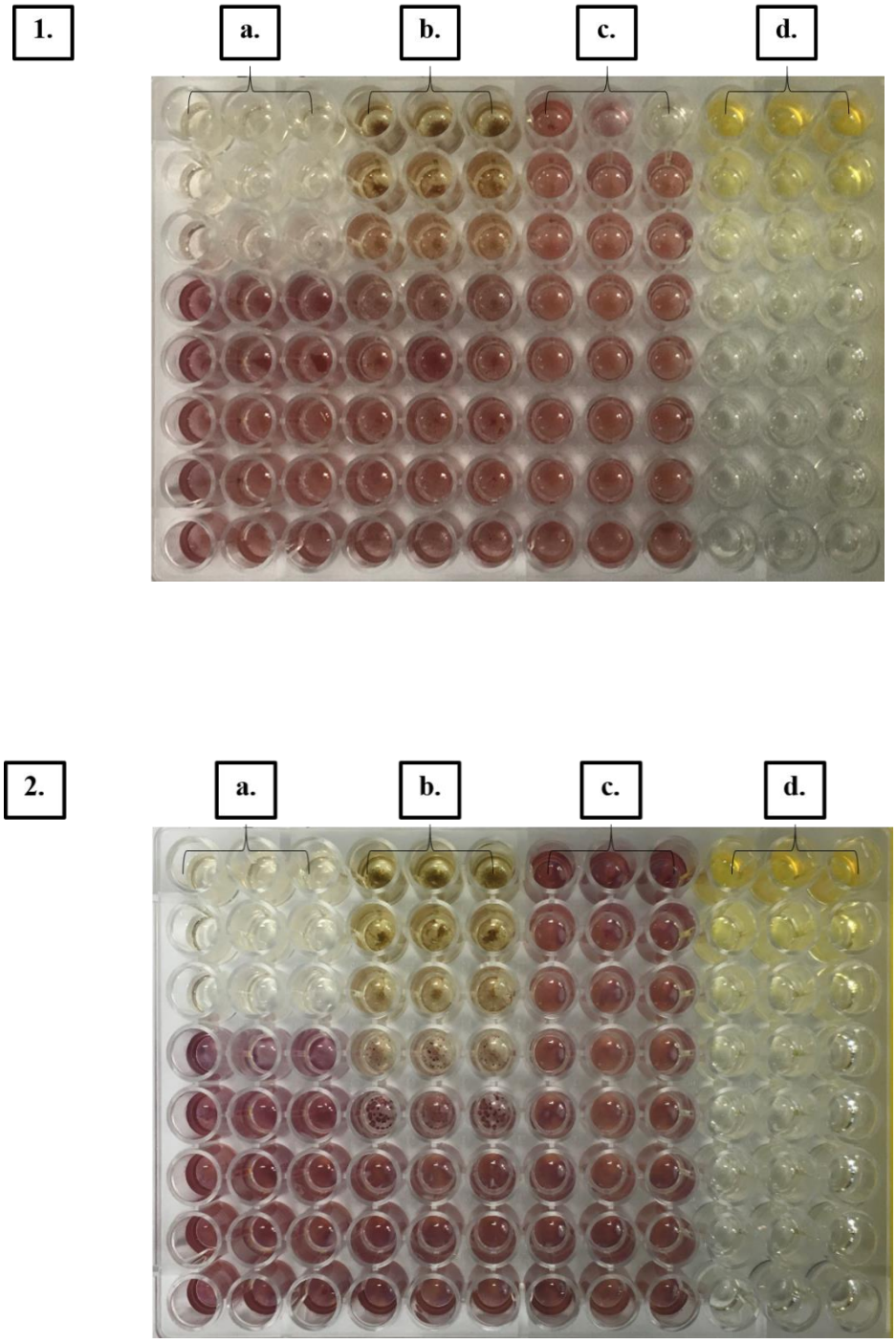

Figure 6.4.1b: Minimum inhibitory concentration testing of *Cannabis indica/sativa* matured inflorescence against **1.** *Candida auris* and **2.** *Candida parapsilosis* using extracts **a**. n-hexane **b**. ethyl acetate **c.** ultra-pure water and positive control **d.** amphotericin B.
(Source: Pictures were taken during the process of investigation).

Table 6.4.1b: Minimum inhibitory concentration (µg/mL) of *Cannabis indica/sativa* mature inflorescence against *Candida* species and positive control Amphotericin B.

	Candida auris	***Candida parapsilosis***	***Candida glabrata***	***Candida tropicalis***
n-hexane	500	500	250	500
Ethyl acetate	500	250	250	500
Ultra-pure water	> 2 000	>2 000	>2 000	>2 000
Amphotericin B (AMB)	250	250	250	250

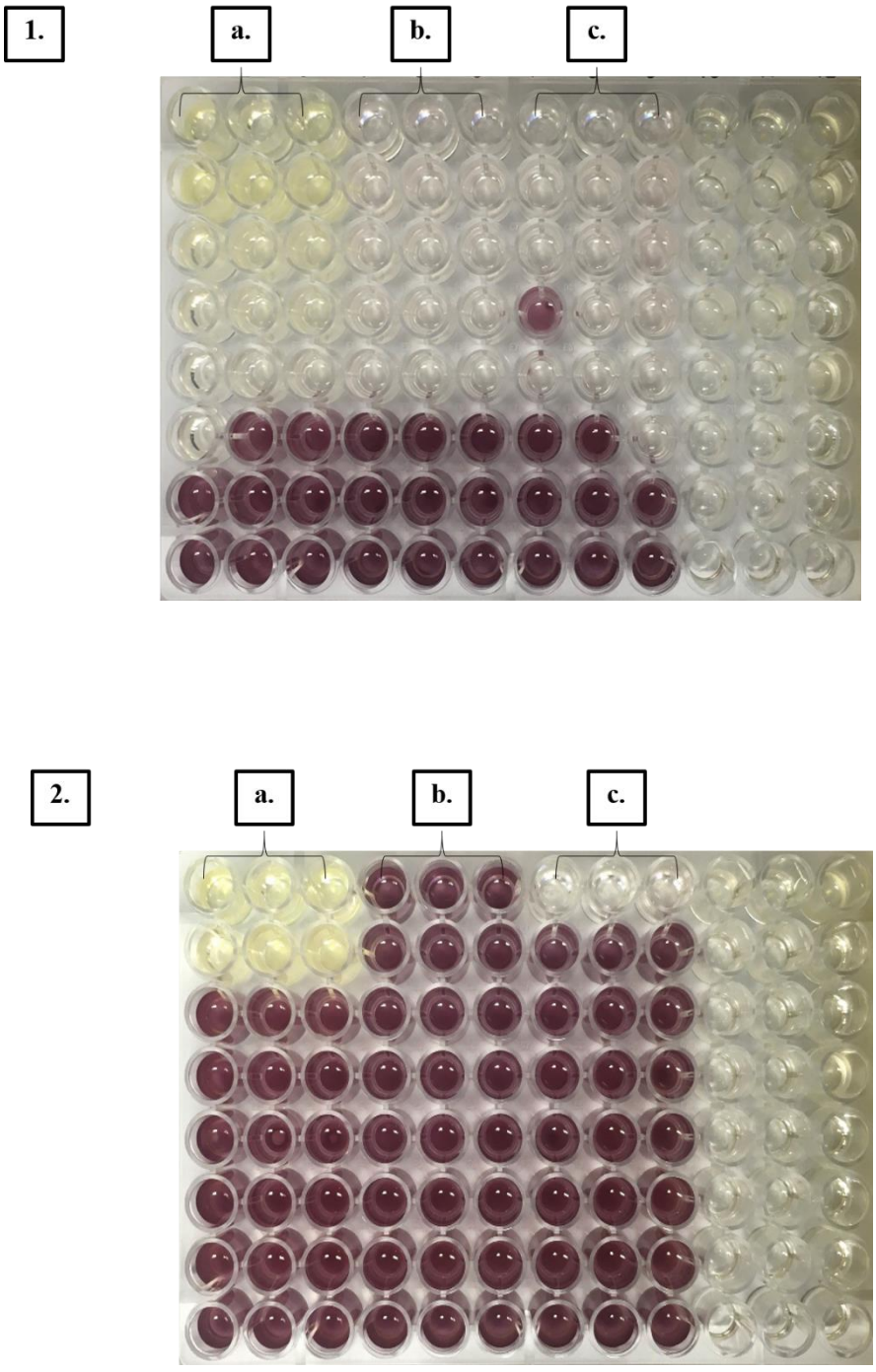

Figure 6.4.1c: Minimum inhibitory concentration testing of modified isoniazid derivatives against **1.** *Candida auris* and **2.** *Candida tropicalis* using **a**. IBS 008 **b**. IBS 013 **c.** IBS 014 coded derivatives.

(Source: Pictures were taken during the process of investigation).

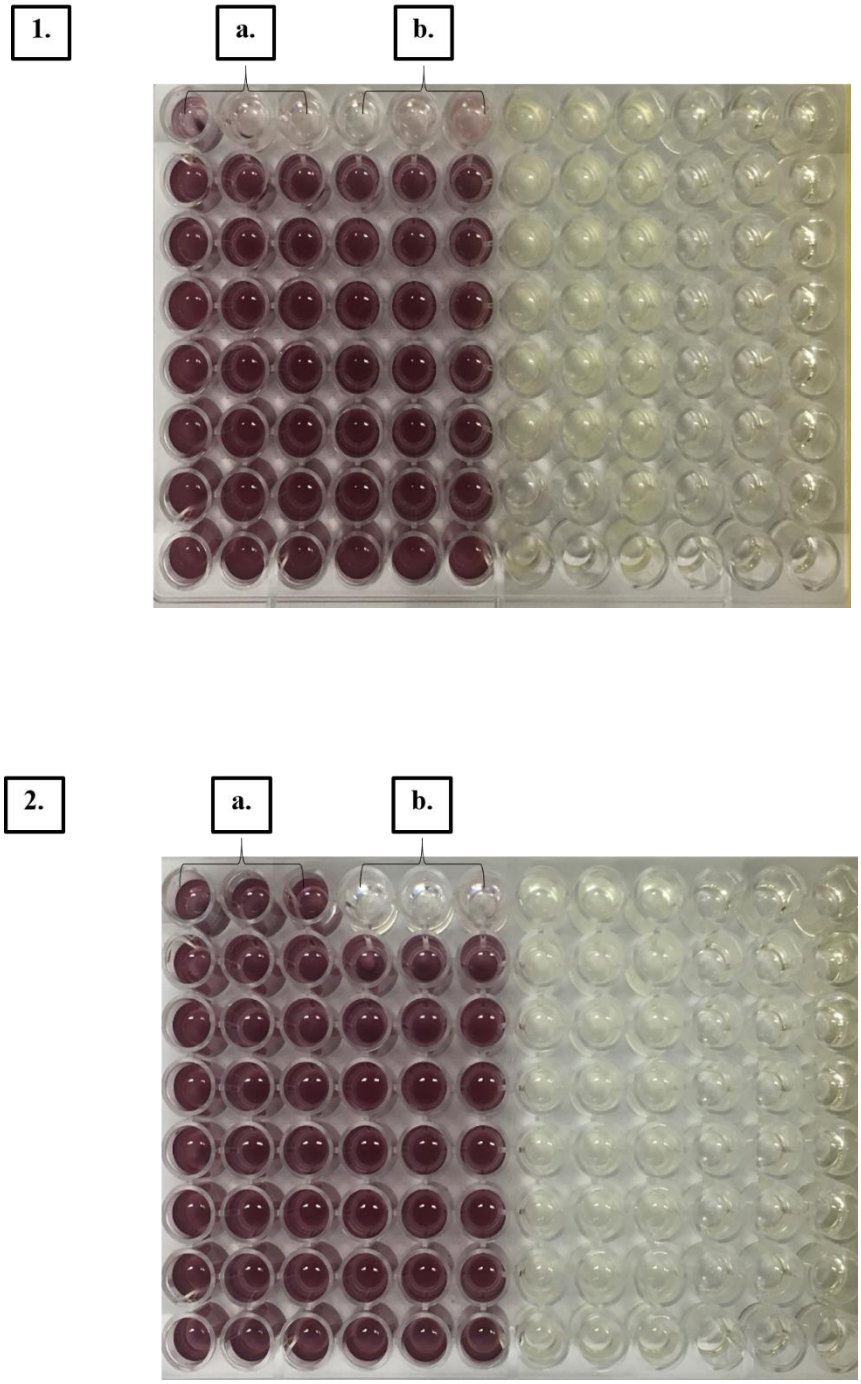

Figure 6.4.1d: Minimum inhibitory concentration testing of modified isoniazid derivatives against **1.** *Candida glabrata* and **2.** *Candida tropicalis* using **a**. IBS 017 **b**. IBS 018 coded derivatives.

(Source: Pictures were taken during the process of investigation).

Table 6.4.1c: Minimum inhibitory concentration (µg/mL) of modified isoniazid derivatives against *Candida* species and positive control Amphotericin B.

	Candida auris	***Candida parapsilosis***	***Candida glabrata***	***Candida tropicalis***
IBS 007	62.50	500	62.5	31.25
IBS 008	31.25	62.5	125	250
IBS 009	250	500	250	62.50
IBS 010	>500	500	500	250
IBS 013	31.25	125	500	>500
IBS 014	31.25	125	500	500
IBS 017	62.50	125	500	>500
IBS 018	62.50	125	500	500
Amphotericin B (AMB)	250	250	250	250
Isoniazid (INH)	>500	>500	>500	>500
Rifampicin (RMP)	0.98	0.98	0.98	0.49

6.4.2 Flow cytometry

In this experiment the *Candida* species were stained using a PI stain which is a membrane permeable stain and is used due to its ability to exclude viable cells and stain dead cells, this is done by its ability to bind to double stranded DNA by intercalating between base pairs and providing precise and rapid labelling of DNA content evaluation (Riccardi and Nicoletti, 2006). Analysis of the stained samples was done using the forward (FSC-H) and side scatter (SSC-H) height lasers. As per indications lead by light refraction, the forward scatter and side scatter gating is commonly used for the identification of cell size and complexity, respectively (Lee, Kiupel and Soboll Hussey, 2017).

The aim of this experiment was to assess the possible *in vitro* antifungal susceptibility of the *Candida* species to the drug its being tested against after 24-hours. The tested concentrations were retrieved from the minimum inhibitory concentrations (MIC) of the antifungal activity. The MIC values of amphotericin B (AMB) at 250µg/mL and rifampicin (RMP) at 0.49µg/mL for *Candida tropicalis* and 0.98µg/mL for the rest of the *Candida* species, were used as a positive control. The cells were gated in a FSC-H and SSC-H dot plot to identify cells which have undergone apoptosis. The gated-parent percentage cells indicate cells which have both morphological and cell component changes (phenotypic frequencies). After a 24-hour incubation with the respective drugs against the various *Candida* species the samples were evaluated. A forward and side scatter dot plot was plotted along with histograms, graphs that indicate a high concentration of granulated cells with a large size are within quadrant two (Q_2). The cells presented in Q_2 are those which were able to be stained by the PI stain and are considered to have experienced cell apoptosis.

The statistical analysis from this experiment was read from Q_2 as it is where the largest phenotypic frequencies occurred. *Candida* species treated with 50% ethanol were quantified as a positive control, *Candida auris* exhibited a 78.6% (**Appendix: Figure 10.1A**) of all cells have experienced apoptosis. *Cannabis indica/sativa* vegetative leave extract with an MIC of 62.5µg/mL showed statistical data similar to the control with 79.2% (**Appendix: Figure 10.1B**) of cells dead. Some of the modified isoniazid derivatives performed better than both controls, IBS 008 at 31.25µg/mL (93.6%) (**Appendix: Figure 10.2B**), IBS 013 at 31.25µg/mL (90.1%) (**Appendix: Figure 10.3B**), IBS 014 31.25µg/mL (95.0%) (**Appendix: Figure 10.4A**) and IBS 017 at 62.5µg/mL (83.5%) (**Appendix: Figure 10.4B**), respectively. The extract and derivatives exhibited parent percentages greater than the control. The derivatives exhibited

greater apoptotic capacity in comparison to the positive control Amphotericin B at 250µg/mL (45.4%) (**Appendix: Figure 10.5A**), but were on par with Rifampicin at 0.98µg/mL (91.5%) (**Appendix: Figure 10.5B**).

Cells treated with 50% ethanol were used as a positive control for the flow cytometry evaluation of *Candida glabrata*, a total of 96.8% (**Appendix: Figure 10.6A**) of cells experienced apoptosis. Two *Cannabis indica/sativa* extracts performed better in comparison to the positive control, the ethyl acetate vegetative leaves extracts (97.6%) (**Appendix: Figure 10.6B**) at 250µg/mL and n-hexane mature inflorescence extract (98.6%) (**Appendix: Figure 10.7B**) at 250µg/mL**.** IBS 007 (91.4%) (**Appendix: Figure 10.8A**) at 62.5µg/mL was the derivative which showed activity closer to the gated-parent percentage of the positive control. Both extracts and derivatives exhibited better activity in comparison to amphotericin B (48.1%) (**Appendix: Figure 10.9A**) at 250µg/mL, however Rifampicin had the best activity in comparison to all tested agent (98.8%) (**Appendix: Figure 10.9B**) at 0.98µg/mL.

Candida parapsiolosis cells were also treated with 50% ethanol as a positive control, with that 97.6% (**Appendix: Figure 10.10A**) of the cells experienced cell apoptosis. The *Cannabis indica/sativa* ethyl acetate vegetative leaves (96.6%) at 250µg/mL (**Appendix: Figure 10.10B**) had activity closely related to that of the positive control. The derivatives IBS 008 (91.0%) at 62.5µg/mL (**Appendix: Figure 10.11B**), IBS 013 (91.8%) at 125µg/mL (**Appendix: Figure 10.12A**) and IBS 017 (90.6%) at 125µg/mL (**Appendix: Figure 10.13A**) also had results closely related to the positive control. Amphotericin B had a result with the least activity with only (47.4%) at 250µg/mL (**Appendix: Figure 10.14A**), Rifampicin had the best activity compared to all the tested agents and an apoptosis rate similar to the reference drug (97.6%) at 250µg/mL (**Appendix: Figure 10.14B**).

6.5 Discussion

Antimicrobial resistance can easily be acquired by many fungal species due to the change in their genomic encoding which range from point mutations to assembly of pre-existing genetic elements to horizontal gene imports from the environment (Miró-Canturri *et al.,* 2019). *Candida* species are responsible for numerous opportunistic infections which have become difficult to treat due to their increased resistance to many of their antifungals (Martins *et al*., 2017). It has become an urgent case for the development of new antimicrobial strategies to use individ-

ually or in synergy with one another to tackle the antimicrobial agent resistance of fungi. Repurposing of drugs has become a popular field of interest as seen in many studies, where a drug designed for one disease has been investigated to treat another (Miró-Canturri *et al.*, 2019). Another popular direction which has been taken is the use of the kingdom *Plantae* as a source of natural compounds and bioactive secondary metabolites which have been characterized as having antifungal properties (Mishra *et al*., 2020).

The aim of this chapter was to compare the effectiveness of the repurposing of modified isoniazid derivatives and *Cannabis* plant extracts against non-*Candida albicans Candida* species. To assess the antifungal potential of the tested agents, the minimum inhibitory concentration testing assay was performed to determine this and from the results it can be deduced that both derivatives and extracts show antifungal activity, many of which showed better activity than the positive control amphotericin B. The modified isoniazid derivatives exhibited excellent antifungal activity in comparison to both *Cannabis* extracts and the positive control. For an MIC to be considered noteworthy, it would have to have an activity of MIC ≤ 1 000µg/mL for essential oils, MIC ≤ 16µg/mL for compounds and MIC ≤ 160µg/mL for plant extracts as per van Vuuren and Holl's (2017) antimicrobial testing MIC goldestandard guidelines. The *Cannabis indica/sativa* vegetative ethyl acetate leaf extract had interesting activity against *Candida parapsilosis* presenting with an MIC = 125 µg/mL which is lower than what is considered a golden standard MIC ≤ 160µg/mL (Pauw and Eloff, 2014). The *Cannabis indica/sativa* ultra pure water vegetative leaves showed a strong and significant activity against *Candida auris* with an MIC < 100µg/mL (van Vuuren and Holl, 2017). All of the vegetative leaves extracts showed moderate activity where the MIC was 100 – 625µg/mL against *Candida auris*, *Candida parapsilosis* and *Candida glabrata*, respectfully. The n-hexane and ethyl acetate *Cannabis indica/sativa* mature inflorescence extracts were also considered to have moderate activity against *Candida auris*, *Candida tropicalis*, *Candida parapsilosis* and *Candida glabrata* as they all had activity between 100 – 625µg/mL. According to van Vuuren and Holl, (2017), MIC values between 64 – 100µg/mL are considered to be clinically relevant, the modified isoniazid derivatives all showed inhibitory concentrations which are between the standardised concentrations against all the *Candida* species tested. Thus the reults obtained were considered noteworthy when compared to the recommended MIC golden standard for antimicrobial testing as recommended by van Vuuren and Holl, (2017). Cannabinoids such as canabigerol (Brenneisen, 2007) and terpenes such as δ-limonene, linanool, β-myrcene (Karas *et al*., 2020) are known to

have potent antifungal activity, activity seen in these extracts can be attributed to such compounds as they were identified in Chapter 9.

Flow cytometry has been used to quantitatively determine single-cell properties, granularity of the cells, cell size measurement, aspects of cell death or viability or the amount of cell components available in less than one minute. The principle behind this lies in the use of fluorescent probes which allows the flow cytometer to distinguish between living and dead cells, microbial viability and membrane integrity (Martins *et al*., 2017). Monitoring of membrane damage and trans-membrane electrochemical potential provides practicable and swift indications of microbial injury induced by antimicrobial agents (Martins *et al*., 2017). To assess cell apoptosis, flow cytometry analysis was conducted, the experiment proved that there was cell apoptosis at a given stage after the fungi were incubated with the respective drugs for 24 hours. The programmed cell death was a clear indicator that the respective agents used in this experiment, whether plant extract or repurposed drugs show inhibitory activity towards respective *Candida* species.

6.6 Conclusion

Many plants and drugs have been discovered, designed and tested for fungal infections. It has always been thought that candidiasis was only caused by *Candida albicans*; however, it is now speculated that most candidiasis infections are caused by non-*Candida albicans Candida* species. Therefore, the investigation of therapeutic agents both from synthetic drugs and medicinal plants is warranted. The aim of this chapter was to prove that *Cannabis indica/sativa* extracts and repurposed isoniazid derivatives are able to be potential drugs for these fungal species. This chapter has achieved its goal to prove that *Cannabis indica/sativa* extracts and modified isoniazid derivatives have antifungal activity. Between the two, the study has shown that repurposed isoniazid derivatives have better activity in comparison to the plant extracts. The isoniazid compounds and *Cannabis indica/sativa* extracts which were tested all fell within the range of being considered noteworthy to be studied further and/ or considered for clinical significance. With that being said, the study has highlighted an area to look into and an area which might be of interest by using anti-mycobacterial drugs to aid in *Candida* fungal infections.

6.7 References

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Chapter 7

Anti-mycobacterial activity of *Cannabis*

7.1 Introduction

Mycobacterium other than tuberculosis (MOTT) are a group of *Mycobacterium* found in environments, such as natural water and soil (Walsh *et al*., 2019). MOTT are very diverse Gram positive bacteria presenting with dissimilar virulence features. Some species of mycobacteria are pathogenic to humans and have the potential to cause severe pulmonary and non-pulmonary diseases such as device related infections (central lines and bloodstream infections) and infections of the skin and soft tissue (Hu *et al*., 2019) while others are found to be infectious to animals (Jagielski *et al*., 2014). The key to the proliferation and survival of MOTT in the environment is their lipid-rich outer membrane; this causes these organisms to be acid-resistant, resistant to high temperatures, disinfectants and antibiotics (Joen, 2019).

Drugs such as amikacin are antibiotics commonly used for MOTT (Egelund *et al.*, 2015), this drug binds to 30S ribosomal subunits, this interferes with protein synthesis inhibition and protein termination (Sizar *et al*., 2021). Another common antibiotic is linezolid which serves as an excellent potential against other species of MOTT and multidrug-resistant tuberculosis (MDR)-TB by the interruption of bacterial growth through inhibition of protein synthesis by binding the bacterial 23S ribosomal ribonucleic acid (RNA) (Ryu *et al*., 2016). The first-line of treatment against tuberculosis is isoniazid, however its activity against latent bacilli is suboptimal, thus this promotes resistance of the drug if it is administered alone. Drug resistance is therefore opposed with combinations of drugs along-side isoniazid (Battah *et al*., 2019). Rifampicin is another potent drug which is used for various mycobacterial infections such as *Myobacterium avium* complex, *Mycobacterium leprae* and more commonly *Mycobactrium tuberculosis* (Siu *et al*., 2011).

Mycobacterium other than tuberculosis are naturally resistant to treatment such isoniazid that is commonly used for the treatment of tuberculosis, this poses as a major challenge for the discovery of new drugs (Wu *et al.*, 2018). Due to the chemical diversity of treatment agents used for *Mycobacterium* and their significant role in the development and sighting of the drug, medicinal plants have been considered more favourable choice when coming to the discovery

of drugs. Medicinal plants such as *Croton marcostachyus, Searsia undulata* and *Oenanthe procumbens* to name a few, have also been recognized for their highly active metabolites such as terpenes, flavonoids, and alkaloids and their ability to be a useful agent containing anti-mycobacterial activity (Gemechu *et al*., 2013).

The rapid increase in antibiotic resistant non-tuberculous *Mycobacterium* opportunistic infections has become a global health concern (Sengupta *et al.*, 2017). The growing antibiotic resistance coupled with expensive and slow discovery of *Mycobacterium* drugs has also aggravated the situation further (Jyoti *et al*., 2020). Drug repurposing is a cost savy strategy which aims to treat various communicable diseases, by classifying known drugs with a predicated efficiency for diseases other than its target diseases. This method of drug discovery has the potential in the treatment of tuberculosis, particularly drug resistant and multidrug resistant tuberculosis (An *et al*., 2020).

It is worth mentioning that the results of the anti-mycobacterial activity of the modified isoniazid derivatives will not be reported as part of the results of this chapter. These results are part of an ongoing study which this study was funded under (Grant UID: 118127-), because of this the results will be withheld and will be published as part of another project. The aim of this chapter is to evaluate the purity of six strains of *Mycobacterium* namely *Mycobacterium avium, Mycobacterium aurum, Mycobacterium bovis, Mycobacterium fortuitum, Mycobacterium smegmatis* and *Mycobacterium tuberculosis* (H37Ra) as well as the anti-mycobacterial activity of vegetative leaves and mature inflorescence *Cannabis indica/sativa* extracts.

7.2 Sample collection and preparation

As described in Chapter 3, the preparation of the *Cannabis indica/sativa* plant extracts is described in section **3.2 Preparation of extracts**. The *Mycobacterium* species culturing method is described in the section **3.6.1 Mycobacterium species cultures** and the characterization was done using **3.6.3 Acid-fast characterization**.

7.3 Anti-mycobacterial activity

7.3.1 Minimum inhibitory concentration testing

The minimum inhibitory concentration of each extract was conducted according to Schönfield *et al*., (2012) with modifications. Middlebrook 7H9 broth (Sigma Aldrich, France) was prepared with the addition of 5mL of OADC growth supplement (Sigma Aldrich, India), 125µL

of 20% Tween80 (Sigma Aldrich, France) and 250µL of Tryptone (Melford, United Kingdom). Each well was seeded with 120µL of broth and this was followed by 80µL aliquots of plant extracts (5mg/mL), into the first row (row A) of the 96-well plate and was two-fold serially diluted. For the testing of the derivatives, 100µL of M7H9 broth, followed by 100µL of isoniazid derivatives (2mg/mL) were prepared in a 5mL centrifuge tube, this was then added into the first row with 100µL broth and then two-fold serially diluted. Rifampicin (Sigma-Aldrich, Israel) and Isoniazid (Sigma-Aldrich, Germany) were used as a positive controls prepared at a concentration of 1mg/mL, 40µL of the reference drug was added into 160µL of broth and was also two-fold serially diluted. Aliquots of 100µL of the *Mycobacteria spp*., *Mycobacterium fortuitium* (ATCC 6841) and *Mycobacterium smegmatis* (ATCC 14468); were then added to each well of the respective 96-well plate, the plate, sealed with parafilm then incubated at 37ºC overnight, where *Mycobacterium bovis* (BCG) (ATCC 27290), *Mycobacterium aurum* (NCTC 10437) *Mycobacterium avium* (ATCC 25291) and *Mycobacterium tuberculosis* (H37-Ra) (ATCC 25177) were incubated for 7 days to allow sufficient growth. A stock solution of 0.2% Resazurin (Sigma Aldrich, USA) was used to create a 0.02% resazurin working solution, 20µL of this solution was added to every well and incubated at 37ºC for 24 hours. The plates were then visualized, any well which turned pink indicated microbial growth while blue wells indicated microbial growth inhibition.

7.4 Results

7.4.1 Acid-fast characterization

Auramine-O staining is a flurochromatic staining process which is used to identify and detect acid-fast *Mycobacterium*. Auramine-O is shown to be more sensitive for the detection of *Mycobacterium tuberculosis* from direct sputum, but has shown to present false positives for the specificity of *Mycobacterium* other than tuberculosis and other weaker acid fast *Mycobacterium* (Alnour *et al.*, 2012). Auramine O is mainly used to test acid-fast mycobacteria, any bacteria which is acid fast as seen on **Figure 7.4.1** fluoresces yellow or orange under ultraviolet light. The *Mycobacterium* species which were stained were observed using a UV-lamp (**Figure 7.4.1**) and due to the cells absorbing the Auramine O stain, it can clearly be deduced that the species were entirely acid-fast bacteria. As pure acid-fast organisms fluoresce and those which are not acid-fast do not.

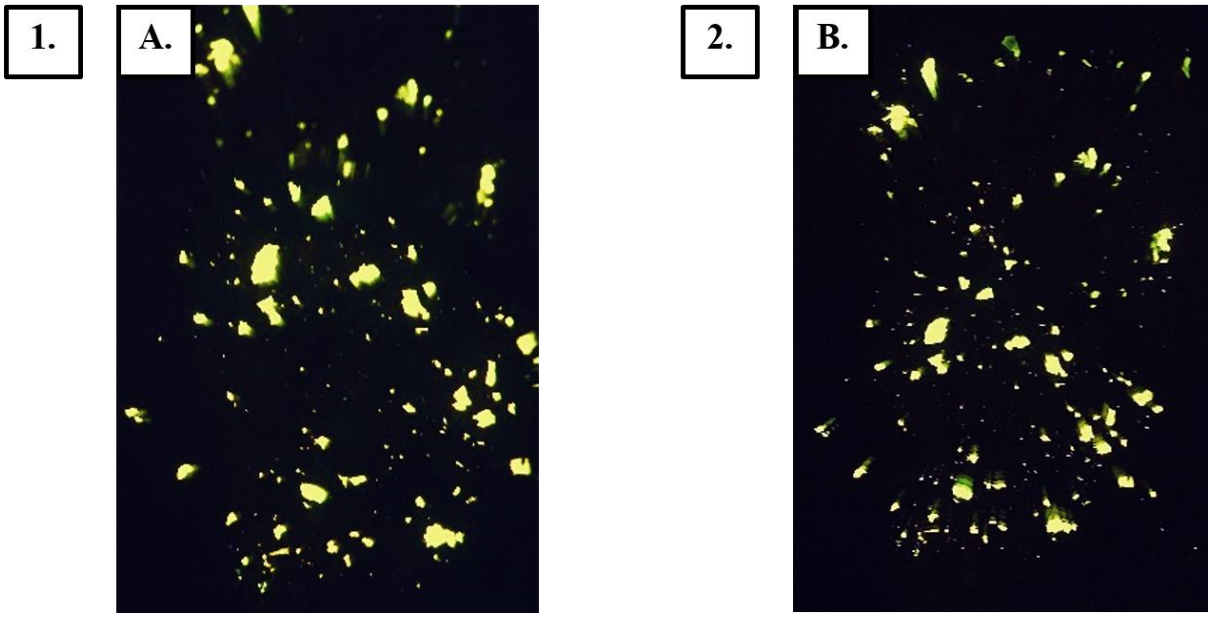

Figure 7.4.1: 1a. Pure colonies of *Mycobacterium tuberculosis* (H37Ra) and **2b.** *Mycobacterium smegmatis* stained with Auramine-O and observed under UV-lamp.

7.4.2 Minimum inhibitory concentration

In this study, six *Mycobacterium* species were tested against *Cannabis indica/sativa* and vegetative mature inflorescence; these species are *Mycobacterium avium, Mycobacterium aurum, Mycobacterium bovis, Mycobacterium fortuitum, Mycobacterium smegmatis* and *Mycobacterium tuberculosis* (H37Ra). The extracts were tested at concentration range of 15.626 – 2 000µg/mL (**Table 7.4.2a**). The *Mycobacterial* species were also tested with their respective positive controls isoniazid and rifampicin. The ethyl acetate vegetative leaves exhibited the best activity for *M. aurum* and *M. smegmatis* with an inhibitory concentration of 31.25µg/mL. The vegetative leaves extract had an inhibitory concentration of 250µg/mL against *M. avium* and *M. smegmatis*. The vegetative leaves of ultra-pure water extract only showed activity towards *M. avium* and *M. aurum* with an inhibitory concentration of 1 000µg/mL and showed no activity towards the rest of the microbes at the tested concentrations with MIC values >2 000µg/mL. The *n*-hexane vegetative leaves extract only exhibited activity towards *M. aurum, M. bovis* and *M. smegmatis* with a concentration of 1 000µg/mL.

The mature inflorescence of ethyl acetate extract showed inhibitory activity towards five of the six *Mycobacerium* species (**Table 7.4.2b**) with the following minimum inhibitory concentrations on *M. avium* (MIC = 1 000µg/mL), *M. aurum* (MIC = 500µg/mL), *M. bovis*

(MIC = 62.5µg/mL), *M. fortuitum* (MIC = 1 000µg/mL) and *M. smegmatis* (MIC = 250µg/mL), respectively. The *n*-hexane mature inflorescence extract showed activity towards *M. aurum* (500µg/mL), *M. bovis* (MIC = 500µg/mL) and *Mycobacterium smegmatis* (MIC = 1 000µg/mL). The ultra-pure water extract only showed activity against *M. bovis* at an MIC activity of 1 000µg/mL. The reference drug isoniazid had a minimum inhibitory concentration >100µg/mL against the species *M. avium*, *M. aurum* and *M. smegmatis*. It had an MIC activity of 6.25µg/mL against both *M. fortuitum* and *M. tuberculosis* (H37Ra) and <0.78µg/mL for *M. bovis*. In comparison to the other reference drug and the extracts, rifampicin displayed noteworthy activity against the respective *Mycobacterium*, it had an MIC activity <0.78µg/mL against *M. avium*, *M. aurum* and *M. bovis.* Rifampicin also showed an activity of 1.56µg/mL against *M. fortuitum*, 3.25µg/mL against *M. smegmatis* and 12.5µg/mL against *M. tuberculosis.*

Table 7.4.2a: Minimum inhibitory concentration values (µg/mL) of *Cannabis indica/sativa* vegetative leaves against *Mycobacteria* species.

	Mycobacterium avium	***Mycobacterium aurum***	***Mycobacterium bovis***	***Mycobacterium fortuitum***	***Mycobacterium smegmatis***	***Mycobacterium tuberculosis***
***n*-Hexane**	>1 000	1 000	1 000	>1 000	1 000	>1 000
Ethyl acetate	250	31.25	>1 000	250	31.25	1 000
Ultra-pure water	1 000	1 000	>1 000	>1 000	>1 000	>1 000
Isoniazid (INH)	>100	>100	<0.78	6.25	>100	6.25
Rifampicin (RMP)	<0.78	<0.78	<0.78	1.56	3.25	12.5

Table 7.4.2b: Minimum inhibitory concentration values (µg/mL) of *Cannabis indica/sativa* mature inflorescence against *Mycobacteria* species.

	Mycobacterium avium	***Mycobacterium aurum***	***Mycobacterium bovis***	***Mycobacterium fortuitum***	***Mycobacterium smegmatis***	***Mycobacterium tuberculosis***
n-Hexane	>1 000	500	500	>1 000	1 000	>1 000
Ethyl acetate	1 000	500	62.5	1 000	250	>1 000
Ultra-pure water	>1 000	>1 000	1 000	>1 000	>1 000	>1 000
Isoniazid (INH)	>100	>100	<0.78	6.25	>100	6.25
Rifampicin (RMP)	<0.78	<0.78	<0.78	1.56	3.25	12.5

7.5 Discussion

In this chapter, the mature inflorescence and vegetative leaves of *Cannabis indica/sativa* were tested against MOTT to determine if they have anti-mycobacterial activity. The vegetative leaves ethyl acetate extract showed to have the most activity in comparison to the other vegetative leaves (**Table 7.4.2a**). Acid fast bacilli are those bacteria which have the physical property to resist acid decolourization caused by acid staining (Bayot *et al.*, 2021). The process of acid staining is important as it enables the identification of many acid fast organisms including *Mycobacterium* species. These organisms have a high mycolic acid content in their cell wall (Wanger *et al*., 2017). Mycolic acids are long chains of fatty acids found in the cell walls in a group of bacteria called *Mycolata taxon* and is responsible for the low susceptibility of bacteria against their respective agents and ensures the survival of many bacterial genera including *Mycobacterium* (Marrakchi *et al.*, 2014).

New anti-mycobacterial agents are constantly needed for the treatment of drug resistant infections in both animals and humans. The resistance of *Mycobacterium* species to many of its antibiotics has prompted many researchers to find new candidates for anti-mycobacterial purposes. The use of plants has led to the optimization of strategies to improve the efficiency of new drug discoveries. The antibacterial activity of various parts of *Cannabis* has been evaluated against many Gram-positive and Gram-negative microbes, most of which are multi-drug resistant (Schofs *et al.*, 2021). In a study performed by Borchardt *et al*., (2008) extracts of *Cannabis sativa* stems and leaves were tested using the disc diffusion method against *Staphylococcus aureus* which showed evidence of hollow areas with a diameter of 25mm, indicating growth inhibition against the bacteria. According to Schofs *et al.*, (2021), *Cannabis sativa* var. *indica* has also shown mycobactericidal effects against *Mycobacteria tuberculosis*. The organic solvent extraction of *C. sativa* was evaluated for antibacterial activity against *S. aureus, Bacillus subtilis, Pseudomonas aeruginosa* and *Escherichia coli.* The results of the experiment showed more activity towards the Gram-positive bacteria in comparison to the Gram-negative bacteria (Ali *et al.*, 2012). The susceptibility testing of *C. indica* seeds, stems and leaves against the multi-drug resistant strains *E. coli, Bacillus cereus, Klebsiella pneumoniae, Proteus mirabilis, P.s aeruginosa* and *S. aureus* also showed a wide range of antimicrobial activity by using the broth diffusion method (Isahq *et al.*, 2015).

From these studies it can be seen that there is an array of species which *Cannabis* has exhibited

antibacterial activity against. The potential in using *Cannabis* as treatment agent for *Mycobacterium* other than tuberculosis has been observed in this chapter. The aim of this chapter was to evaluate the activity of *Cannabis* extracts for their anti-mycobacterial activity against six *Mycobacterium* species. The minimum inhibitory testing method was used to determine the results and compare them against the positive controls, isoniazid and rifampicin respectively. The *Cannabis* ethyl acetate extracts of both mature inflorescence and vegetative leaves in this study showed potential for anti-mycobacterial activity. The best activity exhibited against all the *Mycobacterium* species was from the ethyl acetate extracts. The vegetative leaves gave an MIC = 250µg/mL for *M. avium*, MIC = 31.25µg/mL for *M. aurum*, MIC = 250µg/mL for *M. fortuitum* and MIC = 31.25µg/mL for *M. smegmatis.* The Cannabis mature inflorescence had MIC = 500µg/mL for *M. aurum*, MIC = 62.5µg/mL for *M. bovis* and MIC = 250µg/mL for *M. smegmatis*. The n-hexane and ultra-pure water extracts exhibited MICs = 1 000µg/mL or MIC >1 000µg/mL for *Mycobacterium* strains. The *Cannabis indica/sativa* vegetative leaves ethyl acetate extracts showed significant activity with MICs which are <100µg/mL (van Vuuren and Holl, 2017) against *Mycobacterium aurum* and *Mycobacterium smegmatis* and moderate activity against *Mycobacterium avium* and *Mycobacterium fortuitum* with MIC which were between 100 – 625µg/mL (van Vuuran and Holl, 2017). *Cannabis indica/sativa* mature inflorescence n-hexane extracts showed moderate activity against *Mycobacterium aurum* and *Mycobacterium bovis* the ethyl acetate extracts also displayed moderate activity against *Mycobacterium aurum* and *Mycobacterium smegmatis* with MICs ranging between 100 – 625µg/mL which is considered to be modest (van Vuuran and Holl, 2017). Minimum inhibitory concentrations which are <100µg/mL are considered to have favourable and significant activity (van Vuuran and Holl, 2017), the *Cannabis indica/sativa* mature inflorescence ethyl acetate extract had what is deem as significant activity against *Mycobacterium boivs*. The amount of activity seen from the *Cannabis indica/sativa* extract can be attributed to the presence of compounds such as cannabigerol which were identified during the phytochemical analysis seen in Chapter 9. This compound is known to have antibacterial activity against Gram-positive organisms, another compound known to have antibacterial activity is the terpenes α-humulene, caryophyllene and caryophyllene oxide.

7.6 Conclusion

The anti-mycobacterial activity of the plant was tested against six non-tuberculosis *Mycobacterium* species. All six extracts exhibited anti-mycobacterial activity which probes more investigations into the potential of the plant to be a possible treatment agent for *Mycobacterium*

infections. Although *Cannabis indica/sativa* has shown great potential with inhibiting the growth of Gram positive organisms as displayed, the extracts also displayed activity which was reasonably within the golden standard, these concentrations may not be clinically relevant in comparison to their reference drugs. However, these results are not significant enough to further the study.

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Chapter 8

Phytochemical analysis of Cannabis *indica/sativa* crude extracts using GC x GC – TOF – MS

8.1 Introduction

Medicinal plants have been the key point of interest in the treatment of human ailments and diseases due to their varied and vast assortment or organic compounds that produce physiological action in the human body. Such compounds of importance include tannins, terpenoids, alkaloids, flavonoids, saponins and phenolic compounds. Due to their low toxicity and therapeutic performance, these compounds have caused an interest in pharmacists and pharmacologists (Akhtar *et al.*, 2018).

Phytochemical screening of metabolites in an organism including plants using advanced high resolution analytical instruments such as Nuclear Magnetic Resonance (NMR), ultra-high performance liquid chromatography-quadrupole time-of-flight mass spectrometry (UHPLC/Q-TOF-MS) and Gas chromatography–time-of-flight mass spectrometry (GCxGC-TOF-MS) these are unbiased, analytical approaches used to quantify and identify a wide array of metabolites found in biological material. Chromatographic techniques are biophysical techniques which allow the separation, identification and purification of chemical components for quantitative and qualitative analysis (Coskun, 2016). This technology conducts analysis based on a variety of variables such as binding affinities, charge, size amongst other properties to enable separations and analyses of mixtures (Coskun, 2016).

To investigate the various phytochemicals of the *Cannabis indica/sativa* plant in this study, the GCxGC-TOF-MS technique was utilized. These two separate techniques coupled as one are often used together to yield two fragments of information for each analysis – mass spectral data and retention time (Smith, 2013). This method is a powerful technique used to acquire a wide range of metabolites with low molecular weights such as sterols, sugars, fatty alcohols, amino acids, fatty acids and organic acids (Ohta *et al.*, 2017). Gas chromatography is a "gas-liquid" technique, that uses nitrogen (N_2) or helium (He) gas as a carrier gas. It is highly sensitive, rapid and simple to use for excellent separation of miniscule molecules (Coskun, 2016). Mass spectrometry on the other hand is an analytical technique used for the measurement of mass-to-charge (*m/z*) ion ratio, this provides information on chemical structures and molecular

weights of the analysed sample (Büyükköroğlu *et al*., 2018) and has become a gold standard technique in the analysis of many biological molecules (Glish and Vachet, 2003). Gas chromatography – gas chromatography – time of flight – mass spectrometry or GC x GC –TOF-MS; uses a two dimensional retention plane where the GC x GC makes an enhanced possible precise separation apparatus and typical linearity, with a time-of-flight mass spectrometry (TOFMS) detection used for compound identification using mass analysis which identifies molecules by flight-time in a field-free tube measurement (Song *et al*., 2004). The GC x GC –TOF-MS technique is able to identify a higher number of compounds as compared to the traditional one-dimensional chromatographic techniques such as gas chromatography (GC) (dos Santos *et al*. 2015).

In this chapter, an annotated phytochemical analysis was conducted on the six *Cannabis* extracts viz. *n*-hexane, ethyl acetate and ultra-pure water vegetative leaves and mature inflorescence extracts. This was done to investigate the various tentative constituents which have potentially played an active biological role in the study. The annotated analysis was conducted using a two dimensional gas chromatography – time of flight – mass spectrometry (GC x GC –TOF-MS).

8.2 Plant sample preparation for GC-MS annotated analysis

As described in Chapter 3, the preparation of the *Cannabis indica/sativa* plant extracts is described in section **3.2 Preparation of extracts.**

8.2.1 Gas chromatography – gas chromatography – time of flight – mass spectrometry (GC x GC –TOF-MS) sample preparation

Approximately 1 mg of each extract was mixed with 1mL LC-grade acetonitrile, ultrasonicated for 5 min and the homogenates were centrifuged for 15 min. The filtration of the mixture was done using a 0.22µm Millipore syringe filter. The filtrates were pipetted into 2mL HPLC glass vials. Aliquots of extracts were prepared in triplicates and stored at -20°C prior to analysis.

8.3 Gas chromatography – gas chromatography – time of flight – mass spectrometry (GC x GC –TOF-MS) analysis

The analysis of the phytochemical constituents of the six *Cannabis* extracts was evaluated using the gas chromatography – gas chromatography – time of flight – mass spectrometry (GC x

GC –TOF-MS) method by Ohta *et al.*, (2017) with modifications. An Agilent 7890A Gas Chromatographer (Agilent Science, Santa Clara, California, USA) coupled with a LECO Pegasus HT Flight Mass Spectrometry Time (TOF-MS) (LECO Corporation, St Joseph, Michigan, USA) was used for the analysis. The samples were encumbered onto a Gerstel MPS2 Liquid/HS/SPME auto-sampler and a J and W capillary column HP-5MS 30 m × 0.25 mm I.D. with 0.25µm film thickness was used for the chromatographic separation.

The separation took place under these chromatographic conditions: the injection of a 1µL sample was carried out at a temperature of 250 °C with a splitless injector, the GC oven was automated as follows: 1 min at 80 °C, then a ramp of 10 °C min^{-1} to 280 °C kept for 20 minutes. Helium with a high purity (99.999 %) (Afrox, Johannesburg, RSA) was used as the carrier gas (constant flow at 1.0 mL min^{-1}). The transfer line and ion source temperature of the GC/TOFMS was set at 280 °C and 200 °C respectively, while the mass spectra were obtained in full scan mode at 70eV (*m/z* scan varying from 50 to 550). The collection of data was accomplished using the program ChromaTOF® Software.

ChromaTOF was used for processing and analysing the large amounts of data acquired by the time-of-flight instruments. The NIST library integrated into the ChromaTOF® Software generated library matches. A positive identification in the sample extracts was assumed for compounds with a NIST library match superior to 90%.

8.4 Results

The *n*-hexane, ethyl acetate and ultra-pure water extracts of both vegetative leaves and mature inflorescence were analysed using two-dimensional gas chromatography – mass spectrometry (GC x GC – TOF – MS) to annotate metabolites using their respective molecular weights (**Table 8.4**). The metabolite 9-Octadecenamide, (Z)- is seen in all six extracts which were tentatively analysed at the retention time of 18:47,3min, with a unique mass of *m/z* = 59 this compound has an exact mass of m/z = 281.2719. The *Cannabis indica/sativa* mature inflorescence of *n*-hexane extract had most of the compounds detected as shown in **Table 8.4** such as D-Limonene which was detected at 04:01,2min with a unique mass of *m/z* = 68 and an exact mass of *m/z* = 136.1252 (**Appendix: Figure 10.17**); Linalool was spotted at 04:52,0min with a unique mass of *m/z* = 71 and an exact mass of *m/z* = 154.1358; Trifluoroacetyl-lavandulol was then detected at 18:35,7min at a unique mass of *m/z* = 176 and an exact mass of *m/z* = 324.3028. Other compounds which were seen to be unique to both *n*-hexane mature inflorescence and

vegetative leaves include; Isothiourea, 2-methyl-1-(2,4-dimethylphenyl)-3-(1,1-dimethyl-2-propynyl)- identified at 18:29,7min with a unique mass of *m/z* = 245 with an exact mass of *m/z* = 260.1347; 1H-4-Oxabenzo(f)cyclobut(cd)inden-8-ol-1a-à,2,3,3a,8b-à,8c-à-hexahydro-1,1,3a-trimethyl-6-pentyl- found at 18:35,7min with a unique mass of *m/z* = 231 and an exact mass of *m/z* = 314.2246; 4,8,12,16-Tetramethylheptadecan-4-olide detected at 18:39,8min with a unique mass of 99 and an exact mass of *m/z* = 324.2246 as well as cannabispiran found at 19:14,9min with a unique mass of *m/z* = 176 and an exact mass of *m/z* = 26.1256.

Some cannabinoids which were found in the *Cannabis indica/sativa n*-hexane extracts such as Δ^9-Tetrahydrocannabinol, TMS derivative detected at 19:47,6min with a unique mass of *m/z* = 303 and exact mass of *m/z* = 38.2641 and Cannabinol, TMS derivative detected at 20:26,4min with a unique mass of *m/z* = 367 and exact mass of *m/z* = 382.2328. Cannabichromene was also a metabolite found in both leaves and inflorescence n-hexane extracts and we detected at the periods of 16:15,6min; 18:08,6min and 18:52,6min with a unique mass *m/z* = 231 and an exact mass of *m/z* = 314.2246.

Putative identification of compounds such as Neophytadiene found at the 13:51,7min retention time with a unique mass *m/z* = 68 and exact mass *m/z* = 278.2974 and 2-Pentadecanone, 6,10,14-trimethyl- detected at 13:55,8min with a unique mass *m/z* = 58 and exact mass of *m/z* = 268.2766 can be found in both the *n*-hexane and ethyl acetate mature inflorescence extracts. 2-Pentadecanone, 6,10,14-trimethyl- can also be found in the vegetative leaves ethyl acetate extracts. Phytol which was detected at 16:32,6min with a unique mass of *m/z* = 71 and exact mass of *m/z* = 296.3079 as well as Hexadecanamide detected at 18:57,4min with a unique mass *m/z* = 267 and an exact mass of *m/z* = 255.2562 were found in both vegetative leaves and mature inflorescence n-hexane and ethyl acetate extracts. Hexadecanamide was also found at 17:10,0min in the ultra-pure water leaves and inflorescence extracts. At the period point 15:13,5min and 17:08,5min Dodecanamide was found with a unique mass of *m/z* = 59 and exact mass of *m/z* = 199.1936 as well as Oleanitrile was found at 16:16,5 with a unique mass of *m/z* = 55 and exact mass of *m/z* = 263.2613, in both the leaves and inflorescence ultra-pure water extracts.

Table 8.4: A list of compounds annotated from *Cannabis indica/sativa* vegetative leaves and mature inflorescence samples analysed by two-dimensional gas chromatography – time of flight – mass spectrometry (GC x GC –TOF-MS), showing the retention time (R.t), name of the compound, unique mass (mass-to ratio: *m/z*), proposed area and the exact mass (mass-to ratio: *m/z*). The table also displays the *Cannabis* extracts where the tentative metabolites were present: vegetative leaves *n*-hexane (LHEX), mature inflorescence *n*-hexane (IHEX), vegetative leaves ethyl acetate (LEA), mature inflorescence ethyl acetate (IEA), vegetative leaves ultra-pure water (H_2O) and mature inflorescence ultra-pure water (H_2O).

R.t (min:sec)	**Name**	**Unique Mass (*m/z*)**	**Area**	**Exact Mass (*m/z*)**	**LHEX**	**IHEX**	**LEA**	**IEA**	**LH$_2$0**	**IH$_2$0**
04:01,2	D-Limonene	68	5952646	136,1252		✓				
04:19,1	4-Methyl-1,5-Heptadiene	69	377108	110,1096		✓				
04:48,9	Fenchone	81	931731	152,1201		✓				
04:52,0	Linalool	71	9955465	154,1358		✓				
04:55,6	Nonanal	57	8661030	142,1358		✓				
05:07,7	Fenchol, exo-	95	7628548	154,1358		✓				
06:07,8	Terpineol	59	9034054	154,1358		✓				
06:54,6	2,6-Octadien-1-ol, 3,7-dimethyl-, (Z)-	69	1278018	154,1358		✓				
08:36,2	3-Methyl-3-buten-1-ol, acetate	68	4205205	128,0837		✓				
08:37,4	Hexanoic acid, hexyl ester	117	4205205	200,1776		✓				
09:14,2	Caryophyllene	93	1,48E+08	204,1878		✓				

09:21,3	trans-à-Bergamotene	93	13582431	204,1878	✓
09:27,3	Methyl 11,12-tetradecadienoate	68	886342	238,1933	✓
09:32,9	(E)-á-Famesene	69	7469962	204,1878	✓
09:39,6	Humulene	93	28643159	204,1878	✓
09:45,3	Aromandendrene	105	1195413	204,1878	✓
09:54,2	ç-Muurolene	161	5329293	204,1878	✓
09:55,1	6-Epishyobunone	150	5329293	220,1827	✓
09:58,1	Acetamide, 2-phenylamino-N-(4-phenyl-3-furazanyl)-	106	105936	294,1117	✓
10:52,0	Nerolidol	69	2578951	222,1984	✓
10:56,5	5,9-Undecadien-2-one, 6,10-dimethyl-, (E)-	55	2774578	194,1671	✓
10:56,8	(3S,4aR,5S,8aS)-4a,5-Dimethyl-3-(prop-1-en-2-yl)-2,3,4,4a,5,6-hexahydronaphthalen-1(8aH)-one	81	2774578	218,1671	✓
10:59,5	(R,Z)-2-Methyl-6-(4-methylcyclohexa-1,4-dien-1-yl)hept-2-en-1-ol	132	659504	220,1827	✓
11:09,1	Cyclohexene, 4-isopropenyl-1-methoxymethoxymethyl-	134	3861997	196,1463	✓

11:15,1	Caryophyllene oxide	79	14496108	220,1827	✓
11:21,4	Bicyclo[5.1.0]octane, 8-(1-methylethylidene)-	135	1222351	150,1409	✓
11:29,9	Trifluoroacetyl-lavandulol	69	21574	250,1181	✓
11:33,1	(1R,3E,7E,11R)-1,5,5,8-Tetramethyl-12-oxabicyclo[9.1.0]dodeca-3,7-diene	96	4991214	220,1827	✓
11:37,1	Cyclopentene, 1,2,3,3,4-pentamethyl-	123	2653034	138,1409	✓
11:37,6	Pyrimidine, 4-(2-hydroxy-5-methoxyphenyl)-	187	2653034	202,0742	✓
11:38,3	2-Cyclopenten-1-one, 3-methyl-2-(2,4-pentadienyl)-, (Z)-	59	2653034	162,1045	✓
11:42,2	(E)-2-((8R,8aS)-8,8a-Dimethyl-3,4,6,7,8,8a-hexahydronaphthalen-2(1H)-ylidene)propan-1-ol	202	3719551	220,1827	✓
11:45,1	cis-Z-à-Bisabolene epoxide	125	4822052	220,1827	✓
11:45,4	4,4-Dimethyl-3-(3-methylbut-3-enylidene)-2-methylenebicyclo[4.1.0]heptane	136	4822052	202,1722	✓
11:48,4	Caryophylla-4(12),8(13)-dien-5à-ol	123	2788501	220,1827	✓

11:50,7	11,11-Dimethyl-4,8-dimethylenebicy-clo[7.2.0]undecan-3-ol	136	4524301	220,1827	✓
11:51,8	Tetracyclo[6.1.0.0(2,4).0(5,7)]nonane, 3,3,6,6,9,9-hexamethyl-, cis,cis,trans--	161	4524301	204,1878	✓
11:55,0	1-Adamantanemethanol	135	248185	166,1358	✓
11:56,6	trans-Valerenyl acetate	187	6907802	262,1933	✓
11:57,1	Caryophyllene oxide	84	6907802	220,1827	✓
11:57,8	Octanoic acid, 3-hexenyl ester, (Z)-	82	8056784	226,1933	✓
11:59,3	Bicyclo[2.2.2]oct-5-en-2-ol	80	698936	124,0888	✓
12:01,5	3,5,11-Eudesmatriene	187	11169273	202,1722	✓
12:04,0	Caryophyllene oxide	137	2024808	220,1827	✓
12:05,4	(3S,4R,5R,6R)-4,5-Bis(hydroxyme-thyl)-3,6-dimethylcyclohexene	100	3507507	170,1307	✓
12:09,5	4a,5-Dimethyl-3-(prop-1-en-2-yl)-1,2,3,4,4a,5,6,7-octahydronaphthalen-1-ol	202	455189	220,1827	✓
12:12,0	8-Decen-2-one, 9-methyl-5-meth-ylene-	95	4612973	180,1514	✓

12:12,8	Caryophyllene oxide	92	4612973	220,1827	✓				
12:13,6	Naphthalene, 1,2,4a,5,8,8a-hexahydro-4,7-dimethyl-1-(1-methylethyl)-, (1à,4aá,8aà)-(ñ)-	204	4612973	204,1878	✓				
12:17,8	à-Bisabolol	109	14223904	222,1984	✓				
12:21,6	Methoxyolivetol	138	388465	194,1307	✓				
12:39,8	2,6,10-Dodecatrien-1-ol, 3,7,11-trimethyl-, (Z,E)-	69	82758	222,1984	✓				
13:15,3	Methyl 10,11-tetradecadienoate	82	452331	238,1933	✓				
13:48,6	2-Hexadecene, 3,7,11,15-tetramethyl-, [R-[R*,R*-(E)]]-	70	908380	280,313		✓			
13:51,7	Neophytadiene	68	1859427	278,2974	✓	✓	✓		
13:55,8	2-Pentadecanone, 6,10,14-trimethyl-	58	932781	268,2766	✓		✓		
14:57,8	Isophytol	71	318486	296,3079		✓			
15:09,2	Phthalic acid, monoamide, N-ethyl-N-(3-methylphenyl)-, hexyl ester	149	398913	367,2147	✓				
15:12,7	Butanal, oxime	59	71859	87,0684	✓				
15:13,5	Dodecanamide	72	956	199,1936				✓	✓
16:16,5	Oleanitrile	55	3011225	263,2613				✓	✓

17:08,5	Dodecanamide	59	7860638	199,1936					✓	✓
16:15,8	Cannabichromene	231	290982	314,2246		✓				
16:23,0	Eicosane	57	188170	282,3287		✓				
16:32,6	Phytol	71	2146624	296,3079	✓	✓	✓	✓		
16:54,8	Furo[2,3-H]coumarine, 2-(1-hydroxy-ethyl)-1,6-dimethyl-	175	1606516	258,0892		✓				
17:10,0	Hexadecanamide	59	8110885	255,2562	✓	✓			✓	✓
18:08,6	Cannabichromene	231	3179595	314,2246		✓				
18:25,9	ë9-Tetrahydrocannabivarin	203	5208839	286,1933		✓				
18:29,7	Isothiourea, 2-methyl-1-(2,4-dime-thylphenyl)-3-(1,1-dimethyl-2-propynyl)-	245	3614474	260,1347	✓	✓				
18:35,7	1H-4-Oxabenzo(f)cyclobut(cd)inden-8-ol, 1a-à,2,3,3a,8b-à,8c-à-hexahydro-1,1,3a-trimethyl-6-pentyl-	231	1936523	314,2246	✓	✓				
18:39,8	4,8,12,16-Tetramethylheptadecan-4-olide	99	326694	324,3028	✓	✓				
18:47,3	9-Octadecenamide, (Z)-	59	95255499	281,2719	✓	✓	✓	✓	✓	✓
18:52,6	Cannabichromene	231	3135992	314,2246	✓	✓				
18:57,4	Hexadecanamide	267	3237269	255,2562	✓	✓	✓	✓		

19:14,9	Cannabispiran	176	1049629	246,1256	✓	✓	
19:18,5	Cannabidiol	246	6346797	314,2246		✓	
19:47,6	Δ^9-Tetrahydrocannabinol, TMS derivative	303	4824131	386,2641	✓	✓	
19:54,9	Δ^8-Tetrahydrocannabinol	314	4172349	314,2246		✓	
20:08,0	1-Triisopropylsilyloxyheptadecane	369	2,24E+08	412,41		✓	
20:09,4	11-Hydroxy-.DELTA.9-tetrahydrocannabinol	299	4,37E+08	330,2195	✓	✓	
20:12,2	Δ^8-Tetrahydrocannabinol	313	1,89E+09	314,2246		✓	
20:13,5	Dronabinol	81	1,86E+09	314,2246	✓	✓	✓
20:16,3	Morphinan-3-ol, 6,7,8,14-tetradehydro-4,5-epoxy-6-methoxy-17-methyl-, (5à)-	297	1,05E+08	297,1365		✓	
20:26,4	Cannabinol, TMS derivative	367	9317849	382,2328		✓	
21:40,4	11-Hydroxycannabinol	311	3909444	326,1882	✓	✓	
22:25,3	Farnesol isomer a	69	4026026	331,81	✓		
22:47,0	Norgestrel	245	2636972	312,2089		✓	
22:50,1	1-Docosene	57	796695	308,3443		✓	
22:54,8	Nonacos-1-ene	55	3805764	406,4539		✓	

23:02,9	Octacosane	57	5767587	394,4539		✓
23:51,8	Octadecane, 2-methyl-	57	509358	268,313		✓
25:20,9	Nonacos-1-ene	83	33132	406,4539		✓
29:51,7	á-Sitosterol	55	8750318	414,3862	✓	

8.5 Discussion

Thus far, there are more than five hundred (500) chemical constituents identified from the *Cannabis* plant (Radwan *et al.*, 2021), one hundred and four (104) metabolites were identified in this experiment. Many compounds which were detected in this chapter are known to posess biological activities as seen in previous studies. The annotated analysis of this experiment detected all one hundred and four (104) metabolites from the mature inflorescence hexane extract alone. These metabolites included terpenes, terpenoids, cannabinoids, fatty acids, fatty nitriles and sterol lipids which are known to be biologically active.

According to dos Reis *et al*. (2019), a study by (Hameed *et al.*, 2016), observed that 9-Octadecenamide, (Z)- has antibacterial and antifungal activity against *Klebsiella pneumonia* and *Aspergillus flavus* from *Cinnamomum Zeylanicum* methanol extracts. Dos Reis *et al*., (2019) found that 9-Octadecenamide, (Z)- was the main compound eluded by chemical characterization using gas chromatography in hexane: ethyl acetate (50:50) solvent system and ethyl acetate solvent from *Diaporthe schini*. These extracts showed strong antibacterial activity against *Staphylococcus epidermidis* with a minimum inhibitory concentration (MIC) of 7.81µg/mL and antifungal activity against *Candida krusei* of 3.9µg/mL. This statement can be seen throughout this study, with extracts containing these compounds displaying antifungal activity against various *Candida* species as well as antibacterial activity. This is also true for the 9-Octadecenamide, (Z)- compound, (**Appendix: Figure 10.16 – Figure 10.20**) which was found in all six *Cannabis* extracts. Extracts such as the ethyl acetate vegetative leaves and inflorescence showed both good antifungal activity against *Candida* species with low MIC values = 31.25µg/mL and anti-mycobacterial activity with low MIC values = 31.25µg/mL against *Mycobacterium aurum* and *Mycobacterium smegmatis.*

Cannabinoids are a large and complex group of phytochemicals distinct to the *Cannabis* plant, these compounds are not only abundant in the plant but are also said to have large antimicrobial activity (Karas *et al*., 2020). Compounds such as cannabidiol (CBD), cannabichromene (CBC), Δ^9-tetrahydrocannbinol (Δ^9-THC) and cannabinol (CBN) (**Appendix: Figure 10.16 and Figure 10.17**) (**Appendix: Table 10.1**) are a few examples of these compounds and are a few which were found in this study. In a study reported by Wassmann *et al.*, (2020), it was found that CBD has effective antibacterial activity against *Staphylococcus aureus* with an MIC of 4µg/mL. According to Klahn, (2020), Δ^9-THC was evaluated for antibacterial activity against *Streptococcus pyogenes* with an MIC of 5µg/mL, *Streptococcus anginosis* group with an MIC

of 25µg/mL and *Enterococcus faecalis* with a MIC of 5µg/mL. These results from previous studies are able to support the antibacterial and antifungal activities seen in the study. These compounds are found mainly in both hexane inflorescence and leaves, this act as the driving force behind the antimicrobial activity which can be seen in both *Candida spp.* and *Mycobacterium spp.* However, the activity was better in *Candida glabrata* where the MIC = 250µg/mL, which is equivalent to the value found for amphotericin B.

Terpenes are a large group of hydrocarbons consisting of 5-carbon isoprenes. Most terpenes have been reported to possess a reduced antimicrobial activity in comparison to their derivative terpenoids which have powerful bacteriostatic activity due to the modification of a functional group (Mahizan *et al*., 2019). For example, the removal of a methyl group and addition of an oxygen plays to the derivatization of the terpene molecule, this becomes the determining factor for its antimicrobial activity (Mahizan *et al*., 2019). A few notable terpenes and terpenoids which were found in the extracts include α-Humulene which is known to show antifungal activity against *Aspergillus niger and* antibacterial activity against *Staphylococcus aureus* and *Bacillus cereus* (Ali *et al*., 2017). Limonene which is an excellent antibacterial against food pathogens such as *Listeria monocytogenes* with a minimum inhibitory concentration (MIC) of 0.039 ± 0.3 mg/mL (Han *et al*., 2019), β-Caryophyllene has antibacterial activity against *Staphylococcus aureus* with an MIC 3 ± 0.4µM, *Escherichia coli* 9 ± 2.2µM, *Bacillus subtillis* 8 ± 2.1µM, *Bacillus cereus* 9 ± 1.1µM, *Klebsiella pneumonia* 14 ± 2.7µM, *Pseudomonas aeruginosa* 14 ± 2.7µM and antifungal activity against *Aspergillus niger 6 ± 0.8µM* as well as *Penicillium citrinum 7 ± 1.2µM* (Dahham *et al., 2015)*. These compounds are found in all of the tentatively analysed extracts and supports their antimicrobial potential and results observed in this study for both *Candida spp.* and *Mycobacterium spp.*

In a study conducted by Dahham *et al*., (2015) it was found that β-Caryophyllene had radical scavenging activity similar to that found in ascorbic acid by using the DPPH assay where β-Caryophyllene had an inhibitory concentration (IC_{50}) of 1.2 ± 0.06µM and ascorbic acid 1.5 ± 0.03µM. Caryophyllene was also detected in the n-hexane inflorescence extract of this study (**Appendix: Figure 10.17**) (**Appendix: Table 10.1**) which also showed antioxidant activity respectable in a DPPH assay was conducted and an IC_{50} of 56.0 ± 2.8µg/mL was established. Although the activity is thought to be far off as compared to the positive control used in the study it is still a value that is able to play a significant role in radical scavenging. A common compound which was found in both vegetative leaves and mature inflorescence ethyl acetate

extracts was phytol. Phytol is an acyclic monosaturated diterpene alcohol abundantly found in all green vegetables, vitamin E and K (de Moraes *et al*., 2014) (**Appendix: Figure 10.18**). According to Baskar *et al*., (2012), β-Sitosterol (**Appendix: Figure 10.16**) is a sterol which is a naturally occurring molecule, this compound is known to exhibit moderate antioxidant activity by decreasing the levels of reactive oxygen species. β-Sitosterol, was identified in vegetative leaves n-hexane extracts, these extracts showed 356.8 ± 11.5µg/mL which is what can be measured as a considerable moderate antioxidant activity in comparison to the other tested *Cannabis* extracts. In a study by Santos *et al*., (2013), there was evidence which showed that this compound is an excellent hydroxyl radical remover, a hydroxyl radical scavenging assay was performed and showed a 9.66% removal increase at a concentration of 0.9ng/mL in comparison to other tested concentrations. When referring to the current study performed it can be demonstrated that claim of ethyl acetate inflorescence extract antioxidant potential by the IC_{50} as revealed in a DPPH scavenging assay by the with a IC_{50} = 34.8 ± 12.3µg/mL.

Cannabinoids have been known to be useful tools for the apoptotic effect of cancer cells leading to their death and the inhibition of cancerous cell proliferation. Through the signalling pathways of non-receptors and receptors, cannabinoids tend to show cytotoxicity towards cancerous cells and protect healthy tissue from apoptosis during cancer research (Cerretani *et al*., 2020). African Green Monkey kidney cells (Vero) and murine macrophages (RAW264.7) were evaluated in this study, both of which experienced no toxic effects from any of the tested *Cannabis* extracts. Terpenes such as linalool have also exhibited low cytotoxic effects against normal human skin fibroblast cells (BJ cells) with a lethal concentration (LC_{50}) = 0.599 ± 0.010nM and green money kidney cells (GMK) with LC_{50} = 1.139 ± 0.012mM (Wojtunik-Kulesza *et al*., 2017), this is supported in this study as the *n*-hexane mature inflorescence extract which contains this compound has also shown low probability of being toxic with a LC_{50} = 1 119.75 ± 25.6µg/mL for Vero cells and LC_{50} = 783.54 ± 18.6µg/mL for RAW264.7 cells.

8.6 Conclusion

Although there is an array of studies based on the beneficial effects of *Cannabis* and its multitude of chemical constituents, research on the plant is continuous as well as progressive. In this chapter, the various phytochemical constituents found in *Cannabis* were evaluated using two dimentional gas chromatography – time of flight – coupled with mass spectrometry. Six *Cannabis inidca/sativa* extracts, both vegetative leave and mature inflorescence *n*-hexane, ethyl acetate and ultra-pure water extracts were evaluated. Many of the compounds found in this

analysis have been proven by other studies to have antimicrobial activity. Their activity is confirmed and supported by the results found in previous chapters that the *Cannabis indica/sativa* plant has antioxidant, antifungal and anti-mycobacterial activity, as well as demonstrates non-cytotoxic activity.

8.7 References

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Chapter 9

Discussion and conclusion

9.1 Discussion

The emergence of antimicrobial resistance (AMR) has become one of the principle health problems preventing the effective treatment of microbial infections (Prestinaci *et al.*, 2015). This phenomenon occurs when parasites, fungi, viruses and bacteria no longer respond to medicines used to treat them due to their change in genetic makeup (World Health Organisation, 2020). According to Read and Woods (2014), judicious use of antibiotic treatments is necessary for antimicrobial resistance management as an attempt to decrease the rate at which the resistance spreads. Thus resulting in the negative impact on disease control instead of treatment (Ventola, 2015).

Fungal and bacterial species are prominent cases in antimicrobial resistance. Species classified in the genus *Mycobacterium* and *Candida* are amongst many microbial species which are causative agents of global significant diseases – these species can be non-pathogenic or pathogenic and live in aquatic settings and soil as well as animal insect and plant surfaces (Robinson and Huppler, 2017). Candidiasis is an invasive fungal infection caused by the fungi *Candida* (Hazen and Howell, 2007). *Candida* species usually exist as commensal fungi and often present as pathogenic organisms (Silva *et al*., 2012). Although *Candida albicans* is known as the common cause of candidiasis, *Candida* infections caused by non-*Candida albicans Candida* (NCAC) species have become more common (Turner and Butler, 2014). *Mycobacteria* is a genus of bacteria belonging to the family Mycobacteriaceae which is known to cause debilitating infections found in humans (Vilchèze *et al*., 2016), animals and is also found in soil (Walsh *et al*., 2019). Historically, *Mycobacterium* infections are known to be caused by *Mycobacterium tuberculosis* (MTB), however more infections have now been attributed to *Mycobacterium* other than tuberculosis (MOTT) (Johnson and Odell, 2014). Although, *Mycobacterium* and *Candida* species do not share any commonalities from a biological perspective; however, they do share a chronic colonization trait in humans and prompt disease in a number of colonized humans, this can be seen in the Goldilocks model of both microorganisms (Robinson and Huppler 2017).

Both medicinal plants and repurposing of drugs play a vital and important role in drug discovery as both methods have aided in casting a wider net to find compatible drugs in order to aid

in the treatment of common and rare diseases. Medicinal plants are those species of plants traditionally used for medicinal purposes and contain beneficial chemical constituents that are important in healing diseases in both animals and humans. Ethnopharmacology is the study of the use of plants as forms of medicine binding knowledge from indigenous people (Yeung *et al.*, 2018). The medicinal plant used in this study is *Cannabis*; a single genus of plants that belongs to the family Cannabaceae (Elsohly and Slade, 2005). Outside of its psychoactive characteristics, *Cannabis* has been used for centuries for its medicinal properties; it is known for managing an assortment of ailments such headaches, neuralgia, dysuria, mania (Biosci *et al*., 2014), muscle spasms, whooping cough, pain, inflammation, (McPartland, 2018) anxiety and epilepsy (Gloss, 2015) to name a few. This study aimed to identify drugs that validate traditional medicinal plant use and simultaneously treat or manage diseases in patients (Anand *et al*., 2019). There are a few Food and Drug Administration (FDA) approved drugs which use *Cannabis* derived constituents, one of them being Sativex® which is synthesised from cannabidiol and delta-9-tetrahydrocannabinol (Δ^9-THC), this drug was approved as an analgesic for patients with advanced cancer and multiple sclerosis (Novotnaa *et al.*, 2011). Another approved drug is Marinol® which is derived from dronabinol and has been approved for Acquired Immune Deficiency Syndrome (AIDS) related anorexia, appitite stimulation and nausea (Gaisey and Narouze, 2021).

The study also focused on the repurposing of isoniazid derivatives, isoniazid has been deemed one of the most effective drug used for latent tuberculosis since its introduction for the treatment of tuberculosis in 1952 (Ando *et al*., 2011). According to Pareek *et al.*, (2020), drug repositioning or repurposing is the process of exploring new uses for existing drugs which have been developed. Drug development and discovery is a slow and tedious process, high costs and rates of abrasion are the main cause of this, a popular trend for rare and common diseases is the repurposing of drugs (Pushpakom *et al*., 2019). The justification of drug repurposing is based on the fact that some diseases share similar molecular pathways enabling one drug to work for a disease it wasn't originally designed for (Sultana *et al*., 2020). One of the most successful and well-known drugs which were repurposed include minoxidil, valproic acid, sildenafil, aspirin and methotrexate to name a few. Sildenafil was originally designed and developed for the treatment of angina pectoris and hypertension, however this drug is currently used for the treatment of erectile dysfunctions (Rudrapal *et al.*, 2020). Apirin was originally designed for the treatment of inflammation, pain and fever; however, this drug has been repurposed for the management of various cardiovascular diseases (Kumar and Bhusan, 2019).

Medicinal and dietary plants have the ability to produce natural antioxidants (Qasim *et al*., 2017), antioxidants are defined as bioactive compounds that cause inhibition or limitation of nutrient oxidation and can be categorized as synthetic or natural (Altemimi *et al*., 2017). Antioxidant compounds can either be natural or synthetic, these compounds fall under a few known chemical classes; these natural antioxidative classes of compounds include flavonoids, phenols, carotenoids, tannins and alkaloids which may aid in the prevention of free radical oxidative damage (Qasim *et al*., 2017). It is also important to note that compounds with various functional groups tend to have stronger antioxidant activity. For example, phenolic groups are linked to antioxidant activity, this is justified by claims made by Chen *et al*., (2020), suggesting that the position of phenolic hydroxyls in related possess compounds with antioxidant ability. In addition to this; Flora, (2009) claims that antioxidant activity is owed to the free hydroxyl group attached to an aromatic ring.

Antioxidants prevent and decrease the effects of oxidative stress. It is thus understandable why food and drugs possessing antioxidants are regarded as important for health management. Oxidative stress can be characterized by an increase in intracellular oxidizing species concentration. These species include reactive oxygen species which is accompanied by antioxidant defence capacity (Schumacher *et al*., 2011). Antioxidant capacity of both the *Cannabis* plant extract and isoniazid compounds was measured with inhibitory concentration at 50% (IC_{50}). At this concentration, the compound or plant extracts had scavenged or reduced 50% of the free radicals. The *Cannabis indica/sativa* plant extract tested in this study also showed antioxidant activity, the mature inflorescence of ethyl acetate extract displayed the best inhibitory concentration with an IC_{50} = 34.8 ± 12.3µg/mL in comparison to all the extracts and the best performing compound, the co-crystal *N*'-((4-aminophenyl) phenyl) methylene) isonicotinohydrazide · (salicylic acid) (IBS 010) where IC_{50} = 105.4 ± 1.1µg/mL was observed. When comparing the two, in this aspect the mature inflorescence *Cannabis* extracts had better activity in comparison to the compounds, but not to the positive control – ascorbic acid. The *Cannabis indica/sativa* extracts have good activity as the various compounds at their respective concentrations are able to synergistically work together to form good antioxidants. The results do not necessarily prove that the isoniazid derivatives are not worth pursuing due to their low antioxidant inhibitory concentration, if anything it prompts inquisition of how many compounds have been disregarded due to their weak antioxidant activity yet possessing powerful inhibitory properties against other microorganisms.

Drugs could be therapeutic agents; however, they are of no use if they are toxic to cells. It is important to test drugs against various cells to ensure that they do not cause adverse and irreversible toxicity to cells. Therefore, cell-based assays are used, these assays are important to screen a collection of plant extracts and/ or drug compounds in order to assess the effects of cell proliferation and exhibit cytotoxicity inevitably leading to cell death (Riss *et al*., 2013). Both cell lines evaluated in this study play a very important role in the body, as part of the innate immune system – macrophages play an important role in the detection and destruction of pathogens by the exhibition of phagocytosis (Viola *et al*., 2019). Drugs can be excreted in many ways by the human body, one of the most notable excretory routes is through urination (Taft, 2009). The kidney is a specialized organ responsible for waste excretion in the human body through the production of urine (Lote, 2012). Therefore, determining the toxic effects of a drug on kidney cells also play a crucial role in ensuring non-toxic excretion which may ultimately lead to kidney dysfunction. A (3- [4,5-dimethylhiazol-2-yl]-2,5-diphenyl tetrazolium bromide) (MTT) assay was performed to analyse cell proliferation concentrations and a Real-Time Cell Analysis (RTCA) assay was conducted to evaluate the rate at which these cells proliferate. The results obtained in a study conducted by Mooko *et al*., (2021), where extracts of *Cannabis sativa* L. flower and bud were evaluated for their cytotoxic properties and exhibited non-toxic effects to Vero cells which corresponds with what was obtained in the study. The *Cannabis* extracts did not show any toxicity towards both RAW264.7 and Vero cells, the same applies to the tested derivatives. The least toxic plant extract in this study was the n-hexane vegetative leaves extracts with LC_{50} = 2 404.6 ± 25.3µg/mL and the least toxic isoniazid derivative tested in the study was *N*'- ((4-aminophenyl) phenyl) methylene) isonicotinohydrazide (IBS 009) with LC_{50} = 300.27 ± 18.03µg/mL both these results were against Vero cells. The rate at which both cell lines proliferate was measured using the RTCA assay in the results it can be observed that both cell lines took between 4-10 hours before the proliferation process can begin. After 10 hours the cells began to detach and die, after this time frae the n-hexane vegetative leaves showed a plateau for the Vero cells until the experiment was terminated. The matre inflorescence ethyl acetate extract presented good activity for the plant extracts against the RAW264.7 cells with an LC_{50} = 1 073.16 ± 10.60µg/mL. Both *Cannabis* extracts and isoniazid derivatives were not toxic to either cell line, this is important as it creates a platform where both can be considered for prospective drug use without causing more harm than doing good.

Candida species are common fungal species and are causative of a wide range of non-life-

threatening communicable diseases and life-threatening invasive worldwide (Taei *et al.*, 2019). The rise in fungal infection occurrence has initiated the necessity for drugs as the present available drugs used for these infections have undesirable side effects or are ineffective against new and re-emerging fungal strains (Weitzman and Summerbell, 1995). The antifungal potential of both *Cannabis* extracts and the isoniazid derivatives was appraised using the minimum inhibitory concentration (MIC) testing assay. The vegetative leaves of ultra-pure water extract had the best activity against *Candida auris* with MIC = 62.5µg/mL. According to van Vuuren and Holl, (2017), the MICs obtained from the *Cannabis indica/sativa* extracts and modified isoniazid derivatives, they all meet the criteria to be considered as extracts with moderate and significant activity. The modified isoniazid derivatives *N*'-(bis(2-hydroxyphenyl) methylene) isonicotinohydrazide· (salicylic acid) (IBS 008), *N*'-(propan-2-ylidene) isonicotinohydrazide (IBS 013) and *N*'-(propan-2-ylidene) isonicotinohydrazide· (salicylic acid) (IBS 014) all had the best activity of MIC = 31.25µg/mL against *Candida auris* and *N*'-(bis(2-hydroxyphenyl) methylene) isonicotinohydrazide (IBS 007) with MIC = 31.25µg/mL against *Candida tropicalis*. A flow cytometry assay was conducted in order to effectively quantitate the effects of test samples on single-cell properties, cell granularity, cell size measurement, aspects of cell death or viability and most importantly cell apoptosis (Martin *et al*., 2017). These results validate the use of *Cannabis indica/sativa* and the repurposing of modified isoniazid derivative as antifungal agents as the PI stained gated percentage of cells confirmed that there were cells which exhibited proliferation. These results probe more investigation for not only using *Cannabis indica/sativa* as a possible treatment for candidiasis caused by non-*Candida albicans Candida* species, but also highlights the probability of repurposing of modified isoniazid derivatives for these fungal species. The modified isoniazid derivatives exhibited excellent antifungal activity in comparison to both *Cannabis indica/sativa* extracts, even better than the positive control used in the study. The isoniazid derivatives which were originally designed to be anti-mycobacterial drugs make great candidates to use as antifungal drugs, even more so than the agent – amphotericin B – currently used for the treatment of candidiasis and in this study.

Mycobacteria other than tuberculosis (MOTT) are a group of *Mycobacterium* primarily found in environmental places, such as natural water and soil (Walsh *et al*., 2019). Some species of *Mycobacteria* are found to be transmittable to animals (Jagielski *et al*., 2014) while others are pathogenic to humans and have the potential to cause stark non-pulmonary and pulmonary diseases such as central lines and bloodstream infections as well as infections of the skin and soft tissue (Hu *et al*., 2019). Anti-mycobacterial agents are in continuous need for the treatment

of drug resistant infections in both animals and humans, respectively. *Mycobacterium* antibiotic resistance has incited researchers to find new candidates for mycobacterial infections. (Schofs *et al.*, 2021). The antibacterial activity of various parts of *Cannabis indica/sativa* has been evaluated against many Gram-negative and Gram-positive and other microorganisms, most of which are multi-drug resistant (Schofs *et al.*, 2021). The minimum inhibitory testing method was used to determine the results of the *Cannabis* extracts to compare them against the positive controls, isoniazid and rifampicin, respectively. The *Cannabis indica/sativa* ethyl acetate extracts of both mature inflorescence and vegetative leaves in this study showed potential for anti-mycobacterial activity. The *Cannabis indica/sativa* vegetative leave ethyl acetate extracts showed the best activity against both *Mycobacetrium smegmatis* and *Mycobacterium aurum* with an MIC = 31.25µg/mL. The mature inflorescence ethyl acetate had good activity against *Mycobacterium bovis* with MIC = 62.5µg/mL. Although, the *Cannabis indica/sativa* did show anti-mycobacterial activity it wasn't enough to spark interest to continue investigating as the results were not better or equivalent to the positive control. This could be due to compounds not proving to be potent enough to show any inhibitory activity against MOTTS in synergy. It does not necessarily mean that the plant doesn't have activity as can be seen with the antioxidant testing, but the plant doesn't exhibit satisfactory anti-mycobacterial activity.

Medicinal plants have been the forefront of drug discovery due to their infinite number of compounds. Due to the low toxicity and therapeutic performance of medicinal plants, phytochemicals have been a key point of interest to pharmacists and pharmacologists (Akhtar *et al.*, 2018). Phytochemical analysis plays a significant role in the antioxidant, antimicrobial and cyto-protective properties of medicinal plants. Gas chromatography – gas chromatography – time of flight – mass spectrometry or GC x GC – TOF – MS; is a technique able to identify a higher number of compound and was used to conduct phytochemical investigation of the *Cannabis indica/sativa* plant. (dos Santos *et al*. 2015). Many of the compounds detected in this chapter are known to have biological activity as observed in earlier studies. A study conducted by Lewis *et al*., (2017) is one example where compounds such as Δ^8-tetrahydrocannabinol, Δ^9-tetrahydrocannabinol, cannabigerol and β-caryophyllene oxide to name a few were detected, this was done using the ultra-high performance liquid chromatography – mass spectrometry (UPLC-MS), these are also compounds which were identified in the annotated analysis conducted in this study. The annotated analysis of this experiment had detected a large number of metabolites from the mature inflorescence hexane extract, however the other extracts also proved to have very important metabolites. These metabolites included terpenes, terpenoids,

cannabinoids, fatty acids, fatty nitriles and sterol lipids which are known to have biological activity. Compounds such as terpenes (Mahizan *et al*., 2019) and fatty acids (Desbois and Smith, 2010) are known to have bacteriostatic and bacteriocidal activity when tested against various bacteriological pathogens and also displays antioxidant activity (González-Burgos and Gómez-Serranillos, 2012). Compounds under the class cannabinoids are also known to be potent antimicrobials (Karas *et al*., 2020) and antioxidants (Hacke *et al*., 2019). Terpenoids are also known to have potent antifungal activity (Rao *et al*., 2010).

In conclusion, the aim of the study was to test the antimicrobial activity of *Cannabis indica/sativa* extracts and modified isoniazid derivatives. The agents were also evaluated for antioxidant activity and cytotoxic activity. The results in the study show that both *Cannabis indica/sativa* mature inlorescence and vegetative leaves extracts had better antioxidant activity and a lower cell toxicity in comparison to the modified isoniazid derivatives. However, the plant could be studied for other microorganism as it had impressive antioxidant and cytotoxic potential. More cellular biology work and ancer studies can be based off of these results, mechanisms would be studied with one of the many compounds extracted from these extracs, this could open a new chapter on cell renewal and rejuvenation. The modified isoniazid compounds did display better antifungal activity in comparison to the standard positive control amphotericin B. This prompts and shifts interest to the repurposing of anti-mycobacterial drugs for the treatment of *Candida* fungal infections. The modified isoniazid derivatives which were used in this study had impressive activity and this activity has prompted an interest to investigate the mechanism and mode of action, it is recommended that the investigation on which they work to act as anti-*Candida* drugs be undertaken. The study also evaluated the potential use of *Cannabis indica/sativa* extracts for treatment of various infections caused by *Mycobacteria*. Although, this could have played out as a novel way to treat these infections as *Cannabis indica/sativa* has been dubbed an instigator for *Mycobacterium* pulmonary infections. *Cannabis indica/sativa* was limited in its potential to be an effective anti-mycobacterial, the results obtained from the study were not significant enough to call for it to be studied further. The current synthetic drugs were better inhibitors in comparison to the plant extracts. Overall, the study had met the main objectives it had set out to and a new path has been impressed to further investigate the treatment and management of *Candida* and *Mycobacterium* infections, respectively.

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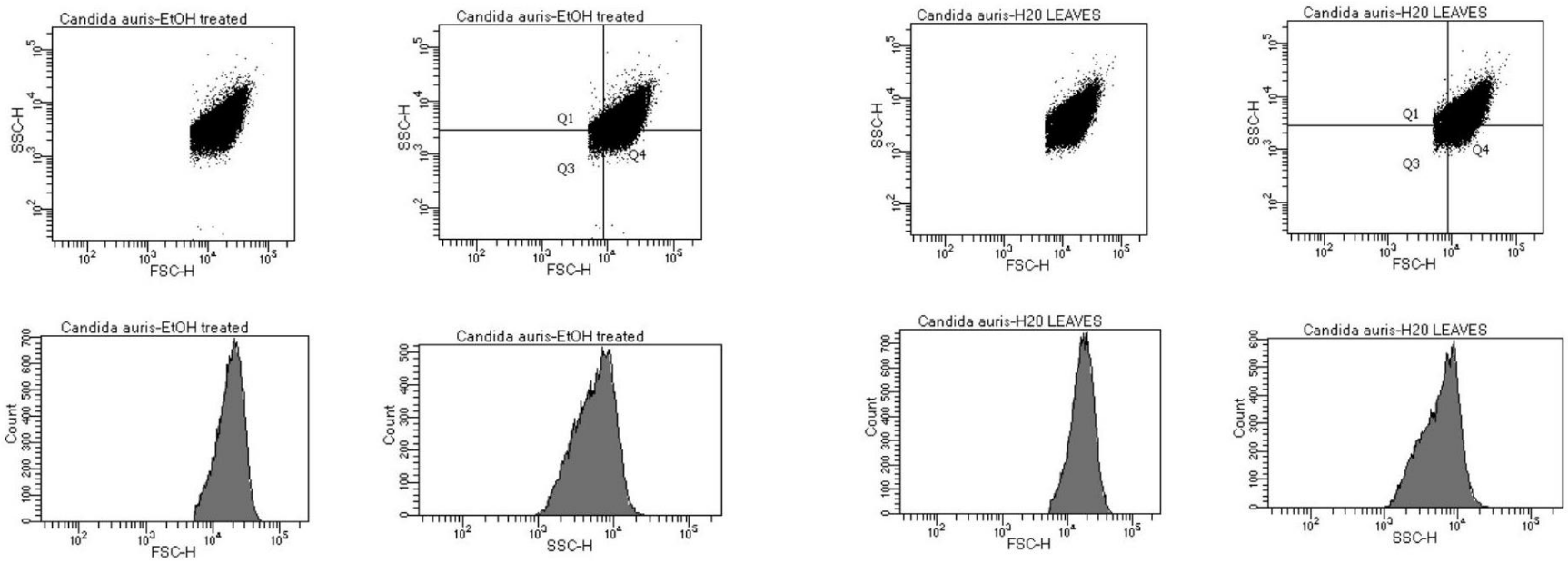
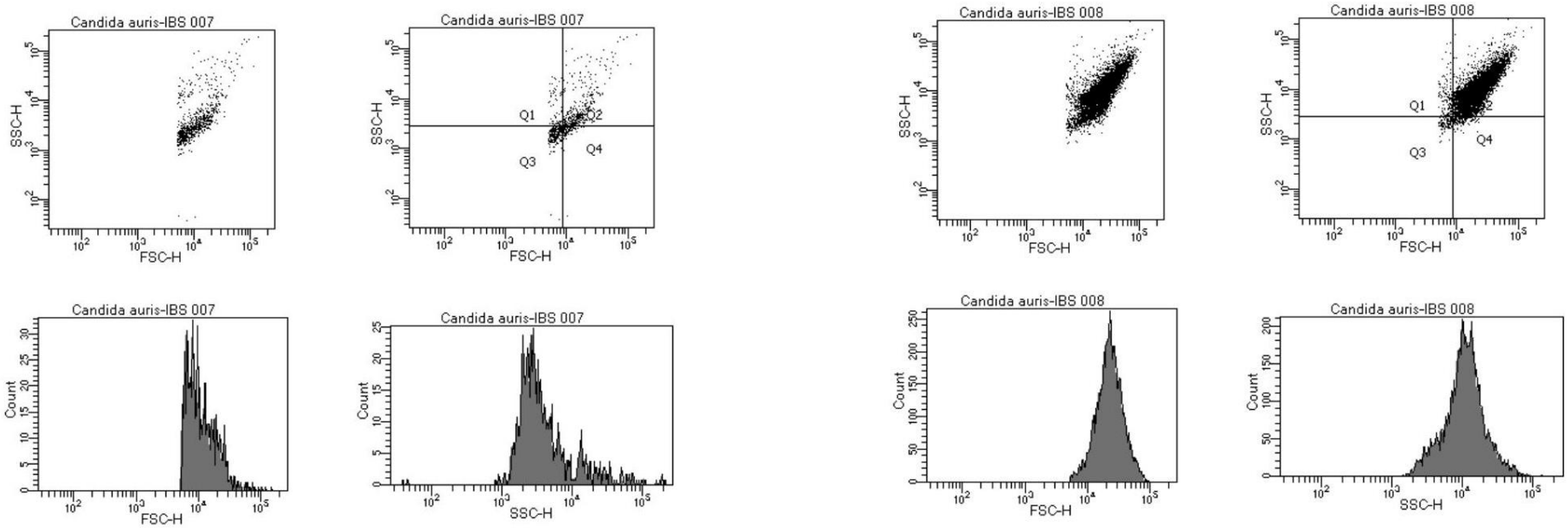
Chapter 10
Appendix



Tube Name:	EtOH treated			
Population	#Events	%Parent	FSC-H Mean	SSC-H Mean
All Events	20,000	####	18,414	6,101
Q1	912	4.6	7,153	3,857
Q2	15,711	78.6	20,219	7,102
Q3	862	4.3	6,849	1,889
Q4	2,515	12.6	15,183	2,105

Tube Name:	H20 LEAVES			
Population	#Events	%Parent	FSC-H Mean	SSC-H Mean
All Events	20,000	####	16,947	6,268
Q1	970	4.9	7,203	4,396
Q2	15,841	79.2	18,468	7,225
Q3	775	3.9	6,957	1,919
Q4	2,414	12.1	14,085	2,132

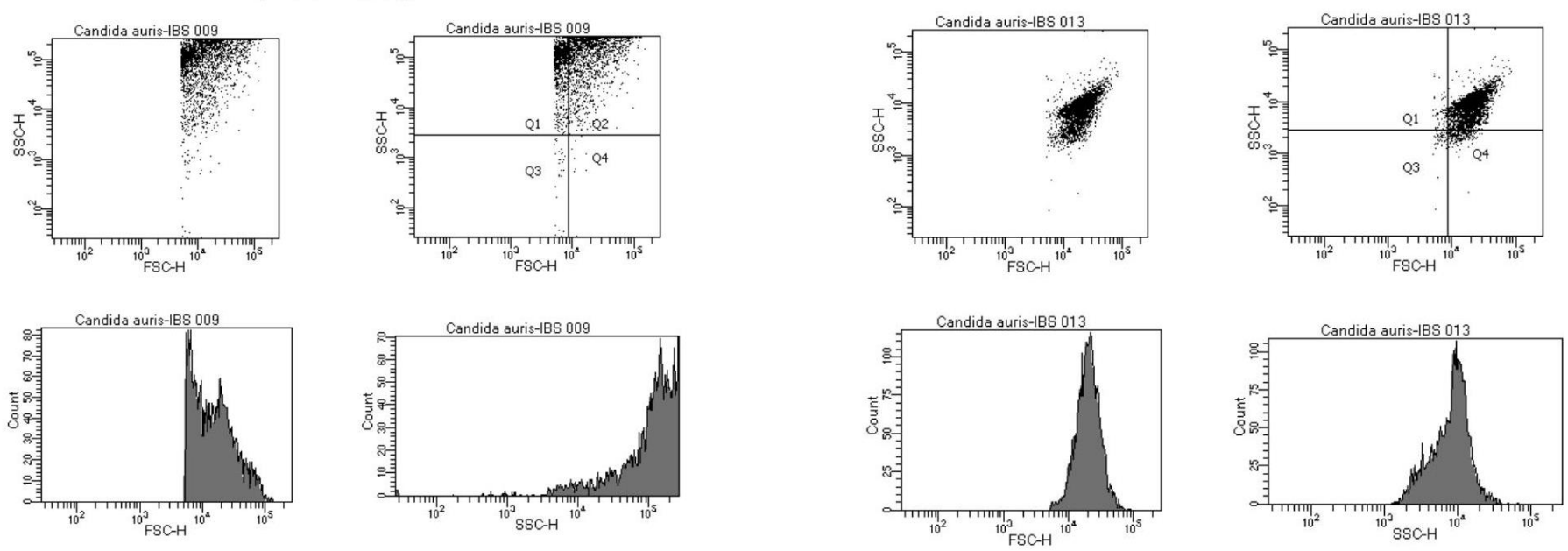
Figure 10.1: Flow cytometry scattergram obtained for *Candida auris* (CDC B11903) A. Positive control ethanol treated cells and B. Ultra-pure water vegetative leaves after 24-hour incubation.



Tube Name:	IBS 007			
Population	#Events	%Parent	FSC-H Mean	SSC-H Mean
All Events	746	####	13,126	8,345
Q1	76	10.2	6,781	8,587
Q2	335	44.9	19,931	14,598
Q3	243	32.6	6,616	1,937
Q4	92	12.3	10,782	2,302

Tube Name:	IBS 008			
Population	#Events	%Parent	FSC-H Mean	SSC-H Mean
All Events	7,001	####	24,111	12,943
Q1	165	2.4	7,032	9,178
Q2	6,551	93.6	25,169	13,504
Q3	139	2.0	6,836	2,103
Q4	146	2.1	12,412	2,364

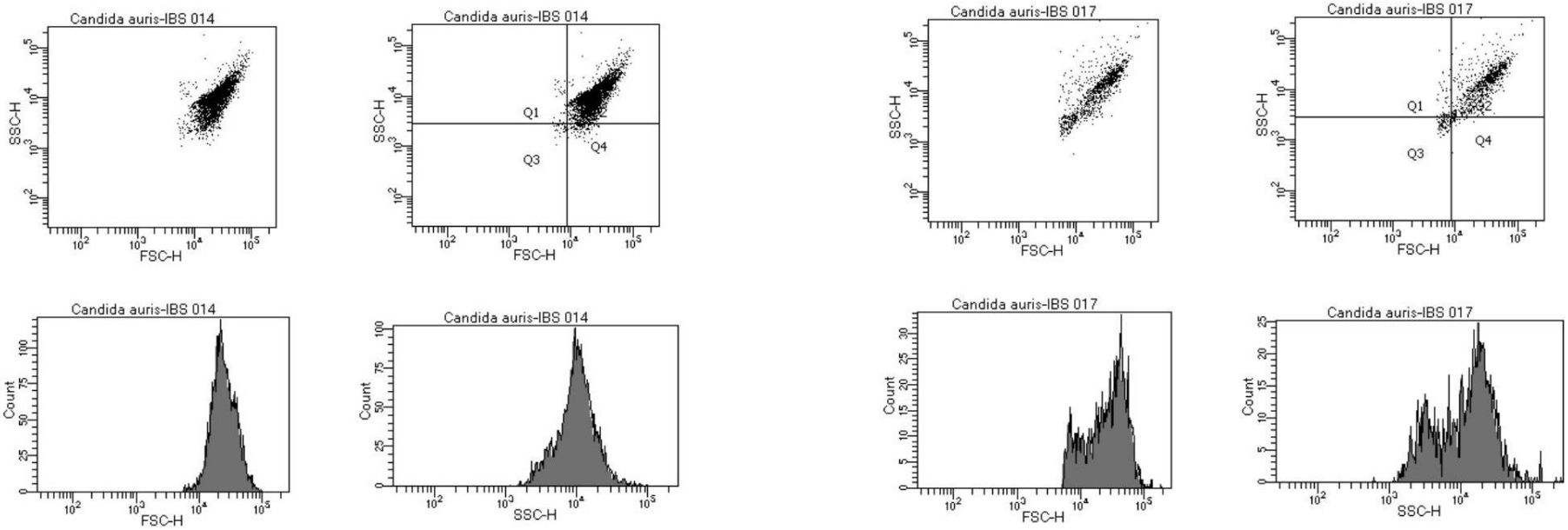
Figure 10.2: Flow cytometry scattergram obtained for *Candida auris* (CDC B11903) A. IBS 007 and B. IBS 008 after 24-hour incubation.



Tube Name:	IBS 009			
Population	#Events	%Parent	FSC-H Mean	SSC-H Mean
All Events	3,000	####	19,603	147,800
Q1	926	30.9	6,464	105,005
Q2	2,035	67.8	25,803	170,088
Q3	28	0.9	6,084	874
Q4	11	0.4	13,048	1,092

Tube Name:	IBS 013			
Population	#Events	%Parent	FSC-H Mean	SSC-H Mean
All Events	3,011	####	20,783	9,038
Q1	61	2.0	6,953	7,732
Q2	2,713	90.1	21,781	9,669
Q3	48	1.6	6,873	1,920
Q4	189	6.3	14,461	2,211

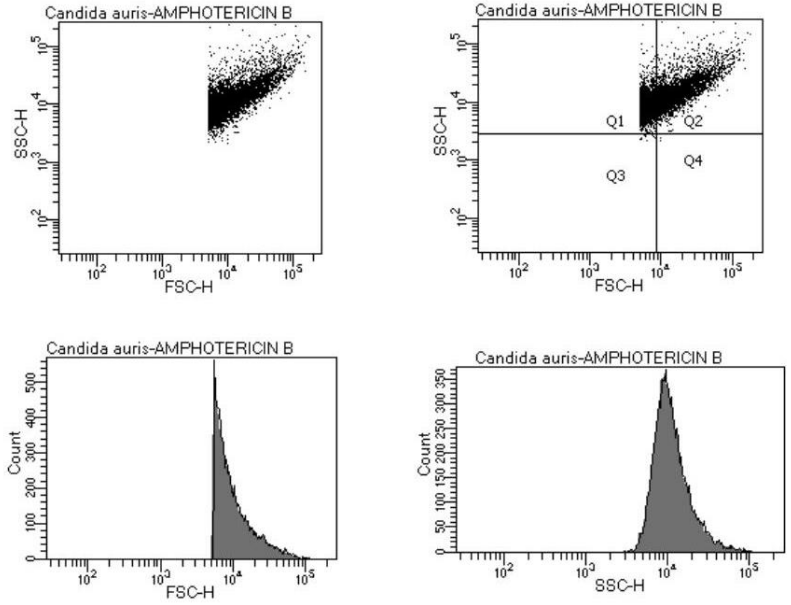
Figure 10.3: Flow cytometry scattergram obtained for *Candida auris* (CDC B11903) A. IBS 009 and B. IBS 013 after 24-hour incubation.



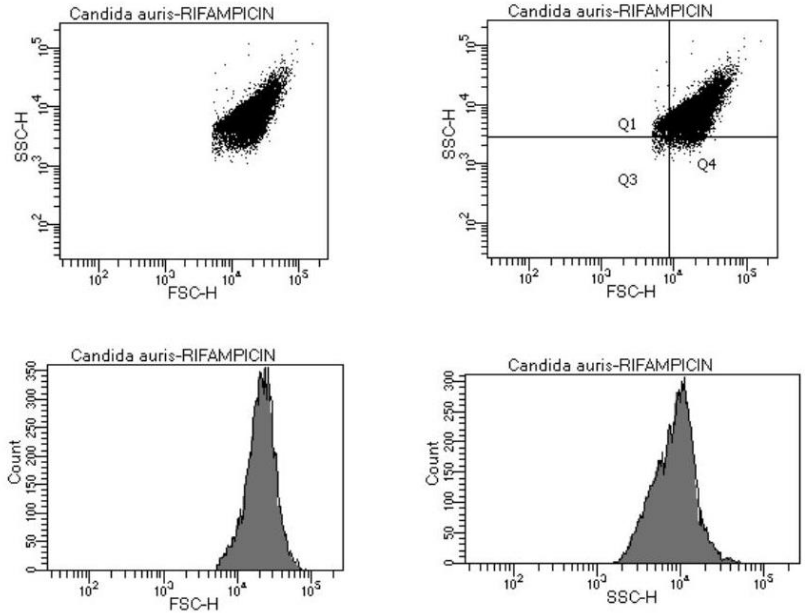
Tube Name:	IBS 014			
Population	#Events	%Parent	FSC-H Mean	SSC-H Mean
All Events	3,029	####	25,720	11,555
Q1	34	1.1	7,026	9,008
Q2	2,878	95.0	26,416	11,963
Q3	24	0.8	6,822	2,179
Q4	93	3.1	15,908	2,289

Tube Name:	IBS 017			
Population	#Events	%Parent	FSC-H Mean	SSC-H Mean
All Events	1,019	####	29,778	16,247
Q1	59	5.8	6,837	7,605
Q2	851	83.5	34,226	18,658
Q3	85	8.3	6,592	2,015
Q4	24	2.4	10,550	2,430

Figure 10.4: Flow cytometry scattergram obtained for *Candida auris* (CDC B11903) A. IBS 014 and B. IBS 017 after 24-hour incubation.

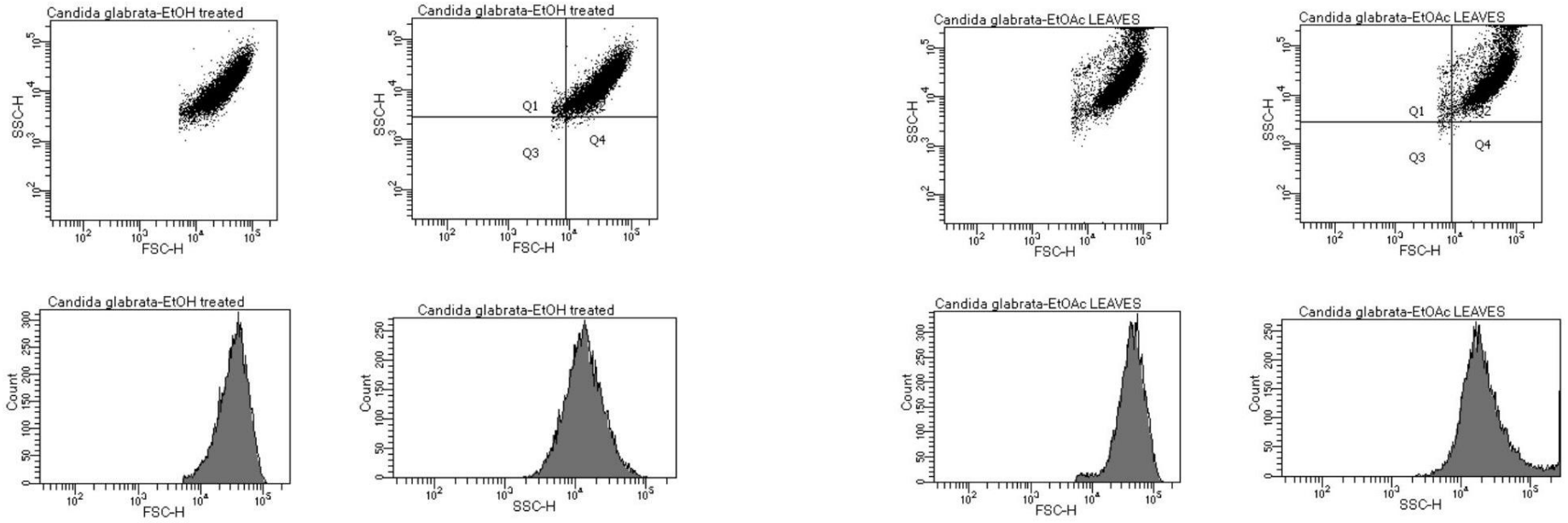


Tube Name:	AMPHOTERICIN B			
Population	#Events	%Parent	FSC-H Mean	SSC-H Mean
All Events	10,000	####	12,976	13,280
Q1	5,453	54.5	6,389	8,994
Q2	4,535	45.4	20,912	18,461
Q3	10	0.1	6,274	2,483
Q4	2	0.0	9,824	2,297



Tube Name:	RIFAMPICIN			
Population	#Events	%Parent	FSC-H Mean	SSC-H Mean
All Events	10,000	####	21,473	9,366
Q1	435	4.4	7,080	4,878
Q2	9,148	91.5	22,529	9,901
Q3	99	1.0	6,757	2,177
Q4	318	3.2	15,357	2,358

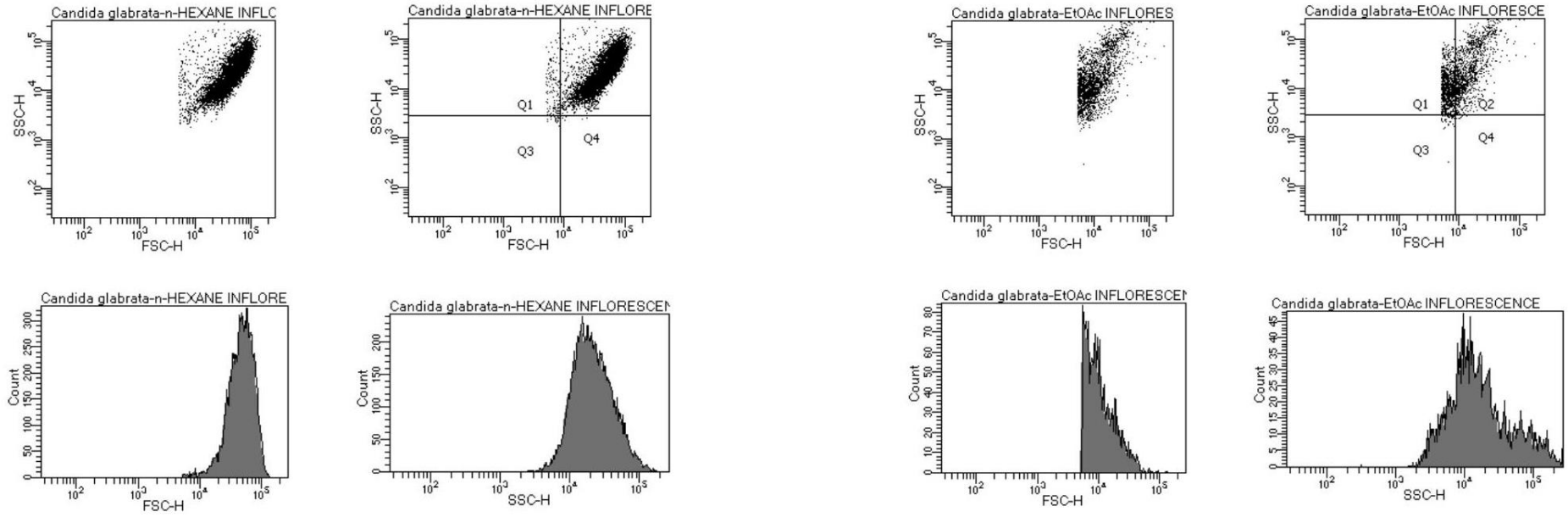
Figure 10.5: Flow cytometry scattergram obtained for *Candida auris* (CDC B11903) reference drugs A. Amphotericin B and B. Rifampicin after 24-hour incubation.



Tube Name:	EtOH treated			
Population	#Events	%Parent	FSC-H Mean	SSC-H Mean
All Events	10,000	####	35,044	15,595
Q1	239	2.4	6,982	4,497
Q2	9,682	96.8	35,947	15,978
Q3	43	0.4	6,272	2,231
Q4	36	0.4	12,850	2,409

Tube Name:	EtOAc LEAVES			
Population	#Events	%Parent	FSC-H Mean	SSC-H Mean
All Events	10,000	####	44,391	36,214
Q1	203	2.0	6,694	13,185
Q2	9,755	97.6	45,336	36,840
Q3	35	0.4	6,311	2,147
Q4	7	0.1	10,858	1,516

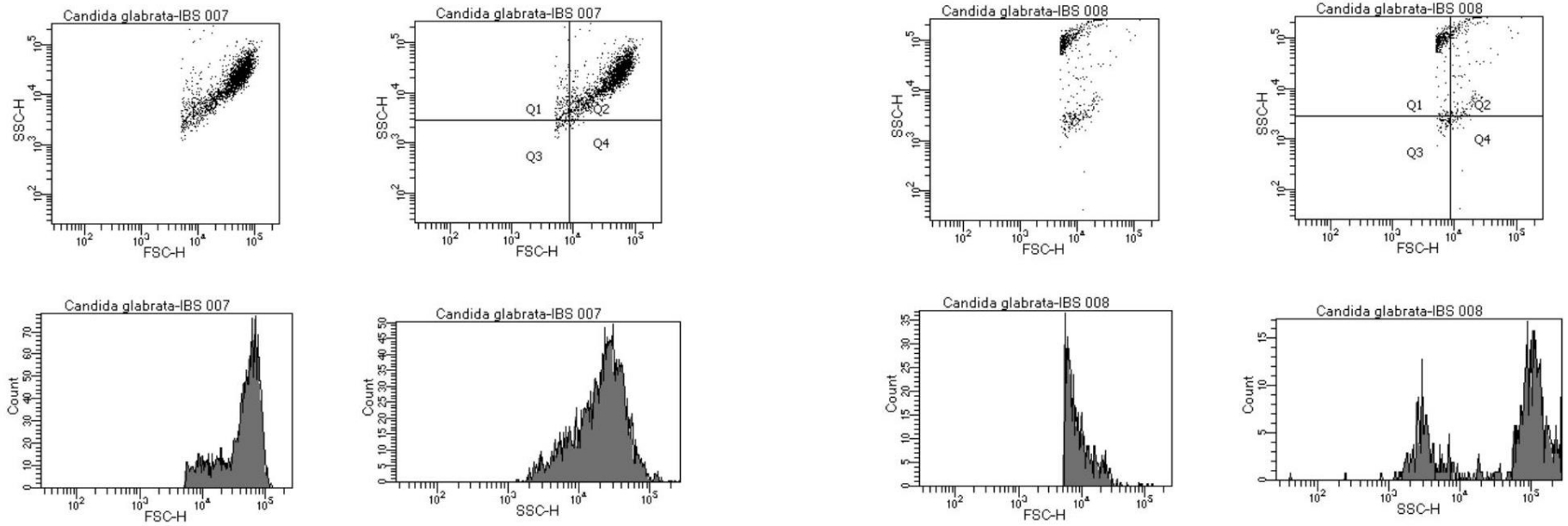
Figure 10.6: Flow cytometry scattergram obtained for *Candida glabrata* (ATCC 15126) A. Positive control ethanol treated cells and B. Ethyl acetate vegetative leaves after 24-hour incubation.



Tube Name:	n-HEXANE INFLORESCENCE			
Population	#Events	%Parent	FSC-H Mean	SSC-H Mean
All Events	10,000	####	47,753	25,820
Q1	117	1.2	6,586	18,748
Q2	9,858	98.6	48,341	25,964
Q3	20	0.2	6,980	2,415
Q4	5	0.1	14,810	2,497

Tube Name:	EtOAc INFLORESCENCE			
Population	#Events	%Parent	FSC-H Mean	SSC-H Mean
All Events	2,133	####	12,516	29,974
Q1	946	44.4	6,524	13,928
Q2	1,144	53.6	17,673	44,284
Q3	36	1.7	6,540	2,245
Q4	7	0.3	10,466	2,520

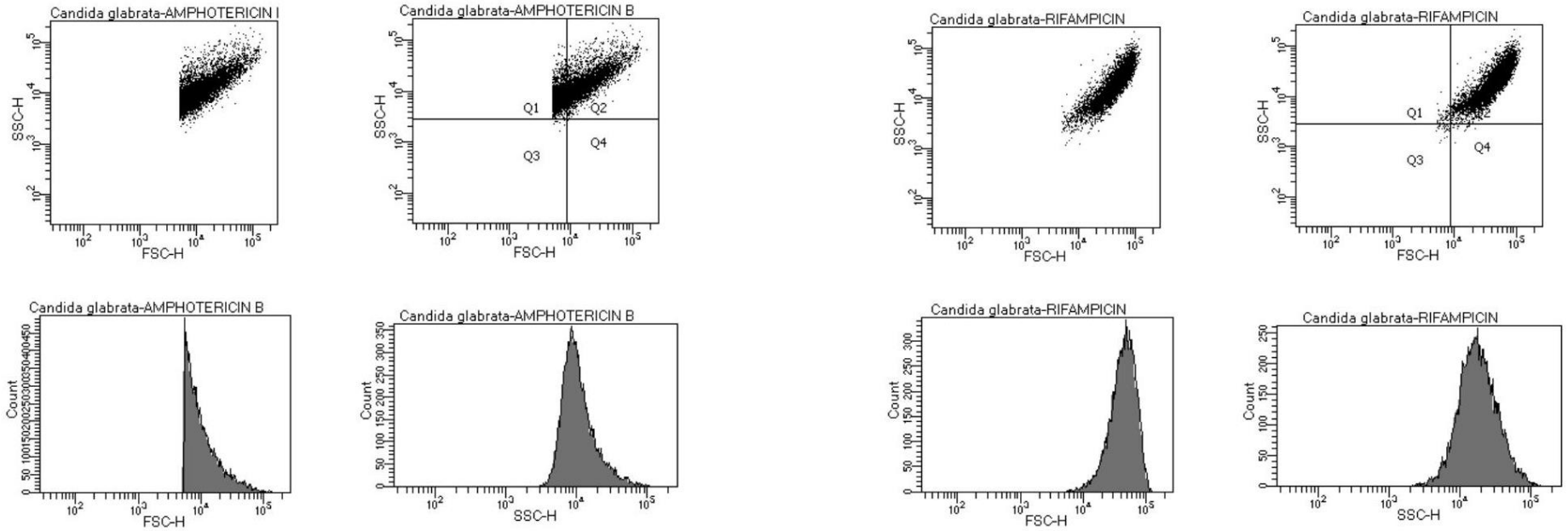
Figure 10.7: Flow cytometry scattergram obtained for *Candida glabrata* (ATCC 15126) A. n-Hexane mature inflorescence and B. Ethyl acetate mature inflorescence after 24-hour incubation.



Tube Name:	IBS 007			
Population	#Events	%Parent	FSC-H Mean	SSC-H Mean
All Events	2,019	####	46,280	24,898
Q1	118	5.8	6,928	11,299
Q2	1,846	91.4	49,966	26,442
Q3	43	2.1	6,144	2,198
Q4	12	0.6	10,158	2,408

Tube Name:	IBS 008			
Population	#Events	%Parent	FSC-H Mean	SSC-H Mean
All Events	543	####	10,561	82,001
Q1	282	51.9	6,197	86,900
Q2	194	35.7	17,723	102,490
Q3	45	8.3	6,762	2,055
Q4	22	4.1	11,112	2,066

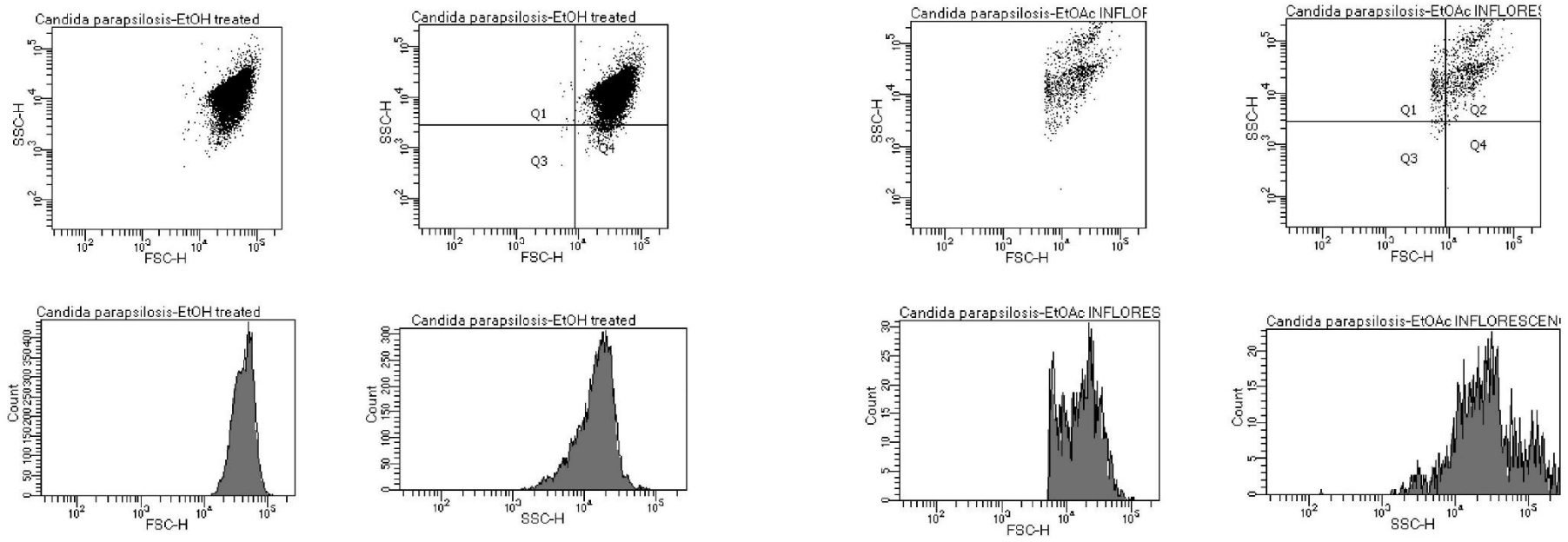
Figure 10.8: Flow cytometry scattergram obtained for *Candida glabrata* (ATCC 15126) A. IBS 007 and B. IBS 008 after 24-hour incubation.



Tube Name:	AMPHOTERICIN B			
Population	#Events	%Parent	FSC-H Mean	SSC-H Mean
All Events	10,000	####	13,432	12,893
Q1	5,178	51.8	6,423	8,400
Q2	4,813	48.1	20,985	17,746
Q3	8	0.1	6,393	2,335
Q4	1	0.0	9,044	2,668

Tube Name:	RIFAMPICIN			
Population	#Events	%Parent	FSC-H Mean	SSC-H Mean
All Events	10,000	####	44,081	20,949
Q1	73	0.7	7,004	4,347
Q2	9,883	98.8	44,510	21,154
Q3	26	0.3	6,650	2,218
Q4	18	0.2	12,925	2,382

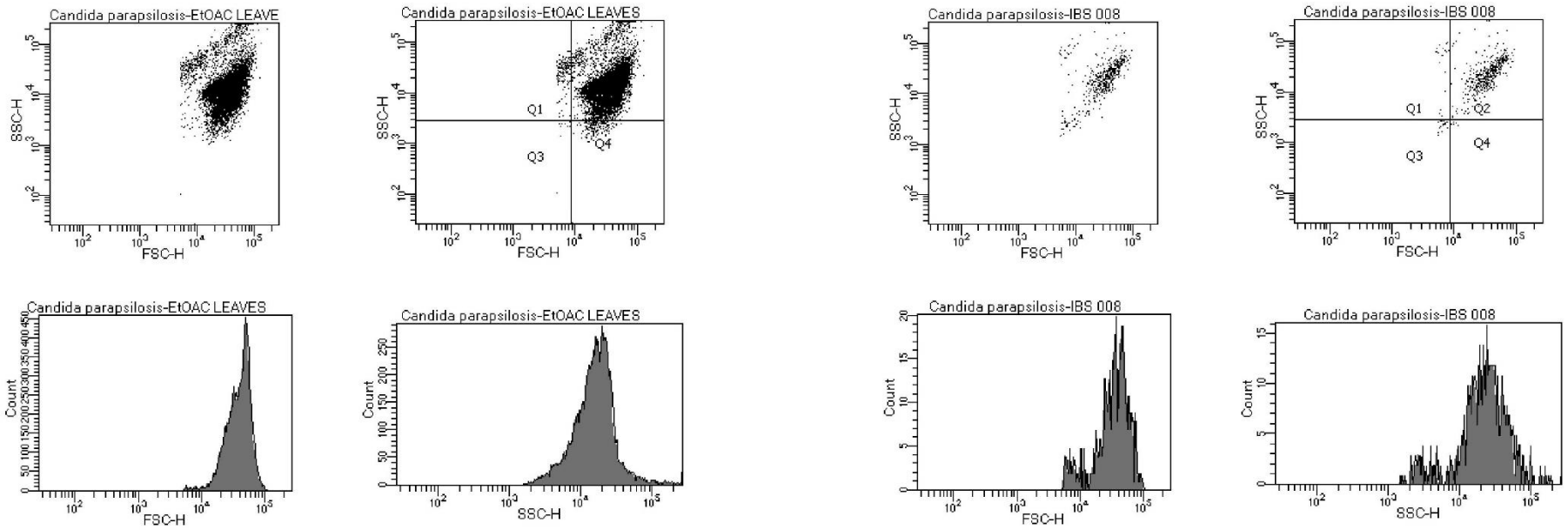
Figure 10.9: Flow cytometry scattergram obtained for *Candida glabrata* (ATCC 15126) reference drugs A. Amphotericin B and B. Rifampicin after 24-hour incubation.



Tube Name:	EtOH treated			
Population	#Events	%Parent	FSC-H Mean	SSC-H Mean
All Events	10,000	####	41,347	15,707
Q1	9	0.1	6,668	11,752
Q2	9,755	97.6	41,749	16,040
Q3	7	0.1	5,979	1,869
Q4	229	2.3	26,678	2,103

Tube Name:	EtOAc INFLORESCENCE			
Population	#Events	%Parent	FSC-H Mean	SSC-H Mean
All Events	1,040	####	19,068	42,262
Q1	239	23.0	6,566	18,051
Q2	778	74.8	23,265	50,886
Q3	19	1.8	6,537	2,182
Q4	4	0.4	9,212	1,882

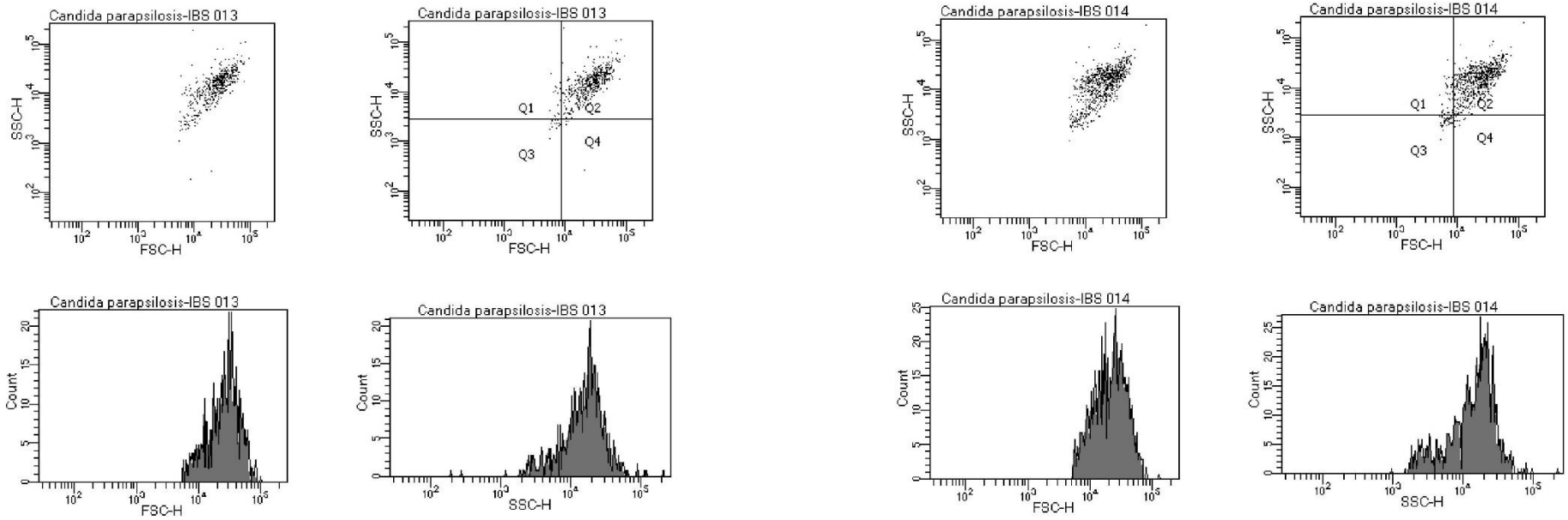
Figure 10.10: Flow cytometry scattergram obtained for *Candida parapsilosis* (ATCC 22019) A. Positive control ethanol treated cells and B. ethyl acetate mature inflorescence after 24-hour incubation.



Tube Name:	EtOAC LEAVES			
Population	#Events	%Parent	FSC-H Mean	SSC-H Mean
All Events	10,000	####	38,695	21,033
Q1	155	1.6	6,562	30,297
Q2	9,655	96.6	39,526	21,256
Q3	9	0.1	6,516	2,007
Q4	181	1.8	23,498	2,142

Tube Name:	IBS 008			
Population	#Events	%Parent	FSC-H Mean	SSC-H Mean
All Events	523	####	34,959	27,926
Q1	23	4.4	6,585	50,003
Q2	476	91.0	37,717	28,153
Q3	18	3.4	6,457	2,213
Q4	6	1.1	10,432	2,393

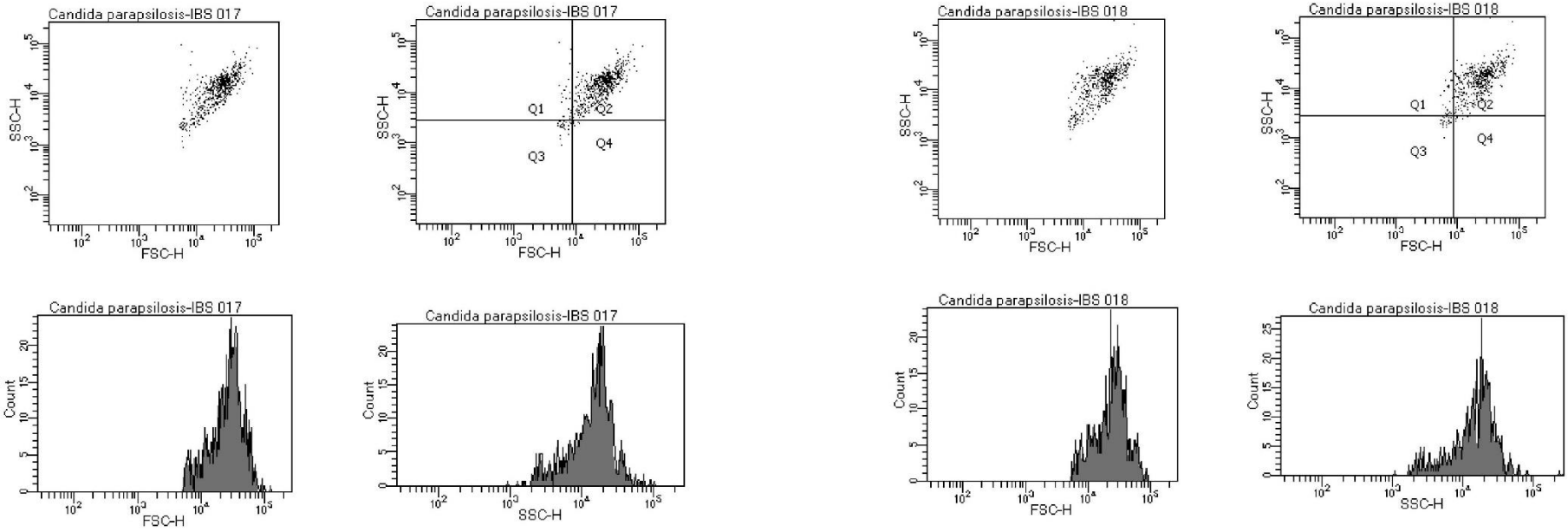
Figure 10.11: Flow cytometry scattergram obtained for *Candida parapsilosis* (ATCC 22019) A. Ethyl acetate vegetative leaves and B. IBS 008 after 24-hour incubation.



Tube Name:	IBS 013			
Population	#Events	%Parent	FSC-H Mean	SSC-H Mean
All Events	514	####	27,948	17,790
Q1	21	4.1	7,301	8,397
Q2	472	91.8	29,753	18,906
Q3	17	3.3	6,818	2,142
Q4	4	0.8	13,137	1,889

Tube Name:	IBS 014			
Population	#Events	%Parent	FSC-H Mean	SSC-H Mean
All Events	777	####	23,182	16,205
Q1	45	5.8	7,197	9,189
Q2	683	87.9	25,365	17,676
Q3	39	5.0	6,614	2,151
Q4	10	1.3	10,630	2,116

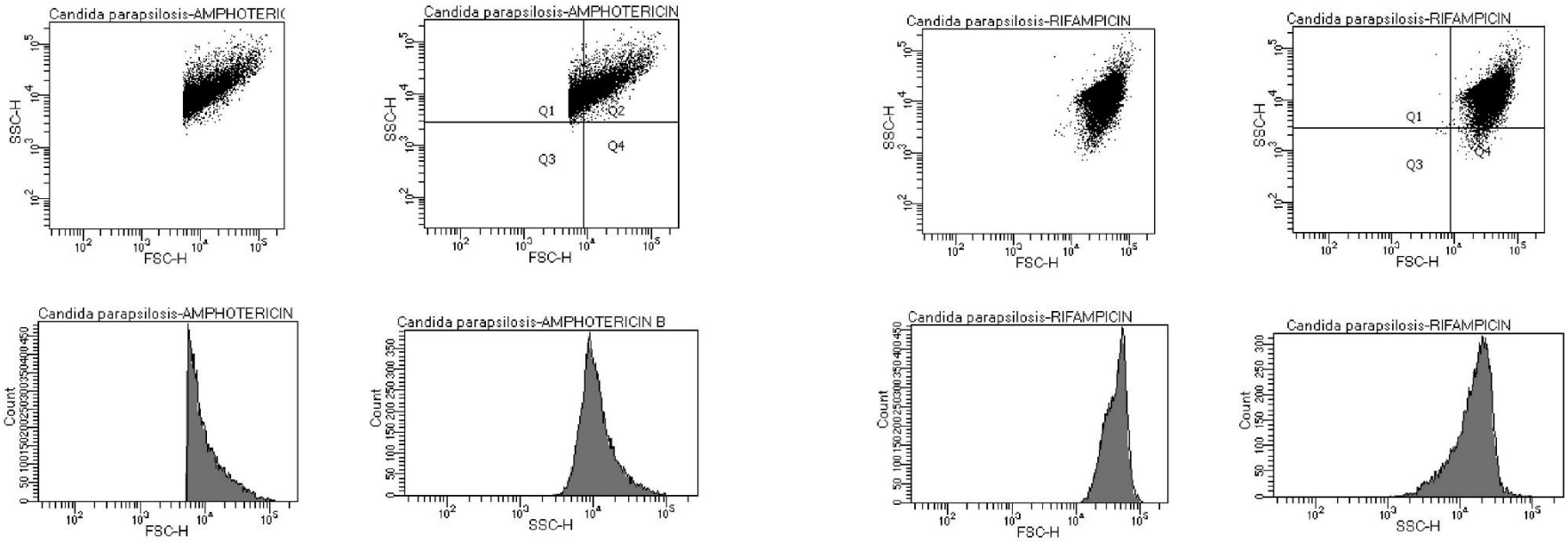
Figure 10.12: Flow cytometry scattergram obtained for *Candida parapsilosis* (ATCC 22019) A. IBS 013 and B. IBS 014 after 24-hour incubation.



Tube Name:	IBS 017			
Population	#Events	%Parent	FSC-H Mean	SSC-H Mean
All Events	631	####	27,792	15,831
Q1	24	3.8	7,006	15,335
Q2	572	90.6	29,940	16,684
Q3	28	4.4	6,227	2,153
Q4	7	1.1	9,747	2,482

Tube Name:	IBS 018			
Population	#Events	%Parent	FSC-H Mean	SSC-H Mean
All Events	554	####	25,411	17,320
Q1	25	4.5	7,011	8,007
Q2	498	89.9	27,457	18,728
Q3	25	4.5	6,564	2,143
Q4	6	1.1	10,754	2,476

Figure 10.13: Flow cytometry scattergram obtained for *Candida parapsilosis* (ATCC 22019) A. IBS 017 and B. IBS 018 after 24-hour incubation.



Tube Name:	AMPHOTERICIN B			
Population	#Events	%Parent	FSC-H Mean	SSC-H Mean
All Events	10,000	####	13,548	13,489
Q1	5,255	52.6	6,382	8,752
Q2	4,736	47.4	21,514	18,766
Q3	9	0.1	5,818	2,534
Q4	0	0.0	####	####

Tube Name:	RIFAMPICIN			
Population	#Events	%Parent	FSC-H Mean	SSC-H Mean
All Events	10,000	####	40,791	16,548
Q1	6	0.1	6,850	18,356
Q2	9,759	97.6	41,216	16,897
Q3	6	0.1	5,913	2,374
Q4	229	2.3	24,490	1,998

Figure 10.14: Flow cytometry scattergram obtained for *Candida parapsilosis* (ATCC 22019) reference drugs A. Amphotericin B and B. Rifampicin after 24-hour incubation.

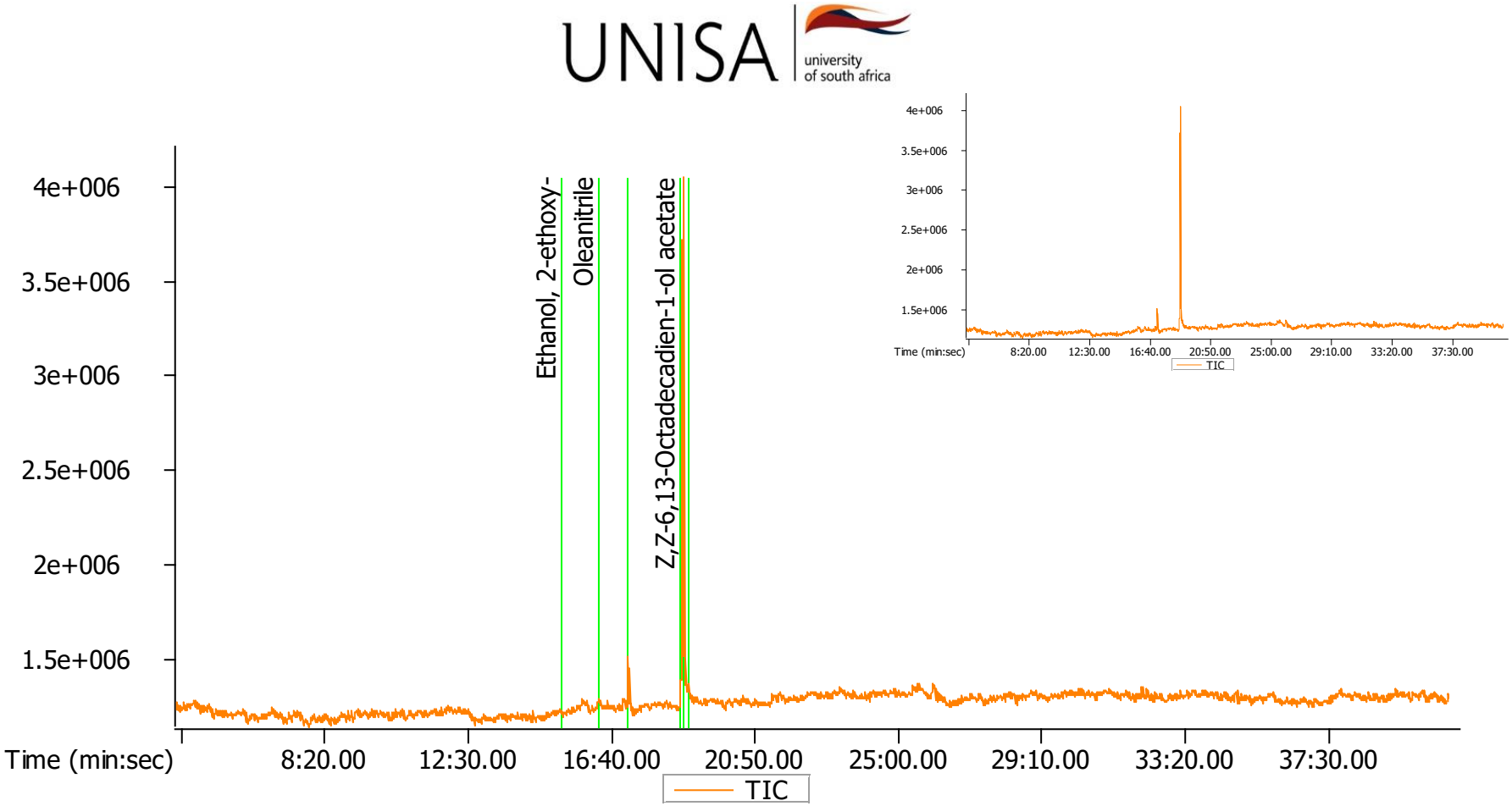


Figure 10.15: Gas chromatography – gas chromatography – time of flight – mass spectrometry (GC x GC –TOF-MS) spectrum of the extraction solvent acetonitrile with a 90% NIST library superior match.

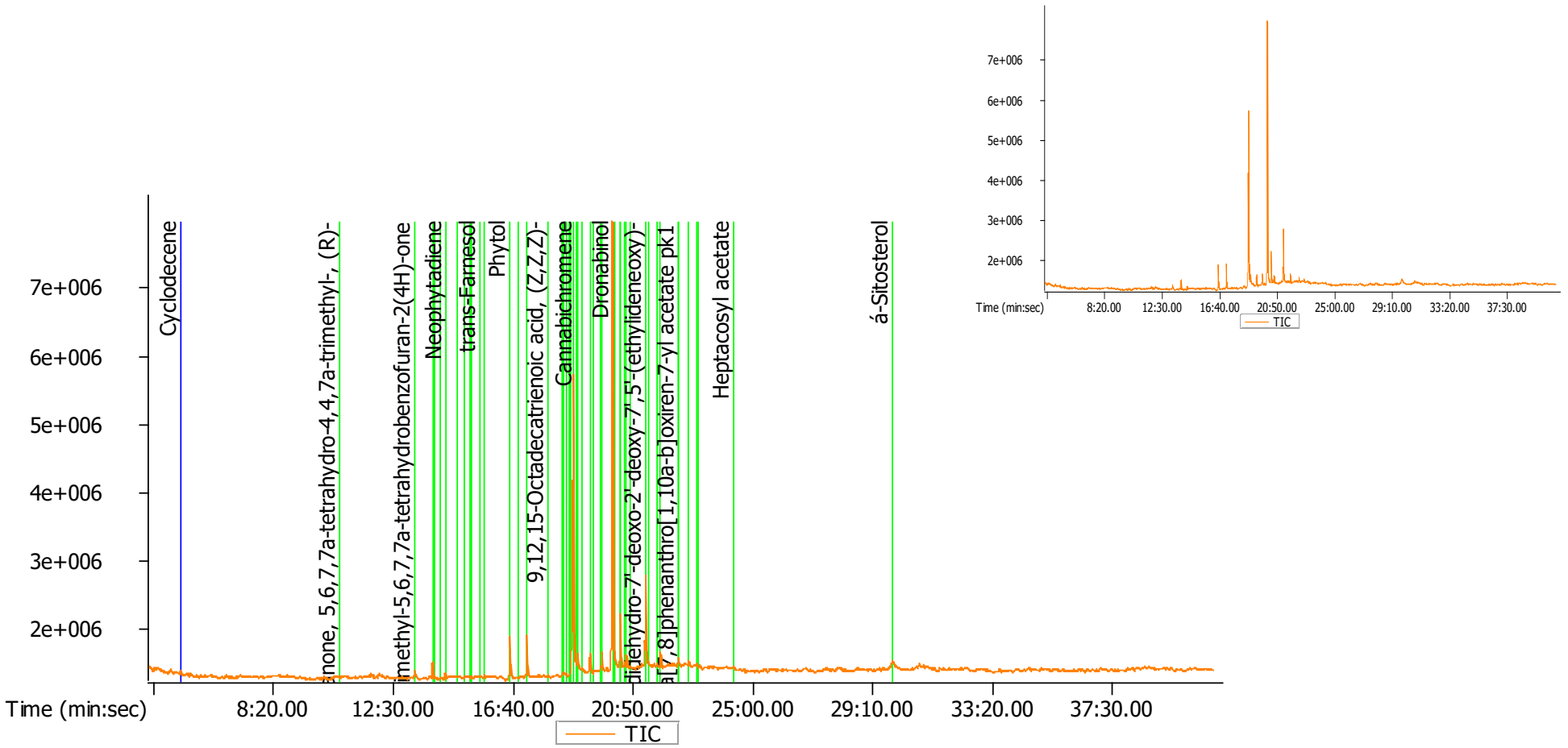


Figure 10.16: Gas chromatography – gas chromatography – time of flight – mass spectrometry (GC x GC -TOF-MS) spectrum of n-hexane *Cannabis sativa* vegetative leaves extract with a 90% NIST library superior match.

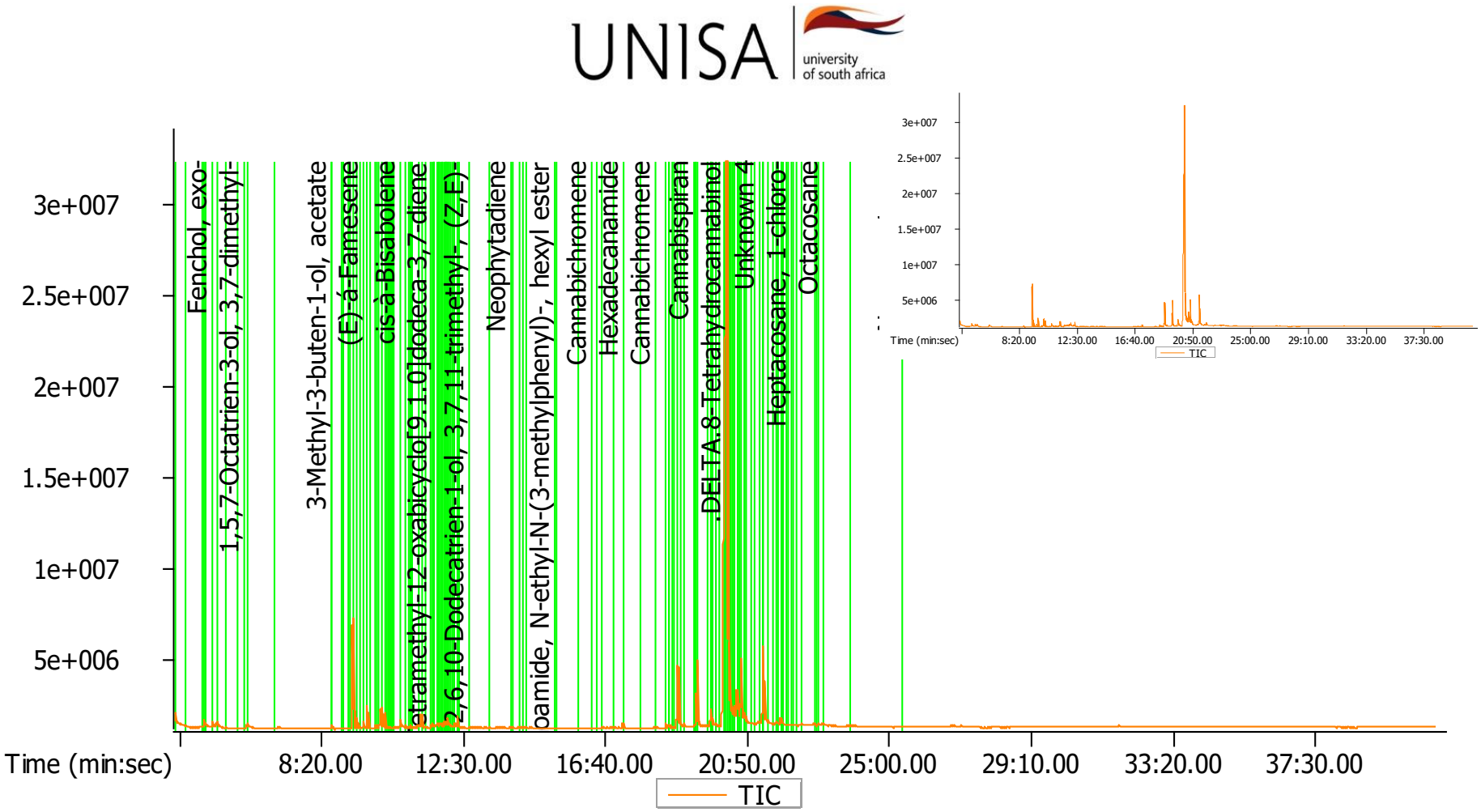


Figure 10.17: Gas chromatography – gas chromatography – time of flight – mass spectrometry (GC x GC –TOF-MS) spectrum of n-hexane *Cannabis sativa* mature inflorescence extract with a 90% NIST library superior match.

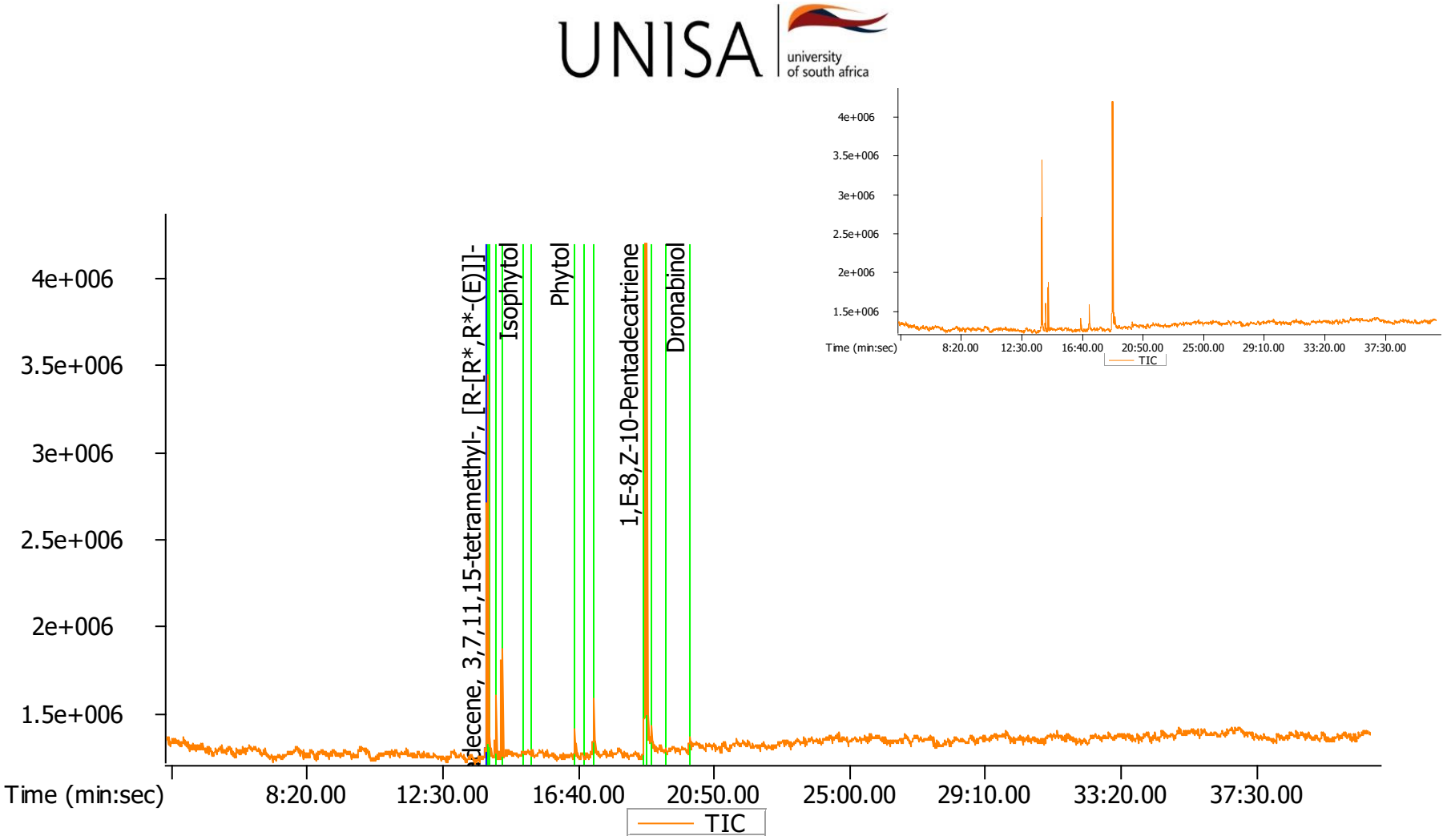


Figure 10.18: Gas chromatography – gas chromatography – time of flight – mass spectrometry (GC x GC –TOF-MS) spectrum of ethyl acetate *Cannabis sativa* vegetative leaves extract with a 90% NIST library superior match.

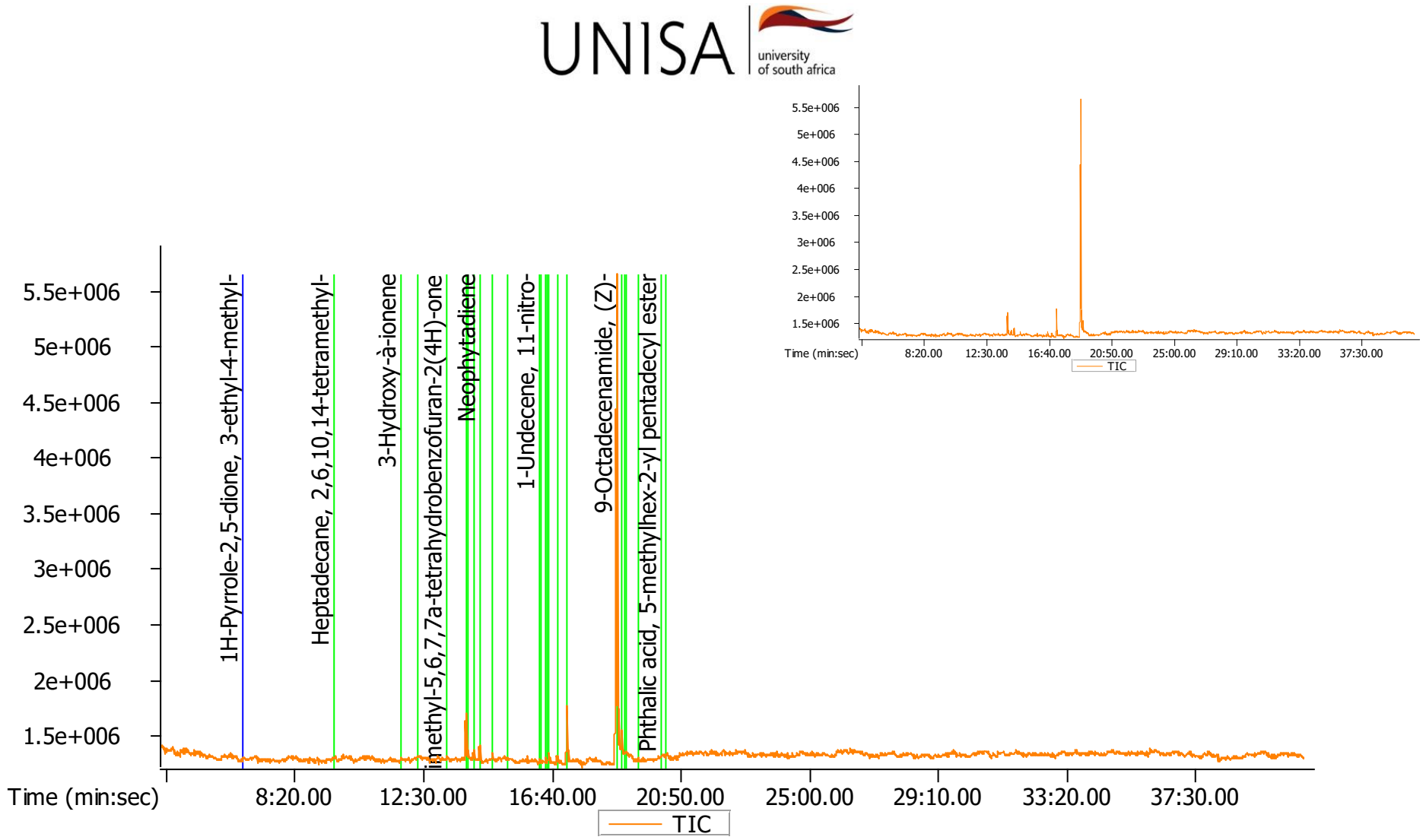


Figure 10.19: Gas chromatography – gas chromatography – time of flight – mass spectrometry (GC x GC –TOF-MS) spectrum of ethyl acetate *Cannabis sativa* mature inflorescence extract with a 90% NIST library superior match.

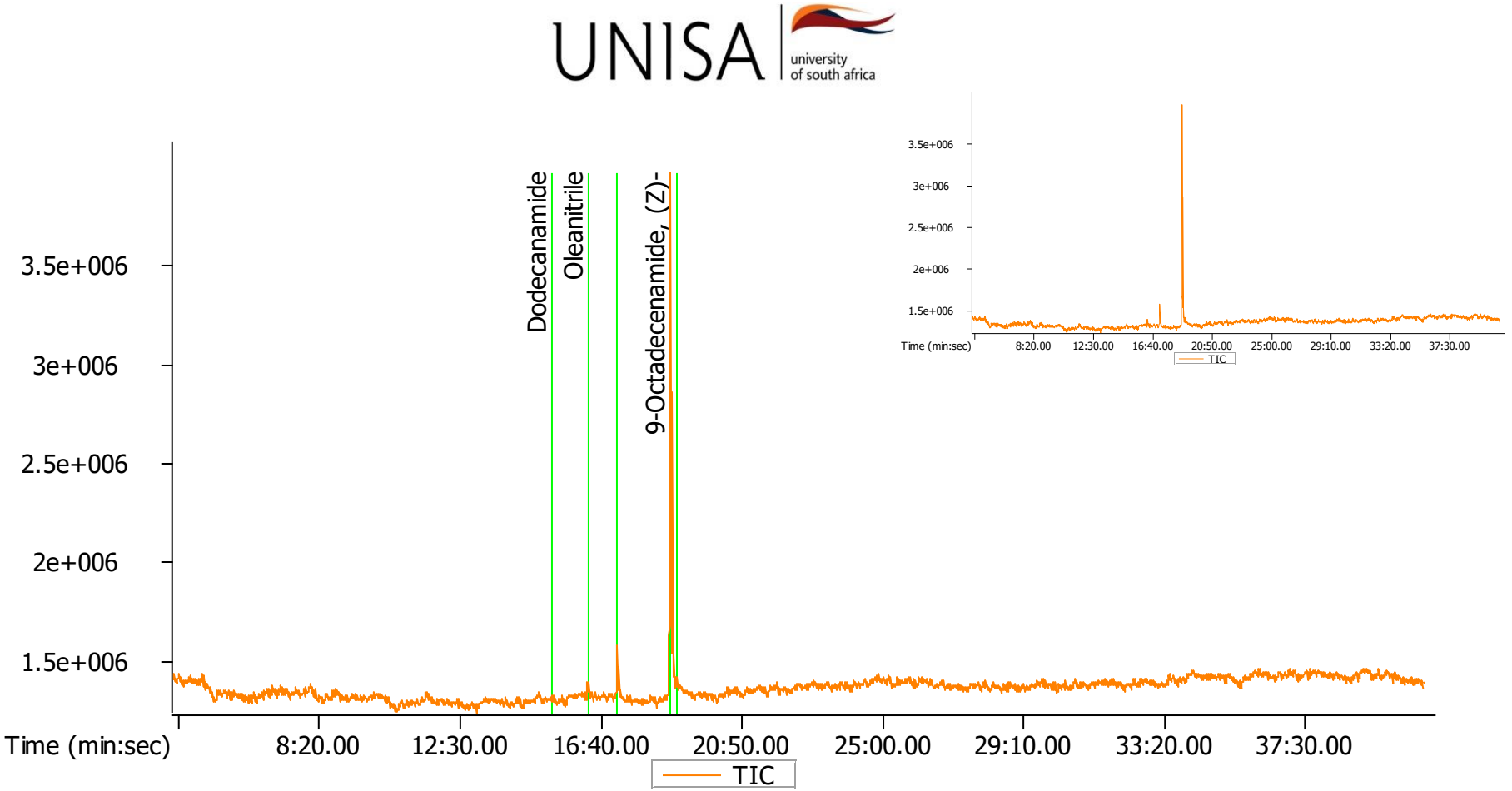


Figure 10.20: Gas chromatography – gas chromatography – time of flight – mass spectrometry (GC x GC –TOF-MS) spectrum of ultra-pure water *Cannabis sativa* vegetative leaves extract with a 90% NIST library superior match.

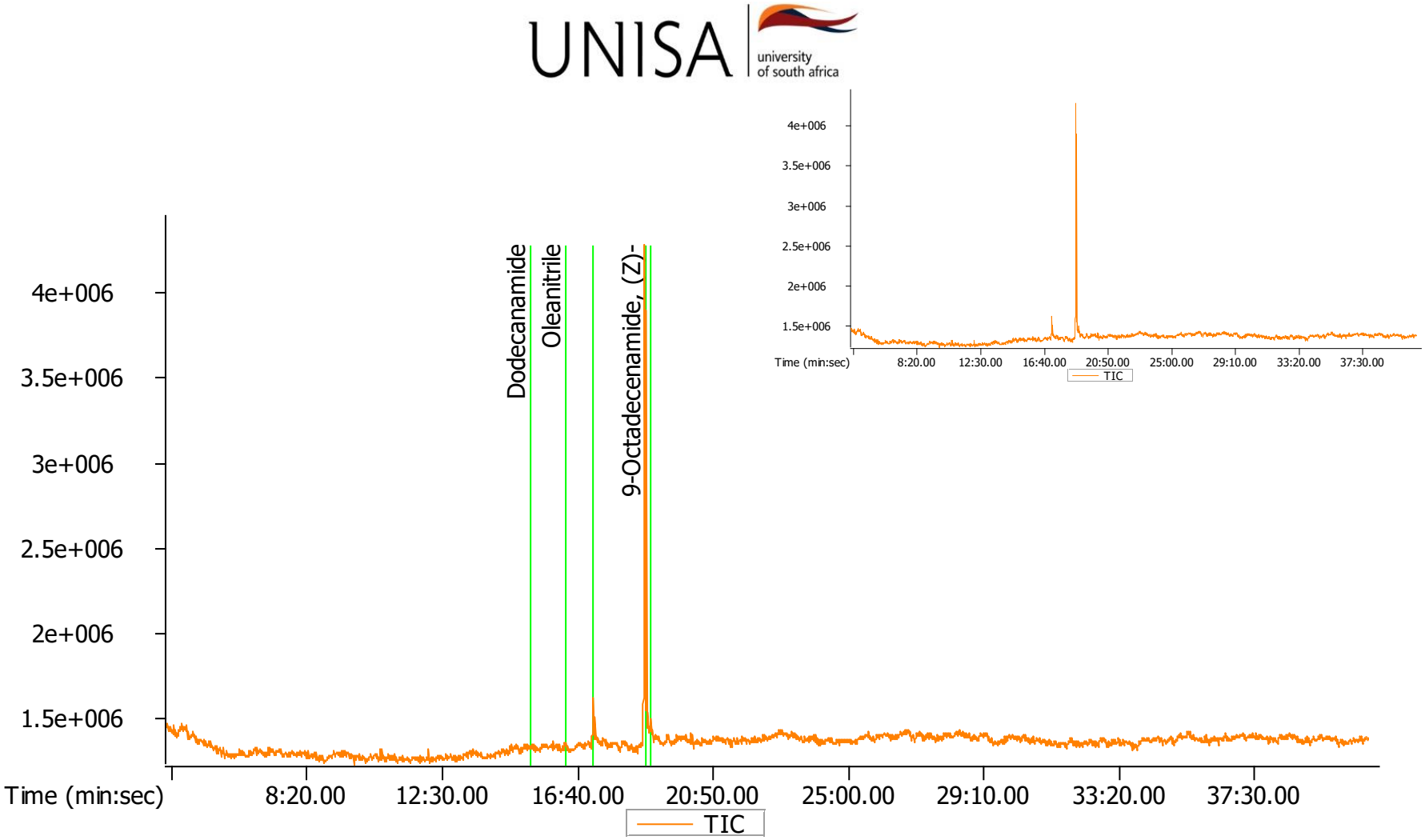
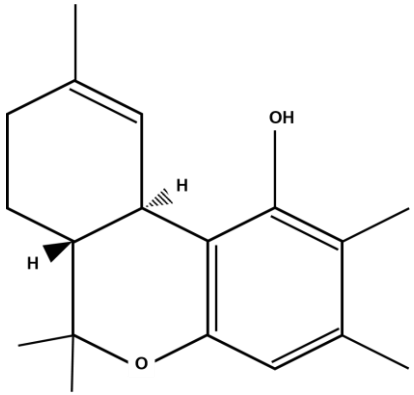
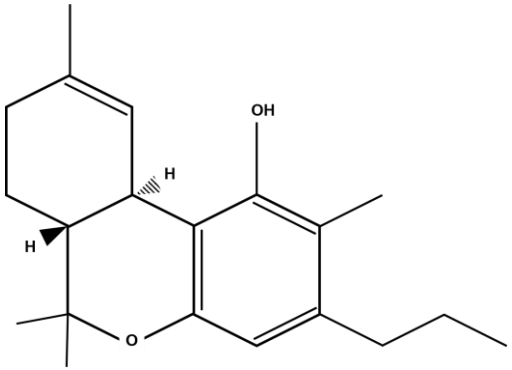
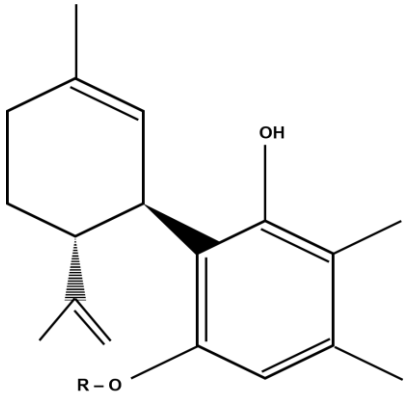
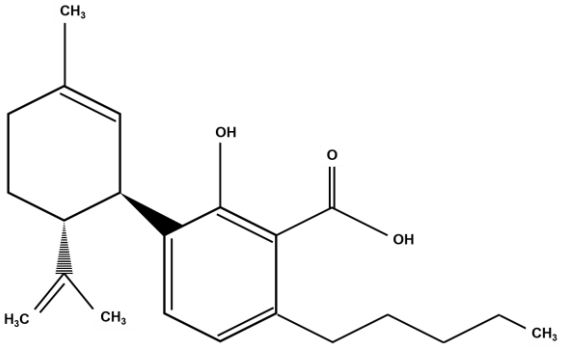
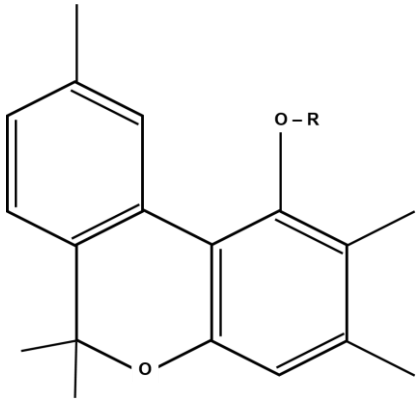
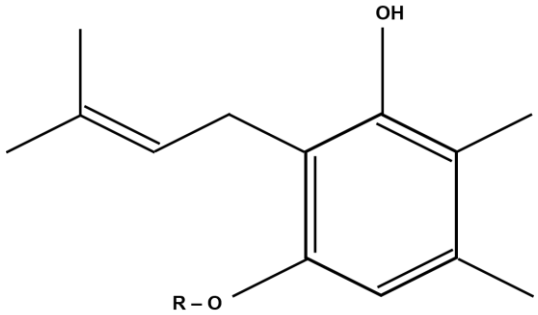


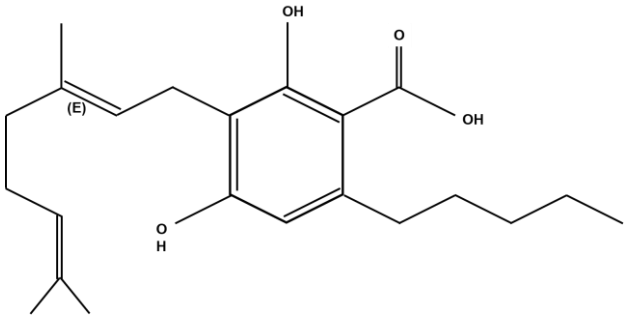
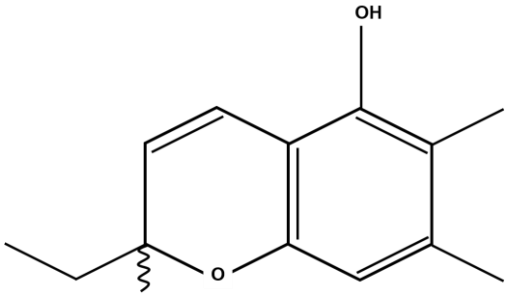
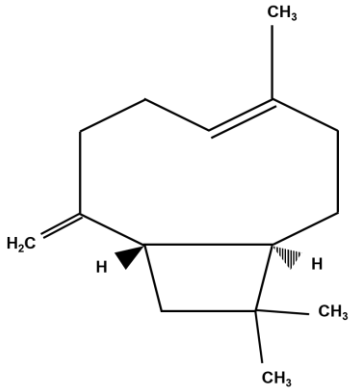
Figure 10.21: Gas chromatography – gas chromatography – time of flight – mass spectrometry (GC x GC –TOF-MS) spectrum of ultra pure water *Cannabis sativa* mature inflorescence extract with a 90% NIST library superior match.

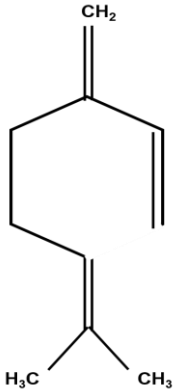
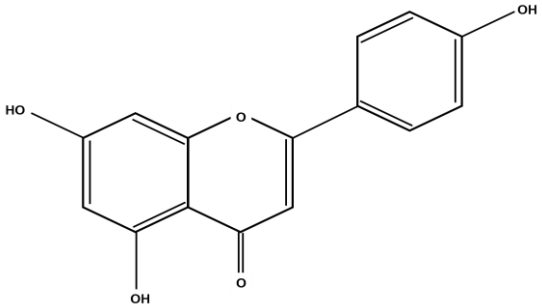
Table 10.1: Common constituents found in the *Cannabis* species with their chemical structures and known pharmaceutical properties.

Compound	Structure	Main pharmacological characteristics
Δ^9-tetrahydrocannabinol (THC)		Euphoriant Analgesic Anti-inflammatory Antioxidant Antiemetic
Δ^9-tetrahydrocannabivarin (THCV)		Analgesic Euphoriant

Compound	Structure	Main pharmacological characteristics
Cannabidiol (CBD)		Anxiolytic Anti-psychotic Analgesic Anti-inflammatory Antioxidant Antispasmodic
Cannabidiolic acid (CBDA)		Antibiotic

Compound	Structure	Main pharmacological characteristics
Cannabinol (CBN)		Sedative Antibiotic Anticonvulsant Anti-inflammatory
Cannabigerol (CBG)		Antibiotic Antifungal Anti-inflammatory Analgesic

Compound	Structure	Main pharmacological characteristics
Cannabigerolic acid (CBGA)		Antibiotic
Cannabichromene (CBC)		Anti-inflammatory Antibiotic Antifungal Analgesic
β-Caryophyllene		Anti-inflammatory Cytoprotective Antimalarial

Compound	Structure	Main pharmacological characteristics
β-Myrcene		Analgesic Anti-inflammatory Antibiotic Anti-mutagenic
Apigenin		Anxiolytic