Exploring Agave *americana L.* as a Promising Alternative for Dermatophytosis Management

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DECLARATION

I, Rethabile Lesole nee Banda, hereby declare that the research project submitted to the Central University of Technology, Free State, was completed by me, independent of any other person and has not previously been submitted by me or any other person, to any institution in fulfilment of attaining any qualification.

_____ 15 March 2024

Signature **Date**

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LIST OF ABBREVIATIONS

AChE	Anti Acetylcholinesterase
AG	Aminoguanidine
CAM	Complimentary Alternative Medicine
DAPI	4',6-diamidino-2-phenylindole
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic Acid
DPPH	2,2-diphenyl-1-picrylhydrazyl
EGCG	Epigallocatechin-3-gallate
FRAP	Ferric Reducing Ability of Plasma
GMP	Good Manufacturing Practices
HaCaT	Human Keratinocyte Cell Line
HMG-CoA	3-hydroxy-3-methylglutaryl coenzyme A
LCMS	Liquid Chromatography-Mass Spectrometry
LPS	Lipopolysaccharide
MIC	Minimum Inhibitory Concentration
MTT	3-(4,5-Dimethylthiazol-2-yl)-2-5-diphenyltetrazolium bromide
NO	Nitric Oxide
PFB	Pseudo Folliculitis Barbae
RSA	Republic of South Africa
THP	Traditional Health Practitioners
TPTZ	Tripyridyltriazine

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ABSTRACT

The role of traditional medicine in South Africa has altered drastically in the past few decades. This could be a result of colonization and westernisation of culture. Poor lifestyle choices including grooming and diet lead to compromised immune systems.

Consequentially, the body becomes vulnerable to recuring illnesses and susceptible to numerous skin infections including fungal infections. The folkloric use of medicinal herbs in the treatment and management of dermatological conditions is well documented. Folkloric medicine has been shown to possess some of the best biological and/ or antipathogenic activities. However, some of these activities are yet to be validated and/ or documented. Therefore, a wide literature search was executed to bring to the fore, some of the dermatologically significant medicinal plants that are utilised by the Sotho speaking people of Bloemfontein with a view to select an understudied species for further studies. Agave *americana L.* was thus selected for further studies. Some South African companies use this plant in the production of skin care products, indicating its potential commercial significance. However, the plant is classified as a category three invasive species in South Africa, posing a threat to local ecosystems. Utilizing the plant for dermatological purposes can offer a dual benefit by addressing prevalent skin conditions in the population and controlling the spread of the invasive species. To minimize ecological impact, it is suggested to promote the controlled utilization of the plant. Exploring the dermatological effects of Agave *americana L.* not only contributes to scientific knowledge but also validates and preserves traditional practices. This research has the potential to bridge the gap between traditional wisdom and modern science, providing effective skincare solutions while addressing ecological concerns related to the plant's invasive nature.

Agave *americana L.* growing in Bloemfontein was profiled for secondary metabolites using LC-MS techniques. The samples were collected near Bloemdustria in Bloemfontein, Free State South Africa, at a height of approximately 1395m above sea level on the Old Thaba Nchu Road. Two triterpenoids, including Piscidic and Eucomic acid and a glycoside, were profiled and identified. This plant is thus potentially rich in terpenes. Terpenes are well recognized for their ability to promote transdermal absorption and possess anti-inflammatory qualities, in addition to other physiological

roles in humans. Because of their unique chemical structures and the tissues or organs they interact with, glycosides have a variety of roles. Although a host of other secondary metabolites could not be identified for various reasons, these results could be more promising. Perhaps essential oil extracts can potentially reveal other secondary metabolites that can then be linked to their supposed medicinal properties and therefore validate this plant's use in folkloric medicine.

The study analysed the potential of Agave *americana L.* acetone, methanol, and water extracts in treating dermatological infections in somatology. The extracts were therefore assayed for biological activities including antioxidant (FRAP and DPPH), anti-and-pro-inflammatory as well as antifungal activity (against *C. albicans, T. interdigit, T. rubrum* and *T. tonsurans*). The water extract showed the most potential, followed by methanol and acetone extracts. The water extract showed the most activity, with concentrations of 250 and 500 µg/ml being more active than Trolox and EGCG. The acetone extract was inactive, while methanol was active but not significantly different. The water extract showed the best potential for DPPH scavenging, with IC_{50} values between 15.625 and 31.25 µg/ml. The water extract showed pro-inflammatory activity, with high concentrations of nitrite at low extract concentrations, which decreased in a dose-dependent manner. None of the extracts exhibited anti-inflammatory potential. The study also found that *C. albicans*, *T. interdigit*, *T. rubrum*, and *T. tonsurans* were resistant to all dilutions tested. This study therefore can conclude that the Agave *americana L.* did not possess any antifungal activity against the tested pathogens. This is also not very surprising considering the very low secondary metabolites constituents as reported in chapter 3.

The genotoxicity and cytotoxicity of Agave *americana L.* leaf extracts on the kidney cell line (Vero cells) in-vitro were assayed using standard methods. The study found that acetone and water extracts did not increase micronuclei formation at all concentrations except for 200 µg/ml. However, the methanol extract showed an increase in micronuclei formation, indicating potential genotoxicity. The water extract showed mild toxicity against Vero cells, with an estimated IC_{50} value of 200 µg/ml. However, these results are negligible as they are below the minimum concentration required to inhibit biological response. Due to some indications of toxicity, further studies are therefore recommended.

CHAPTER 1
GENERAL INTRODUCTION

1.1 Background

Fungal infections serve as an absolute contra-indication for all treatments administered by somatologists, making it a concern for somatologists worldwide. The concerns result from the highly contagious nature of fungal infections (Al-Khikani, 2020). This has a negative impact on training facilities where a lot of equipment is shared and the risk of infection as well as cross-contamination increases, not only to other areas of the body but to other people as well. Additionally, in the business sector, daily revenue is also negatively impacted, as fungal infections limit spa treatments that can be administered. Somatologists have the responsibility to educate and refer clients to a medical doctor when there is any suspicion of a fungal infection. There are several strategies to enhance health and safety in the somatology environment such as the use of sterilisation techniques and medicinal plants.

Somatologists have an obligation to enhance health and safety by reducing any risk factors associated with dermatophytosis. When trying to uncover potential therapies for fungal infections, the interest in investigating medicinal plants and obtaining plant extracts with anti-microbial qualities arose (Al-Khikani, 2020). Should medicinal plant extracts indeed possess the necessary antioxidant, antimicrobial, and anti-inflammatory properties among others, they can then be incorporated into various somatic and Complimentary Alternative Medicine (CAM) therapies as preventative measures to reduce the risk of fungal infections in a somatology environment (Quiroga et al., 2001).

Antifungal and antibacterial properties are some of the antimicrobial properties that have been reviewed in medicinal plants. These properties possess a range of beneficial qualities, including anti-inflammatory and antioxidant properties, among others. These properties are synergistic in wound healing and consequently, effective medicinal plant extracts often possess more than one beneficial antimicrobial property (Sharma et al., 2020). Antifungal properties of medicinal plant extracts have previously been reported around the world (Mandhar et al., 2019); Samoisy and Mahomoodally, 2016; Webster et al., 2008). There have been reports of plant extracts, including

essential oils from traditionally used medicinal plants in Brazil, being used to treat infectious skin conditions (Rabe & van Staden, 1997). Also, in other parts of the world, such as Australia, the aboriginal community is exploring possibilities of amalgamating traditional practices with biomedical health care, illustrating that there is a world of knowledge still to be explored with Indigenous knowledge and plants (Oliver, 2013). There has been a general shift globally and locally in the field of health and skin care in particular. Health and environmentally friendly products are becoming more and more important to people all over the world. There has been a significant increase in consumers opting for healthier, less synthetic and more natural or organic skincare and hair care products. This can be attributed to various factors such as the Y generation being interested in generational knowledge as well as reports documenting cancer-causing ingredients in skin care products over the years (Boon et al., 2020). Generation Y are adults born in the early 1980's-1990's, making them the general consumers and greater contributors to the economy today (Torsello, 2019).

According to the World Health Organization (WHO), approximately 66% of the global population consults THPs, of which 80% of the African population makes use of traditional medicine equating a large proportion of the South African population still relying on traditional healing practices, mainly based on plant extracts (WHO), 2001). The scrutinization of traditional healing practices and medicines is aimed at elucidating pharmacological properties in folkloric medicinal plants (Webster et al., 2008). Numerous studies have been conducted to screen commonly used plants for active compounds, as well as harness scientific support for traditional practices and medicine (Arulselvan, et al., 2016; Che, et al., 2004; Jo Siu et al., 2013; Soomro, et al., 2007), and yet there is still a lot to be learnt and documented.

South Africa has a rich plant biodiversity. This can be attributed to the geographical positioning as well as the vast change in climate during various seasons of the year. Due to the impact of colonisation and global migration, it is evident that South Africa has adopted numerous plant species, some of which have been naturalised over the past few decades and centuries (Hutchinson, 2021). Some of the plants have positively naturalised and blended with the prevailing environment, and yet others have become invasive and damaging to the local ecology, even though they may be aesthetically appealing. For example, *Jacaranda mimosifolia*, a sub-tropical tree Native to South America but now found throughout South Africa notably in the long

streets of Pretoria. Agave *americana L.* is another plant species that has adapted very well to central South Africa and the greater parts of Lesotho, although its origins can be traced to Mexico (van Wilgen et al., 2020).

1.2 Agave *americana L.*

Figure 1.1: Agave *americana L. plant*

(Source: Baloyi and Klopper, 2017)

Agave *americana L.* (Figure 1.1), commonly known as the American aloe, century plant and/or maguey, is a succulent, evergreen flowering plant belonging to the *Agavaceae* family. Although it is known as the American aloe, it is not botanically related to the common Aloe, which belongs to the Asphodelaceae family. The leaves of this plant are thick, rigid, spine-tipped, sword-shaped and can grow up to 180 cm long or even longer. The Sotho-speaking people of Bloemfontein and Thaba Nchu, some of the regions where it is found, call this plant '*lekhala la America'*. *A. americana* is native to the semi-arid regions of Central America, southern North and northern South America. However, this plant has been naturalized in several regions of the world for ornamental purposes including Lesotho and South Africa (Coleman-Derr et al., 2016; Good-Avila et al., 2006). It was introduced to South Africa in the 1960s by

the settlers and has spread in most provinces of the country, with the Western Cape listing it as invasive (Baloyi & Klopper, 2017).

Several varieties of agave have been utilized for millennia in Mexico, where the plant is important both commercially and culturally as a source of fibre, textiles, food, shelter, fuel, medicine, compost, adornment, as well as alcoholic beverages, including bacanora, mezcal, and tequila (Herrera-Ruiz et al., 2022). Furthermore, according to the previous authors, the exploration of this plant as a potential prebiotic, natural sweetener, biofuel, and nutraceutical has been growing.

Some of the folkloric uses of *A. americana* indicate the treatment of trauma, wounds, limb paralysis, sores, snake bites, cancer, scurvy, inflammation, syphilis, rheumatoid arthritis, fractures, postpartum abdominal, and psoriasis while its uses as a laxative and/or diuretic have also been reported (Herrera-Ruiz et al., 2022). Furthermore, this plant has been reported in the treatment of bacterial etiological infections such as wounds and gastrointestinal conditions as well as hypertension, diabetes, and urological disorders (Ahumada-Santos et al., 2013).

Due to this plant's traditional uses not only in the Americas but the world over, interest in *A. americana* has revealed it is antioxidant and anti-inflammatory (Misra et al., 2018), antibacterial (Shegute & Wasihun, 2020), anticancer (Santos-Zea et al., 2016), and anti-neuroinflammatory (Herrera-Ruiz et al., 2019) activity among others.

Although the *A. americana* extracts have been found to possess saponins, and its leaf extracts are used as a soap (Baloyi & Klopper, 2017), there is a lack of information on the plant's role in somatology.

The local traditional health practitioners of Lesotho and some parts of Bloemfontein and Thaba Nchu use this plant for various health-related purposes. This is potentially because this plant's medicinal properties especially wound healing have been reported (Misra & Varma, 2017). Thus, the present study aims to investigate this role in the somatology environment.

1.3 Choice of the plant

Dermatological conditions are prevalent worldwide, impacting the quality of life for individuals affected. Traditional remedies, rooted in cultural knowledge, often hold

untapped potential in addressing skin-related issues. Agave *americana L.*, a plant concentrated in the Bloemfontein area and extensively used by the Sotho-speaking people, presents an intriguing avenue for research in dermatology. Despite its widespread use, scientific investigations into its effects on skin conditions are notably lacking in South Africa. The significance of Agave *americana L.* in traditional dermatological practices emphasizing its therapeutic role in managing skin diseases among the Sotho-speaking communities is well-documented (Asong et al., 2019; Kambizi & Bvenura, 2021; Moteetee & Kose, 2017). However, the lack of comprehensive scientific studies on this plant in relation to dermatology creates a critical gap in our understanding of its potential benefits and applications.

The Bloemfontein area, one of the locations where Agave *americana L.* is widely distributed, serves as a unique geographical context for this investigation. By focusing on a region where the plant is abundantly available and culturally significant, we can uncover valuable insights into its effectiveness in treating local dermatological conditions. This localized approach is essential for developing targeted solutions that resonate with the community's specific needs.

Furthermore, the plant is actively utilized by some South African companies in producing skincare products, signifying its potential commercial importance. Validating the traditional use of Agave *americana L.* in skin-related conditions could contribute to the development of evidence-based skincare solutions and enhance the credibility of existing products in the market. This validation is particularly relevant given the increasing consumer interest in natural and traditional skin care and treatment remedies.

Baloyi and Klopper (2017) previously classified Agave *americana L.* as a category 3 invasive species in South Africa, suggesting that it threatens local ecosystems. Harnessing its dermatological potential provides a dual benefit, i.e., addressing skin conditions prevalent among the population and curbing the spread of this invasive species. Promoting the controlled utilization of the plant can minimize its ecological impact while maximizing its societal benefits.

Exploring the dermatological effects of Agave *americana L.* is a pursuit of scientific knowledge and a venture into validating and preserving traditional practices. This research holds the potential to bridge the gap between traditional wisdom and modern

science, contributing to the development of effective skincare solutions while addressing ecological concerns associated with this plant's invasive nature.

1.4 Problem statement

The spread of dermatophytosis in the somatological environment involves several risk factors, including the use of public ablution facilities, compromised immune function, excessive sweating as well as obesity, among others. These variables serve as an indication for the administration and application of complementary and alternative medicine (CAM) and certain somatic treatments that somatologists provide (Ilkit & Durd, 2014; Nordman, 2011; Wairagade et al., 2020). The skin is the largest human organ and serves as a primary barrier for protection against infection (Kambizi & Bvenura, 2021). However, its risk of infection and cross-contamination from superficial dermatophytosis is very high. This is because the skin is often exposed in the somatology environment. This is a concern, especially where there are poor clinical practices under the backdrop that symptoms often occur long after infection (Mahajan, 2020). Thus, exploring medicinal plants with potential therapeutic anti-microbial properties that may help prevent and/or ameliorate infection and cross-contamination of equipment is highly prioritised in the somatology industry (Quiroga et al., 2001). The implication of Agave *americana L.* in traditional practices highlighting its therapeutic role in managing skin diseases among the Sotho-speaking communities is well-documented (Asong et al., 2019; Kambizi & Bvenura, 2021; Moteetee & Kose, 2017). However, the lack of comprehensive scientific studies on this plant in relation to dermatology created a critical gap in our understanding of its potential benefits and uses, thus the interest in exploring Agave *americana L.* as a potential alternative for dermatophytosis management in the somatology environment.

1.5 Broad aim

The broad aim of the current study was to investigate the therapeutic properties of Agave *americana L.*, a medicinal plant used by traditional health practitioners to manage fungal infections in Bloemfontein, Free State province, South Africa.

1.5.1 Specific objectives

The specific objectives of this study were to:

1. Collect Agave *americana L.* plant samples from Bloemfontein for analysis.
2. Characterise the phytochemical constituents of Agave *americana L.*
3. Evaluate the anti-fungal activity of Agave *americana L.*
4. Evaluate the anti-inflammatory activity Agave *americana L.*
5. To determine the cytotoxicity of Agave *americana L.*

The intended above-mentioned objectives were achieved through the following steps, as shown in Figure 1.2:

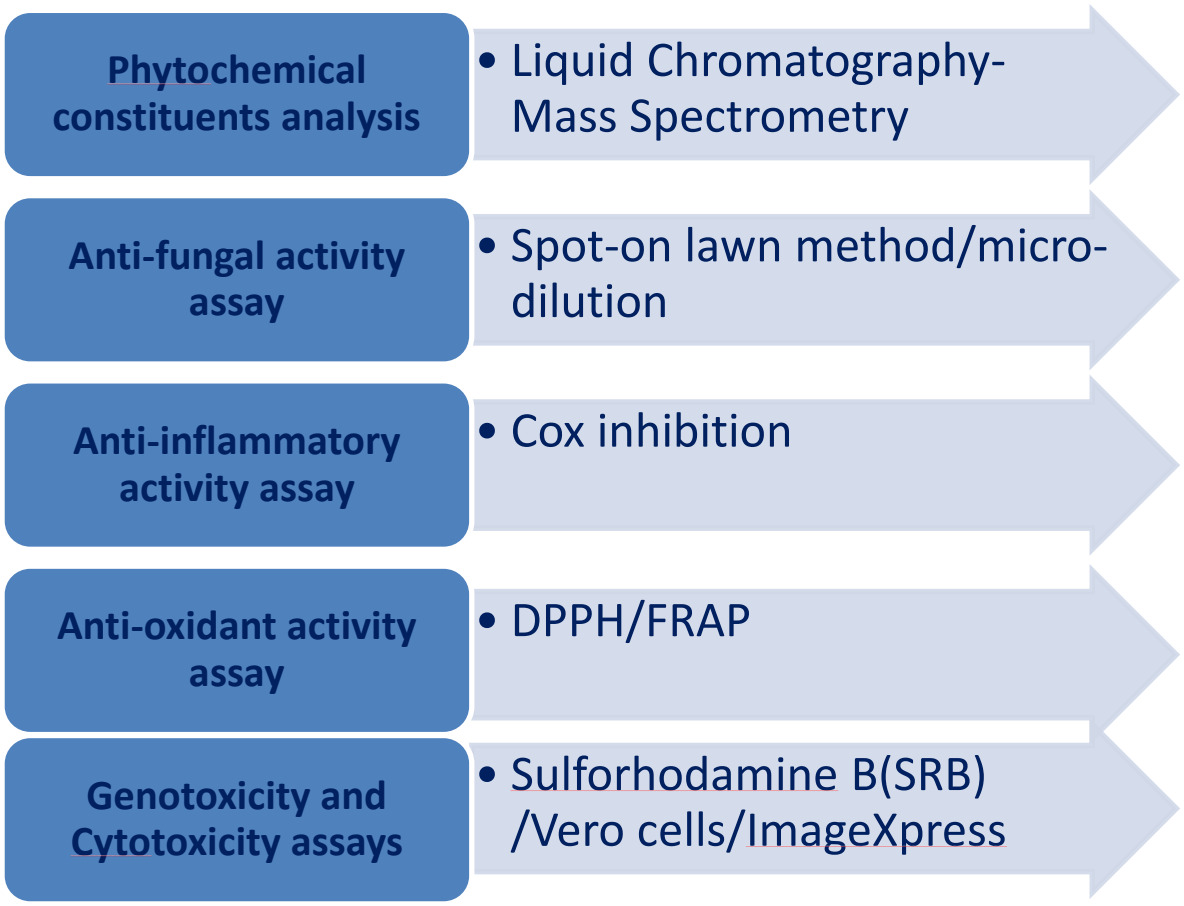

Figure 1.2: Summary of the research

1.6 Conclusion

The aim of the current study was to investigate the therapeutic properties of Agave *americana L.*, a medicinal plant used by traditional health practitioners to manage fungal infections in Bloemfontein, Free State province, South Africa. The methods

used to achieve this aim were carefully selected to achieve accurate, reliable results to complete a comprehensive scientific study on Agave *americana L.* in relation to dermatology, filling a critical gap in our understanding of the plant's potential benefits and uses as a potential alternative for dermatophytosis management in the somatology environment. The study was in pursuit of scientific knowledge venturing to validate and preserve existing traditional practices relevant to the somatology environment, potentially bridging the gap between traditional wisdom and modern science for effective skincare solutions whilst addressing ecological, economic as well as scientific factors concerning the selected plant and the somatology environment.

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CHAPTER 2
LITERATURE REVIEW

2.1 Introduction

The present study reviewed the risk factors involved with fungal infections affecting various somatic treatments somatologists offer. Furthermore, clinical features of fungal infections, causative organisms, and the management thereof were reviewed.

2.1.1 Somatology

Somatology explores the anatomy and physiology of the human body. As a branch of anthropology, somatology considers various physiological characteristics and classifications according to gender, race, and social practices (Rammanhor, 2014). Somatologists are health professionals concerned with health and skin care, as well as improving an individual's general well-being and aesthetic appearance (Richterl & JoosteII, 2013). This is achieved by providing a wide range of healthy lifestyle choices, information on correct product selection and usage, and administering professional clinical treatments. Somatologists apply and provide relevant prophylactic, corrective, and educative strategies that promote and maintain the holistic well-being of an individual (RichterI & JoosteII, 2013; Nordmann, 2010). These strategies are carried out in a range of somatic as well as complementary alternative medicine (CAM) therapies (Slabbert & Friedrich-Nel, 2015).

2.1.2 Somatic therapies & complimentary alternative medicine

Like traditional healing practices, somatic therapies and CAM are treatments that are not approved as conventional allopathic medicine (Esmonde & Long, 2008). According to the World Health Organisation (2023), traditional healing practices (THP) are an amalgamation of healthcare practises, skills and knowledge based on beliefs, theories and experiences that are indigenous to different cultures. In contrast, CAM focuses on health care practices that may not necessarily be a part of the indigenous peoples traditional or cultural practices or conventional medicine and may therefore not be a part of the health care system (WHO, 2023). But in some countries, THP and CAM

are used interchangeably. Nonetheless, today, somatic therapies and CAM are increasingly being used in combination with allopathic medicine. These therapies have been proven to reduce stress and improve immune function. As a result, somatic therapies and CAM enhance the effect of allopathic medicine (Esmonde & Long, 2008; Oleson & Flocco, 1993; Reyes et al., 2015). However, traditional healing practices are as old as humanity. In South Africa's healthcare system, STATISTA (2023) estimates that about 8.09 registered physicians service 10 000 people. The WHO recommends 25 physicians per 10 000 people to provide adequate primary healthcare (Anyangwe & Mtonga, 2007). These statistics indicate that South Africa can barely meet a third of these requirements. Alternative healthcare therapies to complement the existing conventional healthcare system are thus a necessity. However, as of 2007, approximately 200 000 traditional healers servicing nearly 80% of the black population were recorded, a figure that needs to be revised as there has been a potential change (Truter, 2007). Somatic therapies and CAM include but are not limited to numerous types of hydro, massage, slimming as well as facial treatments that can be invasive and non-invasive (Esmonde & Long, 2008). The treatments are offered by a somatologist in accordance with a set of health and safety principles. When administering various treatments, somatologists need to exercise caution through thorough and broad consultations. To provide the appropriate treatment plan or referral, the therapist is required to consider the patient's lifestyle, indications as well as contra-indications (Nordmann, 2010). One of the guiding principles for therapists' states that to improve occupational health, safety and environmental performance, therapists need to employ appropriate action to correct hazards or conditions that endanger health and safety of the patient as well the environment (Nordman, 2011). One such hazard often encountered by somatologist is fungal infections.

2.2 Fungal infections

Fungi are ubiquitous and occur only as moulds and yeasts. Although some fungi can propagate successfully in their natural environments without the need for mammalian substrate, some of the species are adventitious human pathogens causing systemic, subcutaneous, and superficial infections (Gaber, 2001). As such, over 600 different types of fungi have been reported to cause a wide range of systemic, subcutaneous, and superficial infections in humans. Dermatophytosis, also known as ringworm, is a

common superficial fungal infection caused by a group of fungi that invade and manifest on dead keratin such as hair, skin, and nails (Hayette & Sacheli, 2015) Dermatophytosis is a contra-indication that is commonly encountered during consultations in somatic therapies. This contra-indication can however go unnoticed initially, as signs and symptoms are not always immediately visible.

Although fungal infections infect billions of people per year, the many of these are minor infections are not mortal, millions of people contract diseases arising from these fungal infections that potentially claim the same number of lives as those claimed by tuberculosis and malaria (Brown et al., 2012). Although the exact mortality rates are evasive owing to a paucity of epidemiological studies, the incidence of invasive fungal infections are on the rise. According to Gaber (2001), this rise is closely linked to the rise in the number of immunocompromised people due to modifications to medical practice, including the use of immunosuppressive medications and intense chemotherapy. According to the previous author, illnesses that impair immunity, including HIV, have also contributed to this rise.

Fungi can metabolize many substances, resulting in cross-contamination of equipment, new infections, and re-infections in the human body. As a result, fungi can cause serious human diseases such as fungal meningitis (Oladele & Denning, 2014).

2.2.1 Dermatophytosis in the somatology environment

Dermatophytosis is a very contagious, common fungal skin illness that results in a rash on the skin or scalp that resembles a red or silvery ring. This infection can easily spread by contact with an infected person, contaminated equipment and/or surfaces (Badiee & Hashemizadeh, 2014). Various types of dermatophytosis, classified according to the site of infection, can occur in a somatology environment (Ely et al., 2014). The five types of dermatophytosis often encountered in a somatology environment include *Tinea capitis*, *Tinea barbae*, *Tinea circinata/ Tinea corporis (TC)*, *Tinea pedis* and *Tinea unguium*.

2.2.1.1 Tinea unguium

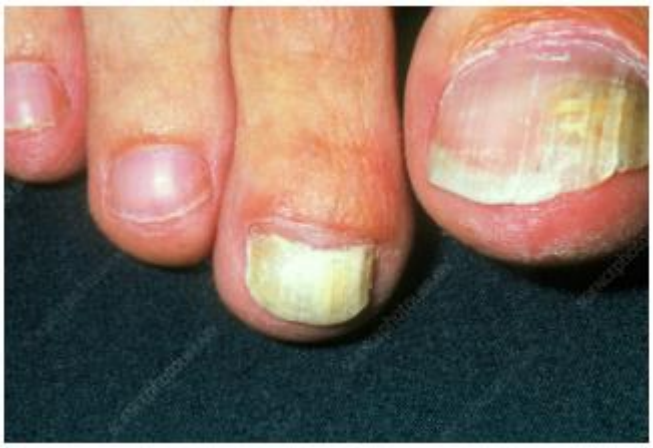

Figure 2.1: *unguium*-infected toenails (Source: Marazzi, 2023)

T. unguium, also known as toenail fungus, turns nails thick and yellow with resultant white spots and streaks as shown in Figure 2.1 (Westerberg & Voyack, 2013). This fungus *affects* both fingernails and toenails, which is the most difficult superficial fungal infection to treat, as most topical applications do not penetrate the nail plate easily (Ely et al., 2014). *Tinea unguium i*s a chronic fungal infection that can be identified by deformity of the nail, thickening and discolouration of the nail and surrounding tissue, as well as the nail becoming brittle and breaking off (Nordmann, 2010). *Tinea unguium,* also known as onychomycosis*,* is thought to be the most common dermatophytosis worldwide. About 70% of the world's population is affected by onychomycosis at some point in their life (Jo Siu et al., 2013). This could be because onychomycosis can be caused by dermatophytes, non-dermatophytes, and yeasts (Smijs et al., 2013). There is usually no pain associated with *T. unguium* unless the condition is very severe. Onychomycosis is not a life-threatening condition; nonetheless, it can impact a person's psychosocial behaviour negatively due to the deformed appearance of the affected nails, particularly when the nails on the hands are affected, resulting in negative self-perceptions and depression (Guptaa & Maysa, 2018; Jo Siu et al., 2013).

2.2.1.2 Tinea barbae

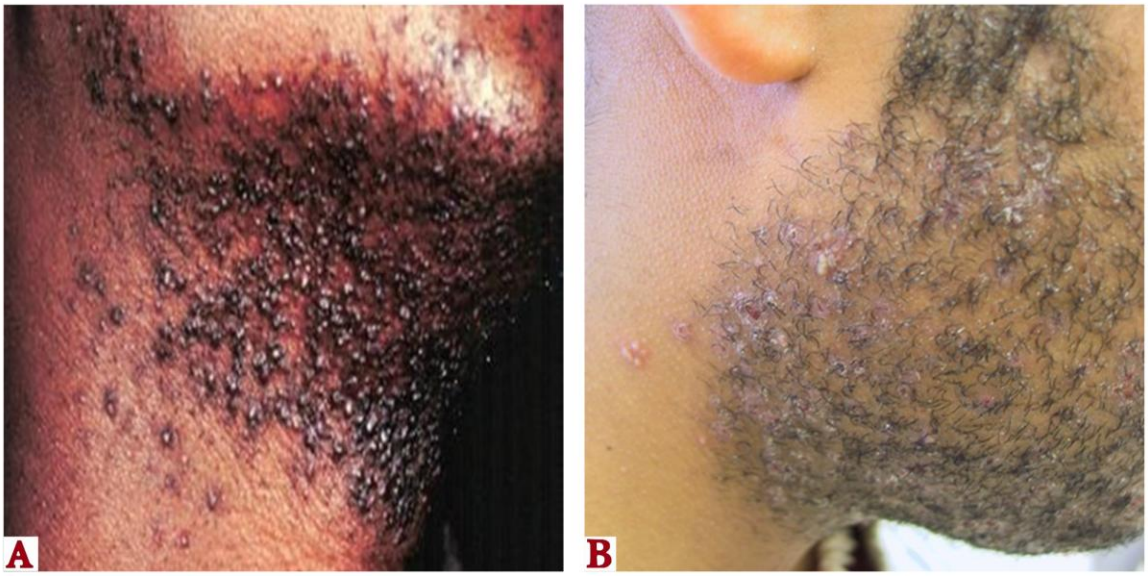

Figure 2.2 A & B: *T. barbae* and *B-P. barbae* (Source: Gray & McMichael, 2016)

T. barbae is an infection of the hair follicle on the face, appearing as small pustules and abscesses (Furlan et al., 2017). As shown in Figure 2.2 A and B, *Tinea barbae* can easily be confused with *Pseudofolliculitis barbae* (PFB), which has similar clinical features to Tinea barbae (Gray & McMichael, 2016). PFB indicates facial treatments (Gray & McMichael, 2016). The misapprehension of the two conditions increases the risk of infection and cross-contamination of equipment and working surfaces (Singh et al., 2016)

2.2.1.3 Tinea circinata

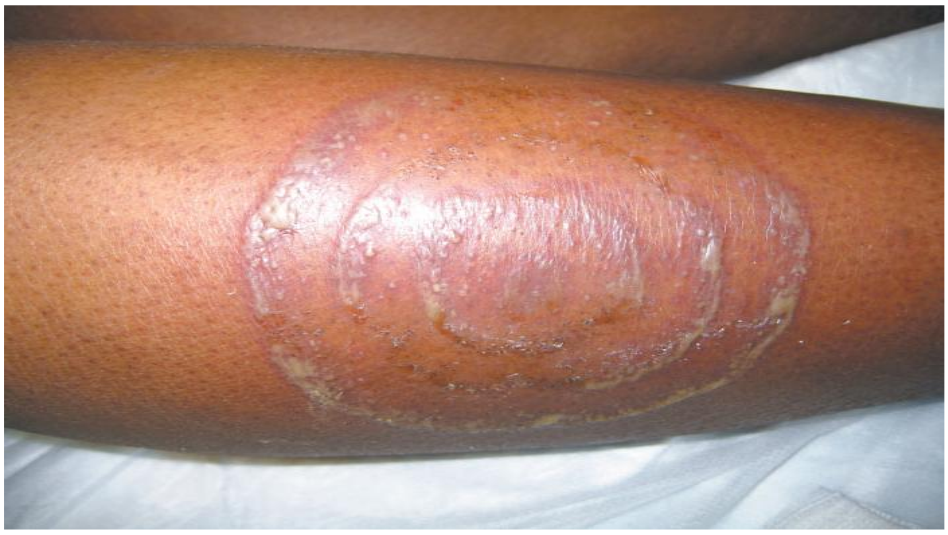

Figure 2.3: *Tinea circinata* (Kimberly et al., 2005)

T. circinata, also known as *Tinea Corporis* (TC), appears as a superficial rash accompanied by mild itching and skin peeling on various body parts (Kimberly et al., 2005). It is commonly known as a ringworm due to its spherical appearance, as shown in Figure 2.3 (Kimberly et al., 2005).

2.2.1.4 Tinea capitis

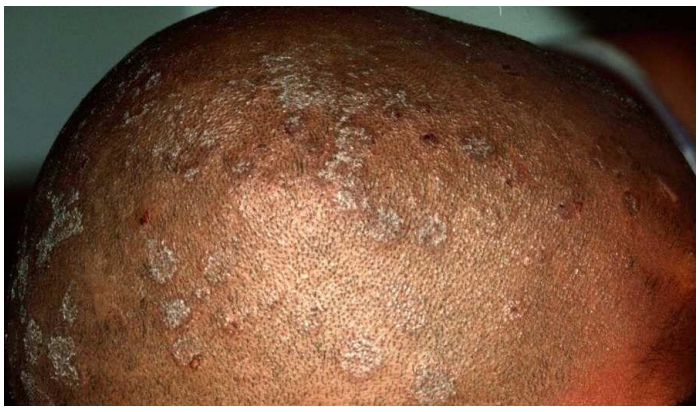

Figure 2.4: *Tinea capitis* infection (Ely et al., 2014)

Figure 2.4 below shows an infection of the scalp caused by a zoophilic dermatophyte, namely *Microsporum canis* causing hair loss, dry scaly rashes, swollen red patches, and itchiness (Auchus et al., 2016; Ely et al., 2014).

2.2.1.5 Tinea pedis

T. pedis is an infection of the feet (Figure 2.5). It is the most typical dermatophyte infection, especially in hot, tropical cities (Badiee & Hashemizadeh, 2014).

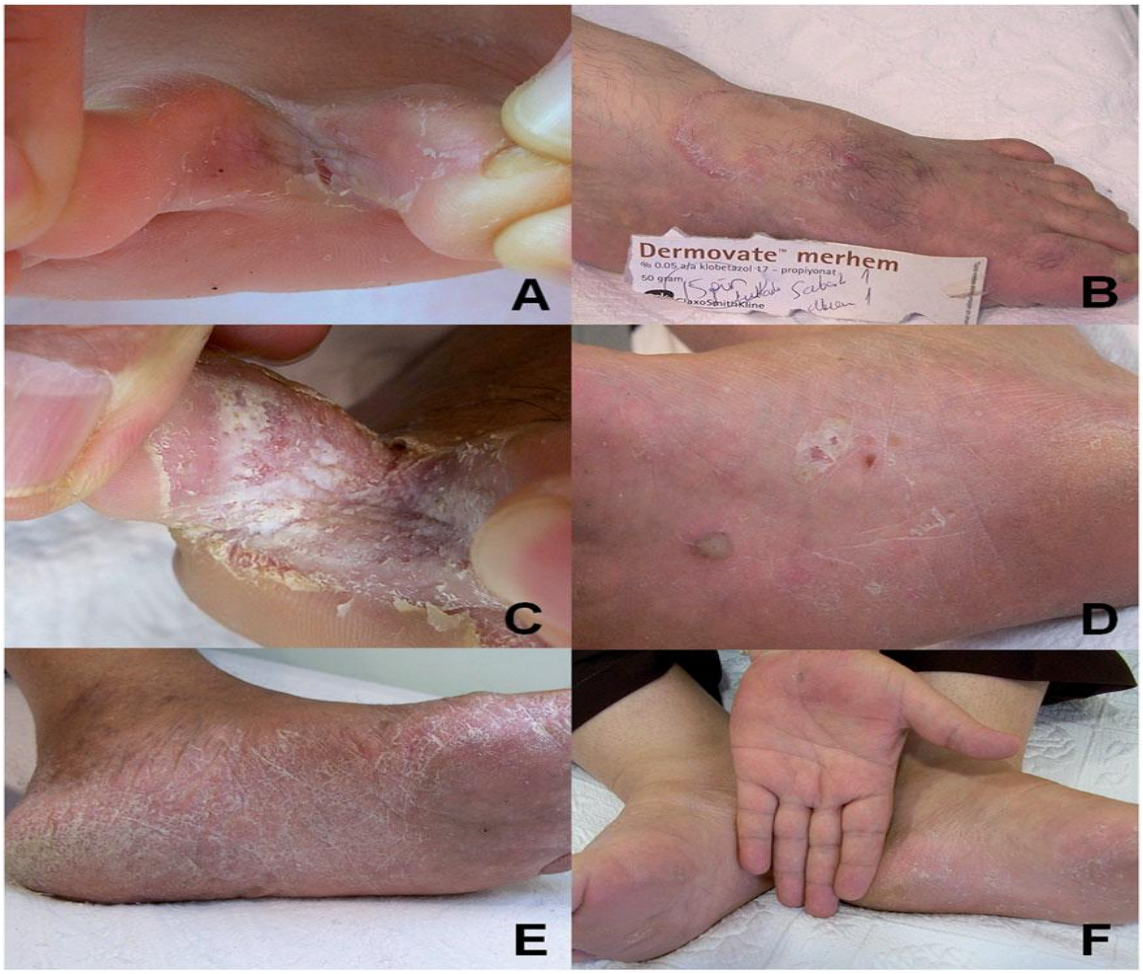

Figure 2.5: *Tinea pedis* clinical forms

Clinical forms of *Tinea pedis* include interdigital scaling, maceration and fissuring in a patient with interdigital *Tinea pedis* (A); an annular erythematous scaly plaque on the back of the foot in a patient with Tinea incognito (B); maceration with scaling borders between the toes in a patient with dermatophytosis complex (C); scaling, crust and pustules on the plantar surface of a foot in a patient with vesicular *Tinea pedis* (D); dry

hyperkeratotic scale involving the entire plantar surface in a patient with chronic hyperkeratotic *Tinea pedis* (E); and erythematous scaly plaques in ‘‘two feet-one hand’’ syndrome (F) (Ilkit & Durd, 2014).

Somatologists need to be cautious of the symptoms associated with *Tinea pedis* and *Tinea circinata* when administering complementary alternative therapy such as hot-stone therapy, aromatherapy, and reflexology. This is because the clinical features of *Tinea pedis* may occasionally be mistaken for dry, flaky skin or eczema.

2.2.2 Diagnosis of fungal infections

Diagnosis of fungal infections is often based on appearance and symptoms. Somatologists, therefore, need to familiarise themselves with these infections so that they can refer clients to a medical doctor. Diagnosis is confirmed by microbiological culturing or skin scraping under a microscope. It may take up to fourteen days after exposure for any symptoms to occur. Thus, symptoms may not be visible even though the condition is communicated from the initial infection (Ely et al., 2014).

Most infections caused by fungi are superficial. However, a fungal infection can cause fatal systemic reactions (Oladele & Denning, 2014). The severity of infection thus depends on the type of fungi and its level of toxicity. Thus, somatologists often implement educative and prophylactic strategies.

2.2.3 Management of fungal infections

Somatologists have a duty to uphold health and safety by reducing the risk of exposure to fungal infections in their environment. People with fungal infections such as dermatophytosis often suffer physical and psychological discomfort (Jo Siu et al., 2013). The most prevalent dermatophytosis in the somatology industry is *Tinea unguium.* However, symptoms of dermatophytosis often differ considerably among individuals. Therefore, various treatment options, including tablets, creams, lotions, and shampoos, are available to the affected area (Ely et al., 2014).

The type of treatment is determined by the area affected and the severity of the infection (Smith, 2015). The most effective treatment for dermatophytosis currently rendered by allopathic health practices is efinaconazole ointment. This topical

ointment is a highly effective anti-fungal with a broad spectrum of activity that can be applied on various types of dermatophyte infections (Jo Siu et al., 2013).

Efinaconazole is ostensibly a very effective option for treatment with very insignificant side effects. The side effects reported included erythema, desquamation and mild dermatitis. These occur in the localised area where efinaconazole has been applied (Lipner & Scher, 2015). Currently, somatologists are required to recognize the various types of dermatophytosis, refer the client to an allopathic practitioner, and sanitize all equipment appropriately. Furthermore, Somatologists must educate their clients by offering appropriate after-care advice that can help prevent the spread of various forms of dermatophytosis and not treat the condition themselves.

2.3 Medicinal plants

If evidence of pharmacological anti-fungal activities could be demonstrated, treating fungal illnesses with medicinal plants would provide a feasible and environmentally friendly approach. Several plants with antifungal activity have been documented. *Tacca chantrieri*, an indigenous vegetable commonly used in Thailand households, has been proven effective in treating *Tinea corporis* (Rujjanawate, 2016).

Evidence of the use of both indigenous knowledge systems and allopathic medicine has been documented in most parts of Africa, including Nigeria, Ghana, Zimbabwe, Malawi, and Tanzania, among others (Abdullahi, 2011; Peltzer & Mngqundaniso, 2008). It has been shown that rural and urban dwellers use medicinal plants, as they often consult traditional healers for health care. For example, it has been reported that five out of six HIV patients with fungal infections consult traditional healers rather than visit a clinic or hospital for primary health care in Tanzania (Hamza et al., 2006). The study of Hamza et al. (2006) found that 45% of plant species utilized in Tanzania, including *Clausena anisata,* were effective against fungal infections in addition to oral infections and gonorrhoea (Hamza, et al., 2006). This indicates the significance of medicinal plants in indigenous knowledge systems and the need to explore these species further to substantiate claims of efficacy in treating fungal infections and other diseases (Afolayan et al., 2014).

More than 27 million people living in South Africa rely on traditional healers for physiological, reproductive, emotional, and psychological healthcare (Twilley et al.,

2020) (Semenya & Potgieter, 2014). Some traditional medicine systems are supported in literature; however, a lot still needs to be scientifically verified and documented as these concepts and practical skills were verbally passed down from generation to generation (Twilley et al., 2020). The traditional medicine often used is from medicinal plants, which can be used in various ways, administered orally or topically. Some of the commonly used plants that are effective in treating numerous skin conditions include *Xysmalobium Udulatum*, *Artemisia Afra* and *Datura Stramonium* (Muggomeri et al., 2016). In South Africa, documented scientific knowledge on ethnobotany and the antimicrobial activity of many plants is still limited. Irrespective of the scarcity of documented information, the larger population still prefers to consult traditional healers for primary health care, potentially because of past good experiences, family traditions, testimonials from networks, accessibility, and affordability of herbal remedies, among others (Afolayan et al. 2014; Mcfarlane, 2015).

Conventional treatments, derived from traditional wisdom, frequently have unrealized promise in treating skin-related problems. Agave *americana L.* is a folkloric medicinal plant that is widely used by the Sotho-speaking community and is widely spread in the Bloemfontein area. It offers dermatologists an exciting new field of study. In South Africa, there are surprisingly few scientific studies on the effects of this plant on skin disorders despite its widespread use. It is commonly known that Agave *americana L.* plays a significant role in traditional dermatological treatments, particularly in skincare and the treatment of skin problems among Sotho-speaking communities (Asong et al., 2019; Kambizi & Bvenura, 2021; Moteetee & Kose, 2017). Nevertheless, a significant knowledge gap regarding this plant's possible advantages and uses in dermatology is caused by the dearth of thorough scientific research on it.

Thus, the present study aimed to explore traditional phytochemistry and biological activity, including potential cytotoxicity of Agave *americana L.* growing in Bloemfontein, a town situated in the Free State province of South Africa.

2.4 Medicinal baths

Medicinal baths date back as far as 2500BC years, with great contribution from the Greek physician Hippocrates, who is also known as “the father of medicine” (Battaglia, 2008). The conception of the Hippocratic Oath, which is still relevant today, is also

credited to this Greek physician who stated: “The way to health is to have an aromatherapy bath and scented massage every day.” Hippocrates used essential oils to rid Athens of the plague and wrote about using plants, such as frankincense, myrrh, roses, and opium, for medicinal purposes (Boekstein, 2014).

Medicinal baths are a form of Hydrotherapy, a term accepted internationally as any treatment incorporating water for its therapeutic properties (Reyes et al., 2015). Native communities globally have unique remedies for medicinal baths. In China, the Yao people are famous for their strong immune systems and give credit to their knowledge of medicinal baths (Samoisy & Mahomoodally, 2016). It has become a cultural practice for the Yao people to add herbal medicine, which they obtain from plants by various extraction methods, including expression, hydro diffusion, and maceration. This does not only treat various skin abrasions and diseases, but also helps in maintaining good health (Samoisy & Mahomoodally, 2016).

Adding anti-microbial herbal extracts as a medicinal bath for patients and clients before a reflexology treatment, for instance, could be beneficial in reducing the risk of dermatophyte infections. Thus, it is critical to explore local medicinal plants and their potential anti-microbial properties for possible incorporation in somatic and CAM therapies to manage and prevent fungal infections in the somatology environment.

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CHAPTER 3

PHYTOCHEMICAL ANALYSIS *OF AGAVE americana L.*

3.1 Introduction

Interest in medicinal plants has been on the increase in recent times because of their supposed therapeutic properties. Consumers are demanding to know the phytochemical constituents of their herbal medicines. In general, remedies from plants in their most natural form are inexpensive, easily accessible, and effective while rarely possessing side effects (Mhaladi et al., 2013)Traditional medical practitioners have long chosen certain plants for their medicinal properties, and these choices have resulted in the development of novel, therapeutically effective medications, including anti-cancer, antibacterial, and anti-hepatotoxic substances (Mhaladi et al., 2013; Sahu et al., 2013)

It is common knowledge that medicinal plants are the best source of various therapeutics for combating various ailments. As such, about 80% of individuals from developing countries such as Asia and Africa use traditional herbal medicines for primary health care globally (Oyebode et al., 2016). Nevertheless, these medicinal plants need to be investigated and validated to provide a better understanding of their pharmacological properties, safety, as well as their efficacy. Medicinal plants contain some polyphenolic compounds which provide certain physiological actions on the human body, including tannins, phenolic acids, hydrogen-containing groups, flavonoids, alkaloids, carbohydrates, Piscidic Acid, tannins, and steroids, among others (Bvenura et al., 2018). These compounds are produced by naturally existing organisms' primary or secondary metabolism. They are widely used in somatic therapies, agriculture, scientific research involving pharmaceuticals and numerous other areas. Many phytochemicals have been shown to possess inhibitory effects on various types of pathogenic microorganisms (Abachi et al., 2016). Most of these compounds are obtained from medicinal plants. The phytochemicals can be derived from the whole plant or various parts such as the bark, leaves, flowers, roots, fruits, and/or seeds.

The folkloric uses of Agave *americana L.* for various medicinal purposes, including skin-related treatments and care, have been documented (Asong et al., 2019;

Mansoor et al., 2023; Misra & Varma, 2017; Moteetee & Kose, 2019). These medicinal properties can potentially be linked to the plant's anti-inflammatory (Misra et al., 2018), antimicrobial, antibacterial, antiseptic, diaphoretic, diuretic, laxative (Misra & Varma, 2017; Shegute & Wasihun, 2020), antioxidant (Pandey et al., 2019), and antiproliferative (Khade et al., 2011) activity among others. Although these activities and findings have been reported in other parts of the world, none of these studies have focussed on the species growing in South Africa, more so in Bloemfontein. This is regardless of the importance of the plant and its prevalence, especially in the study area. Therefore, the present study aimed to reveal the secondary metabolites in Agave *americana L.* growing in Bloemfontein. This could potentially link these compounds with the various biological activities that have been linked with this important folkloric medicinal plant.

3.2 Materials and methods

3.2.1 The study site

The samples were collected near Bloemdustria in Bloemfontein, Free State South Africa, at a height of approximately 1395m above sea level and coordinates of (29.1464590, 26.3884376), located 21km from the CBD. The herbaria of plant specimens were prepared and confirmed by the Free State Botanical Garden.

3.2.2 Sample collection

The century plant was cut at the core using a hoe and handsaw. The plant was further cut into smaller pieces for drying. The spiky thorn edges were cut away using a sharp knife. The plant was air-dried at a temperature of 20°C, and portions of 200g were ground and shaken for extraction in water, methanol, acetone and DMSO. The extracts were then filtered through Whatman no.1 filter paper, and each of the filtrates was then concentrated to dryness under reduced pressure using the rotary evaporator. The water solution was extracted using the sublimation process of lyophilization. The lyophilization method, otherwise known as dry freezing, was used due to the increased quality of the obtained dried material in comparison to other methods (Assegehegn et al., 2019; Pikal et al., 1983).

3.2.3 Chemicals

All chemicals and solvents, including acetonitrile, formic acid, methanol, and DMSO, among others, which were used in this study were of LC-MS/analytical grade. Ultrapure water with a resistivity of 18.2 MΩ cm−1 at 25ºC was obtained using the Millipore water purification system. The chemicals and standards were purchased from Sigma Chemicals in Johannesburg, South Africa.

3.2.4 Sample extraction

The Hoodia inlet apparatus was used for the organic solvent extraction. About 2 g of Agave *americana L.* was accurately weighed into a 50 ml centrifuge tube and extracted using 15 ml of 50% Methanol/ 1% formic acid. A second extraction was also performed using 15 ml of 25% DMSO/25% MeCN/25% water. Extensive vortexing and sonication was used to maximize extraction of phytochemicals.

3.2.5 Liquid Chromatography-Mass Spectrometry (LCMS) analysis

High-resolution UPLC-MS analysis was performed using a Waters Synapt G2 Quadrupole time-of-flight (QTOF) mass spectrometer (MS) coupled to a Waters Acquity ultra-performance liquid chromatograph (UPLC) (Waters, Milford, MA, USA) as shown in Figure 3.1 below. Additionally, UV data for each chromatographic peak was simultaneously collected using a Waters Acquity photo diode array (PDA) detector. An electrospray source with a cone voltage of 15 V and a capillary voltage of 2.5 kV was used to inject samples into the MS. Both positive and negative approaches of data collection were used. The desolvation temperature was set to 275 °C and the desolvation gas employed was nitrogen at 650 l h^{-1}. The remaining MS settings were adjusted for maximum sensitivity and resolution. Data were obtained by scanning in both MSE and resolution modes between 150 and 1500 m/z. Both low collision energy (4 V) and high collision energy ramp (40−100 V) were used to gather two channels of MS data in MSE mode to obtain fragmentation data as well. For precise mass measurement, Leucine enkephalin was utilized as the lock mass (reference mass), and sodium formate was employed to calibrate the device. On a Waters BEH C18 2.1 × 100 mm, 1.7 µm column, separation was accomplished. A 2 µL injection volume was employed, and the solvents A and B for the mobile phase

were 0.1% formic acid and acetonitrile containing 0.1% formic acid, respectively. The gradient began at 100% solvent A for 0.5 minutes and changed linearly over 12 minutes to 100% solvent B. Following a 0.5-minute wash step at 100% B, a 2-minute re-equilibration period to initial conditions is conducted. The column temperature was kept at 55 °C, and the flow rate was 0.4 mL/min.

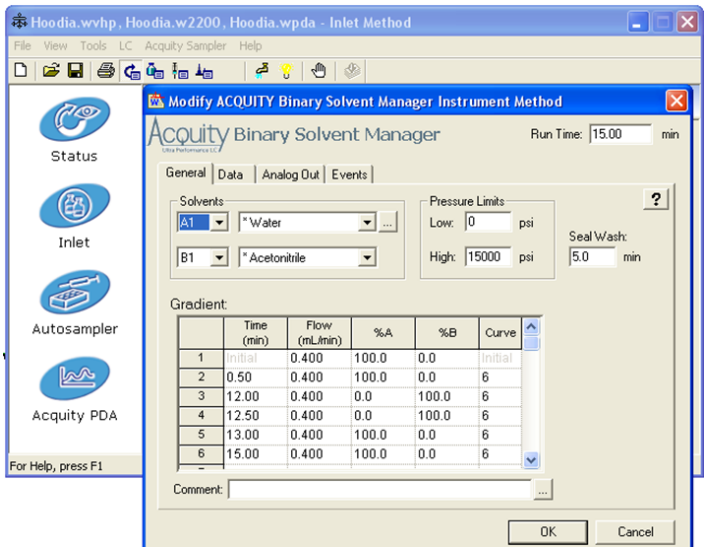

Figure 3.1: Analysis interface - Where: A1: 0.1% formic acid in water and B1: 0.1 % formic acid in acetonitrile

3.3 Results

Table 3.1: Secondary metabolites in Agave *americana L.* by LC-MS

Peak	Rt (min)[a]	*m/z* [M-H]⁻	Tentative Identification	Molecular Formula	Class
1	2.49	255.0504	Piscidic acid	$C_{11}H_{12}O_7$	Triterpenoid
2	1.79	179.0336	Eucomic acid	$C_{11}H_{12}O_6$	Triterpenoid
3	2.40	166.0868	2,3,4,5,6-Pentahydroxy-1-methoxy-7,8,9-trioxabicyclo [4.1.0] heptane-3,4,5-triol	$C_{24}H_{40}O_{20}$	Glycoside

As shown in Table 3.1 above, only three compounds were profiled and identified in *A. americana*. These compounds belong to two main classes, i.e., Triterpenoids and Glycosides. Furthermore, triterpenoids belong to a larger group of compounds known

as lipids. However, as shown in Appendix 1, there are several compounds that were not successfully identified, even though their elemental compositions were obtained. This includes the following peaks: Peak at 3.5 minutes, which gives a 427.2185 peak in ESI positive (m/z [M-H] $^{+}$) corresponding to the 425.2013 m/z peak in esi neg, with elemental composition $C_{18}H_{34}O_{11}$. The peak is at 3.571 minutes with m/z 337.1498 Da, and the elemental composition is C14H25O9 (ESI negative, i.e., m/z [M-H]$^{-}$). Also, the peak is at 3.85 minutes with m/z 1227.5637, which is possibly a saponin. In addition, the peak is at 4.2 minutes with m/z 1357.6279, which is also possibly a saponin. About five alkaloids that have not been seen before were also detected.

3.4 Discussion

The results of the present study indicate that Agave *americana L.* possessed two terpenoids (Piscidic and Eucomic Acid) and a glycoside. Our study significantly falls short of other studies. For example, the Tunisian study of Bouhlel et al. (2021) found apigenin, terpenoids, anthraquinones, tannins, puerarin, vitamin C, carbohydrates, and phlorotannin in methanolic leaf extracts of the same plant. Some authors, also from Tunisia, could detect flavonoid glycosides of kaempferol, quercetin, isorhamnetin, and ellagic acid hexosides (Maazoun et al., 2019). Furthermore, these previous authors detected some fatty acids, although Piscidic Acid which we profiled in the present study was absent. Sahnoun et al. (2018) found apigenin in the leaf extract while Sahnoun et al. (2018) found p-coumaric acid and puerarin also from Tunisian samples. Some samples from Mexico have been found to contain saponins and sapogenins (Leal-Díaz et al., 2015). Interestingly, some related *A. americana* species such as *A. rzedowskiana*, *A. impressa*, *A. ornithobroma*, *A. schidigera* and *A. angustifolia* and *A. tequilana* from Mexico were found to possess some impressive triterpenes (Ahumada-Santos et al., 2013). The present study showed that triterpenes dominated the secondary metabolites. The Indian study of Kadam et al. (2012) showed that the *A. americana* ethanolic root extracts possessed some glycosides. The present study revealed the presence of these compounds in methanolic leaf extracts. In Nepal, Pandey et al. (2019) *A. americana* leaf extracts tested negative for glycosides and coumarins. However, flavonoids, alkaloids, saponins and reducing sugars indicated a presence. In contrast, our study tested positive for glycosides.

The role of Piscidic Acid in human physiology is understudied. A few docking studies have been conducted to elucidate its role better. For example, the study of Ressaissi et al. (2017) evaluated the effects of Piscidic Acid as an inhibitor of cholesterol permeation through a Caco-2 cell monolayer and as 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitor. These authors showed that Piscidic Acid could adequately bind to the HMG-CoA. If Piscidic Acid can adequately bind to the active site and modulate the enzyme's activity, it may have implications for cholesterol regulation or other physiological processes associated with HMG-CoA. These previous authors had previously shown that isorhamnetin glycosides and Piscidic Acid can interact with the antiacetylcholinesterase (AChE) active site, but with later accounting for about 14% enzyme inhibitory effect (Ressaissi et al., 2016).

Like Piscidic Acid, the role of Eucomic acid in human physiology remains speculative. Non the less, some authors have reported the antioxidant activity of this compound (De Santiago et al., 2018). In another study, Simmler et al. (2011) studied the effect of Eucomic acid from *V. teres* stems on cytochrome *c* oxidase isolated from a human keratinocyte cell line (HaCaT) and found that this compound increased cytochrome *c* oxidase activity without enhancing cellular mitochondrial constituents. These authors, therefore, concluded that this compound has great anti-ageing activity potential for further studies. In the somatology environment, this could potentially be of great interest. In a similar study, Lee et al. (2023) reported that some compounds, including Eucomic acid from *Ginkgo biloba* fruits, potentially prevent damage in TNF-α-Stimulated normal human dermal fibroblasts, therefore indicating this compound’s potential against skin damage as well as ageing.

Aruwa et al. (2019) hypothesised that the high *Opuntia ficus-indica* antimicrobial activity was due to the presence of Piscidic and Eucomic acids, among others. In general, the presence of Piscidic Acid indicates the presence of coumaric acid and vice versa, as shown in numerous studies that report on these compounds. This theory also applies to our study.

But, in addition to promoting transdermal absorption, terpenoids generally possess anticancer, anti-inflammatory, antibacterial, antiviral, and antimalarial properties (Lee et al., 2020). According to the same authors, they also have hypoglycaemic properties and can prevent and treat cardiovascular disorders. According to earlier research,

terpenoids also offer a wide range of possible uses, including insect resistance, immunoregulation, antioxidation, antiaging, and neuroprotection (Paduch et al., 2007).

Conversely, glycosides constitute a large group of secondary metabolites, too many to mention. All glycosides are composed of two parts: a hydrophilic glycone unit comprising one or more sugar components and an aglycone (genin) unit mostly lipophilic (Bartnik & Facey, 2024). In human physiology, glycosides can have various roles and effects depending on their specific types and properties. However, some antioxidant, anti-inflammatory, and antiproliferative, and their role in detoxification, among others, are some of their roles in human physiology (Bartnik & Facey, 2024). However, the effects of glycosides can vary widely based on their chemical structures and the specific tissues or organs they interact with. While some glycosides have beneficial effects, others may have toxic properties.

Perhaps we report the low number of secondary metabolites in this study because the plant has not been well studied using LCMS. Thus, very little literature can help identify the various compounds obtained. Furthermore, geographical regions, species variations, and assay principles, among others, may also be the leading causes for this low number.

3.5 Conclusion

The present study profiled Agave *americana L.* secondary metabolites in the species growing in Bloemfontein by LC-MS techniques. Three compounds were detected: triterpenoids (Piscidic and Eucomic acid) and a glycoside. In addition to other human physiological functions, terpenoids are well known for promoting transdermal absorption and anti-inflammatory properties. Glycoside functions are diverse based on their chemical structures and the specific tissues or organs they interact with. These results do not present much promise; however, perhaps biological activities in the following chapters will shed light on this.

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CHAPTER 4

BIOLOGICAL ACTIVITY OF Agave *americana L.* LEAF EXTRACTS

4.1 Introduction

Medicinal plants used for therapeutic and/or cosmetic purposes contain numerous bioactive compounds with copious health benefits. Antioxidants, one of the most significant secondary metabolites/ compounds in medicinal plants, are essential nutraceuticals, as they protect cells on an intracellular as well as extracellular level (Droge, 2002). When there is a natural antioxidant imbalance in the system, the inflammatory response of various cells is triggered. Thus increasing the risk of various chronic and/or reoccurring skin conditions, such as dermatitis and fungal infections, among others (Arulselvan et al., 2016).

On the other hand, macrophages perform a variety of complex microbicide functions, including surveillance, chemotaxis, phagocytosis, and destruction of pathogenic micro-organisms (Mahla et al., 2021). Therefore, the development of therapeutics which can non-specifically augment the innate immune response represents a promising strategy to combat classical and emerging infectious agents (Korkina et al., 2008). Macrophages represent a highly heterogeneous group of hematopoietic cells, which are present in almost all tissues, including adipose tissue (Novak & Koh, 2013). Depending on the trigger, macrophage responses can be divided into two distinct and mutually exclusive activation programs: classical and alternative. Classical activation produces a highly inflammatory phenotype, mainly in response to bacterial products such as Lipopolysaccharide (LPS) and IF-γ. These classically activated macrophages produce a myriad of pro-inflammatory signals, which can alter the functionality of their surrounding cells. In addition, these activated cells produce various highly reactive oxidants, including nitric oxide (NO), a product of the catalysis of arginine by the enzyme inducible nitric oxide synthase (iNOS) (Novak & Koh, 2013) (Vladimir et al., 2010).

Although inflammation is usually associated with a protective or healing response, many chronic diseases are characterised by persistent inflammation, ultimately resulting in tissue dysfunction (Eming et al., 2007). For this reason, the anti- or pro-inflammatory activity of plant extracts needs to be considered within the context of the

disease and/or condition in question as well as the disease progression stage at which intervention will be considered, to evaluate potential therapeutic significance accurately (Novak & Koh, 2013). This is particularly important when dealing with re-occurring skin conditions such as fungal infections, as there is a wide spectrum of dermatophytosis, which increases the risk of infection, re-infection and contamination of equipment and surrounding area (Badiee & Hashemizadeh, 2014).

Reports from various parts of the world indicate that *A. americana* is rich in a wide range of secondary metabolites including steroidal saponins, sapogenins, flavonoids, homoisoflavonoids, phenolic acids, fatty acids, fatty acid amides, alkaloids, tannins, glycosides, and terpenoids among others (El-Hawary et al., 2020; Shegute & Wasihun, 2020; Sidana et al., 2016). These secondary metabolites are potentially linked to the antioxidant and anti-inflammatory (Misra et al., 2018), antibacterial (Shegute & Wasihun, 2020), anticancer (Santos-Zea et al., 2016), and anti-neuroinflammatory (Herrera-Ruiz et al., 2019) activity that has been previously reported in *A. americana*. Various secondary metabolites confer different health benefits. However, steroidal saponins have been shown to possess antifungal activity against some pathogens (Dimoglo et al., 1985), while other reported activities for this class of compounds include antitumor, hypoglycaemic, immunoregulatory and cardiovascular disease prevention and treatment (Sparg et al. 2004).

Leaf extracts of Agave *americana L.* were thus evaluated for antifungal activity against four fungal specimens including *Tinea tonsurans, Tinea rubrum, Tinea interdigitalis* and *Candida albicans.* The fungal specimens selected are the various types of dermatophytosis classified according to the site of infection, which can occur in a somatology environment (Ely et al., 2014). Different solvents are known to extract different metabolites in plants based on solvent polarity. As a result, having a variety of standard investigation techniques is essential for assessing and contrasting findings and conclusions. Therefore, in the present study, water, 2,2-diphenyl-1-picrylhydrazyl (DPPH) and Ferric reducing ability of plasma (FRAP) methods were used to evaluate antioxidant, anti-inflammatory as well as anti-fungal effects activity in *A. americana*.

4.2 Materials and methods

4.2.1 Antioxidant activity

4.2.1.1 FRAP

About 50 µL of extract (0.9765625 – 500 µg/ml) were aliquoted into a 96-well plate, followed by the addition of 200 µL of the FRAP reagent. The FRAP reagent was prepared using acetate buffer (300 mM, pH 3.6), 10 mM TPTZ in 40 mM HCl and 20 mM $FeCl_3$ in water at 10:1:1 (v/v/v). The plate was incubated for 30 min at room temperature, and the absorbance was measured at 593 nm using a BioTek Powerwave XS spectrophotometer (Winooski, VT, USA). A standard curve was prepared using different concentrations of ferrous sulphate (3.906 – 125.3 µM), and the results were then expressed as ferrous sulphate equivalents (µM).

4.2.1.2 DPPH

About 5 µL of extract (0.9765625 – 500 µg/ml) was transferred to the wells of a 96-well plate, followed by the addition of 120 µL of Tris-HCl buffer and 120 µL of 0.1 mM DPPH. The plate was incubated at room temperature for 20 minutes, after which the absorbance was measured at 513 nm using a BioTek Powerwave XS spectrophotometer (Winooski, VT, USA). The antioxidant activity was calculated as % DPPH radical scavenging activity using the following equation:

4.2.2 Anti-inflammatory activity

4.2.2.1 Reagents

RAW 264.7 mouse macrophages were purchased from Cellonex in South Africa. Lipopolysaccharide (LPS), Greiss reagent and aminoguanidine were purchased from Sigma-Aldrich in St. Louise, MO, USA.

4.2.2.2 Sample preparation

Extracts were solubilised in DMSO to a final 100 mg/ml concentration and further diluted into a culture medium. Aminoguanidine (100 µM) was used as a positive control to indicate anti-inflammatory activity.

4.2.2.3 Anti-inflammatory screening protocol

RAW 264.7 cells were seeded into 96-well plates at a density of 25,000 cells per well and allowed to attach overnight. The following day, the spent culture medium was removed, and the samples (diluted in Dulbecco's Modified Eagle Medium [DMEM] complete medium) were added to give final concentrations of 25, 50, 100 and 200 µg/mL (50µl per well at double the desired final concentration). To assess the anti-inflammatory activity, 50 µl of LPS (final concentration of 500 µg/mL) containing medium was added to the corresponding wells. Cells were incubated for a further 18 hours. To quantify NO production, 50 µl of the spent culture medium was transferred to a new 96-well plate, and 50 µl Griess reagent was added. Absorbance was measured at 540 nm, and the results expressed relative to the appropriate untreated control. A standard curve using sodium nitrite dissolved in a culture medium determined the concentration of NO production in each sample. Cell viability was assessed using MTT to confirm the absence of toxicity as a contributory factor. This was achieved by removing the remaining medium and treatments in each well, replacing them with medium containing 0.5 mg/mL MTT, and further incubating for 30 min at 37°C. MTT was removed, and 200 µL DMSO was added to each well to solubilise the formazan crystals. Absorbance was read at 540 nm using a BioTek® PowerWave XS spectrophotometer (Winooski, VT, USA).

4.2.2.4 Pro-inflammatory screening protocol

The procedure as described for the anti-inflammatory screening protocol above was followed. However, LPS was not added to any well.

4.2.3 Anti-fungal assay

4.2.3.1 Technique

A widely utilized method for studying anti-fungal and other anti-microbial activity is the spot-on-lawn technique. A modified version of Fleming et al. (1975) was used to evaluate the antifungal activity of three fungal strains and a yeast (Schillinhger & Lucke, 1989). The spot-on-lawn technique is based on the diffusion through solid or semi-solid culture media to inhibit the growth of probing organisms. The advantage of

this method is that it is reasonably simple and inexpensive, which is why, despite it being categorically less technical, it is still a reliable method widely used for the detection of antimicrobial activity (Fleming et al., 1975; Soomo et al., 2007).

4.2.3.2 Sample preparation

The streak plate method was used to grow the fungi and yeast. The following cultures were purchased from Thermo Fischer Scientific (Waltham, Massachusetts, USA):

- *Tricophyton rubrum* [ATCC 28188]
- *Tricophyton interdigitalis/metagrophyt* [ATCC 9533]
- *Tricophyton tonsurans* [ATCC 28942]
- *Candida albicans* [ATCC 10231]

The media evaluated and utilized was purchased from Merck (Pty) Ltd, South Africa:

- CM0041 Sabouraud Dextrose Agar (500g).

The four species strains were grown on the prepared agar according to standard methods and incubated at 37ºC for seven (7) days, with evaluation and measurement of growth on day one (1), (3) and (7), respectively. To measure the minimum inhibitory concentration (MIC), the broth microdilution method was implemented with the extracts to be evaluated (Eloff, 1998). The starting concentration for the three extracts was Hundred (100) mg/mol. These were streaked and overlaid over the SD agar plates. The plates were incubated at 37ºC for one (1) hour. A total of one hundred and eighty (180) of these strains were plugged and spotted onto the overlaid surface for evaluation of MIC. The plates were incubated at 37ºC for eight (8) days and were subsequently examined on Days 4 and 8 for inhibition zones (Hewitt & Vincent, 1989).

4.3 Results

4.3.1 Antioxidant activity

4.3.1.1 FRAP

From the FRAP assay, the water extract (Figure 4.3) showed the most potential, followed by the methanol (Figure 4.2) and acetone (Figure 4.1) extracts, respectively. In fact, 250 and 500 µg/ ml of the water extract were significantly more active than

Trolox and EGCG. However, the activity of these two concentrations (250 and 500 µg/ml) did not significantly differ. Although statistically significant results were obtained for the acetone and methanol extracts with the FRAP assay, these effects are negligible as these differences are relatively small compared to Trolox and EGCG.

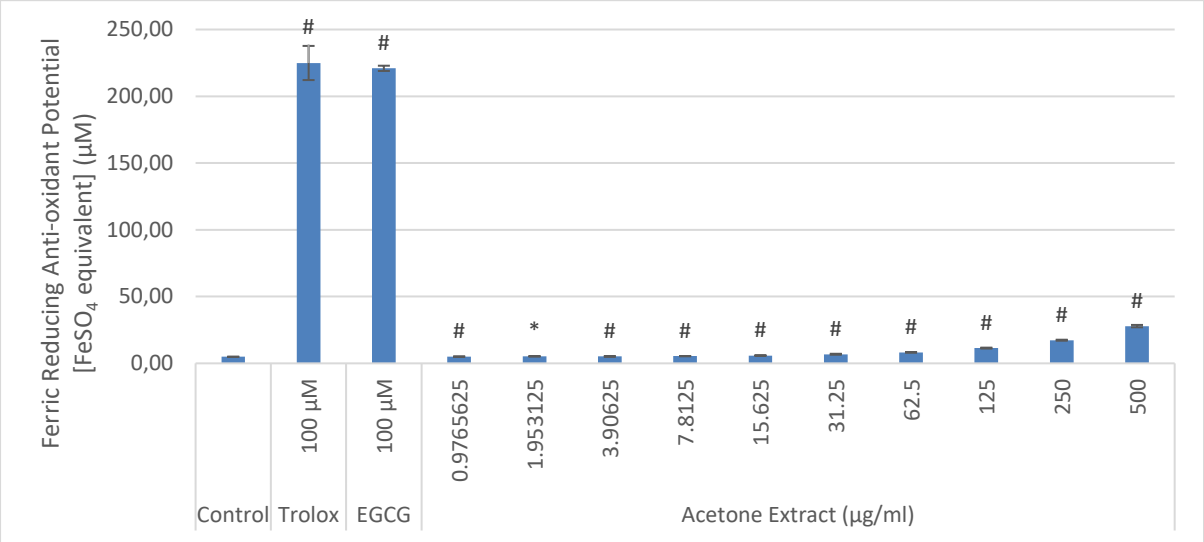

Figure 4.1: FRAP antioxidant potential of *A. americana acetone* extract

Error bars indicate the standard deviation (SD) of quadruplicate values. Significance was determined relative to the control using the two-tailed Student t-test: *p<0.05; #p<0.005.

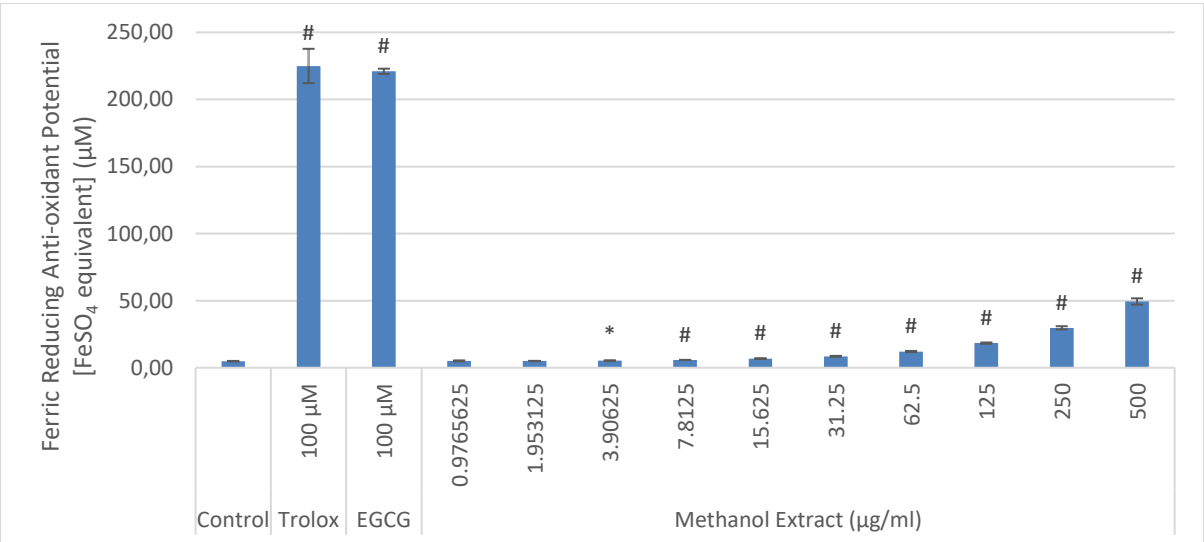

Figure 4.2: FRAP antioxidant potential of *A. americana* methanol extract

Error bars indicate the SD of quadruplicate values. Significance was determined relative to control using the two-tailed Student t-test: *p<0.05; #p<0.005.

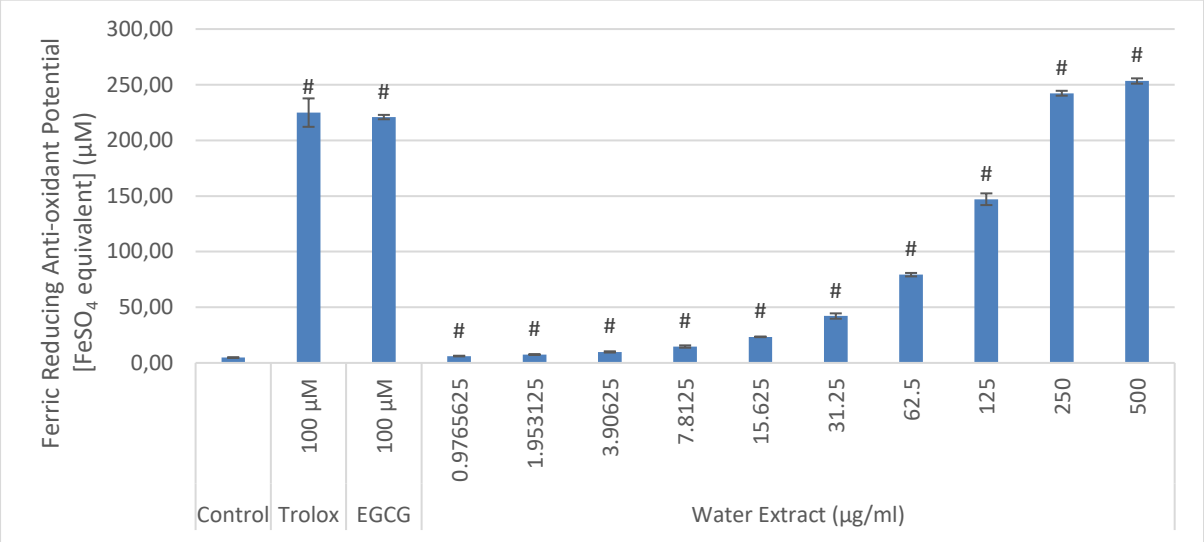

Figure 4.3: FRAP antioxidant potential of *A. americana* water extract

Error bars indicate the SD of quadruplicate values. Significance was determined relative to control using the two-tailed Student t-test: *p<0.05; #p<0.005.

$$\%\ \text{DPPH Radical Scavenging Activity} = \frac{\text{Abs. Control} - \text{Abs. Extract}}{\text{Abs. control}} x\ 100$$

4.3.1.2 DPPH

The results of the present study showed that the acetone extract (Figure 4.4) was completely inactive. However, the methanol extract (Figure 4.5) was active, although this was negligible because this activity was relatively small compared to Trolox and EGCG. This is also beside the fact that most of the concentrations were significantly higher than the control. However, the water extract (Figure 4.5) showed favourable activity with concentrations of 125 and 250 µg/ ml, showing promise as they did not significantly differ from Trolox and EGCG. These results are somewhat comparable with the FRAP assay. Overall, the water extract showed the best potential with IC_{50} values between 15.625 and 31.25 µg/ml for the DPPH Scavenging. It is also very clear

that the Water extract is more effective in DPPH scavenging than reducing ferric ions to ferrous ions.

Therefore, the difference between the FRAP and DPPH results is most likely due to the principles of the assays, respectively.

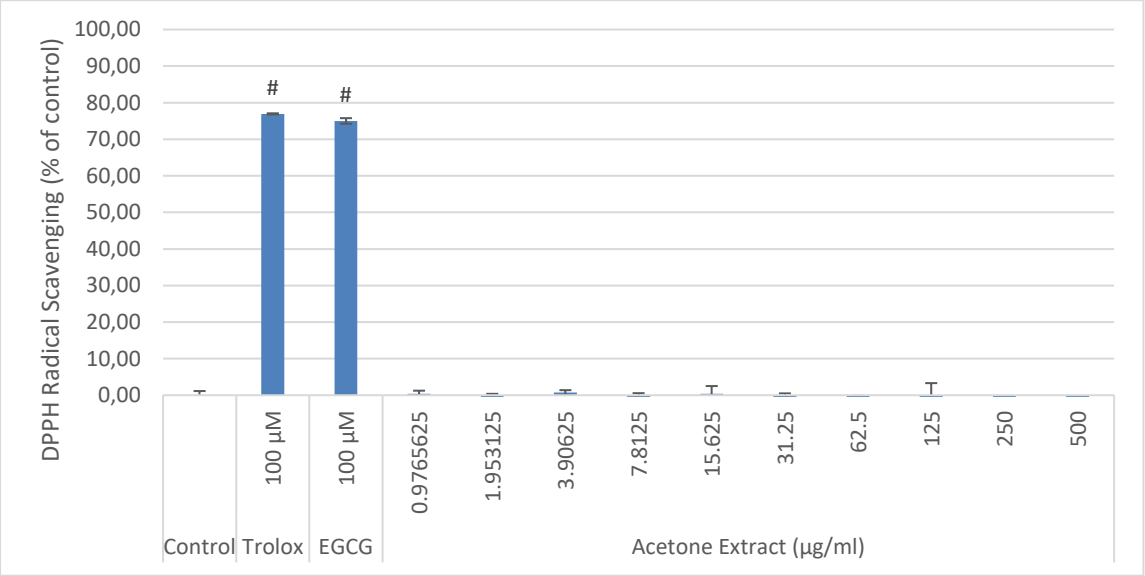

Figure 4.4: DPPH scavenging antioxidant potential of A. americana acetone extract

Error bars indicate the SD of quadruplicate values. Significance was determined relative to control using the two-tailed Student t-test: *p<0.05; #p<0.005.

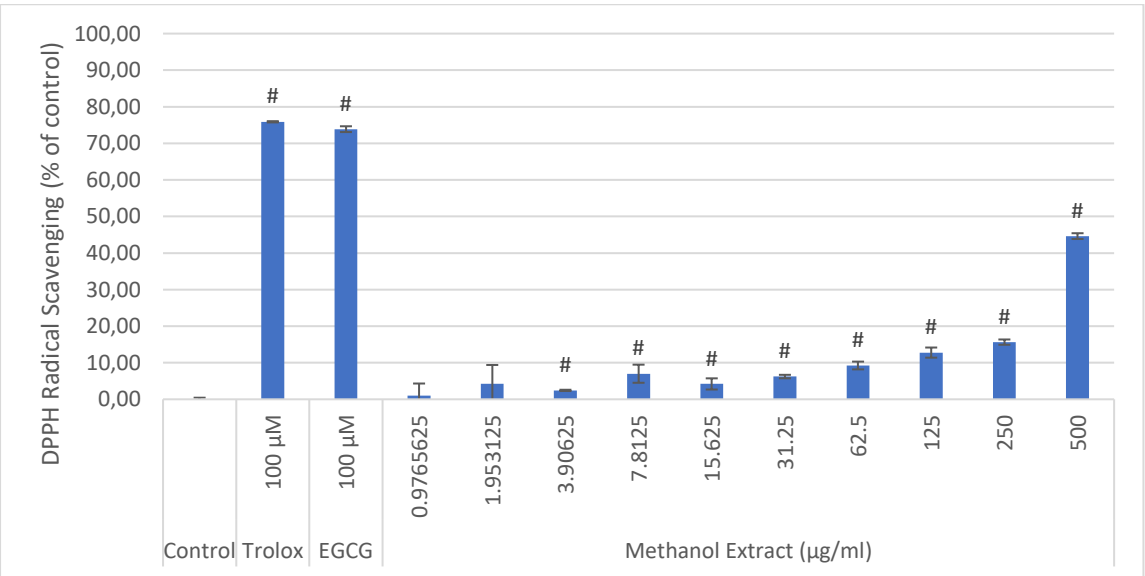

Figure 4.5: DPPH scavenging antioxidant potential of A. americana methanol extract

Error bars indicate the SD of quadruplicate values. Significance was determined relative to control using the two-tailed Student t-test: *p<0.05; #p<0.005.

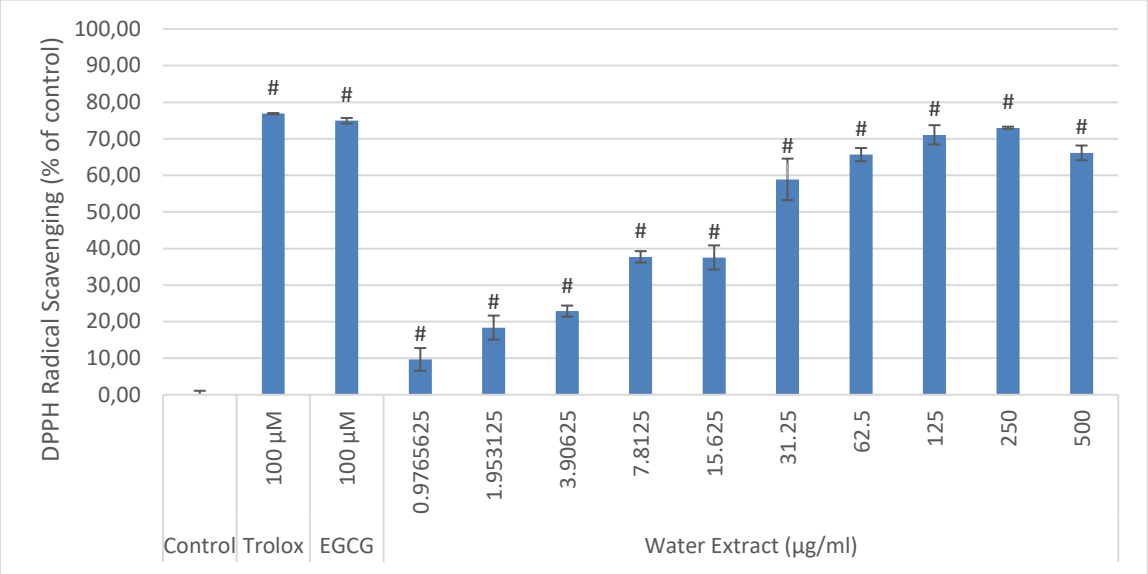

Figure 4.6: DPPH scavenging antioxidant potential of A. americana water extract

Error bars indicate the SD of quadruplicate values. Significance was determined relative to control using the two-tailed Student t-test: *p<0.05; #p<0.005.

4.3.2 Anti-inflammatory activity

Pro- and anti-inflammatory potential of Agave *americana L.* acetone, methanol and water extracts are represented in Figures 4.7 and 4.8, respectively. The results indicate that only the water extract showed pro-inflammatory activity, as seen by the high nitrite concentrations at the low extract concentrations, which decreased dose-dependent (Figure 4.7B). Furthermore, the results indicated a corresponding decrease in cell viability in the water extract-treated cells (Figure 4.7A), indicating toxicity. The decrease in pro-inflammatory activity at high extract concentrations can, therefore, be ascribed to the toxicity of the extract at those higher concentrations. None of the extracts exhibited anti-inflammatory potential. Although a decrease in nitrite concentration is seen for the water extract and at the highest concentration of the methanol extract (Figure 4.8 B), this is due to the decrease in cell viability seen in Figure 3.8A.

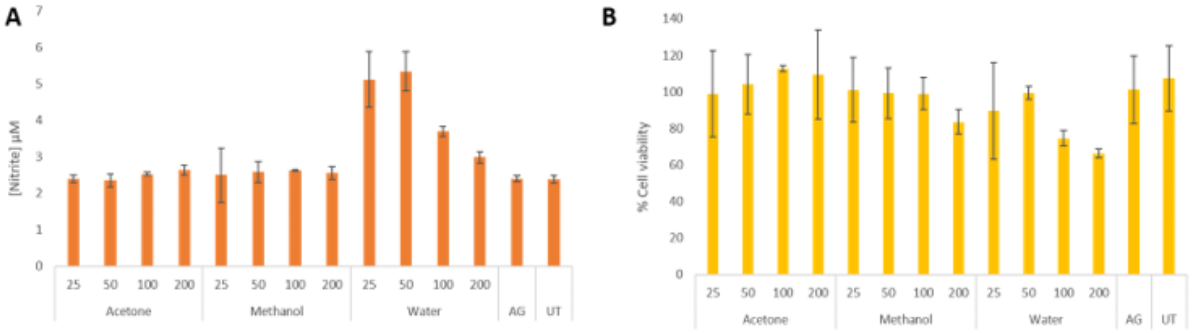

Figure 4.7: Pro-inflammatory effect of extracts at four concentrations (µg/mL) in RAW 264.7 mouse macrophages

Where: (A) Nitric oxide (NO) production after 18hrs of treatment, and (B) Cell viability assessment after NO analysis. AG; aminoguanidine (100 µM). UT; untreated control. Error bars denote the standard deviation of the mean (n=3).

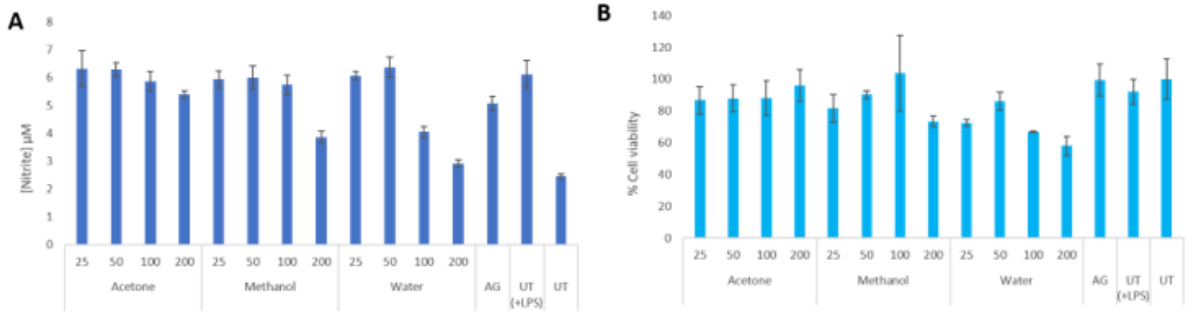

Figure 4.8: Anti-inflammatory effect of extracts at four concentrations (µg/mL) in RAW 264.7 mouse macrophages

Anti-inflammatory effect of extracts at four concentrations (µg/mL) in RAW 264.7 mouse macrophages. Where: (A) Nitric oxide (NO) production after 18hrs of treatment, and (B) Cell viability assessment after NO analysis. AG; aminoguanidine (100 µM). UT; untreated control. Error bars denote the standard deviation of the mean (n=3).

4.3.3 Antifungal activity

The results of the effect of acetonic, methanolic and aqueous Agave *americana L.* extracts on different fungi that have been linked to dermatological infections in somatology are shown in Table 4.1.

4.3.3.1 Acetone extract

The results indicate that *C. albicans, T. interdigit, T. rubrum* and *T. tonsurans* were resistant to all dilutions tested.

4.3.3.2 Water extract

The results indicate that there was some inhibition of *C. albicans* within 48 hours but showed resistance in 72 hours. The rest of the organisms were resistant to the dilutions tested, however, a bacterial growth from solution the water extract caused inhibition of the test organisms.

4.3.3.3 Methanol extract

Like the acetone extract, *C. albicans, T. interdigit, T. rubrum* and *T. tonsurans* were resistant to all dilutions tested.

Table 4.1: Antifungal effects of Agave *americana L.* extracts on different dermatological pathogens

SAMPLE	*C. albicans*			*T. Interdigit*			*T. rubrum*			*T. tonsurans*		
	48h	72h		48h	72h	144 h	48h	72h	144 h	48h	72h	144 h
A: 0.3	0	0	-	n/g	0	0	n/g	0	6	n/g	n/g	0
0.6	0	0	-	n/g	0	0	n/g	0	0	n/g	n/g	0
1.25	0	0	-	n/g	0	0	n/g	0	0	n/g	n/g	0
2.5	0	0	-	n/g	0	3	n/g	0	0	n/g	n/g	0
5.0	0	0	-	n/g	0	4	n/g	0	5	n/g	n/g	0

H: 0.3	0	0	-	n/g	0	2	n/g	0	0	n/g	0	0
0.6	0	0	-	n/g	0	0	n/g	0	0	n/g	0	20*
1.25	0	0	-	n/g	0	0	n/g	0	0	n/g	0	0
2.5	8	4	-	n/g	0	3	n/g	0	8	n/g	0	10*
5.0	6	2	-	n/g	0	4	n/g	14*	18*	n/g	0	0
M: 0.3	0	0	-	n/g	0	0	n/g	0	0	n/g	0	0
0.6	0	0	-	n/g	0	0	n/g	0	0	n/g	0	0
1.25	0	0	-	n/g	0	0	n/g	0	0	n/g	0	0
2.5	0	0	-	n/g	0	0	n/g	0	0	n/g	0	0
5.0	0	0	-	n/g	0	0	n/g	0	0	n/g	0	0
Nystatin control	28	26	-	n/g	14	10	n/g	18	12	n/g	32	28
20µg (100 IU)												

Where: * Bacterial growth causing inhibition (gram positive bacilli); n/g = no growth; A = Acetone extract; H = Water extract; M = methanol extract; Zones of inhibition in are in mm

4.4 Discussion

4.4.1 Antioxidant activity

A substance's ability to convert a ferric (Fe^3+) tripyridyl triazine (TPTZ) complex into a ferrous (Fe^2+) form, resulting in a colour shift, is measured by the FRAP assay (Zhong, Y., & Shahidi, 2015). The reduction potential is indicative of the antioxidant capacity. Therefore, the results of the present study indicate that the water extract was active compared to the acetone and methanol extracts. Antioxidant studies on *A. americana* using the FRAP method are scarce. However, Lo´pez-Romero et al. (2018) reported activity in ethanol extracts of Agave *angustifolia*, a closely related species. However, the present study did not extract using ethanol. However, the study of Mohanalakshmi et al. (2013) showed that the methanolic extracts of Agave cantala, another closely related species, were active. This study contrasts with the current study that found the methanol extract inactive.

The DPPH results of the present study showed that the acetone extract was completely inactive, the methanol extract negligibly active, and the water extract significantly active. The DPPH assay is commonly used to measure the antioxidant activity of a substance. DPPH, a stable free radical, can accept an electron or a hydrogen radical to create a stable diamagnetic molecule. DPPH changes from purple to yellow because of antioxidants, which give it electrons or hydrogen atoms, a colour shift that spectrophotometry can measure (Ionita, 2021). In other studies, Pandey et al. (2019) found *A. americana* methanol leaf extract negligibly active in comparison with our study. Ahumada-Santos et al. (2013) found the DPPH assay to be highly active in six Agave species, excluding *A. americana*. The study by Lo´pez-Romero et al. (2018) revealed some ethanolic extract antioxidant activity in Agave *angustifolia*, a closely related species.

Although the present study showed that methanolic and acetonic extracts of Agave *americana L.* negligibly possess some antioxidant activity, other studies and some closely related species have shown considerable activity. The water extract, however, proved to be more active, and this justifies the use of water extracts by the local traditional healers and herbalists. Water is a polar solvent, while acetone and methanol are polar aprotic solvents. Antioxidants with polar characteristics, such as certain phenolics, flavonoids, and ascorbic acid, may be more effectively extracted by water. The choice of solvent, therefore, influences which types of compounds are extracted. Perhaps more importantly, variations in species, geographical location, climatic conditions, and plant age, among other factors, are known to affect the antioxidant potential of plants (Zargoosh et al., 2019). These factors, therefore, need to be taken into consideration.

In summary, the difference between the FRAP and DPPH results is most likely due to the principles of the assays, among other factors.

4.4.2 Pro- and anti-inflammatory potential

The results of the pro-and-anti-inflammatory assays indicate that only the water extract showed pro-inflammatory activity. Furthermore, the results indicated a corresponding decrease in cell viability in the water-extract-treated cells. A decrease in cell viability, along with a decrease in NO levels, may suggest a cytotoxic effect of the compounds.

On the other hand, if cell viability is maintained or improved while NO levels decrease, it could indicate anti-inflammatory effects without significant cytotoxicity.

However, it is unclear if the observed results are due to the plant extract constituents or whether it is due to the presence of endotoxins in the sample (originating from bacterial contamination of the plant material and/or during extraction), a common feature of water extracts. The decrease in pro-inflammatory activity at high extract concentrations can, therefore, be ascribed to the toxicity of the extract at those higher concentrations. None of the extracts exhibited anti-inflammatory potential. Although a decrease in nitrite concentration is reported for the water extract and at the highest concentration of the methanol extract, this is due to the decrease in cell viability seen in Figure 4.8A.

In contrast to the present study, numerous previous pharmacological studies have demonstrated the anti-inflammatory properties of various agave species, primarily due to the presence of terpenes and steroidal saponins (Augustin, 2005; Da Silva et al., 2022; 2005; Hernández-Valle et al., 2013; Riaz et al., 2023). However, Monterrosas-Brisson et al. (2013) reported that *A. americana* can reduce the inflammatory process brought on by various chemicals. As previously noted, perhaps variations in plant extract constituents, geographical location, species, and principles of the assay, among other factors, are probable causes for the lack of anti-inflammatory activity of *A. americana* observed in the present study. Or perhaps the plant itself did not possess such activity, as shown by the previously observed lack of antioxidant activity (except in water extracts).

4.4.3 Antifungal activity

The study results indicate that *C. albicans, T. interdigit, T. rubrum* and *T. tonsurans* were resistant to the acetone and methanol extracts. Therefore, these extracts do not possess any antifungal activity. Although the water extract showed some activity at some point, this was related to bacterial growth. This means that samples were contaminated. However, in other studies, Gularia and Kumar (2009) found that the methanolic extracts of A. americana leaves possess antifungal activity that is against *A. brassicae*. In another study, *A. americana* leaf extracts showed antifungal activity against *Aspergillus parasiticus* (Rosas-Taraco et al., 2011). The study of Siddhapura

et al. (2011) also showed that *A. americana* exerted some antifungal activity against the *Postia placenta*. Some species that are closely related to *A. americana* have also been shown to possess antifungal activity against some plant pathogenic fungi (Maharshi and Thaker, 2014). Like the antioxidant and anti-inflammatory studies, perhaps the lack of activity shown by the present extracts may be linked to geographical regions, species variations, and principles of the assay, among others.

4.5 Conclusion

Extract cytotoxicity was evaluated after treatment of cells with different concentrations of the three extracts. This ensured that any response observed was not due to a decrease in cell number, as dead or unhealthy cells would not produce nitric oxide (NO). An anti-inflammatory response of an extract will show a decrease in number but not a decrease in the percentage of viable cells. The water extract thus showed the best DPPH scavenging potential. It is also very clear that the water extract is more effective in DPPH scavenging than the reduction of ferric ions to ferrous ions. There is a difference between the FRAP and DPPH results which could be due to the principles of the assays. Only the water extract showed pro-inflammatory activity, as evidenced by the high nitrite concentrations at low extract concentrations, which decreased dose-dependently. However, it is not clear if this was due to the plant extract constituents or the presence of endotoxins in the sample that possibly originated from bacterial contamination of the plant material and/or during extraction. This is a common feature of water extracts. There is also a corresponding decrease in cell viability in water extract-treated cells, indicating toxicity. The decrease in pro-inflammatory activity at high extract concentrations can, therefore, be ascribed to the toxicity of the extract at those higher concentrations. None of the extracts exhibited any anti-inflammatory potential. Although a decrease in nitrite concentration was observed for the water extract and at the highest concentration of the methanol extract, this was due to the decrease in cell viability. The antifungal activity results showed that *C. albicans, T. interdigit, T. rubrum* and *T. tonsurans* were resistant to the acetone and methanol extracts. However, there was some form of resistance in the water extract, which was then attributed to bacterial growth. This study, therefore, can conclude that Agave *americana L.* did not possess any antifungal activity against the tested

pathogens. This is also not very surprising considering the very low secondary metabolite constituents, as reported in Chapter 3 above.

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CHAPTER 5

CYTOTOXICITY AND GENO TOXIC POTENTIAL OF *Agave americana L.* LEAF EXTRACTS

5.1 Introduction

Natural products such as plant extracts and essential oils are important components of many somatic therapies. Thus, the determination of phytochemical constituents and bioactivity of plant extracts, natural products, and synthetic compounds is crucial in detecting and incorporating new and improved therapeutic entities. More so, a crucial element in the discovery of new and improved therapeutic entities is the toxicity level to establish safe administration methods.

Various pharmacological and bioactive compound studies of medicinal plants report that the unguided use of extracts from medicinal plants can cause undesired consumer effects (Boukandou Mounanga et al., 2015). This is because certain therapeutic plants have inherent toxicity due to their ingredients, and if administered improperly, can cause negative effects (Anywar et al., 2021). Adverse occurrences in their use may also be caused by other reasons, such as adulteration, insufficient regulatory measures, noncompliance with good manufacturing practices (GMP), and interactions between herbs and drugs (Mensah et al., 2019).

Solvents rarely extract all phytoconstituents in a plant; hence, different solvents with different polarities are used. In general, varying quantities of secondary metabolites in medicinal plants lead to varying extraction values, which are highly dependent on the polarity of the solvent. However, organic solvents tend to extract more compounds (Elfalleh et al., 2019).

However, studies show that aqueous extracts are less toxic than their alcoholic counterparts. A possible reason for this phenomenon could be the different phytochemical structures of the aqueous and alcoholic types of extracts (Dhawan & Gupta, 2017). Aqueous extracts are also mostly recommended, as water is a readily available resource for traditional health practitioners. As a result, aqueous extracts provide a mimic overview of what is being practised by traditional health practitioners, resulting in more accurate scientific evidence worth documenting.

Medicinal plant toxicity may also occur because of incorrect preparation and plant extract application (Boukandou Mounanga et al., 2015). Another possible cause of toxicity could be the prolonged use of the more popular medicinal plants, following poor regulation in prescribing and using medicinal plants. Prolonged use of the same medicinal plant extract may result in chronic toxicity, which is the constant use of a relatively safe substance over a long period of time (Anywar et al., 2021). According to the previous authors, chronic toxicity may cause serious health complications such as organ tissue damage and degenerative changes in organs, particularly the liver and the skin.

Numerous adverse skin reactions can occur due to toxicity because of the topical application of medicinal plant extracts (Lovell et al., 2021). According to the previous authors, the common types of skin reactions that can occur include sensitization, allergies, irritation, and photosensitization. Sensitization is particularly common in the somatology environment. Sensitization occurs when the initial contact with a substance such as a bioactive plant extract is applied to the skin, and no adverse reaction is noted (Gądarowska et al., 2022). Thereafter, continued use of the same substance for a prolonged period causes sensitivity (Gądarowska et al., 2022). Sensitization can happen to anyone, even those who do not particularly have sensitive skin. Once a person is sensitized, symptoms such as itching, sneezing or more severe reactions such as breathing difficulties may occur as cell degeneration occurs.

Cytotoxicity is the ability of an agent to produce a toxic effect on a cell (Skrzydlewski et al., 2022). Cytotoxicity assays are used to test the ability of cells to continue proliferating in the presence of a test compound or substance over a specific period. This is an important preliminary stage when investigating possible new therapies to be applied topically to treat various skin conditions to determine any possible harmful effects.

A genotoxin is an agent or chemical that can potentially destroy a cell's genetic details by causing mutations that may result in cancer (Phillips & Arlt, 2009). Not all genotoxic substances have a mutagenic effect (Ren et al., 2017). According to the same authors, all mutagenic substances can damage and destroy the genetic detail of a cell and have potential carcinogenic effects. However, not all genotoxic substances can multiply. Thus, one of the objectives of this study was to determine toxicity, particularly

cytotoxicity, of Agave *americana L.* leaf extracts on the kidney cell line (Vero cells) in-vitro, which further warranted the exploration of genotoxicity.

5.2 Materials and methods

5.2.1 Genotoxicity

5.2.1.1 Sample preparation

Vero cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) low glucose supplemented with 10% foetal bovine serum (FBS) at 37°C in a humidified incubator with 5% CO_2. Cells were seeded at 3000 cells/well of a 96-well plate in 100 µl aliquots and incubated overnight to promote attachment. Cells were further treated with extracts at concentrations of 12.5, 25, 50, 100 and 200 µg/ml and incubated for 48 hrs. Cells were then stained with NucRed Live 647 (Thermo Fisher Scientific).

5.2.1.2 Image acquisition and analysis

Images were acquired using the ImageXpress Micro XLS Widefield High-Content Analysis System (Molecular Devices). A 10 X objective was used, and nine sites per well were imaged using the Cy5 filter cube. Image analysis was performed using the Micronuclei analysis module of the MetaXpress version 6.1 High-Content Image Acquisition and Analysis Software.

5.2.2 Cytotoxicity

5.2.2.1 Reagents

Vero cells were purchased from Cellonex, South Africa. Dulbecco's Modified Eagle Media (DMEM) and Foetal Bovine Serum (FBS) were purchased from GE Healthcare System Life Sciences (Logan, UT, USA). Phosphate Buffered Saline (PBS) with and without Calcium (Ca^{2+}) and Magnesium (Mg^{2+}) and trypsin was purchased from Lonza (Wakersville, MD, USA). Bis-benzamide H 33342 trihydrochloride (Hoechst) and Propidium Iodide (PI) were purchased from Sigma (St. Louis, MO, USA).

5.2.2.2 Sample preparation

Test extracts were reconstituted in dimethyl sulfoxide (DMSO) to give a final 100 mg/mL concentration. Samples were subjected to ultrasonic vibration to fragment the cells, where solubility was a problem. Samples were stored at 4°C until required.

5.2.2.3 Treatment protocol

The African green monkey kidney cell line (Vero cells) was used for cytotoxicity screening. The cells were maintained at 37°C in a humidified incubator with 5% CO_2 in 10cm culture dishes. The complete growth medium consisted of DMEM supplemented with 10% FBS. Cells were seeded into 96 well microtiter plates at a density of 4000 cells/well using a volume of 100 µl in each well. The microtiter plates were incubated at 37°C, 5% CO_2, and 100% relative humidity for 24 hrs before the addition of test compounds to allow for cell attachment.

The cells were then treated with 50, 100 µg/mL and 200 µg/mL of each extract diluted in culture medium. One hundred microliter aliquots of the diluted extract in the fresh medium were used to treat cells. Cells were then incubated for a further 48 hrs.

The treatment medium was aspirated from all wells, replaced with 100 µL of Hoechst 33342 nuclear dye (5 µg/mL), and incubated for 10 mins at room temperature. Thereafter, cells were stained with propidium iodide (PI) at 100 µg/mL to quantify the proportion of dead cells within the population. Cells were imaged immediately after the addition of PI using the ImageXpress Micro XLS Widefield Microscope (Molecular Devices) with a 10x Plan Fluor objective and 4,6-diamidino-2-phenyli indole dihydrochloride (DAPI) and Texas Red filter cubes. Nine image sites were acquired per well, representing about 75% of the surface area of the well.

5.2.2.3 Data quantification

Quantifying live and dead cells for the screening assay was performed using the ImageXpress Micro XLS Widefield Microscope (Molecular Devices). The acquired images were analysed using the MetaXpress software and Multi-Wavelength Cell Scoring Application Module. Acquired data was transferred to an EXCEL spreadsheet, and data was analysed and processed.

5.3 Results

5.3.1 Genotoxicity

The results of the present study indicated that the acetone (Figure 5.1) and water (Figure 5.3) extracts generally showed that there was no increase in the formation of micronuclei at all concentrations except for 200 µg/ml in both cases. However, the methanol extract (Figure 5.2) showed an increase in micronuclei formation at all concentrations, indicating genotoxic potential. Unsurprisingly, the samples exposed to Griseofulvin (an antifungal medication commonly used to treat fungal infections of the skin, hair, and nails) exerted genotoxic effects on the Vero cells. When there is no increase in micronuclei formation, an increase in multi- and bi-nucleated cells is negligible.

Table 5.1: Summary of the effects of the acetone extract on Vero cells after 48 hours

Parameter	Acetone Extract (µg/ml)					
	Control	12.5	25	50	100	200
% Micro-nucleated cells	3.05 ± 0.21	3.99 ± 1.26	2.82 ± 0.37	2.90 ± 0.10	2.86 ± 0.24	3.42 ± 0.20 (*)
% Multi-nucleated cells	0.38 ± 0.07	0.53 ± 0.27	0.51 ± 0.18	0.52 ± 0.03 (*)	0.52 ± 0.07 (*)	0.62 ± 0.10 (#)
% Bi-nucleated cells	3.05 ± 0.23	3.37 ± 0.42	3.00 ± 0.45	3.49 ± 0.18 (*)	3.41 ± 0.24	4.03 ± 0.38 (#)
Total cells	1204.94 ± 70.65	938.61 ± 65.21 (#)	1116.14 ± 65.05	973.97 ± 40.91 (#)	1003.78 ± 84.65 (*)	1029.25 ± 40.36 (#)

***Where*:** Black = no effect relative to control; blue = decreased; red = increased; *: $p<0.05$; #: $p<0.005$

Table 5.2: Summary of the effects of the methanol extract on Vero cells after 48 hours

Parameter	Methanol Extract (µg/ml)					
	Control	12.5	25	50	100	200
% Micro-nucleated cells	3.05 ± 0.21	4.00 ± 0.51 (*)	4.91 ± 0.30 (#)	5.54 ± 0.40 (#)	6.32 ± 0.46 (#)	5.52 ± 0.33 (#)
% Multi-nucleated cells	0.38 ± 0.07	0.68 ± 0.06 (#)	0.65 ± 0.07 (#)	0.69 ± 0.12 (#)	0.95 ± 0.21 (#)	1.07 ± 0.12 (#)
% Bi-nucleated cells	3.05 ± 0.23	3.60 ± 0.21 (*)	3.93 ± 0.19 (#)	4.06 ± 0.25 (#)	5.51 ± 0.91 (#)	5.72 ± 0.35 (#)
Total cells	1204.94 ± 70.65	1067.56 ± 35.90 (*)	1072.03 ± 75.25 (*)	1000.44 ± 80.87 (*)	912.30 ± 61.72 (#)	735.72 ± 69.32 (#)

***Where*:** Black = no effect relative to control; blue = decreased; red = increased; *: $p<0.05$; #: $p<0.005$

Table 5.3: Summary of the effects of the water extract on Vero cells after 48 hours

Parameter	Water Extract (µg/ml)					
	Control	12.5	25	50	100	200
% Micro-nucleated cells	3.05 ± 0.21	2.83 ± 0.31	2.61 ± 0.27	3.18 ± 0.37	2.58 ± 0.25	5.60 ± 0.96 (#)
% Multi-nucleated cells	0.38 ± 0.07	0.57 ± 0.07 (*)	0.58 ± 0.05 (#)	0.65 ± 0.02 (#)	0.89 ± 0.10 (#)	3.06 ± 0.61 (#)
% Bi-nucleated cells	3.05 ± 0.23	3.66 ± 0.32 (*)	4.00 ± 0.29 (#)	4.43 ± 0.21 (#)	4.60 ± 0.28 (#)	11.82 ± 0.92 (#)
Total cells	1204.94 ± 70.65	1109.17 ± 52.59	1111.50 ± 42.69	1008.42 ± 49.68 (#)	878.11 ± 57.60 (#)	100.03 ± 31.18 (#)

***Where*:** Black = no effect relative to control; blue = decreased; red = increased; *: $p<0.05$; #: $p<0.005$

Table 5.4: Summary of the effects of Griseofulvin on Vero cells after 48 hours

Parameter	Griseofulvin (µM)					
	Control	6.25	12.5	25	50	100
% Micro-nucleated cells	3.05 ± 0.21	3.73 ± 0.63 (#)	3.92 ± 0.17 (#)	6.03 ± 0.50 (#)	7.88 ± 0.67 (#)	10.79 ± 1.44 (#)
% Multi-nucleated cells	0.38 ± 0.07	0.56 ± 0.11 (*)	0.56 ± 0.09 (*)	1.43 ± 0.07 (#)	2.92 ± 0.29 (#)	6.15 ± 0.46 (#)
% Bi-nucleated cells	3.05 ± 0.23	3.15 ± 0.36	4.65 ± 0.23 (#)	10.92 ± 0.29 (#)	18.24 ± 1.21 (#)	23.04 ± 0.67 (#)
Total cells	1204.94 ± 70.65	929.86 ± 60.12 (#)	810.44 ± 55.34 (#)	438.25 ± 16.78 (#)	238.81 ± 14.79 (#)	193.69 ± 24.98 (#)

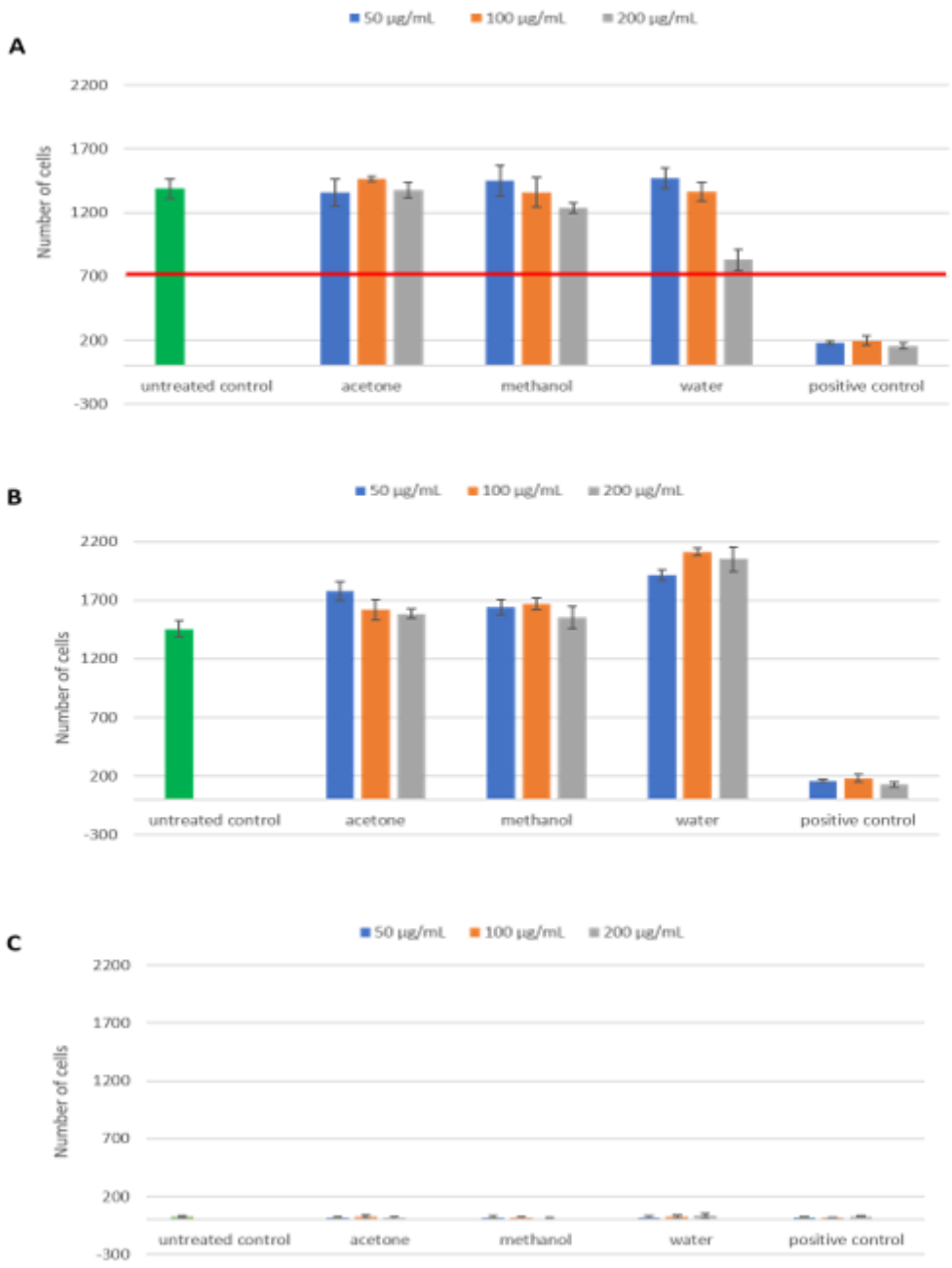
***Where*:** Black = no effect relative to control; blue = decreased; red = increased; *: $p<0.05$; #: $p<0.005$

5.3.2 Cytotoxicity

Cytotoxicity was determined using the dual staining procedure with a nuclear dye, Hoechst 33342 and propidium iodide (PI). All live cells stained positive with Hoechst 33342, but only dead/dying cells stained positive with PI.

The three extracts, namely methanol, acetone, and water, were screened against Vero cells (Figure 5.1). Melphalan was used as a positive control (PC) for the series of experiments. The red line in Figure 4.1A represents half the total number of untreated control (UT) cells and is used as an indication for potential cytotoxic extracts. Extracts exhibiting relevant cytotoxicity must have a total cell number below this red line. The live cell number is the most important parameter which tells us whether there was toxicity. From the live cell numbers (Figure 5.1A), only the water extract showed mild toxicity against Vero cells, with an estimated IC50 value of around 200 µg/ml. The water extract was the most cytotoxic of the three extracts. Followed by the methanol and acetone extracts. However, these results are negligible as they are below the minimum concentration required to inhibit biological response.

Table 5.5: Cytotoxicity of A. americana extracts against Vero cells after 48 hours of exposure



Cell viability was determined using the Hoechst 33342/PI dual staining method. Results displayed as A, total number of cells; B: number of cells stained either with Hoechst 33342 only (Live) or C, Hoechst 33342 and PI (Dead). Error bars indicate the standard deviation (SD) of quadruplicate values done as a single experiment.

5.4 Discussion

5.4.1 Genotoxicity

The results of this study indicate that the acetone and water extracts did not generally pose genotoxic effects on Vero cells. In contrast, the methanol extract had the potential to be genotoxic. The present study determined genotoxicity by in vitro testing. An increase in micronuclei indicated genotoxicity. The percentage of multi- and bi-nucleated cells was also examined in conjunction with micronuclei formation. When there is no increase in micronuclei formation, an increase in multi- and bi-nucleated cells can be deemed negligible. From the results, the methanol extract had the greatest potential of being genotoxic. The term "potential" is used because all in vitro assays have limitations, and a substance cannot be deemed genotoxic unless it is confirmed in an in vivo model. Due to the lack of genotoxic studies of *A. americana* leaf extracts, we compare them with studies conducted on members of the same species, which are also scarce. For example, in contrast to the present study (Araldi et al., 2022) showed that the acid hydrolysis extract of Agave *sisalana* induced DNA double-strand breaks and, therefore, was genotoxic to MRC-5 and A549 cell lines. In a previous study, Araldi et al. (2018) showed that the acid hydrolysis extract of A. sisalana was not genotoxic both in-vitro and in-vivo against Vero cells and mice, respectively. These findings agree with those of the present study.

5.4.2 Cytotoxicity

The cytotoxicity results indicate that the water extract was the most cytotoxic of the three extracts, followed by the methanol and acetone extracts. However, these results are negligible as they are below the minimum concentration required to inhibit biological response. In other studies, the ethanolic extract of *A. americana* leaves was shown to be cytotoxic (Khade et al., 2011). This is in contrast with the present findings. Also, in further contrast to the present study, Araldi et al. (2018) showed that acid hydrolysis of Agave *sisalana* had cytotoxic effects on both Vero cells and mice in-vitro and in-vivo, respectively. in addition, another study showed that acid hydrolysis of *A. sisalana* had varied responses to A549 and H450 cell lines (Araldi et al., 2022). Comparing the results of the two experiments conducted to determine the harmful effects of the Agave *americana L.* leaf extracts, specifically cytotoxicity and

genotoxicity, there is inconsistency in the results. However, this can be justified by adding up live cells and dead cells in the cytotoxicity experiment. This total is higher than the total number in the genotoxicity experiment. This is because when determining genotoxicity, the lowest possible seeding density is used to start for the Genotoxicity experiment (3000 cells versus 4000 per well in the cytotoxicity test). This is necessary to enable accurate micronucleus detection - if the density is too high initially, the micronuclei cannot be seen accurately. The method of analysis is another contributing factor. In the cytotoxicity assay, the software simply counts the number of nuclei in the image. If there is a binucleated cell, it will count it as two cells. In the micronucleus assay, the software can distinguish between mononucleated, binucleated or multinucleated cells, and it counts the two nuclei in a binucleated cell as a single cell. Thus, it can be seen in the genotoxicity results that the water extract increased the number of bi- and multinucleated cells, so the total cell number reported in the micronucleus assay, would be lower than the total number of nuclei, which is reported in the cytotoxicity assay.

5.5 Conclusion

Agave *americana L.* is a commonly used plant among traditional health practitioners in the Bloemfontein area, with various benefits and application methods. However, there are some indications of toxicity which warrant further studies.

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CHAPTER 6
GENERAL DISCUSSION AND CONCLUSION

The present study revealed that Agave *americana L.* contains two terpenoids (Piscidic and Eucomic acid) and a glycoside, which is different from previous research. Previous studies found apigenin, tannins, and fatty acids in methanolic leaf extracts of the same plant, but Piscidic Acid was absent. Furthermore, the present study found that triterpenes dominated the secondary metabolites (Bouhlel et al., 2021). Piscidic acid's role in human physiology is understudied but docking studies have shown its potential in cholesterol regulation and other physiological processes (Ressaissi et al., 2017). This previous study found that Piscidic Acid could bind to the HMG-CoA active site, potentially influencing cholesterol regulation. Eucomic acid, a compound found in Opuntia ficus-indica fruits, has antioxidant activity and potential anti-ageing properties (Simmler et al., 2011). These studies have shown that it increases cytochrome c oxidase activity without enhancing cellular mitochondrial constituents, suggesting the potential for skin damage and ageing. Terpenoids, which include Piscidic and Eucomic acids, have anticancer, anti-inflammatory, antibacterial, antiviral, and antimalarial properties (Paduch et al., 2007). They also have hypoglycaemic properties and can prevent and treat cardiovascular disorders. Glycosides, a large group of secondary metabolites, have various roles in human physiology, including antioxidant, anti-inflammatory, antiproliferative, and detoxification. However, their effects can vary based on their chemical structures and the specific tissues or organs they interact with. The low number of secondary metabolites in this study may be due to the plant's lack of well-studied studies using LCMS, geographical region, species variations, and assay principles. Furthermore, the present study's findings highlight the need for further research on the plant's terpenoids and glycosides.

The FRAP assay measures a substance's ability to convert a ferric tripyridyl triazine (TPTZ) complex into a ferrous form, indicating its antioxidant capacity. This study found water extract to be more active than acetone and methanol extracts. Antioxidant studies on Agave *americana L.* using the FRAP method are scarce, but some studies have shown activity in ethanol extracts of Agave angustifolia. The DPPH assay, which measures antioxidant activity, showed that acetone was inactive, methanol negligible,

and water significantly active. Water extracts were found to be more active, which justifies their use by local traditional healers and herbalists. The choice of solvent influences the extraction of antioxidants, and factors such as species, geographical location, climatic conditions, and plant age also affect plant antioxidant potential.

The water extract showed pro-inflammatory activity, decreasing cell viability in treated cells. This could suggest a cytotoxic effect of the compounds. However, if cell viability is maintained while NO levels decrease, it could indicate anti-inflammatory effects without significant cytotoxicity. The results are unclear if these are due to the sample's plant extract constituents or endotoxins. None of the extracts exhibited anti-inflammatory potential. Previous studies have shown the anti-inflammatory properties of various agave species (Augustin, 2005; Da Silva et al., 2022; Hernández-Valle et al., 2013; Riaz et al., 2023), but this study suggests variations in plant extract constituents, geographical location, species, and assay principles may be responsible.

According to the findings through this study and the literature review, we have found the prevalence of antioxidant and anti-inflammatory activity without significant cytotoxicity. These properties are synergistic in wound healing and can thus be explored for home care as well as pre- and post-treatment for professional treatments in a somatology environment to prepare the client for further treatment and a prophylactic strategy to maintain good skin health. The various extracts can be incorporated in hydrotherapy as medicinal baths or for topical application in the form of a moisturizer used to reduce inflammation post-treatment, thus reducing the risk of dermatophytosis infection. The plant extracts may also be considered for hygiene practice in the form of a pre-treatment for equipment used, which can further curb the potential of cross-contamination.

C. albicans, T. interdigit, T. rubrum, and T. tonsurans were resistant to acetone and methanol extracts, indicating no antifungal activity. However, other studies have found antifungal activity in *A. americana* leaf extracts against Alternaria brassicae (Gularia & Kumar, 2009), Aspergillus parasiticus (Rosas-Taraco et al., 2011), and Postia placenta (Siddhapura et al., 2011). The lack of activity may be due to geographical regions, species variations, and assay principles.

The present study further found that acetone and water extracts did not generally cause genotoxic effects on Vero cells, while the methanol extract had the greatest

genotoxic potential. In vitro testing indicated an increase in micronuclei, indicating genotoxicity. The water extract was the most cytotoxic of the three extracts, followed by methanol and acetone. However, these results are negligible as they are below the minimum concentration required to inhibit a biological response. The cytotoxicity results were higher in the water extract than in the genotoxicity experiment, as the lowest seeding density was used to start the cytotoxicity experiment. The analysis method may also have contributed to the inconsistency in the results. The water extract increased the number of bi- and multinucleated cells, so the total cell number reported in the micronucleus assay would be lower than the total number of nuclei reported in the cytotoxicity assay. Following these indications of toxicity, further studies are recommended.

6.1 Recommendation and Limitations

The water extract was the most cytotoxic of the three extracts, followed by the methanol and acetone, respectively, which were considered negligible as they are below the maximum concentration required to inhibit biological response. Thus, higher concentrations are recommended in further studies with careful consideration for the intended application method considering the somatology scope and limitations for invasive and non-invasive treatments.

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