A COMPARATIVE INVESTIGATION OF THE PHYTOCHEMICAL PROFILES AND BIOACTIVITIES OF SELECTED *AGAVE* SPECIES IN KWA-ZULU NATAL, SOUTH AFRICA

NTOMBIKAYISE GLORIA MKHIZE

Dissertation submitted in fulfilment of the requirements for the Degree

MASTER OF HEALTH SCIENCES: ENVIRONMENTAL HEALTH

in the

Department of Life Sciences

Faculty of Health and Environmental Sciences

at the

Central University of Technology, Free State

Supervisor: Dr MC Achilonu (PhD)

Co-supervisor: Dr IT Manduna (DSc)

BLOEMFONTEIN

July 2024

© Central University of Technology, Free State

DECLARATION OF INDEPENDENT WORK

I, NTOMBIKAYISE GLORIA MKHIZE, identity number _____ and student number _____, do hereby declare that this research project submitted to the Central University of Technology, Free State for the Master of Health Sciences in Environmental Health, is my independent work; and complies with the Code of Academic Integrity, as well as other relevant policies, procedures, rules, and regulations of the Central University of Technology, Free State; and has not been submitted before to any institution by myself or any other person in fulfilment (or partial fulfilment) of the requirements for the attainment of any qualification.

July 2024

_____ _____

SIGNATURE OF STUDENT DATE

© Central University of Technology, Free State

DEDICATION

This research work is dedicated to Jesus, my redeemer, to whom all things are possible, and to my late mother, who encouraged me to study.

I also dedicate this study to my family and friends for their encouragement, prayer, and support, as well as to the Mangosuthu University of Technology - Technology Station in Chemicals for all the support throughout my studies.

© Central University of Technology, Free State

ACKNOWLEDGEMENTS

I would like to express my appreciation to the Mangosuthu University of Technology-Technology Station in Chemicals (MUT-TSC) for funding my studies.

Thank you, Doctor Matthew Achilonu, Dr Idah Manduna, Ms V.X. Ngubane and Mr S'busiso Nkosi, for the guidance, support, and contributions to the successful accomplishment of this research work, as I would not have made it by myself—the TSC team for all the support that was provided for my studies.

© Central University of Technology, Free State

SUMMARY

In northern KwaZulu-Natal, South Africa, as informed by indigenous knowledge, local people use different species of *Agave plants* for cosmetic and therapeutic purposes. Various kinds of the *Agave* genus are claimed to have comprehensive traditional uses, such as treating health disorders such as inflammations and wound infections. There is also a growing number of cosmetic products incorporating plant extracts that are claimed to alleviate specific ailments and ease skin conditions (such as dryness/ itchiness, acne, etc.). Small businesses are producing *Agave*-enriched cosmetic products, which claim soothing and nourishing properties, yet there is no substantiation of their claims. Hence, this study investigated the phytochemical profile, efficacy, and safety use of *Agave angustifolia* Haw. var. *angustifolia* and *Agave sisalana* Perrine leaves in the cosmetic industry.

Fresh wild *Agave* leaves were collected and transported to the laboratory, where they were cleaned, chopped, dried, and cold macerated in four solvents of different polarities, starting from the non-polar to the polar solvent. The extracts were then subjected to phytochemical screening, UV analysis, antioxidant, antimicrobial, and *vitro* cytotoxicity studies. All plant extracts contained flavonoids, glycosides and saponins, while alkaloids were not found in all *A*. *angustifolia* extracts. All *A. sisalana* plant extracts were found to be non-cytotoxic, and this plant can be used to manufacture cosmetic products. The results proposed the possible use of oil-extracted *Agave* leaves from both plants in the manufacture of cosmetic products intended for use in the reduction of the appearance of symptoms associated with *S*. *pyogenes* infections, as well as products claiming antioxidant, anti-ageing, anti-inflammatory, and broad-spectrum anti-microbial activity. *Agave angustifolia* has the potential to be used in antitumor studies.

© Central University of Technology, Free State

TABLE OF CONTENTS

© Central University of Technology, Free State

© Central University of Technology, Free State

© Central University of Technology, Free State

LIST OF TABLES

© Central University of Technology, Free State

LIST OF FIGURES

© Central University of Technology, Free State

LIST OF ABBREVIATIONS/ACRONYMS

AA	*Agave angustifolia* Haw. *var. angustifolia*
AS	*Agave sisalana* Perrine
DPPH	2,2-diphenyl-1-picrylhydrazyl
EAF	Ethyl acetate fraction
HF	Hexane fraction
ME	Methanol extract
MF	Methanol fraction
SMME	Small, medium, and micro enterprises
TPC	Total phenolic content
TPTZ	Tripyridyltriazine (TPTZ)
UV	Ultraviolet

© Central University of Technology, Free State

CHAPTER 1 :
INTRODUCTION TO THE STUDY

1.1 Introduction

South Africa is mandated to exploit its vast plant biological diversity for the benefit of the people and to address the national imperatives of poverty, unemployment, and inhibited economic growth. A high rate of poverty and unemployment poverty challenges South Africa. The government has developed many tools to try and figure out these challenges, such as the establishment of the technology station program, whose mandate is to accelerate the growth of Small, Micro, and Medium Enterprises (SMMEs) so that they create employment opportunities and partake in the financial growth. The South African government has put aside funds to assist with Indigenous knowledge systems, where higher educational institutions would be funded to work with SMMEs on research projects that will see Indigenous knowledge research outputs enter the market in the form of finished products, such as cosmetics, medicine, etc. Some of the plants used in the indigenous knowledge research projects are originally alien plants that have found their way into South African soil and are therapeutically utilised by local communities.

In northern KwaZulu-Natal, as informed by indigenous knowledge, local people use different species of *Agave* for cosmetic and medicinal purposes. The *Agave* genus is Indigenous to America, distributed almost worldwide, and commonly found in most parts of the KwaZulu-Natal province (Cruz-Magalhães *et al.,* 2020). Diverse species of the *Agave* genus have extensive traditional applications, such as treating health disorders caused by bacteria and free radical toxicity induced by xenobiotics (Bhattacharyya *et al.,* 2014). The ailments may include inflammations, wound infections, cancer, and others (Ahumada-Santos *et al.,* 2013).

Research reveals that wild plants contain phytoconstituents exhibiting health-beneficial properties (Araldi *et al.,* 2018). Accordingly, there is growing utilization of products fortified with wild plant phytonutrients capable of alleviating specific ailments that are purported to ease dermatoses, such as acne, ageing, dry skin, eczema, and inflammation (Aburjai & Natsheh, 2003), with an added benefit of skin softening, nourishing and/or soothing. This assertion agrees with the claims of cottage industries

© Central University of Technology, Free State

and folkloric medicine, which use plant parts to manufacture specific products for topical applications to treat various skin ailments, exemplified by the small businesses supported by the Technology Station in Chemicals (MUT-TSC). These MUT-TSC clients, Somkhanda Trading (PTY) and Cyberchem (PTY) Ltd, produce and sell *Agave*-infused cosmetic products. Both companies claim that using *Agave* leaf extracts in their cosmetics products soothes and nourishes the users' skin. Hence, this research aimed to explore the possible use of the *Agave* species (*Agave angustifolia* Haw*. var. angustifolia* and *Agave sisalana* Perrine) as a source of bioactive phytochemicals for cosmetics enrichment. The study was conducted at the MUT-TSC laboratories, based at Umlazi, KwaZulu-Natal and at the Life Science laboratories of the Central University Technology, Free State (Bloemfontein Campus). The investigation concentrated on a comparative investigation of the phytochemical profiles, efficacy, and safety use of the leaves from *Agave angustifolia* (*AA*) and *Agave sisalana* (*AS*) plants found in Kwa-Zulu Natal, South Africa, in the cosmetic industry.

1.2 Motivation for the study

Agave plants are alien to South Africa. Though widespread, the plant has not received much research interest; hence, little scientific literature on its phytochemistry and bioactivity is available. Several *Agave* plant species are physiologically too similar but exhibit different biological traits. Examples comprise *Agave angustifolia var. angustifolia, Agave americana, Agave cupreata, Agave tequilana* and *Agave sisalana*. The close similarity makes it difficult for local users to differentiate between the species. The species' close physiological similarity often confuses selecting the appropriate species for the treatments, resulting in occasional patient poisoning rather than curing. Literature has not shown any proven safe or effective dose for *Agave* medicaments and personal care applications. Thus far, usage is based on moderation.

1.3 Aims and objectives

1.3.1 Primary objectives

The research project aimed to conduct a comparative investigation of the phytochemical profiles and bioactivities of selected *Agave* species (*Agave angustifolia var. angustifolia* and *Agave sisalana*) in KwaZulu-Natal, South Africa.

© Central University of Technology, Free State

1.3.2 Specific objectives

The project aim was attained through these precise objectives:

- Solvent extraction of pulverised *Agave* leaves to obtain solvent extracts and extract fractions.
- Qualitative and quantitative phytochemical evaluation of Agave leaves' solvent extracts and solvent extract fractions.
- Determination of the antioxidant potential of the solvent extracts from *Agave* leaves.
- *In vitro* cytotoxicity screening of the solvent extracts and extract fractions of *Agave* leaves.
- Screening the *Agave* solvent extracts and solvent extract fractions for microbial activity.
- Comparing the phytochemical profiles and the biological activities of *A. angustifolia* and *A. sisalana* leaves.

1.4 Literature review

1.4.1 The *Agave* Family

The *Agave* genus (Agavaceae family) comprises more than 200 species native to North America and widely distributed worldwide (Villanueva-Rodríguez *et al.,* 2016). They are commonly found in China, Brazil, Mexico, Tanzania, and most parts of Southern Africa (Cruz-Magalhães *et al.,* 2020). Few of the species of *Agave* are *abrupta, aciculatis, affinis, amaniensis, americana, angustifolia, angustissima, anomala, aspera, attenuata, aurea, bracteosa, brauniana, breedlovei, brevipetala, brevispina, brittonia, bromeliaefolia, brunnea, bulbifera, cantala, caymanensis, chiapensis, cupreata, durangensis, regia, revoluta, rigida, rupicola, salmiana, sisalana, stricta, stringens, subinermis, tequilana, vivipara* (Tewari *et al.,* 2014).

Diverse types of the *Agave* genus have extensive, scientifically supported traditional uses(Villanueva-Rodríguez *et al.,* 2016). Folk medicine has employed *Agave* species for the treatment of bacterial aetiology and oxidative strain ailments like digestive and wound infections, with *Agave cupreata*, *Agave tequilana*, *Agave angustifolia var. angustifolia*, *Agave sisalana* and *Agave americana* used to treat inflammatory-related ailments. *Agave* species are also noted to exhibit antifungal, antihypertensive, anti-

© Central University of Technology, Free State

inflammatory, antiparasitic and immunomodulatory actions (Ahumada-Santos *et al.,* 2013; Saxena *et al.,* 2013). This claim is supported by studies of Monterrosas-Brisson (Monterrosas-Brisson *et al.,* 2013; Salazar-Pi *et al.,* 2017), which established the antibacterial activity of different solvent fractions against sensitive strains and clinical isolates. Research has suggested the use of a few *Agave* species (*angustifolia, asperrima, potatorum, salmiana, tequilana* and *weberi*) to produce alcoholic beverages (bacanora, mezcal and tequilana) (Velázquez-Martínez *et al.,* 2014).

Agave plants have crassulacean acid metabolism and fructans, biosynthesized and stored in the stems, and serve as osmoprotectants during drought. The branched fructans (gaming and gaming neo-series) have attachments of fructose residue of sucrose and glucoside, called “agavins” (Hernández-Valle *et al.,* 2014). Agavins (fructans mixture) are fermentable dietary fibre with the potential to reduce disease risk. The short degree of polymerization fructans from *A*. *angustifolia* has demonstrated the ability to promote the secretion of peptides, which aid obesity control and its associated metabolic disorders. Hence, agavins are food supplements for diabetes (Velázquez-Martínez *et al.,* 2014). The present study focused on two wild *Agave* plants commonly found in Durban, South Africa: *Agave angustifolia* Haw. var. *angustifolia* and *Agave sisalana* Perrine.

1.4.2 *Agave angustifolia* Haw. var. *Angustifolia*

A. angustifolia (*AA*), also known as *Agave vivipara L*., is an evergreen, long-lived (perennial) shrubby, succulent plant with slim, rigidly erect leaves with moderately spaced spikes and sharp prickle at the end (Verloove *et al.,* 2019; Verloove & Pascual, 2021). *AA* is a variable species native to Central America (Vargas-Ponce *et al*., 2009; Verloove & Pascual, 2021). *AA* is grown in Mexico, Southern Europe, and the West Indies and is used to produce sisal hemp, the most potent natural fibre highly durable and resistant to mould and mildew (Franck, 2012).

AA mainly produces mezcal, a traditional Mexican alcoholic beverage (Vera-Guzmán *et al.*, 2018). Other uses include making ropes, fuel, beverages, and ornaments. In traditional medicine, roots stems, and leaves are cooked to produce juice, which is used for bandages for inflammation and bruises, and the roots are diuretic and diaphoretic (García-Mendoza & Chiang, 2003).

© Central University of Technology, Free State

AA is used for sprains and /or broken bones for people and animals (Hernández-Valle *et al*., 2014; Monterrosas-Brisson *et al*., 2013). The *AA* leaves contain phytosterols (Hernández-Valle *et al*., 2014). In their study, (Ahumada-Santos *et al*., 2013) observed that *AA* extract had antioxidant activity. Studies by Potenza *et al.* (2006) and Bermúdez-Bazán *et al.* (2021) confirmed established steroidal saponins as the active compounds in *A. angustifolia* extracts. The *A. angustifolia* and its thorny leaves are presented in Figure 1.1.

Figure 1.1: (a) *Agave angustifolia* Haw. var. *angustifolia* (b) A. *angustifolia* thorny leaf edge

© Central University of Technology, Free State

1.4.3 *Agave Sisalana Perrine*

A. sisalana Perrine, also known as sisal, has other vernacular names, like ngwengwe (Angola), garingboom, or uhalibhoma (South Africa) (Tewari *et al*., 2014). *A. sisalana* is from the Agavaceae and grows up to 2 m (Zwane *et al*., 2010). It has rigid, smooth-edged grey-green leaves in the shape of a sword (about 1.5 m long), with a sharp spike at the end. The matured plant has yellowish-green flowers (7-9 m flower stalk) (Tewari *et al*., 2014). *A. sisalana* originates from Mexico and Central America and has spread to other tropical countries such as South Africa, Swaziland, Angola, Tanzania, Uganda, Mozambique, and Brazil (Zwane *et al*., 2010). *A. sisalana* is planted chiefly to make ropes from its fibrous leaves (Chandramohan & Marimuthu, 2011; Debnath *et al*., 2010).

A. sisalana contains secondary metabolites (alkaloids, alkaloidal amines, sterols, steroidal alkaloids, steroidal sapogenins) attributed to the plant's therapeutic properties. They are used to synthesise corticosteroids (Araldi *et al*., 2018). *A. sisalana* is used as an antiseptic and an internal antibacterial agent. The acknowledged plant botanical name is *A. sisalana* (Debnath *et al*., 2010). According to Araldi's report (Araldi *et al*., 2018), the phytochemical extracts of *A. sisalana* indicated an antioxidant activity. Dilute *A. sisalana* extract was noted to have cytotoxic potential. Invariably, results indicated the dose-dependence of *A. sisalana's* antioxidant action (Araldi *et al*., 2018; Ribeiro *et al*., 2013). *A. sisalana* leaves have been reported to be rich in sapogenins. The pharmaceutical industry uses Hecogenin, isolated from AS leaves, in steroidal hormone synthesis, such as cortisone (Wu *et al*., 2021). The *A. sisalana* plant and its smoothly-edged- leaf are shown in Figure 1.2.

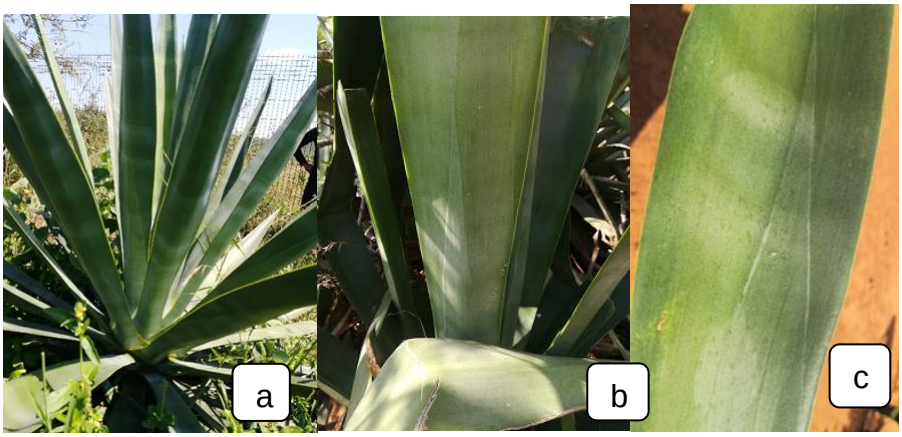


Figure 1.2: (a) A. sisalana (b) A. sisalana smooth leaf; (c) the leaf design

© Central University of Technology, Free State

1.4.4 Phytochemicals

Phytochemicals occur naturally in plants as metabolites (Mazid *et al*., 2011; Saxena *et al*., 2013). Saxena describes phytochemicals as plant-derived, naturally occurring, and biologically active chemical compounds that benefit humans more than micro- and macronutrients. Phytochemicals may exist in flowers, fruits, leaves, roots, seeds or stems (Saxena *et al*., 2013).

The bioavailable compounds help the plant's growth or defence mechanisms against pathogens and predators (López-Romero *et al*., 2018; Mazid & Mohammad, 2011; Patle *et al*., 2020; Rizwan *et al*., 2012). Some phytochemicals can protect against abiotic stress (Mazid *et al*., 2011). It has been reported that the safety and effectiveness of alkaloids, flavonoids, glycosides, terpenes, and other natural compounds in treating atopic dermatitis have been scientifically proven (Wu *et al*., 2021). Phytochemicals exhibit bioactivities against health conditions such as arthritis, cancer, etc.

Phytochemicals are known to demonstrate anti-inflammatory, antioxidant, and DNA repair properties (anti-ageing) (Michalak, 2022; Tabassum & Hamdani, 2014), which can be utilized to prevent photo-ageing and other skin health issues. According to a review paper (Michalak, 2022; Tabassum & Hamdani, 2014), there is evidence of the protective effects of phytochemicals on the biochemical processes caused by UV radiation. Reports suggest regular oral and topical antioxidant phytocompounds (flavonols) protect the skin against UV radiation and consequent ageing (Michalak, 2022; Tabassum & Hamdani, 2014). Antioxidants neutralize oxidative stress from the inside, thereby protecting the skin from deterioration and premature ageing (Michalak, 2022; Pizzino *et al*., 2017; Tabassum & Hamdani, 2014), while topical application of the phytochemicals improves the skin's elasticity and tonus (Gasser *et al*., 2008). Another study (Anbualakan *et al*., 2022) noted that besides significant improvement of skin texture by ingestion of procyanidin (flavonoids) rich diets, oral or topical application of the flavanols also inhibits UV radiation-induced Erythema (redness of the skin) (Anbualakan *et al*., 2022; Pizzino *et al*., 2017; Tabassum & Hamdani, 2014). The plant's healing effects of plants are ascribed to their bioactive phytochemicals, which include flavonoids, glycosides, alkaloids, corticosteroids, and coumarins (Roy *et al*., 2022). Phytochemicals are purported to be the most significant source of new

© Central University of Technology, Free State

antimicrobials as there is a growing number of bacterial-resistant strains that antibiotics fail to cure (Monte *et al*., 2014).

These naturally occurring and biologically active biomolecules can be categorised into four (4) major categories: alkaloids, glycosides, polyphenols, and flavonoids. Flavonoids are subdivided into anthocyanins, flavanols (catechins, epicatechins, and proanthocyanidins), flavanones, flavones and isoflavones (Achilonu & Umesiobi, 2015). Different types of phytochemicals and structures are exhibited in Figure 1.3.

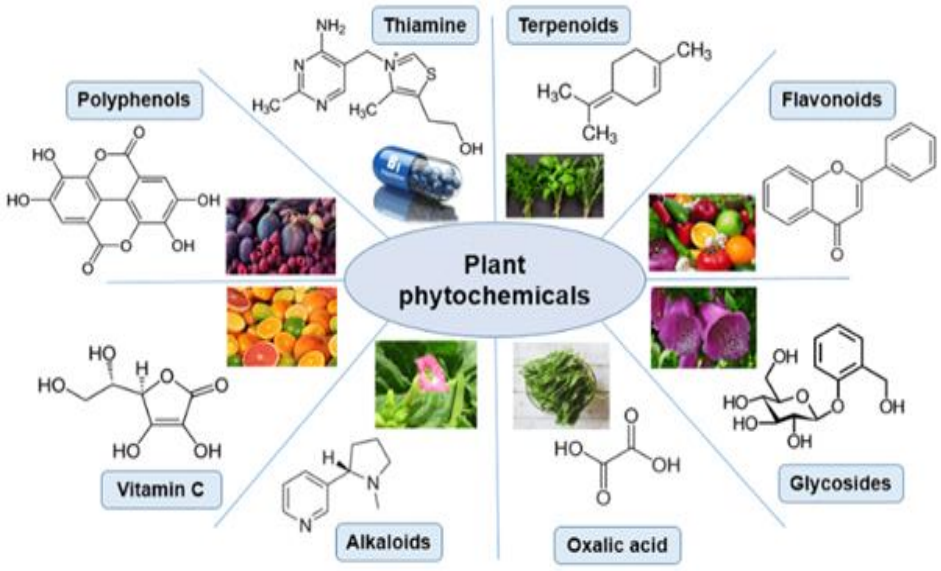


Figure 1.3: Plant phytochemicals (Soltys et al., 2021)

1.4.4.1 Alkaloids

Alkaloids are heterogeneous plant metabolites composed of amine compounds biosynthesised from amino acid building blocks. They are mainly found in the seeds and roots, usually combined with vegetable acids (Achilonu & Umesiobi, 2015; Doughari, 2012; Wink, 2007).

The basicity of the alkaloid is based on the presence of one or more nitrogen atoms as primary, secondary, or third amines, and it differs based on the molecular structure and the location of the functional group (Doughari, 2012).

The solutions are incredibly bitter. Alkaloids are found in nutraceuticals and are recommended for managing diseases and oxidative strain caused by inflammation (Alasvand *et al*., 2019). Alkaloids have been reported to protect plants from pathogens

© Central University of Technology, Free State

and herbivores, have antibacterial, antifungal, cytotoxicity, and carcinogenic activities, and have toxicity in vertebrates (Bribi, 2018; Heinrich *et al*., 2021). They have also found strong use in pharmaceuticals as stimulants, narcotics, and poisons. Although they are said to be toxic, they were seen to be used in treating health conditions and recreational drugs (Achilonu & Umesiobi, 2015). The cosmetic industry uses alkaloids, such as caffeine, piperine, capsaicin, berberine, and spilanthol, as antioxidants, skin lighteners/even skin tone, antimicrobial, anti-cellulite, anti-ageing, soothing and anti-inflammatory agents (Stepniowska, 2021). The alkaloids can, therefore, be utilised in the manufacture of cosmetic products, like tonics, creams, lotions, face and hair masks, anti-cellulite and antiaging products, and compresses for skin problems with numerous inflammations and discolouration (Stepniowska, 2021). In many cases, a single alkaloid can exhibit more than one biological function, such as caffeine, which has anti-ageing, anticellulite and antioxidant properties (Stepniowska, 2021), making it one of the essential cosmetic ingredients. Figure 1.4 shows the structure of caffeine.

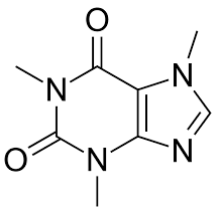


Figure 1.4: The chemical structure of caffeine

1.4.4.2 Phenolic compounds

Plant phenolics are secondary metabolites subdivided into coumarins, phenolic acids, and polyphenols.

Polyphenolics are organic compounds categorized by their significant structural diversity, such as large multiples of phenol structures, which form the bases for their unique bioactivity. They are the most popular phytochemicals, subdivided into flavonoids and phenolic acids (Achilonu & Umesiobi, 2015; Mera *et al*., 2019). They are classified into (i) flavonoid polyphenols (flavanones, flavones, xanthones, and catechins), (ii) non-flavonoids (lignin) and (iii) phenolic acids. Caffeic acid (a whitening agent in cosmetics) is widely distributed in plants (Doughari, 2012). Plant phenolics are defensive phytochemicals against adverse environmental conditions, including UV radiation, microbial infections, and predators. Hence, phenolics manage disease-

© Central University of Technology, Free State

causing microbes (Puupponen-Pimiä *et al*., 2001). The caffeic acid structure is presented in Figure 1.5.

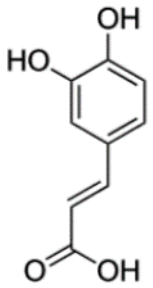


Figure 1.5: The chemical structure of caffeic acid

1.4.4.3 Flavonoids

Flavonoids are polyphenols ubiquitous in different plant parts, particularly the floras and the fruits (Panche *et al*., 2016). They are derived from flavans and found in the form of aglycones (no sugar-bound), glycosides (sugar-bound) and their derivatives (Saxena *et al*., 2013). Flavonoids comprise a 15-carbon skeleton of flavans, C6-C3-C6 compounds (Achilonu & Umesiobi, 2015). The structure of a particular flavonoid will depend on the unsaturation of the C3 ring, the hydroxyl position in the benzene rings, and the occurrence/ absence of the hydroxyl group at the C3 position. Hence, flavonoids are sub-grouped into five (5): flavones, flavonols, flavanones, flavanols, and anthocyanidins (Achilonu & Umesiobi, 2015). Flavone and flavonol are dietary glycoside-bound flavonoids(Achilonu & Umesiobi, 2015; Panche *et al*., 2016). Flavonoids exhibit anti-ageing, anti-inflammatory, antioxidant, and broad-spectrum antimicrobial properties (Maheshwari *et al*., 2022; Sen *et al*., 2022). The commonly reported flavonoids are quercetin, kaempferol, and quercitrin, found in about 70% of plants. Quercetin has been identified as an ingredient with anti-inflammatory, antimicrobial, antioxidant, and antineoplastic activities that treat various oral diseases (Wang *et al*., 2020). The therapeutic uses of quercetin are depicted in Figure 1.6 (Wang *et al*., 2020), where the conventional delivery form and chemical structural formula are shown in the centre.

© Central University of Technology, Free State

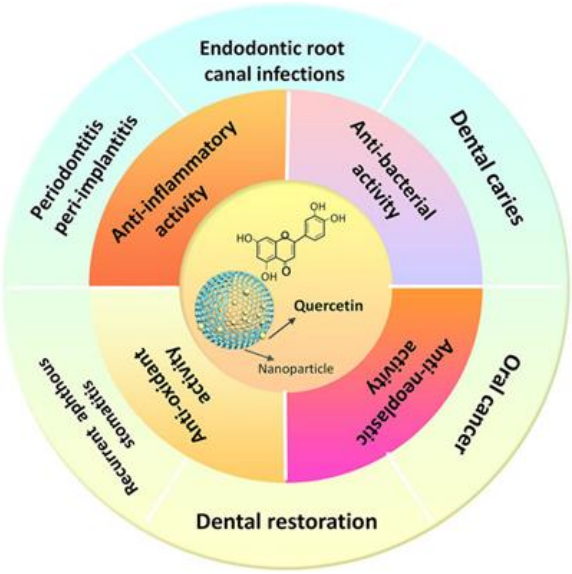


Figure 1.6: The chemical structure and therapeutic applications of Quercetin (Wang et al., 2020)

1.4.4.4 Saponins

The word ‘saponin’ (‘Sapo’ means ‘soap’) is known for foaming, detergency, wetting and emulsifying properties (Guclu-Ustundag & Mazza, 2007; Sharma *et al*., 2023). Saponins are terpenoids with steroidal or terpenoid aglycones attached to the sugar chain group (Guclu-Ustundag & Mazza, 2007; Yu *et al*., 2022). They are claimed to be responsible for the defence mechanism against plant pathogens due to their antimicrobial properties.

Saponins are described as antimicrobial, anti-inflammatory, and antitumor agents (Cheok *et al.,* 2014*;* Sharma *et al.,* 2023*;* Yu *et al.,* 2022*)*. In cosmetics, they are utilised as emulsifiers and foaming agents (Nizioł-Łukaszewska & Bujak, 2018). In medicine, they are used as the starting materials in steroid production (Tewari *et al*., 2014). Steroidal saponin isolated from *A. sisalana* leaves (hecogenin) can be used to prepare cortisones and hydrocortisone. Saponins derived from the *Agave* genus are reported to have anti-inflammatory, antimicrobial, antioxidant, and cytotoxicity activities (Sharma *et al*., 2023; Sidana *et al*., 2016). Figure 1.7 presents a structural example of hecogenin.

© Central University of Technology, Free State

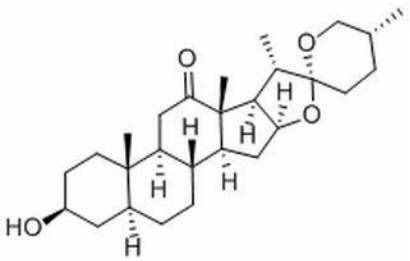


Figure 1.7: The chemical structure of saponin and hecogenin (Sharma et al., 2023)

1.4.4.5 Glycosides

Glycosides include all phytochemicals that have saccharide moieties. Leisegang (2021) described glycosides as a diverse and heterogeneous secondary metabolite group with substantial bioactivity. Glycosides may have one or more sugar components (Awuchi & Amagwula, 2020). Glycosides are classified based on the glycone or glycosidic bond, the chemical nature of aglycone structure or biological activity. Flavonoids, phenolics (saponins), cyanogenic, steroidal cardiac, and coumarins are some examples of clinically useful groups of glycosides (Awuchi & Amagwula, 2020; Leisegang, 2021). Glycosides are bitter and comprise a group of lactones (diterpene lactone or triterpenoids). As shown in Figure 1.8, Vanillin is one of the glycosides used as a perfume in the fragrance industry to mask foul odours and tastes in medicine and cleaning products.

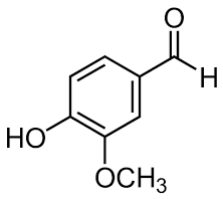


Figure 1.8: The chemical structure of vanillin (Olatunde et al., 2022)

1.4.4.6 Tannins

Tannins are described as widely distributed high molecular weight phenolic antioxidants in various vegetables, fruits, leaves, and barks and have been reported to possess medicinal and therapeutic potentials (Ghosh, 2015). They are protein-complexing compounds and enzyme inhibitors (Lattanzio *et al*., 2012). Tannins have two (2) divisions: hydrolysable (gallic acid and gallotannins) and condensed tannins

© Central University of Technology, Free State

(flavonoids). Tannins are generally antiseptics (opposing microbial infection, especially preventing or inhibiting microbial growth) because of the existence of the phenolic group. One of the essential hydrolysable tannins is gallic acid, which exhibits antimicrobial, anti-inflammatory and antioxidant actions and is mainly used by the cosmetic industry for anti-ageing properties.

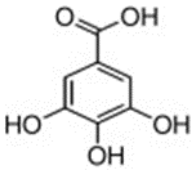


Figure 1.9: The chemical structure of gallic acid (Ghosh, 2015)

1.4.4.7 Terpenes

Terpenes are unsaturated, flammable hydrocarbons generally found in essential oils (Cox-Georgian *et al*., 2019; Paduch et al., 2007). The terpene structure has repeating five-carbon isoprene units. Terpenes may be mono-, di-, tri-, and sesquiterpenes, which depict isoprene unit numbers in the compound. Some examples of terpenes are monoterpene (camphor & menthol), diterpene (taxol, an anticancer agent), and triterpenes (ursolic acid: anti-inflammatory, cytotoxicity activity). Menthol, depicted in Figure 1.10, is used in the cosmetic industry for its antiseptic and topical cooling effect (Farco & Grundmann, 2013). Adding oxygen to the terpene structure results in terpenoids (Cowan, 1999).

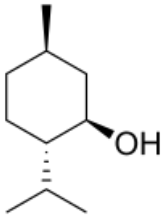


Figure 1.10: The chemical structure of menthol (Farco & Grundmann, 2013)

1.4.4.8 Steroids

Plant steroids (or steroid glycosides) are commonly found in various plant parts and comprise a basic four-carbon ring structure known as a steroid nucleus. They are reported to exhibit antioxidant, anti-tumour, antibacterial, cytotoxicity, and anti-

© Central University of Technology, Free State

inflammatory actions (Li *et al*., 2022; Patel & Savjani, 2015). The addition of different functional groups at other positions on the backbone structure forms various steroidal compounds. This results in different functionalities based on the functional group, such as anti-inflammatory steroids (like corticosteroids) and arbutin (naturally occurring hydroquinone derivative that is used in personal care as a skin-lightening agent), to name a few (Patel & Savjani, 2015). Alpha arbutin (Figure 1.11) is reported to be an antioxidant skin-lightening cosmetic ingredient that reduces melanin production.

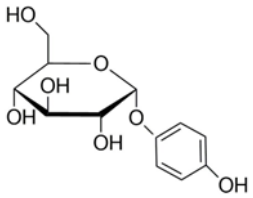
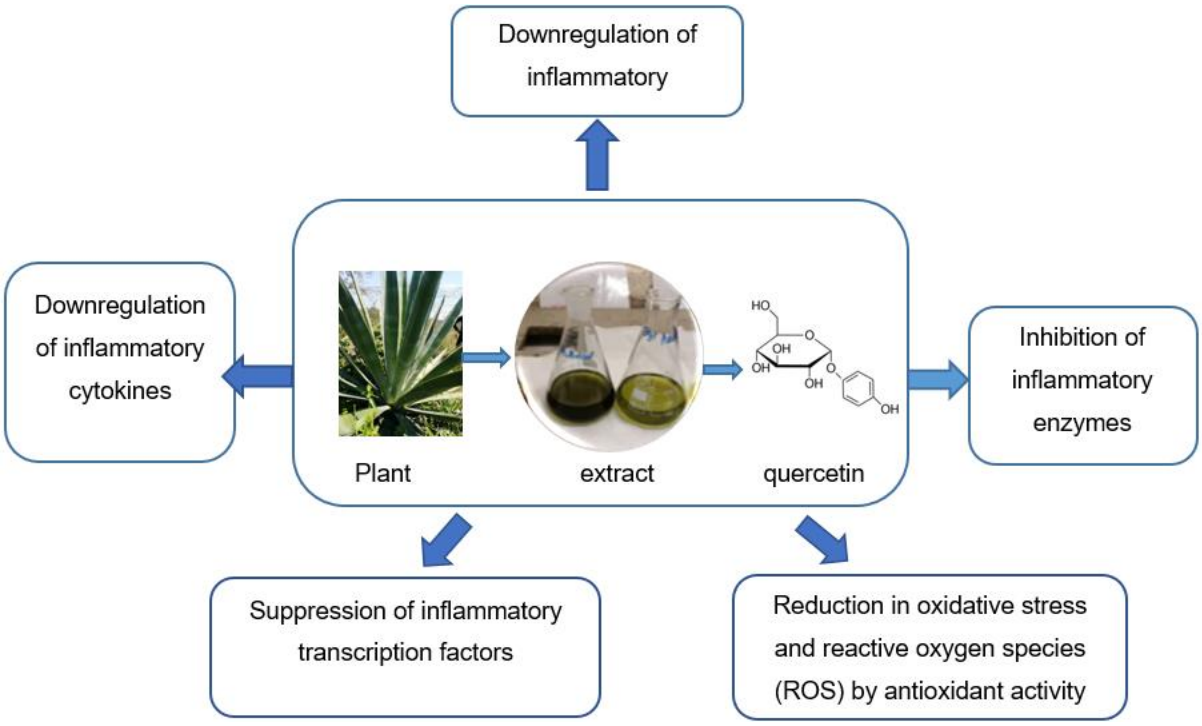


Figure 1.11: Structure of arbutin (Patel & Savjani, 2015)

1.4.5 The phytochemical mode of action

There are various suggested phytochemical mechanisms, such as inhibiting the growth of microorganisms, antioxidants (flavonoids), anti-carcinogenesis (phenols), anti-inflammatory, etc. (Doughari, 2012). The schematic diagram for the anti-inflammatory action of phytochemicals is shown in Figure 1.12, where quercetin is used as an example.



© Central University of Technology, Free State

Figure 1.12: The anti-inflammatory and antioxidant attributes of phytochemicals

When the human skin is excessively exposed to the sun without applying skin-protecting products (e.g., cosmetics infused with sun-protecting agents), the UV radiation acts on the keratinocytes of the epidermis, resulting in neoplastic transformation. As the epidermal layer constantly turns over, the basal layer stem cells undergo cell division. During this process, the keratinocytes differentiate into squamous cells, producing keratin and other proteins.

This results in skin damage such as erythema, oxidative stress, DNA damage, immunosuppression, signal transduction pathways, formation of sunburn cells (inflammation), and extracellular matrix degradation, leading to photoaging and skin cancer (Ouhtit & Ananthaswamy, 2001). Figure 1.13 shows the pathways for carcinogenesis from excessive sunlight exposure.

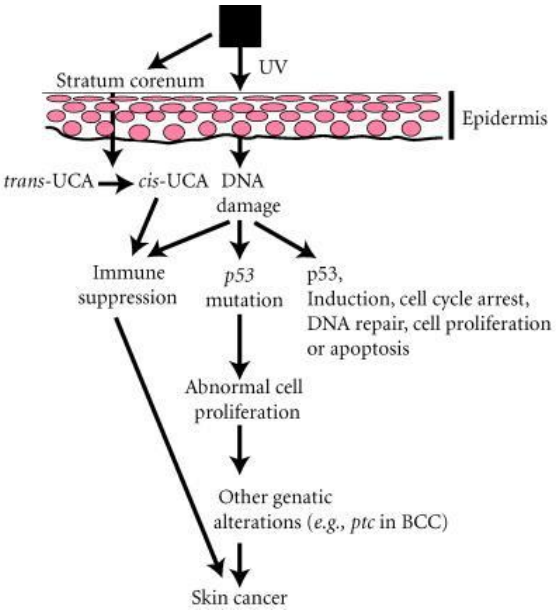


Figure 1.13: Pathways for skin cancer development (Ouhtit & Ananthaswamy, 2001)

However, when phytochemicals are topically applied to the problem skin (acne-prone/ pigmented), the polyphenol oxidase (Tyrosinase) catalyses two types of reactions. These are monophenolase activity, where tyrosinase triggers the ortho-hydroxylation of tyrosine (monophenols), converting them into o-diphenols (L-DOPA), and the

© Central University of Technology, Free State

diphenolase activity, where tyrosinase catalyses the oxidation of o-diphenols, transforming them into o-quinones (Chandorkar *et al*., 2021). The tyrosinase contributes to melanin production, as depicted in Figure 1.14. The mechanism of action for α-arbutin is used to manufacture cosmetic skin care products with skin-lightening properties.

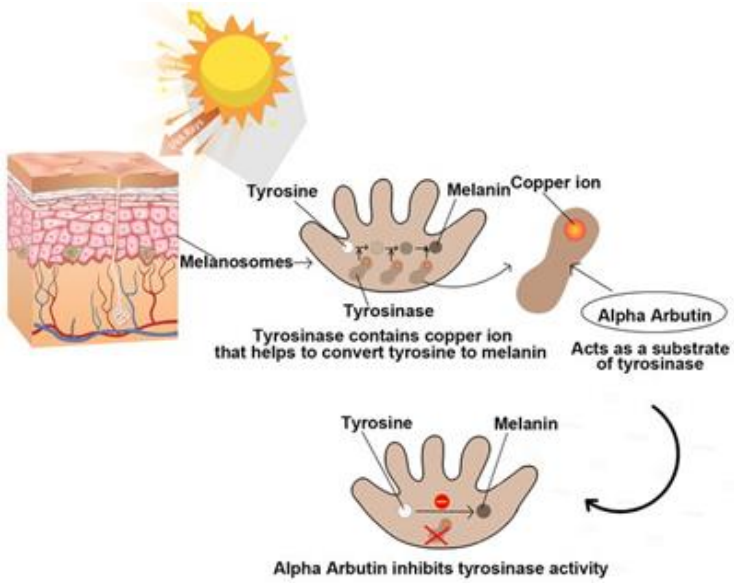


Figure 1.14: The skin-lightening activity of α-arbutin (Chandorkar et al., 2021)

1.4.6 Phytochemical extraction from solids

Plant material can be extracted using different methods, with maceration being the most uncomplicated cold extraction process, where the sample material is soaked in a solvent for some time (De Silva *et al*., 2017; Ong, 2004; Sasidharan *et al*., 2011).

Plant material maceration can be done via serial or selective extraction. The resulting extract contains a lot of solvent, which needs to be removed to get the extract fraction for a particular solvent. Therefore, solvent removal is required without destroying the biological components of the extract.

If the compound is thermo-labile, the solvent removal is carried out on a rotary evaporator (rotavapor), whereby applying vacuum (reducing pressure) results in the reduction of the boiling point of the solvent.

© Central University of Technology, Free State

1.5 References

Aburjai, T., & Natsheh, F. M. (2003). Plants used in cosmetics. *Phytotherapy Research*, *17*(9), 987–1000. https://doi.org/10.1002/ptr.1363

Achilonu, M. C., & Umesiobi, D. O. (2015). Bioactive phytochemicals: Bioactivity, sources, preparations, and/or modifications via silver tetrafluoroborate mediation. *Journal of Chemistry,* 2015, 1–23. https://doi.org/10.1155/2015/629085

Ahumada-Santos, Y. P., Montes-Avila, J., Uribe-Beltrán, M. de J., Díaz-Camacho, ç S. P., López-Angulo, G., Vega-Aviña, R., López-Valenzuela, J. Á., Heredia, J. B., & Delgado-Vargas, F. (2013). Chemical characterization, antioxidant, and antibacterial activities of six *Agave* species from Sinaloa, Mexico. *Industrial Crops and Products*, *49*, 143–149. https://doi.org/10.1016/j.indcrop.2013.04.050

Alasvand, M., Assadollahi, V., Ambra, R., Hedayati, E., Kooti, W., & Peluso, I. (2019). Antiangiogenic effect of alkaloids. *Oxidative Medicine and Cellular Longevity*, *2019*, 1–16. https://doi.org/10.1155/2019/9475908

Anbualakan, K., Tajul Urus, N. Q., Makpol, S., Jamil, A., Mohd Ramli, E. S., Md Pauzi, S. H., & Muhammad, N. (2022). A Scoping Review on the Effects of Carotenoids and Flavonoids on Skin Damage Due to Ultraviolet Radiation. *Nutrients*, *15*(1), 92. https://doi.org/10.3390/nu15010092

Araldi, R. P., dos Santos, M. O., Barbon, F. F., Manjerona, B. A., Meirelles, B. R., de Oliva Neto, P., da Silva, P. I., dos Santos, L., Camargo, I. C. C., & de Souza, E. B. (2018). Analysis of antioxidant, cytotoxic and mutagenic potential of *Agave sisalana* Perrine extracts using Vero cells, human lymphocytes, and mice polychromatic erythrocytes. *Biomedicine & Pharmacotherapy*, *98*, 873–885. https://doi.org/10.1016/j.biopha.2018.01.022

Awuchi, C. G., & Amagwula, I. O. (2020). The Biochemistry, Toxicology, and Uses of the Pharmacologically Active Phytochemicals: Alkaloids, Terpenes, Polyphenols, and Glycosides. *Merit Research Journal of Food Science and Technology*, *5*(1), 1–17. https://doi.org/10.5281/zenodo.3967809

Bermúdez-Bazán, M., Castillo-Herrera, G. A., Urias-Silvas, J. E., Escobedo-Reyes, A., & Estarrón-Espinosa, M. (2021). Hunting bioactive molecules from the *Agave*

© Central University of Technology, Free State

genus: An update on extraction and biological potential. *In Molecules* (Vol. 26, Issue 22). MDPI. https://doi.org/10.3390/molecules26226789

Bhattacharyya, A., Chattopadhyay, R., Mitra, S., & Crowe, S. E. (2014). Oxidative Stress: An Essential Factor in the Pathogenesis of Gastrointestinal Mucosal Diseases. *Physiological Reviews*, *94*(2), 329–354. https://doi.org/10.1152/physrev.00040.2012

Chandramohan, D. and Marimuthu, K., 2011, November. Applications of natural fibre composites for replacement of orthopaedic alloys. *International Conference on Nanoscience, Engineering and Technology (ICONSET 2011),* 137-145).

Cheok, C. Y., Salman, H. A. K., & Sulaiman, R. (2014). Extraction and quantification of saponins: A review. *In Food Research International* (Vol. 59, pp. 16–40). https://doi.org/10.1016/j.foodres.2014.01.057

Cowan, M. M. (1999). Plant Products as Antimicrobial Agents. *Clinical Microbiology Reviews*, *12*(4), 564–582. https://doi.org/10.1128/CMR.12.4.564

Cox-Georgian, D., Ramadoss, N., Dona, C., & Basu, C. (2019). Therapeutic and medicinal uses of terpenes. *Medicinal plants: from farm to pharmacy*, 333-359. Springer International Publishing. https://doi.org/10.1007/978-3-0 30-31269-5_15

Cruz-Magalhães, V., Pereira Andrade, J., Freitas Figueiredo, Y., Arthur Santos Marbach, P., & Teodoro de Souza, J. (2020). Sisal Bole Rot: An Important but Neglected Disease. *In Plant Diseases - Current Threats and Management Trends (p. 879).* IntechOpen. https://doi.org/10.5772/intechopen.86983

Debnath, M., Pandey, M., Sharma, R., Thakur, G. S., & Lal, P. (2010). Biotechnological intervention of Agave sisalana: A unique fiber-yielding plant with medicinal property. *Journal of Medicinal Plants Research*, *4*(3), 177–187. http://www.academicjournals.org/JMPR

Delage, B. (2015). Flavonoids. *Linus Pauling Institute, Oregon State University, Corvallis, Oregon.*

De Silva, G. O., Abeysundara, A. T., & Aponso, M. M. W. (2017). Extraction methods, qualitative and quantitative techniques for screening of phytochemicals from plants. *American Journal of Essential Oils and Natural Products*, *5*(2), 29-32. www.essencejournal.com

© Central University of Technology, Free State

Doughari, J. H. (2012). Phytochemicals: Extraction Methods, Basic Structures and Mode of Action as Potential Chemotherapeutic Agents. *In Phytochemicals - A Global Perspective of Their Role in Nutrition and Health (pp. 1–33). www.intechopen.com*. www.intechopen.com

Farco, J. A., & Grundmann, O. (2013). Menthol-Pharmacology of an Important Naturally Medicinal "Cool." *Reviews in Medicinal Chemistry*, *13*, 124–131.

Franck, A. R. (2012). Guide to *Agave*, Cinnamomum, Corymbia, Eucalyptus, Pandanus, and Sansevieria in the flora of Florida. *Phytoneuron*, *102*, 1–23. https://doi.org/10.13140/2.1.3641.4081

Freidberg, R. (2009). An investigation into the antimicrobial and anticancer activities of Geranium incanum. *Artemisia afra and Artemisia absinthium:* Nelson Mandela Metropolitan University, *13*, 1-245.

García-Mendoza, A., & Chiang, F. (2003). *The Confusion of Agave vivipara L. and A. angustifolia Haw., Two Distinct Taxa* (Vol. 55, Issue 1).

Gasser, P., Lati, E., Peno-Mazzarino, L., Bouzoud, D., Allegaert, L., & Bernaert, H. (2008). Cocoa polyphenols and their influence on parameters involved in *ex vivo* skin restructuring. *International Journal of Cosmetic Science*, *30*(5), 339–345. https://doi.org/10.1111/j.1468-2494.2008.00457.x

Ghosh, D. (2015). Tannins from Foods to Combat Diseases. *International Journal of Pharma Research & Review*, *4*(5), 40.

Guclu-Ustundag, Ö., & Mazza, G. (2007). Saponins: Properties, applications, and processing. *Critical Reviews in Food Science and Nutrition*, *47*(3), 231–258. https://doi.org/10.1080/10408390600698197

Gutiérrez, A., Rodríguez, I. M., & del Río, J. C. (2008). Chemical composition of lipophilic extractives from sisal (Agave sisalana) fibres. *Industrial Crops and Products*, *28*(1), 81–87. https://doi.org/10.1016/j.indcrop.2008.01.008

Heinrich, M., Mah, J., & Amirkia, V. (2021). Alkaloids used as medicines: Structural phytochemistry meets biodiversity—An update and forward look. *Molecules,* 26(7), 1-18. MDPI AG. https://doi.org/10.3390/molecules26071836

Hernández-Valle, E., Herrera-Ruiz, M., Salgado, G., Zamilpa, A., Ocampo, M., Aparicio, A., Tortoriello, J., & Jiménez-Ferrer, E. (2014). Anti-Inflammatory Effect

© Central University of Technology, Free State

of 3-O-[(6'-O-Palmitoyl)-β-D-glucopyranosyl Sitosterol] from *Agave angustifolia* on Ear Edema in Mice. *Molecules*, *19*(10), 15624–15637. https://doi.org/10.3390/molecules191015624

Lattanzio, V., Cardinali, A., & Linsalata, V. (2012). Plant Phenolics: A Biochemical and Physiological Perspective. *In Recent Advances in Polyphenol Research* (pp. 1–39). Wiley-Blackwell. *https://doi.org/10.1002/9781118299753.ch1*

Leisegang, K. (2021). Herbal pharmacognosy: An introduction. In *Herbal Medicine in Andrology* (pp. 17–26). Elsevier. https://doi.org/10.1016/B978-0-12-815565-3.00003-5

Li, X., Xin, Y., Mo, Y., Marozik, P., He, T., & Guo, H. (2022). The bioavailability and biological activities of phytosterols as modulators of cholesterol metabolism. *Molecules*, *27*(2), 523. https://doi.org/10.3390/molecules27020523

Maheshwari, S., Kumar, V., Bhadauria, G., & Mishra, A. (2022). Immunomodulatory potential of phytochemicals and other bioactive compounds of fruits: A review. *Food Frontiers*, *3*(2), 221-238. https://onlinelibrary.wiley.com/doi/abs/10.1002/fft2.129

Mazid, M., Khan, T. A., & Mohammad, F. (2011). Role of secondary metabolites in defense mechanisms of plants. *Biology and Medicine Journal, 3(2)*, 232-249. http://biolmedonline.com/Articles/MAASCON-1/Vol3_2_232-249.pdf

Mera, I. F. G., Falconí, D. E. G., & Córdova, V. M. (2019). Secondary metabolites in plants: Main classes, phytochemical analysis, and pharmacological activities. *Bionatura*, *4*(4), 1000–1009. https://doi.org/10.21931/RB/2019.04.04.11

Michalak, M. (2022). Plant-Derived Antioxidants: Significance in Skin Health and the Ageing Process. *International Journal of Molecular Sciences*, *23*(2), 585. https://doi.org/10.3390/ijms23020585

Monte, J., Abreu, A., Borges, A., Simões, L., & Simões, M. (2014). Antimicrobial Activity of Selected Phytochemicals against Escherichia coli and Staphylococcus aureus and Their Biofilms. *Pathogens*, *3*(2), 473–498. https://doi.org/10.3390/pathogens3020473

Monterrosas-Brisson, N., Ocampo, M. L. A., Jiménez-Ferrer, E., Jiménez-Aparicio, A. R., Zamilpa, A., Gonzalez-Cortazar, M., Tortoriello, J., & Herrera-Ruiz, M. (2013).

© Central University of Technology, Free State

Anti-inflammatory activity of different agave plants and the compound Cantalasaponin-1. *Molecules*, *18*(7), 8136–8146. https://doi.org/10.3390/molecules18078136

Nizioł-Łukaszewska, Z., & Bujak, T. (2018). Saponins as Natural Raw Materials for Increasing the Safety of Bodywash Cosmetic Use. *Journal of Surfactants and Detergents*, *21*(6), 767–776. https://doi.org/10.1002/jsde.12168

Olatunde, A., Mohammed, A., Mohammed, A. I., Tajuddeen, N., & Mohammed, N. S. (2022). Vanillin_ A food additive with multiple biological activities. *European Journal of Medicinal Chemistry Reports*, *5*(100055), 1–16. https://doi.org/https://doi.org/10.1016/j.ejmcr.2022.100055

Ong, E. S. (2004). Extraction methods and chemical standardization of botanicals and herbal preparations. In *Journal of Chromatography B: Analytical Technologies in the Biomedical and Life Sciences* (Vol. 812, Issues 1-2 SPEC. ISS., pp. 23–33). https://doi.org/10.1016/j.jchromb.2004.07.041

Ouhtit, A., & Ananthaswamy, H. N. (2001). A Model for UV-Induction of Skin Cancer. *Journal of Biomedicine and Biotechnology*, *1*(1), 5–6. https://doi.org/10.1155/S1110724301000031

Paduch, R., Kandefer-Szerszeń, M., Trytek, M., & Fiedurek, J. (2007). Terpenes: Substances useful in human healthcare. In *Archivum Immunologiae et Therapiae Experimentalis* (Vol. 55, Issue 5, pp. 315–327). https://doi.org/10.1007/s00005-007-0039-1

Panche, A. N., Diwan, A. D., & Chandra, S. R. (2016). Flavonoids: An overview. *Journal of Nutritional Science,* 5, e47. Cambridge University Press. https://doi.org/10.1017/jns.2016.41

Patel, S. S., & Savjani, J. K. (2015). Systematic review of plant steroids as potential anti-inflammatory agents: Current status and future perspectives. In *The Journal of Phytopharmacology* (Vol. 4, Issue 2). www.phytopharmajournal.com

Patle, T. K., Shrivas, K., Kurrey, R., Upadhyay, S., Jangde, R., & Chauhan, R. (2020). Phytochemical screening and determination of phenolics and flavonoids in Dillenia pentagyna using UV–vis and FTIR spectroscopy. *Spectrochimica Acta -*

© Central University of Technology, Free State

Part A: Molecular and Biomolecular Spectroscopy, *242*. https://doi.org/10.1016/j.saa.2020.118717

Pizzino, G., Irrera, N., Cucinotta, M., Pallio, G., Mannino, F., Arcoraci, V., Squadrito, F., Altavilla, D., & Bitto, A. (2017). Oxidative Stress: Harms and Benefits for Human Health. *Oxidative Medicine and Cellular Longevity*, *2017*, 1–13. https://doi.org/10.1155/2017/8416763

Ribeiro, B. D., Coelho, M. A. Z., & Marrucho, I. M. (2013). Extraction of saponins from sisal (*Agave sisalana*) and juá (*Ziziphus joazeiro*) with cholinium-based ionic liquids and deep eutectic solvents. *European Food Research and Technology*, *237*(6), 965–975. https://doi.org/10.1007/s00217-013-2068-9

Rivas, F., Parra, A., Martinez, A., & Garcia-Granados, A. (2013). Enzymatic glycosylation of terpenoids. In *Phytochemistry Reviews* (Vol. 12, Issue 2, pp. 327–339). https://doi.org/10.1007/s11101-013-9301-9

Rizwan, K., Zubair, M., Rasool, N., Riaz, M., Zia-Ul-Haq, M., & de Feo, V. (2012). Phytochemical and biological studies of *Agave attenuata*. *International Journal of Molecular Sciences*, *13*(5), 6440–6451. https://doi.org/10.3390/ijms13056440

Roy, A., Khan, A., Ahmad, I., Alghamdi, S., Rajab, B. S., Babalghith, A. O., Alshahrani, M. Y., Islam, S., & Islam, Md. R. (2022). Flavonoids a Bioactive Compound from Medicinal Plants and Its Therapeutic Applications. *BioMed Research International*, *2022*, 1–9. https://doi.org/10.1155/2022/5445291

Salazar-Pi, D. T., Castro-Ala, N., Moreno-God, Ma. E., Nicasio-To, M. del P., Perez-Hern, J., & Alvarez-Fi, P. (2017). Antibacterial and Anti-inflammatory Activity of Extracts and Fractions from *Agave cupreata*. *International Journal of Pharmacology*, *13*(8), 1063–1070. https://doi.org/10.3923/ijp.2017.1063.1070

Sasidharan, S., Chen, Y., Saravanan, D., Sundram, K., & Latha, L. (2010). Extraction, Isolation and Characterization of Bioactive Compounds from Plants' Extracts. *African Journal of Traditional, Complementary and Alternative Medicines*, *8*(1), 1-10. https://doi.org/10.4314/ajtcam.v8i1.60483

Saxena, M., Saxena, J., Nema, R., Singh, D., & Gupta, A. (2013). Phytochemistry of Medicinal Plants. *Journal of Pharmacognosy and Phytochemistry*, *1*(6), 1-15. www.phytojournal.comwww.phytojournal.com

© Central University of Technology, Free State

Sen, A. K., Sen, D. B., Zanwar, A. S., Balaraman, R., Shah, U., & Maheshwari, R. A. (2022). Catechins and Theaflavins: An Overview on Therapeutic Application. In *Journal of Natural Remedies* (Vol. 22, Issue 3, pp. 330–346). Informatics Publishing Limited. https://doi.org/10.18311/jnr/2022/30181

Sharma, K., Kaur, R., Kumar, S., Saini, R. K., Sharma, S., Pawde, S. V, & Kumar, V. (2023). Saponins: A concise review on food-related aspects, applications, and health implications. *Food Chemistry Advances*, *2*, 100191. https://doi.org/10.1016/j.focha.2023.100191

Sidana, J., Singh, B., & Sharma, O. P. (2016). Saponins of Agave: Chemistry and bioactivity. In *Phytochemistry* (Vol. 130, pp. 22–46). Elsevier Ltd. https://doi.org/10.1016/j.phytochem.2016.06.010

Soltys, L., Olkhovyy, O., Tatarchuk, T., & Naushad, M. (2021). Green synthesis of metal and metal oxide nanoparticles: Principles of green chemistry and raw materials. In *Magnetochemistry* (Vol. 7, Issue 11). MDPI. https://doi.org/10.3390/magnetochemistry7110145

Stepniowska, A. (2021). Selected Alkaloids Used in the Cosmetics Industry. *Journal of Cosmetic Science, 72, 229-249*. https://www.researchgate.net/publication/359692130

Tewari, D., Tripathi, Y. C., & Anjum, N. (2014). *Agave sisalana*: a plant with high chemical diversity and medicinal importance. *World Journal of Pharmaceutical Research,* 3(8), 238-249. www.wjpr.net

Vargas-Ponce, O., Zizumbo-Villarreal, D., Martínez-Castillo, J., Coello-Coello, J., & Colunga-GarcíaMarín, P. (2009). Diversity and structure of landraces of *Agave* grown for spirits under traditional agriculture: A comparison with wild populations of *A. angustifolia* (Agavaceae) and commercial plantations of *A. tequilana*. *American Journal of Botany*, *96*(2), 448–457. http://www.jstor.org/stable/27793102

Velázquez-Martínez, J. R., González-Cervantes, R. M., Hernández-Gallegos, M. A., Mendiola, R. C., Aparicio, A. R. J., & Ocampo, M. L. A. (2014). Prebiotic potential of Agave angustifolia haw fructans with different degrees of polymerization. *Molecules*, *19*(8), 12660–12675. https://doi.org/10.3390/molecules190812660

© Central University of Technology, Free State

Vera-Guzmán, A., Guzmán-Gerónimo, R., López, M., & Chávez-Servia, J. (2018). Volatile Compound Profiles in Mezcal Spirits as Influenced by *Agave Species* and Production Processes. *Beverages*, *4*(1), 9. https://doi.org/10.3390/beverages4010009

Verloove, F., & Pascual, M. S. (2021). Notes on genuine Agave vivipara (Agavaceae), a poorly known Caribbean species, recently introduced in the Canary Islands (Spain). *Bradleya*, *2021*(39), 259-264. https://doi.org/10.25223/brad.n39.2021.a28

Villanueva-Rodríguez, S. J., Rodríguez-Garay, B., Prado-Ramírez, R., & Gschaedler, A. (2016). Tequila: Raw Material, Classification, Process, and Quality Parameters. In *Encyclopedia of Food and Health* (pp. 283–289). Elsevier. https://doi.org/10.1016/B978-0-12-384947-2.00688-7

Wang, Y., Tao, B., Wan, Y., Sun, Y., Wang, L., Sun, J., & Li, C. (2020). Drug delivery-based pharmacological enhancement and current insights of quercetin with therapeutic potential against oral diseases. *Biomedicine and Pharmacotherapy*, *128,* 110372. https://doi.org/10.1016/j.biopha.2020.

Wink, M. (2007). Chapter 1 Molecular Modes of Action of Cytotoxic Alkaloids: From DNA Intercalation, Spindle Poisoning, Topoisomerase Inhibition to Apoptosis and Multiple Drug Resistance. In Geoffrey A. Cordell (Ed.), *The Alkaloids: Chemistry and Biology* (Vol. 64, pp. 1–47). Academic press. https://doi.org/10.1016/S1099-4831(07)64001-2

Wu, S., Pang, Y., He, Y., Zhang, X., Peng, L., Guo, J., & Zeng, J. (2021). A comprehensive review of natural products against atopic dermatitis: Flavonoids, alkaloids, terpenes, glycosides, and other compounds. *Biomedicine and Pharmacotherapy,* 140, 111741. https://doi.org/10.1016/j.biopha.2021.111741

Wu, S., Pang, Y., He, Y., Zhang, X., Peng, L., Guo, J., & Zeng, J. (2021). A comprehensive review of natural products against atopic dermatitis: Flavonoids, alkaloids, terpenes, glycosides, and other compounds. *Biomedicine and Pharmacotherapy,* 140, 111741.https://doi.org/10.1016/j.biopha.2021.111741

Yu, B., Patterson, N., & Zaharia, L. I. (2022). Saponin Biosynthesis in Pulses. *Plants*, *11*(24), 3505. https://doi.org/10.3390/plants11243505

© Central University of Technology, Free State

Zwane, P. E., Dlamini, A. M., & Nkambule, N. (2010). Antimicrobial Properties of Sisal (*Agave sisalana*) Used as an Ingredient in Petroleum Jelly Production in Swaziland. *Current Research Journal of Biological Sciences*, *2*(6), 370–374.

© Central University of Technology, Free State

CHAPTER 2 :
A COMPARATIVE PHYTOCHEMICAL EVALUATION OF *AGAVE ANGUSTIFOLIA VAR. ANGUSTIFOLIA* AND *AGAVE SISALANA* LEAVES

2.1 Introduction

Plants contain secondary metabolites that assist in their growth and/ or protection from predators and pathogens (López-Romero *et al*., 2018; Mazid *et al*., 2011; Patle *et al*., 2020; Rizwan *et al*., 2012). These naturally occurring and biologically active chemical compounds benefit humans more than micro- and macronutrients (Saxena *et al*., 2013). Phytochemicals perform crucial functions against several skin conditions and are increasingly exploited due to their numerous medicinal purposes. *Agave* species are reported to contain anti-inflammatory, antihypertensive, immunomodulatory, antiparasitic, and antifungal actions (Ahumada-Santos *et al*., 2013; Monterrosas-Brisson *et al*., 2013; Salazar-Pi *et al*., 2017; Saxena *et al*., 2013), and the investigation of the phytochemicals present in each plant would assist in choosing the best *Agave* plant for a particular biological function, such as antioxidant, antibacterial, etc. These results would also confirm if the plants can be interchangeable. This chapter, therefore, focused on the comparative phytochemical evaluation of *Agave angustifolia* (*AA*) and *Agave sisalana* (*AS*) leaves collected from two KwaZulu-Natal municipalities using visual calorimetric methods. The overall view of the study is presented in Figure 2.1.

Figure 2.1: Overall view of the *Agave* leaves analysis

© Central University of Technology, Free State

2.2 Experimental

2.2.1 Preparation of plant material

Fresh leaves of *Agave* plants utilised in this study were harvested from eThekwini Metropolitan municipality and the iLembe district municipality in KwaZulu-Natal. The *AA* leaves were collected from the Isipingo Beach suburb, located on the southern coast of KZN, under the eThekwini Metropolitan Municipality. In contrast, the *AS* leaves were collected from the veld along the R34 road under the iLembe district municipality. The taxonomic identity of the plants was confirmed by the South African National Biodiversity Institute (SANBI), and the two samples were placed at the KwaZulu-Natal Herbarium, with the accession numbers *Agave sisalana* – 152364 and *Agave angustifolia* – 152365.

For the analysis, the fresh leaves of *Agave* plants were collected from each municipality area where the atopic dangerous spiky tips were cut off before cutting both *AA* and *AS* leaves to ensure the protection of the harvester. The collected leaves were taken to the laboratory, where the side leaf thorns of *AA* were removed. All the leaves were cleaned to remove dirt with soapy tap water, rinsed with de-ionized water, spread on the laboratory workbench, lined with a laboratory paper towel, and dried overnight at laboratory ambient temperature. On the next day, the cleaned fresh leaves were neatly chopped into small pieces, spread on clean trays lined with paper towels, placed in the fume cupboard, and allowed to dry for one (1) week. The dried *Agave* leaves were pulverised into a fine powder utilizing a Waring Commercial Xtreme blender, set at 100% speed for 5 minutes. The cycle was repeated until no further crushing was observed. Only 200g of dried *Agave* leaves were pulverised for 5 minutes to produce the fine *Agave* powder to ensure maximum blender effectiveness, as adding too many dried *Agave* leaves would result in only the bottom leaves being pulverised while the top remained the same. The pulverised leaves were then transferred into a washed and sanitised air-tight plastic zip-lock bag and stored in the cabinet for the next step of the analysis.

2.2.2 Preparation of *Agave* plant extracts by gradient maceration

The finely crushed *Agave* leaves sample was sequentially extracted by cold maceration in solvents with increasing polarity for 72 hours at room temperature,

© Central University of Technology, Free State

starting with non-polar hexane, polar ethyl acetate and most polar methanol (Dharajiya *et al*., 2017). Two (2) kg of the pulverized *Agave* sample was soaked in 1.5 litres of hexane and covered with foil to prevent solvent evaporation and ingress of foreign matter. The solvent covered the powdered material to ensure maximum extraction.

The leaves were macerated for 72 hours in the fume cupboard, with daily stirring to ensure maximum extraction, filtered, rinsed with hexane, and the filtrate was put aside. The residue was soaked in ethyl acetate for 72 hours, filtered, washed with ethyl acetate, and put aside with filtrate. The residue was soaked in methanol for another 72 hours, filtered, washed with methanol, and the filtrate was set aside.

While waiting for 72 hours of ethyl acetate extraction, the hexane solvent was removed from the hexane filtrate by concentrating the filtrate under reduced pressure using the IKA RV10 digital rotatory evaporator to obtain the hexane extract fraction. A similar procedure was used for the methanol extract fraction. The methanol extract was obtained by soaking pulverized *Agave* leaves in methanol for 72 hours. The sample was filtered, rinsed with methanol, and concentrated under reduced pressure using the IKA RV10 rotatory evaporator. The methanol extract and solvent extract fractions were kept at 4 °C for the next step of the experiment.

2.3 Phytochemical evaluation of *Agave* plant extracts

The preliminary phytochemical evaluation of the *Agave* leaves extract was carried out by testing different classes of compounds using standard methods to identify the available phytochemicals (Dharajiya *et al*., 2017). The extracts were assessed for the existence of alkaloids, cardiac glycosides, flavonoids, phenolics, saponins, steroids and tannins, as per standard procedures for the detection of available phytochemicals within the tested plants as defined by earlier researchers (Dharajiya *et al*., 2012; 2015), with slight modifications. All chemicals used for both qualitative and quantitative analysis were of analytical grade.

2.3.1 Qualitative tests

All *A. angustifolia and A. sisalana* extracts were subjected to screening tests for flavonoids, tannins, phenols, terpenoids, saponins, steroids, glycosides, and alkaloids.

© Central University of Technology, Free State

2.3.1.1 Test for flavonoids

a) *Concentrated Sulphuric acid test*: 5 mL of dilute ammonia solution was mixed with a solution of the *Agave* extract filtrate. Undiluted sulphuric acid was added to this solution, and the solution was examined for yellow colouration in the extract mixture, which vanished on standing to confirm the existence of flavonoids.
b) *Sodium Hydroxide test*: Adding 2 mL of 2.0% NaOH solution to 1 mL of *Agave* extract resulted in a strong yellow colour, which turned colourless when adding two drops of weak acetic acid, confirming the existence of flavonoids.
c) *Ferric chloride test*: one (1) mL of the *Agave* extract was boiled in de-ionized water and filtered. Adding 10% ferric chloride solution (a few drops) to 2 mL of the *Agave* filtrate produced a violet colour, signifying the existence of a phenolic hydroxyl group (flavonoids).

2.3.1.2 Test for tannins

10 mL water and 0.5g Agave extract were added to a test tube and mixed. The solution was boiled and filtered, and then 0.1% ferric chloride (a few drops) was added and mixed. Then, the colour was checked for blue-black or greenish-brown, confirming the existence of tannins.

2.3.1.3 Test for phenols

Ellagic test: To 1 mL *Agave* extract, 5% $FeCl_3$ (5 drops) was added, and a yellow-green fluorescence was observed, showing the presence of a certain phenol called resorcinol.

2.3.1.4 Test for terpenoids

To one (1) mL *Agave* extract, a mixture of 1 mL each of chloroform acetic anhydride and undiluted Sulphuric acid was added. The mixture was observed for a red-violet colouration, confirming terpenoids' existence.

© Central University of Technology, Free State

2.3.1.5 Test for saponins

To one (1) mL *Agave* extract, 3 mL distilled water and five drops olive oil were added, then shaken vigorously for about 1-2 minutes. A foam was observed in the solution, confirming saponins' existence.

2.3.1.6 Test for steroids

Two (2) ml acetic anhydride and undiluted sulphuric acid were mixed with 1 mL *Agave* extract. The violet solution turned green, confirming the existence of steroids.

2.3.1.7 Test for glycoside

a) *Liebermann's test*: two (2) mL chloroform was mixed with 5 mL *Agave* plant extract. Then, 2 mL of acetic acid was added to the above mixture and cooled before adding 1 mL of undiluted sulphuric acid. The detected green colour revealed the existence of aglycone (the steroidal part of glycosides).
b) *Keller-Killian Test*: two (2) mL glacial acetic acid and five drops of 2.0% $FeCl_3$ were added to 5 mL *Agave* extract, and the solution was shaken. To this, 1 mL of undiluted sulphuric acid. The brownish ring that was seen between the layers suggests the existence of cardiac steroidal glycosides.
c) *Salkowski's test*: when two (2) mL concentrated sulphuric acid was mixed with 5 mL *Agave* extract, the observed mahogany colour revealed the existence of steroidal aglycone (part of the glycoside).

2.3.1.8 Test for alkaloid (Wagner's test)

Two (2) mL of *Agave* extract were mixed with 1,5% hydrochloric acid, adding a few drops of Wagner's reagent. The appearance of a brownish precipitate or yellow colour precipitate confirmed the existence of alkaloids.

2.3.2 Quantitative analysis of *Agave* leaves (UV analysis)

Ultraviolet /Visible (UV/Vis) spectroscopy is an inexpensive, simple, flexible, non-destructive analytical method appropriate for a comprehensive class of organic

© Central University of Technology, Free State

compounds and some inorganic species. UV-vis spectrophotometers measure the absorbance or transmittance of light passing through a medium as a function of the wavelength.

The UV/Vis procedure was utilised to establish the comprehensive phytochemical constituents in *AA* and *AS leaves* by measuring the total contents of flavonoids, tannins, vitamin C and phenolics in triplicate. The standards for calibration curves were prepared for the total contents of phenolics, Vitamin C, tannins, and flavonoids.

2.3.2.1 Total phenolic content

To prepare the stock solution for the phenolic content, 50 mg gallic acid was weighed and transferred into a 50 mL volumetric flask. Methanol was used to dilute the solution to the mark. Water was used to dilute 5 mL stock solution into a 100 mL volumetric flask to make 100 mL of 50 µg/mL working gallic acid standard solution. Then, 2 mL Folin-Ciocalteu reagent was diluted with 2 mL water and put aside. Into five (5) clean 50 mL volumetric flasks were added 0.2, 0.4, 0.6, 0.8 and 1 mL standard gallic acid solution, followed by the adding 2 mL sodium carbonate (5%) and 0.5 mL Folin-Ciocalteu reagent solution prepared above, into each flask.

The flasks were mixed and then incubated in the dark at 40 °C for an hour. The absorbance of the dark blue solution was determined with the UV/Vis at 725 nm against water, which acted as a blank. The calibration curve was constructed from the average measured absorbances from different gallic acids. All the analyses were done in triplicate.

2.3.2.2 Vitamin C content

For the total Vitamin C content, 25 mg ascorbic acid was measured and transferred into a 25 mL volumetric flask, which was then diluted with 0.4% oxalic acid to the mark, resulting in 100 ppm ascorbic acid working standard solution. Then, 0.5, 0.75, 1.0, 1.25- and 1.5-mL ascorbic acid solution were pipetted into each 25 mL volumetric flask, into which 5 mL of 10% H_2SO_4 was added, mixed, and diluted with 5% ammonium molybdate solution to the mark. The flasks were mixed and incubated for 30 minutes, and the absorbance was then determined at 494 nm. The calibration curve for Vitamin C was then plotted.

© Central University of Technology, Free State

2.3.2.3 Total tannic acid content

For the total tannin content, the standard tannic acid stock solution was prepared by weighing 50 mg tannic acid, transferring it into a 50 mL volumetric flask, and diluted with methanol to the mark. Then, 5 mL standard tannic acid stock solution was diluted with distilled water into a 100 mL volumetric flask to make 50 µg/mL tannic acid. To prepare the working tannic acid standard solution, 1,2,3,4 and 5 mL of the working tannic acid standard solution were pipetted into each 50 ml volumetric flask, followed by the addition of 1 mL sodium carbonate (5%) and 0.5 mL Folin-Ciocalteu (1N), then mixed well. After 30 minutes, the absorbance of the solutions was measured at 700 nm against the blank (de-ionized water).

2.3.2.4 Total flavonoids content

For the total flavonoid content, 100 mg quercetin was weighed into a 100 mL volumetric flask and diluted to the mark using deionized water to make 1000 µg/mL quercetin standard stock solution. 0.5, 2.5, 5, 10 and 20 mL of this stock solution were transferred into each 50 mL volumetric flask and then diluted with distilled water to the mark. Then, 2 mL sodium hydroxide (4%) and 10% aluminium chloride solutions were added into each 50 mL quercetin solution, which was mixed well and incubated for 15 minutes at 40 °C. The mixture was cooled, and the calibration curve was obtained by measuring the absorbance at 510 nm.

© Central University of Technology, Free State

2.4 Results and discussion

The result for *Agave* extracts phytochemical screening using calorimetric methods is shown in Table 2.1, where the colour intensity, indicated by the (+) sign, is directly proportional to the abundance of the tested phytochemical.

Table 2.1: The phytochemical screening of *Agave angustifolia* and *Agave sisalana* extracts

Phytochemical test		AA HF	AS HF	AA EAF	AS EAF	AA MF	AS MF	AA ME	AS ME
Flavonoids	NaOH test	+	+	++	+++	+++	+++	-	-
	$FeCl_3$ test	-	++	+++	+	++	+++	+	+++
	H_2SO_4	+	+++	+	+++	-	-	-	++
Tannins		+	++	-	+	+	-	++	++
Phenolics		+	+++	-	++	-	+	++	++
Terpenoids		+++	+	-	-	+	+	+	-
Saponins		+	+++	+	+	++	++	++	+
Steroids		-	++	-	-	-	-	+++	+++
Test for Glycosides	Lieberman's test	++	+++	-	+++	+	+++	+++	+++
	Keller- Kilian test	-	+	-	-	++	+	+	+
	Salkowski's test	+	+++	+	+	+	-	-	++
Alkaloids	Wagner's test	-	-	-	+++	-	+++	-	+++

Notes: HF= Hexane fraction, EAF=ethyl acetate fraction, MF = methanol fraction & ME= methanol extract. (+) = Slightly intense colour present, (++) = moderately intense colour, (+++) = extremely intense colour, and (-) = not detected

For *A. angustifolia*, phytochemical screening confirmed the existence of flavonoids, glycosides, phenols, saponins, tannins and terpenoids in the hexane fraction. In contrast, flavonoids, glycosides, and saponins were confirmed in the ethyl acetate fraction. The methanol fraction indicated the existence of flavonoids, glycosides, saponins, tannins and terpenoids. Flavonoids, glycosides, phenolics, saponins, steroids, tannins and terpenoids were detected from the methanol extract. These results agreed with Hernandez-Valle's study, which reported the separation of steroidal saponins from the leaves of *A. angustifolia* (Hernández-Valle *et al*., 2014).

© Central University of Technology, Free State

Plant phenolics are reported to be natural antioxidants, antimicrobials, anti-inflammatory, and anti-ageing (Maheshwari *et al*., 2022; Sen *et al*., 2022) and known to play a significant role as compounds that protect against environmental strains, such as UV radiation, pathogenic infection, and predators, (Puupponen-Pimiä *et al*., 2001), their availability in plant extracts indicated the potential of the plant for use as antioxidants and antimicrobials in cosmetic products. However, antioxidant and antimicrobial activities had to be investigated to substantiate these claims.

No alkaloids were found in all *A. angustifolia* extracts. As alkaloids are known to defend plants against herbivores and entrants and act as repellents against pathogens (López-Romero *et al*., 2018; Mazid *et al*., 2011; Patle *et al*., 2020; Rizwan *et al*., 2012), these results suggested that the saponins could have provided the plant protections they are also claimed to be responsible for the defence mechanism against pathogens in plants owing to their antifungal, antimicrobial and anti-inflammatory properties (Cheok *et al*., 2014). Saponins are known to contain anti-inflammatory, antioxidant, and antitumour activities (Yu *et al*., 2022; Sharma *et al*., 2023).

The UV results indicated higher phenolics in the hexane fraction of *AS* than *AA*. The quantification of phytochemicals in the *Agave* leaves samples using a UV/Vis spectrophotometer is shown in Table 2.2 and Figures 2.2, 2.3 and 2.4. The flavonoid presence was determined by measuring quercetin content in the *Agave* leaves. The calibration curves for the UV analysis are shown in Figures 2.2, 2.3, and 2.4.

Table 2.2: Phytochemical quantification of *Agave* extracts by UV analysis

Phytochemical content	Total phenolics (ppm)	Ascorbic acid (ppm)	Tannins (ppm)	Quercetin ppm)
AA HF	305,70	0,05	0,70	133,36
AA EAF	771,00	0,07	96,23	84,07
AA MF	1211,00	0,01	122,39	86,93
AA ME	771,00	0,09	1378,15	1280,50
AS HF	22410,00	0,19	348,54	951,93
AS EAF	7311,00	0,03	76,92	580,50
AS MF	1261,00	0,01	215,46	161,93
AS ME	691,00	0,13	1963,15	1895,86

Notes: AA HF & AS HF = hexane fractions, AA EAF & AS EAF = ethyl acetate fractions, AA MF & AS MF = methanol fractions, AA ME & AS ME = methanol extracts.

© Central University of Technology, Free State

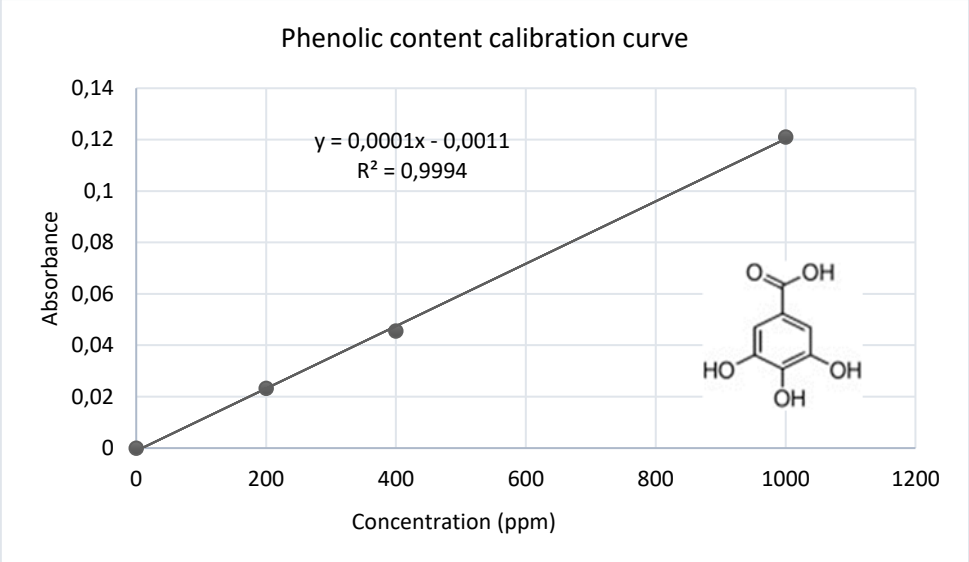


Figure 2.2: Calibration curve for the phenolic content and the structure of gallic acid

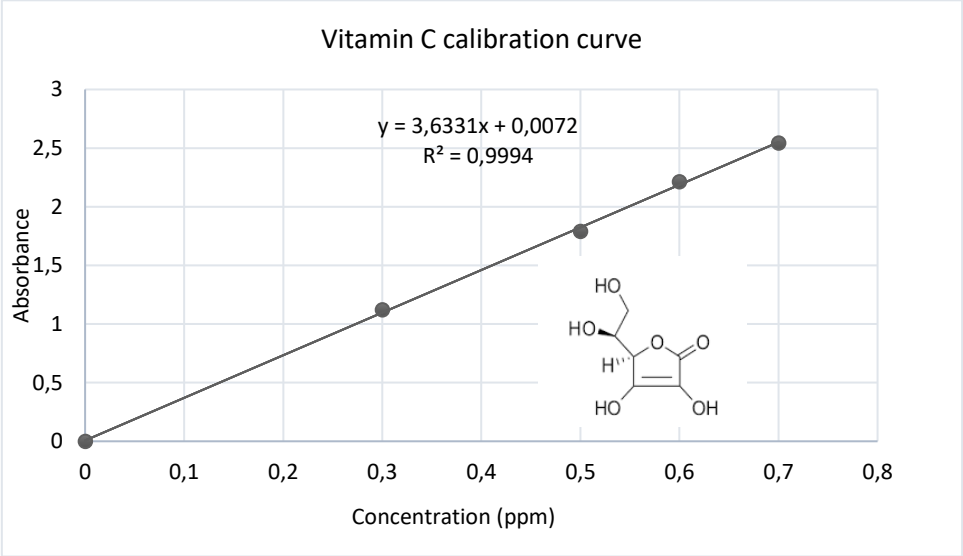


Figure 2.3: Calibration curve for vitamin C content and the structure of vitamin C

© Central University of Technology, Free State

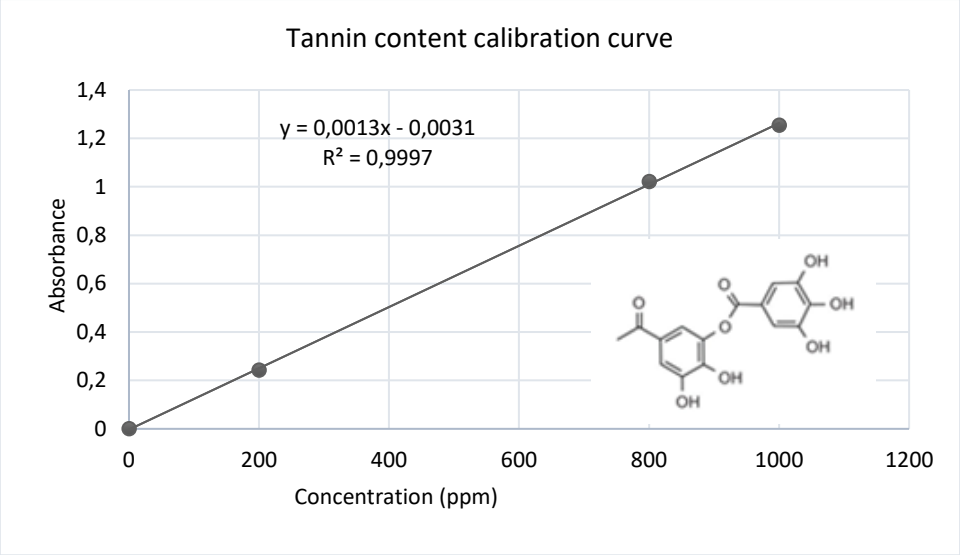


Figure 2.3: Calibration curve for the tannin content and structure of tannic acid

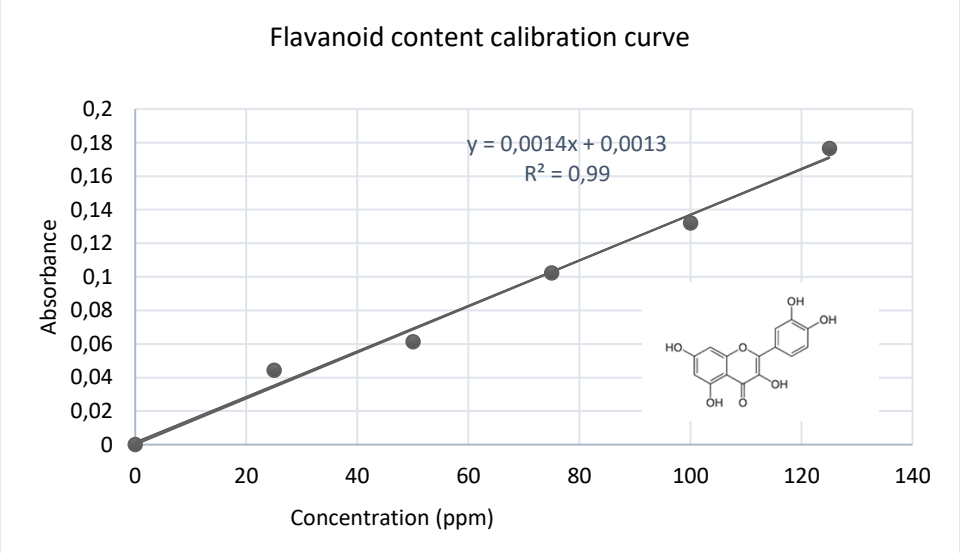


Figure 2.4: Calibration curve for the flavonoid content and structure of quercetin

© Central University of Technology, Free State

2.5 References

Ahumada-Santos, Y. P., Montes-Avila, J., Uribe-Beltrán, M. de J., Díaz-Camacho, ç S. P., López-Angulo, G., Vega-Aviña, R., López-Valenzuela, J. Á., Heredia, J. B., & Delgado-Vargas, F. (2013). Chemical characterization, antioxidant, and antibacterial activities of six *Agave* species from Sinaloa, Mexico. *Industrial Crops and Products*, *49*, 143–149. https://doi.org/10.1016/j.indcrop.2013.04.050

Cheok, C. Y., Salman, H. A. K., & Sulaiman, R. (2014). Extraction and quantification of saponins: A review. In *Food Research International* (Vol. 59, pp. 16–40). https://doi.org/10.1016/j.foodres.2014.01.057

Dharajiya, D., Moitra, N., Patel, B., & Patel, R. K. (2012). Preliminary phytochemical analysis of the Indian medicinal plants for antibacterial activity against bovine mastitis pathogens. *Wayamba Journal of Animal Science, 4, 332-342*. http://www.wayambajournal.com

Dharajiya, D., Patel, P., & Moitra, N. (2015). Antibacterial activity of *Emblica officinalis* (Gaertn.) Fruits and *Vitex negundo* (L.) Leaves. *Current Trends in Biotechnology and Pharmacy*, *9*(4), 357–368.

Dharajiya, Darshan., Patel, Payal., Jasani, Hitesh., & Pagi, N. (2017). Antimicrobial activity and phytochemical screening of Aloe vera (*Aloe barbadensis* Miller). *International Journal of Current Microbiology and Applied Sciences*, *6*(3), 2152–2162. https://doi.org/10.20546/ijcmas.2017.603.246

Hernández-Valle, E., Herrera-Ruiz, M., Salgado, G., Zamilpa, A., Ocampo, M., Aparicio, A., Tortoriello, J., & Jiménez-Ferrer, E. (2014). Anti-Inflammatory Effect of 3-O-[(6'-O-Palmitoyl)-β-D-glucopyranosyl Sitosterol] from *Agave angustifolia* on Ear Edema in Mice. *Molecules*, *19*(10), 15624–15637. https://doi.org/10.3390/molecules191015624

López-Romero, J. C., Ayala-Zavala, J. F., Peña-Ramos, E. A., Hernández, J., & González-Ríos, H. (2018). Antioxidant and antimicrobial activity of Agave angustifolia extract on overall quality and shelf life of pork patties stored under refrigeration. *Journal of Food Science and Technology*, *55*(11), 4413-4423. https://doi.org/10.1007/s13197-018-3351-3

© Central University of Technology, Free State

Maheshwari, S., Kumar, V., Bhadauria, G., & Mishra, A. (2022). Immunomodulatory potential of phytochemicals and other bioactive compounds of fruits: A review. *Food Frontiers*, *3*(2), 221–238. https://doi.org/10.1002/fft2.129

Mazid, M., Khan, T.A. & Mohammad, F. (2011). Role of secondary metabolites in defence mechanisms of plants. *Biology and Medicine*, 3(2), 232–249. http://biolmedonline.com/Articles/MAASCON-1/Vol3_2_232-249.pdf

Monterrosas-Brisson, N., Ocampo, M. L. A., Jiménez-Ferrer, E., Jiménez-Aparicio, A. R., Zamilpa, A., Gonzalez-Cortazar, M., Tortoriello, J., & Herrera-Ruiz, M. (2013). Anti-inflammatory activity of different agave plants and the compound Cantalasaponin-1. *Molecules*, *18*(7), 8136–8146. https://doi.org/10.3390/molecules18078136

Patle, T. K., Shrivas, K., Kurrey, R., Upadhyay, S., Jangde, R., & Chauhan, R. (2020). Phytochemical screening and determination of phenolics and flavonoids in Dillenia pentagyna using UV–vis and FTIR spectroscopy. *Spectrochimica Acta - Part A: Molecular and Biomolecular Spectroscopy*, *242, 118717*. https://doi.org/10.1016/j.saa.2020.118717

Puupponen-Pimiä, R., Nohynek, L., & Meier, C. (2001). Antimicrobial properties of phenolic compounds from berries. *Journal of Applied Microbiology*, *90*(4), 494–507. https://doi.org/10.1046/j.1365-2672.2001.01271.x

Rizwan, K., Zubair, M., Rasool, N., Riaz, M., Zia-Ul-Haq, M., & de Feo, V. (2012). Phytochemical and biological studies of *Agave attenuata*. *International Journal of Molecular Sciences*, *13*(5), 6440–6451. https://doi.org/10.3390/ijms13056440

Salazar-Pi, D. T., Castro-Ala, N., Moreno-God, Ma. E., Nicasio-To, M. del P., Perez-Hern, J., & Alvarez-Fi, P. (2017). Antibacterial and Anti-inflammatory Activity of Extracts and Fractions from *Agave cupreata*. *International Journal of Pharmacology*, *13*(8), 1063–1070. https://doi.org/10.3923/ijp.2017.1063.1070

Saxena, M., Saxena, J., Nema, R., Singh, D., & Gupta, A. (2013). Phytochemistry of Medicinal Plants. *Journal of Pharmacognosy and Phytochemistry*, *1*(6), 1-15. www.phytojournal.comwww.phytojournal.com

Sharma, K., Kaur, R., Kumar, S., Saini, R. K., Sharma, S., Pawde, S. V, & Kumar, V. (2023). Saponins: A concise review on food-related aspects, applications, and

© Central University of Technology, Free State

health implications. *Food Chemistry Advances*, *2*, 100191. https://doi.org/10.1016/j.focha.2023.100191

Wang, Y., Tao, B., Wan, Y., Sun, Y., Wang, L., Sun, J., & Li, C. (2020). Drug delivery-based pharmacological enhancement and current insights of quercetin with therapeutic potential against oral diseases. *Biomedicine and Pharmacotherapy*, 128, 110372. https://doi.org/10.1016/j.biopha.2020.110372

Yu, B., Patterson, N., & Zaharia, L. I. (2022). Saponin Biosynthesis in Pulses. *Plants*, *11*(24), 3505. https://doi.org/10.3390/plants11243505

© Central University of Technology, Free State

CHAPTER 3 :
ANTIOXIDANT ACTIVITY OF EXTRACTS OF *AGAVE ANGUSTIFOLIA VAR. ANGUSTIFOLIA* AND *AGAVE SISALANA* PLANTS USING DPPH AND FRAP METHODS

3.1 Introduction to the antioxidant activity of *Agave* leaves

Investigating the biological activities of extracts from natural products, plants, or synthetic mixtures, be they antimicrobial, antioxidant, cytotoxic, etc., is critical in discovering new and/ or enhanced beneficial entities.

Plants contain secondary metabolites with bioactivities, such as phenolics, carotenoids, vitamins, unsaturated fatty acids, etc. (Munteanu & Apetrei, 2021). Polyphenols have been promised to be among the natural products vital to human well-being. Research has reported that they have good activity as antioxidants, anti-inflammatory, and anti-cancer agents that prevent cardiovascular diseases (Zhang *et al*., 2006; Zhang *et al*., 2006). Antioxidants are any substance that prevents reactive oxygen species from oxidising vital biomolecules (Benzie & Choi, 2014). The cells create potentially harmful reactive oxygen species (ROS) through metabolising oxygen. If they are high, they disrupt the functioning of the biological cells, which results in deficient cell operation, ageing, or disease (Munteanu & Apetrei, 2021).

Antioxidants contribute to plant protection against pathogens and are claimed to provide many health benefits and protection against oxidative stress-related illnesses, as they act as either hydrogen donors or acceptors of free radicals. For this reason, more research is being undertaken to investigate plants' total phenolic content and possible antioxidant activity(Munteanu & Apetrei, 2021; Pisoschi & Negulescu, 2011).

Determining antioxidant activity is essential in reviewing the effectiveness of antioxidants in averting and treating diseases associated with oxidative strains, as well as for the meaningful evaluation of the antioxidant content from plant extracts. Generally, the antioxidant tests incorporate hydrogen atom transfer (HAT)-based assessment and electron transfer (ET)-based evaluations. For ET-based evaluations, the antioxidants react with an oxidizing agent instead of peroxyl radicals. Spectrophotometric ET-based evaluations measure the capability of an antioxidant to

© Central University of Technology, Free State

reduce an oxidant, as it is accompanied by a colour change if reduced. The extent of colour change (either increase or decrease of oxidizing agent's absorbance at a specific wavelength) is equivalent to the number of antioxidants in the sample (Benzie & Choi 2014). The ET-based assays include cupric reducing antioxidant power (CUPRAC) assay, 2,2-diphenyl-1-picryl-hydrazyl-hydrate (DPPH) assay, ferric reducing/antioxidant power (FRAP) assay, ferric thiocyanate (FTC) assay, ferrous oxidation-xylenol orange (FOX) assay and potassium ferricyanide reducing power (PFRAP) assay (Benzie & Choi, 2014; Xiao *et al*., 2020).

The DPPH free radical is a long-lived organic nitrogen radical with a deep purple colour. When a DPPH solution reacts with an antioxidant, the purple colour changes to colourless (equivalent hydrazine, see Figure 3.1).

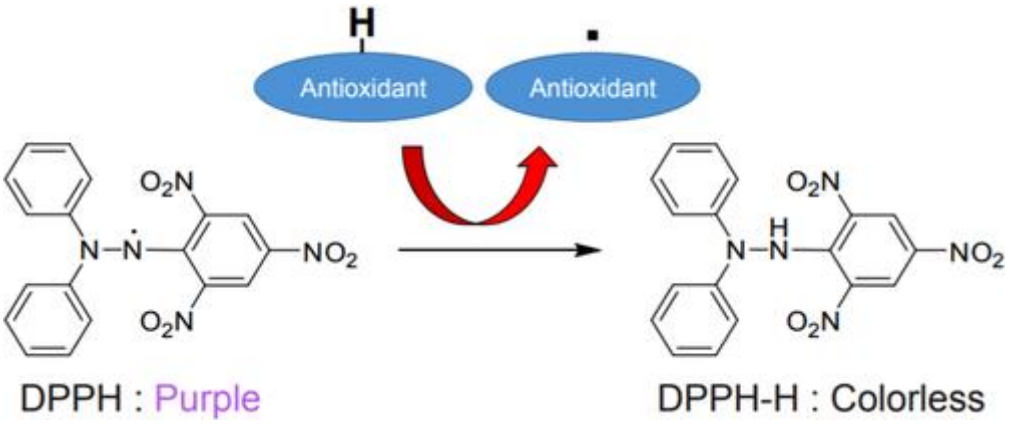


Figure 3.1: DPPH and antioxidants reaction to produce DPPH samples (Benzie & Choi, 2014; Xiao et al., 2020)

The reducing capacity of antioxidants toward DPPH can be assessed by observing the reduction of its absorbance at 515 – 528 nm. The results are expressed as (50% inhibitory concentration), IC_{50} or % scavenging of $DPPH^{\bullet}$ at a fixed antioxidant concentration for all the samples (Benzie & Choi, 2014).

The FRAP assay is amongst the most generally utilised methods to estimate the antioxidant capability, founded on an antioxidant composite's reducing ability. It is comparatively inexpensive, quick, simple, and sensitive to perform. The evaluation is established on the reducing ability of an antioxidant, and it provides a supple instrument to measure the collective activity of the oxidation-reduction-active

© Central University of Technology, Free State

antioxidants in the test sample, be it a food or a plant extract. In FRAP, the assay reaction involves the reduction of the ferric ion (Fe^{3+})-ligand complex to the deep, blue-coloured ferrous (Fe^{2+}) complex by antioxidants in an acidic medium to maintain iron solubility, as well as to encourage the transfer of electrons. As the oxidation-reduction potential rises, it causes a change in the primary reaction mechanism (I. F. Benzie & Strain, 1996). Tripyridyltriazine (TPTZ) is used as the iron-binding ligand. The test reaction encompasses the reduction of Fe^{3+}—TPTZ (iron [III]-2,4,6-tripyridyl-S-triazine) to Fe^{2+}—TPTZ (intense blue) via the single electron transfer (SET) with an antioxidant compound (Amarowicz & Pegg, 2019), as per the following reaction:

$$\text{Antioxidant} + Fe^{3+} \rightleftarrows Fe^{2+} + \text{oxidized antioxidant}$$

Or

$$Fe^{3+} - TPTZ + ArOH \rightarrow Fe^{2+} - TPTZ \text{ (blue at 595 nm)} + AROH^{\cdot} + SET$$

The antioxidant capacity is measured as a rise of absorbance at 593 nm, and the results are reported as micromolar Fe^{2+} equivalents. The FRAP value is determined from the next equation:

$$\text{FRAP value} = [(A_1 - A_0)/ (Ac - A_0)] \times 2,$$

Where:

Ac is the absorbance of the positive control,

A_1 is the absorbance of the sample, and

A_0 is the absorbance of the blank.

This study evaluated the antioxidant activity of Agave angustifolia var. angustifolia and Agave sisalana using the DPPH and FRAP methods.

3.2 Experimental

3.2.1 Sample preparation

Agave angustifolia (*AA*) and *Agave sisalana* (*AS*) plant material were prepared and extracted as described in Chapter 2 of this dissertation to yield a hexane fraction, an ethyl acetate fraction, a methanol fraction, and a methanol extract. These were reconstituted in dimethyl sulphoxide (DMSO) to provide 100 mg/ mL. The

© Central University of Technology, Free State

extracts/fractions with solubility challenge were sonicated. The completed samples were at 4 ˚C until required.

3.2.2 Evaluation of the antioxidant action using the DPPH method

Five (5) µL *Agave extract* sample was mixed with 120 µL of freshly prepared 0.1 mM DPPH (in methanol) and 120 µL Tris-HCl buffer solution (50 mM, pH 7.4), and then they were added into a 96-well plate. The mixture was incubated at room temperature for 20 minutes in the dark. The BioTek® PowerWave XS spectrophotometer (Winooski, VT, USA) was used to determine the absorbance at 513 nm. The percentage of radical scavenging action was determined as follows:

$$\% \text{ DPPH scavenged} = [(A_B - A_A)/ A_B] \times 100\%$$

Where A_B is the absorbance of blank at t= 0 minutes, A_A is the absorbance of the antioxidant at t= 30 minutes.

For the blank (control), the buffer was replaced by the 5 µL sample. The *Agave* extracts were evaluated at 250 and 500 µg/mL final concentrations.

3.2.3 Assessment of antioxidant activity using the FRAP method

The FRAP antioxidant assay method was used to evaluate the possible antioxidant action of the *Agave* extract fractions. The ferric-reducing capacity of the *Agave* extracts and antioxidant controls was assessed as per the method described by Benzie and Strain (1996) with adjustments as follows: Sample stock solutions (100 mg/mL) and positive control Trolox (10 mM) were prepared in DMSO. The freshly made FRAP reagent comprised of 20 mL sodium acetate buffer (300 mM), freshly prepared 2 mL TPTZ solution (10 mM TPTZ and 40 mM HCl dissolved at 50 ºC in a water bath), freshly made 2 mL $FeCl_3$ solution (20 mM ferric chloride in deionized water) and 2.4 mL deionized water. Fifty (50 µL) *Agave* samples were added into a 96-well plate along with 200 µL FRAP reagent and incubated at 37 °C for 30 minutes. Absorbance was read at 593 nm using a BioTek® PowerWave XS spectrophotometer (Winooski, VT, USA). The ferrous sulphate (Fe_2SO_4) (1.25 – 200 µmol/L) standard curve was constructed and used to calculate and express the results as [Fe_2SO_4 (µmol/L) / mg sample].

© Central University of Technology, Free State

3.3 Results and discussion

3.3.1 DPPH antioxidant activity

An antioxidant activity of eight (8) *Agave* extracts is shown in Figure 3.1, where the DPPH assay is expressed as % DPPH scavenged. Trolox acted as a positive control. Error bars represent the standard deviation of quadruple values done as a solo experiment. *Agave angustifolia (AA)* and *Agave sisalana (AS)* samples, AA-3 (methanol fraction), *AA*-4 (methanol extract), *AS*-1 (hexane fraction), and *AS*-4 (methanol extract) showed little DPPH scavenging activity. In contrast, samples *AA*-1 (hexane fraction), *AA*-2 (ethyl acetate fraction), *AS*-2 (ethyl acetate fraction), and *AS*-3 (methanol fraction) showed more than 50% DPPH scavenging activity at their higher concentrations.

For *A. angustifolia*, the hexane and ethyl acetate fractions exhibited more than 50% DPPH scavenging activity. In contrast, the methanol fraction and methanol extract exhibited little DPPH capacity, meaning that the antioxidant compounds in these extracts do not have much polarity and, therefore, could not be extracted by the methanol, both as a fraction and an extract.

For *A. sisalana*, the hexane fraction and the methanol extract showed little DPPH activity. In contrast, the fractions from ethyl acetate and methanol showed more than 50 % DPPH scavenging activity at their highest concentrations. *A. sisalana* demonstrated the presence of polar antioxidant constituents, which were extracted from ethyl acetate and methanol fractions. Both plants had potential antioxidant activities in the ethyl acetate extract fraction. These results agree with the investigation performed by Araldi (Araldi *et al*., 2018), where AS was reported to have an antioxidant activity. The difference was that the compounds with antioxidant potential were extracted by hexane in *A. angustifolia*, while they were extracted by methanol from the *A. sisalana* plant. These compounds proved to be non-polar in *A. angustifolia* and polar in *A. sisalana*.

© Central University of Technology, Free State

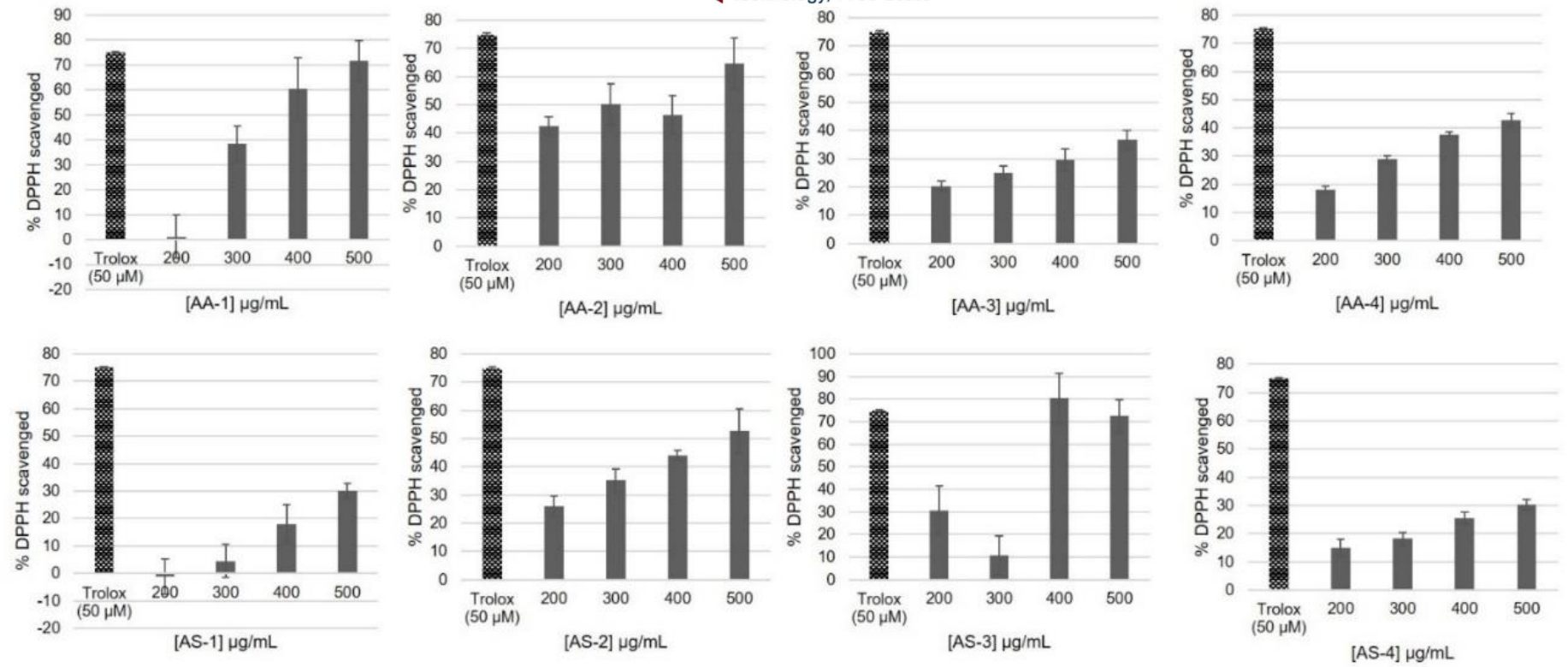


Figure 3.2: The DPPH assay of *Agave* leaves, where AA1 & AS1 = hexane fractions; AA2 & AS2 = ethyl acetate fractions. AA3 & AS3 = methanol fractions; & AA4 & AS4 = methanol extracts

© Central University of Technology, Free State

3.3.2 The FRAP assay

The outcomes of the FRAP method for investigating *Agave* leaf extracts are depicted in Figure 3.2. Trolox acted as a positive control. Error bars represent the standard deviation of quadruple quantities as a solo experiment. While using the FRAP assay method, all plant extracts showed little antioxidant activity, with the maximum antioxidant capability observed in the methanol fraction for both *A*. *angustifolia* and *A*. *sisalana* plants, and the hexane fraction displayed the minimum antioxidant capacity. The methanol extract fraction had similar results for both plants, with the ethyl acetate fraction showing higher activity for *AS* than *AA*, and the hexane fraction showed more radical scavenging activity in the *AA* extract than the *AS*. All the results were observed at the highest treatment concentration. However, both these solvents cannot be used for cosmetic applications due to their toxicity and should, therefore, be substituted with ethanol, which is safe for cosmetic applications.

Both *Agave* plants showed higher antioxidant activity potential in the methanol extract fractions, meaning that this fraction has polar antioxidants that participated in the reactions of the FRAP assay method. Therefore, the acceptable polar solvent for cosmetics, ethanol, can extract both *Agave* plants for potential cosmetic applications. All the tested *Agave* leaf samples showed little antioxidant potential, with *AA*-3 and *AS*-3 showing the highest concentration of Fe_2SO_4 at the maximum treatment concentration of 500 µg/mL.

© Central University of Technology, Free State

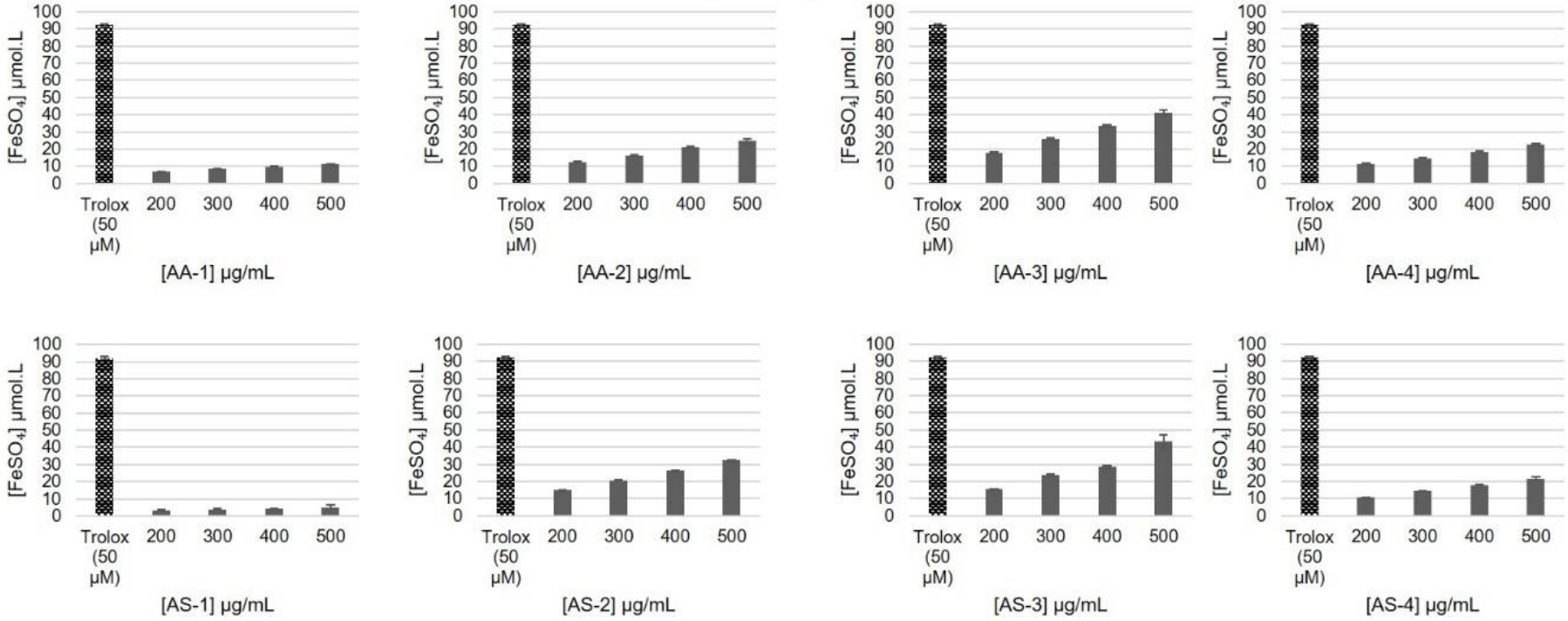


Figure 3.3: The FRAP assay of *Agave* leaves, where AA1 & AS1 = hexane fraction; AA2 & AS2 = ethyl acetate fraction. AA3 & AS3 = methanol fraction & AA4 & AS4 = methanol extract

© Central University of Technology, Free State

The DPPH and FRAP methods showed similar results even though the DPPH showed higher antioxidant activity, and these variations might result from different types of antioxidants found in the samples, each of which reacts differently with the applied radicals.

The DPPH method measures the ability of various antioxidants to donate either an electron or hydrogen radical to the stable DPPH free radical. In contrast, the FRAP method assesses the ability of a substance to reduce ferric (Fe^{3+}) to ferrous (Fe^{2+}) ions through the donation of an electron, resulting in the formation of ferrous ion (Fe^{2+}), which can then be measured to determine the antioxidant capacity of the tested substance (Shah & Modi, 2015). The plant samples might have contained more antioxidants that cannot reduce the Fe^{3+} to $Fe^{2+,}$ but their antioxidant activity is based on either hydrogen or electron donation to the DPPH radical. Such antioxidants include polyphenols and vitamin C (Santos-Sánchez *et al*., 2019). The DPPH is, therefore, the preferred method for the antioxidant assay of the *Agave* plants, as one would report the plants to have less antioxidant activity when only using the FRAP method. The FRAP antioxidant activity was low for all the samples; even at the highest tested concentrations, the observed Fe_2SO_4 concentrations were below 50%.

The DPPH antioxidant activity assay reported *that AA MF, AA ME, AS HF, and AS ME* contain less than 50% antioxidant activity. In contrast, *AA* HF, *AA* EAF, *AS* EAF and *AS* MF contained more than 50% DPPH antioxidant activity. Those with more than 50% DPPH have substantial antioxidant activity, while *AA* MF, *AA* ME, *AS* HF and *AS* ME showed little activity. Even though the DPPH had higher concentrations, generally, the results show a similar trend.

Munteanu reported that both the FRAP and the DPPH reactions yield the same outcomes, even though the kinetics and reaction stages are different, as these reactions depend on the solvent system, solubility and the structure and properties of the antioxidant compound (Munteanu & Apetrei, 2021). The results of the quantitative analysis for AA HF also showed a higher antioxidant assay than the DPPH antioxidant assay. The quantitative results for AAF reported a high total phenolic content, corresponding with the DPPH results, agreeing with the literature, which reported phenolic compounds as hydrogen donors to the DPPH radicals (Santos-Sánchez *et al*., 2019).

© Central University of Technology, Free State

As the FRAP had lower results compared to the DPPH method, the antioxidant compounds from these two plants must be reacting through either an electron or hydrogen atom transfer. However, determining the actual phytoconstituents accountable for the antioxidant activity is recommended to confirm this theory. Based on these antioxidant activity results, the *Agave angustifolia* should be extracted with non-polar or slightly polar solvents, while the *Agave sisalana* should be extracted with polar solvents. Therefore, this indicated that the two plants contain different groups of antioxidant phytochemicals. Now that the potential antioxidant activity of both *Agave* pants has been confirmed, antimicrobial and cytotoxicity profiles of the two *Agave* plants had to be investigated to verify the literature as well as to ensure the safety of *Agave* plants for cosmetic application, as required by the cosmetic legislation (CTFA, 2023; Gazette & Notice 1976, 2008, 2012; Pellevoisin *et al*., 2018; SANS16128, 2018).

© Central University of Technology, Free State

3.4 References

Amarowicz, R., & Pegg, R. B. (2019). Natural antioxidants of plant origin. *Advances in Food and Nutrition Research*, 1–81. https://doi.org/10.1016/bs.afnr.2019.02.011

Araldi, R. P., dos Santos, M. O., Barbon, F. F., Manjerona, B. A., Meirelles, B. R., de Oliva Neto, P., da Silva, P. I., dos Santos, L., Camargo, I. C. C., & de Souza, E. B. (2018). Analysis of antioxidant, cytotoxic and mutagenic potential of *Agave sisalana* Perrine extracts using Vero cells, human lymphocytes, and mice polychromatic erythrocytes. *Biomedicine & Pharmacotherapy*, *98*, 873–885. https://doi.org/10.1016/j.biopha.2018.01.022

Benzie, I. F. F., & Strain, J. J. (1996). The Ferric Reducing Ability of Plasma (FRAP) as a Measure of "'Antioxidant Power'": The FRAP Assay. *Analytical Biochemistry*, *239(1)*, 70–76.

Benzie, I. F. F., & Choi, S. W. (2014). Antioxidants in food: Content, measurement, significance, action, cautions, caveats, and research needs. *Advances in Food and Nutrition Research,* 71, 1–53. https://doi.org/10.1016/B978-0-12-800270-4.00001-8

CTFA. (2023). Bridging the Gap 2023. *Cosmetic compendium,* 1–407. www.ctfa.co.za

Gazette, G. (2017). Foodstuffs, Cosmetics, Disinfectants Act (54, 1972) Regulations_ draft v.Dec 2017. *Government Notice,* 41351, 11–349.

Gazette, G. (2012). Health Professions Act: Regulations: Defining scope of profession of environmental health officers: Amendment: Draft. *Government Notice,* 35354, 3–9.

Gazette, Government. (2008). Health Professions Act: Regulations: Scope of profession of environmental health: Amendment: Draft. *Government Notice,* 30722, 14–24.

Munteanu, I. G., & Apetrei, C. (2021). Analytical methods used in determining antioxidant activity: A review. *International Journal of Molecular Sciences,* 22(7). https://doi.org/10.3390/ijms22073380

Pellevoisin, C., Bouez, C., & Cotovio, J. (2018). Cosmetic industry requirements regarding skin models for cosmetic testing. *Skin Tissue Models for Regenerative Medicine,* 3–37. https://doi.org/10.1016/B978-0-12-810545-0.00001-2

© Central University of Technology, Free State

Pisoschi, A. M., & Negulescu, G. P. (2011). Methods for total antioxidant activity determination: a review. *Biochemistry & Analytical Biochemistry*, 1(1), 106.

Xiao, F., Xu, T., Lu, B., & Liu, R. (2020). Guidelines for antioxidant assays for food components. *Food Frontiers*, 1(1), 60–69. https://doi.org/10.1002/fft2.10

Zhang, A., Jiang, M., Zhang, J., Tan, M. & Hu, X., 2006, Mitogen-activated protein kinase is involved in abscisic acid-induced antioxidant defense and acts downstream of reactive oxygen species production in leaves of maize plants. *Plant Physiology*, 141(2), 475–487. https://doi.org/10.1104/pp.105.075416

Zhang, H., Chen, F., Wang, X., & Yao, H. Y. (2006). Evaluation of antioxidant activity of parsley (*Petroselinum crispum*) essential oil and identification of its antioxidant constituents. *Food Research International*, *39*(8), 833-839. https://doi.org/10.1016/j.foodres.2006.03.007

© Central University of Technology, Free State

CHAPTER 4 :
THE *IN VITRO* CYTOTOXICITY SCREENING OF DIFFERENT EXTRACTS FROM *AGAVE ANGUSTIFOLIA VAR. ANGUSTIFOLIA* AND *AGAVE SISALANA* LEAVES

4.1 Introduction to the *in vitro* cytotoxicity screening of different extracts from *Agave* leaves

Cytotoxicity is described as the capability of a compound to create a poisonous impact on a cell, and any compound or extract that is poisonous to a cell is said to be cytotoxic (Chavda & Socha, 2021). Cytotoxicity assessment is commonly used to screen therapeutic products and artificial organic mixtures. Screening plant extracts for their essential and relative toxicities before human use is imperative as this helps determine the potential harmful or toxic effects those extracts would pose to human health on application (Anywar *et al*., 2022). As all ingredients that are to be used in both cosmetic and pharmaceutical products are required to be safe for use, cytotoxicity studies are one of the critical tests that need to be conducted for each ingredient, which should either have minimal or no toxicity (Chavda & Socha, 2021). Cytotoxicity tests are employed to assess the capability of cells to remain growing in the existence of a test material after a defined time. When evaluating possible innovative remedies or creating novel remedies for a disorder, the imperative preliminary stage is evaluating their cytotoxic capability and establishing their detrimental properties. Several bioassays and diverse cell lines have been used to determine the cytotoxicity of African therapeutic plants (Chavda & Socha, 2021). When cells are treated with a cytotoxic mixture, the cells can lose membrane veracity and quickly die, or the cell viability can decline as the cells halt growing and aggressively dividing (Chavda & Socha, 2021; Gertsch, 2009).

The rapid colourimetric tetrazolium dye technique, usually called the MTT (3-(4,5-dimethylthazolk-2-yl)-2,5-diphenyl tetrazolium bromide) assay, is amongst the most common and suitable techniques to investigate the viable cell numbers in 24-well or 96-well microtiter plates (Konstantin & Engelhardt, 2017). This method is based on the cleavage of the tetrazolium ring of MTT through dehydrogenases in dynamic mitochondria of living cells as an estimation of viable cell numbers (van Meerloo &

© Central University of Technology, Free State

Kaspers, 2011). The universal aim of the MTT procedure is to determine viable cells in comparatively high throughput (96-well plates) with no necessity for excessive cell count.

Therefore, MTT is mainly used to assess the cytotoxicity of various drugs at diverse concentrations. The MTT test concept is that the mitochondrial action is constant for most viable cells, and therefore, change in the number of viable cells (increase or decrease) is directly correlated to mitochondrial action. This cell activity is demonstrated by changing the tetrazolium salt MTT into formazan crystals, which can be solubilised for standardized measurement. Therefore, any decrease or increase in viable cell quantity can be identified by determining the formazan quantity displayed in optical density (OD) using a plate reader at 540 and 720 nm. The OD values of wells with incubated drug-containing cells are compared to the OD values of wells with cells that were not subjected to drugs to measure the responsiveness of drugs.

For dividing cells, the decrease in cell number indicates the hindrance of cell growth (Chavda & Socha, 2021). This e-drug responsiveness is typically stated as the drug concentration needed to attain 50% growth hindrance, contrasted to the growth of the untreated control (50% inhibitory concentration, IC_{50}) (van Meerloo *et al*., 2011). This chapter discusses the MTT assay *in vitro* cytotoxicity screening of different extracts from *Agave angustifolia (AA)* and *Agave sisalana (AS)* leaves to assess the potential danger these solvent extracts pose or their safety in manufacturing cosmetic products.

4.2 Experimental

4.2.1 Sample preparation

The preparation of plant material and extractions are detailed in Chapter 2. The *Agave* extracts were reconstituted in dimethyl sulfoxide (DMSO), resulting in 100 mg/mL as the final concentration. Sonication was utilised for the samples with solubility problems, and the extracts were kept at 4 °C until the next step.

4.2.2 Treatment procedure

The cytotoxicity screening was performed using Vero cells, the African green monkey kidney cell line. The Vero cells were kept in 10 cm culture dishes at 37 °C in a

© Central University of Technology, Free State

humidified incubator with 5% CO_2. The comprehensive growth medium comprised Dulbecco's modification of Eagle medium (DMEM) enhanced with 10% fetal bovine serum (FBS). The cells were seeded into 96-well microtiter plates at 4000 cells/well density, using 100µL in each well. The microtiter plates were then incubated at 37 °C, 5% CO_2, and 100% comparative humidity for 24 hours before adding the *Agave* extracts to permit cell attachment.

For cytotoxicity assessment, the Vero cells were treated with 100 µL aliquots of the diluted *Agave* extract in the fresh medium at 12.5, 25, 50, 100 and 200 µg/mL concentrations, then incubated for 48 hrs. The treatment medium was aspirated from all wells and replaced with a medium containing 0.5 mg/mL MTT, then further incubated at 37 °C for 30 minutes. MTT was removed, and 200 µL DMSO was added to each well to dissolve the formazan crystals. Melphalan was used as a positive control, and the cells were treated with 5, 10, 20, 40 and 80 µM concentrations. Absorbance was read at 540 nm using a BioTek® PowerWave XS spectrophotometer (Winooski, VT, USA).

4.2.3 Data quantification

The data was quantified using Microsoft Excel, and the number of viable cells was considered a proportion of the average unprocessed control cell population after 48 hours of treatment.

4.3 Results and discussion

The cytotoxicity of *Agave* extract samples at varying concentrations against Vero cells was determined 48 hours after exposure to the reaction. Cell viability was estimated using the MTT cell viability assay (Figure 4.1). *AA*1 caused a decrease in cell viability at 12.5 –100 µg/mL; however, at 200 µg/mL, less cytotoxicity was evident than at the lower concentrations. *AA*2 and *AA*3 exhibited cytotoxicity, resulting in approximately 40% cell death. However, a significant dose dependency was not evident. *AA*4 also caused a decrease in cell viability but not below 70% and is thus considered not toxic at physiologically relevant concentrations. *AS*1, *AS*2, *AS*3 and *AS*4 were not cytotoxic.

© Central University of Technology, Free State

For the *Agave angustifolia,* the hexane fraction showed less cytotoxicity at the highest treatment concentration than at lower concentrations, which decreased the viable cells. This fraction can, therefore, be used as an anti-tumour in cancer studies at low concentrations, such as 12.5 to 25 µg/mL. The ethyl acetate and methanol fractions exhibited cytotoxicity, resulting in approximately 40% cell death; however, the results were not dependent on the used concentration (no clear dose dependency). These fractions could also be used for cancer studies at 12.5 to 200 µg/mL. The methanol extract showed some toxicity but was not below 70% and is therefore not cytotoxic. This fraction, thus, showed the potential to be used in the manufacture of cosmetic products. However, methanol is known to harm human health and is not allowed to manufacture cosmetic products (CTFA, 2023). The *Agave* plant extraction for cosmetic application should be done using ethanol, which is cosmetically acceptable.

For the *Agave sisalana* plant, no samples were found to be cytotoxic. Therefore, this plant can be extracted by both polar and non-polar solvents, which means that the plant can be either extracted by oil or ethanol, depending on the cosmetic application. The determination of cytotoxicity for the *Agave* extracts is depicted in Figure 4.1.

© Central University of Technology, Free State

Figure 4.1: Cytotoxicity of *Agave* extract samples at different concentrations against Vero cells after 48 hours of exposure

1-5 denotes increasing concentrations of the sample (12.5, 25, 50, 100 and 200 µg/mL for extracts and 5, 10, 20, 40 and 80 µM for melphalan). UC = Untreated control. Error bars show the standard deviation of quadruple values done as a single experiment. *AA*1 & *AS*1 = hexane fraction; *AA*2 & *AS*2 = ethyl acetate fraction. *AA*3 & *AS*3 = methanol fraction & *AA*4 & *AS*4 = methanol extract.

© Central University of Technology, Free State

4.4 References

Anywar, G. U., Kakudidi, E., Oryem-Origa, H., Schubert, A., & Jassoy, C. (2022). Cytotoxicity of Medicinal Plant Species Used by Traditional Healers in Treating People Suffering From HIV/AIDS in Uganda. *Frontiers in Toxicology*, 4,832780. https://doi.org/10.3389/ftox.2022.832780

Chavda, B., & Socha, B. N. (2021). Cytotoxicity Study. *In Cytotoxicity - New Insights into Toxic Assessment*, 1-8. https://doi.org/10.5772/intechopen.96875

Gertsch, J. (2009). How scientific is the science in ethnopharmacology? Historical perspectives and epistemological problems. *Journal of Ethnopharmacology*, *122*(2), 177–183. https://doi.org/10.1016/j.jep.2009.01.010

Präbst Konstantin and Engelhardt, H. and R. S. and H. H. (2017). Basic Colorimetric Proliferation Assays: MTT, WST, and Resazurin. In O. Gilbert Daniel F. and Friedrich (Ed.), *Cell Viability Assays: Methods and Protocols* (pp. 1–17). Springer. https://doi.org/10.1007/978-1-4939-6960-9_1

van Meerloo, J. K., Gertjan, J. L., & Cloos, J. (2011). Cell Sensitivity Assays: The MTT Assay. In I. A. Cree (Ed.), *Cancer Cell Culture: Methods and Protocols* (pp. 237–245). Humana Press. https://doi.org/10.1007/978-1-61779-080-5_20

© Central University of Technology, Free State

CHAPTER 5 :
DETERMINATION OF THE ANTIMICROBIAL ACTIVITY OF *AGAVE ANGUSTIFOLIA VAR. ANGUSTIFOLIA* AND *AGAVE SISALANA*

5.1 Introduction

Cosmetic products are substances or preparations (except a medicine) that tend to be applied to external parts of the human body for beautification, correcting body odours, changing appearance, etc. (CTFA, 2023; Gazette & Notice, 1976). Cosmetic products sold to the South African market and the European Union must tested for microbial contamination, as microbial safety ensures consumer safety (CTFA, 2023; SANS 11930, 2020). Therefore, the cosmetic industry utilizes preservatives to protect the products from microbial contamination. With the development of cosmetic products fortified with biologically active plant extracts, it has become imperative to check the microbial action of these extracts to ensure consumer safety, as per cosmetic regulations.

Diverse procedures can be employed to establish the antimicrobial action for any extract, such as the diffusion method and the micro broth dilution susceptibility method. The diffusion process is based on the rule that the reservoir having an extract is transported into interaction with an inoculated medium. The solute will disperse into the agar. The diameter of the area with no growth around the reservoir, after incubation, is determined with the ruler and regarded as the antimicrobial capability of that extract. Diffusion essays are proper for first evaluating contaminated constituents, such as alkaloids, flavonoids and terpenoids (Cowan, 1999; Nsele, 2019). Evaluating the zones of inhibition for natural products based on synthetic antibiotic disc assay is profitable for determining the susceptibility of the tested material. These estimations cannot produce a correlation between the antimicrobial effectiveness of the naturally tested substances and the synthetical antimicrobial agent (Nsele, 2019). This is based on several factors, such as diffusion capacity, which can affect the magnitude of the inhibition zones and result in ambiguous decisions. The ideal efficiency of the disc diffusion technique is therefore gained from using the Mueller-Hinton (MH) agar and

© Central University of Technology, Free State

homogenous microorganisms American Type Culture Collection (ATCC) (Nsele, 2019).

Another commonly used method is the broth microdilution assay method, where the bacterial propagation is determined by the turbidness of the solution, which is regarded as an unswerving relationship to the amount of microbial growth (Nsele, 2019). These examinations can generate the minimum inhibitory concentration for the antibacterial specimen. The dilution assessments are usually more complex, tedious, and costly to execute than the disc diffusion techniques and require a homogeneous specimen spreading (Balouiri *et al*., 2016). The micro broth dilution vulnerability procedure in a 96-well microtiter plate is ideal for assessing drug sensitivity based on its high throughput rate, low cost, and tiny sample requirements (Baser & Buchbauer, 2015).

Another method that could be used for drug-susceptibility testing is a Microplate Alamar Blue assay (MABA), also known as the CellTiter-Blue® assay. This is a single-addition, regular assay that assesses cell capability using a redox indicator dye resazurin (also referred to as CellTiter-Blue® or Alamar blue) to determine the cell's metabolic capability, a display of their viability. The viable cells maintain the capacity to reduce resazurin (dark blue) into resorufin (pink), which is exceptionally incandescent. The pink colour indicates microbial growth, and the colour variation can be inspected and measured fluorometrically by excitation at 530 nm and transmission at 590 (Cowan, 1999; Baser & Buchbauer, 2015). The nonviable cells quickly lose metabolic capability and do not produce the incandescent signal as they do not reduce the indicator dye. This method allows users to determine changes using either absorbance or incandescence (preferred).

This chapter, therefore, discusses the assessment of the antimicrobial action of solvent extracts from the leaves of *Agave angustifolia* (*AA*) and *Agave sisalana* (*AS*) against selected common Gram-negative and Gram-positive bacteria and fungi to confirm literature, as well as investigate the potential use of the two *Agave* plants for cosmetic purposes.

© Central University of Technology, Free State

5.2 Experimental

5.2.1 Preparation of culture media

To prepare the Mueller-Hinton (M/H) agar, 38 g M/H agar was weighed and transferred into one-litre glass bottles, adding distilled water to the mark, using a measuring cylinder, and then mixing to dissolve the powder completely. This was done in triplicate, and the bottles were sterilised by autoclaving at 121 ˚C for 15 minutes. The bottles were removed from the autoclave and cooled to about 55 ˚C, then the agar was poured into plates to solidify and then conserved at 4 ˚C until use.

The nutrient broth was prepared by weighing 40 g powder into a one-litre glass bottle, then made to the mark by diluting it with distilled water, which was mixed to dissolve the powder utterly. The nutrient broth solution was then transferred into bijou bottles before autoclaving. The solution was sterilised by autoclaving at 121 ˚C for 15 minutes, cooled to room temperature and stored at 4 ˚C until use.

One (1) litre of deionized water was used to dissolve 111 g mannitol salt agar, which was frequently shaken and heated to boil for one minute until the powder dissolved completely. Then, the solution was autoclaved to sterilize for 15 minutes at 121 °C.

One (1) litre of distilled water was used to dissolve 37 g broth powder, which was frequently shaken and heated to boil for one minute until the powder dissolved completely. Then, the solution was transferred into final containers and autoclaved to sterilize for 15 minutes at 121 °C.

The blood agar was prepared by adding 40 grams to 1000 mL of deionized water. The solution was obtained by heating the dispersion to boiling and then autoclaving to sterilize for 15 minutes at 121 °C.

The solution was removed from the autoclave and cooled to about 40-45 ˚C. Then, 5% v/v of sterile defibrinated blood was aseptically added with vigorous mixing. The blood agar was then transferred into sterile petri plates, solidified under sterilised conditions, and maintained at 4 °C until use.

5.2.2 The growth conditions of the media and microorganisms

Klebsiella pneumoniae and *Staphylococcus aureus* were initially grown on selective Mannitol salt agar and MacConkey agar. The Mueller-Hinton agar plates were used to

© Central University of Technology, Free State

obtain overnight streak plates of the selected organisms, such as *Enterococcus faecalis* (*E. faecalis*), *Escherichia coli* (*E. coli*), *Klebsiella pneumoniae (K. pneumoniae*), *Pseudomonas aeruginosa (P. aeruginosa*) and *Staphylococcus aureus* (*S. aureus*), and the bacterial strains were grown at 37 °C.

Streptococcus pneumoniae (S. pneumoniae) and *Streptococcus pyogenes (S. pyogenes)* were grown on blood agar plates, while *Candida albicans* were grown on Malt extract agar. One (1) microorganism colony, obtained from an overnight streak plate, was inoculated in broth (10 mL) and incubated at 37 °C for 16 hours (log growth phase). The microorganisms *E. faecalis, S. aureus, E. coli, P. aeruginosa, and K. pneumoniae* were grown in Mueller-Hinton (MH) broth (Merck, USA). The *S. pneumoniae* and *S. pyogenes* were grown in the Brain-Heart Infusion broth, and the *C. albicans* were grown in the Malt extract broth.

The positive controls (control drugs) against Gram-negative and Gram-positive bacteria were gentamicin sulfate and vancomycin hydrochloride (Sigma, USA), respectively. For *C. albicans*, Fluconazole (Sigma, USA) was used as a positive control. These broad-spectrum antibiotics (control drugs) were chosen due to their effectiveness against the Gram-negative (gentamycin) and Gram-positive (vancomycin) microorganisms.

Two (2) mg/mL stock solutions of the control drugs were prepared in distilled water and filter sterilized (0.2 µM filter). The fluconazole (5 mg/mL stock solution) was dissolved in DMSO, and broth was used to dilute the working concentrations.

5.2.3 Sample preparation

The stock solutions (100 mg/mL) were prepared by dissolving samples in DMSO. The working concentrations of the samples were prepared in MH broth/ Brain-Heart infusion broth/ Malt extract broth. The stock solution of 100 mg/mL was prepared in DMSO, and the working solutions were obtained from diluting with broth (Muller-Hinton/ Brain-Heart infusion broth/ Malt extract broth), depending on the organism.

© Central University of Technology, Free State

5.2.4 Bacterial sensitivity testing (screening) of plant extracts

A slightly modified agar diffusion assay was used, as proposed by Nsele (2009). Using a sterile swab, an inoculum containing 1 x 106 colony-forming units (CFU) /mL was inoculated through even distribution on the Mueller-Hinton (MH) Agar plates. The inoculums were prepared by comparing the bacterial suspension with one MacFarland turbidity standard, as reported by Nsele (2009).

Sequential dilutions were made for the antibiotics (Vancomycin/Gentamycin: 2 to 0.5 µg/mL and Fluconazole: 1 to 0.25 mg/mL). The cultures were evaluated and adjusted to 0.5 McFarland standard (absorbance at 600 nm = 0.08 - 0.1; equivalent to about 1.5 x108 cells/mL), and 50 µL was added to each test well. The following controls were made as antibiotic/medium control (50 µL broth + 50 µL of maximum antibiotic). Sample colour control (50 µL broth + 50 µL of maximum sample); Microorganism/Growth control (50 µL broth + 50 µL microorganism). The plates were wrapped with microplate sealing tape and incubated for 24 hours at 37 °C.

5.2.5 Micro broth dilution method to investigate MIC values of active samples

MH broth/ Brain-Heart infusion broth or Malt extract broth (50 µL) was included in all test wells (i.e., sample and antibiotics), except for the maximum sample concentration and antibiotic concentration wells, to which 50 µL of the working concentrations were added. Serial dilutions were made for the samples (based on initial screening results) and antibiotics (Vancomycin/gentamicin: 64 to 0.25 µg/mL). The cultures were checked and corrected to 0.5 McFarland standard (absorbance at 600 nm = 0.08-0.1, corresponding to about 1.5 x108 cells/mL), and 50 µL was added to each test well. The following controls were made: The antibiotic/ medium control (50 µL broth + 50 µL maximum antibiotic strength), sample colour control (50 µL broth + 50 µL of maximum sample strength) and the microorganism/ growth control (50 µL broth + 50 µL microbe). The plates were wrapped with microplate sealing tape and incubated at 37 °C for 24 hours.

© Central University of Technology, Free State

5.2.6 CellTiter-Blue® assay (Microplate Alamar blue assay; MABA)

After sample treatment, 20 µL of CellTiter-Blue® was introduced into each well and incubated for one (1) hour. The CellTiter-Blue® was made from dissolving resazurin sodium salt in deionized water (700 µM; Sigma-Aldrich [St. Louis, MO, USA]). The wells were checked for colour change, where a blue colour signified no growth (non-viable cells) and a pink colour denoted growth (viable cells). The fluorescence was read at 560 nm (excitation) and 590 nm (emission) wavelengths, respectively, utilizing a BioTek® SYNERGY Mx fluorometer (Winooski, VT, USA).

The fluorescence measurements of 96-well plates were determined at 560 nm (excitation) and 590 nm (emission) wavelengths after adding CellTiter-Blue®. The percentage inhibition for the samples was estimated as follows:

1-[Fluorescence at 560/590 nm of the tested sample (fluorescence corrected by detracting the fluorescence generated by the sample)/ Fluorescence at 560/590 nm microorganism growth control] *100.

5.3 Results and discussion

The *Agave* sample extracts were denoted *AA* for *Angustifolia, and AS* for *sisalana,* where numbers 1 to 3 represented hexane, ethyl acetate and methanol fraction, respectively, and the number 4 was the methanol extract, *AS*-1, *AS*-2, and *AA*-2 showed antimicrobial action against *E. faecalis* with MIC values of 0.5 mg/mL, and *AA*-1 showed action with a MIC value of 0.25 mg/mL; *AS*-1, *AA*-1 and *AA*-2 showed antimicrobial activity against *S. pyogenes*, where the MIC of *AS*-1 (0.25 mg/mL), *AA*-1 (0.125 mg/mL) and *AA*-2 (0.5 mg/mL). *AS*-1, *AS*-2, *AA*-1, *AA*-2, and *AA*-4 exhibited antimicrobial activity against *S. pneumoniae*, with the following MICs: *AS*-1 showed - (0.0625 mg/mL), *AS*-2 (0.25 mg/mL), *AA*-1 (≤ 0.031 mg/mL), *AA*-2 (0.0625 mg/mL) and *AA*-4 (0.5 mg/mL).

All extracts were not effective against the Gram-negative bacteria (*E.coli, K. pneumoniae and P. aeruginosa*), Gram-positive bacteria (*S. aureus*) and fungus *(C. albicans*) at the maximum treatment strength of 2 mg/mL. The minimum inhibitory concentrations (MIC) of the screened *Agave* samples and the used antibiotics (positive controls) are shown in Tables 5.1 and 5.2.

© Central University of Technology, Free State

For the current study, the discussion will focus more on skin-related bacterial strains as we investigate the potential of *Agave* extracts in manufacturing cosmetic products. The other microbial strains related to ingestion will be considered in the later studies where internal use of the Agave plants will be investigated, as they are some of the common strains tested in medicinal plant extracts.

The antimicrobial results indicated that the hexane and ethyl acetate extract fractions of *Agave Angustifolia*, along with the hexane fraction of the *Agave Sisalana* extract, had the potential to hinder the microbial growth of the Gram-positive bacteria, *Streptococcus pyogenes. S. pyogenes* causes several infectious diseases, such as mild, rapid infections of the throat (pharyngitis, or 'strep throat') and impetigo (skin sores) (Barnett *et al*., 2019; García-Solache & Rice, 2019; Olafsdottir *et al*., 2014). Impetigo is an itchy, sometimes painful, and highly contagious bacterial infection of the skin that is most common in young children but can affect people of all ages. The infection can either be through an insect bite, a broken skin, such as a cut, or an injury and may lead to cellulitis. The infected skin develops sores and blisters, which can also be caused by *Staphylococcus aureus* (*S. aureus*) (Darmstadt & Lane, 1994; Pereira, 2014). Cellulitis is a common and potentially severe skin contamination caused by bacteria. It causes pain, swelling, and redness, sometimes hot and tender, in the infected area, which can rapidly spread, causing severe bacterial skin infection if not treated (Bystritsky, 2021; Gupta, 2018; Raff & Kroshinsky, 2016).

The hexane and ethyl acetate extract fractions of *Agave angustifolia* and the hexane fraction of the *Agave sisalana* extract also showed potential bacterial activity against *Streptococcus pneumoniae* (*S. pneumoniae*), and *Enterococcus faecalis (E. faecalis),* a ubiquitous Gram-positive non-spore-forming bacteria, usually found in the gastrointestinal tract. It also acts as an opportunist pathogen and translocates across the mucosal barrier, causing infections ranging from superficial to more severe human infections, such as wound infections, urinary tract infections, and bacteremia (fungal infection of the blood) (Bolocan *et al*., 2019; García-Solache & Rice, 2019), therefore indicating the potential use of non-polar and slightly polar solvent extracts in the production of cosmetic products intended to reduce the appearance of skin sores and cellulitis caused by the infection with the *S*. *pyogenes* and *E. faecalis* bacteria. These extracts could also manufacture medicinal products targeting the sinuses and the

© Central University of Technology, Free State

nasal cavity. The hexane fraction from the *Agave angustifolia* Haw. var. *angustifolia* had the lowest extract concentration required for the bacterial sensitivities.

Pseudomonas aeruginosa (P. aeruginosa) is a disease-causing microorganism. It is a Gram-negative bacterium that can readily be found in almost any human/animal-impacted environment and does not cause any threat to the skin unless the skin is broken, in which case, it can cause disease, such as folliculitis, an infection of one or more follicles, resulting in an inflammation that can be caused by either *P. aeruginosa*, *Staphylococcus aureus (S. aureus)* or both. Its symptoms include pain, itching, or burning sensations and are characterised by pustules, bleeding, and crusts that can be found in the body, face, and hair scalp (Durdu & Ilkit, 2013; Otberg *et al*., 2008). *S. aureus* is also responsible for Impetigo, cellulitis, and skin abscesses, which are common in atopic dermatitis (Hulshof *et al*., 2018; Murota *et al*., 2018). Further investigations are essential to investigate the minimum quantity of the selected *Agave* extracts required to hinder the growth of different microorganisms, especially those related to skin infections, such as *Candida albicans*, *P. aeruginosa* and *Klebsiella pneumoniae.*

Candida albicans is a yeast fungus usually found on skin, gastrointestinal, and genital mucosal surfaces. It is responsible for invasive fungal infections, such as candidiasis (Alshanta *et al*., 2022; Talapko *et al*., 2021). When it affects the vagina, it may be referred to as a yeast infection or thrush. It can be avoided by maintaining good oral hygiene and clean, dry, odour-free skin (Ho *et al*., 2021; Lopes & Lionakis, 2022; Talapko *et al*., 2021). Plant extracts that are effective against *C. albicans* could be used as antifungal actives in cosmetic manufacture, especially in products that contain water, which acts as a growth medium for antimicrobials.

Klebsiella pneumoniae (K. pneumonia) is reported to cause skin, wound or surgical site infections, bloodstream infections, and meningitis. Bacteria can also be transported from person to person or when someone touches a contaminated surface, but the bacteria do not spread through the air (Ahmad *et al*., 2018).

Escherichia coli (*E. coli*) The potential sources of exposure include person-person contact and contaminated water. The activity against this bacterium will be investigated further in studies intended for internal consumption. The MICs for the

© Central University of Technology, Free State

extracts of *Agave* leaves against selected microorganisms are shown in Tables 5.1 and 5.2.

© Central University of Technology, Free State

Central University of Technology, Free State

Table 5.1: MIC values for *Agave* leaf extracts screened against both Gram-positive and Gram-negative bacteria

Sample	*E. faecalis*	*S. aureus*	*S. pneumoniae*	*S. pyogenes*	*E. coli*	*K. pneumoniae*	*P. aeruginosa*	*C. albicans*
AA-1	0.25 mg/mL	> 2 mg/mL	≤ 0.031 mg/mL	0.125 mg/mL	> 2 mg/mL	> 2 mg/mL	> 2 mg/mL	> 2 mg/mL
AA-2	0.5 mg/mL	> 2 mg/mL	0.0625 mg/mL	0.5 mg/mL	> 2 mg/mL	> 2 mg/mL	> 2 mg/mL	> 2 mg/mL
AA-3	> 2 mg/mL	> 2 mg/mL	> 2 mg/mL	> 2 mg/mL	> 2 mg/mL	> 2 mg/mL	> 2 mg/mL	> 2 mg/mL
AA-4	> 2 mg/mL	> 2 mg/mL	0.5 mg/mL	> 2 mg/mL	> 2 mg/mL	> 2 mg/mL	> 2 mg/mL	> 2 mg/mL
AS-1	0.5 mg/mL	> 2 mg/mL	0.0625 mg/mL	0.25 mg/mL	> 2 mg/mL	> 2 mg/mL	> 2 mg/mL	> 2 mg/mL
AS-2	0.5 mg/mL	> 2 mg/mL	0.25 mg/mL	> 2 mg/mL	> 2 mg/mL	> 2 mg/mL	> 2 mg/mL	> 2 mg/mL
AS-3	> 2 mg/mL	> 2 mg/mL	> 2 mg/mL	> 2 mg/mL	> 2 mg/mL	> 2 mg/mL	> 2 mg/mL	> 2 mg/mL
AS-4	> 2 mg/mL	> 2 mg/mL	> 2 mg/mL	> 2 mg/mL	> 2 mg/mL	> 2 mg/mL	> 2 mg/mL	> 2 mg/mL

Table 5.2: MIC values for used antibiotics

Microorganism	Positive control	MIC (µg/mL)
Enterococcus faecalis	Vancomycin hydrochloride	2 µg/mL
S. aureus	Vancomycin hydrochloride	2 µg/mL
S. pneumoniae	Vancomycin hydrochloride	≤ 0.5 µg/mL
S. pyogenes	Vancomycin hydrochloride	1 µg/mL
E. coli	Gentamicin sulfate	1 µg/mL
K. pneumoniae	Gentamicin sulfate	1 µg/mL
P. aeruginosa	Gentamicin sulfate	1 µg/mL
C. albicans	Fluconazole	0.5 mg/mL

© Central University of Technology, Free State

5.4 References

Ahmad, Nafees., Alspaugh, J. Andrew., Drew, W. Lawrence., Lagunoff, Michael., Pottinger, Paul., Reller, L. Barth., Reller, Megan. E., Sterling, Charles. R., & Weissman, S. (2018). *Sherris medical microbiology.* (K. J. Ryan, Ed.; 4th ed., Vol. 1). McGraw Hill Education. https://booksca.ca/wp-content/uploads/XPreview/Pharmacology/3/sherris-medical-microbiology-7th-edition-by-kenneth-j-ryan.pdf

Alshanta, O. A., Albashaireh, K., McKloud, E., Delaney, C., Kean, R., McLean, W., & Ramage, G. (2022). *Candida albicans* and *Enterococcus faecalis* biofilm frenemies: When the relationship sours. *Biofilm*, *4,100072*. https://doi.org/10.1016/j.bioflm.2022.100072

Balouiri, M., Sadiki, M., & Ibnsouda, S. K. (2016). Methods for in vitro evaluating antimicrobial activity: A review. *Journal of Pharmaceutical Analysis,* 6(2), 71–79. https://doi.org/10.1016/j.jpha.2015.11.005

Barnett, T. C., Bowen, A. C., & Carapetis, J. R. (2019). The fall and rise of Group A Streptococcus diseases. *Epidemiology and Infection*, 147(e4), 1-6. https://doi.org/10.1017/S0950268818002285

Bolocan, A. S., Upadrasta, A., De Almeida Bettio, P. H., Clooney, A. G., Draper, L. A., Ross, R. P., & Hill, C. (2019). Evaluation of phage therapy in the context of enterococcus faecalis and its associated diseases. *Viruses*, *11*(4), 366. https://doi.org/10.3390/v11040366

Bystritsky, R. J. (2021). Cellulitis. *Infectious Disease Clinics of North America*, *35*(1), 49–60. https://doi.org/10.1016/j.idc.2020.10.002

Catterall, J. R. (1999). Streptococcus pneumoniae. *Thorax,* 54(10), 929–937. https://doi.org/10.1136/thx.54.10.929

Cowan, M. M. (1999). Plant Products as Antimicrobial Agents. *Clinical Microbiology Reviews*, *12*(4), 564–582. https://doi.org/10.1128/CMR.12.4.564

CTFA. (2023). Bridging the Gap 2023. *Cosmetic compendium,* pp. 1–407. www.ctfa.co.za

Darmstadt, G. L., & Lane, A. T. (1994). Impetigo: An Overview. *Pediatric Dermatology*, *11*(4), 293–303.

© Central University of Technology, Free State

Durdu, M., & Ilkit, M. (2013). First step in the differential diagnosis of folliculitis cytology. *Critical Reviews in Microbiology*, *39*(1), 9—25. https://doi.org/10.3109/1040841x.2012.682051

García-Solache, M., & Rice, L. B. (2019). The *Enterococcus*: A model of adaptability to Its environment. *Clinical Microbiology Reviews*, *32*(2), 10-1128.

Gazette, G. (2012). Health Professions Act: Regulations: Defining scope of profession of environmental health officers: Amendment: Draft. *Government Notice,* 35354, 3–9.

Gazette, G. (2017). Foodstuffs, Cosmetics, Disinfectants Act (54, 1972) Regulations_ draft v.Dec 2017. *Government Notices,* 41351, 11–349.

Gazette, Government. (2008). Health Professions Act: Regulations: Scope of profession of environmental health: Amendment: Draft. *Government Notice,* 30722, 14–24.

Gertsch, J. (2009). How scientific is the science in ethnopharmacology? Historical perspectives and epistemological problems. *Journal of Ethnopharmacology*, *122*(2), 177–183. https://doi.org/10.1016/j.jep.2009.01.010

Gupta, R. (2018). Cellulitis. *PET/MR Imaging*, 33–34. https://doi.org/10.1007/978-3-319-65106-4_13

Ho, J., Camilli, G., Griffiths, J. S., Richardson, J. P., Kichik, N., & Naglik, J. R. (2021). *Candida albicans* and candidalysin in inflammatory disorders and cancer. *Immunology*, *162*(1), 11–16. https://doi.org/10.1111/imm.13255

Hulshof, L., Overbeek, S. A., Wyllie, A. L., Chu, M. L. J. N., Bogaert, D., de Jager, W., Knippels, L. M. J., Sanders, E. A. M., van Aalderen, W. M. C., Garssen, J., van't Land, B., & Sprikkelman, A. B. (2018). Exploring immune development in infants with moderate to severe atopic dermatitis. *Frontiers in Immunology*, *9*(630), 1-11. https://doi.org/10.3389/fimmu.2018.00630

Husnu Can Baser, K., & Buchbauer, G. (2010). *Handbook of Essential Oils: Science, Technology, and Applications*, 2, 1–994.

Lopes, J. P., & Lionakis, M. S. (2022). Pathogenesis and virulence of Candida albicans. In *Virulence*, 13(1), 89-121. https://doi.org/10.1080/21505594.2021.2019950

© Central University of Technology, Free State

Monte, J., Abreu, A., Borges, A., Simões, L., & Simões, M. (2014). Antimicrobial Activity of Selected Phytochemicals against *Escherichia coli* and *Staphylococcus aureus* and Their Biofilms. *Pathogens*, *3*(2), 473–498. https://doi.org/10.3390/pathogens3020473

Murota H., Yamaga K., Ono E., & Katayama I. (2018). Sweat in the pathogenesis of atopic dermatitis. *Allergology International*, *67*(4), 455–459.

Nsele, N. Wiseman., & Adam, J. K. (2019). Synergistic Effects of Plant Extracts and Penicillins on *Staphylococcus aureus* and *Enterococcus faecalis,* 1-206.

Olafsdottir, L. B., Erlendsdóttir, H., Melo-Cristino, J., Weinberger, D. M., Ramirez, M., Kristinsson, K. G., & Gottfredsson, M. (2014). Invasive infections due to *Streptococcus Pyogenes*: Seasonal variation of severity and clinical characteristics, Iceland, 1975 to 2012. *Eurosurveillance*, *19*(17), 20784. https://doi.org/10.2807/1560-7917.ES2014.19.17.20784

Otberg, N., Kang, H., Alzolibani, A. A., & Shapiro, J. (2008). Folliculitis decalvans. *Dermatologic Therapy*, *21*(4), 238–244. https://doi.org/10.1111/j.1529-8019.2008.00204.x

Pereira, L. B. (2014). Impetigo - Review. In *Anais Brasileiros de Dermatologia*, 89(2), 293-299. https://doi.org/10.1590/abd1806-4841.20142283

Raff, A. B., & Kroshinsky, D. (2016). Cellulitis: a review. *JAMA - Journal of the American Medical Association,* 316(3). https://doi.org/10.1001/jama.2016.8825

SANS 17516. (2023). CTFA Standards - SANS 17516_2020 – Cosmetics – Microbiology – Microbiological limits. *Cosmetic compendium, 1-407*. www.ctfa.co.za

SANS 11930. (2020). CTFA Standards - Cosmetics – Microbiology – Evaluation of the Antimicrobial Protection of a Cosmetic Product, *Cosmetic compendium,* 1-407. www.ctfa.co.za

SANS 16128-1. (2018). CTFA Standards - Guidelines on Technical Definitions and Criteria for Natural and Organic Cosmetic Ingredients and P, Cosmetic compendium 1-407. www.ctfa.co.za

SANS 17516. (2020). CTFA Standards – Cosmetics – Microbiology – Microbiological Limits, *Cosmetic compendium*, 1-407. www.ctfa.co.za

© Central University of Technology, Free State

Talapko, J., Juzbašić, M., Matijević, T., Pustijanac, E., Bekić, S., Kotris, I., & Škrlec, I. (2021). Candida albicans—The Virulence Factors and Clinical Manifestations of Infection. *Journal of Fungi*, *7*(2), 79. https://doi.org/10.3390/jof7020079

Vogt, R. L., & Dippold, L. (2005). *Escherichia coli* O157:H7 Outbreak associated with consumption of ground beef, June–July 2002. *Public Health Reports*, *120*(2), 174–178. https://doi.org/10.1177/003335490512000211

© Central University of Technology, Free State

CHAPTER 6 :

COMPARATIVE INVESTIGATION OF THE PHYTOCHEMICAL PROFILE AND BIOACTIVITIES OF *AGAVE ANGUSTIFOLIA HAW. VAR. ANGUSTIFOLIA* AND *AGAVE SISALANA*

6.1 Introduction

This study evaluated the solvent extracts from A. angustifolia (AA) and A. sisalana (AS) for cosmetics enrichment's qualitative and quantitative phytochemical profiles. The *AA* and *AS* bioactivities were investigated through the antioxidant, antimicrobial and cytotoxicity studies. The antioxidant activity was determined using the DPPH and FRAP assays. The antimicrobial studies were analysed using Gram-positive and Gram-negative bacteria and fungus, while the cytotoxicity was investigated using the MTT assay. These results would help guide the decision on whether these plants should be used in cosmetics as they should be following the legislation, which requires that the cosmetic ingredients be not toxic to human skin, have a microbial load of not more than 100 colony-forming units (cfu) (CTFA, 2023; Gazette & Notice 1976, 2008, 2012; Pellevoisin *et al*., 2018; SANS16128, 2018).

6.2 Comparative phytochemical and bioactivity profiles for *A. angustifolia* and *A. sisalana*

The results indicated that both plants contained flavonoids, tannins, phenolics, terpenoids, saponins and glycosides in the hexane fraction, while other fractions had different phytochemicals per plant. The alkaloids were only detected in *A. sisalana* extracts, and none of the *A. angustifolia* solvent extracts contained alkaloids. Steroids were only detected in the *A. angustifolia* methanol extract.

For *A. sisalana,* only the hexane fraction did not have alkaloids, while steroids were only detected in the methanol extract. In quantitative UV analysis, the TPC was found to be higher in all *A. sisalana* extracts except for the methanol extract, compared to *A. angustifolia* extracts.

© Central University of Technology, Free State

The DPPH antioxidant assay method reported higher antioxidant activity in *A. angustifolia* than in *A. sisalana* extracts, except for methanol extract fraction.

Agave angustifolia Haw*. var*. *angustifolia* indicated more antimicrobial activity against the *S*. *pyogenes* (in the hexane and ethyl acetate extract fractions), *S*. *pneumoniae* (ethyl acetate fraction extract and methanol extract) and *E. faecalis* (hexane fraction), while the *Agave sisalana* Perrine showed more antimicrobial activity than the *Agave angustifolia* Haw. var. *angustifolia* against *S*. *pneumoniae* (ethyl acetate fraction). Notable differences were observed when comparing the phytochemical screening and the UV quantification of the tested extracts, such as when the UV results reported more tannins in the *Agave angustifolia* Haw*. var*. *angustifolia* ethyl acetate extract fraction than in *A. sisalana*, while the qualitative phytochemical screening had not detected the tannins from the *Agave angustifolia* Haw*. var*. *angustifolia* but only from the *Agave sisalana* Perrine. The methanol fraction phytochemical screening also reported results opposite to the UV measurements.

The comparative phytochemical and bioactivity profiles for *AA* and AS are shown in Table 6.1.

© Central University of Technology, Free State

Table 6.1: The comparative phytochemical and bioactivity profiles for *A. angustifolia* and *A. sisalana*

	Agave angustifolia* Haw. *var. angustifolia				***Agave sisalana* Perrine**			
	Hexane extract fraction	Ethyl acetate extract fraction	Methanol extract fraction	Methanol extract	Hexane extract fraction	Ethyl acetate extract fraction	Methanol extract fraction	Methanol extract
Phytochemical contents	Flavonoids Tannins Phenolics Terpenoids Saponins Glycosides	Flavonoids Saponins Glycosides	Flavonoids Tannins Terpenoids Saponins Glycosides	Flavonoids Tannins Phenolics Terpenoids Saponins Steroids Glycosides	Flavonoids Tannins Phenolics Terpenoids Saponins Glycosides	Flavonoids Tannins Phenolics Saponins Glycosides Alkaloids	Flavonoids Phenolics Terpenoids Saponins Glycosides Alkaloids	Flavonoids Tannins Phenolics Saponins Steroids Glycosides Alkaloids
Total phenolic content	305,7	771	1211	771	22410	7311	1261	691
Vitamin C	0,05	0,07	0,01	0,09	0,19	0,03	0,01	0,13
Antioxidant activity (%DPPH scavenged)	>50%	>50%	<50%	<50%	<50%	>50%	<50%	>50%
Microbial activity (MIC in mg/mL)	*E. faecalis* (0.25); *S. pyogenes* (0.125); *S.* pneumoniae (≤ 0.031)	*E. faecalis* (0.5); *S. pyogenes* (0.5); *S.* pneumoniae (0.0625)	None	*S. pneumoniae* (0.5)	*E. faecalis* (0.5); *S. pyogenes* (0.25); *S.* pneumoniae (0.0625)	*E. faecalis* with (0.5); *S.* pneumoniae (0.25)	None	None
Cytotoxicity	Decrease in cell viability (12.5 – 100 µg/mL), but less cytotoxicity at 200 µg/mL.	40 % cell death, no dose-dependency.	40 % cell death, no evident significant dose dependency.	Not considered toxic at physiologically relevant concentrations.	not cytotoxic.	not cytotoxic,	not cytotoxic,	not cytotoxic

© Central University of Technology, Free State

6.3 Conclusion and recommendations

The ambiguity of different secondary metabolites in a therapeutic plant indicates a variety of biological applications (Alamgir, 2018). All tested plant extracts contained flavonoids, saponins and glycosides. Flavonoids indicate a potential antioxidant, anti-ageing, anti-inflammatory and broad-spectrum microbial activity. Therefore, the investigated plant extracts can be used as antioxidants, anti-ageing, anti-inflammatory, and for broad-spectrum microbial activity. The methanolic fraction from both plants showed the potential to be used for emulsifying, antioxidant, anti-ageing, antifungal, anti-inflammatory, astringent, antiseptic, anti-tumour, and antimicrobial activities due to the presence of reported phytochemicals.

The *Agave Angustifolia* hexane and ethyl acetate extract fractions and *Agave sisalana* ethyl acetate and methanol extract fractions have the potential to be used in cosmetics for moisturizing, anti-ageing, and UV protection, as they have substantial antioxidant activity.

Epidermal degeneration, or physiological ageing of the skin, is a growing concern among adults. The cause of skin deterioration is majorly attributed to harmful environmental conditions, such as UV radiations from sunlight, which produce reactive oxygen species (ROS) that trigger inflammation and consequent accelerated physiological ageing and epidermal deterioration (Scapagnini *et al*., 2014). Therefore, the Agave angustifolia leaves can be used for cosmetic products that claim moisturizing, UV protection, and anti-ageing properties when extracted with a cosmetically approved oil that will not interfere with the plant's properties. Laboratory trials will have to be done to determine the best oil. It is proposed that further studies be conducted on these plants to identify the current antioxidant phytoconstituents. Cytotoxicity studies will have to be done on those phytochemicals to ensure their safety in cosmetic products, as required by cosmetic legislation and the environmental health scope of practice and the cosmetic legislation (CTFA, 2023; Gazette & Notice 1976, 2008, 2012; SANS16128, 2018).

Hexane fractions from both plants could inhibit microbial infection from *S. pyogenes* at 0.125 mg/mL (*A. angustifolia)* to 0.25 mg/mL (*A. sisalana*) concentrations. The ethyl acetate fractions had a MIC of 0.5 mg/mL (*A. angustifolia*) and >2 mg/mL (*A. sisalana*). The non-polar extract showed higher activity than the polar ones, with the *AA* requiring

© Central University of Technology, Free State

the lowest concentration. These results, therefore, suggest the potential use of oil-extracted *Agave* leaves from both plants in the manufacture of cosmetic products intended for use in the reduction of the appearance of symptoms associated with *S. pyogenes* infections, which are common amongst children, especially from rural areas and informal settlements, where many children play together, at times without access to medical facilities and clean water. Further studies are recommended to evaluate the actual MIC above the treatment concentration of 2 mg/mL used in this study, as well as to determine other bacterial strains that the Agave extracts can inhibit. Isolation and characterisation of the active compounds responsible for these microbial activities are recommended.

The cytotoxicity of the plant indicates the existence of antitumor compounds in the plant extracts. The crude extract that exhibits an LC_{50} value below 250 µg/mL indicates that the extract is significantly active and could be further investigated (Alamgir, 2018). The *Agave angustifolia* methanol extract showed some toxicity but not below 70% and is therefore not considered cytotoxic. This fraction showed the potential to be used to manufacture cosmetic products. However, methanol is not allowed for use in cosmetic products (CTFA, 2023; Gazette & notice 1976; 2012), and it is therefore, these Agave leaves should be extracted with a cosmetic-grade solvent, such as ethanol. Hence, *Agave angustifolia* leaves can be used in cancer studies to reduce the number of viable cancer cells, as informed by the cytotoxicity results. All *Agave sisalana* plant extracts were found not to be cytotoxic. Therefore, this plant can be used to manufacture cosmetic products.

Steroids are reportedly used as a starting material in manufacturing corticosteroids, such as hydrocortisone (cortisol), a medication that reduces inflammation when applied topically to the human body, as it controls redness, swelling, and itching. The cortisol alleviates rashes and treats extremely dry skin conditions like eczema. The results indicated the presence of steroids, flavonoids, phenolics, vitamin C, tannins and terpenes associated with anti-inflammatory, antioxidant, antiseptic and antimicrobial properties. The antimicrobial studies indicated the actual microbial strains that the *Agave* leaf extract showed the potential to inhibit. Therefore, the Agave leaf extracts from both plants indicate their potential medical use in replacing corticosteroids, as the plant extracts would provide a safer option. However, additional

© Central University of Technology, Free State

investigations are required to establish the actual concentrations of the plant extracts that will produce the necessary effects.

© Central University of Technology, Free State