Tyrosol Production of *Candida* Species by Plant Extracts Used for Skin Treatment Remedies

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KEYWORDS

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ABSTRACT

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Key Words: *Candida,* tyrosol, *Aloe ferox, Bulbine frutescens, Galenia africana*

The fungal genus *Candida* is a collection of approximately 150 asporogenous yeast species which are able to cause life-threatening diseases when the normal host defences have been compromised. The most commonly isolated species is *Candida albicans* (*C. albicans*), which is a dimorphic fungus that exists in the blastopore and mycelial phase. Factors which are critical for the pathogenicity of *Candida* are morphological transition between yeast and hyphal forms, thigmotropism, biofilm formation, expression of adhesins, invasins on the cell surface and the secretion of hydrolytic enzymes such as phospholipases, proteases, and haemolysin. The compound tyrosol stimulates transformation of *Candida* species from a yeast form to a hyphal form, which enables it to infiltrate epithelial surfaces and cause disease. *Candida* biofilm formation is of particular interest because the *Candida* involved exhibits a poor response to antifungal agents. Despite the introduction of improved treatments, resistance to antifungal drugs continues to increase. The use of numerous plant extracts and their constituents may provide an alternative way to prevent fungal growth and production of their pathogenicity factors and compounds. The purpose of this study was to evaluate the efficiency of plant extracts, namely *Aloe ferox* (*A. ferox), Bulbine Frutescens* (*B. frutescens)* and *Galenia africana (G. africana),* on the growth and tyrosol production of several *Candida* species.

Firstly, all samples of *Candida* were subjected to API 20C AUX and VITEK 2 testing for confirmation of species which revealed that the most commonly identified species was *Candida dubliniensis*, followed by *Candida albicans*, *Candida tropicalis*, and *Candida glabrata.* Following species confirmation, the tyrosol production of each species was quantified to determine the highest producers of tyrosol. The so-called high producers of tyrosol were treated with each of the plant extracts and then subjected to HPLC for tyrosol quantification. Statistical analysis provided inconclusive results on the inhibition of tyrosol production by the three plant species.

Secondly, the pharmacological importance and relevance of phytochemicals present in one of the plant extracts, *G. africana,* was determined to assess the safety of the plant's use in traditional medicine. This plant was chosen due to previous studies describing the plant's use as a skin remedy in traditional medicine. Further, there was a lack of literature detailing a comprehensive phytochemical screening and toxicological assessment of *G. africana*. The phytochemical analysis of *G. africana* revealed that the use of this extract as an alternative pharmaceutical agent should not cause any undue health risks and as such may be further investigated for its use in medicine.

DECLARATION

I declare that *"Tyrosol Production of Candida Species by Plant Extracts Used for Skin Treatment Remedies"* is my own work, that it has not been submitted for any degree or examination in any other university, and that all the sources that I have used or quoted have been indicated and acknowledged by complete references.

Dario Gabriel Heredia ..... February 2022

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CHAPTER 1

GENERAL INTRODUCTION AND LITERATURE REVIEW

1.1. Introduction

The fungal genus *Candida* is a collection of approximately 150 asporogenous yeast species (Scully and Ei-Kabir, 1994). Although many of these species form part of the normal flora of humans, they are able to cause life-threatening diseases when the normal host defences have been compromised (Klotz, 1994; Kühbacher et al., 2017). The most commonly isolated species is *Candida albicans* (*C. albicans*), which is a dimorphic fungus that exists in the blastopore and mycelial phase. Other frequently isolated pathogens include *Candida glabrata* (*C. glabrata*), *Candida parapsilosis* (*C. parapsilosis*), *Candida tropicalis* (*C. tropicalis*), and *Candida krusei* (*C. krusei*), where these species in conjunction with *C. albicans* were reported in 95% of patients with known *Candida* infections (Diekema et al., 2012).

Factors which are critical for the pathogenicity of *Candida* are morphological transition between yeast and hyphal forms, thigmotropism, biofilm formation, expression of adhesins, invasins on the cell surface and the secretion of hydrolytic enzymes such as phospholipases, proteases, and haemolysin (Calderone and Fonzi, 2001; Mayer et al., 2013). The majority of *Candida*-infections are linked to its ability to form biofilms on the host tissue (Gulati and Nobile, 2016). It has been reported that the level of tyrosol is higher in biofilms than in planktonic cells (Kruppa, 2009). Various studies revealed that tyrosol stimulates transformation of

Candida species from a yeast form to a hyphal form, which enables it to infiltrate epithelial surfaces and cause disease (Costa et al., 2015). These biofilms protect the organisms from the host immune system by providing a stable environment, which allows the microbe to thrive (Jabra-Rizk et al., 2016). *Candida* biofilm formation is of particular interest because the *Candida* involved exhibits a poor response to antifungal agents (Gulati and Nobile, 2016). Despite the introduction of improved treatments, resistance to antifungal drugs continues to increase (Pfaller, 2012).

The use of numerous plant extracts and their constituents may provide an alternative way to prevent fungal growth and production of their pathogenicity factors and compounds (Savoia, 2012). This is evident from multiple studies which have exposed various fungal and yeast pathogens to different plant extracts and have elicited some form of antimicrobial activity via different mechanisms (Doddanna et al., 2013; Khan et al., 2017; Espino et al., 2019; Czerwińska and Szparaga, 2015). *Aloe ferox* (*A. ferox*) has been used by indigenous South African people as a treatment for gonorrhoea, candidiasis, arthritis and inflammation (Chen et al., 2012; Kambiz and Afolayan, 2008; Thring and Weitz, 2006). *Bulbine frutescens* (*B. frutescens*) is commonly used as a treatment for wounds, burns, rashes, itching, ringworm, and cracked lips (Pather and Kramer, 2012; Pather et al., 2011), and *Galenia africana* (*G. africana*) is used for the treatment of wounds, toothache, pimples, rashes, dandruff, and skin infections (Watt and Breyer-Brandwijk, 1962; Vries et al., 2005; Van Wyk, 2011; De Beer and Van Wyk, 2011).

1.2. Statement of the Problem

Previous literature has shown that *Candida* infections are of growing concern, particularly among individuals with compromised immunity who are at risk of developing serious and sometimes fatal disease. Due to the emergence of pathogens which are resistant to current treatments it is of importance that alternate therapies be investigated. Currently there is limited data concerning the efficacy of commonly used plant extracts, *A. ferox, B. frutescens,* and *G. africana,* for the treatment of fungal infections. This is especially true regarding the downregulation of tyrosol production by *Candida* species.

1.3. Aim

The primary aim of this study is to evaluate the efficiency of plant extracts, namely *A. ferox, B. frutescens* and *G. africana*, on the growth and tyrosol production of several *Candida* species. Tyrosol is a compound which is produced by *Candida* species and it increases the pathogenicity of the organism by promoting the conversion of *Candida* from a yeast form to a more pathogenic hyphal form. The hyphal form is of greater concern as it encourages invasion of host barriers. Therefore, the downregulation of tyrosol production by *Candida* species could potentially be beneficial in reducing the risk of severe fungal infections. Plant extracts have shown potential to remedy various skin ailments and it is important to find alternative therapies resulting from an increase in antifungal resistance to existing treatments.

1.4. Objectives

The objectives of this study were to identify the species of *Candida* which produce the highest amounts of tyrosol and to determine the effect of selected plant

extracts, *A. ferox, B. frutescens,* and *G. africana,* on the production of tyrosol by *Candida* species. Further investigation on the pharmacological importance and relevance of phytochemicals present in one plant extract, *Galenia africana,* was determined to assess the plant's use in traditional medicine.

1.5. Identification of Candida Species

The genus *Candida* is a collection of approximately 150 asporogenous yeast species (Scully et al., 1994). These multitudes of organisms have been placed into different species based mainly on their physiological and biochemical profiles, while occasionally taking into account their morphological characteristics (Lehmann et al., 1992). The species of *Candida* that are of major medical importance include *Candida albicans, Candida tropicalis, Candida glabrata, Candida parapsilosis, Candida stellatoidea, Candida guilliermondii, Candida krusei, Candida lusitaniae* and *Candida pseudotropicalis* (Anaissie et al., 2009; Scully et al., 1994). Although many of these species form part of the normal flora of humans, they are able to cause life threatening diseases in humans when the normal host defences have been compromised (Klotz, 1994; Kühbacher et al., 2017). The most commonly isolated species is *C. albicans,* which is a dimorphic fungus that exists in the blastopore and mycelial phase. Other frequently isolated pathogens include *C. glabrata, C. parapsilosis, Candida tropicalis,* and *C. krusei,* where these species in conjunction with *C. albicans* were reported in 95% of patients with known *Candida* infections (Diekema et al., 2012). The infections they cause are classified into three types, namely mucosal candidiasis, cutaneous candidiasis, and systemic candidiasis (Dabas, 2013). Mucosal candidiasis affects the oral, gastrointestinal, and vaginal mucosa which usually presents as white

patches of microbial growth at the site of infection. Cutaneous candidiasis is the infection of the skin and is a more common cause amongst newborns. This usually results from the skin of the infant being in contact with urine for extended periods, which allows the *Candida* to become embedded in the epidermis. Invasive systemic candidiasis is a potentially lethal infection which is caused by different species of *Candida* and can affect the blood, heart, brain, eyes, and bones (Sternberg, 1994).

Conventional methods of identifying *Candida* species may take 24 - 48 hours, where it is possible to distinguish between different isolates by employing methods such as the germ tube test (GTT), chlamydospore formation, as well as its ability to ferment sugars (Alam et al., 2014). The GTT is used for the presumptive identification of *C. albicans* that includes a loopful of the test strain inoculated in 0.5 ml of citrated rabbit serum. After inoculation a drop of the suspension is placed on a microscope slide and is observed for formation of germ tubes, where a positive result is presumed to be *C. albicans* (Sheppard et al., 2008). It is important to note that *C. dubliniensis* may also yield a positive GTT result. The differentiation between these two species is done based on their ability to grow on sabouraud dextrose agar or broth that contains 6.5% sodium chloride (Alam et al., 2014). Chromogenic media for the identification of *Candida* may also be used to distinguish between species based on colony colour and morphology resulting from the cleavage of colour producing substrates by enzymes which are species specific (Murray et al., 2005). Automated systems for identification of yeasts such as the VITEX 2 ID YST which employ biochemical tests may also be used and have the advantage of being less time consuming than

conventional methods. It uses growth-based technology and employs colorimetric reagent cards which are incubated with the *Candida* and interpreted automatically (Ligozzi et al., 2002). Each of the reagent cards has 64 wells, where the wells contain individual test substrates that measure various metabolic activities. Such activities include acidification, alkalinisation, enzyme hydrolysis and growth in the presence of inhibitory substances. The cards are read by an optical system in the machine which calculates the probabilities of the sample being a certain species. The machine does this by analysing the results of the various tests, and then comparing it to the database which has been programmed into it (Aubertine et al., 2006).

1.6. *Candida* Pathogenicity and the Production of Tyrosol

Factors which are critical for the pathogenicity of *Candida* are morphological transition between yeast and hyphal forms, thigmotropism, biofilm formation, expression of adhesins, invasins on the cell surface and the secretion of hydrolytic enzymes such as phospholipases, proteases, and haemolysin (Calderone and Fonzi, 2001; Mayer et al., 2013). *Candida* species may occur and multiply either as planktonic cells, or as structured communities which are enclosed in a gel-like polysaccharide matrix known as a biofilm (Nett et al., 2010). Biofilms are defined as biological communities with a high-level configuration, in which micro-organisms form structured coordinated and functional communities (Donlan and Consterton, 2002). The formation of biofilms is associated with a high level of antimicrobial resistance (Patel, 2005). Fungal infections, particularly in immunocompromised persons are of concern as they are known to be capable of

forming biofilms on medical implants (Araújo et al., 2017). Recent evidence suggests that the majority of infections produced by this pathogen are associated with biofilm growth (Araújo et al., 2017). A biofilm typically develops over four sequential steps as shown in figure 1.6.1. Firstly, the microorganism adheres to the surface, followed by colony formation and organisation. The next step includes the secretion of extracellular polymeric substances (EPS) and maturation into a three-dimensional structure. Lastly the dissemination of progeny biofilm cells takes place (Seneviratne et al., 2008).

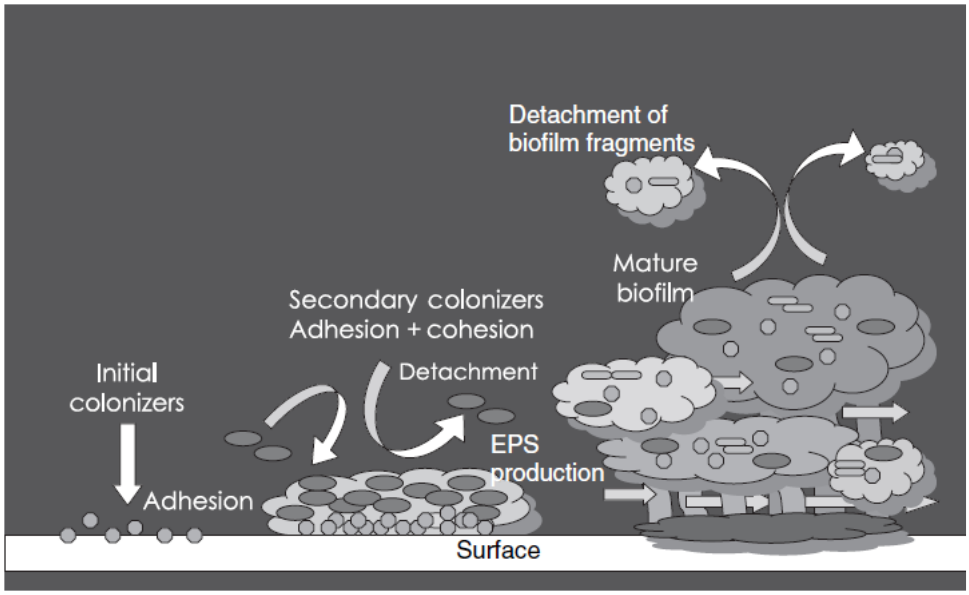

Figure 1.6.1 Sequential steps of biofilm development: adhesion; individual colonisation, and organisation of cells; secretion of EPS and maturation; dissemination of progeny biofilm cells (Seneviratne et al., 2008).

The mechanisms of biofilm resistance to antimicrobial agents are not yet fully understood. Possible mechanisms that have been proposed, include the restricted penetration of drugs through the biofilm matrix, phenotypic changes which result

from nutrient limitations or a decreased growth rate, and the expression of resistance genes induced by contact with a surface (Mah and O'toole, 2001, Donlan and Costerton, 2002).

A major metabolite that is of importance to the pathogenicity of *Candida* is tyrosol, as indicated in the structure below, which is a product derived from tyrosine (Dufour and Rao, 2010).

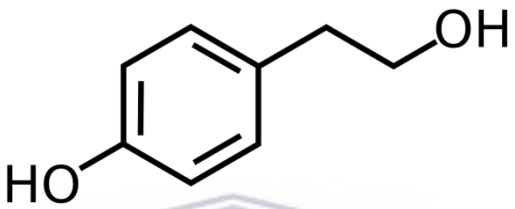

Figure 1.6.2 The chemical structure for tyrosol (Rezaei-Sadabady et al., 2013).

Tyrosol causes a more rapid change from yeast to hyphal form cells which results in germ tube formation (Chen et al., 2004). Evidence suggests that hyphae of *C. albicans* which form germ tubes grow along grooves and through pores, which may aid infiltration of epithelial surfaces during tissue invasion (Gow, 1997). This suggests that germ tube growth of *C. albicans* is well suited for the invasive growth of the fungus in *vivo*, which increases its pathogenicity (Gow, 1997). Furthermore, it has been reported that the level of tyrosol is 1.5 times higher in biofilms than in planktonic cells (Kruppa, 2009). Tyrosol also reduces the long lag phase in diluted cultures, thus allowing exponential growth to proceed sooner (Dufour and Rao, 2010).

C. albicans is a polymorphic fungus which is able to grow either as ovoid-shaped budding yeast, as elongated cells with constrictions at septa (pseudohyphae) or as

true hyphae (Berman and Sudbery., 2002). While yeast and true hyphae are regularly observed during infection and have distinct functions, the role of pseudohyphae and switching in *vivo* is rather unclear (Soll, 2009). A range of environmental cues affect *C. albicans* morphology. For example, in acidic conditions *C. albicans* cells predominantly grow in the yeast form, while under alkaline conditions hyphal growth is induced (Odds, 1987). Morphogenesis has also been shown to be regulated by quorum sensing, a mechanism of microbial communication, where the main quorum sensing molecules are farnesol, tyrosol and dodecanol (Hornby et al., 2001). *C. albicans* has a specialized set of proteins (adhesins) which mediate adherence to other *C. albicans* cells to other microorganisms, to abiotic surfaces and to host cells. The most commonly studied *C. albicans* adhesins are the agglutinin-like sequence (ALS) proteins (Verstrepen and Klis, 2006). One of the most important environmental cues that trigger hypha and biofilm formation in *C. albicans* is contact sensing. Upon contact with a surface, yeast cells will switch to hyphal growth. On certain surfaces, such as on agar or mucosal surfaces, the hyphae are able to invade into the substratum (Kumamoto, 2008).

C. glabrata grows without hyphae or pseudohyphae and lacks some of the known virulence factors such as those present in *C. albicans*. Despite this, *C. glabrata* is able to cause serious diseases in humans (Krcmery and Barnes, 2002). *Candida glabrata* is generally regarded as less pathogenic but more severe than *C. albicans*. Although little is known of proteinase production by *C. glabrata*, a single study has shown that isolates of *C. glabrata* are at least capable of proteinase production, but the type of proteinase was not specified. *C. glabrata* was shown to have

comparable cell surface hydrophobicity (CSH) to *C. albicans*, however the CSH of the *C. glabrata* was less influenced by environmental and growth conditions than that of *C. albicans*. It was recently found that phenotype switching occurs in *C. glabrata*, however the relationship of this phenotype switching to virulence is unknown. It is possible that it enhances virulence and plays a role in causing symptomatic infections (Poláková et al., 2009).

1.7. *Candida* Species and Skin Infections

Cutaneous candidiasis is not common in healthy adults and has been reported to be one of the most prevalent opportunistic yeast infections in immunocompromised individuals such as HIV positive patients. The most frequently isolated species are *C. albicans*, *C. tropicalis*, and *C. dubliniensis* (Anwar et al., 2012). It has also been reported that there has been an increase in the isolation of *C. glabrata*, which is of particular concern due to it being less susceptible to commonly used antifungals (Ruhnke, 2006). Cutaneous candidiasis is of particular importance amongst infants as it causes nappy rash or napkin dermatitis. This is a form of cutaneous candidiasis which is a common affliction amongst babies (Jain et al., 2010). This occurs when *Candida* becomes embedded in the epidermis and when the skin of the infant is sensitive due to prolonged exposure to urine, thus allowing the *Candida* to infect the skin (Odds, 1994; Wolf et al., 2000). This rash normally presents as red patches with clearly defined borders, where a series of small red spots may be present in close proximity to the larger patches (Jain et al., 2010).

Congenital cutaneous candidiasis (CCC) is a far more serious infection amongst neonates. It results from a *Candida* infection that is acquired in utero and it

presents within 6 days after birth as a skin eruption, as shown in the image below (Kaufman et al., 2017).

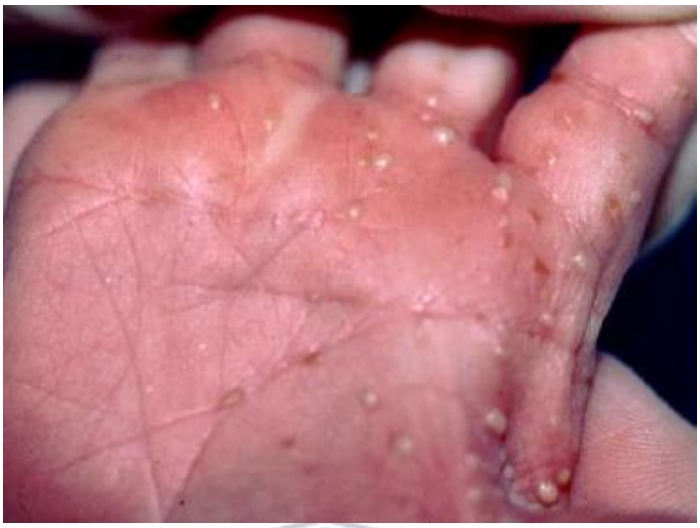

Figure 1.7.1 Discrete superficial pustules on the hand of a newborn with congenital cutaneous candidiasis (Scheinfeld, 2018).

The cutaneous infection manifests differently in adults where symptoms include erythematous and macerated skin, particularly in regions where two skin areas may touch or rub together. When these pustules break open they leave a red, macular base and this infection can result in severe systemic disease which could lead to the death of the individual (Havlickova et al., 2008; Darmstadt et al., 2000).

1.8. *Candida* Species and Mucosal Infections

Candidal yeasts cause mucosal disease in approximately 40% of immunocompromised patients, with the majority of these individuals experiencing superficial mucosal candidiasis (Moyes and Naglik, 2011; Sobel, 1992). This may affect the oral, gastrointestinal, and vaginal mucosa which

represents the most common forms of superficial mucosal candidiasis which can lead to serious disease, particularly when left untreated (Calderone and Clancy, 2012; Spellberg et al., 2006). Infection of the oral cavity is commonly referred to as thrush, and may present as white patches on the tongue or other areas of the oral cavity as shown in figure 1.8.1. This growth may be accompanied by soreness or dysphagia (Lynch, 1994).

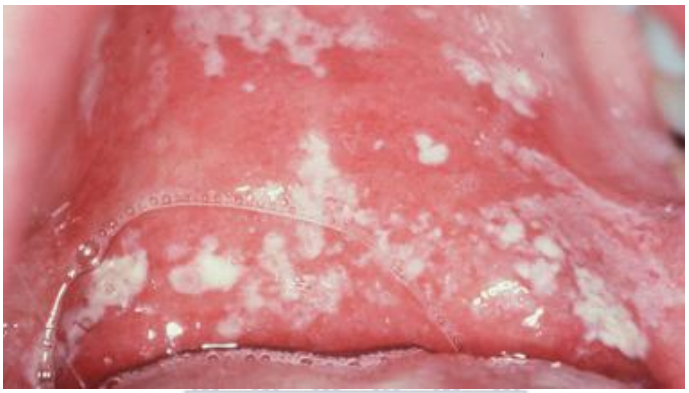

Figure 1.8.1 Pseudomembranous candidiasis presenting as white patches of microbial growth on the soft palate (Gupta and Wilson, 2020)

This growth (Figure 1.8.1) can be scraped off to reveal an erythematous base, which may present with bleeding (Akpan and Morgan, 2002). Gastrointestinal candidiasis manifests as discomfort of the bowel and may cause intestinal hypersensitivity responses, which could be misdiagnosed as irritable bowel syndrome (Birdsall, 1997; Azpiroz, 2002). This is of particular concern because the infection is able to cross the intestinal wall and spread systemically even in individuals with an intact immune system (Birdsall, 1997). Genital candidiasis occurs in both males and females; however, it is more commonly observed

amongst women. Vulvovaginal candidiasis (VVC) is the most common genital yeast infection and causes an itching, white discharge, which may be watery or thick, and dysuria (Martins et al., 2014). Often there is swelling of the labia and vulva accompanied by discrete pustulopapular peripheral lesions (Vazquez and Sobel, 2002). In men genital candidiasis may cause balanitis which is the swelling of the foreskin or head of the penis (Ono and Yasumoto, 2009). It is characterized by a prickly rash which is followed by the formation of small pustules (Martins et al., 2014). The mucosal surfaces are not only the points of entry for the invading microbes but also the ideal sites for their transmission within the host (Beninati et al., 2000).

1.9. *Candida* Species and Systemic Infections

Invasive candidiasis includes acute or chronic bloodstream candidiasis, and infection of single or multiple deep organs, either by seeding through the bloodstream or direct inoculation. Systemic candidiasis is a serious infection which may affect multiple sites of the human body such as the blood, heart, brain and eyes (Pappas, 2006). Infection of the bloodstream (candidemia) is the most common cause of systemic candidiasis and is extremely dangerous because there may be overgrowth of the yeast which may spread to other parts of the body (Antinori et al., 2016). The most common cause of systemic candidiasis is *C. albicans*, however it has been observed that *C. tropicalis* and *C. glabrata* have also been isolated from patients more frequently (Leleu et al., 2002).

Candidaemia is associated with the highest crude mortality of all bloodstream infections, where it exceeds 50% in many studies (Mavor et al., 2005). Risk factors for *Candida* infections include prior colonisation with *Candida* species,

HIV infection, cancer chemotherapy, neutropaenia, organ transplantation, indwelling catheters and devices and previous infection. Diagnosis of systemic candidiasis is challenging due to non-specific and differing clinical presentations (Eggimann et al., 2003). If this infection persists for an extended period it leads to the formation of micro-abscesses in multiple organs. Once this stage is reached it becomes extremely difficult to eradicate, thus therapy is aimed at resolving the infection prior to the onset of chronic disseminated candidiasis (Vincent et al., 1998). Mortality rates of systemic candidiasis depend on the time until diagnosis, severity, and location of the infection, where infection of vital organs results in a higher probability of mortality (Antinori et al., 2016).

1.10. *Candida* Species and Cancer

Another disease that is increasingly being associated with *Candida* infection is cancer. This disease is of particular importance as cancer is regarded as one of the most serious health problems which accounted for approximately 8.2 million deaths worldwide in 2012 (Ramirez-Garcia et al., 2016). Recent studies provide evidence that *Candida* is able to stimulate the onset of cancer through different mechanisms, the first of which is through the induction of inflammatory responses, which may favour metastatic progression (Rodriguez-Cuesta et al., 2010). Another mechanism that promotes cancer is the endogenous production of carcinogens by *Candida*, such as nitrosamines and acetaldehydes (Krogh, 1990). These are able to act alone or in combination with other cancer-promoting chemical compounds, which activate certain proto-oncogenes which could lead to the development of a cancerous lesion (Ramirez-Garcia et al., 2016). This is supported by a study, which found that prolonged exposure to *Candida* and the

production of nitrosamine was related to the development of esophageal cancer (Domingues-Ferreira et al., 2009). Furthermore, candidal yeasts are able to invade oral epithelium and play a causal role in oral leukoplakia changes, which have been estimated to develop into carcinomas in up to 40% of cases (Meurman, 2010). A study by Bakri et al., (2010) showed that *C. albicans* is the most commonly isolated species which has the potential to stimulate the onset of cancer, although other species such as *C. dubliniensis*, *C. tropicalis*, *C. pintolopesii*, and *C. glabrata* may also be implicated. In a study conducted by Chung et al. (2017) it was observed that patients with *Candida* infection had significantly higher risks of overall cancer as well as hematologic malignancy, head and neck cancer, pancreatic cancer, skin cancer, and thyroid cancer.

1.11. *Candida* species and Antifungal Plant Extract Studies

A study conducted by Motsei et al. (2003) screened an array of traditionally used South African plants for their antifungal activity against *C. albicans.* They demonstrated that plants such as *Bulbine frutescens, Bulbine natalensis, Warburgia salutaris* and others could be used as home remedies for candidiasis. The various plant extracts were tested for their antifungal activity against two isolates and one type strain of *C. albicans* by analysing the MIC of the extracts. However, many of the extracts, including *B. frutescens*, were not further investigated as the aim the study was to produce a simple remedy by mixing plants in water, which could be used in indigenous settings. This resulted in the exclusion of certain promising extracts such as *B. frutescens* based on the unpleasant taste or overpowering smell of the simple remedy. The exclusion of these extracts based solely on taste and smell resulted in a gap in the knowledge of efficacy of the

extracts against *Candida* infections. This necessitates further investigation using these extracts.

Another study conducted by Okigbo and Mmeka (2008) in Nigeria aimed to assess the antimicrobial effects of tropical plant extracts on different organisms, including *Candida albicans*. The antimicrobial activity was assessed using the disk-diffusion method and measuring zones of inhibition after incubation. They found that *Garcinia kola* had no effect on *C. albicans*. The plant extracts *Vernonia amygdalina* and *Cymbopogon citratus* were found to be less effective on *Candida albicans* than on the Gram-positive and Gram-negative bacteria. The zone of inhibition for the yeast ranged from 6 mm to 10 mm while the zone of inhibition for the bacteria ranged from 7 mm to 26 mm.

A study by Shai et al. (2008) tested the antifungal activity of seven traditionally used South African plant species against *C. albicans.* Leaf extracts of *Cussonia zuluensis, Vepris reflexa, Curtisia dentata, Trichilia emetica, Terminalia phanerophlebia, Terminalia sambesiaca* and *Kigelia africana* were used. The MIC of the different extracts was determined using the serial microplate dilution method. It was found that all the extracts had an effect on the growth of the yeast. The most potent extracts were those of *C. dentate* and *T. sambesiaca* with a MIC value of 0.12 mg/ml.

In a study by Hammer et al. (1999) the antimicrobial activity of various essential oils, such as lemongrass and oregano, was assessed on a variety of organisms, one of which was *C. albicans*. Using the agar dilution method and by measuring the MIC, it was found that many of the essential oils that were selected were able to

inhibit the growth of *C. albicans* (Hammer et al., 1999). Upon treatment with the lemongrass, a MIC of 0.06% (v/v) was observed, and a MIC of 0.12% (v/v) was observed when *C. albicans* was treated with oregano. This shows promise for the use of plant extracts and essentials oils as a means of preventing disease caused by *Candida* species. However, many of the selected plants used in the study are not abundant in South Africa. This highlights the need to further investigate the antimicrobial activities of more efficient extracts made from other South African indigenous plants.

1.12. South African Skin Care Medicinal Plants

1.12.1 *Aloe ferox*

Aloe ferox is a species of arborescent aloe that belongs to the family Asphodelaceae, and is indigenous to South Africa (Chen et al., 2012). This plant is a single-stemmed aloe which is commonly found in the Cape coastal regions of South Africa (Shackleton and Gambiza, 2007). It grows to a length of 3m and has thick, fleshy leaves which are arranged in rosettes, while its orange-red flowers stand approximately 1m above the leaves (Chen et al., 2012). It is locally referred to as the "Cape aloe" or "bitter aloe" due to the bitter exudate from its leaves. This exudate is collected and then boiled to remove moisture which forms a solidified black mass known as aloe lump. This is used in the production of remedies which are used to treat arthritis and inflammation by direct application to the affected area (Van Wyk, 2011; Thring and Weitz, 2006).

Extracts of this plant have been shown to exhibit antifungal activity, with an inhibitory effect being recorded on the growth of *C. albicans* (Kambiz and Afolayan, 2008). Aloin, which is a pure compound isolated from *A. ferox*, was

found to inhibit the growth of multiple strains of *Neisseria gonorrhoea* at a concentration of 0.1 mg/ml (Kambiz and Afolayan, 2008). The activity of aloin against Gram-positive and Gram-negative bacteria shows its potential as a broad-spectrum antimicrobial with MIC ranging from 62.5 µg/ml to 250 µg/ml (Kambizi et al., 2005). Furthermore, cytotoxicity studies on aloin have demonstrated that the compound does not display any toxic effects in cell culture at a concentration of 0.1 mg/ml (Kambizi et al., 2007). Antifungal screening showed that aloin was active against all strains of *C. albicans* at a concentration of 5 mg/ml (Kambiz and Afolayan, 2008).

1.12.2. *Bulbine frutescens*

Bulbine frutescens is a southern African species of flowering plant belonging to the family Asphodeloideae (Pather and Kramer, 2012). It is a fast-growing succulent perennial which is widespread throughout the Northern, Western and Eastern Capes of South Africa. It has fleshy green leaves laid out in opposite rows, and forms spreading clumps with a greyish stem. During spring 6-petaled star-shaped flowers bloom and have a yellow-orange colour (Harris, 2018). This plant has been used by traditional healers in the treatment of wounds, burns, rashes, itches, ringworm and cracked lips (Pather et al., 2011).

Studies conducted by Rabe and Van Staden have shown that extracts of *B. frutescens* has little to no antibacterial effect on both Gram-positive and Gram-negative bacteria (Rabe and Van Staden., 1997). Aloctin A and Aloctin B have been isolated from the leaves of *B. frutescens*, and they demonstrate some antibacterial effects against Gram-positive bacteria with a MIC of 2 mg/ml to 3 mg/ml (Ndhlala et al., 2013). Phytochemical analysis showed that the crude

extracts contained saponins, tannins, flavonoids, and alkaloids. Furthermore *B. frutescens* demonstrated antifungal properties against clinical isolates of *C. albicans* at a MIC of 6.9 mg/ml (Rachuonyo et al., 2016). Cytotoxicity studies show that ethanol extracts of *B. frutescens* displays toxic effects in cell culture at a concentration of 62.5 µg/ml (Van Huyssteen et al., 2011).

1.12.3. *Galenia africana*

Galenia africana is a Southern African plant species belonging to the family Aizoaceae. This plant is an aromatic, woody perennial sub-shrub common throughout Namaqualand, South Africa (Van der Lugt et al., 1992). It grows to a height of 0.5m – 1.5m and has oppositely arranged green leaves, which turn yellow with age (Simons and Allsopp, 2007). This change in leaf colour resulted in the plant being known locally as "kraalbos" or "yellowbush". Traditional uses of this plant for medicinal purposes includes creating a decoction which is used to create a lotion for treatment of wounds, and the Khoi-San indigenous people chew the plant to relieve toothache (Watt and Breyer-Brandwijk, 1962). This extract has been proven as having antifungal activity on many fungal isolates namely, *Alternaria sp., Botrytis cinerea, Cylindrocarpon sp., Cylindrocladium sp., Eutypa lata, Fusarium equiseti, Fusarium graminearum, Fusarium pseudograminearum, Fusarium oxysporum, Fusarium solani, Fusarium trincintum, Fusarium verticillioides, Penicillium expansum and Phaeomoniella chlamydospora* (Vries et al., 2005). In this study it was found that rechromatographed fractions of this extract produced zones of inhibition ranging from 0.3 mm to 4.2 mm depending on the fungal isolate (Vries et al., 2005). Other uses include making a leaf infusion

to treat pimples, rashes, dandruff, and lice, while topical products are made to treat skin ailments and infections (Van Wyk, 2011; De Beer and Van Wyk, 2011).

In a previous study, extracts of this plant were subjected to spectroscopic analysis which resulted in the identification of flavonoid structures as major components (Vries et al., 2005). The broth microdilution assay revealed that *G. africana* extracts had an antibacterial effect against methicillin-sensitive *Staphylococcus aureus* and methicillin-resistant *Staphylococcus aureus*, with a MIC of 3.12 mg/ml (Ng'uni., 2017). The extract displayed fungicidal properties at concentrations ranging from 6.25 mg/ml to 12.5 mg/ml (Ng'uni., 2017). Cytotoxicity studies involving the analysis of whole blood cultures utilizing lactate dehydrogenase leakage showed that *G. africana* does not display toxic effects at a concentration of 625 µg/ml (Ng'uni, 2017). It has further been reported that use of *G. africana* extract in combination with fluconazole had a synergistic effect with regards to antifungal activity against *Candida* strains (Ng'uni et al., 2021).

1.13. Increase of Antifungal Drug Resistance

Studies have shown that significant mortality has been observed in patients with invasive candidiasis, thus it poses a serious risk to public health (Antinori et al., 2016). Antifungal compounds which are commonly used for the treatment of *Candida* infections include azoles which inhibit ergosterol biosynthesis, such as itraconazole, ketoconazole, miconazole and clotrimazole. Other compounds which are used may include polyenes, allylamines, thiocarbamates, morpholines

and echinocandins (for instance, caspofungins) (Spampinato and Leonardi, 2013). The growing use of broad-spectrum antimicrobial drugs has led to the increase in fungal infections, which has caused emerging resistance to treatment (Bremenkamp et al., 2011). A lack of surveillance of *Candida* prevalence along with uncontrolled distribution of medications and limited resources in African laboratories and clinical settings may have contributed to the emergence of *Candida* resistance to various classes of antifungal drugs (Abrantes et al., 2014). Despite the introduction of new antifungal agents, antifungal resistance continues to rise (Pfaller, 2012). Tyrosol plays an important role in the resistance of *Candida* species to antifungals because of its ability to promote the formation of biofilms. This indicates that in biofilms, which have high cell densities, the resistance of *Candida* to antifungals is greatly increased; especially its resistance to azole drugs (Taff et al., 2013). This, combined with the increase in antifungal resistance, demands alternative methods of treating resistant infections. Such methods include the development of novel classes of antifungal medications.

1.14. References

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CHAPTER 2

CHARACTERISATION AND TREATMENT OF *CANDIDA* SPECIES WITH *ALOE FEROX, BULBINE FRUTESCENS,* AND *GALENIA AFRICANA* EXTRACTS

2.1. Introduction

Candida species are progressively becoming a predominant microorganism isolated in clinical environments (Sardi et al., 2013). It is important to identify *Candida* to the species level due to their differing antifungal drug susceptibility patterns and degrees of pathogenicity (Lockheart et al., 2017). Candidiasis is the fungal infection caused by *Candida* and is typically found on the skin or mucous membranes. The clinical manifestations of an infection with *Candida* species are described in relation to the site of infection, namely oropharyngeal candidiasis, oesophageal candidiasis, cutaneous candidiasis, vulvovaginal candidiasis, and others (Vasquez and Sobel, 2011). There are a range of non- *albicans Candida* species which are being increasingly observed, however *Candida albicans* remains the most frequently isolated species of *Candida*. Conventional methods of characterising and identifying *Candida* species may take 24 - 48 hours, where it is possible to distinguish between different isolates by employing methods such as the germ tube test (GTT), chlamydospore formation, as well as its ability to ferment sugars (Alam et al., 2014). Automated systems for identification of yeasts such as the VITEX 2 ID YST which employ biochemical tests may be used and have the advantage of being less time consuming than conventional methods. It uses growth-based technology and employs colorimetric reagent cards which are

incubated with the *Candida* and interpreted automatically (Ligozzi et al., 2002). Rapid identification of *Candida* species can enable appropriate treatment against candidiasis to be administered swiftly, thereby reducing the severity of fungal infections which are often related to patient mortality and morbidity (Guiver et al., 2001).

A major metabolite that is of importance to the pathogenicity of *Candida* is tyrosol, which is a product derived from tyrosine (Dufour and Rao, 2010). With the increasing incidence of *Candida* infections in African countries (Ghazi et al., 2019; Van Schalkwyk et al., 2019), the understanding of their pathogenicity and its relation to tyrosol production is needed. Assessing tyrosol production by *Candida* is of importance since tyrosol may promote the formation of *Candida* biofilms leading to a form of resistance to medications and increasing the pathogenicity of the organism. It has been found that tyrosol serves as a quorum sensing molecule in pathogenic *Candida* species (Albuquerque and Casadevall, 2012; Wongsuk et al., 2016). A study by Tarkka et al. (2009) described the use of quorum sensing that affects pathogenicity by many fungi. Quorum sensing is a method of microbial communication whereby an increase in signalling molecules enables microbes to sense cell density and then regulate certain traits such as biofilm formation (Padder et al., 2018).

The use of plant extracts and their constituents may provide an alternative way to prevent fungal growth and production of their pathogenicity factors and compounds (Savoia, 2012). This is evident from multiple studies which have exposed various fungal and yeast pathogens to different plant extracts and have elicited some form of antimicrobial activity via different mechanisms (Doddanna

et al., 2013; Khan et al., 2017; Espino et al., 2019; Czerwińska and Szparaga, 2015). In South Africa, *Aloe ferox* (*A. ferox*) has been used by Indigenous Peoples as a treatment for gonorrhoea, candidiasis, arthritis and inflammation (Chen et al., 2012; Kambiz and Afolayan, 2008; Thring and Weitz, 2006). *Bulbine frutescens* (*B. frutescens*) is commonly used as a treatment for wounds, burns, rashes, itching, ringworm, and cracked lips (Pather and Kramer, 2012; Pather et al., 2011), and *Galenia africana* (*G. africana*) is used for the treatment of wounds, toothache, pimples, rashes, dandruff, and skin infections (Watt and Breyer-Brandwijk, 1962; Vries et al., 2005; Van Wyk, 2011; De Beer and Van Wyk, 2011).

The objectives of this study were to characterise *Candida* isolates from clinical sample collections from hospitals in South Africa and Cameroon, to check the levels of tyrosol production among the different *Candida* species, and to confirm whether *A. ferox*, *B. frutescens*, and *G. africana* plant extracts could inhibit tyrosol production.

2.2. Methodology

2.2.1. Sample Collection

Twenty isolates of *Candida* species were obtained as a gift from the Microbial Endogenous Infections Studies (MEnIS) Research Laboratories, Department of Medical Biosciences, University of the Western Cape, South Africa. These samples were previously isolated from South African and Cameroonian patients by Abrantes et al. (2012). The samples received were stored in vials containing *Candida*-embedded microbeads which were preserved at -80°C. They were resuscitated by thawing the vials, following which the *Candida* embedded microbeads were placed onto and streaked across Sabouraud Dextrose agar (SDA)

plates. These plates were incubated for 48 hours at 37°C to allow sufficient time for recovery. These samples were then subcultured and maintained on SDA plates for the duration of the experiments.

2.2.2. Identification of *Candida* Samples

Presumptive identification was performed using API® 20 C AUX test strips. The test strips consist of 20 cupules, of which 19 contain dehydrated substrates which are used for assimilation tests. The remaining "0" cupule serves as a negative control. This identification procedure was performed according to the manufacturer's instructions. A single colony of young growth was transferred to a test tube containing 2 ml of 0.85 % NaCl. This suspension was adjusted to a final turbidity equal to McFarland standard 2 which corresponds to an approximate cell density of 6×10^8 colony forming units per ml. Thereafter 200 µl of this suspension was transferred into a manufacturer supplied ampule of C medium containing compounds allowing for yeast growth. The composition of this medium is detailed in Appendix 1. This mixture was homogenised to create an evenly dispersed solution. Each cupule of the strip was filled with approximately 200 µl of the inoculum suspension, and the test strip was placed in an incubator at 29 ºC ± 2 ºC for a period of 48 h – 72 h. Test strips were observed for growth after 48 h incubation, if clear results were not observed then the strip was incubated for an additional 24 h and then observed again (Appendix 2). A positive result was indicated by a cupule that had growth which was more turbid than the growth present in the "0" cupule. Conversely no growth / less growth than the "0" cupule indicated a negative result. The result (+, -) of each test was recorded on the supplied result sheet (Appendix 3). Identification of species was

done by comparing the result of each test to the manufacturer provided identification table (Appendix 3).

2.2.3. Species Confirmation using VITEK 2 Compact System

To ensure that species identification using the API 20 C AUX test strips were accurate and confirm the results theereof, the 20 samples of *Candida* were identified to a species level using the VITEK 2 system (BioMerieux). It is an automated microbiology system which uses growth-based technology and employs colorimetric reagent cards which are incubated and interpreted automatically. Each of the reagent cards has 64 wells, where the wells contain individual test substrates which measure various metabolic activities such as acidification, alkalinisation, enzyme hydrolysis and growth in the presence of inhibitory substances. Each card was sealed on both sides with an optically clear film which allows for appropriate amounts of oxygen to pass through to the organism-substrate admixtures whilst maintaining the seal.

There were four different reagent cards available for identification of different organism classes. The classes were Gram-negative fermenting and non-fermenting bacilli (GN), Gram-positive cocci and non-spore forming bacilli (GP), yeasts and yeast-like organisms (YST), and Gram-positive spore-forming bacilli (BCL).

For the identification of the *Candida* samples, the YST card was used because the tests it contains were designed to be used for the automated identification of 49 taxa of the most significant yeasts. This card has 46 different biochemical tests which have been adapted from established biochemical methods. The YST card

was inoculated with a suspension of the organism which was prepared by using a sterile swab to pick up pure colonies from a blood agar plate and suspending it in 3.0 ml of sterile saline (aqueous 0.45% to 0.50% NaCl, pH 4.5 to 7.0) in a 12 mm x 75 mm clear polystyrene test tube. The turbidity of this suspension was adjusted to a McFarland standard of between 1.80 and 2.20 using the nephelometer (DensiChek^{TM}) that was supplied with the VITEK 2 compact machine. The YST cards were inoculated with the organism suspension via a vacuum apparatus that is integrated into the VITEK 2 compact machine. The test tube containing the suspension was placed into a cassette and the identification card was placed into the slot next to it, with the transfer tube being inserted into the corresponding test tube. The cassette was placed into the vacuum chamber and a vacuum was applied, after which air was re-introduced into the chamber. This caused the organism suspension to be forced through the transfer tube and into micro channels, thereby filling all the wells of the card. Once the cards have been inoculated, the transfer tubes were cut off, and the cards were sealed before being placed in to the carousel of the VITEK 2 machine. These cards were incubated at 35.5 ± 1.0ºC, and each card was removed from the incubator once every 15 minutes and transported to the optical system of the machine where the reactions were read. The system then calculated the probabilities of the sample being different species by analysing the results of the various tests, and then comparing it to the database. This information was then accessed using a software programme which generated a report (Appendix 4) detailing the identified species, probability of correct identification, and results for each of the tests.

2.2.4. Tyrosol quantification

The *Candida* samples were sub-cultured from the SDA plates into 100 ml Erlenmeyer flasks containing 25 ml of yeast nitrogen base (YNB) supplemented with 50 mM glucose. The inoculated culture media was incubated at 37 ℃ for 24 h in an orbital shaker at 60 rpm. Under these conditions, all strains grew exclusively in the budding yeast phase. After incubation, cells were harvested and washed twice in 0.15 M phosphate-buffered saline (PBS) with a pH of 7.2. A standardised suspension was made with the washed cells to an optical density of 0.8 at 520 nm. Using 100 µl of the standardised cell suspension, 50 ml of YNB was inoculated to give a cell density of 4 x 10^4 cells per ml. These cultures were incubated at 37 ℃ in an orbital shaker at 60 rpm for 48 h. The samples were decanted into centrifuge tubes and centrifuged at 3000 rpm. The supernatants were decanted and filter sterilized through a 0.2 µm filter. Tyrosol was isolated from the culture supernatants by solid-phase extraction (SPE) and quantified by reverse-phase high-pressure liquid chromatography (HPLC). To give working standards, a 1 mM stock solution of tyrosol in acetonitrile was prepared. For SPE the 50 ml culture supernatants were acidified by adding 0.2 ml of a 0.1 M sulfuric acid.

A C-18 Sep-Pak Plus cartridge was conditioned with 10 ml of methanol, followed by 10 ml of 1 mM sulfuric acid. This acidified supernatant was then loaded onto the cartridge. Tyrosol was eluted with 10 ml of 7.5% acetonitrile in 1 mM sulfuric acid. The loading and eluting flow rates were controlled to be 2 to 4 ml per min in an SPE vacuum manifold.

The signal and UV spectra were processed by chromatography data station software. HPLC analyses were carried out using a 15 mm C-18 analytical column. The elution profile consisted of three mobile phase compositions over 30 min of run time. For the first 10 min, the mobile phase consisted of acetonitrile-1 mM H_2SO_4 (10:90, vol/vol) for eluting tyrosol. The mobile-phase composition for the second 10 min was acetonitrile-water (50:50, vol/vol) to clean up the column after some late-eluted compounds. The column was then reconditioned using acetonitrile-1 mM H_2SO_4 (10:90, vol/vol) for 10 min to be ready for the next injection. The flow rate was set at 1 ml per min, and the analysis was carried out at room temperature. An aliquot (20 µl) of the purified extract was injected into the HPLC system. The photodiode array was programmed to record data from 200 to 400 nm.

Using this data, the concentrations of tyrosol present in each sample was calculated to identify the 10 species of *Candida* which produced the greatest amount of tyrosol. This was done by taking the area of the peaks for the samples and dividing it by the area of the tyrosol standard (245), multiplied by the concentration of the standard (20 mg/L). These 10 isolates were then cultured in YNB and treated with commercially purchased ethanolic extracts of *A. ferox, B. frutescens,* and *G. africana* at a concentration of 25 mg/ml was selected based on the results of a previous study by Ng'uni et al. (2018), which found that antifungal activity started to appear at concentrations above 15.62 mg/ml. A concentration at the lower end of the range of antifungal activity was selected so as not to entirely inhibit the growth of the *Candida,* but rather to disrupt the quorum sensing mechanism of the fungus by inhibiting tyrosol production. These cultures were

incubated as described previously and run in triplicate using HPLC to determine the impact of the treatment on the tyrosol production. The results are presented as an average of the triplicate analyses.

2.2.5. Statistical Analysis

Data generated in the present study were recorded and analysed statistically using GraphPad Prism for Windows, version 8.4.3 (686) (GraphPad Software, San Diego, California, United States of America). Controls and samples were run in triplicate and sample sizes were kept to 9. After calculating the summary statistics, including the Kolmogorov-Smirnov test for normal distribution, data were analysed by means of an ordinary one-way ANOVA if normally distributed. If the samples were not normally distributed, the Kruskal-Wallis ANOVA test was used. To test for a difference between untreated and treated samples, the Dunn's multiple comparisons test was used. A P-value of less than 0.05 was considered significant.

2.3. Results

2.3.1. Identification of *Candida* Samples

Table 2.1 shows the results obtained by the API® 20 C AUX test strips. The samples were identified by comparing the result of the test strip (Appendix 1) to the identification table (Appendix 2).

Table 2.1: Species level identification of samples using API® 20 C AUX test strips.

Sample	Isolate / Strain	Identified Species
A	ATCC 90028	*C. albicans*

B	SA 3	*C. dubliniensis*
C	SA 14	*C. albicans*
D	SA 48	*C. albicans*
E	SA 85	*C. albicans*
F	NCPF 3949a	*C. dubliniensis*
G	SA 7	*C. dubliniensis*
H	SA 51	*C. dubliniensis*
I	SA 105	*C. dubliniensis*
J	SA 136	*C. dubliniensis*
K	ATCC 26512	*C. glabrata*
L	SA 11	*C. glabrata*
M	SA 72	*C. lusitaniae*
N	SA 92	*C. glabrata*
O	C 237	*C. glabrata*
P	ATCC 950	*C. neoformans*
Q	C 85	Unidentified
R	C 141	*C. tropicalis*
S	C 245	*C. tropicalis*
T	C 250	*C. tropicalis*

The preliminary identification of the samples revealed that *C. dublininesis* was the most common species (n=6) and *C. lusitaniae* and *C. neoformans* were the least common of the identified species (n=1). Sample Q could not be identified by the API 20 C AUX test strip, but did indicate that the sample was not a yeast.

2.3.2. Species Confirmation using VITEK 2 Compact System

Table 2.2 shows the results obtained by the VITEK 2 system. This was performed to provide a more accurate identification of the samples down to a species level. The results were automatically generated by the VITEK 2 system (Appendix 3).

Table 2.2: Species level identification of samples using the VITEK 2 system.

Sample	Isolate / Strain	Identified Species
A	ATCC 90028	*C. albicans*
B	SA 3	*C. dubliniensis*

C	SA 14	*C. albicans*
D	SA 48	*C. albicans*
E	SA 85	*C. albicans*
F	NCPF 3949a	*C. dubliniensis*
G	SA 7	*C. dubliniensis*
H	SA 51	*C. tropicalis*
I	SA 105	*C. dubliniensis*
J	SA 136	*C. dubliniensis*
K	ATCC 26512	*C. dubliniensis*
L	SA 11	*C. glabrata*
M	SA 72	*C. glabrata*
N	SA 92	Unidentified
O	C 237	*C. glabrata*
P	ATCC 950	*C. tropicalis*
Q	C 85	Unidentified
R	C 141	*C. tropicalis*
S	C 245	*C. tropicalis*
T	C 250	*C. tropicalis*

These results confirmed that *C. dubliniensis* is the most commonly identified species (n=6) within this sample group. The least commonly identified species was *C. glabrata* (n=3). Samples N and Q were both classed as unidentified by the VITEK 2 system using the YST cards and is likely due to the samples not being a species of *Candida*. Therefore these samples were excluded from further testing. Samples H, K, M, and N were identified as different species compared to the identification using the API 20 C AUX test strips. It is possible the results between the API 20 C AUX and VITEK 2 differed due to the higher sensitivity of the VITEK 2 system. Due to the discrepancies between the methods of identification, the results of the VITEK 2 system were used in further studies as this method is more sensitive than the API 20 C AUX and therefore produce more reliable results (Darbandi, 2011).

2.3.3. Tyrosol quantification

HPLC analysis was used to determine the strains which produced the highest amounts of tyrosol (>0.500 mg/L) among the samples tested. A tyrosol standard (20 mg/L) was used to generate a strong peak on the graph and this was used to determine the concentration of tyrosol in the samples (Table 2.3).

Table 2.3: Highest producers of Tyrosol

Sample	Species	Tyrosol Concentration (mg/L)
M	*C. glabrata*	2.041
E	*C. albicans*	1.314
Q	*Unidentified*	1.061
P	*C. tropicalis*	0.865
C	*C. albicans*	0.841
D	*C. albicans*	0.816
A	*C. albicans*	0.694
J	*C. dubliniensis*	0.612
H	*C. tropicalis*	0.539
F	*C. dubliniensis*	0.522

The results show that sample M (*C. glabrata*) was the isolate that produced the highest amount of tyrosol. Additionally, it should be noted that all the *C. albicans* isolates were identified as high producers of tyrosol. This is of importance as this species has been described as the major fungal pathogen of humans and the most common cause of mucosal and systemic fungal infection (Vazquez and Sobel, 2011).

Table 2.4 shows the level of tyrosol present before and after treatment with each of the three plant extracts.

Table 2.4: Comparison of tyrosol levels before treatment and after treatment with plant extracts.

Sample	Species	Pre-treatment tyrosol concentration (mg/L)	Post-treatment tyrosol concentration (mg/L)		
			A. ferox	***B. frutescens***	***G. africana***
M	*C. glabrata*	2.041	2.024	2.065	2.000
E	*C. albicans*	1.314	1.306	1.322	1.282
P	*C. tropicalis*	0.865	0.898	0.816	0.824
C	*C. albicans*	0.841	0.841	0.816	0.808
D	*C. albicans*	0.816	0.833	0.776	0.767
A	*C. albicans*	0.694	0.735	0.710	0.669
J	*C. dubliniensis*	0.612	0.653	0.571	0.604
H	*C. tropicalis*	0.539	0.571	0.539	0.539
F	*C. dubliniensis*	0.522	0.514	0.522	0.539

Kolmogorov-Smirnov normality test was performed to assess whether the data was normally distributed or not. Results of this test revealed that data was not normally distributed at α=0,05. This informed that subsequent tests that should be performed would be non-parametric. To compare the results of the treated samples obtained to the results of the untreated samples as shown in Table 2.4 a Kruskal-Wallis ANOVA test was performed which showed that there was no significant difference ($p > 0.05$) between the medians of the treatment methods. Following this a Dunn's multiple comparisons test was performed whereby the mean rank of each treatment was compared to the mean rank of the control to

determine if a significant difference was present before and after treatment with each of the plant extracts (Figure 2.1).

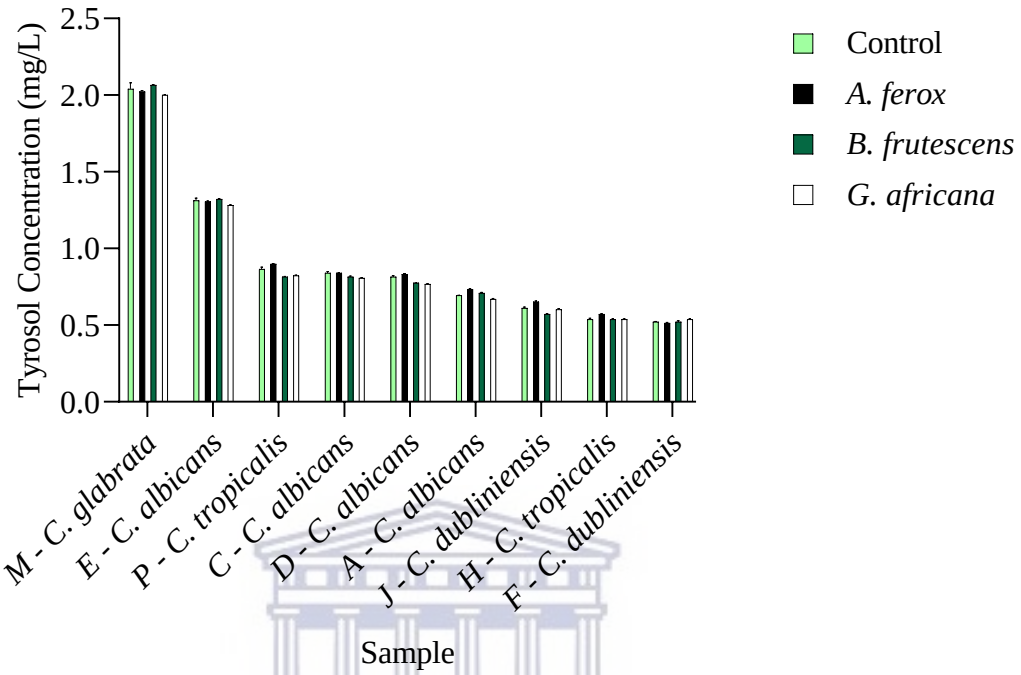

Figure 2.1: Tyrosol concentration (mg/L) of the control (untreated) and after exposure to each of the three plant extracts (*A. ferox, B. frutescens, and G. africana*) for each sample.

In comparison to control samples, there was no significant difference ($p > 0.05$) in the tyrosol concentration pre- and post- treatment. Furthermore, there was also no significant difference ($p > 0.05$) observed in the concentration of tyrosol when comparing treatments with different extracts to each other.

2.4. Discussion

Three southern African medicinal plants, namely *A. ferox, B. frutescens,* and *G. africana* were selected due to their traditional use in the treatment of various skin

ailments. The aim was to expose cultures of *Candida* species to extracts of these three plants and to determine the effect it had on the production of tyrosol by the *Candida* species. A study by Zapata et al. (2013) found that the major antifungal compound present in *A. ferox* was aloin, which has also been confirmed as having an inhibitory effect on different strains of *C. albicans* (Kambiz and Afolayan, 2008). There is limited information available regarding the specific antifungal compounds present in *Bulbine frutescens*, however there are many studies that describe antifungal activity from extracts of this plant (Ghuman and Coopoosamy, 2011; Maninjwa, 2020). In terms of antifungal activity of *G. africana,* flavonoids were found to be the most active antimicrobial compounds (Vries et al., 2005; Ticha et al., 2015; Ng'uni, 2017).

Candida is a fungus that frequently causes mucosal and systemic infections, which may be lethal, in humans by becoming opportunistic despite being part of the normal flora (Vincent et al. 2009). Typically, *Candida* infections are superficial and only infect the oral cavity, skin, genitalia, and respiratory system, however it could further develop into invasive candidiasis which could lead to fatal conditions such as candidemia, meningitis, and endocarditis (De Rosa et al., 2009).

The pathogenicity of *Candida* species is related to its change between the commensal yeast form and its invasive hyphal form (Jacobsen and Hube, 2017). This ability is termed dimorphism and once the conversion from yeast form to hyphal form is triggered, the filaments from the hyphae can penetrate deeper into the host tissues (Mba and Nweze, 2020). One of the mechanisms which is employed by yeasts to enact this change is known as quorum sensing. This makes

use of quorum-sensing molecules, namely farnesol and tyrosol to initiate its morphological transition (Albuquerque and Casadevall, 2012). The specific function of tyrosol in fungi is to stimulate the transition from spherical cells to a germ tube form in a concentration-dependant manner (Rodrigues and Černáková, 2020).

The comparison of tyrosol production results obtained from the untreated samples (Table 2.3) and the results of the samples treated with the plant extracts (Table 2.4) shows that the extracts exerted no apparent effect on the amount of tyrosol that was produced (Figure 2.1). This indicates that the selected plant extracts were unable to disrupt the quorum sensing mechanism of *Candida* and may not be a viable alternative for a therapy to decrease the pathogenicity of the organism. This is in contrast to other studies which found that certain compounds extracted from plants exerted a modulatory effect on the quorum sensing molecules and pathogenic factors of *Candida* (Singh et al., 2015; Bacha et al., 2016; Ta and Arnason, 2016).

Although this study provides inconclusive results on the inhibition of tyrosol production by the three plant species, innovative therapies need to be developed which specifically targets this mechanism and reduces risk of severe infection by *Candida* without the need for more toxic antifungal drugs. It would be beneficial for further studies to be conducted using a greater number of plants and *Candida* species. Additionally, future research could include a combination of reduction of tyrosol and increase in farnesol levels due to their antagonistic effects, where high levels of farnesol promotes the growth of budding yeast instead of the more invasive hyphal form.

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CHAPTER 3

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IMPORTANCE AND RELEVANCE OF PHYTOCHEMICALS PRESENT IN *GALENIA AFRICANA*

3.1. Introduction

Approximately 70-95% of the population in developing countries rely on medicinal plants as their primary source of healthcare (WHO, 2011). These medicinal plants are highly sought after due to their apparent ability to treat burns and promote wound healing (WHO, 2011). The use of such plants as a treatment option for skin infections has become common practice in many rural areas (Naidoo and Coopoosamy, 2011). It has been reported that approximately 27 million people in South Africa rely on the use of medicinal plants to treat skin infections, in particular those caused by pathogens such as *Staphylococci* species and *Candida albicans* (Ndhlala et al., 2013).

The *Galenia africana* plant species also known as 'kraalbos' or 'geelbos', belonging to the family Aizoaceae is endemic to Southern Africa, where it is most commonly found in the Namaqualand region of South Africa, but has recently

become more widespread in the Western and Southern Karoo (Kellerman et al, 1988; Van der Lugt et al., 1992; De Beer and Van Wyk, 2011). Kraalbos is an aromatic, woody perennial sub-shrub which grows to a height of 0.5 – 1.5 m high, having oppositely arranged green leaves of about 5 cm long and hairless, which tend to change from green to yellow with age. The terminal ends of the twigs are the sites at which numerous small yellow flowers, about 1.5 mm in diameter are born in large loose heads, during their blooming season; between October and December (Van der Lugt et al., 1992; Foden and Potter, 2005; Simons and Allsopp, 2007). Kraalbos is considered a highly invasive pioneer plant, being the first perennial to regrow after soil disturbances, and the only remaining species after the veld has been heavily overgrazed. Some local farmers claim that during the summer months the plant is poisonous to goats and sheep (Kellerman et al., 1988). According to other farmers, if kraalbos is green, it is palatable and not poisonous, but if it is yellow and dry it is non-palatable and poisonous (Le Roux et al., 1994).

Traditional uses of *G. africana* by the southern African indigenous Khoi-San people involves chewing the plant to relieve toothache or preparing a decoction which is used to make a lotion for the treatment of wounds (Watt and Breyer-Brandwijk, 1962; Sharma, 2014). It has been shown that frying *G. africana* with other medicinal plants in butter to create an ointment has been used to treat wounds, particularly on the legs of women (Watt and Breyer-Brandwijk, 1962). Other uses which have been described in ethnobotanical surveys, include making a leaf infusion to wash their heads for the purpose of treating pimples and rashes on the affected area. The same infusion was also used in the treatment of dandruff,

lice, dry skull, leg pains and swollen legs. In addition, topical products are prepared for the treatment of venereal diseases as well as skin infections and ailments such as ringworm (De Beer and Van Wyk, 2011; Dubey et al., 2013). There have also been reports of *G. africana* leaves being used for chest pains, and conditions such as asthma and tuberculosis (Sharma, 2014). The medicinal potential of botanicals and natural ingredients derived from *G. africana* could be attributed to the plant being a major source of polyphenolic flavonoids, and other compounds, which are associated with antioxidant activities (Ticha et al., 2015).

The aim of this study is to report on the phytochemical composition of *G. africana* and provide a comprehensive background on the pharmacological importance and relevance of these phytochemicals. The valuable information gained from such a study of the phytochemicals present in *G. africana* will highlight the importance and relevance for treatment of skin diseases described through traditional applications and ethnobotanical surveys. A review on dietary intake of the phytochemicals was used to inform potential health risks after accidental intake of the *G. africana* extract.

3.2. Materials and Methods

3.2.1. Identification of Phytochemicals of *Galenia africana*

It is important that medicinal plants be analysed to determine their phytochemical constituents, which would be beneficial in understanding the pharmacological importance and health risks. The first preparation of a *G. africana* extracts for anti-fungal and anti-bacterial fractionation including phytochemical identification studies was employed by Vries et al. (2005) and Mativandlela et al. (2009). A

study by Ticha et al. (2015) analysed a representative 20% extract (80% ethanol:water) of *G. africana* 80% solution of ethanol: water, obtained from Parceval Pharmaceuticals (Pty) Ltd (South Africa), using gravity liquid column chromatography (GLCC) to afford several fractions, which differ in levels of purity, and using EtOAc:hexane as a gradient eluent (hexane, hexane:EtOAc and EtOAc). In this study six of these fractions (A-F) was received as a gift from Dr. Lawrence Ticha, prepared during his post-graduate studies at the Chemistry Department, University of the Western Cape, and these fractions were subjected to a direct gas chromatography–mass spectrometry (GC – MS) analysis, as described by Al-Asmari et al. (2015). The GC-MS was performed at the Central Analytical Facilities, University of Stellenbosch with results illustrated in Table 3.1. Nuclear magnetic resonance (^{1}H and ^{13}C NMR) spectra were recorded in either $CDCl_3$ or acetone-d6 on a Varian Gemini 2000 spectrometer at 200.05 MHz for ^{1}H and 50.3 MHz for ^{13}C spectra, respectively. All chemical shifts are expressed in parts per million (ppm) relative to trimethylsilane (TMS) as the internal reference standard. GC-MS analysis were carried out in a GC system (Agilent 7890A series, USA) equipped with split/splitless injector and auto-sampler attached to an apolar 5-MS (5% phenylpolymethyl siloxane) capillary column (Agilent 19091S-43; 30 m×0.25 mm i.d. and 0.25- μm film thickness) and fitted to Mass Detector (Agilent 5975C series, USA). The flow rate of the carrier gas, helium (He) was set to be at 1 ml.min−1, split ratio is 1:50. The injector temperature was adjusted at 250°C, while the detector temperature was fixed to 280°C. The column temperature was kept at 40°C for 1 min followed by linear programming to raise the temperature from 40° to 120°C (at 4°C min−1 with 2

min hold time), 120°C to 170°C (at 6°C min−1 with 1 min hold time) and 170°C to 200°C (at 10°C min^{-1} with 1 min hold time). The transfer line was heated at 280°C. Two microliter of sample was injected for analysis. Mass spectra were acquired in scan mode (70 eV); in the range of 50 to 550 m/z (Al-Asmari et al., 2015).

Positive identification of compounds was made by matching mass spectra against a reference library database, with results illustrated in the Chromatogram (Figure 3.1) and compound structures in Table 3.1. Various flavonoids, isolated by gravity chromatographic separation and NMR analysis (Figure 3.2), and reported by Ticha et al. (2015) are also included in Table 3.1. These clearly demonstrate that flavonoids were the major secondary plant metabolites present and possible activity was expected to result from these compounds as several previous studies have shown these compounds to have beneficial effects (Ticha et al., 2015). The relative concentration percentage (%) of any compound was calculated by the individual GC-MS peak area divided by the total peak area of the fraction and multiplying the result by 100.

3.2.2. Dietary Risk Analysis

APC Pharmaceuticals and Chemicals, United Kingdom was contracted by the University of the Western Cape to conduct a toxicological review and health risk assessment of the components of the *G. africana* extract for agriculture operators and consumers. Since the extract is composed of about 61 compounds, most of which are already present in the human diet, an alternative to conventional toxicity testing procedures was proposed, thereby reducing laboratory animal use and minimising development costs and timeline. An alternative proposed by APC was

to utilise peer reviewed literature to assess background dietary exposures for each component. The background dietary intake of the constituents of *G. africana* was analysed using the European Union Pesticide Residue Intake Model (EU PRIMO) consumer risk assessment model which informed a risk assessment for consumers. All compounds approved by the risk assessment were deemed to be non-toxic at the indicated concentration intakes and therefore did not pose a risk to human health, except cinnamic acid, the coumarins, equol, and 2-methoxy-4-vinyl phenol. The dietary intakes for the phytochemicals, as far as could be established from the literature, is summarised and referenced in Table 3.1 and the discussion.

3.2.3. Literature Search

An initial literature search was conducted with a combination of keywords such as *Galenia africana*, distribution, traditional use, medicinal uses, ethnomedicinal uses, phytochemistry, and chemical composition. After data for the phytochemical composition of *Galenia africana* was established, an extended literature search was performed to determine the natural occurrence, phytochemical and toxicological relevance of the individual constituents. This was done using a combination of the phytochemical name and keywords such as pharmacology, antioxidant, anti-proliferative, antifungal, antibacterial, anti-inflammatory, wound healing, and toxicity. These searches were conducted using major databases, including Science Direct, Google Scholar, BioMed Central (BMC), Web of Science, Springer link, Scopus and PubMed.

3.3. Results and Discussion

Results about the phytochemical composition of *Galenia africana* extract fractions and dietary intake risk in humans are shown in the chromatogram (Figure 3.1) and Table 3.1. Figure 3.2 details the calculated spectrum and 1H NMR spectrum of the flavonoid 2',4'-Dihydroxydihydrochalcone.

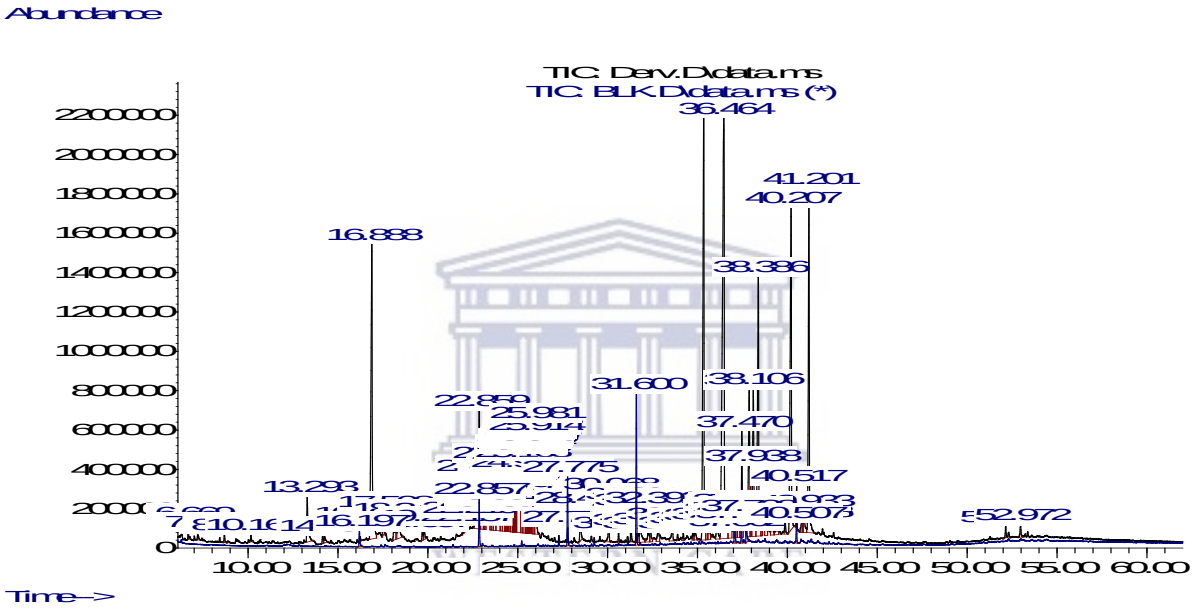


Figure 3.1: Chromatogram of the *Galenia africana* extract sample.

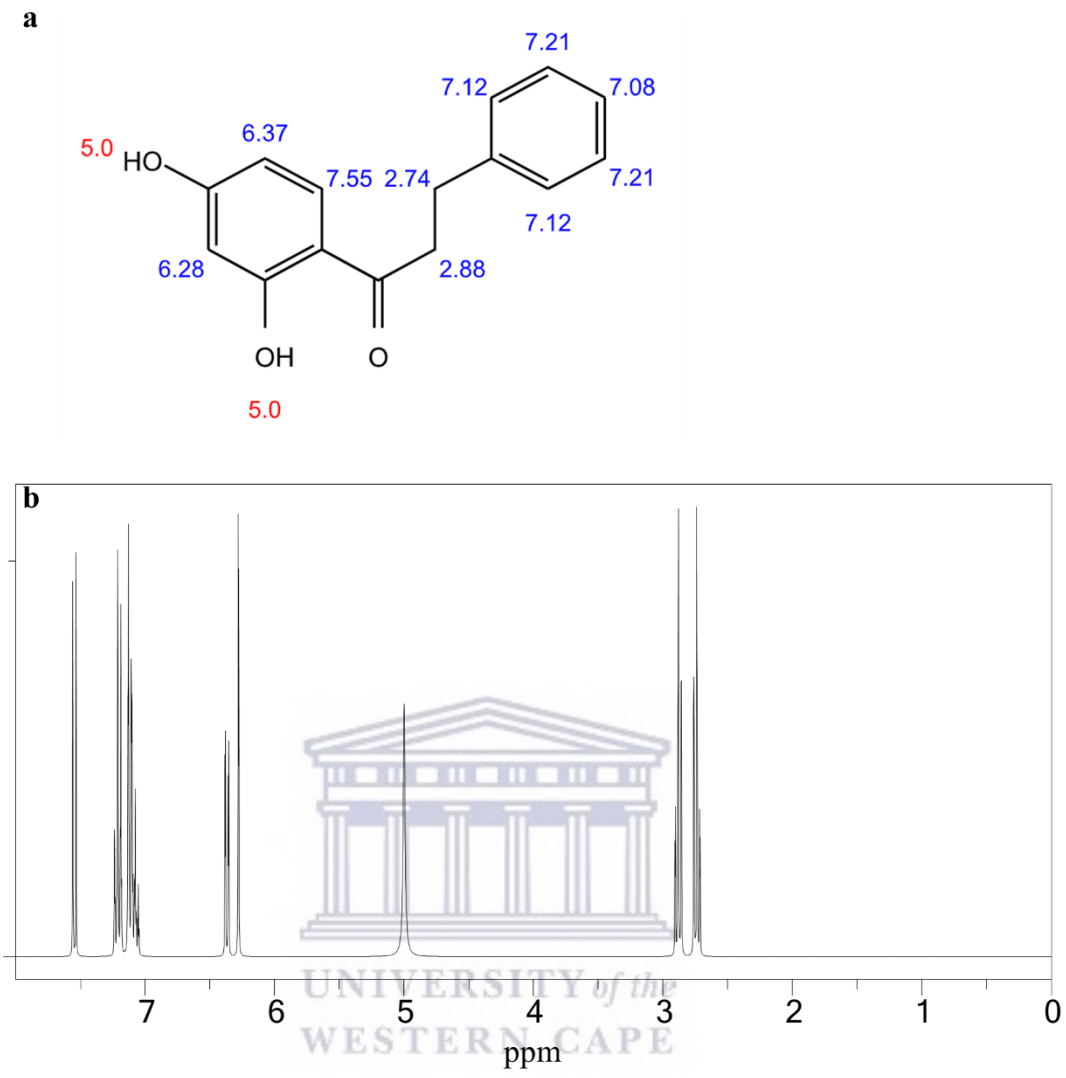
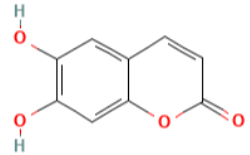
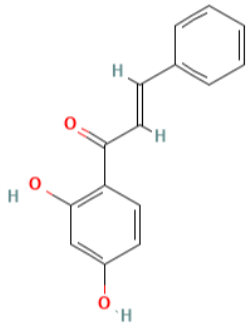
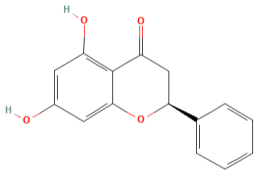
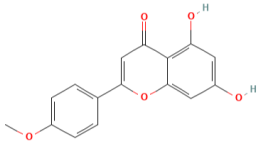
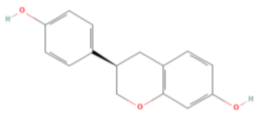
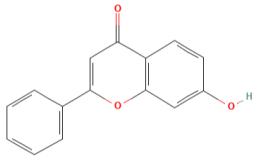
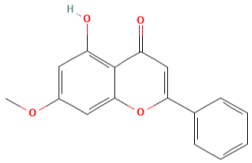
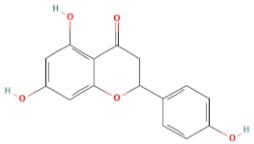
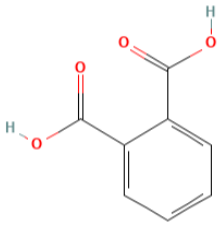
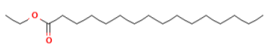
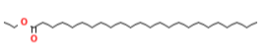
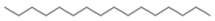
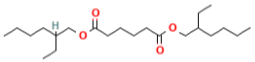
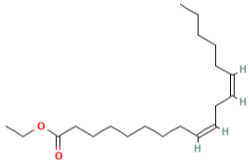
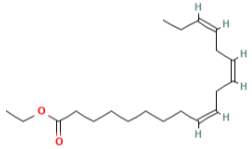
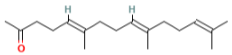


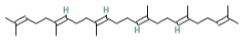
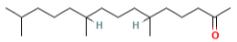
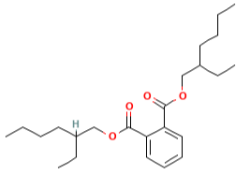
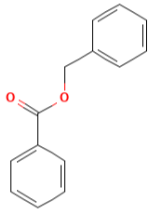
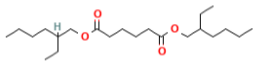
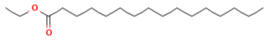
Figure 3.2: (a) Calculated spectrum and chemical structure of the flavonoid 2',4'-Dihydroxydihydrochalcone and (b) 1H NMR spectrum of 2',4'-Dihydroxydihydrochalcone.

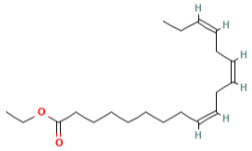
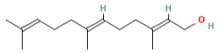
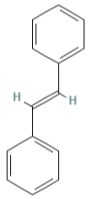
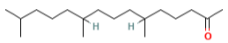
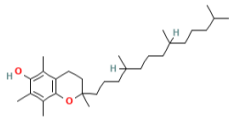
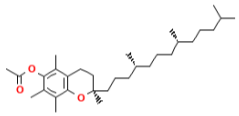
Table 3.1: Phytochemical composition of *Galenia africana* extract fractions and dietary risk intake risk in humans.

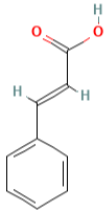
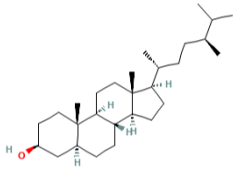
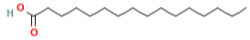
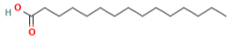
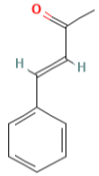
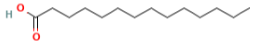
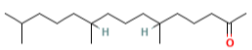
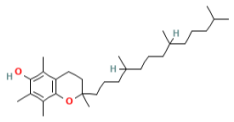
CAS Number[+]	Chemical Structure[*]	Common or IUPAC name	Relative concentration (%)[++]	Chemical Class	Dietary Intake (mg/day)[+++]	Dietary Intake Reference
Flavonoids in 20% extract						
305-01-1		Chrysin or Aesculetin	0.54	Flavonoid	0.008	(Chang et al., 2001)
1776-30-3		2',4'-Dihydroxychalcone	14.00	Flavonoid	300	(WHO, 2021)
480-39-7		5,7- Dihydroxyflavanone /Dihydrochrysin /Pinocembrin)	23.10	Flavonoid	0.0043	(Schimdhuber, 2007; Chang et al., 2001)
480-44-4		5,7-Dihydroxy-4'-methoxyflavone / Acacetin	0.40	Flavonoid	0.4	(Gattuso et al., 2007)

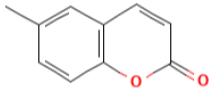
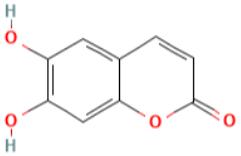
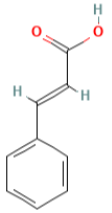
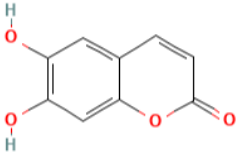
531-95-3		Equol / 4',7-Isoflavandiol	3.87	Flavonoid	9.9	(Arai et al., 2000, Boker et al., 2002)
6665-86-7		7- Hydroxyflavone	10.20	Flavonoid	No Data	
520-28-5		5-Hydroxy-7-methoxyflavone (Tectochrysin)	7.07	Flavonoid	0.001	(Chang et al., 2001)
67604-48-2		5,7,4'-Trihydroxyflavanone / Naringenin	0.44	Flavonoid	45	Skibola and Smith, 2000)
FRACTION A obtained from 20% extract						
88-99-3		Benzene 1,2-dicarboxylic acid (mono 2-ethylhexyl ester)	0.65	Other	30	(EFSA, 2017)
628-97-7		Ethyl hexadecanoate	1.88	Fatty Acid	5000	(Beare-Rogers et al., 2001)
24634-95-5		Ethyl tetracosanoate (C24 ester)	0.58	Fatty Acid	100	(Beare-Rogers et al., 2001)

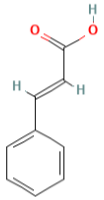
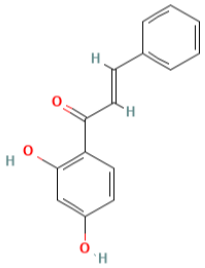
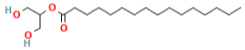
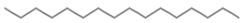
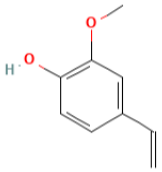
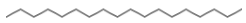
593-49-7		Heptacosane	5.98	Aliphatics	56.4	(WHO, 2021)
7796-19-2		2-Heptacosanone	0.55	Aliphatics	56.4	(WHO, 2021)
630-01-3		Hexacosane	1.12	Aliphatics	56.4	(WHO, 2021)
544-76-3		Hexadecane	0.77	Aliphatics	56.4	(WHO, 2021)
103-23-1		Hexanedioic acid, bis (2-ethylhexyl) ester	0.96	Fatty Acid	5	(Beare-Rogers et al., 2001)
544-35-4		Linoleic acid ethyl ester	1.01	Fatty Acid	17000	(WHO, 2021)
630-03-5		Nonacosane	10.36	Aliphatics	56.4	(WHO, 2021)
593-45-3		Octadecane	0.80	Aliphatics	56.4	(WHO, 2021)
1191-41-9		9,12,15-Octadecatrienoic acid, ethyl ester (ZZZ)-	0.85	Fatty Acid	1600	(WHO, 2021)
629-99-2		Pentacosane	0.90	Aliphatics	56.4	(WHO, 2021)
1117-52-8		5,9,13-Pentadecatrien-2-one- (6,10,14)-trimethyl (E,E)	1.28	Aliphatic Triterpenoid	0.540	(EFSA, 2017)

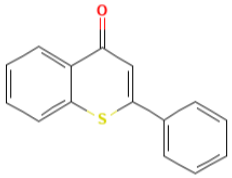
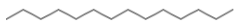
111-02-4		Squalene	0.62	Aliphatic Triterpenoid	400	(Newmark, 1997; Wolosik et al., 2013)
646-31-1		Tetracosane	3.11	Aliphatics	56.4	(WHO, 2021)
638-68-6		Triacontane	0.66	Aliphatics	56.4	(WHO, 2021)
502-69-2		6,10,14-trimethyl-2-pentadecanone	1.72	Aliphatics	0.540	(WHO, 2021)
FRACTION B obtained from 20% extract						
117-81-7		1,2-Benzene dicarboxylic acid mono 2-ethylhexyl ester	2.19		30	(EFSA, 2017)
120-51-4		Benzylbenzoate	1.52	Other	300	(WHO, 2021)
103-23-1		Bis (2-ethylhexyl) hexanedioic acid	1.62		300	(WHO, 2021)
628-97-7		Ethyl hexadecanoate	1.35	Fatty Acid	5000	(Beare-Rogers et al., 2001)

1191-41-9		Ethyl Z,Z,Z- 9,12,15-Octadecatrienoate	1.03	Fatty Acid	1600	(WHO, 2021)
4602-84-0		Farnesol	3.56	Aliphatic Triterpenoid	0.009	(Goldberg and Williams, 1991; Rapior et al., 1997; Rapior et al., 1996)
103-30-0		(E)-stilbene	1.45	Flavonoid	200	(Zamora-Ros et al., 2008)
646-31-1		Tetracosane	0.79	Aliphatics	56.4	(WHO, 2021)
		α-Tocopherolquinone	1.15	Tocopherol	15	(WHO, 2021)
502-69-2		6,10,14-trimethyl-2-pentadecanone	5.29	Aliphatics	0.540	(EFSA, 2017)
10191-41-0		Vitamin E	1.33	Tocopherol	15	(WHO, 2021)
58-95-7		Vitamin E-acetate	1.71	Tocopherol	15	(WHO, 2021)
FRACTION C obtained from 20% extract						

140-10-3		*trans*-cinnamic acid	0.32	Flavonoid	2.7	(EFSA, 2017)
6538-02--9		Ergostanol	0.42	Flavonoid	Not Absorbed	
57-10-3		Hexadecanoic acid	0.49	Fatty Acid	5000	(WHO, 2021)
1002-84-2		Pentadecanoic acid	1.25	Fatty Acid	100	(Beare-Rogers et al., 2001)
122-57-6		4-Phenylbut-3-en-2-one	0.24	Carbonyl	No data	
544-63-8		Tetradecanoic acid	0.19	Fatty Acid	1300	(Beare-Rogers et al., 2001)
502-69-2		6,10,14-trimethyl-2-pentadecanone	1.25	Aliphatics	0.540	Chung et al., 1989; EFSA, 2017)
10191-41-0		Vitamin E	0.20	Tocopherol	15	(WHO, 2021)

FRACTION D obtained from 20% extract						
92-48-8		2H-1-benzopyran-2-one	0.39	Flavonoid	No data	
305-01-1		Esculetin	0.46	Flavonoid	6.0	(WHO, 2021)
FRACTION E obtained from 20% extract						
140-10-3		*trans*-cinnamic acid	0.47	Flavonoid	2.7	(EFSA, 2017)
305-01-1		Esculetin	1.05	Flavonoid	6.0	(WHO, 2021)
544-76-3		Hexadecane	0.32	Aliphatics	56.4	(WHO, 2021)
629-59-4		Tetradecane	0.22	Aliphatics	56.4	(WHO, 2021)
126-33-0		Tetrahydrothiophene 1,1-dioxide	1.03	Other	No data	
FRACTION F obtained from 20% extract						

140-10-3		*trans*-cinnamic acid	2.05	Flavonoid	2.7	(EFSA, 2017)
1776-30-3		2,3-dimethoxy-2',4'-dihydroxy chalcone	2.86	Flavonoid	300	(WHO, 2021)
23470-00-0		Ethylhexadecanoic acid-2-hydroxy ester	3.46		5000	(Shmidhuber, 2007; Beare-Rogers et al., 2001)
544-76-3		Hexadecane	0.83	Aliphatics	56.4	(WHO, 2021)
7786-61-0		2-methoxy-4-vinyl phenol	0.94	Phenolic	0.29	(Janes et al., 2010)
593-45-3		Octadecane	1.19	Aliphatics	56.4	(WHO, 2021)

784-62-3		2-phenyl-4-H-1-benzothiopyran-4-one	2.73	Other	No data	
629-59-4		Tetradecane	0.81	Aliphatics	56.4	(WHO, 2021)
126-33-0		Tetrahydrothiophene 1,1-dioxide	8.92	Other	No data	

[a] Direct GC-MS analysis of chromatography fractions of differing purity after CC fractionation of 20% *G. africana* extract
[b] Mass spectrum of compounds match with 360000 memory banks.
[+]The CAS number is a unique numerical identifier assigned by the Chemical Abstracts Service (CAS) to chemical substances described in the open literature to create a link to information about a specific chemical substance.
[++]The relative concentration percentage (%) of any compound was calculated by the individual GC-MS peak area divided by the total peak area of the fraction and multiplying the result by 100.
[+++]The dietary intake data for the phytochemicals was obtained from data found in peer reviewed articles and reference databases.
[*]Chemical structures were obtained by using the CAS number to search for the chemicals on the PubChem database.

3.3.1. Natural occurrence and pharmacological relevance of phytochemicals identified in the *Galenia africana* extract

The components contained in the *G. africana* extracts and their relative concentrations are listed in Table 3.1. Additionally, each of these phytochemical constituents was placed into one of the following groups: aliphatics, aliphatic triterpenoids, fatty acids, flavonoids, tocopherols, and phenolic compounds. The natural occurrence and pharmacological relevance for the compounds as far as could be established from the literature is outlined below.

3.3.1.1. Aliphatics

Aliphatics are a class of hydrocarbons, where medium and long chain alkanes are components of the surface waxy cuticle of plants and fruits. The function of this waxy layer is to control the rate of moisture loss and to provide a first line of defence against pathogenic organisms (Brigelius-Flohé and Traber, 1999). Humans are also exposed to paraffins and waxes from industrial origin, mainly *via* transfer of these compounds from packaging materials and through the pharmaceutical uses of paraffins. Heptacosane, nonacosane, and pentacosane have all been shown to possess antibacterial activity (Konovalova et al., 2013). Hexacosane extracted from *Sanseveria liberica* was shown to exhibit moderately high antimicrobial activities against *Salmonella typhi, Candida albicans, Streptococcus pyogenes, Staphylococcus aureus, Escherichia coli, Pseudomonas fluorescence, Klebsiella pneumoniae, Proteus vulgaris* and *Candida krusei* (Rukaiyat et al., 2015). Due to limited information from the literature, specific pharmacological relevance of the remaining compounds classed under the aliphatic group could not be determined.

3.3.1.2. Aliphatic Triterpenoids

Farnesol, farnesyl acetone, hexahydrofarnesyl acetone, pristine, and squalene are all classified as aliphatic triterpenoids. These are commonly found in plant matter and fruits which are frequently consumed by humans. Squalene is an aliphatic triterpenoid, present in many foods including amaranth oil and olive oil. In human skin physiology, squalene is used as an antioxidant and moisturizer. It has also been reported to be used for treating skin disorders such as acne, atopic dermatitis, psoriasis, and seborrheic dermatitis (Wolosik et al., 2013). Farnesyl acetone is found in the medicinal herb *Costus pictus*, also in tomato and watermelon (Jose and Reddy, 2010). In mushrooms, farnesyl acetone has been detected as a volatile component, accounting for up to 1% of volatiles in *Suillus granulatus* and up to 12% of *Suillus luteus* (Rapior et al., 1997; Rapior et al., 1996). Farnesol has been detected in plants and fungi, accounting for up to 16% of total volatiles in *Chroogomphus rulitus* (Staines et al., 2004). However, farnesyl compounds are also endogenous in humans where they are involved in the production of cholesterol and protein tagging (Goldstein and Brown, 1990). A study conducted by Chaudary et al. (2009) demonstrated the chemo preventative effect of farnesol on skin tumorigenesis.

3.3.1.3. Fatty Acids

Fatty acids are common components found in nature, where they are present in animal or vegetable fats, oils and waxes (Lusas et al., 2017). They form chains and are classified according to the length and number of carbon atoms present in the chain, such as short, medium, long, or very long (Lusas et al., 2017). Humans can synthesise the full range of essential dietary fatty acids used for energy besides

the linoleic and alpha linolenic acids. Dietary fatty acids are used for energy through the beta-oxidation pathway in addition to assisting in cell wall synthesis and hormone manufacture (Kumari, 2018).

Ethyl hexadecanoate (also known as ethyl palmitate) is the ethyl ester of palmitic acid. It is formed in humans in the non-oxidative metabolite pathway of ethanol and has been proposed as a biomarker for ethanol consumption (Soderberg et al., 2003). It has also been listed as an emollient used in cosmetic products (PubChem, 2021). Ethyl tetracosonate is the ethyl ester of tetracosanoic acid which is present in peanut oil accounting for 1.1-2% mass fraction of total fatty acids (Beare-Rogers et al., 2001). Ethyl linoleate and ethyl linolenate are the ethyl esters of linoleic and alpha linolenic acid, respectively. According to PubChem (2021), hexadecanoic acid (palmitic acid) is used as an emollient in cosmetic products. A study by Uddin et al. (2012) demonstrated that tetracosane had significant cytotoxic activity against HT-29 colon cancer cells, and some toxicity against gastric cancer and estrogen-dependant breast cancer cells. A recent study by Gao et al., (2019) demonstrated the use of hexanedioic acid (adipic acid) as a component of a hydrogel which shows potential for wound healing applications. Additionally, adipic acid is used in cosmetics as a buffering agent (PubChem, 2021).

Linoleic acid ethyl ester (Ethyl linoleate) is an unsaturated fatty acid resulting from formal condensation of the carboxyl group of linoleic acid with the hydroxyl group of ethanol. It is used in many cosmetic products for its antibacterial and anti-inflammatory properties (Park et al., 2014). A recent study by Ko and Cho (2018) demonstrated the potential of ethyl linoleate as a non-cytotoxic and skin

whitening agent in medicine and cosmetic products. Pentadecanoic acid has an uneven number of carbon atoms, and is thought to be synthesised primarily by gut microflora in the rumen of cattle. Hence the major dietary source of this fatty acid is milk, where it accounts for approximately 1% of milk fats (Smedman et al., 1999). It has been shown that pentadecanoic acid could serve as a signalling inhibitor in breast cancer cells (To et al., 2020). Tetradecanoic acid (also known as myristic acid) is present in coconut oil and palm kernel fat accounting for approximately 16% mass fraction of total fatty acids (Beare-Rogers et al., 2001). It is also present in milk (11% mass fraction of total fatty acid), meat and meat products (Beare-Rogers et al., 2001). Tetradecanoic acid is used as a cleansing and emulsifying agent in cosmetic products (PubChem, 2020).

3.3.1.4. Flavonoids

The flavonoids comprise a group of low molecular weight compounds, of which roughly 4000 are known, and are separated into distinct subgroups: anthocyanidins, chalcones, flavanols, flavanones, flavones, isoflavones, and stannols (Weber et al., 1997). They are ubiquitous in plants and have several biological functions. For example, they are responsible for pigmentation of many plants and fruits (Kennedy et al., 1999). The flavonoids, *trans*-cinnamic acid, chalcone, chrysin, and tectochrysin, are associated with a variety of health-promoting effects and are an important component in many nutraceutical, pharmaceutical, medicinal, and cosmetic applications (Hseu et al., 2018; Modzelewska et al., 2006; Khoo et al., 2010; Oh et al., 2014; Lee et al., 2003). This is due to their anti-carcinogenic, anti-inflammatory, anti-mutagenic and antioxidative properties (Kennedy et al., 1999). Flavonoids are not classified as

essential human nutrients per se, but a high dietary intake of flavonoids is thought to be associated with lowered cancer and cardiovascular disease risk (Weber et al., 1997). 2,4,-Dihydroxychalcone is a flavonoid abundant in the leaves of *Oxytropis falcata*, a leguminous plant also known as locoweed. This plant has been widely used in Chinese/Tibetan herbal medicine. In addition to its antioxidant activity, it also exhibits anti-tumour activity (Popoola et al., 2015; Lou et al., 2009). Equol is the major intestinal bacterial metabolite of the isoflavanone diadzin in 25-60% of the population, and is influenced by diet (Setchell and Cole, 2006). Those consuming a diet high in diadzin (e.g., vegetarian diets and/or those containing soya) were more likely to metabolise the material to equol (Setchell and Cole, 2006). Equol has been shown to have anti-aging properties and antiandrogen activity (Lephart et al., 2014; Magnet et al., 2017). Naringenin is found in many citrus fruits and has been measured in grapefruit juice at concentrations of 100-800 mg/L (Skibola and Smith, 2000). There is growing evidence showing the pharmacological effects of naringenin which include anticancer, anti-inflammatory, antimicrobial, anti-mutagenic, and hepatoprotective properties (Salehi et al., 2019). Pinocembrin is a major flavanone found in honey and propolis, the resinous substance used by bees to seal honeycombs and used as a health food (Medić-Šarić et al., 2009). Pinocembrin has shown potential for use as an antioxidative, anti-inflammatory, antimicrobial, and antitumour agent (Rasul et al., 2013).

3.3.1.5. Phenolic compounds

Results reveal that 2-methoxy-4-vinylphenol to be present in many products where production involves the use of yeast and/or cooking. It is also a component

of beer, especially wheat beer, formed by the conversion of ferulic acid during the fermenting process (Coghe et al., 2004). In a study by Kim et al. (2019) it was shown that 2-methoxy-4-vinylphenol possesses anticancer properties as well as exhibits anti-aging and antioxidant activity (Jung et al., 017).

3.3.1.6. Tocopherols

Alpha-tocopherols are part of a group of fat-soluble compounds known as vitamin E. Along with being the most common form of vitamin E present in nature, alpha-tocopherols are also the most biologically active (Colosio et al., 1999). These can be found in foods such as avocados, nuts, and seeds. In a study by Weber et al., (1997) it was shown that a topical application of alpha-tocopherol to mouse skin prior to exposure of UV-irradiation resulted in the preservation of antioxidants (Van Wijk and Nierkins, 2006). Conversely, without prior application of the tocopherol, the antioxidants present in the skin were destroyed after the irradiation. Alpha-tocopherolquinone is a metabolite of alpha-tocopherol (Descotes, 2004). Vitamin E derivatives such as alpha-tocopherolquinone act as an important physiological antioxidant. Alpha-tocopherolquinone has further demonstrated its potential as a biomarker for oxidative stress (Niki and Noguchi, 2020).

Vitamin E is an essential nutrient obtained by external sources such as fresh vegetables, vegetable oils, cereals, and nuts. Vitamin E has been demonstrated to be of importance in recent dermatological studies due to its antioxidant properties. Experimental evidence suggests that topical application and oral consumption of vitamin E has anticarcinogenic, photoprotective, and skin barrier–stabilizing properties (Thiele et al., 2005). Vitamin E acetate has elicited a significant interest

for its role in assisting in curing burn injuries, particularly for its antioxidant action which occurs during tissue reperfusion. Reactive oxygen species and free radicals are produced during the phase of reperfusion of ischemic tissues, damaging numerous cell components, including nucleic acids, lipids and proteins. It has been shown that a bio adhesive film containing vitamin E acetate could facilitate skin regeneration and wound healing through the controlled release of the vitamin E acetate (Pereira et al., 2014).

3.3.2. Potential health risk of phytochemicals identified in the *Galenia africana* extract

Information from the US Dietary Reference Intake (DRI), Joint FAO/WHO Expert Committee on Food Additives (JECFA), and Beare-Rogers et al. (2009) IUPAC Technical Report were used to establish average daily background dietary intake values (Table 3.1). The background dietary intake of the constituents of *G. africana* was analysed using the European Union Pesticide Residue Intake Model (EU PRIMO) consumer risk assessment model, which informed a risk assessment for consumers (EFSA et al., 2019; EFSA 2021).

Based on the *G. africana* phytochemical data, it was observed that most compounds were already present in the general human diet, and therefore should not pose a risk to human health. Potentially relevant components, besides cinnamic acid, the coumarins, equol, and 2-methoxy-4-vinyl phenol, were all found to be non-toxic following preliminary chronic and acute consumer risk assessments. The dietary intakes and potential health risks for the phytochemicals in Table 3.1, as far as could be established from the literature, is summarised below:

For aliphatics in general, Tennant (2004) has estimated that the average intake of each of the mixed alkanes is 0.01 – 0.02 mg/kg/day. Based on this data and information from the European Food Safety Authority, which, considered and listed paraffin oils for use as a pesticide active ingredient, it was deemed that no risk assessment would be necessary as the straight chain alkanes were toxicologically non-relevant.

The aliphatic triterpenoid, squalene is especially prevalent in olive oils, and as a result human dietary intake varies greatly according to the geographical location where intake is estimated at 30 mg/day in the USA but increases to 200-400 mg/day in Mediterranean countries (Newmark, 1997). No quantitative information was available regarding the dietary intakes of faresyl acetone and hexahydrofarnesyl. However, hexahydrofarnesyl acetone undergoes beta-oxidation and is unlikely to pose greater hazards than fatty acids or aliphatics. Farnesyl acetone intake can be approximated as it is approved as a flavouring agent by JECFA with an estimated daily intake of 9 µg/day.

According to Madigan et al., (1994) the phenolic compound 2-methoxy-4-vinylphenol is present in wheat beer at a concentration of 0.68 mg/L. The average Czech Republic intake of beer is 156 L/year giving rise to an average daily intake of 2-methoxy-4-vinylphenol from beer at 0.29 mg/day. Consuming 2 L of beer in one day, as might occur occasionally, results in consumption of 1.36 mg/day.

According to Kennedy *et al*., (1999) the dietary intake of fatty acids in the USA is between 41-117 g/day with saturated fat accounting for 14-42 g of this amount (Ching and Mohamed, 2001). Given that fatty acids are a significant part of the

normal human diet, they are not toxicologically relevant components of the extract and may be excluded from risk assessments.

The potential link between flavonoid intake and health has led to a number of studies measuring the dietary intake of flavonoids in humans. These have produced differing results ranging from 23 mg/day to 1 g/day (Hertog et al., 1993; Somerset and Johannot et al., 2008; Kuhnau, 1976). As such, the potential dietary intake of the many flavonoids may account for up to 600 mg/day if all flavonoids are considered.

As a tocopherol, the recommended daily intake of vitamin E has been set by many countries. According to the US DRI of alpha-tocopherol, intake is set at 15 mg/day with an upper tolerable intake of 1,000 mg/kg (Krinsky et al., 2000).

3.4. Conclusions

Galenia africana is a plant of traditional medicinal and commercial relevance in South Africa. In this review the importance and relevance of the phytochemical constituents and potential risks relating to exposure to the *G. africana* extract was concluded in a descriptive manner. Phytochemical screening results indicate the relative chemistry and concentrations of the compounds in the plant extract. Literature also shows that several compounds present in the *G. africana* extract exhibit anti-inflammatory, antioxidant, anti-microbial, and wound healing which supports the traditional medicinal use of the plant by indigenous people. Furthermore, it was revealed that none of the potentially relevant compounds in *G. africana* were expected to cause undue risk to human health. This data regarding the potential risks related to the ingestion of *G. africana* extract could

be used to infer the potential risks relating to the accidental exposure to these phytochemical compounds when exposed to human skin upon topical application. On this basis, the effect of the *G. africana* extract on human skin in quantities similar to those described in this review should not cause undue risk to human health. However, it is important that further studies be performed using this plant to determine pharmacological action and mechanism of action, which has the potential to lead to the development of *G. africana* as a therapeutic agent.

3.5. References

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CHAPTER 4

GENERAL SUMMARY AND CONCLUSION

In Chapter 1 a review of literature has revealed that *Candida* infections are of concern among people who are immunocompromised, due to their increased likelihood of developing severe and possibly fatal disease. This is in part due to the host's inability to prevent the invasion of *Candida* upon its conversion from a less pathogenic yeast form to a more invasive hyphal form. This conversion process has been found to be facilitated by tyrosol, which is a quorum sensing compound produced by *Candida.* Tyrosol has an antagonistic effect to another quorum sensing molecule, farnesol. These quorum sensing molecules allow the microbe to sense the cell density and in response it modulates its pathogenic factors such as biofilm formation. Another factor influencing the invasion of the pathogen is the expression of adhesin and invasion proteins which facilitates induced endocytosis. In addition, an increase in the emergence of pathogens which are resistant to current therapies necessitates the exploration of alternative remedies to treat skin conditions such as fungal infections.

In Chapter 2, three southern African medicinal plants, *A. ferox, B. frutescens,* and *G. africana,* were selected based on literature, describing their traditional use as treatments for skin-related ailments, to study their effect on the production of tyrosol by *Candida* species such as *C. albicans, C. dubliniensis, C. glabrata,* and *C. tropicalis.* Upon analysis of the results obtained, it was found that none of the

three selected plant extracts exerted a significant effect of the amount of tyrosol produced by the different isolates of *Candida*. This was contrary to what was expected due to literature describing the potential of these plants as alternatives to existing fungal therapies. It is possible that the limitations of this study impacted the final results. These include the limited range of concentrations of plant extracts that were used in the experimental phase of the study. The concentration used was based on the concentrations of extracts in other studies, which tested for antifungal activity. Concentrations were kept as low as possible to avoid complete inhibition of fungal growth, however it is possible the concentrations were too low to elicit an observable effect. Furthermore, tyrosol is not the only regulatory compound produced by Candida species, as such the selected plant extracts may have exerted an effect on other regulatory compounds besides tyrosol.

In Chapter 3 the pharmacological importance and relevance of phytochemicals present in one of the plant extracts, *Galenia africana,* was determined to assess the safety of the plant's use in traditional medicine. This plant was chosen due to previous studies describing the plant's use as a skin remedy in traditional medicine. Further, there was a lack of literature detailing a comprehensive phytochemical screening and toxicological assessment of *G. africana*. The phytochemical analysis of *G. africana* revealed that the use of this extract as an alternative pharmaceutical agent should not cause any undue health risks and as such may be further investigated for its use in medicine.

The phytochemical analysis of *G. africana* revealed that the use of this extract as an alternative pharmaceutical agent should not cause any undue health risks and as such may be further investigated for its use in medicine.

Despite the limitations, this study has laid the groundwork for future research pertaining to alternative therapies for fungal infections through the down-regulation of specific compounds which increase pathogenicity of commensal organisms. Based on the outcomes of the study and its limitations, it is possible for future studies to be performed to provide a more comprehensive body of research related to this topic. Similar studies could be performed looking at the impact of the same plant extracts on other regulatory molecules produced by *Candida* species, such as farnesol. It would also be beneficial to include the use of additional plant extracts which have been described in literature for their use as skin remedies as well as using a greater range of concentrations of plant extracts in order to elicit a response.

LIST OF APPENDICES

APPENDIX 1 – API C Medium Composition

Medium

API C Medium 7 ml	Ammonium sulfate	5 g
	Monopotassium phosphate	0.31 g
	Dipotassium phosphate	0.45 g
	Disodium phosphate	0.92 g
	Sodium chloride	0.1 g
	Calcium chloride	0.05 g
	Magnesium sulfate	0.2 g
	L-Histidine	0.005 g
	L-Tryptophan	0.02 g
	L-Methionine	0.02 g
	Gelling agent	0.5 g
	Vitamin solution	1 ml
	Trace elements	10 ml
	Demineralized water	to make 1000 ml
	final pH : 6.4-6.8 (at 20-25°C)	

Figure A1.1: Chemical composition of API 20 C Aux C medium

APPENDIX 2 – API 20 C AUX Test Strips

Figure A1 shows the raw results of the API® 20 C AUX test strips. Cupules which appear to be more turbid than the "0" cupule is indicative of a positive reaction, whereas cupules that are less turbid indicate a negative reaction. These results are recorded on the result sheet (Figure A3.1, Addendum 3).

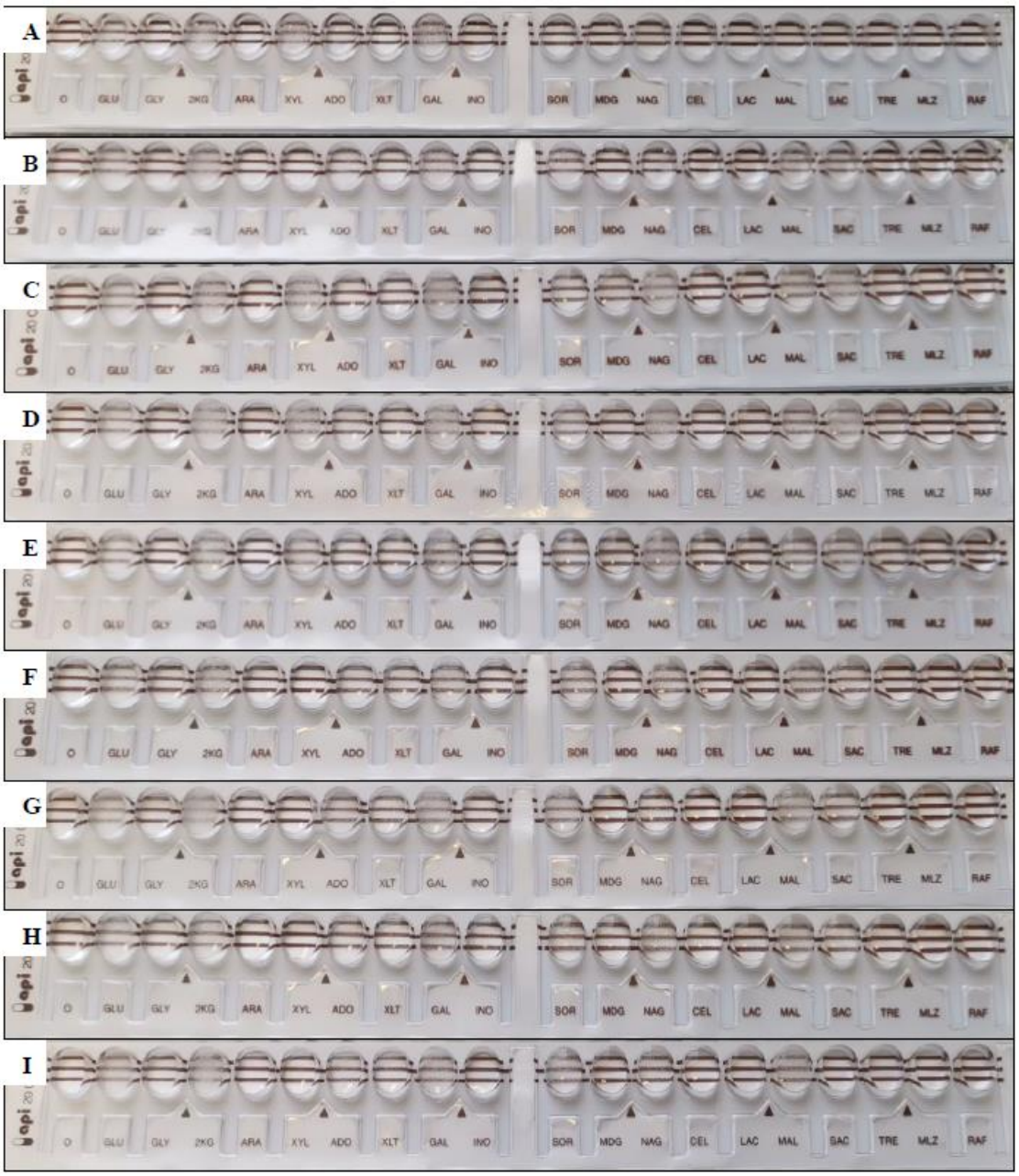

Figure A2.1: Raw results of the API® 20 C AUX test strips after 48 h to 72 h incubation.

APPENDIX 3 – API 20 C AUX Result Sheet and Identification Table

Figure A3.1 shows the result sheet which is used to record the results (+, -) of the API® 20 C AUX test strips. There are two rows for recording results, one for results after 48 h, and one row for results after 72 h. Once the results have been inserted into the sheet, it can be manually compared to the identification table (Figure 2) for confirmation of species. An alternative method of confirming the species would be to enter the results into APIWEB™, which is a software program developed by Biomériuex. This program will automatically provide the possible species, as well as the probability of accurate identification.

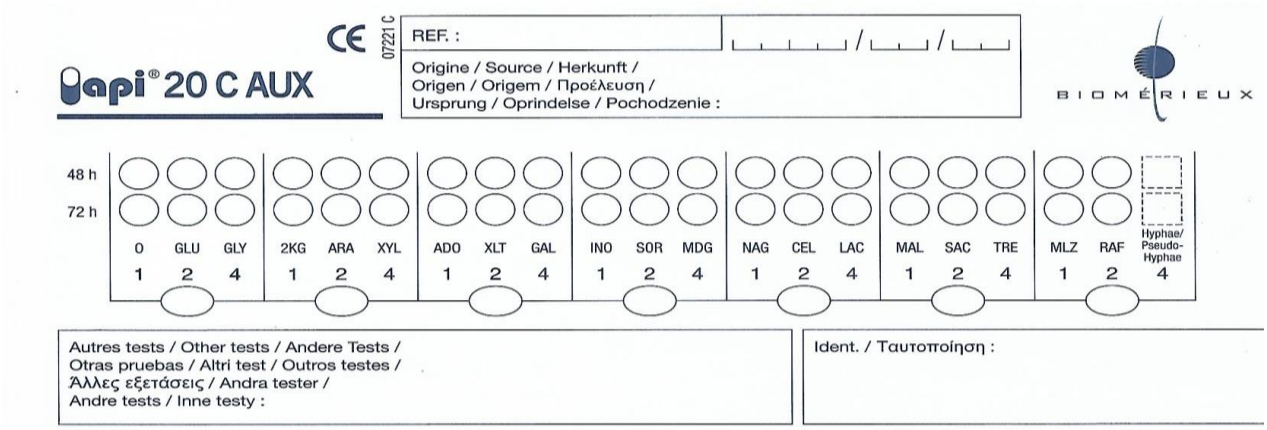

Figure A3.1: API® 20 C AUX result sheet.

The identification table for the API® 20 C AUX test strip, which is included in the package insert, is shown in Figure A3.2. The results that were recorded on the results sheet are compared to the table to identify the species of the sample. A positive result is indicated on the table by a shaded block, conversely a lack of shading is a block indicates a negative result. Each block also has a number which

shows the percentage of reactions that are positive for a specific test between 48 h and 72 h.

api® 20 C AUX 07628H - xl - 2010/02

TABLEAU D'IDENTIFICATION / IDENTIFICATION TABLE / PROZENTTABELLE / TABLA DE IDENTIFICACION / TABELLA DI IDENTIFICAZIONE / QUADRO DE IDENTIFICAÇÃO / ΠΙΝΑΚΑΣ ΤΑΥΤΟΠΟΙΗΣΗΣ / IDENTIFIERINGSTABELL / IDENTIFIKATIONSTABEL / TABELA IDENTYFIKACJI

% de réactions positives après 48-72 h (± 6 h) à 29°C ± 2°C / % of reactions positive after 48-72 hrs. (± 6 hrs) at 29°C ± 2°C / % der positiven Reaktionen nach 48-72 Std. (± 6 Std) bei 29°C ± 2°C / % de las reacciones positivas después de 48-72 H (± 6 H) a 29°C ± 2°C / % di reazioni positive dopo 48-72 ore (± 6 ore) a 29°C ± 2°C / % das reacções positivas após 48-72 H (± 6 H) a 29°C ± 2°C / % θετικών αντιδράσεων μετά από 48-72 ώρες (± 6 ώρες) στους 29°C ± 2°C / % positiva reaktioner efter 48-72 h. (± 6 h) vid 29°C ± 2°C / % af positive reaktioner efter 48-72 timer ved 29°C ± 2°C / % pozytywnych reakcji po 48-72 godzinach (± 6 godzin) w 29°C ± 2°C

API 20 C AUX V4.0	0	GLU	GLY	2KG	ARA	XYL	ADO	XLT	GAL	INO	SOR	MDG	NAG	CEL	LAC	MAL	SAC	TRE	MLZ	RAF	HYPH
Candida albicans 1	0	100	14	99	2	88	94	90	99	0	94	85	99	0	0	99	97	97	5	0	99
Candida albicans 2	0	100	1	99	1	90	1	75	99	0	70	1	99	0	0	90	1	5	1	0	99
Candida boidinii	0	100	55	1	0	89	70	89	25	0	95	1	55	0	0	1	1	1	0	0	100
Candida colliculosa	0	100	96	100	0	0	0	5	13	0	60	1	0	0	0	3	99	60	0	96	25
Candida dubliniensis	0	100	96	99	0	1	99	50	100	1	99	0	40	0	0	100	60	1	0	0	99
Candida famata	0	100	96	98	60	60	98	75	99	0	100	99	99	89	70	100	100	96	78	75	1
Candida glabrata	0	100	20	0	0	0	0	0	0	0	0	0	0	0	0	0	0	94	0	0	1
Candida guilliermondii	0	100	99	97	79	85	97	92	99	0	97	88	99	95	0	94	100	99	90	95	46
Candida kefyr	0	100	27	0	1	18	1	25	100	0	34	0	0	1	95	1	100	1	1	96	75
Candida krusei/inconspicua	0	99	73	0	0	0	0	0	6	0	2	0	64	0	0	0	0	0	0	0	79
Candida lusitaniae	0	100	90	95	1	65	95	20	30	0	99	60	95	80	0	100	99	100	99	0	75
Candida magnoliae	0	100	32	50	0	0	0	0	10	0	60	0	0	0	0	2	97	10	1	75	1
Candida norvegensis	0	100	85	0	0	0	0	0	2	0	0	0	0	25	0	0	0	0	0	0	93
Candida parapsilosis	0	100	94	88	89	89	93	3	99	0	99	89	99	0	0	100	100	93	99	1	99
Candida pelliculosa	0	100	99	0	0	67	1	1	56	0	70	95	1	70	0	97	99	87	96	30	70
Candida rugosa	0	100	74	0	1	70	1	26	99	0	94	0	59	0	0	0	0	0	0	0	99
Candida sphaerica 1	0	100	31	2	0	2	0	62	99	0	99	68	0	35	1	95	100	99	29	76	99
Candida sphaerica 2	0	100	88	1	0	1	0	36	94	0	99	50	0	31	99	80	100	53	80	64	1
Candida tropicalis	0	100	9	99	1	96	99	12	99	0	99	69	99	17	1	99	73	100	72	5	99
Candida utilis	0	100	99	0	0	60	0	1	5	0	1	3	0	37	0	98	96	16	72	79	69
Candida zeylanoides	0	100	100	87	0	0	1	0	1	0	99	0	99	0	0	0	0	74	0	0	75
Cryptococcus albidus	0	100	0	98	80	81	0	0	6	30	60	65	0	99	10	98	100	82	81	51	1
Cryptococcus humicola	0	100	82	100	100	100	36	64	100	100	95	100	100	98	100	100	99	99	95	99	99
Cryptococcus laurentii	0	100	6	92	99	99	69	76	99	84	53	76	92	96	99	92	99	92	96	99	25
Cryptococcus neoformans	0	100	0	100	14	91	71	1	93	97	100	99	88	10	0	99	99	75	97	88	25
Cryptococcus terreus	0	100	0	100	87	100	0	0	45	50	99	0	96	96	36	0	0	54	0	0	1
Cryptococcus uniguttulatus	0	100	3	99	99	99	3	0	1	99	50	99	100	0	0	100	100	75	100	7	25
Geotrichum capitatum	0	95	92	0	0	0	0	0	25	0	10	0	2	0	0	0	0	0	0	0	95
Geotrichum klebahnii	0	100	100	0	0	92	0	0	75	0	88	0	0	0	0	0	0	0	0	0	92
Kloeckera spp	0	100	0	50	1	0	0	0	0	0	0	0	0	96	0	0	0	0	0	0	1
Kodamaea ohmeri	0	100	99	96	0	0	66	0	84	0	93	98	99	56	0	99	99	93	0	80	84
Pichia angusta	0	100	84	0	1	1	66	36	0	0	90	1	1	20	0	94	90	46	97	0	2
Prototheca wickerhamii	0	100	100	0	0	0	0	0	55	0	0	0	0	0	0	0	0	100	0	0	1
Rhodotorula glutinis	0	100	15	91	0	0	8	0	50	0	84	3	0	1	0	91	100	59	84	96	1
Rhodotorula minuta	0	100	100	100	98	95	3	0	0	0	5	0	85	60	1	0	95	95	95	0	1
Rhodotorula mucilaginosa 1	0	100	5	4	15	33	92	61	10	0	5	0	0	0	0	33	100	5	1	87	25
Rhodotorula mucilaginosa 2	0	100	60	1	80	80	64	52	80	0	60	1	0	1	0	98	100	95	86	98	25
Saccharomyces cerevisiae 1	0	100	8	0	0	0	0	0	78	0	1	13	0	0	0	75	90	2	1	62	30
Saccharomyces cerevisiae 2	0	100	1	0	0	0	0	0	99	0	1	29	0	0	0	99	99	99	85	81	25
Sporobolomyces salmonicolor	0	100	1	0	0	0	0	0	5	0	80	0	0	0	0	0	100	85	0	70	90
Stephanoascus ciferrii	0	100	80	80	100	100	71	60	100	100	43	0	99	60	0	99	100	99	0	99	100
Trichosporon asahii	0	100	20	100	100	100	0	5	100	0	1	94	100	100	100	100	98	66	20	0	95
Trichosporon inkin	0	100	4	100	0	98	0	0	95	98	0	100	57	100	95	100	100	95	89	0	95
Trichosporon mucoides	0	100	40	99	74	100	53	65	100	92	78	100	94	100	100	100	100	78	82	99	95

bioMérieux SA II

Figure 3.2: API® 20 C AUX identification table.

APPENDIX 4 – VITEK 2 Raw Data

CPUT Technology

bioMérieux Customer:
System #:

Laboratory Report

Printed Sep 13, 2018 14:45 CAT
Printed by: Rose

Isolate: A-1 (Approved)

Card Type: YST Bar Code: 2430316403018386 Testing Instrument: 000017227F7D (13707)
Setup Technologist: Rose Gwanpu(Rose)

Bionumber: 4102544065327371
Organism Quantity: **Selected Organism: Candida albicans**

Comments:	

Identification Information	**Card:** YST		**Lot Number:** 2430316403	**Expires:**	Oct 8, 2018 12:00 CAT
	Completed:	Sep 8, 2018 04:10 CAT	**Status:** Final	**Analysis Time:**	17.82 hours
Organism Origin	VITEK 2				
Selected Organism	97% Probability		**Candida albicans**		
	Bionumber:	4102544065327371		**Confidence:**	Excellent identification
SRF Organism					
Analysis Organisms and Tests to Separate:					
Analysis Messages:					
Contraindicating Typical Biopattern(s)					
Candida albicans	NAGA1(91),				

Biochemical Details																	
3	LysA	-	4	IMLTa	(-)	5	LeuA	+	7	ARG	+	10	ERYa	-	12	GLYLa	-
13	TyrA	-	14	BNAG	-	15	ARBa	-	18	AMYa	-	19	dGALa	+	20	GENa	-
21	dGLUa	+	23	LACa	-	24	MAdGa	+	26	dCELa	-	27	GGT	-	28	dMALa	+
29	dRAFa	-	30	NAGA1	-	32	dMNEa	+	33	dMELa	-	34	dMLZa	-	38	ISBEa	-
39	IRHAa	-	40	XLTa	+	42	dSORa	+	44	SACa	+	45	URE	-	46	AGLU	+
47	dTURa	+	48	dTREa	+	49	NO3a	-	51	IARAa	-	52	dGATa	+	53	ESC	-
54	IGLTa	+	55	dXYLa	+	56	LATa	+	58	ACEa	+	59	CITa	+	60	GRTas	-
61	IPROa	+	62	2KGa	+	63	NAGa	+	64	dGNTa	+						

Installed VITEK 2 Systems Version: 08.01
MIC Interpretation Guideline:
AES Parameter Set Name:

Therapeutic Interpretation Guideline:
AES Parameter Last Modified:

CPUT Technology

bioMérieux Customer:
System #:

Laboratory Report

Printed Sep 13, 2018 14:47 CAT
Printed by: Rose

Isolate: B-1 (Approved)

Card Type: YST Bar Code: 2430316403018382 Testing Instrument: 000017227F7D (13707)
Setup Technologist: Rose Gwanpu(Rose)

Bionumber: 6103544061305370
Organism Quantity: **Selected Organism: Candida dubliniensis**

Comments:	

Identification Information	**Card:**	YST	**Lot Number:**	2430316403	**Expires:**	Oct 8, 2018 12:00 CAT
	Completed:	Sep 8, 2018 04:09 CAT	**Status:**	Final	**Analysis Time:**	17.80 hours
Organism Origin	VITEK 2					
Selected Organism	99% Probability		**Candida dubliniensis**			
	Bionumber:	6103544061305370			**Confidence:**	Excellent identification
SRF Organism						
Analysis Organisms and Tests to Separate:						
Analysis Messages:						
Contraindicating Typical Biopattern(s)						

Biochemical Details

3	LysA	-	4	IMLTa	+	5	LeuA	+	7	ARG	+	10	ERYa	-	12	GLYLa	-
13	TyrA	-	14	BNAG	-	15	ARBa	-	18	AMYa	(+)	19	dGALa	+	20	GENa	-
21	dGLUa	+	23	LACa	-	24	MAdGa	+	26	dCELa	-	27	GGT	-	28	dMALa	+
29	dRAFa	-	30	NAGA1	-	32	dMNEa	+	33	dMELa	-	34	dMLZa	-	38	ISBEa	-
39	IRHAa	-	40	XLTa	+	42	dSORa	+	44	SACa	+	45	URE	-	46	AGLU	-
47	dTURa	+	48	dTREa	+	49	NO3a	-	51	IARAa	-	52	dGATa	-	53	ESC	-
54	IGLTa	+	55	dXYLa	-	56	LATa	+	58	ACEa	+	59	CITa	+	60	GRTas	-
61	IPROa	+	62	2KGa	+	63	NAGa	(+)	64	dGNTa	-						

Installed VITEK 2 Systems Version: 08.01
MIC Interpretation Guideline:
AES Parameter Set Name:

Therapeutic Interpretation Guideline:
AES Parameter Last Modified:

CPUT Technology

bioMérieux Customer:
System #:

Laboratory Report

Printed Sep 13, 2018 14:48 CAT
Printed by: Rose

Isolate: C-1 (Approved)

Card Type: YST Bar Code: 2430316403018383 Testing Instrument: 000017227F7D (13707)
Setup Technologist: Rose Gwanpu(Rose)

Bionumber: 4102544065327771
Organism Quantity: **Selected Organism: Candida albicans**

Comments:	

Identification Information	**Card:**	YST	**Lot Number:**	2430316403	**Expires:**	Oct 8, 2018 12:00 CAT
	Completed:	Sep 8, 2018 04:09 CAT	**Status:**	Final	**Analysis Time:**	17.80 hours
Organism Origin	VITEK 2					
Selected Organism	97% Probability		**Candida albicans**			
	Bionumber:	4102544065327771			**Confidence:**	Excellent identification
SRF Organism						
Analysis Organisms and Tests to Separate:						
Analysis Messages:						
Contraindicating Typical Biopattern(s)						
Candida albicans	NAGA1(91),					

Biochemical Details

3	LysA	-	4	IMLTa	-	5	LeuA	+	7	ARG	+	10	ERYa	-	12	GLYLa	-
13	TyrA	-	14	BNAG	-	15	ARBa	-	18	AMYa	-	19	dGALa	+	20	GENa	-
21	dGLUa	+	23	LACa	-	24	MAdGa	+	26	dCELa	-	27	GGT	-	28	dMALa	+
29	dRAFa	-	30	NAGA1	-	32	dMNEa	+	33	dMELa	-	34	dMLZa	-	38	ISBEa	-
39	IRHAa	-	40	XLTa	+	42	dSORa	+	44	SACa	+	45	URE	-	46	AGLU	+
47	dTURa	+	48	dTREa	+	49	NO3a	-	51	IARAa	-	52	dGATa	+	53	ESC	-
54	IGLTa	+	55	dXYLa	+	56	LATa	+	58	ACEa	+	59	CITa	+	60	GRTas	+
61	IPROa	+	62	2KGa	+	63	NAGa	+	64	dGNTa	+						

Installed VITEK 2 Systems Version: 08.01
MIC Interpretation Guideline:
AES Parameter Set Name:

Therapeutic Interpretation Guideline:
AES Parameter Last Modified:

CPUT Technology

bioMérieux Customer:
System #:

Laboratory Report

Printed Sep 13, 2018 14:49 CAT
Printed by: Rose

Isolate: D-1 (Approved)

Card Type: YST Bar Code: 2430316403018384 Testing Instrument: 000017227F7D (13707)
Setup Technologist: Rose Gwanpu(Rose)

Bionumber: 6102546065327371
Organism Quantity: **Selected Organism: Candida albicans**

Comments:	

Identification Information	**Card:**	YST	**Lot Number:**	2430316403	**Expires:**	Oct 8, 2018 12:00 CAT
	Completed:	Sep 8, 2018 04:08 CAT	**Status:**	Final	**Analysis Time:**	17.80 hours
Organism Origin	VITEK 2					
Selected Organism	99% Probability		**Candida albicans**			
	Bionumber:	6102546065327371			**Confidence:**	Excellent identification
SRF Organism						
Analysis Organisms and Tests to Separate:						
Analysis Messages:						
Contraindicating Typical Biopattern(s)						

Biochemical Details

3	LysA	-	4	IMLTa	+	5	LeuA	+	7	ARG	+	10	ERYa	-	12	GLYLa	-
13	TyrA	-	14	BNAG	-	15	ARBa	-	18	AMYa	-	19	dGALa	+	20	GENa	-
21	dGLUa	+	23	LACa	-	24	MAdGa	+	26	dCELa	-	27	GGT	-	28	dMALa	+
29	dRAFa	-	30	NAGA1	+	32	dMNEa	+	33	dMELa	-	34	dMLZa	-	38	ISBEa	-
39	IRHAa	-	40	XLTa	+	42	dSORa	+	44	SACa	+	45	URE	-	46	AGLU	+
47	dTURa	+	48	dTREa	+	49	NO3a	-	51	IARAa	-	52	dGATa	+	53	ESC	-
54	IGLTa	+	55	dXYLa	+	56	LATa	+	58	ACEa	+	59	CITa	+	60	GRTas	-
61	IPROa	+	62	2KGa	+	63	NAGa	+	64	dGNTa	+						

Installed VITEK 2 Systems Version: 08.01
MIC Interpretation Guideline:
AES Parameter Set Name:

Therapeutic Interpretation Guideline:
AES Parameter Last Modified:

CPUT Technology

bioMérieux Customer:
System #:

Laboratory Report

Printed Sep 13, 2018 14:49 CAT
Printed by: Rose

Isolate: E-1 (Approved)

Card Type: YST Bar Code: 2430316403018385 Testing Instrument: 000017227F7D (13707)
Setup Technologist: Rose Gwanpu(Rose)

Bionumber: 4102544065327571
Organism Quantity: **Selected Organism: Candida albicans**

Comments:	

Identification Information	**Card:**	YST	**Lot Number:**	2430316403	**Expires:**	Oct 8, 2018 12:00 CAT
	Completed:	Sep 8, 2018 04:07 CAT	**Status:**	Final	**Analysis Time:**	17.78 hours
Organism Origin	VITEK 2					
Selected Organism	98% Probability		**Candida albicans**			
	Bionumber:	4102544065327571			**Confidence:**	Excellent identification
SRF Organism						
Analysis Organisms and Tests to Separate:						
Analysis Messages:						
Contraindicating Typical Biopattern(s)						
Candida albicans	CITa(82),					

Biochemical Details

3	LysA	-	4	IMLTa	-	5	LeuA	+	7	ARG	+	10	ERYa	-	12	GLYLa	-
13	TyrA	-	14	BNAG	-	15	ARBa	-	18	AMYa	-	19	dGALa	+	20	GENa	-
21	dGLUa	+	23	LACa	-	24	MAdGa	+	26	dCELa	-	27	GGT	-	28	dMALa	+
29	dRAFa	-	30	NAGA1	(-)	32	dMNEa	+	33	dMELa	-	34	dMLZa	-	38	ISBEa	-
39	IRHAa	-	40	XLTa	+	42	dSORa	+	44	SACa	+	45	URE	-	46	AGLU	+
47	dTURa	+	48	dTREa	+	49	NO3a	-	51	IARAa	-	52	dGATa	+	53	ESC	-
54	IGLTa	+	55	dXYLa	+	56	LATa	+	58	ACEa	+	59	CITa	-	60	GRTas	(+)
61	IPROa	+	62	2KGa	+	63	NAGa	+	64	dGNTa	+						

Installed VITEK 2 Systems Version: 08.01
MIC Interpretation Guideline:
AES Parameter Set Name:

Therapeutic Interpretation Guideline:
AES Parameter Last Modified:

CPUT Technology

bioMérieux Customer:
System #:

Laboratory Report

Printed Sep 13, 2018 14:50 CAT
Printed by: Rose

Isolate: F-1 (Approved)

Card Type: YST Bar Code: 2430316403018329 Testing Instrument: 000017227F7D (13707)
Setup Technologist: Rose Gwanpu(Rose)

Bionumber: 6002544061305370
Organism Quantity: **Selected Organism: Candida dubliniensis**

Comments:	

Identification Information	**Card:** YST		**Lot Number:** 2430316403	**Expires:**	Oct 8, 2018 12:00 CAT
	Completed:	Sep 8, 2018 04:07 CAT	**Status:** Final	**Analysis Time:**	17.78 hours
Organism Origin	VITEK 2				
Selected Organism	97% Probability		**Candida dubliniensis**		
	Bionumber:	6002544061305370		**Confidence:**	Excellent identification
SRF Organism					
Analysis Organisms and Tests to Separate:					
Analysis Messages:					
Contraindicating Typical Biopattern(s)					
Candida dubliniensis	ARG(84),				

Biochemical Details

3	LysA	-	4	IMLTa	+	5	LeuA	+	7	ARG	-	10	ERYa	-	12	GLYLa	-
13	TyrA	-	14	BNAG	-	15	ARBa	-	18	AMYa	-	19	dGALa	+	20	GENa	-
21	dGLUa	+	23	LACa	-	24	MAdGa	+	26	dCELa	-	27	GGT	-	28	dMALa	+
29	dRAFa	-	30	NAGA1	-	32	dMNEa	+	33	dMELa	-	34	dMLZa	-	38	ISBEa	-
39	IRHAa	-	40	XLTa	+	42	dSORa	+	44	SACa	+	45	URE	-	46	AGLU	-
47	dTURa	+	48	dTREa	+	49	NO3a	-	51	IARAa	-	52	dGATa	-	53	ESC	-
54	IGLTa	+	55	dXYLa	-	56	LATa	+	58	ACEa	+	59	CITa	+	60	GRTas	-
61	IPROa	+	62	2KGa	+	63	NAGa	+	64	dGNTa	-						

Installed VITEK 2 Systems Version: 08.01
MIC Interpretation Guideline:
AES Parameter Set Name:

Therapeutic Interpretation Guideline:
AES Parameter Last Modified:

CPUT Technology

bioMérieux Customer:
System #:

Laboratory Report

Printed Sep 13, 2018 14:51 CAT
Printed by: Rose

Isolate: G-1 (Approved)

Card Type: YST Bar Code: 2430316403017161 Testing Instrument: 000017227F7D (13707)
Setup Technologist: Rose Gwanpu(Rose)

Bionumber: 4002544061305370
Organism Quantity: **Selected Organism: Candida dubliniensis**

Comments:	

Identification Information	**Card:**	YST	**Lot Number:**	2430316403	**Expires:**	Oct 8, 2018 12:00 CAT
	Completed:	Sep 8, 2018 04:06 CAT	**Status:**	Final	**Analysis Time:**	17.77 hours
Organism Origin	VITEK 2					
Selected Organism	95% Probability		**Candida dubliniensis**			
	Bionumber:	4002544061305370			**Confidence:**	Very good identification
SRF Organism						
Analysis Organisms and Tests to Separate:						
Analysis Messages:						
Contraindicating Typical Biopattern(s)						
Candida dubliniensis	IMLTa(96),ARG(84),					

Biochemical Details

3	LysA	-	4	IMLTa	-	5	LeuA	+	7	ARG	-	10	ERYa	-	12	GLYLa	-
13	TyrA	-	14	BNAG	-	15	ARBa	-	18	AMYa	-	19	dGALa	+	20	GENa	-
21	dGLUa	+	23	LACa	-	24	MAdGa	+	26	dCELa	-	27	GGT	-	28	dMALa	+
29	dRAFa	-	30	NAGA1	-	32	dMNEa	+	33	dMELa	-	34	dMLZa	-	38	ISBEa	-
39	IRHAa	-	40	XLTa	+	42	dSORa	+	44	SACa	+	45	URE	-	46	AGLU	-
47	dTURa	+	48	dTREa	+	49	NO3a	-	51	IARAa	-	52	dGATa	-	53	ESC	-
54	IGLTa	+	55	dXYLa	-	56	LATa	+	58	ACEa	+	59	CITa	+	60	GRTas	-
61	IPROa	+	62	2KGa	+	63	NAGa	+	64	dGNTa	-						

Installed VITEK 2 Systems Version: 08.01
MIC Interpretation Guideline:
AES Parameter Set Name:

Therapeutic Interpretation Guideline:
AES Parameter Last Modified:

CPUT Technology

bioMérieux Customer:
System #:

Laboratory Report

Printed Sep 13, 2018 14:52 CAT
Printed by: Rose

Isolate: H-1 (Approved)

Card Type: YST Bar Code: 2430316403017160 Testing Instrument: 000017227F7D (13707)
Setup Technologist: Rose Gwanpu(Rose)

Bionumber: 6112544265323771
Organism Quantity: **Selected Organism: Candida tropicalis**

Comments:	

Identification Information	**Card:** YST	**Lot Number:** 2430316403	**Expires:** Oct 8, 2018 12:00 CAT
	Completed: Sep 8, 2018 04:05 CAT	**Status:** Final	**Analysis Time:** 17.77 hours
Organism Origin	VITEK 2		
Selected Organism	98% Probability **Candida tropicalis** **Bionumber:** 6112544265323771		**Confidence:** Excellent identification
SRF Organism			
Analysis Organisms and Tests to Separate:			
Analysis Messages:			
Contraindicating Typical Biopattern(s) Candida tropicalis TyrA(21),			

Biochemical Details

3	LysA	-	4	IMLTa	+	5	LeuA	+	7	ARG	+	10	ERYa	-	12	GLYLa	-
13	TyrA	+	14	BNAG	-	15	ARBa	-	18	AMYa	-	19	dGALa	+	20	GENa	-
21	dGLUa	+	23	LACa	-	24	MAdGa	+	26	dCELa	-	27	GGT	(-)	28	dMALa	+
29	dRAFa	-	30	NAGA1	-	32	dMNEa	+	33	dMELa	-	34	dMLZa	+	38	ISBEa	-
39	IRHAa	-	40	XLTa	+	42	dSORa	+	44	SACa	+	45	URE	-	46	AGLU	+
47	dTURa	+	48	dTREa	+	49	NO3a	-	51	lARAa	-	52	dGATa	+	53	ESC	-
54	IGLTa	+	55	dXYLa	+	56	LATa	-	58	ACEa	+	59	CITa	+	60	GRTas	+
61	IPROa	+	62	2KGa	+	63	NAGa	(+)	64	dGNTa	+						

Installed VITEK 2 Systems Version: 08.01
MIC Interpretation Guideline:
AES Parameter Set Name:

Therapeutic Interpretation Guideline:
AES Parameter Last Modified:

CPUT Technology

bioMérieux Customer:
System #:

Laboratory Report

Printed Nov 21, 2018 12:18 CAT
Printed by: Rose

Isolate: I-1 (Approved)

Card Type: YST Bar Code: 2430790203629737 Testing Instrument: 000017227F7D (13707)
Setup Technologist: Rose Gwanpu(Rose)

Bionumber: 6102546061325371
Organism Quantity: **Selected Organism: Candida dubliniensis**

Comments:	

Identification Information	**Card:**	YST	**Lot Number:**	2430790203	**Expires:**	Jan 25, 2020 12:00 CAT
	Completed:	Nov 10, 2018 05:49 CAT	**Status:**	Final	**Analysis Time:**	17.80 hours
Organism Origin	VITEK 2					
Selected Organism	99% Probability		**Candida dubliniensis**			
	Bionumber:	6102546061325371			**Confidence:**	Excellent identification
SRF Organism						
Analysis Organisms and Tests to Separate:						
Analysis Messages:						
Contraindicating Typical Biopattern(s)						

Biochemical Details																	
3	LysA	-	4	IMLTa	+	5	LeuA	+	7	ARG	+	10	ERYa	-	12	GLYLa	-
13	TyrA	-	14	BNAG	-	15	ARBa	-	18	AMYa	-	19	dGALa	+	20	GENa	-
21	dGLUa	+	23	LACa	-	24	MAdGa	+	26	dCELa	-	27	GGT	-	28	dMALa	+
29	dRAFa	-	30	NAGA1	+	32	dMNEa	+	33	dMELa	-	34	dMLZa	-	38	ISBEa	-
39	IRHAa	-	40	XLTa	+	42	dSORa	+	44	SACa	+	45	URE	-	46	AGLU	-
47	dTURa	+	48	dTREa	+	49	NO3a	-	51	IARAa	-	52	dGATa	(+)	53	ESC	-
54	IGLTa	+	55	dXYLa	-	56	LATa	+	58	ACEa	+	59	CITa	+	60	GRTas	-
61	IPROa	+	62	2KGa	+	63	NAGa	+	64	dGNTa	(+)						

Installed VITEK 2 Systems Version: 08.01
MIC Interpretation Guideline:
AES Parameter Set Name:

Therapeutic Interpretation Guideline:
AES Parameter Last Modified:

CPUT Technology

bioMérieux Customer:
System #:

Laboratory Report

Printed Nov 21, 2018 12:20 CAT
Printed by: Rose

Isolate: j-1 (Approved)

Card Type: YST Bar Code: 2430790203629736 Testing Instrument: 000017227F7D (13707)
Setup Technologist: Rose Gwanpu(Rose)

Bionumber: 6102544061305370
Organism Quantity: **Selected Organism: Candida dubliniensis**

Comments:	

Identification Information	**Card:**	YST	**Lot Number:**	2430790203	**Expires:**	Jan 25, 2020 12:00 CAT
	Completed:	Nov 10, 2018 05:48 CAT	**Status:**	Final	**Analysis Time:**	17.80 hours
Organism Origin	VITEK 2					
Selected Organism	99% Probability		**Candida dubliniensis**			
	Bionumber:	6102544061305370			**Confidence:**	Excellent identification
SRF Organism						
Analysis Organisms and Tests to Separate:						
Analysis Messages:						
Contraindicating Typical Biopattern(s)						

Biochemical Details

3	LysA	-	4	IMLTa	+	5	LeuA	+	7	ARG	(+)	10	ERYa	-	12	GLYLa	-
13	TyrA	-	14	BNAG	-	15	ARBa	-	18	AMYa	-	19	dGALa	+	20	GENa	-
21	dGLUa	+	23	LACa	-	24	MAdGa	+	26	dCELa	-	27	GGT	-	28	dMALa	+
29	dRAFa	-	30	NAGA1	-	32	dMNEa	+	33	dMELa	-	34	dMLZa	-	38	ISBEa	-
39	IRHAa	-	40	XLTa	+	42	dSORa	+	44	SACa	+	45	URE	-	46	AGLU	-
47	dTURa	+	48	dTREa	+	49	NO3a	-	51	lARAa	-	52	dGATa	-	53	ESC	-
54	IGLTa	+	55	dXYLa	-	56	LATa	+	58	ACEa	+	59	CITa	+	60	GRTas	-
61	IPROa	+	62	2KGa	+	63	NAGa	+	64	dGNTa	(-)						

Installed VITEK 2 Systems Version: 08.01
MIC Interpretation Guideline:
AES Parameter Set Name:

Therapeutic Interpretation Guideline:
AES Parameter Last Modified:

CPUT Technology

bioMérieux Customer:
System #:

Laboratory Report

Printed Nov 21, 2018 12:21 CAT
Printed by: Rose

Isolate: k-1 (Approved)

Card Type: YST Bar Code: 2430790203629735 Testing Instrument: 000017227F7D (13707)
Setup Technologist: Rose Gwanpu(Rose)

Bionumber: 6102546061325371
Organism Quantity: **Selected Organism: Candida dubliniensis**

Comments:	

Identification Information	**Card:** YST	**Lot Number:** 2430790203	**Expires:** Jan 25, 2020 12:00 CAT
	Completed: Nov 10, 2018 05:47 CAT	**Status:** Final	**Analysis Time:** 17.78 hours
Organism Origin	VITEK 2		
Selected Organism	99% Probability **Candida dubliniensis**		
	Bionumber: 6102546061325371		**Confidence:** Excellent identification
SRF Organism			
Analysis Organisms and Tests to Separate:			
Analysis Messages:			
Contraindicating Typical Biopattern(s)			

Biochemical Details

3	LysA	-	4	IMLTa	+	5	LeuA	+	7	ARG	+	10	ERYa	-	12	GLYLa	-
13	TyrA	-	14	BNAG	-	15	ARBa	-	18	AMYa	-	19	dGALa	+	20	GENa	-
21	dGLUa	+	23	LACa	-	24	MAdGa	+	26	dCELa	-	27	GGT	-	28	dMALa	+
29	dRAFa	-	30	NAGA1	+	32	dMNEa	+	33	dMELa	-	34	dMLZa	-	38	ISBEa	-
39	IRHAa	-	40	XLTa	+	42	dSORa	+	44	SACa	+	45	URE	-	46	AGLU	-
47	dTURa	+	48	dTREa	+	49	NO3a	-	51	lARAa	-	52	dGATa	(+)	53	ESC	-
54	IGLTa	+	55	dXYLa	-	56	LATa	+	58	ACEa	+	59	CITa	+	60	GRTas	-
61	IPROa	+	62	2KGa	+	63	NAGa	+	64	dGNTa	+						

Installed VITEK 2 Systems Version: 08.01
MIC Interpretation Guideline:
AES Parameter Set Name:

Therapeutic Interpretation Guideline:
AES Parameter Last Modified:

CPUT Technology

bioMérieux Customer:
System #:

Laboratory Report

Printed Nov 21, 2018 12:21 CAT
Printed by: Rose

Isolate: L-1 (Approved)

Card Type: YST Bar Code: 2430790203629888 Testing Instrument: 000017227F7D (13707)
Setup Technologist: Rose Gwanpu(Rose)

Bionumber: 4000104000201111
Organism Quantity: **Selected Organism: Candida glabrata**

Comments:	

Identification Information	**Card:** YST	**Lot Number:** 2430790203	**Expires:** Jan 25, 2020 12:00 CAT
	Completed: Nov 10, 2018 05:47 CAT	**Status:** Final	**Analysis Time:** 17.78 hours
Organism Origin	VITEK 2		
Selected Organism	99% Probability **Candida glabrata**		
	Bionumber: 4000104000201111		**Confidence:** Excellent identification
SRF Organism			
Analysis Organisms and Tests to Separate:			
Analysis Messages: See product information for additional information.			
Contraindicating Typical Biopattern(s)			

Biochemical Details

3	LysA	-	4	IMLTa	-	5	LeuA	+	7	ARG	-	10	ERYa	-	12	GLYLa	-
13	TyrA	-	14	BNAG	-	15	ARBa	-	18	AMYa	-	19	dGALa	-	20	GENa	-
21	dGLUa	+	23	LACa	-	24	MAdGa	-	26	dCELa	-	27	GGT	-	28	dMALa	-
29	dRAFa	-	30	NAGA1	-	32	dMNEa	+	33	dMELa	-	34	dMLZa	-	38	ISBEa	-
39	IRHAa	-	40	XLTa	-	42	dSORa	-	44	SACa	-	45	URE	-	46	AGLU	-
47	dTURa	-	48	dTREa	+	49	NO3a	-	51	IARAa	-	52	dGATa	-	53	ESC	-
54	IGLTa	+	55	dXYLa	-	56	LATa	-	58	ACEa	+	59	CITa	-	60	GRTas	-
61	IPROa	+	62	2KGa	-	63	NAGa	-	64	dGNTa	+						

Installed VITEK 2 Systems Version: 08.01
MIC Interpretation Guideline:
AES Parameter Set Name:

Therapeutic Interpretation Guideline:
AES Parameter Last Modified:

CPUT Technology

bioMérieux Customer:
System #:

Laboratory Report

Printed Nov 21, 2018 12:23 CAT
Printed by: Rose

Isolate: M-1 (Approved)

Card Type: YST Bar Code: 2430790203629755 Testing Instrument: 000017227F7D (13707)
Setup Technologist: Rose Gwanpu(Rose)

Bionumber: 4000104000200111
Organism Quantity: **Selected Organism: Candida glabrata**

Comments:	

Identification Information	**Card:** YST	**Lot Number:** 2430790203	**Expires:** Jan 25, 2020 12:00 CAT
	Completed: Nov 10, 2018 05:46 CAT	**Status:** Final	**Analysis Time:** 17.77 hours
Organism Origin	VITEK 2		
Selected Organism	99% Probability **Candida glabrata** **Bionumber:** 4000104000200111		**Confidence:** Excellent identification
SRF Organism			
Analysis Organisms and Tests to Separate:			
Analysis Messages: See product information for additional information.			
Contraindicating Typical Biopattern(s)			

Biochemical Details

3	LysA	-	4	IMLTa	-	5	LeuA	+	7	ARG	-	10	ERYa	-	12	GLYLa	-
13	TyrA	-	14	BNAG	-	15	ARBa	-	18	AMYa	-	19	dGALa	-	20	GENa	-
21	dGLUa	+	23	LACa	-	24	MAdGa	-	26	dCELa	-	27	GGT	-	28	dMALa	-
29	dRAFa	-	30	NAGA1	-	32	dMNEa	+	33	dMELa	-	34	dMLZa	-	38	ISBEa	-
39	IRHAa	-	40	XLTa	-	42	dSORa	-	44	SACa	-	45	URE	-	46	AGLU	-
47	dTURa	-	48	dTREa	+	49	NO3a	-	51	lARAa	-	52	dGATa	-	53	ESC	-
54	lGLTa	-	55	dXYLa	-	56	LATa	-	58	ACEa	+	59	CITa	-	60	GRTas	-
61	lPROa	+	62	2KGa	-	63	NAGa	-	64	dGNTa	(+)						

Installed VITEK 2 Systems Version: 08.01
MIC Interpretation Guideline:
AES Parameter Set Name:

Therapeutic Interpretation Guideline:
AES Parameter Last Modified:

CPUT Technology

bioMérieux Customer:
System #:

Laboratory Report

Printed Nov 21, 2018 12:23 CAT
Printed by: Rose

Isolate: N-1 (Qualified)

Card Type: YST Bar Code: 2430790203629756 Testing Instrument: 000017227F7D (13707)
Setup Technologist: Rose Gwanpu(Rose)

Bionumber: 6102544675327371
Organism Quantity: **Selected Organism: Low Discrimination**

Comments:	

Identification Information	**Card:**	YST	**Lot Number:** 2430790203	**Expires:**	Jan 25, 2020 12:00 CAT
	Completed:	Nov 10, 2018 05:46 CAT	**Status:** Final	**Analysis Time:**	17.77 hours
Organism Origin	VITEK 2				
Selected Organism	**Low Discrimination**				
	Bionumber:	6102544675327371		**Confidence:**	Low discrimination
SRF Organism					

Analysis Organisms and Tests to Separate:

Low Discrimination Organism

Candida lusitaniae	IRHAMNOSEa(100),
Candida tropicalis	IRHAMNOSEa(0),

Analysis Messages:

Contraindicating Typical Biopattern(s)

Candida lusitaniae	ARBa(91),GENa(76),dCELa(95),
Candida tropicalis	IRHAa(1),LATa(1),

Installed VITEK 2 Systems Version: 08.01
MIC Interpretation Guideline:
AES Parameter Set Name:

Therapeutic Interpretation Guideline:
AES Parameter Last Modified:

CPUT Technology

bioMérieux Customer:
System #:

Laboratory Report

Printed Nov 21, 2018 12:23 CAT
Printed by: Rose

Isolate: N-1 (Qualified)

Card Type: YST Bar Code: 2430790203629756 Testing Instrument: 000017227F7D (13707)
Setup Technologist: Rose Gwanpu(Rose)

Bionumber: 6102544675327371
Organism Quantity: **Selected Organism: Low Discrimination**

Biochemical Details																
3	LysA	-	4	IMLTa	+	5	LeuA	+	7	ARG	+	10	ERYa	-	12	GLYLa	-
13	TyrA	-	14	BNAG	-	15	ARBa	-	18	AMYa	-	19	dGALa	+	20	GENa	-
21	dGLUa	+	23	LACa	-	24	MAdGa	+	26	dCELa	-	27	GGT	-	28	dMALa	+
29	dRAFa	-	30	NAGA1	-	32	dMNEa	+	33	dMELa	-	34	dMLZa	+	38	ISBEa	+
39	IRHAa	+	40	XLTa	+	42	dSORa	+	44	SACa	+	45	URE	-	46	AGLU	+
47	dTURa	+	48	dTREa	+	49	NO3a	-	51	IARAa	-	52	dGATa	+	53	ESC	-
54	IGLTa	+	55	dXYLa	(+)	56	LATa	+	58	ACEa	+	59	CITa	+	60	GRTas	-
61	IPROa	+	62	2KGa	+	63	NAGa	+	64	dGNTa	+						

Installed VITEK 2 Systems Version: 08.01
MIC Interpretation Guideline:
AES Parameter Set Name:

Therapeutic Interpretation Guideline:
AES Parameter Last Modified:

CPUT Technology

bioMérieux Customer:
System #:

Laboratory Report

Printed Nov 21, 2018 12:25 CAT
Printed by: Rose

Isolate: P-1 (Approved)

Card Type: YST Bar Code: 2430790203629887 Testing Instrument: 000017227F7D (13707)
Setup Technologist: Rose Gwanpu(Rose)

Bionumber: 6112544265323771
Organism Quantity: **Selected Organism: Candida tropicalis**

Comments:	

Identification Information	**Card:** YST	**Lot Number:** 2430790203	**Expires:** Jan 25, 2020 12:00 CAT
	Completed: Nov 10, 2018 06:08 CAT	**Status:** Final	**Analysis Time:** 17.98 hours
Organism Origin	VITEK 2		
Selected Organism	98% Probability **Candida tropicalis**		
	Bionumber: 6112544265323771		**Confidence:** Excellent identification
SRF Organism			
Analysis Organisms and Tests to Separate:			
Analysis Messages:			
Contraindicating Typical Biopattern(s)			
Candida tropicalis	TyrA(21),		

Biochemical Details

3	LysA	-	4	IMLTa	+	5	LeuA	+	7	ARG	+	10	ERYa	-	12	GLYLa	-
13	TyrA	+	14	BNAG	-	15	ARBa	-	18	AMYa	-	19	dGALa	+	20	GENa	-
21	dGLUa	+	23	LACa	-	24	MAdGa	+	26	dCELa	-	27	GGT	-	28	dMALa	+
29	dRAFa	-	30	NAGA1	-	32	dMNEa	+	33	dMELa	-	34	dMLZa	+	38	ISBEa	-
39	IRHAa	-	40	XLTa	+	42	dSORa	+	44	SACa	+	45	URE	-	46	AGLU	+
47	dTURa	+	48	dTREa	+	49	NO3a	-	51	IARAa	-	52	dGATa	+	53	ESC	-
54	IGLTa	+	55	dXYLa	+	56	LATa	-	58	ACEa	+	59	CITa	+	60	GRTas	+
61	IPROa	+	62	2KGa	+	63	NAGa	+	64	dGNTa	+						

Installed VITEK 2 Systems Version: 08.01
MIC Interpretation Guideline:
AES Parameter Set Name:

Therapeutic Interpretation Guideline:
AES Parameter Last Modified:

CPUT Technology

bioMérieux Customer:
System #:

Laboratory Report

Printed Nov 21, 2018 12:27 CAT
Printed by: Rose

Isolate: Q-1 (Qualified)

Card Type: YST Bar Code: 2430790203629757 Testing Instrument: 000017227F7D (13707)
Setup Technologist: Rose Gwanpu(Rose)

Bionumber: 4002505001004150
Organism Quantity: **Selected Organism: Low Discrimination**

Comments:	

Identification Information	**Card:**	YST	**Lot Number:** 2430790203	**Expires:**	Jan 25, 2020 12:00 CAT
	Completed:	Nov 10, 2018 06:08 CAT	**Status:** Final	**Analysis Time:**	17.98 hours
Organism Origin	VITEK 2				
Selected Organism	**Low Discrimination**				
	Bionumber:	4002505001004150		**Confidence:**	Low discrimination
SRF Organism					

Analysis Organisms and Tests to Separate:

Low Discrimination Organism

Candida spherica	LACTOSEa(80),dCELLOB.a(95),dXYLOSEa(80),dMALf(25),
Saccharomyces cerevisiae	LACTOSEa(0),dCELLOB.a(0),dXYLOSEa(0),dMALf(80),

Analysis Messages:

Contraindicating Typical Biopattern(s)

Candida spherica	LACa(94),dSORa(83),
Saccharomyces cerevisiae	dMALa(80),dTURa(87),lPROa(1),

Installed VITEK 2 Systems Version: 08.01
MIC Interpretation Guideline:
AES Parameter Set Name:

Therapeutic Interpretation Guideline:
AES Parameter Last Modified:

CPUT Technology

bioMérieux Customer:
System #:

Laboratory Report

Printed Nov 21, 2018 12:27 CAT
Printed by: Rose

Isolate: R-1 (Approved)

Card Type: YST Bar Code: 2430790203629886 Testing Instrument: 000017227F7D (13707)
Setup Technologist: Rose Gwanpu(Rose)

Bionumber: 6102544245323771
Organism Quantity: **Selected Organism: Candida tropicalis**

Comments:	

Identification Information	**Card:** YST	**Lot Number:** 2430790203	**Expires:**	Jan 25, 2020 12:00 CAT	
	Completed: Nov 10, 2018 06:07 CAT	**Status:** Final	**Analysis Time:**	17.97 hours	
Organism Origin	VITEK 2				
Selected Organism	99% Probability	**Candida tropicalis**			
	Bionumber: 6102544245323771		**Confidence:**	Excellent identification	
SRF Organism					
Analysis Organisms and Tests to Separate:					
Analysis Messages:					
Contraindicating Typical Biopattern(s)					

Biochemical Details

3	LysA	-	4	IMLTa	+	5	LeuA	+	7	ARG	+	10	ERYa	-	12	GLYLa	-
13	TyrA	-	14	BNAG	-	15	ARBa	-	18	AMYa	-	19	dGALa	+	20	GENa	-
21	dGLUa	+	23	LACa	-	24	MAdGa	+	26	dCELa	-	27	GGT	-	28	dMALa	+
29	dRAFa	-	30	NAGA1	-	32	dMNEa	+	33	dMELa	-	34	dMLZa	+	38	ISBEa	-
39	IRHAa	-	40	XLTa	-	42	dSORa	+	44	SACa	+	45	URE	-	46	AGLU	+
47	dTURa	+	48	dTREa	+	49	NO3a	-	51	IARAa	-	52	dGATa	+	53	ESC	-
54	IGLTa	+	55	dXYLa	+	56	LATa	-	58	ACEa	+	59	CITa	+	60	GRTas	+
61	IPROa	+	62	2KGa	+	63	NAGa	+	64	dGNTa	+						

Installed VITEK 2 Systems Version: 08.01
MIC Interpretation Guideline:
AES Parameter Set Name:

Therapeutic Interpretation Guideline:
AES Parameter Last Modified:

CPUT Technology

bioMérieux Customer:
System #:

Laboratory Report

Printed Nov 21, 2018 12:28 CAT
Printed by: Rose

Isolate: T-1 (Approved)

Card Type: YST Bar Code: 2430790203629863 Testing Instrument: 000017227F7D (13707)
Setup Technologist: Rose Gwanpu(Rose)

Bionumber: 6102544245323771
Organism Quantity: **Selected Organism: Candida tropicalis**

Comments:	

Identification Information	**Card:** YST	**Lot Number:** 2430790203	**Expires:**	Jan 25, 2020 12:00 CAT	
	Completed: Nov 10, 2018 06:05 CAT	**Status:** Final	**Analysis Time:**	17.95 hours	
Organism Origin	VITEK 2				
Selected Organism	99% Probability	**Candida tropicalis**			
	Bionumber: 6102544245323771		**Confidence:**	Excellent identification	
SRF Organism					
Analysis Organisms and Tests to Separate:					
Analysis Messages:					
Contraindicating Typical Biopattern(s)					

Biochemical Details

3	LysA	-	4	IMLTa	+	5	LeuA	+	7	ARG	+	10	ERYa	-	12	GLYLa	-
13	TyrA	-	14	BNAG	-	15	ARBa	-	18	AMYa	-	19	dGALa	+	20	GENa	-
21	dGLUa	+	23	LACa	-	24	MAdGa	+	26	dCELa	-	27	GGT	-	28	dMALa	+
29	dRAFa	-	30	NAGA1	-	32	dMNEa	+	33	dMELa	-	34	dMLZa	+	38	ISBEa	-
39	IRHAa	-	40	XLTa	-	42	dSORa	+	44	SACa	+	45	URE	-	46	AGLU	+
47	dTURa	+	48	dTREa	+	49	NO3a	-	51	IARAa	-	52	dGATa	+	53	ESC	-
54	IGLTa	+	55	dXYLa	+	56	LATa	-	58	ACEa	+	59	CITa	+	60	GRTas	+
61	IPROa	+	62	2KGa	+	63	NAGa	+	64	dGNTa	+						

Installed VITEK 2 Systems Version: 08.01
MIC Interpretation Guideline:
AES Parameter Set Name:

Therapeutic Interpretation Guideline:
AES Parameter Last Modified:

APPENDIX 5 – Journal Article

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Review Article

Importance and Relevance of Phytochemicals Present in *Galenia africana*

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Many people in developing countries rely primarily on medicinal plants as their main source of healthcare, particularly for the treatment of skin infections. Despite the widespread use of medicinal plants, there is a lack of literature describing the relevance and risks of exposure of the phytochemicals present. *Galenia africana* has been used traditionally in the form of pastes, decoctions, and lotions to treat wounds and other skin-related ailments. This is a report on the phytochemical composition of *G. africana* and a review on the pharmacological importance and relevance of these phytochemicals. The major groups of phytochemicals identified in *G. africana* extracts were aliphatics, aliphatic triterpenoids, fatty acids, flavonoids, and phenolic and tocopherol compounds. These have been found to exhibit medicinal properties, thus highlighting the need to assess the safety of *G. africana* for topical application. The information related to the safety of the various compounds could indicate the potential risks related to accidental intake of the extract upon topical product applications. This report concludes that the quantities of the phytochemicals present in *G. africana* should not cause undue risk to human health, which provides comfort to pursue future work on using and developing *G. africana* as a therapeutic agent.

1. Introduction

Approximately 70–95% of the population in developing countries rely on medicinal plants as their primary source of healthcare [1]. These medicinal plants are highly sought after due to their apparent ability to treat burns and promote wound healing [1]. The use of such plants as a treatment option for skin infections has become common practice in many rural areas [2]. It has been reported that approximately 27 million people in South Africa rely on the use of medicinal plants to treat skin infections, in particular those caused by pathogens such as *Staphylococci* species and *Candida albicans* [3].

The *Galenia africana* plant species also known as 'kraalbos' or 'geelbos', belonging to the family Aizoaceae, is endemic to Southern Africa, where it is most commonly found in the Namaqualand region of South Africa, but has recently become more widespread in the Western and Southern Karoo [4–6]. Kraalbos is an aromatic, woody perennial sub-shrub which grows to a height of 0.5–1.5 m high, having oppositely arranged green leaves of about 5 cm long and hairless, which tend to change from green to yellow with age. The terminal ends of the twigs are the sites at which numerous small yellow flowers, about 1.5 mm in diameter, are born in large loose heads, during their blooming season; between October and December [5, 7, 8]. Kraalbos is considered a highly invasive pioneer plant, being the first perennial to regrow after soil disturbances, and the only remaining species after the veld has been heavily overgrazed. Some local farmers claim that during the summer months the plant is poisonous to goats and sheep [4]. According to other farmers, if kraalbos is green, it is palatable and not poisonous, but if it is yellow and dry, it is non-palatable and poisonous [9].

Traditional uses of *G. africana* by the southern African indigenous Khoi-San people involve chewing the plant to

relieve toothache or preparing a decoction which is used to make a lotion for the treatment of wounds [10, 11]. It has been shown that frying *G. africana* with other medicinal plants in butter to create an ointment has been used to treat wounds, particularly on the legs of women [10]. Other uses which have been described in ethnobotanical surveys include making a leaf infusion to wash their heads for the purpose of treating pimples and rashes on the affected area. The same infusion was also used in the treatment of dandruff, lice, dry skull, leg pains, and swollen legs. In addition, topical products are prepared for the treatment of venereal diseases as well as skin infections and ailments such as ringworm [6, 12]. There have also been reports of *G. africana* leaves being used for chest pains, and conditions such as asthma and *tuberculosis* [11]. The medicinal potential of botanicals and natural ingredients derived from *G. africana* could be attributed to the plant being a major source of polyphenolic flavonoids and other compounds which are associated with antioxidant activities [13].

The aim of this study is to report on the phytochemical composition of *G. africana* and provide a comprehensive background on the pharmacological importance and relevance of these phytochemicals. The valuable information gained from such a study of the phytochemicals present in *G. africana* will highlight the importance and relevance for treatment of skin diseases described through traditional applications and ethnobotanical surveys. A review on dietary intake of the phytochemicals was used to inform potential health risks after accidental intake of the *G. africana* extract.

2. Materials and Methods

2.1. Identification of Phytochemicals of Galenia africana. It is important that medicinal plants be analysed to determine their phytochemical constituents, which would be beneficial in understanding the pharmacological importance and health risks. The first preparation of a *G. africana* extracts for antifungal and antibacterial fractionation including phytochemical identification studies was employed by Vries et al. (2005) and Mativandlela et al. (2009) [14, 15]. A study by Ticha et al. (2015) analysed a representative 20% extract (80% ethanol : water) of *G. africana*, obtained from Parceval Pharmaceuticals (Pty) Ltd (South Africa), using gravity liquid column chromatography (GLCC) to afford several fractions, which differ in levels of purity, and using EtOAc : hexane as a gradient eluent (hexane, hexane : EtOAc, and EtOAc) [13]. In this study, six of these fractions (A-F) were received as a gift from Dr. Lawrence Ticha, prepared during his postgraduate studies at the Chemistry Department, University of the Western Cape, and these fractions were subjected to a direct gas chromatography-mass spectrometry (GC-MS) analysis, as described by Al-Asmari et al. (2015) [16]. The GC-MS was performed at the Central Analytical Facilities, University of Stellenbosch, with results illustrated in Table 1. Nuclear magnetic resonance (^{1}H and ^{13}C NMR) spectra were recorded in either $CDCl_3$ or acetone-d6 on a Varian Gemini 2000 spectrometer at 200.05 MHz for ^{1}H and 50.3 MHz for ^{13}C spectra, respectively. All chemical shifts are expressed in parts per million (ppm) relative to trimethylsilane (TMS) as the internal reference standard. GC-MS analysis was carried out in a GC system (Agilent 7890A series, USA) equipped with split/splitless injector and autosampler attached to an apolar 5-MS (5% phenylpolymethyl siloxane) capillary column (Agilent 19091S-43; 30 $m \times 0.25$ mm i.d. and 0.25- μm film thickness) and fitted to mass detector (Agilent 5975C series, USA). The flow rate of the carrier gas, helium (He) was set to be at 1 ml.min–1, split ratio is 1 : 50. The injector temperature was adjusted at 250°C, while the detector temperature was fixed to 280°C. The column temperature was kept at 40°C for 1 min followed by linear programming to raise the temperature from 40° to 120°C (at 4°C min–1 with 2 min hold time), 120°C to 170°C (at 6°C min–1 with 1 min hold time), and 170°C to 200°C (at 10°C min^{-1} with 1 min hold time). The transfer line was heated at 280°C. Two microliters of sample was injected for analysis. Mass spectra were acquired in scan mode (70 eV), in the range of 50 to 550 m/z [16].

Positive identification of compounds was made by matching mass spectra against a reference library database, with results illustrated in the chromatogram (Figure 1) and compound structures in Table 1. Various flavonoids, isolated by gravity chromatographic separation and NMR analysis (Figure 2), and reported by Ticha et al. (2015) [13], are also included in Table 1. These clearly demonstrate that flavonoids were the major secondary plant metabolites present and possible activity was expected to result from these compounds as several previous studies have shown these compounds to have beneficial effects [13]. The relative concentration percentage (%) of any compound was calculated by the individual GC-MS peak area divided by the total peak area of the fraction and multiplying the result by 100.

2.2. Dietary Risk Analysis. APC Pharmaceuticals and Chemicals, United Kingdom, was contracted by the University of the Western Cape to conduct a toxicological review and health risk assessment of the components of the *G. africana* extract for agriculture operators and consumers. Since the extract is composed of about 61 compounds, most of which are already present in the human diet, an alternative to conventional toxicity testing procedures was proposed, thereby reducing laboratory animal use and minimising development costs and timeline. An alternative proposed by APC was to utilise peer reviewed literature to assess background dietary exposures for each component. The background dietary intake of the constituents of *G. africana* was analysed using the European Union Pesticide Residue Intake Model (EU PRIMO) consumer risk assessment model which informed a risk assessment for consumers. All compounds approved by the risk assessment were deemed to be nontoxic at the indicated concentration intakes and therefore did not pose a risk to human health, except cinnamic acid, the coumarins, equol, and 2-methoxy-4-vinyl phenol. The dietary intakes for the phytochemicals, as far as could be established from the literature, are summarised and referenced in Table 1 and the discussion.

Table 1: Phytochemical composition of *Galenia africana* extract fractions and dietary risk intake risk in humans.

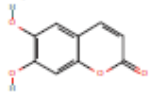
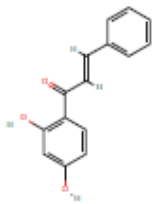
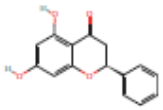
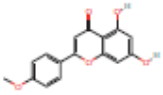
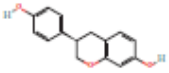
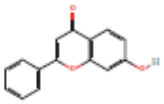
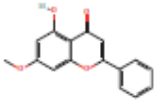
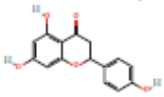
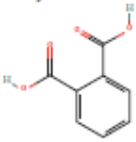
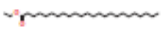
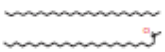
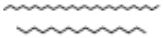
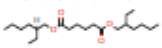
CAS number[+]	Chemical structure[*]	Common or IUPAC name	Relative concentration (%)[++]	Chemical class	Dietary intake (mg/day)[+++]
Flavonoids in 20% extract					
305-01-1		Chrysin or aesculetin	0.54	Flavonoid	0.008 [17]
1776-30-3		2′,4′-Dihydroxychalcone	14.00	Flavonoid	300 [18]
480-39-7		5,7-dihydroxyflavanone/dihydrochrysin/ Pinocembrin)	23.10	Flavonoid	0.0043 [17,19]
480-44-4		5,7-Dihydroxy-4′-methoxyflavone/ Acacetin	0.40	Flavonoid	0.4 [20]
531-95-3		Equol/4′,7-isoflavandiol	3.87	Flavonoid	9.9 [21,22]
6665-86-7		7-Hydroxyflavone	10.20	Flavonoid	No data
520-28-5		5-Hydroxy-7-methoxyflavone (tectochrysin)	7.07	Flavonoid	0.001 [17]
67604-48-2		5,7,4′-trihydroxyflavanone/naringenin	0.44	Flavonoid	45 [23]
Fraction a obtained from 20% extract					
88-99-3		Benzene 1,2-dicarboxylic acid (mono 2-ethylhexyl ester)	0.65	Other	30 [24]
628-97-7		Ethyl hexadecanoate	1.88	Fatty acid	5000 [25]
24634-95-5		Ethyl tetracosanoate (C24 ester)	0.58	Fatty acid	100 [25]
593-49-7		Heptacosane	5.98	Aliphatics	56.4 [18]
7796-19-2		2-Heptacosanone	0.55	Aliphatics	56.4 [18]
630-01-3		Hexacosane	1.12	Aliphatics	56.4 [18]
544-76-3		Hexadecane	0.77	Aliphatics	56.4 [18]
103-23-1		Hexanedioic acid, bis(2-ethylhexyl) ester	0.96	Fatty acid	5 [25]

Table 1: Continued.

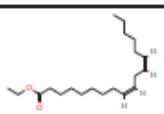
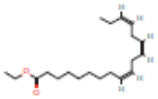
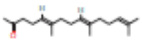
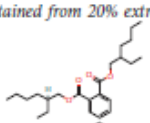
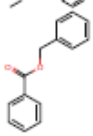
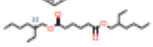
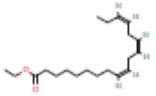
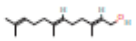
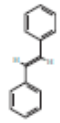
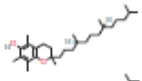
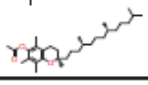
CAS number[+]	Chemical structure*	Common or IUPAC name	Relative concentration (%)[++]	Chemical class	Dietary intake (mg/day)[+++]
544-35-4		Linoleic acid ethyl ester	1.01	Fatty acid	17000 [18]
630-03-5		Nonacosane	10.36	Aliphatics	56.4 [18]
593-45-3		Octadecane	0.80	Aliphatics	56.4 [18]
1191-41-9		9,12,15-Octadecatrienoic acid, ethyl ester (ZZZ)-	0.85	Fatty acid	1600 [18]
629-99-2		Pentacosane	0.90	Aliphatics	56.4 [18]
1117-52-8		5,9,13-Pentadecatrien-2-one- (6,10,14)-trimethyl (E,E)	1.28	Aliphatic triterpenoid	0.540 [24]
111-02-4		Squalene	0.62	Aliphatic Triterpenoid	400 [26,27]
646-31-1		Tetracosane	3.11	Aliphatics	56.4 [18]
638-68-6		Triacontane	0.66	Aliphatics	56.4 [18]
502-69-2		6,10,14-Trimethyl-2-pentadecanone	1.72	Aliphatics	0.540 [18]
Fraction B obtained from 20% extract					
117-81-7		1,2-Benzene dicarboxylic acid mono 2-ethylhexyl ester	2.19		30 [24]
120-51-4		Benzylbenzoate	1.52	Other	300 [18]
103-23-1		Bis (2-ethylhexyl) hexanedioic acid	1.62		300 [18]
628-97-7		Ethyl hexadecanoate	1.35	Fatty acid	5000 [25]
1191-41-9		Ethyl Z,Z,Z- 9,12,15-octadecatrienoate	1.03	Fatty acid	1600 [18]
4602-84-0		Farnesol	3.56	Aliphatic triterpenoid	0.009 [28–30]
103-30-0		(E)-stilbene	1.45	Flavonoid	200 [31]
646-31-1		Tetracosane	0.79	Aliphatics	56.4 [18]
		α-Tocopherolquinone	1.15	Tocopherol	15 [18]
502-69-2		6,10,14-Trimethyl-2-pentadecanone	5.29	Aliphatics	0.540 [24]
10191-41-0		Vitamin E	1.33	Tocopherol	15 [18]
58-95-7		Vitamin E-acetate	1.71	Tocopherol	15 [18]

Table 1: Continued.

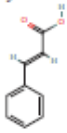
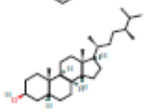
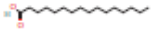
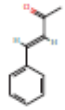
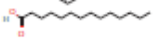
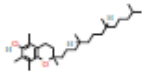
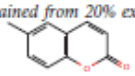
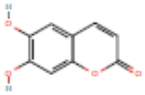
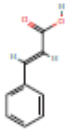
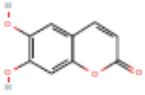
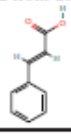
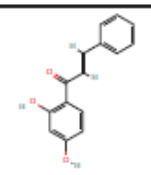
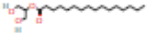
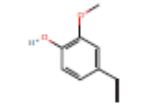
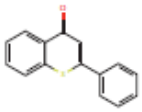
CAS number[+]	Chemical structure*	Common or IUPAC name	Relative concentration (%)[++]	Chemical class	Dietary intake (mg/day)[+++]
Fraction C obtained from 20% extract					
140-10-3		*trans*-Cinnamic acid	0.32	Flavonoid	2.7 [24]
6538-02--9		Ergostanol	0.42	Flavonoid	Not absorbed
57-10-3		Hexadecanoic acid	0.49	Fatty acid	5000 [18]
1002-84-2		Pentadecanoic acid	1.25	Fatty acid	100 [25]
122-57-6		4-Phenylbut-3-en-2-one	0.24	Carbonyl	No data
544-63-8		Tetradecanoic acid	0.19	Fatty acid	1300 [25]
502-69-2		6,10,14-Trimethyl-2-pentadecanone	1.25	Aliphatics	0.540 [24,32]
10191-41-0		Vitamin E	0.20	Tocopherol	15 [18]
Fraction D obtained from 20% extract					
92-48-8		2H-1-benzopyran-2-one	0.39	Flavonoid	No data
305-01-1		Esculetin	0.46	Flavonoid	6.0 [18]
Fraction *E* obtained from 20% extract					
140-10-3		*trans*-Cinnamic acid	0.47	Flavonoid	2.7 [24]
305-01-1		Esculetin	1.05	Flavonoid	6.0 [18]
544-76-3		Hexadecane	0.32	Aliphatics	56.4 [18]
629-59-4		Tetradecane	0.22	Aliphatics	56.4 [18]
126-33-0		Tetrahydrothiophene 1,1-dioxide	1.03	Other	No data
Fraction F obtained from 20% extract					
140-10-3		*trans*-Cinnamic acid	2.05	Flavonoid	2.7 [24]

Table 1: Continued.

CAS number[+]	Chemical structure[*]	Common or IUPAC name	Relative concentration (%)[++]	Chemical class	Dietary intake (mg/day)[+++]
1776-30-3		2,3-Dimethoxy-2′,4′-dihydroxy chalcone	2.86	Flavonoid	300 [18]
23470-00-0		Ethylhexadecanoic acid-2-hydroxy ester	3.46		5000 [19,25]
544-76-3		Hexadecane	0.83	Aliphatics	56.4 [18]
7786-61-0		2-Methoxy-4-vinyl phenol	0.94	Phenolic	0.29 [33]
593-45-3		Octadecane	1.19	Aliphatics	56.4 [18]
784-62-3		2-Phenyl-4-H-1-benzothiopyran-4-one	2.73	Other	No data
629-59-4		Tetradecane	0.81	Aliphatics	56.4 [18]
126-33-0		Tetrahydrothiophene 1,1-dioxide	8.92	Other	No data

[a]Direct GC-MS analysis of chromatography fractions of differing purity after CC fractionation of 20% *G. africana* extract. [b] Mass spectrum of compounds match with 360000 memory banks. [+]The CAS number is a unique numerical identifier assigned by the Chemical Abstracts Service (CAS) to chemical substances described in the open literature to create a link to information about a specific chemical substance. [++]The relative concentration percentage (%) of any compound was calculated by the individual GC-MS peak area divided by the total peak area of the fraction and multiplying the result by 100. [+++]The dietary intake data for the phytochemicals was obtained from data found in peer reviewed articles and reference databases. [*]Chemical structures were obtained by using the CAS number to search for the chemicals on the PubChem database.

2.3. Literature Search. An initial literature search was conducted with a combination of keywords such as *Galenia africana*, distribution, traditional use, medicinal uses, ethnomedicinal uses, phytochemistry, and chemical composition. After data for the phytochemical composition of *Galenia africana* was established, an extended literature search was performed to determine the natural occurrence and phytochemical and toxicological relevance of the individual constituents. This was done using a combination of the phytochemical name and keywords such as pharmacology, antioxidant, antiproliferative, antifungal, antibacterial, anti-inflammatory, wound healing, and toxicity. These searches were conducted using major databases, including Science Direct, Google Scholar, BioMed Central (BMC), Web of Science, Springer link, Scopus, and PubMed.

3. Results and Discussion

Results about the phytochemical composition of *Galenia africana* extract fractions and dietary intake risk in humans are shown in the chromatogram (Figure 1) and Table 1. Figure 2 details the calculated spectrum and 1H NMR spectrum of the flavonoid 2′,4′-dihydroxydihydrochalcone.

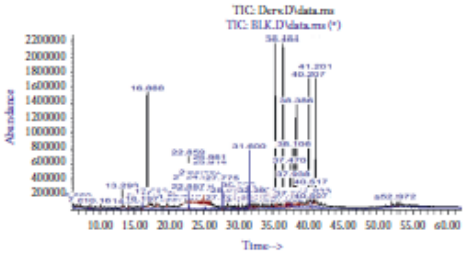


Figure 1: Chromatogram of the *Galenia africana* extract sample.

4. Natural Occurrence and Pharmacological Relevance of Phytochemicals Identified in the *Galenia Africana* Extract

The components contained in the *G. africana* extracts and their relative concentrations are listed in Table 1. Additionally, each of these phytochemical constituents was placed into one of the following groups: aliphatics, aliphatic triterpenoids, fatty acids, flavonoids, tocopherols, and phenolic compounds. The natural occurrence and

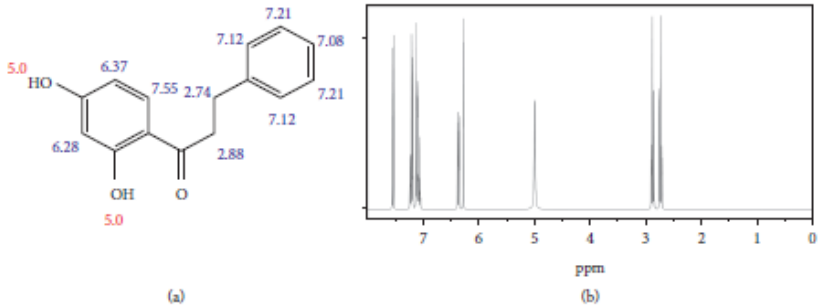


FIGURE 2: (a) Calculated spectrum and chemical structure of the flavonoid 2′,4′-dihydroxydihydrochalcone and (b) 1H NMR spectrum of 2′,4′-dihydroxydihydrochalcone.

pharmacological relevance for the compounds as far as could be established from the literature are outlined below.

4.1. Aliphatics. Aliphatics are a class of hydrocarbons, where medium and long chain alkanes are components of the surface waxy cuticle of plants and fruits. The function of this waxy layer is to control the rate of moisture loss and to provide a first line of defence against pathogenic organisms [34]. Humans are also exposed to paraffins and waxes from industrial origin, mainly via transfer of these compounds from packaging materials and through the pharmaceutical uses of paraffins. Heptacosane, nonacosane, and pentacosane have all been shown to possess antibacterial activity [35]. Hexacosane extracted from *Sanseveria liberica* was shown to exhibit moderately high antimicrobial activities against *Salmonella typhi, Candida albicans, Streptococcus pyogenes, Staphylococcus aureus, Escherichia coli, Pseudomonas fluorescence, Klebsiella pneumoniae, Proteus vulgaris,* and *Candida krusei* [36]. Due to limited information from the literature, specific pharmacological relevance of the remaining compounds classed under the aliphatic group could not be determined.

4.2. Aliphatic Triterpenoids. Farnesol, farnesyl acetone, hexahydrofarnesyl acetone, pristine, and squalene are all classified as aliphatic triterpenoids. These are commonly found in plant matter and fruits which are frequently consumed by humans. Squalene is an aliphatic triterpenoid, present in many foods including amaranth oil and olive oil. In human skin physiology, squalene is used as an antioxidant and moisturizer. It has also been reported to be used for treating skin disorders such as acne, atopic dermatitis, psoriasis, and seborrheic dermatitis [27]. Farnesyl acetone is found in the medicinal herb *Costus pictus*, also in tomato and watermelon [37]. In mushrooms, farnesyl acetone has been detected as a volatile component, accounting for up to 1% of volatiles in *Suillus granulatus* and up to 12% of *Suillus luteus* [29, 30]. Farnesol has been detected in plants and fungi, accounting for up to 16% of total volatiles in *Chroogomphus rulitus* [38]. However, farnesyl compounds are also endogenous in humans where they are involved in the production of cholesterol and protein tagging [38, 39]. A study conducted by Chaudary et al. (2009) demonstrated the chemopreventative effect of farnesol on skin tumorigenesis [40].

4.3. Fatty Acids. Fatty acids are common components found in nature, where they are present in animal or vegetable fats, oils, and waxes [41]. They form chains and are classified according to the length and number of carbon atoms present in the chain, such as short, medium, long, or very long [41]. Humans can synthesise the full range of essential dietary fatty acids used for energy besides the linoleic and alpha linolenic acids. Dietary fatty acids are used for energy through the beta-oxidation pathway in addition to assisting in cell wall synthesis and hormone manufacture [42].

Ethyl hexadecanoate (also known as ethyl palmitate) is the ethyl ester of palmitic acid. It is formed in humans in the nonoxidative metabolite pathway of ethanol and has been proposed as a biomarker for ethanol consumption [43]. It has also been listed as an emollient used in cosmetic products [44]. Ethyl tetracosonate is the ethyl ester of tetracosanoic acid which is present in peanut oil accounting for 1.1–2% mass fraction of total fatty acids [25]. Ethyl linoleate and ethyl linolenate are the ethyl esters of linoleic and alpha linolenic acid, respectively. According to PubChem (2021), hexadecanoic acid (palmitic acid) is used as an emollient in cosmetic products [45]. A study by Uddin et al. (2012) demonstrated that tetracosane had significant cytotoxic activity against HT-29 colon cancer cells and some toxicity against gastric cancer and estrogen-dependant breast cancer cells [46]. A recent study by Gao et al. (2019) demonstrated the use of hexanedioic acid (adipic acid) as a component of a hydrogel which shows potential for wound healing applications [47]. Additionally, adipic acid is used in cosmetics as a buffering agent [48].

Linoleic acid ethyl ester (ethyl linoleate) is an unsaturated fatty acid resulting from formal condensation of the carboxyl group of linoleic acid with the hydroxyl group of ethanol. It is used in many cosmetic products for its

antibacterial and anti-inflammatory properties [49]. A recent study by Ko and Cho (2018) demonstrated the potential of ethyl linoleate as a noncytotoxic and skin whitening agent in medicine and cosmetic products [50]. Pentadecanoic acid has an uneven number of carbon atoms and is thought to be synthesised primarily by gut microflora in the rumen of cattle. Hence, the major dietary source of this fatty acid is milk, where it accounts for approximately 1% of milk fats [51]. It has been shown that pentadecanoic acid could serve as a signalling inhibitor in breast cancer cells [52]. Tetradecanoic acid (also known as myristic acid) is present in coconut oil and palm kernel fat accounting for approximately 16% mass fraction of total fatty acids [25]. It is also present in milk (11% mass fraction of total fatty acid), meat, and meat products [25]. Tetradecanoic acid is used as a cleansing and emulsifying agent in cosmetic products [53].

4.4. Flavonoids. The flavonoids comprise a group of low molecular weight compounds, of which roughly 4000 are known, and are separated into distinct subgroups: anthocyanidins, chalcones, flavanols, flavanones, flavones, isoflavones, and stannols [54]. They are ubiquitous in plants and have several biological functions. For example, they are responsible for pigmentation of many plants and fruits [55]. The flavonoids, *trans*-Cinnamic acid, chalcone, chrysin, and tectochrysin, are associated with a variety of health-promoting effects and are an important component in many nutraceutical, pharmaceutical, medicinal, and cosmetic applications [56–60]. This is due to their anticarcinogenic, anti-inflammatory, antimutagenic, and antioxidative properties [55]. Flavonoids are not classified as essential human nutrients per se, but a high dietary intake of flavonoids is thought to be associated with lowered cancer and cardiovascular disease risk [54]. 2,4-Dihydroxychalcone is a flavonoid abundant in the leaves of *Oxytropis falcata*, a leguminous plant also known as locoweed. This plant has been widely used in Chinese/Tibetan herbal medicine. In addition to its antioxidant activity, it also exhibits antitumor activity [61, 62]. Equol is the major intestinal bacterial metabolite of the isoflavanone diadzin in 25–60% of the population and is influenced by diet [63]. Those consuming a diet high in diadzin (e.g., vegetarian diets and/or those containing soya) were more likely to metabolise the material to equol [63]. Equol has been shown to have antiaging properties and antiandrogen activity [64, 65]. Naringenin is found in many citrus fruits and has been measured in grapefruit juice at concentrations of 100–800 mg/L [23]. There is growing evidence showing the pharmacological effects of naringenin which include anticancer, anti-inflammatory, antimicrobial, antimutagenic, and hepatoprotective properties [66]. Pinocembrin is a major flavanone found in honey and propolis, the resinous substance used by bees to seal honeycombs and used as a health food [67]. Pinocembrin has shown potential for use as an antioxidative, anti-inflammatory, antimicrobial, and antitumor agent [68].

4.5. Phenolic Compounds. Results reveal 2-methoxy-4-vinylphenol to be present in many products where production involves the use of yeast and/or cooking. It is also a component of beer, especially wheat beer, formed by the conversion of ferulic acid during the fermenting process [69]. In a study by Kim et al. (2019), it was shown that 2-methoxy-4-vinylphenol possesses anticancer properties and exhibits antiaging and antioxidant activity [70, 71].

4.6. Tocopherols. Alpha-tocopherols are part of a group of fat-soluble compounds known as vitamin E. Along with being the most common form of vitamin *E* present in nature, alpha-tocopherols are also the most biologically active [72]. These can be found in foods such as avocados, nuts, and seeds. In a study by Weber et al. (1997), it was shown that a topical application of alpha-tocopherol to mouse skin prior to exposure of UV-irradiation resulted in the preservation of antioxidants [57, 73]. Conversely, without prior application of the tocopherol, the antioxidants present in the skin were destroyed after the irradiation. Alpha-tocopherolquinone is a metabolite of alpha-tocopherol [74]. Vitamin *E* derivatives such as alpha-tocopherolquinone act as an important physiological antioxidant. Alpha-tocopherolquinone has further demonstrated its potential as a biomarker for oxidative stress [75].

Vitamin *E* is an essential nutrient obtained by external sources such as fresh vegetables, vegetable oils, cereals, and nuts. Vitamin *E* has been demonstrated to be of importance in recent dermatological studies due to its antioxidant properties. Experimental evidence suggests that topical application and oral consumption of vitamin *E* have anticarcinogenic, photoprotective, and skin barrier-stabilizing properties [76]. Vitamin *E* acetate has elicited a significant interest for its role in assisting in curing burn injuries, particularly for its antioxidant action which occurs during tissue reperfusion. Reactive oxygen species and free radicals are produced during the phase of reperfusion of ischemic tissues, damaging numerous cell components, including nucleic acids, lipids, and proteins. It has been shown that a bioadhesive film containing vitamin *E* acetate could facilitate skin regeneration and wound healing through the controlled release of the vitamin *E* acetate [77].

5. Potential Health Risks of Phytochemicals Identified in the *Galenia africana* Extract

Information from the US Dietary Reference Intake (DRI), Joint FAO/WHO Expert Committee on Food Additives (JECFA), and Beare-Rogers et al. (2009) IUPAC Technical Report was used to establish average daily background dietary intake values (Table 1) [18,25,78]. The background dietary intake of the constituents of *G. africana* was analysed using the European Union Pesticide Residue Intake Model (EU PRIMO) consumer risk assessment model, which informed a risk assessment for consumers [79, 80].

Based on the *G. africana* phytochemical data, it was observed that most compounds were already present in the general human diet and therefore should not pose a risk to

human health. Potentially relevant components, besides cinnamic acid, the coumarins, equol, and 2-methoxy-4-vinyl phenol, were all found to be nontoxic following preliminary chronic and acute consumer risk assessments. The dietary intakes and potential health risks for the phytochemicals in Table 1, as far as could be established from the literature, are summarised below.

For aliphatics in general, Tennant (2004) has estimated that the average intake of each of the mixed alkanes is 0.01–0.02 mg/kg/day [81]. Based on this data and information from the European Food Safety Authority, which considered and listed paraffin oils for use as a pesticide active ingredient, it was deemed that no risk assessment would be necessary as the straight chain alkanes were toxicologically nonrelevant.

The aliphatic triterpenoid, squalene, is especially prevalent in olive oils, and as a result human dietary intake varies greatly according to the geographical location where intake is estimated at 30 mg/day in the USA but increases to 200–400 mg/day in Mediterranean countries [26]. No quantitative information was available regarding the dietary intakes of faresyl acetone and hexahydrofarnesyl. However, hexahydrofarnesyl acetone undergoes beta-oxidation and is unlikely to pose greater hazards than fatty acids or aliphatics. Farnesyl acetone intake can be approximated as it is approved as a flavouring agent by JECFA with an estimated daily intake of 9 μg/day.

According to Madigan et al. (1994), the phenolic compound 2-methoxy-4-vinylphenol is present in wheat beer at a concentration of 0.68 mg/L [82]. The average Czech Republic intake of beer is 156 L/year giving rise to an average daily intake of 2-methoxy-4-vinylphenol from beer at 0.29 mg/day. Consuming 2 L of beer in one day, as might occur occasionally, results in consumption of 1.36 mg/day.

According to Kennedy *et al.*, (1999) the dietary intake of fatty acids in the USA is between 41 and 117 g/day with saturated fat accounting for 14–42 g of this amount [55, 83]. Given that fatty acids are a significant part of the normal human diet, they are not toxicologically relevant components of the extract and may be excluded from risk assessments.

The potential link between flavonoid intake and health has led to a number of studies measuring the dietary intake of flavonoids in humans. These have produced differing results ranging from 23 mg/day to 1 g/day [84–86]. As such, the potential dietary intake of the many flavonoids may account for up to 600 mg/day if all flavonoids are considered.

As a tocopherol, the recommended daily intake of vitamin *E* has been set by many countries. According to the US DRI of alpha-tocopherol, intake is set at 15 mg/day with an upper tolerable intake of 1,000 mg/kg [87].

6. Conclusions

Galenia africana is a plant of traditional medicinal and commercial relevance in South Africa. In this review, the importance and relevance of the phytochemical constituents and potential risks relating to exposure to the *G. africana* extract were concluded in a descriptive manner. Phytochemical screening results indicate the relative chemistry and concentrations of the compounds in the plant extract. Literature also shows that several compounds present in the *G. africana* extract exhibit anti-inflammatory, antioxidant, and antimicrobial activities and wound healing which supports the traditional medicinal use of the plant by indigenous people. Furthermore, it was revealed that none of the potentially relevant compounds in *G. africana* were expected to cause undue risk to human health. This data regarding the potential risks related to the ingestion of *G. africana* extract could be used to infer the potential risks relating to the accidental exposure to these phytochemical compounds when exposed to human skin upon topical application. On this basis, the effect of the *Galenia africana* extract on human skin in quantities similar to those described in this review should not cause undue risk to human health. However, it is important that further studies be performed using this plant to determine pharmacological action and mechanism of action, which has the potential to lead to the development of *G. africana* as a therapeutic agent.

Disclosure

Any opinion, findings, and conclusions or recommendations expressed in this material are those of the authors and, therefore, the NRF does not accept any liability in regard thereto.

Conflicts of Interest

The authors declare that there are no conflicts of interest regarding the publication of this paper.

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