ANTI-DERMATOPHYTIC ACTIVITY OF *Salvia nilotica* METHANOLIC LEAF EXTRACT AGAINST *Trichophyton mentagrophytes*

BY

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A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE IN BIOCHEMISTRY IN THE SCHOOL OF SCIENCE

UNIVERSITY OF ELDORET, KENYA

2021

DECLARATION

DECLARATION BY STUDENT

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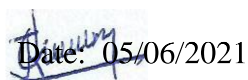
Chepkwony Joseph Kiprop: Signature:  Date: 05/06/2021

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DEDICATION

To my parents Mr. Erick Chepkwony Aengwo and Mrs. Mary Kobilo Aengwo for their constant support throughout my academic journey.

To my wife Flora Kiprop and Sons Jayden Kangogo Kiprop and Jason Chebii Kiprop for their patience, love and understanding for all the time I put into my research.

ABSTRACT

Conventional medicine used against dermatophytosis has resulted in treatment failure, relapses of the fungal infection and side effects due to its use. Herbalist in the Tugen community in Kenya claim that *Salvia nilotica* leaves have anti-dermatophytic effects however, there is no scientific documentation for these claims. This study therefore sought to determine the phytochemical constituents in *S. nilotica* methanolic crude leaf extract and its anti-dermatophytic activity against the dermatophyte, *Trichophyton mentagrophytes* and probable mode of action through the effects on Metalloprotease 2 (MEP2), Sulphite efflux pump (SSU1), Subtilisin 3 (SUB3) and dipeptidyl-peptidases V (DDPV) target genes. The phytochemical constituents of methanolic crude leaf extract of *S. nilotica* were determined using standard methods. Food- poisoned technique was used to determine anti-dermatophytic activity of *S. nilotica* extract at different concentrations ranging from 7.76 mg/mL to 77.59 mg/mL. Plausible mode of action was determined using quantitative real time polymerase chain reaction to establish the effect of *S. nilotica* methanolic leaf extract treatment on MEP2, SSU1, SUB3 and DDPV genes of *Trichophyton mentagrophytes* versus the standard drug fluconazole. Qualitative phytochemical analysis of the methanolic crude leaf extracts of *S. nilotica* indicated the presence of tannins, saponins, flavonoids, terpenoids, steroids, alkaloids, carbohydrates, amino acids and glycosides but there was absence of phlobatannins and anthraquinones. This study also found that *S. nilotica* crude leaf extract has anti-dermatophytic activity against *Trichophyton mentagrophytes*. The activity of crude leaf extract of *S. nilotica* on *Trichophyton mentagrophytes* was not significantly different ($p < 0.05$) when compared with fluconazole. In addition, all the *Trichophyton mentagrophytes* genes, which were targeted in this study, were down regulated by different folds depending on the concentration of the antifungal agent used. The down regulation noted was -1.7, -1.9, -1.1 and -1.1 folds for MEP2, SSU1, SUB3, and DPPV genes respectively at 0.30 mg/mL of fluconazole. At 0.50 mg/mL of fluconazole, the genes were down regulated by -4.2, -2.9, -1.6 and -34.4 folds for MEP2, SSU1, SUB3 and DPPV respectively. *S. nilotica* at 13.97 mg/mL down regulated the target genes by -1, -1.2, -1.2 and -38.4 folds for MEP2, SSU1, SUB3, and DPPV genes respectively. Similarly at the concentration of 77.59 mg/mL of *S. nilotica* the genes were down regulated by -1.3, -7.9, -2.3 and -2211.8 folds for MEP2, SSU1, SUB3 and DPPV respectively. In conclusion, this study has shown that *S. nilotica* crude leaf methanolic extract could offer a potential alternative medicine for dermatophytosis treatment.

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LIST OF ABBREVIATIONS

ANOVA	Analysis Of Variance
BB4	Binding Buffer 4
CB4	Clean Buffer 4
cDNA	Complementary Deoxyribonucleic Acid
CMR	Centre for Microbiology Research
CO	Control
C_T	Cycle Threshold
CTMDR	Centre of Traditional Medicine and Drug Research
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic Acid
dNTP	Deoxyribonucleotide triphosphate
DPPV	Dipeptidyl-Peptidase V
FP	Forward Primer
HOXD10	Homeobox D10 gene
KEMRI	Kenya Medical Research Institute
MEP	Metalloproteases
mRNA	Messenger Ribonucleic Acid

PCR	Polymerase chain reaction
PDT	Photodynamic Therapy
PS	Photosensitizer
qPCR	Quantitative Polymerase chain reaction
RNA	Ribonucleic acid
rRNA	Ribosomal Ribonucleic acid
RP	Reverse Primer
RPM	Revolutions per minute
SDA	Sabouraud dextrose agar
SN	Salvia nilotica
SSU1	Sulphite efflux pump, coding for SSU1 gene
SUB	Subtilisin
TM	Trichophyton mentagrophytes
UOE	University of Eldoret
WB4	Wash Buffer 4
WHO	World Health Organization

ACKNOWLEDGEMENTS

I wish to thank the Almighty God for his sufficient grace and provision during my research and thesis writing. I acknowledge with gratitude all those who have assisted in my thesis studies. Special thanks go to the National Research Fund, Kenya (NRF-Kenya) for the Masters' research grant they awarded me in the 2016/2017 financial year. I register my appreciation to my supervisors, Dr Vivian .C. Tuei, Department of Chemistry and Biochemistry at University of Eldoret, Kenya and Dr. Peter Mwitari, Centre for Traditional Medicine and Drug Research, Kenya Medical Research Institute (KEMRI). My appreciation also goes to research guidance from Dr. Pixley Kipsumbai of Department of Biological Sciences, University of Eldoret and Dr. Christine Bii, Senior Principal Research Scientist, Centre for Microbiology Research (CMR), Kenya Medical Research Institute (KEMRI), Nairobi. I would also like to acknowledge the herbalists from Tugen community of Kenya for their botanical knowledge that they shared.

My sincere and heartfelt appreciation goes to my dear wife Flora Kiprop, and our sons, Jayden Kiprop and Jason Kiprop for their constant moral support during my research writing. To all those mentioned and unmentioned your support is highly appreciated and may the almighty God bless you abundantly.

CHAPTER ONE

INTRODUCTION

1.1 General Background

Dermatophytes are among the oldest groups of pathogens significant in human health and disease burden in the population (de Hoog *et al.*, 2017). There has been huge confusion of dermatophyte classification until scientific advances of the mid-20th century provided standardized nomenclature with the acceptance of *Microsporum,* *Trichophyton,* and *Epidermophyton* genera (de Hoog *et al.*, 2017) . Dermatophytes consist of 40 species of fungi derived from three genera, namely, *Trichophyton, Epidermophyton* and *Micropsorum* (Azrad *et al.*, 2019). They can also be divided into three groups based on the source of infection, the first group of dermatophytes is Zoophilic dermatophytes which comprise the fungi transmitted from animals, domestic or wild, to human or other animals (Al-Janabi, 2014) as with *Microsporum canis* and *Trichophyton mentagrophytes*, which commonly infect dogs and cats in Italy (Mancianti *et al.*, 2003) . Anthropophilic is the second group of dermatophytes when infection is transmitted from human to another through the direct contact, (Al-Janabi, 2014) as with *Microsporum langeronii* which was found to cause tinea corporis in most children of a public primary school of Antananarivo (Madagascar) (Carod *et al.*, 2011) and *Trichophyton interdigitale* which is a causative agent of tinea faciei. The third group of dermatophytes is geophilic which is found in soil living on keratinous materials as saprophytes and can transmit to humans after contact with contaminated soil as with *Microsporum gypseum* (Al-Janabi, 2014).

As fungal pathogens, dermatophytes attack keratinized structures with most effects seen in the integumentary system consisting of nails, skin, and hair, causing dermatophytosis

(Gnat *et al.*, 2019). Dermatophytes cause a disease called dermatophytosis or tinea ,it can can be found on the skin of different parts of the human body which may take various names based on the infected area such as, tinea capitis on the scalp, tinea unguium on the nails, tinea pedis on the feet tinea corporis on the body and tinea cruris on the groin (Andrews & Burns, 2008).

Dermatophytes have attracted public health attention resulting from the growing concern of increasing human fungal infections in the global population (Kohler *et al.*, 2017). Results of observational studies conducted in the last century show high rates of dermatophytes infections across individuals of diverse demographic characteristics and epidemiological statuses with human-animal contagion being a primary element of the cycle) (Abdel-Rahman & Nahata, 1997; Gnat *et al.*, 2019). These fungal organisms utilize human and animal nutritional processes by production of lytic enzymes and uptake systems for the released nutrients that serve as a pathogenesis process (Gnat *et al.*, 2020).

The dermatophytes are confined to the epidermis and skin appendages' stratum corneum, located in moist body regions such as toes, breasts, and groin (Chuang *et al.*, 2007). During the invasion process, these fungi secrete specific enzymes that guide the host tissues (Kadhim *et al.*, 2015). Although dermatophytic infections occur majorly around the epidermis, they sometimes invade and cause serious infections in immunosuppressed patients, resulting in granulomas' development (Peres *et al.*, 2010). Therefore, host immunity is crucial in disease progression, which may occur as minor infection limited to subcutaneous and cutaneous invasions or disseminate to life-threatening infections (Gnat *et al.*, 2019).

In 1955, amphotericin B, a notable drug, was discovered, produced, and recommended for the dermatophytes infections, despite unpleasant side effects such as harsh dose-dependent toxicity, hypokalaemia, and renal impairment (Negri *et al.*, 2014). At present, amphotericin B's lipid-based formulations have demonstrated a wide spectrum of action against the fungi with a considerably superior success rate and a lower nephrotoxicity occurrence. However, a fundamental problem with the lipid formulations is their high pricing, limiting use in therapeutic practice (Gupta & Tomas, 2003).

Research on forty-six Eurasian region derivatives and extracts from 25 plants of the *Rubiaceae Asteraceae*, *Solanaceae,* and *Euphorbiaceae* botanical families (Niño *et al.*, 2012), found activity against filamentous fungi (Zabka *et al.*, 2011). *Nandina domestica* Thunb essential oil contained ingredients that displayed high antifungal activity *in vitro* with an adverse effect on spore germination against *Trichophyton rubrum, Trichophyton mentagrophytes,* and *Microsporum canis* (Bajpai *et al.*, 2009). Other studies have reported antimicrobial and antioxidant effectivity of terpenoids against fungal pathogens, including dermatophytes and *Candida* (Negri *et al.*, 2014). Negri *et al.* (2014) reported similar antifungal activity with propolis extract exhibiting considerable effect against dermatophytes and *C. neoformans*.

S. nilotica is a perennial shrub thriving in Eastern Africa between 900-3600 m altitudes. It belongs to the family Lamiaceae, genus *Salvia* and its species is *nilotica*. Different countries have documented various uses of *S. nilotica.* For instance, in Rwanda, ashes of *S. nilotica,* together with *Dichrocephala integrifolia, Rhoicissus tridenta,* and *Dracaena afromontana,* were found to affect lacerations in rheumatic patients (Kayonga & Habiyaremye, 1987). In Ethiopia, it has been used together with *Veronica amygdalina* to

cure fever, headaches, and lip sores (Giday *et al.*, 2009). According to Pamo *et al.*, (2004), plant oils possess anti-inflammatory, healing, antipyretic, bactericidal activities antiseptic, antispasmodic, and insecticidal effects. According to Nyabayo *et al.*, (2015), preceding studies on the essential oil of *S. nilotica* have shown that they hold potent pesticidal activity and antiradical properties.

1.2 Statement of the Problem

Fungal pathogens have developed significant resistance against antifungal agents over the last few decades of the 21st century. *Candida albicans* and *Trichophyton rubrum* are among the largest fungal pathogens, responsible for a wide range of infections, ranging from minor ailments such as superficial mycoses to life-threatening conditions (Richardson & Warnock, 2012). The amount of money used by families in treating superficial skin infections is soaring and frequently unsuccessful. Paying for these treatments may lessen a household's ability to buy basic commodities such as food and clothing (Jamison *et al.*, 2006). Dermatophytic infections are highly prevalent because of the extensive number of reservoirs such as the skin, hair, nails, scalp, and elevated resistance of dermatophytes microbes to unfavorable ecological circumstances (De Respinis *et al.*, 2013) that also vary with the infectious nature from one host to another. Dermatophytic infections are the leading cause of morbidity-associated superficial mycoses (Gupta & Tomas, 2003). Dermatophytes are to blame for severe fungal human pathogens, causing diseases that have been increasing in the past few decades (Arif *et al.*, 2009).

Although antifungal drugs' topical application shows effective activity in eliminating dermatophytes and the majority of anthropophilic infections, it is rarely sufficient in treating zoophilic dermatophytes, especially Tinea unguium and Tinea capitis, which

requires systemic treatment (Nenoff *et al.*, 2018). Another common problem with antifungal medications is the long dose period and associated side effects, which considerably influence patients' compliance (Martinez-Rossi *et al.*, 2018). Amphotericin B, characterized as a broad-spectrum drug, shows high antifungal efficacy making it significant in the antifungal armamentarium (Lanza *et al.*, 2019). However, its dose-limiting effects and nephrotoxic potential restricts its use in clinical practice (Groll *et al.*, 2019; Lanza *et al.*, 2019). Consequently, dermatophytoses are real health conditions across all age groups. Still, they affect mostly elderly patients, children, and pregnant women, who, without special attention, experience life-threatening adverse effects from antifungal agents (Kaul *et al.*, 2017). Therefore, discovering and developing new antifungal drugs is crucial in the therapeutic management of fungal infections.

However, this therapeutic approach faces two major hurdles. First, most dermatomycoses affect people with compromised immunity, who suffer more adverse effects from antifungal drugs than those with normal immunity (Angadi *et al.*, 2019; Dai *et al.*, 2019; Kalita *et al.*, 2019). Secondly, the conservative physiological functions between humans and fungal pathogens make it difficult to develop a high safety profile and effective antifungal drugs (Mercer *et al.*, 2019). Havlickova *et al.* (2008) concluded that dermatophytic infections are among the most common forms of human diseases. They are projected to infect greater than 20-25% of the global citizens, and their prevalence is continually rising.

1.3 Justification of the Study

Millions of people endure infections caused by dermatophytes, which can be acute or chronic and are difficult to treat in most cases, due to inadequate knowledge of these

filamentous fungi (Staib *et al.*, 2010). Based on the findings of plant derivatives' therapeutic properties, the value of plants in medical practice is undisputable (Gnat *et al.*, 2020). Active antimicrobial molecules from plant extracts are forms of natural compounds essential in developing new therapeutic agents (Negri *et al.*, 2014). The adverse effects of prolonged use of existing drugs in medical practice for the management of dermatophytes infections highlight the need to develop new safe profile formulations (Subha & Gnanamani, 2009).

This study was carried out to establish the anti-dermatophytic activity and possible mode of action of methanolic *S. nilotica* crude leaf extract. Herbalists in the Tugen community in Kenya use *S.nilotica leaves* to treat nail and skin infections (Tinea unguium / onychomycosis and tinea corporis). Scientific evidence has not been documented for these medicinal claims; these claims were learned through observation and verbal ethnobotanical knowledge. *Trichophyton mentagrophytes* strain was subjected to methanolic *S. nilotica* crude leaf extract because they are among fungal strains known to cause dermatophytosis. Iwu et al. (1999), in his comparison of the use of synthetic and anti-dermatophytes from herbs, found that antimicrobials of plant sources are adequate in the management of dermatophytic infectious diseases and at the same time alleviating many of the side effects that are always linked with synthetic drugs. Toxicity of presently available antifungal treatments and the rising drug-resistance amid the causative microbial agents have caused research towards the study of identifying and developing new antimicrobial agents from natural products (Beatriz *et al.*, 2012). Therefore, this study investigated *in vitro* susceptibility of *Trichophyton mentagrophytes* to *S. nilotica*

methanolic crude leaf extracts and determined its possible mode of anti-dermatophyte actions.

1.4 Study Objectives

1.4.1 General Objective

The overall objective was to determine the anti-dermatophytic effect of *Salvia nilotica* crude methanolic leaf extract against *Trichophyton mentagropytes*.

1.4.2 Specific Objectives

The specific objectives of this study were:

(i) To qualitatively determine the classes of phytochemicals present in methanolic crude leaf extracts of *Salvia nilotica.*

(ii) To determine anti-dermatophytic activity of *Salvia nilotica* crude leaf extract against *Trichophyton mentagrophytes.*

(iii) To examine gene expression changes of virulence genes in *Trichophyton mentagrophytes* when treated with methanolic *S. nilotica* crude leaf extract.

1.5 Null hypothesis (H_0)

H_{01}. Methanolic *Salvia nilotica* crude leaf extract does not have any phytochemical constituents.

H_{02}. Dermatophytes (*Trichophyton mentagrophytes)* are not susceptible to the effect of methanolic *Salvia nilotica* crude leaf extract.

H_{03}. Methanolic *Salvia nilotica* crude leaf extract doesn’t affect the expression profiles of *T. mentagrophytes* virulence genes.

1.6 Overall Study Significance

The results obtained from this study have provided useful insights on anti-dermatophytic effects and plausible mode of action of methanolic *Salvia nilotica* crude leaf extract against *Trichophyton mentagropytes.* Thus, this study indicates the potential of *S. nilotica* leaf extract for alternative medicinal use for dermatophytosis caused by *T. mentagrophytes.*

CHAPTER TWO

LITERATURE REVIEW

2.1 Dermatophytes

Dermatophytes are anatomically and physiologically associated with molds, several of which are responsible for causing dermatophytosis called tineas, a common contagious disease (Mihali *et al.*, 2012). This group of microorganisms possesses two unique properties: keratinophilic and keratinolytic; this means they can digest or break down the keratin in tissues like hair, nails, and epidermis among others *in vitro* (Simpanya, 2000). Dermatophytes are deeply specialized filamentous fungi responsible for superficial fungal infections in humans (anthropophilic species) and animals (zoophilic species) (Zaugg *et al.*, 2009). They fit into the genera *Trichophyton*, *Microsporum,* and *Epidermophyton*, which infect the nails, stratum corneum, and hair and rarely infects dermis and subcutaneous tissues. Achterman and White (2011) noted that dermatophytes classification falls into three groups based on their natural habitat; anthropophilic species (adapted to humans), zoophilic (adapted to animal), and a few geophilic species (adapted to the soil and maybe parasitic).

2.2 Etiology of dermatophytosis

Dermatophytes are closely related fungal organisms with a high affinity for keratinized body structures, utilizing keratinase enzymes to infect the hair, skin, and nails (Mercer & Stewart, 2019; Mercer & Verma, 1963). Dermatophytosis's causative agents fall into three genera consisting of *Epidermophyton, Trichophyton,* and *Microsporum* (Ouf *et al.*, 2016). There are two most common dermatophyte pathogens in the population: *T. rubrum* and *Trichophyton mentagrophytes,* which rank first and second most common

causative agents (Nenoff *et al.*, 2007; Rivas & Mühlhauser, 2015). These two organisms cause benign infections confined to the stratum corneum, which is generally referred to as dermatophytosis or tineas, which include tinea pedis, tinea corporis, tinea unguium (onychomycosis), tinea cruris, and tinea capitis (Seebacher *et al.*, 2008). Usually, infections caused by dermatophytes have been named by affixing the Latin name of the afflicted body part after the word "tinea," such as infections of the nails caused by *Trichophyton rubrum* is called tinea unguium (Andrews & Burns, 2008). Circulatory disorders, psoriasis, ichthyosis, diabetes mellitus, and conditions affecting cellular immunity are common host factors associated with tinea pedis and onychomycosis (Chang *et al.*, 2008). According to Weitzman & Summerbell (1995), anthropophilic infestation often causes chronic forms of dermatophytosis affecting humans, which has limited inflammation. In contrast, geophiles and zoophiles infection lead to acute, but self-limiting inflammation.

2.3 Epidemiology of dermatophytosis

Dermatophytosis is a disease occurring worldwide, and every year, 20–25 % of humans and animals are infected (Gnat *et al.*, 2018). The epidemiologic transition has seen the rapid change in clinical patterns of dermatophyte infections with increased incidences and prevalence of the population's disease, especially in developing countries (Ginter-Hanselmayer *et al.*, 2007). Since mammalian skin mycoflora is not the natural reservoir of dermatophytes, the infection pattern follows the vulnerable population's exposure to infected animals, humans, or soil (Ilkit & Durdu, 2015). Dermatophytosis is caused by dermatophytes, which has established itself as a big public health problem in

underdeveloped countries and among elderly and immuno-suppressed patients worldwide (Ouf *et al.*, 2016).

Globally, dermatophytes are the most frequent causes of fungal infections resulting in treatment expenditure of close to five hundred million dollars every year in the United States of America (USA) (Achterman & White, 2011). In studying the global disease burden, The World Health Organization (WHO) reported a 19.7% prevalence of dermatomycoses in middle and low-income countries (Dogra *et al.*, 2019). The most common fungal infections in living hosts are those involving the skin and nails, and they affect between 20 to 25% of the global populations and serve as the fourth most common human diseases (Havlickova *et al.*, 2008). Since dermatophytosis can be transmitted from one person to another or from animals to humans, high costs of treatment, the difficulty of control, and the public health consequences remain highly important (Bokhari, 2009). Dermatophytes are the major causes of acute or chronic infections in humans, chiefly recurrent mucosal, cutaneous, or nail infections that can be very harsh in immunocompromised individuals (Beatriz *et al.*, 2012). Climatic conditions, the vulnerability of the population, cultural practices, lifestyle, migration patterns, and socioeconomic conditions influence the distribution of dermatophytosis and their etiological agents (Gebreabiezgi, 2016). Geography is a factor in the global distribution of dermatophytes, while a few species appear uniformly distributed (Ndunge, 2014). The distribution of dermatophytoses in advanced countries has presented notable changes over the past few decades as an aftermath of changes in some environmental conditions and the distribution of the etiological agents, which usually reflects the variation in clinical patterns dermatophytosis (Mohammed, 2013). According to Rashidian et al. (2015), tinea capitis is

a common infection in children, while tinea cruris is dominant in adults while tinea ungium is normally regarded as a chronic infection of nails in adults.

2.4 Pathogenesis of dermatophytosis

Fungal characteristics and host factors are important conditions for dermatophyte pathogenesis, facilitating infection and progression of diseases in host tissues (Faway *et al.*, 2018). Dermatophytes cannot invade and spread deeply into the skin, as it is countered by the host's immune system (Vermout & Tabart 2008). However, disease progression is dependent on agent ability to break barriers of host immunity, including an attack by phagocytic cells, skin desquamation, acidic nature of the skin, and action of inhibitory molecules such as antimicrobial peptides and fatty acids (Seite & Misery, 2018). Rapid attachment to the host tissues following exposure is necessary to avoid elimination and breakdown of the infection process (Faway *et al.*, 2018). Several factors have been linked to dermatophytosis's pathogenesis, which includes the release of keratinolytic enzymes, genetic characteristics, and host factors (Nenoff *et al.*, 2014).

Understanding the pathogenesis involved in dermatophytosis helps develop new prophylactic and therapeutic remedies (Baldo *et al.*, 2012; Vermout & Baldo *& et al.*, 2008). The first step in the infection process involves contact and attachment of the fungal agents to the hosts, which occurs through exposure to infected animals or fomites, followed by germination of arthrocodium and penetration of stratum corneum by hypha. During the invasion, dermatophytes utilize keratinolytic enzymes to digest the highly keratinized tissues into nucleotides, which are assimilated via the cell transport system.

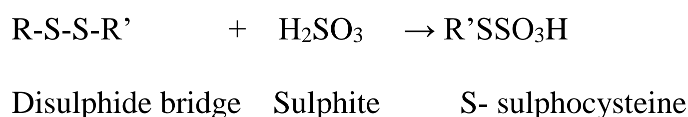
Broadening of knowledge on factors that participate in the pathogenesis of dermatophytoses such as proteases, secreted enzymes, adhesion possibilities and capacity

to fine-tune defense mechanisms of the host is one of the most important steps in dealing with this illness (Vermout & Baldo *& et al.*, 2008). Although there is inadequate knowledge of physiological processes for dermatophyte–host interaction, the pathological process of dermatophytosis involving hydrolytic enzymes, particularly proteases, is well documented (Mercer *et al.*, 2019). Proteases (including keratinases) are a significant fungal virulence component, essential during the invasive stage of the infection (Monod, 2008). Genes responsible for the synthesis of endoproteases involving subtilisins (SUB; S8 family), fungalysins (MEP; M36 family), deuterolysins (M35), and exoproteases, such as dipeptidyl peptidases (S9 family), amino-carboxypeptidases (M14 and S10) play a crucial role in the physiological pathway of protease action during the degradation of proteins to nucleotides in keratinized tissues (Gräser *et al.*, 2018).

Endoproteases serve as first-line proteases in the breakdown of sulphite-cleaved keratin substrate resulting in the release of large peptides (Monod, 2008). Some of the major endoprotease genes fundamental in dermatophyte action include the seven and five putative genes responsible for encoding subtilisins and fungalysins, respectively (Mercer & Stewart, 2019). Following inoculation, arthrospores adhere to the keratinocytes within two hours, followed by proteases secretion by dermatophytes such as the subtilisins and metalloproteases, and finally perform a key function of penetration by digesting keratin (Rouzaud *et al.*, 2015). Dermatophytes also produce reducing agents and sulphites to allow proteases to break down keratin, enabling these microorganisms' to degrade keratin being a major virulence attribute. Once they have adhered to human keratinocytes, dermatophytes penetrate the stratum corneum and cause infection (Baldo *et al.*, 2012).

The fungi utilize the host tissue macromolecules as a source of essential substances such as phosphorus, carbon, sulfur, and nitrogen (Peres *et al.*, 2010). Some enzymes released during the invasion process have also been linked with acting on antigens, consequently inducing various degrees of inflammation (Jensen *et al.*, 2007). The secretion of sulphite, a reducing agent, facilitates efficient keratin degradation through cleaving of keratin-stabilizing cystine bonds (disulphite bridges) responsible for the hard keratin structure (Lechenne *et al.*, 2007). Reduced proteins emerging from sulphitolysis, which cleaves disulphide bridges, undergo further physiological processes facilitated by exo- and endoproteases secreted by the fungal agents. Cysteine metabolism is an important biological process in filamentous fungi, including dermatophytes, utilizing sulphite efflux pump encoded by the gene SSU1 to produce sulphite (Lechenne *et al.*, 2007) as shown in Figure 2.1. Thus, high expression of SSU1 is a major physiological characteristic of dermatophytes, facilitating fungal efficiency in pathological processes such as the damage of hair, stratum corneum, and nails.

Sulphite facilitates cysteine breakdown to cysteine and S-sulphocysteine, reducing proteins available for hydrolysis (Peres *et al.*, 2010). Some researches highlight the significance of sulphite efflux pump encoded by the SSU1 gene in this reduction process, which is a member of the dicarboxylate or tellurite-resistance transporter family (Lechenne *et al.*, 2007). These synergistic processes make sulphitolysis an essential step in the breakdown of keratinized tissues, serving as an antecedent to protease actions as shown in reduction reaction below;



The release of subtilisin and metalloproteases enzymes in *in-vivo* experiments indicates the fungal virulence's significance in infecting healthy individuals. Proteases fall into two subclasses; endo-protease, which acts on peptide bonds of a polypeptide and exo-protease that targets peptide bonds of the N- or the C-terminus in a polypeptide chain.

The dermatophyte genome comprises a collection of genes of hydrolytic enzymes, particularly proteases, highly similar across species (Martinez *et al.*, 2012). In addition, aminopeptidases are another essential enzyme in the dermatophytic process, with dipeptidyl-peptidases (DppIV and DppV) and leucine aminopeptidases (Lap1 and Lap2) showing similar physiological characteristics to *A. fumigatus* orthologues (Monod *et al.*, 2005).

Keratinized tissues

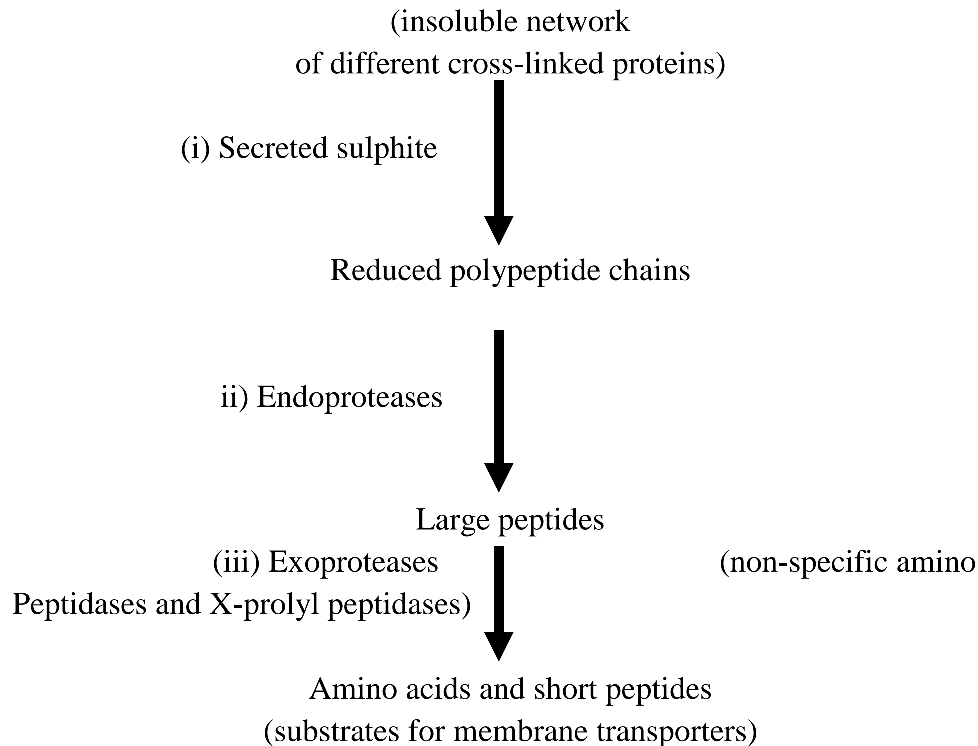


Figure 2.1. **The process of hard keratin degradation by dermatophytes (Gräser *et al.*, 2000)**

(i). Sulphite excretion complements enzymatic activity and is mandatory for sulphide bridge reduction.

(ii)Endoproteases catalyzes the production of large peptides with free ends targeted by exoproteases.

(iii). Exoproteases degrade peptide chains in proline-rich proteins to short (2-5-mer) peptides and amino acids via complementary catalysis by prolyl peptidases.

2.5 Diagnosis of dermatophytosis

Dermatophyte diagnosis entails mycological identification of fungal strain, which involves the correlation of microscopic examination with clinical manifestations (Gnat *et al.*, 2018).

However, medical mycology's evolution saw the emergence of molecular diagnostics, facilitating the analysis of biological markers for accurate identification of the fungal agents (Brillowska-Dabrowska *et al.*, 2010). Nevertheless, despite this promising therapeutic progress, data reliability remains a diagnostic problem in dermatophyte identification. The time-dependent conventional mycological methods fail test validity in some instances (Łagowski *et al.*, 2019a). Subjective evaluation of fungal morphology based on macro- and microscopic structure, time-based variability of these characteristics, and extensive staff experience are major challenges limiting species identification (de Hoog *et al.*, 2017).

Another significant problem is obtaining negative culture results, despite the observed presence of arthrospores in preparation (Gnat *et al.*, 2020). Consequently, the isolation of non-dermatophyte fungi including *Alternaria* spp., *Penicillium* spp., *Aspergillus* spp., and *Fusarium* spp adds to the challenges of conventional diagnostics (Gnat *et al.*, 2020).

In contrast, the high selectivity of PCR methods limits the identification of diverse dermatophytes species using the same primers (Piri *et al.*, 2018). Clinical studies have demonstrated the high specificity and sensitivity of PCR methods, highlighting the diagnostic reliability of 40-100% (Piri *et al.*, 2018). The utilization of the PCR technique is limited to few mycological laboratories, with routine use only in pioneer facilities. The development of molecular-based methods has already improved a lot during the last years, from only being able to detect fungi in cultures to now being able to detect fungi directly in clinical samples (Graeser & Saunte, 2020). The large taxonomic variance between dermatophytes and the significance of species-level identification in scientific practice raises concern on the utilization of “gold standard” for routine identification with the

ongoing debate projected in the inconsistencies of microbiologists' opinion (Gnat *et al.*, 2019).

Although most developed countries utilize cost-effective phenotypic techniques for gold standard diagnosis, this identification approach is often inaccurate and time-consuming (Łagowski *et al.*, 2019a). However, this mycological method's significant characteristic is its flexibility, allowing subjection of isolated dermatophyte to downstream application involving identification, drug susceptibility testing, and epidemiological establishment of the infection source (Ombelet *et al.*, 2019). Therefore, given the current diagnostic challenges of a considerable number of dermatophyte species, the standard method for reliable identification is indisputable (Kalita *et al.*, 2019).

2.6 Treatment and management of dermatophytosis

Despite the advancements of science and technology, the innovation of novel and efficient antifungal drugs still lags, mainly due to the similarity of eukaryotic mechanism between fungi and human beings (Berdy, 1989). These cellular characteristics make it difficult to develop more specific antifungal agents targeting only fungi and avoiding unintended damage to humans (Lakshmipathy & Kannabiran, 2010). Treatment duration depends entirely on both infection type and its symptoms with skin lesions requiring a two-three week therapy and a four-six week period for feet inflammation (Elewski & Hazen, 1989). Generally, most dermatophytic infections are superficial, but immune-compromised patients can experience the disease's cruel spread, including systemic infection (Rodwell *et al.*, 2008). Although dermatomycosis is treatable by the available antifungal drugs, there is an increased re-infection rate and remains unresolved whether this phenomenon is a relapse or a new infection (Gupta & Cooper, 2008). There is a fundamental need for an

accurate identification of causative agent at species level during diagnosis using molecular techniques to accomplish an ultimate and fruitful treatment of dermatophytosis (Dingle & Butler-Wu, 2013). The cell wall acting echinocandin anti-dermatophytic agent was the first primary class of systemically acting anti-dermatophytic agent to target unique β 1, 3-glucan synthase. *Penicillium griseofulvu* derived compound called griseofulvin, was the first widely used antifungal agent that showed selective inhibitory effect against superficial fungal infections (Negri *et al.*, 2014).

However, fluconazole remains the preferred drug of choice for the management of onychomycosis and dermatophytosis because of its great affinity for keratinized tissues (Kathiravan *et al.*, 2012). Azoles' mechanism of action against fungus is positioned on the ergosterol biosynthetic pathway, where the fungal cell wall's inhibition occurs at different steps (Odds *et al.*, 2003). Ergosterol is the dominant compound of the fungal cell membrane, functioning as a bio-regulator of membrane activity. This structural property is crucial in maintaining biosynthetic steps such as fluidity and integrity of the membrane, making it a major target of the clinically available drugs (Khan *et al.*, 2010), such as allylamine and azole derivatives (Carrillo-Munoz *et al.*, 2006). Previously, imidazole derivatives showed an enhanced mechanism of action targeting several membrane-bound enzymes and lipid biosynthetic pathways (Carrillo-Munoz *et al.*, 2006). A major side effect of azole use is its ability to inhibit cytochrome p-450 enzymes responsible for cholesterol secretion in mammalian liver cells (Carrillo-Munoz *et al.*, 2006). New triazole derivatives, including fluconazole, voriconazole, itraconazole, posaconazole, teraconazole, and ravuconazole have higher target specificity, Cytochrome P-450 lanosterol 14-alpha-demethylase and encoded by the *ERG11* gene for Erg11p, have been developed (Carrillo-

Munoz *et al.*, 2006). According to various studies, several types of ringworms show little response to topical medication. As a result, many kinds of ringworms need to be treated with systemic anti-dermatophytic agents such as amphotericin, azoles (clotrimazole, fluconazole, itraconazole, ketoconazole, miconazole, voriconazole), griseofulvin and terbinafine (Behzadi *et al.*, 2014).

Amphotericin B, which is fungicidal in high doses, has a complex action mechanism involving the biochemical interaction of the antifungal molecule with ergosterol membrane. This interactive model hydrophobically links amphotericin B molecules to ergosterol through the pores in the membrane, altering permeability and leakage of vital cytoplasmic components leading to cytolysis (Carrillo-Munoz *et al.*, 2006).

Fungi derived chemical molecules of the class Echinocandins include caspofungin, a derivative of pneumocandin Bo produced by *Glarea lozoyensis*, anidulafungin and micafungin from echinocandin B modified from *Aspergillus nidulans* and *Coleophoma empetri*, respectively (Carrillo-Munoz *et al.*, 2006). Echinocandins inhibit the synthesis of β -1,3-D-glucan, an essential compound in maintaining cell wall functionality (Eshwika *et al.*, 2013). The mechanism of action includes specific targeting of FKS1 genes in fungal organisms encoding glucan synthase components, an enzyme responsible for synthesizing 1,3-β -D glucan fundamental in cell wall susceptibility antifungal agents (Gubbins & Anaissie, 2009).

2.7 Complications and challenges of management of dermatophytosis

Dermatophytic infections are largely considered a nuisance in the healthy population, with itching and discomfort being some of the most common symptoms (Chadwick, 2013). Bacterial secondary illness and allergies can also make matters worse on concealed chronic

ringworm (Kim *et al.*, 2008). Dermatophytes invade keratinized tissues for the obvious reason that they utilize them as a source of food, and their lesions are characterized by inflammation, irritation, swelling, local redness, and scaling (Miron *et al.*, 2014). In extraordinary cases, dermatophytes access the deeper layer of the dermis and other organs (Rezvani *et al.*, 2010). Though dermatomycosis is hardly life-threatening, they cause significant morbid effects such as discomfort, social isolation, deformity, and can incline to secondary bacterial infection (Brown *et al.*, 2012).

The treatment of dermatophytosis needs about 2–4 weeks to be cured in many types and may require many months or even years in cases of tinea capitis and onychomycosis (Hay, 2018). Cases of treatment failure (with topical or systemic treatment) and disease relapses have been reported (Rouzaud *et al.*, 2015). Itraconazole turns out to be a safe drug, the incidence of side effects appearing to depend on the duration of therapy but tends to occur in 7% to 12% of patients (Del Palacio *et al.*, 2000). Skin dermatophytic lesions are fungus and metabolites-linked inflammatory reactions, clinically erythematous, itchy, and round spots. Onychomycosis causes nail thickening with the fungal mass separating the nail bed, occasionally developing white spots and dystrophy (Martinez-Rossi *et al.*, 2016).s

2.8 Alternative therapies for management of dermatophytosis

2.8.1 Photodynamic therapy (PDT)

Photodynamic therapy (PDT) involves localized oxidative photodamage of a target lesion through the dual administration of a photosensitizer (PS) and selective illumination resulting in cell death (Plaetzer *et al.*, 2009). The interaction between photons of visible light with the PS's intracellular molecules provides the mechanism responsible for the underlying PDT effects (Hamblin & Hasan, 2004). Reactive species form from oxidative

stress arising from the visible light effect on biological tissues causing cell damage occurring following the breakdown of a cellular biochemical defense mechanism by reactive oxygen species (Henderson & Dougherty, 1992).

Microbial targeted selectively delivery and irradiation with light of suitable wavelength causes PS's activation in the cells (Maisch, 2009). Type I and type II oxidative mechanisms may occur following this PS activation process, responsible for releasing free radicals and single oxygen molecules, respectively (Wilson & Patterson, 2008). The type I chemical process involving electron-transfer from the PS triplet state to a substrate releases radical ions causing oxygen reaction and cytotoxic species release, including superoxide, lipid-derived, and hydroxyl radicals (Athar *et al.*, 1988). In the type II pathway, energy transfer from the PS triplet state to the ground state breaks down molecular oxygen (triplet), releasing excited-state singlet oxygen that oxidizes various chemical substances, including lipids, proteins, and nucleic acids (Phoenix & Harris, 2006). These biological components can damage cellular physiological processes inactivating microbes (Redmond & Gamlin, 1999), largely via the photo-oxidation of proteins and nucleic acids (Jori & Coppellotti, 2007) and membrane lipids (Smijs & Schuitmaker, 2003).

Photodynamic therapy (PDT) targeting ergosterol production is an alternative treatment option for antifungal medications. Antimicrobial photodynamic therapy (aPDT) combines a PS, specifically pharmacologically inert chromophore, with a suitable light wavelength (Tegos *et al.*, 2012). Given its high selectivity, the limited incidence of drug-resistant strains, and cost-effectiveness (Maisch *et al.*, 2005), PDT's observed effects on dermatophytes and yeasts have raised concern on its potential use in the management of skin mycoses (Hamblin & Hasan, 2004). Therefore, based on the *in vitro* and *ex*

vivo results, successful trials of PDT in clinical practice may turn out to be a valuable alternative antifungal medication (Ragàs *et al.*, 2010). The secretion of harmful radicals, such as nitrogen and reactive oxygen (ROS) following interaction of chromophore with specific light wavelength, is responsible for cell death (Hamblin & Hasan, 2004).

2.8.2 Use of lasers for treatment of dermatophytosis

Recent advances in fungal treatment have seen the development of laser-based therapy to control dermatophytosis in nails (do Espírito Santo & Deps, 2018). This treatment method is temperature-dependent, with a better outcome in inhibiting fungal growth achieved at high temperature (Gnat *et al.*, 2020). Although knowledge of therapy duration is limited for different laser rays at varying temperatures, the significance of homogenous heat distribution for antifungal activity is apparent (Gnat *et al.*, 2020). However, laser treatment inconsistencies border its use as a standalone therapy, with reported cure rates lower than topical or oral methods (Gupta *et al.*, 2019). Nonetheless, a combination of laser rays and drug therapy provides quicker resolution in onychomycosis with reported lower rates of relapse (Bonhert *et al.*, 2019). Widespread use of this combination therapy can be a solution in dermatophyte management; however, more studies on the optimal effective dose, frequency of laser exposure, and treatment duration are necessary (Gnat *et al.*, 2020).

2.8.3 Botanicals for management of dermatophytosis

Plants have been a key foundation of greatly effective conventional drugs for treating many forms of dermatophytes (Shrivastav *et al.*, 2013). According to Ghasemi Pirbalouti *et al.* (2014) the antifungal activity of *Hypercom perforatum* essential oil is effective against *Epidermophyton floccosum*, *T. mentagrophytes* var. mentagrophytes, *Microsporum gypseum*, *T. mentagrophytes var. interdigital*, *Microsporum canis*, *Trichophyton*

tonsurans and *T. rubrum*. Terpineol, the main component of *H. perforatum* essential oil, plays a significant role in anti-dermatophytic activity (Ghosh *et al.*, 2014). The mechanism of action in *H. perforatum* oils involves cytoplasm and cell wall metabolism. Monoterpenes cause swelling of the cytoplasmic membrane and increase permeability, leading to poor regulation of surrounding proteins, altering ion transportation processes, and inhibiting cell respiration (Arora *et al.*, 2013).

Salvia nilotica, a flowering shrub, is a perennial plant in the Eastern African highlands spanning Ethiopia to Zimbabwe, thriving between 900 and 3600 m elevation. Its flowers are arranged in whorls of 6-8 with a color range from white to purple to rose (Clebsch, 2003). In Kenya it can be found in different places like the Mt. Kenya region, Mt Elgon, Mau summit, Nyanza region, Cherangani Hills, Aberdare national park, and Murang' a County. The plant is found in different parts in the East African region like Rungwe district in Tanzania, Gisenyi district in Rwanda, Burundi, and Ethiopian highlands (Vorontsova *et al.*, 2014).

The taxonomy of *S. nilotica,* according to (Mayr & Scharnhorst, 2015), shows that the plant belongs to the unranked asteroids, order Lamiales, family Lamiaceae and the genius *Salvia* which contains *Salvia nilotica* species which was under investigation in this study for anti-dermatophytic potential. *S. nilotica* Jacq and *Salvia schimperi* Benth (Lamiaceae) are plants containing essential oils which are extensively used in the Ethiopian folkloric medicine (Asfaha *et al.*, 2008). *Salvia nilotica* was known by various vernacular names in Ethiopia including "fereshei," "hulegeb," and "sokoksa" is locally used for the treatment of skin warts (by topically applying the fresh leaves and massaging on the wart), wounds (by applying the fresh leaf juice on the affected part), joint pain and

UV skin reactions (by boiling it together with other medicinal plants and passing the steam over the affected part (Abebe *et al.*, 2003). In Kenya, it's known by the name 'sirar' among the Tugen community, and it has been used as an anti- dermatophyte alternative form of treatment for dermatophytosis, by harvesting the leaves, dry in the shade, crushed then mixed with petroleum jelly as a vehicle and applied on the affected part of the body. There is no known scientific work, which has been done on *S. nilotica* plant.

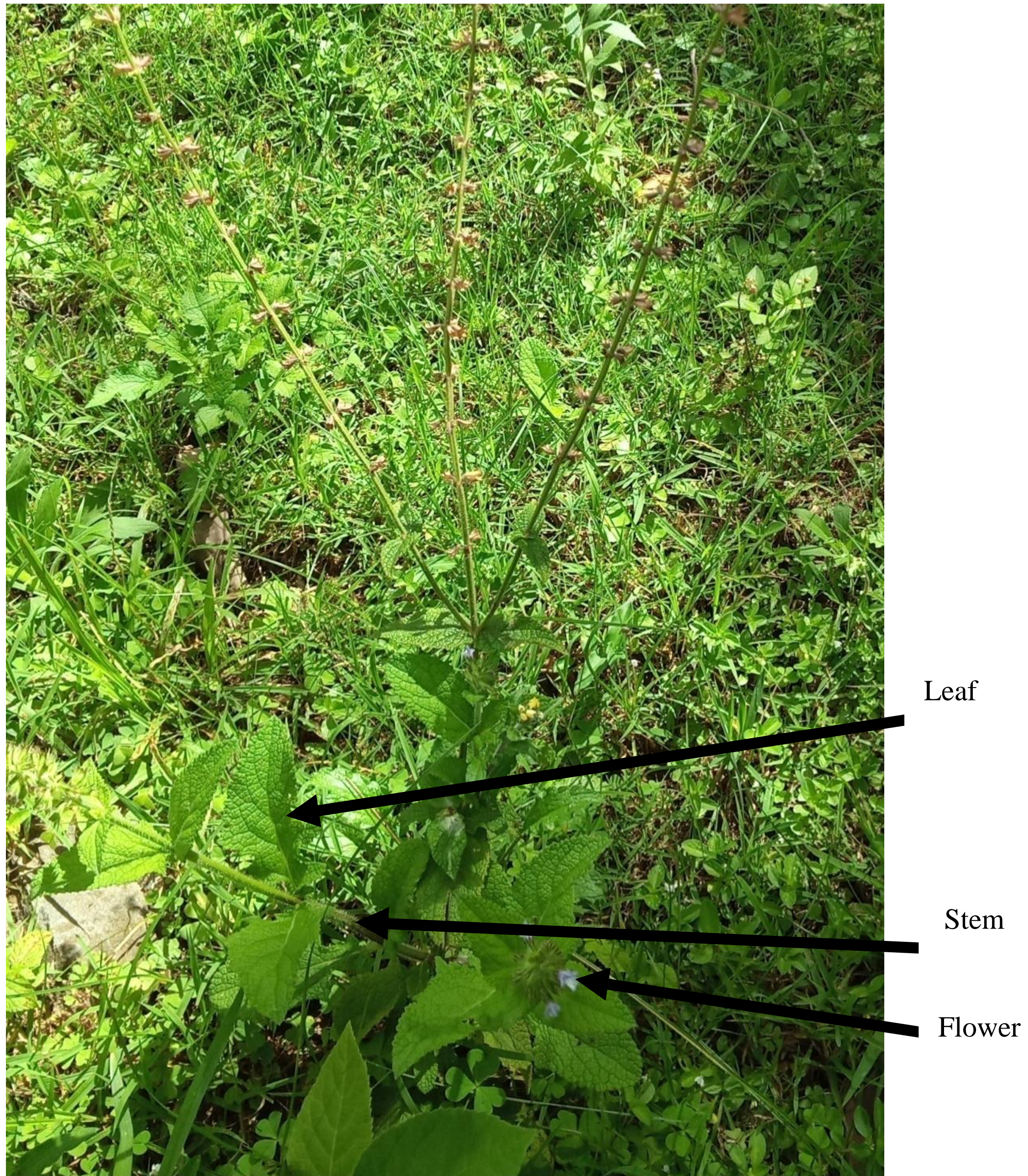


Plate 1. ***Salvia nilotica*** **on its natural habitat in Katimok forest, Kenya.**

(Source: Author, 2016).

2.8.4 Anti-dermatophytic Effect and Mode of Action of Botanicals

Plant extracts involving metabolites possess a range of anti-infective agents (Martinez-Rossi *et al.*, 2018). A significant anti-infective activity exhibited by these compounds includes immunoregulatory effects toward macrophages by Coumarins and cell wall polypeptides and adhesion binding by quinones, causing physiological impairment (Martinez-Rossi *et al.*, 2018). Saponin categories comprising steroidal glycoalkaloid, steroid, and triterpenoid interferes with cellular integrity by disrupting sterol bound membranes (Sinha *et al.*, 2019). Phenolic compounds have broader physiological activity on various cellular targets along different biological pathways evident in Candida sp. For example, cinnamic acid immune properties involve monocytes activation (Conti *et al.*, 2013), while isoquercitrin and curcumin physiological effect include cellular membrane impairment (Lee & Lee, 2014; Yun *et al.*, 2015). Licochalcone A impairs and inhibits hyphae development (Teodoro *et al.*, 2015) with caffeic acid causing isocitrate lyase (Cheah *et al.*, 2014). Thymol, carvacrol, and baicalein have exhibited blocking effects on drug transporters in candida using rhodamine 6G dye, by inhibiting efflux transporters and causing accumulation of antifungal agents and susceptibility of candida to the fungal compounds (Teodoro *et al.*, 2015). These phytochemical effects prompt investigating these compounds on dermatophytes (Martinez-Rossi *et al.*, 2018).

Some studies have demonstrated monoterpene linalool inhibition of *T. rubrum*, including thymol and carvacrol fungitoxicity (de Oliveira Lima *et al.*, 2017). Other studies have reported antidermatophytic activity of essential oils from *Lippia gracilis* genotypes (LGRA106 and LGRA-109) similar to fluconazole (de Melo *et al.*, 2013). The 12-methoxy-4-methylvoachalotine alkoloid (MMV) is another significant compound with a

therapeutic effect against *T. rubrum* (Medeiros *et al*., 2011). Some antifungal agents with open-chain flavonoids, such as chalcones, exhibit fungitoxicity against dermatophytes inhibiting cell wall biosynthesis (Bitencourt *et al.*, 2013; Boeck *et al.*, 2005) through blockage of fatty acid synthase (Bitencourt *et al.*, 2013).

Other researches focus on assessing the specific mechanism of action of biochemicals against *T. rubrum*. For example, the keratinocyte cell line co-culture with *T. rubrum* facilitated the evaluation of the antifungal activity of glycoalkaloid, solanine, which showed down-regulation of the erg1, erg11, mep4, and mdr2 genes (Komoto *et al.*, 2015). Caffeic acid biomolecular activity shows a similar antifungal activity against *T. rubrum*, associated with a decrease in ergosterol content and modest hindrance of isocitrate lyase catalytic activity, impairing the cellular membrane (Cantelli, 2017).

Similarly, luteolin and flavonoids quercetin are other compounds with antifungal activity against *T. rubrum*. Quercetin's comprehensive assessment demonstrated a similar mechanism involving ergosterol reduction, inhibition of fatty acid synthase (FAS) activity, and membrane damage (Bitencourt *et al.*, 2013). A trans-chalcone substance showed a potent inhibitory effect against *T. rubrum* strains to inhibit fatty acid and ergosterol synthesis (Bitencourt *et al.*, 2013; Komoto *et al.*, 2015).

The licochalcone A fungitoxic effect against *T. rubrum* involves suppression of gene for cell wall synthesis and ergosterol production, including those involved in the synthesis of putative virulence factors malate synthase, citrate synthase, and isocitrate lyase (Martinez-Rossi *et al.*, 2018). Other fungitoxic activities include impaired hyphal development and suppression of isocitrate lyase function (Cantelli, 2017). In addition, chalcone treatment

exhibits the down-regulation of genes involved in membrane transporters. These include those encoding for the drug efflux pump, as demonstrated during *T. rubrum* conidia and keratinocyte cell line co-culture (Komoto *et al.*, 2015). These pharmacological activities of chalcones and their presence as natural molecules make them components of increasing interest in the medical field for their potential use in therapeutic practice (Nowakowska, 2007). Notably, these compounds' simple chemical structures make them interesting molecules for cost-effective and safe synthesis for pharmaceutics (Narender & Reddy, 2007). Therefore, considering the increasing demand and challenges in developing new drugs, these natural compounds and their derivatives emerge as promising targets for the development of alternative antifungal agents (Martinez-Rossi *et al.*, 2018).

2.8.5 Amplification of cDNA using quantitative polymerase chain reaction by comparative C_T method

Quantitative real-time polymerase chain reaction (qPCR) has been proven to be a powerful tool in the quantification of DNA and RNA sequences in molecular biology and biomedical fields (Gingeras *et al.*, 2005; Heid *et al.*, 1996). The principle of the method is based on classical PCR, where the employment of fluorescent dyes or probes and fluorescent signal measurement enables quantification of starting DNA material in the sample during amplification (Valasek & Repa, 2005). The main advantages of qPCR include its high sensitivity, accuracy, and the ability to detect and quantify rare transcripts and alterations in gene expression, producing reliable and rapid quantification results ((Pfaffl, 2001; Yuan *et al.*, 2006). When analyzing gene expression, qPCR data can be subjected to absolute or relative quantification (Livak, 2001; Pfaffl, 2001; Yuan *et al.*, 2006). Comparative C_T chosen in this research has the advantage of simplicity and

effective presentation of data as 'fold change' in expression instead of copy number (Schmittgen & Livak, 2008). This absolute expression of data provides the exact transformation of the data via a standard curve (Chen *et al.*, 2005). Secondly, this procedure limits the need for a standard calibration curve or formulations with known concentrations (Fraga *et al.*, 2008). Finally, relative quantification is more comfortable to perform, requiring less set up time than absolute quantification given the dispensability of the standard curve (Fraga *et al.*, 2008; Livak & Schmittgen, 2001). Furthermore, gene expression assessment in biological applications does not require absolute knowledge of the amount of mRNA (Bustin, 2002; Huggett *et al.*, 2005).

CHAPTER THREE

MATERIALS AND METHODS

3.1 Ethical considerations

Research protocols were approved by the CTMDR Centre Scientific Committee of the Kenya Medical Research Institute (Reference Centre number KEMRI / CTMDR / CSCPO90) as shown in Appendix I.

3.2 Collection, identification and processing of plant material

Salvia nilotica leaves were collected from Katimok forest located in Kabartonjo Division, Baringo North Sub-county, Baringo County, Kenya (Latitude 000 37’ 00” N, Longitude 350 47’ 00” E). The whole plant of S. nilotica was collected in November 2016 and identified at the Department of Biological Sciences at the University of Eldoret (UoE) with the assistance of a qualified taxonomist. The plant material was allocated voucher number M.U.H/Salni/008/16 and deposited in the herbarium in the Department of Biological Sciences at the University of Eldoret. The leaves were chosen for the experiment because it is the commonly used part of the plant by the herbalist in the Tugen community in Kenya against dermatophytic infections (Skin and nail infections). The harvested leaves of S. nilotica were air-dried at room temperature for two weeks to a moisture content of between 12-13%. After drying, it was ground into a fine powder using a hammer mill and weighed.

3.3 Materials

Sabouraud dextrose agar (SDA) was obtained from HiMedia Laboratories Pvt. Limited, Mumbai, India. Fluconazole 2mg/mL was obtained from Pfizer Inc New York, USA. Easy pure® RNA Kit was obtained from Zhongguancun Dongsheng International Science Park, Beijing China, and RNeasy® Mini Kit (Qiagen) magBio genomics was obtained from

Gaithersburg inc USA. Primers were obtained from Africa's Genomics Company, trading as Inqaba Biotechnical Industries (Pty) Ltd, Pretoria South Africa. All other reagents used were of standard analytical grade.

3.4 Extraction of the leaf crude extract

The extraction of the leaf was done based on the method described by (Cakir *et al.*, 2004) with little modifications of the procedure. Two hundred grams of ground *S. nilotica* leaves were soaked in 300 mL of methanol for 24 hours then filtered. A further 24-hour re-extraction process was done with 300 mL of methanol. Filtrate concentration was achieved under low pressure at 50 ℃ using Rotary Evaporator (Rotavapor type EL 30; model AG CH-9230, Germany). *Salvia nilotica* crude leaf extract in the form of a paste was stored at 4 ℃ until it was required for qualitative phytochemical evaluation and further investigation.

3.5 Qualitative phytochemical screening

Qualitative phytochemical evaluations were carried out using 30 mL of distilled water mixed with 10 g of methanolic leaf extract of *S. nilotica* paste and stirred thoroughly to form 0.3 g/ mL concentration of the stock solution. The tests to identify the phytochemical constituents of *S. nilotica* extract involved the standard procedures described by Edeoga *et al.* (2005), Simbo (2010), and Trease & Evans (1989), which is outlined in the subsequent subsections.

3.5.1 Test for tannins

For the test of tannins, one milliliter of the *S. nilotica* 0.3 mg/mL solution was taken and stirred with one milliliter of distilled water in a test tube, and three drops of $FeCl_3$ solution were added. The mixture was observed for the presence of green precipitate

3.5.2 Test for saponins

Saponins were tested by taking two milliliters of distilled water and adding two milliliters of *S. nilotica* solution in a test tube, shaken vigorously, and warmed. Formation of stable foam, which lasted for three minutes, indicated the presence of saponins.

3.5.3 Test for phlobatannins

Phlobatannins presence was established by adding 2 mL of one percent hydrochloric (HCl) into 2 mL of *S. nilotica* solution in a test tube and the mixture boiled for 3 minutes. Formation of a red precipitate confirmed the presence of phlobatannins.

3.5.4 Test for flavonoids

One milliliter of 10% lead acetate solution was added to 1 mL of *S. nilotica* solution in a test tube and shaken lightly and observed. The deposition of a yellow precipitate confirmed a positive test for flavonoids.

3.5.5 Tests for anthraquinones

Two milliliters of *S. nilotica* solution was placed in a test tube, and 5 mL of 10% hydrochloric acid was added, boiled for 5 minutes in a hot water bath, then filtered and allowed to cool for 10 minutes. The filtrate was shaken with 5mL of chloroform for one minute. A few drops of 10% ammonia were then added to the mixture and heated until a color change was noted.

3.5.6 Test for steroids and terpenoids (Salkowaski test)

The terpenoids presence was determined using the Salkowaski test. Two milliliters of *S. nilotica* solution was poured into a test tube and dissolved in 2 mL of chloroform. The chloroform was then evaporated to dryness by passing it over Bunsen burner. Then two

milliliters of concentrated sulphuric acid were added and heated for 2 minutes. The reddish-brown color at the interface indicated the presence of both terpenoids and steroids.

3.5.7 Test for alkaloids

The alkaloids test was carried out by taking two milliliters of 1% HCl and stirring 2 mL of *S. nilotica* solution on a steam bath. Mayer's reagent was then added to the mixture. The formation of creamy color precipitate served as evidence for the presence of alkaloids.

3.5.8 Tests for carbohydrates

The carbohydrate presence was tested by taking three milliliters of Molisch's reagent added to 2 mL of *S. nilotica* solution. With the shaking of the resulting mixture, two milliliters of concentrated H_2SO_4 were carefully added down the test tube's side. The formation of a violet ring at the inter-phase indicated the presence of carbohydrates.

3.5.9 Tests for glycosides

This test was performed using Liebermann's test; In Liebermann test, 2 mL of the *S. nilotica* solution was dissolved in 2 mL of chloroform with 2 mL of acetic acid added, and the solution cooled in ice. Sulphuric acid was then added carefully down the side of the test tube. Color change from violet to blue to green indicates a steroidal nucleus (glycone portion of glycoside).

3.5.10 Phenolic compounds

Ferric chloride test was used to test for the presence of phenolic compounds. Three milliliters of *S. nilotica* paste was dissolved in 3 mL of distilled water. Few drops of neutral 5% ferric chloride solution were then added to the mixture. The resulting dark green color indicated the presence of phenolic compounds.

3.5.11 Amino acids

Two milliliters of freshly prepared 0.2% ninhydrin reagent was added to 2 mL of *S. nilotica* solution, and the development of violet or purple color demonstrated amino acids' presence.

3.6 Anti-dermatophytic activity of *Salvia nilotica* methanolic crude leaf extract

The dermatophytic microorganism used in this study was *Trichophyton mentagrophytes*. A clinical isolate was obtained from the Centre for Microbiology Research (CMR) at Kenya Medical Research Institute (KEMRI) with a Log/reference number; KMR/MYCL/TM100. The *T. mentagrophytes* from the stock culture in CMR bank was recovered by cutting a 2 mm of mycelia from preserved isolate and placed on the bench to thaw at room temperature before being inoculated into neutral freshly sterilized solidified Sabouraud dextrose agar (SDA) in a Petri plate and further incubated at 28˚C for five days. Hyphal tip transfer was done to SDA media to obtain pure cultures and incubated at 28 ˚C for five days.

The anti-dermatophytic efficacy of *S. nilotica* crude leaf extract was determined by poisoned food technique as described by (Jakatimath *et al.*, 2017). SDA, which was prepared by dissolving 65 g of powder in 1L of distilled water and supplemented with two capsules of chloramphenicol each 250 g to inhibit the growth of bacteria, is sterilized autoclaving at 15 Ibs pressure and 121˚C for 15 minutes was used. The SDA media was amended into required concentrations by mixing in Eppendorf tubes with *S. nilotica* crude leaf extract, which had been dissolved in 0.25% dimethyl sulfoxide (DMSO). The extract concentrations of 7.76 mg/mL, 9.31 mg/mL, 10.86 mg/mL, 12.41 mg/mL, 13.97 mg/mL, 15.52 mg/mL, 31.04 mg/mL, 46.56 mg/mL, 62.07 mg/mL and 77.59 mg/mL was used in

this experiment. A total of 20 mL concentration amended with SDA was poured into each labeled 90 mm diameter sterile Petri plates from each concentration. Similarly, the antifungal drug, fluconazole (2mg /mL) (Intravenous infusion stock) with concentrations of 0.05 mg/mL, 0.06 mg/mL, 0.07 mg/mL, 0.08 mg/mL, 0.09 mg/mL, 0.10 mg/mL, 0.20 mg/mL 0.30 mg/mL, 0.40 mg/mL and 0.50 mg/mL in the medium was used as positive control.

Equally, SDA amended with 0.25% Dimethyl sulphoxide (DMSO) diluents was used as a negative control. Each plate was inoculated aseptically at the center with 5 mm diameter mycelial discs of clinical isolates of *Trichophyton mentagrophytes* cut from the periphery of 5 days old actively growing cultures and incubated at 28˚C. Colony diameter was recorded by measuring the two different radii of the colony. The highest figure recorded after every 2-3 days until the colony in the negative control filled a 90 mm Petri plate as shown in appendix II. Three replications were maintained for each treatment. The colony diameter of the tested fungus in comparison with negative control was noted, and the following formula calculated percent growth inhibition;

$\text{Growth inhibition (\%)} = \frac{C-T}{C} \times 100$ (Vincent, 1947)

Where,

C = Mean growth in mm of fungal colony in control plates

T = Mean growth in mm of fungal colony in treated plates

3.7 Gene expression analysis

Dermatophyte cells of *Trichophyton mentagrophytes* that were exposed to *S. nilotica* extract at concentrations of 13.97 mg/mL and 77.59 mg/mL and fluconazole at

concentrations of 0.30 mg/mL and 0.50 mg/mL) and those not exposed to any treatment (negative control), were harvested and kept in an Eppendorf bottle with 3 mL of distilled water and stored at –80˚C at CMR in KEMRI, located next to Kenyatta National Hospital (KNH). This sample was later transferred to the Center of Traditional Medicine and Drug Research (CTMDR) in KEMRI headquarters located in Mbagathi, Kenya and stored again at - 80 ˚C until required for RNA extraction.

3.7.1 Targeted genes and primers

The primers used for the test on the genes of interest in this study, namely DPPV, SUB3, MEP2, SSU1, and the housekeeping gene, β-actin 1, were designed using primer 3 software. The nucleotide sequence of the targeted genes and their accession numbers are as shown in Table 3.1. The primers were designed as per the protocol of (Rozen & Skaletsky, 2000).

Table 3.1. Primers for qPCR analyses of target gene expression and reference gene

Gene symbol for *Trichophyton mentagrophytes*	Gene name	Nucleotide Sequence (5´-3´)	Accession Numbers
DDPV-MRNA_2_F.P	dipeptidyl-peptidases V	ATTCACCCCAGAGGACTTCATC	KR018393.1
DDPV-MRNA_2_R.P		ACGGTCCTTCTTGTCGAAGTTG	
SUB3_F.P	Subtilisin 3	GGCCAAGGTATCACCATCTATG	KF146901.1

SUB3_R.P		GTTGCCATCAGTGTTGTCGTTG	
ACTIN_1_F.P	Beta-actin	TGTCCCCATCTACGAAGGTTTC	AF152229.1
ACTIN_1_R.P		GGCCAAGATCTTCATCAGGTAG	
SSU1_F.P	Sulphite efflux pump	ATCACCATCCTCGTCTGCTATG	HM231281.1
SSU1_R.P		TCGAGGAACCAGCTTGTGTATG	
MEP2_F.P	Metalloprotease 2	AGAACAACTACCGCCCAGAAAG	AY283575.1
MEP2_R.P		AGGTGTTGGTGGTGTAGAAGAG	

F.P- Forward Primer   R.P - Reverse Primer

3.7.2 RNA extraction

RNA extraction was done according to the manufactures instruction of Easy pure® RNA Kit. The mycelia were harvested from *T. mentagrophytes* treated with two concentrations of *S. nilotica* methanolic crude leaf extract and fluconazole, as described in section 3.7 to study gene expression.

Trichophyton mentagrophytes cell culture was centrifuged at 12,000 RPM for 2 minutes at 4˚C (refrigerated centrifuge Model-5402, serial number -5402-08801 Japan) to form a pellet. The supernatant was then discarded. To the pellet, 100 µl of TE/lysozyme buffer was added and vortexed at 2800 RPM to completely resuspend the pellet. Binding buffer 4 (BB4) (350 µl) with mercaptoethanol was added and mixed thoroughly by vortexing and incubated at room temperature for 5 minutes. Pipetting up and down five times with RNase free tip to homogenize the solution was done. The supernatant was then centrifuged at

12,000 RPM using table centrifuge (Model KR 1,000, serial number 5070 Japan) for five minutes at room temperature and transferred to a clean RNase-free tube.

3.7.3 RNA purification

Two hundred and fifty microliters of 98% ethanol were added to the lysate. Vortexing was done thoroughly to disperse the precipitate. Centrifugation was done for one minute at room temperature, and the lysate transferred into spin columns and centrifuged at 12,000 RPM for 30 seconds. The supernatant was discarded (if the volume of lysate was more significant than the spin column can hold, this step was repeated). Clean buffer 4 (CB4) (500 µl) was added into the spin column and centrifuged at 12,000 RPM for 30 seconds, and the supernatant was again discarded. This step was repeated once. Then 80 µl of Dnase I working solution was added to the spin column to eliminate genomic DNA (the working solution was prepared by mixing 10 µl of Dnase I and 70 µl of reaction buffer). Into the spin column, 500 µl of wash buffer 4 (WB4) with ethanol was added and centrifuged at 12,000 RPM for 30 seconds at room temperature, then flow through was discarded.
This step was repeated until there was no more flow through. Centrifugation of empty column at 12,000 RPM for 2 minutes at room temperature was done to remove ethanol residue, and the column was air-dried for 10 minutes. The spin column was placed into a 1.5 mL RNase free tube, and 50 µL of RNase-free water was added into the spin column matrix and incubated at room temperature for 1 minute. Centrifugation at 12,000 RPM for 2 minutes was done to elute RNA. The absorbance of RNA was measured at 260/280 nm (a ratio of 2.0 is generally accepted as pure for RNA) to determine the quality and quantity of RNA using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Inc.,

Wilmington, DE, USA) and concentration (ng/µL) recorded. Storage of the RNA isolates was done -80˚C awaiting cDNA synthesis.

3.7.4 Complementary Deoxyribonucleic Acid (cDNA) synthesis

After thawing the components (reagents) from -20˚C of the Thermo scientific kit (cDNA synthesis), they were mixed by vortexing at 2800 RPM and briefly centrifuged and placed in the icebox. As ordered, the following reagents were added into a sterile, nuclease-free tube on icebox and mixed gently; template RNA, random hexamer primer, nuclease-free water, 5X reaction buffer, riboLock RNase inhibitor, 10 mM dNTP mix, and revertAid M-MuLV RT. The composition was then centrifuged briefly and incubated for 5 min at 25˚C, followed by 60 min at 42˚C. The reaction was terminated by heating at 70˚C for 5 min, with the reverse transcription reaction product stored at -70˚C awaiting PCR amplification. cDNA amplification was done according to the RNeasy® Mini Kit (Qiagen). The expression of the following genes in *T. mentagrophytes* was targeted for amplification; ß-actin 1 (housekeeping gene), (SUB3), (MEP2) and (DPPV). The primers were designed using Primer3 (Rozen & Skaletsky, 2000). With the synthesized cDNA template, quantitative real-time PCR was carried out according to the manufacturer's instructions using the SYBR green PCR kit and specific forward and backward primers for the targeted genes (Table 3.1). A single narrow peak from each qPCR product was obtained by melting curve analysis at each primer (appendix V). Relative expression levels were estimated using standard methods as described by (Sowndhararajan *et al.*, 2015).

The gene expression data were interpreted according to the comparative C_T method (Schmittgen & Livak, 2008). Where, if the first ΔC_T is greater than the second ΔC_T, then the value of $2^{-\Delta\Delta CT}$ will be <1. This outcome implied that there was a reduction in the gene

expression due to the treatment. The negative inverse of $22^{-\Delta\Delta CT}$ provided the fold change reduction in expression. (Appendix VI)

For example; if the mean C_T of e.g., gene HOXD10 in experimental and control samples is 26.5 and 24.9, respectively and the mean CT of the 18S rRNA as an internal control in both samples is 9.7 and 9.9, respectively, then the fold change in expression of the HOXD10 gene due to treatment will be;

Fold change of HOXD10 due to treatment = $22^{-\Delta\Delta C_T}$

$$= 2^{-[(26.5-9.7) - (24.9-9.9)]}$$

$$= 0.287$$

$$= -1/0.287 = -3.5$$

The value of -3.5 is thus interpreted as a reduction in gene expression of HOXD10 by 3.5 fold due to treatment.

3.8 Data management and statistical analysis

Data analysis for the treated and untreated samples was analyzed by two-way analysis of variance (ANOVA) using the R studio computer software 14th Edition, R version 3.6.2. Two way ANOVA was used because two parameters were tested, comprising of different concentrations of *S. nilotica* and fluconazole and days of treatment. The value with $p < 0.05$ was considered statistically significant.

CHAPTER FOUR

RESULTS

4.1 Qualitative phytochemical screening of *S. nilotica* leaf extract

Several tests were used to qualitatively analyze the phytochemical components present on the methanolic leaf extracts of *S. nilotica*. The tests revealed that *S. nilotica* possesses tannins, saponins, flavonoids, terpenoids, steroids, alkaloids, carbohydrates, amino acids, and glycosides (Table 4.1). Phlobatannins and anthraquinone were absent in the extract.

Table 4.1: Phytochemicals in methanolic crude leaf extract of *S. nilotica*

Phytochemicals	**Type of test**	**Results (+ or -)**
Tannins	Ferric chloride	+
Saponins	Frothing	+
Phlobatannins	Hydrochloric acid	-
Flavanoids	Lead acetate	+
Anthroquionones	Borntrager's	-
Steroids and Terpenoids	Salkowski	+
Alkaloids	Mayer's	+
Carbohydrates	Molish	+
Amino acid	Ninhydrin	+
Glycosides	Liebermann's	+

Key “+” indicates present and”-”indicates absent.

4.2. The efficacy of *S. nilotica* leaf extracts against *T. mentagrophytes*

The methanolic crude leaf extracts of *S. nilotica* inhibited the growth of *T. mentagrophytes* when tested under *in vitro* conditions. The trend of inhibition showed that the effect of the lowest concentration (7.76 mg/mL) showed initial growth inhibition of 42.8% after the 7th day, which then reduced to 20.8% on the 15th day (Fig 4.1). Similar observations were recorded on the concentrations of 9.31 mg/mL up to 12.41 mg/mL of the *S. nilotica* methanolic crude leaf extracts. At a higher concentration of 13.97 mg/mL, the inhibition percent fluctuated, initially increasing to 61.9% inhibition in day three before decreasing to 51.8% on day 13 and finally increasing gradually to a maximum of 74.7% on the 21st day.

The concentration of 15.52 mg/mL to 77.59 mg/mL of the extract showed no difference in their inhibition across all the days of exposure. The trend of inhibition was initially showing low inhibition. After the 5th day, the highest inhibition percent of 98.9% was achieved, which indicated a Minimum Inhibition Concentration (MIC) of *S. nilotica* against *T. mentagrophytes* at 15.52 mg/mL. The data indicated an association between the concentration, day of exposure, and inhibition, which was significant ($P \leq 0.05$).

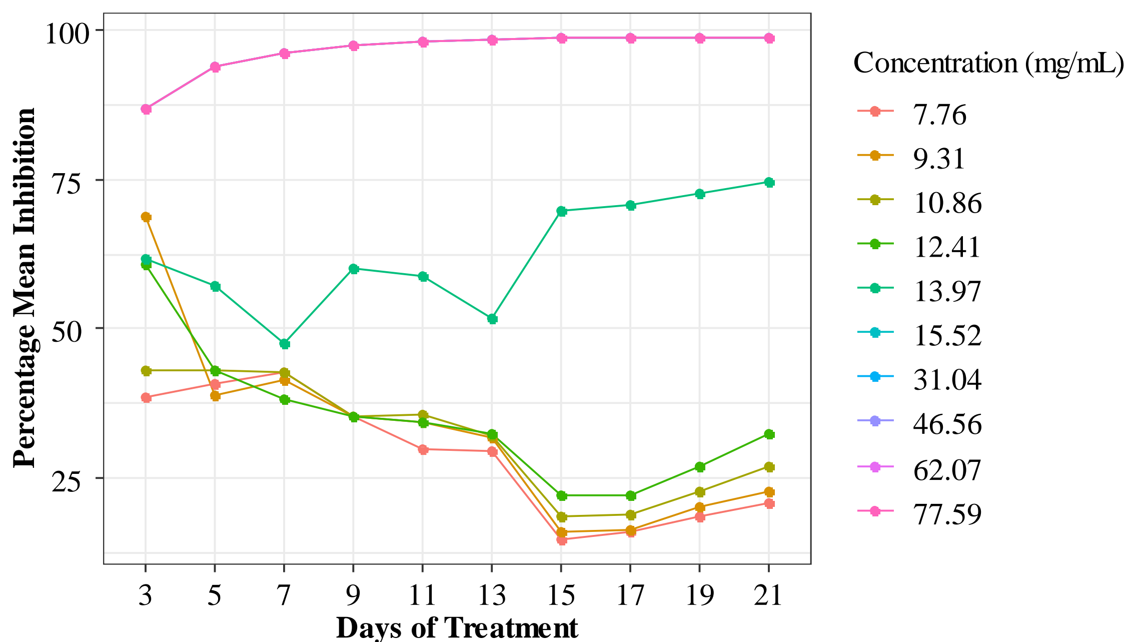


Fig 4.1. The trends of the efficacy of *S. nilotica* leaf extract against *T. mentagrophyte*s

4.3 Concentration against percentage mean inhibition of *S. nilotica*

When the mean percent inhibition was compared on day 21 of exposure, it was noted that inhibition increased as the concentration increased to a maximum inhibition of 98.9% at a concentration of 15.52 mg/mL, which did not change up to a maximum concentration of 77.59 mg/mL (Fig. 4.2). This effect indicates that lower concentration is not sufficient to inhibit the growth of *T. mentagrophytes* completely.

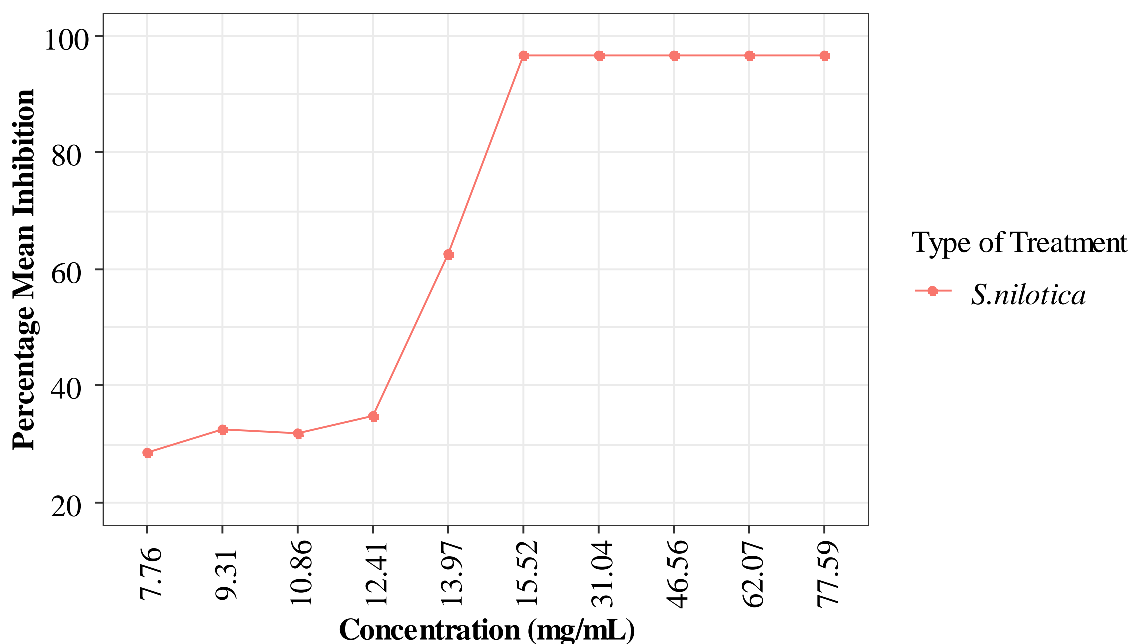


Fig 4.2. **The effect of the concentration of *S. nilotica* on percentage means inhibition against *T. mentagrophytes*.**

4.4 The effects of fluconazole on *T. mentagrophytes*

The exposure of *T. mentagrophytes* to fluconazole under *in vitro* conditions (recommended commercial drug) at different concentrations showed sensitivity. At a concentration of 0.05 mg/mL, initial inhibition of 16.7% was recorded on day 3, which reached 40.4% on day 7 and reduced to 39.0% on day 21. Similar observation was recorded for concentrations 0.06 mg/mL to 0.09 mg/mL (Fig. 4.3).

The concentration of between 0.10 mg/mL and 0.50 mg/mL showed a similar observation, initially showing an increase on inhibition percentage which peaked at the 5th day and then slowed to inhibition of 84.4% for the concentration of 0.50 mg/mL on the 21st day as shown in Figure 4.3. However, the increasing inhibition percentage as the concentration

increased, showed an association between the days of exposure, concentration, and inhibition which was significantly different (*P≤ 0.05*).

It was further observed that a maximum inhibition percentage of 94.1% was recorded for concentration 0.40 mg/mL on the 5th day of exposure and 96.4% on the 7th day of exposure for the concentration of 0.50 mg/mL, but both had the same concentration of 94.1 mg/mL on the 5th day of exposure. The trend was reduced and finally stabilized at 84.4% on the 21st day compared with control, as indicated in Fig 4.3.

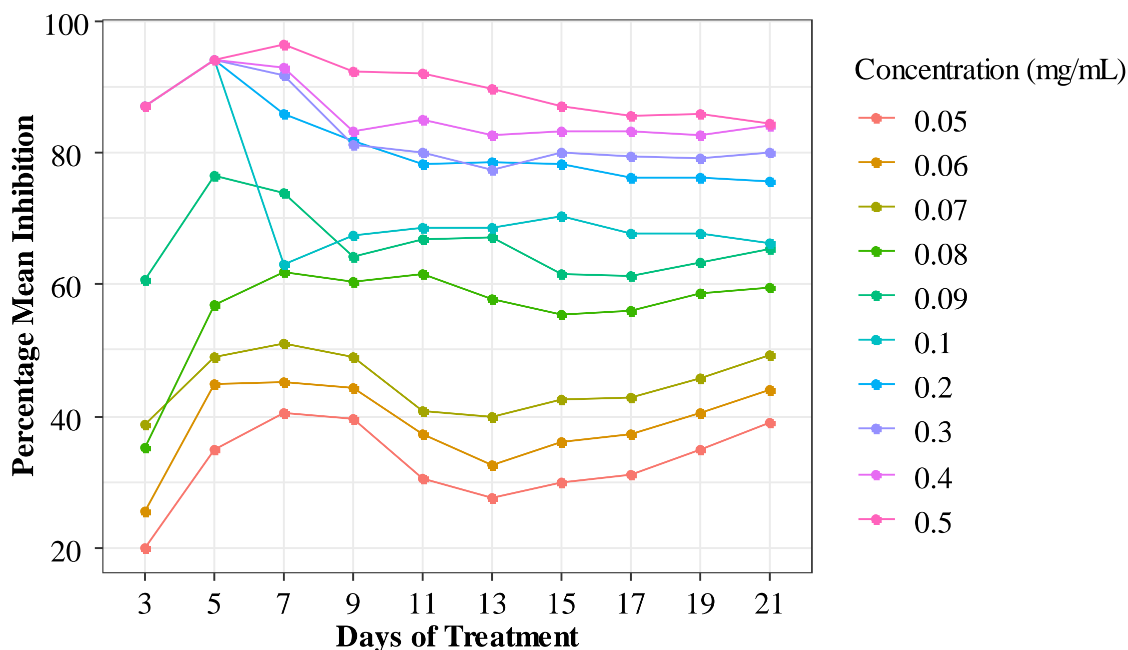


Fig 4.3 Trends of the efficacy of fluconazole against *T. mentagrophytes* across the days of exposure

4.5. Concentration against percentage mean inhibition of fluconazole

Figure 4.4 shows that the day 21 fluconazole effect was found to be directly proportional to the concentration as an increase in the inhibition percentage was noted when the

concentration was also increased. However, a maximum inhibition percentage of 84.4% was noted against 0.5 mg/mL of fluconazole.

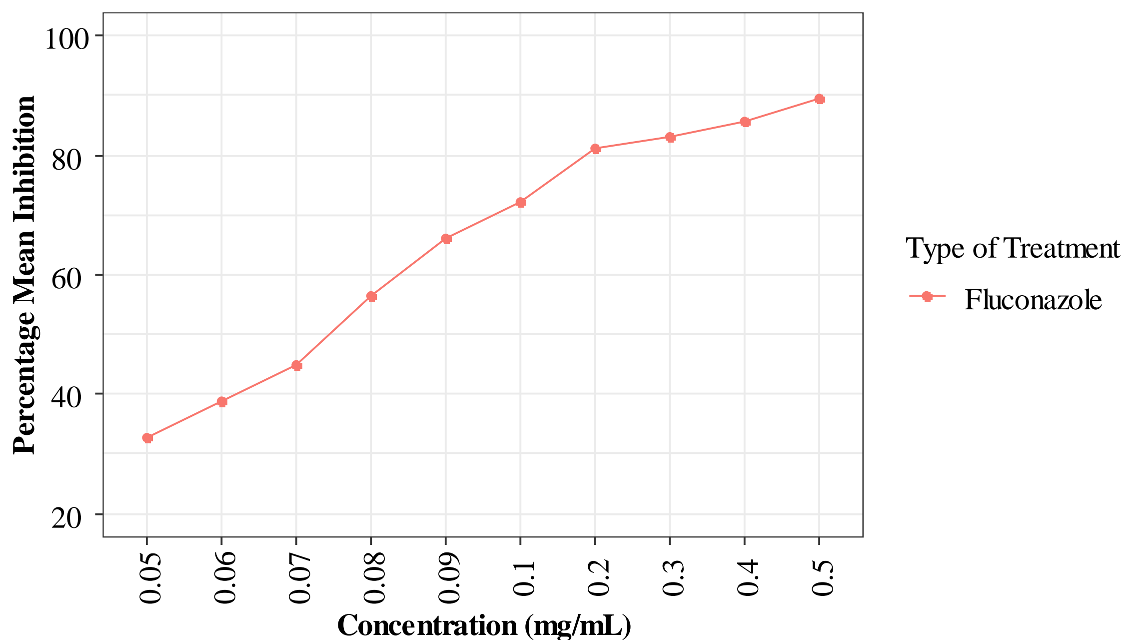


Fig. 4.4. The effect of concentration of fluconazole on percentage means inhibition against *T. mentagrophytes*.

4.6 Mean growth inhibition per day

When the mean growth inhibition per day of exposure for the two treatments of *S. nilotica* and fluconazole were considered, it was found that *S. nilotica* crude leaf extracts were more efficient in the first five days. After the 5th day, the inhibition showed similar trend for both the *S. nilotica* crude leaf extract and fluconazole, as shown in Figure 4.5. The highest inhibition was recorded on day 3 for both treatments but was found to be at its minimal on day 21. This percentage rate inhibition was found not to differ significantly at $P<0.05$ for both treatments.

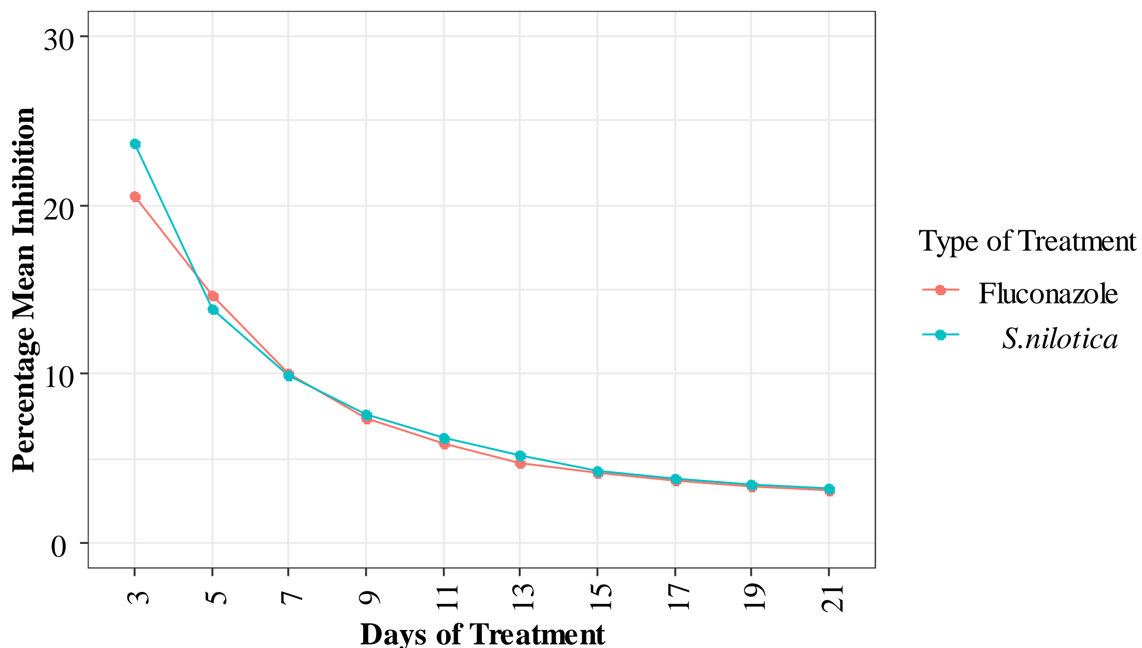


Fig 4.5. Mean growth inhibition per day by fluconazole and *S. nilotica* treatments

4.7 Gene expression on treatment of *T. mentagrophytes* with fluconazole and *S. nilotica*

The differential expression of the four genes; SSU1, SUB3, MEP2 and DDPV of *T. mentagrophytes* and a housekeeping gene β-actin under two different concentrations of 0.30 mg/mL and 0.50 mg/mL, 13. 97 mg/mL and 77.59 mg/mL for both fluconazole and *S. nilotica* crude leaf extracts respectively was determined. The results shown in Table 4.3. Downregulation of all the genes of interest was observed when fluconazole and *S. nilotica* crude leaf extracts were exposed against *T. mentagrophytes*.

Table 4.2 Effect of *S. nilotica* and fluconazole treatments on selected gene expression of *T. mentagrophytes*

Treatment type and concentration	**Gene type**	**Fold change in gene expression**
Fluconazole (0.30 mg/mL)	Beta actin 1	1
Fluconazole (0.50 mg/mL)	Beta actin 1	1
S. nilotica (13.97 mg/mL)	Beta actin 1	1
*S. nilotica (*77.59 mg/mL)	Beta actin 1	1
Fluconazole (0.30mg/mL)	MEP2	-1.7
Fluconazole (0.50 mg/mL)	MEP2	-4.2
S. nilotica (13.97 mg/mL)	MEP2	-1
S. nilotica (77.59 mg/mL)	MEP2	-1.3
Fluconazole (0.30 mg/mL)	SSU1	-1.9
Fluconazole (0.50 mg/mL)	SSU1	-2.9
*S. nilotica (*13.97 mg/mL)	SSU1	-1.2
S. nilotica (77.59 mg/mL)	SSU1	-7.9
Fluconazole (0.30 mg/mL)	SUB3	-1.1
Fluconazole (0.50 mg/mL)	SUB3	-1.6

S. nilotica (13.97 mg/mL)	SUB3	-1.2
S. nilotica (77.59 mg/mL)	SUB3	-2.3
Fluconazole (0.30mg/ml)	DPPV	-1.1
Fluconazole (0.50 mg/mL)	DPPV	-34.4
S. nilotica (13.97 mg/mL)	DPPV	-38.4
S. nilotica (77.59 mg/mL)	DPPV	-2211.8

Key: SSU1- Sulphite efflux pump, MEP 2 –Metalloprotease 2, SUB3- Subtilisin 3 and DPPV- dipeptidyl-peptidase V.

CHAPTER FIVE

DISCUSSION

The global health burden of dermatophytes and the rising public health concern on the increase in fungal strains resistant to the current antifungals (Cowen, 2008) significantly raises the urgency for the development of new therapeutics. Traditionally, medicinal plants have been used from time immemorial to cure various diseases. However, most of these plants have no scientific data on their phytochemical constituents and bioactivity (Mwitari *et al.*, 2013). Some studies have highlighted the need to prioritize research into medicinal plant mechanisms of action in therapeutic practice (Rios & Recio, 2005). This study sought to examine the qualitative phytochemical constitution, anti-dermatophytic activity, and identified the biochemical effect of methanolic leaf extracts of *S. nilotica* through the effect of select gene expression of *T. mentagrophytes*.

The various phytochemical constituents present in the crude leaf extract of *S. nilotica* indicated potential properties to actualize several plant-related medicinal functions. The qualitative screening conducted on methanolic leaf extract of *S. nilotica* showed alkaloids, tannins, flavonoids, glycosides, saponins, carbohydrates, and amino acids terpenoids and steroids and absence of anthraquinones and phlobatannins. Based on the researchers' understanding, this is the first report on the qualitative identification of phytochemical constituents in methanolic leaf extracts of *S. nilotica*. Some of these phytochemicals present in the methanolic leaf extracts of *S. nilotica* have been found to exist in several other plant species (Suurbaar *et al.*, 2017). This phytochemical distribution is because plants synthesize several chemical substances, which are essential for biological activities (Stafford *et al.*, 2005).

Phytochemicals in plants have been known to have antifungal properties. Therefore, they could be responsible for the effect of *S. nilotica* against *T. mentagrophytes* tested in this study, which agrees with the report by Deivasigamani (2018). Thus, this is the first report to show the phytochemical constituents and the antifungal activity of *S. nilotica* on *T. mentagrophytes*. The phytochemicals include tannins that have been reported to have antifungal activity, as reported by (Biasi-Garbin *et al.*, 2016), where they attributed the tannins present in several plant species to the inhibition of two dermatophyte fungi *T. Mentagrophytes* and *T. rubrum*. Similarly, (Negri *et al.*, 2014) reported the antifungal effect of tannins from *Mimosa tenicflora* on dermatophytic fungi. The tannins present, along with other constituents detected in the present study could be responsible for the observed high growth in inhibition. The synergistic effect as antimicrobial agents of several constituents has been reported in several plant species such as *Psidium guajava, Parapiptadenia rigida,* and *Libidibia ferrea (*de Araújo et al., 2014*).*
Similar results were also reported by (Fernandes *et al.*, 2014) from extracts of *psidium guajava*. Flavonoids detected in a variety of Leguminosae species such as *Mimosa pigra,* C. *nictitans,* and *E. heterophyllum* showed good anti-dermatophytic activity, which is related to the fungicidal and fungistatic activities of these secondary metabolites (De Morais *et al.*, 2017). The presence of flavonoids in the *S. nilotica* obtained from the current study could be attributed to the antifungal effect on *T. mentagrophytes,* as shown elsewhere for other plants. Several mechanisms have been documented on the antifungal activities of these phytochemicals. Tannins and flavonoids have the same mechanism of action facilitated by the release of stable free radical and formation of complex compounds with nucleophilic amino acids to inactivate proteins leading to loss of function, targeting

microbial cell surface-exposed adhesions and cell wall polypeptides (Suurbaar *et al.*, 2017).

Terpenoids have been associated with the weakening of the membrane tissues leading to the microorganisms' cell wall dissolution, as reported by Hernández *et al.* (2000). Saponins have been reported to be responsible for the leakage of certain enzymes and proteins from the cell of the microorganisms (Katsambas *et al.*, 2015). The other bioactive constituents isolated from methanolic crude leaf extracts of *S. nilotica,* such as alkaloids, phenols, steroids, carbohydrates, amino acids, and glycosides have been reported by several researchers to have high growth inhibition ability against *T. mentagrophytes* and *T. rubrum* (Zayed & Samling, 2016). These bioactive constituents could show antifungal effects singly, as in the case of tannins and flavonoids (Biasi-Garbin *et al.*, 2016). (Kumar & Bhadauria, 2009) reported alkaloids' ability as anti-dermatophytic against *T. rubrum, T. mentagrophytes,* and *M. gypseum,* which could contribute to the anti-dermatophytic activity of *S. nilotica* crude extracts reported in this current study.

In this study, a minimum inhibition was at 15.52 mg/mL for the *S. nilotica* methanolic crude leaf extracts. The MIC values obtained in this study showed that the methanolic extracts of *S. nilotica* have very high potency effect against *T. mentagrophytes* as reported by other workers, that the lower the MIC the more potent the extracts are (Boyejo *et al.*, 2019) in their study on the effects of the leaf extracts of *Vitellaria paradoxa* components against several dermatophytes.

The treatment of *T. mentagrophytes* with extract of *S. nilotica* resulted in the inhibition of its growth, which showed a fungicidal tendency of the crude leaf extracts. This occurrence

could be due to the suppression of expression of specific genes. Previous studies (Mwitari *et al.*, 2013) have reported plant extracts on the gene expression of fungi, which have been associated with the antifungal phenomenon. In the current study, the expressions of four virulence genes were studied compared to the housekeeping gene beta-actin, significant in quantifying the transcription expression of functional genes (Zhao *et al.*, 2018). Glyceraldehyde-3-phosphate dehydrogenase, β-tubulin, and β-actin gene (*actb*) are frequently used as reference genes in gene expression studies involving filamentous fungi for their ability to provide consistent expression levels (Zampieri *et al.*, 2014). The results of the housekeeping gene in a study obtained from the two software programs, *BestKeeper* and *geNorm*, proved that the *actb* gene as the most stable among the four candidate genes (Zampieri *et al.*, 2014).

The expression DPPV, MEP2, SSU1 and SUB3 genes was determined by exposing *T. mentagrophytes* to two concentrations, (13.97 mg/mL and 77.59 mg/mL) of *S. nilotica* methanolic crude leaf extract and two concentrations (0.30 mg/mL and 0.50 mg/mL) of fluconazole as a way of determining the possible mode of action. The expressions of the tested genes in *T. mentagrophytes* were reduced by the two treatments of fluconazole and methanolic crude leaf extracts of *S. nilotica.* All the genes under observation showed downregulation or reduction by treating S. *nilotica* methanolic crude leaf extracts and fluconazole in *T. mentagrophytes*. SSU1 gene was down-regulated which was similar to the findings of (Lechenne *et al.*, 2007).

They showed that SSU1 is a gene responsible for the process of pathogenicity in *T. mentagrophytes. It* reduces cysteine disulphide bridges because they act by breaking the hard keratin into cysteine and *S*-sulphocysteine. This phenomenon could be responsible

in the present study because it was established that *S. nilotica* crude leaf extract inhibited the growth of *T. Mentagrophytes* as the concentration of the crude leaf extracts increased as well as for fluconazole.

Elsewhere a direct correlation has been established between the keratinases and pathogenicity in dermatophytes as described by Viani *et al.*, (2001), and this could explain the current observation in the effect of *S. nilotica* extract on *T. Mentagrophytes*. Lechenne *et al.*, (2007) indicated that the SSU1 gene encodes surface efflux pump, which necessitates sulfitolysis and therefore a possible detoxification pathway, hence could have been the target by *S. nilotica* crude leaf extract.

DPPV belongs to the S9 family and is one of the exoproteases which plays a role in the hydrolysis of peptides to amino acids in dermatophytes. When *T. mentagrophytes* was exposed to fluconazole and *S. nilotica* treatments, this particular gene was down-regulated. Although both treatments reduced DPPV gene expression, *S. nilotica* reduced by over 2,000 fold than fluconazole. This difference in bioactivity means DPPV can be a more potent drug target against *T. mentagrophytes*, which this report is the first to demonstrate. The DPPV gene is conserved in most microorganisms such as bacteria, protozoans, and fungi and is known to be upregulated by the microorganism during infection on a host (Kaufman *et al.*, 2005). The observed gene downregulation explains the fact that there was inhibition on the fungus's growth during the treatment, which then interfered with its expression. Further, it has been shown that the DPPV gene in fungi acts by secreting exoproteases that biodegrade the host's keratin cells and act on the free ends of glycopeptides, therefore leading to pathogenicity. The downregulation of the DPPV gene

suggests that phytochemicals present in methanolic crude leaf extracts present in *S. nilotica* could interfere with the functioning of DPPV gene.

Treatment of *T. mentagrophytes* with methanolic crude leaf extracts of *S. nilotica* and fluconazole showed downregulation of the MEP2 gene. The trend of down-regulation of this gene shows that this gene (MEP2) is targeted by the bioactivity of methanolic crude leaf extracts of *S. nilotica;* therefore, there is a possibility to reduce the activity of *T. mentagrophytes* in the host infection under *in vivo* condition. However, its downregulation was more pronounced on fluconazole treatment, which indicates that *S. nilotica* is a weak agent against MEP2 in *T. mentagrophytes*.

Since dermatophytes infect healthy and unhealthy individuals, subtilisin and metalloproteases production during *in vivo* experiments serve as evidence of the fungal virulence as true pathogenic fungi (Brouta *et al.*, 2002; Descamps *et al.*, 2002). The significance of MEP2 protease in fungal pathogenicity has been observed in *in-vivo* experiments highlighting its fundamental role in the infection process (Brouta *et al.*, 2002). Although the productions of MEP3 and MEP2 during animal infection, shown in reverse nested PCR, outlines the significant function of the 43.5 kDa MEP gene in host invasion (Brouta *et al.*, 2001), insufficient characterization of MEP2 limits determination of its specific role during dermatophyte infection (Lemsaddek *et al.*, 2010).

Subtilisin genes' fundamental role in *T. mentagrophytes* virulence is evident during the invasion of the host epidermal barrier, with subtilisin 3 gene (SUB3) belonging to the seven-member gene family (*SUB1–SUB7*) that encodes the subtilisin serine proteases (Shi *et al.*, 2015). Fluconazole and *S. nilotica* treatment had a similar effect by reducing SUB3 gene expression. However, fluconazole was reduced by more folds than *S. nilotica* at

concentrations of 0.50 mg/mL and 77.59 mg/mL. It has been observed that subtilisins digest proteins present into large peptides then later into amino acids with other synergistic effects with other gene products (Baldo *et al.*, 2012). Mercer & Stewart, (2019) have shown that sulfitolysis is essential, and the secretion of subtilisins degrades keratin. The effect of the activity of S. *nilotica* crude leaf extracts and fluconazole, which down-regulated this gene could offer the fungicidal and fungistatic activity against *T. mentagrophytes*.

The comparative C_T technique presents real-time PCR data facilitating its extensive use in molecular and biological processes (Schmittgen & Livak, 2008). Similar shapes of the amplification plots demonstrate identical PCR efficiency (Schmittgen & Livak, 2008). In the current study, the amplification plot showed similar shapes meaning there was PCR efficiency during amplification. The CT values that were obtained in this study ranged between 19 and 31 Cts < 29 indicate strong positive reactions owing to abundant target nucleic acid in the sample. Cts of 30-37 show positive reactions associated with moderate amounts of target nucleic acid, while Cts of 38-40 indicates weak reactions linked to the presence of minimal amounts of target nucleic acid in the sample. Due to the limitation of experimental scope on possible mode of action of the *S. nilotica* extract in this study, there is still need to further research on all other plausible mechanisms of anti-dermatophytic activity of this extract and also on studies with other common dermatophytes.

CHAPTER SIX

CONCLUSION AND RECOMMENDATIONS

6.1 Conclusion

The methanolic crude leaf extract of *S. nilotica* was found to be phytochemicals-rich containing tannins, saponins, flavonoids, terpenoids, steroids, alkaloids, carbohydrates, amino acids, and glycosides but phlobatannins and anthraquinone were absent.
The exposure of *T. mentagrophytes* at the concentrations of 7.76 mg/mL, 9.31 mg/mL, 10.86 mg/mL, 12.41 mg/mL (appendix III), 13.97 mg/mL, 15.52 mg/mL, 31.04 mg/mL, 46.56 mg/mL, 62.07 mg/mL and 77.59 mg/mL of methanolic crude leaf extracts of *S. nilotica* showed inhibitory effect under *in vitro* conditions with Minimum Inhibitory Concentration of 15.52 mg/mL.

It was also established that DPPV, MEP2, SUB3, and SSU1 genes were down-regulated by different folds which differed for fluconazole and methanolic crude leaf extracts of *S. nilotica* treatments against *T. mentagrophytes*. The results obtained from this study have provided useful insights on the anti-dermatophytic effects and plausible mode of action of methanolic *Salvia nilotica* crude leaf extract against *Trichophyton mentagropytes.* Thus, this study indicates the potential of *S. nilotica* leaf extract as alternative medicine for dermatophytosis caused by *T. mentagrophytes*.

6.2 Recommendations

From the findings of the efficacy of methanolic crude leaf extracts of *S. nilotica* on *T. mentagrophytes,* the following recommendations are suggested;

- There is a need to characterize and quantify the phytochemical constituents in *S. nilotica* leaf extract and establish each constituent's role in the fungicidal effect.
- The effect of methanolic crude leaf extracts of *S. nilotica* on *T. mentagrophytes* showed high potential for dermatophytosis management; hence, there is a need for extraction and formulation of antifungal drug.
- Following the notable effect of the methanolic crude leaf extracts of *S. nilotica* in downregulating the expression of the DPPV gene, there is need to formulate *S. nilotica* leaf extract to target this gene in the management of dermatophytosis caused by *T. mentagrophytes* and further test against DPPV gene in other dermatophytes causing dermatomycosis.

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APPENDICES

Appendix I: Ethical clearance on research protocols

KENYA MEDICAL RESEARCH INSTITUTE

Centre for Traditional Medicine and Drug Research, P.O. Box 54840, NAIROBI 00200, Kenya,
Tel: (020) 2722541, E-mail: ctmdr@kemri.org, Website. www.kemri.org

20th July, 2018

Joseph Chepkwony Kiproop
Department of Chemistry and Biochemistry
University of Eldoret, Kenya

Dear Mr. Chepkwony,

RE: CTMDR/CSCP090: ANTI-DERMATOPHYTIC EFFECTS OF *SALVIA NILOTICA* LEAF EXTRACT

Following your revision of the above-mentioned proposal as required, the CTMDR CSC approves this proposal.

I wish you the best in your work.

Yours sincerely,

Joyce Ondicho
CTMDR CSC SECRETARY

In Search of Better Health

Appendix II

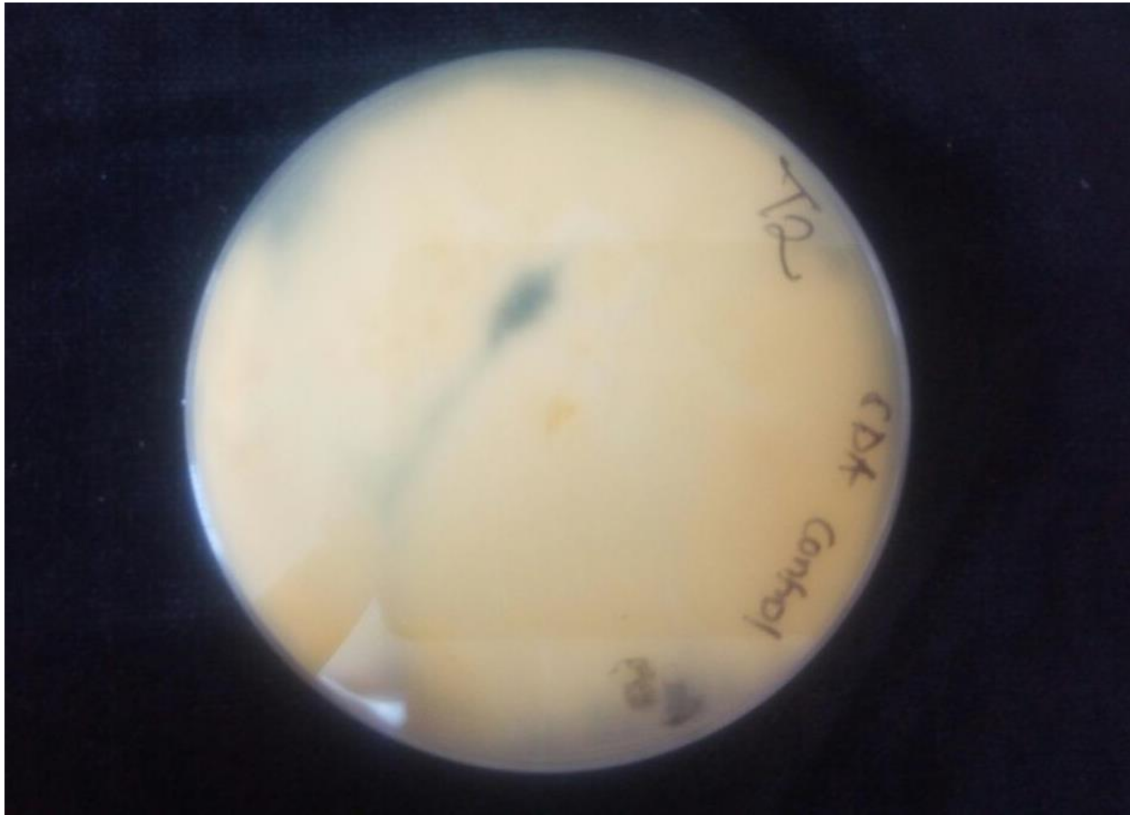


Plate 2 - : Control plate of *T. mentagrophytes* (SDA+ 0.25 % DMSO) after 21 days

Appendix III

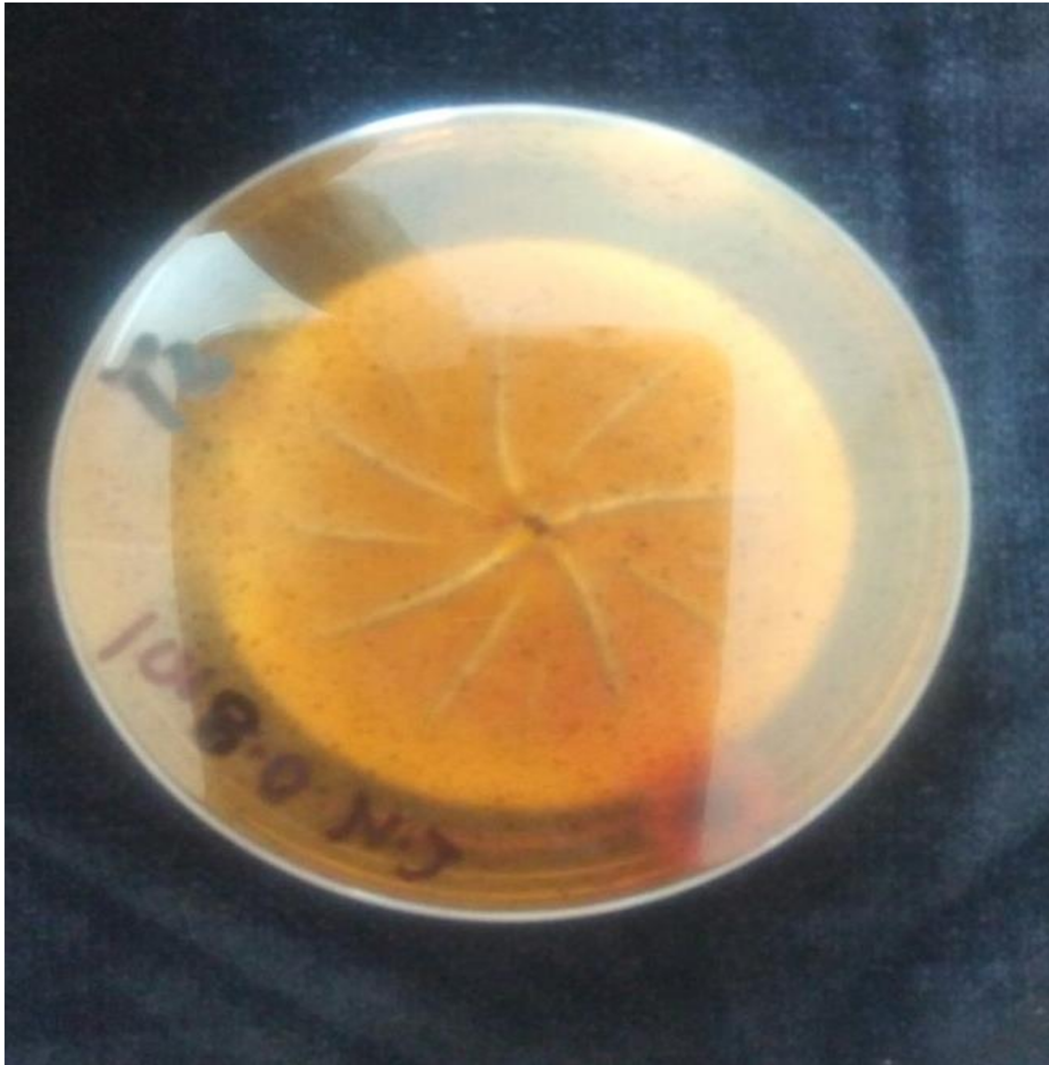
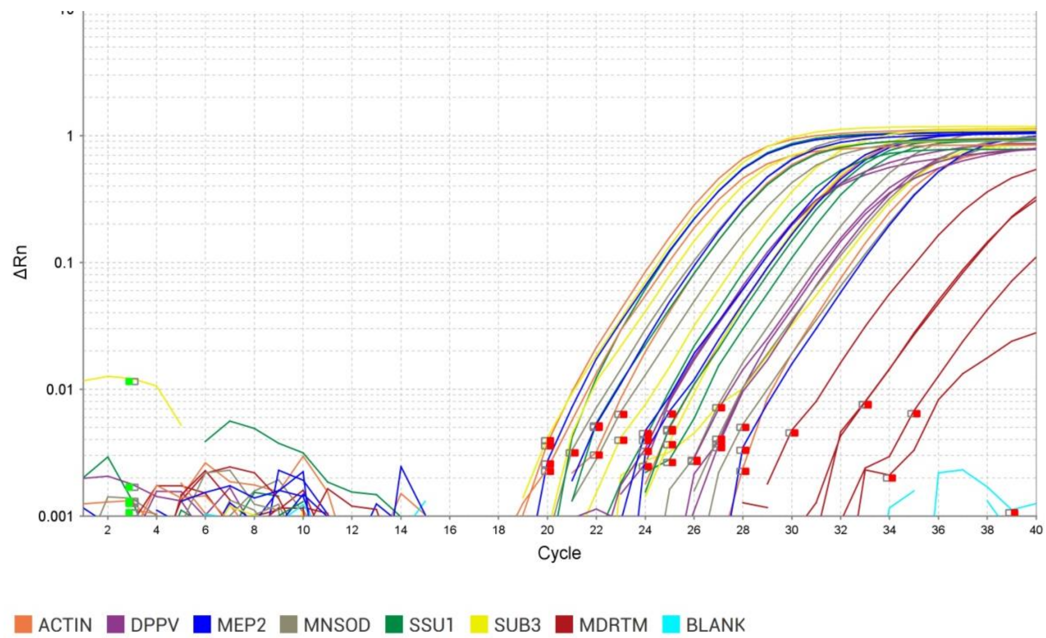


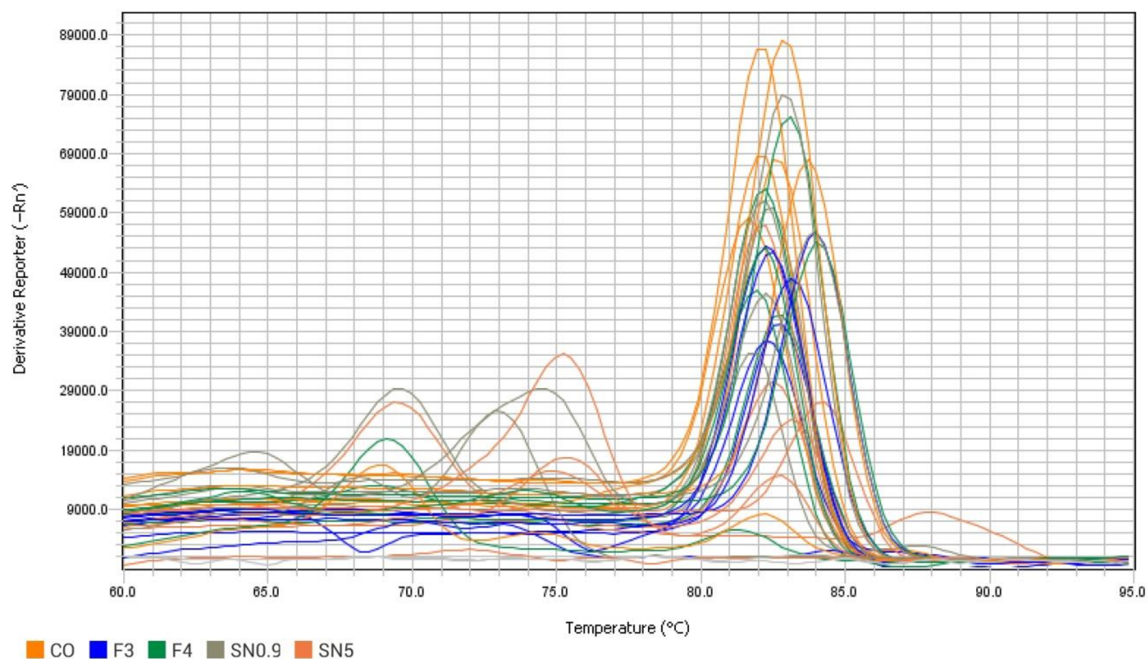
Plate 3: Plate of *T. mentagrophytes* treated with *Salvia nilotica* (12.41 mg/mL) after 21 days

Appendix IV: Amplification plot of targeted genes



MEP2 Metalloprotease, DPPV dipeptidyl-peptidases V, MNSOD Manganese superoxide dismutase, MDRTM Multi drug resistance gene, ACTIN β Actin, SUB3 Subtilisin3, SSU1 Sulphite efflux pump and Blank

Appendix V: Melting curve plot of targeted genes



CO Control, DMSO dimethyl sulphuroxide, F3 fluconazole 0.30 mg/mL, F4 fluconazole 0.50 mg/mL, SN *Salvia nilotica* 0.9 13.97 mg/mL and SN 5 *Salvia nilotica* 77 mg/mL

Appendix VI: Calculation of Relative Quantification of Gene Expression

SERIAL NUMBER	**REFERENCE GENE**				**GENE OF INTEREST**							
1.0	β-ACTIN	CT1	CT2	AVERAGE OF C_T1 AND C_T2	MEP 2	CT1	CT2	AVERAGE OF C_T1 AND C_T2	ΔC_T	$\Delta\Delta C_T$	$2^{-\Delta\Delta CT}$	FOLD CHANGE $(-1/2^{-\Delta\Delta CT})$
	F3	24.5	24.5	24.5	F3	26.3	26.3	26.3	1.8	0.8	0.6	-1.7
	CO	23.8	23.8	23.8	CO	24.9	24.9	24.9	1.1	0.0	1.0	
2.0	β-ACTIN				SSU 1							
	F3	24.5	24.5	24.5	F3	26.4	26.4	26.4	1.9	0.9	0.5	-1.9
	CO	23.8	23.8	23.8	CO	24.8	24.8	24.8	1.0	0.0	1.0	
3.0	β-ACTIN				SUB 3							
	F3	24.5	24.5	24.5	F3	25.5	25.5	25.5	1.0	0.2	0.9	-1.1

	CO	23.8	23.8	23.8	CO	24.7	24.7	24.7	0.9	0.0	1.0	
4.0	β-ACTIN				DPPV							
	F3	24.5	24.5	24.5	F3	30.6	30.6	30.6	6.1	-1.1	0.5	-2.2
	CO	23.8	23.8	23.8	CO	31.1	31.1	31.1	7.3	0.0	1.0	
5.0	β-ACTIN				β-ACTIN (housekeeping gene)							
	F3	24.5	24.5	24.5	F3	24.5	24.5	24.5	0.0	0.0	1.0	
	CO	23.8	23.8	23.8	CO	23.8	23.8	23.8	0.0	0.0	1.0	
6.0	β-ACTIN				MEP 2							
	F5	25.9	25.9	25.9	F5	29.0	29.0	29.0	3.1	2.1	0.2	-4.2
	CO	23.8	23.8	23.8	CO	24.9	24.9	24.9	1.1	0.0	1.0	
7.0	β-ACTIN				SSU 1							

	F5	25.9	25.9	25.9	F5	28.4	28.4	28.4	2.5	1.5	0.3	-2.9
	CO	23.8	23.8	23.8	CO	24.8	24.8	24.8	1.0	0.0	1.0	
8.0	β-ACTIN				SUB 3							
	F5	25.9	25.9	25.9	F5	27.9	27.9	27.9	2.0	1.2	0.4	-2.3
	CO	23.8	23.8	23.8	CO	24.7	24.7	24.7	0.9	0.0	1.0	
9.0	β-ACTIN				DPPV							
	F5	25.9	25.9	25.9	F5	28.0	28.0	28.0	2.2	-5.1	0.0	-34.4
	CO	23.8	23.8	23.8	C5	31.1	31.1	31.1	7.3	0.0	1.0	
10.0	β-ACTIN				β-ACTIN (housekeeping gene)							
	F5	25.9	25.9	25.9	F5	25.9	25.9	25.9	0.0	0.0	1.0	
	CO	23.8	23.8	23.8	CO	23.8	23.8	23.8	0.0	0.0	1.0	

11.0	β-ACTIN				MEP2							
	Sn 0.9	28.7	28.7	28.7	Sn 0.9	29.4	29.4	29.4	0.7	-0.4	0.8	-1.3
	CO	23.8	23.8	23.8	CO	24.9	24.9	24.9	1.1	0.0	1.0	
12.0	β-ACTIN				SSU 1							
	Sn 0.9	28.7	28.7	28.7	Sn 0.9	29.5	29.5	29.5	0.7	-0.3	0.8	-1.2
	CO	23.8	23.8	23.8	CO	24.8	24.8	24.8	1.0	0.0	1.0	
13.0	β-ACTIN				SUB 3							
	Sn 0.9	28.7	28.7	28.7	Sn 0.9	29.3	29.3	29.3	0.5	-0.3	0.8	-1.2
	CO	23.8	23.8	23.8	C0	24.7	24.7	24.7	0.9	0.0	1.0	
14.0	β-ACTIN				DPPV							
	Sn 0.9	28.7	28.7	28.7	Sn 0.9	30.7	30.7	30.7	2.0	-5.3	0.0	-38.4
	CO	23.8	23.8	23.8	CO	31.1	31.1	31.1	7.3	0.0	1.0	

15.0	β-ACTIN				β-ACTIN							
	Sn 0.9	28.7	28.7	28.7	Sn 0.9	28.7	28.7	28.7	0.0	0.0	1.0	
	CO	23.8	23.8	23.8	CO	23.8	23.8	23.8	0.0	0.0	1.0	
16.0	β-ACTIN				MEP2							
	Sn 5	32.0	32.0	32.0	Sn 5	33.1	33.1	33.1	1.1	0.0	1.0	-1.0
	CO	23.8	23.8	23.8	CO	24.9	24.9	24.9	1.1	0.0	1.0	
17.0	β-ACTIN				SSU 1							
	Sn 5	32.0	32.0	32.0	Sn 5	30.0	30.0	30.0	-2.0	-3.0	0.1	-7.9
	CO	23.8	23.8	23.8	CO	24.8	24.8	24.8	1.0	0.0	1.0	
18.0	β-ACTIN				SUB 3							
	Sn 5	32.0	32.0	32.0	Sn 5	32.1	32.1	32.1	0.2	-0.7	0.6	-1.6
	CO	23.8	23.8	23.8	CO	24.7	24.7	24.7	0.9	0.0	1.0	

19.0	β-ACTIN				DPPV							
	Sn 5	32.0	32.0	32.0	Sn 5	28.1	28.1	28.1	-3.8	-11.1	0.0	-2211.8
	CO	23.8	23.8	23.8	CO	31.1	31.1	31.1	7.3	0.0	1.0	
20.0	β-ACTIN				β -ACTIN (housekeeping gene)							
	Sn 5	32.0	32.0	32.0	Sn 5	32.0	32.0	32.0	0.0	0.0	1.0	
	CO	23.8	23.8	23.8	CO	23.8	23.8	23.8	0.0	0.0	1.0	

F3-fluconazole 0.30 (mg/mL) F5- Fluconazole (0.50 mg/mL)

Sn 0.9-*Salvia nilotica* (13.97 mg/mL) *Sn 5-Salvia nilotica* (77.59 mg/mL)

CO-Control C_T-Cycle Threshold

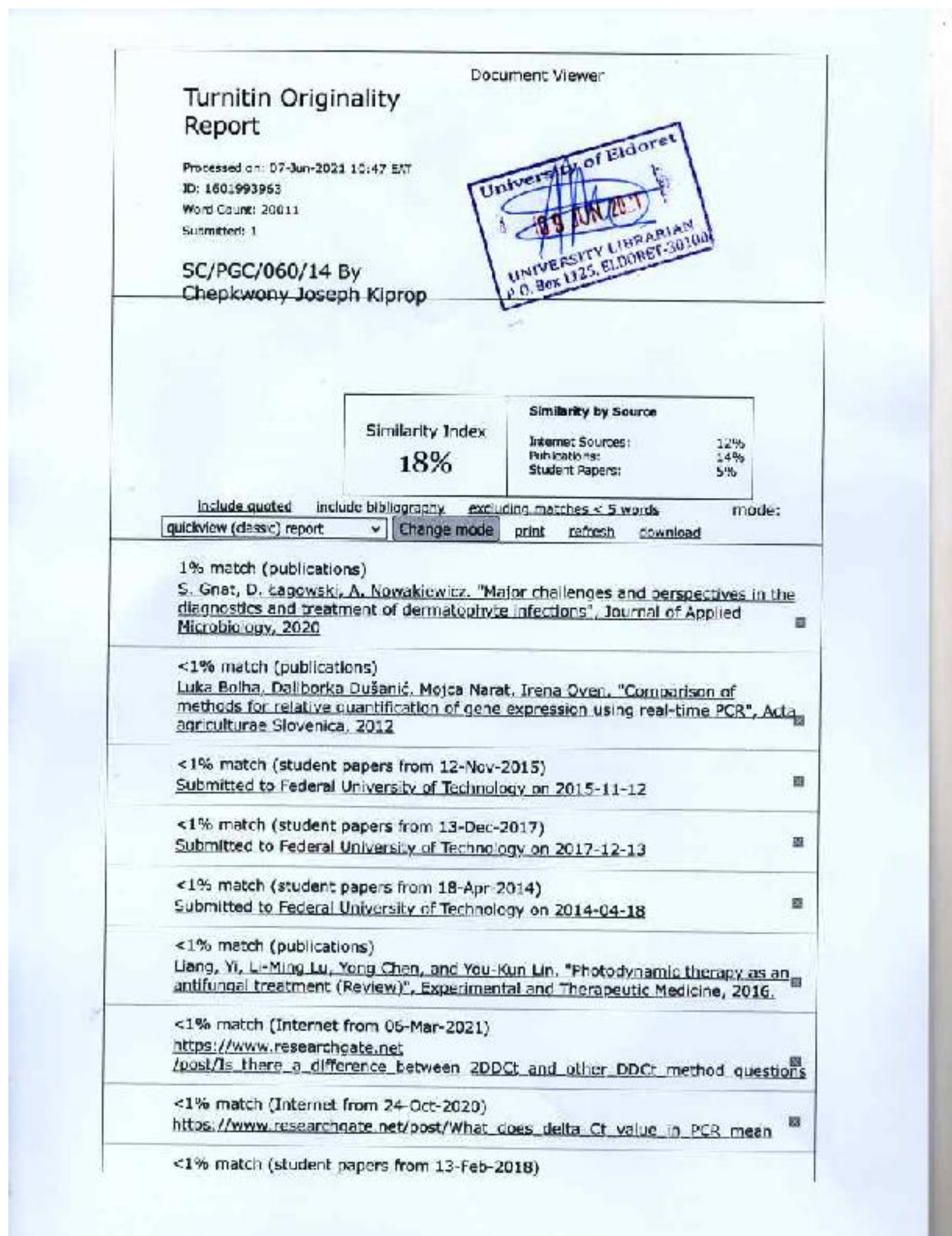
ΔC_T-change in cycle threshold $\Delta\Delta C_T$-change change in cycle threshold

MEP2 gene-Metalloprotease 2 SUB3 gene-Subtillisin 3

DPPV gene-Dipeptidyl-Peptidase V SSU1 gene-Sulphite efflux pump

β Actin gene-Beta

Appendix VII : Similarity Report



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