The Journal of Phytopharmacology 2021; 10(6):433-438
Online at: www.phytopharmajournal.com

The Journal of Phytopharmacology
(Pharmacognosy and phytomedicine Research)

Research Article

ISSN 2320-480X
JPHYTO 2021; 10(6): 433-438
November- December
Received: 23-10-2021
Accepted: 06-11-2021
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doi: 10.31254/phyto.2021.10602

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Anti-dermatophytic activity of *Salvia nilotica* methanolic crude leaf extract against *Trichophyton mentagrophytes*

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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 but there is no scientific documentation for these claims. This study determined the phytochemical constituents in *Salvia nilotica* methanolic crude leaf extract, its anti-dermatophytic activity against *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 *S. nilotica* leaf extract were determined using standard methods. Food-poisoned technique was used to determine the anti-dermatophytic activity of extract at different concentrations ranging from 7.76 mg/mL to 77.59 mg/mL versus the standard, fluconazole. Comparative C_T analysis of real time quantitative PCR data was utilized for gene expression analysis. The Phytochemical-rich *Salvia nilotica* leaf extract was found to have anti-dermatophytic activity against *Trichophyton mentagrophytes which* was not significantly different ($p < 0.05$) when compared with fluconazole treatments. In addition, MEP2, SSU1, SUB3, and DPPV genes of *Trichophyton mentagrophytes* were down regulated by different folds by the extract. This study has shown that *Salvia nilotica* crude leaf methanolic extract could offer a potential alternative medicine for dermatophytosis treatment.

Keywords: *Salvia nilotica*, leaf, *Trichophyton mentagrophytes*, Fluconazole, Anti-dermatophytic, RT-qPCR.

INTRODUCTION

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 [1]. Dermatophytes consist of 40 species of fungi derived from three genera, namely, *Trichophyton, Epidermophyton* and *Micropsorum* [2]. These fungi possesses two unique properties: keratinophilic and keratinolytic to digest or break down the keratin in tissues like hair, nails, and epidermis [3].

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 in keratinized tissues [4]. Cysteine metabolism is an important biological process in filamentous fungi, including dermatophytes, utilizing sulphite efflux pump encoded by the gene SSU1 to produce sulphite [5]. Aminopeptidases such as dipeptidyl-peptidases (DPPIV and DPPV) are another essential enzymes in the dermatophytic process [6].

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 [7]. This therapeutic approach faces two major hurdles, because most dermatomycoses affect people with compromised immunity, who suffers more adverse effects from antifungal drugs [8]. Further, the conservative physiological functions between humans and fungal pathogens make it difficult to develop a high safety profile for effective antifungal drugs.

Therapeutic properties of plant derivatives offer active antimicrobial molecules in forms of natural compounds, which provide undisputable essential potential in developing new therapeutic agents [9]. Plants have been a key foundation of greatly effective conventional drugs for treating many forms of dermatophytes [10]. For instance, *Salvia nilotica*, a perennial shrub thriving in Eastern Africa has various medicinal uses by itself or with combination with other plants. *Particularly, h*erbalists in the Tugen community in Kenya use *Salvia nilotica* leaves to treat nail and skin infections but scientific evidence has not been documented for these medicinal claims. This study was therefore carried out to establish the anti-dermatophytic activity and possible mode of action of *Salvia nilotica* methanolic crude leaf extract *against Trichophyton mentagrophytes*.

MATERIALS AND METHODS

Collection and identification of plant

Salvia nilotica leaves were collected from Katimok forest located in Baringo North Sub- County, Baringo County, Kenya (Latitude 00° 37' 00" N, Longitude 350 47' 00" E). The whole plant of *S. nilotica* was collected and identified, allocated voucher number M.U.H/Salni/008/16 and deposited in the herbarium of the Department of Biological Sciences at the University of Eldoret.

Preparation of crude leaf extract

The extraction of the leaf extract was done based on the method described by [11] with little modifications. Two hundred grams of ground *S. nilotica* leaves were soaked in three hundred milliliters 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 50ºC using Rotary Evaporator (Rotavapor type EL 30; model AG CH-9230, Germany). *S. nilotica* crude leaf extract in the form of a paste was stored at 4ºC until it was required for qualitative phytochemical evaluation and further investigations.

Qualitative phytochemical screening

The methanolic leaf extract of *Salvia nilotica* was subjected to qualitative phytochemical tests to identify classes of phytochemical constituents present using standard methods as described by [12].

Ethical clearance

Research protocols were approved by the Centre for Traditional Medicine and Drug Research Scientific Committee of the Kenya Medical Research Institute (Reference Centre number KEMRI/ CTMDR/CSCPO90).

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

A clinical isolate of *Trichophyton mentagrophytes* with a log/reference number; KMR/MYCL/TM100 was obtained from the Centre for Microbiology Research (CMR) at Kenya Medical Research Institute (KEMRI), Nairobi. The *T. mentagrophytes* from the stock culture in CMR bank was recovered by cutting a 2mm of mycelia from preserved isolate and thawed at room temperature before being inoculated into a freshly sterilized solidified sabouraud dextrose agar (SDA) in a petri plate 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 [13].

The percent growth inhibition was calculated using the formula;

$$\text{Growth inhibition } (\%) = \frac{C-T}{C} \times 100 \text{ [14]}$$

Where,

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

Gene expression analysis

The 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 selected and harvested for RNA extraction. RNA extraction and purification was done according to the protocol of Easy pure® RNA Kit (Zhongguancun Dongsheng International Science Park, Beijing China). Thermo scientific kit (cDNA synthesis) was used to synthesize cDNA and cDNA amplification was done according to the RNeasy® Mini Kit (Qiagen). The expression of the genes in *T. mentagrophytes* that were targeted for amplification were beta-actin 1 (housekeeping gene), MEP2, SSU1, SUB3 and DDPV. The primers were designed using Primer 3 software [15] and obtained from Inqaba Biotechnical Industries (Pty) Ltd, Pretoria, South Africa. With the synthesized cDNA template, quantitative real-time PCR (RT-qPCR) was carried out using the SYBR green PCR kit and specific forward and backward primers for the targeted genes (Table 1). A single narrow peak from each RT-qPCR product was obtained by melting curve analysis at each primer. Relative expression levels were estimated using standard methods as described by [16]. The gene expression data were interpreted according to the comparative C_T method described by [17].

Table 1: Primers for RT-qPCR analyses of target gene expression in *Trichophyton mentagrophytes* and the reference gene

Gene symbol for *Trichophyton mentagrophytes*	Gene name	Primer sequence (5′→3′)	Accession numbers
DDPV-F.P	Dipeptidyl-peptidases V	ATTCACCCCAGAGGACTTCATC	KR018393.1
DDPV-R.P		ACGGTCCTTCTTGTCGAAGTTG	
SUB3_F.P	Subtilisin 3	GGCCAAGGTATCACCATCTATG	KF146901.1
SUB3_R.P		GTTGCCATCAGTGTTGTCGTTG	
ACTIN_1_F.P	Beta-actin (Reference)	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.

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 and the value with $p < 0.05$ was considered statistically significant.

RESULTS

Phytochemical screening and anti-dermatophytic activity of *S. nilotica* leaf extracts against *T. mentagrophytes*

The qualitative phytochemical analysis of *S. nilotica* crude leaf extract indicated the presence of tannins, saponins, flavonoids, terpenoids, steroids, alkaloids, carbohydrates, amino acids and glycosides while phlobatannins and anthraquinone were absent as shown in Table 2. On the other hand, 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 of 7.76 mg/mL up to 12.41 mg/mL of extract was initially higher but reduced (Figure 1). At a higher concentration of 13.97 mg/mL, the inhibition percent fluctuated, but finally increased 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. 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 following extract treatment indicated an association between the concentrations, days of exposure, and inhibition, which was statistically significant ($p < 0.05$). When the exposure of *T. mentagrophytes* to fluconazole under *in vitro* conditions at different concentrations was investigated, it showed similar trend from the lowest concentration of 0.05 mg/mL to 0.09 mg/mL (Figure 2). The concentration of between 0.10 mg/mL and 0.50 mg/mL showed a similar observation on inhibition percentage which peaked on the 5th day and then slowed to inhibition of 84.4% for the concentration of 0.50 mg/mL on the 21st day. There was an association between the days of exposure, concentration, and inhibition which was statistically significantly ($p < 0.05$) for fluconazole treatments.

Table 2: Phytochemicals in methanolic crude leaf extract of *S. nilotica*

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

'+' indicates present and '-' indicates absent.

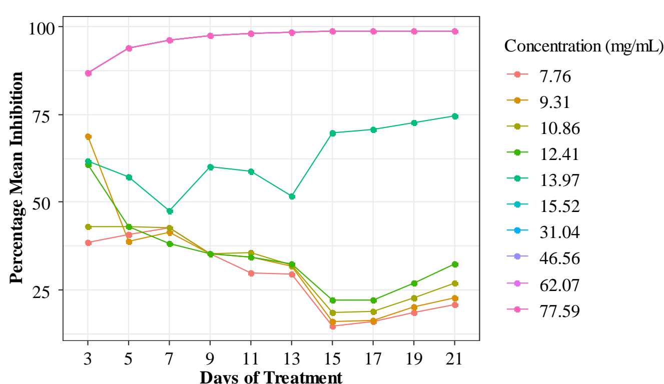

Figure 1: The trends of the efficacy of *S. nilotica* leaf extract against *T. mentagrophytes*. Values represent mean and *n*=3.

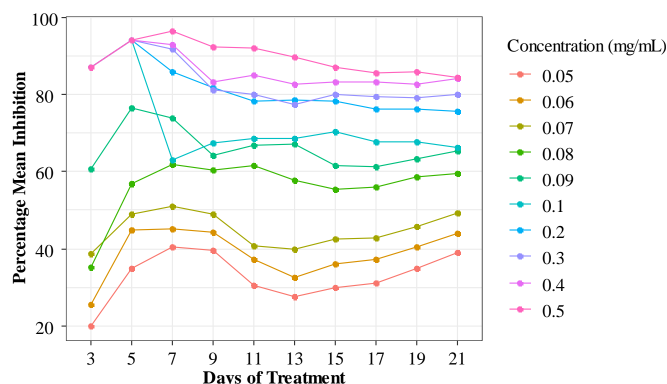

Figure 2: The trends of the efficacy of fluconazole against *T. mentagrophytes* across 21 days of exposure. Values represent mean and *n*=3.

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

The effect on the four target genes; SSU1, SUB3, MEP2, and DDPV of *T. mentagrophytes* showed different extents in gene expression following treatment. Down regulation of all these genes was observed when fluconazole and *S. nilotica* crude leaf extracts were exposed to *T. mentagrophytes* (Table 3). The amplification plot showed similar shapes meaning there was PCR efficiency during amplification. The cycle threshold (Ct) values that were obtained ranged between 19 and 31 as shown in *Figure 3*. 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.

Table 3: Effect of *S. nilotica* and fluconazole treatments on the expression of selected virulence genes 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 (0.30 mg/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 (0.30 mg/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

MEP 2-metalloprotease 2, SSU1-sulphite efflux pump, SUB3-subtilisin 3, DPPV-dipeptidyl-peptidase V. Fold changes values were normalized to the reference gene (beta-actin 1) and were calculated by comparative C_T analysis of quantitative real time PCR data.

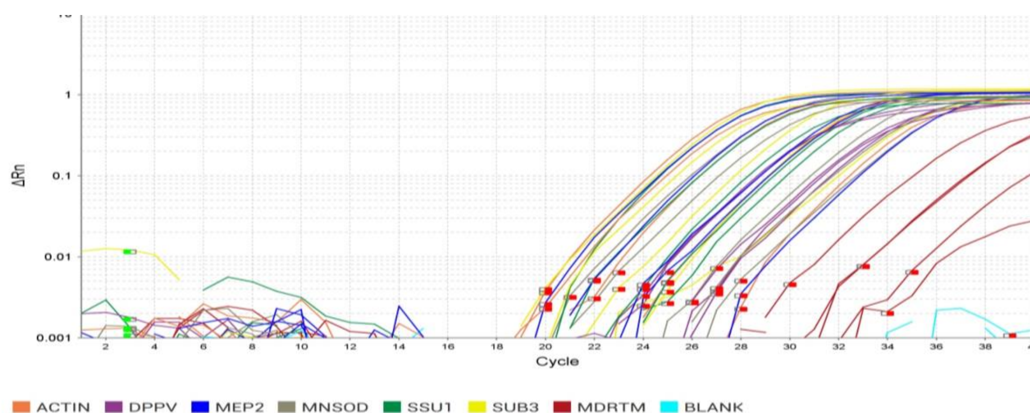

Figure 3: Amplification plot of targeted genes of *T. mentagrophytes*; metalloprotease 2 (MEP2), dipeptidyl-peptidases V (DPPV), subtilisin 3 (SUB3), sulphite efflux pump (SSU1), the reference gene (beta actin 1) and the blank.

DISCUSSION

The global health burden of dermatophytosis and the rising public health concern on the increase in fungal strains resistant to the current antifungals [18] significantly raises the urgency for the development of new therapeutics. Traditionally, medicinal plants have been used from time immemorial to cure various diseases. However, there are no scientific data on their phytochemical constitution and bioactivity. In this study, the qualitative screening on methanolic leaf extract of *S. nilotica* showed alkaloids, tannins, flavonoids, glycosides, saponins,

carbohydrates, amino acids, terpenoids, steroids and absence of anthraquinones and phlobatannins. Some of these phytochemicals present in the methanolic leaf extracts of *S. nilotica* have been found to exist in several other plant species [19]. Therefore, they could be responsible for the anti-dermatophytic effect of *S. nilotica* against *T. mentagrophytes* tested in this study. For instance, flavonoids detected from 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 [20]. Additionally, terpenoids have been associated with the weakening of the membrane tissues leading to the microorganisms' cell wall dissolution as reported by Hernández *et al.,* [21] while saponins have been reported to be responsible for the leakage of certain enzymes and proteins from the cell of the microorganisms [22]. The other bioactive constituents that were present in the 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* [23].

A MIC of 15.52 mg/mL for the *S. nilotica* methanolic crude leaf extracts against *T. mentagrophytes* was found in this study. These MIC values indicate that the methanolic extracts of *S. nilotica* have very high potency effect against *T. mentagrophytes*. The treatment of *T. mentagrophytes* by extract of *S. nilotica* resulted in the inhibition of its growth, which showed a fungicidal tendency of the *S. nilotica* crude leaf extracts. This phenomenon could be due to the suppression of expression of specific genes. Previous studies [24] have reported association of gene expression of fungi with the antifungal phenomenon. In the current study, the expression of four virulent genes; DPPV, MEP2, SSU1, and SUB3 were studied. There was down regulation of these four target genes in this study.

SSU1 gene was down-regulated which was similar to the findings by Lechenne *et al*. [5], who showed that SSU1 is a gene responsible for the process of pathogenicity in *T. mentagrophytes. It* reduces cysteine disulphide bridges by breaking the hard keratin into cysteine and *S*-sulphocysteine. On the other hand, 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. Although both treatments in this study 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*. The trend of down-regulation of MEP2 gene shows that this gene is targeted by the bioactivity of methanolic crude leaf extracts of *S. nilotica*. However, its down regulation was more pronounced on fluconazole treatment, which indicates that *S. nilotica* is a weak agent against MEP2 in *T. mentagrophytes*. Additionally, 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 [25]. Fluconazole and *S. nilotica* treatment had a similar effect by reducing SUB3 gene expression, which was more pronounced as the treatment concentration increased. 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*. There is still need however to further research on all other plausible mechanisms of anti-dermatophytic activity of this extract and also on studies with other common dermatophytes as these were limitations of this study.

CONCLUSION

The methanolic crude leaf extract of *S. nilotica* was found to be phytochemically-rich and showed high growth inhibitory effect on *T. mentagrophytes* under *in vitro* conditions. Gene expression of DPPV, MEP2, SUB3, and SSU1 genes were down-regulated by different folds which differed for fluconazole and methanolic crude leaf extract 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 *S. nilotica* methanolic 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*.

Conflicts of interest

The authors declare no conflicts of interest.

Funding

This work was supported by the National Research Fund (NRF), Kenya (2016/2017 Masters' Research Award to Joseph K. Chepkwony).

Acknowledgements

The authors are grateful to Anthony Odero of University of Eldoret, Kenya and Regina Mbugua and Ngule Mutuku of KEMRI, Nairobi for their technical support. The authors also acknowledge that this manuscript is a presentation of a Master of Science Thesis by the first author, Joseph K. Chepkwony at University of Eldoret, Kenya in 2021.

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HOW TO CITE THIS ARTICLE

Chepkwony JK, Mwitari PG, Kipsumbai PK, Bii CC, Tuei VC. Anti-dermatophytic activity of *Salvia nilotica* methanolic crude leaf extract against *Trichophyton mentagrophytes*. J Phytopharmacol 2021; 10(6):433-438. doi: 10.31254/phyto.2021.10602

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