Original Research Article

In vitro Susceptibility Studies of the Extract, Fraction and Cream Formulation of the Leaf of *Musa paradisiaca* Linn against Known Dermatophytes

ABSTRACT

~~As man is constantly confronted with pathogens resistance to available antimicrobials, medicinal plants continue to serve as viable alternatives for controlling these agents~~. This study was aimed at investigating the *invitro* susceptibility of known dermatophytes to *Musa paradisiaca* leaf extract. The antifungal activity of the methanol extract, aqueous fraction and cream formulation of *Musa paradisiaca* leaf was established by agar well diffusion method while minimum inhibitory concentration (MIC) was determined by broth microdilution and broth macrodilution methods respectively. Results obtained showed~~ed~~ that all the test organisms had varying degrees of ~~susceptibility~~susceptibility, which is comparable to that of a standard drug, ketoconazole. *Microsporum audounii* exhibited the lowest MIC of 25 mg/mL for the aqueous fraction and 50 mg/mL for the cream formulation. The MFC/MIC ratio determined indicates the fungicidal potential of the plant. The *in vitro* susceptibility of *M. paradisiaca* leaf extract to known dermatophytes is an indication of the possible use of the leaf as a potential for the treatment of skin infections due to the tested organisms.

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1-aims
2-study design
3-place and duration of study
4-methodology
5-results
6-conclusion

INTRODUCTION

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Dermatophytes are a group of closely related fungi that have the capacity to invade keratinized tissue (skin, hair and nails) of humans and other animals to produce an infection (Weitzman and Summerbell, 1995). They belong to the small category of disease organisms that almost every human alive will be infected by at some point over the course of his or her lifetime (Graser*et al.*, 2008). Diseases caused by this group of organism is referred to as dermatophytoses (Masri-Fridling and Gayle 1996), or dermatomycoses (Lakshmipathy and Kannabiran, 2010). These organisms colonize the keratin tissues and in response to their metabolic products, host experiences inflammatory reactions. They are usually restricted to the non-living cornified layer of the epidermis because of their inability to penetrate viable tissue of an immune-competent host (Sarika *et al.*, 2014). The colonization of the keratinized layers of the body is brought about by the organisms belonging to the three genera namely:

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Trichophyton, Microsporum and Epidermophyton. Reactions to a dermatophyte infection may range from mild to severe as a consequence of the host's reactions to the metabolic products of the fungus, the virulence of the infecting strain or species, the anatomic location of the infection and local environmental factors (Weitzman and Summerbell, 1995). *Tinea* infections can often be severe and recurrent (Gupta *et al.*, 2004).Infections affecting the keratinized tissues are of serious concerns worldwide and are increasing on a global scale. Although dermatophyte infections are treatable, there is a high rate of reinfections and recurrent infections, and often visible scars remain after treatment and sometimes deformities.

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In recent years, drug resistance to human pathogens has been commonly reported from all over the world. Multiple drug resistance has developed due to the indiscriminate use of commercial antimicrobial drugs commonly used in the treatment of infectious diseases among other reasons. In addition to this problem, antifungal agents in particular are sometimes associated with adverse effects on the host including hypersensitivity, immunosuppression and allergic reactions. This problem has forced scientists to continue to search for new antifungal agents from other sources including plants (Kolawole *et al.*, 2011). Antimicrobials of plant origin have enormous therapeutic potential. They are effective in the treatment of infectious diseases while simultaneously mitigating many of the side effects that are often associated with synthetic antimicrobials (Blake, 2004).

Medicinal plants are frequently used in traditional medicine to treat different diseases. The world health organization (WHO) estimates that 80% of the earth's inhabitants depend on traditional medicine for their primary health care needs, and most of this therapy involves the use of plant extracts or their active components. This has helped in the exploration of different medicinal plants to find the scientific basis of their traditional uses.

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Musa paradisiaca L. is a very common plant, known as plantain. It belongs to the Musaceae family and is indigenous to both tropical and subtropical countries (Ghani, 2003). It has been cultivated for more than 4000 years, and its numerous varieties are staple food in the tropical regions of the world where they are found especially in the Sub-Saharan Africa (Imam and Akter, 2011). The plant is an evergreen plant with an aerial pseudo stem, an underground rhizome and a height of 2 to 9 metres (Ekunwe and Ajayi, 2010). ~~The leaves are oblong, deep green and narrowed to the base, the fruits are edible and contains about 220 calories.Traditionally, the fruit, Stem juice, flowers of the plant have reportedly been used for~~

~~treating diarrhoea, dysentery, menorrhagia, diabetes (Yusuf *et al.*, 2009), inflammation, pain & snakebite (Krishnan and Vijayalakshmi, 2005).~~Pharmacological investigations revealed that plantain fruits, Stem juice, flowers are known to possess antidiarrhoeal activity (Rabbani *et al.,* 2001), hypoglycemic activity (Singh *et al.,* 2007); hypocholesterolaemic activity (Vijayakumar *et al.,* 2008), antioxidant activity (Yin *et al.,* 2008), diuretic activity (Jain *et al.,* 2007), wound healing activity (Agarwal *et al.,* 2009), Anti-allergic activity (Tewtrakul*et al.,* 2008), antimalarial activity (Kaou*et al.,* 2008), antiulcer (Gupta *et al*., 2003), antidiabetic and anti-snake venom. *Musa paradisiaca* has also been documented to have beneficial effects in the management of other disease conditions including atherosclerosis, diabetes mellitus, hypertension, hyperlipidaemia, sexual dysfunction (Yakubu *et al.,* 2007) and thyroid dysfunction (Mallick *et al.,* 2006; Parmar and Kar, 2007), and additionally offers protective effects on organs of the body such the kidneys in certain clinical conditions (Vinaykumar*et al.,* 2010). Other Literatures indicate that plantain fruits, leaves, peels, sap, stalk and flowers contain antimicrobial principles. Okorondu*et al* (2012) reported the antifungal properties of the peel and stalk extracts of *M. paradisiaca* while Ige *et al.* (2015) reported the antidermatophytic activities of *Musa sapientum* leaf, a variety of *M. paradisiaca*.

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METHODS

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Plant Collection, Authentication, Preparation and Extraction

Fresh plantain leaves were collected from the botanical garden of the Department Pharmacognosy, Faculty of Pharmacy, University of Uyo, Nigeria.The taxonomic keys for the identification of the plant were provided by the Herbarium of the same department, where a specimen sample with the specimen number UUPH51(a) was deposited.

The leaves were cut into smaller pieces for easy air drying. The dried leaves were powdered using a mortar and pestle. The powdery sample was packed into screw-capped bottles and labelled appropriately.

The leaf of *Musa paradisiaca* L. was extracted by maceration using methanol as a solvent.This was prepared by soaking 500 g of the dried powdery samples in 5,000 ml of methanol for 72 hours, during which the mixture was intermittently shaken. It was later filtered through Whatman No. 42 filter paper. The extract was concentrated with a freeze dryer (FD-10N CLIFTON)

Fractionation of the extract

The methanol extract of the leaf of *Musa paradisiaca* was partitioned with petroleum ether chloroform and water according to the method of Udobi*et al.* (2008). Twenty grams of the extract was dissolved in 200 mL of water before shaking vigorously in a separating flask. The mixture obtained was filtered using a filter paper to remove debris. Petroleum ether (200 mL) was then added to the mixture in a separating funnel, shaken vigorously and allowed to settle. The petroleum ether layer (on top) was removed and concentrated while a further 200 mL of chloroform was added to the aqueous layer and also vigorously shaken and allowed to settle. The aqueous and the chloroform layers was further separated, the chloroform portion was concentrated to dryness by allowing to stand on the laboratory bench until all the solvent evaporated, while the aqueous layer was concentrated to dryness using a freeze dryer..

Test Organisms

The test organisms, *Microsporum audounii, Trichophyton rubrum, Trichophyton verrucosum*, *Trichophyton tonsurans* and *Epidermophyton floccosum* were obtained from the National Veterinary Research Institute, Vom, Plateau State, Nigeria.

Susceptibility Testing

Determination of the antifungal activity of the aqueous fraction of the methanol extract of the leaf of *Musa paradisiaca* L. was done using the agar well diffusion method as described by Asuquo and Udobi, (2016). Sabouraud dextrose agar (SDA) was used as the culture medium while ketoconazole was used as control. The fungal inoculum was prepared by suspending five distinct fungal colonies from a 72 hour culture on SDA in 5 mL of sterile distilled water. The turbidity was adjusted to the turbidity of 0.5 McFarland standard by serial dilution, equivalent to 5 x 10^6Cfu/mL. A 1 in 10 dilution of the standardized suspension was carried out in sterile distilled water to obtain an inoculum size of 5 x 10^5Cfu/mL. About 0.1mL of the final dilution of the test organism were aseptically introduced into labelled petri dishes. Twenty millilitres (20 mL) of sterilized and cooled Sabouraud dextrose agar medium was aseptically poured into each Petri dish and gently swirled to mix. The plates were allowed to set and equidistant wells were created on the surface of the agar using a sterile 4 mm standard cork borer. Different concentrations (400 mg/mL, 200 mg/mL, 100 mg/mL, 50 mg/mL, 25 mg/mL, 12.5 mg/mL 6.25 mg/mL, 3.125 mg/mL) of the extract diluted in sterile distilled

water was introduced into the different wells and labelled appropriately. The plates were incubated at room temperature for 72 hours after which diameters of inhibition zones were measured in millimetres.

Determination of minimum inhibitory concentration (MIC)

The minimum inhibitory concentration (MIC) of the aqueous fraction against test dermatophytes were determined by the broth microdilution method as recommended by EUCAST (Sanguinetti and Posteraro, 2018) using a 96-well round-bottom plate. The aqueous fraction of the methanol extract of the leaf of *M. paradisiaca* L. was prepared four times the highest concentration required for the test in Sabouraud dextrose broth (SDB) prepared as double strength. With the help of a micropipette, 100 µL of the extract was aseptically introduced into column 1 containing 100µL of SDB and mixed. About 100 µL was withdrawn from column 1 to carry out a twofold serial dilution to other columns. The fungal inoculum was prepared by suspending five distinct fungal colonies from a 72 hour culture on SDA in 5ml of sterile distilled water. The turbidity was adjusted to the turbidity of 0.5 McFarland standard by serial dilution, equivalent to 5 x 10^6Cfu/mL. A 1 in 10 dilution of the standardized suspension was carried out in sterile distilled water to obtain an inoculum size of 5 x 10^5Cfu/mL. About 100µL of the 5 x 10^5Cfu/mL fungal suspension was introduced into the different wells containing 100 µL of extract accordingly, resulting in a final inoculum concentration of 2.5 x 10^5Cfu/mL. The inoculated plates were incubated for 72 hours at room temperature. The lowest concentration of aqueous fraction of the methanol extract of *M. paradisiaca* leaf that showed no visible turbidity after the period of incubation was recorded as the minimum inhibitory concentration (MIC).

Determination of the minimum fungicidal concentration (MFC)

MFCs were determined by spreading 10 µL of the contents from wells showing no visible growth on Sabouraud dextrose agar plates. The plates were incubated for 72 hours at room temperature. The lowest concentration of the aqueous fraction of the methanol extract of *M. paradisiaca* leaf that showed no growth after the period of incubation was recorded as the minimum fungicidal concentration (MFC).

Formulation of cream

Aqueous Cream (BP) used as the base was prepared using BPC 1979 method. Briefly, in a beaker placed on water bath the emulsifying ointment (30%w/w) was melted (I) and in another beaker, chlorocresol (0.1% w/w) was dissolved in purified water (69.9% w/w) with the aid of gentle heat (II). Solution (II) was added to the melted wax (I) while still hot with continuous stirring until it became cold. The aqueous fraction of the methanol extract of *M. paradisiaca* was used to prepare creams at concentration of 10- 50%w/w by mixing the required quantity of extract with the aqueous cream BP with continuous stirring until an elegant product was formed. The product was emptied into properly labelled containers and stored in a cool dry place.

Susceptibility testing of the Cream formulation

The antifungal activity of the formulated cream against dermatophytes was carried out by the agar well diffusion method as described by Asuquo and Udobi, 2016. Sabouraud dextrose agar (SDA) was used as the culture medium while ketoconazole 2% cream was used as control. The fungal inoculum was prepared by suspending five distinct fungal colonies from a 72 hour culture on SDA in 5 mL of sterile distilled water. The turbidity was adjusted to the turbidity of 0.5 McFarland standard by serial dilution, equivalent to 5×10^6Cfu/mL. A 1 in 10 dilution of the standardized suspension was carried out in sterile distilled water to obtain an inoculum size of 5×10^5Cfu/mL. About 0.1mL of the final dilution of the test organism were aseptically introduced into labelled petri dishes. Twenty millilitres (20 mL) of sterilized and cooled Sabouraud dextrose agar medium was aseptically poured into each Petri dish and gently swirled to mix. The plates were allowed to set and equidistant wells were created on the surface of the agar using a sterile 4 mm standard cork borer. Different concentrations (200 mg/mL, 100 mg/mL, 50 mg/mL, 25 mg/mL, 12.5 mg/mL 6.25 mg/mL) of the cream formulation diluted in sterile distilled water introduced into the different wells and labelled appropriately. The plates were incubated at room temperature for 72 hours after which diameters of inhibition zones were measured in millimetres.

Determination of minimum inhibitory concentration (MIC) of the cream

The minimum inhibitory concentration (MIC) of the cream formulation against test dermatophytes was determined by the standard broth macrodilution method as described by Kulkarni et al 2018. Sabouraud dextrose broth (SDB) was prepared according to the manufacturers instruction and aseptically dispensed 5ml each, into 6 sterile test tubes. The cream formulation was diluted with sterile distilled water to a concentration of 800 mg/mL. This concentration was used to carry out a twofold serial dilution to the 6 test tubes to obtain a concentration of 400mg/ml-12.5mg/ml. The fungal inoculum was prepared by suspending five distinct fungal colonies from a 72 hour culture on SDA in 5ml of sterile distilled water. The turbidity was adjusted to the turbidity of 0.5 McFarland standard by serial dilution, equivalent to 5 x 10^6Cfu/mL. A 1 in 10 dilution of the standardized suspension was carried out in sterile distilled water to obtain an inoculum size of 5 x 10^5Cfu/mL. About 0.1ml of the fungal suspension was aseptically transferred into the different test tubes containing the different concentrations of the cream formulation accordingly. 2% ketoconazole cream was used as the control. The inoculated test tubes were incubated for 72 hours at room temperature. The lowest concentration of the cream formulation that showed no visible turbidity after the period of incubation was recorded as the minimum inhibitory concentration (MIC).

Determination of the minimum fungicidal concentration (MFC) of the cream

MFCs were determined by inoculating 0.1ml from test tubes with no visible growth after the minimum inhibitory concentration determination on Sabouraud dextrose agar (SDA) plates. The plates were incubated for 72 hours at room temperature. The lowest concentration of the cream formulation that showed no visible turbidity after the period of incubation was recorded as the minimum fungicidal concentration (MFC).

RESULTS

A. Susceptibility testing of the crude extract and aqueous extract

The susceptibility of the test organisms to the crude extract of *M. paradisiaca* leaf is presented in table 1, while the result of the aqueous fraction is presented in table 2. The aqueous fraction is observed to be more active than the crude extract. The MIC and MFC is presented in table 3 and 4 respectively. The MFC/ MIC is presented in table 5.

Table 1: The Antifungal Assay of the Methanol Extract of the leaf of *M. paradisiaca* L.

Conc. (mg/mL)	Zones of inhibition (mm)				
	M. audouinii	*T. rubrum*	*T. verrucosum*	*T. tonsurans*	*E. floccosum*
400	18	17	-	10	13
200	14	12	-	8	12
100	11	10	-	-	8
50	8	-	-	-	-
25	-	-	-	-	-
12.5	-	-	-	-	-

Inhibition zones are average of double determination

Table 2: **The Antifungal Assay of the Aqueous Fraction of the Methanol Extract of the leaf of *M. paradisiaca* L.**

Conc. (mg/mL)	Zones of inhibition (mm)				
	M. audouinii	*T. rubrum*	*T. verrucosum*	*T. tonsurans*	*E. floccosum*
400	22	21	12	15	18
200	18	18	8	12	16
100	15	12	-	10	12
50	12	10	-	-	-
25	10	-	-	-	-
12.5	-	-	-	-	-
Control	30	32	18	26	22

Inhibition zones are average of double determination
Control: ketoconazole 5mg/mL

Table 3: Minimum Inhibitory Concentrations (MIC) and minimum fungicidal concentrations of the Aqueous Fraction of the Methanol Extract of the leaf of *M. paradisiaca* L. against test Dermatophytes

Test organisms	Concentration of extract (mg/mL)

M. audouinii	25
T. rubrum	50
T. verrucosum	200
T. tonsurans	100
E. floccosum	100

Table 4: Minimum Fungicidal Concentrations (MFC) of the Aqueous Fraction of the Methanol Extract of the Leaf of *M. Paradisiaca* L. against Test Dermatophytes

Test Organisms	Concentration of Extract (mg/mL)
M. audouinii	50
T. rubrum	100
T. verrucosum	400
T. tonsurans	200
E. floccosum	100

Table 5: MFC/MIC Ratio of the Aqueous Fraction of the Methanol Extract of the Leaf of *M. Paradisiaca* L. against Test Dermatophytes

Test organisms	MIC (mg/mL)	MFC(mg/mL)	MFC/MIC ratio
M. audouinii	25	50	2:1
T. rubrum	50	100	2:1
T. verrucosum	200	400	2:1
T. tonsurans	100	200	2:1
E. floccosum	100	100	1:1

B. Susceptibility of the cream formulation

The susceptibility of the test organisms to the different concentrations of the cream formulation of *M. paradisiaca* leaf is presented in tables 6-10. The MIC is presented in table 11, while the MFC is presented in table 11.

Table 6: Antifungal Assay of the 50 % (w/w) of the cream formulation

Conc. (mg/mL)	Zones of inhibition (mm)				
	M. audouinii	*T. rubrum*	*T. verrucosum*	*T. tonsurans*	*E. floccosum*
400	20	17	14	15	16
200	18	16	12	13	12
100	15	12	-	10	10
50	12	10	-	-	-
25	-	-	-	-	-
12.5	-	-	-	-	-
Control	28	32	18	25	24

Inhibition zones are average of double determination

Control: 2% ketoconazole cream

Table 7: Antifungal Assay of the 40 % (w/w) of the cream formulation

Conc. (mg/mL)	Zones of inhibition (mm)				
	M. audouinii	*T. rubrum*	*T. verrucosum*	*T. tonsurans*	*E. floccosum*
400	18	15	11	13	14
200	15	14	-	11	11
100	14	12	-	-	10
50	10		-	-	-
25	-	-	-	-	-
12.5	-	-	-	-	-

Inhibition zones are average of double determination

Table 8: Antifungal Assay of the 30 % (w/w) of the cream formulation

Conc. (mg/ml)	Zones of inhibition (mm)				
	M. audouinii	*T. rubrum*	*T. verrucosum*	*T. tonsurans*	*E. floccosum*
400	15	13	-	-	11
200	13	11	-	-	10
100	-	-	-	-	-
50	-	-	-	-	-
25	-	-	-	-	-
12.5	-	-	-	-	-

Inhibition zones are average of double determination

Table 9: Antifungal Assay of the 20 % (w/w) of the cream formulation

Conc. (mg/ml)	Zones of inhibition (mm)				
	M. audouinii	*T. rubrum*	*T. verrucosum*	*T. tonsurans*	*E. floccosum*
400	10	-	-	-	-
200	-	-	-	-	-
100	-	-	-	-	-
50	-	-	-	-	-
25	-	-	-	-	-
12.5	-	-	-	-	-

Inhibition zones are average of double determination

Table 10: Antifungal Assay of the 10 % (w/w) of the cream formulation

Conc. (mg/ml)	Zones of inhibition (mm)				
	M. audouinii	*T. rubrum*	*T. verrucosum*	*T. tonsurans*	*E. floccosum*
400	-	-	-	-	-
200	-	-	-	-	-
100	-	-	-	-	-
50	-	-	-	-	-
25	-	-	-	-	-
12.5	-	-	-	-	-

Table 11: Minimum Inhibitory Concentrations (MIC) of the cream formulation against test Dermatophytes

Test organisms	MIC (mg/mL)				
	50%	40%	30%	20%	10%
M. audouinii	50	50	200	-	-
T. rubrum	50	100	200	-	-
T. verrucosum	200	400	-	-	-
T. tonsurans	100	200	-	-	-
E. floccosum	100	200	-	-	-

Table 12: Minimum fungicidal Concentrations (MFC) of the cream formulation against test Dermatophytes

Test organisms	MFC (mg/mL)				
	50%	40%	30%	20%	10%
M. audouinii	50	100	200	400	-
T. rubrum	100	100	400	-	-
T. verrucosum	400	400	-	-	-
T. tonsurans	200	400	-	-	-
E. floccosum	200	200	400	-	-

DISCUSSION

The susceptibility of some dermatophytes was tested against the aqueous fraction of the methanol extract of the leaf of *Musa paradisiaca* L. The extract exhibited sufficient antifungal activity against *Microsporum audouinii*, *Trichophyton rubrum*, *Trichophyton verrucosum*, *Trichophyton tonsurans* and *Epidermophyton floccosum* as presented in table 2. *Microspurum audouinii* and *Trichophyton tonsurans* are anthropophilic fungi that causes infection of the scalp known as *Tinea capitis* which have been reported to be the most predominant fungal infection in school children (Nweze, 2010). *Trichophyton rubrum* colonizes the upper layers of dead skin and is the most common cause of tinea pedis and also implicated in tinea corporis and onychomycosis. *Trichophyton verrucosum*, commonly known as the cattle ringworm fungus, is largely responsible for fungal skin disease in cattle but also causes ringworms in other animals. *Epidermophyton floccosum*, one of the two known species of the *Epidermophyton* genus, causes superficial and cutaneous mycoses such as *Tinea corporis*, *Tinea cruris*, *Tinea pedis* and *Tinea unguium.*

The Minimum inhibitory concentration (MIC) of an agent is the lowest concentration of that substance that inhibits the visible growth of a microorganism (fungus) after the incubation period while the minimum fungicidal concentration (MFC) is the lowest concentration that results in the death of the fungus. In this study, *M. audouinii* exhibited the lowest MIC of 25mg/mL (table 3) and MFC of 50mg/mL (table 4). The nature of the antifungal effect of the extract with respect to inhibition of the test organism is important, hence the MFC/MIC ratio is used to evaluate the activity of the an agent. Antimicrobials are usually regarded as fungicidal if the MFC/MIC ratio is ≤4 and fungistatic if >4 (Appiah *et al.*, 2017). This study shows MFC:MIC ratiio of 1:1 and 2:1 respectively against the test organisms (table 5), thus, suggesting that the aqueous fraction of the methanol extract of the leaf of *Musa*

*paradisiaca*L. has fungicidal properties. These activities may be due to the presence of secondary metabolites such as saponins and flavonoids in the leaf extract as previously reported by Asuquo and Udobi 2016. These secondary metabolites have been reported to possess antifungal activities (Mebude*et al.*, 2017).

The invitro antidermatophytic activity of the cream formulation of the aqueous fraction of the methanol extract of the Leaf of *M. paradisiaca* L. against test dermatophytes exhibited sufficient inhibitory effects. However, the observed antidermatophytic activity was dose-dependent with 50%w/w having increased activity (table 6) and compared well with the activity of the control (2% ketoconazole cream). Only *M. audounii* had a slight activity at 20%w/w (table 9), no activity was observed at 10%w/w (table 10) for any of the test organisms. Therefore, the cream formulation of the aqueous fraction of the methanol extract of the Leaf of *M. paradisiaca* L can be employed in the topical management of dermatophytosis due to the studied fungi.

Although this work has shown the susceptibility of the selected dermatophytes to the extracts of Musa paradisiaca leaf, the susceptibility pattern is not fully established. Thus, an ongoing research in this regard which will aid in establish the plant as a candidate for in vivo testing.

CONCLUSION

This study has demonstrated the susceptibility of known dermatophytes to the extract and aqueous fraction of *Musa paradisiaca* leaf. The ability of the cream formulation to inhibit the growth of these organisms in vitro, indicates its potential for use in the treatment of skin infections and hence, improve the quality of health. Further studies are needed to isolate and elucidate the compound responsible for the observed activity.

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Comment [MA9]: AcknowledgEments

A brief acknowledgement section may be given after the conclusion section just before the references. The acknowledgments of people who provided assistance in manuscript preparation, funding for research, etc. should be listed in this section. All sources of funding should be declared as an acknowledgement. Authors should declare the role of funding agency, if any, in the study design, collection, analysis and interpretation of data; in the writing of the manuscript. If the study sponsors had no such involvement, the authors should so state.

Comment [MA10]: Competing interests

Declaration of competing interest should be placed here. All authors must disclose any financial and personal relationships with other people or organizations that could inappropriately influence (bias) their work. Examples of potential conflicts of interest include employment, consultancies, honoraria, paid expert testimony, patent applications/registrations, and grants or other funding. If no such declaration has been made by the authors, SDI reserves to assume and write this sentence: "Authors have declared that no competing interests exist.".

Comment [MA11]: Ethical approval Should be added

Comment [MA12]: References must be listed at the end of the manuscript and numbered in the order that they appear in the text. Every reference referred in the text must also present in the reference list and vice versa. In the text, citations should be indicated by the reference number in brackets [3].

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Comment [MA13]: Amend the reference.

Comment [MA14]: Reference to a journal:
For Published paper:

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