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ABSTRACT

The inhibitory activity of the essential oils (EO) of mint and thyme on Candida albicans are well known, however, their valorization in a dosage form for the management of cutaneous candidiasis has been little explored. This study aimed to formulate innovative and cost-effective dermatological topicals based on mint and thyme essential oils for the treatment of cutaneous candidiasis. Thyme essential oil was obtained commercially, while that of mint was obtained by hydrodistillation. In order to determine their antifungal activity, both essentials oils were tested alone and in combination against a reference strain of Candida albicans (strain MHMR) and clinical strains of Candida albicans and Candida tropicalis. Three creams were formulated using natural (shea butter, palm oil) and chemical (cetomacrogol) excipients. The efficacies of these creams were assessed in vivo using Wistar rats infected with Candida albicans MHMR. The in vitro antimicrobial study showed that C. albicans MHMR ...

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Development of new dermatological formulations for the treatment of cutaneous candidiasis

Aude Zinsou[a,b], Assogba G Assanhou[a,b,*], Habib Ganfon[c], Hope Sounouvou[a,b,d], Urbain C Kassehin[a], Roland F Lawson[e], Sèdami Medegan[a], Brigitte Evrard[d], Joëlle Quetin-Leclercq[f], Fernand A Gbaguidi[a,*]

[a] Laboratoire de Chimie Organique te Pharmaceutique, UFR Pharmacie, Faculté des Sciences de la Santé, UAC, 01 BP 188, Cotonou, Benin
[b] Laboratoire de Pharmacie Galénique et de Technologie Pharmaceutique, Ecole de Pharmacie, Faculté des Sciences de la Santé, Université d'Abomey-Calavi, Campus du champ de Foire, 01BP 188, Cotonou, Benin
[c] Laboratoire de Pharmacognosie et de Phytothérapie, UFR Pharmacie, Faculté des Sciences de Santé de Cotonou, Université d'Abomey Calavi, Benin
[d] Laboratoire de Technologie Pharmaceutique et Biopharmacie, CIRM, ULiège, Avenue de l'Hôpital 15, B-4000 Liège, Belgium
[e] Laboratoire de Pharmacologie, UFR Pharmacie, Faculté des Sciences de Santé de Cotonou, Université d'Abomey Calavi, Benin
[f] Pharmacognosy, Louvain Drug Research Institute, UCLouvain, Avenue E. Mounier 72, 1200 Bruxelles, Belgium

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ABSTRACT

The inhibitory activity of the essential oils (EO) of mint and thyme on *Candida albicans* are well known, however, their valorization in a dosage form for the management of cutaneous candidiasis has been little explored. This study aimed to formulate innovative and cost-effective dermatological topicals based on mint and thyme essential oils for the treatment of cutaneous candidiasis. Thyme essential oil was obtained commercially, while that of mint was obtained by hydrodistillation. In order to determine their antifungal activity, both essentials oils were tested alone and in combination against a reference strain of *Candida albicans* (strain MHMR) and clinical strains of *Candida albicans* and *Candida tropicalis*. Three creams were formulated using natural (shea butter, palm oil) and chemical (cetomacrogol) excipients. The efficacies of these creams were assessed *in vivo* using Wistar rats infected with *Candida albicans* MHMR. The *in vitro* antimicrobial study showed that *C. albicans* MHMR was more sensitive to thyme essential oil with a MIC of 310 μgmL^{-1}, which was higher than the reference fluconazole used for the *in vitro* study (32 μgmL^{-1}). The antimicrobial synergy study of both essential oils using checkerboard test demonstrated an additive effect of mint and thyme essential oils on *C. albicans* MHMR. GC/FID and GC/MS analyses led to the identification of thymol and menthol respectively as the main components of thyme and mint essential oils. A shea butter cream base and a shea butter and palm oil cream both containing 5% of EO (mint and thyme) exhibited the best *in vivo* antimicrobial activities, inducing optimal wound healing in infected rats compared with ketoconazole commercial cream used for the *in vivo* study. These results provide a solid basis at least in part, for the use of essential oil in creams formulated with natural excipients for the management of cutaneous candidiasis.

* Corresponding authors.
E-mail addresses: gassogba1983@gmail.com (A.G. Assanhou), ahokannou@yahoo.fr (F.A. Gbaguidi).

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Introduction

In sub-Saharan Africa, skin diseases are often trivialized because of the low mortality attributed to them [1]. The prevalence of dermatoses in tropical environments is estimated between 21 and 87% [2]. The microbial agents responsible are mainly yeast-type fungi (50.8%), in particular, *Candida spp*. In Benin, skin diseases constitute a major reason for medical consultation. Therapeutic management of cutaneous candidiasis involves the use of antifungals, especially nystatin, ketoconazole and fluconazole [3]. Moreover, there is increasing resistance problems [4]. It is then necessary to find new alternatives that are both less expensive and effective. Essential oils (EOs) from medicinal plants fall under this category of alternative medicines for handling infectious diseases.

EOs are considered as broad-spectrum antimicrobial agents [5] which are hardly subject to microbial resistance because they do not have specific pharmacological targets [6]. The activities of *Thymus vulgaris* [7–9] and *Mentha piperita* L, [10] on the *in vitro* growth of *Candida albicans* strains have been demonstrated in previous studies. However, assessment of their activities in appropriate dosage forms against cutaneous candidiasis has received little attention. This could probably be due to the highly concentrated nature of the EOs components, their high volatility and hydrophobicity. Some preparations based on EOs are available in West Africa, particularly in Benin in the form of hydrophobic ointments. Although these ointments reduce the possibilities of evaporation of the hydrophobic active constituents of the essential oils, they tend to trap these actives making their diffusion through the skin difficult and, thereby affecting their pharmacological effects.

The optimization of the use of EOs in the management of cutaneous candidiasis requires a stable pharmaceutical formulation based on effective EOs against *Candida* strains, responsible for cutaneous candidiasis. In this context, this research was conducted to enhance the bioactivities of the essential oils of mint and thyme by formulating them into hydrophilic creams using local excipients such as shea butter and palm oil.

Materials and methods

Materials

Mint samples were collected in Benin (Cotonou) and extraction of mint essential oil from dry leaves was done by hydrodistillation using a Clevenger-type apparatus. Thyme EO used is the European thyme EO, "*Thymi aetheroleum*" from Fagron Laboratories, France. Ketoconazole was purchased from Sigma Aldrich. The strains of *Candida spp* used were *C.tropicalis* and *C.albicans* (clinical isolates) from the Hubert Maga National Hospital Center of Cotonou (Benin), *Candida albicans* MHMR (reference strain) was obtained from the Laboratory of Biology and Molecular Typing in Microbiology (LBTMM) of the Faculty of Science and Technology of the University of Abomey–Calavi.

Methods

Phytochemical characterization of mint and thyme EO

Chemical composition of the essential oils was analysed by gas chromatography with flame ionization detector (GC / FID) for quantitative analysis and mass spectrometer (GC/MS) for qualitative analysis. GC/MS analyses were performed on the TRACE GC 2000 series (Thermo Quest, Rodano, Italy), equipped with an AS2000 autosampler (Thermo Quest). The GC system was coupled to a Trace MS (Thermo Quest) type mass spectrometer operating in electronic impact mode. Helium was used as a carrier gas at a constant flow rate of 1.3 mL/min. The coupling temperature of the GC was 260 °C. The identification of the compounds was confirmed by the determination of the Kovats Index which depends on the column used. The values obtained were compared with reference values of NIST (National Institute of Standards and Technology) and European Pharmacopoeia data. For GC/FID analysis, the vector gas used was helium gas stream leaving the column and arriving in a flame of hydrogen and air (T = 210 °C). We followed the same method described by Ahouansou *et al.*, [11].

MICs determination

For the tests we used mint EO, thyme EO, fluconazole, ketoconazole, combination of mint and thyme EOs. The method used for MIC determination was REMA (Resazurin Microtiter Assay) microdilution in Sabouraud broth using 96-well microplates. We prepared a series of two-fold dilutions from each sample, so as to obtain a final concentration range of 0.031% and 1% $^{v}/_{v}$. The microplates were treated with yeast suspension (10^6 CFU/mL). After incubation of the plate for 48 h, 30 μL of a solution of 0.02% $^{w}/_{v}$ resazurin was introduced into each of the wells. Fungal growth was attested by a change in color from blue to pink. The last well where there was no color change represented the MIC.

Activity of the combination of mint and thyme EOs on *C. albicans* MHMR was performed by the Checkerboard method [12,13] using 96-well microplates. Dilutions of both EOs were made in liquid Sabouraud in separate microplates. Subsequently, the EOs were transferred to a main microplate containing on the X axis 50 μL series of 2 of dilutions of mint essential oil and on the Y axis 50 μL, the dilution series of 2 of the thyme essential oil. The Fractional Inhibitory Concentration Index (FICI) was determined by the following formulae:

FICI = FIC (mint) + FIC (thyme);

FICI = MIC (mint combined with thyme)/MIC (mint alone) + MIC (thyme combined with mint)/MIC (thyme alone).

Table 1
Composition of cream.

	Cetomacrogol cream	Shea butter cream	Shea butter and Palm oil cream
Lipidic phase	Cetostearyl alcohol 7.2%; cetomacrogol 1000 1.84%; white vaseline 15%; liquid paraffin 6%; thyme EO 2.5%; mint EO 2.5%	Cetostearyl alcohol 7.2%; cetomacrogol 1000 1.84%; shea butter 15%; liquid paraffin 6%; thyme EO 2.5%; mint EO 2.5%	Cetostearyl alcohol 7.2%; cetomacrogol 1000 1.84%; shea butter 15%; palm oil 6%; thyme EO 2.5%; mint EO 2.5%
Aqueous phase	Monosodium phosphate dihydrate 0.3%; purified water 6%;	Monosodium phosphate dihydrate 0.3%; purified water 6%;	Monosodium phosphate dihydrate 0.3%; purified water 6%;
In addition to the final phase	Purified water q.s. ad 100%	Purified water q.s. ad 100%	Purified water q.s. ad 100%

The combination of the two EO was assessed according to Mulyaningsih et al., [14] as: synergistic when the FICI value is ≤ 0.5; additive when $0.5 < FICI < 1$; indifferent when $1 < FICI < 4$; antagonistic when $FICI > 4$.

Development of the hydrophilic creams

Different hydrophilic creams were prepared on the basis of the cetomacrogol cream formulation. Cetomacrogol cream is a non-greasy hydrophilic cream containing a non-ionic surfactant and a phosphate buffer. The pH value of this O/W emulsion was 5.5 allowing a good stability of active ingredients unstable at higher pH values.

When applied to the skin, cetomacrogol cream provides a thin layer of oil on the surface of the skin, which prevented water evaporating from the skin surface. In this work, we tested the feasibility to replace white vaseline and liquid paraffin, two hydrocarbons present in the cetomacrogol cream composition by naturally occurring substances from West Africa such as shea butter and palm oil.

Composition of the 3 developed formulations are shown in Table 1. They contained 5% of the mixture of mint and thyme EOs in equal proportions.

Emulsification was done by the inversion phase technique, which consists in incorporating under continuous stirring the aqueous phase heated at 70 °C in the lipidic phase heated at 70 °C. The preparation was completed to 100% by purified water kept to 70 °C. Manual stirring was maintained until the temperature was below 30 °C, essentials oils were incorporated with stirring after cooling.

The different formulations were evaluated. The pH value of the preparations was measured using a DOSATEST brand pH paper and the stability of the emulsion was assessed by the centrifugation technique whereby 10 mL of the cream was centrifuged for 15 min at 1500 rpm.

In vivo *efficacy evaluation of formulated creams against* Candida *spp*

The animal studies were performed according to OECD principles and the internationally accepted principles for laboratory animal use and care (NIH publication No 85–23, revised 2010).

An adapted method of Ray and Wuepper [14] was used. Eighteen adult male and female Wistar rats weighing 220 ± 30 g on average were used. After anesthesia, their backs were manually shaven. Injury was induced on the shaven back of each rat using a hot copper metal material of 1.5 cm^2 area. After induction of the injury, each animal was inoculated with 0.5 mL broth containing 10^8 Colony-Forming Units (CFU) of *C. albicans* MHMR by swabbing. Depending on their weight, the animals were divided into six groups and then subjected to different treatments from the third day. The groups were as follows: Group 1 ($n = 3$) sham-operated control group with injury but not infected with *C. albicans* MHMR; Group 2 ($n = 3$) rats with injury and infected with the fungus but without treatment; Group 3 ($n = 3$) rats with injury, infected with *C. albicans* MHMR and treated with ketoconazole cream as reference, unfortunately, on our market, fluconazole cream was not available; Group 4 ($n = 3$) rats with injury, infected with *C. albicans* MHMR and treated with the classic cetomacrogol cream with 5% EO; Group 5 ($n = 3$) rats with injury, infected with *C. albicans* MHMR and treated with shea butter cream based on 5% EO; Group 6 ($n = 3$) rats with injury, infected with *C. albicans* MHMR and treated with shea butter and palm oil cream with 5% EO. Sterile gauzes were placed on the rats and all dressings were fixed with adhesive plaster. The dressings were changed every day. The wound area was measured with rule technique (length by width) and the weight of the rats taken for each group on days, 0,3,6,9,12 and 15 in order to examine the evolution of the wound surface and compare the treated with untreated groups.

Statistical analysis

The one-way ANOVA test was performed with Graph Pad Prism 7.00 software to identify the significant differences between the groups. The level of probability for rejection of the null hypothesis was 5% ($P < 0.05$).

Table 2
Results of phytochemical analysis of Thyme and mint EO.

EO	GC/MS	Kovats Index	GC/FID	pH Eur requirement
Thyme	Components		%	
	δ-Terpinene	754. 66	5.48	4.0–12.0
	p-Cymene	780.48	20.77	14–28
	Linalol	1054.87	4.11	1.5–6.5
	Thymol	1669.71	44.76	37–55
	Carvacrol	1692.51	1.91	0.5–5.9
	Limonene	1113.39	–	–
	Carophyllene	1092.82	1.50	–
	Total		79.13	–
Mint	Limonene	705.42	1.71	1.0–3.5
	Eucalyptol	716.70	4.347	–
	Menthone	967.31	20.13	14–32
	Iso-menthone	991.35	2.45	1.5–10
	Menthyl acetate	1063.85	3.37	2.8–10
	Menthol	1144.77	32.28	30–55
	Total		64.28	

Table 3
Minimal inhibitory concentrations of mint and thyme EOs (μgmL^{-1}) against *Candida spp*.

	C. tropicalis	*C. albicans*	*C. albicans* MHMR
Thyme EO	1250	310	310
Mint EO	>10,000	>10,000	2500
Fluconazole	32	32	32
Ketoconazole	–	32	32

Table 4
MIC (μgmL^{-1}) of the combination of mint and thyme EOs.

	MIC of EO alone	MIC combination (mint EO)	MIC combination (thyme EO)
Thyme EO	310	150	–
Mint EO	2500	–	625

Results and discussion

Results

Chemical analysis of essential oil

The results of the chemical characterization showed that the major components of the essential oils of thyme were thymol and *p*-cymene with respective percentages of 44.76% and 20.77% while caryophyllene was a minor component (1.5%). All identified compounds represented 79.13%. These data on the chemical composition of the sample of thyme provided information on the chemotype concerned: the thymol chemotype. The results of the GC/FID phytochemical analysis of mint EO showed that the major components were menthol (32.28%) and menthone (20.13%). Limonene was present at 1.71%. The identified compounds represented 64.28% of the essential oil. The mint chemotype used was *Mentha piperita* menthol (Table 2)

In vitro *efficacy of EO*

The minimum inhibitory concentration values of the EOs against the three strains of *Candida spp* are presented in Table 3 below and expressed in μgmL^{-1}.

Fluconazole displayed the same MIC value for all three yeasts (32 μgmL^{-1}). The MIC value for ketoconazole was the same as fluconazole against *C. albicans* and *C. albicans* MHMR. The lowest MIC (310 μgmL^{-1}) was obtained with thyme EO against *C. albicans* strain MHMR. The MICs of mint EO were greater than 10,000 μgmL^{-1} against *C. tropicalis* and *C. albicans* (clinical strains). However, the mint EO showed a non-negligible inhibitory activity against *C. albicans* MHMR with a MIC of 2500 μgmL^{-1}.

The results of the antifungal activity of the combination of EOs of mint and thyme against *C. albicans* MHMR are shown in Table 4

The index of the fractional inhibitory concentration gave a value of 0.75, which was within the range of 0.5–1. This indicates that the combination of mint and thyme EOs showed an additive effect against *C. albicans.*

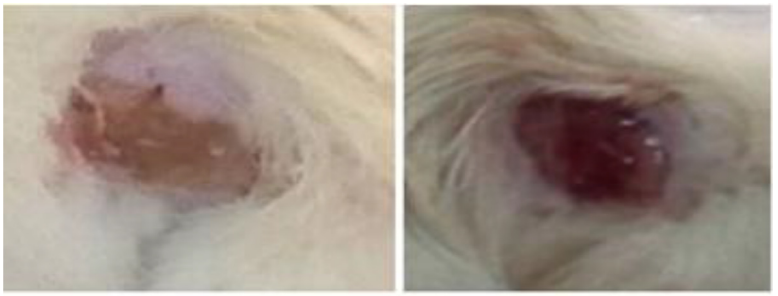

Fig. 1. Appearance of the wounds of an uninfected rat (A) and an infected rat (B) on the third day.

Characterization of the hydrophilic creams

All the creams were stable whitish o/w emulsions with different viscosities due to the modification of the chemical nature of the consistency excipients (vaseline or shea butter and paraffine or palm oil). The viscosity of the base increases in the following manner cetomacrogol < shea butter < shea butter and palm oil. pH values were 5–5.5 and no phase separation was observed after centrifugation. All the creams were whitish however, the shea butter cream and the shea butter + palm oil cream were less whitish compared to the cetomacrogol cream.

In vivo *efficacy evaluation*

Remarkable differences in wound appearance were observed in groups of rats exposed to *C. albicans* MHMR, group of unexposed rats, and exposed and treated groups. The wounds of infected rats were deep, gaping, suppurative, and red, whereas those of uninfected rats were superficial, non-suppurative, and less reddish. Fig. 1 shows the appearance of the wounds of an uninfected and infected rat on the third day when treatment began.

Along with this remarkable difference in wound appearance of infected and uninfected rats, weight loss and increased wound area were observed in infected rats before treatment began.

Weight change. Fig. 2A shows the changes in the weight of the rats by days after infection. Changes are epitomized by a biphasic graph, where one notes a fall of the weight from day 0 to day 3, then a recovery from day 3 until day 15. The analysis of variance (ANOVA) showed that between day 0 and day 3 the weight losses were statistically significant ($p < 0.0001$). The overall change in weight from day 0 to day 15 was statistically significant ($p < 0.0001$).

On day 3, the rats from groups 3, 4,5 and 6 were respectively treated with ketoconazole, the classic cetomacrogol cream with 5% EO, the shea butter cream with 5% EO and the shea butter and palm oils cream with 5% EO. In general, recovery began as soon as treatment was initiated, thus, accounting for the gradual increase in the weight of the rats from day 3 to day 15 (Fig. 2A).

Change in wound area. At day 0 the wounds of all rats had the same area (1.5 cm^2). Groups 1 and 2 experienced an increase in wound area between day 0 and day 6. And it is only from day 6 that the reduction of the surface of their wounds began (Fig. 2B). Groups 3, 4, 5 and 6 experienced an increase in wound area between day 0 and day 3. We also noted that when treatment began on day 3, the wound surface of the rats from these groups began to decrease in size. In the group 3 which received ketoconazole treatment (reference antifungal), the wound area decreased from 2.39 cm^2 on day 3 to 0.31 cm^2 on day 15. The wounds of groups 4 and 5 decreased respectively from 3.96 to 1 cm^2 and 4 cm^2 to 0.01 cm^2 from day 3 to day 15. Finally, for the group 6, we noted a decrease from 3.86 cm^2 on day 3 to 0.11 cm^2 on day 15. Based on these results, the best cream which controlled the regression of the wounds was found to be the cream with shea butter added. Fig. 2C shows the appearance of wounds of infected and uninfected rats at day 15.

Discussions

The essential oil of thyme exhibited a good inhibitory ability against all three strains of *Candida* tested with a MIC of 310 μgmL^{-1} against *C. albicans*. These results are similar to those obtained by Aligiannis *et al.*, [15] and by Duarte *et al.*, [16]. According to their findings only essential oils with MICs lower than 0.5 $mgmL^{-1}$ can be considered as powerful antifungal agents. This good antifungal activity can be explained by the chemical composition of the essential oil of thyme, the major compounds of which are thymol (44.76%) and *p*-cymene (20.77%). A study conducted in France reported a higher antifungal activity of thyme EO with a MIC of 0.016 μgmL^{-1} against *C. albicans* ATCC 90,029 [7]. Another study in Iran even found a MIC value of 0.30 μgmL^{-1} against *C. albicans* ATCC 10,231 [8]. This concordance of results could be explained by the fact that the essential oils used in these different studies are from the thymol and carvacrol chemotypes -thymols and carvacrol being phenolic alcohols with strong antifungal effects. *Mentha piperita* showed a significant inhibitory effect against *C. albicans*

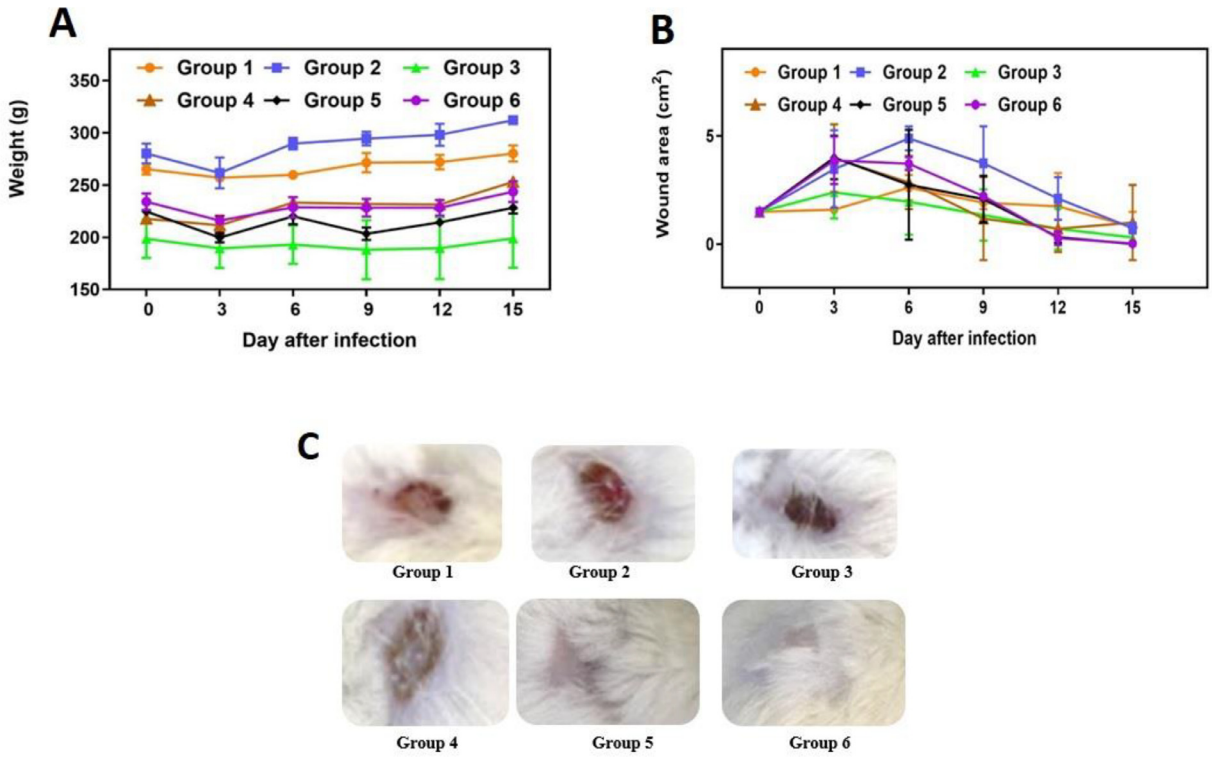

Fig. 2. A: Change in weight of the rats, B: Change in wound area of the rats and C: Wounds appearance on day 15. Group 1: rats injured but were not infected with *C. albicans* MHMR; Group 2: rats injured and infected with the fungus but without treatment; Group 3: rats injured, infected with *C. albicans* MHMR and treated with ketoconazole cream; Group 4: rats injured, infected with *C. albicans* MHMR and treated with the classic cetomacrogol cream with 5% EO; Group 5: rats injured, infected with *C. albicans* MHMR and treated with shea butter cream based with 5% EO; Group 6: rats injured, infected with *C. albicans* MHMR and treated with shea butter and palm oil cream with 5% EO. 5% EO (2.5% of mint and 2.5% of thyme).

MHMR with a MIC of 2500 μgmL^{-1}, this result is confirmed by previous studies which showed similar trends of MICs (3120 μgmL^{-1} [17] and 6250 μgmL^{-1} against *C. albicans* [10]). This antifungal activity of the essential oil of mint can be explained by the presence of menthol and menthone identified as major components in the respective proportions of 32.28% and 20.13%. Differences in the MIC values obtained by other authors can be attributed to the source of *C. albicans* strains, the conditions of extraction of EO which could influence the chemical composition, and the analytical technique used. The checkerboard antimicrobial analysis conducted to evaluate the effectiveness of the combination of mint and thyme essential oils showed an additive effect on *C. albicans* MHMR. We could therefore combine these oils to formulate antifungal cream.

All the hydrophilic creams formulated had a pH range of 5.0–5.5, corresponding to that of the skin. On the basis of pH, all formulated creams were therefore suitable for cutaneous use. No phase separation was observed after centrifugation. All the creams were stable whitish o/w emulsions with increasing viscosities: cetomacrogol < shea butter < shea butter and palm oil. The difference in viscosity of shea butter/palm oil formulation compared to conventional formulation could be explained by the chemical nature of the constituent excipients (vaseline or shea butter and paraffin or palm oil). Shea butter and palm oil had optimal stability and organoleptic characteristics compared to conventional bases.

On day 3, the wounds of the infected rats had a deep, gaping, suppurative, and reddish appearance, whereas those of uninfected rats had a superficial, non-suppurative appearance indicating *C. albicans* infection. This hypothesis was confirmed by rat weight and wound area. An extremely significant weight loss was recorded in all groups in varying proportions between 2.94% and 11.05%. This general weight loss which affected both infected and uninfected rats could be explained by experimental conditions that indisputably caused stress in the animals. According to Walker and Mason [18], a weight loss of more than 7.5% under this instance of disease induction, is indicative of a systemic disease. This could imply that the rats of groups 5 and 6 exposed to C. *albicans*, with respective weight losses of 11.11% and 7.65%, probably had systemic infection.

When we compared the evolution of the wounds of all groups (Fig. 2B), the group treated by the creams containing shea butter (5 and 6) displayed better healing of the wound by better cicatrisation with hair growth (Fig. 2C) compared to the group treated by the ketoconazole cream (group 3), where the wound was healed but there were no growth of hairs in the area. Compared to the untreated group (group 2), all the creams showed better healing of the wound with *in vivo*short period of epithelialization in treated rats. This activity could be due to the presence of the essential's oils in their composition.

In this study, the approach used made it possible to establish a link between the evolution of wound healing and the effectiveness of the formulated topical creams. Some authors explained the relevance of this approach by the fact that, the topical application of drugs is an efficient method of destroying microbial populations due to their increased availability at the infected wound site leading to enhanced wound healing [19]. As explained by Barku *et al.,* "Wound healing is a complex and dynamic process of restoring cellular structures and tissue layers in damaged tissue as closely as possible to its normal state". The essential oils of mint and thyme have anti-*candida* properties which contributed to good wound healing compared to untreated rats. It is known that the control of microbial infection is necessary for better wound healing and its management [20]. The slow rate of wound healing in the infected and untreated group (group 2) might be explained by the presence of *Candida* and its metabolites, which inhibit wound contraction and deteriorates the wound healing activity. This confirmed the observations made by Sasidharan *et al.,* [19]. Likewise, it has been shown there was delayed healing of wounds infected with *Candida* [21]. Therefore, to accelerate wound healing, the source of infection needs to be dealt with [22].

The effectiveness of the creams in groups 5 and 6 was a proof that the excipients used (shea butter and palm oil) had enabled good release of essential oils promoting wound healing in these groups. Our observations confirmed those made by Barku [23] who claimed that shea butter acting as a vehicle in their ointment preparation was able to potentiate the wound healing activity. Thioune *et al.,* also found that shea butter released aureomycin easily and at a faster rate than other excipients [24]. In addition, the inherent anti-inflammatory properties of shea butter were proven [25]. However, for better understanding of the effect of essential oil, the investigation of shea butter cream alone should be conducted.

Conclusion

Essential oils of mint and thyme have been found to possess an *in vitro* activity against *C. albicans*. Shea butter and palm oil proved to be good excipients in the formulation of hydrophilic antifungal creams based on essential oils of mint and thyme. Furthermore, *in vivo* tests showed an optimal recovery of Wistar rats infected with *C. albicans* MHMR after treatment with the formulated creams. This work opens a new platform for the management of cutaneous candidiasis.

Authors contribution

AZ and **AGA contribute**d equally to the work, by conducting all the experiments. **HG, SM** and **UCK** contributed for the phytochemical analysis. **HS** contributed for the microbiology and the formulation parts. **AGA** and **RFL** designed the animal study. **BE, JQL** and **FAG** were the promotor of this study. All the authors contributed for the editing of the manuscript.

Declaration of Competing Interest

All authors have participated in (a) conception and design, or analysis and interpretation of the data; (b) drafting the article or revising it critically for important intellectual content; and (c) approval of the final version. This manuscript has not been submitted to, nor is under review at, another journal or other publishing venue. The authors have no affiliation with any organization with a direct or indirect financial interest in the subject matter discussed in the manuscript

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