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Title:

In vitro evaluation of antifungal activity of virgin coconut oil and white palm kernel oil on *Candida* species-Experimental study

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ABSTRACT

Aims: The aim of this study is to evaluate *in vitro* the antifungal activity of virgin coconut oil and the white palm kernel oil on the growth of six species of *the genus Candida.*

Study design: This study is an experimental study

Place and duration of the study: Department of Microbiology, Laboratory of Microbiology of the University of Yaoundé I. Laboratory of Bacteriology, Yaoundé University teaching Hospital between April to September 2017.

Methodology: We included six species of *Candida*. The method of diffusion discs in agar medium was used for Sensitivity tests and macro-dilution in liquid medium was used for dilution.

Results: Majority of the species tested were resistant to conventional antifungals used. *Candida hoemulonii* was the most sensitive species to virgin coconut oil with percentages of inhibitions higher than 50 % when the concentration of oil was only 3.125 mg/mL and a maximum inhibition percentage of 90.10 % when the concentration of oil was 100 mg/mL. *Candida albicans,* was the least susceptible species to virgin coconut oil with a maximum inhibition percentage of 59.85 % when the concentration of oil was 100 mg/mL. *Candida lipolytica* was the most sensitive specie to white palm kernel oil with a maximum inhibition percentage of 90,26 % when the concentration of oil was 100 mg/mL and *Candida parapsilosis* was the least susceptible species to white palm kernel oil with a maximum inhibition percentage of 52,69 % at the same concentration. In addition, the white palm kernel oil which was more active ($P<0.05$) than virgin coconut oil was introduced in the Sabouraud broth and the MIC and MFC obtained with Fluconazole was lower on *Candida albicans* and *Candida parapsilosis* compared to activity of Fluconazole without palm kernel oil in the medium. Nystatin showed also lower MIC and MFC values on *Candida parapsilosis* when palm kernel oil was present in the culture medium.

Conclusion: These results prove that these oils can be used to develop antifungals drugs.

Key words: candidiasis, vegetable oils, combinations, antifungal activity, species**.**

Abbreviations

PIg: growth inhibition percentages (%);

SEM : Standard error of the mean

R: Resistant; I: Intermediate; S: Sensitive

Ne_{48h}: Number of cells obtained from experimental fungal cultures after 48 hours;

Ne_{0h}: Number of initial cells of experimental fungal cultures;

Nc_{48h}: Number of cells obtained from the fungal culture control after 48 hours.

Nc_{0h}: Number of initial cells of the control fungal culture

Introduction

During the past decade, the incidence of opportunistic fungal infections has steadily increased. Among these diseases candidiasis constitute real public health problems in the world, this more especially as the prevalence of both superficial and deep candidiasis has not ceased increasing in recent years [1]. During their life 70 to 75% of healthy women have at least one episode of vulvovaginal candidiasis [2]. The situation is still very worrying in Cameroon among HIV-positive patients, who have 28.3% gastrointestinal candidiasis, 39.7% oesophageal candidiasis and 79.6% oral candidiasis [3]. The management of candidiasis is based on the use of antifungals. However, their use is not without side effects. Moreover, the emergence of new pathogenic strains and the increase in resistance to these antifungals increases the risk of infection and predisposes patients to life-threatening relapses.

It is therefore necessary to research and develop new therapeutic approaches that can be used as an alternative to conventional therapies. One strategy is the use of white palm kernel oil and virgin coconut oil used in traditional medicine and potential sources of bioactive compounds (lauric acid, caprylic acid, capric acid). The objective of this work is to evaluate the antifungal activity of virgin coconut oil and white palm kernel oil on *Candida* species.

Material and methods

Microorganisms

Candida parapsilosis, *Candida hoemulonii*, *Candida tropicalis*, *Candida lipolytica* are clinical isolates from the Centre Pasteur of Yaoundé. *Candida albicans ATCC* 37037 and *Candida krusei* (clinical isolate) were obtained from the Laboratory of Microbiology of the University of Yaoundé I.

Plants

Palm kernel were collected at Melong in Ouest region of Cameroon and coconut were collected at Kribi in Sud region of Cameroon. They were identified at the national herbarium of Cameroon.

Preparation of vegetable oils

Extraction of virgin coconut oil

The endocarp of the nut was first removed, then the fresh coconut was grated and pressed. The resulting coconut milk was left at rest during 48 hours in a sterile and closed container. After this time, the liquid and solid parts were separated. The oil found on the surface was then extracted and stored in a dark and sterile bottle at 4^0 C until use.

Extraction of white palm kernel oil

The palm kernel nuts were previously sun dried for 72 hours. The palm kernels were then introduced into a machine which exerts a mechanical pressure on these nuts until obtaining the crushed product which is conveyed into a filter system. After filtration the oil was collected in a sterile bottle and stored at 4^0 C until use. For each oil, the yield was determined.

Sensitivity tests of Candida species to antifungals

Sensitivity tests was carried out on the basis of direct examination with the presence of fungic shapes under microscopy and colonies in the culture medium. The following six antifungal disks were used: Fluconazole 25, Nystatin 100, Cotrimazole 10, Amphotericin B 100, Ketoconazole 50, 5-Fluorocytosine 1. The method of diffusion discs in agar medium was performed according to the recommendations of the Antibiogram Committee of the French Society for Microbiology [4].

The standardized inoculum was prepared from a pure culture of 24 at 48 hours of yeast obtained on Sabouraud + Chloramphenicol medium. Turbidity was adjusted to 3 McFarland by addition of physiological saline. After having streaked the Sabouraud + Chloramphenicol medium by swabbing, the preparation was incubated at 37° C. The reading was performed after 24 hours of incubation for *Candida albicans*, *Candida krusei*, *Candida tropicalis* and 48 hours later for *Candida hoemulonii*, *Candida lipolytica* and *Candida parapsilosis*.

Macrodilution

The effects of each vegetable oil and antifungal were evaluated on the growth of *Candida* species by the method of macrodilution according to the protocol of Clinical Laboratory Standard Institute [5] adapted to this work with some modifications.

Effect of white palm kernel and virgin coconut oils

To determine growth inhibition percentages, yeast cultures were harvested and then suspended in sterile saline (0.8% NaCl) and the cell density was adjusted to 2.10^5 cells/mL. Yeasts were allowed to grow in 1 mL of Sabouraud broth (supplemented with 1% tween 80) at different concentrations of each vegetable oil. The doubling dilution technique was used for vegetables oil. This led to a decreasing concentration range of the vegetable oils varying from 100 mg / mL to 1.56 mg / mL. In parallel, a growth control tube and a sterility control tube of the medium were made. All tubes were incubated at 37^0 C for 48 hours. After incubation each tube was homogenized, and the number of yeasts was determined by counting using the Malassez cell. The tests were repeated 3 times. The growth inhibition percentages (PIg) were determined according to the formula below:

$$PIg\ (\%) = 100 - \frac{Ne_{48h} - Ne_{0h}}{Nc_{48h} - Nc_{0h}} \times 100$$

PIg: growth inhibition percentages (%);

Ne_{48h}: Number of cells obtained from experimental fungal cultures after 48 hours;

Ne_{0h}: Number of initial cells of experimental fungal cultures;

Nc_{48h}: Number of cells obtained from the fungal culture control after 48 hours.

Nc_{0h}: Number of initial cells of the control fungal culture.

Effect of Nystatin and Fluconazole on Candida Growth

For MIC (Minimal inhibitory concentration) determination, yeast of *Candida* species at 2.10^5cells/mL were allowed to grow in 1 mL of Sabouraud broth at different concentration of each antifungal. The stock solutions of these antifungals were prepared at 20 mg/mL followed by a geometric progression of 2 until the 7th dilution. In parallel, the yeast growth control and the sterility control of the medium were made. All tubes were incubated at 37^0 C for 48 hours.

MFC (Minimal fungicidal concentration) were determined by inoculating 10µL of culture medium at greater than or equal to the MICs on to Sabouraud dextrose agar and incubated for 48h. The tests were repeated three times.

Effects of Nystatin and Fluconazole with the presence of palm kernel oil in the culture medium

The effects of Nystatin and Fluconazole were evaluated with the presence of palm kernel oil on *Candida* growth to appreciate their activities compared to activities without palm kernel oil in the culture medium. To do this, palm kernel oil was introduced into Sabouraud broth (supplemented with 1% tween 80) medium for a final concentration equal to IC_{50} in each tube. Successives dilutions of geometric reason 2 were carried out with each antifungal in Sabouraud + palm kernel oil. The 7 experimental tubes and the growth control tube were inoculated with 1 ml of the inoculum at 2.10^5 cells / ml. This led to a decreasing range of antifungal concentration ranging from 5mg / mL to 0.078mg / mL. All tubes were incubated at 37^0 C for 48 hours. The MIC was determined for each antifungal. The tubes of concentrations greater than or equal to the MICs were seeded in Sabouraud + chloramphenicol agar with a volume of 10 µL to determine the MFC. The tests were repeated 3 times.

Statistical analysis

Statistical analysis and graphs were performed with the GraphPad software, version 5.03. Mean ±SEM values were indicated on the graphs. The student test was used to compare the activities of virgin coconut oil and white palm kernel oil on all *Candida* species studied.

Results and Discussion

Extraction yield

Extraction of the virgin coconut oil gave a mass of 239.09 g, a yield of 23.90% per 1000 g of coconut. As for white palm kernel oil, for 1000 g of palm kernel nut we obtained a mass of 233.8g, a yield of 23.38%. These two fruits belong to the same family and have similar compounds. This could justify this similarity of yields.

Sensitivity profile of Candida species to antifungals

The observation of the clinical categorization in table 1 reveals several resistances of the different species studied to antifungals. However, Nystatin and Amphotericin B were the most effective antifungals with their activities observed in the majority of species except *Candida hoemulonii*, which proved more resistant. This activity is thought to be due to the mechanism of action of the polyenes (Nystatin, Amphotericin B), which act by altering the functioning of the cell membrane, resulting in death of the fungal cell [6]. These results corroborate those of [7] who showed that *Candida albicans, Candida tropicalis* and *Candida parapsilosis* are sensitive to Amphotericin B. 5-Fluorocytosine is the least active antifungal because the diameters of the zones of inhibition obtained on all species not exceeding 10 mm and fluconazole is the second weakest antifungal after 5-fluorocytosine because two species only, namely *Candida parapsilosis* and *Candida tropicalis* were sensitive to this azole with respective inhibition diameters of 30 mm and 20 mm.

Table I: Correspondence diameter and clinical categorization

Antifungals	*Candida* species	Diameters of the zones of inhibition measured (mm)	Critical diameters (mm) [9], [10],			Clinical categorization
			R	I	S	
Fluconazole	*Candida albicans ATTC 37037*	0	≤14		≥19	Resistant
	Candida parapsilosis	30				Sensitive
	Candida tropicalis	20				Sensitive
	Candida hoemulonii	0				Resistant
	Candida lipolytica	0				Resistant
	Candida krusei	12				Resistant
Nystatin	*Candida albicans ATTC 37037*	20	≤10		>10	Sensitive
	Candida parapsilosis	21				Sensitive
	Candida tropicalis	21				Sensitive
	Candida hoemulonii	10				Resistant
	Candida lipolytica	20				Sensitive
	Candida krusei	20				Sensitive
Amphotericin B	*Candida albicans ATTC 37037*	11	<10		>10	Sensitive
	Candida parapsilosis	11				Sensitive
	Candida tropicalis	12				Sensitive
	Candida hoemulonii	0				Resistant
	Candida lipolytica	12				Sensitive
	Candida krusei	11				Sensitive
5-Fluorocytosine	*Candida albicans ATTC 37037*	0	≤10		≥20	Resistant
	Candida parapsilosis	0				Resistant
	Candida tropicalis	10				Resistant
	Candida hoemulonii	0				Resistant
	Candida lipolytica	0				Resistant
	Candida krusei	0				Resistant
Kétoconazole	*Candida albicans ATTC 37037*	12	≤10		≥20	Intermédiate
	Candida parapsilosis	35				Sensitive
	Candida tropicalis	13				Intermédiate
	Candida hoemulonii	60				Sensitive
	Candida lipolytica	11				Intermédiate
	Candida krusei	22				Sensitive
Clotrimazole	*Candida albicans ATTC 37037*	7	≤10		≥20	Resistant
	Candida parapsilosis	30				Sensitive
	Candida tropicalis	25				Sensitive
	Candida hoemulonii	0				Resistant
	Candida lipolytica	0				Resistant
	Candida krusei	15				Intermédiate

R: Resistant I: Intermediate S: Sensitive

Effect of Palm kernel oil on the growth of Candida species

The antifungal activity of palm kernel and coconut oils was evaluated *in vitro* against the six *Candida* species by determining percent inhibition of fungal growth (PIg) after 48 hours of culture. The yeast count was carried out under an optical microscope on a Malassez cell. The results obtained are represented in graphs expressing the percentages of inhibition of the growth according to the concentration of each oil.

It appears from figure 1 that white palm kernel oil exerts antifungal activity on all six *Candida* species and this activity increases with the concentration of palm kernel oil. Organization of African Unity [11] proved that palm kernel oil has antimicrobial activities and is used in the treatment of dermatoses. This activity is due to the presence of bioactive compounds such as capric acid and lauric acid. Studies have evaluated the antifungal activities of capric acid and lauric acid (major components of palm kernel oil) *in vitro* on *Candida albicans* and have shown that these acids are fungicidal [12].

The inhibiting capacity of fungal growth is greater with concentrations ranging from 50 to 100 mg / mL in the majority of species except *Candida parapsilosis*, the least sensitive species that showed a maximum inhibition percentage of 52, 69% at the maximum concentration of palm kernel oil which is 100 mg / mL.

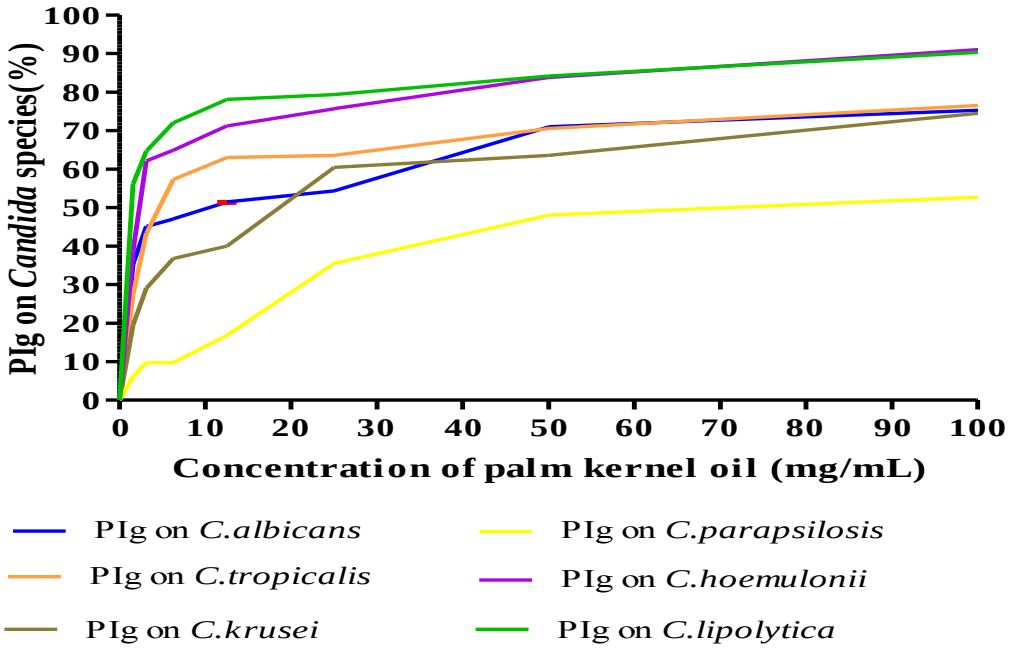

Figure 1: Percentages of inhibition of white palm kernel oil on the growth of *Candida* species.

Effect of virgin coconut oil on the growth of Candida species

Figure 2 shows the inhibition percentages of virgin coconut oil on the growth of *Candida* species. An evolution of inhibition percentages is observed on the species when the concentration of the virgin coconut oil increases. Inhibition percentages greater than 50% are observed from concentrations of 3.125 mg / mL of oil on *Candida hoemulonii*, 12.5 mg / mL of oil on *Candida lipolytica*, 25 mg / mL of oil on *Candida tropicalis*, 50 mg / mL of oil on *Candida krusei* and *Candida parapsilosis*. *Candida hoemulonii* appears to be the most sensitive species to virgin coconut oil with a maximum inhibition percentage of 90.10% at the concentration of 100 mg / mL. *Candida albicans* is the least sensitive species to virgin coconut oil with a maximum growth inhibition percentage of 59.85% at the maximum concentration of virgin coconut oil. The inhibitory power of coconut oil might be due to its composition of capric and lauric acid which exert antifungal activities by disintegrating the cell wall [13].

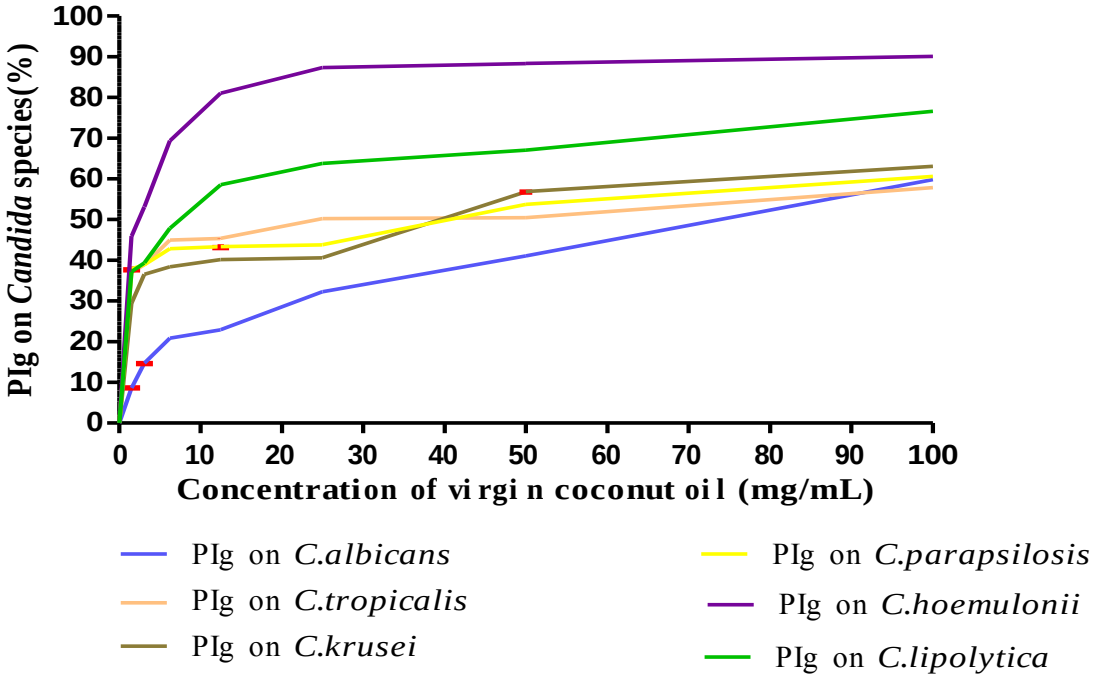

Figure 2: Percentages of inhibition of the virgin coconut oil on the growth of *Candida* species.

Minimal Inhibitory Concentration (MIC) and Minimal Fungicidal Concentration (MFC) of Nystatin and Fluconazole

The antifungal susceptibility was determined with a presence and absence of palm kernel oil in the culture medium by using the macrodilution technique. This consisted to determining the MIC and MFC. The results summarised in table 2 shows a strong fungicidal effect of Nystatin in the absence of palm kernel oil, with MICs ranging from 0.078 mg / mL to

0.625 mg / mL apart from *Candida parapsilosis*, where the antifungal activity was fungistatic. Fluconazole exerted fungistatic effect in the absence of palm kernel oil with MICs ranging from 0.07 mg / mL to 1.25 mg / mL. This was predictable on *Candida krusei* because this species has an intrinsic resistance to Fluconazole [14]. This further confirms the observation that Fluconazole appears as a weakly active antifungal on *Candida species*. The activity of Fluconazole is limited [8].

Regarding the outcome in the presence of palm kernel oil in the culture medium, The CMI of Fluconazole are 4 times weaker on *Candida tropicalis* and *Candida albicans* compared to the CMI obtained without palm kernel oil in the culture medium. Moreover on *Candida parapsilosis* the CMI decreased with a value lower than 0,078 mg/mL. In light of the problems posed by the use of dispersants [15], we chose tween as an emulsifier because it is used in several studies. Tested in our study at a concentration of 1%, it proved to be devoid of any detrimental effect on fungal growth.

Table II: Minimal inhibitory concentrations, Minimum fungicidal concentrations of Nystatin and Fluconazole with presence / absence of palm kernel oil in the medium.

Candida species	Fluconazole (mg/mL)		Nystatin (mg/mL)	
	Absence of palm kernel oil MIC/MFC	Presence of palm kernel oil MIC/MFC	Absence of palm kernel oil MIC/MFC	Presence of palm kernel oil MIC/MFC
Candida tropicalis	0,625 /5	0,156 /5	0,156 /0,312	0,156 /0,312
Candida parapsilosis	0,078 /1,25	<0,078 / < 0,078	0,625 /5	< 0,078 /< 0,078
Candida krusei	0,312 /2,5	0,312 /2,5	0,078 /0,156	0,078 /0,156
Candida albicans	0,625 /5	0,156 /2,5	0,156 /0,312	0,156 /0,312
Candida Hoemumonii	0,625 /5		0,625 /1,25	
Candida lipolytica	1,25 /5		0,625/1,25	

MIC, minimal inhibitory concentration; MFC, minimal Fungicidal concentration; (*), not evaluated.

Statistical analysis

ANOVA analysis was performed to compare the activities of the two oils. The student test was used with $\alpha = 0.05$.

Table III: Comparison of the activities of white palm kernel and virgin coconut oils on *Candida albicans*

Indicator	Groups	Means	t test	P value	< 0,05 = *
Maximum inhibition percentage	Palm kernel oil	75,28	3,493	0,025	*
	Virgin coconut oil	59,85			

Table IV: Comparison of the activities of white palm kernel and virgin coconut oils on *Candida lipolytica*

Indicator	Groups	Means	t test	P value	< 0,05 = *
Maximum inhibition percentage	Palm kernel oil	90,26			
	Virgin coconut oil	76,60	2,229	0,089	

Table V: Comparison of the activities of white palm kernel and virgin coconut oils on *Candida hoemulonii*

Indicator	Groups	Means	t test	P value	< 0,05 = *
Maximum inhibition percentage	Palm kernel oil	90,99			
	Virgin coconut oil	90,10	0,760	0,489	

Table VI: Comparison of the activities of white palm kernel and virgin coconut oils on *Candida krusei*

Indicator	Groups	Means	t test	P value	< 0,05 = *
Maximum inhibition percentage	Palm kernel oil	74,44			
	Virgin coconut oil	63,10	6,544	0,002	*

Table VII: Comparison of the activities of white palm kernel and virgin coconut oils on *Candida parapsilosis*

Indicator	Groups	Means	t test	P value	< 0,05 = *
Maximum inhibition percentage	Palm kernel oil	52,69			
	Virgin coconut oil	60,59	1,355	0,246	

Table VIII: Comparison of the activities of white palm kernel and virgin coconut oils on *Candida tropicalis*

Indicator	Groups	Means	t test	P value	< 0,05 = *
Maximum inhibition percentage	Palm kernel oil	76,50			
			1,107	0,330	
	Virgin coconut oil	57,87			

Conclusion

Vegetable oils used in this work have displayed the capacity to inhibit fungal growth of six *candida* species. Among the vegetable oils tested, palm kernel oil appears to be more active compared to virgin coconut oil. Furthermore, its presence in the culture medium improves the antifungal effect of Fluconazole and Nystatin. Nevertheless, further studies must be conducted on others yeasts.

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Competing interests

The authors declare that they have no competing interests.

Authors' contributions

Hortense Gonsu Kamga., Sara Riwom Essama. and Yves Le Grand Napa Tchuedji designed the study, performed the statistical analysis, wrote the protocol, and wrote the first draft of the manuscript. Maurice Boda, Stève Voundi Olugu and Patrick Betote Diboue acquired, analyzed, interpreted data. Francis Ekwin, Emilia Lyonga Mbamyah, William Abange Baiye, Anicette Chafa Betbeui managed the literature searches and revised article critically. Francois-Xavier Etoa revised article critically. All authors have seen and approved the final version of the manuscript.

Ethics approval and consent to participate

Not applicable to this work.

Consent for publication

Not applicable to this work.

References

1. Sardi L, Scorzoni T, Bernardi AM, Fusco A, Mendes MJSG. *Candida* species: current epidemiology, pathogenicity, biofilm formation, natural antifungal products and new therapeutic options. *Journal of Medical Microbiology.* 2013;62,10–24.

2. Kechia FA, Dohbit JS, Kouotou EA, Iwewe SY, Dzoyem JP, Mbopuwouo NM, Monamele CG, Moyou SR. Profil épidémiologique et étiologique de la candidose vulvo-vaginale chez la femme enceinte à Yaoundé (Cameroun). *Health Sci. Dis*. 2015;16 (4): 1-6.

3. Yongabi KA, Mbacham WF, Nubia SKK, Singh RM. Yeast strains isolated from HIV-seropositive patients in Cameroon and their sensitivity to extracts of eight medicinal plants. African Journal of *Microbiology Research*. 2009;3(4):133-136.

4. CA-SFM. Recommandations. 2019; V 1.0.1-14.

5. (CLSI) Clinical and Laboratory Standards Institute. Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically. *Approved standard.* 2012; 32, 1 – 68.

6. Aderiye, Babatunde I, Oluwole and Olusola A. Disruption of fungi cell membranes by polyenes, azoles, allylamines, amino acids and peptides. *Academia Journal of Microbiology Research.* 2015;3(2): 022-030.

7. Dhraief S, Trabelsi S, Sellem M, Bouchekoua M, Bouhlel S, Aloui D and Khaled S. Sensibilité de levures aux antifongiques : évaluation des souches isolées de prélèvements

profonds. *Journal de mycologie médicale*. 2016;26:1-25. 8.

8.Gonsu KH, Kechia AFA, Tegankam D, Toukam M, Moyou SR. Profil de sensibilité aux antifongiques des *Candida* spp. isolés dans les candidoses digestives chez les sujets séropositifs au VIH à Yaoundé-Cameroun. Health Sci. Dis. 2014;15 (3): 6p.

9. Koudougou K. Etude de la chimie et de l'activité anti-mycosique des extraits de ***Biophytum petersianum*** Klotzsch (Oxalidaceae). Université de Ouagadougou. 2000;53:6-26.

10. Mallié M. Surveillance des traitements antifongiques: les antifongigrammes et les dosages d'antifongiques sont-ils utiles? 2010. 1-9.

11. Mboui OSE. Huile de palmiste traditionnelle. 2003. 63:1-74.

12. Thormar H, Bergsson G, Gunnarsson E, Georgsson G, Witvrouw M, Steingrimsson O, DeClercq E, Kristmundsdottir T. Hydrogels containing monocaprin have potent microbicidal activities against sexually transmitted viruses and bacteria *in vitro*. Sex *Transm Infect*. 1999.75:181–185.

13. Bergsson G, Arnfinnsson J, Steingrimsson O, Thormar H. *In vitro* killing of *Candida albicans* by fatty acids and monoglycerols. *Antimicrob Agents Chemother*. 2001;45:3209–3212.

14. Sheehan DJ, Hitchcock CA., Sibley CM. Current and emerging azole antifungal agents. *Clin Microbiol Rev*. 1999;12:40–79.

15. Remmal A, Tantaoui-El Araki A, Bouchikhi T. Improved method for the determination of antimicrobial activity of essential oil in Agar medium. *J Essenti Oils Res.* 1993;5: 179-184.