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OPTIMIZED COMBINATIONS OF OCIMUM ESSENTIAL OILS INHIBIT GROWTH OF FOUR *Candida albicans*

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Abstract- Drug combinations against candidiasis and other fungal infections have been considered as alternatives for mono-therapy. However, there are no data available on the combination of essential oils from Ocimum genus.The aim of this work is the optimization of essential oils derived from Cameroon-grown Ocimum against four *Candida albicans*.

The essential oils were extracted by hydrodistillation. The anti-candidal activities were assessed using broth dilution technique and the combinatorial analysis was done using central composite design, the latter being maximised by a multiple response optimization approach. The optimums were tested for their efficiency using the time kill kinetic approach.

Linalool (53.34%), 1,8 Cineol (55.32%), γ-Terpinene (24.54%), Eugenol (26.01%) were the major componentsfor *Ocimum basilicum, Ocimum canum, Ocimum gratissimum* and *Ocimum urticaefolium* respectively. The most active oil was *Ocimum gratissimum* followed by *Ocimum basilicum* and *Ocimum urticaefolium,* the least active being *Ocimum canum*. The optimum values were 0.62/0.14mg/ml with desirability of 96%for the combination of *Ocimum basilicum/Ocimum gratissimum* and 0.58/0.14mg/ml with the desirability of 98% for *Ocimum urticaefolium/ Ocimum gratissimum.* The optimum values of 0.56/0.63mg/ml and the desirability of 100%for the combination of *Ocimum urticaefolium/ Ocimum basilicum*. All these combinations inhibited the growth of the fungal strain for 20 hours.

Our research shows that the Ocimum essential oils have an antifungal activity and that this potential is increased when the essential oils are combined. More research is needed to extend this potential to other microbial strains .

Keywords- *Ocimum*, Essential oil, Chemical Composition, *Candida albicans*, Multiple Response Optimization

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Introduction

During the past three decades, a drastic rise in severe systemic fungal infections has been observed due to the increased size of the immune-compromised population, mainly resulting from organ transplantation, cancer treatment, and HIV infection [1]. The genus *Candida* is responsible for 64% of these infections [2], and the case fatality exceeds 40% despite the use of available antifungal drugs. Unfortunately, only a few classes of antifungal drugs are currently available, and these either lack potency, have little spectrum of activity, or are toxic to humans [3-5]. The emergence of antifungal resistance, exacerbated by the long-term usage of antifungal in high risk immunocompromised individuals [6], further complicates the treatment of fungal infections. It is essential to develop new drugs for which there would be little microbial resistance [4,7], low or no adverse effects [3] and large spectrum of activity. New antimicrobial agents will have to be developed as resistance to current antibiotics spreads [4]. However, the past record of rapid and widespread emergence of resistance towards newly introduced single antimicrobial molecules indicates that, even if new families of antimicrobial agents are discovered, they may have a short life expectancy [8].

These difficulties have driven recent efforts to determine the efficacy of combination therapy in the treatment and management of invasive infections. The response surface methodology was used recently in order to overcome the limitations of studies on combination antimicrobial agents *in vitro*. Here, the drug effect is measured by the proportion of growth with respect to a drug-free control and is related to any combination of drugs, showing a surface response when this relationship is plotted tri-dimensionally. The goodness of the fit of the model was checked by the determination coefficient (R^2). The closer the R^2 value is to 1, the model is stronger and the it predicts better the response [9]. In fact, this test is perform by comparing the variability of the current residuals to the variability between observations at the replicate setting of the factor and designs that perform well with respect to lack-of-fit detection also perform

reasonably well with respect to the bias, but the opposite is not necessarily true [10]. The essential oil from *Ocimum* genus were studied for their pharmacological properties and to enhance the efficacy of essential oils, the combined use of different oils were evaluated recently for potential synergistic effects [11]. In this regard, this paper aims to evaluate the antifungal activities of four Cameroon-grown *Ocimum* essential oils and their combinations.

Materials and Methods

Plant Material and Extraction

The leaves of *Ocimum gratissimum, Ocimum basilicum*, *Ocimum canum* and *Ocimum urticaefolium* were collected at Nkolodom II, Yaoundé and Bali, Bamenda on the 08 and 23 of August 2012 respectively.

The plants were identified at the National Herbarium of Cameroon (HNC) as *Ocimum gratissimum* L. 5817/SRF/Cam. *Ocimum basilicum L* 428782 HNC*, Ocimum canum L* 15866/SRF/Cam*, Ocimum urticaefolium* L. 49085 HNC*.*

Fungal Strains

The four yeast strains used were *Candida albicans* ATCC12C, *Candida albicans* ATCCL26, *Candida albicans* ATCC37037*and Candida albicans* ATCC37039.

Essential Oil Extraction and Characterization

Extraction of Essential Oils

The plant samples were hydro-distilled for 5 hours using a Clevenger-type apparatus. Essential oils obtained were dried over anhydrous sodium sulphate and stored at 4°C until use for further experiments. The extraction yields were calculated in percentage (w/w).

Chemical Analysis of the Essential Oils

The essential oils were analysed by gas chromatography (GC) and gas chromatography coupled with mass spectrometry (GC/MS). As describe earlier [12].

Minimal Inhibitory Concentration (MIC) Determination

The broth dilution method was performed as previously described [13] with slight modifications. The modifications were at the end point determination. Spectrophotometric reading of each wells were performed with a Biokit EL 800 automated plate reader set at 490 nm after the well had been agitated. The Minimal Inhibitory Concentration (MIC) were determined as the first concentration of the antifungal agent at which turbidity in the well was 90% less than that in control wells [14].

Combinatorial Analysis

The response surfaces methodology (RSM), which is a group of mathematical and statistical techniques used in the development of an adequate functional relationship between a response of interest and a number of associated variables, were used for the combinatorial analysis. Central composite design plus star were used to fit second order models in order to optimize the response of yeasts to essential oil combinations. In the aim of having a general view of the response of all the tested yeasts on the essential oil combination, the multiple response optimizations were used [15].

The RSM was used to estimate the effect of essential oils, considered here as independent variable, on the growth of each yeasts estimate as percentage of inhibition. Central composite design with replication at the center point was employed to design the experimental data.

The following second order binomial model was applied.

$$\% I = \gamma_0 + \alpha A + \beta B + (\alpha A)^2 + (\beta B)^2 + \alpha\beta AB + \varepsilon$$

Where %I is the percentage of inhibition obtained from each pair of drug combination; γ_0= constant, α and β are coefficients defining the magnitude of individual and interactive effects of the drugs;

A and *B* are the concentration of different essential oils involved in the combination.

The goodness of the model was checked with a variety of diagnostic tests, such as R^2, adjusted R^2, the lack of fit test, analysis of variance, residual and average plot analysis, diagonalization of parameter matrices and standard error of parameters.

The optimal inhibitory concentrations were maximized over the studied region by the Multiple Response Optimization (MRO) process using desirability function with the expression of Derringer and Suich [16] as recently described [15] using the following equation:

$$D = \left(d_1^{w1} x d_2^{w2} x \ldots\ldots\ldots x d_i^{wi}\right)^{1/\sum wi}$$

Where D is the overall desirability function to be maximized, *di* is the individual desirability function of each response function and *w* is the number of responses.

Statistical Analysis

All data were analyzed using STATGRAPHICS 5.0 (for Windows) and $p \leq 0.05$ were considered as significant.

Results

Essential Oil Extraction

The essential oil was obtained with yield from 0.038% for *Ocimum basilicum* to 0.349% for *Ocimum canum,* while the density yields were similar [Table-1].

Table 1- Physical characteristics of the *Ocimum* essential oils.

Essential oil	Color	Yield (%w/w)	Density
Ocimum basilicum	Colorless	0.039	0.89
Ocimum canum	Yellowish	0.349	0.91
Ocimum gratissimum	Colorless	0.203	0.88
Ocimum urticaefolium	Yellowish	0.222	0.96

Cameroon-grown *Ocimum canum* the yield and chémotype obtained follows the straight line with that obtained previously [17-19]. The yield of extraction of essential oil from fresh leaves of *Ocimum basilicum* was 0.039%. This yield is very low compared to that obtained elsewhere [17] and could be greater with drying; Ndoye [17] obtained from semi dry leaves of this plant an essential oil with yield of 0.2%, and up to 1.25% when the material is completely dry and ground [20]. Regarding *Ocimum urticaefolium*, this is the first report on the essential oil extraction, chemical composition as well as antimicrobial activity.The yield of extraction is 0.203%, which is within the range usually obtained from essential oil extraction from the Ocimum genus [17,19,21,22]. The extraction of fresh leaves of *Ocimum gratissimum* yielded 0.222% of essential oil. This yield is within the range of yield obtained previously from Cameroon-grown *Ocimum gratissimum* by several authors [17,18,23].

Chemical Analysis of the Essential Oil

Essential oil from *Ocimum basilicum* revealed 38 compounds Linal-

ool (53.34%) and dihydro-eugenol (21.79%) were the major compounds. The chemotype obtain here is the same as previously obtained from Cameroon-grown *Ocimum basilicum* [17] Essential oil from *Ocimum canum* contained30 compounds which were all identified. 1,8-cineol (55.32%) and Caryophyllene oxide (11.01%) were the major compounds of this essential oil. This chemotype has also been described in other Cameroonian *Ocimum canum* [17]. γ-terpinène (24.55%), Thymol (22.61%), 2.3 dihydrofarnesol (14.18%) were the major compound from the 33 compounds obtained with the essential oil of *Ocimum gratissimum*. This chemotype is the same as previously described [17,22]. The essential oil of *Ocimum urticaefolium* contained 26 compounds. The major compounds of *Ocimum urticaefolium* oil were Eugenol (26.02%), Farnesal (17.1%),Elemol (14.28%) and α-cadinol (11.30%). It can be classified as Eugenol/Farnesal/Elemol chemotype. This the first chemotype described for this essential oil [Table-2].

Table 2- Chemical Composition of essential oil from four Cameroon-grown Ocimum

KI	Compounds	*O. basilicum*	*O. canum*	*O. gratissimum*	*O. urticaefolium*
921	α thujene	-	0,1543	0,64	-
940	α pinène	0,27	2,53	0,95	-
969	α-fenchene	-	-	0,55	-
974	sabinene	-	-	0,32	-
980	β-pinene	0,26	4,53	3,04	-
982	transmeta menthene-2,8-diene	0,17	-	-	-
983	Myrcene	-	0,68	-	-
984	transisolimonène	0,6	-	-	-
995	déshydrotranslinalool oxyde	0,91	-	-	-
1000	δ-2-carene	0,04	-	0,34	-
1012	α-Phellandrene	-	0,32	0,16	-
1013	1,4 cineol	-	-	3,31	-
1021	α terpinene	-	-	0,52	-
1025	p-cymene	-	-	0,86	-
1030	Limonene	-	-	0,71	-
1031	**1,8 cineole**	0,32	**55,32**	-	5,33
1038	(Z) -β-Ocimène	7,08	-	-	-
1041	(Z) -β-Ocimène	-	-	0,20	0,28
1051	(E) -β-Ocimène	0,72	-	-	-
1053	(E) -β-Ocimène	-	0,54	-	-
1062	**γ-Terpinene**	-	0,47	**24,55**	26,02
1063	trans-4-thujanol	-	-	0,13	-
1083	m cymenene	-	0,18	0,79	-
1086	fenchone	-	0,27	-	-
1093	p cymenene	0,17	0,33	-	-
1093	linalol	-	-	0,15	-
1111	**Linalol**	**53,34**	-	-	-
1115	transThujone	0,08	-	-	-
1152	menthone	0,59	-	-	-
1163	β-terpineol	-	0,71	-	-
1163	1,3,8-p-menthatriene	-		0,12	-
1173	menthol	0,21	1,19	-	-
1174	trans-thujone	-	-	1,00	-
1184	p-cymen-8-ol	0,24	-	-	-
1188	α-terpineol		3,12	-	-
1197	γ-terpineol	0,99	-	-	-
1214	iso dihydrocarveol	-	0,18	-	-
1259	2-methyphenol	0,31	-	-	-
1293	**Thymol**	0,26	-	**22,61**	-
1296	carvacrol	-	-	0,27	-
1352	thymol acetate	-	0,14	-	-
1371	**DihydroEugenol**	**21,79**	0,47	-	-
1376	α copaene	-	-	0,38	0,97
1387	β-cubebene	-	-	-	0,24
1390	β-Elemene	-	-	-	0,48
1402	z-Caryophyllene	0,22	-	-	-
1412	E-caryophyllene	0,14	-	-	-
1420	β-copaene	-	0,47	-	-
1422	β-caryophyllene	-	-	2,60	-
1423	4,8 epoxy β-caryophyllane	-	-	-	5,02
1433	β-Gurjunene	-	0,83	-	-
1447	α-Humulene	4,42		-	-
1450	E-β-Farnesene	0,1		-	-
1451	β-(E)-farnesene	-	1,35	-	0,66
1456	(Z)-β-farnesene	-	-	0,23	0,40
1467	γ-Gurjunene	0,14	-	-	-
1476	γ-curcumène	0,11	-	-	-

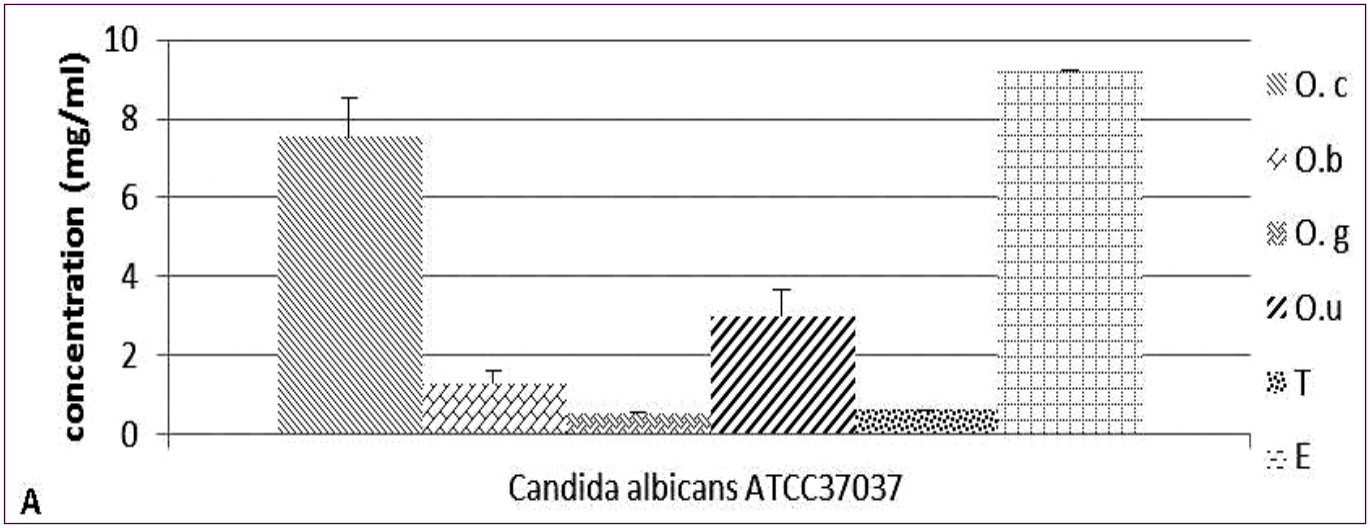
Table 2- Continues

KI	Compounds	*O. basilicum*	*O. canum*	*O. gratissimum*	*O. urticaefolium*
1477	γ-gurjunene	-	-	-	0,22
1481	Germacrene D	-	0,58	0,16	4,42
1488	α selimène	-	-	0,84	-
1495	α-Muurolene	1,44	-	-	-
1496	α-curcumene	-	-	0,29	-
1498	α-selimène	-	-	-	0,75
1503	α-Muurolene	-	0,14	-	-
1509	**germacrene A**	-	-	-	**17,10**
1511	γ-Z-Bisabolene	0,31	-	-	-
1515	β-curcumene	-	-	-	0,22
1519	δ-cadinene	0,3	0,51	-	-
1522	δ-cadinene	-	-	0,21	-
1523	δ-cadinene	-	-	-	0,78
1528	α-cadinene	1,14	-	-	-
1530	γ-(E)-bisabolene	-	-	-	0,12
1538	δ-cuprene	0,32	2,49	-	-
1556	**trans dauca-4(11),7 diene**	-		-	**14,28**
1564		-	0,97	-	-
1584	**caryophylleneoxide**	-	**11,01**	-	0,19
1588	β-cis-elemone	-	-	-	0,50
1593	viridiflorol	-	5,46	-	-
1616	8-hydroxy-linalool	-	3,67	-	-
1630	γ-Eudesmol	0,2	0,20	-	-
1643	cubenol	-	-	-	0,15
1656	α-cadinol	-	-	-	0,28
1666	14-hydroxy-(Z)-Caryophyllene	-	-	1,14	1,06
1666	dihydroEudesmol	1,57	-	-	-
1686	**2,3-dihydrofarnesol**	-	-	**14,18**	**11,31**
1695	(2Z,6Z)farnesol	-	-	-	5,43
1696	β-farnesol	-	-	6,39	-
1698	(2E,6Z) farnesal	0,73	-	-	-
1713	(2E,6Z) farnesal	-	-	0,12	-
1718	(2E,6Z) farnesol	-	-	4,31	3,67
1735	(2E,6Z) farnesol	0,04	-	-	-
1738	(2E,6E)3,7,11-trimethyl-2,6,10-dodecatrienal	-	-	0,19	0,12
1859	(Z-Z)Farnesylacetone	0,17	-	-	-
2200	Docosane	0,18	-	-	-

KI= Kovats Index; O. basilicum= Ocimum basilicum; O. canum= Ocimum canum; O. gratissimum= Ocimum gratissimum; O. urticaefolium= Ocimum urticaefolium

Determination of the Ocimum Essential Minimal Inhibitory Concentrationon Four *Candida albicans*

The MIC of the essential oil from *Ocimum canum* essential oil was up to 9.6mg/ml on *Candida albicans* ATCCL26 [Fig-1C] and the best MIC obtained was 5.31 ± 0.18mg/ml [Fig-1D] on *Candida albicans* ATCC12C. The activity of this essential oil was not significantly different from that of 1,8-cineol (Eucalyptol)($p \leq 0.05$) but this activity could not be linked to the presence of 1.8cineol as this compound represented 55.32% of the overall essential oil composition and the essential oil did not exert any inhibitory potential on *Candida albicans* ATCCL26 [Fig-1C]. This poor inhibitory potential has been described elsewhere [24].



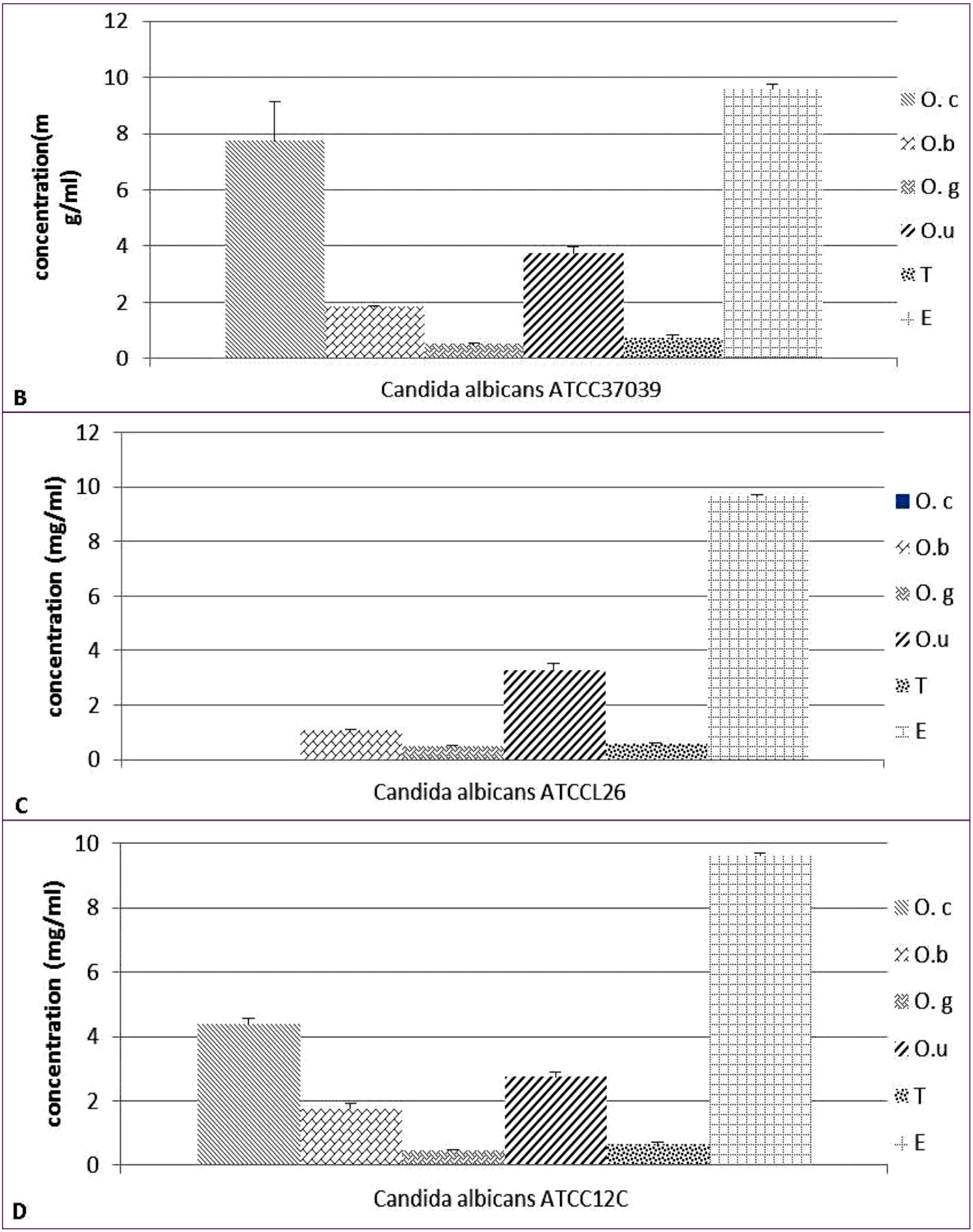


Fig. 1- Minimal Inhibitory Concentration of Ocimum essential oils *on Candida albicans ATCC37037* **(A)**, *Candida albicans ATCC37039* **(B)**, *Candida albicans ATCCL26* **(C)**, *Candida albicans ATCC12C* **(D).**

O. C=essential oil from Ocimu mcanum; O. b= essential oil from Ocimum basilicum; O. g= essential oil from Ocimum gratissimum; T= thymol; E= eucalyptol

Ocimum urticaefolium showed significant inhibitory potential on all the strains tested and was the third most active essential oil ($p \leq 0.05$). The most sensitive yeast to this essential oil was *Candida albicans* ATCC12C (MIC= 2.98±0.15mg/ml) [Fig-1D] and the most resistant strain was *Candida albicans* ATCC37039 (MIC = 4.06 ± 0.25mg/ml) [Fig-1B]. There is no data available in the literature regarding the antimicrobial potential of this Ocimum. The essential oil from *Ocimum basilicum* was the second most active oil on all strains ($p \leq 0.05$). The most sensitive strain was *Candida albicans* ATCCL26 (MIC = 1.37 ± 0.04µg/ml) [Fig-1C] and the most resistant was *Candida albicans* ATCC37039 (MIC = 2.31 ± 0.07mgl/ml) [Fig-1B] respectively on the essential oil from *Ocimum basilicum* [25,26]. The overall active essential oil was that from *Ocimum gratissimum* ($p \leq 0.05$). The most sensitive yeast was *Candida albicans* ATCC12C (MIC = 0.62 ± 0.018mg/ml) [Fig-1D] and the most resistant yeast was *Candida albicans* ATCCL26 (MIC = 0.67 ± 0.017mg/ml) [Fig-1C]. Thymol was also active on all the strains with its activity similar to that of *Ocimum gratissimum.*The essential oil from Brazilian eugenol rich oil from *Ocimum gratissimum* by Sartoratto, et al. [25] has revealed that the MIC was 2µl/ml, which is similar to that obtained in this study [Fig-1]. These observations have also been confirmed with 11 specimens of *Ocimum gratissimum* collected from Kenyan regions by Matasyoh, et al. [22].

From the following statistical classification, *Ocimum basilicum*, *Ocimum gratissimum* and *Ocimum urticaefolium* were selected for the combinatorial studies.

Combinatorial Analysis

The results of each combination and the desirability estimate of the optimization are in [Tables-3], [Tables-4] & [Tables-5] and [Fig-2], [Fig-3] & [Fig-4].

Table 3- Combination parameters of *Ocimum basilicum* and *Ocimum gratissimum* on yeasts growth inhibition.

Strains	Optimization					Multiple Response Optimization		
							Optimums (mg/ml)	
	R^2	R^2adj	P (LFT)	P (Durbin-Watson)	Regression equation	Desirability%	*O. b*	*O. g*
Candida albicans ATCC37037	97.68	94.78	0.52	0.1713	47.67 + 15.83*Ob+ 13.13*Og+ 4.73*Ob^2 + 3.50*Ob*Og +5.91*Og^2.	96,35	0.629	0.147
Candida albicans ATCC37039	95,77	90,49	0.07	0,0013	61,65 + 16,4*Oba + 0,85*Og +1,58*Ob^2 + 1,44*Ob*Og + 3,98269*Oga^2			
Candida albicans ATCCL26	96,83	92,86	0.37	0,2097	52,84 + 15,56*Oba + 2,86*Og +1,53*Ob^2 - 6,83*Ob*Og + 4,28*Og^2			
Candida albicans ATCC12C	96,89	93,01	0.14	0,0644	52,94 + 17,64*Oba + 11,38*Oga -2,16*Ob^2 + 3,28*Ob*Og + 6,76*Og^2			

R^2adj= R square adjusted, LFT= lack of fit test, Ob= Ocimum basilicum, Og= Ocimum gratissimum. a= significantly equal to 0.

Table 4- combination parameters of *Ocimum urticaefolium* and *Ocimum.gratissimum* on yeasts growth inhibition.

Strains	Optimization					Multiple Response Optimization		
							Optimums (mg/ml)	
	R^2	R^2adj	P (LFT)	P (Durbin-Watson)	Regression equation	Desirability%	*O. u*	*O. g*
Candida albicans ATCC37037	95,06	88,90	0.92	0,3945	49,62 + 13,83*Ou + 2,24*Og +4,26*Ou^2 + 3,19*Ou*Og + 2,99*Og^2	97,86	0.583	0.147
Candida albicans ATCC37039	98,96	97,67	0.23	0,3760	43,66 + 20,1*Oua - 0,95*Og -1,77*Ou^2 + 3,39*Ou*Og + 2,53*Og^2			
Candida albicans ATCCL26	96,65	92,48	0.29	0,3743	77,94 + 24,2925*Oua + 4,48*Og -5,42*Ou^2 - 10,38*Ou*Og -1,03*Og^2			
Candida albicans ATCC12C	91,72	81,37	0.33	0,2946	84,84 + 20,93*Ou + 2,00*Og-12,57*Ou^2 + 2,07*Ou*Og - 2,44*Og^2			

R^2adj= R square adjusted, LFT= lack of fit test, O u= Ocimum urticaefolium, O g= Ocimum gratissimum.

Table 5- Combination parameter of *Ocimum urticaefolium* and *Ocimum basilicumon* yeast growth inhibition.

Strains	Optimization					Multiple Response Optimization		
							Optimums (mg/ml)	
	R^2	R^2adj	P (LFT)	P (Durbin-Watson)	Equation of regression	Desirability%	*O. u*	*O. g*
Candida albicans ATCC37037	94,94	88,61	0.1231	0,0267	38,60 + 18,26*Oua + 12,35*Oba+3,14*Ob^2 + 1,81*OU*Ob - 3,55*Ob^2	100	0.562	0.629
Candida albicans ATCC37039	91,70	81,33	0.451	0,4453	28,65 + 15,79*Ou + 8,59*Ob +5,65*Ou^2 + 2,15*Ou*Ob + 0,423*Ob^2			
Candida albicans ATCCL26	92,83	83,86	0.1036	0,2659	68,47 + 8,19*Oua + 18,81*Oba -4,94*Ou^2 + 6,64*Ou*Ob -7,19*Ob^2			
Candida albicans ATCC12C	93,78	86,01	0.1802	0,3570	31,49 + 6,89*Ou + 20,75*Oba +2,62*Ou^2 + 8,00*Ou*Ob + 8,91*Ob^2			

R^2adj= R square adjusted, LFT= lack of fit test, O.urticaefolium= Ocimum urticaefolium, O. basilicum= Ocimum basilicum.

Combination of *Ocimum basilicum* and *Ocimum gratissimum*

The R^2 and adjusted R^2 were up to 90% for all the strains [Table-4], meaning that the models as fitted have at least 90% chance to describe properly the response of the yeast to the combination of *Ocimum basilicum* and *Ocimum gratissimum*. The lack of fit tests was not significant. *Ocimum basilicum* as parameter were significantly equal to 0 in all the models except in the case of *Candida albicans* ATCC37037 showing that the response of this strain is not influenced by the *Ocimum basilicum* as parameter. Also, *Ocimum gratissimum* did not influence the response of *Candida albicans* ATCC12C as well as *Ocimum gratissimum*[2] in the case of *Candida albicans* ATCC37039. The combination parameter *Ocimum basilicum* Ocimum gratissimum* was significantly different from 0, demonstrating that the combination of both essential oils influenced the response of all strains tested [Table-3].

The optimum value of *Ocimum basilicum* and *Ocimum gratissimum* that could inhibit 90% of all the tested yeast growth was 0.629mg/ml and 0.147mg/ml respectively. The desirability in this case was 97.86% [Fig-2] meaning that there is 97.86% of chance that the concentrations of two essential oils will show described inhibition.

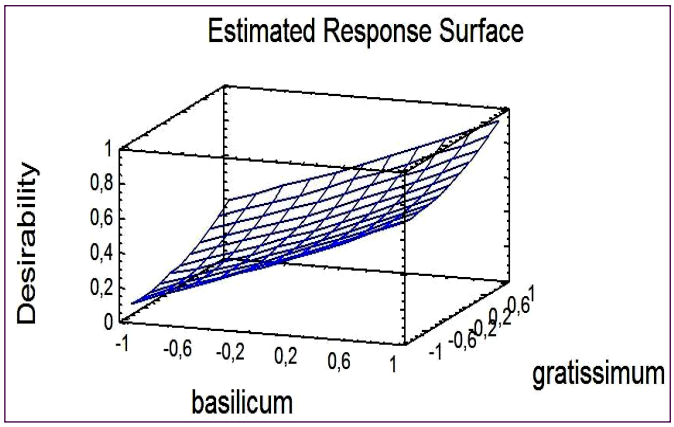


Fig. 2- Multiple Response Optimization desirability for combination of essential oil of *Ocimum basilicum* and *Ocimum gratissimum*.

Combination of *Ocimum urticaefolium* and *Ocimum gratissimum*

The combination parameters for combination of *Ocimum urticaefolium* and *Ocimum gratissimum* are shown in [Table-4]. For all the tested strains, R^2 and adjusted R^2 were up to 90%, meaning that the model as fitted has at least 90% chance to describe properly the response of the yeast to the combination of *Ocimum urticaefolium* and *Ocimum gratissimum*. The lack of fit tests was not significant showing that the models appear to be adequate for the observed data at the 95% confidence interval. *Ocimum urticaefolium* as parameter were significantly equal to 0 in the case of *Candida albicans* ATCC37039 and *Candida albicans* ATCCL26 while *Ocimum gratissimum* were significantly different to 0 in all models. The combination parameter *Ocimum basilicum*Ocimum gratissimum* were up to 0.05 indicating that the combination of these two essential oils was significantly different from 0 [Table-4].

The optimum value of *Ocimum basilicum* and *Ocimum gratissimum* that could inhibit 90% of all tested yeast growth was 0.583mg/ml and 0.147mg/ml respectively. The desirability in this case was 96.35% [Fig-3] meaning that there is 96.35% of chance that the concentrations of these two essential oils will show the described inhibition.

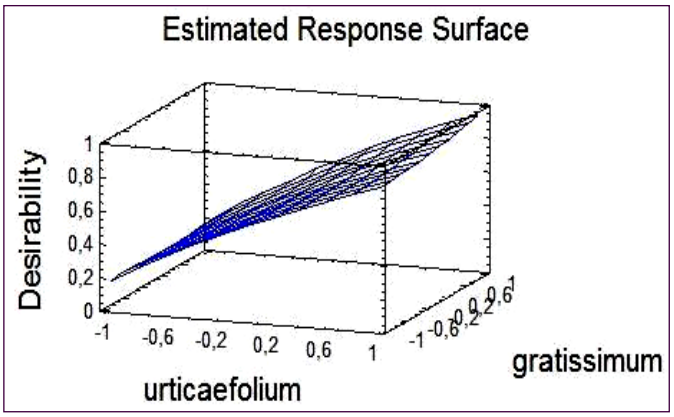


Fig. 3- Multiple Response Optimization desirability for combination of essential oil of *Ocimum urticaefolium* and *Ocimum basilicum*.

Combination of *Ocimum urticaefolium* and *Ocimum basilicum*.

The R^2 were up to 91.70% and adjusted R^2 were between 83, 86% to 88.61% [Table-5] which means that the model as fitted has at least 91.70% of chance to describe properly the response of the yeast to the combination of *Ocimum urticaefolium* and *Ocimum basilicum*. The lack of fit tests was not significant showing that the model appears to be adequate for the observed data at the 95% confidence interval. *Ocimum basilicum* as parameter did not influence the response of *Candida albicans* ATCC12C.*Ocimum urticaefolium* and *Ocimumbasilicum* as parameters did not influence the response of *Candida albicans* ATCC37037 and *Candida albicans* ATCCL26. The parameters *Ocimum basilicum*Ocimum urticaefolium* were up to 0.05 indicating that the combination of these two essential oils was significantly different from 0 [Table-5].

The optimum value of *Ocimum basilicum* and *Ocimum urticaefolium* that could inhibit 90% of all tested yeast growth is 0.629mg/ml and 0.147mg/ml respectively. The desirability in this case was 100% [Fig-4] meaning that it is highly probable that the concentrations of two essential oils will show the described inhibition.

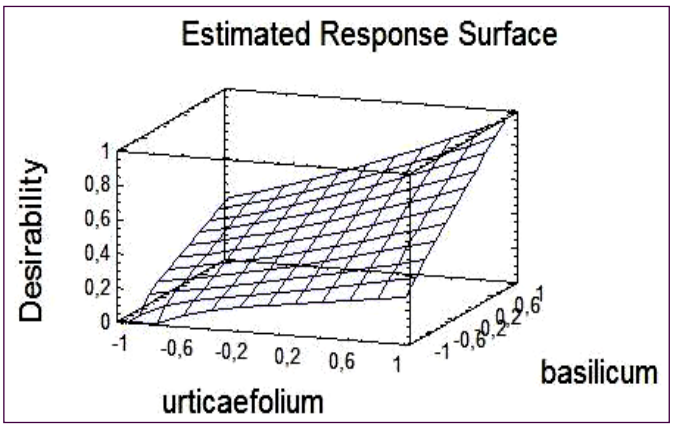


Fig. 4- Multiple Response Optimization desirability for combination of essential oil of *Ocimum urticaefolium* and *Ocimum basilicum*.

In this case, the value of the determination coefficient ($90\% \leq R^2$) indicates that the model could explain at least 90% of the variability in the inhibition of the growth of yeast. The variability of the R^2 for the same combination on different yeasts could be explained by the yeast species and /or intrinsic yeast characteristics. The lack of fit test is non-significant at the 95% confidence interval [Table-3], [Table-4] and [Table-5]. This indicates that the regression model

provides an excellent explanation of the relationship between the independent variables (essential oils) and the response (yeast growth inhibition) [10,27]. The p-values of the Dublin-Watson tests are in most of the case up to 0.05 indicating that there is no auto-correlation in the residuals.These results show that the results obtained are realistic, as the responses were not correlated [16].

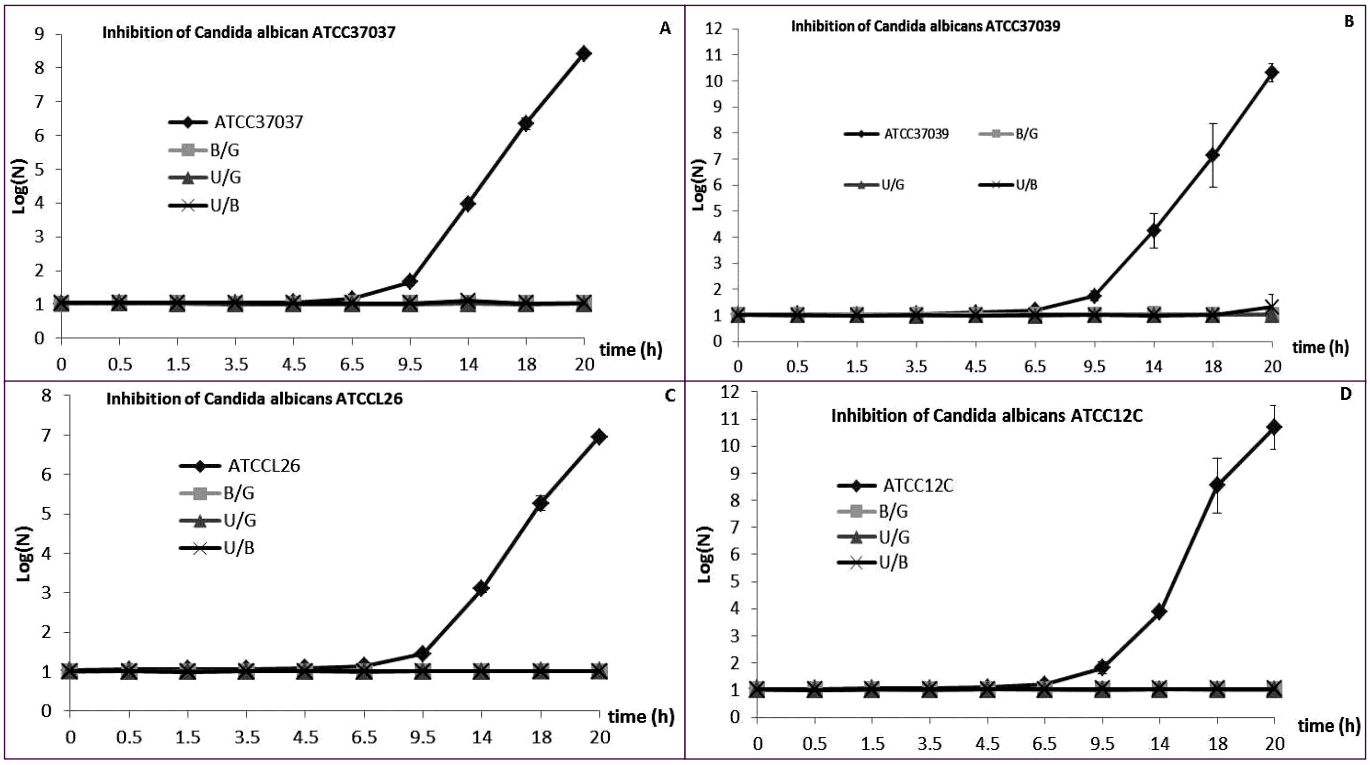


Fig. 5- Optimized combination of essential oil from most active essential oil on. *Candida albicans* ATCC37037 **(A)**, *Candida albicans* ATCC37039 **(B)**, *Candida albicans* ATCCL26**(C)**, *Candida albicans* ATCC12C**(D)**.

C/G= optimized combination of essential oil from *Ocimum basilicum* and *Ocimum gratissimum;* U/G = optimized combination of essential oil from *Ocimum urticaefolium* and *Ocimum gratissimum*; U/C = optimized combination of essential oil from *Ocimum urticaefolium* and *Ocimum basilicum.*ATCC37037= *Candida albicans* ATCC37037, ATCC37039= *Candida albicans* ATCC37039, ATCCL26= *Candida albicans* ATCCL26, *Candida albicans* ATCC12C

The Multiple Response Optimization was drawn and the optimum concentrations obtained were at least twofold lower than the MIC of essential oil alone. The desirability coefficient varied from 0.9 to 1, this result indicating that the optima obtained were as ideal as possible [16]. This shows the important pharmacological potential as the optimum obtained inhibited the growth of all the tested yeast strains within 20 hours [Fig-5]. These observations are in accordance with those obtained by combining amiodarone with azoles (Fluconazole, Itraconazole and Voriconazole) [28].

The Time Kill Kinetic

The optimized combination of essential oils inhibits the growth of the tested yeast within 20 hours.The fact that the optical density did not decrease can be due to the fungistatic potential of the essential oil. But Nakamura, et al. [29] has previously shown that the essential oil from *Ocimum gratissimum* at certain concentrations kills the yeast without disintegration of the yeast cells. We observed the change in the cell morphology at the subinhibitory concentrations (unpublished data) and this later observation was also observed by Nakamura, et al. [29]. All these observations demonstrate that the combination presented here are fungicidal. Further research is needed to elaborate these mechanisms.

Conclusion

Our research demonstrates that the essential oil of *Ocimum canum*, *Ocimum basilicum, Ocimum gratissimum*, and *Ocimum urticaefolium* has good antifungal potential. When combined with each other, their activities were increased. Further research is needed on the optimal combinations for use as anti-Candidal drugs.

Abbreviations

%w/w: percentage weight per weight

ATCC: American Type Culture Collection

CG/SM: Gas Chromatography coupled with Mass Spectrometry

GC: Gas Chromatography

mg/ml: milligram per milliliter

MIC: Minimal Inhibitory Concentration

MRO: Multiple Response Optimization

NHC: National Herbarium of Cameroon

RSM: Response Surface Methodology

SRF/Cam: Service de la Recherché Forestière du Cameroun

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