Preservative efficacy of the combination of essential oils of *Cymbopogon citratus* (DC.) Stapf (Poaceae) and *Citrus sinensis* (l.) Osbeck (Rutaceae) in a cream formulation

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ABSTRACT

Background: The search for so called green chemicals is on the increase. This is particularly important in antimicrobials, given the current trend in the development of antimicrobial resistance and the questions concerning the possible carcinogenic effect of traditional cosmetic preservatives like the parabens.

Objectives: This study is aimed at investigating the combined antimicrobial properties of essential oils extracted from *Cymbopogon citratus* and *Citrus sinensis* peels, and to evaluate their preservative efficacy in aqueous creams.

Methods: The essential oils were obtained by steam distillation. Their antibacterial and antifungal activities were evaluated using the disc agar diffusion method while their minimum inhibitory concentrations (MIC) were determined by the agar dilution method. The combined antimicrobial effects of the essential oils were also evaluated using a modified checkerboard method. Various decimal combinations of the oils were included informulatedcreams, and the physicochemical and microbiological stability *(*preservative activities) of the creams assayed.

Results: Antimicrobial screening showed that*C. citratus* and *C. sinensis* both had strong antifungal activities against the species tested but have variable antibacterial activities against different bacterial species. All the decimal combinations of the essential oils had synergistic effects against the studied bacteria as shown by the fractional inhibitory concentration (FIC) index against the various test isolates. The results of the challenge tests revealed that the oils produced significant reduction of the microbial inocula, satisfying the criterion A of the European Pharmacopoeia.

Conclusions: Due to the stability and preservative efficacy of essential oils of *C. citratus* and *C. sinensis*, they could be useful adjuvants in creams for topical application as antimicrobial agent to minimize microbial contamination caused by these organisms in multidose cream formulations.

Keywords: *Cymbopogon citratus*, *Citrus sinensis*, essential oil, preservative, stability.

Efficacité conservatrice de la combinaison d'huiles essentielles de *Cymbopogon citratus* (DC.) Stapf (Poaceae) et *Citrus sinensis* (l.) Osbeck (Rutaceae) dans une formulation de crème

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RESUME

Contexte: La recherche de soi-disant substances chimiques vertes (écologiques) est en hausse. Ceci est particulièrement important dans les antimicrobiens, compte tenu de la tendance actuelle au développement de la résistance aux antimicrobiens et des questions concernant l'effet cancérogène possible des conservateurs cosmétiques traditionnels comme les parabènes.

Objectifs: Cette étude vise l'étude des propriétés antimicrobiennes combinées des huiles essentielles extraites des peaux *de Cymbopogon citratus* et *Citrus sinensis* et l'évaluation de leur efficacité conservatrice dans les crèmes aqueuses.

Méthodes: Les huiles essentielles ont été obtenues par distillation à la vapeur. Leurs activités antibactériennes et antifongiques ont été évaluées à l'aide de la méthode de diffusion d'agar de disque alors que leurs concentrations minimales inhibitrices (MIC) étaient déterminées par la méthode de dilution d'agar. Les effets antimicrobiens combinés des huiles essentielles ont également été évalués à l'aide d'une méthode de damier modifiée. Diverses combinaisons décimales des huiles ont été incluses dans les crèmes informées, et la stabilité physicochimique et microbiologique (activités conservatrices) des crèmes testées.

Résultats: Le dépistage antimicrobien a montré que C*. citratus* et *C. sinensis* ont tous deux eu des activités antifongiques fortes contre les espèces testées mais ont des activités antibactériennes variables contre différentes espèces bactériennes. Toutes les combinaisons décimales des huiles essentielles ont eu des effets synergiques contre les bactéries étudiées, comme en témoigne l'indice de concentration inhibitrice fractionnaire (FIC) par rapport aux différents isolats de test. Les résultats des tests de provocation ont révélé que les huiles ont produit une réduction significative des inoculations microbiennes, satisfaisant au critère A de la Pharmacopée Européenne.

Conclusions: En raison de la stabilité et de l'efficacité conservatrice des huiles essentielles de *C. citratus* et *C. sinensis*, elles pourraient être des adjuvants utiles dans les crèmes pour une application topique en tant qu'agent antimicrobien afin de minimiser la contamination microbienne causée par ces organismes dans les formulations de crème multi-dose.

Mots-clés: *Cymbopogon citratus*, *Citrus sinensis*, huile essentielle, conservateur, stabilité.

INTRODUCTION

Creams are described as emulsions of a semi-solid consistency or emulsions of a high apparent viscosity [1,2] with a typical creamy white appearance [3] manufactured for topical applications. Pharmaceutical creams may contain one or more active ingredients dissolved or dispersed in either an o/w or a w/o system. [4] Microbial contamination and growth in creams leads to a degradation and physical instability in such formulations and constitutes a potential toxic hazard. There is therefore a need to include appropriate preservatives to prevent microbial growth and to maintain the quality and shelf life of topical formulations. [1]

The microbiological safety of pharmaceutical and cosmetic products have always been of special interest to industries as microbial contamination can lead to product degradation or, in the case of pathogens, constitute a risk for the health of the consumer and potentially spread infection. [5,6] Antimicrobial agents are included in pharmaceutical and cosmetic formulations to protect them from deleterious effects of multiplications of microorganisms that may be introduced in the product during manufacture and/or fromraw materials. A recent trend in cosmetic preservation is to avoid the use of synthetic chemical agents, leaving scientists in the search for natural antimicrobial alternatives. [7,8] Volatile oils, also known as essential oils, are odoriferous oils occurring in buds, flowers, leaves, fruits, seeds, roots, etc. of plants. [9] They represent the total odour principle of any single botanical species and are generally isolated from non-woody plant materials by distillation methods, solvent extraction or cold expression. They are used in perfumes, cosmetics, flavouring food and drinks and for scenting. They are claimed to have antimicrobial activities. Medicinal uses proposed by marketers of essential oils vary from skin treatments to remedies for cancer. [10] Cymbopogon *citratus* (DC,) Stapf (Poaceae) commonly known, as lemon grass is native to warm regions and grows in almost all tropical and subtropical countries. [11] *C. citratus* essential oil have been reported to have medicinal value to cure acne, oily skin, scabies, and also used systemically to treat flatulence, headaches, and blood circulation problems. Therehave also been claims of its stimulant, diuretic [12] and anti-diarrhoeal effect. [13] The oil has been found to possess anti-fungal and bactericidal properties, which is comparable to that of penicillin. [14] In Nigeria, lemongrass is used as a carminative and as an admixture in combination with other plants totreattyphoid and malaria infections. [15]

Sweet orange (*Citrus sinensis*), a native plant of Southern Asia, is the most commonly grown tree fruit in the world. The citrus species are famous as reliable source of essential oil. *Citrus sinensis* is the same as other citrus species that contain essential oil gland in peel of their fruit. The citrus peels have a strong and desirable aroma with refreshing effect. They have been used as flavouring additivein pharmaceutical products, [16] foods, and beverages. [17]

This work therefore aims to determine the antimicrobial activity of essential oils extracted from *C. citratus* and *C. sinensis* and evaluate their efficacy as preservatives in cream formulations.

MATERIALS AND METHODS

Materials

Clotrimazole (Canesten)® tablet (Bayer AG, Germany), chloramphenicol capsules (May & Baker, Nigeria), Ampicillin disc (Oxoid, Hampshire, UK), Dimethyl sulfoxide (BDH Chemicals Ltd, Poole, England). All other materials used were analytical grade.

Test organisms

The bacterial and fungal cells usedwere clinical isolates obtained from the laboratory of the Department of Pharmaceutical Microbiology and Biotechnology, Faculty of Pharmaceutical Sciences, Nnamdi Azikiwe University, Awka, Nigeria). These are*Staphylococcus aureus, Escherichia coli, Salmonella typhi, Klebsiella pneumonia, Pseudomonas aeruginosa, Bacillus subtillis, Candida albicans and Aspergillus niger*

Experimental

2.2.1 **Collection and identification of plant materials**

The leaves of lemon grass *(Cymbopogon citratus*) and orange peels (*Citrus sinensis*) were harvested locally in Enugu State, Nigeria inApril 2012. Mr A.O. Ozioko at the Bioresources Development and Conservation Programme (BDCP), Nsukka, Enugu State confirmed the taxonomic identification of the plants.

Extraction of Essential oils

The essential oil was extracted from lemon grass leaves and orange fruit peels by steam distillation [18] using the Clevenger type apparatus (Pyrex Coring Inc., USA). Sample (2 kg) was weighed out, washed with clean water, placed in 1000 ml round bottom glass flask, and filled with distilled water and connected to a distillation apparatus. The trap arm was also filled with distilled water to allow the oil to condense on the water layer. Distillation was continued until there was no more difference in successive readings of the oil volume. The

distillate (a mixture of oil and water) was then poured into a separating funnel where the mixture separated. Essential oil extracted from *C. citratus* was labelled as CCEO while that from *C. sinensis* was labelled as CSEO. The yield from each plant source was calculated as a percentage of the mass of the plant material used.

Characterisation of essential oils

Yield and sensory analyses of the essential oils

The percentage yield of essential oils was determined after measurement in a measuring cylinder while the sensory analysis was done using the sense organs of sight, smell and touch. The yield was calculated using the equation 1

$$\text{Yield (\%)} = \frac{\text{Weight of oil recovered}}{\text{Weight of crude sample}} \times 100\ \% \quad \text{.....Equation 1}$$

Antimicrobial testing

Inoculum preparation

Cultures of the bacteria and fungi were maintained on nutrient and Sabouraud dextrose agar respectively at 4^{o}C. The bacterial cultures were sub-cultured in nutrient broth at 37^{o}C for 24 h while the culture of *C. albicans* was sub-cultured in liquid Sabouraud dextrose medium for 48 h at 25^{o}C. The turbidity of the broth culture was then equilibrated to 0.5 McFarland standards using normal saline as diluent. Mature culture of *A. niger was* inoculated into Sabouraud dextrose broth to prepare the test inocula that were similarly equilibrated with 0.5 McFarland standard.[19]

Preparation of 0.5 McFarland standards

McFarland's standard was prepared using standard methods. The preparation was stored in a well-sealed container in the dark at room temperature (20 – 28 ^{o}C); the standard was kept for up to 6 months for further test.

Antimicrobial Assay of essential oils

Antibacterial and antifungal properties of CCEO and CSEO were evaluated by measuring the zone of inhibition (IZD) [20]using the Kirby-Bauer method. [21] Mueller Hinton agar and Sabouraud dextrose agar plates were prepared by pouring 20 ml each in sterile petri dishes for bacterial and fungal assay respectively and allowed to solidify. Exactly 0.1 ml of overnight bacterial, yeast and 4 – 8days old mould cultures were used so as to ensure the concentration of these organisms to contain approximately 1 x 10^{6} CFU/ml. Sterile cotton swabs dipped in respective cultures were swabbed on solidified agar surface. Different concentrations (0.4, 2, 10, 50, 250, µl/ml) of each essential oil was prepared, using dimethylsulfoxide as the diluents. Pre-sterilized filter paper discs of 6 mm diameter, which absorb 10 - 12 µl sample/ disc, were dipped into individual oil separately and placed on the swabbed agar plates before incubation. Similar process was followed for the controls using ampicillin disc (10 µg drug/ disc) and clotrimazole disc (100 µg drug/ Disc) as standard against antibacterial and antifungal test discs respectively. Assay was also performed separately for evaluatingthe antimicrobial activity of dimethyl sulfoxide, the solvent used to dilute the essential oil. The plates were then left at room temperature for 1h to equilibrate, and then incubated overnight at 37 ^{o}C for bacteria and for 48 - 72h at 25 ^{o}C for yeast/fungi. At the end of incubation period, diameter of inhibition zones formed in all three replicates were measured and the results were expressed as mean ± SD. P values <0.05 were considered as significant.

Determination of minimum inhibitory concentration (MIC) of essential oils

The agar dilution method recommended by the National Committee for Clinical Laboratory (NCCL) Standards[19, 20,21]was used to determine the minimum inhibitory concentration. Series of five-fold dilution of each oil (CCEO and CSEO) ranging from 0.4 to 250 µl/ml, was prepared in Mueller-Hinton and Saboraud dextrose agar at 48 °C. The plates were dried at room temperature for 30 min prior to spot inoculation with 3 µl aliquots of culture containing approximately 10^{6} CFU/ml of each organism. Inoculated plates were incubated at 37 °C for 18 h for bacteria and 48 - 72 h at 25 ^{o}C for yeast/fungi and the MIC were determined. Experiments were carried out in triplicates. Inhibition of bacterial growth in the plates containing test oil was compared togrowth in blank control plates. The MICs were determined as the lowest concentration of oil inhibiting visible growth of each organism on the agar plate. [22]

Evaluation of combined antimicrobial effect

Stock solution of CCEO and CSEO were prepared in nutrient and Saboraud's dextrose broth. Varying

proportions of the oils; CCEO: CSEO designated as A and B respectively, ranging from 0:10 to 10:0 were mixed according to the continuous variation checkerboard method of Bonapase *et al.*[23] Each combination of the extract was serially diluted with single strength nutrient or sabouraud's dextrose broth medium. Then 1 ml of each of the extract combination was seeded into a petri dish together with 19 ml of molten sterile nutrient or Saboraud's dextrose agar and allowed to stand for 1 h to solidify and pre-diffuse. An aliquot equivalent to 0.5 McFarland standards of test organisms was streaked on the surface of the agar plate. The set up was done in triplicates with a control containing no oil extract and these were incubated overnightat 37^{o}C for bacteria and for 48 - 72 h at 25^{o}C for yeast/fungi and observed for growth. The MICs of the various combinations were determined. The interactions between the antimicrobial agents were assessed by determining their fractional inhibitory concentration (FIC) index according to the equations 2 to 5

FIC index = FIC A + FIC B..... Equation 2

Where A = CCEO, B = CSEO

$$\text{FIC A} = \frac{\text{MIC of CCEO in combination with CSEO}}{\text{MIC of CCEO alone}} \quad \text{.....Equation 3}$$

$$\text{FIC B} = \frac{\text{MIC of CSEO in combination with CCEO}}{\text{MIC of CSEO alone}} \quad \text{.....Equation 4}$$

The Activity index (AI) = log FIC index .....Equation 5

Formulation of Cream

Preparation of Emulsifying Ointment B.P

This was prepared according to the formula specified in B.P. [24] This served as the base for the formulation of the essential oil-loaded creams.

Preparation of essential oil-loaded creams

The formula used for the preparation of the essential oil creams is shown in Table 1. A quantity of emulsifying ointment B.P (18 g) was melted over a hot water bath on a porcelain dish to 70^{o}C. A combination of methyl- and propyl- parabens (3:1) or 1 ml of combinations of CCEO and CSEOwas dissolved in 41 g of purified water. The solution was brought to the same temperature as the water bath before mixing with the emulsifying ointment. The mixture was stirred occasionally over a cool water bath to cool. The cream was transferred quantitatively into a wide mouthed jar. Eight batches of creams were formulated: cream containing a combination of CCEO and CSEO (10:0, 8:2, 6:4, 4:6, 2:8, 0:10), cream containing methyl- and propyl- parabens (3:1) as positive control and cream without any essential oil as negative control.

Table 1: Composition of different batches of cream

Batch	INGREDIENTS (QUANTITIES)			
	Emulsifying ointment (g)	M.paraben: P. paraben (%)	CCEO: CSEO (1ML)	Water(g)
C1 (positive control)	18	0.1	_	41
C2	18	_	10:0	41
C3	18	_	8:2	41
C4	18	_	6:4	41
C5	18	_	4:6	41
C6	18	_	2:8	41
C7	18	_	0:10	41
C8 (negative control)	18	_	_	41

Evaluation of essential oil-loaded creams

Cream morphology

The colour and odour of the creams were observed and perceived respectively. The creams were also tested for homogeneity and the presence of lumps.

Determination of antimicrobial activities in creams

The agar diffusion method [25] was employed to assess the antimicrobial activity of the formulated creams. Overnight bacterial, yeast and 4 – 8 days old mould

cultures were used so as to ensure the concentration of these organisms to contain approximately 1 x 10^6 CFU/ml. Exactly 0.1 ml bacteria of the overnight culture were used to inoculate 20 ml of molten sterile nutrient agar at 45 °C and allowed to set. For the fungi, 20 ml of molten sterile Saboraud dextrose agar containing 0.5mg/ml chloramphenicol was poured into sterile plates and allowed to set. The surface was inoculated with 0.1 ml of the culture. The surface of the set agar was allowed to dry and equidistant 6 – mm wells were cut into the agar at equal separation distances. Cream prepared with methyl- and propyl- parabens (3:1) were used as positive controls for the bacteria and fungi, respectively. One gram of each formulation (batchesC1-C8) was mixed with 1 ml of 2 % tween 80 using a whirl mixer until slurry was formed. Exactly 100 µl of each cream slurry was placed into pre-labelled wells. The experiment was repeated for all the test microorganisms. The tests were performed in triplicates. The creams were allowed to diffuse for 1 h before incubating the bacteria plates at 37 °C for 24 h, and the fungal plates were incubated at 25 °C for a minimum of 48 h. The diameter of zone of inhibition was measured after the incubation period for each formulation.

Stability Studies

The formulated creams were stored in tightly sealed wide-mouthed jars in the laboratory and the pH was determined every 4 weeks over a period of 12 weeks. The antimicrobial activity screened before and after 12 weeks to determine the change in pH and the microbial stability of the creams.

Thermal cycle test

Portions of each formulation were stored at 4 ^oC for 48 h and then 25 ^oC for 48 h. The test was repeated and the creams were observed for visible changes in colour, homogeneity and consistency.

Microbial challenge test

The micro-organisms used in this study include the following: *E. coli, P. aeruginosa, S. aureus, C. albicans* and *A. niger.* Bacteria were cultured on nutrient agar for 24 h at 37 ^oC; *C. albicans* and *A. niger* were grown on Sabouraud dextrose agar at 25 ^oC for 48 h and 5 days respectively. For microbial inoculants, the cells were harvested in a sterile fluid containing 0.9 % w/v of sodium chloride and 0.1 % w/v of peptone for dispersal. The suspension was adjusted to yield suspensions of approximately 10^8 CFU/ml. A portion from each suspension was removed to determine the number of CFU/ml in each suspension by plate count method. The value serves to determine the inoculums and the baseline to use in the test. The microbial challenge test was performed following a modification of the standards proposed by the European Pharmacopoeia Commission (1996) [26] concerning topical preparations. Formulations (10g) were placed in sterile containers and separately inoculated with 0.05 ml bacterial and fungal suspensions to give a final level of approx. 10^6 CFU /g. After a contact time of 0, 2.0, 7.0, 14, and 28 days, at 20 – 25 ^oC, the samples (1.0 g) were mixed with 1.0 ml of 2 % tween 80 to form slurry. Cell viability was determined by the plate count method in nutrient or sabouraud's dextrose agar and colony-forming units were counted after 5 - day incubation at 37 and 25^oC for bacteria and mycetes respectively. The number of colony forming units per gram were counted using colony counter. The results were expressed as Log CFU /g. Assay was also performedseparately for evaluating the anti-microbial activity of 2 % tween 80, the solvent used to mix the cream.

Statistical analysis

The results were subjected to analysis of one way (ANOVA) followed by Duncan's post hoc multiple comparison test using SPSS software package version 16.0. The results were expressed as mean ± SD. P values <0.05 were considered as statistically significant.

RESULTS

Oil yield and physical test

The percentage yields were 0.76 and 0.53 for CSEO and CCEO respectively. The result of the sensory evaluation showed that CSEO was yellow in colour, had fresh, fruity, citrusy and pleasant smell, while CCEO was amber to reddish in colour and had lemony sweet smell. Generally, the oils were insoluble in water but soluble in dimethyl sulfoxide (DMSO).

Antimicrobial assay of essential oils

The results obtained from the antimicrobial assay of CCEO and CSEO are shown in Figures 1 and 2.

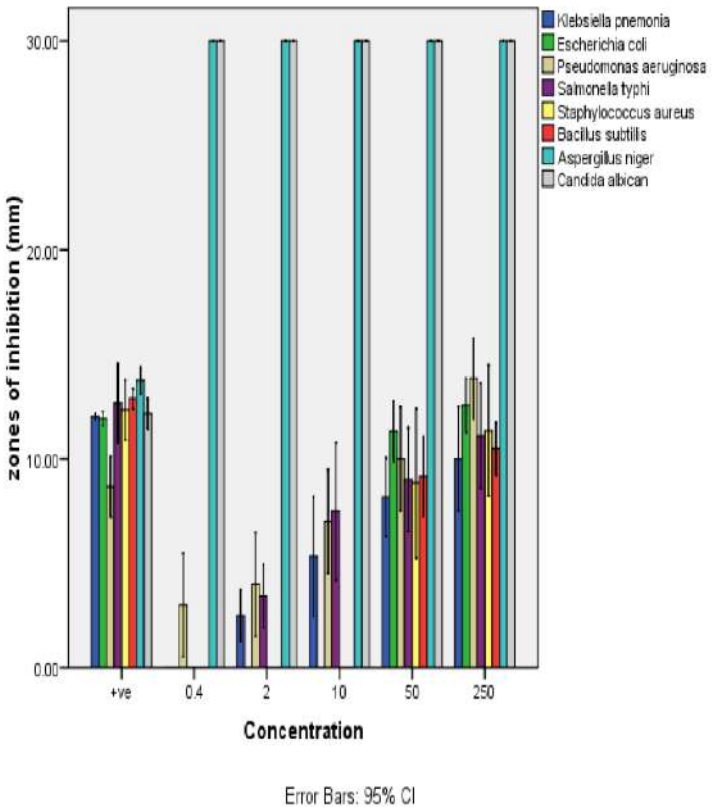

Figure 1 Zones of inhibition (mm) of *C. citratus* essential oil against different organisms

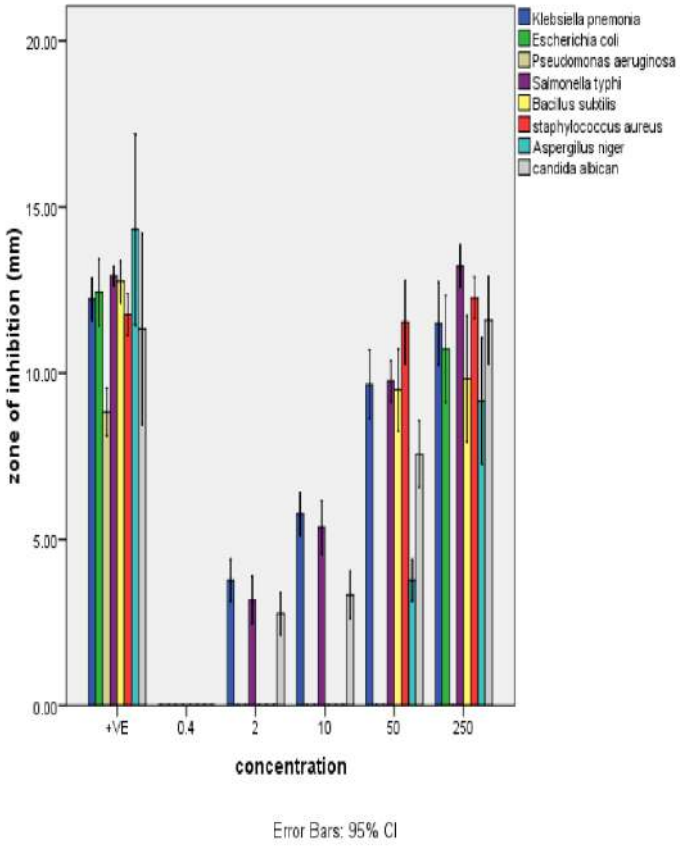

Figure 2 Zones of inhibition (mm) of *C. sinensis* essential oil against different organisms

Minimum inhibitory concentration (MIC) of essential oils.

The results obtained from determining the minimum inhibitory concentrations of CCEO and CSEO against different organisms presented in Table 2.

Antimicrobial Combination studies

The results of the modified checkerboard method for evaluating combined antimicrobial effects against the organisms (*S. aureus*, *E. coli, C. albicans and A. niger)* are presented in Tables 3 and 4.

Table 2 MIC of CCEO and CSEO against different organisms

Test organism	MIC (µl/ml)	
	CCEO	CSEO
Bacteria		
Klebsiella pneumonia	0.10	0.10
Escherichia coli	2.63	13.15
Pseudomonas aeruginosa	0.02	-
Salmonella typhi	0.10	0.10
Bacillus subtillis	2.63	2.63
Staphylococcus aureus	2.63	2.63
Fungi		
Aspergillus niger	0.02	2.63
Candida albicans	0.02	2.63

(Key: - = No activity, CCEO = *C. citratus* essential oil, CSEO = *C. sinensis* essential oil).

Evaluation of volatile oil creams

Physical examination

The formulated creams had the sweet characteristic aroma of the essential oils of orange peel and lemongrass. Physical assessment showed that the creams had a homogenous, uniform consistency and were white in colour. They were also spreadable and without lumps.

Table 3: Combined antimicrobial effects of various decimal combinations of *C. citratus* and *C. sinensis* essential oils against *E. coli* and *S. aureus.*

Combination Ratio	MIC A:B	FIC A:B	FIC (A.I)* Index	Effect
		Escherichia coli		
10:0	2.63 : -	1.0 : -	-	-
8:2	0.26:0.32	0.098:0.024	0.122(-0.913)	Synergism
6:4	0.39:0.13	0.148:0.009	0.157(-0.804)	Synergism
4:6	0.52:3.94	0.197:0.299	0.494(-0.306)	Synergism
2:8	0.06:0.13	0.022:0.009	0.031(-1.508)	Synergism
0:10	- :13.15	- :1.0	-	-
		Staphylococcus aureus		
10:0	2.63: -	1.0 : -	-	-
8:2	1.05:0.26	0.399:0.098	0.497(-0.303)	Synergism
6:4	0.39:0.26	0.148:0.098	0.246(-0.609)	Synergism
4:6	0.52:0.09	0.197:0.034	0.231(-0.636)	Synergism
2:8	0.26:1.05	0.098:0.399	0.497(-0.303)	Synergism
0:10	- :2.63	- : 1.0	-	-

Antimicrobial activity

The results of evaluating the anti microbial activity of the essential oils in cream formulation are shown in Table 5.

Stability Studies

The result of the effect of storage on pH at ambient temperature is shown in Table 6.

Table 4: Combined antimicrobial effects of various decimal combinations of *C. citratus* and *C. sinensis* essential oil against *A. niger* and *C. albicans*

Combination Ratio	MIC A:B	FIC A:B	FIC (A.I)* Index	Effect
		Aspergillus niger		
10:0	0.021: -	1.0 : -	-	-
8:2	0.008:0.26	0.380:0.099	0.479(-0.319)	Synergism
6:4	0.006:0.39	0.285:0.148	0.433(-0.363)	Synergism
4:6	0.002:0.26	0.095:0.098	0.193(-0.714)	Synergism
2:8	0.001:0.39	0.047:0.148	0.195(-0.709)	Synergism
0:10	- :2.63	- :1.0	-	-
		Candida albicans		
10:0	0.021: -	1.0 : -	-	-
8:2	0.004:0.005	0.190:0.047	0.237(-0.625)	Synergism
6:4	0.006:0.021	0.285:0.200	0.485(-0.314)	Synergism
4:6	0.004:0.031	0.190:0.295	0.485(-0.314)	Synergism
2:8	0.001:0.021	0.047:0.200	0.247(-0.607)	Synergism
0:10	- :0.105	- :1.0	-	-

Table 5 Preservative activities of *C. citratus* and *C. sinensis* essential oil in cream formulations.

Testorganism	Zone of inhibition (mm)							
Batch	**C2**	**C3**	**C4**	**C5**	**C6**	**C7**	**C8**	**C1**
Bacteria								
K. pneumonia		13.3± 1.5	13.6± 0.5	13.3± 1.1	10.5± 1.1	3.6± 1.5	-	13.0± 1.0
E. coli	6.6± 1.1	7.3± 1.1	8.6± 0.5	8.0± 1.0	5.6± 1.1	6.0± 1.0	-	12.3± 0.5
P. aeruginosa	10.6± 1.1	8.6± 1.1	4.0± 1.0	2.3± 0.5	-	-	-	12.6± 0.5
S. typhi	13.0± 1.0	13.0± 1.7	13.3± 0.5	14.0± 1.0	11.3± 1.5	6.3± 1.5	-	14.0± 1.0
B. subtillis	6.3± 1.1	7.6± 0.5	5.3± 1.1	7.0± 1.0	6.0 ±1.0	7.6 ±1.1	-	13.0± 1.7
S. aureus	7.6± 1.0	7.0± 1.0	12.0± 1.0	12.6± 0.5	7.0± 1.0	8.6 ±1.5	-	11.6 ±1.5
Fungi								
A. niger	14.3± 1.1	13.3± 2.8	13.3± 1.5	12.6± 0.5	11.3± 1.1	9.3± 1.1	-	18.6 ±1.1
C. albicans	15.3± 0.5	13.6± 1.5	11.3± 1.1	9.3± 0.5	10.0± 2.0	3.6± 1.5	-	17.0 ±1.7

Key - = No activity. Data presented as Mean ± SD.

Table 6. pH of *C. citratus* and *C. sinensis* essential oil cream

Batch	**pH value (mean ± SD)**				**F value**
Storage (week)	**1**	**4**	**8**	**12**	
C2	5.30 ± 0.1[a]	5.36 ± 0.3[a]	5.26 ± 0.4[a]	5.23 ± 0.2[a]	0.057NS
C3	5.10 ± 0.2[a]	5.13 ± 0.2[a]	5.23 ± 0.2[a]	5.25 ± 0.1[a]	1.638NS
C4	5.36 ± 0.4[a]	5.73 ± 0.5[a]	5.33 ± 0.4[a]	5.30 ± 0.3[a]	0.795NS
C5	5.30 ± 0.2[a]	5.53 ± 0.2[a]	5.30 ± 0.4[a]	5.30 ± 0.4[a]	0.304NS
C6	5.83 ± 0.3[a]	6.10 ± 0.1[a]	6.20 ± 0.5[a]	6.22 ± 0.2[a]	0.695NS
C7	6.40 ±0.4[a]	6.40 ± 0.2[a]	6.63 ± 0.2[a]	6.65 ± 0.3[a]	0.746NS
C8	6.06 ± 0.3[a]	6.00 ± 0.2[a]	6.13 ± 0.1[a]	6.14 ± 0.1[a]	0.676NS
C1	7.16 ± 0.3[a]	7.16 ± 0.1[a]	7.20 ± 0.2[a]	7.22 ± 0.3[a]	0.600NS

Key: Mean ± SD in the same column for the same pH of the cream, followed by the same superscript letters in a row do not differ significantly at p= 0.05 (Duncan test). NS =not significant p>0.05.

Thermal cycle test

The result of the thermal cycle test displayed in Table 7.

Microbial challenge test of creams

The result of microbial challenge test is displayed in Table 8 and graphical representation of the effects of the various batches on the individual test isolates are represented in the Figures 3 to 7.

Table 7: Effect of thermal cycle on the formulated creams

Batch	**4 ^{o}C (48 h)**	25 ^{o}C (48 h)
C2	Stable, homogenous	Stable, homogenous
C3	Stable, homogenous	Stable, homogenous
C4	Stable, homogenous	Stable, homogenous
C5	Stable, homogenous	Stable, homogenous
C6	Stable, homogenous	Stable, homogenous
C7	Stable, homogenous	Stable, homogenous
C8	Stable, homogenous	Stable, homogenous
C1	Stable, homogenous	Stable, homogenous

Table 8: Preservative efficacies of CCEO and CSEO in cream formulations

Batch	Time (day) (log CFU g^{-1})				
	0	2	7	14	28
Escherichia coli					
C2	3.7	1.5	1.4	1.0	1.0
C3	3.7	1.6	1.61.1	1.0	
C4	3.7	1.4	1.2	1.2	1.0
C5	3.7	1.5	1.3	1.3	1.2
C6	3.7	1.5	1.5	1.2	1.0
C7	3.7	1.6	1.6	1.5	1.2
C1	3.1	1.0	1.0	-	-
C8	3.7	3.6	3.6	3.2	3.0
Staphylococcus aureus					
C2	3.7	1.4	1.4	1.4	1.2
C3	3.7	1.6	1.5	1.2	1.2
C4	3.7	1.5	1.4	1.1	1.0
C5	3.7	1.4	1.2	1.2	1.1
C6	3.7	1.6	1.5	1.1	1.1
C7	3.7	1.6	1.6	1.2	1.2
C1	3.2	1.0	-	-	-
C8	3.7	3.7	3.6	3.2	3.0
Pseudomonas aeruginosa					
C2	3.7	1.4	1.2	1.0	1.0
C3	3.7	1.4	1.3	1.0	1.0
C4	3.7	1.6	1.5	1.3	1.0
C4	3.7	1.6	1.6	1.4	1.1
C6	3.7	3.6	2.8	2.2	2.0
C7	3.7	3.7	3.0	2.8	2.2
C1	3.0	1.0	1.0	-	-
C8	3.7	3.5	3.5	3.0	3.0
Candida albicans					
C2	3.6	2.2	1.0	-	-
C3	3.6	2.2	1.0	1.0	-
C4	3.6	2.3	1.1	1.0	1.0
C5	3.6	2.4	1.2	1.0	1.0
C6	3.6	2.4	1.3	1.2	1.0
C7	3.6	2.5	1.5	1.2	1.0
C1	2.2	1.0	-	-	-
C8	3.6	3.0	3.0	2.6	2.0
Aspergillus niger					
C2	3.4	2.0	1.0	-	-
C3	3.4	2.2	1.0	1.0	1.0
C4	3.4	2.2	1.1	1.1	1.0
C5	3.4	2.3	1.3	1.0	1.0
C6	3.4	2.3	1.2	1.2	1.0
C7	3.4	2.2	1.2	1.0	1.0
C1	2.5	1.0	-	-	-
C8	3.4	3.4	3.0	2.6	2.0

Key : - No growth

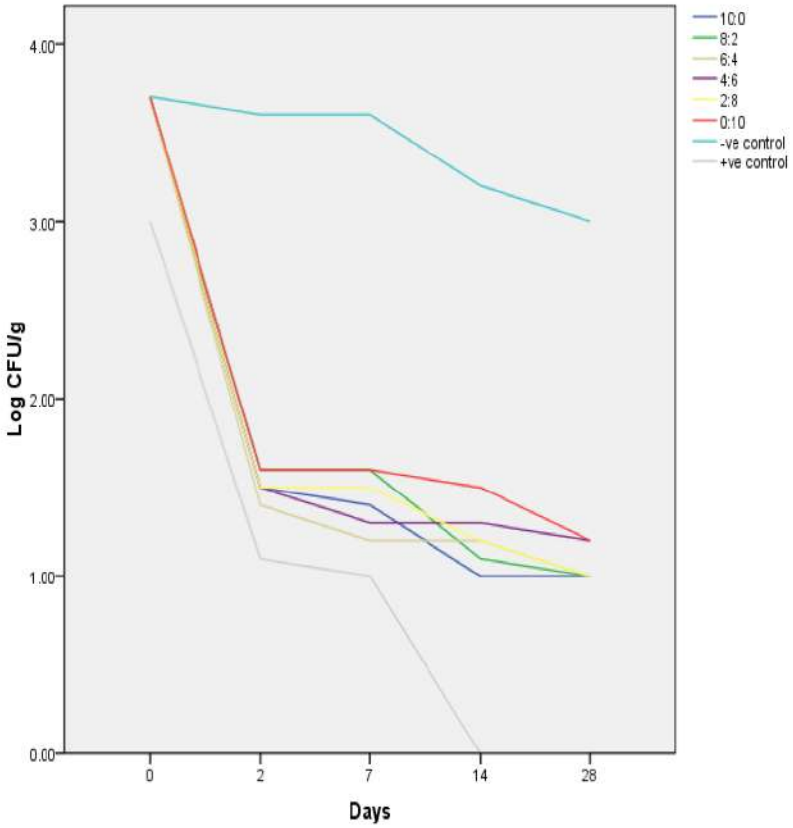

Figure 3: Microbial challenge tests of different batches against *E. coli*

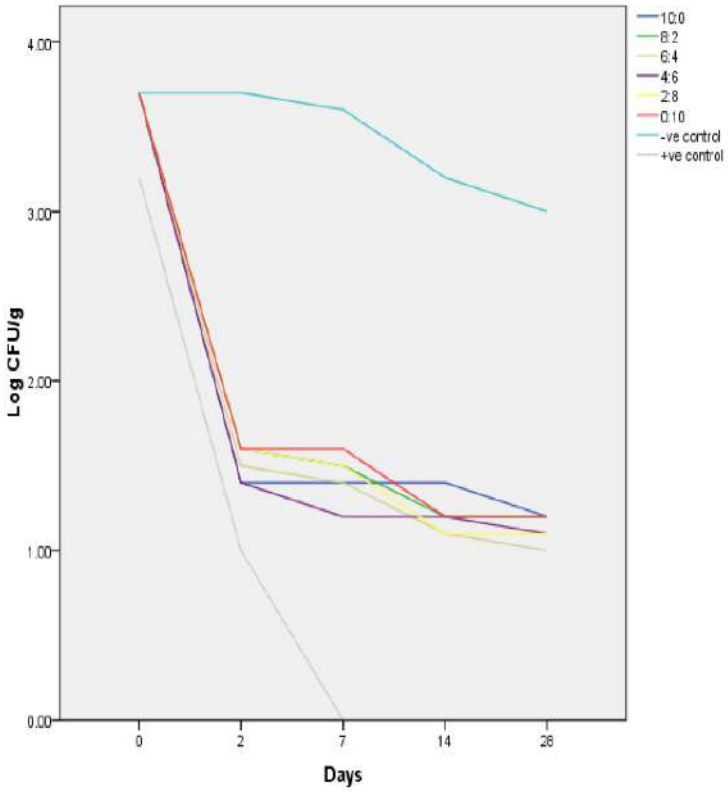

Figure 4: Microbial challenge tests of various batches of cream against *S. aureus.*

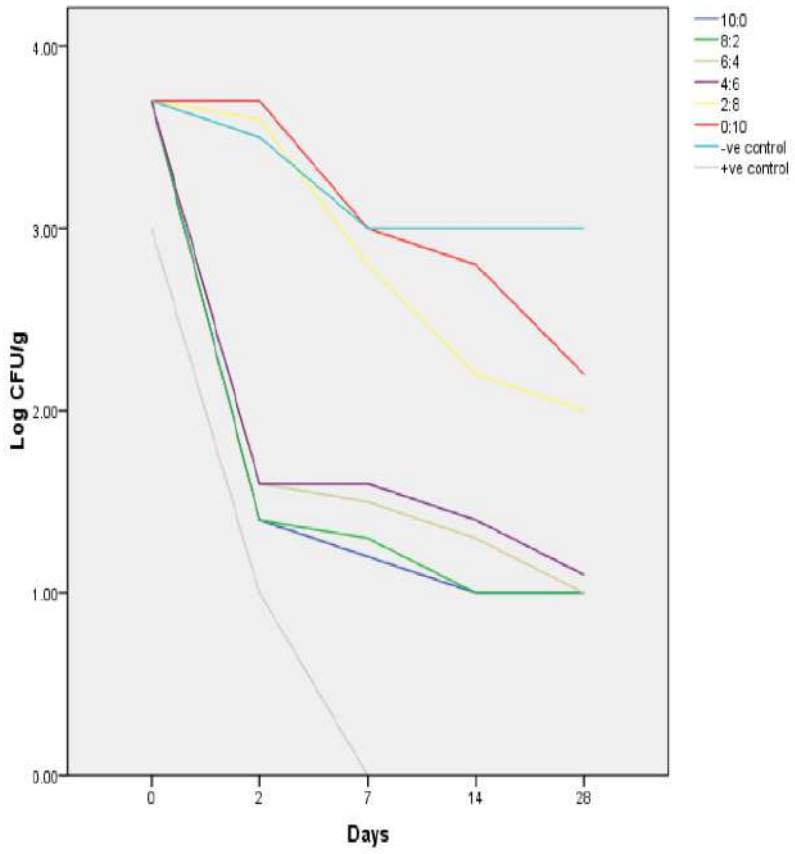

Figure 5: Microbial challenge test of different batches of cream against *P. aeruginosa*

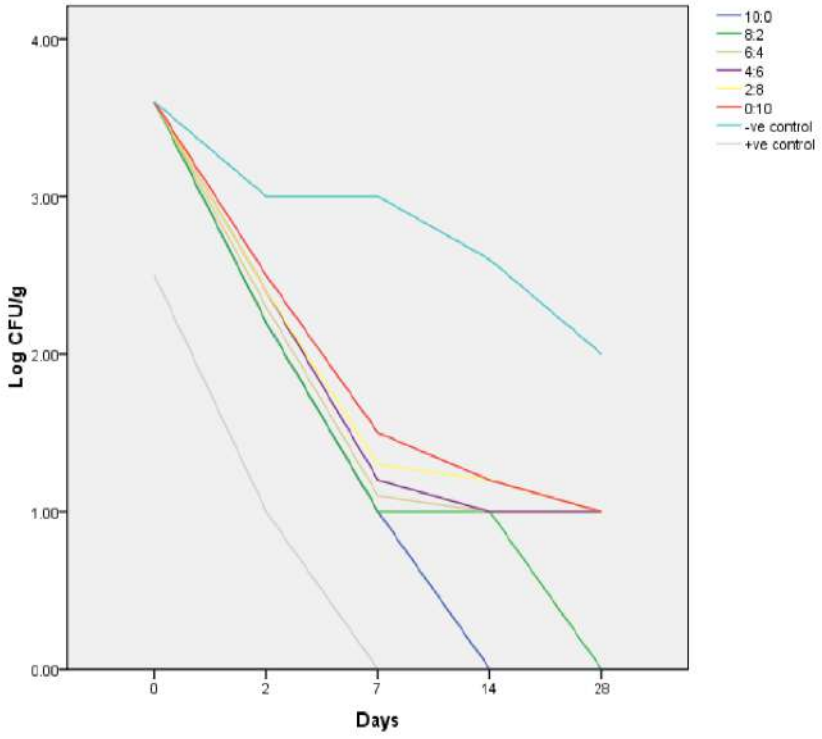

Figure 6: Microbial challenge tests of different batches of cream against *C. albicans*

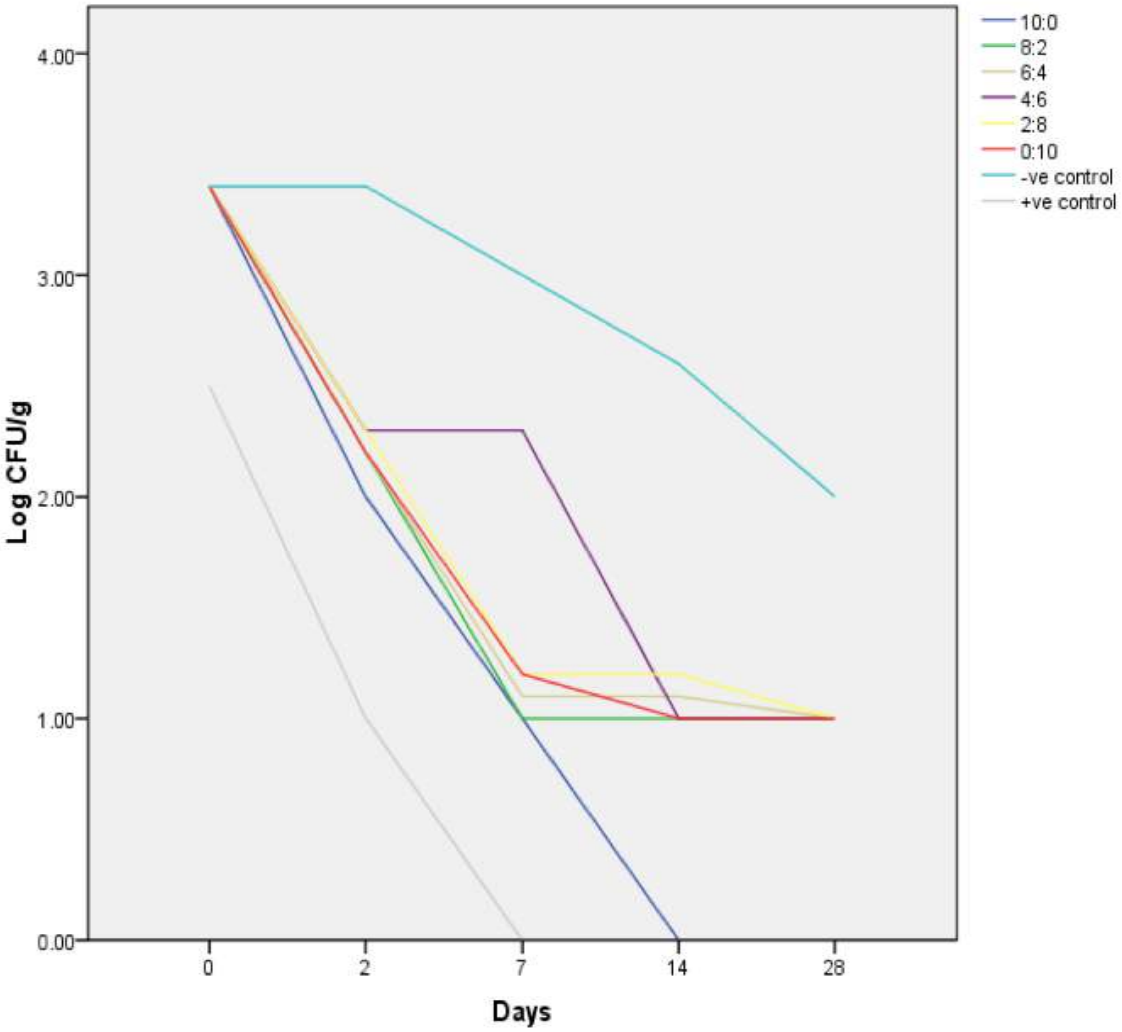

Figure 7: Microbial challenge tests of different cream formulations against *A. niger.*

DISCUSSION

CCEO showed variable but significant ($p<0.05$) effects against the tested strains of bacteria (Gram +ve and -ve) and fungi respectively, with clear zones of inhibition. The results displayed in

Figure 1revealed that at a concentration of 250 µl/ml, CCEO exhibited maximum inhibitory effect on the growth of *P. aeruginosa* with an inhibition diameter of 13.80 mm while it showed the least inhibition against *B. subtilis* with an inhibition diameter of 10.5 mm. Generally, the ranking of the antibacterial activity of CCEO against organisms tested is *P. aeruginosa>E. coli>S. aureus>S. typhi>K. pneumonia>B. subtilis*. CCEO also showed very strong antifungal activity against *A. niger* and *C. albicans*, with zones of inhibition of >30.00 mm each. These antibacterial and antifungal actions are probably due to the presence of the mono-terpene hydrocarbon, limonene, present in the oils. Limonene has been shown to possess strong antimicrobial activities.[27]Pratt and co-workers suggested that the antifungal activity of CCEO might be due to the presence of flavonoids, [28, 29] and phenolic compounds[29] in the oil. Due to this antifungal effect, CCEO has been proposed as an effective fungi control agent suitable for protection of food products[30]

Figure 2 shows that *P. aeruginosa* was not susceptible to the activity of CSEO at concentration of 250 µl/ml. Although in earlier studies, antimicrobial activity of CSEO has been reported against *P. aeruginosa,* it was reported forCSEO obtained from commercial fruits from Spain.[31]As such the extraction process (cold press)and cultivation method simply may have influenced the composition and consequently the antimicrobial activity. Other factors that have been found to affect antimicrobial activity of essential oil extracts include inoculation method, [32] geographical locations from which the plants were harvested [33]age and condition of the fruit and time of harvest. [34]CSEO showed the highest antibacterial activity against *S. typhi* exhibiting inhibition diameter of 13.2 mm. The least effect was

against *B*. *subtilis* with an inhibition diameter of 9.8 mm. The antibacterial activity of CSEO could be generally ranked thus: *S*. *typhi*>*S*. *aureus*>*K*. *pneumonia*>*E*. *coli*>*B*. *subtilis*. These results are shown in Fig. 2. CSEO also showed strong antifungal activity against *C. albicans* and *A. niger* strains with zones of inhibition of 11.60 mm and 9.10 mm respectively although this was below the activity exhibited by CCEO. The reasons for these activities are similar to those advanced for CCEO, however, Janssen *etal* summarized that the culture medium, the technique (s) of testing, botanical source and age of the plant, the state of plant material used (dried or fresh) and the isolation technique are also factors implicated in the variations of the activities.[35]

Determination of the minimum inhibitory concentration of the essential oils against various organisms showed that generally, CCEO has MIC values ranging from 0.02 − 2.36 µl/ml against the bacterial cells, and MIC value of 0.02 µl/ml against the fungal cells. CCEO showed MIC of0.02 µl/ml against *P. aeruginosa* and MIC value of 0.10 µl/ml. against both *K. pneumonia* and*S. typhi.* A relatively weak inhibitory effect of the volatile oil was observed against *E. coli, S. aureus* and *B. subtillis* as shown by the MIC value of 2.63 µl/ml. Though an earlier study by Helal and co-workers reported the antimicrobial activity of CCEO to be higher against bacteria than fungi[36] with MIC for fungi being 2 µl/ ml, our study showed MIC of 0.02µl/ ml against. *C. albicans and A. niger*. This might be due to the use of different strains of the organisms in earlier studies, which might be more resistant than the strains currently used in this report. It could also be as a result of variability in the compositionsofthevolatileoils as stated earlier on.

On the other hand, the MIC of CSEO ranged from 0.10 − 13.15 µl/ml for bacteria and MIC value of 2.63 µl/ml for fungi. *S. typhi* showed the highest susceptibility to CSEO with an MIC value of 0.10 µl/ml while *E. coli* showed the least susceptibility with an MIC of 13.15 µl/ml. *P. aeruginosa* did not show any activity. These results clearly show that the test organisms (bacteria and fungi) have the highest susceptibility to CCEO while they show the least to CSEO. This variation could be as a result of the differences in the concentrations of phenolic compounds in the oils due to their innate antimicrobial property. It might be the effect of the age or sources of the plant materials. Also, MIC figures from microbial assays might be influenced by other factors extraneous to the oils such as the strain, age and number of organisms, the nature and pH of the culture medium, the temperature and time of incubation.

All the various (batches C2-C7) combinations of the CCEO and CSEO yielded synergistic effects against all the susceptible organisms. It should be noted that very often, varying ratios of antibiotic in combination produce different types of synergies which can not only be a function of the nature of the antibiotics involved, but also that of their combination ratios. Fractional inhibitory concentration (FIC) index values < 1 were considered synergistic and the degree of synergy increases as the value tends towards zero. Synergism is defined as a further decrease in MIC of two agents in combination compared with their individual values.[37,38] This implies that the ethno-medicinal combination of CCEO and CSEO would yield greater effectiveness in treatment of infections in which any of *S. aureus*, *E. coli, C. albicans and A. niger* is implicated than when either of the oils is used individually.

From the physical evaluation of the creams, the fragrances of the essential oils were predominant. It should be noted that the fragrance of essential oils could vary according to the geo-climatic locations and growing conditions, season, and time when they were harvested. The homogeneity of the creams shows that the formulation ingredients were evenly mixed with the oils and properly distributed in the creams.

The observed anti microbial effects of the essential oils were evaluated in cream preparations to determine if the antimicrobial effect will persist. The CCEO and CSEO creams still retained their original antimicrobial (antibacterial and antifungal) effects as elicited by the oils. The negative control cream containing no essential oil had no activity against the test organisms while the positive control cream containing methyl- and propyl-parabens produced activities against bacteria and fungi respectively. All the formulated creams containing different combinations of CCEO and CSEO had activity against all the test organisms except batches C6 and C7.These did not show any activity against *P. aeruginosa.* The susceptibility differences between the test organisms to the creams might be due to the variations in the rate of limonene penetration through the cell wall and cell membrane structures of the test organisms. The disruption of the permeability barrier of the cell membrane factors followed by the loss of chemiosmosis functions could account for the killing effect of the oil-loaded creams.[39] All the cream formulations showed higher antifungal activity when compared to their antibacterial activity. This, in addition to the above reasons, might be due to the establishment of a pH gradient across the cytoplasmic membrane and the blocking of energy production in the

yeast cells by the oil-loaded creams.[40]

All the formulated creams were weakly acidic with pH values between 5 and 6. There were slight increases in pH after 4 weeks of storage in all the formulations. These decreased by the12th week of storage. The positive control cream, prepared with methyl- and propyl-parabens was neutral while the negative control cream, containing no essential oil, was weakly acidic. The change in the pH of each formulation over the 12 weeks period was not significant ($p > 0.05$). The implication of these findings is that the oil-loaded creams would be compatible with the human skin that has a normal pHof 5.5 and hence will be suitable for topical application. It is known that essential oil compounds are fat-soluble, thus they have the ability to permeate the membranes of the skin before their uptake by the microcirculation and drained into the systemic circulation which reaches all target organs.[39]

Stability test results showed that after 48 h of exposure of the creams to varying test temperatures, all the creams were stable, of uniform colour, homogenous and consistent. Since this test was designed to explore and observe possible changes in the physical and chemical properties, as well as the aesthetics of the oil-loaded creams on storage. The results presented did not indicate any significant change(s) in the integrity of the creams. This further indicated that the creams were stable on storage within the limits of experimental conditions.

From the result of the bacterial challenge test presented inTable 8, it could be seen that cream formulations were preserved with CCEO and CSEO when they were combined in the ratios of10:0, 8:2, 6:4, and 4:6 and thus satisfied the European Pharmacopoeia (E.P) criteria A which makes for reduction of the bacterial inoculum by a factor of 10^2 within 2 days of challenge with no increase up to the 28th day. On the other hand, CCEO and CSEO when combined in the ratios of (2:8 and 0:10) failed the test being ineffective as regards the criteria A and B for *P. aeruginosa* whose growth decreased gradually during the incubation time. This might be due to the large proportions of CSEO in the combinations to which *P. aeruginosa* was resistant as shown in the antimicrobial screening of the oils. Furthermore, the affinity of the essential oil for liquid paraffin probably reduced the essential oil bioavailability in aqueous phase. This is in agreement with the report of Orafidiya *et al.* who demonstrated that the antimicrobial activity of *Ocimum gratissimum* essential oil was influenced by the presence of liquid paraffin.[41] The examination of European Pharmacopoeia criteria A for mycetes (i.e. reduction by a factor of 10^2 within 14 days of challenge with no increase up to the 28th day) revealed that the log reduction in the number of viable microorganisms was acceptable for all the batches. All the preserved creams at various ratios reduced the fungal inoculums by a factor of 10^2 for *A. niger* and *C. albicans* within 14 days. The control composed of cream preserved with methyl- and propyl- parabens satisfied the European Pharmacopoeia criteria A whereas the unpreserved base cream failed the test because of the absence of the preservative effect of the oils. It should be noted that the positive control cream produced total clearance of all the test isolates after 14 days while the oil-loaded creams did not. Though there is a wide acceptance of the challenge test as the logical test of preservative efficacy, there is no guarantee that a preservative that satisfies the conditions of the challenge test will preserve the product throughout its shelf life and other conditions of use. The study has some limitations in that only one strain of each microbe is used so the results obtained cannot be extrapolated to all strains of the microorganisms tested. Secondly, the age and time of harvest of the plants was not determined as they were purchased from the market. As such the effect of these parameters on the concentration and consequently the efficacy of the extracts could not be established

CONCLUSION

The results show that a combination of *C. citratus and C. sinensis* essential oils at ratios of 10:0, 8:2, 6:4 and 4:6 adequately preserved the cream formulation and exhibited a broad spectrum of antimicrobial activities that were synergistic. These essential oils can equally be used as fragrance to mask the pungent odour and improve the aesthetic value of creams formulated with some medicinal plants. The results demonstrate that combination of CCEO and CSEO in cream formulation is a safer and alternative means to minimise microbial contamination and to improve their quality. Looking forward, these essential oils can be utilized as green chemicals in pharmaceutical product preservation that is gaining popularity because of their environmental safety and bio-rational mode of action is promulgated.

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REFERENCES

1. Barry BW. (1983). Formulation of dermatological

vehicles. In: Barry BW (editor): *Dermatological Formulations: Percutaneous Absorption*. Mercel Dekker, Inc.; New York, pp. 296-350.
2. Billany M. (2002). Suspensions and emulsions. In: Aulton ME (editor): *Pharmaceutics: The Science of Dosage Form Design*. Churchill Livingstone; New York. pp. 334-359.
3. Flynn GL. (1990). Topical Drug Absorption and Topical Pharmaceutical Systems. In: Banker GS, Rhodes CT, (Eds.), *Modern Pharmaceutics*. New York: Marcel Dekker, pp. 298-302.
4. Allen LV, Popovich NG, Ansell HC. (2005). Semi-solid dosage forms and transdermal systems: ointments, creams, and gels. In: Allen LV, Popovich NG, Ansel HC (Eds), *Ansel's Pharmaceutical Dosage Forms and Drug Delivery Systems*. Baltimore: Lippincott Williams & Wilkins, pp. 276-297.
5. Bloomfield SF. (1990). Microbial contamination: spoilage and hazard. In: Danyer S, Baird R (Eds.), Guide to Microbiological Control in Pharmaceuticals. Chichester: Ellis Horwood. pp. 29–52.
6. Wong S, Street D, Delgado SI, Klontz KC (2000). Recalls of foods and cosmetics due to microbial contamination reported to the U.S. Food and Drug Administration. *Journal of Food Protection* 63: 1113–1116
7. Johnson C, Lods L, Scholz D, Dres C, Dashnaw RA, Linstra L, Brooks G (2000). Maintaining the integrity of cosmetic formulations via the utilization of endogenous signal molecules from plants. *Agro Food Industry Hi-Tech* (11): 23-25.
8. Seo SB, Ryu CS, Ahn GW, Kim HB, Jo BK, Kim SH, Lee JD, Kajiuchi T (2002) Development of natural preservative system using the mixture of chitosan-Inula helenium L. extract. *International Journal of Cosmetic Science*. 24: 195–206.
9. Heath HB (1981). Source book of flavours. Westport, Connecticut AVI. Pp. 560-570.
10. Iwu WM, Duncan AR, Okunji CO (1999). New antimicrobials of plant origin In: Janick J (Eds.), Perspectives on new crops and new uses. Alexandria: ASHS Press, pp. 457-462.
11. Cheel J, Theoduloz C, Rodríguez J and Schmeda-Hirschmann G. (2005). Free radical scavengers and antioxidants from Lemongrass (*Cymbopogon citratus* (DC.) Stapf.). *Journal of Agricultural and Food Chemistry*. 53: 2511-2517.
12. Halberstein RA (2012). Botanical medicines for diuresis: cross cultural comparisons In: Atta-Ur-*Rahman* (Eds.). Studies in natural product chemistry. Elsevier. Pp 31
13. Lawless J (1995). Illustrated Encyclopaedia of Essential Oil: The Complete Guide to use of Oil in Aromatherapy and Herbalism. Element Books: Rockport, MA, pp. 56-67.
14. Caccioni DRL, Guizzardi M. Biondi DM, Renda A, and Ruberto G (1998). Relationship between volatile components of citrus fruit essential oils and antimicrobial action on *Peniccilium digitatum and Penicillium italicum*. *International Journal of Food Microbiology* 43 (1-2): 73-79
15. Aibinu I, Adenipekun T, Adelowowtan T, Ogunsanya T and Odugbemi T (2007). Evaluation of the antimicrobial properties of different parts of *Citrus aurantifolia* (lime fruit) as used locally. *Africa Journal of Traditional Contemporary and Alternative medicine* 2: 185-190.
16. Lis-balchin M, Hart S (1999). Studies on the mode of action of the Essential Oil Lavender. *phytotherapy Research* 13: 540-542.
17. Reische DW, Lillard DA, Eitenmiller RR (1998). Antioxidants in food lipids. In CC Ahoh and DB Min (Eds.), Chemistry, Nutrition and Biothecnology, New York: Marcel Dekker Pp. 423-448.
18. Harbone JB (1998). Essential oils In: *Phytochemical Methods: A guide to modern techniques in plant analysis.* 3rd ed. Chapman & Hall, PA, USA. pp. 110-124.
19. Lemma H, Debella A, Addis G, Kunert O, Geyid A, Teka F, Yersaw K (2002). Antibacterial activity of *Plumbago zeylenica*L. roots on some pneumonia causing pathogens. SINET: *Ethiopian Journal of Health Sciences*. 25: 285-294.
20. Bauer A, Kirby WMM, Sherric JC and Turck N (1966). Antibiotic susceptibility testing by a standardized single disk method. *American Journal of Clinical Pathology* (45): 493-496.
21. Smith MD and Navilliat PL (1997). A new protocol for antimicrobial testing of oils. *Journal of Microbiological Methods*. 28: 21-24.
22. Prudent D, Perineau F, Bessiere JM, Michel GM, Baccou JC (1995). Analysis of the essential oil of wild oregano from Martinique (*Coleus aromaticus* Benth.): Evaluation of its bacteriostatic and fungistatic properties. *Journal of Essential Oil Research* 7:165–173.
23. Bonapace CR, Bosso JA, Friedrich LV, White RL (2002). Comparison of methods of interpretation of checkerboard synergy testing. *Diagnostic, Microbiology and Infectious Disease.* 44(4): 363-366

24. British Pharmacopoeia. (2003). Her Majesty Stationery office, Vol. 2; London. Pp. 1905- 2010.
25. Rios JL, Recio MC, Villar A (1988). Screening methods for natural antimicrobial products with antimicrobial activity: a review of the literature. *Journal of Ethnopharmacology* 23:127– 149.
26. European Pharmacopoeia Commission. (1996). Efficacy of Antimicrobial Preservation. Group of Experts No. 1CM (Microbial Contamination). Strasbourg: Council of Europe.
27. Chanthaphon S, Chanthachum S, Hongpattarakere T (2008). Antimicrobial activities of essential oils and crude extracts from tropical *Citrus* spp. against-food related microorganisms. *Songklanakarin Journal of Science and Technology* 30 (Suppl. 1): 125-131.
28. Shahidi F, Wanasundara PKJPD, Hong C. (1992). Antioxidant activity of phenolic compounds in meat model systems. In: CT Ho, CY Lee, MT Huang (Eds) *Phenol compounds in food and their effects on health 1. ACS* Symposium Series vol 506; American Chemical Society., Washington, DC, Pp. 214-222.
29. Abu-Seif FA, Abdel-Fattah SHM, Abo Sreia YH, Shaaban HA, Ramadan MM (2009). Antifungal properties of some medicinal plants against undesirable and mycotoxin-producing fungi. *Journal of Agricultural Sciences Mansuora University* 34: 1745-1756
30. Patker KI, Usha CM, Shetty SH, Paster N, Lacey J (1993). Effect of spice oils on growth and aflatoxin B1 production by *Aspergillusflavus*. *Letters in Applied Microbiology* 17: 49-51.
31. Espina L, Somolinos M, Loran S, Conchello P, Garcia D, Pangan R (2011). Chemical composition of commercial citrus fruit essential oils and evaluation of their antimicrobial property acting alone or in combined processes. *Food Control* 22(6): 890-902.
32. Aberkane A, Cuena-Estrella M (2002). Comparative evaluation of two different mehods of inoculum preparation on the antifungal susceptibility testing of filamemtous fungi. *Journal of Antimicrobial Chemotherapy* 50(6): 719-722.
33. Toure D, Bikoffi F, Kouame P, BediG, Joseph A, Guessennd N, Oussou R, Chalchit J, Dosso M, Tonzibo F (2014). Effect of geographical location on antibacterial activity of essential oil from Ivoirian *Chromolaena odorata* (L). R.M. King and Robinson (Asteraceae). *Journal of Pharmacognosy and phytotherapy* 6(6): 70-78
34. McCalley D, Torres-Grifol JF (1992). Analysis of volatiles in good and bad conditions by gas chromatography and gas chromatography-mass spectrophotometer. *Analyst.* 117: 721-725
35. Janssen AM, Scheffer JJC, Baerheim SA (1986). Antimicrobial activity of essential oils: A 1976-1986 literature review. Aspects of test methods. *Planta Medica* 53: 395-398.
36. Helal GA, Sarhan MM, Abu Shahla ANK and El-Khai EKA (2006). Antimicrobial activity of some essential oils against microorganisms deteriorating fruit juices. *Mycobiology* 34: 219-229.
37. Ofokansi KC, Adikwu MU, Esimone CO, Nwodo NM (2004). Antimicrobial properties of leaf extract of *Dissotis theifolia* (Melastomateceae). *Journal of Pharmaceutical and Allied Sciences* (1): 173-178.
38. Tallarida RJ. (2001) Drug synergism: Its detection and applications. *Journal of Pharmacology and Experimental Therapeutics.* 298 (3): 865-872
39. Matasyoh JC, Kiplimo JJ, Karubiu NM, Hailstorks TP (2007). Chemical composition and antimicrobial activity of the essential oil of *Saturejabiflora* (Lamiaceae). *Bulletin of* Chemical Society*of Ethiopia* 21(2): 249-254
40. Djilani A, Dicko A (2012). The therapeutic benefits of essential oils, nutrition, well being and health, Dr. Jaouad Bouayed (Ed), InTech, available from: http://www.intechopen.com/books/nutrition-well-being-and-health/the-therapeutic-benefits-of-essential-oils.
41. Orafidiya LO, Oyedele AO, Shittu AO, Elujoba AA (2001). The formulation of an effective topical antibacterial product containing *Ocimum gratissimum* leaf essential oil. *International Journal of Pharmaceutics* 22 (4): 177–183