



ORIGINAL RESEARCH

CitruTox[®] Hand Sanitizer - An Innovative Essential Oil and Alcohol-Based Sanitizer for preventing COVID-19 infections.

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ABSTRACT

Background: Hand sanitizers are alternatives to handwashing with soap and water for personal hygiene and are helpful when there is poor access to flowing water. Essential oil-based hand sanitizers are more effective than soap as the essential oils contain compounds that are microstatic and/or microbicidal and stay longer after application. In folk applications, limes were used to relieve common cold, flu, and other diseases and has potential to inhibit the growth of pathogenic microorganisms.

Objective: To formulate an essential oil and alcohol-based hand sanitizer from fruit peel extract of *Citrus aurantifolia* and evaluate the antimicrobial properties of the formulations against known clinical isolates found on human hands.

Methodology: CITRUTOX[®] was prepared via hydrogel formulation utilizing alkyl acrylate polymer cross polymerization with triethanolamine as the cross-linking agent. *C. aurantifolia* essential oil was analyzed via Gas Chromatography Mass Spectrometry (GCMS) and formulation evaluated for its antimicrobial activity using Agar Disc Diffusion method on common pathogenic bacteria (*B. subtilis* and *S. aureus*) and fungi (*Aspergillus ficuum* and *Aspergillus niger*).

Results: Phytochemical screening showed presence of several compounds representing 99.92% of the total oil out of which 24 have been identified. The extract showed significant results against the tested micro-organisms at different concentrations. Antimicrobial activity of the formulation increases as the concentration of the extract increases against the tested microorganisms

Conclusion: CITRUTOX[®] was most effective against *S. aureus* while lowest activity was recorded against *Escherichia coli*. The antibacterial and antifungal potency of the sanitizer could be because of presence of antimicrobial compounds in the oils which showed high content percentage of β -bisabolene and Caryophyllene that are known to have antimicrobial and antiviral effects.

Keywords: Caryophyllene; COVID-19 prevention; Essential oils; Hand sanitizer; Herbal Medicine

INTRODUCTION

Hand washing and applying hand sanitizers are important precautions to protect the skin

from pathogenic microorganisms and to prevent the spreading of many bacterial and viral infections. Coronavirus disease 2019 (COVID-19) pandemic is an ongoing

disease caused by the coronavirus and with symptoms such as severe acute respiratory syndrome, onset of fever, generalized weakness and dry cough¹. To date, most effective hand sanitizer products are alcohol-based formulations containing 62%–95% of alcohol as it is capable of denaturing the proteins of microbes and inactivating viruses². The outbreak which was first identified in Wuhan, China, in December 2019 has since spread to most parts of the world including sub-Saharan Africa with Nigeria recording over 20,000

cases as at the time of this writing. Hand sanitizers or hand antiseptics are alternatives to hand washing with soap and water, usually applied in settings with limited access to flowing water and soap. There are typically 2 types of hand sanitizers; alcohol based such as Sanex[®] and alcohol free such as Kutol[®] (Figure 1). It is also necessary to note that some of the chemicals employed as excipients and antimicrobials in alcohol-free formulations are not environmentally friendly e.g. Triclosan³.

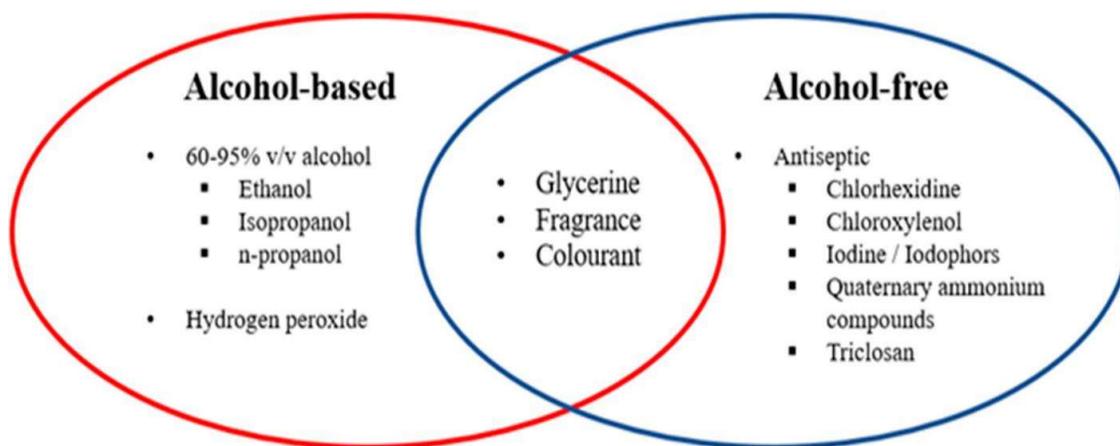


Figure 1. Alcoholic, non-alcoholic compounds, and other commonly used antimicrobials in hand sanitizers³.

C. aurantifolia (Swingle), family Rutaceae, is commonly known as lime. It is a shrubby tree of about 5m (16 ft), with many thorns. Flowers and fruit appear throughout the year⁴. The oils from the peels are mainly utilized in the perfume industry⁴. Essential oil-based hand sanitizers are more effective as antimicrobials because the essential oils contain compounds like Citral and Germacrene which have been reported to be microstatic or microcidal⁵. Currently pathogenic microorganisms exhibit wide ranging drug resistance⁶ typified by the dreaded superbugs, MRSA, and essential oils have been reported to be gaining in the struggle to defeat them as the phytocompounds in the essential oil are understood to act synergistically in killing the microbes⁷. A previous study showed

that essential oil sanitizers kill most bacteria, fungi and inactivate some viruses⁸. They contain at least 70% alcohol (mainly ethyl alcohol) and kill 99.9% of the bacteria on the hands within 30 seconds after application and 99.999% within 1 minute². Plant volatile oils are generally isolated from non woody plant material by distillation methods, usually steam or hydro distillation, and are variable mixtures of principally terpenoids, specifically monoterpenes (C₁₀H₁₆) and sesquiterpenes (C₁₅H₂₄) although diterpenes (C₂₀H₃₂) may also be present⁹. A variety of low molecular weight aliphatic hydrocarbons (linear, ramified, saturated and unsaturated), acids, alcohols, aldehydes, acyclic esters or lactones and exceptionally nitrogen and sulphur-containing compounds, coumarins

and homologues of phenylpropanoids are also present⁹. Terpenes are amongst the chemicals responsible for the medicinal, culinary and fragrant uses of aromatic and medicinal plants¹⁰. Investigations into the antimicrobial activities, mode of action and potential uses of plant volatile oils have regained momentum since their chemical analysis has become improved with GC/MS. The use of higher plants and their extracts for treating the infectious diseases has long been practiced in many parts of the world⁸. The plant derived medicines may be used in many different forms including powders, liquids or mixtures which could be raw or boiled such as, liniments, ointments and incisions⁹.

Use of ineffective sanitizers may lead to a false sense of security, despite a label claim of reducing “germs and harmful bacteria” by 99.9%. Hence, there still exists a need for verification of these claims by the regulatory authorities for the enforcement of good quality. We aimed to formulate an essential oil-based alcohol hand sanitizer from Lime fruit peel extract of *Citrus aurantifolia*, evaluating the antimicrobial properties of the formulation against known clinical isolates found on human hands.

MATERIALS AND METHODS

Plant Collection

Unripe Lime fruits (*Citrus aurantifolia*) were identified and purchased from the local market of Mile 12 Ketu, located at N 6° 34' 56.1252", E 3° 22' 29.3592" Lagos state, Nigeria. The plant was authenticated by the Taxonomist at the Botany Department of the University of Lagos herbarium and assigned Voucher number LUH 5787. The peels were manually removed using a sharp knife, weighed and stored in the -20°C freezer.

Materials

Carbopol[®] 940, Absolute Ethanol and triethanolamine were obtained from (Sigma-Aldrich, St. Louis, USA), Glycerin USP (Merck Darmstadt, Germany)

Clevenger apparatus (Pyrex), Nutrient agar, Muller Hinton agar, Water bath Uniscope (serial no: L.908805), Unified national inventory database incubator (serial no: 065782), Uniscope hot air oven (serial no: N-701341), DV-E digital viscometer (serial no: 1202M15), *DET* Standard Manufactured 2016, Expiry 2018. *PUR* standard manufactured 2016, Expiry 2019. *DET* and *PUR*; coded names of commercially available hand sanitizers.

Extraction of Samples with Steam Distillation

The unripe peels of *C. aurantifolia* fruits were used for extraction of lime oil by steam distillation using Clevenger apparatus as described by Harbone¹⁰. Essential oil measuring 12ml was obtained from 2.5kg fruit material. The oil was drained out and dried over anhydrous sodium sulphate to eliminate remaining water, placed in an amber bottle and stored at 4°C.

Hand Sanitizer Formulation

The formulation of the sanitizers applying different concentrations of extracted lime oil was carried out following Table 1. The extract was added to absolute alcohol along with glycerine and was then mixed together with other ingredients. Prepared product was stored in air-tight containers. Volume was made up to 100ml with distilled water.

Gas Chromatography - Mass Spectrometry (GC/MS)

The lime oil was submitted for GC/MS, sample was injected into a GC model 7890 (Agilent Technologies) coupled to a MS model 5975c (Agilent Technologies), column 30x0.25um Hp-5m sec fused silica capillary with a (5% phenyl)-methylpolysiloxane stationary phase (Agilent part No. 19091S-433). The mobile phase was helium gas with a flow rate of 1ml/min. The injector temperature was 250°C, the injection volume was 1µl and the oven temperature was initially programmed at 300°C for 2min. This was

then increased by 10°C per minute to a final temperature of 240°C for 6 minutes.

Table 1: Composition of Essential Oil-Based Hand Sanitizer Formulation.

COMPONENT	PURPOSE	Concentration in v/v (%)					
		F1	F2	F3	F4	F5	F6
Deionized water	Vehicle	30	30	30	30	30	30
Carbopol	Thickening agent	1.0	1.0	1.0	1.0	1.0	1.0
Alcohol	Antimicrobial	65	65	65	65	65	65
Glycerine	Emollient	0.7	0.7	0.7	0.7	0.7	0.7
Lime oil	Extract	NIL	0.2	0.4	0.6	0.8	1.0
Tri-ethanolamine (TEA)	Solubilizing agent	0.2	0.2	0.2	0.2	0.2	0.2

GC/MS was interpreted using data base of National Institute Standard and Technology (NIST) and Wiley spectra libraries. Spectrum of the unknown component was compared with spectrum of known components stored in the NIST library. Name, molecular weight and structure of the components of the test materials were recorded.

Antimicrobial Activity of *C. aurantifolia* Oil

The agar disc diffusion method was employed for the determination of antibacterial and antifungal activity of *C. aurantifolia* peel oil following a revised Baydar *et al.*,¹¹ protocol against bacteria (*B. subtilis* and *S. aureus*) and fungi (*Aspergillus ficuum* and *Aspergillus niger*) and *Candida utilis*). Standard culture media (CM139, CM271, CM145, CM69, CM7 and CM201) from Oxoid were employed for the purpose of culture maintenance at their respective temperatures that is 25°C for fungi and 37°C for bacteria as used in the food studies of Baydar *et al.*,¹¹. A final concentration of 0.5% (v/v) Tween-20 (Sigma) was incorporated into the agar after autoclaving to increase oil solubility. Briefly, a series of two-fold dilutions of each oil, amounting to 2% (v/v), was prepared in Mueller Hinton agar with 0.5% (v/v) Tween-20. The plates were dried at 35°C for 30min prior to inoculation with 1–2µl spots containing approximately 10⁴ cfu of each organism, using a multipoint replicator¹². The diameter of the cork borer

used was 11mm and zones of inhibition was measured using a pair of callipers. Mueller Hinton agar, with 0.5% (v/v) Tween-20 but no oil, was used as a positive growth control. Inoculated plates were incubated at 35°C for 48 hours. Minimum inhibitory concentrations (MICs) were determined after 24h for the bacteria and after 48h for *C. albicans*¹³. MICs were determined as the lowest concentration of oil inhibiting the visible growth of each organism on the agar plate. McFarland turbidity standard was used for optical density adjustment. The liquid culture was added drop by drop to already sterilized normal saline until the turbidity matches the turbidity of McFarland 0.5. Standards, PUR (active ingredient: Ethyl Alcohol, 70% v/v) and DET (Batch no: 385768) were used as formulated. All experiments were done in triplicate.

Statistical Analysis

Statistical comparison with the controls was carried out by One-Way ANOVA. Data presented represent means ± SEM, and values of P <0.05 were considered statistically significant.

RESULTS

The essential oil from the Lime fruit peel (*C. aurantifolia*) contained sixty (60) constituents (Table 2) which were identified using the GC analysis by means of their retention times and mass spectral fragmentation pattern.

TABLE 2: Chemical constituents of *Citrus aurantifolia* essential oil as recovered from GC/MS

R_t (min)	Area (%)	Library/Identity	Ref No.
11.149	10.37	β-bisabolene	68576
9.673	6.02	Geranyl isobutyrate	87579
10.245	5.91	α farnesene	68625
10.073	5.52	Caryophyllene	68509
9.398	4.13	2, 6-octadien-1-ol	74325
8.334	3.91	Geraniol	27449
7.973	3.85	Citral	25779
8.460	3.04	Citral	25778
10.955	2.78	Octahydronaphthalene	68739
10.760	2.78	γ-murolene	68585
12.848	2.72	1, 2, 4-methenoazulene	68897
13.003	2.62	β-bisabolene	68576
9.079	2.47	2-carene	16030
13.203	2.37	α-bisabolene	68575
9.724	2.20	Cyclohexane, 1-ethenyl-1-methyl-2, 4 bis (1-methyl ethenyl)	68686
8.171	2.04	Citral	25779
10.445	1.90	Humulene	68480
5.816	1.82	Cyclohexane	16238
12.316	1.81	1 H-benzocycloheptane	68795
6.789	1.74	Terpinen-4-ol	27505
5.599	1.68	γ-Terpinene	16078
8.248	1.66	o-gerany 2, 6-octadiene-1-ol	174836
9.998	1.60	2-decen-1-ol	29188
7.916	1.46	2, 6, 10-dodecatrien-1-ol	85761
6.881	1.41	P-menth-8-en-1-ol	27617
6.194	1.28	1, 4-hexodiene, 5-methyl-3-(1-methylidene)	16246
8.620	1.22	Thymol	24335
8.414	1.20	Geranyl vinyl ether	47881
6.829	1.18	P-menth-8-en-1-ol	27617
6.119	1.17	Fenchol	27443
6.005	1.01	Linalool	27447
7.837	0.98	Cyclopropane-carboxamidine	1372
12.528	0.97	Isospathulenol	83495
10.834	0.95	Aromandendrene	68525
5.931	0.87	Linalool	27447
9.198	0.80	Citronellyl butyrate	89552
18.450	0.76	α-murolene	68582
11.487	0.75	Cis-α-bisabolene	68617
10.680	0.71	Naphthalene	68819
11.309	0.70	Hexahydronaphthaene	68698
8.723	0.61	Dodecanal	51251
11.681	0.61	γ-elemene	68546
6.051	0.59	β-ocimene	16062
11.452	0.53	1 H-cyclopropa naphthalene	68921
10.874	0.48	α farnesene	68625
10.491	0.46	Bicyclo heptane, 2-methyl-3-methylene-2-(4-methyl-3-pentenyl)	68804
13.312	0.42	1-naphthalenol	85840
6.262	0.40	1, 3, 6, octatriene	16181
8.700	0.40	Cycloheptane 1, 3, 5, tri methylene	15241

14.594	0.40	Tetradecanal	76509
21.208	0.37	Hexacosane	217890
12.076	0.37	Naphthalene	68902
12.391	0.35	Selin-6-en-4- α -ol	85728
12.465	0.31	2-isopropyl-5-methyl-9-methylene	68697
8.889	0.27	2, 6-octadienoic acid	49449
8.832	0.24	2-methoxy-4-Vinyl phenol	25129
11.584	0.22	Cyclohexane methanol	85864
5.759	0.22	2-Furanmethanol 5-ethenyltetrahydro- α , α 5-trimethyl-cis	39800
12.665	0.20	Agarospinol	85688
3.516	0.11	α -pinene	16063

Total identified=99.92%; Monoterpenes=15.58%; Sesquiterpenes=36.54%; Alcohols=18.83%; Acids=0.27%; Ether=1.20%; Other hydrocarbons=27.5%

The chromatogram from the GC/MS (Figure 2) show the abundance of compounds contained in the oil due to the numerous

peaks observed. Most of the compounds are highly volatile and were detected before 15 minutes (arrow) of GC/MS run time.

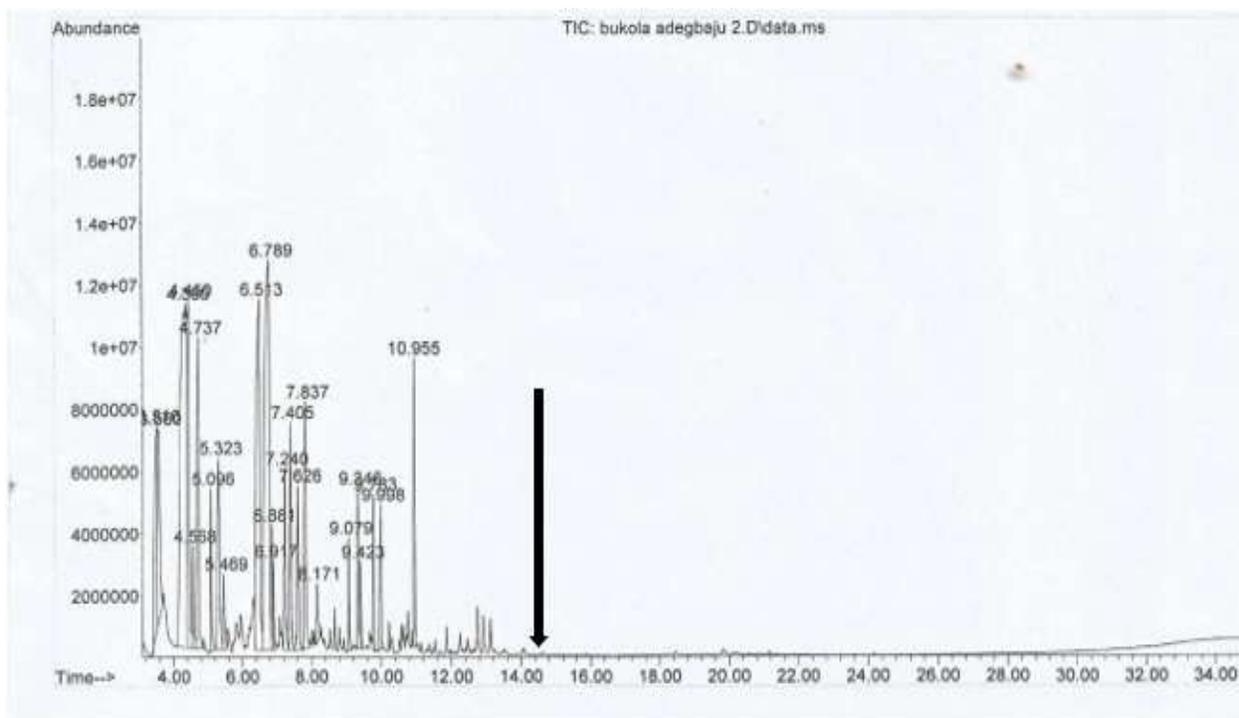


Figure 2: GC/MS Chromatogram of *C. aurantifolia* peel essential oil

F1 - F6 indicates the concentration of the formulations. F1 is negative control without any oil extract but includes alcohol as

antimicrobial which explains the zones of inhibition obtained from it from some of the cultures (Figure 3).

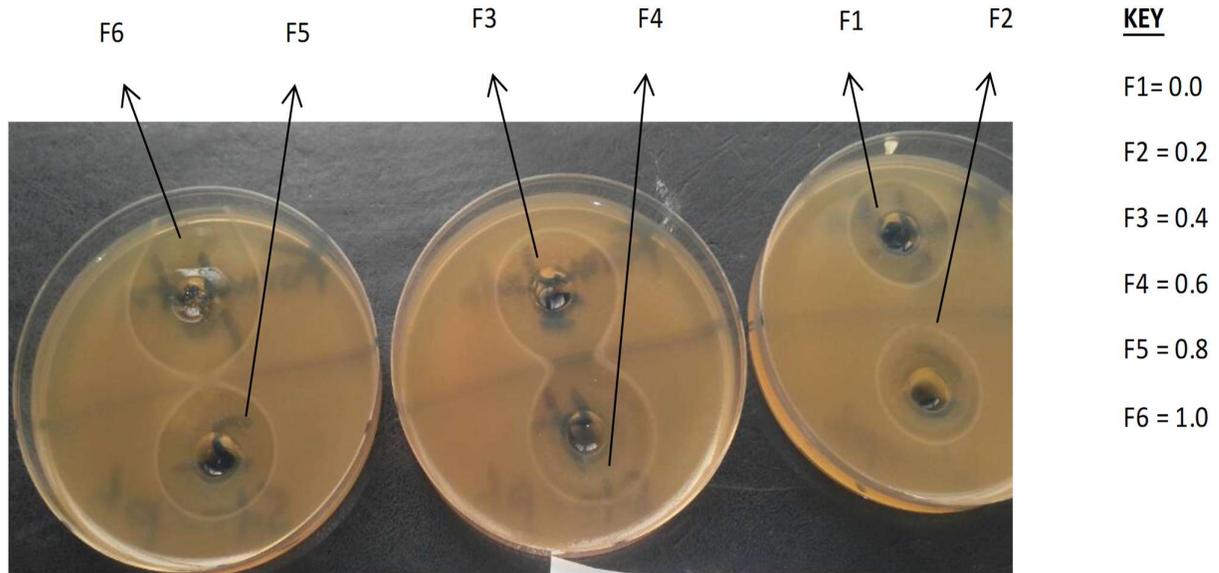


Figure 3: Zone of inhibition of essential oil-based hand sanitizer on *S. aureus*

DET is STD 1 and PUR is STD 2 are both commercial hand sanitizers. DET is locally produced and PUR is imported. It is

obvious that PUR performed better as from this figure, it had larger zones of inhibition on *S. typhi* cultures (Figure 4)

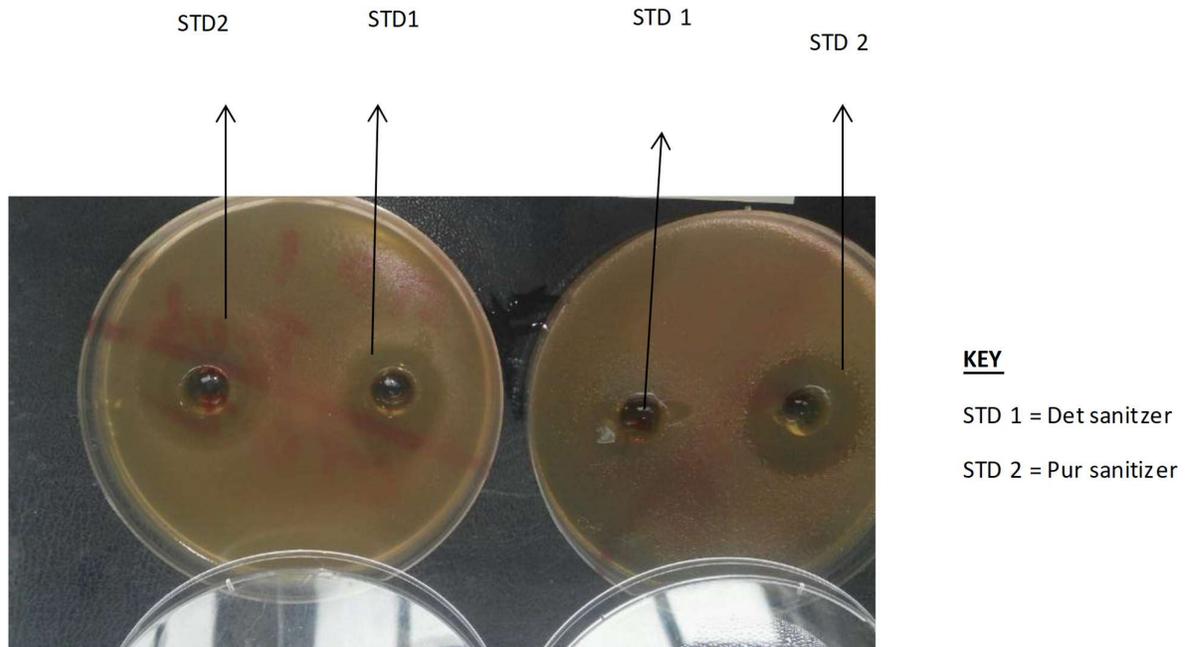


Figure 4: Minimum zone of inhibition of *DET* and *PUR* on *S. typhi*

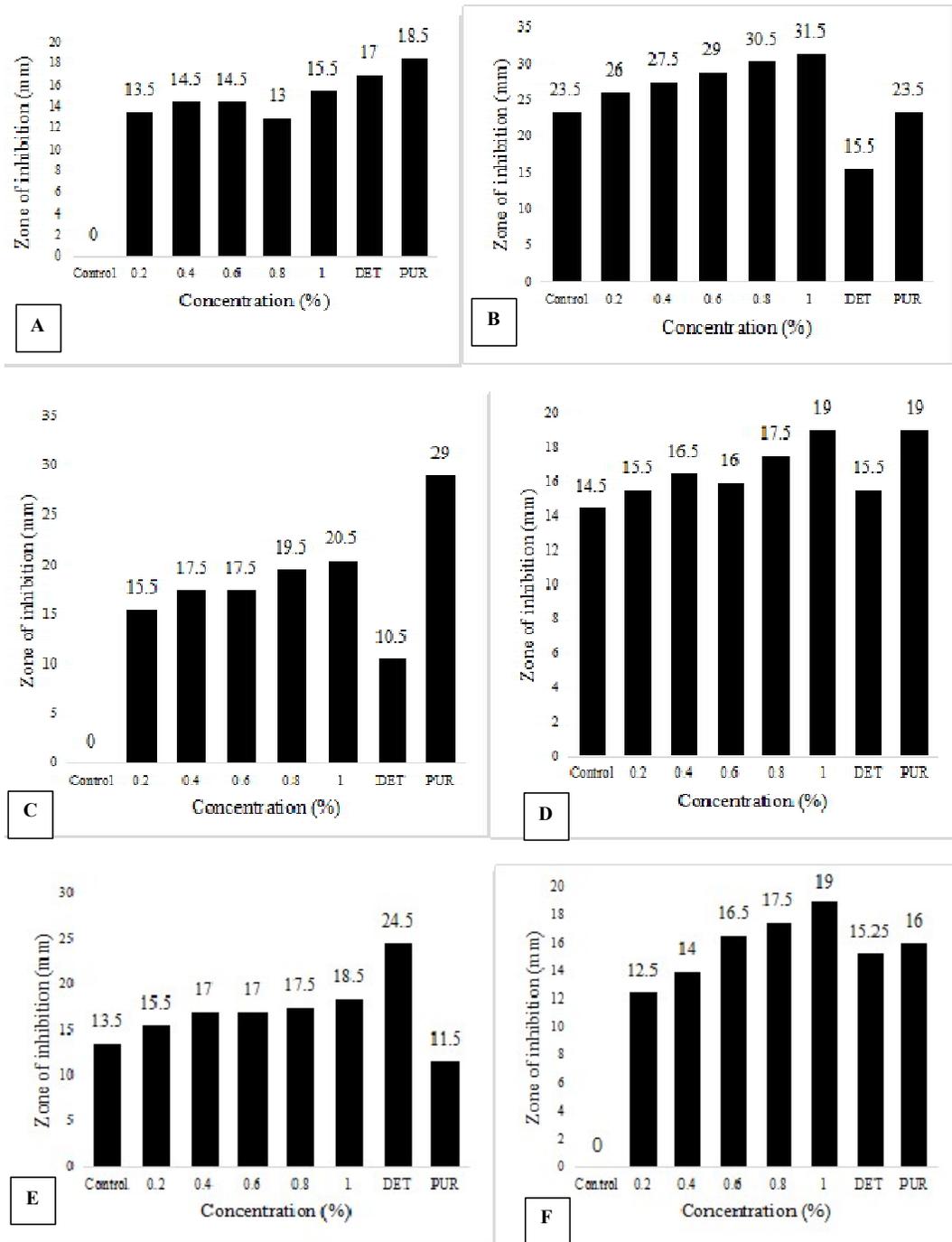


Figure 6: [A] Antimicrobial activity of essential oil-based hand sanitizer against *E. coli* [B] Antimicrobial activity of essential oil-based hand sanitizer against *S. aureus* [C] Antimicrobial activity of essential oil-based hand sanitizer against *B. subtilis*. [D] Antimicrobial activity of essential oil-based hand sanitizer against *S. typhi* [E] Antimicrobial activity of essential oil-based hand sanitizer against *P. aeruginosa*. [F] Antimicrobial activity of essential oil-based hand sanitizer against *C. albicans*.

A. Data are expressed in Mean±S.E.M (n=5); ****p<0.0001 is statistically significant compared with control. B. Data are expressed in Mean±S.E.M (n=5); *p<0.05, **p<0.01, ***p<0.001 are statistically significant compared with control C. Data are expressed in Mean±S.E.M (n=5); *p<0.05 is statistically significant compared with control. D. Data are expressed in Mean±S.E.M (n=5); *p<0.05, **p<0.01 and ****p<0.0001 are statistically significant compared with control. E. Data are expressed in Mean±S.E.M (n=5); *p<0.05, **p<0.01 and ****p<0.0001 are statistically significant compared with control. F. Data are expressed in Mean±S.E.M (n=5); ****p<0.0001 are statistically significant compared with control.

DISCUSSION

Essential oils are complex compounds, exhibiting powerful antimicrobial effects; they usually have a strong odour and are formed by plants as secondary metabolites^{14,15,16}. Human skin provides optimum growth conditions for most disease-causing micro-organisms and opportunistic pathogens. The aim of hand hygiene is sufficient reduction of microorganisms on the skin though washed hands can become re-contaminated from sinks and water faucets. *Pseudomonas aeruginosa*, commonly found in tap water, is a water-borne-bacteria from the plumbing system which may be present in the tap water being used for handwashing. In contrast, essential oil-based hand sanitizer eliminates the risk of hand contamination because the composition of the formulation kills microorganisms present on the hands, on application. The essential oil-based hand sanitizers have excellent, rapid germicidal activity against bacteria, fungi, and antimicrobial activity is based on protein denaturation of microorganisms¹⁷.

GC/MS analysis of the essential oil *C. aurantifolia* revealed the presence of 60 components representing 99.92% of the total oil, out of which 24 of these compounds have been identified from their fragmentation pattern by mass spectrometry. *Citrus aurantifolia* oil was found to be a mixture of monoterpenes, sesquiterpenes and oxygenated hydrocarbons. The sesquiterpenes, β -bisabolene was identified as a major component in terms of quantity followed by Caryophyllene, 2-carene and humulene making the sesquiterpenes the more abundant component in the essential oil studied. However, Jafari's work found monoterpene hydrocarbons to be more abundant compounds in *C. aurantifolia* essential oil¹⁸. β -bisabolene is a known antiviral compound¹⁹ which is found in the oils from *C. aurantifolia*. Among the monoterpenes, Citral has been reported to

have antimicrobial effects, especially against MRSA methicillin resistant *Staphylococcus aureus*, where it was found to have similar activity as current drugs like Ampicillin²⁰.

Citral is a naturally occurring mixture of neral and geranial geometric isomers of 3, 7-dimethyl-2, 6-octadien-1-al, and an important monoterpene aldehyde that produce some of the most intense aromas and are key aroma impact compounds in many essential oils²⁰. Other monoterpenes identified were γ -terpinen, thymol, fenchol and linalool. Limonene, a major component of all citrus oils was not found in the essential oil of *Citrus aurantifolia*, the absence of limonene in the oil increased its importance among citrus oils. This observation was supported in a similar work where limonene was also not present²¹. Thus, these compounds might have several invasive targets which could lead to the inhibition of bacterial pathogens²². Environmental contamination has been demonstrated to play an important role in the transmission of certain communicable pathogens, such as MRSA²⁴. Gram-negative *Bacillus subtilis* with multiple-drug resistance and MRSA are major nosocomial pathogens. Data from a previous study²⁵ suggests that these pathogens do not exhibit decreased susceptibility to disinfectants²⁶. However, this study provided evidence that Gram-positive *Staphylococcus aureus* is susceptible to essential oil-based hand sanitizer with a large zone of inhibition (31.5mm). The investigations carried out on essential oil-based hand sanitizer showed significant $p < 0.05$, $p < 0.01$ and $p < 0.001$ results against the tested micro-organisms at different concentrations. Antimicrobial activity of the formulation increases as the concentration of the extract increases against the tested microorganisms. The mean zone of inhibition of *C. albicans* by the essential oil-based hand sanitizer is significantly $p < 0.0001$ higher at 1.0% but significantly lower at 0.6% when compared to that of the standard *PUR*. The zone of

inhibition of *S. aureus* by the essential oil-based hand sanitizer showed significantly high values at 0.2%, 0.4%, 0.6%, 0.8%, and 1.0%, while the formulation (F1) without the essential oil extract which served as control showed the least value as same as the reference standard and this is as a result of the absence of the extract (*Citrus aurantifolia* oil). Synergistic effects against pathogens from the complex mixture of chemically different components of the extract may be the explanation for the result obtained²⁶.

The composition of *Citrus aurantifolia* has been attributed to properties like free radical scavenging, anti-helminthic, antimicrobial, anti-inflammatory and analgesic activity²⁷.

The antimicrobial activity of the essential oil-based hand sanitizer was found highest against *S. aureus* while lowest activity was found against *Escherichia coli* which are facultative anaerobic, Gram-negative bacteria. The low activity of the Gram-negative bacteria *Escherichia coli* could be due to the complexity of the cell wall of this microorganism. The external membrane of Gram-negative bacteria causes highly hydrophilic surfaces whereas the negative charge of the surface of the Gram-positive wall may reduce their resistance to antibacterial compounds. A previous research work showed effective antimicrobial activity of Basil essential oil against *Staphylococcus aureus*²⁸ utilizing activities of the oils against diverse Gram positive and Gram-negative bacteria. The results from the investigation showed that essential oil based-hand sanitizer was more effective as an antimicrobial agent than when compared to the alcohol-only based sanitizer controls. The potency could be as a result of higher percentage compositions of β -bisabolene, Caryophyllene, Geranyl isobutyrate and a significant number of monoterpenes present in the extracted oil. The active ingredients responsible for antimicrobial potential of Citrus peel oils are monoterpene components and sesquiterpenes²⁹. Linalool or citral is a

major attributor for the antimicrobial capacity of Citrus peel oils. Previous work revealed that the inhibitory influence of Citrus peel essential oils is owed to the presence of linalool rather than limonene³⁰. However, it has been found that antimicrobial activity is not only produced by one particular major component but also due to the antagonistic and synergistic effects of the variety of compounds present³¹.

Limitations

The limitation to this study is the very low yield of essential oils from the lime peels which makes collection a tedious process.

CONCLUSION

Contact with the hands is the most common mode of transmission of pathogens, and in the current COVID-19 pandemic, the use of essential oil-based hand sanitizer plus personal hand hygiene has the ability to serve as an efficient prevention tool to curb the spread of bacterial and viral infections. The results showed that CITRUTOX[®] has a significant antimicrobial effect on the tested microorganisms. Thus, there is immense potential in establishing the use of antimicrobial herbal products as a measure to prevent the spread of communicable diseases like COVID-19.

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