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Formulation Of Pine Oil And Pine Oil - Chlorhexidine Gluconate Disinfectants And Their Antibacterial Activities Against Staphylococcus Aureus

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Abstract
Background *The outbreak and transmission of infectious diseases can be prevented by the regular disinfection of high-touch surfaces. Such surfaces often harbour disease-causing microorganisms (pathogens) but through disinfection, the pathogens are eliminated or inactivated, thereby decontaminating the surfaces. A disinfectant is a chemical agent or substance that destroys or irreversibly inactivates pathogens like bacteria, viruses and fungi on surfaces of inanimate objects. Thus, disinfectant formulations contain various biocidal active agents responsible for their antimicrobial activities.*
Materials and Methods *In this study, a total of twelve liquid disinfectant variants were formulated using aromatic pine oil (PO) alone and PO in combination with chlorhexidine gluconate (PO-CG) as the active antimicrobial agents. The PO disinfectants were produced using varied aromatic pine oil levels of 12, 14, 16 and 20 weight % in sodium ricinoleate soap solution. The PO-CG disinfectant variants were also formulated using a combination of pine oil and chlorhexidine gluconate (CG) at CG levels (weight %) of 0.3 (PO-CG1) and 0.5 (PO-CG2). The antibacterial activities of the disinfectant variants were evaluated by determination of their minimum inhibitory concentrations (MICs) and inhibition zone diameters using agar well diffusion method. The bacterial species, Staphylococcus aureus was used as the test microorganism.*
Results *All the twelve disinfectant variants displayed bacteriostatic (inhibitory) activity against S. aureus to varying degrees as they all had zones of inhibition where there was no visible growth of S. aureus after incubation. The inhibitory properties of the PO disinfectants were found to increase with increasing pine oil levels as observed in their MICs of 1:30, 1:40, 1:50 and 1:60 obtained for 12, 14, 16 and 20 weight% pine oil respectively. The PO-CG1 variants containing 0.3 wt% CG showed greater potency than the PO variants from their MICs of 1:70, 1:80, 1:100 and 1: 115 for PO-CG1a, PO-CG1b, PO-CG1c and PO-CG1d, respectively. However, an optimum antibacterial activity was attained at PO level of 20% in combination with CG level of 0.3% (PO-CG1d). At 0.5% CG (PO-CG2) there was no further improvement in the potency of the PO variants as the MICs of PO-CG2 were similar to those of PO-CG1.Thus, all the disinfectants formulated with 12-20 weight % of pine oil had antibacterial activity against S. aureus which was enhanced when 0.3% CG was combined with the various levels of pine oil. The disinfectants all possessed odour-masking property as an additional benefit.*
Keywords: *antibacterial, aromatic pine oil, chlorhexidine gluconate, disinfectant, minimum inhibitory concentration*

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I. Introduction

The consciousness of the need to take preventive measures against the outbreak and transmission of infectious diseases, has increased worldwide following the COVID 19 pandemic (2020-2023) which had devastating socio-economic impact globally with horrifying number of fatalities. One of such measures is the regular cleaning and disinfection of frequently-touched surfaces which help to prevent the transmission of infectious diseases contracted through contact with contaminated surfaces. [1, 2] A disinfectant is a chemical agent or substance that destroys or irreversibly inactivates disease-causing microorganisms (pathogens) like bacteria, viruses and fungi on surfaces of inanimate objects. [3, 4, 5.] Effective disinfection involves eliminating or reducing harmful microorganisms from surfaces, water, air, and objects.[6] Thus, a disinfectant does not necessarily kill all microorganisms but reduces them to a level that is not harmful to health or that would not quickly degrade perishable goods. Disinfectants function by destroying the cell walls of microbes or by disrupting their metabolism. [3] The popular commercial antiseptics and disinfectants found in the Nigerian market contain different biocidal actives and combinations of biocides which are responsible for their antimicrobial activities against pathogens. The brand names of six popular commercial disinfectants and the active biocidal agents they contain according to their product labels are : *Dettol* Antiseptic- Disinfectant (chloroxylenol + aromatic pine oil); *Septol* Aniseptic Disinfectant (chloroxylenol + pine oil); *Carat* Disinfectant (chloroxylenol + aromatic pine oil); *Savlon* Antiseptic Liquid (chlorhexidine gluconate + cetrimide), *Purit* Antiseptic Liquid (chlorhexidine gluconate + cetrimide + aromatic pine oil) and Carex Antiseptic liquid (chloroxylenol). From the aforementioned active agents, it is evident that while some of the antiseptics and disinfectants contain pine oil in combination with chloroxylenol as antimicrobial agents, none of them contains only pine oil and pine oil + chlorhexidine gluconate only as the active agents. Aromatic pine oil serves the dual purpose of inactivating pathogens and imparting odour-masking effect when used in disinfectants. In this work, therefore twelve alternative f disinfectants composed of aromatic pine oil alone and aromatic pine oil combined with chlorhexidine gluconate as the active antimicrobial agents were formulated, and the levels of their antibacterial potency against *Staphylococcus aureus* were determined. An antimicrobial agent is any of the many different chemical substances and physical agents used to eliminate or stop the growth of microorganisms.[7] Pine oils, derived by steam distillation of wood from pines, consist of complex mixtures of monoterpene hydrocarbons (alpha, beta-pinene) and oxygenated monoterpenes (terpineol, borneol, bornyl acetate). [8,9] Disinfectant liquids produced with pine oil have the advantages of being inexpensive, biodegradable, environment-friendly, low in toxicity and when mixed with soap, form good cleansers. [8, 9, 10] Concentrated formulations of pine oil disinfectants may contain over 50% pine oil with soap/anionic surfactant and alcohol to yield white, milky emulsions when diluted with water (blooming effect) with a characteristic pine odour.[8] Pine oil disinfectants inactivate rather than kill bacteria and this limits their use to general home-cleaning activities where a supplementary advantage is offered by their odour-masking action.[3] Chlorhexidine gluconate (CG) is a relatively rapid-acting bactericidal agent that is effective against both gram-positive and gram-negative, bacteria, and some fungi. [3,11,12] It is probably one of the most widely used biocides in antiseptic products, particularly in hand washing and oral products, and is also used as a disinfectant and preservative.[13,14] Despite the advantages of CG, its activity is pH-dependent and its potency is reduced in the presence of organic matter.[14] It is envisaged that the addition of CG to the aromatic pine-oil based disinfectants will boost the antimicrobial efficacy of the disinfectant since CG is a very effective broad spectrum biocide. Sodium ricinoleate is the sodium salt of ricinoleic acid, an unsaturated hydroxyl fatty acid which is the major component (about 90%) of castor oil. It is a biodegradable, non-toxic anionic surfactant widely used in cosmetics and pharmaceutical products. [15, 16] Early studies have shown that sodium ricinoleate also possesses antimicrobial activity. [17, 18] It is used in making soaps where its molecular structure causes it to lather more easily than comparable sodium soaps derived from fatty acids. [19] *Staphylococcus aureus, a* major pathogen of humans is one of the most clinically significant bacterial pathogens responsible for infections ranging from superficial skin lesions; to life-threatening conditions like pneumonia. [20]

II. Materials And Methods

Materials

The materials used in the formulation of the disinfectant liquid variants are aromatic pine oil, sodium ricinoleate, chlorhexidine gluconate and isopropyl alcohol. The materials were all of pharmaceutical grade and obtained from a reputable importer of pharmaceutical grade chemicals in Lagos, Nigeria.

The microorganism used in the study was *Staphylococcus aureus.* Pure cultures of *S, aureus* were obtained from the Department of Microbiology & Parasitology, David Umahi Federal University of Health Sciences, Uburu, Ebonyi State, Nigeria.

Disinfectant Formulation Design & Experimental Trials

Several formulations of the disinfectant variants were designed by systematically varying the levels of the components of the disinfectants. Series of experimental trials were carried out using the designed formulae until clear, homogeneous PO and PO-CG liquid disinfectant variants which showed good stability over time were obtained. A total of twelve disinfectant variants were formulated comprising four PO, four PO-CG1 and four PO-CG2 disinfectant variants.

Formulation 1: Pine Oil (PO) Disinfectant Variants (DV) Formulae

The formulae used in the production of the disinfectant liquid variants based only on aromatic pine oil is given in Table 1.

Table 1: Formulae for the Production of Aromatic Pine Oil (PO) Disinfectant Liquid Variants

Component	Weight%			
	POI	**PO2**	**PO3**	**PO4**
Aromatic pine oil	12.00	14.00	16.00	20.00
Isopropyl alcohol	5.00	5.00	5.00	5.00
Sodium ricinoleate	16.00	17.00	18.00	19.00
Distilled water	67.00	64.00	61.00	56.00
Total	100.00	100.00	100.00	100.00

Production procedure

The required quantity of aromatic pine oil was weighed into a beaker and isopropyl alcohol was added to give a homogenous solution (A). Sodium ricinoleate was added to A to give a clear liquid (B). Distilled water was gradually added to B with stirring to give a clear, homogeneous, golden yellow liquid, which is the disinfectant.

Formulation II: Pine Oil- Chlorhexidine Gluconate (PO-CG 1) Disinfectant Variants

The formulae used in the production of Pine Oil-Chlorhexidine Gluconate Disinfectant variants using CG level of 0.3wt% (PO-CG 1) is given in Table 2

Table 2: Formulae for Pine Oil - Chlorhexidine Gluconate Disinfectant Variants at 0.3 wt% CG Level (PO-CG1)

Components	Weight%			
	PO-CG1a	**PO-CG1b**	**PO-CG1c**	**PO-CG1d**
Aromatic pine oil	12.00	14.00	16.00	20.00
Isopropyl alcohol	5.00	5.00	5.00	5.00
Sodium ricinoleate	16.00	17.00	18.00	19.00
Distilled water	66.70	63.70	60.70	55.70
Chlorhexidine gluconate	0.30	0.30	0.30	0.30
Total	100.00	100.00	100.00	100.00

Production Procedure

The required quantity of aromatic pine oil was weighed and mixed with about 50% of the required isopropyl alcohol (A). Chlorhexidine gluconate was mixed with the remaining quantity of IPA and added to the pine oil solution (A). Sodium ricinoleate was added to solution (A) to give a clear liquid (B). Distilled water was gradually added to B with stirring to give a clear, homogeneous, golden- yellow liquid, which is the disinfectant.

Table 3: Formulae for Pine Oil-Chlorhexidine Gluconate Disinfectant Liquid Variants at 0.5 wt% CG Level (PO-CG2)

Component	Weight %			
	PO-CG2a	**PO-CG2b**	**PO-CG2c**	**PO-CG2d**
Aromatic pine oil	12.00	14.00	16.00	20.00
Isoprophyl alcohol	5.00	5.00	5.00	5.00
Sodium ricinoleate	16.00	17.00	18.00	19.00
Distilled water	66.50	63.50	60.50	55.50
Chlorhexidine gluconate	0.50	0.50	0.50	0.50
Total	100.00	100.00	100.00	100.00

Production Procedure: Same as for PO-CG1

Determination of Minimum Inhibitory Concentrations (MICs) /Bacteriostatic Activity of the Disinfectants

The minimum inhibitory concentration (MIC), which is indicative of the bacteriostatic property of disinfectants, is the lowest concentration (highest dilution) at which the disinfectant is active against microbial activity under the test conditions. The bacteriostatic property tests were carried out on the formulated disinfectant samples to determine the degree of their antimicrobial effectiveness against *Staphylococcus aureus.* The MIC test is therefore simply a means of assessing the bacterial inhibitory activity of the disinfectant samples using serial dilution technique or in some cases, a customised dilution technique as was used in this work. Thus, in this study, the highest dilution (or lowest concentration) at which the disinfectant inhibited the visible growth of *Staphylococcus aureus* was taken as its MIC. The MIC is usually expressed in the ratio 1: x, which represents volume of the disinfectant (1) to volume of water + disinfectant (x), which is the dilution ratio.

Collection of Test Organism

The test microorganism used in this study was *Staphylococcus aureus*. Pure cultures of *S. aureus* identified using standard microbiological and biochemical methods, were obtained from the Department of Microbiology and Parasitology, David Umahi Federal University of Health Sciences, Uburu, Ebonyi State, Nigeria. The isolates were sub-cultured on agar slants and stored at 4^0 C in the refrigerator until needed for the study.

Preparation of Test Inoculum: The test inoculum was prepared using a standard method as described by Monica Cheesbrough. [21] Fresh pure cultures of the test organism were prepared by incubation overnight at 37^0C on appropriate culture media. The inocula were adjusted to the same level. Normal saline was prepared by dissolving 8.5g of sodium chloride (NaCl) in 1000ml of distilled water and sterilised by autoclaving at 121^0C for 15 min. Subsequently, 4ml aliquots of the sterile saline were dispensed into sterile test tubes. Overnight cultures of the test organisms were sub-cultured into the saline solution using a sterile wire loop and aseptically inoculated into separately labelled test tubes. The turbidity of the suspensions was adjusted by adding more bacterial colonies or sterile saline until it reached the 0.5 McFarland standard, approximately equal to 1.0 x 10^6 CFU/ml.

Determination of Antimicrobial Activity of Diluted Disinfectants Using Agar Well Diffusion Method

a) Dilution of the Disinfectant Variants: A customised dilution technique was employed for the determination of the MICs of the disinfectants. 1ml of each disinfectant variant was added to increasing volumes of distilled water (ml) as follows, 10, 20, 30, 40, 50, 60, 70, 80,90,100……..130. The same dilution procedure was carried out for all the disinfectant variants of PO, PO-CG1 and PO-CG2.

b) Agar Well Diffusion Method: The inhibitory activities of the diluted disinfectants against the test organism were determined using Agar well diffusion technique according to the Clinical and Laboratory Standard Institute [22] on Mueller-Hinton Agar (Oxoid) medium. Mueller-Hinton agar (20 ml) was poured into sterile Petri dishes (plates) and allowed to solidify, then properly labelled. Each *S. aureus* isolate was then streaked on agar surface of the Petri dishes using a sterile swab stick. Thereafter, a sterile 6mm cork borer (Fischer Scientific) was used to make wells on the inoculated plates. A total of five wells were made on each plate, one for each concentration of the diluted disinfectant variant**.** The disinfectant dilutions were delivered into the respective wells using sterile micro pipettes. Thereafter, the plates were allowed to stand for 30 min before incubation at 37°C for 24h.

c) Measurement of Inhibition Zone Diameters (IZDs): The incubated agar plates were placed upside down on a flat surface. The clear, circular area surrounding the disinfectant (Zones of growth inhibition) were identified and their diameters measured in millimetres (mm) using a transparent metric ruler.

Physical Properties of the Disinfectant Variants: The physical properties of the formulated disinfectant variants were evaluated in terms of physical appearance, pH, foamability. Their odour-masking ability was assessed organoleptically.

III. Results

Physical Properties of the Disinfectant Variants

All the twelve disinfectant variants were clear, homogenous golden-yellow liquids. Their pH values were in the range of 8.0 - 8.5.They all exhibited the phenomenon of turning water milky/cloudy due to micro-emulsification which is a common occurrence when pine oil and chloroxylenol-based disinfectants containing soap/anionic surfactant and alcohol come in contact with water.[8] The disinfectants all possessed odour-masking property as an additional benefit.

Antibacterial Activity of the Disinfectant Variants (DVs)

The zones of inhibition as well as the results of the determined MICs of the disinfectant variants (DVs) at different pine oil and chlorhexidine gluconate levels are presented in Table 4 and Fig.1. The presence of inhibition zones in all the DVs is an indication that all of them successfully inhibited the activity of *S. aureus,* though the degree of inhibitory activity varied with the composition and concentration of the disinfectants.

Table 4: Zones of inhibition (mm) and Minimum Inhibitory Concentration (MIC) of Disinfectant Variants

Disinfectant Variant	Inhibition Zone Diameters (mm)	MIC/Highest Dilution Ratio
PO1	+	1: 30
PO2	+	1: 40
PO3	+	1: 50
PO4	++	1: 60
PO-CG1a	++	1 : 70
PO-CG1b	++	1 : 80
PO-CG1c	+++	1: 100
PO-CG1d	+++	1:115
PO-CG2a	++	1: 70
PO-CG2b	++	1 : 80
PO-CG2c	++	1: 90
PO-CG2d	+++	1 : 110

Zones of inhibition: Below 6mm (-) Inactive; 6-9mm (+) slightly active; 9-12mm (++) Moderately active; 12-16mm (+++) Highly active

A comparison of the MICs of the twelve DVs containing different amounts of pine oil, chlorhexidine gluconate is given in Table 5.

Table 5: Comparison of the Composition and MICs of the Disinfectant Liquid Variants

Disinfectant Variant	Aromatic Pine Oil Weight%	Cl-G Weight%	Highest Dilution Ratio / MIC
PO1	12.00	0.00	1:30
PO-CG1a	12.00	0.30	1:70
PO-CG2a	12.00	0.50	1:70
PO2	14.00	0.00	1:40
PO-CG1b	14.00	0.30	1: 80
PO-CG2b	14.00	0.50	1:80
PO3	16.00	0.00	1: 50
PO-CG1c	16.00	0.30	1: 100
PO-CG2c	16.00	0.50	1: 90
PO4	20.00	0.00	1: 60
PO-CG1d	20.00	0.30	1 : 115
PO-CG2d	20.00	0.50	1 : 100

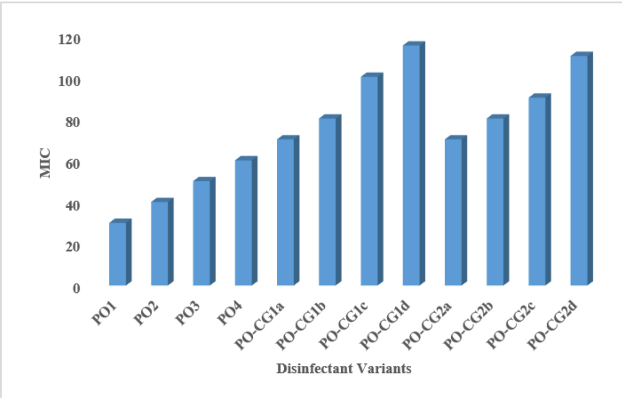

Fig I: MICs of the 12 Disinfectant Variants PO, PO-CG1 and PO-CG2

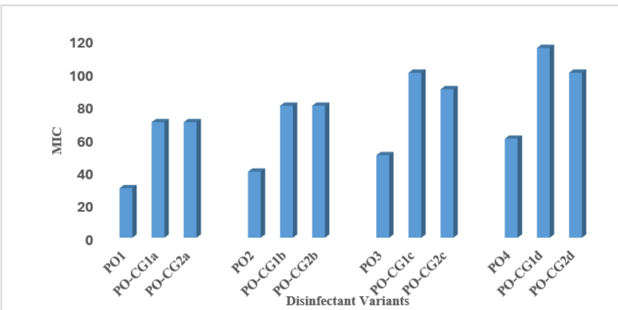

Fig I: Comparison of the MICs of the 12 Disinfectant Variants based on composition

IV. Discussion

Physical Properties: The odour-masking ability of the disinfectant variants is due to the fresh, woody fragrance of aromatic pine oil. The odour-masking effect was found to increase with increase in the concentration of pine oil in the disinfectant such that the variant containing 20% pine oil had the greatest impact and 12%, the least. All the disinfectant variants showed very good foamability when compared with ordinary soap solutions. This can be attributed to the presence of pine oil which boosts the lathering ability of sodium ricinoleate, $CH_3 (CH_2)_5 CH(OH)CH_2CH=CH(CH_2)_7COONa$, a soap with intrinsically good lathering ability.[6] Similarly, the twelve DVs all possessed slight cleansing property which is also attributable to the presence of sodium ricinoleate (SR) in the formulations. Sodium ricinoleate, like all soaps, has a hydrophilic (polar) head and a hydrophobic tail which make it amphipathic (amphiphilic) and thus effective in removing both non-oily and oily dirt.[15]

Antibacterial Activity All the twelve disinfectant variants displayed inhibitory (bacteriostatic) activity against S. *aureus* indicating susceptibility of *S. aureus* to all the variants. This was evident in their inhibition zone diameters (IZDs) and MICs which confirmed their antibacterial activity against *Staphylococcus aureus,* but to varying degrees. The zones of inhibition are clear, circular areas on agar plates where the test organism did not grow. The efficacy of a disinfectant is dependent on the concentration at which it is used, which is determined from the MIC. Thus, the twelve DVs had the ability to inhibit the activity and growth of *S. aureus***,** thereby suppressing its potential pathogenicity.

The PO disinfectants were found to have relatively low antibacterial activity compared to the PO-CG variants. The antibacterial activities of the variants were found to increase with increasing pine oil levels as evident in their highest dilution ratios of 1:30, 1:40, 1:50 and 1:60 obtained for 12, 14, 16 and 20 weight% pine oil, respectively. The PO-CG1 variants containing 0.3wt% CG in addition to pine oil, exhibited inhibitory properties appreciably superior to those of POs as shown in their MICs (highest dilution ratios) of 1:70, 1:80, 1:100 and 1: 115 for PO-CG1a, PO-CG1b, PO-CG1c and PO-CG1d, respectively. For instance comparing the MICs of PO1 (1: 30) and POCG1a (1: 70), it shows that PO-CG1a is much more potent than PO1 because it is more effective at a lower concentration (higher dilution), which means a smaller amount is needed for antibacterial efficacy than PO1. This greater effectiveness observed by the incorporation of CG, a highly efficacious, broad spectrum antimicrobial agent into pine oil disinfectants evidently enhanced the antibacterial property of the PO-CG1 disinfectant liquids until an optimum level was attained. Pine oil disinfectants generally have low antibacterial efficacy compared to other disinfectants [8] thus the incorporation of a stronger biocidal agent improved its efficacy. This increased bacteriostatic efficacy of PO-CG1 variants is suggestive of synergistic interaction between pine oil and chlorhexidine gluconate at the specific concentrations investigated. This optimum bacteriostatic effect was attained at PO level of 20% in combination with CG level of 0.3% (PO-CG1d) having a highest dilution ratio of 1:115. At 0.5% CG, there was no further improvement in inhibitory properties as the PO-CG2 had MICs similar to those of PO-CG1. This is evident in PO-CG1a versus PO-CG2a and PO-CG1b versus PO-CG2b. In the cases of PO-CG 1c vs PO-CG 2c and PO-CG1d vs PO-CG2d, there was a slight drop in bacteriostatic activity as observed in their reduced MICs indicating that the optimum level of CG has been exceeded. This shows that the use of chlorhexidine gluconate beyond 0.3wt% in combination with pine oil has reduced potency and is not cost-effective.

The significance of the MIC test is that in diluting the disinfectant with water for practical use, it provides the correct amount of water to be added by users to ensure that the disinfectant is still potent after dilution. This is why the appropriate dilution levels for disinfectants that will ensure their effectiveness are always specified by manufacturers since over-dilution can render the disinfectant ineffective. Under-dilution, on the other hand, can make the product hazardous, uneconomical and it may have a damaging effect on surfaces by corroding, staining or degrading surfaces. [23, 24i]

The dilution ratios of the disinfectants (1:30 to 1:115)were found to be within the range of MIC's of the commercial disinfectants in the Nigerian market as stated on their labels based on the type of application. This shows that all the twelve disinfectant variants can be used as disinfectants as they all inhibited the activity of *S. aureus*

V. Conclusion

This study has proven that all the twelve disinfectants formulated using aromatic pine oil alone and aromatic pine oil combined with chlorhexidine gluconate have antibacterial activity against the test bacterial species, *Staphylococcus aureus*. The bacteriostatic efficacy of the PO variants increased with increasing levels of pine oil. The PO-CG1 disinfectants had greater bacteriostatic effectiveness than those of PO with an optimum attained at PO-CG1d which contained 20 wt% PO and 0.3wt% CG. The PO-CG2 variants had antibacterial properties similar to those of PO-CG1 as there was no enhanced antibacterial potency with the increased level of CG from 0.3 % to 0.5%. All the twelve variants were found to possess odour-masking property provided by the fresh, woody scent of aromatic pine oil as additional benefit.

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