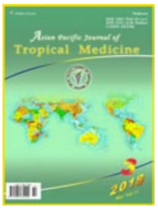

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Effects of some common additives on the antimicrobial activities of alcohol−based hand sanitizers

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ABSTRACT

Objective: To study the effects of some common additives on the antimicrobial activities of alcohol-based hand sanitizers. **Methods:** The antibacterial activities of varying aqueous concentrations of ethanol and isopropyl alcohol were tested by the agar well diffusion method. The influences of different concentrations of glycerin was similarly tested. Finally, isopropyl alcohol and benzalkonium chloride were combined in different ratios within the safe use concentrations of each, and the effects of these combinations were compared with values obtained for the two agents used alone. Statistical methods, such as student *t* test and one-way ANOVA were used when appropriate to evaluate the differences in activity. **Results:** The activities of the alcohols showed marked concentration dependence, and both showed peak activity at 85%–95% concentration range. Over the concentration range of 60%–100%, isopropyl alcohol inhibited more bacterial and fungal organisms than ethanol, though the inhibition zone diameters it produced were not statistically different from those of ethanol for organisms which were sensitive to both of them. Addition of glycerin reduced the antimicrobial activities of the isopropyl alcohol, as shown by reduction in the inhibition zone diameters produced *in vitro*, which may be due to reduced drug diffusion with increase in viscosity. Addition of benzalkonium to isopropyl alcohol systems improved the activity of the alcohol, but the overall activity of the combination was not superior to that seen in the use of benzalkonium alone. **Conclusion:** Alcohol-based hand sanitizers should not be used outside the concentration range of 85%–95% and isopropyl alcohol inhibits more bacterial and fungal organisms than ethanol for most concentrations. Inclusion of benzalkonium improves the antimicrobial spectrum and activity of isopropyl alcohol, and the combination may justifiably be used to achieve both immediate and long lasting effect. Glycerin may adversely affect the antimicrobial activities of isopropyl alcohol-based hand sanitizers and should be used with caution.

1. Introduction

The purpose of handwashing using any of the available methods is to remove germs and prevent their spread, with or without their inactivation[1]. Hand hygiene is very important and has been reported to be able to achieve up to 31% reduction in the incidence of gastrointestinal illness and up to 21% reduction in respiratory illness globally[2]. This translates into millions of lives saved. In some cases like Ebola where vaccines do not yet exist, non-pharmaceutical interventions such as handwashing and use of hand sanitizers become very important interventions, especially in school-aged children, who can become heavily affected by viruses[3]. This is also true for other infectious diseases including cholera and tuberculosis. The increasing importance of hand sanitizers is tied to scarcity of water and soap in many locations, fueling a quest for alternative hygiene methods not based on water but equally effective or even more effective[4-7].

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The components of hand rubs or hand sanitizers are either bactericidal or bacteriostatic, though the biological outcome may depend on concentration[8,9]. A number of agents including phenolics, alcohols and quaternary ammonium compounds are used in hand disinfection preparations. On the other hand, the efficacy of any particular hand sanitizer is affected by bioload, the concentration of agent used, presence of biofilms, environmental pH, presence of organic matter and debris, sporulation, presence of lipid envelopes, and other factors[10].

Alcohols are popular in hand sanitizer preparations owing to a number of advantages such as low cost and rapid time of action. In addition, they are volatile, leave no residues and have a high safety margin. Alcohol can penetrate normal intact skin, and even though this can be an advantage in many instances, there is a risk of unwanted systemic penetration[11], with the risk of intoxication. Alcohols are reputed to kill up to 99.9% in as short as 15 s of application[12]. Even though alcohols are affected by organic matter, they do not dry out the hands as is the case with soaps.

The use of alcohol has some draw backs. Irritation and defatting of the hands can occur, thereby creating cosmetic concerns. For instance, due to its high volatility, it has very little residual antimicrobial effects[13]. Combination with more persistent agents such as benzalkonium chloride represents an attractive way of improving the activities of hand rubs to provide for both immediate and persistent actions. When antimicrobials are combined, a broader spectrum of activity can be achieved, and the risk of resistance can also be lowered. Such combination if resulting in synergism can help to reduce concentration-dependent toxicity.

The compositions of hand sanitizer formulations vary, and there are more and more preparations including herbal extracts intending to serve different purposes. Though several reports on the effects of type and concentration of alcohol on the antimicrobial activity of hand sanitizers exist, the effects of inclusion of all the key basic ingredients likely to be encountered in a hand sanitizer formulation have not been presented together in any report known to us. The aim of this research was to identify and quantify the influences of common additives encountered in alcohol-based hand sanitizers on the antimicrobial activity of the resulting formulation. This can enhance activity and enable the ingredients to be used more effectively, thereby also lowering cost and potential toxicities.

2. Materials and methods

2.1. Chemicals and bacteria

The following chemicals were procured from their manufacturers: ethanol (Sigma-Aldrich), isopropyl alcohol (Sigma-Aldrich), benzalkonium chloride (Loba Chemie, India), and glycerin (JHD). Media including nutrient agar, nutrient broth, Mueller Hinton agar, Sabouraud dextrose agar, and Sabouraud dextrose broth were all from Oxoid Limited, England.

Escherichia coli (*E. coli*), *Staphlococcus aureus* (*S. aureus*), *Klebsiella pneumonia* (*K. pneumonia*), *Bacillus subtilis* (*B. subtilis*), *Candida albicans* (*C. albicans*) and *Aspergilus niger* (*A. niger*) were from laboratory stocks kept in the Department of Pharmaceutical Microbiology of Nnamdi Azikiwe University, Awka, Nigeria.

2.2. Evaluation of the antibacterial activities of ethanol and isopropyl alcohol

Several concentrations (60%, 70%, 80%, 90% and 100%) of ethanol and isopropyl alcohol were separately prepared by diluting the respective pure alcohol using distilled water to the various concentrations. The antimicrobial activities of all the formulations were then determined against strains of *E. coli*, *S. aureus*, *K. pneumonia*, *B. subtilis*, *C. albicans* and *A. niger*.

The *in vitro* antibacterial and antifungal activities of the samples were determined by the agar well diffusion method. Briefly, 20 mL of molten Mueller Hinton agar and Sabouraud dextrose agar (for bacterial and fungal isolates, respectively) were poured into sterile petri dishes (90 mm diameter) and allowed to set. Standardized concentrations (McFarland 0.5) of overnight cultures of test isolates were swabbed aseptically on the agar plates, and holes (6 mm) were made in the agar plates using a sterile metal cock-borer. Then, 50 μL of the various samples and controls were introduced in each hole under aseptic conditions, kept at room temperature for 1 h to allow the agents to diffuse into the agar medium, and incubated accordingly. Ciprofloxacin (5 μg/mL) and miconazole (50 μg/mL) were used as positive controls in the antibacterial and antifungal evaluations respectively, while DMSO or sterile water was used as the negative control. The Mueller Hinton agar plates were incubated at 37 ℃ for 24 h, and the Sabouraud dextrose agar plates were incubated at room temperature (25±2) ℃ for 2 d. The inhibition zone diameters (IZDs) were then measured and recorded. The size of the cork-borer (6 mm) was deducted from the values recorded for the IZDs to get the actual diameter. This procedure was conducted in triplicates, and the mean IZDs were calculated and recorded.

Several concentrations of isopropyl alcohol (75%, 85% and 95%) were further tested alone using the same procedure outlined above.

2.3. Influence of glycerin on activity of isopropyl alcohol

Based on the results of preceding tests, three isopropyl alcohol-glycerin-water ratios were prepared: 85:5:10, 85:10:5 and 85:15:0. In the three preparations, the concentration of isopropyl alcohol was kept constant at 85%. The antimicrobial activities of the formulations were then evaluated as described in the subsection 2.2.

2.4. Influence of benzalkonium chloride on the antimicrobial activity of isopropyl alcohol

Two batches (B1 and B2) were formulated as described in the subsection 2.3 with the stated ratio of isopropyl alcohol, glycerin and distilled water. The samples were each placed in five test tubes and equal volumes (1.5 mL) of varying concentrations of benzalkonium chloride (0.02%, 0.04%, 0.06%, 0.08% and 0.10%) were then introduced to respective tubes. The antimicrobial activities of the 10 resulting samples were then evaluated as described in the subsection 2.2. A control sample consisted of 0.06% benzalkonium chloride alone.

2.5. Statistical analysis

The data were analyzed using student's t test and ANOVA when appropriate. Results are presented as mean ± standard deviation. Charts show percentage error. Values of $P<0.05$ were considered as statistically significant.

3. Results

3.1. Influence of type of alcohol and concentration

The antimicrobial activities of the two alcohols are presented in Figure 1. The activities of the agents were strongly concentration-dependent with the amount and variety of microorganisms inhibited rising with concentration. At 60%, ethanol was only active against *C. albicans* and *E. coli*. In addition to these two, isopropyl alcohol was also active against *B. subtilis* at this concentration. The activities peaked at 90%, where the greatest inhibition was recorded against *S. aureus*. Higher concentrations like 95% (Figure 2) did not enhance the activity but led to a loss of activity against *A. niger*. The comparison of the IZDs using two-tail *t* test showed that the activity of ethanol was comparable to that of isopropyl alcohol. The respective *P* values for comparisons carried out at the different concentrations were: P=0.539 for 60%, P=0.433 for 70%, P=0.138 for 80%, P=0.179 for 90% and P=0.178 for 100%, and this established that the activities of the two alcohols were comparable whenever both exhibited activity against an organism.

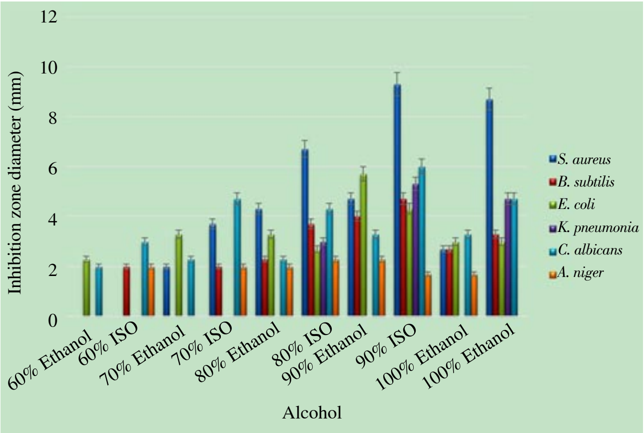

Figure 1. Effect of concentrations of ethanol and isopropyl alcohol (ISO) on inhibition zone diameter produced against some test microorganisms.

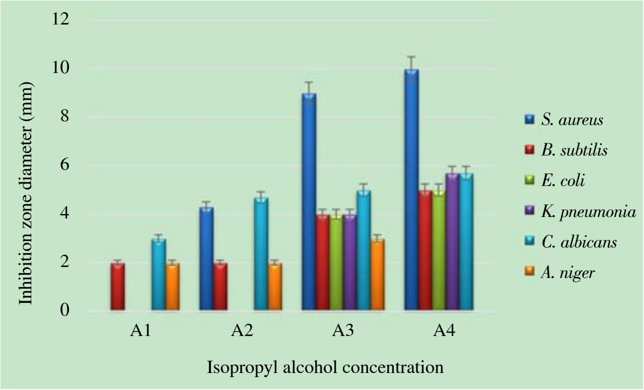

Figure 2. Effect of concentration of isopropyl alcohol on the inhibition zone diameter produced against test organisms.

A1, A2, A3 and A4 represent 60%, 75%, 85% and 95% aqueous isopropyl alcohol respectively.

3.2. Influence of glycerin

As shown in Figure 3, a general reduction in IZDs was observed in the alcohol-glycerin systems relative to results (Figures 1 and 2) obtained for alcohol alone. The highest IZDs were obtained with the preparation containing 10% glycerin and 5% water, being the only glycerin-containing preparation that recorded activity against *B. subtilis*. The least was seen with the system containing 15% glycerin. The activity against *A. niger* was also higher than those of the other concentrations tested. Differences in the concentration of glycerin did not affect the pH of the formulations, maybe because the pH of glycerin itself (7.5) is close to neutral. All the preparations demonstrated poor activity against *E. coli*, a common contaminant of the hands due to poor toilet habits. The control system of ciprofloxacin demonstrated no activity against the strain of *S. aureus* used.

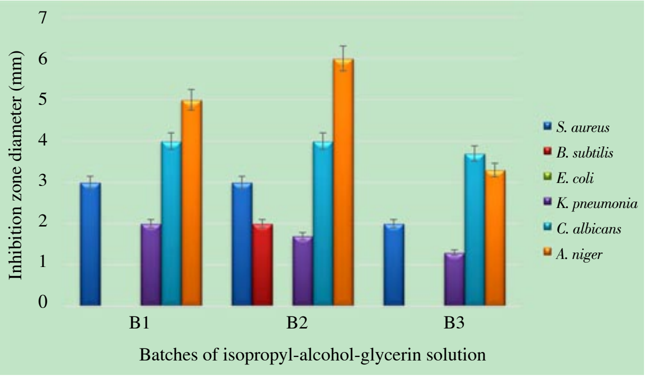

Figure 3. Effect of isopropyl alcohol-glycerin-water ratios on inhibition zone diameter produced against test organisms.

B1: 85% isopropyl alcohol, 5% glycerin, and 10% distilled water (pH 6.98±0.08); B2: 85% isopropyl alcohol, 10% glycerin, and 5% distilled water (pH 7.12±0.05); B3: 85% isopropyl alcohol, 15% glycerin, and 0% distilled water (pH 7.14±0.10).

3.3. Influence of benzalkonium on the antimicrobial activity of isopropyl alcohol

The effects of inclusion of benzalkonium chloride were demonstrated using a preparation containing either 85% isopropyl alcohol, 5% glycerin and 10% water (Figure 4A) or 85% isopropyl alcohol, 10% glycerin and 5% water (Figure 4B). The inclusion of benzalkonium generally increased the IZDs of the preparations (P<0.05) and promoted activity against *B. subtilis* and *E. coli*. Even with the inclusion of benzalkonium, higher activities were still obtained with samples containing 10% glycerin than those with 5% glycerin, indicating a need to maintain a balance in the concentration of water relative to glycerin.

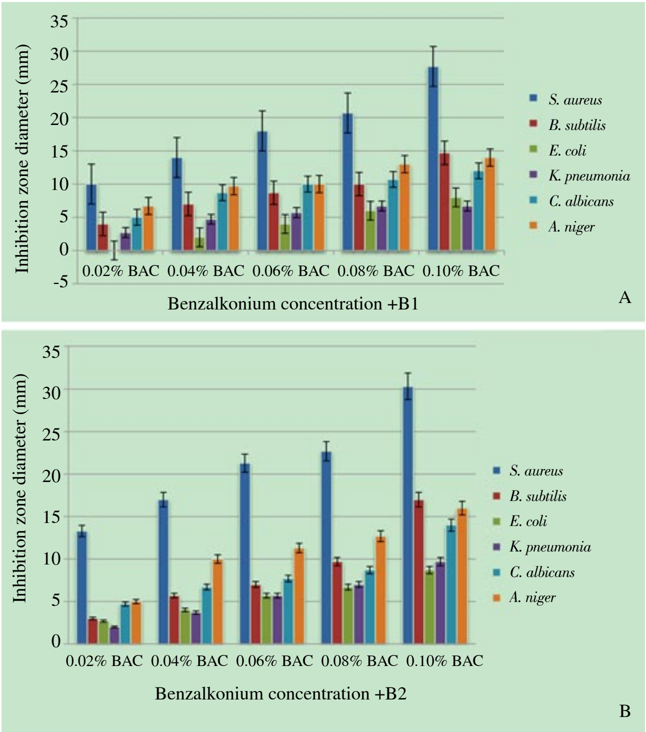

Figure 4. Effect of concentration of benzalkonium chloride (BAC) on the inhibition zone diameter produced by (A) B1: isopropyl alcohol-glycerin-water (85:5:10) solution and (B) B2: isopropyl alcohol-glycerin-water (85:10:5) solution.

The addition of benzalkonium generally increased the pH of the formulation from 6.75±0.02 for the solution containing 0.02% to 7.10±0.02 for the system containing 0.10%. Though benzalkonium–alcohol systems generally achieved better activities than isopropyl alcohol alone, the combination was not superior to benzalkoinum used alone at the same concentration (Figure 5), in that there were no statistically significant differences. The control antibacterial agent used (ciprofloxacin at 5 μg/mL) had generally poor activity against *S. aureus*, but the antifungal agent miconazole at 50 μg/mL demonstrated excellent activity against both fungal organisms tested.

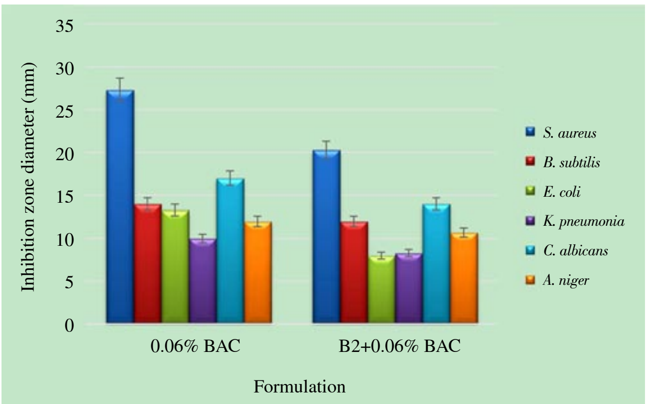

Figure 5. Comparison of the inhibition zone diameter produced by benzalkonium chloride (BAC) alone and with isopropyl alcohol.

The concentration of BAC was fixed at 0.06%. The isopropyl alcohol preparation (B2) contains 85% isopropyl alcohol, 10% glycerin and 5% water.

4. Discussion

Though alcohols are not active against spores, they are very bactericidal against vegetative forms of bacteria with their activity dropping profoundly on dilution beyond 50%[14,15]. Even though both agents are used in hand disinfection, isopropyl alcohol demonstrated an edge over ethanol in terms of spectrum of activity across the concentration range tested. This result may be because isopropyl alcohol has a lower minimum inhibitory concentration than ethanol. However, at the optimum use concentration (85%–95%) for both agents, there were no statistically significant differences in their activities against sensitive organisms. The results obtained here agree with earlier reports on the relative antibacterial activity of the two alcohols[16]. Against viruses, the results may be different and ethanol is often considered more potent. Distinctly lower activities were recorded at 100% and this could be because proteins are denatured more efficiently and quickly in the presence of water. The concentration range for activity obtained here agrees with previously quoted values[17-19]. The increase in the concentration of alcohol led to a reduction in pH, from 6.50±0.08 for 60% alcohol to 6.00±0.04 for 95% alcohol. The statistical analysis (two tail ^{t} test) indicated that 95% isopropyl alcohol did not have a better activity than 85% isopropyl alcohol. Therefore, 85% isopropyl alcohol was used in subsequent tests. The relatively high concentration of alcohol used in hand sanitizers is a potential public health concern because of the possibility of wilful ingestion and subsequent intoxication[19,20].

Glycerin was deliberately tested in the concentration range of 5%–15%, being lower than the concentrations encountered in some commercial leave-on products. The sample containing 15% glycerin and no water recorded the least activity, most probably due to increase in viscosity or other effects. The use of glycerin in hand sanitizers is purely cosmetic, and the WHO recommends very low concentrations (1.45%) in the final product[21]. The reduced activity is likely a product of increased viscosity of the samples due to the high concentrations of glycerin used, thereby impeding diffusion through the culture medium. Suchomel *et al.* reported that glycerol reduced the 3-hour efficacy of alcohol-based hand rubs[22]. In the circumstances, the use of glycerin should be kept to a minimum and should be restricted to situations where there are overriding cosmetic reasons[23-25].

The increased activity seen in benzalkonium–alcohol systems as against isopropyl alcohol alone suggests that blending of the two (with benzalkonium being at a very low concentration) would be beneficial, especially in handling risk organisms for gastrointestinal conditions like *E. coli*. This gives rise to the concept of double barrel hand sanitizers which combine alcohol with a less volatile agent like benzalkonium[26]. The rapid activity of the alcohol ensures fast onset of action, while the less volatile component ensures persistent activity. This kind of formulation would be ideal for conditions where the risk of infection is high, such as hospital wards and toilets. However, though this combination has the prospect of enhancing the killing rate, it does not extend the spectrum of activity over that of benzalkonium chloride used alone. The above results indicate general susceptibility of the organisms to benzalkonium chloride, which achieves its antibacterial activity using multiple mechanisms as against alcohol which exerts activity by denaturing proteins.

We conclude that with the two alcohols (ethanol and isopropyl alcohol), the optimum antimicrobial activities were seen at a concentration range of 85%–95%, where there were no statistically significant differences in their inhibition zone diameters. In the range of 60%–100%, isopropyl alcohol had a generally wider spectrum than ethanol at equal concentration, maybe because it had lower minimum inhibitory concentrations than ethanol against most organisms. Addition of glycerin in the concentrations tested here adversely affected the activity of the alcohol, though mechanical spreading action encountered in real use may lead to improved drug diffusion. Addition of varying concentrations of benzalkonium chloride improved the activity of isopropyl alcohol and such combination can result in a formulation having both immediate and persistent actions.

Conflict of interest statement

The authors declare they have no conflicts of interest.

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