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Research Article

Efficacy of World Health Organization-Recommended Homemade Hand Sanitizer Against Bacteria and Fungus

Moehamad Orliando Roeslan[1§], Mario Richi[2], Aradhea Monica Drestia[2], Armelia Sari Widyarman[3]

[1] *Department of Oral Biology, Faculty of Dentistry, Trisakti University, Indonesia*
[2] *MiCORE Laboratory, Faculty of Dentistry, Trisakti University, Indonesia*
[3] *Department of Microbiology, Faculty of Dentistry, Trisakti University, Indonesia*

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ABSTRACT

Introduction: Handwashing is among the best practices to prevent the transmission of various diseases especially during the pandemic. Handwashing with soap can reduce respiratory tract infections. The World Health Organization (WHO) released guidelines for making homemade hand sanitizer. **Objective:** This study aimed to analyze the effectiveness of antibacterial hand-rub, as recommended by WHO, containing ethanol, hydrogen peroxide, and glycerol in varying concentrations against bacteria and fungus. **Methods:** This experimental laboratory study was designed to assay the efficacy of hand sanitizer ingredients—96% ethanol, 3% hydrogen peroxide, and 98% glycerol, as recommended by WHO—against *Escherichia coli*, *Staphylococcus aureus*, and *Candida albicans*. Commercial hand rubs were used in this study for comparison. **Result:** Minimum inhibitory concentration (MIC) testing showed that the WHO hand rub at 25% concentration inhibited *E. coli* and *S. aureus* growth, while 12,5% concentration inhibited *C. albicans*. **Conclusion:** The WHO-recommended homemade hand sanitizer containing ethanol, hydrogen peroxide, and glycerol at a concentration of 50% is effective in eliminating *Escherichia coli, Staphylococcus aureus*, and concentration of 25% is effective on *Candida albicans*. Further study is needed to analyse these materials against other bacteria and viruses.

[§] Corresponding Author
E-mail address: orliandoichol@gmail.com (Roeslan MO)

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INTRODUCTION

The awareness level of Indonesian people on maintaining general health and hygiene is still low, especially in densely populated slum communities. These will increase the spread of infectious diseases.[1] Handwashing is among the best practices to prevent the transmission of various diseases. Handwashing with antimicrobial agents is a recommended sanitation procedure that can effectively clean your hands and break the chain of microbial infections.[2] Hand is one of the main carriers of pathogenic microbes from one person to another, directly through handshake and indirectly by touching objects such as towel, glass, or the surfaces of public places and public transportation.[2,3] Unwashed hands that have been in contact with human or animal feces or other body fluids, such as mucus or sputum, and contaminated food/drink will transmit the pathogens to another person who is unaware of the transmission.[4,5]

A study on interventions to prevent infectious disease was conducted by teaching proper handwashing techniques to one community and comparing the incidence of infections to a similar community that received no intervention. Results showed that the number of diarrhea patients was reduced by 50%. Study showed the risk of unwashed hands in the intervention study; 95% of patients had diarrhea. Handwashing with soap reduced the diarrhea risk significantly.[6] A well-known diarrhea-causing microbe is *Escherichia coli (E. coli)*, a Gram-negative bacteria commonly found in human and animal gut. *E. coli* infection can cause severe intestinal infection, which leads to diarrhea, abdominal pain, and fever.[7]

Another bacterium whose transmission can be prevented by handwashing is *Staphylococcus aureus (S. aureus)*. Due to the yellow pigment grape xanthin, this facultative anaerobic pathogen appears as a golden colony when it grows in a dense, enriched medium.[8,9] *S. aureus* is an adaptable Gram-positive bacteria that can proliferate in blood vessels and tissue.[8] The mechanism underlying bacterial transition and adaptation depends mostly on sigma B regulon, the transcription factor that modulates the stress response of various Gram-positive bacteria.[10] Oxygen is required for the growth and virulence of *S. aureus*. Once infected by these bacteria, the atmospheric oxygen level will decrease, and in under hypoxic conditions, the *S. aureus* cytotoxins will increase.[11] *S. aureus* originates from a flora colony on mucous membrane.[12]

Fungal infections, most commonly caused by Candida, can also be prevented by washing hands. This microorganism is known as an opportunistic pathogen because it can be an etiologic factor for several diseases in patients with immune deficiency or known to be immunocompromised. *Candida sp.* is a polymorphic fungus that can present as a beginner yeast in an ovoid shape, an ellipsoid cell with elongated constriction in the septa, or as a hypha with parallel walls. Most *Candida* species have the morphological appearance of white and opaque cells formed at the time of transfer and can also form spore-like structures called chlamydospores.[13,14]

Many other infections can also be prevented by hand washing, including respiratory tract infections, the cause of many deaths in toddlers and mortality in early childhood worldwide.[8,15] Handwashing with soap can reduce respiratory tract infections in two phases: by removing the respiratory pathogens from the palms and by removing other pathogens, especially enteric bacteria, that cause diarrhea as well as other respiratory symptoms. A recent study proved that handwashing with soap can reduce more than 50% of respiratory tract infections related to pneumonia in toddlers.[16]

Handwashing with water alone is common, however, it is ineffective in eliminate the pathogens compared to handwashing with soap. Nevertheless, handwashing with soap is often underestimated and ignored, even at important times, especially before eating.[17] A recent review found that handwashing behavior and awareness are very low in several countries; the review mentioned especially that only 17% of child caretakers wash their hands with soap after using the toilet.[17] Thus, using a hand sanitizer has become the main alternative at present due to its practicality—it can be performed anywhere without water, and it is quick.[18]

As infectious diseases have spread and become more critical, the price of hand sanitizers has risen due to high demand. In the interest of savings, we can create our own hand sanitizer as an alternative. An effective hand sanitizer must contain at least 60% alcohol to eradicate pathogenic bacteria and a moisturizer to prevent potential over-drying and irritation of the skin.[19] Unfortunately, preparation of making hand sanitizers requires precise measurements with special equipment and chemical materials. Making hand sanitizer requires high profile accuracy to produce an effective hand sanitizer with an alcohol level capable of eliminating microbes on the hands. Thankfully, The World Health Organization (WHO) released simpler guidelines for making homemade hand sanitizer.[20] Currently, there are no in vitro scientific studies regarding the effectiveness of this handmade hand sanitizer based on WHO recommendations. Therefore, this study aimed to analyze the effectiveness of hand-rub, containing ethanol, hydrogen peroxide, and glycerol as recommended by WHO, in varying concentrations against bacteria and fungus.

MATERIALS AND METHODS

This experimental laboratory study was designed to assay the efficacy of hand sanitizer ingredients—96% ethanol, 3% hydrogen peroxide, and 98% glycerol, as recommended by WHO[20]—against *Escherichia coli, Staphylococcus aureus,* and *Candida albicans*. Commercial handrubs (Fresco antiseptic handrub, Padalarang, Indonesia) were used in this study as a positive control.

Homemade Hand Sanitizer Preparation

Following the practical guide of WHO-recommended handrub formulations,[20] by using a measuring cylinder, the homemade hand sanitizer was prepared with 833.3 ml of 96%-ethanol, 41.7 ml of 3%-hydrogen peroxide, and 14.5 ml of 98%-glycerol. Distilled water was added to the bottle to reach one liter, and it was closed with a screw cap as quickly as possible to prevent evaporation. The solution was mixed by gently agitating the bottle, and it was left for 72 hours at room temperature before testing. This was considered a 100% hand sanitizer solution. Subsequently, gradual dilution was carried out to obtain concentrations of 50%, 25%, 12.5%, 6.25%, and 3.125% by adding sterilized distilled water and applied in the minimum inhibitory concentration assay with dilution method.

Bacterial Culture

E. coli ATCC 25922 and *S. aureus* ATCC 25923 were cultured anaerobically in Brain Heart Infusion (BHI) (Oxoid, Hampshire, UK) broth for 24 hours at 37°C. The bacterial cells were collected by centrifuging the tubes and discarding the medium. After adding phosphate-buffered saline (PBS), the bacterial cell concentration was measured with a microplate reader at 600 nm to achieve 0.5 McFarland standard.

Candida albicans Culture

C. albicans ATCC 10231 was cultured in autoclaved Saboraud's Dextrose Broth (SDB) (Oxoid, Hampshire, UK) (pH 5.6) and incubated at 37°C for 24 hours. Cells were collected by centrifugation, and the cells were suspended in PBS. Assessment for optical density (OD) was performed with a 1.5×108 CFU/ml McFarland standard.

MIC Assay with Dilution Method

The handrub at various concentrations and the bacterial or fungal cultures were inserted into a 15 ml tube using a micropipette. For each concentration, 2 ml of bacterial or fungal culture and 2 ml of handrub were inserted into the tube. The same procedure was performed for the control group. The tubes were incubated for 24 hours at 37°C. After incubation, the mixture from each tube was spread on the agar medium. Then, the agar plates were incubated for 24 hours at 37°C. Bacterial or fungal colony that formed on the agar plate was counted using total plate count method.

RESULTS

The MIC test showed that the WHO hand sanitizer inhibited the growth of *E. coli* at a concentration of 25%, and the minimum bactericidal concentration (MBC) that completely eliminated it was observed at a concentration of 50%. Meanwhile commercial hand sanitizers were proven to be effective Inhibition can eliminate *E. coli* even at a concentration as low as 3.125%, as shown in Table 1 and Fig. 1.

Results showed that the WHO handrub at 25% concentration inhibited *S. aureus* growth, and the MBC to eliminate it was 50%. The results for the commercial handrub against *S. aureus* showed it to be very effective, with the MBC to eliminate *S. aureus* at the smallest concentration tested—3.125%. (Table 2 and Fig.2)

Table 1. Results of MIC tests on handrubs against *E. coli*

Concentrations	Commercial handrub Average ±Standard Deviation (CFU/mL)	WHO handrub Average ±Standard Deviation (CFU/mL)
100%	0	0
50%	0	0
25%	0	100 ± 10
12.5%	0	59.000 ± 9400
6.25%	0	200.000 ± 1000
3.125%	0	200.000 ± 1100
Negative control	200.000 ± 1100	200.000 ± 900

Table 2. Results of MIC tests on handrubs against *S. aureus*

Concentrations	Commercial handrub Average ±Standard Deviation (CFU/mL)	WHO handrub Average ±Standard Deviation (CFU/mL)
100%	0	0
50%	0	0
25%	0	11.000 ± 400
12.5%	0	73.200 ± 2400
6.25%	0	200.000 ± 1000
3.125%	0	200.000 ± 1300
Negative control	200.000 ± 1200	200.000 ± 1100

Results also showed that WHO handrub inhibited *C. albicans* growth at 12.5% concentration and the MBC to eliminate it was 25%. The results of the commercial handrub against *C. albicans* showed it to be very effective with the MBC to eliminate *C. albicans* at the smallest concentration tested—3.125%. (Table 3 and Fig. 3).

Table 3. Results of MIC tests on handrubs against *C. albicans*

Concentrations	Commercial handrub Average ±Standard Deviation (CFU/mL)	WHO handrub Average ±Standard Deviation (CFU/mL)
100%	0	0
50%	0	0
25%	0	0
12.5%	0	61500 ± 5700
6.25%	0	200000 ± 2100
3.125%	0	200000 ± 1800
Negative control	200000 ± 2200	200000 ± 2000

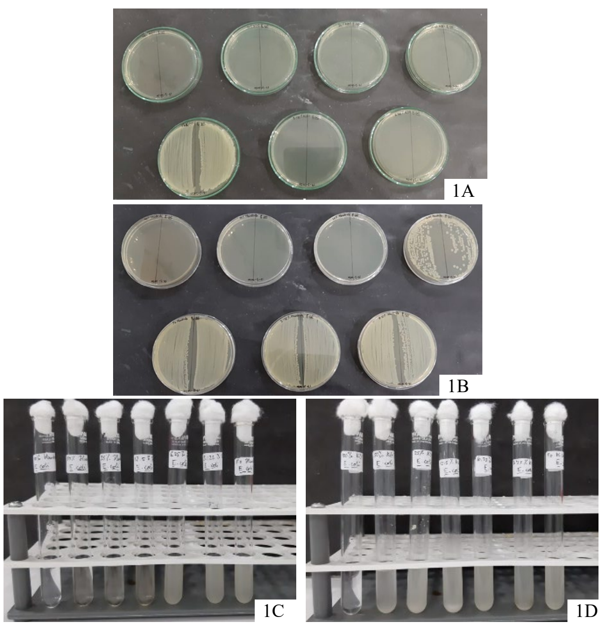

Figure 1. (A) Results of MIC tests for *E. coli* after 24 h incubation for commercial handrub at 100%, 50%, 25%, 12.5%, 6.25% and 3.13% concentrations compared to the negative control. (B) Results of MIC test for *E. coli* after 24 h incubation using WHO handrub at 100%, 50%, 25%, 12.5%, 6.25% and 3.13% concentrations compared to the negative control. (C) MIC test results of WHO handrub showing inhibitory effects on all tested materials. (D) Results of MIC test using commercial handrub showing inhibitory effects at all concentrations. Note that the commercial handrub causes a turbid appearance in the tube.

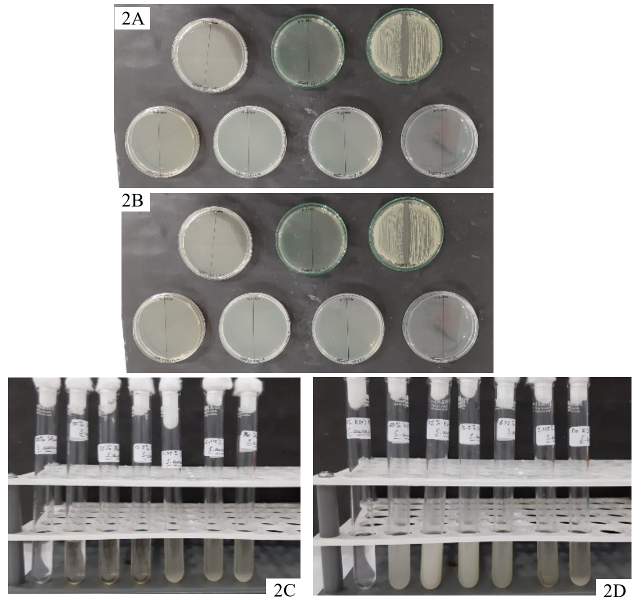

Figure 2. (A) Results of MIC test for *S. aureus* after 24 h incubation for WHO handrub at 100%, 50%, 25%, 12.5%, 6.25%, and 3.13% concentrations, compared to untreated medium as negative control. (B) Results of MIC test for *S. aureus* after 24 h incubation for commercial handrub at 100%, 50%, 25%, 12.5%, 6.25% and 3.13% concentrations, compared to untreated medium as negative control. (C) MIC test results of commercial handrub showing inhibitory effect on all tested materials. (D) MIC test results of WHO handrub showing inhibitory effects at 100% and 50% concentrations.

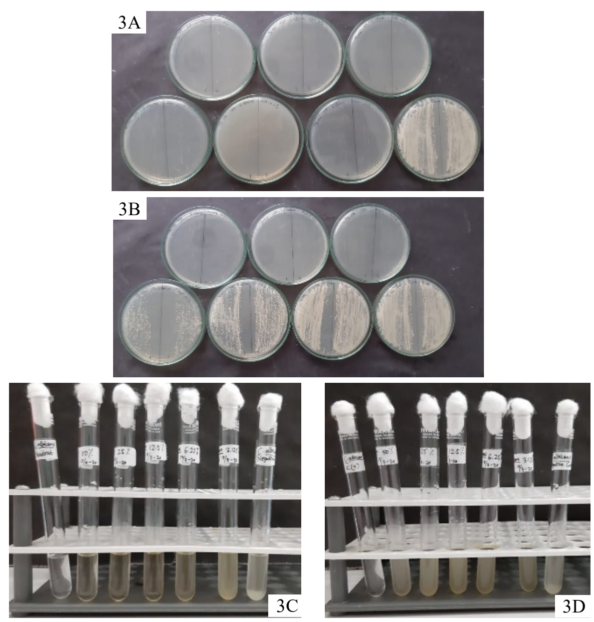

Figure 3. (A) Results of MIC test for *C. albicans* after 24h incubation for WHO handrub at 100%, 50%, 25%, 12.5%, 6.25%, and 3.13% concentration, compared to untreated medium as negative control. (B) Results of

MIC test result for *C. albicans* after 24 h incubation for commercial handrub at 100%, 50%, 25%, 12.5%, 6.25%, and 3.13% concentrations, compared to untreated medium as negative control. (C) MIC test results for WHO handrub showing inhibitory effect on all tested materials. (D) MIC test results for commercial handrub showing inhibitory effects at 100% and 50% concentrations.

DISCUSSION

In this study, testing for minimum inhibitory concentration (MIC) was performed to determine the minimum concentration of handrubs required to prevent the proliferation of bacteria, and the minimum bactericidal concentration (MBC) was set one level above the MIC value. Furthermore, the statistical analysis was performed to observe the significance and distribution of data. Based on MIC tests, the WHO handrub recipe was found to be effective at eliminating bacteria growth, whilst not as effective as the commercially produced hand sanitizer. In spite of the less efficacy from the WHO-recommended hand sanitizer, the authors highlight that the formula for creating the WHO hand sanitizer was easy and economically efficient. Non-specialized individuals can easily make the WHO-recommended hand sanitizer to mitigate the scarcity of commercial hand sanitizers, due to the current pandemic that caused hardships all over the world.[21] From these results, a 2-fold dilution (50% concentration) of WHO-recommended hand sanitizer retains its capacity to eradicate bacteria. Having the same effectiveness at lower concentrations can help common people create their own hand sanitizer using the WHO recommendation at relatively low expense, and therefore maintaining hygiene and cleanliness at all times.

Aldehydes are reactive to thiol and amine, thus leading to inactivation of protein and micro-molecules in the microbe tested.[22] This was proven by MIC test results. Based on the MIC results, the MIC value for *C. albicans* was higher than that for *S. aureus*. The pathogenicity mechanism and virulence factor of *S. aureus*, the thiol group, which is bacilli thiol (BSH), plays an important role in oxidative response.[23] Regarding the pathogenicity of *C. albicans*, it metabolizes glutathione (GSH), a thiol substance, which plays an important role in increasing *C. albicans* resistance against antimicrobial and antifungal compounds.[24] This result was in line with the ethanol mechanism basic theory, as a reactive antimicrobial compound against the thiol group. *C. albicans* is a fungus that has a specific protein that functions as an adhesive; thus, it can mediate adhesion to a biotic or abiotic surface, namely Als3 (Agglutinin-like sequence) and Hwp1 (Hyphal wall protein), and can cause *C. albicans* to develop drug resistance.[14]

With the specific protein, *C. albicans* cells can mediate adherence to other *C. albicans*, to other microorganisms, to abiotic or biotic surfaces, and to different types of tissue. *C. albicans* can form a drug-resistant biofilm.[25] Environmental pH value affects the morphology of *C. albicans*; at a pH <6, *C. albicans* will grow in yeast form, and at pH >7, the hyphae will grow.[26] A specific protein in *C. albicans*, the Als3, acts as an adhesive which mediates the adhesion on the epithelial cell, endothelial cell, and extracellular protein matrix.[27] Another specific protein that acts as an adhesive is Hwp1 or hyphal wall protein 1, in hyphae form.[28]

S. aureus is an antibiotic-resistant Gram-positive bacteria, but the results of this study showed that *S. aureus* can be eliminated by using a low concentration hand sanitizer. The color of the *S. aureus* colony is gold in dense media. *S. aureus* can adapt and proliferate in blood vessels and tissue.[10] The growth of *S. aureus* depends on oxygen; in hypoxic conditions, the *S. aureus* cytotoxin will increase. On the formation of *S. aureus* biofilm, the agrC mutation gene will phosphorylate the response of the agrA regulator and later initiate the transcription process and produce an RNA III molecule that regulates the virulence factor of *S. aureus* biofilm.[29] This study showed that the WHO handrub was effective in eliminating these pathogens.

There are several mechanisms involved in ethanol's ability to kill bacteria. A previous study showed that ethanol can interfere with the cell division of *E. coli*.[30] Another study showed the role of ethanol during the synthesis of peptidoglycan, which can prevent osmotic lysis in bacteria. In the presence of ethanol, peptidoglycan weakens, and the cell becomes swollen and lysed because of differences in osmotic pressure in the plasma membrane.[31,32] Another study showed that ethanol can inhibit the synthesis rate of the DNA, RNA, and protein of *E. coli*,[32] and at the same concentration, ethanol can also suppress the synthesis of the outer membrane protein of *E. coli*.[33] Several studies of ethanol mechanisms on microorganisms use *E. coli* as the model system. The fatty acids of *E. coli* include palmitic, palmitoleic, and vaccenic acids. Previous research has shown that ethanol may alter the composition of fatty-acyl groups of *E. coli* by decreasing the synthesis of palmitic acid. These changes take place during the synthesis of lipid.[31]

The main way ethanol eliminates bacteria is by disrupting the cellular permeability barrier. Lipid solubilization and protein denaturation lead to destruction of the cell membrane.[30,34] Ethanol is also reported to increase the leakage of nucleotides, which leads to cell death in *E. coli*. Besides nucleotides, potassium and protons, which are smaller than nucleotides, are also susceptible to leakage.[35] Ethanol has the ability to fluidize the membrane, and then generate uncontrolled solutes that cause a decrease in proton flux across the membrane and the leakage of cofactors such as Mg^{2+}. ATPase and glycolytic enzymes can be activated by ethanol, resulting in inhibition of cell growth.[33] Seventy-percent ethanol concentration is more effective in killing *Staphylococcus* species than methanol. Fifty-percent ethanol was effective in a 10-second exposure time, while methanol was only 30% effective compared to ethanol. This study also showed that Gram-positive bacteria were more resistant to ethyl alcohol; concentrations of 60%–95% ethyl alcohol were required in contrast with Gram-negative, while on *Pseudomonas aeruginosa*, starting from a concentration of 30%, ethanol can kill the bacteria. *Serratia marcescens*, *E. coli*, and *Salmonella typhi* can be eradicated by ethanol starting from a concentration of 40%.[36]

A previous study showed that exposure to 30% ethanol for four hours could kill and inhibit the growth of *C. albicans*. Another study showed that to eliminate fungal spores takes a higher ethanol concentration compared to the effective concentration against bacteria. The mechanism of ethanol's ability to kill *C. albicans* is mainly through rapid disruption of the membrane function. Interaction of ethanol with cellular membranes increases membrane permeability, causing solutes to leak and cell lysis.[30,37]

Since hydrogen peroxide is an oxidizing agent, the main mechanism by which hydrogen peroxide kills bacteria is oxidation of macromolecules, such as protein, lipids, carbohydrates, and nucleic acids that make up the structure and function of microorganisms. Protein has a significant role in the surface and internal structures of bacteria. Hydrogen peroxide in the form of solution has proven capable of oxidizing certain amino acids and proteins.[38] Another study showed that hydrogen peroxide could break the phosphodiester bonds in nucleotide molecules due to the localized generation of short-lived hydroxyl radicals. That mechanism could also inhibit growth and lead to cell death. However, the low concentration of H_2O_2 in this experiment may also play a role in removing spores in the solutions.[39]

Glycerol contains hydroxyl groups that bind and retain water, causing it to act as a humectant for the skin. Glycerol has the ability to diffuse into the stratum corneum and retain water in that layer.[40] A mixture of water and glycerol can hydrate the skin, prevent further dehydration, and have a smoothing effect on the skin,[41] thereby promoting reduction of tissue scattering,[41,42] helping in the stabilization of collagen,[43,44] and protecting the skin against irritation caused by washing procedures.[45] Glycerol has physical effects on the water in the outer layer of the stratum corneum. Moreover, glycerol's interaction with lipid or protein structures in the stratum corneum may change their water-binding properties.[40] However, previous research has shown that 1.45% glycerol may inhibit the 3-hour efficacy of alcohol-based handrubs.[46]

The limitation of this study is that it was done on limited bacteria. However, the background of this research was assessed during the COVID-19 pandemic, in which the availability of hand sanitizer on the market at the time was very scarce. Further study is needed by testing the WHO-recommended handrub's efficacy to eliminate viruses, especially SARS-CoV2 in this trying time of the current COVID-19 pandemic.

CONCLUSION

The WHO-recommended hand sanitizer containing ethanol, hydrogen peroxide, and glycerol at a concentration of 50% is effective in eliminating *Escherichia coli, Staphylococcus aureus*, and *Candida albicans*. Further study is needed to test these materials against other bacteria and viruses.

Conflict of Interest

All authors declared that there was no conflict of Interest related to this study.

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