



Evaluating antimicrobial potential of ozonated water in preliminary trials

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In response to the growing demand for sustainable disinfection solutions, ozonated water emerges as a promising antimicrobial agent. This study evaluates the efficacy of ozonated water against microbial strains such as *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Candida albicans* under standardized laboratory conditions. Sterile distilled water was ozonated using a portable ozone generator of 13 W for 30 min. Ozone concentration was confirmed using test strips. Nutrient broth and peptone water were used for bacterial and fungal growth, respectively. The study had three groups: negative control (uninoculated media), positive control (media inoculated with microbes) and test group (ozonated media inoculated with microbes). Microbial growth was assessed by solution turbidity after 24 h for bacterial species and 48 h for fungi. Additionally, the bactericidal and fungicidal activities were determined by transferring 10 µL of the test group sample into fresh nutrient broth and peptone water, followed by incubation. The results confirmed antimicrobial activity of ozonated water. Also, the bactericidal and fungicidal activity of ozonated group confirmed by the clear broth in the test group, like the negative control, suggests its applications in various domains including healthcare. Further research is necessary to evaluate its compatibility with various materials for optimal applications.

Keywords: *Candida albicans*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*

Over the past few years, an increasing focus on antimicrobial agents has been developed due to the necessity of sustainable disinfection procedures. Ozone is one such agent, which is a triatomic form of oxygen,

renowned for its oxidizing properties and effective as an antimicrobial agent for a broad spectrum of microorganisms including bacteria, viruses and fungi¹. It is effective in various forms such as ozone gas, ozonated oil and ozonated water. Among these ozonated water gathered attention as a disinfecting solution as it is considered most biocompatible and environment friendly agent that can be generated by dissolving ozone gas into water². Due to the easiness in handling, it has been employed in several fields including water treatment, food processing, sterilization procedures and healthcare sector¹⁻³.

Maintaining a quality standard of hygiene is essential to inhibit the prevalence of healthcare associated infections. Conventional disinfection procedures depend on chemical agents which may increase the possibility of toxicity and foster the proliferation of antimicrobial resistance⁴. Another traditional practice to inhibit microbial contamination is through heat sterilization. However, it is not suitable for equipment and materials sensitive to high temperature⁵. In such instances, ozonated water can be an effective disinfection agent that offers both bio-tolerant and renewable characteristics². It is useful in decontaminating surgical instruments, endoscopes and contact lenses. Moreover, irrigation of oral cavity with ozonated water reduces the risk of developing periodontal disease and caries⁶. Beyond surface disinfection and sanitization, ozonated water is employed in management of several problems such as diabetes related wound care, cancer treatment, skin diseases. This is due to its ability to enhance blood circulation, improve oxygen delivery and better activation of autacoids, cytokines and growth factors⁷⁻¹⁰. Despite the multifaceted usage and benefits of ozonated water, its antimicrobial activity for disinfecting dental impression materials has not been extensively investigated in a controlled laboratory environment. Therefore, the present pilot study seeks to assess the effectiveness of ozonated water against common bacterial and fungal strains such as *Pseudomonas aeruginosa*, *Staphylococcus aureus*, and *Candida albicans* found in oral conditions. By evaluating the efficacy under standardized conditions, this research offers insight into the possible use of ozonated water for dental health care procedures and facilitates the way for future research in this area.

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Materials and Methods

Study settings

This study was conducted in a controlled laboratory environment at the microbiology department, Applied Medical Science College, Jouf University, Saudi Arabia. The laboratory was equipped with peptone water, nutrient broth, standard 10 μL inoculating loops, glass bottles of varying sizes and incubators for microbiological analysis. Also, a sterile and contamination free zone was used for all experimental procedures. Three microorganisms such as *Staphylococcus aureus* (ATCC 43300), *Pseudomonas aeruginosa* (ATCC 10145) and *Candida albicans* (ATCC 60193) were used for this study.

Preparation of ozone water

A portable ozone generating machine was used to produce ozone (O_3) and the ozonated water was prepared based on the manufacturer's instruction with a power of 13 W for 30 min. The tube of the machine was connected to a bottle containing 200mL of sterile distilled water.

Evaluation of the concentration of ozone in the water

The concentration of ozone in water evaluated using the test strips (Fig. 1A) purchased from the manufacturer. 50 mL of the prepared ozonated water was taken in a container and test strip dipped into it for 10 seconds with a gentle back and forth motion as per the manufacturer's recommendation. The test strip was removed and shaken briskly to remove the excess water. After 20 seconds, the colour developed in the strip was matched with the chart provided by the manufacturer for understanding the ozone concentration. The reading was completed within 30 seconds. The temperature of the ozonated water was measured using a digital thermometer.

Antimicrobial testing of ozonated water

A nutrient broth and peptone water served as the growth medium for microbial cultures. The study involved testing the antimicrobial effectiveness of ozonated water against organisms such as *Pseudomonas aeruginosa*, *Staphylococcus aureus*, and *Candida albicans*. To initiate the experiment, 10 μL of microbial inoculum was introduced into the prepared media, forming the positive control group. Additionally, a negative control group comprised the media without any microorganisms, while the test group consisted of the microbe-containing media treated with ozonation (Table 1). Following a 24 h

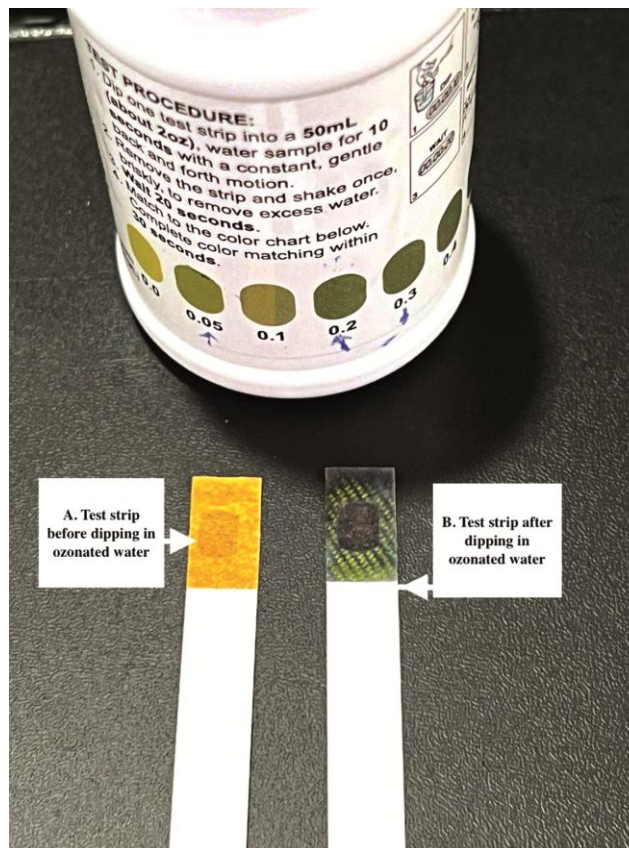


Fig.1 — Ozone concentration test strip before dipping in the ozonated water (A) and Evaluation of ozone concentration using test strip after dipping in the ozonated water (B).

incubation period, visual examination was conducted to discern any observable differences among the negative control, positive control, and test groups' appearances in the media¹¹⁻¹⁴. Additionally, the bactericidal and fungicidal activity of ozonated water was confirmed by transferring 10 μL samples from each test group into nutrient broth and in peptone water for 24 h and 48 h incubation periods for the bacterial and candida species respectively.

Statistical analysis

Descriptive statistics were used to summarize the data. Categorical variables were presented as frequencies and percentages. The association between negative control, positive control, test groups and turbidity status was analyzed using the Chi-square test of independence using SPSS version 23. The Pearson Chi-square test was initially performed; however, since some expected cells counts were below 5, violating the assumptions of the Chi-square test, the likelihood ratio test was also considered. To further validate the results, Fisher's exact test was performed

Table 1 — Description of study groups

Negative control groups	Positive control groups	Test groups
Nutrient broth	Nutrient broth inoculated with <i>Staphylococcus aureus</i>	Ozonated nutrient broth inoculated with <i>Staphylococcus aureus</i>
Nutrient broth	Nutrient broth inoculated with <i>Pseudomonas aeruginosa</i>	Ozonated nutrient broth inoculated with <i>Pseudomonas aeruginosa</i>
Peptone water	Peptone water inoculated with <i>Candida albicans</i>	Ozonated peptone water inoculated with <i>Candida albicans</i>

using R (version 4.4.2) as it is more suitable for small sample size and low expected counts. A *P* value less than 0.05 was considered statistically significant for all tests.

Results and Discussion

The concentration of the ozone in water confirmed with test strips (Fig. 1B). The colour of the test strip indicated more than 0.5 ppm ozone concentration within 30 seconds of removal from the ozonated water, confirmed the efficacy of the ozone generating machine. The water temperature measured as 20±3°C.

Microbial growth in the study groups

Upon visual examination after the 24 h incubation period, noticeable differences observed among negative control, positive control and test groups in nutrient broth medium for organisms such as *Pseudomonas aeruginosa* and *Staphylococcus aureus*. The *Candida albicans* growth was observed in peptone water after 48 h. Negative control group doesn't exhibit microbial growth as expected. Abundant microbial load was observed in positive control group which indicate successful inoculation of microbial cultures into the media (Fig. 2A & B). Significant reduction was observed in the microbial growth for the test group compared to the positive control group both in nutrient broth and peptone water indicate the antimicrobial effectiveness of the ozonated water against *Pseudomonas aeruginosa*, *Staphylococcus aureus*, and *Candida albicans* (Fig. 2A & B). The study examined the association between experimental groups (negative control, positive control and test groups) and turbidity status (turbid or non-turbid). The negative control and test group each had non-turbid samples, while the positive control group had turbid samples. Statistical analysis using Pearson Chi-Square test showed a significant association between group type and turbidity status ($\chi^2 = 9$, *df*= 2, *P* = 0.01). Fisher's Exact test further confirmed this significance with a two-sided *p*-value of 0.03571 (*P* < 0.05), indicating a statistically significant relationship between the groups and turbidity status.

Bactericidal/Fungicidal activity of the ozonated water

The bactericidal activity of the ozonated water confirmed (Fig. 2C & D) on nutrient broth after 24 h of incubation by the inoculated sample from the test groups for *Pseudomonas aeruginosa* and *Staphylococcus aureus*. Similarly, the fungal growth was also nil after 48 h of incubation from test group for *Candida albicans* on peptone water (Fig. 2C). The clear broth obtained like the negative control group for each microorganism confirmed the bactericidal and fungicidal activity of ozonated water.

The results obtained for the current study on the antimicrobial efficacy of ozonated water against *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Candida albicans* provided a valuable insight into the use of ozonated water as disinfection medium. *Pseudomonas aeruginosa* is a ubiquitous gram-negative bacterium, that causes healthcare associated infectious diseases due to its ability to form biofilm on surfaces. It is a nosocomial pathogen poses significant threat among individuals particularly immunocompromised individuals¹⁵. The oxidizing property of ozone enables the penetration into bacterial cell wall and interaction with cellular components. This interaction disrupts the integrity of cell membrane in *Pseudomonas aeruginosa* resulting in cell lysis and subsequent bacterial inactivation¹⁶. Ozone inactivates the key enzymes and proteins present in the bacterial cell wall causing the alteration of metabolic pathways and cellular functions¹⁷. Presence of triatomic oxygen molecules produces an oxidative stress environment resulting in the accumulation of reactive oxygen species and causing damage to the deoxyribonucleic acids (DNA) and proteins¹⁶. *Pseudomonas* family is known for its ability to form biofilms which may lead to the protection of this organisms against antimicrobial agents. However, ozonated water exhibited destruction of these biofilms causing the enhanced susceptibility to bacterial cells¹⁸. Santos *et al.* and Sakowron *et al.* also showed the high mortality rate of *Pseudomonas* when ozonized water used as biocidal agent for microbial control^{19,20}. The findings from the

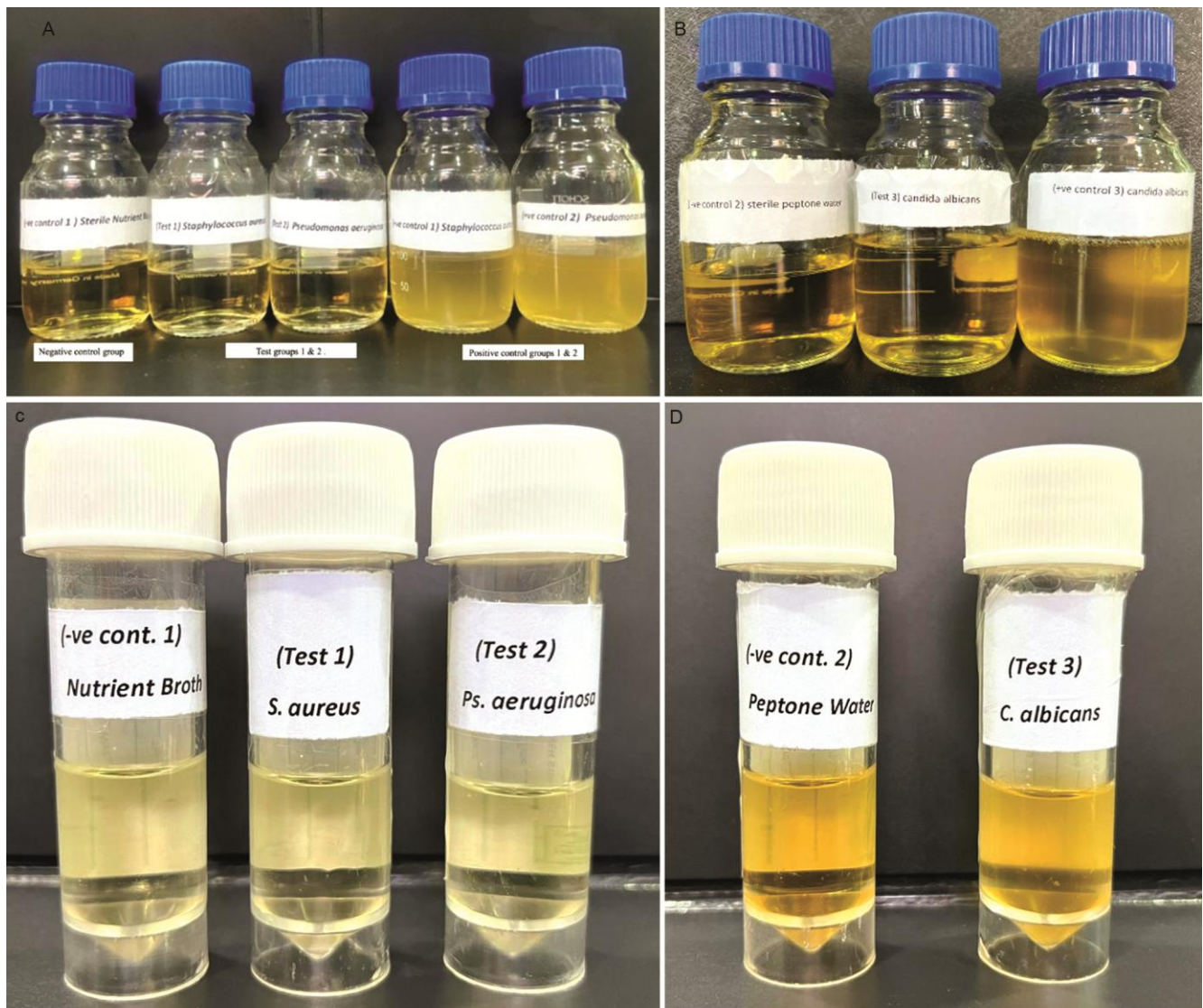


Fig.2 — Effectivity of the ozonated water against; (A) *S. aureus* and *P. aeruginosa*; (B) *C. albicans*; (C) Bactericidal activity of ozonated water; (D) Fungicidal activity of ozonated water.

current study agree with these studies highlighting the ability of ozone against this pathogen¹⁵⁻²⁰.

Staphylococcus aureus is a gram-positive bacterium, characterized by its spherical or round shape and occur in clusters, commonly present on human skin and mucous membranes. It causes various infections including those affecting skin, blood stream and bone. It produces a diverse array of virulence factors including toxins, enzymes and cell surface proteins, which contributes to its resistant to many chemicals. Ozonated water can be active against *Staphylococcus* species like that of *Pseudomonas* by generating leakage of intracellular contents, damage to the cell membrane, destruction of cellular macromolecules through induction of oxidative stress,

inactivation of bacterial enzymes, proteins and disruption of biofilms^{16,21-23}. The present study revealed substantial antibacterial activity of ozonated water against *Staphylococcus aureus* supports the investigations made by other researchers²¹⁻²³.

Candida albicans is considered as a causative organism for oral candidiasis manifests as white lesion on the tongue, inner cheeks and palate often accompanied by pain and discomfort. In addition, *Candida* species are the reason for angular cheilitis, xerostomia, denture stomatitis, periodontal disease and dental caries^{12,13}. Moreover, organic acids produced by *Candida* cells contribute to enamel demineralization and progression of dental caries particularly in immunocompromised individuals with

poor salivary flow²⁴. Ozonated water proposed as an adjunctive therapy for the management of fungal related dental problems due to its oxidative properties^{25,26}.

Furthermore, ozonated water offer additional properties in terms of easiness in application, safety and sustainability^{1,3}. Conventional antifungal medications possess adverse toxic interactions making them not suitable for vulnerable populations²⁷. In contrast, ozonated water does not leave chemical residues or alter taste, thus enhances the patient's compliance. Ability of ozonated water to penetrate fungal cells leads to dissociation and reduction of *Candida* colonization, thus suggested to manage fungal related dental problems²⁸. The antifungal activity of ozonated water from the current study also recommends the efficacy of ozonated water against *Candida albicans*. The disruption of fungal cell membranes and cellular components by ozonated water promises the avenue for management of candida-related infections²⁹.

The findings of the study reveal the broad-spectrum antimicrobial efficacy of ozonated water against clinically relevant pathogens, including bacteria and fungi. However, further research is necessary to elucidate the optimal conditions for ozonated water application, its compatibility with different materials and surfaces. Additional analysis into the potential cytotoxic effects and long-term safety considerations of ozonated water are essential for its application into clinical practice. Despite that, the results of the present study bring out the potential of ozonated water as a versatile disinfection agent with implications for infection control and prevention strategies in healthcare settings.

Conclusions

The study confirmed that ozonated water possesses antimicrobial activity, successfully eliminating the studied organisms. Results parallel the uninoculated media, underscore the potential of ozonated water for use in various fields including healthcare, where robust disinfection is critical. However, further research is required to assess how ozonated water interacts with different materials and in real-world conditions.

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Conflict of interest

Authors declare no conflict of interest related to this study.

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