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Research Article

Antimicrobial properties of hydrogen peroxide and potash alum alone and in combination against clinical bacterial isolates

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Abstract

Hydrogen peroxide (H_2O_2) and potash alum (ALM) are considered important for wide range of biological activities including microbial disinfection. However, comparison of H_2O_2 and ALM antibacterial effect alone and in combination on different clinical bacterial isolates with respect to post treatment time is still not well studied. Thus, in present study we tested susceptibility of five bacterial isolates; *Enterococcus faecalis* (*E. faecalis*), *Enterococcus faecium* (*E. faecium*), *Escherichia coli* (*E. coli*), *Staphylococcus aureus* (*S. aureus*) and *Klebsiella pneumoniae* (*K. pneumonia*) against H_2O_2 and ALM alone and in combination which showed concentration dependent and incubation dependent effect. The H_2O_2 bacterial susceptibility trend was *E. coli*>*K. pneumoniae*>*S. aureus*>*E. faecium*>*E. faecalis* and ALM susceptibility trend was as *K. pneumoniae*>*S. aureus*>*E. faecalis*>*E. coli* >*E. faecium* at highest tested concentration (35mg/ml) and 24hr of incubation period. Comparatively, antimicrobial activity was higher with H_2O_2 than ALM however in the order of 24hr > 48hr >72hr. Both H_2O_2 and ALM showed more zones of inhibition at highest tested concentration than positive control azithromycin (AZI) against *E. faecalis*, *E. faecium* and *S. aureus* at all incubation periods. Moreover, H_2O_2 and ALM in combination (1:1) showed increased zone of inhibition than ALM alone (against all bacteria), H_2O_2 alone (against *E. faecalis* and *E. faecium*) and, AZI (against *E. faecalis*, *E. faecium* and *S. aureus*) without increasing final concentration. Thus, combination treatment might be more effective disinfection and antisepsis strategy which may help us in minimizing dose dependent side effects without compromising efficacy.

Keywords: Combination; Hydrogen peroxide; Potash alum; Zones of inhibition

Introduction

Antimicrobial agents are extensively utilized in health care, industry and the environment to control and treat microbial infection [1, 2]. Combining antimicrobial agent may improve their activities by synergism and may provide an effective alternative to microbial resistance against single chemical [1, 3]. Additionally, a combination strategy is cost effective and time-saving which does not require long laboratory work to design, synthesize or discover new drugs more frequently. Thus, encouraging the use of drugs in combination for the management of bacterial infections.

Hydrogen peroxide (H_2O_2), our study compound, retains bactericidal efficacy which is being observed in H_2O_2 producing bacteria and phagocytic cells that inhibit other bacterial species and can kill invading microorganism, respectively [4]. The ability of hydrogen peroxide to produce hydroxyl radical contributes in the oxidation process of biomolecules. Hydrogen peroxide conversion to cytotoxic compounds is supported by peroxidases and by reducing agents [4]. Hydrogen peroxide along with produced free hydroxyl radicals may contribute in microbial management [1]. H_2O_2 is common disinfectant used to control gingival plaques [5], treating biofilms [6] and Fournier's gangrene [7]. It is also used for the sterilization of other surfaces [8] including industrial fish egg [9, 10] and fruits treatments [11].

Alum (ALM), our second study compound is a salt known as aluminium potassium sulfate ($KAl(SO_4)_2$) [12] and is suggested as a class 1 dynamic compound in mouthwashes [13], and used for the treatment of pediatric cough, hemorrhagic cystitis [14] ulcers, oral cavities and burns with anticarcinogenic effect [15], cosmetics [16], and domestic and industrial water treatments [17]. It has potential inhibitory effects on microbes [18].

In infection treatment, side effects associated with an overload of drugs in the human body and reduced microbial susceptibility to a single drug could be controlled by using them in combination even at lower concentrations. A synergistic or additive effect in mixtures is a key point in decreasing concentrations of drugs without compromising outcomes. Similarly, the synergy of hydrogen peroxide was found with many compounds including rifampicin [19], chlorhexidine [20], neucoproine [21], hypothiocyanite [22], sodium bicarbonate [23], iodine [24-26], different organic acids [27, 28], as well as with UV-irradiation [29]. Likewise, alum in combination with leaf extract of guava (*Psidium guajava*) has been utilized topically as an antiperspirant, an antibacterial and for astringent purposes [30]. However, alum and H_2O_2 incubation wise and concentration wise in combination against various microorganisms were not studied. Thus, in this study, we compared incubation-dependent and concentration-dependent inhibitory effects of alum and hydrogen peroxide alone and in mixtures against five clinical bacterial isolates.

Materials and methods

Culture media and chemicals

Nutrient agar (Neogen Company, UK), nutrient broth (Merck, Germany), and dimethyl sulfoxide (BIOCHEM) were used in experiments. Test compounds hydrogen peroxide (Sigma) and potash alum (purchased from the local market of Mirpur, AJK) were diluted with saline to acquire desired concentrations.

Strains and culture conditions

The clinical bacterial isolates *Enterococcus faecalis* (*E. faecalis*), *Enterococcus faecium* (*E. faecium*), *Escherichia coli* (*E. coli*), *Staphylococcus aureus* (*S. aureus*) and *Klebsiella pneumoniae* (*K. pneumonia*) were taken from the Combined Military Hospital (CMH) Rawalpindi (Pakistan) for this study. Bacteria were sub-cultured on

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nutrient agar plates and incubated at 37°C for 24hr in this experiment.

Anti-bacterial activity of alum and hydrogen peroxide separately

Fresh bacterial cultures were prepared by spreading 70µl bacterial suspension (0.060 OD at 600 wavelengths) on agar plate homogeneously. After short air dry (10min), 4 wells (6mm diameter) were made on each plate and loaded with 50µl of test compounds having 4.4mg/ml, 8.7mg/ml, 17.5mg/ml and 35mg/ml concentrations, respectively. Later, the plates were incubated at 37°C for 24hr, 48hr and 72hr. Finally, plates were photographed and zones of inhibition were measured in cm.

Anti-bacterial activity of alum and hydrogen peroxide in combination

In combination test, alum and hydrogen peroxide were used in 1:1 ratio with two-fold increasing concentration from well-1 to well-4, respectively. Briefly, well-1, well-2, well-3 and well-4 were loaded with 50µl of alum and hydrogen peroxide in 1:1 ratio from 4.4mg/ml, 8.7mg/ml, 17.5mg/ml and 35mg/ml concentrations for testing their synergistic effects. Later, the plates were incubated, photographed and zones of inhibition were measured the same as in the screening of alum and hydrogen peroxide separately.

Statistical Analysis

All experiments were performed thrice in triplicates. Student's t-test was applied to check significant differences using the lowest zone of inhibition (except zero) VS each tested zone of inhibition ($^{a}p<0.05$; $^{b}p<0.005$, $^{c}p<0.0001$).

Results and Discussion

The present study was carried out to evaluate the antibacterial activity of hydrogen peroxide (H_2O_2) and potash alum (ALM) alone and in combination. For this, well diffusion method was used against five pathogenic bacterial isolates namely *E. coli, E. faecium, E. faecalis, S. aureus* and *K. pneumoniae.* Later, zones of inhibition were measured as a representative of bacterial susceptibility at different concentrations and different incubation periods at 24hr, 48hr and 72hr.

Individual antibacterial effect of alum and hydrogen peroxide

The hydrogen peroxide tested concentrations, 35mg/ml, 17.5mg/ml, 8.7mg/ml and 4.4mg/ml showed 0.783cm, 0.693cm, 0.581cm, 0.461cm zones of inhibition against *E. faecalis,* 0.872cm, 0.725cm, 0.612cm, 0.522cm zones of inhibition against *E. faecium,* 0.891cm, 0.712cm, 0.583cm, 0.421cm zones of the inhibition against *S. aureus,* 1.129cm, 0.976cm, 0.806cm, 0.647cm zones of inhibition against *E. coli* and 0.998cm, 0.821cm, 0.705cm, 0.579cm zones of inhibition against *K. pneumoniae,* respectively at 24 hr incubation (Table 1). Hydrogen peroxide is known as an effective disinfectant and it retains the ability to quickly kill microorganisms **[31]**. In addition to antimicrobial activity of hydrogen peroxide because of its oxidative properties it generates more powerful oxidant, called hydroxyl radical **[32]**. Explaining hydrogen peroxide's mode of action, it is reported that hydrogen peroxide exposure to logarithmically growing *E. coli* to kill by damaging DNA at low concentration whereas higher concentration (> 10 mM) may kill by damaging all macromolecules **[33-36]**.

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Table 1. Antibacterial susceptibility of selected bacteria against H_2O_2, ALM and control AZI at indicated concentrations and incubation periods. Student's t-test was applied to check significant where [a]p<0.05; [b]p<0.005, [c]p<0.0001

Tested compound	Concentration (mg/ml)				Concentration (mg/ml)				Concentration (mg/ml)				Test bacteria
	4.4	8.7	17.5	35	4.4	8.7	17.5	35	4.4	8.7	17.5	35	
	Zone of inhibition (cm) at 24 hr incubation				Zone of inhibition (cm) at 48 hr incubation				Zone of inhibition (cm) at 72 hr incubation				
AZI	0.00±0.00	0.09±0.02	0.28±0.01 c	0.33±0.01 **c**	0.00±0.00	0.09±0.02 **a**	0.23±0.00 **b**	0.29±0.03**c**	0.00±0.00	0.13±0.02 **a**	0.22±0.00 **b**	0.29±0.01 **c**	***E. faecalis***
H_2O_2	0.46±0.01	0.58±0.01 **c**	0.69±0.01**c**	0.78±0.01 **c**	0.46±0.01	0.58±0.01 **c**	0.69±0.01 c	0.78±0.01**c**	0.45±0.01	0.53±0.02 **a**	0.63±0.02 **c**	0.71±0.02 **c**	
ALU	0.01±0.00	0.09±0.02 a	0.26±0.02c	0.44±0.02 c	0.01±0.01	0.10±0.02 a	0.23±0.02 c	0.40±0.02c	0.01±0.00	0.09±0.03 a	0.25±0.02 c	0.43±0.02 c	
AZI	0.00±0.00	0.11±0.02	0.20±0.02	0.33±0.03 **a**	0.00±0.00	0.10±0.02	0.19±0.02	0.29±0.01**a**	0.00±0.00	0.10±0.01	0.19±0.03	0.28±0.03 **a**	***E. faecium***
H_2O_2	0.52±0.01	0.61±0.01 **a**	0.73±0.01**c**	0.87±0.01 **c**	0.52±0.01	0.61±0.01 **a**	0.71±0.01 **c**	0.86±0.00c	0.51±0.01	0.60±0.01 **a**	0.70±0.01 **c**	0.82±0.01 **c**	
ALU	0.00±0.00	0.02±0.02	0.18±0.02**c**	0.42±0.02 **c**	0.00±0.00	0.03±0.01	0.17±0.02	0.42±0.02**c**	0.01±0.00	0.03±0.01	0.16±0.02 **c**	0.39±0.02 **c**	
AZI	0.00±0.00	0.10±0.02 c	0.26±0.00**b**	0.34±0.03 c	0.00±0.00	0.09±0.01 **a**	0.24±0.01 **a**	0.31±0.03c	0.00±0.00	0.09±0.01 **b**	0.3±0.01**a**	0.31±0.03 **b**	***S. aureus***
H_2O_2	0.42±0.01	0.58±0.01 **c**	0.71±0.02**c**	0.89±0.02 **c**	0.42±0.01	0.58±0.01 **c**	0.70±0.01 **c**	0.87±0.01 **c**	0.41±0.01	0.54±0.01 **a**	0.66±0.02 **c**	0.80±0.02 **c**	
ALU	0.01±0.00	0.12±0.02 a	0.23±0.02**c**	0.46±0.02 **c**	0.01±0.00	0.11±0.03 a	0.23±0.02 **c**	0.43±0.02 **c**	0.01±0.01	0.09±0.02 **a**	0.21±0.01 c	0.42±0.02 **c**	
AZI	1.09±0.03	1.29±0.02 **b**	1.42±0.01**c**	1.56±0.01 **c**	1.02±0.02	1.24±0.02 **a**	1.36±0.02 **b**	1.52±0.01 **c**	1.04±0.00	1.24±0.02 **a**	1.38±0.03 **c**	1.50±0.04 **c**	***E. coli***
H_2O_2	0.65±0.01	0.81±0.02 **b**	0.98±0.02**c**	1.13±0.02 **c**	0.64±0.01	0.80±0.01 **b**	0.96±0.01 **c**	1.11±0.01 **c**	0.64±0.01	0.79±0.01 **c**	0.96±0.02 **c**	1.11±0.02 **c**	
ALU	0.01±0.01	0.13±0.03 **a**	0.26±0.02**c**	0.43±0.03 **c**	0.01±0.00	0.11±0.02 **a**	0.25±0.02 **c**	0.42±0.02 **c**	0.01±0.01	0.09±0.02 **a**	0.21±0.02 **c**	0.40±0.01 **c**	
AZI	1.10±0.03	1.23±0.02 **a**	1.38±0.02**c**	1.49±0.02 **c**	1.04±0.01	1.19±0.03 **a**	1.33±0.01 **b**	1.46±0.03**c**	0.10±0.03	1.18±0.04 **a**	1.30±0.02 **b**	1.43±0.02 **b**	***K. pneumoniae***
H_2O_2	0.58±0.02	0.71±0.02 **c**	0.82±0.01**c**	1.00±0.02 **c**	0.57±0.02	0.69±0.00 c	0.81±0.01 **c**	0.99±0.02 **c**	0.42±0.01	0.55±0.02 **a**	0.66±0.01 **c**	0.81±0.03 **c**	
ALU	0.03±0.00	0.11±0.02 **a**	0.26±0.02 c	0.49±0.02 c	0.02± 0.00	0.1±0.03	0.29±0.02 b	0.47±0.02 c	0.01± 0.00	0.07±0.02	0.21±0.03 b	0.45±0.03 c	

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Similarly, alum concentrations, 35mg/ml, 17.5mg/ml, 8.7mg/ml and 4.4mg/ml showed 0.438cm, 0.263cm, 0.089cm, 0.005cm zones of inhibition against *E. faecalis*, 0.421cm, 0.184cm, 0.019cm, 0cm zones of inhibition against *E. faecium*, 0.455cm, 0.234cm, 0.122cm, 0.011cm zones of inhibition against *S. aureus*, 0.430cm, 0.258cm, 0.126cm, 0.013cm zones of inhibition against *E. coli*, and 0.485cm, 0.264cm, 0.106cm, 0.025cm zones of inhibition against *K. pneumoniae*, respectively at 24hr incubation (Table 1). These results concur with the previous findings which reported protective effect of the 100 PPM alum solution as less than 1000 PPM alum or more **[37]**. In 2014, Bnyan et al **[38]**, additionally observed a paramount bactericidal effect of alum however, the mechanism of bactericidal effect of alum is not prominent **[39]**. Some postulations attribute the antibacterial effect of alum to reduction in acidity or deleterious effects on bacterial cell wall **[40]**.

Thus, H_2O_2 bacterial susceptibility trend was *E. coli*>*K. pneumoniae*>*S. aureus*>*E. faecium*>*E. faecalis* and ALM susceptibility trend were as *K. pneumoniae*>*S. aureus*>*E. faecalis*>*E. coli* >*E. faecium* at highest tested concentration and 24hr of incubation period (Table 1). However, H_2O_2 showed more zone of inhibition than alum against all tested bacteria at all tested concentrations and incubation. Interestingly, zones were higher at lower incubation time which reduced later in the order of 24hr > 48hr >72hr incubation times even at same H_2O_2 and ALM concentration (Table 1). Similarly, Saranraj *et al*., 2012, **[31]** reported hydrogen peroxide as an effective disinfectant but this effect was a short term which means hydrogen peroxide has no long-term or preserving effect. Similar, incubation effect was observed for azithromycin (AZI), used as a positive control as shown in (Table 1). However, both H_2O_2 and ALM showed more zones of inhibition at the highest tested concentration (35mg/ml) than AZI against *E. faecalis*, *E. faecium* and *S. aureus* at 24hr, 48hr and 72hr of incubation.

Combined antibacterial effect of alum and hydrogen peroxide

To check combination effect, alum and hydrogen peroxide were used in 1:1 ratio with two-fold increase in concentration from well-1 to well-4 in well diffusion method, respectively, as explained in method section. The combination of alum and hydrogen peroxide in 1:1 ratio forming 4.4mg/ml, 8.7mg/ml, 17.5mg/ml and 35mg/ml total concentrations showed 0.508cm, 0.635cm, 0.779cm and1.052cm zones of inhibition against *E. faecalis*, 0.497cm, 0.656cm, 0.802cm and 0.979cm zones of inhibition against *E. faecium*, 0.406cm, 0.552cm, 0.672cm and 0.843cm zones of inhibition against *S. aureus*, 0.414cm, 0.547cm, 0.735cm and 0.870cm zones of inhibition against *E. coli* and, 0.397cm, 0.575cm, 0.770cm and 0.968cm zones of inhibition against *K. pneumoniae*, respectively at 24hr of inhibition (Table 2). In combination test, zones were higher at lower incubation time which reduced later in order of 24hr > 48hr >72hr incubation times same as H_2O_2 and ALM in individual test.

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Table 2. Antibacterial susceptibility of selected bacteria against H_2O_2 and ALM in combination (1:1) at indicated concentrations and incubation periods. Student's t-test was applied to check significance where [a]p<0.05; [b]p<0.005, [c]p<0.0001

Combination	Concentration (mg/ml)				Concentration (mg/ml)				Concentration (mg/ml)				Test Bacteria
	4.4	8.7	17.5	35	4.4	8.7	17.5	35	4.4	8.7	17.5	35	
	Zone of inhibition (cm) at 24 hr incubation				Zone of inhibition (cm) at 48 hr incubation				Zone of inhibition (cm) at 72 hr incubation				
H_2O_2+ ALM	0.51±0.02	0.64±0.01[a]	0.78±0.01[c]	1.05±0.02[c]	0.46±0.02	0.57±0.02[a]	0.68±0.02[c]	0.92±0.00[c]	0.45±0.05	0.53±0.02	0.64±0.02[a]	0.88±0.02[c]	*E. faecalis*
H_2O_2+ ALM	0.50±0.05	0.66±0.06[a]	0.81±0.03[b]	0.98±0.04[c]	0.48±0.03	0.63±0.06[a]	0.75±0.04[c]	0.91±0.02[c]	0.48±0.04	0.62±0.04[a]	0.73±0.03[c]	0.88±0.03[c]	*E. faecium*
H_2O_2+ ALM	0.41±0.02	0.55±0.03[a]	0.67±0.02[b]	0.84±0.02[c]	0.38±0.03	0.51±0.03	0.61±0.01[b]	0.78±0.01[c]	0.39±0.02	0.51±0.02	0.60±0.02[a]	0.76±0.01[c]	*S. aureus*
H_2O_2+ ALM	0.41±0.01	0.55±0.01	0.74±0.01[c]	0.87±0.03[c]	0.37±0.01	0.49±0.01	0.66±0.012[c]	0.79±0.02[c]	0.39±0.01	0.53±0.01[a]	0.68±0.01[c]	0.78±0.03[c]	*E. coli*
H_2O_2+ ALM	0.40±0.03	0.58±0.04[c]	0.77±0.02[c]	0.97±0.02[c]	0.37±0.04	0.50±0.04[a]	0.69±0.02[c]	0.86±0.011[c]	0.36±0.02	0.50±0.03[a]	0.69±0.03[c]	0.83±0.01[c]	*K. pneumonia*

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Interestingly, H_2O_2 and ALM in combination showed more zones of inhibition at the highest tested concentration (35mg/ml) than AZI against *E. faecalis, E. faecium* and *S. aureus at* 24hr, 48hr and 72hr of incubation (Table 1 & 2). Moreover, H_2O_2 and ALM in the combination showed more zone of inhibition than H_2O_2 alone against *E. faecalis* and *E. faecium* and, also more effect than ALM alone against all tested bacteria *i;e E. faecalis, E. faecium, S. aureus, E. coli* and *K. pneumoniae* at all incubations (Table 1 & 2), summarized in (Table 3). Amadi *et al*. **[30]** found similarly increased antibacterial impacts of alum with guava leaf extract in combination. Another study reported that the combination of alum with antibiotics (tetracycline and cefotaxime) was more effective than antibiotic combination and the combination effect was more efficient than an individual effect **[40]**. Similarly, hydrogen peroxide in combination with lactoperoxidase and thiocyanate is reported much more effective than hydrogen peroxide alone as an inhibitor of bacterial metabolism and growth **[41]**. Thus, the combination strategy provides us with an opportunity to improve their efficacy at low concentration. Therefore, side effects associated with a higher load of drugs can be reduced without compromising their efficacy **[42]**. Due to these advantages, combination therapies have become a standard in many areas including cancer treatment **[43]**, hypertension **[44]**, asthma **[45]**, and AIDS **[46, 47]** which open a new horizon for research and new hope for disease control including bacterial infections in future.

Table 3. Comparison of antibacterial effect at highest tested concentration (35 mg/ml) of test compounds and their combinations against selected bacteria

Bacteria	Susceptibility trend against tested compounds
E. faecalis	H_2O_2+ALU > H_2O_2 > ALU > AZI
E. faecium	H_2O_2+ALU > H_2O_2 > ALU > AZI
S. aureus	H_2O_2 > H_2O_2+ALU > ALU > AZI
E. coli	AZI > H_2O_2 > H_2O_2+ALU > ALU
K. pneumoniae	AZI > H_2O_2 > H_2O_2+ALU > ALU

Conclusion

This study found that the increasing concentration of H_2O_2 and ALM have increasing antibacterial effects on tested bacteria which reduced with time. Moreover, H_2O_2 and ALM in combination showed increased zone inhibition than ALM alone (against all bacteria) and H_2O_2 alone (against *E. faecalis* and *E. faecium*) without increasing final concentration. Thus, a combination strategy might help us in minimizing dose-dependent side effects of drugs without compromising effectiveness.

Authors' contributions

Conceived and designed the experiments: A Ali & H Khurshid, Performed the experiments: H Khurshid & A Ali, Analyzed the data: A Ali & B Akbar, Contributed materials/ analysis/ tools: M Rafiq, F Nazir & M Ahmed, Wrote the paper: I Ali & M Ahmed.

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