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EVALUATION OF ANTIMICROBIAL PRESERVATIVE EFFICACY OF SODIUM PERBORATE IN SODIUM CHLORIDE NASAL DROPS IN COMPARISON WITH BENZALKONIUM CHLORIDE

Syeda Rima Ishaq and Tasneem Adam Ali

Department of Microbiology, University of Karachi, Karachi 75270, Pakistan
reemaishaq@yahoo.com

ABSTRACT

Benzalkonium chloride (BKC), an antimicrobial preservative, commonly used in nasal drops, has been reported for its many adverse effects especially when used in pediatrics formulations. Therefore it is the need to find out safe and effective antimicrobial preservative. The aim of this study was to evaluate antimicrobial preservative efficacy of sodium perborate in 0.9% sodium chloride nasal drops. Trials A, B and C were prepared with 0.01, 0.02, and 0.04%, w/v sodium perborate at pH between 5.5- 6.5. Trials D, E and F, manufactured at pH ranges 6-7, 7-8 and 8-9.Trial H was made with benzyl alcohol and disodium EDTA in addition to sodium perborate, Trial K was with addition of benzyl alcohol and sodium perborate. Antimicrobial preservative efficacy was determined as per United State Pharmacopoeia (USP). Marketed formulation, sodium chloride nasal drops with 0.02% BKC (Trial G, I and J) were used as control. All the trial samples complied with acceptance criteria except A and B. Thus sodium perborate can be used in sodium chloride nasal drops at 0.04% concentration at all pH ranges as substitute of BKC. Furthermore, the addition of benzyl alcohol and disodium EDTA also enhanced the antimicrobial preservative efficacy of sodium perborate.

Keywords: Sodium chloride nasal drops, antimicrobial preservative, benzalkonium chloride, sodium perborate.

INTRODUCTION

Most of the nasal drug products are manufactured in aqueous forms such as solutions, emulsions and suspensions. These liquid dosage forms are particularly prone to microbial contamination that may be introduced during manufacturing and/ or use by the patients as they are packed in multi-dose containers. Multiple time uses of drug from the same bottle can cause microbial contamination, resulting in product degradation or infection to the user (Aulton, 2013). To overcome this contamination, antimicrobial preservatives are used as additives. These are used in:

a) Non-sterile Dosage Forms to protect from microbial growth or from microorganisms that are introduced during or subsequent steps of the manufacturing process.

b) Sterile Dosage Forms (packaged in multi-dose containers) to inhibit growth of microorganisms that might be introduced from repeated withdrawing of doses (USP 36, 2013).

Though antimicrobial preservatives are used to control microbial contaminants in drugs however, due to the emergence of drug resistance, many preservatives have become less effective against certain microorganisms. An effective concentration of preservative may also produce a physiological response and undesirable clinical side effects. Benzalkonium chloride (BKC), the most commonly used antimicrobial preservative in nasal drugs has many reported side effects such as, its ciliotoxic effect at concentrations used in normal saline. At higher concentrations, it causes nasal irritation (Riechelmana *et al.*, 2004), potentially toxic to the mucosa in vivo (Berg *et al.*, 1997), causes complete standstill of ciliary beat frequency in human nasal mucosa (Boek *et al.*, 1999), toxic to human neutrophils (Boston *et al.*, 2003), induce irreversible cessation of ciliary movement observed in all cells exposed to nasal sprays containing benzalkonium chloride in a 50% solution, causes rhinitis medicamentosum. The mucosa exposed to benzalkonium chloride showed squamous cell metaplasia in which normal cells changed to abnormal cells (Grossan, 2003).

As sodium chloride nasal drops are widely used in neonates so they are high risk group due to low age and less developed immunity. Therefore, it is essential to investigate other substances which should be having good antimicrobial preservative action and safe in use. Sodium perborate tetrahydrate ($NaBO_3.4H_2O$) is a preservative that alters protein synthesis in bacterial cells by oxidizing cell membranes and altering membrane-bound enzymes thus inhibiting the microbial cell growth. In aqueous environment, it is catalyzed into hydrogen peroxide (H_2O_2), oxygen and water, and the resulted hydrogen peroxide has cidal activity for microbes (Freeman and Kahook, 2009,

Freeman, 2008). Though sodium perborate is being used in eye drops, however, there is no reference data in the literature found on attempts to use it in nasal drops. Therefore, in the current investigation antimicrobial preservative efficacy of sodium perborate was determined in 0.9% sodium chloride nasal drops at different concentrations and pH. The antimicrobial preservative efficacy of sodium perborate was also evaluated alone and in combination with other compounds such as benzyl alcohol and disodium EDTA.

MATERIALS AND METHODS

Benzalkonium chloride solution (50% Pharma grade, Novo Nordisk Pharmatech A/S Denmark), and sodium perborate tetrahydrate (Merck KGaA) were received complimentary from M/s Instrochem Services Pakistan. Sodium chloride (Fischer Scientific), benzyl alcohol (Hubei Greenhome China), disodium EDTA (Riedel –de Haen) and HCl were also gifted by AsianContinental Pvt. Ltd. Trypticase soya agar (TSA) and sabouraud dextrose agar (SDA) were purchased from Oxoid, High density polyethylene (HDPE), white opaque 30 mL bottles with nozzles and cap were donated by National Care Pack, Pakistan.

Preparation of Microbiological Media

The culture media were prepared as per manufacturer's instructions and sterilized at 121ºC at 15 lbs pressure for 15-20 minutes. Its suitability was checked by growth promotion tests as mentioned in USP.

Inoculum preparation and standardization

From a freshly grown stock culture of each of the test microorganisms, the respective media slants were inoculated and incubated as mentioned in Table **1**. Cultures were harvested after incubation period in 0.9% sterilized normal saline by properly shaking the slants to get bacterial and fungal suspensions. The concentrate of culture suspension was adjusted by McFarland solution to get 1 x 10^8 cfu/mL.

Preparation of trial formulations

a) **Evaluation of antimicrobial preservative efficacy of sodium perborate at different concentrations.**

Trials A, B and C of 0.9% sodium chloride nasal drops were prepared in distilled water by using 0.01, 0.02 and 0.04% sodium perborate, respectively. Commercial product, 0.9% sodium chloride nasal drop (available in the market), containing 0.02% BKC was prepared as trial G and used as control sample. The pH was adjusted in the range 5.5 - 6.5. All trial preparations were filtered through 0.45µ sterile polyvinylidene flouride (PVDF) syringe filter and filled in 30mL HDPE plastic bottles.

b) **Evaluation of the antimicrobial preservative efficacy of sodium perborate at different pH**

Trials C, D, E and F were formulated with 0.9% sodium chloride and 0.04% sodium perborate in distilled water. The pH was adjusted between 5-6, 6-7, 7-8 and 8-9, respectively. For comparison purpose, trial G was used.

c) **Evaluation of the antimicrobial preservative efficacy of sodium perborate with benzyl alcohol and disodium EDTA**

For evaluating the synergistic effect of other ingredients with sodium perborate in formulation, trial H was included in the study, formulated with 0.9% sodium chloride, 0.04% sodium perborate, 0.02% disodium EDTA and 1% benzyl alcohol. In order to compare it with the marketed product, trial I was also included having the same formulation, except instead of using 0.04% sodium perborate, 0.02% BKC was added.

d) Evaluation of the antimicrobial preservative efficacy of sodium perborate with benzyl alcohol

Another group of trials was prepared to evaluate the synergistic effect of sodium perborate with benzyl alcohol. Trial J was replicated as marketed product with 0.9% sodium chloride, 0.02% BKC and 1% benzyl alcohol. Trial K was prepared with the same ingredients, except instead of using 0.02% BKC, 0.04% sodium perborate was used. The pH of the trials was 5.5-6.5.

Antimicrobial effectiveness testing

Antimicrobial effectiveness test was carried out as per USP <51> monograph for product category 2 (USP 36, 2013). Each of the trial sample (20mL) was taken in 30 mL HDPE bottles. Samples were challenged with standardized inoculum of *Escherichia coli* (ATCC 8739), *Staphylococcus aureus* (ATCC 6538), *Pseudomonas aeruginosa* (ATCC 9027), *Candida albicans* (ATCC 1023) and *Aspergillus niger* (ATCC 16404) in order to achieve final concentration between 1 x 10^5 -1 x 10^6 cfu/mL of sample. The initial concentration of inoculums in samples was determined by plate count method. Ten fold serial dilutions were prepared, using sterile normal saline (0.9 % w/v) with peptone (0.1 % w/v) for all organisms except *A. niger*, for which sterile saline with polysorbate 80

(0.05%) was prepared. All the challenged samples were incubated at 20-25^0 C for 28 days. Total viable count (cfu/mL) was determined and log reduction was calculated at 14 and 28 days from initial count.

Statistical analysis

All results were expressed as the mean ± standard deviation (SD) of three independent experiments carried out in triplicates. Statistical analysis for all the tests was performed using one-way ANOVA with post hoc Bonferoni's test. Values of $p < 0.05$ were considered significant.

Acceptance Criteria

For bacteria, the log reduction should not be less than 2.0 from the initial count at 14 days, and no increase from the 14 days' count at 28 days. For yeast and molds there should be no increase from the initial calculated count at 14 and 28 days.

RESULTS

a) **Antimicrobial preservative efficacy of sodium perborate at different concentrations**

In trial A, no reduction in growth (cfu/mL) of the microorganisms was observed at 14 and 28 days. All strains showed increase in count. In trial B, when concentration of sodium perborate was increased up to 0.02%, it failed to reduce the growth of *E. coli* and *P. aeruginosa*, however in case of *S. aureus* , 2.7 $\pm$ 0.314 log reduction was observed at 14 days but again increase in growth was observed at 28 days as log reduction found 0.774 $\pm$ 0.533 calculated from initial count, thus not meeting the acceptance criteria. This concentration of sodium perborate was found effective only for *C. albicans* and *A. niger* as no increase in growth observed at 14 and 28 days.

Trial C with 0.04% sodium perborate concentration was found to meet the acceptance criteria. In case of challenged bacteria, more than 3.0 log reduction was observed from the initial count at 14 and 28 days. Maximum log reduction was observed in case of *E. coli* that is 4.5 $\pm$ 0.2 and 4.7 $\pm$ 0.1 at 14 and 28 days respectively. For yeast and molds, sodium perborate was found to possess fungicidal activity as in *c.albicans* 1.8 $\pm$ 0.7 and 2.1 $\pm$ 0.6, and in *A.niger* 1.0 $\pm$ 0.3 and 1.2 $\pm$ 0.5 log reduction observed at 14 and 28 days respectively. The results were compared with trial G (control sample) which showed more than 4 log reduction in all test strains (Fig. **1).**

Table 1. Microorganisms and their incubation requirements.

No.	Name of microorganisms	Medium	Incubation requirements
1.	*Candida albicans* ATCC 10231	Sabouraud dextrose agar	20-25°C for 3 days
2.	*Aspergillus niger* ATCC 16404	Sabouraud dextrose agar	20-25°C for 7 days
3.	*Escherichia coli* ATCC 8739	Trypticase soya agar	30-35°C for 24 to 48 hours
4.	*Pseudomonas aeruginosa* ATCC 9027	Trypticase soya agar	30-35°C for 24 to 48 hours
5.	*Staphylococcus aureus* ATCC 6538	Trypticase soya agar	30-35°C for 24 to 48 hours

*ATCC. American Type Culture Collection

b) **Antimicrobial preservative efficacy of sodium perborate at different pH**

Though the recommended pH for nasal solutions is 5.5- 6.5 but in order to evaluate the effect of pH on antimicrobial preservative efficacy of sodium perborate, trials C, D, E and F were prepared in pH range 5-6, 6-7, 7-8 and 9-10 respectively. All trials were found to meet the acceptance criteria. More than 2 log reduction in the count of all bacterial strains, while in yeast and mold, more than 1 log reduction at pH 5-6 (Trial C) and 6-7 (Trial D) was observed. The trials (E and F) formulated at alkaline pH (7-8 and 9-10) showed more than 4 log reduction in all strains (Fig. **2**).

c) **Synergistic effect of sodium perborate with benzyl alcohol and disodium EDTA.**

Trial H was prepared with sodium perborate, benzyl alcohol and disodium EDTA at pH 5-6 in order to evaluate the synergistic effect of other compounds used in the formulation. The results revealed > 5 log reduction in all strains except *A.niger* which showed < 2 log reduction, however meeting the acceptance criteria since the count did not increase from the initial count. The results were compared with the marketed formulation (Trial I) and it also showed more than 5 log reduction in all strains except *A.niger* showing 3 log reduction in growth (Fig. **3**).

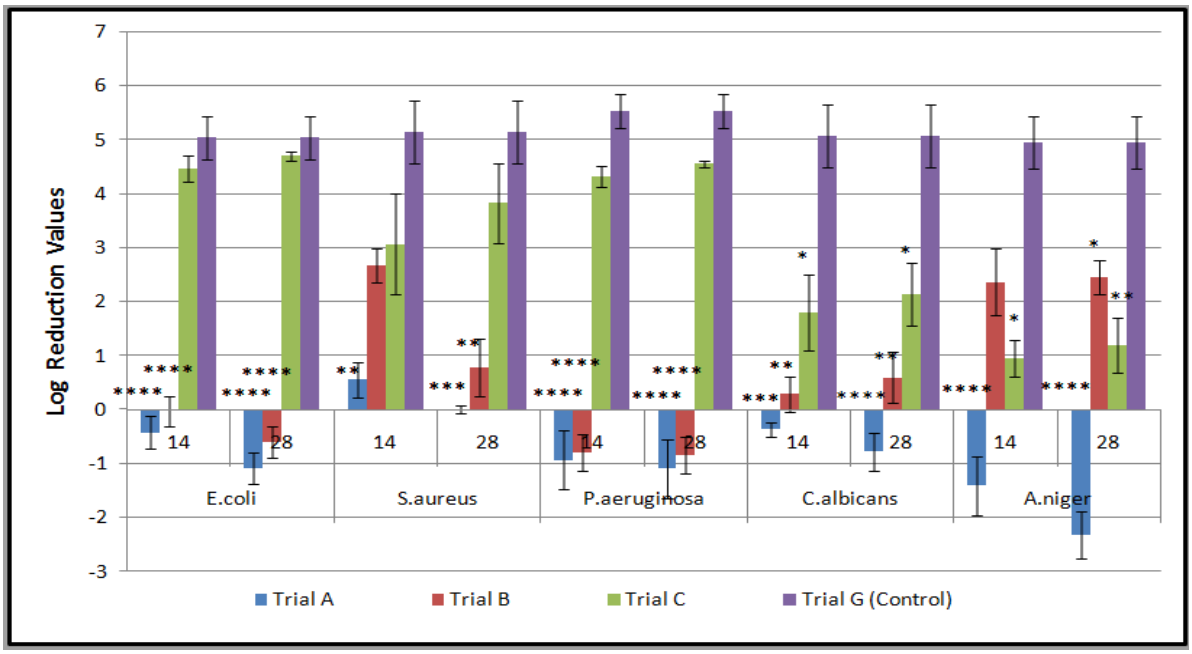

Fig.1. Evaluation of antimicrobial preservative efficacy of sodium perborate at different concentrations. Trial A and B found failed in antimicrobial effectiveness test and found significantly different ($P<0.05$) while comparing with trial G. Negative log reduction values indicated the increase in growth. Trial C was complied with the test and not found significantly different as $P > 0.05$. Each bar represent mean ± SD of three replicates. *Significant at $p < 0.05$; ** $p < 0.005$; *** $p < 0.001$;**** $p < 0.0001$

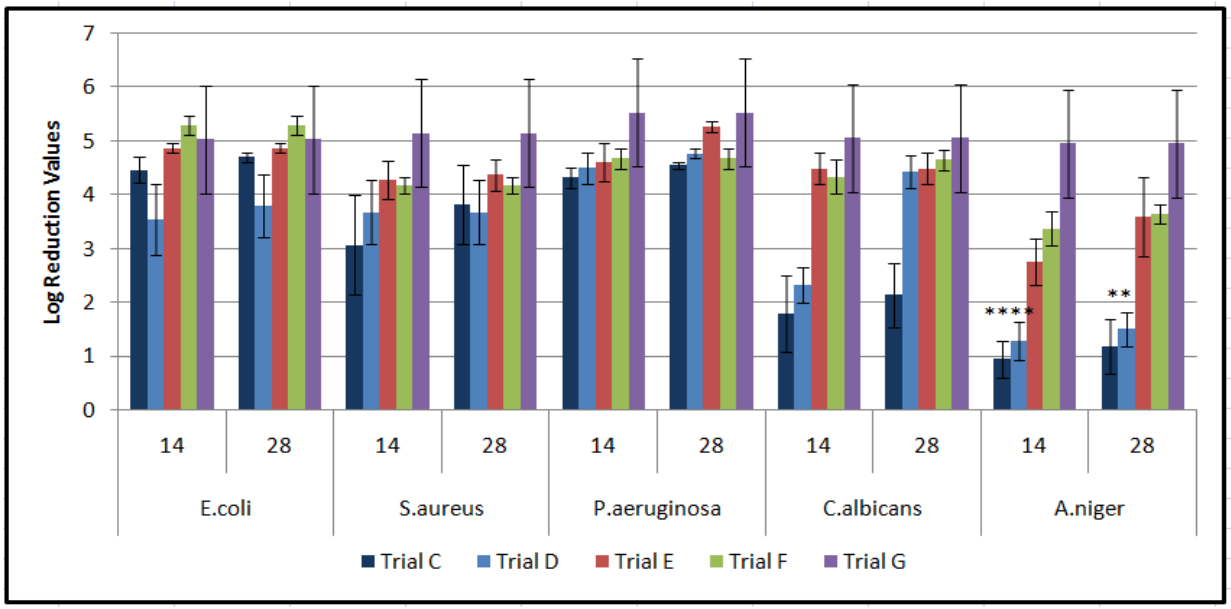

Fig.2. Evaluation of antimicrobial preservative efficacy of sodium perborate at different pH. All trials (C, D, E, and F) passed in antimicrobial effectiveness test. Level of significance of all trials was found $P> 0.05$ except in *A.niger* where $P < 0.05$ observed.

d) **Synergistic effect of sodium perborate with benzyl alcohol.**

Trial K formulated with sodium perborate and benzyl alcohol at pH 5-6, results showed more than 5 log reduction in all strains while in case of *A.niger*, 3 log reduction was observed, indicating its compliance with the acceptance criteria. Marketed formulation (Trial J) was also depicted the same behavior (Fig. **4**).

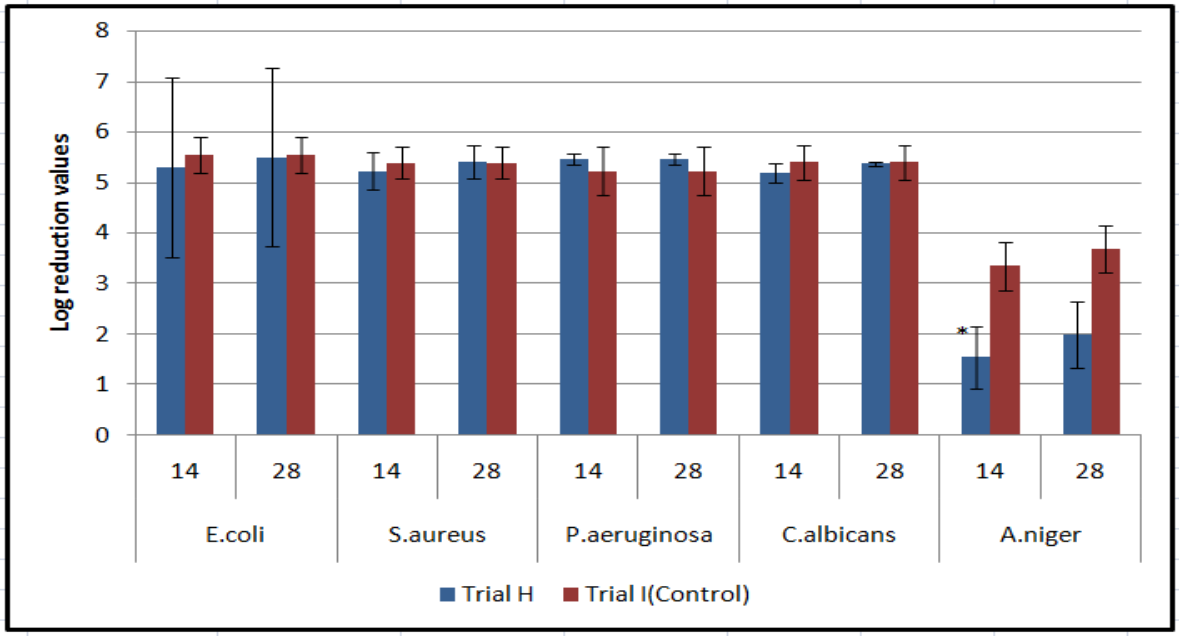

Fig.3. Evaluation of synergistic effect of sodium perborate with benzyl alcohol and disodium EDTA. Trial H and I found equal as P >0.05 except in case of *A.niger*

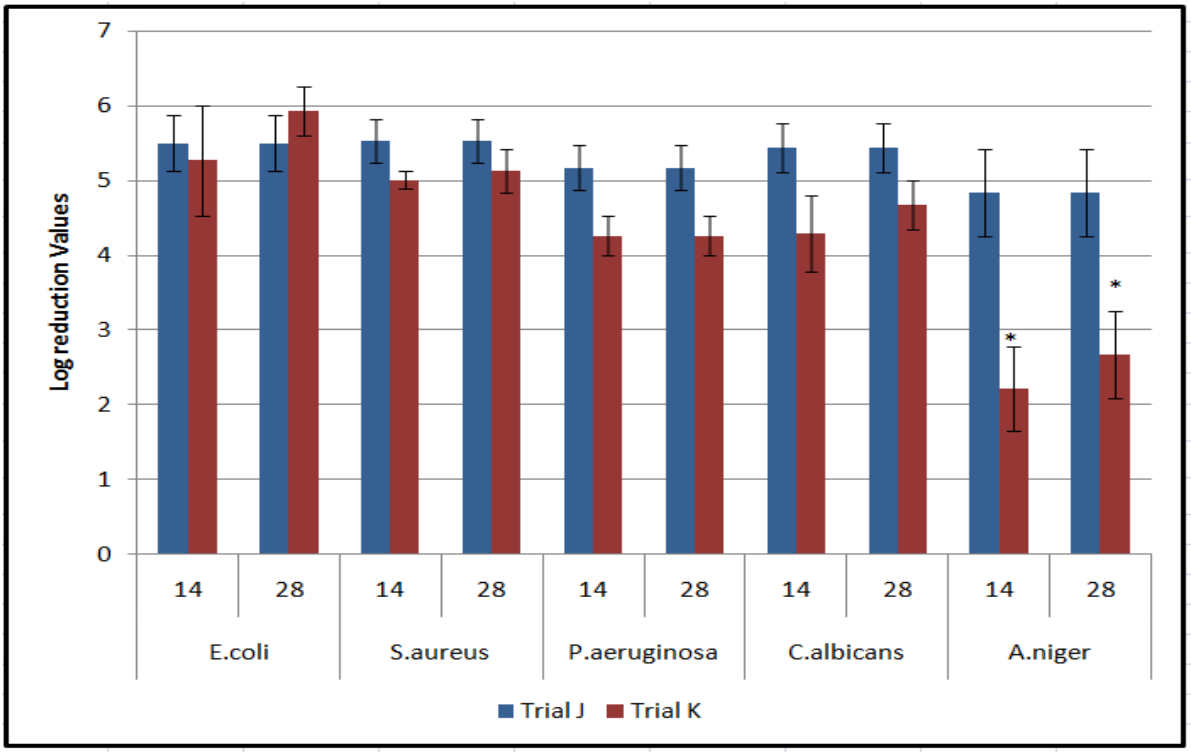

Fig.4. Evaluation of synergistic effect of sodium perborate with benzyl alcohol. No significant difference is found between trial J and K as P >0.05 except in *A.niger*.

DISCUSSION

Non sterile pharmaceutical liquid dosage forms such as nasal drops require antimicrobial preservatives to be added in formulation to control the microbial contamination, normally introduced during manufacturing, filling, packing or use from multidose packaging. The most frequently used antimicrobial preservative in the nasal drops is

benzalkonium chloride. Many studies have revealed the adverse effects of benzalkonium chloride (Berg *et al*., 1997, Riechelmana *et al*., 2004). As these nasal drops are also being used in pediatrics, so there is much concern about their safety. Sodium perborate is relatively a new preservative in ophthalmic solutions but has not been used in nasal drug delivery system so far. It generates hydrogen peroxide in the formulation, which is highly effective antimicrobial agent and sodium perborate after coming in contact with endogenous enzymes, convert hydrogen peroxide into water and oxygen (Furrer *et al.*, 2001). In this study sodium perborate tetrahydrate was evaluated for its antimicrobial efficacy in sodium chloride nasal drops to replace BKC. Effect of different concentrations of sodium perborate, pH of the formulations and synergistic effects of other ingredients have also been evaluated. The results clearly depict that sodium perborate tetrahydrate was found to be best effective in 0.04% concentration at pH 5.5-6.5. More than 3 log reduction in bacteria and more than 1 log reduction was observed in yeast and mold. Despite the fact that the results of trial C are in accordance with the acceptance criteria but the log reduction values were found slightly low in all microorganisms, when compared with the log reduction values of trial G (Marketed product). However, its acceptability is endorsed since a compound with less antimicrobial activity also possesses less toxicity to host cell. Sodium chloride nasal drops with 0.04% sodium perborate at pH ranges 5-6, 6-7, 7-8 and 9-10 were also found effective and met the acceptance criteria of the test. It is obvious from the results that in alkaline pH, the antimicrobial efficacy of sodium perborate tetrahydrate also increases. An enhanced antimicrobial preservative efficacy was observed by incorporating benzyl alcohol and disodium EDTA, showing at least 4 log reduction in case of all microorganisms except in *A. niger*. The trials H and K showed almost same log reduction in microbial count as observed in the commercially available formulation with BKC (Trial I and J).

CONCLUSION

From the present study, it can be concluded that in sodium chloride nasal drops, sodium perborate at concentration 0.01 and 0.02% was not found effective as antimicrobial preservative. Sodium perborate at 0.04% concentration at different pH range can be used as antimicrobial preservative in sodium chloride nasal drops. However its antimicrobial preservative efficacy increased with the increasing pH values (towards alkaline side). The addition of benzyl alcohol and disodoium EDTA in the formulation enhanced the antimicrobial preservative efficacy of sodium perborate. In order to check the toxicity of the trial formulations, a study is underway to evaluate the effects on cell lines in our lab.

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