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EVALUATION OF PRESERVATIVE EFFECTIVENESS IN ANTACID, COUGH SYRUP AND OPHTHALMIC SOLUTION BY MICROBIAL CHALLENGE TEST

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ABSTRACT: Pharmaceutical preparations having high water content face the problem of microbial spoilage which affects consumer safety. Control on such problem is generally done by the addition of a specific preservative to these pharmaceutical preparations, but sometimes some physical changes and clinical hazards are observed even after the addition of preservatives. The present study aimed to evaluate and compare the effectiveness of preservatives in market samples of antacids, cough syrups, and ophthalmic solutions through a microbial challenge test. The samples of antacids, cough syrups, and ophthalmic solutions were challenged with 3 bacterial, and 2 fungal strains and results were periodically (0, 7, 14 and 28 day) investigated. The number of surviving microorganisms were determined using standard microbiological dilution pour-plate technique. More than 1 log reduction of microbial counts were observed in all samples at 7 day. Moreover, the log reduction in microbial counts was significantly increased up to 28 day. Results showed that preservatives Sorbitol, Sodium Citrate, and Benzalkonium Chloride present in antacids, cough syrups, and ophthalmic solutions respectively were effective against all the challenged microorganisms. Benzalkonium chloride as a preservative in ophthalmic solution was found to be most effective. Hence, from this study, it is concluded that preservatives present in all tested liquid pharmaceutical preparations are effective in preventing contamination of the product during their use and storage.

INTRODUCTION: Pharmaceutical products having a high degree of water faces the problem of microbial spoilage which affects consumer safety [1]. Liquid pharmaceutical preparations are susceptible to microbial contamination because of the nature of their ingredients. More often, microorganisms are the cause of organoleptic alterations, such as offensive odors, changes in viscosity and color [2]. Such preparations are protected by the addition of antimicrobial preservatives that prevent the alteration and degradation of product formulations [3].

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Preservatives have been commonly used as an additive in pharmaceutical preparations such as antacids, cough syrups, ophthalmic solutions, and multiple dose parenteral. Cough syrups and other oral liquid preparations are not manufactured under aseptic conditions because they are intended for oral use. However, manufacturers usually add preservatives as well as other formulation materials such as alcohol to maintain a minimum or no accidental microbial growth upon use and storage of oral solutions [4]. Ophthalmic preparations like eye drops are required to be sterile.

However, the accidental contamination of such products while in use and home storage might adversely affect the health of the patient. So, preservative is added in them. Besides their use in pharmaceuticals preparations, preservatives are also used as additives in foods and cosmetics [5].

The primary purpose of adding antimicrobial preservatives to pharmaceutical dosage forms is to prevent adverse effects arising from contamination by microorganisms that may be introduced inadvertently during or after the manufacturing process [6]. However, antimicrobial agents should not be used solely to reduce the viable microbial count as a substitute for good manufacturing procedures [6]. To prevent microbial contamination, the addition of preservatives is needed according to the microbial sensibility of the pharmaceutical product and its use by consumers. Preservatives are effective for control of yeast, molds and bacterial growth.

The challenge test (Preservative effectiveness test) is designed to measures the level of biological activity possessed by the preservative system of pharmaceutical products. Preservative efficacy test includes artificial contamination of a formulation with a predetermined number of microorganisms followed by periodic removal of samples at fixed time intervals which, after recovery in suitable media, are used for the viable count of the microorganisms present in the formulation. The organism specified for used in the tests is intended to be representative of those that might be expected to be found in the environment in which the preparation is manufactured, stored and used [6]. Manufacturers usually test the ability of liquid preparation to maintain minimum microbial growth by deliberately inoculating the final product with a suitable micro-organism such as *Escherichia coli, Pseudomonas aeruginosa, Staphylococcus aureus, Candida albicans, Aspergillus niger* and monitor the level of contamination at several time intervals [7−11]. The test then compares the level of microorganisms found on a control sample versus the test sample throughout 28 days.

Any antimicrobial agent may show the protective properties of a preservative. However, for the protection of the consumer, the concentration of the preservative shown to be effective in the final packaged product should be considerably below the concentrations of preservative that may be toxic to human beings [6, 12, 13]. The high amount of preservative in liquid preparations are hazardous for human health.

An ideal preservative should have a broad spectrum of activity against microorganisms and be compatible with different ingredients of a product and its packaging [14]. Preservative should be effective at low concentration against all possible microorganisms and nontoxic [15]. Additionally, a preservative must be active in the complete formulation with its lowest concentration and be effective and stable over the range of pH values [16, 17]. Hence, the objective of this study was to evaluate the efficacy of preservative in three liquid pharmaceutical preparations, *i.e.* antacid, cough syrup and ophthalmic solution which are most commonly used. The samples randomly collected from local stakeholders for study which is required for quality control as well as for patient safety analysis.

MATERIALS AND METHODS:

Chemicals and Reagents: Microbiological dehydrated media, Soybean casein digest agar, and Sabouraud dextrose agar were procured from HiMedia, Mumbai. 0.9% NaCl solution used for harvesting of microorganisms from media slants was obtained from Merck Ltd. Double distilled water was used to dissolve dehydrated media and to prepare a 0.9% NaCl solution. The commercial samples of Antacids, Cough Syrups and Ophthalmic Solutions having preservatives Sorbitol, Sodium Citrate, and Benzalkonium Chloride respectively were randomly collected from the local market. The ingredients of these products are mentioned in **Table 1**.

TABLE 1: TESTED ANTACID, COUGH SYRUP AND OPHTHALMIC SOLUTION AND THEIR COMPOSITION AND NATURE OF PACKAGING

Product	Composition	Nature of packaging
Antacid	Each 5 ml contains- Aluminium hydroxide gel IP 300 mg; Magnisium hydroxide IP 250 mg; Activated Dimethicone 40 mg; Sorbitol solution (70%) IP 1000 mg	Plastic
Cough syrup	Each 5 ml contains- Diphenhydramine HCl 14.08 mg; Ammonium Chloride 138 mg; Sodium Citrate 57.03 mg; Menthol 1.14 mg	Plastic
Ophthalmic Solution	Each 5 ml contains-Timolol maleate IP 0.5% w/v; Benzalkonium chloride IP 0.01% w/v	Plastic

Challenged Microorganisms: The Gram-negative bacteria *Escherichia coli* ATCC-8739, and *Pseudomonas aeruginosa* ATCC-9027; Gram-positive bacteria *Staphylococcus aureus* ATCC-6538; yeast *Candida albicans* ATCC-10231 and mold *Aspergillus niger* ATCC-16404 were used as challenged microorganisms in preservative effectiveness test as prescribed in different Pharmacopoeias [6, 12, 13]. All standard microbial strains collected from American Type Culture Collection (ATCC) were preserved as lyophilized and glycerol stocks.

Preparation of Microbiological Media: Primary objective of microbiological media is to support the rapid growth of the microorganism being used in the preservative effectiveness test. Soybean casein digest agar media was used for the recovery of bacteria having ingredients; pancreatic digest of casein (15.0 g/l), a peptic digest of soybean meal (5.0 g/l), sodium chloride (5.0 g/l) and agar (15.0 g/l). On the other hand, Sabouraud dextrose agar media having ingredients; peptones (10.0 g/l), dextrose monohydrate (40.0 g/l) and agar (15.0 g/l) was used for the recovery of yeast and moulds. Dehydrated media were dissolved in the double distilled water and pH was adjusted as per instructions on the dehydrated media container. The media were sterilized in the autoclave at 121 ºC and 15 psi for 15 min. Sterilized media were tested for growth promotion test for best recovery of challenged microbial strains.

Preparation of Inoculums and its Standardization: The effective and fully characterised microbial strain is required for antimicrobial effectiveness testing. Fresh microbial strains preserved on glycerol stock were revived and then sub-cultured on Soybean casein digest agar slants for bacterial growth and Sabouraud dextrose agar slants for fungal growth. The slants of *Escherichia coli*, *Pseudomonas aeruginosa* and *Staphylococcus aureus* were incubated at the 30-35 ºC for 24 h. The slants of *Candida albicans* were incubated at 20-25 ºC for 48 h whereas; the slants of *Aspergillus niger* were incubated at 20-25 ºC for 5 days. After the incubation period sterilized 0.9% NaCl solution was used to harvest the bacterial and fungal cultures from agar slant through proper shaking and then the suspensions of microorganisms were diluted with the sterile 0.9% NaCl solution to gave a microbial count of 1×10^8 cfu/ml [6]. The number of cfu was determined by dilution pour-plate method.

Methodology of Challenge Test: The challenging test for preservative effectiveness was performed by using the microorganism *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Candida albicans and Aspergillus niger*. The 20 ml of all tested formulations (Antacids, Cough syrups and Ophthalmic Solutions) were taken in sterile tubes (100 ml capacity) for each challenged microorganisms. Each product tube was inoculated with one of the prepared and standardized inoculums in such a way that after inoculation the final concentration of microorganism remains between 1×10^5 and 1×10^6 cfu/ml and the volume of the inoculum does not exceed 1 percent of the volume of the product. All the inoculated tubes were incubated at 20-25 ºC for 28 day, and viable counts were periodically determined by the pour-plate method at 0, 7, 14, and 28 days after the inoculation. The preservative effectiveness test was performed by following the standard protocol described in Indian Pharmacopoeia and the United States Pharmacopoeia.

RESULTS: Growth of challenged microbes was periodically observed at 0, 7, 14 and 28 days by counting the colony forming units (cfu) of microorganisms by pour-plate method after the inoculation. From the calculated concentration of cfu/ml present at the start of the test, the log reduction in cfu/ml for each microorganism at the different time intervals (0, 7, 14, and 28 day) were calculated.

Antacid: The challenge of Antacid samples with tested microbes, heavy growth of *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Candida albicans*, and *Aspergillus niger* were found on zero-day. The counts of challenged microorganisms were continuously decreasing from the initial count when observed at 7, 14 and 28 day.

Moreover, the counts of *Candida albicans* and *Aspergillus niger* were found nil at 28 days. The results of the challenge test for the Antacid sample are mentioned in **Table 2**. Microbial testing of Antacid sample (negative control) indicated no growth of any of the tested microbes.

TABLE 2: ANTIMICROBIAL EFFECTIVENESS TESTING OF ANTACID

Name of Organisms	Inoculum Concentration	Microbial Count (cfu/ml)				Log Reduction (from 0 day count)		
		0 day	7th days	14th days	28th days	7th days	14th days	28th days
Escherichia coli ATCC-8739	7×10^8	2×10^6	8×10^4	2×10^4	8×10^3	1.39	1.99	2.39
Pseudomonas aeruginosa ATCC-9027	5×10^8	3×10^6	2×10^5	4×10^4	7×10^2	1.18	1.87	3.63
Staphylococcus aureus ATCC-6538	8×10^8	4×10^6	2×10^5	3×10^4	4×10^3	1.30	2.12	2.99
Candida albicans ATCC-10231	9×10^8	6×10^5	4×10^4	5×10^3	Nil	1.17	2.07	5.77
Aspergillus niger ATCC-16404	4×10^8	5×10^5	3×10^4	Nil	Nil	1.22	5.69	5.69

Cough Syrup: The microbial challenge test of Cough Syrup showed the intensive growth of *Escherichia coli, Pseudomonas aeruginosa, Staphylococcus aureus, Candida albicans* and *Aspergillus niger* on zero-day. The counts of these microorganisms were significantly decreased at 7, 14 and 28 day. The numbers of all tested microorganism at 14 days were at least 1 log lower than the initial (zero-day) counts. Whereas, at 28 days their numbers had decreased by more than 3 log reduction from the zero-day counts. The negative control showed no growth of all the tested microorganisms. The results are depicted in **Table 3**.

TABLE 3: ANTIMICROBIAL EFFECTIVENESS TESTING OF COUGH SYRUP

Name of Organisms	Inoculum Concentration	Microbial Count (cfu/ml)				Log Reduction (from 0 day count)		
		0 day	7th days	14th days	28th days	7th days	14th days	28th days
Escherichia coli ATCC-8739	7×10^8	4×10^6	2×10^5	6×10^4	2×10^3	1.30	1.82	3.30
Pseudomonas aeruginosa ATCC-9027	5×10^8	6×10^6	3×10^5	2×10^4	3×10^3	1.30	2.47	3.30
Staphylococcus aureus ATCC-6538	8×10^8	3×10^6	1×10^5	4×10^3	3×10^2	1.47	2.87	4.00
Candida albicans ATCC-10231	9×10^8	5×10^6	4×10^4	3×10^3	1×10^2	2.09	3.22	4.69
Aspergillus niger ATCC-16404	4×10^8	3×10^5	3×10^3	3×10^2	8×10^1	2.00	3.00	3.57

Ophthalmic Solution: Ophthalmic Solution showed heavy growth of *Escherichia coli, Pseudomonas aeruginosa, Staphylococcus aureus, Candida albicans* and *Aspergillus niger* on zero-day. The counts of *Escherichia coli* and *Pseudomonas aeruginosa* were found nil at 7, 14 and 28 days. However, the numbers of *Staphylococcus aureus, Candida albicans,* and *Aspergillus niger* decreased more than 1 and 3 log reduction at 7 and 14 days respectively. At 28 day the counts of all tested microorganisms were found nil. The negative control showed no microbial growth. The results of the challenge test for the Ophthalmic Solution are presented in **Table 4**.

TABLE 4: ANTIMICROBIAL EFFECTIVENESS TESTING OF OPHTHALMIC SOLUTION

Name of Organisms	Inoculum Concentration	Microbial Count (cfu/ml)				Log Reduction (from 0 day count)		
		0 day	7th days	14th days	28th days	7th days	14th days	28th days
Escherichia coli ATCC-8739	7×10^8	6×10^5	Nil	Nil	Nil	5.77	5.77	5.77
Pseudomonas aeruginosa ATCC-9027	5×10^8	8×10^6	Nil	Nil	Nil	6.90	6.90	6.90
Staphylococcus aureus ATCC-6538	8×10^8	1×10^6	3×10^4	2×10^2	Nil	1.52	3.69	6.00
Candida albicans ATCC-10231	9×10^8	7×10^5	1×10^3	3×10^2	Nil	2.84	3.36	5.84
Aspergillus niger ATCC-16404	4×10^8	2×10^5	2×10^3	1×10^1	Nil	1.99	4.30	5.30

Normal Saline (Control): Normal Saline (0.9% w/v NaCl) was used as a control in this experimental study. The normal saline was challenged with the tested microbes. The heavy growth of all tested microbes was observed at zero-day and was slightly declined at 7, 14 and 28 days **Fig. 1**.

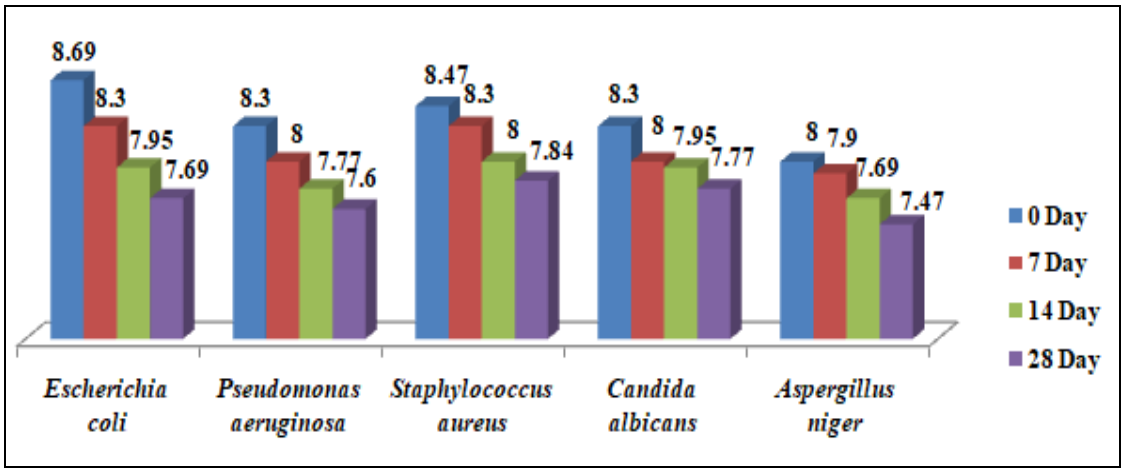

FIG. 1: GRAPH SHOWING THE NUMBERS (LOG_{10} VALUE) OF CHALLENGED MICROORGANISMS PERIODICALLY (0, 7, 14 AND 28 DAY) INVESTIGATED AFTER INOCULATION INTO NORMAL SALINE

DISCUSSION: The liquid pharmaceutical products are easily contaminated with microorganisms that result in spoilage of product with loss of its therapeutic properties, and if they are pathogenic, serious infection can arise. To minimize the risk of spoilage of pharmaceutical product by microbial contaminants, an antimicrobial preservative is incorporated in a formulation which preferably kills low level of microbial contaminants introduced during the manufacturing process, storage or repeated use of multiple-dose containers [18]. In the present study, we investigated the growths of *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Candida albicans* and *Aspergillus niger* inoculated into three different samples of antacids, cough syrups and ophthalmic solutions collected from local stack holders.

The cough syrups and antacids contain a high amount of water which make them easily contaminated with microorganisms. Therefore, the preservative is added in cough syrups and antacids that will prevent and minimize microbial growth. The effectiveness of parabens as a preservative in liquid antacid suspension was evaluated by preservative effectiveness test, and more than 4 log reduction of the bacterial strains were observed on 7 days whereas, the numbers of *Candida albicans* and *Aspergillus niger* were found 2 and 3 log reduction lower than the initial count [19].

In the present experiment antacid samples containing sorbitol as a preservative were challenged with selected microorganisms. The numbers of added microorganisms were significantly showed more than 1 log reduction at 7 days from initial day count. The numbers of all the challenged microorganisms were fount continuously decreasing up to 28 days. Additionally, the count of *Candida albicans* and *Aspergillus niger* were observed nil at 28 days.

AbuTaha *et al*., assess and compare the vulnerability of cough syrups through preservative effectiveness test. The growth of microorganisms into syrups was compared by counting the colony forming units (cfu) from a subculture of inoculated syrups at zero, 3, 6, 24 and 48-h intervals and excellent results were found [4]. In the present study, the cough syrups samples having sodium citrate were challenged with selected microorganisms and results were observed and compared at zero, 7, 14, 28 days. The numbers of microbial challenge count were continuously decreased from zero to 28 day. More than 1 log and 3 log reductions of all challenged microorganisms were observed at 7 and 28 days respectively.

Ophthalmic preparations like eye drops are required to be sterile. However, the accidental contamination of such products while in use might adversely affect the health of the patient.

It was observed that market samples of an ophthalmic solution containing benzalkonium chloride as a preservative showed massive growth of challenged microbes at zero-day which significantly decreased up to 28 days. No growths of *Escherichia coli* and *Pseudomonas aeruginosa* were observed on and after 7 days. Whereas, the counts of *Staphylococcus aureus*, *Candida albicans*, and *Aspergillus niger* showed more than 3 log reduction at 14 days from the initial count (zero-day). No growths of *Staphylococcus aureus*, *Candida albicans*, and *Aspergillus niger* were observed at 28 days. All three liquid pharmaceutical preparations, *i.e.* antacids, cough syrups, and ophthalmic solutions were having efficient preservatives which are effective against the tested microorganisms.

CONCLUSION: Microbial contamination of liquid pharmaceutical preparations is a matter of great importance to the industry, and it can become a major cause of both product and economic losses. Such preparations are protected by the addition of antimicrobial preservatives that prevent the alterations and degradation of the product formulations. An ideal preservative should have a broad spectrum of activity against microorganisms.

Present study aimed to evaluate and compare the effectiveness of preservatives through microbial challenge test in antacids, cough syrups and ophthalmic solutions randomly collected from local stack holders. These samples were challenged with 3 bacterial, and 2 fungal strains and results were periodically (0, 7, 14 and 28 day) investigated. The numbers of microbial challenge count were found continuously decreasing from the initial day (zero-day) to 28 day in all the three tested liquid pharmaceutical preparations. In antacids, cough syrups and ophthalmic solutions more than 1 log reduction in the numbers of added microorganisms were observed at 7 days from initial day count. Whereas, up to 28 days the counts of challenged microorganisms were drastically decreased. Results showed that preservatives, *i.e.* sorbitol, sodium citrate, benzalkonium chloride present in antacid, cough syrup and ophthalmic solution respectively were found effective against all challenged microorganisms. However, benzalkonium chloride in ophthalmic solution was found to be most effective.

From this study, it is concluded that preservatives present in all tested liquid pharmaceutical preparations are effective in preventing contamination of the product during their storage and use.

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