Trends in Applied Sciences Research

ISSN 1819-3579

Academic
Journals Inc.

www.academicjournals.com

Trends in Applied Sciences Research 6 (2): 198-203, 2011
ISSN 1819-3579 / DOI: 10.3923/tasr.2011.198.203

Assessment of the Preserving Efficacy of the Pharmaceutical Syrups to Identified Air-Borne Microorganisms

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ABSTRACT

The purpose of the current study was a continuation of the evaluation of the efficacy of different preservatives ingredients of different cough syrups and which previously assessed to Air-borne microorganisms. The airborne microorganisms were characterized firstly according to their colony color and then to their DNA sequences. The identified microorganisms were inoculated into syrup A (glycerol and propylene glycol), syrup B (propylene glycol and glycerin), syrup (C) (glycerin, propylene glycol and butyl paraben), syrup D (methyl paraben and propyl paraben) and normal saline as a control which were then incubated for 24 h. Growth of microorganisms into syrup was compared by counting the CFUs from a subculture of inoculated syrup at different time. The data showed that the genome of the three isolated air-borne microbes revealed three different species and that all the combinations of the preservatives in the four studied cough syrups behaved similarly in term of efficacy towards the microbial contamination. The results revealed that the preservatives mixtures of propylene glycol with glycerol or with glycerin or with butyl paraben preservatives and the methyl paraben with propyl paraben are have efficient antimicrobial activity against Airborne microbes during the 24 h studied period.

Key words: Antimicrobial, preservatives, 16S rRNA

INTRODUCTION

Syrups are concentrated solutions of sugar such as sucrose in water or other aqueous liquid. They have unusual opportunities as vehicles in extemporaneous compounding and are readily accepted by both children and adults. Because they contain no or very little alcohol, they are vehicles of choice form many of the drugs that are prescribed by pediatricians. They possess remarkable masking properties for bitter and saline drugs. The USP states that syrups may contain preservatives to prevent bacterial and mold growth (Parfitt, 1999).

Preservatives are substances that commonly added to various foods and pharmaceutical products in order to prolong their shelf life. The addition of preservatives to such products especially to those that have higher water content is essential for avoiding alteration and degradation by microorganisms during storage (Parfitt, 1999).

Different types of preservatives could be added one of the most common is the antimicrobial preservatives which work by inhibiting the growth of microorganisms inadvertently introduced during manufacture or use (Rosenthall *et al*., 2006; Fahelelbom and El-Shabrawy, 2007).

Various approaches to Preservative Efficacy Testing (PET) have been developed over the years by different regulatory agencies and companies. Microbial challenge test has evolved as the most commonly used and accepted evaluation criterion. The fundamental principle of the microbial challenge is based on the concept of measuring the survival ability of selected microorganism that is purposely introduced into a preserved test product system. Conventional preservative efficacy testing or preservative challenge test methods generally require microbial assays at multiple test points over extended periods of time and based on a sample inoculation using a microbial suspension with a determined amount of Colony Forming Units (CFUs). After that the number of survivors is investigated by periodic evaluations and the results are compared with specifications (Souza and Ohara, 2003; Cremieux *et al*., 2005; Yablonski *et al*., 2007).

Since the air does not, under normal conditions, contain the nutrients and moisture for growth, maintenance and multiplication of microorganisms, it could not be considered their natural environment. Nevertheless, air normally abounds in their numbers as microorganisms gain entry into it from soil and other dry decomposed material including excrete exposed to the action of wind. Since air is in contact with almost all animate and inanimate objects, air-borne microorganisms become an important source of contamination in laboratories, hospitals, industries and of exposed food material and drinks. Depending upon the nature of microorganisms, some contaminations may cause spoilage of contaminated products and diseases when ingested. By mere sneeze and cough, infection from mouth and lungs may be discharged into the air around.

In view of this, knowledge of quantity and quality of air microorganisms seems essential because we need pure air for respiration. As stated earlier, air is not a medium for microorganisms but is a carrier of particulate matter, dust and droplets which remain generally laden with microorganisms. These carrier transport microorganisms and the ultimate fate of such microorganisms is governed by a complex set of conditions such a sunlight, temperature, humidity, size of microbe laden particulates, degree of susceptibility or resistance of a particular microbe to the new physical environment and the ability of microbe to form resistant spores or cysts.

As long as microorganisms remain in the air they are of little importance. When they come to rest they may develop and become beneficial or harmful. Knowledge of the microorganisms in air is of importance in several aspects (Polymenakou *et al*., 2008).

This study is a continuation of the work which was carried out in our labs regarding preservative efficacy of different cough syrups. Accordingly the aims of the current study were Molecular identification of the environmental isolated Air-borne microorganisms and the evaluation of the efficacy of different preservatives against those microorganisms (Khanfar *et al*., 2009).

MATERIALS AND METHODS

All of the experiments were carried out at the Biotechnology Department, Faculty of Science at Philadelphia University during the period October 2009-February 2010.

Molecular identification of the environmental air-borne microorganisms

Microorganism isolation and DNA isolation: Three environmental isolated air-borne microorganism colonies were picked up from nutrient agar plates exposed to air for 24 h. Firstly, the colonies were chosen according to their colony. The colonies color was white, yellow and orange.

Then the molecular identification of the three pure Air-borne microorganisms was performed by preparing the genomic DNA from overnight culture using Wizard genomic DNA purification kit according to manufacturer's instruction (Promega, USA). The purity of the DNA was measured spectroscopy while their quality was determined by gel electrophoresis.

16S rRNA amplification and sequencing: The Polymerase Chain Reaction (PCR) was used to amplify 16s rRNA genes of the three genomic DNA (Qasem *et al*., 2010). The PCR reaction including Taq polymerase and its buffer, $MgCl_2$, deoxyribonucleotids (dNTPs), forward and reverse primers Fd1 for 16S rRNA and chromosomal DNA. The PCR was carried out in Thermal Cycler (BioRad). The PCR condition was 1 cycle of 94°C for 3 min; 30 cycles of 94°C for 30 sec, 55°C for 40 sec and 72°C for 45 sec was carried out in thermal cycler (BioRad, USA). The PCR product was checked out by agarose gel electrophoresis.

Purification of PCR products: PCR products were purified using QIA quick PCR purification kit as per manufacturer's instructions (QIAGEN, Inc., Valencia, CA, USA).

16S rRNA partial DNA sequencing and sequencing and sequencing analysis: The sequencing analysis was performed according manufacturer's instruction by using ABI 310 Genetic analyzer.

The obtained DNA sequences were assembled using Bio edit and assembled sequences were analyzed and compared with known sequences of microbial genome using BLASTn at the NCBI server (http://www.ncbi.nlm.nih/gov).

Evaluation the preservatives efficacy by CFUs counting: The methodology was followed as previously reported (Wachowski *et al*., 1999; Crowther *et al*., 1996). Overnight cultures of three identified Air-borne microorganisms white, yellow and orange. The cultures were then diluted to a density of 0.5 McFarland units with 0.9% sterile non bacteriostatic saline using spectrophotometer (Cecil, England). Each organism solution was further diluted 1:50 with sterile 0.9% saline. Each diluted organism was then added to sterile sealed culture vials containing the following solutions A, B, C and D and which were used previously by Khanfar *et al*. (2009). The solutions are: syrup (A) (glycerol and propylene glycol); syrup (B) (propylene glycol and glycerin); syrup(C) (glycerin, propylene glycol and butyl paraben); syrup D (methyl paraben and probylparaben) and 0.9% nonbacteriostatic saline as a control. After the organisms were added, each vial was vortexed and subplated to three plates of Trypticase Soy Agar (TSA). Vials were subplated out at zero, 3-, 6-, 12- and 24 h intervals for a total of nine plates per solution per sampling period and stored at 20°C between samplings. The plates were then incubated at 37°C for 24 h. Each plated medium was read and numbers of Colony Forming Units (CFUs) were counted and recorded using colony counter (Galaxy 230, USA).

For each microorganism, the number of CFUs per plate was averaged for each sample period. Data are presented as the mean of nine replicate assays. A probability of p-value at 0.05 was taken to indicate statistical significance.

RESULTS

Molecular identification of the air-borne microorganisms: Comparison of nucleotide sequences of the three Airborne microorganisms colonies white, yellow and orange with the

microbial genomics using BLASTn at the NCBI server (http://www.ncbi.nlm.nih/gov) revealed that the white colonies were closely homologous to the *Streptomyces flavogriseus* genes (gb|ACH01000001.1), the yellow colonies were highly homologous to the *Streptomyces viridochromo* genes (gb|ACEZ01000201.1) and the orange colonies showed moderate homologous to the *Mcycobacterium* sp.(ref|NC_009077.1).

Efficacy assessment of the preservatives: The results obtained from the assessment analysis of the antimicrobial efficacy of the different preservatives constituents of the 4 different cough syrups to the three different Air-borne microbes; white, yellow and orange colonies; *Streptomyces flavogriseus*, *Streptomyces viridochromo* and *Mcycobacterium* sp., respectively, revealed similar antimicrobial efficiency.

With White colonies (*Streptomyces flavogriseus*) inculcated in syrups A, B, C and D showed significant reduction in the mean CFUs after 3 h of incubation time compared to baseline time and in compare to 0.9% saline. The observed high growth in the inoculated syrup D with white colonies was similar to the inoculated 0.9% saline at zero time. The syrups A, B, C and D which consist of propylene glycol with glycerol or with glycerin or glycerin with butyl paraben and paraben and probylparaben combinations, respectively, suppressed the growth of white colonies during the study period (Table 1).

The inoculated syrups A, B and C showed no significant CFUs count of yellow colonies (*Streptomyces viridochromo)* during the 24 h study period. Nonetheless, a significant decline in the mean of CFUs of yellow colonies was observed after 6 h in syrup D. Furthermore, the growth of yellow colonies in the inoculated saline and syrup D were significantly greater at zero time compared with the remaining syrups (Table 2).

While propelyne glycol with different combination additives in syrups A, B and C reduced the mean of CFUs, while the methyl paraben and probylparaben in syrup D enhanced the formation of yellow colonies CFUs at zero time.

With orange colonies (*Mcycobacterium* sp.) in syrups A, B and C significant reduced CFUs was observed at 3, 6, 12 and 24 h. The inoculated syrup D showed significant changes in the mean of CFUs after 6 h incubation with orange colonies (Table 3).

Table 1: Number of CFUs of White colonies (*Streptomyces flvogriseus*) counted versus time (h) after inoculation in various cough syrups (A, B, C, D and 0.9% saline as a control)

Time of incubation (h)	Saline	A	B	C	D
0	225	62	39	29	166
3	151	45	20	35	100
6	120	12	0	0	7
12	70	0	0	0	1
24	34	0	0	1	0

Table 2: Number of CFUs of Yellow colonies (*Streptomyces viridochromo*) counted versus time (h) after inoculation in various cough syrups (A, B, C, D and 0.9% saline as a control)

Time of incubation (h)	Saline	A	B	C	D
0	380	1	45	11	350
3	141	1	20	5	247
6	100	1	4	3	100
12	44	0	1	0	5
24	26	0	0	0	4

Table 3: Number of CFUs of Orange colonies (*Mycobacterium* sp.) counted versus time (h) after inoculation in various cough syrups (A, B, C, D and 0.9% saline as a control)

Time of incubation (h)	Saline	A	B	C	D
0	300	10	35	8	150
3	299	11	27	10	86
6	307	12	13	10	75
12	365	10	7	4	18
24	86	2	4	3	10

DISCUSSION

The currant study was continuation to the previous study reported by Khanfar *et al.* (2009). Since, the preserving efficacy of the four preservatives mixtures A, B, C and D of the four different cough syrups, were tested against control microorganism, this study and based on the frequent usage of the syrups by individual, the efficacy of preservatives were tested against environmental air-borne microorganisms.

Present experimental were divided into two parts the first part was the identification of airborne- microorganisms using molecular techniques. Three microbes were found to be clearly homologous to three different microbial genomes *Streptomyces flavogriseus*, *Streptomyces viridochromo* and *Mcycobacterium* sp., for white, yellow and orange colonies, respectively.

The first Airborne microbe was *Streptomyces flavogriseus*, which is a bacteria producing β-xylosidase enzyme that is responsible for breakdown of xylo-oligosaccahrides to xylose (El-Sawah *et al.*, 1999). *Streptomyces viridochromo* producing antibiotic phosphoinothrieyl-alanyl-alanine (PTT) that is transported into bacterial cells via oligopeptide transport system and cleaved intracellularly by peptidases and release Phosphinothrin (PT) which is a structural analogue to glutamic acid and competitively inhibit glutamine synthesis system (Behrmann *et al.*, 1990). The third type of Airborne microorganism was from the *Mycobacterium* species which is classified as acid-fast gram positive bacterium, these types of bacteria are wide spread organisms.

Diseases caused by species of the genus Mycobacterium are major sources of morbidity and mortality in the world today particularly in developing and tropical countries (Plikayatis *et al.*, 1992). And since this type is very dangerous and wide spread it is crucial for the type of preservative added to food, pharmaceutical products especially those products which contain water to have good antibacterial preservative to counteract any accidental entrance of airborne microorganisms.

The results led to the second objective which was determination of the efficacy of the already present preservatives combinations in different syrups. Several preservative are added to prevent accidental entrance of microorganisms like glycerol (Biswas *et al.*, 2002) borax, boric acid (Wahab *et al.*, 2005). In this study, it was found that all preservatives present in the syrups have the capability of suppression the CFUs during the first 3 h, But by comparing between the types of preservatives used and their efficacy it was found that the combination of (propylene glycol and glycerol, syrup A) showed equivalent activity to both (glycerin, syrup B) and (butyl paraben, syrup C). While the preservative combination present in syrup D (methyl paraben and propyl paraben) showed lower ability to suppress CFUs at zero time but after 3 h the results were comparable meaning that onset of action of this combination is a little bit longer which is expected since the those combinations seem to be more hydrophilic than butyl paraben which is more lipophillic and can enter the membrane of the cell wall of bacteria easily and suppress its multiplication.

In conclusion this study supported the results obtained from Khanfar *et al.* (2009). The results revealed strong evidence that the preservatives combinations used in this study have efficient

antimicrobial activity towards the selected contaminated air-borne microbes and are strongly recommended to be an ingredient for a variety of other manufactured cough syrups.

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