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Antimicrobial Efficacy of Preservatives used in Skin Care Products on Skin Micro Biota

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Abstract: ***Bacteria are the dominant group of skin microbiota that colonize the human skin and protect from invasions. Variations in skin microbiota because of the use of skin care products containing preservatives have been studied by testing three organisms - Pseudomonas aeruginosa, Micrococcus luteus and Staphylococcus epidermidis against the six selected preservatives: Phenoxyethanol, Methyl paraben, Propyl paraben, Sorbic acid, Potassium sorbate and Sodium benzoate. The Minimum Inhibitory concentrations of the six preservatives were determined followed by challenge tests and have shown desired results of Antimicrobial Efficacy in between 0.2, 0.3, 0.4, 0.5& 0.6 percent concentrations by achieving 3 log reductions for 14 days & 7 log reductions at 21 days respectively. The study revealed that the combination of two or more preservatives at 0.1% concentration was found to be more than the effect of single preservative at higher concentration. The behavior of the three organisms with the three acid preservatives Sorbic acid, Potassium sorbate and Sodium benzoate at pH 5.5 was examined. This indicated that lowering the pH of personal/skin care products will not only be beneficial for the control of microbial growth in the products but also bring down the pH of the skin to the recommended levels.***

Keywords: Skin microbiota, Preservatives, Antimicrobial Efficacy, skin care products,low pH.

1. Introduction

Over the past two decades, the world has witnessed increased concern over environmental health problems and one of the major challenges being ***protection of skin*** from pollution and other exogenous problems. The consortium of microorganisms that reside on human skin is called ***skin microbiota***. The total number of bacteria on an average human has been estimated at 10^{12} or 1 trillion (Todar, 2008) residing upon 2 m^2 surface of human skin (Grice *et al.,* 2009). The benefits bacteria can offer include preventing transient pathogenic organisms from colonizing the skin surface, either by competing for nutrients, secreting chemicals against them, or stimulating the skin's immune system (Cogen, 2008). The microbial communities present on skin are determined by skin conditions, the host's hormonal status, age, gender, and ethnicity(Fierer *et al.,* 2008; Fredricks, 2001; Grice *et al.,* 2009; Roth and James, 1988). Altered skin microbiota diversity may result in disease, from 'species diversity / microbial community structure' to 'health outcomes', include inflammation, absence of necessary members of the microbial community, and a decrease in microbial antagonistic interactions (Stecher & Hardt, 2008).

Personal care products and Skin care products: Both personal care products and skin care products fall under the general category of cosmetics. The present work is restricted to the effect skin care products on skin microbiota. Cosmetics, soaps, hygienic products and moisturizers alter the conditions of the skin barrier but their effects on skin microbiota remain unclear. The effect of antibiotic treatment on the gut microbiota has been examined using molecular methods (Dethlefsen & Relman, 2011) but, a similar assessment of skin microbiota in healthy individuals does not exist.

Preservatives and preservatives used for topically used products: The objective of cosmetic preservation is clearly to maintain microbiological quality. Cosmetic products can be contaminated with all kinds of microorganisms capable of growing in the formulation. This means that the preservatives in the formulation must be able to withstand contamination from Gram-negative and Gram-positive bacteria as well as yeast and mold. The efficacy of preservatives and other antimicrobials is measured as the Minimum Inhibitory Concentration (MIC). The MIC obviously inhibits the growth of the microorganism, but it is not known whether it still proliferates in the media at a slower rate. There are many factors that can cause variations in MIC values, primarily inoculum size, incubation time and growth media (Madigan, 2003 & Schuurmans, 2009). MIC tests for aerobic bacteria, filamentous fungi and yeast have been standardized by the Clinical and Laboratory Standards Institute (CLSI) in order to ensure low variability and comparable results from different departments (NCCLS, 2002 and 2006).In U.S.A the cosmetic industry employs about 60 preservatives (FDA's voluntary cosmetic registration program, Steinberg, 2003) of which fewer than 20 have high frequency use.

Regulations for preservatives: Three organizations serve as sources for guidelines covering testing of preservation efficacy in cosmetic and toiletry products in the United States. These are the Cosmetic, Toiletry, and Fragrance Association (CFTA), the American Society for Testing and Materials (ASTM), and the U.S. Pharmacopeia (USP). All three involve challenging cosmetic formulations with microorganisms. However, specific differences among the procedures are intended to address the concerns of the parent organizations (Brannan, 1995; Orth, 1993; Ray, 1989; Madden, 1984; Orth, 1981; CTFA, 1973). The U.S. Food and Drug Administration has revised the microbiological methods for cosmetics, Chapter 23, of its *Bacteriological Analytical Manual* (U.S. FDA, 2001).

Although the continuous use of skin care products is associated with microbial resistance, no systematic and

scientific studies have been under taken on the antimicrobial efficacy of preservatives used in skin care products on skin microbiota, especially in tropical countries.The objectives of the present study are – 1) To study the changes on skin microbiota by the use of skin care products containing preservatives. 2) To assess the role of preservatives in skin care products on antimicrobial activity against bacteria and other side effects on skin. 3) To identify the optimum and safe doses of preservatives to reduce the effect on skin flora as well as to reduce the risk of side effects.

2. Methodology

The present work is related to the study of variations of skinmicrobiota, antimicrobial activity of skin care products on skin microbiota and the effect of preservatives on that are normal inhabitants of skin.To procure the information in case of human microbota and efficacy of preservatives different types of protocols are used. Most of the procedures implemented in this study are incurred from predecessor studies and some are modified according to the convenience of the available laboratory.The methodology involves the following steps:

Isolation of different bacteria from skin donors : Facial swabbing was done to the donors of Visakhapatnam city and the samples were inoculated on Nutrient Agar. Colonies developed within 24 – 48 hours were processed for identification.

Identification of isolates based on the cultural and biochemical characters : Bacteria were identified by using Bergey's Manual of determinative bacteriology (John et al., 1994). These involved staining techniques such as Gram staining, spore staining and acid-fast staining. Motility was observed by hanging drop method. Biochemical tests such as IMViC, Catalase, Oxidase, Urease, Starch hydrolysis, gelatin liquefaction, nitrate reduction test, Oxidative-fermentative test (O/F) (Hugh and Leifson test), coagulase test, novobiocin sensitivity test and fermentation tests with glucose, fructose & lactose were performed.

Study of Antimicrobial Activity of Personal Care Products on skin isolates by using Kirby-Bauer's method: In this method, Muller-Hilton Agar plates were inoculated with the isolated skin flora and discs containing 50 µl of the skin care product samples (fairness cream, deodorant and talc-cum-powder) were placed. The plates were observed for the Antimicrobial activity of personal care products on skin flora.

High Performance Liquid Chromatography: The actual concentration of cosmetic preservative in the product was studied by HPLC.

The fairness cream was analyzed for the fraction of preservatives – methyl paraben, propyl paraben and phenoxyethanol as per the ingredient label of the product using gradient method.The talc-cum-powder was analyzed for the fraction of triclosan using Isocratic method.

Preservative Efficacy tests or challenge tests :PEThavebeen carried out by 3 pure cultures of bacteria isolated from skin(test organisms) were procured from IMTECH, Chandigarh. *Pseudomonas aeruginosa* –MTCC 1688 , *Micrococcus luteus* – MTCC 4428 and *Staphylococcus epidermidis* – MTCC 435. Six preservatives are selected to conduct the efficacy tests. They are Phenoxyethanol, methyl paraben, propyl paraben ,sorbic acid, potassium sorbate and sodium benzoate.

Testing the preservative efficacy in the Skin cream(USP method): The test preservatives were analyzed for their capability of reducing the viable bacterial count to less than 0.1% of the initial concentration by the 14th day in a skin cream of known concentration. According to the USP method within 14days the bacteria should decrease by 3-log reduction (i.e., 99.9%).

A 20-ml sample of the product is transferred to a sterile, capped bacteriological tube. Inoculation of the test sample with the suspension is done using a ratio of 0.01 ml inoculum to 20 ml test sample in such a way that the concentration of microorganisms in solution should be between 1×10^5 and 1×10^6 CFU/ml. The preservatives of 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9 and 1.0 concentrations were added to the cream samples. The inoculated containers are incubated at 20 to 25°C and examined on 7, 14, 21, and 28 days after inoculation. Microbial numbers (CFU/ml) are determined by the plate count method at each of these intervals. Change in growth was estimated by comparison to initial viability using cream with inoculum and no preservative. A control containing cream with no inoculum and no preservatives was also set up.

A cream without preservatives was also included in the challenge test as control. Each cream was inoculated with a standardized suspension of each microorganism and incubated at 25^0C for 28 days.

Challenge tests with combination of preservatives: The same work has been repeated with preservatives in combinations of 0.1%. 15 possible combinations with 2 compounds were used for the testing of *P.aeruginosa*, *S.epidermidis*, and *M.luteus*.

Challenge tests with the 3 test organisms and the three organic acid preservatives at pH 5.5: The growth patterns of the three organisms with the three organic acids Sorbic acid, Potassium sorbate and Sodium benzoate were also studied at 5.5 pH.

3. Results

Bacteria isolated from the human skin : Several bacterial colonies were isolated from the 20 donors. About 56 colonies were identified.Among the 56 colonies *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *S.epidermidis*, *S.saprophyticus*, *Staphylococcus sp.*, *Bacillus cereus*, *Bacillus subtilis*, *Corynerbacteriumsp.* and *Micrococcus lutues*were present.

Biochemical characters of the identified bacteria: The outcomes of biochemical tests were mentioned in the table 1.

Table 1: Results of the biochemical tests performed with skin isolates

Isolate→ / Test↓	1	2	3	4	5	6	7	8	9	10
IMViC tests										
Indole	-	-	-	-	-	-	-	-	+	-
Methyl Red	-	-	-	-	-	-	-	-	-	-
Voges Proskeur	-	-	+	+	+	+	+	+	+	-
Citrate utilization	+	+	-	-	+	+	+	+	-	+
Nitrate reduction	+	+	-	+	+	-	+	+	+	-
Catalase	+	+	+	+	+	+	+	+	+	+
Oxidase	+	+	-	-	-	-	-	-	-	+
Urease	-	-	-	-	+	+	-	-	-	-
Gelatin	+	+	+	+	-	-	+	+	-	+
Starch hydrolysis	-	-	-	+	+	-	+	+	-	-
Sugar Fermentation										
Lactose	-	-	A	-	A	AG	-	-	AG	A
Glucose	-	-	-	A	A	A	A	A	A	-
Sucrose	-	-	AG	A	A	A	A	A	A	A

'-' negative for the test ; '+' for the test
A- acid production; AG-acid & gas production.

Antimicrobial Activity (AMA) of personal care products on Skin isolates: The antimicrobial test was performed on the 10 isolated bacterial samples with the three personal care productstalc-cum-powder, fairness cream and deodorant sprayand the results are shown in Table 2.

Isolates 1,2,5, 6 &10 have shown AMA with both talc and deodorant.Isolates 4&5 have not shown AMA with both talc and deodorant.The experimental results indicate that the topical application of the three personal care products - talc-cum-powder and deodorant spray on the body can restrict the colonization of skin microbiota.

Table 2 :AMA of talc-cum-powder, fairness cream and deodorant spray with the bacterial samples isolated from the skin.

Isolate. No.	Diameter of inhibition zone (in cms) on Muller-Hilton agar medium		
	Talc-cum-powder	Fairness cream	Deodorant spray
1	1.2	#	1.3
2	1.2	#	1.5
3	#	#	0.8
4	#	#	0.8
5	1.0	#	1.8
6	2.0	#	2.0
7	#	#	#
8	#	#	#
9	1.5	#	#
10	2.0	#	1.8
'≠' – indicates that no zone of inhibition was observed.			

The products talc-cum powder and fairness cream were analyzed for their preservative compounds by **HPLC** and found that the talc contained triclosan (0.71%) while the fair ness cream contained phenoxyethanol (0.417%), methylparaben(0.354%) & propyl paraben (0.137%). This finding endorses that the combination of phenoxyethanol, methyl paraben and propyl paraben at very lower concentrations are used to maintain the cream stability and reduce the spoilage without showing any inhibitory effect on the skin isolates.

The Minimum Inhibitory concentrations of the six preservatives were determined based on the turbidity and the color change, but the results are completely not accurate since it is based on a visual definition of an endpoint. The values determined for each preservative against the test organism are shown in Table 3 in the percentage of their concentration taken:

Table 3: showing Minimum inhibitory concentrations of preservatives with the 3 test cultures

	P.aeruginosa	*M.luteus*	*S.epidermidis*
Phenoxyethanol	0.3	0.3	0.3
Methyl paraben	0.5	0.6	0.4
Propyl paraben	0.4	0.5	0.4
Sorbic acid	0.2	0.2	0.2
Potassium sorbate	0.6	0.4	0.4
Sodium benzoate	0.5	0.4	0.4

The challenge tests were carried out to assess the effect of the six selected preservatives on the three test organisms using a basic cream. The desired concentrations of the six preservative are summarized in Table 4:

Table 4: Desired concentrations of preservatives

	PE	**MP**	**PP**	**SA**	**PS**	**SB**
P.aeruginosa	$C_{0.4}$	$C_{0.6}$	$C_{0.5}$	$C_{0.3}$	$C_{0.5}$	$C_{0.6}$
M.luteus	$C_{0.4}$	$C_{0.5}$	$C_{0.5}$	$C_{0.2}$	$C_{0.5}$	$C_{0.5}$
S.epidermidis	$C_{0.4}$	$C_{0.5}$	$C_{0.5}$	$C_{0.3}$	$C_{0.5}$	$C_{0.8}$

Combinations of preservatives: No colony formation was detected from 7 to 28 days of incubation with all combinations except for the combination with potassium sorbate.

Optimum dose of organic acids for the three test organisms at pH 5.5:

Generally , the three test organic acids- sorbic acid, potassium sorbate and sodium benzoate are used as preservatives in the personal care products like shampoos, face wash and other washing products in which the pH will be less because they work best below pH of 6.5 The optimum working pH of these three organic acids is 5.5. Hence, the growth patterns of the three organisms were observed with the three organic acids at pH of 5.5.

No colonies were recovered from the cream inoculated with the three test organisms for 7, 14, 21, and 28 days when each of the three preservatives used at 0.1% concentrations. This may be possibly due to

1) Retardation in the growth of bacteria in cream at pH 5.5.(Essodolom, 2013).
2) The three organic preservatives might have worked efficiently in controlling the growth of bacteria.
3) The combined effect of both pH at 5.5 and activity of organic acids.

The other three preservatives phenoxyethanol, methyl paraben and propyl paraben exhibit their antimicrobial activity at a wide pH range from 4.5 -7.5(Soni et al., 2002.).

Hence, the pH of the cream was adjusted to 6.0 so as to observe the growth patterns of the three bacteria in the presence of six preservatives under similar conditions.

The skin surface pH acts as an antimicrobial barrier. At pH 5.0 the growth of pathogenic bacteria is inhibited. Species of the normal resident flora are positively affected by the physiological, acidic milieu (Kurabayashi, 2002). Further, the dissociation of endogenous bacteria from skin surface is enhanced at alkaline conditions (Lambers, 2006). The superficial layers of the skin are naturally acidic (pH 4-4.5) due to lactic acid in sweat and produced by skin bacteria. At this pH, mutualistic flora grows but not the transient flora. Another factor affecting the growth of pathological bacteria is that the antimicrobial substances secreted by the skin are enhanced in acidic conditions. In alkaline conditions, bacteria cease to be attached to the skin and are more readily shed. It has been observed that the skin also swells under alkaline conditions and opens up allowing move to the surface (Schauber & Gallo, 2008). Hence, lowering the pH of personal/skin care products will not only be beneficial for the control of microbial growth in the products but also bring down the pH of the skin to the recommended levels.

4. Conclusions

The study reveals that the six preservatives-Phenoxyethanol, Methyl paraben, Propyl paraben, Sorbic acid, Potassium sorbate and Sodium benzoate shown antimicrobial activity with the three test organisms at various concentrations and time periods. The data also supports that combinations of preservatives are more effective than individual preservatives used at higher concentrations. Lowering of pH to 5.5 in personal/skin care products not only addressing the microbial growth reduces the pH of the skin to recommended levels by US and EU pharmacopeia.

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