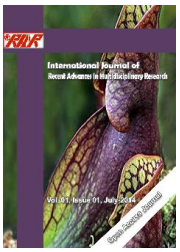

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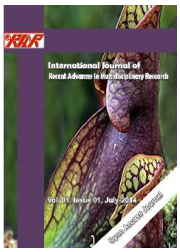

Research Article

STUDY OF BACTERIAL CONTAMINANTS IN LOCAL AS WELL AS BRANDED LIPSTICKS BEFORE AND AFTER CONSUMER USE

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ABSTRACT

Lipstick, is a face-care cosmetic that commands a unique market as it is one of the most affordable cosmetic products available with as many as 80% of women using it regularly. Lipsticks need not be sterile and may contain low levels of microbial load during or prior to use. Lipsticks are often inadvertently consumed by the users and hence it is imperative that the health regulators have a microscopic look at the ingredients as well as the microbial flora (if any) in the lipsticks. This study was performed to determine the bacterial load in terms of colony forming units in addition to the type and concentration of a paraben preservatives used in lipsticks. Twelve brands of lipsticks were selected for the study of which, four were taken from the Indian market (unused samples), four (Multinational brands) used for over a year and four (multinational brands) used for over two years. The bacteria in these samples were isolated and identified by 16s rDNA sequencing and the amount of preservatives quantified by HPLC (High Performance Liquid Chromatography). Our results indicated that all the products were contaminated to varying degrees depending on their usage. Besides the skin normal flora *Staphylococci,* gram negative organisms of *Pseudomonas, Proteus*, *Morganella*, *Providencia* species also featured prominent among the isolates. The HPLC data obtained indicated the presence of parabens at a concentration of 2740 ppm and 6960 ppm which is higher than 100-3000 ppm of parabens as stated by the US-FDA. The work cautions the end user about the quality of lipsticks a widely used cosmetic product.

INTRODUCTION

According to Federal food and Drug Cosmetic Act, 'Cosmetics are articles that are intended to be rubbed, sprinkled, sprayed or introduced into or applied to the human body or any part thereof for cleansing, beautifying, promoting attractiveness or altering the appearance or any or the article that is intended for use as a component for any such article' (Nigam 2009). Lipsticks fall under the face-care cosmetics category and are composed of waxes, oils, emollients, emulsifiers, pigments/colorants, binders in varying concentrations which determines the characteristics of the final product. Lipsticks when designed to remain on the lips for a prolonged period are composed of high percentage of wax and pigment concentration along with low concentration of oils**.** On the other hand, lipsticks designed for smooth creamy feel have a low concentration of wax and a high concentration of oils (Arifin *et al*., 2002). Acosmetic product including lipsticks need not be sterile (Mwambete and Simon 2010) however, the microbiological limit for finished lipcare products as per Bureau of Indian Standards is 1000 cfu/gm and require absence of *Staphylococcus aureus* and gram negative organisms (Bureau of Indian Standards 2011). Lipsticks should remain in this state until used by the consumers (Mwambete and Simon 2010). The composition of the lipsticks together with the warm and humid climatic conditions support as well as encourage the survival and growth of many micro-organisms. This could potentially lead to biodegradation of the product and as well as increase the risk of infection to the users (Hugbo *et al*., 2011). Lipsticks are used in contact with human skin thereby, easily being contaminated with the normal flora as well as those that may be carried from drinks or any other edible sources consumed by the individual using the cosmetic. The moment a lipstick is opened the chances of contamination due to air flora and these fluids goes on increasing with use until the product is discarded by the consumer (Brian 2001). Commonly isolated microorganisms from poorly preserved cosmetic preparations are *Klebsiella*, *Enterobacter, Staphylococcus, Bacillus species, Pseudomonas, Penicillium* and *Candida albicans* (Muhammed 2011). In order to lower the microbial loads and to increase the shelf life of the lipsticks, preservatives capable of inhibiting the immediate postproduction contamination to maintain the microbial counts

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to a lower level are used in varying concentrations and combinations in formulations (Council of Europe Guidelines). Many different preservatives are available but those that are commonly used are the parabens, formaldehyde, methylisothiazoline (Muhammed 2011), Of the various preservatives, parabens and its derivatives are the most widely used chemical preservatives in cosmetics due to their cost effectiveness, preservative efficiency and biodegradability (Rajagopal and Agrawal 2011). The amount of parabens in cosmetics as per US-Food and Drug Administration should be in the range of 100 – 3000 ppm. Indian market is dominated with multinational as well as local brands of lipsticks being widely used by women from all socio-economic strata. According to a report in Economic times 2013, most of the cosmetic companies reported that lipstick sales go on the rise even during an economic crisis. The lipstick market is the largest contributor to the cosmetics sale, amounting to almost 42 % of the total cosmetics. The growth reported for lipstick market between January – June 2013 is 25-30% as compared to 13 % of face-care cosmetics and 10 % of eye care. Hence with this background and considering the number of users of lipstick in the country it was thought worthwhile to explore and correlate the efficacy of the preservatives and bacterial count of used as well as unused lipsticks.

MATERIALS AND METHODS

Sample selection and processing

12 samples categorized according to their usage and brands were procured for the study. Four of these lipstick samples from Multinational brands (A-D) were used for one to less than a year, the four lipstick samples (I-L) which were also from the from Multinational brands were used for about two to more than two years while 4 unused lipstick samples (E-H) purchased from the local (Mumbai, India) market. It was worthwhile to note that none of the samples irrespective of the brands came with a manufacturing date or an expiry date. The sample was prepared as described by Onurdurg 2010. 0.1 gm of lipstick sample was homogenized in 2ml of Tween 80 (S d Fine, India) and used for further analysis. .

Table 1. Container label information on the collected lipsticks

Sample	Manufacturing date	Expiry Date	Manufacturer's Details	Batch Number	Preservative indicated
A	NA	NA	A	A	NA
B	NA	NA	A	A	NA
C	NA	NA	A	A	NA
D	NA	NA	A	A	NA
E	NA	NA	A	NA	NA
F	NA	NA	NA	NA	NA
G	NA	NA	NA	NA	NA
H	NA	NA	A	NA	NA
I	NA	NA	NA	A	NA
J	NA	NA	NA	A	NA
K	NA	NA	NA	A	NA
L	NA	NA	NA	A	NA

(Key NA –Not available, A – Available)

Isolation and identification of bacteria

All the media used were purchased from HiMedia laboratories pvt ltd, India. Sterile Nutrient broth (NB) was added to the homogenized lipstick emulsion to make up the volume to 10ml and the same was serially diluted to 10^{-6}. 0.1 ml of appropriate dilutions were spread on sterile nutrient agar (NA), mannitol salt agar (MSA), cetrimide agar (CM), salmonella shigella agar (SSA) and eosin methylene blue (EMB) agar plates and were incubated at at 37^0C for 24 hours unless otherwise required (Onurdurg *et al.*, 2010). After incubation the number of colonies counted and the bacterial load was expressed in terms of colony forming units (CFU) per gram lipstick. The identification of the bacteria, was based on their gram nature, biochemical characterization and 16s rDNA sequencing. Genomic DNA isolation was carried out by the method described by Sambrook *et al*., 1989.

The PCR amplification was carried as described in the Bangalore Genei Kit using Universal primers (Lau *et al*., 2002) and Bioer XP cycler. The sequence of the forward primer was 5' – GGA GGC AGC AGT AAG GAA T - 3' whereas that of the reverse primer was 5' – CTA CCG GGG TAT CTA ATC C – 3'. Primers were obtained from Allied Scientific, Kolkata India.

The PCR products were subjected to 1.2% agarose (Genei, India) gel electrophoresis stained with ethidium bromide and visualized under gel documentation system (Biorad) for the presence of 454 bp PCR product. The PCR products were sent for sequencing to Allied Scientific laboratories, Mumbai India. The homology of the 16s rDNA gene sequences was compared with the 16s rDNA gene sequences of other organisms in the GenBank database using BLASTN.

Estimation of parabens

All the reagents used were of HPLC grades and purchased from s d fine, India. 0.1 gm of lipstick samples were macerated and vortexed with 10 ml of methanol. The samples were subsequently centrifuged at 5000 rpm for 10 minutes and the supernatant (10 mg/ml) used for HPLC analysis (Nijes Pedije). 1000 ppm stock solutions of methyl and propyl parabens were diluted to obtain a range from 200 – 800 ppm in methanol. Reverse phase C18 column, Inertsil ODS-3 125mm with a pore size -5 um was used for HPLCanalysis. The parabens were detected at 254 nm using 0.1% ammonium formate: formic acid in 30:70 ratio (Port A: Port B).

RESULTS

Lipsticks are often eaten away by the users and hence it is imperative that health regulators have a microscopic look at the ingredients that go into the lipsticks (Deepali *et al*., 2011). As per Bureau of Indian Standards (BIS), lipcare cosmetics should not contain gram negative organisms nor *S.aureu.s* Our study

however reveals the presence of atleast four different species of gram negative bacteria belonging to *Pseudomonas* species, coliform group of bacteria like *Proteus, Providencia* and *Morganella* as well as one species of gram positive bacteria belonging to *Staphylococcus.* The *Staphylococcus* species was isolated from both used and unused samples, whereas organisms belonging to *Morganella* were isolated only from unused sample whilst *Providencia* and *Proteus* species were obtained only from used samples. The bacterial count for all the samples studied is far beyond the permissible microbiological limit of the BIS.

Table 2. Bacterial counts in terms of cfu/gm lipsticks on Nutrient agar as well as growth on differential as well as selective media

Sample	NA Cfu/gm * 10^9	EMB	CM	SSA	MSA
A_b	0.46	-	-	-	>300
B_b	0.25	-	-	-	5
C_b	0.19	-	-	-	8
D_b	4.55	-	-	-	11
E_l	2.09	-	-	-	-
F_l	5.9	>300	62	48	57
G_l	5.21	6	4	-	-
H_l	0.94	>250	41	124	9
I_b	24.7	4	37	2	>300
J_b	27.4	6	-	-	>400
K_b	2.14	9	-	-	15
L_b	2.18	17	-	-	58

(Key: A_b, B_b, C_b, D_b - Multinational lipsticks used for one to less than a year, E_l, F_l, G_l, H_l - Local lipsticks purchased from the market, I_b, J_b, K_b, L_b - Multinational lipsticks in used for about two years, -ve - No growth).

Table 3. Colony characteristics of bacteria selected for sequencing

Source	Name of the bacteria	NCBI accession no	Colony character	Media for isolation
Used lipsticks	*Proteus penneri*	KP031695	Gram negative 2 -3 mm pink colony	Eosin Methylene Blue agar
	Providencia vermicola	KP031698	Gram negative 3 mm white colony	Cetrimide medium
Unused lipsticks	*Proteus vulgaris*	KM220899	Gram negative 2-3 mm pink colony	Salmonella Shigella agar
	Morganella morganii	KP031696	Gram negative 3-4 mm pink black nucleated colony.	
	Pseudomonas species		Gram negative 2 mm fluorescent green colony	Cetrimide medium
Used as well as unused lipsticks	*Staphylococcus arlettae*	KP031697	Gram positive 1-2 mm golden yellow colony	Mannitol Salt agar

Comparison of the sequences obtained by 16s rDNA sequencing with those in NCBI revealed the presence of *Proteus penneri, Proteus vulgaris, Providencia vermicola, Staphylococcus arlettae* and *Morganella morganii* in addition to the *Pseudomonas species* obtained on Cetrimide medium.

Proteus penneri

TCTTTGTCCAGGGGGCCGCCTTCGCCACCGGTATTCCT
CCACATCTCTACGCATTTCACCGCTACACGTGGAATT
CTACCCCCCTCTACAAGACTCTAGCCAACCAGTTTCA
GATGCAATTCCCAAGTTAAGCTCGGGGCTTTCACATC
TGACTTAATTGACCGCCTGCGTGCGCTTTACGCCCAG
TAATTCCGATTAACGCTTGCACCCTCCGTATTACCGC
GGCTGCTGGCACGGAGTTAGCCGGTGCTTCTTCTGCG
GGTAACGTCAATTGATAAAGGTATTAACTTTATCACC
TTCCTCCCCGCTGAAAGTACTTTACAACCCTAAGGCC
TTCTTCATACACGCGGCATGGCTGCATCAGGCTTGCG
CCCATTGTGCAATATTCCTTACTGCTGCCTCCCA

Proteus vulgaris

CGTCAGTCTTTGTCCAGGGGGCCGCCTTCGCCACCGG
TATTCCTCCACATCTCTACGCATTTCACCGCTACACGT
GGAATTCTACCCCCCTCTACAAGACACTAGCCAACCA
GTTTCAGATGCAATTCCCAAGTTAAGCTCGGGGCTTT
CACATCTGACTTAATTGACCGCCTGCGTGCGCTTTAC
GCCCAGTAATTCCGATTAACGCTTGCACCCTCCGTAT
TACCGCGGCTGCTGGCACGGAGTTAGCCGGTGCTTCT
TCTGCGGGTAACGTCAATTGATAAAGGTATTAACTTT
ATCACCTTCCTCCCCGCTGAAAGTACTTTACAACCCT
AAGGCCTTCTTCATACACGCGGCATGGCTGCATCAGG
CTTGCGCCCATTGGGCAATATTCCTTACTGCTGGCTCC

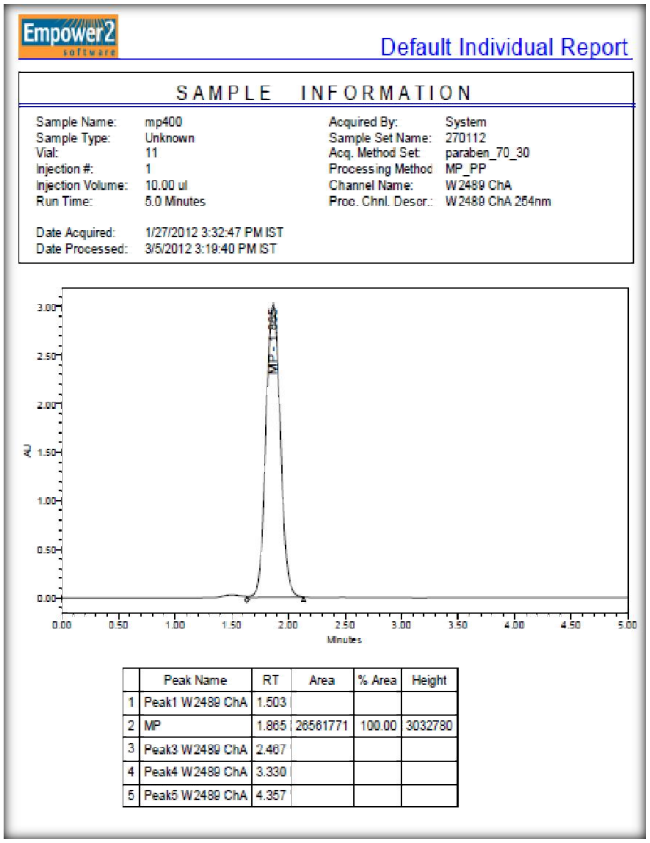
Morganella morganii

CGTCAGTCTTTGTCCAGGGGGCCGCCTTCGCCACCGG
TATTCCTCCACATCTCTACGCATTTCACCGCTACACAT
GGAATTCTACCCCCCTCTACAAGACTCTAGCTGACCA
GTATCAGATGCAATTCCCGGGTTAAGCCCGGGGATTT
CACATCTGACTCAATCAACCGCCTGCGTGCGCTTTAC
GCCCAGTAATTCCGATTAACGCTTGCACCCTCCGTAT
TACCGCGGCTGCTGGCACGGAGTTAGCCGGTGCTTCT
TCTGTCGGTAACGTCAATTGATGAGCGTATTAAGCTC
ACCACCTTCCTCCCGACTGAAAGTACTTTACAACCCG
AAGGCCTTCTTCATACACGCGGCATGGCTGCATCAGG
CTTGCGCCCATTGTGCAATATTCCTTACTGCTGCCTCC

Staphylococcus arlettae

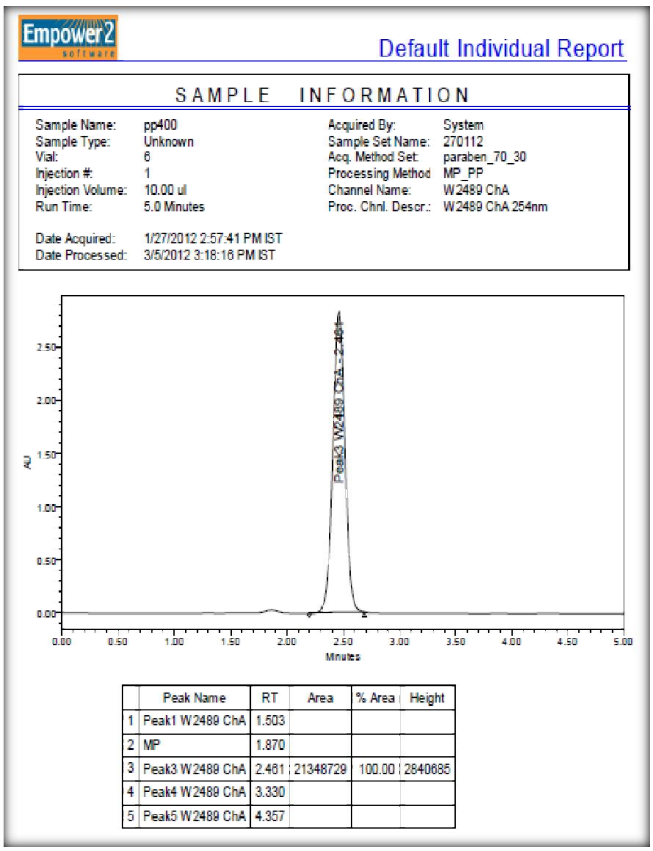
GGTCCTTTCGCAATTAGCGTCAGTGACTGAGCAAGAA
AGGCTGCTTCCCCACTGGTGTTCCTCCCTAACTCTGCG
CATTTCCCGCTACCATGGGATTCCACTTTCCTCTTCTG
CACTCTAGTCTCCCAGTTTCCAATGACCCTCCCAAGTT
GAGCTGGGGGATTTCACATTTGACTTAATAAACCGCC
TACGCGCGCTTTACGCCCAATAATTCCGAATAACGCT
TGCCCCCTCTGTATTACCGCGGCTGCTGGCACGTAGT
TAGCCGTGGCTTTCTGATTAAGTACCGTCAAGAATTG
CTAGGTTACTTACACGTTTGTTCTTCCCTAATAACAAA

GTTTTACGAGCCAAAACCCTTCCTCACTCACGCGGCG
TTGCTCCGTCAGGGTTTGCCCCATTGGGGAAAAATCC
TTACTGGTGCCTCCA



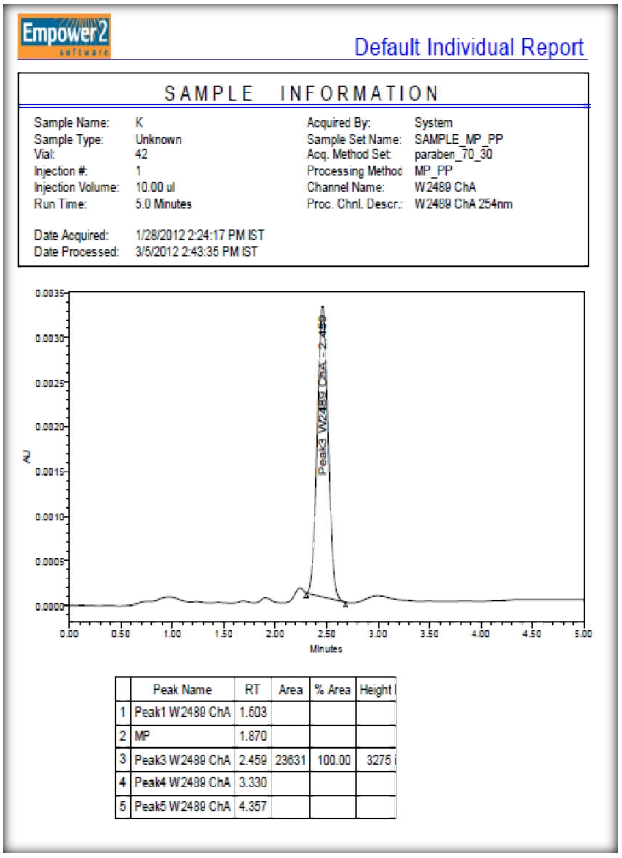
	Peak Name	RT	Area	% Area	Height
1	Peak1 W2489 ChA	1.503			
2	MP	1.865	26561771	100.00	3032780
3	Peak3 W2489 ChA	2.467			
4	Peak4 W2489 ChA	3.330			
5	Peak5 W2489 ChA	4.357			

Fig. 1A. Chromatogram for 400 ppm methyl paraben



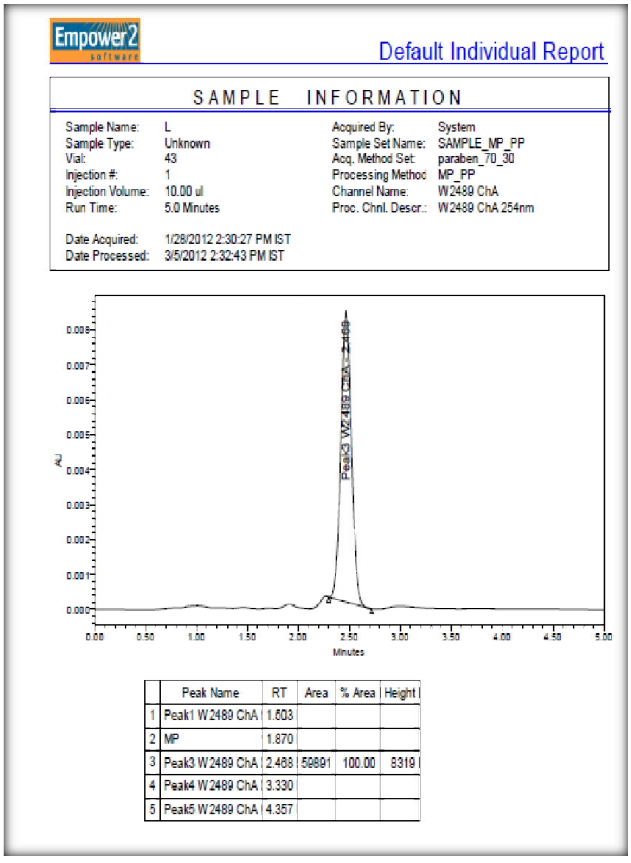
	Peak Name	RT	Area	% Area	Height
1	Peak1 W2489 ChA	1.503			
2	MP	1.870			
3	Peak3 W2489 ChA	2.461	21348729	100.00	2840685
4	Peak4 W2489 ChA	3.330			
5	Peak5 W2489 ChA	4.357			

Fig. 1B.Chromatogram for 400 ppm propyl paraben



	Peak Name	RT	Area	% Area	Height
1	Peak1 W2489 ChA	1.503			
2	MP	1.870			
3	Peak3 W2489 ChA	2.459	23631	100.00	3275
4	Peak4 W2489 ChA	3.330			
5	Peak5 W2489 ChA	4.357			

Fig. 2A. Chromatogram for sample K



	Peak Name	RT	Area	% Area	Height
1	Peak1 W2489 ChA	1.503			
2	MP	1.870			
3	Peak3 W2489 ChA	2.468	59891	100.00	8319
4	Peak4 W2489 ChA	3.330			
5	Peak5 W2489 ChA	4.357			

Fig. 2B. Chromatogram for sample L

Providencia vermicola

GTCAGTCTTTGTCCAGGGGGCCGCCTTCGCCACCGGT
ATTCCTCCACATCTCTACGCATTTCACCGCTACACATG

GAATTCTACCCCCCTCTACAAGACTCTAGCTGACCAG
TCTTAGATGCCATTCCCAGGTTAAGCCCGGGGATTTC
ACATCTAACTTAATCAACCGCCTGCGTGCGCTTTACG
CCCAGTAATTCCGATTAACGCTTGCACCCTCCGTATT
ACCGCGGCTGCTGGCACGGAGTTAGCCGGTGCTTCTT
CTGTCGGTAACGTCAATCGTTGATGATATTAGCATCA
ACGCCTTCCTCCCGACTGAAAGTACTTTACAACCCTA
GGGCCTTCTTCATACACGCGGCATGGCTGCATCAGGC
TTGCGCCCATTGTGCAATATTCCTTACTGCTGCCTCC

HPLC analysis and interpretation

Our study revealed the presence of propyl parabens at concentrations of 2740 ppm in case of sample K_b and 6960 ppm in sample L_b which was far beyond the average amount of paraben levels in cosmetics as per US-FDA and still ineffective in controlling the bacterial growth in the product which was evident from the colony count of $2*10^9$ cfu / gm for both the samples. This calls for a remedial measure to reduce the bacterial load in the product using an alternative or combinations of preservatives in specified concentrations to inhibit or minimize the microbial load. Parabens as such are known to be effective against gram positive bacteria but need to be used in combination with other preservatives for inhibition of the gram negative load (Kenith Walters). From the chromatograms it was evident that sample K_b and L_b had propyl parabens at a concentrations of 2740 ppm and 6960 ppm respectively. Samples A to J contained neither methyl paraben nor propyl paraben indicating use of alternative preservatives.

DISCUSSION

In similar studies on different cosmetic products such as eye care cosmetics, lip care, powders and baby shampoos *Staphylococcus species, Escherichia coli* have been commonly isolated. In addition, to these two Samiah and Al-Mijalli 2013, reported the presence of pathogenic bacteria like *Pseudomonas aeroginosa, Enterobacter cloacae, Salmonella typhimurium, Providencia stuartii, Flavimonas oryzihabitans, Brucella spp., Chryseobacterium indologenes, Klebsiella oxytoca* in different brands of baby shampoos. Mohammed 2011, reports the presence of *Klebsiella pneumonia* and *Escherichia coli* ($1.3 * 10^4$ - $1.5 *10^5$ cfu/ml) as well as *Staphylococcus species* ($9.1 * 10^3$ - $1.5 * 10^5$ cfu/ml) in mascara, lip pencils and eye pencils.

Omorodion et.al 2014 reported the total viable count for adult powders as ranging from $3.50 * 10^8 – 1.35 * 10^9$ cfu/gm whereas for the baby powders as $4.90 * 10^8 – 1.37 * 10^9$ cfu/gm. *Staphylococcus spp.,Micrococcus spp., Streptococcus spp.*were isolated from both the baby powders as well as adult powders whereas *Escherichia coli* was isolated only from the baby powders. Osungunna *et al.,* 2010, isolated *Staphylococcus aureus*, *Pseudomonas spp, Klebsiella spp.*and *Bacillus species* from the unused creams and lotions. 13 of the 15 unused samples showed bacterial contamination ranging from $0.24 * 10^3 – 2.56 * 10^3$ cfu/ml. *Staphylococcus aureus*, *Pseudomonas spp, Klebsiella spp.*and *Bacillus spp.* were isolated from them. Parabens have been used in cosmetics since 1930s. Amongst personal care products tested in US, lipsticks were found to contain highest concentration of methyl parabens ranging from 0.15 % – 1% i.e 1500 to 10000 ppm (Kirchoff and Gannes 2013). Parabens are known to penetrate the skin in inverse proportion to the ester chain length (Cosmetic Ingredient review 2008) and increase the expression of the genes responsible for growth of human breast cancer cells. Parabens are also associated with reproductive toxicity, irritation, immunotoxicity and neurotoxicity (Praveen 2014). The European Scientific Committee on Consumer Safety in 2010 concluded that the levels of propyl and butyl parabens in cosmetics should be reduced to 0.19% i.e 1900 ppm when used individually or combined for them to be safe for the health of the consumers (Cosmetic Ingredient Report 2012). The amount of paraben preservative which should be added to the final formulation is therefore, equally important for health of the consumers. It is desirable to develop an effective amount of a single /multiple preservatives to be added to the final formulation. The current study is one of the first to report the bacterial count (cfu/gm) in lipsticks alongwith the quantification of the paraben preservatives.

The presence of *Proteus, Providencia and Morganella, Staphylococcus and Pseudomonas species* is alarming and calls for stringent means of testing and analyzing of lipsticks by the regulatory agencies. It also gives room for suspicion as some of the products may be fake/ misbranded as per the fair packaging and labelling act of US-FDA for cosmetic products. For lipstick samples, the manufacturer's are required to give the content label in decreasing order of their concentration, though the concentration may not be revealed. Also the antioxidant mixture and the color additives have to be listed in the product formulation below the other ingredients (Fair Packaging and Labelling Act). However, it is disheartening to see none of the lipsticks used for the study had either of the details. The study thus emphasizes the need for improvization of the production procedures to minimize the microbial contaminants assuring safety of the end users.

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