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Microbial and Heavy Metal Contamination in Five Brands of Kohl Used in Sulaimani City

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Abstract

Kohl is a traditional eyeliner widely used among Middle East people. There is a growing concern about using Kohl due to its potential human health risks. Over the last decades, Kohl has gained a bad reputation due to its high contamination with microorganisms and the high concentration of heavy metals.

This descriptive study aims to detect the microbial content and some heavy metals concentration such as lead and Antimony in kohl samples available in the local markets of Sulaimani city.

Detection of microbial content was done by using six different types of culture media and gram staining techniques with some biochemical tests, and the final identification was done by the automated system VITEK 2. The analytical test for heavy metals was performed by using an Inductively Coupled Plasma Optical Emission Spectrometer (ICP-OES).

The results showed that two samples were contaminated with uncommon gram-negative bacteria (*Pantoea agglomerans* and *Dilftia acidovorans*) and one with gram-positive bacteria (*Bacillus mycoides*) while there is no evidence for fungal contamination. The concentrations of lead in the two samples were over the limit with a remarkable concentration of 1491 and 1117 ppm. Also, an unacceptable concentration of Antimony was recorded in one sample with 10.65 ppm.

According to our results, the brands of the kohl that are sold in Sulaimani city are not safe for use because it might serve as a vehicle for the transmission of potentially pathogenic organisms or loaded with heavy metal. Therefore, this cosmetic product must be thoroughly evaluated for safety before marketing.

1. Introduction

Kohl is a fine powder that is applied to the conjunctive and outer surface of the eye. Its usage among Middle East people is rooted in deeply held customs that have cultural and religious connotations. Kohl mainly originates from manufactures based in Saudi Arabia, India, and Pakistan. Traditionally, it is given high importance in the field of ophthalmology for the protection and cure of eye diseases. In most cases, it is prepared at home or sold in markets without regulation [1]. Because kohl is commercially successful and popular, it may be reasonable to think that it is safe. But over the last decades, Kohl has gained a bad reputation due to its high contamination with microorganisms and a high concentration of heavy metals [2,3].

Kohl is usually applied as eyeliner to the upper lid close to the lash line and the periocular skin of the lower eyelid, external to the lash line. Likewise, it can be placed on the surface of the lower eyelid margin within the lash line directly onto the mucocutaneous junction. It is worth mentioning that many types of research demonstrated, with clinical evidence, the migration of externally applied eye cosmetics onto the ocular surface [4–6]. Furthermore, studies have confirmed that tear film contamination can also result from the migration of eyeliner across the eyelid utilizing: eye rubbing, direct accidental ocular instillation, and poor application technique [4–6].

Due to the frequency of Kohl usage and the possibility of ocular infection and heavy metal toxicity which is consistently reported [1–3,7,8], we decided to evaluate the microbial content and determine some of the heavy metals (such as lead and Antimony) found in traditional eye cosmetics known as kohl (also known as Kajal or Surma) that is available in Sulaimani markets. To our knowledge, this is the first study to investigate both the microbial and heavy metal contamination of Kohl used in Iraq.

2. Materials and methods:

2.1 Collection of samples:

Five widely used brands of commercially available kohl powder were collected from local markets at Sulaimani city, Kurdistan Region, Iraq. They are: 1) Kohl al-asmad al-aswad, 2) Kohl original stone with zamzam water, 3) Al-asmad kohl, 4) Hashmi kohl Aswad, 5) Surma kohl.

2.2 Detecting Microbial content

The samples were collected separately in sterilized glass tubes and transferred to a microbiological lab; two measurements of 0.1 gram for each sample were taken. The first 0.1 grams were aseptically placed into 100 ml of nutrient broth and incubated aerobically at 37°C for 24 hours. The other 0.1 grams were placed into 100 ml of Sabouraud dextrose broth and incubated at 25°C for 72 hours. After incubation, a loop-full of the broth cultures from each sample were streaked on the surfaces of our solid media: nutrient agar, MacConkey agar, blood agar, and mannitol salt agar for bacterial detection. The cultures from Sabouraud dextrose broth were inoculated onto Sabouraud dextrose agar to detect fungal growth. For the identification of isolates, the pure bacterial cultures obtained from the samples were primarily identified (as gram-negative or positive) by phenotypic characterization on selective media and by gram staining. Gram-negative pure isolates were identified by using the automated system VITEK 2 compact (bioMérieux, France) while gram-positive isolate was identified by conventional methods like motility test and some biochemical tests; starch hydrolysis, catalase, indole, gelatinase and nitrate reduction test.

Viable cells were counted followed by a calibrated loop transfer method(9). From a mixture of 0.01g of Kohl sample in 9.9 ml of normal saline, two quantitative calibrated loops were used to spread 0.001 and 0.010 ml to nutrient agar plates. After incubation for 24 hours, the colonies are counted and the number of CFU per gram is determined by multiplying the number of colonies by the dilution factor. Also, Antibiotic sensitivity was tested by the disc diffusion method (10) against seven antibiotic discs: ciprofloxacin (CIP), Meropenem (MEM), Cefotaxime (CFM), Doxycycline (DO), cefotaxime (CTX), gentamycin (CN), and Ampicillin (AMP).

2.3 Quantitative determination of heavy metals concentration

Digestion of the samples was done by weighting 1 gram of each sample and dissolving them in 6 ml of HNO_3 and 3 ml of HCl and heating them to 140°C for about 30 minutes by multiwave 3000 microwave digestion system (Anton Paar, Austria) [11]. Then, samples of kohl were analyzed for assessment of nine heavy metals: Magnesium (Mg), Chromium (Cr), Manganese (Mn), Iron (Fe), Copper (Cu), Zink (Zn), Tin (Sn), Antimony (Sb), and Lead (Pb) by Optima 2100 DV Inductively Coupled Plasma Optical Emission Spectrometer (ICP-OES) (PerkinElmer, USA).

3. Results

3.1 Detection of microbial content

Mycological evaluation of the five brands of Kohl indicated that there is no evidence of contamination by fungi. The bacteriological evaluation indicated that the second and fourth brands tested were completely sterile, while Kohl al-asmad al-aswad, Al-asmad kohl, and Surma kohl were contaminated with pure growth.

The result of the Gram stain demonstrated that the three bacterial isolates were rod in shape. Two of the bacterial growths belong to the group gram-negative bacteria (growth appeared on nutrient agar, MacConkey agar, Eosin methylene blue agar, and blood agar but not on mannitol salt agar), while the third growth belongs to the group gram-positive bacteria (growth appeared on nutrient and blood agar only).

The final identification of the two Gram-negative isolates which were done by using the automated system VITEK 2 recognized the first growth as *Pantoea agglomerans* and the second as *Delftia acidovorans.*

Regarding the identification of the Gram-positive isolate, the morphological characteristics of the colonies on nutrient agar were mucoid, large, and irregular with lobate margins (Fig. 1). The gram staining technique identified the cells as rod-shaped with small clumps, short chains, or single cells (Fig. 2), while in old culture the presence of spores was dominating (Fig. 3). The feature of spore-forming cements the identification of the isolate as a member of the genus Bacillus.

Fig. 1. Colonies of *Bacillus* on nutrient agar

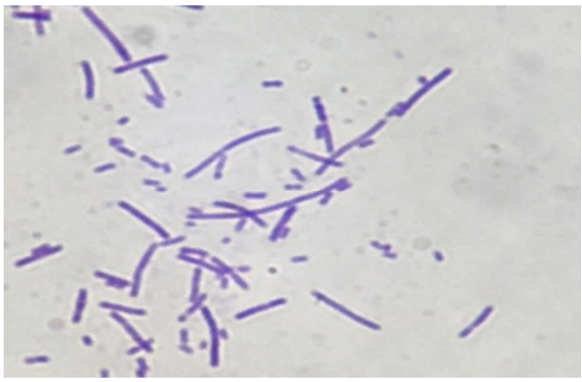

Fig. 2. Vegetative form of Bacillus at 1000X magnification

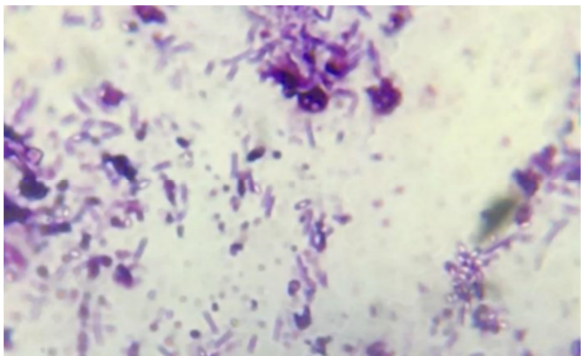

Fig. 3. Sporulated form of Bacillus at 1000X magnification

For the identification of this isolate to the species level, Bergey's Manual of Determinative Bacteriology [12] was followed. The characteristic of aerobic growth of mucoid colony appearance with the positive result of starch hydrolyses test and negative result of motility test along with the following finding: catalase (+), indole (-), gelatinase (+), nitrate (+) supported the final identification of the gram-positive isolate as Bacillus mycoides.

The results of the identification and viable cell count of the three contaminated samples are shown in table 1.

Table 1: Identification and viable cell count of the three contaminated samples

Kohl brand	Isolate identity	Viable cell count
1. KOHL AL-ASMAD AL-ASWAD	*Pantoea agglomerans*	$8x10^5$ (CFU/g)
2. AL-ASMAD KOHL	*Bacillus mycoides*	$2x10^6$ (CFU/g)
3. SURMA KOHL	*Dilftia acidovorans*	$2x10^5$ (CFU/g)

The three isolates showed different antibiotic resistance patterns in table 2.

Table 2: The antibiotics susceptibility pattern of the isolates

Isolate identity	CFM	CTX	MEM	CN	DO	CIP	P
Pantoea agglomerans	S	S	S	S	R	S	-
Bacillus mycoides	R	S	S	S	S	S	R
Dilftia acidovorans	S	S	S	R	S	S	-

3.2 Quantitative determination of heavy metals concentration

The results displayed in table 3 show that the mean of the heavy metals content in decreasing order was the following: Fe>Pb>Zn>Sn>Mn>Cr>Cu>Sb>Mg. A high concentration of iron was noticed in sample 1. The second predominant element is lead with a high concentration in samples 3 and 4. It was notable that AL-ASMAD KOHL (sample 3) was contaminated with Antimony and lead in addition to bacterial contamination with Bacillus.

Table 3: The elemental concentration of kohl samples in ppm

Sample no.	The concentration of heavy metal in ppm								
	Mg	**Cr**	**Mn**	**Fe**	**Cu**	**Zn**	**Sn**	**Sb**	**Pb**
1	5.152	19.8	27.89	9115	9.133	6.154	14.35	0	0.727
2	0.898	0.419	0.037	1.563	0.083	0.078	5.509	0.599	1.727
3	1.08	0.569	0.13	5.491	7.53	10.87	5.99	10.65	1491
4	0.599	0.401	0.043	6.243	2.617	18.3	5.257	4.582	1117
5	0.689	0.536	0.047	22.09	0.132	34.38	5.935	0.774	2.879
Mean	**1.6836**	**4.345**	**5.6294**	**1830.08**	**3.899**	**13.9564**	**7.4082**	**3.321**	**522.667**

4. Discussion

Although cosmetics are usually applied to the skin, systemic exposure to their ingredients cannot be excluded [7]. Prolonged use of Kohl may pose human health risks due to microbial contamination and toxic metal loading through dermal contact with the possibility of the migration of eyeliner across the eyelid margin onto the ocular surface which causes ocular infection and conjunctivitis or can increase the absorption of heavy metals into the human body and elevate the health hazards [5].

In the Kingdom of Saudi Arabia, about 35% of kohl sellers claimed that they prepare kohl themselves, with most of this kohl sold without proper labels and packing [1] this proves the uncertainty of safety around these unevaluated kohl products.

Microbial contamination of cosmetics is a serious public health concern [13,14]. According to the Scientific Committee on Consumer Safety (SCCS), microbial infection is of specific concern when cosmetics are used around the eyes [15]. However, isolation of potential bacterial pathogens from cosmetics, including eye-cosmetics, is not rare [7].

Many studies conducted in countries like Saudia Arabia, India, and Libya mentioned the isolation of bacteria from Kohl [2,3,16,17]. All these studies identified the isolated bacteria by using conventional methods like selective media and some biochemical tests which is not enough for accurate identification, especially at the level of species. Misidentification at the level of the genus could also happen due to insufficient tests. In this study, however, an advanced automated system (VITEK 2) is used to identify the gram-negative isolates at the level of species which leads to the isolation of two uncommon bacteria: *Pantoea agglomerans* (formerly known as *Enterobacter agglomerans*) and *Dilftia acidovorans* (formerly known *Comamonas acidovorans*). The third isolate is identified as a member of the genus Bacillus according to the colony and cell morphology. These three microorganisms are known to be opportunistic and can cause infections to immunosuppressive patients.

Pantoea agglomerans is a Gram-negative non-spore-forming, aerobe, rod-shaped bacterium of the family *Enterobacteriaceae*. All species of the genus *Pantoea* can be isolated from plants, soil, and feculent material where they can be either pathogens or commensals. *Pantoea agglomerans* and other *Pantoea* species can cause eye infections [18,19].

Delftia acidovorans is a Gram-negative non-spore-forming, aerobe, rod-shaped bacterium of the family *Comamonadaceae*. Species of this genus can be isolated from soil, water, and hospital environment, infection occurs frequently in hospitalized or immunocompromised patients, there are also many reports documenting infections in immunocompetent patients [20]. Also, many types of research have recorded that ocular infection is associated with Delftia sp. [21].

The isolation of members of the genus *Bacillus* was expected since most of the previous studies recorded this bacteria as one of the common causes of eye product contamination [3,16,17].

From the result of previous studies and this study, we noticed that bacterial contamination is more likely to occur than fungal contamination, maybe because bacterial growth is optimum at neutral pH and most cosmetic products are in this range.

Also, this study revealed that the number of viable cells (CFU/g) in Kohl samples is two to three times higher than the number accepted according to the SCCP (Scientific Committee on Consumer Products) [22], in which cosmetic products are divided into two different categories: the first category includes products specifically intended for children under three years or to be used in the eye area and on mucous membranes, while the second category includes all other products. Products intended for use on babies and the eye area should not exceed 100 CFU/g or ml of aerobic mesophilic microorganisms.

As for the antibiotic sensitivity of the bacterial isolates, the three isolates showed an all-around sensitivity to most of the antibiotic disks. No discernable pattern of resistance to the antibiotic discs can be identified.

Heavy metal impurity in cosmetic products is common due to their natural presence. However, they should be kept to a minimum wherever technically possible [4].

Contamination of cosmetic preparations with heavy metals occurs during the production process of, or as a result of, inadequate purification of the natural raw materials used as ingredients. An unrestrained application of cosmetic products with considerable amounts of heavy metals can potentially increase exposure levels [23].

Unfortunately, there are no current international standards for heavy metals concentration in cosmetics. The American FDA states that the maximum limit of lead concentration in cosmetics should be 20 ppm. [24]. However, the Canadian regulatory limits disagree, stating that lead and antimony concentration should not go over 10 and 5 ppm respectively [25].

In this study, two out of the five brands investigated exceeded the US FDA limits with outstanding concentrations (1419 and 1117 ppm) while the others were beyond Canadian regulations. This result induces fear since the relation between using kohl and lead poisoning is affirmed by many types of research. A study done in Kuwait reported that among 20 patients (ages between 1 and 18 months) suffering from lead encephalopathy, the blood levels in 19 children ranged between 60 and 257 mg/dl (equal to 600-2570 ppm). The source of lead in 11 patients was confirmed to be from kohl [26]. In another study, a seven-month-old baby was found to have a high blood lead level of 39 mg/dl (equal to 390 ppm) due to the use of kohl [27].

Regarding the content of Antimony, which according to Health Canada must be less than 5 ppm [22], our result shows that only one sample (Al-asmad kohl) was over the limit with 10.65 ppm. However, the European Commission states that any amount of Antimony (and its compounds) in cosmetics should be prohibited as it's classified as carcinogenic, mutagenic, or toxic. Only one of our samples appeared to be clear of Antimony [28].

Although the presence of iron in cosmetics is permitted under European parliament Regulation No 1223/2009 [28], a concentration of 9115 ppm in the sample (1) was noticeable. An approximate result of 8780000 ppb (equal to 8780 ppm) of iron concertation was recorded in face powder used in Saudi Arabia [7].

Also, we noticed that the sample (Surma kohl) has the highest concentration of Zinc among the samples with 34.38 ppm, however, there is no permissible limit for Zn in cosmetics currently available.

In general, heavy metal toxicity can result in reduced or damaged mental and central nervous function, lower energy levels, and damage to blood composition, liver, kidneys, lungs, and other vital organs. Long-term exposure may result in slowly progressing muscular, physical, and neurological degenerative processes that mimic Alzheimer's or Parkinson's disease, multiple sclerosis, and muscular dystrophy. Allergies are not uncommon and repeated long-term contact with some metals or their compounds may even cause cancer [29].

5. Conclusion

According to our results, Kohl sold in Sulaimani city is not safe for use because it might serve as a vehicle for the transmission of potentially pathogenic organisms that could have contaminated the product during the producing and packaging process. So, it is important to keep monitoring cosmetic products for contamination because an increasing number of cosmetic products are recalled each year and the majority of which is contaminated with potentially pathogenic microorganisms [16]. Also, heavy metal impurity in cosmetic products is common and should be kept to a minimum wherever technically possible. According to our results, the original stone with Zamzam water is the safest kohl among the others.

Our study recorded this impurity in three samples of which the concentration of some heavy metal was higher than the acceptable concentration limit, and that might be a critical public health hazard, especially for women and children. Therefore, this cosmetic product must be thoroughly evaluated for safety before marketing.

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