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In vitro Evaluation of *Spirulina Platensis* Extracts Against Pathogenic Bacteria Isolated from some Cosmetic Products.

Mariam, A. Elzoughby; Abdel-Rahman., H.M; Tewfike, T.A. and Salem, A.A.
Agric. Microbiology Dept., Fac. Agric. Moshtohor, Benha Univ., Egypt.
Mariam.elzoughby@fagr.bu.edu.eg

Abstract

Cosmetic products support microbial growth due to the presence of variable amounts of nutrients. Pathogens can be reached to cosmetic products by many sources, but antibiotics-resistant bacteria are the most dangerous. Moreover, there is an urgency to look for a natural antimicrobial agent that is safer and has fewer side effects. This research was investigated antibacterial activity of *S. platensis* extracts against antibiotics-resistant pathogenic bacteria isolated from some cosmetic products. From microbiological analysis of collected cosmetic sample 18 pathogenic bacterial isolates possess different virulence factors (protease, rhamnolipid and hemolysin production) were obtained. Four of them high resistant to tested antibiotic and these isolates were genetically identified as *Bacillus cereus*, *Staphylococcus* sp., *Pseudomonas aeruginosa* and *Stenotrophomonas maltophilia*. Results showed that methanol and hexane extracts of *S. platensis* (S2) at 0.5 mg/mL were highly active against four pathogenic isolates with inhibition zone ranged between 2 ±0.5 mm to 23±0.4 mm. This result indicated that the methanol and hexane of the cyanobacteria *S. platensis* have the potential as a natural antibacterial agent.

Keywords: Cosmetic products, cyanobacteria, antibiotics-resistant bacteria, virulence factors, *S. platensis* extracts.

Introduction

Many cosmetic products have optimal conditions for microbial growth, due to water and nutrients contents, pH and other factors, which implies a risk for consumers. Moreover, microbial contamination of cosmetics possesses a great problem to the cosmetics manufacturing process, especially from an economic point of view (**Alvarez-Rivera *et al*., 2018).** Several studies have revealed that cosmetic products may be contaminated with pathogenic microorganisms to different levels. *Escherichia coli*, *Pseudomonas* species, *Staphylococcus* species and *Bacillus* species were the most commonly recovered bacteria from cosmetics (**Hosny *et al*., 2017).** Preservatives are essential ingredients widely added to cosmetics and personal care products that are daily used, with the primary purpose of preventing spoilage from microbial growth.

The resistance of pathogenic microorganisms to the current preservatives highly increased. Accordingly, seeking for safe, lower toxic, biodegradable and biocompatible active preservatives is considered an important target **(Orús *et al*., 2015).**

The interest in natural antimicrobial preservatives is rapidly grown due to their exceptional properties such as biodegradability, biocompatibility and lower toxicity. Alongside with plants, algae and eukaryotic microalgae, cyanobacteria have been explored for the isolation of compounds with potential application in the cosmetic and cosmeceutical field **(Alagawany *et al*., 2021 and Reda *et al*., 2021).**

Numerous studies reported that *Spirulina* contains biological properties such as immunomodulation, antioxidant, anticancer, antimicrobial and probiotic effects. The presence of phytochemical compounds such as protein, carbohydrates, flavonoids, phenols, terpenoids and steroids exhibits the relation to the antimicrobial activity of *Spirulina platensis* against human pathogens (**Sowmya *et al*., 2021).** *Spirulina platensis* has been studied as a valuable source of antimicrobial, antiviral and antioxidant compounds but its activity is variable and dependent on the extractive solvent. These compounds can be utilized for the development of natural antibiotics against multi drug resistant bacteria. *Spirulina* is a potential source of bioactive compounds and recently used in skincare products (**Elshouny *et al*., 2017**; **Abdel-Moneim *et al*., 2022 and Hidhayati *et al*., 2022).**

The aim of the research was to measure *in vitro* the antibacterial activity of different extracts of *Spirulina platensis* against different cosmetics microbial isolates and characterize the structure of active compound using different methods including HPLC and GC-MS analysis.

Materials and methods

Cosmetic samples

Eighty-seven commercial cosmetic samples (shampoo, hair gels, liquid soaps, lotions, creams, balsam, scrub and conditioners) were collected from pharmacies, supermarkets and spices shops. Samples were collected during 2018 - 2019 and stored at 4°C

for determination of pathogens and total microbial counts.

Antibiotics

Twenty antibiotics belonging to different groups were used in this study and purchased from Oxoid, UK as shown in **Tables 5.** The antibiotics in the present study were selected based on common antibiotics used in medical practice, health treatment and the recommended dose **(Andrews and Howe, 2011** and **Etebu and Arikekpar, 2016).**

Cyanobacteria sources

Pure crude of *Spirulina platensis* powder was purchased from biotechnological international laboratory for researches and development (BIRD) and the algal biotechnology unit, National Research Centre (NRC), Dokki-Cairo, Egypt.

Table 1. Media used for microbial estimation of cosmetic samples.

Media	Usage	Source	Reference
Tryptone soy broth	Was used for pathogenic bacteria activation and for determination of the MIC of the antimicrobial agents	Himedia Co., Germany	**(Atlas, 2005)**
Mannitol-salt agar medium and Baird-Parker agar medium.	Used for selective cultivation of *Staphylococcus* sp.	Neogen/Lab M	**(Atlas, 2005)**
Cetrimide agar medium and *Pseudomonas* agar medium	Used for selective cultivation of *Pseudomonas* sp.	Himedia Co., Germany	**(Atlas, 2005)**
***Streptococcus* selection agar medium**	Used for selective cultivation of *Streptococcus* sp.	TM media	**(Atlas, 2005)**
***Salmonella* and *Shigella* agar (SSA) medium**	Used for selective cultivation of *Salmonella* sp.	Himedia Co., Germany	**(Atlas, 2005)**
MacConkey agar medium	Used for selective cultivation of pathogens Enterococcaceae	Himedia Co., Germany	**(Atlas, 2005)**
Mueller Hinton agar medium	Was used as a test medium for antimicrobial susceptibility test.	Himedia Co., Germany	**(Atlas, 2005)**
Sabouraud dextrose agar medium	Was cultured for other yeasts and filamentous fungi.	Neogen/Lab M	**(Atlas, 2005)**
Plate count agar	A general-purpose medium for bacteria.	Merck	**(Atlas, 2005)**

Microbial counts

The collected samples of cosmetic products were analyzed for the determination of total bacterial count, yeast and fungal count, which is based on the analytical method described in the FDA's BAM Chapter 23 **(Huang *et al.*, 2017).** For detection of pathogens in cosmetics samples the tests are performed according to the guidelines given in the technical publications of International Organization for Standardization (ISO) 18415 **(Food and Drug Administration, 2016 and International Organization for Standardization, 2017).**

Bacterial isolates Identification

The bacterial isolates were initially identified with the colony morphology appearance on specific agar media also according to hemolytic type on blood agar medium.

After observing colony morphology of bacterial isolates, microscopic examinations were achieved throughout gram staining to determine shape, size of cell and arrangement **(Alfred, 2005).** The four selected bacterial isolates were genetically identified according to **(Khedr *et al.*, 2017)**

Antibiotic susceptibility testing

The susceptibility of the bacterial isolates to twenty different antibiotics was performed by modified Kirby-Bauer single-disk diffusion technique on Müller Hinton agar according to **Robert *et al.*, (2003).** Different antibiotics were used for determination of antibiotic resistance profiles of the isolates.

Results of the susceptibility tests were interpreted according to the criteria established by the Clinical and Laboratory Standards Institute **(CLSI, 2017&2018).** Selected multidrug resistant bacteria were used in antibacterial assay.

Preparation of *Spirulina* extracts

The dried *Spirulina platensis* biomass (20 g) was separately homogenized in water and different organic solvents such as methanol, ethanol, acetone, chloroform, diethyl ether, ethyl acetate and hexane (HPLC grade, Sigma-Aldrich). Each homogenized biomass was sonicated for 20 min using ultrasonic micro tip probe of 400 watt (ULTRASONIC Get 750), then centrifuged at 4500 rpm for 10 min (SIGMA Laborzentrifugen Gmbh). Supernatants were collected separately and the pellets were re-extracted twice as mentioned before. Combined supernatants were evaporated to dryness at 40°C using rotary evaporator. Dried extracts were kept in labeled sterile vials in a deep freezer at -20 ºC till further use **(Marrez *et al*.,2019).**

Antimicrobial activity of *Spirulina platensis* extracts

The antibacterial activity evaluation of *S. platensis* extracts was performed using the well diffusion method **(Shanmuga *al*., 2002).** The inoculum of the microorganisms was prepared from pure bacterial isolates. Fifteen milliliters of nutrient agar medium were poured in clean sterilized Petri-plates and allowed to cool and solidify. Hundred microliters of bacterial broth were pipetted out and spread over the medium evenly with a spreading rod till it dried properly. Wells of 9 mm in diameter were bored using a sterile cork borer. Solutions of the extracts (0.5 mg/mL) in dimethyl sulphoxide (DMSO) were prepared. 100µL of *S. platensis* extract solutions was added to the wells. The Petri plates were incubated at 37°C for 24 h. Tetracycline (1 mg/mL) was used as a positive control to determine the sensitivity of the microbial species used and DMSO was taken as negative control. Antibacterial activity was evaluated by measuring the diameters of the zones of inhibition (ZI). All the determinations were performed in triplicates.

Minimum inhibitory concentration (MIC) of different extracts of *Spirulina platensis*

Spirulina platensis extracts (hexane, methanol extract and their combination) were tested for minimum inhibitory concentration (MIC) against bacterial isolates by broth microdilution method **(NCCLS, 1999).** All tests were performed in Mueller– Hinton broth medium. The inocula of bacterial strains were prepared from overnight Mueller–Hinton broth cultures at 37 °C. Pathogenic tested strains were suspended in Mueller–Hinton broth to give a final density 10^8 (CFU/mL). The *S. platensis* extracts dilutions in DMSO were ranging from 0.5 mg/mL to 25 mg/mL.

The volume of the agent in each tube ranges from 2 -5 mL. All the test tubes were incubated at 37 °C for 24 h for bacteria. Negative controls were prepared using 100 µL of DMSO, the solvent used to dissolve *Spirulina* extracts. After incubation for 24 h at 37 °C, 30 µL of resazurin (0.015% w/v) aqueous working solution was added, and further incubated for 30 min. After incubation, the observation of resazurin color change from blue to pink specifies the reduction of the resazurin indicator and thus microbial growth. The MIC tubes showed no color change (blue resazurin color remained unchanged) was recorded as the MIC value, according to **Elshikh *et al*., (2016)** with some slight modifications.

Statistical analysis

The obtained data were statistically analyzed Using CoStat version 6.400 (CoHort software, Monterey, CA, 93940, USA). For comparison between means, standard deviation (SD) was used.

Results and discussions

Total microbial counts

A total of 87 cosmetics and personal care products were analyzed in this study. Of the 87 samples, 41.38 % were hair care preparations, 16.09% were skin care preparations, 11.49% were face care preparations, 6.90% were hand care preparations, 5.75% were foot care preparations and 18.39% were body care products. Out of 87 cosmetic samples collected from pharmacies, supermarkets and spices shops in Egypt, were tested for their total microbial counts and yeast & mold counts.

It has been found that only 63 (approximately 73.6%) of the tested samples were contaminated with bacteria while only 33 (38%) were contaminated with yeast and mold. The incidences and level of microbial contamination are present in **Table 2.**

Data revealed that the maximum bacterial counts were found in body washing and cream samples compared to other cosmetic samples. Total bacterial counts in contaminated samples varied from 6.89±0.51 to 11.15±1.51 (log CFU/g). Moreover, the maximum total yeast and mold counts were found in face washing and hair cream samples compared to other cosmetic samples. Total yeast and mold count in contaminated samples were varied from 3.68±1.35 to 9.07±1.04 (log CFU/g).

Cosmetics samples showed high load of total viable bacteria up to 11.15±1.51 log CFU/g. The highest bacterial count was found in body washing and cream samples. However, the lowest bacterial count was observed within shampoo and conditioner samples. The highest yeast and mold count was found in face washing and hair cream samples. Whereas, the lowest yeast and mold count was observed for hand soap sample.

Table 2. Total microbial counts of some commercial cosmetic samples.

Cosmetic products		Counts (log CFU/g)	
		Total bacterial count	Total yeast and mold count
Hair care cosmetics			
1H	**Shampoo & conditioner**	6.89±0.51	ND
2H	**Shampoo & conditioner**	7.05±0.34	ND
3H	**Shampoo Co-creations**	7.26±0.53	4.28±2.86
4H	**Anti-dandruff Shampoo**	7.07±0.55	5.4±0.48
5H	**Anti-dandruff nourishing Shampoo**	7.11±0.44	5.61±0.53
6H	**Shampoo with keratin & conditioner**	7.74±0.34	5.23±0.48
7H	**Shampoo with natural Shea butter**	7.68±0.52	5.71±0.63
8H	**Hair conditioning cream moisturizing**	ND	ND
9H	**Protecting shampoo**	10.47±1.50	9.05±0.66
10H	**Baby Shampoo**	10.48±1.45	ND
11H	**Peeling Shampoo**	7.95±5.40	ND
12H	**Hair shampoo**	ND	ND
13H	**Shampoo & conditioner with Shea butter**	7.47±5.13	5.93±4.04
14H	**Hair shampoo with Almond and Olive oil**	10.85±1.51	ND
15H	**Advanced Keratin Balsam**	7.77±5.40	ND
16H	**Hair Shampoo**	ND	ND
17H	**Hair styling cream with garlic oil & marrow extract**	10.67±1.49	ND
18H	**Anti-Hair Fall shampoo**	ND	ND
19H	**Hair Cream**	10.72±1.66	8.81±1.02
20H	**Green grass oil shampoo and conditioner Shampoo**	10.3±1.15	6.07±4.13
21H	**Anti-dandruff Shampoo**	ND	ND
22H	**Shampoo and conditioner rich in olive oil**	ND	ND
23H	**Shampoo and conditioner with Pizzeria and green tea**	ND	ND
24H	**Hair conditioner**	10.85±1.60	8.39±0.93
25H	**Hair shampoo**	10.67±1.67	8.67±1.03
26H	**Hair Mix - Shampoo**	10.40±1.62	8.51±0.88
27H	**Anti-dandruff spray**	10.48±1.64	ND
28H	**Hair Anti-dandruff styling cream**	10.69±1.70	8.71±0.94
29H	**Wax for Hair & Skin**	10.19±1.66	ND
30H	**Intense Conditioning Cream**	10.66±1.60	8.35±0.78
31H	**Ultimate Repair Shampoo**	10.89±1.59	9.02±1.02
32H	**Keratin Smooth hair conditioner.**	10.44±1.62	ND
33H	**Hair Conditioner with Keratin and Herbal Extracts**	10.24±1.53	8.91±0.91
34H	**Hair cream**	11.05±1.68	9.06±1.01
35G	**Gel cream with Rosemary & Lanolin (2*1)**	10.88±1.62	8.73±1
36G	**Gel cream with Rosemary & Bees wax (3*1)**	10.77±1.54	8.42±1.02
Skin care cosmetics			
37S	**Skin Softener Body Cream**	9.86±1.72	ND
38S	**Body Lotion with Glycerin and Almond**	10.85±1.51	ND
39S	**Baby Lotion**	6.92±4.82	ND

Cosmetic products		Counts (log CFU/g)	
40S	**Nourishing Milk & Honey Hand & Body Lotion**	10.81±1.63	6.15±4.14
41S	**Milk and Honey Gold Nourishing Hand and Body Cream.**	10.99±1.36	ND
42S	**Baby cream with Zinc and Castor oil**	10.2±1.65	8.57±0.96
43S	**Skin Moisturizing Cream with Coconut Butter**	ND	ND
44S	**Whitening skin Cream golden**	10.46±1.56	ND
45S	**Deep Moisturizing Skin Cream with Coconut**	ND	ND
46S	**Moisturizing Skin Cream rich with vitaminB3 - Even Tone**	10.26±1.60	ND
47S	**Makeup Remover -With Almond Oil**	10.85±1.64	9.03±0.98
48S	**Skin Scrub**	ND	ND
49S	**Skin Cream with Yogurt**	10.28±1.56	ND
50S	**Cream for acne-prone skin is rich in salicylic acid**	9.87±1.59	8.29±0.85
	Face care products		
51F	**Face day cream.**	10±1.56	ND
52F	**Face scrub with coconut water.**	ND	ND
53F	**Face scrub with honey and pineapple extract.**	ND	ND
54F	**Cooling after sun cream.**	10.43±1.62	8.43±0.85
55F	**Daily face wash.**	10.80±1.68	9.07±1.04
56F	**Face cleaner with milk protein & honey.**	10.97±1.63	8.86±0.89
57F	**Refreshing Face Wash.**	ND	ND
58F	**Face wash rich with lemon extract.**	9.96±1.61	ND
59F	**Face and hand cream.**	9.98±1.51	ND
60F	**Face and hand cream.**	ND	ND
Hand care			
61D	**Anti-wrinkles moisturizer hand cream.**	10.16±1.60	5.98±4.05
62D	**Natural fairness hand cream.**	9.86±1.51	ND
63D	**Extra nourishing cream for dry & cracked hands.**	10.42±1.62	8.62±0.75
64D	**Hand moisturizing cream.**	ND	ND
65D	**Hand soap.**	9.85±1.35	3.68±1.35
66D	**Hand wash.**	ND	ND
Foot care products.			
67T	**Foot powder deodorant.**	9.85±1.42	ND
68T	**Refreshing foot spray.**	9.52±1.46	ND
69T	**Moisturizing cream for cracked heels.**	ND	ND
70T	**Cracked feet cream.**	10.05±1.156	8.35±1.56
71T	**Cracked heels cream.**	11.05±1.59	ND
Body care products.			
72B	**Shower Gel, Fresh Pure**	10.77±1.7	ND
73B	**Body Wash**	11.15±1.51	8.46±1.26
74B	**Shower Gel with Bouquet Scent**	10.68±1.69	ND
75B	**Bath a0 Shower Foam with Flora**	9.44±1.29	ND
76B	**Baby shower gel for baby's deep hydration**	ND	ND
77B	**Shower Gel Creamy with Coconut Frag**	ND	ND
78B	**Skin cream.**	10.83±1.64	9.03±0.98
79B	**Shower Gel Amber**	10.95±1.61	6.21±4.20
80B	**Shower Gel for Face & Body**	10.46±1.63	ND
81B	**Hair Remover cream for Sensitive Skin with Honey**	ND	ND
82B	**Hair Remover cream with yogurt**	9.81±1.44	ND

Table 2. continued

Cosmetic products		Counts (log CFU/g)	
		Total bacterial count	Total yeast and mold count
83B	**Skin Smoothing Cream**	9.69±1.51	ND
84B	**Hair minimizing cream and stop growth**	9.76±1.48	ND
85B	**Body whitening cream with milk protein and pearl powder**	ND	ND
86B	**Body lotion**	ND	ND
87B	**Body scrub**	ND	ND

(ND) Not detected, Means ± standard deviation

The obtained results of this study are similar to those of many other studies on commercial cosmetics, which reported microbial counts in creams, lotions, bath foam, and shampoo ranging from 10^2 to 10^4 CFU/g or mL. **Zaghloul *et al*, (2015) and Jairoun *et al*, (2020)** found that out of 140 cosmetic samples, only 31 (22.14%) samples were contaminated with bacteria or fungi or both. Data reveal that the maximum bacterial counts were found in shampoo samples compared to other cosmetic samples, followed by gel, solution, cream and oil samples.

The highest levels of contamination by yeast and mold and aerobic mesophilic bacteria in cosmetic creams due to the fact that cosmetic creams have rich textures created using growth factors, essential minerals, and high moisture levels; with a wide spread of organic and inorganic compounds, this creates a good environment for microbes to grow (**Zaghloul *et al*., 2015 and Desouky *et al*., 2017).**

Generally, data revealed that bacterial contamination of collected samples was higher than yeast and mold contamination. This result is in agreement with **Hugbo *et al*, (2003)** who found that 90% of examined cosmetic cream products were contaminated with bacteria at levels more than 10^2 and 10^3 CFU/mL and 70% were contaminated with moulds at less level than bacteria. **Mwambete and Simon (2010)** found 70% of cosmetic products investigated yielded bacterial contaminants, while 40% yielded fungal contaminants at levels more than 10^3 CFU/mL. The microbiological contaminants may come from raw materials, or the contamination may occur during the processes of producing, packing, and storing of cosmetic products this was confirmed by (**Taha, 2019).**

Total pathogens count

The samples are qualitatively examined for the presence of some potential pathogens. Of the 87 samples, 26.4 % were contaminated with bacterial pathogens using different selective and differentiated media and data are tabulated in **Table 3.**

All detected pathogenic bacteria group contaminants were found in the hair care, skin care and body care, face care and foot care products. Using selective bacterial agar medium (MacConkey agar medium, Mannitol salt agar medium, Streptococcus selection agar medium, Cetrimide agar and Salmonella & shigella agar media), data showed that approximately 9.2%, 13.8%, 10.3%, 14.9% and 5.7% of all used products were contaminated with typical bacterial colony, respectively.

Table 3. Total pathogens count of some commercial cosmetic samples.

Cosmetic products code	Total pathogens Counts (CFU/g)				
	MacConkey agar medium	Mannitol salt agar medium	Streptococcus selection agar medium	Cetrimide agar medium	Salmonella & shigella agar medium
Hair care cosmetics					
6H	**ND**	**68±7.6**	**3±1.5**	**45±4.5**	**ND**
8H	**ND**	**1.3±0.6**	**ND**	**3±1.5**	**ND**
9H	**ND**	**ND**	**ND**	**1.3±0.6**	**ND**
10H	**ND**	**2±1**	**ND**	**ND**	**ND**
13H	**ND**	**4±1**	**1.3±0.6**	**ND**	**ND**
15H	**ND**	**44±5.2**	**ND**	**12.7±3**	**ND**
19H	**3±1**	**ND**	**ND**	**ND**	**ND**
20H	**ND**	**ND**	**4±1.5**	**71±4**	**3±1**
24H	**ND**	**24±5**	**ND**	**13±3**	**7±2**
34H	**18±3**	**13±3.4**	**ND**	**36±3**	**52±3.5**
Skin care cosmetics					
37S	**ND**	**26±3**	**ND**	**45±8**	**ND**
38S	**ND**	**2±0**	**ND**	**2±0.6**	**ND**
39S	**ND**	**48±8**	**27±4**	**ND**	**ND**
40S	**3±1**	**ND**	**5±1.5**	**5±1.5**	**ND**
41S	**6±1.5**	**ND**	**ND**	**ND**	**ND**
44S	**ND**	**ND**	**ND**	**ND**	**8±1**
Face care products					
56F1	**ND**	**29±1**	**5±0.6**	**27±3**	**ND**
Foot care products.					
71T	**15±2.5**	**ND**	**ND**	**8±1**	**ND**
Body care products.					
76B	**4±1**	**ND**	**3±0.6**	**ND**	**ND**
78B	**8±1.7**	**ND**	**2±1**	**1±0**	**ND**
79B	**ND**	**4±1.2**	**10±1.7**	**ND**	**ND**
83B	**ND**	**ND**	**ND**	**ND**	**7±1.5**
84B	**16±2**	**ND**	**ND**	**ND**	**ND**

(ND) Not detected, Means ± standard deviation

Screening of bacterial pathogens and virulence factors

Pathogenic bacteria that isolated from collected sample were tested for production of virulence factors and resistance to antibiotics. These tests were conducted for detection of multi-drug resistant isolates. The bacterium that possess different virulence factors indicates that it has different mechanisms of infection and may enhance its ability to cause diseases in humans and other organisms. Therefore, some important virulence factors were detected in bacterial isolates from cosmetic products as shown in **Table 4.**

The ability of bacterial isolates to produce protease, rhamnolipid and blood hemolysis were estimated. Obtained results clear that diverse pattern of virulence markers were observed in different isolates. Among the pathogenic bacterial isolates, 39 isolates (69.64%) were able to produce rhamnolipid while, 36 isolates (64.3%) were able to produce protease, as well as 34 isolates (60.7%) showed hemolysin activity. Only 18 (32.14%) bacterial isolates were found to possess the three virulence factors. Potential to lyse red blood cell is considered as a pathogenic marker (**Liaqat *et al*, 2019)** so any isolates showed hemolysis on sheep blood agar indicating that these organisms may also cause other systematic infections.

Iron limiting condition provide a signal for the induction of virulence genes including genes for toxins e.g. hemolysin. One of the most abundant source of iron in the body is haem and so it is not surprising to find that pathogenic bacteria can use haem as an iron source. Haemolysis can be defined as a known virulence factor among pathogenic microorganism. Moreover, lysis of the red blood cells

indicates the presence of rhamnolipid (**Jakobsen *et al.*, 2013).** In many cases, the virulence factors are secreted proteins or enzymes, sometimes performing very specific functions. Proteases are probably the most effective of all bacterial compounds in the establishment of an infection. As enzymes, proteases present a large turnover, processing enormous amounts of substrate in little time (high kcatm value), while proteins and other molecules that act by binding to targeted receptors or tissues, even with high affinity, are limited to single or few events.

Table 4. Production of virulence factors by bacterial isolates.

Isolates code	Virulence factors		
	Blood hemolysis	Proteolytic activity	Rhamnolipid production
6H4i	+	+	+
6H2i	+	+	-
6H3i	-	-	+++
40S4i	+	+	+++
40S1i	+	+	+
40S3i	+	+	+++
38S2i	-	-	+
38S4i	+	+	-
34H4i	+	+	+++
34H5i	+	+	+++
34H2i	+	+	-
34H1i	+	+	-
34H2II	+	+	-
19H1i	+	+	-
9H4i	-	-	+++
25H1i	-	-	++
76B3i	-	+	+
76B1i	+	+	-
79B3i	-	-	+
79B2i	+	+	+++
37S2i	-	+	+++
37S4i	+	+	-
13H3i	+	-	+
13H2i	+	-	+++
10H2i	+	+	+++
15H4i	+	+	++
15H2i	+	+	+++
39S2i	+	+	-
39S3i	+	-	+
8H4i	+	+	-
8H2i	+	+	-
24H5i	-	+	+++
24H2i	-	-	+++
24H4i	-	-	++
7H2i	+	+	-
78B4i	-	-	+
78B3i	-	-	+++
78B1i	-	-	+++
71T1i	+	+	+++
71T4i	+	+	-
56F2i	+	+	++
56F2II	+	+	++
56F4i	+	+	+++
56F4II	-	-	++

Table 4. continued

Isolates code	Virulence factors		
	Blood hemolysis	Proteolytic activity	Rhamnolipid production
56F3i	+	+	+
41S1i	+	+	+++
20H3i	-	-	+++
20H5i	+	+	+
20H4i	+	+	+
44S5i	-	-	-
44S5II	-	-	+
44S5III	-	-	-
83B5i	-	-	+
83B5II	-	+	-
83B5III	-	-	+
84B1i	-	+	+

(-) no formation of clear zone, (+) positive, the degrees of Rhamnolipid activity are as follows: + + + > + + > + (a slight formation of clear zone).

Additionally, proteases can open their own ways to get into the host tissues and cells, thus representing a huge and valuable tool for colonization and spreading of the bacteria. Most secreted bacterial endoproteases are highly specific against their substrates in the host and as opposed to exoproteases, they cleave at very specific sites within the target molecule, which confers a characteristic symptom, typical of the disease caused by the pathogen secreting it. In many cases, they also constitute a defense mechanism against the host's immune system (**Mittal *et al*., 2006 and Lebrun *et al*., 2009).**

Antibiogram of pathogenic bacterial isolates

Antibiotics sensitivity of twenty different antibiotics were tested with disc diffusion assay against eighteen bacterial isolates and the results observed in **(Fig 1 and Table 5).** Wide variability was observed in the susceptibility of tested isolates. Obtained data showed that the bacterial isolate **(**56F4i**)** showed highest resistance (95%) to most of the tested antibiotics followed by 20H4i (65%), 15H2i (60%) and 34H5i (55%). Whereas, 40S4i showed the lowest resistance to antibiotics (30%). Also, most bacterial isolates showed highest resistance rates to ampicillin, ceftazidime, cefotaxime, ceftriaxone, cefamandole, cefoxitin, cefadroxil, and aztreonam belonging to three groups of antibiotics penicillin, cephalosporins and Monobactams. Result of antibiotic susceptibility highlights the possibility of permeating resistant bacterial isolates in cosmetic products.

Antimicrobial resistance (AMR) is a worldwide public health concern that has drawn attention in the recent time. Multi-drug resistant pathogens are the major threat to the doctors treating an infected patient. Indiscriminate use of antibiotics is the main reason behind it (**Abedin *et al*., 2020).** There are many contributing factors for development of antibiotic resistance bacteria in cosmetic products. For example, untreated wastewater from antibiotic industry may aid in developing the reservoir of antibiotic resistance gene pool in environmental bacteria. These resistance genes may be transfer to human microbiome including pathogens **(Li *et al*., 2010; Cabello *et al*., 2013 and Taha, 2019).** Additionally, the development of antibiotic-resistant microorganisms due to the selective pressure from preservatives included in cosmetic products could be a risk for the emergence and spread of bacterial resistance in the environment (**Orús *et al*., 2015).**

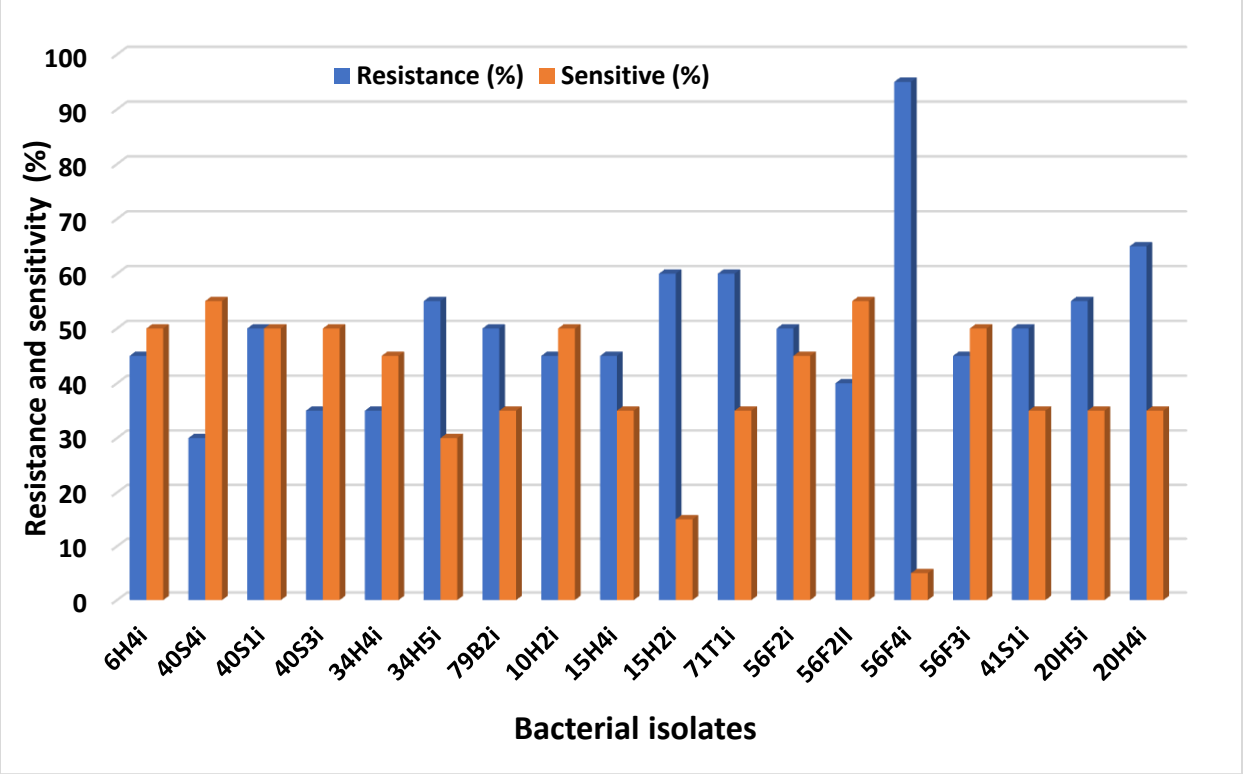

Fig 1. Resistance and sensitivity percentage of bacterial isolates against tested antibiotics.

Table 5. Antibiotic susceptibility test of the bacterial isolates

Antibiotic	Disk content (µg)	Bacterial isolates									
		6H4i	40S4i	40S1i	40S3i	34H4i	34H5i	79B2i	10H2i	15H4i	15H2i
		Diameter of inhibition (mm).									
Penicillins											
Ampicillin	10 µg	4±0.3**(R)**	24±0.1**(S)**	29±0.1**(S)**	ND**(R)**	ND**(R)**	ND**(R)**	ND**(R)**	5±0.5**(R)**	9±0.5**(R)**	5±0.1**(R)**
Ampicillin-sulbactam	10/10 µg	16±0.1**(S)**	15±0.2**(S)**	24±0.2**(S)**	14±0.2**(I)**	14±0.1**(I)**	ND**(R)**	14±0.3**(I)**	ND**(R)**	12±0.2**(I)**	24±0.3**(S)**
Cephalosporins											
Cefadroxil	30 µg	22±0.5**(R)**	26±04**(S)**	24±0.3**(R)**	26±0.5**(S)**	19±0.1**(R)**	9±0.2**(R)**	24±0.3**(R)**	24±0.4**(R)**	24±0.3**(R)**	9±0.4**(R)**
Cefaclor	30 µg	34±0.2**(R)**	39±0.1**(S)**	34±0.5**(R)**	39±0.4**(S)**	39±0.3**(S)**	ND**(R)**	34±0.4**(R)**	39±0.2**(S)**	39±0.1**(S)**	3±0.5**(R)**
Cefoxitin	30 µg	9±0.4**(R)**	4±0.5**(R)**	5±0.4**(R)**	ND**(R)**	6±0.4**(R)**	ND**(R)**	10±0.1**(R)**	19±0.3**(S)**	7±0.4**(R)**	6±0.2**(R)**
Cefamandole	30 µg	17±0.3**(I)**	15±0.4**(I)**	9±0.2**(R)**	15±0.1**(I)**	15±0.2**(I)**	ND**(R)**	ND**(R)**	ND**(R)**	4±0.3**(R)**	ND**(R)**
Ceftazidime	30 µg	ND**(R)**	ND**(R)**	ND**(R)**	9±0.4**(R)**	ND**(R)**	ND**(R)**	ND**(R)**	ND**(R)**	ND**(R)**	9±0.3**(R)**
Cefotaxime	30 µg	ND**(R)**	ND**(R)**	ND**(R)**	ND**(R)**	ND**(R)**	ND**(R)**	ND**(R)**	ND**(R)**	ND**(R)**	ND**(R)**
Ceftriaxone	30 µg	7±0.1**(R)**	6±0.3**(R)**	ND**(R)**	9±0.5**(R)**	4±0.3**(R)**	ND**(R)**	9±0.2**(R)**	ND**(R)**	21±0.5**(I)**	4±0.2**(R)**
Carbapenems											
Imipenem	10 µg	59±0.5**(S)**	54±0.2**(S)**	54±0.5**(S)**	54±0.1**(S)**	54±0.5**(S)**	39±0.4**(S)**	54±0.3**(S)**	59±0.4**(S)**	49±0.5**(S)**	34±0.5**(S)**
Aminoglycosides											
Gentamicin	10 µg	9±0.2**(R)**	14±0.3**(I)**	19±0.1**(S)**	4±0.5**(R)**	14±0.2**(I)**	14±0.5**(I)**	14±0.2**(I)**	14±0.2**(I)**	14±0.2**(I)**	14±0.5**(I)**
Amikacin	30 µg	19±0.4**(S)**	19±0.4**(S)**	20±0.2**(S)**	19±0.4**(S)**	19±0.4**(S)**	16±0.1**(I)**	19±0.5**(S)**	19±0.4**(S)**	14±0.4**(R)**	14±0.2**(R)**
Fluoroquinolones											
Norfloxacin	10 µg	25±0.3**(S)**	25±0.5**(S)**	24±0.2**(S)**	29±0.2**(S)**	24±0.5**(S)**	19±0.3**(S)**	22±0.2**(S)**	21±0.2**(S)**	19±0.1**(S)**	14±0.3**(I)**
Levofloxacin	5 µg	31±0.3**(S)**	29±0.4**(S)**	31±0.4**(S)**	34±0.1**(S)**	34±0.4**(S)**	24±0.3**(S)**	24±0.4**(S)**	29±0.4**(S)**	29±0.2**(S)**	24±0.5**(S)**
Nalidixic acid	30 µg	34±0.1**(S)**	34±0.2**(S)**	34±0.4**(S)**	34±0.3**(S)**	34±0.1**(S)**	24±0.5**(S)**	34±0.5**(S)**	34±0.1**(S)**	37±0.4**(S)**	9±0.1**(R)**
Tetracyclines											
Tetracycline	30 µg	21±0.2**(S)**	18±0.3**(I)**	21±0.1**(S)**	36±0.2**(S)**	36±0.5**(S)**	21±0.1**(S)**	33±0.3**(S)**	41±0.4**(S)**	33±0.2**(S)**	18±0.4**(I)**
Lincosamide											
Clindamycin	2 µg	24±0.5**(S)**	34±0.4**(S)**	ND**(R)**	19±0.5**(I)**	19±0.5**(I)**	9±0.2**(R)**	19±0.2**(I)**	14±0.5**(R)**	16±0.5**(I)**	9±0.2**(R)**
Miscellaneous											
Nitrofurantoin	300 µg	21±0.4**(S)**	6±0.5**(R)**	19±0.2**(S)**	24±0.3**(S)**	24±0.2**(S)**	16±0.4**(I)**	9±0.4**(R)**	19±0.3**(S)**	12±0.2**(R)**	19±0.3**(S)**
Monobactams											
Aztreonam	30 µg	ND**(R)**	ND**(R)**	ND**(R)**	ND**(R)**	ND**(R)**	ND**(R)**	ND**(R)**	ND**(R)**	ND**(R)**	ND**(R)**
Sulfonamides Folate pathway inhibitors											
Trimethoprim/sulfamethoxazole	1.25/23.75 µg	24±0.4**(S)**	29±0.5**(S)**	ND**(R)**	34±0.4**(S)**	19±0.2**(S)**	24±0.5**(S)**	29±0.1**(S)**	24±0.2**(S)**	29±0.5**(S)**	14±0.2**(I)**

(ND) Not detected, Means ± standard deviation

Table 5. Continued

Antibiotic	Disk content (µg)	Bacterial isolates							
		71T1i	56F2i	56F2II	56F4i	56F3i	41S1i	20H5i	20H4i
		Diameter of inhibition (mm).							
Penicillins									
Ampicillin	10 µg	ND**(R)**	ND**(R)**	ND**(R)**	ND**(R)**	14±0.2**(I)**	ND**(R)**	19±0.2 **(S)**	ND**(R)**
Ampicillin-sulbactam	10/10 µg	ND**(R)**	17±0.5**(S)**	17±0.4**(S)**	ND**(R)**	24±0.5**(S)**	24±0.1**(S)**	14±0.5**(I)**	ND**(R)**
Cephalosporins									
Cefadroxil	30 µg	24±0.3**(R)**	26±0.3**(S)**	24±0.4**(R)**	ND**(R)**	20±0.5**(R)**	24±0.3**(R)**	24±0.1**(R)**	19±0.5**(R)**
Cefaclor	30 µg	ND**(R)**	29±0.4**(R)**	39±0.3**(S)**	ND**(R)**	39±0.3**(S)**	34±0.1**(R)**	29±0.2**(R)**	34±0.2**(R)**
Cefoxitin	30 µg	ND**(R)**	11±0.5**(R)**	14±0.5**(R)**	ND**(R)**	14±0.3**(R)**	14±0.5**(R)**	ND**(R)**	ND**(R)**
Cefamandole	30 µg	ND**(R)**	14±0.1**(R)**	ND**(R)**	ND**(R)**	14±0.5**(R)**	14±0.2**(R)**	ND**(R)**	ND**(R)**
Ceftazidime	30 µg	ND**(R)**	4±0.3**(R)**	ND**(R)**	ND**(R)**	4±0.4**(R)**	ND**(R)**	ND**(R)**	ND**(R)**
Cefotaxime	30 µg	ND**(R)**	ND**(R)**	ND**(R)**	ND**(R)**	ND**(R)**	ND**(R)**	ND**(R)**	ND**(R)**
Ceftriaxone	30 µg	ND**(R)**	5±0.5**(R)**	27±0.2**(S)**	ND**(R)**	ND**(R)**	ND**(R)**	ND**(R)**	4±0.4**(R)**
Carbapenems									
Imipenem	10 µg	44±0.5**(S)**	54±0.4**(S)**	54±0.2**(S)**	24±0.1**(S)**	54±0.4**(S)**	39±0.5**(S)**	49±0.2**(S)**	ND**(R)**
Aminoglycosides									
Gentamicin	10 µg	4±0.1**(R)**	19±0.5**(S)**	14±0.4**(I)**	4±0.4**(R)**	9±0.5**(R)**	14±0.2**(I)**	9±0.2**(R)**	19±0.5**(S)**
Amikacin	30 µg	20±0.2**(S)**	25±0.5**(S)**	21±0.5**(S)**	10±0.2**(R)**	21±0.2**(S)**	19±0.5**(S)**	20±0.4**(S)**	19±0.3**(S)**
Fluoroquinolones									
Norfloxacin	10 µg	24±0.4**(S)**	26±0.2**(S)**	29±0.1**(S)**	10±0.2**(R)**	29±0.2**(S)**	25±0.5**(S)**	24±0.3**(S)**	19±0.2**(S)**
Levofloxacin	5 µg	29±0.3**(S)**	30±0.3**(S)**	39±0.3**(S)**	10±0.5**(R)**	31±0.1**(S)**	34±0.3**(S)**	24±0.2**(S)**	26±0.4**(S)**
Nalidixic acid	30 µg	24±0.1**(S)**	9±0.4**(R)**	34±0.2**(S)**	ND**(R)**	34±0.5**(S)**	24±0.1**(S)**	24±0.5**(S)**	19±0.3**(S)**
Tetracyclines									
Tetracycline	30 µg	21±0.4**(S)**	18±0.3**(I)**	31±0.5**(S)**	ND**(R)**	21±0.4**(S)**	17±0.2**(I)**	17±0.5**(I)**	23±0.4**(S)**
Lincosamide									
Clindamycin	2 µg	16±0.5**(I)**	14±0.4**(R)**	ND**(R)**	ND**(R)**	14±0.4**(R)**	ND**(R)**	ND**(R)**	14±0.2**(R)**
Miscellaneous									
Nitrofurantoin	300 µg	19±0.4**(S)**	21±0.2**(S)**	29±0.2**(S)**	ND**(R)**	19±0.1**(S)**	16±0.3**(I)**	19±0.2**(S)**	ND**(R)**
Monobactams									
Aztreonam	30 µg	ND**(R)**	ND**(R)**	ND**(R)**	9±0.2**(R)**	ND**(R)**	ND**(R)**	ND**(R)**	ND**(R)**
Sulfonamides Folate pathway inhibitors									
Trimethoprim/sulfamethoxazole	1.25/23.75 µg	ND**(R)**	34±0.4**(S)**	39±0.1**(S)**	ND**(R)**	39±0.5**(S)**	29±0.2**(S)**	ND**(R)**	34±0.5**(S)**

Identification of multi-drug resistant isolates

More resistant bacterial isolates to tested antibiotic (56F4i, 20H4i, 15H2i and 34H5i) were selected for identification tests. These isolates were phenotypically and genetically identified as *Pseudomonas aeruginosa, Stenotrophomonas maltophilia, Staphylococcus* sp. and *Bacillus cereus* respectively.

Antibacterial activity of *spirulina* extracts

The different solvents of two *Spirulina platensis* strains extracts (S1 and S2) showed varying degrees of antibacterial activity against all tested pathogenic bacteria. On a general note, methanolic extracts exhibited higher degree of inhibitory activity than other used solvents. The antibacterial activity of *S. platensis* was determined against two pathogenic bacteria and the findings were recorded in **Table 6**. Inhibition zone of *S. platensis* extracts against bacteria was ranged between 2 ±0.5 mm to 23±0.4 mm at 0.5 mg/mL. When the concentration of the extract increased more than 0.5 mg/mL an increased inhibitory activity was observed. The methanol and hexane extracts of *S. platensis* (S2) were highly active against gram-positive and gram-negative bacteria with different ranges of inhibition zones.

Table 6. Antibacterial activity of *S. platensis* in organic solvent extracts against pathogenic bacteria.

Strains of *Spirulina platensis*	**Tested extracts.**	**Bacterial strains**	
		***Staphylococcus* sp.** MZ314089	***Stenotrophomonas maltophilia*** MZ314086
		Diameter of effective zone of inhibition (mm)	
Strain 1 **(S1)**	Control negative (DMSO)	ND	ND
	Control positive (Tetracycline)	18±0.4	23±0.4
	Methanol	11±0.2	ND
	Ethanol	ND	ND
	Chloroform	ND	ND
	Diethyl ether	ND	ND
	Ethyl acetate	ND	ND
	Acetone	ND	ND
	Hexane	ND	ND
	Aqueous	ND	ND
Strain 2 **(S2)**	Control negative (DMSO)	ND	ND
	Control positive (Tetracycline)	18±0.4	23±0.4
	Methanol	10±0.5	11±0.3
	Ethanol	ND	2 ±0.5
	Chloroform	ND	ND
	Diethyl ether	ND	ND
	Ethyl acetate	ND	ND
	Acetone	ND	ND
	Hexane	6±0.5	ND
	Aqueous	ND	ND

(ND) Not detected, Means ± standard deviation

The methanol crude extract of *Spirulina platensis* (S2) showed the highest inhibition zone (11±0.3 mm) against *Stenotrophomonas maltophilia* followed by (mm), *Staphylococcus* sp. (10±0.5 mm).

However, the minimum inhibition zone obtained from chloroform, diethyl ether, ethyl acetate, acetone and aqueous extract of *Spirulina platensis* against bacterial pathogens when compared to other solvent extracts. No inhibition zone was seen in DMSO blind control, and the positive control Tetracycline (30 µg) showed zone of inhibition was ranging from 18±0.4 mm to 23±0.4 mm against the tested bacterial pathogens.

In case of hexane extract against *S. maltophilia* and *Staphylococcus* sp. showed complete resistance against hexane extract of *S. platensis.* Hexane extract was lower effective against isolated pathogenic bacteria than methanolic extract.

The stated results indicated that the most promising extract against all tested pathogenic bacteria was extract of strain 2 of *S. platensis*. Therefore, it was selected for further study.

These results were in accordance with data obtained by several workers **(Martelli *et al.*, 2020 and Kavisri *et al.*, 2021)** who found that methanol seemed to be the best solvent for extracting the bioactive compounds. Also, the obtained data was correlated with the findings of **Usharani *et al*, (2015) and Elshouny *et al*, (2017)** who found that the methanol extract of *Spirulina platensis* showed maximum inhibition zone against all pathogenic bacterial and fungal isolates. On the other hand, the hexane extract of *Spirulina platensis* showed minimum inhibition zone against bacterial and fungal pathogens when compared to other solvent extracts.

Generally, the methanolic extracts showed the highest antimicrobial activity followed by ethyl acetate and then hexane extracts **(Gheda and Ismail, 2020).** However, variation in inhibition zones using methanol, ethyl acetate and hexane might be ascribed to the difference in the active metabolite's composition dissolved in these extracts **(Rajishamol *et al.*, 2016).**

The obtained results showed that the antimicrobial effect against all tested pathogenic bacteria and fungi was varied among the different extracts. The strong antimicrobial activity of methanolic extract may be attributed to its high total phenolic content. It has been reported that pathogens colonize humans and animals' gut with the same mechanism of adhesion and invasion. The antimicrobial activity of *Spirulina* might be attributed to its potential to disrupt attachment and invasion, motility, biofilm formation and quorum sensing of pathogens **(Abou-Kassem *et al.*, 2021 and Saleh *et al.*, 2021).** The bioactive compounds in *Spirulina* can impair bacterial cell integrity and increase cell permeability, which leads to cytoplasmic content leakage.

Minimum inhibitory concentration

The minimum inhibitory concentration (MIC) values of *Spirulina platensis* methanol and hexane extract and their mixture against tested pathogenic bacteria was ranged between 1 mg/mL to 20 mg/mL as showed in **Table 7.** The lowest MIC (2 mg/mL) value of methanol extract was recorded against *Stenotrophomonas maltophilia*. However, the lowest MIC (15 mg/mL) value of hexane extract was recorded against *Stenotrophomonas maltophilia* and *Staphylococcus* sp. Data revealed that the methanolic extract was more effective than hexane extract.

Table 7: The Minimum inhibitory concentration (MIC) values of *Spirulina platensis* methanol and hexane extract and their mixture.

Bacterial strains	Minimum inhibitory concentration (MIC) (mg/mL)		
	Methanol extract	Hexane extract	Methanol +Hexane extract
	Concentration (mg/mL) *Spirulina* extracts		
***Staphylococcus* sp.**	15	20	2.0
Stenotrophomonas maltophilia	5.0	20	1.0

The combination of methanol and hexane extract (1:1 v/v) exhibited a clear synergistic effect against tested pathogens, where the low MIC was recorded at (1 mg/mL) value. Regarding the MIC methanolic extract **Usharani *et al*, (2015)** found that the (MIC) value of *Spirulina platensis* against tested pathogenic bacteria was ranged between 1.25 mg/mL to 80 mg/mL. The lowest MIC (1.25 mg/mL) value of methanol crude extract was recorded against *Staphylococcus aureus, Streptococcus pyogenes, Streptococcus epidermidis, Proteus mirabilis, Bacillus cereus, Klebsiella pneumoniae* and *Shigella flexneri*. Moreover, **Abdel-Moneim *et al*, (2022)** reported that results of MIC confirmed the obtained results in the current study, where *Spirulina* methanolic extract exhibited the lower MIC (1–2 mg/mL) against tested pathogenic bacteria compared to other extracts.

Conclusion and recommendation

This study indicated that all collected cosmetic samples were contaminated with varying degree. Of the 87 samples, 26.4 % were contaminated with bacterial pathogens. The ability of pathogenic isolates to produce protease, rhamnolipid and blood hemolysis were estimated, and the results were clear that diverse pattern of virulence markers were observed in different isolates. Only 18 (32.14%) bacterial isolates were found to possess the three virulence factors. Four isolates were selected for identification which were considered more resistant against most tested antibiotics. The effect of various extracts of *Spirulina platensis* using different solvents (methanol, ethanol, chloroform, ethyl acetate, acetone, diethyl ether and hexane) are antimicrobial agents against two bacterial pathogens: *Staphylococcus* sp. and *S. maltophilia*. Results indicate that among the various used extracts, methanol and hexane extracts of *Spirulina platensis* appeared to be the most effective ones, since they are showing maximum antibacterial activity against the selected bacterial pathogens.

From the obtained results, it could be concluded and recommended that the methanol, hexane and their combination extract of cyanobacterium, *S. platensis*, contains potential bioactive compound with an effective antibacterial activity. This compound can be utilized for the development of natural agent against multi drug resistant bacteria that found in cosmetic products.

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