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Safe Application and Preservation Efficacy of Low-toxic Rhamnolipids Produced from *Ps. aeruginosa* for Cosmetics and Personal Care Formulation

Atef M. Diab, Sami I.A. Ibrahim#, Hesham M. Abdulla
Botany Department, Faculty of Science, Suez Canal University, Ismailia, Egypt.

THE INTEREST in natural antimicrobial preservatives is rapidly grown due to their exceptional properties such as biodegradability, biocompatibility, and lower toxicity. On the contrary, common synthesized preservatives are more toxic, partially or non-degradable, and have undesirable side effects on human health and the environment. Furthermore, the resistance of microorganisms to current antimicrobial preservatives is increasing. In the present study, rhamnolipid was produced from *Pseudomonas aeruginosa* ATCC 9027, and twelve congeners of rhamnolipids were elucidated by ESI-MS. Additionally, rhamnolipids reduced the surface tension of water to 26mN/m with a critical micelle concentration value of 22mg/L. The produced rhamnolipids showed prominent lower toxicity against the brine shrimp *Artemia salina* than those of positive control and methylisothiazolinone. A significantant antimicrobial effect of rhamnolipids was achieved *in vitro*, whereas the disc diffusion assay showed noticeable inhibition zones around the paper discs saturated with different concentrations of the rhamnolipid produced in this study, with MICs ranged between 8 and 336µg/ml against *Escherichia coli*, *Salmonella typhimurium*, *Pseudomonas aeruginosa, Staphylococcus aureus*, *Bacillus subtilis, Saccharomyces cerevisiae*, *Candida albicans* and *Aspergillus brasiliensis*. Moreover, a promising preservation efficacy of the produced rhamnolipid was obtained when applied to a personal care formulation model even after 28 days of incubation, with reduction values of about 3 and 1 expressed in log units for bacteria and fungi, respectively. However, rhamnolipids could be recommended as a powerful nontoxic bio-preservative for personal care formulations.

Keywords: Bio-preservative, Personal care formulation, *Pseudomonas aeruginosa,* Rhamnolipids, Toxicity.

Introduction

Cosmetics and personal care products are considered suitable media for microbial proliferation due to, availability of microbial growth requirements in most formulations (Halla et al., 2018). These products just like foods could be contaminated, leading to product spoilage and could cause irritation or infections for human skin and eye area resulted in a health risk to the consumer (Orús et al., 2015). Also, Physico-chemical properties change for products that will be occurred such as discoloration, viscosity changes, and bad smell. Consequently, preservatives are necessary active components in cosmetics and personal care formulations to control microbial proliferation and assure the hygienic quality of products (Orús et al., 2015). Recently, the interest in natural antimicrobial preservatives is rapidly grown and driven by the fact that national and international regulatory foundations are very strict about the toxicological effect of synthetics additives (Halla et al., 2018). The cosmetic and personal care industries suffer from a considerable lack of less-toxic preservatives (Halla et al., 2018). Moreover,

#Corresponding author emails: sami.ibrahim@Arma.com.eg; sami.ibrahim2010@yahoo.com Mobile: +20 1002564889
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the resistance of pathogenic microorganisms to the current preservatives highly increased (Orús et al., 2015; Diab et al., 2002). Accordingly, seeking for safe, lower toxic, biodegradable and biocompatible active preservatives is considered an important target.

At present, there are 56 different synthetic preservatives listed in European Regulation (EU No 1223/2009) which permitted to add to cosmetic formulations. Despite the regulation allowed a lot of preservatives, only a few numbers of preservatives are dominant in the commercial market such as parabens, formaldehyde donor's compounds, phenolic compounds, and isothiazolinones. Among these preservatives, the isothiazolinone group widely used in personal care and household detergent formulations, namely methylisothiazolinone (MI) and Chloromethylisothiazolinone (CMIT). These compounds are heterocyclic derivatives chemicals of isothiazolinones (Garcia-Hidalgo et al., 2017). Despite their powerful antimicrobial effect on different types of microorganisms, the isothiazolinones have undesirable side effects, in particular, they have high aquatic toxicity and its derivatives MI and CMIT causing allergy (Basketter et al., 2003; Van Huizen et al., 2017). However, synthetic preservatives and fragrances are the most common causative agents for allergens in cosmetic and personal care products (Paye et al., 2009).

Rhamnolipids are surface-active biomolecules synthesized mainly by *Pseudomonas aeruginosa* (Mawgoud et al., 2010). Moreover, rhamnolipid biosurfactants have potential applications in various industrial fields (Banat et al., 2010; Mnif & Ghribi, 2015; Irorere et al., 2017; Jiang et al., 2020). Beside their definite antimicrobial activities, the interest in rhamnolipids has steadily increased due to their special advantages over their synthetic chemical counterparts such as lower toxicity, biodegradability, friendly for ecosystems, and high stability under extreme environmental conditions (Banat et al., 2010; Ozdal et al., 2017). Despite the potential applications of biosurfactants mentioned in a lot of literature (Banat et al., 2010; Shekhar et al., 2015), their evaluations as active components in different applications still not sufficient to stand out their characteristics in comparison with corresponding synthetic chemicals. Accordingly, biosurfactants still face challenges to be used as alternate for chemically synthetic ones worldwide. However, the application of biosurfactants as antimicrobials represents a promising area of evolution, in particular, after a global call from world health organization concerning seeking for alternates for current antimicrobials due to antimicrobial resistance (Naughton et al., 2019; Diab et al., 2000, 2008).

This study aimed to produce rhamnolipids from *Ps. aeruginosa* ATCC 9027. To evaluate the effectiveness of rhamnolipids as an antimicrobial biocide *in vitro*. Furthermore, to examine the preservation efficacy of rhamnolipids in a personal care formulation model in comparison with a commonly synthetic counterpart of MI.

Materials and Methods

Microorganisms

Microorganisms that were used in this study are, *Pseudomonas aeruginosa* (*Ps. aeruginosa*) ATCC 9027, *Escherichia coli* (*E. coli*) ATCC 25922, *Salmonella typhimurium* (*Sal. typhimurium*) ATCC 14028, *Pseudomonas aeruginosa* (*Ps. aeruginosa*) ATCC 27853, *Staphylococcus aureus* (*St. aureus*) ATCC 25923, *Bacillus subtilis* (*B. subtilis*) ATCC 6633, *Candida albicans* (*C. albicans*) ATCC 10231, *Aspergillus brasiliensis* (*A. brasiliensis*) ATCC 16404 (Obtained from BD Diagnostics, Germany) and *Saccharomyces cerevisiae* (*Sacch. cerevisiae*) NCPF 3178 (Obtained from Liofilchem s.r.l., Italy).

Culture medium and growth conditions for rhamnolipids production

Mineral Salt Medium (MSM) was used as a culture medium for the production of rhamnolipids by *Ps. aeruginosa* (ATCC 9027). At first, MSM broth containing (g/L): Magnesium sulfate 0.2, potassium nitrate 1.0, monopotassium phosphate 1.0, diammonium hydrogen phosphate 1.0, calcium chloride 0.02 and ferric chloride 0.05, in addition 3% glycerol was added as a carbon source. Then, fresh culture (1%) of the corresponding bacterial suspension (1X 10^8 cfu/ml) was inoculated to a 250ml Erlenmeyer flask containing MSM and incubated in a shaker incubator at 200rpm and 35^{o}C for 96hrs.

Extraction of rhamnolipids

Broth culture was centrifuged (at 10,000x g for 15min) to remove cells. Subsequently, the supernatant was separated and acidified to pH 2.0

with 1M HCl for precipitation of rhamnolipids and kept standing in a refrigeratorat 4°C till 24hrs for further precipitation. The obtained precipitate was collected at 10,000x g for 15min, the precipitate was dissolved in 0.05M sodium bicarbonate, re-acidified (pH 3.0), and re-centrifuged at 12,000x g for 20min. Then, the precipitate was extracted with chloroform/ methanol (2:1, v/v) mixture. Afterwards, the extract was collected under vacuum using a rotary evaporator till a yellowish oily residue was obtained at 40°C (De Rienzo et al., 2016a).

Surface tension and critical micelle concentration (CMC) measurements

The surface tension was measured in a 10ml cell-free supernatant using a ring tensiometer (krüss tensiometer – K6) using the ring method at 25°C. The CMC of rhamnolipid was determined using surface tension method, whereas the CMC was obtained by measuring the surface tension at different concentrations of rhamnolipid in distilled water until a constant value was achieved (Gudiña et al., 2015).

Emulsification index determination

It was carried out by adding 5ml of vegetable oil to a tube containing 5ml cell-free supernatant then the tube was shaken on a vortex for 5min to confirm the mixing of both liquids. The emulsification activity was noticed after 24hrs and calculated as the emulsification index (E24, %) using formula (1) (Zhao et al., 2018):

$$E24= \frac{\text{(Volume of the emulsion layer)}}{\text{(Total liquid volume)}} \times 100 \quad (1)$$

Rhamnolipids characterization

Fourier transform infrared spectroscopy (FTIR)

The extracted rhamnolipid was firstly subjected to the FTIR instrument for chemical characterization, where the spectra of the dried yield were recorded on Shimadzu FTIR-4200 spectrometer using 100mg KBr disc as reference. The sample was analyzed by FTIR in the wavenumber range of 4000–500cm^{-1}, and then its IR spectrum was recorded.

Electrospray ionization mass spectra (ESI-MS)

The produced rhamnolipid was subjected for further characterization using ESI-MS positive ion acquisition mode (XEVO TQD triple quadruple instrument, Waters Corporation, Milford, MA01757 U.S.A). The mass spectrometer, Column: ACQUITY UPLC-BEH C18 1.7µm - 2.1× 50mm Column, flow rate: 0.2ml/min, the solvent system consisted of eluent A (water acidified with 0.1% formic acid) and eluent B (methanol acidified with 0.1% formic acid). Mass spectra were detected in the ESI positive ion mode between m/z 100–1000 (source temperature 150°C, cone voltage 30V, capillary voltage 3kV, desolvation temperature 440°C, cone gas flow 50L/hr and desolvation gas flow 900L/hr). The spectra, data acquisition, and treatment of results were processedusing the Masslynx™ 4.1 software.

Temperature stability of the produced rhamnolipids

To evaluate the influence of temperature on the stability of the produced rhamnolipids, rhamnolipids solutions (1%) were treated under different temperatures (5, 25, 45, 70, 100, and 121°C). Surface tension and emulsification index were measured at 35°C to evaluate the stability of the produced rhamnolipids (Zhao et al., 2020).

Toxicity testing for rhamnolipids

For determining the toxicity effect of rhamnolipids, initially, the brine shrimp *Artemia salina* dried cysts (Brine shrimp eggs obtained from Marine Science Department, Faculty of Science, Suez Canal University, Ismailia, Egypt)were placed in a bottle containing natural seawater for hatching larvae and incubated at room temperature (25-30°C) under conditions of strong aeration and continuous illuminations till 36-48hrs. Serial concentrations of rhamnolipids (from 0.01 to 5000ppm) were papered in sterile tubes containing 1ml of seawater. After that, 10 hatched larvae were added to each tube, and a tube containing 10 larvae and seawater only was left as control, all tubes were incubated at room temperature for 24hrs. In consequence, the numbers of surviving larvae in each tube were counted, and the lethality percentages were calculated using formula (2) to obtain the Lethal Concentration 50 (LC_{50}) (Rajabi et al., 2015). Likewise, the same steps were carried out for evaluating the toxicity of MI (95%, obtained from Sigma Aldrich) against *A. salina*. The experiments were conducted in triplicate for each tested material.

$$\text{Lethality \%} = (\text{Test-Control})/(\text{Control}) \times 100 \quad (2)$$

Antimicrobial activity of rhamnolipids

Disc diffusion assay: The antimicrobial

activity of rhamnolipids was carried out by disc diffusion method. Inoculum of tested strains was grown on tryptic soy broth (Oxoid Ltd, UK), incubated at 35-37°C for 24hrs, the culture was adjusted with sterile saline (NaCl 0.85%) to give turbidity equivalent to the McFarland 0.5 standard (Remel INC, USA) to have a concentration very near to (1 to 2X 10^8 cfu/ml). Subsequently, microorganisms were streaked over the entire Muller–Hinton agar surface's (Oxoid Ltd, UK), and then, 6mm sterile discs (Biolife Italiana s.r.l) were applied onto the agar plate's surfaces, these discs were saturated with 25, 50 and 100µg of rhamnolipids after evaporation of the loading solvent. Eventually, plates were inverted and incubated at 35±2 °C till 18 ±2hrs (Zhao et al., 2020). The antimicrobial activity of rhamnolipids against *Sacch. cerevisiae* and *C. albicans* were tested using yeast peptone dextrose agar culture medium (Difco Laboratories, Sparks, Md), plates were incubated for 72hrs at 28°C (Christova et al., 2011). After the incubation period, the clear diameter zones around discs were observed, measured in millimeters from edge to edge and compared to blank discs.

Minimum inhibitory concentrations (MICs): MICs of rhamnolipids were determined by the Macro-broth dilution method using Muller–Hinton broth (Oxoid Ltd, UK) based on Clinical and Laboratory Standards Institute (CLSI), the international standard for antimicrobial susceptibility guidelines. Stock dilutions of rhamnolipids were prepared (from 0.5 to 512µg/ml). Subsequently, in each dilution tube, a volume from standardized inoculum equivalent to the McFarland 0.5 standard was added to an equal volume of the diluted rhamnolipid, bringing the microbial concentration to approximately 500,000 cells/ml. Afterwards, incubated at 35±2ºC for 18±2hrs (Elshikh et al., 2017). Whilst, the MIC of rhamnolipids against yeast and molds was determined on potato dextrose broth (Difco Laboratories, Sparks, Md.) and incubated for 2-4days at 28°C (Kim et al., 2000). Eventually, microbial growth was observed, indicated by turbidity with no growth in un-inoculated negative control tube and the MIC value is the corresponding lowest concentration of rhamnolipid that completely inhibits the growth of microorganisms in the tube.

Time-kill method (bactericidal and fungicidal activities of rhamnolipids): The bactericidal and fungicidal activities of rhamnolipids were determined by a time-kill method according to the American Society for Testing and Materials (ASTM**)** standard method E2315-16 (2016). At first, overnight broth cultures of microorganisms grown on nutrient broth (Oxoid Ltd, UK) at 37°C were standardized to reach approximately 1 to 3x 10^6 cfu/ml for bacteria and 1 to 5x 10^5 cfu/ml for fungi. Afterwards, 100µl from overnight standardized inoculum suspension was added to a sterile tube containing 1ml of 1% rhamnolipids solution, and then a portion was removed after 0, 1, 2, 3, 4 and 5hrs, respectively by taken 100µl from the suspension to 9ml tube containing Dey/Engley (D/E) neutralizing broth (Liofilchem s.r.l., Italy). Subsequently, microbial enumeration was carried out by pour plate technique using Tryptic Soy Agar (TSA, Oxoid Ltd, UK) for bacteria and Sabouraud Dextrose Agar (SDA, Difco Laboratories, Sparks, Md) for fungi. The above steps were repeated for each tested microorganism. Finally, plates were inverted and incubated at 37ºC till 48hrs for bacteria and 28ºC till 120hrs for fungi. After the incubation period, all plates were counted and expressed as a number of colonies per plate, multiply by the dilution factor corresponding to the contact time of microorganisms that were exposed to rhamnolipids. Accordingly, the percentage and log reductions were calculated using formula (3, 4):

$$\text{Percent reduction} = ((B-A)/B) \times 100 \quad (3)$$

$$\text{Log reduction} = \text{Log}(B/A) \quad (4)$$

Where;
B = Number of viable test microorganisms in the control substance immediately after inoculation.
A = Number of viable test microorganisms in the test substance after the contact time.

Determination the preservation efficacy of rhamnolipids

The preservation efficacy of rhamnolipids was evaluated according to the international standard ISO 11930 (2019), whereas personal care formulations containing rhamnolipids and MI were prepared as below sets:

A. Shower gel personal care formulation sample containing 0.2% of rhamnolipids.
B. Shower gel personal care formulation sample containing 0.2% of MI.

C. Shower gel personal care formulation sample containing 0.1% of rhamnolipids + 0.1% of MI.

Concurrently, from each sample, 20g was dispensed into a sterile container. Then, inoculated with 0.2ml from reference type strains to obtain between $1X10^5$ cfu/g to $1X\ 10^6$ cfu/g for bacteria (*E. coli, Sal. typhimurium, St. aureus* and *B. subtilis*), and between $1X\ 10^4$ cfu/g to $1X\ 10^5$ cfu/g for *C. albicans* and *A. brasiliensis* in the formulation final concentration. At the same time, the initial count of microorganisms (N_0) present in the inoculated samples at a time (t_0) was calculated using the results of the enumeration of the calibrated inoculum (N) by the formula; $N_0 = N/100$. Subsequently, inoculated samples were stored at 25°C. After each specified interval time 7 days (T7), 14 days (T14), and 28 days (T28), 1g from each inoculated formulation sample was transferred to 9ml tube containing D/E neutralizing broth, and tubes were left at room temperature till 30min. To carry out microbial enumeration, pour plate technique was used, whereas a serial dilution was made by tryptone sodium chloride solution for bacteria and *C. albicans*, and polysorbate 80 diluent for *A. brasiliensis* using culture media of TSA for bacteria, SDA for *C. albicans* and potato dextrose agar (Oxoid Ltd, UK) for *A. brasiliensis*. Eventually, plates were incubated at 32.5°C for 48-72hrs for bacteria and *C. albicans* and at 25°C for 3 days to 5 days for *A. brasiliensis*. After incubation time, the number of colonies was counted, and the number of surviving microorganisms (Nx) in the inoculated formulation samples was calculated in cfu/g at each sampling interval time. Subsequently, the reduction values (Rx) was calculated using formula (5) expressed in log units:

$$Rx = \log N_0 - \log N_x \quad (5)$$

Where;
N_0: Is the initial number of microorganisms inoculated at time t_0
N_x: Is the number of surviving microorganisms at each sampling time t_x

Results

Production and characterization of rhamnolipids

Ps. aeruginosa ATCC 9027 was able to produce rhamnolipid after 96hrs at 35°C using MSM supplemented with 3% of glycerol as a carbon source. However, rhamnolipid was extracted from MSM broth culture by acid precipitation and solvent extraction, and the surface activity of rhamnolipid was determined. Rhamnolipid mixture reduced the surface tension of water to 26mN/m with a CMC value of 22mg/L. Also, a percentage of a stable emulsion of about 63% was obtained when the cell-free broth was mixed with vegetable oil.

The FTIR spectra for the produced rhamnolipids was estimated and illustrated in Fig. 1, which shows the strong symmetric stretching C=O band of a carboxylate group of a broad peak at 3393cm^{-1}, characteristic of O–H stretching vibrations. Absorption around 2928cm^{-1} is assigned to the asymmetric C-H stretch of CH_2 and CH_3 groups. The corresponding symmetric stretch is recorded at 2855cm^{-1}. The deformation C–OH band at 1402cm^{-1}, the O–H in-plane deformation at 1317cm^{-1}, the O–C–O symmetric band at 1064cm^{-1}, the C–O is stretching at 1172, 1128 and 1064cm^{-1}, C–H deformations at 1462, 1238 and 810cm^{-1} and CH_3 rocking at 983cm^{-1}.

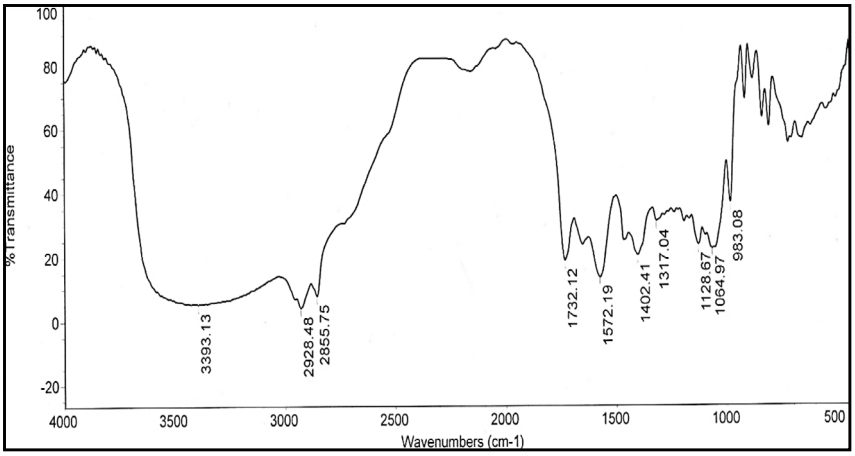

Fig. 1. The infrared spectra for the produced rhamnolipids from *Ps. aeruginosa* ATCC 9027.

The congeners of the rhamnolipid mixture produced by *Ps. aeruginosa* ATCC 9027 were presented in Table 1, where twelve rhamnolipid congeners were detected and confirmed in the presence of $[M+Na]^+$ ions. The congeners were Rha-Rha-C_8 (m/z 329.2), Rha-C_{10} (m/z 357.2), Rha-C_{12} (m/z 385.2), Rha-$C_{8:2}$(m/z 325.3), Rha-Rha-C_8-C_{10} or Rha-Rha-C_{10}-C_8 (m/z 645.3), Rha-C_8-C_{10} or Rha-C_8-C_{10} (m/z 499.3), Rha-C_{10}-C_{10}:1/Rha-$C_{10:1}$-C_{10}(m/z 525.3), Rha-C_{10}-C_{10} or Rha-C_8-C_{12}/Rha-C_{12}-C_8(m/z 527.3), Rha-C_{10}-C_{10}-CH_3(m/z 541.3), Rha-C_{10}-$C_{12:1}$ or Rha-$C_{12:1}$-C_{10}(m/z 553.3), Rha-Rha-C_{10}-C_{12} or Rha-Rha-C_{12}-C_{10}(m/z 701.4) and Rha-C_{10}-C_{12} or Rha-C_{12}-C_{10} (m/z 555.4), these congeners represented ten mono-rhamnolipids and two di-rhamnolipids. However, the major mono-rhamnolipid detected were Rha-C_{10} with a relative abundance of about 17.12%and Rha-C_{10}-C_{10} or Rha-C_8-C_{12}/Rha-C_{12}-C_8 with relative abundance about 23.79% (Fig. 2). On the other hand, the two di-rhamnolipids congeners were Rha-Rha-C_8-C_{10} or Rha-Rha-C_{10}-C_8 and Rha-Rha-C_{10}-C_{12} or Rha-Rha-C_{12}-C_{10} with a relative abundance of about 3% for each congener (Fig. 3).

Temperature stability of rhamnolipids

Results showed that the produced rhamnolipids were stable at different temperatures from 5 to 121°C (Table 2) during incubation for 60min. Despite there is a little reduction in E24 percentages at a temperature higher than 70°C, rhamnolipids still reduced the surface tension of water lower than 28mN/m. However, the temperature influence on surface activity and emulsifying property of rhamnolipids were quite stable at different treatment temperatures.

Toxicity evaluation of rhamnolipids against A. salina

Cytotoxicity of rhamnolipids was evaluated against the brine shrimp *A. salina* and determined from the cell viability after 24hrs of exposure, as lethality percentages. Screening different concentrations of rhamnolipids proved a weak toxicity effect on *A. salina*, where the LC_{50} was obtained at 100ppm. In contrast, MI showed a strong toxicity effect on *A. salina,* where the LC_{50} was obtained at 0.35ppm. Furthermore, when *A. salina* was exposed to a little concentration of rhamnolipids (5%), the LC_{50} was obtained at 4000ppm, whilst the LC_{50} was obtained at 16ppm when *A. salina* was exposed to 5% of MI.

The antimicrobial activity of rhamnolipids

At first, the antimicrobial effect of rhamnolipids was examined against *Sal. typhimurium, Ps. aeruginosa, E. coli, St. aureus, B. subtilis, C. albicans,* and *Sacch. cerevisiae* by disc diffusion method. Results obtained showed varied sensitivities to rhamnolipids concentrations, where the strongest antimicrobial effect of rhamnolipids was reported on Gram-positive bacteria, while a moderate effect was noticed on both Gram-negative bacteria and yeasts (Table 3). Moreover, from the obtained MIC values, the strongest antimicrobial effect of rhamnolipids was noticed against Gram-positive bacteria with values of 8 and 16µg/ml for *St.aureus* and *B. subtilis*, respectively. On the other hand, the MIC values for Gram-negative strains were found 80µg/ml for *E.coli*, 80µg/ml for *Sal.typhimurium* and 96µg/ml for *Ps. aeruginosa*. Whilst, the highest MIC values were reported for yeasts and mold, whereas the MIC values were found 288µg/ml for both *C. albicans* and *Sacch. cerevisiae* and 336µg/ml for *A. brasiliensis* (Table 3).

Subsequently, the antimicrobial activity of rhamnolipids against bacteria and fungi was evaluated by the time-kill method.The maximum reduction rates were noticed for both Gram-positive bacterial strains *St. aureus* (99.6%) and *B. subtilis* (99.7%) within 5hrs of exposure (Fig. 4a). Whilst, the minimum reduction rates were reported for fungal strains *A. brasiliensis* (86.3%) and *C. albicans* (85.4%) after 5hrs of exposure. Furthermore, moderate reduction percentages against Gram-negative bacteria were observed, where the reduction rates were found at 96.5%, 94.5% and 95.7% for *E. coli, Ps. aeruginosa,* and *Sal. typhimurium*, respectively, after 5hrs of exposure.In a like manner, the log reduction values for tested bacterial and fungal strains were observed (Fig. 4b) whereas the maximum log reduction expressed in log units was noticed for *St. aureus* (2.44) and *B. subtilis* (2.5). However, the log reduction values were reached to more than one log cycle for *E. coli* (1.45)*, Ps. aeruginosa* (1.26) and *Sal. typhimurium* (1.37). In contrast, the lower log reduction values were noticed for fungal strains, whereas the log reduction values of 0.86 and 0.84 were reported for *C. albicans* and *A. brasiliensis*, respectively.

TABLE 1. Major rhamnolipid congeners identified by ESI–MS spectra in positive ion mode $[M+Na]^+$ produced by *Ps. aeruginosa* ATCC 9027.

Rhamnolipid congeners	Retention time (min)	Relative abundance (%)	m/z $[M+Na]^+$
Rha-C_8	8.18	1.18	329.2
Rha-C_{10}	9.64	17.12	357.2
Rha-C_{12}	10.93	0.53	385.2
Rha-$C_{8:2}$	11.92	1.65	325.3
Rha-Rha-C_8-C_{10} or Rha-Rha-C_{10}-C_8	12.21	3.04	645.3
Rha-C_8-C_{10} or Rha-C_8-C_{10}	12.32	8.98	499.3
Rha-C_{10}-C_{10}:1/Rha-$C_{10:1}$-C_{10}	12.77	1.38	525.3
Rha-C_{10}-C_{10} or Rha-C_8-C_{12}/Rha-C_{12}-C_8	13.22	23.79	527.3
Rha-C_{10}-C_{10}-CH_3	13.51	5.09	541.3
Rha-C_{10}-$C_{12:1}$ or Rha-$C_{12:1}$-C_{10}	13.74	5.09	553.3
Rha-Rha-C_{10}-C_{12} or Rha-Rha-C_{12}-C_{10}	13.95	3.25	701.4
Rha-C_{10}-C_{12} or Rha-C_{12}-C_{10}	14.12	5.64	555.4

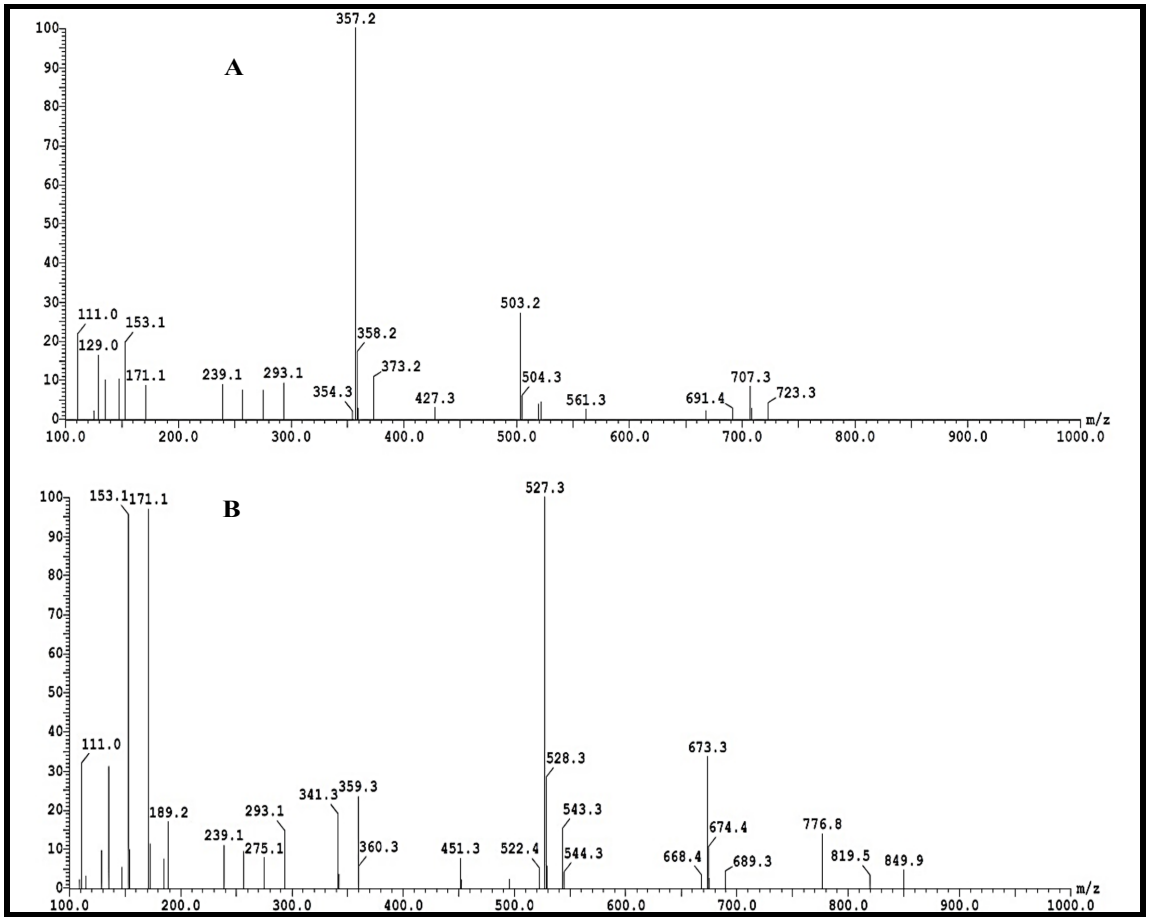

Fig. 2. ESI-MS/MS spectra in positive ion mode of most abundant mono-rhamnolipids congeners produced by *Ps. aeruginosa* ATCC 9027; (A) Mono-rhamnolipid (Rha-C_{10}) (m/z 357.2), (B) Mono -rhamnolipid (Rha-C_{10}-C_{10} or Rha-C_8-C_{12}/Rha-C_{12}-C_8) (m/z 527.3).

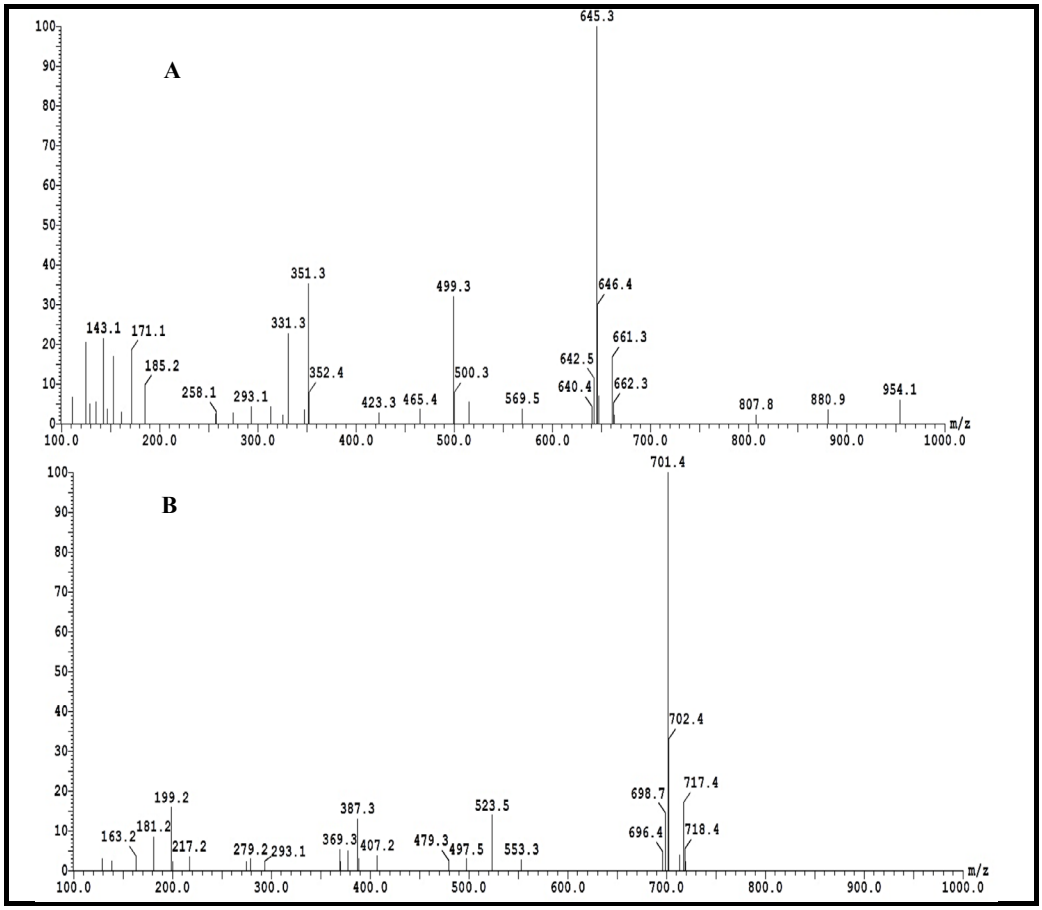

Fig.3. ESI-MS/MS spectra in positive ion mode of di-rhamnolipids congeners produced by *Ps. aeruginosa* ATCC 9027; (A) Di-rhamnolipid (Rha-Rha-C_8-C_{10}) (m/z 645.3), (B) Di-rhamnolipid (Rha-Rha-C_{10}-C_{12}) (m/z 701.4).

TABLE 2. Temperature influence on surface activity and emulsifying activity (E24%) of rhamnolipids.

Treatment temperature (°C)	Surface tension reduction (mN/m)	E24 (%)
5	25.5	60.7
25	26.0	63.0
45	26.0	63.4
70	27.0	62.6
100	27.5	59.8
121	28.0	58.6

TABLE 3. MIC values and sensitivity diameters of inhibition zones corresponding to concentrations of rhamnolipids against tested microbial strains

Microorganisms	MIC values (µg/ml)	Diameters of inhibition zones in mm		
		20µg	50µg	100µg
E. coli	80	11	14	19
Sal. typhimurium	80	12	14	21
Ps.aeruginosa	96	10	13	18
St. aureus	8	21	28	34
B. subtilis	16	19	27	33
C. albicans	288	9	11	16
Sacch.cerevisiae	288	8	11	15
A.brasiliensis	336	-	-	-

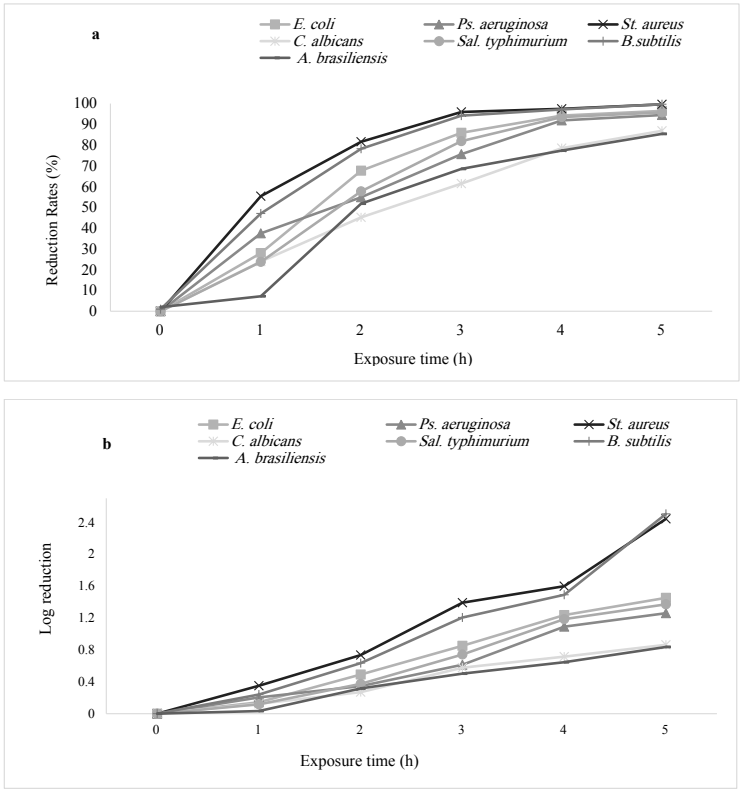

Fig. 4.The antimicrobial effect of 1% rhamnolipids on microbial growth after exposure to 5hrs; (A) In the form of reduction percent, (B) In the form of log reduction rates.

The preservation efficacy of rhamnolipids

The preservation efficacy of rhamnolipids was evaluated in a personal care formulation model. According to Table 4, the log reduction values were found more than 3 log units for bacterial strains (*E. coli, S. typhimurium, St. aureus* and *B. subtilis*) and almost more than 1 log unit for fungal strains (*C. albicans* and *A. brasiliensis*) at all examined time. However, the maximum log reduction values of rhamnolipids were reported against *St. aureus* and *B. subtilis*, where 3.84 and 3.69 log reduction units were obtained, respectively after 28 days of exposure. Furthermore, the obtained preservation log reduction values of rhamnolipids were almost similar to preservation log reduction values obtained by the synthetic preservative MI with the same concentration (0.2%). Notwithstanding, the higher preservation efficacy was reported when rhamnolipids and MI were supplemented to the personal care formulation at 0.1% from each. Accordingly, the combination of rhamnolipids and MI has a synergistic preservation effect. The influence of the combined preservatives is more active than that of the individual preservatives used alone, in other words, the combination was more active against a broad spectrum of microorganisms than when rhamnolipids and MI were used lonely.

TABLE 4. Preservation efficacy of rhamnolipids in personal care formulation represented in log reduction values .

Microorganisms	Log reduction values								
	0.2% Rhamnolipids			0.2% MI			0.1% Rhamnolipids + 0.1% MI		
	T7*	T14*	T28*	T7	T14	T28	T7	T14	T28
E. coli	3.04	3.08	3.09	3.09	3.18	3.23	3.44	3.57	3.61
Sal. typhimurium	3.01	3.06	3.06	3.05	3.17	3.21	3.45	3.61	3.68
St. aureus	3.76	3.81	3.84	3.65	3.71	3.77	3.77	3.84	3.86
B. subtilis	3.56	3.64	3.69	3.46	3.49	3.54	3.52	3.66	3.71
C. albicans	1.09	1.11	1.12	1.12	1.14	1.15	2.06	2.14	2.19
A. brasiliensis	-	1.01	1.03	-	0.96	1.02	-	1.28	1.48

*T7, T14, and T28: Sampling time after 7, 14 and 28 days.

Discussion

The capability of *Ps. aeruginosa* ATCC 9027 to produce rhamnolipids was reported in the current study using glycerol as a carbon source. However, *Ps. aeruginosa* is a ubiquitous bacterium and could consume different types of substrates for rhamnolipid production such as glycerol, carbohydrates, and vegetable oils (Tan & Li, 2018; George & Jayachandran, 2013). *Pseudomonas* was the most commonly used bacteria for rhamnolipids production in previous investigations (Haba et al., 2003; Mawgoud et al., 2010; Rahman et al., 2010; Nguyen & Sabatini, 2011; De Rienzo et al., 2016a; Ozdal et al., 2017). Recently, *Pseudomonas* sp. is still the most frequently rhamnolipid producer with high yields after short incubation periods (Zhao et al., 2020).

In the current investigation, rhamnolipid was produced from *Ps. aeruginosa* ATCC 9027, and the chemical characterization was harmonized by FTIR. The IR spectra of the produced rhamnolipids were found in accordance with similarity to some extent significantly that recorded by Leitermann et al. (2008) and Rahman et al. (2010). Furthermore, the chemical configuration of rhamnolipids was confirmed by ESI-MS, where twelve congeners were detected, despite two di-rhamnolipids congeners (Rha-Rha-C_8-C_{10} and Rha-Rha-C_{10}-C_{12}) were detected, the mono-rhamnolipids were found the most predominant congeners, in particular, both Rha-C_{10} and Rha-C_{10}-C_{10} or Rha-C_8-C_{12}. The length β-hydroxyfatty acid chains were varied among minor rhamnolipids homologues including C_8 (with two unsaturated $C_{8:2}$), C_{10} (with one unsaturated $C_{10:1}$) and C_{12} (with one unsaturated $C_{12:1}$). According to previous studies (Grosso-Becerra et al., 2016; De Rienzo et al., 2016a), *Ps. aeruginosa* ATCC 9027 produced predominant mono-rhamnolipid congeners in accordance with the results presented in the current study. However, *Ps. aeruginosa* produced different rhamnolipid congeners depends on different strains, culture media, and cultivation conditions. However, the most common rhamnolipid congeners were produced from *Ps. aeruginosa* is Rha-C_{10}- C_{10} and Rha-Rha C_{10}-C_{10} (Gudiña et al., 2015).

The ability of biosurfactants to reduce the surface tension mainly emphasizes the activity of the specific surface-active compound. The produced rhamnolipids in the current study drastically reduced the surface tension of water to 26mN/m with a CMC value of 22mg/L, and these values are like those previously reported for *Ps. aeruginosa* sp. (De Rienzo et al., 2016a; Liu et al., 2017). The CMC is a significant property during the evaluation of the activity of biosurfactants. Because it is the point at which surfactant molecules aggregate together in the liquid to form micelles and the surface-active properties are at the optimum performance (Zhao et al., 2018; Rahman et al., 2010). Accordingly, surface tension is not further reduced above this concentration. In addition to surface tension activity, the formation of stable emulsions by mixing water and hydrophobic compounds commonly used as an indicator of surface activity of biosurfactants. It worth noting that, the produced rhamnolipids in the current study showed good emulsifying activity, whereas, a stable emulsion of hydrophobic vegetable oil and water was formed. The ability of rhamnolipids to form stable emulsions supports a potential application in the cosmetic industries. The produced rhamnolipids in the current study were stable at different treatment temperatures during incubation till 1hr. The stability of rhamnolipids under extreme environmental conditions such as temperature has been reported (Silva et al., 2010; Zhao et al., 2018). This property will enhance the application of rhamnolipids in the industrial fields, which requires heat-stable products for operational and environmental conditions (Silva et al., 2010).

The produced rhamnolipids from *Ps. aeruginosa* ATCC 9027 was tested for its toxicity against brine shrimp (*A. salina*). The results indicate the low toxicity of rhamnolipids. Moreover, the toxicity effect of rhamnolipids against *A. salina* was found much lower than the toxicity effect of commonly used synthetic preservative (MI). *Ps. aeruginosa* ATCC 9027 was reported as a non-virulent strain for mice models suitable for the industrial production of mono-rhamnolipids (Grosso-Becerra et al., 2016). Therefore, the produced rhamnolipid in the current investigation seems to be an excellent potential candidate for cosmetics and personal care formulations in the current investigation. However, many investigators studied the toxicity effect of rhamnolipids against various organisms. Johann et al. (2016) reported a low embryotoxic and teratogenic potential of rhamnolipids on a fish embryo, also reported low toxicity effect of

rhamnolipids on the germination of *Aspergillus niger* spores and the growth of *C. albicans.* Silva et al. (2010) reported that rhamnolipids from *Pseudomonas aeruginosa* UCP0992 caused 50% lethality for brine shrimp after 24hrs at 525mg/L. Haba et al. (2003) evaluated the ocular, skin toxicity and the biocompatibility of rhamnolipids in male albino rabbits, they reported that rhamnolipids had no superficial damages effect for the cornea, and no dermal irritation or edema was noticed. Lang & Wagner (1993) observed there is no toxicity of pure rhamnolipids on *Daphnia magna* at a concentration of 200ppm. According to the US Environmental Protection Agency (EPA), rhamnolipid consisting of a rhamnose sugar unit(s) and fatty acid molecules, which are considered nontoxic components in nature. Rhamnose approved as a food additive by the US Food and Drug Administration, and fatty acids are found ubiquitous in living organisms. Therefore, the breakdown yield of rhamnolipids is nontoxic components at all. Furthermore, the EPA classified the 9.5% of rhamnolipids in category (IV) toxicity, in which there is no acute toxicity was reported when oral, dermal, and inhalation toxicity were studied.

The antimicrobial potentiality of rhamnolipids was repeatedly studied against wide variety of pathogenic bacteria, yeast, and filamentous molds (Christova et al., 2011; Lotfabad et al., 2013; De Rienzo et al., 2016b; Elshikh et al., 2017), which have been significantly confirmed by the current study results. In the current study, the mono-rhamnolipids were detected as the most predominant homologues from *Ps. aeruginosa* ATCC 9027 with significant antimicrobial activity against all types of tested microorganisms. It is well documented that the antimicrobial susceptibility of rhamnolipids against microorganisms is dependent on different rhamnolipid homologues (Samadi et al., 2012; Das et al., 2014; Elshikh et al., 2017). Haba et al. (2003) reported a satisfactory antimicrobial activity of rhamnolipids mixture with dominant di-rhamnolipid congeners against bacteria and have a lower antimicrobial effect on tested yeasts and molds. Nitschke et al. (2010) reported antimicrobial activity of a mixture of mono and di-rhamnolipids against *Bacillus cereus, St. aureus, Micrococcus luteus, Mucor miehei* and *Neurospora crassa*. Ndlovu et al. (2017) reported the antimicrobial activity of a mixture of mono and di-rhamnolipids against various environmental and clinical bacteria and fungi. Das et al. (2014) reported that mono-rhamnolipids have antimicrobial activity against different types of bacteria and fungi better than di-rhamnolipids. Also, Samadi et al. (2012) reported that mono-rhamnolipids were found more potent antibacterial agents than di-rhamnolipids in agreement with our results in the current study. Other investigators indicated that rhamnolipids were active against Gram-positive bacteria and had weak or no effect on other Gram-negative bacteria (Christova et al., 2011; Lotfabad et al., 2013; De Rienzo et al., 2016b). However, variations in susceptibility testing of rhamnolipids against microorganisms may be reported due to the differences in the lipid membrane composition of examined microbial cells, also the composition of rhamnolipid (mono or di), the microbial origin, the extraction method and the purity of rhamnolipids (Nitschke et al., 2010; Elshikh et al., 2017).

In the current investigation, the preservation efficacy of rhamnolipids was evaluated to verify the suitability and the biocompatibility in the preservation system. However, from the obtained results in the current study rhamnolipids proved to be an excellent candidate as a safe preservative for shower gel personal care formulation. Many factors could impair the preservation efficacy in cosmetics and personal care formulations such asthe pH of the formulation, interactions occurring between formulation components, formulation homogeneity and solubility, mechanisms of ingredient degradation, and possible incompatibilities (Palefsky, 2014). Nevertheless, a promising preservation efficacy of rhamnolipid in the personal care formulation was obtained even after a long period of preservation. Moreover, the obtained log reduction values of rhamnolipids were found in accordance with the acceptance criteria required in ISO 11930 (2019) at preservation period up to 28 days. However, based on the obtained high reduction log values of rhamnolipids, we could postulate that synergistic interactions occurred among rhamnolipids and other personal care formulation components resulted in increasing the preservation efficacy of rhamnolipids. However, rhamnolipid could be used alone or in combination with MI to maintain the preservation efficacy of personal care formulations. Besides the lower toxicity and potent antimicrobial activities of rhamnolipid in the current investigation, also rhamnolipid was produced from a non-virulent strain of *Ps.*

aeruginosa ATCC 9027 (Grosso-Becerra et al., 2016), which confer an additional safe property for commercial uses.

Conclusion

In conclusion, the current investigation showed that the obtained rhamnolipid is an excellent potential candidate as bio-preservative for cosmetics and personal care formulations which could be safer and biocompatible in comparison with synthetic chemicals. However, rhamnolipid could be used alone or in combination with MI to increase the microbiological quality of cosmetics products and decreasing the potential undesirable side effects caused by the synthetic preservatives.

Conflict of interest: The authors declare that they have no conflicts of interest.

Ethical approval: This article does not contain any studies with human participants or animals performed by any of the authors.

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