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Chromatic intervention and biocompatibility assay for biosurfactant derived from *Balanites aegyptiaca* (L.) Del

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Extraction of biosurfactants from plants is advantageous than from microbes. The properties and robustness of biosurfactant derived from the mesocarp of *Balanites aegyptiaca* have been reported. However, the dark brown property of biosurfactant and lack of knowledge of its biocompatibility limits its scope. In the present work, the decolorization protocol for this biosurfactant was optimized using hydrogen peroxide. The hemolytic potential and biocompatibility based on cell toxicity and proliferation were also investigated. This study is the first report on the decolorization and toxicity assay of this biosurfactant. For decolorization of biosurfactant, 3^4 full factorial design was used, and the data were subjected to ANOVA. Results indicate that 1.5% of hydrogen peroxide can decolorize the biosurfactant most efficiently at 40 °C in 70 min at pH 7. Mitochondrial reductase (MTT) and reactive oxygen species (ROS) assays on M5S mouse skin fibroblast cells revealed that decolorized biosurfactant up to 50 µg/mL for 6 h had no significant toxic effect. Hemolysis assay showed ~ 2.5% hemolysis of human RBCs, indicating the nontoxic effect of this biosurfactant. The present work established a decolorization protocol making the biosurfactant chromatically acceptable. Biocompatibility assays confirm its safer use as observed by experiments on M5S skin fibroblast cells under in vitro conditions.

Abbreviations

ANOVA	Analysis of variance
DCF	2′,7′-Dichlorofluorescin
DCF-DA	2′-7′Dichlorofluorescin diacetate
DMSO	Dimethyl sulfoxide
DW	Distilled water
FBS	Fetal bovine serum
H_2O_2	Hydrogen peroxide
MTT	Mitochondrial reductase
PBS	Phosphate buffer saline
PSN	Penicillin, streptomycin, and neomycin
ROS	Reactive oxygen species

Surfactants (surface-active materials) belong to a diverse group of chemicals and may be divided into three categories, namely cationic, anionic and neutral, depending upon their polar moiety[1]. Currently, surfactants are manufactured from petrochemicals, and they are used in various industries like pharmaceuticals, detergents, cosmetics, petroleum, textile, food, agriculture, paper, and water treatment[2–4]. However, some harmful effects

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of synthetic surfactants present in shampoos have been observed, like hair loss, irritation to the scalp, and drying out of follicles[5,6]. Further, current strict environmental regulations across the countries and apprehensions among consumers for its safe use have drawn researchers' attention to exploring alternative options to synthetic surfactants[7]. The natural surfactants can be obtained easily from many marine organisms and various plants[8–11]. Biosurfactants possess high-foaming capacity, high-biodegradability, environment-friendly, low-toxicity, low-cost[12], and par with chemically-synthesized surfactants[8]. They are also used for environmental clean-up like bioremediation of heavy metals and oil-recovery from water and soil[9,13].

Commercially, *Pseudomonas* and *Bacillus* bacterial groups are the largest producers of biosurfactants[14,15]. *Lactococcus lactis* mediated cost-effective biosurfactant has also been reported[16]. Production of biosurfactants from bacteria is not cost-efficient due to cumbersome downstream processing, the requirement of a skilled workforce, and instrumentation for maintenance of bacterial strain under specific conditions. Besides, uncertainty in production output per batch is a point of concern[1]. Biosurfactants obtained from plants are proteins or protein hydrolysates, phospholipids, and saponins. Saponins have some biological and physiochemical properties and therefore are suitable for various biotechnological applications[1,5,11,17]. Saponins are nonionic and could be used in folk remedies and employed as detergents for textile cleaning[1,18].

Balanites aegyptiaca has excellent detergent potential in its mesocarp. Its biosurfactant properties, including density, surface tension, viscosity, pH, electric conductivity, foaming potential, foam stability, detergency, dirt dispersal potential, and sebum removal potential, have been explored[19]. Sharma et al.[20] reported the robustness of the detergent at varying pH, temperature, and salinity. However, the biosurfactant's brown color and a lack of biosafety evaluation limit its application in various industrial sectors. Decolorization and biocompatibility investigation of the biosurfactant can improve its market acceptance. Hydrogen peroxide (H_2O_2) is a chlorine-free oxidizing agent, and by providing free •OH radicals, it mediates the breakdown of several organic substances and bleaches the substances[21–24]. Bleaching efficiency depends on the dose of H_2O_2, the temperature, pH of the medium, and duration of the interaction[23,25]. Zhang and Zheng (2009) conducted experiments to optimize the decolorization of acid green dye (AG20) using various pH, H_2O_2 concentration, and ultrasonic power density. They found that the optimum conditions for decolorization of AG20 were 4.85 of initial pH, 1.94 mM of H_2O_2, and 1.08 W/mL of ultrasonic power density[23]. Similarly, the decolorization process has also been optimized for polysaccharides obtained from *Cyclocarya paliurus*, where 0.5 mg/mL polysaccharides, 0.623 mM H_2O_2, 40℃ temperature, and 9.0 pH were found to be the best combination[24].

Biosurfactants find applications in various fields such as cosmetics, skincare, laundry, utensils and floor cleaning, etc. Biosurfactants can enhance drug availability to the target cells and can facilitate the absorption of active principles. To test the biosurfactant for its efficacy in cosmetics, skincare, and pharmaceuticals, the in vitro toxicity assays are essential before conducting any in *vivo* tests. The toxicity assay includes preliminary tests like hemolysis and cell viability assays.

In the present study, the decolorization of biosurfactant derived from *B. aegyptiaca* was optimized using H_2O_2. Further, the toxicity of the biosurfactant was investigated by hemolysis and in vitro biocompatibility assays. The present investigation findings could be advantageous in developing a market acceptable, safe biosurfactant with a wide range of applications.

Materials and methods

Plant material. Mature fruits of *B. aegyptiaca* were collected from Jaipur and nearby areas (N 26°44.260′ E 075°43.174′ and 338 m altitude). After removing the pericarp, the mesocarp was collected by scratching the fruit surface with a sharp knife. This mesocarp was dried in a hot air oven at 65 °C for 48 h. The dried mesocarp was further powdered using the mortar-pestle and stored in an airtight container at room temperature until further use.

Preparation of biosurfactant. Dried mesocarp powder (10 g) was mixed in 100 mL distilled water to get 10% (w/v) suspension of the biosurfactant.[19] The suspension was shaken for 10 min and then filtered through blotting paper followed by Whatman No. 1 filter paper. The filtrate was used as the biosurfactant solution in further experiments.

Decolorization of biosurfactant, experimental design, and statistical analysis. The biosurfactant solution (400 µL) was mixed with 20 µL H_2O_2 and 580 µL distilled water. Then the pH of this reaction mixture was adjusted to 6 using either 1 N NaOH or 1 N HCl. The final volume of this reaction mixture was raised to 4000 µL using distilled water. The reaction mixture was then incubated at 30℃ for 60 min (This is referred to as decolorization at 0.5% H_2O_2, pH 6, 30℃ for 60 min incubation period). The mixture was cooled to room temperature, and the absorption of light at 495 nm wavelength (A_{495}) was recorded using a spectrophotometer (Thermo Fisher Scientific, USA), taking distilled water as a blank. Reduction in absorbance was considered as a measure of decolorization. Experiments were carried out to evaluate the effect of four factors, namely dose of H_2O_2, pH, temperature, and duration of treatment on the decolorization (Supplementary Table S1). Each aspect was studied at three different levels (H_2O_2 at 0.5, 1.0 and 1.5% v/v; pH at 6, 7 and 8; the temperature at 30, 40, and 50 °C and duration of incubation of 60, 70, and 80 min, respectively; Supplementary Table S1) as described by Wang et al.[25]. A complete 3^4 factorial experiment was conducted in a randomized block design with three replicates (blocks), resulting in 81 treatment combinations and 243 observations (Supplementary Table S1). The data were subjected to analysis of variance (ANOVA) for testing various hypotheses regarding the biosurfactant's decolorization. The main effects and interactions of factors were tested to determine the most optimum treatment combinations for decolorization of biosurfactant.

The biosurfactant was decolorized using 1.5% H_2O_2 at 40 °C for 70 min and tested for biocompatibility.

Biosafety assay. *Materials.* M5S mouse skin fibroblast (JCRB1322) was obtained from the National Institute of Biomedical Innovation Health and Nutrition (NIBIOHN, Ibaraki, and Osaka), Japan. Cell culture medium alpha-MEM was purchased from Sigma Aldrich Co. (St. Louis, MO), USA. Fetal bovine serum (FBS) and PSN antibiotic cocktail mixture (penicillin 5 mg/mL, streptomycin 5 mg/mL, neomycin 10 mg/mL) were bought from Gibco Co Ltd (Gibco, Tokyo), Japan. Oxiselect TM intracellular ROS assay kit was procured from Cell Biolabs Inc. (San Diego, CA), USA. Cell viability and proliferation assay kit were purchased from Dojindo Laboratories (Dojindo, Molecular Technologies Inc. Kumamoto), Japan. All other analytical grade chemicals were bought from Wako (Wako analytical, Osaka) or Nacalai Tesque Inc. (Nacalai Tesque, Tokyo), Japan.

Methods. Cell toxicity assay. m5S skin fibroblast cells were cultured in alpha-MEM medium with 10% FBS and 1% PSN at 37 °C in a 5% CO_2 animal cell incubator up to the sub-confluent stage. Cells were treated with different doses of biosurfactant (0, 5, 10, 25, 50, 100, and 200 µg/mL) for 12 h in 96 well culture plate under conditions as stated above. After the incubation period, cells were washed in cold (4℃) phosphate buffer saline (PBS), and MTT (1 mg/mL) was added to the wells and incubated further at 37 °C with standard incubation chamber for 3 h.

After the second incubation time, MTT was removed from each well, and 50 µL DMSO was added to dissolve the formazan crystal. The resulting intracellular formazan content was quantified by taking absorbance at 570 nm using a spectrophotometer (Multi-scan FC, Thermo Fisher Scientific, Inc., Pittsburg, PA, USA).

Percentage cell viability data were tested for normality based on the histogram, and data were not found normal. Then data were transformed to log10, and then they were subtracted from the highest value. These values were then subjected to a normality test, and the coefficients of skewness and kurtosis were found to be 0.376 and 0.921, respectively. Similarly, Kolmogorov–Smirnov and Shapiro–Wilk coefficient was found to be 0.082 and 0.024 (degree of freedom = 55). These findings proved that the data is not significantly different from normal data. Then the transformed data were subjected to one-way analysis of variance (ANOVA) at $p < 0.05$. $F(48, 6) = 35.365$, $p = 0.000$). Further, Duncan's multiple range test was applied to compare two treatment means (Degree of freedom = 6).

The decolorized biosurfactant effect on cell viability was also investigated. The percentage of cell viability was tested for normality based on the histograms, and data were found to be statistically normal (skewness = 0.391 and kurtosis = 1.639). Then the transformed data were subjected to one-way analysis of variance (ANOVA) at $p < 0.05$.

Cell proliferation assay. Sub confluent m5S cells were treated with biosurfactant (50 µg/mL) for various time intervals of 0, 2, 6, 10, 15, 24, and 48 h. Untreated cells at each time were used as the control group. Following incubation, the cells were processed by MTT assay to calculate the percentage of live cells, as per the manufacturer's instructions.

Data were subjected to a normality test and were found to be not significantly different from a normal distribution statistically. Then proliferation was compared after 6 and 10 h based on an independent sample t-test using SPSS version 16 at the significance level $P < 0.05$. Further analysis of skewness and kurtosis (6 h skewness z value: 0.289, 6 h kurtosis z value: 0.590, 10 h skewness z value: 0.2996, and 10 h kurtosis z value: 1.787) indicated that these values are in a range of ± 1.96, justifying that data is normal.

Similarly, the effect of decolorized biosurfactant (50 µg/mL) was also studied. The data obtained were subjected to the normality test and were found to be statistically normal. Then proliferation was compared after 6 and 10 h based on an independent sample t-test using SPSS ver. 16 at the significance level $P < 0.05$.

Quantification of intracellular reactive oxygen species (ROS). Intracellular reactive oxygen species (ROS) content was measured using a standard commercial kit (Cell Biolabs, Inc. San Diego, CA, USA). Cell population at 1×10^4 cells/mL density were treated with different doses of biosurfactant (0, 5, 10, 25, 50, 100 and 200 µg/mL) for 6 h in 96 well culture plate. Following treatment, cells were washed with Hank's balanced salt solution (Gibco. Co. Ltd., Milliwake, USA) and incubated in 10 µM of dichlorofluorescin diacetate (Sigma. Aldrich. Inc. MO.USA) for 30 min at 37 °C. The fluorescence signal was quantified using a spectrophotometer (DTX800, Beckman Coulter, Inc. Brea, CA, and USA) at excitation and emission of 485 and 530 nm.

The same procedure was used to evaluate the influence of a 6 h incubation with the decolorized biosurfactant (0, 5, 10, 25, 50, 100, and 200 µg/mL) on cell viability.

The data obtained as percentage fluorescent units based on biosurfactant effect were subjected to normality test. They were not statistically significantly different from a normal distribution (Degree of freedom = 147, Kolmogorov–Smirnov coefficient = 0.086, skewness z value = 0.659, and kurtosis z value: 0.753). The data were further subjected to one-way analysis of variance (ANOVA) at $p < 0.05$ using SPSS ver. 16.

Similarly, the data on the effect of decolorized biosurfactants were also subjected to normality test. They were statistically significantly different from a normal distribution (Degree of freedom = 21, Sig. = 0.000, skewness z value = 1.258, and kurtosis z value: 0.200). The difference could be due to the small data size. The data were subjected to a non-parametric Kruskal–Wallis analysis of variance (ANOVA) test using SPSS ver. 16. The results (Chi-square test with Degree of freedom = 6, $p = 0.003 < 0.005$) indicated that the treatment with decolorized biosurfactants yielded statistically significant differences in cell viability percentage. The Mann–Whitney U test was applied using SPSS ver. 16 for comparison of each possible pair of treatments (21 pairs of treatments).

Immuno-cytochemistry of DCF-DA (2′-7′dichlorofluorescin diacetate). Cells (3×10^4/mL) were seeded on culture compatible coverslips in 6 well culture plates and incubated for 6 h and 10 h with biosurfactant (0, 50 µg/mL) or decolorized biosurfactant (0, 50 µg/mL). The cells were washed with cold (4℃) PBS solution and fixed with 4% paraformaldehyde solution at room temperature for 10 min. After fixing, cells were

Treatment		Mean $A_{495} \pm SE$
Temperature	30	0.3518 ± 0.18065
	40	0.2255 ± 0.06764
	50	0.2083 ± 0.05481
Duration	60	0.2549 ± 0.11100
	70	0.2663 ± 0.15593
	80	0.2644 ± 0.12624
Percentage hydrogen peroxide	0.5%	0.3016 ± 0.15255
	1%	0.2596 ± 0.08539
	1.5%	0.2244 ± 0.05939
pH	6	0.2895 ± 0.14385
	7	0.2673 ± 0.14875
	8	0.2287 ± 0.08565

Table 1. Descriptive statistics for the effect of temperature, time, pH and percentage hydrogen peroxide on decolorization of biosurfactant solution.

again washed with PBS and mounted on glass slides. Intracellular green fluorescence was observed under a fluorescence microscope at excitation and emission of 485 and 530 nm, respectively, for 1/5 s (BZ-9000, Keyence, Osaka, Japan). The fluorescence intensity was obtained as histograms using the Image J software (Fig. 5).

Haemolysis assay. Biosurfactant's in vitro toxicity was evaluated by a hemolytic assay using human red blood cells (RBCs) as described earlier[26]. This assay has been used to study the toxic nature of silver nanoparticles (AgNPs)[27]. The magnitude of hemolysis of RBCs indicates the level of toxicity of the material under study. Five percent biosurfactant solution was prepared by suspending the dried mesocarp powder in PBS and sterilized distilled water separately. The suspension was filtered through a syringe filter (0.22 µm). This filtrate was used as a biosurfactant solution. Approx. 10^8 cells of RBCs were mixed with biosurfactant solution and incubated for 1 h at 37 °C under shaking conditions. The mixture was centrifuged at 2000 rpm for 10 min at room temperature to remove the debris. The absorbance of the supernatant was recorded at 540 nm. Triton X-100 (1% w/v) was used as a positive control. The percentage of hemolysis was calculated as follows:

$$\% \text{ of hemolysis} = \frac{(A - B) * 100}{(C - B)}$$

where A, B, C are absorbance at 540 nm in the presence of biosurfactant, PBS, and 1% Triton X-100, respectively.

Results and discussion

Effect of variable parameters on decolorization of biosurfactant solution. The results obtained from various experiments to understand the effect of percentage H_2O_2, temperature, pH, and duration of treatment individually and in combination, are explained as follows.

Temperature. Temperature shows a highly significant effect on the decolorization of biosurfactant solution (Tables 1 and 2). Interaction of temperature parameter with other independent factors also indicates a significant impact. Homogenous subsets obtained by performing post-hoc test revealed that 40 and 50 °C contribute equally, but 50 °C contributes maximum ($p \leq 0.05$) for the decolorization of biosurfactant (Table 1). Similar observations were recorded in decolorization of polysaccharides derived from *Cyclocarya paliurus,* where decolorization increased with an increase in temperature from 20 to 60 °C, with more than 80% decolorization efficiency at 40°C[24]. Salem et al.[28] reported that an increase in temperature increased decolorization up to 40 °C. However, 30 °C is reported as an optimum condition for decolorization of acid blue dye.

Time duration. Different time durations (60, 70, and 80 min) were used to study the effect of time as an independent variable on decolorization of biosurfactant solution. However, the data did not reveal a significant impact ($p = 0.624$) (Table 2). On the other hand, interactions of the duration of treatments with temperature, pH, and doses of H_2O_2 yielded significant effects (Table 2). All three variables of these time-durations contributed equally to the decolorization of biosurfactant. But 80 min duration was the most effective towards decolorization at $p \leq 0.05$ level of variation (Table 1). Similar results were reported for decolorization of polysaccharides derived from *C. paliurus,* where decolorization increased with time up to 40 min. No significant bleaching effect was found beyond this time[24].

Effect of pH. The statistical analysis indicates that pH plays a significant role in decolorizing biosurfactant solution (Table 2). Further, it has also been found that its combinations with the other three factors are also crucial (Table 2). pH 8 was the most suitable for decolorization with a mean value of absorbance as 0.2887 (Table 1). These findings conform with earlier published scientific reports where decolorization increased with an increase

Tests of between-subjects effects					
Dependent variable: absorbance at 495 nm					
Source	**Type III Sum of squares**	**df**	**Mean square**	**F**	**Sig**
Corrected model	3.169[a]	80	0.040	6.150	0.000
Intercept	16.662	1	16.662	2.587E3	0.000
Temperature	0.995	2	0.497	77.233	**0.000**
Time	0.006	2	0.003	0.473	0.624
Percentage H_2O_2	0.242	2	0.121	18.811	**0.000**
pH	0.153	2	0.077	11.913	**0.000**
Temperature × time	0.213	4	0.053	8.255	**0.000**
Temperature × percentage H_2O_2	0.072	4	0.018	2.783	**0.029**
Temperature × pH	0.067	4	0.017	2.582	**0.039**
Time × percentage H_2O_2	0.104	4	0.026	4.031	**0.004**
Time × pH	0.090	4	0.022	3.488	**0.009**
Percentage H_2O_2 × pH	0.076	4	0.019	2.965	**0.021**
Temperature × time × percentage H_2O_2	0.319	8	0.040	6.197	**0.000**
Temperature × time × pH	0.288	8	0.036	5.583	**0.000**
Temperature × percentage H_2O_2 × pH	0.249	8	0.031	4.842	**0.000**
Time × percentage H_2O_2 × pH	0.094	8	0.012	1.829	0.075
Temperature × time × Percentage H_2O_2 × pH	0.200	16	0.013	1.944	0.020
Error	1.043	162	0.006		
Total	20.874	243			
Corrected total	4.212	242			

Table 2. Effect of temperature, time duration, pH and hydrogen peroxide % on the decolorization of biosurfactant (ANOVA table for univariate analysis, $p < 0.05$). [a]R Squared = 0.752 (Adjusted R Squared = 0.630)

in pH from 5 to 9 and decreased at pH 10. The highest decolorization efficiency (~ 77%) was observed at pH 9 in polysaccharides derived from *C. paliurus*[24].

Hydrogen peroxide (H_2O_2). The dose of H_2O_2 as an individual variable and its interaction with the three factors plays a significant role (Table 2). Among all the doses, 1.5% had the maximum effect on decolorization of biosurfactant solution (Table 1). Decolorization of polysaccharides derived from *C. paliurus* increased with an increase in H_2O_2 dosage, with maximum decolorization obtained at 0.623 mM H_2O_2[24]. An increase in H_2O_2 dose from 0.02 M to 0.4 M enhanced the decolorization for Acid Blue 29 dye[28]. Decolorization increased from ~ 30% to ~ 60% when the dose of H_2O_2 was increased from 0.2 to 1.6 mM[29]. H_2O_2 has various advantages as a bleaching agent, like high stability to decolorize different substances, can quickly destroy the dye, high efficiency, and does not require any special equipment.[25] Decolorization of textile effluents is typically carried out using H_2O_2. Wang et al.[25] reported efficient decolorization of *Sapindus mukorossi* pericarp (dark brown) using 2.5% H_2O_2 at pH 6 and 80 °C after 80 min incubation period.

Effect of combination of various treatments towards decolorization of biosurfactant solution. The most effective combination towards decolorization was found with 50 °C and 60 min with a mean value of absorption of 0.180 (Table 3). Out of the nine combinations of temperature and percentage H_2O_2, the most effective combination towards decolorization was 40 °C and 1.5% H_2O_2 with a mean value of A_{495} of 0.183 (Table 3). Among the nine combinations of temperature and pH, 50 °C with pH 7 is the most effective combination towards decolorization with a mean value of A_{495} of 0.197 (Table 3). 70-min time duration with 1.5% H_2O_2 was the most effective combination with a minimum A_{495} of 0.218 (Table 3). Another combination of treatment duration and pH reveals that 60 min treatment at pH 8 was the most effective combination towards decolorization with a mean value of A_{495} of 0.211 (Table 3). Among the nine combinations of H_2O_2 and pH that were examined, 1.5% H_2O_2 at pH 8 was the most effective combination towards decolorization with a mean value of A_{495} of 0.207 (Table 3). In twenty-seven different combinations of temperature, duration of treatment, and percentage H_2O_2, the best combination was identified as 40 °C at 1.5% H_2O_2 for 80 min with the absorption of 0.159 (Table 3, Fig. 1). Among the twenty-seven different combinations of temperature, duration of treatment, and pH, the best combination was 50 °C for 60 min at pH 8 with the absorption of 0.160 (Table 3). Almost similar observation (0.161) has been observed at 40 °C for 80 min at pH 8 (Table 3). Among twenty-seven combinational approaches involving the duration of treatment, percentage H_2O_2, and pH, the best combination was identified as 70 min treatment with 1.5% H_2O_2 at pH 7 with absorption of 0.187 (Table 3). Lastly, among the eighty-one combinations of temperature, duration of treatment, percentage H_2O_2, and pH, the best combination has been identified as treatment with 1.5% H_2O_2 at pH 7 and 40 °C for 70 min with absorption of 0.139 (Table 3). From these observations, it can be inferred that

Temperature	Time duration (minutes)	Hydrogen peroxide (%)	pH	Mean A_{495}	Range of A_{495}
50	60	–	–	0.180	0.150–0.211
40	–	1.5	–	0.183	0.152–0.213
50	–	–	7	0.197	0.166–0.227
	70	1.5		0.218	0.187–0.248
–	60	–	8	0.211	0.181–0.242
–	–	1.5	8	0.207	0.176–0.237
40	80	1.5	–	0.159	0.107–0.212
50 (2)	60	–	8	0.160	0.108–0.213
40	–	1.5	8	0 .171	0.118–0.224
–	70	1.5	7	0.187	0.134–0.239
40	70	1.5	8	0.139	0.048–0.230

Table 3. Effect of combination of temperature, duration of treatment, percentage hydrogen peroxide and pH on decolorization of biosurfactant solution. The data presented in this table are based on full factorial analysis.

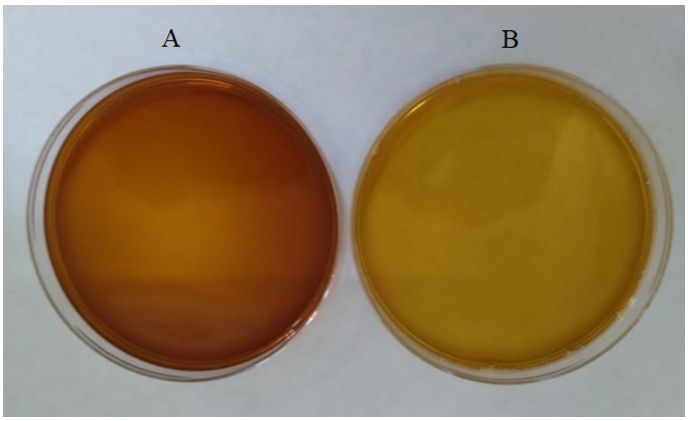

Figure 1. Biosurfactant solution: (**A**) before decolorization and (**B**) after decolorization.

the dose of H2O2 highly influences biosurfactant decolorization. 1.5% H_2O_2 treatment consistently resulted in better decolorization.

Biosafety aspects. *In vitro cell toxicity assay.* Observations recorded for MTT assay for cell viability have been presented in Fig. 2. It has been observed that there is no statistically significant difference in cell viability up to 50 µg/mL of biosurfactant treatment as observed in Fig. 2. Further, no significant difference in cell viability was found when cells were treated with 5 µg/mL, 25 µg/mL, and 50 µg/mL of biosurfactant with cell viability of 105%, 113%, and 110%, respectively. However, 100 µg/mL and 200 µg/mL of biosurfactant resulted in a drastic reduction in cell survival by 43% and 13%, respectively, indicating the biosurfactant's toxic effect on cells at higher concentration (Fig. 2). From these observations, it is inferred that the biosurfactant could be used up to 50 µg/mL without compromising in vitro cell viability. These results prove the safety of the material up to 50 µg/mL under in vitro conditions. The effect of 50 µg/mL biosurfactant and decolorized biosurfactant on cell proliferation was studied under different incubation times. The findings are presented in Fig. 3 A,B. Up to 6 h, the cell proliferation was similar between the groups, without statistically significant difference ($p = 0.013$, which is less than 0.05; Degree of freedom = 14). However, after 10 h, cell proliferation was significantly reduced ($p = 0.000$; Degree of freedom = 14). It can be concluded that the use of biosurfactant at 50 µg/mL is safe for in vitro cell proliferation. To our knowledge, there is no in vitro cell proliferation toxicity study available in the literature regarding aqueous extract of mesocarp (biosurfactant used in the present study) of *B. aegyptiaca*.

The results of cell viability following treatment with decolorized biosurfactant is presented in Fig. 2B. No significant difference in cell viability was observed on treatment with 0, 5, 10, 25, 100 µg/mL decolorized biosurfactant (Posthoc, Degree of freedom = 14). However, slightly better growth of cells was observed at 50 µg/mL treatment. Treatment with 200 µg/mL decolorized biosurfactant resulted in a drastic reduction in cell survival by ~ 66%, indicating its cytotoxic effect (Fig. 2B). Results show that up to 100 µg/mL decolorized biosurfactant could be used without compromising in vitro cell viability. It also proves the biocompatibility of decolorized biosurfactant up to 100 µg/mL under in vitro conditions. Decolorized biosurfactant (50 µg/mL) was used to study the effect of treatment duration on percentage cell viability (Fig. 3B). The findings indicate that up to 6 h of the incubation period, the proliferation of cells was similar to that of treatment with 50 µg/mL, without statistically significant difference (Levene's test is applied to test the equality of variances, $F(2,2) = 0.029$, $p = 0.873 > 0.05$).

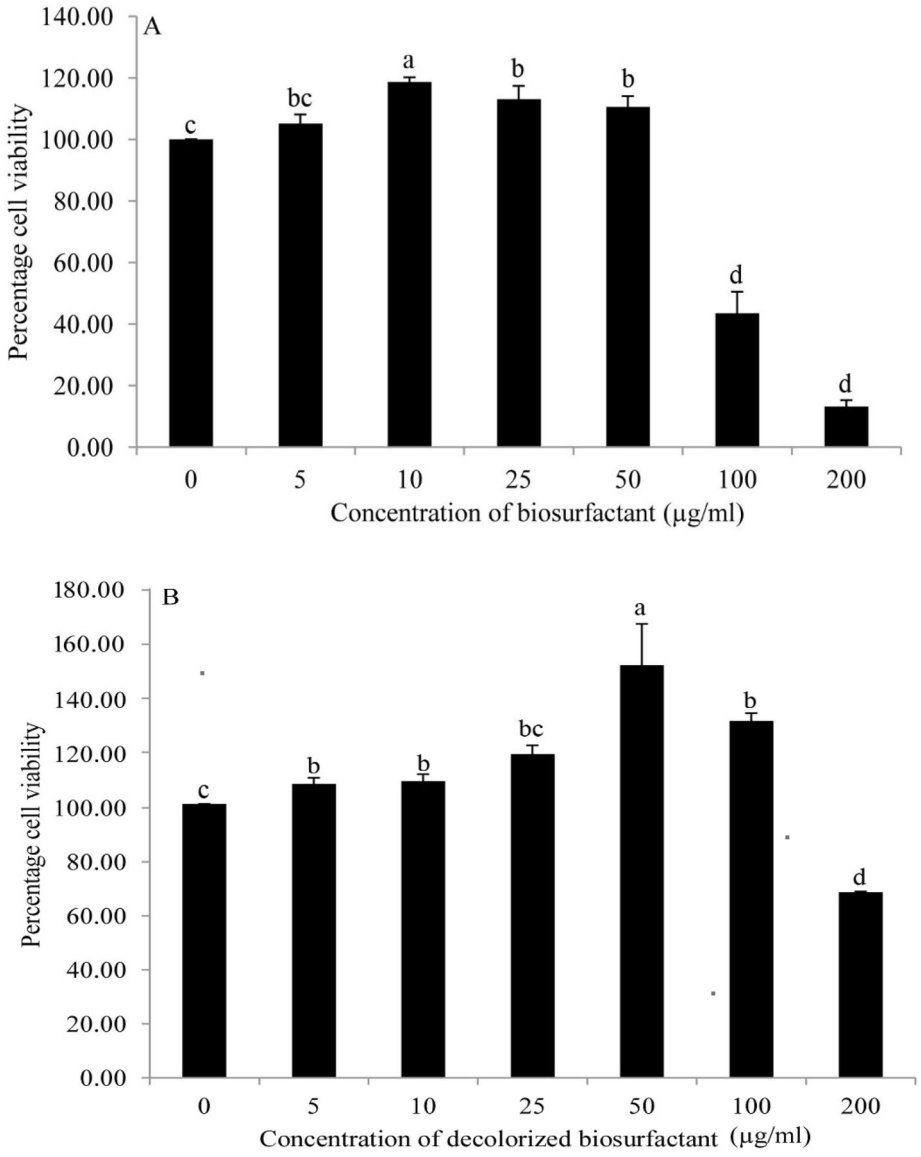

Figure 2. Toxicity assay of (**A**) biosurfactant and (**B**) decolorized biosurfactant at various concentrations on the mouse skin fibroblast (M5S) cell line (Results of the cell survival based on MTT assay). (Number of observations for biosurfactant and decolorized biosurfactant are 55 (df:6) and 21 (df:6) respectively. Error bars presents standard error. Similar letters given on the columns indicate that there is no statistically significant difference at $p > 0.05$) and the different letters on the column present statistically significant difference ($p < 0.05$) according to Duncan's multiple range test.

The T-test showed that the difference between control and 50 µg/mL was not statistically significant (t = 0.391, degree of freedom = 4, $p = 0.716 > 0.05$). It can be inferred that 50 µg/mL decolorized biosurfactant does not pose any cytotoxic effect. The ethanolic extracts of *B. aegyptiaca* fruit mesocarp did not exhibit any significant toxic effect when given orally at a concentration up to 4000 mg/kg body weight to Wistar albino rats[30]. The aqueous extract of mesocarp of *B. aegyptiaca* exhibited molluscicidal activities with LC_{50} values 65.51 mg/L and 83.52 mg/L, respectively, against *Biomphalaria pfeifferi* and *Lymnaea natalensis*[31]. A strong correlation between the saponin content of *B. aegyptiaca* mesocarp extracts and *Aedes aegypti* larval mortality was revealed[32]. Various studies have highlighted the presence of saponins, balanitoside and diosgenyl saponins, terpenoides, phenolic compounds and alkaloids[32–37]. These biochemicals may have direct and indirect effects on the survival of cells.

Reactive oxygen species (ROS). Biosurfactants induce ROS formation, which oxidizes the reduced form of $DCFH_2$, resulting in $DCFH_2$-mediated fluorescence. Fibroblast cells were treated with biosurfactant (0 µg/mL to 200 µg/mL) and evaluated for intracellular ROS generation (Fig. 4). The ROS was recorded in terms of relative fluorescent units. Biosurfactant at 5 µg/mL did not produce any significant ROS quantity (Fig. 4). An increase in the dose of biosurfactant increased the ROS generation (Fig. 4). Overall, the effect seems significant from a

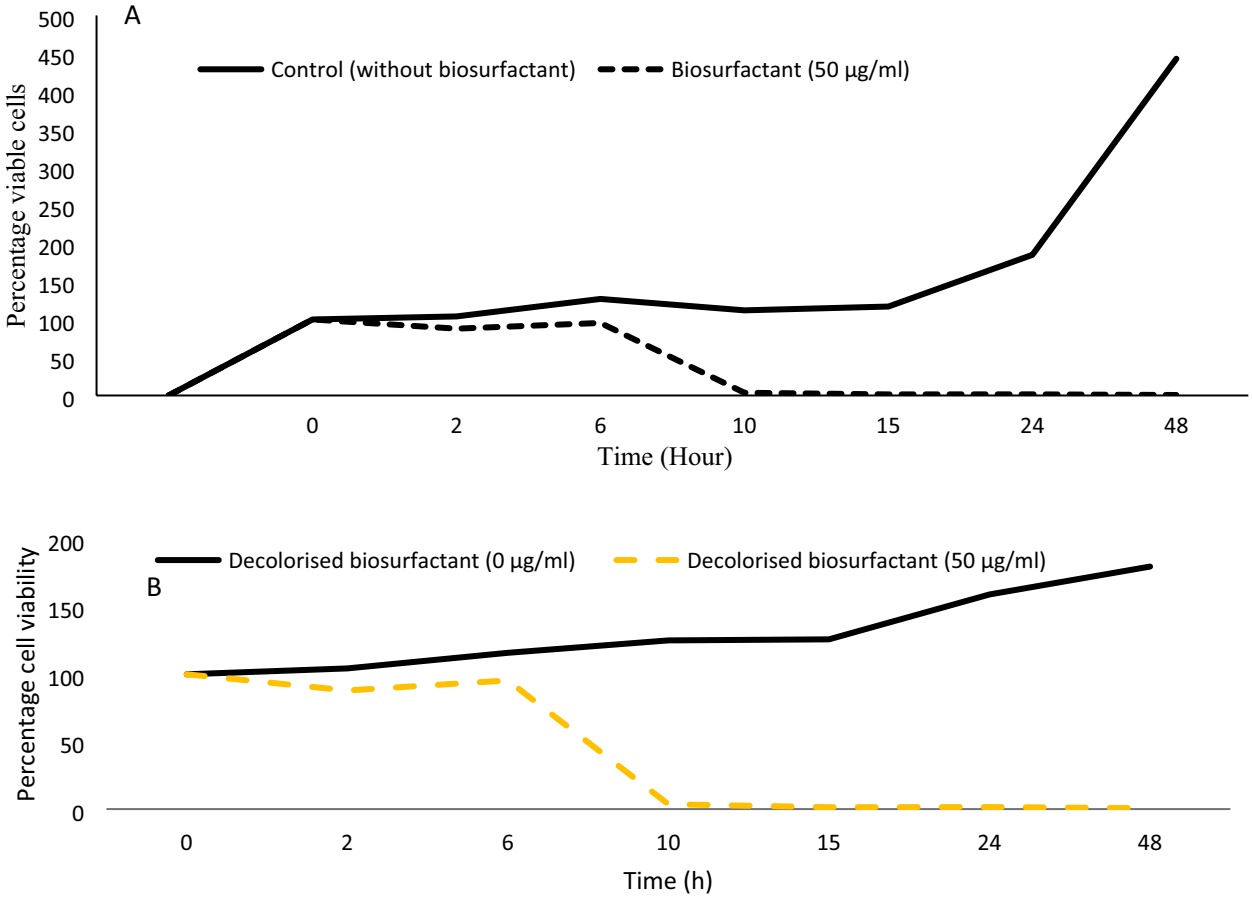

Figure 3. (**A**) Effect of biosurfactant on proliferation of mouse skin fibroblast (M5S) cell line (sample size:56) (**B**). Effect of decolorized biosurfactant on proliferation of mouse skin fibroblast (M5S) cell line. (**B**) (sample size: 42).

cytotoxicity perspective when 50 µg/mL biosurfactant was used. Again, the inference remains towards safe use of biosurfactant at a dose of 50 µg/mL. There is little literature available regarding the cytotoxicity of biosurfactants. Treatment of HepG2 cells with aqueous fruit extract of *B. aegyptiaca* resulted in intrinsic cell death mediated by down-regulation of the BCL2 gene[37]. Similarly, Beit-Yannai et al.[38] reported that steroidal saponin extracted from roots and fruits of *B. aegyptiaca* caused the generation of ROS in h MCF-7 breast and HT-29 human colon cancer cells.

Fibroblast cells were treated with different concentrations of decolorized biosurfactant and analyzed for ROS generation (Fig. 4B). The results indicate that decolorized biosurfactant had a statistically significant effect on ROS quantity based on Mann–Whitney U tests (coefficient: $0.000 < 0.05$) except for one pair of treatments (10 µg/mL and 50 µg/mL, Mann–Whitney U tests coefficient: $1.000 > 0.05$). As the concentration of decolorized biosurfactant increased, the ROS generation amount also increased, as evident from the correlation coefficient of 0.965. The decolorization process does not pose any significant toxic effect, as evident from the treatment up to 50 µg/mL (Fig. 5A,B).

The treatment of cells for 6 h with 50 µg/mL biosurfactant resulted in ROS generation (Fig. 5A,B). However, the amount of fluorescence increased after 10 h incubation significantly, as presented in Fig. 5C. It reflects that an increase in treatment duration enhances the increment in ROS generation and cell toxicity. Therefore, it can be inferred that cells should be safe if exposed to biosurfactants for ≤ 6 h.

The treatment of cells with 50 µg/mL decolorized biosurfactant resulted in ROS generation, with similar fluorescence intensity after 6 h and 10 h of incubation compared to the untreated cells (Fig. 5D–F). Therefore, it can be inferred that exposure to biosurfactant for ≤ 6 h and decolorized biosurfactant up to 10 h at 50 µg/mL concentration is not toxic to cells. The intensity of fluorescence obtained through Image J analysis indicates a significant increase in fluorescence intensity when cells are incubated with biosurfactant for 10 h at 50 µg/mL. However, there was no considerable accumulation of ROS in cells exposed to decolorized biosurfactant at 50 µg/mL up to 10 h, as evident from the histograms (inset of Fig. 5D–F).

Haemolysis assay. The observations recorded as percentage hemolysis due to biosurfactant treatment are presented in Fig. 6. It indicates that biosurfactant in DW and PBS causes 2.48% and 2.22% hemolysis, respectively.

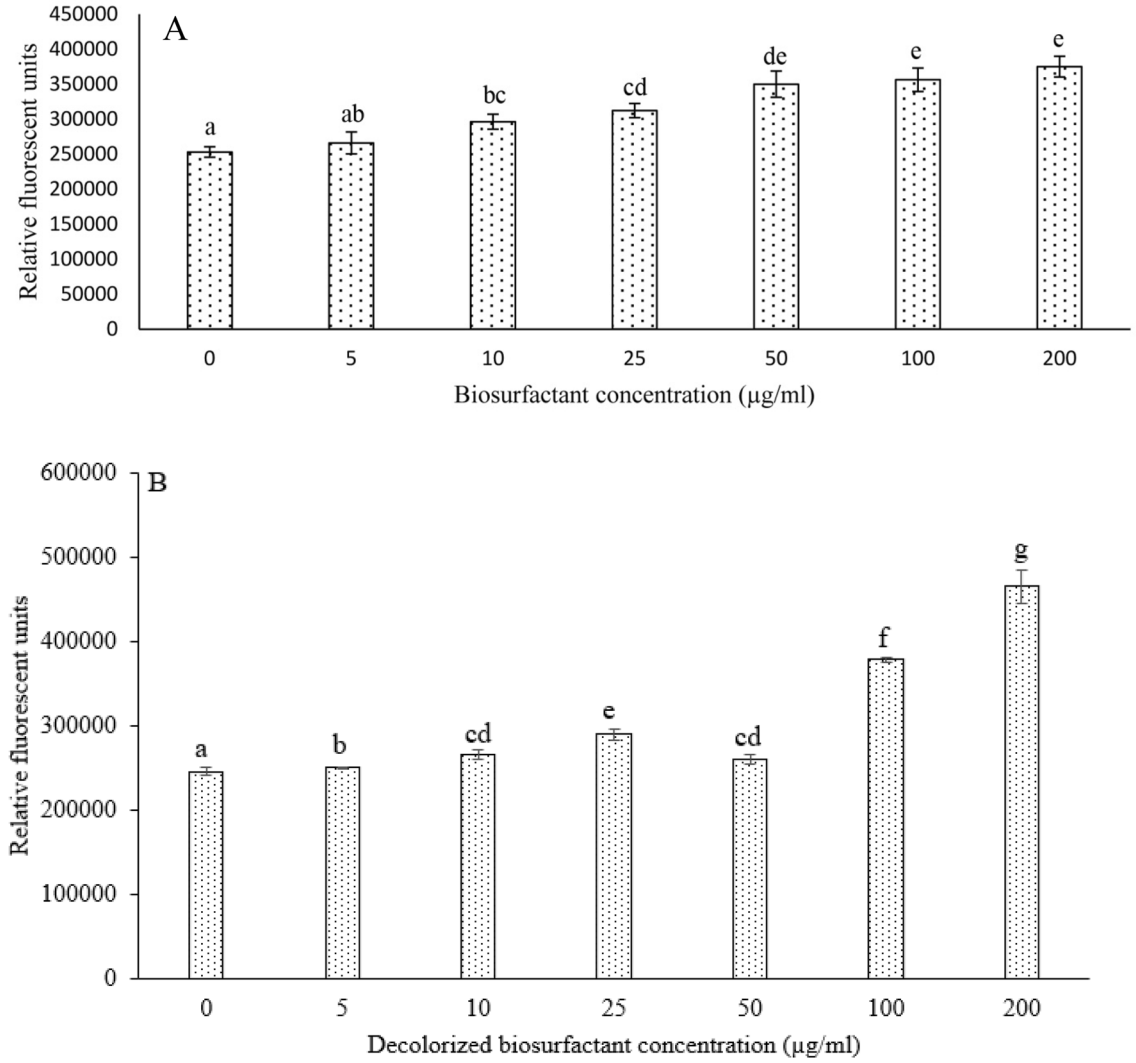

Figure 4. (**A**) Effect of biosurfactant on generation of reactive oxygen species (in terms of relative fluorescent units) on mouse skin fibroblast (M5S) cell line (sample size is147, Duncan's multiple range test at df = 6) and (**B**) effect of decolorized biosurfactant on generation of reactive oxygen species (in terms of relative fluorescent units) on mouse skin fibroblast (M5S) cell line (sample size is 21, non-parametric Mann–Whitney test). Error bars in the graphs presents standard deviation. Similar letters on the columns indicate that there is no statistically significant difference at $p > 0.05$. Whereas dissimilar letters on the columns indicate that there is statistically significant difference at $p < 0.05$.

While 100% hemolysis was observed in Triton X-100 (1%), no hemolysis was observed in DW. It indicates that the biosurfactant is relatively safer as the safer range is below 5% haemolysis.[39].

Conclusion

The advantages of biosurfactants over synthetic detergents have attracted researchers to the exploration of newer sources. The present investigation is the first study to reveal the cytosafety property of biosurfactant and decolorized biosurfactant derived from the mesocarp of *B. aegyptiaca*. Experiments were conducted to decolorize the biosurfactant to make it more acceptable to the market. The findings revealed that the most suitable combination of the factors studied is biosurfactant treatment with 1.5% H_2O_2 at 40 °C and pH 7 for 70 min. These findings bring out a strong base for in *vivo* study to explore this biosurfactant application as shampoo/detergent (hair growth, hair fall, microbial infection, etc.), laundry detergent, dishwasher, etc. Neutral pH, easy and economical method bleaching of the biosurfactant and retention of nontoxic nature (at 50 µg/mL for 6 h under in vitro conditions) even after decolorization of the present biosurfactant opens a wide range of applications of the biosurfactant derived from the mesocarp of *B. aegyptiaca*. It may find pharmacology applications as drug formulation components that can facilitate drug delivery and better access of the drug to the target cells. Further studies are required to establish applications of this biosurfactant in various fields.

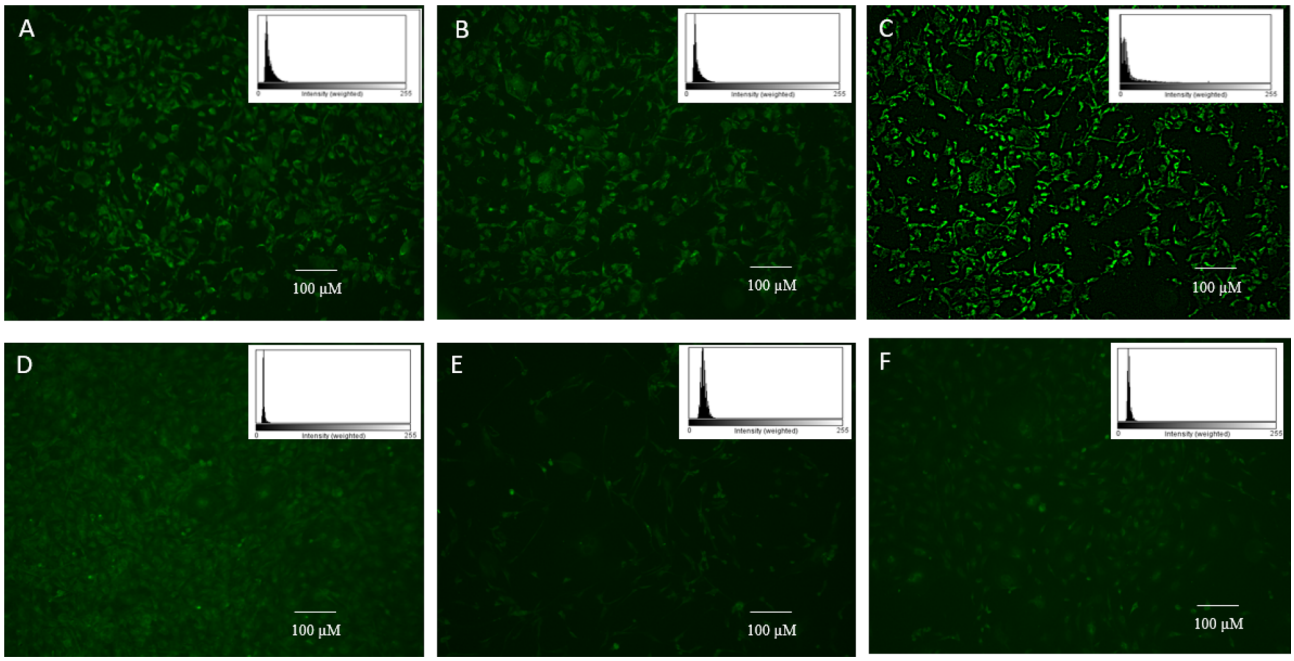

Figure 5. Intracellular ROS analysis by DCFH-Da Fluorescence assay. (**A**–**C**) Biosurfactant. (**A**) Wild control group. (**B**) cells treated with 50 µg/mL for 6 h and (**C**) 50 µg/mL for 10 h. (**D**–**F**) Decolorized biosurfactant. (**D**) Wild control group. (**E**) Cells treated with 50 µg/mL for 6 h and (F) 50 µg/mL for 10 h. Intensity based histogram (as calculated by image J) is presented in inset for each treatment.

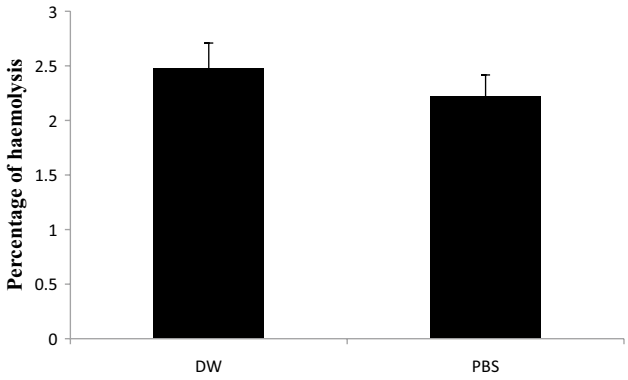

Figure 6. Percentage haemolysis by biosurfactant in distilled water and phosphate buffer saline (PBS). Number of observations: 6, Error bars in the graphs represents standard error.

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Author contributions

V.P., V.B., H.M., Y.N., and R.M. conducted experiments and recorded the observations. J.P., V.S.G., M.K.S. and P.S.: carried statistical analysis and intrepreted the results. V.S.G.: Conceptualized the idea. V.S.G. and P.S.: written first draft of the manuscript. P.S., Ha., V.D.R. and S.L.K.: Reviewed the manuscript and improved the manuscript by their valuable suggestions

Competing interests

The authors declare no competing interests.

Additional information

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