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Antiviral Potential of Lipopeptide Biosurfactant Isolated from *Acinetobacter junii* B6

Amir Mohammad Bagheri[1,2], Hamid Forootanfar[3], Gholamreza Dehghannoudeh[2,4], Mohammad Amin Raeisi Estabragh[2,4], Ibrahim M. Banat[5], Hosnieh Sokhandan[6], Hadiseh Shokouhi[6], Mandana Ohadi[4*], Parvaneh Mehrbod[6,7*]

[1]Herbal and Traditional Medicines Research Center, Institute of Pharmaceutical Sciences, Kerman University of Medical Sciences, Kerman, Iran; [2]Department of Pharmaceutics, Faculty of Pharmacy, Kerman University of Medical Sciences, Kerman, Iran; [3]Pharmaceutical Sciences and Cosmetic Products Research Center, Institute of Pharmaceutical Sciences, Kerman University of Medical Sciences, Kerman, Iran; [4]Pharmaceutics Research Center, Institute of Pharmaceutical Sciences, Kerman University of Medical Sciences, Kerman, Iran; [5]School of Biomedical Sciences, Faculty of Life & Health Sciences, Ulster University, Coleraine BT52 1SA, N. Ireland, UK; [6]Influenza and Respiratory Viruses Department, Pasteur Institute of Iran, Tehran, Iran; [7]Autophagy Research Center, Shiraz University of Medical Sciences, Shiraz, Iran

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ABSTRACT

Background: Influenza A virus (IAV) is a global health concern, increasing the exploration of alternative therapeutics. Biosurfactants (BSs) are biodegradable, non-toxic biomolecules with favorable biological activities. *Acinetobacter junii* B6, known for utilizing crude oil as a carbon and energy source, produces BSs. This study evaluated the antiviral potential of the lipopeptide biosurfactant (LPB) against H1N1/A/PR8/34 infection.

Methods: *A. junii* B6 LPB was extracted and characterized in our previous study. The 50% cytotoxic concentration and non-cytotoxic concentration (NCTC), defined as the concentration with no toxicity on cells, were determined by the MTT assay on MDCK cells. The NCTC was exposed to the cells in the presence of PR8 (100 $TCID_{50}$) under simultaneous, pre- and post-exposure combination treatments for 1 h. After 48 h of incubation, the hemagglutination and MTT assays assessed viral propagation and cell protection, respectively. Amantadine and oseltamivir served as antiviral control drugs.

Results: The extracted LPB caused a 1 $\log_2$ reduction in pre- and post-exposure treatments, whereas amantadine and oseltamivir reduced viral titers by more than 1 $\log_2$. Cell protection was favorable in all combination treatments except for LPB co-treatment. LPB extract showed limited but notable anti-IAV activity, alongside cell protection.

Conclusion: While LPB is less potent than amantadine and oseltamivir, its non-toxic and environmentally friendly nature warrants further study. As surfactants act on the lipid envelope, viral proteins, and nucleocapsid proteins, LPB could be potentially used as an adjuvant with other antiviral agents to control the prevalence of viral diseases and improve therapeutic outcomes. ***DOI: 10.61882/ibj.5241***

Keywords: Acinetobacter junii, Antiviral agents, Influenza A virus, Lipopeptides

Corresponding Authors:

Mandana Ohadi
Pharmaceutics Research Center, Institute of Pharmaceutical Sciences, Kerman University of Medical Sciences, Kerman, Iran;
E-mail: m.ohadi@kmu.ac.ir, mandanaohady@gmail.com; ORCID ID: 0000-0001-7251-3751

Parvaneh Mehrbod
Influenza and Respiratory Viruses Department, Pasteur Institute of Iran, Tehran, Iran, Autophagy Research Center, Shiraz University of Medical Sciences, Shiraz, Iran; Tel.: (+98-21) 64112176; E-mail: mehrbode@yahoo.com, mehrbode@pasteur.ac.ir; ORCID ID: 0000-0002-8391-9228

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1. INTRODUCTION

Numerous viral diseases are considered global health issues[1]. Influenza A virus (IAV), an important respiratory infection, is a high-risk disease that affects human and animal health universally. Current vaccines are challenging, and conventional drugs have shown severe side effects and resistance. Accordingly, introducing alternative approaches may improve access to better therapeutic options to control this infection[2].

Surfactants are amphiphilic molecules, abundantly used in daily life. These surface-active agents reduce the interfacial tension between different phases[3]. Although surfactants are traditionally used as detergents and cleansing agents, they have attracted interest from various industrial sectors, including petroleum, environmental, agricultural, medical, biomedical, and pharmaceutical sciences[4].

Since the beginning of the 20th century, the production of synthetic surfactants from petroleum sources has significantly increased[5]. However, concerns about sustainable processes to reduce environmental issues have encouraged scientists to focus on bio-based alternatives from natural sources, commonly known as biosurfactants (BSs)[6]. These green source surfactants are secondary metabolites produced within a wide range of microorganisms, such as bacteria (*Bacillus cereus* and *Staphylococcus aureus*), fungi (*Rhizomucor miehei*), and yeast (*Candida bombicola* and *Candida apicola*)[7]. The most important bacteria that produce surfactants include *Pseudomonas* sp., *Bacillus* sp., and *Acinetobacter* sp.[8]. Due to their natural source, BSs are fundamentally biodegradable, non-irritating, and non-toxic with interesting biological activities, including antioxidant, anti-inflammatory, antibiofilm, antimicrobial, and wound healing effects, which accelerate their superiority over synthetic counterparts[9,10].

Acinetobacter junii B6, an alkane-degrading bacterium isolated from the oil-contaminated samples, produces a lipopeptide biosurfactant (LPB) that has significant interfacial activities, as well as notable antioxidant, antimicrobial, and antibiofilm activities[11,12]. Further studies on such BS-producing microorganisms may provide superior applications in biomedical fields. BSs, due to their amphiphilic nature, can enter the lipid bilayer membrane and disrupt viral membranes. They have shown efficacy against membranous viruses such as SARS-CoV-2[13,14], hepatitis C virus, and herpes simplex virus[15].

In our previous studies, we focused on the isolation and characterization of *A. junii* B6 bacteria, optimization of LPB production, and assessments of its antioxidant potential and wound-healing activities[16,17]. This LPB attenuated lung inflammation and histopathological alterations in a rat model of asthma[18]. Following this capacity, the current study was designed to screen the potential antiviral capacity of LPB derived from *A. junii* B6 against H1N1/PR8 as a viral model.

2. MATERIALS AND METHODS

2.1. Chemicals

Dulbecco’s Modified Eagle’s Medium (DMEM) was purchased from Gibco, USA. Fetal bovine serum (FBS), tosylamide phenylethyl chloromethyl keton-treated trypsin (Trypsin-TPCK), penicillin/streptomycin, and MTT [3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide] were purchased from Sigma-Aldrich, USA. Influenza and Respiratory Viruses Department, Pasteur Institute of Iran, prepared influenza vaccine strain (A/Puerto Rico/8/1934 [ATCC VR-1469™]) and Madin-Darby Canine Kidney (MDCK) cells. Amantadine hydrochloride and oseltamivir carboxylate, prepared by Sigma-Aldrich, were also tested in parallel as control drugs.

2.2. Preparation and characterization of BS

A. junii B6 (GenBank accession no. KT946907) was isolated from the BiBi Hakimeh area in Ahvaz (N, 50°24′0″ E, 30°10′48″), the Southwest of Iran. Detailed information on bacterial source identification, species characterization, optimal culture conditions, and lipopeptide extraction, along with purification methods, has been reported in our previous studies[16,17,19,20]. The bacterium was cultivated in an optimal medium (mineral salt medium) containing (g/L): $MgSO_4$, 0.1; KH_2PO_4, 0.5; $CaCl_2 \cdot 2H_2O$, 0.01; $FeSO4 \cdot 7H_2O$, 0.001; $NaNO_3$, 2; and K_2HPO_4, 0.5, supplemented with Iranian light crude oil[17]. The produced LPB was purified using acid precipitation followed by solvent extraction and characterized using FT-IR and ultraviolet (UV)-visible spectroscopy, as well as thermogravimetric and differential thermal analyses[11].

2.3. Cell culture and virus propagation

MDCK cells were cultured in a humidified incubator with 5% CO_2 at 37°C, using DMEM supplemented with FBS (10%) and penicillin/streptomycin (1%), and were sub-cultured once a week. Afterwards, 80% confluent cells were inoculated with influenza virus (A/PR/8/34) for 1 h. As the maintenance medium, DMEM containing Trypsin-TPCK (1 µg/mL) was used during the antiviral experiment for 48 h. Then, the cell-free supernatant was harvested as the virus stock and stored at -80°C. To assess the infectivity dose of the virus, a cell culture infectious dose 50 ($CCID_{50}$) assay was performed. Briefly, semi-confluent cells in a 96-well microplate (3 × 10^4 cells/well; Nunc, Denmark) were exposed to 10-fold serial dilutions of the virus stock in quadruplicates. Following 48 h of incubation, a hemagglutination assay (HA) using chicken red blood cells (cRBCs), together with the Karber formula, was used[21,22].

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2.4. Cytotoxicity evaluation and selectivity index (SI) calculation

MDCK cells were seeded in a 96-well microplate (3 × 10^4 cells/well) and incubated in 5% CO_2 at 37°C for 24 h to reach 80-90% confluency. LPB was dissolved in DMEM + P/S + 1% DMSO (10 mg/mL) at room temperature, followed by 15 min sonication to ensure complete dissolution and dispersion[23,24]. The solution was passed through a 0.45 µM filter. Amantadine and oseltamivir were dissolved in dH_2O (2 mg/mL). The semi-confluent cells were exposed to two-fold serial dilutions of LPB, amantadine, and oseltamivir in DMEM and incubated at 37°C for 48 h. The cells without compound exposure and those exposed to DMEM + P/S + 1% DMSO were considered negative and vehicle controls, respectively. The experiment was repeated twice in duplicate. The colorimetric MTT assay was carried out to evaluate cell viability as previously described[25]. Briefly, the culture medium was replaced with MTT 1× in PBS and incubated at 37°C for 3-4 h. To release the purple color of formazan crystals, the MTT solution was replaced with DMSO, and the absorbance (OD) was measured at 570 nm using a microplate reader (StataFax 2100, USA). Cell viability was calculated using the following formula: (mean OD of treated cells/mean OD of control cells) × 100. The 50% cytotoxic concentration (CC_{50}) was calculated by plotting a dose-dependent response curve. Then, two-fold serial dilutions of CC_{50} were prepared and added to MDCK cells. After 48 h incubation under the same conditions, the non-cytotoxic concentration (NCTC) was calculated using ODs obtained from the MTT assay and analyzed by SPSS. Besides, the 50% effective concentration (EC_{50}) was also obtained[22], and the SI was measured as dividing CC_{50} by NCTC (EC_{50})[26].

2.5. Antiviral evaluation

For antiviral evaluation, FBS-supplemented media were removed, and the cells were washed with PBS. Cells were then exposed to different combinations of NCTCs and PR8 (100 $CCID_{50}$). In the simultaneous (co-exposure) procedure, PR8 was primarily mixed with NCTCs at room temperature for 30 min, then added to MDCK cells and incubated at 37°C for 1 h. In the other two (pre- and post-exposure) approaches, PR8 was added to the cells before and after compound treatment, respectively. Following 1 h of incubation, unabsorbed viruses were washed. Then, TPCK-containing medium (0.5 µg/mL) was added to the cells and incubated at 37°C for 48 h. In this respect, as described earlier, cell viability was assessed using the MTT assay. The percentage of protection was calculated as follows: percentage of protection = [A-B]/[C-B] ×100, where A, B, and C indicate the absorbance of the sample, the virus-infected control (no compound), and mock-infected control (no virus and no compound), respectively[27]. The virus titer was simultaneously evaluated by the HA assay using cell supernatants. The HA activity was assessed by adding different dilutions of supernatants to the washed cRBCs using U-bottom 96-well microplates. Accordingly, plates were added with two-fold serial dilutions of supernatants. Afterwards, each well received 0.5% cRBCs. In haemagglutinin titration, HA units were measured as the reciprocal of the highest virus dilution, which causes complete agglutination. In this regard, RBC precipitation indicates virus absence, whereas diffuse lattice formation (HA) demonstrates virus presence[21,22,25]. The experiment was repeated twice in duplicate. The antiviral activity of amantadine and oseltamivir was also measured as antiviral control drugs.

2.6. Statistical analysis

Data were expressed as mean ± SD, and SPSS software (SPSS for Windows, Version 26, IBM, SPSS Inc., USA) was used for the statistical analysis. One-way analysis of variance (ANOVA) and Tukey's post-hoc test were applied to compare the sample values, with a significance level of 0.5 ($p < 0.05$).

3. RESULTS

3.1. Characterization of LPB

The physicochemical properties of LPB derived from *A. junii* were assessed as described in our previous study[16]. Briefly, according to the FT-IR spectroscopy results, LPB exhibited two strong absorption bands at 1655 cm^{-1} and at 1546 cm^{-1}, which were characterized as amide I and amide II vibrations in peptides, respectively[16]. In addition, UV-visible spectroscopy showed maximum absorption in the range of 200–240 nm, indicating the presence of π–σ* and/or π –π* transitions typically found in functional groups such as amine, carboxyl, carbonyl, and ester[28]. The results of thermogravimetric and differential thermal analyses also confirmed the production of LPB[11]. The BS production was also confirmed based on the changes in medium components (carbon and nitrogen sources) and physical factors (aeration rate, temperature, and inoculum size), as previously highlighted[29]. Moreover, LPB was found to reduce the surface tension of water to 36 mN m^{-1}, with a critical micelle concentration (CMC) of approximately 300 mg/L[16,18].

3.2. Cytotoxicity results

The cytotoxicity of the compounds on MDCK cells was investigated to determine the NCTCs for use in the antiviral assay. The EC_{50} and NCTC values were found to be similar at 10, 0.09, and 0.39 mg/mL for LPB, amantadine, and oseltamivir, respectively. The CC_{50}, NCTC, and SI values for LPB, amantadine, and oseltamivir are shown in Table 1.

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Table 1. CC_{50}, NCTC, and SI of the tested compounds

Compound	CC_{50} (mg/mL)	NCTC (mg/mL) (equal to EC_{50})	Selectivity index ($SI = CC_{50}/NCTC$)
LPB	13.93 ± 0.10	10.00 ± 0.00	1.40
Amantadine	0.19 ± 1.53	0.09 ± 0.00	1.90
Oseltamivir	0.79 ± 6.01	0.39 ± 0.00	2.02

Data presented as mean ± SD are averages of two independent assays.

3.3. Antiviral evaluation results

In an in vitro screening, the potential antiviral activity of LPB derived from *A. junii* B6 was determined in treatments combined with PR8. The experiment was repeated twice in duplicate, and the results were compared to the viral control group. Table 2 shows the $\log_{10}$ HA titers. As shown in the table, the highest Log_{10} HA titer was observed for PR8 (2.56 ± 0.15). Amantadine in co-exposure (0.90 ± 0.00), followed by oseltamivir in co-exposure (1.96 ± 0.15), pre-exposure (1.36 ± 0.16), and post-exposure (0.75 ± 0.15), showed the lowest Log_{10} HA titers, respectively. LPB in pre-exposure and post-exposure (both at 2.26 ± 0.15) showed the next highest titers. Figure 1 also represents the Log_2 HA decrement from the HA assay. Figure 2 shows the cellular percentage of protection obtained from MTT cell viability data. The cellular percentages of protection for LPB in co-, pre-, and post-exposure treatments were 26.17%, 83.33%, and 86.34%, respectively. For amantadine and oseltamivir, across all combination treatments, values ranged from the lowest (63.27%) to the highest (92.69%). The antiviral activity of LPB, although not significant, was notable. On average, LPB caused a 1-log reduction in pre- and post-exposure treatments. Cellular protection was favorable in these two combination treatments.

4. DISCUSSION

In this study, we evaluated the anti-influenza capacity of LPB isolated from *A. junii* B6 bacteria. Compared to conventional drugs, the antiviral capacity of LPB was relatively weak; however, it was non-toxic to the cells, which supports its application as an adjuvant with other antiviral agents.

IAV infection remains a high-risk disease affecting human health worldwide. It cannot be easily controlled by current vaccines and conventional drugs, which are associated with antigenic changes in the viral genome. Recently, conventional antiviral agents have faced significant challenges due to viral resistance. Therefore, the introduction of new alternative approaches to combat such viral complications is an utmost need[30,31]. It is always recommended to explore compounds of natural origin due to their low side effects. In this context, natural microbial BSs have also shown antiviral properties[32]. The global market size of surface-active biomolecules is expected to grow to over 6.3 billion USD by 2026[33].

Known as friendly alternatives to chemical counterparts, BSs are attracting attention in various fields such as oil recovery, agriculture, medicine, and drug delivery. They have been reported to enhance the oral bioavailability of poorly water-soluble drugs, improve drug penetration through biological barriers, and protect encapsulated materials from harsh environments[34,35]. Accordingly, the continuous increase in market demand and scope of applications has encouraged scientists to identify new BS-producing strains and evaluate further biological applications for existing molecules. In this context, potential applications of BSs to fight against viral outbreaks have been discussed[36]. This study therefore provided new information about the potential antiviral properties of LPB derived from *A. junii* B6 against the H1N1/PR8 infection model. Although the antiviral capacity in this design was moderate, the MTT assay confirmed the non-cytotoxicity of LPB on MDCK cells, even at high concentration, while the tested antiviral drugs

Table 2. Log_{10} HA titer from the HA assay in the combined treatments, as compared to the virus control

Compound	Log_{10} HA (mean ± SD)		
	Co-exposure	Pre-exposure	Post-exposure
LPB	2.56 ± 0.15	2.26 ± 0.15	2.26 ± 0.15
Amantadine	0.90 ± 0.00**	2.26 ± 0.15	2.26 ± 0.15
Oseltamivir	1.96 ± 0.15*	1.36 ± 0.16*	0.75 ± 0.15**
PR8	**2.56 ± 0.15**	**2.56 ± 0.15**	**2.56 ± 0.15**

Data presented as mean ± SD are averages of two independent HA titrations. * and ** show significant ($p \leq 0.05$) and highly significant ($p \leq 0.01$) differences for values obtained from compound-treated samples compared to PR8, analyzed by SPSS, Tukey's post-hoc test.

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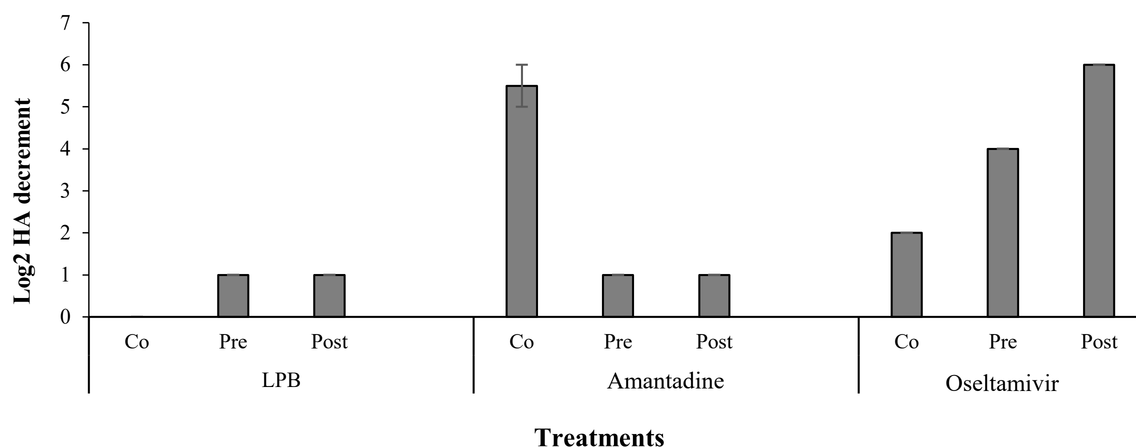

Fig. 1. Log_2 HA decrements in the combination treatments. The chart shows Log_2 HA reduction of compounds in different combination treatments (co-, pre-, and post-exposure procedures). The results were more pronounced with amantadine in co- and oseltamivir in all combination treatments (n = 2).

(amantadine and oseltamivir) showed cytotoxic effects at very low concentrations. Cermelli et al. have investigated the antiviral activity of hyaluronic acid against several viral species, including both RNA and DNA viruses, in vitro. Since distinct viral species have different replication strategies and structures (with or without an envelope), the inhibitory effect of this substance may involve the cell membrane-virus interaction steps through a non-specific mechanism. This hypothesis is supported by the results of the kinetic experiments[37]. Subramaniam et al. have shown that BSs can disturb the membrane structure of SARS-COV-2. Indeed, because of their amphiphilic nature, BSs can enter the lipid bilayer membrane and change its permeability. At higher concentrations of BSs, viral disintegration accelerates. Furthermore, complete inactivation of viral infection could be achieved by encapsulation of the virus into the micelles[38]. Later, Sanches et al. have used rhamnolipid-based liposomes (derived from *Pseudomonas aeruginosa* strain LBI) to improve the antibacterial activity of ParELC3 (a synthetic peptide). Their findings showed the potential use of BS-based nanocarriers as promising delivery systems to enhance the bioactivity of peptides[39]. Chowdhury et al. have evaluated the potential antiviral properties of seven different LPBs (tsushimycin, daptomycin, surfactin, bacillomycin, iturin, srfTE, and LPD-12) to interact with viral fusion proteins of SARS-CoV-2. Among the tested BSs, LPD-12 was shown to interact with the S

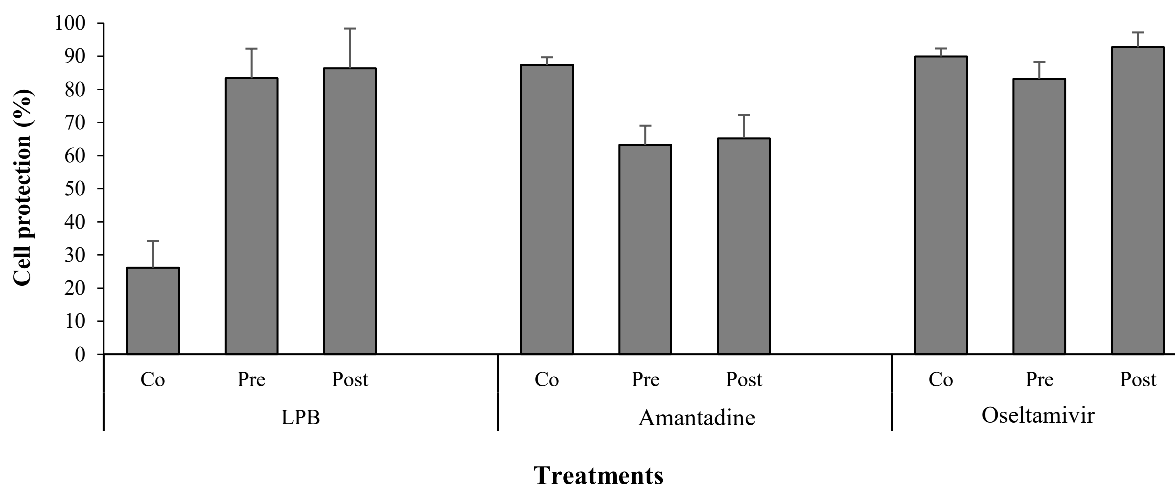

Fig. 2. Cell protection in the combination treatments. The chart shows the percentage of cell protection against PR8 using compounds in different combination treatments (co-, pre-, and post-exposure procedures). Cellular protection was favorable in the majority of combination treatments except for the LPB co-exposure treatment (n = 2).

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protein of the virus and strongly inhibited the binding of the virions to the host cell receptors[40], thus serving as a potential therapeutic drug[41]. Daverey et al. have reviewed the potential of sophorolipids as an antiviral treatment for the inactivation of SARS-CoV-2. Accordingly, sophorolipids were able to solubilize the lipid envelope of the virus due to their surface-active nature. Apart from that, sophorolipids could also suppress the virus-induced cytokine storm because of their immunomodulatory effects[14].

The potential antiviral capacity of surfactin, an LPB generated by *Bacillus subtilis*, was assessed by Crovella et al. against SARS-CoV-2 in Vero E6 cells. In that study, a two-hour co-exposure treatment of surfactin and virus substantially reduced viral infectivity, whereas no antiviral effect was detected in already infected cells. Therefore, the inhibitory effect of surfactin on Vero cells can probably be explained by damage to the integrity of the viral membrane. Surfactin is a macromolecule that may be unable to enter the cells efficiently[13]. The potential virucidal activity of a BS produced by the *haloarchaeon Natrialba* sp. M6 was investigated against H1N1 and other pulmonary viruses by Hegazy et al. The tested BS revealed high radical-scavenging activity as well as suppression of H1N1 hemagglutinin. They also evaluated the potential antiviral properties of this BS against the hepatitis C virus and herpes simplex virus[15]. In 2024, Pavia et al. assessed the biological activity of *Bacillus subtilis*-produced lipopeptide BSs against four main target proteins of SARS-CoV-2 in silico and in vitro. The in silico evaluation showed more interaction of the studied BSs with the S protein of the virus, which was in line with a previous study[42]. Also, researchers have evaluated several families of microbial lipopeptides with various lengths, peptide moieties, compositions, structures, and charges in terms of structure-activity relationship against SARS-COV-2. They have highlighted lipopeptides with a high number of amino acids, especially the anionic amino acids; surfactin, WLIP, fengycin, and caspofungin were the most promising lipopeptides against SARS-CoV-2 fusion and budding processes[43].

All tested viruses contain membranes that have similar morphological properties to those of PR8 in our study, which can support the antiviral capacity outcome of our LPB against H1N1. Studies have shown potential applications of BSs to protect loaded drugs from environmental damage, as they can form micellar aggregations above the CMC level. CMC is defined as the surfactant concentration at which micelles form spontaneously[44,45]. Jeon et al. have used nanostructured lipid carriers to improve the efficacy of the loaded drug (remdesivir) against SARS-CoV-2. As a beneficial strategy to enhance antiviral effects, this nanosized spherical formulation was shown to effectively increase bioavailability and cellular uptake of remdesivir while preventing the cellular entry of the virus[46].

The LPB derived from *A. junii* has shown potential antioxidant and anti-inflammatory properties, as described in our previous studies[20,28]. Regarding its mechanism of action in the current study, the reduction in HA detection of the virus may not be attributed to the physical interaction between LPB and the virus, as the co-exposure treatment revealed no substantial changes. However, it may be involved in virus-host interaction signaling pathways in the virus-infected cells. Research has indicated that viral infection can uncontrollably elevate pro-inflammatory cytokines and cause severe complications[47,48]. IAV can cause over-generation of ROS and nitric oxide[21,49,50]. It can also induce tissue injuries through oxidation and nitration of biological molecules. Accordingly, effective anti-inflammatory and immunomodulatory agents can be used to attenuate its virulence factor as an alternative therapy[21]. Apparently, the tested LPB might exert antiviral properties through similar approaches. It has been demonstrated that compounds with an SI value higher than three are considered potentially safer[26]. In the current study, the SI value of LPB was measured to be 1.4, indicating that it may be associated with some dose-related side effects. However, BSs are fundamentally considered suitable candidates for the development of lipid-based drug delivery systems.

The weak efficacy of LPB tested in this study might be related to our limitations. The limitations of this study were a short viral exposure time (which could have been extended), a lack of mechanistic investigations (which would have revealed a detailed mechanism of LPB effect), and a low number of replications (which could have decreased the probability of error). Understanding the exact inhibitory mechanism of LPB needs further investigation. Bio-based surfactants often have several interesting biological properties and may offer potential antimicrobial applications to combat infectious outbreaks and improve therapeutic outcomes.

5. CONCLUSION

In the current study, the potential antiviral effect of LPB derived from *A. junii* B6 was investigated against H1N1/IAV infection. Moderate antiviral properties were detected, with favorable effects on cell viability upon exposure to LPB and PR8. Accordingly, this LPB could potentially be used synergistically with other antiviral agents to reduce their dosage and enhance their bioavailability, which would reduce related side effects and improve their therapeutic potential. Further investigations on the structure of this LPB, its encapsulation in nanostructures for better dispersion and delivery, and the mechanism of its effects may help improve the treatment outcomes.

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DECLARATION

Acknowledgments

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Generative AI and AI-assisted technologies

In this study, no artificial intelligence technology was used in the production of the submitted work.

Ethical approval

Not applicable.

Consent to participate

Not applicable.

Consent for publication

All authors reviewed the results and approved the final version of the manuscript.

Authors' contributions

AMB: drafting the manuscript; HF: comprehensive reading and editing, GhD: study concept and design; MARE and IMB: Drafting the manuscript and editing; HS and HSh: performing antiviral experiments; MO: contributing materials, study concept and design, drafting the manuscript, and comprehensive reading and editing; PM: contributing materials, analyzed data, drafting the manuscript, and comprehensive reading and editing.

Data availability

Data that support findings are available from the corresponding authors (MO and PM) upon reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Supplementary information

The online version does not contain supplementary material.

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