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ORIGINAL PAPER

Evaluation of the antiviral potential of gemini surfactants against influenza virus H1N1

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Abstract

Influenza A virus (IAV) affects human health worldwide as a high-risk disease. It can neither be easily controlled by current vaccines and nor be treated by conventional drugs. Gemini surfactants (GS) have shown several properties including antiviral activity. In this study, the antiviral capacity of some GS compounds with different levels of hydrophobicity was examined. The 50% cytotoxic (CC_{50}) and non-cytotoxic (NCTC) concentrations of the compounds were determined by MTT method. The NCTCs, the same as effective concentrations (EC_{50}s), were tested for the antiviral capacity against IAV in different combination treatments for 1 h incubation on MDCK cells. The HA and MTT assays were used to evaluate the virus titer and cell viabilities, respectively. The hemolytic activity of the compounds was also assessed using an HA inhibition assay. To evaluate the apoptotic effect of GS compounds, Annexin V-PI kit was used. The HA titers decreased between 1–6.5 logs, 1–4.5 logs, and 1–5.5 logs in simultaneous, pre- and post-penetration combination treatments, respectively. The cell viability values in all combination treatments were favorable. The HI assay indicated the hemolytic potential of GSs and their physical interaction with viral HA. The apoptosis test results highlighted anti-apoptotic capacity of the GS compounds alone and in the presence of influenza virus especially for the hydrophobic ones. Gemini surfactants were generally more efficacious in simultaneous treatment. Their antiviral potential may be attributed to their physical interaction with viral membrane or HA glycoprotein that disrupts viral particle or blocks viral entry to the cell and inhibits its propagation.

Keywords Antiviral · Apoptosis · Gemini surfactant · H1N1 · Influenza virus

Abbreviations

Ama	Amantadine
ATCC	American Type Culture Collection
cRBC	Chicken red blood cells
CMC	Critical micelle concentrations
CPE	Cytopathic effects
CC_{50}	Cytotoxic concentration 50%
DMEM	Dulbecco's Modified Eagle's Medium
EC_{50}	Effective concentration 50%
FBS	Fetal Bovine Serum
GS	Gemini surfactants
HA	Hemagglutinin
HI	Hemagglutination inhibition
HLB	Hydrophilic-lipophilic balance
IAV	Influenza A virus
MOI	Multiplicity of infection
NA	Neuraminidase
NCTC	Non-cytotoxic concentrations
OD	Optical density
Ose	Oseltamivir
PS	Phosphatidylserine

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QSR	Quantitative-structure-activity-relationships
QAC	Quaternary alkyl ammonium compounds
SI	Selectivity index
TMEG	Thermo-magneto-electric generator
$TCID_{50}$	Tissue culture infectious dose 50
Trypsin_TPCK	Tosylamide Phenylethyl Chloromethyl Keton-treated Trypsin

Background

Influenza A virus (IAV) belongs to the *Orthomyxoviridae* family. These viruses undergo antigenic changes including antigenic drift and antigenic shift, which are minor and major modifications of the virus surface antigens, respectively. The latter causes the emergence of new viral strains with no immunity against it (Blut 2009; Iuliano et al. 2018; MohammadEbrahimi et al. 2022). Therefore, despite widespread access to vaccines and antiviral remedies, influenza virus infection cannot be eradicated from human populations (MohammadEbrahimi et al. 2022). From billion cases of influenza incidence annually worldwide, almost 500,000 cases are expected to death (Iuliano et al. 2018). It results in an estimated 31,000 deaths and 200,000 hospitalizations each year in the United States (Thompson et al. 2003; Simonsen et al. 1997). In the Eastern Mediterranean region, influenza outbreaks represent a potential threat to a global pandemic due to the situations in the climate and economic burdens (Baghi and Soroush 2018). Iran is one of the Mediterranean countries with a significant burden of influenza. According to the global burden of disease estimations in 2017, the influenza burden in Iran had an incidence of 587/100,000 people (Mozhgani et al. 2018; Troeger et al. 2019). Thus, influenza epidemics and pandemics impose a significant burden on the healthcare system that highlights the need for effective control of this infection.

Apoptosis (programmed cell death) plays a major role in the pathogenesis of influenza viruses through the destruction of alveolar epithelial cells, leading to pneumonia and leukocyte destruction, which ultimately leads to leukopenia (leukocyte depletion), which is a prominent clinical feature of influenza virus in humans (Uiprasertkul et al. 2007). It is characterized by specific symptoms such as DNA fragmentation, bruising, cell surface phosphatidylserine exposure, and formation of apoptotic bodies (Kar and Sivamani 2015).

Due to the severity of this infection, ineffectiveness of vaccines against new strains, and emerging resistance to conventional drugs, several compounds are being tested for the antiviral capacity against this infection.

Gemini surfactants (GS), which are environmental-friendly chemically-synthesized compounds, have shown antiviral capacity in previous studies against different viruses like HIV (Wong et al. 2002), SARS-CoV-2 19 (Kaur and Gupta 2020), Norovirus (Bolton et al. 2013), influenza virus (Gerba 2015), HBV (Zhang et al. 2016) and HSV (Thevenin et al. 2013).

The GS compounds contain at least two hydrophobic chains (generally organic halves such as aliphatic chains or aromatic groups) and two cationic or anionic groups (polar heads) linked covalently by a rigid or flexible spacer, which can be aromatic or aliphatic groups (Brycki et al. 2017).

There are different classes of gemini surfactants like quaternary ammonium salts (Obłąk et al. 2014), random cationic amphiphilic copolymers (Kuroda et al. 2009), and Gemini alkyl ammonium salts (Brycki 2010a). The classification of gemini surfactants is based on the different physicochemical properties of the hydrophobic tails and the spacer. With an increase in the number of carbon atoms, the surface activity of the surfactants is increased (Mondal et al. 2015). The hydrophobicity of compounds reinforces their attachment to the lipid bilayers and induces compound chain breakdown (Kuroda et al. 2009). Gemini surfactants also play a role in reducing adhesion, which depends on the length of the alkyl chain and the spacer distance (Chun et al. 2017) and it might be due to its hydrophobic nature (Obłąk et al. 2014). Among the counterions, dichlorides are less active than dibromides, and the introduction of an organic anion instead of a halide reduces the critical micelle concentration (CMC) value (Islam et al. 2017). They also show hemolytic activity. This activity is defined as the equilibrium binding of the compound to the lipid bilayer membrane. The new generations have shown less toxicity/ more compatibility with human cells (Kuroda et al. 2009). Gemini alkyl ammonium salts are a new class of gemini surfactants. Their spacers are hydrophobic (aliphatic or aromatic) or hydrophilic (polyester, hydroxyalkyl). Symmetrically gemini alkyl ammonium salts are represented as [m–s–m], where m is the number of carbon atoms in the hydrophobic chain and s is the number of methylene groups in the spacer. These salts have unique micelle formation and adsorption properties in aqueous solutions. For these surfactants, low CMC values and increased spacer length are desirable (Brycki 2010a)

Due to the valuable capacities of gemini surfactants at surface activity, this research was designed to study and compare the antiviral potential of a few synthesized gemini surfactants with different hydrophobic properties against influenza virus H1N1.

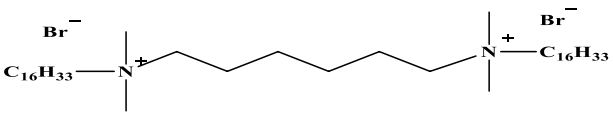
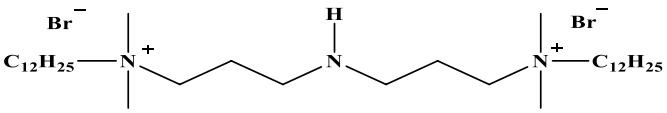
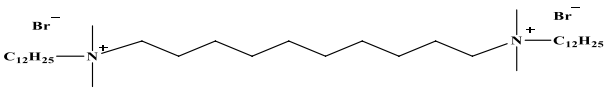
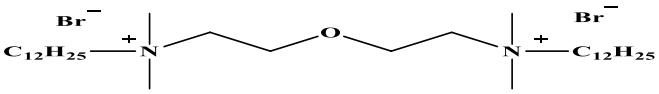
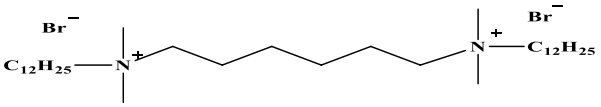
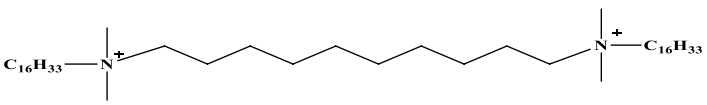
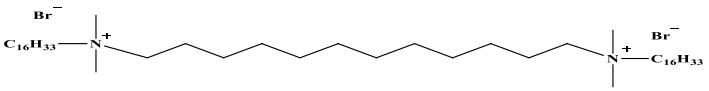
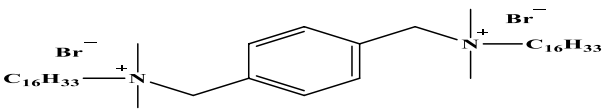
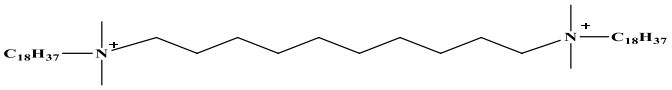
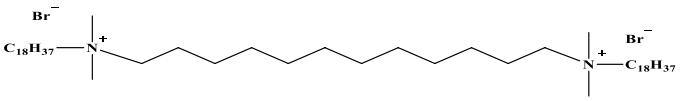
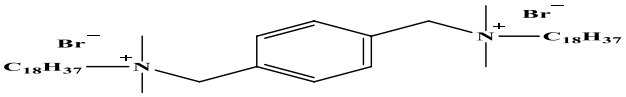
Methods

Preparation of gemini surfactants

Synthesis of gemini surfactants was performed with hydrophilic-lipophilic balance (HLB) at Adam Mickiewicz

University (AMU, Poland). The reactions were proceeded according to the S_N2 nucleophilic substitution mechanism. These reactions rate depends on the concentration of the substrates and the type of solvent. Usually, the synthesis of gemini surfactants occurs in alcohol (Cao et al. 2021; Sharma et al. 2017), acetone (Kuperkar et al. 2012; Dani et al. 2018a) or acetonitrile (Brycki et al. 2011; Laschewsky et al. 2005). A new method developed in our laboratory is the solvent-free reaction at room temperature (Brycki et al. 2017, 2020). In the case of a solvent-free reaction, the reactants concentration is as high as possible. Such reactions occur with high efficiency and without loss. These reactions are consistent with the green solvent method, which reduces solvent use and minimizes cost (Mondal et al. 2015, 2016). This method is suitable only for liquid reagents; therefore, in the case of the synthesis of gemini surfactants presented in this context, we decided to carry out the reaction in the minimum amount of acetonitrile. Due to the specificity of antiviral testes, all syntheses were carried out in an analogous way. The chemical structure, formula, melting point, molecular weight and CMC of the gemini surfactants used in this study are shown in Table 1. The detail protocols for the GS compounds preparation are available in Supplementary File 1.

Table 1 Gemini surfactants specifications

GS	Symbol	Chemical structures	mp (°C)	MW (g/mol)	CMC
(GSA)	16–6-16		220–221	782.99	0.034
(GSB)	12-N-12		133–136	685.79	1.047
(GSC)	12–10-12		143–144	726.88	0.63
(GSD)	12-O-12		248–249	658.72	1.047
(GSE)	12–6-12		223–224	670.77	0.98
(GSF)	16–10-16		164–166	839.11	0.032
(GSG)	16–12-16		155–157	867.17	0.022
(GSH)	16-ph-16		215–216	802.99	0.48
(GSI)	18–10-18		172–174	895.22	0.0213
(GSJ)	18–12-18		133–135	923.27	0.0331
(GSK)	18-ph-18		213–215	859.10	0.29

Mp melting point, *MW* molecular weight, *CMC* critical micelle concentration

All the GS compounds were prepared in 5 ml/ml dH2O. After complete dissolve and getting a clear solution they were used for the toxicity assay.

Cell culture and influenza virus propagation

MDCK cells were cultured in cell culture media (Dulbecco's Modified Eagle's Medium (DMEM) (ICN)), including 10% Fetal Bovine Serum (FBS) and 1% Pen/Strep (Sigma Co.) at 37 °C in a humidified incubator. The influenza A/PR/8/34 (H1N1) virus with American Type Culture Collection (ATCC) Ref NO VR-897™ was propagated in MDCK cells in the presence of Trypsin_TPCK (Tosylamide Phenylethyl Chloromethyl Keton-treated Trypsin) (Sigma, USA) to prepare the working stock. Tissue culture infectious dose 50 ($TCID_{50}$) assay and the Karber formula were conducted to measure virus infectious dose (Karber 1931; Mehrbod et al. 2012).

Cytotoxicity assay

The cells were seeded (3×10^4 cell/well) in 96-well flat-bottom micro-plates with 10% FBS and incubated for 24 h at 37 °C to reach semi-confluency. Then, they were exposed to twofold serial dilutions of the GS compounds (from 2.5 to 0.001 mg/ml) (4 wells/each dilution; 100 µl/well) for 48 h to obtain the cytotoxicity effect of different concentrations. After incubating for the determined time, the cells media were removed and 1X MTT [3-(4,5-dimethyl-2-thiazolyl)-2,5- diphenyl-2H-tetrazolium bromide; Sigma, USA] was added to each well. Following 2–3 h incubation at 37 °C in dark, MTT was discarded and DMSO was added and mixed thoroughly to release the color of formazan precipitations. Optical densities were measured using a Plate reader (StataFax 2100, USA) at 540 nm and the viabilities of the cells were evaluated in Excel using the following formula: (mean OD of treated cells/mean OD of control cells) × 100.

The CC_{50} of the compounds were calculated by drawing dose-dependent response curves.

The twofold serial dilutions of CC_{50} were added to MDCK cells in quadruplicates. Following 48 h the NCTCs were calculated using MTT assay data based on SPSS analysis, One-way ANOVA, *Post-hoc* Tukey test. The EC_{50} of the compounds were also obtained. In brief, the cells were exposed to the virus ($100TCID_{50}$) for 1 h. Then, removed and twofold serial dilutions of the compounds from NCTC were added to the cells. After 1 h incubation and removal of the supernatant, TPCK-containing medium was added and incubated at 37 °C for 48 h. Following incubation time, the HA test was performed to find the EC_{50} of the compounds.

Selectivity index

The selectivity index (SI) was measured by dividing CC_{50} to NCTC (EC_{50}), which represents the compound relative safety. The compounds with a selectivity index higher than 3 are considered potentially safe reagents (Chattopadhyay et al. 2009).

Antiviral evaluation

During antiviral evaluations, media supplemented with FBS was removed, the cells were washed with PBS and then treated as needed.

Co-inoculation treatment (simultaneous treatment assay)

The semi-confluent MDCK cells were exposed to the combination of H1N1 (100 $TCID_{50}$) and NCTC of the GSs for 1 h at 37 °C in a 96-well flat-bottom micro-plate. Following the incubation time, the supernatants were removed and TPCK-containing medium was added to each well.

Pre-inoculation treatment (pre-penetration assay)

The cells were exposed to NCTC of the GSs for 1 h before exposure to 100 $TCID_{50}$ of the virus. Followed by 1 h incubation at 37 °C, TPCK-containing media were added to the wells.

Post-inoculation treatment (post-penetration assay)

The cells were exposed to 100 $TCID_{50}$ of the virus for 1 h before exposure to NCTC of the GSs. Followed by 1 h incubation at 37 °C, TPCK-containing media were added to the wells.

Amantadine hydrochloride and oseltamivir carboxylate were tested in parallel as standard antiviral control groups (Nguyen et al. 2010). The cells with only H1N1 inoculation and the cells with only media (no treatment) were considered as positive and negative controls, respectively. All the plates with different combined treatments were incubated at CO_2 incubator for 48 h. The virus titration was carried out by hemagglutination assay (HA). The viability of the cells was evaluated by MTT assay (Mehrbod et al. 2009).

Percentage of protection

Viability of the cells (infected and non-infected) was obtained using absorbance values of formazan. The percentage of protection was calculated as follows:

$$\text{Percentage of protection} = [A - B]/[C - B] \times 100,$$

where the 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 (Shigeta et al. 1997).

Hemagglutination assay (HA)

To quantify the virus titer in combination with treatment cell supernatants, the HA assay was conducted as mentioned before (Hirst 1942). Briefly, serial dilutions of the supernatants were added to the 96-well U-shaped microplates in two separate duplicates. The HA units were measured as the highest dilution giving complete agglutination with 1% chicken red blood cells (cRBC). The absence and the presence of the virus are demonstrated by the precipitation and diffuse lattice formation of the RBCs, respectively. The test was conducted twice.

Hemagglutination inhibition assay (HI)

To investigate the effect of the compounds on the hemagglutinin activity, the GS compounds were twofold serially diluted from CC_{50} of the compounds till 8 more dilutions. Then, 4 HA unit of the virus was added to each well. After 45 min exposure at room temperature, 1% cRBC was added to the combinations. The physical interaction between compounds and virus surface HA glycoprotein was evaluated after 1 h using the agglutination inhibition pattern.

Apoptosis assay

The protocol of Annexin V- propidium iodid Apoptosis Detection Kit (IQ products) was performed by Flowcyt Science-Based Company, Tehran, Iran (Rieger et al. 2011). Briefly, upon completion of treatments after 48 h, the cells were harvested and washed with calcium-binding buffer. Then, Annexin V and PI were added in turn and incubated in dark for 20 and 10 min on ice, respectively. Subsequently, washing was performed with binding buffer twice. Finally, the samples were analyzed with Bacton Dickson FacsCalibur Flow Cytometer (BD Biosciences, USA).

Statistical analysis

The data expressed as mean ± SD was analyzed by analysis of variance (ANOVA) (SPSS 18.0) Tukey *post-hoc* test. Sample values with $p \leq 0.05$ and $p \leq 0.01$ were considered statistically significant and highly significant, respectively.

Results

Cytotoxicity test results

The CC_{50} values obtained from different compounds were broadly different which can be attributed to their different capacities of them with regard to their structure. The NCTCs which had no significant toxicity on the cell viability were calculated using MTT data and one-way ANOVA as compared to the negative control. The EC_{50} of the compounds were obtained by HA test. No more dilutions of the compounds except for NCTC could give HA positive. Thus, NCTC was selected as EC_{50}. The selectivity index values for the GS compounds were all about 3 and above with the highest and lowest values of 85.340 and 2.667 for J and B compounds, respectively, which shows the safety of the compounds. These results are shown in Table 2.

Antiviral assay outcome

The antiviral capacity of the GS compounds was assessed by calculating the viral titer and cellular percentage of protection in different combination treatments. The effect of the compounds on viral titer was measured by decrements in the Log HA titer (Fig. 1). The upper and lower panels in Fig. 1 show the Log HA decrements from different compound's point of view, and different combination treatments point of view, respectively. As shown in the upper panel, among all the compounds, the highest log HA decrements were observed at 6.5 logs for GSA and GSC (hydrophobic ones) in co-penetration, 4.5 logs for GSC in pre-penetration, and 5.5 logs for GSD (hydrophilic) in post-penetration treatments. Amantadine hydrochloride and oseltamivir carboxylate showed 7 logs of decrement in co-penetration (Ama), and 8 logs of decrement in co-, pre-, and post-penetration treatments (Ose). And from a brief look at the lower panel, it is taken that most of the compounds were effective in lowering the viral titer in co-penetration treatments. Data presented as mean ± SD are averages of three independent HA titrations.

The viability of the cells exposed to the treatments was evaluated as the compounds protection on the cells against

Table 2 CC_{50} and NCTC (EC_{50}) of the compounds

Compound	CC_{50} (μg/ml)	NCTC (μg/ml)	Selectivity index ($SI = CC_{50}/EC_{50}$)
A	7.7 ± 0.0	2.6 ± 1.7	2.9
B	1.9 ± 0.1	0.7 ± 0.3	2.7
C	4.0 ± 0.0	1.2 ± 0.6	3.4
D	4.9 ± 0.1	1.7 ± 0.6	3.0
E	3.4 ± 0.0	0.9 ± 0.5	3.4
F	8.8 ± 0.0	1.7 ± 0.5	5.3
G	11.9 ± 0.0	1.7 ± 1.3	7.1
H	522.2 ± 0.0	36.7 ± 28.6	14.2
I	16.4 ± 0.0	2.6 ± 1.5	6.4
J	48.6 ± 0.0	0.6 ± 0.2	85.3
K	1229.2 ± 0.0	307.3 ± 0.0	4.0
Ama	197.0 ± 1.5	98.5 ± 0.0	2.0
Ose	788.5 ± 6.0	394.2 ± 0.0	2.0

Ama amantadine hydrochloride, *Ose* oseltamivir carboxylate

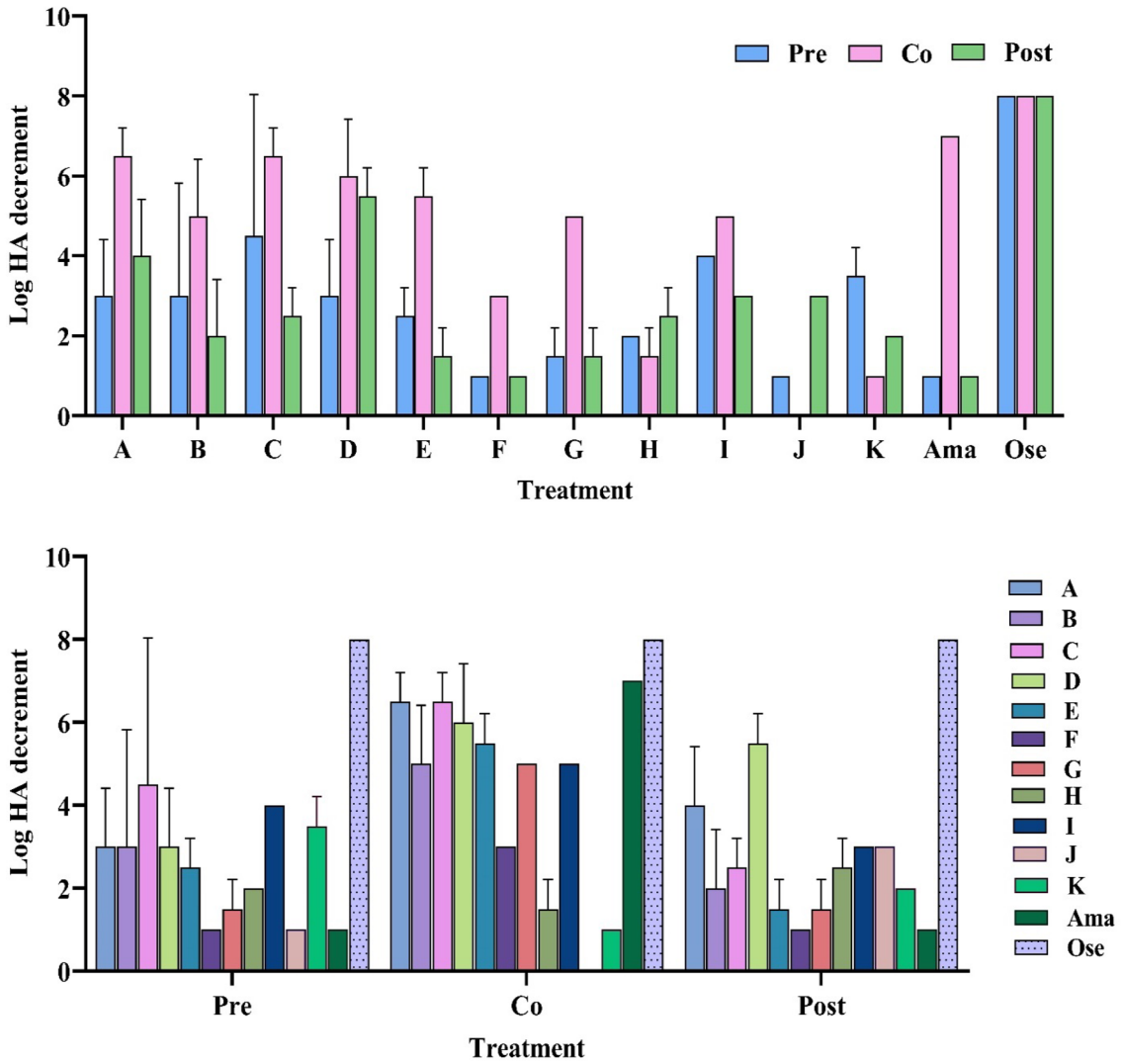


Fig. 1 Log HA decrement of different GS compounds, amantadine and oseltamivir in different combination treatments. Upper panel: Log HA decrement from different compounds treatments point of view. Lower panel: Log HA decrement from different combination treatments point of view

the virus infectivity (Fig. 2). The upper and lower panels in the figure show the cellular protection from different compound's viewpoint and combination treatments viewpoint, respectively. Based on both panels, all compounds in all combination treatments showed favorable protection against virus cytopathic effects (CPE). Data presented as mean ± SD are averages of three independent tests in duplicate. The values of these tests are shown in Supplementary Tables 1 and 2.

Surface interaction results

In this assay, the influenza virus was exposed to GS compounds at different concentrations with no cell (from CC_{50} and continued for eight more twofold dilutions). As shown in Table 3, Some GS compounds no matter whether hydrophobic or hydrophilic, showed hemolytic effect on RBCs in higher concentrations (CC_{50} and 1–2 more dilutions) that is typical for these compounds. Since a certain dilution, they showed physical interaction with virus surface HA glycoprotein, as RBCs precipitated in those wells. Generally, treatment of the influenza virus with GS compounds reduced the heamagglutination activity of the virus in all dilutions,

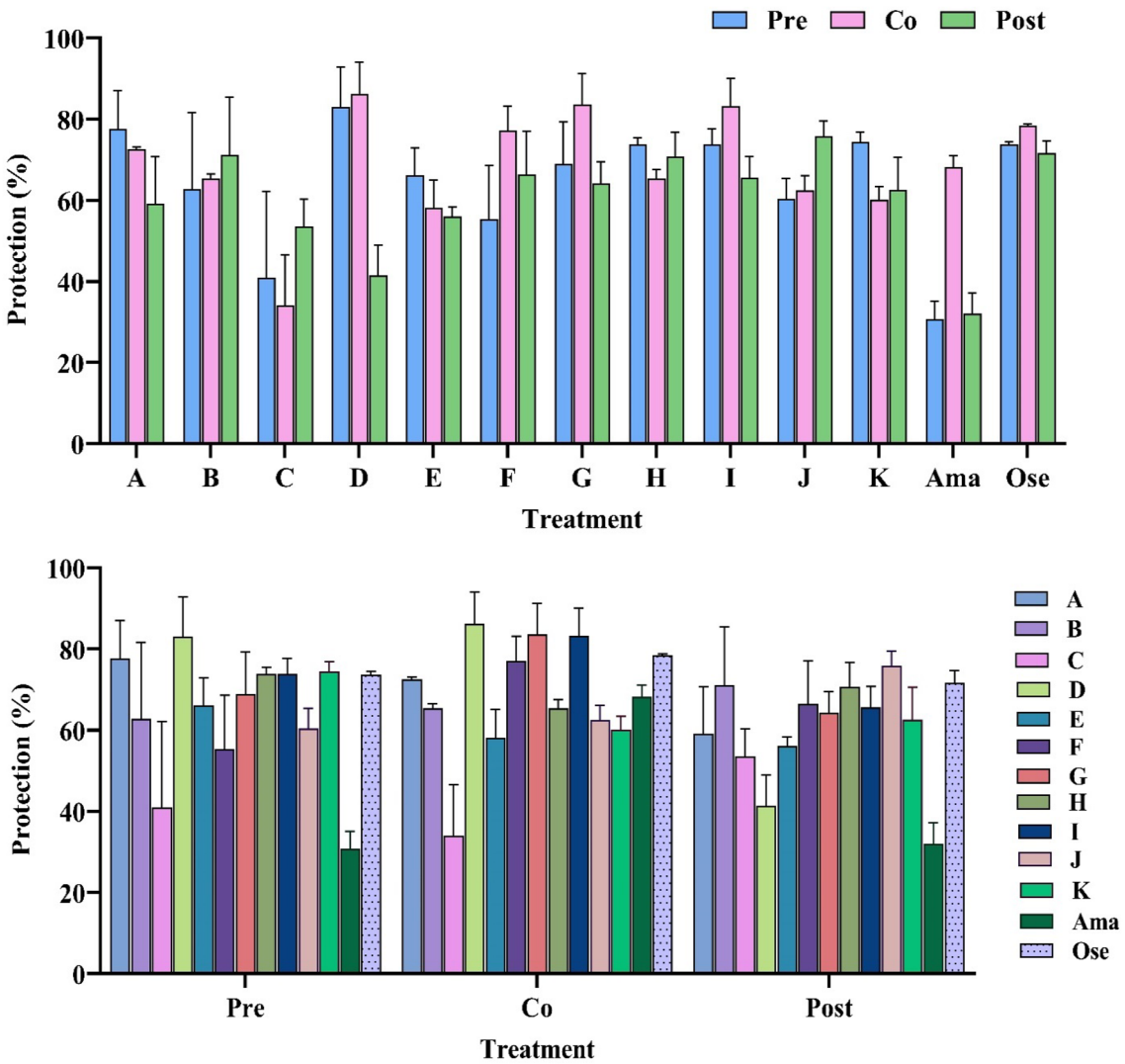


Fig. 2 Percentage of cell protection against H1N1 in combination treatments. Upper panel: Protection from different compounds treatments point of view. Lower panel: Protection from different combination treatments point of view

which is against a dose-dependent manner. This test was repeated four times with similar results. Amantadine and oseltamivir showed HA interaction from their 2nd and 1st dilutions from CC_{50}, respectively.

Apoptotic activity of the compounds

The annexin V-FITC double staining was performed to calculate the percentage of cell death in different treatments. The cells were grouped and revealed in a dot plot (Fig. 3). The results and data analysis are shown in Supplementary Table 3. Late apoptosis (Q2) was found to be the major cell death mechanism in H1N1-infected cells for 48 h (59.60%). Generally, the GS compounds treatments with no H1N1 did not cause any apoptotic event which was similar to the healthy negative control (Q4). In the cells challenged with H1N1 virus, the hydrophobic compounds effectively reduced late apoptotic injury to the healthy condition (Q4) by GSA (54.85%), GSC (37.50%), GSF (70.50) GSG (70.10), GSH (68.05), GSI (77.90), GSJ (68.75), GSK (75.20) and early apoptosis (Q3) by GSE (54.10%) compared to the infected group. But the hydrophilic ones; GSB (49.50%) and GSD (46.70%) were not effective to reverse the late apoptotic injury caused by the virus.

Discussion

In this research we evaluated the antiviral capacity of gemini surfactants looking at their apoptotic effects and surface interactions with virus hemagglutinin glycoprotein.

Table 3 HI results

Compound twofold dilutions from CC_{50}	Dilution to give HI +
A Hydrophobic	Since 1st HI +
B Hydrophilic	1st hemolysis, Since 2nd HI +
C Hydrophobic	Since 1st HI +
D Hydrophilic	1st hemolysis, Since 2nd HI +
E Hydrophobic	1st hemolysis, Since 2nd HI +
F Hydrophobic	Since 1st HI^+
G Hydrophobic	1st hemolysis, Since 2nd HI^+
H Hydrophobic	Since 1st HI^+
I Hydrophobic	1st hemolysis, Since 2nd HI^+
J Hydrophobic	2nd hemolysis, Since 3rd HI^+
K Hydrophobic	2nd hemolysis, Since 3rd HI^+
Ama	2nd
Ose	1st

Ama amantadine hydrochloride, *Ose* oseltamivir carboxylate

Influenza is an infectious disease that causes several morbidities and mortalities in various epidemics and pandemics. However, it can be considered a controllable and preventable disease (Watanabe et al. 2010). One of the most important pathogenic mechanisms in influenza virus infection is apoptosis (Lee et al. 2018). The specific viral molecules synthesis at the early stage of infection may play a crucial role in the apoptosis induction mechanism (Uchide et al. 2002) that can be targeted for some antiviral treatments.

Drugs currently available in the market for the treatment of influenza are oseltamivir and amantadine which inhibit neuraminidase and M2 ion channel, respectively (Razonable 2011). The clinical efficacy of all these drugs is unclear and several cases of drug resistance have been reported (Collins et al. 2009). The virus continuous genetic changes; antigenic drifts and antigenic shifts, cause widespread and dangerous epidemics and pandemics (Blut 2009). The resistance to common drugs and the lack of effective vaccines are the reasons for the unsuccessful treatment of this virus. Therefore, research on new materials to identify anti-influenza strategies for the preparation of medical therapy against influenza virus will be very beneficial.

Amphiphilic compounds with two alkyl chains (hydrophobic tails) and two cationic head groups (hydrophilic groups) separated by a spacer with a covalent bond are called twin or gemini surfactants (GS). The spacer can be either hydrophobic or hydrophilic and can be rigid or flexible (Brycki et al. 2017). The properties of gemini surfactants are related to the balance between hydrophilic-lipophilicity balance (HLB). The hydrophobicity of polymers increases their binding to the lipid bilayers and causes the polymer chains collapse in solution and reduces the affinity of polymers for membranes (Brycki et al. 2017; Kuroda et al. 2009). The cumulative behavior of gemini surfactants in solution is controlled by the cooperation of intermolecular and intra-molecular interactions of the GSs (Han and Wang 2011; Garcia et al. 2017)

The unique properties of the GSs that have a wide range of hydrophilicity-lipophilicity balance make them very useful and innovative materials in detergents, cosmetics, personal care products and additives for paints and coatings, pesticides, in materials science, organic syntheses, pharmaceuticals, textiles, advanced oil recycling, nanotechnology, petroleum and many other branches of life (Brycki et al. 2017; Tyagi and Tyagi 2014).

Gemini surfactants are less toxic to the aqueous environment than monomeric analogues. Compared to their monomer counterparts, the GSs, especially the cationic ones, are more efficient in reducing surface tension and have better wetting properties. The mechanism of their biocidal activity starts with the adsorption of negatively charged quaternary ammonium cations on the cell surface. This is due to the fact that GSs not only have two positively charged nitrogen atoms but also two lipophilic substituents. Consequently, their absorption in the cell wall of microorganisms and subsequent penetration in the bilayer lipid membrane is more efficient (Brycki 2010b). The cationic GSs also have hemolytic activity, which depends on the length of their alkyl chain, while compounds with 10 and 12 carbon atoms show the highest hemolytic activity, surfactants with shorter alkyl chains induce hemolysis only in very high concentration (Łuczyński et al. 2013).

They can easily change their morphological structure with pH, temperature and salt (Brycki et al. 2017; Brycki 2010b). Their reversible conversion from micelle form to other structures, especially vesicles, by changing pH is very useful for their drug delivery capacity. It has been found that GSs are able to form aggregates of micelles or bilayers (Wang et al. 2004).

Various studies have been conducted to design and synthesize the GSs to help treat several life-threatening diseases such as viral diseases (Brycki et al. 2017). The GSs are known as highly active surface compounds (Brycki et al. 2017). They show very good antimicrobial activity against bacteria (Dani et al. 2018b; Maneedaeng et al. 2018), yeasts (Kuperkar et al. 2012; Taleb et al. 2017) and viruses (Mondal et al. 2015). Quaternary ammonium salts have been shown to be effective antiviral compounds against enveloped viruses such as HSV and HIV (Wong et al. 2002; Thevenin et al. 2013), noroviruses (Bolton et al. 2013; Gerba 2015) and influenza virus (Gerba 2015). The efficacy of alkaloid matrine dimer surfactants was investigated against hepatitis B virus (Zhang et al. 2016). Recently, in 2020, the effect of gemini surfactants was

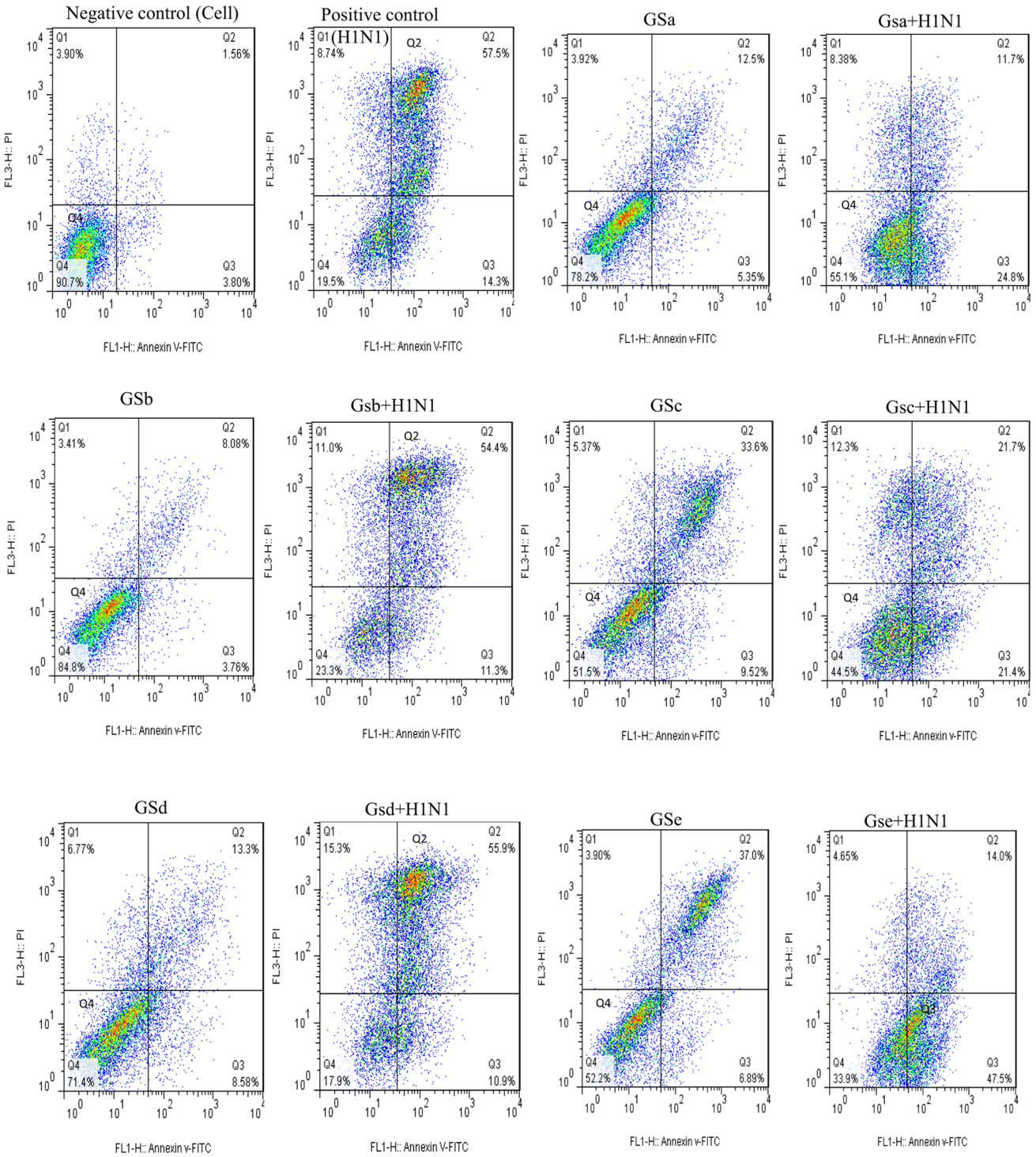


Fig. 3 The results of apoptosis. Cell death evaluation was performed after the effect of different GS compounds on H1N1-induced apoptosis in MDCK cells using a Flow Cytometry analysis. The plots are representative of two independent experiments

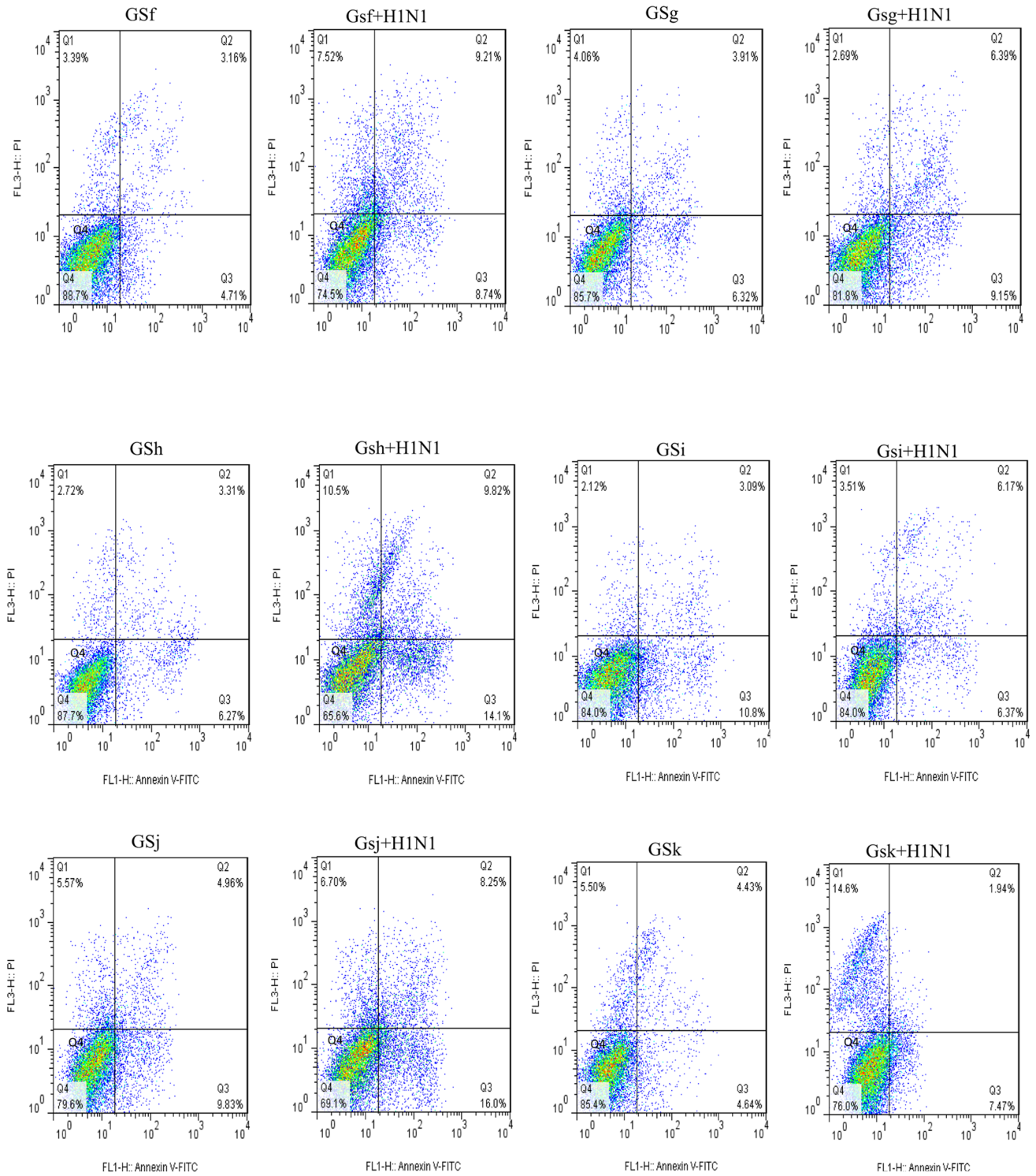


Fig. 3 (continued)

investigated against SARS-CoV-2. The results showed that gemini surfactants perform their antiviral properties by acting on the lipid envelope of the virus (Kaur and Gupta 2020).

In this project, the antiviral capacity of a few surfactants of the fourth generation of QACs with different degrees of hydrophobicity was studied against H1N1, PR/8/34 influenza A virus. First, CC_{50} (cytotoxic concentration 50%), NCTC (non-toxic concentration) and EC_{50} (effective concentration

50%) of the compounds were obtained by the MTT method. The NCTC and EC_{50} values were the same. The selectivity Index (SI) of the compounds was also calculated. The SI value indicates the safety of the compounds for use in cellular experiments (Pritchett Joshua et al. 2014). The SI levels of the GSs were higher than conventional antiviral drugs; amantadine and oseltamivir, indicating the GS compounds safety compared to the conventional drugs.

In the antiviral test, which consisted of three co-, pre- and post-penetration combination treatments, the HA titer decreased in the majority of the tests, especially and mostly in co-penetration treatment to 6.5 logs for hydrophobic ones and then in post-penetration treatment to 5.5 logs for hydrophilic one, which may indicate that hydrophobic GS compounds might be more effective on the surface of the virus that may affect the surfcae glycoproteins of the virus or compete with the virus in binding to the cellular receptors and hydrophilic ones may affect the viral life cycle after adsorption to the cell.

The cell viabilities and percentages of protection were favorable in all treatments that confirms the safety of the compounds.

In total, the compounds with longer hydrocarbon chains, which tend to be more hydrophobic and the presense of counterion like Br, had a greater effect on reducing the HA titer and maintaining cell viability such as A, C and I compounds. The presence of a short and hard spacer with a benzene ring and a double bond had a negative effect on the performance of the surfactants such as H and K compunds.

Apoptosis evaluation was performed using simultaneous treatments of the GS compounds with the virus. The apoptotic cells were resulted from phosphatidylserine (PS) translocation outward in the plasma membrane and loosing cell membrane integrity. Annexin V-FITC−/PI− (Q4) are classified as healthy cells, the cells that are considered early apoptotic showed Annexin V-FITC+/PI− (Q3), whereas late apoptotic (Q2) represents as Annexin V-FITC+/PI+ as well as necrotic (Q1) which represents as Annexin V-FITC−/PI+. The positive control (H1N1 sample) showed late apoptosis which is not unexpected. According to previous studies, influenza virus induces apoptosis (Uiprasertkul et al. 2007; Kar and Sivamani 2015; Atkin-Smith et al. 2018). In the treatment of the cells with gemini surfactants alone and in the presence of the influenza virus, cells showed a healthy pattern. In fact, gemini surfactants in non-toxic concentrations had the potential to inhibit apoptosis induced by the virus, which can be attributed to the hydrophobicity and antiviral properties of the compounds (this property depends on the length of the alkyl chain, spacer and canterion).

Generally, the highest percentage of viable cells was observed in the treatments with GSI (84.850 ± 1.202) and GSF (82.950 ± 8.132) in virus-free treatments. They have long hydrocarbon chains, which increase their hydrophobicity that may increase the percentage of anti-apoptotic cells. The lowest percentages of anti-apoptotic cells belonged to compounds C (37.500 ± 9.900) and E (62.800 ± 18.668) in the virus-free treatments. They have short hydrocarbon chains and short spacer, which can reduce their hydrophobicity and the percentage of anti-apoptotic cells. The results suggest that hydrophobic gemini surfactants but not hydrophilic ones are strong potential anti-apoptotic agents, which act by inhibiting the apoptosis pathway.

The results of the HI test revealed that some gemini surfactants at high concentrations have hemolytic effect on erythrocytes (RBCs) but at a certain dillution they showed physical interaction with virus surface glycoproteins, as RBC precipitation continued even till eight more twofold dilutions from CC_{50}, which is indicative of GS compounds physical interaction with virus HA glycoprotein even in much more diluted concentrations, or it might be attributed to the destructive effect of the GSs on the viral membrane effect.

In total, our compounds potential was consistent with previous researches outcome. Mostly, the hydrophobic GS compounds used in the current project showed more antiviral capacity, which was consistent with Kuroda et al., study that stated hydrophobic nature of surfactants plays an important role in their antiviral and hemolytic activity against lipid and non-lipid viruses (Kuroda et al. 2009). Gerba in a study on influenza virus, HAV, Norovirus, Rotavirus and Enterovirus stated that quaternary alkylammonium compounds reduce the total number of microorganisms and their infectious titer (Gerba 2015), which was confirmed in our study.

Also, among the gemini surfactants used in this project, the ones with long spacer, showed a better antiviral effect that was in accordance with Pisarcik et al., study that showed better antiviral properties for the long-spaced cationic gemini surfactants (Pisárčik et al. 2017).

Regarding the spacer properties, the compounds with flexible and hydrophilic spacers had lower CMC. It was in consistent with previous researches that mentioned gemini surfactants with flexible spacers (methylene units) decrease the CMC values (Kaur and Gupta 2020; Brycki et al. 2017; Garcia et al. 2017; Hussain et al. 2019). Wang and Brycki mentioned that a short hydrophilic spacer with an ether or ester bond reduces CMC (Brycki et al. 2017; Wang et al. 2004). The gemini surfactants with longer hydrophobic chains used in this project also had lower CMC and greater antiviral capacity. This property was in accord with previous studies that highlighted increasing the length of hydrophobic chains reduces CMC (Brycki et al. 2017; Han and Wang 2011; Garcia et al. 2017; Zou et al. 2015).

Also regarding the association between counterions and CMC, researchers mentioned counterions such as dichloride and dichromate reduce CMC (Kaur and Gupta 2020; Brycki et al. 2017). Our compounds contained dibromide and had low CMC, resulting in good antiviral activity.

Conclusion

In summary, the gemini surfactants with long hydrocarbon chains and hydrophobic properties showed the most antiviral capacity against H1N1 by decreasing the viral titer while keeping cell viability at a high level against viral CPE. Conversely, the compounds with short hydrocarbon chain and short/rigid spacer with a benzene ring and double bond showed less antiviral activity. Regardless of the GS compounds hydrophobicity or hydrophilicity, they may have physical interaction with the viral HA surface glycoprotein in any dilution that indicates the ability of the compounds in inhibiting viral attachment to the cell and propagation. The apoptitic evaluation of the GS compounds highlighted their anti-apoptotic potential especially for the hydrophobic ones. Consequently, compounds with long side chains, ion pairs, flexible spacers, and hydrophobicity have higher antiviral capacity. The assessment of these GS compounds on the other cellular pathways such as autophagy and inflammation are under consideration.

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Author contributions Conceived and designed the experiments: PM, performed the experiments: MKh, PM; Analyzed the data: MKh, PM, ZK; Contributed reagents/materials: PM, BB, AS; Wrote the paper: PM, MKh; Comprehensive reading the manuscript: all authors.

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Availability of data and material All data are available in case of need.

Declarations

Competing interests Authors declare that they have no conflicts of interest.

Ethics approval Not applicable.

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