

Development of adjuvant nanocarrier systems for seasonal influenza A (H3N2) vaccine based on Astragaloside VII and gum tragacanth (APS)

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ABSTRACT

Adjuvants are chemical/biological substances that are used in vaccines to increase the immunogenicity of antigens. A few adjuvants have been developed for use in human vaccines because of their limitations including lack of efficacy, unacceptable local or systemic toxicity, the difficulty of manufacturing, poor stability, and high cost. For that reasons, novel adjuvants/adjuvant systems are under search. Astragaloside VII (AST-VII), isolated from *Astragalus trojanus*, exhibited significant cellular and humoral immune responses. The polysaccharides (APS) obtained from the roots of *Astragalus* species have been used in traditional Chinese medicine and possess strong immunomodulatory properties. In the present study, the immunomodulatory effects of a newly developed nanocarrier system (APNS: APS containing carrier) and its AST-VII containing formulation (ANS: AST-VII + APNS), on seasonal influenza A (H3N2) vaccine were investigated. Inactivated H3N2 alone or its combinations with test compounds/formulations were intramuscularly injected into Swiss albino mice. Four weeks after immunization, the immune responses were evaluated in terms of antibody and cytokine responses as well as splenocyte proliferation. APNS demonstrated Th2 mediated response by increasing IgG1 antibody titers, whereas ANS showed response towards Th1/Th2 balance and Th17 by producing of IFN- γ , IL-17A and IgG2a. Based on these results, we propose that APNS and ANS are good candidates to be utilized in seasonal influenza A vaccines as adjuvants/carrier systems.

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1. Introduction

Vaccination is the most effective way to control influenza outbreak, but the inability to produce safe and effective vaccines on a worldwide scale in a short period brings the antigen shortage problems with low immunogenic products. Adjuvants have a crucial role by increasing the vaccine potency, reducing the antigen dose, diminishing the number of immunizations and overcoming stability, safety and bioavailability problems [1–6]. Alum, widely used adjuvant in human vaccines, has been used in inactivated influenza vaccines with significant levels of protection and antibody responses [7]. However, its side effects and safety problems have prompted vaccine industry and researchers to investigate novel adjuvants/adjuvant systems. MF59, an oil-in-water emulsion containing squalene, Tween 80 and Span 85, was approved for use

in flu vaccine (FLUAD) in elderly people [8,9]. The potency of MF59 enhanced the development attempts towards emulsion-based adjuvants. AS03 adjuvant system, composed of squalene and α -tocopherol, was developed and demonstrated HA-specific antibody response and Th1/Th2/Th17 mediated cytokine responses for influenza H3N2 split vaccine [10]. MF59 and AS03 adjuvant systems were used in H1N1 influenza pandemics in 2009, but side effects were reported in some European countries [11].

Saponin based adjuvants/systems such as QS-21, AS01 (liposome, MPL, QS-21) and AS02 (MPL, QS-21, squalene) have been investigated in influenza vaccines because of their cellular and humoral boosting properties, and CD8⁺ T cell-mediated immune response [12,13]. QS-21 and its semi-synthetic analog, GPI-0100, which is synthesized through conjugation of dodecylamine onto glucuronic acid, are clinically tested in a trivalent influenza vaccine (TIV). The results stated that GPI-0100 failed to increase HI titers, but it enhanced antibody and T cell response compared to the unadjuvanted group [7,14]. Moreover, ISCOMs (immune stimulating complexes), which contain a purified fraction of *Quillaja*

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saponin, cholesterol and phospholipid, and Matrix-M™ have also demonstrated antibody and T cell responses in split influenza and pandemic virosomal influenza vaccines, respectively [15,16]. APS, as known as tragacanth gum, is the major component in the water extract of *Astragalus*. Besides it acts as encapsulation/carrier agent, it has widely studied because of their immunomodulatory properties including adjuvanticity, promotion of dendritic cell maturation and activation of B cells [17,18]. AST-VII, a triterpenoid saponin isolated from *Astragalus trojanus*, promotes Th1/Th2 balanced immune response with antigen-specific antibody response and splenocyte proliferation [19–24]. Besides induction capabilities on cellular and humoral immune responses, its higher solubility in water, lesser hemolytic activity at high concentrations, higher stability, and appropriateness to lyophilization make AST-VII a valuable and potent adjuvant candidate.

In the aim of developing novel adjuvant carrier system by using APS and AST-VII, we prepared two formulations, and made an attempt to investigate their effects on seasonal influenza A (H3N2) vaccine in Swiss albino mice. The activities of APNS (*Astragalus* polysaccharide-based adjuvant nanocarrier system) and its AST-VII integrated formulation (ANS: adjuvant nanocarrier system) were evaluated preliminarily concerning induction of antibody titers, cytokine responses, and their splenocyte proliferation capacity.

2. Materials and methods

2.1. Materials

RPMI-1640 (Gibco, USA) and 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma, USA), penicillin-streptomycin (Biological Industries, Israel), fetal bovine serum (Gibco, USA), concanavalin A (Con A) (Biochrome, Germany), lipopolysaccharide (LPS) (Sigma, USA), IL-17A, IL-2, IFN- γ ELISA kits (e-BioScience, CA, USA), QS-21 (Desert King, San Diego, CA) were purchased. Goat anti-mouse IgG, IgG1 and IgG2a peroxidase conjugates were obtained from Southern Biotech. Assoc., Birmingham, AL, USA. A/duck/Hokkaido/5/77 Influenza A (H3N2) was provided from Avian Influenza OIE /FAO Reference Lab., Hokkaido University, Japan as a gift. AST-VII, gum tragacanth (APS), and alum were donated by Bionorm Natural Products and ATA-FEN, respectively (Izmir, TURKEY).

2.2. Experimental animals

Male-female Swiss albino mice (5–7 weeks old) weighing 20–26 g was purchased from Application and Research Center of Laboratory Animal of Ege University (Bornova-Izmir, Turkey). Mice were maintained in groups of 5 under standard conditions of temperature $22 \pm 1^\circ\text{C}$ with regular 12 h light and 12 h dark cycles, and had free access to standard laboratory food and water. The experimental protocol was approved by the Local Ethics Review Committee for Animal Experimentation of Ege University (2016/069).

2.3. Preparation, isolation and inactivation of influenza A virus (H3N2)

H3N2 subtype of Influenza A virus was selected as a model antigen. H3N2 was propagated at 9–10 days old embryonated SPF (specific pathogen-free) chicken eggs. Allantoic fluid was collected at the end of incubation period, centrifuged at 6000 rpm for 10 min and then tested for hemagglutinating activity (HA). For this purpose, serial 10-fold dilutions of allantoic fluid were prepared and injected into embryonated SPF chicken eggs to calculate EID₅₀ by the Spearman-Kärber method [25]. The titer of H3N2 was determined as 1:264 HA. H3N2 was inactivated with 8% of BEI (binary

ethyleneimine) and then, inoculated into 5 SPF eggs and incubated for 3–4 days to confirm the inactivation of virus by HA.

2.4. Preparation of adjuvant nanocarrier system (ANS) based on AST-VII and *Astragalus* polysaccharide (APS)

A PBS solution (10 $\times$, pH 7.4) containing 1% ethanol and 3% glycerol was prepared by mixing on a hot plate. Once the solution reached 80 $^\circ\text{C}$, 3 mg/ml of APS was transferred to the PBS solution and vortexed vigorously for over 5 min. The mixture was incubated for 1 h in a sonic bath at 80 $^\circ\text{C}$ and then left at the room temperature for cooling. 1 mg/ml AST-VII solution in PBS (10 $\times$, pH 7.4) was transferred into the mixture and vortexed until a homogenous solution was obtained. pH of the APS/AST-VII preparation (ANS) was adjusted to 11 with NaOH (10 mM) and then dropped to 5.0 by HCl (10 mM). APNS was prepared following the same methodology as ANS (Fig. 1) without adding AST-VII into the mixture. Particle size distribution and surface zeta potential of the samples were measured via Malvern Nanosizer/Zetasizer Nano-ZS ZEN 3600 (Malvern Instruments, USA). Morphological analysis was done using JEM1230 transmission electron microscope (JEOL) (80 kv). Samples were negatively stained with 2% uranyl acetate.

2.5. Immunizations

Alum was prepared by mixing $\text{KAl}(\text{SO}_4)_2$ with NaOH and incubated overnight with inactivated H3N2 by permanent agitation. AST-VII and QS-21 adjuvants were dissolved in saline. All adjuvant preparations were filtered through a 0.22 μm sterile filter and added into inactivated H3N2 (256 HA) with simple admixture. Mice were immunized intramuscularly with following vaccination groups: control (saline), inactivated H3N2 alone (256 HA), APNS

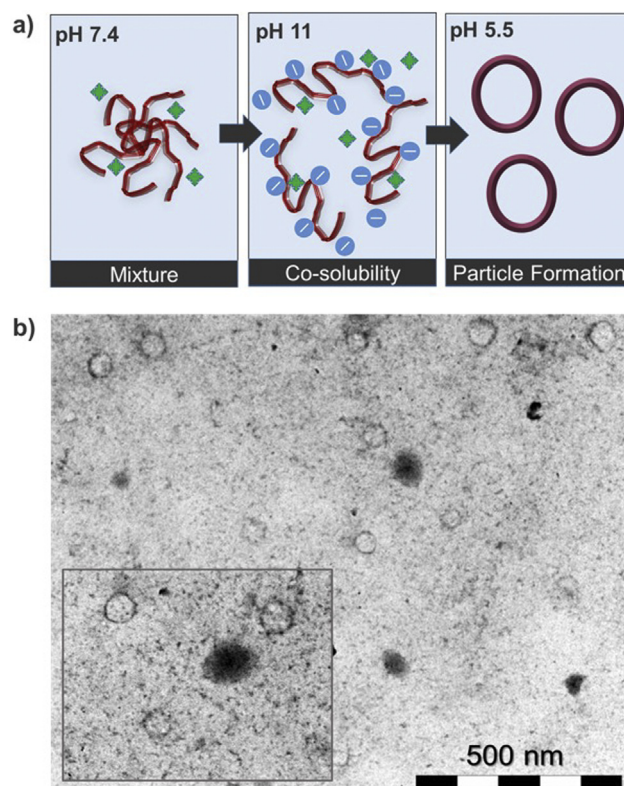


Fig. 1. (a) Schematic illustration of ANS preparation through pH driven electrostatic interaction and (b) TEM image of as prepared adjuvant carrying nanoparticles. Inset: magnified image of ANS.

alone, inactivated H3N2 (256 HA) combined with different adjuvants such as QS-21 (10 µg/ml), AST-VII (10; 25; 75 µg/ml), APNS and ANS. Four weeks after immunizations, mice were sacrificed by cervical dislocation. Spleens and sera were collected for splenocyte proliferation assay, measurement of neutralizing antibody, and H3N2 specific antibody, and cytokine response.

2.6. Splenocyte proliferation assay

Splenocyte proliferation assay was performed according to a modified method by Nalbantsoy et al. [22]. Single splenocyte suspension was obtained by gently mincing and grinding the spleen fragment through steel mesh in complete RPMI-1640 medium (10% FBS, 100 U/ml penicillin/streptomycin, 2 mM L-glutamine, 0.05 mM β-mercaptoethanol). The splenocytes were treated with Concanavalin A (Con A-final concentration 5.0 µg/ml), lipopolysaccharide (LPS-final concentration 1.0 µg/ml), inactivated H3N2 (4 HA) and incubated for 72 h at 37 °C. The stimulation index (SI) was calculated based on the following formula: SI = The absorbance value for mitogen-stimulated cultures/The absorbance value for non-stimulated cultures. Each experiment was performed in triplicate.

2.7. Determination of H3N2-specific antibody response

H3N2 specific antibodies (IgG, IgG1, IgG2a) were detected by ELISA according to a modified method by Nalbantsoy et al. [22]. ELISA plates were coated with 100 µl inactivated H3N2 (8HA) in the coating buffer for overnight at +4 °C. After washing steps, 100 µl of 1:100 diluted samples were added into the wells and incubated for 1 h at 37 °C incubator. 100 µl of horseradish peroxidase-conjugated antibodies for IgG, IgG1 or IgG2a (diluted in 1:8000) were added into the wells followed by washing three times and incubated 1 h at 37 °C. After washing steps, 100 µl substrate solution (3,3',5,5'-Tetramethylbenzidine: TMB) was added and incubated in room temperature for 30 min. The reaction was terminated by adding 50 µl/well stop solution (H₂SO₄). The optical density (OD) was measured in ELISA plate reader at 492 nm. Data were calculated by subtracting the mean OD value of the control from the mean OD value of the samples. The results were expressed as log 2 titers.

2.8. Assessment of cytokine response

IL-2, IFN-γ, IL-4 and IL-17A concentrations in the sera of mice were determined by using commercial ELISA kits (e-Bioscience, Austria). All steps were performed in accordance with the manufacturer's instructions. Sensitivity of ELISA kits were 5.3 pg/ml, 5.3 pg/ml, 2 pg/ml, 4 pg/ml for IL-2, IFN-γ, IL-4, IL-17A, respectively.

2.9. Hemagglutination inhibition (HI) assay for determination of post-vaccination antibody production in sera

The following procedure was applied to measure HI titers [26]. Briefly, 25 µl of PBS was added to all V-bottom 96-well microplate. Then, 25 µl of sera obtained from vaccination groups was transferred into first wells and serially diluted 1:2. After that, 25 µl of inactivated H3N2 virus (4 HA) and 1% mouse RBC's (red blood cells) (25 µl) were added into the wells. The plate was shaken and incubated at 22 °C for 40 min. Finally, hemagglutination inhibition titers were measured at the dilution in which RBC did not settle and the final titers of hemagglutination were present.

Hemagglutination assay was performed by same procedure with HI assay. 25 µl inactivated virus was added to first wells of V-bottom 96-well microplate and serially diluted 1:2. After addition of RBC's, the hemagglutination (HA) titer was determined as the last titer that gives the lace like appearance.

2.10. Evaluating the protective response of mice sera obtained from H3N2 vaccine formulations in chicken egg model

The protection studies were made in embryonated hen's eggs. The sera of each vaccination group were collected, pooled and mixed with 4 HA live H3N2 virus at a 1:1 ratio and incubated at 37 °C for 30 min. Then, 100 µl of mixture was inoculated into 10 days-old embryonated hen's egg and incubated at 37 °C for 5 days. After 5 days, the development of embryos was analyzed, and allantoic fluids were collected for HA assay.

2.11. Statistics

The data were measured with mean standard errors. Statistical significance of differences was examined by using Student-*t* test, One-way ANOVA, and Tamhane's T2 as the post hoc test (GraphPad Prism 5.01 and SPSS for Windows). P values of less than 0.05*, 0.01** and 0.001*** were stated as statistically significant.

3. Results

3.1. Adjuvant nanocarrier system preparation and characterization

In the pH range of 5–6, APS (gum tragacanth) solutions are acidic and negatively charged due to carboxylic groups of galacturonic acid (pKa = 3.48 at 25 °C) [27]. The chemical structure of gum tragacanth and AST-VII was shown in [Supplementary Fig. 1](#).

APS is used mostly for food stabilization where steric repulsion forces controlled by pH is the main mechanism. At pHs closed to the isoelectric point of the polysaccharide, the insoluble complex size decreases until reached to the critical structure forming pH [28]. Herein, APNS and ANS were obtained based on molecular aggregation of the polysaccharides solubilized at pH 11 by turning the pH back to pH 5.5 where nanosized insoluble complexes [29] were formed as schematized in [Fig. 1a](#).

Synthesized particles containing AST-VII were first analyzed with TEM and as depicted in [Fig. 1b](#), finely shaped nanovesicles with defined borders were observed. Particle size distribution of APNS, ANS, and alum were then measured with DLS (Dynamic Light Scattering) analysis. Particulates with 23.02 ± 2.24 nm, 47.16 ± 4.93 nm and 39.78 ± 5.05 nm in diameter were achieved, respectively ([Fig. 2](#)). Integration of AST-VII into APS resulted in 51 percent increase in nanoparticle size compared to APNS which was consistent with the TEM results. All formulations showed negative surface zeta potential (−22.97 mV, −8.2 mV and −13.13 mV, respectively) in PBS. Negatively charged surface of APNS and ANS is due to the presence of carboxylic acid groups of galacturonic acids. AST-VII addition caused a decrease on the surface charge ([Fig. 2d](#)) substantiating successful integration of AST-VII. All groups showed smaller polydispersity index below 0.5 that indicated homogeneous size distribution of the formulations in aqueous solution.

3.2. Assessment of cytokine response

The type of immune response (Th1/Th2/Th17) induced by adjuvant-antigen formulations was investigated based on the cytokine profile (IL-2, IFN-γ, IL-4 and IL-17A) in the sera. There was no statistically significant difference in the production of IL-2 compared to the inactivated H3N2 group. IL-4 titers were similar in each vaccination group compared to the control. QS-21 (1.17-fold) and ANS (1.32-fold) boosted the production of IFN-γ, APNS and AST-VII did not make an additive effect on IFN-γ production. AST-VII administration triggered the IL-17A production at all concentrations [10 µg/ml 1.20-fold, 25 µg/ml 1.15-fold (*p* < 0.05), 75 µg/ml 1.19-fold (*p* < 0.05)] compared to the inactive H3N2

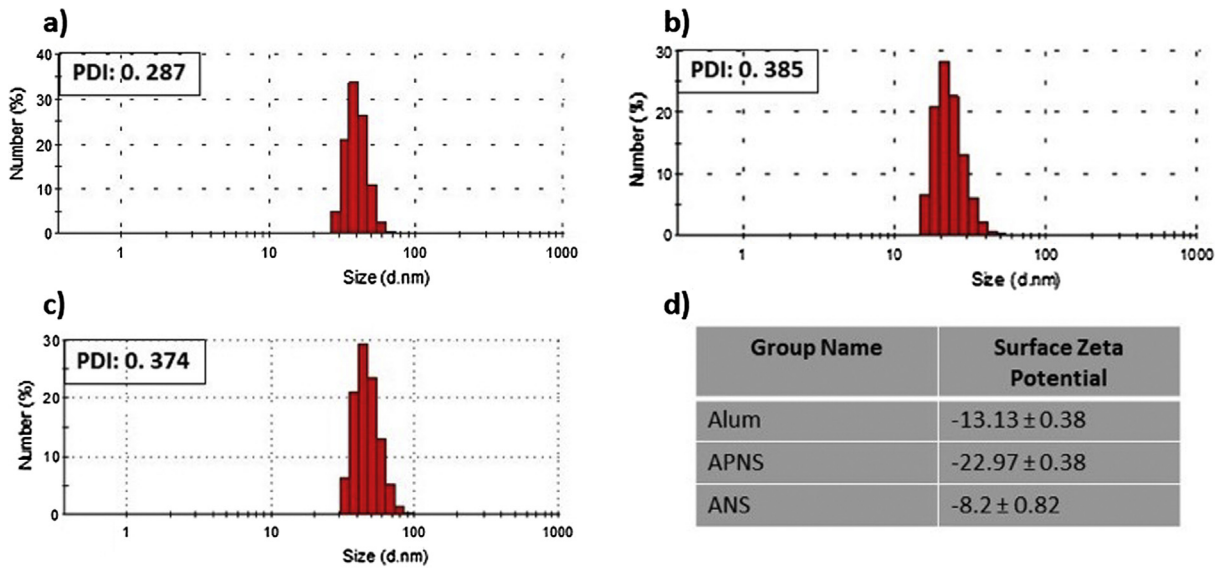


Fig. 2. Particle size distribution and PDI values of nanocarriers made of (a) Alum, (b) APNS, (c) ANS and (d) surface zeta potentials of Alum, APNS, ANS.

group. Moreover, QS-21 (1.22-fold, $p < 0.05$) stimulated higher levels of IL-17A than the inactivated virus as shown in Fig. 3. ANS slightly increased the IL-17A titers (1.14 fold).

3.3. Evaluation of H3N2 specific antibody response

H3N2 specific IgG, IgG1 and IgG2a antibody levels in the sera were measured by ELISA. QS-21, APNS and ANS demonstrated H3N2 specific IgG, IgG1 and IgG2a antibody response as shown in Fig. 4. Alum did not exhibit antibody response in this study. AST-VII at the concentration of 10, 25 and 75 µg/ml increased IgG1 antibody titers (1.17-fold, 1.5-fold and 1.34-fold, respectively). AST-VII at the concentration of 25 µg/ml was responsible for the statistically significant enhancement of IgG1 production ($p < 0.05$). Induction of the IgG2a titers by AST-VII was lower than inactivated H3N2; however, the response increased in a dose-dependent manner. QS-21 increased the production of IgG (1.24-fold, $p < 0.001$), IgG1 (1.89-fold, $p < 0.01$) and IgG2a (1.20-fold) compared to the inactivated H3N2 group. APNS with inactivated H3N2 boosted the titers of IgG (1.25-fold, $p < 0.01$) and IgG1 (1.81-fold, $p < 0.01$) as same as QS-21. ANS induced higher IgG1 (1.43-fold, $p < 0.05$) levels than inactivated H3N2. IgG2a titers treated by ANS augmented by 1.33-fold regarding inactivated H3N2, but this response was not statistically significant.

3.4. Investigating the potential of vaccine-induced splenocyte proliferation

The effects of adjuvanted/non-adjuvanted H3N2 vaccines on mitogens (LPS and Con-A) and H3N2 stimulated splenocyte proliferation are shown in Fig. 5. ANS induced splenocyte proliferation 1.49-fold ($p < 0.001$) and 2.17-fold ($p < 0.01$) compared to the inactivated H3N2 group in LPS and Con A re-stimulated splenocytes, respectively. QS-21 stimulated proliferation by 1.24-fold and 1.88-fold ($p < 0.05$) in LPS and Con-A re-stimulated splenocytes, respectively. Co-administration of APNS or AST-VII with H3N2 did not elicit any stimulation on splenocytes. However, integration of both in a system (ANS) altered their stimulatory capacity in inactivated H3N2 vaccine.

3.5. Hemagglutination inhibition (HI) assay for determination of post-vaccination antibody production in sera

The sera from each vaccination group were collected and tested by HI assay to evaluate neutralizing antibodies against H3N2. HI titers, which have threshold of 1:40 is indicated as a 50% protection against influenza [30]. Alum (1:64), QS-21 (1:256), APNS (1:128) and ANS (1:64) with inactive H3N2 provided sero protection

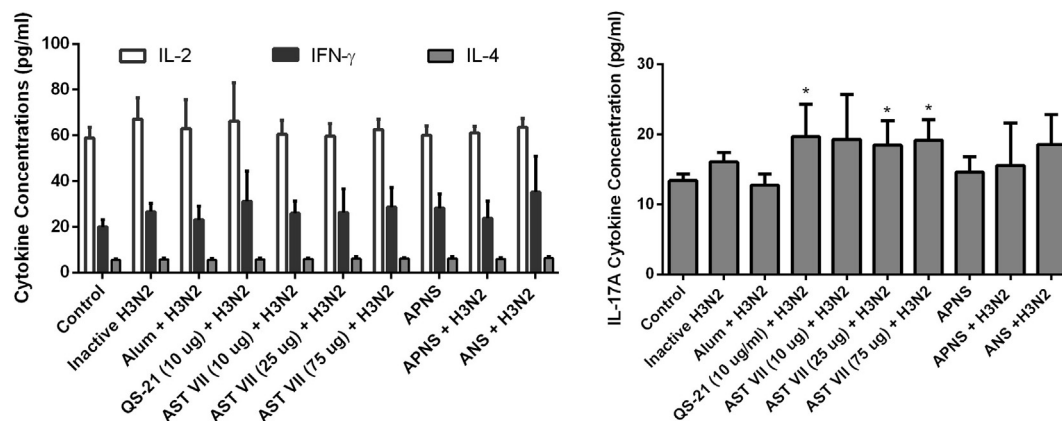


Fig. 3. The effects of adjuvanted/non-adjuvanted H3N2 vaccine on the production of IL-2, IFN- γ , IL-4 and IL-17A. Swiss albino mice were immunized intramuscularly with inactivated H3N2 alone or combined with alum, QS-21 (10 µg/ml), AST-VII (10, 25, 75 µg/ml), APNS and ANS. Four weeks after immunization, the sera were collected and analyzed regarding cytokine production by ELISA. The significant difference was defined between sample groups and inactivated H3N2: * $p < 0.05$.

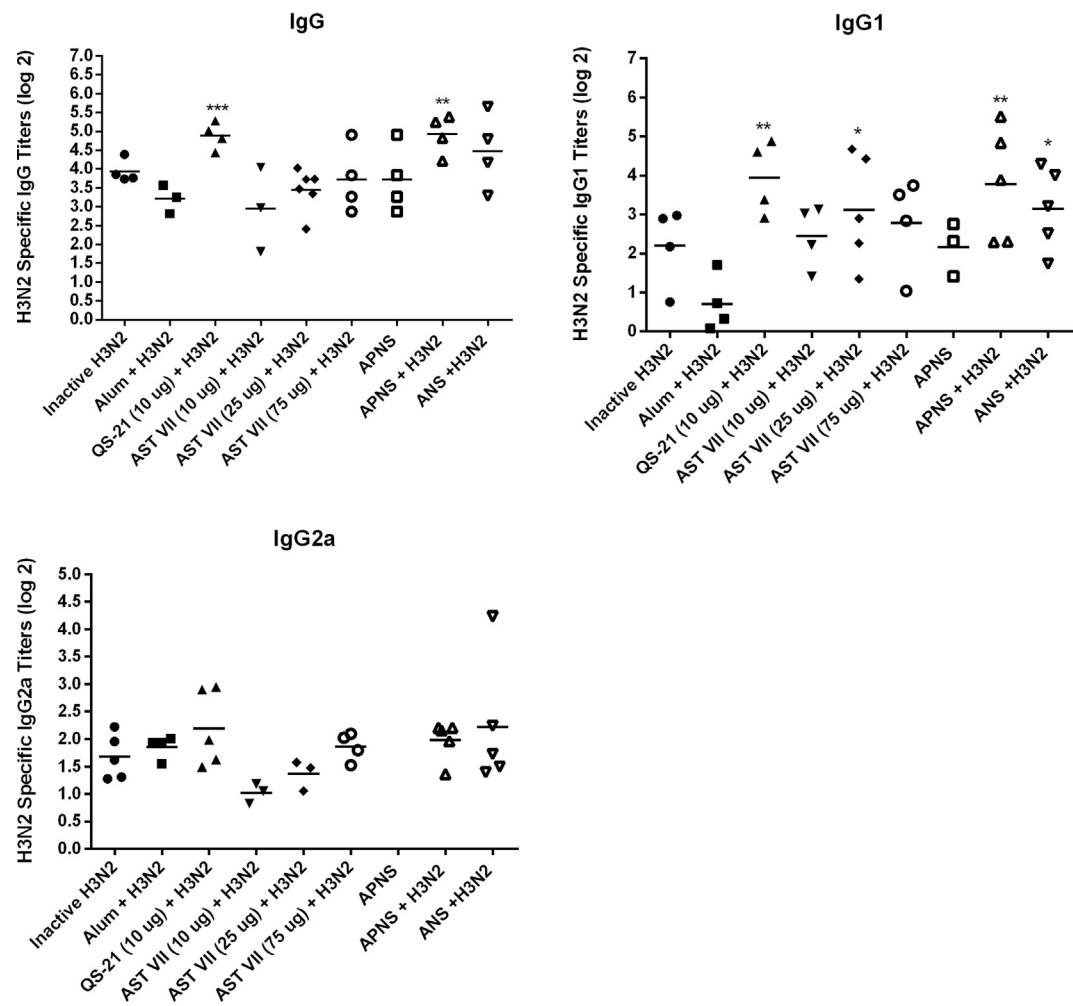


Fig. 4. The effects of adjuvanted/non-adjuvanted H3N2 vaccine on the production of H3N2 specific IgG, IgG1 and IgG2a. Swiss albino mice were immunized intramuscularly with inactivated H3N2 alone or combined with alum, QS-21 (10 µg/ml), AST-VII (10, 25, 75 µg/ml), APNS and ANS. Four weeks after vaccinations, the sera were collected and analyzed by ELISA to detect H3N2 specific antibodies. The significant difference was defined between sample groups and inactivated H3N2: **p* < 0.05, ***p* < 0.01, ****p* < 0.001.

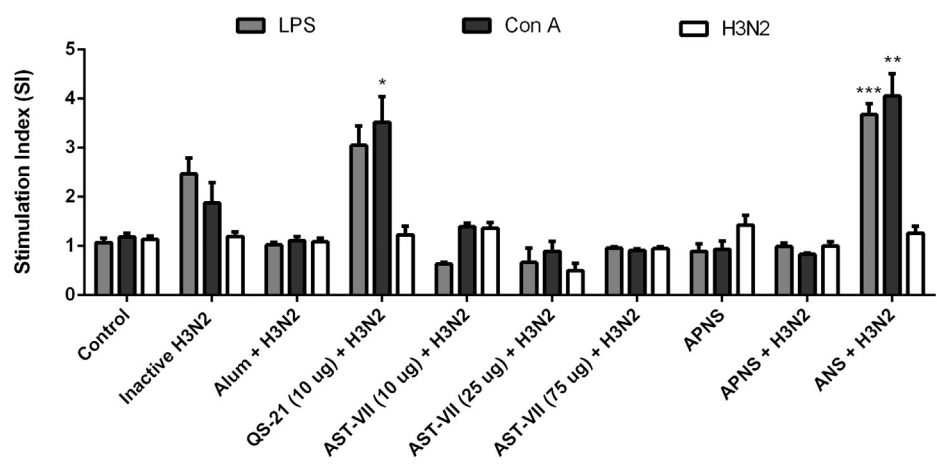


Fig. 5. Stimulation indexes of LPS, Con A and H3N2 re-stimulated splenocytes collected from mice immunized with H3N2 alone or formulated with adjuvants. Four weeks after immunizations, the spleens were harvested and passed through a sterile mesh to obtain single splenocyte suspension. The splenocyte suspension was re-stimulated with Con-A, LPS and inactivated H3N2 and incubated for 72 h. The significant difference was defined between sample groups and inactivated H3N2: **p* < 0.05, ***p* < 0.01, ****p* < 0.001.

Table 1
Hemagglutination inhibition titers of different vaccination groups.

Vaccination group	HI titers
Control	–
Inactive H3N2	1:64
Inactive H3N2 + Alum	1:64
Inactive H3N2 + QS-21 (10 µg)	1:256
Inactive H3N2 + AST VII (10 µg)	1:16
Inactive H3N2 + AST VII (25 µg)	1:32
Inactive H3N2 + AST VII (75 µg)	1:32
APNS	–
Inactive H3N2 + APNS	1:128
Inactive H3N2 + ANS	1:64

against H3N2 (Table 1). Based on the HI titers, H3N2 *ex-vivo* challenge studies were performed.

3.6. Evaluating the protective response of mice sera obtained from H3N2 vaccine formulations in chicken egg model

The sera of each vaccination group, which had higher HI titers than 1:40, were collected, pooled and mixed with 4 HA live H3N2 virus. This mixed solution was inoculated into 10 days-old embryonated hen's egg and incubated for 5 days. After 5 days, the allantoic fluids and embryos were examined in terms of protective response induced by H3N2 vaccine formulations. In all vaccinated groups, growth deficiencies, early death and hemorrhage were observed in the embryos. However, these development defects were slightly lower in inactive H3N2 + APNS and inactive H3N2 vaccination groups than other groups (Supplementary Fig. 2). The rapid hemagglutination assay was performed on allantoic fluids as shown in Supplementary Fig. 3. In inactive H3N2 vaccinated group, exposure of the sera to live and killed H3N2 virus did not lead to hemagglutination. Alum adjuvanted vaccine resulted in hemagglutination inhibition in killed virus exposure, whereas APNS adjuvanted vaccine elicited no hemagglutination in live H3N2 virus along with slight hemagglutination in killed H3N2 exposure. Compared to HI titers and *ex-vivo* challenge studies, APNS was found to be the most promising formulation in H3N2 vaccine to provide protection against H3N2.

4. Discussion

Annual influenza vaccination by administering TIV/QIV (trivalent or quadrivalent inactivated influenza vaccine) is the most effective way to prevent influenza infection. These vaccines induce antibody response to prevent initial infection, but they lack cell-mediated immune response. CD8⁺ T cell and CD4⁺ T cell mediate the viral clearance after infection in mice [31]. Novel vaccination strategies aim to induce humoral and cellular immune responses. Utilization of the right adjuvant(s) in the vaccine formulations determines the type and size of immune response elicited by vaccinations [32].

In this study, an adjuvant nanocarrier system was prepared by using AST-VII, the first tridesmosidic saponin isolated from natural sources, and APS, a major polysaccharide in the root tissue coexisting together with AST-VII. We aimed to develop novel adjuvant system, which induces humoral and cellular immune responses, enhances the production of neutralizing antibody response and provides protection against influenza A (H3N2) *ex vivo*. The physical properties of the newly formulated APNS and ANS, demonstrated similar particle size (23 and 47 nm, respectively) and surface charge (negatively charged) as saponin based particulate adjuvants, viz. ISCOMATRIX and ISCOMs (40–50 nm). ISCOMATRIX adjuvanted recombinant influenza nucleoprotein demonstrated high anti-IgG antibody and strong Th1 immune response [33–35].

The type of immune response (Th1/Th2/Th17) induced by AST-VII, APNS and ANS were examined by measuring cytokine and antibody levels in the sera. There was no enhancement of IL-4 titers in all vaccinated groups. IL-2 response in the adjuvanted vaccine demonstrated similar levels of augmentation as inactivated H3N2 vaccine. IFN- γ titers were increased by the administration of QS-21 and ANS, but the response is not statistically significant. IL-17A response was induced by immunization of QS-21, AST-VII at the concentrations of 25 and 75 µg ($p < 0.05$). APNS did not alter IL-17A levels in the sera, whereas the integration of AST-VII into the nanocarrier system led to slight increase in IL-17A production. In previous studies, AST-VII co-administrated with LPS and BSA (bovine serum albumin) was able to induce the production of IL-2, IFN- γ , TGF- β [22,23]. However, AST-VII did not increase IL-2 or IFN- γ production over inactivated H3N2 vaccine. When ANS was compared with other particulate adjuvants tested in influenza vaccine, Th1 and Th17 mediated immune response in terms of induction of IFN- γ and IL-17A titers compared to the other cytokine subtypes can be specified. Saponin based particulate adjuvant Matrix M, having 40 nm particle size and containing the mixture of two purified *Quillaja* saponins fractions Matrix-ATM and Matrix-CTM, combined with virosomal H5N1 vaccine induced Th1/Th2 balanced immune response with high levels of IL-2, IFN- γ , IL-5, IL-10 and high antibody titers *in vivo* [36,37]. Cationic liposome adjuvant system CAF01, combined with trivalent influenza vaccine (TIV) enhanced influenza-specific serum antibody titers and Th1/Th17 immune response in terms of secreting IL-1 β , IL-2, IL-12, IFN- γ , TNF- α and IL-17 cytokines *in vivo* [38]. AS03 adjuvanted H1N1 vaccine administrated to Balb/c mice, and elicited Th1/Th2 mixed and Th17 immune response with high levels of IL-2, IL-17 cytokines [39]. The role of Th17 response in influenza vaccine is unclear, but its effect can be beneficial or detrimental. Previous studies pointed that IL-17 protects mice against lethal influenza A (H1N1 and H3N2) infections by recruiting B cells to the pulmonary site of the infection and contributing better survival percentage [40,41]. On the other hand, the IL-17 response could be the result of severe pulmonary immunopathology after influenza infection [42]. Enhancement of IL-17 titers induced by QS-21 and ANS were parallel to IFN- γ levels. Th17 and Th1 immune cells recruited each other for the activation of virus-fighting T cells and removal of the virus [43]. According to these results, Th1/Th17 immune response induced by ANS could play a role in the viral clearance. The reason for the low level of IL-2 following treatment of AST-VII and ANS could be the presence of IL-17A leading to a negative regulation on IL-2 production [44].

QS-21, APNS and ANS increased H3N2 specific IgG, IgG1, IgG2a antibody response. IgG1 is an indicator of Th2 whereas IgG2a antibody represents Th1 mediated immune response. While alum and MF59 adjuvanted influenza vaccines generally promote Th2 immune response, Quil-A saponin fraction demonstrates Th1/Th2 balanced immune response [45]. MF59 and CpG combination in influenza vaccine enhanced Th1 immune profile with the induction of IFN- γ and IgG2a antibody response [46]. It is clear that ANS reveals Th1/Th2 balanced immune response via IgG1 and IgG2a production. The levels of IgG2a induced by ANS was slightly higher than the levels of QS-21 and APNS. This response is consistent with ISCOM mediated IgG1 and IgG2a antibody response in influenza [47]. AdvaxTM, a natural plant-derived polysaccharide adjuvant, demonstrated Th2 driven immune response with IgG1 antibody and IL-13 cytokine production in influenza split vaccine [48]. Similar to AdvaxTM, APNS demonstrated Th2 bias immune response by amplifying IgG1 antibody titers, but it did not exhibit any effect on IL-4 production.

The spleens were collected four weeks after administration of adjuvanted/non-adjuvanted H3N2 vaccines to evaluate cell-mediated immune response. ANS significantly enhanced the

splenocyte proliferation in LPS and Con A re-stimulated splenocytes. It was more effective than QS-21 in LPS (1.21-fold) and Con A (1.15-fold) stimulated splenocyte proliferation. There were contradictory results in AST-VII induced splenocyte proliferation. In the previous study, AST-VII exhibited a stimulatory effect on LPS and BSA induced splenocytes, but co-administration of AST-VII with H3N2 did not alter this response [22,23]. APNS with H3N2 did not elicit splenocyte proliferation, although there was a report explaining the enhancement of cell proliferation by APS [44]. Reduced splenocyte proliferation following administrations of AST-VII and APNS could result from immune suppressive properties of influenza virus [49].

The H3N2 virus protection of our vaccine formulations was evaluated by *ex vivo* challenge studies in embryonated hen's egg and hemagglutination inhibition assay. In all vaccination groups, inactive H3N2 (1:64), inactive H3N2 + alum (1:64) and inactive H3N2 + QS-21 (1:256), inactive H3N2 + APNS (1:128), inactive H3N2 + ANS (1:64) give higher HI titers than 1:40, an accepted titer for protection generally. These groups were evaluated for H3N2 virus protective response by inoculating vaccine formulations into 10 days-old embryonated hen's egg. The morphological alterations in the embryos and hemagglutinating activity of the collected allantoic fluids revealed no/slight protection against H3N2 virus. AST-VII (75 µg) was not protective as the QS-21 adjuvanted vaccine. The inactive H3N2 and inactive H3N2 + APNS vaccine formulations displayed hemagglutination in the allantoic fluid and slight protection in the embryos compared to the other formulation groups.

In conclusion, APNS demonstrated significant antibody response driven Th2 mediated immunity, neutralizing antibody response and modest protection in the embryos. Besides its encapsulation properties, APNS can be used as an immunomodulatory agent in vaccines where Th2 type immune response is needed. ANS elicited Th1/Th2 balanced antibody response along with lesser quantities of Th17 type cytokine production, and it exhibited a neutralizing antibody response with remarkable splenocyte proliferation. Based on these data, APNS and ANS have a potential to be used as an adjuvant in influenza vaccines in terms of mediating cellular and humoral immune responses and induction of neutralizing antibody response. One should take into consideration that this is an initial attempt to develop a novel adjuvant system, and further optimization studies with formulations will be carried out to obtain desired immune response. It is also warranted to test the efficiency of the developed nanocarrier system including AST-VII on the other viral vaccines to put forward its potential as a new candidate for clinical use.

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Conflict of interest

The authors declare that they have no conflict of interest.

Appendix A. Supplementary material

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.vaccine.2019.05.038>.

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