Innocuity and anti-Newcastle-virus-activity of *Cladosiphon okamuranus* fucoidan in chicken embryos

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ABSTRACT This study evaluated the potential toxicity and antiviral activity of fucoidan from *Cladosiphon okamuranus* against Newcastle disease virus (NDV), one of the most serious threats to the poultry industry in the world. Toxicity was assayed on chicken embryo fibroblast (CEF) secondary cultures at concentrations ranging from 0.1 to 1500 μg per mL culture medium, assessing the cell viability by the yellow tetrazolium MTT (3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide) assay, and on 9-day-old embryonated chicken eggs by inoculation of 2 to 500 μg doses in the allantoic cavity, assessing the embryos morphology and liver histology. At 48 h post-inoculation, viability of CEF exposed to concentrations up to 10 μg/mL was not significantly affected, and the 50% cytotoxic concentration was estimated as of 1062 μg/mL; after exposure in ovo, some chick embryos showed liver steatosis when treated with fucoidan doses over 20 μg per egg (15 to 28% at 200 μg, 27 to 56% at 500 μg), but no change was detected in their size or aspect. Antiviral activity was tested by treating 9-day-old embryos via the allantoic route with 0.25 to 16 μg fucoidan doses that were applied at different times (−1, 0 and +1 h) relative to the inoculation of 10,000 folds the 50% Tissue Culture Infective Dose ($TCID_{50}$) of the NDV, La Sota strain. At 72 h post infection, virus titration in the allantoic fluid by hemagglutination assay (HA) showed a considerable and significant inhibition of infectivity for all doses, the best result (a 90% decrease) being obtained in embryos treated with 1 μg fucoidan one hour before infection. Viral RNA semi-quantification in pooled liver and small intestine of embryos that had been treated with 4 and 16 μg fucoidan 1 h before the infection showed reductions of the virus replication by 60 and 99.8%, respectively. Since this high anti-NDV activity in ovo was obtained with quite innocuous doses, fucoidan from *C. okamuranus* could be a potential low-toxicity antiviral compound to be used in areas exposed to NDV.

Key words: antiviral, NDV, fucoidan, *Cladosiphon okamuranus,* citotoxicity

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INTRODUCTION

Newcastle disease (**ND**) is one of the most serious infectious diseases affecting birds, particularly poultry, and has caused serious economic losses worldwide (Alexander, 1988; Aldous et al., 2003). The Newcastle disease virus (**NDV**), etiological agent of this disease, is a single-stranded negative-sense RNA virus, member of the Paramyxoviridae family in the genus Avulavirus (Mayo, 2002). Due to variation in viral virulence and host susceptibility, the symptoms of NDV infection in domestic species (chicken, turkey, goose, duck, and pigeon) range from unapparent to severe, the later including respiratory, enteric, and nervous system disease (Alexander, 2000). Even though vaccination using live and killed vaccines is widespread as a management practice, the velogenic strains are endemic in the commercial poultry of many countries and are responsible for direct and indirect economic losses (Villegas, 1998; Alexander, 2001). Unresolved disease events have been reported in Botswana, Israel, Costa Rica, and Nicaragua in 2015, and the disease was present the previous year in more than 50 countries (OIE, 2015).

Many natural products can inhibit viruses. Marine organisms are a promising source of antiviral compounds, especially those that have high efficacy on resistant mutant viral strains and low toxicity to host. Several sulfated seaweed polysaccharides exhibit antiviral activities against a wide spectrum of viruses (Witvrouw and De Clercq, 1997). An advantage of the algae

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sulfated polysaccharides is their high content in the extracellular matrix, which allows their preparation and availability at a very low cost. Algal sulfated polysaccharides are effective to inhibit a wide range of enveloped viruses, such as HIV types 1 and 2, HSV types 1 and 2, human cytomegalovirus, pseudorabies virus, dengue virus type 2, vaccinia virus, hepatitis B virus, influenza A virus, respiratory syncytial virus, parainfluenza virus, vesicular stomatitis virus, Junin virus, Tacaribe virus, Sindbis virus, and Semliki Forest virus (Damonte et al., 2004; Cheung et al., 2014). The fucoidan (sulfated polysaccharide) from *Cladosiphon okamuranus* potently inhibits dengue virus type 2 infection (Hidari et al., 2008) and significantly inhibits the growth of HTLV-1-infected T-cells (Haneji et al., 2005).

In a previous work we evaluated the potential antiviral activity and the mechanism of action of fucoidan from *C. okamuranus* against NDV in the Vero cell line; our results supported the concept that fucoidan acts on early stages of viral infection so as to inhibit viral induced syncytia formation, probably by blocking the F protein (Elizondo-Gonzalez et al., 2012). The aim of the present study was to test the inocuity of the fucoidan from *C. okamuranus* in chick embryos and evaluate its effect on the virus yield when added at different times of the NDV infection cycle.

MATERIALS AND METHODS

Fucoidan

Fucoidan was purchased as a dried powder from Kadoya & Co., Kobe, Japan (lot A03012), extracted (as described by Tako et al., 2000) from cultured kelp *Cladosiphon okamuranus* harvested off the coast of Okinawa Island, Japan. The polysaccharide preparation was certified to contain 90.4% fucoidan (anthrone-sulfuric acid method) with a mean molecular weight of 92.1 kDa (HPLC method). The fucose and sulfate contents of fucoidan were of 38.6 and 15.9% respectively, with ash comprising 19.6% of the content and other sugars comprising 23% (glucuronic acid and traces of xylose). Dried samples of fucoidan were suspended in Dulbecco's modified Eagle's medium (DMEM/F12 GiBCO, New York, NY) at a concentration of 2.5 mg/mL and passed through a membrane filter (Filter Paper, Whatman, MA, USA, 0.22 μm).

Preparation of Primary Chicken Embryo Fibroblasts

Chicken embryo fibroblasts (**CEF**) were prepared using 11-day-old chick embryos; embryo's head, viscera, and limbs were removed first; the remaining tissues were homogenized and washed three times with PBS, trypsinized with 0.05% trypsin-EDTA (Gibco/BRL Life Technologies, Carlsbad, CA) in PBS three times for 15 min at 37°C, and the trypsin was neutralized with fetal bovine serum (**FBS**) (GIBCO-BRL Carlsbad, CA, USA). The cells were then centrifuged, re-suspended in fresh DMEM/F12 supplemented with 10% FBS, 100 units/mL penicillin, 100 μg/mL streptomycin (GIBCO-BRL) and 2 mM L-glutamine (GIBCO-BRL), and filtered through double gauze into a funnel. Cells dissociated from embryos were grown in 25 cm^2 tissue culture flasks (Nunc, Thermo Scientific, Denmark) at 37°C in a humid incubator with a 5% CO2 atmosphere, until cells reached confluent monolayers (2 to 4 days); primary CEF cells were passaged every 3 to 4 days, and frozen stocks of cells were prepared from each passage at a density of 3×10^6 cells and stored in liquid nitrogen (secondary cultures). Cell freezing medium was prepared by the addition of 40% FBS to growth media supplemented with 10% DMSO (Corning Cellgro, Mediatech, Inc, Manassas, VA). Research Ethics and Animal Welfare Committee (CEIBA) of the "Facultad de Ciencias Biológicas, Universidad Autónoma de Nuevo León", approved the procedures for chicken embryos handling, based on the principles of the 3Rs on the NC3Rs (National Center for the Replacement, Refinement & Reduction of Animal in Research).

Cells and Viruses

Secondary CEF cells were grown in medium DMEM/F12 supplemented with 10% (v/v) FBS and 1% (v/v) PES. The flasks were maintained in a humidified atmosphere with 5% CO_2 at 37°C. The La-Sota-NDV-strain (FORT DODGE, Pfizer, New York, NY) was propagated in 9-day-old chicken embryo eggs. Stock viruses were harvested, titrated, and stored at −70°C until used. La sota NDV stocks were titrated by hemagglutination and hemadsortion assays.

Toxicity Assay in vitro

The fucoidan cytotoxicity was evaluated by the yellow tetrazolium MTT (3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide) reduction assay. CEF were seeded in 96-well plates at an initial density of 5×10^3 cells per well; the medium was removed and a 100 μL test sample with different concentrations (suspended with DMEM supplemented with 2% FBS) was added in each well. The dilution medium without the sample was used as control; all the plates were incubated at 37°C in a humidified 5% CO_2 atmosphere. After 48 hrs of incubation, 20 μL of the MTT solution (5 mg/mL in PBS) (Life technologies, Invitrogen, Carlsbad, CA) were added, and the cells incubated again for 4 hrs. After removing the medium, 100 μL of dimethylsulfoxide (DMSO) (Baker, Phillipsburg, NJ) were added to each well and incubated for 5 min to dissolve the formazan crystals. The optical densities were then read in a microplate spectrophotometer (Autoreader EL311; BIOTEK

Instruments Inc. Winooski, VT, USA) at a wavelength of 570 nm. Each fucoidan concentration was tested in sextuplicate and experiments were repeated three times. Half cell-toxicity value ($\mathbf{CC_{50}}$) was calculated as the extract concentration able to diminish the MTT signal (optical density) to 50% compared with the untreated cells.

Toxicity Assay in ovo

Fucoidan was diluted in DMEM as follows: a constant volume of 200 μL DMEM containing crescent amounts of fucoidan (2, 10, 20, 100, 200, 300, 400, and 500 μg) was inoculated into the allantoic cavities of three 9-day-old embryonated chicken eggs, constituting eight groups according to the fucoidan dose; a control group was inoculated with sterile DMEM. The eggs were incubated in a humidified incubator at 37ºC for 2 days; the viability of the chick embryos was checked by candling the eggs daily, and embryo death was recorded. At 48 hours post-inoculation, the size and aspect of chicken embryo were recorded and the liver removed, fixed and preserved in 10% phosphate-buffered formalin. Fixed tissues were embedded in paraffin, subsequently sectioned and stained with hematoxylin and eosin, and examined with a light microscope at CIMA Hospital, Monterrey, N.L. México by Dr. Ricardo Fuentes Pensamiento.

Time of Adition and Hemagglutination Assays

Fucoidan solution in DMEM (0.1 mL) at different doses (0.25 to 16 μg per egg) was inoculated into fertilized eggs (9-day-old) via the allantoic route at different times: 1 hour before infection [**H -1**], at same time as infection [**H 0**], and 1 hour after infection [**H 1**]); the eggs were inoculated with 10,000 NDV-$TCID_{50}$ (50% Tissue Culture Infective Dose) in 0.1 mL DMEM, followed by gentle rocking every 15 min during 1 hour. Eggs were further incubated at 37°C for 3 days. Allantoic fluids were harvested at 3 days post-inoculation (**dpi**) and the viral presence in the allantoic fluid of each egg was determined according to the viral hemagglutination assay (**HA**) (OIE, 2012): briefly, 2-fold dilutions of allantoic fluid in PBS were mixed with an equal volume of a 1% (v/v) erythrocyte suspension in a V-bottomed 96-well microtiter plate; the plates were then incubated for 30 min at room temperature; the titres were expressed in hemagglutinating units as the reciprocal of the highest dilution of virus that demonstrated erythrocyte agglutination. Viral infectivity in the fucoidan treated embryos was then calculated as the ratio of HA titers in treated embryos to the HA titer of infected but untreated controls. The results were the average of three independent experiments, each one involving triplicate embryos per treatment.

RNA Extraction

Total RNA was extracted from allantoic fluids or liver and small intestine from chicken embryo by Trizol method according to the manufacturer's protocol (Invitrogen, Carlsbad, CA). RNA was washed once in 75% alcohol and resuspended in 30 μL of RNase-free-water. These extractions were carried out on samples collected from embryos that had been treated by fucoidan at [H -1].

RT-PCR for NDV-RNA Semiquantification

The RNA samples were subjected to reverse transcription (**RT**) using High-Capacity cDNA Archive Kit (Applied Biosystems Foster, CA, USA) according to the manufacturer's specification. After reverse transcription, PCR was done for a genomic region of 316 nucleotides (**nt**) of the fusion gene (**F**) of NDV with Taq DNA Polymerase (Invitrogen. Carlsbad, CA, USA) and a set of specific primers (Farkas et al., 2007). The program used for allantoic fluid samples consisted of 7 min at 94°C followed by 32 cycles of 95°C for 30 seconds, 58°C for 30 seconds, and 72°C for 1 min, and finally 10 min at 72°C. For semiquantitative analysis, RT-PCR for actin was performed in parallel to show an equal amount of total RNA in each sample. The set of primers, FW 5′- GTTGGTGACAATACCGTGTTCA-3′ Rev 5′- CCCAGACATCAGGGTGTGATG-3′ were used to amplify a portion of actin gene (123 bp). The amplified PCR products were electrophoresed on a 1% agarose gel and were visualized in UV light.

Data Analysis

All variables were tested in triplicate and experiments were repeated three times and analyzed by SPSS 10. Results are reported as means±standard deviation (**SD**). The 50% cytotoxic concentration (CC_{50}) was determined by Probit regression analysis. The antiviral activity was compared using one-way analysis of variance (ANOVA) followed by Duncan test ($\alpha = 0.05$).

RESULTS

Cytotoxicity in vitro

Cell viability determinations indicated that low fucoidan concentrations (up to 10 μg/mL) had no significant cytotoxicity to chick embryo fibroblast cells, and lead to an estimate of CC_{50} at 1062 μg/mL (Figure 1).

Toxicity in ovo

Nine-day-old chicken embryos, inoculated with different concentrations of fucoidan and evaluated 48 hrs after for external features, did not show any abnormality or change in size and aspect due to treatment when compared with the control (Figure 2). Liver tissue was

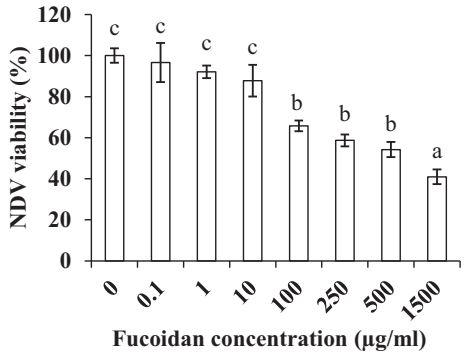

Figure 1. Evaluation of fucoidan cytotoxicity to chick embryo fibroblasts. The cell viability was determined by MTT assay and compared with that of untreated cells (100% of viability). Bars represent mean values and vertical lines represent the standard error of mean (n = 9, ANOVA $p < 0.05$).

evaluated for the presence of histological alterations, such as steatosis, necrosis, and fibrosis: control group showed a normal morphology (Figure 3 A); Figures 3 B, C, and D depict fucoidan treatments, obtaining an increase of lipid accumulation associated with crescent fucoidan doses. Chicken embryos treated with 20 μg fucoidan presented a normal morphology while those treated with 200 μg showed 15 to 28% steatosis, and those treated with the highest dose (500 μg) showed 27 to 56% steatosis. For antiviral assays, activity of fucoidan was tested at doses below 20 μg fucoidan to avoid any compound-related toxicity in the embryos.

Antiviral Activity in ovo

The highest viral inhibition was found when the compound was added 1 hour before the infection [H -1]: the HA titer decreased by 89.5% at a dose of 1 μg of fucoidan per embryo; this is a considerable and significant inhibition of infectivity (HA titer of infected yet untreated embryos was considered as 100% infectivity)(Figure 4). Fucoidan induced the highest percentages of viral inhibition when added before infection [H -1], but a good antiviral activity was also obtained when 1 μg compound was added at time [H 0] (73.6%) or at time [H 1] (66.7%)(Figure 4).

Effect of Fucoidan on NDV-RNA Level in Chicken Embryos

In ovo delivered fucoidan was able to reduce viral replication. Chicken embryos treated with 0.25 to 16 μg fucoidan per embryo for one hour and then infected with NDV (10,000 $TCID_{50}$) did express NDV-RNA in the allantoic fluid (as detected by RT-PCR of the "F" NDV fragment), but only for fucoidan doses from 0.25 to 2 μg per embryo, while a considerable reduction of the viral RNA production could be observed for those treated with 4 and especially 16 μg per embryo (Figure 5).

RT-PCR semiquantitative analyses of the viral RNA level in liver and small intestine of chick embryos showed that 4 μg fucoidan/embryo have already a significant effect on NDV-RNA level (a 60% decrease), while 16 μg fucoidan/embryo of fucoidan dramatically reduced NDV-RNA level (by 99.8%), compared with infected untreated embryos (Figure 6A). The ratio of NDV/Actin mRNA from RT-PCR detection was quantified with Phoretix 1D v2003.02 software (Figure 6B). The size and overall appearance of embryos treated with 4 and 16 micrograms of fucoidan before infection,

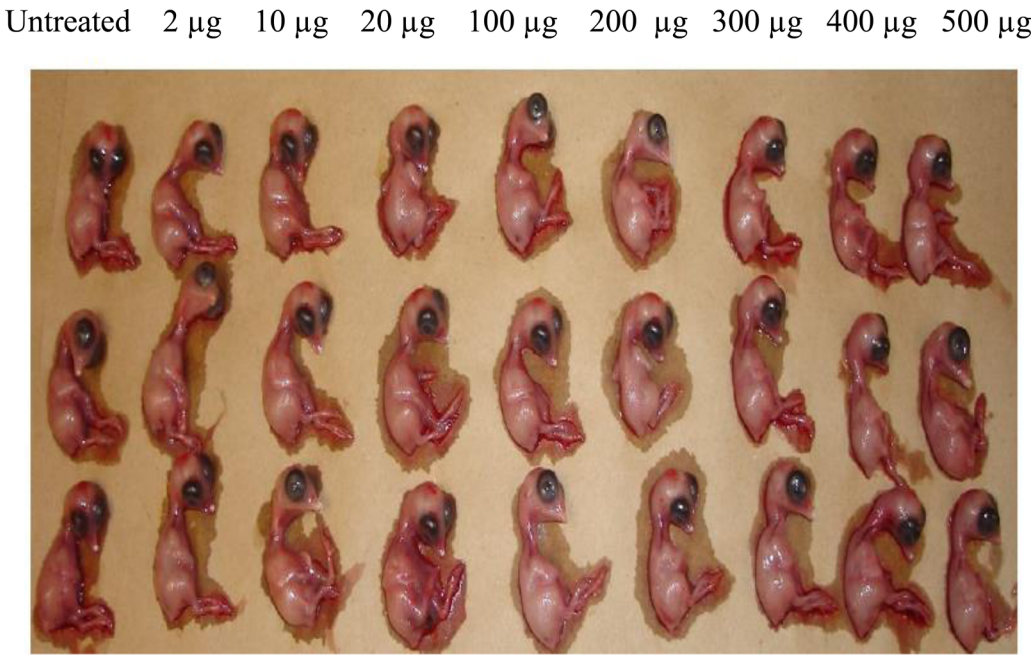

Figure 2. Effect of fucoidan on size and aspect of chicken embryos. Chicken embryos were exposed to different doses of fucoidan for 48 hours. The assay was repeated three times.

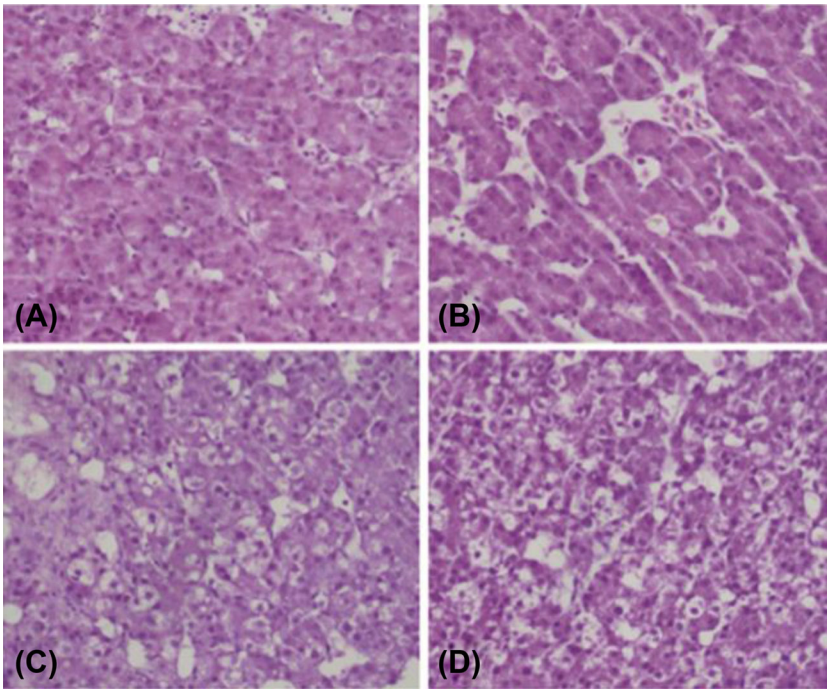

Figure 3. Effect of fucoidan in liver of chicken embryos, analyzed by histopathology: A) untreated; B) treated with 20 μg of fucoidan; C) treated with 200 μg fucoidan; D) treated with 500 μg fucoidan (hematoxylin & eosin stain, 20X).

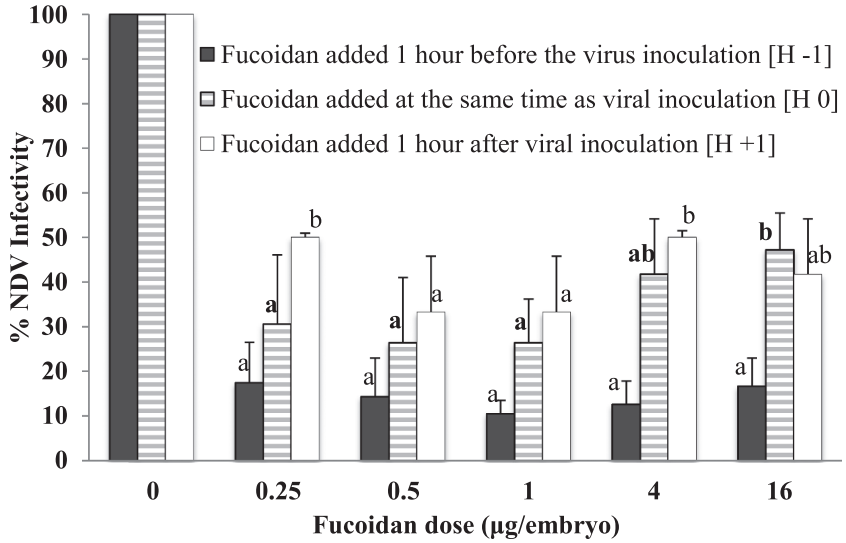

Figure 4. NDV infectivity in chicken embryos treated with single doses of fucoidan added in the allantoic cavity at different times. Values are given as mean ±SD (n = 9). Letters indicate homogeneous subsets as determined by a Duncan test among doses for a given time ($\alpha = 0.05$).

or treated but not infected afterwards, was equal to untreated control embryos (data not shown).

DISCUSSION

In recent years consumers are becoming interested into more natural food and it seems clear that this phenomenon is requiring a change in the sanitary additives used in food production. At present, many research groups are investigating seaweed compounds for their antiviral, antibacterial, antiparasitic, antioxidant, or growth promoting effects (Damonte et al., 2004; Lee et al., 2004; Queiroz et al., 2006; Yasuhara-Bell and Lu, 2010; Costa et al., 2011).

The potential of using sulfated polysaccharides from algae in treatment of viral diseases has aroused a great deal of interest in the scientific community (Damonte et al., 2004; Lee et al., 2004; Hidari et al., 2008; Wang et al., 2012; Ngo and Kim, 2013). Fucoidans, the sulfated polysaccharides isolated from brown seaweeds, have been shown in many studies to inhibit different virus as dengue virus type 2 (Hidari et al., 2008) and HTLV (Haneji et al., 2005). The fucoidan we used in present study, a sulfated polysaccharide isolated from the brown seaweed *Cladosiphon okamuranus*, showed

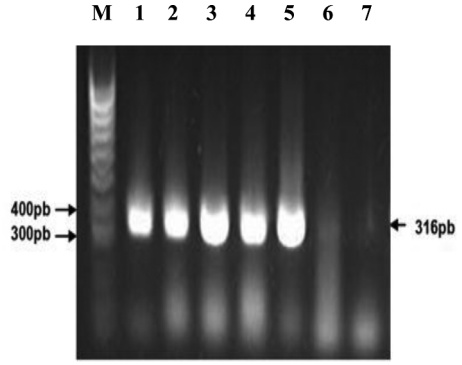

Figure 5. Reduction of viral RNA in allantoic fluid from embryos treated by fucoidan**.** Lane 1: Untreated. Lanes 2 to 7: 0.25, 0.5, 1, 2, 4, and 16 μg fucoidan /embryo.

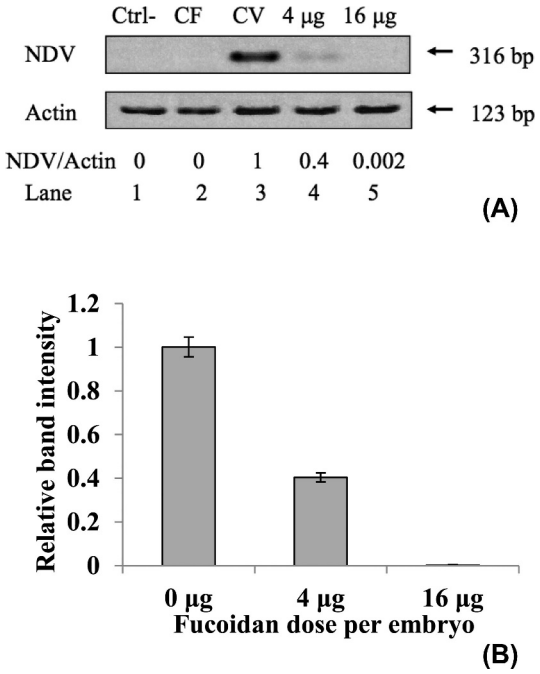

Figure 6. In ovo inhibition of NDV replication by fucoidan. A) Nine-day-old chicken embryos were treated with 4 and 16 μg fucoidan/embryo and infected 1 h later with 10,000 NDV-$TCID_{50}$. After 72 h incubation, total RNA of pooled liver and small intestine was extracted and analyzed by semi-quantitative RT-PCR. PCR was performed at 28 cycles. RT-PCR for actin was performed in parallel to show an equal amount of total RNA in each sample. Ctr- = untreated, uninfected embryos; CF = fucoidan treated, uninfected embryos; CV infected untreated embryos. B) Relative ratio of NDV/actin mRNA from RT-PCR detection was quantified with Phoretix 1D v2003.02 software. Data are expressed as relative band intensities to control (embryo infected without treatment), which is defined as 1.0. The data shown are the mean ± SD of three separate experiments, each one involving triplicate embryos per fucoidan doses.

in a previous study properties of an antiviral agent against NDV, being able both to inhibit the penetration of NDV to Vero cells and to suppress the cell-to-cell spread between NDV-infected cells and uninfected cells (a process that drastically enhances NDV spread and infectivity) (Elizondo-Gonzalez et al., 2012).

Our results in the present study clearly show the absence of significant toxicity for fucoidan in vitro (CEF) at concentrations of 0.1, 1, and 10 μg/mL, which were able to inhibit 42%, 45%, and 68% of syncytia formation in Vero cells in the previous study (op. cit.); moreover, we found a CC50 of 1062 μg fucoidan/mL DMEM that is far higher than the 50% effective concentrations of 0.75 μg fucoidan/mL against syncytia formation in Vero cells or 58 μg fucoidan/mL against plaque formation in Vero cells (Elizondo-Gonzalez et al., 2012). Also we have shown that fucoidan did not induce a significant cytotoxicity in ovo at doses with antiviral effect (0.25 to 16 μg/embryo). Our findings regarding toxicity in ovo revealed that fucoidan did not cause change in size, weight, or macroscopical features of the chicken embryos. When evaluating histological alterations of liver, we found no difference between control embryos and those treated with 20 μg of fucoidan, although at higher concentrations such as 200 and 500 μg we found evidence of steatosis; however the doses of fucoidan considered for an antiviral effect were below 20 μg/embryo. These findings are consistent with the earlier findings by Guideon and Rengasamy (2008), who reported no change at necropsy, pathological examination in several organs, or histopathological examination of Wistar rats treated with low doses (600 mg/kgBW/day) of *Cladosiphon okamuranus* during 3 months.

The antiviral activity of fucoidan in ovo was demonstrated in the current study by a dose-dependent diminution of the allantoic fluid hemagglutination ability, as an indicator of the amount of viruses produced by live chicken embryos. Fucoidan doses from 0.25 to 16 μg per embryo induced a significant reduction of infectivity for the three different times of addition, although we obtained the best response when fucoidan was added before infection, the dose of 1 μg per embryo resulting in 89.5% of NDV infectivity diminution. Surprisingly, we found a lower degree of inhibition of HA with higher concentrations of the compound (Figure 4); notwithstanding, the highly significant reduction of viral population in fucoidan-treated embryos was further confirmed by reduction of the viral RNA expression: the diminition by 99.8% of viral RNA in chicks embryos treated with 16 μg fucoidan/embryo is an indication of the efficiency of the compound against NDV (Figure 5).

The HA apparently contradictory results regarding the level of viral RNA by RT-PCR are likely due to hemagglutination of "aggregates of fucoidan". We performed a hemagglutination assay with different concentrations of fucoidan and found spontaneous hemagglutination of this compound with concentrations greater than 2 μg/mL, perhaps by direct carbohydrate-carbohydrate interaction, forming aggregates as described in marine sponges (Vilanova, Coutinho, Mourão, 2009). This is possible due to carbohydrate−carbohydrate interactions by the chain ramifications (Spillmann and Burger, 1996); the existence and character of species-specific carbohydrate−carbohydrate recognition are fundamental to adhesion events (Bucior and Burger, 2004).

Other compounds derived from natural products have been assessed against NDV in ovo. Neem tree (*Azadirachta indica*) extract had a significant inhibitory action against NDV, at concentrations ranging from 3 to 9 μg/mL (Wafaa et al., 2007). *Adansonia digitata* root bark methanol extract was able to inhibit NDV growth at concentrations of 200 and 250 mg/mL (Sulaiman et al., 2011). *Commiphora swynnertonii* (various extracts) at the concentration of 50 μg/mL inhibited 50% of HA in the allantoic fluid of chicken embryo infected with NDV (Bakari et al., 2012). *Achillea millefolium* and *Thymus vulgaris* extracts reduced the NDV potency by 56-fold (Rezatofighi et al., 2014). *Toussantia orientalis* and *T. patriciae* (Annonaceae) leaf extracts exhibited antiviral activity in embryos infected with NDV or Infectious Bursal Disease Virus (Nyandoro et al., 2014). Cranberry juice-enriched and cranberry pomace polyphenol-enriched soy protein isolate, decreased reproduction of Influenza, NDV, and Sendai viruses in ovo (Turmagambetova et al., 2015). The current study has clearly demonstrated the antiviral activity of fucoidan from *C. okamuranus* against ND virus in ovo. Therefore, it is speculated that the chickens at risk of NDV can be successfully treated by this compound. However, in vivo assays are needed to validate the use of fucoidan from *Cladosiphon okamuranus* to help control Newcastle disease in chickens.

CONCLUSIONS

Previously, we revealed that fucoidan from *Cladosiphon okamuranus* possesses anti-NDV activity through its ability to block the entry of the virus to VERO cells and to suppress the NDV cell-cell fusion process supported by its F-protein. Here, our results showed that fucoidan applied in ovo is also active against NDV, while not toxic at therapeutic doses. The fucoidan appears to act mainly by interfering with the initial steps of NDV infection. Taken together, these results suggest that the administration of this compound could be a preventive measure in areas exposed to NDV.

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