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Assessment of Antiviral Activity for Ethanolic *Chlorella vulgaris* Extract Against Newcastle Disease Virus (NDV) Infection in Sasso Chicken

Reda R.Fathy[1*], Reem A.Soliman[2] , Abdalla El-Hadary[3], Mai Osman[4], Yasmeen Magdy[5], Sawsan S. El basuni[1]

[1]Department of Avian and Rabbit Diseases, Faculty of Veterinary medicine, Benha University, Egypt
[2]Evaluation of Inactivated Viral Poultry Vaccines Department, Central Laboratory for Evaluation of Veterinary Biologics, Agriculture Research Center (ARC), Cairo, Egypt
[3]Biochemistry Department, Faculty of Agriculture, Benha University, Benha 13736, Egypt
[4]Biochemistry, Toxicology and Nutritional Deficiency Diseases Department, Animal Health Research Institute, Benha Branch, Agriculture Research Center (ARC), Egypt
[5]Department of Anatomy and Embryology, Faculty of Veterinary Medicine, Benha University, Egypt

***Corresponding Author:** Reda R.Fathy, **E-Mail:** reda.megahed@fvtm.bu.edu.eg

ABSTRACT

Newcastle disease (ND) is an extremely viral disease that has tremendous impacts on poultry production worldwide. Increasing and repeating ND outbreaks and suspecting of fifth pandemic occurrence demonstrated the need for novel medicines to control the disease. *Chlorella vulgaris* (CV) microalgae have recently emerged as natural alternatives with antiviral activity, in current study, ethanolic *Chlorella vulgaris*) ECV) extract was prepared and evaluated as an effective antioxidant and antiviral agent against Newcastle disease virus (NDV) in vitro and in vivo stages. The effect of ECV extract with three concentrations (50µL, 100µL, and 200µL) was estimated on embryonated chicken eggs (ECEs). The data revealed that 100 µL of ECV extract is a nontoxic dose, as evidenced by the absence of embryo deaths, and has effective antiviral activity by using a mixture of 0.2 mL of NDV with $10^{8.5}$ EID_{50}/ml with 0.2 mL of ECV extract, which led to decrease of viral titer to $10^{3.4}$ EID_{50}/ml with complete inhibition of NDV replication and loss of haemagglutination (HA) activity. Later on, in vivo study was applied in 28 days old Sasso chickens to evaluate the activity of ECV extract at 1 g/kg concentration according to invitro assessment as it is nontoxic effective antiviral dose in the drinking water before and after the NDV challenge. The used assessment parameters in this study were clinical signs, post-mortem (PM) lesions and histopathological pictures and it showed the effective role of ECV extract in viral replication inhibition in the treated groups when compared to control ones. Also, Real-time PCR was conducted to estimate NDV titer after challenge, in the group (III) and group (IV) showed a decrease in viral shedding at 3rd and 5th day post challenge (dpc) and a complete absence of viral titer at 7th dpc in the prophylactic group (III) in comparison with the positive control (II). These findings illustrated the potential role of ECV extract in overcoming NDV infection under field conditions and advised using it as an antiviral agent.

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INTRODUCTION

The poultry industry is facing huge problems that economically affect meat and egg production through a fight with foreign microbes especially viral infection, among which is NDV. Newcastle disease (ND) is a very transmissible disease with devastating impacts on poultry flocks that cause high economic losses worldwide, such as high morbidity, high mortality and a drastic drop in egg production **(Alexander, 2011).** Also, it is one of the notifiable diseases by OIE, and in many countries, velogenic strains are endemic in poultry farms **(OIE, 2012).** NDV, causes it, is grouped under the family Paramyxoviridae, Subfamily avulavirinae, avian orthoavulavirus type-1 (NDV) **(Ferreira *et al*., 2019)**.

An enveloped virus containing single-stranded, non-segmented linear RNA with negative sense **(Naguib *et al*., 2022).** Vaccination is the suggested way for ND control and prevention using inactivated and live vaccines, and it is greatly practiced **(Villegas, 1998; Ezema, 2009)** but does not confer complete protection. Detection of natural antiviral agents is an important alternative to overcome the risks of emerging new viral strains **(Elizondo *et al*., 2012).** Scientific researches highlight photosynthetic organisms such as microalgae with a store of bioactive secondary compounds, making microalgae valuable awareness in seeking novel agents with antiviral impacts **(Carbone *et al*., 2021).**

Microalgae are recognized as promising antiviral compounds that contain many derivatives with biological activities **(Khaligh *et al*., 2022).** The presence of secondary therapeutic metabolites, inclusive alkaloids, photosynthetic pigments, sterols, terpenes, carotenoids, polysaccharides and lectins, give the capability of marine algae to display a broad extent of biological efficacies like antioxidant, anti-inflammatory, antimicrobial and antitumor **(Val *et al*., 2001 and Adhoni 2016).** The green algae, chlorella vulgaris is related to the order Chlorococcales, family Oocytaceae, genus Chlorella, it has commercial uses are clinical treatments, food supplements, heavy metals removal and production of cosmetics **(Coronado-Reyes *et al*.,2020).**

Within the past decades, numerous studies have demarcated the antiviral role of algal polysaccharides with various mechanisms of action, such as blocking the binding or entrance of viruses into the cellular host or inhibiting protein synthesis and DNA replication, their results refer to the favorable impacts of effective antiviral of choice for pharmaceutical experiments, such as laminarin, navicular, fucan, carrageenan, and alginate **(Ahmadi *et al*., 2015).**

The investigation for natural components emerging from algal species has gained many crude solvents and efficient aqueous extracts that have antiviral action against a broad range of viruses, including paramyxoviruses like Respiratory syncytial virus (RSV), togaviruses like-Sindbis virus and Semliki Forest virus, rhabdoviruses like Vesicular stomatitis virus (VSV), retroviruses like both human and simian immune deficiency viruses (HIV and SIV) and herpes viruses **(Yasuhara-Bell *et al*., 2010)**. More than 50 years ago, the effective role of algal extracts and their polysaccharides component was evaluated in chicken embryos against mumps and influenza B virus as a potent antiviral agent **(Gerber *et al*., 1958; Lee *et al*., 2006).** Using dried microalgal biomass in a broiler ratio potentially affects the growth rate, weight gain, and immune system **(Abdo *et al*., 2019).** In the current study, we trial for using micro algal therapy in vitro by different doses of ECV extract in ECEs and the application of its role as an antiviral agent against challenge of virulent NDV in the preclinical and clinical study under field conditions in drinking water on Sasso chicken.

MATERIALS AND METHODS

1. Preparation and phytochemical analysis of Ethanolic Chlorella vulgaris extract

Chlorella vulgaris were obtained from the retail store in Benha, Egypt. The used standards were gallic acid for the determination phenolic compounds and Qurecitine for determination flavonoid compounds as well as the solvent is ethanol and Folin-Ciocalteu reagents were gained from Sigma-Aldrich Company (Louis, Missouri, USA). The recommended algae are chlorella vulgaris were grinded and saturated in ethanol and water at a ratio of 1:5 (80:20 v/v) for 24 hr at room temperature. Using a rotary evaporator (IkA-WERKE, Germany) , extracts were settled under pressure at 40°C in the dim, then dried and frozen (LABCONCO Free Zone 2.5 Liter -50C Benchtop, India). For the following studies, the harvested ECV extract was emulsified in 20% dimethyl sulfoxide (DMSO) **(Hassanin *et al*., 2020).** The phytochemical analysis estimated the phenolic and flavonoid contents according to **(Meda *et al*., 2005)** and expressed them as the gallic acid and quercetin equivalents, respectively. A s well as the antioxidant activities of the harvested extract were calculated according to **(El-Hadary and Mohamed, 2019).**

2. Invitro study

2.1. Cytotoxicity (CC_{50})

We carried out the test for determine the safe and effective antiviral dose by estimating cytotoxic concentration as follows: firstly, preparation of working solution was performed by 5 mL of dimethyl sulphoxide (DMSO) for dissolving 0.5 g of ECV extracts to make 10% w/v with some modification **(Priya *et al*., 2022).** Then, different concentrations of chlorella vulgaris extract (50 µL, 100µL, and 200µL) were inoculated into five Specific Pathogen Free (SPF) embryonated chicken eggs (ECEs) via allantoic cavities separately for each concentration. The other five SPF-ECEs were used to control un-inoculated embryos **(Hong *et al*., 2015).** Incubation of inoculated embryos for 6 days at 37ºC with 60-80% humidity.

2.2. Antiviral effect of Ethanolic chlorella vulgaris extract

Antiviral properties of ethanolic chlorella vulgaris extract were checked against NDV in SPF-ECEs as follows: Firstly, 0.2 mL of the NDV with $10^{8.5}$

EID_{50}/ml were mixed with 0.2 mL of nontoxic dose ECV extract and then left for one hour at room temperature **(Song *et al.*, 2015).** Secondly, serial dilutions of mixtures were performed in a ten-fold manner, and each dilution was inoculated into five SPF-ECEs of 9-11 days old with 0.2mL per embryo via allantoic route, then incubated at 37 ºC for 6 days with humidity 60-80%. Five un-inoculated SPF-ECEs were kept as control. Thirdly, the inoculated embryos were examined daily, and mortalities within 24 hrs post-inoculation were discarded, while deaths from the second day to six-day post inoculation were collected and included. Allantoic fluid of each embryo was tested for presence of NDV by haemagglutinating (HA) test with recording of positive and negative results for each dilution. NDV infectivity in ECEs (Virus titer) was determined by the method of **(Reed and Muench, 1938).** Finally, the NDv infectivity in ECE was determined by the haemagglutinating activity of the allantoic fluid of the inoculating eggs as measured by slide haemagglutinating (HA) test **(Takasty,1966).**

3. In vivo study

3.1. Experimental birds

Sixty Sasso chicks, one day old, were gained from the local hatchery at Qalubia governorate- Egypt. They are reared on a deep litter system within a high level of sanitary conditions in segregated, cleaned, and disinfected rooms at the center of animal research at the faculty of veterinary medicine, Benha University, Egypt. Chicks were supplied with commercial broiler ration. They were treated in this study according to the care research ethics board and using the committee of Benha university and institutional animals and followed approval to use the animals in research in Egypt with ethical number as **BUFVTM 08-11-21**. They have been used to evaluate ECV extract's efficacy under field conditions

3.2. Viral strain

Velogenic NDV related to class II (genotype VII) with code in gene bank as (NDv/CH/EG-Q/11/2018) and its accession No is MN137991.The virus was kindly obtained from the Newcastle disease vaccines Department, Veterinary Serum and Vaccine Research Institute. The challenge virus in this study was propagated in SPF-ECEs and harvested as infectious allantoic fluid, and then titrated and its infectivity titer was $10^{8.5}$ EID_{50} / dose according to the manual of World Organization of Animal Health **(OIE, 2021).**

3.3. Assessment of viral shedding

Viral shedding can be estimated through a collection of tracheal swabs from four groups at the 3rd, 5th, and 7th day post challenge (dpc). NDV shedding was done by using Real time-reverse transcription-polymerase chain reaction (RRT-qPCR) as follow, total RNA was extracted from 500 µL of the prepared tracheal swabs using the RNeasy® Mini Kit (Qiagen). RT-qPCR was applied in a Bio-Rad real-time thermal cyclerCFX96™, and the prepared primers and probes specific for matrix (M) gene of genotype VII NDV strain were designed (table1) according to **(Wise,2004)** and were obtained from Metabion (Germany). TOPreal™ One-step RT-qPCR kit was used in the amplification. The reverse transcription step for the primer set was 30 minutes at 50°C, then directed by 15 minutes at 95°C. The polymerase chain reaction's thermal conditions are as follows: denaturation step at 94°C for 10 seconds (s), annealing temperature at 58°C for 30 s, and 10 s for an extension at 72°C for 40 cycles. RNA concentration was quantified by the cycle threshold (Ct) method and interpreted versus the standard curve to NDV EID_{50} **(Tan,2004).** All steps followed the manufacturer's instructions.

Table 1: The design of probe and primers used in Real-Time RT-PCR for the detection of NDV matrix protein gene

Primer	Sequence
Probe M+4169	5′FAM - TTCTCTAGCAGTGGGACATGC-TAMRA3′
ForwardND-M+4220	5′CCTGAGGAGAGGATTTGCTA3′
Reverse ND-M-4100	5′AGTGATGTGCTCGGACCTTC3′

3.4. Experimental design

Sixty Sasso chicks of one-day-old were divided into 4 groups with fifteen birds for each group and there no group ECV extract alone as it evaluated as antiviral dose according to invitro test and later on we will determine other aim as immunostimulant or growth promotor .Group I was a control negative and kept without any treatment, and Group II was a control positive and kept untreated but challenged. Groups III and IV were received the prepared ECV extract with one dose (1gm) in the drinking water for five days before and after infection. At 28^{th} days old, chicks in three groups (II, III, and IV) were challenged by 0.2 mL of virulent NDV with a titer of $10^{6.5}$ /chick **(Pohuang *et al.*, 2013).** The recording data from the clinical investigation, post-mortem examination, and the number of live and dead birds were estimated daily. Three birds per groups were humanly killed at 3^{rd} ,5^{th} and 7^{th} day post challenge and scoring lesions from internal organs such as the trachea, lung, intestine, caecal tonsils, spleen, kidneys, and proventriculus, which were evaluated by naked eye, according to **(Hussein *et al.*, 2018)**. Five tracheal swabs were taken

from each group at the 3rd, 5th and 7th dpc to evaluate the shedding of the virus using RRT-qPCR.

3.5. Histopathological examination

The collected tissue samples were also used as a section of the trachea, cecal tonsils, spleen, and proventriculus embedded in 10% of formalin and dehydrated in the degrees of alcohol concentrations purified by xylene, then fixed in molten wax. Parts 5 µm thick were made and finally stained with H&E stain. The slides were examined microscopically, and the lesion score was performed according to **(Hussein *et al*., 2018)**.

3.6. Statistical analysis

Variance analysis of ANOVA was applied by the Statistical Analysis System software **SPSS (2004)** for presenting the results through the means and standard errors of the mean (SEM), and the results were shown when statistically significant with ($P>0.05$).

RESULTS

1. Extraction yield , antioxidant properties and phytochemical components of ethanolic *Chlorella Vulgaris*

The extraction yield percentage of CV was found to be 13.73 g /100 g based on the dry basis. Also results in Table (2) illustrate the DPPH and ABTS free radical scavenging activity of CV . The ethanolic *Chlorella Vulgaris* has strong anti-radical activities were adjusted to be 43.15 and 41.33 % in DPPH and ABTS, respectively.

2. Cytotoxicity activity and antiviral effect of Ethanolic chlorella *vulgaris* extract on ECE

Different concentrations of inoculated *Chlorella vulgaris* extract on ECEs showed that 200 µL was toxic. In comparison, 50 µL and 100 µL have less or no toxic effect on the embryo that causes less or no mortalities of inoculated embryos, as illustrated in the table (3). Using a mixture of 0.2 mL of the NDV with $10^{8.5}$ EID_{50}/mL and 0.2 mL of nontoxic dose (100 µL) of ethanolic chlorella vulgaris extract in ECEs, the data revealed that the collected allantoic fluid from treated inoculated embryos showed decrease in viral titer to $10^{3.4}$ in comparison with control group as shown in Fig (1).

Table 2: Extracts output, antioxidant activities, total phenolic and flavonoid contents of ECV extract

Parameters		Results
Extraction output	(g/100 g material)	13.73 ± 1.12%
Antioxidant activity	(% DPPH*)	43.15±1.11
Antioxidant activity	(% ABTS**)	41.33±0.96
Phenolic contents	(mg GAE*** / g extract)	40.7±0.86
Flavonoids contents	(mg QE****/g extract)	420.8±3.75

*DPPH (2,2-diphenylpicryIhydrazyl **ABTS (2,2-azino-bis(3ethylbenzothiazoline 6-sulfonic acid
GAE (Gallic acid equivalent). * QE (Quercetin equivalent)

Table 3: Cytotoxicity effect of different concentrations of ECV extract on ECEs for seven days post-inoculation

Dose of ECV extract	No of Dead embryos/ total No of inoculated eggs per day post inoculation						
	1st dpi	2nd dpi	3rd dpi	4th dpi	5th dpi	6th dpi	7th dpi
50 µL	0/5	1/5	0/5	0/5	0/5	0/5	0/5
100 µL	0/5	1/5	0/5	0/5	0/5	0/5	0/5
200 µL	5/5	-	-	-	-	-	-

Data showed that 50 µLand 100 µL were nontoxic dose as one embryo dead from total number of inoculated eggs while 200 µL was toxic dose as cause death in all inoculated embryos at first day post inoculation (dpi).

Fig. 1: Effect of different dilutions for example , B: (dilution no 4), C: (dilution no 7) and D:(dilution no 11) of mixture of ECV extract and NDv on inoculated embryos that loss viral titer and infectivity A: control negative.

3. Haemagglutination activity

Treatment with ECV extract showed a reduction of viral titer from $10^{8.5}$ to $10^{3.4}$, which led to losing its ability for HA in the treated group compared to the control group, as presented in Fig. (2). Titer $10^{3.4}$ by Reed and Muench equation , virus lose its antiviral activity , slide HA used for detection HA activity

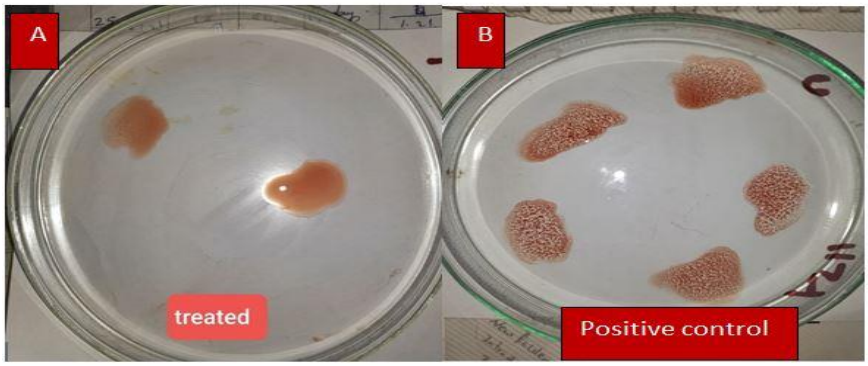

Fig. 2: Haemagglutination activity of NDV genotype VII in the treated group with ECV extract and control one: Comparison between (A): treated group showed loss of haemagglutination activity and (B): control positive group in which NDV has the ability to agglutinated RBCs

4. Investigation of clinical and post-mortem findings

The experimental study of NDV challenge at 28 days old showed depression, ruffled feathers, conjunctivitis, and nasal discharge presented in both infected group (II) and treated groups (III) & (IV) at 3rd dpc. Birds in control positive (II) showed depression, ruffled feathers, conjunctivitis, nasal discharge, swollen head, nervous signs such as torticollis (twisting of head), and paralysis of legs also greenish diarrhea, and morbidity rates recorded at 100%. In comparison, the mortalities reached 58 % at 5th dpc, as shown in table 4 and Fig. 3.

Table 4: The protective effect of ECV extract on the clinical finding of chickens after VNDV genotype VII challenge

Item / Group	Morbidity rate	Mortality rate	No of challenged birds	No of dead birds	No of survived birds	Depression	Conjunctivitis	Nasal discharge Swollen head	Greenish diarrhea	Nervous signs
Group I (-ve)	0	0	15	0	15	0	0	0	0	0
Group II (+ve)	100%	58%	15	8	7	100%	100%	98%	46.6%	52%
Group III Before infection	43%	25%	15	4	11	60%	60%	55%	12%	16%
Group (IV) After infection	66.3 %	34%	15	5	10	66.6%	66.6%	62%	20%	23%

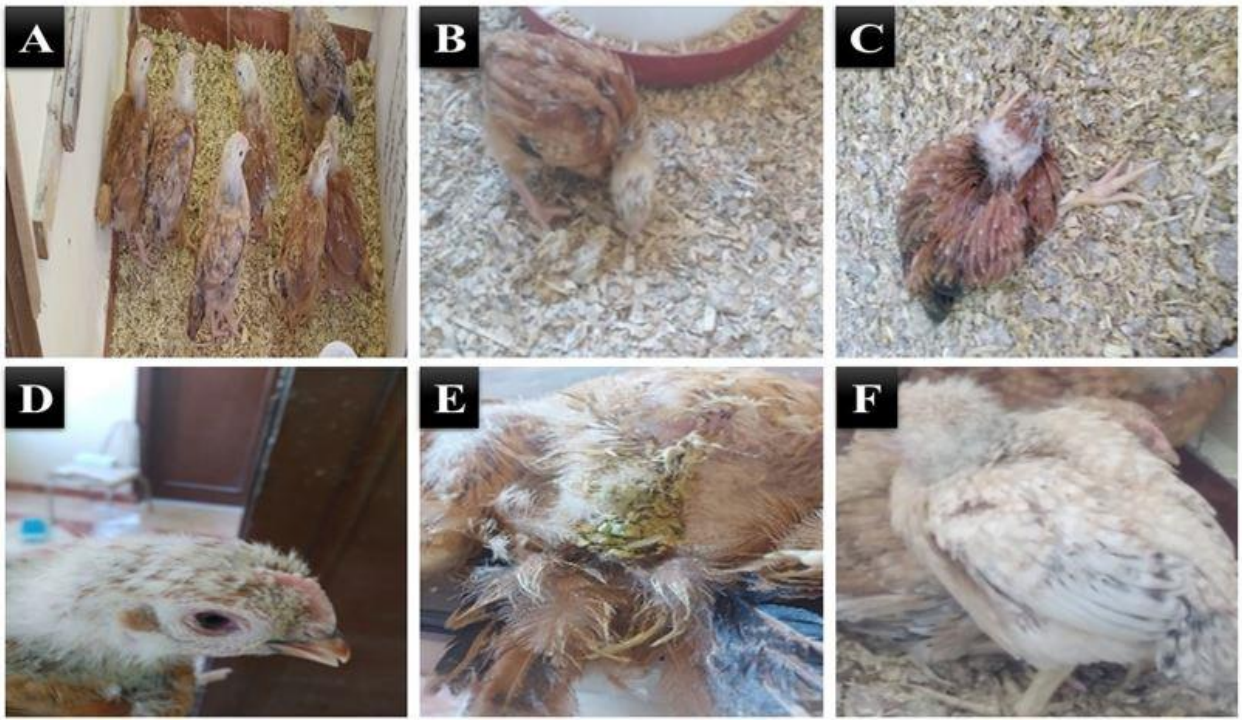

Fig 3: Clinical investigation of Sasso chickens in control and treated groups with ECV extract challenged with VNDV genotype VII. (A): Group (I) with the active condition (B): Nervous signs (torticollis), (C): Recumbent with paralysis of legs (D): Conjunctivitis, nasal discharge and swollen head (E): Greenish diarrhea (F): Depression with ruffled feathers

Necropsy findings of dead birds in the control non-treated group (II) revealed significant hemorrhage and ulcers on the tip of proventricular glands, small intestine, and caecal tonsils, and also congestion of lung and trachea is congested with catarrhal exudate in comparison with control negative group (I) appeared clinically normal with no mortality. At 7th dpc, the autopsy of the humanly slaughtered birds from the control positive group (II) reported notable PM changes with the highest lesion score as edematous and hemorrhagic proventriculus, hemorrhagic ulcers along the intestine and coecal tonsils, congestion in trachea and lungs with mottled spleen and congested kidneys. On the other hand, the administration of 1gm ECV extracts in drinking water resulted in a decrease in morbidity and mortality ratio in the group (III), 43% &25%, and in group (IV), 66.6% & 34 % and gross lesion scoring of examined organs revealed obvious results in treated group III and IV in comparison with those positive control group (II) as shown in table (5) and Fig. 4.

Table 5: Effect of 1 gm of ECV extract on post-mortem lesion scores in Sasso chickens after challenge with NDv

Examined organ	Group I (-ve)	Group I (-ve)	Group III Before infection	Group (IV) After infection
Trachea	0^{c}	$2.6^{a}\pm0.33$	$1.3^{b}\pm0.33$	$2.3^{a}\pm0.33$
Lung	0^{c}	$2.3^{a}\pm0.33$	$1.3^{b}\pm0.33$	2^{ab}
Proventriculus	0^{c}	$3.3^{a}\pm0.33$	$2^{bc}\pm0.33$	$3.1^{ab}\pm0.33$
Intestine	0^{c}	3^{a}	$1.3^{b}\pm0.33$	$2.5^{a}\pm0.5$
Cecal tonsils	0^{c}	3^{a}	$1.6^{b}\pm0.33$	2.6^{a}
Spleen	0^{c}	3.2^{a}	$2.6^{b}\pm0.33$	$3^{a}\pm0.33$
Kidney	0^{c}	2^{a}	$1.2^{b}\pm0.33$	1.5^{b}

Post-mortem lesion scores mean the following: (0) no, (1) mild, (2) moderate, and (3) severe lesions

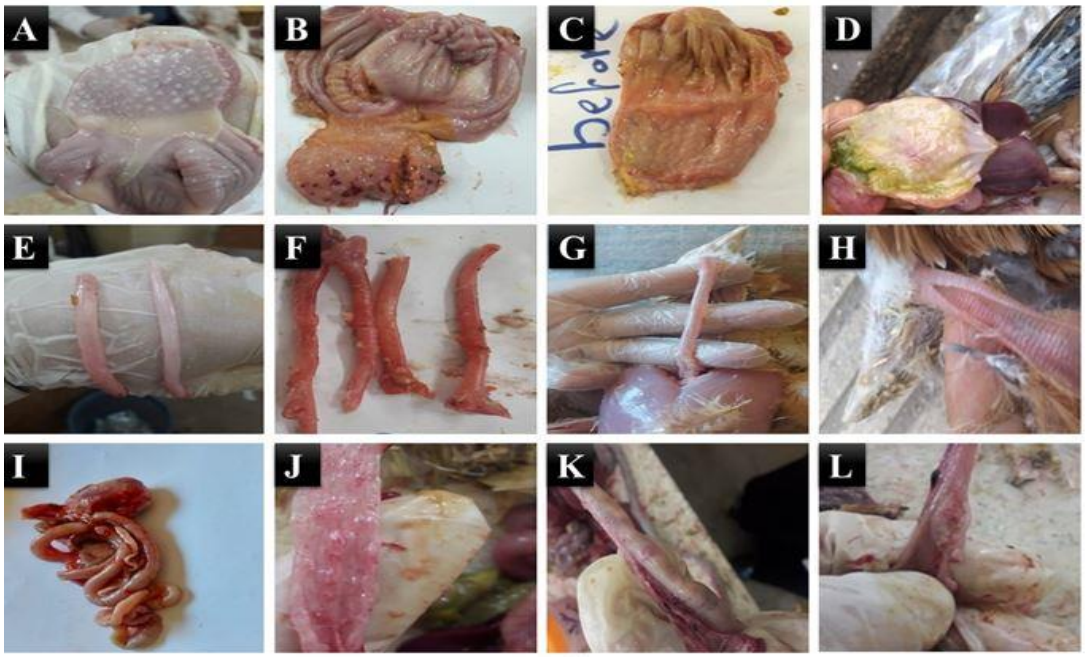

Fig. 4: Effect of ECV extract on post -mortem findings in prophylactic, therapeutic groups and, control groups at 5 dpc and 7 dpc NDv challenge. Comparison in pathological picture of proventriculus A: control group (I) normal anatomical structure and B: control group (II) showed marked petechial hemorrhage at tip of proventricular gland C: group edematous (III) and D: edematous and increased accumulated gland secretions in group IV at 5-day post challenge . E:Trachea of control negative (I) showed normal structure. F: congested trachea with catarrhal exudate of control positive (II) . G: group (III) showed mild tracheitis. H: congested trachea in group (IV).I: intestinal tract of control negative showed no alternation. J: in control group positive (II) showed hemorrhagic ulcers along the intestine and hemorrhagic ulcers in caecal tonsils. K: congested intestine wall. L: hemorrhagic raised ulcer at caecal tonsils of group (II) at 7day post challenge.

5. Effect of Ethanolic Chlorella vulgaris extract on viral shedding

The viral replication and shedding were evaluated efficiently at the 3rd dpc from tracheal swabs of the control positive group (II) with mean titers approximately 4.2^{a} x10^{4}±0.45 EID_{50}/ml, the mean titer persistent to 5th and 7th dpc was 7.2^{a}x10^{6}±0.55 and 4.3^{a}x10^{5}±0.33 EID_{50}/ml, respectively. While no tracheal viral shedding was detected in the group (I) control negative in comparison with the highest titer that was generated by group (II) control positive at all three interval times. Treatment with the ECV extract showed a significant reduction of viral titers, especially in birds of prophylactic group (III) than group (IV) receiving the ECV extract after the challenge. At 7th dpc, preclinical group (III) has no viral titer with considerable variation in the treated groups that indicate application of ethanolic extract in DW has an influential role on viral shedding in comparison with a group (IV) and control positive (II) as shown in table (6) and Fig. 5.

Table 6: Efficacy of ECV extract on viral shedding at the three-time interval after NDV challenge by using RRT-PCR

Group / Time interval	Group I	Group II	Group III	Group IV
3dpc	0^{c}	4.2^{a} x10^{4}±0.45	1.6^{b}×10^{4} ±0.23	3.2^{a}×10^{4}±0.50
5dpc	0^{c}	7.2^{a}x10^{6}±0.55	2.7^{b}×10^{4}±0.32	4.4^{b}×10^{4}±0.46
7dpc	0^{c}	4.3^{a}x10^{5}±0.33	0^{c}	1.6^{b}×10^{3}±0.57

Real-time RT-PCR tested viral shedding, and a, b, and c represent the statistical analysis significance

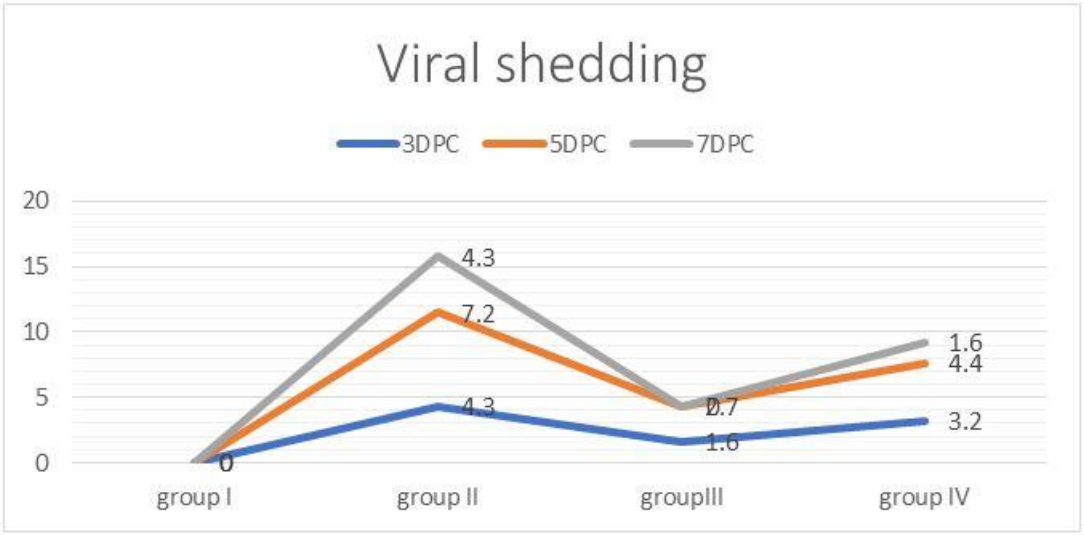

Fig. 5: Efficacy of ECV extract on viral shedding at three-time intervals in the prophylactic group (III) and therapeutic group (IV), and control group (I) and (II)

6. Microscopic lesions

The microscopic changes of control and treated challenged groups were demonstrated at 7 dpc. All examined organs from the negative control group (I) have typical histological alterations. While in the control positive group (II), tracheal sections showed significant congestion, deciliation, and necrosis of tracheal epithelium associated with dense lymphocytic infiltration, proventricular sections showed desquamation of proventricular epithelium and epithelial gland, hyperemia of the muscular layer with lymphocytic infiltration and catarrhal exudation. The spleen section showed thickening of blood vessels wall and diffuse lymphoid follicles depletion. Caecal tonsils are characterized by necrosis of lymphoid follicles. In the treated challenge group (III), the trachea showed deciliation, mononuclear inflammatory cell infiltration, and edema. Proventricular sections showed epithelial cell degeneration and lymphocytic infiltration. The splenic section showed mild to moderate lymphocytic degeneration. Section of the caecal tonsils showed mild lymphocytic depletion. In group (IV), the Trachea showed prominent epithelial desquamation and hyperplasia, the proventriculus showed lymphocytic infiltration, and increased accumulated gland secretions. Splenic section showed lymphoid depletion and thickening of blood vessels wall. Section of caecal tonsils showing focal necrotic lymphoid follicle. Microscopic lesion scores are illustrated in table (7) and Fig. 6.

Table 7: Effect of 1 gm of ECV extract on histopathological lesion scores after VNDV challenge in Sasso chickens from different organs

Examined organ	Group I (-ve)	Group I (-ve)	Group III Before infection	Group (IV) After infection
Trachea	0^{c}	5^{a}	$3.6^{b}\pm0.33$	5^{a}
Proventriculus	0^{c}	4^{a}	$2.3^{b}\pm0.33$	$3.6^{a}\pm0.33$
Cecal tonsil	0^{c}	5^{a}	$3.6^{b}\pm0.33$	$4.3^{ab}\pm0.33$
Spleen	0^{c}	3^{a}	$2^{b}\pm0.33$	$3^{a}\pm0.33$

Histopathological lesion scores mean the following: (0) no, (1) mild, (2,3) moderate, and (4,5) severe lesions

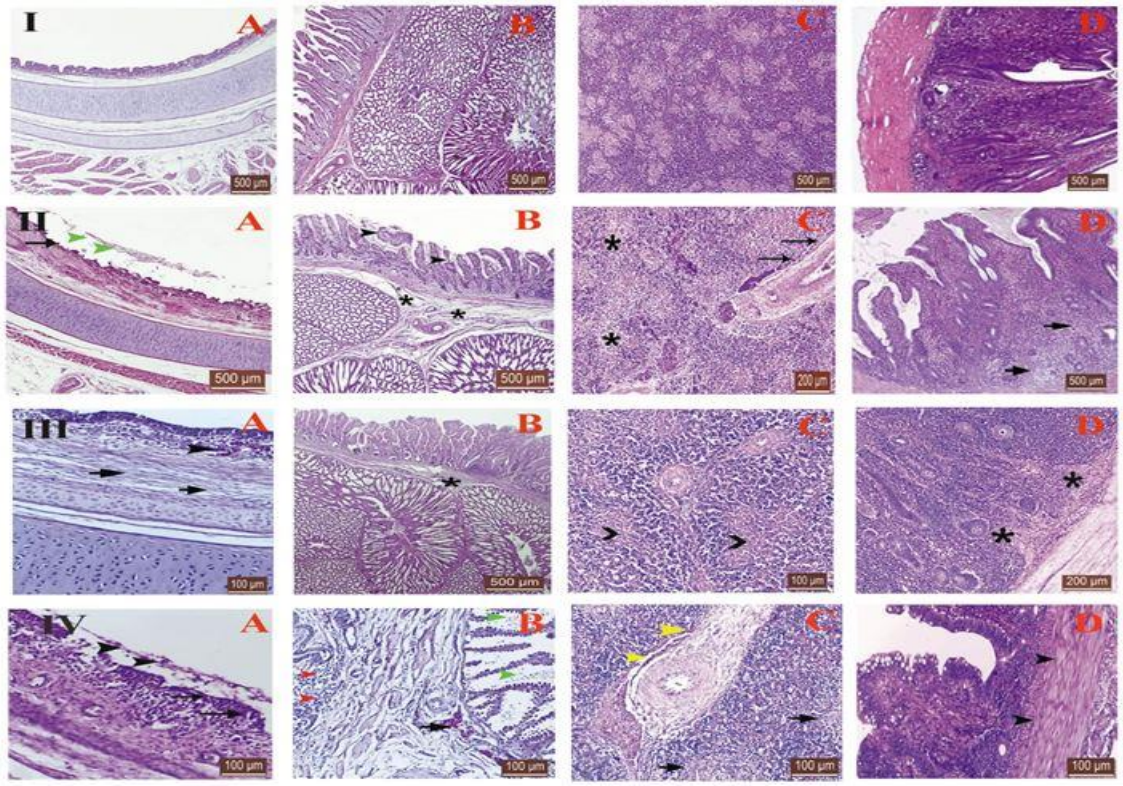

Fig. 6: Photomicrographs for histological sections of the trachea, proventriculus, spleen, and cecal tonsil of experimental groups (I, II, III, and IV) H&E stain, bar indicates magnification. Group (I) (A) trachea, (B), Proventriculus, (C) Splenic sections and (D) cecal tonsils, they showed normal histological structure. Group (II) (A) Trachea showed severe haemorrhage (black arrow) and severe cellular desquamation (green arrowhead), (B) Proventriculus showed severe haemorrhage, extensive epithelial cell degeneration (black arrowhead), and lymphocytic infiltration (black star), (C) Splenic section showed thickening of blood vessels wall (black arrow) and diffuse lymphoid follicles depletion (black star), (D) Section of cecal tonsils showed necrosis and lymphoid depletion (black arrow). Group (III) (A) Trachea showed deciliation (black arrowhead) and infiltrated lymphocyte (black arrow), (B) Proventriculus showed epithelial cell degeneration and lymphocytic infiltration (black star), (C) Splenic section showed mild to moderate lymphocytic degeneration (black arrowhead), (D) Section of cecal tonsils showed mild lymphocytic depletion (black star). Group (IV) (A) Trachea showed prominent epithelial desquamation (black arrowhead) and hyperplasia (black arrow) (B) Proventriculus showed lymphocytic infiltration (red arrowhead) and increased accumulated gland secretions (green arrowhead). (C) Splenic section showed lymphoid depletion (black arrow) and thickening of blood vessels wall (yellow arrowhead). (D) Section of caecal tonsils showed focal necrotic lymphoid follicle (black arrowhead).

DISCUSSION

NDV is a destructive virus causing serious economic losses in poultry industry, especially velogenic strains, which have negative impacts on respiratory, gastrointestinal, and nervous systems that result in high deaths rate that can be the extent to 100% and also lead to a reduction in egg production **(Ali *et al.*, 2022).** The recurrent and emerging new viral strains due to several mutations in NDV complicate control measures and the need to seek alternatives **(Raza *et al.*, 2015).** They were increasing more challenges as a wide range of diseases needed to demonstrate that algae reservoir of effective medicinal effect which have many benefits such as cheap, potential supply, and lower cytotoxic effect **(Liu *et al.*, 2012).** Currently, the wide extent of experimental research identifies the antiviral efficacy of seaweed recap. Increasing concern about its role may serve as a stimulus for the first steps of massive trials in this field. Also, veterinary medical sections urged the application of naturalistic derivatives that have antiviral and immunostimulant effects **(El-Kheir *et al.*, 2016 and Abotaleb *et al* ., 2020) .**

In our study, we trial the evaluation of ethanolic chlorella vulgaris extract in vitro and Sasso chickens as an antiviral agent in drinking water before and after the NDV genotype VII challenge. Using a mixture of 0.2 ml of the NDV with $10^{8.5}$ EID_{50}/ml and 0.2 ml of (100µl) *Chlorella vulgaris* extract in ECE led to a decrease of viral titer to $10^{3.4}$ and loss of viral HA ability as presented in the treated group in comparison with the control group. The results come in similarity with **Ibraheem *et al.*, (2012)** who assessed the

ethanolic extracts of ten marine macroalgae for their antimicrobial against bacteria, fungi, and also NDV, the results showed that ethanolic extract of tested algal had no toxic effect on chicken embryos and seven of them had a strong effect on NDV and led to decrease in the viral titer. Our data explained by (**Reynolds *et al*.,2021 and Carbone *et al*.,2021)** who stated that the sulphate polysaccharides and acidic polysaccharides derived from extracted algae had strong antiviral activities mainly against enveloped viruses through interaction between positively charged sites of viral glycoprotein and their molecules, which have negatively charges resulted in a production of fixed complex, so they take up the adsorption sites for virus and make inhibitory effects which ban viral attachment at the site in the cellular membrane.

The phytochemical analysis of 100 g ECV contains 3.73 g of extract based on dry weight; antioxidant activities were adjusted as 43.15 and 41.33 % in DPPH and ABTS, respectively, as well as its total phenolic and flavonoid contents, were detected as 40.7 mg/GAE and 420.8 mg/QE, respectively. The present findings are similar to the data of **(Mtaki *et al.*, 2020)** who reported a significant assessment of total extraction yield, antioxidants contents, phenolics contents (8.53 ± 0.10 mg/g GAE), flavonoids, β-carotene (2.887 ± 0.121 mg/g) and lycopene present in C. vulgaris (13.73 ± 0.121%). Among different secondary active metabolites, lectins are called carbohydrate-binding agents (CBAs), which block the entrance of microbes into the cells by preventing their attachment with the cellular membrane **(Mitchell *et al*., 2017).**

For those reasons, we investige the role of ECV extract on the response of Sasso chicken to the challenge with NDV, which resulted in significant clinic-pathological findings in the control positive group (II) as ruffled feathers, depression, prostration, swollen head, greenish diarrhea, torticollis and paralysis of the leg, the investigated post mortem lesions were pinpoint hemorrhages at the proventricular gland tips, prominent congested ulcers in caecal tonsils and along intestinal wall, congested trachea with and catarrhal exudates and congested lungs, similar results demonstrated by **(Hasan *et al*., 2010; Yune and Abdela, 2017).** The administration of 1gm of ECV extract in drinking water of groups III and IV resulted in a decrease in morbidity and mortality percent to 43% & 25 % and 66.6% & 34 %, respectively, and the estimated clinical signs, lesion scorings of gross and histopathological revealed significant results in treated groups in comparing with the positive control (II).

On the other hand, the application of ECV before the infection has a potential role in reducing clinical signs and both micro and macroscopic lesions. The preclinical study of the addition of ECV extract in DW potentially stimulates the immune system for facing viral infection. Our results matched with **(Halle *et al*., 2009),** who found that the numerical improvement in the immune system could be related to Chlorella contain high level of selenium.

Also **(Lee *et al*., 2003)** illustrated that Chlorella assists in the construction of a robust and strong immune response as it is a rich source of amino acids, trace element as zinc, selenium, calcium, nucleic sugars such as arabinose, xylose, galactose, mannose, rhamnose beta-carotene, and carotenoids. As well as, the supplementation of ECV extract prophylactically has a potential effect on viral infection, as explained by **(Nair *et al*., 2003).** They supplemented chlorella tablets to normal (uninfected) people and showed a beneficial immunomodulatory activity by stimulating the NK cell, Th-1 cell-induced cytokines, and enhanced INF-γ, IL-1β, and IL-12. Also **(Queiroz *et al*., 2002; Guzmán *et al*., 2003 and Halperin, *et al*.,2003)** illustrated that dietary chlorella supply in humans and animals has a potential role in the immune system by enhancement of immune cells and macrophages in raising interferon levels to fight pathogens and abnormal proteins.

Our data explained by **(Daman *et al*., 2021; Dora *et al*.,2021)** that the sulphate polysaccharides and acidic polysaccharides derived from extracted algae had strong antiviral activities mainly against enveloped viruses through interaction between positively charged sites of viral glycoprotein and their molecules, which have negatively charges resulted in produce a fixed complex, so they take up the adsorption sites for virus and make inhibitory effects which ban viral attachment at the site in the cellular membrane as illustrated in Fig. 7.

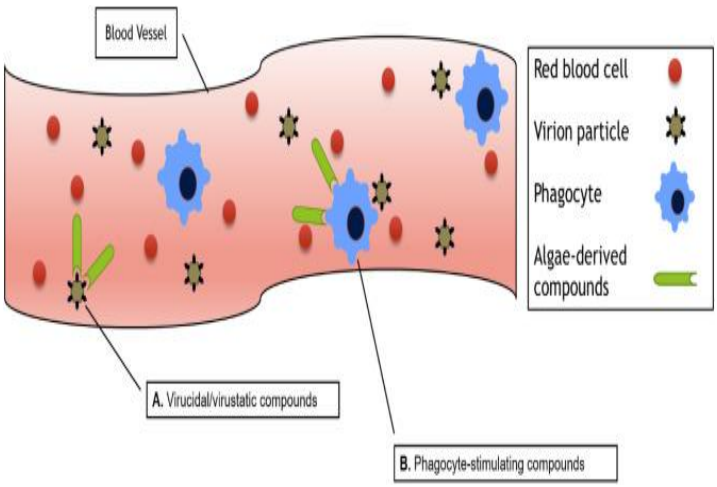

Fig. 7: Diagram illustrate the mechanism of action in which algae have an antiviral effect through block viral attachment A. formation of virucidal or virustatic structure and B. stimulating phagocytic activity **(Daman *et al*., 2021)**.

Treatment with the ECV extract showed a significant reduction of viral shedding in which real-time PCR revealed a decrease of viral titer at 3rd dpc and 5th dpc in the group (III) and group (IV) and a complete absence of viral shedding at 7th dpc in the prophylactic group (III). As well as, considerable variation in the treated groups indicate that the application of ECV extract in DW has an influential role in viral shedding compared with other challenge group and control positive (II). Our findings agree with **(Abotaleb *et al*.,2020)** who suggested that 1gm of spirulina has immunomodulatory impacts on the immune response in SPF chickens and can minimize NDV shedding dietary supplementation of β-Caryophyllene (BCP) at week intervals decreased mortality rate, reduced viral shedding, and enhanced humoral immune response after challenge **(Hassanin *et al*., 2020).**

CONCLUSION

Viral pathogens are still a dangerous threat inside poultry farms. Using various means for control and prevention can be applied but does not confer complete protection, so searching for natural alternatives measures with desirable results is urged. In our study, ECV extract has a potential role in NDV infectivity in ECEs, which cause loss of viral HA activity and also showed reduction in viral titer compared with the control group. In vivo study, the administration of 1 gm of ECV extract in DW before the challenge had a significant effect on both clinical signs, postmortem lesions, and histopathological findings, which resulted in a decrease in both morbidity and mortality percent, the severity of clinical signs and post-mortem, and microscopic lesions, and real-time PCR revealed favorable feedback as decrease of viral titer at 3rd dpc and 5th dpc in the group (III) and group (IV) and complete absence of viral shedding at 7th dpc in the prophylactic group (III). From the obtained data either in vitro and in vivo studies, we recommend and advise the using of ECV extract as a prophylactic measure before occurrence of viral outbreak as well as curative measures during viral infection in poultry farms to overcome destructive viral agents.

Conflicts of interest

The authors declare no conflict of interest

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