



The potential inhibitory impact of curcumin, epigallocatechin gallate and their combinations on infectious bronchitis virus *in vivo*

Fadwa Amin¹, Sabry Tamam², Abdou Allayeh^{3*} and Mai Raslan¹

¹Biotechnology and Life Sciences Department, Faculty of Postgraduate Studies for Advanced Sciences, Beni-Suef University, Egypt.

²Virology Department, Faculty of Veterinary Medicine, Beni-Suef University, Egypt.

³Virology Lab 176, Environmental Research Division, National Research Centre (NRC), El-Buhouth St., Dokki, 12622-Cairo, Egypt.

Email: abdou.kamal@nawah-scientific.com

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ABSTRACT

Aims: Infectious bronchitis virus (IBV) is a highly contagious, acute viral respiratory disease that mostly affects chickens. The poultry sector has suffered enormous losses as a result of IBV. Currently, live attenuated vaccines are routinely used to prevent and control IBV. However, due to the enormous genetic variety, vaccinations are becoming ineffective, with low cross-protection effects among vaccine serotypes. The present study aimed at investigating the possible antiviral effects of curcumin, epigallocatechin gallate (EGCG) and their mixtures against IBV *in vivo*.

Methodology and results: Curcumin, EGCG and their combinations were administered to infected and uninfected chicken groups and viral load titers were determined by real-time PCR. The clinical symptoms of both the negative and positive control groups were also compared. Finally, the trachea tissues of each group were examined histopathologically. According to our findings, the viral titer and the clinical signs dropped significantly during the pretreatment infection procedure. Curcumin, EGCG and their combinations also show significant antiviral activities.

Conclusion, significance and impact of study: This study clearly shown that natural compounds and their combinations, such as curcumin or/and EGCG can reduce viral pathogenicity *in vivo*, suggesting that they might have therapeutic implications in the poultry sector.

Keywords: Infectious bronchitis virus, curcumin, EGCG, coronavirus, real time PCR

INTRODUCTION

The infectious bronchitis virus (IBV) is the causative agent of infectious bronchitis (IB), a highly contagious, acute viral respiratory illness that affects chicken. Globally, IBV has a significant direct negative impact on the poultry sector due to high mortality, poor egg quality and meat output, as well as a significant indirect impact owing to obstacles in IBV prevention (Liang *et al.*, 2019; Wu *et al.*, 2019). IBV is a member of the Coronaviridae family and the genus *Coronaviruses*. The IBV genome is surrounded by a positive sense RNA strand. Its replication is distinguished by a high rate of genetic recombination (Wu *et al.*, 2019). IBV infects chickens of all ages, however it causes severe sickness in newborn chickens and the severity lessens with age (Liu *et al.*, 2005). IBV can be transferred to chickens by direct contact with aerosols as well as indirect contact with contaminated equipment, egg packing material, farm utensils and litter items (OIE, 2012). Currently, live attenuated vaccines are routinely

utilized for IBV prevention and control. However, due to the high rate of genetic variability among IBV strains, vaccinations are becoming ineffective with poor cross-protection effects among various serotypes (Yan *et al.*, 2018). Meanwhile, because of a lack of concerted effort to avoid IBV, as well as a lack of adequate surveillance and the introduction of foreign strains to battle IBV in specific locations, IBV prevention and control has become extremely challenging. As a result, finding efficient antiviral substances for IBV prevention is critical.

Turmeric, *curcuma longa*, is an essential plant in traditional Chinese medicine because it contains curcumin [1,7-bis(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione], a key active component (Wei *et al.*, 2017). The Food and Drug Administration (FDA) has determined that it is generally regarded as safe (GRAS). During the clinical trials, a healthy amount of curcumin up to 12 g/day was known to be safe for human consumption without provoking adverse effects (Gupta *et al.*, 2013). Curcumin has been recognized as analgesic, antioxidant,

*Corresponding author

antimicrobial, anti-inflammatory and anticarcinogenic in many previously published reports (Çıkırıkçı *et al.*, 2008). Curcumin, as antimicrobial agent has antibacterial activity against a wide range of bacterial strains, parasites and fungi (Moghadamtousi *et al.*, 2014; Ali and Banerjea, 2016). Several studies with curcumin against human viruses such as herpes simplex virus (HSV), hepatitis C virus (HCV) and respiratory syncytial virus (RSV) have been undertaken (Yang *et al.*, 2016b; Wei *et al.*, 2017). According to Yang *et al.* (2014), curcumin bioavailability in tissues is quite low due to its poor absorption, rapid metabolism, chemical instability and rapid systemic elimination; hence the inclusion of various substances such as piperine or liposome can boost that bioavailability (Kakaral *et al.*, 2010). The processes involving interference with viral life cycle step(s) have been outlined in Table 1 based on the literature on curcumin's antiviral action.

Green tea, on the other hand, is one of the world's most popular beverages. Catechin, epicatechin, epigallocatechin, galocatechin and epigallocatechin

gallate are quaternary polyphenolic substances found in green tea that have been shown to help reduce lipid peroxidation in the liver, kidney and blood vessels (Okuda *et al.*, 1983). These natural polyphenols have piqued the interest of researchers due to the possible health advantages stemming from their antioxidant properties. Among the five types of catechins, epigallocatechin-3-gallate (EGCG), accounts for roughly 59% of the total polyphenols in dried green tea leaves (Schinzari *et al.*, 2015). Many reactive oxygen/nitrogen species (ROS/RNS) such as superoxide radical anions, peroxy and hydroxyl radicals, singlet oxygen, nitric oxide and peroxynitrite are scavengers for EGCG. EGCG has the potential to capture peroxy radicals and disrupt the chain reaction of free radicals, so halting lipid peroxidation (Thangapandiyar and Miltonprabu, 2014). Several studies have been undertaken using green tea catechins or EGCG against human viruses such as adenovirus (Ad). Table 1 summarizes the processes involving interference with viral life cycle steps in the literature on green tea catechins' antiviral activity.

Table 1: A summary of antiviral activities of curcumin and green tea against several human viruses, as reported in the literatures.

Compound	Virus	Antiviral activity mode	Reference
Curcumin	Human immunodeficiency virus (HIV)	Replication inhibitors	Ali and Banerjea (2016)
	Hepatitis C virus (HCV)	Adsorption inhibitors	Anggakusuma <i>et al.</i> (2014)
	Human cytomegalovirus (HCMV)	Adsorption inhibitors	Lv <i>et al.</i> (2015)
	Epstein-Barr virus (EBV)	Replication inhibitors	Ferreira <i>et al.</i> (2015)
	Bovine herpesvirus 1 (BHV 1)	Adsorption inhibitors	Zhu <i>et al.</i> (2015)
	Chikungunya virus	Adsorption inhibitors	Narayanan <i>et al.</i> (2012)
	Ebola virus	Adsorption inhibitors	Setlur <i>et al.</i> (2017)
	Enterovirus 71 (EV71)	Replication inhibitors	Qin <i>et al.</i> (2014)
	Rift Valley fever virus (RVFV)	Replication inhibitors	Narayanan <i>et al.</i> (2012)
	Human norovirus (HuNoV)	Adsorption inhibitors	Yang <i>et al.</i> (2016a)
	Respiratory syncytial virus (RSV)	Virucidal effect	Yang <i>et al.</i> (2016b)
Influenza A virus (IAV)	Adsorption/replication inhibitors	Chen <i>et al.</i> (2010; 2013)	
Green tea or EGCG	Hepatitis B virus (HBV)	Adsorption/replication inhibitors	Xu <i>et al.</i> (2016)
	Herpes simplex virus (HSV)	Adsorption/replication inhibitors	Colpitts and Schang (2014)
	Epstein-Barr virus (EPV)	Adsorption/replication inhibitors	Liu <i>et al.</i> (2013)
	HIV	Adsorption/replication inhibitors	Hartjen <i>et al.</i> (2012)
	Hepatitis C virus (HCV)	Adsorption/replication inhibitors	Calland <i>et al.</i> (2015)
	Influenza virus	Adsorption/replication inhibitors	Yang <i>et al.</i> (2014)
	Dengue virus (DENV)	Adsorption/replication inhibitors	Carneiro <i>et al.</i> (2016)
	Chikungunya virus (CHIKV)	Entry inhibitor	Weber <i>et al.</i> (2015)
	Human T-lymph virus type 1 (HTLV-1)	Inhibition of viral gene expression	Harakeh <i>et al.</i> (2014)
	Rotavirus	Entry inhibitor	Mukoyama <i>et al.</i> (1991)
	Enterovirus EV71	Entry inhibitor	Ho <i>et al.</i> (2009)
	Ebola virus (EBOV)	Release/replication inhibitors	Reid <i>et al.</i> (2014)
	Porcine reproductive & respiratory syndrome virus (PRRSV)	Entry inhibitor	Zhao <i>et al.</i> (2015)
	Viral hemorrhagic septimia virus (VHSV)	Replication inhibitor	Estepa and Coll (2015)
	Grass carp reovirus (GCRV)	Replication inhibitor	Wang <i>et al.</i> (2016)
Zika virus (ZIKV)	Adsorption/replication inhibitors	Carneiro <i>et al.</i> (2016)	

Natural antimicrobials or plant extracts (phenolics, flavonoids, tannins) can have a negative influence on oxidative stress, assisting the organism in protecting itself from the harmful effects of reactive oxygen species (Hwang *et al.*, 2016). Natural antimicrobial combinations have previously been shown to be effective in reducing bacterial infections in chicken and mouse animal models (Sima *et al.*, 2018; Stratakos *et al.*, 2019). We propose that combining natural antimicrobials can successfully minimize clinical signs of sickness while having no negative impact on animal health. The activity of curcumin, EGCG and their combination against IBV *in vivo* was investigated in this work.

MATERIALS AND METHODS

Viruses and natural compounds

Infectious bronchitis virus (IBV) was graciously donated by Egypt's Animal Health Research Institute. IBV was propagated in pathogen-free embryonated chicken eggs that were 10 days old. The allantoic fluids were extracted, titrated and kept at $-80\text{ }^{\circ}\text{C}$ 48 h after infection. The median infectious dosage of embryos (EID₅₀) was estimated using a previously described approach (Reed and Muench, 1938). The EGCG and curcumin were purchased from Sigma-Aldrich, St. Louis, MO and kept at $4\text{ }^{\circ}\text{C}$ until further use.

Preparation of pepperin

A total of 10 g of black pepper powder were extracted in a solvent extractor for two hours with 150 ml of 100% ethanol. The solution was filtered and concentrated in a $60\text{ }^{\circ}\text{C}$ water bath. With constant stirring, 10 ml of 10% alcoholic potassium hydroxide was added to the filtrate. The insoluble residue was filtered and the alcoholic solution was allowed to stand overnight before being filtered through a membrane filter. Pepperin was isolated and mixed with curcumin to increase curcumin bioavailability.

Cytotoxicity assay

The cytotoxicity test was performed on accessible cell lines in our laboratory, including Hep-2 and vero cell lines (Nawah-Scientific, Cairo, Egypt). The cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) with 10% FBS, 100 units/mL penicillin and 100 mg/mL streptomycin. Cultures were kept at $37\text{ }^{\circ}\text{C}$ in a humidified environment with 5% CO₂. To make a stock solution, each chemical was pre-solubilized in dimethyl sulphoxide (DMSO) at $37\text{ }^{\circ}\text{C}$. Serial two-fold dilutions were conducted (100, 50, 25, 12.5, 6.25, 3.125, 1.5625 $\mu\text{g}/\text{mL}$). The sulforhodamine B colorimetric (SRB) test was used to assess cell viability. Confluent monolayers of each cell line were cultivated for 24 h in 96 well microtitre plates. Cells were cultured in triplicate with varying doses of the test substances for 72 h at $37\text{ }^{\circ}\text{C}$ in a CO₂ atmosphere. After that, 150 μL cold 10% trichloro acetate (TCA) was

added to each well and incubated at $4\text{ }^{\circ}\text{C}$ for 1 h. Plates were washed four times with slow-running tap water before being blotted dry with paper towels. 100 μL of 0.057% (w/v) SRB solution was added and incubated at room temperature for 30 min before washing with 1% (v/v) acetic acid to remove unbound pigment followed by air dry of the plates. The SRB stain was dissolved in each well with 200 μL of 10 mM Tris base solution. Finally, the OD was measured at 570 nm in a microplate reader (BMG LABTECH® FLUOstar Omega, Germany) and cell survival percentages were estimated using the following formula:

$$\text{Survival rate} = \frac{\text{OD}_{570} \text{ Drug}}{\text{OD}_{570} \text{ Control (untreated)}} \times 100\%$$

Study design and experimental model

This study followed the rules of the Local Ethical and Animal Welfare Committee of the Faculty of Postgraduate Studies for Advanced Sciences at Beni-Suef University in Egypt. One-day old chickens (n=100) were purchased from a nearby hatchery and raised to the fourth week of life. Under typical management settings, 90 two weeks old mixed-sex hens were maintained in separate high efficiency particulate arresting isolators with wire flooring. Each chicken received commercial chicken feed containing 23% crude proteins, 4.93% fats, 70% carbohydrates and 3027 kcal/kg calories. The chickens were sorted into nine groups of ten chickens (A, B, C, D, E, F, G, H and I). Curcumin and EGCG were diluted in 1% carboxy methyl cellulose and sprayed into the diet (Naderi *et al.*, 2014). Group A served as the untreated and uninfected negative control. The positive control group, Group B was infected with IBV. Groups C and D were infected with IBV and treated with curcumin or EGCG at doses of 5 mg/kg and 400 mg/kg, respectively. Group E was infected with IBV and given a diet containing 2.5 mg/kg curcumin and 200 mg/kg EGCG. The F and G groups were fed for five days with curcumin and EGCG at 5 mg/kg and 400 mg/kg of feed, respectively, before being infected with IBV. Group H was given a five-day treatment with 2.5 mg/kg curcumin and 200 mg/kg EGCG before being infected with IBV. The tested concentrations of curcumin and EGCG were selected based on previously published reports (Umar *et al.*, 2016; Aydogan *et al.*, 2018). At the second week of life, 0.1 ml of IBV (10^{5.6} EID₅₀/bird) was injected intra-nasally (IN) into infected groups. Acyclovir (40 mg/kg) was given to Group I as a positive control. Table 2 summarizes the study's design.

Clinical signs

The chickens were monitored daily for clinical indications such as illness, sneezing, nasal discharge, diarrhea, head swelling and death. Clinical indications were quantified and expressed as a proportion of total chicken population (Table 3). The percentage difference was utilized to determine the efficacy of curcumin, EGCG and their

Table 2: The study groups utilized to evaluate the *in vivo* antiviral activity of curcumin and EGCG against IBV in chicken. The table indicates the number of birds in each group, the concentration of each drug employed and the alternative modes for chicken treatment by compounds.

Group	Number	Treatment
Group A	10	No compounds + no virus
Group B	10	10 ^{5.6} EID ₅₀ /100 µl of IBV
Group C	10	10 ^{5.6} EID ₅₀ /100 µl of IBV + curcumin (5 mg/kg feed)
Group D	10	10 ^{5.6} EID ₅₀ /100 µl of IBV + EGCG (400 mg/kg feed)
Group E	10	10 ^{5.6} EID ₅₀ /100 µl of IBV + (50% curcumin/EGCG mixture)
Group F	10	Curcumin (5 mg/kg feed) + 10 ^{5.6} EID ₅₀ /100 µl of IBV
Group G	10	EGCG (400 mg/kg feed) + 10 ^{5.6} EID ₅₀ /100 µl of IBV
Group H	10	(50% curcumin/EGCG mixture) + 10 ^{5.6} EID ₅₀ /100 µl of IBV
Group I	10	10 ^{5.6} EID ₅₀ /100 µl of IBV + Acyclovir (40mg/kg feed)

Chicken were classified into 9 groups as follow: Groups A, B and I were negative and positive controls, respectively; Groups C, D and E were challenged by IBV then treated with different compounds; Groups F, G and H are treated with different compounds and then challenged by IBV.

Table 3: The clinical signs scoring in all chicken groups. The number of chickens in each group that have one or more clinical signs represents the incidence percentage of each sign inside the group.

Group	Clinical signs			Odds ratio	p-value
	Sneezing	Mouth breathing and trachea rale	Conjunctivitis		
A	NA	NA	NA	NA	NA
B	10/10	8/10	6/10		
C	8/10	5/10	5/10	1.333	0.4773
D	8/10	6/10	2/10	1.500	0.3266
E	5/10	4/10	2/10	2.182	0.0805
F	2/10	1/10	NA	24.000	0.0018*
G	2/10	NA	NA	12.000	0.0014*
H	1/10	NA	NA	24.000	0.0025*
I	2/10	NA	NA	12.000	0.0014*

*Significant difference between test group and control group ($p < 0.05$). NA means no clinical sign appeared.

Table 4: Sequences of the primers and probe used in this study.

Name	Gene	Sequence	Reference
Forward Primer		5'-ATGCTCAACCTTGTCCTAGCA-3'	
Reverse Primer	N Gene	5'-TCAAAGTGGGATCATCACGT-3'	Meir <i>et al.</i> (2010)
Probe		5'-FAM-TTGAAGTAGAGTGACGCCAAACTTCA-TAMRA-3'	

mixtures in reducing clinical symptoms of illness. Chickens from each group were humanely slaughtered after 28 days and tracheal samples were preserved at -80 °C for viral RNA quantification through RT-qPCR and histological exams.

Quantification of viral titer using RT-qPCR

The RNA was isolated using the QIAamp viral RNA isolation kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. The viral titer was measured using the previously established quantitative real time reverse transcriptase polymerase chain reaction (qRT-PCR) (Meir *et al.*, 2010). In brief, RT-qPCR experiment

targeting the IBV N gene was carried out with the RT-qPCR Kit (Qiagen, Düsseldorf, Germany) using the primers and probe (Table 4). The quantitative RT-qPCR reactions were carried out using the following temperature program on a Real-Time PCR machine (Stratagene MX3005P). For primer sets, the reverse transcriptase step settings were 50 °C for 30 min, 94 °C for 15 min, followed by 40 cycles of 94 °C for 15 sec and 60 °C for 45 sec. With RNA isolated from the challenge virus, standard curve was generated with viral control and the amount of RNA in the samples was converted into EID₅₀/mL by interpolation (Umar *et al.*, 2015). The virus titer was presented by the mean ± SD of the virus titer per mL.

Histopathology examination

Representative trachea samples were preserved in 10% formalin solution for 48 h before being processed by paraffin embedding, sectioned 5 and stained with Haematoxylin and Eosin (H&E) according to Bancroft and Gamble (2002). The trachea was then inspected microscopically and scored using the prior histopathologic scoring system for trachea (Levisohn *et al.*, 1986; Todd *et al.*, 1991) with slight modifications. The following morphological alterations were observed: mucous epithelial cilia loss, balloon-like cell extrusion and necrotic exfoliation of the mucous layer, lymphocyte and heterophil infiltration in the submucosal layer, hyperemia and/or bleeding.

Statistical analysis

GraphPad software was used for statistical analysis. The data were reported as mean \pm SD. *p*-values less than 0.05 were deemed statistically significant. Viral titers obtained were analyzed for significance using student's *t*-

test. An ANOVA test was used to compare the percentage reduction to the untreated control.

RESULTS

The cytotoxicity of curcumin and EGCG on vero and Hep-2 cell lines

The vitality of the cells was determined using an optical microscope and validated using the SRB technique. The cytotoxicity was computed using GraphPad Prism software (version 5.01) nonlinear regression analysis by graphing log inhibitor versus normalized response. The cytotoxic concentration of the compounds to cause death to 50% (CC₅₀) of viable vero cells of curcumin and EGCG were found to be 93.19 and 88.82 μ g/mL, respectively. For Hep-2 cells, the CC₅₀ of curcumin and EGCG were found to be 52.86 and 41.90 μ g/mL, respectively. As indicated in Figure 1, the cell survival rate evaluated by the SRB technique was close to 100%, indicating that there was no substantial influence on the cells.

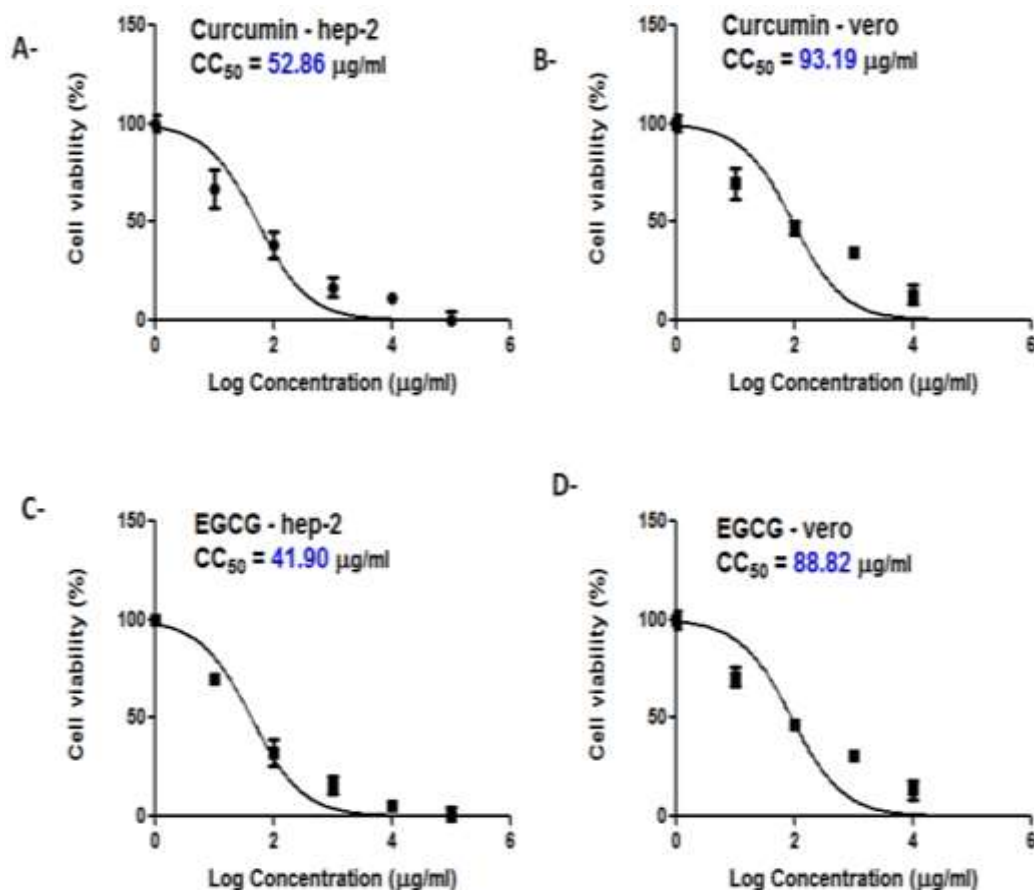


Figure 1: Effect of curcumin and EGCG on the viability of Hep-2 and vero cell lines. Sulforhodamine B colorimetric (SRB) staining was used to assess cell viability. The survival rates of Hep-2 and vero cells were given at different concentrations of curcumin and EGCG, and cell survival rates of more than 50% was considered to be the maximum non-toxic concentration of curcumin and EGCG. Data are expressed as mean \pm SD of 3 independent experiments.

Effect of curcumin and EGCG on the clinical signs of IBV in chicken groups

In order to quantify the favorable benefits stated above, we extensively examined the clinical symptoms in the challenged chicken during curcumin or EGCG treatment following 6 days of viral infection (Table 3). The A, G, H and I groups had no symptoms, the F group had light symptoms, the E group had moderate symptoms and the B, C and D groups had severe symptoms. Sneezing, tracheal wet rale, mouth breathing and conjunctivitis (coughing, ruffled feathers and frequent shaking signals) were noted in IBV-infected groups. In the C and F groups, the effectiveness of curcumin administration was assessed before and after viral infection, in contrast to the control group B. While the efficacy of EGCG therapy was compared in the D and G groups before and after viral infection. The efficacy of the curcumin/EGCG combination was also examined in the E and H groups before and after viral infection. Sneezing was decreased in the curcumin-treated group before viral infection (F) compared to the curcumin-treated group after viral infection (C), (50% vs. 80%, respectively). Sneezing was reduced also in the EGCG-treated groups before (G) and after viral infection (D), (20% vs. 80%, respectively). For sneezing in mixed curcumin/EGCG groups before (H) and after (E) treatment, there is also markedly change (10% vs. 50%, respectively). For mouth breathing and conjunctivitis clinical symptoms, all treated curcumin, EGCG and mixed groups before viral infection were significantly reduced more than all groups treated with tested compounds after viral infection as shown in Table 3.

Effect of the curcumin and EGCG on the viral load in the trachea of chickens with IBS

We then investigated the antiviral effects of curcumin and EGCG in a chicken model infected with the IBS virus. As shown in Table 5, the viral RNA copies in group B was approximately $\log_{10}^{6.6394}$ copies/g tissue, with a considerable decrease in groups F, G and H ($\log_{10}^{3.3922}$,

$\log_{10}^{3.5822}$ and $\log_{10}^{3.5807}$ copies/g tissue, respectively) similar to the Acyclovir-treated control group (I). The viral load levels were reduced to those found in the Acyclovir-treated group (I) ($\log_{10}^{3.581}$ copies/g tissues). The levels of significance were computed using group I as a reference. These findings suggest that curcumin, EGCG and their combination reduced the virus load in tracheal tissue, particularly during pretreatment prior to viral infection by reduction fold up to 10^3 , while groups (C, D and E) have a minor reduction fold on viral load up to 10^1 .

Effect of the curcumin and EGCG on the pathological injury trachea of chickens with IBS

The ratings for the distribution and degree of the aforementioned lesions in each chicken's trachea are classified as follows: 0, no lesion; 1, lesion represented in less than 10% of tissue involvement; 2, lesion represented in 10-50% involvement; 3, lesion represented in 50-90% involvement; and 4, lesion represented in more than 90% involvement (Table 6). The majority of the tracheal cilia in the IBV-infected group (B) broke off sever mucous epithelial cilia loss, balloon-like cell extrusion and necrotic exfoliation of the mucous layer, lymphocyte and heterophil infiltration in the submucosal layer. The mucosal epithelial cells exfoliated, vanished and the submucosal structure was jumbled (Figure 2-A). While moderated pathogenicity in some areas were observed in the treatment groups (C, E and D), where compounds were injected after viral infection (Figures 2-B, C and D), the mucosa is thickened and infiltrated, with degeneration and desquamation of epithelial cells. For treatment groups (F, G and H), where the compounds were injected prior to viral infection (Figures 2-E, F and G), mild pathogenicity in some areas were seen, the mucosa is thickened with degeneration and desquamation of epithelial cells. Totally, the tracheal lesions in the treated groups prior to viral infection were grade 1, the same as in the Acyclovir-treated group (I). They all had low vacuolar degeneration, with only a few mononuclear cells in the submucosal tissue.

Table 5: The average of RNA copies per group using quantitative real time PCR.

Group	Average titer (EID ₅₀ /mL)	Log ₁₀	Reduction fold	Odds ratio	p-value
Group A (Negative control)	-	-	-	-	-
Group B (Positive control)	4358700 ± 128	10 ^{6.6394}	-	-	-
Group C (Virus + Curcumin)	313850 ± 165	10 ^{5.4967}	10 ¹	13.8878	0.0001*
Group D (Virus + EGCG)	535780 ± 558	10 ^{5.729}	10 ¹	8.1352	0.0001*
Group E (Virus + Curcumin/EGCG)	39394 ± 442	10 ^{4.5954}	10 ²	110.6438	0.0001*
Group F (Curcumin + virus)	2468 ± 131	10 ^{3.3922}	10 ³	1766	0.0001*
Group G (EGCG + virus)	3822 ± 107	10 ^{3.5822}	10 ³	1140	0.0001*
Group H (Curcumin/EGCG + virus)	3808 ± 80	10 ^{3.5807}	10 ³	1144	0.0001*
Group I (Virus + Acyclovir)	3811 ± 180	10 ^{3.581}	10 ³	1144	0.0001*

Each number represents the mean ± SD of three independent experiments.

*Significant difference between test group and control group ($p < 0.05$).

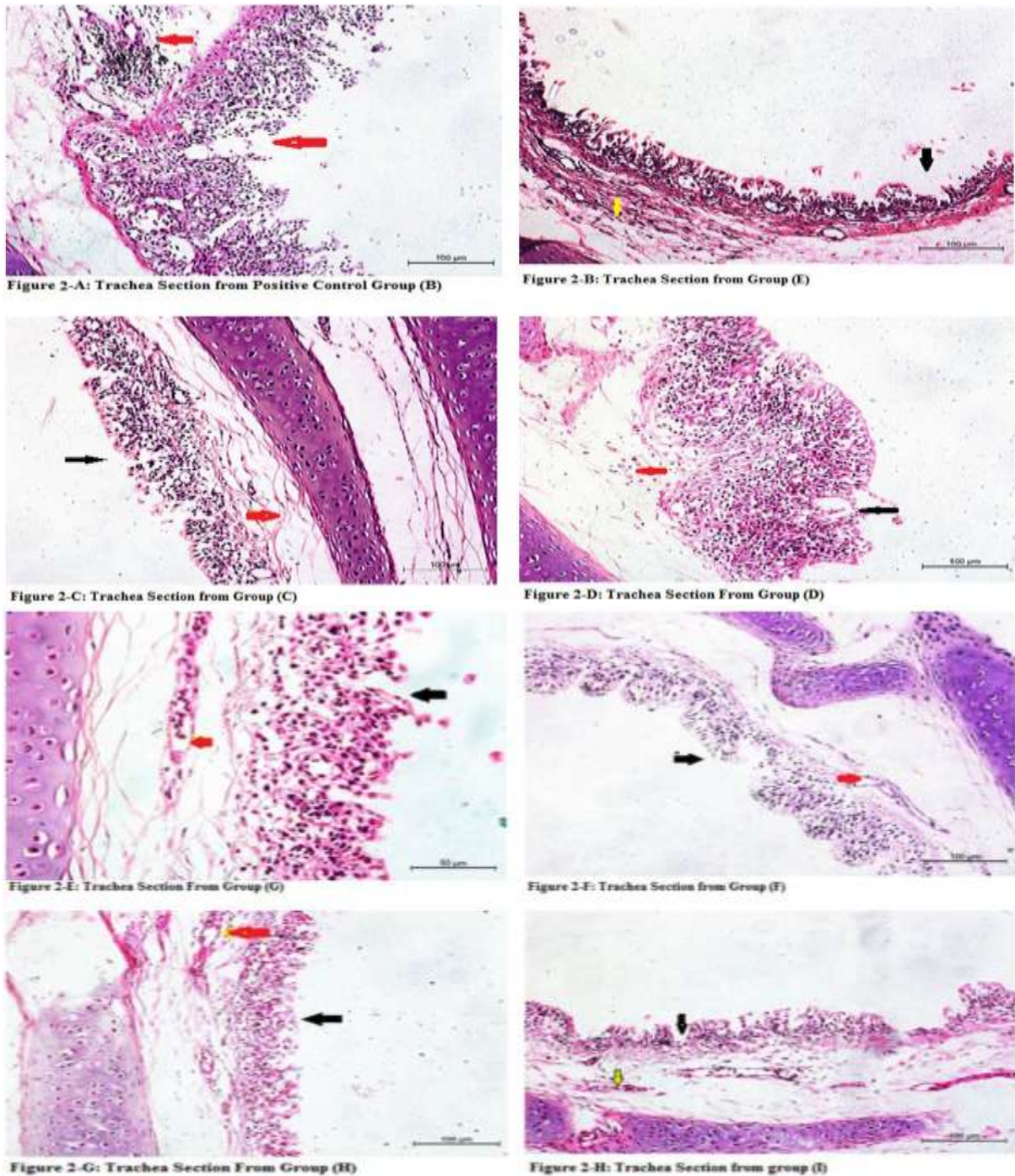


Figure 2: Effects of curcumin and EGCG on IBV-induced tracheal injury of chicken. Trachea was processed for histological evaluation at day 6 after viral infection. Black arrow refers to the degree of loss cilia and degeneration of epithelial cell, while red arrow indicates mucosal inflammatory infiltrates or epithelial hyperplasia infiltration. (2-A) Trachea section from the IBV-infected group B shows severe mucous epithelial cilia loss, balloon-like cell extrusion and necrotic exfoliation of the mucous layer, lymphocyte and heterophil infiltration in the submucosal layer. (2-B), (2-C) and (2-D) show moderate pathogenicity and the mucosa is thickened, infiltrated with degeneration and desquamation of epithelial cells. (2-E), (2-F) and (2-G) show mild pathogenicity in some areas and the mucosa is thickened with degeneration and desquamation of epithelial cells. (2-H) The trachea section from treated group I shows low vacuolar degeneration, with only a few mononuclear cells in the submucosal tissue.

Table 6: Scoring of trachea tissues histopathology.

Group	Loss of cilia or epithelial cells	Degeneration or necrosis of epithelial cells	Mucosal inflammatory infiltrates or epithelial hyperplasia	Grade
A	-	-	-	0
B	+++	++++	+++	3
C	+	++	+	2
D	+	++	+	2
E	+	+	++	2
F	+	-	+	1
G	+	-	+	1
H	+	-	+	1
I	+	-	+	1

(-) indicates no apparent clinical disease sign in trachea tissue; + Mild, ++ Moderate, +++Severe.

DISCUSSION

There are several known mutant strains of infectious bronchitis virus (IBV), resulting in more varied and complicated genotypes (Laconi *et al.*, 2019). It is difficult to prevent or control IBV infection due to poor cross-protection across various serotypes of vaccines. As a result, developing an effective antiviral medication is a critical strategy for IBV therapy. Previous research has revealed that curcumin's antiviral action is mediated by a variety of mechanisms. Curcumin, for example, inhibits the Japanese encephalitis virus via a dysregulated ubiquitin-proteasome pathway (Dutta *et al.*, 2009). The inhibition of NF- κ B signalling by curcumin limits Rift Valley fever viral multiplication (Narayanan *et al.*, 2012). Curcumin inhibits SREBP-1, an essential transcription factor in sterol synthesis regulation that is susceptible to Hepatitis C virus (Kim *et al.*, 2010).

We reported here how IBV may be suppressed by curcumin or/and EGCG, components of turmeric and green tea, respectively, using three approaches: qRT-PCR, clinical symptoms and histological investigation. In this study, two alternative experimental designs were investigated: (1) pretreatment of chickens prior to infection and (2) direct treatment of virus-infected chickens. Our findings suggested that curcumin or/and EGCG may markedly reduce the amount of IBV titer, the clinical manifestations of this respiratory disease and the pathological destruction of the trachea produced by IBV in chickens, particularly in pretreatment of hens prior to infection experiment design.

In particular, with 0.1 mL of $10^{5.6}$ EID₅₀ infective units of IBV inoculated into each chicken, the curcumin, EGCG and curcumin/EGCG mixture treatments reduced viral load ($\log_{10}^{3.3922}$, $\log_{10}^{3.5822}$ and $\log_{10}^{3.5807}$ copies/g tissue, respectively) compared to infected non-treated chickens ($\log_{10}^{6.6394}$), representing a 3-log reduction of infective units during pretreatment mechanism. This might be because curcumin or EGCG decreased virus-cell binding considerably in the cells of infected chickens. These findings are consistent with previous reports on the antiviral properties of curcumin and EGCG. Curcumin was demonstrated to have antiviral activity against zika and

chikungunya viruses by inhibiting viral-cell binding in a pretreatment experiment according to (Mounce *et al.*, 2017). Additionally, curcumin was shown to impact HCV replication through binding and fusion (Anggakusuma *et al.*, 2014), similar to the results we have obtained. EGCG proved to be more likely to inhibit early stages of infections, such as attachment, according to (Ogawa *et al.*, 2020).

Chickens are an important natural host of the infectious bronchitis virus which targets the upper respiratory tract and intensive virus replication leads to clinical manifestation characteristic signs such as sneezing and conjunctivitis. When curcumin or/and EGCG were included in the diet of chicken, the characteristic clinical signs were significantly decreased by curcumin or/and EGCG during the pretreatment mechanism. The efficacy of curcumin or/and EGCG to prevent respiratory illness progression was validated by the reduced presence of clinical symptoms in infected birds, as in Table 2. The improved immune and antiviral activities of the studied substances, as reported earlier, may account for the decreased severity of clinical symptoms in EGCG and curcumin fed chickens (Chen *et al.*, 2013; Moghadamtousi *et al.*, 2014; Umar *et al.*, 2015). As a result, it's reasonable to believe that the compounds studied can reduce the severity of IBV clinical indications, particularly when administered prior to viral infection. To further confirm the inhibitory effect of curcumin or/and EGCG on infected chickens, the pathological injury of trachea tissues was examined. Our results confirmed the curcumin or/and EGCG had anti-IBV activity, such as reducing the alleviating the pathological injury of trachea caused by IBV in chickens (Figure 2). When came to evaluation of antiviral effects, Acyclovir was used as a control drug. The inhibitory effect of 5 g/kg curcumin and 400 mg/kg EGCG was comparable to that of 40 mg/kg Acyclovir *in vivo*. Alterations associated with IBV infection were observed specifically in trachea ranged from loss of cilia, degeneration of epithelial cells, to necrosis and mucosal infiltrate. However, for most of these abnormalities, severity was lower in the curcumin- or EGCG-treated groups before viral infection.

CONCLUSION

The poultry industry needs to find and test new and natural antiviral combinations that might minimize the severity of clinical symptoms and maybe prevent viral infections. Our research convincingly demonstrated that antiviral drugs like curcumin and/or ECGC, as well as their combination, can reduce IBV pathogenicity *in vivo*.

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