German Journal of Veterinary Research

eISSN:2703-1322

Research article

Efficacy of *Olea europaea* leaves and propolis extracts in the control of experimentally induced infectious bronchitis in broiler chickens

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Article History:
Received: 29-Jan-2023
Accepted: 12-Mar-2023
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Abstract

Infectious bronchitis (IB) is a viral disease that causes serious economic losses in the broiler industry. This study evaluated the effectiveness of *Olea europaea* leaves and propolis extracts (OLP) mixture at a rate of 400 μg and 100 mg/mL, respectively, in curing IB in broiler chickens. One-day-old Ross broiler chicks were randomized into four groups (G) of twenty-one chicks; G1 (control negative; no infection and treatment); G2 (no infection, treatment only), G3 (control positive; infection only and no treatment), and G4 (infection and treatment) that infected with IBv (10^6 EID_{50}/mL) at 21 days old. The OLP treatment was applied for birds in G2 and G4 at a dose of 0.5 mL/liter drinking water for three successive days. The growth performance, clinical and pathological examinations, and viral shedding were evaluated. The use of the OLP resulted in protection from IB infection through the significant improvement of performance parameters such as weight gain and feed conversion ratio, decrease in mortality rate, lowering disease severity, and rapid recovery from the observed clinical signs (mainly respiratory signs), gross and microscopic lesions in the trachea, lung, and kidneys as compared to those in the positive control (G3). Moreover, the viral shedding in the OLP-treated chicks (G4) was significantly decreased in tracheal and cloacal swabs to a rate less than 3×10^3 IBv genome copy number and became not detectable at 14-days post-infection (dpi) in their cloacal swabs. In conclusion, OLP can potentially display an antiviral effect against IB in broiler chickens. Therefore, adding OLP to the chicken drinking water is recommended to prevent and control IB.

Keywords: Broiler chick, Infectious bronchitis, *Olea europaea*, Olive leaves extract, Propolis, Virus shedding

Citation: Elbasuni, S. S., Bahgat, H., Nada, M. O., Khattab, M. S., Abugomaa, A., Hamam, H. and Elbadawy, M. 2023. Efficacy of *Olea europaea* leaves and propolis extracts in the control of experimentally induced infectious bronchitis in broiler chickens. Ger. J. Vet. Res. 3 (2):1-10. https://doi.org/10.51585/gjvr.2023.2.0052

Introduction

Infectious bronchitis (IB) is a prevalent disease in chickens caused by the IB virus (IBv), leading to pathological alterations in the respiratory, urinary, and reproductive tracts, reducing poultry production, and increasing mortalities (Lau et al., 2008; Zhuang et al., 2009; Jackwood et al., 2010; Chen et al., 2014). IBv is a *Gammacoronavirus* (γ-CoV), with a 27-kb positive-sense single-stranded RNA genome that encodes four structural and four non-structural proteins (Müller et al., 2018). IBv was the first coronavirus found in domestic chickens, causing a serious economic impact, and continues to emerge and re-emerge despite various efforts in disease prevention (Lau et al., 2008; Chen et al., 2014). Outbreaks of IBv are reported worldwide in chickens and other birds with high morbidity, mortality, and adverse changes in the quality and quantity of egg production (Jackwood et al., 2010; Lelešius et al., 2019; Mani et al., 2020; Mansour et al., 2021). To control IB, live attenuated and inactivated vaccines

are often used in broilers, layers, and breeders (Sultan et al., 2019; Toro, 2021), although lately, immunization efficacy has been declining due to reports of viral genetic diversity and continuous emergence of serotypes (Franzo et al., 2016).

Medicinal plants have served as a source of motivation for developing numerous therapeutic pharmaceuticals (Shinohara et al., 2019; Elkomy et al., 2020; Elbadawy et al., 2021; Shinohara et al., 2022; Soliman et al., 2022a,b). These plants are of interest as immune modulators with antiviral against certain viral infections due to their content of various chemical ingredients that could stop the replication cycle of many types of DNA or RNA viruses (Jassim and Naji, 2003). Extensive research was conducted on the antiviral efficacy of the different plant species extracts against avian IBv strains (Jackwood et al., 2010; Chen et al., 2014; Lelešius et al., 2019). Most plant-derived compounds are direct-targeting antivirals through direct inhibition of some part of the virus, such as proteases or spike proteins (O'Keefe et al., 2010; Müller et al., 2018). Moreover, another important group of plant-derived compounds is host-targeting antivirals that prevent viral entry to the host cells (Zhuang et al., 2009) or stimulate the immune response against it (Lau et al., 2008). Recently, a docking analysis guided to have antiviral effects of naturally occurring phytochemicals that may boost their potency and/or selectivity (Takayasu et al., 2020). Oleuropein, quercetin, and kaempferol appeared to have the best potential to act as viral protein inhibitors (Khaerunnisa et al., 2020; Saif et al., 2021).

The *Olea europaea* is an important medicinal plant whose leaves have antioxidant, hypoglycemic, antihypertensive, and antimicrobial effects (Wang et al., 2008; Abugomaa and Elbadawy, 2020; Elbadawy et al., 2021). Previous studies on the olive leaf extract reported its antiviral activity against viral hemorrhagic septicemia rhabdovirus and Newcastle disease virus, as it has a lot of polyphenols, especially oleuropein, rutin, verbascoside, apigenin-7-glucoside, and luteolin-7-glucoside, which may be related to these properties (Micol et al., 2005; Salih et al., 2017). Hence, it could be a source of antiviral agents. Recently during the COVID-19 pandemic, *Olea europaea* considered as one of the most important medicinal plants used for prevention purposes against COVID-19 with a frequency rate of 72.7% in Morocco (El Alami et al., 2020).

It is worth mentioning that bee products with high amounts of quercetin, kaempferol, propolis, and their derivatives can be a natural alternative to fight against the important viral affections of humans and animals (Rzepecka-Stojko et al., 2015; Lima et al., 2021). Propolis has important pharmacological properties and can be used for various purposes. It has shown a tendency to be effective against a variety of viruses due to its content of natural protein kinase enzyme blockers and strengthens the immune system so that it can better fight off infections (Maruta and He, 2020). Several *in-vitro*, *in-silico*, animal models, and human clinical trials have shown that propolis can be used as a pharmaceutical compound. Based on the weight and strength of the available experimental and clinical evidence, propolis treats respiratory tract diseases as a main or complementary treatment (Zulhendri et al., 2022).

Moreover, the Food and Drug Administration (FDA) approved some safe polyphenolic compounds for drugs that produce safe, effective antivirus treatment medicines from naturally generated chemicals (Takayasu et al., 2020). From these points, the present study aimed to investigate the potential use of *Olea europaea* leaves and propolis extracts mixture as a potential therapy for the IBv and evaluate their efficacy on measurable parameters such as growth performance, clinical and pathological findings, and viral shedding in the broiler chickens.

Materials and methods

Challenge virus and used medical products

The challenge virus is IBv variant 2 (IBv/CH/GIZA/A-2019) obtained from the Animal Health Institute, Agriculture Research Center, Giza, Egypt. Moreover, Olivera® is a commercial product containing a mixture of *Olea europaea* leaves (400 μg/mL) and bee propolis extracts (100 mg/mL); obtained freely from Enhance Vet Company, a private veterinary pharmaceutical company in Egypt.

Experimental design

The Research Committee in the faculty of veterinary medicine at Benha University reviewed and authorized the experiments conducted here (BUFVTM02-12-22). One-day-old Ross broiler chicks from the commercial hatchery in EL-Qalyubia governorate were randomized into four groups, each of 21 chicks, and housed in an isolated room with free access to food and water. Group one (G1), labeled as control negative) was orally administered normal saline. Group two (G2), labeled as OLP-treated) was orally administered a mixture containing *Olea europaea* leaves extract and propolis (OLP; Olivera® 0.5 mL/liter drinking water) (Vahidi-Eyrisofla et al., 2019; Daza-Leon et al., 2022). Group three (G3), labeled as IBv-infected) was intranasally given 0.1 mL of IBv (10^6 EID_{50}/mL) at 21 days old (Cheng et al., 2018) to induce IBv infection. Group four (G4), labeled as IBv + OLP) was infected as in G3 but administered OLP simultaneously at the same dosage as in G2 for three successive days after IBv infection. A strict biosecurity program without vaccination was followed in the study.

Parameters used to evaluate the efficacy of *Olea europaea* leaves and propolis extracts mixture

Growth performance

The chicks were weighed individually before and after the challenge (0 and 14 dpi) to calculate body weight gain (BWG). Consequently, feed intake (FI) and feed conversion ratio (FCR) were estimated in the different groups (Conway and McKenzie, 2007).

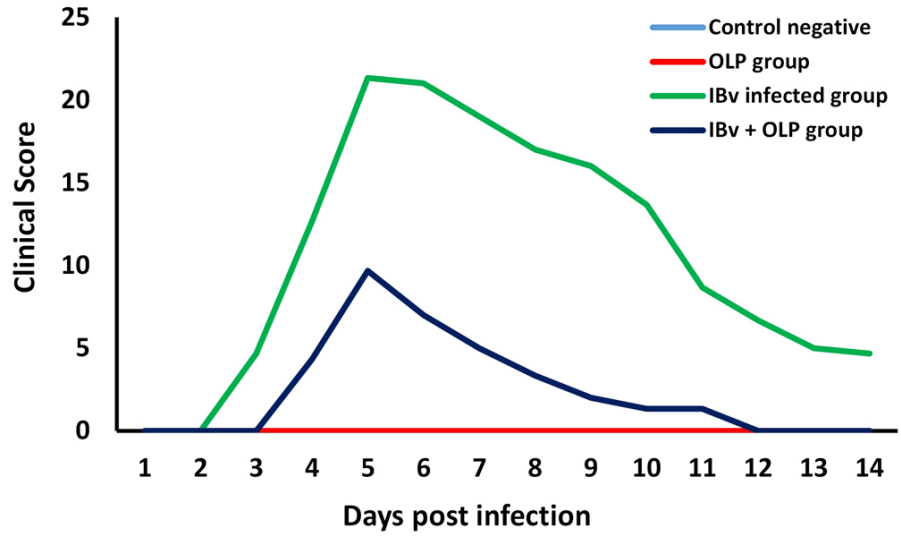

Figure 1: Impact of *Olea europaea* extract and propolis mixture on the clinical score of the IBv-infected Ross broiler chickens compared with other groups as recorded for 14 days post-infection. Clinical scores according to Lin et al. (2016): 0 = no clinical signs; 1 = slight lacrimation, slight shaking, watering feces or tracheal rales; 2 = lacrimation, presence of nasal exudate, depression, watery feces, apparent sneezing or cough; 3 = same as two but stronger with severe watery whitish feces; 4= death.

Clinical and pathological examinations

All chicks in the experimental groups were seen daily for 14 dpi. The clinical signs and postmortem lesions were recorded for respective groups when required (Jackwood and Wit, 2020). The chicks in the different groups were examined daily and scored according to Lin et al. (2016) as 0 (no clinical signs); 1 (slight lacrimation, slight shaking, watering feces or tracheal rales); 2 (lacrimation, presence of nasal exudate, depression, watery feces, apparent sneezing or cough); 3 (same as two but stronger with severe watery whitish feces); 4 (death). The mortality and survival rates were recorded. At 7 and 14 dpi, three chicks from each experimental group were euthanized and examined grossly. Samples from the trachea, lung, and kidneys were collected and fixed in phosphate-buffered formalin for at least 24 hrs and embedded in paraffin. Sections of 5 mm were cut and stained with hematoxylin and eosin (Gamble, 2008). The stained sections were microscopically examined for histopathological changes.

Viral shedding from the infected birds

Tracheal and cloacal swabs were obtained from three chicks in each group at 4, 7, 10, and 14 dpi in 1.5 mL of phosphate-buffered saline containing gentamicin (200 mg/mL) and penicillin G (1.000 units/mL). The obtained swabs were centrifuged at 600 g for 10 min; the supernatants were collected and stored at -80°C until use. The Viral RNA was extracted from the supernatant fluids using an extraction kit following the manufacturer's protocol. Reverse transcription was carried out with a reverse transcriptase PCR kit (Qiagen, Inc. Valencia CA, cat. no. 204443), and genome segments were amplified by PCR (MX3005P machine, Strata gene, USA) using IBv N gene primer Sequence (F; 5'-CAAGCTAGGTTTAAGCCAGGT-3' and R: 5'-TCTGAAAACCGTAGCGGATAT-3') (Metabion, Germany) (Yin et al., 2022).

The amplification reaction of RT-PCR assays was conducted according to the following thermal profile: pre-incubation one cycle at 95°C for 5 s, then two-step amplification: 40 cycles at 95°C for 20 s and 56°C for 30 s, followed by melting: one cycle at 56°C for 15 s, ended by cooling one cycle at 37°C for 30 s. The relative mRNA expression levels were calculated by the 2Ct method. For shedding titer, RT-PCR was used for virus quantitation (Yu et al., 2017). A standard curve for the assay was used to estimate the approximate genome copy number for each sample with real-time RT-PCR test components and thermocycler parameters that were previously described (Callison et al., 2006).

Statistical Analysis

The experimental data were analyzed with SPSS 16 software (SPSS Inc., Chicago, IL, USA). The results are expressed as the Means ± Standard Errors (S.E.M.). Differences between groups were evaluated using the one-way analysis of variance (ANOVA) $p\text{-}value < 0.05$ and Duncan's multiple comparison Post Hoc tests.

Results

Effect of *Olea europaea* leaves, and propolis extracts mixture on growth performance of broiler chicks infected with IBv

The body weights of chicks before IBv challenge at 21 days old were not significantly different. At 35 days old, which was 14 dpi, the body weight gain, feed consumption, and feed conversion ratio of the infected chicks in G3 were adversely affected compared to the non-infected chicks (G1) (Table 1). On the other hand, the

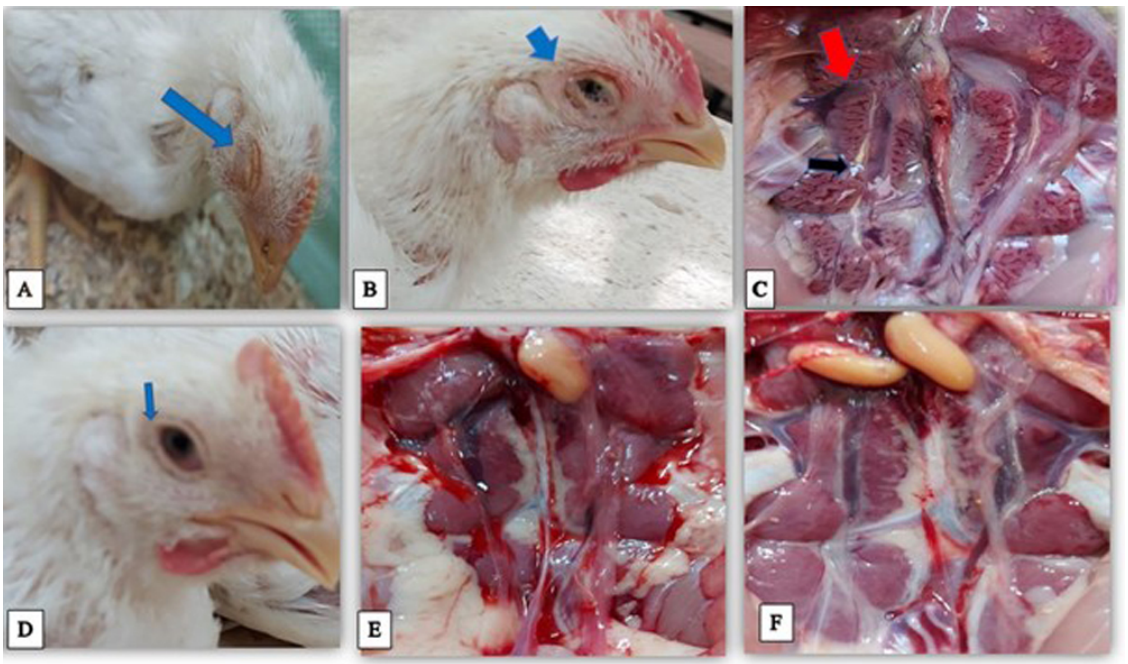

Figure 2: Efficacy of *Olea europaea* extract and propolis mixture on clinical and pathological findings of IBv-infected chickens. Pathological examination of the IBv-infected chicks (G3) showed severe depression, ruffled feather (A; blue arrow), swollen eyelids, and lacrimation (B; blue arrow) in addition to swollen pale kidneys and distended ureters with urates (C; red and black arrows). The OLP-treated chicks (G4) showed alert and apparently healthy states with mild eye lesions (D; blue arrow) and normal kidneys (E and F).

Table 1: Effect of *Olea europaea* leaves and propolis extracts (OLP) mixture on growth performance parameters, mortality and survival rate of broiler chicks infected with infectious bronchitis virus (IBv).

Groups	Parameters*				
	BWG (g)	FI (g)	FCR	Mortality %	Survival %
G1 (Control negative)	1281.3±65.9^{ab}	1696±0.00^{c}	1.33±0.07^{b}	0.00	100.0
G2 (OLP-treated)	1381±51.1^{a}	1800±12.9^{b}	1.30±0.05^{b}	0.00	100.0
G3 (IBv-infected)	953.3±29.1^{c}	1866.7±33.8^{a}	1.96±0.03^{a}	14.3	85.7
G4 (IBv + OLP)	1183.3±66.7^{b}	1478.8±10.4^{d}	1.27±0.07^{b}	0.00	100.0

* BWG: body weight gain, FI: feed intake, FCR: food conversion rate. The means within a raw with different superscripts are considered significantly different.

OLP treatment showed a significant improvement in the growth performance of the infected chicks (G4) and non-infected chicks (G2) when compared with those of G3 and G1, respectively (Table 1). During the daily examination, clinical signs were observed early after three days of infection in the IBv-infected group (G3) and continued for 14 days. The highest recorded score compared with the OLP-treated group (G4) that showed lesser abnormal signs with lower scoring started from 4 dpi and recovered early at 12 dpi (Figure 1).

Effect of *Olea europaea* leaves and propolis extracts mixture on clinical and pathological findings of broiler chicks infected with IBv

All chicks experimentally infected intranasally with IBv at 10^6 EID_{50} exhibited at 3 dpi respiratory signs, including sneezing, rales, gasping, nasal and ocular discharge accompanied by depression, and watery diarrhea (Figure 2 A, B). During the 14-day observation period, IBv-infected chicks (G3) showed the lowest survival rate (85.7%) as compared to other treated chicks (G2 and G4), and control negative G1 (100%) (Table 1). The earliest death from the infected group (G3) was found on 3 dpi and continued until 10 dpi with a rate of 14.3 (Table 1). The PM findings of the dead birds revealed pale, mottled, and swollen kidneys and distended renal tubules and ureters with excess urate (Figure 2 E). In addition, slight hemorrhaging with serous catarrhal exudates was observed in the tracheas of infected chickens. Clinicopathological assessment for the different experimental groups revealed that the in-

Table 2: Effect of *Olea europaea* leaves and propolis extracts (OLP) mixture on viral shedding (Viral RNA copies (log)) from the trachea of the infected broiler chicks with infectious bronchitis virus (IBv).

Groups	Days post-infection (dpi)*			
	4 dpi	7 dpi	10 dpi	14 dpi
G1 (Control negative)	0.00[c]	0.00[b]	0.00[b]	0.00[b]
G2 (OLP-treated)	0.00[c]	0.00[b]	0.00[b]	0.00[b]
G3 (IBv-infected)	52.5±0.29[a]	40.3±0.84[a]	12.40±0.26[a]	6.50±0.26[a]
G4 (IBv + OLP)	1.84±0.02[b]	1.43±0.24[b]	0.26±0.26[b]	0.00[b]

*The means within a raw with different superscripts are considered significantly different.

Table 3: Effect of *Olea europaea* leaves and propolis extracts (OLP) mixture on viral shedding (Viral RNA copies (log)) from the cloaca of the infected broiler chicks with infectious bronchitis virus (IBv).

Groups	Days post-infection (dpi)*			
	4 dpi	7 dpi	10 dpi	14 dpi
G1 (Control negative)	0.00[b]	0.00[c]	0 .00[c]	0.00[b]
G2 (OLP-treated)	0.00[b]	0.00[c]	0.00[c]	0.00[b]
G3 (IBv-infected)	20.1±0.01[a]	79.4±0.23[a]	40.7±0.41[a]	44.4±0.23[a]
G4 (IBv + OLP)	0.00[b]	22.4±0.26[b]	11.0±1.15[b]	0.00[b]

*The means within a raw with different superscripts are considered significantly different.

fected chicks of G3 beginning at 2 dpi exhibited severe respiratory signs persisting through 14 dpi. At 4 dpi, the OLP-treated chicks (G4) were alert and active with mild respiratory signs (Figure 2 C). At 7 dpi, more than half of the OLP-treated chicks in G4 became recovered, and clinical signs disappeared completely after 10 dpi. The recovery of OLP-treated chicks (G4) regained earlier and faster than the control positive (G3). Conversely, no abnormal signs were seen in the non-infected chicks (G1 and G2) throughout the observation period.

The pathological lesions of experimentally infected chickens were evaluated at 7 dpi. The IBv-infected chicks presented gross pathological lesions, including catarrhal exudates in the nasal passages and trachea, areas of pneumonia, and swollen pale kidneys with distended urates in the tubules and ureters (Figure 1). In addition, histopathological lesions were recorded such as desquamation of ciliated cells, mononuclear cell infiltration, and epithelial hyperplasia in the trachea. Further, severe diffuse mononuclear cell infiltration in the wall of the bronchi and alveolar wall was observed (Figure 3 A, B). Moreover, renal tubule dilation, necrotic tubular cells, and urates in the tubular cavity were observed in the kidneys (Figure 3 C). On the other hand, the OLP-treated chicks (G4) showed grossly mild tracheal lesions and normal kidneys (Figure 2 D, F) that were nearly like the control negative (G1). Also, the microscopic lesions in the trachea, lung, and kidneys in the OLP-treated chicks (Figure 3 D-F) were less severe than those in G3. Abnormal signs and pathological lesions were completely absent in the non-infected groups (G1 and G2).

Effect of *Olea europaea* leaves and propolis extracts mixture on viral shedding from the respiratory and digestive tracts of the infected broiler chicks with IBv

To further investigate the viral shedding of IBv in Ross broiler chicks, tracheal and cloacal swabs were collected from virus-infected chicks (10^6 EID_{50}, intranasal) at various time points. Viral detection was conducted by reverse transcription polymerase chain reaction (RT-PCR). As indicated in Table 2 & Table 3, 100% of the tracheal and cloacal swabs from the infected chicks (G3) were detected positive at 4, 7, 10, and 14 dpi with a significantly higher number of RNA copies exceeding 5×10^4 and 7×10^5, respectively, whereas viral shedding was decreased and remained with a rate less than 3×10^3 for the OLP-treated chicks (G4). More importantly, for the cloacal swabs, the virus was detected from the OLP-treated chicks (G4) as late at 7 dpi and became not detectable at 14 dpi. There was no detectable level of virus in any of the swab samples from the non-infected chicks (G1 and G2).

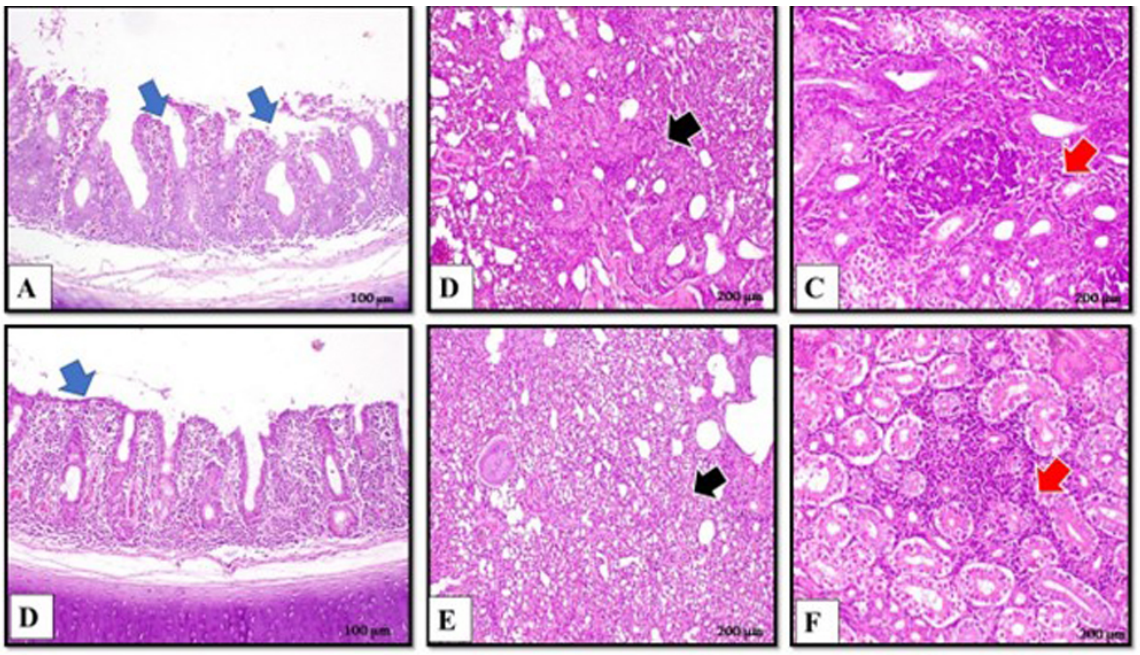

Figure 3: Effect of *Olea europaea* extract and propolis mixture on the pathological findings in the trachea, lung, and kidney from IBv experimentally infected chicks. IBv-infected chicks (G3) presented different pathological lesions such as focal cilia desquamation, edema, and severe diffuse lymphocytes infiltration in the trachea (A; H&E ×100; Blue arrow); severe diffuse mononuclear cells infiltration in the wall of bronchi and alveolar wall (B; H&E stain, ×200; Black arrow), and foci of mononuclear cells with lymphocytolysis and necrosis of the renal tubular epithelium (C; H&E stain, ×200; Red arrow). Panels D, E, and F refer to pathological lesions in the IBv-infected tissues of the OLP-treated chicks (G4) presented as mild mononuclear cells infiltration in the trachea (D; H&E ×100; Blue arrow) and lung (E; H&E stain, ×200; Black arrow), and mild leukocytes infiltration in the interstitial tissue of kidney (F; H&E stain, ×200; Red arrow).

Discussion

Infectious bronchitis is one of the important viral diseases in the poultry industry, associated with significant economic losses due to high mortalities and the expenses of vaccination and treatments (Chen et al., 2014). IBv has numerous known strains, these strains continue to evolve, and more mutants continue to recombine, increasing the diversity and complexity of IBv genotypes and serotypes (Franzo et al., 2016). In the current study, the infection with IBv induced significant clinical outcomes and high morbidity with a mortality rate of 14.3% in addition to dramatically reduced BWG and FCR in broiler chickens, compared with control non-infected chicks (G1 and 2), which is generally consistent with the results of previous studies (Albassam et al., 1986; Butcher et al., 1989; Feng et al., 2012; Huang et al., 2020; Wu et al., 2022) and also agreed with Jackwood and Wit (2020) who mentioned that chicks infected with the IBv showed respiratory symptoms, weight loss, and less feed efficiency with the morbidity rate of 100% and variable mortalities based on age and immune status of the birds, the specific virus strain, and the presence of secondary bacterial or viral infections.

On the other side, the tissue affections due to the IBv infection appeared clearly in the chicks of (G3) in terms of serious pathological lesions in the respiratory system, including grossly catarrhal exudates in the nasal passages, and trachea, areas of pneumonia that correlated with the histological findings in the trachea as the desquamation of ciliated cells, mononuclear cell infiltration, and epithelial hyperplasia; and in the lung with severe diffuse mononuclear cell infiltration of the bronchi and alveolar walls. The kidneys of the IBv-infected chicks were also affected and observed to be grossly swollen and pale with distended urates in the tubules and ureters and microscopically with signs of renal tubule dilatation, necrotic tubular cells, and urates in the tubular cavity. Overall, this is consistent with earlier research findings (Riddell, 1987; Chen et al., 1996; Chen and Itakura, 1997; Ziegler et al., 2002). Moreover, IBv was detected for the first time in the tracheal and cloacal swabs of the infected chicks (G3) at 4 dpi, and their highest concentrations were determined at 4 and 7 dpi, respectively. After that, the virus levels decreased but were still detectable. The IBv was excreted through the respiratory and digestive tracts, as previously reported by Alexander and Gough (1977, 1978); Chong and Apostolov (1982); Naqi et al. (2003). They were the main sources of the virus infection by direct and indirect transmission and explained the contagious nature of IBv (de Wit et al., 1998; De Wit, 2000; Matthijs et al., 2008).

The prevention of IBv infection is difficult and continues to be challenging because of the limited cross-protection of vaccinations of different serotypes. Con-

sequently, developing an efficient antiviral therapy is essential. When figuring out how well an antiviral drug works, the ability of the drug to get rid of pathogens in the body usually looks at the target proteins involved in their replication. This is done by measuring the viral load in infected birds and using clinicopathological examinations to figure out how well a drug can repair tissue damage induced by an infection (Jackwood et al., 2010; Chen et al., 2014; Lelešius et al., 2019; Kausar et al., 2021). In the present study, the inhibitory effect of the *Olea europaea* leaves and propolis extracts mixture against IBv infection was evaluated through three parameters, including protection against weight loss (as an important economical parameter), virological shedding, and microscopic lesions in the tracheas and kidneys (as the site of IBv replication).

The *Olea europaea* extract and propolis protected the broiler chicken from IBv by showing a significant improvement in the performance, rapid recovery from the observed signs, and no mortalities, in addition to limiting the progress of the pathological damage of IBv multiplication to the trachea, lungs, and kidneys and finally, stop the viral spread by making it less likely that the viruses would shed. Several *in-vitro* and *in-vivo* trials demonstrated the potential separate benefits for each *Olea europaea* extract and propolis against viruses, bacteria, and parasites. They further seem helpful for viral infections and spreading these viruses by inactivating them and the ability to penetrate infected cells and stop viral replication. The *Olea europaea* extract and propolis have a broad power to kill viruses by interfering with essential amino acid production. So, they are used separately to control several diseases for the presumed ability to act as natural killers of infectious agents by inhibiting their replication process (Sudjana et al., 2009; Omar, 2010; Ożarowski and Karpiński, 2023).

This corresponds to the data previously published, which demonstrated that the antiviral activity of *Olea europaea* extract and propolis against coronaviruses might be due to holding enrichment of the main effective substances as oleuropein, and quercetin, respectively (Khaerunnisa et al., 2020; Saif et al., 2021). Moreover, the positive protection effect of OLP mixture from IBv infection and getting rid of the virus has been linked to the potency of cellular immunity at the mucosal location, mainly in the respiratory tract but also in the gastrointestinal mucosal linings which prohibited the invasion of the challenge virus in the mucosa (Maruta and He, 2020; Ganapathy, 2021; Pojero et al., 2022).

Conclusion

Our study provides, for the first time, significant evidence that the mixture having the *Olea europaea* leaves and propolis extracts possess antiviral activities against IBv. Furthermore, its anti-IBv effect may be associated with reducing virus shedding levels, related to decreasing pathological injuries caused by IBv in the trachea, lung, and kidney, improved bird growth and health, and could be useful for the development of new antiviral agents. Even though more research is needed to figure out exactly how a mixture of *Olea europaea* and propolis extracts work to fight viruses, this new medicine has much potential for research and development.

Article Information

Funding. The present work did not receive any funding from any source.

Conflict of Interest. The authors declare that the research was conducted without any commercial or financial relationships that could be construed as a potential conflict of interest.

Authors Contributions. All authors contributed to the design, conception, data gathering, analysis, and interpretation. Authors drafted or edited the manuscript. The authors gave final approval of the published version and accepted to handle all aspects of the design, including investigating and resolving concerns about accuracy or integrity.

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