Tropical Journal of Pharmaceutical Research February 2025; 24 (2): 185-193
ISSN: 1596-5996 (print); 1596-9827 (electronic)

Available online at http://www.tjpr.org
http://dx.doi.org/10.4314/tjpr.v24i2.6

Original Research Article

Antiviral activity of *Eucalyptus globulus* and *Eucalyptus citriodora* essential oils against H5N1 avian influenza virus and infectious bronchitis virus

Ni Luh Putu Indi Dharmayanti[1]*, Risa Indriani[2], Diana Nurjanah[2], Harimurti Nuradji[2], Teguh Suyatno[2], Fadjry Djufry[3]

[1]Research Organization for Health, National Research and Innovation Agency, Indonesia, Jl. Raya Jakarta-Bogor, Pakansari, Kec. Cibinong, Kabupaten Bogor, West Java Indonesia 16911, [2]Research Center for Veterinary Science, Research Organization for Health, National Research and Innovation Agency, Indonesia, Jl. Raya Jakarta-Bogor, Pakansari, Kec. Cibinong, Kabupaten Bogor, West Java Indonesia 16911, [3]Agricultural Instruments Standardization Agency, Ministry of Agriculture, Jakarta, Indonesia

For correspondence:* ***Email:* *niluhputuindi@gmail.com;* ***Tel:*** *+62-81386000575*

Sent for review: 3 August 2024 *Revised accepted: 14 February 2025*

Abstract

Purpose: *To explore the antiviral potential of Eucalyptus citriodora and Eucalyptus globulus essential oils against zoonotic highly pathogenic avian influenza (HPAI) H5N1 and infectious bronchitis virus.*
Methods: *The extraction process of eucalyptus leaves was carried out using the steam distillation method. The physicochemical properties of both essential oils were determined by gas chromatography (GC) injection. The essential oils were then tested for toxicity and antiviral activity by liquid phase and vapour phase methods in ovo. In addition to specific pathogen-free (SPF)-egg testing, the infectivity of HPAI H5N1 and effectivity of the essential oils in the vapour phase in inhibiting the growth of H5N1 were also tested using MTT assays.*
Results: *The result showed 100 % inhibition by both essential oils against 10^3 EID_{50} of H5N1 AI and IBV for all concentrations that were administered through direct contact. In the vapor phase, complete inhibition of 10^2 EID_{50} of HPAI H5N1 and IBV viruses was observed at 8 % concentration for both essential oils, as confirmed by the absence of lesions on the embryos and negative HA test result.*
Conclusion: *Eucalyptus essential oils from E. citriodora and E. globulus may serve as potential alternative natural products for combating zoonotic HPAI H5N1 and infectious bronchitis virus (IBV). Further studies are needed to characterize their phytopharmaceutical activity and pathways as novel antiviral agents against these viruses.*

Keywords: *Essential oils, Eucalyptus, Viruses, Poultry, H5N1, Anti-infectious bronchitis virus (IBV)*

Tropical Journal of Pharmaceutical Research is indexed by Scopus, Chemical Abstracts, Embase, Index Copernicus, EBSCO, African Index Medicus, JournalSeek, Directory of Open Access Journals (DOAJ), African Journal Online, Bioline International, Open-J-Gate and Pharmacy Abstracts

INTRODUCTION

Highly Pathogenic Avian Influenza (HPAI) Virus A (H5N1) was first isolated and characterized from poultry in China in 1996 and has now become endemic in domesticated poultry, wild birds, and even humans in more than 60 countries in the world. Human-to-human transmission of H5N1 was first reported in 1997 causing severe health conditions in infected patients [1]. In Indonesia, an endemic HPAI H5N1 virus was reported to infect avian population which then led to sporadic infection in humans since 2005 [2]. This virus has been

reported to have the second-highest infection rate, with 200 reported cases, of which 168 were fatal. Furthermore, the death rate in Indonesia has been reported as the highest globally [3]. The infection of H5N1 in humans showed several clinical manifestations, such as acute respiratory syndrome, systemic organ failure and high mortality rate. It remains the biggest threat to economic losses among large poultry farms and small farm holders. However, vaccination, the main preventive program, results in highly variable outcomes. One of the limitations of these vaccines is vaccination failures resulting in insufficient long-term protective immunity in birds [4]. The rapid evolution of H5N1 virus by antigenic drift and antigenic shift (reassortment) has led to a decline or loss of vaccine efficacy. In addition, mutations in viral surface proteins have also resulted in antiviral drug resistance.

Infectious Bronchitis Virus (IBV), which is often found to be co-infected with H5N1 infection, originated from *Gammacoronavirus* genus and is also responsible for huge economic loss in poultry farms by causing respiratory system disorders, decrease in egg quality and quantity in the infected birds [5]. This infection affects the respiratory tract, as well as the reproductive, renal and digestive systems of chickens with various clinical manifestations.

The virus is an enveloped virus with a single-stranded positive-sense RNA genome of approximately 27 kb. The estimates of IBV substitution rate (10^{-4} – 10^{-5} substitutions/site/year) are still remarkable and yield notable evolutionary potential. This reflects the heterogeneity of variants that have led to the emergence of new serotypes and genotypes globally. Due to its evolutionary ability, the effectiveness of vaccination is very limited. This is indicated by the continued occurrence of IBV outbreaks in poultry farms that received vaccination [6]. This condition is worsened when infection between IBV and avian influenza virus (AIV) occurs simultaneously. Co-infection with the viruses might increase the pathogenicity by increasing the activation of NLRP3 inflammasome and inducing lethal inflammatory response, resulting in lesions that are found in several vital organs [7].

Indonesia is known for its enormous herbal plant diversity which empirically has been utilized for treatments such as anti-cancer, antimalarial, anti-parasite, anti-diabetes, antimicrobial, antifungal, anti-allergy, immunostimulants and also antiviral [8]. Most herbal extracts and compounds have been shown to regulate intercellular immune function and proteins, as well as directly inhibit the virus [9]. One of such product which originated from herbal plants is in the form of essential oils. Eucalyptus essential oils (EEO) contain both major and minor phytoconstituents which have long been used as traditional medicine across the globe, including as an antiviral therapy against influenza and other respiratory diseases [10].

Indonesia's herbal plants from the family *Myrtaceae* namely *Eucalyptus citriodora* (*E*. *citriodora*) and *Eucalyptus globulus* (*E*. *globulus*) have the potential to be utilized as new antiviral candidates. Therefore, this study explores the potential of *E. citriodora* and *E. globulus* essential oils as alternative natural products against zoonotic HPAI H5N1, which poses significant health risks to humans and poultry in Indonesia, as well as against IBV, a major viral agent of poultry infection that causes high mortality and reduced egg production.

EXPERIMENTAL

Virus isolation and propagation

Local isolates of HPAI H5N1 (A/Muscovy Duck/Banten/BR7/2013) and Infectious Bronchitis Virus (IBV) were collected from Banten Province, Indonesia and characterized as reported [11]. Both IBV and HPAI H5N1 viruses were propagated in Specific Pathogen Free (SPF) embryonated chicken eggs following standard procedure [12]. The viral titration test was conducted according to established protocol [13] (Table 1). Furthermore, Hemagglutination Assay (HA) was performed to confirm the presence of HPAI H5N1 using the OIE procedure [14]. Molecular assay using reverse transcription-polymerase chain reaction (RT-PCR) was also conducted to confirm both IBV and HPAI H5N1 as described previously [11]. The isolated virus was aliquoted in 1.8 mL cryotube and stored at -80 ^{o}C for further testing.

Table 1: Viral particle of Gammacoronavirus (IBV) and AI H5N1 virus

Virus	Lesion or Embryonic death	Hemagglutination Assay (Titre)	Titer (Embryo infectious dose 50)	RT-PCR Test
Infectious bronchitis virus	Positive	Not done*	$10^{6.9}$ EID_{50} / 1 mL	Positive
HPAI subtype H5N1	Positive	Positive (258/8log2)	10^{9} EID_{50} /1 mL	Positive

*Note: IBV does not spontaneously agglutinate chicken red blood cells [15]. EID: Embryo infectious dose

Plant materials and extraction methods

Selected concentrations (2, 4, 8, 10, 20 and 30 %) of leaf essential oils from *E. citriodora* and *E. globulus* were used for toxicity testing, diluted with mineral oil. The *Eucalyptus* leaf was collected from the Indonesian Spice and Medicinal Crops Research Institute (Balittro), Bogor, Indonesia and extraction process was carried out using steam distillation method for the leaf parts. First, leaf collection was carried out by picking leaves from the 5th-year-old *Eucalyptus* tree. Collected leaves were separated from stalks and twigs and then inserted into the distillation kettle without withering process to avoid the loss of volatile compound. Furthermore, the distillation apparatus was set to above 200 ^{o}C inside the kettle and machine pressure was set at 1 atmosphere. The distillation process was carried out for four hours and production of essential oils commenced after two hours running. The average yield of essential oils was ~1.3 % of the total leaves extracted (w/w). The essential oils were further tested for quality checking in the Quality Assurance Laboratory (Balittro, Ministry of Agriculture).

Direct contact/liquid phase methods of essential oils

The toxicity test for essential oils (2, 4, 8, 10, 20 and 30 %) was conducted using embryonated chicken Specific Pathogenic Free (SPF) eggs (9 to 11 days old). A total of 0.1 mL of each essential oil was injected into the eggs, with five replicates for each concentration, including a positive control (10^3 EID_{50} of IBV and H5N1 AI virus) [16]. Based on the cytotoxicity results, the SPF eggs were subsequently inoculated with a mixture of 0.1 mL of each essential oil (2, 4 and 8 %) and 0.1 mL of 10^3 EID_{50} of IBV and HPAI H5N1, followed by incubation at room temperature (22 °C) for 45 min. Furthermore, 0.2 mL of the mixed essential oil and virus solution was injected into the allantoic cavity of the SPF eggs, with five replicates for each group. The inoculated eggs were incubated at 37 °C for 5 days and monitored twice daily (morning and evening).

The result was analyzed as follows: (A) death or defect of the embryo after treatment with the Eucalyptus essential oil (EEO); (B) death or defect caused by viral infection as a positive control; and (C) death of the embryo in the negative control. viability confirmation from HPAI H5N1 was performed using the Hemagglutination Test (HA/HI). Allantoid fluids from treated SPF chicken eggs were aseptically harvested and tested using 10 % normal chicken Red Blood Cells (RBC).

Vapour phase method

Testing of the essential oils' activity against AI H5N1 and IBV was conducted at three different concentrations (2, 4 and 8 %) following a modification of the method by Vimalanathan and Hudson [17]. Aliquots (10 µL) of virus (10^2 EID_{50}) were individually dried for 10 min on the underside of the caps of sterile microcentrifuge tubes within a biosafety cabinet. Test oils (500 µL) were added to each tube, and caps, containing the dried viral film, were replaced. The tubes were then exposed to the oil for 45 min at 37 °C. Phosphate-buffered saline (PBS) containing Penicillin-Streptomycin (Pen-Strep, 100 IU) was used as negative control. After exposure, the caps were removed, and the dried viral film was reconstituted in 1 mL of PBS with Penicillin-Streptomycin (Pen-Strep, 100 IU; 200 µL/mL) and injected into the allantoic cavity of SPF eggs (200 µL per egg) with five replicates. The eggs were then incubated at 37 °C for 5 days and monitored twice daily (morning and evening).

The analysis was carried out by using indicators as follows: (A) Death or defect of the embryo after *Eucalyptus* treatment; (B) Death or defect of the embryo caused by the viral infection as a positive control; (C) Death of the embryo without any treatment; and (D) Hemagglutination (HA) Test against HPAI H5N1 virus.

Cell culture and microculture tetrazolium technique (MTT) assay

In addition to SPF-egg testing, the infectivity of HPAI H5N1 and the efficacy of essential oils in the vapor phase to inhibit the growth of H5N1 were tested on MDCK cells using MTT assays [18]. The MDCK cells were cultured in 96-well flat-bottom microplates. MTT results were confirmed using HA test according to OIE standards [19]. Unfortunately, attempts at viral isolation using Vero cells were unsuccessful for IBV. For the cell culture experiments, 0.5 mL of *E. citriodora* and *E. globulus* essential oils at concentrations of 2, 4 and 8 % were added to 2 mL microcentrifuge tubes. As a negative control, 0.5 mL of PBS containing Pen-Strep (100 IU) was used. HPAI H5N1 virus, at a concentration of 10^2 $TCID_{50}$/10 µL (Tissue Culture Infectious Dose), was placed in the cap of each tube, which was then tightly sealed and incubated at 37 °C for 45 min. After incubation, the contents of each tube cap were re-suspended in 1 mL of Dulbecco's Modified Eagle Medium (DMEM)

containing 2 % fetal calf serum (FCS) and added to MDCK cells in the 96-well plate once confluence reached 85 – 90 % (200 µL/well). Five replicates were used for each concentration. The treated MDCK cells were then incubated at 37 °C in a carbon dioxide (CO_2) incubator for 48 h and monitored twice daily (morning and evening). After 48 h the culture media in the microplate were examined using the Hemagglutination Assay (HA) for HPAI H5N1. The MTT assay was performed using the Cell Proliferation Kit I (MTT) from Sigma (Cat no. 11465007001), following the standard protocol, to assess the metabolic activity or cell death of HPAI virus-infected cells. Absorbance was measured at 620 nm using an ELISA microplate reader.

RESULTS

Gas chromatography (GC) analysis revealed that citronellal was the major constituent of E. citriodora (76.07 %), followed by 1,8-cineole at 0.05 %. In contrast, *E. globulus* primarily contained 1,8-cineole (83 %). The GC retention time for citronellal peaked at 17.9 min, confirming it as the main component, while the retention time for 1,8-cineole peaked at 8.336 minutes. The detailed parameters are presented in Table 2 and Table 3.

The toxicity of essential oils from *E. citriodora* and *E. globulus*, administered via direct injection into the allantoic cavity of 9- to 11-day-old SPF-embryonated chicken eggs, was tested at three different concentrations (2, 4 and 8%). The results, presented in Table 4, indicate that the essential oils at concentrations of 2 to 8 % did not exhibit toxic effects on the embryonated chicken eggs. However, at concentrations of 10 to 30 %, both *E. citriodora* and *E. globulus* essential oils were toxic to the eggs.

Table 2: Physicochemical properties of *Eucalyptus citridora* essential oil

Parameter	Result	Standard quality range
Citronellal	76.07%	≥ 70%
Color	Transparent	Transparent to yellowish
Specific gravity	0.865	0.86-0.87
Absorbance	-2°	-(0°) to 20°
Refractive Index	1.4530	1.4510 -1.4640
Odor	Specific to *E. citriodora*	Specific to *E. citriodora*
Solubility in ethanol 70 %	1:2	<1:3

Table 3: Physicochemical properties of *Eucalyptus globulus* essential oil

Parameter	Result	Standard quality range
1,8-Cineole	83%	≥ 80%
Color	Transparent	Transparent to greenish-yellow
Specific gravity	0.915	0.905-0927
Absorbance	+5°	-(0°) to 18°
Refractive Index	1.4637	1.4580 – 1.4700
Odor	Specific to *E. globulus*	Specific to *E. globulus*
Solubility in ethanol 70 %	1:4	1:5

Table 4: Toxicity test result of *E. citriodora* and *E. globulus* essential oils

Essential oils concentration	Presentation of embryonic lesion in SPF eggs (embryonic lesion/total embryonic eggs)	Toxicity test result
PBS+Ab* (control)	0% (0/5)	Normal embryo, no toxicity
E. citriodora (2%)	0% (0/5)	Normal embryo, no toxicity
E. citriodora (4%)	0% (0/5)	Normal embryo, no toxicity
E. citriodora (8%)	0% (0/5)	Normal embryo, no toxicity
E. citriodora (10%)	80% (4/5)	80% toxicity
E. citriodora (20%)	100% (5/5)	100% toxicity
E. citriodora (30%)	100% (5/5)	100% toxicity
E. globulus (2%)	0% (0/5)	Normal embryo, no toxicity
E. globulus (4%)	0% (0/5)	Normal embryo, no toxicity
E. globulus (8%)	0% (0/5)	Normal embryo, no toxicity
E. globulus (10%)	60% (3/5)	60% toxicity
E. globulus (20%)	100% (5/5)	100% toxicity
E. globulus (30%)	100% (5/5)	100% toxicity

*Ab: Antibiotics (0.5 mL PBS containing Pen-Strep 100 IU)

Thus, the 2 to 8 % concentrations of the essential oils were selected for further analysis. Following the toxicity test, the direct contact (liquid phase) test was conducted using 10^3 EID_{50} of both the HPAI H5N1 and IBV viruses (Table 5). Results from the direct contact test showed that both essential oils successfully inhibited 100 % of the growth of 10^3 EID_{50} of both viruses, which infected SPF-egg embryos within 45 min. Additionally, the infected eggs treated with the essential oils showed no lesions on the embryos, and the HA test for the HPAI H5N1 virus was negative. Furthermore, the antiviral activity of the essential oils from *E. citriodora* and *E. globulus* was tested using the vapor phase (Table 6). The results of MTT assay are presented in Table 7, while the cytopathic effect (CPE) is shown in Figure 1.

Table 5: The direct contact or liquid phase test result of *E. citriodora* and *E. globulus* essential oils against 10^3EID_{50} AI H5N1 and IBV virus

Essential oils concentration	**Presentation of embryonic lesion in SPF eggs (embryonic lesion/total embryonic eggs) HA test result (total positive result)**		**Result**
	10^3 EID $_{50}$ AI H5N1	***10^3 EID $_{50}$ IBV***	
E. citriodora (2%)	0% (0/5) Negative	0% (0/5) n.d	Inhibits 100% of the growth of AI H5N1 and IBV viruses
E. citriodora (4%)	0% (0/5) Negative	0% (0/5) n.d	Inhibits 100% of the growth of AI H5N1 and IBV viruses
E. citriodora (8%)	0% (0/5) Negative	0% (0/5) n.d	Inhibits 100% of the growth of AI H5N1 and IBV viruses
E. globulus (2%)	0% (0/5) Negative	0% (0/5) n.d	Inhibits 100% of the growth of AI H5N1 and IBV viruses
E. globulus (4%)	0% (0/5) Negative	0% (0/5) n.d	Inhibits 100% of the growth of AI H5N1 and IBV viruses
E. globulus (8%)	0% (0/5) Negative	0% (0/5) n.d	Inhibits 100% of the growth of AI H5N1 and IBV viruses
Virus control/ Positive control (AI H5N1 and IBV Virus)	100% (5/5) Positive (5)	100% (5/5) n.d	Gammacoronavirus (IBV) and AI H5N1 Viruses were detected (100% growth of viruses)
Embryo control / Negative control (PBS+Ab)	n.d	n.d	100% embryo was normal, HA test result was negative

EID_{50}: Embryo Infected Doses $_{50}$; n.d: not done; HA: Hemagglutination

Table 6: The vapour phase test result of *E. citriodora* and *E. globulus* essential oils against 10^2 EID_{50} AI H5N1 and IBV virus

Essential oils concentration	**Presentation of embryonic lesion in SPF eggs (embryonic lesion/total embryonic eggs) HA test result (total positive result)**		**Result**
	10^2 EID_{50} AI H5N1	***10^2 EID_{50} IBV***	
E. citriodora (2%)	100% (5/5) Positive (5)	100% (5/5) n.d	Unable to inhibit the growth of AI H5N1 and IBV viruses
E. citriodora (4%)	100% (5/5) Positive (5)	100% (5/5) n.d	Unable to inhibit the growth of AI H5N1 and IBV viruses
E. citriodora (8%)	0% (0/5) Negative	0% (0/5) n.d	Inhibit 100% the growth of AI H5N1 and IBV viruses
E. globulus (2%)	100% (5/5) Positive (5)	100% (5/5) n.d	Unable to inhibit the growth of AI H5N1 and IBV viruses
E. globulus (4%)	100% (5/5) Positive (5)	100% (5/5) n.d	Unable to inhibit the growth of AI H5N1 and IBV viruses
E. globulus (8%)	0% (0/5) Negative	0% (0/5) n.d	Inhibit 100% the growth of AI H5N1 and IBV viruses
Virus control (AI H5N1 and IBV Virus)	100% (5/5) Positive (5)	100% (5/5) n.d	Gammacoronavirus (IBV) and AI H5N1 Viruses were detected (100% growth of viruses)
Embryo control (PBS+Ab)	n.d	n.d	100% embryo was normal, HA test result was negative

EID_{50}: Embryo Infected Doses $_{50}$; n.d: not done; HA: Hemagglutination

Table 7: MTT and HA test results of *E. citriodora* and *E. globulus* essential oils vapour phase on MDCK cells

Treatment	Absorbance value	HA Titer (log2)	Description	CPE
10^2 $TCID_{50}$ AI H5N1 virus Positive control	≤ 0.107	3log2	Positive	+
MDCK cell control/Negative control	≥ 0.145	0log2	Negative	-
E. citridora 8%	0.134	0log2	Negative	-
E. citridora 4%	0.106	3log2	Positive	+
E. citridora 2%	0.102	2log2	Positive	+
E. globulus 8%	0.119	0log2	Negative	-
E. globulus 4%	0.105	3log2	Positive	+
E. globulus 2%	0.103	3log2	Positive	+

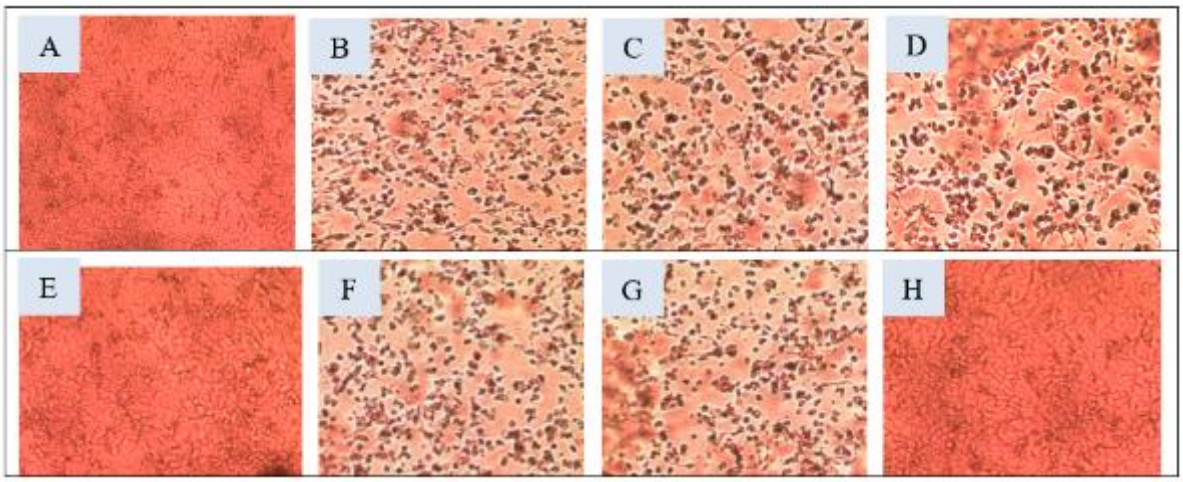

Figure 1: MDCK cells (A) normal MDCK Cells and (B) MDCK cells infected with AI virus subtype H5N1; (C, D, F and G) corresponds to *E. citriodora* 2 %, *E. citriodora* 4 %, *E. globulus* 2 %, *E. globulus* 4 % against AI virus subtype H5N1 in MDCK cell, respectively. E and H are *E. citriodora* 8 % and *E. globulus* 8 %, respectively, against AI virus subtype H5N1 in MDCK cell

Results from the vapor phase test, using both essential oils at the same concentrations and with 45 min of exposure to both viruses, showed that 2 and 4 % concentrations were unable to inhibit viral growth. However, complete inhibitory activity was observed at 8 % concentration for both essential oils against 10^2 EID_{50} of HPAI H5N1 and IBV viruses, as confirmed by the absence of lesions on the embryos and negative HA test result. A similar outcome was obtained from the vapor phase test on MDCK cells, where both essential oils effectively inhibited 10^2 $TCID_{50}$ of H5N1 virus. According to the MTT assay results, E. citriodora and E. globulus at concentrations of 2 and 4 % showed absorbance (A) values similar to that of the viral control (H5N1 10^2 $TCID_{50}$; ≤ 0.107), with values of 0.102, 0.106, 0.103, and 0.105, respectively. These results indicate that the metabolic activity of MDCK cells treated with E. citriodora and E. globulus essential oils at 2 and 4 % concentrations was comparable to that of the positive control.

This result was further confirmed by positive HA test, with titers ranging from 2 log2 to 3 log2, indicating growth of the HPAI H5N1 virus in MDCK cells exposed to the vapor phase of essential oils at 2 and 4 % concentrations. These findings suggest that both concentrations were unable to effectively inhibit HPAI H5N1 virus growth, as evidenced by the presence of CPE in the MDCK cells. In contrast, the 8% concentration of essential oil successfully inhibited 100 % of the growth of 10^2 TCID50 HPAI H5N1 virus. This was further corroborated by an average mean absorbance value close to that of the MDCK control or negative control (≥ 0.145), with values of 0.134 and 0.119, respectively. Additionally, the negative HA test results and the absence of CPE in MDCK cells after exposure to both essential oils at the 8% concentration further support these findings.

DISCUSSION

Herbal plants are potential sources for new viral drug development since they have diverse phytoconstituents that potentially inhibit viral replication and control virus infection by specific mechanisms. The constituents of various essential oils have significant antimicrobial

activities, which make them potential agents useful in the treatment of respiratory infections caused by influenza or coronavirus [20]. The essential oils from herbal plants have been reported to suppress viral activity with diverse mechanisms, one of which is via inhibition of viral replication, or by preventing the spread of the virus from cell to cell [20]. Antiviral activity of essential oils has been reported on Herpes simplex virus type-1 and type-2 (HSV-1 and HSV 2), pseudorabies virus (PrV), A3 influenza virus, Junin virus (JUNV) and type-2 dengue virus (DEN-2).

Essential oils generally contain a variety of constituents with different chemical structures, each of which has been reported to exhibit distinct scents, properties, and specific mechanisms of action in the human body [20]. In this study, the essential oil of E. citriodora, which contains citronellal as its primary constituent, demonstrated 100 % inhibitory activity against 10^3 EID_{50} of both the HPAI H5N1 and IBV viruses when tested using the direct contact method with 45 minutes of exposure. In contrast, the oils at concentrations of 2 – 4 % in the vapor phase test were ineffective in inhibiting the growth of 10^2 EID_{50} and 10^2 $TCID_{50}$ of the HPAI H5N1 and IBV viruses. However, the vapor phase test showed significant inhibitory effects at the 8 % concentration, achieving 100 % inhibition of HPAI H5N1 and IBV viruses. Previous studies have reported that ethanol extracts of *E. citriodora* leaves possess antiviral, anti-inflammatory, and antioxidant activities [21].

Essential oil from *E. citriodora* is reported to contain 32 active compounds, including citronellol, linalool, isopulegol, spatulenol and β-eudesmol. Despite its rich composition, only one or two compounds are responsible for its physiological function [20]. Some of these compounds, such as citronellol, citronellal and eucalyptol have been reported to have anti-tuberculocidal activity, while citronellal, the main component of the essential oil from *E. Citriodora,* is known to have lower tuberculocidal activity. This result shows the importance of synergistic effects between the major and minor phytoconstituents in developing new drugs or medicines [21]. Several *in vitro* studies have shown that essential oils have limited antiviral activity, seen only on the viral envelope and in the free-state virus, before viral attachment or entry into the host. Hence, the essential oil of *E. citriodora* (2, 4 and 8 % concentrations) may act synergistically to exert antiviral activity against AI H5N1 and IBV viruses. Moreover, both viruses have an envelope structure which becomes the cause of antiviral inhibition by the EEO [22].

The phytocompound 1.8-cineole (1,3,3-Trimethyl-20-oxabicyclo (2.2.2) octane, also known as Eucalyptol, 1,8-Epoxy-p-menthane; European Pharmacopoeia Reference Standard) is a major constituent of *E. globulus* essential oil. It exhibited 100 % inhibitory activity against both HPAI H5N1 and IBV viruses in the direct contact test at all concentrations tested within 45 min of exposure. In contrast, the vapor phase test only showed significant inhibitory effects against the HPAI H5N1 and IBV viruses at the 8% concentration. Previous studies have demonstrated that 1.8-cineole possesses diverse pharmacological activities, including antiviral, anti-inflammatory, anti-nociceptive, and antimicrobial properties [24]. An in vitro study by Cermelli *et al* [25] also reported that *E. globulus* exhibits antiviral activity against the mumps virus. Furthermore, the 1.8-cineole compound, being the dominant constituent of *E. globulus* essential oil, has demonstrated antiviral activity, including against IBV. This anti-IBV activity may result from chemical interactions between 1.8-cineole and the RNA or nucleocapsid (N) protein of the IBV virus. Another study found that 1.8-cineole reduced influenza virus infection in experimental mice by decreasing levels of interleukins (IL-4, IL-5, IL-10) and Monocyte Chemoattractant Protein-1 (MCP-1) in nasal lavage fluid, and IL-1β, IL-6, TNF-α, and IFN-γ in lung tissue. These findings suggest that 1.8-cineole enhances resistance to influenza virus infection by modulating the inflammatory response in the lungs [26].

Essential oil from *Eucalyptus* consists of a 1,8-cineole compound (88 %) and is known to be active towards HSV-1 *in vitro*. *Eucalyptus* suppresses the HSV-1 multiplication up to greater than 96 %. This result shows that the antiviral activity against HSV-1 by the EEO may be by directly inactivating the free-viral particle and interfering with the virion envelope structure which is required by the virus to enter the host cell. These activities might be produced by the synergistic effect of 1,8-cineole and other phytoconstituents which triggers antiviral activity against IBV and AI H5N1 viral envelope, both through direct contact test or liquid phase and vapour phase test [26]. The result is in tandem with another study that reported that the antiviral activity of 1,8-cineole may be by directly inactivating the free-viral particle and interfering with the virion envelope structure before the virus enters the host cells [25].

The administration of essential oil may be through direct contact (injection, dermal absorption, etc) oral or inhalation. Administration through the dermal route is usually used to treat

skin infections caused by bacteria or yeast. Meanwhile, both inhalation and oral administration are used to treat many respiratory infections such as bronchitis, asthma and Chronic Obstructive Pulmonary Disease (COPD). The volatility of oil extract enables them to reach vast regions of the respiratory system via the inhalational route. The administration through inhalation of the EEO is considered a safe method and has been applied in traditional medicine. This procedure is carried out by mixing a few drops of EEO in a hot-water bowl. Nowadays, some invented devices have been commercially produced to facilitate essential oil administration through the inhalational route. A recent study showed that essential oil of *Eucalyptus* administered by direct contact or liquid phase at various tested concentrations (2 – 8 %) was able to inhibit the 10^3EID_{50} of H5N1 and IBV viruses before viral penetration (pre-treatment) into the host cells. On the other hand, the vapour phase test of EEO was only effective at 8 % concentration at lower viral concentration i.e. 10^2 EID_{50}.

Limitations of this study

This result might be affected by the concentration, time duration and optimal distance between the administered EEO and viral location. Hence, further study is required to explore the potential antiviral activity of essential oil from both species of *Eucalyptus*, administered either through direct contact or inhalation, against AI H5N1 or IBV virus. Also, this study emphasized Indonesia's herbal plant potential as a source of natural products for further exploration for possible drug development, especially antiviral drugs. Further studies are necessary to explore the mechanism and interaction of the natural product compounds with the viruses, such as the viral life cycle which encompasses the entry mechanism, replication, assembly and release mechanism. Specific target of the natural compounds and the biological relationship between the viruses and the hosts also requires further studies.

CONCLUSION

Essential oils from *E. citriodora* and *E. globulus* show potential as antiviral candidates for the treatment of HPAI H5N1 and IBV infections. The study demonstrates that both essential oils completely inhibit viral growth at concentrations of 2, 4 and 8 % when applied via direct contact or liquid-phase methods. In contrast, inhibition of viral growth was only observed at 8 % concentration when the oils were applied in the vapor phase for 45 min. In this *in vitro* study, 1.8-cineole likely contributes to the antiviral activity of both essential oils, in combination with other phytoconstituents. Essential oils of *E. citriodora* and *E. globulus* could serve as complementary treatments for viral diseases such as zoonotic HPAI H5N1, which poses a significant public health risk, and IBV, a persistent challenge for poultry farms in Indonesia.

DECLARATIONS

Acknowledgement/Funding

The authors would like to thank the Indonesian Medicinal and Aromatic Crop Research Institute, Ministry of Agriculture for their assistance. This study was funded by the Ministry of Agriculture, Indonesia.

Ethical approval

None provided.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Conflict of interest

No conflict of interest is associated with this work.

Contribution of authors

We declare that this work was done by the author(s) named in this article, and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. Conceptualization: Dharmayanti NLPI, Risa I, Nurjanah D; Project administration: Dharmayanti NLPI; Resources: Dharmayanti NLPI, Djufry F; Software: Nurjanah D; Supervision: Dharmayanti NLPI; Validation: Dharmayanti NLPI, Risa I, Suyatno T; Visualization: Risa I, Nurjanah D; Writing - original draft: Nurjanah D; Writing - review & editing: Dharmayanti NLPI, Nurjanah D, Indriani R, Nuradji H, Suyatno T.

REFERENCES

1. *Claas EC, Osterhaus AD, van Beek R, De Jong JC, Rimmelzwaan GF, Senne DA, Krauss S, Shortridge KF, Webster RG. Human influenza A H5N1 virus is related to a highly pathogenic avian influenza virus. Lancet 1998; 351: 472-477.*

2. Dharmayanti NLPI, Thor SW, Zanders N, Hartawan R, Ratnawati A, Jang Y, Rodriguez M, Suarez DL, Samaan G, Pudjiatmoko, et al. Attenuation of highly pathogenic avian influenza A(H5N1) viruses in Indonesia following the reassortment and acquisition of genes from low pathogenicity avian influenza A virus progenitors. Emerg Microbes Infect 2018; 7: 147.
3. WHO. Cumulative number of confirmed human cases of avian influenza A(H5N1) reported to WHO 2003-2020 2020.
4. Tarigan S, Wibowo MH, Indriani R, Sumarningsih S, Artanto S, Idris S, Durr PA, Asmara W, Ebrahimie E, Stevenson MA, et al. Field effectiveness of highly pathogenic avian influenza H5N1 vaccination in commercial layers in Indonesia. PLoS One 2018; 13: e0190947.
5. Fadhilah AS, Kai TH, Lokman HI, Yasmin NAR, Hafandi A, Hasliza AH, Tengku Rinalfi TA, Hezmee MNM. Molecular and pathogenicity of infectious bronchitis virus (Gammacoronavirus) in Japanese quail (Coturnix japonica). Poult Sci 2020; 99: 2937-2943.
6. Setiawaty R, Soejoedono RD, Poetri ON. Genetic characterization of S1 gene of infectious bronchitis virus isolated from commercial poultry flocks in West Java, Indonesia. Vet World 2019; 12: 231-235.
7. Kong L, You R, Zhang D, Yuan Q, Xiang B, Liang J, Lin Q, Ding C, Liao M, Chen L, et al. Infectious bronchitis virus infection increases pathogenicity of H9N2 avian influenza virus by inducing severe inflammatory response. Front Vet Sci 2021; 8: 824179.
8. Elfahmi, Woerdenbag HJ, Kayser O. Jamu: Indonesian traditional herbal medicine towards rational phytopharmacological use. J Herbal Med 2014; 4: 51-73.
9. Lee JY, Abundo MEC, Lee CW. Herbal medicines with antiviral activity against the influenza virus, a systematic review. Am J Chin Med 2018; 46: 1663-1700.
10. da Silva AP, Aston EJ, Chiwanga GH, Birakos A, Muhairwa AP, Kayang BB, Kelly T, Zhou H, Gallardo RA. Molecular characterization of Newcastle disease viruses isolated from chickens in Tanzania and Ghana. Viruses 2020; 12.
11. Dharmayanti N, Indriani R. identifikasi dan karakterisasi virus infectious bronchitis (IBV) di Indonesia. Jurnal Biologi Indonesia. 2017; 13: 53-59.
12. OIE. Avian influenza. OIE Terrestrial Manual 20092009.
13. Reed LJ, Muench H. A simple method of estimating fifty percent endpoints. American J Epidemiol 1938; 27: 493-497.
14. OIE. Avian influenza (including infection with high pathogenicity avian influenza viruses). OIE Terrestrial Manual 2021. p. 1-26.
15. Zhou JY, Zhang DY, Ye JX, Cheng LQ. Characterization of an avian infectious bronchitis virus isolated in China from chickens with nephritis. J Vet Med B Infect Dis Vet Public Health 2004; 51: 147-152.
16. Niknafs S, Meijer MMY, Khaskheli AA, Roura E. In ovo delivery of oregano essential oil activated xenobiotic detoxification and lipid metabolism at the hatch in broiler chickens. Poult Sci 2024; 103: 103321.
17. Vimalanathan S. Anti-influenza virus activity of essential oils and vapors. American J Essential Oils Nat Prod 2014; 2: 47-53.
18. Ghasemi M, Turnbull T, Sebastian S, Kempson I. The MTT Assay: Utility, limitations, pitfalls, and interpretation in bulk and single-cell analysis. Int J Mol Sci 2021; 22.
19. OIE. World Animal Health Information Database (WAHIS Interface) – Version 1. 2018.
20. Tisserand R, Young R. 2 - Essential oil composition. In: Tisserand R, Young R, editors. Essential Oil Safety (Second Edition). St. Louis: Churchill Livingstone; 2014. p. 5-22.
21. Ramos Alvarenga RF, Wan B, Inui T, Franzblau SG, Pauli GF, Jaki BU. The airborne antituberculosis activity of Eucalyptus citriodora essential oil. J Nat Prod 2014; 77: 603-610.
22. Schnitzler P, Schuhmacher A, Astani A, Reichling J. Melissa officinalis oil affects infectivity of enveloped herpesviruses. Phytomed 2008; 15: 734-740.
23. Li Y, Lai Y, Wang Y, Liu N, Zhang F, Xu P. 1, 8-Cineol protect against influenza-virus-induced pneumonia in mice. Inflam 2016; 39: 1582-1593.
24. Cermelli C, Fabio A, Fabio G, Quaglio P. Effect of eucalyptus essential oil on respiratory bacteria and viruses. Curr Microbiol 2008; 56: 89-92.
25. Yang Z, Wu N, Fu Y, Yang G, Wang W, Zu Y, Efferth T. Anti-infectious bronchitis virus (IBV) activity of 1,8-cineole: effect on nucleocapsid (N) protein. J Biomol Struct Dyn 2010; 28: 323-330.
26. Astani A, Reichling J, Schnitzler P. Comparative study on the antiviral activity of selected monoterpenes derived from essential oils. Phytother Res 2010; 24: 673-679.