

# The Effect of Flavonoid Fraction from *Vitex trifolia* Leaves on Pandemic-2009 H1N1 Influenza A Virus Propagated in Embryonated Chicken Eggs

Neny Purwitasari<sup>1</sup>, Krisma Agung Subarka<sup>2</sup>, David Fransnado Grismana Putra<sup>2</sup>, Herra Studiawan<sup>3</sup>, Kuncoro Puguh Santoso<sup>4</sup>

<sup>1</sup>Researcher and Lecture, <sup>2</sup>Student, <sup>3</sup>Researcher And Lecture, Department of Pharmacognosy and Phytochemistry, Faculty of Pharmacy, Universitas Airlangga, Surabaya, 60115, Indonesia, <sup>4</sup>Associate Professor, Faculty of Veterinary Medicine, Universitas Airlangga, Surabaya, 60115, Indonesia

## Abstract

The aim of this study is to investigate antiviral activity of flavonoid fraction of *Vitex trifolia* leaves towards influenza A viruses H1N1 pandemic-2009 that were propagated in embryonated chicken eggs (ECEs). Fraction that rich of flavonoid compounds was isolated from the crude ethanolic extract using column chromatography, prior to be subjected to antiviral inhibition assay. The assay was evaluated by using hemagglutination (HA) test. The HA titre reduction of the virus was counted after being treated with the flavonoid fraction, compared to untreated one. The result showed that percentage of inhibition of this flavonoid fraction against pandemic-2009 H1N1 influenza A virus are 37.50%, 71.25%, 71.25% at concentration 125 µg/mL, 500 µg/mL, and 750 µg/mL, respectively. In conclusion, the flavonoid fraction of *Vitex trifolia* leaves showed effect to reduce viral hemagglutinine, the enzyme that has a crucial role in the initiating step of virus infection, without possessed any toxicity to ECEs.

**Keywords:** flavonoid, influenza A H1N1 pandemic 2009 virus, *Vitex trifolia*, hemagglutinin

## Introduction

Influenza virus is a member of Orthomyxoviridae, one genera of Infuenza A with a complex pathogenesis due to its genetic variability. One of the specific mutation of influenza virus is due to the antigenic drift and mutation<sup>[1]</sup>. The current influenza A subtype H1N1 is a combination from influenza virus gen from swine, bird and human, that has an ability to mutate by the mechanism of antigenic drift and antigenicshift to produce the new resistant influenza virus<sup>[2]</sup>. However, throughout the world until August 4, 2009, 168 countries reported cases of influenza A H1N1 with 162,388 positive cases, 1,154 of them died. And data on the cumulative number of H1N1 infections in Indonesia up to August 23, 2009 were 1,005 people with 5 of them died. FDA has recommend two types of drugs to cure

and to prevent Influenza A, which are M2 inhibitors (Amantadine and Rimantadine) and Neuraminidase inhibitors (Oseltamivir and Zanamivir). Those drugs also can be use for prophylaxis and treatment for seasonal influenza.

The concern about this virus is emergent due to the resistance of the common influenza drug such as oseltamivir due to the mutation of H274T<sup>[3,4]</sup>. The resistance of adamantane derivatives is also detected due to the substitution of amino acid in the order 26 (Leu→Phe), 27 (Val→Ala atau Thr), 30 (Ala→Thr atau Val), 31 (Ser→Asn atau Arg), and 34 (GàE) in the domain of M2 trans membrane<sup>[5]</sup>. Thus, the alternative treatment for influenza infection is strongly needed. One of the sources for searching a new potent drug is herbal medicine. One suggestion used for antiviral drugs is chemotaxonomy. In fellow genus *Vitex* studies, extracts and fractions rich in flavonoids from fruits and leaves of *Vitex polygama* Cham (Vebenaceae) can increase dose-dependent antiviral activity against type 1 herpes simplex virus (ACV-HSV-1). Leaf extract shows

---

**Corresponding author:**

**Neny Purwitasari**

Email: neny-p@ff.unair.ac.id

antiviral activity while the fruit extract has a virucidal effect. Some fractions of ethyl acetate extract can inhibit the virus by blocking HEP-2 receptor cells<sup>[6]</sup>.

In this study, a fractionation method from the methanol extract of the leaves of *Vitex trifolia* was carried out to obtain sub-fractions which contained many flavonoids using ethyl acetate solvents. Ethyl acetate solvents are semi-polar solvents and can dissolve compounds such as flavonoids<sup>[7]</sup>. In addition, one of the flavonoid compounds that has the ability to inhibit viruses with the mechanism of neuraminidase inhibitors is vitexin. Vitexin compound is owned by *Vitex trifolia*<sup>[8]</sup>. Based on these facts it is possible that the sub flavonoid fraction of *Vitex trifolia* also has activity as an antiviral.

## Materials and Method

The test material used was sub flavonoid fraction of *Vitex trifolia* leaves which was isolated by fractionation using vacuum column chromatography. The fraction that rich of flavonoid compounds was used to the assay.

### Virus and Testing Media

The influenza virus used was influenza A virus pandemic H1N1 subtype 2009 strain A/Unair-367/2010 and provided by Avian Influenza Research Center (AIRC) Laboratory, Universitas Airlangga, Surabaya, Indonesia. The virus is grown in ECES obtained from Pusvetma, Surabaya, Indonesia. The hemagglutination test (HA) is carried out using guinea pig red blood cells.

### Chemicals and Reagents

Silica gel GF254, silica gel 60G for thin-layer chromatography, Silica Gel 60, technical methanol (Brataco), Ethyl Acetate (Brataco), n-hexane (Brataco), Chloroform Pa (Fulltime), Phosphate Buffer Saline (PBS) (GIBCO), Dimethylsulfoxide (DMSO) (Merck), Sterile Water For Irrigation U.S.P. (Otsuka), red blood cell (RBC) guinea pig, an antibiotic solution of Penicillin-Streptomycin (Gibco).

### Tools

Vacuum column chromatography, filter paper, separating funnel, UV scanner (CAMAAG), Analytical scales (Ohaus), Chamber, autoclave (HL-340), Biosafety cabinet (NuAire), incubator, micropipette (Eppendorf), glassware, 96 Centrifuge (Tommy) "U and V" plate, refrigerator 4 °C (Sanyo), -80 °C freezer, Vortex (Barstead Thermolyne), 1 mL injection syringe,

Buchner (Schott) funnel, rotary evaporator (Buchi).

### Extraction Method

The thick methanol extract of leaves of *Vitex trifolia* was weighed 143 g then added with water as much as put in a separating funnel. Prepare as much n-hexane with volume and put it in a separating funnel, shake until homogeneous after that separated between water fraction and n-hexane. The n-hexane fraction is present. This process is repeated until the n-hexane fraction is not dark in color. In this process, the n-hexane fraction is obtained. In the water fraction that is still in the separating funnel, ethyl acetate is added as much, and then shaken until homogeneous, and the ethyl acetate fraction is accommodated. This step is repeated until the ethyl acetate fraction is colorless. Then the ethyl acetate fraction was obtained, the ethyl acetate fraction was subjected to a rotary evaporator at 40 °C until thick ethyl acetate fraction was obtained. The fraction was subjected to vacuum column chromatography. Gradient volume of chloroform and ethyl acetate was used as mobile phase to separate the flavonoid compounds. The sub fraction was diluted in 5% of DMSO and phospat buffer saline until reach the final concentration 1000 µg/mL, 500 µg/mL, 250 µg/mL, and 125 µg/mL.

### Toxicity test

Toxicity test and determination of safe concentration of the tested samples on 11 days old ECEs. 15 ECEs then disinfected the outside of the eggs by using 70% of alcohol. Four concentrations of the fractions that have been made 1000 µg/mL, 500 µg/mL, 250 µg/mL and 125 µg/mL each concentration is taken as much as 100 µL+50 µL of penicillin streptomycin solution. Each concentration was injected into the ECEs, replicated 3 times. After entering the fraction+penicillin streptomycin solution, the hole in the egg is covered with tape until it is completely tight. ECEs was incubated at 37 °C incubator for 2 days. Every day the eggs are observed for the embryo. Embryos that die before two days, are removed from the incubator then stored in a refrigerator at 40 °C. After 2 days the non-dead ECE was transferred to the refrigerator at 40 °C for one night. From this process, it can be determined which concentration will be used for the activity test. The virus that will be used for the activity test has a 2<sup>12</sup> titre.

### Control Groups

a. Inject 100 µL of the pandemic-2009 H1N1



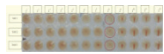


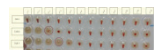
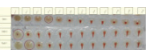
Cont... Table 1. Result of toxicity test on ECEs.

250	+	+	+	+	+	+	+	+	-
500	+	+	+	+	+	+	-	+	+
1000	+	+	+	+	+	+	-	+	-
2000	+	+	+	+	+	+	-	+	+

Note: (+) Embryo no died

(-) Embryo died

Table 2. Result of HA Assay.

	Negative Control	Positive Control	125 µg/mL	250 µg/mL	500 µg/mL
HA test					
Mean HA titer	28	25	25	22.3	22.3
2Log2 HA titer	8	5	5	2.3	2.3
% of titer reduction	0	37.5	37.5	71.25	71.25

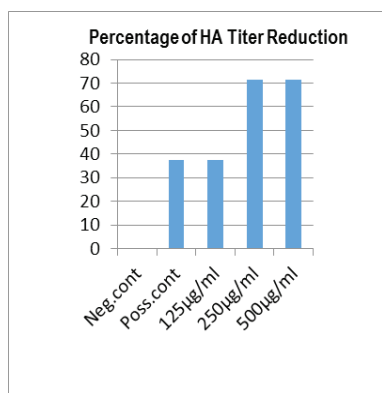


Figure 3. Percentage of HA titer reduction.

**Discussion**

Treatment and prevention of influenza can be done through administration of antiviral and vaccination. At present there are 2 classes of influenza antiviral drugs, namely adamantane groups and neuraminidase inhibitors. However, infection control of influenza A

virus using antivirals has begun to experience problems due to the emergence of new strains that are resistant to existing antiviral drugs<sup>[10]</sup>. This study used sub fraction of flavonoids from *Vitex trifolia* leaf with chemotaxonomic approach to determine the effect on the swine flu virus (H1N1 Pandemic 2009).

In previous studies it has been shown that the methanol extract of *Vitex trifolia* leaves can reduce H1N1 virus titers carried out by the hemaagglutination (HA) test and *in silico* through Molegro Virtual Docker (MVD) software based on docking results can be known if vitexin compared to zanamivir have ties hydrogen on Arginine 152 (Arg 152), Arginine 292 (Arg 292), Arginine 371 (Arg 371), Aspartic Acid Asp 151, Tyrosine 406 (Tyr 406). Based on the rerank score, vitexin had a score of -95.6056 kcal/mol and zanamivir -111,423 kcal/mol. The lower (more negative) value of the rerank score, the more stable the receptor ligands interaction. Previous

research also proved that the methanol extract of *Vitex trifolia* leaves can reduce H5N1 virus titers carried out by the hemagglutination (HA) and neuraminidase inhibitor (NAI) tests<sup>[11]</sup>. To determine the effect of sub-fraction on ECEs as a viral growth medium, a non-toxic dose of sub-fraction in ECEs was tested in several fraction concentrations which are, 125 µg/mL, 250 µg/mL, 500 µg/mL, 1000 µg/mL, 2000 µg/mL. There is no limit in the choice of these concentrations as long as the dose is safe in ECEs<sup>[12,13]</sup>. ECEs used are free from antibody to fight viruses will be inoculated<sup>[10]</sup>.

After 72 hours of inoculation, embryonic death was found in concentration 2000 µg/mL and 1000 µg/mL for two ECEs. This can be occurred due to individual variations, different egg immunity systems and ECEs used in replication. So, in this study, only concentration of 125 µg/mL, 250 µg/mL, and 500 µg/mL that were used. To determine the growth of viruses in ECEs, can be determined in two ways, namely by observing the embryonic death and testing HA. Based on the results of the observation, embryonic death did not occur at the same time. Individual variations in ECEs greatly affect the ability of the embryo to survive so that hemagglutination tests are carried out to determine the virus titers in ECEs<sup>[14]</sup>.

The data showed that at concentration of 250 µg/mL and 500 µg/mL, the inhibition of HA titer is 71.25 %, and indicated a strong inhibition of HA titer. From the previous study, it was also proved that the extracts and fractions of several plants such as *Garcinia mangostana*, *Eurycoma longifolia*, *Tabernaemontana divaricata*, *Brucea javanica*, and *Momordica charantia* had good inhibition percentages at concentrations of 250 µg/mL<sup>[15]</sup>. In this study, the compound which is suspected to give inhibition for the growth of H1N1 virus is vitexin, one of flavonoid compounds presence in *Vitex* genera. In a similar study, the use of embryonic chicken eggs as a learning model to determine the effect on influenza A H9N2 virus using oseltamivir carboxylate with ten ECEs per replication<sup>[13]</sup>.

### Conclusion

In conclusion, the flavonoid fraction of *Vitex trifolia* leaves showed effect to reduce viral hemagglutinin, the enzyme that has a crucial role in the initiating step of virus infection, without possessed any toxicity to ECEs.

**Conflict of Interest:** The authors declare that they have no conflict of interest.

**Source of Funding:** This work is funded by Sceme of Penelitian Dosen Pemula RKAT, Faculty of Pharmacy, Universitas Airlangga, F.Y. 2015.

**Acknowledgement:** We would like to thank Prof. C. A. Nidom for providing the virus and equipment to handle the virus assay. We thank Arif Nur Muhammad Ansori for editing the manuscript.

**Ethical Approval:** This study was approved by the Animal Care and Use Committee, Faculty of Veterinary Medicine, Universitas Airlangga, Surabaya, Indonesia.

### References

1. Lombardo T, Chiapponi C, Baioni L, et al. Protein mutations following adaptation of avian influenza viruses in different biological systems. *Research in Veterinary Science*. 2015; 103(2015): 176-178.
2. Spackman E. *Methods in Molecular Biology Avian Influenza Virus*. Humana Press; 2008.
3. Calatayud L, Lackenby A, Reynolds A, et al. Oseltamivir-resistant pandemic (H1N1) 2009 virus infection in England and Scotland, 2009-2010. *Emerg Infect Dis*. 2011; 17(10): 1807-1815.
4. Mahardika IGNK, Sukada IM, Antara MS, et al. Motif for amino acid sequence forming oseltamivir binding bag on avian influenza (H5N1) protein neuraminidase from humans and animals from Indonesia. *Jurnal Veteriner*. 2008; 9(4): 204-206.
5. Dharmayanti NLPI, Hewajuli DA, Ratnawati AK, et al. Genetic character of viral membrane proteins from avian influenza virus subtype H5N1. *JITV*. 2010; 15(3): 231-239.
6. Gonçalves JL1, Leitão SG, Monache FD, et al. In vitro antiviral effect of flavonoid-rich extracts of *Vitex polygama* (Verbenaceae) against acyclovir-resistant herpes simplex virus type 1. *Phytomedicine*. 2001; 8(6): 477-480.
7. Yuswantina R. Radical Capture Activity of Rhizoma Binahong (*Anredera cordifolia* (tenore) steen) Ethanol Acetate and Ethanol Extract with DPPH Method. Surakarta: Fakultas Farmasi Universitas Muhammadiyah Surakarta; 2009.
8. John KMM, Enkhtaivan G, Ayyanar M, et al. Screening of ethnic medicinal plants of South India against influenza (H1N1) and their antioxidant activity. *Saudi Journal of Biological Science*. 2014; 22(2): 191-197.

9. Ramadhani RA, Dewi K, Enny F. Isolation, identification and antioxidant test of flavonoid compounds from ethyl acetate extract of tempuyung (*Sonchus arvensis* L.) leaf. *Jurnal Kimia, Fakultas Sains dan Matematika*. 2013; 1(1): 247-255.
10. Purwitasari N, Studiawan H, Rahmawati K. Antivirus influenza activity from methanolic extract of *Momordica charantia* fruit. *Jurnal Farmasi dan Ilmu Kefarmasian*. 2015; 2(2): 34-40.
11. Lestari WIP. Activity of *Vitex trifolia* Leaf Extract as An Anti-H5N1 (Bird Flu). Surabaya: Fakultas Farmasi Universitas Airlangga; 2015.
12. Murtini S, Murwani R, Satrija F, Malole MBM. Determination of the route and dose of inoculation in embryonated chicken eggs as a test medium for the efficacy of benalu tea (*Scurrula oortiana*) extracts. *JITV*. 2006; 11(2).
13. Tare DS, Pawar SD. Use of embryonated chicken egg as a model to study the susceptibility of avian influenza H9N2 viruses to oseltamivir carboxylate. *Journal of Virological Methods*. 2015; 224(2015): 67-72.
14. Wang JX, Zhou JY, Yang QW, et al. An improved embryonated chicken egg model for the evaluation of antiviral drugs against influenza A virus. *Journal of Virological Method*. 2008; 153: 218-222.
15. Ikram NKK, Durrand JD, Muchtaridi M, et al. A Virtual screening approach for identifying plants with anti H5N1 neuraminidase activity. *J Chem Inf Model*. 2015; 55: 308-316.