Activity of Ethyl Acetate Fraction of *Merremia mammosa* Hall as Anti-Influenza A (H1N1)

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Abstract

The outbreak of influenza A viruses (H1N1 and H5N1) has raised a global concern on the future risk of a pandemic. Oseltamivir, the current neuraminidase inhibitor, could not meet the demand if there is a major outbreak. Thus, there is a need to find alternative treatment for influenza A, especially from natural herbs. The objectives of this study were to determine antiviral influenza A activities of *Merremia mammosa* Hall against influenza A H1N1. People in Madura island, Indonesia used this rhizome to cure tuberculosis. In this research, *Merremia mammosa* Hall rhizome was extracted gradually using n-hexane to remove the non-polar compound. The residue was extracted using ethyl acetate to obtain semi-polar extract. The ethyl acetate fraction of *Merremia mammosa* Hall was subjected to *in vitro* antiviral assay against influenza A (H1N1) virus using Hemagglutinin Assay. This is a method for titering influenza viruses based their ability to attach molecules present on the surface of chicken red blood cell. The virus was incubated in embryonated chicken eggs and treated by ethyl acetate fraction of *M. mammosa* for 3 x 24 hours. Allantoic fluid was harvested and subjected on hemagglutinin assay to measure the titre of virus after treated with the fraction. The ethyl acetate extract of *Merremia mammosa* Hall can reduce the titer of hemagglutinin virus by 97.39% at concentration 1000 µg/mL In conclusion*, Merremia mammosa* Hall has the potential to be developed as agent of antiinfluenza A infection.

Keywords: *Merremia mammosa Hall, influenza A, H1N1.*

Introduction

As 1st August 2010, there were over then 18.449 deaths in more than 214 countries that have reported laboratory confirmed cases of pandemic influenza H1N1 2009, for the period of April 2009 to August 2010. However, WHO stated that the total death (including unreported deaths) from the H1N1 strain was actually higher[1]. In fact, Dawood reported that the estimate global deaths associated with H1N1 pandemic 2009 influenza were 15 times higher, which were 284,400 deaths[2].

The existed vaccines against seasonal influenza virus to control this disease have been ineffective due to its rapid variable mutation. In the period of pandemic, vaccine supplies would not be adequate[3]. Thus, the development of effective and safe anti-influenza becomes a matter of certainty in drug discovery[4]. People in Madura island, Indonesia used the tuber of *Merremia mammosa* (Convolvulaceae) to treat tuberculosis infection *Merremia mammosa* is one Indonesian folk medicine use as treatment of cough and sorethroat[5].

However, there is still limited of scientific data can be found about this plant. The chemical component of this plant are Mammoside A and B[6]. This plant also contain polyphenol, triterpenoid, terpenoid, and flavonoid[7]. It has reported that this plant has pharmacologycal activity as anti-HIV. *Merremia peltata*, another plant from the same family, has antimicrobial activity and anti-HIV activity by inhibiting HIV-1 reverse transcriptase and gp120-CD4 binding *in vitro*[8]. Thus, the objectives of this study were to determine the neuraminidase inhibition (curative) and hemagglutinin inhibition (preventive) influenza A antiviral activities of the tuber of *Merremia mammosa* Hall against influenza A H1N1.

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Materials and Method

Chemicals and Reagents

In this study, the solvents used were methanol, *n*-hexane, *n*-butanol purchased from Merck (Germany), ethyl acetate and cloroform purchased JT Backer (USA), all the solvent used was analytical reagent grade. Phospate Buffer Saline (PBS) from Nacalai Tesque (Japan), Penicilline-Streptomycin from Gibco (USA), sterile distilled or deionised H_2O, physiologycal saline (0.85% NaCl) from Otsuka (Japan). The dimethyl sulfoxide (DMSO) was purchased from Merck. The virus used in this experiment was A/Indonesia/Unair/2011 (H1N1) pandemic strain which was propagated in 11-days embryonated chicken eggs.

Plant Extraction

Merremia mammosa Hall tuber was obtained from Sumenep, Madura, Indonesia. The tuber was cut into slice, dried, and grinded into powder. Dried powder of *Merremia mammosa* Hall (1900 gr) was subjected on maceration in room temperature using methanol as the solvents (3×5 L). The supernatant was collected and subjected on rotary evaporator to obtain a thick crude extract. The methanol extract was added by deionised water in the same amount and added by non-polar solvent (*n*-hexane) to obtain non-polar fraction. The mixture of methanol-water and *n*-hexane was shaken vigorously for 10 minutes. This mixture was allowed to stand for some time to allow a complete separation and formed into two layers (water portion and *n*-hexane portion). The *n*-hexane portion was collected. This step was repeated for 3-5 times until the n-hexane layer was clear. The *n*-hexane portion was concentrated *in vacuo*, while the water portion was added by ethyl acetate to obtain the ethyl acetate fraction. The ethyl asetate fraction was subjected to *in vitro* antiinfluenza againts H1N1 influenza virus.

Toxicity Assay

In order to obtain the MNTD (Minimum Non Toxic Dose), the toxicity test was carried out using the host which was embryonic chicken egg. This assay was aimed to determine the highest concentration of the sample which was not caused the death of chicken embryo. Ethyl acetate fraction was added to PBS solution with final concentration 1000, 500, and 62.5 µg/mL (High, moderate, low). DMSO was added in concentration 2.5% to improve the solubility. In this experiment, the solution of the samples were injected carefully to the broad pole of the 11-days-old embryonic chicken egg. This experiment was conducted in 72 hours, the death of the embryo was a checked every 24 hours. The highest concentration that not caused lethality of chicken embryo is proceed for the next assay. All the experiment were ruuning in triplicate.

Treatment of the Virus

11-days-old embryonic chicken egg were injected with 0.01 MOI of H1N1 virus solution (50 µL). The solution of the sample were injected 30 minutes prior to the virus infection to give chance of the samples to spread to the protein of the egg. Zanamivir 10 µg/mL was used as a positive control and the H1N1 virus without treatment extract is used as a negative control. In each extract solution, 50 mL penicillin-streptomycin were added to avoid bacterial infection. The eggs were then incubated for 72 hours at 37 ºC. Embryo mortality observed every 24 hours. After an incubation period is complete, the eggs stored at 4 ºC for 12-24 hours and allantoic fluid was harvested to be subjected on hemagglutination inhibition activity.

Hemmaglutinin Assay

The first opportunity to prevent influenza virus replication is when the virus attaches to the host cell membrane. Influenza virus hemagglutinin oligosaccharides attached to the host cell membrane associated with sialic acid terminal position. The agents act to inhibit the viral replication by interfering the first attachment step of the virus to the host cell, resulting the reducing of HA titre in culture specimen[9]. Guinea Pig's whole blood 5 mL+PBS 10 mL, was subjected on centrifuge 1200 rpm for 10 min. The supernatant was removed and this step was repeated until the PBS was clear. 0.75% of RBC was prepared by adding 37.5 µL of RBC+462.5 µL of PBS. To do this assay, U-shaped 96 well of micro-plates were taken. Using a micropipette, 50 µL of PBS (pH 7.2) was added to all wells except first well of each row. Harvested fluid from virus growth inhibition assay (100 µL) was added to the first two wells of each row. Twofold dilution was made by transferring 50 µL from the second well of each column A2-H2 to A3-H3 by using a multi-channel micropipette. This step was preceded until the 12th column and the remaining 50 µL was discarded after the 12th column. Last, 50 µL of 0.75% RBCs was added to all wells and incubated for 30 minutes at 4ºC. Control well was checked for

complete settling of the RBCs. Results were recorded in HA sheet. The dilution also can be done vertically, with the same principle of procedure[10]. The percentage inhibition was calculated by applying the equation below:

$$\%\ HA\ titer\ reduction = A - BA \times 100\%$$

Note: A. Hemagglutinin titer without the treatment; and B. Hemagglutinin titer with the treatment.

Results

Table 1. Result of toxixity test.

Toxicity Test									
Concentration (µg/ml)	Incubation Time								
	24 h			48 h			72 h		
	I	II	III	I	II	III	I	II	III
1000	+	+	+	+	+	+	+	+	+
500	+	+	+	+	+	+	+	+	+
62.5	+	+	+	+	+	+	+	+	+

(+) means there is no mortality of chicken embryo expossed by the sample. Note: I, II, III = a triplicate experiment.

From this assay, it can be concluded that in the highest concentration, there was no letality of chicken embryo. Therefore, the next assay will be started at concentration 1000 µg/mL.

Table 2. Result of hemagglutinin assay.

Hemagglutinin Assay		
Grup concentration (µg/mL)	Mean of HA unit	Percentage of inhibition (%)
F.EtOAc 1000 µg/mL	10.67	93.75
F.EtOAc 500 µg/mL	9.3	92.73
F.EtOAc 62.5 µg/mL	32	75
Control (Zanamivir 10)	0	100
Control (virus only)	128	-

Discussion

In vitro antiviral assay was performed using A/Indonesia/Unair/2011 (H1N1) virus which was propagated in embryonated chicken egg. Ethyl acetate fraction was proved to have a specific interaction with HA protein. The reduction of HA titre and 93.75% of H1N1 infectious titre. From the result of the present study, it is possible to suggest that the treatment given was affecting the viral replication especially in the early stage of infection[11]. Ethyl acetate fraction reduced HA titer near to zero in allatoic fluid of embryonated chicken egg specimens and showed high protection against viral infection. Ethyl acetate fraction is rich in flavonoid and terpenoid compounds that has the possibility to inhibit the growth of the virus. In conclusion, ethyl acetate fraction of *Merremia mammosa* Hall is able to inhibit viral at early step of infection especially when giving before the infection.

Conclusion

In sum, *Merremia mammosa* Hall has the potential to be developed as antiviral agent of influenza A (H1N1) infection.

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

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

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