

Research Article

INFLUENCE OF *Morinda citrifolia*, L. FRUIT EXTRACT AS ADJUVANT ON IG-Y PRODUCTION RAISED IN LAYING CHICKENS AGAINST AVIAN INFLUENZA VACCINE

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ABSTRACT

Immunized avian with vaccine, produces specific antibody in the serum as well as in the egg yolk. An adjuvant is used to augment the effects of a vaccine by stimulating the immune response. *Morinda citrifolia*, L., especially the fruit contains of some chemical compounds such as polysaccharide, scopoletin, damnacanthol, proxeronine and proxeroninase. All those of active compounds, are soluble in different solvents and have different activities. Some of those compounds have adjuvant activity. The aim of our study was to observe the capability of *M.citrifolia* fruit extracts as adjuvant to enhance the specific antibody (IgY) production in immunized laying chickens Lohmann strain (28 weeks old) with a commercially available avian influenza (H5N2) vaccine. To the groups (@6) of laying chickens were orally administered once a day, through out of the study, with *n*-hexane (group I; divided into 3 sub groups), ethanolic (group II; divided into 3 subgroups) and aqueous extract (group III; divided into 3 subgroups) of *M.citrifolia* (prepared in capsules) on dose of 29 mg; 58 mg; and 116 mg/chicken/day, respectively, and group IV as plasebo control and group V as normal control. All of the groups were induced with avian influenza vaccine, except the normal control. Eggs of 3, 6, and 10 weeks after immunized, were collected. Isolation of IgY was performed by repeated polyethylene glycol 6000 precipitation steps. IgY obtained was analyzed by indirect ELISA method. The serum was tested for antibody specificity against the influenza H5N2 virus by hemagglutination-inhibition (HI) method. The result of this study showed that the dose of 58 mg/chicken/day of aqueous extract of *M.citrifolia* fruit had an optimal capability to enhance specific antibody (IgY) as well as IgG serum production against avian influenza (H5N2) vaccine .

Key words: Adjuvant, extract, *Morinda citrifolia*, L., H5N2 vaccine, immunoglobulin yolk (IgY)

INTRODUCTION

Avian influenza is an infection caused by avian (bird) influenza (flu) viruses. These influenza viruses occur naturally among birds. Wild birds worldwide carry the viruses in their intestines, but usually do not get sick from them. However, avian influenza is very contagious among birds and can make some domesticated birds, including chickens, ducks, and turkeys, very sick and kill them (Anonymous, 2007a).

It was declared, most H5N1 viruses that have caused human illness and death appear to

be resistant to amantadine and rimantadine, two antiviral medications commonly used for treatment of patients with influenza. Two other antiviral medications, oseltamivir and zanamivir, would probably work to treat influenza caused by H5N1 virus, but additional studies are needed to demonstrate their effectiveness.

Several studies concerning with oral immunotherapy with yolk antibodies from hyper-immunised hens, have been reported (Lu *et al.*, 2006; Carlander *et al.*, 2000; Mine, and Kovacs-Nolan., 2002). Oral immunotherapy with yolk antibodies is a new promising

treatment strategy, primarily for infections in the mouth and gastrointestinal tract. Oral treatment could potentially be used against many frequently encountered diseases (e.g. common cold, tonsillitis and caries).

Chickens transfer high quantities of IgY (IgG) into the egg yolk and harvesting antibodies from eggs eliminates the need for the invasive bleeding procedure. Avian IgY more resistant to temperature, pH and ion strength of environment than IgG. The importance of eggs as a source of specific antibodies is well known. Egg yolk contains 8-20 mg of immunoglobulin (IgY) per mL (Szabo *et al.*, 1998).

An adjuvant is a substance that is added to a vaccine to enhance the body's immune response to the vaccine's active constituent, called the antigen. The studies found that, without the adjuvant, a range of doses of vaccine did not induce protection. With the adjuvant, however, the vaccine induced protective antibody levels against the original H5N1 strain, including when used at low doses (Stephenson *et al.*, 2003).

Use of adjuvant in the vaccine preparation is a long standing practice. Despite major advances in our understanding of vaccine adjuvants, both old and new vaccines seem likely to depend on aluminium salts. However, these adjuvants can lead to serious adverse effects. Herbal immunomodulators are paving its way as a safe alternative. These herbal modulators can be administered along with the vaccine to elicit a faster and stronger immune response. Various herbal preparations have been shown to exert strong immunomodulatory. Use of herbal immunomodulators perhaps might be helpful in the future (Sakure *et al.*, 2008).

Study about the use of herbal as adjuvant has been done. From the study, was reported, that ethanolic extract of *C.mangga* could enhance the IgY produced in induced avian influenza vaccine-duck (Ediati *et al.*, 2008)

In this our study, *M.citrifolia* was one alternative herbal chosen as adjuvant, because of its ability as immunomodulator. The fruit of *M.citrifolia* contains of some chemical compounds such as polysaccharide, scopoletin,

damnacanthal, proxeronine and proxeroninase. All those of active compounds, soluble in different solvents and have different activities. Some of those compounds have adjuvant activities. Further more, it has been reported by (Sundvall, 2007), in Pohnpei, Micronesia, native fruit of *M.citrifolia* boosters immune systems in wild birds and used by locals to treat Avian Flu-like diseases. According to Ellis *et al.*, (2004), vaccination of chickens with a commercially available killed H5N2 vaccine enhanced biosecurity measures and intensive surveillance for control of highly pathogenic avian influenza subtype H5N1 disease in Hong Kong in 2002. Because of that reason, H5N2 vaccine used in our study.

From all those reasons, the aim of our study was to observe the capability of *M.citrifolia* fruit extracts as adjuvant to enhance the specific antibody (IgY) production in immunized laying chickens Lohmann strain (28 weeks old) with a commercially available avian influenza H5N2 inactivated vaccine.

METHODOLOGY

Animals

Lohmann strain chickens, 28 weeks old, weighing about 1,7 kg were obtained from Faculty of Animal Sciences, Universitas Gadjah Mada, Yogyakarta, Indonesia. All Chickens were housed under standard conditions through out of the study.

Preparation extracts of *M.citrifolia* fruit

The fruit of *M.citrifolia* before ripening state were collected from Sleman, Yogyakarta, washed, sliced, dried in oven 45°C, powdered. About 2 kg of dried powder; was macerated for 5 days with n-hexane. The n-hexane supernatant was filtered and the drying residu then macerated 5 days with ethanol. The ethanol supernatant was filtered and the drying residu was then was infundated with aquadest. The aqueous supernatant was filtered. Each of the supernatant obtained was evaporated under vaccum condition up to viscous mass. Each of the viscous mass was then mixed with pollard powder to get doses of 29 mg; 58 mg; and 116 mg/capsul. The placebo was prepared by filling capsuls with pollard powder.

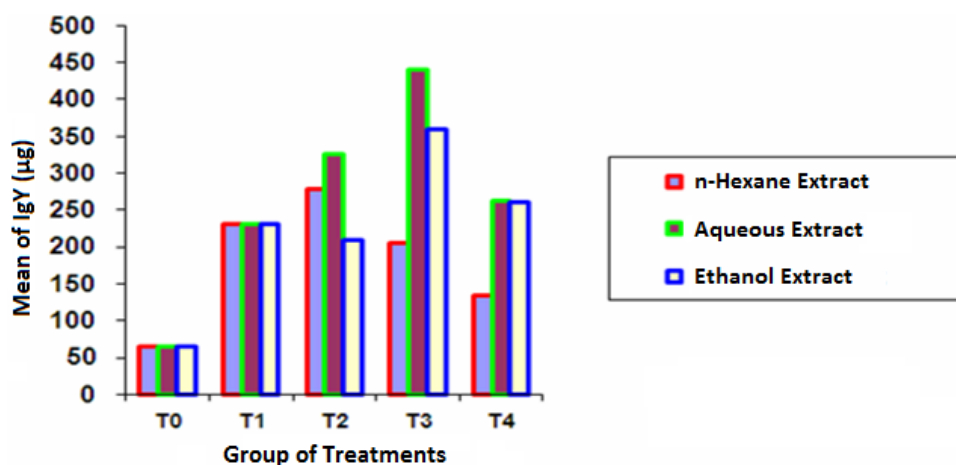


Fig 1. Mean \pm SD of IgY level, was isolated from immunized laying chickens by H5N2 vaccine after the treatment of *M. citrifolia* extracts.

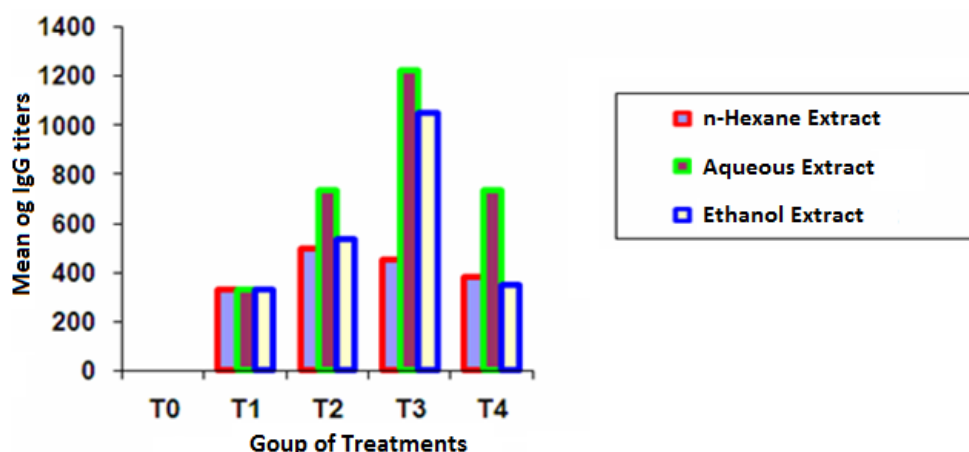


Fig 2. Mean \pm SD of IgG serum titers, was isolated from immunized laying chickens by H5N2 vaccine after the treatment of *M.citrifolia* extracts.

Notes :

T0 = normal control

T1 = negative control

T2 = extract dose of 29 mg/chicken

T3 = extract dose of 58 mg/chicken

T4 = extract dose of 116 mg/chicken

Experimental design

The chickens were divided into 5 groups. Group I (6 of chickens) as normal control (without vaccine+placebo capsul); group II (6 chickens) as negative control (vaccine +placebo capsul); group III, group IV and group V (each consist of 3 subgroup of 6 chickens) treatment with vaccine + *M.citrifolia*

n-hexane; ethanol; and aqueous extract (with 3 varieties of doses for each extract), respectively. The dose were 29 mg; 58 mg; and 116 mg/capsul/chicken/day. After per condition time (7 days), chickens were immunized with commercial H5N2 inactivated vaccine (Medivax®). The treatments p.o. of *M.citrifolia* capsulated-extract, except the normal controls,

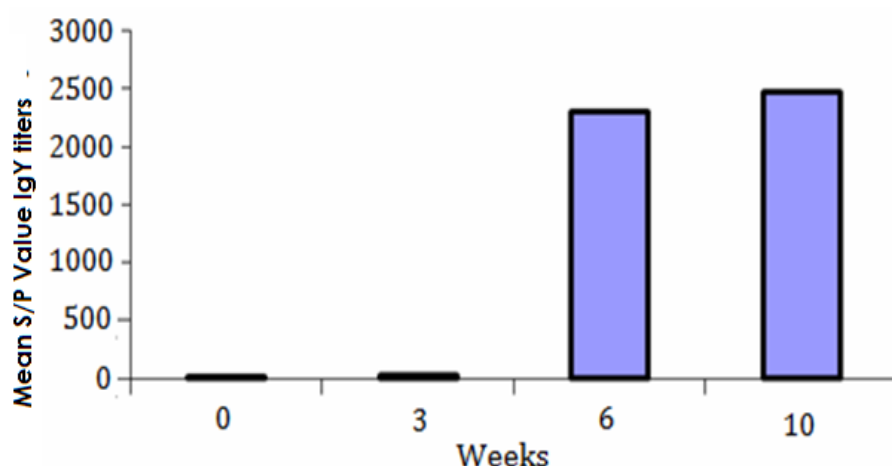


Fig 3. Mean of S/P value of IgY was isolated after immunized by H5N2 vaccine and aqueous extract dose of 0.58 mg/chicken treated

were given through out of the study. All the chicken, except normal control were given one shot intramuscular with 0.5 mL chicken/vaccine. Eggs and serum were collected on the weeks of 3, 6 and 10. Isolation of IgY was performed by repeated polyethylene glycol 6000 precipitation steps as described by Gasmann *et al.*, (1990). IgY obtained was analyzed by indirect ELISA method, according to Bock *et al.*, (1986). The IgG serum was tested for antibody specificity against inactive antigen subtype H5N1 from Pusvetma by HI (hemagglutination-inhibition) method Beard, (1989). The time sampling (3, 6 and 10 weeks) was also evaluated to find out which was the best time sampling during 10 weeks experiment.

Statistical analysis

The data obtained were statistically analyzed with Split plot variance, followed by DMRT (Duncan Multiple Range Test and Tuckey test, using a significance level of $P < 0.05$).

RESULTS AND DISCUSSION

The isolated of antibody (IgY) was determined by yolk indirect ELISA method. The result presented in figure 1. From figure 1 is indicated that the aqueous extract of

M.citrifolia fruit with the dose 58 mg/chicken has an optimal capability to enhance the IgY produced in egg yolk of immunized chickens by H5N2 vaccine. This condition followed by the IgG serum titer which was determined by HI test presented in figure 2. This may be due to the aqueous extract contains of polar active compounds, for example polysaccharide which is well known as natural adjuvant. Immunopotentiating effects of four Chinese herbal polysaccharides administered at vaccination in chickens, also has been reported by Qiu *et al.*, (2007). The result of the study showed that those herbal at individual doses could enhance the Newcastle disease (ND) antibody titers.

From the data of time sampling stated with the value of S/P IgY titers. It can be proposed that the 10 weeks time sampling of extract aqueous treated showed the highest value comparing with 3 and 6 weeks time sampling. However there was no significantly ($P > 0.05$) different Result at the week 6 and 10, but significantly ($P < 0.05$) to 3 weeks time sampling. It means that after more than 3 weeks (may 4 weeks) after immunization, was started producing antibody. The value of S/P IgY titer of 6 weeks and 10 weeks time sampling were 2.31 and 2.48 (value of > 0.5 it means there is a specific antibody against avian

influenza). The antibody production lasted until 10 weeks. There was a relation between the result of time sampling observation with the state of *Prosedur Operasional Standar Pengendalian Penyakit Avian Influenza, Dirjen Peternakan, Departemen Pertanian RI* (Anonymous, 2006), that the successful of vaccination can be tested by finding antibody production within 3-4 weeks and the repetition can be administered every 12-16 weeks. The data of mean \pm SD S/P value vs time sampling detected 3, 6, and 10 weeks after vaccination based on HI antibody determined, was given in figure 3.

CONCLUSION

The result of the study concluded that the dose of 58 mg/chicken of aqueous extract of *M.citrifolia* fruit had an optimal capability to enhance specific antibody as adjuvant of IgY as well as IgG serum production against avian influenza (H5N2) vaccine.

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REFERENCES

- Anonymous, 2007a, About Avian Influenza (Bird Flu) and Avian Influenza A (H5N1). Virus, Available at <http://www.cdc.gov/flu/avian/gen-info/facts.htm>
- Anonymous, 2006, Prosedur Operasional Standar (SOP) Pengendalian Penyakit AI, Direktorat Jenderal Peternakan, Departemen Pertanian RI, Jakarta.
- Anonymous, 007b, Treatment and Vaccination for H5N1 virus in Human, Available at <http://www.cdc.gov/flu/avian/gen-info/qa.htm>
- Beard, C.W., 1989, Serologic Procedures in a Laboratory Manual for the Isolation and Identification of avian Pathogens, 3rd ed, p. 192-200. *Am. Ass. Av. Path.*
- Bock, R.E., Burgess, G.W., Douglas, I.C., 1986, Development of an enzyme linked immunosorbent assay (ELISA) for the detection of bovine serum antibody to bovine viral diarrhoea virus, *Aust. Vet. J.*, 63(12):406-8.
- Carlander, D., Kollberg, H., Wejaker, P.E, and Larsson, A., 2000, Peroral Immuno-therapy with Yolk Antibodies for the Prevention and Treatment of Enteric Infections, *Immunologic Research*, 21/1:1-6. Mary Ann Libert, Inc.
- Ediati S., Shaum S., Pratiwi, G., Debritha A.D., and Danang P.H., 2008, The Effect of Ethanolic Extract of *Curcuma mangga*, Val. on Egg Yolk Immunoglobulin (IgY) Raised in Duck against Avian Influenza Vaccine, Presented at The International Symposium on Molecular Pathogenesis: Recent Advances on Molecular Pathogenesis Applications to Pharmaceutical Product Development, *ITB.*, Bandung, Januari 2008.
- Ellis, T.M., Leung, C.Y., Chow, M.K., Bisset, L.A., Wong, W., Guan, Y., and Peiris, M., 2004, Vaccination of chickens against H5N1 Avian influenza in the face of an outbreak interrupts virus trans-mission, *Avian Pathol.* 33(4):405-412.
- Gasmann, M., Thommes, P., Weiser, T., and Hubscher, U., 1990, Efficient production of chicken egg yolk antibodies against a conserved mammalian protein, *FASEB*, 4 : 2528-2532
- Lu, J., Guo, Z., Pan, X., Wang, G., Zhang, D., Li, Y., 2006, Passive immunotherapy for influenza A H5N1 virus infection with equine hyperimmune globulin F(ab')₂ in mice, *Respiratory Research*, 7:43.
- Mine, Y. and Kovacs-Nolan, J., 2002, Chicken Egg Yolk Antibodies as Therapeutics in Enteric Infectious Disease : A Review, *J. Med. Food*, 5(3):159-169.
- Qiu, Y., Hu, Y.L., Ciu, B.A., Zhang, H.Y., Kong, X.F., Wang, D.Y., and Wang Y.G., 2007, Immunopotentiating effects of Four Herbal Polysaccharides Administered at Vaccination in Chickens, *Poult Sci*, 86:2530- 2535.
- Sakure, S., Negi, V.D., and Mitra, S.K., Nandakumar, K.S. and Chakravortty, D. 2008, Vaccine with herbal adjuvant-A better cocktail to combat the infection, *Vaccine*, 26 (25), 3387-3388.
- Stephenson, I., Nicholson, K.G., Colegate, A., Podda, A., Wood, J., Ypma, E., and Zambon, M., 2003, Boosting immunity to influenza H5N1 with MF59-adjuvanted H5N3, *Vaccine*, 21(15): 1687-93

- Sundvall, C., 2007, *Morinda Citrifolia* bolsters immune systems in wild birds and is used by locals to treat Avian Flu-like diseases, In http://newfarm.rodale-institute.org/depts/readermail/2007/0107/0112_5.shtml, Postings 2007.
- Szabo,Cs., Bardos,L., Lozonczy,S. and Karchesz, K., 1998, Isolation of Antibodies from Chicken and Quail Eggs, Presented at INABIS'98. Available at URL <http://www.mcmaster.ca/inabis98/immunology>