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Anti Marek's Disease Virus Activity *of Scurrula oortiana* (Tea Mistletoe) Stem Extract in Embryonated Chicken Eggs*

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Abstract: *S. oortiana* extract has been previously shown to have anti-tumour and antiviral activity *in vitro*. This study was carried out to investigate an antiviral effect of *S. oortiana* stem extract against Marek's Disease Virus (MDV) *in ovo*. The antiviral effect was studied in embryonated chicken eggs (n = 5 per treatment) by inoculating 0, 0.1, 0.2, or 0.4 mg extract/egg before or after infection with MDV. In the "before" treatment, the extract was administered on day 9 with MDV infection on day 12. In the "after" treatment, the extract was administered on day 12 after infection with MDV on day 9. Histopathological examinations were performed on day 20 to determine the number of inclusion bodies, pock formation, macrophage infiltration and immunohistochemistry was used to determine the frequency and intensity of viral antigen expression. Inoculation of the extract before MDV infection significantly ($p<0.05$) reduced the frequency and intensity of viral antigen expression. There was also a significantly lower level of epithelial destruction on chorioallantoic membrane of the groups given 0.2 and 0.4 mg extract/egg than the control group given no extract. This study provides the first evidence of anti MDV viral activity of *S. oortiana* extract *in vivo* and may provide an alternative method to prevent or alleviate Marek's disease.

Key words: Marek virus, *s.oortiana*, tea mistletoe, embryo, chicken, egg

INTRODUCTION

Marek's Disease Virus (MDV) is a poultry disease characterized by invasive lymphomas due to α-herpes virus infection which is highly contagious in chicken. Marek viral infections lyze B lymphocytes and transform T cells into tumours (Witter, 2001). Lymphocytes destruction leads to immunosuppression indicated by a decrease in the number of active lymphocytes and lymphoid organ weight. Animals are more sensitive to subsequent infection and antibody titres following vaccination could not reach a protective level. Economic loss due to MDV in a form of death and growth depression in broilers reached 1.3-1.7% (Tabbu, 2001) and death and drop in egg production in layer reached 3-5% and 60% respectively (Fatimah, 2000). Economic losses in layer are higher not only due to the afore mentioned reasons but also to high cost of vaccination and disease control (Witter, 2001).

Prevention of MDV can be done by several methods one of which is in ovo vaccination. *In ovo* vaccination can stimulate protective antibody formation in Day Old Chicks (DOC). However due to high cost and inability to provide protection against the constantly mutating virus, alternative attempts should be made (Schat, 2004). Naturally occurring bioactive substance found in medicinal plants have been shown to have antiviral and anti tumour activities. However such evidence has not been explored to be used in livestock to prevent or alleviate viral disease. Therefore, this study was carried out to investigate such potential of medicinal herbs.

S. oortiana is mistletoe that grows in tea tree and has been used for generation to treat tumour and have been shown to have anti tumour and anti viral activity (Kusumoto *et al*., 1992; Murwani, 2003). The extract has immunomodulating activity, increasing sensitivity of tumour cells to TNFα mediated lysis, increasing IgG in C_3H mice and inhibit DNA isomerase of tumour cells (Winarno *et al*., 2000; Murwani, 2003). Earlier studies have also shown that this extract can increase the number of active lymphocytes in bursa of fabricius and thymus (Murtini *et al*., 2006). The following studies explored further the ability of the extract to alleviate MDV infection *in ovo* embryonated chicken eggs.

MATERIALS AND METHODS

Embryonated chicken eggs (ECE): This study used ECE from Specific Pathogen Free (SPF) White Leghorn eggs. Eggs were obtained from PT. Biofarma, Bandung city. All

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eggs were placed in egg tray in egg incubator at 37-38°C until 9 days of age. All eggs were checked for fertility by candling.

Tea mistletoe extracts: *S. oortiana* was collected from the Centre of Tea and Quinine Research, Ciwedey, West Java. The collected materials were taken to Herbarium Bogorensis for species identification (collection no 26250, collector: Bacher). Five grams of dried leaves and stem were extracted with 100 ml of water three times under reflux for 3 h. The resulting filtrates were evaporated to give dried extracts (Murwani, 2003).

Inoculum of *S. oortiana* extract for *in ovo* inoculation: The extracts were weighted and dissolved in sterile distilled water to reach the desired concentration of 0.1 mg, 0.2 mg or 0.4 mg per 0.2 ml. The distilled water contained 10,000 IU penicillin and 10,000 µg. streptomycin per ml.

Marek virus preparation: Marek virus was obtained from Veterinary Drug Quality and Certification Assurance Institute, Gunung Sindur Bogor. The virus were propagated in Laboratory of Virology Department of Animal Diseases and Vet. Public health, Faculty Veterinary Medicine, Bogor Agriculture University, Bogor in SPF-ECE. Infection was carried out at 10^3 EID_{50}.

Preparation of polyclonal antibody to marek virus: Antibody was raised in 3 months old White New Zealand rabbit obtained from PT. Biofarma Bandung. Two rabbits were each immunized with Marek virus at 10^3 EID in 0.5 ml volume with Freud complete adjuvant. The viral preparation was injected subcutaneously and booster injection was carried out two weeks after the first injection with Marek virus at 10^3 EID in 0.5 ml volume with Freud incomplete adjuvant. Two weeks after booster blood was sampled to detect antibody to Marek virus formation by gel precipitation. When antibody was already detected, rabbits were re-immunized with the same antigen without adjuvant intravenously. Five days following this re-immunization, rabbits were sacrificed and blood was collected as a source of polyclonal antibodies and stored frozen until use.

Forty five (45) SPF-ECE were randomly assigned into 9 groups, each group consisted of 5 eggs. *S. oortiana* extract was inoculated via allantois and Marek virus was inoculated via CAM (Murtini *et al*., 2006). The extract was inoculated at 0, 0.1, 0.2, or 0.4 mg extract/egg before or after infection with MDV. In the "before" treatment, the extract was administered on day 9 with MDV infection on day 12 (designated as B1 to B3). In the "after" treatment, the extract was administered on day 12 after infection with MDV on day 9 (designated as A1 to A3). Following inoculation ECE were incubated at 37°C until they were 20 days of age. The treatments were summarized in Table 1.

The ability of the extract in preventing Marek virus growth was examined based on pock formation, the presence of inclusion bodies, mononuclear cells and macrophages on CAM and Bursa Fabricius. The presence of Marek virus in ECE organ was detected by immunohistochemistry. Positive immunoactivity using polyclonal antibodies to Marek virus as described previously is an indicator of the presence of the virus.

Samples of embryos, histopathological preparations and pathological and anatomical observations: ECE were open aseptically and CAM and the embryos were collected separately into sterile Petri dish and examined for Pathological and Anatomical (PA) observations. Embryos were then rinsed with Phosphate buffer saline and Bursa Fabricius (BF) and CAM were preserved in 10% Buffered Neutral Formalin (BNF). The samples were processed further for histopathological and immunohistochemical preparations.

Samples on slides were deparafinized using xylol I, II and III and rehydrated with graded alcohol. Finally it was washed with deionized water. Samples containing glass slides were then dipped in 3% methanol-H_2O_2 for 15 min to eliminate endogenous peroxides, then washed with deionized water and PBS. Normal goat serum was added and incubated for 60 min at 37°C, after which it was washed and incubated overnight at 4°C with anti Marek polyclonal antibodies. It was further incubated in secondary antibody (peroxidase conjugated) (Dako DEPS K1491) for 30 minutes in the dark at 37°C. Antigen was visualized by adding 3,3 amino H_2O_2 as substrate for peroxidase. Finally it was counter stained by Haematoxylin for 10 second.

Table 1: Treatments of SPF-ECE

	Treatments			
Groups	9 days	12 days	19 days	21 days
A1	Marek virus infection	*S. oortiana* extract 0.1 mg/egg	Eggs were stored	Embryos collection
A2	Marek virus infection	*S. oortiana* extract 0.2 mg/egg	at 4°C temperature	
A3	Marek virus infection	*S. oortiana* extract 0.4 mg/egg		
B1	*S. oortiana* extract 0.1 mg/egg	Marek virus infection		
B2	*S. oortiana* extract 0.2 mg/egg	Marek virus infection		
B3	*S. oortiana* extract 0.4 mg/egg	Marek virus infection		
Positive control (K+)	-	Marek virus infection		
Negative control (K-)	-	Placebo		
Mistletoe control	*S. oortiana* extract 0.4 mg/egg	-		

Histopathological observation (HP): The number of inclusion bodies and macrophages in CAM were taken from the average of 5 observation fields, while that in Bursa Fabricius (BF) were taken from the average of 30 follicles for each BF.

BF was observed for the intensity and spread of cells which are immunoreactive against polyclonal antibody. This observations were analyzed descriptively according to Cho *et al*. (1999) method. The observations was scored as 1) negative when there was no infected cells, 2) mild infection when positive immunoreactivity in cells are low, 3) medium infection when the number of positive cells are medium and 4) heavy infection when there were large number of positive immunoreactivity cells.

Quantitative analyses of immunohistochemistry in CAM were determined by counting the number of cells which reacted against polyclonal antibodies. The CAM were negative from infection when there was no immunoreactivity, positive when immunoreactivity was weak and strong positive when immunoreactivity was strong and cell lyses occurred. Observations were done on 100 CAM epithelial cells. Each sample was observed 5 times on different 100 cells.

Data analysis: The data of embryos weight, histopathological observations, the number of inclusion bodies, pock formation in CAM and Bursa of Fabricius were analyzed by variance using GLM software. Further test was carried out if there was significant difference ($p<0.05$).

RESULTS AND DISCUSSION

Anatomical observation of CAM showed that viral inoculation was successfully infecting the ECE. The viral infection and replication was shown as pock formation i.e. thickening of CAM in focal or diffuse form (Fig. 1). Observation in groups inoculated with *S. oortiana* extract before or after viral infection showed a significantly lower number of pock formation compared to positive control with no extract ($p<0.05$) (Table 2). Pock scores in group inoculated with extract prior to infection had lower number than group receiving the extract after viral infection and positive control groups. PA observation was followed by HP observations on CAM. The observation showed that inclusion bodies were formed in all groups infected with Marek virus. This inclusion bodies are part of virus or intact viral particles which remained within the cell after it is infected (Fig. 2).

Observation on BF showed lymphocytes depletion in all groups except in negative control groups (Table 3). This depletion might be due to Marek virus infection itself. The percentage of lymphoid follicles in lymphocytes depleted groups were not significantly different. However in groups inoculated with the extract prior to infection the depletions is lower than the positive control. The number of active lymphoid follicles of BF was also higher in group receiving the extract than positive control. Statistical analysis showed that a significant difference was found at 0.2 mg extract inoculation per egg after infection compared to inoculation of the extract prior to infection and the positive control. MDV is an oncogenic

Table 2: Histopathological changes of ECE-CAM infected with Marek virus and inoculated with *S. oortiana* extract

	Histopathological changes of CAM	
Groups	Pock scores	The number of Marek viral inclusion body (in each observation of microscopic field)
A1	0.17±0.41^{b}	5.88±2.09^{cd}
A2	0.25±0.50^{b}	2.25±2.22^{ab}
A3	0.80±1.79^{b}	3.16±2.08^{bc}
B1	0.00±0.00^{b}	7.70±1.27^{ed}
B2	0.20±0.45^{b}	3.55±0.84^{bc}
B3	0.50±1.00^{b}	5.47±0.81^{cd}
Control positive	4.00±0.00^{a}	9.50±0.71^{e}
Control negative	0.00±0.00^{b}	0.00±0.00^{a}

Different superscript in the same column showed significant difference among treatments ($p<0.05$)

Table 3: Histopathological changes ECE-BF infected with Marek virus and inoculated with *S. oortiana* extract

	The percentage of histopathological changes in BF		
Groups	Lymphocyte depletion	Active lymphoid follicles	Macrophage and lymphoblast
A1	30.67±5.96^{bc}	24.00±10.38^{ab}	45.33±18.25^{bc}
A2	26.67±18.86^{bc}	61.67±35.35^{c}	11.67±16.49^{a}
A3	34.17±1.67^{c}	30.00±18.66^{b}	35.83±17.29^{b}
B1	16.67±4.72^{bc}	16.67±14.14^{ab}	66.67±9.42^{c}
B2	15.55±6.94^{b}	20.00±12.02^{ab}	64.44±18.36^{c}
B3	24.44±6.94^{bc}	24.44±18.35^{ab}	51.11±13.87^{bc}
K+	28.66±7.67^{bc}	14.66±5.06^{ab}	56.67±6.23^{bc}
K-	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}
Mistletoe control	7.95±9.77^{b}	62.90±12.40^{c}	29.15±6.98^{b}

Different superscript in the same column showed significant difference among treatments ($p<0.05$)

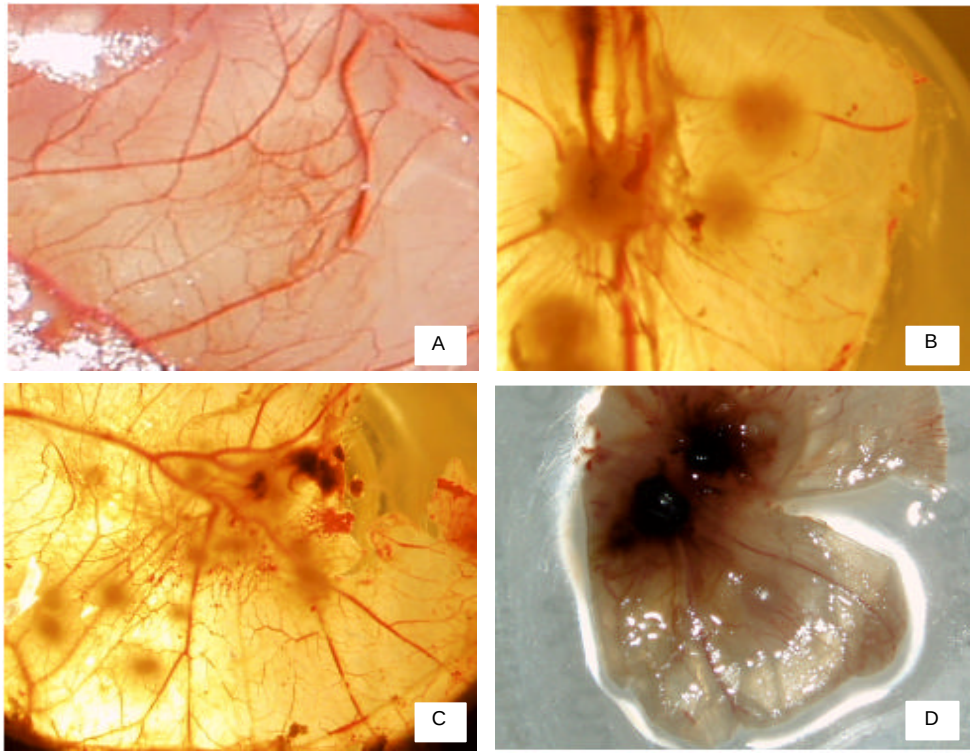

Fig. 1: Pock formation on CAM after Marek virus infection. A) Pock formation on CAM with score 0. B) Pock formation on CAM with score 1. C) Pock formation on CAM with score 2. D) Pock formation on CAM with score 3

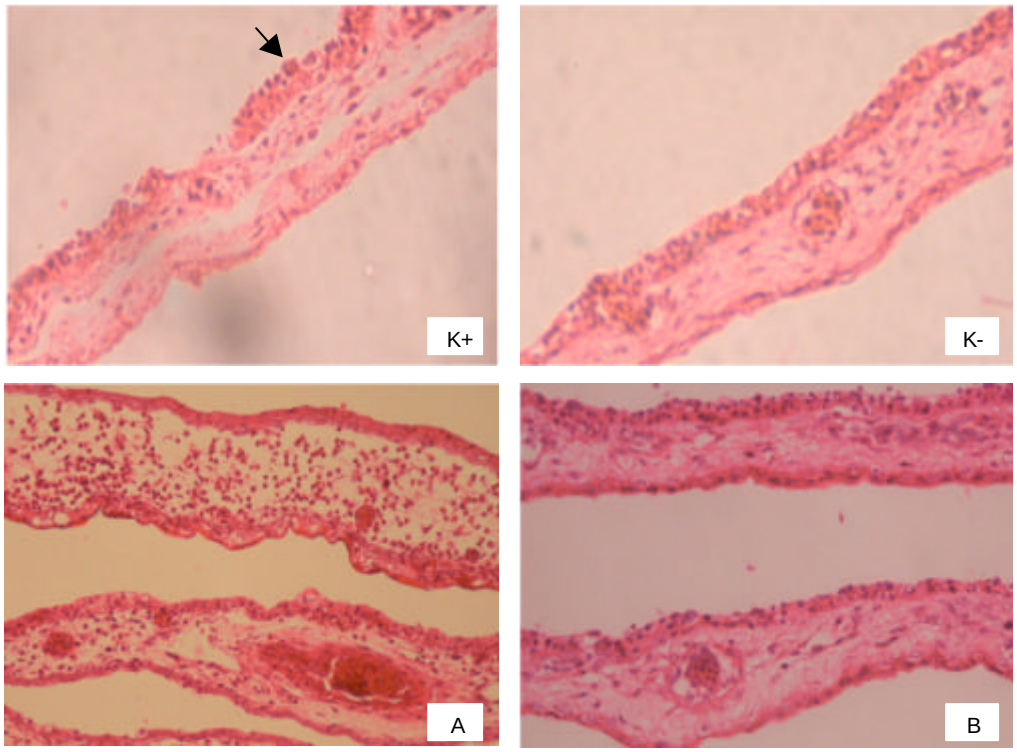

Fig. 2: Photomicrograph of Chorio Allantois Membrane (CAM) inoculated with or without *S. oortiana* extract and infected with MDV. HE stained with 100x magnification. Arrow: inclusion body

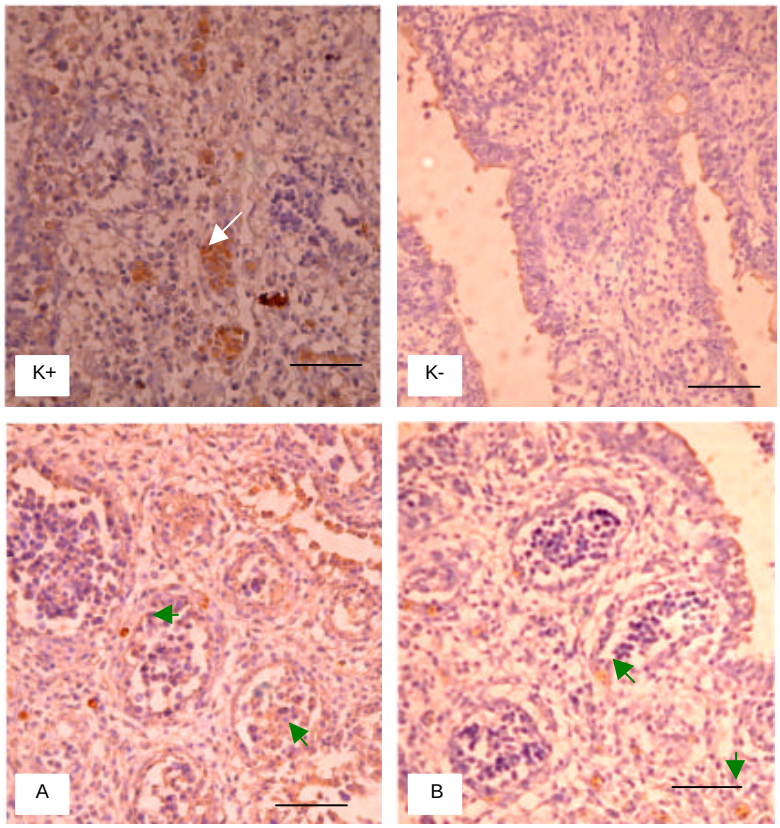

Fig. 3: Photomicrograph of immunohistochemistry staining of ECE-BF against Marek virus positif immunoreactivity to Marek antibody in positive control (K+, white arrow), negative control (K-), inoculated with the extract after MDV infection (A), inoculated with the extract before MDV infection (B). Bar = 50 µm. Green Arrow: Immunoreactivity against Marek antigen

virus which cause lymphocyte proliferation (Witter, 2001). Result of this study showed that administration of the extract prior to infection prevent lymphocyte proliferation is more effective than after infection.

Antigen of Marek virus in BF was detected by immunohistochemistry and positive immunoreactivity in cells reflects the presence of the virus. No reactivity was found in uninfected negative control groups (Fig. 3). In BF, immunoreactivity was found on the epithelial surface of plicas, intrafolliculer lymphoid and lymphoid follicles. In control groups, viral infection of low to medium degree was found in the epithelial cells of plicas surface. In lymphocytes, intrafollicular and lymphoid follicles infection was found from medium to high degree. Low viral infection on epithelial cells of plicas was found in groups inoculated with 0.1 mg extract prior to infection. No infection was found in the intrafolliculer lymphoid cells while low infection was found in lymphoid follicles cells. In groups inoculated with 0.2 mg extract, low infection was found only in epithelial cells of plicas and intrafolliculer lymphoid, however no infection was found in the follicles. CAM epithelial cells undergoing lysis in groups inoculated with extract before or after Marek infection are lower than positive control groups.

The observed parameters described above indicated the ability of *S. oortiana* extract in suppressing Marek viral growth in ECE. The extract suppressing effect is possibly associated with bioactive contents of *S. oortiana* extract. The extract were identified to contain 6 different conjugated fatty acid, 2 xanthines, 2 flavonol glycoside, 4 flavonols and lignan (Ohashi *et al*., 2003; Winarno *et al*., 2003; Murwani and Simanjuntak, 2002). Flavonoids have been shown to exert immunomodulating activity which can stimulate maturation of lymphocytes and macrophages and could lead to increase number of the cells in ECE. Increase number of mature lymphocytes in turn could increase effector molecules such as cytokine which enhance phagocytosis and lysis of viral infected cells.

Other study reported that quercetin can act as an antiviral by stimulating macrophage development into active macrophage (Veckenstedt and Pusztai, 1981). These active macrophage could induce cytokines production such as IFNγ which is important molecules that can

activate Natural Killer Cell (NKC), enhancing expression of MHC I and II and other accessories molecules in antigen presentation. IFNγ alone or in combination with TNFα can stimulate the production of iNOS which is catalyzed to form Nitric Oxide (NO) that inhibit Marek virus replication. IFNγ alone can also inhibit viral replication (Xing and Schat, 2000a,b) therefore together with NO could enhance inhibition of viral replication. Marek virus replication induce tumourgenesis in T lymphocytes. The active constituents in *S. oortiana* extract posses antitumor activity which may reduce tumor cells number. This reduction may occur via apoptosis which is induced by the active constituents in the extracts. Quercetin, one of the active substance was shown to be lethal to human melanoma by inducing apoptosis (Rosner *et al*., 2006). Another study showed that *S. oortiana* extract can enhance the sensitivity of tumor cells to TNFα mediated lysis so that elimination of tumor cells or viral infected cells such as macrophage and T cells can be executed effectively (Murwani, 2003). In groups receiving the extract after viral inoculation, cytokine induction is low or absent as virus already infect the cells. Therefore response to viral infection showed similar pattern to that of viral infected control group which did not receive the extract. This response indicated that the extract must be administered early before viral infection occurs so that modulation of the extract on somatic or immune cell membrane has been formed and when viral infection took place, the cells are ready to response to viral-induce changes.

From the three dosages administered, 0.2 and 0.4 mg/egg before viral infection showed the highest inhibition of viral growth compared to other groups. This was reflected by pock score, the number of inclusion body, the degree of infection in BF and the presence of low Marek antigen in CAM compared to the rest of the groups. These results showed that 0.2 and 0.4 mg/egg are the effective dose that can prevent viral growth. When the relation of extract dosage and the number of inclusion body are plotted into graph it forms U shape curve. This U shape curve is termed "Hormesis" (Calabrese and Baldwin, 1999) and provides additional evidence of hormesis for medicinal plants.

Conclusion: Administration of *S. oortiana*extract prior to Marek virus infection can inhibit viral growth and cell destruction due to viral infection. 0.2 and 0.4 mg extract/egg showed the most effective level to inhibit Marek virus infection and to reduce cell destruction.

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