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Effect of 4-Hydroxycinnamic Acid on Chickens Infected with Infectious Bursal Disease Virus

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Abstract: 4-hydroxycinnamic acid was firstly isolated from Fructus mume and identified to be one of the main components. The studies aimed to investigate the immunotherapeutic potential of 4-hydroxycinnamic acid on SPF chickens infected with IBDV. The results showed that the bird survival rate of 3 dosage groups of 4-hydroxycinnamic acid was 87.5, 62.5 and 42.86%, respectively while body weight gain rate was 25.68, 25.28 and 21.37%, respectively. As for the survival rate and body weight gain, no significant difference was found between 35 mg kg^{-1} dosage and high immune yolk antibody group ($p>0.05$). The histopathology score of the high dosage group 3 was lower than that of the high immune yolk antibody group 6. The Stimulate Index (SI) of spleen lymphocytes of chickens fed 4-hydroxycinnamic acid (SI = 1.69) was significantly higher than that of untreated chickens (SI = 1.36) ($p<0.05$). These results showed that chickens fed with 35 mg kg^{-1} 4-hydroxycinnamic acid for 5 days could effectively induce a high protection against IBDV and its related mechanism may enhance cell immunity.

Key words: 4-hydroxycinamic acid, Fructus mume, infectious bursal disease, spleen lymphocytes proliferation, screaning, spleen, China

INTRODUCTION

In the screening for antiviral effects of natural products, infectious bursal disease virus model continues to be useful *in vivo* system. Infectious bursal disease is a highly contagious disease of young chickens and it will lead to high mortality amounting to 50-70% in intensive farm and huge economical losses in poultry industries (Muller *et al*., 2003). Once the very virulent strain occurs, IBD will involve in the high mortality. In the past decades, Chinese traditional medicines and herbal extracts have been widely used against IBDV as well as other avian viruses (Sun *et al*., 2006; Gao *et al*., 2009). The antiviral activity is due to the balance of the body immune function and the improvement of the cellular responses (Sun *et al*., 2006; Huang *et al*., 2008). Unfortunately, it is unclear what elements play in the main role during the virus infection which hinders the commercialization of herbal medicines in the world. In the previous experiments, the 3-hydroxy-3-carboxy-methyl glutaric acid dimethyl ester from Fructus mume was confirmed to be effective against the infection of avian *E. coli* (Zhang *et al*., 2008). Meanwhile, the extract of Fructus mume could enhance the protection rate of chickens against IBDV. By now, 4-hydroxycinnamic acid was isolated and identified using biological tracking method and modern chromatographic methods.

MATERIALS AND METHODS

Virus strain and reagents: IBDV CJ801 was donated from professor Jue Liu, Beijing Institute of Husbandry and Veterinary Research. The LD_{50} was confirmed to be 100 ID_{50} in SPF chickens in the previous study (Liu *et al*., 2002). The following reagents, such as ethanol (EtOH, purity>99%), chloroform (purity>99%), methanol (purity>99%), silica gel (100-200 mesh) and silica GF_{254} for TLC were purchased from Sinopharm Chemical Reagent

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Co., Ltd. Polyamide (80-100 mesh) and Sephadex LH-20 were purchased from Pharmacia Company. High immune yolk antibody (No. 2009100701, 99.0% purity) was purchased from China Institute of Veterinary Drug Control.

Plant material: Fructus mume was purchased from Sichuan, China in July, 2008 and identified by the Institute of Medicinal Plant Development, Chinese Academy of Medical Sciences and Peking Union Medical College. A voucher specimen (No. 2008070815) was deposited at the Herbarium of the Institute of Medicinal Plant Development.

Extraction and isolation: Fructus mume (4.4 kg) was successively extracted with 75% EtOH (3×3000 mL, 3 h each) and yielded 0.85 kg syrup. Subsequently, the above syrup was diluted in five portions using N-hexane, chloroform, acetone, 95% EtOH and 50% EtOH, respectively. Five fractions were dried under vacuum and the acetone extract (330 g) was submitted to vacuum liquid chromatography on silica gel (11×70 cm) using a gradient of $CHCl_3$-MeOH 100:0 (3 L), 9:1 (6 L), 8:2 (4 L) and 5:5 (2L) then 150 fractions were collected and monitored by Thin Layer Chromatography (TLC). The fractions 11-50 (1100-5000 mL, 118 g) were combined and separated on a silica gel column (5×50 cm, eluted with $CHCl_3$-MeOH, flow rate 3 mL min^{-1}). Fractions of 9-21 (450-1050 mL, 570 mg) were combined and separated on a Polyamide column (2×30 cm) eluted with EtOH-H_2O, approx flow 1 mL min^{-1}. Then the fractions 8-10 (200-250 mL, 70 mg) were harvested. The final element of 4-hydroxycinnamic acid (57 mg) was purified using Sephadex LH-20 column (2×30 cm) with MeOH-H_2O at 1 mL min^{-1} flow.

Structure determination of 4-hydroxycinnamic acid: The structure was identified by Infrared (IR) spectroscopy, Electron Ionization Mass Spectrometry (EI-MS), Distortionless Enhancement by Polarization Transfer (DEPT), ^1H and ^{13}C Nuclear Magnetic Resonance (NMR) spectroscopy and 2-Dimensional (2D) NMR spectroscopy. Infrared (IR) spectra were recorded on a Nicolet MX-1 spectrophotometer and EI-MS was measured with VG ZAB-HS spectrometer. ^1H and ^{13}C NMR spectra were respectively recorded on a Bruker 400 M Hz with TMS as internal standard and $CDCl_3$ as solvent.

Chickens and protocols: All studies were performed by 3 weeks old SPF chickens (Beijing Vital Bridge Co. Ltd). All animals were maintained under pathogen-free conditions and treated in accordance with the guidelines issued by the Beijing Laboratory Animal Administration Committee on animal care. The 47 chickens were randomly divided into 6 groups. Three group chickens were administered with 35, 25 and 15 mg kg^{-1}, 4-hydroxycinnamic acid twice daily for 5 days, respectively. The positive control group was injected with 0.5 mL high immune yolk antibody for consecutive 3 days. The remaining 2 groups were used as challenge group and healthy group. All chickens were nasally challenged with 0.2 mL of 100 ID_{50} 2 days later than administered with drugs except the healthy group. Chickens of the healthy group were separately kept in minus pressure isolator. Mortality and histopathology score were recorded for 7 days post challenge.

MTT colorimetric assay: Ten SPF chickens were randomly divided into 2 groups and 1 group was orally administered with 4-hydroxycinnamic acid (40 mg kg^{-1} body weight, once daily) for 5 days while another group had no treatment.

The proliferation of spleen lymphocytes isolated from experimental chickens was determined by MTT dye [3-(4, 5-dimethyl thiazol-2-yl)-2, 5-diphenyl tetrazolium bromide] according to the method described by Mosmann (1983), Bounous *et al.* (1992) and Carmichael *et al.* (1987) with slight modifications. The assay was carried out as follows. Chicken spleen lymphocytes were isolated from spleen by Ficoll-hypague density gradient centrifugation following the manufacture's introduction. The viable cells were counted by 0.4% trypan blue dye exclusion test and the cell count was adjusted to 5×10^6 cells mL^{-1}. A pilot experiment was performed using a cell concentration of 5×10^6 cells mL^{-1} (175 μL $well^{-1}$) in 6 wells in a 96 well microculture plate, three for 25 μL, 200 μg mL^{-1} Concanavalin A, three for 25 μL culture media. The culture plate was then incubated at 37°C in a CO_2 incubator (Queue, USA) for 66 h. Then 10 μL of MTT dye (5 mg mL^{-1} of phosphate buffered saline (pH 7.2), sigma) was added to all the wells and the plate was then incubated at 37°C in a CO_2 incubator again. After 4 h, 100 μL of 10% SDS-0.01M HCl was added to all the wells to dissolve the formazan crystals and the Optical Density (OD) was measured at a test wavelength of 570 nm and reference wavelength of 630 nm on microplate reader (Multiskan MK3, Thermos Labsysterm) after the plate was placed in 37 μL incubator for at least 2 h. Stimulate index was expressed as follows:

$$SI = \frac{\text{Absorbance of Con. A-treated cells}}{\text{Absorbanceof Con. A-untreated cells}}$$

Statistical analysis: The results were expressed as the mean±SD. Statistical evaluation for differences between groups was carried out using one way Analysis of Variance (ANOVA) by SPSS for windows (SPSS 13.0; SPSS Inc., Chicago, IL, USA). A $p<0.05$ was considered significant statistically.

RESULTS AND DISCUSSION

Structure determination of the 4-hydroxycinnamic acid: The compound was isolated and purified to be a white needle crystal power with a mp. 214~217. Uvλmax (MeOH): 228, 309.IR (Kbr) cm-1:3390, 1672, 1630, 1600, 1520, 1450, 1250, 1220, 1180. EI-MS m/z (%): 164 [M]+(100), 147, 119, 107, 91, 77, 65.1H-NMR (500 M Hz, acetone-d6): 7.58 (1H, d, J = 16 Hz, H7), 7.51(2H, dd, J1 = 8Hz, J2 = 2 Hz, H2, 6), 6.87 (2 H, dd, J1 = 8 Hz, J2 = 2Hz, H3, 5), 6.30 (H, d, J = 16 Hz, H8).13C-NMR (125 MHz, acetone-d6) δ ppm: 168.3 (C9), 160.5 (C4), 145.5 (C7), 130.8 (C2, 6), 127.0 (C1), 116.6 (C3, 5), 115.8 (C8). The above data was consistent with the previous report of 4-hydroxycinnamic acid (Zhou and Li, 2006). Therefore, the active element was identified to be 4-hydroxycinnamic acid and the structure was shown in Fig. 1.

Bioactivity of 4-hydroxycinnamic acid *in vivo*: The chicken body weight gain rate was 25.68, 25.28 and 21.37%, respectively in 35, 25 and 15 mg kg^{-1} of 4-hydroxycinnamic acid compared to 31.09% in high immune yolk antibody group (Fig. 2). The body weight gain of high-dose group was significantly higher than that of challenge group ($p<0.01$) while there was no difference between the high-dose group and the high immune yolk antibody group ($p>0.05$). The survival rate was 87.5, 62.5 and 42.86%, respectively in three dosages of 4-hydroxycinnamic acid as compared to 50% of challenge group (Table 1). Both body weight gain rate and survival rate showed some dose-dependent effects during the preventive experiment. Histopathology scores of drug-treated groups were obviously lower than that of high immune yolk antibody and challenge control group even though it showed no dose-dependent effect.

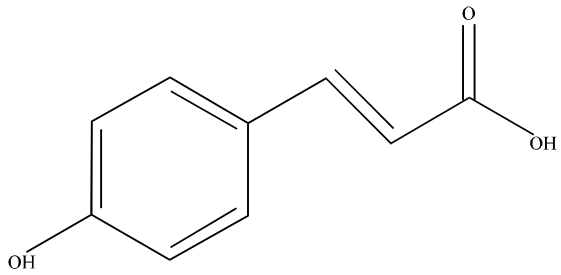

Fig. 1: Structure of 4-hydroxycinnamic acid

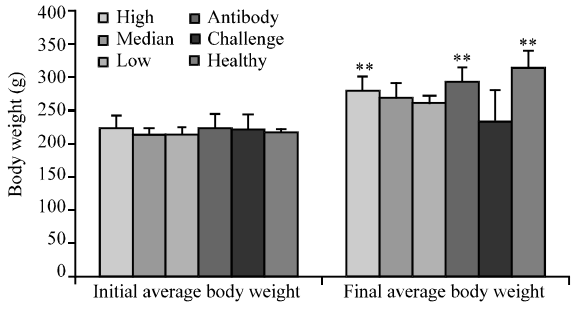

Fig. 2: Body weight change in the preventive experiment

MTT colorimetric assay: Mean±SE stimulation index of chicken fed 4-hydroxycinnamic acid or not are shown in Fig. 3. Comparison of means revealed significant ($p<0.01$) differences between the healthy control group and 4-hydroxycinnamic acid treated groups. The SI increased significantly ($p<0.01$) in the 4-hydroxycinnamic acid treated group (SI = 1.69) as compared to the healthy control group (SI = 1.36).

In the past years, the antiviral and antibiotic activities of Fructus mume have been recorded in related articles and several active elements have been identified (Perez-Alvarez *et al*., 2001; Jeong *et al*., 2006). Almost active elements are effective against bacterial infections, such as *E. coli*, Salmonella and *Pseudomonas aeruginosa* (Zhang *et al*., 2008; Kwon *et al*., 2008). Other reports even described the antiparasite infection characterized by diarrhea, fever and abdominal pains in human being (Liu *et al*., 2009). New flavonol oligoglycosides and polyacylated sucroses from Fructus mume exhibited inhibitory effects against aldose reductase and platelet aggregation (Yoshikawa *et al*., 2002). Up to now, immunity

Table 1: Survival rate, histopathology score of experimental groups

Groups	Number	No. of survive	Survive rate (%)	Histopathology score
35 mg kg^{-1}	8	7	87.50	3
25 mg kg^{-1}	8	5	62.50	1
15 mg kg^{-1}	7	3	42.86	3
Antibody group	8	8	100.00	6
Challenge group	8	4	50.00	10

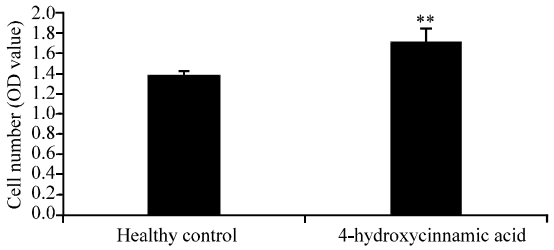

Fig. 3: Mean stimulation index of spleen lymphocytes

enhancement of 4-hydroxycinnamic acid has not been recorded although, Fructus mume beverage is widely accepted as the anti-inflammation beverage. The results in animal protection experiment against IBDV showed that 4-hydroxycinnamic acid could effectively improve survival rate of high dosage group chickens compared with the challenged group. Meanwhile, body weight gain of 4-hydroxycinnamic acid groups was significantly higher than that of challenged group and histopathology score was obviously lower. However, 4-hydroxycinnamic acid did not show expected results when chickens were inoculated with IBDV before they were fed with 4-hydroxycinnamic acid (data not shown). These results motivated us that 4-hydroxycinnamic acid might enhance chickens' cell immunity rather than kill directly IBDV. The lymphocytes proliferation determination results showed that SI values of the birds treated with 4-hydroxycinnamic acid were significantly higher than that of untreated chickens which revealed that 4-hydroxycinnamic acid possessed stronger effect on enhancing cell immunity. The ability of 4-hydroxycinnamic acid was of important clinical significance for prevention of infectious bursal disease.

CONCLUSION

It is a new method to extract effective components of traditional Chinese medicine to seek compound prescription and bioactive compound. Fructus mume is rich in organic acid and the content amounts to 40.5% (Ruan, 2008). There are phenolic acids, fatty acid, triterpenoidic acids and other organic acids in Fructus mume. We have evaluated the bioactivity of ursolic acid and some fatty acids (data not shown) and they did not improve cell immunity. There are no records related to ursolic acid to enhance the survival rate of chickens infected with IBDV by now and we easily get the idea that triterpenoidic acids in Fructus mume are not the immunopotentiator component. In another word, phenolic acids including 4-hydroxycinnamic acid are mainly preventive agent against IBDV in Fructus mume.

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