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-hydroxycinnamic acid, Fructus mume, infectious bursal disease, spleen lymphocytes proliferation, screening, 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., 2005). 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 C1801 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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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.

 Extration 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 CHCl3-MeOH-H2O (100:3:2), 9:1 (6 L), 8:2 (4 L) and 5:5 (2 L) 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 CHCl3-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-H2O, approximate flow 1 mL min⁻¹. Then the fractions 8-10 (200-250 mL, 70 mg) were harvested. The final element of 4-hydroxyinnamic acid (57 mg) was purified using Sephadex LH-20 column (2×30 cm) with MeOH-H2O at 1 mL min⁻¹ flow.

 Structure determination of 4-hydroxyinnamic acid: The structure was identified by Infrared (IR) spectroscopy, Electron Ionization Mass Spectrometry (EI-MS), Distortionless Enhancement by Polarization Transfer (DEPT), ¹H and ¹³C 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 ¹³C NMR spectra were respectively recorded on a Bruker 400 MHz with TMS as internal standard and CDCl₃ 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-hydroxyinnamic 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 LD₅₀ 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-hydroxyinnamic 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 Carnichael et al. (1987) with slight modifications. The assay was carried out as follows. Chicken spleen lymphocytes were isolated from spleen by Ficoll-hypaque 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 5×10⁸ cells mL⁻¹. A pilot experiment was performed using a cell concentration of 5×10⁸ 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 CO₂ 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 CO₂ incubator again. After 4h, 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 Labsystem) after the plate was placed in 37 µL incubator at least 2 h. Stimulate index was expressed as follows:

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SI = \frac{\text{Absorbance of Con. A-treated cells}}{\text{Absorbance of 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-hydroxyxycinnamic acid: The compound was isolated and purified to be a white needle crystal powder with a mp. 214–217. UV/IR (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 MHz, acetone-d6): 7.58 (1H, d, J = 16 Hz, H7), 7.51(2H, dd, J1 = 8 Hz, J2 = 2 Hz, H2, 6), 6.87 (2H, dd, J1 = 8 Hz, J2 = 2 Hz, H3, 5), 6.53 (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-hydroxyxycinnamic acid (Zhou and Li, 2006). Therefore, the active element was identified to be 4-hydroxyxycinnamic acid and the structure was shown in Fig. 1.

Bioactivity of 4-hydroxyxycinnamic 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-hydroxyxycinnamic acid compared to 31.06% in high immune yolk antibody group (Fig. 2). The body weight gain of high-close 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-hydroxyxycinnamic 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.

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

<table>
<thead>
<tr>
<th>Groups</th>
<th>Number</th>
<th>No. of survival</th>
<th>Survive rate (%)</th>
<th>Histopathology score</th>
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<td>3</td>
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<tr>
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<td>8</td>
<td>100.00</td>
<td>6</td>
</tr>
<tr>
<td>Challenge group</td>
<td>8</td>
<td>4</td>
<td>50.00</td>
<td>10</td>
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</tbody>
</table>

Fig. 1: Structure of 4-hydroxyxycinnamic acid

Fig. 2: Body weight change in the preventive experiment

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, triterpenoid acids and other organic acids in Fructus mume. We have evaluated the bioactivity of ursoic acid and some fatty acids (data not shown) and they did not improve cell immunity. There are no records related to ursoic acid to enhance the survival rate of chickens infected with IBDV by now and we easily get the idea that triterpenoid 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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REFERENCES


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