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การทดสอบคุณสมบัติการต้านเชื้อไวรัสของสารสกัดสมุนไพรผสมสี่ชนิดต่อเชื้อไวรัสโลหิตจางติดต่อในไก่ในเซลล์เพาะเลี้ยง

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บทคัดย่อ: เชื้อไวรัสโลหิตจางติดต่อในไก่เป็นสาเหตุของโรคโลหิตจางติดต่อในไก่ โรคนี้เป็นโรคกดภูมิคุ้มกันที่สำคัญในไก่ อย่างไรก็ตาม วัคซีนที่ได้รับการขึ้นทะเบียนไม่สามารถใช้ได้กับไก่ทุกช่วงอายุและไม่มียาต้านไวรัสที่มีประสิทธิภาพในการรักษาโรคโลหิตจางติดต่อในไก่ สมุนไพรจีนจำนวนมากถูกรายงานว่ามีฤทธิ์ต้านเชื้อไวรัส ในการศึกษานี้ สารสกัดผสมถูกทดสอบฤทธิ์ในการต้านเชื้อไวรัสโลหิตจางติดต่อในไก่ในเซลล์เพาะเลี้ยง MSB-1 ค่าความเป็นพิษต่อเซลล์ถูกทดสอบด้วยวิธี MTT สารสกัดถูกทดสอบฤทธิ์ทั้งในระยะก่อนที่มีการติดเชื้อเข้าสู่เซลล์ และหลังจากติดเชื้อเข้าสู่เซลล์แล้วเป็นเวลา 30 นาที 1, 2 และ 4 ชั่วโมง ผลการทดสอบความเป็นพิษ พบว่าความเข้มข้นที่ทำให้เซลล์ตายไป 50% ของเซลล์ทั้งหมดคือ 84.77 ไมโครกรัม/มิลลิลิตร ผลการต้านไวรัสก่อนที่มีการติดเชื้อ พบว่า สารสกัดที่ความเข้มข้น 15.625 ไมโครกรัม/มิลลิลิตร สามารถยับยั้งเชื้อไวรัสได้หลังจากบ่มเป็นเวลา 2 และ 4 ชั่วโมง โดยพบการลดลงของปริมาณเชื้อไวรัสอย่างมีนัยสำคัญทางสถิติ ในขณะที่ ไม่ผบผลการต้านไวรัสหลังจากติดเชื้อเข้าสู่เซลล์ ฤทธิ์ต้านเชื้อไวรัสของสารสกัดชนิดเดียว ในการยับยั้งการติดเชื้อนั้นได้ถูกอธิบายแล้ว อย่างไรก็ตาม กลไกการเสริมฤทธิ์หรือการรบกวนการต้านเชื้อของสารสกัดผสมนั้นยังไม่ถูกศึกษา

คำสำคัญ: สารสกัดสมุนไพร การต้านเชื้อไวรัส ไวรัสโลหิตจางติดต่อในไก่

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Anti-viral Activity of Four Mixed Herbal Extracts Against Chicken Anemia Virus (CAV) in Cell Lines

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Abstract: Chicken anemia virus (CAV) causes chicken infectious anemia (CIA), the major immunosuppressive disease in chickens. However, licensed vaccines could not be used for all ages of chickens, and there are no effective anti-viral drugs to treat CIA. Chinese medicinal herbs have been reported for anti-viral activity. In this present study, mixed extracts were tested for anti-CAV activities in the MSB-1 cell line. The cytotoxicity was carried out through an MTT assay. The extracts were tested for pre- and post-infection anti-viral activities at 30 min, 1, 2, and 4 hrs. The results showed that 50% cytotoxic concentration was 84.77 µg/ml. Our pre-treatment results demonstrated that the mixed extracts (15.625 µg/ml) showed anti-viral activity against CAV in vitro with incubated 2 to 4 hrs and significantly reduced virus titers. We suggested that these extracts act during the adsorption phase. On the other hand, the post-treatment results exhibited that the mixed extracts did not have anti-viral activity. The potent anti-viral effect of a single active component was implicated. However, their anti-viral action's possible synergistic or interference effect mechanisms of mixed compounds have not been studied yet.

Keywords: Anti-viral activity, Herbal extract, Chicken anemia virus

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Introduction

Chicken anemia virus (CAV) is a member of the Anelloviridae family. It could be vertically transmitted to young chicks, and the virus is also spread through feces to older chickens. CAV is economically important because they cause immunosuppressive disease, Chicken infectious anemia (CIA). The disease affects the poultry industry worldwide and cannot treat by any medicine. It has been reported that CIA caused 14 to 24% loss in broiler flocks (Davidson et al., 2004). The vaccine is only one way to protect chickens from the virus, but it still found limitation; not all age was safe to use. Some herbal extracts were reported, and they have immunomodulation activity against viral infection in poultry (Lv et al., 2019). Herbal-based medicine is traditionally practiced in Asia for its therapeutic efficacies against various diseases, including viral

infections. However, few traditional compounds were documented to have indirect anti-CAV activity (Latheef et al., 2017). Therefore, it is necessary to find direct anti-viral compounds against CAV. *Coptidis Rhizoma* dose-dependently inhibited hepatitis C virus infection in vitro by blocking the viral attachment and entry into Huh-7.5 cells (Hung et al., 2018). *Scutellariae Radis* has anti-tumor and antibacterial activity. *Radix Astragali* has an antioxidant activity. *Cnidii Fructus* has been proven by USDA, which has anti-parasitic activity (Yu et al., 2022). This study assessed the anti-viral effects of four herbal preparations, namely *Coptidis Rhizoma, Scutellariae Radis, Radix Astragali, and Cnidii Fructus* mixed extract in resisting the viral replication by CAV in MSB-1 cell.

Materials and methods

Cell line

The MDCC-MSB1 cell line was provided by the poultry disease laboratory of the Veterinary medicine department National Pingtung University of Science and Technology University. MSB-1 cells (1 mL) were added to a 25 cm^2 culture flask (5×10^5 cells/mL), and fresh RPMI-1640 culture solution (10%FBS) was added to 5 mL. The culture was conducted at 37°C, 5% CO_2, and 85% humidity.

Virus

The CAV isolate 1537TW (GenBank access number MT795930) was provided by our laboratory and stored at -80 °C. Virus titers were determined by the Reed-Muench method and expressed as a tissue culture infectious dose of 50% ($TCID_{50}$) (10^3 $TCID_{50}$).

Tested compound

The mixed extracts were supplied from Zhongchengbencao Co., Ltd., Taiwan. The tested compound of *Coptidis Rhizoma*, *Scutellariae Radis*, *Radix Astragali*, and *Cnidii Fructus* were dissolved in sterile water. The stock solutions were filtered through a 0.22 μm millipore membrane filter and stored at 4°C for further use.

Cytotoxicity test of mixed extracts against MSB-1

The cytotoxicity test of mixed extracts against MSB-1 was performed. The tested compound was diluted in a 2-fold-series from 1000, 500, 250, 125, 62.5, 31.3, 15.6, and 7.8 μg/mL, respectively, at each concentration was added to a 96 well plate (50 μL/well) in duplicate. Freshly MSB-1 cells were suspended and adjusted to 5×10^3 cells/mL, then added to the same plate (50 μL/well) to mix with the tested compound at different concentrations. RPMI-1640 control and cell control were prepared in the same way. The cells were incubated under 5% CO_2 at 37 °C for 72 h. After that, the medium was removed, and 20 μL of 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT) solution was added to each well with a final MTT concentration of 5 mg/mL. After additional 4 h incubation, the solution was removed, and the cell medium was replaced with 100 μL DMSO. The plate was shaken for 10 minutes, and absorbance was measured on a

microplate reader at 570 nm to determine the viability relative to the untreated control. Each compound's 50% cytotoxic concentration (CC50) was calculated by nonlinear regression analysis using GraphPad Prism 5 software. The maximum non-cytotoxic concentration (MNTC) was defined as the concentration of each compound required to maintain cell viability with no significant difference compared with the cell control ($p < 0.05$)

Pre-treatment assay

MSB-1 cell suspensions (10^5 cells/mL) were seeded into 24 well plates (500 μL/well) and incubated in a 5% CO_2 humidified incubator at 37°C. Serial two-fold dilutions of each compound were added and incubated for 30 minutes, 1, 2, and 4 hours. After that, the medium-containing compound was removed; 100 $TCID_{50}$ CAV was added and incubated for 2 h. Next, the virus inocula was removed, and fresh media was added and incubated for another 72 h.

Post-treatment assay

An MSB-1 cell in 24 well plates was pre-incubated with 100 $TCID_{50}$ CAV for 2 h in a 5%CO_2 humidified incubator at 37°C. The medium was then removed, and fresh RPMI-containing compound was added and incubated for 30 min, 1, 2, and 4 h. Serial two-fold dilutions diluted each compound with RPMI. The compound solution was then removed, and fresh media was added and incubated for 72 h.

Determination of viral load by real-time time PCR

After 72 h continuous incubation, the viral suspension was harvested and 3 freeze-thaw cycles for qPCR assays. Total DNA extraction from the cell culture supernatants was performed using the DNeasy®blood and tissue kit (Qiagen, Germany). The CAV DNA was amplified using qPCR (Kaffashi et al., 2006). To quantify the viral load of each sample, a standard curve was made using LightCycler® 480 (Roche, Switzerland) (Tongkamsai et al., 2019). The amount of DNA in each sample was used, expressed as viral concentration.

Statistical Analysis

Statistical analysis of all data was performed using GraphPad Prism 5 software. The data of each group treated with mixed extracts were compared using a one-way analysis of variance of mean comparison. All values were expressed as means±SD. $P<0.05$ was considered a significant difference. All results in the study represent four repeatabilities.

Results

Maximum non-toxic dose of mixed extracts to MSB-1 cells

The MTT assay was utilized to assess the drug- induced toxicity to optimize the concentration of the mixed extract. As a result, as mixed extracts concentration decreased from 62.5 ug/ml to 31.25 ug/ml, the cell survival rate gradually increased from 63.38% to 80.65%, and a further decrease in concentration did not induce significant cytotoxicity. Thus, 15.62 ug/ml was considered a safe concentration for mixed extracts and could be used for the following studies (Table 1). According to the result, the

Table 1 Effect of different concentrations of mixed extracts on MDCC-MSB1 cells'viability (mean±SD, n=5)

Group (µg/ml)	Samples	Cell viability (%)
1000	5	21.55 ± 0.98
500	5	21.67 ± 0.69
250	5	22.22 ± 2.17
125	5	22.96 ± 0.86
62.5	5	63.38 ± 9.45
31.25	5	80.65 ± 7.67
15.625	5	100.42 ± 10.16
7.8125	5	103.06 ± 17.45
Negative control	5	100.67 ± 1.04

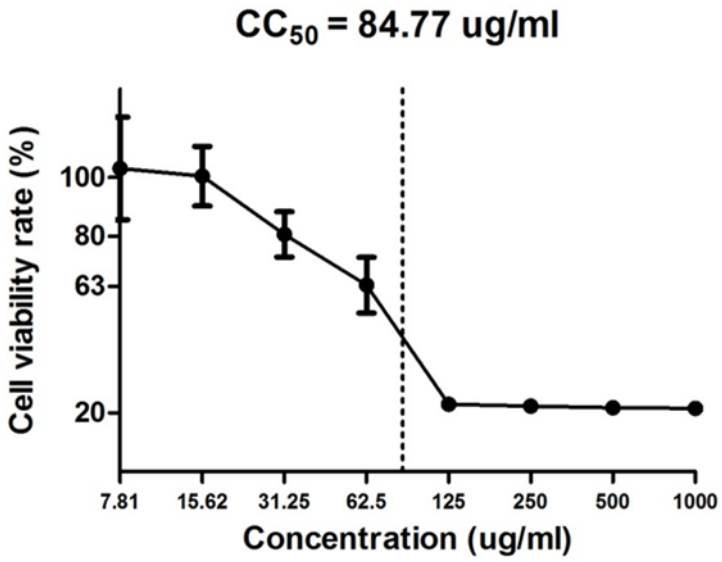

Figure 1 Dose-response curves of tested compounds in cytotoxicity assay.

CC50 of the tested compound was 84.77 ug/ml, and the MNT was 15.62 ug/ml. In addition, the tested compound was shown to have an" S" shape of the dose- response curve; with increasing compound concentration, the viability decreased (Fig.1).

Mixed extracts inhibit CAV proliferation in vitro

The mixed extracts were diluted using cell growth medium from the maximum non-toxic dose to two levels: 15.625 and 7.8125 µg/ml. The results are shown in Fig. 2A. Incubated infected cells with the dosing of 7.8125 µg/ml had no significant inhibitory effect on CAV adsorption ($P>0.05$). However, at 4 hours incubated cells with the dosing of 15.625 ug/ml of mixed extracts could significantly inhibit CAV adsorption in MSB1-cells compared with the negative control ($P<0.05$). And at 2 hours,

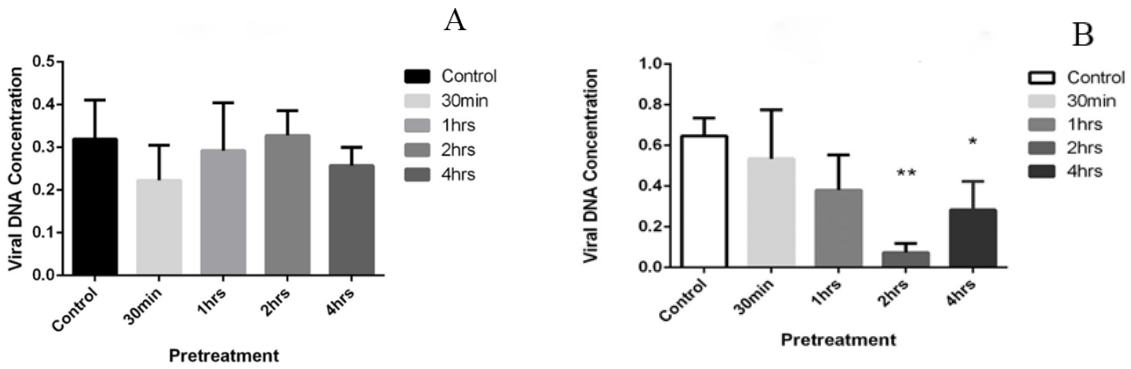

Figure 2 The inhibition of CAV adsorption. A: 7.8125 µg/ml, B: 15.625 µg/ml.

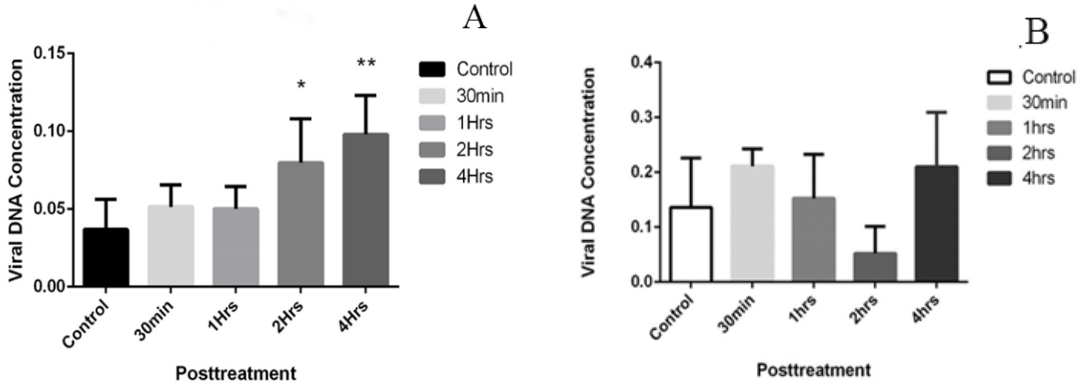

Figure 3 The inhibition of CAV replication. A: 7.8125 µg/ml, B: 15.625 µg/ml.

incubated with the same dose could strong significantly effective (P<0.001) (Fig 2B).

The effect of mixed extracts on viral replication was determined by using MSB-1 cells post-treated with the tested compound. Under the post- treatment condition, 7. 8125 µg/ ml concentration exerted significant reduction at 2 hours of incubation onwards (Fig 3A.) While at a concentration of 15.625 µg/ml. had no significant anti-CAV effect on viral replication (P>0.05) (Fig 3B).

Discussion

Chicken infectious anemia causes the severe economic problem, and immune-suppression makes the disease a concern. Traditionally, herbs have been used against various viral diseases, including chicken disease (Lelesius et al., 2019). Therefore, herb-based anti-viral drugs might be an alternative way to treat this immunosuppressive disease. The present study tested mixed extracts composed of *Coptidis Rhizoma*, *Scutellariae Radis*, *Radix Astragali*, and *Cnidii Fructus* were tested for their anti-viral activity against CAV. The 15.625 and 7.8125 ug/ml were selected for this study. Before establishing the anti-viral activities of mixed extracts, cytotoxicity is tested to determine the concentration that can be used. In this study, we reported cytotoxicity as CC50, the CC50 of mixed extracts was 84.77 ug/ml. Three of four high-

possible herbs extracts were found to be *R. astragalis*, *C. rhizoma* and *S. radix* extracts, with CC50 of 338, 1,031 and 370 ug/ml, respectively (Jia et al., 2010; Shi et al., 2016; Wang et al., 2009). Probably different cell lines in the assays indicated a difference in CC50. Therefore, two non-toxic concentrations from the mixed extract were incubated separately. There has been only one report on the anti-CAV properties of mixed extracts in vivo. There was no publication on the in vitro anti-CAV study with mixed extracts. Here, the dose of 15.625 ug/ml, with 2 or 4 h after challenge incubation in pre-treatment, had an inhibitory effect, that mixed extracts could block CAV absorption to MSB-1 cells. Previously, they investigated the inhibitory effect of *R. astragali* on the hepatitis-B virus in HepG2 2.2.15 cells. They reported that *R. astragali* reduced hepatitis-B antigen secretion dose-dependently (Wang et al., 2009). While *C. fructus* extract has been single or combination used as an anti-pruritic agent (Zhong et al., 2017). Another report shows that *C. rhizoma* significantly reduced respiratory syncytial virus titers compared to untreated control in Hep2 cells (Lee et al., 2017). *S. radix* presented that it may inhibit HIV-1 absorption by interfering with viral binding (Li et al., 2000). The anti-viral effect of each herb was examined, similar to our report. In addition, the 7.8125 ug/ml dose, with 2 or 4 h after challenge incubation in post-treatment, had the stimulatory effect that mixed extracts may act MSB-1cell highly permissive to CAV. The previous study showed that *S. radix* did not affect viral replication (Li et al., 2000). In conclusion, the mechanism of anti-CAV activity of mixed extracts shown in the present study needs to be further explored. However, the active compounds have not yet been identified. Further studies are required to investigate the exact mechanism of their anti-viral activity, purification, and characterization of their active compounds.

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