Effects of Star Anise and Eucommia Leaf Extracts on Growth Performance, Serum Enzyme Activity, and Hepatic Tumor Necrosis Factor-α Distribution and Expression in Weaned Piglets (Postprint)

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Abstract

This study aimed to investigate the effects of dietary supplementation with star anise and Eucommia ulmoides leaf extracts on growth performance, serum enzyme activities, and hepatic tumor necrosis factor-α (TNF-α) distribution and expression in weaned piglets. A single-factor experimental design was adopted, in which 48 healthy "Duroc × Landrace × Large White" weaned piglets were selected and randomly assigned to 4 groups with 3 replicates per group and 4 piglets per replicate. The control group was fed a basal diet, while the experimental groups were supplemented with star anise extract (500 mg/kg), Eucommia ulmoides leaf extract (250 mg/kg), and chlortetracycline (50 mg/kg) to the basal diet, respectively. The pre-trial period lasted 7 days, and the formal trial period lasted 42 days. The results showed that compared with the control group, dietary supplementation with star anise extract, Eucommia ulmoides leaf extract, and chlortetracycline significantly increased the average daily gain of weaned piglets ($P<0.05$), while dietary supplementation with star anise and Eucommia ulmoides leaf extracts significantly decreased serum alanine aminotransferase, alkaline phosphatase activities, and hepatic TNF-α mRNA relative expression ($P<0.05$). TNF-α immunopositive results were mainly observed in the interlobular areas and hepatic sinusoids of the liver. It can be concluded that dietary supplementation with star anise and Eucommia ulmoides leaf extracts can improve growth performance in weaned piglets and possess the ability to resist hepatic inflammatory responses and oxidative stress.

Full Text

Effects of Illicium verum and Eucommia Leaf Extracts on Growth Performance, Serum Enzyme Activity and Distribution and Expression of Tumor Necrosis Factor-α in the Liver of Weaned Piglets

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Abstract: This study investigated the effects of dietary supplementation with Illicium verum and Eucommia leaf extracts on growth performance, serum enzyme activity, and the distribution and expression of tumor necrosis factor-α (TNF-α) in the liver of weaned piglets. Using a single-factor experimental design, 48 healthy "Duroc × Landrace × Large White" weaned piglets were randomly allocated into four groups, with three replicates per group and four piglets per replicate. The control group received a basal diet, while the experimental groups received the basal diet supplemented with Illicium verum extract (500 mg/kg), Eucommia leaf extract (250 mg/kg), or aureomycin (50 mg/kg), respectively. The trial consisted of a 7-day adaptation period followed by a 42-day experimental period. The results demonstrated that compared with the control group, dietary supplementation with Illicium verum extract, Eucommia leaf extract, and aureomycin significantly increased average daily gain ($P<0.05$). Supplementation with Illicium verum and Eucommia leaf extracts significantly reduced serum glutamic-pyruvic transaminase and alkaline phosphatase activities, as well as hepatic TNF-α mRNA relative expression levels ($P<0.05$). Immunohistochemical staining for TNF-α was predominantly observed in the interlobular regions and hepatic sinusoids of the liver. These findings indicate that dietary supplementation with Illicium verum and Eucommia leaf extracts can improve growth performance in weaned piglets and enhance their capacity to resist hepatic inflammatory responses and oxidative stress.

Keywords: Illicium verum extract; Eucommia leaf extract; weaned piglets; growth performance; serum enzymes

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Introduction

In recent years, driven by heightened concerns over meat product safety, the need for sustainable livestock development, and the negative effects of antibiotics, the development of "safe, efficient, stable, and controllable" novel feed additives has become a research hotspot worldwide. Plant extracts, with their advantages of safety, efficiency, lack of residue, and no induction of drug resistance, have been widely applied in livestock production as a new type of green

antibiotic alternative [1-2]. Plant extracts not only possess antimicrobial [3] and antioxidant [4] properties but also enhance animal immunity [5], promote growth [6], improve intestinal microbial environments [7], and enhance meat quality [8]. Both Illicium verum and Eucommia possess multiple biological functions and are abundant resources in China. In vitro studies have demonstrated that extracts from Illicium verum and Eucommia leaves exhibit significant effects in antibacterial activity [9-10], antioxidant capacity [11-12], and immune enhancement [13-14]. However, the effects of these extracts on the distribution and expression of nuclear factor erythroid 2-related factor 2 (Nrf2) and tumor necrosis factor-α (TNF-α) in the liver of different breeds of weaned piglets have not been reported. Therefore, this study aimed to investigate the effects of Illicium verum and Eucommia leaf extracts on growth performance, serum enzyme activity, and hepatic TNF-α distribution and expression in weaned piglets, providing a scientific basis for their application in livestock production.

1. Materials and Methods

1.1 Experimental Materials **Illicium verum extract:** Extracted by the Animal Nutrition Research Laboratory of Shandong Agricultural University using ethanol extraction. Main components: trans-anethole $ $55.50%, chavicol $ $6.17%, and anisaldehyde $ $4.56%.

Eucommia leaf extract: Provided by Shandong Longchang Animal Health Products Co., Ltd. Main components: Eucommia polysaccharides $ $20.00%, flavonoids $ $8.00%, and chlorogenic acid $ $5.00%.

Antibiotic: Aureomycin, provided by Shandong Yuzeyinfen Animal Pharmaceutical Co., Ltd.

1.2 Experimental Design A single-factor experimental design was employed. Forty-eight 28-day-old "Duroc × Landrace × Large White" weaned piglets were randomly divided into four groups with three replicates per group and four piglets per replicate, with no significant difference in body weight among groups ($P>0.05$). The basal diet was formulated according to the Chinese "Feeding Standard of Swine" [15], with composition and nutrient levels shown in Table 1 . The control group received the basal diet only, while the experimental groups received the basal diet supplemented with 500 mg/kg Illicium verum extract (Illicium group), 250 mg/kg Eucommia leaf extract (Eucommia group), or 50 mg/kg aureomycin (antibiotic group). The trial included a 7-day adaptation period and a 42-day experimental period, during which piglets had ad libitum access to feed and water.

Table 1 Composition and nutrient levels of the basal diet (air-dry basis) %

Items	Content
Ingredients	

Items	Content
Corn	61.00
Wheat middling	5.00
Soybean meal	24.00
DDGS	3.00
Soybean oil	2.00
Premix[1]	5.00
Total	**100.00**
Nutrient levels[2]	
DE/(MJ/kg)	14.27
CP	20.00
Ca	0.80
AP	0.40
Lys	1.30
Met	0.40
Met+Cys	0.74
Thr	0.80
Try	0.23

[1] The premix provided the following per kg of diet: VA 3,300 IU, VD_3 330 IU, VE 24 IU, VK 2.00 mg, VB_1 0.75 mg, VB_2 5.25 mg, VB_6 2.25 mg, VB_{12} 0.026 mg, D-pantothenic acid 15.00 mg, biotin 0.075 mg, folic acid 0.45 mg, niacin 22.50 mg, Cu ($CuSO_4 \cdot 5H_2O$) 9.00 mg, Zn ($ZnSO_4 \cdot H_2O$) 150 mg, Se (Na_2SeO_3) 0.45 mg, I (KI) 0.21 mg, Mn ($MnSO_4 \cdot H_2O$) 6.00 mg, Fe ($FeSO_4 \cdot H_2O$) 150 mg.

[2] CP was a measured value, while other nutrient levels were calculated values.

1.3 Experimental Procedures 1.3.1 Growth Performance

Daily feed intake was recorded for each group, and body weight was measured weekly to calculate average daily feed intake (ADFI), average daily gain (ADG), and feed-to-gain ratio (F/G).

1.3.2 Blood Collection, Processing, and Serum Enzyme Activity Determination

On day 43 of the experiment, one piglet per replicate was randomly selected before morning feeding. Blood samples (15 mL) were collected using vacuum coagulation-promoting tubes, left to stand in a 37°C water bath for 10 minutes, then centrifuged at 3,000 r/min for 10 minutes to separate serum, which was stored at -20°C until analysis. Serum activities of glutamic-pyruvic transaminase (ALT), glutamic-oxaloacetic transaminase (AST), and alkaline phosphatase (ALP) were measured using an automatic biochemical analyzer (COBAS MIRA Plus, Roche, Switzerland).

1.3.3 Piglet Slaughter and Sample Collection

After blood collection, piglets were euthanized by electric shock. Following exsanguination, the thoracic and abdominal cavities were opened, and the liver was aseptically removed. One portion of liver tissue was immediately fixed in Bouin' s solution, while another portion was placed in a cryovial and stored in liquid nitrogen at -80°C.

1.3.4 Immunohistochemistry (Strept Avidin-Biotin Complex Method)

Liver tissue fixed in Bouin' s solution was dehydrated through graded ethanol series, cleared in xylene, and embedded using a BMJ23 embedding machine. Sections (5 m) were cut using a microtome (LEICA RM2135, Germany), dewaxed, and rehydrated. Antigen retrieval was performed using citrate buffer (0.01 mol/L, pH 6.0), followed by three washes with phosphate-buffered saline (PBS) (0.01 mol/L, pH 7.2) for 5 minutes each. Endogenous peroxidase activity was blocked by incubating sections with 3% hydrogen peroxide (H_2O_2) at room temperature in the dark for 30 minutes, followed by three PBS washes. Sections were then blocked with 10% fetal bovine serum at 37°C for 1 hour. Primary rabbit anti-TNF-α polyclonal antibody (bs-0078R, Beijing Biosynthesis Biotechnology Co., Ltd.) was applied at 1:50 dilution and incubated overnight at 4°C, followed by three PBS washes. Biotinylated goat anti-rabbit immunoglobulin G secondary antibody (bs-0295Gs, Beijing Biosynthesis Biotechnology Co., Ltd.) was applied at 1:50 dilution and incubated at 37°C for 1 hour, followed by three PBS washes. Horseradish peroxidase-conjugated streptavidin (1:100) was applied and incubated at 37°C for 45 minutes, followed by three PBS washes. Color development was achieved using diaminobenzidine (DAB) with microscopic monitoring to control reaction time. Sections were counterstained with hematoxylin, dehydrated, cleared, mounted, and observed under a microscope to determine the distribution pattern of immunopositive cells.

1.3.5 Determination of TNF-α mRNA Relative Expression

Liver samples (50-100 mg) stored at -80°C were used for total RNA extraction following the Trizol reagent kit protocol (Invitrogen, USA). RNA quality and concentration were assessed using a UV spectrophotometer, with all samples showing OD values between 1.8 and 2.0. Extracted RNA was immediately reverse-transcribed using the PrimeScript® RT Master Mix Perfect Real Time kit according to the manufacturer' s instructions. Fluorescent quantitative PCR was performed in a 20 L reaction volume with the following cycling conditions: pre-denaturation at 95°C for 30 seconds, 40 cycles of denaturation at 95°C for 5 seconds and annealing/extension at 60°C for 34 seconds, followed by a final step at 95°C for 15 seconds and 60°C for 60 seconds with fluorescence detection. Each sample was run in triplicate, and results were analyzed using the $2^{-\Delta\Delta Ct}$ method to determine relative TNF-α mRNA expression levels in the liver.

Table 2 Primer sequences

Genes	Accession No.	Primer sequences (5' —3')	Product length/bp
Glyceraldehyde-3-phosphate dehydrogenase			
GAPDH	NM_{001206359}.1	F: GAAGGTCGGAGTGAACGCATR: CCTGGGTCGAATCATACTGGAACA	146
Tumor necrosis factor-α			
TNF-α	NM_{214022}.1	F: CGTGAAGCTGAAAGACAACCAGR: GATGGTGTGAGTGAGGAAAACG	114

1.4 Data Processing Experimental data were analyzed using SAS 9.3 software. Differences among treatments and piglet breeds were analyzed using two-factor analysis of variance, with Duncan' s multiple range test used for pairwise comparisons. Data are expressed as means and SEM, with $P<0.05$ considered statistically significant.

2. Results

2.1 Effects of Illicium verum and Eucommia Leaf Extracts on Growth Performance of Weaned Piglets As shown in Table 3 , there were no significant differences in average daily feed intake among the control, Illicium, Eucommia, and antibiotic groups ($P>0.05$). However, average daily gain was significantly higher in the Illicium, Eucommia, and antibiotic groups compared with the control group ($P<0.05$). No significant differences in feed-to-gain ratio were observed among groups ($P>0.05$), though the Illicium and antibiotic groups showed a decreasing trend.

Table 3 Effects of Illicium verum and Eucommia leaf extracts on growth performance of weaned piglets

Items	Control group	Illicium verum group	Eucommia group	Antibiotics group	P-value
ADG/(kg·d)	0.52	0.59	0.58	0.58	<0.05

Items	Control group	Illicium verum group	Eucommia group	Antibiotics group	P-value
ADFI/(kg/d)	1.96	1.00	0.99	0.99	>0.05
F/G	1.85	1.69	1.71	1.71	>0.05

In the same row, values with different superscript letters indicate significant difference (P<0.05), while values with the same or no superscript letters indicate no significant difference (P>0.05). The same applies below.

2.2 Effects of Illicium verum and Eucommia Leaf Extracts on Serum Enzyme Activity of Weaned Piglets As shown in Table 4 , serum ALT activity was significantly lower in the Illicium and Eucommia groups compared with the control and antibiotic groups (P<0.05). No significant differences in serum AST activity were observed among groups (P>0.05). Serum ALP activity was significantly reduced in the Illicium, Eucommia, and antibiotic groups compared with the control group (P<0.05).

Table 4 Effects of Illicium verum and Eucommia leaf extracts on serum enzyme activity of weaned piglets (U/L)

Items	Control group	Illicium verum group	Eucommia group	Antibiotics group	P-value
ALT	23.55	18.06	19.11	24.24	<0.05
AST	45.33	46.00	44.67	45.67	>0.05
ALP	94.47	84.35	82.01	85.73	<0.05

2.3 Effects of Illicium verum and Eucommia Leaf Extracts on TNF-α Distribution in Liver of Weaned Piglets Immunohistochemical analysis revealed that TNF-α immunopositive staining in the liver of weaned piglets appeared yellow, brownish-yellow, or brown, while negative cells showed no coloration, confirming the specificity of the SABC method used in this study.

As shown in Figure 1 [Figure 1: see original paper], TNF-α showed strong positive staining with relatively uniform distribution in hepatic lobules. Based on staining intensity and distribution patterns, the antibiotic group exhibited the strongest immunopositive reaction, primarily localized in hepatocyte cytoplasm. The staining intensity in other groups, in descending order, was control group, Illicium group, and Eucommia group, with positive signals mainly distributed in hepatic sinusoidal endothelium and hepatocyte membranes.

A, C, E, and G are low-magnification images; B, D, F, and H are high-magnification images. I: central vein; J: interlobular connective tissue; K: interlobular artery; L: interlobular bile duct; M: interlobular vein; N: hepatocytes; O: hepatic sinusoid. Red arrows indicate TNF-α-positive cells; green arrows

indicate TNF-α-negative cells. Control: control group; Illicium: Illicium verum group; Eucommia: Eucommia group; Antibiotics: antibiotics group.

Figure 1 Effects of Illicium verum and Eucommia leaf extracts on liver TNF-α distribution of weaned piglets

2.4 Effects of Illicium verum and Eucommia Leaf Extracts on TNF-α mRNA Expression in Liver of Weaned Piglets As shown in Figure 2 [Figure 2: see original paper], hepatic TNF-α mRNA relative expression was significantly lower in the Illicium and Eucommia groups compared with the control group ($P<0.05$), while no significant difference was observed in the antibiotic group ($P>0.05$).

Columns with different letters differ significantly ($P<0.05$).

Figure 2 Effects of Illicium verum and Eucommia leaf extracts on liver TNF-α mRNA relative expression level of weaned piglets

3. Discussion

3.1 Effects of Illicium verum and Eucommia Leaf Extracts on Growth Performance of Weaned Piglets Song Zhenshuai [16] reported that Illicium powder (10 g/kg) had no significant effect on average daily gain or feed intake in finishing pigs but improved feed conversion ratio. Li Xiaomei et al. [17] demonstrated that dietary Eucommia supplementation (250 mg/kg) improved average daily gain and feed conversion ratio. In the present study, dietary supplementation with Illicium verum extract (500 mg/kg) and Eucommia leaf extract (250 mg/kg) enhanced growth performance in weaned piglets, as evidenced by increased average daily gain. Both extracts possess antibacterial and anti-inflammatory properties [18-20], which may improve growth performance by inhibiting competition for nutrients from harmful pathogens and enhancing nutrient digestion and absorption [21]. Additionally, these extracts contain components that inhibit acetylcholinesterase (AchE), which may excite gastrointestinal smooth muscle, promote intestinal motility, and increase gastric secretion [22-23], representing another potential mechanism for improved growth performance. However, the molecular mechanisms underlying these effects require further investigation.

3.2 Effects of Illicium verum and Eucommia Leaf Extracts on Serum Enzyme Activity of Weaned Piglets The liver is a vital immune organ with detoxification and hematopoietic functions. Hepatic injury from inflammatory responses or drug enzyme inducers can increase hepatocellular enzyme secretion, making serum ALT, AST, and ALP activities important indicators of liver function and stress status [24-25]. ALT participates in transamination between glutamate and pyruvate, while AST catalyzes transamination between glutamate and oxaloacetate [26]. These enzymes facilitate amino acid synthesis through deamination and transamination, promoting protein synthesis capacity.

Their activity levels reflect protein metabolism status and correlate positively with average daily gain in piglets [27]. ALP plays a crucial role in bone formation and participates in lipid and protein metabolism [28]. Wang Dingfa et al. [29] reported that Piper sarmentosum extract (50 mg/kg) reduced serum ALT and AST activities. Liu Hejun et al. [30] found that Acanthopanax senticosus extract (1,000 mg/kg) decreased serum ALP activity. In this study, both Illicium and Eucommia groups showed reduced serum ALT and ALP activities compared with the control group, consistent with previous findings and suggesting hepatoprotective and anti-inflammatory effects. However, the cellular mechanisms regulating serum ALT, AST, and ALP activities by these extracts require further elucidation.

3.3 Effects of Illicium verum and Eucommia Leaf Extracts on TNF-α Distribution and Expression in Liver of Weaned Piglets Tumor necrosis factor is a critical inflammatory regulator that induces release of superoxide anions and nitric oxide (NO) and promotes secretion of other inflammatory cytokines such as interleukin-6 (IL-6) and interleukin-8 (IL-8), thereby participating in disease pathogenesis [31-32]. Xu Lamei et al. [33] reported that TNF-α immunopositivity in rat liver was primarily distributed in hepatocyte cytoplasm, with some distribution in mononuclear cells and few portal areas, consistent with our findings. TNF-α mRNA expression is significantly upregulated in many inflammatory processes [34-35]. In this study, the antibiotic group showed the highest TNF-α mRNA expression, indicating potential hepatic damage from antibiotics. The Eucommia group exhibited the lowest TNF-α mRNA expression, suggesting anti-inflammatory effects that may be attributed to chlorogenic acid in Eucommia leaf extract. Chlorogenic acid can inhibit activation of nuclear factor- B (NF- B) and Toll-like receptor 4 (TLR4) signaling pathways, thereby suppressing expression of inflammatory cytokines including TNF-α, IL-1β, and IL-6 [36]. The distribution and expression patterns of TNF-α in the liver were consistent in this study; however, which components in Illicium verum and Eucommia leaf extracts play dominant roles and their molecular mechanisms affecting TNF-α distribution and expression require further investigation.

4. Conclusions

1. Dietary supplementation with Illicium verum and Eucommia leaf extracts improved average daily gain in weaned piglets.
2. Dietary supplementation with Illicium verum and Eucommia leaf extracts reduced serum ALT and ALP activities.
3. Dietary supplementation with Illicium verum and Eucommia leaf extracts decreased hepatic TNF-α mRNA relative expression levels.

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Note: Figure translations are in progress. See original paper for figures.