

Influences of dietary *Eucommia ulmoides* extract on growth, flesh quality, antioxidant capacity and collagen-related genes expression in grass carp (*Ctenopharyngodon idellus*)

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Abstract

Background: The study investigated the effects of dietary *Eucommia ulmoides* extracts on growth, flesh quality and collagen-related genes expression of grass carp (*Ctenopharyngodon idella*). *Eucommia ulmoides* bark extract (EBE) and *Eucommia ulmoides* leaf extract (ELE) were individually supplemented into basal diet (control) at an inclusion of 4 g/kg, and then the three diets were fed to grass carp (59.7 ± 0.3g) for 60 days.

Results: The results indicated that dietary ELE significantly increased weight gain (+4.22%), and decreased feed conversion ratio (-0.07) ($P < 0.05$), while EBE did not significantly affect the growth performance of grass carp. Compared to the control, dietary ELE increased apparent digestibility coefficient of dry matter, crude protein and intestinal digestive enzyme activity ($P < 0.05$), and the contents of calcium, total collagen, heat-insoluble collagen and free amino acids level (including delicious amino acids) in flesh were also increased ($P < 0.05$). In both extracts groups, the flesh centrifugal loss, steaming loss, drip loss^{24h}, protein carbonyl and malondialdehyde were significantly lower, and superoxide dismutase and glutathione peroxidase activities were higher than the control ($P < 0.05$). No significant differences in the amount of saturated, monounsaturated and polyunsaturated fatty acids in flesh were found among the three groups, but DPA, DHA, n-3 polyunsaturated fatty acids and n-3/n-6 ratio in flesh were increased by dietary EBE and ELE ($P < 0.05$). In collagen-related genes expression, the ELE supplementation significantly promoted the expression of collagen type I (COL1A1, COL1A2), proline hydroxylase, lysine oxidase in muscle, while the expression of matrix metalloproteinase-2 and matrix metalloproteinase-9 was decreased ($P < 0.05$).

Conclusion: In conclusion, dietary ELE improved the growth, muscle antioxidant capacity and flesh quality of grass carp.

Background

The development of intensive aquaculture produced a large amount of aquatic foods for human, but on the other hand, it also brought water pollution and the increased harmful microorganisms, which damaged the health and adversely affected the growth and flesh quality of fish [1]. Antibiotics have been banned or strictly restricted in various countries for its accumulation in flesh and drug resistance, thus herbal plants are becoming effective alternatives for antibiotics [2]. As a traditional herb widely used in East Asia, *Eucommia ulmoides* (EU) has many functions in human including enhancing bones strength, antioxidant capacity, immunity and reducing body fattiness[3]. In recent years, EU has been reported as feed additive in rabbit [4], pig [5, 6], chicken [7, 8] and fish [9–11]. Studies have shown that EU promoted the flesh quality by increasing muscle collagen of aquatic animals, such as eel *Anguilla japonica*[12], crucian carp *Carassius auratus gibelio* [13], grass carp *Ctenopharyngodon idellus* [9, 14] and white shrimp *Penaeus vannamei* [15], but the mechanism is unclear.

When herbal plants were directly added in diets, the inclusion levels were usually high with relatively higher anti-nutritional factors. Thus, extracting active compounds from the raw herbs is an important strategy for the application of herbal plants in the future. The traditional medicinal part of EU is the bark, but in recent years, the leaves have attracted more attentions in feed industry due to the scarcity of bark resources. The inclusion of *Eucommia* leaf extracts (ELE) in diets has been found in lamb [16], rabbit [17], chicken [18] and pig [19–20].

Grass carp (*Ctenopharyngodon idellus*) is an important cultured freshwater fish in China with a production of 5.50 million tons in 2018 [21]. In past years, we have conducted a series trials to investigate the effects of dietary EU (bark) [9] and its active compounds including chlorogenic acid [22], quercetin [23], geniposide [24] and geniposidic acid [25] on the growth and flesh quality of grass carp, but the mechanism of flesh quality-improving and collagen-promoting effects is still unclear. Moreover, how about the effects of EU leaf extract when compared to EU bark extract? Therefore, *Eucommia* bark extracts (EBE) and ELE were supplemented in feed to investigate their effects on growth, flesh quality and collagen-related genes expression of grass carp by biochemical, molecular analysis and flesh quality evaluation.

Materials And Methods

Experimental design and feeding management

The basal diet was designed to contain 300 g/kg crude protein with the inclusion of fish meal, soybean meal, rapeseed meal and cottonseed meal as the protein sources. Then, EBE and ELE were supplemented in basal diet (control) with an inclusion of 4 g/kg and 4 g/kg, respectively. EBE and ELE were supplied by Xi'an Huilin Bio-Tech Co., Ltd (Xi'an, China), and 1 kg of EBE and ELE was produced from 5 kg of bark and 10 kg of leaves, respectively. The composition of both extracts is shown in Table 1. The supplemental level of EBE and ELE was calculated by referring to the studies of Sun et al. [9] and Leng et al. [26], respectively. In addition, Y_2O_3 was used as marker to measure the digestibility. All ingredients were ground, sifted, mixed and pelleted as described by Sun et al. [22], and then all diets were air-dried and stored at 4 °C until use. The ingredients and proximate composition of experimental diets are shown in Table 2.

Table 1
The composition of *Eucommia* extracts
(g/kg)

Parameters	EBE	ELE
Total phenolic acids	50.1	85.8
Total flavonoids	36.3	95.4
Total polysaccharides	81.0	112.3

Table 2
Ingredients and proximate composition of experimental diets (g/kg)

Ingredients	Control	EBE	ELE
Fish meal	20.0	20.0	20.0
Soybean meal	180.0	180.0	180.0
Cottonseed meal	150.0	150.0	150.0
Rapeseed meal	190.0	190.0	190.0
Wheat bran	102.5	98.5	98.5
Defatted rice bran	100.0	100.0	100.0
Wheat middling	220.0	220.0	220.0
Soybean oil	10.0	10.0	10.0
Ca(H ₂ PO ₄) ₂	15.0	15.0	15.0
Vitamin premix	2.0	2.0	2.0
Choline chloride (500 g/kg)	5.0	5.0	5.0
Mineral premix	5.0	5.0	5.0
Y ₂ O ₃	0.5	0.5	0.5
EBE	-	4.0	-
ELE	-	-	4.0
Total	1000.0	1000.0	1000.0
Proximate composition			
Moisture	94.2	93.5	94.5
Crude protein	300.4	302.2	299.5
EBE, <i>Eucommia</i> bark extract; ELE, <i>Eucommia</i> leaf extract			
The ingredients were purchased from the Yuehai Feed Company (Zhejiang, China), and the protein contents of ingredients are as follow: fish meal (630.0 g/kg), soybean meal (442.0 g/kg), cottonseed meal (500.0 g/kg), rapeseed meal (377.0 g/kg), wheat middling (169.0 g/kg), defatted rice bran (143.0 g/kg).			
Vitamin premix (mg or IU/kg diet): VA, 10 000 IU; VD ₃ , 3 000 IU; VE 150 IU; VK ₃ , 12.17 mg; VB ₁ , 20 mg; VB ₂ , 20 mg; VB ₃ , 100 mg; VB ₆ , 22 mg; VB ₁₂ , 0.15 mg; VC, 1000 mg; biotin, 0.6 mg; folic acid, 8 mg; inositol, 500 mg.			
Mineral premix (mg/kg diet): I, 1.5 mg; Co, 0.6 mg; Cu, 3 mg; Fe, 63 mg; Zn, 89 mg; Mn, 11.45 mg; Se, 0.24 mg; Mg, 180 mg.			

Ingredients	Control	EBE	ELE
Crude lipid	42.9	42.7	43.2
Crude ash	70.4	69.8	69.8
EBE, <i>Eucommia</i> bark extract; ELE, <i>Eucommia</i> leaf extract			
The ingredients were purchased from the Yuehai Feed Company (Zhejiang, China), and the protein contents of ingredients are as follow: fish meal (630.0 g/kg), soybean meal (442.0 g/kg), cottonseed meal (500.0 g/kg), rapeseed meal (377.0 g/kg), wheat middling (169.0 g/kg), defatted rice bran (143.0 g/kg).			
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Mineral premix (mg/kg diet): I, 1.5 mg; Co, 0.6 mg; Cu, 3 mg; Fe, 63 mg; Zn, 89 mg; Mn, 11.45 mg; Se, 0.24 mg; Mg, 180 mg.			

Grass carp were obtained from Jinshan Aquaculture Farm (Shanghai, China). One hundred and sixty-two grass carp with an average initial weight of 59.7 ± 0.2 g were randomly distributed into 9 cages ($1.5 \times 1.0 \times 1.2$ m) with 18 fish per cage. During the experimental period, the fish were fed with a daily feeding rate of 3–5% of body weight with three meals (7:00, 12:00, 17:00) per day for 60 days. The feed intake of all cages was appropriately adjusted according to water temperature, and maintained with a similar amount to ensure no feed residue left. The waste in the pools was cleared by siphoning every 5 days, and 1/3 cultured water was renewed with pond water. The dissolved oxygen was not less than 5 mg/L, and water temperature and pH were maintained at $27 \pm 2^\circ\text{C}$ and 7.7 ± 0.2 , respectively. The feeding experiment was conducted at Binhai Aquaculture Station (Shanghai, China).

Sample Collection And Analysis

Growth performance and physical indices

When the feeding trial ended, all fish were starved for 24 h, and measured total final body weight to calculate weight gain (WG) and feed conversion ratio (FCR). Three fish per cage were randomly selected and anesthetized with MS-222 (30 mg/L) to individually measure body weight, body length, liver weight, visceral weight and mesenteric lipid weight, then the indices of condition factor (K), hepato-somatic index (HSI), viscero-somatic index (VSI) and mesenteric lipid-somatic index (MSI) were calculated as follows:

Weight gain (WG, %) = $100 \times [\text{final weight} - \text{initial weight}] / \text{initial weight}$

Feed conversion ratio (FCR) = feed intake / wet weight gain

Condition factor (K, g/cm³) = $100 \times [\text{body weight (g)} / \text{body length (cm)}^3]$

Viscero-somatic index (VSI, %) = $100 \times [\text{visceral weight} / \text{body weight}]$

Hepato-somatic index (HSI, %) = $100 \times [\text{liver weight} / \text{body weight}]$

Mesenteric lipid-somatic index (MSI, %) = $100 \times \text{Mesenteric fat weight} / \text{final weight}$

Water holding capacity of flesh

Five blocks of flesh (about 3 g) were sampled from the dorsal muscle of the left side of the body per fish to determine water-holding capacity (WHC) immediately as follows:

The first and second flesh sample (W1) was steaming in pot for 5 min or centrifuging at 3 500 r/min for 10 min, then wiped off the surface liquid and weighed (W2) to calculate steaming loss and centrifugal loss. The other three flesh sample (W1) at 4°C for 6, 12 and 24 h, respectively, then wiped off the surface liquid and weighed (W2) to calculate drip loss.

Steaming (centrifugal, drip) loss (%) = $100 \times (W1 - W2) / W1$

Flesh And Diets Proximate Composition

The rest of dorsal muscle was stored at -20 °C for the analysis of crude protein, crude lipid, crude ash, moisture, calcium, phosphorus, total collagen, heat-soluble collagen, free amino acids and fatty acid composition.

The proximate composition in flesh (moisture, crude lipid, crude protein and crude ash) was analyzed according to AOAC [27] methods described in our previous study [22]. The calcium, phosphorus content was determined by methylthymol blue colorimetry and phosphomolybdic acid colorimetry.

Total collagen content were calculated by multiplying the hydroxyproline content by 8 [22], and the hydroxyproline test kits were supplied by Nanjing Jiancheng Bioengineering Institute (Nanjing, China). Heat-soluble collagen was determined according to the method of Kong et al. [28]. Muscle sample (2 g) was homogenized with four times Ringer's solution (0.86% NaCl, 0.03% KCl, and 0.033% CaCl₂) (10 000 rpm, 1 min), then the homogenate was heated at 77 °C for 70 min and centrifuged (12 000 r/min) for 30 min at 4 °C. The extraction was repeated twice with supernatants combined. The collagen content of supernatants was measured as heat-soluble collagen.

Heat-insoluble collagen = Total collagen - Heat-soluble collagen

For determination of free amino acid in muscle, samples were homogenized with 30 volumes of extract liquid (Methanol:water = 4:1) and centrifuged at 12 000 r/min and 4 °C for 30 min. The supernatants were analyzed using Ultra Performance Liquid Chromatography, UPLC (Waters Acquity, USA). The fatty acids

composition of the muscle was determined with Boron trifluoride method according to the description of Zuo et al. [29] with GC-MS (7890B gas chromatograph- mass spectrometer, Agilents Technologies, USA).

Antioxidant Capacity Of Flesh

The muscle samples were thawed at 4 °C, and then homogenized with four times ice-cold distilled water at 4 °C and centrifuged for 10 min (6 000 r/min). The supernatant was preserved at 4 °C, and determined antioxidation index in 24 hr. Lactic acid (LC), malondialdehyde (MDA), protein carbonyl (PC), catalase (CAT), superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px) were measured by kits (Shanghai Haling Biotechnology Co., Ltd, Shanghai, China)

The Histology Of Flesh

Muscle samples were immersed in 4% methanol solution for at least 48 hr. The tissue was dehydrated, paraffin-embedded, sectioned (8 µm), stained (Picrosirius Red) and sealed with a neutral gum. The collagen distribution in flesh was observed using an imaging microscope (Nikon YS100).

Digestive Enzyme Activity

The anterior intestine was sampled from three fish per cage after they were dissected on ice, and then stored at -80 °C until use. The 2.0 g samples were thawed at 4 °C, and then homogenized with 8.0 ml ice-cold distilled water at 4 °C and centrifuged for 10 min (6 000 r/min). The supernatant was collected and preserved at 4 °C until the use in 24 hr.

The measurement of amylase activity and soluble protein concentration were used by kits (Nanjing Jiancheng Bioengineering Institute) with iodine-starch colorimetric method and Coomassie brilliant blue method, respectively, and protease activity was analyzed by Folin-Ciocalteu method. The methods were referred to the description of Yang et al. [30].

Digestibility

After the sampling, all fish continued to keep their original feeds for one week, then the intact faeces was siphoned 2 hr after feeding and stored at -20 °C for analysis. Yttrium contents in diets and faeces were analyzed using inductively coupled plasma (ICP) emission spectroscopy (Vista MPX; Varian). Faeces protein was measured as the above method (2.2.3). Apparent digestibility coefficient (ADC) of dry matter (DM) and crude protein (CP) was calculated as follows:

$$\text{ADC of DM} = [1 - \text{Dietary Y}_2\text{O}_3 / \text{Faecal Y}_2\text{O}_3] \times 100\%$$

$$\text{ADC of CP} = [1 - (\text{Faecal CP} / \text{Dietary CP} \times \text{Dietary Y}_2\text{O}_3 / \text{Faecal Y}_2\text{O}_3)] \times 100\%$$

Real time quantitative PCR analysis of gene expression in flesh

Total RNA was isolated from muscle samples using an RNAiso Plus Kit (Takara, Dalian, China) and assessed by agarose gel electrophoresis and by spectrophotometric analysis. Subsequently, cDNA synthesis was performed using the PrimeScript™ RT reagent Kit (Takara, Dalian, China), and then was stored at -80 °C until use. Based on the sequence of grass carp 18S (EU047719.1) and type I collagen (COL1A1, COL1A2), matrix metalloproteinase-2 (MMP-2) and matrix metalloproteinase-9 (MMP-9) in GenBank, and the cloned lysine oxidase (LOX) and proline hydroxylase (PHD) sequence of full length cDNA (they will be published separately) in our laboratory, the PCR primers were designed (Table 3). All real-time quantitative PCR analysis was performed using the SYBR® Premix Ex Taq (Perfect Real-Time) kit (TaKaRa) according to the manufacturer's instructions. The total reaction volume was 20 µl, containing 10 µl SYBR® Premix Ex Taq™ (Tli RNaseH Plus), 0.5 µl upstream primer, 0.5 µl downstream primer, 1 µl cDNA template and 8 µl ddH₂O. The thermocycling conditions of real-time PCR were presented as follows: denaturing at 95 °C for 3 min and 39 cycles of denaturing at 95 °C for 5 s, annealing at 60 °C for 10 s and extension at 72 °C for 30 s, then the melting curve was created after the extension. The expression results of COL1A1, COL1A2, PHD, LOX, MMP-2 and MMP-9 in flesh was calculated using the $2^{-\Delta\Delta C_t}$ method.

Table 3
The primer for real-time PCR

Primer name	Sequence from 5' to 3'	usage
18srRNA-F	GGAATGAGCGTATCCTAAACCC	qRT-PCR
18srRNA-R	CTCCCGAGATCCAACCTACAAGC	qRT-PCR
COL1A1-F	ACGCACACAAACAATCTCAAGT	qRT-PCR
COL1A1-R	GCATGGGGCAAGACAGTCA	qRT-PCR
COL1A2-F	ACTCCGATAGAGCCCAGCTT	qRT-PCR
COL1A2-R	ACATTGGTGGCGCAGATCA	qRT-PCR
LOX-F	GTTATCAGGTGGCGATGGAG	qRT-PCR
LOX-R	GTAGACTCGTATGGGTTGTAAGG	qRT-PCR
PHD-F	CTCGAAACCCAACGGACAGA	qRT-PCR
PHD-R	AGCTGTCCGTCTGTAAAGCC	qRT-PCR
MMP2-F	GAGCTGTGGACATTAGGAGAAG	qRT-PCR
MMP2-R	GAACAAGAGCTCATGAGGACAG	qRT-PCR
MMP9-F	ACTTGGAGTTGTGGCTTTTC	qRT-PCR
MMP9-R	AGGGCTCTGTCATCAGGTTA	qRT-PCR

Statistical analysis

The experimental data were carried out using SPSS 22.0 software, and presented as the mean \pm standard deviation (SD). All data were subjected to a one-way analysis of variance (ANOVA), and combined with Duncan's multiple range test to identify the differences among treatments. The significance level for differences was determined at $P < 0.05$.

Results

Growth performance and physical indices

During the feeding period, no mortality was recorded. Compared to the control, WG was increased by 4.22% ($P < 0.05$), and FCR was decreased by 0.07 ($P < 0.05$) by the ELE supplementation in diet, but the growth performance was not significantly affected by dietary EBE ($P > 0.05$). HSI, VSI, MSI, K and survival showed no significant differences among all the treatments ($P > 0.05$) (Table 4).

Table 4
Growth performance and physical indices of grass carp fed *Eucommia* extracts diets

Parameters	Control	EBE	ELE
IBW (g)	59.67 ± 0.32	59.67 ± 0.38	59.67 ± 0.65
FBW (g)	229.04 ± 1.25 ^a	229.98 ± 2.51 ^a	236.18 ± 2.87 ^b
WG (%)	283.86 ± 2.09 ^a	285.44 ± 4.19 ^a	295.84 ± 4.82 ^b
FCR	1.73 ± 0.02a	1.72 ± 0.03a	1.66 ± 0.02b
K (g/cm ³)	1.83 ± 0.05	1.81 ± 0.04	1.82 ± 0.04
VSI (%)	8.46 ± 0.34	8.32 ± 0.44	7.94 ± 0.44
HSI (%)	1.88 ± 0.10	1.82 ± 0.07	1.82 ± 0.11
MSI (%)	2.14 ± 0.08	2.04 ± 0.10	2.06 ± 0.07
Survival (%)	100.00	100.00	100.00
Values in the same row with different superscripts alphabets indicate significant differences ($p < 0.05$)			
IBW, initial body weight (g); FBW, final body weight (g); WG, weight gain (%); FCR, feed conversion ratio; HSI, hepato-somatic index (%); VSI, viscero-somatic index (%); MSI, mesenteric lipid-somatic index (%); K, condition factor (g/cm ³).			

Muscle Composition

In Table 5, there were no significant differences in the contents of muscle moisture, crude lipid, crude protein, crude ash and phosphorus among all the groups ($P > 0.05$), but dietary ELE significantly increased the contents of calcium, total collagen and heat-insoluble collage when compared to the control ($P < 0.05$).

Table 5

Proximate composition in muscle of grass carp fed *Eucommia* extracts diets (g/kg, wet weight)

Parameters	Control	EBE	ELE
Moisture	775.98 ± 8.94	777.20 ± 5.87	775.60 ± 9.85
Crude ash	11.83 ± 0.21	11.73 ± 0.15	12.10 ± 0.32
Crude protein	194.90 ± 2.08	195.65 ± 1.34	196.87 ± 3.40
Crude lipid	12.80 ± 0.85	12.70 ± 0.56	12.17 ± 1.89
P	3.23 ± 0.10	3.04 ± 0.14	3.02 ± 0.15
Ca	1.96 ± 0.20 ^a	2.11 ± 0.27 ^{ab}	2.38 ± 0.12 ^b
T collagen	3.10 ± 0.19 ^a	3.09 ± 0.15 ^a	3.41 ± 0.16 ^b
HS collagen	0.43 ± 0.03	0.43 ± 0.02	0.40 ± 0.03
HIS collagen	2.67 ± 0.13 ^a	2.66 ± 0.17 ^a	3.01 ± 0.12 ^b
Values in the same row with different superscripts alphabets indicate significant differences ($p < 0.05$)			
T collagen: total collagen; HS collagen: heat-soluble collagen; HIS collagen: heat-insoluble collagen; Ca: calcium; P: phosphorus.			

In Fig. 1, the red area relected the distribution of collagen in muscle, and the collagen in muscle mainly distributed in the endomysium (EM) and perimysium (PM). The ELE group rather than EBE group showed significantly larger red area (collagen) than the control group.

Digestibility and digestive enzyme activity

The dry matter and protein digestibility, the activity of protease and amylase in ELE group, but not in EBE group, were significantly higher than those of the control group ($P < 0.05$) (Table 6).

Table 6

The nutrient digestibility and digestive enzyme activities of grass carp fed *Eucommia* extracts diets

Parameters	Control	EBE	ELE
Dry matter digestibility (%)	57.28 ± 0.52 ^a	57.75 ± 1.17 ^a	59.73 ± 1.09 ^b
Crude protein digestibility (%)	84.69 ± 0.76 ^a	85.17 ± 0.45 ^{ab}	86.33 ± 0.63 ^b
Amylase activity (U/mg prot)	4.81 ± 0.33 ^a	5.38 ± 0.26 ^{ab}	6.50 ± 0.39 ^b
Protease activity (U/mg prot)	77.06 ± 4.34 ^a	80.17 ± 5.90 ^{ab}	86.65 ± 4.55 ^b
Values in the same row with different superscripts alphabets indicate significant differences ($p < 0.05$)			

Water holding capacity and antioxidant capacity of flesh

In Table 7, the supplementation of EBE and ELE in diets significantly decreased the flesh steaming loss, centrifugal loss and the contents of LC, PC and MDA, while increased the activity of SOD and GSH-Px ($P < 0.05$). No significant difference was found in CAT activity among the three groups ($P > 0.05$).

Table 7

The flesh water-holding capacity and antioxidant parameters of grass carp fed *Eucommia* extracts diets

Parameters	Control	EBE	ELE
Steaming loss (%)	11.29 ± 0.62 ^a	7.61 ± 0.85 ^b	8.45 ± 0.75 ^b
Centrifugal loss (%)	8.71 ± 0.52 ^a	6.79 ± 0.60 ^b	7.11 ± 0.73 ^b
Drip loss ^{6h} (%)	13.99 ± 0.56	13.86 ± 1.24	13.09 ± 0.75
Drip loss ^{12h} (%)	22.53 ± 2.54	22.01 ± 0.52	22.06 ± 1.53
Drip loss ^{24h} (%)	37.02 ± 1.18 ^a	32.20 ± 1.55 ^b	31.88 ± 0.97 ^b
LC (µmol/g)	6.49 ± 0.40 ^a	5.97 ± 0.19 ^b	5.98 ± 0.08 ^b
PC (µmol/g prot)	1.05 ± 0.05 ^a	0.98 ± 0.01 ^b	0.96 ± 0.02 ^b
MDA (nmol/g prot)	16.24 ± 1.01 ^a	13.40 ± 1.56 ^b	12.82 ± 0.47 ^b
SOD (µ/g prot)	522.4 ± 17.7 ^a	600.3 ± 33.8 ^b	607.6 ± 23.1 ^b
CAT (µ/g prot)	1673.5 ± 21.3	1672.2 ± 13.3	1685.4 ± 10.7
GSH-Px (nmol/min/g prot)	2744.5 ± 151.0 ^a	3424.7 ± 445.3 ^b	3344.8 ± 137.1 ^b
Values in the same row with different superscripts alphabets indicate significant differences ($p < 0.05$)			
lactic acid (LC), malondialdehyde (MDA), protein carbonyl (PC), catalase(CAT), superoxide dismutase (SOD), glutathione peroxidase (GSH-Px)			

Free Amino Acids In Muscle

In Table 8, a total of 17 free amino acids were detected in the muscle. Compared to the control, dietary ELE significantly increased Gly, Ala, delicious amino acids (DAAs) and total free amino acids (TFAAs), while decreased Cys in flesh ($P < 0.05$). Thr, Leu and Ser were significantly higher in EBE group, while Arg and Cys were lower than those of the control ($P < 0.05$).

Table 8

Muscle free amino acid of grass carp fed *Eucommia* extracts diets (mg/kg, wet weight)

Parameters	Control	EBE	ELE
Aspt	8.07 ± 1.01	8.03 ± 2.70	7.17 ± 3.22
Glut	28.87 ± 2.66	28.03 ± 3.11	28.6 ± 2.50
Glyt	125.67 ± 9.25 ^a	139.67 ± 6.65 ^a	178.70 ± 23.9 ^b
Alat	73.17 ± 5.71 ^a	82.63 ± 4.29 ^{ab}	91.20 ± 8.40 ^b
Cys	10.40 ± 0.61 ^a	4.87 ± 1.77 ^b	3.37 ± 0.75 ^b
Tyr	11.60 ± 0.53	12.27 ± 0.90	10.77 ± 2.0
Pro	138.40 ± 7.89	128.13 ± 8.01	137.27 ± 3.49
Ser	9.83 ± 1.08 ^a	14.27 ± 0.40 ^b	11.97 ± 1.68 ^{ab}
Val	9.93 ± 1.02	10.36 ± 0.70	9.90 ± 0.76
Met	4.70 ± 1.45	3.50 ± 1.08	3.93 ± 0.71
Ile	5.17 ± 1.03	6.83 ± 1.51	4.63 ± 1.57
Leu	7.73 ± 0.76 ^a	10.87 ± 1.86 ^b	7.90 ± 1.13 ^a
Thr	99.27 ± 16.83 ^a	111.40 ± 6.83 ^b	95.40 ± 10.96 ^a
His	335.57 ± 7.62	334.90 ± 9.57	333.87 ± 9.66
Lys	30.77 ± 3.68	29.63 ± 3.88	31.40 ± 5.15
Arg	32.73 ± 2.40 ^a	19.00 ± 1.47 ^b	36.77 ± 4.89 ^a
Phe	7.13 ± 1.00	7.53 ± 2.19	6.37 ± 1.04
DAAAs	235.80 ± 9.47 ^a	258.30 ± 3.68 ^{ab}	305.70 ± 30.00 ^b
TFAAs	938.97 ± 12.07 ^a	951.83 ± 16.20 ^{ab}	986.01 ± 26.14 ^b
Values in the same row with different superscripts alphabets indicate significant differences ($p < 0.05$)			
DAAAs, delicious amino acids (†); TFAAs, total free amino acids			

Fatty Acids Composition In Muscle

As shown in Table 9, a total of 14 fatty acid were detected in the muscle. There were no significant differences in saturated fatty acids (SFAs), monounsaturated fatty acids (MUFAs) and polyunsaturated fatty acids (PUFAs) among all the treatments ($P > 0.05$), but the supplementation of EBE and ELE significantly increased DPA, DHA, n-3PUFAs and n-3/n-6 ratio in flesh ($P < 0.05$).

Table 9

Muscle fatty acid composition of grass carp fed *Eucommia* extracts diets (percentage of fatty acids, %)

Parameters	Control	EBE	ELE
C14:0	1.23 ± 0.02	1.31 ± 0.07	1.23 ± 0.12
C16:0	14.27 ± 0.27	14.38 ± 0.22	14.38 ± 0.61
C17:0	0.35 ± 0.05	0.38 ± 0.08	0.37 ± 0.11
C18:0	8.92 ± 0.37	8.59 ± 0.11	8.08 ± 0.29
SFAs	24.75 ± 0.48	24.67 ± 0.13	24.06 ± 1.08
C16:1	2.01 ± 0.10	2.16 ± 0.08	2.23 ± 0.05
C18:1	32.39 ± 0.66	30.25 ± 1.22	31.55 ± 1.54
C20:1	1.12 ± 0.06	1.15 ± 0.01	1.19 ± 0.07
MUFAs	33.52 ± 0.64	32.41 ± 1.22	32.73 ± 1.5
C18:2	25.36 ± 1.33	24.56 ± 0.69	24.16 ± 1.35
C20:2	0.97 ± 0.03	1.03 ± 0.03	1.03 ± 0.06
C20:3	1.33 ± 0.16	1.43 ± 0.08	1.50 ± 0.17
ARA	6.59 ± 0.86	6.48 ± 1.02	6.52 ± 1.27
n-6PUFA	34.26 ± 0.51	33.50 ± 0.65	33.21 ± 0.55
EPA	0.42 ± 0.08	0.47 ± 0.05	0.52 ± 0.10
DPA	2.31 ± 0.43 ^a	3.33 ± 0.63 ^b	3.63 ± 0.41 ^b
DHA	2.74 ± 0.51 ^a	3.44 ± 0.19 ^b	3.60 ± 0.08 ^b
n-3PUFAs	5.48 ± 0.97 ^a	7.23 ± 0.63 ^b	7.75 ± 0.4 ^b
PUFAs	39.73 ± 1.09	40.73 ± 1.21	40.97 ± 0.65
n-3/n-6	0.16 ± 0.03 ^a	0.21 ± 0.02 ^b	0.23 ± 0.01 ^b
Values in the same row with different superscripts alphabets indicate significant differences ($p < 0.05$)			
SFAs, saturated fatty acids; MUFAs, monounsaturated saturated fatty acids; PUFAs, polyunsaturated fatty acids			

Gene Expression In Muscle

As shown in Fig. 2, the gene expression levels of COL1A1, COL1A2, PHD and LOX in flesh were significantly increased, and MMP-2, MMP-9 expression was decreased by dietary ELE ($P < 0.05$), while these genes showed no significant differences between EBE group and control group ($P > 0.05$).

Discussion

Effects of *Eucommia* extracts on the growth performance

In land animals, the supplementation of 0.08% ELE (containing total phenolic acid 542.9 g/kg, total flavonoids 64.4 g/kg) in diet increased the average daily gain (ADG) of pig by 18.6% and decreased FCR by 0.49 [19]. Zhao et al. [18] also reported that dietary ELE (containing 820 g/kg chlorogenic acid) (0.1%) significantly increased ADG (+ 11.39%) and decreased FCR (-0.15) of chicken. In grass carp [31] and crucian carp [13], the WG was also reported to be increased (+ 8.57%, + 12.9%) by the supplementation of 0.15% ELE (containing 50 g/kg chlorogenic acid, 80 g/kg total flavonoids and 200 g/kg crude polysaccharide). In the present study, the WG of grass carp was increased by 4.22% and FCR decreased by 0.07 ($P < 0.05$) by the supplementation of 4 g/kg ELE, which was consistent with the above results. However, the present inclusion level of ELE was higher than that in others studies, which may be related to the lower active compounds content in this extract (Table 1).

The improvement of intestinal digestive enzymes and nutrients utilization may be directly related to the promote growth-promoting effect of ELE. It has been reported that intestinal digestive enzymes and nutrient digestibility were significantly increased by dietary ELE, therefore improving growth performance of crucian carp [13] and chicken [32]. In general, the intestinal digestion and absorption is closely related to the intestinal microflora. In the study of Shi et al. [33], dietary ELE inhibited the pathogenic bacteria by promoting the growth of probiotics and balancing the intestinal floracommunity. Lv et al. [8] found that ELE reduced the number of intestinal *Escherichia coli* in chicken, which may be concerned with the active compounds such as phenolic acids, flavonoids and polysaccharides in ELE. Studies have shown that phenolic acids [34], flavonoids [35], and polysaccharides [36] inhibited the colonization of pathogenic bacteria in the intestines, increased the number of beneficial bacteria and enhanced the intestinal nutrient absorption. In grass carp, the EU active compounds such as chlorogenic acid [22], quercetin [23] and baicalein [37] have been reported to significantly promote the growth performance. Therefore, the growth improvement by dietary ELE is the combined effect of various active compounds.

However, the supplementation of EBE did not improve the growth of grass carp in this study. The similar result was also reported in rainbow trout that was fed diet containing water extract of *Eucommia* bark [38]. As shown in Table 1, the active compounds contents in EBE are lower than those of the ELE, which may be the major reason that the growth-promoting effect was observed in ELE diet rather than in EBE diet. If more EBE was supplemented, or the EBE contained higher concentration of active compounds, it would promote the growth performance of grass carp. In addition, the active compounds composition of

EU bark was different from the leaf. Generally, barks contain more lignin and iridoids, while leaves contain more phenolic acids and flavonoids [39]. Previous studies in our laboratory have shown that dietary phenolic acids (chlorogenic acids) [9] and flavonoids (quercetin) [23] produced more positive effects on growth of grass carp than iridoids (geniposide and geniposidic acid) did [24–25].

Effects of *Eucommia* extracts on the flesh quality

Water-holding capacity (WHC) is directly related to the flesh quality, and it is generally evaluated by steaming loss, centrifugal loss and drip loss [40]. The supplementation of both *Eucommia* extracts significantly promoted the flesh WHC by decreasing steaming loss, centrifugal loss and drip loss in this study, and the similar results were also reported in pig [41], chicken [18] and lamb [16]. The improvement of flesh WHC mainly resulted from the reduction of oxidative damage, thus promoting the structural integrity of muscle tissue [42]. In fish, MDA and PC contents are widely used for evaluating the oxidative damage of lipid and protein [43]. Generally, the oxidation of protein and lipid is related to the increased levels of superoxide free radicals ($O_2^{\cdot-}$) and hydroxyl free radicals (OH^{\cdot}) in the body, while SOD, CAT, and GSH-Px are non-enzymatic antioxidants that can clear $O_2^{\cdot-}$ and OH^{\cdot} , and play an important role in the self-protection system of organisms [44]. In this study, the MDA and PC contents in EBE and ELE groups were significantly decreased, and SOD and GSH-PX activities were significantly increased. Similarly, *Eucommia* leaf extracts was also reported to improve the muscle antioxidant capability in chicken [18] and lamb [18]. Zhao et al. [45] reported that flesh WHC is negatively correlated with MDA and PC levels, and positively correlated with SOD and GSH-PX activities, and the similar results was also found in the present study. In addition, the lactic acid content of muscle is another important factor affecting the flesh WHC. After the slaughtering, muscle metabolism mode was changed from aerobic to anaerobic status. The anaerobic respiration of muscle glycogen generated a large amount of lactic acid, which decreased muscle pH and caused protein denaturation, thereby affecting flesh WHC [46]. In the current study, *Eucommia* extracts significantly decreased the lactic acid content in flesh of grass carp. Maybe *Eucommia* extracts promote the aerobic metabolism in muscle, and the similar results have been found in chicken [18] and pig [19]. Fatty acid composition is also an important indicator of flesh quality. As an important part of unsaturated fatty acids, n-3PUFAs play important roles in preventing cardiovascular and cerebrovascular diseases, and in lowering serum lipid, blood pressure and inflammation [47–48]. When used for the nutrition evaluation of flesh, the ratio of n-3/n-6 PUFA may be more important than the content of n-3PUFAs or n-6PUFAs [1]. In the current study, dietary EBE and ELE increased DPA, DHA, the n-3 PUFAs and n-3/n-6 ratio in flesh, which may be related to the active compounds such as phenolic acids and flavonoids. The studies of Andrés et al. [49] and Starčević et al. [50] have shown that phenolic acids (gallic acid) and flavonoids (quercetin) increased the n-3/n-6 ratio in lamb and chicken, respectively. The changes in fatty acid composition may result from it's the lipid intake, transport, metabolism and storage processes, and the modulating mechanism of *Eucommia* extracts for fatty acids needs a further study in the future.

Free amino acids, especially delicious amino acids, are the major flavor substance in the flesh of fish. In the study, the free delicious amino acid and total free amino acid contents in ELE group were significantly increased, especially glycine and alanine. The increased free amino acid in muscle could provide sufficient raw materials for the body to synthesize protein [51]. So, it is speculated that dietary ELE promoted the protein transport and metabolism, then increased the content of free amino acids including delicious amino acids, which needs further study.

As the most abundant metal element in the body, calcium is widely involved in various physiological processes such as muscle contraction, neurotransmitter synthesis and release [52], and it is also closely related to the muscle fiber development [52], muscle fiber type [53] and muscle hardness [54]. In the study of Oikawa et al. [55], ELE was reported to promote calcium accumulation in human osteoblasts through calcium signaling pathways. Therefore, the increased calcium content in flesh by dietary ELE was also found in the present study. Maybe calcium signaling pathway induced by ELE modulate the calcium accumulation in muscle.

Effects of *Eucommia* extracts on collagen-related genes expression

As the major component of connective tissues, collagen influences the functional and structural properties of muscle, and makes a great contribution to the adhesion and tensile strength of muscle [56]. In fish, it has been reported that the collagen content was positively correlated with flesh hardness [57]. Dietary ELE significantly increased the flesh collagen in crucian carp [13] and shrimp [15]. Collagen can be divided into two groups, soluble and insoluble collagen. Sun et al. [9] found that dietary EU (2%) increased alkaline-insoluble collagen rather than the soluble collagen in muscle of grass carp. The similar results were also found in the present study with the increased total collagen and heat-insoluble collagen in flesh, which indicated that the increased total collagen in muscle mainly comes from the insoluble collagen, the structural part of collagen in muscle.

The collagen in fish muscle was mainly type I collagen, containing two $\alpha 1$ chains (COL1A1) and one $\alpha 2$ chain (COL1A2). The biosynthesis of collagen begins from the transcription of COL1A1 and COL1A2. Guan et al. [58] once reported that ELE promoted the expression of COL1A1 gene in osteoblast cell of rats. In grass carp, Xu et al. [14] found that dietary EU (bark) significantly increased the expression of COL1A1 and COL1A2 in muscle and skin. In the present study, ELE also promoted the mRNA expression of COL1A1 and COL1A2 in muscle. The active compounds in EU such as chlorogenic acid, geniposide and geniposidic acid may be involved in the up-regulation of genes, which has been proved in the previous studies in grass carp, where COL1A1 expression in muscle was promoted by the three active compounds supplementation in diets [22].

The biosynthesis of collagen involves a series of processes, including the transcription and translation of genes, the post-translational modification and covalent cross-linking. Proline hydroxylase (PHD) is an

important hydroxylase for post-translational modification, and it can increase the stability of intermolecular hydrogen bonds. If the hydroxylation was incomplete, a triple helix cannot be formed, which affects the secretion of procollagen molecules outside the cell [59]. Lysine oxidase (LOX) is the only enzyme involved in covalent cross-linking, and the insufficient secretion will affect the elasticity and toughness of collagen [60]. At present, the effects of dietary nutrients on PHD and LOX gene expression have not been reported in aquatic animals. We cloned the full-length sequences of PHD and LOX genes (it will be published in another paper) for the first time, and found that dietary ELE significantly increased the expression of LOX and PHD in grass carp muscle. In the future, we will continue investigating what active compounds in *Eucommia* extracts up-regulate the genes expression.

The decomposition process of collagen also affects the muscle collagen content. The major enzymes involved in collagen degradation are matrix metalloproteinases (MMPs). MMPs are highly conserved metal ion-dependent enzymes, and they can degrade almost all components in extracellular matrix [61]. In fish, type I and V collagen are the two main types of collagen in muscle [62], and MMP-2 and MMP-9 showed a significant degradation effect on collagen, especially type I and V collagen [63]. In the study of Chen et al. [64] and Wang et al. [65], ELE decreased the mRNA expression of MMP-2 in endothelial cells and MMP-9 in ankle of rat. Therefore, the increased collagen in flesh by dietary ELE is the combined results of increased synthesis and decreased decomposition of collagen.

Conclusion

In the present study, dietary ELE (4 g/kg) promoted the growth performance and antioxidant capacity of grass carp, and improved the flesh quality by increasing the expression of collagen synthesis-related genes and decreasing the expression of collagen degradation-related genes.

Declarations

Availability of data and materials

All data generated or analyzed during this study are included in this published article.

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Contributions

Hang Yang and Sumei Tan completed the experiment and prepared the manuscript. Xiaoqin Li, Zhen Xu and Zhuo Cheng carried out the growth experiment and analysed the samples. Xiangjun Leng designed the experiment and revised the manuscript.

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Ethics declarations

Ethics approval and consent to participate

All the procedures were strictly carried out in accordance with the Regulations of the Experimental Animal Ethics Committee of Shanghai Ocean University and in compliance with regulations by Institutional Animal Care and Use Committee.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Figures

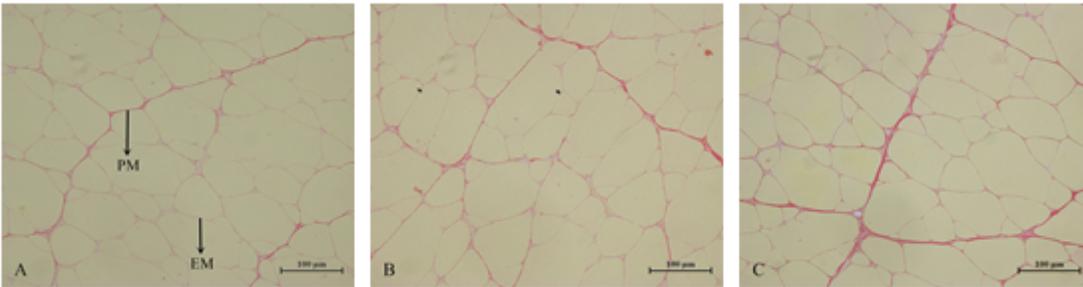


Figure 1

Muscle tissue transverse sections of grass carp. A: Control; B: EBE; C: ELE; PM: Perimysium; EM: Endomysium

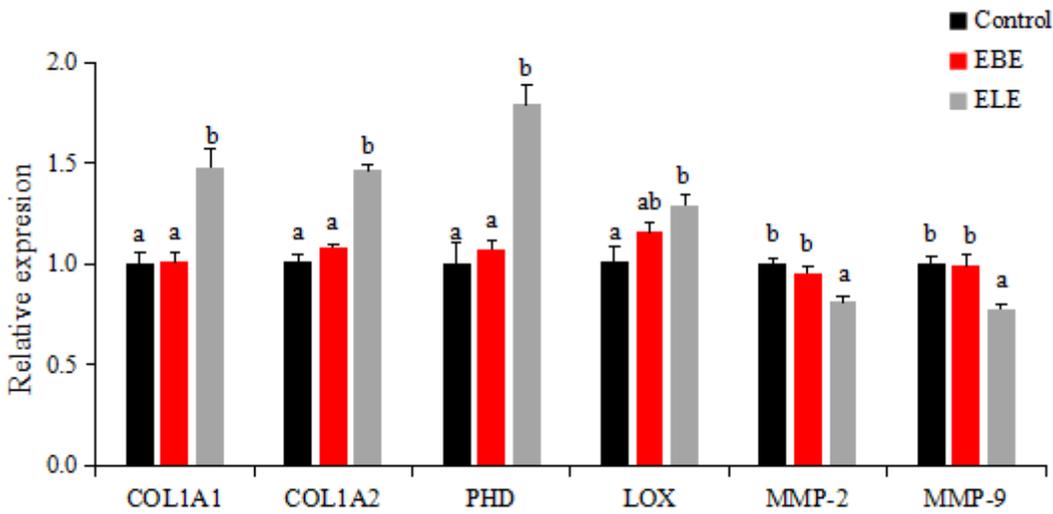


Figure 2

Relative collagen-related genes expression levels in flesh of grass carp fed *Eucommia* extracts diets. Values in the same row with different superscripts alphabets indicate significant differences ($p < 0.05$)