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UNIVERSITÀ DEGLI STUDI DI TORINO

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by:

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Antioxidative activity of the polyphenols from the involucre of *Castanea mollissima* Blume and their mitigating effects on heat stress

S. Dong,^{*} H. Li,^{*} L. Gasco,[†] Y. Xiong,^{*} K. J. Guo,^{*,1} and I. Zoccarato[†]

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ABSTRACT Polyphenols extracted from plants have multiple functions in animal production. To explore new sources of tannin-rich extracts, which have potential benefits for animal health, this study focused on the effects of polyphenolic extracts from involucre of *Castanea mollissima* Blume (PICB) on heat-stressed broilers. *In vitro* experiments were first performed using intestinal cryptlike epithelial cell line-6 (IEC-6) cells to evaluate the effects of PICB on cell proliferation and antioxidative parameters under normal and heat-stress conditions. Then *in vivo* experiments were carried out with 2 trials: in trial 1, 400 one-day-old male Arbor Acres (AA) broilers were randomly assigned to 5 groups (4 replicates/group, 20 chicks/replicate): group 1 was a normal control group fed the basic ration; groups 2 to 5 were fed the basic ration supplemented with 0.2% vitamin C and 0.2%, 0.3%, and 0.4% PICB, respectively. Trial 1 lasted 42 d, and growth performance was monitored every week. At the end of the trial, the chicks

were sacrificed and sampled. In trial 2, 400 twenty-eight-day-old chicks were randomly assigned to 5 groups as described in trial 1. After 1 week of adaptation, heat stress was applied for 7 consecutive days. On days 3 and 7 of heat stress, the chicks were sacrificed and sampled. The results showed that PICB could stimulate IEC-6 cell proliferation and had strong *in vitro* antioxidant activity. PICB had no effect on the growth performance and carcass parameters of AA broilers in trial 1, whereas in trial 2, group 4 saw improved growth performance and antioxidant activity compared to the first three groups ($P < 0.05$). In conclusion, PICB had no effects on the growth performance of IEC-6 cells and AA broilers under normal conditions, whereas it could mitigate heat-stress effects on the growth performance and antioxidant activity of IEC-6 cells and AA broilers, implying that PICB could be used as a suitable additive to improve animal production under heat-stress conditions.

Key words: antioxidative activity, heat stress, *Castanea mollissima* Blume involucre polyphenol, intestinal cryptlike epithelial cell line-6, broiler

INTRODUCTION

Polyphenols, also known as tannins, are widely found in plants and are the fourth most abundant component in plant tissues after celluloses, hemicelluloses, and lignin. Because of their specific structures, polyphenols may have antimicrobial, antiparasitic, and antioxidant activities, which could improve animal performance (Jouany and Morgavi, 2007). Song et al. (2000) and Quideau (2011) also reported that polyphenols have strong radical-scavenging and antioxidant activities. The ethanol extracts from various parts of chestnut were mainly composed of polyphenols (Ogawa et al., 2008; Reinoso et al., 2012; Shi et al., 2013c). Nut, flower, leaf, and fruit extracts from the chestnut tree have shown a strong *in vitro* antioxidant capacity

(Ferreira et al., 2007; Barreira et al., 2008; Dinis et al., 2012). Liu et al. (2011) reported that *Castanea sativa* Mill. wood extracts reduced the malondialdehyde (MDA) content in rabbits while increasing total superoxide dismutase (T-SOD), total antioxidant capacity (T-AOC), and glutathione peroxidase (GSH-Px) activities. However, there is little information on the *in vivo* antioxidative activity of polyphenolic extracts of the involucre of *Castanea mollissima* Blume (PICB).

Because the amount of chestnut involucre was estimated to be 2.5 to 3 times that of chestnut production and most of these were not used properly, which caused environmental pollution, the aims of this paper were to study the antioxidative activity of PICB and its effects on IEC-6 cells and broilers in order to evaluate the possibility of utilizing it as a feed additive. The antioxidant activity of PICB was evaluated through growth performance and some antioxidative parameters, for example, T-SOD and GSH-Px activity, MDA content, and

T-AOC of *in vitro* cell trials and *in vivo* animal trials under normal and heat-stress conditions.

MATERIALS AND METHODS

Chemicals

The involucre of *Castanea mollissima* Blume were collected from Huairou Chestnut Station. The PICB used for animal trials was extracted using the method described by Shi et al. (2013a) and the polyphenol content was approximately 52.5%, while the PICB used for IEC-6 cell trials was purified using the method described by Shi et al. (2013b) with a polyphenol content of approximately 85.2%. The PICB also included non-tannins, crude fiber, ash, and water (Schiavone et al., 2008).

All chemicals and reagents were of analytical grade and were obtained from commercial sources. All water was treated in a water purification system. Vitamin C (VC, ascorbic acid) was obtained from Sigma Chemical Co. (St. Louis, MO, USA).

In Vitro Cell Trials

IEC-6 Cell Culture. IEC-6 cells (CRL21592, obtained from Peking Union Medical College, Beijing, China) were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 5% (v/v) fetal bovine serum (HyClone, Logan, UT, USA), 2 mg/L insulin, 50 IU/mL penicillin, and 50 g/mL streptomycin (complete medium) at 37°C in a 5% (v/v) CO₂ atmosphere. The medium was changed 24 h post-plating.

Dosage Screening of PICB. When IEC-6 cells reached a greater than 95% confluence, they were digested with 0.25% pancreatic enzyme (GIBCO, New York). The PICB was adjusted to concentrations of 0, 0.020, 0.040, 0.060, 0.080, 0.100, and 0.120 mg/mL. 90 µL of the IEC-6 cell suspension was loaded into a 96-well microplate and cultured in DMEM containing a 5% fetal bovine serum at a density of 1×10^5 cells/mL. The PICB solutions (10 µL) at different concentrations were added to the cell suspension. The cells were divided into 14 groups, which were treated with 10 µL of PICB at the different concentrations mentioned previously under normal temperature conditions (NTC) or heat-stress conditions (HSC). Cells in NTC were cultured at 37°C for 44 h, while cells in HSC were first cultured at 37°C for 40 h and then incubated at 41°C for the final 4 h (HSC, according to Guo et al., 2011). At the end of the culture, a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was performed using the method of Yu et al. (2010). All groups were analyzed in triplicate, and the optimal concentration was determined by reading the absorbance at 570 nm. Furthermore, the effects of PICB on IEC-6 cells were compared with those of VC (0.080 mg/mL) (Ma and Liu, 2001) under NTC and HSC.

Effects of PICB on Antioxidative Parameters of IEC-6 Cells. IEC-6 cells were cultured in 75-mL culture bottles. After reaching a confluence of greater than 95%, cells were digested with 0.25% pancreatic enzyme. After centrifugation at 1,000 × g for 10 min at room temperature, cells were collected into 1.5-mL centrifuge tubes and washed twice with PBS. The effects of the predetermined concentrations of PICB and VC on T-AOC, T-SOD, and GSH-Px activities and the MDA and GSH contents of the IEC-6 cells were assayed using colorimetric methods with a spectrophotometer (Leng Guang SFZ1606017568, Shanghai, China). The T-AOC was measured using a ferric-reducing antioxidant power assay (Benzie and Strain, 1996) and detected at 520 nm with the spectrophotometer (Liu et al., 2011). The T-SOD and GSH-Px activities and MDA and GSH contents were measured spectrophotometrically according to the descriptions of Guo et al. (2011) using commercially available kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). The protein content in the cells was determined using the Coomassie brilliant blue method. The measurement units were expressed as units or nanomoles per milligram (U/mg or nmol/mg) of protein.

In Vivo Experiment

Ethical Approval. All experimental protocols were approved by the Committee for the Care and Use of Experimental Animals, Beijing University of Agriculture.

Feed and Bird Husbandry. The trials were carried out at the experimental base of the state key laboratory of the Beijing Institute of Animal Husbandry and Veterinary Medicine, Chinese Academy of Agricultural Science, located in the Changping District of Beijing. Group 1 was fed a basal diet as the control group (reported in Table 1). Group 2 was fed the basal diet supplemented with VC (0.2% of the diet, Jang et al., 2014), which is most widely used in practical feeding, as the positive control group; groups 3 to 5 were fed the basal diet supplemented with 0.2%, 0.3%, and 0.4% PICB, respectively. VC and PICB were initially mixed with 1 kg basal diet and subsequently mixed into an appropriate quantity of basal diet to obtain the prefixed inclusion level. The chicks were managed according to AA broiler feeding and immunization procedures. Broilers were fed and watered ad libitum.

Broilers Feeding under Normal Temperature Conditions. In trial 1, 400 one-day-old male Arbor Acres (AA) broilers (41.36 ± 0.18 g) were randomly assigned to 5 groups (4 replicates/group, 20 chicks/replicate), as described earlier. Involuntary mortality was recorded daily. Individual BW and feed consumption per replicate were recorded weekly from the beginning. Subsequently, the overall BW, ADG, ADFI, and feed conversion ratio (FCR) per replicate were calculated. Trial 1 lasted 42 d. On day 42, 4 chicks per group were randomly chosen from each replicate and fasted for 12 h.

Table 1. Composition and nutrient levels of basic diets (%).

Ingredient	Content	
	1 to 3 weeks	4 to 6 weeks
Corn	59.4	61.9
Soybean meal (46% CP)	25	22.4
Fish meal (68% CP)	8	7
Soybean oil	4	5.5
Limestone	1.2	1.3
CaHPO ₄	1	0.5
NaCl	0.3	0.3
Premix ¹	1	1
DL-Met	0.1	0.1
Total	100.00	100.00
Nutrient levels ²		
ME (MJ/kg)	3.12	3.19
CP	22.05	20.23
Ca	1.02	0.93
AP	0.48	0.39
Lys	1.25	1.13
Met	0.49	0.46

¹Premix provided the following per kilogram of diets: Zn (as zinc sulfate), 75 mg; Fe (as ferrous sulfate), 100 mg; Mn (as manganese sulfate), 100 mg; Cu (as copper sulfate), 8 mg; I (as potassium iodide), 0.45 mg; Se (as sodium selenite), 0.3 mg; vitamin A, 8,800 IU; vitamin D₃, 3,000 IU, vitamin E, 30 IU; vitamin K, 1.65 mg; vitamin B₁₂, 22 µg; vitamin B₁, 1.1 mg; vitamin B, 26.6 mg; pantothenate, 11 mg; vitamin B₃ nicotinic, 66 mg; vitamin B₆, 4.4 mg; biotin, 0.15 mg; choline 1,300 mg.

²Values calculated based on NRC (1994) ingredient composition and NY/T33-2004 AA broiler nutrient requirements.

with ad libitum access to water. Blood samples were collected by radial venipuncture (wing vein) using a syringe with a 25-gauge needle containing 100 µL 0.1M EDTA (anticoagulant) and used for the analyses of antioxidative parameters. The chicks were then weighed and killed by intravenous sodium pentobarbital injection and dissected to measure the weight of the carcass, liver, thigh, and breast and the length of the jejunum. Then the dressing percentage, eviscerated yield percentage, breast muscle percentage, leg muscle percentage, abdominal fat percentage, and hepatosomatic index were calculated.

Broilers Feeding under Heat-Stressed Conditions. In trial 2, 400 twenty-eight-day-old chicks (1,245.87 ± 22.19 g) were randomly assigned to the 5 groups described earlier. After 1 week of adaptation (temperature 24 ± 0.5°C, relative humidity 65 ± 5%), heat stress was applied for 7 consecutive days for all groups (regime: 9:00 to 17:00 temperature 34 ± 0.5°C, relative humidity 85 ± 5%; 17:00 to 9:00 temperature 24 ± 0.5°C, relative humidity 65 ± 5%). Chick feeding and management were arranged in the same way as in trial 1. On days 3 and 7, the chicks were sampled and sacrificed following the same procedure as in trial 1.

Effects of PICB on Antioxidative Parameters of Serum. Sampled serums were centrifuged at 3,000 × g for 15 min. The parameters, including T-AOC, MDA content, T-SOD, and GSH-Px activities, were measured using a commercial kit (Nanjing Jiancheng Bioengineer-

ing Institute, Nanjing, China) (Zhang et al., 2009; Guo et al., 2011; Liu et al., 2011).

Statistical Analysis

The data were expressed as means ± SE. The data set of *in vitro* cell trials was first analyzed using GLM of SPSS19.0 (SPSS Inc., Chicago, IL, USA). When there was no interaction between temperature and treatments, the data, together with those of the *in vivo* trials, were analyzed using one-way ANOVA, followed by Tukey's test using SPSS19.0 for multiple comparisons. The effects of different inclusion levels of PICB were evaluated using a polynomial procedure of ANOVA. A mortality comparison was carried out using a chi-squared procedure of SPSS19.0. $P < 0.05$ was considered significant and $P < 0.01$ was considered highly significant.

RESULTS

Effects of PICB on Proliferation of IEC-6 Cells

Dosage Screening. IEC-6 cells in the HSC groups generally exhibited reduced cell proliferation compared with the NTC groups (Table 2). In the HSC groups, the optical density (OD) values initially increased with increases in PICB concentration and then decreased. The group containing 0.080 mg/mL PICB showed a higher OD value than other HSC groups ($P < 0.05$) while showing no differences with the NTC groups. There were no differences among the NTC groups. Therefore, a concentration of 0.080 mg/mL was used in the subsequent trials.

Comparisons of Cell Proliferations among PICB, VC, and control group (CG). The proliferations of cultured IEC-6 cells are shown in Table 3. For cells cultured at NTC, the OD values of PICB, VC, and CG

Table 2. Effect of different concentrations of PICB on proliferation of IEC-6 cells.^{1,2}

PICB dosage	NTC ³ (OD570)	HSC ⁴ (OD570)
0 mg/mL	0.8359 ± 0.0203	0.7563 ± 0.0030 _{b,c}
0.020 mg/mL	0.9042 ± 0.0136	0.8019 ± 0.0079 _b
0.040 mg/mL	0.8569 ± 0.0046	0.8548 ± 0.0124 _b
0.060 mg/mL	0.8746 ± 0.0173	0.8378 ± 0.0087 _a
0.080 mg/mL	0.8958 ± 0.0046	1.0983 ± 0.0230 _b
0.100 mg/mL	0.9645 ± 0.0047	0.8336 ± 0.0220 _c
0.120 mg/mL	0.9890 ± 0.0302	0.6979 ± 0.0263

^{a-c}Means within a column lacking a common superscript differ ($P < 0.05$).

¹Data are means ± SE for 6 replicates.

²PICB: polyphenolic extract of involucre of *Castanea mollissima* Blume.

³NTC: normal temperature condition where cells were cultured at 37°C for 44 h.

⁴HSC: heat-stressed condition where cells were cultured at 37°C for 40 h then heat-stressed at 41°C for 4 h.

Table 3. Effects of PICB on cell proliferation, MDA and GSH contents, T-SOD and GSH-Px enzyme activities, and T-AOC of IEC-6 cells.^{1,2}

Item	Group ³ Condition ⁴	CG		PICB		VC	
		NTC	HSC	NTC	HSC	NTC	HSC
OD570 nm		0.8052 ± 0.0201 ^a	0.7001 ± 0.0026 ^b	0.8184 ± 0.0426 ^a	0.7906 ± 0.0251 ^a	0.8086 ± 0.0404 ^a	0.7378 ± 0.0095 ^b
MDA (nmol/mg protein)		255.35 ± 13.80 ^c	377.27 ± 27.89 ^a	202.44 ± 3.98 ^a	296.76 ± 24.88 ^{b,c}	213.44 ± 6.90 ^a	310.56 ± 6.90 ^b
T-SOD (U/mg protein)		1271.68 ± 17.03 ^{b,c}	970.48 ± 61.54 ^d	1565.39 ± 68.69 ^a	1248.61 ± 13.99 ^{b,c}	1339.15 ± 39.23 ^{a,b}	1159.84 ± 39.07 ^{c,d}
GSH-Px (U/mg protein)		1737.33 ± 93.19 ^c	1326.92 ± 96.29 ^d	2178.73 ± 55.35 ^a	1622.63 ± 66.88 ^c	1994.46 ± 6.92 ^b	1539.47 ± 82.06 ^{c,d}
GSH (nmol/mg protein)		37.57 ± 0.54 ^b	33.22 ± 1.09 ^d	43.88 ± 1.09 ^a	36.80 ± 1.16 ^b	43.14 ± 1.06 ^a	35.18 ± 0.84 ^{b,c}
T-AOC (U/mg protein)		45.027 ± 6.88 ^{b,c}	32.50 ± 1.79 ^d	59.71 ± 6.16 ^a	42.48 ± 0.89 ^c	51.28 ± 4.56 ^{a,b}	40.33 ± 2.37 ^c

^{a-d}Means within a row lacking a common superscript differ ($P < 0.05$).

¹Data are means ± SE for 6 replicates.

²PICB: polyphenolic extract of involucre of *Castanea mollissima* Blume; MDA: malondialdehyde; GSH: glutathione; T-SOD: total superoxide dismutase; GSH-Px: glutathione peroxidase; T-AOC: total antioxidant capacity.

³CG: control group; PICB: 0.080 mg/mL; VC: Vitamin C, 0.080 mg/mL.

⁴NTC: normal temperature condition where cells were cultured at 37 °C for 44 h; HSC: heat-stressed condition where cells were cultured at 37 °C for 40 h then heat-stressed at 41 °C for 4 h.

were not different among groups. For cells cultured at HSC, the PICB group showed larger absorbance values than the VC group ($P = 0.034$) and CG ($P = 0.015$), while there were no differences between PICB groups at HSC and CG at NTC ($P = 0.232$).

Effects of PICB on Antioxidant Status of IEC-6 Cells

The antioxidant status of different groups was measured. Heat stress reduced T-AOC and GSH contents and T-SOD and GSH-Px activities but increased MDA content compared with the corresponding values observed in cells cultured at NTC ($P < 0.05$, Table 3). The application of PICB and VC mitigated these changes at different levels and the PICB group at HSC was no different from CG at NTC for all the parameters concerned. At both NTC and HSC, the MDA contents of the PICB and VC groups were less than that of the CG group ($P < 0.05$). The application of PICB and VC could increase the T-SOD activity, and the T-SOD activities of the PICB and VC groups at HSC were greater than that of CG at HSC ($P = 0.016$ and 0.036 , respectively) and similar to that of CG at NTC. The GSH-Px activity of CG at HSC was less than that at NTC ($P = 0.021$). The GSH-Px activity of the PICB group was greater than that of CG with the VC group in the middle. At NTC, the GSH content of the PICB group was no different from that of the VC group ($P = 0.778$), and both were larger than that of the CG group ($P = 0.026$ and $P = 0.037$ respectively). At HSC, only the GSH content of the PICB group was higher than that of the CG group ($P = 0.045$). At HSC, the T-AOC activities of the PICB group were no different from those of the VC group, but both were higher than those of the CG group ($P = 0.023$ and $P = 0.030$, respectively). Therefore, PICB demonstrated to have strong *antioxidative activities on cells*.

Effects of PICB on Growth Performance of AA Broilers under Normal Conditions (Trial 1)

The effects of PICB on the growth performance of AA broilers are shown in Figure 1. The weights of different groups at different stages were in the ranges of AA broilers (Figure 1a). There were no differences among groups ($P > 0.05$).

The addition of PICB did not affect the ADG during the first 3 weeks (Figure 1b). In the fourth week, the ADG of group 3 was less than that of group 4 ($P = 0.043$). In weeks 5 and 6, the ADGs of group 3 were greater than those of the control group ($P = 0.036$ and $P = 0.032$, respectively). There were no differences in the ADFI during the first 4 weeks (Figure 1c). The ADFI of group 3 at week 5 was greater than that of the control group ($P = 0.037$). It was greater than those of both groups 1 and 2 ($P = 0.041$ and $P = 0.045$, respectively) in week 6. The FCR generally showed no differences among the groups at different stages (Figure 1d), except that in week 4, the FCR of group 3 was greater than that of the other groups ($P < 0.05$).

Even though there were oscillations for the values of ADG, ADFI, and FCR among the different groups during the second half of the trial, the effects of PICB on the growth performance of AA broilers in trial 1 showed no differences at different stages (Table 4). From day 1 to 21, the ADFI had a negative linear response to the increasing levels of PICB inclusion ($P = 0.037$). The ADFI of the entire trial 1 tended to have a quadratic response to the increasing PICB level ($P = 0.070$). The death rate was no different among the groups.

Analysis of the antioxidative parameters showed that there were no differences among the groups for T-AOC (9.00 to 11.01 U/ml), T-SOD (44.85 to 51.28 U/ml), MDA (1.31 to 2.24 nmol/ml), and GSH-Px (254.21 to 295.11 U/ml). At dissection, no gross anatomic-pathological lesions were observed on the organs. The average value for carcass weight was 2,117.72 g, and

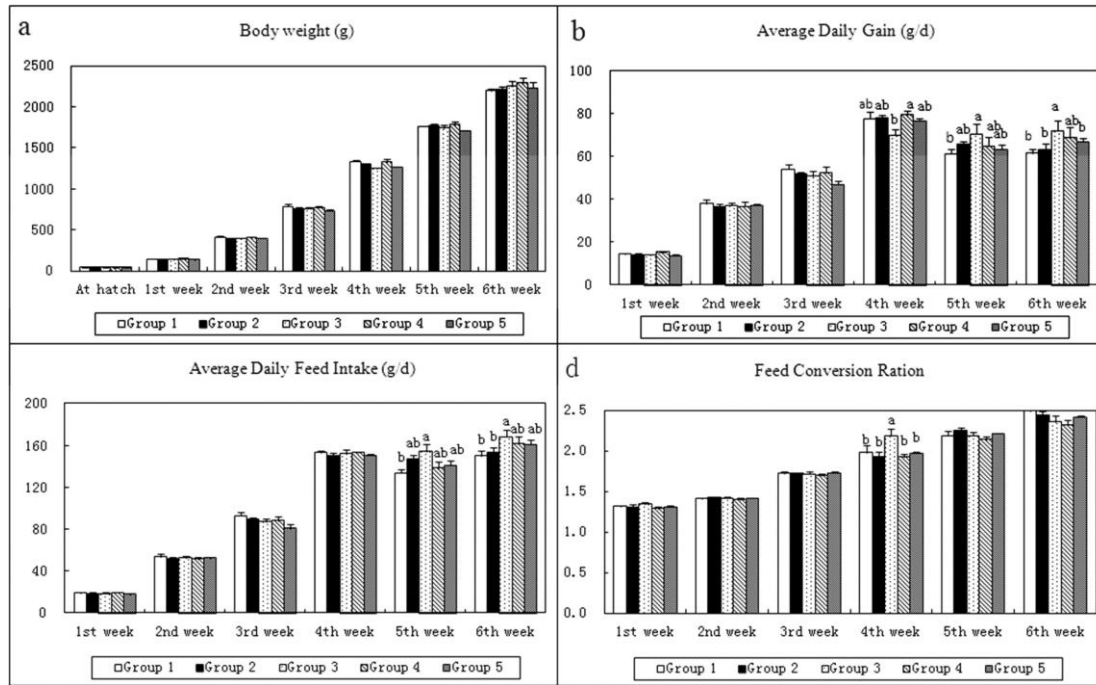


Figure 1. Effects of PICB on growth performance of AA broilers at different weeks. PICB: polyphenolic extract of the involucre of *Castanea mollissima* Blume. Group 1: chicks fed basal diet; Group 2: chicks fed the basal diet supplemented with vitamin C; Groups 3–5: chicks fed basal diet supplemented with 0.2%, 0.3%, and 0.4% PICB, respectively. Value of columns with different lowercase letters indicate difference ($P < 0.05$).

Table 4. Effects of PICB on growth performance of AA broilers at different growth stages in trial 1.^{1,2}

Items ³	Group ⁴					Polynomial ⁵
	1	2	3	4	5	
Initial body weight (g)	41.22 ± 0.48	41.26 ± 0.53	41.38 ± 0.47	40.88 ± 0.45	41.40 ± 0.42	
D 21 BW (g)	786.07 ± 26.78	763.47 ± 17.80	757.58 ± 13.75	774.57 ± 22.45	729.45 ± 15.58	
1 to 21 d						
ADG (g)	35.47 ± 1.23	34.39 ± 0.65	34.10 ± 0.72	34.94 ± 1.02	32.76 ± 0.69	
ADFI (g)	55.07 ± 1.52	53.59 ± 1.36	52.95 ± 0.99	53.59 ± 1.17	50.68 ± 1.36	1
FCR	1.55 ± 0.01	1.56 ± 0.01	1.55 ± 0.01	1.53 ± 0.01	1.55 ± 0.01	
D 42 BW (g)	2189.25 ± 15.75	2165.05 ± 25.53	2249.57 ± 62.48	2265.85 ± 62.59	2230.08 ± 57.36	
22 to 42 d						
ADG (g)	66.82 ± 1.20	66.74 ± 1.15	71.05 ± 3.48	71.01 ± 2.89	71.46 ± 3.71	
ADFI (g)	115.86 ± 2.46	116.94 ± 2.07	126.85 ± 3.65	119.78 ± 3.18	120.05 ± 2.03	
FCR	1.73 ± 0.03	1.75 ± 0.02	1.79 ± 0.04	1.69 ± 0.03	1.68 ± 0.06	
1 to 42 d						
ADG (g)	51.14 ± 0.58	50.57 ± 0.76	52.58 ± 1.02	52.98 ± 1.27	52.11 ± 1.41	
ADFI (g)	85.56 ± 1.46	85.03 ± 1.52	89.68 ± 1.41	87.03 ± 1.32	85.15 ± 0.32	
FCR	1.67 ± 0.02	1.68 ± 0.01	1.71 ± 0.02	1.64 ± 0.02	1.63 ± 0.04	
Mortality rate (%)	7.20 ± 1.15	5.23 ± 0.96	4.59 ± 1.81	3.54 ± 1.22	3.96 ± 1.09	

¹Data are means ± SE for 4 replicates of 20 chicks/pen.

²PICB: polyphenolic extract of involucre of *Castanea mollissima* Blume.

³FCR = feed conversion ratio.

⁴Group 1: chicks fed basal diet; group 2: chicks fed basal diet supplemented with vitamin C; groups 3–5: chicks fed basal diet supplemented with 0.2%, 0.3%, and 0.4% PICB, respectively.

⁵1 = linear effect of dietary inclusion of PICB (group 1 vs. groups 3–5; $P < 0.05$).

dressing percentages ranged from 94.59 to 95.84%. The percentages of eviscerated yield were approximately 79.50%. The abdominal fat percentage oscillated from 1.88 to 1.36%. Thigh percentages of carcass varied from 24.18 to 26.06, while breast percentages of carcass varied from 29.66 to 31.60. With the increase in PICB inclusion level, all the foregoing parameters had a numerical trend toward increasing, except that abdominal

fat percentage tended to decrease ($P = 0.067$), while no differences were observed. Mean length of the jejunum was $1,110.55 \pm 9.58$ mm, which was in the normal range for AA broilers. No differences ($P > 0.05$) were observed in the hepatosomatic index (HSI; liver weight/BW × 100) among the 5 groups (1.97 ± 0.39 , 2.04 ± 0.31 , 1.76 ± 0.14 , 1.83 ± 0.08 , 1.64 ± 0.14 , respectively). These results showed that PICB inclusion

Table 5. Effects of PICB on growth performance of heat stressed AA broilers in trial 2.^{1,2}

Items ³	Group ⁴					Polynomial ⁵
	1	2	3	4	5	
Initial BW (g)	1,249.93 ± 27.54	1,265.29 ± 18.39	1,221.54 ± 29.37	1,268.48 ± 21.48	1,224.11 ± 30.25	
BW after adaptation (g)	1,737.92 ± 38.49 _c	1,743.26 ± 27.99	1,720.08 ± 39.26	1,751.06 ± 25.37	1,708.82 ± 16.56	
Final BW (g)	1,962.12 ± 39.62	2,013.89 ± 10.91 _b	2,000.65 ± 5.75 _{bc}	2,137.51 ± 16.58 ⁺	2,057.26 ± 7.14 _b	L, Q, C
ADG (g)	32.03 ± 1.81 _c	38.66 ± 0.82 _b	40.08 ± 2.25 _b	55.21 ± 2.50 _a	49.78 ± 1.25 _a	L, Q, c
ADFI (g)	115.06 ± 6.32 _a	136.95 ± 3.14 _a	137.89 ± 3.34 _{a,b}	167.82 ± 2.00 _c	156.03 ± 2.22 _{b,c}	L, Q, c
FCR	3.59 ± 0.02	3.54 ± 0.01	3.44 ± 0.10	3.04 ± 0.10	3.13 ± 0.05	L, Q
Mortality rate (%)	15.63 ± 0.44	12.25 ± 0.27	12.10 ± 0.79	10.23 ± 0.56	9.14 ± 0.93	L

^{a-c}Means within a row lacking a common superscript differ ($P < 0.05$).

¹Data are means ± SE for 4 replicates of 20 chicks/pen.

²PICB: polyphenolic extract of involucre of *Castanea mollissima* Blume.

³FCR = feed conversion ratio.

⁴Group 1: chicks fed basal diet; group 2: chicks fed basal diet supplemented with vitamin C; groups 3–5: chicks fed basal diet supplemented with 0.2%, 0.3%, and 0.4% PICB, respectively.

⁵L = linear effect of dietary inclusion of PICB (group 1 vs. groups 3–5; $P < 0.01$); Q = quadratic effect of dietary PICB inclusion level ($P < 0.01$); C = cubic effect of dietary PICB inclusion level ($P < 0.01$); c = cubic effect of dietary PICB inclusion level ($P < 0.05$).

had no effect on chicken growth performance and health under suitable conditions.

Effects of PICB on Growth Performance of Heat-Stressed AA Broilers

In trial 2, the BWs of different groups were no different following adaptation ($P > 0.05$, see Table 5). At day 7 of heat stress, broilers' BW of the control group was smaller than that of other groups except for group 3. Group 4 had a value larger than all other groups ($P < 0.05$). BWs showed linear, quadratic, and cubic responses to the increasing levels of PICB inclusion ($P < 0.001$, $P < 0.001$, $P < 0.001$, respectively). The ADG of group 4 was highest among the groups and that of group 1 was lower than other groups ($P < 0.05$). A similar situation held for ADFI. For both ADG and ADFI, there were linear, quadratic ($P < 0.001$, $P < 0.001$, respectively), and cubic responses ($P = 0.022$) with the increasing levels of PICB inclusion. The FCRs of groups 4 and 5 were smaller than those of groups 1 and 2 ($P < 0.05$). With the increasing levels of PICB inclusion, FCR showed linear ($P < 0.001$) and quadratic responses ($P = 0.039$). Mortality tended to show a linear response ($P = 0.059$) to the increasing levels of PICB, with the highest supplementation of PICB leading to the lowest mortality rate.

At dissection, no gross anatomic-pathological lesions were observed on the organs. The dressing percentages, abdominal fat percentage, percentage of eviscerated yield, breast muscle percentage, and leg muscle percentage were in the normal ranges and were no different at day 3 or 7 of heat stress.

Effects of PICB on Antioxidant Parameters of AA Broilers under Heat Stress

Supplementation with PICB affected T-AOC, T-SOD, and GSH-Px activities and the MDA con-

tent in the serum of AA broilers under heat stress (Figure 2). T-AOC in broiler serum underwent some oscillation without differences among groups ($P > 0.05$) at day 3 of heat stress, while at day 7 of heat stress, the T-AOC in group 4 was higher than those of groups 1 ($P = 0.029$) and 2 ($P = 0.014$). T-AOC in group 3 was greater than in group 2 ($P = 0.020$). T-AOC had linear responses to the increasing levels of PICB inclusion ($P = 0.019$). At both days 3 and 7 of heat stress, T-SOD activity showed a linear response ($P = 0.042$ and $P = 0.013$, respectively) to the increasing PICB inclusion levels. At the former date, group 4 had higher T-SOD values than group 1 ($P = 0.048$), while at the latter date, T-SOD in the other four groups was greater ($P < 0.05$) than that of the control group. At day 3 of heat stress MDA content in group 1 was greater than in groups 3 to 5 ($P < 0.05$), with the value of group 2 in the middle showing no difference between the two groups. MDA decreased linearly with increases in PICB inclusion ($P < 0.001$), while at day 7 of heat stress, there were no differences among groups ($P > 0.05$). Regarding GSH-Px activity, the values in groups 2 to 5 were greater than that of the control group ($P < 0.05$) at day 3 of heat stress. At day 7 of heat stress, only the value in group 3 was greater than that in group 5 ($P = 0.048$). GSH-Px activity had highly quadratic responses to the increasing levels of PICB inclusion ($P = 0.003$).

DISCUSSION

Antioxidative Effects of PICB on IEC-6 Cells

Effects of PICB on Proliferation of IEC-6 Cells.

Since the establishment and characterization of the IEC-6 cell line, these cells have been used extensively to elucidate the mechanisms of cell growth and wound healing, to investigate the effects and actions of cytokines and growth factors, and to study the extra-cellular matrix regulation of differentiation and the effects of endotoxins and infection (He et al., 1993; Meyer

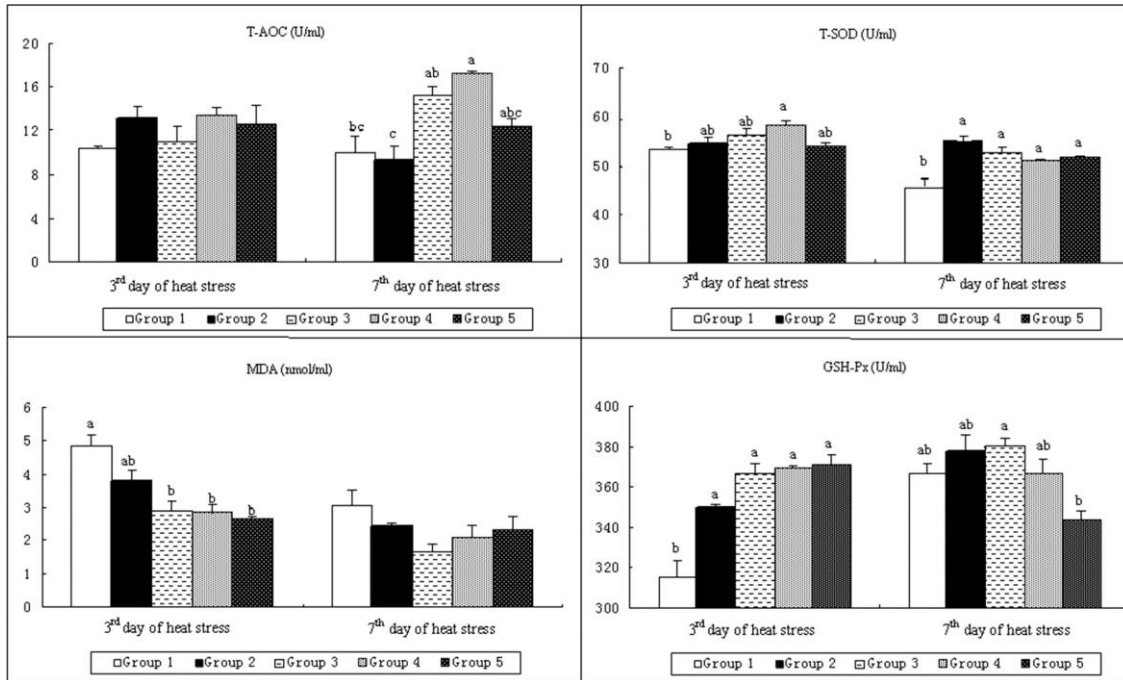


Figure 2. Effects of PICB on antioxidant parameters of AA broilers. PICB: polyphenolic extract of involucre of *Castanea mollissima* Blume. Group 1: chicks fed basal diet; Group 2: chicks fed basal diet supplemented with vitamin C; Group 3–5: chicks fed basal diet supplemented with 0.2%, 0.3%, and 0.4% PICB, respectively; T-AOC: total antioxidant capacity; T-SOD: total superoxide dismutase; MDA: malondialdehyde; GSH-Px: glutathione peroxidase. Value of columns with different lowercase letters mean difference ($P < 0.05$).

et al., 1994; Wood et al., 2003). Because heat stress can easily cause disorders in the oxidative balance of cells and animals, the heat-stress model established by Guo et al. (2011) was employed as the culturing condition to evaluate the regulating effects of PICB on antioxidant status and lipid peroxidation in IEC-6 cells. With the increasing concentration of PICB, cell proliferation increased under NTC and HSC. However, for the HSC groups, when the PICB concentration exceeded a certain threshold (0.080 mg/mL), cell proliferation decreased, which could be explained by the possible toxicity of extra PICB toward the IEC-6 cells. PICB enhanced the proliferation of IEC-6 cells impaired by heat stress, while VC had no effect on the proliferation of IEC-6 cells under heat stress.

Effects of PICB on Antioxidant Property of IEC-6 Cells. Morrison et al. (2005) and Zhang et al. (2006) reported that high temperatures led to changes in cell oxidative status and the accumulation of reactive oxygen species (ROS), causing decreases in T-AOC, T-SOD, and GSH-Px and increases in MDA content (Circu and Aw, 2010). With respect to the antioxidant status of IEC-6 cells treated with PICB and VC, PICB can improve IEC-6 cells' antioxidative capacity both under normal conditions and heat-stress conditions for all parameters concerned, while VC shows good antioxidative capacities in certain cases. It is important to point out that the antioxidant status of heat-stressed IEC-6 cells treated with PICB was no different from that of IEC-6 cells under normal conditions without any treatment, which implied that PICB was a good antioxidant and worthy of being used *in vivo*.

Effects of PICB on Growth Performance of AA Broilers under Normal Temperatures

The decision to use a particular feed additive should be based on whether or not it affects animal growth performance under suitable conditions. In this study, even though there were some oscillation in ADG, AFDI, and FCR values in different weeks, neither the additions of various doses of PICB nor the supplementation of VC affected the growth performance of AA broilers. These results were similar to those of Schiavone et al. (2008), who concluded that chestnut wood extract (containing a polyphenol content similar to that of PICB) had no effect on the final weight of broilers, but it did improve daily gain during the first 2 weeks. A similar conclusion was drawn by Zoccarato et al. (2008) feeding chestnut wood extract to meat rabbits without affecting production performance.

Subsequent analyses showed that PICB had no effect on the slaughtering parameters and antioxidant parameters of AA broilers, and no visible pathological lesions were found in the organs of AA broilers, which implied that PICB could be used as a safe feed additive.

Effects of PICB on Growth Performance of AA Broilers under Heat Stress. Oxidative stress is common in poultry production, and heat stress is one of the main causes of oxidative stress. Since broilers have no sweat glands and are fully covered with feathers, thermoregulation is challenged in hot weather. Under heat stress, the accumulated ROS could damage intestinal mucosa and cause increased muscle protein hydrolysis. Feed intake, BW gain, and FCR would be reduced as a

result of adaptive responses to the high environmental temperatures (Gu et al., 2008). Exogenous antioxidants may help animals maintain healthy intestinal mucosa, restore oxidative balance, and improve growth performance. Wang et al. (2008) reported that *Forsythia suspensa* extract could improve the growth performance of broiler chickens under high temperature conditions. Selim et al. (2013) indicated that among the examined natural additives, aqueous extract of beetroot improved overall BW gain, while adding ginger root extract to broiler diets decreased feed consumption under environmental temperatures of 35 to 41°C and humidity of 30 to 45%. Liu et al. (2009) found that diet supplemented with chestnut wood extract could improve ADG and reduce the FCR of rabbits during summertime.

In this study (trial 2), most broilers showed typical symptoms of heat-stress syndrome, such as, for example, depression, stretching neck with open mouth, fever, panting, and accelerated breathing, which is in accord with Wang et al. (2008), Yang et al. (2011) and Jang et al. (2014), who reported that at similar temperatures and humidity broiler chicks suffered heat stress as evidenced by the behavior, physiology, and mRNA expression of antioxidant parameters, proinflammatory cytokines, and heat-shock protein 70. Compared with the control group with no additive and the positive control group supplemented with VC, 0.3% PICB improved ADG and AFDI values while decreasing the FCR. Because VC is widely used as an antioxidant in commercial applications, this result implied that PICB could be used as a plant extract substitution of chemical antioxidants. However, further verification should be carried out.

Under heat stress, broiler mortality tended to decrease with the supplementation of PICB, which was in agreement with Zoccarato et al. (2008), who drew a similar conclusion regarding the mortality of rabbits fed chestnut wood extract. The reason for this could be that heat stress modified the metabolism and enzymatic response of broilers (Yang et al., 2011) and supplementation of PICB mitigated these modifications.

Ain Baziz et al. (1996) reported that heat stress could reduce protein deposition and improve fat synthesis capacity, thereby increasing abdominal fat percentage. However, in this study, no difference was found among groups with respect to dressing percentage, abdominal fat percentage, percentage of eviscerated yield, breast muscle percentage, or leg muscle percentage. The reason for this could be that the 7-d period of heat stress was too short to demonstrate any difference.

Effects of PICB on Antioxidant Parameters of AA Broilers under Heat Stress

Heat stress can cause the body to produce excess ROS, which could affect animal antioxidant status and lipid peroxidation levels (Gu et al., 2008). In this study, chicks supplemented with 0.3% PICB had T-AOC val-

ues that were higher than chicks in the control group supplemented with VC at day 7 of heat stress. For T-SOD, GSH-Px, and MDA, chicks supplemented with PICB had improved values over chicks in the control group and chicks supplemented with VC at both the third and seventh days of heat stress. This indicated that PICB could mitigate oxidative stress, which could be the main reason for the better performance of the treated broilers.

In conclusion, purified PICB can stimulate IEC-6 cell proliferation and has strong *in vitro* antioxidant activity under normal temperatures and heat-stressed conditions. The optimal concentration of purified PICB was 0.080 mg/mL. The addition of PICB in broiler diets had no effects on ADG, AFDI, FCR, or mortality under suitable conditions, which indicated that PICB had no negative effects on AA broilers. Under heat-stress conditions, PICB could improve the growth performance of broilers and mitigate the effects of heat stress. A dosage of 0.3% PICB could improve ADG and AFDI and restore the redox status of AA broilers.

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