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Use of a hydroalcoholic extract of *Salix alba* L. bark powder in diets of broilers exposed to high heat stress

M. Saracila[1,2#], T.D. Panaite[1], C. Soica[1], C. Tabuc[1], M. Olteanu[1], C. Predescu[2], C.M. Rotar[1] & R.D. Criste[1]

[1] National Research-Development Institute for Animal Biology and Nutrition (IBNA), 1, Calea Bucuresti, Balotesti, 077015, Ilfov, Romania

[2] University of Agronomic Sciences and Veterinary Medicine, Faculty of Veterinary Medicine, 105 Splaiul Independentei, 050097, Bucharest, Romania

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Abstract

A study was conducted to determine the effects of dietary hydroalcoholic willow bark extract powder (HWE) supplemented to broilers (14–42 days old) that were exposed to heat stress, on the performance, serum biochemical parameters, liver oxidative status and caecal microflora. The feeding trial was conducted on 120 Cobb 500 broilers (14 days old), assigned to three treatments (T0, T25, and T50), each treatment consisting of eight replicates (five chicks per replicate). The broilers were housed in an experimental hall at a 32 °C constant temperature and 23 hours light regimen. Unlike the dietary control treatment (T0), the experimental treatments were supplemented with 25 g HWE powder/100 kg diet (T25), and 50 g HWE powder 100 kg diet (T50), respectively. Dietary HWE powder did not affect the broilers' performance significantly (14–42 days). A significantly lower amount of malondialdehyde was noticed in the liver of broilers from T25 and T50 treatments in comparison with broilers from T0. Also, the serum cholesterol, triglycerides and alanine aminotransferase were significantly lower in broilers fed with T50, compared with those fed with T0. At 35 and at 42 days, the broilers from T25 and T50 recorded a significantly lower number of *E. coli* and staphylococci and a higher number of lactobacilli in the caecum than those of T0. It could be concluded that supplementation of dietary HWE powder reduced some of the adverse effects of heat stress, the most effective being the level of 50 g/100 kg diet.

Keywords*:* biochemical parameters, caecal microflora, high temperature, liver oxidative status
[#]Corresponding author: mihaela.saracila@yahoo.com

Introduction

Global warming as a result of increased industrialization and environmental degradation has led to a continuous increase in ambient temperature, thereby making heat stress a major problem of livestock farming, particularly in the poultry sector (Daghir, 2009; Pirgozliev *et al.*, 2019). Modern poultry species such as broiler chickens are highly sensitive to heat stress because of feather cover, lack of sudoriferous glands and fast growth (St-Pierre *et al.*, 2010; Piestun *et al.*, 2013). A temperature above 30 °C represents a heat-stressed condition for birds and is one of the most common stressors that affect the production criteria in poultry (Kamboh *et al.*, 2013; Ma *et al.*, 2015). Studies on broilers showed that heat stress disrupted the equilibrium between antioxidants and reactive oxygen species (Gu *et al.*, 2012; Nisar *et al.*, 2013), increased tissue damage (Abidin & Khatoon, 2013; Huang *et al.*, 2018), impaired metabolic function (Habibian *et al.*, 2014), and even changed the bacterial composition in the intestine (Wang *et al.*, 2018; Shi *et al.*, 2019).

An additional pressure factor for the European poultry producers, besides heat stress, is the ban on antibiotic growth promoters in poultry diets in Europe (Castanon, 2007). Thus, the industry must look for viable alternatives that can improve performance, protect animal health and maintain profit margins (Basmacıoğlu *et al.*, 2004; Yegani & Korver, 2008; Tugay *et al.*, 2015). In recent years, poultry producers and nutritionists have paid greater attention to the use of bioactive compounds from natural resources in broiler diets to alleviate the impact of high environmental temperature, such as changes in feeding and drinking water management. Seidavi *et al.* (2018) reported that many products in the form of powders,

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extracts and essential oils from many trees and shrubs have been included in broiler diet because of their bacterial, antioxidant, and cholesterol lowering properties. In this context, feed additives that contain phenolics could improve the resistance of broilers to heat stress (Chang Song *et al.*, 2017), lower the rectal temperature (Al-Fataftah & Abdelqader, 2013) and reduce pathogens such as *C. perfringens* and *E. coli* in the broiler gut (Jakubcova *et al.*, 2014). These effects are due largely to their well-known antioxidant and antibacterial activity (Gavris *et al.*, 2019). Many phytoadditives have been used in the diets of broilers that are reared under heat stress, such as grape seed extract (Hajati *et al.*, 2015), oregano (Criste *et al.*, 2017), peppermint (Arab Ameri *et al.,* 2016), artemisia (Panaite *et al.*, 2018; Saracila *et al.*, 2018), rosehip (Criste *et al.*, 2017; Vlaicu *et al.*, 2017), and willow bark hydroglyceroalcoholic extract (Saracila *et al.*, 2018). These feeding studies aimed to determine the effects of these sources on broiler performance, serum biochemical parameters and the balance of gut microflora.

A liquid extract obtained from boiling willow bark *(Salix alba)* has been used by southern Romanian farmers as a traditional remedy for chicks with enteritis. The European Pharmacopoeia defines willow bark as the whole or fragmented dry bark of young shoots or dry branches of various *Salix* species, including *Salix purpurea* L., *Salix daphnoides ViLL.* and *Salix fragilis* L. (Nahrstedt *et al.*, 2007). Its use has been widely accepted following the positive monographs of European Scientific Cooperative on Phytotherapy (ESCOP) and the European Commission, and is supported by many clinical studies. Zabihi *et al.* (2018) show that although salicin is the main active compound of the willow bark, its polyphenols and flavonoids must receive due attention. Other authors (Sulaiman *et al.*, 2013; Shara & Stohs, 2015) showed that willow bark contains flavonoids and polyphenols that contribute synergistically to the beneficial effects of *Salix alba* and may be more important than those of synthetic salicylic acid. *Salix alba* extract has free radical scavenging activity and can be used as a radical inhibitor or scavenger, with the possibility of acting as a primary antioxidant (Sulaiman *et al.*, 2013). When willow bark is used, its toxicity is much less than aspirin toxicity, owing to the low levels of salicylates in the plant products (Altınterim, 2013). Implications of human use of Salix as a source of acetylsalicylic acid have been studied (Mahdi, 2010; Vlachojannis *et al*., 2011; Ishikado *et al*., 2013). However, only a few studies have been conducted over the last 5-10 years on the effect of using Salix species extracts on broilers that are raised under heat-stress conditions. For example, Al-Fataftah & Abdelqader (2013) found that *Salix babylonica* extract improved heat tolerance, feed intake, body weight gain, and feed conversion ratio, and reduced mortality of heat-stressed broilers (35 °C) with similar efficacy to acetylsalicylic acid. El-Soud *et al.* (2006) observed significant decreases in serum cholesterol levels of Japanese quail under heat stress that were fed diets containing 0.025%, 0.05% or 0.1% acetylsalicylic acid.

Effects of diet supplementation with powdered hydroalcoholic willow bark extract on performance, health and oxidative status and caecal microflora of broilers exposed to heat stress were not found in the literature. Therefore, the present study was conducted to investigate whether dietary supplementation of broiler chicks between the ages of 14 and 42 days with HWE powder alleviates the negative effects of heat stress on performance, serum biochemical parameters, liver oxidative status and the caecal microflora of broilers.

Materials and Methods

The feeding trial was conducted in an experimental hall at the Laboratory of Chemistry and Nutrition Physiology of the National Research Development Institute for Animal Biology and Nutrition (IBNA-Balotesti, Romania) according to an experimental protocol (no. 3005/15.05.2018). This protocol was approved by the Ethics Commission of the Institute.

The feeding trial used a total of 120 Cobb 500 broiler chicks, males and females, which were purchased from a local hatchery (SEMAR Trading S.R.L., Ploiesti, Romania). Upon arrival, the chicks were weighed individually and randomly housed in three-tiered digestibility cages (5 chicks/cage), allowing a daily recording of the feed intake and excreta. Throughout the experimental period, the environmental temperature of the experimental hall was kept constant at 32 °C. The humidity was 36%, with 0.38% ventilation/broiler, and 899 ppm CO_2 emission. The light regimen was appropriate to the age of the chicks, that is, 23 hours light/1 hour darkness. Up to the age of 14 days, they received a commercial starter diet (3000 kcal/kg metabolizable energy, 22% crude protein). At 14 days, the chicks were weighed individually and assigned to three treatments (T0, T25, and T50), each treatment consisting of 8 replicates of 5 chicks/ replicate and thus 40 chicks/treatment. At the onset of the experiment (14 days) the chicks assigned to each treatment were similar in bodyweight: 400.64 ± 11.85 g (T0), 400.67 ± 10.35 g (T25), and 400.36 ± 9.56 g (T50). Table 1 shows the composition of the grower (24–35 days) and finisher (35–42 days) diets. Unlike the dietary control treatment (T0), the chicks that were assigned to the treatments T25 and T50 were supplemented with 25 g HWE powder/100 kg diet and 50 g HWE powder/100 kg diet, respectively (Table 1).

Table 1 Formulation of diets for broiler chicks to test the efficacy of hydroalcoholic willow bark extract powder in mitigating the effects of heat stress

Ingredient	Grower stage (14–35 days)			Finisher stage (35–42 days)		
	T0	T25	T50	T0	T25	T50
%						
Corn	62.000	61.975	61.950	60.500	60.475	60.000
Soybean meal	26.580	26.58	26.580	25.460	25.460	25.460
Gluten	4.000	4.000	4.000	6.000	6.000	6.000
Oil	2.500	2.500	2.500	3.750	3.750	3.750
Hydroalcoholic willow bark extract powder		0.025	0.050		0.025	0.050
Calcium carbonate	1.400	1.400	1.400	1.330	1.330	1.330
Monocalcium phosphate	1.360	1.360	1.360	1.130	1.130	1.130
Salt	0.370	0.370	0.370	0.330	0.330	0.330
Methionine	0.260	0.260	0.260	0.250	0.250	0.250
Lysine	0.480	0.480	0.480	0.20	0.200	0.200
Choline	0.050	0.050	0.050	0.050	0.050	0.050
Vitamin-mineral premix*	1.000	1.000	1.000	1.000	1.000	1.000
Ration composition						
Metabolizable energy, kcal/kg	3250.00	3250.00	3250.00	3108.00	3108.00	3108.00
Dry matter, %	90.16	90.44	90.30	89.54	89.71	89.50
Crude protein, %	22.41	22.19	19.92	21.65	21.49	21.67
Ether extractives, %	4.16	4.78	4.60	5.67	5.57	5.70
Crude fibre, %	4.70	4.25	4.62	3.68	3.49	3.89
Ash, %	6.43	5.67	6.26	5.63	5.82	5.25
Calcium, %	0.84	0.83	0.83	0.82	0.83	0.81
Phosphorus, %	0.72	0.75	0.72	0.74	0.73	0.71

*1kg premix contains 1100000 IU/kg vitamin A; 200000 IU/kg vitamin D3; 2700 IU/kg vitamin E; 300 mg/kg vitamin K; 200 mg/kg Vit. B1; 400 mg/kg vitamin B2; 1485 mg/kg pantothenic acid; 2700 mg/kg nicotinic acid; 300 mg/kg vitamin B6; 4 mg/kg Vit. B7; 100 mg/kg vitamin B9; 1.8 mg/kg vitamin B12; 2000 mg/kg vitamin C; 8000 mg/kg manganese; 8000 mg/kg iron; 500 mg/kg copper; 6000 mg/kg zinc; 37 mg/kg cobalt; 152 mg/kg iodine; 18 mg/kg selenium

The powdered hydroalcoholic willow bark (Salix alba L.) extract containing 98% salicin was purchased from a commercial company in China (Changsha Vigorous-tech Co., Ltd). The extract had been obtained from dry bark, using hydroalcoholic extraction in grain alcohol and water. Feed and water were provided for ad libitum consumption. Diet formulations were calculated to meet or exceed the minimum requirements for broiler chicks as recommended by Cobb-Vantrese, Inc. (Anonymous, 2015). All diets were fed in mash form.

Throughout the experimental period (14–42 days of broiler age) bodyweight (g), average daily feed intake (g feed/broiler/day) were monitored and average daily weight gain (g/broiler/day), and feed conversion ratio (FCR, g feed/g gain) were calculated. Individual bodyweight was recorded weekly. The experiment protocol stipulated that mortality should be recorded daily throughout the experiment.

At 42 days of age, and before slaughter, eight chicks per treatment with bodyweight within ± 10 g standard deviation of the mean treatment weight had their blood aseptically collected from the brachial vein into 9-mL Vacutainers containing 14.3 U/mL of lithium heparin (Vacutest®, Arzergrande, Italy). These samples were used to determine the serum lipid, protein, and enzyme profiles.

One bird from each replication (eight birds per treatment), with bodyweight within ± 10 g standard deviation of the mean treatment weight, was slaughtered on days 35 and 42 by cervical dislocation and bled immediately. Carcasses were eviscerated manually and the gut was carefully excised from the oesophagus to the cloaca. Caecal contents (two caeca per bird) were collected aseptically in sterilized plastic tubes and preserved at -20 °C until the bacteriological analyses (*E. coli*, staphylococci, lactobacilli, *Salmonella spp*). Any digesta that remained in the two caeca was removed with gentle pressure. Liver tissue was collected

after removing fat and connective tissue. Liver samples were vacuum packed and stored at −80 °C until analysis.

The total phenol content of HWE powder was measured spectrophotometrically according to the Folin-Ciocalteu method, as described by Untea *et al.* (2018). The principle is to record the absorbance of a basic extract which, by complexation with the Folin-Ciocalteu reagent, absorbs in Vis domain at λ = 732 nm, wavelength. The methanol extract was obtained by extracting 2 g of sample in 20 mL methanol (80%), and kept on a rotary shaker for 24 hours in the dark. The extract was centrifuged at a relative centrifugal force of 1500 times gravity for 10 min and the supernatant was considered for analysis. The calibration curve of gallic acid was used to determine total phenol compounds, and the results (n = 2) were reported as mg gallic acid equivalents (GAE) per gram sample (mg GAE/g).

The total antioxidant capacity of the willow bark extract was evaluated by the phosphomolybdenum method of Prieto *et al.* (1999), based on the reduction of Molybdenum(VI) (Mo(VI)) to Mo(V) and the further formation of a phosphate-Mo(V) green complex at an acidic pH. The results (n = 2) were expressed as mmol equivalent ascorbic acid/kg sample and as mmol equivalent vitamin E/kg sample.

Feed samples were taken from each batch of compound feeds and assayed for the chemical proximate composition using the chemical methods from Commission of the European Communities (2009). Dry matter (ISO 6496/2001), crude protein (ISO 5983-2/2009), ether extractives (SR ISO 6492/2001), crude fibre (ISO 6865/2002), and ash (ISO 2171/2010) were determined. Calcium was analysed according to SR ISO 6490-1/1996 and phosphorus according to SR ISO 6491:1983.

Blood samples were centrifuged at 775 × g for 25 min at 4 °C. The supernatant was used to determine these biochemical markers: energy profile (cholesterol, triglyceride), protein profile (albumin, total protein), and enzyme profile (alanine aminotransferase and aspartate aminotransferase). The biochemical parameters were determined on an automatic BS-130 chemistry analyser (Bio- Medical Electronics Co., Ltd, China).

Thiobarbituric acid reactive species assay from liver was carried out according to the method reported by de Zwart *et al.* (1999) based on the absorbance measurement of the rose complex formed from the reaction of malondialdehyde and two molecules of thiobarbituric acid. The results were expressed as µM malondialdehyde (MDA)/100 mL.

The microbiological analyses of *E. coli*, staphylococci, lactobacilli and *Salmonella spp*. were determined as Criste *et al.* (2017) described. The Scan 300 colony counter (Interscience, Paris, France) was used to determine the colony count of *E. coli*, staphylococci and lactobacilli. The results were expressed as log base 10 colony-forming units (CFU) per gram of caecal contents.

A complete randomized model was used to analyse the data for growth performance, serum biochemical parameters, liver oxidative status and caecal microflora. The effects of treatments were tested by analysis of variance using the GLM procedure of Minitab® Statistical Software, version 17 (State College, Pennsylvania, USA), with treatment as fixed effect. When the overall F-test was significant, differences between means were declared significant at $P < 0.05$ using the Tukey test. The comparative graph was done using free software R version 3.5.1 (R Core Team, 2013).

Results and Discussion

The proximate analysis, total polyphenol content and antioxidant capacity of HWE powder are presented in Table 2. The dietary HWE powder contained a concentration of polyphenols of 4.67 mg gallic acid equivalent per g. Sulaiman *et al.* (2013) reported that *Salix alba* bark extract in boiled ethanol has more polyphenols (162 mg gallic acid equivalent per g). The differences between the present results and those reported by Sulaiman *et al.* (2013) may be because differences in solvents that were used in extraction (grain/water as opposed to boiled ethanol), the extraction method (hydro-alcoholic extraction as opposed to Soxhlet), extraction time, and environmental factors. Many researchers have reported major differences in the concentration of polyphenols of willow bark extracts (Durak & Gawlik-Dziki, 2014; Zabihi *et al.*, 2018; Gligoric *et al.*, 2019). Among the causes of their differences are the part of product that was used and the *Salix* species. The powder from hydroalcoholic willow bark extract had a total antioxidant capacity of 35.13 mM equivalent ascorbic acid, 35.97 mM equivalent vitamin E, respectively. Enayat & Banerjee (2009) showed the importance of the type of solvent used for the extraction of *S. aegyptiaca* bark in the antioxidant assay capacity. However, *S. aegyptiaca* bark had the highest antioxidant capacity when extracted in ethanol (169 ± 28 mg quercetin equivalence /g dried sample), followed by water (78 ± 4 mg quercetin equivalence /g dried sample) and cyclohexane (10 ± 0.1 mg quercetin equivalence /g dried sample). Saracila *et al.* (2018) compared several types of plant extracts (rosehip, buckthorn, grape seeds, sesame and willow buds) in terms of their antioxidant capacity, expressed in inhibition percentages. They showed that the hydroglyceroalcoholic extract of white willow bark has a free radical inhibitory capacity similar to that of grape seed and sesame seed extracts and greater than willow bud extract.

Table 2 Composition of hydroalcoholic willow bark extract (*Salix alba* L.*)*

Measurements	Composition (n = 2 samples)
Dry matter, %	99.26
Crude protein, %	0.18
Crude fat, %	0.01
Crude fibre %	1.89
Ash, %	0.46
Total polyphenols, mg gallic acid equivalent /g	4.67
Total antioxidant capacity, mM equivalent ascorbic acid	35.13
Total antioxidant capacity, mM equivalent vitamin E	35.97

The bodyweight of T25 and T50 broilers was not significantly different (P >0.05) from that of T0 group (Figure 1). However, the bodyweights attained by the broilers in all three treatments at 14, 21, 28, 35, and 42 days were less than those put forth in the management guide (Anonymous, 2015). This difference was anticipated due to the imposition of heat stress. As Figure 1 shows, this difference increased over time with continued the exposure to heat stress. The results are in agreement with those reported by Saracila *et al.* (2018), who used 1% HWE in a diet for broilers (14–42 days old) reared under heat stress (32 °C).

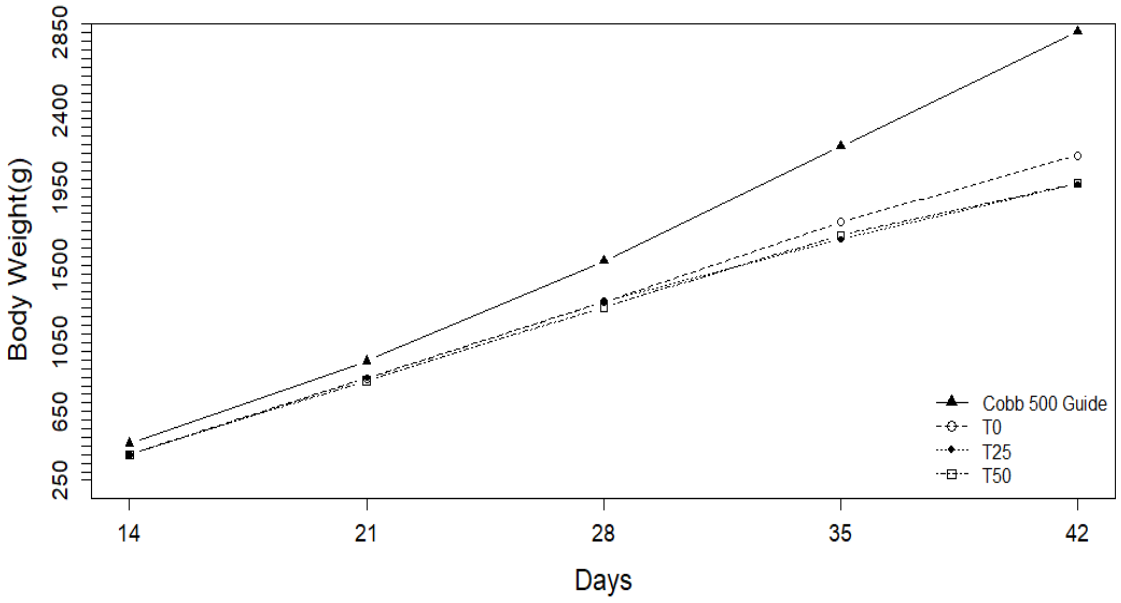

Figure 1 Changes in bodyweight over time of broilers exposed to high heat stress compared with the growth projected for Cobb 500 broilers reared under a recommended temperature regime (Anonymous, 2015)

Table 3 Effects of diets supplemented with hydroalcoholic willow bark extract powder on the performance of broilers between 14 and 42 days of age

Parameter	T0	T25	T50	SE	*P*-value
BWG (g/broiler/day)					
14–35 days	57.99	42.85	53.08	3.780	0.5344
35–42 days	64.07	60.59	61.37	2.045	0.5377
14–42 days	62.05	56.16	56.72	2.053	0.3765
AFI (g feed /broiler/day)					
14–35 days	109.19	101.82	93.96	5.578	0.802
35–42 days	74.32	71.25	70.10	3.474	0.8791
14–42 days	80.59	76.71	74.49	3.098	0.7216
FCR (g feed/g gain)					
14–35 days	2.00	2.37	2.20	0.103	0.5970
35–42 days	1.16	1.18	1.17	0.059	0.9389
14–42 days	1.33	1.39	1.35	0.058	0.9079

BWG: bodyweight gain; AFI: average feed intake; FCR: feed conversion ratio
T0: dietary control treatment; T25: dietary control treatment supplemented with hydroalcoholic willow bark extract powder at the level of 25 g/100 kg diet; T50: dietary control treatment supplemented with hydroalcoholic willow bark extract powder at the level of 50 g/100 kg diet

No significant differences among diets were observed for average daily feed intake (Table 3). Al-Fataftah & Abu-Dieyeh (2007) showed that broilers that were exposed to 30 ± 2 °C and 35 ± 2 °C had a reduced feed intake (by 12.4% and 28%) and a decreased weight gain (by 18% and 44%) when compared with broilers exposed to constant or variable thermoneutral temperature regimes (24 – 28 °C and 25 ± 2 °C.

There was no effect (P >0.05) of HWE powder on FCR (14–42 days). The addition of HWE powder to the diet (25 g/100 kg and 50 g/100 kg) resulted in higher average daily weight gain and improved FCR (Table 3) compared with the results reported by Saracila *et al.* (2018) when they used 1% hydroglyceroalcoholic willow bark extract in broiler diets (14–28 days). Although under thermoneutral conditions dietary herbs (Bampidis *et al.*, 2005; Cross *et al.*, 2007) and plant extract supplements (Demir *et al.*, 2005) may be expected to stimulate the growth performance of broilers, there are studies on broilers under heat stress that provide contradictory results (Lee *et al.*, 2003; Hernández *et al.*, 2004).

Many studies have documented lower growth performances for broilers exposed to heat stress (Attia *et al.*, 2011; Ghazi *et al.*, 2012; Zhang *et al.*, 2012; Imik *et al.*, 2013; Olfati *et al.*, 2018). Sohail *et al.* (2012) showed decreased bodyweight, average daily weight gain, and average feed intake in broilers reared under heat stress compared with those reared under normal temperatures. The data of the current study are in agreement with a recent study by Pirgozliev *et al.* (2019), which showed that Ross 308 chickens (20–35 days old) that were reared at 35 °C had reduced feed intake and growth rate and higher FCR compared with those reared at 21 °C. Animals reduce their feed intake under acute and chronic heat stress because of the stimulation of the hypothalamic axis by increasing leptin and adiponectin levels (Lu *et al*., 2007). Pena *et al.* (2008) showed that under cyclic heat stress (32 °C for 5 hours and 19 hours at thermoneutral temperature), the addition of some antioxidants (citric flavonoids and ascorbic acid) to the diet did not affect live performance over the first 32 days of life. Marchini *et al.* (2011), who studied the effect of cyclic heat stress on performance and carcass yield of broiler chickens, found similar results.

There were no mortalities in the present study. This finding may result from the broilers being kept in a sanitary environment, and thereby possibly leading to diminished efficacy of the dietary additives (Ocak *et al.* 2008).

The serum profiles (Table 4) revealed several benefits of feeding the broilers reared under heat stress with HWE powder. These serum profiles reflect the health, nutrition, climate and management conditions to which the animals are subjected (Minafra *et al.*, 2010). Levels lipid, protein and enzymatic activity in the blood could indicate the productive performance of the birds and metabolic diseases (Rotava *et al.*, 2008). The blood components in birds are particularly sensitive to changes in ambient temperature, being important indicators of physiological responses to stressing agents (Tawfeek *et al.*, 2014).

Table 4 Effects of diets supplemented with hydroalcoholic willow bark extract powder on the serum profile of broilers at 42 days of age

	Serum lipid profile		Serum protein profile		Serum enzyme	
Treatment	Cholesterol, mg/dL	Triglyceride, mg/dL	Albumin, g/L	Total protein, g/dL	ALT, U/L	AST, U/L
T0	123.6[a]	50.51[a]	1.00	2.286	5.99[a]	261.47
T25	124.1[ab]	43.94[ab]	1.00	2.708	5.53[a]	243.63
T50	91.1[b]	35.03[b]	1.17	2.090	3.98[b]	229.45
SE	6.5	2.50	0.06	0.106	0.314	18.63
p-value	0.0461	0.0218	0.3911	0.0381	0.0078	0.8075

[a,b] Means in the same column with different superscripts differ significantly (P <0.05), n = 8 / treatment
T0: dietary control treatment; T25: the dietary control treatment supplemented with hydroalcoholic willow bark extract powder at the level of 25 g/100 kg diet; T50: the dietary control treatment supplemented with hydroalcoholic willow bark extract powder at the level of 50 g/100 kg diet; ALT: alanine aminotransferase, AST: aspartate aminotransferase

Under heat stress conditions, the cholesterol and triglycerides in the serum of broilers that were fed T50 were significantly less compared with T0. This result could be a consequence of HWE powder leading to a lower catabolic effect in broilers exposed to heat stress. However, serum cholesterol and triglyceride were not affected (P >0.05) by the level of addition of HWE powder to broiler diets. Serum albumin and total protein (Table 4) also did not differ significantly as a function of the treatments. These results partially contradict those reported by Saracila *et al.* (2018). In the cited study, a significantly lower level of albumin was recorded in the treatment containing willow bark extract (1%) than in the dietary control treatment, but the level of total protein in the serum of Cobb 500 broilers showed no significant differences between treatments. The alanine aminotransferase (ALT) level (-50.5 %) in broiler serum from T50 treatment was significantly decreased (-50.5%) compared with T25 and T0. The literature shows contradictory effects of heat stress on the general health state of broilers. For instance, Jaiswall *et al.* (2017) showed significant increases in cholesterol (187.42 mg/dL vs. 128.43 mg/dL) and triglycerides (165.31 mg/dL vs. 116.15 mg/dL) in the serum of heat challenged broilers, compared with broilers reared under thermoneutral temperatures. On the other hand, the present results are in agreement with those reported by El-Soud *et al.* (2006), who noted significant decreases in the levels of serum cholesterol of Japanese quail under heat stress that were fed diets containing 0.025%, 0.05%, and 0.1% acetylsalicylic acid. Dudzińska *et al.* (2015) studied the hypercholesteraemic effect of willow bark and related it to its content of polyphenols. Tawfeek *et al.* (2014) explained that during heat stress there was greater catabolic effect and concentration of adrenocorticotropic hormone yielding more triglycerides in broiler serum. In a study conducted on the same hybrid (Cobb 500), Attia *et al.* (2017) showed that the serum cholesterol and serum protein (total protein, albumin) increased significantly in the broilers reared under chronic heat stress (36 ± 2 °C) compared with broilers reared under normal temperature. They showed that among the transaminases, only aspartate aminotransferase (AST) increased significantly in the serum of broilers exposed to heat stress compared with the broilers reared under normal temperature, ALT not being affected. Furthermore, Imik *et al.* (2013) showed that the heat stress did not significantly affect the cholesterol, triglycerides, serum protein (total protein, albumin), AST or ALT. The differences in the numerical values that characterize the serum profile in these studies could be due to the analytical methods and equipment, breed, management and diet formulation.

Heat stress increases fat deposits in broilers and induces oxidative damage in tissues. One of the tissues that is most affected is the liver. Levels of malonaldehyde (MDA) in blood and tissues have been used as biomarkers of lipid peroxidation (Yousef *et al.*, 2009; Ismail *et al.*, 2013). The MDA level is directly proportional to the degree of lipid peroxidation (Ismail *et al.*, 2013). Table 5 shows the effect of the supplement of HWE powder on liver MDA concentration in broiler chickens reared under heat stress conditions. In the livers of broilers treated with HWE powder there was a lower (P <0.05) amount of MDA compared with those that were fed the conventional diet (Table 5). This could be because of the antioxidant action of HWE powder to alleviate the oxidative stress caused by the heat. According to Altan *et al.* (2000), changes in MDA concentration might be regarded as a heat stress response of broilers. Therefore, the level of MDA, as a major product of lipid peroxidation, is an important variable in evaluating oxidative stress (Sim *et al.*, 2003). Erol *et al.* (2017) showed that a hot environment (34 °C) led to increased MDA concentration in the liver tissues of Ross 308 broilers (24–39 days old) fed a low protein diet. The inclusion of various sources of antioxidants in broiler diets has led to significant reductions of MDA in the liver (Erdoğan *et al.*, 2005; Ismail *et al.*, 2013). At 42 days, Ismail *et al.* (2013) showed a decrease in liver MDA from 53.2 µM MDA

(untreated heat-stressed group) to 18 µM MDA, following the addition of zinc bacitracin and ascorbic acid to the diet of broilers exposed to 39 °C. Other studies that investigated the effect of willow bark extract on animal performance showed that it reduced oxidative stress and increased glutathione (which was not determined in this experiment) in various species of animals with arthritis (Khayyal *et al.*, 2005; Sharma *et al.*, 2011). These studies suggest that willow bark extract might suppress oxidative stress by inducing antioxidant enzymes in addition to annihilating free radicals. Ishikado *et al.* (2013) showed that willow bark extract stimulates the expression of antioxidant enzymes and prevents oxidative stress by activating Nrf2 in the vascular endothelial cells. Thus, the results in tables 4 and 5 indicate an improvement in liver function because of the supplementation.

Table 5 Effects of levels of supplementation with hydroalcoholic willow bark extract powder on liver malonaldehyde concentration (µM MDA) in liver of 42-day old broilers

Treatment	MDA (µM)
T0	5.850[a]
T25	3.820[b]
T50	4.14 [b]
SE	0.242
P-value	<0.0001

[a, b] Means in the same column with different superscripts differ significantly (P <0.05), n = 8 / treatment
T0: dietary control treatment; T25: the dietary control treatment supplemented with hydroalcoholic willow bark extract powder at the level of 25 g/100 kg diet; T50: the dietary control treatment supplemented with hydroalcoholic willow bark extract powder at the level of 50 g/100 kg diet

At 35 and 42 days of age, the supplements of HWE powder influenced the *E. coli* count in the caecal content of the broilers significantly (Table 6). Thus, *E. coli* colony forming units were lower (P <0.05) in the caecal content of broilers in T25 and T50 than in T0. The explanation for this effect might be that *Salix alba* extract had the largest inhibition zones on agar plate against *Bacillus cereus* (2.72 mm), *Staphylococcus aureus* (1.10 mm), *Listeria monocytogenes* (3.08 mm), and *E. coli* (0.85 mm) (Pop *et al.,* 2013). At 35 and at 42 days, broilers in T25 and T50 also had lower (P <0.05) staphylococci colony forming units than broilers in T0. Akbarian *et al.* (2013) highlighted that the antimicrobial efficacy of phytoadditives arose directly from an antimicrobial action or was indirectly mediated by phytogenics to affect the microbiota. Burkholder *et al.* (2008) noted that heat stress (30 °C) significantly decreased the intestinal bacterial populations of Ross 308 broilers (44 days). Saracila *et al.* (2018) supported the idea that under heat stress, the dietary hydroglyceroalcoholic willow bark extract (1%) significantly decreased *E. coli* and *Staphylococci* populations in broiler caecums at 42 days. However, at 28 days of age, the 1% hydroglyceroalcoholic willow bark extract did not influence (P >0.05) the caecum staphylococci populations. A reason for the reduced pathogen populations in the caecum of T25 and T50 broilers may be an antimicrobial effect of the HWE powder. Other studies found antimicrobial effects of willow bark (Pop *et al.*, 2013; Sulaiman *et al.*, 2013), although the antimicrobial effects of HWE powder were not determined in the present study. Sulaiman *et al.* (2013) observed the greater efficacy of willow bark extract against gram-positive bacteria than against gram-negative bacteria, possibly owing to the differences in their cell wall structure. The gram-negative organisms seem to be more resistant because of their outer membrane, which acts as a barrier to many environmental substances including antibiotics (Kaye *et al.*, 2004). Pop *et al.* (2013) analysed hot willow bark extract in acidulated water containing 1% HCl. They found that the extract proved to be rich in eriodyctiol, naringenin and quercetin glucosides, and in catechins, salicylate and isorhamnetin, with all these bioactive molecules being responsible for its antibacterial activity. Additionally, Sulaiman *et al.* (2013) showed that the antimicrobial and cytotoxic activities of the willow bark extract were positively associated with its antioxidant potentials. Others explain the significant antibacterial property of crude ethanol extract of *S. alba* by the constituents, such as tannins and glycosides, which enable the extract to overcome the barrier of the bacterial cell wall (Scalbert, 1991; Senthamilselvi *et al.*, 2012). Although a great number of articles deal with the antioxidant inhibitory effect on various species of bacteria, only few refer to the effect of antioxidants on probiotic bacteria such as lactobacilli (Välimaa *et al.*, 2007).

The data in Table 6 show that at 35 and at 42 days, more (P <0.05) lactobacilli populations were present in the caecal content of broilers in T25 and T50 than those in T0. At the same time, the greatest

number of lactobacilli colony forming units (P <0.05) was noted in the caecal content of T50 broilers. The positive effect of plant extracts on gut microbiota include increasing the number of lactic acid bacteria and decreasing the coliform counts in the ileum and caecal contents of broiler chickens (Vidanarachchi *et al.*, 2005). Criste *et al.* (2019) found that dietary 0.01% oregano essential oil led to lactobacilli multiplication in the intestinal and caecal segment of Cobb 500 broilers (14–35 days) reared under high temperature.

Table 6 Effects of levels of supplementation with hydroalcoholic willow bark extract powder on caecal bacterial populations (lg10 colony forming units/ g wet caecal content)

Treatment	*E. coli*	Staphylococci	Lactobacilli	*Salmonella spp.*
Determination at 35 days				
T0	9.936[a]	8.161[a]	9.557[a]	absent
T25	9.922[b]	7.914[b]	9.618[b]	absent
T50	9.923[b]	7.900[b]	9.672[c]	absent
SE	0.002	0.029	0.013	NA
P-value	0.0153	<0.0001	<0.0001	NA
Determination at 42 days				
T0	10.366[a]	8.652[a]	10.662[a]	absent
T25	10.341[b]	8.415[b]	10.715[b]	absent
T50	10.340[b]	8.353[b]	10.785[c]	absent
SE	0.004	0.033	0.014	NA
P-value	0.0001	<0.0001	<0.0001	NA

[a, b,c] Means in the same column with different superscripts differ significantly (P <0.05). NA: not calculated, n = 8;
T0: dietary control treatment; T25: the dietary control treatment supplemented with hydroalcoholic willow bark extract powder at the level of 25 g/100 kg diet; T50: the dietary control treatment supplemented with hydroalcoholic willow bark extract powder at the level of 50 g/100 kg diet

There are several limitations to the generalization of these results as they are specific to the breed, management, diet formulation, analytical methods and equipment that were used in this study. The differences between the concentration of polyphenols in HWE from the present study and that reported by Sulaiman *et al.* (2013) may in part be due to the solvent used for extraction, the extraction method, the extraction length, and laboratory conditions. Since the most definitive results were obtained at the highest level of HWE supplementation of the broiler diet, further investigations using higher levels of HWE powder (75 g and 100 g/100 kg diet) will be conducted under the same environmental conditions.

Conclusions

Supplementing the diet of broilers with HWE powder did not affect broiler performance (14–42 days old). The treatment supplemented with HWE powder at 50 g/100 kg of diet significantly decreased cholesterol and triglyceride levels in serum and improved the liver oxidative status. Also, adding HWE powder to the diet had a positive effect in reducing the number of pathogenic bacteria and in increasing the number of lactobacilli in the broiler caecum at 35 and 42 days of age. Because of its antioxidant and antipyretic properties, supplementing each 100 kg of the diet with 50 g HWE powder could be a promising solution to the harmful effects of heat-stress on broiler performance, liver oxidative status and caecal microflora.

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Authors' Contributions

All the authors contributed equally and commented on the early and final version of manuscript.

Conflict of Interest Declaration

The authors have no conflict of interest to declare.

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