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Research article

THE *IN VITRO* ANTIMICROBIAL EFFECTS OF HYDROLYSABLE AND CONDENSED TANNIN EXTRACTS ON *ESCHERICHIA COLI* ISOLATED FROM PATHOLOGICAL SAMPLES OF DECEASED POULTRY

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Colibacillosis, caused by avian pathogenic *Escherichia coli* (APEC), is one of the most prevalent and economically damaging bacterial diseases affecting poultry globally. Managing colibacillosis is difficult and frequently ineffective because APEC strains have developed widespread resistance to antibiotics, and the strict regulations and public concerns towards using antimicrobial agents in poultry further complicate the situation. This study aimed to evaluate the antibacterial properties of hydrolysable (sweet chestnut extract) and condensed (quebracho extract) tannins on *Escherichia coli* (*E. coli)* isolates from poultry, exploring their potential as antibiotic alternatives in managing colibacillosis. *E. coli* was isolated from the internal organs of deceased poultry across 18 farms, including layers, broilers, and broiler breeders. Each isolate was assessed for the presence of APEC strain predictors (virulence genes *iutA*, *hlyF*, *iss*, *iro*N, and *ompT*), antimicrobial resistance to 14 antibiotics using the disc diffusion method, and the presence of resistance genes for specific antibiotics (ampicillin, gentamicin, tetracycline, and quinolones). Out of 43 isolates, 27 (62.8%) were classified as APEC, 30 (69.8%) showed resistance to three or more antibiotic classes, and 32 (74.4%) carried at least one AMR gene. The minimum inhibitory concentrations (MICs) for the hydrolysable tannins from sweet chestnut extract (*Castanea sativa* Mill.) (SwCh) ranged from 0.5 to 3 mg/mL, while for the condensed tannins from quebracho extract (*Schinopsis lorentzii*) (Que), the MICs ranged from 1.5 to 4.5 mg/mL. The results indicate that both hydrolysable and condensed tannins possess significant *in vitro* antimicrobial activity against APEC, offering a potentially valuable alternative for controlling colibacillosis in the poultry industry.

Keywords: antimicrobial resistance, APEC, poultry, quebracho, sweet chestnut

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INTRODUCTION

Colibacillosis, caused by avian pathogenic *Escherichia coli* (APEC), significantly impacts poultry producers globally due to its severe economic consequences. The disease is identified by the triad of lesions: perihepatitis, pericarditis, and airsacculitis, which often lead to septicaemia and elevated mortality rates [1,2]. While *E. coli* is a normal component of the intestinal microbiota, certain virulent strains trigger colibacillosis. The differentiation between APEC and avian fecal commensal *E. coli* (AFEC) is possible through specific genes found in APEC pathotypes, including *iutA* (aerobactin siderophore receptor gene), *hlyF* (putative avian hemolysin), episomal *iss* (increased serum survival gene), *iroN* (salmochelin siderophore receptor gene), and episomal *ompT* (outer membrane protease gene) [2,3]. The severity of the disease caused by APEC varies based on the host's health, the virulence of the *E. coli* strain, and other predisposing factors such as stress [4]. Effective control of avian colibacillosis hinges on management practices, infection control measures, and vaccination strategies.

The antibiotics most commonly utilized in the poultry industry include β-lactams (such as penicillins and cephalosporins), aminoglycosides, tetracyclines, sulphonamides, and fluoroquinolones. The frequent administration of these antibiotics exerts selective pressure, which contributes to the development and spread of antimicrobial resistance in APEC strains, complicating the management of colibacillosis [5]. In the Republic of Serbia, numerous studies in recent years have shown a high prevalence of multi-drug resistant (MDR) *E. coli* strains isolated from poultry. The resistance rates reported include up to 94.7% for tetracycline, 75.0% for ampicillin, 48.3% for trimethoprim with sulfamethoxazole, and 16.7% for fluoroquinolones (such as ciprofloxacin or enrofloxacin) [6,7]. Although third-generation cephalosporins are infrequently used in Serbian poultry farming, resistance to these antibiotics has still been observed in *E. coli* isolates, likely due to the conjugative transfer of multidrug-resistant plasmids [8]. Additionally, resistance to colistin has recently been detected for the first time in Serbia in an *E. coli* strain isolated from turkeys [9].

As antibiotic treatments for colibacillosis become increasingly ineffective, and in alignment with the global effort towards reducing antibiotic use in domestic animals, there is a pressing need to explore alternative strategies for managing colibacillosis. These alternatives include the use of prebiotics, probiotics, enzymes, acidifiers, immunostimulants, and phytobiotics such as essential oils and tannins [10]. Phytobiotics offer multiple benefits, including promoting the growth of beneficial gut bacteria and providing antioxidative, antimicrobial, and anti-inflammatory effects.

Tannins, which are plant secondary metabolites present in seeds, bark, leaves, or the skin of fruits, act as chemical defense agents against pathogens and insect attacks [11]. They are classified into three primary groups based on their water solubility, molecular weight, structure, polyphenolic composition (containing 12–16 phenolic groups and 5-7 aromatic rings), and their chemical interactions with alkaloids, gelatin, and other proteins. These groups are hydrolysable tannins, condensed tannins, and

phlorotannins, the latter being unique to marine brown algae [12]. Hydrolysable tannins, which consist of a polyol core, typically D-glucose, have molecular weights ranging from 500 to 3,000 Da and are easily broken down by hydrolysis with weak acids, bases, or esterases, making them readily degradable in the digestive tract. In contrast, condensed tannins are flavonoid oligomers or polymers, more structurally complex and larger with molecular weights between 1,000 to 20,000 Da, resistant to breakdown by oxidative and acidic hydrolysis and not susceptible to anaerobic enzyme degradation [12,13].

Tannins exhibit several biological activities including antimicrobial, anti-parasitic, antioxidative, and anti-inflammatory properties [12]. Their antimicrobial mechanisms are thought to include the inhibition of microbial extracellular enzymes, depriving microbes of essential growth substrates, disrupting microbial metabolism through the inhibition of oxidative phosphorylation, depriving microbes of essential metal ions, or by binding to bacterial cell membranes which alters cell wall morphology and increases membrane permeability [12,14].

The antimicrobial effectiveness of tannins is specific to microbial species and closely linked to their chemical structure and composition [12]. Tannins extracted from various plants have shown potent activity against major poultry pathogens like *E. coli* O157:H7, *Salmonella* spp., *Campylobacter* spp., and *Clostridium perfringens* [15-18]. It has been demonstrated experimentally that sweet chestnut tannins at doses of 500 and 1000 mg per kg of feed significantly reduce *E. coli* populations in the small intestines of 28-day-old chicks [16]. Furthermore, the antimicrobial action of hydrolysed tannins from sweet chestnut, both alone and combined with short-chain organic fatty acids, has been proven *in vitro* and *in vivo* against *E. coli* O45 isolated from broiler chickens that succumbed to colibacillosis [19].

For over sixty years, antibiotics have been used in sub-therapeutic doses as growth promoters in animal feed, enhancing feed conversion rates and reducing production costs. However, their extensive use has led to the rise of antibiotic-resistant pathogens from animal sources, posing a serious threat to food safety and public health. In response to this critical issue, many countries have prohibited the use of antibiotics as growth promoters, underscoring the urgent need for alternative growth-promoting agents (AGP). Hydrolysable tannins from chestnut and condensed tannins from quebracho are among the most commercially available products that meet these needs [20].

This study aimed to assess the *in vitro* antimicrobial properties of hydrolysable (sweet chestnut extract) and condensed (quebracho extract) tannins against *E. coli* strains from laying hens, broilers, and broiler breeders in the Republic of Serbia. The samples were collected from September 2020 to May 2021. The effectiveness of these tannins was evaluated based on their impact on various *E. coli* strains, distinguished by their pathogenic profiles (APEC and AFEC) and patterns of antimicrobial resistance.

MATERIAL AND METHODS

The conducted research was not related to animals use. No ethical approval was obtained because this study did not involve laboratory animals and only involved non-invasive procedures, as collection of internal organs of deceased poultry.

Samples were gathered from deceased poultry suspected of having colibacillosis due to pathomorphological alterations, across 18 farms in Serbia, including 10 layer, 6 broiler, and 2 broiler breeder farms. A total of 123 internal organs, such as the liver, intestines, spleen, and egg follicles, were collected for analysis.

Isolation of *E. coli* was performed after inoculation of the samples on MacConkey agar (Becton Dickinson, USA) and Columbia agar with 5% sheep blood (Becton Dickinson, USA). The inoculated plates were incubated for 24 hours at 37°C. After obtaining pure culture, conventional biochemical tests and commercial kit API-20E system (bioMérieux, France) were used for *E. coli* identification.

DNA extraction was performed using GeneJET Genomic DNA Purification Kit (Thermo Scientific, USA) following the manufacturer's Gram-Negative Bacteria Genomic DNA Purification Protocol. The extracted DNA was stored at – 20° C until further testing. For the purpose of differentiating APEC strains, a multiplex PCR was employed for identification of *iroN, ompT, hlyF, iss* and *iutA* genes as minimal APEC predictors previously described by Johnson et al. [2]. Nuclease-free water served as the negative control, while in-house *E. coli* strains were used as positive control. DNA fragments were analyzed by horizontal gel electrophoresis (Serva, Germany) in 0.5× TBE buffer on a 1.5% agarose gel stained with 0.5 μg/mL ethidium bromide and visualized on transilluminator (Vilber Lourmat, France).

Disk diffusion antimicrobial susceptibility testing was performed according to the standard and interpretive criteria described by EUCAST [21] using the following antimicrobial susceptibility disks (Bio-Rad, France): ampicillin 10 μg (AMP), amoxicillin with clavulanic acid 20 μg + 10 μg (AMC), cefotaxime 30 μg (CTX), ceftazidime 30 μg (CAZ), meropenem 10 μg (MEM), nalidixic acid 30 μg (NAL), ciprofloxacin 5 μg (CIP), tetracycline 30 μg (TET), colistin 10 μg (CL), gentamicin 10 μg (GEN), trimethoprim 5 μg (TMP), chloramphenicol 30 μg (CHL), tigecycline 15 μg (TGC), and trimethoprim with sulfamethoxazole 1.25 μg + 23.75 μg (SXT). Multidrug resistance (MDR) was characterized, following the criteria established by Magiorakos et al. [22], as the resistance exhibited by bacteria against a minimum of 3 distinct antibiotic classes. When the phenotypic antimicrobial resistance of the *E. coli* isolates was determined, the presence of genes encoding AMR was tested with multiplex PCR. The detection of the following resistance genes to ampicillin, gentamicin, tetracyclines, and quinolones was conducted: *blaTEM, aac3VI, tetB,tetA, aph(3)IA, qnrA, qnrB,* and *qnrS* genes [23, 24].

During the investigation of the antimicrobial effects of tannin extracts on *E. coli* isolates, we used commercially available extracts of sweet chestnut (*Castanea sativa* Mill.

with 73.5% hydrolysed tannins) (SwCh) and red quebracho (*Schinopsis lorentzii* Griseb. with 72.5% condensed tannins) (Que), supplied by Tanin d.d. Sevnica (Slovenia). Both extracts were dissolved in sterile water, incubated for 30 minutes at 60 ° C, and then filtered through a 0.45 μm membrane filter. The susceptibility of *E. coli* isolates to tannin extracts was tested using broth micro-dilution reference method for testing the *in vitro* activity of antimicrobial agents according to ISO 20776-1:2019 standard [26]. Before performing this method, solutions of magnesium chloride ($MgCl_2$) at a concentration of 20–25 mg/L and calcium chloride ($CaCl_2$) at a concentration of 10–12.5 mg/L were added to the Mueller-Hinton broth. Tannin extracts were added at 12 different concentrations ranging from 0.5 to 6 mg/mL to sterile Mueller-Hinton broth. 100 μL of the prepared tannin dilutions were poured into the wells followed by 10 μL of a bacterial suspension with a density of 5 x 10^6 CFU/mL. The microtiter plates were incubated in a thermostat at 35° C ± 1° C for 16 to 20 hours. Turbidity indicated microbial growth and the MIC was the lowest concentration where no growth is visually observed. The MIC values were confirmed by reading the absorbance on ELISA reader (LKB, Austria) using a 620 nm filter.

In statistical analyses, the program XLSTAT for Microsoft Excel (v. 2023) was used. Shapiro Wilk, Anderson Darling, Lilliefors and Jarque-Bera tests were used to test the normality of the distribution of MIC values for both tested tannins. The Mann-Whitney (MW) U test coupled with Kolmogorov-Smirnov (KS) test was used to determine the difference in the antimicrobial activity of hydrolysable and condensed tannins on *E. coli* isolates from poultry, as well as the difference in the antimicrobial effect of tannin extracts on MDR and non-MDR *E. coli* isolates. They were also used in testing the difference in the antimicrobial activity of tannin extracts on APEC and AFEC (non-APEC) isolates of *E. coli*. Differences in P-values less than 0.05 were considered statistically significant.

RESULTS

A total of 123 organ samples from deceased poultry across 18 farms were analyzed, resulting in the confirmation of 43 *E. coli* isolates originating from the liver (15), intestines (12), spleen (11), and egg follicles (5). The presence of key predictive genes for APEC strains (*iroN*, *ompT*, *iss*, *hlyF*, and *iutA*) was assessed using multiplex PCR (Figure 1). Out of all the isolates tested, 27 (or 62.79%) carried four or more genes from the APEC gene subset. The genes *iroN*, *ompT*, *iss*, and *hlyF* were identified in 27 isolates, while the *iutA* gene was found in 6 isolates. The results of the multiplex PCR tests for the APEC subset genes are summarized in Table 1.

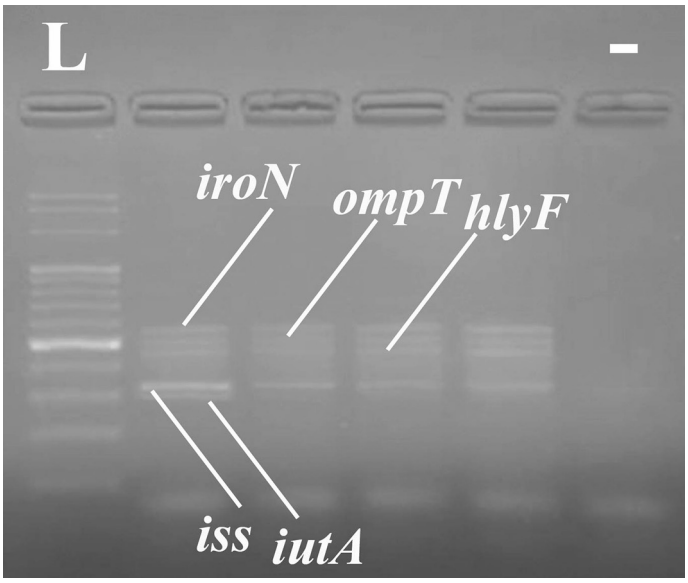

Figure 1. Multiplex PCR results for the presence of the minimal predictor genes of the APEC strains (*iroN, ompT, iss, hlyF*, and *iutA*)

Table 1. Multiplex PCR results for the genes included in the APEC subset

Virulence genes	*iroN*	*ompT*	*iss*	*hlyF*	*iutA*
Number of isolates and % of all *E.coli* isolates	27 (62,8%)	27 (62,8%)	27 (62,8%)	27 (62,8%)	6 (13,9%)

The results of the disc diffusion antimicrobial susceptibility tests of *E. coli* isolates are shown in Table 2a.

Out of the examined *E. coli* isolates, the highest percentage of resistance was found to ampicillin (79.1%), tetracycline (69.8%), and nalidixic acid (55.8%).

From the total of 43 isolates, five were sensitive to all antibiotics, and four isolates were resistant to one or two antibiotics, respectively. Twenty-four isolates were resistant to three antibiotics, four isolates to four antibiotics, one isolate to five antibiotics, and one to seven antibiotics. The number of *E. coli* isolates characterised by resistance to a particular number of examined antibiotics were shown in Table 2b. Thirty tested isolates of *E. coli* exhibited MDR to three or more classes of antibiotics, which represented 69.8% of the total number of isolates.

The presence of genes encoding antimicrobial resistance to ampicillin, gentamicin, tetracycline, and quinolones was tested with multiplex PCR (Figure 2).

Table 2a. Results of antimicrobial susceptibility tests of *E. coli*

	All isolates n (%)			APEC isolates n (%)			AFEC/NON-APEC isolates n (%)		
Antibiotic	S*	I**	R***	S*	I**	R***	S*	I**	R***
Ampicillin 10 µg (AMP)	9 (20.9%)	0	34 (79.1%)	4 (14.8%)	0	23 (85.2%)	5 (31.2%)	0	11 (68.7%)
Amoxicillin with clavulanic acid 20 µg + 10 µg (AMC)	40 (92.5%)	0	3 (7.5%)	25 (92.6%)	0	2 (7.4%)	15 (93.7%)	0	1 (6.2%)
Cefotaxime 30 µg (CTX)	40 (92.5%)	3 (7.5%)	0	25 (92.6%)	2 (7.4%)	0	15 (93.7%)	1 (6.2%)	0
Ceftazidime 30 µg (CAZ)	41 (95.4%)	1 (2.3%)	1 (2.3%)	26 (96.3%)	1 (3.7%)	0	15 (93.7%)	0	1 (6.2%)
Meropenem 10 µg (MEM)	43 (100%)	0	0	27 (100%)	0	0	16 (100%)	0	0
Nalidixic acid 30 µg (NAL)	18 (41.9%)	1 (2.3%)	24 (55.8%)	12 (44.4%)	0	15 (55.6%)	6 (37.5%)	1 (6.2%)	9 (56.2%)
Ciprofloxacin 5 µg (CIP)	37 (86.1%)	1 (2.3%)	5 (11.6%)	23 (85.2%)	1 (3.7%)	3 (11.1%)	14 (87.5%)	0	2 (12.5%)
Tetracycline 30 µg (TET)	13 (30.2%)	0	30 (69.8%)	9 (33.3%)	0	18 (66.7%)	4 (25%)	0	12 (75%)
Colistin 10 µg (CL)	43 (100%)	0	0	27 (100%)	0	0	16 (100%)	0	0
Gentamicin 10 µg (GEN)	36 (83.7%)	0	7 (16.3%)	21 (77.8%)	0	6 (22.2%)	15 (93.8%)	0	1 (6.2%)
Trimethoprim 5 µg (TMP)	39 (90.7%)	0	4 (9.3%)	24 (88.9%)	0	3 (11.1%)	15 (93.8%)	0	1 (6.2%)
Chloramphenicol 30 µg (CHL)	42 (97.7%)	1 (2.3%)	0	26 (96.3%)	1 (3,7%)	0	16 (100%)	0	0
Tigecycline 15 µg (TGC)	43 (100%)	0	0	27 (100%)	0	0	16 (100%)	0	0
Trimethoprim with sulfamethoxazole 1,25 µg + 23,75 µg (SXT)	39 (90.7%)	0	4 (9.3%)	24 (88.9%)	0	3 (11.1%)	15 (93.8%)	0	1 (6.2%)

Legend: * Sensitive, **Intermediate, *** Resistant

Table 2b. Number of isolates that exhibit resistance to a particular number of examined antibiotics

Number of antibiotics	All isolates	APEC isolates	AFEC/NON-APEC isolates
0	5	2	3
1	4	2	2
2	4	2	2
3	24	17	7
4	4	4	0
5	1	0	1
6	0	0	0
7	1	0	1

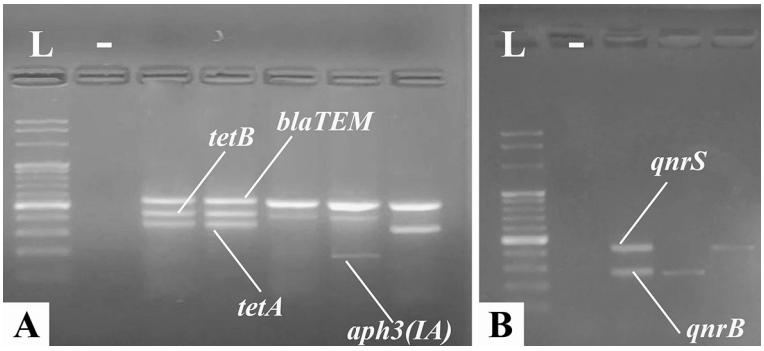

Figure 2. Multiplex PCR results for presence of genes encoding (A) antimicrobial resistance to ampicillin (*blaTEM*), gentamicin (*aph(3)IA*), tetracycline (*tetA, tetB*), and (B) quinolones (*qnrB and qnrS*)

Six different antimicrobial resistance genes (AMR genes) were detected in 32 isolates in ten combinations. Ampicillin resistance genes were found in the majority of isolates (29 isolates), subsequently tetracycline (25 isolates), quinolones (11 isolates), and aminoglycosides (7 isolates). Out of the total number of isolates carrying antimicrobial resistance genes, 4, 7, 18, and 3 isolates were found to harbour 1 (9.3%), 2 (16.3%), 3 (41.9%), and 4 (7.0%) AMR genes, respectively. Ten isolates were found to have *blaTEM+tetB+tetA*, the most common combination of AMR genes. The number of *E. coli* isolates with a certain AMR gene was shown in Table 3.

Table 3. Number of *E. coli* isolates with AMR genes

Antimicrobial resistance genes	***blaTEM*** ampicillin	***aac3VI*** gentamicin	***aph(3)IA*** gentamicin	***tetB*** tetracycline	***tetA*** tetracycline	***qnrA*** quinolones	***qnrB*** quinolones	***qnrS*** quinolones
Number of isolates and % of all *E. coli* isolates	29 (67.4%)	0	7 (16.3%)	20 (46.5%)	16 (37.2%)	0	2 (4.6%)	10 (23.2%)

The susceptibility of *E. coli* isolates to SwCh and Que was tested using the microdilution method in broth on flat-bottomed polystyrene microtiter plates.

MIC values for SwCh ranged from 0.5 to 3 mg/mL, while these values for Que were 1.5 to 4.5 mg/mL. The MIC of SwCh and Que against *E. coli* isolates were shown in Table 4.

Table 4. MIC values of sweet chestnut (SwCh) and quebracho (Que) against *E. coli* isolates (mg/mL)

N^0	Isolate	APEC	AMR N^0 of antibiotics	MIC Sweet chestnut	MIC Quebracho
1	EC spl 1	-	2	2	3
2	EC intst 1	+	2	1,5	3
3	EC spl 2	+	3	2	3
4	EC ylk 2.1	-	3	2.5	3
5	EC ylk 2.2	-	3	2.5	3
6	EC ylk 2.5	+	3	3	3
7	EC intst 2.2	-	3	1.5	1.5
8	EC spl 3	-	3	2	4
9	EC spl 4.1	-	0	1.5	1.5
10	EC hpt4.1	-	0	2	4
11	EC spl 5.3	-	7	1.5	4
12	EC hpt6	-	5	1.5	4
13	EC spl7	+	3	1.5	4
14	EC hpt 7	+	4	1.5	2.5
15	EC intst 8	+	0	1.5	3.5
16	EC ylk 8	-	0	1.5	3
17	EC intst 9	+	1	2	4.5
18	EC hpt 9	+	3	2.5	4.5
19	EC spl 10.1	+	3	2	4.5
20	EC spl 10.2	-	3	1.5	4.5
21	EC hpt 10	+	3	1.5	4
22	EC intst 11.2	-	3	2.5	2.5
23	EC hpt 11.1	+	3	2.5	3
24	EC hpt 11.2	+	3	3	3
25	EC intst 11.1	-	3	3	3

Continuation of table 4.

26	EC ylk 11.2	+	3	2	3
27	EC spl 11	+	3	2.5	3
28	EC intst 12	-	1	2.5	3.5
29	EC hpt 12	+	3	2	3.5
30	EC spl 12	+	3	3	3
31	EC intst 13	+	4	2.5	3
32	EC hpt 13	+	3	3	3
33	EC spl 14	+	3	3	3
34	EC hpt 14	+	3	3	3
35	EC intst 14	+	3	2	3.5
36	EC intst 15	-	2	3	4.5
37	EC hpt 15	+	3	0.5	3
38	EC hpt 16.1	+	1	2.5	3
39	EC hpt 16.2	+	4	2	3.5
40	EC intst 16	+	0	1	3
41	EC hpt 17	+	4	1.5	3
42	EC intst 17	+	2	2.5	3
43	EC hpt 18	-	1	2.5	3.5
44	EC ATCC 25922	-	0	1	3

Legend: EC-*E. coli*, spl-spleen, intst-intestines, ylk-yolk, hpt-liver (origin of the isolate).

The average MIC value for SwCh was 2.06 mg/mL, while for Que it was higher at 3.28 mg/mL. The two extracts' levels of MIC values (sweet chestnut extract and quebracho extract) have been found to differ significantly ($P<0.0001$) (Figure 3).

The Kolmogorov–Smirnov test and the Mann-Whitney test proved that the action of both tannin extracts from sweet chestnut and quebracho is not found to be related to the presence of APEC virulence genes (KS: $D=0.114$, $P=0.999$ and $D=0.202$, $P=0.7935$; MW: $U=167$, $P=0.628$ and $U=195.5$, $P=0.737$) (Figures 4 and 5).

Also showed that there is no difference in terms of the effects of tannins from sweet chestnut and quebracho on multi-resistant *E. coli* isolates and those that are not multi-resistant, i.e., that the presence of antibiotic resistance genes does not lead to the development of resistance to tannins (KS: $D=0.156$, $P=0.98$ and $D=0.095$, $P=1.0$; MW: $U=163$, $P=0.409$ and $U=209.5.5$, $P=0.798$) (Figures 6 and 7).

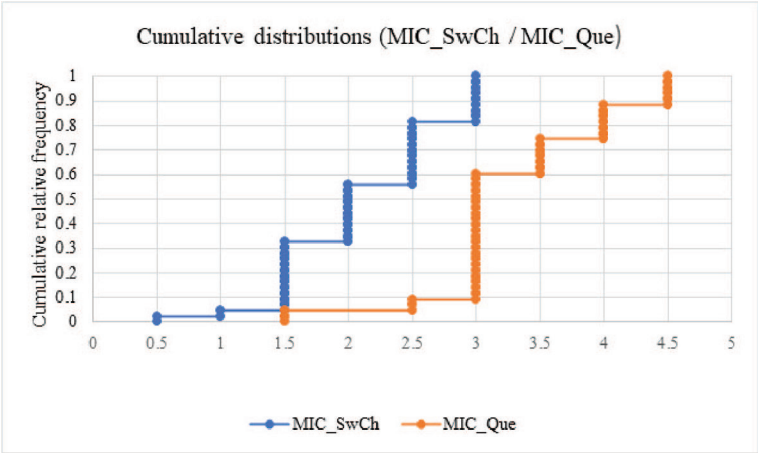

Figure 3. Cumulative distributions of MIC values frequencies for sweet chestnut (SwCh) and quebracho (Que) tannin extracts (mg/mL)

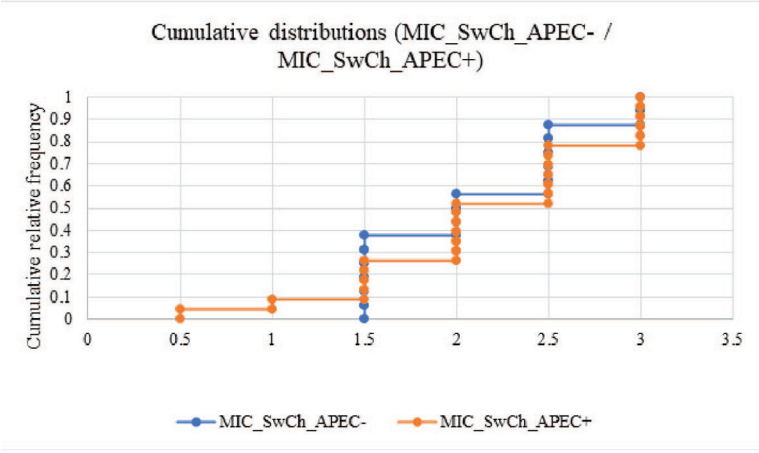

Figure 4. Cumulative distributions of MIC values frequencies for sweet chestnut (SwCh) for commensal AFEC (APEC-) and APEC (APEC+) isolates (mg/mL)

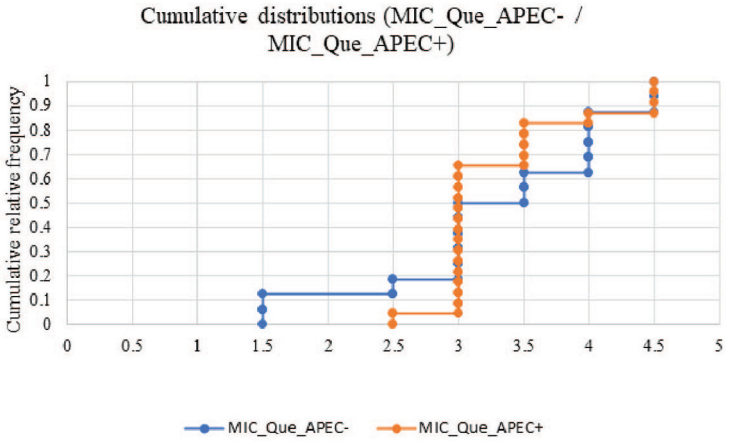

Figure 5. Cumulative distributions of MIC values frequencies for quebracho (Que) extract for commensal AFEC (APEC-) and APEC (APEC+) isolates (mg/mL)

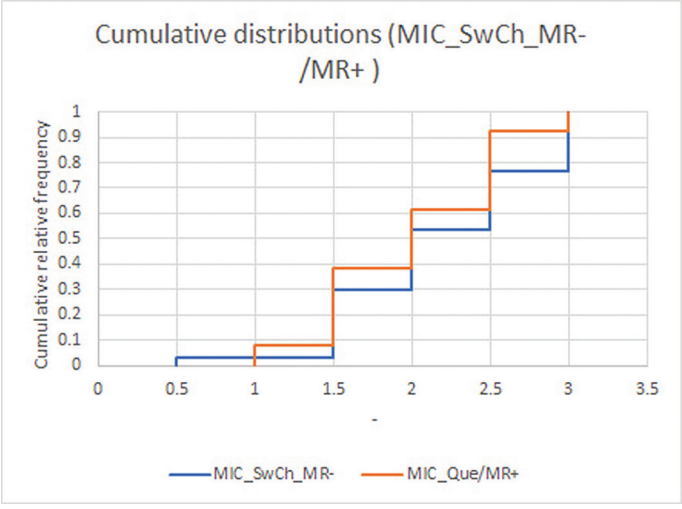

Figure 6. Cumulative distributions of MIC values frequencies for sweet chestnut (SwCh) for multi-resistant (MR+) and not multi-resistant (MR-) *E. coli* isolates (mg/mL)

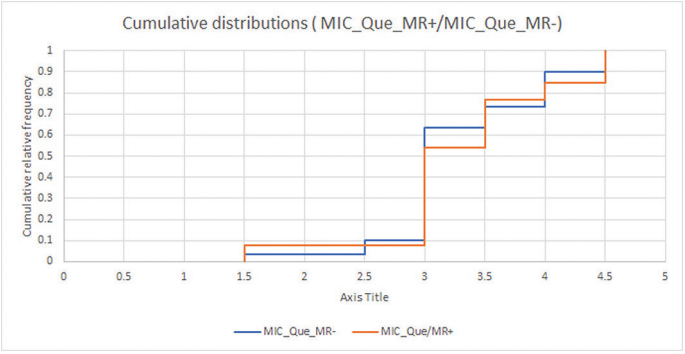

Figure 7. Cumulative distributions of MIC values frequencies for quebracho (Que) extract for multi-resistant (MR+) and not multi-resistant (MR-) *E. coli* isolates (mg/mL)

DISCUSSION

Out of 123 examined organ samples from deceased poultry across 18 farms, including layers, broilers, and broiler breeders, 43 *E. coli* strains were isolated, of which 27 (62.8%) were identified as APEC. As noted by other researchers, traditional biochemical reactions and ELISA methods for APEC identification were not only costly but also time-consuming. Consequently, the PCR method has emerged as a quick and efficient technique for detecting APEC [26,27].

Our findings indicate that virulence genes associated with the APEC subset are omnipresent among *E. coli* isolates from poultry farms in the Republic of Serbia. This prevalence suggests a substantial risk of colibacillosis, particularly under stress conditions prevalent on these farms.

Effective prevention and control of APEC infections hinge on identifying and mitigating predisposing factors. Thus, the primary goal is to minimize exposure to APEC through enhanced biosecurity measures, as well as improving litter and ventilation conditions in poultry environments [1,8,10].

Antibiotics are commonly used in both prevention and treatment of APEC infections, which contributes to the rapid emergence of multidrug-resistant bacteria [28]. In our analysis of 18 chicken farms, a significant majority (69.8%) of the *E. coli* isolates exhibited resistance to three or more antibiotics. The highest resistance rates were observed for ampicillin (79.1%), tetracycline (69.8%), and nalidixic acid (55.8%). Additionally, resistance was found in 16.3% of isolates for gentamicin, 11.6% for ciprofloxacin, 9.3% for trimethoprim with sulfamethoxazole, and 7.5% for amoxicillin with clavulanic acid.

Compared to previous studies in the Republic of Serbia, the resistance levels of *E. coli* isolated from poultry have remained high for ampicillin and tetracycline, decreased

for trimethoprim with sulfamethoxazole, and increased for gentamicin [6,7,29]. In summary, our study found high levels of antimicrobial resistance in *E. coli* isolates particularly to ampicillin, tetracycline, and nalidixic acid, while lower resistance rates were noted for the other antibiotics tested.

Our analysis of antimicrobial resistance in APEC and AFEC isolates revealed that APEC isolates had a higher rate of resistance to amoxicillin (85.2% vs. 68.7%), gentamicin (22.2% vs. 6.2%), and trimethoprim as well as trimethoprim with sulfamethoxazole (11.1% vs. 6.2%). These findings align with global research, which indicates that APEC isolates are often resistant to multiple antibiotics [30]. Consequently, it is critical to accurately identify the *E. coli* strain responsible for colibacillosis and select an effective treatment based on the results of antimicrobial susceptibility testing.

The *blaTEM* gene is commonly found among *Enterobacteriaceae* and is the most prevalent resistance gene in bacterial populations worldwide. In our study, 29 isolates (67.4% of all isolates) tested positive for the *blaTEM* gene. Additionally, ten isolates (23.3% of all isolates) were found to carry the antimicrobial resistance (AMR) gene combination of *blaTEM*+*tetB*+*tetA*.

AMR genes such as *blaTEM*, *aph(3)IA*, *tetB*, *tetA*, *qnrA*, *qnrB*, and *qnrS* in *E. coli* can be horizontally transferred via mobile genetic elements, and these bacteria are commonly found on poultry farms in Serbia. The high levels of antimicrobial resistance in APEC are concerning as antibiotic-resistant bacteria and their genes can be transmitted to humans through the food chain. The emerging resistance of APEC to important antibiotics like carbapenems, fluoroquinolones, β-lactams, and colistin highlights the impending challenges in using antibiotics to manage APEC infections in poultry [31].

It's also crucial to recognize that APEC is part of the extraintestinal pathogenic *Escherichia coli* (ExPEC) subgroup, similar to uropathogenic *E. coli* (UPEC) and neonatal meningitis *E. coli* (NMEC). Numerous studies have identified APEC as both a potential foodborne zoonotic pathogen and a source of extraintestinal infections in humans [32, 33].

Given the widespread prevalence of APEC strains and the common occurrence of predisposing factors for poultry colibacillosis, coupled with the inefficacy and strict limitations on antibiotic use, there is a pressing need for effective alternatives to prevent and control this disease. Managing APEC infections in poultry depends on robust biosecurity and management practices, the use of antibiotics, and vaccination strategies, including those against viral and immunosuppressive diseases [1]. However, there currently is no effective vaccine to protect chickens from APEC infections, largely due to the diversity of APEC serotypes involved in field outbreaks of colibacillosis.

Various potential alternatives such as probiotics, prebiotics, bacteriophages, innate immune stimulants, virulence and growth inhibitors, antimicrobial peptides, and phytobiotics have been explored with the aim of developing effective preventative and therapeutic measures to manage colibacillosis in chickens [34]. Furthermore, the antibacterial, anti-inflammatory, and antioxidant properties of natural extracts,

including tannins, have been extensively studied. Numerous investigations have examined the impact of tannin-rich feed on growth performance, feed efficiency, and antimicrobial activity in poultry [12, 13].

Our study of the antimicrobial *in vitro* activity of tannin extracts on *E. coli* isolated from deceased poultry resulted in MIC values ranging from 0.5 to 3 mg/mL for SwCh and from 1.5 to 4.5 mg/mL for Que. The average MIC value for SwCh was 2.06 mg/mL, while for the Que was 3.28 mg/mL. The MIC values of SwCh and Que were statistically significantly different (KS: $D=0.721$, $P<0.0001$; MW: $U=185$, $P<0.0001$), which could have important implications for the practical use of tannins in poultry production. The MW (Two-tailed test) and KS tests showed that there are statistically significant differences regarding the effects of tannin obtained from Quebracho and that obtained from Sweet Chestnut on the growth of *E. coli*. Although in both cases of application of these different tannins, a positive effect of stopping the growth of *E. coli* was achieved, it is evident that there are differences at the level of MIC values. This can certainly have potential significance in terms of the practical application of tannins in clinical practice or the food industry, taking into account other properties of tannins, such as taste, pH and the like.

Our results suggest that the effectiveness of the antimicrobial action of tannins does not depend on the pathogenicity of *E. coli* isolates (APEC or AFEC strains) or the presence of AMR genes.

The obtained MIC values are very similar to the results of other authors: for example, the susceptibility of strains of *E. coli* was determined on sweet chestnut extract, and the MIC values for hydrolysable tannins were 0.3–1.2 mg/mL [35]. In another experiment, it was demonstrated that chestnut and mimosa tannins have growth-inhibitory and bactericidal effects in vitro against *E. coli* O157:H7. Quantitatively, mimosa tannins had higher growth-inhibitory activity, but chestnut tannins had greater bactericidal activity [36].

In scientific studies dealing with the determination of the extent of the antimicrobial properties of plant extracts, there are various threshold values for the MIC, which define the strength of a certain activity.

Thus, if we compare the MIC values obtained in our research with the breakpoint MIC value of strong activity plant extracts of less than 5 mg/ml defined by Bussmann et al. [38], we can conclude that SwCh and Que are characterized by strong antibacterial activity against the tested isolates of *E. coli*. On the other hand, if we apply the breakpoints values of the antimicrobial activity of plant extracts established by Saraiva et al. [39], the determined MIC values of SwCh and Que belong to moderately active and low active.

It has already been mentioned that the type of microorganism and the chemical structure of tannins influence the tannins antimicrobial activity. Due to the vast sources of tannins, there is a great diversity in their chemical composition and, consequently, different antimicrobial activities.

In general, it has been found that tannins have a greater antimicrobial effect against Gram-positive than against Gram-negative bacteria. That is a consequence of the structure of Gram-negative bacteria cell wall, which possesses an outer membrane consisting of a lipid bilayer envelope. Nevertheless, tannins, especially condensed tannins isolated from several plants, have been shown to possess strong activity against Gram-negative bacteria. It is important to note that pathogenic bacteria such as *E. coli* O157:H7, *Salmonella* spp., *Shigella* spp., *Staphylococcus* spp., *Pseudomonas* spp., and *Helicobacter pylori* all demonstrate sensitivity to tannins [12].

One study examined the antimicrobial effectiveness of hydrolysable tannins on *E. coli*, *Staphylococcus aureus*, *Clostridium perfringens*, and *Lactobacillus plantarum* [39]. Ellagitannins were shown to exhibit antimicrobial effects, and the strength for the growth inhibition was in the following order: *S. aureus* > *E. coli* > *C. perfringens*. Interestingly, ellagitannins lacked antimicrobial activity against probiotics and had no or minimal negative effect against *L. plantarum*.

Furthermore, it has been reported that a combination of quebracho and chestnut tannins can influence the diversity of chicken cecal microbiota by reducing the presence of the genus *Bacteroides* and increasing certain members of the order Clostridiales, mainly from the *Ruminococcaceae* and *Lachnospiraceae* families [40]. These findings suggest that the selective antimicrobial properties of tannins could positively affect the microbiome in poultry.

Several studies have also shown that tannins exhibit a notably stronger antibacterial effect against *Salmonella* spp. compared to *E. coli* [41, 42]. Beyond *in vitro* assessments, the antibacterial effectiveness of chestnut and quebracho wood extracts was tested in experimental infection trials with laying hens and broilers, specifically targeting *S.* Gallinarum and *S.* Enteritidis. Notably, the excretion of *S.* Enteritidis was significantly lowered on days 5 and 12 in the quebracho-treated group. In a fowl typhoid infection model, hens treated with chestnut extract demonstrated a significantly lower mortality rate [42].

The findings from our study on the antimicrobial effects of hydrolysable and condensed tannins extracts against *E. coli*, isolated from the pathological samples of deceased poultry, highlight the significant potential of extracts from sweet chestnut and quebracho in preventing and treating colibacillosis. It's crucial, however, to extend this research beyond *in vitro* studies to *in vivo* experiments where the impact of tannins as a feed additive on the poultry microbiome could be assessed. Given their antimicrobial properties against *Salmonella* spp., tannins could not only enhance poultry health but also positively influence public health. Recognizing tannins as promising growth promoters, it is evident that their use could serve as an effective strategy to address current challenges in poultry production [43].

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Authors' contributions

MĆ, MI and DK carried out sample collection, culturing bacteria, biochemical analysis, antimicrobial susceptibility testing and antimicrobial testing of hydrolysable and condensed tannins. AR, IP and NZ performed the molecular analysis. DK, MĆ, MŠ, RR, and VK conceived the study, participated in its design and coordination, and revised the final version of the manuscript. SS and DK conducted data analysis and statistical interpretation of data. MĆ, DK, AR and IP came up with the draft of the manuscript. All authors read and approved the final manuscript.

Declaration of competing interests

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ISPITIVANJE *IN VITRO* ANTIMIKROBNE AKTIVNOSTI EKSTRAKATA HIDROLIZABILNIH I KONDENZOVANIH TANINA PREMA IZOLATIMA *ESCHERICHIA COLI* POREKLOM OD UGINULE ŽIVINE

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Kolibaciloza, uzrokovana patogenim sojevima *Escherichia coli* kod živine (APEC), predstavlja jedno od najrasprostranjenijih i ekonomski najznačajnijih bakterijskih oboljenja živine širom sveta. Kontrola kolibaciloze je veoma kompleksna i često neefektivna zbog široke rasprostranjenosti APEC sojeva i njihove rezistencije na antibiotike, kao i zbog zabrinutosti javnosti i strogih propisa koje ograničavaju upotrebu antimikrobnih

sredstava u živinarstvu. Ova studija je imala za cilj ispitivanje uticaja hidrolizabilnih (ekstrakt pitomog kestena) i kondenzovanih (ekstrakt kebrača) tanina na izolate *E. coli* poreklom od živine, u cilju analize mogućnosti njihove primene kao alternative antibioticima. *E. coli* je izolovana iz unutrašnjih organa uginule živine poreklom sa 18 farmi, uključujući farme kokošaka nosilja, brojlera i brojlerskih roditelja. Svaki izolat je ispitivan na prisustvo prediktora sojeva APEC (gena virulencije iutA, hlyF, iss, iroN i ompT), na osetljivost prema 14 antibiotika metodom disk difuzije, kao i na prisustvo gena rezistencije na antibiotike (ampicilin, gentamicin, tetraciklin i fluorohinolone). Od ukupno 43 izolata, 27 (62,79%) je klasifikovano kao APEC, 30 (69,8%) je ispoljavalo rezistenciju prema tri ili više klasa antibiotika, a 32 (74,4%) je posedovalo bar jedan gen antimikrobne rezistencije. Minimalne inhibitorne koncentracije (MIK) za hidrolizabilne tanine ekstrakta pitomog kestena kretale su se od 0,5 do 3 mg/mL, dok su za kondenzovane tanine ekstrakta kebrača iznosile od 1,5 do 4,5 mg/mL. Dobijeni rezultati ukazuju da i hidrolizabilni i kondenzovani tanini ispoljavaju značajnu *in vitro* antimikrobnu aktivnost protiv APEC što ukazuje na njihov potencijal kao alterative antibioticima u kontroli kolibaciloze u živinarskoj industriji.