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Feasibility of including a phytobiotic containing cinnamon oil in the diet to reduce the occurrence of neurodegenerative changes in broiler chicken tissues

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ABSTRACT. It was assumed that the addition of a phytobiotic preparation containing cinnamon oil and citric acid to water, at a properly selected dose and time of application, could reduce the occurrence of metabolic disorders of the nervous tissue leading to neurodegenerative lesions. The aim of the study was to compare the effect of selected doses of the phytobiotic administered over different periods of time on the level of parameters indicating the occurrence of neurodegenerative changes in selected tissues of broiler chickens. All doses of the phytobiotic reduced the formation of β-amyloid deposits both after its continuous administration and during selected rearing periods. The levels of neurometabolism indicators were analysed to assess the effect of the phytobiotic containing cinnamon oil. The addition of the highest and medium doses of the phytobiotic (0.25 and 0.1 ml/l) resulted in a beneficial increase in the concentration of acetylcholinesterase (AChE) and low-density lipoprotein receptor-related protein 1 (LRP1), as well as a decrease in the concentration of hyperphosphorylated Tau protein and cholesterol levels, especially during continuous application of the cinnamon preparation. The highest dose of phytobiotic (0.25 ml/l) also favourably reduced glycosylated acetylcholinesterase (GAChE) and Tau protein levels, and the strongest effect was obtained during continuous application of the cinnamon oil formulation. The strongest neuroprotective effect was obtained using a phytobiotic containing cinnamon oil and citric acid at a dose of 0.25 ml/l water for 42 days of rearing broiler chickens, manifested as a reduction in the formation of toxic amyloid-β, phosphorylated Tau protein and GAChE, as well as increased levels of LRP1 and AChE proteins.

Introduction

Among the natural poultry feed additives, phytobiotics have recently become increasingly important. Plant additives could contain dried, fermented or freeze-dried plant parts or isolated essential oils (Krauze, 2021). Generally, phytogenic feed additives are used to improve the overall health of birds, aid in digestive processes and help detoxify the body (Krauze, 2021). However, phytogenic additives can also have far more specific effects, i.e. inhibit the development of pathogenic microorganisms, regulate the composition and abundance of the gastrointestinal microbiome, promote the regeneration

of the intestinal epithelium and villi, as well as exert antioxidant, immunostimulatory, anti-inflammatory, antimicrobial and neuroprotective effects (Mueller et al., 2010; Khasnavis and Pahan, 2012).

Cinnamon oil, extracted from the bark or leaves of cinnamon tree (*Cinnamomum cassia*), contains many bioactive substances, but the presence of eugenol and flavonoids, as well as cinnamaldehyde, cinnamic acetate, and cinnamic alcohol is responsible for the stimulation of anti-inflammatory, immunostimulatory and antioxidant effects, making cinnamon oil a neuroprotective substance (Khasnavis and Pahan, 2012; Adarsh et al., 2020; Ali et al, 2021). By protecting structural and enzymatic components in nerve cells from oxidation, cinnamon oil prevents adverse changes in their structure, as well as enhances metabolic activity in neurons (Ali et al., 2021). The presence of tannins, fibre and iron improves blood flow to the brain, allowing more oxygen and nutrients to reach nerve cells (Abd El-Hack et al., 2020), as well as improves carbohydrate metabolism, thereby increasing insulin effectiveness in neurons. The components of cinnamon oil also support neuronal lipid metabolism by reducing the synthesis of cholesterol, which in excess has neurotoxic effects. Cinnamon oil strengthens poultry health and performance, and a study by Ali et al. (2021) showed that the administration of cinnamon preparations was safe for bird health and did not impair the quality of animal products. Citric acid present in the phytobiotic preparation supports cellular metabolism in all animal tissues, and being a natural component of the Krebs cycle, it mediates oxidative metabolism (Ali et al., 2021).

Neurodegenerative changes caused by metabolic disorders in neurons and the accumulation of toxic peptides and protein complexes may result in deterioration of health and performance of broiler chickens. Therefore, it was considered reasonable to identify parameters indicating the occurrence of changes in neuronal metabolism in specific tissues. Due to the superior function of the brain, the metabolic function of the liver, and the transport role of blood plasma, these tissues were considered to be most susceptible to neurodegenerative changes that may result in impaired performance during commercial rearing. Neurodegenerative changes result from the interplay between neuronal metabolic disorders caused by mitochondrial dysfunction and oxidative damage and inflammation in neurons. In addition, neuronal damage results from the accumulation of β-amyloid, formation of Tau protein clusters, decreased acetylcholinesterase (AChE) levels, and the generation of neurotoxic glycosylated acetylcholinesterase (GAChE). The GAChE complex consists of compounds formed from the combination of AChE with amyloid-β (Aβ) and Tau protein (Angelopoulou et al., 2021). Pathological neurodegenerative lesions may also result from epigenetic changes in the brain, resulting from methylation reactions of selected DNA sequences in neurons (Princz et al., 2020).

It was assumed that the occurrence of metabolic disorders of the nervous tissue leading to neurodegenerative changes could be reduced by adding a phytobiotic preparation containing cinnamon oil and citric acid to water, in a properly selected dose and application time window. The aim of the study was to compare the effect of selected doses of phytobiotics administered for different periods of time on the level of neurodegenerative change indices in selected tissues of broiler chickens.

Material and methods

Experimental procedure

Animal experiments were approved by the Second Local Ethics Committee for Experiments with Animals in Lublin (approval No. 38/2018). The experimental factor was a commercial phytobiotic preparation containing cinnamon oil, which in this study was administered to chickens in two modes: continuous and periodic, at doses of 0.05, 0.1 and CT-0.25 ml/l (Table 1). A full description of the experimental procedures and nutritional, zoohygienic and slaughter recommendations were described in detail in our earlier publication concerning other parameters (Krauze et al., 2021).

Phytobiotic and chemical analysis of volatile oils in the phytobiotic preparation

The experiment used a commercial preparation for poultry (EW Nutrition, Visbek, Germany) containing 3000 mg/l cinnamon oil and 150000 mg/l citric acid. Due to the fact that the manufacturer does not declare the full content of biologically active substances on the label, we have quantitatively and qualitatively analysed this preparation in our earlier study (Krauze et al., 2021) for the content of the main bioactive substances by capillary gas chromatography using an Agilent 7890A GC apparatus (with an autosampler) (Agilent Technologies, Inc., Detroit, MI, USA). In this work, we showed the results regarding only the composition of the phytobiotic preparation, which were as follows: 78.08% cinnamaldehyde, 2.17% cinnamon acetate, 0.09% camphene, and 3.25% eugenol (Krauze et al., 2021).

Table 1. Experimental scheme for the administration of phytobiotic preparation to chickens

Heading	Treatment						
	G-C	CT-0.05	CT-0.1	CT-0.25	PT-0.05	PT-0.1	PT-0.25
Administration cycles of phytobiotic containing cinnamon oil and citric acid	0	6 × 7	6 × 7	6 × 7	3 × 7	3 × 7	3 × 7
Total intake of phytobiotic, ml/bird	0	0.33	0.63	1.66	0.14	0.28	0.72
Total intake of cinnamon oil, mg/bird	0	0.99	1.89	4.97	0.42	0.84	2.16
Total intake of citric acid, mg/bird	0	0.50	0.95	2.49	0.21	0.42	0.11

6 × 7 – intake at 1–42 days of age, 3 × 7 – intake at 1–7, 15–21 and 29–35 days of age; G-C – control group, receiving water without phytobiotic supplementation; CT – continuous administration of phytobiotic during all 6 weeks of chicken rearing; PT – temporary administration of phytobiotic – chickens received the phytobiotic preparation only during the selected periods at days 1–7, 15–21, and 29–35 of rearing, 21 days in total; CT-0.05 and PT-0.05 – groups receiving water with 0.05 ml/l of phytobiotic; CT-0.1 and PT-0.1 – groups receiving water with 0.1 ml/l of phytobiotic; CT-0.25 and PT-0.25 – groups receiving water with 0.25 ml/l of phytobiotic (according to Krauze et al., 2021)

Determination of metabolic indicators of nervous tissue

AChE levels were determined in brain and liver tissue homogenates and chicken plasma using the Chicken Acetylcholinesterase ELISA Kit (Qayee Bio-Technology Co., Shanghai, China), and amyloid-β levels using the Chicken Total β Amyloid Protein (Aβ) ELISA Kit (Qayee Bio-Technology Co., Shanghai, China). GAChE was determined using the Chicken glycosylated acetylcholinesterase (GAChE) ELISA kit (Qayee Bio-Technology Co., Shanghai, China). LRP1 was determined using the Chicken low-density lipoprotein receptorrelated protein 1 (LRP1) ELISA kit (Qayee Bio-Technology Co., Shanghai, China). Tau protein levels were assessed using the Chicken Tau Protein (Tau) ELISA kit (Qayee Bio-Technology Co., Shanghai, China), and phosphorylated Tau protein using the Chicken Phosphorylated Tau 231 (pTau231) ELISA kit (Qayee Bio-Technology Co., Shanghai, China). Cholesterol concentration was assessed using the Chicken Cholesterol (CH) ELISA Kit (My BioSource, San Diego, CA, USA). The level of epigenetic changes was determined by global DNA methylation (methylome) analysis using Merck diagnostic kits (Sigma-Aldrich, Saint Louis, MO, USA).

Statistical analysis

The results were analysed using one-way ANOVA; model assumptions of normality and homogeneity of variance were verified by the Shapiro-Wilk and Levene tests. Comparison of the control group with the experimental groups was performed using contrast analysis. A two-way ANOVA was performed in the model without the G-C group to examine the following effects: D – dose effect, T – time effect, and D × T – dose and time interaction. A full description of the statistical analysis was included in our previous publication, which reported the results concerning other parameters (Krauze et al., 2021).

Results

The application of three different doses (0.05, 0.1 and 0.25 ml/l) and two application modes (continuous and periodic) of the cinnamon oil preparation affected parameter variation, indicating reduced abnormalities in neural tissue metabolism, in the context of the neuroprotective effect of the phytobiotic.

Contrast analysis revealed increased AChE enzyme levels ($P = 0.009$ and $P = 0.026$, respectively) in the plasma and brain of chickens both in the continuous (CT) and periodic treatment (PT) compared to the control group (Table 2 and Table 3). Elevated levels of this enzyme were particularly evident when the cinnamon oil preparation was applied at higher doses of 0.1 and 0.25 ml/l (plasma: $P = 0.05$; brain: $P = 0.026$), as shown by two-way ANOVA. For liver, two-way ANOVA showed that AChE levels increased only in chickens treated with CT-0.25 and PT-0.25 ($P = 0.041$) (Table 4).

Contrast analysis showed that plasma and brain GAChE levels ($P = 0.007$ and $P < 0.001$) decreased in chickens from the CT and PT groups compared to the control group. Two-way ANOVA showed that the strongest reduction in the level of this enzyme was observed when the phytobiotic preparation containing cinnamon oil was applied at the highest dose of 0.25 ml/l (plasma: $P = 0.039$; brain: $P = 0.032$). The implementation of two different application regimes of the cinnamon oil preparation (continuous and periodic) resulted in differences in the level of GAChE in the brain. Regardless of the application dose, continuous (CT) application of the phytobiotic caused a marked reduction ($P = 0.032$) in the level of this complex. GAChE levels also decreased ($P < 0.001$) in the liver after treatment with the cinnamon oil preparation. For hepatic GAChE levels, a dose × time interaction ($P = 0.024$) was noted, which was due to the fact that the effect

Table 2. Plasma levels of selected neural tissue metabolism indices

Item		AChE, ng/ml	GAChE, ng/ml	Aβ, pg/ml	LRP 1, pg/ml	Tau, ng/ml	p-Tau, ng/ml	Chol, mmol/l
G-C		20.92	0.491	232.5	128.7	41.42	4.97	3.94
CT-0.05		26.62*	0.244*	166.5*	148.8*	34.87*	4.41	2.46*
CT-0.1		28.52*	0.194*	131.4*	202.5*	31.47*	3.74*	2.44*
CT-0.25		26.71*	0.133*	128.9*	241.4*	26.78*	2.51*	2.28*
PT-0.05		19.56	0.375	224.3*	146.3*	39.89	5.99*	2.66
PT-0.1		26.91*	0.251*	201.5*	183.5*	36.39	4.47	2.21*
PT-0.25		26.19*	0.192*	189.8*	227.2*	27.91*	3.29*	2.04*
SEM		0.121	0.03	0.56	0.17	1.13	0.11	0.08
D	0.05 ml/l	23.09^{b}	0.310^{a}	195.4^{a}	147.6^{b}	37.38^{a}	3.59	2.56^{a}
	0.1 ml/l	27.72^{a}	0.223^{ab}	166.4^{b}	193.0^{a}	35.43^{ab}	3.36	2.33^{b}
	0.25 ml/l	26.45^{a}	0.170^{b}	159.5^{b}	243.3^{a}	27.35^{b}	3.18	2.16^{c}
T	CT	27.28	0.190	142.27^{b}	197.57^{a}	31.04	3.39	2.39
	PT	24.22	0.273	205.19^{a}	185.67^{b}	34.73	3.36	2.30
P-value								
G-C vs. all other		0.009	0.007	0.032	<0.001	0.011	0.024	0.018
D		0.005	0.039	0.043	0.011	0.025	0.038	0.015
T		0.076	0.004	0.042	0.049	0.058	0.078	0.031
D × T interaction		0.088	0.054	0.104	0.125	0.087	0.128	0.392

CT – continuous administration of phytobiotic during all 6 weeks of chicken rearing; PT – temporary administration of phytobiotic during the selected periods at days 1–7, 15–21, and 29–35 of rearing, 21 days in total; CT-0.05 and PT-0.05 – groups receiving water with 0.05 ml/l of phytobiotic; CT-0.1 and PT-0.1 – groups receiving water with 0.1 ml/l of phytobiotic; CT-0.25 and PT-0.25 – groups receiving water with 0.25 ml/l of phytobiotic; AChE – acetylcholinesterase, GAChE – glycosylated acetylcholinesterase, Aβ – β amyloid protein, LRP1 – low-density lipoprotein receptor related protein 1, Tau – tau protein, p-Tau – phosphorylated tau protein, Chol – cholesterol, D – dose effect, T – time effect; * – means within the same column differ significantly from control at $P \leq 0.05$; ^{abc} – means within the same column differ significantly ($P \leq 0.05$) according to Newman-Keuls mean comparison (only for significant D × T interaction); data represent mean values of seven replications per treatment; SEM (standard error of the mean) = SD (standard deviation) divided by the square root of the number of replicates, n = 7

Table 3. Levels of selected neural tissue metabolism indices in the brain

Item		AChE, ng/ml	GAChE, ng/ml	Aβ, pg/ml	LRP 1, pg/ml	Tau, ng/ml	p-Tau, ng/ml	Chol, mmol/l
G-C		19.11	0.710	297.4	105.8	153.5*	12.77	4.25
CT-0.05		22.21	0.558*	269.7	223.6*	136.5*	10.89	4.05
CT-0.1		26.12*	0.239*	205.9*	332.2*	70.03*	8.540*	3.26*
CT-0.25		30.87*	0.222*	175.3*	469.1*	55.45*	5.510*	2.58*
PT-0.05		22.48	0.604*	298.5	225.5*	148.8	13.78	4.44
PT-0.1		23.94	0.369*	243.4	302.1*	86.12*	10.99	4.10*
PT-0.25		29.77*	0.258*	209.3*	436.8*	53.44*	6.780*	3.69*
SEM		0.08	0.04	0.38	0.11	1.87	0.09	0.02
D	0.05 ml/l	22.35^{b}	0.596^{a}	284.1^{a}	224.6^{c}	142.6^{a}	13.34^{a}	4.23^{a}
	0.1 ml/l	26.03^{ab}	0.304^{b}	224.7^{b}	317.72^{b}	78.01^{b}	9.771^{ab}	3.68^{ab}
	0.25 ml/l	30.32^{a}	0.240^{c}	192.3^{c}	453.0^{a}	54.45^{c}	6.152^{b}	3.14^{b}
T	CT	26.40	0.34^{b}	217.0^{b}	341,6	87.30	8.31	3.29^{b}
	PT	25.39	0.41^{a}	250.4^{a}	321.5	96.11	10.52	4,07^{a}
P-value								
G-C vs. all other		0.026	<0.001	0.026	0.047	<0.001	0.049	0.019
D		0.041	0.032	0.022	0.009	<0.001	0.006	0.025
T		0.064	0.035	0.008	0.087	0.062	0.233	0.017
D × T interaction		0.063	0.072	0.069	0.057	0.064	0.07	0.25

G-C – control group, receiving water without phytobiotic supplementation; CT – continuous administration of phytobiotic during all 6 weeks of chicken rearing; PT – temporary administration of phytobiotic during the selected periods at days 1–7, 15–21, and 29–35 of rearing, 21 days in total; CT-0.05 and PT-0.05 – groups receiving water with 0.05 ml/l of phytobiotic; CT-0.1 and PT-0.1 – groups receiving water with 0.1 ml/l of phytobiotic; CT-0.25 and PT-0.25 – groups receiving water with 0.25 ml/l of phytobiotic; AChE – acetylcholinesterase, GAChE – glycosylated acetylcholinesterase, Aβ – β amyloid protein, LRP1 – low-density lipoprotein receptor related protein 1, Tau – tau protein, p-Tau – phosphorylated tau protein, Chol – cholesterol, D – dose effect, T – time effect; * – means within the same column differ significantly from control at $P \leq 0.05$; ^{abc} – means within the same column differ significantly ($P \leq 0.05$) according to Newman-Keuls mean comparison (only for significant D × T interaction); data represent mean values of seven replications per treatment; SEM (standard error of the mean) = SD (standard deviation) divided by the square root of the number of replicates,, n = 7

Table 4. Level of selected metabolic neural tissue indices in the liver

Item		AChE, ng/ml	GAChE, ng/ml	Aβ, pg/ml	LRP1, pg/ml	Tau, ng/mL	p-Tau, ng/ml	Chol, mmol/l
G-C		14.44	0.671	587.1	125.8	177.4	18.78	7.25
CT-0.05		14.98	0.574	346.9*	132.2	178.5	17.25	5.66*
CT-0.1		16.87	0.247*	308.5*	210.6*	170.6	17.11	5.04*
CT-0.25		21.10*	0.258*	258.3*	287.7*	125.6*	16.47*	4.55*
PT-0.05		16.47	0.614	566.5	123.6	178.4	17.58	5.16*
PT-0.1		16.65	0.588	545.8	158.7*	156.8*	17.44	4.54*
PT-0.25		20.44*	0.369*	308.7*	233.8*	123.7*	16.58*	3.47*
SEM		0.11	0.03	0.43	0.12	1.25	0.14	0.12
D	0.05 ml/l	15.73^{b}	0.59^{a}	465.7^{a}	127.9^{b}	178.5^{a}	17.42	5.41^{a}
	0.1 ml/l	16.67^{b}	0.412^{b}	427.1^{a}	184.7^{a}	163.7^{b}	17.28	4.79^{b}
	0.25 ml/l	20.77^{a}	0.314^{c}	283.5^{b}	260.8^{a}	124.5^{c}	16.53	4.01^{c}
T	CT	17.65	0.367^{b}	304.53^{b}	210.2^{a}	158.23	16.94	5.08^{a}
	PT	17.85	0.524^{a}	473.65^{a}	172.3^{b}	152.95	17.09	4.39^{b}
P-value								
G-C vs. all other		0.002	<0.001	0.047	<0.001	0.021	0.026	0.014
D		0.034	0.039	0.028	0.017	0.025	0.093	0.024
T		0.084	0.021	0.008	0.038	0.063	0.067	0.013
D × T interaction		0.066	0.024	0.091	0.054	0.071	0.069	0.127

G-C – control group, receiving water without phytobiotic supplementation; CT – continuous administration of phytobiotic during all 6 weeks of chicken rearing; PT – temporary administration of phytobiotic during the selected periods at days 1–7, 15–21, and 29–35 of rearing, 21 days in total; CT-0.05 and PT-0.05 – groups receiving water with 0.05 ml/l of phytobiotic; CT-0.1 and PT-0.1 – groups receiving water with 0.1 ml/l of phytobiotic; CT-0.25 and PT-0.25 – groups receiving water with 0.25 ml/l of phytobiotic; AChE – acetylcholinesterase, GAChE – glycosylated acetylcholinesterase, Aβ – β amyloid protein, LRP1 – low-density lipoprotein receptor related protein 1, Tau – tau protein, p-Tau – phosphorylated tau protein, Chol – cholesterol, D – dose effect, T – time effect; * – means within the same column differ significantly from control at $P \leq 0.05$; ^{abc} – means within the same column differ significantly ($P \leq 0.05$) according to Newman-Keuls mean comparison (only for significant D × T interaction); data represent mean values of seven replications per treatment; SEM (standard error of the mean) = SD (standard deviation) divided by the square root of the number of replicates, n = 7

of continuous (CT) application of the phytobiotic preparation became apparent in the form of lower GAChE levels, while no such effect was observed for periodic (PT) application (Table 4).

The results of the contrast analysis demonstrated that the administration of the cinnamon oil formulation at doses of 0.05, 0.1 and 0.25 ml/l significantly reduced the plasma ($P = 0.032$) and brain ($P = 0.026$) β-amyloid levels in broiler chickens compared to the control group (Table 2 and Table 3). The results of two-way ANOVA showed that the strongest reduction ($P = 0.043$) in plasma β-amyloid levels was obtained when higher doses of the phytobiotic (0.1 and 0.25 ml/l) were used in both CT and PT treatments. On the other hand, in the brain ($P = 0.022$) and liver ($P = 0.028$), similar results were obtained for all CT treatment doses, and for the 0.25 ml/l dose from the PT treatment. A two-way ANOVA showed that the use of two different modes of phytobiotic administration to chickens (CT and PT) resulted in a more significant reduction of β-amyloid levels in the plasma ($P = 0.042$), brain ($P = 0.008$), and liver ($P = 0.008$) of broiler chickens after continuous (CT) phytobiotic administration than with periodic (PT) application, regardless of the dose size (Table 4).

Moreover, contrast analysis proved that the addition of cinnamon oil increased LRP1 levels ($P < 0.001$) in the plasma, brain and liver of broiler chickens, following continuous and periodic application of the preparation compared to the control group. A two-way ANOVA showed that of the doses applied, the strongest effects were observed for 0.1 and 0.25 ml/l ($P = 0.011$, $P = 0.009$ and $P = 0.009$, respectively). It was also found that plasma and liver LRP1 levels increased ($P = 0.049$ and $P = 0.038$, respectively) after continuous application of the phytobiotic, regardless of the dose administered, which was not observed with periodic application.

Toxic Tau protein levels decreased ($P = 0.011$) in the plasma of chickens in all CT and PT-0.25 treatments compared to the control group. A similar effect was found in the brain, for the CT and PT groups administered the highest doses (0.1 and 0.25 ml/l) compared to the control group. In the liver, such a correlation was observed for the same doses in the PT group, while only the highest dose of 0.25 ml/l significantly reduced Tau levels in CT treatment compared to the control group. A two-way ANOVA indicated that the application of different

doses of cinnamon oil preparation differentiated Tau protein levels in the plasma, brain and liver. It was observed that irrespective of the duration of administration, the level of Tau protein in the brain and liver decreased most strongly after 0.1 and 0.25 ml/l doses (brain $P < 0.001$; liver $P = 0.025$) (Table 3 and Table 4). It was also recorded that regardless of the application time, plasma Tau levels were most significantly reduced by the 0.25 ml/l dose ($P = 0.025$).

In the plasma and brain of broiler chickens from CT-0.1, CT-0.25 and PT-0.25 treatments, the level of phosphorylated Tau protein was lower ($P = 0.024$ and $P = 0.049$) than in the control group. At the same time, plasma p-Tau levels were significantly higher ($P = 0.024$) in the PT-0.05 group compared to the control group. A comparison with the control values obtained for the liver clearly indicated that a reduction ($P = 0.026$) in p-Tau protein levels was achieved only at the highest doses. The most pronounced reduction in plasma and brain levels of this indicator was observed at the highest phytobiotic dose of 0.25 ml/l ($P = 0.038$), as demonstrated by the result of a two-way ANOVA. For p-Tau, there was no effect of different administration mode on the final results of the analysis.

A one-way ANOVA showed that plasma cholesterol levels in chickens from the experimental groups treated with 0.1 and 0.25 ml/l doses were lower ($P = 0.019$) than in the control group. Additionally, it was observed that of the doses applied, the 0.25 ml/l dose most significantly lowered brain cholesterol levels ($P = 0.025$), especially during the continuous application ($P = 0.017$) of the phytobiotic preparation. Moreover, cholesterol levels in the liver were found to decrease the most ($P = 0.024$) after continuously administered dose of 0.25 ml/l.

There was no effect of cinnamon oil on methylation changes of histone proteins in DNA.

Discussion

In the present study, we assessed the levels of β-amyloid, which forms senile plaques that alter the integrity of neuronal cell membranes, resulting in increased production of reactive oxygen species and disruption of calcium homeostasis, leading to nerve cell death (Dhouafli et al., 2018). We also analysed the levels of Tau protein, a major component of neurofibrillary tangles, as well as the concentrations of AChE, a crucial enzyme in nerve impulse conduction and neuronal protection processes. Other authors have also determined the levels of toxic GAChE complexes, which are responsible for neuronal apoptosis, as well as the amount of low density LRP1, which promotes the degradation of toxic amyloid-β (Krauze et al., 2022). The level of cholesterol that can be oxidized by free radicals was also measured, as the products of these transformations exert neurotoxic effects causing neuronal cell membrane dysfunction (Princz et al., 2020). Assessing the extent of histone protein methylation resulting from covalent modifications of lysine and arginine residues may also be helpful in diagnosing the severity of neurodegenerative changes (Rowe et al., 2019).

In our opinion, the novelty of this study is the broadening of knowledge on the use of cinnamon-based phytobiotics in the context of reducing the occurrence of neurodegenerative changes. Due to the lack of similar studies in the world literature, both in animal models and in humans, it is difficult to accurately compare our results on neurodegenerative changes in broiler chicken tissues with the results of other researchers. The different duration of phytobiotic administration to chickens is another complication in our work.

The evaluation of production performance reported in our earlier publication (Krauze et al., 2021) showed that the final mean body weight of chickens from the CT-0.1, CT-0.25 and PT-0.25 groups, at 42 days of age, was statistically higher (2.72, 2.74 and 2.67 kg, respectively) compared to the G-C group (2.65 kg).

The results of our study indicated that the bioactive components of the phytobiotic containing cinnamon oil and citric acid, reaching the brain and liver with blood, could inhibit the development of degeneration processes of nervous tissue in these organs. Yulug and Cankaya (2019) demonstrated that cinnamon oil could prevent metabolic disorders, reduced inflammation and oxidative changes in nerve tissue. Furthermore, according to the latter researchers, the components of the oil not only reduced cognitive changes in the brain, but also inhibited the loss of physiological function of a specific organ (e.g., brain or liver), which was classified as non-cognitive symptoms (Yulug and Cankaya, 2019). The results of a mouse model study by Qubty et al. (2021) showed that the use of cinnamon extract could also inhibit neuronal degradation and alleviate behavioural disorders.

Moreover, Yulug and Cankaya (2019) reported that bioactive compounds of *C. cassia* prevented the formation of undesirable protein clusters in neural tissue, especially senile plaques, built from deposits of toxic amyloid-β and excessive accumulation

of Tau protein, which can cause extensive neuronal death through a number of toxic pathways (Yulug and Cankaya, 2019; Hajinejad et al., 2020). Although Tau protein physiologically determines the normal function of synapses and neurons, when this compound accumulates excessively and oxidative stress occurs in neurons, it begins to act pathologically. In addition, dysregulation of phosphorylation, abnormal folding of Tau protein and its excessive aggregation result in the formation of toxic neurofibrillary tangles, i.e. deposits of over-phosphorylated Tau protein. The findings of Emamghoreishi et al. (2019) have indicated a strong, neuroprotective nature of cinnamaldehyde, as it protects neuronal cells from amyloid-β neurotoxicity, reduces Tau protein oligomerization and aggregation in neurons and its phosphorylation. According to the latter authors, the neuroprotective effect of cinnamaldehyde could be related to the inhibition of the N-methyl-D-aspartate receptor, which transports Ca^{2+} ions inside the cell.

In our study, the application of all doses (0.05, 0.1 and 0.25 ml/l) of a phytobiotic containing cinnamon oil resulted in a very beneficial reduction of amyloid-β levels in the brain, plasma and liver of chickens, especially when applied continuously. Ahmed et al. (2021) and Akbar et al. (2021) reported that cinnamic aldehyde and eugenol raised the levels of brain-derived neurotrophic factor (BDNF) in the hippocampus, which in turn increased the number of neuronal dendrites, improved neuronal plasticity, and helped reduce the inflammation caused by amyloid-β accumulation in neurons.

The administration of the cinnamon oil phytobiotic at a dose of 0.25 ml/l, regardless of the application mode, most strongly reduced Tau protein levels especially in the liver, although these changes were also evident in the plasma and brain. Yulug and Cankaya (2019) concluded that the neuroprotective effects of cinnamon oil, associated with reduced amyloid-β cluster formation and Tau protein aggregation, were due to the suppression of inflammation and oxidative damage in neurons. According to these researchers, cinnamaldehyde, cinnamic acetate, and cinnamic alcohol, which are converted through hydrolysis and oxidation reactions to cinnamic acid, a potent neuroprotective agent, are mainly responsible for such effects. Cinnamic acid is subsequently β-oxidized to sodium benzoate or benzoyl-CoA in the liver of birds. Khasnavis and Pahan (2012) found in their study that it was sodium benzoate and benzoyl-CoA that prevented neural tissue inflammation by stimulating the production of anti-inflammatory cytokines, improving mitochondrial function, reducing free radical formation and preventing oxidative stress. Sodium benzoate can also inhibit the expression of pro-inflammatory molecules in activated glial cells and reduce epigenetic changes in neural tissue (Adarsh et al., 2020). Moreover, sodium benzoate reduces neuropathological changes by producing neurotrophic factors and inhibiting neuroinflammation, and neurotrophins facilitate these processes. Sodium benzoate activates the protein kinase A and cyclic adenosine-3′,5′-monophosphate (cAMP) response element-binding pathway, resulting in the production of brain-derived neurotrophic factor and neurotrophin-3 (Hajinejad et al., 2020).

In a study of Zhao et al. (2019), cinnamon oil was effective in reducing the deposition of β-amyloid, which accumulate in the central nervous system and initiate nerve cell disorders. These researchers reported that the neurotoxic effects of amyloid-β were caused by competitive binding to the insulin receptor and disruption of glucose metabolism in the brain (Hajinejad et al., 2020). Kawatra and Rajagopalan (2015) identified stimulation of insulin signalling, increased glucose transport into cells, as well as reduced oxidative stress and increased AChE synthesis as the reasons for the improvement in neuronal metabolism after cinnamon oil application (Kawatra and Rajagopalan, 2015). Amyloid plaques deposited in the brain may also cause calcium metabolism disturbances and mitochondrial damage, which contributes to the overproduction of free radicals that not only promote inflammation, but also exacerbate DNA and cellular protein degeneration (Valera et al., 2016). In our study, citric acid, which can suppress the initiation of oxidative stress and lipid peroxidation, as well as reduce inflammation severity by inhibiting inflammatory marker production, could have supported the neuroprotective effect (Ali et al., 2021). Another important fact in terms of neurodegenerative disease development is that large amounts of copper can accumulate in the amyloid plaques themselves (Cendrowska-Pinkosz et al., 2021), which, as reported by Bush and Tanzi (2008), further exacerbate the toxicity of amyloid-β to neuronal cells. However, Martinez et al. (2018) has shown that citric acid can chelate excess copper, which may contribute to the neuroprotective effect of this compound as a decontamination additive in cinnamon oil (Martinez et al., 2018). Moreover, Krauze et al. (2021) reported that the combination of cinnamon oil and citric acid had a positive effect on poultry performance, because it lowered the pH of the intestinal contents and reduced bacterial growth due to pH changes. In addition, undissociated organic acids can penetrate the lipid

membrane of the bacterial cell and lower its pH, leading to bacterial cell death.

Valera et al. (2016) showed that cinnamic oil inhibited Tau protein aggregation and destroyed existing Tau protein clusters. Moreover, Momtaz et al. (2017) stated that cinnamaldehyde could form strong bonds with two cysteine residues on Tau protein surface, thereby protecting it from oxidative changes and reducing its excessive accumulation in neurons (Momtaz et al., 2017). Findings of multiple research teams have also suggested that amyloidβ may stimulate the formation of neurotoxic deposits of hyperphosphorylated Tau protein, and thus form neurotoxic neurofibrillary tangles (Pradeepkiran and Reddy, 2019). In our study, a dose of 0.25 ml/l applied continuously most highly inhibited Tau protein hyperphosphorylation, suggesting the strongest neuroprotective effect of this dose and mode of phytobiotic administration. Moreover, the results of Yulug and Cankaya (2019) on the therapeutic efficacy of cinnamon in neurodegenerative diseases showed that *C. cassia* bioactive compounds could inhibit Tau protein hyperphosphorylation. However, only the results of Dhouafli et al. (2018) demonstrated that cinnamon oil could inhibit this process by limiting β-amyloid aggregation itself and inhibiting protein kinases responsible for regulating protein phosphorylation and dephosphorylation in neurons. Reducing excessive phosphorylation of Tau protein prevents destabilization and degradation of this protein. According to George et al. (2013), limiting the formation of phosphorylated Tau protein molecules is possible through a protective binding to the thiol side chain of cysteine present in the Tau protein structure. This reversible type of binding allows Tau protein to retain its biological function, which determines the affinity of Tau protein for microtubules, thereby preventing the formation of neurodegenerative lesions. Importantly, as demonstrated by Valera et al. (2016), cinnamon oil components reduce the formation of hyperphosphorylated Tau protein deposits without compromising the normal cellular Tau function, i.e. the synthesis of tubulin, which forms the cytoskeleton of nerve cells (Valera et al., 2016). Phosphorylated Tau can damage the neuronal cytoskeleton, thereby contributing to the impaired transport of nutrients in these cells, as well as disturbed transmission of electrical signals between synapses. A study by Zhao et al. (2019) showed that components of cinnamon oil also reduced the expression of genes responsible for the formation of phosphorylated Tau protein.

The results of the present study concerning the level of AChE in the brain, blood plasma and liver indicated that the highest dose (0.25 ml/l) of the phytobiotic, both in continuous and periodic application, was the most beneficial for practical use. On the other hand, a lower dose (0.1 ml/l) already had a neuroprotective effect on brain tissue and blood plasma. Many authors have reported that decreased AChE levels are an early symptom of a neurodegenerative disease. Such a condition leads to the accumulation of large amounts of acetylcholine, resulting in pathological lesions caused by the hyperactivation of the cholinergic system (Husain et al., 2017). Underhill and Amara (2021) showed that a significant increase in acetylcholine levels in the blood and nervous tissue stimulated adverse myofibroblast proliferation and collagen gene expression, as well as enhanced protein phosphorylation, including Tau (Underhill and Amara, 2021).

On the other hand, the application of the highest dose of the phytobiotic (0.25 ml/l) most strongly reduced the formation of toxic GAChE complexes, especially in the case of continuous application. According to some researchers, both AChE deficiency and GAChE formation promote mitochondrial dysfunction and the formation of large amounts of reactive oxygen species, which in turn induce apoptotic neuronal death (Underhill and Amara, 2021). The mechanism of GAChE-mediated neuronal apoptosis involves biochemical changes associated with the activation of glutamatergic NMDA (N-Methyl-D-Aspartate Receptor), opening of calcium channels, and activation of calcium-dependent kinase II and calmodulin. This causes the overproduction of free radicals and the release of lactate dehydrogenase, whose presence in the neuronal cytoplasm indicates cell membrane damage. Excessive increase in the cell's calcium ion concentration decreases the rate of oxidative phosphorylation, reduces intracellular ATP, promotes reactive oxygen species formation and mitochondrial mega channel opening; this in turn results in caspase 3 activation and apoptotic cell death (Underhill and Amara, 2021). A study by Yang and Lai (2021) demonstrated that a reduction in Ca^{2+} ion accumulation in the cell can be achieved by the addition of citric acid. This is because citric acid chelates excess extracellular Ca^{2+} (Yang and Lai, 2021), which in our study may have contributed to the neuroprotective effect of the phytobiotic (Martinez et al., 2018).

In our study, beneficial increases in LRP1 levels were obtained in the brain, plasma and liver after all doses of the phytobiotic. Among the doses and application modes used, the strongest neuroprotective

effect was recorded for continuously applied doses of 0.1 and 0.25 ml/l. An increase in LRP1 levels after cinnamon oil application was also observed by Momtaz et al. (2017). The results of Hajinejad et al. (2020) demonstrated that cinnamaldehyde alleviated neuroinflammatory conditions by increasing LRP1 synthesis, which in turn reduced free radical generation, improved mitochondrial function and blocked nuclear factor kappa B.

The results of our study show that the lack of a significant increase in cholesterol levels after the application of cinnamon oil, found in all tissues assessed, should be considered a highly favourable phenomenon. As with other indicators of neurodegenerative changes, the most effective were 0.1 ml/l and 0.25 ml/l doses applied continuously, and the 0.25 ml/l dose applied periodically. Khasnavis and Pahan (2012) argued that it was sodium benzoate and benzoyl-CoA, formed by the oxidation of cinnamic acid contained in cinnamon oil, that inhibited excessive cholesterol synthesis. As reported by Hussain et al. (2019), high blood cholesterol levels facilitated the attachment of amyloid-β to cell membranes and also promoted calcium influx into the cells, which accelerates accelerated nerve cell death. In our study, the application of cinnamon oil did not increase DNA methylation, which should be considered a positive phenomenon. This proved that DNA methylation pathways were not disturbed, and the disorders occurred at the levels of action of individual enzymes responsible for the functioning of the nervous system (Adarsh et al., 2020).

Conclusions

The strongest neuroprotective effect was obtained using a phytobiotic containing cinnamon oil and citric acid at a dose of 0.25 ml/l water for 42 days of rearing broiler chickens. This was manifested by a reduced formation of toxic amyloid-β, phosphorylated Tau protein and GAChE, as well as increased LRP1 and AChE levels.

Conflict of interest

The Authors declare that there is no conflict of interest.

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