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Tolerance Study for Standardized *Macleaya cordata* Extract Added to Chicken Layer Diet

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Abstract

The objective of this study was to assess the effects of Sangrovit®, a standardized preparation of *Macleaya cordata* extract (MCE), on the health status and egg laying parameters of layer chickens. Layer chickens ($N = 360$) were randomly divided into four groups (90 birds/treatment, 10 birds/pen) and fed either a standard basal feed (T1) or a basal diet that was supplemented with 100 mg/kg (T2), 500 mg/kg (T3) or 1000 mg/kg (T4) Sangrovit® (providing 0, 3.7, 18.5, and 37.0 mg MCE/kg feed, respectively) for 56 consecutive days. Live Weight (LW), Average Daily Feed Intake (ADFI) and the Feed Conversion Ratios (FCR) were calculated during the study, and biochemical and hematological endpoints were obtained at the end of the study (Day 56). Eggs were analyzed for the isoquinoline alkaloids sanguinarine and chelerythrine. No statistically significant ($P > 0.05$) differences were found between control and treatment groups for LW, ADFI and FCR. There was a significant increase in the % laying in groups T3 and T4 for the study overall, but no significant differences in egg size during the study. Blood biochemical analyses showed a near-significant trend for decreased bilirubin in the T2 and T4 groups, but this was not dose-dependent and not considered treatment-related. The percent hemoglobin was significantly decreased in the high dose group, but was not considered treatment-related as it was not a dose-dependent effect. No treatment-related changes were found after necropsy of the selected organs. No quantifiable sanguinarine or chelerythrine was found in the eggs after 56 days administration of the MCE preparation to the hens. The results of this study show that consumption of a standardized MCE preparation at up to 1000 mg/kg feed in laying hens had no adverse effect on the hen or eggs, and no residual sanguinarine or chelerythrine was transferred to the eggs.

Keywords

Macleaya cordata, Chicken Layer, Tolerability, Alkaloids, Residues

1. Introduction

Novel ingredients to be added to animal feed or drinking water, even those that are to provide taste, aroma or nutritive value, must undergo a safety assessment following consumption by the target species and an assessment of the safety of consumers of the animal-derived products. A feed-stable version of a standardized *Macleaya cordata* extract preparation (MCEP; trade name Sangrovit®) has previously been fed to chicken broilers as a flavoring agent, with no adverse effects at levels up to 10 times the recommended consumption level [1]. Other studies have evaluated the effects of MCEP inclusion into poultry feed. MCEP fed to male chickens for fattening for five weeks at 15 mg/kg feed decreased β-glucuronidase and β-glucosidase caecal activities, while increasing production of certain caecal short chain fatty acids [2]. Other research found that feeding MCEP to chickens for fattening at 20 mg/kg feed may optimize nutrient absorption by reducing excessive caecal fermentation pathways without increasing the pH of the caecal contents or diminishing glycolytic activity [3]. MCEP fed to chickens for fattening at 30 mg/kg feed resulted in no adverse effects [4]. However, the evaluation of the safety of the standardized MCEP in the study reported here, when provided to layer chickens and the assessment of the potential of *M. cordata* residues in eggs has not previously been available in the published literature. Residual flavor components in eggs could alter the taste and acceptability profile for consumers. The objective of the present study was to evaluate the safety and potential residual levels in eggs of a standardized MCEP when consumed by layer chickens *via* the feed at 100, 500 and 1000 mg MCEP/kg feed for 56 days.

2. Material and Methods

2.1. Test Substance

The test substance was a standardized *Macleaya cordata* extract (MCE) preparation (MCEP; trade name of Sangrovit®), containing the MCE combined with a carrier, standardized to provide at least 1.5% sanguinarine. The test substance was provided by Phytobiotics Futterzusatzstoffe GmbH (Eltville, Germany) and met analytical characteristics previously described [1] [5].

2.2. Animals

Three hundred-sixty female laying hen pullets of the Isa Brown breed that had not been previously laying (Farm: Az. Agr. Loca Dario, Via Zappellazzo 157/1-Carpaneto P.no, Italy) with average weight of 1886 ± 108 g and an age range of 130 - 140 days, were employed for this study. The hens were vaccinated at the breeding farm for: pseudopest-bronchitis-coryza, laryngotracheitis, infectious bronchitis virus, rhinotracheitis, *Salmonella gallinarum*, encefalomielitis, and variola virus. They were reared according to Recommendations 526/2007 CE and Italian D. Lgs (2014) and then placed in pens with 0.22 square meters/animal, staying in the same initial pen and house during the entire study.

The study evaluated a control and three treatment groups (four groups total), with 9 replicates/treatment and 10 animals/pen. The pen was the experimental unit for this study.

2.3. Housing and Diets

The testing site (CERZOO S.r.l., Piacenza, Italy) was equipped with a dynamic ventilation system, with a ventilation rate that varied from 0 m^3/hour to the maximum ventilation rate required (up to 2500 m^3/hour) to maintain optimal conditions, according to the desired temperature and the age of the layers. The temperature and relative humidity were recorded daily at a 30 minute interval for the duration of the trial using a computerized automatic system. The lighting period varied during the study: the pre-lay period (Day-60 to Day 0) provided a 16:8 light:dark ratio with a light increase of 30 minutes once a week until a 18:6 ratio was achieved; the study period (Day 0 - Day 56) maintained an 18:6 ratio of light: dark. The animals were fed a blank basal (control) feed without a zootechnical feed additive as defined in (EC) No 1831/2003 during the pre-layer period (from arrival to the start of laying); the feed was provided in meal form (Table 1). The pre-layer diet and the basal diet of the layer period were prepared by Ferrari Luigi Feed Mill, Piacenza, Italy. No antibiotics, growth promoters, probiotics, organic acids or enzymes, with the exception of the test product, were added to the experimental diets. Feed was analyzed for: moisture, crude ash, starch, crude protein, crude fat and fiber, and sugar. The metabolizable energy was

Table 1. Composition (%) and calculated analysis (% as feed) of the basal diet.

Diet composition (%)		Calculated analysis (% as feed)	
Corn meal	50.00	Dry Matter	90.47
Soybean meal 48%	23.00	Crude Protein	17.41
Wheat meal	13.00	Crude fiber	2.64
Calcium gritted	6.00	Crude fat	4.70
Animal fat	2.00	Ash	11.96
Calcium carbonate	3.00	Starch	42.74
Monocalcium phosphate	2.00	Calcium	3.88
Salt	0.30	Phosphorous	0.74
Sodium bicarbonate	0.15	Methionine + Cystine	0.75
DL methionine	0.15	Lysine	0.89
Vitamins and minerals[1]	0.40	Metabolizable Energy[2] (MJ/kg)	11.41

[1]Content of vitamins and Oligo minerals/kg premix provided by Istituto delle Vitamine (Segrate-MI, Italy): Vit. A: 2,700,000 UI; Vit. D3: 1,000,000 UI; Vit. E: 15,000 mg; Vit. B1: 1000 mg; Vit. B2: 1600 mg; Vit B6: 1400 mg; Vit. B12: 4 mg; Vit K: 1000 mg; niacin: 15,000 mg; folic acid: 600 mg; biotin: 60 mg; D-pantothenic acid: 5000 mg; choline chloride: 120,469 mg; Mn: 24,000 mg; F3 10,000 mg; Cu: 3500 mg; Co: 18 mg; I: 300 mg; Zn: 20,000 mg; Se: 60 mg; anticaking (meerschaum): 225,000 mg; excipient (limestone): 39.98%. [2]Metabolizable Energy=calculated according to the equation proposed by Legislation (G.U. CE n. L54, February 22, 2009).

calculated according to G.U. CE n. L54, February 22, 2009. A sufficient amount for the global study period of experimental diets were produced in the CERZOO feed mill. The basal and experimental diets were produced and stored at room temperature under dry conditions. Control and treatment animals were fed using one feeder per pen, and drinking water (fit for human consumption) was provided *ad libitum*. The water quality was analytically evaluated annually. Feed intake was measured per pen.

2.4. Study Design

The animals were assigned to receive one of the four feed treatments: T1—Control group received basal feed without treatment; T2—Treatment group supplemented with 3.7 mg MCE/kg feed refers to 100 mg/kg Sangrovit® (Highest Intended Dose—HID); T3—Treatment group supplemented with 18.5 mg MCE/kg feed refers to 500 mg/kg Sangrovit® (5X HID); and T4—Treatment group supplemented with 37.0 mg MCE/kg feed refers to 1000 mg/kg Sangrovit® (10× HID). The treatment period was 56 days (between Day 0 and Day 56) with a 60-day pre-experimental period that was initiated to start egg laying and included the adaptation period. All feed added to or removed from each pen during the study was weighed. Control and treatment groups were treated in the same manner throughout the study.

The number of eggs laid/day/pen was obtained from D0 - D56. Mean weight of eggs was obtained every day/pen during the periods D0 - D28, D28 - D56 and D0 - D56, and the percent dirty, cracked, broken, shell-less or otherwise unsalable eggs were recorded per treatment. On D28 and D56, every egg laid in a 24-hour period was collected, individually weighed, then classed per egg size according to commercial egg classifications (Reg CE 557/2007 of 23 May 2007) as small (S; egg weight < 53 g), medium (M; 53 g ≤ egg weight < 63 g), large (L; 63 g ≤ egg weight < 73 g) or extra-large (XL; egg weight ≥ 73 g). Data were expressed as mean egg weight/pen and the mean egg class/pen.

Feed and egg analysis for sanguinarine and chelerythrine levels in the four experimental groups was conducted. The feed was analyzed for each lot of production for each treatment group, while the eggs were analyzed for those laid at D0, D28 and D56, as a pool of not less than 7 eggs/replicate/time of sampling.

The general health status of the chickens and the correct performance of the equipment were evaluated twice a day by the Study Director and stockmen. Individual Live Weights (LW) of the chickens were obtained on D0, D28 and D56. Dead animals were weighed on the date of death and the weight recorded. The feed intake by pen was defined as the difference between the feed offered and the refused and measured back for the growing periods (*i.e.*, D0 - D28, D28 - D56 and D0 - D56 for feed). The Average Daily Feed Intake (ADFI) per pen was determined during the treatment period at D28 and D56 and was used with the egg mass output/pen to calculate the average Feed Conversion Ratio (FCR) during the periods D0 - D28, D28 - D56, and D0 - D56, considering the total feed intake

per pen divided by the sum of egg mass output for each replicate.

2.5. Hematology and Biochemistry Analysis

On Day 56 blood was collected from one randomly selected animal from each pen for a total of 36 chickens, nine per treatment group. The blood samples were collected by wing vein puncture into Vacuette® vacuum tubes (Greiner bio-one; Cassina de Pecchi, Italy). For biochemical parameters, 9 cc. capacity disposable vacuum tubes without anticoagulant but containing Vacuette® Z Serum Sep Clot Activator (Breiner bio-one; Cassina de Pecci, Italy), an inert separator gel that forms a stable barrier between the serum and the blood clot after centrifugation, were used. The samples were centrifuged and frozen in the primary tube for later routine biochemistry analysis of the parameters: glucose, calcium, inorganic phosphorus, cholesterol, triglycerides, phospholipids, uric acid, urea, creatinine, lactate dehydrogenase (LDH), alkaline phosphatase (ALP), aspartate transaminase (AST), alanine transaminase (ALT) and total bilirubin.

For hematological parameters, 6 cc. capacity disposable vacuum tubes with K_3EDTA (Greiner bio-one; Cassina de Pecchi, Italy) as anticoagulant were utilized. The following hematological parameters were analyzed or calculated: hematocrit, hemoglobin concentration, erythrocyte count, total and differential leukocyte count, platelet count, mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), and red cell volume distribution width (RDW). The blood samples were analyzed in an ISO9001-2000 certified laboratory (La Fontana; Piacenza, Italy).

2.6. Gross Pathology Analysis

The animals utilized for blood analysis were necropsied and evaluated by a veterinary surgeon for gross pathology of: external skin, eyes and any injuries, feet, ears, head and tail, mouth and anus, gut (oral cavity, esophagus, stomach, upper, mid and lower small intestine, caecum and colon), pancreas, spleen, liver and gallbladder, kidneys, genitals, abdominal fat, omentum, heart and lungs, skeletal muscle and fat.

2.7. Feed and Egg Analyses for Sanguinarine/Chelerythrine

Sanguinarine/chelerythrine, as markers for the MCEP, were extracted from the eggs and feed (dried and ground through a 1 mm sieve) using acidified methanol (1% HCl) (Carlo Erba, LC-MS grade) and analyzed by high pressure liquid chromatography-triple quadrupole tandem mass spectrometry (HPLC-MS/MS). Standard solutions were prepared utilizing sanguinarine and chelerythrine standards supplied by Extrasynthese (Genay, France) and were injected for calibration (external standard method). The method was validated for specificity, accuracy, precision, detection and quantification limits, linearity and range (data not shown). Method validation yielded a limit of detection (LOD) of 0.03 mg/kg and a limit of quantification (LOQ) of 0.05 mg/kg.

2.8. Statistical Analysis

The raw data of egg deposition were tested for normality with the Shapiro-Wilk test. Data were analyzed as repeated measurements in a completely randomized design using the MIXED procedure of SAS (SAS, 2002-2010, release 9.3; Cary, NC, USA). Measured variables were subjected to two covariance structures: compound symmetry and autoregressive. The Akaike information criterion and the Schwarz Bayesian criterion were used to find out the covariance structure that best fit the model for the considered parameter.

The parameters of LW, ADFI, FCR, egg classification, number of eggs laid, hematological and biochemistry parameters and necropsy data were analyzed by the General Linear Model procedure of SAS (SAS, 2002-2010, release 9.3) using ANOVA as the main statistical test. Student "t" and Tukey tests were used to compare the means of each group. The level of significance stated in the ANOVA model was $P \leq 0.05$ when the difference was statistically significant, while $0.05 < P \leq 0.10$ when the difference was a near-significant trend. The raw data were analyzed for outliers. The SAS program found some outliers, but these data were not excluded from the statistical analysis because the animals were in good health and did not require removal from the study, and because the raw data were not indicated as outliers in two subsequent measurements. The results of the diet chemical analysis, egg laying (%) and daily egg weight are provided as mean values ± the standard error of the mean (SEM). All other data provided as mean values ± standard deviation (SD).

3. Results

All animals were considered healthy during the course of the study and husbandry was generally good. Fecal consistency was normal. Results from the control group were unremarkable. No veterinary drugs were provided to the animals during the study, and no mortality/culling occurred.

3.1. Chemical Analysis of Feed

The chemical analysis of the diets showed that the diets were within expected values for general nutrients and alkaloid (sanguinarine and chelerythrine) levels (Table 2). Parameters including crude protein, crude fiber, sugar and metabolizable energy were consistent between control and treatment groups.

3.2. Live Weight, Egg Output and Egg Characteristics with Feed Intake

The LW and the ADFI were not statistically different between control and treatment groups (Table 3). The total egg mass output from period D0 - D28 trended higher in Groups T3 and T4, compared to the control group (T1 *vs.* T3 at $P = 0.0940$ and T1 *vs.* T4 at $P = 0.0697$). There were no statistical differences between control and treatment groups in the total egg mass output and FCR parameters for the periods D28 - D56 and D0 - D56 (Table 3).

Table 2. Analytical characteristics of the experimental diets (% as feed).

Parameter	Control Group (T1)	MCEP 100 mg/kg feed (T2)	MCEP 500 mg/kg feed (T3)	MCEP 1000 mg/kg feed (T4)
Dry matter (%)	90.28	90.32	90.29	90.31
Crude protein (%)	17.37	17.55	17.56	17.46
Ether extract (%)	4.69	4.62	4.71	4.59
Crude fiber (%)	3.09	3.05	3.12	3.08
Ash (%)	12.35	12.35	12.38	12.36
Starch (%)	41.21	41.01	41.20	41.34
Sugar (%)	3.57	3.62	3.59	3.58
Metabolizable energy (MJ/kg)	11.65	11.62	11.68	11.65
Sanguinarine (mg/kg)#	<0.05	1.81 ± 0.08	9.21 ± 0.22	18.40 ± 0.66
Chelerythrine (mg/kg)#	<0.05	0.59 ± 0.03	3.31 ± 0.41	5.97 ± 0.88

#Mean ± Standard deviation.

Table 3. Live weight, egg mass output and feed intake (mean ± standard deviation).

Experimental period	Control Group (T1)	MCEP 100 mg/kg feed (T2)	MCEP 500 mg/kg feed (T3)	MCEP 1000 mg/kg feed (T4)
Live weight (g)				
D0	1880.44 ± 28.30	1892.22 ± 33.15	1884.11 ± 22.96	1885.56 ± 33.97
D28	2000.67 ± 42.09	2007.89 ± 28.76	1999.22 ± 24.28	2007.00 ± 36.60
D56	2031.89 ± 37.29	2040.11 ± 45.78	2027.00 ± 28.48	2032.78 ± 32.28
Average daily feed intake (g)				
D0 - D28	125.18 ± 5.63	126.27 ± 4.71	127.64 ± 4.67	129.71 ± 5.69
D28 - D56	126.01 ± 6.57	126.51 ± 7.70	126.71 ± 8.75	128.75 ± 3.97
D0 - D56	125.60 ± 5.65	126.39 ± 6.16	127.18 ± 5.58	127.73 ± 2.65
Total egg mass output (g)				
D0 - D28	16180.33 ± 689.06	16441.78 ± 657.02	16945.89 ± 836.16	16990.56 ± 446.64
D28 - D56	15673.00 ± 783.78	15721.89 ± 856.21	15990.33 ± 1036.84	16297.22 ± 350.66
D0 - D56	31853.33 ± 1236.07	32163.67 ± 1403.14	32936.22 ± 1819.44	33287.78 ± 687.29
Feed conversion ratio				
D0 - D28	2.17 ± 0.13	2.15 ± 0.08	2.11 ± 0.16	2.09 ± 0.12
D28 - D56	2.26 ± 0.18	2.25 ± 0.07	2.22 ± 0.19	2.21 ± 0.06
D0 - D56	2.21 ± 0.14	2.20 ± 0.07	2.17 ± 0.17	2.15 ± 0.07

The laying percent was higher in the T3 and T4 groups during the D0 - D28 period when compared to the control group ($P < 0.05$), while the T2 group trended higher ($P = 0.0805$) (Table 4). During D28 - D56, the laying percent trended higher in the T4 group when compared to the control group ($P = 0.0520$). The laying percent was higher in the T3 and T4 groups ($P < 0.05$) when compared to the control group for the complete D0 - D56 study period. The weight of the eggs was not statistically different between groups for the period D0 - D28, but the egg weight from group T2 was lower than the control group ($P < 0.05$), while the egg weights in the T3 and T4 groups were not different from the control group during this period. For the entire study period (D0 - D56), the mean egg weight trended lower in the T2 group when compared to the control group ($P = 0.0564$).

No differences were found between treatment and control groups for the percent small (S) and extra-large (XL) eggs at D0, D28 and D56. At D56 the percentage of large (L) eggs tended to be lower in the T4 *vs.* T1 group ($P = 0.0647$), with no differences at D0 and D28 (Table 5). No statistical differences were found at D28 between control and treatment groups for the percent medium (M) eggs, although they tended to be higher in the T4 group when compared to the control group ($P = 0.0794$).

No statistically significant differences among treatments were found in the percentage of cracked, shell-less or other egg anomalies (Table 6). The percentage of dirty eggs was significantly lower ($P < 0.05$) in all treatment groups, compared to the control group on D0 - D28 and D0 - D56, but no difference in this parameter was seen for the period D28 - D56. The percentage of total faults tended to be lower in the T4 *vs.* T1 group during the period D28 - D56 ($0.05 < P \leq 0.10$), while for the entire study period (D0 - D56) the percentage of total faults was lower in the T4 *vs.* T1 group ($P < 0.05$) and tended to be lower in the T3 and T2 *vs.*T1 group ($0.05 < P \leq 0.10$) (Table 6).

Table 4. Egg laying and egg characteristics.

Experimental period	Control Group (T1)	MCEP 100 mg/kg feed (T2)	MCEP 500 mg/kg feed (T3)	MCEP 1000 mg/kg feed (T4)	SEM
Laying (%)					
D0 - D28	90.56ax	93.61aby	95.71by	95.24by	1.20
D28 - D56	91.74x	93.73xy	94.52xy	95.87y	1.45
D0 - D56	91.15a	93.67ab	95.12b	95.56b	1.22
Daily egg weight (g)					
D0 - D28	61.69	60.77	61.67	61.45	0.40
D28 - D56	63.58b	62.37a	62.80ab	62.99ab	0.37
D0 - D56	62.64y	61.57x	61.98xy	62.22xy	0.38

All data mean values ± SEM=Standard error of the mean. a, b = Different letter in the same row = significant difference ($P \leq 0.05$). x, y = Different letter in the same row = differences near significant trend ($0.05 < P \leq 0.10$).

Table 5. Egg characteristics (mean ± standard deviation).

Experimental period	Control Group (T1)	MCEP 100 mg/kg feed (T2)	MCEP 500 mg/kg feed (T3)	MCEP 1000 mg/kg feed (T4)
Egg size (% laid eggs)				
Small eggs (S < 53 g)				
D0	9.18 ± 11.21	5.58 ± 10.16	3.58 ± 5.38	10.51 ± 8.90
D28	2.50 ± 5.00	2.35 ± 4.66	4.44 ± 7.26	0.00 ± 0.00
D56	0.00 ± 0.00	2.22 ± 4.41	0.00 ± 0.00	0.00 ± 0.00
Medium eggs (M from 53 to 63 g)				
D0	71.28 ± 9.71	78.70 ± 11.70	81.35 ± 9.61	68.90 ± 9.99
D28	49.97 ± 17.12	56.60 ± 10.75	55.67 ± 20.45	62.47 ± 14.85
D56	35.19 ± 13.91	51.88 ± 12.53	44.67 ± 18.29	53.28 ± 20.44
Large eggs (L from 63 to 73 g)				
D0	19.54 ± 11.76	13.50 ± 8.71	10.93 ± 9.09	16.00 ± 6.70
D28	46.30 ± 17.02	38.83 ± 12.95	37.64 ± 19.90	37.53 ± 14.85
D56	59.01 ± 15.81	43.67 ± 12.88	51.99 ± 16.57	39.93 ± 16.85
Extra-large eggs (XL > 73 g)				
D0	0.00 ± 0.00	2.22 ± 4.41	4.14 ± 6.70	4.59 ± 7.80
D28	1.23 ± 3.70	2.22 ± 4.41	2.24 ± 4.48	0.00 ± 0.00
D56	5.80 ± 10.27	2.22 ± 4.41	3.33 ± 5.00	6.79 ± 13.27

Table 6. Egg faults.

Experimental period	Control Group (T1)	MCEP 100 mg/kg feed (T2)	MCEP 500 mg/kg feed (T3)	MCEP 1000 mg/kg feed (T4)	SEM
Dirty eggs (% layed egg)					
D0 - D28	0.18b	0.00a	0.00a	0.00a	0.035
D28 - D56	0.09	0.00	0.00	0.04	0.035
D0 - D56	0.13b	0.00a	0.00a	0.02a	0.019
Cracked eggs (% layed egg)					
D0 - D28	0.08	0.04	0.04	0.04	0.046
D28 - D56	0.21	0.08	0.16	0.17	0.083
D0 - D56	0.15	0.06	0.10	0.10	0.052
Broken eggs (% layed egg)					
D0 - D28	0.22	0.38	0.20	0.12	0.125
D28 - D56	0.31	0.21	0.01	0.00	0.099
D0 - D56	0.26*xy*	0.30*x*	0.12*xy*	0.06*x*	0.066

Continued

Shell-less eggs (% layed egg)					
D0 - D28	0.14	0.08	0.13	0.04	0.089
D28 - D56	0.05	0.13	0.12	0.04	0.062
D0 - D56	0.09	0.11	0.12	0.04	0.055
Other anomalies (% layed egg)					
D0 - D28	0.00	0.13	0.00	0.00	0.045
D28 - D56	0.05	0.04	0.00	0.00	0.031
D0 - D56	0.02	0.08	0.00	0.00	0.025
Total faults (% layed egg)					
D0 - D28	0.62	0.64	0.37	0.21	0.166
D28 - D56	0.70 *y*	0.47*xy*	0.32 *xy*	0.25 *x*	0.127
D0 - D56	0.66a*x*	0.55ab*xy*	0.35ab*yz*	0.23b*z*	0.087

All data mean values ± SEM = Standard error of the mean. a, b = Different letter in the same row = significant difference ($P \leq 0.05$). x, y, z = Different letter in the same row = differences near significant trend ($0.05 < P \leq 0.10$).

3.3. Hematology and Biochemistry Analysis

The results of plasma analysis are summarized in Table 7. There were no statistically significant differences between control and treatment groups for any of the biochemical parameters, other than a near-significant trend ($P = 0.0695$) for a decrease in bilirubin in the T2 and T4 groups, when compared to the T1 (control) group. However, the bilirubin response was not dose-dependent and therefore was not considered treatment-related. A statistically significant decrease in hemoglobin occurred in the T4 group when compared to the control (T1) group ($P < 0.05$), although this response was not dose-dependent and was not considered treatment-related (Table 8). No other hematological parameters were statistically different between the control and treatment groups.

3.4. Sanguinarine and Chelerythrine Residues in Eggs

Analysis of the eggs concentrations of sanguinarine and chelerythrine found that the residue levels in the control group was negligible, as expected with the LOD set to 0.03 mg/kg and the LOQ set at 0.05 mg/kg for both sanguinarine and chelerythrine. The linear dynamic range was satisfactory ($R^2 > 0.99$ in the range of 0.03 - 10 mg/kg) while the accuracy was 92% and 88% for sanguinarine and chelerythrine, respectively. The precision (RSD) was 17% and 19% for sanguinarine and chelerythrine, respectively. No sanguinarine or chelerythrine residues above the LOQ were found in the eggs of the control or treatment groups (Table 9).

4. Discussion

This study is in agreement with work [6] showing that consumption of feed supplemented with 20 mg MCEP/kg feed for five weeks had no significant effect on

Table 7. Blood biochemical parameters (mean ± standard deviation).

Experimental period	Control Group (T1)	MCEP 100 mg/kg feed (T2)	MCEP 500 mg/kg feed (T3)	MCEP 1000 mg/kg feed (T4)
Glucose (mg/dl)	196.67 ± 13.27	189.56 ± 16.19	182.44 ± 14.72	188.00 ± 17.98
Urea (mg/dl)	6.90 ± 1.98	5.44 ± 2.26	6.73 ± 2.21	5.74 ± 2.74
Uric acid (mg/dl)	10.48 ± 1.66	9.62 ± 2.32	9.12 ± 1.82	8.87 ± 1.88
Creatinine (mg/dl)	0.25 ± 0.04	0.30 ± 0.05	0.27 ± 0.04	0.26 ± 0.05
Cholesterol (mg/dl)	146.67 ± 31.35	154.44 ± 32.71	149.56 ± 33.16	139.00 ± 25.08
Triglycerides (mg/dl)	631.22 ± 4.82	628.78 ± 2.59	627.22 ± 5.54	629.22 ± 2.05
Bilirubin (mg/dl)	0.87 ± 0.22y	0.61 ± 0.17x	0.64 ± 0.23xy	0.61 ± 0.23x
Aspartate Transaminase (U/l)	147.33 ± 15.73	144.89 ± 12.21	148.22 ± 12.54	142.33 ± 15.06
Alanine Transaminase (U/l)	40.22 ± 6.24	40.00 ± 8.00	40.56 ± 6.21	37.11 ± 4.48
Alkaline phosphatase (U/l)	811.67 ± 185.62	894.78 ± 323.97	992.33 ± 235.15	912.22 ± 171.48
Lactate dehydrogenase (U/l)	1022.44 ± 63.84	1006.44 ± 50.02	1005.11 ± 85.91	999.67 ± 103.76
Calcium (mg/dl)	18.12 ± 0.41	17.04 ± 1.16	17.14 ± 1.87	18.05 ± 1.42
Inorganic P (mg/dl)	6.78 ± 1.36	6.60 ± 0.94	6.86 ± 1.45	6.58 ± 0.99
Phospholipids (mg/dl)	85.63 ± 4.04	88.04 ± 4.58	86.71 ± 6.97	86.30 ± 6.73

x, y: different letter on the same row indicates near significant trend ($0.05 < P \leq 0.10$).

Table 8. Blood hematological parameters (mean ± standard deviation).

Experimental period		Control Group (T1)	MCEP 100 mg/kg feed (T2)	MCEP 500 mg/kg feed (T3)	MCEP 1000 mg/kg feed (T4)
WBC	1000/ml	21.90 ± 3.09	21.67 ± 2.29	21.03 ± 2.10	20.40 ± 1.90
RBC	1,000,000/ml	2.54 ± 0.26	2.49 ± 0.20	2.53 ± 0.21	2.40 ± 0.11
Hemoglobin	%	13.07 ± 0.52b	12.66 ± 0.51ab	13.06 ± 0.44b	12.42 ± 0.32a
Hematocrit	%	30.58 ± 2.79	29.99 ± 2.70	29.97 ± 2.29	28.59 ± 1.61
MCV	Fl	120.67 ± 4.53	120.44 ± 2.79	118.56 ± 2.46	107.13 ± 35.67
MCH	Pg	51.77 ± 4.52	50.88 ± 2.41	51.07 ± 4.18	51.91 ± 2.38
MCHC	%	42.91 ± 2.30	42.36 ± 2.55	43.68 ± 2.54	43.51 ± 2.36
Neutrophils	%	29.00 ± 3.66	29.02 ± 4.31	27.72 ± 3.53	30.44 ± 4.87
Lymphocytes	%	65.48 ± 3.85	65.83 ± 4.27	67.07 ± 2.95	64.56 ± 5.29
Monocytes	%	2.53 ± 0.35	2.11 ± 0.56	2.13 ± 0.48	2.03 ± 0.51
Eosinophils	%	1.32 ± 0.54	1.37 ± 0.49	1.23 ± 0.35	1.09 ± 0.50
Basophils	%	1.67 ± 0.52	1.67 ± 0.43	1.84 ± 0.36	1.88 ± 0.45
Platelets	1000/ml	59.70 ± 2.34	57.68 ± 5.50	61.98 ± 2.48	61.57 ± 4.45

WBC = white blood cells; RBC = red blood cells; MCV = mean corpuscular volume; MCH = mean corpuscular hemoglobin; MCHC = mean corpuscular hemoglobin concentration; RDW = Red Cell Volume Distribution Width. a, b: different letter on the same row indicates significant difference ($P < 0.05$).

Table 9. Sanguinarine and chelerythrine residue in eggs (mg/kg).

Experimental period	Control Group (T1)	MCEP 100 mg/kg feed (T2)	MCEP 500 mg/kg feed (T3)	MCEP 1000 mg/kg feed (T4)
Sanguinarine (mg/kg)				
D0	<0.05	<0.05	<0.05	<0.05
D28	<0.05	<0.05	<0.05	<0.05
D56	<0.05	<0.05	<0.05	<0.05
Chelerythrine (mg/kg)				
D0	<0.05	<0.05	<0.05	<0.05
D28	<0.05	<0.05	<0.05	<0.05
D56	<0.05	<0.05	<0.05	<0.05

ADFI, live weight gain or FCR of fattening chickens. Ross 308 broilers fed MCEP at 500 and 1000 mg/kg feed (0.05% and 0.1% of the diet, respectively) also did not significantly increase feed intake, FCR or small intestinal morphology during the entire 42 day study period [7]. However, transient increases in LW and cumulative MCEP effects were found [8] when MCEP was consumed at 50 mg/kg feed from D1 - D21 and MCEP at 25 mg/kg feed from D22 - D42 by male Cobb broiler chicks, corresponding to increased feed intake. Sangrovit® added to the diet at 20 or 50 mg/kg feed [9] was also found to significantly ($P < 0.05$) increase Ross broiler final LW, daily live weight gain and FCR, when compared to the control group (feed intake was also significantly improved at Days 22 - 35 of the study). When evaluated for effects on the sensory attributes of eggs when fresh or stored for 28 days, the addition of Sangrovit® to layer feed did not alter albumen or yolk taste, odor or texture [10].

The lack of sanguinarine or chelerythrine residues in the eggs from consumption of Sangrovit® is in agreement with the previous research on Sangrovit® consumed by chickens for fattening when added to feed [1], who did not find sanguinarine or chelerythrine residues in muscle tissues when Sangrovit® was added to the feed at up to 1000 mg/kg feed, although sanguinarine (but not chelerythrine) was found in the fat + skin samples of birds consuming 500 or 1000 mg Sangrovit®/kg feed. The current work found that Sangrovit® or its main components do not concentrate in the eggs of laying hens. The absence of sanguinarine or chelerythrine in the eggs is consistent with studies finding that the majority of sanguinarine and chelerythrine administered to rats (98%) is directly excreted in the feces and only approximately 2% is absorbed through the gastrointestinal tract [11].

Recent residue studies demonstrated that neither sanguinarine nor chelerythrine could be found in the tissues or organs of swine fed a MCE preparation (*i.e.*, Sangrovit®) at 100 mg/kg feed for 28 days [12], consistent with the current study. Swine fed a MCE at 2 and 100 mg/kg feed for 90 days did not result in sanguinarine or chelerythrine in muscle tissue, but sanguinarine was found in

the plasma (ng/ml), liver, gingiva, tongue, stomach and intestine (4 - 79 ng/g range) when MCE was consumed at the 2 mg/kg feed level, which is greater than MCEP consumption levels in the current study [13]. The study utilized a MCE that contained approximately 64% sanguinarine, which provided a higher concentration of sanguinarine in the feed [13], compared to the concentration of sanguinarine in the drinking water in the current study.

The results of the current study are in agreement with a previous poultry study that found that the consumption of Sangrovit® by broilers at up to 1000 mg/kg in the feed had no adverse effect on blood plasma or feed intake parameters [1], as also seen in the current study.

Statistical evaluation of the necropsy results showed no statistically significant differences between feeding treatments for all parameters (data not shown). No dose-dependent differences were noted for any of the parameters analyzed and no statistically significant effects occurred. Therefore, it was concluded that there were no test-article related effects on the tissues. The veterinary surgeon who conducted the necropsy stated that all the carcasses were fit for human consumption.

5. Conclusion

In conclusion, the results of the study showed no adverse effects of consumption of the standardized MCEP provided to layer chickens when administered in the feed at 100, 500 and 1000 mg/kg feed for 56 days, as compared with control birds. No residual levels of sanguinarine or chelerythrine were found in the eggs. Previous work evaluated the safety of the standardized MCEP when added to feed for broilers [1] and for evaluating the health of swine consuming MCEP when added to feed [13], but this is the first published tolerance study of the evaluation of the MCE preparation when administered to layer chickens. The current work confirms that consumption of this MCE preparation when added at up to 1000 mg/kg feed for 56 days is well tolerated by laying chickens and that neither sanguinarine nor chelerythrine are transferred to the eggs.

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