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Influence of *Artmisia absinthium* essential oil on broilers experimentally infected with *Eimeria* oocysts

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ABSTRACT

The aim of this study was to investigate the effects of *Artemisia absinthium* essential oil (AAEO) on enzymatic activity of superoxide–dismutase (SOD), glutathione–peroxidase (GSHPx), glutathione–reductase (GR), peroxidase (POD) and xanthine–oxidase (XOD) and content of lipid peroxides (LPx) and glutathione (GSH) in broilers infected with oocysts mixture of *Eimeria tenella*, *Eimeria mitis* and *Eimeria necatrix,* compared to coccidicide salinomycine. Investigation was carried out on 240 Arbour acres broilers of both sex. Broilers were completely random distributed into four treatments: Treatment A was uninfected and untreated; treatment B was infected and was kept untreated; Treatment C preventively received coccidicide salinomycine in quantity of 60 mg/kg of feed and inoculated with oocysts mixture at 21st day-of-age; Treatment D in feed received AAEO in quantity of 3 g/kg and infected with oocysts mixture at 21st day of age. During the study, the bloody diarrhoea was observed from 3th to 9th day after the challenge. After six days of infection, the most intensive bloody diarrhoea was noticed in unmedicated treatment. In order to evaluate the effects of essential oil on poultry coccidiosis induced by *Eimeria* spp. oocysts per gram of faeces (OPG) was also investigated in all treatments. During the experiment, the oocysts output and mortality rate were significantly lower ($P<0.05$) in AAEO treatment (D_2) in comparison to positive control (B), while significant excretion of oocysts were noticed in faeces of non-treated broilers infected with *Eimeria* spp. The broilers treated with salinomycin (C_2) showed complete reduction of oocysts in faeces at 30 days of age. The results obtained in this study indicate changes in the content and the activity of the non-enzymatic and enzymatic antioxidative protective systems in blood hemolysated of infected chickens. Positive preventive effects of applied AAEO in concentration of 3g/kg of feed were high on the antioxidative system of erythrocytes.

Based on the obtained results, it was concluded that AAEO was effective in lowering the bloody diarrhoea intensity as well in reducing the oocyst output of the preventive treated and infected broilers; hence it can be used as prophylactic feed additive. Moreover, AAEO showed important role in activation of antioxidative protection systems in infected broilers, which is of great interest since free radicals and lipid peroxides, formed as a result of smaller food intake and exhaustion of the organism induced by diarrhoea, could cause cellular membrane damages.

Key words: *Artemisia absinthium*, coccidiosis, prophylactic feed additive, antioxidative system, salinomycin

Introduction

Coccidiosis is acute invasion and destruction of intestinal mucosa by protozoa of the genus *Eimeria,* with the oocysts often present in the environment wherever poultry are raised (CHAPMAN et al., 2010). Coccidiosis is one of the most economically damaging disease of the poultry industry, resulting in major economic losses by reducing poultry performance and lowering productivity (CHAPMAN et al., 2010; McDONALD and SHIRLEY, 2009; PEEK and LANDMAN, 2011). Chickens are hosts to seven species of *Eimeria* that develop at specific sites along the digestive tract (McDONALD and SHIRLEY, 2009). These pathogens may cause damage of the intestinal tissue, decrease feed intake and absorption of nutrients, and also increases the susceptibility to secondary bacterial infections (MORRIS et al., 2007; COOPER and SONGER, 2009; KOSTADINOVIĆ et al., 2015a).

Coccidiosis is mainly controlled using prophylactic coccidicides administered in the feed (CONSTANTINOIU et al., 2008; SHIRLEY et al., 2005). These coccidicides are now in widespread use on chicken farms, bringing high levels of development and prosperity to the poultry industry. The prevention/treatment of chicken coccidiosis relies on the availability and

effective use of coccidicides. Therefore, coccidicides plays an important role in coccidiosis prevention in the commercial broiler industry. However, the extensive use of these compounds over the past 50 years has resulted in the development of drug resistance by *Eimeria* spp. (BEREZIN et al., 2008; MOLAN et al., 2009; WILLIAMS, 2006; YADAV and GUPTA, 2001). Cross-resistance and multi-drug resistance have reduced the effectiveness of the coccidicides.

Subsequently, with increasing demands for high-protein meat and increased consumer concerns over the side effects of conventional anticoccidial drugs on poultry, toxicity of some of these drugs on other animal species, and public health concerns about tissue residues of anticoccidial drugs, have intensified the search for alternative strategies against coccidiosis. One of the potential candidates is the use of medicinal plants such as *Artemisia* species, or their extracts (KOSTADINOVIĆ et al., 2015a; KOSTADINOVIĆ et al., 2015b)**.** The genus *Artemisia* belongs to the family *Compositae* (*Asteraceae)* with over 300 species spread worldwide. The essential oil obtained from wild plant *Artemisia absinthium* shows antibacterial (JUTEAU et al., 2003; LOPES-LUTZ et al., 2008; SENGUL et al., 2011), antifeedant (as naturally occurring substance in certain plants that adversely affects insects or other animals that eat them), antipyretic, fertility increasing, cytostatic and antimalarial activities (KHATTAK et al., 1985).

Considering the aforementioned positive aspects of *Artemisia absinthium* essential oil, the aim of this study was to compare prophylactic efficacy of the conventional coccidicide (salinomycin) and *Artemisia absinthium* essential oil in artificially infected broilers with coccidiosis. The comparative assessment was based on the clinical symptoms and changes in catalytic activity of the important oxidative protection enzymes in blood hemolysates of healthy and artificially infected broilers.

Material and methods

Chickens and housing. The experimental protocol was approved by the Ethics Committee of University of Novi Sad, Faculty of Medicine (EC/15/05/432-6) and the principles of animal protection and welfare were strictly followed. Experiments under *in vivo* conditions were performed on 240 broilers of both sexes of the heavy Arbour acres strain. One day old chicks were raised in a clean and disinfected room under standard conditions. Broilers were fed standard basal diet with the access to water and food *ad libitum.* Faecal samples were taken daily in order to monitor the possibility of infection. Temperature and lighting regimen were in accordance with the recommendation of the breeder. The initial room temperature (32-33 °C) was reduced weekly 1 °C to a final temperature of 28 °C.

The broilers were randomly divided into non-infected and infected treatments. The broilers in infected treatments were exposed to mixture of sporulated oocysts of *E. tenella*, *E. mitis* and *E. necatrix* genus, collected from infected chicken farms. Coccidial oocysts of *E. tenella, E.mitis* and *E. necatrix* were obtained from the guts of infected chickens and they were preserved in 2.5 % potassium dichromate solution to induce sporulation and kept in a refrigerator at 2-5 °C until use. Oocyst mixture consisted of 20000 oocysts per ml (5000 *E. tenella* oocysts per ml; 5000 *E. mitis* oocysts per ml and 10000 *E. necatrix* oocysts per ml). The challenge infection of 21–day–old chickens was performed by oral administration of 1 ml oocyst suspension.

Artemisia absinthium essential oil was obtained from the Institute for Medicinal Plant Research „Dr Josif Pancic“, Belgrade, Serbia.

Experimental protocol. One-day-old broilers, randomly selected, were divided into four treatments (Table 1), each containing 60 individuals, further divided in three replicates each, respectively:

Treatment A. uninfected and unmedicated broilers – negative control treatment. Blood sampling and decapitation of 10 broilers was carried out at 30th day-of-age.

Treatment B. infected and unmedicated broilers – positive control treatment. Inoculation of 21-day-old broilers was performed by p.o. application of 1 ml of oocysts mixture. Nine days later (30th day-of-age), when first clinical signs of disease appeared (broilers were bristling, showed decreased food conversion, white mucous, later bloody diarrhoea appeared, appetite decreased etc.), blood sampling and decapitation of 10 broilers were carried out.

Treatment C. broilers which received preventively coccidicide salinomycine in quantity of 60 mg/kg of feed (Group C_1) and the remaining broilers inoculated with laboratory derived coccidian species at 21st day-of-age. Blood sampling and decapitation of 10 broilers were carried out at 30th day-of-age (Group C_2).

Treatment D. broilers which received AAEO in quantity of 3 g/kg (Group D_1) and the remaining broilers infected with *Eimeria* oocysts mixture at 21st day-of-age. Blood was collected at 30th day-of-age (Group D_2). The essential oil was given to the broilers three times a day.

During the experiment broilers were regularly controlled, autopsies were performed and all findings were carefully recorded. The oocyst output, after the infection, was measured every third day during the period from 21st to 30th day of age in each group.

The means of oocysts per gram of faeces (OPG) in treated treatments were compared with OPG values for non-treated control treatments in order to evaluate the effects of the plant essential oil on avian coccidiosis induced by *Eimeria* spp.

Bloody diarrhoea was investigated from 3th to 9th day after the challenge. Bloody diarrheal score was described using numerical values from 0 to 3. Zero corresponded to normal status, whereas 1, 2 and 3 corresponded to 33; 33-66; 66-99 % of blood in total faeces, respectively.

Commercial test (”Dialab“, Vienna, Austria) was used for determination of haemoglobin level which is important indicator of enzymes activity in haemolysed blood. This method was performed on spectrophotometer (Multiscan MCC 340, Finland). Protein content was determined by the method of PRAKASH et al. (2010).

Preparation of blood haemolysate. Blood was collected by heart puncture of broilers into heparinized test tubes. After centrifugation (10 min at 3500 rpm and 4 °C) and plasma removal, the erythrocytes were rinsed 3 times in saline. The resulting erythrocyte pellet was suspended in an equal volume of double distilled water and vortexed. After incubation for 1 hour at room temperature, the haemolysate was centrifuged for 15 min at 3500 rpm and supernatant was collected for further analysis (KOSTADINOVIĆ, 1998).

Sample preparation for glutathione (GSH) determination. Proteins from freshly prepared haemolysates were separated by adding half the volume of 10% sulphosalicylic acid and centrifuged at 5000 rpm, for 5 min, at 4 °C. The supernatant was stored at 4 °C, without freezing, and GSH determined within 24 hours. The GSH content in the blood haemolysate was determined from the amount of sulfhydryl residues by means of Ellmann’s reagent (KAPETANOVIC and MIEYAL, 1979).

Determination of enzymatic activity. Superoxide–dismutase (SOD) (EC 1.15.1.1) activity was determined by the spectrophotometric method based on the inhibition of adrenaline reduction to adrenochrome at pH 10.2 (KOSTADINOVIĆ et al., 2001). The GSHPx (EC 1.11.1.9) activity was determined by spectrophotometric measurement of absorbance at 412 nm with cumenhydroperoxide as the substrate (CHIU et al., 1976).

Activity of glutathione–reductase (GR) (EC 1.6.4.2.) was determined from the rate of NADPH oxidation and it was monitored by measuring the absorbance at 340 nm (LUKASZEWICZ-HUSSAIN and MONIUSZKO-JAKONIUK, 2004).

Content of lipid peroxides (LPx) was determined by thiobarbituric acid (TBA) test. The oxidation of cellular membrane lipids was measured via reaction of lipid peroxides with thiobarbituric acid (PIRONI et al., 2003).

The determination of peroxidase (POD) (EC 1.11.1.7) activity was based on the catalytic oxidation of guayacole by hydrogen peroxide as an electron acceptor (KOSTADINOVIĆ et al., 2011). The reaction of xanthine oxidation of uric acid was used for determination of xanthine–oxidase (XOD) (EC 1.17.3.2) activity. Spectrophotometric measurement was performed in 0.1 mmol/dm^3 phosphate buffer, pH 7.5, at 295 nm (KOSTADINOVIĆ et al., 2011).

Data analysis. The results given in tables are reported as the mean ± standard deviations (SD) of a number (n) of independent determinations. The one way ANOVA analysis and Tukey post hock test were performed to assess data differences between various groups using Statistica software version 12 (STAT SOFT inc. 2013; USA). All the analyses were carried out in triplicate for each experimental treatment. The data means were considered different at $P<0.05$.

Results

Bloody diarrhoea was observed from the third to the ninth day after the infection with *Eimeria* spp in all experimental groups, except the uninfected experimental treatments.

It was observed that the bloody diarrhoea was of the same intensity in all infected treatments, except in negative control treatment, third day of infection (Table 2). Six days after the infection the most intensive bloody diarrhoea was noticed in the unmedicated treatment (B). The intensity of bloody diarrhoea was lower in the treatment treated with salinomycine (C_2) compared to other treatments at the 27 day of age.

(Position of TABLE 2)

188 During the experiment, the non-treated broilers infected with *Eimeria* spp. showed
189 significant excretion of oocysts in faeces, which is showed on Table 3. The broilers treated
190 with salinomycin (C_2) showed complete reduction of oocyst in faeces at 30th day. In AAEO
191 treatment (D_2) the oocysts output and mortality rate were significantly lower ($P<0.05$) in
192 comparison to positive control treatment (B). Hence, it can be concluded that AAEO was
193 effective in reducing the oocyst output of the preventive treated and infected broilers.

194 **(Position of TABLE 3)**

195 *Enzymatic activity in blood haemolysates.* The GSH and LPx levels and enzymatic
196 activity of blood haemolysates from the control treatment (A and B) and the experimental
197 treatments (C_1, C_2, D_1, D_2) are shown in Table 4.

198 **(Position of TABLE 4)**

199 The obtained results indicate a significant ($P<0.05$) increase of GSH content and higher
200 catalytic activity of GR in blood haemolysates of infected broilers. Moreover, the increase in
201 the GSHPx and POD activity was also significant ($P<0.05$) in group C_1 compared to group
202 C_2. The only exception was the catalytic activity of XOD and SOD which showed a
203 statistically very significant reduction in positive control treatment compared to the negative
204 control treatment.

205 The preventive doses of coccidicide salinomycin indicated a statistically significant
206 ($P<0.05$) decrease of GSH content, statistically significant ($P<0.05$) increase of activity of
207 GSHPx and statistically significant ($P<0.05$) reduction of catalase-activity of SOD and POD
208 compared to treatment A. Increase of LPx content and activity of GR were not statistically
209 significant ($P>0.05$) in treatment C_1 compared to treatment A.

210 Infection in treatment of broilers C_2, nine days later (30th day–of–age) resulted in
211 statistically very significant ($P<0.05$) increase of GSH content and higher catalase-activity of
212 XOD compared to the treatment B. Decrease of LPx content were also statistically significant

213 ($P<0.05$) and amounted 0.4 and 0.2 in treatments C_1 and C_2, respectively. The activity of
214 other investigated enzymes (GSHPx, POD, SOD) were statistically very significant ($P<0.05$)
215 in treatment C_1 compared to treatment C_2. Induction and inhibition of the catalytic activity of
216 antioxidant defence in blood haemolysates of treatment C_2 were carried out to achieve a basic
217 level of activity characteristic in broilers of a control treatment.

218 The content of erythrocyte GSH and activity of GSHPx and GR in blood haemolysates
219 of broilers fed diet supplemented with AAEO in quantity of 3g/kg (Group D_1) were
220 significantly higher compared to the treatments A and C_1. Addition of AAEO did not affect
221 the LPx content and activity of POD and XOD in haemolysates of broilers. Broilers of AAEO
222 treatment had greater ($P<0.05$) activity of SOD than broilers in control and salinomycine
223 treatment. Comparing the results of the effects of preventive doses of salinomycin or AAEO
224 on the activity of antioxidative enzymes in blood haemolysate, it is concluded that a good
225 agreement was achieved.

226

227 Discussion

228 Some herbal extracts used as a feed additives have been applied to control of coccidiosis
229 on some chicken farms, obtaining a satisfying results (DU and HU, 2004). Medicinal herbs
230 and their extracts are of interest for coccidiosis since several studies have shown substantial
231 antimicrobial and antioxidative activity (ALIYU et al., 2012). The biological activity of this
232 extracts have been mainly attributed to phenolic components. *In vivo* and *in vitro* tests have
233 shown (WILLIAMS and LOSA, 2001) that phenols can be specifically used as oocysticides
234 against *Eimeria* spp. It is known that phenols interact with the cytoplasmic membrane by
235 changing its permeability for captions, like H^+ and K^+. The dissipation of ion gradients leads
236 to the impairment of essential processes in the cell, allows leakage of cellular constituents,

resulting in water unbalance, collapse of the membrane potential and inhibition of ATP synthesis, and finally cell death (ULTEE et al., 1999).

The most likely explanation for the observed phenomena presented in Table 4, is that the pathological alterations intensify free radical processes by stimulating catalytic activities of enzymes involved in the antioxidative protection, POD, GSHPx and GR. However during the disease period lipolysis from the lipid depots is increased due to smaller food intake and exhaustion of the organism by diarrhoea which leads to intensification of free radical processes and formation of larger quantities of lipid peroxides in blood. Newly formed lipid peroxides and their degradation products are transported by blood stream to inactive organs and tissues having toxic effect on them and generating cellular membrane damages. In order to protect itself the organism activates its antioxidative protection system. Reduction of catalytic activity of SOD is expected and in agreement with literature data (SHANKER et al., 2011). Concomitantly with the increased risk of lipid peroxidation in blood, there is an increase in the enzymatic activity of GSHPx. GSH plays an important role in reduction the acute toxicity of xenobiotic and products of lipid peroxidation as a substrate for GSHPx. Addition of salinomycin in feed increase of GSHPx activity and reduces the need for high levels of GSH content, which took part in the detoxification of harmful compounds in the body. A statistically significant decrease of POD activity compared to the corresponding control group was expected, since POD catalyses the oxidation of various proton donors with hydrogen peroxide. Salinomycine is ionophore coccidicide and does not act as a proton donor.

Conclusions

Based on the obtained results, it can be concluded with certainty that the addition of *Artemisia absinthium* essential oil in broilers nutrition has positive effect on lowering the bloody diarrhoea intensity. Also, it can be concluded that significant reduction of the oocyst

number by these medical herb supplementation in broiler diet indicate that *Artemisia absinthium* essential oil could be used as prophylactic feed additive. Moreover, *Artemisia absinthium* essential oil showed important role in antioxidative protection of broilers infected with coccidiosis, which is also of great importance in terms of treating coccidiosis.

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361 Table 1. Experimental design with broilers

Experimental treatment	**Components received by broilers**		
	Coccidiostatic salynomicine (60 mg/kg)	*Artemisia absinthium* essential oil (3 g/kg)	Eimeria oocysts* (1 ml oocyst suspension)
A - Negative control treatment	-	-	-
B - Positive control treatment	-	-	+
C_1 -Preventively coccidicide salinomycine	+	-	-
C_2 - Broilers inoculated with laboratory derived coccidia species	+	-	+
D_1 -Preventively *Artemisia absinthium* essential oil	-	+	-
D_2 - Broilers infected with *Eimeria* oocysts	-	+	+

362 *Broilers were infected with *Eimeria* oocysts at 21st day-of-age

363

364 Table 2. Intensity of bloody diarrhoea of chickens challenged with *Eimeria* spp. mixture and
365 treated with prophylactic dose of salinomycine and AAEO

Treatment	Day of infection	After infection		
	21	24	27	30
A	-	-	-	-
B	-	1	3	1
C_1	-	-	-	-
C_2	-	1	+	-
D_1	-	-	-	-
D_2	-	1	2	-

366 (0) - normal status; (1) - 33%, (2) - 33-66%, (3) - 66 -99% blood in total faeces;
367 AAEO - *Artemisia absinthium* essential oil

368 Table 3. Effectiveness of salinomycine and AAEO on faecal oocyst counts (means±SE) and
369 mortality rate in different treatment group of broilers

Treatment	Average oocyst count (per g)				Mortality rate (%)
	Day of infection	After infection			
	21 day	24 day	27 day	30 day	
A	0	0	0	0	3
B	21025.4±838^{b}	34536.1±177^{c}	37747.0±420^{c}	39485.0±364^{b}	12
C_2	10538.0±1220^{a}	1019.2±23.8^{a}	106.1±18.3^{a}	0	5
D_2	17031.0±1050^{b}	11200.0±156^{b}	4200.8±140^{b}	106.8±12^{a}	7

370 Results are given as means ± standard deviation (n = 3);
371 $^{a-c}$ Means within a column with no common superscript differ significantly at $P < 0.05$;
372 AAEO - *Artemisia absinthium* essential oil; A - negative control; B-positive control; C_2 - salinomycin 60 mg/kg
373 of feed and infected; D_2 – AAEO 3g/kg of feed and infected

374

375 Table 4. GSH and LPx content and the activity of GSHPx, POD, SOD, GR and XOD in blood
376 haemolysates

Treatment	GSH (µmol/g Hb)	LPx (µmol/g Hb)	GSHPx (µmol/g Hb min)	POD (µmol/g Hb min)	SOD (µmol/g Hb min)	GR (µmol/g Hb min)	XOD (µmol/g Hb min)
A	5.3 ± 1.2^{c}	0.4 ± 0.1^{a}	8.2 ± 2.4^{a}	64.8 ± 3.9^{b}	81.4 ± 7.3^{d}	13.0 ± 6.1^{a}	27.1 ± 2.9^{c}
B	2.4 ± 0.2^{a}	6.4 ± 0.2^{c}	13.8 ± 6.8^{e}	98.3 ± 5.8^{d}	55.4 ± 7.0^{c}	19.4 ± 3.9^{c}	11.0 ± 6.6^{a}
C_1	4.1 ± 0.8^{b}	0.4 ± 0.1^{a}	10.7 ± 4.3^{c}	56.7 ± 3.0^{a}	57.1 ± 2.0^{c}	13.8 ± 1.5^{a}	25.1 ± 7.5^{b}
C_2	5.9 ± 0.2^{c}	0.2 ± 0.1^{b}	9.2 ± 1.3^{b}	58.1 ± 9.6^{a}	21.2 ± 3.9^{a}	20.5 ± 7.5^{c}	25.4 ± 8.7^{b}
D_1	6.1 ± 1.1^{d}	0.4 ± 0.03^{a}	11.7 ± 0.6^{d}	59.8 ± 2.5^{a}	35.8 ± 9.5^{b}	17.0 ± 9.1^{b}	27.0 ± 3.2^{c}
D_2	7.9 ± 1.3^{e}	0.3 ± 0.1^{b}	11.9 ± 4.2^{d}	78.3 ± 2.8^{c}	22.0 ± 7.5^{a}	23.6 ± 5.9^{d}	28.6 ± 7.4^{c}

377 Results are given as means ± standard deviation (n = 3);
378 $^{a-d}$ Means within a column with no common superscript differ significantly at $P < 0.05$;
379 GSH- glutathione; LPx - lipid peroxides; GSHPx - glutathione–peroxidase; POD – peroxidase;
380 SOD -superoxide–dismutase; GR – glutathione-reductase; XOD – xanthine-oxidase
381