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Research Article

Anticoccidal Efficacy of Usnic Acid in Broilers [1]

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Abstract

This experiment was conducted to investigate if usnic acid, a lichen metabolite, exerts therapeutic action against coccidiosis. A total of 160 one-day-old male Ross 308 broiler chicks were divided into 5 experimental groups (A-E), each replicated in 4 pens of 8 chicks. At 16 days of age Groups B-E were infected orally with a mixture of purified oocysts including 30 x 10^4 sporulated oocysts from field isolates of *E. acervulina, E. brunetti, E. maxima, E. mitis, E. praecox* and *E. tenella*, whereas Group A was remained uninfected. Seven days after the coccidiosis induction, infected birds were divided into 4 groups to receive orally with 20 mg usnic acid (Group C), 100 mg usnic acid (Group D) and 7 mg toltrazuril (Group E) per kg body weight. The birds in Group B were untreated and served as infected control. The experiment was terminated at 29 days of age. As a result of the study it was evaluated that infected birds had lower feed intake and body weight and worse feed conversion, higher intestinal lesion score, longer small intestine and cecum, and higher fecal oocyst count than healthy birds. The anticoccidial effect of usnic acid at 100 mg/kg application dose was comparable to toltrazuril as reflected by alleviations in performance and pathology findings. In conclusion, it is demonstrated that usnic acid possesses some anticoccidial effects, but not nearly as good as toltrazuril.

Keywords: *Lichen, Usnic acid, Toltrazuril, Broiler, Coccidiosis*

Usnik Asitin Broylerlerdeki Anticoccidial Etkinliği

Özet

Bu çalışmada, bir liken metaboliti olan usnik asitin coccidiosise karşı terapötik etkinliğinin araştırılması amaçlanmıştır. Toplam 160 adet, 1 günlük yaştaki erkek Ross 308 civciv, her bir grup 4 tekerrürlü olacak şekilde 5 deneysel gruba (A-E) ayrılmıştır. Hayvanlar 16 günlük yaşa geldiğinde Grup B-E'dekiler *E. acervulina, E. brunetti, E. maxima, E. mitis, E. praecox* ve *E. tenella*'nın saha izolatlarından elde edilmiş inokulumdan 30 x 10^4 sporlanmış oocyst ile enfekte edilmiştir. Grup A'da yer alanlar civcivler ise enfekte edilmeden kontrol olarak bırakılmıştır. Hastalık oluşturulduktan 7 gün sonra, enfekte hayvanlara ağız yolu ile 20 mg/kg usnik asit (Grup C), 100 mg/kg usnik asit (Grup D) ve 7 mg/kg toltrazuril (Grup E) uygulanmış; B grubundaki enfekte hayvanlara tedavi uygulanmadan enfekte kontrol olarak bırakılmıştır. Broylerler 29 günlük olduğunda çalışma sona erdirilmiştir. Enfekte hayvanların sağlıklı hayvanlara kıyasla daha düşük yem tüketimi, vücut ağırlığı ve yemden yararlanma oranına; daha yüksek lezyon skoruna, daha uzun bağırsak uzunluğuna ve daha yüksek oocyst sayısına sahip olduğu tespit edilmiştir. Performans ve patolojik bulgulardaki değişimler göz önünde bulundurulduğunda 100 mg/kg dozda uygulanan usnik asitin toltrazuril ile karşılaştırılabilecek derecede anticoccidal etkinliğe sahip olduğu belirlenmiştir. Sonuç olarak; usnik asitin bazı anticoccidial etkinliklere sahip olduğu ancak bu etkilerin toltrazuril kadar güçlü olmadığı kanısına varılmıştır.

Anahtar sözcükler: *Liken, Usnik asit, Toltrazuril, Broyler, Coccidiosis*

INTRODUCTION

Coccidiosis is a widespread poultry disease caused by *Eimeria* parasites. Primarily, seven species of *Eimeria (E. acervulina*, *E. brunetti*, *E. maxima*, *E. mitis*, *E. necatrix*, *E. praecox* and *E. tenella)* cause coccidiosis in chickens [1]. The disease is associated with reduced growth rate, impaired feed conversion leading to poor performance, increased susceptibility to other diseases and increased mortality [2].

Traditionally, coccidiosis control is largely dependent on anticoccidial drug usage and on live vaccines on a limited scale at intensive poultry production systems. However, problems with drug resistance in *Eimeria* strains

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in the field, withdrawal period for drugs prior to slaughter, consumer pressure for poultry products free of drug residues, restriction of using antibiotics as growth promoters by European Union and the cost of expensive vaccines urged researchers to explore cheap and safe alternative agents [3,4]. There are a number of research articles proving anticoccidial activity of natural products including plants [5], pre- and probiotics [4,5] and fungi [6,7]. Natural products are expected to be alternative in the coccidiosis control promisingly by including new therapeutic molecules to which *Eimeria* strains have not yet developed resistance [8-10].

Lichens are symbiotic associations between an exhabitant fungus and one or more inhabitant photosynthetic partners (algae or cyanobacteria) and they synthesize more than 800 types of metabolites [11,12]. Numerous biological investigations have showed that secondary lichen metabolites have a broad range of biological activities including antibiotic [13], antiviral [14], analgesic and antipyretic [15], antifungal [16,17], anti-inflammatory [18], cytotoxic and antimicrobial [19], antiulcerogenic and antioxidant [20] and immunologic modulator [21].

Usnic acid is one of the most extensively studied lichen metabolites and present in the lichen genera *Alectoria*, *Cladonia*, *Evernia*, *Lecanora*, *Ramalina* and *Usnea* [22]. It is shown to exert a number of biological activities including anti-inflammatory, analgesic and antipyretic, antibiotic, antiviral, antimicrobial, antiproliferative, gastroprotective, antitumor, antioxidant, antimycotic, antigrowth and anti-insect properties [22,23]. Although few studies [9,24-27] dealing with antiprotozoal effect of usnic acid are available, its anticoccidial effect is largely unknown. The present experiment was set out to investigate therapeutic action of usnic acid against coccidiosis in broilers.

MATERIAL and METHODS

Animals and Management

A total of 160 one-day-old male Ross 308 broiler chicks were purchased from a commercial hatchery and housed in an experimental house from 1 to 29 days of age. They were reared altogether in a large pen from d 1 to 7 for adaptation. Chicks were then divided randomly into the final 5 groups (A, B, C, D and E) in 4 replicate subgroups containing of 8 chicks per subgroup. Each subgroup was housed in a separate floor pen (1.5 m x 2.5 m) equipped with one hanging bell drinker, two tube-type feeders and electrical heater. Wood shavings were used as bedding material with a depth of 5 cm. The room temperature was gradually decreased from 33ºC on d 1 to 22ºC on d 21, and then remained unchanged. The chicks were vaccinated against infectious bronchitis and Newcastle disease with Nobilis MA5+Clone30 (Intervet, Boxmeer, Netherlands) at 1 d of age via drinking water. The feed contained no anticoccidials or growth enhancers. Birds were offered feed and water *ad libitum*. The experiments were conducted according to the ethical norms approved by the Atatürk University Ethic Committee of Experimental Animal Teaching and Researcher Center (No: 2012-49).

Parasites and Usnic Acid

The reference parasite stock was provided by the Department of Parasitology at the Veterinary Medicine Faculty of Ataturk University, Turkey. The stock, containing 30 x 10^4 sporulated oocysts from field *Eimeria* isolates *(E. acervulina*, *E. brunette*, *E. maxima*, *E. mitis*, *E. praecox* and *E. tenella)*, was passaged in *Eimeria*-free chickens to keep them infective.

(+)- Usnic acid (Sigma-Aldrich, Steinheim, Germany) was suspended in 1% carboxymethyl cellulose (CMC) water solution and then had been filtered through a 0.2-µm-pore-size filter.

Acute Toxicity Test

An acute toxicity test was conducted on 10-day-old broiler chicks. The birds were divided into 5 groups (CMC and 10, 100, 500 or 1.000 mg (+)-usnic acid extract per kilogram diet, respectively), each consisting 5 birds. The birds were observed for 24 h for signs of toxicity or death.

Experimental Design

After one-week adaptation period, chicks in Group A were not infected and served as uninfected control, whereas those in Group B-E were infected orally with a mixture of purified oocysts including 30 x 10^4 sporulated oocysts from field isolates of *E. acervulina, E. brunetti*, *E. maxima, E. mitis, E. praecox* and *E. tenella* on 16 days of age. The oocyst inoculum was washed several times with tap water and then a 2 ml suspension of 30 x 10^4 sporulated oocysts administered directly into the crop via oral gavage.

After observation of typical lesions of coccidiosis at d 7 post-infection (PI), one chick from each subgroup was chosen randomly and euthanized to inspect lesion scores in order to confirm success of the infection. Group B was untreated and served as infected control. Starting from d 8 PI the birds in Group C, D and E were dosed orally with 20 mg usnic acid, 100 mg usnic acid and 7 mg toltrazuril (Baycox 2.5%, Bayer, Leverkusen, Germany) per kg BW for 5, 5, and 2 days, respectively. The experiment was terminated on d 13 PI.

Fecal oocysts were enumerated a day before infection and performed daily between 20-29 days of age. For this purpose, approximately 200-300 g fecal samples were collected daily from each replicate pen in several spots. Representative fecal samples for each pen were placed in screw cap containers and stored at +4ºC until oocyst counts were performed (oocyst per gram of feces, OPG) using the McMaster counting technique [28].

At the end of the experiment, all birds were slaughtered

for scoring intestinal lesions caused by *Eimeria* species according to the method of Johnson and Reid [29], 0 indicating normal and 1, 2, 3 or 4 indicating the degree of severity of infection. The upper, middle and cecal sections of the chick intestine were examined for lesions. Because the mixed infection was induced, the sectional data were averaged by the group prior to statistical analysis.

Performance Measurements

Feed intake (FI) and body weight (BW) were measured and performance variables (BW gain, BWG and feed conversion ratio, FCR) were calculated at d 1, 16, 23, and 29 on a pen basis.

Statistical Analysis

Considering 5% reduction in oocyst count upon treatment with usnic acid to be significant, sample size was calculated to be 4 replicates. Data were analyzed by one-way ANOVA in a completely randomized design. The oocyst count data were log transformed prior to statistical analysis. The model to test effect of treatments included treatment effect as fixed effect and treatment within group as random effect. Time and group by time interactions were also fixed factor for FI and oocyst data. Statistical significance was considered at $P<0.05$.

RESULTS

No acute toxic effect or mortality was detected in the toxicity test.

Typical clinical signs of coccidiosis including inappetence, wing drooping, distorted feathers, huddling and bloody droppings were observed in all the infected groups of chickens at day 7 PI which were inferred the success of the experimental infection. Reduction in severity of the clinical signs was observed in groups C-E, conspicuously in group E, after the treatment. On the contrary, severity of clinical signs in group B was increased progressively and resulted with a mortality of 10% by day 13 PI.

Table 1 summarizes performance parameters in response to treatment effects in broilers subjected to the coccidiosis induction. After oocyst inoculation, the coccidiosis-induced birds had depressed FI (by 12.1%) and BWG (by 12.2%) and elevated FCR (by 9.9%). Toltrazuril treatment alleviated FCR as compared to the healthy control group. The high level of usnic acid treatment was as effective as toltrazuril treatment *(Table 1, Fig.1)*.

Responses of changes in intestinal pathology and fecal oocyts count to the treatments were ambiguous. Comparing with the healthy control group, the oocyst inoculation caused an increase in lesion scores and elongation of intestine sections as well as presence of oocyst in feces *(Table 2)*. Toltrazuril and usnic acid treatments considerably reduced intestinal lesion score at a similar extent as compared to the untreated groups. However, usnic acid was not as effective as toltrazuril to recover intestinal length. Both usnic acid and toltrazuril caused reduction in fecal oocyst count at a similar level as compared to the untreated group *(Fig. 2)*.

DISCUSSION

In acute toxicity test, neither mortality nor toxicity signs were recorded. This result confirms the previous report [20], which indicated that usnic acid is well tolerated up to 1000 mg/kg BW

Table 1. *Performance parameters in response to treatments in broilers subjected to coccidiosis induction*
Tablo 1. *Coccidiosis oluşturulan broylerlerde tedavilere karşı gelişen performans parametreleri*

Parameter/Stage	Groups[1]					P<
	Group A	Group B	Group C	Group D	Group E	
Body weight (BW), g						
Before trial (d 7)	----------189.4±1.3----------					
Before infection (d 16)	----------320.4±2.5----------					
End of infection (d 23)	661.2±3.1[a]	----------581.4±10.2[b]----------				0.0005
End of treatment (d 28)	1399.2±29.4[a]	1190.0±13.4[c]	1159.7±39.5[c]	1331.7±17.0[ab]	1293.3±39.2[b]	0.0002
Feed intake (FI), g						
Before trial	----------59.0±0.8----------					
During infection period cumulative	520.3±6.9[a]	----------456.7±10.0[b]----------				0.002
During treatment period cumulative	1127.9±31.4[a]	1056.2±23.2[ab]	994.6±33.2[b]	1110.4±37.6[a]	1070.8±16.8[ab]	0.05
Feed conversion ratio (FCR, Feed: BW Gain)						
Before trial	----------0.31±0.004----------					
During infection	1.58±0.02[a]	----------1.74±0.03[b]----------				0.01
During treatment	1.53±0.02[a]	1.68±0.04[b]	1.65±0.07[ab]	1.51±0.05[a]	1.59±0.04[ab]	0.10

Superscripts among columns indicate group differences at P<0.05

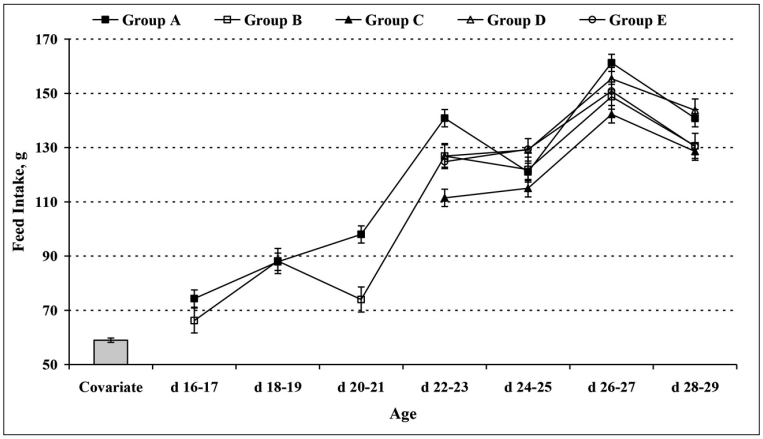


Fig 1. Effect of treatment on feed intake in broilers subjected to coccidiosis induction. Covariate represents feed intake on d 7. The birds in Group A were not infected and served as positive control, the birds in Groups B-E were infected and then subdivided into untreated and served as negative control (Group B) or treated with 20 mg usnic acid (Group C), 100 mg usnic acid (Group D) and 7 mg toltrazuril. Pooled SE was 3.79

Şekil 1. Coccidiosis oluşturulan broylerlerde tedavinin yem tüketimine etkisi. Eşdeğişken 7. gündeki yem tüketimini gösteriyor. A grubundaki hayvanlar enfekte edilmeyerek pozitif kontrol olarak ayrılırken B-E gruplarındakiler enfekte edilip daha sonra tedavi uygulan-mayan negatif kontrol (Grup B), 20 mg/kg usnik asit ile tedavi edilen (Grup C), 100 mg/kg usnik asitle tedavi edilen (Grup D) ve 7 mg/kg toltrazuril uygulanan alt gruplara ayrılmıştır. Ortalama standart hata 3.79

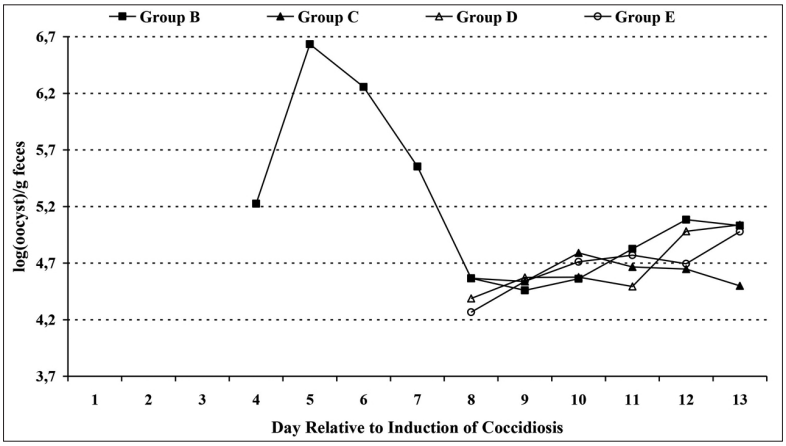
Table 2. *Intestinal lesion score and fecal oocyst count in response to treatments in broilers subjected to coccidiosis induction*
Tablo 2. *Coccidiosis oluşturulan broylerlerde tedaviler sonrası bağırsak lezyon skorları ve oocyst sayıları*

Parameter	Part/Period	Groups[1]					P<
		Group A	Group B	Group C	Group D	Group E	
Lesion score		0.00±0.00[a]	2.96±0.12[c]	0.9±0.04[b]	0.6±0.03[b]	0.5±0.13[b]	0.0001
Length (mm)	Small Intestine	156.5±1.8[b]	177.2±5.6[ab]	186.2±9.3[a]	187.2±11.6[ab]	158.5±7[b]	0.03
	Cecum	16.2±0.7[b]	19.12±0.5[a]	18.0±0.5[ab]	18.1±0.9[ab]	16.9±0.6[b]	0.05
Oocyst count[2]	Infection Period	0.00±0.00[a]	----------5.92±0.07[b]----------				0.0000
	Treatment Period	0.00±0.00[a]	5.27±0.12[c]	5.13±0.12[b]	5.17±0.12[b]	5.12±0.13[b]	0.0001

[1] The birds in Group A were not infected and served as uninfected control, the birds in Groups B-E were infected and then subdivided into untreated control (Group B) or treated with 20 mg usnic acid (Group C), 100 mg usnic acid (Group D) and 7 mg toltrazuril (Group E) per kg body weight. Superscripts among columns indicate group differences at P<0.05; [2] Time effect, P<0.0001. Group x Time effect, P<0.0001

Fig 2. Effect of treatment on fecal oocyst count in broilers subjected to coccidiosis induction. The birds in Groups B-E were infected and then subdivided into untreated control (Group B) or treated with 20 mg usnic acid (Group C), 100 mg usnic acid (Group D) and 7 mg toltrazuril (Group E) per kg body weight. Time effect, P<0.0001. Group x Time effect, P<0.0001. Pooled SE was 0.12

Şekil 2. Coccidiosis oluşturulan broylerlerde tedavinin fekal oocyst sayısına etkisi. B-E gruplarındakiler enfekte edilip daha sonra tedavi uygulanmayan negatif kontrol (Grup B), 20 mg/kg usnik asit ile tedavi edilen (Grup C), 100 mg/kg usnik asitle tedavi edilen (Grup D) ve 7 mg/kg toltrazuril uygulanan alt gruplara ayrılmıştır. Zaman etkisi, P<0.0001. Grup x Zaman etkisi, P<0.0001. Ortalama standart hata 0.12



In the present experiment, poor performance *(Table 1, Fig. 1)* as well as intestinal lesions, prolonged intestine and OPG count *(Table 2, Fig. 2)* confirm success of the coccidiosis induction [30-33]. These are related to significant damage to the intestinal mucosa and enterocytes during the progression of *Eimeria* lifecycle after the *Eimeria* challenge [34]. It appears that body responds to the challenge through elongation of the intestine.

As an alternative to antibiotics, anticoccidial effect of various herbal extracts has been reported [31,34-39]. Their effects were related to protection and/or relieve of intestinal mucosa. To our knowledge, no data on anticoccidial effect of usnic acid are available. However, few investigations have been performed on its antiprotozoal activity. Wu et al.[27] stated that (K) usnic acid exhibited a strong effect against *Trichomonas vaginalis in vitro*. Intralesional administration of (+) usnic acid in mice infected with *Leishmania* promastigotes produced a significant reduction of cutaneous lesions and parasite loads [40]. Luz et al.[40] also determined the antileishmanial activity of usnic acid on *L. infantum chagasi* promastigotes and suggested that usnic acid as a possible phytotherapic agent in the treatment of visceral leishmaniasis. It appears that the

antileishmanial action mode is linked to a complete lysis of promastigotes of the *Leishmania* species [17]. Lichen constituents (thallus, methyl evernate, tenuiorin and three hopane triterpenoids) exerted a weak trypanocidial effect in comparison with the conventional drug in use against epimastigotes of *Trypanosoma cruzi* [20]. De Carvalho et al.[24] investigated the effects of usnic acid against *Trypanosoma cruzi* epimastigotes and trypomastigotes, and reported that usnic acid treatment resulted in growth inhibition in a dose-dependent manner. Lauinger et al.[26] reported antiplasmodial effect of some lichen compounds (*e.g.*, evernic acid, vulpic acid, psoromic acid and (+)-usnic acid) against liver stages of *Plasmodium berghei*.

Anticoccidial effect of usnic acid was comparable to toltrazuril that is a well-known anticoccidial agent [32,41]. In this study, usnic acid increased FI and BWG and alleviated FCR *(Table 1, Fig. 1)*. These could be consequence of its relieve effects on intestinal mucosa *(Table 2)*, which was associated with decreased OPG *(Fig. 2*) and partially shortened cecal length *(Table 2)*.

In summary, data showed that usnic acid (100 mg/kg) was effective in the treatment of coccidiosis as reflected by performance and pathology parameters but regrettably not as good as toltrazuril. Further studies are needed to substantiate our findings and elucidate its action in detail to suggest usnic acid as an alternative anticoccidial agent.

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