

***Artemisia annua* improves chickens performances but has little anticoccidial effect in broiler chickens**

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Abstract. Coccidiosis is a parasitic disease that inflicts severe economic losses for the poultry industry. The decades of anticoccidials usage for control of this disease has led to the development of resistant *Eimeria* strains to all known drugs so botanicals seem to represent a good alternative. *Artemisia annua*, a herb native from China, has been intensively studied especially for its anticoccidial activities against *E. tenella*.

In this study we aimed to assess the anticoccidial effect of dried leaves of *A. annua* administered in diet of broiler chickens infected with a suspension containing *E. acervulina*, *E. tenella* and *E. maxima* sporulated oocysts. For this purpose we designed a battery trial by randomly dividing 147 chickens into 7 groups of 21 chickens each. The experimental groups consisted in: two control groups (one positive, infected group and one negative, uninfected group), one group infected and supplemented with 125 ppm monensin in diet, two groups infected and supplemented with *A. annua* German cultivar, with a concentration of 5 and 50 ppm of artemisinin and two groups infected and supplemented with *A. annua* Romanian cultivar, with a concentration of 5 and 21.3 ppm of artemisinin. The Romanian cultivar of *A. annua* improved significantly the weight gain, even than uninfected chickens ($p \leq 0.004$). These chickens had also an efficient use of feed, comparable or superior to the uninfected chickens. However, the decrease of number of oocysts/g of faeces and the reduction of intestinal lesion score in the chickens receiving the *A. annua* diets, no matter what cultivar, was not satisfactory.

A. annua could be used with success as a feed additive in broiler chickens by improving the production performances, but its anticoccidial effect needs to be further studied.

Keywords: *Artemisia annua*; *Eimeria* spp.; Chickens; Coccidiosis.

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Introduction

Coccidiosis is a devastating disease for the poultry industry. It is produced by seven *Eimeria* species (*E. acervulina*, *E. maxima*, *E. tenella*, *E. necatrix*, *E. brunetti*, *E. mitis* and *E. praecox*), with localization in the chicken's gut, of which *E. acervulina*, *E. tenella* and *E. maxima* are the most prevalent (Györke et al., 2013). Although this disease has been controlled for many years with anticoccidial drugs, resistant strains of *Eimeria* have developed due to the irrational use, and nowadays no synthetic drug has the ability to combat this strains (Chapman, 1997). Also, the consumer concerns about the presence of drug meat residues and the demand for organic products have grown in the last years (Viegas et al., 2012; Greene, 2013).

In view of these issues, a greater interest is being expressed in using natural feedstuffs or botanicals as alternative means of control of chicken's coccidiosis. In the recent years, a wide variety of plants have been studied for their anticoccidial activity, some of them (*Sophora flavescens*, *Gyamopsis tetragonoloba*, *Tulbaghia violacea*, *Saccharum officinarum*, *Aloe vera*, *Echinacea purpurea*, *Bidens pilosa* and many others) showing important characteristics that can be used against infection with various species of *Eimeria* in chickens (Youn et al., 2001; Hassan et al., 2008; Naidoo et al., 2008; Awais et al., 2011; Akhtar et al., 2012; Orengo et al., 2012; Yang et al., 2015).

One of the most promising herbs studied for its anticoccidial properties was *Artemisia annua* along with its bioactive compound artemisinin. *A. annua* is an herb that has been used for many centuries in treating fevers and it's the main source of artemisinin, the component that has an important antimalarial activity. *Artemisia* spp. are also composed of flavonoids, terpenes and antioxidant phenolics which are immunostimulatory and together with artemisinin can suppress free radicals that form in the blood of parasitized animals, thus growing the organism defense against the parasites located in the gastrointestinal tract (Ferreira, 2009). Oh et al. (1995) was the first to conduct a study regarding the effects of *A.*

annua extracts on *E. tenella* infection in broiler chickens, showing that this plant extracts can improve chickens performance characteristics and reduce lesion scores. Many other researchers followed this path. Allen et al. (1997) has expanded the research also on *E. acervulina* and *E. maxima*, but succeeded to prove the anticoccidial effects of dried leaves of *A. annua* only on *E. tenella*. Youn and Noh (2001) used a plant decoction supplied in chickens' water and showed the same anticoccidial effect on *E. tenella*. Brisibe et al. (2008) proved that dietary inclusion of dried leaves of *A. annua* enhance growth in chickens and has anticoccidial effect on *E. tenella*, improves egg characteristics and production in layers. De Almeida et al. (2012) showed that *A. annua* has anticoccidial effect in a pen-trial infection with *E. acervulina* and *E. maxima*. Drăgan et al. (2010; 2014) used *A. annua* as leaf powder in feed or oil in water administered to chickens challenged with low and high infection of *E. tenella* and proved also the anticoccidial effect of the herb and the positive effect on production performances.

Based on this solid background, in the present study we aimed to investigate the effect of *A. annua* in chickens experimentally infected with a suspension of *E. acervulina*, *E. tenella* and *E. maxima*, to verify if this herb has anticoccidial activity in mixed infection.

Materials and methods

Experimental animals

One day-old broiler chickens Ross 308 hybrids were purchased from Vis Avis Vadu Crișului and housed in batteries at the University of Agricultural Sciences and Veterinary Medicine Cluj-Napoca. Permanent lighting and *ad libitum* feed and water were provided during the entire housing period. Anticoccidial-free standard starter feed was ensured until 12 days-old.

Artemisia product

The crops of *A. annua* were established at Research and Development Station for Cattle Breeding Târgu-Mureș in 2013. Seeds of *A. annua* were purchased from Anamed company

Germany and the hybrid Anamed A3 was used due to its high contents of artemisinin, accordingly to the manufacturer's recommendations. An indigenous variety, rich in volatile oils, was purchased from SC Transapicola. The seeds were initially sowed in boxes in March 2013. When the seedlings reached the height of 2-3 cm they were transplanted in trays with nutrient blocks. When the plants had 10-12 cm in height they were transplanted in the main field. The plants were harvested three times: first on 23.08.2013, second time on 05.09.2013 and third on 28.09.2013. After harvesting, the plants were artificially air-dried and then they were minced, sieved and packed in paper bags.

Determination of artemisinin concentration

The resulted plant material was analyzed by high-performance liquid chromatographic (HPLC) method to determine the concentration of artemisinin from both German (all three harvesting periods) and Romanian cultivars. The extraction of artemisinin was done from 1 g of plant refluxed with petroleum ether at 45°C for one hour, vacuum filtration, evaporation to dryness, the resumption of residue in 10 mL acetonitrile and filtration by micro filters HPLC, according to the protocol of Ferreira and Gonzalez (2009).

Experimental design

At 12 days-old, the chickens were randomly divided into 7 groups of 21 chickens, each group with 3 replicates of 7 chickens. The experimental groups consisted of two control groups, one infected (positive control group – PC), one uninfected (negative control group – NC), both of them received standard growing feed with no anticoccidials added. The other groups were designed as follows: four groups infected and in-feed supplemented with *A. annua* German cultivar (with concentration of 5 and 50 ppm artemisinin – AaG5; AaG50), respectively *A. annua* Romanian cultivar (concentrations of 5 and 21.3 ppm artemisinin – AaR5; AaR21.3), and one group infected and fed with standard feed in which 125 ppm of monensin was added (Mon).

At 14 days-old chickens were infected by oral gavage with a suspension of 3.2×10^4 *Eimeria* spp. oocysts which was comprised of 2×10^4 *E. acervulina* oocysts, 2×10^3 *E. tenella* oocysts and 1×10^4 *E. maxima* oocysts.

In order to verify the effect of *A. annua* on mixed infection with *Eimeria* spp. in broiler chickens the following factors were recorded: the performance characteristics, mortality percentage, number of oocysts shedded/g of faeces (OPG) and lesion score.

Performance parameters

The chickens were weighted in the day of the experimental infection (considered as day 0 of the experiment) and also in days 7 and 14 after the infection for determining the body weight gain (BWG) in two different periods: 0-7 days post-infection and 7-14 days post-infection. The weight gain was calculated also for the total period of 14 experimental days. The feed administered to the chickens was weighted daily beginning with day 0 until day 14 and the feed conversion ratio (FCR) was established for the same periods as the weight gain.

Mortality percentage

The chickens were monitored each day in order to record any death that could occur due to coccidial infection.

OPG and lesion score

Faecal samples from each group were collected in days 4 and 5 post-infection and flotation technique (Willis method) was applied in order to verify if the chickens started to shed oocysts. The faecal samples from days 5-14 post-infection were collected and processed for counting the number of oocysts per gram of faeces by McMaster method. In day 7 post infection 10 chickens from each group were euthanized and necropsy was performed. From each chicken the entire intestine was removed and separated into duodenum, jejunum and caecum. Grades from 0 to 4 were given for each intestinal segment depending on the severity of lesions caused by the site specific *Eimeria* species (Johnson and Reid, 1970).

Statistical interpretation

The data were analyzed in Excel 2007 and Medcalc software. The standard deviation and arithmetic mean were calculated in Excel. All the data were subjected to analysis of normal distribution in Medcalc using Shapiro-Wilk test. The normally distributed data were analysed by Independent samples T test, whilst the data not normally distributed by Mann-Whitney test. The differences between experimental groups were considered significant if the *p* value was equal or lower than 0.05.

Results

Artemisinin content

The German cultivar of *A. annua* had different concentrations of artemisinin accordingly to the time of harvesting: 1st period (23.08.2013) – 0.899 g/100 g dried plant, second period (5.09.2013) – 0.667 g/100 g dried plant and third period (28.09.2013) – 0.848 g/100 g. For the experiments the plant material harvested in the first period was used due to its higher content of artemisinin. The Romanian cultivar of *A. annua* had a very low content of artemisinin, 0.071 g/100 g dried plant.

Weight gain

The chickens that received in their diet 5 ppm of *A. annua* Romanian cultivar recorded the highest weight gain in the period 7-14 days after infection and also in the total period ($p \leq 0.004$). In the first period, when the coccidian parasites were just beginning to cause damage to the intestine, the chickens from the groups AaR5 and AaG50 recorded similar weight gains, but this were lower than the positive control group, which recorded a higher weight gain even than the uninfected group ($p = 0.005$). The German cultivar of *A. annua* didn't seem to have the same positive effect on the chickens' growth, the weight gains of the chickens fed with this diet being similar or even lower than the chickens from the positive control group (table 1).

Feed conversion ratio

The best feed conversion ratio in the first period of recording was obtained in the AaG50 group, of the chickens which diet was supplemented with *A. annua*. However, in the second period, this group had the worst use of feed. The chicken supplemented with 5 ppm of Romanian cultivar had feed conversions similar or even superior to the positive control group (table 1).

Mortality percentage

None of the chickens died during the experimental period.

Oocysts per gram of faeces

The dynamics of oocysts shed by chickens from the experimental groups showed a tendency to increase in day 7 post infection in the groups that received *A. annua* Romanian cultivar and also the control group. On the contrary, the chickens which diet was supplemented with German cultivar, showed a decreased tendency in day 7 after infection, the number of oocysts shed being significantly lower than the control group ($p \leq 0.003$). After day 8, until day 12 post infection, the chickens from all the groups supplemented with *A. annua* shed higher number of oocysts than the positive control group. In day 12 post-infection the number of oocysts shed increased again in all groups, but the *A. annua* medicated groups recorded lower values of OPG than the control group, statistically significant only for groups AaG5 and AaR21.3 ($p \leq 0.02$). However, in the last two days of recording, the chickens medicated with *A. annua* German cultivar with a concentration of 50 ppm artemisinin shed significantly more oocysts than the other groups ($p \leq 0.001$) (figure 1).

Lesion score

The supplementation of chicken's diet with *A. annua* had no effect on lesions produced by *E. acervulina* in the duodenum. The lesion score for all the *A. annua* treated groups was higher than the positive control group ($p \leq 0.6$), whilst the chickens treated with the classical

anticoccidial monensin had no duodenal lesions.

However, the lesions caused by *E. maxima* in the jejunum were reduced in all *A. annua* treated groups, but not significantly compared with the control group ($p \leq 0.8$).

In the caecum, the lesion score was the same as in the control group for all groups medicated with *A. annua*, except the group AaG5, but the reduction of lesions caused by *E. tenella* in this group was not marked enough ($p = 0.3$).

By summing up the lesion score from all segments, we can observe that monensin reduced to almost half the intestinal lesions, whilst only the German cultivar of *A. annua* with a concentration of 5 ppm artemisinin, manage to reduce the lesions, but this reduction was unsatisfactorily ($p = 0.3$) (figure 2).

Discussions

For centuries, *A. annua* has been an herb used in treating fevers associated with malaria. Many other therapeutic attributes of this plant have been discovered, even antimicrobial or anticancer properties (Tajehmiri et al., 2014; Breuer and Efferth, 2014). All this actions seem to be attributed to the main bioactive compound named artemisinin (Sertel et al., 2010). Regarding artemisinin anticoccidial activity, it's believed that the production of free radicals due to the cleavage of artemisinin's endoperoxide bridge leads to inhibition of the coccidian sarco/endoplasmic reticulum calcium ATP-ase (Del Cacho et al., 2010). However, there are hypotheses that state that other components of *A. annua* like flavonoids or essential oils enhance the anticoccidial effect of the plant (Ferreira et al., 2010).

Table 1. The effect of *A. annua* German and Romanian cultivars on chicken's performance characteristics compared with monensin and control groups

Group	BWG (g/day)			FCR (kg feed/kg weight gain)		
	I	II	III	I	II	III
NC	20.44±5.31	26.14±6.98	23.29±4.77	2.74±0.40	2.62±0.18	2.66±0.06
PC	25.12±3.32	24.40±5.48	24.45±3.91	2.26±0.18	3.34±0.45	2.69±0.01
AaG5	17.67±5.98	24.66±6.22	21.23±6.29	3.13±0.52	2.65±0.41	2.86±0.50
AaG50	23.75±6.10	16.43±5.05	20.81±4.23	2.56±0.31	4.56±1.19	3.04±0.22
AaR5	23.94±4.76	32.24±4.39	28.80±2.28	2.74±0.25	2.91±1.05	2.68±0.43
AaR21.3	18.34±3.53	20.77±5.86	19.31±4.46	2.97±0.11	3.42±0.08	3.21±0.04
MON	21.84±4.19	29.12±6.31	24.97±3.52	2.73±0.25	2.60±0.71	2.56±0.24

I: 1st period = 0-7 days; II: 2nd period = 7-14 days; III: total period = 0-14 days; results are expressed as means ± SD.

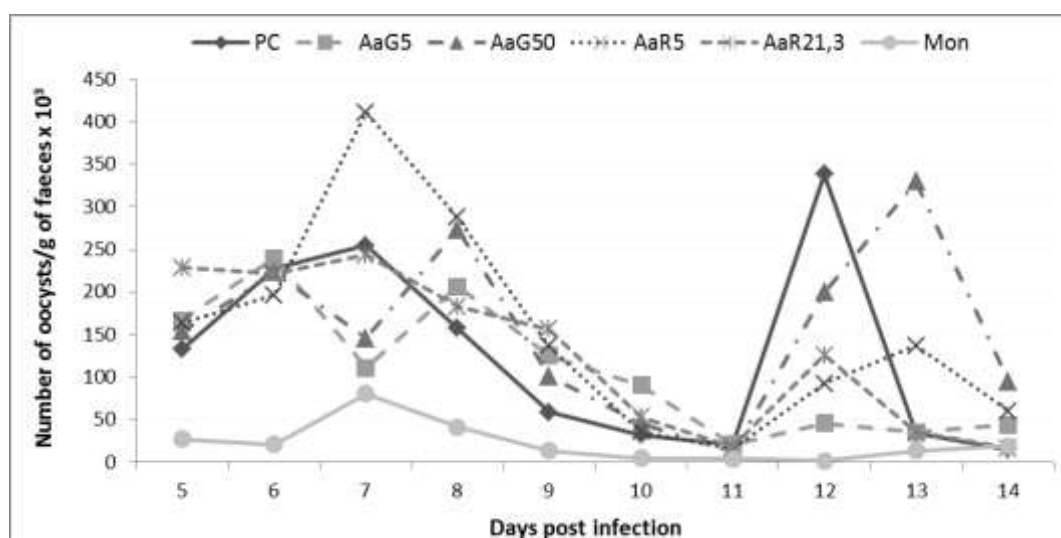


Figure 1. Dynamic of mean number of oocysts/g of faeces in groups of chickens fed with *A. annua* diets compared with chickens infected and fed with anticoccidial-free and monensin diets

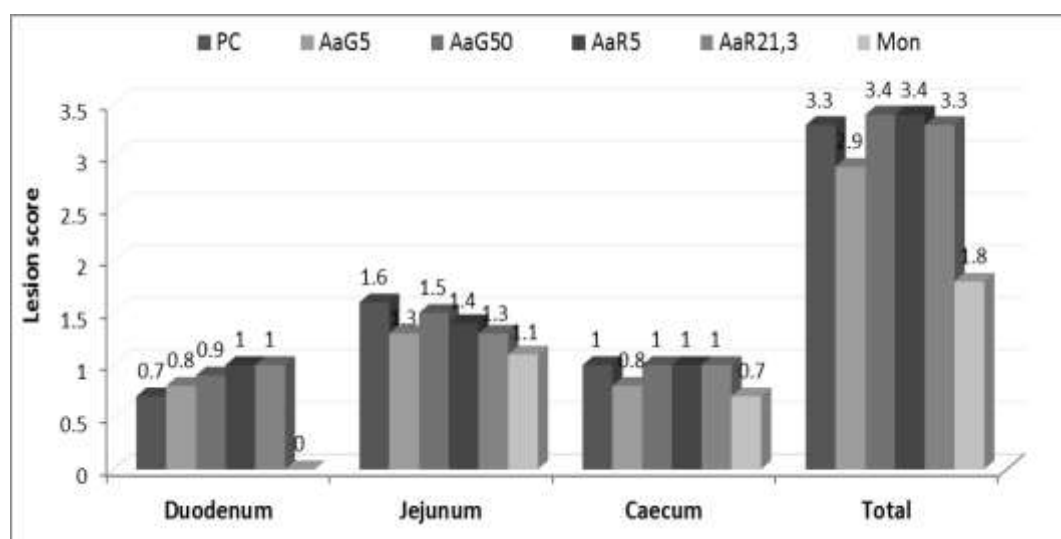


Figure 2. The mean lesion score in different parts of the gut from groups of chickens fed with *A. annua* diets compared with chickens infected and fed with anticoccidial-free and monensin diets

In the present study *A. annua* (with a known amount of artemisinin) was tested for anticoccidial activity against mixed infection with *E. acervulina*, *E. tenella* and *E. maxima* in a battery trial.

Two cultivars of *A. annua* (German and Romanian) were supplemented in chickens' diet in the form of minced dried leaves, the content of artemisinin being verified before establishing the dosages. The administration of a drug in feed is a simple and effective way to prevent or cure diseases in large groups of animals and along with water administration this could represent the only practical solution in poultry. The experimental feed was introduced in the chickens' diet two days prior infection, in order to check if this could protect the chickens against the *Eimeria* challenge. The broiler chickens were infected at 14 days-old with a mixed suspension of *E. acervulina*, *E. tenella* and *E. maxima* oocysts, this species being the most prevalent in the field (Györke et al., 2013). For a product to be considered effective against coccidiosis it should reduce the parasite multiplication and as consequence attenuate the lesions produced in the intestine, should not affect the chicken's abilities to build up a natural immunity and should not impair the production performances. On this grounds, the number of oocysts/g of faeces were counted from the first day of shedding until the end of the experiment, a representative

number of birds were euthanatized for the lesion score evaluation and the weight gain and feed conversion ratio were assessed at one-week interval after infection.

The positive effect of *A. annua* in increasing the production performances of broiler chickens demonstrated in the present study was also noticed by De Almeida et al. (2012) but only in a late period after the coccidial challenge. However, Allen et al. (1997) observed a reduction in weight gain in the chickens challenged with only *E. maxima* or *E. acervulina* + *E. tenella* and supplemented with 0.5 and 1% *A. annua* leaves, but they recorded the weight gain only at 6 days post infection. This positive effect of the plant on the performance characteristics of broiler chickens was also recorded in our previous studies, in *E. tenella* infection (Drăgan et al., 2010; 2014).

In this study, the number of oocysts shed by the chickens fed with *A. annua* diets was significantly lower than that of the control group only in day 7 and 12 after the experimental infection, in the rest of the shedding time the OPG values were higher in the *A. annua* treated groups than in the infected untreated chickens. This was in contradiction with the results obtained by Allen et al. (1997) but they tested pure artemisinin in concentrations of 2, 8.5 and 17 ppm against single and mixed infection with *E.*

tenella and *E. acervulina* and observed a significantly reduced oocyst output in the chickens which diet was supplemented with artemisinin. In the study of De Almeida et al. (2012) powder of *A. annua* dried leaves administered at 3% of the expected daily food intake in the diet of broiler chickens reared in a free-range system decreased the number of excreted oocysts of *E. acervulina* and *E. maxima*, but the infection was performed by the introduction of seeders in the chickens groups and it was subclinical. In our previous study, the administration of 1.5% powder of *A. annua* in the diet of chickens challenged with a similar dose of *E. tenella* reduced the oocyst output by 90.76% compared to the infected unmedicated group, which could signify that a higher dose of the plant could be much more effective (Drăgan et al., 2010). When the chickens received nearly ten times more oocysts of *E. tenella*, the capability of reducing the oocyst counts was still maintained for the same administration protocol of the plant (Drăgan et al., 2014).

The reduction of intestinal lesions by the supplementation of *A. annua* in the chickens' diet in the present study was not satisfactory for any *Eimeria* infection. This is not consistent with the previously reported results only for the infection with *E. tenella*. Thus, Allen et al. (1997) using *A. annua* supplementation at a level of 5% in the diet recorded a significantly reduction in the caecal lesion score in the treated chickens compared with the control group. This was also recorded in our previous study using 1.5% powder of *A. annua* (Drăgan et al., 2014).

In conclusion, our results suggest that *A. annua* could be a promising additive in chickens' diet for its beneficial effect on the performance attributes, but the plant still needs to be studied in order to find the mechanism involved and also the effective dosage against coccidiosis.

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