

Original Article**Effects of essential oils combination on sporulation of turkey
(*Meleagris gallopavo*) *Eimeria* oocysts****Isakakroudi¹, N., Talebi^{1,*}, A., Allymehr¹, M., Tavassoli², M.***1. Department of Poultry Health & Diseases, Faculty of Veterinary Medicine, Urmia University, Urmia, Iran**2. Department of Pathobiology, Faculty of Veterinary Medicine, Urmia University, Urmia, Iran*

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ABSTRACT

Avian coccidiosis is the most important parasitic disease in poultry production, which inflicts numerous losses to the industry. The extensive use of anticoccidial drugs leads to parasite resistance and drug residue in poultry products. In the present study, we aimed to investigate the effects of three famous essential oils (EOs) and their combination on inactivation of mixed oocysts of *Eimeria adenoides*, *Eimeria dispersa*, *Eimeria meleagrimitis*, and *Eimeria meleagridis*. The EOs of *Thymus vulgaris*, *Artemisia sieberi*, and *Mentha pulegium* were prepared. After inoculation of each turkey with 7×10^5 sporulated oocysts, fresh unsporulated oocysts were harvested from their feces. To evaluate the sporulation inhibition effect, 5×10^4 oocysts were used in each treatment. Each EO was used in increasing concentrations. Half maximal inhibitory concentration (IC_{50}) was determined for each EO and they were blended in pairs based on IC_{50} line. Our results showed that the IC_{50} values for mentha, artemisia, and thyme were 22.92, 40.5, and 53.42 mg/ml, respectively. According to our results, artemisia and thyme combination has a synergistic effect, whereas the combination of a high concentration of mentha with a low concentration of thyme had an antagonistic effect. During this study, no interactions were observed between mentha and artemisia.

Keywords: Essential oils, Coccidiosis, Turkey, *Eimeria*, Sporulation inhibition**Effets d'une combinaison d'huiles essentielles sur la sporulation des oocystes d'*Eimeria* chez les dindes (*Meleagris gallopavo*)**

Résumé: La coccidiose aviaire est la maladie parasitaire la plus importante dans l'industrie aviaire infligeant des pertes importantes. La forte utilisation de médicaments anticoccidiens entraîne des résistances aux parasites et la persistance des résidus de médicaments dans les produits de volaille. Cette étude visait à étudier l'effet de trois célèbres huiles essentielles (EO) et leur combinaison sur l'inactivation d'oocystes mixtes d'*Eimeria adenoides*, *Eimeria dispersa*, *Eimeria meleagrimitis* et *Eimeria meleagridis*. Les EO de *Thymus vulgaris*, *Artemisia sieberi* et *Mentha pulegium* ont été extraites. Après inoculation de chaque dinde avec des oocystes sporulés de 7×10^5 , des oocystes frais non sporulés ont été récoltés à partir de leurs fèces. Pour évaluer l'effet inhibiteur sur la sporulation, un nombre de 5×10^4 oocystes ont été utilisés dans chaque traitement. Chaque EO a été utilisé dans des concentrations croissantes. La concentration inhibitrice demi-maximale (CI_{50}) a été déterminée pour chaque EO ainsi que sur les mélanges de deux huiles essentielles. Les résultats de cette étude ont montré que la CI_{50} pour l'origan, l'armoise et le thym était respectivement de 22,92, 40,5 et 53,42 mg/ml. Selon nos résultats, l'interaction de l'armoise et du thym conduit à un effet synergique alors que la combinaison d'une forte concentration d'origan et d'une faible concentration de thym a des effets antagonistes. Au cours de cette étude, aucune interaction entre l'origan et l'armoise n'a été observée.

Mots-clés: Huiles essentielles, Coccidiose, Turquie, *Eimeria*, Inhibition de la sporulation

INTRODUCTION

Avian coccidiosis, caused by protozoa belonging to the genus *Eimeria*, is considered as the major parasitic disease in the poultry industry. The parasite infects the intestinal tract and leads to extensive damages to the intestinal epithelium resulting in poor performance (e.g., reduced nutrimental absorption dynamics, feed conversion rate, and body weight gain), dehydration, blood loss, and eventually, increased susceptibility to other pathogenic agents (McDougald and Fitz-Coy, 2013; Fatemi et al., 2015). Avian coccidiosis as a cosmopolitan disease is associated with more than 3×10⁹ US\$ annual loss in the poultry industry (Shivaramaiah et al., 2014). In turkey (*Meleagris gallopavo*), as the lesions of coccidiocidal infection are less spectacular in comparison to chicken, therefore, it often goes undetected (McDougald and Fitz-Coy, 2013). Seven *Eimeria* species infect turkeys, but only four species are common (McDougald and Fitz-Coy, 2013; Vrba and Pakandl, 2014). Coccidiosis is transmitted through sporulated oocysts that are resistant to mechanical damages and chemical disinfectants. Some factors such as temperature, humidity, and oxygen are essential for oocyst sporulation (Guimaraes et al., 2007). The effective use of chemoprophylaxis in the past decades has been the traditional preventive measure for coccidiosis. Since drug resistance and people's interest in antimicrobial-free products are ever increasing, an alternative strategy has been suggested for the management of coccidiosis (Peek, 2010; Remmal et al., 2011). Medicinal herbs and their products such as essential oils (EOs) and extracts have been used for a long, and their antimicrobial and antioxidant activities have been proven (Brenes and Roura, 2010; Solórzano-Santos and Miranda-Novales, 2012; Bozkurt et al., 2013). Several surveys have investigated the in vivo and in vitro effects of herbal extracts and EOs in the treatment of chicken coccidiosis, but the available data on the efficacy of these products in the treatment of turkey coccidiosis are limited. The EOs from thyme (*Thymus vulgaris*), artemisia (*Artemisia sieberi*), and mentha (*Mentha*

pulegirum), which are natural harmless products, are currently being used in the medicine and food industries. The aim of this study was to investigate the effects of these EOs on the inhibition of sporulation process of turkey *Eimeria* oocysts alone and in combination. EO combinations may lead to synergistic, antagonistic, or additive effects (Delaquis et al., 2002). This study may provide a basis for the application of these EOs as an alternative for anticoccidiosis drugs or disinfectants.

MATERIALS AND METHODS

Isolation and purification of *Eimeria* oocysts. The oocysts collected from litter of a domestic turkey farm in Northwest of Iran were brought to the Department of Pathobiology, Faculty of Veterinary Medicine, Urmia University, Urmia, Iran. After isolating the oocysts by saturated NaCl solution (Shirley, 1992), they were transferred to 2.5% potassium dichromate and sporulated. A number of mixed sporulated oocysts were inoculated to turkeys (7×10⁵/turkey) in order to obtain sufficient number of oocysts. Six to ten days post-infection (PI), fecal samples were collected in water, homogenized with a vortex mixer for 5 min, and passed twice through a 60-mesh (250 μm aperture) sieve, and subsequently, through 100-mesh (150 μm aperture) and 200-mesh (75 μm aperture) sieves. After 24 h of keeping the slurry at 4 °C, the supernatant was discarded and the residue was centrifuged. The sediment was re-suspended in saturated NaCl solution and centrifuged for 10 min at 1500 g. After removing the oocysts-containing floating film, fresh unsporulated oocysts were washed twice in tap water to remove the remaining salt and stored in refrigerator at 4 °C. The number of oocysts was adjusted to 10⁵ oocyst/ml using haemocytometry (Conway and McKenzie, 2008) in order to be applied in in vitro experiments. In order to identify the isolated *Eimeria* species based on shape and index, oocysts' length and width were measured via light microscope using calibrated ocular lens. The oocysts were identified as *Eimeria adenoides*, *E.*

dispersa, *E. meleagrimitis*, and *E. meleagridis* in the final suspension.

Preparation of Eos. The EOs and gas chromatography (GC) analysis were supplied by Barij Esans Kashan Corporation (Mashade Ardehal, Kashan, Iran). The relative content of major components in artemisia EO were α -thujone (32.8%), β -thujone (11.3%), and camphor (20.3%). In mentha EO, the main compositions were pulegone (54.6%), 1, 8-cineole (10.7%), and α -isopulegone (8.2%), and in thyme, they were thymol (27.9%), carvacrol (24.4%), and p-cymene (8.41%). The EOs were diluted in 0.2% agar solution and the liquid medium was stirred vigorously until being homogenized according to a method developed by Remmal et al. (1993). EOs were kept in refrigerator until being used.

Effect of ammonia and diclazuril. As a positive control, anti-sporulation effects of ammonia and diclazuril were tested. Two percent ammonia solution and water soluble diclazuril (Rouyandaroo pharmaceutical company) were used at the dose recommended by the manufacturer (1 ppm).

Sporulation inhibition assay (the first phase). To evaluate the effects of aromatic EOs on turkey *Eimeria* oocysts, 5×10^4 fresh unsporulated oocysts were used in each treatment. The test groups consisted of three EOs at the final concentrations of 0, 1, 2, 4, 8, 10, 20, 40, 80, and 800 mg/ml with three replications. The control group contained 5×10^4 oocysts in pure water. Tubes were capped tightly in order to prevent evaporation of the Eos, and they were put in an incubator at 25-29 °C providing 60-80% humidity with continuous aerating and homogenizing with an air pump using a needle transferring the air straight to the bottom of the tubes. After 72 h of incubation, the oocysts were washed twice in tap water and re-incubated to confirm the constant coccidiocidal effect (Williams, 1997). Finally, the oocysts were washed again and stored at 4 °C until being counted. The hemocytometer method was used for counting the sporulated oocysts. Only the oocysts containing four differentiated sporocysts and

stieda body were considered as sporulated (del Cacho et al., 2010).

Combination of EOs (the second phase). After counting the sporulated oocysts in each treatment, concentrations that could inhibit the sporulation of 50% of the oocysts (IC_{50}) were determined. In this phase, EOs were mixed based on IC_{50} line (Gessner, 1995) in order to evaluate the effect of the EO combinations on oocysts. The incubation process and counting methods were similar to those in the first phase.

Statistical analysis. Statistical analysis was carried out by GraphPad Prism software, version 6.00, for Windows (La Jolla California USA, www.graphpad.com). For statistical analysis, non-linear modeling (non-linear regression) and one-way ANOVA were performed.

RESULTS

Results of this study are presented in tables 1 and 2, as well as in figures 1, 2, 3, 4, 5, and 6 as follows: Log dose-response curve of inhibition of oocysts sporulation was drawn. According to IC_{50} , mentha, artemisia, and thyme could inhibit sporulation at 22.92, 40.5, and 53.42 mg/ml concentrations, respectively (Table 1). The 800 mg/ml concentration had inactivation effects but could not inactivate all the oocysts.

Table 1. Comparative values of sporulation inhibition efficacy in log dose response curve

	Mentha	Thyme	Artemisia
Log IC_{50}	1.360	1.728	1.607
IC_{50}	22.92	53.42	40.50
	Std. Error		
Log IC_{50}	0.02647	0.03512	0.04171
	95% Confidence Intervals		
Log IC_{50}	1.306-1.415	1.656 to 1.800	1.522 to 1.693
IC_{50}	20.22 to 25.97	45.24 to 63.09	33.24 to 49.34

The EOs were combined at the ratios of 25:75, 50:50, and 75:25 according to IC_{50} line. The mixed concentrations for artemisia and thyme were 10.1:40 mg/ml, 20.25:26.7 mg/ml, and 30.4:13.35 mg/ml (Figure 4). For mentha and thyme, they were 5.75:40 mg/ml, 11.5:26.7 mg/ml, and 17.2:13.35 mg/ml (Figure

5). The mixed concentrations for mentha and artemisia were 5.75:30.4 mg/ml, 11.5:20.5 mg/ml, and 17.2:10.1 mg/ml (Figure 6).

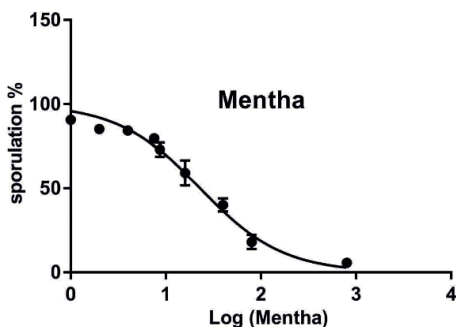


Figure 1. The effect of mentha essential oil on turkey *Eimeria* oocysts sporulation

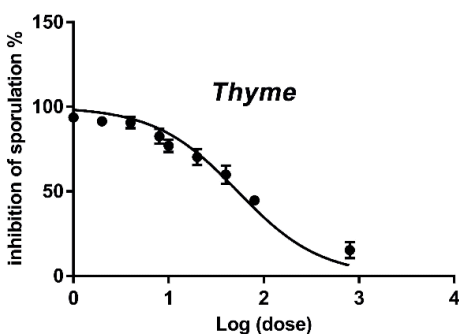


Figure 2. The effect of thyme essential oil on turkey *Eimeria* oocysts sporulation

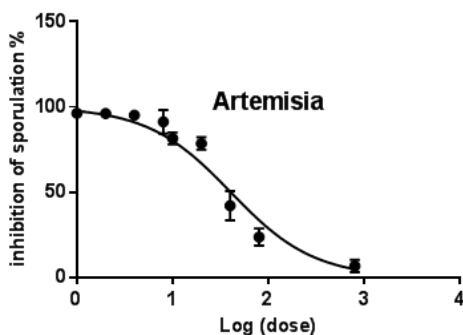


Figure 3. The effect of artemisia essential oil on turkey *Eimeria* oocysts sporulation

Sporulation inhibition rates for the three mixtures of artemisia and thyme were 65.3%, 67.2%, and 70.6% ($P < 0.05$). For the mentha and thyme combination, the inhibition rates were 37.3%, 50.9%, and 51.6%

($P < 0.05$). Inhibition rates of 50.6%, 49.7%, and 49.9% were recorded for the mentha and artemisia combination (Table 2).

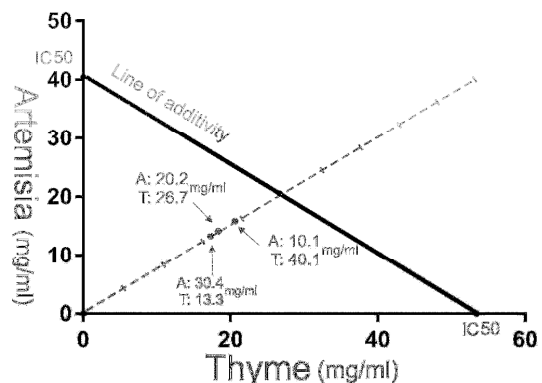


Figure 4. Combination of artemisia and thyme essential oils based on IC₅₀ line

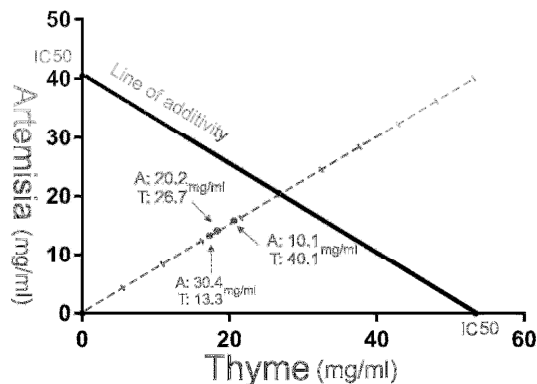


Figure 5. Combination of mentha and thyme essential oils based on IC₅₀ line

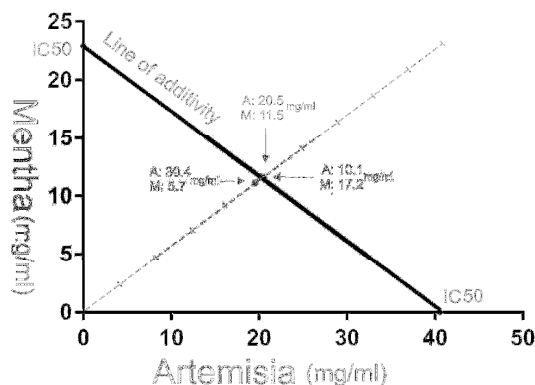


Figure 6. Combination of mentha and artemisia essential oils based on IC₅₀ line

In comparison of the positive and negative controls, sporulation inhibition effects of ammonia and diclazuril

relative to the control were 97.5% and 19.5%, respectively.

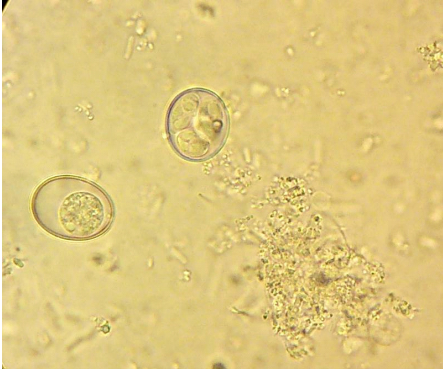


Figure 7. Comparison of unsporulated (left) and sporulated oocysts (right)

Table 2. Essential oil combination and sporulation inhibition rate

Artemisia-Thyme (mg/ml)	SI (%)	Mentha-Thyme (mg/ml)	SI (%)	Mentha-Artemisia (mg/ml)	SI (%)
10.1-40	65.3	5.75-40	37.3	5.75-30.4	50.6
20.25-26.7	67.2	11.5-26.7	50.9	11.5-20.5	49.7
30.4-13.35	70.6	17.2-13.35	51.6	17.2-10.1	49.9

DISCUSSION

Poultry litter provides the appropriate conditions for sporulation of *Eimeria* oocysts, which are resistant to many chemical and mechanical damages. The principal control and treatment programs against this disease are coccidiostatic drugs or anticoccidial vaccine generally administered in food or water. Resistance of the parasite to treatment and drug residues in poultry production are major concerns in the poultry industry (Stephen et al., 1997; Kennedy et al., 1998). For this reason, alternative substances with similar anticoccidiocidal effects and lacking harmful residues are preferred. Several in vitro and in vivo studies have reported the antimicrobial effects of aromatic plants. Allen et al. (1997) reported that artemisia extract could be effective in protecting the chicken from intestinal lesions induced by coccidiosis. A study carried out by Fatemi et al. (2015) demonstrated that PE extract of

artemisia decreased sporulation of chicken *Eimeria* oocysts. Saini et al. (2003) showed the prevalence of coccidiosis in broiler and pathogenicity of *Clostridium* prefringens were reduced by mentha EOs. In a study by Remmal et al. (2011), in vitro destruction of chicken *Eimeria* oocysts by EOs was reported. Recently, other studies on the use of EO combinations in poultry against coccidiosis were documented (Bozkurt et al., 2013). This is the first study on anti-sporulation properties of EOs and their combination on turkey *Eimeria* oocysts. Sporulation of *Eimeria* oocyst in litter and ingestion by poultry is an important factor in the epidemiology of coccidiosis. To evaluate the efficacy of EOs, sporulation inhibition was set as a criterion (Figure 7). The most effective EO for sporulation inhibition was mentha followed by artemisia and thyme. Our results are in agreement with those of previous reports, in that, the EO extracted from *T. vulgaris* was able to destroy both oocysts and sporozoites (Muthamilselvan et al., 2016). The bioactive cytotoxicity of these oils is mostly related to phenol, aldehyde, and alcohol constituents. High concentrations of pulegone in mentha EO may be the reason for its anticoccidial activity. Although the exact mechanism remains inconspicuous, antibacterial and antifungal activities of pulegone have been demonstrated in several studies (Oumzil et al., 2002; Duru et al., 2004). Moreover phenolic compounds like thymol and carvacrol may penetrate the oocyst wall and damage its cytoplasm (Williams, 1997; Molan et al., 2009). It can be assumed that these phytochemicals may interfere with the sporulation process by inhibiting the responsible enzymes or preventing oxygen accessibility (Zaman et al., 2012). This inactivation was coccidiocidal due to the insignificant difference between sporulations at the first and second assessments ($P > 0.05$). It has been suggested that some parts of unmetabolized EOs in treated birds that are in close contact with oocysts in litter, which could impair sporulation (Fatemi et al., 2015). During counting oocysts, some morphological changes and deformed

oocysts were noticed, which is in line with previous reports (Molan et al., 2009; Remmal et al., 2011; Fatemi et al., 2015). In parallel with EOs, abilities of ammonia and diclazuril to inactivate oocysts were tested. Ammonia is a potentially dangerous chemical, the anticoccidiocidal activity of which has been demonstrated by several studies (Williams, 1997; Samaha et al., 2013). Our 2% ammonia solution inactivated more than 97% of *Eimeria* oocysts that may indicate the ability of ammonia to destroy turkey *Eimeria* oocysts. It has been suggested that small molecules of ammonia could penetrate into the oocyst wall and destroy the nucleus (Belli et al., 2006). The obtained results from diclazuril may also indicate its anticoccidiocidal effects in *in vitro* conditions, but it may be more efficacious in poultry intestine conditions to affect sexual and asexual stages (Bozkurt et al., 2013). The anticoccidiocidal activity of EO combinations has been less documented. EOs consist of various components and their combination may lead to synergistic, antagonistic, or additive effects. According to our results, *A. sieberi* and *T. vulgaris* combination exhibited synergistic effects against oocysts sporulation in each combination (sporulation inhibition rate > 50%). The interaction between phenolic and monoterpene alcohol components of EOs leads to synergistic effects. Increased membrane permeability via a component and easier transportation of the other one has also been proposed (Zhou et al., 2007). Antimicrobial activity of EOs may depend on their major constituents, but there is increasing evidence that minor components of EOs and their interaction with the main constituents are more critical for their activity (Burt, 2004; Bozkurt et al., 2013). However, a study carried out by Mahboubi et al. (2015) showed that *Artemisia sieberi* EO with different chemical compositions in its major components had the same antimicrobial activity against some bacteria. In combination of *A. sieberi* and *M. pulegium*, no interaction was recorded (sporulation inhibition almost 50%). In high concentration of *T. vulgaris* in combination with low concentration of *M. pulegium*, as

well as in equal ratio, the effects were additive (sporulation inhibition rate close to 50%), but in high concentration of *M. pulegium* with low concentration of *T. vulgaris*, an antagonistic effect was observed (sporulation inhibition rate < 50%). The mechanism of antagonistic interaction is not thoroughly known, however, it has been suggested that the agents may act on the same target of the microorganism, which leads to less activity due to competition. It has been reported that the combination of mentha and thyme has synergistic effects (Guimaraes et al., 2007; Molan et al., 2009).

In conclusion, combination of EOs like artemisia and thyme can increase their efficacy in comparison to their individual use against turkey *Eimeria*. These EOs can be used as food additives or disinfectants in poultry production. Further *in vivo* studies are suggested to evaluate the effect of EOs on sporozoites and merozoites, as well as syngamy process. Meanwhile, it should be kept in mind that multiple factors, including origin of the plants, environment, genetics, season of collection, and methods of isolation, can influence the chemical composition of EOs. Variations in bioactive compounds and their interaction may be considered as a major restriction in this field of research.

Ethics

I hereby declare all ethical standards have been respected in preparation of the submitted article.

Conflict of Interest

The authors declare that they have no conflict of interest.

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Authors' Contribution

Alireza Talebi and Nima Isakakroudi developed the original idea, acquired and analyzed the data, and prepared the manuscript. Manoochehr Allymehr and

Mousa Tavassoli contributed to the development of the protocol and technical support.

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