

Mulberry extract as an ecofriendly anticoccidial agent: *in vitro* and *in vivo* application

Extrato de amoreira como agente anticoccidiano ecologicamente correto:
aplicação *in vitro* e *in vivo*

Felwa Abdullah Thagfan^{1*} ; Wafa Abdullah Al-Megrin¹; Saleh Al-Quraishy²; Mohamed Abdel Monem Dkhil^{2,3}

¹Department of Biology, College of Science, Princess Nourah Bint Abdulrahman University, Riyadh, Saudi Arabia

²Department of Zoology, College of Science, King Saud University, Riyadh, Saudi Arabia

³Department of Zoology and Entomology, Faculty of Science, Helwan University, Cairo, Egypt

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Abstract

Natural products are ecofriendly agents that can be used against parasitic diseases. *Eimeria* species cause eimeriosis in many birds and mammals and resistance to available medications used in the treatment of eimeriosis is emerging. We investigated the *in vitro* and *in vivo* activity of *Morus nigra* leaf extracts (MNLE) against sporulation of oocysts and infection of mice with *Eimeria papillata*. Phytochemical analysis of MNLE showed the presence of seven compounds and the *in vitro* effects of MNLE, amprolium, Dettol™, formalin, ethanol, and phenol were studied after incubation with oocysts before sporulation. Furthermore, infection of mice with *E. papillata* induced an oocyst output of approximately 12×10^5 oocysts/g of feces. MNLE significantly decreased oocyst output to approximately 86% and the total number of parasitic stages in the jejunum by approximately 87%. In addition, the reduction in the number of goblet cells in the jejunum of mice was increased after treatment. These findings suggest that mulberry exhibited powerful anticoccidial activity.

Keywords: *Morus nigra* extract, sporulation, eimeriosis, oocysts, mice, jejunum.

Resumo

Os produtos naturais são agentes ecologicamente corretos que podem ser usados contra doenças parasitárias. As espécies de *Eimeria* causam eimeriose em muitas aves e mamíferos e a resistência aos medicamentos disponíveis usados no tratamento da eimeriose está emergindo. Foram investigadas as atividades *in vitro* e *in vivo* dos extratos de folhas de *Morus nigra* (MNLE) contra esporulação de oocistos e infecção de camundongos com *Eimeria papillata*. A análise fitoquímica do MNLE mostrou a presença de sete compostos e os efeitos *in vitro* do MNLE, amprolium, Dettol™, formalina, etanol e fenol foram estudados após incubação com oocistos antes da esporulação. Além disso, a infecção de camundongos com *E. papillata* induziu uma produção de oocistos de aproximadamente 12×10^5 oocistos / g de fezes. O MNLE reduziu significativamente a produção de oocistos para aproximadamente 86%, e o número total de estágios parasitários no jejuno em aproximadamente 87%. Além disso, a redução no número de células caliciformes no jejuno de camundongos aumentou após o tratamento. Esses achados sugerem que a amoreira exibiu uma poderosa atividade anticoccidiana.

Palavras-chave: Extrato de *Morus nigra*, esporulação, eimeriose, oocistos, camundongos, jejuno.

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*Corresponding author: Felwa Abdullah Thagfan. E-mail: fafa-85@hotmail.com



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Introduction

Coccidiosis, one of the most serious diseases affecting many animals (Mehlhorn, 2014), is caused by infection with *Eimeria* spp. (Andrews et al., 2004) and leads to gastrointestinal problems characterized by diarrhea, poor growth performance, and in some cases death (Collier et al., 2008; Orengo et al., 2012). Furthermore, infections induced by *Eimeria* spp. can foster opportunistic infections with other pathogens such as bacteria (Collier et al., 2008). Consequently, this pathogen causes massive economic losses worldwide (Shirley et al., 2007; Chapman, 2014).

Eimeria oocysts are relatively resistant to environment conditions, which makes control measures difficult (Stephen et al., 1997). Therefore, disruption of the sporulation process is a critical point where this parasite can be controlled (Mai et al., 2009). In addition, the prevalent prophylactic use of anticoccidial feed additives has led to widespread resistance (Stephen et al., 1997), which has currently been reported against available drugs (Williams, 1999; Chapman, 2014).

Medicinal plants are the major resources of all alternative or indigenous systems of medicine and are considered promising sources for discovery of new chemical compounds (Kalia, 2009). In addition to targeting parasites, these products also have organ-protecting properties in hosts infected with *Eimeria* (Wunderlich et al., 2014). *Morus nigra* (black mulberry) is a perennial woody plant (Pan & Lou, 2008) that belongs to the family Moraceae (Yang et al., 2010). The genus *Morus* is found in warm and temperate regions and subtropical regions of Asia, Africa, North America (Pérez-Gregorio et al., 2011) including the US (Abbasi et al., 2014), and South Europe. The fruits, bark, and leaves of black mulberry are used medicinally as analgesic, antipyretic and anti-diabetic (Rodrigues et al., 2019) and, in particular, the fruits are used against dysentery (Ercisli & Orhan, 2007).

Additionally, the antiparasitic activity of *Morus alba* (Riffat et al., 1986; Nguyen-Pouplin et al., 2007) and anthelmintic activity of *Morus indica* (Mughal et al., 2013) were reported. The present study was conducted to investigate the *in vitro* and *in vivo* effects of *M. nigra* leaf extracts on *Eimeria papillata* oocysts sporulation and viability.

Materials and Methods

Extract preparation of extract

The *M. nigra* leaf extract (MNLE) was prepared using leaves obtained from Riyadh, Saudi Arabia and the botanical identity of the plant was confirmed by a taxonomist at the Department of Botany, University of King Saud. The leaves (100 g) were air-dried at 40 °C, ground into a powder, and then extracted with methanol (70%) for 24 h at 4 °C. The obtained extract was concentrated and dried in a rotary vacuum evaporator (Yamato RE300, Japan) as previously reported by Dkhil et al. (2013). Distilled water was used to dissolve the powder for the various experiments.

Phytochemical analysis

The phytochemical analysis of MNLE was performed according to the recommended protocol of Kanthal et al. (2014). The gas chromatography-mass spectrometry (GC-MS) analysis was performed using a Thermo Scientific, Trace GC Ultra and ISQ single quadrupole MS (Miami, CA, USA).

Oocyst sporulation

Fresh *E. papillata* unsporulated oocysts were originally obtained from Prof. Mehlhorn at Duesseldorf University (Duesseldorf, Germany) and the parasite was maintained by passaging in mice (Dkhil et al., 2011). The unsporulated oocysts (1×10^5) were incubated in 5 mL potassium dichromate containing one of the following: MNLE (100, 200, and 300 mg/mL), 8.3 mg amprolium (Veterinary Agriculture Products Company [VAPCO], Jordan), 109 µL Dettol™, or 25 µL phenol while 5 mL potassium dichromate (2.5%) alone was used as the control. In addition, unsporulated oocysts (1×10^5) were incubated in 5 mL distilled water, ethanol (70%), and formalin (5%). We used three replicates for each treatment and all Petri dishes used incubated for 48 and 90 h at 25-29 °C (Gadelhaq et al., 2018). The sporulated oocysts were counted using a McMaster chamber and the percentage sporulation was calculated using the following equation:

$$\text{Sporulation (\%)} = \left(\text{Number of sporulated oocysts} / \text{total number of oocysts} \right) \times 100 \quad (1)$$

Infection of animals

Adult male C57BL/6 mice (9-11 weeks old) were used. The experiments were approved by the institutional review board of the Princess Nourah Bint Abdulrahman University (IRB Approval Number: 19-0259). Fresh faecal pellets were collected and weighed for each mouse once every 24 h and the bedding was changed to eliminate reinfection. The output of oocysts was calculated as mentioned previously by Schito et al. (1996).

For oocyst flotation, the fecal pellets from each individual mouse were suspended and diluted using 2.5% (w/v) potassium dichromate in saturated sodium chloride (NaCl). Each mouse was inoculated orally with 100 μ L sterile saline solution containing 10^3 *E. papillata* sporulated oocysts. A McMaster chamber was used to count the oocysts, and the results are expressed as the number per gram of wet feces (Dkhil et al., 2011).

Experimental design

The mice were divided into six groups of eight animals each group. The first group (control) was daily gavaged with 100 μ L 0.9% NaCl for 5 days. The second group was daily treated with 100 μ L MNLE (200 mg/kg) by oral gavage, while the third, fourth, fifth, and sixth group were orally infected with 10^3 *E. papillata* oocysts. The last three groups were daily treated for 5 days with 200, 400, and 800 mg/kg of MNLE, respectively.

Sample collection

Fresh feces samples were collected from the mice on day 5 after infection, where each mouse was separated in this day in a small cage to collect its faeces and the oocysts shed per gram of feces was calculated (Schito et al., 1996). Then, all the mice were euthanized and parts of the jejunum were collected and fixed in formalin (10%) for the histological and histochemical examination.

Number of oocysts in jejunum

Pieces of the jejunum were fixed in 10% neutral formalin buffered, dehydrated in ethanol, embedded in paraffin wax, and then cut into 5- μ m thick sections. The sections were stained with hematoxylin and eosin (H&E) (Drury & Wallington, 1980). Oocysts were counted in 10 well-oriented villous-crypt units (VCU) for each animal using Olympus BX61 light microscope (Tokyo, Japan).

Number of goblet cells

The number of goblet cells was determined in Alcian blue-stained sections. The number was expressed as mean goblet cells per 10 VCU (Allen et al., 1986).

Statistical analysis

Analysis of ANOVA was carried out in one way, and statistical comparisons between groups were made using the Duncan method. Values have been expressed as mean \pm SD, at a significance level of ≤ 0.05 . SigmaPlot software (version 11) was used for statistical analysis.

Results

Figure 1 shows the GC-MS chromatogram of MNLE. The phytochemical components identified were 1,3-benzenediamine, 2,4-dinitro-N3, N3-dipropyl-6-(trifluoromethyl)-, β -carotene, gamabufotalin, ricinoleic acid, cholesteryl benzoate, tetradecanoic acid, and methotrexate (Table 1). After a 48 h incubation with MNLE (200 and 300 mg/mL), formalin, or ethanol, the *E. papillata* unsporulated oocysts showed no sporulation. However, oocysts incubated with potassium dichromate (2.5%), MNLE (100 mg/mL), amprolium, and Dettol™ showed different levels of sporulation (Table 2).

Incubation with MNLE (300 mg/mL), formalin, and ethanol for 90 h inhibited sporulation by approximately 100% (Table 2). MNLE (100 and 200 mg/mL), amprolium, Dettol™, and phenol induced average sporulation levels of 90.4%, 31%, 81.1%, 87% and 28%, respectively. On day 5 post-infection, the highest oocyst output was level was $12.1 \pm 6.2 \times 10^5$ oocysts/g of feces in infected mice. Treatment with different doses of MNLE (200, 400, and 800 mg/kg) significantly ($P < 0.01$) reduced the oocyst output to 86.8%, 86%, and 93.8%, respectively (Figure 2). For further

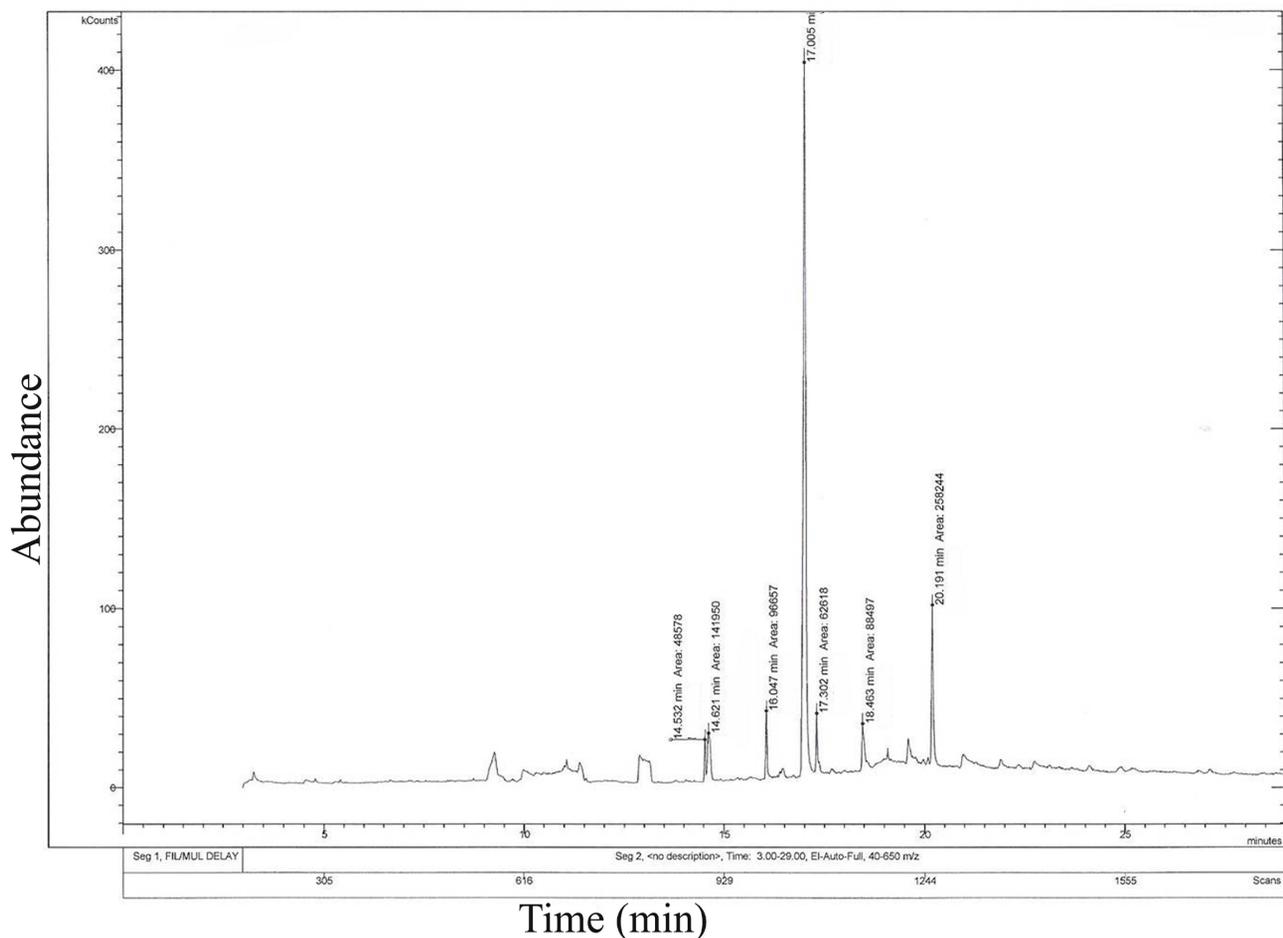


Figure 1. GC-MS Chromatogram of methanolic leaf extract of *Morus nigra*.

Table 1. Identification of phytochemical compounds by GC-Mass in *Morus nigra* leaf extracts. RT: Retention time, M-H: Protonated molecule, MS: Mass acquired range.

RT	Assignment	Molecular formula	[M-H] ⁺ (m/z)	MS (m/z)	Peak area (%)
14.5	1,3-Benzenediamine, 2,4-dinitro-N3, N3-dipropyl-6-(trifluoromethyl)-	C ₁₃ H ₁₇ F ₃ N ₄ O ₄	349	18-321	2.87
14.6	β-Carotene	C ₄₀ H ₅₆	535	43-536	8.4
16.1	Gamabufotalin	C ₂₄ H ₃₄ O ₅	401	43-366	5.7
17.0	Ricinoleic acid	C ₁₈ H ₃₄ O ₃	297	55-166	58.8
17.3	Cholesteryl benzoate	C ₃₄ H ₅₀ O ₂	489	43-368	3.7
18.5	Tetradecanoic acid	C ₂₈ H ₅₆ O ₂	423	41-229	5.23
20.2	Methotrexate	C ₂₀ H ₂₂ N ₈ O ₅	453	41-176	15.3

investigations, we have chosen the dose 200 mg/kg as a lower dose that can help to avoid adverse side effects, drug diversion, and toxicity where there was no significant difference in effect between the other treated groups.

MNLE (200 mg/kg) significantly ($P < 0.01$) decrease the oocyst number in the mouse jejunum by approximately 86% (Figure 3). Finally, examination of Alcian blue-stained sections showed that the infection significantly reduced the number of goblet cells in the jejunum villi (Figure 4). Compared to the infected group, mice treated with 200 mg/kg MNLE showed a significant ($P < 0.01$) increase in the number of goblet cells by approximately 71% (Figure 5).

Table 2. Effect of *Morus nigra* on sporulation of *E. papillata* oocysts. MNLE: *Morus nigra* leaf extracts, P: Probability.

Groups	Time	Unsporulated oocysts (%)	Inhibition of sporulation (%)	P value
Potassium dichromate (2.5%)	48 h	83.03 ± 2.9	0	-
	90 h	5.5 ± 1	0	-
MNLE (100 mg/ml)	48 h	95 ± 1	70.5 ± 1	0.01
	90 h	9.6 ± 1	3.3 ± 1	0.01
MNLE (200 mg/mL)	48 h	100 ± 0.1	100 ± 0.2	0.01
	90 h	69 ± 1	67.2 ± 1	0.01
MNLE (300 mg/mL)	48 h	100 ± 0.2	100 ± 0.2	0.01
	90 h	100 ± 0.2	100 ± 0.2	0.01
Amprolium	48 h	88.1 ± 1	29.7 ± 1	0.01
	90 h	18.9 ± 1	14.1 ± 1	0.01
Formalin	48 h	100 ± 0.2	100 ± 0.2	0.01
	90 h	100 ± 0.1	100 ± 0.2	0.01
Dettol™	48 h	99 ± 0.9	94.1 ± 0.9	0.01
	90 h	13 ± 1	7.9 ± 1	0.01
Ethanol	48 h	100 ± 0.2	100 ± 0.2	0.01
	90 h	100 ± 0.2	100 ± 0.2	0.01
Phenol	48 h	100 ± 0.2	100 ± 0.2	0.01
	90 h	72 ± 1	70. ± 1	0.01

Values were expressed as mean ± SD.

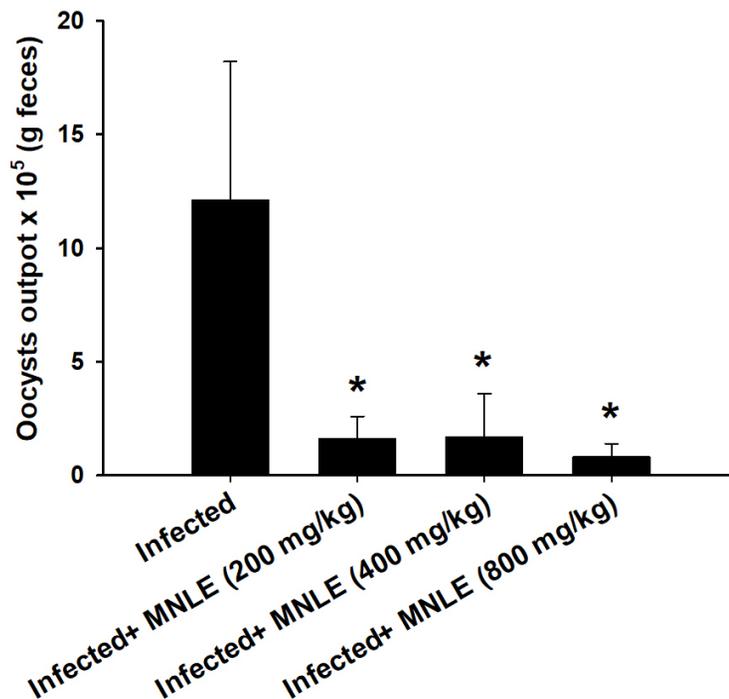


Figure 2. Effect of *M. nigra* extract on oocyst output on day 5 postinfection with *E. papillata* oocysts. *Significance against the infected group (P < 0.01).

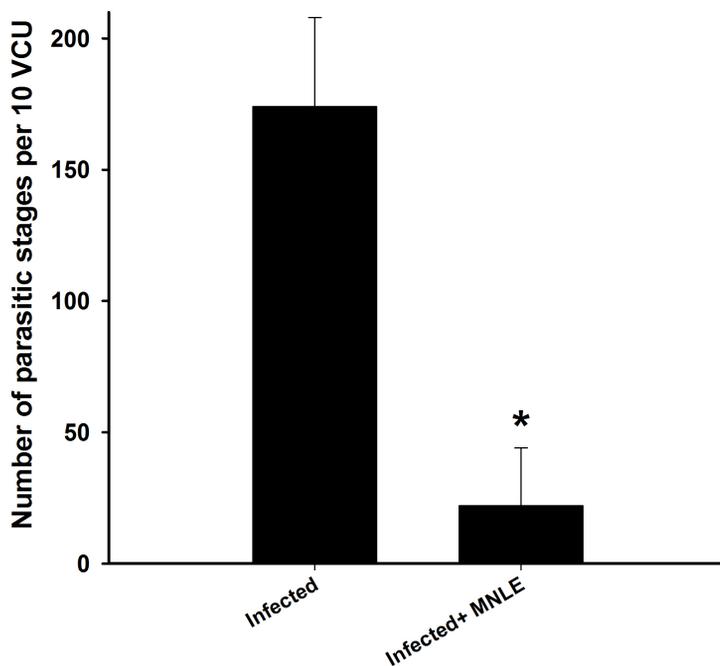


Figure 3. Effect of *M. nigra* extract on the number of parasitic stages in ten well-orientated villous-crypt units (VCU) for each mouse, on the fifth day of infection with *E. papillata*. *Significant at $p < 0.01$. MNLE: *Morus nigra* leaf extracts.

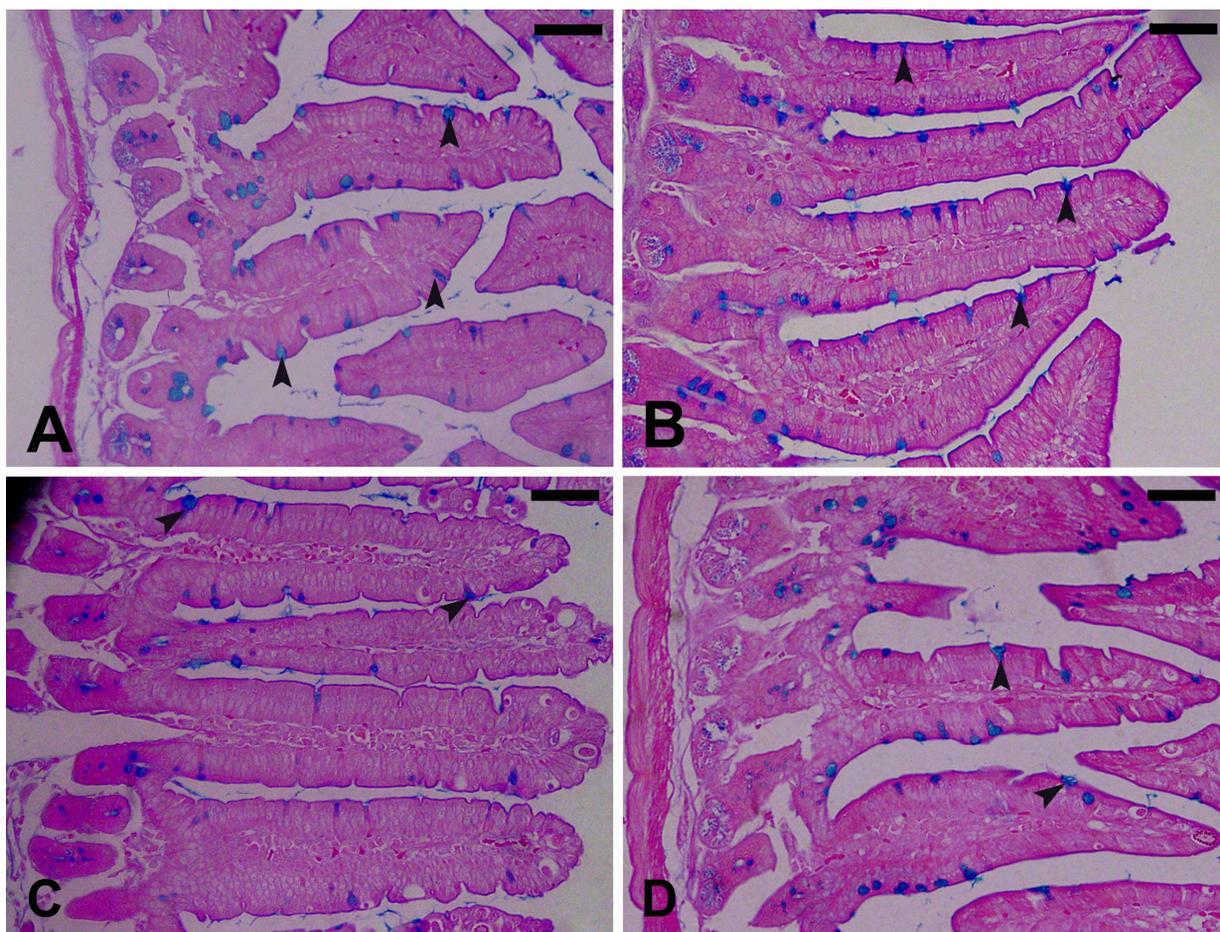


Figure 4. Effect of *M. nigra* extract on mice jejunal goblet cells (arrow head) infected with *E. papillata*. (A) non-infected control group (B) MNLE treated group (C) *E. papillata* infected group with decreased number of goblet cells (D) infected-MNLE treated group. Sections were stained with Alcian blue. Bar = 50 μ m. MNLE: *Morus nigra* leaf extracts.

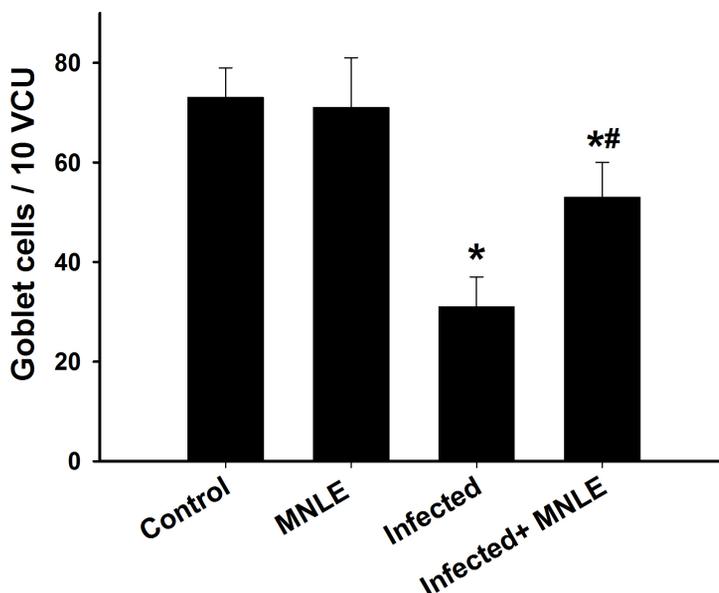


Figure 5. Effect of *M. nigra* extract on the number of goblet cells on day 5 p.i. with *E. papillata*. *Significance against non-infected control group ($p \leq 0.01$). #Significance against *E. papillata*-infected group ($p < 0.01$). MNLE: *Morus nigra* leaf extracts.

Discussion

Eimeriosis affects most animal species, causing considerable economic loss in many countries (Andrews et al., 2004). *E. papillata* infections occur when sporozoites of ingested sporulated oocysts are released, invade the jejunal epithelial cells, and rapidly multiply before oocysts are formed. Within the oocysts, outside the host sporogony occurs and they become infectious (Pakandl, 2005). Drug resistant eimeriosis has been reported (Williams, 1999; Chapman, 2014) and, therefore, research into the use natural of products as antiparasitic agents has been the focus of attention because these products are more effective, less harmful, and exhibit fewer less side effects than conventional chemical agents (Wunderlich et al., 2014).

Several studies have reported the effects of mulberry in treating many diseases (Ody, 2000; Naderi et al., 2004; Wang et al., 2009) and its potential effectiveness as an antiparasitic agent (Riffat et al., 1986; Ercisli & Orhan 2007; Nguyen-Pouplin et al., 2007). In this study, MNLE (300 mg/mL) affected the oocysts sporulation, which is attributable to the presence of numerous bioactive phytochemical constituents (Sharma et al., 2010). In addition, our data showed that formalin (5%) as a hazard chemical completely inhibited sporulation of *E. papillata* and Gadelhaq et al. (2018) reported that formalin (10%) completely inhibited sporulation of *Eimeria tenella*. In addition, Chroustová & Pinka (1987) reported that formalin (2%) significantly affected sporulation of *E. tenella* oocyst. Formalin contains a highly reactive chemical (40% formaldehyde in water) (Power, 1995) that interacts with proteins *in vitro* (Fraenkel-Conrat et al., 1945) to inhibit sporulation.

Ethanol (70%) inhibited sporulation and oocyst wall deterioration, considering that the most potent concentration of antimicrobial alcohol is between 60 to 90% (McDonnell & Russell, 1999). Ethanol causes rapid denaturation of proteins, disrupts membranes, and causes subsequent interference with metabolism and cell lysis (Morton, 1983; Larson & Morton, 1991). In addition, phenol (10%) has been reported to inhibit sporulation (Samaha et al., 2013) whereas, in contrast, Dettol™ had no effect on oocyst sporulation. These findings may be because the oocyst wall is impermeable to water-soluble substances and resistant to proteolysis (Kuticic & Wikerhauser, 1996; Mai et al., 2009).

Jejunal infection with *E. papillata* in mice results in oxidative damage and serious local and systemic inflammatory responses (Dkhil et al., 2013). *M. nigra* is a plant that possesses anti-inflammatory (Yildirim et al., 2019), antioxidant (Lee et al., 2018), and antiparasitic (Ercisli & Orhan, 2007) activity. The leaves of black mulberry contain flavonoids, ascorbic, and phenolics (Iqbal et al., 2012; Chen et al., 2016). MNLE showed anticoccidial activity following treatment of mice, as evidenced by a significant reduction in the production of *E. papillata* oocysts in infected mouse feces and oocysts in the jejunal villi. These results are in agreement with those of other studies that investigated the *Punica granatum* plant (Amer et al., 2015) and *Azadirachta indica* (Dkhil et al., 2013) as potential sources of anticoccidial agents.

Parasitic infections cause a decrease in the number of goblet cells, which are known to be significant immunocompetent intestinal cells that secrete mucus (Linh et al., 2009). These cells may be reduced by the parasite-induced damage to stem cells (Cheng, 1974). A decrease in the number of goblet cells encourages an increase in opportunistic pathogens or their penetration of the local epithelium (Yunus et al., 2005). MNLE significantly increased the number of goblet cells, which was likely mediated by the numerous bioactive phytochemical constituents present in mulberry leaves (Sharma et al., 2010).

Different plant or herbal extracts are widely used as poultry diets to promote growth rates and animal health, particularly when health challenges are required. Quite enough research has confirmed the beneficial effects of plant extract on poultry productivity (Alçiçek et al., 2004; Gracia et al., 2016). Due to the anticoccidial activity of MNLE, we assumed that it could be used as a food additive in poultry feed.

Our results indicate that MNLE possessed a powerful antiparasitic and anti-sporulation activity. Additional studies are needed to elucidate the histological and molecular mechanism of sporulation inhibition by MNLE and its protective effects against *E. Papillata*-induced intestinal injury.

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