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Original article

Rumex nervosus leaf extracts enhance the regulation of goblet cells and the inflammatory response during infection of chickens with *Eimeria tenella*

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ABSTRACT

Eimerian parasites are the main intestinal tract pathogens in many animals, which invade and damage the intestinal epithelium causing severe injuries and economic loss. Our study was planned to examine the ameliorative effect of *Rumex nervosus* leaf extracts (RNE) against *Eimeria tenella*-induced changes in caecal goblet cells and cytokines of chickens. The infected chickens with *E. tenella* were treated with 50, 100, 200 mg/Kg RNE, respectively. Amprolium was used as a reference drug. Our result showed that RNE contained 8 phytochemical components that were able to decrease the number of oocysts in bird's faeces. Also, the number of goblet cells was decreased after infection. This number was increased after RNE treatment. In addition, RNE caused upregulation of the mucin gene, MUC2 after *E. tenella* infection. Moreover, the infection caused upregulation in the inflammatory cytokines IL1β, IL 6, INF-γ and LiTAF. This increase in mRNA expression of IL1β, IL 6, INF-γ and LiTAF was about 5, 5.6, 4 and 5.8 fold, respectively. Collectively, *R. nervosus* is a promising medicinal plant with anticoccidial, and anti-inflammatory properties and could be used for the treatment of eimeriosis.

1. Introduction

In recent decades, the demand for poultry meat has increased greatly by consumers especially broiler chickens, thus many countries use intensive breeding systems for increasing the level of production, where over 50 billion chickens are produced annually in allover world (Quiroz-Castañeda and Dantán-González, 2015). However, the huge production leads to increase of diseases either microbial or parasitic.

Coccidia is a renowned parasitic and infectious disease, caused by the eimerian parasites which represent the principal pathogens of the intestinal tract in poultry and many other domestic animals, caused major economic losses (Long, 1990; Yunus et al., 2007; Mehlhorn, 2014). Among poultry, Eimeria parasites infect numerous species-specific sites in the intestine, triggering intestinal coccidia except for *Eimeria tenella*, which invades caeca cells. (Daugschies and Najdrowski, 2005; Chapman et al., 2010; Blake and Tomley, 2014). *E. tenella* is the most dangerous chicken *Eimeria sp* because it is very virulent and leading to severe hemorrhage and very high mortality (Fossum et al., 2009).

The goblet cells represent an immunological defense line in the intestinal tract; it is responsible of secreting mucus, so when *E. tenella* invade the caecal epithelial cells, it increase the rate of mucus production and changes the constituents to protect the epithelium as immunological reaction against the parasite (Sharma and Schumacher, 1995; Meslin et al., 1999). In addition, immune cells trigger and generate cytokines that are proteins or peptides such as interferons (IFN), tumor necrosis factors (TNF), interleukins (IL), transforming growth factors (TGF) once infection occurs for chickens. (Wigley and Kaiser, 2003).

Notwithstanding effective control of avian coccidiosis with various anticoccidial chemical drugs (Sharma and Ranjan, 2015) such as Amprolium, Diclazoril, Monincine and others, the incidence of

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drug resistance from parasites and high costs as well as consumers prefer residue-free meats have prompted researchers to propose and establish alternative control programs (Chapman et al., 2010; Abbas et al., 2011; Wunderlich et al., 2014).

Today, researchers consider the medicinal plants (e.g., garlic, neem, moringa, pomegranate, and others) and their derivatives to be the main point of interest. They obtain bioactive phytochemical compounds against the induced eimeriosis and also innocuous of the consumers for chicken's meat (Abbas et al., 2012; Wunderlich et al., 2014; Bauri et al., 2015; Muthamilselvan et al., 2016; Thagfan et al., 2017).

Rumex nervosus is a medicinal herb that belongs to the Polygonaceae family and has pharmacological and biological properties (Alwashli et al., 2012), used as a traditional therapy for many diseases, and functions as an antiparasitic (Awadh Ali et al., 2016). However, it is not yet tested as anticoccidial therapy in broiler chickens. Therefore, in this study, we assessed the efficacy of *R. nervosus* leaf extracts (RNE) as anticoccidial and anti-inflammatory agent in *E. tenella*- experimentally infected broiler chickens.

2. Material and methods

2.1. Collection, extract preparation and gas chromatography-mass spectrometry (GC–MS) analysis of R. nervosus

Fresh leaves of *R. nervosus* were harvested during the fully developed green stage of the plant from Taif region, Saudi Arabia. The plant was authenticated at the herbarium in Department of Botany, Faculty of Science, King Saud University. The leaves were air-dried, and milled into powder by using an electric blender then cold extracted in 70% methanol with mixing continuously by shaker for 24 h (Amer et al., 2015). The dissolved contents were filtered and evaporated at 50 °C in rotary evaporator. The solvent was evaporated to obtain dark hydro-alcohol extractions (Chikoto and Eloff, 2005), and to obtain liquid extract again, it was dissolved in dimethyl sulfoxide (DMSO) (Al Yahya et al., 2018) for inoculating chickens.

Thermo Scientific, Trace GC Ultra and ISQ Single Quadruple MS were used to analyze the extract of *R. nervosus*. The used flow rate was 1.5 mL/min. The identification of mass spectrum was conducted using the database of the Wiley9, replib, and National Institute Standard and Technology.

2.2. Infection of birds

Oocysts of *E. tenella* strain were collected from the cecum of naturally infected chickens. Identification of the species was based on the morphological characteristics described by Thienpont et al. (1979), as well as the site of lesions. The collected oocysts were sporulated (using 2.5% potassium dichromate at 25 °C 72 h), washed with distilled water, and passaged from two to three times in healthy chickens to confirm the site of lesions. After the confirmation of the parasite species, oocysts were also collected, sporulated, washed and dose was adjusted to 1×10^4/100 μL/bird.

3. Experimental design

Seventy broiler chicks of one-day-old were purchased from a commercial hatchery. They were bred until reaching 21 days' age at the animal facility of Zoology Department, King Saud University. Then, all chickens were individually weighted and allocated into 7 groups (10 birds per group). The first two groups as are the non-infected and the non-infected-treated group with 200 mg/kg RNE, respectively. The third to the seventh groups were infected with 10,000 *E. tenella* oocysts (Lee et al., 2012). Groups 4, 5 and 6 were treated after infection with 50, 100, 200 mg/Kg RNE, respectively. The seventh group was treated with 25 mg/Kg Amprolium 60% (Veterinary and Agricultural Products Company (VAPCO), Jordan) (EMEA, 2001). The treated groups were medicated orally and daily with the extract until day 6 post infection (Wang et al., 2016).

3.1. Counting of goblet cells

In order to obtain cecum samples, 5 birds of each group were slaughtered on day 6 post infection with *E. tenella*. Small parts of cecum were freshly prepared and fixed in 10% neutral buffered formalin. Sections were stained with Alcian blue for counting of the caecal goblet cells. For each chicken, the number of goblet cells in the cecum was counted on at least ten well-orientated villous-crypt units (VCU). Results were expressed as the mean number of goblet cells per ten VCU (Allen et al., 1986).

3.2. Quantitative real-time PCR

Total RNA from caecum tissue was extracted by using Trizol reagent and then converted to cDNA using RevertAid™ H Minus Reverse Transcriptase (Fermentas, Thermo Fisher Scientific Inc., Canada) in accordance with the manufacturer's instructions. For gene expression analysis, quantitative real-time PCR was employed by using QuantiFast SYBR Green RT-PCR kit (Qiagen, Hilden, Germany). Sense and antisense primers were obtained from Sigma-Aldrich and are listed in Table S1. All reactions were performed in duplicate by using ViiA™ 7 System (Thermo Fisher Scientific, CA, USA). The PCR cycling conditions were set as follows: initial denaturation at 95 °C for 2 min, followed by 45 cycles of denaturation at 94 °C for 60 s, annealing at 60 °C for 20 s, and extension at 70 °C for 20 s, with a final extension at 70 °C for 10 min. The relative differences in gene expression between different groups were determined by using Ct method ($2^{-\Delta\Delta ct}$) (Livak and Schmittgen, 2001). Glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*) was used as the reference gene.

3.3. Statistical analysis

All obtained data were analyzed using computerized statistical program (SPSS version 17.0). Statistical comparison among the studied groups was carried out using one-way analysis of variance (ANOVA). The significant levels for means were tested by Duncan's *t*-test when $P \leq 0.05$.

4. Results

The phytochemical analysis carried out by GC-Mass (Fig. 1) showed that RNE contained 8 phytochemical components (Table S2) at different peak areas and retention time.

RNE was able to suppress the shedded oocysts by about 72% when treating the *E. tenella* infected chickens with 200 mg/kg (Fig. 2), while in the group treated with the reference drug Amprolium, the oocyst output was still high and reached $35.77 \pm 8.69 \times 10^6$/g faeces.

Our results concerning the change in goblet cells number (Fig. 3) revealed that the infection of animals with *E. tenella* induced a decrease in the number of goblet cells (26.72 ± 2.27/10 VCU) when compared to the non-infected control chickens (60.60 ± 1.60). Treatment of the birds with RNE significantly increased ($P \leq 0.05$) the number of goblet cells (53.90 ± 2.81) more than when the chickens treated with Amprolium (41.89 ± 3.70/10 VCU) (Fig. 4). These data were obtained from the examination of the caecum sections stained with alacian blue (Fig. 3). The goblet cells were arranged in the villi of chicken caecum.

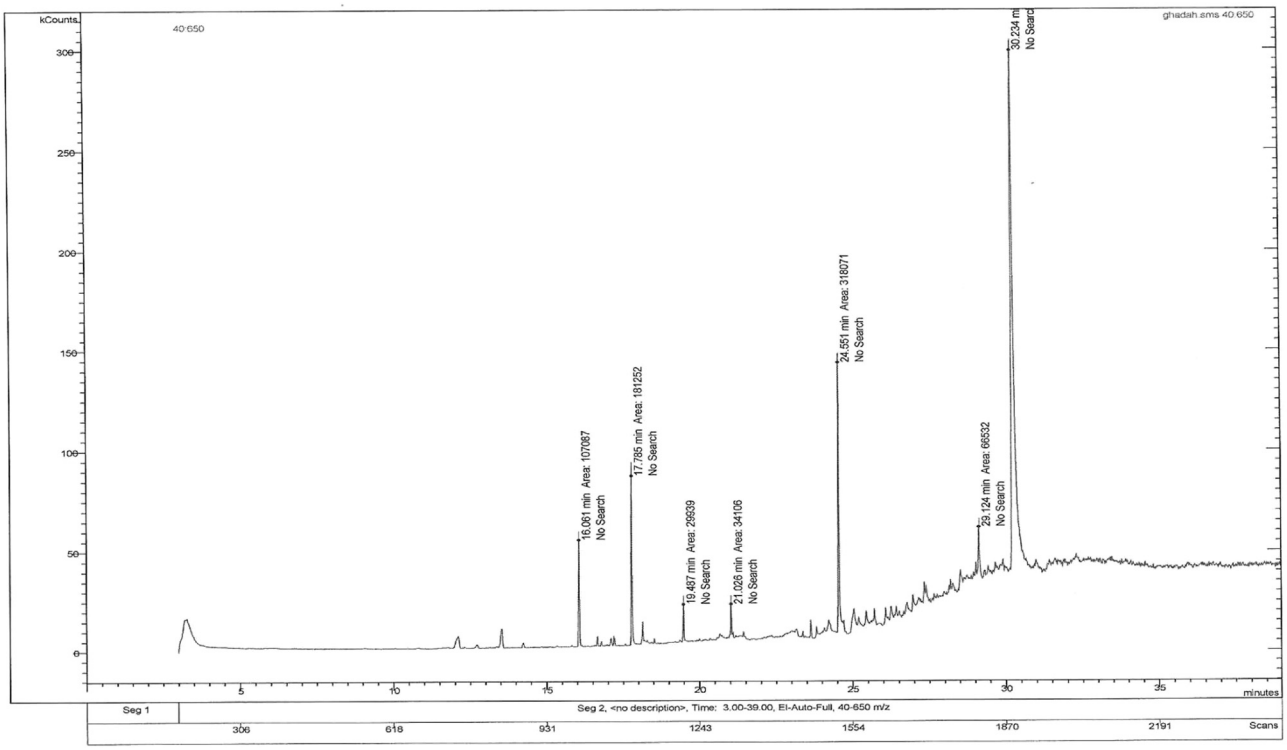

Fig. 1. GC–MS chromatogram for phytochemical compounds of *Rumex nervosous* leaf extracts.

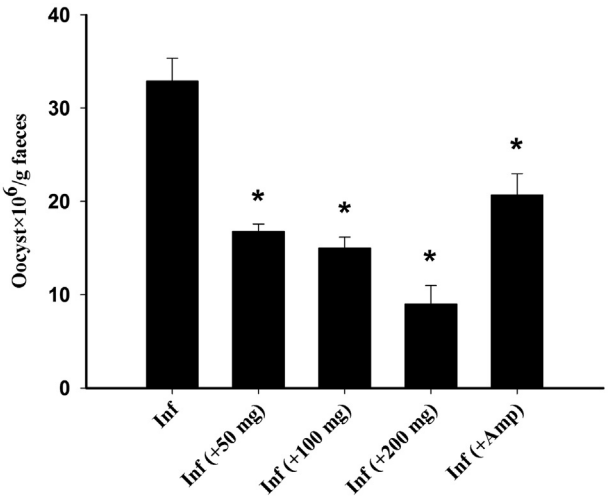

Fig. 2. RNE treatment induced changes in the number of *E. tenella* oocysts. Different doses (50, 100 and 200 mg/kg) of RNE indicated changes in oocysts output in chickens' faeces. Values are expressed as means ± SEM. *p < 0.05 with respect to the infected group.

Fig. 5 showed that the mucin gene, Muc2 was downregulated by the *E. tenella* infection. This downregulation is about one fold compared to the control group. RNE could upregulate the expression of Muc2 in the infected-treated group nearly similar to that induced by the reference drug, Amprolium (Fig. 5).

The infection caused upregulation in the inflammatory cytokines IL1β, IL 6, INF-γ and LiTAF (Fig. 6). This increase in mRNA expression of IL1β, IL 6, INF-γ and LiTAF was about 5, 5.6, 4 and 5.8 fold, respectively when compared to the non-infected control animals (Fig. 5). RNE could significantly downregulate the expression of these genes nearly similar to Amprolium (Fig. 6).

5. Discussion

The infection of bird with *E. tenella* began with the oral uptake of the sporulated oocysts that invade the intestinal caecum resulting in destruction of the caecal epithelium due to the multiplication of *E. tenella* stages and finally the developed oocysts were released in faeces (Mehlhorn, 2014).

Researchers are seeking to find multiple prevention methods to monitor the effects of eimeriosis, this is to reduce the millions of dollars paid to overcome the induced damage in poultry industry (Ott et al., 2018). The consequences of damage vary from local, degeneration of the intestinal tissue to death in more severe cases (Vermeulen et al., 2001).

To ovoid adverse effects on animal performance, there's a need to develop new agents with low cost and reduced side effects against eimeriosis. Through this study, RNE was able to control the increased oocysts output in chicken's faeces. This may be due to the presence of some active components in RNE acting against eimeriosis. Previous studies reported the effective role of another herbal extracts like *Curcuma longa* (Abbas et al., 2010), *Azadirachta indica*, *Nicotiana tabacum*, *Calotropis procera* and *Trachyspermum ammi* (Zaman et al., 2012), *Piper sarmentosum* (Wang et al., 2016) against coccidiosis.

RNE contained some active phytochemical compounds. One of these compounds is isophorone that reported to possess biological activity against a variety of microorganisms, and also had antioxidant properties (Kiran et al., 2013). Also, the phenolic compound, eugenol present in RNE was found to have antioxidant and antiapoptotic properties (Pandey and Mishra, 2004). Moreover, Sutili et al. (2014) and El-kady et al. (2019) confirmed the antiparasitic activity of eugenol. Interestingly, the anticoccidial activity of eugenol was reported by Remmal et al. (2013). The phytochemical compound, 2,5-Cyclohexadiene-1,4-dione, 2,6-bis(1,1-dimethylethyl)- present in RNE is related to Benzoquinones that possess potent pharmacological properties against inflammatory

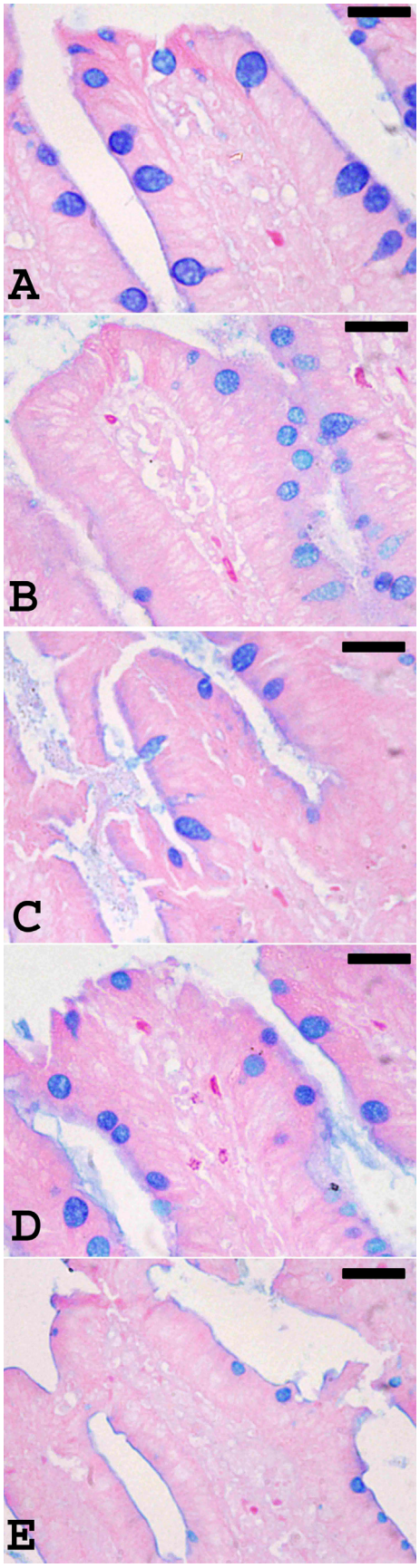

Fig. 3. Alcian blue staining of caecum sections showing goblet cells in control chickens (A), RNE-treated chickens (B), *E. tenella*-infected chickens (C), and infected chickens treated with 200 mg/Kg RNE (D) and Amprolium (E). Scale bar = 50 μm.

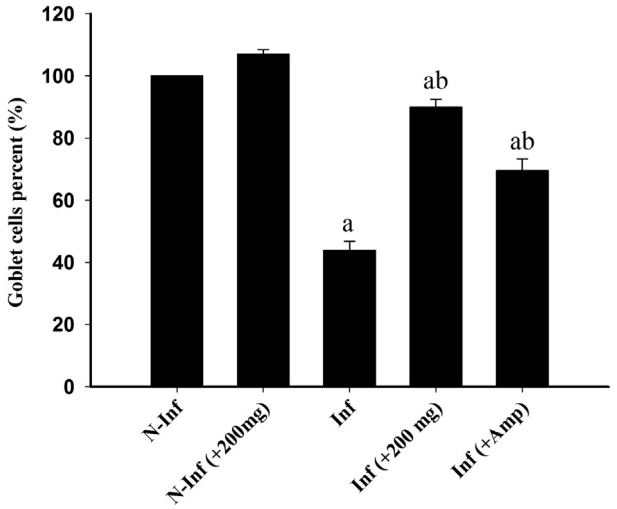

Fig. 4. Changes in the number of caecal goblet cells in control chickens (A), RNE-treated chickens (B), *E. tenella*-infected chickens (C), and infected chickens treated with 200 mg/Kg RNE (D) and Amprolium (E). Values are means ± SEM. a: $p < 0.05$, significant change with respect to control group; b: $p < 0.05$, significant change with respect to infected group.

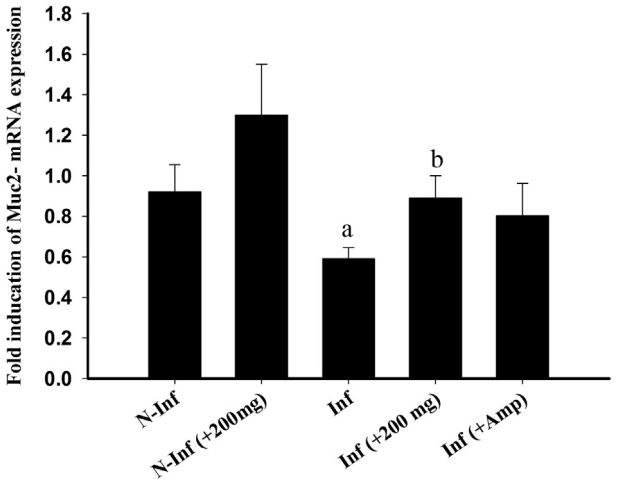

Fig. 5. Effect of RNE on the mRNA expression of *MUC2* in the caecum samples from *E. tenella*-infected chickens. The expression values obtained by RT-PCR analysis were normalized to the *GAPDH* mRNA level and are shown as fold induction (in log 2 scale) relative to the mRNA level in the control. a: $P < 0.01$, significant change with respect to the control group; b: $P < 0.01$, significant change with respect to the infected group.

and microbial diseases (Pangal et al., 2013). Retinal is also called 9-*cis*-Vitamin A aldehyde, this compound could regulate the intestinal mucosal immune response (Sun et al., 2007).

Animal infected with Eimeria show a significant weight loss, due to the poor nutrient absorption and decreased immune response resulting in intestinal tissue damage (Chapman et al., 2010).

In this study, the caecal villi were damaged and also the goblet cells affected. The decrease in goblet cells number due to infection was previously studied not only in infected chicken with *E. tenella* (Yunus et al., 2007) but also in rodents infected with *Eimeria*. Dkhil et al. (2013) reported that the reason of the decreased goblet cells on mice jejunum infected with *E. papillata* is that the parasite destroys the stem cell producing goblet cells leading to the reduced number in the intestinal villi (Cheng, 1974).

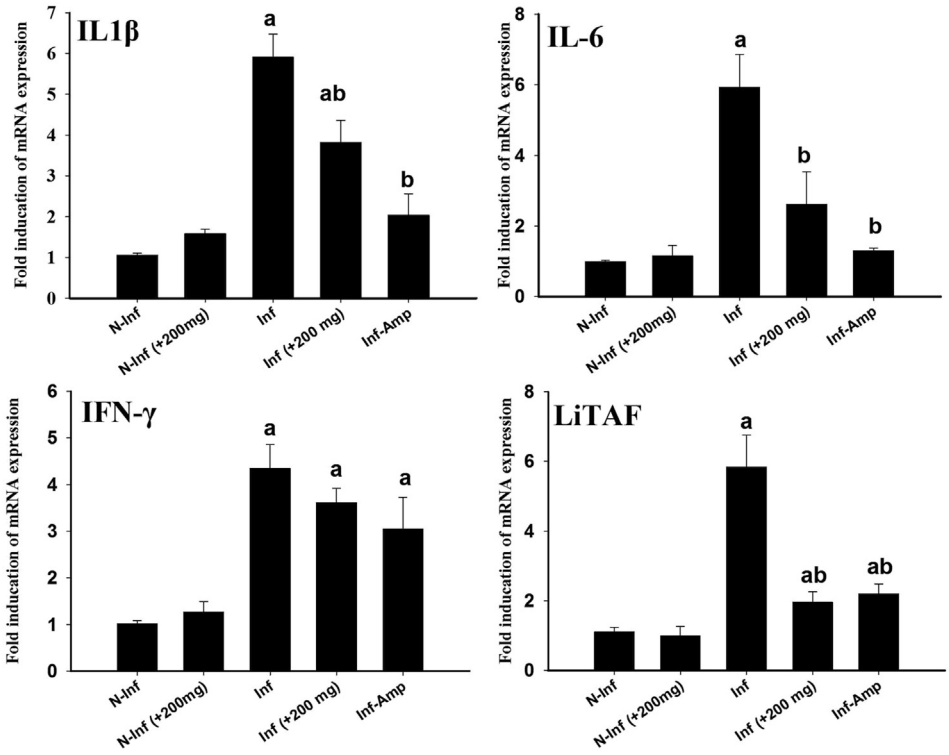

Fig. 6. Effect of RNE on the mRNA expression of *IL1β*, *IL 6*, *INF-γ* and *LiTAF* in the caecum samples from *E. tenella*-infected chickens. The expression values obtained by RT-PCR analysis were normalized to the *GAPDH* mRNA level and are shown as fold induction (in log 2 scale) relative to the mRNA level in the control. a: $P < 0.01$, significant change with respect to the control group; b: $P < 0.01$, significant change with respect to the infected group.

Due to eimeriosis there was a disturbance in the population of some immune cell like lymphocytes, mast cells, goblet cells and macrophages (Huntley et al., 1985; Vervelde et al., 1996).

E. tenella infection induces tissue inflammation due to the invasion of the parasite to the caecal epithelium. These inflammations increase the expression of the inflammatory cytokines (Laurent et al., 2001; Hong et al., 2006). These cytokines were produced by the leucocytes as a regulatory response (Hong et al., 2006).

Chickens infected with *E. tenella* develop strong immune response through T-lymphocytes (Hong et al., 2006). Cytokines are considered to be one of the major molecules controlling the host response to poultry infection with *Eimeria* (Wang et al., 2016). Hong et al. (2006) reported that upregulation of cytokine mRNA levels after *E. tenella* infection. In this study, IL 1β was upregulated after infection. It played a critical role in initiating inflammation (Laurent et al., 2001; Dalloul et al., 2006). Also, our data showed that there was upregulation of IFN-γ after *E. tenella* infection. This result was supported by the previous observations carried out by Laurent et al. (2001).

Similarly, for homeostasis the proinflammatory cytokine, IL-6 was upregulated during infection (Lynagh et al., 2000). Moreover, LiTAF mRNA expression was increased due to the induced eimeriosis by *E. tenella*. This lipopolysaccharide induced TNF- γ factor was also found to be more expressed during *Eimeria* infection (Hong et al., 2006).

Collectively, *R. nervosus* is a promising medicinal plant with anticoccidial, and anti-inflammatory properties and could be used for the treatment of eimeriosis as a food additive but further studies are required to understand the metabolic activity and the mechanism of action of each phytochemical component during the infection.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jksus.2020.01.024.

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