

# *Salvadora persica* protects mouse intestine from eimeriosis

## *Salvadora persica* protege o intestino do rato da eimeriose

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### Abstract

Eimeriosis is a global poultry health problem. In the current study, we investigated the role of *Salvadora persica* leaf extracts (SE) against murine eimeriosis induced by *Eimeria papillata*. The infection induced an oocyst output of  $6242 \pm 731$  oocysts/g feces. After treatment with 300 mg/kg SE, the oocysts expelled in feces decreased by approximately 3-fold. In addition, the total number of *E. papillata* in the parasitic stage decreased in the jejunum of mice after treatment with SE. In addition, SE significantly reduced the number of apoptotic cells by approximately 2-fold in the infected jejunum. SE ameliorated the changes in glutathione, malondialdehyde, and catalase due to *E. papillata* infection. Finally, SE regulated the cytokine genes, interleukin (IL)-1 $\beta$ , IL-6, interferon- $\gamma$ , and tumor necrosis factor- $\alpha$ , and the apoptotic genes, B-cell lymphoma-2, Bax, and Caspase-3. SE protects the jejunum from *E. papillata* induced injury and may have potential therapeutic value as a food additive during eimeriosis.

**Keywords:** *Salvadora persica*, jejunum, apoptosis, oxidative stress, Eimeriosis.

### Resumo

A eimeriose é um problema global de saúde avícola. No presente estudo, investigou-se o papel dos extratos de folhas de *Salvadora persica* (SE) contra a eimeriose murina induzida por *Eimeria papillata*. A infecção induziu uma produção de oocistos de  $6242 \pm 731$  oocistos/g de fezes. Após o tratamento com 300 mg/kg SE, os oocistos eliminados nas fezes diminuíram em aproximadamente 3 vezes. Além disso, o número total de *E. papillata* no estágio parasitário diminuiu nos jejunos de camundongos após o tratamento com SE. Da mesma forma, o SE reduziu significativamente o número de células apoptóticas em aproximadamente 2 vezes no jejuno infectado. O estudo mostrou que o SE melhorou as alterações na glutatona, malonaldeído e catalase devido à infecção por *E. papillata*. Finalmente, o SE regulou os genes das citocinas, interleucina (IL) -1 $\beta$ , IL-6, interferon- $\gamma$  e fator de necrose tumoral  $\alpha$ , e os genes apoptóticos, linfoma-2, Bax e Caspase-3. Assim, o SE protegeu os jejunos das lesões induzidas por *E. papillata* e pode ter potencial valor terapêutico como aditivo alimentar durante a eimeriose.

**Palavras-chave:** *Salvadora persica*, jejuno, apoptose, estresse oxidativo, Eimeriose.

## Introduction

Eimeriosis is a dangerous parasitic disease caused by the infection of animals with a protozoan parasite belonging to genus *Eimeria* (MEHLHORN, 2014). It causes severe problems in poultry and livestock that can cause huge economic losses (LONG, 1990). Although the drugs used against eimerioses like toltrazuril, monensin and paromomycin are effective, they cause some side effects to the infected host and resistance to the drugs can develop (WUNDERLICH et al., 2014). Natural sources

such as pomegranate, neem, and garlic have been evaluated as an alternative control to eimeriosis (WUNDERLICH et al., 2014). These agents are more effective and less harmful because they cause negligible side effects, exhibit low mammalian toxicity, and can be handled easily (WUNDERLICH et al., 2014; DESHPANDE et al., 2011).

*Salvadora persica* is a traditional medicinal plant used in Islamic countries for oral hygiene. It belongs to the family Salvadoraceae and is commonly termed the Meswak tree. The evergreen desert shrub is found in different regions of the world including the Middle East and Africa. Its roots and twigs have been used for teeth

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cleaning since ancient times (ELVIN-LEWIS, 1980; EID et al., 1990; SHER et al., 2010).

The medicinal properties attributed to *S. persica* include anti-microbial, anti-plaque, analgesic, anti-inflammatory, diuretic, alexiteric, anti-pyretic, aphrodisiac, astringent, and bitter stomach therapeutic activities (GALLETTI et al., 1993; DARMANI et al., 2006). The antihelmintic activity of aqueous and alcoholic root extract of *S. persica* has been evaluated (ABDUL MAJEED, 2011), against *Pheretima posthuma*. Recently, Dkhil et al. (2019) studied the antihelmintic and anticoccidial activities of *S. persica* root extracts in mice and reported the excellent modulation of oxidative damage induced by *E. papillata* infection in mice jejunum.

Most of the previous studies focused only on the root extract. In this study, *S. persica* leaf extract (SE) was used as an anti-eimerial, antioxidant, and anti-apoptotic agent in male C57Bl/6 mice.

## Materials and Methods

### Leaf extraction and gas chromatography-mass spectrometry analysis

Fresh leaves of *S. persica* were collected from Jazan Region, Saudi Arabia. The leaves were authenticated at the herbarium of the Botany Department, King Saud University. A methanolic extract was prepared as described previously (AMER et al., 2015). Briefly, the leaves were dried, ground to a powder using an electronic blender, and extracted with 70% methanol. The extract obtained was dissolved in water as a preparation to inoculate mice. Trace GC Ultra and ISQ Single Quadrupole mass spectrometry (Thermo Fisher Scientific, Waltham, MA, USA) were used to analyze SE as previously described (HUANG et al., 2012). The flow rate was 1.5 mL/min. Identification of mass spectra was conducted using the Wiley 9, replib, and National Institute Standard and Technology databases.

### Animals

Adult male C57Bl/6 mice (9-12 weeks old) were obtained from the animal facility of King Faisal Specialist Hospital Research Center. Mice were fed a standard diet and water *ad libitum*. The experiments (project number 198) were approved by state authorities and Saudi Arabian rules for animal protection were observed.

### Infection of mice

Unsporulated oocysts of *E. papillata* were collected from feces of mice after several passages. This strain was kindly provided by Prof. Heinz (Heinrich Heine University, Germany). Oocyst sporulation was carried out in potassium dichromate. The sporulated oocysts were washed with sterile saline (ABDEL MONEIM, 2013). Using an epigastric tube, mice were orally gavaged with 1000 sporulated oocysts (AMER et al., 2015). The mice were allocated to five groups (n=5 per group). The first group was the non-infected control group. They received 100  $\mu$ L saline daily for 5 days. Mice

in the remaining groups were infected with 1000 sporulated *E. papillata* oocysts. One hour later, group three, four, and five began daily treatment with 300, 600 and 900 mg/kg of SE for 5 days. On day 5 postinfection with *E. papillata* sporulated oocysts, feces were collected and the oocyst output was measured as previously described (SCHITO et al., 1996). The oocyst number was expressed per gram feces.

### Parasitic stages

Tissue paraffin sections were prepared as previously described (DRURY & WALLINGTON, 1980). To differentiate the different parasitic stages in the jejunum of mice, the sections were stained with hematoxylin and eosin then enumerated for meronts, gamonts, and developing oocysts in the infected and infected-treated groups. Values were expressed in 10 VCU.

### Apoptotic changes by the TUNEL assay

The terminal deoxynucleotidyl transferase mediated deoxyuridine triphosphate "dUTP" nick end labeling assay (TUNEL) was performed in mice jejunum using the TUNEL Apoptosis Detection Kit (GenScript, Piscataway, NJ, USA) according to the manufacturer's protocol. The number of TUNEL positive cells was counted as previously described (DKHIL et al., 2013; DKHIL, 2013).

### Oxidative status in the jejunum

The jejunum from all the groups of mice were weighed and then immediately homogenized in phosphate buffer then centrifuged at 1000 $\times$ g for 10 min at 4°C. The supernatant was collected and 10% of the volume was used for the assays. GSH and MDA levels were determined in the jejunum according to Ellman (1959) and Satoh (1978), respectively. In addition, catalase activity was estimated in mice jejunum as previously described (AEBI, 1984).

### Gene expression

RNA was isolated from frozen jejunum samples following the standard RNA extraction protocol with Trizol (Peqlab Biotechnology, Erlangen, Germany) (DELIC et al., 2010). Using the DNA-free TM Kit (Applied Biosystems, Darmstadt, Germany), all RNA samples were reverse transcribed into cDNA following the manufacturer's protocol using the QuantiTect™ Reverse Transcription Kit (Qiagen, Hilden, Germany). Amplifications were performed using QuantiTect™ SYBR® Green PCR kit (Qiagen) and gene-specific QuantiTect™ primers (Qiagen) according to the manufacturer's instructions. Relative quantitative evaluation of the amplification data was done using Taqman75000 system (Applied Biosystems) normalized to 18S rRNA. The following primers were purchased from Qiagen: IL-1 $\beta$  (Mm\_Il1b\_2\_SG, Cat. No. QT01048355), IL-6 (Mm\_Il6\_1\_SG, Cat. No. QT00138663), INF- $\gamma$  (Mm\_Iifng\_1\_SG, Cat. No. QT01038821), TNF- $\alpha$  (Mm\_Tnf\_1\_SG, Cat. No. QT00104006), Bax (Mm\_Bax\_1\_SG, Cat. No. QT00102536), Bcl2 (Mm\_Bcl2\_3\_SG, Cat. No. QT00156282), and caspase-3

(Mm\_Casp3\_1\_SG, Cat. No. QT00260169). The  $2^{-\Delta\Delta CT}$  method was used to determine the fold-change in mRNA expression (LIVAK & SCHMITTGEN, 2001).

### Statistical analyses

Statistical comparison among the studied groups was carried out using one-way analysis of variance (ANOVA). Duncan's t-test and a statistical package program (SPSS version 17.0) were used. The statistical significance for all data was set at  $p \leq 0.05$ . The checks were made via tests and / or graphs of the residuals for normality and heterogeneity.

## Results

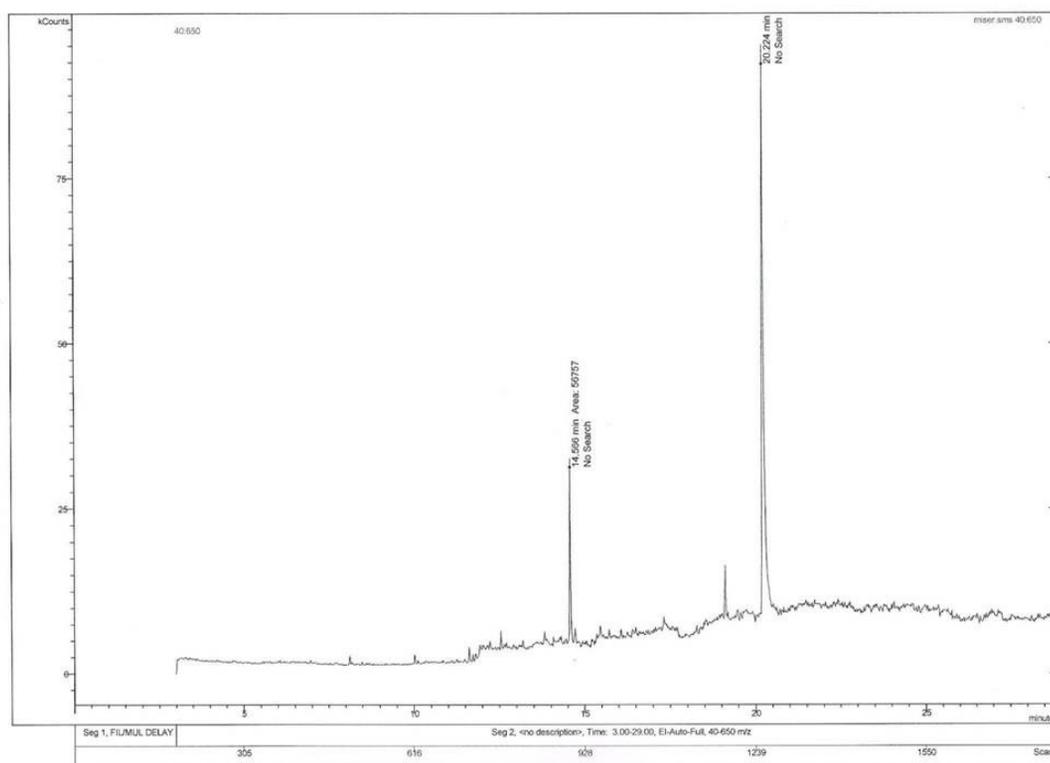
Figure 1 shows the mass spectrum of SE. The phytochemical components present included  $11\alpha$ -hydroxy- $17\alpha$ -methyl testosterone, 2,5-cyclohexadiene-1,4-dione,2,6-bis(1,1-dimethylethyl)-, prednisolone acetate, and bis(2-ethylhexyl) phthalate (Table 1).

On day 5 post-inoculation (p.i.), the expelled oocysts from the *E. papillata* infected mice were greatest ( $6242 \pm 731$  oocysts/g feces). Treatment of infected mice with different doses of SE produced a significant decline in the total number of expelled oocysts, especially after treatment with 300 mg/kg SE ( $2070.2 \pm 540.7$  oocysts/g feces; Figure 2).

The total number of intracellular *E. papillata* stages (meronts, gamonts, and developing oocysts) in the jejunal villi of the hematoxylin and eosin stained sections (Figure 3) was significantly decreased in infected mice treated with different doses of SE, especially with 300 mg/kg *S. persica* leaf extract (Table 2).

The numbers of TUNEL positive cells (Figure 4) were significantly higher during infection ( $30 \pm 5$  apoptotic cells / 10 villous crypt units, VCU) compared to the non-infected jejunum ( $3 \pm 0.3$  apoptotic cells/10 VCU). The group treated with 300 mg/kg SE displayed a significant reduction in apoptotic cell number by approximately  $10 \pm 2$  apoptotic cells / 10 VCU (Figure 5).

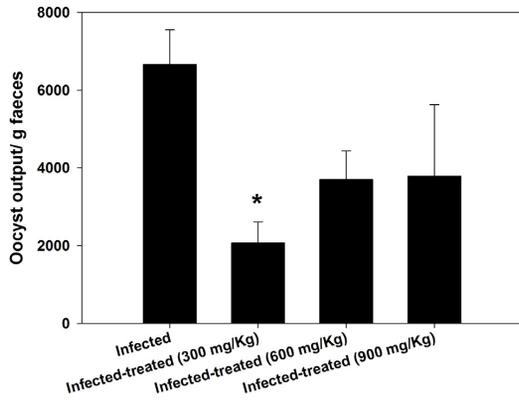
*E. papillata* infection of mice caused a significant decrease in the level of glutathione (GSH) and catalase (Figure 6). Treatment



**Figure 1.** Gas chromatography-mass spectrometry chromatogram plot of aqueous leaf extract of *S. persica*.

**Table 1.** Identification of phytochemical compounds by GC-Mass in *S. persica* leaf extracts.

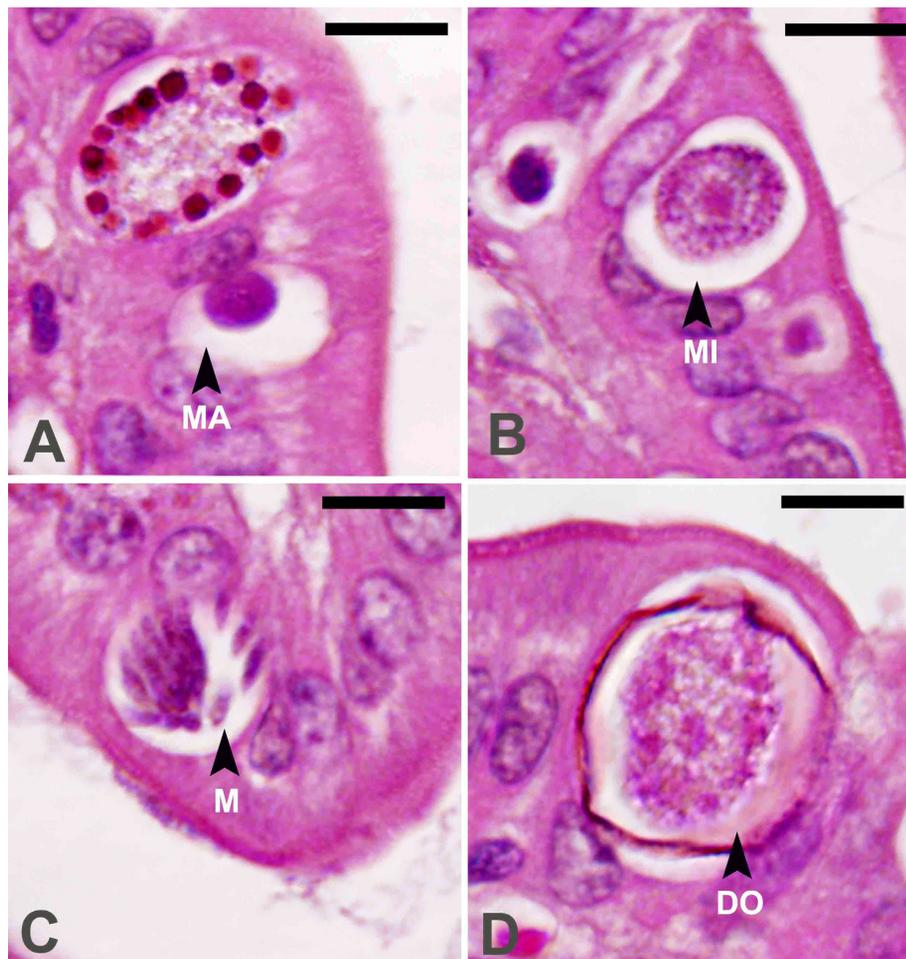
RT	Assignment	Molecular formula	[M-H] <sup>-</sup> (m/z)	MS (m/z)	Peak area (%)
12.547	11 $\alpha$ -Hydroxy-17 $\alpha$ -methyl testosterone	C <sub>20</sub> H <sub>30</sub> O <sub>3</sub>	317	43-137	22.6
14.568	2,5-Cyclohexadiene-1,4-dione,2,6-bis(1,1-dimethylethyl)-	C <sub>14</sub> H <sub>20</sub> O <sub>2</sub>	219	41-205	22.4
19.111	Prednisolone acetate	C <sub>23</sub> H <sub>30</sub> O <sub>6</sub>	401	15-404	44.33
20.221	Bis(2-ethylhexyl) phthalate	C <sub>24</sub> H <sub>38</sub> O <sub>4</sub>	389	41-279	7.18



**Figure 2.** Changes in oocyst output after treatment of *E. papillata* infected mice with *S. persica*. (\*) Significant change at  $P \leq 0.01$  with respect to the infected group.

with 300 mg/kg SE produced a significant increase in the levels of GSH and catalase compared with both the non-infected and infected groups (Figure 6). Moreover, the infection induced a significant increase in the level of malondialdehyde (MDA) (Figure 6). Treatment with SE could improve this change in MDA (Figure 6).

Gene expression analysis of jejunum cytokines including interleukin (IL)-1 $\beta$ , IL-6, tumor necrosis factor-alpha (TNF- $\alpha$ ), and interferon-gamma (IFN- $\gamma$ ) revealed upregulated expression of the genes in infected mice (Figure 7). Treatment of mice with SE induced the down-regulation of these genes (Figure 7). In addition, the apoptotic genes B-cell lymphoma 2 (Bcl2), BCL2-associated X (Bax), and caspase 3, which were upregulated in *E. papillata* infection, were significantly downregulated after treatment of mice with SE (Figure 8).

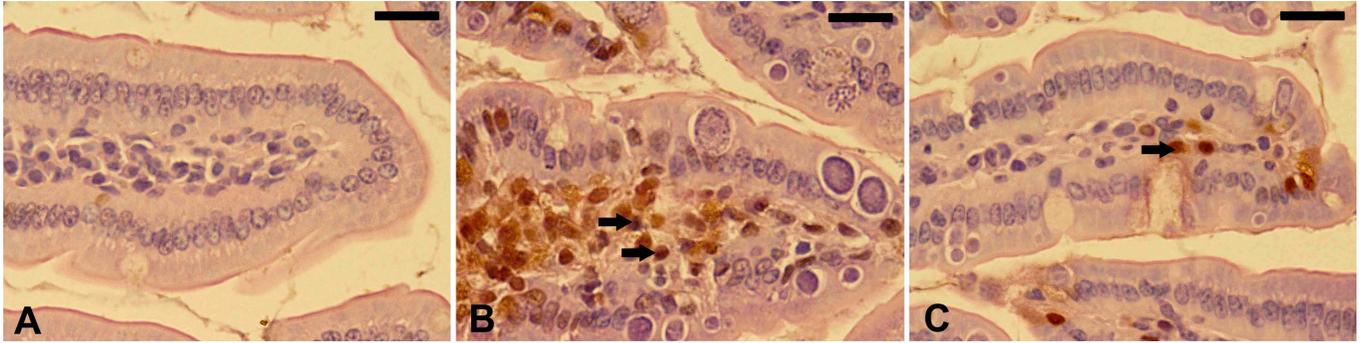


**Figure 3.** Parasitic stages of *E. papillata* in jejunum of mice. Meronts (M), microgamonts (MI), macrogamonts (MA), and developing oocysts (DO). Sections stained with eosin and hematoxylin. The bar=10  $\mu$ m.

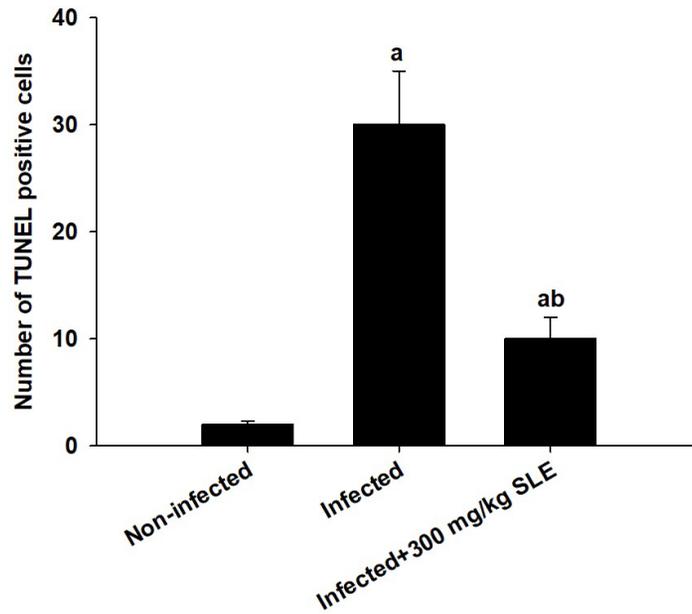
**Table 2.** Parasitic stages in jejunum of *E. papillata*-infected and infected-treated mice with *S. persica*.

Group	Meronts / 10 VCU	Male and female gamonts/ 10 VCU	Developing Oocysts / 10 VCU	Total number of parasitic stages
Infected	80.25 $\pm$ 4	25 $\pm$ 3.5	11.3 $\pm$ 2.5	116.5 $\pm$ 10
Infected-treated (300 mg/Kg)	35.7 $\pm$ 4*	8.3 $\pm$ 1.1*	6.75 $\pm$ 2.5*	50.8 $\pm$ 7.6*

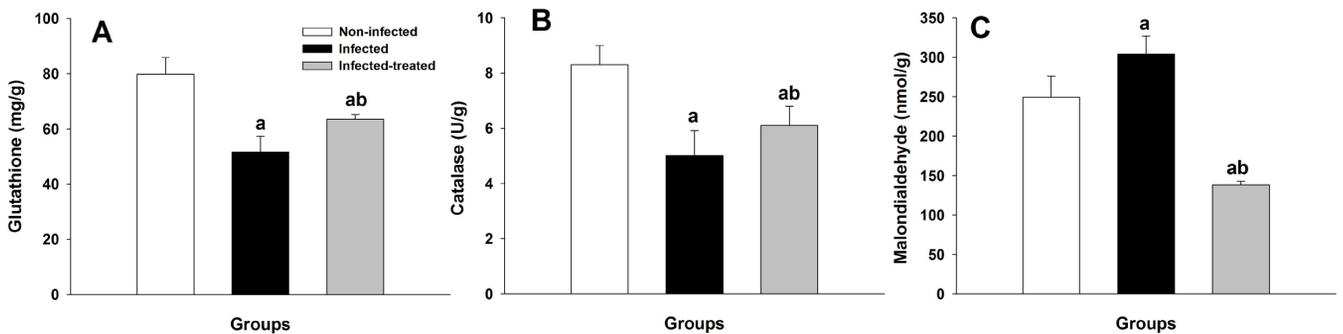
(\*) Significance against infected group at  $p < 0.01$ .



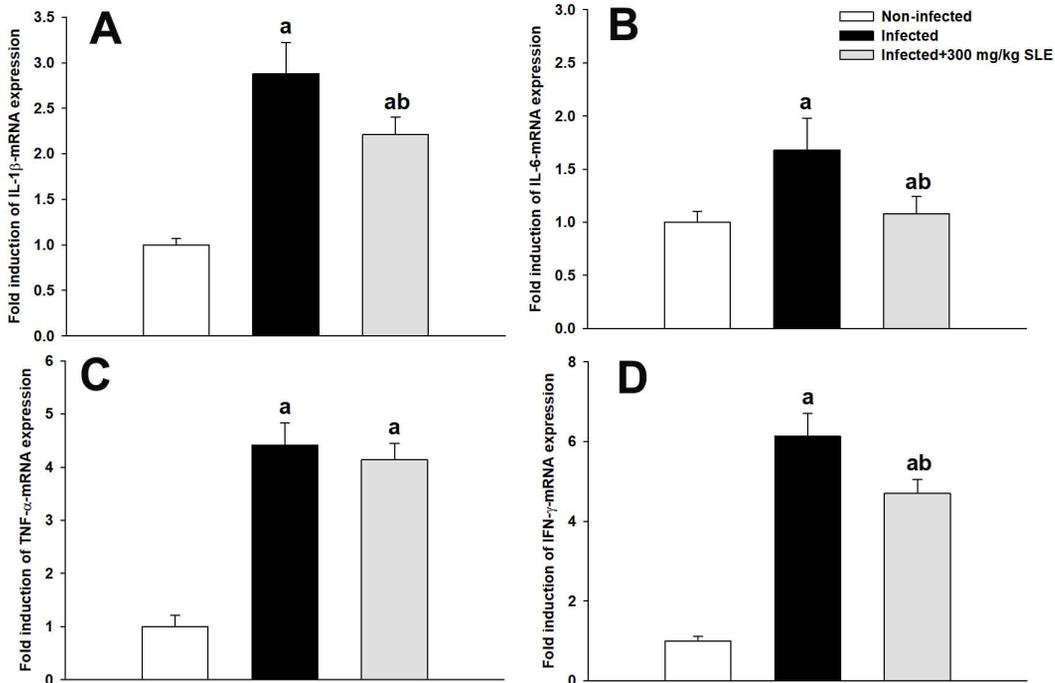
**Figure 4.** Changes in apoptotic cells in control (A), *E. papillata* infected (B), and infected-treated (C) mice jejunum. Brown cells represent a TUNEL positive cells. The bar=50 μm.



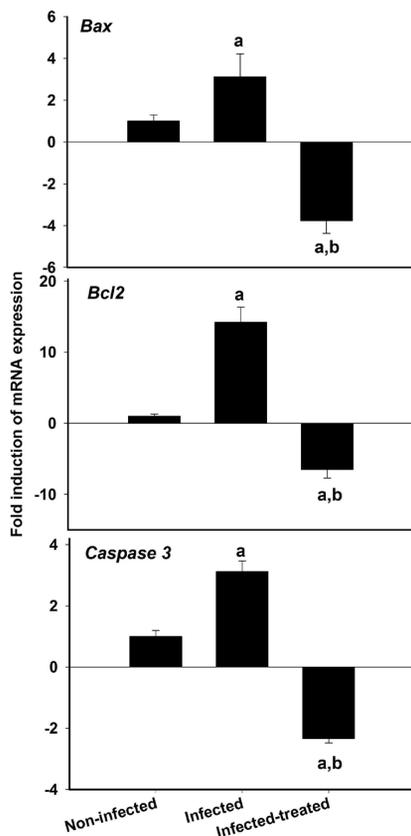
**Figure 5.** *S. persica*-induced changes in the apoptosis level in jejunum of mice. (a) Significant change at  $P \leq 0.05$  with respect to the non-infected group; (b) Significant change at  $P \leq 0.05$  with respect to the infected group.



**Figure 6.** Effect of *S. persica* leaf extracts on glutathione (A) and malondialdehyde (B) levels, and catalase (C) activity in mouse jejunum infected with *E. papillata*. Data expressed as mean and standard deviation. (a) significant change against control animals; (b) significant change against infected animals.



**Figure 7.** The level of jejunal IL-1 $\beta$  (A), IL-6 (B), IFN- $\gamma$  (C), and TNF- $\alpha$  (D) gene expression of control, *E. papillata* infected and infected-treated mice jejunum. (a) Significant change at  $P \leq 0.05$  with respect to the non-infected group; (b) Significant change at  $P \leq 0.05$  with respect to the infected group.



**Figure 8.** Apoptotic gene (*Bcl2*, *Bax* and *Caspase 3*) expression in control and *E. papillata* infected and infected-treated mice jejunum. (a) Significant change at  $P \leq 0.05$  with respect to the non-infected group; (b) Significant change at  $P \leq 0.05$  with respect to the infected group.

## Discussion

Several studies reported the anti-parasitic activities of several plant extracts (AMER et al., 2015; ALZHRANI et al., 2016; DKHIL et al., 2019). The results of the present study demonstrate the potent anti-eimerial activity of SE against murine intestinal *E. papillata* infection. This effect may be due to the active ingredients present in the extract. Khan et al. (2010) found that SE contains flavonoids, glycosides, alkaloids, saponins, carbohydrates, tannins, and steroids. *S. persica* reportedly has beneficial therapeutic properties and the potency for use as an effective adaptogenic herbal therapy (AHMAD & RAJAGOPAL, 2013).

Ahmad et al. (2011) reported significant anti-inflammatory activity of *S. persica* extract at a dose of 300 mg/kg in rats with induced paw edema. In our gas chromatography-mass spectrometry analysis, prednisolone acetate was identified as a component in SE. This compound is used as an anti-inflammatory agent (DANNI et al., 2019).

Presently, infection with *E. papillata* in mice caused intestinal tissue injury at the site of infection because of parasites at the developmental stages, especially merozoites, breaking out of the gut cells and invading other cells of the gut. The anti-eimerial activity of the extract resulted in a significant decrease in the number of the parasitic stages inside the jejunum. Palm pollen was reported to induce the same effect (METWALY et al., 2014). Moreover, selenium significantly reduced the number of meronts, gamonts, and developing oocysts of *E. papillata* (DKHIL et al., 2014).

*Eimeria papillata* infection is associated with oxidative damage in the jejunum of mice and severe local and systemic inflammatory responses (DKHIL et al., 2015). The authors further reported that

this oxidative damage within the jejunum of infected mice was associated with a reduction in the activity of antioxidant enzymes, catalase, and glutathione peroxidase and depletion in the reduced GSH level. Intestinal infection with *E. papillata* in mice caused potential oxidative and cytotoxic damage within infected mucosal tissue as revealed by increased levels of nitric oxide and hydrogen peroxide, protein oxidation, and enhanced lipid peroxidation and depletion of the intracellular antioxidant defense system (DKHIL et al., 2015). Moreover, MDA, the most frequently used biomarker of oxidative stress, was significantly increased due to *E. papillata* infection. The SE was able to ameliorate the altered MDA level in mice jejunum.

Natural antioxidants obtained from *S. persica* have medicinal value in *E. papillata* infection. Some studies have suggested that two-thirds of the plant species on Earth have medicinal value, with several medicinal plants having significant antioxidant potential (KRISHNAIAH et al., 2011). The high therapeutic potential of *S. persica* reflects the anti-inflammatory (EZMIRLY et al., 1979) and antioxidant (MOHAMED & KHAN, 2013) activities of the plant extract components. Ramadan and Alshamrani (RAMADAN & ALSHAMRANI, 2015) described the potential anti-stress activity of *S. persica* aqueous extract. *Salvadora persica* inhibited stress-induced abnormalities in hematological parameters, glucose level, and lipid profile, indicating its protective effect against stress. The treatment of *E. papillata* infected mice with *S. persica* resulted in the pronounced modulation of oxidative damage and enhanced antioxidant capacity in the jejunum of mice.

The infection of mice with *E. papillata* caused jejunal inflammation as evidenced by the increased expression of mRNAs of the jejunal inflammatory cytokines, IL-1 $\beta$ , IL-6, TNF- $\alpha$ , and INF- $\gamma$ . *Salvadora persica* treatment regulated the induced changes in these cytokines, indicating the potential as a host defense against parasite-induced inflammation (SCHITO et al., 1998).

Apoptosis helps to eliminate cells infected by parasites (LÜDER et al., 2001; BALAMURUGAN et al., 2002). In addition, the upregulation of apoptotic genes within the jejunum of control non-infected mice is part of the normal function to maintain tissue homeostasis for the renewal of intestinal cells. *E. papillata* induced the regulation of Bcl2, Bax, and caspase-3. Bax activity stimulates the release of cytochrome c, which in turn activates caspases to finally induce cell death (ROSSÉ et al., 1998). *E. papillata* invasion and replication might impose considerable stress on host cells (ALZAHIRANI et al., 2016), which in turn triggers apoptosis (GREEN & REED, 1998; GREEN, 2000). Other factors that stimulate apoptosis are the elevation of nitrogen intermediates, reactive oxygen species, and inflammatory cytokines within the intestinal epithelium (RAMACHANDRAN et al., 2000; MAJOR et al., 2011). Presently, SE administration significantly reduced the number of apoptotic cells in the jejunum of treated mice. Aqueous extracts of *S. persica* contain important molecules, such as benzyl isothiocyanate (ALALI & AL-LAFI, 2003), which induce cell cycle arrest at the G2/M phase and apoptosis in human melanoma A375.S2 cells through reactive oxygen species and both mitochondria-dependent and multiple death receptor-mediated signaling pathways (HUANG et al., 2012). *Salvadora persica* possesses potent anticoccidial, antioxidant, anti-inflammatory, and antiapoptotic activities. Further studies are required to determine the mechanism of action of the extract on the parasite and the host.

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