# ANTIPARASITIC ACTIVITY AND THE MODES OF ACTION OF NATURAL PRODUCTS AND TRADITIONAL MEDICINES

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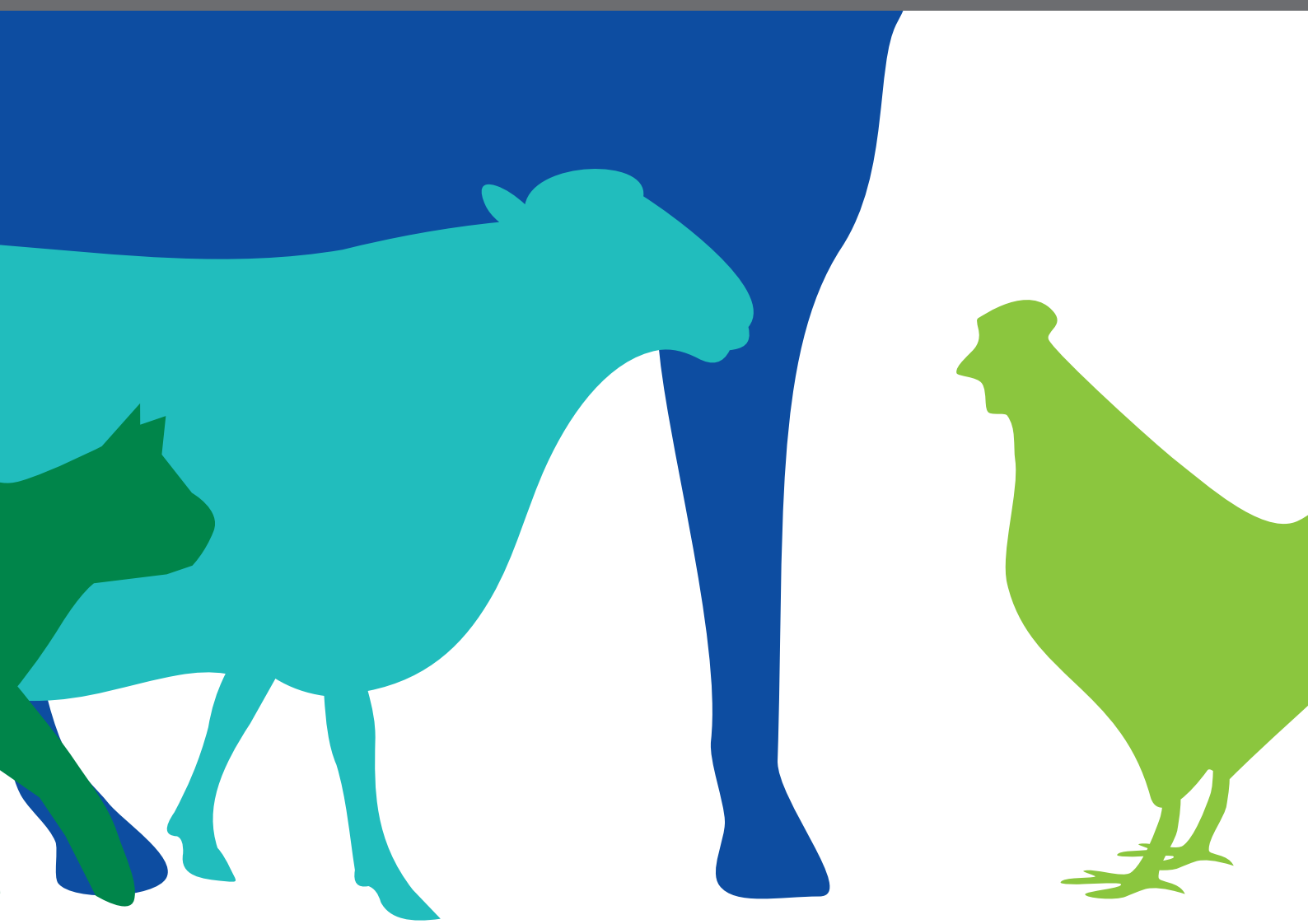

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# ANTIPARASITIC ACTIVITY AND THE MODES OF ACTION OF NATURAL PRODUCTS AND TRADITIONAL MEDICINES

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EDITORIAL
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# Editorial: Antiparasitic Activity and the Modes of Action of Natural Products and Traditional Medicines

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**Keywords: antiparasitic activity, natural product, traditional medicines, modes of action (MOA), lead compounds**

## Editorial on the Research Topic

## Antiparasitic Activity and the Modes of Action of Natural Products and Traditional Medicines

Worldwide parasites pose a serious threat to the health of both humans and animals, leading to large economic losses. Currently, the treatment of parasitic diseases mainly relies on chemotherapy, and antiparasitic drugs represent the second largest segment of the global animal health market with 23% of the market share (1). During the past half century, significant treatments have been advancing, however, they remain a major threat to livestock farming and cause large deficits for the agricultural economy (1). In addition, the long-term use of chemical drugs has resulted in more serious problems, such as pesticide resistance, environmental contamination, environmental persistence, resurgence, and other side effects. These problems are not only destroying the healthy community structure of husbandry but also bring risks for humans (1, 2). Hence, more efforts are now being directed toward the global control of parasites in intensive livestock production.

In the past 50 years, traditional medicines and natural products have been considered an important alternative strategy for the sustainable management of parasitic diseases, and great progress has been achieved. Between January 1981 and September 2019, 20 antiparasitic agents were approved by the FDA, and nine agents (45%) were derived from natural products (3). Notably, the Nobel Prize in Physiology or Medicine 2015 was awarded jointly to William Campbell, Satoshi Omura, and Tu Youyou for their discoveries concerning a novel therapy against infections caused by roundworm parasites and Malaria, respectively (4). Considerable effort has been made to exploit the active compounds that occur naturally as secondary metabolites of plants, animals, and microorganisms, and some traditional medicines and natural products have been proven to have strong antiparasitic activity in both animals and humans *in vivo* and *in vitro*, especially for ectoparasites. After searching the web of science database, 284 papers related to anti-ectoparasite agents were published by *Veterinary Parasitology, Frontiers in Veterinary Sciences,* and other international journals worldwide from January 2015 to June 2020, and 204 papers (71.83%) aimed to find the active natural products or extracts from plants, 74 papers (26.06%) focused on essential oils (5).

Recently, along with the development of tremendous technologies in structural biology, computational chemistry, structure-based drug design, and multi-omics, coupled with enhanced automation in high-throughput screening platforms and affinity strategies, the paradigms for screening lead compounds have led to a shift toward more mechanism-based screening (6). For

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the first time, our group found that cardiac glycosides may be active compounds of *Adonis coerulea* in terms of its acaricidal activity using proteomics and surface plasmon resonance technology, which also is a sensitive and environmentally friendly analytical method (7). In addition, compared with the traditional empirical methods of screening lead compounds from the synthetic compound libraries (million compounds), traditional medicines with antiparasitic activity or natural products also have been attracting many people's interest as a way of finding new agents. More and more active compounds from plants or traditional medicines have been discovered, and the potential targets or mechanisms of action explored.

Our group established this Research Topic to present the development of antiparasitic activity and the modes of action of natural products and traditional medicines. Nine papers by 73 authors from nine different countries were published to perform the latest progress in their field, which will help us find more interesting topics. The anthelmintic activity against *Teladorsagia circumcincta* of Nordic bark extracts (Athanasiadou et al.) and *Diospyros anisandra* (Flota-Burgos et al.) are explained respectively, and the leishmanicidal activity of licochalcone A *in vitro* and in an experimental model of *Leishmania* (Leishmania) infantum was also proven (Souza et al.). S-Methylcysteine (SMC) presented the ameliorate the intestinal damage Induced by Eimeria tenella Infection (Elmahallawy et al.) and silver nanoparticles biosynthesized with *Salvia officinalis* leaf performed a protective effect on the hepatic tissue injury induced by *Plasmodium chabaudi* (Metwally et al.). Silva Bello et al. proved the prophylactic effects of ivermectin and closantel on *Oestrus ovis* infestation in sheep. Arfuso et al. and Li et al. also made contributions to this Research Topic. We are aware that the selection of papers is incomplete and does not do justice to the importance of the research field "*Antiparasitic Activity and the Modes of Action of Natural Products and Traditional Medicines*". However, it does reflect to some extent where we currently are knowledge-wise, and the topic should receive more attention and be discussed by people.

Currently, the development of new veterinary drugs is slow worldwide. It is important to explore new ways of finding more rational and effective strategies to screen novel active compounds: a crucial but not easy task. The discovery of new and promising antiparasitic agents from natural products and traditional medicines will be important for controlling parasites in the future.

## AUTHOR CONTRIBUTIONS

XS wrote the manuscript. AP and GI revised the manuscript. JZ supervised the manuscript. All authors contributed to the article and approved the submitted version.

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# Anthelmintic Activity of Extracts and Active Compounds From *Diospyros anisandra* on *Ancylostoma caninum*, *Haemonchus placei* and Cyathostomins

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The present study aimed to evaluate the anthelmintic activity of leaf and bark extracts of *Diospyros anisandra* collected during different seasons and their major constituents on eggs of *Ancylostoma caninum*, *Haemonchus placei,* and cyathostomins. Specifically, the eclosion inhibition of the methanolic extracts of the leaves and bark of *D. anisandra* collected during the dry and rainy seasons (600–37.5 μg/ml) were evaluated in addition to the fractions, sub-fractions (300–37.5 μg/ml) and active major constituents (150–2.3 μg/ml). The rainy season bark extract had the highest percentage of eclosion inhibition (PEI) against the evaluated nematodes (≥ 90% at 75 μg/ml) along with high ovicidal activity (90.0 to 93.4% at 75 μg/ml). The purification of the rainy season bark extract showed that its biological activity came from the non-polar *n*-hexane fraction (≥ 93% at 75 μg/ml). The bioguided fractionation pointed to sub-fraction 5 as having the highest anthelmintic activity against the three evaluated genera of nematodes (PEI ≥ 93% at 37.5 μg/ml). Gas chromatography and mass spectrometry revealed that the major constituent in sub-fraction 5 was plumbagin. Upon evaluation, plumbagin was confirmed to be responsible for the anthelmintic activity of *D. anisandra*, with a PEI ≥ 90% at 2.3 μg/ml on the three evaluated nematodes. Additionally, the compounds betulin and lupeol in the bark of *D. anisandra* were evaluated but presented low anthelmintic activity (PEI ≤ 5.3% at 2.3 μg/ml). In conclusion, the rainy season bark extract of *D. anisandra* exerts a high ovicidal activity against the eggs of the three studied nematodes. Plumbagin is the active compound responsible for this activity and represents a potential alternative for the control of different genera of gastrointestinal nematodes given the current scenario of anthelmintic resistance.

**Keywords: active compounds, bioguided fractionation, gastrointestinal nematodes, plant extracts, plumbagin**

## INTRODUCTION

Gastrointestinal nematodes (GINs) are a serious threat to the health and well-being of domestic animals and negatively affect the economy of animal production. Also, they can negatively affect public health because of their zoonotic potential. Some examples of GINs include *Ancylostoma caninum*, *Haemonchus placei* and cyathostomins, which are the most prevalent and pathogenic in dogs, bovines and horses, respectively (1–4).

Traditionally, the control of GINs has been based on the intensive administration of anthelmintic drugs. However, this has generated anthelmintic resistance, mainly to benzimidazoles and macrocyclic lactones. There are also reports of *Ancylostoma caninum* resistance to pyrantel, *Haemonchus placei* resistance to salicylanilides and imidazothiazoles and cyathostomin resistance to tetrahydropyrimidines (4–7). The situation is exacerbated by multi-resistance to numerous anthelmintics, which has been documented in the aforementioned three genera (8–11).

The lack of effectiveness of current anthelmintic treatments has prompted the search for control alternatives, including the use of plant extracts with anthelmintic properties and their secondary metabolites. Plant extracts and their natural derivatives have long been used as an additional or alternative treatment to conventional chemical products and have also served as important sources of new anthelmintic molecules for the development of alternative treatments (12). Among the plants reported to have broad biological activity in the Mexican tropics is *Diospyros anisandra* (13–15). Arjona-Cambranes et al. (16) found that the bark extract of *D. anisandra* inhibited more than 98% of *Ancylostoma caninum* eclosion (the act of hatching from the egg) *in vitro* at a concentration 1,200 μg/ml and that the leaf extract showed a similar percentage of eclosion inhibition (PEI) at triple the concentration (3,600 μg/ml). Thus, the bark extract of *D. anisandra* appears to exert an ovicidal effect. Another study confirmed the effects of the methanolic extract of *D. anisandra* against cyathostomin eggs, finding a PEI > 90% at a concentration of 75 μg/ml. Specifically, two effects were noted: an ovicidal effect from using the bark extract and a larval effect using the leaf extract in which larvae failed to hatch (15).

Despite the demonstrated anthelmintic potential of *D. anisandra* in these previous studies, its extract was only evaluated in each case against one genus of nematode. The question remains as to whether *D. anisandra* extracts have a broad spectrum of action or exert effects against two or more genera of nematodes. Also, only the methanolic extracts of *D. anisandra* were evaluated without determining the active compound(s) that confers its biological activity. Hence, the objective of the present study was to evaluate the anthelmintic activity of leaf and bark extracts of *D. anisandra* collected during different seasons of the year and their major constituents on eggs of *Ancylostoma caninum*, *Haemonchus placei*, and cyathostomins.

## MATERIALS AND METHODS

The present study was carried out at the Parasitology Laboratory of the Faculty of Veterinary Medicine (Facultad de Medicina Veterinaria) of the Autonomous University of Yucatán (Universidad Autónoma de Yucatán). The region has a warm, sub-humid climate (85.7% of the territory) characterized by a rainy (June-October) and dry (February-May) season, average annual temperature of 26°C, relative humidity between 65 and 95% and an average annual rainfall of 902 mm (17).

### Information From *Diospyros anisandra* and the Collection Site

*Diospyros anisandra* S. F. Blake is a quasi-endemic species of Peninsula of Yucatan, commonly known as k'aakalche', k'ab che' or xanob che', of the Ebenacea family; It can measure up to 7 meters in height, uses vary depending on the locality, such as wood, instrument making, firewood and skin diseases such as pimples, scabies and inflammation (18, 19). And it is widely distributed in the Yucatan Peninsula. *D. anisandra* is a shrub or small tree, up to 7 meters tall, short branches, glabrous. Leaves subfasciculate at the tips of the branches, obovate, rounded and reticulous at the apex, cuneate at the base, glabrous or almost glabrous, 2 to 6 cm long and 1.2 to 3 cm wide. Axillary inflorescence, from 1 to 2 staminate flowers, hanging, pedicels from 1 to 2 mm long; funelform calyx, about 4 mm long, 4 lanceolate, acuminate lobes, and about 1.5 mm long; corolla urceolada, about 14 mm long, tube 7 mm long, acuminate lobes of similar length, 1 to 2 pistillate flowers. Globose fruits, about 1 cm in diameter, black and shiny when ripe. It blooms in February (the dry season), May, June, July, and September (the rainy season), although its fruits can be seen almost all year round with the exception of April and May.

The site of collection was the municipality of Yaxcabá, wich is located in the central-southern region of the state of Yucatan, Mexico. The geographical coordinates are 20° 19' and 20° 49' north latitude and 80° 36' and 88° 56' west longitude, with an average altitude of 29 meters above sea level. The average annual temperature is 26°C, with an average annual rainfall of 1,118 mm, subject to variations due to the presence of hurricanes (20). The prevailing climate is warm sub-humid (Aw) with rains in summer, where humidity decreases from south to north with record temperatures in May and the lowest in January. The annual rainfall is 1,111 mm, with an annual relative humidity of 89%. The characteristic soil types of the municipality are Cambisols. Calcisols and in low proportion Luvisols (21). The vegetation is a sub deciduous tropical forest in different stages of succession (22).

### Plant Extracts

Leaves and bark of *D. anisandra* were collected in the rainy and dry season in Yaxcabá, Yucatán (for obtaining their extracts). Then, the aforementioned plant parts were separated and placed in a drying oven at 40°C for 48 h. The dry plant material was ground in an electric mill to reduce the size of particles to 5 mm. Two extractions were performed, each for 24 h, using methanol (MeOH) at a ratio of 30 ml for every 25 g of ground plant material. After each extraction, the methanolic solvent was separated from the plant material through filter paper and deposited in glass flasks. Using a rotary evaporator (Rotavapor, Büchi®), the solvent was eliminated under reduced pressure,

concentrating the dry extract. The obtained products were placed in glass vials and stored at 4°C until use (23).

## Obtainment of GIN Eggs

Feces samples were obtained from naturally infected animals. The McMaster technique was used to determine the GINs present and quantify the number of eggs per gram of feces (24). Consecutive coprocultures were carried out to morphologically identify the $L_3$ larvae of *Ancylostoma caninum* (canine), *Haemonchus contortus* (bovine) and cyathostomins (equine) (*Cyathostomum* spp type G, *Posteriostomum* spp, *Gyalocephalus capitatus Cylicoclyclus* spp type B, *Triodontophorus* spp) (25–27). Prior to processing samples for egg recovery, a centrifugal flotation of each sample was performed to ensure that eggs were in the morula stage and that larvae had not begun to form (28). The feces were macerated with purified water at a ratio of 100 ml for every 50 g of feces. The resulting mixture was filtered through non-sterile gauze and deposited in 45-ml plastic tubes, which were centrifuged at 1,500 rpm for 5 min. The supernatant was discarded, and saturated sugar solution was added (density: 1.280). The mixture was homogenized in a vortex and once again centrifuged. The eggs were recovered from the superficial portion with an inoculation loop and deposited in phosphate buffer solution (PBS). Afterwards, three washes with PBS were performed, eliminating the residues of the saturated sugar solution. The concentration of recovered eggs (eggs/ml) was estimated, and the suspension was diluted to obtain a final solution of 400 eggs/ml (15, 29).

## Egg Hatch Assay

The methanolic extracts were evaluated by hatching inhibition tests carried out according to the guidelines of the World Association for the Advancement of Veterinary Parasitology (WAAVP) (30). The evaluated concentrations were 600, 300, 150, 75, and 37.5 μg/ml. To dilute the extracts and create a negative control, a solution of PBS 0.01M (Sigma®) plus 5% absolute ethanol was used. Thiabendazole (0.1 μg/ml) was used as a positive control. Three repetitions were performed for each evaluated concentration. The extracts were diluted to the aforementioned concentrations in an ultrasonic bath (Branson®). Culture plates with 24 wells were used; in each, 0.5 ml of solution containing eggs (200 eggs approximately) was deposited in addition to 0.5 of diluted extract for a total volume of 1 ml in each well. Then, the plates were incubated in a bacteriological oven at 28°C for 48 h. At following, Lugol solution (20 μg) was added to disrupt the hatching process. The contents of each well were deposited in McMaster chambers and observed through a microscope at 10× to count the number of morulated eggs, eggs with larvae inside and hatched larvae. All plates whose negative controls obtained an eclosion percentage equal to or greater than 80% were included in the study (15, 29).

The PEI was calculated with the following formulas proposed by Peachey et al. (31) and Flota Burgos et al. (15):

$$\%\ hatched = \left(\frac{L_1}{L_1 + eggs}\right) \times (100)$$

$$\%\ egg\ hatch\ inhibition = 100 - \%\ hatch$$

The effect of the extracts on larval development was calculated according to the formulas proposed by Vargas-Magaña et al. (28) and Flota Burgos et al. (15). Two effects were recorded: ovicidal activity (OA) for eggs that did not form larvae or eggs with $L_1$ larvae failing eclosion (LFE) during the incubation time.

$$\%\ OA = \left(\frac{morulated\ eggs}{morulated\ eggs + eggs\ containing\ a\ larva + L_1\ larvae}\right) \times (100)$$

$$\%\ LFE = \left(\frac{eggs\ containing\ a\ larva}{morulated\ eggs + eggs\ containing\ a\ larva + L_1\ larvae}\right) \times (100)$$

## Fractionation of the Crude Extract of *Diospyros anisandra*

The methanolic extract of *D. anisandra* was partitioned with *n*-hexane, ethyl acetate and methanol (23). The obtained fractions (*n*-hexane, ethyl acetate and residual methanol) were evaluated by the same eclosion inhibition assays described in section Obtainment of GIN Eggs at concentrations of 300, 150, 75, and 37.5 μg/ml.

The partition with the highest anthelmintic activity was then sub-fractionated using a glass column (4 × 5 cm) with 140 g of sodic bentonite. Each sub-fraction was eluted (liquid-liquid partition) with 200 ml of *n*-hexane and then with volumes of *n*-hexane and acetone of increasing polarity (ratio of 100:0 to 0:100). Based on similar Rf values in TLC (60F254 aluminum plates coated with silica gel, Merck®) developed with the eluent *n*-hexane: acetone (8:2) and sprayed with a solution of phosphomolybdic acid, 11 fractions were grouped (23). Each sub-fraction was evaluated by hatching inhibition tests at concentrations of 300, 150, 75, and 37.5 μg/ml.

## Analysis by Gas Chromatography-Mass Spectrometry

The active sub-fraction was analyzed in a gas chromatograph (Agilent Technologies 6890N) coupled with a selective mass detector with a HP-5MS column (5% phenyl-methylpolysiloxane, 25 m × 0.2 mm internal diameter). A split injection was performed on 1 μl of a 1% solution of the F5 sample at a flow rate of 1.0 ml/min (helium as the carrier gas) and column temperature of 100°C for 3 min; then, the temperature was increased 10°C per min along a gradient until reaching a final temperature of 280°C.

The constituents in the extract were identified by searching commercial reference libraries. The fragmentation patterns of the mass spectra were compared with those in the NIST05 libraries. The major constituent (plumbagin) was then evaluated at concentrations of 150, 75, 37.5, 18.7, 9.3, and 2.6 μg/ml. Additionally, the major constituents of the bark extract of *D. anisandra*, betulin and lupeol, were isolated according to the methodology reported by Uc-Cachón et al. (32) and evaluated at concentrations of 150 to 2.6 μg/ml.

## Statistical Analysis

ANOVAs (generalized linear models) were carried out to identify significant differences between the evaluated concentrations and controls with respect to the eclosion inhibition tests (StatgraPEIcs 5.1). The lethal concentrations of the evaluated extracts were

**TABLE 1 |** Averages and standard deviation (±) of the percentages of eclosion inhibition of the methanolic extracts of *Diospyros anisandra* against eggs of *Ancylostoma caninum*, *Haemonchus placei*, and cyathostomins.

| Plant part | Concentration (μg/ml) | *Ancylostoma caninum* | | *Haemonchus placei* | | Cyathostomins | |
|---|---|---|---|---|---|---|---|
| | | Rainy season | Dry season | Rainy season | Dry season | Rainy season | Dry season |
| Bark | C– | 8.5 (0.9)[a] | 8.6 (2.8) | 1.2 (1.5)[a] | 1.9 (0.5)[a] | 3.4 (0.8)[a] | 5.5 (1.1)[a] |
| | C+ | 98.8 (1.3)*[b] | 99.2 (0.6)[a] | 97.1 (0.8)*[b] | 99.6 (0.5)*[b] | 98.4 (0.9)*[b] | 99.0 (0.1)*[b] |
| | 600 | **99.1 (0.8)*[b]** | **97.4 (3.0)*[b]** | **99.0 (0.4)*[b]** | **94.9 (0.5)*[b]** | **99.2 (0.7)*[b]** | **98.0 (1.0)*[b]** |
| | 300 | **99.1 (0.7)*[b]** | **97.3 (0.7)*[b]** | **98.0 (1.8)*[b]** | 71.9 (7.5)*[c] | **99.0 (0.9)*[b]** | **97.4 (1.7)*[b]** |
| | 150 | **98.8 (0.6)*[b]** | 52.8 (4.4)*[c] | **96.7 (1.2)*[b]** | 58.9 (6.1)*[d] | **98.8 (0.5)*[b]** | 83.9 (4.9)*[c] |
| | 75 | **98.8 (1.2)*[b]** | 46.1 (6.2)*[d] | **95.5 (0.1)*[b]** | 35.5 (2.9)*[e] | **99.5 (0.7)*[b]** | 69.9 (7.7)*[d] |
| | 37.5 | 40.4 (5.5)*[c] | 40.9 (5.2)*[e] | 62.2 (2.4)*[e] | 11.3 (2.0)*[f] | **97.4 (1.6)*[b]** | 34.5 (2.0)*[e] |
| Leaves | C– | 1.0 (0.5)[a] | 1.6 (1.5)[a] | 1.7 (0.5)[a] | 2.2 (1.9)[a] | 6.4 (1.6)[a] | 5.1 (1.6)[a] |
| | C+ | 99.0 (1.7)*[b] | 99.3 (1.2)*[b] | 99.1 (0.8)*[b] | 99.0 (0.9)*[b] | 99.0 (1.5)*[b] | 97.1 (0.9)*[b] |
| | 600 | 25.9 (0.4)*[c] | 5.3 (0.2)*[c] | 81.6 (2.3)*[c] | 87.9 (8.8)*[c] | **99.0 (0.1)*[b]** | **97.1 (1.3)*[b]** |
| | 300 | 6.7 (0.9)*[d] | 3.0 (0.9)[d] | 61.3 (1.4)*[d] | 64.2 (8.6)*[d] | **98.8 (1.0)*[b]** | **96.0 (1.9)*[b]** |
| | 150 | 5.8 (1.9)*[e] | 2.9 (0.8)[e] | 52.6 (0.9)*[e] | 59.6 (5.0)*[e] | **98.7 (0.4)*[b]** | 87.1 (2.4)*[d] |
| | 75 | 3.6 (0.7)*[f] | 2.7 (1.9)[f] | 28.9 (0.8)*[f] | 37.7 (1.0)*[f] | 84.4 (1.4)*[c] | 73.8 (4.1)*[e] |
| | 37.5 | 1.6 (0.7)[g] | 1.4 (0.4)[g] | 9.2 (0.7)*[g] | 3.5 (2.3)[g] | 44.3 (5.5)*[d] | 17.3 (2.5)*[f] |

*Significant differences were found with respect to the negative control.*
*Different letters among columns indicate significant differences ($P < 0.05$).*
*C- Negative control; C+ Positive control.*
*Standard deviation (±).*
*Bold values indicate Highlighted results.*

determined at 50% ($LC_{50}$) and 99% ($LC_{99}$) in addition to their 95% confidence intervals through Probit analysis (POLO Plus, LeOra Software®) (15).

# RESULTS

## GIN Eclosion Inhibition by Methanolic Extracts of *D. anisandra*

The bark methanolic extract of *D. anisandra* had a greater PEI compared to the leaf extract. Considering both the bark and leaf extracts, the material collected in the rainy season (RS) had a greater PEI compared to that collected in the dry season (DS) (**Table 1**). The RS extract exerted the highest activity against the three genera of nematodes, with a PEI > 90% from a concentration of 75 μg/ml. For cyathostomins, the most notable effect was reached at 37.5 μg/ml (97.4%) with the RS bark extract, which was statistically similar to the PEI of thiabendazole (≥ 98.0%) ($P > 0.05$), whereas for *Ancylostoma caninum* and *Haemonchus placei*, the most notable effects were reached at 75 μg/ml, which were also statistically similar to that of thiabendazole (≥ 97.1%) ($P > 0.05$). The DS bark extracts had a PEI > 97% against *Ancylostoma caninum* and cyathostomins at 300 μg/ml and a PEI of 94.9% against *Haemonchus placei* at 600 μg/ml. Meanwhile, the leaf extract had a PEI ≥ 90% only on cyathostomin eggs, with the RS extract exerting a greater effect (98.7% at 150 μg/ml) compared to the DS extract (96.0% at 300 μg/ml).

Overall, the lowest $LC_{50}$ and $LC_{99}$ were obtained with the RS bark extract ($P < 0.05$) (**Table 2**). Specifically, for cyathostomins, the lowest $LC_{50}$ and $LC_{99}$ were 11.3 and 38.1 μg/ml, respectively, which significantly differed from the higher lethal concentrations required by *Ancylostoma caninum* (60.0 and 76.7 μg/ml, respectively) and *Haemonchus placei* (43.1 and 128.7 μg/ml, respectively). Notably, the $LC_{50}$ of all extracts for cyathostomins was lower than 62 μg/ml. In contrast, the $LC_{50}$ and $LC_{99}$ of the RS leaf extracts, DS bark extracts and DS leaf extracts were at least two times higher for all three GINs.

## GIN Eclosion Inhibition by Fractions and Compounds of *D. anisandra*

Based on the results obtained for the crude extracts, the RS bark extract was selected for the bioguided fractionation process. The evaluation of the fractions of the partition revealed that the active fraction with the greatest efficacy (PEI) against the three nematodes was that of *n*-hexane (PEI ≥ 90% from 75 μg/ml) (**Table 3**). Also, the residual methanol fraction at 600 μg/ml had a PEI of 86.6% and 59.7% against *Ancylostoma caninum* and *Haemonchus placei*, respectively. Against cyathostomins, the residual methanolic fraction and ethyl acetate showed a PEI ≥ 80% at 150 μg/ml.

Similarly, the *n*-hexane fraction had the highest efficacy ($P < 0.05$) according to the $LC_{50}$ and $LC_{99}$ values (3.1 to 62.1 μg/ml and 22.2 to 133.9 μg/ml, respectively). The lethal concentrations obtained with the ethyl acetate and residual methanol fractions were at least six times higher.

The sub-fractionation of the *n*-hexane fraction showed that sub-fraction 5 had the greatest anthelmintic activity (**Table 3**). At 18 μg/ml, sub-fraction 5 had a PEI of 95.6, 89.8, and 96.7% against *Ancylostoma caninum*, *Haemonchus placei*, and cyathostomins, respectively. The remaining sub-fractions

**TABLE 2 |** Lethal concentrations at 50% and 99% (μg/ml) and confidence intervals (95%) of the methanolic extracts of *Diospyros anisandra* against eggs of *Ancylostoma caninum, Haemonchus placei* and cyathostomins.

| Plant part | Season | *Ancylostoma caninum* | | *Haemonchus placei* | | Cyathostomins | |
|---|---|---|---|---|---|---|---|
| | | LC 50 | LC 99 | LC 50 | LC 99 | LC 50 | LC 99 |
| Bark | Rainy | **60.0$^{a}$ (52.0–66.1)** | **76.7$^{a}$ (69.5–103.3)** | **43.1$^{a}$ (27.0–68.0)** | **128.7$^{a}$ (92.4–247.0)** | **11.3$^{a}$ (9.1–13.3)** | **38.1$^{a}$ (33.1–45.6)** |
| | Dry | 132.5$^{ba}$ (59.2–176.1) | 410.3$^{b}$ (341.0–560.1) | 197.6$^{b}$ (138.7–247.7) | 694.5$^{b}$ (583.3–890.3) | **61.2$^{b}$ (23.5–84.9)** | 249.6$^{b}$ (193.6–398.4) |
| Leaves | Rainy | 811.6$^{c}$ (788.5–835.4) | 1583.2$^{c}$ (1524.3–1650.3) | 297.5$^{bc}$ (182.3–412.1) | 1443.9$^{c}$ (1141.5–2036.7) | **45.2$^{bc}$ (41.2–48.6)** | 118.4$^{bc}$ (110.3–128.8) |
| | Dry | 2316.3$^{d}$ (2217.8–2.424.1) | 4950.2$^{d}$ (4676.4–5277.1) | 295.4$^{bcd}$ (147.2–444.2) | 1152.6$^{cd}$ (859.9–1912.3) | **57.3$^{bd}$ (50.5–63.3)** | 111.5$^{bcd}$ (97.5–138.9) |

*Different letters among columns indicate significant differences ($P < 0.05$).*
*Bold values indicate Highlighted results.*

**TABLE 3 |** Average percentages of eclosion inhibition and 50% and 99% lethal concentrations (95% confidence intervals) of the products obtained from the bioguided fractionation against eggs of *Ancylostoma caninum*, *Haemonchus placei* and cyathostomins.

| Nematode | Evaluated partition | Percentage of eclosion inhibition (μg/ml) | $LC_{50}$ (CI) (μg/ml) | $LC_{99}$ (CI) (μg/ml) |
|---|---|---|---|---|
| *Ancylostoma caninum*. | Methanol | 80.6% (600) | 418.7 (384.5–458.7)$^{a}$ | 875.0 (788.4–995.5)$^{a}$ |
| | Ethyl acetate | 3.1% (600) | ND | ND |
| | ***n*-hexane** | **≥ 93.7% (75)** | **62.1 (50.5–79.3)$^{b}$** | **133.9 (107.0–197.0)$^{b}$** |
| | **SF5** | **95.6 (18.7)** | **—** | **—** |
| *Haemonchus* | Methanol | 59.7% (600) | 139.6 (115.4–181.2)$^{c}$ | 311.5 (246.9–454.4)$^{c}$ |
| *placei* | Ethyl acetate | 15.5% (600) | ND | ND |
| | ***n*-hexane** | **≥ 98.0% (31)** | **10.9 (–17.0–22.5)$^{d}$** | **85.3 (68.8–125.5)$^{bd}$** |
| | **SF5** | **89.8 ((18.7)** | **—** | **—** |
| Cyathostomins | Methanol | ≥ 80.0% (150) | 49.5 (8.4–77.5)$^{de}$ | 222.8 (170.3–353.0)$^{bce}$ |
| | Ethyl acetate | ≥ 80.0% (150) | 107.6 (92.6–119.8)$^{cf}$ | 230.1 (206.5–268.5)$^{cdf}$ |
| | ***n*-hexane** | **≥ 96.0% (18.7)** | **3.1 (–0.53–5.5)$^{g}$** | **22.2 (17.8–30.8)$^{g}$** |
| | **SF5** | **96.7 (18.7)** | **—** | **—** |

*SF5, active sub-fraction 5. CI: confidence intervals.*
*The $LC_{50}$ and $LC_{99}$ of sub-fraction 5 could not be determine due to the high percentages of eclosion inhibition obtained at low concentrations.*
*Bold values indicate Highlighted results.*
*Different letters among columns indicate significant differences ($P < 0.05$).*

obtained a PEI of 5.9 to 11.8% against *Ancylostoma caninum*, 5.7 to 14.0% against *Haemonchus placei* and 3.3 to 18.2% against cyathostomins.

Gas chromatography-mass spectrometry revealed that the major constituent present in sub-fraction 5 was plumbagin (72.69% abundance). This compound was evaluated against the three nematode genera, obtaining a PEI ≥ 91% at 2.3 μg/ml. The $LC_{50}$ and $LC_{99}$ of sub-fraction 5 and its active compounds could not be determined because of the high PEI (≥ 90%) reached at all evaluated concentrations (150 to 2.3 μg/ml). In addition, the constituents betulin and lupeol found in the bark of *D. anisandra* (**Table 4**) were evaluated. These had low activity against the eggs of *Ancyolostoma caninum* (PEI of 3.6 and 3.2%, respectively), *Haemonchus placei* (PEI of 1.4 and 1.9%), and cyathostomins (PEI 5.0 and 5.3%) at 2.3 μg/ml. Even at the highest evaluated concentration (150 μg/ml), low PEIs were even observed against the eggs of *Ancylostoma caninum* (5.0 and 3.1%), *Haemonchus placei* (4.3 and 1.5%), and cyathostomins (8.8 and 5.5%).

**TABLE 4 |** Average percentages of eclosion inhibition of the major constituents of the bark of *Diospyros anisandra* on *Ancylostoma caninum*, *Haemonchus placei* and cyathostomins (at a concentration of 2.3 μg/ml).

| Compound | *Ancylostoma caninum*. | *Haemonchus placei* | Cyathostomins |
|---|---|---|---|
| Plumbagin | **91.3 (0.8)** | **92.6 (1.4)** | **92.4 (2.4)** |
| Betulin | 3.6 (0.3) | 1.4 (0.8) | 5.0 (1.5) |
| Lupeol | 3.2 (1.0) | 1.9 (1.2) | 5.3 (0.9) |

*Bold values indicate Highlighted results.*

## Ovicidal Effect of Extracts, Fractions and Major Constituents of *D. anisandra*

The methanolic extracts of *D. anisandra* bark from the RS in addition to the hexanic fraction, sub-fraction 5 and plumbagin contained within produced an ovicidal effect on the eggs of the evaluated nematodes. Inhibition of larval development was observed: After the incubation period, the

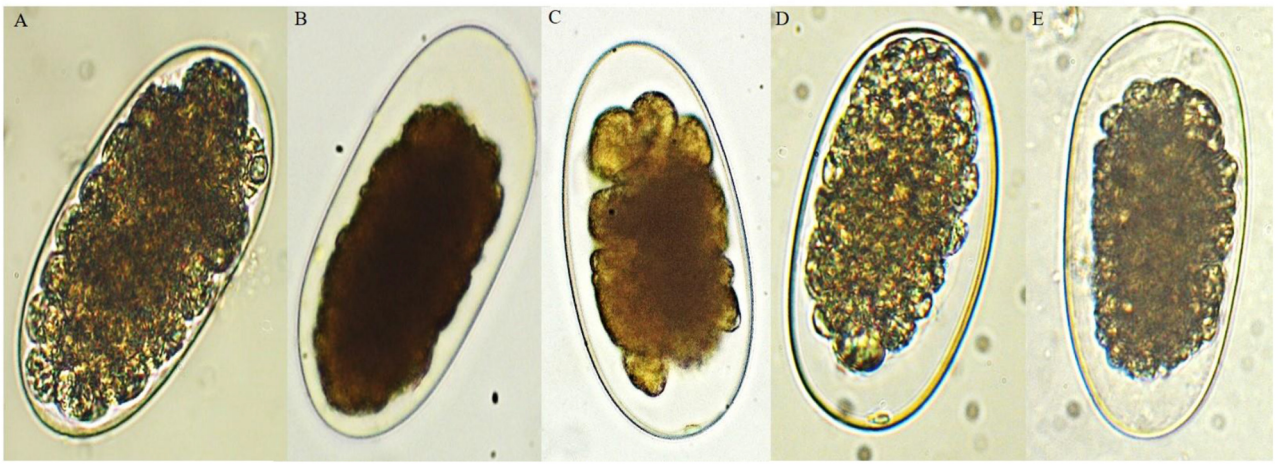


**FIGURE 1 |** Ovicidal effect of the bark extract of *D. anisandra* collected during the rainy season (40×): **(A)** negative control with developing morula, **(B)** positive control, **(C)** *Ancylostoma caninum*, **(D)** *Haemonchus placei*, and **(E)** cyathostomins.

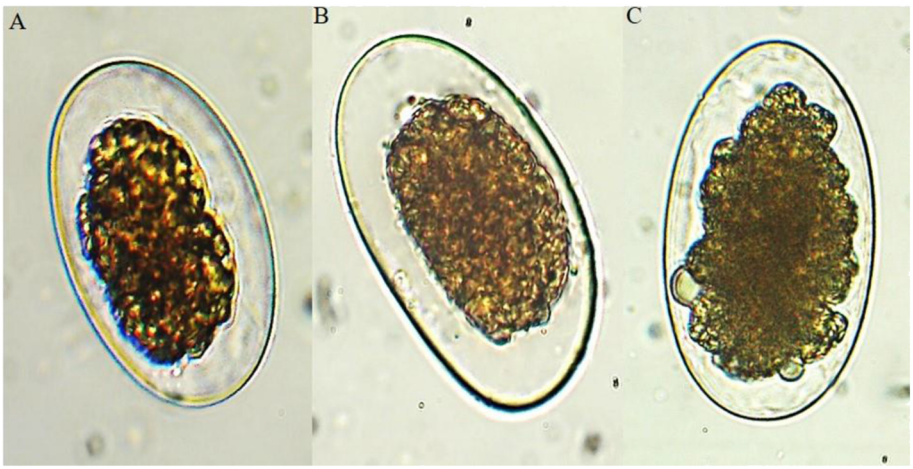


**FIGURE 2 |** Ovicidal effect of plumbagin on eggs of the evaluated gastrointestinal nematodes (40×): **(A)** *Ancylostoma caninum*, **(B)** *Haemonchus placei*, and **(C)** cyathostomins.

morula of the eggs exposed to the extract showed signs of degeneration and a dehydrated appearance (**Figure 1**) (15, 28). Specifically, the RS bark extract had a percentage of ovicidal activity (POA) of 92.5, 90.0, and 95.5% at 75 μg/ml against the eggs of *Ancylostoma caninum*, *Haemonchus placei* and cyathostomins, respectively. At 37.5 μg/ml, it also had a high POA (92.9%) against cyathostomins. On the other hand, the DS bark extract reached a POA ≥ 86% at 600 μg/ml against *Haemonchus placei* and at 300 μg/ml against *Ancylostoma caninum*; against cyathostomins, a POA of 94.2% was reached at 300 μg/ml. Using the DS bark extract, a concentration three times was required to reach the same POA as the RS bark extract.

The POAs of the leaf extracts against *Ancylostoma caninum* were lower than 22% independently of the collection season. Against *Haemonchus placei*, a POA ≥ 50% was observed at 300 μg/ml of the leaf extracts from both seasons. However, against cyathostomins, a higher POA of 97.8% was recorded with the RS leaf extract (150 μg/ml) and 94.2% with the DS leaf extract (300 μg/ml).

The hexane fraction, fraction 5 and plumbagin only exerted an ovicidal effect on treated eggs, so the reported PEIs (**Tables 3**, **4**) also reflect the ovicidal effect (**Figure 2**). With the hexane fraction, a POA of 93.7 and 98% was observed at 75 μg/ml against eggs of *Ancylostoma caninum* and *Haemonchus placei*, respectively; against cyathostomins, a similar POA (96.0%) was obtained at 18.7 μg/ml. Notably, sub-fraction 5 had a POA of 95.6, 89.8, and 96.7% against *Ancylostoma caninum*, *Haemonchus placei* and cyathostomins, respectively, at 18.7 μg/ml. At only 2.3 μg/ml, plumbagin had a POA of 91.3, 92.6, and 92.4% against *Ancylostoma caninum*, *Haemonchus placei* and cyathostomins, respectively.

## DISCUSSION

Different plants of the genus *Diospyros* (family Ebenaceae) have been used in traditional medicine because of their high biological activity, including their antioxidant, anti-inflammatory, antipyretic, analgesic, antimicrobial, antifungal, antiprotozoal and insecticidal activities (33–36). In particular, *D. anisandra* has been attributed with numerous properties, including antimicrobial activity against multi-resistant strains of *Mycobacterium tuberculosis*; ixodicidal activity against the tick *Rhipicephalus microplus*; antiviral potential against the influenza virus; and anthelmintic activity (13–15, 32, 37).

Despite the anthelmintic potential of *D. anisandra*, previous studies have mostly focused on a single genus of GIN. So, the question remained as to whether the plant extracts of *D. anisandra* had wide-spectrum activity against two or more genera of GINs affecting different animal species. Hence, in the present study, the anthelmintic activity of *D. anisandra* was evaluated against three genera of GINs belonging to the order strongylida. Also, a modification was made to the extract dilution technique used by Arjona-Cambranes et al. (16) and Flota-Burgos et al. (15), adding 5% absolute ethanol to the solvent and using an ultrasonic bath to ensure the complete dilution of the extracts and augment their activity (38, 39).

Arjona-Cambranes et al. (16) evaluated *D. anisandra* extract against *Ancylostoma caninum* eggs and reported that the RS bark extract had a PEI of 94.1% at 1,200 μg/ml., whereas the DS bark extract had a 98.7% at 2,400 μg/ml. The leaf extracts had a PEI ≥ 90% at 2,400 μg/ml independently of the season when plant materials were collected. In contrast, in the present study, higher PEIs were obtained for a RS bark extract 15 times less concentrated (≥ 95% at 75 μg/ml) and a DS bark extract 6 times less concentrated (≥ 94% at 300 μg/ml). With respect to the RS and DS leaf extracts, a low PEI (25.9 and 5.3%, respectively) was obtained at 600 μg/ml against *Ancylostoma caninum* Notably, Arjona-Cambranes et al. (16) only used PBS as a solvent, which could have influenced the dissolution of the extracts and the obtained PEIs. Chagas (39) mentioned the importance of using suitable solvents for evaluating plant extracts *in vitro*, suggesting that an unsuitable solvent could be toxic to eggs, resulting in false positives and thereby masking the real effect of the extract. Meanwhile, (40) advised that the potential effects of plant extracts might be underestimated or discarded due to external factors, such as the poor dilution of extracts in unsuitable solvents, which could result in the rejection of potential alternative sources of anthelmintic agents.

The anthelmintic activity of other species of Diospyros or other representatives of the Ebenaceae family on eggs of *Ancylostoma* spp. has not been reported. The ethanolic extracts of *Canthium manii* (1,000 μg/ml), *Mikania laevigata*, *M. glomerata,* and *Euterpe edulis* (10,000 μg/ml) demonstrated a PEI of 90, 21.8, 25.9, and 21.1% (41, 42). To obtain PEIs similar to those reported in the present study, Wabo et al. (41) required at least 10 times more concentrated. On the other hand, the bark extracts of *D. anisadra* have demonstrated better results than other control alternatives for *Ancylostoma caninum* eggs, such as mushroom extracts. Hofstätter et al. (43) reported PEIs against *Ancylostoma caninum* of 59.5 to 68.3% with *Paecilomyces lilanicus* extract, 52.2 to 53.5% with *Trichoderma harzianum* extract and 56.3% with *Trichoderma virens* extract.

Several studies have evaluated the use of plant extracts for the control of *Haemonchus contortus.* For instance, the acetone extract of *Diospyros whyteana* was evaluated against *H. contortus* eggs and was shown to have great potential for inhibiting eclosion, with a $LC_{50}$ range of 73.77 to 175.2 μg/ml, even though the PEI and the observed effect on eggs were not reported (44). Luka et al. (45) administered *in vivo* the ethanolic extract of *D. mespiliformis* to sheep and observed a 34.05 and 55.08% reduction in the excretion of *H. contortus* eggs at doses of 100,000 and 200,000 μg/kg, respectively. Ngaradoum et al. (46) evaluated the methanolic extract of *Ziziphus mucronate* bark, obtaining a PEI of 40 to 50% on eggs at 4,000 μg/ml. De Jesús-Martínez al. (47) examined the methanolic extracts of *Caesalpinia coraria* fruits, observing high ovicidal activity and a PEI > 98% at 780 μg/ml. Váradyová et al. (48) found that the methanolic extract of *Artemisia absinthium* had an ovicidal activity of 100% against eggs at 1,024 μg/ml. Notably, in the present study, the RS bark extract of *D. anisandra* demonstrated a high PEI (95.5% at 75 μg/ml) against *Haemonchus placei* at lower concentrations than the previous methanolic extracts.

With respect to cyathostomins, Flota-Burgos et al. (15) evaluated the methanolic extracts of *D. anisandra* collected during the RS and DS against these parasites, reporting a PEI of 95% from 37.5 μg/ml, similar to the results of the present study. No other species of *Diospyros* or member of the same family (Ebenaceae) has been reported to exert anthelmintic activity against cyathostomins. However, the anthelmintic activities of the extracts of *Acacia baileyana*, *A. melanoxylon*, *A. podalyriifolia*, *Alectryon oleifolius*, *Duboisia hopwodii*, *Eucalyptus gomphocephala* and *Santalum spicatum* were evaluated against cyathostomin eggs and shown to have a PEI of 100% at 1,400 μg/ml (49). Meanwhile, Peachey et al. (31) documented the anthelmintic activities of *Acacia nilotica*, *Cucumis prophetarum* and *Allium savitum*. Concentrations of 1,900 μg/ml were required to obtain a PEI higher than 90%. In this latter study, a high PEI (96.4%) was reported for cyathostomins at 37.5 μg/ml, around half the concentration required to reach a similar PEI for *Ancylostoma caninum* and *Haemonchus placei*. Despite the evaluated GINs belonging to the same order (strongylida) and sharing the same basic structure of the egg membrane, there is considerable variability in the thickness, composition and vulnerability of eggs, which can explain the differing degrees of susceptibility among GINs (50, 51).

It is also important to highlight that the ovicidal activity of the methanolic extracts of *D. anisandra* in the present study was similar to that of thiabendazole (52). No significant differences ($P > 0.05$) were found between the PEI of the RS bark extract at 75 μg/ml and that of thiabendazole against *Ancylostoma caninum* and *Haemonchus placei*. In the case of cyathostomins, no significant differences ($P > 0.05$) were found between the PEI of thiabendazole and that of RS and DS bark extract from 37.5 to 300 μg/ml, respectively, or the RS bark extract from 150 μg/ml and the DS leaf extract from 300 μg/ml. These results confirm that, at the aforementioned concentrations, the

methanolic extracts of *D. anisandra* can reach similar efficacies as a commercial anthelmintic such as thiabendazole.

Overall, with respect to the lethal concentrations of the *D. anisandra* extracts, the lowest $LC_{50}$ and $LC_{99}$ were obtained with the RS bark extract. These values significantly differed ($P < 0.05$) from the lethal concentrations obtained with the DS bark extract, RS leaf extract and DS leaf extract. Arjona-Cambranes et al. (16) reported a $LC_{50}$ and $LC_{99}$ of 500 μg/ml and 1,700 μg/ml, respectively, for RS bark extract against *Ancylostoma caninum*, which is at least seven times higher than the values reported herein for the same extract. Specifically, a $LC_{50}$ of 60.0, 43.1, and 11.3 μg/ml and a $LC_{99}$ of 76.7, 128.7, and 38.1 μg/ml were found herein for *Ancylostoma caninum*, *Haemonchus placei,* and cyathostomins, respectively. Sakong et al. (44) reported a $LC_{50}$ of 73.77 to 175.2 μg/ml against *Haemonchus contortus* with the acetone extract of *Diospyros whyteana*; this range is greater than that obtained herein with the RS bark extract of *D. anisandra* against *Haemonchus placei*, as well as against *Ancylostoma caninum* and cyathostomins. Meanwhile, Flota-Burgos et al. (15) documented a $LC_{50}$ of 10.2 μg/ml with the RS bark extract of *D. anisandra* against cyathostomins, similar to the value obtained herein ($LC_{50}$ of 11.3 μg/ml). In the present study, the $LC_{50}$ and $LC_{99}$ of the RS bark extract, RS leaf extract and DS leaf extract required for cyathostomins were the lowest of all the evaluated GINs; however, only the $LC_{50}$ and $LC_{99}$ of the RS bark extract were significantly lower. This agrees with the proposals of Bird and McClure (50) and Averlar et al. (51) concerning the structural differences and differential susceptibility of eggs, even in GINs of the same order.

In regard to the season when plant materials were collected, the RS extract had better results compared to the DS extract independently of the evaluated GIN. The concentration, composition and expression of active compounds can vary between species of the same genus, plant parts and development stages and can also be influenced by environmental factors (53, 54). Kubec and Musah (55) mentioned that the content of active compounds in plants varies depending on the climate and season of the year in which plants are collected. Valares (56) measured the flavonoid and diterpene content of the leaves and stems of *Cistus ladanifer* and found that these compounds were present in greater proportion in stems. Similarly, the total studied compounds were present in higher proportion in plants collected during the summer and in lesser proportion in plants collected during the winter. Ahmad and Mahmund (57) mentioned that plumbagin, the active compound of *Diospyros* with high biological activity, is found in greater proportion in the bark of this plant. These factors could explain the variation in PEI values and lethal concentrations obtained herein between plant parts and collection seasons.

The hexanic fraction had a higher PEI and lower $LC_{50}$ and $LC_{99}$ ($p < 0.05$) compared to the ethyl acetate and methanolic fractions, indicating that non-polar compounds are responsible for the activity of the bark extract of *D. anisandra* collected during the RS (**Table 3**). The hexanic fraction of other plants has also been reported to contain active compounds with high biological activity. For example, Rosado-Aguilar et al. (23) observed that the hexanic fraction of *Petiveria alliacea* caused the 93.6% mortality of *Rhipicephalus microplus* larvae. Other studies have shown that the compounds responsible for the biological activity of plants of the genus *Diospyros* are found in low-polarity fractions. Trongsakul et al. (33) found that the hexanic fraction of *D. variegata* Kruz exhibited a significant anti-inflammatory effect on rats in an induced oedema model as well as an antinociceptive and antipyretic effect. Meanwhile, Borges-Argáez et al. (13) reported the antifungal activity of the hexanic fraction of *D. anisandra* on *Candida albicans*, *Aspergillus niger*, and *Colletotrichum gloeosporioides.* Finally, Germann et al. (58) showed that the hexanic fraction of *D. kaki* lead to a notable reversion of resistance to multiple pharmaceuticals comparable to the efficacy of the positive control verapamil.

The presence of other bioactive compounds such as terpenoids, flavonoids, naphthoquinones, polyphenols, tannins, steroids, and coumarins has been reported in other plants of the genus *Diospyros* (34, 59, 60). In particular, the presence of naphthoquinones, a group of highly reactive phenolic compounds, can be highlighted. These are important for the development of new agrochemical pharmaceuticals because of their broad antiparasitic effect, which has recently generated increasing research interest (61). Also, the antimicrobial, antifungal, anti-inflammatory, anticarcinogenic, antiprotozoal and acaricidal activities of *Diospyros* have been attributed to the presence of the 1,4-naphthoquinones, specifically plumbagin (13, 32, 62–64).

Fetterer and Fleming (65) evaluated the activity of plumbagin on *H. contortus* and *Ascaris suum* and found that it inhibited 100% of the motility of $L_1$ larvae at a concentration of 10.0 μg/ml; however, only 44% of egg eclosion was inhibited, with non-hatched eggs being observed as partially embryonated. Likewise, plumbagin was found to cause the death of larvae ($L_1$ to $L_4$) and adults of *Caenorhabditis elegans* exposed to 100 μg/ml for 24 h. Exposure to plumbagin (25 and 50 μg/ml) also had an adverse effect on the fertility of females, decreasing up to 80% the average number of eggs laid and the further development of larvae. Against eggs, plumbagin inhibited 95% of eclosion at 100 μg/ml; however, it did not present a complete ovicidal effect since eggs were still found in several stages of development, including eggs containing $L_1$ larvae (66). These results differ from those of the present study in which plumbagin had a PEI higher than 90% from 2.3 μg/ml against the three evaluated genera of GINs and also exerted a notable ovicidal effect on treated eggs. However, it is worth noting that the dissolution of plumbagin in the studies of Fetterer and Fleming (65) and Chaweeborisuit et al. (66) was carried out using only DMSO, possibly influencing the results and reinforcing the importance of selecting suitable solvents for *in vitro* studies. Even so, Chaweeborisuit et al. (66) observed that strains of *C. elegans* resistant to levamisole, albendazole and ivermectin presented 100% mortality after exposure to 100 μg/ml of plumbagin for 24 h, reinforcing the potential value of plumbagin as an alternative control for GINs resistant to current anthelmintics.

Other major compounds found in the bark of *D. anisandra* are the triterpenes betulin and lupeol (32). Betulin and its derivatives have been shown to have anti-inflammatory, antimicrobial,

antiviral, antifungal, antimalarial, anticarcinogenic and anthelmintic activity (67–73). However, in the present study, betulin had a low PEI against the three evaluated genera of GINs ($\leq$ 5.0%). Meanwhile, lupeol has been shown to have anti-inflammatory, antitumoral, antimicrobial and antiprotozoal activity (74–77). However, lupeol similarly had a low PEI against the three evaluated GINs ($\leq$ 5.3%).

Similar to the methanolic extracts, the products obtained from the bioguided fractionation of *D. anisandra* herein, including plumbagin, had an ovicidal effect on the eggs of the three evaluated GINs. This finding agrees with the reports of Arjona-Cambranes et al. (16) and Flota-Burgos et al. (15), who also evaluated the ovicidal effects of *D. anisandra* extract on the eggs of *Ancylostoma caninum* and cyathostomins. It is important to note that several studies evaluating anthelmintic activity do not make a distinction between the type of effect observed, whether ovicidal or causing $L_1$ larvae failing eclosion. However, their data are still valuable and enable us to better understand how the active compounds of *D. anisandra* extract exert their effects. The observed ovicidal effect suggests that the plumbagin present in the extract is capable of penetrating the membrane of a high proportion of treated eggs, damaging the morula and halting the development of larvae (40, 78). From 2.3 μg/ml, plumbagin had a similar PEI as that obtained with thiabendazole against the three evaluated GINs. Although the concentrations of the *D. anisandra* extracts that obtained a PEI similar to thiabendazole are higher than the discriminating dose, GIN resistance to benzimidazoles has been widely reported. Hence, the extracts of *D. anisandra* and plumbagin are a potential control alternative with a similar efficacy to commercially available anthelmintics. Due to their strong anthelmintic activity, future studies should examine the mechanisms of action of the methanolic extracts of *D. anisandra* and plumbagin against GIN eggs within the order strongylida and carry out *in vivo* evaluations.

In conclusion, the bark extract of *D. anisandra* collected in the rainy season had the highest anthelmintic activity against eggs of *Ancylostoma caninum*, *Haemonchus placei* and cyathostomins. Plumbagin was demonstrated to be the active compound responsible for the anthelmintic activity and ovicidal effect of *D. anisandra*. Because of its wide-spectrum anthelmintic activity, *D. anisandra* extract could be a potential alternative control of different genera of GINs given the current scenario of anthelmintic resistance.

## DATA AVAILABILITY STATEMENT

All datasets presented in this study are included in the article/supplementary material.

## AUTHOR CONTRIBUTIONS

JR-A, RR-V, RB-A, and GF-B proposed the study framework, designed and developed the experiment and analyzed the results. CM-O-M and MG-A participated in the experimental design and monitored the progress of the study. All authors participated in the writing of the manuscript.

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# Nutritional Supplements Containing *Cardus mariano, Eucalyptus globulus, Gentiana lutea, Urtica urens*, and *Mallotus philippinensis* Extracts Are Effective in Reducing Egg Shedding in Dairy Jennies (*Equus asinus*) Naturally Infected by Cyathostomins

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The increasing levels of anthelmintic resistance together with the restrictions in the use of drugs in food producing animals have enforced the search for sustainable alternative approaches for parasite control. The current study aimed to investigate the safety and the efficacy of a commercially available phytotherapic formulation against gastrointestinal strongyles in donkeys. Twenty-two Ragusana jennies (2.6 ± 0.5 years old) were assigned to two equal groups. One group was treated with two doses of a phytotherapic supplement Paraxitebio® containing *Cardus mariano*, *Eucalyptus globulus*, *Gentiana lutea*, *Urtica urens*, and *Mallotus philippinensis*, 14 days apart (Group A). One group was used as negative control (Group B). Individual fecal samples were collected at the beginning of the study ($T_{-1}$), and after 7, 14, and 28 days ($T_7$, $T_{14}$, $T_{28}$). Blood samples were collected on $T_{-1}$ and $T_{28}$ in order to assess changes in donkeys' hematological profile. After the initial rise in EPG values observed on $T_7$, Group A showed a significant EPG decrease with lower eggs per gram (EPG) count compared to Group B on $T_{28}$ and an overall fecal egg count reduction of 56.9% on the same time-point. Hematological parameters were within the normal physiological ranges for enrolled donkeys. However, significant differences in the values of RBCs, Hb, MCHC, MCV, WBCs, eosinophils, and basophils were recorded between groups after phytotherapic treatments, with Group A showing a general improvement in the hemogram picture. The phytotherapic supplement used in the current study was helpful in controlling intestinal parasites allowing a significant reduction in the fecal egg count 28 days after treatment. Further studies are needed to better explore the specific mode of action of the plant-derived formulation herein tested as well as to encourage their use as tool for the control of equine strongylosis under multimodal integrated approach in dairy donkey farms.

**Keywords: donkey, Cyathostominae, *Equus asinus*, gastrointestinal parasites, phytotherapy, strongyles**

## INTRODUCTION

In recent years, donkey farming gained popularity in several countries, such as Italy, France, and Belgium, where these equids are mainly reared for milk production (1). Thanks to its special properties, donkey milk is suitable for infants who cannot be breast-fed and people suffering from cow's milk protein allergies (2–4). Donkeys reared for milk production needed to be continuously managed and monitored to maintain optimal general health conditions (5), avoiding nutritional deficiencies (6). Worldwide, gastrointestinal parasite infestations represent a major issue for donkey farming systems. As a matter of facts, helminthiasis is a serious health hazard, inducing poor body condition, poor productive performance, diarrhea, colic, and potentially death in severe cases (7).

Equids are usually coinfected with different nematode species, rather than a single helminth species (8), and cyathostomins, also known as small strongyles, often represent the 95–100% of the total worm burden. These nematodes show a cosmopolitan diffusion, and they are considered as the most important intestinal parasite group in wild and domestic equids for their pathogenic potential at both larval and adult stages (9, 10). The immature cyathostomins can encyst in the large intestinal wall, and it is thought that these stages can persist for years (11). These stages, in particular early third-stage larvae, are relatively insensitive to most anthelmintics available (12). When these larvae reemerge in large numbers from the gut wall, a fatal colitis, named larval cyathostominosis, can occur (13).

Several other nematode species infect equids, although their prevalence is usually lower than cyathostomins (14). Among non-cyathostomin species affecting equids and causing clinical disease included *Parascaris equorum*, *Strongylus vulgaris*, *S. edentatus*, *S. equinus*, and the tapeworm *Anoplocephala perfoliata* [reviewed by (15)]. Also, the pinworm *Oxyuris equi* is relatively common in equids. Donkeys are also susceptible to the fluke, *Fasciola hepatica*, which can be transmitted via snails and the environment, from ruminants. Moreover, the lungworm *Dictyocaulus arnfieldi* is relatively common in donkeys which usually show no disease and can be silent carriers and/or shedders of this parasite, which causes clinical signs in horses [reviewed by (16)].

In the past years, the control of gastrointestinal parasites was based on regular and frequent administration of anthelmintic drugs as preventive treatment strategy. However, the recurring onset of anthelmintic resistance together with the restrictions in the use of drugs in food-producing animals has enforced the search for sustainable alternative approaches for parasite control (17). Among the nutritional supplements used for the control of internal parasites in equine husbandry, promising results have been gained with the employment of plant-derived compounds (18, 19). Although many plants have been listed as having anthelmintic activity in animals (20, 21) and the use of plant-derived anthelmintics would be preferable to synthetic drugs in dairy farming, scientific data demonstrating the real efficacy of these compounds against gastrointestinal parasites are still scarce.

On the basis of the above considerations, the main goal of the current study was to assess the efficacy of 14-day interval administration of a plant-derived product against gastrointestinal nematodes in dairy donkeys. The obtained evidence-based data would improve the current knowledge on the potential use of phytotherapic products for the control of equine strongylosis under the multimodal integrated approach.

**Abbreviations:** RBCs, Red Blood Cells; WBCs, White Blood Cells; Hb, Hemoglobin concentration; Hct, Haematocrit; MCV, Mean Corpuscular Volume; MCH, Mean Corpuscular Hemoglobin; MCHC, Mean Corpuscular Hemoglobin Concentration; PLTs, Platelets; EPG, eggs per gram; FECR, fecal egg count reduction test.

## MATERIALS AND METHODS

### Animals and Study Design

The study was performed in a donkey farm located in Sicily (latitude: 37°23′10″ N; longitude: 14°41′32″ E) in July and August 2019. A total of 22 non-pregnant, non-lactating Ragusana jennies, mean age 2.6 ± 0.5 years, mean body weight 272 ± 27 kg, were enrolled in the study. The study protocol and procedures were approved by the Animal Ethics Committee of Camerino University registration number: E81AC.11/A.

All the donkeys were fed polyphyte meadow hay and had access to pasture 6 h daily, and water was provided *ad libitum*. No anthelmintic treatment had been performed over the 6 months preceding the study.

Fecal samples were collected in the morning (9.00 a.m.), directly from the rectum of each donkey on−1, 7, 14, and 28 days ($T_{-1}$, $T_7$, $T_{14}$, $T_{28}$). Samples were transported in a cooled box and analyzed within 8 h from collection. Blood samples (5 mL) were collected by jugular venipuncture into K3-EDTA anticoagulant tubes before phytoterapic administration ($T_{-1}$) and at the end of the experimental period ($T_{28}$). EDTA whole blood samples were delivered to the laboratory and processed within 2 h.

The body weight of each donkey was measured by means of a weighting platform (PS3000HD Heavy Duty Floor Scale, Breckwell, UK).

Enrolled donkeys were firstly grouped into blocks according to their age, body weight, and fecal egg count estimated on $T_{-1}$ and then randomly allocated to two homogenous groups (i.e., Group A and Group B) of 11 animals each.

Animals of Group A were treated with the commercially available phytotherapic product PARAXITEBIO® (BIOEQUIPE SRL, Lombardy, Italy) composed by standardized extracts of *Cardus mariano*, *Eucalyptus globulus*, *Gentiana lutea*, *Urtica urens*, and *Mallotus philippinensis*, and by analytical components including crude protein (0.62%), crude fat (0.22%), crude fiber (0.09%), crude ash (0.32%), moisture (91.32%), and nitrogenous extracts (7.43%).

According to the manufacturer's instructions, the product was administered two times at fortnight interval (i.e., $T_{-1}$, $T_{14}$) using the dose of one syringe (50 g) per donkey.

Donkeys included in Group B were left untreated and served as negative control animals. During the study, the animals were subjected to clinical examinations at each sampling and at the

treatment days ($T_{-1}$, $T_7$, $T_{14}$, $T_{28}$). The donkeys included in Group A were observed for 12 h after the administration of the phytotherapic supplement in order to record any side effects potentially related with the treatment.

## Hematological Analysis

On blood samples, Red Blood Cells (RBCs), White Blood Cells (WBCs), hemoglobin concentration (Hb), hematocrit (Hct), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), and platelets (PLTs) were assessed with an automated hematology analyzer (HeCo Vet C, SEAC, Florence, Italy).

For each blood sample, two smears were done and, after air drying the obtained slides, were stained by MGG Quik stain kit (Bio-Optica Milano s.p.a., Milan, Italy). After washing the excess dye from the blood smears and air-drying, the slides were viewed under oil immersion at 100× by using an optical microscope (Nikon Eclipse Y100; Nikon Instruments Europe BV, Amsterdam, The Netherlands). A manual 100-cell differential count on each blood film was performed by the same laboratory professional. For each animal, the leukocyte differential count was calculated by averaging the data recorded from each blood film of the same sample, and the percentage of lymphocytes, neutrophils, monocytes, basophils, and eosinophils was reported.

## Coprological Examinations and Fecal Egg Count Reduction Test

Fecal egg counts were performed on individual fecal sample by Mini-FLOTAC® technique according to Noel et al. (22) and Went et al. (23). Briefly, five weighed grams of feces were placed into a Fill-FLOTAC homogenizer and suspended in 45 mL of the NaCl flotation medium (specific gravity 1.25). After homogenization, the liquid was transferred into the two 1-mL chambers on Mini-FLOTAC slides. After a 10-min wait time, the top piece of the reading disc was rotated allowing the translocation of the floating eggs to lecture area of the chambers and their counting under 10× magnification (24, 25).

At each time point, two pooled fecal samples per group were incubated at 25°C for 7–10 days for larval development. Third-stage larvae (L3) were recovered using the Baermann–Wetzel technique and identified at species level according to morphological keys proposed by Cernea et al. (26) and by Bowman et al. (27). When a coprocolture had 100 or less L3, all were identified; when a coprocolture had more than 100 L3, only 100 were identified.

Mean values of eggs per gram (EPG) obtained from individual fecal samples on $T_{-1}$, $T_{14}$, and $T_{28}$ were used to estimate the fecal egg count reduction test (FECR) using guidelines established by the World Association for Advancement of Veterinary Parasitology (28) and by the American Association of Equine Practitioners (AAEP) guidelines (29). FERC was calculated according to the formula:

$$\mathrm{FECR}(\%) = 100[(\mathrm{C} - \mathrm{T})/\ \mathrm{C}]$$

where C is the geometric mean of EPG before the treatment and T is the geometric mean of EPG after the treatment. The geometric mean was calculated by averaging the log counts (x+1) of the single EPG values, taking the anti-logarithm and then subtracting 1.

## Statistical Analysis

All data were expressed as mean values ± standard deviation (±SD).

Data were tested for normality using the Shapiro–Wilk normality test. A normal distribution of the data was found ($P > 0.05$). Two-way repeated-measures analysis of variance (ANOVA) was applied to evaluate the possible significant effects of the phytoterapic treatment and time on the values of EPG and hematological parameters. When significant differences were found, Bonferroni *post-hoc* comparisons were conducted. *P*-values $< 0.05$ were considered statistically significant. Data were analyzed using statistical software program Prism v. 5.00 (GraphPad Software Ltd., CA, USA).

# RESULTS

None of the animals included in the study showed clinical signs of disease during the experimental period. No adverse reaction nor side effects were observed in animals of Group A following phytotherapic treatments. As shown in **Table 1**, no differences in body weight values between the two groups were observed at each study time-point.

EPG values showed no difference between Groups A and B on $T_{-1}$, whereas dynamic changes were observed between the two groups at subsequent time-points and in Group A following plant-derived anthelmintic administration (**Table 1**).

In Group A, EPG values were higher ($P < 0.001$) on $T_7$ than $T_{-1}$ and $T_{14}$, and on $T_{28}$ compared to $T_{-1}$, $T_7$, and $T_{14}$. FECR percentages calculated for Group A were −21.6% on $T_{14}$ and 56.9% on $T_{28}$. The EPG values recorded in Group B showed an unchanged trend for most of the study, but not on $T_{28}$ whose values resulted statistically higher ($P < 0.05$) than on $T_{-1}$. Statistical analysis highlighted a positive significant effect of treatment on $T_{28}$ ($P < 0.001$) with Group A showing lower EPG values compared to Group B.

All the L3 harvested at pooled coprocultures carried out on fecal samples of Group A and Group B were identified as Cyathostominae (*Trichonema* spp. and *Poteriostomum* spp.) (**Table 2**).

As shown in **Table 3**, hematological parameters evaluated at the beginning and at the end of the study fall within the physiological ranges for donkeys (30). However, significant differences were found for some of these parameters between groups A and B on $T_{28}$. In particular, RBC, Hb, and MCHC values were higher in Group A with respect to Group B on $T_{28}$ ($P < 0.05$), whereas MCV, WBC, and eosinophil values were lower in donkeys from Group A compared to Group B on $T_{28}$ ($P < 0.001$). An increase in RBC values and a decrease in MCV values were found in Group A on $T_{28}$ vs. $T_{-1}$ ($P < 0.01$), while an opposite trend was observed in Group B with lower RBC, Hb, and MCHC values, and higher MCV levels ($P < 0.05$) on $T_{28}$ vs. $T_{-1}$.

**TABLE 1 |** Mean values and standard deviation (SD) of egg per gram of feces (EPG) and body weight (BW) recorded in treated (Group A, $n = 11$) and untreated (Group B, $n = 11$) donkeys at study time-points ($T_{-1}$-$T_{28}$).

| | $T_{-1}$ | | $T_7$ | | $T_{14}$ | | $T_{28}$ | |
|---|---|---|---|---|---|---|---|---|
| | **EPG ± SD (Range)** | **BW ± SD (Range)** | **EPG ± SD (Range)** | **BW ± SD (Range)** | **EPG ± SD (Range)** | **BW ± SD (Range)** | **EPG ± SD (Range)** | **BW ± SD (Range)** |
| Group A | 634.5 ± 280.5 (200–1000) | 264.5 ± 29.8 (210–310) | 1157.7 ± 429.9 (590–2250) | 267.7 ± 28.4 (215–310) | 612.7 ± 199.8 (315–950) | 270.5 ± 26.8 (220–310) | 272.5 ± 84.4 (150–375) | 271.4 ± 26.8 (225–315) |
| Group B | 629.1 ± 287.0 (224–985) | 280.0 ± 26.1 (250–330) | 806.4 ± 475.8 (200–1600) | 278.2 ± 26.0 (250–330) | 745.0 ± 337.7 (250–1150) | 275.0 ± 32.2 (230–320) | 835.0 ± 371.3 (350–1400) | 274.5 ± 32.7 (230–320) |

*Animals in Group A were treated with a phytoterapic product on $T_{-1}$ and $T_{14}$ using the same dose rate.*

**TABLE 2 |** Cyathostominae third-stage larvae developed in pooled fecal samples of treated (Group A, $n = 11$) and untreated (Group B, $n = 11$) donkeys at study time-points ($T_{-1}$-$T_{28}$).

| **Cyathostominae species** | | $T_{-1}$ (%) | $T_7$ (%) | $T_{14}$ (%) | $T_{28}$ (%) |
|---|---|---|---|---|---|
| *Trichonema* spp. | Group A | 97.5 | 99.5 | 99.4 | 100 |
| | Group B | 96.5 | 97.5 | 98.5 | 97.5 |
| *Poteriostomum* spp. | Group A | 2.5 | 0.5 | 0.6 | 0 |
| | Group B | 3.5 | 2.5 | 1.5 | 2.5 |

*Animals in Group A were treated with a phytoterapic product on $T_{-1}$ and $T_{14}$ using the same dose rate.*

A significant decrease in WBC, eosinophil, and basophil values together with an increase in lymphocyte number were found on $T_{28}$ vs. $T_{-1}$ in Group A ($P < 0.001$). No time-dependent change was found in the leukocyte population of Group B ($P > 0.05$).

## DISCUSSION

The current study provides data on the usefulness of a commercially available phytoterapic supplement to control intestinal strongyle infection in donkeys.

Few published *in vivo* data on the use of phytotherapeutic drugs against gastrointestinal parasites in equids are available in scientific literature, and unsatisfactory results are often found (31). To the best of our knowledge, only one study by Papini et al. (32), investigated the effect of a plant-derived product against gastrointestinal parasites in donkeys but no efficacy has been observed in that report. On the contrary, the phytotherapic supplement used in the current study allowed a 56.9% reduction of intestinal strongyle egg shedding in naturally infected donkeys treated two times at fortnight interval (i.e., T-1, T14).

Recently, the aqueous extracts of *Achillea millefolium* L. (flowers), *Artemisia absinthium* L. (aerial parts), *Centaurium erythraea* Rafn. (flowers), *Gentiana asclepiadea* L. (rhizomes and roots), *Inula helenium* L. (rhizomes and roots), and *Tanacetum vulgare* L. (aerial parts) have been tested *in vitro* for their potential ovicidal and larvicidal activity against donkey nematodes. Except for *C. erythraea*, all tested plant extracts showed significant anthelmintic effects against donkey gastrointestinal nematodes (33). Also, the efficacy of a plant compound containing *Medicago* saponins was tested *in vitro* showing a 90% hatching reduction of donkey gastrointestinal parasite eggs (34). However, the real deworming effectiveness of the *Medicago* saponins has not been assessed in *in vivo* studies, and many factors related to the host and nematode species may alter the bioavailability of the active compound. For instance, host pharmacokinetics may limit the amount of active ingredient reaching the nematodes. The only *in vivo* study carried out on horses infected by strongyles showed that the garlic-derived compound has no effect in reducing the egg shedding (31).

The analysis of pooled coprocoltures indicated small strongyles (Cyathostominae) as the only gastrointestinal parasites infecting the studied donkey population. Specifically, *Trichonema* spp. were found at higher percentage (>96%) compared to *Poteriostomum* spp. (<4%). No difference in Cyathostominae species was found between treated and control groups on $T_{-1}$ and $T_{14}$. Nevertheless, on $T_{28}$ only *Trichonema* spp. were found in coprocoltures of treated animals. Although only these two Cyathostominae genera have been identified in the studied population, the real presence of gastrointestinal strongyle species may have been suffered for underestimation, since only one-hundred third-stage larvae were identified for each coprocolture. As a consequence, the less spread species belonging to the Cyathostominae subfamily (35–37) may not have been included in the one-hundred larvae identified.

At the beginning of the study, both groups showed very similar EPG mean values, while after two administrations of the plant-derived anthelmintic supplement, a significant reduction in EPG values was observed in Group A (i.e., 272.5 ± 84.4) compared to control Group B (i.e., 835.0 ± 371.3). The results herein gained showed a dynamic EPG trend in treated donkeys. In particular, a significant increase in EPG values was found 7 days after the first treatment compared to the starting pretreatment values, whereas EPG values recorded 14 days after the first product administration showed a decrease with respect to $T_7$. Interestingly, a notable EPG reduction was recorded after the second phytoterapic administration estimated on the final time point ($T_{28}$). In the current study, the efficacy of the phytoterapic supplement was evaluated using the method proposed by Nielsen et al. (29) for the synthetic anthelmintic drugs, based on the percentage of egg reduction in fecal samples before and after treatment. The FECR value obtained in the current study is lower compared to other anthelmintic drugs

**TABLE 3 |** Mean values ± standard deviation (M ± SD) of red blood cells (RBCs), hemoglobin (Hb), hematocrit (Hct), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), white blood cells (WBCs) together with the leukocyte sub-population percentages, and platelets (PLTs) determined in treated (Group A, $n = 11$) and untreated (Group B, $n = 11$) donkeys before the first administration of the phytoterapic product ($T_{-1}$), and at the end of the trial ($T_{28}$).

| Parameters | | $T_{-1}$ M ± SD | $T_{28}$ M ± SD | Reference range for donkey species[b] |
|---|---|---|---|---|
| RBCs ($\times 10^6/\mu L$) | Group A | 5.6 ± 1.0 | 6.4 ± 0.8*[a] | 4.7–9.0 ($\times 10^6/\mu L$) |
| | Group B | 6.2 ± 1.1 | 5.3 ± 1.0[a] | |
| Hb (g/dL) | Group A | 10.7 ± 1.2 | 11.0 ± 1.3* | 10.6–18.9 (g/dL) |
| | Group B | 10.5 ± 1.7 | 9.6 ± 0.9[a] | |
| Hct (%) | Group A | 34.5 ± 4.9 | 34.8 ± 4.9 | 34–49 (%) |
| | Group B | 35.5 ± 5.3 | 36.0 ± 5.2 | |
| MCV (fL) | Group A | 61.4 ± 2.5 | 54.3 ± 6.1*[a] | 46–67 (fl) |
| | Group B | 59.8 ± 5.2 | 69.4 ± 11.0[a] | |
| MCH (pg) | Group A | 19.4 ± 4.1 | 17.1 ± 1.3 | 16–23 (pg) |
| | Group B | 17.2 ± 0.7 | 18.6 ± 3.1[a] | |
| MCHC (%) | Group A | 32.4 ± 5.4 | 31.7 ± 2.8* | 32–36 (%) |
| | Group B | 28.9 ± 2.3 | 26.8 ± 2.3 | |
| WBCs ($\times 10^3/\mu L$) | Group A | 10.8 ± 1.5 | 8.9 ± 0.9*[a] | 5.4–15.5 ($\times 10^3/\mu L$) |
| | Group B | 11.2 ± 2.3 | 12.0 ± 1.5 | |
| Lymphocytes (%) | Group A | 40.3 ± 2.8 | 47.1 ± 6.4[a] | 19–67 (%) |
| | Group B | 40.0 ± 6.3 | 42.8 ± 6.9 | |
| Neutrophils (%) | Group A | 42.0 ± 6.2 | 45.3 ± 5.5 | 23–69 (%) |
| | Group B | 42.2 ± 5.2 | 43.8 ± 6.6 | |
| Monocytes (%) | Group A | 1.4 ± 1.0 | 1.6 ± 1.3 | 0–11 (%) |
| | Group B | 2.2 ± 1.3 | 2.0 ± 1.7 | |
| Eosinophils (%) | Group A | 14.3 ± 6.0 | 5.5 ± 2.8*[a] | 0–14 (%) |
| | Group B | 13.9 ± 2.7 | 10.1 ± 4.0 | |
| Basophils (%) | Group A | 2.0 ± 1.4 | 0.5 ± 0.3[a] | 0–1.4 (%) |
| | Group B | 1.7 ± 1.1 | 1.3 ± 0.8 | |
| PLTs ($\times 10^3/\mu L$) | Group A | 266.1 ± 70.5 | 278.7 ± 55.5 | 160–584 ($\times 10^3/\mu L$) |
| | Group B | 268.8 ± 64.7 | 273.3 ± 51.6 | |

*Animals in Group A were treated with a phytoterapic product on $T_{-1}$ and $T_{14}$ using the same dose rate.*

**Statistically significant different vs. Group B ($P < 0.05$).*

*[a]Statistically significant different vs. $T_{-1}$ ($P < 0.01$).*

*[b]Weiss and Wardrop (30).*

tested in donkeys as ivermectin (96%) (38) and eprinomectin (99%) (39); moreover, according to the AAEP parasite control guidelines, the FECR value herein found is lower than the suggested cutoff values for interpreting results of strongyle FECR in horse (Fenbendazole/Oxibendazole, >95%; Pyrantel, >90%; Ivermectin/Moxidectin, >98%). However, the egg shedding reduction over the fifty percent suggests a potential of the phytoterapic product herein tested as a useful tool for the control of intestinal strongyles in dairy donkeys under the multimodal integrated approach. Furthermore, these findings suggest that the potential anthelmintic efficacy of *Cardus mariano*, *Eucalyptus globulus*, *Gentiana lutea*, *Urtica urens*, and *Mallotus philippinensis* extracts is worthy of investigation also in other equids such as horses, where alternative strategies to chemical products are strongly demanded.

The plant extracts contained in the tested supplement are known to have several anthelmintic properties thanks to their content in terpenoids, steroids, flavonoids, coumarins, and phenols. Particularly, the anthelmintic activity of *Eucalyptus globulus* extracts was investigated *in vivo* in naturally infected sheep showing a FECR of 66% 21 day post treatment (40). *Gentiana lutea* extracts have properties stimulating the immune system and mid-level validity as anthelmintic (41), whereas *Carduus marianus* extracts have choleretic and hepatoprotective actions. Antioxidant, antiviral, and cytotoxic activities have been recognized to the extracts of *Mallotus philippinensis* (42). Indeed, *M. philippinensis* is traditionally used for antifilarial (43), antibacterial, anti-inflammatory, immune regulatory (44), purgative, and anthelmintic (45–47) activities. Scientific reports on the phytochemical analysis of *Urtica urens* have revealed compounds exhibiting anthelmintic, antiviral, immunomodulatory, antioxidant, and anti-inflammatory activities (48–50).

Despite the well-recognized properties of the plant extracts contained in the phytotherapic supplement herein tested, the mode of action of these plant compounds on gastrointestinal nematodes is not well-established. As suggested for other plants constituents like *Allium sativum* extracts, the mode of action

could be involved in stimulating the host's immune system in controlling parasites rather than directly killing the nematodes. This hypothesis could explain the rise in EPG values recorded 1 week after the first supplement administration that may reflect an adaptive response of adult nematodes to the changes occurring in their habitat as a result of the potentiated host immune response. The hypothesis that plant-derived supplement used in the current survey can play a role in the immunomodulation of the host seems to be encouraged by the hematological profile shown by treated animals.

Hematological parameters evaluated in the control group and in treated animals before and after phytoterapic product administration fall within the physiological ranges for donkeys. However, at the beginning of the study, in both groups the values of Hb, Hct, MCH, and MCHC were close to the lower limit of the reference range, while the values of MCV, WBCs, eosinophils, and basophils were close to the upper limit of the reference range established for donkey species (30). As previously observed in a mule infected by Cyathostominae (51), also the hemogram picture obtained in this study could be related to parasitism. In fact, the high values of eosinophils and basophils could indicate an active inflammatory response to parasitic invasion and larval migration (51–53). Noteworthily, after the second administration of the phytotherapic supplement, treated donkeys exhibited an improvement of the general hemogram picture on $T_{28}$ with a slight increase in RBC and Hb values and a marked decrease in eosinophil and basophil number, suggesting a moderate attenuation of inflammatory response.

According to the above findings, it can be speculated that the phytotherapic supplement used in the current study has an immunomodulatory effect on host's immune response either by attenuating inflammation or by increasing the host's ability to cope with parasitic growth and proliferation.

## CONCLUSIONS

Nowadays, the recurring onset of resistance to existing drugs by intestinal strongyles of equids and the increased public awareness for drug residues in animal products compel the scientific community to investigate novel strategies to control parasitic diseases in domestic animals.

According to the findings herein obtained, the use of phytotherapic supplement could represent a useful tool in the integrated/biological control of parasites in dairy donkeys. Two administrations of phytotherapic supplement at fortnight interval were successful in reducing 56.9% intestinal strongyle egg shedding in naturally infected donkeys, causing no adverse reaction in treated animals throughout the experimental period. Further studies are, however, needed to better explain the mode of action of plant-derived products and to suggest their proper employment as tool for the control of equine strongylosis under the multimodal integrated approach.

## DATA AVAILABILITY STATEMENT

All datasets presented in this study are included in the article/supplementary material.

## ETHICS STATEMENT

The study protocol and procedures were approved by the Animal Ethics Committee of Camerino University registration number: E81AC.11/A. Written informed consent was obtained from the owners for the participation of their animals in this study.

## AUTHOR CONTRIBUTIONS

FA, MB, and EB conceived and designed the study. MB performed the veterinary examinations and sampling. FA and GG carried out the laboratory work. FA drafted the first version of the manuscript. MB, EB, GG, BT, AP, and FL critically reviewed the manuscript. All the authors read and approved the final manuscript.

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# Licochalcone a Exhibits Leishmanicidal Activity *in vitro* and in Experimental Model of *Leishmania* (*Leishmania*) *Infantum*

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The efficacy of Licochalcone A (LicoA) and its two analogs were reported against *Leishmania (Leishmania) amazonensis* and *Leishmania (Leishmania) infantum in vitro*, and in experimental model of *L. (L.) infantum in vitro*. Initially, LicoA and its analogs were screened against promastigote forms of *L. (L.) amazonensis*. LicoA was the most active compound, with $IC_{50}$ values of 20.26 and 3.88 μM at 24 and 48 h, respectively. Against amastigote forms, the $IC_{50}$ value of LicoA was 36.84 μM at 48 h. In the next step, the effectivity of LicoA was evaluated *in vitro* against promastigote and amastigote forms of *L. (L.) infantum*. Results demonstrated that LicoA exhibited leishmanicidal activity *in vitro* against promastigote forms with $IC_{50}$ values of 41.10 and 12.47 μM at 24 and 48 h, respectively; against amastigote forms the $IC_{50}$ value was 29.58 μM at 48 h. Assessment of cytotoxicity demonstrated that LicoA exhibited moderate mammalian cytotoxicity against peritoneal murine macrophages; the $CC_{50}$ value was 123.21 μM at 48 h and showed about 30% of hemolytic activity at concentration of 400 μM. *L. (L.) infantum*-infected hamsters and treated with LicoA at 50 mg/kg for eight consecutive days was able to significantly reduce the parasite burden in both liver and spleen in 43.67 and 39.81%, respectively, when compared with negative control group. These findings suggest that chalcone-type flavonoids can be a promising class of natural products to be considered in the search of new, safe, and effective compounds capable to treat canine visceral leishmaniosis (CVL).

**Keywords: cutaneous leishmaniasis, visceral leishmaniasis, canine visceral leishmaniasis, licochalcone A, leishmanicidal activity**

## INTRODUCTION

Leishmania parasites are the etiological agents that cause leishmaniasis, and the parasites are transmitted by the bite of infected female phlebotomine sandflies (1). Human disease is classified into three main clinical forms: cutaneous leishmaniasis (CL), mucocutaneous leishmaniasis (MCL), and visceral leishmaniasis (VL) (1). CL is the most common form of the disease, and the manifestations can depend of the parasite species and host immune responses (2). *Leishmania (Viannia) braziliensis* and *Leishmania (Leishmania) amazonensis* are the main species responsible by CL in Brazil, and also by diffuse cutaneous leishmaniasis (DCL) (2). On the other hand, VL exists in two forms, zoonotic and anthroponotic, which are caused by *Leishmania (Leishmania) infantum* and *Leishmania (Leishmania) donovani*, respectively (3). Among the different mammal species, dogs can be considered as the main reservoirs of *L. (L.) infantum,* and this parasite also is responsible by canine visceral leishmaniasis (CVL) (4). Due the extension of CVL in urban regions in South American countries, this parasite has the attention of public health and the scientific community (4, 5).

The chemotherapy of leishmaniasis on human depends on three drugs, pentavalent antimonials, such as meglumine antimoniate (MA), amphotericin B (AmpB), and miltefosine (6). These drugs have high cost, limited efficacy, long administration protocols, severe side-effects, and development of drug resistance upon repeated use. In relation on CLV, miltefosine is an alternative for treatment of animals in regions where this drug is not used in humans; however, studies shown that miltefosine reduces the parasitic load of dogs infected with *L. (L.)infantum,* but no parasitological cure (4, 7, 8).

Licochalcone A (LicoA, **1**) (**Figure 1**) is a naturally occurring chalcone-type flavonoid isolated from roots of the Chinese licorice (*Glycyrrhiza inflata*) that can be considered an antiparasitic hit compound (9). It can chemically describe as an α,β-unsaturated bisphenylic ketone (chalcone skeleton) substituted with two phenolic hydroxyl groups, one methoxy moiety, and an isoprenoid side chain. Previous works described the significant *in vitro* and *in vivo* effect of LicoA against *Leishmania (L.) major* and *L. (L.) donovani*, which are, respectively, associated with the CL and VL forms (10, 11). Another chalcone-type flavonoid compound from licorice roots extracts is Echinatin (**2**) (**Figure 1**). It has been reported to have a broad range of bioactivities, such as hepatoprotective (12) anti-inflammatory, antioxidant (13, 14), antimicrobial (15), and cardioprotective (16).

Considering that LicoA has demonstrated its potential against other forms of the parasite and that other analogs of LicoA could also present promising results, the aim of this study is to evaluate the *in vivo* leishmanicidal effect of LicoA and its two analogs against *L. (L.) amazonensis* and *Leishmania (L.) infantum in vitro* and in an experimental model of *Leishmania (Leishmania) infantum* in hamster. The analogs were synthesized and constitute structures similar to the natural products LicoA (**1**) and also Echinatin (**2**) (**Figure 1**). The analog LLA1 (**1a**) (**Figure 2**) is a derivate of LicoA that belongs to the class of chalcones containing the group *O*-prenil (17). In another study, the evaluation of a series of prenyloxy and geranyloxy chalcones against *Leishmania (Leishmania) mexicana* and *Trypanosoma cruzi* demonstrated that the position of the substituent has an influence on the activity and selectivity of these compounds (18). The antiparasitic activity of the analog LLA2 (**2a**) (**Figure 2**) has not been investigated yet.

## MATERIALS AND METHODS

### Reagents and Compounds

The reagents and solvents used in the synthesis of the analogs of LicoA (LLA1 **1a** and LLA2 **2a**) were obtained from commercial sources and used directly without further purification. $NbCl_5$ was supplied by Companhia Brasileira de Metalurgia e Mineração (CBMM). Thin layer chromatography (TLC) was performed on Sigma-Aldrich silica gel matrix, pre-coated plates with fluorescent indicator 254 nm (Sigma-Aldrich, St Louis, USA). $^1H$ and $^{13}C$ NMR spectra were recorded on Varian equipment (500 MHz) from Federal University of ABC or Bruker equipment (DPX-300 and DPX-400) from University of São Paulo, Ribeirão Preto-SP, using the solvent deuterated chloroform ($CDCl_3$) or dimethylsulfoxide (DMSO-d6). HRMS were obtained in instrument micrOTOFfrom University of São Paulo, Ribeirão Preto-SP. AmpB was purchased from Sigma-Aldrich, and MA (Glucantime®) was obtained from Sanofi-Aventis (Sanofi-Aventis, São Paulo, Brazil), which each 5 mL of aqueous solution contained 1.5 g of MA and represented 405 mg of pentavalent antimonial (Sanofi-Aventis).

### Chemistry

#### Isolation of LicoA (1)

The isolation and identification of LicoA was performed as previously described (19) and its chemical structure was identified by $^1H$- and $^{13}C$-NMR analysis data in comparison with the literature (20). Purity of LicoA (**1**) was estimated to be higher than 95% by both $^{13}C$ NMR and HPLC analysis using different solvent systems.

#### Synthesis of Compound LLA1 (1a) and LLA2 (2a) (Scheme 2)

***3-methoxy-4-((3-methylbut-2-en-1-yl)oxy)benzaldehyde (4)***

The potassium carbonate was previously macerated with a mortar and pistil. Vanillin (**3**) (0.4526 g; 3 mmol), potassium carbonate (1.6970 g; 12 mmol), prenyl bromide (0.353 mL, 3 mmol), tetrabutyl ammonium chloride (0.0816 g; 0.3 mmol), and dichloromethane (DCM) were added to a round-bottom flask. The reaction was kept under stirring and at room temperature. The reaction was monitored by TLC until the practically total consumption of vanillin was verified (26 h). Then, the material was dissolved in dichloromethane and transferred to a separating funnel. The organic layer was separated and the aqueous layer extracted twice with dichloromethane. The combined organic extracts were washed with brine and the solution obtained was dried over anhydrous magnesium sulfate, filtered, and the solvent was evaporated under reduced pressure. The crude product obtained was purified over silica gel column chromatography using the mixture of 80% *n*-hexane in ethyl acetate as eluent to

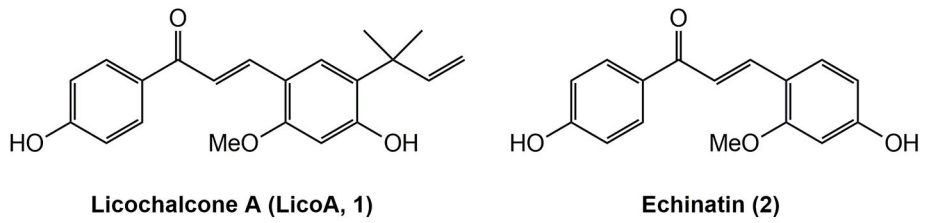


**FIGURE 1 |** Structures of natural products Licochalcone A (LicoA **1**) and Echinatin (**2**).

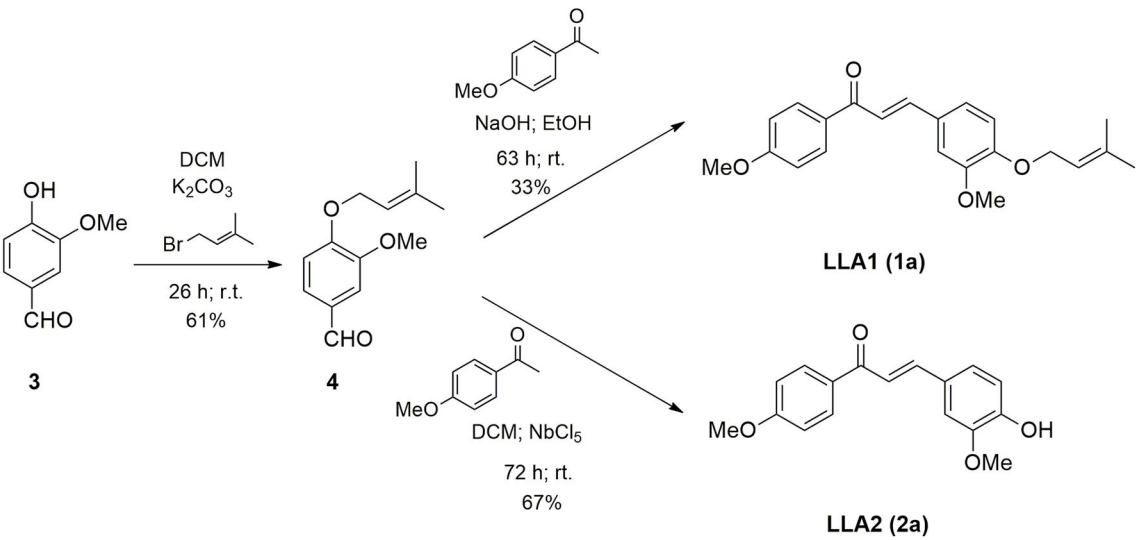


**FIGURE 2 |** Scheme of synthesis of LLA1 **(1a)** and LLA2 **(1a)**.

yield 0.4007 g of the pure product (61%) of **4**. **$^1$H-RMN ($CDCl_3$, 300 MHz) δ:** 1.75 (d, 3H, $J = 1.2$ Hz); 1.80 (d, 3H, $J = 1.2$ Hz); 4.58 (d, 2H. $J = 6.7$ Hz); 5.48 (tsept, 1H, $J = 6.7$ Hz; $J = 1.2$ Hz); 7.00 (d, $J = 8.7$ Hz); 7.82 (d, $J = 8.7$ Hz); 9.87 (s, 1H). **$^{13}$C-RMN ($CDCl_3$, 75 MHz) δ:** 18.18 ($CH_3$), 25.72 ($CH_3$); 65.17 ($CH_2$); 114.95 (CH); 118.87 (CH); 129.86 (C); 131.88 (CH); 138.89 (C); 163.98 (C), 190.64 (CH).

### *(E)-3-(3-methoxy-4-((3-methylbut-2-en-1-yl)oxy)phenyl)-1-(4-methoxyphenyl)prop-2-en-1-one (LLA1 1a)*

To a round-bottom flask, sodium hydroxide (0.0462 g; 0.9 mmol), ethanol (4 mL), and *p*-methoxyacetophenone (0.0754 g; 0.45 mmol) were added. The mixture was kept in an ice bath for 20 min. Then, the prenylated vanillin (**4**) obtained in the previous reaction (0.1001 g; 0.45 mmol) was added and the temperature was spontaneously raised to room temperature. The reaction was kept under stirring at room temperature until the total reagent consumption was observed by TLC (63 h). Ethanol was removed under reduced pressure. Then the material was dissolved in ethyl acetate and transferred to a separatory funnel. The aqueous phase was extracted twice with ethyl acetate. The combined organic phases were washed with brine. The organic extract was dried over anhydrous magnesium sulfate, filtered, and the solvent was evaporated under reduced pressure. The crude product obtained was purified over silica gel column chromatography using the mixture of 30% ethyl acetate in *n*-hexane as the eluent to yield 0.0498 g of the pure product (33%) of **1a** as a yellow powder. **$^1$H-RMN ($CDCl_3$, 500 MHz) δ:** 1.76 (d, 3H, $J = 1.0$ Hz); 1.80 (d, 3H, $J = 1.0$ Hz); 3.89 (s, 3H); 3.94 (s, 3H); 4.64 (d, 2H, $J = 6.8$ Hz); 5.52 (thept, 1H. $J = 6.8$ Hz; $J = 1.0$ Hz); 6.90 (d, 1H, $J = 8.2$ Hz); 6.99 (d, 2H, $J = 8.8$ Hz); 7.17 (d, 1H, $J = 2.1$ Hz); 7.21 (dd, 1H, $J = 8.2$ Hz; $J = 2.1$ Hz); 7.41 (d, 1H, $J = 15.6$ Hz); 7.76 (d, 1H, $J = 15.6$ Hz); 8.04 (d, 2H, $J = 8.8$ Hz). **$^{13}$C-RMN ($CDCl_3$, 125 MHz) δ:** 18.28 ($CH_3$); 25.84 ($CH_3$); 55.47 ($CH_3$); 55.97 ($CH_3$); 65.77 ($CH_2$); 110.27 (CH); 112.58 (CH); 113.77 (CH); 119.42 (CH); 119.66 (CH); 122.87 (CH); 127.91 (C); 130.71 (CH); 131.34 (C); 138.19 (C); 144.27 (CH); 149.55 (C); 150.62 (C); 163.25 (C); 188.81 (C).

### *(E)-3-(4-hydroxy-3-methoxyphenyl)-1-(4-hydroxyphenyl)prop-2-en-1-one (LLA2 2a)*

To a round bottom flask fitted with a drying tube were added niobium pentachloride (0.8998 g; 1.35 mmol) and dichloromethane (10 mL). Then, p-methoxyacetophenone

(0.1346 g, 0.9 mmol) and prenylated vanillin (0.2085 g, 0.9 mmol) were added. The reaction mixture was stirred at room temperature for 72 h. After the period, an extraction with dichloromethane was carried out, then the solvent was eliminated under reduced pressure and the crude residue was purified over silica gel column chromatography using the mixture of n-hexane and ethyl acetate (2:1) as eluent to yield 0.1814 g (67%) of **2a**. **$^{1}$H NMR (CDCl3, 500 MHz)** δ: 3.87 (s, 3H); 3.93 (s, 3H); 6.95 (d, 1H, J = 8.3 Hz); 6.97 (d, 2H, J = 9.0 Hz); 7.12 (d, 1H, J = 1.9 Hz); 7.20 (dd, 1H, J = 1.9 Hz; J = 8.3 Hz); 7.39 (d, 1H, J = 15.4 Hz); 7.74 (d, 1H, J = 15.4 Hz); 8.03 (d, 2H, J = 9.0 Hz). **$^{13}$C NMR (CDCl3, 125 MHz) δ:** 55.46 (CH3); 55.97 (CH3); 110.11 (CH); 113.78 (CH); 114.92 (CH); 119.42 (CH); 123.16 (CH); 127.59 (C); 130.73 (CH); 131.27 (C); 144.46 (CH); 146.88 (C); 148.22 (C); 163.28 (C); 188.90 (C). HRMS m/z [M+H]+ calcd: 285.1121; found: 285.1124.

## Animals

Male Balb/c mice (*Mus musculus*) were acquired from the animal houses of the University of São Paulo (Ribeirão Preto, São Paulo, Brazil) and male golden hamsters (*Mesocricetus auratus*) (110 g) were acquired from ANILAB–Laboratory Animal Creation and Trade Ltd. (Paulínia, São Paulo, Brazil). The experiments were conducted in accordance with the Brazilian legislation regulated by the National Council for the Control of Animal Experimentation (CONCEA) and approved by the University of Franca's Ethics Committee for Animal Care under protocol number 046/15 (Approval Date: November 09, 2015).

## Parasites and Mammalian Cell Maintenance

*L. (L.) amazonensis* (IFLA/BR/67/PH8.) was maintained as promastigote forms in Roswell Park Memorial Institute medium (RPMI 1640 medium) (Gibco, Grand Island, NY, USA), supplemented with antibiotics (penicillin 10.000 UI/mL and streptomycin 10 mg μg/mL) (Cultilab, Campinas, BR), and 10% bovine fetal serum (FBS) (Cultilab), at 25°C. *L. (L.) infantum* (MHOM/BR/1972/LD) was maintained as promastigote forms in M199 medium (Gibco), supplemented with 10% FBS (Cultilab), antibiotics (Cultilab), 5% human urine and 0.25% hemin (Sigma-Aldrich) at 25°C, and amastigote forms were obtained from the spleen of golden hamsters after 60 to 70 days of infection (21). Peritoneal murine macrophages were obtained by washing the peritoneal cavity of BALB/c mice with RPMI 1640 medium (Gibco) after 72 h of the application of 5 mL of the sodium thioglycolate (Sigma-Aldrich) at 3%, and then were cultivated in RPMI 1640 medium (Gibco) supplemented with antibiotics and 10% FBS (Gibco), at 37°C in a 5% $CO_2$ humidified incubator.

## *In vitro* Leishmanicidal Activity

Initially, a screening was performed against promastigote forms of *L. (L.) amanzonensis*. Briefly, promastigotes ($1 \times 10^6$ parasites/well) were distributed in 96-well plates, and the compounds LicoA (**1**), LLA1 (**1a**), LLA2 (**2a**), and MA were added at concentrations 0.78–400.0 μM, and AmpB was added at concentrations 0.0025–1.56 μM. The plates were incubated at same conditions previously described during 48 h, and the leishmanicidal activity was determined by the inhibition of growth of the promastigote forms by counting the total number of live promastigote in Neubauer's chamber (Global Glass, São Paulo, BR) using an optical microscope (Nikon New York, USA). The compound (LicoA, **1**) that exhibited activity against promastigote forms of *L. (L.) amazonensis* also was evaluated against promastigote forms *of L. (L.) infantum* as previously described and against amastigote forms of both *Leishmania* parasites. To evaluate the leishmanicidal activity against amastigote forms, peritoneal murine macrophages were seemed ($2 \times 10^5$ cells per well) in 24-well plates on slide chambers and incubated at 37°C for 24 h. After the 24 h, the cells were infected with promastigote forms of *L. (L.) amazonensis*, previously acidified (22) or with amastigote forms of *L. (L.) infantum* at a ratio of 10:1 (parasites/macrophage) for 4 h, and subsequently the cells were incubated with the LicoA (**1**) (6.25–100 μM), AmpB (0.095–0.005 μM), or MA (12.5–400 μM) 48 h. The slides were stained with Giemsa (Synth, Diadema, BR), analyzed using an optical microscopy (Nikon), and the parasite load was defined by the number of infected macrophages X number of intracellular amastigotes/number of total macrophages (23). Parasites cultured in medium with 0.1% were used as negative control and parasites cultured in medium with MA or AmpB were used as positive controls.

## *In vitro* Cytotoxic Activity

Peritoneal murine macrophages were seemed ($2 \times 10^5$ cells per well) in 96-well plates and LicoA (**1**), AmpB, and MA were added at concentrations 12.5–400 μM. The cells were incubated at 37°C in a 5% $CO_2$ humidified incubator at 37°C during 48 h, and the cytotoxic activity was determined using MTT assay (23). Cells cultured in medium with 0.1% DMSO were used as negative control and cells cultured in medium with 25% DMSO were used as positive control.

## *In vitro* Hemolytic Activity

Defibrinated sheep blood (Newprov, Pinhais, PR, BR) was diluted in 0.9% saline solution and a suspension at 3% of erythrocytes was transferred (100 μL) in 96-well plates. LicoA (**1**) and AmpB were added (12.5–400 μM), and the plates were incubated for 30 min at 37°C. The hemolytic activity was determined in the cell supernatant by optical density reading at 415 nm (Libra S12–Biochrom–Cambridge, RU) Distilled water was used as a positive control, and 0.9% saline solution with 0.1% DMSO was used as negative control.

## *In vivo* Leishmanicidal Activity of LicoA

The *in vivo* leishmanicidal activity of LicoA (**1**) was assessment in experimental model of *Leishmania (L.) infantum* and conducted as described by (21) with adaptations. Male golden hamsters were infected by intraperitoneal route with $1.0 \times 10^8$ *L. (L.) infantum* amastigotes. Four weeks after infection, the animals were randomly separated in 4 groups of 6 animals each. Two groups of treated animals received intraperitoneal doses of 20 and 50 mg/kg of body weight of LicoA (**1**), respectively, for eight consecutive days. Negative control group received the same number of injections of phosphate buffered saline (PBS)

and animals from positive control group was treated with MA (Sanofi-Aventis) at dose 50 mg/kg of body weight during eight consecutive days by intraperitoneal route. Thirty five days after treatment the animals were sacrificed and the parasite burden was evaluated both in spleen and liver by limiting dilution method (24).

In order to evaluate the hepatotoxicity, groups with 6 male golden hamsters no-infected were treated intraperitoneally with LicoA (**1**) (20 mg/kg or 50 mg/kg of body weight per day) or MA (50 mg/kg of body weight per day) for 15 days. After this period, serum concentrations of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were determined using sets of commercial reagents (Labtest, Minas Gerais, Brazil). Negative control group received the same number of injections of PBS.

## Statistical Analysis

The analyses were performed using GraphPad Prism 7 program (GraphPad Software, San Diego, CA, USA). Both $IC_{50}$ and $CC_{50}$ values were calculated with the aid of sigmoid dose–response curves, and the selectivity index (SI) values were determined by $CC_{50}/IC_{50}$. *In vitro* experiments were performed in triplicate and repeat two times. *In vivo*, statistical analysis was performed using one-way ANOVA followed by Dunnett's multiple comparison test.

# RESULTS

## Synthesis of LLA1 (1a) and LLA2 (2a)

According to **Figure 2**, the chalcone containing *O*-prenyl (LLA) was synthesized in two steps. The vanillin prenylation reaction was carried out under conditions of phase transfer catalysis (PTC) that allows the use of aqueous potassium carbonate solution and a transfer catalyst (25). The most commonly used catalysts for this type of reaction are quaternary ammonium salts. Tetrabutyl ammonium chloride (TBAC) was used because it has large substituent groups (*n*-butyl) that favor the reaction. Despite the long reaction time, the procedure was simple, at room temperature and product **4** was obtained with a yield of 61%. The prenylated chalcone (LLA1) (**1a**) was prepared via the Claisen-Schmidt condensation between the prenylated vanillin and commercial *p*-methoxyacetophenone. The reaction was carried out under basic conditions and the yield of the product LLA1 (**1a**) was 33% after purification. The synthesis of LLA2 (**2a)** was carried out through the Claisen-Schmidt condensation by acid catalysis (**Figure 2**). The reaction between the prenylated vanillin and the *p*-methoxyacetophenone promoted the formation of chalcone, together with the hydrolysis of the prenyl group in the presence of Lewis acid (niobium pentachloride), providing the product **2a** with 67% yield, after purification by chromatography on silica gel column. The use of niobium pentachloride as an acid catalyst is an alternative for the synthesis of chalcones through Claisen-Schmidt.

## *In vitro* Leishmanicidal Activity

Initially, the leishmanicidal activity of LicoA (**1**) and analogs LLA1 (**1a**) and LLA2 (**2a**) was evaluated against promastigotes, and the active compound was subsequently evaluated against amastigote forms of *L. (L.) amazonensis*. LicoA (**1**) exhibited promising leishmanicidal activity *in vitro* with $IC_{50}$ values of 20.26 and 3.88 μM at 24 and 48 h, respectively, against promastigote and $IC_{50}$ value of 36.84 against amastigote forms of *L (L.) amazonensis*. LLA1 (**1a**) was the second most active against promastigote forms with $IC_{50}$ values of 74.94 and 67.16 μM at 24 and 48 h, and LLA2 (**2a**) exhibited modest activity *in vitro* against promastigote forms of *L. (L.) amazonensis* ($IC_{50}$ values $> 300$ μM). AmpB, used as positive control against *L. (L.) amazonensis*, exhibited $IC_{50}$ values against promastigote forms of 0.27 and 0.065 μM at 24 and 48 h, respectively; and against amastigote forms the $IC_{50}$ value was 0.022 at 48 h. As LicoA (**1**) was the most active, in the next step, the effectivity of LicoA (**1**) was evaluated *in vitro* against promastigote and amastigote forms of *L. (L.) infantum*. LicoA (**1**) exhibited leishmanicidal activity *in vitro* against promastigote forms with $IC_{50}$ values of 41.10 and 12.47 μM at 24 and 48 h, respectively; against amastigote forms the $IC_{50}$ value was 29.58 μM after 48 h. MA, used as positive control exhibited $IC_{50}$ values $> 400$ μM in both promastigote and amastigote forms (**Table 1**).

## *In vitro* Cytotoxicity and Hemolytic Activities

LicoA (**1**) at concentrations evaluated exhibited moderate mammalian cytotoxicity against peritoneal murine macrophages, the $CC_{50}$ values was 123.21 μM at 48 h. AmpB, exhibited mammalian cytotoxicity with $CC_{50}$ values of 21.90 μM. On the other hand, MA showed no cytotoxicity at concentrations evaluated (**Table 1**). In the relationship between activity against intracellular amastigotes, and cytotoxicity in peritoneal murine macrophages, it was possible to determine that selectivity index (SI) of the LicoA (**1**) was 3.85 to *L (L.) amazonensis* and 4.16 to *L. (L) infantum* (data not shown). The hemolytic activity also was evaluated, and LicoA (**1**) showed about 30% of hemolytic activity at highest concentration evaluated (400 μM). On the other hand, AmpB showed 60% of hemolysis at concentration 100 μM and at higher concentrations ($\geq$ 200 μM) was observed 100% of hemolysis.

## Treatment in Experimental Model of *Leishmania (L.) infantum*

*L. (L.) infantum*-infected hamsters were treated during eight consecutive days at doses of 20 and 50 mg/kg of body weight per day of LicoA (**1**) 4 weeks after infection, and 35 days after treatment the animals were sacrificed. LicoA (**1**) at 20 and 50 mg/kg of body weight per day was able to significantly reduce the parasite burden in liver in 15.27 and 43.67%, respectively, and in spleen in 20.69 and 39.81%, respectively, when compared with negative control group. Positive control group that received 50 mg/kg of body weight per day of MA during eight consecutive days also showed significant reduction of parasite burden in 55.87% (liver) and of 63.57% (spleen) (**Figures 3A,B**). Besides, the average body weight animals showed no significant variation among negative, LicoA (**1**), and MA groups. However, after the treatment, 100% survival rate was registered in LicoA and MA groups, but in the negative group, the mortality of 33% of animals

**TABLE 1 |** Leishmanicidal activity and mammalian cytotoxicity of LicoA and its analogs.

| Compounds | $IC_{50}$-μM[a] (95% CI)[b] *L (L.) amazonensis* | | | $IC_{50}$-μM[a] (95% CI)[b] *L (L.) infantum* | | | $CC_{50}$-μM[c] (95% CI)[b] Peritoneal murine macrophages |
|---|---|---|---|---|---|---|---|
| | Promastigote | | Amastigote | Promastigote | | Amastigote | |
| | 24 h | 48 h | 48 h | 24 h | 48 h | 48 h | 48 h |
| LicoA (1) | 20.26 (17.26–24.54) | 3.88 (2.84–4.89) | 36.84 (32.25–40.02) | 41.10 (37.55–44.98) | 12.47 (10.02–15.06) | 29.58 (26.03–33.06) | 123.10 (119.87–128.65) |
| LLA1 (1a) | 74.94 (71.39–78.42.) | 67.16 (62.66–71.74) | nd | nd | nd | nd | >400 |
| LLA2 (2a) | 300.6 (297.05–304.08) | 287.26 (283.71–290.74) | nd | nd | nd | nd | >400 |
| AmpB | 0.27 (0.19–0.42) | 0.065 (0.054–0.078) | 0.022 (0.018–0.026) | nd | nd | nd | 21.90 (18.23–26.87) |
| MA | nd | nd | nd | >400 | >400 | >400 | >400 |

[a]$IC_{50}$, 50% Inhibitory Concentration; [b]95% CI, Confidence interval; [c]$CC_{50}$: 50% Cytotoxic Concentration; nd, no determined; AmpB, amphotericin B; MA, meglumine antimoniate.

was observed (data not shown). Also, the animals no-infected and treated intraperitoneally at doses 20 and 50 mg/kg of body weight per day of LicoA (**1**) showed no significant alterations in the serum levels of aminotransaminases when compared with negative control group, but in the MA group, a significant increase in the serum levels of aminotransaminases was observed (**Figures 4A,B**).

## DISCUSSION

Secondary metabolites from plants have been demonstrated promising leishmanicidal activity *in vitro* and *in vivo*, including compounds from chalcone class (8). Then, our decision to investigate LicoA (**1**) and its two analogs [**LLA1 (1a), LL2 (2a)**] was triggered by our previous study which demonstrated that LicoA (**1**) possess leishmanicidal activity against *L. (L.) major* and *L. (L.) donovani in vitro* and *in vivo*, which are parasites that cause CL and VL, respectively (10, 11). Besides, a search of the literature reports that LicoA (**1**) is of interest due to be inexpensive. Thus, this information encouraged us to evaluate the effect of LicoA and its two analogs against *L. (L.) amazonensis* and *L. (L.) infantum*, which are parasites involved in the DCL and CVL, respectively.

In this study, the synthesis of LLA1 and LLA2 was performed. As described, the prenylated chalcone LLA1 (**1a**) was prepared via the Claisen-Schmidt condensation between the prenylated vanillin and commercial p-methoxyacetophenone (**Figure 2**). The yield of this reaction was low; however, the difficulty in preparing prenylated chalcones was also found in another study that reports 16% yield for condensation of prenylated benzaldehydes (26). The analog LLA2 (**2a**) was obtained when the Claisen-Schmidt condensation between the prenylated vanillin and the *p*-methoxyacetophenone was performed using Lewis acid niobium pentachloride in dichloromethane. Niobium pentachloride promoted the formation of chalcone and hydrolysis of the prenyl group. Niobium pentachloride ($NbCl_5$) has a wide variety of applications in organic synthesis, for example, in Diels-Alder reactions, multicomponent reactions, and polymerization reactions. In addition, niobium compounds are more readily available in Brazil due to the presence of the largest niobium reserves in the world (27). The obtained result shows that niobium pentachloride can be an efficient alternative for the synthesis of chalcones.

Among the compounds evaluated, LicoA (**1**) was the most promising compound against *Leishmania* parasites under the *in vitro* experimental conditions used. In general, compounds with $IC_{50}$ values $< 10\,\mu M$ *in vitro* in infective protozoan forms are considered as a hit and lead candidates for further *in vivo* evaluations (28). Thus, this study demonstrated that LicoA (**1**) possess moderate activity against amastigote forms of *L (L.) amazonensis* and *L. (L.) infantum*. On the other hand, previous studies demonstrated that LicoA (**1**) inhibits the *in vitro* growth of *L. (L.) major* and *L. (L.) donovani* promastigotes with $IC_{50}$ value of $\sim 0\,\mu M$ for both parasites in 72 h, and a reduction in the number of amastigotes was observed at $2.5\,\mu M$ after 72 h of incubation with LicoA (**1**) (10, 11). It should be noted that promastigote forms were more susceptible to LicoA (**1**) than amastigotes forms, and this effect can be attributed by distinct metabolism effects, as previous described in the literature (23).

By comparing the leishmanicidal activity observed to LicoA (**1**) with the positive control, the results demonstrated that promastigote and amastigote forms of *L (L.) amazonensis* were susceptible to AmpB. In contrast, promastigote and amastigote forms of *L. (L.) infantum* were not susceptible to the positive control, MA at conditions evaluated in this study. This results reinforces the hypothesis that pentavalent antimonials, such as MA act as prodrugs that require biological reduction to the trivalent to exert anti-leishmanial activity (29, 30). Although MA did not show leishmanicidal activity *in vitro,* this compound was choice to positive control *in vivo* assay, and then was evaluated under *in vitro* conditions. Additionally, we choice MA rather than sodium stibogluconate (SSG) because previous study has demonstrated that MA can be more effective than SSG in the treatment of leishmaniosis (31). As the experiments were performed in different periods, MA was not used as positive control against *L. (L.) amazonensis in vitroI*; however, pentavalent antimonials are considered first line agents in the therapy against leishmaniasis (6).

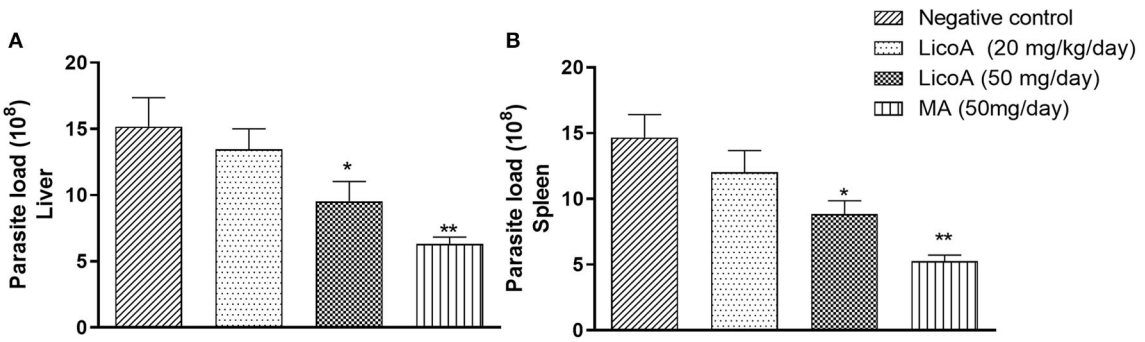


**FIGURE 3 |** Liver **(A)** and spleen **(B)** parasite load of *L. (L.) infantum*-infected hamsters treated intraperitoneal with 20 and 50 mg/kg of body weight of LicoA (**1**) during eight consecutive days 4 weeks after infection. Thirty-five days after treatment the animals were sacrificed and the parasite burden was evaluated both in spleen and liver by limiting dilution method. Negative control group received the same number of injections of phosphate buffered saline (PBS) and animals from positive control group was treated with meglumine antimoniate (MA) (Glucantime®-Sanofi-Aventis) at dose 50 mg/kg of body. An asterisk indicates statistically significant differences as compared to the negative control group (PBS) (*$p < 0.05$, **$p < 0.01$).

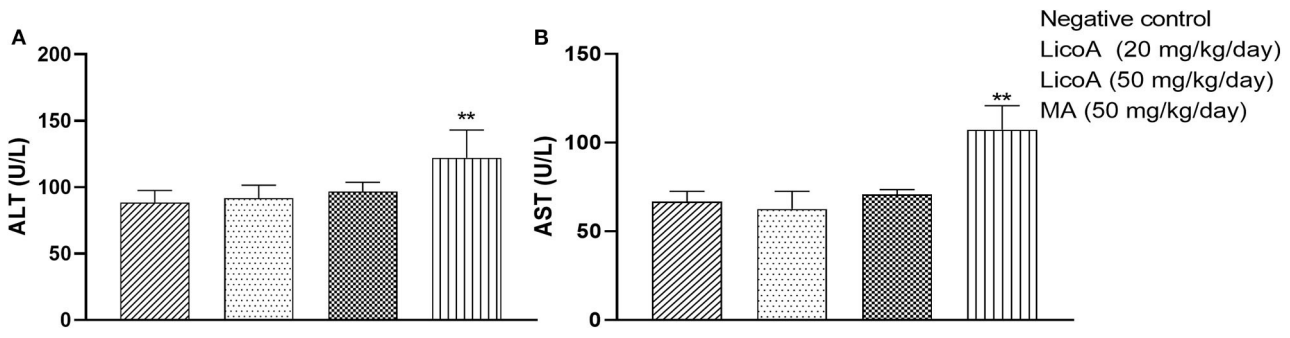


**FIGURE 4 |** Serum concentrations of aminotransaminases ALT (alanine aminotransferase) **(A)** and AST (aspartate aminotransferase) **(B)** in no-infected hamsters and treated intraperitoneal with 20 and 50 mg/kg of body weight per day of LicoA (**1**) or treated with meglumine antimoniate (MA) (Glucantime®-Sanofi-Aventis) at dose 50 mg/kg of body weight per day during 15 days. Negative control group received the same number of injections of phosphate buffered saline (PBS) and animals from positive control group. An asterisk indicates statistically significant difference as compared to the negative control group (PBS) (**$p < 0.01$).

Despite of the moderate cytotoxic effect of LicoA (**1**) observed in our study, previous study has demonstrated that LicoA (**1**) shows low-toxicity in HFF cell *in vitro* (32). Also, study has demonstrated that LicoA (**1**) induces hemolysis to trigger cell shrinkage and phospholipid scrambling of the cell membrane at concentrations higher than 30 μM after 24 h (33).

Although the results *in vitro* have been better against *L. (L.) amazonensis*, the *in vivo* assay was performed against *Leishmania (L.) infantum* because this parasite is responsible by CVL, and also dogs are the main urban domestic reservoir of VL (3, 4). Several advances in the treatment of CVL have been made in the last years, but the pharmacotherapeutic options are still not satisfactory (5–7, 34). MA and allopurinol (alone or in combination) are drugs for CVL treatment and are able to achieve clinical cure (35). Other leishmanicidal drugs such as miltefosine in combination with allopurinol have also demonstrated leishmanicidal efficacy in infected dogs (36). Unfortunately, drug treatment cannot impede disease transmission since treated animals remain carriers of the parasite (5, 7, 34–36). Moreover, other aspects like toxicity and the rise of *Leishmania* strains that are resistant to the current use drugs highlights the importance of the development of new, safe and effective compounds capable to treat CVL and actually to eliminate the infection (5, 21, 34–36). Then, the *in vivo* activity of LicoA (**1**) was evaluated in experimental model of *Leishmania (L.) infantum* in hamster. The doses of LicoA (**1**) and the scheme of treatment were based on previous reports and also on dose of MA (11, 21). The results demonstrated a reduction of parasite burden in both liver and spleen to hamsters infected with *L. (L.) infantum* after treatment with LicoA (**1**). The positive control group, MA, showed better results than compared with LicoA (**1**), but both compounds significantly reduced the parasite burden. Previous study has shown that BALB/c mice infected with *L. (L.) major* and treated with LicoA (**1**) at doses 2.5 and 5 mg/kg of body weight by intraperitoneal administration 7 days after infection during 39 days completely prevented lesion development (11). Also, it was observed that the treatment of hamsters infected with *L. (L.) donovani* with intraperitoneal administration of LicoA (**1**) at a dose of 20 mg/kg of body weight per day for six consecutive days resulted in a >96% reduction of parasite load

in the liver and the spleen compared with untreated control (11). However, our results demonstrated that at a dose of 20 mg/kg of body weight per day resulted in a reduction of parasite load of the 15.25 and 20.69% in liver and spleen, respectively. The intrinsic and/or acquired susceptibility of the parasite species can have contribute to differences of LicoA (**1**) susceptibility among the *Leishmania* species *in vitro* and *in vivo* when compared with the effect of LicoA (**1**) against *L. (L.) donovani* and *L. (L.) major* (10, 11). Previous study has demonstrated that intrinsic variation in miltefosine susceptibility of *Leishmania* clinical isolates was observed for visceral leishmaniasis in Nepal and cutaneous leishmaniasis in Peru (36). Another study demonstrated intraspecies differences in natural susceptibility to AmpB of clinical isolates of *Leishmania* subgenus *Viannia* (37). In this same study, it was demonstrated that isolates and strains maintained in the laboratory were less sensitive to AmpB when compared to clinical isolated (37).

No evidence of hepatotoxicity was found since serum concentrations of aminotransaminases showed no changed between LicoA-treated and non-treated groups. Previous study also demonstrated that LicoA (**1**) at doses higher than 1,000 mg/kg, administered orally once a day for 2 weeks did not cause any signs of toxicity. Also, intraperitoneal administration of LicoA (**1**) at doses of 100 mg/kg in rats and 150 mg/kg in hamsters did not show any toxicity (11). In addition, study has been shown that LicoA (**1**) has a protective effect against Acetaminophen overdose-induced hepatotoxicity (38). These findings suggest the safety of LicoA (**1**) in animal model independently of the route of administration. Notwithstanding positive control group (animal treated with MA) has showed better results than compared with LicoA (**1**) at dose 50 mg/kg of body weight per day, pentavalent antimonials cause serious side effects (4, 7, 8). Also, the MA administration can not be administered by the oral route due irritation of intestinal mucosa (39), but LicoA (**1**) could be evaluated by the oral route in the future research in *L. (L.) infantum*-infected hamsters.

About the action mechanism, studies suggest that the effect of LicoA may be due to the altering of both ultrastructure and function of the mitochondria of *Leishmania* parasites (40). Moreover, studies involving *in silico* approaches also reinforced the antileishmanial potential of LicoA by denoting its capability to inhibit the NADPH-dependent fumarate reductase, which is the key enzyme in the fumarate respiration. This anaerobic process is essential for the growth and survival of the parasite in their host, but is absent in the normal cells of the mammalian hosts (41, 42). In addition, studies of the modeling and docking suggest that LicoA is a potent inhibitor of cysteine proteases A, which are the essential virulence factors in leishmania parasitic (43–45). Also, study has been shown that chalcones act against malarial papain-like cysteine proteases, which is an important mechanism of the hemoglobin degradation (46).

Given in consideration that CVL manifests through splenomegaly possibly associated with hepatomegaly, and keeping in mind the lack of hepatoxicity of LicoA (**1**) and its *in vitro* effect against *L (L.) amazonensis* and *L (L.) infantum*, and *in vivo* effect in hamster-infected with *L (L.) infantum*, further studies to address the effects LicoA (**1**) and other chalcones on pathological changes associate to leishmaniosis are crucial. In addition, the results suggest that chalcone-type flavonoids are a promising class of natural products to be considered in the search of new compounds capable to treat CVL, and the association of such compounds with nanostructured carriers may lead to formulations that can increase the efficacy, selectivity, and safety of the CVL treatment.

## DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be available by request from the corresponding author.

## ETHICS STATEMENT

The experiments were conducted in accordance with the Brazilian legislation regulated by the National Council for the Control of Animal Experimentation (CONCEA) and approved by the University of Franca's Ethics Committee for Animal Care under protocol number 046/15 (Approval Date: November, 09, 2015).

## AUTHOR CONTRIBUTIONS

JS, EC, AC, MF, RM, and FD carried out the biological experiments and maintenance of *Leishmania* parasites. RP, AN, and MC contributed to the isolation, synthesis, and characterization of the compounds. SA, AS, and MS supervised the chemical part of the study. AA, LM, and RV supervised the work and provided the facilities for biological and chemical activities. All authors contributed to the article and approved the submitted version.

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# Silver Nanoparticles Biosynthesized With *Salvia officinalis* Leaf Exert Protective Effect on Hepatic Tissue Injury Induced by *Plasmodium chabaudi*

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Malaria is an important health problem in subtropical and tropical areas around the world. Infection with protozoan parasites of the Plasmodium genus, which grow inside host erythrocytes, causes malaria and may lead to morbidity and mortality. Liver tissue plays an important role in the pathogenesis of malaria and is closely involved in parasitic pre-erythrocytic development. Numerous published studies have demonstrated that the liver is not only the source of Plasmodium parasites prior to erythrocytic growth but is also a primary immune effector toward the blood stage of the malaria life cycle. Despite efforts to improve antimalarial drugs and vaccines, *Plasmodium species* that cause severe malaria are being detected increasingly frequently in endemic regions. In this study, *Salvia officinalis* (*S. officinalis*) leaf extract was employed to synthesize silver nanoparticles (Ag-NPs). This method is eco-friendly and represents a single-step technique for the biosynthetic process; therefore, it has attracted considerable attention. Accordingly, we biosynthesized Ag-NPs with extract of the *S. officinalis* leaf and examined the antimalarial activity of these nanoparticles in a murine model of *Plasmodium chabaudi* malaria (*P. chabaudi* malaria). Forty mice were chosen and classified into four types: infected group, healthy control, pretreated mice infected after treatment with 50 mg/kg of *S. officinalis* leaf extract-biosynthesized Ag-NPs for two weeks, and post-treated mice infected before treatment with 50 mg/kg of *S. officinalis* leaf extract-biosynthesized Ag-NPs (administered daily for 7 d). In this study, both pre-treatment and post-treatment with Ag-NPs produced a substantial reduction in parasitemia relative to the infected group. We investigated the antiplasmodial and hepatoprotective effects of *S. officinalis* leaf extract-biosynthesized Ag-NPs on *P. chabaudi*-induced inflammation and hepatic oxidative stress markers.

**Keywords: sage leaf extract, antioxidant, gene expression, malaria, liver injury**

## INTRODUCTION

Malaria is triggered by parasitic disease caused by infection with protozoan parasites of the genus Plasmodium; hosts become infected through bites from infected female Anopheles mosquitoes (1). Malaria infection causes major health complications, which may lead to mortality and morbidity of the host. During host infection, sporozoites emitted from the mosquito's salivary glands penetrate the blood, and infected sporozoites enter the liver and destroy cells within seconds. The parasites undergo asexual reproduction (schizogony) and initiate division in the hepatocytes to make a massive pre-erythrocytic schizont containing several 100 merozoites. When infected hepatocytes rupture, merozoites enter the bloodstream and can attack erythrocytes (2).

The liver is a key effector site and can eliminate parasite-derived hemozoin during the blood stage of malaria through phagocytosis. One of the most significant pathological effects of malarial infections is oxidative damage (3). A valuable murine model for examining the role of the liver during malaria disease is *Plasmodium chabaudi* (*P. chabaudi*) since it has numerous shared characters to human pathogenic *P. falciparum* (4). The good achievement of chloroquine and its broad use throughout the many years ultimately prompted resistance to chloroquine in *P. falciparum* and *P. vivax*, the two parasite species liable for most instances of human malaria (5). Since early times, medicinal plants have been used to treat malaria as, the Chinese traditional treatment of malaria includes the use of *Artemisia annua* (Compositae) and its active compound, Artemisinin, which has a greater chemotherapeutic index than chloroquine and is effective in chloroquine-resistant strains of human malaria. These plants are promising anti-malarial agents (6).

Few research efforts have attempted to develop drugs from natural products as a means of identifying cost-effective and non-toxic drugs against malaria. Natural products are considered to be rich sources of chemical entities that may be employed to develop new operative drugs for the treatment of neglected diseases. Some metabolites from flavonoids, quinines, terpenes, and alkaloids can be easily utilized to treat diseases caused by protozoan parasites due to the scientific evaluation of medicinal plants (7).

Primaquine (PQ) and chloroquine (CQ) are the two widely used antimalarials in the tropical regions of the world (8). These drugs are used not only for the treatment of malaria but also for the prevention and prophylaxis, as well as for treatment of other diseases Some studies have indicated that antimalarials such as CQ and PQ inhibit cytochrome P450 mediated mixed function oxidase activities both *in vivo* and *in vitro* and lead to oxidative stress by decreasing non-enzymatic and enzymatic antioxidant defenses (9–12).

The antimalarial drugs that are commonly administered leave evidence of degradation after treatment, as demonstrated by memory decline in cerebral malaria (CM). Neurological complication, with the use of the CM model in mice, it has been shown that curcumin have potent activity as an immunomodulator and anti-oxidant activities which may alleviate CM and delay death of animals by about 10 days (13, 14).

Therefore, a number of researchers have specifically focused on the use of antioxidants, either alone or synergistically with antimalarial drugs, as a feasible treatment approaches to relieve Plasmodium-induced oxidative stress and alleviate its related health issues (15, 16).

However, practical implementation of this approach may provide contradictory results, as some antimalarials function by inducing oxidative stress. Vitamins C and E, deferoxamine, N-acetylcysteine, and folate are the most heavily researched antioxidants. Although some studies have evaluated the effectiveness on Plasmodium parasites of direct administration of antioxidants, other studies have employed adjunctive therapy with standardized antimalarials (17). Green synthesis nanoparticles are a great interest, since their large-scale application in the biomedical sector (nanomedicine). This is because particles synthesized by green technologies in the size range from 1 to 100 nanometers exhibit antioxidant, anti-inflammatory, and immunomodulatory activities. A distinctive feature of the nanoparticle's synthesis use plants (phytosynthesis) due to a higher rate of nanoparticle formation and contains a wide range of biomolecules such as, (poly) phenolic and flavonoids compounds (18, 19). Each antioxidant's therapeutic application in the management of malaria depends on the targeted element of malaria pathogenesis. These studies may establish a foundation for future research investigating the medicinal role of antioxidants in malarial pathogenesis (20). In this report, we describe the effectiveness of silver nanoparticles biosynthesized with leaf extract (*Salvia officinalis*) against an *in vivo* model of *Plasmodium chabaudi* malaria.

Scientific evaluation of medicinal plants has made it possible to use some metabolites from polyphenol and flavonoids for the treatment of diseases caused by protozoan parasites. Synthesis of silver nanoparticles (Ag-NPs) utilizing plant extracts has emerged as an alternative approach. There are a number of reasons for interest in green biosynthetic methods for Ag-NPs. They are cost-effective, simple, provide large quantities, are harmless and are environmentally friendly (7, 21). These methods are simple, have large yields and are cost-effective. Silver ions are reduced and stabilized by integrating organic compounds, including amino acids, proteins, tannins, saponins, phenolics, polysaccharides and nutrients, from plant/leaf extracts that have been characterized as having therapeutic benefits (22).

*Salvia officinalis* (sage) (family Lamiaceae) leaves are commonly employed as food flavorings and are a reliable resource for the rapid synthesis of Ag-NPs. Additionally, sage plants include tannins, volatile oils, triterpenes, diterpenes, flavones, steroids, and flavonoids. Sage leaf extracts have exhibited a variety of medicinal effects, including antioxidant effects, inhibitory effects, anti-hyperglycemic effects due to lipid peroxidation and anti-inflammatory properties (23–25).

Therefore, this study assessed the beneficial effect of employing Ag-NPs biosynthesized using *Salvia officinalis* leaf extract to treat a Balb/c mouse model of *P. chabaudi* malarial infection.

# MATERIALS AND METHODS

## Plant Material and Extract Preparation

*Salvia officinalis* leaves were obtained from a sector in Riyadh, Saudi Arabia. The extract of the plant leaves was produced by combining 50 g of the leaves with 500 ml of sterile distilled water followed by boiling for 10 min. Next, the liquid was collected and filtered (Whatman No. 1 filter paper), and the filtrate was employed immediately to prepare Ag-NPs.

## Total Phenolic Content

The complete content of phenolic compounds in methanol extracts from *S. officinalis* was measured using the Folin-Ciocalteu technique as previously explained in (26). In brief, 0.2 mL of the test sample was placed in a test tube with a volume of 2.0 ml of sterile distilled water, preceded by 0.2 mL of unfiltered Folin-Ciocalteu reagent (Sigma-Aldrich, St. Louis, MO, US). The solution was mixed thoroughly and then allowed to stand for 6 min before the addition of 50 mL of 20% sodium carbonate. At room temperature (20°C), the color was allowed to form for 30 min, and then the absorbance was recorded at 760 nm by a spectrophotometer (PD 303 UV spectrophotometer, Apel Co., Limited, Saitama, Japan). A blank solution was formulated using 0.1 mL methanol instead of the extract. The measurement was analyzed with a gallic acid solution calibration graph and was presented as mg gallic acid comparable (eq.) per g of dry weight extract.

## Total Flavonoids

The colorimetric aluminum chloride technique was employed to measure the total content of flavonoids. Extricate of *S. officinalis* was obtained as in (27). In a test tube, 50 μL of the extract mixed with 4 mL of sterile distilled water was added to the mixture, and next, 0.2 mL of 5% $NaNO_2$ solution and 0.2 mL of 10% $AlCl_3.6H_2O$ was added. The mixture was allowed to stand for 6 min, and afterwards, 2 mL of 1 mol/L NaOH solution was added, and sterile distilled water was added to bring the total concentration of the mixture to 10 ml. The mixture was allowed to stand for the next 15 min, and absorbance was evaluated at 510 nm. A calibration curve was employed to assess the flavonoid content, and the measurements were presented as mg eq. rutin on dry weight per g.

## Synthesis of Silver Nanoparticles (Ag-NPs)

Green Ag-NPs were synthesized through the biosorption of Ag+ using the clean suspension of *Salvia officinalis* extract. Five milliliters of the *S. officinalis* sample was incorporated drop-by-drop into the $AgNO_3$ alkaline solution (50 mL, 0.1 mM/mL) and mixed for 30 min at 45–55°C. Next, the mixture was ultrasonicated for 3 h. The color of the silver nitrate changed from colorless solution to brownish color, implying the development of Ag-NP. Dialysis of the retained $AgNO_3$ was performed at 4°C toward deionized water. Using a zeta sizer (ZEN 3600, Malvern, UK), the developed Ag-NPs were characterized employing transmission electron microscopy (TEM) (JEM-1011, JEOL, Akishima, Japan). Additionally, a UV-Vis spectrophotometer was employed to verify green Ag-NP formation within 200–1,000-nm wavelengths. Using 1 cm of aligned quartz cells, the absorbance spectrum was measured using a PerkinElmer Lambda 40 B double-beam spectrophotometer. The consistency of the Ag-NPs was evaluated by monitoring the solution color at 4°C in the refrigerator after 20, 40, 50, and 60 d of processing.

## Infection of Mice

In this research study, female BALB/c mice ($n = 40$; 8 weeks of age) employed for the *in vivo* tests were collected from the animal house of the Female Center for Scientific and Medical Colleges in Riyadh, Saudi Arabia. The mice were reared in pathogen-free circumstances and provided a defined diet and water *ad libitum*. Cryopreserved *P. chabaudi* protozoa were passed through donor mice five times and were injected intraperitoneally into infected mice as initially described (28). The infused dose ($10^5$ parasitized erythrocytes) was determined with the Neubauer chamber. The mice were intraperitoneally injected with 100 μl of phosphate sample buffer containing *P. chabaudi*-parasitized erythrocytes (28). Parasitemia was assessed in smudged-blood smears subjected to Giemsa staining, and complete erythrocytes were measured with a Neubauer chamber. This measurement used the calculation of the proportion of parasitemia: parasitemia (percent) = (number of infected erythrocytes/total number of erythrocytes) multiplied by 100.

## Experimental Design

The mice were randomly subdivided into four groups, with each group having 10 mice as follows:

**Group I:** Negative experimental group – mice were gavaged every day with 100 μL saline (7 days daily).

**Group II:** Positive control group – mice were infected by intraperitoneal injection of $10^5$ *P. chabaudi*-infected erythrocytes (29).

**Group III:** Pretreated group – mice were orally given silver nanoparticles biosynthesized using *S. officinalis* leaf extract at a dosage of 50 mg/kg 2 weeks before infection with $1 \times 10^5$ *P. chabaudi*–infected erythrocytes (30, 31).

**Group IV:** Post-treated group, after 60 min infection, mice were treated with the silver nanoparticles biosynthesized using *S. officinalis* leaf extract oral form and continued to receive the nanoparticles at a dose of 50 mg/kg daily for 7 days (30, 32). On day 7 post-infection, all mice were sacrificed by $CO_2$ oxygen starvation and dissected, and liver tissue samples were frozen at −80°C until they were processed for molecular and biochemical analysis.

## Enzymatic Antioxidant Status

Prepared liver homogenates were analyzed to characterize catalase (CAT) (33), glutathione reductase (GRd) (34) and glutathione peroxidase (GPx) (35).

## Oxidative Stress

To measure lipid peroxidation (LPO), homogenates of the liver were formulated in 50 mM Tris-HCl and 300 mM sucrose to measure the amount of malondialdehyde (MDA) produced using the thiobarbituric acid (TBA) methodology (36), but unlike

the method described by (37, 38), the liver homogenates were employed to measure the concentrations of glutathione and nitrite/nitrate (nitric oxide; NO).

## Measurement of the Inflammation Markers Interleukin (IL)-1β and TNF-α

Quantitative observations of the concentrations of IL-1β (Cat. No. BMS606, Thermo Fisher Scientific) and TNF-α (Cat. No. EZMTNFA, Millipore) were made using enzyme-linked immunosorbent assay (ELISA) kits for mice according to the manufacturers' instructions.

## Mitochondrial Levels of Cytochrome C

An ELISA kit was employed to quantify the mitochondrial levels of cytochrome c (Abcam, ab110172) according to the manufacturer's instructions.

**TABLE 1 |** Primers used to amplify IL-1β and TNF-α genes.

| Name | Sense (5′-3′) | Antisense (5′-3′) |
|---|---|---|
| IL-1β | GACTTCACCATGGAACCCGT | GGAGACTGCCCATTCTCGAC |
| TNF-α | AGAACTCAGCGAGGACACCAA | GCTTGGTGGTTTGCTACGAC |
| GAPDH | GCATCTTCTTGTGCAGTGCC | GATGGTGATGGGTTTCCCGT |

**TABLE 2 |** Experimental determination of total phenolic and flavonoid contents for *S. officinalis* extract.

| Parameters | Mean ± SEM |
|---|---|
| Total phenols (mg eq. gallic acid/g sample) | 12.234 ± 0.988 |
| Total flavonoids (mg eq. rutin/g sample) | 0.934 ± 0.053 |

## Real-Time Polymerase Chain Reaction (RT-PCR)

Total RNA from liver tissue samples was obtained using the RNeasy Plus Minikit (Qiagen, Valencia, CA, USA). RevertAid H Minus Reverse Transcriptase (Fermentas, Thermo Fisher Scientific Inc., Waltham, MA, USA) was used to reverse-transcribe RNA. The PCR analyses were performed continuously utilizing an Applied Biosystems 7500 PCR System. The expression levels of the genes were calculated using Power SYBR Green (Life Technologies, Carlsbad, CA, USA) and the correlating threshold cycle method (39). Jena Bioscience GmbH (Jena, Germany) synthesized the PCR primers for the IL-1β and TNF-α genes. The specific primers were designed using the NCBI program Primer-Blast. Additionally, the mRNA levels were normalized to GAPDH in every sample. The primer sets are presented in **Table 1**.

## Statistical Analysis

The results are presented as mean values ± standard medium error (SEM). The results were calculated using single-way variance analysis (ANOVA). In keeping with the statistical packages for social sciences (SPSS version 20.0 IBM, Armonk, NY, USA), Duncan's test was employed as a *post-hoc* test to compare substantial differences between the groups.

# RESULTS

**Table 2** displays the total flavonoid and phenolic concentrations of the extract under investigation, and the levels were 12.234 ± 0.988 mg eq. gallic acid/g and 0.934 ± 0.053 mg eq. rutin/g.

The spectrophotometer measuring visible to UV light was employed to demonstrate the existence of Ag-NPs. The presence of a band at ∼450 nm implies that Ag-NPs are formed (**Figure 1A**). Zeta analysis was performed to investigate the mean particle length within the nanometer (d.nm) to assess the number of synthesized silver nanoparticles. **Figure 1B** shows

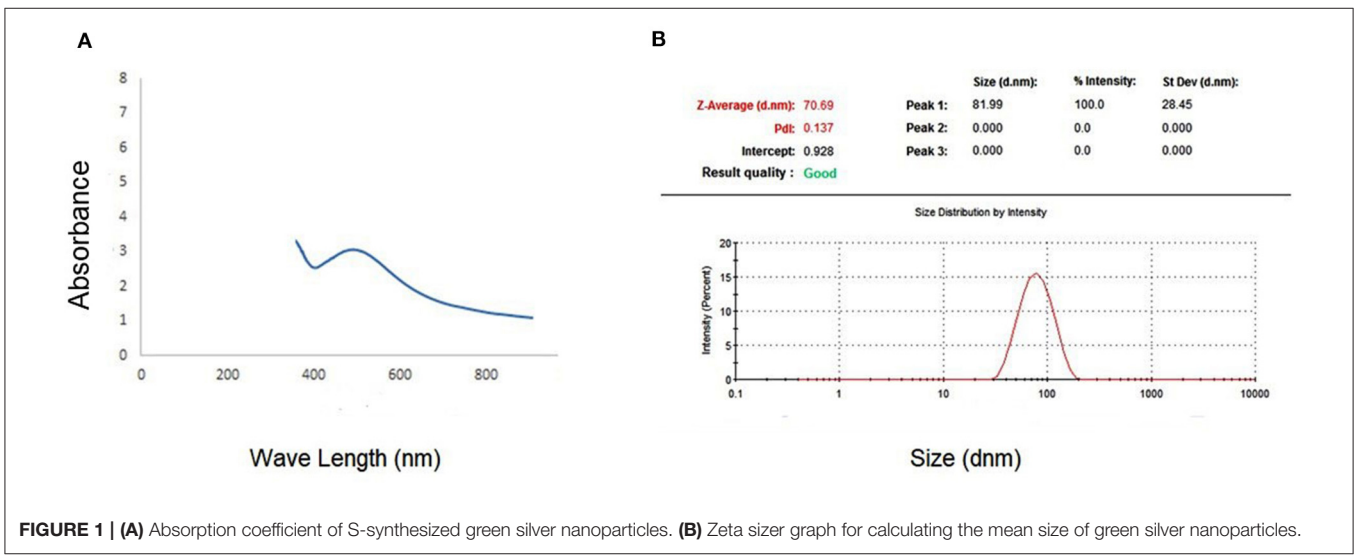


**FIGURE 1 | (A)** Absorption coefficient of S-synthesized green silver nanoparticles. **(B)** Zeta sizer graph for calculating the mean size of green silver nanoparticles.

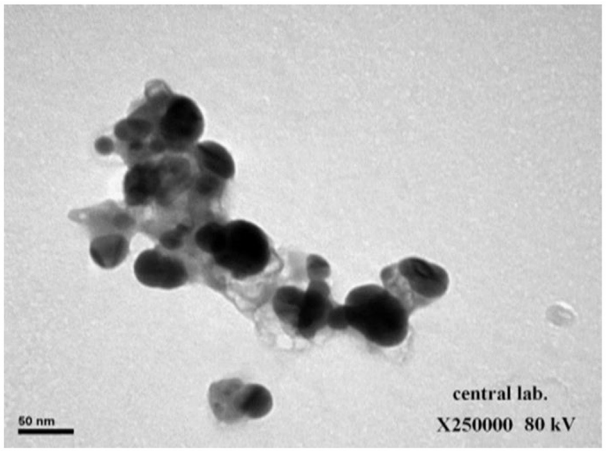


**FIGURE 2 |** Transition electron microscopy (TEM) image showing synthesized green silver nanoparticles (scale bar: 50 nm).

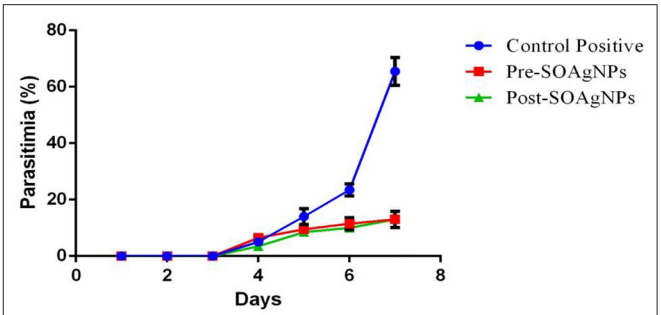


**FIGURE 3 |** Treatment with *S. officinalis* leaf extract (Post-SOAg-NPs and Pre-SOAg-NPs) -biosynthesized AgNPs decreases the proportion of parasitemia at day 7 postinfection. Erythrocytes parasitized with *P. chabaudi*. Values are shown ± SEM.

the dispersion of the size of the Ag-NPs. It was determined that the size of Ag-NPs varied between 51 and 226 d.nm with an optimal size of 81.99 d.nm. Additionally, the TEM image in **Figure 2** shows that the majority of Ag-NPs were morphologically spherical.

Notably, the color of the Ag-NPs aqueous solution barely changed after 40 d of storage, indicating the stability of the Ag-NPs. After 60 d, the color varied to colorless.

Mice infected with *P. chabaudi* malaria showed some clinical signs and symptoms including stiff neck, increased muscle tone, ataxia, local paralysis, delirium, and blood urine. However, mice (pre-treatment and post-treatment with Ag-NPs biosynthesized with leaf extract of the *S. officinalis*) showed less or no clinical signs appeared.

Progressive increases in parasitemia were pronounced in infected mice after being inoculated with $10^5$ parasitized erythrocytes (**Figure 3**). Starting from day 4 postinfection with P. *chabaudi,* the parasitemia was decreased in the experimental groups treated with silver nanoparticles biosynthesized using *S. officinalis* leaf extract (pre-treatment and post-treatment), parasitemia were reduced ∼5-fold on day 7 postinfection in comparison to the control group.

In hepatic homogenates, the levels of lipid peroxidation (LPO) and nitrous oxide (NO) were assessed to estimate the impact of malarial infection on oxidative stress parameters (**Figures 4A,B**). *P. chabaudi* produced a significant ($p < 0.05$) increase in liver NO and LPO concentrations compared to the control group, whereas *S. officinalis*-biosynthesized AgNPs alleviated this increase significantly ($p < 0.05$).

*P. chabaudi* also stimulated hepatic oxidative stress, as depicted by a substantial decrease ($p < 0.05$) in the liver tissue quality of infected mice compared to the control group. This decrease in GSH levels was mitigated by post- and pre-infection treatment with AgNPs biosynthesized with *S. officinalis* leaf extract **Figure 4C**.

To analyze how *P. chabaudi* infection triggered oxidative stress in the tissue samples, possible changes in the antioxidant defense system were evaluated by determining the activities of the enzymes CAT, GPx, and GR. The repression of the activities of these enzymes by *P. chabaudi* infection was significantly alleviated by treatment with *S. officinalis* leaf extract-biosynthesized Ag-NPs ($p < 0.05$) (**Figures 5A–C**).

The level of cytochrome c activity in liver homogenates was increased in infected mice compared with the uninfected control ($p < 0.05$). Pre- and posttreatment with Ag-NPs biosynthesized with *Salvia officinalis* leaf extract significantly decreased cytochrome c activity compared with the untreated infected mice (**Figure 6**).

Inflammation is characterized by the secretion of high levels of pro-inflammatory cytokines, such as IL-1β and TNF-α (**Figures 7A,B**) illustrated the effect of Ag-NPs biosynthesized by *S. officinalis* leaf extract on hepatic IL-1β and TNF-α that showed significantly increase in infected non-treated group level $p < 0.05$. But this increased that inhibited in Ag nanoparticles groups (Pre-SOAg-NPs and Post-SOAg-NPs).

To characterize the transcription of proinflammatory cytokine mRNA, this study determined that *P. chabaudi* triggered substantial expression of TNFα mRNA and IL-1β mRNA. In comparison, treatment with *S. officinalis* leaf extract-biosynthesized Ag nanoparticle groups (Post-SOAg-NPs and Pre-SOAg-NPs) triggered a marked downregulation of IL-1β and TNFα mRNA gene expression levels (**Figures 8A,B**).

## DISCUSSION

Parasitic diseases remain a significant global health problem causing ∼1 million deaths per year and affecting more than 1.7 billion people worldwide. Malaria may be the most life-threatening infectious disease of many diverse parasite infections (40). Due to the extreme complexity of parasite biochemistry and immunogenic variance, no efficient therapy has been recommended to date against malaria. As most commonly produced antimalarial drugs may be utilized to cure malaria, the underlying issue is drug resistance. Resistance to parasitic infections creates inconclusive or delayed parasitic infestation clearance from the patient's bloodstream after treatment with an antimalarial agent (41).

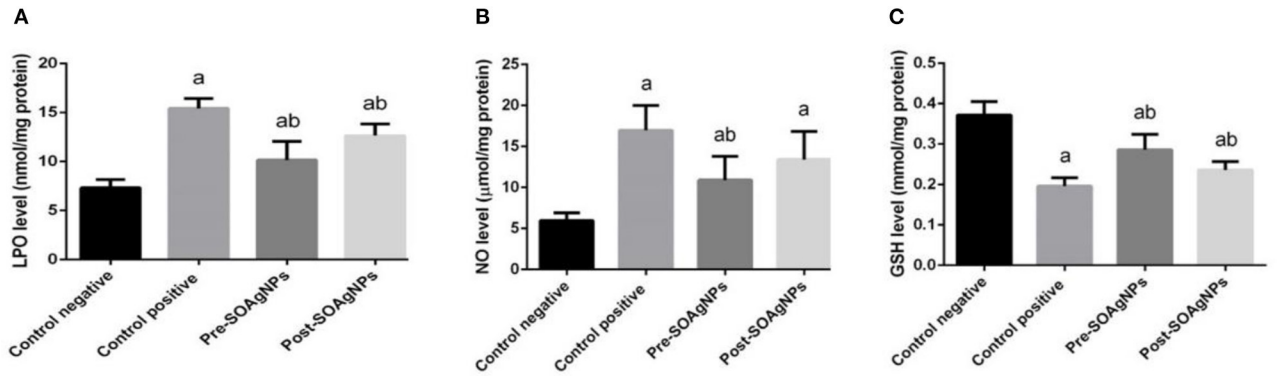


**FIGURE 4 |** Effect of Ag-NPs biosynthesized by *S. officinalis* leaf extract on hepatic oxidative stress markers activities (Pre-SOAg-NPs and Post-SOAg-NPs): **(A)** malonaldehyde, **(B)** nitric oxide, and **(C)** glutathione. Values are mean ± SEM ($n = 10$); $^{a}p < 0.05$, significant change from –ve control group; $^{b}p < 0.05$, notable change from +ve control group.

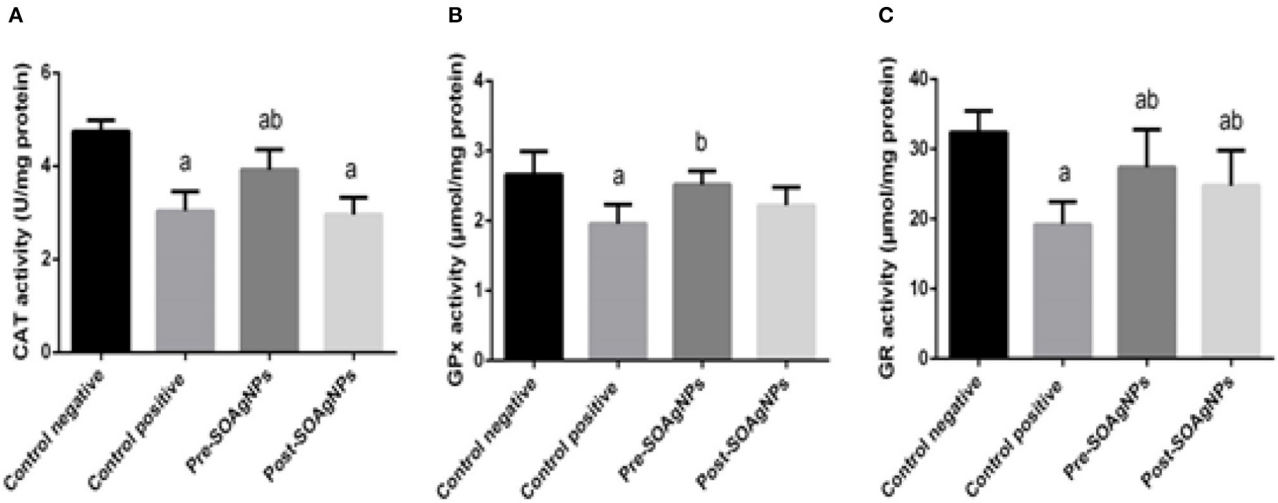


**FIGURE 5 |** Effect of Ag-NPs biosynthesized by *S. officinalis* leaf extract on hepatic antioxidant enzyme activities (Pre-SOAg-NPs and Post-SOAg-NPs): **(A)** catalase, **(B)** glutathione peroxidase, and **(C)** glutathione reductase. Values are mean ± SEM ($n = 10$); $^{a}p < 0.05$, significant change from –ve control group; $^{b}p < 0.05$, notable change from +ve control group.

The liver is the very first pre-erythrocytic source of malarial infection and acts as receptor for malaria in the initial infection, where the liver epithelial system attacks the parasitized erythrocytes, potentially by phagocytosis. *P. chabaudi*-infected mice are characterized by parasitemia that reaches ∼40% of infected erythrocytes and causes inflammation in the liver (42, 43).

One efficient strategy to combat parasite resistance is the detection of various sources of new antimalarial compounds, particularly from traditional plant sources. The use of plant species as production agents to be placed in silver nanoparticles has attracted attention because this method is notably rapid, has less environmental impact and represents a one-step method for a biosynthetic procedure (44).

Regarding the display of flavonoids and phenols, in the biosynthesis of these metal nanoparticles, *S. officinalis* plays an essential role. The synthesis of silver nanoparticles is reliable, replicable, and cost-effective, making it preferable to several other chemical methods (42).

*S. officinalis* leaf extract-biosynthesized Ag-NPs treatment (Pre-SOAg-NPs and Post-SOAg-NPs) may significantly decrease parasitemia, implying the removal of malaria parasites and their antimalarial influence. This clearance may be facilitated by both innate and acquired immune responses. Liver-formed cytokines are not only important for the local response in the liver but also may have an effect on the systemic response to blood-stage malarial infection (43, 45).

Nanobiotechnology's potential benefits began to emerge when nanoparticles were biosynthesized using bioactive ingredients, such as proteins, botanical extracts, vitamins, and biodegradable polymers (46, 47).

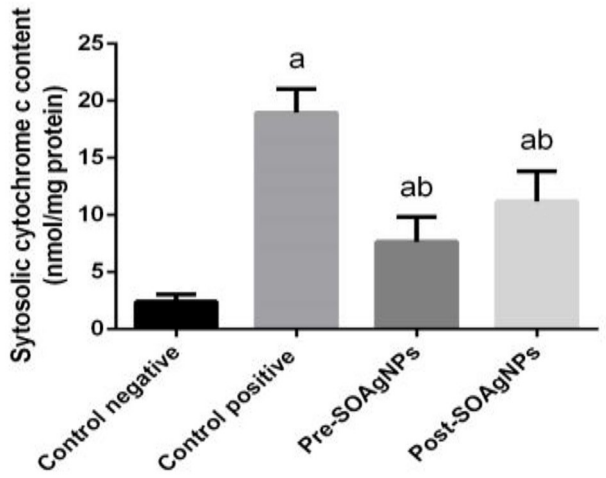


**FIGURE 6 |** Effect of Ag-NPs biosynthesized by *S. officinalis* leaf extracts on hepatic cytochrome c activities (Pre-SOAg-NPs and Post-SOAg-NPs). Values are mean ± SEM ($n = 10$); $^{a}p < 0.05$, significant change from –ve control group; $^{b}p < 0.05$, notable change from +ve control group.

Nuclear factor-κB plays a pivotal role in immunity; this protein translocates to the nucleus and binds to the promoters of target genes as a transcription factor to πB motifs, and it activates the transcription of proinflammatory genes encoding IL-1β and TNF-α (48). The interaction of infected cells with or their encroachment of *P. chabaudi* products often causes IL-1β and TNF-α to induce $CD8^{+}$ T and $CD4^{+}$ T cell activation (46, 48, 49). Nuclear factor-κB has a seminal role in immunity, because it activates proinflammatory genes encoding iNOS, COX-2, TNF-α, IL-1β, and IL-6, it is activated by phosphorylation, ubiquitination and subsequent proteolytic degradation of the IκB protein by activated IκBkinase (IKK) (50). The liberated NF-κB translocate to the nucleus and binds as a transcription factor to κB motifs in the promoters of target genes, leading to their transcription. Aberrant NF-κB activity is linked with various inflammatory diseases, and most anti-inflammatory drugs suppress inflammatory cytokine expression by inhibiting the NF-κB pathway (51).

The evidence presented in this report shows a strong increase in proinflammatory cytokine rates triggered by *in vivo* infection with *P. chabaudi*. This process is initiated by omni-ubiquitination, phosphorylation, and consequent proteolytic deterioration of the I-B protein by I-B kinase (IKK) (52) activation. Nevertheless, *S. officinalis* leaf extract-biosynthesized Ag-NPs repressed the transcription of NF-κB in liver tissue; these results agree with (52), in which Ag-NPs were determined to maintain the repression of proinflammatory cytokines by reducing the phosphorylation of IκBα.

In this study, the leaves of *S. officinalis* are excellent sources for Ag-NPs biosynthesis; the development of Ag-NPs was indicated by a shift in color and stable solution. The color change is directly associated with the emergence of Ag-NPs, which can be attributed to the presence of reducing agents, such as flavonoids and phenols (53, 54). The TEM results demonstrate that the average particle size is ∼81.8 d.nm and has a spherical form. Flavonoids and phenols are present in *S. officinalis* leaf extract, as well as other compounds. The official function of this extract is as the surface stabilizing component for Ag-NPs biosynthesis, as well as antioxidant and anti-inflammatory impacts. The biological components of Ag-NPs are encapsulated in *S. officinalis* extract. These characteristics agree with other *S. officinalis* leaf extract studies, which evaluated the extract for Ag-NPs synthesis using a 1 mM silver nitrate alkaline phase (55).

Liver diseases are among the most serious infections and represent a major public health issue worldwide; however, their management and treatment options are limited, despite major advances in medical technology. The pathophysiology of hepatic infections with the role of the inflammatory response and oxidative injury is well-known, and the chain reactions of the inflammatory response and oxidation reactions may therefore be slowed or blocked by promising therapeutic methods to avoid liver injury (53, 56).

The increased production of NO in reaction to the parasitic infection might be assumed to be an adverse outcomes of tissue damage and oxidative injury. The development of ROS in tissues and cells throughout numerous normal procedures indicates the pathophysiology of parasitic infections, such as *P. chabaudi* infection (37, 57). In the present study, *P. chabaudi* controlled Reactive oxygen species (ROS) synthesis in heavily infected mice in a manner that was inversely correlated with the extent of intracellular parasitization. NO radicals also were observed to play a significant role in the inflammatory process, which degrades biomolecules (58). Our findings show that levels of NO reliably increased in malaria-inoculated mice along with the severity of infection, and NO levels ultimately decreased after treatment with extract-biosynthesized Ag-NPs. Therefore, these results suggest a possible application in which nanoparticles may function as anti-inflammatory agents and thus safeguard the liver.

Recently, considerable experimental and clinical evidence has shown that the main apoptotic stimulus in various types of chronic and acute liver deterioration is chronic inflammation impacted by a discrepancy between the body's oxidant systems and antioxidants in favor of oxidants (59).

This phenomenon was suggested by increased lipid peroxidation correlated with lowered levels of the antioxidant enzymes, which included GPx, SOD, and GR. Antioxidant enzymes have been identified as being a mutually helpful system of defense against ROS. In this study, we reported that infection with *P. chabaudi* causes a substantial reduction in antioxidant enzyme activity. This decrease could result in higher superoxide radical absorption, which could facilitate induced lipid peroxidation. The reduction of enzymatic antioxidants after infection with *P. chabaudi* could be due to protein inhibition due to ROS, since oxidative damage can lead to the loss of a particular protein component, SOD, that catalyzes the $H_2O_2$ and $O_2$ disproportionation of superoxide anion, since $H_2O_2$ is detrimental to cells (2, 60). Therefore, the organized activities of different cellular antioxidants in cell lines are crucial for the detoxification of free radicals. Mice infected with *P. chabaudi* reduced the activity of mouse liver antioxidant enzymes, which

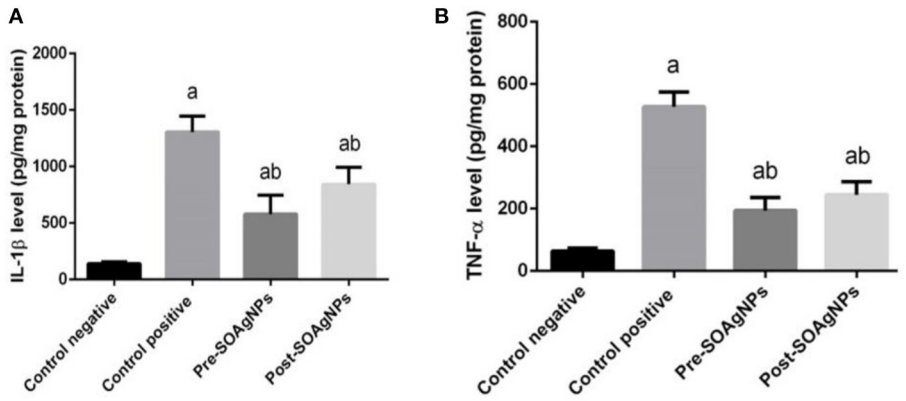


**FIGURE 7 |** Effect of Ag-NPs biosynthesized by *S. officinalis* leaf extract on hepatic proinflammatory cytokines (Pre-SOAg-NPs and Post-SOAg-NPs). **(A)** IL-1β and **(B)** TNF-α. Values are mean ± SEM ($n = 10$); $^{a}p < 0.05$, significant change from –ve control group; $^{b}p < 0.05$, notable change from +ve control group.

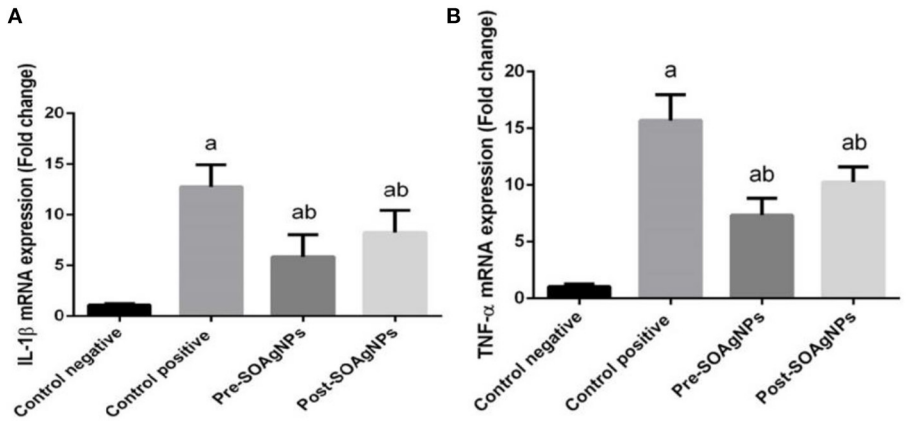


**FIGURE 8 |** Effect of Ag-NPs biosynthesized by *S. officinalis* leaf extract (Pre-SOAg-NPs and Post-SOAg-NPs) on the hepatic proinflammatory cytokines **(A)** IL-1β and **(B)** TNF-α. Findings for the expression of genes are described as means ± SEM of triplicate assays, normalized to GAPDH and expressed as fold change (log2 scale), relative to mRNA levels in controls $^{a}p < 0.05$, significant change from –ve control group; $^{b}p < 0.05$, notable change from +ve control group.

is consistent with the findings of other studies (61, 62). The results obtained with SOD, GPx, and GR demonstrate that the levels of ROS in the mouse liver were significantly changed upon infection, thereby affirming that free radicals and oxidative metabolism could play an essential role in the pathophysiology of liver injury (63).

GSH is the main antioxidant and the most abundant source of non-protein thiol found in liver cells, which acts as a substratum for some enzymes and plays a protective role in the metabolism of a large number of toxic agents. This substratum can act as a free radical trapping agent and safeguard cytochrome P450 by preventing lipid peroxidation (64). Nanoparticles increased substantially in this study and retained hepatic GSH activity after infection. Infection with *P. chabaudi* leads to a considerable decrease in the amount of glutathione, which may be an essential toxic effect alleviated by treatment with *S. officinalis* leaf extract-biosynthesized silver nanoparticles, which may be due to restoration of the GSH level. In this respect, researchers can use nanoparticles with antioxidant effects that are synthesized through the green synthesis method (where plant sublayers are used to prepare environmentally friendly nanoparticles that do not contain harmful chemical compounds). At present, using non-toxic precursors for synthesizing nanoparticles is largely regarded as a means of preventing biological hazards. The green synthesis method is believed to enhance the biocompatibility and performance of metal nanoparticles for biological applications.

Using the *S. officinalis* extract of leaves in silver nanoparticles was observed to attenuate malaria infection-induced apoptosis, and the amount of cytochrome c in the liver homogenates was quantified. ROS were shown to make the mitochondrial membrane more permeable and cause mitochondrial malfunction (47, 63).

The permeation of the mitochondrial membrane varies depending on the transition pore, resulting in cytochrome c being released from the mitochondria to the cytoplasm. Once set to release, cytochrome c may bind to apoptotic proteolytic enzyme-activating factor−1 (Apaf−1) in the cytosol, forming a complex that upregulates caspase 9 with enhanced expression

of death-inducing variables (65, 66). Our results showed that in the infected mice, *S. officinalis* leaf extract-biosynthesized Ag-NPs treatment reversed the change in cytochrome c levels, and may potentially have prevented all detrimental occurrences, solubilized the oxygen-based reaction mixture produced in mitochondria, and stabilized the infection-induced mitochondrial membrane.

## CONCLUSIONS

Our results indicate that Ag-NPs biosynthesized from the *S. officinalis* leaf extract may be involved in the implementation of a new candidate therapeutic agent with greater efficacy in suppressing hepatotoxicity by modulating the cellular redox status while downregulating pro-inflammatory cytokine genes and inflammation. These results suggest that the potential mechanism of the anti-inflammatory properties of the *S. officinalis* leaf extract-biosynthesized silver nanoparticles was the intracellular blockade of inflammatory pathways and downregulation of proinflammatory cytokines.

## DRYAD URL

(https://datadryad.org/stash/share/S15z6f0OtTiUhHwjFOlzZLceHkDjK63EFpUHf0ntX4Y)
Doi (https://doi.org/10.5061/dryad.1ns1rn8s5).

## DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/supplementary material.

## ETHICS STATEMENT

The animal study was reviewed and approved by this research (IRB Number: KSU-SE-20-36) received approval from King Saud University's (Saudi Arabia) Organizational Committee for Postgraduate Research and Studies.

## AUTHOR CONTRIBUTIONS

DM, RA, ME-K, and SA-Q: conceptualization, data curation, formal analysis, funding acquisition, investigation, methodology, project administration, resources, software, supervision, validation, visualization, writing original draft, writing review and editing. All the authors contributed equally to this work.

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# Protective Immunity Against *Neospora caninum* Infection Induced by 14-3-3 Protein in Mice

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*Neospora caninum* is an apicomplexan parasite that infects many mammals and remains a threatening disease worldwide because of the lack of effective drugs and vaccines. Our previous studies demonstrated that *N. caninum* 14-3-3 protein (Nc14-3-3), which is included in *N. caninum* extracellular vesicles (NEVs), can induce effective immune responses and stimulate cytokine expression in mouse peritoneal macrophages. However, whether Nc14-3-3 has a protective effect and its mechanisms are poorly understood. Here, we evaluated the immune responses and protective effects of Nc14-3-3 against exposure to $2 \times 10^7$ Nc-1 tachyzoites. Antibody (IgG, IgGl, and IgG2a) levels and Th1-type (IFN-γ and IL-12) and Th2-type (IL-4 and IL-10) cytokines in mouse serum, survival rates, survival times, and parasite burdens were detected. In the present study, the immunostimulatory effect of Nc14-3-3 was confirmed, as it triggered Th1-type cytokine (IFN-γ and IL-12) production in mouse serum 2 weeks after the final immunization. Moreover, the immunization of C57BL/6 mice with Nc14-3-3 induced high IgG antibody levels and significant increases in $CD8^+$ T lymphocytes in the spleens of mice, indicating that the cellular immune response was significantly stimulated. Mouse survival rates and times were significantly prolonged after immunization; the survival rates were 40% for Nc14-3-3 immunization and 60% for NEV immunization, while mice that received GST, PBS, or blank control all died at 13, 9, or 8 days, respectively, after intraperitoneal *N. caninum* challenge. In addition, qPCR analysis indicated that there was a reduced parasite burden and diminished pathological changes in the mice immunized with Nc14-3-3. Our data demonstrate that vaccination of mice with Nc14-3-3 elicits both cellular and humoral immune responses and provides partial protection against acute neosporosis. Thus, Nc14-3-3 could be an effective antigen candidate for vaccine development for neosporosis.

**Keywords:** ***Neospora caninum*, 14-3-3, extracellular vesicles, cytokines, immunity**

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# INTRODUCTION

*Neospora caninum* is an intracellular protozoan parasite belonging to the phylum Apicomplexa and is the causative agent of Bovine neosporosis (1). Although there is no evidence that *N. caninum* infection occurs in humans, anti-*N. caninum* antibodies have been detected in humans (2), suggesting that it might be a potential zoonotic pathogen. Neosporosis can spread by transplacental transmission from an infected dam to her fetus and brings enormous economic losses to the cattle industry worldwide (3). However, there are no effective drugs or vaccines available to control this disease (4), and developing a potent vaccine against neosporosis is vital.

The 14-3-3 protein family includes highly conserved proteins that are widely expressed in all eukaryotic cells and are involved in many cellular processes, such as cell cycle control, signal transduction, protein trafficking, and responses to environmental stimulation (5). Furthermore, as 14-3-3 proteins have been indicated to be highly immunogenic (6), these proteins from parasites represent a rational approach for the development of effective vaccines against the respective infections. *T. gondii* 14-3-3 (Tg14-3-3) was detected in tachyzoites and found to be present mainly in the cytoplasm as well as within the parasitophorous vacuolar space, inducing the migratory activation of immune cells (7). Our previous research demonstrated that *N. caninum* 14-3-3 protein was included in *N. caninum* extracellular vesicles (NEVs), and the protein was mainly found in the cytosol and cell membrane in *N. caninum* tachyzoites and has shown that the *N. caninum* 14-3-3 protein can induce effective immune responses and stimulate cytokine expression by activating the MAPK, AKT, and NF-κB signaling pathways (8); however, the protective efficacy of 14-3-3 protein as a vaccine antigen against *N. caninum* remains unclear. Here, we purified a recombinant fusion Nc14-3-3 protein to assess its protective efficacy against *N. caninum* infection.

# MATERIALS AND METHODS

## Animals and Parasites

Female C57BL/6 mice (6–8 weeks old) were purchased from the Changsheng Experimental Animal Center (Benxi, China). The mice were housed in isolator cages under specific pathogen-free conditions in the animal house of the Laboratory Animal Center of Jilin University (Changchun, China), and the food and water provided were sterile. All animal experimental procedures were performed in strict accordance with the Regulations for the Administration of Affairs Concerning Experimental Animals approved through the State Council of the People's Republic of China (1988.11.1) and with the approval of the Animal Welfare and Research Ethics Committee at Jilin University.

*N. caninum* tachyzoites (Nc-1 strain) were maintained by serial passage in Vero cells in RPMI-1640 medium, and free tachyzoites were obtained and harvested from Vero cells as described in a previous study (8, 9).

## *N. caninum* EV Preparation

NEVs were purified as previously described (9), and free tachyzoites were collected using Percoll and cultured in exosome-depleted culture medium for 24 h. The NEVs were collected by differential centrifugation. Briefly, the supernatant was first centrifuged at 300 × g for 10 min at 4°C, and then the supernatant was centrifuged at 2,000 × g for 10 min at 4°C and at 10,000 × g for 30 min at 4°C to remove the parasites and debris. Finally, the supernatant was further ultracentrifuged at 100,000 × g for 70 min at 4°C to precipitate the expected NEVs with an ultracentrifuge (Hitachi Micro Ultracentrifuge, Japan). The observed NEV pellets in each tube were collected together and ultracentrifuged once more at 100,000 × g for 70 min at 4°C. The final pellet was resuspended in cold PBS, and protein concentrations were measured using a BCA Protein Assay Kit (Thermo Scientific, Waltham, USA).

## Expression and Purification of Recombinant Nc14-3-3 Protein

The recombinant protein Nc14-3-3 was expressed as a glutathione S-transferase (GST) fusion protein in the *E. coli* expression strain Rosetta DE3a (TIANGEN, Beijing, China). After induction of expression, the recombinant proteins were purified using Proteinlso® GST Resin (TransGen Biotech, Beijing, China) as described previously (8). The induced expression of the pGEX-4T-1 empty vector and purified GST-tagged protein were used as controls.

## Mouse Immunization and Challenge

To assess the immunogenicity of Nc14-3-3, female C57BL/6 mice were randomly divided into five groups (16/group) as follows: Blank, PBS, GST, NEVs, and Nc14-3-3. NEVs were dissolved in sterile PBS to a final concentration of 1 μg/μl, and mice were injected intramuscularly with 50 μl of NEVs or PBS alone. Otherwise, mice were intramuscularly immunized with recombinant Nc14-3-3 protein or GST protein (50 μg) emulsified with Freund's adjuvant (Sigma, St. Louis, USA). Briefly, mice were intramuscularly immunized with Nc14-3-3 protein or GST protein, which was emulsified with Freund's complete adjuvant (Sigma, St. Louis, USA). Boosters were administered at 2, 4, and 6 weeks, and the proteins were emulsified with Freund's incomplete adjuvant (Sigma, St. Louis, USA). Two weeks after the third immunization, each mouse was challenged with a dose of $2 \times 10^7$ Nc-1 tachyzoites, and the survival time, body weight and clinical observations of the mice ($n = 10$) were observed and recorded every day by the same person at similar time points.

## Determination of Serum Antibody and Cytokine Levels

Mouse polyclonal antibodies were prepared as follows: serum was collected from the mice via the tail veins on the day before each vaccination. The antibody levels in mouse serum was detected by indirect enzyme-linked immunosorbent assays (ELISA) as previously described (10). Briefly, plates were coated with 2 μg of *N. caninum* lysate antigen (NLA), and mouse sera diluted in PBST at 1:100 were added. HRP-labeled antibodies (IgG, IgG1, or IgG2a, 1:2,000 dilution) (Proteintech, Wuhan, China) were

used as secondary antibodies. The reaction was detected by TMB (Beyotime, Shanghai, China) and stopped by 2 M $H_2SO_4$ addition. Absorbance at 450 nm was measured with a microplate reader. In addition, NLA was prepared as previously described (9). Briefly, *N. caninum* tachyzoites were resuspended in BAG buffer (116 mM NaCl, 5.4 mM KCl, 0.8 mM MgSO4, 50 mM HEPES, 5.5 mM d-glucose, pH 7.3) with protease inhibitors (KeyGen Biotech, Nanjing, China) and subjected to ultrasonic disruption (60 Hz/30 s) on ice. After centrifugation at 10,000 × g for 30 min at 4°C, the NLA was collected and filtered using 0.22 μm membranes. The protein concentrations were measured using the BCA Protein Assay Kit (Thermo Scientific, Waltham, MA, USA).

To evaluate the concentration of cytokines in serum samples, 2 weeks after the final immunization, six mice were sacrificed, blood was collected from the eyeball, and serum was obtained for cytokine measurements. Cytokine ELISA Ready-SET-Go kits (eBioscience, San Diego, CA, USA) were used to detect the IL-12p40, IFN-γ, IL-10, and IL-4 levels according to the manufacturer's instructions.

## Flow Cytometry Analysis of T Cell Subpopulations

The percentages of T cell subpopulations in the spleens of mice in the experimental groups were detected by flow cytometry. The spleens were obtained 2 weeks after the final immunization from mice ($n = 6$) in each group, and a flow cytometry assay was performed as previously described (11). Briefly, $1 \times 10^6$ splenocytes were suspended in pre-cooled PBS and incubated with anti-mouse CD3-PerCP, anti-mouse CD4-PE, and anti-mouse CD8-APC antibodies (all from BioLegend) at 4°C for 30 min in the dark. Then, the cells were washed twice with pre-cooled PBS, resuspended in PBS and analyzed with a FACSAria flow cytometer (BD Biosciences), with 20,000 total events/sample. Data were analyzed by FlowJo software (Tree Star Inc.).

## Quantification of the Parasite Burden by qPCR

Two weeks after the last immunization, each mouse was challenged with $2 \times 10^7$ Nc-1 tachyzoites. At 5 days post-infection, infected mice were euthanized, and the heart, liver, spleen, lung, kidney, and brain were harvested and stored at −40°C. The parasite replication in tissues were monitored by real-time quantitative PCR (qPCR) as previously described (12). Briefly, the tissues were homogenized and used for DNA extraction (TIANGEN, Beijing, China). Five hundred nanograms of extracted DNA from each sample was amplified with the Nc5 sequence of *N. caninum* (forward: 5′-ACTGGAGGCACG CTGAACAC-3′, reverse: 5′-AACAATGCTTCGCAAGAGGAA-3′) using FastStart Universal SYBR Green Master Mix. To quantify the number of parasites, a standard curve was generated by amplifying 10-fold dilutions of $2.3 \times 10^8$ *N. caninum* tachyzoites in separate reactions.

## Histopathology

Pathological changes were observed by H&E staining, and fresh tissue was fixed with 10% neutral buffered formalin and routinely processed in paraffin. Fixed paraffin-embedded tissues were sectioned at 3–4 μm and stained with haematoxylin and eosin (H&E).

## Statistical Analysis

Statistical significance was determined by one-way ANOVA using SPSS 19.0 software (SPSS Inc., Chicago, IL, USA), and data are shown as the mean ± standard deviation (SD) of triplicate experiments. GraphPad Prism 7 (GraphPad Software, Inc., San Diego, CA, USA) was utilized to generate the graphs. $P < 0.05$ were considered significant.

# RESULTS

## Serum Antibody Responses in C57BL/6 Mice

To assess changes in antibody levels caused by recombinant Nc14-3-3 protein, the total IgG antibody level and the distribution of IgGl and IgG2a isotype 2 were tested after each immunization. As shown in **Figure 1**, significantly higher levels of IgG antibodies were observed in the groups vaccinated with NEVs and Nc14-3-3. In contrast, specific higher IgG1 antibody levels were detected in the above mentioned groups, but IgG2a was not obviously induced. No or low detectable levels of antigen-specific antibodies were observed in the control group receiving PBS or GST, respectively. These results suggested that immunization with NEVs or Nc14-3-3 can induce Th2 immune responses against *N. caninum* in mice.

## Levels of Cytokines in the Sera of Vaccinated Mice

To evaluate the cytokines released in serum samples in immunized groups, mice were sacrificed after the final immunization. As shown in **Figure 2**, mice vaccinated with NEVs or Nc14-3-3 generated significantly higher levels of IFN-γ and IL-12p40 than mice vaccinated with single-gene plasmids, PBS or empty vector ($P < 0.01$). In contrast, high IL-10 levels were observed in Nc14-3-3-immunized mouse serum compared with those in serum from the other groups ($P < 0.01$), and the levels of the cytokine IL-4 were not significantly different among all groups ($P > 0.05$). These results indicated that NEVs and Nc14-3-3 mainly cause Th1-type immune responses in mice, and the cytokines IFN-γ and IL-12 play an important role in protection against *N. caninum* infection after vaccination.

## Nc14-3-3 Immunization Increased $CD8^+$ T Cell Levels

It is well-established that T cells play an important role in protective immunity against protozoan infections. To determine whether NEV or Nc14-3-3 vaccination activated $CD4^+$ or $CD8^+$ T cells, flow cytometry was used, and as shown in **Figure 3**, there was no significant difference in the percentage of $CD4^+$ T cells in the mice immunized with different vaccines, but the percentage

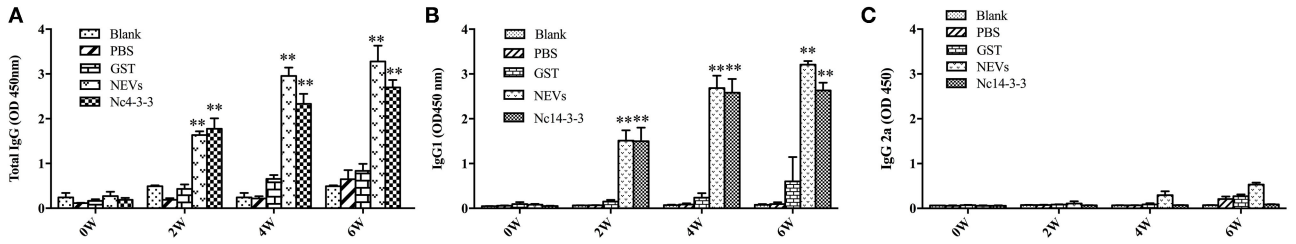


**FIGURE 1 |** Measurement of specific IgG antibodies in the sera of immunized mice. Mouse serum was collected from the tail vein plexus before each vaccination, and antibodies were detected by indirect enzyme-linked immunosorbent assays (ELISAs). Determination of specific IgG antibodies in the serum at 0, 2, 4, and 6 weeks. **(A)** Total IgG; **(B)** IgG1; and **(C)** IgG2a. The results are shown as the means of OD450 ± SDs, and significant differences compared with PBS or GST (**$P < 0.01$) is indicated by asterisks (*).

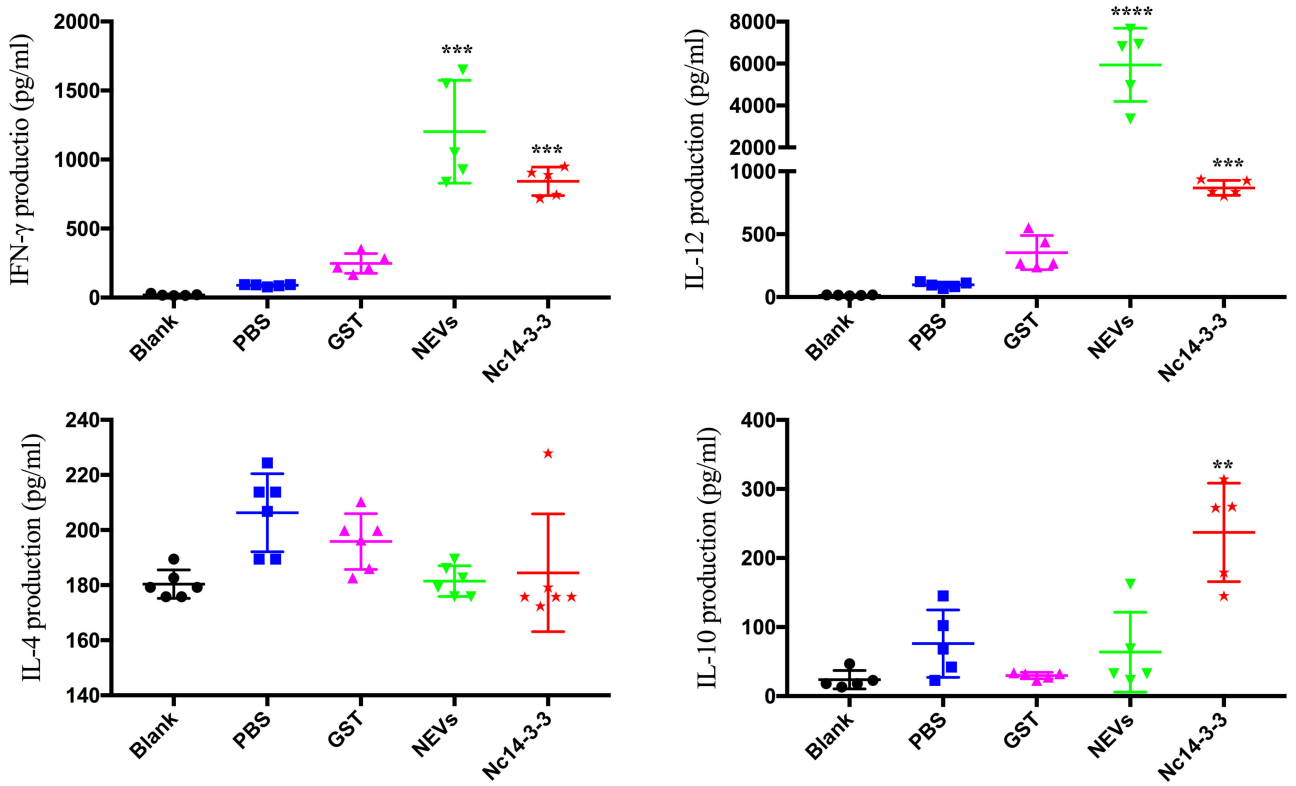


**FIGURE 2 |** Cytokine production in the serum of mice detected using indirect enzyme-linked immunosorbent assays (ELISAs). Data are expressed as the mean ± SD from three separate experiments. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; and ****$P < 0.0001$ for the NEV- and Nc14-3-3-immunized groups vs. the PBS or GST group.

of $CD8^+$ cells in the groups vaccinated with NEVs or Nc14-3-3 was significantly increased compared with that in the control groups that received PBS or GST ($P < 0.05$).

## Experimental Protection After *N. caninum* Infection in Mice

To assess the protection provided by NEVs or Nc14-3-3, 2 weeks after the last immunization, all mice were challenged with $2 \times 10^7$ Nc-1 tachyzoites, and the survival time was monitored daily until 40 days after the challenge. Mice were highly susceptible to acute infection, and increased mortality was observed in the mice that received GST, PBS, or blank control (all of these mice died within 12, 8, or 7 days, respectively) (**Figure 4A**). In contrast, the survival rates for the NEV- or Nc14-3-3-immunized groups were 60 and 40% at the end of the trial, which showed significantly prolonged survival times. Furthermore, the body weight continuously decreased in the control groups vaccinated with PBS, GST or blank control until death, although no significant weight increase in NEV- or Nc14-3-3-immunized mice was found (**Figure 4B**).

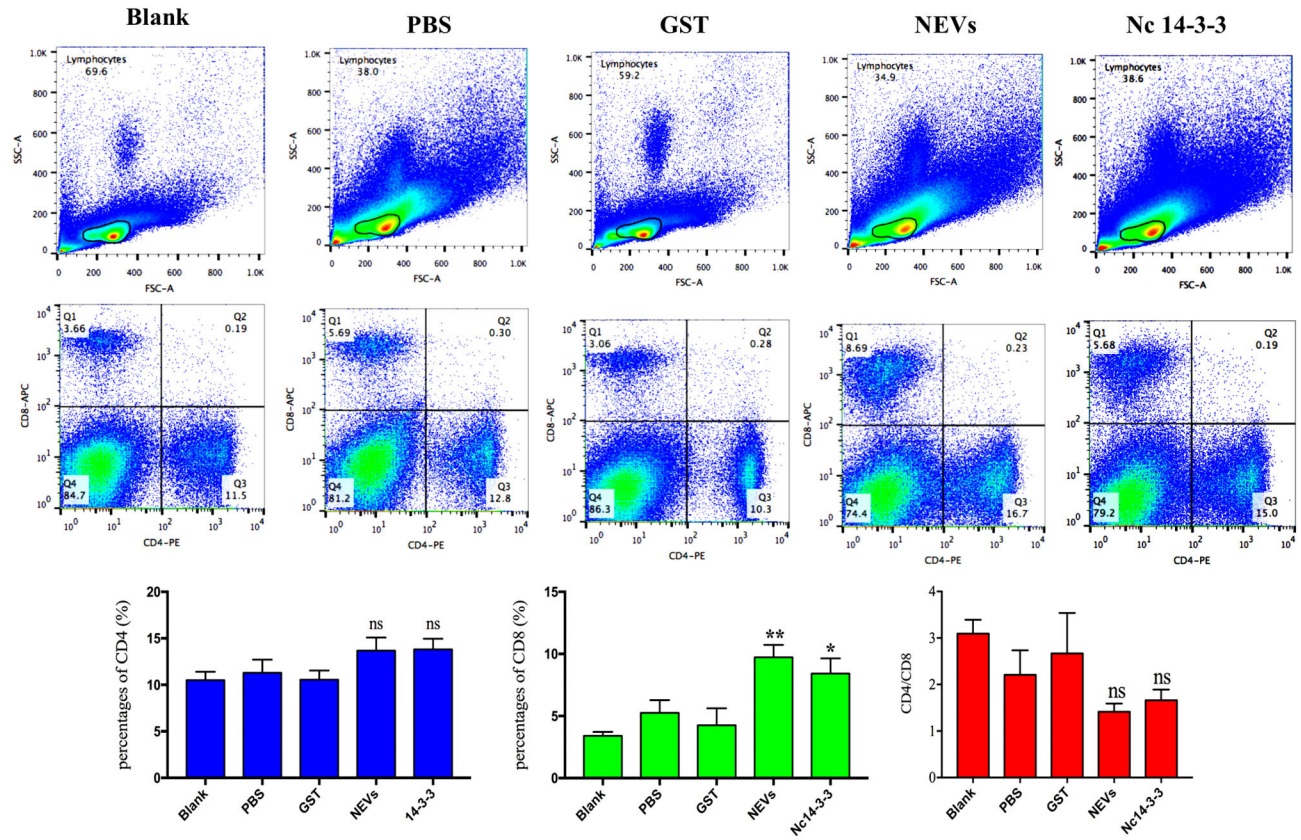


**FIGURE 3 |** Flow cytometry analysis of T cell subsets. The percentages of $CD4^+$ and $CD8^+$ T cells in the spleen of immunized mice 2 weeks after the last immunization are shown, and the results are representative of three independent experiments. $^*P < 0.05$; $^{**}P < 0.01$ for the NEV- and Nc14-3-3-immunized groups vs. the PBS or GST group.

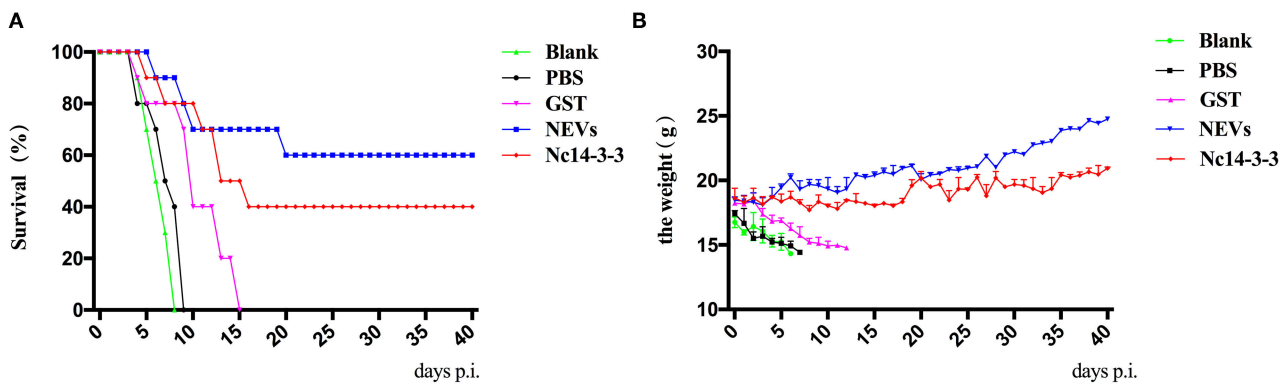


**FIGURE 4 |** Survival rates and clinical observations of mice. **(A)** Survival rates (surviving mice/total mice) of vaccinated mice in response to infection with a dose of $2 \times 10^7$ Nc-1 tachyzoites over 40 days. **(B)**. The body weight of mice was recorded daily before death occurred.

## Vaccination With NEVs or Nc14-3-3 Controlled *N. caninum* Proliferation and Reduced Host Pathological Changes

At 5 days post-infection, infected mice were euthanized, and the heart, liver, spleen, lung, kidney, and brain were harvested to examine the parasite burden by qPCR and pathological changes by H&E staining. As shown in **Figure 5**, the number of parasites in the NEV- or Nc14-3-3-immunized group was significantly lower than that in the other groups vaccinated with PBS, GST or blank control ($P < 0.05$). The pathological changes shown in

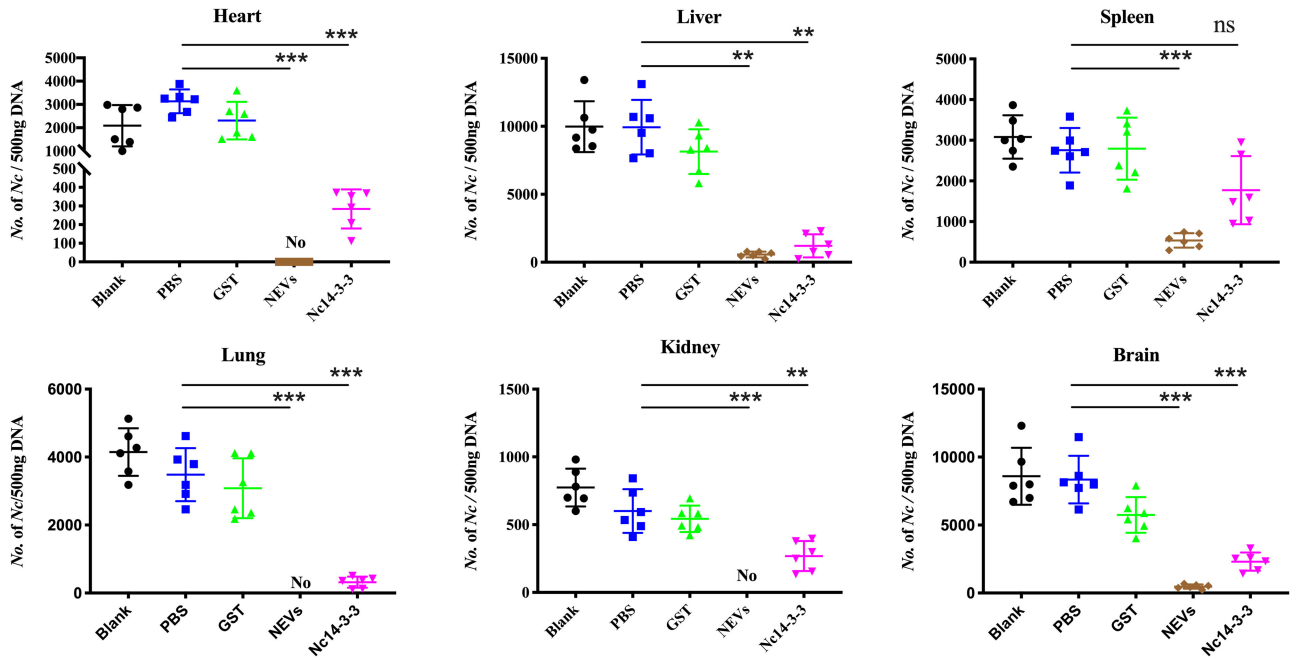


**FIGURE 5 |** Two weeks after the last injection, each mouse was challenged with 2 × $10^7$ Nc-1 tachyzoites. At 5 days post-infection, infected mice were euthanized; the heart, liver, spleen, hung, kidney, and brain were harvested; and parasite loads were measured by qPCR. $^{**}P < 0.01$; and $^{***}P < 0.001$ for the NEV- and Nc14-3-3-immunized groups vs. the PBS or GST group.

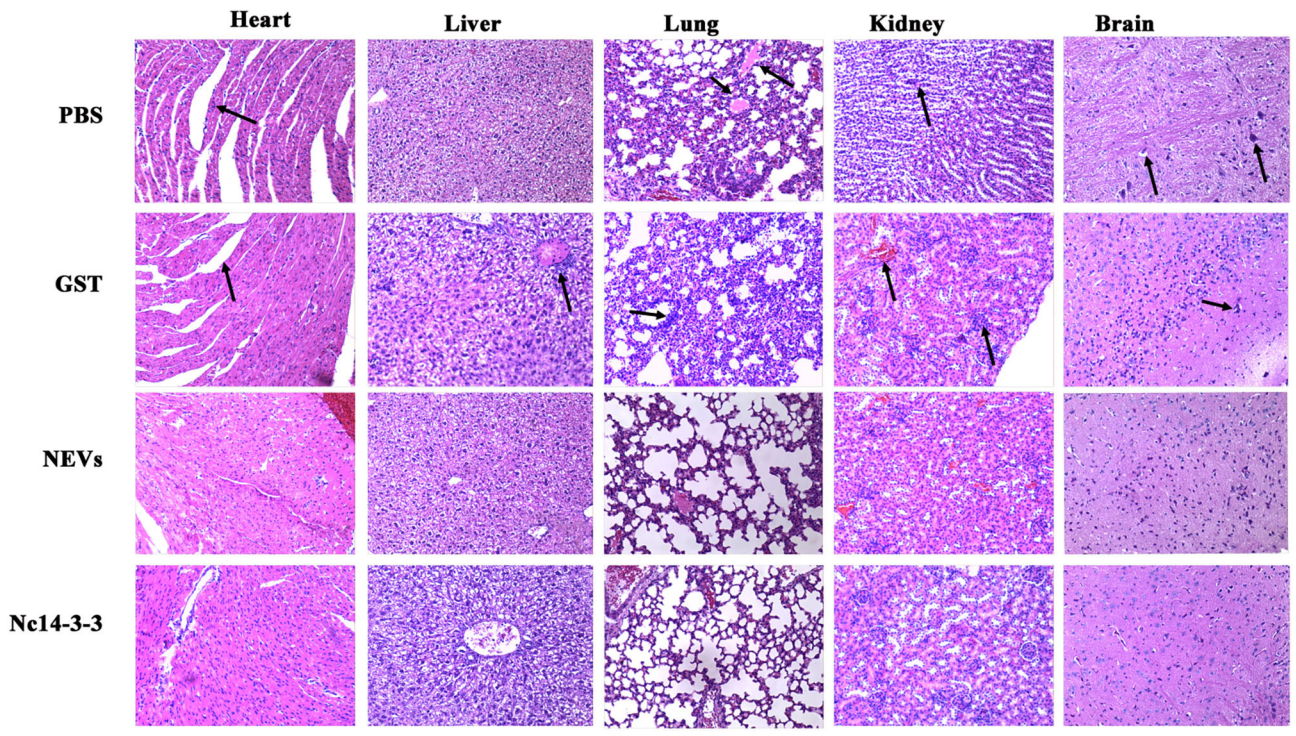


**FIGURE 6 |** Two weeks after the last injection, each mouse was challenged with 2 × $10^7$ Nc-1 tachyzoites. At 5 days post-infection, infected mice were euthanized, and the heart, liver, hung, kidney, and brain were harvested. Pathological changes were observed by H&E staining.

**Figure 6** indicate that tissue lesions in the PBS and GST groups were serious, especially the thickening of the lung interstitium infiltrated with the most inflammatory cells, increased fluid in the alveoli, and widened alveolar septum. The liver structure was disordered and had necrotic foci, accompanied by a large number of inflammatory cells. The brain glial cells increased and exhibited macrophage infiltration, while the NEV- and Nc14-3-3-immunized mice had mild lesions. These results suggest that NEVs and Nc14-3-3 led to effective protection in the mouse model following infection with *N. caninum* tachyzoites.

## DISCUSSION

*Neospora caninum* is an apicomplexan parasite that infects a broad range of warm-blooded animals and leads to neosporosis worldwide (13), and neosporosis often causes abortion especially in dairy cattle and leads to global economic losses (12).

Although many efforts have been made to restrain bovine neosporosis, there are still no effective methods to control this disease (14). Therefore, there is an urgent need to develop a safe and effective *N. caninum* vaccine (3). In recent years, with the identification of new antigens, several dense granule (GRA) and rhoptry (ROP) proteins in *N. caninum* have been identified that could be used in diagnostics or as vaccine candidates (15–17). We have previously demonstrated that the *N. caninum* 14-3-3 protein, which is included in extracellular vesicles (EVs) released by *N. caninum,* induced effective immune responses and stimulated cytokine expression through the MAPK, AKT and NF-κB signaling pathways in murine bone marrow-derived macrophages (BMDMs) (8, 9), but whether Nc14-3-3 can be used as a novel vaccine candidate against neosporosis has not yet been determined.

Currently, the most effective way to control neosporosis is to develop an effective vaccine. The first vaccines developed against *N. caninum* were live or attenuated vaccines because they could elicit both humoral and cellular immunity and provide a variable degree of protection, but their use was limited due to potential safety problems. Inactivated or classical subunit vaccines against neosporosis are safe but currently do not stimulate protective immunity (4). To date, numerous vaccine antigens have been widely evaluated, especially DNA, recombinant protein, or vector-based vaccines, and vaccination using a recombinant antigen triggering appropriate levels of protective immunity for effective protection could offer the most appropriate vaccination tool (15), such as surface proteins and/or those secreted from micronemes, rhoptries or dense granules, which have been the most studied for protection. The 14-3-3 protein is a phosphoserine-binding protein that plays a key role as a regulator of multiple cellular processes in eukaryotes (18, 19), and it has been isolated and sequenced in many apicomplexan parasites, such as *Toxoplasma gondii* (20), *Eimeria tenella* (21), and *Cryptosporidiidae* (22). More importantly, research has shown that 14-3-3 proteins can be used as vaccines in sheep infected with *Fasciola hepatica* (23). *Eimeria maxima* 14-3-3 could significantly reduce jejunal lesions and weight loss, increase the oocyst reduction ratio, and produce an anticoccidial index of more than 165, demonstrating that Em14-3-3 could be used as a promising antigen candidate for vaccine development against *E. maxima* (24). The 14-3-3 protein of *T. gondii* has been shown to be a new candidate vaccine against toxoplasmosis (25). Our previous research indicated that NEVs were enriched for secreted membrane-associated proteins, including 14-3-3, and EVs modulated inflammatory cytokine expression in BMDMs by triggering the TLR2 and MAPK signaling pathways *in vitro* (9). Increasing evidence has indicated that EVs can evoke the innate immune response to control or facilitate infection in parasites (26–28). EVs contain a variety of substances, including proteins, lipids, and RNA, that play multiple roles in intercellular communication, such as delivering signals, regulating cytokine secretion, and regulating the immune response, so EVs are novel vaccine candidates (10, 29, 30); therefore, our previous study also selected NEVs as vaccine controls.

When *N. caninum* invasion occurs, the parasite is first captured and processed by antigen-presenting cells and then presented to T lymphocytes to induce adaptive immunity. Upon subsequent invasion, specific antibodies, as one of the robust protective immune responses, can prevent and inhibit the attachment of *N. caninum* to its host cell receptors, further helping macrophages kill and eliminate the parasite and preventing reactivation (31–33). Humoral immunity is important in eliminating pathogens, strengthening the elimination of invasive microbes, and building immunological memory to protect against reinfection. In this study, we determined the humoral response on the basis of specific anti-*N. caninum* IgG levels; significantly increased levels of IgG were observed in the serum of mice vaccinated with NEVs and Nc14-3-3, which would contribute to strong protective efficacy against subsequent *N. caninum* infection. Higher levels of IgG1 were also detected in the serum of mice in the NEV- and Nc14-3-3-immunized groups than the serum of those in the control groups, and the level of IgG1 antibodies was significantly higher than that of IgG2a, indicating that NEVs and Nc14-3-3 induced a mixed Th1/Th2 immune response. These results are similar to those for other previously reported *T. gondii* vaccines (31, 34, 35). Type 1 immune responses are known to play an important role in protection against intracellular pathogens, and these responses are associated with high levels of IFN-γ and IL-12 (36). Therefore, we subsequently examined the expression of Th1 (IFN-γ and IL-12) and Th2 cytokines (IL-4 and IL-10) (37).

The elimination of intracellular protozoan parasites depends critically on the action of cellular immunity. Through interactions with many effector cells and molecules, a variety of immune cells actively cooperate to fight against infection. Accumulating evidence has shown that IL-12 triggers a Th1-type immune response, is a pivotal proinflammatory cytokine for the control and restriction of acute and chronic protozoan parasite infection, and is required for the long-term maintenance of the cytokine IFN-γ (38, 39). Notably, high levels of the IL-12 cytokine are essential to resistance against *N. caninum* and *T. gondii* infection (40, 41), and blocking or lacking a functional IL-12 receptor leads to high susceptibility to these two parasite infections (42). In addition, IL-12 is a well-known inducer of IFN-γ production in parasite infection, and studies

have shown that IFN-γ and IL-12p40 are important to further explore the host protective mechanism. IL-12/IL-23 p40 chain-deficient (IL-12$^{-/-}$) mice presented elevated parasitic burdens after intraperitoneal infection with *N. caninum* (43). Calves challenged with live tachyzoites could produce a predominant IgG response, and high levels of IFN-γ and TNF-α were also observed in animals (44, 45). Our study demonstrated that mice vaccinated with NEVs or Nc14-3-3 generated significantly higher levels of IFN-γ and IL-12p40 than mice vaccinated with single-gene plasmids, PBS or empty vector. These results suggested that immunization with NEVs or Nc14-3-3 elicited a Th1-type immune response against acute *N. caninum* infection. Furthermore, we also detected the levels of the Th2-type cytokines IL-4 and IL-10. High IL-10 levels were observed in Nc14-3-3-immunized mouse serum but not NEV-immunized mouse serum, and IL-4 levels did not change significantly in any group. This finding was similar to the results from BALB/c mice immunized with *T. gondii* exosomes (10). A large number of studies have shown that many proteins, including GAG1, ROP18, and GRA7, could protect mice after *T. gondii* infection by inducing the production of Th1-biased immune responses (46, 47). BALB/c mouse vaccination with the recombinant protein rNcSRS2 of *N. caninum* promoted the upregulation of IL-10 expression (48). Another study also reported that the IL-10 and IFN-γ cytokines were highly expressed in mouse spleen cells restimulated with *Neospora* antigens. IL-10 is a homodimeric cytokine, which can be produced by most cells of the innate and adaptive immune system, in addition, it functions as a self-limiting mechanism of effector T cells. During infection, it is produced to limit inflammation and collateral tissue damage, such as during *T. gondii* infection, IL-10 produced by Th1 cells is essential to limit an otherwise excessive and detrimental Th1 cell response (49). IL-10 is an essential anti-inflammatory cytokine that plays important roles as a negative regulator of immune responses (50); it is able to regulate the Th1-type response and promote high levels of IFN-γ production (51). Therefore, the high IL-10 level contributed to the prolonged survival time of mice immunized with Nc14-3-3.

Cellular immunity plays an important role in the control of *N. caninum* infection. To determine whether NEV or Nc14-3-3 vaccination activated CD4$^+$ or CD8$^+$ T cells, we determined the percentage of CD4$^+$ and CD8$^+$ T lymphocytes in the spleens of mice from each experimental group ($n = 6$) after the final immunization by flow cytometry. The results indicated that the percentage of CD8$^+$ cells in the NEV- or Nc14-3-3-vaccinated mice was significantly increased, while there was no significant difference in the percentage of CD4$^+$ T cells. These results were similar to those of Li's report, which showed that the percentage of CD8$^+$ T cells was significantly increased in BALB/c mice immunized with *T. gondii* exosomes (10). IL-12 stimulates IFN-γ synthesis by natural killer (NK) cells, and T lymphocytes control intracellular replication of *Toxoplasma* (52). In the acute phase of infection, IFN-γ and IL-12, which involve CD8$^+$ T cells, play critical roles in the detection and elimination of pathogens and, to a lesser extent, CD4$^+$ T cells (53). CD8$^+$ dendritic cells (DCs) are important *in vivo* for cross-presentation of antigens derived from intracellular pathogens (54), in the case of parasitic infection, CD8$^+$ T cells are exposed to persistent antigen and/or inflammatory signals (55). CD8$^+$ T cell contribute to the early production of the pro-inflammatory cytokine IL-12, which stimulates the release pro-inflammatory cytokine of IFN-γ, the generation of I pro-inflammatory is central to host resistance (56). As *T. gondii* is an intracellular parasite, a strong CD8$^+$ T cell response plays an important role in controlling the development and spread of *T. gondii* infection (57). The current research indicated that mice immunized with NEVs and Nc14-3-3 activated specific cellular immunity against *N. caninum*.

Host innate immunity plays an important role in fighting protozoal infections by inhibiting parasite replication and triggering appropriate adaptive immune responses, which control active infections and overcome subsequent re-exposures (58). A number of mechanisms for how the immune system recognizes and responds to pathogens have been defined, including mechanisms regulated by EVs (59). EVs likely play an important role during parasite infection because of their diverse group of biomolecules, which have immunomodulatory properties (60, 61). As a vaccine, exosomes were associated with the development of fewer brain cysts in *T. gondii*-infected CBA/1 mice (62). *T. gondii* exosomes were also found to trigger the immune response and activate partial protective immunity against acute *T. gondii* infection in BALB/c mice (10). *Leishmania* exosomes have also been well-studied, and *Leishmania donovani* exosomes modulate innate and adaptive immune responses in C57BL/6 mice by affecting monocyte and dendritic cell (DC) cytokine production (63). *Leishmania major* releases exosomes with different protein contents, which are known to function in immune modulation (64). DCs pulsed with *Eimeria* antigens could be used as a vaccine against *Eimeria* infection in chickens (65). Mice treated with exosomes derived from DCs pulsed with *T. gondii* antigens were shown to elicit humoral and cellular immune responses and protect mice against subsequent parasite infection (66). Although the above results all indicated that EVs could be potential candidates, a variety of antigen components from pathogens can be loaded in EVs, and they will not cause infection due to the lack of live parasites. However, considering that obtaining high-purity EVs or exosomes is still difficult at present, much work needs to be done to identify and validate EV biomarkers that can be utilized in the diagnosis and therapy of parasitic disease.

## CONCLUSIONS

In the work described here, we examined the immunogenicity and potency of Nc14-3-3 as a vaccine candidate against infection with *N. caninum* in a murine model. Our data demonstrate that the vaccination of mice with Nc14-3-3 elicited both cellular and humoral immune responses and provided partial protection against acute neosporosis. Thus, Nc14-3-3 could be used as an effective antigen candidate for developing vaccines against *N. caninum*.

## DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author/s.

## ETHICS STATEMENT

The animal study was reviewed and approved by all animal experimental procedures were performed in strict accordance with the Regulations for the Administration of Affairs Concerning Experimental Animals approved by the State Council of People's Republic of China (1988.11.1) and with the approval of the Animal Welfare and Research Ethics Committee at Jilin University (IACUC Permit Number: 20160612).

## AUTHOR CONTRIBUTIONS

SLi, NZ, and XZ developed the study protocol. SLi and SLiu carried out the experiments. SLi, XW, XL, and LL performed the data analysis. SLi wrote the manuscript. PG, JL, and XZ revised the manuscript. All authors read and approved the final manuscript.

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# Chemical Analysis and Anthelmintic Activity Against *Teladorsagia Circumcincta* of Nordic Bark Extracts *In vitro*

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Helminth parasitic infections are common in small ruminants in Norway; infection is usually treated with anthelmintic drugs, but anthelmintic resistance is an increasing problem. It is necessary to identify strategies to reduce the use of anthelmintic drugs and mitigate the impact of anthelmintic resistance. Condensed tannin (CT)-rich forages have been shown to reduce the helminth burden in small ruminants, but these forages have limited cultivation potential in Scandinavia. A good source for CT in cold climatic regions may be the bark of several commercially utilized tree species. In the present study, we determined the content and characterized the type of CT in bark extracts of pine (*Pinus sylvestris* L.), spruce (*Picea abies* L.), and birch (*Betula pubescens*). Extracts of selected bark samples were tested for their anthelmintic efficacy against the ovine infectious nematode *Teladorsagia circumcincta.* Total CT content was higher in the bark from younger (10–40 years old) pine and spruce trees; it decreased with tree age in pine, whereas it remained relatively stable in the bark of spruce and birch. Pine trees consisted of 100% procyanidins, whereas prodelphinins were present in most spruce (4–17%) and all birch samples (5–34%). Our studies clearly showed that there is variation in the anthelmintic activity of water and acetone extracts of bark samples collected from various sites around Norway, as this was measured with two independent *in vitro* assays, the egg hatch and larvae motility assays. The anthelmintic activity of some extracts was consistent between the two assays; for example, extracts from the three samples with the highest CT content showed very high activity in both assays, whereas the extract from the sample with the lowest CT content showed the lowest activity in both assays. For other extracts, activity was not consistent across the assays, which could be attributed to the susceptibility of the different stages of the parasitic life cycle. We demonstrated that bark extracts from commercially used trees in Scandinavia have the potential to be used as alternatives to anthelmintics. Further work should focus on refining the associations between bark extracts and anthelmintic activity to identify the best strategies to reduce the input of anthelmintic drugs in livestock production systems.

**Keywords: plant extract, condensed tannins, plant compound, anthelmintic, sheep, pine tree, proanthocynidins, bark**

## INTRODUCTION

Recent research has shown that there is a high treatment frequency with anthelmintic drugs in sheep lambs in Norway, particularly in coastal areas (1), which may be responsible for the development of anthelmintic resistance (2, 3). Strategies to prevent helminth infections are needed to reduce treatments with anthelmintics. Dietary inclusion of plants or feed supplements containing condensed tannins (CT) may be one such strategy, as CT have been shown to have anthelmintic activity (4). CT are complex flavonoid polymers widely distributed in higher plants and hardwood and softwood bark (5, 6). Many common CT-rich forages which have been investigated in other parts of the world, such as birdsfoot trefoil (*Lotus corniculatus*), sulla (*Hedysarum coronarium*), sericea lespedeza (*Lespedeza cuneata*), and sainfoin (*Onobrychis viciifolia*), have cultivation limitations in Norway (7). However, Nordic countries have a strong forest and sawmill industry, which produces a vast amount of bark as a by-product. Especially softwood bark (8), which is rich in CT, is disposed off as fuel or used in horticulture (9). Exploiting the potential use of bark as a feed supplement for ruminants would make the tree industry more economically viable, as well as provide a strategy to mitigate the impact of anthelmintic resistance for sustainable parasite control.

There are very few records of antiparasitic effects of bark and CT extracted from trees, but a study by Min and coworkers showed that dietary inclusion of bark from *Pinus taeda L.*, a species found in the United States of America, improved animal performance and reduced fecal egg count and ruminal ammonia concentration in growing male goats (10). Additionally, Williams et al. (11) observed direct anthelmintic effects of CT from *Pinus sylvestris* bark against the parasitic nematode *Ascaris suum*, which is prevalent in pigs and humans. To our knowledge, there is no published evidence on the concentration and type of CT in bark from common tree species in Norway, although a recent study showed that there is variation in tannin production in cultures of cells originating from Nordic plants (12). In this study, we aimed to (1) quantify the concentration and determine the type of CT in fresh bark from logging sites and in samples from commercial sawmills and (2) test the anthelmintic activity of selected bark extracts *in vitro* against *Teladorsagia circumcincta*, a common abomasal parasitic nematode in sheep and goats.

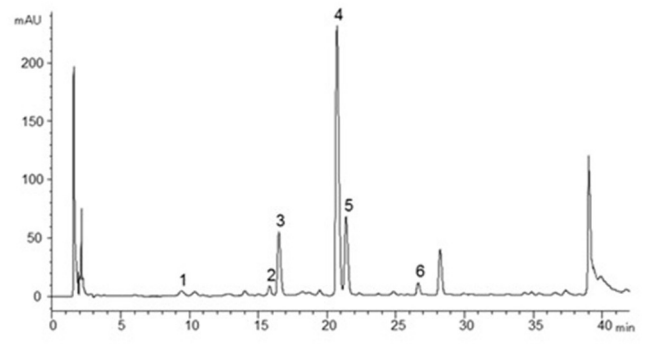


**FIGURE 1 |** HPLC-PDA ($\lambda = 280$ nm) chromatogram of birch bark sample after thiolysis. Peaks are cysteaminyl thioethers of gallocatechin (1), epigallocatechin (2), catechin (3), and epicatechin (4), respectively. Peak 5 corresponds to catechin and peak 6 to epicatechin (pure/non-derivatized compounds).

## MATERIALS AND METHODS

### Bark Sampling

Twenty-nine bark samples were collected for the study. Bark of Scots pine (*Pinus sylvestris* L., $n = 8$), Norway spruce (*Picea abies* L. $n = 8$), and Downey birch (*Betula pubescens* Ehrh., $n = 6$) were sampled from trees felled from January to April in 2013 at different locations in the municipality of Tingvoll. Samples were taken from the lower and upper parts of the tree trunk, and the age of the tree was estimated by tree-ring dating. Additional bark samples of Scots pine (three samples) and Norway spruce (four samples) were provided by the sawmills MøreTre AS (Surnadal) and Kjeldstad Sagbruk & Høvleri AS (Selbu), respectively. All bark samples were composed of both inner and outer bark (not separated). The samples were dried at 65°C for 48 h, cut into smaller pieces, and homogenized to pass a 0.5-mm screen using a rotor mill (Retsch Ultra Centrifugal Mill ZM 200) prior to extraction.

### Condensed Tannins Analysis

CT exist in both free and bound states in the bark (8). Free CT may be extracted with aqueous acetone, aqueous methanol, or alkaline hot water (13–17), whereas non-extractable (or bound) CT require chemical or enzymatic pre-treatment, e.g., thiolysis, to be released (5, 18).

#### Total CT Determination

All bark samples were analyzed for total CT content (free plus bound CT) by high-performance liquid chromatography (HPLC) coupled to a photodiode array (PDA) detector after thiolysis, that is, a depolymerization of the CT in the presence of a nucleophile (cysteamine) (19), at the Biotechnology and Food Research laboratory of LUKE Natural Resources Institute Finland. In short, 10 mg homogenized bark sample was weighed into an Eppendorf tube and 1 ml thiolysis reagent [3 g cysteamine + 56 ml methanol + 4 ml aqueous (37%) HCl] was added. The sample was put in a water bath at 65°C for 60 min and then cooled on ice for 5 min. The sample was homogenized using a Whirli mixer and filtrated (0.45 μm regenerated cellulose syringe filter) into an HPLC vial and analyzed immediately. During the thiolysis reaction, the CT B-type bonds were broken. The extension units were released as flavanol-cysteamine derivatives while the terminal units were released as underivatized free flavanol monomers. These units were determined individually with HPLC-PDA and summed into total CT content (18). The A-type bonds remained intact and produced dimers or higher oligomers, which could be distinguished from the monomeric flavanols by characteristic peaks during the chromatography. Each sample was analyzed in triplicate by HPLC-PDA on an Inertsil ODS-3 (GL Sciences Inc., Torrance, CA) reversed-phase column (150 × 4.0 mm i.d., 3 μm). The mobile phase consisted of (A) 50 mM phosphoric

acid (aqueous), pH 2.5, adjusted by NaOH and (B) acetonitrile. Elution started isocratically with a constant flow of 5% B in A, 5 min, followed by 5–20% B in A, 5–35 min; 20–50% B in A, 35–45 min; and 50% B in A, 45–50 min. Separation was monitored by PDA at $\lambda 1 = 270$ nm for (epi)gallocatechin) and $\lambda 2 = 280$ nm for (epi)catechin and their cysteaminyl derivatives. Reference standards (gallocatechin, epigallocatechin, catechin, epicatechin, procyanidin B2, procyanidin A2) were purchased from Extrasynthese (Lyon, France) and used to set up external calibration curves for quantification. Cysteaminyl derivatives of (epi)gallocatechins were quantified using (epi)gallocatechin calibration curves. Cysteaminyl derivatives of (epi)catechins were quantified using the epicatechin cysteaminyl calibration curve generated from thiolyzed procyanidin B2. A-type dimers and their cysteaminyl derivatives were determined against procyanidin A2. A representative chromatogram is given in **Figure 1**. As the CT oligomers in the bark were cleaved into monomers during thiolysis, the nominal CT polymer sizes—or degree of polymerization (DP)—could not be determined, but the *average* degree of polymerization was calculated by dividing the total CT amount with the amount of terminal units.

### Free CT Determination

Bark samples that were selected for *in vitro* anthelmintic testing were additionally analyzed for free CT content using a gravimetric ytterbium (Yb) precipitation method. Extractable CT were extracted with 70% acetone, according to a previously described method (20). Following the removal of acetone, the aqueous solution was washed three times with petroleum ether. Traces of solvent in the water phase were removed under vacuum before washing it three times with equal amounts of ethylacetate. Traces of ethylacetate in the aqueous phase were removed under vacuum. The remaining solution was separated into portions of 50 mL. Ytterbium(III) acetate (1 M, 2 ml) was added to the aqueous solution and stored overnight at 5°C. The next day, the stored solution was centrifuged at rpm = 3,590 for 10 min at 5°C. The formed pellet was washed and recentrifuged twice with 70 % acetone and once with pure acetone and dried under vacuum. The dry CT-ytterbium precipitation was weighed and heated to ashes at 800°C with a burner, above the oxidizing flame, until the black powder turned into gray ash. Before measuring the weight of the ash, the dish was cooled down to room temperature in a desiccator and the total amount of free CT was corrected for Yb(III) ash (21).

## Preparation of Bark Extracts for *in vitro* Anthelmintic Testing

Following the chemical analysis of the initial 29 bark samples collected for the study, 10 bark samples were selected based on CT content and type for *in vitro* anthelmintic testing. The aim was to cover all three tree species, so we included pine, spruce, and birch bark samples low and high in CT content and with variable proportions of PC and PD. Two types of bark extracts were prepared from these 10 bark samples to test the anthelmintic activity of CT, acetone, and water extracts. Seventy percent aqueous acetone solution is the solvent of choice for CT extraction; such CT extracts have been previously used in parasitological studies (21). To obtain those, 1 g of the dried bark sample was added to 20 ml of 70 % acetone and sonicated (probe sonicator) in ice for 20 sec. This was followed by stirring for 4 min. This procedure was followed five times, and the homogenate was filtered through Miracloth (Millipore, UK). The filtrate was then placed in a rotary evaporator until all acetone evaporated; the extract was resuspended in 20 ml of 1% dimethylsulfoxide (DMSO).

Hot water extraction was included in this study as the simplest extraction procedure and consequently one that may be favored by the industry. Although water is not the preferred solvent for CT extraction, hot water can extract free CT (22). To obtain the water extracts, 1 g of the dry bark sample was added to 20 ml of water, heated at 80°C, and stirred on a magnetic stirrer for 30 min. The homogenate was then filtered through Miracloth (Millipore, UK); the extract was resuspended in 20 ml of 1 % DMSO. Water and acetone extracts from each sample were tested with the assays described below. The two *in vitro* parasitological assays were selected, as they are routinely used to assess the anthelmintic efficacy of drugs and plant extracts.

## Egg Hatch Assays

An egg hatch assay (23) was used to test the anthelmintic efficacy of the selected bark extracts. *Teladorsagia circumcincta* eggs were isolated with a flotation technique (24) from freshly collected feces deriving from donor sheep monospecifically infected with the abomasal nematode. Following their isolation, eggs were washed with distilled water to remove any debris and quantified per ml of suspension. One hundred to 150 eggs were added in each well of 24-well plates, at volumes that did not exceed 500 μl; depending on the egg concentration in the suspension, distilled water was added to make up the appropriate volumes. An equal volume of the bark extract was added to each well. In the negative controls, distilled water was added instead of a bark extract. The plates were cultured at 20°C for 48 h. Hatching was stopped by adding helminthological iodine (10 g iodine, 50 g potassium iodine (KI), 100 ml deionized water in ¼ dilution) to the samples, and the numbers of eggs and first-stage larvae present were counted in each well. Each plant extract was tested in triplicate at 2% concentration (1 g of extract in 20 ml of 1% DMSO), and experiments were repeated. The hatching percentage was calculated as number of first stage larvae/number of first stage larvae + number of eggs per well.

## Larval Motility Assays

A high-throughput larval motility assay was used to test the anthelmintic efficacy of the selected bark extracts. The DP xCELLigence Real-Time Cell Analyzer, which measures electrical impedance-based signals across interdigitated microelectrodes integrated on the bottom of tissue culture e-plates, has previously been developed to diagnose anthelmintic resistance (13). Third-stage (infective larvae) *T. circumcincta* larvae were recovered from fecal cultures of monospecifically infected donor sheep after a 10-day incubation period at 20°C. Larval suspension [3,000 L3 per 100 μl of phosphate-buffered saline (PBS)] was added to the wells of E-plates, and the impedance was monitored every 15 sec, for 24 h at 37°C. All larvae used in the experiments were

freshly produced (within 3 months of development to L3). At the end of this period, bark extracts were added in all wells at the 2 % concentration (1 g of extract in 20 ml of 1% DMSO) except the negative control wells, where 1% DMSO was added; these wells served as negative controls, and the expectation was that these larvae will be maintained alive until the end of the experiment. In addition to the negative controls, positive controls (dead larvae, which were frozen and maintained at −20°C for a month) were also included in three wells. The wells with dead larvae received bark extracts after 24 h, to enable the testing of our hypothesis (see below). Wells with no worms were also included as technical controls. All controls were present in all experiments. Impedance signals were monitored in total for 48 h (24 h prior to and 24 h post extract addition) before stopping the experiments. Each treatment was run in triplicate (technical replicate). Impedance data were analyzed and a motility index was calculated as described by Smout et al. (25). Motility index data were used for the statistical analysis as described below.

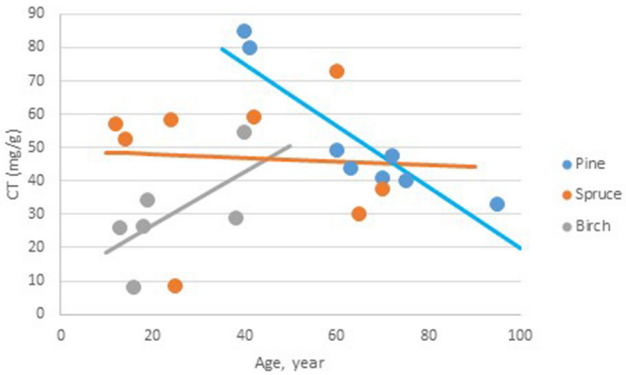


**FIGURE 2 |** The relationship between age of the trees and the total content of condensed tannins (mg CT/g bark) in bark from Norwegian pine, spruce, and birch. Pine CT (g/kg) = 111.8 (SE 21.9, $p < 0.001$, df = 15.9)−0.92 (SE 0.328, $p = 0.013$, df = 16) * Age. Spruce CT (g/kg) = 49.1 (SE 11.49, $p < 0.001$, df = 14.1)−0.05 (SE 0.255, $p = 0.842$, df = 15.3) * Age. Birch CT (g/kg) = 10.7 (SE 15.64, $p = 0.504$, df = 16) + 0.80 (SE 0.591, $p = 0.196$, df=15.5) * Age.

## Statistical Analysis

Statistical analyses on bark total CT concentration and degree of polymerization were carried out using a mixed-model procedure in SAS (Version 9.4; SAS Institute Inc., Cary, NC). The categorical variable tree Species (birch, pine and spruce) and the continuous variable tree age and their interaction were included as fixed effects, and the effect of Location was treated as random effect. LSmeans was used to compare "Species" means, and Tukey's multiple-comparison test was used to determine the significant ($P < 0.05$) difference between treatments. The "estimate" statement in the mixed-model procedure of SAS was used to obtain tree species-specific intercepts and slopes, and the Satterthwaite approximation was used to determine the denominator degrees of freedom for the test of effects.

All egg hatching experiments were duplicated, and the experiments were included in the model as a block. Egg hatching data were analyzed with one-way ANOVA, with treatment as factor. Larval motility data were averaged prior to (24 h) and following the addition of the bark extract (24 h). Motility data following the addition of the bark extract were analyzed with one-way ANOVA, with motility data prior to the addition of the bark extracts used as covariate. Bark extract type (water or acetone) was included in the model as a fixed factor. Each experiment had the following setup: alive larvae treated with water or acetone extract from the same sample, dead control larvae also treated with the two extracts from the same sample, alive controls, which were throughout left untreated, and no worm control wells ($n = 3$). Readings from the no-worm control treatment were not included in the statistical model as these were only included in the experiment as technical controls. Our null hypothesis was that the motility of the larvae exposed to bark extracts was significantly different ($P < 0.05$) than that of the dead control larvae. This was the most appropriate comparison as the environment was the same in these treatments (alive larvae treated with bark extracts and dead larvae treated with bark extracts).

# RESULTS

## Condensed Tannins in the Bark Samples

The pine and spruce bark samples were composed of both inner and outer bark, and the homogenized bark from the older trees (>60 years old) had a higher outer/inner bark ratio and displayed a dark brown appearance, compared to the homogenized bark from the younger trees, which had a yellow appearance. Tree age did not affect color variation of the homogenized birch bark samples.

The LSmeans of bark total CT content, that is free and bound CT determined with the chromatography method, was 72.2 (SE 8.7), 47.2 (SE 13.6), and 46.8 (SE 5.7) mg/g bark in pine, birch, and spruce, respectively. The Tukey test revealed that the content was significantly higher in pine than in spruce, while there were no significant differences between pine and birch and spruce and birch. There was a significant interaction between age of the trees and the species on the CT content ($P = 0.047$); the total CT content decreased [−0.92 (SE 0. 0.328) mg/g bark yearly] with age of the tree in pine (**Figure 2**). For the two other species, there were no significant effects of age on bark total CT content.

Only procyanidins (PC; catechin and epicatechin)—not prodelphinidins (PD; gallocatechin and epigallocatechin)—were detected in the pine bark samples. Procyanidins were also most predominant in spruce, but prodelphinidins were detected at an amount of 4–17% of total CT in 8 out of 12 spruce samples. Prodelphinidins were detected in all birch bark samples, constituting 5–34% of total CT content. Epicatechin was the predominant procyanidin in all species. The ratio of epicatechin:catechin was measured to 4:1 and 5:1 in pine and spruce, respectively, and 2:1 in birch. The average degree of CT polymerization was significantly higher in spruce (8.2 SE 0.38) than in birch (5.7 SE 0.44) and pine (6.6 SE 0.38) samples.

CT characteristics of a selection of the bark samples (corresponding to the 10 samples tested for anthelmintic effects) are displayed in **Table 1**. The bark CT monomers were

**TABLE 1 |** Analysis of total CT in selected bark samples used for anthelmintic screening by high-performance liquid chromatography (HPLC) coupled to a photodiode array detector after thiolysis ($n = 3$).

| Species | ID no. | Location | Age (years) | Total CT | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | | Conc. (mg/g) | SD ($n = 3$) | DP | Procyanidins (%) | Prodelphinidins (%) |
| *Pinus sylvestris* | 2 | Tingvoll | 40 | 85.1 | 1.1 | 5.3 | 100 | 0 |
| | 9 | Tingvoll | 70 | 40.8 | 0.7 | 6.5 | 100 | 0 |
| | 12 | Tingvoll | 41 | 79.8 | 1.4 | 7.2 | 100 | 0 |
| | 41[a] | Surnadal | NA | 35.6 | 1.2 | 6.3 | 100 | 0 |
| *Picea abies* | 7 | Tingvoll | 42 | 59.4 | 2.6 | 7.5 | 94.4 | 5.6 |
| | 15 | Tingvoll | 60 | 72.7 | 0.6 | 7.2 | 100 | 0 |
| | 27 | Tingvoll | 25 | 8.4 | 0.4 | 10.8 | 100 | 0 |
| | 61[a] | Selbu | NA | 26.1 | 0.3 | 10.1 | 83.3 | 16.7 |
| *Betula pubescens* | 3 | Tingvoll | 40 | 54.8 | 2.3 | 5.6 | 84.9 | 15.6 |
| | 52 | Tingvoll | 16 | 8.2 | 0.4 | 6.0 | 65.8 | 34.2 |

[a]*Samples from sawmills.*
*NA, not available.*
*Average degree of polymerization (DP) and content of procyanidins (% (epi)catechin) and prodelphinidins (% (epi)gallocatechin) are also reported.*

predominantly linked by B-type interflavan bonds (i.e., single carbon–carbon bonds), but some A-type bonds (additional interflavonoid ether bond together with a common B-type bond, <1.3 %) were detected in pine samples (data not shown in **Table 1**). The average number of monomers in the CT oligomers, given as the average degree of polymerization (DP), was approximately 7–10 in spruce bark, five to seven in pine bark, and five to six in birch. The spruce bark from Selbu (sample ID 61) had one of the largest average polymer sizes and had a higher content of prodelphinidins (16.7% of CT) than the other spruce bark samples.

The proportion of CT that was extractable with aqueous acetone, that is, the free CT determined with gravimetric method, varied between 9 and 18 mg/g and constituted approx. 17–29% of the total CT in pine ($n = 3$). Free CT appeared to be higher in the younger trees compared to the older trees (15.6 mg free CT/g bark in 40-year-old trees vs. 11.7 mg free CT/g bark in a 70-year-old tree). The free CT concentration in spruce varied between 6 and 18 mg/g, constituting 11–35% of the total CT amount ($n = 2$). Free CT concentration was measured in a single birch sample from a 40-year-old tree and was estimated at 25 mg free CT/g bark.

**TABLE 2 |** Percentage of *T. circumcincta* egg hatching [$n = 6$ (3 reps × 2 experiments per extract)] following incubation in either water extracts or acetone extracts at 2 % concentration (1 g of extract in 20 ml of 1% DMSO) of bark samples for 48 h.

| Species | Id | Egg hatching (%) | |
|---|---|---|---|
| | | Water extract | Acetone extract |
| *Pinus sylvestris* | 2 | 8 | 1 |
| | 9 | 1 | 0 |
| | 12 | 8 | 0 |
| | 41 | 2 | 1 |
| SED | | 2.3 | |
| *Picea abies* | 7 | 11 | 1 |
| | 15 | 13 | 0 |
| | 27 | 0 | 1 |
| | 61 | 12 | 0 |
| SED | | 1.4 | |
| *Betula pubescens* | 3 | 65 | 61 |
| | 52 | 96 | 99 |
| SED | | 3.6 | |

*SED, standard error of difference.*
*Hatching of eggs incubated in water served as control, and hatching was consistently higher than 95%.*

## Egg Hatch Assays

The percentage of egg hatching under control conditions was consistently between 95 and 99%. Overall, incubation in acetone extracts resulted in average hatching of 11% and in water extracts of 20%, although this difference was not significant ($P = 0.199$). Incubation in either water or acetone bark extracts resulted in most cases in significantly reduced hatching compared to the controls (**Table 2**). Extracts from pine samples showed consistently high efficacy, with acetone extracts from most samples inhibiting hatching up to 100% (**Table 2**). Incubation in water and acetone extracts from birch 52 did not affect hatching whereas birch three extracts significantly reduced hatching compared to controls ($P < 0.01$), although the magnitude of the effect was not the same as in extracts from pine or spruce samples.

## Larval Motility Assays

Throughout the duration of all experiments, the motility of alive control larvae was significantly higher than that of dead larvae (motility: 0.012 vs. 0.002, respectively; sed: 0.0002; $P < 0.001$). Our data show that the biological activity of the extracts was variable between samples and between extracts from the same samples (**Table 3**). In all experiments, motility of alive treated larvae (following the addition of the bark extract) was

**TABLE 3 |** Motility of *T. circumcincta* larvae ($n = 3$) following incubation in bark sample extracts at 2% concentration (1 g of extract in 20 ml of 1% DMSO).

| Species | ID no. | Acetone extract | | Water extract | | Alive controls | SED | Rejecting the null hypothesis[a] | |
|---|---|---|---|---|---|---|---|---|---|
| | | Alive treated | Dead control | Alive treated | Dead control | Nontreated | | Acetone extracts | Water extracts |
| *Pinus sylvestris* | 2 | 0.0047 | 0.0038 | 0.0038 | 0.0035 | 0.0089 | 0.0005 | Yes | Yes |
| | 9 | 0.0047 | 0.0024 | 0.0045 | 0.0019 | 0.0099 | 0.0009 | No | No |
| | 12 | 0.0040 | 0.0034 | 0.0041 | 0.0034 | 0.0095 | 0.0009 | Yes | Yes |
| | 41 | 0.0056 | 0.0025 | 0.0038 | 0.0030 | 0.0096 | 0.0007 | No | Yes |
| *Picea abies* | 7 | 0.0061 | 0.0005 | 0.0058 | 0.0006 | 0.0198 | 0.0019 | No | No |
| | 15 | 0.0047 | 0.0014 | 0.0055 | 0.0015 | 0.0187 | 0.0020 | yes | Yes |
| | 27 | 0.0044 | 0.0028 | 0.0033 | 0.0024 | 0.0067 | 0.0013 | No | Yes |
| | 61 | 0.0045 | 0.0015 | 0.0061 | 0.0010 | 0.0194 | 0.0015 | Yes | No |
| *Betula pubescens* | 3 | 0.0045 | 0.0025 | 0.0045 | 0.0026 | 0.0104 | 0.0004 | No | No |
| | 52 | 0.0043 | 0.0013 | 0.0047 | 0.0017 | 0.0107 | 0.0008 | No | No |

[a]*Null hypothesis was that the motility of the larvae exposed to bark extracts was significantly different to the dead control larvae.*
*SED, standard error of difference.*
*The means are adjusted for covariate (motility of worms prior to addition of bark extracts).*

significantly different from that of alive control larvae. **Table 3** shows the full set of data and all the treatments that allow the rejection of our hypothesis. Our hypothesis was rejected following the incubation in four acetone and five water extracts. The motility of larvae treated with acetone extracts from two pine samples (2 and 12) and two spruce samples (15 and 611) was reduced to the level of dead larvae. Similarly, the incubation of larvae in water samples from three pine samples (2, 12, and 41) and two spruce samples (7 and 611) resulted in their motility reduced to the level of dead larvae. None of the remaining of the extracts resulted in reduced motility of L3 to the level of dead larvae, although in some cases motility of L3 following the addition of the extract was much lower than that of alive controls (**Figures 3**, **4**).

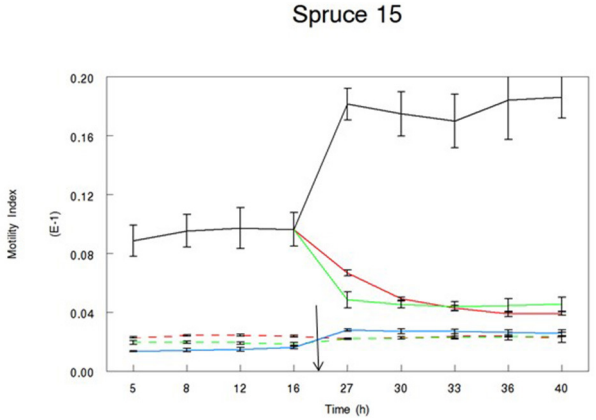


**FIGURE 3 |** Motility Index of L3 *T. circumcincta* larvae incubated in bark extracts of spruce sample no. 15. Acetone (green lines) or water extract (red lines) reconstituted in 1% dimethyl sulfoxide (DMSO) was added in alive (solid line) or dead (dotted line) *T. circumcincta* L3 24 h in the experiment (black arrow). One percent DMSO was added in control wells, where control *T. circumcincta* L3 were maintained alive until the end of the trial (black line). No worm controls were also added in the experiment as technical controls (blue line). Bars indicate standard error ($n = 3$).

# DISCUSSION

## Condensed Tannin Concentration in Bark Samples

In the present study, we determined the variation in the content of total and free CT and characterized the type of CT in the bark extracts of Nordic trees, namely, *Pinus sylvestris* L., *Picea abies* L., and *Betula pubescens*. We showed that tree age is associated with CT content in pine but not in spruce and birch. We quantified the anthelmintic efficacy of bark extracts against the ovine nematode *Teladorsagia circumcincta* and showed that some extracts have the ability to reduce egg hatching and larval motility to almost 100%, which confirms the potential of using bark extracts for parasite control in ruminants.

From the three tree species tested, pine samples showed a decline in the bark content of total CT with tree age. Considering that older trees have more outer bark than younger trees, the expectation is that the outer:inner bark ratio would increase with age. Previous evidence has shown that the outer bark contains lower CT levels than the inner bark, (8). Matthews et al. (8) observed a decline in free CT in pine bark with increasing tree age, due to CT in the dead outer bark being bound to lignocellulose. A small decrease in the content of free CT with age was also observed in our samples (from 16 to 11 mg free CT/g bark), although it is unclear whether the decrease with age in total CT was only attributable to the decrease in free CT.

Commonly, most CT in tree barks are oligomers of procyanidins, with epicatechin and catechin as the monomeric units, followed by prodelphinidins, constituted by gallocatechin and epigallocatechin units. Our pine bark samples contained only procyanidins, which is supported with current literature (6, 8, 18). However, nearly all spruce and birch bark samples also contained both procyanidins and prodelphinidins. Procyanidins have been previously reported in birch bark (8, 11, 18), whereas prodelphinidins have not, although *Betula* leaves are known to

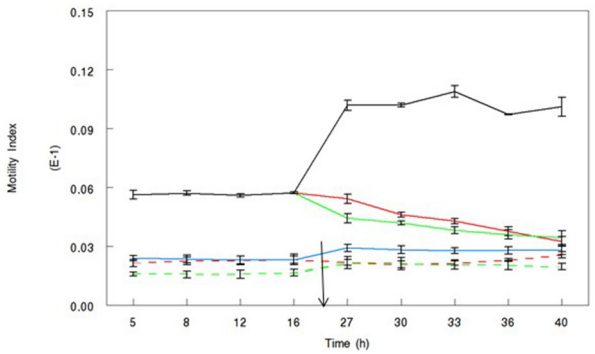


**FIGURE 4 |** Motility Index of L3 *T. circumcincta* larvae incubated in bark extracts of birch sample no 52. Acetone (green lines) or water extract (red lines) reconstituted in 1% dimethyl sulfoxide (DMSO) was added in alive (solid line) or dead (dotted line) *T. circumcincta* L3 24 h in the experiment (black arrow). One percent DMSO was added in control wells, where control *T. circumcincta* L3 were maintained alive until the end of the trial (black line). No worm controls were also added in the experiment as technical controls (blue line). Bars indicate standard error ($n = 3$).

have a high level of prodelphinidins (9). Epicatechin was the most predominant procyanidin in our samples; the pine and spruce bark contained four to five times as much epicatechin as catechin, whereas birch bark contained two times more epicatechin than catechin. This is in agreement with data found in the literature (6, 8). Pine bark samples contained the highest CT levels (mean value of 5 % total CT) when compared to spruce and birch, and the levels are in agreement with the 5–8 % CT levels reported in Scots pine inner bark and 5 % in outer pine bark (8, 18).

## Anthelmintic Activity of Selected Extracts

Our studies clearly showed that there is variation in the anthelmintic activity of water and acetone extracts of bark samples collected from various sites around Norway, as this was measured with two independent assays. Our data support a previously expressed view that the anthelmintic activity of acetone extracts may not always be related to the CT content of these extracts alone, and this will require further investigation. Furthermore, we showed that different stages of nematode parasites may vary in their susceptibility to active compounds contained in extracts from bark samples.

Egg hatching was significantly affected by incubation in most acetone bark extracts. Although this activity may be attributed to CT, other compounds may also be partly responsible for the activity observed, particularly as not all samples tested here had high CT content. The results here agree with previous evidence where incubation in purified extracts from pine bark (*Pinus radiata*) originating from New Zealand resulted in significant reduction in egg hatching and migration ability of *T. circumcincta* (26). In this study, concentrations of total CT of about 2 mg/g resulted in a reduction of egg hatching by 80%. Apart from two extracts, the concentrations of CT contained in our acetone extracts exceeded those previously reported and may be the reason for the high magnitude of activity observed in our egg hatching experiments.

The two extracts with the lowest CT content tested here had a very different activity pattern. Incubation of eggs in spruce 27 extracts significantly reduced hatching compared to controls, whereas incubation in birch 52 extracts did not affect egg hatching. This activity difference could be attributed to the degree of CT polymerization in the two samples. CT present in spruce 27 sample had the highest degree of polymerization, which was not the case for birch 52 samples. Our data support previous evidence where polymer size appeared to have a positive correlation with anthelmintic activity (11, 27). The two samples also differ in their CT profiling, with spruce 27 having 100% PCs, whereas the content of PCs in birch 52 is lower. The evidence related to the efficacy of PCs vs. PDs has been scarce and rather conflicting. PC-rich extracts from pine trees were more effective against parasite development compared to PD-rich ones (28). On the other hand, previous studies showed that PDs may be more efficient in reducing larval exsheathment (29), feeding (30), and adult motility *in vitro* (30). It appears that the issue of monomer efficiency may be more complex than previously thought and may also be affected by other factors, such as total CT content and degree of polymerization. The possibility that other compounds may be responsible for the activity of spruce 27 sample cannot be disregarded.

The results from the larval motility assays showed that incubation of larvae in water and acetone extracts resulted in a reduction in motility, with variable intensity. In some cases, water extracts showed weaker activity compared to acetone extracts, such as extracts from the pine 611 sample. In other cases, acetone extracts showed weaker activity, such as extracts from pine 41 and spruce 27. The three samples with the most CT in the acetone extracts were pine 2, pine 12, and spruce 15. Interestingly, larvae incubated in both acetone and water extracts of these samples reduced their motility to the level of dead larvae. Although CT were not determined in the water extracts in the current study, previous evidence has shown that CT can be extracted with hot water (22). These samples had the highest concentration of CT (73–85 mg Ct/g bark), free CT (14–18 mg free CT/g bark), average degree of polymerization, and no PDs. Consequently, it is possible that the water extraction resulted in free CT extraction, which was responsible for the activity.

The anthelmintic activity of many extracts was consistent between the two assays. Extracts from the three samples with the highest CT content showed very consistent activity in both assays. Similarly, birch 59 samples with the lowest CT content did not show biological activity against any of the parasitic stages. For others, activity was not consistent across the assays. For instance, extracts from pine 9 and spruce 7 samples showed very strong activity against the eggs, but that was not the case for the larvae motility assay. This variation in the magnitude of activities in the two assays could be attributed to the stage of the parasitic life cycle (4). Not unexpectedly, L3 appeared to be the least affected stage as infective larvae are the most resistant form of the parasite during their life cycle.

These results clearly showed that certain extracts from tree bark samples originating from Norway have anthelmintic activity

that varies in intensity *in vitro*. Some of the extracts had substantial biological activity *in vitro*, which if validated *in vivo* could be utilized to reduce the input of anthelmintic drugs in livestock production systems to reduce the development and mitigate the impact of anthelmintic resistance. Further work should focus on the identification of the best tree type and age at felling to achieve maximum biological activity from the bark. Refining the associations between bark extracts and anthelmintic activity *in vivo* is essential to identify the best strategies to reduce the input of anthelmintic drugs in livestock production systems.

## DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author/s.

## AUTHOR CONTRIBUTIONS

HS initiated the study. HS, SA, NS, and MA designed the study. HS conducted the sampling and preparation of the bark. MA, NS, EM, and JH performed the condensed tannin analysis of the bark. SA performed the parasitological assays and analyzed the results. SA, MA, and HS drafted the manuscript. All authors read and approved the manuscript.

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# Molecular Characterization of a Tetraspanin TSP11 Gene in *Echinococcus granulosus* and Evaluation Its Immunoprotection in Model Dogs

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Cystic echinococcosis (CE) is a cosmopolitan zoonosis caused by the larval stage of *Echinococcus granulosus*, which affects humans and a wide range of mammalian intermediate hosts. Parasite tetraspanin proteins are crucial for host-parasite interactions, and therefore they may be useful for vaccine development or disease diagnosis. In the present study, the major antigen coding sequence of tetraspanin 11 (Eg-TSP11) from *E. granulosus* was determined. The results of immunolocalization showed that Eg-TSP11 was mainly located in the tegument of adult worms and protoscoleces. Western blotting analysis showed that the serum from dogs injected with recombinant Eg-TSP11 (rEg-TSP11) could recognize Eg-TSP11 among natural protoscolex proteins. Moreover, the serum from dogs with *E. granulosus* infection also recognized rEg-TSP11. Serum indirect enzyme-linked immunosorbent assays demonstrated that IgG levels gradually increased after the first immunization with rEg-TSP11 compared with those in the control group. Furthermore, the serum levels of interleukin 4, interleukin 5, and interferon gamma were significantly altered in the rEg-TSP11 group. Importantly, we found that vaccination with rEg-TSP11 significantly decreased worm burden and inhibited segment development in a dog model of *E. granulosus* infection. Based on these findings, we speculated that rEg-TSP11 might be a potential candidate vaccine antigen against *E. granulosus* infection in dogs.

**Keywords:** ***Echinococcus granulosus*, cystic echinococcosis, Eg-TSP11, immunogenicity, dog vaccine**

## INTRODUCTION

Cystic echinococcosis (CE) is a serious zoonotic parasitic disease caused by *Echinococcus granulosus* larvae. CE is a major public health concern in developing and developed countries (1, 2). In epidemic areas, the incidence of CE is between 1 and 200 per 100,000 (3, 4). CE seriously endangers human health and the development of animal husbandry and causes the loss of at least 285,500 disability adjusted life years (DALYs) each year (5, 6). The disease is a neglected tropical disease, confirmed by the World Health Organization (WHO) (2). To date, the most successful intermediate

host vaccine is the EG95 recombinant protein, with a protective efficiency of up to 98% (7). However, the recombinant EG95 protein had no protective effect on hosts have been infected with *E. granulosus* and the cysts have been formed. Canids, such as dogs, are the definitive hosts of *E. granulosus,* which play an important role in the life cycle of *E. granulosus.* Unfortunately, there is no commercially available definitive-host vaccine, which has seriously hindered the effective control of CE. Therefore, the development of a dog vaccine against *E. granulosus* is urgently required.

The tetraspanin superfamily (TSP, also known as the transmembrane-4-superfamily, TM4SF) is a hydrophobic plasma membrane-associated protein of 200–350 amino acids. Tetraspanins can be divided into four families, including the cluster of differentiation (CD) family (e.g., CD9, CD81, and CD151), the retinal degeneration slow (RDS) family (e.g., RDS-ROM), the uroplakin family (e.g., UPK1A/1B), and the CD63 family (e.g., CD63 and TSPAN31) (8). The four conserved transmembrane domains of TSPs are called tetraspanin-enriched microdomains (TEMs). TEMs protrude 3–5 nm from the cell surface to form two extracellular rings, one large and one small (EC1 and EC2). In addition, two short N-and C-terminal cytoplasmic tails are formed in the intracellular region. The large extracellular loop (LEL), which contains 2–6 cysteines, is called the "tetraspanin web" which plays a central role in the interaction of TSPs with several other molecules (9, 10).

To date, marked progress has been made in research on tetraspanins as parasite vaccine candidates. Tetraspanin family proteins now occupy an important position in parasite immune interactions, and have been proven to be candidate target proteins for schistosomiasis, Clonorchis sinensis, alveolar echinococcosis, and filariasis (10–13). Tetraspanins are involved in trematode cuticle development, maturation, stabilization, and immune escape (14). The two hydrophilic groups (Sm-TSP-1 and Sm-TSP-2) of the recombinant tetraspanin of *Schistosoma mansoni*, especially Sm-TSP-2, have been shown to be able to induce a >40% immunoprotective effect against *S. mansoni* infection (15). The *TSP1* gene of *E. granulosus* was cloned and expressed for the first time in 2015 and it was demonstrated that TSP1 could stimulate a marked Th1 type immune response in a mouse model. This means that TSP1 may be a possible target in the treatment, prevention, and control of echinococcosis (8). Tetraspanins play important roles in the study of the life cycle of parasites and in mediating parasite signal transduction, immune escape, and other important biological processes. As cell surface molecules, tetraspanins play important roles as bridges in parasite cell signaling pathways and thus represent potential therapeutic targets (9). The present study aimed to characterize the biological characteristics of TSP11 in *E. granulosus*. In addition, the immunoprotective effect of recombinant tetraspanin 11 Eg-TSP11 (rEg-TSP11) was analyzed by evaluating changes in the levels of IgG, and the cytokines Th1 and Th2 in model dogs. Furthermore, we analyzed the worm burden reduction rate and the inhibition of segment development in rEg-TSP11-vaccinated dogs. The results of the present study provide basic immunogenicity data for rEg-TSP11 and pave the way for the development of anti-*E. granulosus* vaccines in dogs.

# MATERIALS AND METHODS

## Parasites

Naturally infected sheep livers, obtained from an abattoir in Urumqi, Xinjiang Province, China, were the source of hydatid cysts. Cyst fertility was checked using light microscopy to determine the presence of protoscoleces (PSCs) within the cysts. PSCs were isolated and treated according to previously described methods (16). PSCs ($n = 2{,}000$) were grown in 1 mL Roswell Park Memorial Institute (RPMI) 1640 medium containing 100 μg/mL streptomycin and 100 U/mL penicillin (Sigma-Aldrich, St. Louis, MO USA) and 10% bovine serum albumin (BSA; Hyclone, Logan, UT, USA). At 28 days after artificial PSC infection in an 8-month-old dog, adult worms were obtained.

## Bioinformatic Analysis

The cDNA sequence of Eg-TSP11 (XP_024352489.1) was downloaded from the NCBI database. The physicochemical parameters of the encoded proteins were analyzed using ProtParam tools on the ExPASY website (https://web.expasy.org/protparam/). SignalP 4.1 (http://www.cbs.dtu.dk/services/SignalP/) was used to check for the presence of a signal peptide, and Novopro tools were used to analyze the transmembrane regions (https://www.novopro.cn/tools/tmhmm.html). SWISS-MODEL (http://swissmodel.expas y.org/) was used to model the tertiary (three-dimensional) structures. The B cell epitopes of Eg-TSP11 were predicted using the IEDB online server (http://tools.immuneepitope.org/main/). MEGA software (version 5.05) used the neighbor-joining (NJ) method to construct phylogenetic trees of aligned proteins (17).

## Expression and Purification of the Eg-TSP11 LEL Region

An RNA-prep Pure Tissue Kit (Nanjing Vazyme Biotech, Nanjing, China) was used to extract total RNA from the PSCs. First-strand cDNA was synthesized from total RNA using a reverse transcription system kit (Nanjing Vazyme Biotech). PCR was then used to amplify the LEL coding sequence from the cDNA using a sense primer (5′- CGC GGA TCC ATG TTT CCA GCA CCG CTT CAA G-3′) comprising a *BamHI* site (underlined) and an antisense primer (5′-CCG CTC GAG TCA TTC ATA GTT TTT CAA GGA G-3′) comprising a *XhoL I* site (underlined). The PCR amplicons were ligated into the pET32a (+) plasmid (Novagen, Darmstadt, Germany) and transformed into *Escherichia coli* BL21 (DE3) cells (Tiangen, Beijing, China). Isopropyl-1-thio-β-D-galactopyranoside (1 mM; IPTG) was used to induce expression from the plasmid for 6 h at 37°C. Inclusion bodies were obtained from the *E. coli* cells, suspended in lysis buffer containing 8 M urea, and incubated for 2.5 h on ice to completely solubilize the recombinant protein. $Ni^{2+}$ affinity chromatography with a His-affinity resin column (Bio-Rad, Hercules, CA, United States) was used to purify the His-tagged rEg-TSP11 protein, which was subjected to 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). A NanoDrop 2000c (Bio-Rad) instrument was used to determine protein concentration.

## Western Blotting

SDS-PAGE (10%) was used to separate rEg-TSP11 and total proteins in extracts from PSCs. The separated proteins were then electrotransferred onto nitrocellulose membranes. Membranes were blocked with 5% (w/v) skim milk for 2 h at 37°C. The membranes were then incubated with *E. granulosus* positive or negative dog sera, anti rEg-TSP11 dog sera, or pre-immunized mouse sera (1:100 v/v dilutions) at 4°C overnight. The next day, the membranes were washed and incubated with horseradish peroxidase (HRP)-conjugated rabbit anti-dog IgG or sheep anti-mouse IgG (1:5,000 v/v dilution) for 2 h. Actin protein of *E. granulosus* was used as an internal control. The membranes were incubated with Pierce ECL western blotting substrate (Thermo Fisher Scientific, Waltham, MA, USA) and then exposed to X-ray films.

## Quantitative Real-Time Reverse Transcription PCR

Quantitative real-time reverse transcription PCR (qRT-PCR) was used to examine the expression profiles of Eg-TSP11 in 28-day strobilated worms and PSCs. Total RNA was isolated from 28-day strobilated worms and PSCs, and cDNA was synthesized from total RNA. The cDNA was then used as a template for qPCR using the TSP11 primers: 5′- GAA GAT AAT GGC TGG GGT GC-3′ and 5′-GTT GTG TGC CCC ATT TGT GA-3′. Actin gene expression was used as an internal control for normalization. Primers for amplification of *E. granulosus* actin were 5′-GAG TCA TGT AGG CCA CG-3′ and 5′-AGA TGG AGG TGG GGA TAG G-3′. The $2^{-\Delta\Delta CT}$ method was used to analyze the data (18).

## Immunolocalization

To identify the location of TSP11 at different developmental stages, adult worms and fresh PSCs were fixed overnight using 4% paraformaldehyde hydrophosphate buffer, permeabilized for 30 min using 1% Triton X-100, and incubated in 0.01% Triton X-100 at 4°C for 1 h. They were then washed three times with 0.01 × phosphate-buffered saline (PBS) and blocked with 5% (w/v) skim milk for 2 h at 37°C. Next, the fixed adult worms and PSCs were incubated with anti-rEg-TSP11 dog IgG (1:100 v/v dilution in PBST) at 4°C overnight. After washing, the sections were incubated with phycoerythrin (PE)-conjugated goat anti-dog IgG (H+L) (1:1,000 v/v dilutions in PBST) for 2 h in the dark at room temperature (25°C). The sections were washed four times with PBST and then examined under a fluorescence microscope (Leica, Wetzlar, Germany). The negative control was comprised of antibodies from the pre-immunized mice.

## Vaccination and *E. granulosus* Challenge

The vaccination experiment included nine Beagles. Group 1 was comprised of three dogs that were vaccinated with rEg-TSP11 mixed with the saponin adjuvant Quil A; Group II was comprised of three dogs that were vaccinated with Quil A only; and Group III was comprised of three dogs that were vaccinated with PBS (control group). Each 350 μL dose of vaccine included 200 μg of soluble rEg-TSP11 and 100 μg of Quil A (Sigma) in PBS. Before vaccination, the mixture was stirred overnight at 4°C. All experiments beagle dogs were immunized through subcutaneous injection in the neck and all the dogs were immunized with the same dose four times, at a 14 days interval. Seven days after the last booster vaccination, all nine dogs were challenged orally with 100,000 *E. granulosus* PSCs. Finally, at 28 days after infection (before eggs appeared), all nine dogs were euthanized and necropsied to collect and count worms as previously described (19, 20). Thirty worms were chosen randomly from each experimental group and the sizes of developed (≥4 segments) vs. underdeveloped (≤3 segments) worms were determined (21).

## Indirect Enzyme-Linked Immunosorbent Assay (ELISA)

The standard checkerboard titration procedure was used to assess the optimal concentrations of the rEg-TSP11 antigen and serum. Carbonate buffer (0.1 M, pH 9.6) was used to dilute the purified rEg-TSP11 protein to 5 μg/mL, which was used as the antigen in the ELISA test. The diluted antigen solution was used to coat ELISA plates at 4°C overnight. The next day, the plate was washed with PBS-Tween-20 (PBST) and then incubated with 5% skim milk at 37°C for 2 h. After thorough washing, 100 μL of serum samples (2-fold dilutions, 1:80) in PBST was added to each well and incubated for 1.5 h at 37°C. After washing, HRP-labeled rabbit anti-dog IgG (1:3,000 dilution; Solarbio, Beijing, China) was added to the plates and incubated for 1.5 h at 37°C. After washing, the substrate, 3, 3′, 5, 5′-tetramethylbenzidine (TMB) (Tiangen, Beijing, China) was added to the wells and incubated for 1.5 h at 37°C. Finally, 1 M $H_2SO_4$ (100 μL) was added to stop the development of color, and the optical density was measured at 450 nm (OD 450).

## Cytokine Detection

Five main cytokines in dogs with *E. granulosus* infection, including interferon gamma (IFN-γ), interleukin (IL)-6, IL-5, IL-4, and IL-1 were determined using an ELISA kit (Janglaibio, Shanghai, China). The required strip was removed from the aluminum foil bag after being left for 60 min at room temperature (25°C). The standard sample well, blank well, and sample well were set, and 50 μL of standard product at different concentrations was added. Then, HRP-labeled antibody (100 μL) was added to each well, which was sealed using a sealing film and incubated for 1 h at 37°C. The liquid was discarded, and the plate was patted dry using absorbent paper. Next, 350 μL of detergent was added to each well and incubated for 1 min, after which the detergent was shaken and the plate was patted dry using absorbent paper. The detergent wash step was repeated five times. Then, 50 μL of substrate A and 50 μL of substrate B were added to each well and incubated for 15 min at 37°C. Finally, 50 μL of termination solution was added to each well, and within 15 min, the OD value of each well was measured at a wavelength of 450 nm.

## Data Analysis

Statistical analyses were performed using SPSS software (version 22.0; IBM Corp, Armonk, NY, USA). All data analyses and graphs were performed using GraphPad Prism 6.0 software package (GraphPad Software Inc., San Diego, CA, USA). Statistical

significance was set at $P < 0.05$. All experiments were repeated a minimum of three separate times.

# RESULTS

## Bioinformatic Analysis

The Eg-TSP11 cDNA sequence was comprised of 765 nucleotides, which encoded a putative protein of 254 amino acids (aa). TSP11 had a predicted pI of 8.91, a predicted mass of 27 kDa, three potential N-terminal glycosylation sites, five potential protein kinase phosphorylation sites, and one predicted tyrosine kinase phosphorylation site (**Figure 1**), including the three typical transmembrane regions (17–39, 59–81, and 94–116). TSP11 contains a NET-5-LIKE-LEL (the largest extracellular loop) located at amino acids 116–120 aa. TSP11 comprises seven predicted B cell linear epitopes (ep1: 48–55 aa; ep2: 123–123 aa; ep3: 143–143 aa; ep4: 162–170 aa; ep5: 186–189 aa; ep6: 210–219 aa; and ep7: 227–240 aa). The three-dimensional structure of TSP11 (judged by structural modeling) revealed multiple helices and folding regions, and its three-dimensional

1 MAFRLRPFVLRIFIHAVNVILGVTNLYLFIVKFFGVFILSLSIFTSLNKSNTPEILGNYL

61 FSGGVYSALFCSIFLIFLPIWGSIALKRYSRLMLILYVIGIATLIIVTFCAGTSLIVFPA

121 PLQAAVKLEMNKTLYHEYGKRGFITDSWDFVQSFLRCCAVEDNGWGAYNGSWWDLSVNAY

181 FYSVDSRLPETSLFYKRVPKSCCLTLVDPLTGWLPTDQYQNVLQCQNWQYGPPRFTNGAHN

241 DALYYRVSSLKNYE

**FIGURE 1 |** The deduced amino acids sequence of TSP11. Underlined: five protein kinase phosphorylation sites; border marked on frame: three potential N-terminal glycosylation sites; shaded: one tyrosine kinase phosphorylation site.

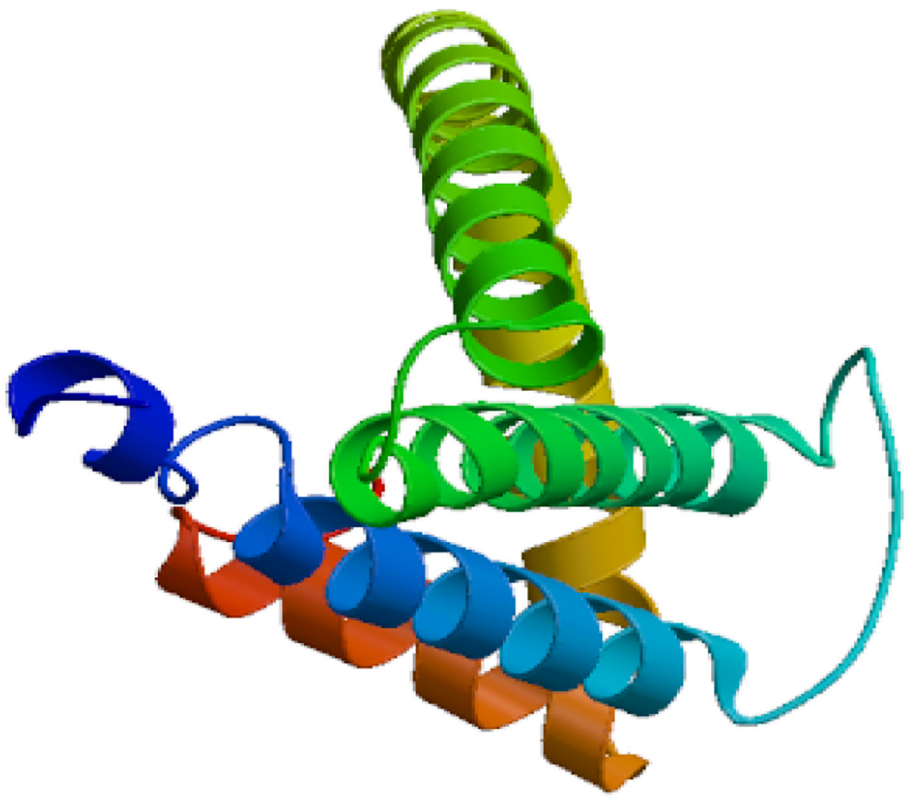

**FIGURE 2 |** Predicted secondary and three-dimensional structure of the Eg-TSP11 protein.

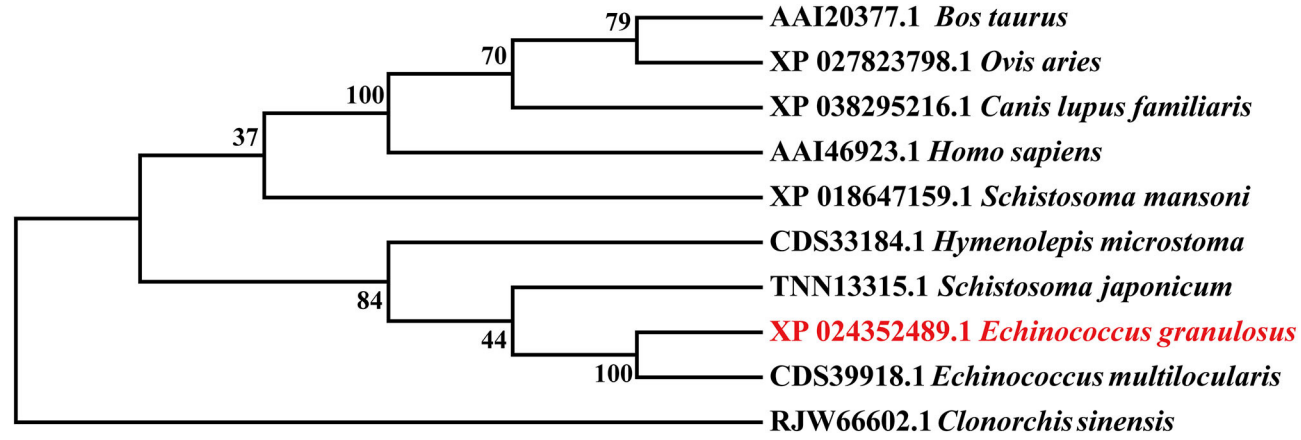


**FIGURE 3 |** Neighbor Joining phylogenetic tree constructed using Eg-TSP11 and TSP proteins from other species.

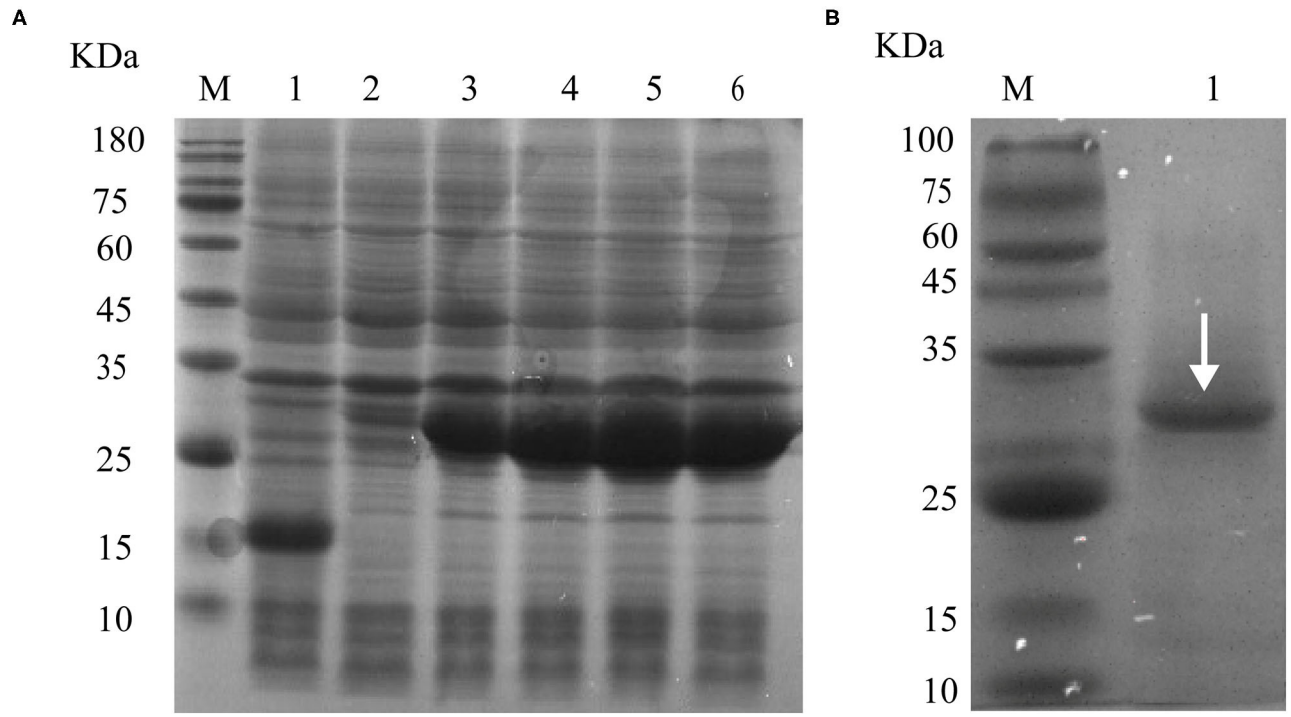


**FIGURE 4 |** rEg-TSP11 expression and purification. **(A)** Lane M: Protein molecular weight markers; Lane 1, The recombinant plasmid was transferred into *E. coli* BL21 (DE3) followed by IPTG induction of pET-32a (+) empty vector for 6 h. Lane 2–6: Recombinant plasmids were transferred into *E. coli* BL21 (DE3) followed by IPTG induction of pET32a-TSP11 for 0, 2, 4, 6, and 8 h. **(B)** Lane M: Protein molecular weight markers. Lane 1: protein sample following purification of the His-tagged rTSP11 protein using an $Ni^{2+}$ column.

structure was mainly comprised of irregular coils (**Figure 2**). In the phylogenetic analysis, Eg-TSP11 was similar to TSP proteins from *Echinococcus multilocularis* (**Figure 3**).

## Expression, Purification, and Recognition of rEg-TSP11

The cDNA encoding the LEL of Eg-TSP11 was PCR-amplified from PSCs, and the recombinant rEg-TSP11 was expressed successfully. The purified rEg-TSP11 protein, which included a His-tag, showed a single band close to the predicted size of 32 kDa on 10% SDS-PAGE (**Figure 4**).

## Western Blotting

Immunoblotting using sera from dogs experimentally infected with *E. granulosus* showed a single ∼32 kDa band. The native Eg-TSP11 protein from PSC extract could be recognized using

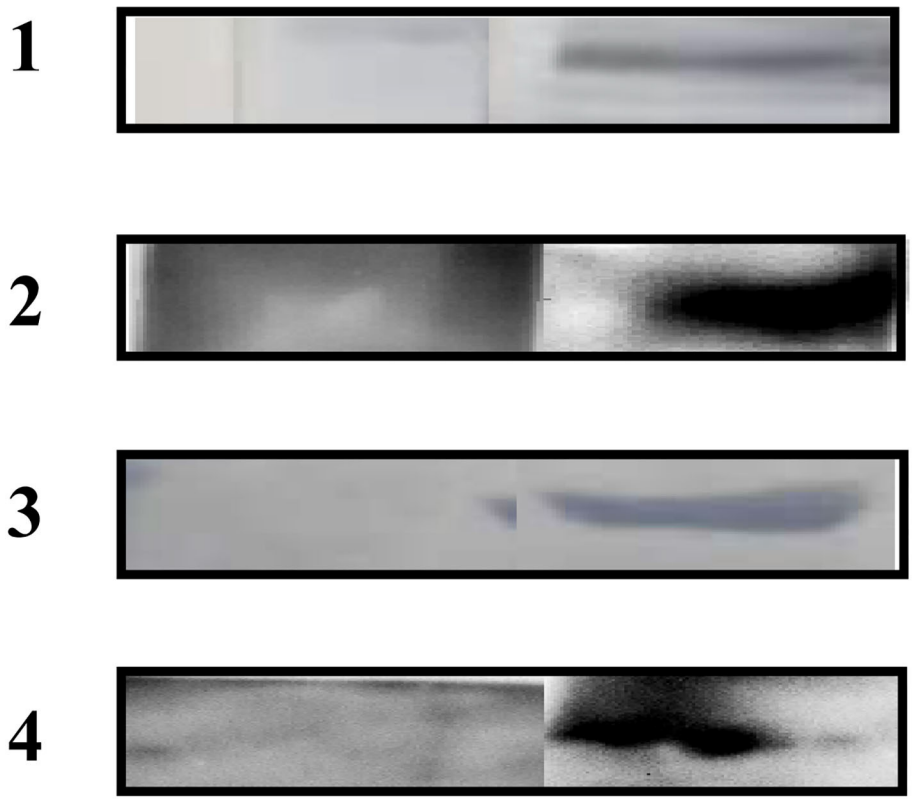


**FIGURE 5 |** Western blotting analysis. **(A)** Lane A1: total protein extracts of PSCs probed with pre-immunized dog sera; Lane A2: total protein extracts of PSCs probed with pre-immunized dog sera; Lane A3: non-infected dog sera was used to probe purified rEg-TSP11; Lane A4: non-infected dog sera was used to probe purified rEg-actin. **(B)** Lane B1: total protein extracts of PSCs probed using anti-rEg-TSP11 dog sera; Lane B2: total protein extracts of PSCs probed using anti-rEg-actin dog sera; Lane B3: purified rEg-TSP11 probed with the serum of *E. granulosus* infected dog; Lane B4: purified rEg-actin probed with the serum of *E. granulosus* infected dog.

anti-rEg-TSP11 dog IgG, with an apparent molecular mass of ∼27 kDa, as expected. As anticipated, when reacted with pre-immunized dog serum or non-infected dog serum, no signal was observed with rEg-TSP11 (**Figure 5**).

## qRT-PCR Analysis of Eg-TSP11 Expression

The relative transcription of Eg-TSP11 was assessed using qRT-PCR. Eg-TSP11 mRNA was detected in *E. granulosus* adult and protoscolex stages, with a significantly higher level in the PSC stage ($P > 0.05$; **Figure 6**).

## Eg-TSP11 Localization During Different Life Stages of *E. granulosus*

The localization of native Eg-TSP11 in PSCs and adult worms was determined by immunofluorescence using specific polyclonal antibodies against rEg-TSP11. In PSCs, the fluorescence signals were mainly localized in the tegument tissues and hook, while weak signals were detected in the parenchymal region (**Figure 7A**). In adult worms, weak signals were detected in tegument tissue (**Figure 7B**). No fluorescence signals were detected in the negative control samples.

## Indirect ELISA

Indirect ELISA was used for a preliminary assessment of serum IgG changes after vaccination with rEg-TSP11. At 0, 14, 28, and 42 days after the first vaccination, sera from nine immunized dogs were tested. In the dogs immunized with rEg-TSP11, the IgG level increased significantly at 14, 28, and 42 days after immunization (14 d $t = 11.35$ $P = 0.0003$, 28 d $t = 7.700$, $P = 0.00151$, 42 d $t = 14.89$, $P = 0.0001$) and at 28 days post-challenge compared with the PBS control group ($t = 4.729$, $P = 0.0091$). IgG levels peaked 42 days post-vaccination (**Figure 8**).

## Response of Canine Cytokines Induced by rEg-TSP11

ELISA was used to detect cytokines IFN-γ, IL-6, IL-5, and IL-4 at 0, 14, 28, and 42 days after immunization and at 28 days post-PSC challenge (**Figure 9**). Compared with that in the PBS

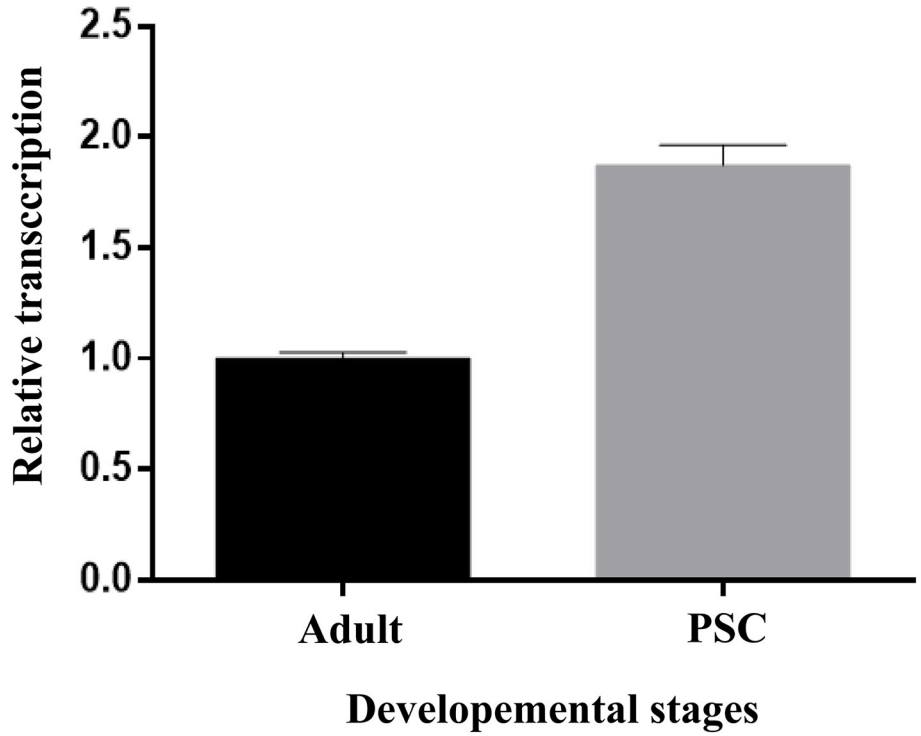


**FIGURE 6 |** Expression analysis of Eg-TSP11 mRNA in different developmental stages of *E. granulosus*.

control group, the serum IL-4 level increased significantly in the rEg-TSP11 vaccinated dogs at 42 days after immunization (14 d $t = 0.09306$ $P = 0.9303$, 28 d $t = 2.129$, $P = 0.1003$, 42 d $t = 5.786$, $P = 0.0045$); however, there was no significant change at day 28 post-PSC challenge ($t = 0.3628$, $P = 0.7351$) (**Figure 9A**). Moreover, the serum IL-5 level increased significantly at 42 days after immunization (14 d $t = 0.3599$ $P = 0.7372$, 28 d $t = 0.3042$, $P = 0.7761$, 42 d $t = 11.08$, $P = 0.0004$) and 28 days post-PSC challenge ($t = 3.104$, $P = 0.0361$) in the rEg-TSP11 vaccinated dogs (**Figure 9B**). Furthermore, the serum IFN-$\gamma$ level increased significantly at 28 days and 42 days after immunization (14 d $t = 2.592$ $P = 0.0605$, 28 d $t = 6.200$, $P = 0.0034$, 42 d $t = 2.803$, $P = 0.0487$). There were no significant changes at day 28 post-PSC challenge ($t = 0.4121$, $P = 0.7014$) in the rEg-TSP11 vaccinated dogs (**Figure 9C**). In addition, there were no significant differences in serum IL-6 (**Figure 9D**) and IL-1 (**Figure 9E**) levels in rEg-TSP11 vaccinated dogs in the different groups.

### Vaccine Efficacy of rEg-TSP11 Combined With Quil A

Vaccination using rEg-TSP11 mixed with Quil A resulted in a 76.80% reduction in the number of worms compared to that in the PBS control group (**Table 1**). This suggested that vaccination with rEg-TSP11 mixed with Quil A induced good protective efficacy (inhibition of worm growth) in Beagles against *E. granulosus* infection 28 days after PSC challenge.

### Worm Development in rEg-TSP11 Vaccinated Dogs

The results showed that in the rEg-TSP11-vaccinated group, the development and maturation of the worms was inhibited by 77.04%. Thus, vaccination with rEg-TSP11 induced a significant protective effect, as indicated by the inhibition of worm growth (**Table 2**).

## DISCUSSION

Tetraspanins are widely distributed in eukaryotes and participate in many cellular biological processes (22). They play a significant downregulatory role, especially in immune interactions with host molecules, including the major histocompatibility complex (MHC) (23, 24). This immune interaction is vital in the process of parasite immune evasion. The downregulation of the host immune response by tetraspanin allows the parasite to hide its non-self-state and evade detection by the host, leading to successful host infection. Therefore, targeting tetraspanins using a variety of methods, including RNA interference or monoclonal antibodies, could lead to the development of anti-parasite vaccines. Certain members of the tetraspanin family have been targeted in schistosomiasis (25–27), Clonorchiasis sinensis (28), and alveolar echinococcosis (29, 30), and as candidate vaccines for filariasis (31). However, there are few reports on the TSPs of *E. granulosus*. In this study, molecular cloning, prokaryotic expression, western blotting, and fluorescence immunolocalization were carried out. The immune protective effect of the rEg-TSP11 in dogs was evaluated by assessing

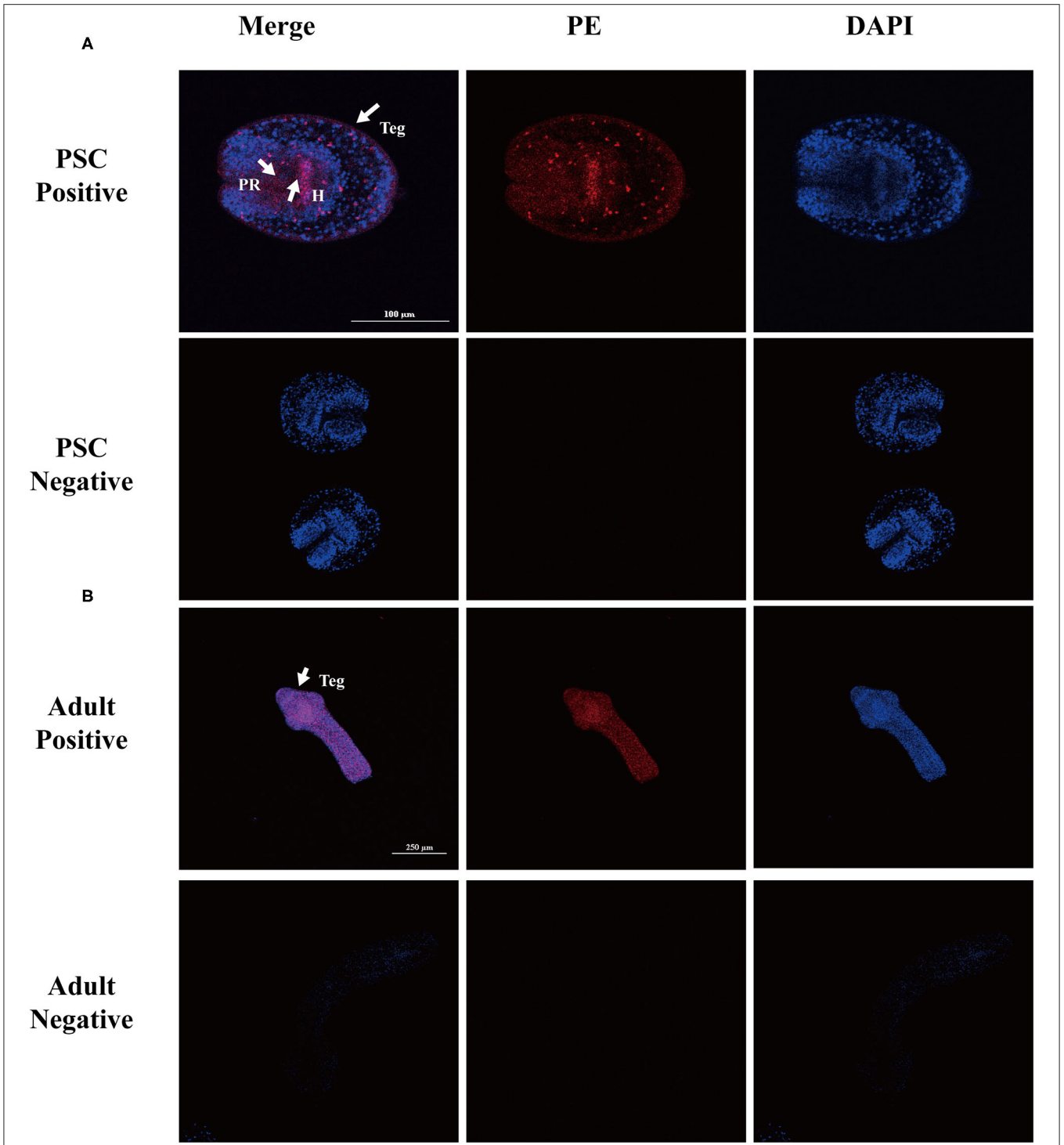


**FIGURE 7 |** Immunofluorescent localization of Eg-TSP11 in different stages of *E. granulosus*. Eg-TSP11 was localized in the protoscolex and adult worm using specific anti-rEg-TSP11 IgG (positive) or pre-immune serum (negative), respectively. The nuclear DNA was stained with DAPI (blue). Teg, tegument; PR, parenchymal region; H, hook; *Scale bars*: 50 μm **(A)**, 250 μm **(B)**.

the changes in antibody levels, the worm reduction rate, the inhibition of worm segmentation, and Th1/Th2 cytokine levels. These results provide the basis for determining the antigenic potential of rEg-TSP11.

The results of bioinformatic analysis showed that TSP11 has five protein kinase phosphorylation sites and one tyrosine kinase phosphorylation site, indicating that phosphorylation of TSP11 plays a pivotal role in *E. granulosus* growth and development

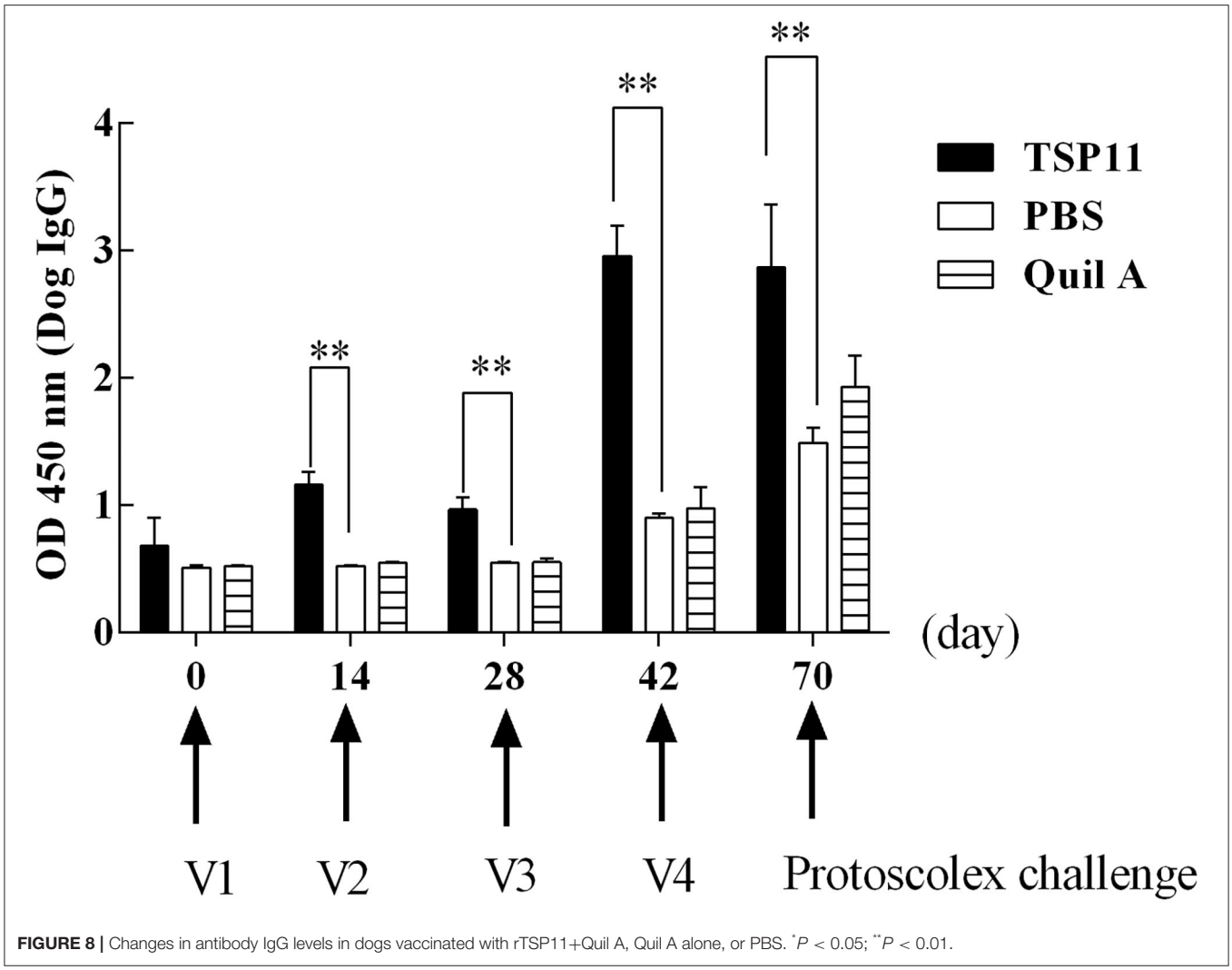


**FIGURE 8 |** Changes in antibody IgG levels in dogs vaccinated with rTSP11+Quil A, Quil A alone, or PBS. $^{*}P < 0.05$; $^{**}P < 0.01$.

(32). Specific epitopes determine the specificity of antigens (33). Therefore, epitope prediction is particularly important in the study of protein structure and function, and for the diagnosis of diseases and the design of vaccines (34). The present study found that there were seven potential B-cell epitopes in Eg-TSP11, among which the random coil regions were 227–240 (amino acid sequence: WQYGPPRFTNGAHN) and 210–219 (amino acid sequence: LTGWPTDQYQ). The results showed that these two regions had strong antigenicity, and most of the epitopes were located in the extracellular loop of tetraspanin LEL, which has a highly conserved cysteine sequence. Studies have shown that the LELs of some TSPs have good immunogenicity and reactivity and are potential vaccine candidate antigens (8); therefore, in the present study, we cloned and expressed the LEL of Eg-TSP11. This analysis indicated that Eg-TSP11 has good antigenic potential and provides a theoretical basis for Eg-TSP11 as a candidate antigen for the development of a vaccine against *E. granulosus* infection. Transcriptional analysis indicated that *Eg-TSP11* is expressed in both adult worms and the protoscolex, suggesting that Eg-TSP11 might play a role in the parasite life cycle. Moreover, we found that Eg-TSP11 was mainly expressed in the epidermis of adult worms and protoscolex. Interestingly, we also found that the serum from dogs with *E. granulosus* infection also recognized rEg-TSP11. In platyhelminths, the tetraspanin families are expanded and are likely to be components of the host-pathogen interface (35). Studies have shown that the tetraspanins could be part of extracellular vesicles that are released by helminths within hosts (36), that they bind to the Fc domain of host antibodies (37), and that they are highly immunogenic (38). Based on these findings, we speculated that Eg-TSP11 might be a component of the host-pathogen interface and could be part of the extracellular vesicles released by *E. granulosus* within hosts.

Quil A, as a saponin adjuvant, is widely used in veterinary vaccines and human and veterinary immunology. Saponins can induce strong adjuvant responses to T cell-dependent antigens (39). Saponins can also induce a strong cytotoxic $CD8^{+}$ lymphocyte reaction and enhance the response to mucosal antigens (40), and when combined with cholesterol and phospholipids, immune stimulating complexes are formed

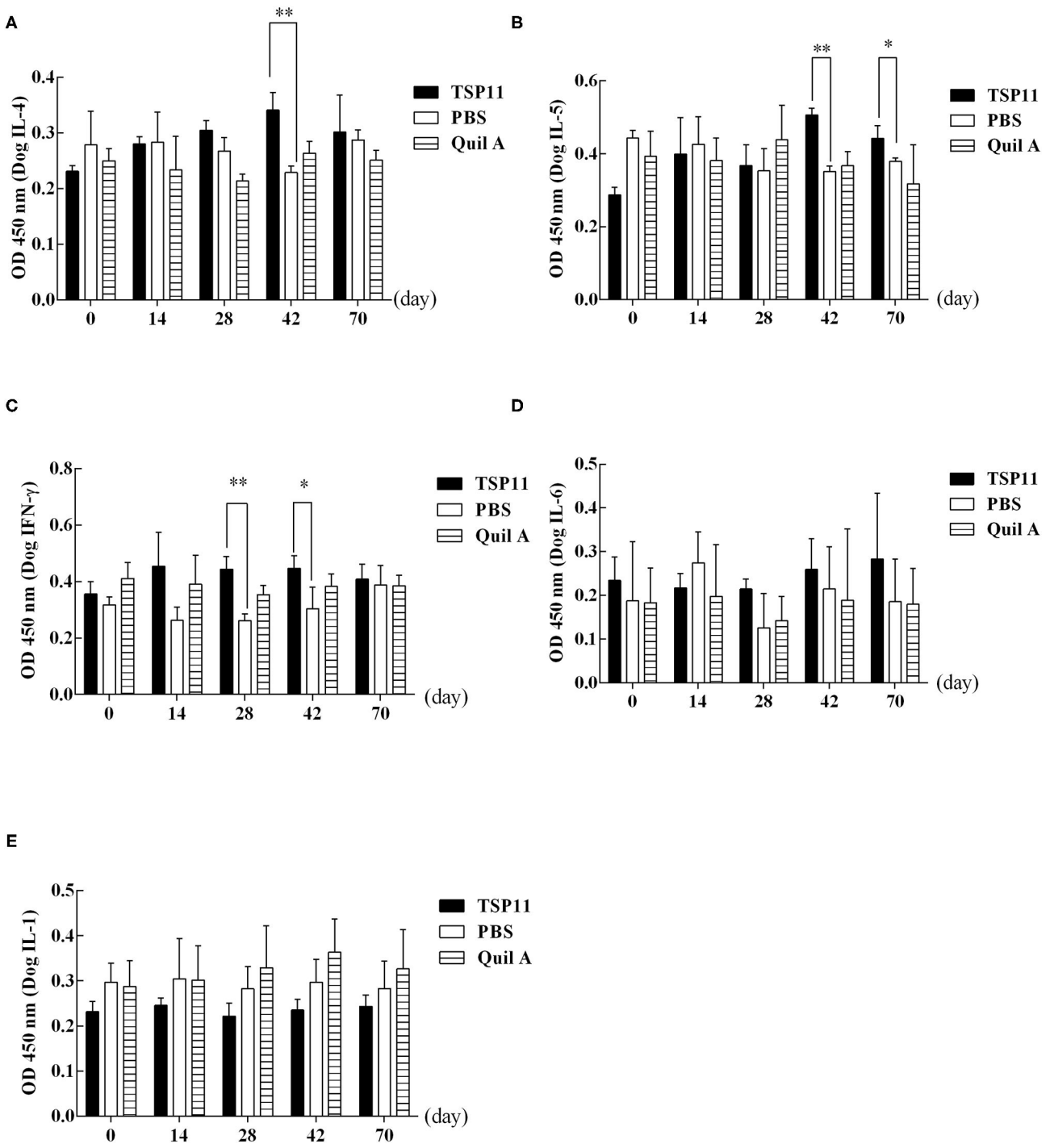


**FIGURE 9 |** ELISA detection of the levels of different cytokines in dogs vaccinated with rTSP11+Quil A, Quil A alone, or PBS. **(A)** IL-4, **(B)** IL-5, **(C)** IFN-γ, **(D)** IL-6, E: IL-1. $^{*}P < 0.05$; $^{**}P < 0.01$.

(41). The Quil A adjuvant can activate cellular and humoral immune responses against a variety of viruses, bacteria, parasites, and tumor antigens (41, 42). Hence, in this study, we used rEg-TSP11 mixed with Quil A to induce a more effective immune response. Indirect ELISA was used to detect changes in IgG levels in dog serum. The results showed that rEg-TSP11 could significantly stimulate the body to produce specific antibodies.

**TABLE 1 |** Number of Worms in vaccinated dogs after PSC challenge.

| Group (protein) | Dog ID number | Number of worms | Reduction %[a] |
|---|---|---|---|
| PBS | 1 | 13,200 | |
| | 2 | 4,800 | |
| | 3 | 5,700 | |
| Average | | 7,900 | - |
| Quil A | 4 | 28,200 | |
| | 5 | 4,200 | |
| | 6 | 4,800 | |
| Average | | 12,400 | |
| Eg-TSP11 | 7 | 1,200 | |
| | 8 | 1,400 | |
| | 9 | 2,900 | |
| Average | | 1,833 | 76.80 |
| *P*-value[b] | | | 0.100 |

[a] *Reduction% = [(average number of worms in the control group—the average number of worms in the experimental group)/(average number of worms in the control group)] × 100%.*

[b] *The Mann-Whitney U test was used to compare the worm burden median; P-value < 0.05 indicates that the reduction is significant between PBS (control) and experimental groups using median analysis.*

**TABLE 2 |** Worm segment development in the experimental groups.

| Group (protein) | Dog ID | Worm development | | |
|---|---|---|---|---|
| | | ≤3 segments | ≥4 segments | Worm inhibition |
| Eg-TSP11 | 1 | 26 | 4 | |
| | 2 | 22 | 8 | |
| | 3 | 28 | 2 | |
| Average | | 25 | 5 | 77.04% |
| PBS | 4 | 9 | 21 | |
| | 5 | 13 | 17 | |
| | 6 | 7 | 23 | |
| Average | | 9 | 21 | – |
| Quil A | 7 | 7 | 23 | |
| | 8 | 9 | 21 | |
| | 9 | 3 | 12 | |
| Average | | 7 | 23 | – |

Echinococcosis is a zoonotic parasitic disease that is also an immune imbalance disease (42). *E. granulosus* infection initiates long-term interactions with the host. In this interaction, the existence of Th1/Th2 type immune responses plays a crucial role. When the worm changes the immune balance of Th1 and Th2 in the host's own system via specific antigens, it produces inhibitory cytokines to escape the host's immune defense (42). Moorhead et al. reported that $CD4^+$ T cytokines in mice can be divided into two subgroups. Th1 type cells mainly secrete IL-1, IFN-γ, and TNF-β, and Th2 cells mainly secrete IL-4, IL-5, and IL-10 (43). A previous study found that in patients with echinococcosis, Th2 type cytokines were mainly increased in their serum (44). Another study showed that the Th1-mediated cellular immune response can enhance the host's anti-infection ability in the early stage of echinococcosis, playing an important role in the control of the initial infection of echinococcosis (45). In the later stages of infection, the main humoral immune response is mediated by Th2 type immunity (45). It has also been suggested that the transformation from a Th1 immune response to a Th2 type immune response is beneficial for the growth and development of *E. granulosus*, such that IL-4, IL-5, IFN-γ, and other cytokines play an important role in the immune interaction between the host and *E. granulosus* (45). In the present study, rEg-TSP11 induced Th1 and Th2 immune responses in dogs, and the changes in IFN-γ cytokine levels were more obvious in the pre-immune period. However, after challenge with PSCs for 28 days, marked changes in IL-5 cytokine levels were observed. Such delayed cytokine responses to PSC Ags have been previously described in infected mice and dogs (20, 46), which indicated that the humoral immune response mediated by Th2 type immunity is the main pathway in the late stage of echinococcosis.

We vaccinated dogs with Eg-TSP11 mixed with Quil A, and used PBS as a control. In terms of worm reduction rates, the rEg-TSP11 immunized group showed a worm reduction of 76.80% compared with that in the PBS control group, representing a significant reduction in the worm burden. Moreover, the rEg-TSP11 immunized group showed a development and maturation inhibition of the worms was 77.04%. However, 70.00 and 76.67% of worms developed to the adult worms in the PBS control groups and Quil A, while only 22.96% of worms developed to the adult worms in the rEg-TSP11 immunized group. This results also suggested that the rEg-TSP11 vaccine induced significant protective efficacy in terms of inhibition of worm growth compared with the control dogs. This was better than the oral vaccine comprising a live carrier of Salmonella EgA31 EGTRP (70–80%), although individual differences were large (21), and slightly lower than that of EgM123 (89.2%) (19). Thus, rEg-TSP11 showed good performance in decreasing worm burden and inhibiting segment development. For humans, cattle, sheep, and other intermediate hosts, the threat comes from the eggs of adult *E. granulosus*. If a vaccine can be developed to reduce the number of parasites and inhibit their development by immunizing the definitive host, we might effectively reduce the amount of ovulation and reduce the threat. In this study, rEg-TSP11 showed good performance in terms of the worm reduction rate and the inhibition of segmental development, and thus is expected to be a good vaccine candidate antigen for the definitive host of *E. granulosus*.

## CONCLUSIONS

An increasing number of studies are focusing on the TSP because of its wide distribution and important biological roles in a variety of organisms. In the present study, we cloned and prokaryotically expressed Eg-TSP11. Furthermore, we evaluated the immunoprotective effects of rEg-TSP11 in model dogs. The results indicated that rEg-TSP11 could induce the production of specific antibodies in dogs and significantly increase or decrease the levels of Th1 and Th2 cytokines. In addition, rEg-TSP11 decreased worm burden and inhibited segment development.

These findings revealed that rEg-TSP11 might be a potential candidate vaccine antigen against *E. granulosus* infection in dogs.

## DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author/s.

## ETHICS STATEMENT

The animal study was reviewed and approved by the Care and Use of Laboratory Animals of the Xinjiang Academy of Agricultural and Reclamation Sciences (Shihezi, China) (Approval No. 2019-012, April 9, 2019). All animals were handled in strict accordance with the animal protection laws of the People's Republic of China (a draft animal protection law was released on September 18, 2009) and the National Standards for Laboratory Animals in China (executed on January 5, 2002).

## AUTHOR CONTRIBUTIONS

JX, PZ, and NW performed the experiments, collected and analyzed the data, and prepared the manuscript. WW and XM contributed to study design and implementation. YZ and JM collected the parasite specimens and performed immunofluorescence experiments. ZW and XB conceived the study, participated in its design, coordinated the project, and contributed to the interpretation of the results. All authors contributed to the article and approved the submitted version.

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# S-Methylcysteine Ameliorates the Intestinal Damage Induced by *Eimeria tenella* Infection *via* Targeting Oxidative Stress and Inflammatory Modulators

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Avian coccidiosis is one of the major parasitic diseases in the poultry industry. The infection is caused by *Eimeria* species, and its treatment relies mainly on the administration of anticoccidial drugs, which can result in drug resistance and side effects. The recent trends in avian coccidiosis treatment is directed to the development of a new therapy using herbal compounds. S-Methylcysteine (SMC) is considered one of the organosulfur compounds in garlic that showed promising activity in the treatment of different pathological conditions *via* a wide range of anti-inflammatory and antioxidant mechanisms. In this study, the anticoccidial activity of SMC was investigated in *Eimeria tenella*-infected chickens compared to diclazuril as a widely used anticoccidial drug. In this regard, 14-day-old broilers were divided into six groups ($n = 18$). The first group (G1) was the healthy control group, while the second group (G2) was the non-infected SMC group treated at a dose of 50 mg/kg b.w. (high dose). Moreover, the third group (G3) was the positive control group (infected and non-treated). The fourth group (G4) was the infected group treated with SMC of 25 mg/kg b.w. (low dose), while the fifth group (G5) was the infected group treated with SMC of 50 mg/kg b.w. (high dose). Conversely, the sixth group (G6) was the diclazuril-treated group. The anticoccidial effects of SMC and diclazuril were evaluated by counting oocysts and recording the body weight gain, feed conversion ratio, clinical signs, lesions, and mortality rate. Interestingly, SMC showed potent anticoccidial activity, which was exemplified by reduction of oocyst count. Furthermore, the biochemical, antioxidant, and anti-inflammatory parameters in the cecal tissues were restored toward their control levels in G4, G5, and G6. Histopathological observation of cecal tissues was consistent with the aforementioned results revealing

the ameliorative effect of SMC against *E. tenella* infection. This study concluded novel findings in relation to the anticoccidial role of SMC as a plant-based compound against the *E. tenella*-induced coccidiosis in broiler chickens combined with its antioxidative and anti-inflammatory properties. Further studies for exploring the mechanistic pathways involved in this activity and the potential benefits from its use in association with conventional anticoccidial drugs are warranted.

**Keywords: S-Methylcysteine (SMC), anticoccidial, *Eimeria tenella*, inflammatory, oxidative stress**

## INTRODUCTION

The poultry industry is considered an emerging food-producing sector in Egypt, reflecting its role in food security and economic development (1). Coccidiosis is the main reason for low performance and productivity in poultry and is a protozoal infection caused by the genus *Eimeria* (2). *Eimeria* invade the intestinal tract's epithelium, resulting in reduced feed intake (FI) and absorption and secondary bacterial infection (3). Coccidiosis was reported as a major worldwide problem and the top broiler-related disease in the United States in 2019 (4). There are seven species of *Eimeria*: *E. tenella, E. necatrix, E. acervulina, E. maxima, E. brunetti, E. mitis*, and *E. praecox*. They differ in their pathogenicity and site in the intestinal tract (5). Among these, *E. tenella* is considered a strong pathogenic species that induces hemorrhagic cecal coccidiosis in chickens, clinically presenting as bloody diarrhea, severe body weight loss, and death. The life cycle of *E. tenella* is monogenetic and host specific. It undergoes two endogenous phases (schizogony and gametogony) and is initiated by the sporozoite infection of the host cecum, leading to hemorrhagic lesions (6). To control avian coccidiosis, both vaccination and prophylactic anticoccidial drug administration (chemotherapy) are the widely used methods (7, 8). However, the used vaccines, either attenuated or non-attenuated live oocysts, show a risk of reaction development in the vaccinated chickens with different degrees (9). The anticoccidial drugs are used to either kill the coccidial population (coccidiocidal drugs) or prevent coccidial replication and growth (coccidiostatic drugs). To be effective, drugs should be used as a prophylactic, not a therapeutic method (10). Unfortunately, anticoccidial drugs (sulfonamides, pyrimidine derivatives, and polyether ionophores) have shown a problem of drug resistance (11, 12). To prevent drug resistance, alternative treatments should be used, such as aromatherapy, plants extracts, and probiotics. Using natural plant-based extracts as a therapy or supplement is currently targeted to obtain their benefits with minimum side effects compared to chemical therapies. Many plant-based compounds have shown protective effects against coccidiosis by inhibition of *Eimeria* species development, such as the extracts of citric fruits, oregano, mushrooms, and turmeric (13–15). Garlic (*Allium sativum*) is rich in bioactive compounds, which were used as a source of different medicinal drugs (16). One of the organosulfur compounds in garlic is S-methylcysteine (SMC), which was reported in different studies as a treatment for different pathological conditions, including cancer, obesity, and neurological disorders (17–19). SMC exhibited many biological reactions, such as anti-inflammatory and antioxidant effects (20). To date, no previous studies investigated the anticoccidial effect of SMC. Therefore, this study aimed to assess the potential of SMC as a plant-based compound to control avian coccidiosis by recording the body weight gain (BWG), feed conversion ratio (FCR), clinical signs, lesions, and mortality rate following the infection and treatment compare with control groups. Furthermore, oocysts were counted; the biochemical, antioxidant, and anti-inflammatory parameters were measured; and the histopathological changes in cecal tissues in response to treatment by SMC were recorded.

## MATERIALS AND METHODS

### Ethical Approval

This study followed the guidance of the Research, Publication, and Ethics Committee of the Faculty of Veterinary Medicine, Kafrelsheikh University, Egypt (ethical approval number: KFS-2019/3).

### Chemicals

All chemicals and reagents used, including SMC, were purchased from Sigma-Aldrich, Egypt. Diclazuril (Diclosol® suspension 10 mg/ml) was purchased from Pharma Swede—Egypt and administered in drinking water at a concentration of 2.5 ppm (1 ml of Diclosol®/4 L of drinking water).

### Birds

Chicks (1 day old, unsexed) of Cobb strain broilers were obtained from a hatchery in Kafr El Sheikh, Egypt. Rearing was initiated in floor pens till 7 days old; then, chickens were moved to wire-floored cages until the end of the experiment following all hygienic measures. Balanced commercial ration free from antibiotics and anticoccidials and tap water were provided to chickens *ad libitum*. Optimum temperature was adjusted using electric radiators and ventilators. Regular examinations of fecal samples were conducted twice daily during the first 14 days of the experiment (before the experimental infection) to confirm the privation of *Eimeria* oocysts by flotation technique using NaCl saturated solution with specific gravity of 1.28. Chickens were vaccinated against Newcastle disease by eye drops of Hitchner B1 strain in drinking water at 7 days old. Moreover, chickens were immunized against infectious bursal disease by Gumboro vaccine at 12 days old.

## *Eimeria tenella* Oocysts

Oocysts of *E. tenella* were isolated from cecal tissues of naturally infected birds following the Chapman and Shirley method (21). Isolated oocysts were then maintained in 4-week-old coccidia-free Cobb strain broilers through two passages at 3-month intervals. The doses used per bird were 4,000 oocysts/1 ml phosphate-buffered saline (PBS) for the first passage and 8,000 oocysts/1 ml PBS for the second passage. Subsequently, oocysts were collected and kept in solution of 2.5% potassium dichromate at 26–28°C for sporulation. The McMaster technique was used to clear and count the sporulated oocysts (22), which were then kept in 2.5% potassium dichromate at 4°C for a maximum of 4 weeks to be used in the experimental infection.

## Experimental Protocol

At the age of 14 days, 108 chicks were divided randomly into six groups ($n = 18$). Then, each group was subdivided into three different cages ($n = 6$); each cage represents a biological replication. The six main groups were as follows:

Group 1 (G1): negative control group; non-infected and non-treated chicks.

Group 2 (G2): SMC-treated group; non-infected chicks and treated at a dose of 50 mg/kg b.w., in drinking water.

Group 3 (G3): positive control group; infected and non-treated chicks.

Group 4 (G4): low-dose SMC-treated group; infected chicks and treated at a dose of 25 mg/kg b.w., in drinking water.

Group 5 (G5): high-dose SMC-treated group; infected chicks and treated at a dose of 50 mg/kg b.w. SMC, in drinking water.

Group 6 (G6): diclazuril-treated group; infected chicks and treated with 2.5 ppm diclazuril in drinking water.

At the age of 14 days, all groups, except G1 and G2, were infected with $4.0 \times 10^4$ sporulated oocysts/chick of *E. tenella* in 1 ml of PBS. Disposable Pasteur pipettes were used for the inoculation directly into the crop. On the 2nd day after infection (at the age of 15 days), SMC and diclazuril were administered for 4 consecutive days.

## Sampling

Blood samples were drawn from the wing vein of five chicks of each group on the 7th and 14th day after infection. Blood was used to separate the serum samples, which were kept at −20°C for further biochemical analysis. The same five chicks were killed by neck dislocation, and both the liver and cecum were collected and trimmed. Both organs were divided into two portions for the antioxidant's assessment and histopathological examination. The experimental treatments and sampling timeline are presented in **Figure 1**.

## Evaluation of Anticoccidial Efficacy of SMC

Clinical signs of coccidia infection, including bloody diarrhea, anorexia, and depression, were observed and recorded after infection in the SMC-treated groups compared to the untreated groups. Moreover, the growth performance was evaluated by recording the chicken's body weights at 14, 21, 28, 35, and 42 days of age. FI, BWG, and FCR were recorded as described previously (23). Moreover, *E. tenella* oocysts were counted using the modified McMaster technique (22) starting from the 4th day after infection until the 16th day. Mortality percentages and gross lesions were scored on the 7th and 14th day according to Johnson and Reid (24).

## Histopathological Examination

On the 7th and 14th day after infection, cecal tissue samples were obtained from the slaughtered chickens. Collected samples were rinsed with neutral PBS and fixed in 10% formalin. Subsequently, tiny blocks were trimmed and processed in an automatic processor containing many chambers with ascending concentrations of alcohol to dehydrate the tissue before paraffinization. Then, 3- to 5-micron-thick blocks were cut using a microtome, stained with hematoxylin and eosin, and allowed to dry before the light microscopical analysis.

## Biochemical Parameters Analysis

In serum samples, aspartate, and alanine amino transferases (AST and ALT, respectively) were measured following Varliy's protocol (25). Moreover, alkaline phosphatase (ALP), total protein (TP), and albumin contents were measured according to the methods of Thirunavukkarasu et al. (26), Domas (27), and Doumas et al. (28), respectively. Serum creatinine and uric acid levels were measured according to the methods of Henry (29) and Haisman and Muller (30), respectively.

## Antioxidative Parameter Analysis

Liver tissues were dissected and cleaned using neutral pH 50 mM Tris–HCl and then homogenized in an ice-cold solution containing neutral 50 mM Tris–HCl. Homogenates were used for the antioxidative parameter analysis after centrifugation at 2,500 rpm for 30 min. The enzymatic activities of hepatic superoxide dismutase (SOD), glutathione peroxidase (GSHPx), and catalase (CAT) were measured in liver homogenates depending on the methods of Nishikimi et al. (31), Paglia (32), and Aebi (33), respectively.

## Gene Expression Analysis of Inflammatory Parameters

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) was used to analyze the gene expressions. Total RNA from the liver was extracted using the TRIzol® reagent (Invitrogen, USA). Then, reverse transcription of the extracted RNA was conducted to create cDNA. **Table 1** shows the employed primer sequences of the housekeeping (β-actin), interleukin 1 β (IL-1β), interferon gamma (IFN-γ), and nuclear factor kappa-light-chain-enhancer of activated B cell 1 (NF-κB1) genes. RT-qPCR reactions were completed by Power SYBR® Green PCR Master Mix (Applied Biosystems, USA). Reactions were monitored by the 7500 Real-Time PCR System (Applied Biosystems, USA). Thermal cycles were performed at 95°C for 4 min, 40 cycles of 10 s at 95°C, 30 s at 60°C, and finally 10 s at 72°C. Data were presented as relative fold changes compared to control's gene expressions.

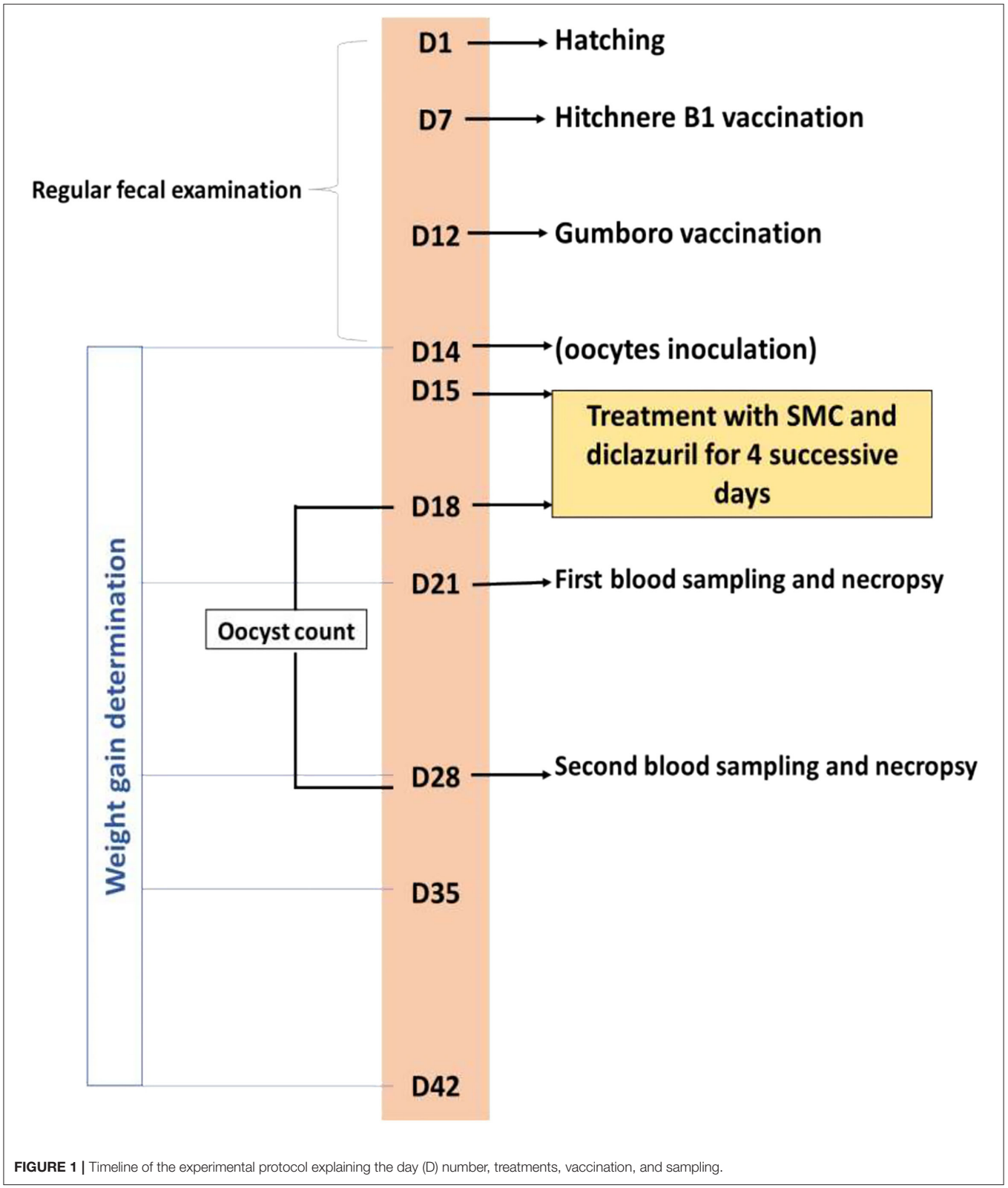


**FIGURE 1 |** Timeline of the experimental protocol explaining the day (D) number, treatments, vaccination, and sampling.

## Statistical Analysis

GraphPad Prism 5 software was used to analyze the data by one-way analysis of variance and Tukey multiple comparisons. Data were presented as means ± SD. Statistical difference was considered significant when $p$-value is $<0.05$.

# RESULTS

## Direct Anticoccidial Effect of SMC

### Clinical Signs, Lesion Scoring, and Mortality Rate

As presented in **Table 2**, data showed that there were no observed abnormalities or recorded lesions and deaths in both control chickens (G1) and SMC-treated group (G2). In contrast, the infected non-treated group (G3) had typical clinical signs of coccidiosis showing dullness, reduced appetite, depression, wasting, bloody diarrhea, and increased weakness with progression resulting in death within 4–5 days of infection, whereas five of 18 chickens died in G3. After treatment of the infected chicken with high dose of SMC (G5), only one bird died, while the low-dose SMC treatment resulted in death of three birds. Diclazuril treatment resulted in death of two chickens. The scored lesions were significantly reduced in chickens treated with high-dose SMC and diclazuril (G5 and G6) with a higher significance than the reduction induced by the low dose of SMC (G4) after 7 days of infection, as shown in **Table 2**. Moreover, after 14 days of infection, treatment with high dose of SMC showed the highest significant improvement in the lesion's score. These data suggested the protective effect of SMC against coccidiosis symptoms and mortality rate.

**TABLE 1 |** Primers used for RT-qPCR analysis.

| Gene | Primer | References |
|---|---|---|
| β-actin | F: ACCTGAGCGCAAGTACTCTGTCT<br>R: CATCGTACTCCTGCTTGCTGAT | NM_205518.1 (34) |
| NF-κB1 | F: TACCGGGAACAACACCACTG<br>R: CAGAGGGCCTTGTGACAGTA | NM_205134 (35) |
| IFN-γ | F: GAACTGGACAGAGAGAAATGAGA<br>R: ATGTGTTTGATGTGCGGCTT | NM_205149 (35) |
| IL-1β | F: CAGCCTCAGCGAAGAGACCTT<br>R: CACTGTGGTGTGCTCAGAATCC | XM_015297469.1 (36) |

## Assessment of Growth Performance

To assess the growth performance, BWGs of all groups were measured and presented in **Table 3**. All treated groups (G4, G5, and G6) showed significant improvements in the BWG compared to the non-treated group (G3). The highest improvements were induced by the high dose of SMC (G5), which showed no significant changes compared to the control healthy chickens (G1) at 21 days of age. In addition to the BWG, FCRs were measured and presented in **Table 4**. Chickens treated with high dose of SMC showed the best significant improvements in the FCRs compared to the other treated and the non-treated groups. Interestingly, the SMC-exposed group (G2) had no significant changes in the BWG and FCRs compared to the healthy control group (G1), indicating that SMC has no side effects. The assessment of growth performance suggested that SMC had a

**TABLE 2 |** The effect of SMC on mortality % and lesion score in broiler chickens experimentally infected with *E. tenella*.

| Parameter | Control | SMC | Infected | | | |
|---|---|---|---|---|---|---|
| | | | Non-treated | SMC low dose | SMC high dose | Diclazuril |
| Number of birds | 18 | 18 | 18 | 18 | 18 | 18 |
| Mortality | 0 | 0 | 5 | 3 | 1 | 2 |
| Percent | 0 | 0 | 27.78 | 16.67 | 5.55 | 11.11 |
| Lesion's score (7 days) | 0 | 0 | $3.70 \pm 0.29$ | $2.40 \pm 0.48^{a}$ | $1.20 \pm 0.29^{ab}$ | $1.70 \pm 0.29^{ab}$ |
| Lesion's score (14 days) | 0 | 0 | $2.70 \pm 0.29$ | $1.40 \pm 0.29^{a}$ | $0.40 \pm 0.41^{ab}$ | $1.00 \pm 0.70^{a}$ |

*Data are expressed as mean ± S.D. a and b indicate statistical significance in comparison with the infected non-treated group (G3) and SMC treated at low dose (G4). Means within rows with different superscripts differ at $p \leq 0.05$.*

**TABLE 3 |** The effect of SMC on body weight gain (in grams) in broiler chickens experimentally infected with *E. tenella*.

| Days of age | Control | SMC | Infected | | | |
|---|---|---|---|---|---|---|
| | | | Non-treated | SMC low dose | SMC high dose | Diclazuril |
| 14th–21st | $349.23 \pm 7.86$ | $357.78 \pm 12.25$ | $138.34 \pm 11.58^{a}$ | $236.37 \pm 12.14^{ab}$ | $307.21 \pm 6.87^{abcd}$ | $270.08 \pm 11.75^{abc}$ |
| 21st–28th | $376.64 \pm 9.42$ | $418.43 \pm 5.62$ | $211.29 \pm 10.96^{acd}$ | $295.70 \pm 12.43^{abcd}$ | $360.20 \pm 26.33^{bcd}$ | $317.39 \pm 10.34^{ab}$ |
| 28th–35th | $357.13 \pm 26.77$ | $368.35 \pm 17.25$ | $271.51 \pm 20.84^{ab}$ | $305.40 \pm 13.28^{ab}$ | $337.30 \pm 11.57^{bc}$ | $311.55 \pm 7.45^{ab}$ |
| 35th–42nd | $406.90 \pm 21.09$ | $447.00 \pm 12.99$ | $304.00 \pm 5.88^{a}$ | $323.50 \pm 14.90^{ab}$ | $408.00 \pm 6.43^{bc}$ | $367.00 \pm 25.19^{ab}$ |

*Data are expressed as mean ± S.D. a, b, c, and d indicate statistical significance in comparison with control, infected non-treated, low dose SMC-treated, and diclazuril-treated groups, receptively. Means within rows with different superscripts differ at $p \leq 0.05$.*

**TABLE 4 |** The effect of SMC on feed conversion ratio (in grams) in broiler chickens experimentally infected with *E. tenella*.

| Days of age | Control | SMC | Infected | | | |
|---|---|---|---|---|---|---|
| | | | Non-treated | SMC low dose | SMC high dose | Diclazuril |
| 14th–21st | 2.28 ± 0.10 | 2.16 ± 0.14 | 4.84 ± 0.17$^{a}$ | 3.26 ± 0.24$^{ab}$ | 2.75 ± 0.17$^{abcd}$ | 3.22 ± 0.11$^{ab}$ |
| 21st–28th | 2.48 ± 0.11 | 2.25 ± 0.18 | 4.25 ± 0.18$^{a}$ | 2.91 ± 0.11$^{ab}$ | 2.43 ± 0.26$^{bcd}$ | 3.04 ± 0.17$^{ab}$ |
| 28th–35th | 2.57 ± 0.08 | 2.31 ± 0.16 | 3.75 ± 0.19$^{a}$ | 2.69 ± 0.26$^{b}$ | 2.36 ± 0.13$^{b}$ | 2.54 ± 0.27$^{b}$ |
| 35th–42nd | 2.14 ± 2.25 | 2.30 ± 2.09 | 2.78 ± 2.8$^{a}$ | 2.16 ± 2.44 | 2.10 ± 2.36$^{b}$ | 2.60 ± 2.51 |

*Data are expressed as mean ± S.D. a, b, c, and d indicate statistical significance in comparison with control, infected non-treated, low dose SMC-treated, and diclazuril-treated groups, receptively. Means within rows with different superscripts differ at $p \leq 0.05$.*

**TABLE 5 |** The effect of SMC on an oocyst count (×$10^6$/g of feces) from the 4th to 14th day post infection in broiler chickens experimentally infected with *E. tenella* oocysts.

| Days post-infection | Control (×$10^6$/g of feces) | SMC (×$10^6$/g of feces) | Infected | | | |
|---|---|---|---|---|---|---|
| | | | Non-treated (×$10^6$/g of feces) | SMC low dose (×$10^6$/g of feces) | SMC high dose (×$10^6$/g of feces) | Diclazuril (×$10^6$/g of feces) |
| 4th day | 0.00 ± 0.00 | 0.00 ± 0.00 | 0.23 ± 34.38 | 0.12 ± 20.33$^{a}$ | 0.10 ± 14.91$^{a}$ | 0.16 ± 21.58$^{a}$ |
| 5th day | 0.00 ± 0.00 | 0.00 ± 0.00 | 0.75 ± 46.04 | 0.40 ± 29.59$^{a}$ | 0.29 ± 28.81$^{ab}$ | 0.34 ± 30.09$^{ab}$ |
| 6th day | 0.00 ± 0.00 | 0.00 ± 0.00 | 1.38 ± 19.75 | 0.77 ± 132.59$^{a}$ | 0.47 ± 53.74$^{abc}$ | 0.67 ± 43.31$^{a}$ |
| 7th day | 0.00 ± 0.00 | 0.00 ± 0.00 | 1.78 ± 94.45 | 0.99 ± 88.78$^{a}$ | 0.62 ± 38.50$^{abc}$ | 0.87 ± 49.44$^{ab}$ |
| 8th day | 0.00 ± 0.00 | 0.00 ± 0.00 | 1.50 ± 80.04 | 0.82 ± 60.76$^{a}$ | 0.56 ± 64.52$^{abc}$ | 0.88 ± 57.44$^{a}$ |
| 9th day | 0.00 ± 0.00 | 0.00 ± 0.00 | 0.99 ± 105.82 | 0.67 ± 38.54$^{a}$ | 0.47 ± 45.27$^{ab}$ | 0.56 ± 85.22$^{a}$ |
| 10th day | 0.00 ± 0.00 | 0.00 ± 0.00 | 0.64 ± 58.00 | 0.47 ± 38.44$^{a}$ | 0.26 ± 25.54$^{abc}$ | 0.40 ± 58.11$^{a}$ |
| 11th day | 0.00 ± 0.00 | 0.00 ± 0.00 | 0.58 ± 30.10 | 0.37 ± 20.09$^{a}$ | 0.21 ± 39.81$^{abc}$ | 0.31 ± 36.90$^{ab}$ |
| 12th day | 0.00 ± 0.00 | 0.00 ± 0.00 | 0.46 ± 38.63 | 0.23 ± 16.04$^{a}$ | 0.18 ± 24.01$^{abc}$ | 0.24 ± 30.07$^{a}$ |
| 13th day | 0.00 ± 0.00 | 0.00 ± 0.00 | 0.40 ± 10.64 | 0.31 ± 26.52$^{a}$ | 0.14 ± 17.82$^{abc}$ | 0.22 ± 21.83$^{a}$ |
| 14th day | 0.00 ± 0.00 | 0.00 ± 0.00 | 0.25 ± 34.90 | 0.16 ± 21.25$^{a}$ | 0.10 ± 7.95$^{abc}$ | 0.13 ± 6.52$^{a}$ |

*Oocyst counts are shown as (no.×$10^6$/g of feces) and the data are expressed as mean ± S.D. a, b, and c indicate statistical significance in comparison with infected non-treated, low dose SMC-treated, and diclazuril-treated groups, receptively. Means within rows with different superscripts differ at $p \leq 0.05$.*

protective role against the coccidiosis-induced reduction in the growth rate.

## Count of Oocysts

On the 4th day after infection, *E. tenella* oocysts started to appear in G3, and the highest count was recorded on the 8th day. Then, the count decreased gradually until the 14th day after the infection, as shown in **Table 5**. In all infected and treated groups (G4, G5, and G6), the oocyst count reduced significantly compared to the infected non-treated G3. Interestingly, treatment with high-dose SMC (G5) induced the highest significance compared to other treatments (G4 and G6). The recorded oocyst count reveals the protective effect of SMC against coccidiosis by reducing the oocyst count.

## Histopathology

The histopathological findings of posterior intestinal segments are shown in **Figure 2**. The cecal segments of birds of G1 and G2 showed normal intestinal mucosal lining with normal glandular epithelium. However, the ceca of the G3 revealed severe necrotic enteritis associated with marked infestation of the intestinal glands with schizont of the coccidial parasites. The diseased birds treated with low dose of SMC showed a decrease in necrotic changes within the glandular epithelium. The high dose showed a marked decrease in degenerative changes associated with a marked decrease in infestation of glandular epithelium with coccidial stages. Diseased birds treated with diclazuril revealed a decrease in necrotic change with a decrease in parasitic stages. The pathological findings of the second sacrifice are presented in **Figure 3**. During the 2nd sacrifice of the study, the diseased birds showed a decrease in the number of parasites within the intestinal epithelium with an obvious chronic inflammatory reaction and hyperplastic changes of the intestinal epithelium. Diseased birds treated with diclazuril revealed a marked decrease in coccidial stages and intestinal inflammatory changes. Interestingly, the high dose of SMC revealed marked decrease of the enteritis and obvious increase of the regenerative and reparative actions within the mucosal epithelium with an increase in intraepithelial lymphocytes.

## Indirect Protective Effect of SMC

### Assessment of Biochemical Parameters in the Serum

As presented in **Tables 6**, **7**, biochemical parameters were measured after 7 and 14 days of the infection. The results revealed that G3 showed significant increases in ALT, AST, ALP,

creatinine, and uric acid levels and significant decreases in TP and albumin levels compared to G1. In G4, G5, and G6, treatment with SMC (low and high doses) and diclazuril could significantly improve the levels of the biochemical parameters compared to those in G3. These data suggested the protective effect of SMC on the disturbed biochemical parameters by the effect of *Eimeria* infection.

### Assessment of Antioxidant Parameters

As presented in **Figure 4**, the levels of the antioxidant enzymes (SOD, GSHPx, and CAT) were significantly reduced in G3 compared to those in G1 on the 7th and 14th day of infection. After treatment with SMC and diclazuril, the enzyme levels showed significant improvement (G4, G5, and G6) compared to the non-treated chickens (G3). Moreover, G2 did not show any significant changes compared to the control group (G1), revealing the antioxidant role of SMC.

### Assessment of Inflammatory Parameters

As presented in **Figure 5**, the gene expressions of IL-1β and IFN-γ were significantly downregulated along with upregulation of NF-κB1 gene expression in G3 compared to G1 on the 7th and 14th day of infection. After treatment with SMC and diclazuril, the dysregulated gene expressions showed significant improvements in G4, G5, and G6 compared to those in G3. These data showed the anti-inflammatory effect of SMC, which is the same effect of diclazuril on the inflammatory response.

## DISCUSSION

Poultry coccidiosis has a great economic significance because of its effect on the production, which is indicated by the low growth rate, high mortalities, and costs of treatment and control (37). Anticoccidial drugs could be classified into four categories depending on their mode of action (38): The first category is drugs that affect an essential cofactor disturbing the biochemical pathway of the coccidia cells, such as sulfonamides and pyrimethamine (39). The second category is drugs that affect the mitochondrial function and inhibit the respiration of coccidian, such as quinolone, meticlorpindol, nicarbazin, and triazinetrione drugs (40). The third category consists of drugs that affect the cell membrane, inducing an osmotic damage, such as ionophores (41). The last category has unknown mechanism of action, such as diclazuril and halofuginone, which inhibit different stages of coccidia differentiation (42). Despite the availability of many anticoccidial drugs, the wide range of their use had developed a loss of sensitivity and high resistance, either quickly, such as quinolones, or after long-term use, such as ionophores (43). Therefore, using plant-based compounds to control the poultry coccidiosis would help avoid the chemical therapy's resistance. Garlic extract compounds, such as SMC, were reported previously to have an antiprotozoal effect against the *Cryptosporidium parvum* infection in mice (44). In this study, the anticoccidial effect of SMC was investigated in poultry for the first time and compared to diclazuril's anticoccidial activity.

In this study, broiler chickens were infected with *E. tenella*, and the infection was confirmed by the oocyst count from the 4th day until the 14th day after infection. SMC affected the oocyst count in a dose-dependent manner, whereas the high dose could reduce the count to a greater extent than the low dose. Moreover, the high dose of SMC had a significant protective effect compared to the diclazuril as a widely used anticoccidial drug. Reduction in the oocyst count by SMC as an organosulfur compound is mostly due to the ability of thiosulfinates to inhibit the microorganism's thiol-containing enzymes, resulting in its antimicrobial effect (45). Furthermore, the allicin content of garlic can inhibit the parasitic RNA synthesis and activate the natural killer immune cells (46). The anticoccidial effect of diclazuril is noted in its ability to disturb the different stages of the *Eimeria* species life cycles by inhibiting the amylopectin synthesis, which is a main component of the cell wall of *E. tenella* parasitic stage (47). Furthermore, diclazuril downregulates the microneme genes, leading to reduction in the second-generation merozoites of *E. tenella* (48), and the enzyme expressions that are responsible for the *E. tenella* cell replication (6). Epithelial cells with *E. tenella* first-generation schizonts are present in the cecal lamina propria and release themselves to develop the second-generation schizonts, which then migrate to the mucosa for the intracellular maturation (49). Here, SMC-treated chickens showed necrotic *E. tenella* schizonts in cecal crypts with migrating cell-containing schizonts as observed in the diclazuril-treated group, showing the anticoccidial effect of SMC by affecting the early schizont stage, similar to that of diclazuril. Damage of the cecal tissue results in release of plasma proteins into the intestine with hemorrhage and absorption disturbance, reducing the TP level in case of coccidiosis. This is in accordance with our results, which could be ameliorated by SMC by interfering with the *Eimeria* life cycle.

In the infected groups treated with SMC (G4 and G5), histopathological examination of cecal tissues showed that the cecal crypts and migrating epithelial cells have necrotic elements of the *E. tenella* schizonts; necrosis occurred in a dose-dependent manner. Furthermore, the alive meronts of *E. tenella* decreased in G4 and G5. The effect of SMC was similar to that of diclazuril (G6), which revealed high dead parasitic stages in the crypts' epithelial cells. The *E. tenella*-induced severe necrotic and hemorrhagic enteritis is in accordance with a previous report showing the existence of different parasitic stages in the intestinal crypts (50). Histopathological results demonstrated that SMC might affect the early schizogony stage, producing an anticoccidial effect. Regarding the induced hepatic damage by *E. tenella* infection, mitochondrial damage was indicated by the elevations of the aminotransferase enzymes (ALT and AST), while the cellular and biliary damage were referred by the elevated ALP activity. Moreover, both TP and albumin were significantly reduced in the infected chickens because of hepatic cell damage, which is responsible for the protein synthesis (51). Significant elevations in creatinine and uric acid levels were also observed because of coccidiosis due to reduced feed and water intake. SMC treatment could restore the ALT, AST, ALP,

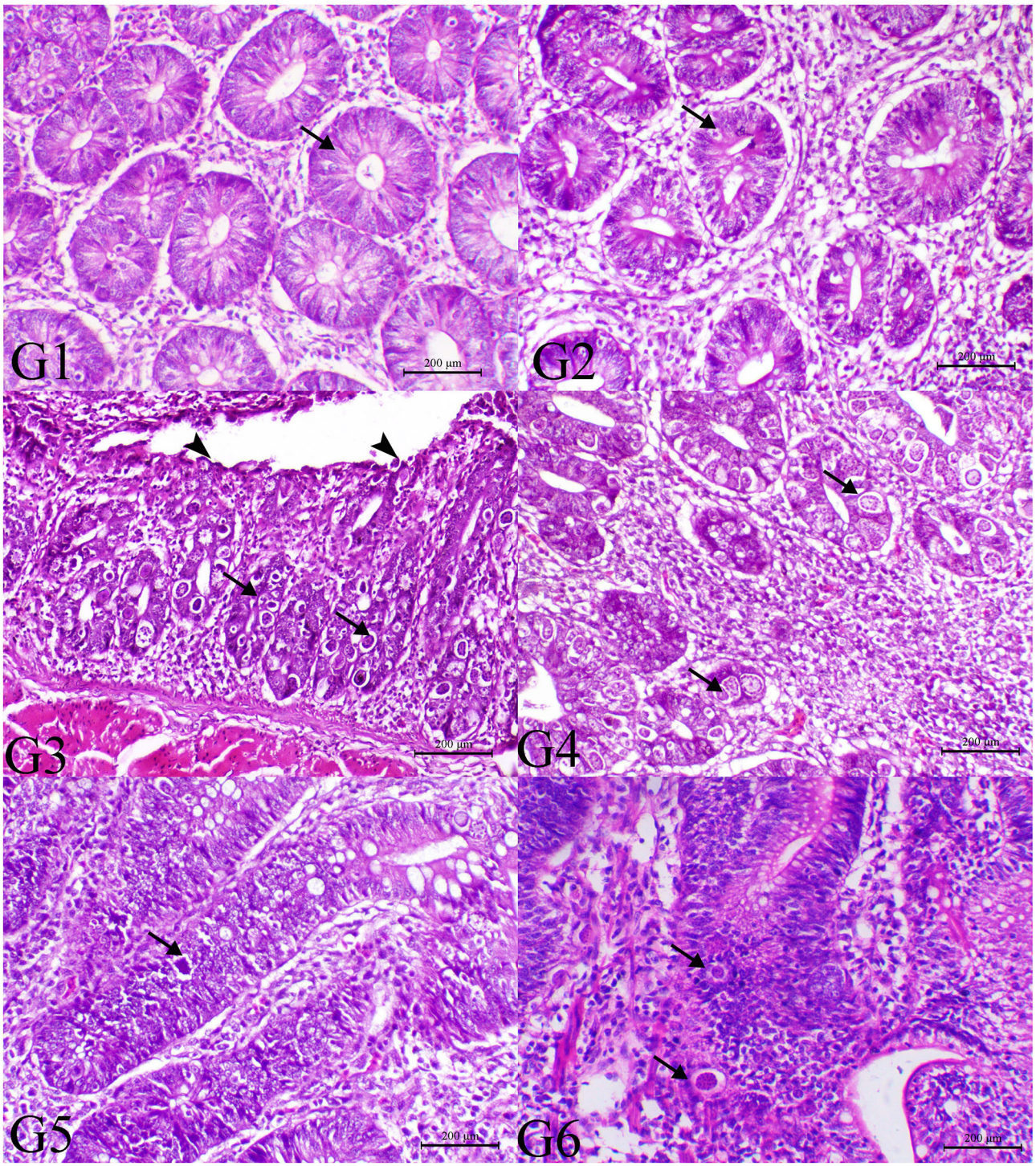


**FIGURE 2 |** Histopathology of cecal tissues of the normal bird (G1), SMC-treated birds (G2), *E. tenella*-challenged birds and examined on the 7th day post infection (G3), challenged and treated birds with SMC low dose (G4), high dose (G5), and diclazuril (G6). G1 and G2 (arrows indicate normal intestinal crypts), G3 (arrowheads indicate desquamation of the intestinal epithelium and arrows reveal severe infection of the intestinal crypts with parasitic schizonts), G4 (arrow indicates decrease the parasites within the intestinal mucosa), G5 (arrow indicates very few parasites), and G6 (arrow indicates moderate number of coccidial parasitic stages), H&E stain, bar = 200 µm.

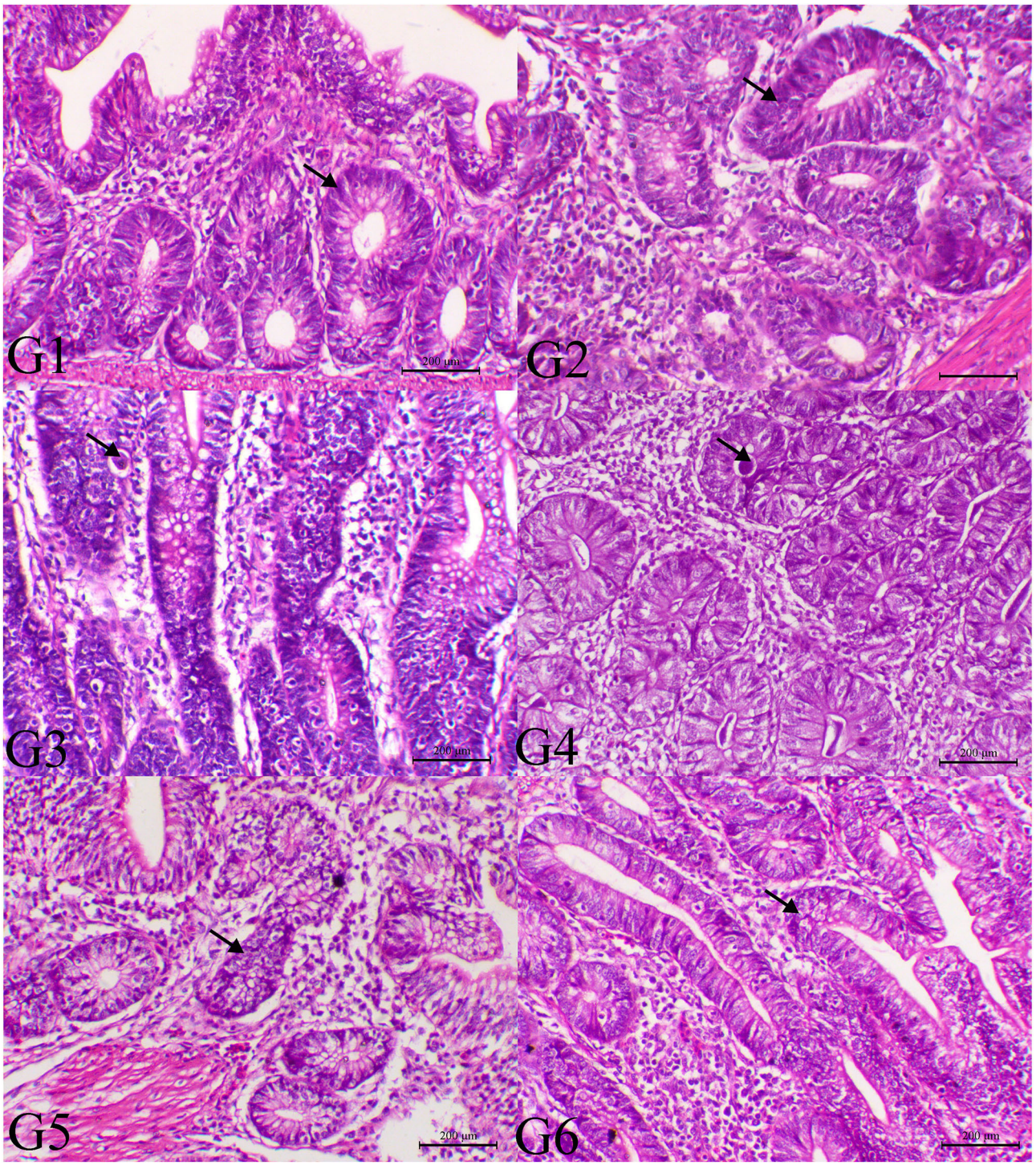


**FIGURE 3 |** Histopathology of cecal tissues of the normal bird (G1), SMC-treated birds (G2), *E. tenella*-challenged birds and examined on the 14th day post infection (G3), challenged and treated birds with SMC low dose (G4), high dose (G5), and diclazuril (G6). G1 and G2 (arrows indicate normal intestinal crypts), G3 (arrow reveals the presence of parasites within the intestinal crypts), G4 (arrow indicates marked decrease of the parasites within the intestinal mucosa), G5 (arrow indicates normal intestinal crypts), and G6 (arrow indicates hyperplastic changes within the intestinal mucosa), H&E stain, bar = 200 µm.

**TABLE 6 |** The effect of SMC on the serum biochemical parameters (values are means ± SD) in broiler chickens experimentally infected with *E. tenella* at 7th day post infection.

| Parameter | Non-infected | | Infected | | | |
|---|---|---|---|---|---|---|
| | Control G1 | SMC G2 | Non-treated G3 | SMC low dose G4 | SMC high dose G5 | Diclazuril G6 |
| ALT (U/L) | $10.33 \pm 1.24^{e}$ | $13.33 \pm 1.24^{a}$ | $19.33 \pm 5.24^{b}$ | $20.33 \pm 3.68^{c}$ | $19 \pm 0.81^{d}$ | $19 \pm 2.16^{d}$ |
| AST (U/L) | $158.67 \pm 25.3^{e}$ | $170.33 \pm 15.45^{a}$ | $278.33 \pm 31.56^{b}$ | $236 \pm 36.24^{c}$ | $198.33 \pm 8.37^{d}$ | $200 \pm 8.16^{d}$ |
| ALP (U/L) | $1{,}908 \pm 274.1^{e}$ | $1{,}907 \pm 210.77^{a}$ | $4266.7 \pm 839.73^{b}$ | $2{,}350 \pm 515.2^{c}$ | $2053.66 \pm 585.71^{d}$ | $1944.33 \pm 189.58^{d}$ |
| T P (g/dl) | $5.26 \pm 0.66^{a}$ | $4.53 \pm 0.41^{e}$ | $3.23 \pm 0.26^{d}$ | $3.66 \pm 0.20^{c}$ | $4.06 \pm 0.09^{b}$ | $4.16 \pm 0.17^{b}$ |
| Albumin (g/dl) | $1.63 \pm 0.13^{a}$ | $1.72 \pm 0.22^{e}$ | $1.21 \pm 0.08^{d}$ | $1.33 \pm 0.09^{c}$ | $1.53 \pm 0.12^{b}$ | $1.73 \pm 0.17^{b}$ |
| Creatinine (mg/dl) | $0.50 \pm 0.08^{e}$ | $0.46 \pm 0.04^{a}$ | $0.85 \pm 0.04^{b}$ | $0.60 \pm 0.08^{c}$ | $0.50 \pm 0.08^{cde}$ | $0.60 \pm 0.08^{cde}$ |
| Uric acid (mg/dl) | $6.78 \pm 0.71^{e}$ | $6.78 \pm 0.76^{a}$ | $9.76 \pm 0.69^{b}$ | $7.64 \pm 3.09^{c}$ | $6.69 \pm 0.85^{de}$ | $7.63 \pm 0.47^{de}$ |

*Means within rows with different superscripts differ at $p \leq 0.05$. a, b, c, and d indicate statistical significance in comparison with control, infected non-treated, low dose SMC-treated, and diclazuril-treated groups, receptively. Means within rows with different superscripts differ at $p \leq 0.05$.*

**TABLE 7 |** The effect of SMC on the serum biochemical parameters (values are means ± SD) in broiler chickens experimentally infected with *E. tenella* at 14th day post infection.

| Parameter | Non-infected | | Infected | | | |
|---|---|---|---|---|---|---|
| | Control G1 | SMC G2 | Non-treated G3 | SMC low dose G4 | SMC high dose G5 | Diclazuril G6 |
| ALT (U/L) | $6.00 \pm 1.41^{e}$ | $5.66 \pm 0.47^{a}$ | $14.66 \pm 1.24^{b}$ | $5.00 \pm 0.81^{c}$ | $7.00 \pm 0.81^{d}$ | $5.33 \pm 0.94^{d}$ |
| AST (U/L) | $213.67 \pm 7.76^{e}$ | $193.66 \pm 7.36^{a}$ | $284.33 \pm 05.31^{b}$ | $223.66 \pm 3.39^{c}$ | $229.33 \pm 18.08^{d}$ | $192 \pm 12.83^{d}$ |
| ALP (U/L) | $1623.3 \pm 107.28^{e}$ | $1623 \pm 128.83^{a}$ | $2{,}277 \pm 189.64^{b}$ | $1{,}491 \pm 91.17^{c}$ | $1.353 \pm 88.58^{d}$ | $1424.33 \pm 68.66^{d}$ |
| T P (g/dl) | $5.00 \pm 0.81^{a}$ | $5.33 \pm 0.47^{e}$ | $4.06 \pm 0.16^{d}$ | $5.00 \pm 0.81^{c}$ | $5.53 \pm 0.41^{b}$ | $4.73 \pm 0.52^{b}$ |
| Albumin (g/dl) | $1.53 \pm 0.17^{a}$ | $1.83 \pm 0.09^{e}$ | $1.76 \pm 0.20^{d}$ | $1.93 \pm 0.18^{c}$ | $1.96 \pm 0.19^{b}$ | $1.76 \pm 0.12^{b}$ |
| Creatinine (mg/dl) | $0.56 \pm 0.04^{e}$ | $0.53 \pm 0.04^{a}$ | $0.70 \pm 0.08^{b}$ | $0.56 \pm 0.04^{c}$ | $0.56 \pm 0.04^{cde}$ | $0.55 \pm 0.05^{cde}$ |
| Uric acid (mg/dl) | $7.08 \pm 1.93^{e}$ | $6.08 \pm 0.33^{a}$ | $6.08 \pm 0.33^{a}$ | $6.18 \pm 0.82^{c}$ | $8.07 \pm 1.14^{de}$ | $8.07 \pm 1.14^{de}$ |

*Means within rows with different superscripts differ at $p \leq 0.05$. a, b, c, and d indicate statistical significance in comparison with control, infected non-treated, low dose SMC-treated, and diclazuril-treated groups, receptively. Means within rows with different superscripts differ at $p \leq 0.05$.*

TP, albumin, creatinine, and uric acid levels toward the normal levels as measured on the 7th and 14th day after infection, revealing the protective effect of SMC, particularly the high dose, which showed a higher protection than diclazuril. The SMC-induced protection is attributed to the organosulfur nature and its antioxidative ability, which reduces hepatic and renal damage by restoring the normal cellular membrane structure and lipid peroxidation inhibition (52, 53).

It is well-known that coccidiosis leads to the release of reactive oxygen species (ROS) because of the damaged tissues (54). As a result, antioxidant enzymes (SOD, GSHPx, and CAT) were highly consumed to overcome the oxidative stress in the infected chickens (G3). SMC treatment (G4 and G5) could restore the activities of the measured antioxidant enzymes toward the normal levels by reducing the cecal and hepatic tissue damage and lipid peroxidation similar to that in diclazuril. These data were in accordance with previous studies that reported the scavenging of free radicals by the organosulfur content of garlic extract (55). Moreover, SMC was able to inhibit the NADPH oxidase enzyme and its signal transduction, leading to reduced ROS release as reported previously (56). The current data of the antioxidant parameters revealed the antioxidant activity of SMC against *Eimeria*-induced oxidative damage. It is noteworthy to state that *E. tenella* infection stimulates the immune cells to express pro-inflammatory cytokines (57). The inflammatory response was indicated by upregulation of NF-κB1 expression in the cecal tissue as observed in G3. NF-κB1 promotes the expression of other cytokines, such as IL-1β and IFN-γ, which activate the signal transduction of NF-κB pathway ending by tissue inflammation as confirmed by histopathological examination. The release of IL-1β was reported previously on the 7th day after coccidia infection (57). In this study, the dysregulated gene expressions of NF-κB1, IL-1β, and IFN-γ were restored to the normal regulation after treatment of infected chickens with SMC by the same significance as that with diclazuril. The anti-inflammatory effect of SMC is probably due to its ability to restrict the phosphorylation of cytochrome p65, reduce the inflammatory cytokine release, and inhibit cyclooxygenase-2 signal transduction, which have a potential role in the inflammatory response (56, 58). These data revealed the anti-inflammatory activity of SMC against the *E. tenella*-induced inflammatory response. Altogether, the recorded oocyst count and biochemical, antioxidant, and anti-inflammatory parameters after treatment of the infected chickens by SMC explain how the clinical signs, lesions, and mortality rate could be improved along with reduced BWG

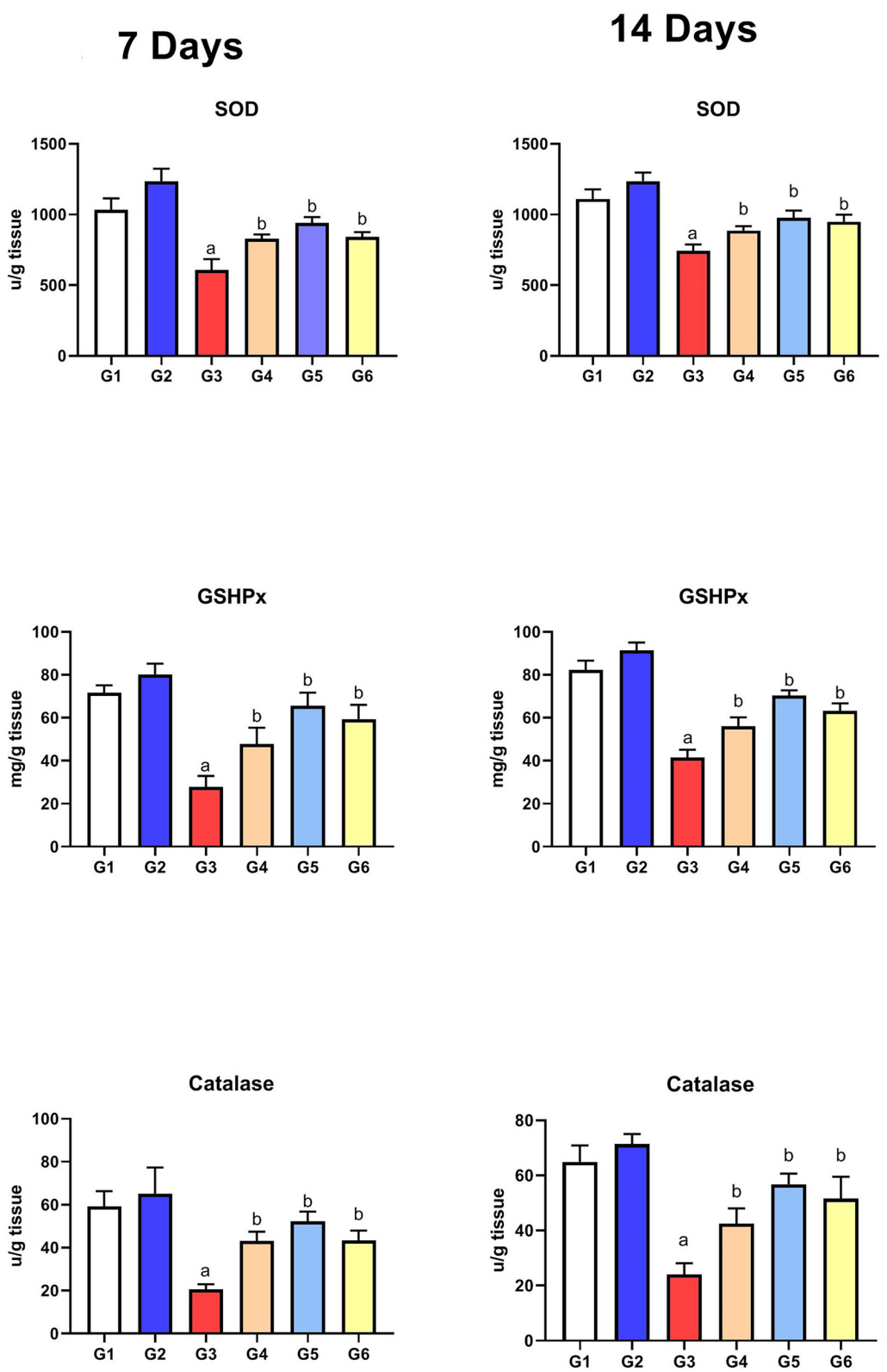


**FIGURE 4 |** The effect of SMC on the antioxidant parameters on the 7th and 14th day post infection in broiler chickens experimentally infected with *E. tenella*. a and b indicate statistical significance ($p \leq 0.05$) in comparison with control (G1) and infected non-treated (G3) groups, receptively.

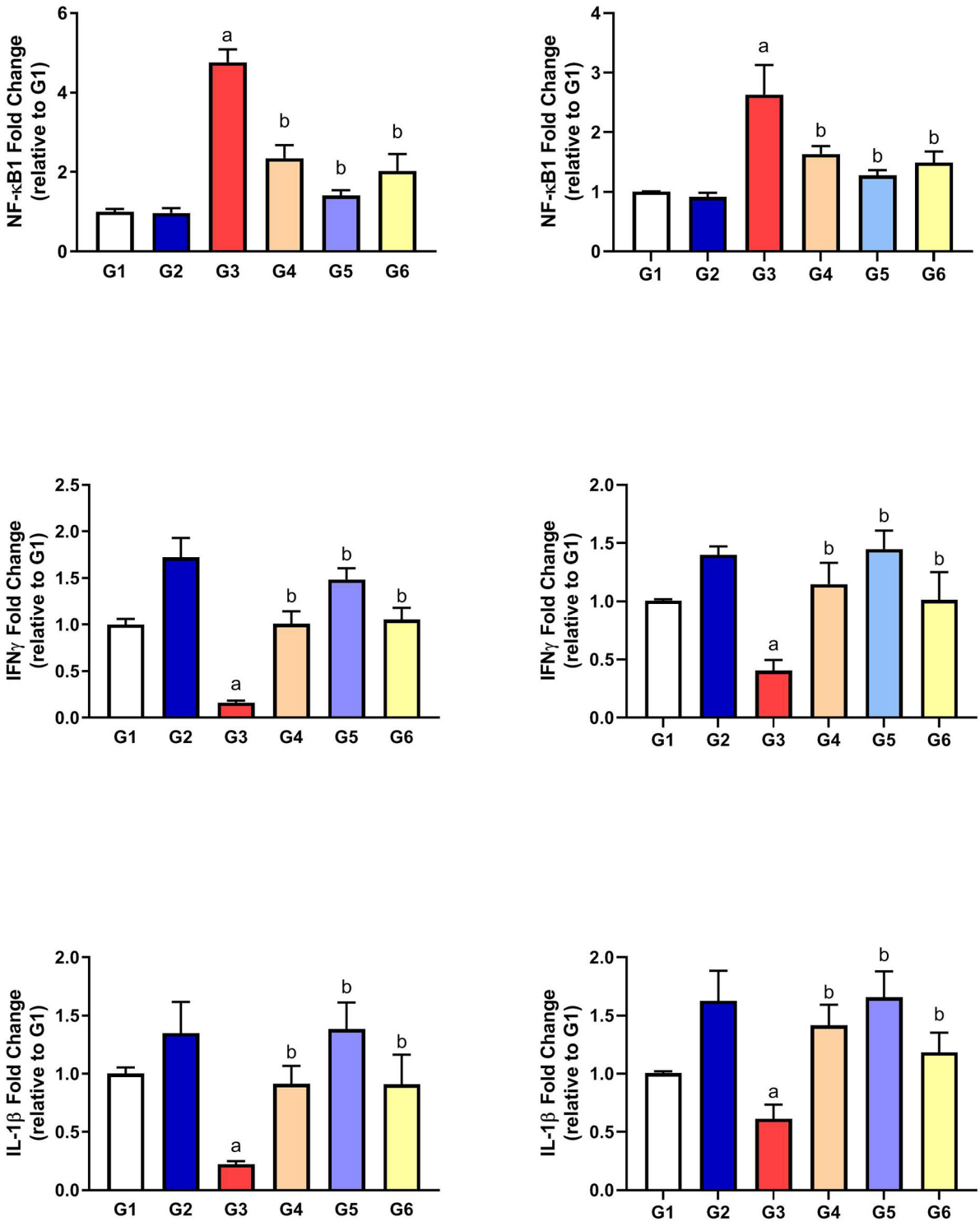


**FIGURE 5 |** The effect of SMC on the inflammatory parameters on the 7th and 14th day post infection in broiler chickens experimentally infected with *E. tenella*. a and b indicate statistical significance ($p \leq 0.05$) in comparison with control (G1) and infected non-treated (G3) groups, respectively.

and FCR, which were all induced by *E. tenella* infection, demonstrating the dose-dependent protective role of SMC against coccidiosis effects.

## CONCLUSION

Taken together, the present study concluded the anticoccidial role of SMC as a plant-based compound against *E. tenella*-induced coccidiosis in broiler chickens. Finally, our conclusion recommends the use of SMC, a garlic component, as a supplementary or alternative therapy to control avian coccidiosis induced by *E. tenella*. Further clinical trials should be conducted to confirm its efficiency, and more studies are needed to determine the detailed mechanism of action of SMCs.

## DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author/s.

## ETHICS STATEMENT

The animal study was reviewed and approved by the Research, Publication, and Ethics Committee of the Faculty of Veterinary Medicine, Kafrelsheikh University, Egypt. The ethical approval number is KFS-2019/3.

## AUTHOR CONTRIBUTIONS

EE and WA were involved in the conception of the idea and methodology design, performed data analysis and interpretation, and prepared the manuscript for publication and revision. AF performed data interpretation, wrote the initial draft of the manuscript, and provided scientific revision of the final manuscript. DE, AR, AA, RM, NN, NA, EH, and KA provided scientific advice and prepared the manuscript for revision. All authors have read and approved the final manuscript.

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# Prophylactic Effects of Ivermectin and Closantel Treatment in the Control of *Oestrus ovis* Infestation in Sheep

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The sheep nasal bots *Oestrus ovis* is parasite of the nasal cavities and sinuses of small ruminants causing oestrosis, one of the most frequent parasitic diseases in sheep and goats. The widely use of ivermectin and closantel by the sheep breeders in the treatment and prophylaxis of gastrointestinal nematodes resulted in widespread cases of anthelmintic resistance. However, there is no report about cases of *O. ovis* with drug-resistance. In this study, we evaluated the prophylactics and therapeutic effects of both antiparasitics in sheep with *O. ovis* natural infestation. The trial was carried out from early December 2019 to March 2020, with 30 crossbred males lambs allocated into three groups of 10 animals each: control (without treatment), treated with ivermectin (0.2 mg/kg subcutaneously) and treated with closantel (10 mg/kg orally). The animals were kept together grazing the same pasture area. The treatment groups were drenched in two occasions 70 days apart: on 5th December 2019 and on 13th February 2020. On 19th March 2020, all lambs were slaughtered. The lamb heads were removed and sectioned along their longitudinal and sagittal axis to search for larvae. Recovered *O. ovis* larvae were counted and identified according to their developmental stage (L1, L2, and L3). Seven of the control lambs were infested with *O. ovis* larvae ranging from six to 17 larvae (11.6 mean infestation intensity). All recovered larvae from control group were intact and active. Three animals treated with ivermectin had *O. ovis* larvae (1–3 larvae), however they were dead and in degeneration. The animals treated with closantel did not have any larvae. The clinical suggestive signs of oestrosis were scarce over the experimental period. The averages of daily weight gain were similar ($p > 0.05$) among groups. Closantel and ivermectin had high efficacy against oestrosis and *O. ovis* parasitism did not hinder the performance of lambs.

**Keywords: myiasis, Ovis aires, lambs, bot fly, Oestridae**

## INTRODUCTION

*Oestrus ovis* L. (Diptera: Oestridae) is a cosmopolitan parasite with a higher prevalence in tropical regions. It causes cavitary myiasis in small ruminants, once the larvae are an obligate parasite of the nasal and sinus cavities of sheep and goats (1).

In endemic areas, the *O. ovis* larvae have been found in humans' eyes and nasopharyngeal airway. Cases of ocular affections are likely to occur more frequently than currently notified, because many cases without complications are unreported (2).

The female fly flies around the head of its host to deposits larvae at a few centimeters from sheep nostrils (3). In case of the fly presence, the animals hide the muzzle on the soil or in the others sheep's wool, swing head and sneezing, which leads to distress. The larvae hooks and spine irritate the nasal mucosal, provoking inflammation and mucous nasal discharge. At the same time, this irritating action secures the production of an inflammatory exudate, which count for larva's feeding (4). Besides frequent sneezes, sheep infested with *O. ovis* larvae may also present difficulty in breathing, hyporexia, and weight loss. In heavy infestation, animals may have related secondary bacterial infection in the lungs (5).

In a trial conducted in Brazil for three consecutive years, the prevalence of *O. ovis* was 50% with the occurrence of the parasite in all seasons. Nevertheless, the highest prevalence (61.1%) were observed in the spring and summer. The climatic conditions of this region during this period are optimal for fly activity, and consequently for a high rate of sheep infestation (6).

The oestrosis therapy is based on the use of antiparasitic drugs that target the larvae. Many systemic antiparasitic drugs may be used for the oestrosis treatment. The most widely used antiparasitics to control *O. ovis* are closantel and the macrocyclic lactones, such as orally or injectable ivermectin (7, 8). Sheep treated with closantel, injectable and oral ivermectin had 100, 100, and 98% of efficacy, respectively considering all instars (7). In a survey conducted by Oliveira et al. (8) the injectable doramectin was 100% effective against all larval stages. Nevertheless, the first instar larvae (L1) is considered less susceptible to these drugs in comparison with the second instar larvae (L2) and third instar larvae (L3) (9) because the L1 larvae feed less thus they are less prone to ingest the systemic parasiticides (10). Martínez-Valladares et al. (11) observed that the long-acting injectable moxidectin had 90.2% of efficacy against L1.

The intensification of sheep farming and emergence of drug-resistant parasites have brought new challenges regarding the prophylaxis and treatment of small ruminant parasitism. Frequent cases of anthelmintic resistance have been reported (12–14) as well as ectoparasites, such as *Dermatobia hominis* (15, 16), *Rhipicephalus microplus* (17) and *Cochliomyia hominivorax* (18), with resistance to the treatments with macrocyclic lactones. For this reason, the aim of this work was to verify whether closantel and ivermectin that have been used for decades are still effective in sheep naturally infested with *O. ovis*. Because animals rarely die due to oestrosis, the economic losses caused by the parasitism may be underestimated. Therefore, the present study aimed also to assess the prophylactic effects of closantel and ivermectin on the productive performance of lambs.

## METHODS

### Statement of Ethics

The study was carried out according to the standards established by the local Ethics Committee on Animal Use (FMVZ/UNESP protocol number 0159/2019).

### Study Area

The experiment was conducted in the experimental area of the São Paulo State University (UNESP), Department of Biostatistics, Plant Biology, Parasitology and Zoology of the Bioscience Institute, Botucatu, SP, Brazil.

The lambs were kept in an area of 3.840 m² divided in 5 paddocks, in rotational grazing on *Urochloa decumbens,* exposed to natural infestation with *O. ovis*, and infection with gastrointestinal nematodes. The area had been grazed previously by sheep with natural infestation by those parasites. The animals had free access to tap water and mineral salt (Supre Ovinos - Coopermota®) and were fed daily with supplement with 16.5% of crude protein (Ração Ovinos Confinamento - Coopermota®) with an amount corresponding to 2% of their body weight.

### Experimental Design

The experiment was conducted from December 2019 to March 2020, during the rainy season. Thirty 2 to 3-month-old uncastrated male lambs crossbred Santa Ines x White Dorper were purchased from a local commercial farm. The lambs arrived in the experimental area on October 29th, and underwent an adaptation period of 37 days, before the beginning of trial on December 5th. Upon the arrival, blood and fecal samples from all the animals were collected for hematological and parasitological procedures, and in addition, all lambs were vaccinated against clostridiosis (Sintoxan Polivalente® - Merial), and were treated with Toltrazuril (Farmacox® - Farmabase, 20 mg/kg, orally) in order to prevent coccidiosis.

The animals were randomly distributed into three groups that were as homogenous as possible regarding body weight: group 1 ($n = 10$), control animals that did not receive any antiparasitic treatment against oestrosis; group 2 ($n = 10$), animals treated with ivermectin (Ivomec® - Merial), 0.2 mg/kg, subcutaneous injection; and group 3 ($n = 10$), animals treated with closantel (Diantel® - Merial), 10 mg/kg, orally. Treatments against oestrosis with closantel or ivermectin were performed on 5th December 2019 and on 13th February 2020.

Additionally, all experimental animals were treated with an anthelmintic combination with monepantel (2.5 mg/kg, Zolvix® - Novartis), albendazole (10 mg/kg Endazol® - Hipra) and levamisole phosphate (9.4 mg/kg Ripercol – Zoetis) for three consecutive days, which were administered in three different occasions: on 12th December, 16th January and 13th February. These anthelminitic treatments were performed to prevent losses due to nematode infections, keeping helminth infection degree similar among groups.

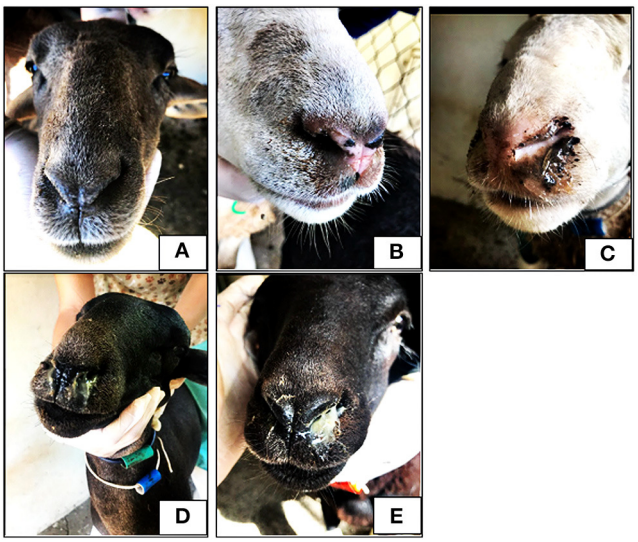


**FIGURE 1 |** Photos of lambs with different nasal discharge score. No discharge **(A)**, serous discharge **(B)**, sero-mucous discharge **(C)**, thick mucous discharge **(D)** and mucopurulent thick discharge **(E)**.

**TABLE 1 |** Nasal discharge score.

| Score | Nasal discharge |
|---|---|
| 1 – **Figure 1A** | No discharge |
| 2 – **Figure 1B** | Serous discharge |
| 3 – **Figure 1C** | Sero-mucous discharge |
| 4 – **Figure 1D** | Thick mucous discharge |
| 5 – **Figure 1E** | Mucopurulent thick discharge |

## Animals Performance and Clinical Observation

Weekly, the lambs were weighed and examined looking for clinical signs of infestation by *O. ovis*, which included their nasal discharge degree (**Figure 1**; **Table 1**), recorded according to Dorchies et al. (7).

## Parasitological Analysis

Feces samples were obtained directly from the rectum of each animal weekly to perform fecal egg counts by modified McMaster technique, in which each worm egg counted represented 100 eggs per gram (EPG) (19). In addition, fecal cultures were performed for each group to obtain infective larvae (L3) of gastrointestinal nematodes, which were identified according to descriptions of Ueno and Gonçalves (19).

## Recovery of *Oestrus ovis* Larvae

The animals were slaughtered 35 days after the last oestrosis treatment. They had their heads removed and then sectioned along their longitudinal and sagittal axis. Nasal cavity (nasal passage, septum, middle meatus, and conchae) (**Figure 2A**) and frontal sinus (**Figure 2B**) were carefully examined, and all larvae found were collected, counted, and identified with regards to their stage of development (1, 20).

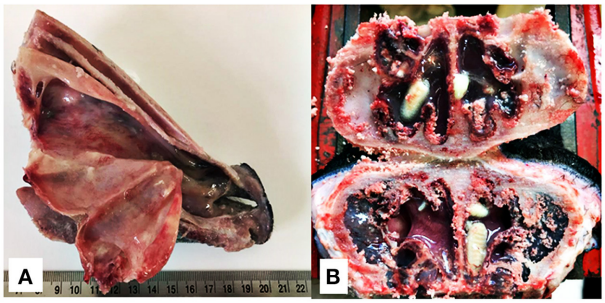


**FIGURE 2 |** Longitudinal section of the rostral portion of the head without the mandible **(A)** and cross section of the nasal sinuses for the recovery of *O. ovis* larvae from naturally infested lambs **(B)**.

## Recovery of Gastrointestinal Nematodes

One lamb from each group, with the highest EPG in the last sampling, was chosen for worm counts. After slaughter, the lambs had their abomasum and small intestine removed and opened longitudinally for content recovery. Their gastrointestinal contents were placed in graduated buckets to obtain aliquots of 10% of the total contents from each organ. The aliquots of 10% were stored in identified plastic flasks and preserved with 5% formaldehyde. All nematodes present in the 10% preserved material were morphologically identified and quantified according to their developmental stages (19).

## Hematological Analyses

Blood samples (5 ml) were obtained every 14 days by jugular vein puncture into Vacutainer® tubes containing anticoagulant (K2 EDTA 7.2 mg, BD, Brazil). Packed cell volume (PCV) was determined by microhematocrit centrifugation (5 min/15,000 g, Fanem 211 microhematocrit centrifuge), and Total Protein Plasmatic (TPP) concentrations were estimated using a handheld refractometer (Refractometer SPR-N, Atago) (21, 22). Eosinophil counts were performed in a Neubauer's chamber after staining with Carpentier's solution, and the counts were expressed as the number of eosinophil cells per μL of blood (23).

## Enzyme-Linked Immunosorbent Assay

Plasma samples collected at 8 time-points were used to determine IgG levels against L2-soluble extract of *O. ovis*. The L2 extract was prepared as described by Silva et al. (24). A previously described protocol was applied to determine the parasite-specific plasma IgG levels (24) with some modifications: the plates were coated with 2 μg of antigen/mL, each wash was done six times which were incubated with peroxidase-conjugated rabbit anti-sheep IgG diluted at 1:5,000 then the plates were read at 450 nm by using an automated ELISA reader (Biotrak II; Amersham Biosciences, Little Chalfont, UK). For the negative control, plasma samples were obtained from young animals that were kept indoors without any contact with adult bot flies as previously described by Silva et al. (24). The plasma positive control sample

used was from a sheep naturally infested with *Oestrus ovis*. Results were expressed as the percentage of plasma sample optical density (OD) value divided by OD of positive control.

## Therapeutic Efficacy

The therapeutic efficacy of each antiparasitic was based on the mean number of *O. ovis* larvae recovered from lambs of each group at necropsy and calculated according to the following Abbot's formula (25).

$$Efficacy = (1 - \text{Treated group/Control group}) \times 100$$

For a therapeutic claim a reduction of larval counts should be at least 90% (25).

## Meteorological Data

The meteorological data was registered daily (temperatures, precipitation and relative humidity). The climatic conditions during the experiment period were similar to those reported in the last years, except for February with total rainfall of 552.2 mm. This high value occurred because on the 9th it rained 299 mm, more than expected for the whole month. Data was obtained from the Lageado Experimental Farm weather station belonging to Unesp - Campus Botucatu at a distance of 8 km from the experimental area.

## Statistical Analyses

All data were submitted to the normality test Shapiro-Wilk and transformed using log10 (x + 1) when necessary, which was the case of EPG, *O. ovis* larvae stages, eosinophils and IgG values. Data were analyzed by one-way analysis of variance (ANOVA) in the case of variables measured only once (carcass weight, daily BWG and larvae stages of *O. ovis*) and by ANOVA with repeated measures in the case of variables measured at several time points (body weight, PCV, TPP, OD and eosinophils) using the General Linear Model (GLM) of the Statistical Analysis System, version 9.2 (SAS Institute, Inc., Cary, NC, USA). The group means were compared by Tukey's test and chi-square test was used for categorical variable (nasal discharge). Values of $p < 0.05$ were considered statistically significant.

Descriptive statistical analyses were used to summarize the data on larval burden, in agreement with Bush et al. (26), using the following terms:

Prevalence: the number of hosts infested with *O. ovis* larvae, divided by the number of hosts examined;

Intensity of infestation: the number of *O. ovis* larvae in a single infested host;

Mean intensity of infestation: the total number of *O. ovis* larvae found divided by the number of hosts infected with that parasite.

# RESULTS

During the study none of sheep showed adverse effects as a consequence of the treatments. Seven animals of the control group were infested with *O. ovis* larvae, which were alive. The control group average, considering also three animals with zero value, was 8.1 larvae per sheep (**Table 2**), which were 1.4 L1, 4.3 L2 e 3.0 L3 per head. An animal from control group with the highest intensity of infestation had 17 larvae (four L2 and 13 L3).

**TABLE 2 |** First (L1), second (L2) and third stage (L3) larvae of *Oestrus ovis* in naturally infested lambs of the control, ivermectin and closantel groups.

| Group | Infested animal | Mean | Min–max | Total | | |
|---|---|---|---|---|---|---|
| | | | | L1 | L2 | L3 |
| Control ($n = 10$) | 7 | 8.1[b] | 0–17 | 14 (17%) | 41 (51%) | 26 (32%) |
| Ivermectin ($n = 10$) | 3 | 0.6[a] | 0–3 | 0 | 2 (33%) | 4 (67%) |
| Closantel ($n = 10$) | 0 | 0[a] | 0 | 0 | 0 | 0 |
| *p*-value | | <0.001 | | | | |

*Different letters in the column indicate a significant difference among means (Tukey test, $p < 0.05$).*

The treatment with closantel was 100% effective against oestrosis, once there were no larvae on animals' heads. The treatment with ivermectin was 93% effective with three lambs presenting two larvae each in total six *O. ovis* larvae (**Table 2**). However, the larvae found were dead and had altered morphology, suggestive of the beginning of larva degeneration process. Differently from the lambs treated with ivermectin, the larvae identified in animals from the control group were active and without any morphological changes. Therefore, considering only alive larvae counting, the efficacy of ivermectin was 100%.

All the L1 observed in control group were in nasal conchae, 56% of the L2 were in ethmoidal conchae, and 44% in frontal sinus, while 23% of the L3 were in ethmoidal conchae and 77% in frontal sinus. Regarding the six dead larvae found in lambs treated with ivermectin, one L2 were in ethmoidal conchae, two L3 were in ethmoidal conchae and two L3 were in frontal sinus.

The clinical suggestive signs of oestrosis were scarce over the experimental period (**Table 3**) and there was no influence of treatment in the characterization of the lamb nasal discharge ($p = 0.060$). Each animal was examined 15 times over the trial. From the 450 nasal discharge exams conducted, 88% of them were serous, 9% were sero-mucous, 1% were very tick mucous, and 2% were mucopurulent thick (**Table 3**). This last score was observed in six animals, two from each group. Two weeks post the second oestrosis treatment, one animal from control group and one from group treated with closantel had muco-purulent discharges. Because lambs treated with closantel did not present larvae, it was possible to deduce that such discharge had no relation with *O. ovis* infestation.

At the beginning of the trial, the average (±standard error) body weight were 23.1 (±1.3) kg, 22.8 (±1.4) kg and 22.9 (±1.6) kg, for non-treated control, ivermectin and closantel groups, respectively. In the last sampling, lambs were about 7–8 months old, and before slaughter, they had the following weight averages: 40.8 (±1.7), 40.5 (±2.0) kg and 40.6 (±1.6) kg for non-treated control, ivermectin and closantel groups, respectively. There was no significant difference among groups ($p = 0.971$) and also no significant group × time interaction ($p = 0.615$). The

control group had daily gain mean (0.193 ± 0.016 kg), similar to ivermectin group (0.192 kg ± 0.01) and closantel group (0.192 kg ±0.02) ($p = 0.994$). In the same way, the means of carcass weight were similar ($p = 0.999$) among the different groups: 19.16 kg (±0.80), 19.11 kg (±1.01), 19.13 kg (±0.92), for control, ivermectin and closantel groups, respectively (**Table 4**).

Because the animals had been kept in feedlot on their commercial farm of origin, EPG values were low and relatively homogeneous with averages similar between groups ($p = 0.611$) at the arrival in the experimental facilities. Lambs from control group had 70 (±39.58) EPG, lambs from ivermectin group had 740 (±322.90) EPG, and lambs from closantel group had 370 (±247.68) EPG.

After the first treatment for oestrosis, closantel group had significantly lower ($p < 0.001$) EPG mean (300 ± 84) than the control (2,220 ± 424) and ivermectin (2,730 ± 925) groups. In order to avoid interference of helminthiasis in the animals' performance, it was chosen to drench all the animals with the combination of three anthelmintics (levamisole + albendazole + monepantel) on three occasions as previously described. By adopting this procedure, the EPG counts remained similar in all groups until the day of slaughter (**Figure 3**). Despite the low EPG values, group mean differences were observed on 19th December 2019, when the EPG average of the control group (390 ± 55) was higher ($p < 0.001$) than the means of the ivermectin (110 ± 48) and closantel (40 ± 22) groups; and on the last evaluation (12th March 2020), when once again the control group presented EPG mean (170 ± 49) significantly higher ($p = 0.024$) than the closantel (40 ± 40) and ivermectin (70 ± 30) groups. There was no significant interaction between the group and time ($p = 0.281$), but there was significant effect of time ($p < 0.001$) in the EPG counts.

It was also observed eggs of *Strongyloides* spp. and *Moniezia* spp. and oocysts of *Eimeria* spp. in fecal exams, however in small amounts. None of lambs had symptoms of parasitic gastroenteritis during the trial.

In fecal cultures, larvae of *Haemonchus* spp., *Cooperia* spp., and *Trichostrongylus* spp. were detected. However, *Haemonchus* spp. predominated during the experiment, being the only genus recorded from February until the end of experiment period (**Table 5**).

In the examination of the gastrointestinal contents, *Haemonchus* spp. was the major genus found, with a high number of immatures stages (**Table 6**). Additionally, ten adult females of *Trichostrongylus* spp. were recovered from a lamb

**TABLE 3 |** Total and percentage of nasal discharge classification of lambs naturally infested with *Oestrus ovis* of the control, ivermectin and closantel groups, in 15 evaluations from December 2019 to March 2020.

| Group | Serous | Sero-mucous | Thick mucous | Mucopurulent | Total |
|---|---|---|---|---|---|
| Control | 140 (94%) | 8 (5%) | 0 | 2 (1%) | 150 |
| Ivermectin | 124 (83%) | 19 (12%) | 3 (2%) | 4 (3%) | 150 |
| Closantel | 133 (89%) | 14 (9%) | 0 | 3 (2%) | 150 |
| Total | 397 (88%) | 41 (9%) | 3 (1%) | 9 (2%) | 450 (100%) |

**TABLE 4 |** Initial and final body weight, daily body weight gain and carcass weight averages (±S.E.) of lambs naturally infested with *Oestrus ovis* of the control, ivermectin and closantel groups.

| Variable | Control | Ivermectin | Closantel | *p*-value |
|---|---|---|---|---|
| Initial | 23.1 (±1.3) | 22.8 (±1.4) | 22.9 (±1.6) | 0.971 |
| Final | 40.8 (±1.7) | 40.5 (±2.0) | 40.6 (±1.6) | 0.990 |
| Daily gain | 0.193 (±0.02) | 0.192 (±0.01) | 0.192 (±0.02) | 0.994 |
| Carcass | 19.16 (±0.80) | 19.11 (±1.01) | 19.13 (±0.92) | 0.999 |

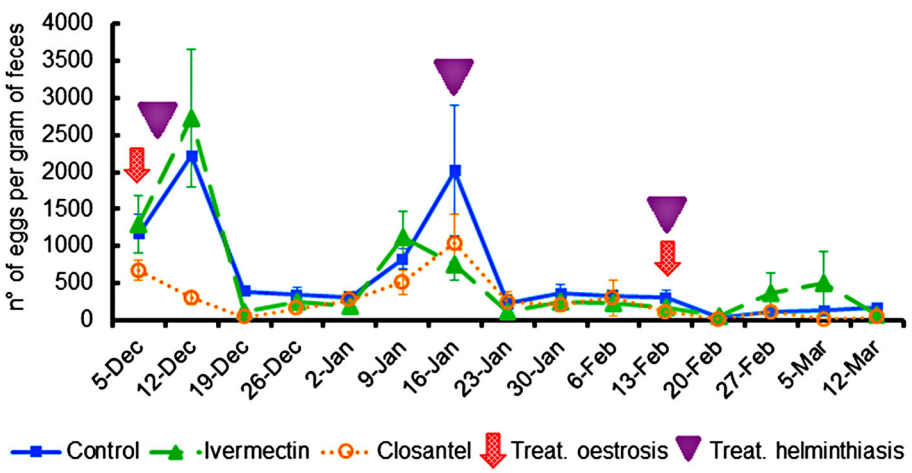


**FIGURE 3 |** Averages of eggs per gram feces (±standard error) of lambs naturally infected with gastrointestinal nematodes and infested with *O. ovis*. The moments of treatments against oestrosis (with ivermectin or closantel) are indicated by arrow and treatments against helminthiasis (with a combination of levamisole + albendazole + monepantel) are indicated by triangle.

**TABLE 5 |** Percentage of infective larvae of *Haemonchus* spp. (*Haem*), *Trichostrongylus* spp. (*Trich*) e *Cooperia* spp. (*Coop*) in fecal cultures from control, ivermectin and closantel groups.

| Date | Control | | | Ivermectin | | | Closantel | | |
|---|---|---|---|---|---|---|---|---|---|
| | *Haem* | *Trich* | *Coop* | *Haem* | *Trich* | *Coop* | *Haem* | *Trich* | *Coop* |
| 5/Dec | 99 | 1 | 0 | 100 | 0 | 0 | 98 | 1 | 1 |
| 12/Dec | 95 | 1 | 4 | 100 | 0 | 0 | 100 | 0 | 0 |
| 19/Dec | 91 | 0 | 9 | 97 | 0 | 3 | 100 | 0 | 0 |
| 26/Dec | 92 | 0 | 8 | 96 | 0 | 4 | 96 | 0 | 4 |
| 2/Jan | 91 | 0 | 9 | 98 | 0 | 2 | 99 | 0 | 1 |
| 9/Jan | 84 | 0 | 16 | 93 | 0 | 7 | 97 | 0 | 3 |
| 16/Jan | 90 | 0 | 10 | 96 | 1 | 3 | 94 | 1 | 5 |
| 23/Jan | 100 | 0 | 0 | 100 | 0 | 0 | 100 | 0 | 0 |
| 30/Jan | 100 | 0 | 0 | 99 | 0 | 1 | 100 | 0 | 0 |
| 6/Feb | 100 | 0 | 0 | 100 | 0 | 0 | 100 | 0 | 0 |
| 13/Feb | 100 | 0 | 0 | 100 | 0 | 0 | 100 | 0 | 0 |
| 20/Feb | 100 | 0 | 0 | 100 | 0 | 0 | 100 | 0 | 0 |
| 27/Feb | 100 | 0 | 0 | 100 | 0 | 0 | 100 | 0 | 0 |
| 5/Mar | 100 | 0 | 0 | 100 | 0 | 0 | 100 | 0 | 0 |
| 20/Mar | 100 | 0 | 0 | 100 | 0 | 0 | 100 | 0 | 0 |

**TABLE 6 |** Total *Haemonchus contortus* and *Trichostrongylus* spp. worm burden from one lamb of each group naturally infected with gastrointestinal nematodes.

| Genera | Stage | Control | Ivermectin | Closantel |
|---|---|---|---|---|
| *Haemonchus* spp. | Early L4 | 2,360 | 1,000 | 1,620 |
| | Female late L4 | 2,540 | 10 | 180 |
| | Male late L4 | 2,630 | 330 | 0 |
| | Female early L5 | 370 | 0 | 0 |
| | Male early L5 | 910 | 0 | 0 |
| | Adult female | 20 | 100 | 0 |
| | Adult male | 0 | 50 | 0 |
| *Trichostrongylus* spp. | Adult female | 10 | 10 | 0 |
| | Total worm burden | 8,840 | 1,500 | 1,800 |

treated with ivermectin and ten in an animal of the control group (**Table 6**).

The values of eosinophils, PVC and TPP (**Figure 4**) were similar among the three groups throughout the trial, and there were no significant interactions between group and time for these variables. The only exception occurred on 19th December 2019, at the beginning of the experiment, when the TTP mean of ivermectin group (6.02 ± 0.109) was significantly higher than the closantel group (5.60 ± 0.126) ($p = 0.017$). The lowest means of PCV (**Figure 4B**) were registered on 16th January 2020 ($p = 0.097$), that were 21.7% (±3.65), 25.2% (±4.10) and 24.2% (±2.86), for the control, ivermectin and closantel groups, respectively. This reduction in the values of PCV coincided with the increase in the EPG means (**Figure 3**). Additionally, in the same date, it was observed the highest means of blood eosinophils (**Figure 4A**).

The mean levels of anti-*O. ovis* L2 IgG in plasma showed changes over time, resulting in significant interaction between time and treatment ($p = 0.007$). The antibody levels increased in the control group on the last 2 weeks of the trial, being significantly higher than values of the treated groups ($p = 0.004$) on 27th February and 12th March (**Figure 4D**). The three animals from control group that did not have any larvae of *O. ovis* presented the lowest averages of antibodies in their group (lamb 1: 49%; lamb 2: 49%; and lamb 4: 45%).

## DISCUSSION

The results of this trial confirmed the high effectiveness of closantel in animals naturally infested by *O. ovis*, with the absence of larvae 35 days after the last treatment. Regarding the ivermectin, some treated animals still hosted larvae of second and third stages. Nevertheless, the larvae recovered from these animals were already dead presumably because of ivermectin action. However, it was not possible to determine when the larvae died. It is possible that the larvae have died soon after the treatment, but, somehow, they remained in the nasal cavities. The dead larvae are possibly expelled by sneezes that combined with the force of gravity cause nasal discharge.

Therefore, the results of the present study were in accordance with Dorchies et al. (7) that reported 100% of efficacy of oral closantel and injectable ivermectin against oestrosis, in sheep treated twice with an interval of 60 days. Likewise, oral ivermectin resulted in 100% efficacy against all stages 12 days after the treatment (9). Different of other Diptera that cause myiasis (*D. hominis* and *C. hominivorax*) with cases of drug-resistance (15-16-18), to the best of our knowledge, there is no report of populations of *O. ovis* with resistance to ivermectin or closantel.

The typical clinical signs of oestrosis were uncommon over trial and during the examination performed at necropsy there were no macroscopic morphological changes in the nasal cavities. There was only a small amount of secretion at the site where parasites were located and around the larvae. The absence of clinical sings in most of the evaluations may be a consequence of the low infestation rate. *Oestrus ovis* clinical manifestation is induced partially by spines on the larval cuticle and oral hooks used by L1 to move and progress toward nasal cavities and to anchor to the mucous surface. In addition, the L3 has large hooks, stout spines and dorsal plates which serve to support its gradual descend in the nasal cavities to the outside environment (4), whereupon this mechanical action provokes irritation in nasal mucosa (27). The pathogenesis of *O. ovis* is also induced by biomolecules (enzymes and antigens) excreted or secreted by the larvae onto the mucosa. These biomolecules comprise a complex array of enzymes necessary to degrade mucus and plasma proteins for larval feeding and nutrition (28). In agreement with our results, Silva et al. (6) observed that most tracer lambs in their study did not show any clinical signs typical of oestrosis after exposed to infestation during 4 weeks, although they hosted among 12 and 66 larvae, mainly L1 and L2. Like in the trial conducted by Silva et al. (6), possibly in our trial the infestation was recent, without enough time to manifestation of the immunopathological changes typical of the disease. Although not all infested sheep show clinical signs of

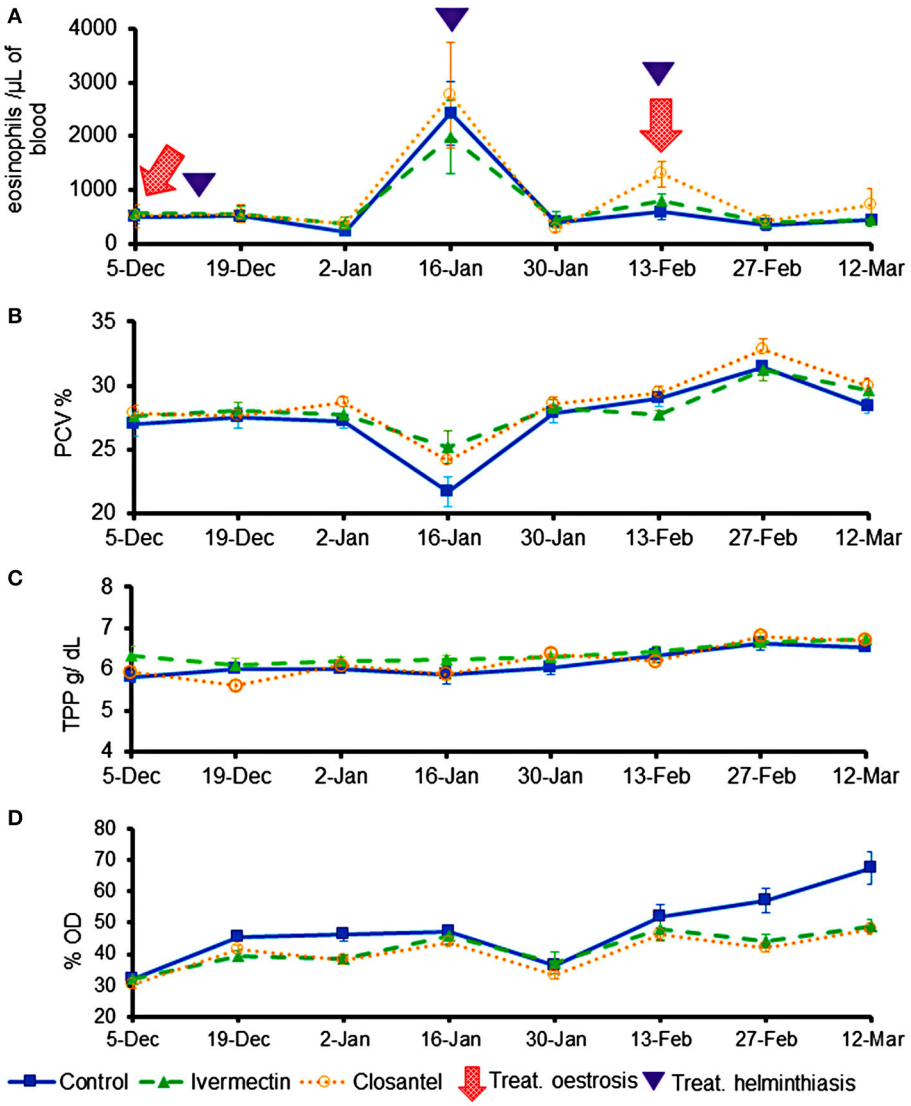


**FIGURE 4 |** Averages (±standard error) of eosinophils (cells/µL) **(A)**, packed cell volume (%) **(B)**, total plasma protein (g/dL) **(C)**, and optical density (%OD) of IgG anti-*O. ovis* L2 **(D)** of lambs treated with ivermectin or closantel. The moments of treatments against oestrosis (with ivermectin or closantel) are indicated by arrow and treatments against helminthiasis (with a combination of levamisole + albendazole + monepantel) are indicated by triangle.

oestrosis, the major symptoms of infestation (nasal discharge and frequent sneezing) are immune-mediated depending on acquisition of an immune response against the parasite (6, 29). Infestation induces a great recruitment of inflammatory cells such as mast cells and eosinophils, to site of host-parasite interaction and also increases the immunoglobulin production (29).

If the trial had a longer duration, perhaps the typical clinical signs would occur, because the ability of sheep to become resistant to oestrosis is limited, different from what usually happens in infection by helminths (24). Also drew attention the fact that the treatment against oestrosis did not influence the weight gain of the animals, demonstrating the benign character of the disease in lambs. The treatment with anthelminthics, including in those lambs from control group, avoided the interference of helminthiasis in the body weight gain of the lambs, which were around 190 g per day, within the expected for grass fed male lambs supplemented with concentrate (30). However, more studies are necessary to evaluate the influence of oestrosis on sheep productivity in our environmental conditions, especially in older animals continuously exposed to the parasitism.

Lopes et al. (31) reported a case of poisoning by closantel in three animals from a group of 15 sheep treated with closantel twice with interval of 28 days. These lambs showed apathy, anorexia, diarrhea and blindness after the second treatment

with 7.5 mg/kg orally. In our trial, lambs did not show any adverse effects post treatment with closantel or ivermectin. Some studies have shown that well-nourished animal, which was the case of our lambs, has less chances of poisoning because the closantel binds to plasmatic proteins, mainly albumin and healthy animals tend to have higher plasmatic protein level that reduce the amount of drug available in tissues (32, 33).

There was an increase in EPG means and a reduction in PCV values caused by *Haemonchus* spp. in all groups on 16th January 2020. After the anthelmintic treatment, PCV means increased and remained within the reference interval for sheep until the end of the study. Concomitantly, it was observed a significant increase in blood eosinophils counts on 16th January. Eosinophilia has been reported as one of the defense mechanisms against the gastrointestinal nematode parasitism (34, 35) and against *O. ovis* infestation (36).

While IgG-anti *O. ovis* means remained low in treated groups, the control lambs showed an increase in averages at the end of the trial, thus indicating that the animals were responding to parasite infestation. Previous studies demonstrated that detection of *O. ovis* antibodies in sheep is a valid diagnosis technique (24, 37–39).

## CONCLUSIONS

Closantel oral drench at a dose rate 10 mg/kg and ivermectin subcutaneously at 0.2 mg/kg remain effective in the prophylaxis of oestrosis. These drugs are effective at the same concentrations used in the previous studies and there is no evidence of increasing trends of resistance. At the early stage of infestation, *O. ovis* parasitism did not cause a negative impact on the performance of lambs.

## DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author/s.

## ETHICS STATEMENT

The animal study was reviewed and approved by Ethics Committee on Animal Use (FMVZ/UNESP Protocol Number 0159/2019).

## AUTHOR CONTRIBUTIONS

HB performed all experiment, collected and analyzed the data, and completed the manuscript preparation. JL, ACA, and GF helped in the collection of data. AFA, JL, ACA, and MA participated in its design and coordination and helped to interpret the results. All authors interpreted the results and substantively revised the manuscript, read, and approved the final manuscript.

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