

Punica granatum and amygdalin extracts plus cobalamin combined with albendazole reduce larval burden and myositis in experimental trichinosis

Extratos de *Punica granatum* e amígdalina mais cobalamina combinado com albendazol reduzem a carga larvar e a miosite na triquinose experimental

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Abstract

Trichinellosis is a zoonosis results from eating raw or semi-cooked meat of infected animals. Medicinal plants have been used lately as alternatives and/or combined therapies to resolve some drawbacks of the current regimens. This work analyzed the effect of albendazole monotherapy on *Trichinella spiralis* experimental infection (group A), in comparison to *P. granatum* and amygdalin extracts +cobalamin (group B), plus its combination with albendazole (group C). The study revealed that the extracts alone or combined with albendazole had an inferior effect to albendazole monotherapy regarding number of adult worms (40.83 ±3.82, 18.67 ±1.86 and 16.83 ±2.32, respectively). However, their effect was more obvious in muscle phase combined with albendazole, achieving the lower number of larvae/mL tissue homogenate (22.33 ±3.27 in comparison to 39.67 ±2.58 achieved by albendazole monotherapy). The extracts exerted a significant immunomodulatory effect by reducing the local CD4+ expression in the intestine as well as in muscle phase (1.15 ±0.25 and 3.80 ±0.65 in comparison to 4.97 ±0.37 and 12.20 ±0.87 with albendazole monotherapy, respectively). So, these extracts improved the therapeutic efficacy of albendazole, specifically in muscle phase and counteracted the inflammatory reaction caused by albendazole monotherapy, thus extensively alleviating the resulting myositis.

Keywords: Trichinosis, *Punica grantaum*, amygdalin, cobalamin, albendazole, muscle phase.

Resumo

Trichinellosis é uma zoonose resultante da ingestão de carne crua ou semicozida de animais infectados. As plantas medicinais têm sido usadas, ultimamente, como alternativas e/ou terapias combinadas, para resolver algumas desvantagens dos regimes atuais. Este trabalho analisou o efeito da monoterapia albendazole na infecção experimental por *Trichinella spiralis* (grupo A), em comparação com extratos de *P. granatum* e amígdalina +cobalamina (grupo B), além de sua combinação com albendazol (grupo C). O estudo revelou que os extratos sozinho ou combinado com albendazol teve efeito inferior à monoterapia albendazol em relação ao número de vermes adultos (40,83 ±3,82, 18,67 ±1,86 e 16,83 ±2,32, respectivamente). No entanto, seu efeito foi mais óbvio na fase muscular combinado com o albendazol, alcançando o menor número de larvas/mL homogeneizado de

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tecido ($22,33 \pm 3,27$ em comparação com $39,67 \pm 2,58$ obtidos pela monoterapia albendazol). Os extratos exerceram um efeito imunomodulatório significativo, ao reduzir a expressão local CD4⁺ no intestino, bem como na fase muscular ($1,15 \pm 0,25$ e $3,80 \pm 0,65$ em comparação com $4,97 \pm 0,37$ e $12,20 \pm 0,87$ com monoterapia albendazol, respectivamente). Assim, esses extratos melhoraram a eficácia terapêutica do albendazol, especificamente na fase muscular e neutralizaram a reação inflamatória causada pela monoterapia albendazol, aliviando extensivamente a miosite resultante.

Palavras-chave: Trichinose, *Punica grantaum*, amígdalina, cobalamina, albendazol, fase muscular.

Introduction

Trichinellosis or trichinosis is an emerging and reemerging zoonotic disease in many countries. Transmission occurs through consumption of the specific nematode larvae in raw or semi-raw meat and meat-derived products of infected animals, most commonly domestic and wild swine (Bai et al., 2017; Conlan et al., 2014). While infection can develop in a broad variety of mammalian hosts, the different cultural eating habits represent the main risk factor for human infections (Pozio, 2007; Gottstein et al., 2009).

Trichinella spiralis, a member of the encapsulated clade, is the most characterized species in the *Trichinella* genus, being of high prevalence and an important cause of human infections. Moreover, it is readily infective for laboratory animals; providing valuable models to understand various aspects of the infection (Pozio & Zarlenga, 2013; Sofronic-Milosavljevic et al., 2015). All developmental stages of the parasite are found in the same host. *T. spiralis* infection starts with a brief intestinal phase, where the infective larvae (L1) mature into adult worms which in turn release the migrating newborn larvae (NBL). During this phase a mixed immune response is induced with initial Th1 control followed by a protective Th2 predominance resulting in the expulsion of adult worms (Ilic et al., 2012). The muscular phase ensues with NBL reaching skeletal muscle, where they develop into L1, and modify the muscle cell architecture in order to survive in the host for several months up to years. The chronic stimulation of the immune system through the parasite excretory-secretory products (ES) is thought to activate regulatory elements; such as regulatory T cells (T-regs) which were observed near the infected cells, furthermore, TGF- β and IL-10 are documented to promote persistence of infection (Ilic et al., 2012; Sun et al., 2019).

Trichinellosis is a serious disease with no satisfactory treatment and the clinical course can range from an asymptomatic form to being fatal. Benzimidazoles: albendazole and mebendazole, are the principal anthelmintics used in treatment of this infection (Gottstein et al., 2009). Both drugs demonstrate weak activity against encapsulated larvae. The effectiveness of therapy depends on the time of administration, being highly effective within 1 week after the infection, during the intestinal phase. The later the drug is administered the greater the probability that larvae will remain viable for years in human hosts despite treatment (Dupouy-Camet et al., 2002; Gottstein et al., 2009). This downside of the current treatment highlights the need for novel effective and safe anti-trichinellosis drugs (Shalaby et al., 2010; Yadav & Temjenmongla, 2012).

The use of medicinal plants has gained increasing attention lately, with various plant extracts being the subject of many pharmacological studies. *Punica granatum*, commonly known as the pomegranate, is a fruit with rich ethnomedical applications frequently grown in the Mediterranean region. Described as the nature's power fruit, *P. granatum* extracts were reported to have potent antioxidant and anti-inflammatory properties. In addition they were introduced as potential anti-microbial and anthelmintic agents (Al-Megrin, 2017; Dkhil, 2013). Another naturally occurring compound, amygdalin, referred to as vitamin B17, is a cyanogenic glycoside found in fruit kernels of members of the Rosaceae family such as almonds, apricots and peaches. Amygdalin and its semi-synthetic derivative, laetrile, have been labelled as controversial antineoplastic agents over the past century (Juengel et al., 2016; Saleem et al., 2018). Amygdalin is reported to be involved in the regulation of apoptosis, adhesion molecules and cell cycle elements in addition to its inhibitory effect on the expression of inflammatory cytokines. Additionally, amygdalin was proposed to have antifibrotic effect, and a stimulatory impact on muscle growth (Liczbiński & Bukowska, 2018). The present study aimed at investigating the effect of pomegranate *P. granatum* and amygdalin extracts on both the intestinal and muscular phases of *T. spiralis* infection in mice in comparison with albendazole monotherapy as a reference drug.

Materials and methods

Experimental animals and investigational infection

Female mice BALB/c mice (25-30 g) aged six-eight weeks were purchased from the Animal Center of Theodor Bilharz research Institute "TBRI", following the institutional code of ethics for animal research.

The experiment was approved by ethical committee at Theodor Bilharz Research Institute (Approval code: 2020/FWA00010609).

Mice were maintained in this study in a controlled environment (12:12 h light/dark photocycle with a temperature of 22 ± 2 °C and a relative humidity of 55%). The mice were infected orally with about 300 larvae per mouse. The strain of *T. spiralis* was originally isolated from the diaphragms of infected mice reared in TBRI, Cairo. The infected diaphragms were minced and digested in 1% pepsin-hydrochloride. After overnight incubation at 37°C, larvae were collected using the sedimentation technique, washed in physiological saline (0.85%) several times, and the number of larvae per mL homogenate was counted and prepared for animal infection (Dunn & Wright, 1985).

Study groups

Infected mice were classified into three main groups (added to a control infected non-treated group/each phase); A, B, and C, each subdivided into two subgroups (I and II) intestinal and muscular phases, 10 mice per subgroup (Cui et al., 2008; Zhang et al., 2010). Group A; infected treated with full dose albendazole, Group B; *P. granatum* + amygdalin + cobalamin and Group C; half dose albendazole + *P. granatum* + amygdalin + cobalamin. Assessment of results was done using parasitological examination by counting adult worms in the intestine on the 7th day post infection (dpi), and larval count in the muscles on the 45th dpi.

Drugs and herbal treatment

Reference drug

Albendazole was supplied as suspension, 20 mg/mL, from the Egyptian International Pharmaceutical Industries Co. in a dose 50 mg/kg. For each mouse, 20 mg/mL was prepared and given orally (50 mg/kg) from the 2nd dpi for 3 successive days.

Plant supplies, extracts and dose preparation

In order to prepare fresh extracts, fresh pomegranates (500 g) were obtained from a public market. Peeling of the pomegranate was performed to be separate the peel from the flesh. The peels were then oven dried at 33°C for 7 days. Following this the dried peels were powdered in an electric grinder and stored in plastic bags at 4 °C for the next step. In concordance with Moneim et al. (2011), powdered plant materials were extracted with 70% ethanol at room temperature by maceration and then filtered. The filtrates were concentrated under vacuum using a rotary evaporator. The obtained solvent-free residue of the plant extract was stored at 4°C for subsequent preparation of the required doses. Plant extracts were freshly prepared before usage by suspending 1 g of each extract in 50 mL 3% tween 80 dissolved in 0.9% saline and 60mg/kg/day was given/mouse orally via gavage at the day of infection and continued every day till the end of the experiment.

Amygdalin

In concordance with Moslehi et al. (2018), the purified powder form of the supplement was given orally 50 mg/kg, diluted in 0.2 mL 0.9% saline solution, LKT Laboratories, Inc. (St. Paul, MN, USA) and the dose was given daily until the end of the experiment.

Parasitological analysis

Adult parasites were collected and counted in intestinal phase subgroups. In concordance with (Wakelin & Lloyd, 1976), following mice euthanasia the small intestines were isolated and washed in physiological saline, then divided into 1cm segments. Intestinal segments were incubated in physiological saline at 37°C for 2 hours, and then washed 3 times, taking

into account that all the solutions from the previous step were collected to be centrifuged for 3 minutes at 2000 rpm. The number of adults in the sediment was counted under an inverted microscope using low power magnification x100. Larvae were collected and counted from muscular phase subgroups. Muscle tissue was digested as mentioned above in 1% pepsin/1% HCl according to (Dunn & Wright, 1985), larvae were recovered and counted under an inverted microscope.

Histopathological examination

Intestinal specimens (1 cm from the small intestine at the junction of the proximal 1/3 and distal 2/3) were taken from mice sacrificed on the 7th dpi, while skeletal muscle specimens from the hind legs were taken from mice sacrificed on the 45th dpi. These specimens were fixed in 10% formalin, dehydrated, cleared, and then embedded in paraffin blocks. Paraffin sections were taken of 5 µm thickness, stained with haematoxylin and eosin (Hx & E) and examined microscopically.

Immunohistochemistry

Automatic immunohistochemical staining was performed, applying a polymer-based detection system (DakoEnVision™ FLEX, K8000). Sections (5µm) from paraffin-embedded specimens were deparaffinized in xylene and then rehydrated in descending grades of alcohol. To block endogenous peroxidase activity, the sections were incubated in hydrogen peroxide 3% for 5 minutes. The sections were then washed twice in PBS for 5 minutes each. For antigen retrieval, tissue sections were retained in 0.01 mol/l citrate buffer (pH 6) in a water bath (Dako PT link) to be followed by incubation with the primary antibody murine anti-human CD4+ monoclonal antibodies (Dako, USA) at room temperature for one hour, then the sections were washed 3 times in PBS/ each for 15 minutes. Then, biotinylated goat anti-polyvalent secondary antibody and streptavidin peroxidase enzyme were added consecutively for 10 min and then washed in PBS. The peroxidase activity was visualized with diaminobenzidine (DAB) chromogen applied for 5 min (Ramos-Vara & Miller, 2014). Tissue sections were then rinsed, counterstained with haematoxylin, dehydrated, cleared in xylene, and mounted by DPX. Sections from the tonsils were used as positive control in concordance with the manufacturer's recommendation. Negative controls were handled as in previous steps except for the use of the primary antibody. T lymphocytes expressing membranous or cytoplasmic brownish immunostaining for CD4+ were considered positive.

Image analysis by real-time quantitative morphocytometry

The specific analysis was performed using the Leica Qwin 500 Image Analyzer (LEICA Imaging Systems Ltd., Cambridge, England). Optical density (OD) and area percentage was automatically calculated in 10 fields for CD4+ marker on a real-time image from the microscope that was connected to a video monitor.

Statistical analysis

Results were presented as mean and standard deviation (SD). Data were analyzed by IBM SPSS software version 28.0 (Armonk, NY: IBM Corp). One way analysis of variance on ranks (Kruskal-Wallis) test and Dunn's-Bonferroni post hoc test were done for multiple pairwise comparisons between the study groups. P-values < 0.05 were considered statistically significant.

Results

Effect of *P. granatum* and amygdalin extracts on number of adult and larval stages of *Trichinella spiralis* infection, in comparison to albendazole:

Superior effect of albendazole in eliminating intestinal adult stages

All the treated groups showed a reduction in the number of adult worms in comparison to the control group. The highest reduction was observed in the albendazole group (A), followed by the combined group (C) and lastly the *P. granatum* and amygdalin+ cobalamin group (B). Pairwise, comparison between the study groups revealed statistically significant difference between the control group, and groups A and C, respectively (Table 1, Figure 1).

Combined therapy significantly decreases larval stage

The number of larvae was reduced in all of the treated groups. The highest diminution in the count larval stages was detected in the combined group (C), followed by the albendazole group (A), then *P. granatum* and amygdalin+cobalamin group (B). Statistically significant differences were demonstrated between the control group, and groups A and C, respectively, and between groups B and C (Table 1, Figure 1).

Table 1. Effect of different treatments on parasitic stages and CD4 cells in intestinal and muscle tissues in the study groups.

	Intestinal (Subgroup I)		Muscle (Subgroup II)	
	Number of adults mean \pm SD/mL intestinal fluid	CD4 ⁺ Area % mean \pm SD	Number of larvae mean \pm SD/mL homogenate	CD4 ⁺ Area % mean \pm SD
Control group	198.33 \pm 15.12	2.95 \pm 0.58	399.67 \pm 16.35	7.63 \pm 0.55
Group A	16.83 \pm 2.32	4.97 \pm 0.37	39.67 \pm 2.58	12.20 \pm 0.87
Group B	40.83 \pm 3.82	1.15 \pm 0.25	68.00 \pm 6.51	3.80 \pm 0.65
Group C	18.67 \pm 1.86	3.08 \pm 0.66	22.33 \pm 3.27	2.47 \pm 0.53

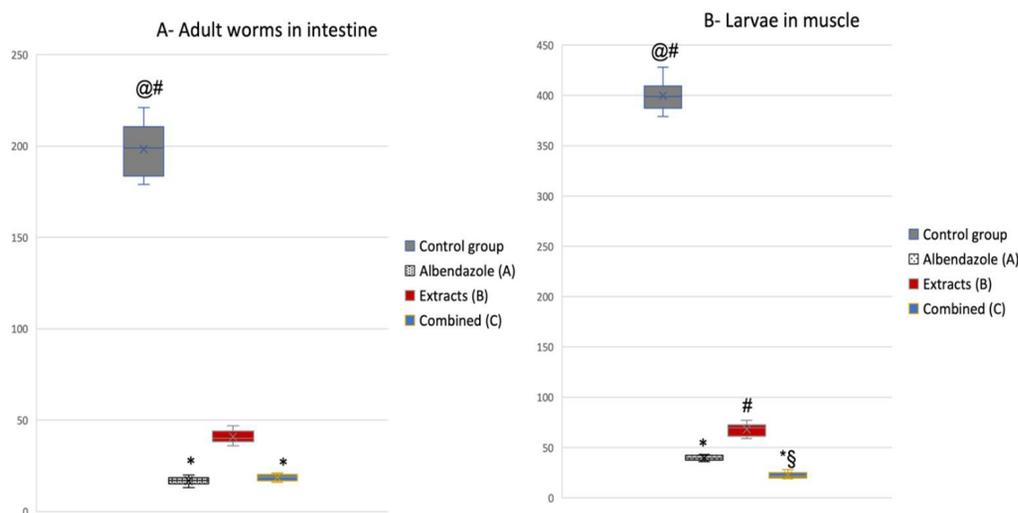


Figure 1. A boxplot representing the number of parasitic stages in different study groups. Statistical significance between groups, determined by Dunn's-Bonferroni post hoc test, is presented as: *, @, §, # in relation to the control; albendazole; extracts; and combined groups, respectively.

Effect of *P. granatum* and amygdalin extracts on histopathological changes of *Trichinella spiralis* infection

P. granatum and amygdalin extracts significantly reduce intestinal inflammatory reaction

By histopathological examination, intestinal tissue of the control group exhibited a severe inflammation with necrotic severely inflamed villi, in addition to vacuolation indicating the degenerative effect of the parasitic stages. Compared to the control group, the albendazole group (A) displayed a relatively improved intestinal epithelium with epithelial vacuolation in some areas and numerous inflammatory cells. In contrast, intestinal tissue in *P. granatum* and amygdalin+cobalamin and the combined groups (B and C) appeared almost healthy with normal villi and a mild inflammatory reaction (Figure 2).

P. granatum and amygdalin extracts significantly diminish local inflammatory reaction within infected muscle tissue

Histopathological examination of muscle tissue in the control group revealed intact larvae with surrounding moderate inflammatory response. In the albendazole group (A), muscle tissue demonstrated a severe inflammatory reaction enclosing the cystic parasitic lesions. While in the *P. granatum*/Amygdalin+cobalamin group (B), the larvae were seen intact with adjacent mild inflammatory response in the muscle tissue. Degenerated larvae with a mild inflammatory reaction were observed in the combined group (C) (Figure 3).

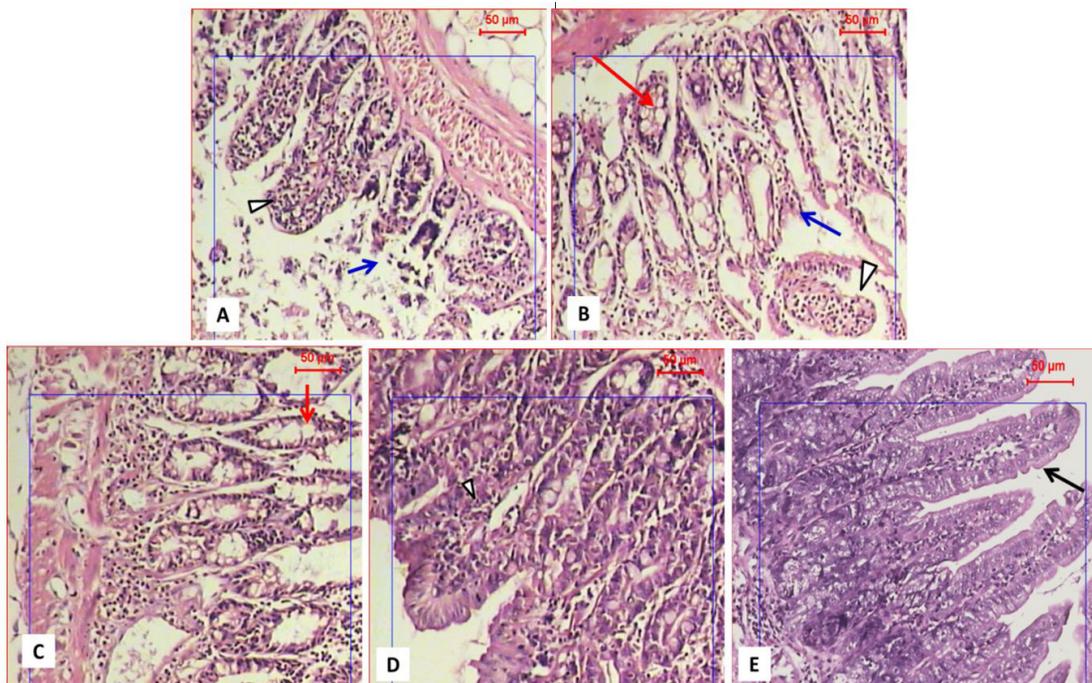


Figure 2. Histopathological examination of intestinal epithelium in different study groups. (A & B) control groups showing shortening and necrosis (blue arrow), severe inflammation in villi (arrowhead), and vacuolation (red arrow); (C) albendazole treated group showing improved epithelium with sporadic vacuolation (red arrow); (D) natural remedies treated group demonstrating almost normal epithelium with mild inflammation (arrowhead). E; combined therapy group revealing normal length of the villi (black arrow) with mild inflammatory reaction (Hx & E stain x200).

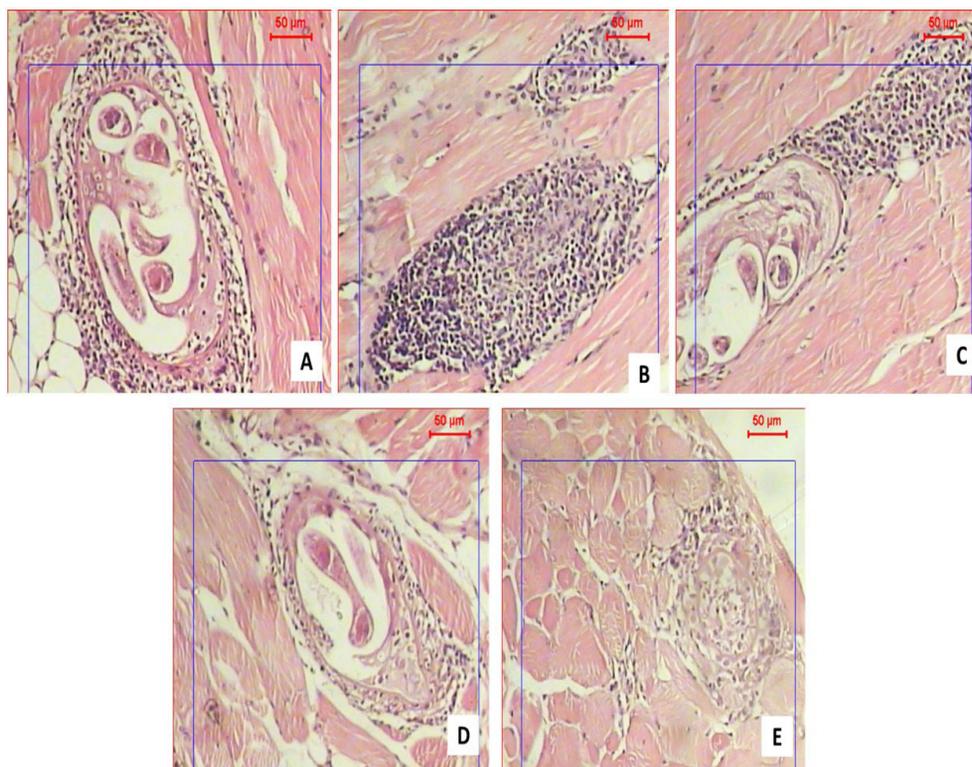


Figure 3. Cystic lesions in infected muscles of different study groups. (A) infected non treated control group showing intact larvae surrounded by moderate inflammatory response; (B & C) albendazole treated group showing severe inflammatory response nearly encompassing the cystic parasitic lesions; (D) natural remedies treated group showing mild inflammation with apparently intact larva; (E) combined therapy group showing degenerated larva enclosed in mild inflammatory reaction (Hx & E stain x200).

Effect of *P. granatum* and amygdalin extracts on immunohistochemical changes of *Trichinella spiralis* infection

P. granatum/amygdalin extracts significantly downgrade local expression of CD4+ cells in infected intestinal tissue

Compared to the control group, albendazole group (A) demonstrated an increase in the expression of CD4+ cells, while *P. granatum* and amygdalin+ cobalamin group (B) showed a reduction in CD4+ expression. The combined group (C) revealed minimal difference from the control group. Pairwise comparison revealed statistically significant difference between group A and B (Table 1, Figures 4 & 5).

P. granatum and amygdalin extracts significantly lower local expression of CD4+ cells in infected muscle tissue

In comparison to the control group, both the combined group (C) and the *P. granatum* and amygdalin+ cobalamin group (B) showed a decrease in the expression of CD4+ cells, while the albendazole group (A) showed an increased expression of CD4+ in muscle tissue. Pairwise comparison analysis revealed statistically significant differences between the albendazole group (A) and groups B and C, respectively, and between the control and group C (Table 1, Figures 4, 5).

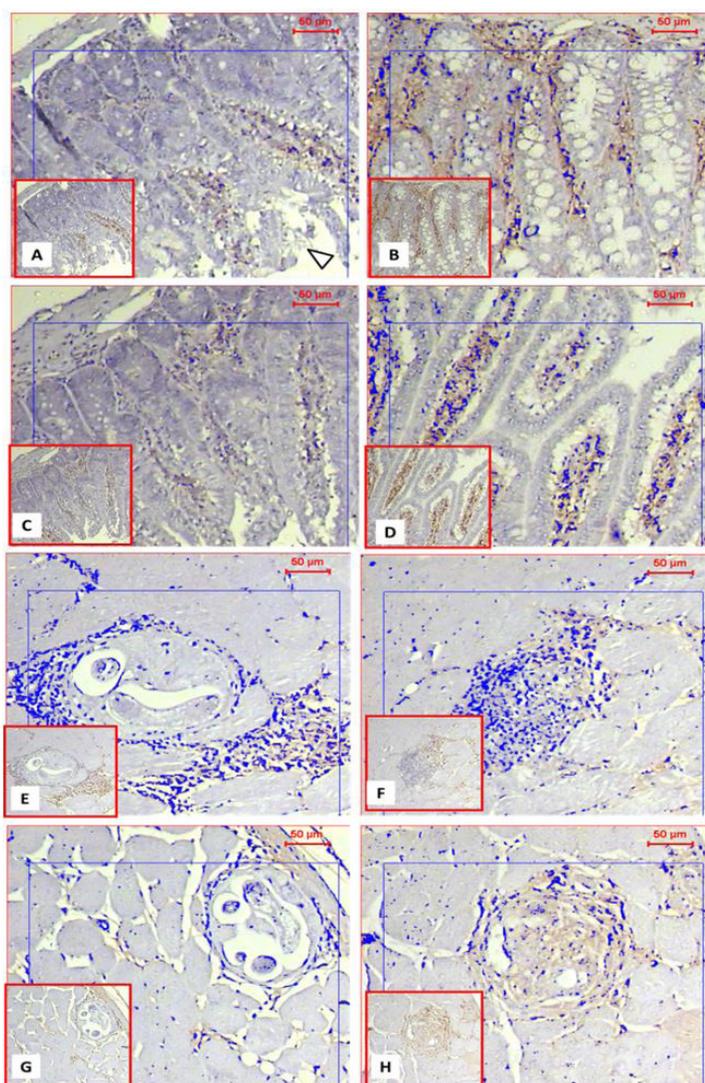


Figure 4. A to D; Local expression of CD4 within infected tissues of different groups. A; infected non treated group shows necrotic areas (arrow head) with moderate expression. Minimum expression appears within group C; group treated with natural remedies, while maximum expression exists in group B; treated with full dose Albendazole. Moderate expression be seen in group D; group treated with combined therapy (half Albendazole plus full dose of natural remedies). E to H; Photographs show local expression of CD4 within infected muscles of different groups. Minimum expression appears within group G; group treated with natural remedies, while maximum expression exists in group E; infected non treated and group F; treated with full dose Albendazole. Moderate expression be seen in group H; group treated with combined therapy (half Albendazole plus full dose of natural remedies) (IHC x200).

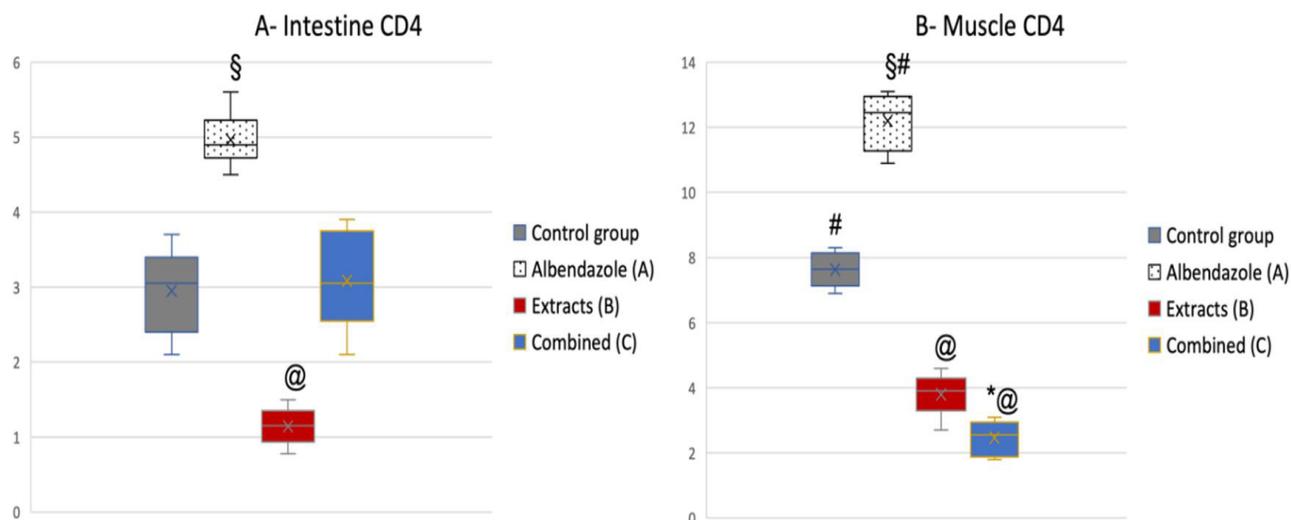


Figure 5. A boxplot representing the expression of CD4 within different study groups. Statistical significance between groups, determined by Dunn's-Bonferroni post hoc test, is presented as: *, @, §, # in relation to the control; albendazole; extracts; and combined groups, respectively.

Discussion

Medical treatment of trichinellosis has been contested and challenged by the lack of a completely effective drug of choice. In order to achieve complete effectiveness, such a drug must act on all developmental stages of the parasite located in body organs (Dupouy-Camet et al., 2002). Since its introduction, albendazole has been pivotal for the treatment of many parasitic diseases; acting through various mechanisms, including inhibition of tubulin polymerization and fumarate reductase (Movahedi et al., 2017). Although being a broad spectrum anthelmintic, the low water solubility, and consequently poor bioavailability are limiting factors in albendazole usage (Hettiarachchi et al., 2016). Moreover, resistance to benzimidazoles, including albendazole, is increasingly reported globally (Dilks et al., 2020; Srivastava & Misra-Bhattacharya, 2015). Over the years, plants have been key players in the development of new drugs (Yones et al., 2016). Plant extracts are a rich source of a large variety of biochemical active compounds which can impact several therapeutic targets (Anand et al., 2019; Ullah et al., 2020).

In the present work, we explored the therapeutic potentials of 2 medicinal plant extracts; *P. granatum* peel extract and amygdalin+ cobalamin *in vivo*, during both the intestinal phase and muscular phase of trichinellosis in comparison or/and in combination with albendazole monotherapy. Our results showed that *P. granatum* peel extract and amygdalin+ cobalamin reduced the number of parasites in intestine and muscle, but their effect was inferior to albendazole monotherapy. However, when these 2 plant extracts were used in combination with albendazole treatment, they achieved a significantly higher effect in muscle phase with more reduction in the larval count than albendazole monotherapy. Yet, the superior effect of plant extracts alone on reducing inflammatory response was clearly evident by the significant down regulation of CD4 within both intestinal and muscle tissues, indicating their potent anti-inflammatory effect.

Among various solvent extracts of *P. granatum* peel, ethanol extract was found to contain the highest antioxidant capacity. Phenols, tannins and flavonoids were noted as main active compounds in the extract that may confer antimicrobial, anthelmintic effects in addition to their antioxidant activity (Barathikannan et al., 2016; Castagna et al., 2020). Since it was first isolated from bitter almonds in 1830s, amygdalin exhibited an expansive range of therapeutic properties such as anti-inflammatory, antiangiogenic, antipyretic, antitussive and analgesic in treating several conditions including leprosy, migraine, hypertension and diabetes. Nevertheless, its benefit and use as an anti-cancer agent remains debatable (Jaswal et al., 2018; Saleem et al., 2018). Upon oral ingestion, amygdalin is hydrolyzed into glucose, benzaldehyde and hydrogen cyanide which can precipitate cyanide toxicity; yet the toxic dose is not clearly defined (Ayaz et al., 2020; Tříšková & Rudá-Kučerová, 2019). Vitamin B12 (cobalamin) has been proposed as a safe and effective antidote, it binds to cyanide forming cyanocobalamin which is excreted safely via the kidneys (Dang et al., 2017; Jaswal et al., 2018).

Several studies have examined the effect of *P. granatum* peel extract on various intestinal helminths, and to a lesser extent on extra-intestinal helminths. For instance, Dkhal (2013) used a methanol extract of *P. granatum* peel to examine its anticoccidial and anthelmintic effects *in vivo* and *in vitro*, respectively. *P. granatum* peel was found to reduce *Eimeria papillate* output, improved the inflammation and vacuolation in jejunal epithelium *in vivo* and caused paralysis and death of adult *Allolobophora caliginosa* *in vitro*. While, (Abdel Aziz et al., 2018) study on *Ascaridia galli* showed that *P. granatum* peel aqueous extract had a closely similar effect to fenbendazole *in vitro* and a lower effect *in vivo*. Similarly, Castagna et al. (2020) highlighted the potential anthelmintic effect of *P. granatum* aqueous macerate, demonstrating an *in vitro* ovicidal activity against sheep intestinal nematodes. For extra-intestinal helminths, *P. granatum* peel and leaf extracts were shown to cause death of *Schistosoma mansoni* worms and schistosomules, both *in vivo* and *in vitro* (Fahmy et al., 2009). Recently, in their work on muscular phase of trichinellosis, Hafez et al. (2020) reported that *P. granatum* extract in methanol demonstrated a promising therapeutic effect, and suggested its use as an adjuvant with radiation-attenuated vaccine. Despite numerous studies having investigated the therapeutic properties of amygdalin, reports on its anti-parasitic effect are extremely limited.

Trichinella infection elicits an adaptive immune response where CD4+ T cells play a key role (Wang et al., 2020). Our findings revealed that *P. granatum* peel extract and amygdalin+ cobalamin treated group decreased local expression of CD4+ cells in the 2 phases of trichinellosis, in contrast to albendazole monotherapy which increased CD4+ expression in both phases. As for the combined group, the effect of *P. granatum* peel extract and amygdalin counteracted the effect of albendazole resulting in almost no change in CD4+ expression in the intestine, compared to the control group, while in muscle phase the resulting combined effect decreased the local expression of CD4+ cells.

Upon antigenic stimulation, activated CD4+ cells can differentiate into one of the following subset populations; Th1, Th2, Th17 or Treg. This differentiation is directed mainly by the cytokines present in the microenvironment. The relative proportions of each subset can change according to the course of infection (Ilic et al. 2012; Mousset et al., 2019; Sun et al., 2019). Early in the intestinal phase of trichinellosis, a strong CD4+ response of mixed Th1/Th2 subsets is induced in intestinal mucosa with predominance of Th2 cytokine profile (Ding et al., 2017). The resulting immunopathological alterations include mastocytosis and its degranulation, breaching the epithelial tight junctions and increased luminal fluid. Recently, TGF- β was found to regulate CD4+ polarization into Th17 thereby enhancing intestinal muscle hypercontractility and propulsive activity leading to worm expulsion (Ilic et al., 2012; Steel et al., 2019). In muscle tissue, a local inflammatory response is formed around infected cells including numerous CD4+ cells with significant Th2, this response is influenced by the intestinal phase of the infection as it enhances myositis (Bruschi & Chiumiento, 2011; Fabre et al., 2009). Parasite antigens induce Treg expression when encapsulation is formed, as a mechanism of immune evasion to ensure its survival within a regulatory microenvironment with high levels of TGF- β and IL-10 (Ilic et al., 2012; Sun et al., 2019; Wang et al., 2020).

Punica granatum peel extract and amygdalin can interfere with *Trichinella* induced immune response through various immunomodulatory mechanisms. Both medicinal plants were found to reduce liver infiltration with CD4+ cells in autoimmune hepatitis (Elsaed, 2019; Wang et al., 2018). Additionally, amygdalin was also found to suppress TGF- β in the context of fibrosis (Guo et al., 2013; Luo et al., 2016) and to inhibit proinflammatory cytokines TNF- α and IL-1 β expression in arthritis (Hwang et al., 2008).

In conclusion, the present study revealed that *P. granatum* and amygdalin, alone and combined with albendazole had an inferior effect to albendazole alone regarding adults' worm burden, yet they evidently amended the intestinal pathology. However, their effect was more evident in muscle phase when combined with albendazole, achieving the highest reduction in larvae. In addition, *P. granatum* and amygdalin exerted a significant immunomodulatory effect in the form of a reduction of local CD4+ expression in the intestines as well as in muscle. These findings suggest that *P. granatum* and amygdalin can improve the therapeutic efficacy of albendazole specifically in muscle phase. Further detailed analysis is recommended regarding the mechanism of action of medicinal plants and their effect on different CD4+ subsets to benefit from their medicinal potentials.

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