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THERAPEUTIC AND IMMUNOMODULATORY EFFECTS OF *ACACIA NILOTICA* ON EXPERIMENTALLY *TRICHINELLA SPIRALIS* INFECTED MICE

By

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Abstract

Trichinosis is a common infection that spreads primarily through food. None of the therapies, including albendazole (ALZ), have significant effectiveness against *Trichinella spiralis* larvae encapsulated in muscle, so there is a need to find new, effective trichinosis medications. *Acacia nilotica* (*A. nilotica*) is linked to many biological activities as anti-inflammatory, anti-microbial and anti-carcinogenic activities. This study determined the efficacy of *A. nilotica* alone or in combination with ALZ for treating intestinal and muscular trichinosis.

In this study, fifty mice were divided into five groups, non-infected control group, *T. spiralis* infected control group, *A. nilotica*-treated group, ALZ treated group and finally *Acacia*-ALZ treated group. Adult count, larval load, intestinal and muscular histology, COX2 expression, and measurement of CD4+ & CD8+ lymphocytes percentages were investigated.

The results showed that mice received the combined therapy achieved the greatest outcomes in terms of the criteria employed. ALZ was better than *Acacia* for intestinal trichinosis, although *Acacia* was better for muscular trichinosis.

Keywords: *Trichinella spiralis*, *Acacia nilotica*, Albendazole; CD4+; CD8+; COX2.

Introduction

Trichinella spiralis is an extremely harmful parasite that can lead to chronic skeletal muscle infection in its host (Abou Rayia *et al*, 2023). A considerable number of human infections are reported because of consuming improperly cooked raw pork flesh that harbors the infective larvae (Albogami, 2023).

Human infection with *T. spiralis* progresses through three stages: the intestinal, migratory, and muscular phases. Once the parasite enters the host's cells, muscle inflammation results (Eissa *et al*, 2022). Clinical signs and symptoms can differ depending on the amount of live larvae swallowed, the host's age, gender, health status, and cultural background. The adult worms in small intestine are commonly associated with nonspecific intestinal symptoms. The brief intestinal phase ends after the larvae are deposited and have gained access to the circulation, and the parenteral phase begins. Myalgia and periorbital edema are common symptoms of parenteral phase on larvae arrival in skeletal muscles (Sharaf-El-Deen *et al*, 2023).

Reducing muscle damage is the primary goal of trichinosis treatment. The primary antiparasitic medications used to treat trichinosis are benzothiazole derivatives, specifically ALZ and mebendazole. By interacting with cuticular tubulin, ALZ causes structural harm to the parasite. Despite the considerable efficacy of ALZ against adult worms, it is less effective at killing encysted larvae in skeletal muscles (Hamed *et al*, 2022). One more drawback of ALZ with limited water solubility, which impedes its absorption (Nada *et al*, 2018).

The increasing threat of anthelmintic resistance and diminishing efficacy against encapsulated parasite stages have led to a search for novel anthelminthics derived from plants, especially those used in traditional medicine in many countries (Shalaan *et al*, 2023). Medicinal herbs hold great promise

as potential new treatments because of the wide variety of bioactive chemicals found in them that could target parasites (Elazab and Arafa, 2022).

Acacia nilotica is a plant that belongs to the family of Mimosaceae, grows in tropical and subtropical regions. It has various biological activities, including anti-inflammatory, antimicrobial, anti-platelet, and anti-carcinogenic activities (Muddathir *et al*, 2020). The phytochemical tests demonstrated that *A. nilotica* pod and leaves are possible sources of polyphenols with antioxidant activity (Sadiq *et al*, 2017). Different types of *Acacia* extracts were reported to have the ability to treat pneumonia, tonsillitis, dysentery, diarrhea, malaria, tuberculosis, and leprosy. Additionally, it has been recognized as a rich source of numerous active metabolites. These compounds have the potentiality to be used as lead compounds in developing new drugs (Sulaiman *et al*, 2021).

This study aimed to evaluate the anti-inflammatory properties of *A. nilotica* and its impact on experimentally infected *T. spiralis* mice. Parameters tested were adult count, larval load, intestinal and muscular histology, COX2 expression, and measurement of CD4+ and CD8+ lymphocytes percentages.

Material and Methods

Theodor Bilharz Research Institute (TBRI) and Department of Parasitology, Faculty of Medicine, Menoufia University served as the site of this experimental study.

Study design: The study was designed to assess *A. nilotica*, ALZ, and combined treatment in both intestinal and muscular trichinosis. The measures employed were adult count, larval load, intestinal and muscular histology, COX2 expression, and measurement of CD4+ and CD8+ lymphocytes percentages.

Mice and study groups: Fifty inbred pathogen-free male Swiss Albino mice (6-8w, ~ 20gm) free from all parasitic infections confirmed by stool examination were used. Mice were divided into five groups of ten mice each. GI: non-infected negative control, GII: *T. spiralis* infected positive control, GIII: *A. nilotica*-treated, GIV: ALZ-treated and GV: *Acacia*-ALZ treated.

Mice infection: mice belonging to groups II-V were infected with *T. spiralis* larvae in a dose of 200±10/mouse through an intraesophageal tube (Dunn and Wright, 1985).

Plant preparation and extraction: After removing the seeds from the dried pods of *A. nilotica*, the pods were rinsed with tap water and then with sterilized distilled water. After a few days of drying in an oven at 50°C, the pods were ground into a fine powder. The powder was then stored in a dark, airtight container until needed. One hundred g of the dried powder was transferred to a sterile glass vial, and then 500 ml of 80% methanol was added gradually. The container was sealed and shaken violently at least three times daily for 7 days. Next, to obtain a dry extract, the fluid was filtered through Whatman filter sheet No. 1; the filtrate was then separated and kept in an incubator at 45°C for up to seven days (Abdallah, 2016).

Treatment regimen: Mice of GIII received *Acacia* orally at a dose of 200 mg/kg/d for 21 days (Sulaiman *et al*, 2021), GIV mice received ALZ, at a dose of 50 mg/kg/d for five successive days (Li and Ko**,** 2012) and lastly GV received both drugs with the same doses as GIII and GVI. Medications were administered daily, on the first day of infection. *A. nilotica* cytotoxicity extract was assessed using a viability assay. A graphic plot of the dose-response curve for each concentration and the 50% inhibitory concentration (IC_{50} μg/ml) was calculated. IC_{50} is the extract's half-maximum inhibitory concentration to kill 50% of cancer cells in culture and to determine drug effectiveness. Dose was within safety margin (Diab *et al*, 2022).

Samples collection: From each group, five mice were euthanized on the seventh-day post-infection (dpi). Adult worms were then collected from the upper two-thirds (duodenum and jejunum) of the small intestine as follows: the small intestines were excised, longitudinally opened, rinsed with saline so-

lution, and divided into small segments. These segments were then placed in a phosphate-buffered saline (PBS) solution and incubated at a temperature of 37°C for 2hrs. Mature worms were gathered and enumerated by binocular microscope. A portion of intestinal tissue was fixed in 10% neutral buffered formaldehyde for histopathological and immunohistochemical analyses.

The remaining mice were sacrificed on the 35^{th} dpi, and muscles including the diaphragm, tongue, triceps, biceps, and quadriceps were dissected into three parts. The first part was digested using 1% pepsin and 1% concentrated HCL in distilled water for larval counting (Blair, 1983). The second part was fixed with 10% neutral buffered formaldehyde for histopathological and immunohistochemical analyses. For flow cytometry, the third part was used fresh.

Intestine and muscle histopathology: Paraffin blocks were prepared from intestinal tissue and skeletal muscle for each mouse of the five studied groups according to the conventional process and then stained with hematoxylin and eosin stain (Kessel, 1998). Small intestine samples were observed for inflammatory and goblet cells proliferation. Degeneration and interstitial infiltration were inspected in muscle sections. Degeneration of encysted larvae was identified when the larval structure was replaced with a homogenized acidophilic material.

The histopathological changes were graded as follows: no alterations were assigned a score of (–), mild changes were assigned a score of (+), moderate changes were assigned a score of (++), and severe modifications were assigned a score of (+++). In each tissue section, ten fields were examined under high-power magnification (× 400) to determine any histopathological alterations.

Immunohistochemical assay for COX2: An immunohistochemical technique to detect cyclooxygenase type 2 (COX2) expression in intestinal and muscle tissues was performed using anti-mouse antibodies for COX2 (Cat 35-8200, Thermo-Scientific, USA) following the instructions of the manufacturer. Sections were embedded in 3% hydrogen peroxide for ten minutes after being deparaffinized, rehydrated, and rinsed in tap water, then dipped in an antigen retrieval solution. Phosphate buffered solution (PBS), which acts as a blocking solution, was used to block nonspecific protein binding. For 2 hours sections were incubated with the diluted primary antibody and PBS at 1/100 dilution. 100 µl of streptavidin peroxidase was applied for 20 min and then washed with phosphate-buffered saline (PBS) for 5 min. Mayer's hematoxylin was utilized as a counterstain, and diamethoxylidine was added as chromogen. PBS was used, in the place of the main antibody, as a negative control.

The presence of brown stains either on cell membrane or membrane and cytoplasm indicated a positive result. By using histoscore (H-score), immunohistochemical staining for COX2 was graded, as 0 (not stained) to 1+, 2+, and 3+ multiplied by stained cells percent. Tissues were graded from 0 to 300 (Fraser *et al*, 2003)

Flow cytometry analysis: To identify the proportion of lymphocytes that are positive for CD4 & CD8 markers, single-cell suspensions were prepared from different muscle tissues of mice (Kumaraswami *et al*, 2020). The single-cell suspensions were adjusted to 1×10^{6}/ml. One hundred µl of cells were treated with 10µl of each of the mouse monoclonal fluorescein isothiocyanate (FITC) anti-CD8 alpha antibody (ab33786, Abcam, UK) and phycoerythrin (PE) anti-rat CD4 antibody (201507, Bio-Legend, USA). To inhibit FC receptors and non-specific protein-protein interactions, cells were incubated in PBS with 10% rat serum for 30 minutes on ice according to the manufacturer's instructions. Analysis was done using Accuri C6 becton Dickinson and the results obtained were subsequently processed using Accuri C6 software.

Statistical analysis: SPSS Version 23 (IBM Corp., Armonk, N.Y., USA) was utili-

zed to tabulate and analyze the data. The means, standard deviations, and ranges of the quantitative variables and the frequencies and percentages of the categorical variables were used for the presentation of data. On the normal distribution of quantitative data, a one-way analysis of variance (*ANOVA*) was used. For data with a non-normal distribution, the *Kruskal-Wallis* test was used. Finally, for categorical variables, chi-squared test was used. The post hoc test was conducted when there was a significant difference in the data. Statistical tests were deemed significant when $P<0.05$. The reduction percentages were calculated as follows: mean value (positive control group) – mean value (treated group)/ mean positive control ×100.

Ethical consideration: The protocol was approved by the Ethics Committee for Scientific Research at the Faculty of Medicine, Menoufia University, Egypt (IRB; number 2/ 2024 PARA 22). All mice experimental studies went with Declaration of Helsinki 2008.

Results

Adult count reduction was highest in mice given combined therapy (0.80±0.84; 99.2%). ALZ-treated mice (GIV) didn't show decrease in adult count (1.4±1.14; 98.6%), followed by GV. There was a significant difference between (GIV) and (GIII), with P < 0.001), or between GIII and GV (P <0.001) with (GIII) showed lowest adult count reduction (18.60±2.51; 80.9%).

The reduction of larval count was highest in GV given combined therapy (92.9 %). Both GIII and GV showed less larval count of 63.8% and 62.3% respectively.

Histopathological sections of small intestine of positive control showed distorted intestinal villi, which became short and broad, with dense inflammation. Moderate subepithelial inflammatory infiltration was in *Acacia*-treated mice (GIII). Mild inflammation in villi core and slight altered villous pattern were in ALZ treated mice (GIV). Combined treated mice treatment (GV) showed almost intact intestinal villi and cells randomly infiltrated inside a core of connective tissue with normal epithelial appearance**.** Combined therapy (GV) gave lowest degree of inflammatory reactions with 100% mild ones. Mice treated with ALZ (GIV) showed a significant decrease in inflammation, second to GV with (80%) mild inflammation & (20%) moderate ones. *Acacia* treated mice (GIII), showed (40%) mild inflammation and (60%) moderate ones. GII muscles showed numerous larvae surrounded by a dense chronic inflammation, primarily lymphocytes, with marked changes in structures. Muscle of *Acacia* treated mice (GIII) showed a slight larval accumulation, and a mild lymphocytes infiltration. In ALZ treated mice (GIV), muscle showed rare larvae within fibers with moderate infiltration of inflammatory cells. Muscles in combined therapy (GV) showed a sparse lymphocyte infiltration around a low larval concentration within fibers, with more or less normal appearance. The inflammation extent in adjacent muscle tissue was the most minimal in mice given combined therapy, with 100% exhibiting mild inflammation. *Acacia*-treated mice showed a notable in inflammation reduction second to GV, but mild inflammation was in 80% of mice, while moderate inflammation was in 20%. ALZ-treated mice showed lowest one, 60% showed severe inflammation, and 40% mild inflammation.

Immunohistochemical assay of COX2 showed a mean H-score (15±2.2) in combined therapy (GV), COX2 expression was much lower than in ALZ-treated (GIV), with significant difference (P <0.001). *Acacia* treated mice (GIII) showed highest COX2 expression level (60.2±8.1), as compared to other treated mice, but was lower as compared to positive control (138.2±12.2), P <0.001.

In combined therapy, COX2 expression decreased (24.6±2.1), followed by *Acacia*-treated mice (141.6±2.7), highly significant difference (P<0.001). ALZ-treated mice showed highest COX2 expression (214.6± 3.3) as compared to other treated mice, but low-

er compared to positive control (276.2±8.1), with (P <0.001).

Flow cytometer of CD4+ & CD8+ lymphocytic cells in skeletal muscles showed a remarked rise in total CD4 & CD8 cells percentages in all treated mice, with highest increase in mice treated with both drugs followed by *Acacia*-treated mice, and then ALZ-treated ones. Total CD8 was higher than total CD4 in all treated groups.

Details were given in table (1) and figures (1, 2, 3, 4, 5, 6, 7, 8, 9, 10, & 11).

Table 1: CD4+ and CD8+ percentages in groups.

Groups	CD4 (%)		CD8 (%)	
	Mean ±SD	Range	Mean ±SD	Range
GI Negative control (n=5)	15.3±1.74	13.3-17.5	19.48±1.70	17.3-21.7
GII Positive control (n=5)	34.18±3.28	31.8-39.8	39.28±2.18	36.8-41.0
GIII *Acacia* (n=5)	53.9±5.56	46.8-59.6	58.4±3.57	54.2-62.9
GIV Albendazole (n=5)	45.74±3.69	43.2-51.6	51.74±1.99	49-53
GV *Acacia*+ Albendazole (n=5)	67.96±1.11	66.6-69.3	77.58±1.66	75.1-79.6
F test	167.38		433.73	
P value	P<0.001		P<0.001	

Relation between every group significant (P <0.001)

Discussion

Since ancient times, people have utilized plants as a source for producing medications with significant therapeutic potential to cure a wide range of illnesses and infections (Gurib-Fakim, 2006). *Acacia* is a fairly big genus, comprising over 1350 species. Most species have abundant secondary metabolites, primarily flavonoids, tannins and gums (Seigler, 2003). *A. nilotica* is a significant source of polyphenols, primarily made up of tannin, along with ellagic acid, gallic acid, and numerous other substances (Singh *et al*, 2009). All plant parts are credited for having promising benefits in traditional medicine (Rather and Shahidul-Islam, 2015).

In the present study found that *A. nilotica* alone or co-infected to enhance the ALZ therapeutic effectiveness, in treating trichinosis, either intestinal or muscular. The mice received *A. nilotica* showed 80% & 63.8% decrease in adult and larval count compared to the negative control. ALZ- treated mice showed reduction in adult (98.6%) and larvae 62.3% respectively.

In the present study, there was decrease in number of aduls and larvae in mice received both drugs (99.2% & 92.9% respectively). Also, *A. nilotica* showed a significant effect on adults and larvae, which may be related to tannins. Tannins are crucial in disrupting reproductive cycle of gastrointestinal parasites, reducing rate of adults expel larvae hindering larval hindering development in subsequent life stages (Rodríguez-Hernández *et al*, 2023). Tannins ability to bind free protein for larval nutrition by decreased nutrient led to starvation or decrease in metabolism to inhibition to oxidative phosphorylation (Athanasiadou *et al*, 2001).

Albendazole inhibits the parasites' ability to polymerize their microtubules by β-tubulin that progressively impair the parasite functioning cells by attaching to tubulin but, its effect was limited to very early stages of infection in intestine with the low bioavailability (García-Rodríguez *et al*, 2012).

In the present study, *A. nilotica* (fruit) showed anthelmintic effects with dose- and time-dependent in adults' elimination and thus eggs and larvae development (Bachaya *et al*, 2009). Sadiq *et al.* (2017) found that *A. nilotica* showed schizonticide against malignant malaria by preventing schizonts formation. Zarza-Albarrán *et al.* (2020) reported that *A. farnesiana* pods control the sheep *Haemonchus contortus* larvae. Sulaiman *et al.* (2021) found that *Acacia* extract markedly decreased *Trypanosoma brucei* burden in in experimentally infected rats. Also, Ali *et al.* (2021) reported that *A. nilotica* bark methanol extract showed potential action against leishmanial amastigotes and promastigotese. Also, Handayanta *et al.* (2023) found that *A. nilotica* leaves and fruits extract on *H. contortus* worms reduced them.

In the present study, *A. nilotica* minimized parasite pathogenesis in the intestines and muscules. *Acasia*-albendazole treated all infected mice with mild inflammation. Many authours reported that *A. nilotica* is an anti-inflammatory agent (Sakthivel and Chandrasekaran, 2014; Stohs and Bagchi, 2015; Jhundoo *et al*, 2021; Wang *et al*, 2022). Also, *in vitro Acacia* gum increased intestinal barrier (Daguet *et al*, 2015).

In the present study, *T. spiralis* infected mice showed a significantly positive immuno-reactivity of COX2 in intestinal mucosa around the encapsulated larvae with maximum peak on 35th day post-infection This agreed with Barbara *et al.* (2001) reported that COX2 impacted the trichinosis muscle dysfunction, and that COX2 mRNA and local protein smoothed jejunum muscles playing an important role in muscle regeneration. Also, data agreed with Burdan *et al.* (2006), who reported that the cellular infiltrates cyclooxygenase is prostaglandin enzyme catalyzed the initial stage of prostanoid biosynthesis. Significantly COX2 is expressed in pathogenesis accompanied by fever, pain, and inflammation (Szweda *et al*, 2019).

In the present study, total percentages of CD4-positive and CD8-positive lymphocytes. This agreed with Karmanska *et al.* (1994), who found that CD4+ & CD8+ cells in *T. spiralis*-infected and cyclosporine A-treated mice's muscles, found that CD4 and CD8+ expression increased in treated and untreated mice, but more significantly in treated mice and the predominance was to CD8+ cells late in the 35th dpi post infection. Also, Morales *et al.* (2002) reported that in trichinosis' muscle phase, increased in CD8+ lymphocytes and a type 2 cytokines pattern, with complemented by a decrease in naïve cells and a rise in memory cells. Piekarska *et al.* (2020) found that CD4+ and CD8+ lymphocytes controlled immune response by *T. spiralis*. There was a marked Th1-mediated component damage response by larvae overcoming rejection at the initial phase promoting a Th2 response (Li and Ko, 2001). Undoubtedly, in early infection infection with *T. spiralis* produced a mixed Th1/Th2 immune response, with Th2 cells predominating, and that immune response changed to Th2 once the majority of nurse cells were fully formed (El-Aswad *et al*, 2020).

In the present study, the CD4+ and CD8+ significantly raise in response to *Acacia*, albendazole treatment with, and both in combination, with improving the inflammation of muscle, indicating that these cells may be involved in the recovery of muscles. Park *et al.* (2011) found that *A. nilotica* reduced allergy caused by trichinosis. Moreover, *A. nilotica* raised the total WBC count (Ahmad *et al*, 2012). Sunil *et al.* (2019) reported that *A. catechu* extracts have immunomodulatory effects on humoral, cell-mediated, and innate immunological activities.

Conclusion

Acacia nilotica combated *T. spiralis* infection. The ability to reduce inflammation and gives boost to the immune system is the mechanism key. The combination of *A. nilotica* and Albendazole gave a synergistic effect in treating trichinosis in intestinal and muscular phases.

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Authors' contributions: The study design, and protocol were prepared by all authors who also shared in writing manuscript and approved publication. Shalaan, FH, Ammar, AI and Al-attar, TAA carried out all the parasitological histopathological, immunohistochemical and flow cytometry assays studies. Abd-Elhafiz, HI, prepared drug and adjusted dose. Badr, NM, did histopathological studies. Abdel Raouf and Al-attar tabulated data.

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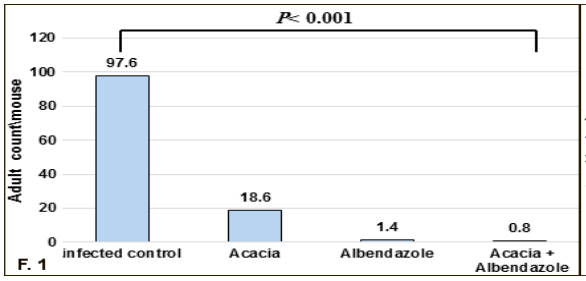
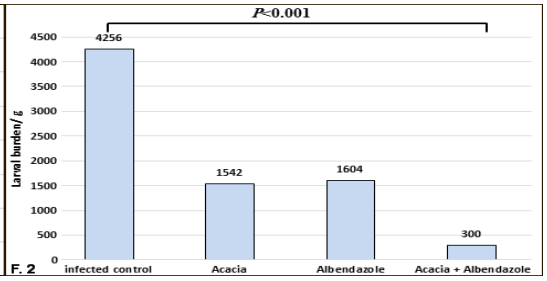
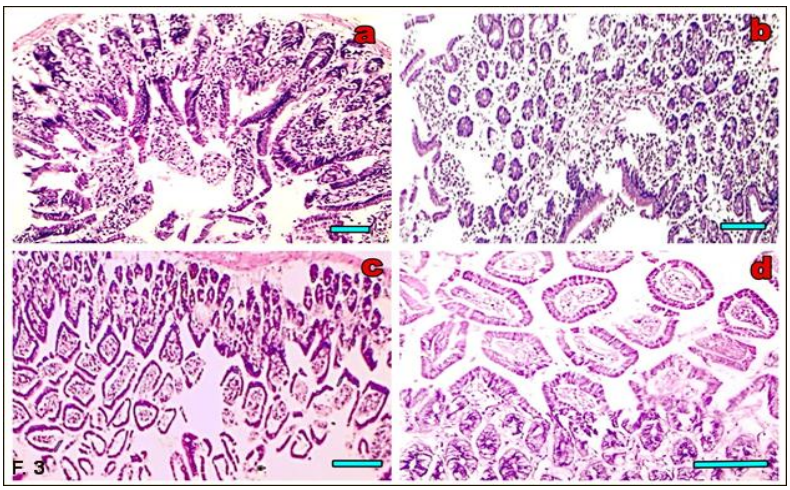
Explanation of figures

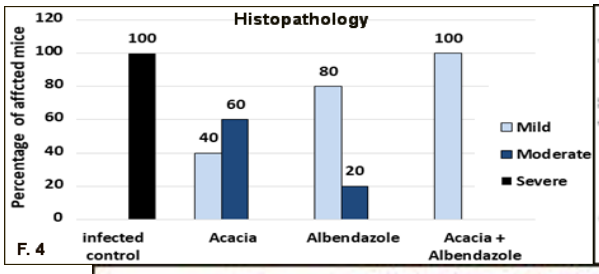
Fig. 1: Adult counts in mice, combined *Acacia*-albendazole exhibited lowest numbers compared to groups, with significant differences.
Fig. 2: Larval burden in groups, compared to other groups, *Acacia*-albendazole treated group, with fewest larvae, with significant differences.
Fig. 3: Histopathological sections of small intestine stained with H&E. a. GII showed dense mononuclear inflammatory infiltration, b. GIII showed moderate sub-epithelial infiltration of inflammatory cells, c. GIV showed mild infiltration with inflammatory cells, & d. GV (Acacia-ALZ- treated) showed almost normal intestinal villi, scattered cellular infiltrate with normal epithelial covering (x100, scale bar = 100μm).
Fig. 4: Pathology of intestinal tissue in groups. Lowest degree in *Acacia*- ALZ treated group and all showed mild inflammation.
Fig. 5: Pathology of muscle tissues. Lowest degree detected in *Acacia*- ALZ treated group, all with mild inflammation.
Fig. 6: Histopathological sections of muscles stained with H&E: a. GII showed larvae (black circle) surrounded by a dense chronic inflammatory reaction, mainly lymphocytes (black arrows) (x100), b. GIII showed mild larval deposition within muscle fibers (black circle) and mild lymphocytic infiltration (x200), c. GIV showed moderate larval accumulation within muscle fibers (black circle) surrounded by a moderate chronic inflammatory reaction (x100), & d. GV showed minimal larval deposition within muscle fibers (black circle) surrounded by sparse lymphocytic infiltrate (x100) (scale bar = 100μm).

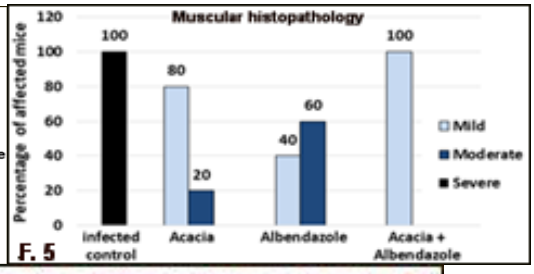
Fig 7: Immunohistochemical expression of COX2. a. GII showed strong positive immunoreactivity in mucosa & inflammatory cells (x200), b. GIII showed moderate expression (x200), & c. GIV showed mild expression (x100). d. GV showed weak expression in lamina propria and epithelial cells (x200) (scale bar = 100μm).
Fig 8: Mean H-scores of COX2 expression in tissues showed lowest mean H-score detected in *Acacia*-ALZ treated group.
Fig 9: Immunohistochemical expression of COX2 in muscle: a. GII showed a strong positive immunoreactivity level around *T. spiralis* larvae and in cellular infiltrates (x200). b. GIII showed mild COX2 immunoreactivity of muscle fibers around vanishing larvae (x200), c. GIV showed moderate COX2 immunostaining of muscle fibers around degenerated larvae (x100), & d. GV showed very minimal COX2 (x200).
Fig 10: Mean H-scores of COX2 expression in muscle tissues of groups, lowest mean H-score in *Acacia*-ALZ treated group.
Fig 11: Flow cytometry assay of CD4+ & CD8+ lymphocytes in muscle at 35th days post-infection showed different lymphocytes.

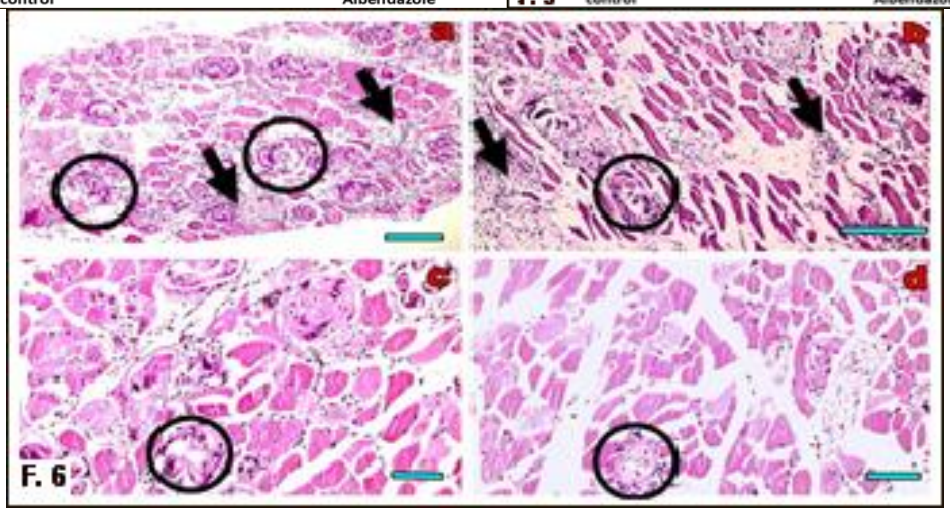


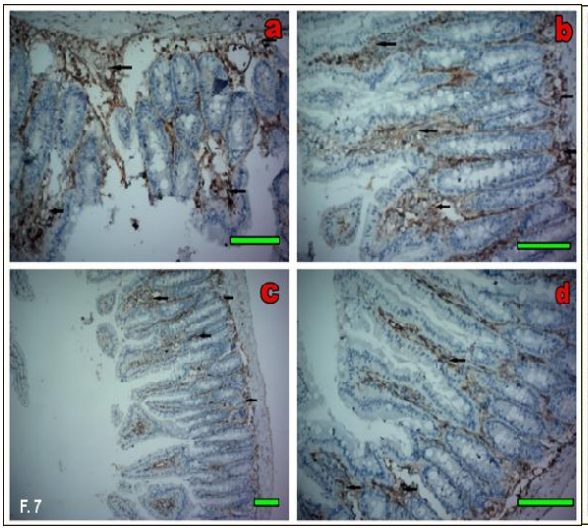


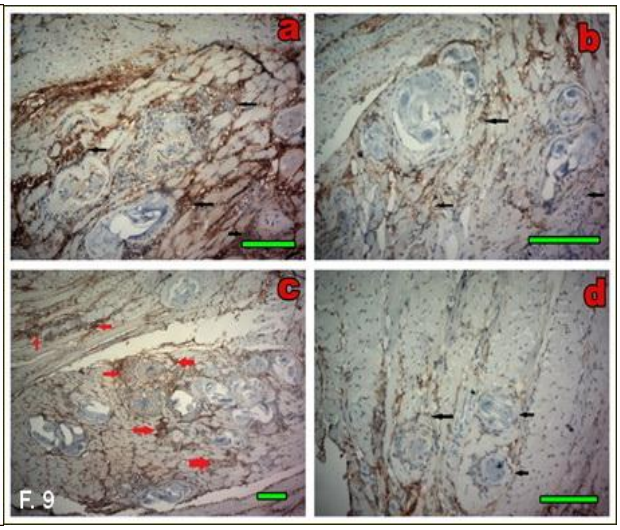


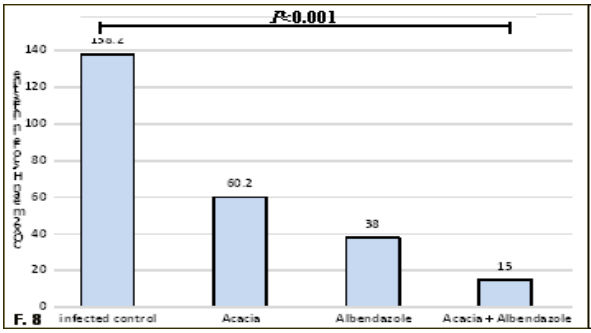


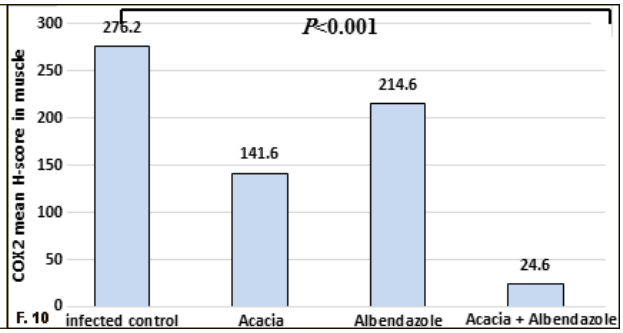


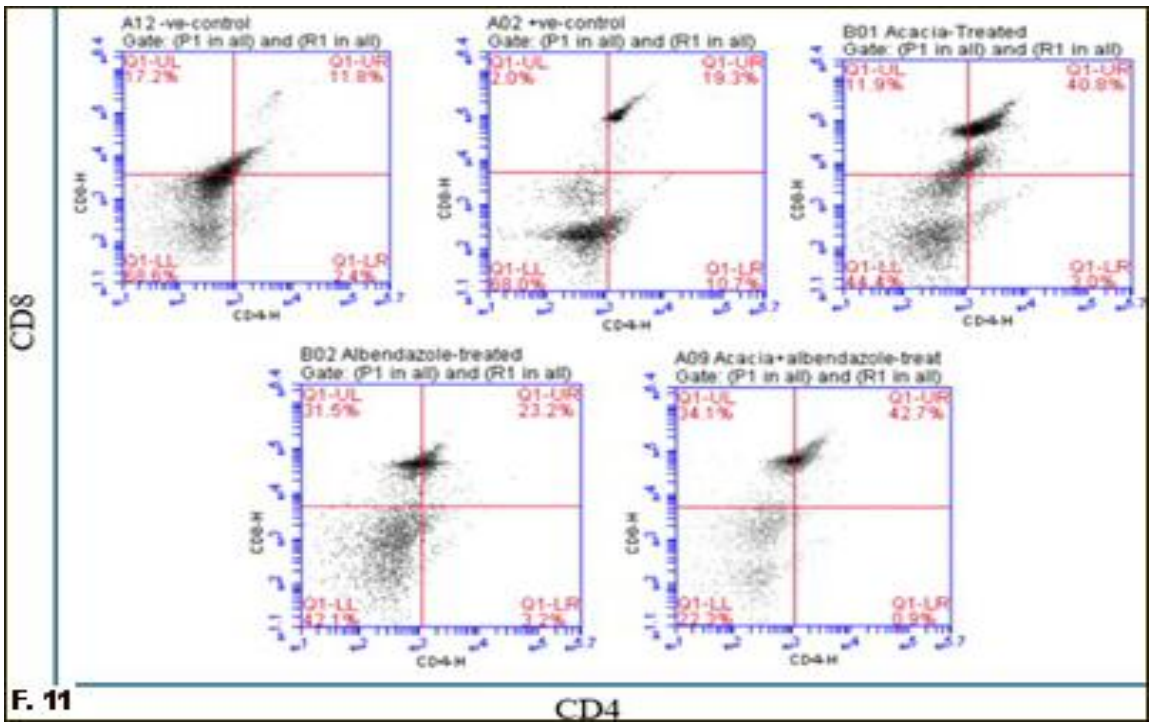



a
b
c
d
F.7


a
b
c
d
F. 9


P≤0.001
156.2
60.2
38
15
infected control
Acacia
Albendazole
Acacia + Albendazole
F. 8


P≤0.001
276.2
141.6
214.6
24.6
COX2 mean H-score in muscle
infected control
Acacia
Albendazole
Acacia + Albendazole
F. 10


A12 -ve-control
Gate: (P1 in all) and (R1 in all)
A02 +ve-control
Gate: (P1 in all) and (R1 in all)
B01 Acacia-Treated
Gate: (P1 in all) and (R1 in all)
B02 Albendazole-treated
Gate: (P1 in all) and (R1 in all)
A09 Acacia+albendazole-treat
Gate: (P1 in all) and (R1 in all)
CD8
CD4
F. 11