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GENISTEIN FOR EXPERIMENTAL MURINE TRICHINELLOSIS: A NOVEL THERAPEUTIC INSIGHT WITH EVALUATION OF IMMUNOLOGICAL AND IMMUNOHISTOCHEMICAL PARAMETERS

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Abstract

Herbal remedies have been studied as alternative or adjuvant treatment options against parasitic infections. Treating trichinellosis with albendazole (ABZ) showed many drawbacks despite its efficacy. This study assessed the effectiveness of genistein (Soybean extract), either with or without ABZ, in treating experimental murine trichinellosis and evaluated the regulatory mechanisms. One hundred Swiss albino laboratory-bred mice were categorized into five groups: G1 uninfected and untreated mice, G2 infected untreated mice, G3 infected and ABZ treated, G4; infected and genistein treated, G5 infected and ABZ &genistein treated. Each group was divided according to the scarification time (7- or 40-days post-infection (dpi), respectively). The treatment efficacy was evaluated parasitological, histopathological, immunological, immunohistochemical (GATA3, glutathione peroxidase 1 (GPX1), and caspase 3) methods and by SEM. Combination of genistein & ABZ in treating trichinellosis showed the best drug efficacy, with reduction of adults and larvae count at 96.73% & 81.56 %, respectively. They showed the highest degree in amelioration of histopathological changes and reduction of inflammation parallel to genistein-treated ones with significant reduction of GATA3 & caspase 3 expressions, but significant elevation of GPX1 expression as well as highest degeneration of adults and larvae by SEM.

Keywords: Albendazole; Caspase 3; GATA3; Glutathione peroxidase 1 (GPX1); Genistein (Soybean extract); Immunopathology; Trichinellosis.

Introduction

Trichinellosis is of great medical importance as it ranked seventh on the list of the top ten food-borne parasites that affect muscle tissues and organs and cause serious health problems published by the WHO and FAO (Muñoz-Carrillo *et al*, 2018). Both the definitive and intermediate hosts harbor adult parasites in intestinal epithelium, and larvae encysted in skeletal muscle cells (Despommier, 2009), and life cycle is classified into enteral and parenteral phases and parenteral one can be subdivided into migrating and muscular phases. Man is infected by eating raw or undercooked meat from many infected animal species (Diaz *et al*, 2020). In zoonotic infection, there is a mixed T helper (Th)-1/Th2 immune response with Th2 predominant in chronic one (Wang *et al*, 2020). Interleukin (IL)-12 promotes the differentiation of naive T cells into INF-γ-producing Th1 cells that enhances the development and differentiation of Th1 cells inducing the inducible nitric oxide synthase (iNOS) expression (Muñoz-Carrillo *et al*, 2017). While Th2 immune response promoting *T. spiralis* expulsion from the intestine via induction of cytokines synthesis such as IL-4, IL-5, and IL-13, by stimulating IgE synthesis, causing mast cell and eosinophil hyperplasia, triggering immediate hypersensitivity reactions (Ilic *et al*, 2011). GATA3 has the ability to interact with the DNA sequence GATA essential for determining the phenotypic characteristics of Th0 cells, favoring their differentiation to the Th2 lineage while inhibiting differentiation into Th1 cells. Also, GATA3 promoted production of IL-4, IL-5, &IL- 13

from Th2 cells (Hafez *et al*, 2020).

The primary contributing factors to pathology are the mechanical injury caused by infection, inflammatory cell accumulation, and disruptions in normal redox status (Elmehy *et al*, 2021). Invasion of host intestinal epithelium by infective larvae in early stages, increases pro-inflammatory cytokines levels and affects antioxidant capacity (Soliman *et al*, 2013), changing the antioxidants levels, as superoxide dismutase (SOD) & glutathione peroxidase (GPX) (Gabrashanska *et al*, 2019). Besides, there was an elevation of apoptotic events during trichinellosis intestinal and muscular phases. The alterations are linked to an increase in apoptosis-related factors expression, including Bcl-2-associated protein X (BAX), tumor necrosis factor-alpha (TNF-α), caspase-3, caspase-8, and caspase-9 (Bruschi *et al*, 2022).

Albendazole is a drug of choice in treating trichinellosis (Paredes *et al*, 2016), but with low bioavailability, exhibits only moderate action to encapsulated muscle larvae with high resistance risk (Codina *et al*, 2015). It was neither allowed for children below three years nor for pregnant women (Yadav and Temjenmongla, 2012), and is carcinogenic (Shalaby *et al,* 2010). These necessitated new, more effective and safe drugs against trichinellosis (El-Wakil *et al*, 2023a).

Genistein (4, 5, 7-trihydroxy isoflavone) is a phytoestrogen naturally present in the soy plants of flavonoid family, subgroup isoflavones (Goh *et al*, 2022). It has various pharmacological activities, such as anticancer, antioxidant, and anti-inflammatory (Tandon and Das, 2018). Also, they showed activity against many zoonotic parasites as *Fasciola gigantica* (Nassef *et al*, 2014), *Schistosoma mansoni* (Sobhy *et al*, 2018), and *Plasmodium falciparum* (Nyandwaro *et al*, 2020).

This study aimed to evaluate anti-*Trichinella* and immunomodulatory effects of Genistein (Soybean extract) either alone or combined with albendazole (ABZ) versus ABZ in experimental infected murine model during intestinal and muscular phases by parasitological, histopathological, immunological, and SEM studies.

Materials and Methods

Ethical consideration: The study was approved by the Institutional Review Board of the National Liver Institute, Menoufia University (NLI IRB protocol N. 00342/2022)

Experimental design: A total of 100 female Swiss albino mice weighing about 20 to 25g laboratory-bred were housed in Theodor Bilharz Research Institute (TBRI) biological unit, Giza, at 24°C and fed a normal diet. Mice were divided into five groups of 20 mice each. G1: uninfected and untreated (negative control), G2: infected untreated mice (positive control), G3: infected and albendazole treated, G4: infected and genistein treated, and G5: infected and ABZ & genistein treated. Each group was subdivided into 2 subgroups (Sga for intestinal phase & Sgb for muscular phase) due to scarification time 7 or 40 days post infection (dpi), respectively (Etewa *et al,* 2018).

Infective inoculum preparation: *T. spiralis* isolates kindly supplied by Medical Parasitology Department, Tanta Faculty of Medicine were used. The isolates were passaged repeatedly in an animal model at TBRI for viability. The infective inoculum was prepared from *T. spiralis*-infected mice, sacrificed five weeks post-infection. Muscles were digested in 1% pepsin and 1% HCl in 200ml distilled water, and mixture was continuously stirred with an electric stirrer for 2hrs at 37°C. The digested product was sieved with a 50mesh/inch to remove large particles. Encysted larvae were collected on a sieve with 200mesh/inch, rinsed twice with tap water, and then suspended in 150ml of tap water in a conical flask. Supernatant fluid was removed, and 200-300 *T. spiralis* larvae were orally given to each mouse (Gamble, 1996).

Drugs were administered orally as followed: In Sga: 3dpi for three consecutive days, and in Sgb 3 dpi for seven consecutive days. One tablet (200mg Albendazole) in 50ml distilled water and given in 50mg/kg/ day orally to G3. Genistein powder (4, 5, 7-trihyd-

roxy isoflavone, AliExpress, CAS NO: 446-72-0) was immediately diluted before in 10% dimethyl sulfoxide (DMSO) and 90% distilled water and given orally as 100mg/kg /day to G4 & G5.

Parasitological assessment of infection and drug effects: To assess infection and drug effects, all Sga were counted for adults of *T. spiralis* on the 7th (Etewa *et al*, 2018) and all Sgb were counted for muscular larvae on 40th dpi (Gamble, 1996). Reduction percentage (Ashour *et al,* 2016):

$$100 = \times \frac{\text{mean No. recovered in controls} - \text{mean No. recovered in treated mouse}}{\text{mean No. recovered in controls}}$$

Compression diagnostic method on 40th dpi in Sgb before artificial digestion, a piece of each mouse's diaphragm was compressed between two slides to a thin layer and microscopically examined for *T. spiralis* larvae (Allam *et al*, 2021).

Histopathological assessment of infection and drug effects: On the 7th dpi, a centimeter was extracted from the mid-intestinal region of Sga. Also, samples of tongue, diaphragm, and hind leg muscles from Sgb were gathered for histopathological processing at Pathology Department, National Liver Institute, Menoufia University. Samples were dehydrated, immersed in xylene, fixed in 10% neutral buffered formalin and processed to paraffin sectioning at 5um thickness stained with haematoxylin and eosin (H & E) to evaluate enteropathy and inflammatory response on the intestinal stages, larval presence and degenerative inflammatory response in the muscular stages.

Larval deposition intensity scoring of each muscle segment and determination of inflammatory response severity in each intestinal and muscle tissue section was done (Othman *et al*, 2016).

Immunological parameters: A- Serologically blood samples were collected on 7th & 40th dpi; sera were separated and stored at -20^{O}C until serum IL-12 & IL-4 levels were evaluted by mouse IL-12(p70) ELISA Kit PicoKine™ (Boster Biological Technology, Pleasanton CA, USA, Catalog #EK0422) & mouse IL-4/Interleukin-4 ELISA Kit PicoKine® (Catalog # EK0584) respectively following the manufacturer instructions. B- Immunohistochemical staining (IHC) all paraffin sections from intestinal and muscular phases underwent de-paraffinization, rehydration and streptavidin-biotin-amplified system was used (Mohammed *et al,* 2022). The primary antibodies were polyclonal GPX1 diluted as 1:100 (Cat. #abx 117034, Abbexa, Milton, Cambridge, United Kingdom), monoclonal active caspase 3 diluted 1:300 (Cat. #bsm 33199 M2, Bioss Antibodies, Woburn, United States), and GATA3 antibody diluted as 1:2000 (Cat. #ab282110, Abcam, Cambridge, UK).

By examining 20 HPF areas of the intestinal and muscle sections of each studied animal, the slides were evaluated for staining status with the proportion of positive cells, and intensity of the stain (+1 for mild, +2 for moderate, and +3 for strong). Histoscore (H score) was used to assess the sections (Pu *et al*, 2017), by using formula; H-score= +1x% of mildly stained cells +2x% moderately stained cells +3x% of strongly stained cells. H score ranged from 0 to 300.

SEM for adult and larvae: After extraction, both were immediately placed into a fresh fixation of 2.5% glutaraldehyde (w/v) in 0.1M sodium cacodylate at pH 7.2. This was done in Electron Microscope Unit, Faculty of Medicine, Tanta University, by a Jeol SEM (Jeol Corp., Mitaka, Japan).

Statistical analysis: The SPSS (Statistical Package for Social Science) program, version 20 Armonk, NY: IBM Corp., Data were tabulated and analyzed as mean SD and % served for descriptive statistics. Chi-square test, Fisher exact test and ANOVA were used for analytical statistics. Post-Hoc evaluated the relationship between two groups for significant associations with Bonferroni correction. If P value was less than 0.05, it was significant.

Results

Death was 1 in each in Sg4a & in Sg4b, and 2 in Sg2b and 1 in each of Sg3b & Sg5b. Mean number of adults and encysted larvae showed significant reduction in treated mice compared to positive ones ($P < 0.0001$) especially in ABZ and genistein treatment (96.73% & 81.56%, respectively), then ABZ treated (95.76% & 79.35%%, respectively) and genistein treated (54.39% & 42.66%, respectively).

Microscopically, in positive control larvae were coiled with increased count and treated ones were straightened with less number.

Histopathological in intestinal phase, positive control showed marked blunting of intestinal villi, ulcerated, moderate to severe epthelial hyperplasia, goblet cell hyperplasia, inflammation, edema, and decreased villous crypt ratio. Amelioration changes and inflammation in treated mice compared to positive control were in genistein-treated, ABZ & genistein-treated and then ABZ treated (P5 >0.05). In muscular, positive one showed moderate to severe larval decomposition intensity score with mild to moderate degradation of internal structure and muscles with moderate to severe inflammation degree. In treated mice, highest reduction in encysted larvae and inflammation degree in ABZ & genistein, ABZ, and then genistein-treated

Serological showed a significant IL-12 level elevation in intestinal phase (7^{th} dpi) in positive control compared to negative one, then muscular phase level reduction (40^{th} dpi). SIL-4 level in positive control didn't different from negative ones ($P> 0.05$), compared to positive controls respectively, level increased significantly in muscular than intestinal one ($P <0.0001$).

Treated mice showed a significant reduction in IL-12 level and significant elevation of IL-4 (except ABZ treated compared to positive control either in both phases. Its level decreased significantly, but IL-4 level increased significantly in muscular phases of infected mice as compared to corresponding ones in intestinal phase. Genistein-treated showed highest reduction of IL-12 and highest elevation of IL-4 levels. In muscular one, highest reduction of IL-12, but highest elevation of IL-4 levels were in ABZ & genistein-treated with least IL-12 reduction & IL-4 levels elevation of in ABZ-treated.

Immunological in both phases; positive control showed high GATA3 & caspase 3 expressions. Treated one showed significant expressions reduction and least one in genistein-treated and then ABZ & genistein treated. Expression of GPX1 increased in positive control than in negative one in phases. In intestinal one, high GPX1 H score in genistein-treated, genistein, & ABZ-treated, but GPX1 H score was v.v. in muscular phase.

SEM: Treated mice showed marked changes in adults and larvae in ABZ & genistein-treated, then ABZ-treated. Control showed sloughing and cuticle destruction, multiple fissures, with complete internal content disappearance and worm flattening. Sg5b showed loss of normal transverse and longitudinal cuticle creases, larva flattening, multiple blebs & cauli-flower masses, and cuticle destruction with mul-titiple fissures.

Details were given in tables (1, 2, 3, 4, 5 & 6) and figures (1, 2, 3, 4 & 5)

Table 1 Comparison of adult and larval count between controls and treated groups in intestinal and muscular phases.

Intestinal phase	Adults count		Reduction %	ANOVA	P-value Post-HOC analysis
	Mean	SD			
Sg1a: (N=10)	0	0	-----	P= <0.0001*	P1=<0.0001*, P2=0.878 P3=<0.0001*, P4=0.967 P5= <0.0001*, P6= <0.0001* P7= <0.0001*, P8= <0.0001* P9= 1, P10= <0.0001*
Sg2a: (N=10)	82.60	9.640	0%		
Sg3a: (N=10)	3.50	2.369	95.76%		
Sg4a: (N=9)	37.67	5.679	54.39%		
Sg5a: (N=10)	2.70	1.947	96.73%		
Muscular phase	Larval count		Reduction %	ANOVA	P-value
Sg1b: (N=10)	0	0	-----	P= <0.0001*	P11=<0.0001*, P12=<0.0001* P13= <0.0001*, P14= <0.0001* P15= <0.0001*, P16= <0.0001* P17= <0.0001*, P18= <0.0001* P19= 0.995, P20= <0.0001*
Sg2b: (N=8)	95218.75	10260.393	0%		
Sg3b: (N=9)	19666.67	3535.534	79.35%		
Sg4b: (N=10)	54600.00	7042.727	42.66%		
Sg5b: (N=9)	17555.56	3205.897	81.56 %		

* Significant value.

P1 Relation between Sgs1a & 2a	P8 Relation between Sgs3a & 4a	P15 Relation between Sgs2b & 3b
P2 Relation between Sgs1a & 3a	P9 Relation between Sgs3a & 5a	P16 Relation between Sgs2b & 4b
P3 Relation between Sgs1a & 4a	P10 Relation between Sgs4a & 5a	P17 Relation between Sgs2b & 5b
P4 Relation between Sgs1a & 5a	P11 Relation between Sgs1b & 2b	P18 Relation between Sgs3b & 4b
P5 Relation between Sgs2a & 3a	P12 Relation between Sgs1b & 3b	P19 Relation between Sgs3b & 5b
P6 Relation between Sgs2a & 4a	P13 Relation between Sgs1b & 4b	P20 Relation between Sgs4b & 5b
P7 Relation between Sgs2a & 5a	P14 Relation between Sgs1b & 5b	

Table 2 Comparison of inflammation degrees between controls and treated groups in intestinal and muscular phases.

Intestinal phase	Degree of inflammation				P-value
	0 No (%)	+1 No (%)	+2 No (%)	+3 No (%)	
Sg1a: (N=10)	10 (100%)	0 (0%)	0 (0%)	0 (0%)	P1= <0.0001*, P2= <0.0001*
Sg2a: (N=10)	0 (0%)	0 (0%)	7 (70%)	3 (30%)	P3= <0.0001*, P4= <0.0001*
Sg3a: (N=10)	0 (0%)	5 (50%)	5 (50%)	0 (0%)	P5= 0.016*, P6=<0.0001*
Sg4a: (N=9)	0 (0%)	9 (100%)	0 (0%)	0 (0%)	P7= 0.003*, P8=0.013*
Sg5a: (N=10)	0 (0%)	7 (70%)	3 (30%)	0 (0%)	P9=0.361,P10=0.073
Muscular phase	Degree of inflammation				P-value
	0 No (%)	+1 No (%)	+2 No (%)	+3No (%)	
Sg1b: (N=10)	10 (100%)	0 (0%)	0 (0%)	0 (0%)	P11= <0.0001*, P12=<0.0001*
Sg2b:(N=8)	0 (0%)	0 (0%)	5 (62.5%)	3 (37.5%)	P13=<0.0001*, P14=<0.0001*
Sg3b: (N=9)	0 (0%)	3 (33.3%)	5 (55.6%)	1 (11.1%)	P15=0.138, P16=0.126
Sg4b: (N=10)	0 (0%)	4 (40%)	4 (40%)	2 (20%)	P17=0.004*, P18=0.765
Sg5b:(N=9)	0 (0%)	7 (77.8%)	2 (22.2%)	0 (0%)	P19=0.143, P20=0.179

Inflammation degree: 0 (no), +1 (mild: up to 10 cells/HPF), +2 (moderate: 11–40 cells/HPF), and +3 (intense< 40 cells/HPF).

Table 3: Comparison of larval deposition intensity score, internal degradation degree in both groups in muscle phase.

Intestinal phase	Epithelial hyperplasia				P-value
	Absent No (%)	Mild No (%)	Moderate No (%)	Severe No (%)	
Sg1a (N=10)	10 (100%)	0 (0%)	0 (0%)	0 (0%)	P1=<0.0001*, P2= <0.0001*
Sg2a (N=10)	0 (0%)	0 (0%)	5 (50%)	5 (50%)	P3= 0.008*, P4= <0.0001*
Sg3a (N=10)	0 (0%)	2 (20%)	6 (60%)	2 (20%)	P5= 0.185, P6= 0.001*
Sg4a (N=9)	3 (33.3%)	5 (55.6%)	1 (11.1%)	0 (0%)	P7= 0.016*, P8= 0.020*
Sg5a (N=10)	0 (0%)	3 (30%)	7 (70%)	0 (0%)	P9= 0.320, P10= 0.019*
Intestinal phase	Villous/crypt ratio				P-value
Sg1a (N=10)	10 (100%)	0 (0%)	0 (0%)	0 (0%)	P1= <0.0001*, P2= <0.0001*
Sg2a (N=10)	0 (0%)	0 (0%)	6 (60%)	4 (40%)	P3= <0.0001*, P4= 0.001*
Sg3a (N=10)	0 (0%)	3 (30%)	4 (40%)	3 (30%)	P5= 0.170, P6=<0.0001*
Sg4a (N=9)	1 (11.1%)	8 (88.9%)	0 (0%)	0 (0%)	P7= 0.028*, P8= 0.017*
Sg5a (N=10)	2 (20%)	3 (30%)	5 (50%)	0 (0%)	P9= 0.164, P10= 0.023*
Muscular phase	Larval deposition intensity score				P-value
Sg1b (N=10)	10 (100%)	0 (0%)	0 (0%)	0 (0%)	P11= <0.0001*, P12=<0.0001*
Sg2b (N=8)	0 (0%)	0 (0%)	3 (37.5%)	5 (62.5%)	P13=<0.0001*, P14=<0.0001*
Sg3b (N=9)	0 (0%)	2 (22.2%)	7 (77.8%)	0 (0%)	P15=0.014*, P16=0.066
Sg4b (N=10)	0 (0%)	0 (0%)	8 (80%)	2 (20%)	P17=0.009*, P18=0.134
Sg5b (N=9)	0 (0%)	4 (44.4%)	5 (55.6%)	0 (0%)	P19=0.317, P20=0.036
Muscular phase	Degradation of larval internal structure				P-value
Sg1b (N=10)	10 (100%)	0 (0%)	0 (0%)	0 (0%)	P11=<0.0001*, P12=<0.0001*
Sg2b (N=8)	0 (0%)	4 (50%)	4 (50%)	0 (0%)	P13=<0.0001*, P14=<0.0001*
Sg3b (N=9)	0 (0%)	4 (44.4%)	5 (55.5%)	0 (0%)	P15=1, P16=1
Sg4b (N=10)	0 (0%)	5 (50%)	5 (50%)	0 (0%)	P17=0.005*,P18=1
Sg5b (N=9)	0 (0%)	1 (11.1%)	1 (11.1%)	7 (77.8%)	P19=0.005*, P20=0.002*

Larval deposition intensity score: 0 (no larva), +1 (mild, <5), +2 (moderate, 5-10), and +3 (intense, >10), * Significant value.

Table 4: Comparison of IL-12 & IL-4 levels between both groups in intestinal and muscular phases.

Intestinal phase	IL- 12		ANOVA	P-value	IL- 4		ANOVA	P-value
	Mean	SD			Mean	SD		
Sg1a	170.50	8.683	P= <0.0001	P1=<0.0001*, P2=<0.0001*	29.90	2.767	P= <0.0001	P1=0.512, P2=0.774
Sg2a	500.70	14.461		P3=<0.0001*, P4=<0.0001*	27.50	2.563		P3=<0.0001*, P4=<0.0001*
Sg3a	427.60	16.015		P5=<0.0001*, P6=<0.0001*	31.80	1.900		P5=0.016*, P6=<0.0001*
Sg4a	282.33	4.500		P7=<0.0001*, P8=<0.0001*	52.33	4.924		P7=<0.0001*, P8=<0.0001*
Sg5a	307.70	4.668		P9=<0.0001*, P10=<0.0001*	35.70	2.163		P9=0.041*, P10=<0.0001*
Muscle	IL- 12		ANOVA	P-value	IL- 4		ANOVA	P-value
Sg1b	170.50	8.683	P= <0.0001	P11=<0.0001*,P12=<0.0001*	29.90	2.767	P= <0.0001	P11=<0.0001*, P12=<0.0001*
Sg2b	433.75	14.646		P13=<0.0001*, P14=<0.0001*	33.50	2.563		P13=<0.0001*, P14=<0.0001*
Sg3b	393.00	6.614		P15=<0.0001*, P16=<0.0001*	37.11	1.900		P15=<0.0001*, P16=<0.0001*
Sg4b	255.30	70718		P17=<0.0001*, P18=<0.0001*	255.30	7.718		P17=<0.0001*, P18=<0.0001*
Sg5b	199.00	3.808		P19=<0.0001*,P20=<0.0001*	199.00	3.808		P19=<0.0001*,P20=<0.0001*

Table 5 Comparison of GATA3 and GPX1 H score between both groups in intestinal and muscular phases

Intestinal	GATA3		P-value	GPX1		P-value
	Mean	SD		Mean	SD	
Sg1a	4.00	3.944	P1=<0.0001*, P2=<0.0001*	20.50	14.615	P1=0.000*, P2=0.002*
Sg2a	171.50	11.797	P3=<0.0001*, P4=0.989	140.00	52.068	P3=<0.0001*,P4=<0.0001*
Sg3a	81.00	11.738	P5=<0.0001*, P6=<0.0001*	124.00	44.020	P5=0.998, P6=<0.0001*
Sg4a	36.11	18.838	P7=<0.0001*, P8=<0.0001*	267.78	62.205	P7=0.008*, P8=<0.0001*
Sg5a	9.50	6.433	P9=<0.0001*, P10=0.003*	232.00	76.420	P9=0.001*, P10=0.842
Muscular	GATA3		P-value	GPX1		P-value
Sg1b	5.00	5.270	P11=<0.0001*, P12=<0.0001*	6.00	6.992	P11=0.080, P12=0.261
Sg2b	105.00	22.039	P13=0.813, P14=0.924	45.63	14.745	P13=<0.0001*, P14=<0.0001*
Sg3b	59.44	28.553	P15=0.002*, P16=<0.0001*	37.22	13.017	P15=0.999, P16=<0.0001*
Sg4b	19.50	22.167	P17=<0.0001*, P18= 0.004*	117.00	36.833	P17=<0.0001*, P18=<0.0001*
Sg5b	17.22	17.159	P19=0.003*, P20=1	132.22	18.559	P19=<0.0001*P20=0.938

*Significant value.

Table 6: Comparison of caspase 3 expression between both groups in intestinal and muscular phases.

Intestinal phase	Caspase 3 expression				P-value
	0 No (%)	+1 No (%)	+2 No (%)	+3 No (%)	
Sg1a (N=10)	8 (80%)	2 (20%)	0 (0%)	0 (0%)	P1=<0.0001*, P2=<0.0001*
Sg2a (N=10)	0 (0%)	0 (0%)	2 (20%)	8 (80%)	P3=0.002*, P4=0.003*
Sg3a (N=10)	0 (0%)	0 (0%)	5 (50%)	5 (50%)	P5=0.160, P6=0.086
Sg4a (N=9)	0 (0%)	2 (22.2%)	4 (44.4%)	3 (33.3%)	P7=0.008*, P8=0.277
Sg5a (N=10)	0 (0%)	6 (60%)	2 (20%)	2 (20%)	P9=0.014*,P10=0.244
Muscular phase	Caspase 3 expression				P-value
Sg1b (N=10)	10 (100%)	0 (0%)	0 (0%)	0 (0%)	P11=<0.0001*, P12=<0.0001*
Sg2b (N=8)	0 (0%)	0 (0%)	2 (25%)	6 (75%)	P13=<0.0001*, P14=<0.0001*
Sg3b (N=9)	0 (0%)	2 (22.2%)	4 44.4%	3 (33.3%)	P15=0.164, P16=<0.0001*
Sg4b (N=10)	0 (0%)	9 (90%)	1 (10%)	0 (0%)	P17=0.001*, P18=0.010*
Sg5b (N=9)	0 (0%)	8 (88.9%)	1 (11.1%)	0 (0%)	P19=0.015*,P20=0.937

Degree of caspase 3 expression: 0 (no), +1 (mild), +2 (moderate), and +3 (intense), * Significant value.

Discussion

In the present study, treatment with ABZ caused significant adult and larval reduction (95.76% &79.35% %, respectively). This agreed with Fahmy and Diab (2021), who reported reduction of 88.7% & 79.6%, respectively Also, El-Wakil *et al*. (2023a), who reported reduction of 86% in adults and 77% in larvae. But, Eid *et al*. (2020) found that ABZ treated murine trichinellosis led a reduction of 61.8% in adults and 42.4 % in larvae. Diversity in count reduction of adults and larvae resulted from the treatment protocols of dosage and scarification time.

In the present study, the muscle sections of ABZ treated mice were same as that of Eissa *et al*. (2022), who found moderate inflammatory cellular infiltration and moderate to severe fibrosis. But, Huang *et al*. (2020) reported that inflammatory cellular infiltration was reduced with pathological damage compared to positive control.

In the present study, genistein not used before in treating trichinellosis caused a significant reduction in adult and larval counts (54.39% & 42.66%, respectively). Also, genistein-treated mice, either alone or combined with ABZ showed better histopathological improvement than ABZ-treated mice with more reduction of inflammation in intestinal and muscular stages.

Nassef *et al*. (2014) reported that genistein in treating fascioliasis caused significant improvement in pathological changes and reduction of eggs in feces. Sobhy *et al*. (2018) used genisteinin treatment of experimental schistosomiasis *mansoni* alone or combined with ABZ or combined soybean oil extract and an avocado extract; reported significant reduction in total worm burden and tissue egg load with eggs marked degeneration, as well as significant reduction in hepatic granulomas diameter in acute or chronic stages. Sutrisno *et al*. (2015) found that genistein inhibits various signaling pathways such as nuclear factor kappa-B (NF-κB) leading to a reduction in the expression of IL-6, IL-1 & TNF-α, proinflammatory cytokines, prostag-

landins (PGs), iNOS, reactive oxygen species (ROS) and macrophage inflammatory protein-1 alpha (MIP-1a). Goh *et al*. (2022) reported that genistein has a strong anti-inflammatory activities.

The present study showed a significant elevation of serum IL-12 level in intestinal phase of positive control, followed by a reduction in muscular phase, but significantly reduced in treated mice intestinal or muscular phases. This agreed with Muñoz-Carrillo *et al*. (2017), who found a significant increase of Th1 cytokines in early stage of *T. spiralis* intestinal infection with increased synthesis of pro-inflammatory mediators, which led to increased eosinophils and intestinal pathology development. Salama *et al.* (2022) also reported a significant reduction in TNF-α on 7^{th} & 35^{th} dpi in ABZ or herbal extracts mice treated. Also, the present study showed increased level of IL-4 in treated mice in intestinal or muscular phases. This agreed with Ilic *et al*. (2011), who found that Th2 cytokines in intestinal phase enhanced IgE synthesis, induced mast cell and eosinophil hyperplasia, and favored *T. spiralis* expulsion from intestine. In the muscular phase, *T. spiralis* activated Th2 response to form nurse cell and declined (Kang *et al*, 2012). Also, Wang *et al*. (2020) reported that in *T. spiralis* rodent a persistent Th2 immune response resulted after a short Th1 immune response.

In the present study, treated mice showed a significant elevation of IL-4 than positive control but reduction in GATA3 expression. This agreed with Hafez *et al*. (2020), they found GATA3 significant increase expression infected control. Also, it agreed with Long *et al*. (2022), who found that as *T. spiralis* induced Th2 response in mouse lung tissue, increased IL-4, IL-5, IL-13 & GATA3 expression. Astry *et al*. (2015) found that *Punica*-treated mice gave a significant decrease in GATA3 expression, due to *Punica's* ability to inhibit all inflammatory mediators.

Genistein treatment with or without ABZ showed a significant reduction in IL-12 level with elevation of IL-4 levels than positive control in both phases. This agreed with Wang *et al*. (2008), they found that genistein modulated a Th1-predominant immune response, by suppressed IFN-γ secretion and IL-4 production. Mace *et al*. (2019) found that genistein inhibit IL-12/IL-18-induced IFN-γ production by natural killer cells. Also, ABZ caused significant reduction of IL-12 levels, and GATA3 expression in both phases that agreed to some extent with Wu *et al*. (2021)**,** who found that ABZ on *Echinococcus multilocularis*-infected mice didn't reduce IL-12 level and messenger ribonucleic acid expressions of GATA3 transcription factor compared to positive control.

In the present study, epithelium of small intestine and skeletal muscles showed pathological changes mediated by ROS caused by parasite and host as a defense mechanism. Gabrashanska *et al*. (2019), in mice trichnosis reported that they protected by antioxidant against oxidant-mediated injury by changing SOD, GPX, levels and non-enzymatic total antioxidant status.

Genistein with or without ABZ activated GPX1 H score in intestinal or muscular phases, as antioxidant protected cells from excessive ROS formation by scavenging free radicals. Suzuki *et al*. (2002) found that GPX1 gene expression level was the most upregulated gene by genistein on human prostate cancer cells. Yon *et al*. (2011) found that genistein enhanced expressions of GPX and SOD mRNAs. ABZ caused a non-significant reduction in GPX1 expression in intestinal or muscular phases than the positive control. This agreed to some extent with Adiang *et al*. (2021), where oral administration of ABZ induced a significant decrease in SOD, antioxidant enzymes catalase, and GPX levels compared to control.

Expression of caspase 3 increased in intestinal or muscular phases in positive control that agreed with Bruschi *et al*. (2022), they reported up-regulation of some apoptosis-related genes such as P_{53} and caspase 3, caspase 8, caspase 9, BAX & TNF-α, in trichinellosis. ABZ treatment didn't cause signific-

ant difference in caspase 3 expression compared to positive control in both phases, which agreed with Zhang *et al*. (2019), they found that the anticancer effect of ABZ in squamous cell carcinoma cells caused apoptosis-related signals including cleaved caspase-3, increased in a dose-dependent manner. Genistein didn't cause significant reduction in apoptosis in intestinal phase, but a significant reduction in muscular phase. This agreed with Nassef *et al*. (2014), they found that fascioliasis was improvement in hepatocyte apoptosis caused by soybean extract. They added that soybean isoflavones antioxidant properties decreased oxidative DNA damage in several cell lines. But, it disagreed with Nazari-Khanamiri and Ghasemnejad-Berenji (2021), as genistein induced apoptosis in human cancer cells in cervix and colon by increasing of caspases 9 and/or 3 enzymes activities. Genistein caused apoptosis in colon cancer cell lines by blocking NF-κB pathway and adjusting anti-apoptotic protein levels (Luo *et al*, 2014).

In the present study, risky degenerative changes in genistein-treated mice were loss of cuticle normal annulations, with complete destruction of all internal contents causing cuticle rupture and flattening of adults and larvae. Larval destruction decreased the secreted toxin that stimulated immune system and decreased cellular infiltration, causing muscles' pathogenesis. Also, ABZ showed the same changes in adults' cuticle but, less effects on muscle larvae. This agreed with Paredes *et al*. (2016); Eid *et al*. (2020), and Abou Hussien *et al.* (2022), who found that genistein with or without ABZ caused adults and larval stages caused severe tegumental destruction. Toner *et al*. (2008) in trematoda, found that genistein caused spines to slough off or deformed without impact on tegumental surface but, decreased its enzymes causing paralysis and even death. Abou Rayia *et al*. (2017) reported that the parasitic blebbing formation was an attempt to repair the damaged tegument in response to drug action.

Conclusion

The best efficacy on *T. spiralis* was combined ABZ and genistein in intestinal and muscular phases followed by ABZ alone that was more active than genistein alone. But, genistein caused a significant reduction in intensity with better histological results than ABZ treated mice in muscular and intestinal stages, especially in reducing inflammation degree. This amelioration is due to significant reduction of IL-12 level but elevation of IL-4 level. Genistein caused a significant reduction of GATA3, caspase-3 and enhancement of GPX1, with more destructive changes in adults & larvae than ABZ.

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Explanation of Figures:

Fig. 1: Compression diagnostic method in muscles showed coiled *T. spiralis* larvae (blue arrows) in infected untreated G2b (1.a) while straightened (non-coiled) larvae in treated groups less in number (1.b) (x100).

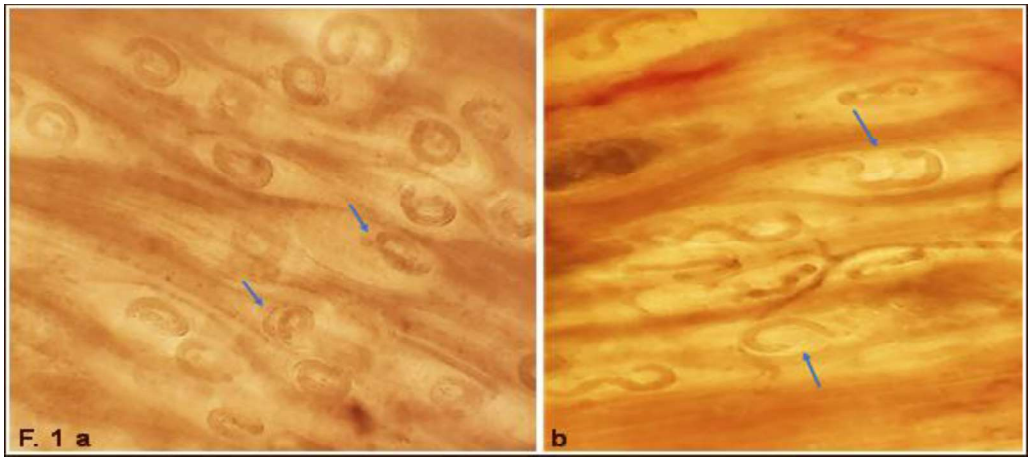
Fig. 2: T.S. in intestine. G1a showed a normal preserved villous/crypt ratio (H&E x40). G2a showed encysted larvae (arrows) (H&E x40) (2b), moderately decreased villous/crypt ratio, and moderate crypt hyperplasia (H&E x40) (2c). G3a showed a moderate decrease in vil-

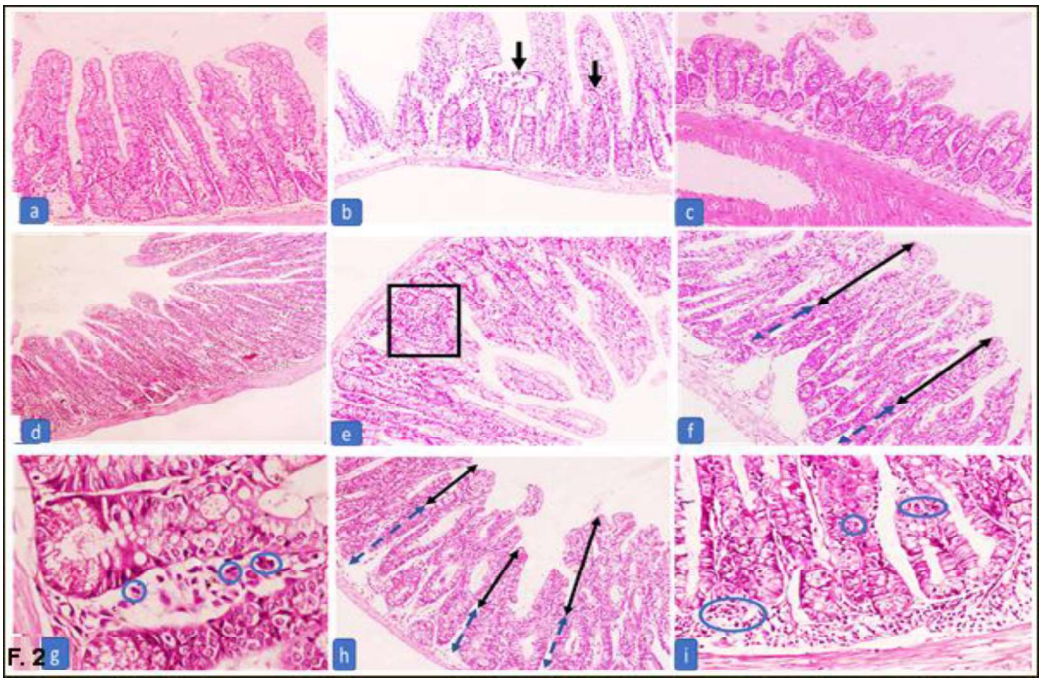
lous/crypt ratio with blunting of villi and moderate oedema (H&E x40) (2d) plus moderate inflammation with lymphoid aggregate formation (black box) (H&E x40) (2e). G4a showed a mild decrease in villous/crypt ratio (black arrows) and mild crypt hyperplasia (blue arrows), mild inflammation, and mild edema (H&E x40) (2f), a same case showed mild inflammation containing few eosinophils (circles) (H&E x200) (2g). G5a showed a moderate decrease in villous/crypt ratio (black arrows) and moderate crypt hyperplasia (blue arrows), mild inflammation, and mild edema (H&E x40) (2h), a same case showed mild inflammation with a moderate eosinophils (circles) (H&E x200) (2i).

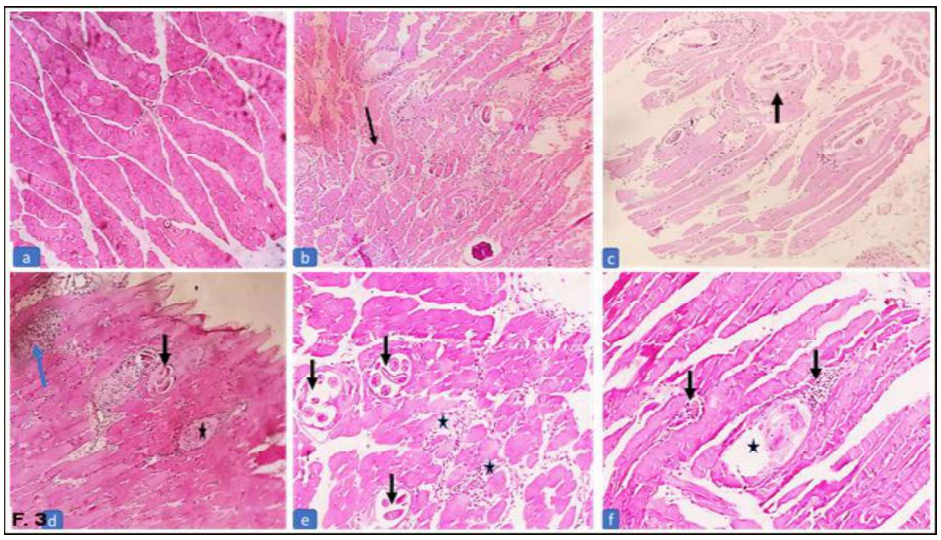
Fig. 3 Muscular phase of G1b showed normal muscle without inflammation (H&E x100) (3a). G2b showed a large density of encysted larvae (+3) associated with moderate inflammation (+2), and mild fragmentation of internal structures (black arrows) (H&E x100) (3b, 3c). G3b showed a moderate density of encysted larvae (+2) associated with moderate inflame-mation (+1) (blue arrow) and severe fragmentation of internal structures (black arrow) with occasional empty capsules (asterisk) (3d). G4b showed a moderate density of encysted larvae (+2) associated with fragmentation of internal structures (black arrows) and mild inflammation (asterisks) (+1) (3e). G5b showed a low density of encysted larvae (+1) with severe fragmentation of internal structures and empty capsule associated with mild inflammation (+1) (arrows) (3f).

Fig. 4: SEM of adult *T. spiralis*. G2a showed anterior end (blue arrow), hypodermal glands opening (green square), fine longitudinal ridges (red arrow), and transverse creases (yellow arrow) (4a). G3a showed widening of hypodermal gland opening (green box), sloughing cuticle parts (red box), and cauliflower masses arising from cuticle (blue arrows) (4b). G4a showed sloughing, cuticle erosion, large blebs with multiple fissures and loss cuticle normal annulations (4c). G5a showed cuticle rupture without internal content (blue arrows), multiple fissures with loss of cuticle normal annulations (red arrow) (4d), and complete large opening due to cuticle rupture with complete disappearance of internal content (blue arrows) (4e).

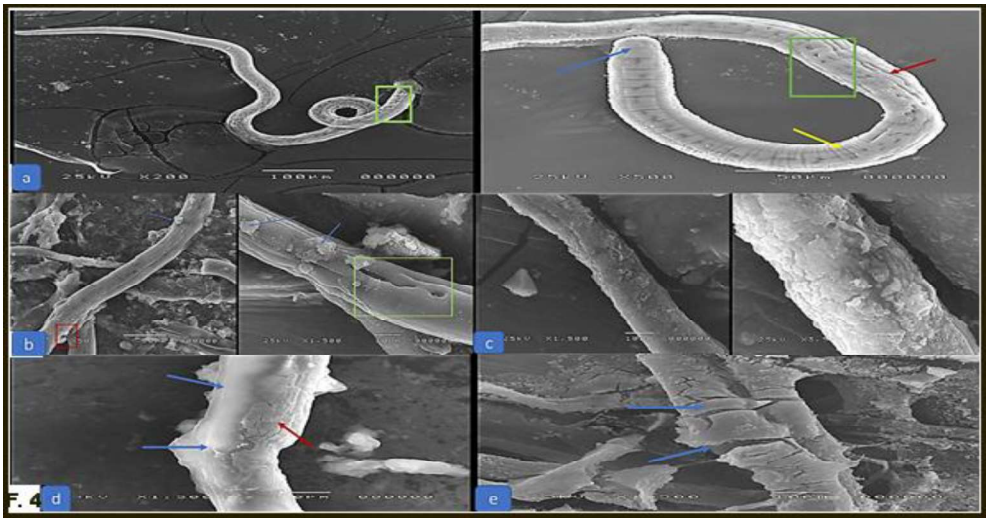
Fig. 5: SEM of *T. spiralis* larva. G2b showed a normal cuticle with fine longitudinal ridges (blue arrow) and transverse creases (red arrow) (5a). G3b showed blebs (red arrows) and cauliflower masses raised from cuticle (blue arrows) (5b). G4b showed multiple cauliflower masses from cuticle (red arrows) (5c). G5b showed loss of normal transverse and longitudinal creases of cuticle, multiple blebs and cauliflower masses, and destruction of cuticle with multiple fissures (red arrows) (5d).



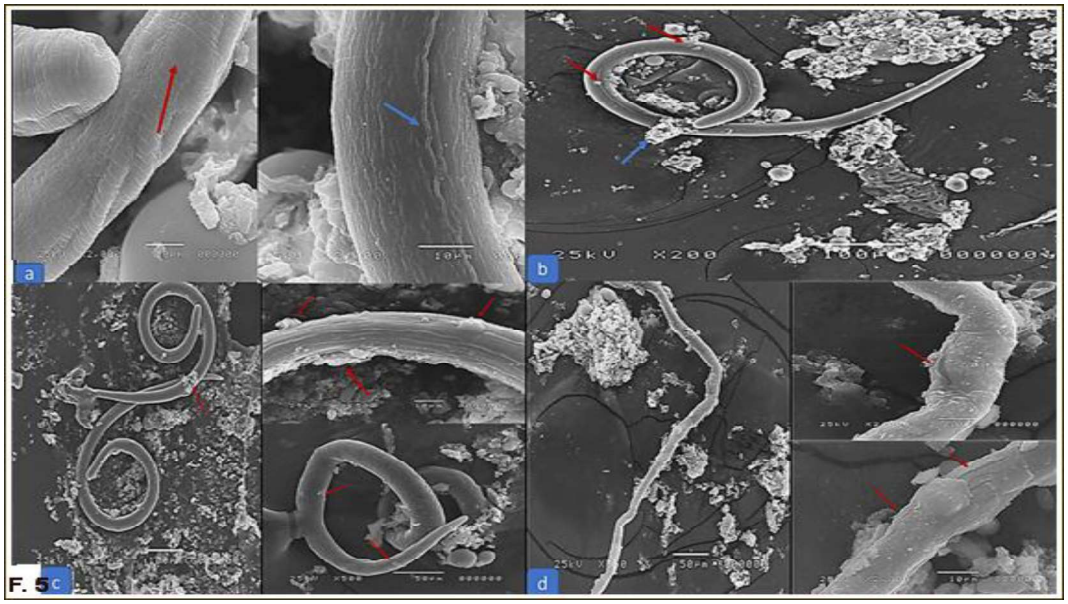




F. 3



F. 4



F. 5