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Garlic oil as a fight against histological and oxidative stress abnormalities in Wistar rats after oral inoculation of *Anisakis* spp. Type II (L3) (Nematoda)

[*O uso do óleo de alho na luta contra anormalidades histológicas e de estresse oxidativo em ratos Winstar após inoculação oral de* Anisakiss*pp. Tipo II (L3) (Nematoda)*]

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ABSTRACT

The consumption of inadequately thermally treated fish is a public health risk due to the possible propagation of *Anisakis* larvae and their antigenic proteins, the causative agent of the zoonotic disease anisakidosis. The present study demonstrated the physiological and histopathological changes that accompanied an oral inoculation of crude extracts from fresh and thermally treated *Anisakis* Type II (L3) in Wistar albino rats. Nematode worms were isolated from the marine fish *Dicentrarchus labrax*. They were examined and taxonomically identified using light and scanning electron microscopy. The study was performed in 6 rat groups: a control group (I), a garlic oil (GO) inoculated group (II), a fresh L3 inoculated group (III), a thermally treated L3 inoculated group (IV), a fresh L3 + GO inoculated group (V), and a thermally treated L3 + GO inoculated group (VI). It was observed that rats inoculated with fresh and thermally treated L3 crude extracts showed abnormal oxidative stress markers associated with the destruction of normal architecture of spleen and thymus. GO produced a protective effect in rat groups inoculated with L3 extracts + GO administration via the amelioration of oxidative stress markers, which was confirmed by the marked normal structure of the organs' histology. Cooking of L3 infected fish induced severe physiological and histopathological alterations compared to uncooked infected fish. The administration of garlic before and after fish eating is recommended to avoid the dangerous effect of anisakids, even if they are cooked.

Keywords: anisakid larvae, histopathology, zoonotic parasites, morphology, biochemistry

RESUMO

O consumo de peixes tratados termicamente de forma inadequada é um risco à saúde pública devido à possível propagação das larvas de Anisakis *e suas proteínas antigênicas, o agente causador da doença zoonótica anisakidose. O presente estudo demonstrou as alterações fisiológicas e histopatológicas que acompanharam a inoculação oral de extratos brutos de Anisakis Tipo II (L3) frescos e termicamente tratados em ratos Wistar albinos. Vermes nematoides foram isolados do peixe marinho* Dicentrarchus labrax *e foram examinados e identificados taxonomicamente usando microscopia óptica e eletrônica de varredura. O estudo foi realizado em 6 grupos de ratos: grupo controle (I), grupo inoculado com óleo de alho (GO) (II), grupo inoculado com L3 fresco (III), grupo inoculado com L3 tratado termicamente (IV), grupo inoculado com L3 + GO fresco (V), e grupo inoculado com L3 + GO tratado termicamente (VI). Observou-se que ratos inoculados com extrato bruto L3 fresco e tratado termicamente mostraram*

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marcadores de estresse oxidativo anormais associados à destruição da estrutura normal do baço e do timo. GO produziu um efeito protetor em grupos de ratos inoculados com extrato L3 + administração de GO através da melhoria dos marcadores de estresse oxidativo, que foi confirmada pela marcante estrutura normal da histologia dos órgãos. O cozimento de peixes infectados com L3 induziu alterações fisiológicas e histopatológicas graves quando comparado com peixes infectados não cozidos. Recomenda-se a administração de alho antes e depois da ingestão do peixe para evitar o efeito perigoso dos anisakídeos, mesmo se cozidos.

Palavras-chave: larvas de anisakídeos, histopatologia, parasitas zoológicos, morfologia, bioquímica

INTRODUCTION

Anisakidosis is a vital fish-borne zoonotic disease caused by third or, infrequently, fourth larval stages of the nematode *A. simplex*, which live as encapsulated larvae embedded in the edible muscle of marine fish (Morsy *et al.*, 2013). The life cycle begins when eggs are passed in the feces of marine mammals into water, where they hatch to release free-living larvae (L2), which are consumed by crustaceans and further develop into L3. When infected crustaceans are ingested by fish, the third larvae enter their body cavity and muscles (Kassem and Bowashi, 2015; Nieuwenhuizen, 2016). Humans may be accidentally infected by the L3 larvae when they ingest raw, undercooked meat of parasitized fish (Mattiucci *et al.*, 2013). The pathological effects appear when the third larvae pierce the mucosal layers of the host's gastrointestinal tract, which directly produces tissue injury, and causes nausea, diarrhea, and abdominal pain (Kassem and Bowashi, 2015).

Some patients develop an IgE–mediated "gastro allergic anisakiasis", which leads to clinical manifestation that range from urticaria to life-threating anaphylactic shock (Nieuwenhuizen, 2016). *Anisakis* larvae pose a health risk to humans even when the fish is thoroughly cooked because dead or thermally treated L3 produce a number of physiological and histopathological changes in humans after fish ingestion, which lead to the concept of acute anisakiasis (Montalto *et al.*, 2005). Traditional medicines worldwide identified the benefits of plants in human health and have taken advantage of the empirical treatment of common human diseases.

Plant phytochemicals act at the molecular and cellular levels to protect human cells against oxidative damage, stimulate enzymes, interfere with DNA replication, and affect infection processes (Seckiner *et al.*, 2014). Garlic (*Alliumsativum* L., Alliiaceae) is one the oldest familiar plants used for health and the treatment of numerous diseases in folk medicine (Lee *et al.*, 2012). Garlic was recently suggested as a promising candidate for maintaining homeostasis. Garlic has a large number of potentially active chemical components, such as amino acids, organosulfates, minerals, and vitamins (World…, 2000). Substantial studies showed that garlic and its bioactive constituents exhibit antioxidant, anti-inflammatory, antibacterial, antifungal, immunomodulatory, cardiovascular protective, anticancer, hepatoprotective, digestive system protective, anti-diabetic, anti-obesity, neuroprotective, and renal protective properties (Yun *et al.*, 2014; Abdel-Daim *et al*., 2017). Abdel-Daim *et al*. (2020) concluded that the antioxidant effect of GO was evidenced by the significant decrease in MDA and NO levels and increase in GSH, T.SOD, and CAT activities in the liver and kidneys.

Also, allicin supplementation and diallylsulfide (DAS) produced from garlic provided a near complete protection in terms of serum and tissues′ biochemical alterations, antioxidant activity and oxidative stress (Abdel-Daim and Abdou, 2015; Abdel-Daim *et al*., 2015, 2018). Garlic exhibits immunomodulatory actions like the modulation of cytokine secretion, phagocytosis promotion, macrophage activation, immunoglobulin production, allergic reactions, and lymphocyte proliferation (Liu *et al.*, 2009; Mahima *et al.*, 2012; Percival, 2016). Therefore, the present study demonstrated the biochemical, oxidative stress activity and histopathological changes in Wistar albino rats after oral inoculation of fresh and thermally treated *Anisakis* spp. type II (L3) larvae isolated from the Red sea fish *Dicentrarchus labrax*. The present study also evaluated the protective and ameliorative effects of garlic oil (GO) in L3 inoculated rats.

MATERIALS AND METHODS

Parasite Collection and Identification: Twenty specimens of the European seabass *Dicentrarchus labrax* (family: Moronidae) were collected alive from fishermen at boat landing sites along the Red sea in Jizan, Saudi Arabia, and sometimes from local markets. Fish were transported to the laboratory of Parasitology; and were identified according to Randall (1992). Worms were collected as encapsulated larvae from the surfaces of visceral organs, such as the stomach, intestines, and muscles, of the examined fish. The worms were rinsed in phosphate buffered saline for further experiments. For the morphological study, some of the collected worms were fixed in hot 70% ethanol for light microscopy (Morsy *et al.*, 2013). For scanning electron microscopy (SEM), 10 worms were fixed in 3% phosphate-buffered glutaraldehyde (pH 7.3) for 3h, washed in the same buffer, and dehydrated in an ascending alcohol series according to Madden and Tromba (1976). After passing through an ascending series of Genosolv–D, worms were processed in a critical point drier, "Bomer–900", with freon 13 and sputter-coated with gold–palladium in a Technic Summer V for examination using an Etec Autoscan (20 kV) scanning electron microscope (SEM).

Garlic oil (GO) and FT-Raman spectroscopy: GO was purchased in the form of capsules. Each capsule contained 10mg/kg pure GO equivalent to 1000mg of fresh garlic bulb and other ingredients (Vitaminshoppe Co., New Jersey, U.S.A.). Using the amount of garlic oil concentration printed on some of the commercial capsules labels and described in the literature (Iberl *et al.*, 1990; Amagase *et al.*, 2001), mixtures were prepared from standard garlic oil. The mass fractions of garlic oil were in the range of 0.00% to 1.75% (0.00%, 0.25%, 0.50%, 0.75%, 1.00%, 1.25%, 1.50%, 1.75%). Using an automatic pipette, the masses of the components of each mixture were weighed on a watch glass in a calibrated analytical balance, and then stirred for 3 minutes to ensure homogeneity. The instrument used for Infrared (IR) analysis was a NICOLET 6700 Fourier transform infrared spectroscopy (FT-IR) optical spectrometer from Thermo Scientific. The spectra were obtained in the medium infrared region, from 4000 to 650cm-1, as an average of 16 scans at a resolution of 4cm-1, under a controlled temperature of 23 ± 2°C.

Experimental Design: Thirty-six adult male Wistar albino rats (*Rattus norvegicus*), 150–170g in weight, were housed in a suitable cage under a 12h light/12h dark cycle. Rats were housed in metal cages under a controlled temperature and humidity with water and food available *ad libitum*. The experimental protocols for animal use were performed in accordance with the regulatory laws of experimental ethics of animal use and collecting permits, NODCAR research ethics Committee for experimental and clinical studies, Egypt (Approval no. NODCAR/III/35/19).

The rats were divided into the following six groups (n=6):

Group I: The control group, inoculated with corn oil only (2ml/kg, p. o) as a vehicle for 21 days (according to Wu *et al*. (2001)).

Group II: GO group, inoculated with GO only (100mg/kg b.wt.) for 21 days (Mohamed *et al.*, 2016).

Group III: The fresh L3 inoculated group: Rats were inoculated with corn oil for 21 days (2ml/kg p. o) and inoculated twice with fresh *Anisakis* spp. (L3) crude extract on the 0^{th} and 14^{th} days (Figure 1).

Group IV: The thermally treated L3 inoculated group: Rats were inoculated with corn oil for 21 days (2ml/kg p. o); and inoculated twice with thermally treated (in 100°C water bath, 10min) *Anisakis* spp. (L3) crude extract at the 0^{th} and 14^{th} days.

Group V: The fresh L3+ GO group: Rats were inoculated with GO for 21 days (100mg/kg b.wt.) and inoculated twice with fresh *Anisakis* spp. (L3) crude extract on the 0^{th} and 14^{th} days.

Group VI: The thermally treated L3 + GO group: rats were inoculated with GO for 21 days (100mg/kg b.wt.) and inoculated twice with thermally treated (in 100°C water bath, 10min) *Anisakis* spp. (L3) crude extract at the 0^{th} and 14^{th} days.

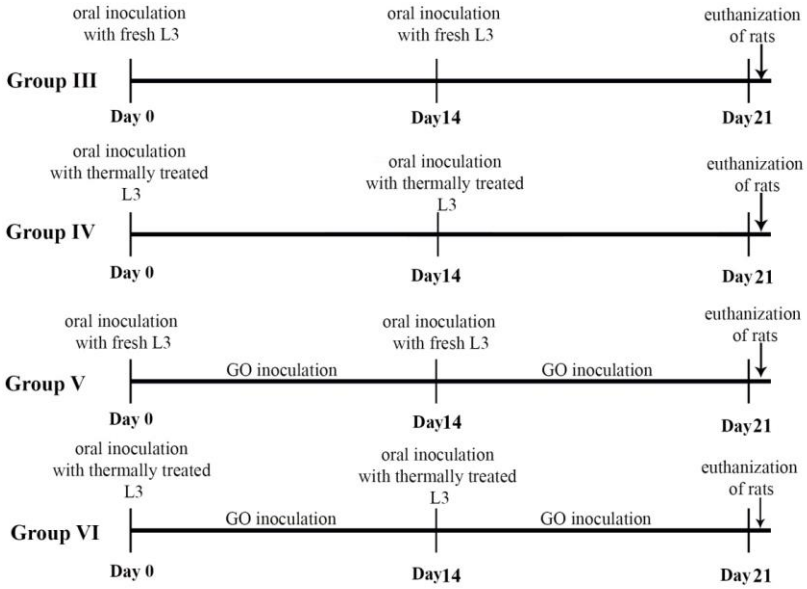

Figure 1. Timeline of experimental rats inoculation and euthanization. Groups III–VI assigned as: (III) fresh L3 inoculated; (IV) thermally treated L3 inoculated; (V) fresh L3+GO inoculated; (VI) thermally treated L3 + GO inoculated. The experiment was designed in 21 days, days 0 and 14 for first and second dose inoculation while day 21 for euthanizing.

Rats were orally inoculated with fresh or thermally treated L3 as crude extracts placed on the pharynx or directly into the stomach. GO extract was given to the rats at a dose of 100mg/kg b.wt. according to Riad *et al.* (2007) using an esophageal tube. Crude extracts were prepared via the homogenization of L3 for each rat separately before inoculation, and 10 portions of fresh or thermally treated L3 of an equal size were ruptured using microtube pestles and sonicated on ice at 100 W five times for 30s using an ultrasonic homogenizer (Abe and Teramoto, 2014).

Tissue Preparation for Oxidative stress markers: The oxidant/antioxidant status was evaluated in the liver and kidney tissue homogenates, Twenty-four hours after the last dose, rats were euthanized, tissues were isolated homogenized in a cold phosphate buffer and then the homogenates were centrifuged at 10,000g for 15min at 4ºC. The following parameters were measured in the supernatants of the tissue homogenates: the level of malondialdehyde (MDA), a marker for lipid peroxidation, was determined by the procedure of Fawcett and Scott (1978). Glutathione reductase (GRD) was measured according to the protocol by Goldberg and Spooner (1983). Catalase activity (CAT**)** was measured according to the method of Kotze (2003). Nitric oxide (NO) was determined according to the method described by Masetti *et al.* (2004). Superoxide dismutase (SOD) was measured according to the protocol of Nishikim *et al.*, 1972).

Histpathological study: A portion of the spleen and thymus tissues was excised from the control and the experimental rat groups at the end of the experiment and fixed in a 10% neutral buffered formalin solution for at least 12h. A phosphate buffer was used to wash the samples 2–3 times for 10min. The specimens were dehydrated in a graded ethanol series, cleared in butanol, and embedded in paraplast at 62 ºC. Sections of 5 µm thickness were prepared using a rotary microtome. After wax removing, sections were hydrated in a descending series of ethanol and stained with hematoxylin and eosin (H&E). The stained sections were examined and photographed using a BX53 microscope (Olympus Corporation, Tokyo, Japan).

Image Preprocessing: The quantification of histopathological changes between the control and experimental rat groups was performed and analyzed in detail using the ImageJ software

(version k 1.45). Histopathological images of the rat thymus, and spleen tissues stained with hematoxylin and eosin were used for image morphometry, which is the best conventional staining for comparisons. Measurements were performed in 10 low power field of (X: 100)/rat, except for the nuclear/cytoplasmic ratio, which was performed in 10high-power fields of (X: 400)/rat.

Statistical Analysis: SPSS for Windows (SPSS Inc., Chicago, IL, USA) was used for data analyses to compare the physiological data between the examined rat groups. Statistical analyses were performed via an analysis of the means using a one-way ANOVA followed by Tukey's post-hoc test (Tukey, 1949). The results are expressed as the means ±standard error of the mean, and different superscript letters symbolize a significant difference at $P<0.05$.

RESULTS

FT-Raman results: Figure 2 presents the FT-Raman spectra of garlic oil. Four major peaks were observed in the region between 3100 and 2900cm^{-1}. The first peak at 3086–3083cm_1 corresponds to the asymmetric stretch vibration of = CH2, the second peak at 3010cm^{-1}shows C–H stretching, the third peak at 2075cm^{-1} is the symmetric stretch vibration of = CH2 and the fourth peak at 2910cm^{-1} corresponds to – CH2 –stretching. The region from 1600 to 1000 showed four peaks. An intense peak at 1630cm^{-1} was assigned as the C = C stretching vibration of the allyl group. The peak at 1401cm^{-1} was assigned to the stretching of a – CH2 –group and the CH2 = CH –stretching shifted to 1300cm^{-1}. The skeletal vibration of diallyl sulfide molecule caused a peak at 1201cm^{-1}.

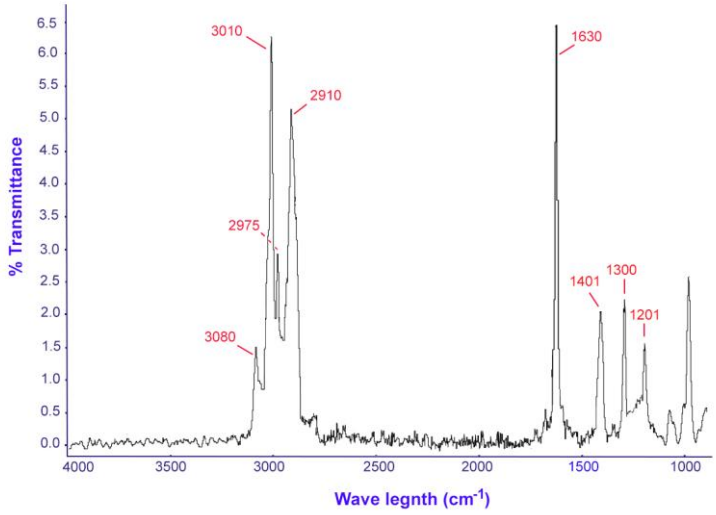

Figure 2. FT-Raman spectrum of garlic oil capsules showed four major peaks between 3100 and 2900cm^{-1}; the first at 3086–3083cm_1 (= CH2 asymmetric stretching), the second at 3010cm^{-1} (C–H stretching), the third at 2075cm^{-1} (= CH2 symmetric stretching) and the fourth peak at 2910cm^{-1} (– CH2 –stretching). The region from 1600 to 1000 showed four peaks; at 1630cm^{-1} (C = C stretching vibration), at 1401cm^{-1} (– CH2 –stretching group) and the CH2 = CH –stretching shifted to 1300cm^{-1}, at 1201cm^{-1} (diallyl sulfide molecule).

Parasitological study: Nine European sea bass out of 20 (45%) were naturally infected by nematode worms which were recovered from the peritoneal cavity as encapsulated larvae. The worm intensity ranged from 5 to 25 worms per fish (Figure 3a). Morphologically (Figure 3b–d), the recovered parasites were identified as *Anisakis* spp. The worms possessed slender bodies (0.67–2.8cm long and 0.2–15mm wide), with prominent boring teeth at the anterior extremity and four small papillae (two dorsolateral and two ventrolateral) surrounding their triangular mouths. Their cuticles had transverse striations that extended from the cephalic region prior to the anus. The postanal tail was rounded without a terminal mucron.

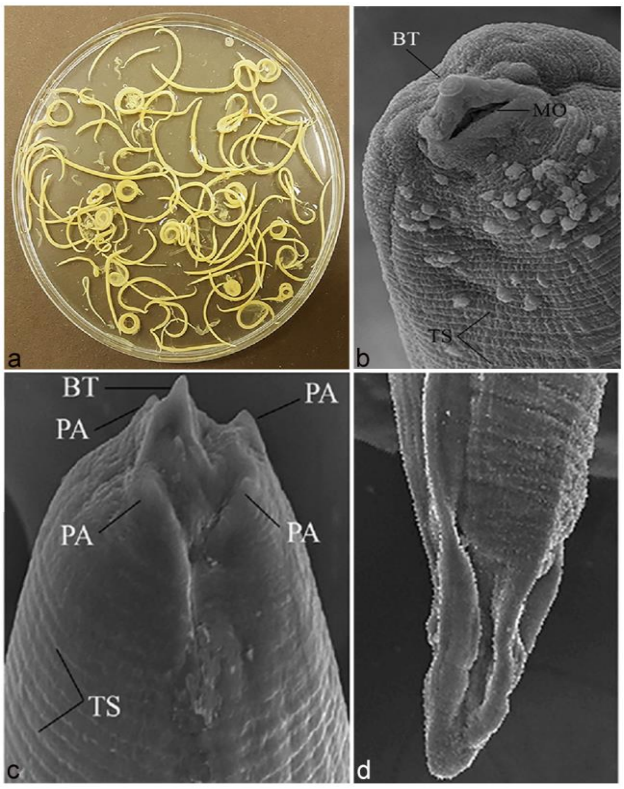

Figure 3. a. Photographs showing *Anisakis* Type II (L3) worms infecting *Dicentrarchus labrax*. b–d. SEM showing; b, c. The anterior part, triangular mouth (MO) with boring tooth (BT) and surrounded by four papillae (PA), TS transverse striation of cuticle, bars, 10µm. d. the posterior rounded end with no mucron, Bar, 100µm.

Oxidative Analyses: It was observed that the inoculation of L3 worms produced abnormal hepatic and renal oxidative changes (Figure 4a–f). These changes are evidenced by a significant elevation ($P<0.05$) in MDA and NO contents accompanied with a significant reduction ($P<0.05$) in the contents of GRD, SOD, and CAT compared with the control group. Conversely, a significant reduction ($P<0.05$) was recorded in the MDA and NO levels, as well as a significant elevation ($P<0.05$) in the GRD, SOD, and CAT in hepatic and renal tissue homogenates of the rat groups inoculated with GO in combination with fresh and thermally treated L3 worms in comparison with the untreated groups.

Histopathology: histopathological examination of the spleen and thymus tissues of the control and GO administrated rats showed normal histological features without concerning pathological evidence.

Thymus: The thymuses of the control and GO inoculated rats showed normal architectures, where the entire organ was lobulated and separated by trabeculae and enclosed by a collagenous capsule. Each lobule consisted of an outer cortex and an inner medulla (Figure 5a–c). In contrast, the thymus tissues from rats orally inoculated with fresh L3 crude extracts revealed a loss of their cortico–medullary distinction accompanied by a decrease in the number of lymphocytes marked by apoptosis and an increase in apoptotic bodies. The proliferation of medullary epithelial cells resulted in tubule formation along with congestion in blood vessels (Figure 5d, e), while the thymus tissues of thermally treated L3 orally inoculated rats were atrophied, with a complete loss of the cortico–medullary junction, a reduction of lymphocytes with an increase in apoptotic bodies and tangible body macrophages, cytoplasmic vacuolization of stromal cells, cyst formation, prominent hassle's corpuscles, and fibrotic changes (Figure 5f–k). In contrast, the thymus

tissues of rats inoculated with GO and fresh L3 extract were similar to the control thymus (Figure. 5l). On the other hand, the thymus tissues of rats inoculated with GO and thermally treated L3 extract were improved, with a mild loss of lymphocytes (Figure 5m). The thymus morphometry, produced by an image analysis of the cortex and medulla thickness, is shown in Table 1.

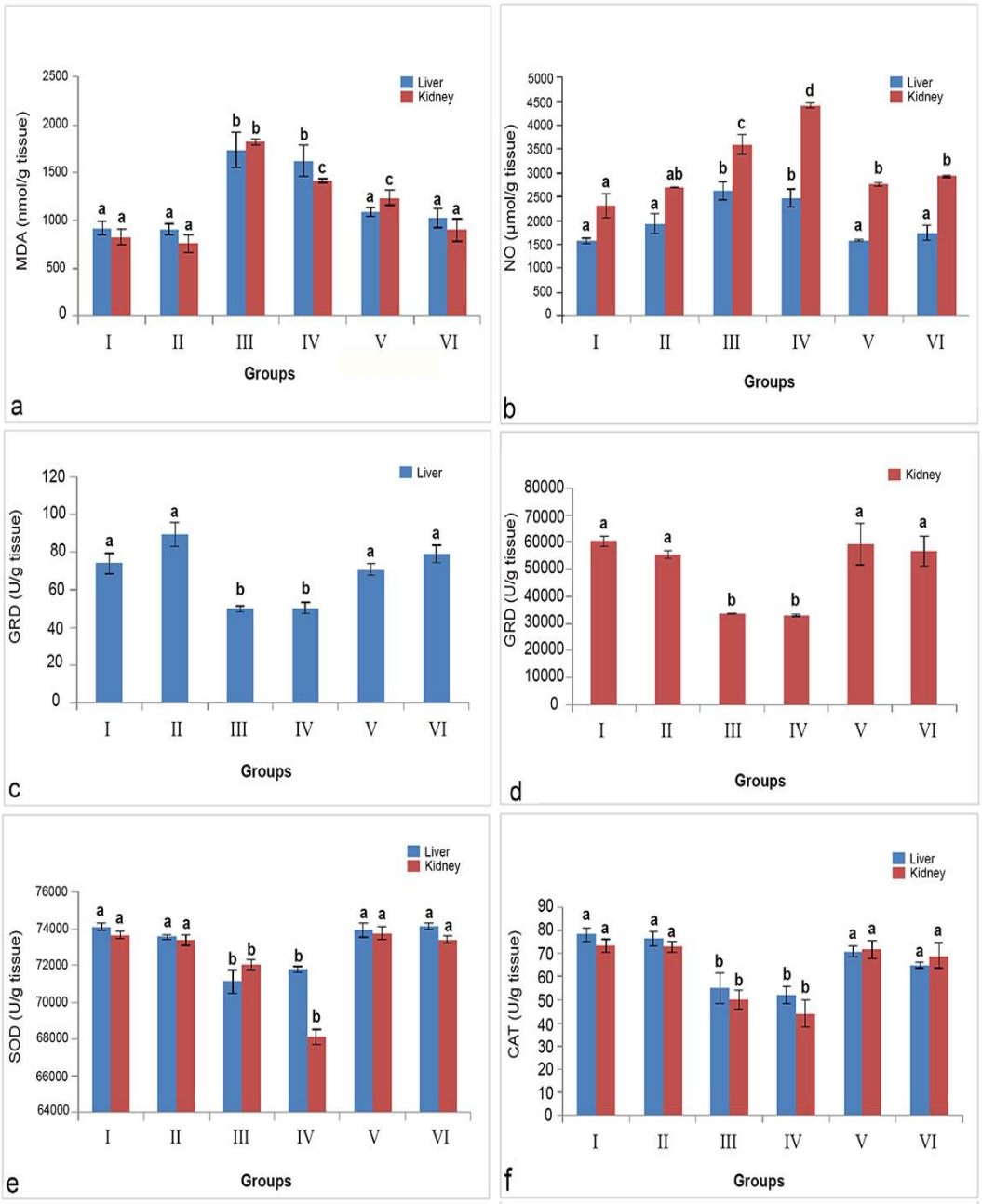

Figure 4. (a–f). Oxidative stress markers measured in liver and kidney of control (groups I, II) and experimental rats (group III–VI): a. Malondialdehyde *(*MDA). b. Nitric oxide (NO). c, d. Glutathione Reductase (GRD). e. Superoxide dismutase (SOD). f. Catalase (CAT).

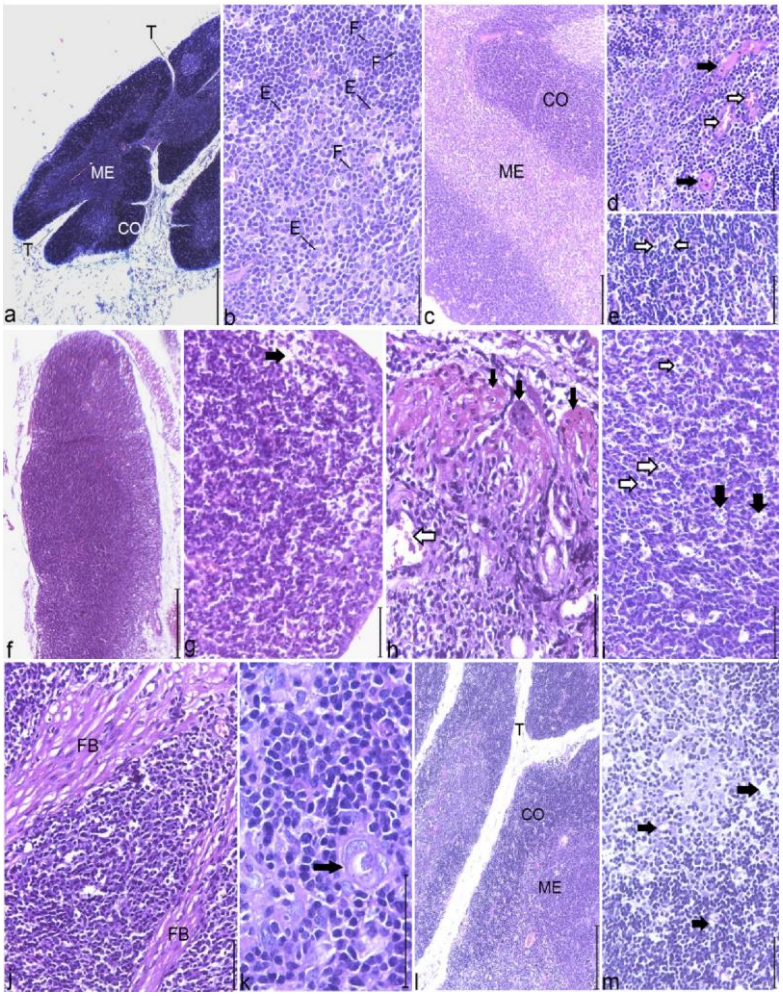

Figure 5.(a–m). Photomicrographs showing thymus sections (H&E) of: a, b. Control rats (group I), bars, 200µm. c: GO rats (group II); cortex (CO), medulla (ME), trabeculae (T), lymphocytes (L), thymic epithelial cell (E). (d, e). Fresh L3 inoculated rats (groups III) showing; d. tubules formation (black arrows), congested blood vessel (white arrows). E. Apoptotic bodies (white arrows). (f–k). Thermally treated L3 inoculated rats (group IV) showing; f. Atrophied thymus lobules with complete loss of cortico–medullary junction. g. Decreased cellularity (black arrows).h. Cytoplasmic vacuolization of stromal cells (white arrows), cyst formation (black arrows), i: Apoptotic bodies (white arrows), tangible body macrophages (black arrows). j. Fibrotic changes (FB) in medulla region. k. hassle's corpuscles (black arrow), bar, 200µm. l. GO + fresh L3 inoculated rats (group V) showing normal showing nearly normal thymus. M. GO + thermally treated L3 (group VI), almost normal with mild decreased cellularity (black arrows). Bars, 50µm.

Spleen: The spleen tissues of the control and GO inoculated rats showed normal architectures, with the surrounding capsule and the normal distribution of white and red pulps and their constituents (Figure 6a, b). The spleen tissues from rats orally inoculated with fresh L3 crude extracts exhibited apoptotic lymphocytes in their white pulp, and vacuolar degenerative changes in their stromal cells and diffused hemorrhagic areas with capsular fibrosis (Figure 6c, d).histological examination of the spleen tissues of thermally treated L3 orally inoculated rats revealed the same histological changes observed in the fresh L3 inoculated rats in addition to disorganized white pulp, scattered deposits of dark brown granular pigment, fibrotic trabeculae, and congested dilated blood vessels, accompanied by perivascular edema and inflammation and

hemorrhaging with prominent mega karyocytes in the red pulp (Figure 6e–h).

In contrast, the spleen tissues of rats inoculated with GO and fresh L3 extract showed a marked restoration and improvement of the spleen compartments (Figure 6i). However, the notable improvement of kidney tissues from the rats inoculated with GO and thermally treated L3 extract showed a mild loss of lymphocytes and less fibrotic trabeculae. (Figure 6j, k). The area percentages (%) of the white and red pulps of spleen tissues isolated from the control and experimental rat groups were analyzed by image analysis, as shown in Table 1.

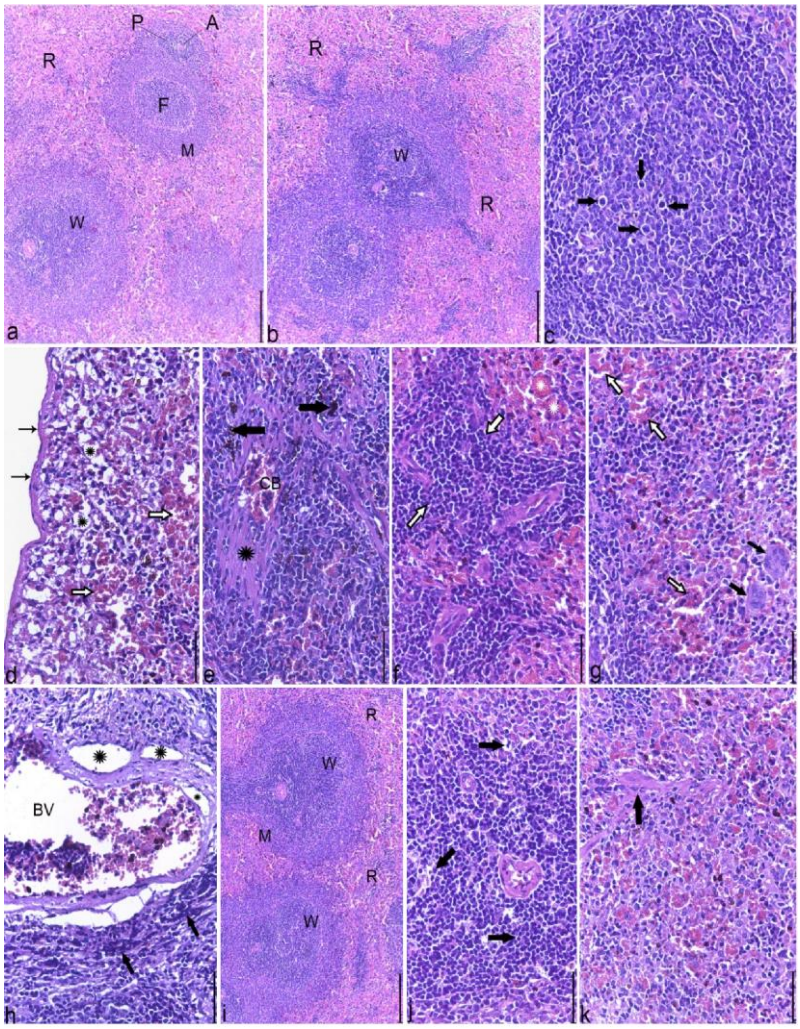

Figure 6. (a–k). Photomicrographs showing spleen sections (H&E) of; a. Control rats (group I), bar, 500µm. b. GO rats (group II); red pulp (R), white pulp (W), lymphoid follicle (F), marginal zone (M), central artery (A), periarteriolar lymphatics heath (P). (c, d). Fresh L3 inoculated rats (groups III) showing; c. apoptotic lymphocytes (black arrows), bar, 200µm. d. Capsular fibrosis (small arrows), vacuolar degenerative changes in stromal cell (*), diffused hemorrhagic areas (white arrows). (e–h). Thermally treated L3 inoculated rats (group IV) showing; e. congested blood vessel (CB), fibrotic trabeculae (*), pigment deposits (black arrows). f. Disorganized white pulp (white arrows), congestion (*). g. hemorrhage (white arrows), megakaryocytes (black arrows),h. Contested and dilated blood vessel (BV), perivascular edema (*) and inflammation (black arrows). i. GO + fresh L3 inoculated rats (group V) showing normal showing normal white and red pulps. (j, k). GO + thermally treated L3 (group VI), almost normal with; j. mild apoptotic lymphocytes (black arrows). k. mild fibrotic trabeculae (black arrow).

Table 1. Quantification of thymus and spleen pathology for the six experimental rat groups

	Organ /group	I	II	III	IV	V	VI
Thymus	Thickness of cortex	4.957 ± 0.541	5.308 ± 0.816	1.733 ± 0.571***	0.876 ± 0.577***	5.291 ± 0.504	4.885 ± 0.157
	Thickness of medulla	12.62± 1.633	13.19 ± 2.184	3.167± 0.995***	1.909 ±0.796***	12.23± 0.743	11.91± 1.083
Spleen	Area % of white pulp	31.39 ± 0.488	34.57± 0.619	15.18± 0.739 ***	11.47± 0.764 ***	29.21± 0.70	28.28 ± 0.421
	Area % of Red pulp	68.61± 0.488	65.43± 0.619	84.82± 0.739***	88.53 ±0.764***	70.79± 0.743	71.72± 0.421

Values are presented as mean ± SD; ***significant difference at $P < 0.05$ compared to control

DISCUSSION

The current study performed a morphological examination of worms recovered from examined host fish using light and scanning electron microscopy and revealed that the worms belonged to the genus *Anisakis* because they possessed all of the characteristic features of the genus according to the guide published by Pippy and van Banning (1975): inconspicuous three lips with a prominent boring tooth on the anterior end around the triangular mouth; a straight anterior gut consisting of an esophagus, ventriculus, and intestines; the posterior end terminating with or without a mucron; and a transverse lystriated cuticle. There are two morphotypes of genus *Anisakis*: Type I and II. The recovered worms in the current study were identified as *Anisakis* Type II, according to Berland (1961), due to the presence of a triangular mouth surrounded by four papillae with no lips and the rounded postanal tail without a terminal mucron. In contrast, Type I *Anisakis*spp. possesses a rounded mouth opening with dorsal and ventrolateral lips equipped with papillae and the postanal region terminates with a small mucron.

The levels of oxidative stress markers are an important indicator of the physiological state of an animal. The significant increase in the MDA level in the fresh and thermally treated rat groups may suggest enhanced lipid peroxidation subsequent to L3 administration, which reflects free radical mediated hepatic cell membrane damage which lead to transaminase leakage into the bloodstream (Dorostkar *et al.*, 2017; Li *et al.*, 2015; Nazarizadeh and Asri–Rezaie, 2016; Rizvi *et al.*, 2012). These findings are in parallel to the hepatocyte degeneration and the other lesions observed in the liver histology, which confirm hepatic lack of integrity and transaminase outflow. NO is essential in different biological functions, such as in muscular relaxation, and it act as an important mediator for a variety of neuropeptides in ionic channels (Dennis and Witting, 2017).

In the present study, an increase was observed in the level of NO in inoculated rats with both fresh and thermally treated L3. This agrees with a previous report, which suggested that NO plays an important role in the detoxification of the free radicals of oxygen resulting from the inoculation of crude parasite extracts (Barrett and Brophy, 2000; Minning *et al.*, 1999). Sod and Grd levels decreased in the rat groups inoculated with fresh and thermally treated L3, which agrees with Shafaquat *et al.* (2017), who stated that foreign bodies inoculated into animal bodies are known to generate reactive oxygen species (ROS). These ROS include superoxides, hydroxide radicals, oxides of nitrogen, and glutathione peroxide. The antioxidant system provides protection against the damage caused by ROS (Adeyemi, 2014). The antioxidant defense system consists of enzymatic and non-enzymatic antioxidants, which have been found to scavenge free radicals and ROS (Kamiński *et al.*, 2009). These enzymatic antioxidants mainly involve SOD, CAT, GRD, glutathione–S–transferase, and glutathione peroxide.

A decline in antioxidants enhanced the formation of ROS, resulting in a condition termed as oxidative stress**.** The increase in superoxide dismutase was observed to be caused by their active involvement in neutralizing the effects of free radicles, as observed in the treated groups. Moreover, the current results explained that the oral input of L3 crude extracts produced a marked increase in creatinine levels compared to the controls. High serum creatinine concentrations may be attributed to lower glomerular filtration in the kidneys and reflects a defect in the kidney

tubes in accordance with Eissa and Zidan (2010). Also, the increase in the levels of urea and uric acid in rat groups inoculated with fresh and thermally treated L3 compared to the control rats agrees with the study by Larsen *et al.* (2014), who claimed that, due to toxicity, ammonia is usually rapidly excreted either directly or is detoxified into other harmful nitrogenous molecules, such as urea and uric acid. According to Hammond *et al.* (1997), the continued and inappropriate uses of synthetic antihelmentic have led to the development of antihelmentic resistance.

This phenomenon has compromised the efficiency of current and future nematode control programs, prompting the use of plant extracts to control nematode infestations in their flocks (Hammond *et al.*, 1997). *A. sativum* is one such plant whose well documented anti-parasitic properties have prompted this research to investigate its antihelmentic ability. In the present study, severe pathological alterations were observed in the thymus, and spleen of rats orally inoculated with *Anisakis* L3 crude extracts. This result is in accordance with Arcos *et al.* (2014) and Fæste *et al.* (2014), who reported that *A. simplex* may cause disease in experimental animals, even without exposure to live worms.

In the present study, most of the thymus sections from the experimental rat groups inoculated with fresh and thermally treated L3 showed severe pathological changes and decreased cellularity, which is consistent with Stefanski *et al.* (1990), Elmore (2006), and AL–Kindi *et al.* (2009), who claimed that rats inoculated with fresh and processed larvae caused atrophy of the thymus with severe lymphocytic depletion and necrosis in both the cortical and medulla areas, with an increased number of apoptotic lymphocytes. Also, Smythe *et al.* (1971) and Dutz *et al.* (1973) stated that temporary thymus atrophy may be due to nutritional or infectious stress in animals and may cause prolonged and profound changes in the immune status and host defense.

Rat groups inoculated with fresh, thermally treated L3showed a severe congestion of the spleen's red pulp, no demarcation between the red pulp and white pulp, and an excessive distension of its sinuses by erythrocytes, in accordance with Stefanski *et al.* (1990), and Ward *et al.* (1999). These observations coincide with those reported by AL–Kindi *et al.* (2009), who concluded that the inflammatory lesions and degenerations of epithelial cells produced as a result of parasitic antigen administration are related to mononuclear cell infiltrates and the migration of white blood cells (mainly macrophages and lymphocytes) to the immune organs to attack and clear parasite antigen debris. The rat groups inoculated with GO and crude L3 extracts (either fresh or thermally treated) showed marked histological recovery with mild changes in the examined tissues.

This agrees with Seckiner *et al.* (2014) and Reem & El–Shinnawy (2015), who stated that GO extract can directly or indirectly stimulate the nitric oxide production of macrophages in an additive manner, thereby generating antifibrogenic substances and decreasing the number and size of the granuloma, which is considered a major pathway in the induction of protective immunity against parasitic disease. The ameliorative and protective effects of GO on the four tissues examined may be related to the design of GO inoculation before and after crude L3 extract administration. This observation is consistent with Reem and El–Shinnawy (2015), who reported that GO administration before and after parasite inoculation induced mild pathological changes that correlated to the statistically significant liver and kidney functions of these groups.

CONCLUSION

To avoid nematode infestations and their implications, fish should be inspected well before eating them by the candling process; this process involves examining fish fillets over lights to detect surface parasites that should be removed. Fish with a heavy infestation of worms on their muscles and internal viscera should not be eaten. Moreover, fish meals should be eaten with garlic, which has a history of tissue protection against parasites. Garlic also detoxifies while gently stimulating elimination and has antioxidant properties to protect against the oxidation caused by parasitic toxins. Also, one should buy fish from a trusted store where they carefully choose healthy fish for consumers.

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