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Abstract:

The current study aimed to evaluate the in vitro scolicidal action of pumpkin seed oil (PSO) alone or combined with nitazoxanide (NTZ). Also, the clinical efficacy and protective effect of PSO alone or combined with NTZ against complications of secondary hydatidosis were detected in vivo. Collected protoscolices (PSCs) from camels were exposed to concentrations of 10 µg/ml and 750 mg/ml from NTZ and PSO, respectively or their combination for 5-, 15-, 30- and 60-min in vitro. Viability reduction and ultrastructural changes were evaluated by light and scanning electron microscopy. Fifty-six rats were divided into eight equal groups: Groups 1, 2, negative and vehicle; group 3, received 500 PSCs of hydatid cyst intraperitoneally; group 4, received NTZ at a dose of 50 mg/kg BW; group 5, received PSO at a dose of 250 mg/kg BW; groups 6-8, animals infected as group 3 and treated as groups 4, 5 and their combination, respectively. Treatments continued orally for 14 days then samples collected for anti-echinococcus immunoglobulin G and cytokines estimation, hemato-biochemical study and histopathological examination. The combination treatment in vitro provoked greater scolicidal action and ultrastructural alterations in PSCs compared to the monotherapy. The combined treatment group produced effective scolicidal action in vivo, inhibited inflammatory condition, induced immunomodulatory effect and improved the alterations in hemato-biochemical parameters and histopathological picture in examined tissues. In conclusion, this treatment strategy may provide a new efficient and safe way for echinococcosis treatment in the future and may recommend the use it before, during and after surgical removal of hydatid cysts.

Keywords: Echinococcus granulosus, hemato-biochemical, nitazoxanide, pumpkin seed oil, scolicidal, ultrastructure

INTRODUCTION

The cystic echinococcosis (CE) or Hydatidosis is an illness that is occurred by Echinococcus granulosus larval stage (Possenti et al., 2016). This disease is zoonotic and causing animals casualties (Torgerson, 2003). Also, it causes high morbidity and mortality rates in human being and is accounting for severe health, social and economic aspects (Torgerson and Macpherson, 2011). CE mainly found in areas where humans, dogs and herbivores in close contact with each other (Eckert and Deplazes, 2004). In this situation, human besides livestock act as intermediate (incidental/ aberrant) host where the cysts develop in the diverse organs, while the dogs act as the final host where adult tapeworm survive in their intestine (Brunetti et al., 2010; Galeh et al., 2018).

Hydatidosis effect on the intermediate host based on the size and site of the cyst, so can say that large cysts in rigid boundaries of the body such as the brain or lungs could be so critical (Daryani et al., 2007). On the
other hand, the spilling of cyst contents due to trauma or surgery may release cyst fluid that can disseminate to develop new other cyst in a new place with a highly antigenic content may induce possible fatal anaphylaxis (Derbel et al., 2012; Bhutani and Kajal, 2018; Salemi et al., 2021).

Treatment options for hydatid cysts are few and depend mainly on surgery followed by prophylactic anti-parasite treatment (Siles-Lucas et al., 2017), but the risk of leak the cyst contents intraoperative and formation of secondary cysts in body organs after surgery, still the fundamental problem of this way of treatment. For that reason, usage of efficient scolicidal agents may be obligatory in postoperative care cases nearly 10% (Brunetti et al., 2010). Till now, a number of studies have been performed on the drug treatment of hydatid cyst in human being and animals, some of them have been successful (Sadeghi et al., 2020). Due to the possible adverse effect of scolicidal agents, developing some new ones that have good efficacy and less side effects is necessary (Smego and Sebanego, 2005; Stamatakos et al., 2009; Kohansal et al., 2017).

Nitazoxanide (NTZ) is a drug that acts as antibacterial, anti-protozoal and even anti-cestode parasites such as Echinococcus species (Gilles and Hoffman, 2002 and Stettler et al., 2003). NTZ Combined with Albendazole were more effective in mice infected with E.multilocularis. (Stettler et al., 2004).

In order to use alternative, sustainable and less toxic agents, phytotherapy became the target (Makkar et al., 2007). Nowadays, different Cucurbita species seeds were used as herbal alternatives for the control of parasitic infection with medical efficacy, which could eliminate of various types of parasites because of their different secondary metabolites contents such as nitrogen-containing compounds such as (cucurbitin and cucurbitacin B), sterols, saponins and primary metabolites contents, such as proteins (curcumosin), sugars and fats (fatty acids) as well as its properties, which enhancing the resistance to infectious disease (Brooker and Acamovic, 2005; Li et al., 2009; Grzybek et al., 2016).

Pumpkin (Cucurbita maxima) as an edible fruit from Cucurbita species are known for their different health advantages and so was used in folk medicine for a long time around the world (Kim et al., 2012). It’s extract or oil has been used worldwide for treatment of different disease condition (Ayaz et al., 2015; Lestari and Meiyanto, 2018).

The presented study aimed to evaluate the scolicidal action of pumpkin seed oil (PSO) on protoscolices (PSCs) of hydatid cysts either alone or combined with nitazoxanide (NTZ), biological and non-biological trials have been done. The viability reduction percentages and light and scanning electron microscopic alterations have been reported. An in vivo assay was designed to prove the clinical efficacy and safety of treated agents as well as the protective effect of PSO alone or combined with NTZ against complications of secondary hydatidosis by determining the anti-echinococcus IgG, hemato-biochemical changes and histopathological alterations.

MATERIAL AND METHODS

Protoscolices (PSCs) collection:

PSCs of E. granulosus were aseptically collected from hydatid cysts from the lungs and livers of infected camels slaughtered in Belbeis, El-Basatin and Kom Hamada abattoirs in Sharkia, Cairo, and Beheira Provinces, Egypt. The samples were transferred directly in an icebox to Parasitology and Clinical Pathology departments, Faculty of Veterinary Medicine, Zagazig University. The fluid and other content were aseptically separated according to (Yones et al., 2014; Maurice et al., 2021).

Nitazoxanide and pumpkin seed oil:

(DMSO) and was reached to optimal used concentration by dilution with normal saline. For in vivo assay, NTZ was dissolved in DMSO and diluted to the desired concentration with normal saline before treatment. Pumpkin (C. maxima) seed oil was purchased from Intenan Health Company, Cairo, Egypt as natural cold pressed oil.
the in vitro assay, the essential oil was solubilized in DMSO in addition to use Tween-20 to promote the dispersion of the essential oil in normal saline to obtain the appropriate used concentration. The concentration of DMSO for in vitro use was below 1.5% and that in vivo use was 1.5%.

Gas chromatography/mass spectrometry (GC/MS) analysis of pumpkin seed oil:
GC analysis was performed using a Trace GC-TSQ mass spectrometer (Thermo Scientific, Austin, TX, USA). The oil components after being GC analyzed, were specified by comparing of their mass spectra with those of WILEY 09 and the National Institute of Standards and Technology 14 (NIST 14) mass spectral database (El-Kareem et al., 2016).

PSCs viability assay and light microscopy (LM) morphological changes:
In the current study, the scolicidal action of NTZ and PSO were tested at concentrations of 10 µg/ml according to Walker et al. (2004) and 750 mg/ml referring to Al-Juboori et al. (2020), respectively. The PSCs-rich sediment from hydatid cysts (500 µl) was transferred to 1.5 ml Eppendorf tubes, then 500 µl of each treatment was added to Eppendorf tubes either separately or in combination. PSCs-incubated in Eppendorf tubes contained normal saline 0.9% solution plus DMSO and the Tween-20 served as vehicle one, while tubes containing non-treated PSCs were used as control one (Yones et al., 2014). The contents of the tube were slowly mixed and incubated at 37°C for 5, 15, 30 and 60 minutes (min). All experiments were performed in triplicate per treatment condition and repeated three times. The PSCs viability was determined by using eosin staining exclusion test via adding 10 µl of 0.1% aqueous solution of eosin stain to 10 µl of collected PSCs pellet and examined microscopically within two min. The dead one absorbed the stain and colored red, while the live remained unstained (Maurice et al., 2021). The viability reduction percentages were calculated by counting red stained PSCs within three hundred tested PSCs at the end of each time point for each treatment condition.

Scanning electron microscopy (SEM) ultrastructure changes:
At the end of each incubation time with different treatments, the treated and control PSCs were prepared for Scanning Electron Microscopy examination according to Maurice et al., 2021.

Experimental rats:
Fifty-six healthy Wistar albino rats (Rattus norvegicus; 10 weeks old; 185–210 g; male) were purchased from the laboratory animal house located in Faculty of Veterinary Medicine, Zagazig University, Egypt. Rats were housed under controlled environmental conditions. They were pliable with free access to standard pelleted feed and drinking water throughout the experiment. All rats were assessed daily for health condition and adapted to the new experimental environment for one week pre-initiation of the study.

In vivo assay:
The fifty-six rats were split at random into eight groups (7 rats/group). Group1, rats were received standard feed and water only (negative control); group 2, rats received DMSO 1 ml/kg BW in saline solution as a vehicle group; group 3, animals received 500 PSCs (>95% viability) intraperitoneally (positive control); group 4, animals received nitazoxanide orally at a dose of 50 mg/kg BW and the dose selection based on recommended dosage used in human (Fox and Saravolatz, 2005; NTZ-treated); group 5, animals received pumpkin seed oil at a dose of 250 mg/kg BW orally, considering that (LD50 oral) for the rat was >2000 mg/kg BW according to EU (1999; PSO-treated); group 6, animals were inoculated with PSCs as group 3 and administered NTZ as same dose and route of group 4 (Inf+ NTZ); group 7, animals were inoculated with PSCs as group 3 and administered PSO as same dose and route of group 5 (Inf+PSO) and group 8, animals were inoculated with PSCs as group 3 plus NTZ and PSO as the same dose and route of groups 3 and 4 (Inf+NTZ+PSO). Treatment administration started 48 hours post inoculation of hydatid cyst PSCs via using the esophageal tube and continued for 14 consecutive days. The rats were monitored for body weight and the visual observations for animal groups were
conducted once daily for physical appearance of animals, presence any signs of illness and any mortality till the end of experiment.

**Sampling:**
Blood samples were collected 14 days post-starting NTZ and PSO treatments via retro-orbital venous sinus puncturing from overnight fasted animals from all experimental groups post-anesthesia by sodium pentobarbital. Collected blood samples were dispensed into clean plain test tubes for serum separation for measuring specific anti-echinococcus IgG, cytokines and the different biochemistry parameters as well as into clean Wasserman tubes containing dipotassium salt of ethylenediamine tetraacetic acid (EDTA) for proceeding distinct hematological tests. Anesthetized experimental animals were euthanized by decapitation and the liver, kidneys and spleen were excised quickly for histopathological assessment.

**Antigen preparation and anti-echinococcus immunoglobulin G estimation:**
Hydatid cyst fluid (HCF) was aseptically separated from hydatid cysts of slaughtered camels and prepared as the test antigen (Fotoohi et al., 2013). The diagnostic specificity, sensitivity, and efficiency of crude HCF Ag were calculated according to (González-Sapienza et al., 2000).

Anti-echinococcus immunoglobulin G (IgG) estimation executed using indirect enzyme-linked immunosorbent assay (ELISA) as described by Golassa et al., 2011.

**Serum cytokines:**
The concentrations of serum interleukin 10 (IL-10) and interferon gamma (IFN-γ) were measured by using sandwich-CLIA kits purchased commercially from Elabscience by following of the manufacturer’s instructions.

**Hematological and biochemistry assays:**
Hematological parameters were determined including the number of red blood cells (RBCs) and thrombocytes, hematocrit (Ht) value, hemoglobin (Hb) content and total and differential white blood cells number were evaluated utilizing an automated blood cell counter (Buttarello and Plebani, 2008).

Serum biochemistry parameters, including activities of alanine aminotransferase (ALT), aspartate transaminase (AST), gamma-glutamyl transferase (GGT) and levels of creatinine, urea, total proteins and albumin were estimated using obtainable diagnostic kits purchased from Spectrum Diagnostics, Egypt according to the manufacturer instructions. The total globulins fraction was estimated basically by subtracting the albumin from the total proteins.

**Histopathological assessment and lesions scoring:**
Fresh tissues were obtained from different animal groups (spleen, liver and kidneys) then fixed in 10% formalin solution, after that the fixed tissues were switched to increasing degrees of ethanol for dehydrating process, then were refined in xylene shifts then were impregnated in paraffin wax and finally the blocks were divided into sections 5-μm thickness and stained with hematoxylin and eosin (H&E). The prepared sections were examined to disclose the histopathological changes and photographed under a light microscope (Suvarna et al., 2013). Scoring the lesions was rated semi-quantitative according to the intensity of the different histopathological alterations that were spotted in inspected tissues (Gibson-Corley et al., 2013).

**Statistical Analyses:**
Data of the present study were analyzed via using SPSS software (version 21, IBM Corporation) with utilized one-way ANOVA and Tukey’s post hoc test according to Snedecor and Cochran (1994). The data were plotted using GraphPad Prism 8. Obtained values were represented as mean ±SE. The differences in values were regarded statistically significant at p <0.05.

**Ethics approval**
All experimental procedures and protocol were approved by Zagazig University Institutional Animal Care and Use Committee “ZU-IACUC”, Egypt (Approval No: ZU-IACUC/2/ F/90/ 2022).

**RESULTS**

**Chemical composition of pumpkin seed oil:** Data on common PSO bioactive compounds which obtained from the
GC-MS analysis are presented in Table 1.

**PSCs viability assay and LM morphological changes:**
Viability loss (stop movements either in invaginated or evaginated PSCs) after exposure to different treatments compared with each other as well as with the control group in various exposure times. PSCs viability was reduced in NTZ treated group to be 43, 59.66, 63.22 and 64.77%, after 5, 15, 30, and 60 min, respectively. As well as in PSO treated group at the same exposure times, it reached 55, 66.11, 68.66 and 79.66 %, respectively. On the other hand, the viability of PSCs after exposure to NTZ and PSO combination at the same exposure times reduced to reach 66.33, 70.33, 75 and 85 %, respectively. Although all treated groups showed noticed viability reduction, the combination of NTZ+PSO triggered a stronger action than each treatment alone. Statistical analysis using the ANOVA test revealed that there was a highly significant difference between the mean scolicidal actions of NTZ and PSO as well as the combined treatment compared to control group at different exposure times (p < 0.001). The results displayed the highest reduction in viability percentage (85%) of PSCs post-exposure to combined treatment of NTZ and PSO at time point (60 min). The vehicle group exhibited an insignificant change in the PSCs viability in comparison with the control group at different exposure times (Fig.1A).

By light microscope, the dead PSCs absorbed the stain and colored red, but the live ones remained colorless with characteristic motility resulting from the activity of the flame cells and movements of rostellar and sucker muscles. PSCs in both negative control and vehicle one revealed normally intact cuticular integrity and arranged hooks on the rostellum with distinct movements (Fig. 2 A, B). They appeared clear with intact calcareous corpuscles, hooks and suckers. By the time, most of the PSCs had become evaginated with obvious suckers. PSCs exposed to NTZ (10 µg/ml) showed hooks disorganization after 15 min. Tegument discontinuity, hook losses, disrupted rostellum and neck region shrinkage were observed after 30 min later. Moreover, 60 min post-exposure tegument discontinuity, complete rostellum and hooks disruption were observed. While no changes were observed after 5 min from exposure (Fig. 2 C-F). PSCs treated with PSO (750 mg/ml) exhibited partial disorganization and loss of hooks after 5 min. Additionally, more disorganization and loss of hooks were exhibited in PSCs after 15 min. After 30 minutes, there was detachment in the tegument, appearance of the knob-like projection and extensive hook losses in treated PSCs. Finally, complete loss of integrity was observed 60 min post-exposure (Fig. 2 G-J). PSCs exposed to the combined treatment of NTZ (10 µg/ml) and PSO (750 mg/ml) showed slight collapse in suckers and rostellum with intact microtriches and flame cells (indicated still viable protoscolex), and appearance of the knob-like projection in the integument after 5 min from the exposure. Partial disorganization and loss of hooks, damaged rostellar cone, detached and discontinued tegument with bright stained suckers and corpuscles were noticed 15 min later. While retracted PSCs with few rostellar hooks were observed after 30 min. After 60 min of exposure, the PSCs showed a damaged sucker region and contracted neck and soma regions (Fig. 2 K-N).

**SEM ultrastructure changes:**
The ultrastructural changes appeared more obvious in evaginated than in invaginated PSCs. PSCs in both negative and vehicle control groups didn’t show any changes (Fig. 3A, B). In case of using NTZ (10 µg/ml), the PSCs showed hooks disorganization and suckers disruption, slightly contracted neck and soma regions after 5 min. More shrinkage in the neck region and hook loss was observed 15 min later. Complete loss of hooks, collapse in the rostellum, suckers, and soma region were noticed after 30 min. On the other hand, after 60 min, complete loss of morphological integrity with multiple small balloon-like blebs formation were
noticed. Microtriches appeared intact especially in suckers and rostellar regions after 5, 15, and 30 min. While they were lost 60 min post-exposure (Fig. 3 C-G).

In PSCs treated with PSO (750 mg/ml) exhibited hooks disorganization and slightly contracted suckers, neck and soma regions after 5 min. Additionally, partial loss of hooks and collapse in soma region with small blebs were noticed in PSCs after 15 min. On the other side, 30 min post-exposure there was complete loss of hooks, rostellar shrinkage with digitiform like extensions, and collapse in the neck and soma regions. Finally, complete loss of integrity with multiple small blebs was observed within 60 min from exposure. Microtriches appeared intact especially rostellar region only after 5 min. While, they were lost after 15, 30 and 60 min post-exposure (Fig. 3 H-K).

Moreover, after exposure to combined treatment of NTZ and PSO, PSCs showed shrinkage in the suckers and soma region with partial hook losses after 5 min, whereas the shrinkage and hook losses increased after 15 min post-exposure. Complete loss of hooks, damaged rostellar cone with blebs appearance and digitiform like extensions, complete loss of microtriches and contacted neck and soma regions were noticed 30 min later. After 60 min post-exposure to treatments, retracted PSCs with complete loss of integrity in the form of holes covering the damaged tegument was clearly obvious. The microtriches were partially lost after 5 min in the rostellar region (above suckers). While they were completely lost after 15-, 30- and 60-min post-exposure (Fig. 3 L-P).

Animals’ health evaluation:

The animals in the control, vehicle, PSO-treated groups showed normal health appearance throughout the entire experimental period. While the treatment of animals with NTZ only provokes signs of weakness and decreased animals’ activity. On the other hand, the inoculated animals with PSCs without any treatment showed various symptoms that represented in loss of appetite, reduction in the body weight, signs of discomfort, movement reduction, arching of the back, labored respiration and rough hair coat till the end of the experiment. Treatment of

inoculated animals with PSCs either with NTZ or PSO or their combination reduced the clinical manifestations, especially in the combined treatment group. No mortality was noticed in any experimental groups during the experimental course, except for the inf only and Inf+NTZ groups, where one rat died from each group.

Anti-echinococcus IgG findings:

ELISA values in experimentally infected rats' sera showed 75% sensitivity and 100% specificity with 83.33% efficiency for crude HCF Ag. A significant increase (p < 0.001) in specific serum anti-echinococcus IgG values in all experimentally infected groups compared with the control non-infected group was observed. The infected group treated with PSO showed an insignificant increase and the infected group treated with a combination of NTZ and PSO showed an insignificant decrease in serum anti-echinococcus IgG values if compared to the infected group only, respectively (Fig.1B).

Serum cytokine findings:

The concentration of serum IL-10 was significantly lower (p < 0.001) than the control group in Inf and Inf+PSO groups and the lowest value was spotted in the Inf group, while Inf+NTZ and Inf+NTZ+PSO groups showed an insignificant decrease compared with control group. However, PSO-treated group showed a significant elevation in IL-10 value and the vehicle and NTZ groups showed a non-significant change in compared to the control group (Table 2).

Serum IFN-γ level showed a significant elevation in (p < 0.001) in Inf and Inf+NTZ+PSO groups compared to the control group, the highest value was predestined in Inf+NTZ+PSO group. On the other side, significant and insignificant decrease were observed in this parameter in Inf+PSO and Inf+NTZ, respectively, in compared with the control one (Table 2).

Hematological and clinical biochemistry findings:

As seen in Table 3, a significant (p < 0.001) elevation was noted in RBCs count, Ht value and Hb content in Inf, Inf+PSO and Inf+
NTZ+PSO groups as well as PSO-treated group showed a significant elevation in RBCs count concerning the control group. Non-significant change was predestined in vehicle, NTZ-treated and Inf+NTZ groups in comparison to control one. Co-administration of NTZ and PSO in infected rats significantly increased RBCs count, Ht value and Hb content in compared with the Inf alone, Inf+PSO and Inf+NTZ groups and on the other side, in the presence of infection administration of NTZ significantly decreased RBCs count, Ht value and Hb content in compare with Inf alone group. Furthermore, thrombocytosis (p < 0.001) was observed in the Inf, Inf+NTZ, Inf+ PSO and Inf+NTZ+PSO groups compared to control group, while non-significant change was noticed in the vehicle, NTZ-treated and PSO-treated groups in comparison to the control group. Receiving NTZ or PSO alone or in combination in the presence of infection significantly reduced the thrombocytes count in compared to the Inf alone group, the lowest value was noted in Inf+NTZ+PSO group.

Leukocytosis, monocytes and eosinophilia were seen in Inf, Inf+NTZ and Inf+ PSO groups compared to the control one (p < 0.001). While rats in Inf, NTZ, Inf+NTZ, Inf+ PSO and Inf+NTZ+PSO groups showed neutrophilia compared to the control group. On the other side, lymphocytosis was observed in the Inf and Inf+PO groups, while lymphopenia was noticed in NTZ and Inf+NTZ+PSO groups in comparison with control group. No significant change in the count of total leukocytes, monocytes and eosinophils were observed in the vehicle, NTZ-treated, PSO-treated and Inf+NTZ+PSO groups in comparison to the control one. Whereas insignificant change in the count of neutrophils and lymphocytes were noticed in the vehicle and PSO-treated groups besides Inf+NTZ group regarding the lymphocytes count compared with the control group (Table 4).

The results shown in Figs. 4,5 represent the changes in different biochemical tests, a significant lowering (p < 0.001) was noticed in the serum activity of ALT in Inf, Inf+NTZ, Inf+ PSO and Inf+NTZ+PSO groups, while the significant increase was noticed in NTZ-treated group in compared to the control one. Administration of NTZ or PSO or their combination in the presence of infection caused a significant elevation in serum ALT activity compared to Inf alone group, the highest value was noticed in Inf+NTZ group (Fig. 4A). On the other hand, serum AST activity was significantly elevated (p < 0.001) in the Inf, NTZ, Inf+NTZ, Inf+ PSO and Inf+NTZ+PSO groups compared to the control animals, the highest value was noticed in Inf+NTZ+PSO group (Fig. 4B). While serum GGT activity significantly increased (p < 0.001) in Inf+NTZ, Inf+ PSO and Inf+NTZ+PSO groups compared with the control (Fig. 4C). Insignificant change in serum activities of ALT, AST and GGT was observed in the vehicle and PSO-treated groups in comparison to control group in addition to a non-significant change in activity of serum GGT was noticed in the Inf and NTZ-treated groups in comparison to the control animals.

Serum creatinine level increased significantly (p < 0.001) in Inf+NTZ+PSO group in relative to the normal control, while a non-significant change in this parameter was observed in other groups in comparison to the control group (Fig. 4D). On the other hand, serum urea level showed a significant increase (p < 0.001) in the Inf, Inf+NTZ, Inf+ PSO and Inf+NTZ+PSO groups compared to control animals. Administration of NTZ or PSO alone in the presence of infection significantly decreased serum urea level compared with the rats that received infection alone, while coadministration of NTZ and PSO in infected rats caused a significant increase in the level of serum urea relative to the Inf only group (Fig. 4E).

Hyperproteinemia (p < 0.001) was observed in Inf, Inf+PSO and Inf+NTZ+PSO groups, the highest value was observed in Inf+NTZ+PSO group. Insignificant increase in this parameter was noticed in Inf+NTZ group in addition to non-significant change in vehicle, NTZ-treated and PSO-treated groups compared to the control animals (Fig. 5A).
Serum albumin level exhibited a non-significant change in all experimental rats’ groups in compare with control rats (p=0.003), while the Inf+NTZ+PSO group showed an insignificant increase in compared to the Inf group as well as Inf+NTZ and Inf+PSO showed insignificant decrease in compare with the Inf group (Fig. 5B). On the other hand, a significant increase (p < 0.001) in serum globulins level was noticed in Inf, Inf+NTZ, Inf+PSO and Inf+NTZ+PSO groups compared to the control animals. Receiving of PSO along with NTZ in the presence of infection significantly increased serum globulins level compared with the rats that were infected alone (Fig. 5C).

**Histopathological findings and lesions scoring:**

Spleen sections of rats in the control, vehicle and PSO groups showed apparently normal splenic architectures including normal sized white pulps with a germinal center and marginal zone around the central arterioles, normal red pulps among trabeculae (Fig. 6A,B,F). While that from parasitic infected animals exhibited marked atrophy due to depletion of the white pulp with permanent trabeculae and very widening of red pulp (Fig. 6C). The high magnification of the previous photomicrograph showed decrease cellularity in periarteriolar area associated with compensatory widening of red pulp sinusoids (Fig. 6D). Other alterations in NTZ-treated and infected-treated groups were reported (Fig. 6 E,G,H,I).

Hepatic tissue in the control, vehicle and PSO groups showed apparently normal hepatic architectures including hepatic lobules arranged among portal triads, including the portal artery, vein and bile ducts. Moreover, central veins and normal hepatocytes arranged as a hepatic cord followed by Kupffer cells individually through hepatic sinusoids (Fig. 7A, B,F). Rat’s liver sections from the parasitic infected group showed multifocal necrotic areas and inflammatory cells (mainly lymphocytes) aggregations around portal triads (Fig. 7C,D). Different changes in NTZ-treated and infected-treated groups were photographed and reported (Fig. 7 E,G-J).

The micro-morphological findings of H&E-stained sections of kidneys in the control and vehicle groups exhibited normal cortical glomeruli among proximal tubules where with magnification showed normal glomerular tufts, spaces and capsules, followed by apparently normal renal tubules (Fig. 8A,B). However, kidneys of the parasitic infected group exhibited degenerated glomeruli, interstitial nephritis characterized by perivascular edema, lymphocytic infiltration among degenerated renal tubules (Fig. 8C). Pathological changes in renal tissues from other groups treated only or infected+ treated were photographed and reported (Fig. 8 D-I).

Lesions score was summarized and illustrated in (Table 5), where marked alterations in examined tissues was observed in infected group and marked amelioration of lesions was observed in the combined treatment group.
Table 1: Identified chemical compounds of pumpkin (C. maxima) seed oil determined by GC-MS

<table>
<thead>
<tr>
<th>Components</th>
<th>R.t (min)</th>
<th>Relative %</th>
</tr>
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<tbody>
<tr>
<td>Hexadecanoic acid, methyl ester</td>
<td>20.77</td>
<td>21.65</td>
</tr>
<tr>
<td>9,12-Octadecadienoic acid (Z,Z)-, methyl ester</td>
<td>23.39</td>
<td>5.90</td>
</tr>
<tr>
<td>9-Octadecenoic acid (Z)-, methyl ester</td>
<td>24.10</td>
<td>40.92</td>
</tr>
<tr>
<td>Methyl stearate</td>
<td>24.25</td>
<td>16.67</td>
</tr>
<tr>
<td>Linoleic acid ethylester</td>
<td>24.69</td>
<td>1.13</td>
</tr>
<tr>
<td>cis-13-Eicosenoic acid</td>
<td>25.28</td>
<td>0.84</td>
</tr>
<tr>
<td>Methyl 9-eicosenoate</td>
<td>26.06</td>
<td>1.22</td>
</tr>
<tr>
<td>Eicosanoic acid, methyl ester</td>
<td>26.44</td>
<td>2.45</td>
</tr>
<tr>
<td>Octadecenoic acid, 9,10-dihydroxy-, methyl ester</td>
<td>26.52</td>
<td>0.70</td>
</tr>
<tr>
<td>Docosanoic acid, methyl ester</td>
<td>28.97</td>
<td>0.21</td>
</tr>
<tr>
<td>Tetraicosanoic acid, methyl ester</td>
<td>31.38</td>
<td>0.61</td>
</tr>
<tr>
<td>2-Oleoylglycerol, 2TMS derivative</td>
<td>31.52</td>
<td>0.34</td>
</tr>
<tr>
<td>Squalene</td>
<td>32.40</td>
<td>94.05</td>
</tr>
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</table>

R.t: Retention time

Table 2: Serum IL-10 and IFN-γ values for various experimental groups post treatment.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Parameters</th>
<th>IL-10 (pg/ml)</th>
<th>IFN-γ (pg/ml)</th>
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<tbody>
<tr>
<td>Control</td>
<td></td>
<td>254.78 ±2.154</td>
<td>175.30±4.837</td>
</tr>
<tr>
<td>Vehicle</td>
<td></td>
<td>253.37± 0.900</td>
<td>179.17±1.090</td>
</tr>
<tr>
<td>Inf</td>
<td></td>
<td>231.54±6.575</td>
<td>234.15±4.053</td>
</tr>
<tr>
<td>NTZ</td>
<td></td>
<td>269.75±2.173</td>
<td>174.32±0.837</td>
</tr>
<tr>
<td>PSO</td>
<td></td>
<td>319.04±2.370</td>
<td>173.30±1.021</td>
</tr>
<tr>
<td>Inf+ NTZ</td>
<td></td>
<td>241.13±3.386e</td>
<td>160.36±6.363e</td>
</tr>
<tr>
<td>Inf+ PSO</td>
<td></td>
<td>236.00±2.309e</td>
<td>150.36±3.497</td>
</tr>
<tr>
<td>Inf+ NTZ+PSO</td>
<td></td>
<td>247.07±0.354e</td>
<td>253.71±2.299</td>
</tr>
</tbody>
</table>

p-value <0.001 <0.001

Table 3: Erythrogram values for various experimental groups post-treatment.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Parameters</th>
<th>RBCs (×10⁶/μl)</th>
<th>Ht (%)</th>
<th>Hb (g %)</th>
<th>Thrombocytes (×10⁹/μl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td></td>
<td>6.77±0.109e</td>
<td>44.26 ±0.206e</td>
<td>14.34±0.097d</td>
<td>635.60±2.400e</td>
</tr>
<tr>
<td>Vehicle</td>
<td></td>
<td>7.02±0.055de</td>
<td>44.54 ±0.134ed</td>
<td>14.49±0.045cd</td>
<td>649.20±2.853d</td>
</tr>
<tr>
<td>Inf</td>
<td></td>
<td>7.56±0.050b</td>
<td>45.63±0.125b</td>
<td>15.14±0.050ab</td>
<td>850.00±20.493a</td>
</tr>
<tr>
<td>NTZ</td>
<td></td>
<td>6.88±0.087e</td>
<td>43.88±0.156e</td>
<td>14.36±0.116e</td>
<td>662.60±3.043d</td>
</tr>
<tr>
<td>PSO</td>
<td></td>
<td>7.22±0.009cd</td>
<td>44.93±0.187bcd</td>
<td>14.80±0.176bcd</td>
<td>600.20±6.086e</td>
</tr>
<tr>
<td>Inf+ NTZ</td>
<td></td>
<td>6.98±0.038de</td>
<td>44.25±0.225de</td>
<td>14.46±0.103cd</td>
<td>754.20±2.034b</td>
</tr>
<tr>
<td>Inf+ PSO</td>
<td></td>
<td>7.36±0.014be</td>
<td>45.22±0.081bc</td>
<td>14.90±0.189abc</td>
<td>790.00±4.472b</td>
</tr>
<tr>
<td>Inf+ NTZ+PSO</td>
<td></td>
<td>7.86±0.030a</td>
<td>47.78±0.086a</td>
<td>15.42±0.073e</td>
<td>694.00±5.099e</td>
</tr>
</tbody>
</table>

p-value <0.001 <0.001 <0.001 <0.001
Data are expressed as mean ± SE. The values in the same column have non-identical superscript letters are significantly different (p < 0.001), the highest value was represented by the letter (a) while the same letters indicate no differences. Inf-infection; NTZ-nitazoxanide; PSO-pumpkin seed oil; RBCs-red blood cells; Ht-hematocrit; Hb-hemoglobin.

Table 4: Leukogram values for various experimental groups post-treatment.

<table>
<thead>
<tr>
<th>Groups</th>
<th>WBCs (×10⁳/μl)</th>
<th>Neutrophils (×10⁳/μl)</th>
<th>Lymphocytes (×10⁳/μl)</th>
<th>Monocytes (×10⁴/μl)</th>
<th>Eosinophils (×10³/μl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>13.56±0.078</td>
<td>3.55 ±0.017</td>
<td>9.31 ±0.040</td>
<td>0.51 ±0.028</td>
<td>0.19 ±0.009</td>
</tr>
<tr>
<td>Vehicle</td>
<td>13.26±0.134</td>
<td>3.36 ±0.081</td>
<td>9.15 ±0.152</td>
<td>0.52 ±0.024</td>
<td>0.23 ±0.009</td>
</tr>
<tr>
<td>Inf</td>
<td>19.92±0.212</td>
<td>7.12 ±0.205</td>
<td>11.42 ±0.047</td>
<td>0.93 ±0.038</td>
<td>0.45 ±0.014</td>
</tr>
<tr>
<td>NTZ</td>
<td>13.36±0.116</td>
<td>4.07 ±0.019</td>
<td>8.43 ± 0.131</td>
<td>0.62 ±0.020</td>
<td>0.24 ±0.029</td>
</tr>
<tr>
<td>PSO</td>
<td>13.78±0.058</td>
<td>3.51 ±0.013</td>
<td>9.54 ± 0.060</td>
<td>0.52 ±0.008</td>
<td>0.21 ±0.011</td>
</tr>
<tr>
<td>Inf+ NTZ</td>
<td>14.80±0.054</td>
<td>4.49 ±0.023</td>
<td>9.38 ± 0.022</td>
<td>0.64 ±0.040</td>
<td>0.29 ±0.022</td>
</tr>
<tr>
<td>Inf+ PSO</td>
<td>15.65±0.122</td>
<td>4.83 ±0.093</td>
<td>9.82 ± 0.019</td>
<td>0.70 ±0.034</td>
<td>0.30 ±0.021</td>
</tr>
<tr>
<td>Inf+ NTZ+PSO</td>
<td>13.82±0.056</td>
<td>4.23 ±0.016</td>
<td>8.77 ± 0.072</td>
<td>0.56 ±0.008</td>
<td>0.26 ±0.008</td>
</tr>
</tbody>
</table>

Data are expressed as mean ± SE. The values in the same column have non-identical superscript letters are significantly different (p<0.001), the highest value was represented by the letter (a) while the same letters indicate no differences. Inf-infection; NTZ-nitazoxanide; PSO-pumpkin seed oil.

Table 5: Histopathologic scoring of different tissues sections for various experimental groups

<table>
<thead>
<tr>
<th>organ</th>
<th>Criteria</th>
<th>Control</th>
<th>Vehicle</th>
<th>Inf</th>
<th>NTZ</th>
<th>PSO</th>
<th>Inf+ NTZ</th>
<th>Inf+ PSO</th>
<th>Inf+ NTZ+PSO</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Spleen</strong></td>
<td>Depletion white pulp</td>
<td>-</td>
<td>-</td>
<td>++</td>
<td>-</td>
<td>-</td>
<td>++</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>Congestions of red pulp</td>
<td>-</td>
<td>-</td>
<td>++</td>
<td>-</td>
<td>-</td>
<td>++</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>Necrosis /apoptosis</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><strong>Liver</strong></td>
<td>Inflammatory Cells Infiltrations</td>
<td>-</td>
<td>-</td>
<td>+++</td>
<td>-</td>
<td>-</td>
<td>++</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>Congestions</td>
<td>-</td>
<td>-</td>
<td>+++</td>
<td>-</td>
<td>-</td>
<td>++</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>Hemorrhages</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Necrosis</td>
<td>-</td>
<td>-</td>
<td>++</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Kupffer cell hyperplasia</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+++</td>
</tr>
<tr>
<td></td>
<td>Hepatocytes degenerations</td>
<td>-</td>
<td>-</td>
<td>+++</td>
<td>-</td>
<td>-</td>
<td>++</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td><strong>Kidney</strong></td>
<td>Inflammatory Cells Infiltrations</td>
<td>-</td>
<td>-</td>
<td>++</td>
<td>-</td>
<td>-</td>
<td>++</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>Congestions renal BV</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
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<tr>
<td></td>
<td>Renal tubules epithelium hyperplasia</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>Necrosis /degeneration glomeruli / tubules</td>
<td>-</td>
<td>-</td>
<td>+++</td>
<td>-</td>
<td>-</td>
<td>++</td>
<td>++</td>
<td>+</td>
</tr>
</tbody>
</table>

Note: −, No detectable histopathological lesion; +, mild; ++, moderate; ++++, severe.
Fig. 1. A The viability reduction percentages of *E. granulosus* protoscolices after exposure to nitazoxanide, pumpkin seed oil and the combination of both agents at different exposure point. B Specific serum anti-echinococcus IgG values in different experimental groups post-treatment. Data are expressed as mean ± SE. The bars have non-identical letters are significantly different (p < 0.001), the highest value was represented by the letter (a) while the same letters indicated no differences. IgG-immunoglobulin G; OD-optical density.

Fig. 2. Optical microscope photographs of *E. granulosus* protoscolices (PSCs) exposed to NTZ, PSO, NTZ+PSO and non-exposed controls. A) negative control; B) vehicle; C-F) PSCs exposed to NTZ: C) 5 min; D) 15 min; E) 30 min and F) 60 min; G-J) PSCs exposed to PSO: G) 5 min; H) 15 min; I) 30 min and J) 60 min; K-N) PSCs exposed to NTZ+PSO: K) 5 min; L) 15 min; M) 30 min and N) 60 min; O) PSCs stained with 0.1% eosin: live (L) and dead (D). CC: calcareous corpuscles; Df: digitiform extension; H: hook; Ho: holes; Mi: microtriches; NR: neck region; RR: rostellar region; S: soma; SR: sucker region; flame cells (red circles) (magnification×400).

Fig. 3. Scanning electron microscopy images of *E. granulosus* protoscolices (PSCs) exposed to NTZ, PSO, NTZ+PSO and non-exposed controls. A) negative control (750X), B) vehicle (350X), C-G) PSCs exposed to NTZ: C) 5 min (600X), D) 15 min (500X), E) 30 min (1000X), F) 60 min (1000X), G) higher magnification showing blebs (B, 5000X), H-K) PSCs exposed to PSO: H) 5 min (800X), I) 15 min (800X), J) 30 min (650X) and K) 60 min (600X), L-P) PSCs exposed to NTZ+PSO: L) 5 min (400X), M) 15 min (350X), N) 30 min (600X), O) higher magnification showing blebs (B, 1500X) and P) 60 min (1000X). CC: calcareous corpuscles; Df: digitiform extension; H: hook; Ho: holes; Mi: microtriches; NR: neck region; RR: rostellar region; S: soma; SR: sucker region; B: blebs (yellow circles); neck region contraction (small yellow arrows).
Fig. 4. Some serum hepatorenal function parameters for various experimental groups post-treatment. Data are expressed as mean ± SE. The bars have non-identical letters are significantly different (p <0.001), the highest value was represented by the letter (a) while the same letters indicate no differences. ALT-alanine aminotransferase; AST-aspartate aminotransferase; GGT-gamma-glutamyl transferase.

Fig. 5. Serum proteinogram for various experimental groups post-treatment. Data are expressed as mean ± SE. The bars have non-identical letters are significantly different (p<0.05), the highest value was represented by the letter (a) while the same letters indicate no differences.
**Fig. 6.** Morphometrical assessment of spleen tissue of albino rats in the various experimental groups stained with hematoxylin & eosin.

**A** Control group reveals apparently normal splenic architectures including normal sized white pulps with germinal center (circle) and marginal zone (black star) around central arterioles (arrow), normal red pulps (red star) among trabeculae (arrow heads), scale bar 100 µm.  
**B** Vehicle group reveals apparently normal splenic architectures including normal sized white pulps with germinal center (star) and marginal zone (arrow) around central arterioles (arrow heads) besides normal red pulp (red star), scale bar 100 µm.  
**C** Inf group reveals marked atrophy due to depletion of the white pulp (black stars) with permanent trabeculae (arrow) and very widening of red pulp (red star), scale bar 100 µm.  
**D** High magnification of the photomicrograph to show decrease cellularity in periarteriolar area (arrow) associated with compensatory widening of red pulp sinusoids (star), scale bar 50 µm.  
**E** NTZ-treated group reveals apparently normal splenic architectures including normal sized white pulps with germinal center (black stars) and marginal zone, normal red pulps (red star) among trabeculae (arrow), scale bar 100 µm.  
**F** PSO-treated group reveals apparently normal splenic architectures including normal sized white pulps with germinal center (black star), marginal zone (arrow) and normal red pulps (red star), scale bar 100 µm.  
**G** Inf treated with NTZ group reveals return architecture to nearly normal spleen white pulp size (black stars) beside surrounded red pulp (red star), scale bar 100 µm.  
**H** Inf treated with PSO group shows nearly normal splenic white pulp size (stars) with still narrowing marginal zone beside surrounded red pulp, scale bar 100 µm.  
**I** Inf treated with NTZ plus PSO group reveals return splenic architecture to normal white pulp size (star) with still widening degree of red pulp, scale bar 100 µm.

**Fig. 7.** Morphometrical assessment of hepatic tissue of albino rats in the various experimental groups stained with hematoxylin & eosin.  
**A** Control group reveals apparently normal hepatic architectures including hepatic lobules (star) among portal triads...
(arrow), scale bar 100µm. B Vehicle group reveals central vein (star), normal hepatocytes arranged as a hepatic cord followed with Kupffer cells individually (thick arrow) through hepatic sinusoids (thin arrow), scale bar 50 µm. C Inf group reveals multifocal necrotic areas (circles) and inflammatory aggregations around portal triads (arrow), scale bar 100µm. D High magnification of the e photomicrograph shows necrotic area replaced with inflammatory cells especially histocytes and a few lymphocytes in the periphery (star), necrotic center with Langhans giant cells (arrow) followed with congested sinusoids and hyperplastic Kupffer cells, scale bar 50µm. E NTZ-treated group reveals hepatic cells degenerations (star) with congested blood vessels (thick arrow) besides minute inflammatory cells infiltration in the portal triad (thin arrow), scale bar 100µm. F PSO-treated group reveals nearly normal hepatic architectures including hepatic lobules (star) among portal triads (arrows), scale bar 100µm. G Inf treated with NTZ group reveals mildly inflammatory cells infiltrations around portal area (circle), narrowing sinusoids and congested blood vessels (thick arrows) besides moderate hyperplastic Kupffer cells, scale bar 50 µm. H Inf treated with PSO group reveals restoring of the most hepatic structures with still numerous periportal hepatic cells degeneration (star), scale bar 100 µm. I Inf treated with NTZ plus PSO group reveals apparently normal hepatic architectures with mild congested blood vessels (arrows) scale bar 100µm. J High magnification of the i photomicrograph shows apparently normal hepatic cords and sinusoids with marked diplocytes (arrows) with still little extravasated erythrocytes, scale bar 50 µm.

**Discussion**

Usage of scolicidal agents is highly recommended before surgery to avoid cyst leakage and the appearance of multiple hydatidosis (Zhang et al., 2003; Lv et al., 2013). Finding effective agents with fewer side effects is still an urgent need (Mahmoudvand et al., 2014). Nowadays, nitazoxanide was recorded to be an antiparasitic agent with marked activity against CE (Soliman et al., 2016), which may indicate the possibility for using it as an alternative option for benzimidazoles, the fundamental treatment used clinically against CE (Stettler et al., 2003; Walker et al., 2004; Vuitton, 2009).
Different natural agents earlier known to be efficient as anti-infectious agents also have been examined in vitro and in vivo models of the Echinococcus sp., but only little numbers of them have reached clinical application (Hemphill et al., 2014). However, it can be said that pumpkin seeds have promising results as scolicidal agent (Moazeni et al., 2012; Babaei et al., 2018). Phytochemical screening of PSO in the current study by using of GC-MS has shown the presence oleic acid, methyl ester (9-Octadecenoic acid (Z)-, methyl ester), which was detected as an anti-inflammatory fatty acid that play a role in the activation of diverse immune competent cells pathways (Carrillo et al., 2012) and palmitic acid, methyl ester (hexadecanoic acid, methyl ester), which has nematicidal and anti-inflammatory activities (Rukshana et al., 2017). As well as squalene, which has a strong antioxidant activity and enhances immune system performance (Owen et al., 2000) and linoleic acid, methyl ester (9,12-Octadecadienoic acid (Z,Z)-, methyl ester) is omega-6 fatty acid which was found to exhibit anti-inflammatory activity and reacts well with thiobarbituric acid reactive substances, inhibited the fenton-reaction and lipid oxidation (Chouhan et al., 2011). Also, it has in vivo and in vitro protective efficacies against some parasitic infections (Alhusseiny and El-Beshbishi, 2020).

In our study, we have established the viability reduction potential of NTZ, PSO and their combination. According to the results, PSO produced a greater viability reduction than that observed by NTZ, while the damage created by their combination was marked and appeared faster than that observed with the drugs acting alone. The high percentage of viability reduction caused by PSO due to the presence of (alkaloids, cucurbitane glycosides and triterpenes), where they exhibit cytotoxic activity (Aniszewski, 2007; Shaban and Sahu, 2017). According to Babaei et al. (2018) pumpkin seed had previously shown a high scolicidal action in vitro. On the other hand, the in vitro scolicidal action of the NTZ had been previously reported (Walker et al., 2004), where its exact mode of action on PSCs was unknown but it had been presumed that NTZ might interfere with the enzyme-dependent anaerobic energy metabolism of parasites (Gilles and Hoffman, 2002). The development of a treatment plan involving combinations of two or more agents with diverse modes of action is an ideal approach to enhance its effectiveness, reduce the treatment period (Lanusse et al., 2015).

In the current study, the highest viability reduction percentage (85%) was detected after 60 min from using NTZ+PSO combination against PSCs (from camels) in Egypt. On the other hand, a higher percentage reached 97% by using Pestalotiopsis sp against PSCs that were collected from cattle in India according to Verma et al. (2013), where lower percentages were reported by Shi et al. (2022) as 60% and 52% by using short interfering RNA (siRNA-1) and (siRNA-2), respectively, where PSCs obtained in this study from Mongolian gerbils in China. Also, Napooni et al. (2019) recorded a lower rate of scolicidal effect (68%) by using chitosan–curcumin nanoparticles for 60 min on PSCs which were collected from sheep in Iran. These differences might be attributed to different strains and localities of Echinococcus sp. in different hosts.

According to results of this study, light microscopy of the treated PSCs in vitro showed distortion of the soma region, disorganization, complete loss of hooks and appearance of several small blebs in the tegument, which was similar to the described changes in PSCs caused by eugenol essential oil and its nano-emulsion (Mauric et al., 2021). Moreover, the incidence of knob-like projection was proved after 15 and 30 min after exposure to PSO and after 5 min post-exposure to NTZ+PSO, which resembled the action of albendazole sulfoxide after 140 h which tested by Shiee et al. (2021). Also, the detached and discontinued tegument induced by NTZ+PSO after 15 min was looked like the noticed effect of bee venom which found by Tawfik (2018). Lastly, most of PSCs observed evaginated with obvious suckers in control groups by the time, which indicated
their development with high viability (Yones et al., 2011).

In the present study, different ultrastructural morphological anomalies were observed also by SEM where microtriches disappeared by the action of NTZ after 60 min. On the other hand, using PSO separately or combined with NTZ caused the disappearance of the microtriches after 15- and 30 min and induced digitiform-like extensions in the rostellar region after 30 min. Such findings were similar to those observed by (Xinga et al., 2019) using sodium arsenite and albendazole, but the appearance of digitiform extensions was noticed in soma region at the 2nd day of the exposure. Also, these extensions in the soma region were observed after exposure to Eupenicillium extract according to Verma et al. (2014).

Loss of microtriches and tegmental integrity in the form of holes detected by Maurice et al. (2021) after 24 h from using eugenol oil (0.2 μl/ml), that was similar to our finding after using a combined mixture from NTZ and PSO for 60 min, where PSCs appeared retracted with holes covering the damaged tegument. Moreover, the ultrastructural morphological changes resulting from the exposure to NTZ+PSO were summarized as hooks disorganization and contracted suckers, digitiform-like tegumental extensions in the rostellar region and microtriches and hook losses. In contrast, the use of NTZ alone produced contraction of the sucker and neck regions as the primary damage with intact microtriches. These alterations were considered markers for the pre-death stage of PSCs, as discussed by (Elissondo et al., 2006; Xinga et al., 2019).

Taking into account that, light microscopical examination of PSCs exposed to NTZ for 5 min did not reveal any changes in comparison with negative or vehicle controls like the description of Yones et al. (2014), which used 0.167 and 16.7 nM/ retinoic acid on human PSCs. In spite of that, the SEM examination revealed suckers disruption, slightly contracted neck and soma regions in our study. The detected tegumental blebs by both LM and SEM were similar to the findings of Yones et al. (2014). By the time, the blebs might be enlarged and ruptured, which resulted from PSCs osmoregulatory system upset (Lv et al., 2013). Lastly, we can say the hooks disarrangement and microtriches loss indicate that PSCs lost their viability as they are responsible for physiological homeostasis, nutrient absorption and defense (Affi and Harba, 2012; Verma et al., 2013). Also, the contraction and shrinkage of suckers and tegument, which were responsible for nutritional, secretory and sensory activities of helminths might increase their susceptibility to anthelmintic (Shalaby et al., 2012).

In this study, different clinical signs have been observed in the groups of animals, which were inoculated with PSCs and its severity reduced after treatment, especially with the combined treatment. This may be related to the generalized reaction of the body to the presence of the parasite antigen as well as the local effect of parasite to the peritoneum, which causing irritation of the adjacent peritoneum, which may reach to inflammation (Ferrer et al., 2022).

Generally, the exposure of animal to parasitic antigen stimulates immunoglobulins production, one of them the IgG, which acts as potent mediator of protective immunity after parasitic infection, but on the other side only a fraction of this increase is regarded to specific antibody and considered a valuable method for screening the efficacy of the treatment (Harris and Gause, 2011). ELISA values in experimentally infected rats sera showed 75% sensitivity and 100% specificity for the prepared HCF Ag in this study. A similar percentage of sensitivity and lower specificity (75%) was recorded by Mousa et al. (2015). However, they reached 93.33% and 70%, respectively by Rashed et al. (2019) and 82.75% and 62.5%, respectively by El-Kattan et al. (2020) from naturally infected camels in Egypt. Such variances explained by the following previous findings as naturally infected animals produced lower antibody reaction than the observed in human infection (Lightowlers and
Gottstein, 1995) as well as the produced immunoglobulins against different HC antigens were undetectable in the sera of some infected sheep (Jenkins and Rickard, 1986), and also seasonal factors, host susceptibility and its immune response might affect. In the presented study, the infected rats in all experimental groups displayed a significant elevation in the specific serum anti-echinococcus IgG, where PSCs could lead to humoral immune response activation in the form of high values of specific IgG (Zhang et al., 2012). Where the immunogenicity of HCF Ag mainly was regarded to the presence of highly immunodominant glycan in molecules of antigen 5 and antigen B, which secreted by the germinal layer of hydatid cyst and PSCs inside HCF (Lorenzo et al., 2005; Diaz et al., 2016). Plus, HCF was considered as a reservoir for lactate and succinate, which affected immune-regulatory responses (Hosch et al., 2008).

On the other hand at looking to the infected group treated with NTZ can observe a significant rise in the specific serum anti-echinococcus IgG in compare with infected group only and this may be related to the action of the drug which results in a transient increase in antigens from dead parasites and from the metabolic products of living ones, where parasite antigens are released at all time points throughout the infection resulting in an increase in the specific immune responses, which aid in the killing of parasitic agent (Davies et al., 1999). Also, treatment the infected animals with PSO showed insignificant increase in specific serum anti-echinococcus IgG compared to infected group only due to the same reason where significantly decrease in combined treated group in compare with infected animals only and this may be consequence of no more antigenic stimuli because of the death of PSCs and clearance of the body from parasite infection (Maizels and McSorley, 2016).

During an infection, both humoral and cellular mechanisms are implicated in fighting the parasitic infestation, where the humoral response involves antibodies and the cellular response includes secreted cytokines and T-cells, which react with parasitic antigens directly (Matowicka-Karna et al., 2011).

IL-10 is a T helper 2 (Th2) anti-inflammatory cytokine, which regulates and inhibit immune responses against any pathogen, therefore embraces the inflammatory processes, which may harm the host tissues (Ouyang et al., 2011). There is a lack of consensus in the studies clarifying the role of anti-inflammatory cytokines during parasitic infestation, where some studies reported high IL-10 level, while others observed low IL-10 levels in association with acute parasitic infection, for example in malaria infection (Boeuf et al., 2012). On the other side, IFN-γ (T helper 1 (Th1) is pro-inflammatory cytokine, which acts an important role in tissue homeostasis, immune and inflammatory responses (Ivashkiv, 2018).

According to the results of a recent study, the infected group only showed a significant decrease in serum IL-10 level in compared to the control animals. This probably due to failure of mechanisms that regulate the cytokine balance, which can lead to higher level of inflammatory cytokines and low level of IL-10 expression, and eventually lead to exaggerate the inflammatory response or the failure to reverse balance the inflammatory response (Mahanta et al., 2015), and also a negative correlation between IL-10 expression and parasitemia was observed according to Mahanta et al. (2015). On the other side, this reduction in IL10 as Th2 cytokine might be due to the effect of infection, which stimulated the cellular immune response more than humoral response (Abo-Aziza et al., 2019).

In the current research, treatment of infected animals with NTZ or PSO showed insignificant increase in the level of serum IL-10 in comparison to infected only group, but its level was still minimal than that of the control animals. This result was attributed to the NTZ potentially exerting...
immunomodulatory effects by rising M2 anti-inflammatory subpopulation of monocytes/macrophages (Clerici et al., 2011). These M2 macrophages can release a different cytokine, such as IL10 (Cassetta et al., 2011), so can say NTZ was also capable of stimulating IL-10 production. On the other side, PSO can induce the production and expression of IL-10 to oppose the inflammatory conditions via increasing the adiponectin, which induces the production of important anti-inflammatory cytokines, such as IL-10 and inhibits the production of interferon-γ (IFN-γ) (Wu and Schauss, 2012; Ghahremanloo et al., 2017). A marked increase in this parameter compared to the infected group was observed when NTZ was administered along with PSO due to the combining of their mixed effects.

Regarding serum IFN-γ level in this study, infected untreated rats revealed a significant increase in its level in compare to control rats and that may relate to marked increase in IFN-γ production after infection, where the innate immune response against infection is characterized by the induction of a cell-mediated response that comprises the production of IFN-γ (by NK and T cells), which required for enhancing iNOS activity by phagocytic cells and priming the adaptive immune response, so that iNOS activation is so important for controlling parasite growth during the infection and thus reduce the level of infection (Dematteis et al., 2003). Our data also implies that treatment of infected animals with NTZ or PSO showed significant decrease in serum IFN-γ level in compare with infected group. This may be due to NTZ may exert anti-inflammatory effects, by inhibiting the production of pro-inflammatory cytokines (Shou et al., 2019). Also, pumpkin seed components act to decrease IFN-γ production and induce potent anti-inflammatory activity due to their complementary immune cell modulation (Wu and Schauss, 2012).

On the other hand, the present work revealed that the highest level of serum IFN-γ level was observed in the combined treatment group than NTZ or PSO treated groups and that could be related to more intensive reduction of parasites load and higher scolicidal effect of the combined therapy that increase in T cell clone reactivity (IFN-γ producing cells) due to increase exposure of released antigens to the immune system following high destruction of parasite. Thus, we can suppose that the differences in IFN-γ production may be more linked to an effect of various antigen loads on differentiating T cells, where IFN-γ identified as a crucial element for parasite control (Dematteis et al., 2003; Dvoroznáková et al., 2004). We must bear in mind that the host response aims fundamentally to eliminate the parasites and during that may lead to uncontrolled inflammation, which can later be modulated by regulatory mechanisms to avoid tissue damage (Perez et al., 2011).

Hematological parameters provide us with crucial information on the different responses of the body against stress, infection, injury and deprivation of nutrients and their values are affected by a complex of factors in the body (Greer et al., 2014). Regarding erythrogram, our investigations recorded a significant increase in its parameters in most infected groups in compare with control group may be due to histamine effect, which is a mediator of allergic inflammation and could take part in the response against the parasite via rise vascular permeability, loss of intravascular permeability and lastly lead to hemoconcentration (González-Muñoz, 1999; Tizard, 2022). On the other hand, infected rats treated with NTZ or PSO showed a lower value of these parameters in compare with control group only and this may be due to decreased parasitic burden and its adverse effect on body post-treatment. Moreover, the pumpkin seeds are rich in different phytochemistry that is important for the formation of RBCs and Hb and consider as haematopoietic factors such as protein, minerals like iron, zinc, calcium, manganese, copper, magnesium and sodium and vitamins such as thiamine.
(vitamin B1), riboflavin (vitamin B2), niacin (vitamin B3), pantothenic acid (B5), vitamin B6, folate (vitamin B9), vitamin C, (Syed et al., 2019), so in this study can observe the significant and insignificant increase in erythrogram parameters in PSO-treated group in compare with control group and also when comparing the Inf+PSO group with Inf +NTZ group and according to Ifeanyi et al. (2014) and Lawal et al. (2015) the rats administered fluted pumpkin leaves extract at different concentrations showed significant increase in the hematological components including RBCs count, Hb content, PCV value.

On the other side, we can observe a significant increase in erythrogram parameters in Inf+NTZ+PSO in compare with control one and all infected groups and this may be due to overlap several factors such as significant decrease in parasitic load by action of combined treatment as well as a good effect of the pumpkin seeds components on erythropoiesis process and the decline of the inflammatory condition, which induced by infection where the inflammation are able to induce a decline in erythrogram parameters with underlying anemia as it happened in other infected groups through various mechanisms as suppression of erythroid precursors directly by inflammation as well as induction of hepcidin as a quick response resulting in a decline in iron levels within hours besides increase red blood cell clearance under inflammatory condition (Straat et al., 2012).

In the present research, thrombocytosis was observed in infected groups either non-treated or treated with NTZ or PSO or their combination in compare with control group. This may be due to the thrombocytes actively share in defense mechanisms of the organism, also the parasitic infection rises thrombocyte production and enhances the cytotoxic function of thrombocytes where they can release significant mediators that expand their function beyond their role in hemostasis (Matowicka-Karna, 2006).

Moreover, the acute inflammation was also associated with an increase in thrombocytes count (Bardaa et al., 2020). On the other hand, the treated groups, especially the combined treatment group showed a significant decline in thrombocytes number compared to the infected group only and that may be related to the activity of anti-parasitic treatments to get rid of the parasite and subsequently its effect on the body, which ends in decline thrombocytes count till normalizing it (Matowicka-Karna et al., 1995).

In this research, the leukocytosis was observed in infected group and that can be related to the induction of a defense mechanism against the injected PSCs of hydatid, which could stimulate the bone marrow to produce a higher number of different sorts of leukocytes (Alsaadawi et al., 2022). Where the neutrophilia and monocytosis were observed in the same group as a secondary or reactive to an underlying inflammatory process that includes infection (Turgeon, 2018). We can say that neutrophils interact with invading parasites and play a function in host defense mechanisms during the induction of experimental secondary hydatidosis in experimental animals as well as its increase as a reaction to local inflammation induced by PSCs (Makepeace et al., 2012). Also, we cannot ignore that the important role of monocytes in the production, mobilization and regulation of immune-effector cells, also its role in infection elimination (Chaplin, 2010). On the other hand, lymphocytosis after PSCs inoculation in infected group may reveal the induction of a high response of the immune system, where the activation of polyclonal lymphocytes occurred by parasite (Alsaadawi et al., 2022). Moreover, the number of eosinophils increased dramatically after PSCs inoculation and this may relate to the main character of host response to parasitic infection, where the eosinophils are fundamental contributors to antiparasitic immunity. Also, eosinophils are elevated
in number and directed into inflamed tissues under the influence of cytokines and chemokines, where they possess numerous cell surface receptors, which may be linked with eosinophil-mediated tissue inflammatory reactions in parasitic infestation (Ramos et al., 2006; Hogan et al., 2008).

On the other side, treating the infected rats with NTZ or PSO or their combination significantly reduced the leukogram parameters in comparison with the infected rats. This may be due to the capacity of treatments to fight the parasite and enhance the normal status of the leukocytes, where the decline in the count of different leukocytes occurred in a way harmonious with the eradication level of the infection and was noticed significantly in the rats group that be given the combined treatment as a result of marked reduction in the severity of the infection. Especially since the PSO components have an immunomodulatory effect either by attenuating the inflammation or rising the host ability to overcome with parasitic growth and proliferation (Shang et al., 2022).

The spleen is considered a key organ in regulating immune response against the infection in the body, so this study was supported by histopathological examination of the spleen in the different groups (Figs. 6 C-I).

Serum ALT and AST activities are used as a sensitive marker for possible tissue damage particularly the liver (Ramaiah, 2007). This study revealed that the infected group only displayed a significant decline in serum ALT activity compared to the control rats. This change may relate to the nutritional fluctuations of infected animals where loss of appetite and decrease food consumption may lead to decrease in serum ALT activity, where the serum ALT is more sensitive than AST to change in the nutritional status of animals (Kobayashi et al., 2010). Also, the pyridoxal 5-phosphate (PLP, the active form of vitamin B6), a co-factor for ALT and AST synthesis, which has consistently been shown to be decreased in inflammatory conditions and consequently lead to decrease ALT (Fischbach and Dunning, 2009). Also, serum ALT activity may misleadingly decrease several days after incidence of massive liver necrosis due to exhausted coenzyme supply (Haschek et al., 2010). Moreover, treatment of infected animals with NTZ or PSO or their combination significantly increased activity of ALT in compare with infected group and this may mean that the treatment may have reduced the factors that led to the decline in ALT activity. Also, it should also be noted that the serum ALT activity was significantly increased in NTZ-treated group in compare with a control group and that may indicate that pathological processes affect hepatic cells integrity (Ray, 2015). According to Hochadel (2011) nitazoxanide can induce increase in serum ALT activity and this also explains the increase in this parameter in Inf+NTZ group in compared with other infected treated groups.

Serum AST activity is usually increased due to origins other than the liver, where injury to non-hepatocytes, particularly cells that contain mitochondria can increase it and should be considered. So can say that because of widespread tissue distribution of enzyme, it would be reasonable to deduce that low AST activity would be less occurrence than low ALT activity (Botros and Sikaris, 2013). In the current study, the increased serum AST activity in the infected group in compare with control group was attributed mainly to tissue injury (Huang et al., 2006), where PSCs invade and destroy tissues and extend beyond organ borders (Lorenzo-Morales, 2012). However, the increase in serum AST activity in the infected group appears lower than that other groups which infected and treated, this may happen due to the effect of treatments in reducing the different factors lowered AST activity similar to reducing factors for ALT activity. Despite, ALT is more specific than AST for hepatic diseases, the latter is more sensitive for the reason that the liver
contains larger amounts of AST so may appear AST higher than ALT at some times (Ferrier, 2014). The increase in serum AST activity in NTZ-treated group in compare with control group for the same cause of increase in ALT activity in this study.

GGT activity in the serum has always been known as a marker of hepatobiliary injury, especially cholestasis and biliary injury (Kaneko et al., 2008), but in fact it also has diagnostic value as a marker of oxidative stress (Xing et al., 2022). In this study, the serum GGT activity was insignificantly increased in the infected group compared with the control animals and this may relate to elevate GGT activity during oxidative stress to facilitate GSH turnover, de novo GSH synthesis and metabolism and detoxification of GSH conjugates that increase cells resistance to coming stress (Sies and Packer, 2005). As the oxidant and antioxidants balance is disrupted after exposure of animal to E. granulosus antigens because of reactive oxygen species (ROS) production in infected animals was high enough to overcome the antioxidant system within the body due to the continuous immune reactions against the parasite, subsequently raised the oxidant stress and ultimately leading to oxidative damage, which in turn has an impact in the complications of the disease (Heidarpour et al., 2013).

On the other hand, Inf+NTZ and Inf+PSO groups showed a significant elevation in this parameter in comparison with control, Inf and Inf+NTZ+PSO groups. This change may be associated with an oxidative stress in addition to that NTZ induced a rapid up-regulation of ROS and induced oxidative stress during its activity as an antiparasitic (Mesquita et al., 2013). Also, in case of Inf+PSO group the increase in GGT activity may be related to oxidative stress under effect of the infection besides that PSO contains a high amount of iron (Ramadan, 2013) and in the same time the high iron intake may lead to increased level of cellular GGT (Lee et al., 2004). Moreover, GGT activity was positively associated with body mass (Hirschfield et al., 2023), so the Inf-group showed the lowest values in comparison with other infected treated groups. On the other side, combined treatment acted on decrease parasites burden in animals and subsequently decrease oxidative stress and serum GGT activity.

The present results obtained for kidney function markers showed insignificant increase in serum creatinine level in the infected group only and infected groups treated with NTZ or PSO in comparison with the control group. This may be because the normal serum creatinine level does not inevitably reflect normal renal function, where the abnormal reduced muscle mass due to high catabolism in states of undernutrition or low dietary protein intake leads to lower serum creatinine, which could mask or cause an underestimation of an insufficiency in renal clearance (Sharkey and Radin, 2010; Klein, 2020). Thus, the kidneys may be injured but this is not significantly reflected in serum creatinine level as observed in these groups. On the other aspect, there is a positive correlation between protein intake and serum creatinine level, where better nutritional condition and higher muscle mass present in animals with higher dietary protein intake (Rifai, 2022). This may explain the significant increase in creatinine level in the serum of the Inf+NTZ+PSO group compared with the control and infection groups, where the better nutritional status and muscle mass occurred in this group after receiving the combined treatment, especially PSO, which is rich in protein (Syed et al., 2019) and hence no occurrence of masked renal injury.

As for the level of the serum urea, the significant increase in their level in the infected group in compare with a control group may reveal renal injury besides the effect of dehydration, which decreased glomerular filtration rate and leads to retention of urea (Beckett et al., 2010). Despite of that the increase in the level of this parameter was less in compare with
Inf+NTZ+PSO group and this may relate to that serum urea level tends to decrease with protein malnutrition, which occurred from low feed intake and in other side, a high protein level in PSO in combined-treated group lead to increase the amount of urea that must be excreted (Beckett et al., 2010). Infected groups treated with NTZ or PSO showed a significant decrease in comparison with Inf or Inf+NTZ+PSO but higher than the control rats and this may relate to the effect of different factors such as a renal injury but in lesser degree than inf only group and the improvement in the state of protein nutrition for animals but in lesser degree than Inf+NTZ+PSO group.

The present results revealed insignificant changes in serum albumin level in different groups either infected only or infected and treated. This may be due to the decrease in body weight, which is often linked with a decline in serum albumin level and increase in the protein requirement secondary to infection that may be masked by dehydration (Jackson, 2007), especially in the infected non-treated group. On the other aspect, albumin synthesis is decreased in inflammation state where it considers as a negative acute phase protein (Jackson, 2007), so combined treatment of infected animals reduced the inflammation and subsequently raised albumin level insignificantly in compare with the inf only group and significantly in compare with the Infected treated with NTZ or PSO groups.

On the other side, hyperglobulinemia was observed in this study in groups infected and infected and treated either in alone or combined treatment form in compare with control group and this may be resulted from an increase in immunoglobulins which could be seen with antigenic stimulation, which triggered by the parasite, but the increase in serum globulin level was lesser in Inf group in compare with Inf+NTZ+PSO due to malnutrition, which can cause a decrease in total globulins due to decrease its synthesis (Bushu, 1990). Also, treatment of infected animals with PSO in Inf+NTZ+PSO may increase globulins level as it contains ascorbic acid, which induce globulins production also we found increase in globulins level in Inf+PSO group in compare with Inf+NTZ group for the same reason (Ranganathan et al., 2013).

The gained results regarded hepatorenal functions in the current study were corroborated by histopathological study of the liver and kidneys in the different groups (Figs. 7C-J, 8C-I). Considering that the normal or decreased serum levels of liver enzymes do not necessarily indicate an absence of liver damage or necrosis (Vasudevan et al., 2019). Also, the kidneys may be injured but this is not markedly reflected in measured renal function tests (Klein, 2020).

**Conclusion**

The outcome results showed that PSO was effective as scolicidal agent and the combinational treatment with NTZ displayed a higher scolicidal action in vitro which was resulted from their ability to reduce PSCs viability, induce tegmental contractions and PSCs evagination. The pervious changes resulted in deformity of the tegument and so failure of tegmental epithelium renewal in vitro. In addition to the therapeutic effects of PSO, especially after combining with NTZ in vivo, which resulted from the interactions between many components of both agents as well as their ability to alleviate hydatid illness or any adverse effects for the drug and that shown through the improvement in hematobiochemical parameters and histopathological picture of the examined tissues. This treatment strategy may provide a new way for treating cystic echinococcosis in humans and animals in the future and this will contribute to recommending the use of the combined mixture of PSO and NTZ as scolicidal agents (less harmful & more effective) before, during and after surgical removal of hydatid cysts.
Acknowledgements

The authors thankfully acknowledge Dr/Naif A. Algabri, Department of Veterinary Medicine, Faculty of Agriculture and Veterinary Medicine, Thamar University, Yemen for his worthy help related to histopathological examination. Also, the authors offer faithful thanks to Dr. Adel Salah Radwan, manager of the Kom Hamada slaughterhouse for his help in collecting the hydatid samples from camels. In addition to grateful thanks from the authors to Future Pharmaceutical Industries (FPI) for giving the nitazoxanide as a gift.

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التأثير المضاد للرؤيسات الأولية لزيت بذور القرع وحده أو مع النيتازوكسانيد في المختبر والجسم الحي وتأثيرهم المخففة للتغيرات الباثولوجية الاكلينيكية في الجرذان المصابة تجريبياً بالرؤيسات الأولية للأكياس المائية

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هدفت الدراسة الحالية إلى تقييم التأثير المضاد للرؤيسات الأولية لزيت بذور القرع بمفرده أو مع النيتازوكسانيد في المختبر. أيضاً، تم الكشف عن الفعالية السريرية والتأثير الوقائي لزيت بذور القرع بمفرده أو مع النيتازوكسانيد ضد مضاعفات الاصابة الثانوية بداء المشوكات في الجسم الحي. تم تجريبياً. تم تقييم انخفاض الحيوية وتأثيرهم المخففة للتغيرات الباثولوجية الاكلينيكية في الجرذان المصابة تجريبياً بالرؤيسات الأولية للأكياس المائية

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المجموعات متساوية: