INTRODUCTION

Aquaculture is one of the fastest-growing industries of the Egyptian agriculture sector, contributing to the national income with a market value of over $2.61 billion (GAFRD, 2016; Wally, 2016). Fish farming plays an indispensable role in guaranteeing food security in Egypt and alleviating poverty by creating job opportunities (Soliman & Yacout, 2016). Egyptian fish farmers strongly contributed 79.6% to the total fish production in 2017 (CAPMAS, 2019). Among other farmed fish, Nile tilapia (Oreochromis niloticus) is considered the keystone and the major farmed fish species of Egyptian aquaculture and constituted 59.4% of the total fish production (GAFRD, 2016). Egyptian fish farmers strongly prefer to cultivate Nile tilapia because of its ability to show a rapid and high growth rate in polyculture and monoculture systems, accept formulated feeds and resist low water quality. Egypt comes first in Nile tilapia production in Africa and the 3rd worldwide after China and Indonesia. Farmed tilapias are highly susceptible to parasitic diseases caused by a variety of ectoparasites, including trichodinids. Because of such economic importance of tilapia, more attention should be shifted to ectoparasites affecting farmed tilapias leading to numerous economic losses.

Trichodiniasis is considered one of the serious parasitic diseases affecting farmed tilapia causing economic losses; therefore, trichodinids should be spotlighted, giving more attention to protecting fish against these detrimental invaders. Trichodinids are a group of peritrichous ciliated protozoans belonging to the family Urceolariidae.
They have a monoxenic life cycle, low host specificity and multiply rapidly via binary divisions on fish surfaces (Martins et al., 2015). Trichodinids are commonly found in different environments as symbionts and act as ectocommensal parasites feeding on external cellular debris (Dias et al., 2009). Heavy parasitism with trichodinids causes mortalities in larval stages and decreased fish growth rate and immunity responses of vaccinated fish (Valladão et al., 2016). Infected fish commonly exhibited white patches appearance, molting of fins and skin with excessive production of bluish mucous (Abdel-Baki et al., 2011).

The pathogenesis of trichodinids is primarily related to the attachment of their adhesive discs to the epithelial cells of infected fish, leading to severe irritation to host tissues and serious damages to the skin, fins and gill surfaces when the parasitism loads are high (Basson & Van As, 2006; Martins & Ghiraldelli, 2008; Valladão et al., 2016). Hyperplasia, oedema, inflammatory cell infiltrations and necrotic cellular changes are commonly noticed in infected gill tissues (Valladão et al., 2013, 2014; Yemmen et al., 2011). Additionally, these lesions can facilitate secondary parasitic, mycotic and bacterial infections, resulting in fish mortality and significant economic losses in fish farms (Shinn et al., 2015).

All trichodinids have a rounded or spherical body covered with a thin membrane (pellicle) and peritrichous cilia (Basson & Van As, 2006). Traditional morphological identification of trichodinids is based mainly on light microscopy examination of silver-impregnated and/or Giemsa-stained smears. However, this method appeared insufficient due to silver nitrate's relative unavailability (Gong et al., 2010). Advanced molecular techniques resolved such deficiencies in morphological identification among various ciliated protozoa and are considered promising tools for protozoal molecular characterization (Utz & Eizirik, 2007). Sequencing and phylogenetic analysis of the 18S rRNA gene is successfully used to identify species of trichodinids (Gong et al., 2006).

Several species of trichodinids have been observed infecting Nile tilapia, such as Trichodina centrostrigeata, T. acuta, T. kalimbeza, T. linyanta, T. pediculus, T. velasquezae, T. nigra, T. minuta, T. heterodentata, T. compacta, T. fultoni, T. salmincola, T. canton, T. magna, Paratrichodina Africana, Trichodinella tilapiae, Tripartiella orthodens and T. migala (Al-Rasheid et al., 2000; Aly et al., 2020; Ghiraldelli et al., 2006; Valladão et al., 2013, 2016). T. centrostrigeata, T. compacta and T. heterodentata are the most important trichodinid pathogens because they cause severe injuries to fish and economic losses to fish farmers. In Egypt, most previous studies were employed morphological and histopathological assays for characterizing T. centrostrigeata, T. compacta and T. heterodentata infections in Nile tilapia, and there are no records for their molecular characterization or their immunological impacts on infected fish except in T. compacta (Abdelkhalek et al., 2018).

Up to date, the application of chemical disinfectant agents such as formalin, hydroxide peroxide and potassium permanganate proved their anthelmintic effectiveness against ectoparasitic infections with a variable level of success. Potassium permanganate (KMnO₄) and hydrogen peroxide (H₂O₂) are commonly applied as strong disinfectants in aquaculture, depending on their oxidizing properties. KMnO₄ is potentially toxic at high concentrations and is not approved by the U.S. Food and Drug Administration (FDA). While H₂O₂ is more toxic at high water temperature and is approved by FDA to be used in aquaculture (Aly et al., 2020; França et al., 2011). Therefore, it is necessary to evaluate the anthelmintic efficacies of both chemical agents' small doses to avoid their serious drawbacks.

Therefore, this study documented the natural coinfection of T. centrostrigeata with T. heterodentata in farmed O. niloticus in Egypt. This study has highlighted that morphomolecular assays are crucial for accurate identification of pathogenic trichodinids. Besides, the immunological assay was further conducted to study the immune responses of infected tissues with trichodinids. Treatment trials using small doses of hydrogen peroxide (H₂O₂ 40%) and potassium permanganate (KMnO₄) have been conducted to evaluate their effectiveness against trichodinid infestations.

2 | MATERIALS AND METHODS

This study was approved by the Institutional Animal Care and Use Committee, Faculty of Veterinary Medicine, Cairo University, Egypt (Vet. CU. IACUC, No: Vet-CU24112020239).

2.1 | Fish samples

From May to September 2020, a total of 250 moribund Nile tilapia Oreochromis niloticus were randomly collected from earthen ponds of a private fish farm in El-Sharkia governorate. The farm's culture system is semi-intensive, and this farm fed their fish with well-prepared commercialized pellets; and the owners of this farm used untreated fowl litter and droppings as fertilizers and/or direct feed to fish. This farm recently experienced a disease outbreak among farmed tilapia that exhibited different respiratory distress and skin irritation signs. The collected fish were transported alive to the Faculty of Veterinary Medicine laboratory, Cairo University, for parasitological and molecular analysis (Abdelsalam et al., 2016, 2020).

2.2 | Parasitological examination

Wet smears from skin, body mucous and gills of infected fish were prepared on clean slides and then investigated under a light microscope an Olympus CX41 (X400). Positive slides for ciliated protozoa were fixed and stained with Giemsa, which is usually used to analyse nuclear characters (Eissa et al., 2020; Kazubski & El-Tantawy, 1986). All measurements were termed as the minimum–maximum (mean ± standard deviation) and expressed with micrometres according to Van As and Basson (1989). The measurements and morphological descriptions of retrieved Trichodina spp. were performed based on 50 specimens.
2.3 | DNA extraction

For DNA extraction, at least 20 Trichodina protozoan specimens were collected by glass micropipette under a binocular stereo microscope and then stored in sterile Eppendorf tubes at −20°C. According to the manufacturer’s protocol, protozoan DNA extraction from the stored samples was performed using the DNeasy Tissue Kit (Qiagen, GmbH). The DNA concentration was measured using spectrophotometer apparatus, and then, the extracted DNA was stored at −20°C for molecular applications.

2.4 | Partial sequencing of 18S rDNA

The gene encoding the small subunit of the nuclear ribosomes (SSU rRNA gene or 18S rRNA) was amplified by PCR using the following primers: forward primer: 5′-AACCTGTTGATCCTGCCAGT-3′, reverse primer: 5′-TGATCCCTCGAGGTTCCACCT-3′ according to Medlin et al. (1988). PCRs were performed using the MyTaq™ Red Mix kit (Bioline) in a total reaction volume of 25 µl containing 12 µl of MyTaq™ Red Mix, 5 µl of DNA, 2 µl for each primer and 4 µl of nuclease-free water. Thermocycling procedures were performed following the protocol of Gong et al. (2006) with minor modification. The thermal cycling operations involved initial denaturation at 94°C for 7 min, with 5 cycles of denaturation at 94°C for 1 min, annealing at 56°C for 2 min and extension at 72°C for 2 min, followed by 35 cycles of the same parameters but annealing temperature was readjusted to 63°C, and ended with a final extension step at 72°C for 10 min then kept at 4°C.

The PCR products were electrophorized in 1.5% agarose gel, stained with ethidium bromide and then visualized by UV transilluminator. According to the manufacturer’s recommendations, the PCR products were purified using the QIAquick Gel Extraction Kit (QiAGEN). The purified amplicons were directly sequenced by Macrogen Inc. with the QIAquick Gel Extraction Kit (QIAGEN). The PCR products were purified using ethidium bromide and then visualized by a UV transilluminator.

2.5 | Gene expression using qRT-PCR

Gills from infected and uninfected fish were collected and aseptically dissected. The selected gills samples were subjected to bacteriological and mycological examination to ensure that the fish free from microbial infections. One hundred milligrams of infected gills was used to extract total RNA using an RNA isolation kit (TRIsure Kit (Bioline), following the manufacturer’s instructions. The RNA quality was checked using NanoDrop (Quawell, Uv-Vis spectrophotometer Q5000/USA) with 1.5% agarose gel electrophoresis. The cDNA was transcribed using the cDNA synthesis kit (SensiFAST; Bioline) from the extracted RNA. Then, the transcribed cDNA was kept at −40°C until to be used.

PCR primer sets used in this study for MHCIIβ specific for O. niloticus were as follows: MHCIIβ-F: 5′-ACTGATTTGTTGGAGGATGC-3′; MHCIIβ-R: 5′-GTGGA GTGAAGCTTCTACTGAGT-3′, and primer set for CD4 specific for O. niloticus was CD4-F: 5′-AGAAGAAGGGATCCGAGA-3′; CD4-R: 5′-CAGAAGAGGGAACGACAGAGAC-3′; β-actin was used as an internal reference control gene, and sample normalization; β-actin-F: 5′-CAGAAGAGGGAACGACAGAGAC-3′; β-actin-R: 5′-GTGGA GTGAAGCTTCTACTGAGT-3′.

Real-time quantitative PCR (RT-qPCR) was performed in a Step One™ Real-Time PCR System (Applied Biosystems). A 10 µl SYBR® Premix Ex Taq™ (Tli RNase H Plus), ROX plus (TaKaRa; 1 µl of synthesized cDNA, 0.5 µl of forward and reverse primer (100 nM) were mixed in a reaction), complete the final volume to 20 µl with ultra-pure water. The adjusted cycling conditions were followed as Younis et al. (2020). The ΔCT value was calculated as recorded in Attia et al. (2020).

2.6 | Treatment trial

This trial was conducted based on field and laboratory experiments performed by França et al. (2011) and Aly et al. (2020), with minor modifications. Hydrogen peroxide (H₂O₂ 40%) and potassium permanganate (KMnO₄) have been used for treating ectoparasites infections in freshwater fish. The recommended dose of hydrogen peroxide was 30 ppm for 72 h as an indefinite bath for three days. Hydrogen peroxide is validated by FDA to be used in aquaculture. Potassium permanganate powder (1000 mg) was dissolved in 1000 ml distilled water to prepare a concentration of 1000 mg L⁻¹ of KMnO₄. The effective treatment of KMnO₄ could be achieved if the water remains purple to pink in colour for 12 h post-application of the chemical.

Three tanks of 2-m diameter were chosen for treatment trial against trichodinid infection. Sixty moribund tilapias were collected from infected ponds, and twenty fish were stock in each tank. The untreated fish group was fed a normal diet and was left without treatment. The second tank was treated with potassium permanganate at a concentration of 2 mg L⁻¹ as an indefinite bath. The third tank was treated with hydrogen peroxide (40%) at a concentration of 30 ppm as an indefinite bath. Moribund fish were examined daily for trichodinid infection and recovery rates.

2.7 | Statistical analysis

The comparison of the gene expression groups means was calculated using the one-way-ANOVA test. When p < 0.05 was considered statistically significant. All statistical analysis was performed on
3 | RESULTS

3.1 | Gross examination of the infested fishes

Moribund O. niloticus showed several skin irritations and respiratory distress signs such as sluggish swimming, lethargy, rubbing its bodies against fixed objects, fish surfacing and rapid movement operculum. Heavily infected fish with trichodinids showed loss of appetite, scales detachment, excessive mucus, skin erosions with blood spots at the base of fins (Figure 1). On the other hand, excessive accumulation of mucus in the gill pouches and around gill filaments was frequently noticed in some infected fish. The gills appeared pale and swollen in some cases and hyperaemic in other cases with the accumulation of excess mucus that indicated a high infestation of trichodinids. The investigated tilapias were found to be infected with trichodinids in the skin and gills. The prevalence rate of trichodinids in O. niloticus from El-Sharkia governorate was 72% (n = 180/250), and the Trichodina load in one microscopic field (X 40) was ranged 30-50 protozoan parasites.

3.2 | Comparative morphological identification

The trichodinids detected were categorized according to many parameters as body diameters, adhesive disc; border membrane; denticulate ring diameter; denticles numbers; length of denticles, ray and blade lengths. Based on these morphological features, the investigated tilapias were ascribed into two species of Trichodina: T. heterodentata and T. centrostrigeata. The main points of differentiation between the two species were recorded in Table 1.

3.3 | T. heterodentata

The identity of the first detected Trichodina species was morphologically ascribed as T. heterodentata (Figure 2). The ciliated protozoan parasites had a disc-shaped body that resembles the sun disc measured 45–65 μm (56 μm ± 3.0) in diameter. The adhesive disc size ranged from 35 to 54 μm (42 μm ± 2.0), and a border membrane surrounded it with finely striated 2–5.5 μm (4.5 μm ± 0.6). The diameter of the denticulate ring 16–34 μm (25 μm ± 1.0) in diameter. The number of denticular rings was 22–29 (25 ± 1.0); each denticle had a length from 7 to 10 μm (9 μm ± 1.0). The denticle is composed of a blade, a ray and a central part. The measurement of this blade ranged from 3.5 to 6 μm (4.7 μm ± 0.8). The denticle ray was stout and along the y-axis side, and its measurement ranged from 4.6-8.5 μm (6.0 μm ± 1.0) in length (Table 1).

3.4 | T. centrostrigeata

The identity of the second Trichodina specimens was morphologically ascribed as T. centrostrigeata (Figure 3). The ciliated protozoan parasites had a disc-shaped body that resembles the sun disc measured 35–56 μm (45 μm ± 1.0) in diameter. The adhesive disc size ranged between 35 and 45 μm (40 μm ± 2.0), and it was circumscribed by a border membrane that was finely striated 2.6-5.6 (4.3 ± 1.5). The diameter of the denticulate ring 18-29 μm (25 μm ± 1.5) in diameter. The number of denticular rings was 25-35 (27 ± 1.0); each denticle had a length from 3.0 to 7.5 μm (5.8 μm ± 1.0). The denticle is composed of a blade, a ray and a central part. The broad blade measured 2.5–6.5 μm (4.0 μm ± 1.0). The denticle ray was stout and was located beside the y-axis, and its measurement ranged between 3.4 and 6.5 μm (5.3 μm ± 0.5) in length (Table 1).

3.5 | Phylogenetic analysis

The two purified PCR products of the 18S rRNA gene were directly sequenced to identify the Trichodinids and yielded 653 and 540 bp, respectively. The obtained 18S rRNA gene sequences of two Trichodinid infecting farmed O. niloticus were deposited in GenBank under the accession numbers MW131534 and MW131535. Based on their sequence alignment, the present
two isolates of Trichodinids are firmly embedded within the family *Trichodinidae*. These sequences’ identities were confirmed as *Trichodina centrostrigeata* (MW131534) and *T. heterodentata* (MW131535), respectively.

The accession number (MW131534) showed 99.54% similarity to that of *T. centrostrigeata* (KP295473), 98.47% similarity to that of *T. truttae* (LC186029), 98.01% similarity to that of *T. domerguei* (KY596034) and 93.01% similarity to that of *T. reticulata* (MH191329). Meanwhile, the accession number (MW131535) exhibited 99.44% identity with *T. heterodentata* (AY788099) and 93.15% identity with *T. reticulata* (AY741784). Interestingly, the phylogenetic analysis of both accession numbers MW131534 and MW131535 showed 100% similarity to the members of the family Trichodinids. Phylogenetic analysis of 18S rRNA genes revealed strong nodal support for two major lineages (Figure 4). The first major clade is monophyletic and comprised *Trichodinidae* species. The second major clade is divided into two subclades. The first subclade was comprised of Nassulidae family species, while the second subclade involved the Sessilidae group of protozoa.

### 3.6 | Gene expression using qRT-PCR

Gene expression of infected gills with *Trichodina* sp. showed that T-cell surface coreceptor (CD4) means was upregulated by 9.5. In contrast, the gill expression of mRNA of major histocompatibility class II (MHC-IIβ) was 13.8 in comparison with the control group (3.00 ± 0.00; *p* = 0.0001; Figure 5).

### 3.7 | Treatment trial

The treated fish in both treated groups showed gradual disappearance of skin irritation and asphyxia signs at the end of one week of treatment. These results were confirmed through microscopical examination of the gills and skin smears from fish treated with

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**TABLE 1** Comparative morphological parameters of *Trichodina* spp. infecting *O. niloticus* (all measurements expressed as micrometres: μ)

<table>
<thead>
<tr>
<th>Diameters</th>
<th><em>T. heterodentata</em></th>
<th><em>T. centrostrigeata</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>Body diameter</td>
<td>45–65 (56 ± 3.0)</td>
<td>35–56 (45 ± 1.0)</td>
</tr>
<tr>
<td>Adhesive disc diameter</td>
<td>35–54 (42 ± 2.0)</td>
<td>35–45 (40 ± 2.0)</td>
</tr>
<tr>
<td>Border membrane</td>
<td>2–5.5 (4.5 ± 0.6)</td>
<td>2.6–5.6 (4.3 ± 1.5)</td>
</tr>
<tr>
<td>Denticulate ring diameter</td>
<td>16–34 (25 ± 1.0)</td>
<td>18–29 (25 ± 1.5)</td>
</tr>
<tr>
<td>Number of denticles</td>
<td>22–29 (25 ± 1.0)</td>
<td>25–35 (27 ± 1.0)</td>
</tr>
<tr>
<td>Denticule length</td>
<td>7–10 (9 ± 1.0)</td>
<td>3.0–7.5 (5.8 ± 1.0)</td>
</tr>
<tr>
<td>Blade length</td>
<td>3.5–6 (4.7 ± 0.8)</td>
<td>2.5–6.5 (4.0 ± 1.0)</td>
</tr>
<tr>
<td>Ray length</td>
<td>4.6–8.5 (6.0 ± 1.0)</td>
<td>3.4–6.5 (5.3 ± 0.5)</td>
</tr>
</tbody>
</table>

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**FIGURE 2** Light microscopic micrograph of *T. heterodentata* showing its morphological and morphometric characteristic with a special denticule shape

**FIGURE 3** Light microscopic micrograph of *T. centrostrigeata* showing its morphological and morphometric characteristic with a special denticule shape and numbers
KMnO₄ and H₂O₂. The investigated slides showed a sharp reduction in trichodinids at the end of the treatment’s third day. Application of KMnO₄ and H₂O₂ reduced the infestation rate from 100% to 55% (no = 11/20) and 65% (no = 13/20), respectively, at the end of the first day of the trial. The infestation rates were gradually decreased to be 30% (no = 6/20) and 45% (no = 9/20), respectively. The infestation rates were further decreased to be 10% (no = 2/20) and 15% (no = 3/20), respectively. Both chemicals accomplished the best clearness results with a 0% infestation rate at the end of the fourth-day post-treatment.

4 | DISCUSSION

In the present study, infected tilapias by trichodinids exhibited excessive mucus secretion and scattered skin haemorrhages in different areas. Gills appeared pale and anaemic. Previous studies showed that infected gill tissue had hyperplasia, hypertrophy, oedema, inflammatory infiltrations and necrotic changes (Abdel-Baki et al., 2011; Yemmen et al., 2011). Infected fish suffered from decreased immunity, decreased growth rate and acute to chronic mortality (Martins et al., 2011; Valladão et al., 2014). Morphological identification of recovered trichodinids was based on the shape of the adhesive disc, the number of denticles as well as the length of the blade and ray (Basson et al., 1983; Duncan, 1977; Van As & Basson, 1992). The denticles and the denticle ring components of the adhesive disc were among the main characteristics used for the taxonomic distinguishing of retrieved Trichodina sp. (Leon & Perez, 2017; Van As & Basson, 1989). Molecular diagnostics methods enabled fast and precise identification of parasitic diseases in fish, facilitating prompt treatment, especially in the intensive fish farming system (Gentekaki et al., 2017). In this study, trichodinids retrieved from moribund Nile tilapia were identified as T. heterodentata and T. centrostrigata based on morphological characteristics and molecular analysis.
The phylogenetic analysis of sequenced 18S rRNA genes was conducted to distinguish the genetic correlation of *T. centrostrigedta* and *T. heterodentata* in this study with other Trichodinids as well as peritrichous. The phylogenetic tree showed the existence of two major lineages. The first major lineage was monophyletic and involved the Trichodiniidae family. The second clade was subdivided into two subclades. The first subclade comprised the mobilia branch and involved Nassulidae family, while the second subclade comprised the Sessilidae branch and included the remaining peritrichous. It is worth mentioning that mobilids peritrichous initially evolved from sessilids peritrichous (Gong et al., 2010), and in our phylogenetic tree, mobilia branch of Nassulidae family was grouped with Sessilidae branch, while Trichodiniidae was grouped separately from other peritrichous. These results coincided with that of Abdelkhalek et al. (2018). Homologs trichodinid coinfections might occur when fish get concomitantly infected by two different species of trichodinids. Parasitic coinfections exhibited synergistic interactions by magnifying the host–parasites dynamics, the severity and course of the disease, leading to elevated pathogenicity of two parasitic pathogens (Abdel-Latif et al., 2020; Eissa et al., 2015; Mahdy et al., 2020).

The immunological responses of Nile Tilapia against *Trichodina* infection were evaluated via the expression of two different genes, MHCII and CD4. Those two genes were upregulated during the infection with Trichodina sp. The previous study on the immunological reaction of Nile tilapia tissues through gene expression of three genes (IL-1β; IL-8 and tgf-β) revealed increasing the expression of IL-1β and decreasing the expression of tgf-β and IL-8 levels (Abdelkhalek et al., 2018). On the other hand, further studies reported that IL-1β, MHCII, TNF-α and proinflammatory cytokines were upregulated during *Ichthyophthirius multifilis* (ICH) infection (Gonzalez et al., 2007; Sigh et al., 2004; Wang et al., 2019).

The immune system of fish is contributing to innate and acquired immunity. The adaptive immune system identifies different pathogens with cell-mediated immune responses by generating B and T lymphocytes. Major histocompatibility class II expressed in antigen-presenting cells (B lymphocytes, macrophages and dendritic cells, as well as the thymic epithelium and in some species, activated T lymphocytes) which increased in the fish lymphoid tissues; its function is presenting the antigen to T helper cell (Nath et al., 2006). CD4 is a glycoprotein transmembrane expressed on the T helper lymphocyte surface and performs a major function in the immune response by presenting the antigen with the help of MHCII β. T helper lymphocyte generates CD4 which synchronize the immunity response via acting either as memory cells or as effector cells (Ashfaq et al., 2019). Therefore, MHCIIβ and CD4 genes were elevated during trichodinid infection to present the antigen to T cell to complete the immunological functions and producing different cytokines.

In this study, fish treated with KMnO4 and H2O2 showed an absence of trichodinid infestation on the fourth day of treatment. Our findings are nearly similar and coincided with those obtained by Aly et al. (2020), who recorded that both chemicals proved their effectiveness in treating trichodinids at small doses. H2O2 quickly decays to water and oxygen through photochemical and biochemical processes during oxidation, and therefore, H2O2 is considered safe and does not pollute the environment with any residues. However, it is a potentially toxic substance at elevated concentrations or high water temperature, causing severe gill damages, including intercellular oedema and swellings of secondary gill lamellae (Gaikowski et al., 1999). Also, H2O2 is comparatively expensive and should be handled with cautious (Aly et al., 2020).

On the other hand, KMnO4 is relatively inexpensive and is readily available in powder form. However, it quickly reacts with the organic matter in fish ponds and becomes unavailable to treat infected fish. Besides, it exerts a toxic effect on treated fish if applied in high concentration, causing gill damage (França et al., 2011).

It should be noted that the therapeutic efficacies of both chemicals are limited if the infection is progressed, causing rise in the mortality of the treated fish. Moreover, the application of these chemicals should be restricted to be used under strict monitoring. It is recommended to conduct a bioassay in small-scale trials before applying them in large-scale treatment trials.

**ETHICS APPROVAL**

This study was approved by the Institutional Animal Care and Use Committee, Faculty of Veterinary Medicine, Cairo University, Egypt.

**CONFLICT OF INTEREST**

All authors declare that they have no conflict of interest.

**AUTHORS CONTRIBUTION**

All authors are equally contributed to this work.

**DATA AVAILABILITY STATEMENT**

Research data are not shared.

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