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# Application of Bead Tree Leaf Extract (*Melia azedarach*) as a Green and Natural Anti-parasitic Drug to Control Trichodinosis in Whitespotted Freshwater Catfish (*Clarias fuscus*) Farming

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Trichodinosis, an ectoparasitic infestation caused by ciliated protozoa-*Trichodina* spp., is commonly found in skins and gills of freshwater fishes and causes a significant economic loss of fish farming. Synthetic chemicals are widely used to eliminate trichodinosis but it poses a potential risk to environment. The aim of this study is to develop of a new alternative approach by using a natural and environment-friendly material, such as bead tree (*Melia azedarach* L.) leaf extract for treatment of trichodinosis in whitespotted freshwater catfish (*Clarias fuscus*). The ethanol leaf extract (EtMA) was obtained via ultrasonic assisted extraction (30 °C, 90 min). The preliminary phytochemical screening confirmed the presence of some bioactive compounds in the extract, including alkaloids, tannins, saponins, phenolic compounds, steroids, terpenoids and flavonoids. In the *in vitro* experiment, all of doses of the extract (50, 100, 150 ppm) could effectively eradicate *Trichodina nigra* after 180 min of treatment (53.03 %-85.35 %). The extract showed no acute toxicity to whitespotted freshwater catfish after bath treatment up to 1,600 ppm for 15 min. The trichodinosis model was established via infection of *Trichodina nigra* in whitespotted freshwater catfish and bath treatment with EtMA (800, 900, 1,000 ppm) not only decreased the infection rate of *T. nigra* (20.00 %-46.67 %) but also enhanced the survival rate of *T. nigra* infected fishes (56.67 %-90.00 %) as compared to untreated fishes. The hematological (red blood cells, white blood cells, and hemoglobin) and biochemical parameters (total protein, blood urea nitrogen, creatinine, and total bilirubin) of all doses of EtMA-treated fishes were improved as compared to those of untreated fishes. These findings suggest that EtMA has potential use as a green and natural treatment to control trichodinosis in whitespotted freshwater catfish farming.

## 1. Introduction

Catfish is a high economic value aquatic product with a delicious taste and high nutrient value. Catfish has a fast growth rate, can tolerate adverse environmental factors, a variety of foods, and high-density fish farming conditions. Vietnam is one of well-known countries for freshwater catfish exportation; for instance, 569 thousand metric tons of catfish which worth approximately 1.2 billion US dollars have been exported by Vietnam in 2021 (FAO, 2022). One of the most popular catfish in Vietnam is whitespotted freshwater catfish (*Clarias fuscus*), which is susceptible to trichodinosis, a protozoal disease caused by *Trichodina* spp. The parasite is frequently found on the skin and gills of fish and causes abrasions, severe skin ulcers, tissue damage, as well as weakens immune system and leads to secondary infections (Roknmani and Riwidiharso, 2020). Synthetic antibiotics and chemicals are common used to eliminate trichodinosis for prevention of economic losses in aquaculture. The use of synthetic antibiotics and chemicals causes many side effects on the environment and health safety, such as development of antibiotic resistance (Pham et al., 2021), potential hazards to animal health, and environmental disadvantages (Reverter et al., 2014). There is a rise of demand for consumption of organic and ecofriendly food in recent days, which drives fish farming toward the organic and sustainable aquaculture

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practices, for example limitation of synthetic chemicals and utilization of alternative treatments and natural bioactive compounds, to control parasitic diseases in aquaculture. Several researcher have studied and isolated bioactive compounds from herbal sources, such as garlic, neem, tulsi, turmeric, which can be used as nutraceuticals, immunostimulants, biopesticides, anti-protozoal agents, and treatments for aquaculture disease (Raman, 2017). Among them, bead tree is a valuable biosource not only for its high-quality timber, but also for feed for some ruminants, treatment of skin infections, scabies, and vaginitis in Vietnam. Several bioactivities of bead tree have also been reported including anti-oxidant, anti-bacterial, anti-fungal, anti-inflammatory, immunomodulatory, anti-ulcer, and analgesic effects (Sharma and Paul, 2013). Recently, Al-Turaihi and Al-Rudainy (2017) proved the anti-parasitic effect of the water and alcohol extracts of bead tree fruits against some monogenean parasites on common carps, which suggests the application of bead tree in aquaculture. However, anti-protozoal activity of the ethanol extract of bead tree leaves against trichodinosis in whitespotted freshwater catfish has not been studied yet. The objective of this study is to investigate the application of the ethanol extract of bead tree leaves, a natural and environment-friendly anti-protozoal agent, for treatment of trichodinosis in whitespotted freshwater catfish and reduction of the use of synthetic chemicals in aquaculture.

## 2. Materials & Methods

### 2.1 Preparation of the extract

Fresh bead tree leaves (*Melia azedarach* L.) were harvested in March 2022 from Song Trau commune, Trang Bom district, Dong Nai province, Vietnam. The voucher specimen was deposited for identification and future reference. To remove impurities, fresh leaves were washed with tap water and dried at 40 $^{o}$C for 12 h. Then the dried leaf powder was obtained by grinding machine. To prepare the extract, 100 g of powdered leaves were extracted with 100 mL of 95 % ethanol using the ultrasonic-assistance extraction according to Mathialagan et al. (2017) method with some modifications (30 $^{o}$C, 90 min) and the extract was kept shaking at room temperatures for 2 d. The filtrate was obtained via filtration through Whatman filter paper and the extraction procedure was repeated once more. The filtrate was combined and evaporated using a rotary evaporator, the resulting extract is referred to as EtMA stored at 4 $^{o}$C until further use.

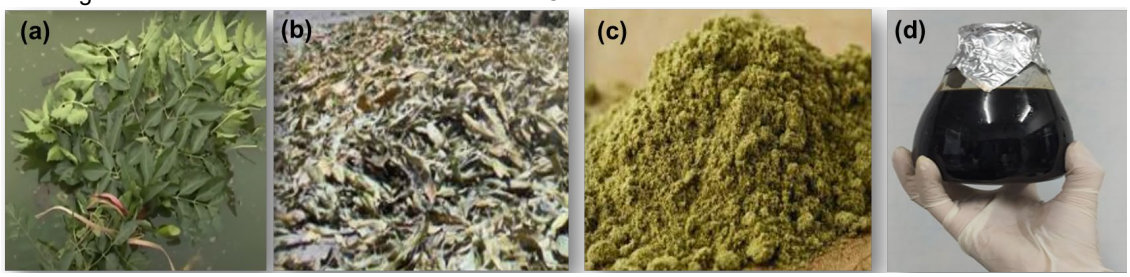


*Figure 1: Illustration of preparation process of* ethanol extract from M. azedarach leaves. (a) Fresh M. azedarach leaves; (b) dried leaves; (c) dried powder; (d) ethanol extract from M. azedarach leaves (EtMA).

### 2.2 Phytochemical screening of the extract

The chemical profile of the extract was preliminarily evaluated via Nugroho et al. (2017) procedures: *Alkaloids (Dragendorff's test), Saponins (Frothing test), Steroids (Salkowski test), Terpenoids (Liebermann-Burchard test), Quinones (Borntrager's test), Phenolic compounds (Ferric chloride test), Tannins (Ferric chloride test), Flavonoids (Shinoda test).*

### 2.3 The *in vitro* anti-protozoal activity of the *M. azedarach* leaf extract against *Trichodina nigra*

*Trichodina nigra* suspension was provided by the Department of Biotechnology, The University of Science, Vietnam National University, Hanoi, Vietnam. The parasites were infected and propagated on catfish, and the mucus of *T. nigra* infected fishes was used in further experiment. The *in vitro* anti-parasitic efficacy of EtMA against *T. nigra* was evaluated using Kumar et al. (2012) method. A volume of *T. nigra* suspension (1 mL) was immediately placed in tubes containing 4 mL of extract solution (50, 100, and 150 ppm) or formalin (100 ppm), or water, respectively, and mixed thoroughly. Cell vitality/ mortality of the samples was detect via eosin-nigrosin staining and anti-protozoal activity of the extract was evaluated via the mortality of the parasites in treated samples versus untreated sample (control) for 60, 120, 180 min, and the experiment was performed in triplicate.

### 2.4 Experimental animal

Whitespotted freshwater catfishes (*Clarias fuscus*) weighing 20 ± 5 g were captured from Tham fish farm, Nguyen Van Linh street, Phong Phu commune, Binh Chanh District, Ho Chi Minh City, Vietnam. The fishes were acclimated to laboratory conditions for one week. In each fish tank, the fish were provided catfish food (F5005 France-Feed, Proconco Can Tho Ltd, Vietnam) and supplemented with frozen bloodworms at a rate of 1 % of body weight per day. Excess food and waste were removed before replacement of water. The water level was maintained at 20 L per tank. Water quality was suitable for fish farming, as follows: temperature 28-30 $^{o}$C, pH 7.55 ± 0.11, dissolved oxygen 4.35 ± 0.21 mg/L, total alkalinity 75.52 ± 0.16 mg/L, with the natural light-dark cycle. The care and uses procedure of experimental fishes were strictly followed the CPCSEA guidelines for testing on fish. All possible measures may have been taken to minimize animal suffering.

### 2.5 Acute toxicity of *M. azedarach* leaf extract on catfish

The acute toxicity test of *Melia azedarach* L. leaf extract on catfish was conducted according to the Guidelines for Testing of Chemicals of the Organization for Economic Cooperation and Development (OECD, 2019). The acute toxicity of *M. azedarach* leaf extract (EtMA) to catfish was determined by bath treatment method at selected test concentrations of 1,000, 1,300, and 1,600 ppm ($EtMA_{1000}$, $EtMA_{1300}$, and $EtMA_{1600}$) for 15 min. Control fishes were treated under identical conditions without any toxicants. The fishes were observed for 96 h. Each concentration was repeated three times, with 10 organisms used in each treatment. Changes in behavior, body equilibrium, swimming speed, fin, and gill movement, abnormal signs such as head standing, slime secretion, hemorrhage on the body, signs of poisoning, and mortality rates were monitored and recorded at 24, 48, 72, and 96 h after exposure of the fishes to different concentrations of EtMA.

### 2.6 The *in vivo* anti-protozoal activity of the *M. azedarach* leaf extract against trichodinosis in catfish

A total of 60 male healthy catfishes were randomly divided into six experimental groups (10 fishes/group). The control group consisted of catfishes which were immersed in ordinary water without any extract treatment or *T. nigra* infection. The remaining fishes were infected with *T. nigra* cell suspension and monitored for 24 h, then they were classified into 5 groups: untreated, formalin (100 ppm), three doses of the extract ($EtMA_{800}$, $EtMA_{900}$, $EtMA_{1000}$) treated groups. All of fishes were bathed in tanks containing ordinary water (control and untreated groups) or formalin (100 ppm), the extract (800, 900, 1,000 ppm of EtMA) for 15 min daily for 3 d. The infection rate (prevalence of *Trichodina* spp. infected samples) and survival rate of the experimental fishes were recorded after 3 d of the last treatment. At the end of experiment (3 d after the last treatment), the fishes were anesthetized with eugenol, blood was collected from caudal vein and transferred to heparinized tube for testing the hematological parameters, such as red blood cells (RBC), white blood cells (WBC), hemoglobin (HGB) via Nwani et al. (2014) method. Aliquot of anticoagulant-free serum was used to detect biochemical parameter, including total protein (TP), and creatinine via the procedure of Adamu and Kori-Siakpere (2011), whereas the other parameters, such as blood urea nitrogen and total bilirubin were determined using the methods of Nguyen et al. (2022). The *in vivo* experiment was performed triplicate and the data were presented as mean ± standard deviation. The data were analyzed using Statgraphics Centurion XVI software with One-way analysis of variance (ANOVA) and Fisher's least significant difference test to evaluate the differences among groups ($p$<0.05).

## 3. Results & Discussion

### 3.1 *M. azedarach* leaf extract could effectively eliminate *T. nigra* at *in vitro* level

The preliminary phytochemical screening of the extract indicated the presence of some bioactive compounds such as alkaloids, tannins, saponins, phenolics, steroids, terpenoids, and flavonoids in chemical profile of the extract, but quinone was not detected in the extract. In previous study, terpenoid fraction of *Eugenia pruniformis* leaves exhibited demonstrated a strong anti-protozoal effect against *Leishmania amazonensis* (Albuquerque et al., 2020). Amaral et al. (2022) suggest saponins as a promising agent to control protozoal diseases. The presence of these compounds give a basis to investigate and explain the anti-protozoal effect of the extract.

The efficacy of anti-protozoal activity of *M. azedarach* leaf extract was presented in Table 1. The results suggested that the extract exhibited a prominent *in vitro* anti-protozoal activity against *T. nigra* in a dose and time-dependent manner. For instance, the efficacy of $EtMA_{150}$ group was 2.15 times higher than that $EtMA_{50}$ group after 60 min of treatment (70.15 ± 6.37 % versus 32.60 ± 5.19 %, respectively, $p$<0.05). In the same dose of the extract, prolongation of the treatment time could more effectively eradicate *T. nigra*. Treatment with 50 ppm EtMA for 180 min produced the most significant efficacy (53.03 ± 2.62 %), followed by those of 120 min and 60 min treatment (43.16 ± 2.59 % and 32.60 ± 5.19 %, $p$<0.05). Among three doses of treatment, only the anti-protozoal effect of the highest dose of the extract (150 ppm) was equivalent with the efficacy of formalin treated groups at three time points ($p$>0.05). This finding suggests the extract as an effective anti-parasitic

remedy to replace formalin treatment against *T. nigra* infection. This study was in line with several reports using herbal remedies such as neem, ginger, rosemary to treat aquaculture diseases related to protozoal infection (Zhu, 2020) and provide more information for application of bead tree as a potential aquaculture drug.

*Table 1: The efficacy of anti-protozoal activity of EtMA extract in vitro (%)*

| Treatment | 60 min | 120 min | 180 min |
|---|---|---|---|
| Formalin group | 72.53 ± 3.96$^{c}$ | 81.20 ± 3.70$^{c}$ | 88.38 ± 4.37$^{c}$ |
| $EtMA_{50}$ group | 32.60 ± 5.19$^{a}$ | 43.16 ± 2.59$^{a}$ | 53.03 ± 2.62$^{a}$ |
| $EtMA_{100}$ group | 57.51 ± 3.86$^{b}$ | 64.96 ± 2.96$^{b}$ | 70.71 ± 3.50$^{b}$ |
| $EtMA_{150}$ group | 70.15 ± 6.37$^{c}$ | 78.42 ± 4.36$^{c}$ | 85.35 ± 4.87$^{c}$ |

*The different superscript letters (a, b, c) denote statistical differences among groups ($p<0.05$)*

### 3.2 The toxicity of *M. azedarach* leaf extract is low and suitable used in catfishes

One of the most importance criteria for an aquaculture drugs is the safety of the drugs. The drugs must not only effectively eradicate the disease but also possess the low toxicity to the aquatic host organisms. The results indicated that treatment with the extract in a variety of doses (1,000, 1,300, and 1,600 ppm of EtMA) did not produce any adverse effects in terms of behavior, body equilibrium, swimming rate, fin and gill movement, mucus secretion, hemorrhage. No lethal effect in the extract treated fishes was found during observation periods (24, 48, 72, 96 h). The extract is safe at the tested doses and can be used to *in vivo* experiment for determination of the *in vivo* anti-protozoal activity of the extract.

### 3.3 Treatment with the extract suppresses *Trichodina* spp. infection and enhances survival rate of catfishes

The extract could hinder *Trichodina* spp. infection in catfishes with the dose dependent manner (Figure 2a). For instance, the lowest infection rate belonged to the highest dose of EtMA treatment ($EtMA_{1000}$ group) with 20.00 ± 10.00 % ($p<0.05$), followed by the medium dose ($EtMA_{900}$) and low dose treated groups ($EtMA_{800}$) accounting for 33.33 ± 5.77 % and 46.67 ± 11.55 %, respectively. Infection model was successful established with 100 % of *Trichodina spp.* infected fishes in untreated group. There is no significant difference between infection rates of $EtMA_{1000}$ treated and formalin treated groups ($p>0.05$). This finding indicates *M. azedarach* leaf extract as a promising tool to control infection of *Trichodina spp.* in catfish farming. Treatment with a variety doses of the extract (800, 900, 1,000 ppm) could improve the survival rate of *Trichodina spp.* infected catfishes (56.67 ± 5.77 %, 73.33 ±11.55 %, 90.00 ± 10.00 %, respectively) with as compared to the untreated catfishes (33.33 ± 5.77 %, $p<0.05$). As shown in Figure 2b, the highest dose of the extract enhanced the survival rate toward the values of formalin treated and control groups ($p>0.05$). That implies the protective effect of *M. azedarach* leaf against trichodinosis on parasite infected catfishes. According to Johnson (2013), saponins, a chemical group found in the extract, exerts its anti-protozoal activity via targeting to cholesterol component on cell membrane of protozoa, which leads to prevention the parasitic infection and management of waterborne diseases. As the consequence, the extract could reduce *Trichodina* spp. infection rate and increase the survival rate of parasites infected catfishes.

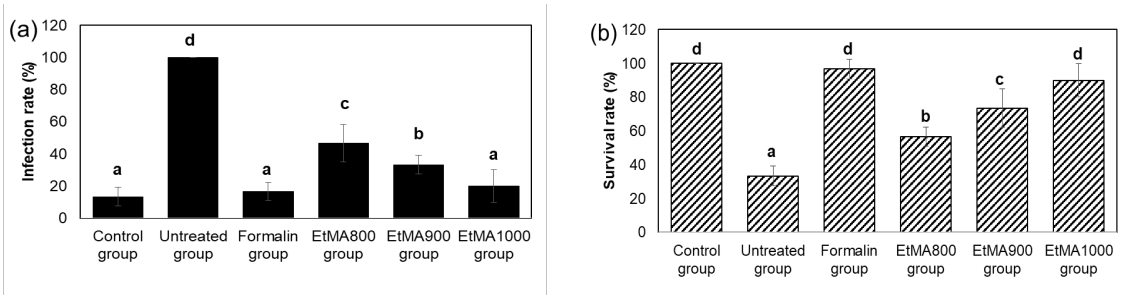


*Figure 2: The changes of (a) infection rate and (b) survival rate of experimental fishes ($p<0.05$). The different superscript letters (a, b, c, d) denote statistical differences among groups ($p<0.05$)*

### 3.4 The extract could improve hematological and biochemical indices of *T. nigra* infected catfishes

Trichodinosis is usually associated with anemia and inflammatory response, which was manifested in the changes of hematological and biochemical profiles of untreated group (Table 2). In untreated groups, erythrocyte count and hemoglobin level were lower than those of normal control group indicating anemia conditions ($p<0.05$). The number of leukocytes in untreated groups was higher than that of control fishes

($p$<0.05), which implied the activation of inflammation response. The biochemical parameters for kidney function test (creatinine, BUN) were noticeably higher than those of control group whereas the other indices for liver function test (total protein, total bilirubin) were lower than those of control fishes ($p$<0.05), which referred the damage in kidney and liver of *Trichodina* spp. infected fishes. All of three doses of the extract treatment could rescue the abnormal changes of hematological and biochemical parameters in dose dependent manner (Table 2). Some indices such as leukocyte count, hemoglobin, total protein, total bilirubin, BUN, creatinine of $EtMA_{1000}$ treated fishes were comparable with those of reference drug treatment ($p$<0.05), formalin. That demonstrates the high effectiveness of *M. azedarach* leaf extract in trichodinosis treatment and its health boosting activities.

*Table 2: The changes of hematological and biochemical indices of experimental fishes*

| | Control | Untreated group | Formalin group | $EtMA_{800}$ group | $EtMA_{900}$ group | $EtMA_{1000}$ group |
|---|---|---|---|---|---|---|
| RBC ($\times10^6$cells/mm$^3$) | $1.83 \pm 0.05^{f}$ | $1.26 \pm 0.04^{a}$ | $1.75 \pm 0.05^{e}$ | $1.42 \pm 0.04^{b}$ | $1.52 \pm 0.06^{c}$ | $1.68 \pm 0.04^{d}$ |
| HGB (g/dL) | $6.35 \pm 0.27^{e}$ | $4.38 \pm 0.22^{a}$ | $5.96 \pm 0.13^{d}$ | $4.78 \pm 0.36^{b}$ | $5.29 \pm 0.15^{c}$ | $5.83 \pm 0.20^{d}$ |
| WBC (x$10^3$cells/mm$^3$) | $6.95 \pm 0.18^{a}$ | $12.64 \pm 0.33^{e}$ | $7.31 \pm 0.16^{b}$ | $9.17 \pm 0.24^{d}$ | $7.82 \pm 0.21^{c}$ | $7.28 \pm 0.17^{b}$ |
| Creatinine (mg/dL) | $1.22 \pm 0.06^{a}$ | $2.13 \pm 0.06^{e}$ | $1.45 \pm 0.04^{b}$ | $1.75 \pm 0.07^{d}$ | $1.67 \pm 0.06^{c}$ | $1.51 \pm 0.05^{b}$ |
| BUN (mg/dL) | $38.28 \pm 2.83^{a}$ | $66.02 \pm 3.41^{e}$ | $44.98 \pm 1.44^{b}$ | $58.02 \pm 3.16^{d}$ | $52.13 \pm 1.89^{c}$ | $46.11 \pm 2.52^{b}$ |
| TP (mg/dL) | $1.59 \pm 0.05^{e}$ | $0.96 \pm 0.04^{a}$ | $1.46 \pm 0.04^{d}$ | $1.31 \pm 0.03^{b}$ | $1.37 \pm 0.05^{c}$ | $1.42 \pm 0.06^{cd}$ |
| TB (mg/dL) | $1.17 \pm 0.05^{a}$ | $1.79 \pm 0.07^{e}$ | $1.22 \pm 0.06^{ab}$ | $1.46 \pm 0.07^{d}$ | $1.39 \pm 0.06^{c}$ | $1.26 \pm 0.03^{b}$ |

*The different superscript letters (a, b, c, d, e, f) denote statistical differences among groups ($p$<0.05)*

Macroscopic observation results showed that trichodinosis caused severe lesions on skin of the parasite infected fishes (Figure 3b and 3c). The skin of untreated fishes was changed into the pale and light-gray color as compared to control fishes along with the excessive production of turbid mucus. The extract could reduce ulceration and mucus production as well as recovered normal color of the skin of *T. nigra* infected fishes (Figure 3d). The data were consistent with those of hematological and biochemical profiles, which indicates the health beneficial effect of the extract on parasite infected fishes. These findings are in line with numerous studies about pharmacological properties and health beneficial effects of *M. azedarach* such as anti-bacterial, anti-fungal, anti-helminthic, anti-inflammatory, immunomodulatory, anti-oxidant, anti-ulcer, and analgesic effects (Sharma and Paul, 2013). They also suggest further applications of *M. azedarach* for management of parasitic diseases in the organic and sustainable aquaculture.

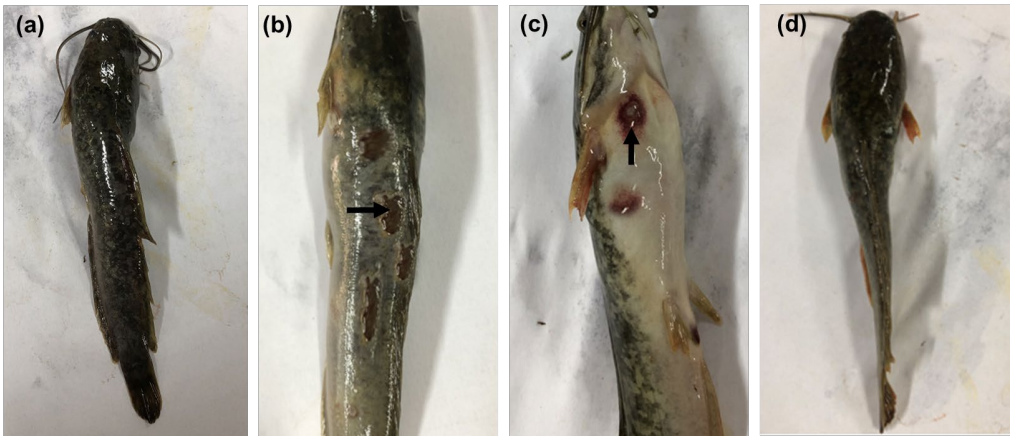


*Figure 3: Macroscopic observation of experimental fishes. Representative images for macroscopic examination of (a) control,(b) and (c) untreated, and (d) $EtMA_{1000}$ treated fishes. Black arrows indicate ulcerative lesions.*

## 4. Conclusions

Replacing synthetic chemical treatment in aquaculture is an urgent demand for prevention of aquatic environment pollution and compliance with organic fish farming practices toward the sustainable and neutral carbon production. In this study, the anti-protozoal activity of the ethanol extract of *M. azedarach* leaves against *Trichodina* spp. at *in vitro* and *in vivo* has been proven at the first time. The *Trichodina nirga* eradication efficacy

of the extract (150 ppm) is comparable with formalin (100 ppm) at three tested time point (60, 120, 180 min), the standard chemical used for trichodinosis treatment at three time points. *M. azedarach* extract is applied in treatment of trichodinosis on catfish at the first time and it proves the anti-protozoal activity of the extract *in vivo*. All three doses of the extract treatment (800, 900, 1,000 ppm) have protective effect on catfishes against trichodinosis not only in term of infection rate and survival rate but also health beneficial effect manifested through hematological and biochemical indices. Bath treatment with 1,000 ppm of the extract daily for 15 min daily in a-3-day- regimen can prevent and treat trichodinosis in catfishes as effective as the formalin treatment (100 ppm). Taken together, this study indicates that *M. azedarach* leaf extract is a natural and effective tool to control trichodinosis in catfish farming, and it can be alternative with synthetic chemical treatment for further application in organic aquaculture and the cleaner production.

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