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## Research Article

# *In vitro* study of short-term antiparasitic effect of alcoholic extract of *Terminalia catappa* L. leaves on *Ichthyophthirius multifiliis* theronts

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**Abstract**

*Ichthyophthirius multifiliis* is one of the parasitic diseases of fish infecting both farmed and wild freshwater fishes at all ages. This parasite penetrates into the fish skin epidermis and creates white spots on the skin accordingly, the disease caused by this parasite is also called white spot disease. Formalin, chloramine-T, malachite green, and other chemical compounds may be used against this disease; however, each of these compounds leads to harm to humans and the environment in some way; hence, it seems necessary to find natural alternative compounds to treat this disease. This study aimed at determining the effectiveness of alcoholic extract of *Terminalia catappa* L. at different concentrations (0-850 mg/L) and exposure times (1-3 h) in vitro. The findings were statistically compared with those of the control and positive control groups (formalin at a dose of 15 ppm). The results of the present study revealed that the effectiveness of *T. catappa* L. alcoholic extract on the theront stage of the *I. multifiliis* parasite is a function of time and concentration and with increasing concentration and time, its effect will enhance. The results of this study indicated that after two hours, a dose of 850 mg/L killed 100% of the theronts and it is the most appropriate dose, even though the possibility of applying this dose to treat live fish should be investigated.

**Keywords**: White spot disease, *Terminali catappa L.*, *Ichthyophthirius*, Acute toxicity, Fatality rate, Antiparasitic effects, Theronts.

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## Introduction

Both in tropical aquatic environments and temperate regions, one of the significant freshwater fish diseases, all over the world, is Ichthyophthiriasis or white spot disease caused by the ciliated Holotrich protozoa called *Ichthyophthirius multifiliis* (Matthews, 2005). This parasite results in significant economic losses in a wide range of farmed and wild fish species (Dickerson and Daw, 2006) and high mortality rate in farmed and ornamental fish (Song *et al.*, 2015); so that it can kill farmed fish at different ages (Aguilar *et al.*, 2005). The rapid replication of this parasite, displacement of infected hosts, as well as the wide range of hosts are among the factors leading to the widespread prevalence of this disease worldwide (Matthews, 2005; Forwood *et al.*, 2015).

The life cycle of this parasite is straightforward. Ich cyclically transforms between an obligate fish-associated trophont and a free-living reproductive stage, the tomont. The theront is the infective stage of the parasite (Woo, 2006). Since the trophonts of this parasite are replaced in deep skin and protect themselves from the drugs commonly used to treat skin parasites and the theronts separate themselves from the fish body, they are the most sensitive step of parasite life against treatment (Noga, 2011).

Various combinations have been reported to use against Ich. Malachite green is the most effective substance among these compounds to combat Ich. Nevertheless, the use of malachite green is prohibited given its toxic and mutagenic effects on humans and the environment (Srivastava *et al.*, 2004). Other compounds like potassium permanganate (Straus and Griffin, 2002); sodium percarbonate (Buchmann *et al.*, 2003); chloramine-T (Rintamaki-kinnunen *et al.*, 2005); hydrogen peroxide (Rintamaki-kinnunenen *et al.*, 2005); copper sulfate (Rowland *et al.*, 2009); formalin (Rowland *et al.*, 2009), peracetic acid (Sudova *et al.*, 2010), and bronopol (Shinn *et al.*, 2012) also have antiparasitic properties; however, these agents are often dangerous to human health and the environment (Tieman and Goodwin, 2001). Hence, we have to find herbal medicine with both high-performance antiparasitic properties and no side effects on human health and environment. In general, compared to chemical compounds, synthetics, and antibiotics, herbal compounds have rare harmful side effects, while lacking toxicity and harmful accumulation in living tissues, in some cases they are cheaper, degradable and reversible, and compounds of plant origin are more environmental friendly compared to the other substances (Citarasu *et al.*, 2003).

With the Persian name Garom Zangi, *Terminalia catappa* L. belongs to the Combretaceae family (Cashew). This plant is widely grown in tropical areas, such as East Asian countries. It is native to countries e.g. India, Malaysia, Cameroon, and Madagascar (Noumi and Yomi, 2001). Found in some southern provinces of Iran, including Hormozgan and Sistan and Baluchestan, Garom Zangi is a tall tree of 25 meters height with horizontal branches (Zargari,



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1988). In various countries, the leaves and bark of this tree are extensively employed in traditional medicine. Antibacterial, antifungal, antiparasitic, anticancer, anti-inflammatory, anti-diabetic, antioxidant, and liver protective properties are of the medical properties which are reported for this plant (Anand, 2015). In addition to the role of this plant in regulating pH, its inhibitory feature against some bacteria and parasites has been proven in aquaculture (Chitmanat *et al.,* 2005; Chansue, 2007; Lee *et al.,* 2016; Nugroho *et al.,* 2017).

Many studies have suggested that plant extracts can eliminate the free-living (floating) stages of Ich parasite (Buchman *et al.,* 2003; Ekanem *et al.,* 2004; Yao *et al.,* 2010, 2011; Ling *et al.,* 2012; Yi *et al.,* 2012). These studies have revealed that some plants, such as herbal medicine, have effective antiparasitic properties. In fish and water, these compounds can be degradable and do not create any dangerous effect in human or environmental health. As Ich disease is very common in aquaculture, the use of antiparasitic compounds extracted from plants is a safe, modern and eco-friendly way to cure Ich disease. Since the effect of Garom Zangi extract on Ich parasite has not been studied but its antiparasitic effect on other parasites such as *Trichodina* has been proven before, this study is aimed to investigae the antiparasitic effect of Garom Zangi on *I. multifiliis* parasite *in vitro*.

## Materials and methods

*Preparation of Terminalia catappa* L. *leaves*

*Terminalia Catappa* L. leaves were collected from Hormozgan province and then, the samples were sent to the herbarium of the Faculty of Pharmacy, University of Tehran to confirm the desired plant, and the plant was definitively identified.

*Preparation of alcoholic extract of Terminalia catappa* L.

In order to get the extract, 175 g of powder of fully dried leaves of the plant with 950 mL of fully soaked 96% methanol was placed in an incubator shaker for 2 hours at 25 °C. Subsequently, the sample was exposed to laboratory temperature for 18 hours. After that, the mixture was filtered using Whatman filter paper, the supernatant was isolated from the precipitate, and 750 mL of 96% methanol was added to the remaining precipitate. This was repeated for up to 4 days and all isolated supernatants were mixed together and after refining, distilled by vacuum distillation.

*Isolation of Ich parasite*

Five fish infected with Ich parasite were bought from ornamental fish supply centers. Subsequently, wet mount was carefully prepared from the infected fish and all the contents of the petri dish were washed. Then, the fish were killed through merciless slaughter and placed in small beaker (Bécher) containing water similar to the original aquarium water. They were examined under



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stereomicroscope. After about an hour, the tomonts detached from the fish's body slowly moved by the freestyle and landed on the beaker floor. Then, the beaker contents were transferred to separate petri dishes. The tomonts were ultimately isolated under a stereomicroscope at 40x magnification and using a sampler.

*Preparation of theronts of Ich parasite*

Petri dishes containing tomonts were incubated at 23.5°C for 22 hours. Tiny theronts with the feature of fast swimming in water were detectable after 22 hours. Density of theronts reached a fixed level by centrifuge (at 4000 rpm for 10 minutes). Toma and Neobaur slides were used to count the theronts.

*Preparation of alcoholic extract concentrations of Terminalia catappa* L.

Given the initial tests and reviewing the references, five different concentrations of alcoholic plant extract (50, 100, 250, 500, and 850 mg/L), formalin concentration (15 ppm, positive control), as well as a control group without extract (containing aquarium water) were prepared. The seven treatments investigated in the present study are as follows:

Group 1) Control, only chlorine-free aquarium water and alcoholic plant extract
Group 2) Positive control containing 15 ppm formalin
Group 3) Concentration of 50 mg/L alcoholic plant extract
Group 4) Concentration of 100 mg/L alcoholic plant extract
Group 5) Concentration of 250 mg/L alcoholic plant extract
Group 6) Concentration of 500 mg/L alcoholic plant extract
Group 7) Concentration of 850 mg/L alcoholic plant extract

*Conditions of exposing theronts to Terminalia catappa* L. *alcoholic extract concentrations*

Fifty microliters of theront suspension (600 theronts) was first poured into three replicates (3 wells) in 96-well plates based on the above seven treatments. The concentration treatment was subsequently applied in accordance with the numbers specified in the previous section with a volume of 50 µl and the mortality rate was calculated and recorded at 1, 2 and 3 hours.

*Calculating the survival and mortality rates of theronts*

At the end of the exposure time of the theronts, their losses were specified by this method. In summary, 10 µl of the contents of each well was placed on the Toma slide. In order to obtain the number of live theronts per cubic millimeter, the number of live theronts in nine large squares of Toma slide was counted and 10% was added to their number. Specification of theronts mortality rate was accordance to a morphologic change in parasitic theronts from an almost cylindrical to rounded-



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oval form caused by cell lysis. Given the initial density of 600 theronts per 100 mL, the results were calculated based on the number of live theronts per 100 mL.

### *Data analysis*

One-way analysis of variance (ANOVA) procedure followed by Duncan's testusing the SPSS software (Version 22, SPSS Inc., Chicago, IL, USA) was applied to determine the significant difference ($p$<0.05) between treatment means.

## Results

Fig. 1 illustrates the results of in vitro investigation of *Terminalia catappa* L. alcoholic extract in various concentrations (50-850 mg/L) at different test times (1, 2, and 3 hours) on the mortality rate of *I. multifiliis*. The results revealed the direct and significant relationship of the mortality rate of parasitic theronts with the concentration of the compound and the exposure time.

The shortest interval to kill more than 80% of parasites is related to the concentration of 850 mg/L. After two hours, concentration of 500 mg/L was able to kill more than 90% of parasites. As observed in Fig. 1, the dose of 850 mg/L after one to three hours does not significantly differ, although killing 100% of parasites over time and after 3 hours. A dose of 500 mg/L kills 100% of parasites after three hours; although the ability to kill the parasites is less than three hours, the way it works after two hours, is not significantly different from how this dose works after three hours. Of course, the rate of killing of parasites at a dose of 500 mg/L after one hour was significantly different from the rate of killing them after two and three hours.

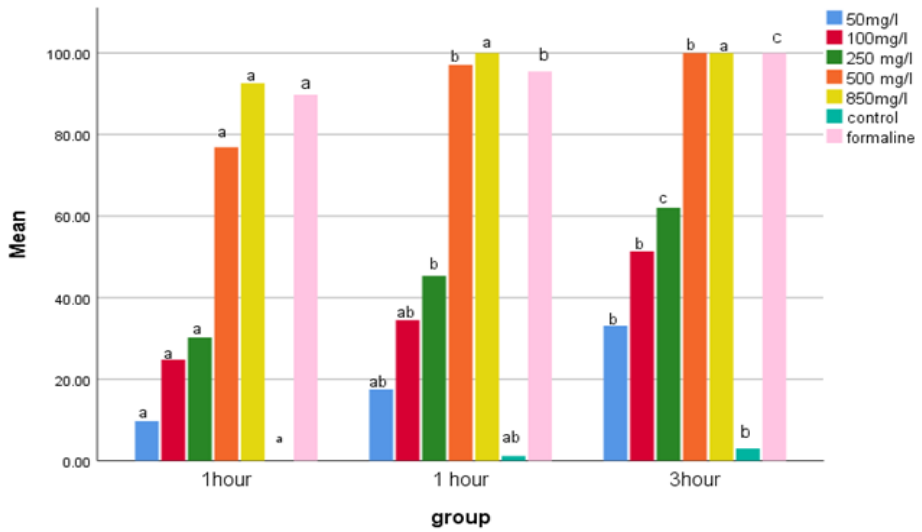


**Figure 1: Percentage of mortality of theronts of Ich parasites is a function of concentration of alcoholic extract (50-850 mg/L) and exposure time of 1-3 hours. Formalin 15 ppm, and aquarium water without any alcoholic extract was considered as positive control and control treatments, respectively. Different statistical letters in each column indicate a significant difference at the 5% level.**



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After one hour of exposure of *I. multifiliis* theronts, *Terminalia catappa* L. at a dose of 850 mg/L showed the best performance, so that it had a significant difference with this performance at a dose of 500 mg/L. After one hour, formalin performance was between 500 and 850 mg/L and did not significantly differ between these two doses.

After two hours of exposure of *I. multifiliis* theronts, *Terminalia catappa* L. at a dose of 850 mg/L killed 100% of the theronts; however, formalin and the dose of 500 mg/L alcoholic extract of *Terminalia catappa* L could not kill 100% of the theronts, no significant difference was observed between the performance of 15 ppm formalin and doses of 500 and 850 mg/L of alcoholic extract of *Terminalia catappa* L. 15-ppm formalin and concentrations of 500 and 850 mg/L of the alcoholic extract of *Terminalia catappa* L. could kill 100% of the parasites after 3 hours and no significant difference was observed.

## Discussion

In the present study, the effect of *Terminalia catappa* L. alcoholic extract at different concentrations (50-850 mg/L) and at different intervals (1, 2, and 3 hours) on the mortality rate of *I. multifiliis* theronts was examined in vitro. Mortality and survival rates of parasitic theronts have been chosen as the indices of the effectiveness of parasites over different intervals. This study was performed at regular intervals since exposure time is another indicator to assess the potential of antiparasites.

The results of the current study revealed the antiparasitic effect of the alcoholic extract of *Terminalia catappa* L. on *I. multifiliis* parasite. Increasing the concentration and exposure time led to a significant increase in the antiparasitic properties of the extract. The highest anti-theront effect (100% decline in the shortest possible time) was related to the concentration of 850 mg/L in 2 hours. Hence, it can be claimed that its antiparasitic effect against the *I. multifiliis* parasite was appropriate.

Previously, Chitmanat *et al.* (2005) employed various concentrations of grounded dried leaf solution of *Terminalia catappa* L. against tilapia pathogens, observing that 800 mg/L of crude extract of this plant after 48 hours killed *Trichodina* in this fish. This may suggest the high resistance of *Trichodina* parasite and show that *I. multifiliis* parasite is much more sensitive to this alcoholic extract compared to *Trichodina*.

In the study on the effect of dried leaf extract of *Terminalia catappa* L. on monogenic parasites of goldfish, Chansue (2007) indicated that the using 5.1 g/L of this substance for two weeks had the greatest effect on killing *Gyrodactylus sp*. and *Dactylogyrus sp.* parasites in goldfish. Moreover, the present study has revealed that the extract of dried leaves of *Terminalia catappa* L. may be effective in killing Ich parasite.

Moreover, studies have shown that this extract may be helpful for improving survival, blood profiles, as well as resistance to *A. hydrophila*. Also the



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antibacterial effects of this extract have been previously assessed. Bacteria such as *S. aureus, E. coli, Corynebacteria, Staphylococci, Enterococci, Escherichia, Salmonella, S. typhi,* and *P. aeruginosa* were the antimicrobial effects of *Terminalia catappa L.* on which were assessed (Shahina *et al.,* 2007; Opera *et al.,* 2012).

Goun *et al.* (2003) reported that methylene chloride and methanolic extracts of *Terminalia catappa* L. have antifungal activity against *Pythium ultimum, Rhizoctonia solani, Sclerotium rolfsii, Aspergillus fumigatus* and *Phytophthora parasitica*, so that the antifungal effects of this extract against *Pythium ultimum* and *Phytophthora parasitica* is very noticeable.

The *I. multifiliis* parasite is one of the parasites causing great damage to the aquaculture industry. Since employing the malachite green and formalin as chemical compounds in the treatment of edible fish to control the *I. multifiliis* parasite has been forbidden due to carcinogenicity, extensive studies have been proposed to introduce safe and effective alternative methods to control this disease.

Liang *et al.* (2015) examined the effect of two flavonoids (kuwanons G, O) extracted from *Morus alba.* Their findings revealed a 100% lethal effect of these flavonoids on the theronts of Ich parasite.

Alavinia *et al.* (2018) investigate the *in vitro* and *in vivo* effect of tannic acid on *I. multifiliis* in zebrafish (*Danio rerio*) to treat ichthyophthiriasis. *In vitro* antiparasitic assays revealed that tannic acid (TA) in a dose- and time-dependent pattern through the damage of parasite plasma membrane could be 100% effective against *I. multifiliis* theronts at concentrations of 8 and 11 ppm during all the exposure times (45-270 min). These authors then showed the short-term effectiveness of TA at different concentrations (0.0-7.0 mg/L) and in various exposure times (1-3 h) on the parasite theronts of *I. multifiliis* (Alavinia *et al.,* 2019).

The results of the study of Yao *et al.* (2011) indicated that the use of an alkaloid called dihydrosanguinarine, in the concentration range of 5.18 to 9.43 mg/L, has a very effective lethal coefficient on theronts of Ich parasite in vitro. They have previously revealed that different concentrations of sanguinarine extracted from a native plant in China, *Macleaya cordata*, could significantly kill the Ich parasitic theronts in vitro (Yao *et al.,* 2010). Shan *et al*. (2014) reported the strong lethal effect of two alkaloid compounds, including chloroxynol and chlorithrin, on Ich parasitic theronts in vitro. The positive effect of garlic extract on the inhibition of parasites and theronts of Ich parasite has been reported by Buchman *et al.* (2003).

Ekanem *et al*. (2004) assessed the effect of crude extracts of *Mucuna pruriens* leaves and *Papaya* seeds rich in phenolic compounds (particularly tannins) and antioxidants on the inhibition of Ich parasite. The number of parasites generally reduced to 90% after exposure to each treatment at a concentration of 200 mg/L compared to the control group.



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A 100% reduction count of this parasite was observed at the 150 mg/L concentration of *Mucuna pruriens* leaf extract and 200 mg/L concentration of papaya seed extract in vitro after 6 hours.

Fu *et al*. (2019) investigated the antiparasitic effect and mechanism of 10-gingerol of *Ziniber officinale* on Ich parasite in grass carp, concluding that gingerol-10 in concentrations of 2, 8, and 16 respectively causes 100% destruction of theronts, unencapsulated and encapsulated tomonts, created by the increased osmotic pressure, accumulation of free radicals, and membrane damage.

Martinez-Ortiz-de-Montellano *et al*. (2010) assessed the impact of tannin-rich tropical plant *Lysiloma latisiliquum* on adult parasite populations. The findings revealed that using this plant in a short interval due to high content of tannic acid could directly affect the biology of adult *Haemonchus contortus* (size of parasitic helminthe and fertility of female types).

The results of the present study suggested that like the other herbal compounds, the alcoholic extract of *Terminalia catappa* L. can be used as an alternative and appropriate drug against *I. multifiliis*. In accordance with the current study, 850 mg/L is the best dose against this parasite in vitro; nevertheless, more studies should be conducted, such as the possibility of using this dose in live fish.

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