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Antiparasitic Efficacy and Toxicity Evaluation of *Zingiber officinale* Roscoe Oil against *Ichthyophthirius multifiliis* in Freshwater Fish

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ABSTRACT

Ichthyophthirius multifiliis (*I. multifiliis*) is a harmful parasite with a negative impact on the health of freshwater fish. This research was operated to identify the chemical compound in the extracted oil *Zingiber officinale* Roscoe (RO) rhizomes using gas chromatography- mass spectrometry (GC-MS) and also to determine the antiparasitic efficacy against *I. multifiliis* isolated from goldfish (*Carassius auratus*). The toxicity of ZO was investigated via a zebrafish (*Danio rerio*) model. The results from GC-MS revealed three main compounds in the oil extracted, namely E-citral, Zingiberene, and Z-citral, respectively. Antiparasitic efficacy against *I. multifiliis* theronts was detected in concentrations of 10, 30, and 50mg/L of ZO. Moreover, after one hour of exposure to treatment, 62.2 and 73.9% mortality was observed in *I. multifiliis* theronts at concentrations of 30 and 50mg/L of ZO, respectively. However, antiparasitic efficacy against *I. multifiliis* protomonts was not detected in all concentrations of ZO treatment. In the cases of 100 and 200mg/L of ZO, 100% mortality was found in zebrafish embryos after one hour of exposure. In addition, after treatment with concentrations of 12.5, 25 and 50mg/L, no coagulation was detected in the embryo, but observed abnormalities included heart oedema, no circulation and yolk abnormalities during the 96-hour test. The results of the present study indicate that the major active compounds in ZO have the potential to eliminate *I. multifiliis* theronts isolated from goldfish. However, further study should be undertaken to determine the appropriate dose of ZO for safe application.

Key words: Antiparasitic; *Zingiber officinale* Roscoe; Essential oil; *Ichthyophthirius multifiliis*

INTRODUCTION

Ichthyophthirius multifiliis (*I. multifiliis*) is a pathogenic protozoan parasite with a negative impact on the health of freshwater fish, resulting in the development of ichthyophthiriasis, more generally referred to as "Ich" or "white spot disease" (Zhang et al. 2013; Valladão et al. 2016; Huang et al. 2022). It has four stages of development: a parasitic trophont, a free-swimming protomont, a reproductive tomont, and an infective theront. The parasites attach to the fish and cause damage to their skin. These parasites feed on fish and grow inside their skin and gills. Moreover, improper handling or lack of treatment during ichthyophthiriasis could enhance the potential for bacterial pathogens to infect the fish, ultimately resulting in death (Valladão et al. 2016; Huang et al. 2022). Typically, several chemicals and/or antiparasitic drugs, such as malachite green, formalin, bronopol, and potassium ferrate, can be used to treat ichthyophthiriasis in fish (Picón-Camacho et al. 2012; Hadfield 2021). However, these substances have some disadvantages, including accumulating in fish tissue, bioaccumulating, and causing harmful effects on the environment and human health (Picón-Camacho et al. 2012; Hadfield 2021; Gharavi-Nakhjavani et al. 2023). Malachite green, which is extensively applied to treat *I. multifiliis*, has been shown to have cytotoxicity, carcinogenicity, and mutagenicity effects and is also reported to be bioaccumulating in the

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environment and fish tissue. In 2000, the EC Directive 90/676/EEC and Regulation 2377/90/EEC banned the use of malachite green within the European Union, leading to its widespread prohibition (Hadfield 2021; Gharavi-Nakhjavani et al. 2023).

Over the past decades, extensive research has been needed to find a new natural alternative treatment to control the *I. multifiliis* infection that is non-toxic to animals and humans and not harmful to the environment (Özil 2023). *Z. officinale* Roscoe is one of the most well-known members of the Zingiberaceae family, also known as "ginger" (Saeed and Tariq 2006; Styawan et al. 2022; Eltaly et al. 2023). Researchers have found that substances extracted from ginger have significant biological and pharmacological activities. These include anti-inflammatory, antibacterial, antiviral, and antiparasitic activities (Saeed and Tariq 2006; Mishra et al. 2012; El-Sayed and El-Saka 2015; Mao et al. 2019; Styawan et al. 2022). Recently, there has been a focus on the development and use of medicinal plants, particularly *Z. officinale* Roscoe, against parasite infections in aquaculture. These treatments can be administered orally or in a bath (El-Sayed and El-Saka 2015; Van et al. 2021). Van et al. (2021) studied carp (*Cyprinus carpio*) and found that the essential oil from *Z. officinale* Roscoe bulb could effectively kill gill fluke (*Dactylogyrus* sp.) parasites.

Although most medicinal plants are safe, there have been reports of negative medication reactions caused by herbal treatments (Alafiatayo et al. 2019; Chahardehi et al 2020; Mubashir et al. 2022; Hegazy et al. 2023; Gul et al. 2024; Iqbal et al. 2024). Thus, the toxic effects of medicinal plants should be considered. Since toxicity tests on interesting plants are important to evaluate, zebrafish (*Danio rerio*) are currently considered the model of choice (Chahardehi et al. 2020). The present study investigates the chemical compound in the essential oil of *Z. officinale* Roscoe (ZO) or ginger oil cultured in Chiang Mai, Thailand, to determine its potential as a practical, alternative treatment for *I. multifiliis* infection in goldfish (*Carassius auratus*). Its toxicity effect is also investigated in a zebrafish model.

MATERIALS AND METHODS

Ethical approval

The Animal Care and Committee of the Faculty of Veterinary Medicine, Chiang Mai University (FVM-ACUC) (Process No. S5/2562 and No. S18/2563) approved the investigation for use in animal studies.

Materials

The fresh rhizomes *Z. officinale* Roscoe used in this experiment were taken from the local market in Chiang Mai, Thailand (Lat 18° 47′ 46.1148″ N and Long 98° 58′ 45.3468″ E), which were cultivated in Chiang Mai, Thailand. The specimens of plants were preserved at the Herbarium of the Faculty of Pharmacy, Chiang Mai University, Thailand. The plant materials were washed, cut into small pieces, and then dried and ground using a dry grinder. The prepared ginger powder was kept in a light-resistant package pending subsequent experiments.

The ZO was extracted by simultaneous stream distillation using a Clevenger-type apparatus for three hours. The collected volatile oil was dried over anhydrous sodium sulphate and stored in a closed, air-tight, light-resistant container at -20°C for later chemical compound analysis and further experiment steps. The chemical constituents of the ZO were carried out by gas chromatography-mass spectrometry (GC-MS) using an HP 6890 gas chromatograph coupled with an HP 5973 mass selective detector (Agilent Technologies, Foster City, CA, USA) on the HP-5MS column (30m × 250µm i.d. × 0.25µm film thickness). The flow rate of the carrier gas was set at 1 mL/min using a split mode (split ratio 500:1). The injection temperature was 250°C and the detector temperature was 280°C. The relative percentage of the essential oil constituents evaluated from the total ion chromatogram (TIC) by apparatus software and the analyzed essential oil constituents were identified based on their relative retention times to those of authentic samples and also confirmed by comparison of their mass spectra with the MS library search (Wiley, NIST, and NBS) and W8N08 library (John Wiley & Sons, Inc., Hoboken, NJ, USA).

Materials and Methods

Fish and parasites

For this experiment, ten goldfish were selected aged 6–12 months, infected with the Ich parasite from a local ornamental fish shop in Chiang Mai. The goldfish was infected with the Ich parasite and screened by viewing clinical signs on the skin. All obtained fish were further examined for the parasite trophont stage using wet mount techniques on their skin and fins under a stereomicroscope (EZ4 W, Leica, Germany). The fish were subsequently separated and distributed into the transparent glass tanks (one fish per tank) containing filtered tap water (15L per tank) with dissolved oxygen at 5.5 mg/mL, pH at 7.4–7.6, a constant temperature of 23°C, and natural daylight conditions at the Aquatic Animal Medicine Laboratory of the Faculty of Veterinary Medicine, Chiang Mai University, Thailand. The fish were fed commercial goldfish pellets according to 2% of their body weight once a day. Water parameters, including the ammonia level and pH value, were monitored twice a week and every day, 50% of the rearing water was renewed.

The goldfish presented high levels of Ich infection, characterized by large white spots on their skin, causing the *I. multifiliis* to fall off the fish at the mature trophont stage. Gentle skin scrapings were performed on the fish to collect the mature parasites for *in vitro* ginger oil efficacy tests (Zhang et al. 2013; Huang et al. 2022). The isolated parasites were separated randomly and used to evaluate the efficacy of ginger oil for parasiticidal effects against both the theront and protomont stages of parasites.

Effect of ZO on *I. multifiliis* theronts and protomonts

In the theront study, the theront concentration was determined before being applied to evaluate the antiparasitic activity of the ZO via a Sedgewick Rafter counting plate and light microscopy (Nikon, Tokyo, Japan). For *in vitro* ZO against the theronts of *I. multifiliis*, approximately 600 parasites were dispensed into each 24-well plate and exposed to various concentrations of ZO (10, 30, and 50mg/L) in a final volume of 500mL. Filtered tap water, formalin (50mg/L), and 0.1% dimethyl sulfoxide

(DMSO) without ZO were used for the negative, positive, and solvent controls, respectively. The killing efficacy of ginger oil in each well was examined using light microscopy at various intervals up to 9 hours after exposure to the treatment. The absence of parasite motility and/or abnormal morphology was considered to indicate dead theront. The experiment was performed at 25.0±0.5°C and repeated three times (n=3) (Zhang et al. 2013; Alavinia et al. 2018; Huang et al. 2022).

For the protomont study, 10 protomonts were put into each well of a 24-well plate using the above-mentioned method for separating parasites and the calculated concentration of parasites. The protomonts had their mucus removed. The test group exposed the parasites on the bottom of a 24-well plate to different concentrations of ZO (10, 30 and 50mg/L) in a final volume of 500mL. In this study, the negative, positive, and solvent controls were water, formalin, and solvent control, respectively. The parasiticidal efficacy of oil in each well was examined during 1, 2, 4 and 6 hours of exposure time and calculated via light microscopy. No motility with or without irregular morphology was classified as a dead protomont. The plates were kept at a temperature of 25.0 ± 0.5°C three times to test for dead protomonts (n=3) (Zhang et al. 2013; Alavinia et al. 2018; Huang et al. 2022).

Zebrafish maintenance and breeding

The zebrafish of both sexes (one male and two female adult zebrafish) were placed in a glass tank and carried during the breeding period. Following a stage of 3–4 hours post-fertilization (hpf), the fertilized eggs were selected under a stereomicroscope (EZ4 W, Leica, Germany) and washed with sea salt egg water (60mg/L sea salt and 2mg/L methylene blue). The healthy fertilized embryos were incubated at 28.5°C in a clean petri dish with previously prepared egg water until an *in vivo* toxicity test was conducted.

Embryonic toxicity and teratogenicity test

In total, 20 fertilized eggs were transferred to individual wells of 24-well plates for each concentration treatment. The embryos were exposed and incubated with various concentrations of ZO (12.5, 25, 50, 100, and 200mg/L) containing 0.1% dimethyl sulfoxide (DMSO) diluted in 2mL of embryo water. Filtered tap water and DMSO were used for the negative and vehicle controls, respectively. The exposed embryos were evaluated under a stereomicroscope at 24, 48, 72, and 96hpf (h post-fertilization). After incubation, the exposed zebrafish embryos were observed for both the morphology according to OECD test guidelines (OECD 2013) and the development of the embryo following the Kimmel et al. (1995) study to compare the normal and defective via a stereomicroscope. All data on abnormal embryos were recorded to evaluate the teratogenic effect of ZO on zebrafish embryos. Coagulation of embryos and/or no observed heartbeat of the embryo indicated death. The mortality rate of zebrafish embryos was determined by calculating the number of dead embryos.

Statistical Analysis

The experiments were performed with three independent replications of each treatment. The number of dead theronts (as mentioned previously, the absence of parasite motility and/or abnormal morphology was considered dead theronts) in different groups. The analysis was conducted using the survival package in the R statistical program R Core Team (Allignol and Latouche 2023). The mortality of zebrafish embryos was compared using a two-way analysis of variance (ANOVA) and Tukey's multiple comparison test using the R analysis program (R Core Team 2020).

RESULTS

Plant extraction and chemical composition of the extracted essential oil

The simultaneous stream distillation of *Z. officinale* Roscoe rhizome generated non-viscous, pale yellow transparent liquids with a 0.24±0.09% yield of essential oil. The chemical composition of ZO was determined using GC-MS. The GC-MS result of the extracted ginger oil identified a total of 18 compounds (Fig. 1). The most interesting components are listed in Table 1. The top three substances in the ZO were E-citral (19.94%), Zingiberene (16.47%) and Z-citral (9.95%), respectively.

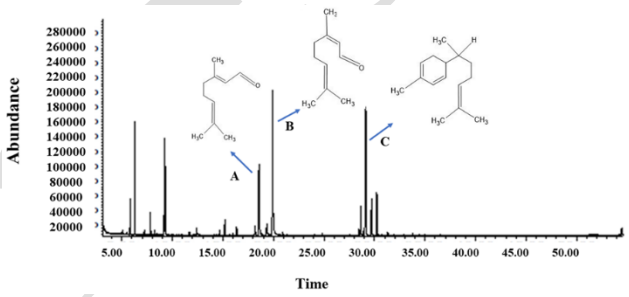

Fig. 1: GC-MS chromatograph for ZO. Chemical structures of the four major constituents identified from the ginger oil:(A) Z-citral (Retention time [RT] 18.57), (B) E-citral (RT 19.90), and (C) Zingiberene (RT 29.14).

Efficacy of ZO against *I. multifiliis* theronts

All studied ZOs demonstrated expressive efficacy against *I. multifiliis* theront (Table 2). In addition, antiparasitic efficacy correlated positively with the essential oil concentration and greater mortality was found in *I. multifiliis* theronts according to rising concentrations and duration of exposure. Interestingly, the mortality of theronts at concentrations of 30 and 50mg/L was 62.2±1.9 and 73.9±3.5% after one hour of exposure to treatment, respectively. However, after seven hours of exposure, the maximum concentration of ZO was effective in killing all theronts (100%). Compared to 50mg/L of formalin, it could not remove 100% of the theronts within nine hours of exposure. Furthermore, after seven hours of exposure, 30mg/L of essential oil also detected more than 90% mortality.

Efficacy of ginger oil against *I. multifiliis* protomonts

During the exposure time, ranging from 1–6 hours, at multiple concentrations of ZO (10, 30, 50mg/L), no efficacy of ZO was observed against *I. multifiliis* protomonts. Furthermore, 50mg/L of formalin could remove 100% of *I. multifiliis* protomonts after one hour of exposure to treatment. Moreover, DMSO could not eliminate *I. multifiliis* protomonts during the exposure time range of 1–9 hours.

Table 1: GC-MS analysis of the chemical composition of ZO, with ginger oil obtained through simultaneous steam distillation of the rhizomes of *Zingiber officinale* Roscoe**.**

Name	IUPAC* name	RT** (min)	Area (%)	Quality
E-citral	2,6-Octadienal, 3,7-dimethyl-, €-	19.90	19.94	96
Zingiberene	(5R)-2-methyl-5-[(2S)-6-methylhept-5-en-2-yl]cyclohexa-1,3-diene	29.14	16.47	96
Z-citral	(2E)-3,7-dimethylocta-2,6-dienal	18.57	9.95	90
Camphene	2,2-dimethyl-3-methylidenebicyclo[2.2.1]heptane	6.28	9.48	98
Sabinene	4-methylidene-1-propan-2-ylbicyclo[3.1.0]hexane	9.20	8.77	95

* IUPAC, International Union of Pure and Applied Chemistry; ** RT, retention time

Table 2: Mortality rate of *I. multifiliis* theront (n=3)

Experiments	Intervals after exposure to the treatment (h)				
	1	3	5	7	9
Control	2.8±2.5a	9.4±3.5a	11.7±5.0ab	20.0±12.0ab	29.4±12.6bc
DMSO	8.3±8.3a	15.6±15.0a	19.4±15.8ab	25.6±14.9ab	29.4±18.6bc
Formalin 50mg/L	80.6±4.2d	85.6±2.5d	91.7±2.9f	94.4±5.1f	98.9±1.0f
ZO 10mg/L	38.3±3.3c	51.1±3.5e	59.4±8.2e	67.8±1.9e	76.1±5.1d
ZO 30mg/L	62.2±1.9e	77.8±3.5d	90.0±7.3f	95.0 ±2.9f	98.3±1.7f
ZO 50mg/L	73.9±3.5d	87.8±2.5df	98.3±1.7f	100.0±0.0f	100.0±0.0f

Values (mean±SD) with the same alphabet in a row/column are not significantly different.

Zebrafish embryonic toxicity test

The zebrafish embryos were exposed to various concentrations of ZO to determine their toxicity. In this study, the toxicity effect was found to be correlated with the concentration of the extracted oils and periods after exposure; increased concentrations and duration of exposure detected a higher mortality rate in embryos (Table 3). Zebrafish embryos showed 100% mortality after one hour of exposure to 100 and 200mg/L of ginger oil. Moreover, 50mg/L of the extracted oil, after 96 hours, also detected 100% mortality in zebrafish embryos. Mortality at 24–96 hours post-treatment was significantly higher at concentrations of 100 and 200mg/L ZO than at the lowest concentration of 12.5mg/L ZO.

Table 3: Mortality rate of zebrafish embryos (n=3)

Experiments	Intervals after exposure to the treatment (h)			
	24	48	72	96
ZO 12.5mg/L	4.0±1.7ad	8.3±2.9ad	8.7±1.2ad	10.0±5.0ad
ZO 25mg/L	6.0±1.7ae	10.0±5.0af	26.7±11.5a	42.3±11.2a
ZO 50mg/L	25.0±5.0b	63.3±2.9b	95.0±5.0b	100.0±0.0bc
ZO 100mg/L	100.0±0.0c	100.0±0.0c	100.0±0.0c	100.0±0.0c
ZO 200mg/L	100.0±0.0c	100.0±0.0c	100.0±0.0c	100.0±0.0c
DMSO	0.0±0.0d	0.7±1.2d	1.0±1.0de	0.0±0.0def

Values (mean±SD) with the same alphabet in a row/column are not significantly different.

Teratogenicity in zebrafish

In addition, at 12.5, 25 and 50mg/L of ZO, no coagulation was detected in the embryo, but heart oedema, no circulation, and yolk abnormalities were observed during the 96 hours of treatment (Table 4). During the experiment, a lack of heartbeat was also discovered in the 25 and 50mg/L groups. Moreover, a lack of somite formation and no detachment of the tail bud were revealed in only 50mg/L of the extracted oil. At the highest concentrations of ZO, all embryos exhibited teratogenicity in both the 100 and 200mg/L ZO groups coagulated at the end of 24 hours. During the 96-hour test, no significant teratogenic abnormalities in embryos were observed for the vehicle control and negative groups. Additionally, both control groups showed normal development (Fig. 2).

Table 4: Teratogenic effects of ZO in zebrafish embryos.

Abnormalities in zebrafish embryos	Concentration of ZO (mg/L)					DMSO
	12.5	25	50	100	200	
Coagulated	N	N	N	P	P	N
Lack of somite formation	N	N	P	N/A	N/A	N
No detachment of tail bud	N	N	P	N/A	N/A	N
Lack of heartbeat	N	P	P	N/A	N/A	N
Heart oedema	P	P	P	N/A	N/A	N
No circulation	N	P	P	N/A	N/A	N
Yolk abnormality	P	P	P	N/A	N/A	N

Abbreviations: N, no effect of morphological characteristics compared to the negative control group; P, presence of abnormalities; N/A, not applicable as the embryo died (all embryos were dead after 24 hours of treatment exposure).

DISCUSSION

Since medicinal plants are used in the traditional treatment of many diseases, both human and animal, increasing research has been conducted to determine their efficacy (Hussain et al. 2022; Jamil et al. 2022; Abbas et al. 2023; Alsayeqh and Abbas 2023; Altaf et al. 2023; Rehman et al. 2023; Jamil et al. 2024). In the present study, ginger oil was extracted from rhizomes through simultaneous stream distillation using a Clevenger-type apparatus, yielding a 0.24% essential oil yield. Similarly, Kamal et al. (2023) extracted ginger rhizomes from Thailand, observing a 0.2% yield of extracted oil by hydro-distillation, while the research by de Souza Junior et al. (2020) in Brazil demonstrated a 0.25% yield of essential oil using the steam distillation extraction method. In contrast, the studies by Abdurahman et al. (2013) in India and Zaid et al. (2022) in Malaysia presented the percentage yield of ginger oil as 0.85%, separated by the microwave-assisted hydro-distillation method, while 0.08 to 0.77% was obtained by the steam-distillation extraction method, respectively. In addition, Kamal et al. (2023) extracted the oil of ginger rhizomes from China, yielding 0.09 to 0.15% by hydro-distillation. Generally, the variation in essential oil yield from plants could be observed. This can be affected by the source of the plant, immaturity of the harvested plant, changes in climatic conditions during cultivation, agricultural practices, soil quality, freshness or dryness, long storage periods before analysis, and extraction methods (Mahboubi 2019; Zaid et al. 2022).

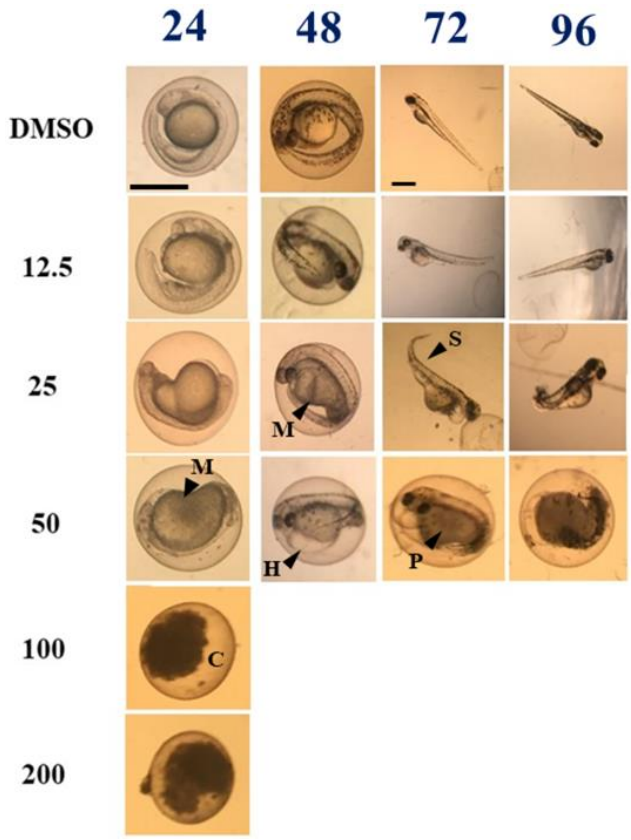

Fig. 2: Morphological features of zebrafish embryos and larvae, examined after exposure to varying doses of ZO at different time intervals (24, 48, 72, and 96 hours after fertilisation). Normal morphology can be observed in the group treated with the lowest doses and DMSO. Typical zebrafish development abnormalities can be observed in embryos treated with ZO at concentrations ranging from 25 to 50mg/L. Abbreviations: M, malformation of yolk sac; S, spinal column curving; H, heart oedema; P, poor reabsorption of yolk sac; and C, coagulated embryo (scale bars = 1µm).

Most of the medical effects of *Z. officinale* Roscoe rhizomes are due to phytochemical compounds (Styawan et al. 2022). Several studies have shown that the chemicals in ginger essential oil have important biological and pharmaceutical effects (Saeed and Tariq 2006; Ganjewala et al. 2012; Styawan et al. 2022; Gutiérrez-Pacheco et al. 2023). GC-MS was also used to examine the chemical compounds in this study, and numerous monoterpenes were found, including citral (29.89%), E-citral (19.94%), and Z-citral (9.95%). Furthermore, the volatile oil extraction from ginger in this study also revealed the presence of sesquiterpene compounds, specifically zingiberene (16.47%). There are two types of citral: neral (also written as Z-citral, β-citral, cis-citral, or citral B) and geranial (also written as E-citral, α-citral, trans-citral, or citral A). It is an important compound in many medicinal plants, especially ginger rhizomes. Many earlier studies have investigated this compound, reporting that it might help prevent and treat diseases caused by bacteria, fungi, and parasites, as well as cancer, inflammation, insects, and pests (Ganjewala et al. 2012; Gutiérrez-Pacheco et al. 2023). Recently, interesting research has revealed that citral could kill microorganisms by stopping the growth and reproduction of harmful microorganisms and delaying oxidative processes (Gutiérrez-Pacheco et al. 2023). Citral was very good at killing parasites because it caused apoptosis, marked by cell shrinkage, cytoplasmic bubbles, the loss of a flagellum, nuclear chromatin condensation, loss of mitochondrial membrane potential, cell membrane lysis with cytoplasm leakage, and DNA fragmentation. This likely occurs due to the loss of mitochondrial function (Santoro et al. 2007; Dos Anjos et al. 2016; Moreno et al. 2018; Pereira et al. 2022). Citral, the primary ingredient in the extracted oil, plays a crucial role in the medicinal properties of *Z. officinale* Roscoe rhizomes.

Previous studies have shown that certain medicinal plants demonstrate effectiveness in protecting fish from *I. multifiliis* (Zhang et al. 2013; Valladão et al. 2016; Huang et al. 2022). Moreover, the literature indicates that theronts are more responsive to parasiticidal compounds from plants and chemical substances (Buchmann et al. 2003; Rowland et al. 2008; Ling et al. 2012; Yi et al. 2012; Zhang et al. 2013; Huang et al. 2022). Interestingly, the present study is the first to report that the ZO cultivated in Chiang Mai, Thailand, performed an antiparasitic effect against *I. multifiliis* theronts isolated from goldfish. This investigation may be based on the chemical composition of oil, as described previously. However, the study did not uncover the efficacy of ginger oil against protomonts. Similarly, a study in China on the parasiticidal effects of berberine revealed that 15mg/L of berberine during a 4-hour exposure time could kill 99.30% of *I. multifiliis* theronts, while it had no effect on protomonts (Huang et al. 2022). According to this study, ZO demonstrates the potential to eliminate an infective theront stage of *I. multifiliis* isolated from goldfish but could not kill protomonts. Due to the protomont stage, the parasite was covered by a cyst, creating a barrier to ZO penetration.

Lin et al. (2016) reported that the minimum concentrations of *Zingiber officinale*, *Cynanchum atratum*, and *Cynanchum paniculatum* extract needed to achieve 100% *I. multifiliis* mortality were 8 (after 131 min of exposure), 16 (after 124 min of exposure), and 16mg/L (after 148.7 min of exposure), respectively. Due to the differences between the plants and the extraction method, the applied dose and exposure time may also vary to achieve 100% mortality of *I. multifiliis*. Zhang et al. (2013) also showed that all theronts could be killed by exposure to 5 and 10mg/L of pentagalloylglucose extracted from *Galla chinensis* for one hour. Furthermore, Valladão et al. (2016) reported that the studied concentrations of *Melaleuca alternifolia*, *Lavandula angustifolia*, and *Mentha piperita* essential oils were 57 to 455mg/L, showing 100% mortality of theronts after two hours of exposure. In comparison to previous studies, ZO was more effective in killing *I. multifiliis* than garlic (*Allium sativum*), which required 15 hours to eliminate all theronts at a concentration of 62.5mg/L (Buchmann et al. 2003).

Although natural active ingredients are considered safe for humans, animals, and the environment, inappropriate use of these compounds can lead to intoxication (Zhang et al. 2013; Valladão et al. 2016; Huang et al. 2022). In this study, the toxicity effects of ZO were also examined using a zebrafish model. Zebrafish serve as a common model for screening the efficacy and toxicity of drugs due to the significant data on vertebrate animals (Chahardehi et al. 2020). Active compounds, particularly in the zebrafish embryo, have gained

popularity in toxicity studies due to their comprehensive and well-defined developmental duration for vertebrate embryos, allowing evaluation in the early life stages of fish. As a result, zebrafish evaluation can be used as a primary toxicity model to scan for toxic drug candidates and as a substitute for vertebrate animal models (Chahardehi et al. 2020; Stachurski et al. 2023). However, few reports exist on the toxicity of ginger oil in zebrafish embryo models. Similarly to other previous experiments, significant morphological damage in zebrafish embryos to extracted oil was discovered in this present study. After exposing the zebra embryos to ginger oil, abnormalities were observed, such as coagulation, lack of somite formation, no tail bud detachment, lack of heartbeat, heart oedema, no circulation, and yolk abnormalities, particularly at high concentrations. In accordance with previous data, morphological abnormalities and developmental impairment were observed in zebrafish embryos exposed to different essential oils obtained from plants, such as lemongrass oil, oregano oil, and thyme oil (Hudaib et al. 2002; Teixeira et al. 2013; Majewska et al. 2019; da Silva Jr et al. 2023). These oil compounds, such as thymol, carvacrol and citral, are known for their ability to pass through zebrafish chorion (Majewska et al. 2019; Piasecki et al. 2021; da Silva Jr et al. 2023). Therefore, it is reasonable to hypothesise that the toxic effects of these extracted oils are associated with the presence of phytochemical substances. Moreover, a study by da Silva et al. (2023) revealed that citral was the likely cause of a reduction in the percentage epiboly occurring when these compounds pass through the chorion, indicating a delay in the gastrulation process. Interestingly, citral is also reported to be used in pharmacology as an inhibitor of retinoic acid biosynthesis, which causes acid reduction (Gur et al. 2022). During the early stages of embryonic development, particularly during gastrulation and the formation of the axis and limbs, retinoic acid plays a crucial role, leading to the detection of cytoskeletal system malformation and development delay (Stafford and Prince 2002; Smith et al. 2003; Gibert et al. 2006). The present study demonstrates that a low dose of ZO has the potential to kill *I. multifiliis* infections with less embryonic toxicity and teratogenicity. However, further research is necessary to determine the safe therapeutic dose of ZO for treating *I. multifiliis* infections.

The findings of this study demonstrate the pharmacological activities of the active compounds in the extract from herbal plants, indicating their potential for animal disease treatment. The active compounds of ZO demonstrate the ability to eliminate parasites and potentially eradicate an infective stage of *I. multifiliis* from goldfish. However, further *in vivo* experiments should be conducted to investigate the appropriate dosage of ZO to prevent adverse effects. In addition, the findings from this initial investigation suggest the use of ZO for alternative treatment of an infective theront stage of *I. multifiliis* in ornamental fish. Nevertheless, in the protomont stage, ZO should be prepared in pharmaceutical dosage form or formulated using pharmaceutical nanotechnology to increase oil penetration through the covered cyst of the parasite. Additionally, herbal plants, unlike conventional chemical treatments, have low bioaccumulation and are decomposable, potentially preventing drug resistance from the use of hazardous chemicals.

Conclusion

There is growing interest in the application of medicinal plant extracts in human and animal treatments because they are safer and contain many active phytoconstituents. In this study, the identified active substances in the ZO exhibited antiparasitic activity, effectively eliminating an infective stage of *I. multifiliis* in an *in vitro* experiment. However, inappropriate use of this compound could still have toxic effects. Further study is required to establish precise therapeutic doses in treatment and safety thresholds, as this will provide valuable information for developing an appropriate pharmaceutical dosage form of ginger oil for ornamental fish aquaculture.

Authors' contributions: BS and SP: Conceptualization and project administration. BS, WC, SO, and S.P: Methodology. BS and TY: Analyzed and interpreted the data. BS, WC and SP: Writing—original draft preparation. BS and SP: writing—review and revised the manuscripts. All authors have read and agreed to the published version of the manuscript.

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