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Evaluation of Anaesthetic Effect of Commercial Basil *Ocimum basilicum* on Zebrafish (*Danio rerio*) Embryos

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Abstract: Essential oils (EOs) are natural compounds used in various fields such as traditional medicine, gastronomy, and agriculture, often used against plant and animal parasites. In the present study, the toxic and pathological effects of basil (*Ocimum basilicum*) essential oil on the development of zebrafish embryos was investigated. The manufacturer datasheet reported linalool and eugenol as major compounds. The effects of the essential oil were evaluated through a zebrafish embryo toxicity test (ZFET) following the OECD n°236 guidelines. Embryos were exposed to different essential oil concentrations (50, 100, and 200 µL/L). Results showed mortality effects in the experimental groups in a dose-dependent manner. Moreover, zebrafish exposed to the higher concentration showed several pathological alterations; particularly, delayed hatching, pericardial edema, and a significant reduction of heart rate after 96 h post-fertilization (hpf) compared to the control group. Results reported here suggest an anaesthetic effect of the *O. basilicum* at low concentrations, due to the possible synergist effect of the main components such as linalool and eugenol which act on the GABAergic system evoking bradycardia.

Keywords: *Danio rerio*; basil oil; anaesthesia; larvae

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1. Introduction

Basil (*Ocimum basilicum*), belonging to the Lamiaceae family, is one of the most popular plants cultivated around the world. The most important use is represented by gastronomy. *O. basilicum* is used as an aromatic and flavour additive in cooking and in treatment for various medical conditions, including headaches, kidney malfunction, diarrhea, and constipation [1]. Basil is known for its nutritional properties by providing essential mineral salts and vitamins of groups B and C in the daily diet [2]. It is used for several purposes such as insect repellent [3] and an antibacterial against various pathogenic Gram-negative and Gram-positive bacteria, including species found in aquaculture such as *A. hydrophila* where it can inhibit its growth [4–6]. Basil oil extracts have been reported to have anti-inflammatory properties, able to modulate the production of several cytokines, such as TNF-α, IL-1β, IL-6, and IL-10 [7]. It has been used as an immunostimulant supplement added to fish feed, promoting growth, and protecting them from various pathogens thanks to a significant increase in RBC and WBC blood elements [8,9]. Basil and other plant species such as clove oil showed a strong ability to significantly modulate the levels of antioxidant activities in a mouse model by increasing CAT, SOD, and GSH enzymes [10–12].

Bioactive compounds from various natural medicinal herbs have a key role in health-care applications and progressively became main components for the development of many biopharmaceutical applications so they could be a new resource for pharmacotherapeutic

discovery [13]. Secondary metabolites are found in different plant species produced by many metabolic functions and are used by the plants themselves as a deterrent for vertebrate and invertebrate parasites. For this reason, many compounds are toxic to a wide range of insects that can parasitize the plant.

Recent studies have provided promising results for essential oils tested as mosquito larvicides [14]. Basil essential oil has been used against mosquito larvae of *Culex tritaeniorhynchus*, *Aedes albopictus,* and *Anopheles subpictus*. The toxic effect of basil essential oil was useful against third-stage mosquito larvae at 20 ppm [15]. In other studies, the use of basil essential oil against *Giardia lamblia* in a mouse model has been investigated. The results showed that the essential oil of *O. basilicum* and its compounds, especially linalool, induce a strong antiparasitic activity at 2 mg/mL [16]. Medicated baths of African basil (*Ocimum gratissimum*) essential oil (10 to 15 mg/L) showed a parasite load reduction of up to 100% on Tambaqui (*Colossoma macropomum*) gills [17]. Treatment with American basil *Ocimum americanum* essential oil was used as antiparasitic against *Gyrodactylus* sp. The essential oil reduced the abundance of parasites in Silver catfish *Rhamdia quelen* at 10 and 20 mg/L. Authors demonstrate the potential use of *O. americanum* against relevant fish pathogens, *Aeromonas hydrophila* and *Gyrodactylus* sp. [18].

Zebrafish embryos are often used as an in vivo model to evaluate the effects of different substances in toxicological assays [19]. Due to the lack of literature regarding the effects of basil essential oil on zebrafish embryos, the aim of the present study was to evaluate the potential toxicity and the anesthetic effect of *O. basilicum* essential oil in zebrafish embryos. This is useful for further study aimed at the possible effect of basil essential oil as an antiparasitic against fish infections and as a valid alternative to common anesthetics used in animal experimental studies.

2. Materials and Methods

2.1. Ocimum basilicum Composition

In the present study, the essential oil, extracted by steam distillation, was purchased from Base Formula (Melton Mowbray, UK). Based on the product datasheet, the essential oil major constituents were linalool (62%), eugenol (15%), Geraniol 0.2%, Citronellol 0.3%, Limonene 1%, and 21.5% was composed of other minor constituents.

2.2. Fish Management

Wild-type adult zebrafish strains, between 4 and 6 months old, were used for egg production. Zebrafish, coming from the Centre for Experimental Fish Pathology (Centro di Ittiopatologia Sperimentale della Sicilia—CISS) at the Department of Veterinary Sciences, were maintained in a stand-alone facility (ZebTec, Tecniplast, Buguggiate, Italy) at standard environmental conditions: temperature 27 ± 1 °C, natural light/dark cycle of 14:10 h, pH stabilized to 7.3, and dissolved oxygen 7.6 ppm. Fish were fed twice a day with artificial food (Gemma micro-300, Skretting, Verona, Italy) at 3% of body weight, and live food composed of *Artemia salina.*

2.3. ZFET Trial

Embryos obtained in this study were used to carry out a Zebrafish Embryo Toxicity test (ZFET) according to OECD guidelines [20]. Eggs were obtained by mating zebrafish at a ratio of 2:1 of female to male. Embryos were collected and transferred to a petri dish (90 mm) containing embryo medium (50 eggs on each plate). Fertilized eggs were selected under a stereomicroscope (Leica M205C, Leica Microsystems Srl, Buccinasco (MI), Italy) and divided into 24-well plates (LABSOLUTE, Th. Geyer GmbH & Co.KG, Renningen, Germany) (1 embryo per well) filled with 2 mL of the respective solutions. The essential oil was dissolved 1:10 with ethanol 96°, reaching final concentrations of 50, 100, and 200 µL/L (OBZ1, OBZ2 and OBZ3 respectively) of basil oil mixed in one litre of sterilized embryo medium (15 mM NaCl, 0.5 mM KCl, 1 mM $CaCl_2$, 1 mM $MgSO_4$, 0.15 mM KH_2PO_4, 0.05 mM 24 Na_2HPO_4, 0.7 mM $NaHCO_3$; pH 7.3). The blank control group was immersed

in only embryo medium. Controls and experimental groups were incubated at 26°C up to 96 h post-fertilization (hpf). According to OECD n. 236, embryos were monitored at 24, 48, 72, and 96 hpf to estimate several endpoints: lack of somite formation, coagulation of the embryo, non-detachment of the tail, detection of the heartbeat, and the percentage of hatched embryos were recorded. Furthermore, the heartbeat (HB) in all experimental groups was detected. Every 24 h and after checking the embryos, solutions were renewed at 90% of the volume of each well. A total number of 60 eggs were used for each concentration (3 replicates, with 20 larvae for each group). The positive control solution was prepared using the highest dosage of 96° ethanol (1.8 mL/L) dissolved in embryo medium.

According to Directive 2010/63/EU and relating Italian DL 26/2014 on the protection of animals used for scientific purposes, experiments on zebrafish larvae up to five days (120 h) post-fertilization are not considered adult animals and thus they do not need ethical approval.

2.4. Anesthetic Trial

To evaluate the potential anaesthetic effects of basil essential oil on the cardiovascular system, an additional assay with healthy zebrafish larvae at 96 hpf was performed. The heart rate was evaluated on 20 larvae per group in 50, 100, and 200 µL/L of essential oil, respectively identified as ZBA1, ZBA2, and ZBA3. Each group in the present study was assessed in triplicate.

2.5. Heart Rate Determination

For the heartbeat, estimation by video recording on zebrafish larvae was defined. Videos were recorded under the stereomicroscope (Leica M205C, Leica Microsystems Srl, Buccinasco (MI), Italy) at a room temperature of 26 °C. The heartbeat was analysed following the methodology reported by other authors [21]. For imaging, the control group was immobilized with MS-222 at 0.003% (*w/v*), which did not have any effect on the heartbeat in zebrafish embryos [22]. Briefly, for the first ZFET trial, contextually the observations of acute endpoints, the heart rate of all larvae directly on 24-well plates was evaluated. For the second trial, healthy larvae were transferred to a petri dish with an essential oil solution. After reaching the state of anaesthesia, larvae were placed in lateral view and video was captured for 1 min at 60 fps, reduced in post-production to 30 fps using Adobe Premiere CC. Video output was changed in AVI format to make it compatible with Fiji software. After opening the video with Fiji software, the region of interest (ROI) was selected and positioned in the atrium and/or ventricle region. Heartbeat intensity was analysed using the Time Series Analyzer V3 plugin and the Find Peak plugin. Data obtained were exported in Microsoft Excel and the beat interval was calculated by subtracting the average time between two consecutive atrial/ventricular peaks. Beat intervals were converted into time by dividing them with the frame rate. Finally, BPM (HR) was obtained by dividing one minute by the time interval [23]. During the trial, the necessary times to reach the anesthesia phases and the recovery times were calculated and recorded.

2.6. Statistical Analysis

Data were presented as mean standard deviation (SD). All data were tested by one-way ANOVA test and Tukey's multiple comparisons of each column with the control and were normally distributed ($p < 0.0001$). Statistical analysis was performed using GraphPad Prism version 9.3.0 (GraphPad Software, San Diego, CA, USA).

3. Results

3.1. ZFET Trial

The positive control with 0.18% ethanol caused no mortality or hatching delay in the zebrafish embryos. Acute toxicity investigations on zebrafish embryos using basil essential oil reveal malformations and mortality in experimental groups. Indeed, at 96 hpf,

essential basil oil caused mortality in the experimental group OBZ3 (200 µL/L) at a rate of 55%. Embryos exposed to 100 µL/L showed a lower mortality rate of 10%. In contrast, OBZ1 and control groups did not cause lethal effects. These embryos hatched at 48 hpf and were well developed. The acute toxicity test caused delayed hatching in all exposed embryos at 200 µL/L, while only 10% in the OBZ2 were unhatched (Figure 1). Statistical analysis confirmed the difference among groups (Figure 2A). Moreover, the treatment with essential oil caused pericardial edema in a dose-dependent manner at a rate of 100% and 80%, respectively for OBZ3 and OBZ2. Other cardiovascular pathologies findings showed blood congestion and un-looped heart for both higher experimental groups. In OBZ3, blood congestion and un-looped heart were found in 90% and 80%, respectively of embryos. Results showed in OBZ2 less alteration of the cardiocirculatory system, eliciting blood congestion and the un-looping of the heart in 60% of the embryos. Noteworthy is the findings of spinal cord deformities in OBZ2 showing kyphosis in 90% of hutched embryos (Figure 1). Unfortunately, it was not possible to evaluate this deformity in OBZ3 because all embryos were unhatched. During ZFET, basil essential oil induced a reduction of the heart rate as shown in Figure 2B. This finding was evaluated in all three replicates.

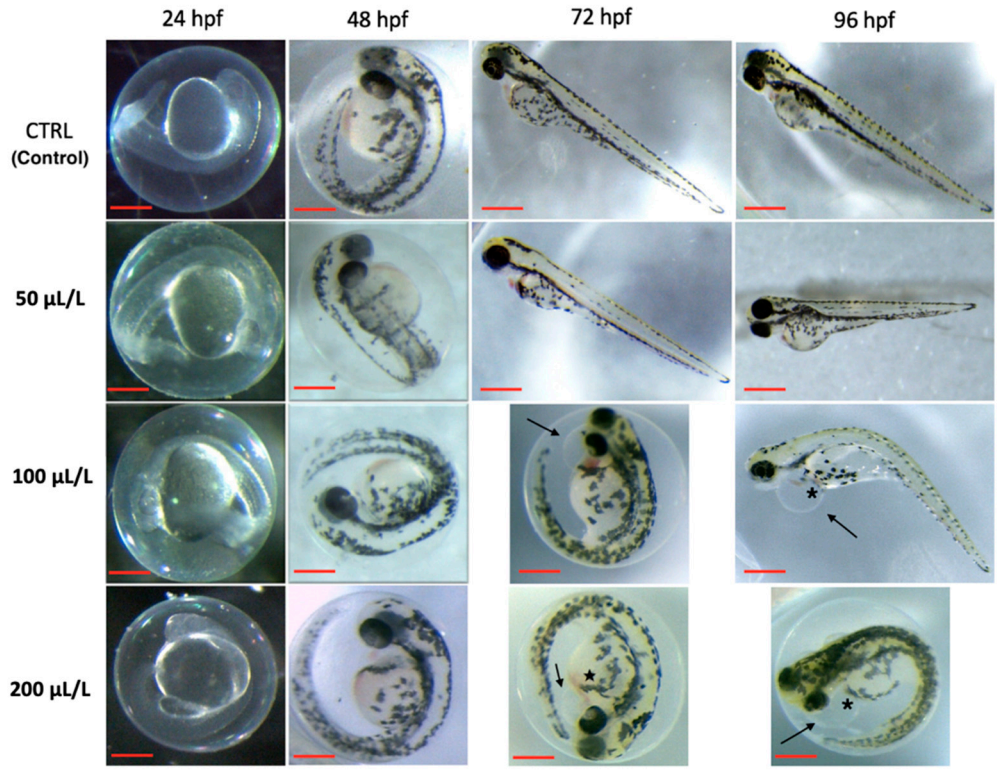

Figure 1. Zebrafish embryos development up to 96 hpf (hours post-fertilization) during exposure to *O. basilicum.* Pericardial edema (arrow); un-looped heart (asterisk); blood congestion (star). Scale bar 500 µm.

3.2. Anesthetic Trial

Regarding anesthetic trials, ZBA1 dipped in an essential oil solution showed no signs of ataxia, loss of balance, or decreased respiratory rate. The average heart rate was 157.9 BPM, comparable to the 160.4 BPM of the control group. Instead, ZBA2 reduced the body movement and the respiratory rate after 252 ± 4.1 s on average, showing a heart rate of 103.2 BPM. Basil oil induced a state of light anaesthesia in larvae (stage III, plane 1 of anesthesia) [24]. The larvae maintained in fresh embryo medium recovered their normal state in an average time of 193.8 ± 3.8 s. Statistical analysis showed a significant difference in ZBA2 compared with the control and ZBA1 groups (Figure 3). In addition, at 96 hpf, larvae exposed to 200 µL/L showed a marked decrease in heart rate after 182.4 ± 3.1 s from immersion in essential oil. The larvae decreased their respiratory rate, lost their balance,

and were unable to react to external stimuli, reaching a stage of deep anaesthesia (stage III, plane 3 of anaesthesia). The mean heart rate of ZBA3 was 82.9 BPM. Time for recovery from anaesthesia was 246 ± 4.6 s. Statistical analysis showed a significant reduction of heartbeat in comparison with the control, ZBA1, and ZBA2 groups (Figure 3).

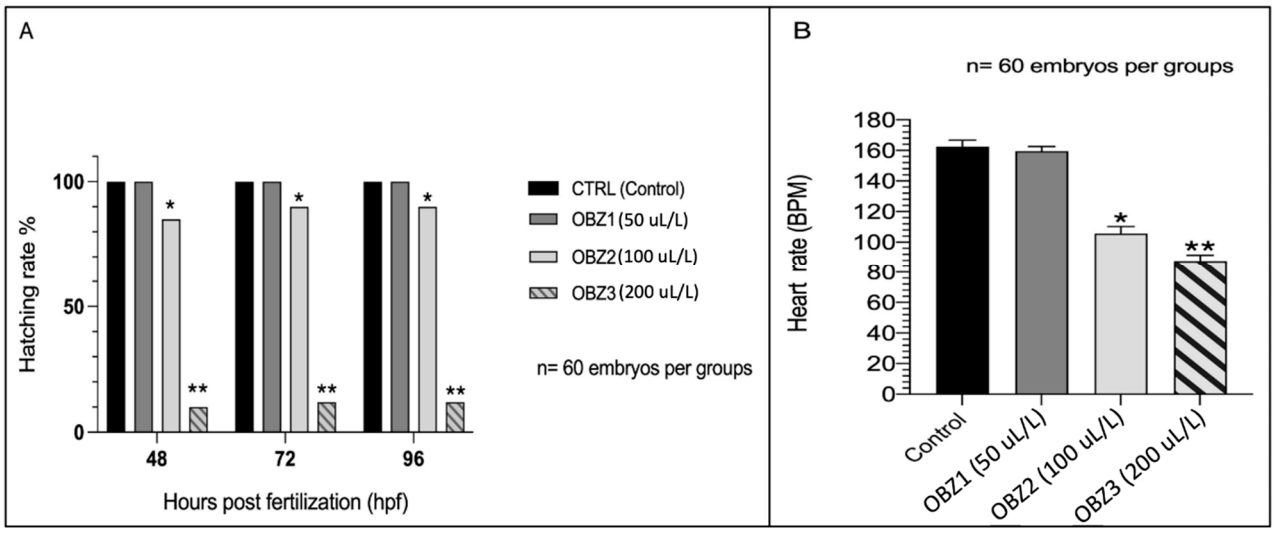

Figure 2. Zebrafish Embryo Toxicity (ZFET) Trial. (**A**) Effect of different concentrations of essential oil on hatching rate. (**B**) Anesthetic effect of basil essential oil on the heart rate at 96 hpf. Significant difference among groups: * OBZ2 vs. CTRL and OBZ1 groups; ** OBZ3 vs. CTRL, OBZ1, and OBZ2 groups. Values are expressed as mean ± SD of three independent experiments. Data are statistically significant for ($p < 0.0001$).

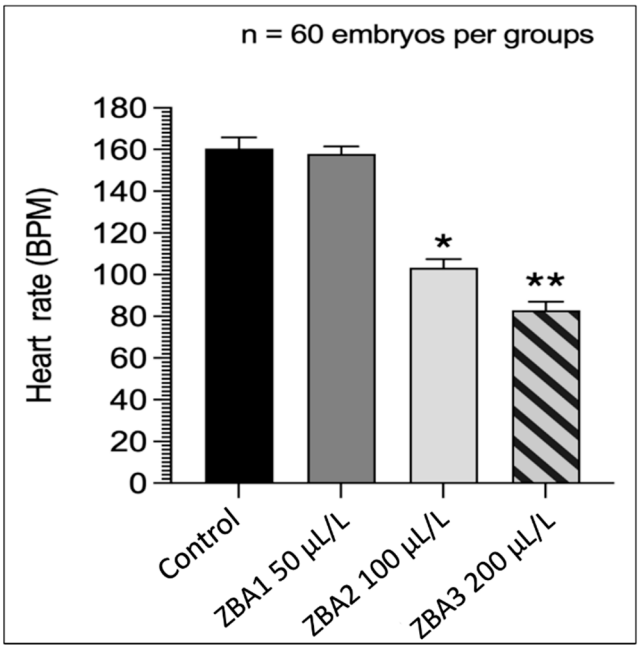

Figure 3. Anesthetic trials. Heartbeat in zebrafish embryos at 96 hpf. Significant difference among groups: * ZBA2 vs. CTRL and ZBA1 groups; ** ZBA3 vs. CTRL, ZBA1, and ZBA2 groups. Values are expressed as mean ± SD of three independent experiments ($n = 60$ embryos); * $p < 0.0001$ when compared to the control group.

4. Discussion

In the present study, a sedative effect was induced at 100 µL/L, while an anesthetic effect of the essential oil of *O. basilicum* at the concentration of 200 µL/L was observed. Lar-

vae did not react to external stimuli and remained lying on their side. Studies reported that the best concentration of *O. gratissimum* essential oil can induce anesthesia in *O. niloctilus* juveniles ranging from 90 to 150 mg/L [25], while other authors reported a concentration between 60 and 100 mg/L [26]. The experimental study showed that the essential oil of *O. basilicum* at 20 µL/L can induce mild sedation without loss of balance and ventilation in Nile tilapia juveniles [27]. Other studies showed an anesthetic effect of eugenol at low dosages of 20 µL/L [28] and at a higher dosage of 60 mg/L [29]. The different concentrations reported in these studies were due to the composition and relative abundance of certain compounds of the essential oil [30]. Moreover, the anesthetic response is strictly dependent on biological factors such as fish species, body size, and stage of life but also due to environmental factors such as salinity and temperature [31].

Several anesthesia methods have been described for fish [32]. Tricaine (MS-222) is widely used and it is also the only anesthesia agent approved in some countries [33]. Concentrations of 25 to 175 mg/L MS-222 in zebrafish larvae caused a loss in equilibrium without showing a decreasing heart rate. However, heart rate was reduced at concentrations of 250 ppm, reaching the state of deep anesthesia [34]. A study conducted on zebrafish embryos exposed at concentrations of 50 to 150 mg/L of MS-222 observed high rates of mortality and skeletal deformities in a dose-dependent manner [35]. A recent study indicates that MS-222 anesthetic overdose is ineffective on zebrafish up to 15 dpf, which can survive after a prolonged absence of heartbeat [36].

Clove oil is used as an alternative to MS-222. Research based on the anesthetic effect of clove oil at different doses (40, 60, 90, 120, 150 ppm) in 24 hpf zebrafish embryos, showed a reduced survival rate at the higher doses. However, in 5 dph larvae, clove oil induced deep anesthesia in a short time when immersed in 120–150 mg/L of clove oil, causing an additional 20% mortality [37]. These findings suggest that natural and synthetic anesthetics used during the early developmental stages lead to toxicity and mortality, however, they can be useful as anesthetic substances if used in the larval stages even at low dosages.

The anesthetic effect of the essential oil tested in this work demonstrated the synergic analgesic effect of linalool and eugenol compounds, which are used as an anesthetic agent in fish, implicating the opioid pathway, resulting in a reduction of the heart rate [38,39]. Many anesthetics have exhibited an influence on GABAA receptor activity, including natural products [40]. GABA is a neurotransmitter that inhibits neuronal activity. The expression of the GABAergic system was also found in zebrafish [41]. The interaction of *O. basilicum* essential oil with the benzodiazepine site of the GABAA receptor was reported as the interaction site of the EO in fish, acting as positive allosteric modulators [26,42,43]. Essential oil compounds such as linalool and eugenol were reported as capable of interacting with the GABAergic system [44,45]. Indeed, EO induces bradycardia by potentiating the binding of GABA to its receptor, increasing the inhibitory effect of GABA regulating the parasympathetic nervous system, leading to a lowering in the excitability of the cardiac vagal neurons in the nucleus ambiguus and decreasing heart rate [46].

The results obtained in this study showed that basil oil represents a natural substance that causes mortality in zebrafish embryos during the developmental stages. In the ZFET assay, heartbeat is an important sublethal endpoint that is routinely measured in zebrafish embryos as an index of toxicity. We found a dose-dependent decrease in the heart rate at 100 and 200 µL/L as compared to the control group. A reduction of the heart rate induces a slow blood flow which can cause a lower level of glucose in the muscles and therefore a lower muscular response and a reduction of the hatching rate [47]. Studies conducted on *O. gratissimum* extracts showed that at higher concentrations than in our study, they do not cause mortality in *R. quelen* [48] and Nile Tilapia *Oreochromis niloticus* [49]. Blood congestion is characterized by the accumulation of blood cells and usually can be observed in concurrence with a reduction of both blood flow and heartbeat rate [50]. Malformation of the zebrafish cardiovascular system such as the failure of heart looping was linked to oxidative stress and lipid metabolism [51]. Hatching is considered an essential stage during zebrafish embryogenesis. Therefore, the delayed hatching rate is often provoked

by structural and functional alterations that occurred during embryonic development induced by xenobiotic compounds which lead to apoptosis or oxidative stress [52]. It is well known that fish take advantage of both mechanical and biochemical ways to break the chorion and hatch. During embryogenesis, to digest and soften the chorion, the embryo secretes the hatching enzyme (ZHE1) produced by the hatching gland placed in the epidermis of the yolk sac. The proteolytic enzymes are secreted in the perivitelline space followed by embryonic muscular movements to provoke the rupture of the outer layer of the membrane [53].

In our study, pathological changes such as skeletal deformities and pericardial edema lead us to assume that basil oil extract has an acute toxic effect during embryogenesis. Therefore, the delay in the hatching rate was induced by an alteration of the proteolytic enzymes ZHE1 and by the larvae's inability to break the chorion by mechanical body movement, induced by the essential oil anesthetic effect. The effective concentrations of anesthetics are depending on the fish species and anaesthetic agent, such as new technologies used to administer active compounds [54–56]. Moreover, many factors such as fish size, temperature, and pH affect the efficiency of anesthetics in fish [57]. These results suggest that the anesthetic effect of *O. basilicum* essential oil is the result of the synergism of different components such as linalool and eugenol which acts on the GABAergic system.

5. Conclusions

In conclusion, exposure to *O. basilicum* essential oil at the concentration of 100 and 200 µL/L on zebrafish embryos promoted a delay in hatching rate, skeletal deformities, and mortality during larvae assay. The present study showed a pharmacological effect, in zebrafish larvae causing cardiotoxicity. ZFET assays revealed essential oil-induced dose-dependent hatching rate and heart rate alterations in zebrafish embryos after 96 h of exposure. This condition is related to the sedative effect of essential oil compounds, suggesting that *Ocimum basilicum* has a tolerable effect on fish after hatching. These findings suggest that essential oils can be used as a viable alternative to the use of chemical anaesthetics in post-hatching zebrafish larvae, commonly used in experimental research.

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