Antioxidant essential oil–based nanoemulsions associated with levothyroxine: Physicochemical characterization and toxicity assessment in zebrafish

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Abstract

This study aimed to develop nanoemulsions containing levothyroxine associated with essential oils with antioxidant activity and to evaluate their toxicity in zebrafish (*Danio rerio*). The essential oils of *Piper aduncum*, *Citrus sinensis*, and *Carum carvi* were characterized by qualitative and quantitative gas chromatography. The oil/water nanoemulsions were prepared using a high-energy emulsification method, and the effect of time and stirring speed was studied by experimental design. The mean size, polydispersity index (PDI), and zeta potential were evaluated as response variables. Antioxidant activity was also analyzed. Toxicity tests were conducted on zebrafish based on the OECD 236 guidelines, and the response variables were mortality, teratogenic effects, and behavioral tests. GC-MS analysis of *C. carvi*, *C. sinensis*, and *P. aduncum* identified 6, 16, and 13 compounds, with carbon (60.44 ± 0.80%), limonene (90.13 ± 1.11%), and dilapiol (78.41 ± 0.98%) being the major constituents. Nanoemulsions were successfully developed, with a mean size ranging from 218.5 to 489.2 nm, PDI of 0.113 to 0.478, and zeta potential of -12.5 to -39.1 mV. All oils exhibited antioxidant activity, with EC_{50} values ranging from 204.3 to 288.5 µg/mL for DPPH and 156.6 to 194.9 µg/mL for ABTS. Toxicity tests revealed that all oils exhibited toxic effects with animals exposed to all nanoemulsions showing teratogenic effects, mostly at the larval stage. Only the *C. carvi* nanoemulsion affected the behavioral tests. In summary, nanoemulsions showed strong antioxidant activity and were well-characterized, but toxicity in zebrafish suggests safety concerns that require further research before therapeutic use.

1. Introduction

Essential oils (EOs) are products of spices used since antiquity due to their medicinal properties. Wide acceptance by consumers requires the natural product to show safety and efficacy (Bilia et al., 2014). EOs are volatile liquids, being soluble in lipid and organic solvents, and synthesized by all plant organs (flowers, leaves, and bark) (Bilia et al., 2014; Pavoni et al., 2020). EO constituents are secondary plant metabolites (Aziz et al., 2019), including monoterpenes, sesquiterpenes, and phenylpropanoids.

Citrus is the genus that includes the world's major fruit-cultivated species, *Citrus sinensis* (sweet orange), and Brazil is the world’s main producer of this fruit (Rezende et al., 2020). According to the Food and Agriculture Organization of the United Nations (FAO), in Brazil, the total production of this fruit was 16.713.534 tons in 2018. Antibacterial, antifungal, and antioxidant properties are reported for *C. sinensis* essential oil (Frassinetti et al., 2011). Popular uses include skin health and promoting detoxification, and pharmacological actions include reduced anxiety, agitation, stress, challenging behaviors, and insomnia (Ali et al., 2015).

Piper is a genus of the Piperaceae family, widely distributed in subtropical regions. *Piper aduncum* is popularly known as long pepper, and *monkey pepper* among other names (Pohlit et al., 2006; Silva et al., 2019a). In Brazil, it is used as a bactericide, leishmanicide, fungicide, antioxidant, anticancer, larvicide, insecticide, antiplatelet (anticoagulant), molluscicide, and antiviral (Pohlit et al., 2006; Silva et al., 2019a).

Carum carvi, also known as caraway and black zeera, is a plant from the Apiaceae family, grown in Northern Africa, western Asia, and Europe. Recently, it was introduced in other regions of the world like Russia, Iran, Iraq, Indonesia, and North America. Its potential biological effects include antioxidant, anti-inflammatory, anti-diabetic, anti-obesity, anti-spasmodic, anti-asthmatic, anti-microbial, anti-hemolytic, anti-foaming, and galactagogue (Obeid et al., 2020; Trifan et al., 2016). In addition, the essential oil of *Carum Carvi* is a potent hypothyroidism treatment (Obeid et al., 2020).

In hypothyroidism, there is an accelerated production of free radicals and low availability of antioxidants that eliminate free radicals (Mancini et al., 2016). As a result of this imbalance, oxidative stress arises, which is implicated in the pathophysiological mechanism of hypothyroidism and other diseases (Mancini et al., 2016). In subclinical hypothyroidism patients, symptoms like memory deficit, despondency, depression, and anxiety are frequently reported (Almeida et al., 2007). Levothyroxine monotherapy is the standard treatment for patients with hypothyroidism. Patients are biochemically well-controlled with levothyroxine, but a substantial proportion of patients have persistent complaints, like symptoms of depression and impaired mental well-being. This raised the question of whether treatment with this synthetic hormone is sufficient for all patients or whether alternative therapies can be adopted to minimize these adverse effects (Chaker et al., 2017). The formulation of nanoemulsions (NEs) containing levothyroxine associated with essential oils with antioxidant activity may be a good alternative and overcome these limitations.

Nanotechnology applied to the pharmaceutical industry is an opportunity for new treatment approaches that overcome the obstacles of conventional therapies (Rojas-Aguirre et al., 2016). Therefore, controlled release systems have been widely investigated to enable delayed and sustained drug release, controlling the site of drug release and reducing the amount of drug required for treatment (Singh et al., 2017). Among the controlled drug release systems, NEs are widely reported in the pharmaceutical research literature. NEs are produced by the mixture of two immiscible liquids (oil and water are usually used), stabilized by the addition of a surfactant suitable for the compounds used, which decreases the surface tension between the immiscible liquids (Singh et al., 2017). Sometimes, a combination of surfactants is necessary to ensure the formation and stabilization of NEs (McClements, 2012). NEs offer better bioavailability and drug release due to vesicle size, which is usually < 500 nm, giving emulsions a clear or hazy appearance (Aziz et al., 2019; Singh et al., 2017).

Emulsification processes are commonly used for essential oils to solubilize and stabilize volatile compounds, which are easily degraded by exposure to environmental factors like heat, light, oxygen, and humidity (Aziz et al., 2019; Pavoni et al., 2020), allowing essential oils to be used in drug development. Emulsification of the *C. sinensis* (Velmurugan et al., 2017)d *aduncum* (Silva et al., 2019a) essential oils are described in the literature.

In silico, *in vitro*, and *in vivo* methods are used to evaluate the toxicity of NEs because the nanoscale can change their toxicity. *In silico* and *in vitro* methods are beneficial for the preliminary toxicological assessment of NEs. However, animal tests are still needed. Zebrafish have become a well-established

animal model for the toxicological and pharmacological screening of new drugs. Zebrafish can also reveal toxic mechanisms of discovery candidates as NEs and provide 3 Rs (replacement, reduction, and refinement) value to animal research (Cassar et al., 2020). In addition, animal model research can reveal the benefit/risk of new products, and nanotechnological products present a new dimension to the pharmacological/toxicological profile as compared with traditional drugs (Pensado-Lopez et al., 2021). Using zebrafish to evaluate the toxicity of new drugs at the pre-clinical stage allows easier and more rapid decisions on the viability of candidates and information about modifying these new drugs to produce safer and more effective alternatives (Cassar et al., 2020; Jayasinghe and Jayawardena, 2019).

Zebrafish embryos are an effective model to study the toxicological effects of a broad spectrum of herbal medicines (Jayasinghe and Jayawardena, 2019). First, zebrafish are externally fertilized, and embryos/larvae are optically transparent during early developmental stages, allowing direct, noninvasive observations (Cassar et al., 2020). The zebrafish genome is 87% conserved with human genes (Jayasinghe and Jayawardena, 2019), and the zebrafish shares 82% of human disease genes (Cassar et al., 2020), but also behavioral similarities. Thyroid receptor proteins have 91% identity with human thyroid receptors (MacRae and Peterson, 2015). This conservation allows an effective comparison of this animal model with the human condition, including hypothyroidism. These advantages can be exploited to evaluate drug toxicity using *D. rerio* in pre-clinical studies to develop safer drugs (Jayasinghe and Jayawardena, 2019).

In the present study, NEs of essential oils associated with levothyroxine were produced and characterized, and antioxidant activity was analyzed. Also, the toxicity of these produced NEs was evaluated in the zebrafish (*Danio rerio*) model. Toxic effects on fish development were tested using mortality, teratogenic effects, and animal behavior assays (thigmotaxis and touch sensitivity). These experiments provide insights into the potential utility of these NEs.

2. Material and methods

2.1. Collection of plant material

Fresh leaves of the *P. aduncum* (8°01'10.1"S 34°56'50.4" W) were collected in the *Mata de Dois Irmãos*, in *Recife*, *Pernambuco*, Brazil. Leaf collections for specimens were performed at around 9:00 am in the dry season, in February 2019. The plants were identified by botanist *Dra. Ângela Maria de Miranda Freitas* (*Universidade Federal Rural de Pernambuco*). Voucher specimens were mounted and deposited in the *Sérgio Tavares* Herbarium of the UFRPE under numbers HST 18177. Fruits of the *C. sinensis* were collected in *Sítio Cigarra*, in the municipality of *Santana do Mundaú*, Alagoas-AL, in May 2019. Fruits collections for specimens were performed at around 11:00 am in the dry season. The plant was identified by *Dra. Suzene Izídio da Silva* from the Biology Department of the *Universidade Federal Rural de Pernambuco* (UFRPE). Voucher specimens were deposited in the *Vasconcelos Sobrinho* Herbarium of the UFRPE, under the number: 48735, *C. sinensis* Osbeck var. *pear*). The oil of the *Carum carvi* was acquired from a local supplier.

2.2. Isolation, chemical analysis, identification, and quantification of the essential oil

The essential oils from fresh leaves of *Piper aduncum*, and shells of the fruit of *Citrus sinensis* (100 g) were separately isolated using a modified Clevenger-type apparatus and hydrodistillation for 2h. The oil layers were separated and dried over anhydrous sodium sulfate, stored in hermetically sealed glass containers, and kept at a low temperature (-5 ºC) until analysis. Total oil yields were expressed as percentages (g/100 g of fresh plant material). All experiments were carried out in triplicate. For the *Carum carvi* oil was acquired from a local supplier (LASZLO, Brazil). Quantitative GC analysis was carried out using a PerkinElmer Clarus 500 GC apparatus equipped with a flame ionization detector (FID) and a non-polar DB-5 fused silica capillary column (30 m x 0.25 mm x 0.25 µm film thickness) (J & W Scientific). The oven temperature was programmed from 60 to 240°C at a rate of 3°C/min. Injector and detector temperatures were 260°C. Hydrogen was used as the carrier gas at a flow rate of 1 mL/min in split mode (1:30). The injection volume was 0.5 µL of diluted solution (1/100) of oil in n-hexane. The amount of each compound was calculated from GC-FID peak areas in the order of DB-5 column elution and expressed as a relative percentage of the total area of the chromatograms. Analyses were carried out in triplicate. The qualitative Gas Chromatography-Mass Spectrometry (GC-MS) analysis was carried out using a Varian 220-MS IT GC system with a mass selective detector, a mass spectrometer in EI 70 eV with a scan-interval of 0.5 s, and fragments from 40 to 550 Da. fitted with the same column and temperature program as that for the GC-FID experiments, with the following parameters: carrier gas = helium; flow rate = 1 mL/min; split mode (1:30); injected volume = 1 µL of diluted solution (1/100) of oil in n-hexane. Identification of the components was based on GC-MS retention indices with reference to a homologous series of C8-C40 n-alkanes calculated using the Van der Dool and Kratz equation (van Den Dool and Dec. Kratz, 1963) and by computer matching against the mass spectral library of the GC-MS data system (NIST 14 and WILEY 11th) and co-injection with authentic standards as well as other published mass spectra (Adams, 2017). Area percentages were obtained from the GC-FID response without the use of an internal standard or correction factors.

2.3. Preparation of levothyroxine stock solution

Commercial levothyroxine (L-T_4) (composition: L-T_4 (37,5 µg), mannitol, maize starch, microcrystalline cellulose, butyl-hydroxyanisole, and magnesium stearate) was used in the experiments. The stock solution of L-T_4 was prepared by dissolving in distilled water, obtaining the final nominal concentration of 250 µg/L, which was stored under refrigeration (4˚C) until further use.

2.4. Preparation of nanoemulsions

The oil/water (O/W) NEs were prepared using a high-energy emulsification method (dos Santos Magnabosco et al., 2022) by magnetic stirring from an oily phase (OP) produced from a mixture of 10–20% (w/w) of individual essential oils (*Carum carvi*, *Citrus sinensis*, or *Piper aduncum*), 10–20% (w/w) of sorbitan monooleate (Tween 80) and/or 10% polysorbate 80 (Span 80). The OP was first homogenized using a magnetic stirrer for 30 min at 22 *g* at room temperature (25 ± 2 ºC). Simultaneously in another

container, water phase (WP) was prepared with or without (empty) levothyroxine diluted in 43.75 mL of phosphate buffer 100 mM, pH 7.4. Finally, 70% of the WP was dripped (flow of 3.5 mL/min) using a burette into the OP under magnetic stirring according to Duarte et al. (2015). The prepared NEs were designated by the initials of each essential oil: *Carum carvi* – CC; *Citrus sinensis* – CS; and *Piper aduncum* - PA.

2.5. Experimental designs

A two-level 3 x 2^2 full experimental design was carried out to study the effect of time and stirring speed in the preparations of NEs. Factors (stirring speed and stirring time) of each formulation that mainly influenced the physicochemical parameters were evaluated at two levels and a central point (Table 1) in authentic triplicate. The mean size, polydispersity index (PDI), and zeta potential (ζ mV) of NEs were used as the response variables of the design study.

Table 1

Two-level 3 x 2^2 full experimental designs of nanoemulsions of each essential oil associated with levothyroxine.

Run	Stirring speed (*g*)	Stirring time (h)	Essential oil (g)	Tween 80 (g)	Span 80 (g)	Phosphate-buffered + Levothyroxine (250 µg/L)
1	10 *g*	1 h	0.5 (*Carum carvi*)	1.0	0.0	3.5
2	39 *g*	1 h	0.5 (*Carum carvi*)	1.0	0.0	3.5
3	10 *g*	2 h	0.5 (*Carum carvi*)	1.0	0.0	3.5
4	39 *g*	2 h	0.5 (*Carum carvi*)	1.0	0.0	3.5
5 (CP)	22 *g*	1 h 30 min	0.5 (*Carum carvi*)	1.0	0.0	3.5
6	10 *g*	1 h	1.0 (*Citrus sinensis*)	1.0	0.0	3.0
7	39 *g*	1 h	1.0 (*Citrus sinensis*)	1.0	0.0	3.0
8	10 *g*	2 h	1.0 (*Citrus sinensis*)	1.0	0.0	3.0
9	39 *g*	2 h	1.0 (*Citrus sinensis*)	1.0	0.0	3.0
10 (CP)	22 *g*	1 h 30 min	1.0 (*Citrus sinensis*)	1.0	0.0	3.0
11	10 *g*	1 h	0.5 (*Piper aduncum*)	0.5	0.5	3.5
12	39 *g*	1 h	0.5 (*Piper aduncum*)	0.5	0.5	3.5
13	10 *g*	2 h	0.5 (*Piper aduncum*)	0.5	0.5	3.5
14	39 *g*	2 h	0.5 (*Piper aduncum*)	0.5	0.5	3.5
15 (CP)	22 *g*	1 h 30 min	0.5 (*Piper aduncum*)	0.5	0.5	3.5

Legend: Runs 1 to 5 are *Carum carvi* samples with a central point (CP – run 5); 6 to 10 are *Citrus sinensis* samples with a central point (CP – run 10); 11 to 15 are *Piper aduncum* samples with a central point (CP – run 15).

2.6. Physicochemical characterization

The NEs were diluted (50x) in double-distilled water before analysis to reduce the turbidity before microscopic analysis. The average size (nm), polydispersity index (PDI), and zeta potential (mV) of the oil vesicles were evaluated using the standard photon correlation spectroscopy (PCS) technique fixed at 90º to 25 ºC using a Zetasizer Nano ZS (Malvern, UK) (Cadena et al., 2013; dos Santos Magnabosco et al., 2022). The analysis was performed at *Laboratórios Associados em Rede de Nanotecnologia* (LARnano) of the *Universidade Federal de Pernambuco* (UFPE). The data were measured in triplicate.

2.7. Antioxidant Activity of nanoemulsions

Antioxidant activity was analyzed by $DPPH^{\cdot}$ and $ABTS^{\cdot+}$ radicals. The experiments were performed at *Laboratório de Produtos Naturais Bioativos* (LPNBio) of the *Universidade Federal Rural de Pernambuco* (UFRPE). The antioxidant activity of the NEs was tested against the free radical DPPH following the methodology of Araujo et al. (2022). Stock solutions were prepared from the extracts and methanol fraction at several concentrations (0.1 to 5.0 mg/mL). Through preliminary analysis, appropriate quantities of stock solutions of the samples and 450 µL of the solution of $DPPH^{\bullet}$ (23.6 mg/mL in EtOH) were transferred to 0.5 mL Eppendorf tubes, and the sample was diluted with EtOH following homogenization. Samples were sonicated for 30 min and the amount of $DPPH^{\bullet}$ was measured in a 96-well plate using a UV-vis spectrophotometer (Biochrom EZ Read 2000) at 517 nm. Ascorbic acid was used as a positive control. The determination of the antioxidant activity in the NEs to detect the radical cation $ABTS^{\cdot+}$ was carried out following the methodology described by Araujo et al. (2022), in a UV-vis spectrophotometer (Biochrom EZ Read 2000) using Trolox as the standard compound. The starting concentrations of the solutions of the samples were 0.1–1.0 mg/mL, with the addition of 450 µL of the radical $ABTS^{\cdot+}$ solution to give final concentrations of 2.5–100.0 µg/mL samples. Samples were protected from light and sonicated for 6 min. The absorbance of the samples and the positive control in a 96-well microplate were measured at a wavelength of 734 nm. In the two tests each concentration was tested in triplicate, and the percentage of free radical scavenging activity of $DPPH^{\cdot}$ or $ABTS^{\cdot+}$ was calculated by the equation: $\%SA = 100 \times (Abs_{control} - Abs_{sample}) / Abs_{control}$, where $Abs_{control}$ is the absorbance of the control containing only the ethanol solution of $DPPH^{\cdot}$ or $ABTS^{+}$ and Abs_{sample} is the absorbance of the radical in the presence of the sample or standard ascorbic acid. Antiradical efficiency was established using linear regression analysis and the 95% confidence interval ($p < 0.05$) obtained using the statistical program GraphPad Prism 5.0.

2.8. Fish husbandry, crossing, and toxicity test

Toxicity tests were performed using zebrafish (*Danio rerio*; WT strain; 7 months old) as an animal model. The experiments were carried out at *Laboratório de Ecofisiologia e Comportamento Animal* (LECA) – *Universidade Federal Rural de Pernambuco* (UFRPE). Adult zebrafish used in the experiments were obtained from a commercial supplier and housed in quarantine to detect or confirm the absence of pathogens or diseases maintained according to OECD 236 guidelines (OECD, 2025). The protocols used in this study were approved by the Ethics Committee in the use of animals of the same University, protocol number 2008150721. Adult male/female zebrafish were isolated in spawning aquariums

(Alesco Zebclean) with a 2:1 ratio (Westerfield, 2000). For fish crossing, dividers between males and females were removed, and they were allowed to breed for 40 min. The embryos were collected and washed with distilled water in Petri dishes. Healthy embryos (normal development of the blastula) were used to form experimental groups.

The experiments were conducted with 5 x 15 animals per experimental group (a total of 825 animals with replicates and repetitions). The assay was based on the OECD guidelines on the Fish Embryo Acute Toxicity (FET) Test (OECD, 2025) and Silva et al. (2019b). The toxicity test started at 1-hour post-fertilization (hpf). Embryos were maintained in sterile polystyrene test chambers (80 mL) and maintained in an incubator with a light-dark cycle of 14:10 h, controlled temperature of 26 ± 1˚C, and pH of 7.5 ± 0.5. Daily water renewal was performed to maintain the NEs levels. The study used a control group and experimental groups that were described in Table 2 with two dilution rates where 4444x and 3200x to evaluate toxicity. These dilution ratios were chosen based on studies showing most of the animals survived at 96 hpf. The final nominal concentration of L-T_4 in all experimental groups was the same as described above (item 2.3). The exposure to NEs started at 1-h post-fertilization (hpf) being renewed daily until 96 hpf, according to OECD 236 (2025). Any residue generated by these compounds during the experiments was treated by the advanced oxidative process (AOP) in a reactor using photo-oxidation UV (16w) and H_2O_2 (Hansen and Andersen, 2012; Silva et al., 2019b).

Table 2
The dilution ratio of the chemical compounds used in the experimental groups for the acute toxicity test.

Experimental groups	***Abbreviations***	***Dilution ratio***
Control	C	-
Levothyroxine	L-T_4	320x
Empty (E) *Carum carvi* (CC) nanoemulsion (0.5 g oil, 1.0 g tween, 3.5 g of phosphate buffer) diluted	ECC	3200x
Carum carvi (CC) nanoemulsion (0.5 g oil, 1.0 g tween, 3.5 g of phosphate buffer with levothyroxine) diluted	CCN44	4444x
Carum carvi (CC) nanoemulsion (0.5 g oil, 1.0 g tween, 3.5 g phosphate buffer with levothyroxine) diluted	CCN32	3200x
Empty (E) *Citrus sinensis* (CS) nanoemulsion (1.0 g oil, 1.0 g tween, 3.0 g phosphate buffer) diluted	ECS	3200x
Citrus sinensis (CS) nanoemulsion (1.0 g oil, 1.0 g tween, 3.0 g phosphate buffer with levothyroxine) diluted	CSN44	4444x
Citrus sinensis (CS) nanoemulsion (1.0 g oil, 1.0 g tween, 3.0 g phosphate buffer with levothyroxine) diluted	CSN32	3200x
Empty *Piper aduncum* (PA) nanoemulsion (0.5 g oil, 0.5 g tween, 0.5 g spam, 3.5 g of phosphate buffer) diluted	EPA	3200x
Piper aduncum (PA) nanoemulsion (0.5 g oil, 0.5 g tween, 0.5 g spam, 3.5 g of phosphate buffer with levothyroxine) diluted	PAN44	4444x
Piper aduncum (PA) nanoemulsion (0.5 g oil, 0.5 g tween, 0.5 g spam, 3.5 g of phosphate buffer with levothyroxine) diluted	PAN32	3200x

2.9. Effects of levothyroxine and nanoemulsions on zebrafish embryonic development

Mortality data were observed at 24, 48, 72, and 96 hours post-fertilization (hpf). The determination of embryo lethality was assayed according to OECD 236 (2025). Determination of mortality as coagulation, tail not detached, no somite formation, absence of heartbeat, and lack of hatching. Teratogenic effects were evaluated according to OECD 236 (2025), Silva et al. (2019b), and Cadena et al. (2020). The teratogenic effects studied were spine deformation, tail deformation, yolk sac edema, and pericardial edema. The effects were identified in images of lateral and dorsal views *in vivo*. The images were captured using a Hayear Mod. HY-2307 digital camera microscope and S-EYE 1.4.2.474 software. We consider larvae affected when at least, mortality or one teratogenic effect was observed under a microscope (Cadena et al., 2020). These results were presented as a percentage of affected animals per experimental group.

2.10. Effects of levothyroxine and nanoemulsions on zebrafish animal behavior

Behavioral tests were used as a method to study the effects of NEs s in zebrafish (*Danio rerio*). The thigmotaxis (TH Test) is the tendency of an animal to move toward the edge or periphery of plates that are introduced. Therefore, this animal avoids the center of this environment. This parameter evaluates the anxiety like-behavior in zebrafish (Basnet et al., 2019). The thigmotaxis test was evaluated at 144 hpf, according to Cadena et al. (2020). The larvae were left at 10 min of acclimatization in 48-well plates (48 larvae per experimental group) in a light environment, and if larvae remained near the wall of plates, the response was recorded. The touch sensitivity test (TS Test) was performed according to Cadena et al. (2020). This test evaluated the larval response to mechanical stimuli. At 144 hpf, the larvae were touched on the tail or head with a pipette tip. The response was recorded if the larva exhibited swimming behaviors (slow or fast), that is, an escape response.

2.11. Statistical analysis

All data were shown as mean ± SD. ANOVA was used to analyze data, and the means were compared by Tukey's test ($p < 0.05$). Statistical analyses were carried out using the Origin Pro Academic 2015 (Origin Lab. Northampton, MA USA) and GraphPad Prism 5.0.

3. Results

3.1. Isolation, chemical analysis, identification, and quantification of the essential oil

The chemical composition of essential oils from the leaves of *P. aduncum*, *C.carvi*, and *C. sinensis* is shown in Table 3. Hydrodistillation of *P. aduncum* leaves provided yellowish oil. The extraction of the oil revealed a yield of 2.52 ± 0.10%. Moreover, for *C. sinensis* hydrodistillation provided a colorless citrus-scented oil. The extraction of the oil revealed a yield of 1.4 ± 0.13%. The GC-MS analysis of *P. aduncum* oils allowed the identification of 13 compounds, representing a total of 98.53 ± 1.01% of the oil. The oil revealed the presence of compounds belonging to the class of monoterpenes (4.03 ± 0.05%), sesquiterpenes (16.09 ± 0.10%), and phenylpropanoid (78.41 ± 0.98%). The phenylpropanoid dilapiol (78.41 ± 0.98%) was characterized as a major constituent in the oil. For *C. carvi* oil the GC-MS analysis allowed the identification of 6 compounds, representing a total of 98.37 ± 0.82% of the oil. The oil revealed a predominance of compounds belonging to the monoterpene class (98.37 ± 0.82%), having as main constituent carbon (60.44 ± 0.80%) followed by limonene (34.14 ± 0.64%). Finally, *C. sinensis*, the analysis by GC-MS allowed the identification of 16 compounds, representing a total of 97.47 ± 1.22% of the oil. The oil revealed the presence of compounds belonging to the class of monoterpenes (96.39 ± 1.08%) and sesquiterpenes (1.09 ± 0.01%). For this oil, limonene (90.13 ± 1.11%) was characterized as a major constituent in the oil.

Table 3
Chemical composition of essential oils of *Piper aduncum, Carum carvi*, and *Citrus sinensis*.

Compounds	**RI[a]**	**RI[b]**	***P. aduncum***	***C. carvi***	***C. sinensis***	**Method of identification**
α-Thujene	921	924	-	-	0.16 ± 0.00	RI, MS
α-Pinene	928	932	-	0.33 ± 0.02	1.25 ± 0.07	RI, MS, CI
Sabinene	966	969	-	-	0.95 ± 0.03	RI, MS
β-Pinene	971	974	0.11 ± 0.00	0.13 ± 0.01	1.87 ± 0.10	RI, MS, CI
iso-Sylvestrene	1005	1007	0.45 ± 0.02	-	-	RI, MS
p-Cymene	1018	1020	-	2.14 ± 0.08	-	RI, MS, CI
Limonene	1023	1024	-	34.14 ± 0.64	90.13 ± 1.11	RI, MS, CI
β-Phellandrene	1025	1025	-	-	0.11 ± 0.00	RI, MS, CI
(Z)-*β*-Ocimene	1031	1032	-	-	0.28 ± 0.01	RI, MS
(*E*)-*β*-Ocimene	1040	1044	1.01 ± 0.05	-	-	RI, MS
γ-Terpinene	1055	1054	-	-	0.35 ± 0.02	RI, MS
Terpinolene	1088	1086	0.13 ± 0.00	-	0.38 ± 0.01	RI, MS
Linalool	1094	1095	-	-	0.22 ± 0.01	RI, MS, CI
cis-*β*-Terpineol	1143	1140	-	-	0.36 ± 0.01	RI, MS
Citronellal	1145	1148	-	-	0.11 ± 0.00	RI, MS
Terpinen-4-ol	1174	1174	0.50 ± 0.01	-	-	RI, MS, CI
(*E*)-Isocitral	1175	1177	-	-	0.22 ±	RI, MS

Legend: RI[a] = Retention indices calculated from retention times in relation to those of a series C_8-C_{40} of n-alkanes on a 30m DB-5 capillary column. RI[b] = Retention indices from literature. SD = Standard deviation. RI = retention indices, MS = mass spectroscopy, and CI = Co-injection with authentic compounds. See supplementary material for chromatograms.

Compounds	RI[a]	RI[b]	*P. aduncum*	*C. carvi*	*C. sinensis*	Method of identification
					0.03	
Dill ether	1180	1184	-	1.19 ± 0.10	-	RI, MS
α-Terpineol	1189	1186	1.83 ± 0.03	-	-	RI, MS, CI
δ-Elemene	1340	1335	0.75 ± 0.04	-	-	RI, MS
Carvone	1235	1239	-	60.44 ± 0.80	-	RI, MS
β-Caryophyllene	1415	1417	3.20 ± 0.09	-	0.08 ± 0.00	RI, MS, CI
α-trans-Bergamotene	1431	1432	-	-	0.11 ± 0.01	RI, MS
α-Humulene	1456	1452	8.32 ± 0.34	-		RI, MS, CI
γ-Gurjunene	1474	1475	-	-	0.9 ± 0.00	RI, MS
γ-Muurolene	1477	1478	0.55 ± 0.02	-	-	RI, MS
γ-Himachalene	1488	1481	1.93 ± 0.06	-	-	RI, MS
Germacrene B	1555	1559	1.34 ± 0.09	-	-	RI, MS
Dillapiole	1620	1620	78.41 ± 0.98	-	-	RI, MS, CI
Total			98.53 ± 1.01	98.37 ± 0.82	97.47 ± 1.22	
Monoterpenes			4.03 ± 0.05	98.37 ± 0.82	96.39 ± 1.12	
Sesquiterpenes			16.09 ± 0.10	-	1.09 ± 0.01	
Phenylpropanoids			78.41 ± 0,98	-	-	

Legend: RI[a] = Retention indices calculated from retention times in relation to those of a series C_8-C_{40} of n-alkanes on a 30m DB-5 capillary column. RI[b] = Retention indices from literature. SD = Standard deviation. RI = retention indices, MS = mass spectroscopy, and CI = Co-injection with authentic compounds. See supplementary material for chromatograms.

3.2. Physicochemical characterization of nanoemulsions

The results of the physicochemical characterization of NEs are shown in Table 4 (A and B). Our data revealed significant results in the zeta potential for the *C. carvi* NE suggesting that the higher agitation speed promotes more negative zeta potential. For the *C. sinensis* NE, a significant result was observed

only in the size of the vesicles, demonstrating that the higher stirring speed reduces the size of the vesicles. In addition, a significant result in PDI values observed in the *P. aduncum* NEs suggested that the shorter stirring time induces the smaller PDI values. Finally, the *C. carvi* nanoemulsions were produced using a high stirring speed and short stirring time (1129 *g*/1 h), *C. sinensis* NE was produced using a low stirring speed, and short stirring time (282 *g*/1 h) and *P. aduncum* NEs produced using low stirring speed and long stirring time (282 *g*/2 h) were chosen for the next experiments. Our choice was based on the best physicochemical parameters for industrial production.

Table 4
Effect of variables stirring speed (*g*) and stirring time (h) in the physicochemical parameters of nanoemulsions of each essential oil associated with levothyroxine. Bold values indicate significant results by the Tukey test ($p < 0.05$).

A) Size, polydispersity index (PDI), and zeta potential data for each nanoemulsion			
Emulsion formulations (g/h)	*Mean size (nm)*	*PDI*	*ζ (mV)*
Carum carvi 282 *g* / 1 h	384.7 ± 4.9	0.333	-12.5 ± 0.6
Carum carvi 1129 *g* / 1 h	277.1 ± 2.5	0.304	-22.7 ± 0.4
Carum carvi 282 *g* / 2 h	408.6 ± 7.8	0.409	-15.1 ± 0.4
Carum carvi 1129 g / 2 h	259.0 ± 12.0	0.323	-14.6 ± 0.4
Carum carvi 635 g / 1 h 30 min	218.5 ± 4.4	0.315	-12.8 ± 0.5
Carum carvi 635 g / 1 h 30 min	223.1 ± 3.3	0.317	-13.3 ± 0.9
Carum carvi 635 g / 1 h 30 min	225.8 ± 6.3	0.257	-14.6 ± 0.6
Citrus sinensis 282 *g* / 1 h	287.3 ± 20.5	0.226	-21.3 ± 2.6
Citrus sinensis 1129 g / 1 h	401.9 ± 45.5	0.113	-18.5 ± 0.3
Citrus sinensis 282 *g* / 2 h	325.3 ± 3.7	0.424	-16.9 ± 0.7
Citrus sinensis 1129 g / 2 h	391.8 ± 35.7	0.223	-20.9 ± 1.6
Citrus sinensis 635 g / 1 h 30 min	200.2 ± 7.4	0.339	-12.9 ± 0.3
Citrus sinensis 635 g / 1 h 30 min	235.2 ± 6.0	0.460	-16.0 ± 0.3
Citrus sinensis 635 g / 1 h 30 min	224.9 ± 10.2	0.235	-18.5 ± 2.0
Piper aduncum 282 *g* / 1 h	489.1 ± 78.1	0.234	-32.2 ± 0.3
Piper aduncum 1129 g / 1 h	445.9 ± 298.0	0.299	-36.8 ± 0.3

A) Size, polydispersity index (PDI), and zeta potential data for each nanoemulsion					
Piper aduncum 282 *g* / 2 h		326.9 ± 15.7	0.406	-30.6 ± 1.3	
Piper aduncum 1129 g / 2 h		401.7 ± 21.1	0.478	-39.1 ± 0.4	
Piper aduncum 635 g / 1 h 30 min		631.3 ± 118.2	0.302	-36.1 ± 1.3	
Piper aduncum 635 g / 1 h 30 min		426.4 ± 42.7	0.343	-31.3 ± 1.6	
Piper aduncum 635 g / 1 h 30 min		489.2 ± 57.4	0.265	-35.7 ± 0.6	
B. ANOVA of 3 x 2^2 full experimental design					
Carum carvi					
Size	*Sum of squares*	*Df*	*Mean square*	*F*	*P*
Stirring speed	16537.96	1	16537.96	2.393784	0.219558
Stirring time	8.41	1	8.41	0.001217	0.974359
PDI	*Sum of squares*	*Df*	*Mean square*	*F*	*P*
Stirring speed	0.003306	1	0.003306	1.011704	0.420410
Stirring time	0.002256	1	0.002256	0.690407	0.493425
Zeta	*Sum of squares*	*Df*	*Mean square*	*F*	*P*
Stirring speed	**77.4400**	**1**	**77.44000**	**89.6988**	**0.010965**
Stirring time	1.4400	1	1.44000	1.6680	0.325658
Citrus sinensis					
Size	*Sum of squares*	*Df*	*Mean square*	*F*	*P*
Stirring speed	**8226.49**	**1**	**8226.49**	**25.42729**	**0.037150**
Stirring time	198.81	1	198.81	0.61450	0.515195
PDI	*Sum of squares*	*Df*	*Mean square*	*F*	*P*
Stirring speed	0.024649	1	0.24649	1.943876	0.297943
Stirring time	0.023716	1	0.023716	1.870298	0.304843

A) Size, polydispersity index (PDI), and zeta potential data for each nanoemulsion					
Zeta	*Sum of squares*	*Df*	*Mean square*	*F*	*P*
Stirring speed	0.36000	1	0.36000	0.045743	0.850467
Stirring time	1.00000	1	1.00000	0.127065	0.755588
Piper aduncum					
Size	Sum of squares	Df	Mean square	F	*P*
Stirring speed	249.64	**1**	249.64	0.022653	0.894171
Stirring time	10650.24	1	10650.24	0.966443	0.429218
PDI	Sum of squares	Df	Mean square	F	*P*
Stirring speed	0.004692	1	0.004692	3.08228	0.221235
Stirring time	**0.030800**	**1**	**0.030800**	**20.23226**	**0.046039**
Zeta	Sum of squares	Df	Mean square	F	*P*
Stirring speed	42.90250	1	42.90250	6.048285	0.133109
Stirring time	0.12250	1	0.12250	0.017270	0.907475

3.3. Antioxidant activity of nanoemulsions (DPPH$^{\cdot}$ and ABTS$^{\cdot+}$)

The antioxidant activity of NEs from the leaves of *P. aduncum*, *C. carvi*, and *C. sinensis* associated with levothyroxine is presented in Table 5. According to our results, all NEs showed antioxidant activity. In the DPPH test, the highest antioxidant activity was observed for *C. carvi* NE (EC_{50} = 204.3 µg/mL). In the test with the radical ABTS, the *P. aduncum* NE (EC_{50} = 156.6 µg/mL) showed the best antioxidant activity.

Table 5
Antioxidant activity of the essential oil of species of *Carum carvi*, *Citrus sinensis*, and *Piper aduncum*. Legend: Ascorbic acid and TROLOX (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) were used as reference antioxidants.

Chemicals	***DPPH***	***ABTS***
	EC_{50} µg/mL (Confidence interval)	***EC_{50} µg/mL (Confidence interval)***
C. carvi	204.3 (199.4-208.2)	194.9 (190.4-199.4)
C. sinensis	288.5 (280.8-295.1)	191.5 (186.7-196.3)
P. aduncum	266.5 (261.4–272.0)	156.6 (151.4-161.8)
Ascorbic acid	1.6 (1.4–1.8)	-
TROLOX	-	4.1 (3.7–5.8)

3.4. Effects of levothyroxine and nanoemulsions on zebrafish embryonic development

Toxicity tests were carried out with zebrafish embryos and larvae to evaluate the toxicity of the NEs. The results are shown in Fig. 1 from the zebrafish embryo and larval stages exposed to different dilution ratios of L-T_4 and NEs of each essential oil. Mortality was the only significant effect observed at 24 hpf in the PAN32 group. Teratogenic effects were observed in embryos still in the chorion. However, toxic effects were most observed in the 72 hpf larvae stage for the following groups: ECS, CSN32, EPA, PAN44, and PAN32, with *P. aduncum* NE being the most toxic. While at 96 hpf, significant teratogenic effects were observed in groups exposed to all NEs. Yolk sac edema is the most common toxic effect presented in zebrafish larvae, followed by pericardial edema.

3.5. Effects of levothyroxine and nanoemulsions on zebrafish animal behavior

The results of the thigmotaxis test (TH test) and touch sensitivity response (TS test) are shown in Fig. 2A and 2B. The data in the TH test represents the number of animals that move to the edge or periphery of plates. We did not observe toxic effects in this test when larvae were exposed to L-T_4 or any of the NEs (Fig. 2A). TS test was also performed by the mechanical stimuli to evaluate if L-T_4 or NEs exposure affects the swimming activity of the larvae. Our results (Fig. 2B) show that this parameter was affected only in the larvae exposed to empty *C. carvi* NE (ECC).

4. Discussion

Essential oils have been used by our ancestors as therapeutic agents for the treatment of various diseases. Their chemical constituents have high volatility. As a result, nanoencapsulation of these oils

has been proposed as a drug delivery system since this formulation technique is capable of improving the solubility, stability, and bioavailability of the compounds. NEs are being studied in recent years using formulations produced from essential oils (Pavoni et al., 2020). In the present study, GC-MS and GC-FID identified 16 chemical constituents in *Citrus sinensis* essential oil. Limonene was found to be the main constituent at 90.13%. Rezende et al. (2020) also found that essential oil from *C. sinensis* had a high limonene content (91.4%), which corroborates our data. In addition, traces of α- and β-pinene (1.25 ± 0.07, 1.87 ± 0.10, respectively), and phellandrene are present. Also, there is aldehyde (E)-isocitral (0.22 ± 0.03), which provides the characteristic odor present in this essential oil, as noted by Ali et al. (2015). *Piper aduncum* essential oil was identified with 13 chemical constituents, and dillapiole was the main constituent (78.41%), corroborating data in studies reported by Pino et al. (2004) and Maia et al. (1998). Finally, *Carum carvi* essential oil showed 6 constituents. The main ones are carvone (60.44 ± 0.80) and limonene (34.14 ± 0.64). This dataset corroborates the findings by Obeid et al. (2020) and Trifan et al. (2016).

Increased therapeutic potential of the essential oil is possible through nanoencapsulation (Aziz et al., 2019). The emulsification technique was successfully used to develop the three NEs from essential oils in our study. The formulation of NEs was affected by the stirring speed and stirring time. Our formulations were characterized as NEs since their vesicle sizes were less than 300 nm (Singh et al., 2017). It is preferable that the polydispersion index (PDI), a measure that characterizes the homogeneity of the vesicle size, have values below 0.2 (Danaei et al., 2018), which indicates a high degree of size homogeneity (Caddeo et al., 2008). PDI values can vary more or less depending on the technique used to produce NEs. The results obtained in our study correspond to PDI values varying between 0.3 and 0.4, which were obtained in our best NEs, demonstrating the homogeneity of the formulations. Additionally, the zeta potential (ζ - mv) parameter measures the surface charge and electrostatic parameters of vesicles (Grisham and Nanda, 2020). Our data revealed that all formulations presented high negative potential, indicating the stability of the NEs by repulsion forces between the vesicles and showing the absence of aggregations of the vesicles.

The use of surfactants with intermediate high hydrophilic-lipophilic balance (HLB) (generally between 11 and 16), such as tween, is the best choice to produce cheaper NEs by dispersing the oily phase in the aqueous phase. In addition, non-ionic surfactants such as Span are important due to their better toxicological safety for oral ingestion (Pavoni et al., 2020). According to Aziz et al. (2019), a higher value HLB surfactant is needed to produce smaller vesicles in the NEs, which was also used in our studies.

NEs containing *C. carvi*, *C. sinensis*, and *P. aduncum* essential oils showed good antioxidant activity, according to our values by EC_{50} (concentration of sample required to scavenge 50% of free radicals). This data confirms findings by Trifan et al. (2016), Rodríguez et al. (2013), and Frassinetti et al. (2011) for *C. carvi*, *P. aduncum*, and *C. sinensis*, respectively. These results can be explained because oils have a large percentage of oxygenated compounds in their compositions, such as limonene, carvone, and dilapiol, besides phenylpropanoid that is present in their structure methoxy and methylenedioxy groups. Furthermore, we cannot rule out the presence of other oxygenated compounds found in lower

percentages than the cited ones. Our findings are important to developing products that reduce the toxicity of monotherapy levothyroxine treatment (Chaker et al., 2017), and we suggest a combination of therapeutics and nanoemultions can be effective.

Keeping this in mind, this study focused on the development of the NEs produced from essential oils, and their characterization and toxicity evaluation. We used the zebrafish (*Danio rerio*) to evaluate the toxicity. The ethical consideration of the use of lower vertebrates such as zebrafish to evaluate the toxicity of NE was taken into account (Jayasinghe and Jayawardena, 2019). Our results revealed that the animals exposed to all NEs presented teratogenic effects. Independent of the type of essential oils used, despite *P. aduncum* NE producing more toxicity, similar teratogenic effects were observed for each NE. This could indicate that the same chemical compound used in the formulation of nanoemulsions has related toxicity effects. We did not observe significant teratogenic effects in the L-T_4 solution. However, all NEs were produced with surfactants (tween 80 and span 80). This idea is supported by Yang et al. (2015), who found that tween 80 induced anaphylactoid reactions in zebrafish. Ginzburg et al. (2018) investigated the influence of surfactants on nanoparticle biocompatibility by studying mixtures of ligand-stabilized gold nanoparticles and polysorbate 80 (span 80) in embryonic zebrafish and observed that the mixtures produced synergistic toxicity at concentrations where the individual components were benign.

The acute toxicity of span 80 was previously studied in zebrafish (Sun et al., 2017). This study revealed that span 80 has high mortality rate on zebrafish. This indicates that pharmaceutical excipients, including mixtures such as span 80, have potential risks when the safety of these new nanotechnological drugs were evaluated in zebrafish. Additionally, tween 80 also could be toxic to zebrafish (Ali et al., 2011). In our study, we saw more toxic effects in the larval stage and the absence of chorion may explain this effect. The chorion functions to protect the embryo against chemical exposure, for example (Kim and Tanguay, 2014), and when it ruptures, the fish are more readily exposed to toxins. Finally, our results indicate these NEs affected morphology rather than behavioral endpoints. This may be because the animals chosen for tests did not show any morphological defects, and probably these animals were more resistant to or less affected by toxic compounds present in NEs. Further research is necessary to reduce adverse effects before the use of these NEs.

5. Conclusion

In this study, antioxidant essential oil–based nanoemulsions associated with levothyroxine were successfully developed using a high-energy emulsification approach and optimized by factorial experimental design. The formulations showed suitable physicochemical characteristics, including nanometric mean size, acceptable polydispersity index, and negative zeta potential, indicating good colloidal stability. All nanoemulsions exhibited relevant antioxidant activity, confirming the contribution of the essential oils and supporting their potential to mitigate oxidative stress associated with hypothyroidism. Despite these promising properties, *in vivo* toxicity assessment using the zebrafish model revealed significant teratogenic effects, particularly during the larval stage, regardless of the essential oil used. Behavioral alterations were limited and formulation-dependent, with effects observed

mainly for the *Carum carvi* nanoemulsion. The absence of toxicity in levothyroxine alone and the occurrence of toxic effects in empty nanoemulsions suggest that surfactants employed in the formulations likely play a major role in the observed toxicity. Further studies focusing on reformulation strategies, surfactant replacement and long-term toxicity are necessary before these nanoemulsions can be considered viable therapeutic alternatives for hypothyroidism.

Declarations

Competing Interests

The research reported in this manuscript is part of a patent (BR102020025513-4) in the course of the process.

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Ethics approval

The protocols used in this study were approved by the Ethics Committee in the use of animals of the *Universidade Federal Rural de Pernambuco* - Brazil, protocol number 091/2018.

Consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and material

The data and data and material used in this study are available from the corresponding author on request.

Declaration of generative AI in scientific writing

During the preparation of this work the author(s) used Copilot in order to correct the language of the manuscript.

Author Contributions

Details of each author with their contribution in this paper: Conceptualization, methodology, validation, formal analysis, Investigation, data curation, writing - original draft, writing - review and editing, and visualization were performed by Thamiris Pinheiro Santos. Investigation and data Curation also were performed by Paulo Eduardo da Silva Bastos. Authors Matheus Victor Viana de Melo, Tiago Queiroz da Mota Bittencourt, Renata Meireles Oliveira Padilha, Renatta Priscilla Ferreira Silva, Niely Priscila Correia da Silva, Carolina Alves de Araujo, Marcilio Martins de Moraes, Cláudio Augusto Gomes da Câmara and Amanda Rodrigues dos Santos Magnabosco performed investigation. Authors Jadson Freitas da Silva and André Lucas Corrêa de Andrade performed the investigation and formal analysis. Co-supervision, project administration, writing - original draft, writing - review & editing were performed by Marilia Ribeiro Sales Cadena, Samara da Silva Gomes and Yuri Mateus Lima de Albuquerque. Conceptualization, methodology, validation, formal analysis, resources, data curation, writing - original draft, writing - review and editing, visualization, supervision, project administration, and funding acquisition were performed by Pabyton Gonçalves Cadena. All authors read and approved of the final manuscript.

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Figures

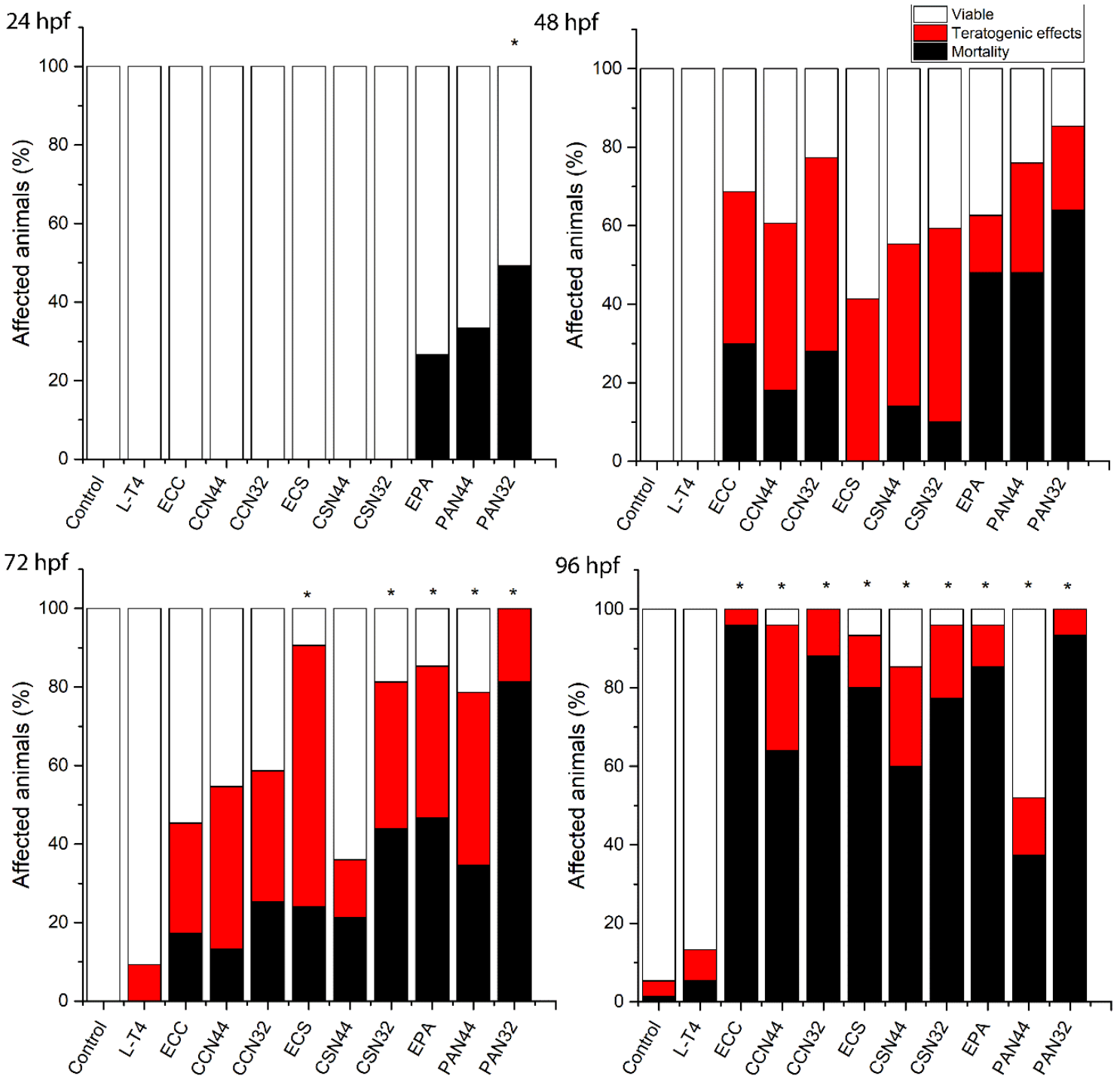

Figure 1

Percentage of affected animals after 24, 48, 72, and 96 h (post-fertilization) in toxicity test. The reduction in the percentage of affected animals was considered significant when * = $p < 0.05$. Legend: L-T_4 - levothyroxine; ECC - Empty *C. carvi* nanoemulsion; CCN44 - *C. carvi* nanoemulsion (4444x dilution rate); CCN32 - *C. carvi* nanoemulsion (3200x dilution rate); ECS - Empty *C. sinensis* nanoemulsion; CSN44 - *C. sinensis* nanoemulsion (4444x dilution rate); CSN32 - *C. sinensis* nanoemulsion (3200x dilution rate); EPA - Empty *P. aduncum* nanoemulsion; PAN44 - *P. aduncum* nanoemulsion (4444x dilution rate); PAN32 - *P. aduncum* nanoemulsion (3200x dilution rate).

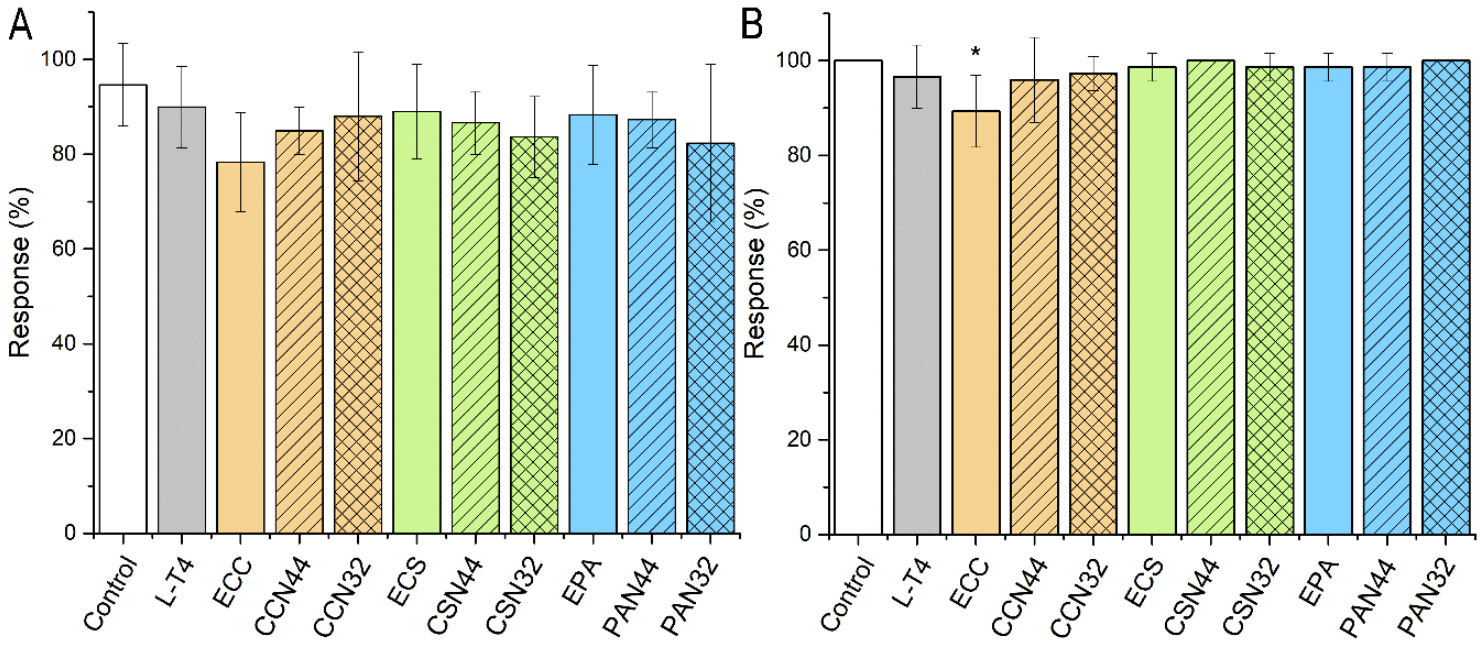

Figure 2

The thigmotaxis test (A) showed that the empty nanoemulsion and levothyroxine nanoemulsion did not affect the thigmotaxis response, but the empty *C. carvi* nanoemulsions affected the touch sensitivity response (B). The response for each group was compared to the control group by one-way ANOVA followed by Tukey's test (thigmotaxis ($F(10, 732) = 1.15$, $p < 0.05$); touch sensitivity response ($F(10, 732) = 2.81$, $p < 0.05$). * = $p < 0.05$. Legend: L-T_4 - levothyroxine; ECC - Empty *C. carvi* nanoemulsion; CCN44 - *C. carvi* nanoemulsion (4444x dilution rate); CCN32 - *C. carvi* nanoemulsion (3200x dilution rate); ECS - Empty *C. sinensis* nanoemulsion; CSN44 - *C. sinensis* nanoemulsion (4444x dilution rate); CSN32 - *C. sinensis* nanoemulsion (3200x dilution rate); EPA - Empty *P. aduncum* nanoemulsion; PAN44 - *P. aduncum* nanoemulsion (4444x dilution rate); PAN32 - *P. aduncum* nanoemulsion (3200x dilution rate).

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