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Assessment of acute toxicity of crude extract rich in carotenoids from Cantaloupe melon (*Cucumis melo* L.) and the gelatin-based nanoparticles using the *zebrafish* (*Danio rerio*) model

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ABSTRACT

Cantaloupe melon is known for its carotenoid-rich orange pulp. However, carotenoids are sensitive to oxygen, light, and heat, potentially reducing their benefits. Nanoencapsulation can preserve these benefits but raises concerns about toxicity. We aimed to assess the safety and bioactive potential of crude extract-rich carotenoids (CE) and nanoparticles based on gelatin loaded with CE (EPG) by investigating parameters such as cardio or neurotoxicity, especially acute toxicity. EPG was obtained by O/W emulsification and characterized by different methods. Zebrafish embryos were exposed to CE and EPG at 12.5 mg/L and 50 mg/L for 96h and were investigated for survival, hatching, malformations, and seven days post fertilization (dpf) larvae's visual motor response. Adult fish underwent behavioral tests after acute exposure of 96h. CE and EPG showed no acute toxicity in zebrafish embryos, and both improved the visual motor response in 7dpf larvae ($p = 0.01$), suggesting the potential antioxidant and provitamin A effect of carotenoids in cognitive function and response in the evaluated model. Adult fish behavior remained with no signs of anxiety, stress, swimming pattern changes, or sociability that would indicate toxicity. This study highlights the safety and potential benefits of carotenoids in zebrafish. Further research is needed to explore underlying mechanisms and long-term effects.

1. Introduction

Cantaloupe melon (*Cucumis melo* L.) is a widely consumed fruit globally, known for its orange flesh and slightly ridged skin, varying in colors from gray to green. This fruit is highly adaptable to different soils and temperate climates (Fundo et al., 2018; Vella et al., 2019).

The flesh of Cantaloupe melon is rich in carotenoids, giving it antioxidant, anti-inflammatory, and antimicrobial properties, besides the potential to act as a precursor for vitamin A (De Oliveira et al., 2021; De Queiroz et al., 2022; Gomes et al., 2023; Sinha et al., 2023). Due to their conjugated double bonds, carotenoids can interact with free radicals, stabilizing unpaired electrons and protecting against lipid peroxidation and oxidation (Young and Lowe, 2018). However, they are sensitive to processing and storage, quickly degrading in the presence of light, oxygen, heat, and pH variations (De Oliveira et al., 2021). Thus, techniques such as nanoencapsulation have been employed to ensure greater stability and preservation of bioactive properties (De Queiroz et al., 2022; Medeiros et al., 2019).

Nanoencapsulation involves coating an active ingredient (core) with an encapsulating agent, forming particles that enhance the active compound's solubility, stability, bioaccessibility, and bioavailability. Furthermore, this technique allows controlled release at a specific site, regulated by the wall material used (Steiner et al., 2018).

The bioactive potential of a crude extract rich in carotenoids from

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Cantaloupe melon has been underscored in preceding studies, as nanoencapsulation produced substantial advantages (Medeiros et al., 2019, 2020; de Oliveira et al., 2021; Gomes et al., 2023; Queiroz et al., 2023). The nanoencapsulation by O/W emulsification using porcine gelatin as an encapsulating agent effectively increased the water solubility and color stability of carotenoids derived from Cantaloupe melon in a food matrix (yogurt) (Medeiros et al., 2019). Moreover, nanoencapsulation conserves properties and optimizes interactions in the biological environment, potentializing the encapsulated carotenoid formulations in various biological applications (Medeiros et al., 2019; de Oliveira et al., 2021).

For instance, a preceding study demonstrated that nanoparticles loaded with a crude extract rich in carotenoids from Cantaloupe melon exhibited the capacity to enhance hepatic retinol levels in a preclinical model of diet-induced obesity, indicating the bioactive potential inherent in these formulations (Gomes et al., 2023). Furthermore, other investigations have explored the safety and bioactive potential of nanoparticles containing carotenoids sourced from Cantaloupe melon within an experimental model of chronic inflammation, accentuating the relevance of these particles in modulating inflammatory processes (Medeiros et al., 2020).

Queiroz et al. (2023) observed a reduction in adipose tissue hypertrophy and inflammatory response, with a significant decrease in plasma levels of IL 6 and leptin, in Wistar rats with chronic systemic inflammation induced by high glycemic index and high glycemic load diet (HGLI diet), evidencing the potential use of these nanoparticles against inflammatory diseases. These antecedent studies provide invaluable insights into the potential of these encapsulated carotenoid formulations across various biological applications. Despite all the studies already carried out with EPG, the various positive effects presented, and the fact that Medeiros et al. (2020) did not find signs of toxicity in Wistar rats, there is still great concern about the clinical use of EPG. Furthermore, one of the major current concerns is related to the neurotoxicity effects of nanoparticles, which, due to their smaller size, can easily cross the blood-brain barrier and lodge in the brain or nervous system, compromising their functions (Zhao et al., 2022). It is worth noting that in the study by Medeiros et al. (2020), EPG was not evaluated for cardio or neurotoxicity, which highlights the unprecedented nature of the present study.

In this context, exploring the interaction with other biological systems is essential for comprehensively understanding encapsulated formulations. Furthermore, considering the common differences between animal species in susceptibility to therapeutic agents in the absorption, distribution, metabolism, and excretion (ADME) processes, it is a differential to use more than one animal model to investigate toxicity and assess risks for new therapeutic agents (Lin et al., 2003). While rodent-based studies have furnished significant insights, appraising embryotoxicity within the zebrafish (*Danio rerio*) model during its embryonic development offers a distinct strategy of investigation (Bailone et al., 2020; Berry et al., 2007). This model facilitates exploration into the safety of encapsulated compounds but also permits the evaluation of pivotal parameters, such as phenotypic changes, and neurotoxicity, indicative of potential impacts on nervous systems.

Moreover, the inherent transparency of zebrafish embryos allows for direct real-time observation of the impacts of encapsulated nanoparticles, offering crucial insights into their biological dynamics (Lin et al., 2013). By monitoring the development of zebrafish embryos exposed to encapsulated nanoparticles carrying carotenoids derived from Cantaloupe melon, we can detect early-stage responses, thus fostering a comprehensive understanding of the potential effects of these compounds over time.

Therefore, this study aims to fill this knowledge gap by leveraging the distinct advantages of the zebrafish model by providing a comprehensive assessment of the biosafety and efficacy of a crude extract rich in carotenoids from Cantaloupe melon and encapsulated nanoparticles. We aim to consider not only the immediate effects but also the implications throughout embryonic development. Through this research, we intend to provide important information on how these compounds interact with other biological systems, thus contributing essential knowledge for the safe and effective development of products containing nanocapsulated carotenoids from Cantaloupe melon.

2. Materials and methods

2.1. Melon pulp processing and extraction of carotenoid-rich crude extract

Cantaloupe melons (*Cucumis melo* L.) of the A5A85DF genetic registration in the National System of Genetic Heritage (SisGen) were obtained from local markets in Natal, Rio Grande do Norte, in excellent states of ripeness, devoid of any signs of deterioration.

The process of obtaining a carotenoid-rich crude extract from melon pulp followed the procedure outlined by Medeiros et al. (2019). Melons were sanitized, their peels and seeds were removed, and the flesh was separated and cut into approximately 2 cm cubes. Subsequently, they underwent a 24-h drying period in a ventilated oven (Tecnal, Piracicaba, Brazil) at 55 °C. After drying, the cubes were processed into flour using an industrial blender.

For the extract preparation, this melon flour was macerated in 95% (v/v) ethanol at a ratio of 1:4 (w/v). The containers were shielded from light, and the solvent was changed every 24 h through vacuum filtration. The resultant ethanolic extract was subjected to a liquid-liquid partition using hexane in a 1:1 (v/v) ratio and a 10% (w/v) NaCl solution. Subsequently, the solvent was removed using a rotary evaporator at 28 °C. This process led to the formation of the crude extract (CE), which was lyophilized (at −57 °C and a pressure of 43 μHg) to ensure the complete removal of any residual solvents from the extract.

2.2. Characterization of the crude extract rich in carotenoids from Cantaloupe melon

2.2.1. UV–vis absorption spectrophotometry

The concentration of total carotenoids present in the pulp extract of fresh melon was determined using UV–visible absorption spectrophotometry analysis. The methodology described by Medeiros et al. (2019) was followed, and the equation developed by Biehler et al. (2009) was employed, utilizing the wavelength of maximum absorption (450 nm) obtained through a scanning spectrophotometer. The concentration (C) of carotenoids (in mol/L) was calculated using the following equation: $C\ (mol/L) = (A450 \times FD)/2592$ **(Equation 1),** where: A450 = Average absorbance measured at the wavelength of maximum absorption, FD = Dilution factor adjusted for absorbance measurements of the dry extract solubilized in hexane, and 2592 = Molar absorptivity coefficient of β-carotene (ε).

The results were expressed in micrograms of carotenoids per gram of fresh cantaloupe melon pulp (μg/g). The experiment was conducted in triplicate.

2.2.2. Ultra-performance liquid chromatography (UPLC)

The determination and quantification of β-carotene present in the extract were carried out using a chromatograph (Shimadzu®) coupled to a diode array detector (SPDM20A) at a wavelength of 450 nm, according to Medeiros et al. (2019). In summary, the chromatograph featured a binary analytical pump (LC-20A3 XR), an automatic injector (SIl-20AD XR), a degasser (DGU-20A3), and a column oven (CTO20AC), all controlled by LC Solution® software. The column employed was an XR-ODS type, Shim-Pak®, C18, 30 × 20 mm; 2.2 μm. The mobile phase consisted of acetonitrile and water as phase A and ethyl acetate as phase B, with the following proportions: 0 min, 100% A, 0% B; 5 min, 75% A, 25% B; 10 min, 30% A, 70% B; 13 min, 0% A, 100% B; 14 min, 100% A, 0% B; 20 min, 100% A, 0% B, all at a flow rate of 0.5 mL/min. A calibration curve of β-carotene standard was generated within

concentrations ranging from 0.005 to 0.2 mg/mL. The result was expressed in micrograms of carotenoids per gram of fresh melon pulp (μg/g).

2.3. Nanoencapsulation and characterization of carotenoid nanoparticles

Gelatin-based nanoparticles loaded with a carotenoid-rich raw extract from Cantaloupe melon pulp (EPG) were synthesized using the oil-in-water emulsification technique, following the methodology outlined by Medeiros et al. (2019). This process involved the preparation of two distinct aqueous phases: the first (FA1) consisted of 1.5% Tween 20 (w/v) dissolved in 90 mL of distilled water, while the second (FA2), comprised of 4% porcine gelatin (w/v) and 1.5% Tween 20 (w/v) dissolved in 100 mL of distilled water. The oil phase, containing 0.5% carotenoid extract (w/v) dissolved in soybean oil, had the extract incorporated into it through magnetic stirring at room temperature for 30 min, mirroring the technique used to solubilize FA1. For the solubilization of FA2, magnetic stirring was applied for 1 h at 40 °C.

The emulsion formation process involved homogenizing FA1 with the oil phase using ultradispersion equipment (Ultra-Turrax, IKA® T18 basic, Guangzhou, China) at 17,000 rpm for 5 min. Subsequently, FA2 was homogenized with the resulting emulsion using the earlier conditions. Following this step, the emulsion was subjected to lyophilization to yield powdered particles, which were later blended.

2.3.1. Fourier Transform Infrared Spectroscopy (FTIR)

For FTIR analysis, the raw extract, encapsulating agent, surfactant, and EPG were separately homogenized with potassium bromide. These mixtures were then macerated and pressed into pellets. The FTIR analysis covered the 400 to 4000 cm^{-1} range using a Shimadzu IRTracer-100 spectrometer from Kyoto, Japan. The scan consisted of 32 scans with a resolution of 4 cm^{-1}.

2.3.2. Carotenoid incorporation efficiency determination (EI)

To assess carotenoid incorporation efficiency (EI), we dissolved 150 mg of EPG in 1 mL of hexane per extraction cycle. Afterward, the dispersion underwent 3 min of ultrasonic treatment using an Ultra Cleaner 1650 Unique® device in Cotia, Brazil. Next, centrifugation (at 947.52×*g* for 20 min) separated the carotenoid-containing supernatant. This process was repeated in triplicate until color saturation, following the method described by Medeiros et al. (2019). Supernatant absorbance at 450 nm was measured using a spectrophotometer, and carotenoid extract concentration was measured using a pre-established calibration curve.

CE over a concentration range of 0.002–0.36 mg/mL was used for calibration. We calculated the incorporation efficiency using Hu et al.'s equation: Incorporation Efficiency (%) = (CE in Nanoparticles/Total CE Used) x100 **(Equation 2)**. This method assessed how efficiently carotenoids were incorporated into nanoparticles relative to the total CE used.

2.3.3. Characterized by scanning electron microscope (SEM)

A silicon wafer fixed onto a stub support using carbon tape was used for SEM analysis. Particles were suspended in acetone, droplets were placed onto the silicon wafer, and analysis was conducted under a high vacuum and a voltage of 2–3 kV using a ZEISS SEM-FEG microscope (AURIGA®, Oberkochen, Germany).

2.3.4. Laser diffraction

According to Medeiros et al. (2019), the experiment was done in triplicate. EPG's average diameters and polydispersity index were determined by dispersing 10 mg of nanoencapsulated material in 4 mL of acetone with magnetic stirring for 2 min at room temperature. Subsequently, 2 mL of formaldehyde (v/v) was added, and the dispersion was stirred for 30 min. Filtration was done using filter paper, and the retained particles were collected for analysis. The retained material was dispersed in 6 mL of distilled water, placed in a glass cuvette, and analyzed at a rate of 10 runs/minute using the NanoBrookZetaPlusZeta Potential Analyzer (Brookhaven Instruments, New York, USA) with BrookhavenInstruments – ZetaPALS Particle Sizing Software.

2.4. Animals' test design

2.4.1. Ethical statement

This study was approved by the Animal Ethics Committee at the Federal University of Rio Grande do Norte (CEUA 301.035/2022). The experimental procedures followed the National Council for the Control of Animal Experimentation (CONCEA) and the guidelines of ARRIVE (Animal Research: Reporting of *In Vivo* Experiments https://arriveguidelines.org/). All zebrafish experiments were conducted in the FishLab within the Department of Physiology and Behavior at the Federal University of Rio Grande do Norte.

2.4.2. Animals housing

Adult zebrafish (*Danio rerio*, wild-type Tübingen lineage (TU), six months old, female and male, weighing approximately 0.4 ± 0.03 g) were housed at the Fish Lab. The fish were maintained in an automated rack system (ZebTEC Active Blue Stand Alone - Tecniplast®) with a 14-10h light-dark cycle (lights on at 6 a.m.). They were housed at a density of 2–3 animals per liter in non-enriched acrylic tanks, with water conditions closely monitored: temperature at 28 °C, pH at 7.2, total ammonia levels below <0.01 mg/L, and a conductivity of 600 μS/cm. The fish were fed a diet consisting of brine shrimp (Artemia salina do RN®, Brazil) and commercial flake food (Alcon Basic®, Brazil) with a composition of 60% protein and 15% fat. Feeding occurred three times a day.

2.5. Fish embryo test [FET]

2.5.1. Embryo collection

Adult fish were randomly chosen and placed in breeding tanks at a ratio of three females to two males. These breeding tanks were filled with system water and maintained under the conditions described. The next day, eggs were collected approximately 60 min after the lights were turned on. Viable eggs, identified by blastula formation at 3 h post-fertilization (hpf), were carefully separated, rinsed with system water, and then transferred to 24-well polystyrene plates, with two embryos placed in each well.

2.5.2. Zebrafish embryos acute toxicity test

The number of animals employed in this study adhered to the guidelines and protocols established by the "Fish Embryo Acute Toxicity (FET)" (OECD 236), with minor modifications. Each well containing two embryos was filled with 2 mL of test solutions at 12.5 mg/L and 50 mg/L for both materials (CE and EPG). This study aimed to complement safety information, considering the previous toxicity study with CE and EPG (Medeiros et al., 2020). Therefore, these concentrations were determined based on previous investigations into toxicity and bioactive potential in an animal model (Wistar rats) with diet-induced chronic inflammation (Medeiros et al., 2020). Additionally, positive controls (4 mg/L 3,4-dichloroaniline) and negative controls (system water from the rack and 0.1% DMSO) were included in the study. Four embryos served as internal controls for each plate.

The embryos were exposed to these different solutions for 96 h. Every 24 h, the embryos were observed under a stereomicroscope at 80X magnification to assess lethality endpoints, which included egg coagulation, absence of somite formation, non-movement of the tail base, and absence of heartbeats. Developmental endpoints were also monitored, encompassing pericardial edema, yolk sac edema, ocular edema, body pigmentation alterations, and malformations in the head, tail, heart, and spinal cord structures. In total, 80 embryos were evaluated per group.

2.6. Optomotor response

Larvae at 7dpf were used to measure motion stimuli through the optomotor response, following the methodology described by Creton (2009). The stimulus was applied in a Petri dish (8.5 cm in diameter) with 30 larvae per group through a PowerPoint projection on a computer screen. A moving stripe pattern was employed, consisting of alternating black and white stripes (24.5 cm × 1.5 cm). Initially, the stripes moved upwards for 1 min, followed by a stimulus-free period (white screen) for 5 s. After this interval, the stripes moved downwards for another 1 min. The moving stripe stimuli were repeated at a constant speed (1 cm/s), while a video recorded the behavior of the larvae (Souza et al., 2023).

For each interval (white screen), images were generated (10 images per group), which were subsequently imported into ImageJ (http://rsb.info.nih.gov/ij/index.html) to assess larval orientation relative to the centroid. The larvae were identified, and X and Y coordinates were exported to Microsoft Excel. The obtained coordinates were standardized based on the centroid value. The number of individuals exhibiting a positive optomotor response (OMR+), indicating alignment with the stripe movement direction, and a negative optomotor response (OMR-), indicating alignment in the opposite direction of the stripe movement, were quantified.

2.7. Adult fish toxicity and behavioral tests

Behavioral tests were conducted in TU adult zebrafish (six months old, females and males in equal proportions). Three distinct tests were performed: the novel tank, social preference, and light/dark preference paradigms. This battery of tests facilitated a comprehensive evaluation of diverse behavioral aspects arising from compound exposure.

Animals were exposed to solutions containing CE and EPG for 96 h at 12.5 and 50 mg/L concentrations and negative controls (system water and 0.1% DMSO). The exposed fish were observed every 24 h post-exposure, and any visible anomalies related to equilibrium, appearance, ventilatory behavior, and swimming behavior were recorded, along with mortality. Each test group consisted of 10 individuals, totaling n = 60. Each fish was gently captured using a net on the behavioral testing day and then transferred randomly to the testing tanks. All tests were conducted between 2 and 5 p.m. and video-recorded using a webcam (Logitech® c920 HD Pro).

2.7.1. Novel tank test

Each fish was introduced individually into test tanks (22 × 15 × 22 cm), pre-filled with system water. The behavior of each fish was recorded over a 6-min using a Logitech c920 HD Pro webcam positioned at a distance of 40 cm from the apparatus. The following behavioral parameters were selected for analysis: mean swimming velocity, immobility duration, time spent at the bottom of the tank, and average distance from the bottom. The behavioral records obtained were analyzed utilizing the ANY-Maze™ tracking software (Stoelting, CO, USA). This software enables precise and comprehensive behavioral data analysis, providing detailed insights into swimming and other behavioral patterns under various experimental conditions. The behavioral parameters extracted from the novel tank test were average speed and distance traveled to infer locomotion, distance from the bottom of the tank, latency to enter the top area, and time spent in the top area to infer anxiety-like behavior.

2.7.2. Light and dark test

The light-dark test was carried out according to Fernandes Silva et al. (2022) with modifications, where a tank was divided into two compartments, one black and the other white, with a s sliding door that restricted free access between the areas.

The experimental subject was initially placed in the black area and allowed to acclimate for 3 min. Subsequently, the sliding door was lifted to enable the zebrafish to move between the black and white areas for 7 min, and behavior was recorded. ANY-Maze™ software was used to track the videos. Parameters analyzed included latency to enter the light area, the time spent in the light area, and the number of transitions between the two areas.

2.7.3. Sociability test

Three rectangular tanks (22 × 15 × 22 cm) were aligned for the sociability test. Each tank was filled with water. The experimental tank was positioned in the middle, housing one zebrafish per recording. One of the two additional tanks contained five zebrafish of the same size and age as the test groups, serving as the social stimulus (conspecific tank); the other tank remained empty. At the test's outset, two white panels were placed between the tanks to prevent the experimental zebrafish from observing the other tanks. The experimental zebrafish was introduced into the tank and allowed to acclimate for 6 min, after which the two panels were removed. Swimming behavior was observed over 6 min using a Logitech c920 HD Pro camera and analyzed using the ANY-Maze™ software, where immobility time, distance traveled in the social area, latency to first entry into the social area, time spent in the social area, and distance from the specific group were verified (Moreira et al., 2022).

2.8. Statistical analysis

Statistical analyses were conducted using GraphPad Prism, version 8.0.1 (GraphPad Software, San Diego, CA), and Jamovi (Version 2.3). The normality of data was assessed using the Shapiro-Wilk and Anderson-Darling tests. Non-parametrically distributed data were compared using the Kruskal-Wallis test. Survival curve comparisons were executed using the log-rank test (Mantel-Cox). For the hatching rate and phenotypic changes analysis, a Two-Way ANOVA was applied considering the factors "time" and "groups" for hatching rate data and "malformations" and "groups" for the phenotypic changes analysis. Post hoc comparisons with the negative control were conducted using Dunnett's post hoc test. The optomotor response was evaluated using the chi-square test to determine neurotoxicity in the larvae through a visual motor test. Behavioral evaluations in adult zebrafish were carried out through One-Way ANOVA, followed by Bonferroni's post hoc test. A significance level of 0.05 was adopted for all statistical inferences.

3. Results

3.1. Characterization of crude extract rich in carotenoids from Cantaloupe melon and Nanoparticles

For the CE, the total carotenoid and β-carotene concentrations determined were 46.80 (±2.57) μg/g and 28.20 (±1.97) μg/g, respectively. Regarding EPG, the SEM revealed a spherical morphology, showcasing a smooth surface devoid of any signs of cracks or depressions. The observed scale was consistently within the nanometric range (<100 nm) (Fig. 1a).

In Laser Diffraction analysis (Fig. 1b), the encapsulated gelatin-based nanoparticles (EPG) demonstrated an average diameter of 88.7 nm (±7.02) and a polydispersity index of 0.41 (±0.03). Notably, the encapsulation efficiency reached 94% (±4.04). For FTIR, distinct changes were observed. Specifically, CE's characteristic bands at 2923 and 2955 cm^{-1} were significantly reduced in the EPG spectrum. Moreover, bands at 1041 and 848 cm^{-1} were absent, while those at 1640, 1095, and 947 cm^{-1} became more pronounced. These FTIR results strongly suggest an interaction between CE's carbon chain and the nonpolar amino acids in gelatin, confirming a compound interaction and successful encapsulation (Fig. 1c).

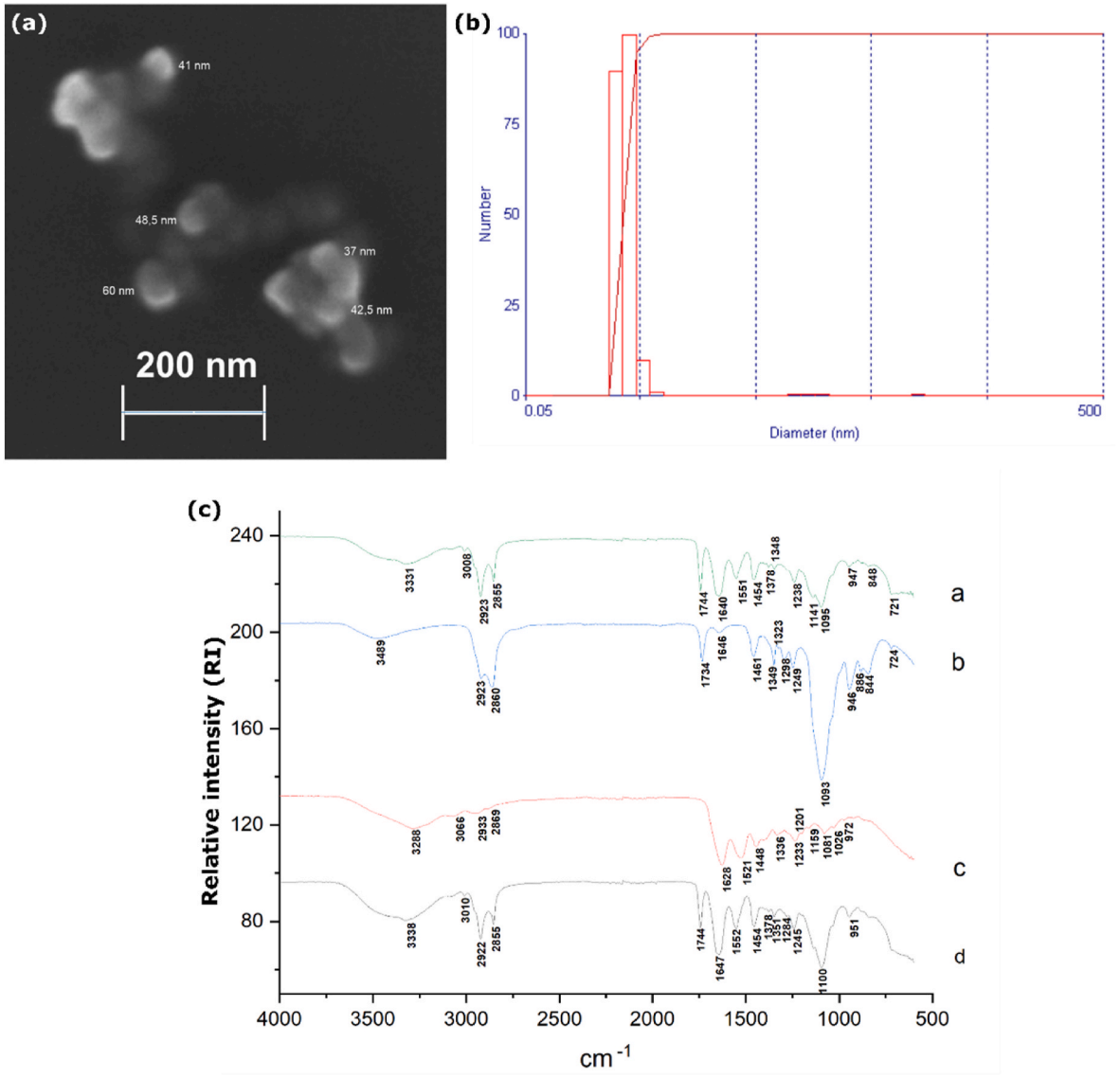

Fig. 1. Characterization of a crude extract rich in carotenoids from Cantaloupe melon pulp nanoencapsulated in porcine gelatin (EPG). a) Scanning Electron Microscopy (SEM); c) Laser diffraction; c) Fourier Transform Infrared Spectroscopy (FTIR) a. crude extract rich in carotenoids (CE); b. Tween 20; c. porcine gelatin; d. nanoparticles based on porcine gelatin loaded with CE (EPG).

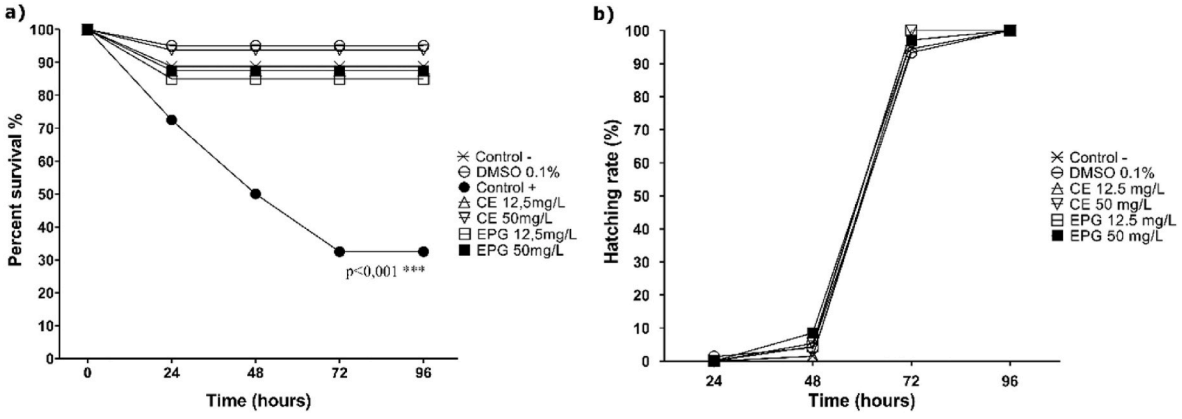

Fig. 2. Survival and Hatch Rate of Exposed Zebrafish Embryos. a) Zebrafish embryos were exposed to various treatments for 96 h: Control (system water), DMSO 0.1%, Control+ (3.4 DCA), CE, and EPG at concentrations of 12 mg/L and 50 mg/L for both groups. Daily mortality was monitored (n = 80 embryos per group). The log-rank test revealed statistical significance for the positive control compared to the other groups ($\chi 2 = 167$, df = 6, $p < 0.001$). b) Hatching rate of surviving zebrafish embryos recorded every 24 h. Two-way ANOVA indicated that only time significantly influenced the hatching rate. ***$p < 0.001$.

3.2. Evaluation of lethal concentration and development endpoints

To evaluate the toxicity of CE and EPG compounds at concentrations of 12.5 and 50 mg/L, we analyzed the survival rate of zebrafish, hatching rate, and gross morphological alterations at each larval developmental stage (24, 36, 48, 72, 96 hpf). The survival and hatching rates remained unaffected by exposure to the analyzed compounds (Fig. 2). The negative control group displayed a survival rate of 88.75%. In contrast, the positive control group exhibited a considerably lower rate of only 36.25%. The survival rates for embryos treated with CE at concentrations of 12.5 and 50 mg/L were 87.5% and 93.75%, respectively. Similarly, for the EPG groups, the survival rates were 85% and 87.5% at 12.5 and 50 mg/L concentrations, respectively (Fig. 2a). The log-rank test demonstrated statistical significance for the positive control group compared to the other groups ($\chi 2 = 167$, df = 6, $p < 0{,}001$). However, no statistical significance in survival rate was observed in the remaining groups compared to the negative control ($p > 0.05$).

The larval hatching rate did not vary significantly among the treatment groups (Fig. 2b). In general, most larvae had already undergone hatching by 72 hpf. At 48 hpf, the hatching rate for the negative control was 1.47%, while for the DMSO control, it was 4.05%. For the CE 12 mg/L, CE 50 mg/L, EPG 12 mg/L, and EPG 50 mg/L groups, the rates were 1.42%, 5.33%, 4.41%, and 8.49%, respectively. At 72 hpf, the hatching rates for the negative control and DMSO control were 94.47% and 93.38%, respectively. Treatment with CE and EPG at concentrations of 12 mg/L and 50 mg/L resulted in hatching rates of 97.14%, 100%, 100%, and 97.13%, respectively. A two-way ANOVA revealed no significant interaction between the treatment groups and time ($F(15, 18) = 1.476$, $p = 0.2141$). But time was the stronger determining factor for hatching rates than the treatments themselves [Time, $F(1{,}396, 8{,}375) = 7870$, $p < 0{,}0001$; Groups, $F(5, 6) = 1{,}729$, $p = 0{,}2615$]. By the end of 96 hpf, larvae from all treatment groups had successfully hatched.

Our results showed no morphological defects in embryos treated with 12.5 mg/L and 50 mg/L of CE and EPG (Fig. 3). Two-way ANOVA revealed statistically significant effects for groups [$F(6, 49) = 2463$; $p < 0.0001$], for malformation [$F(6, 49) = 469.8$; $p < 0.0001$], and for the interaction terms groups vs. malformation [$F(36, 49) = 455.8$; $p < 0.0001$]. However, the Dunnett test showed that only fish exposed to the positive control group exhibited phenotypic alterations ($p < 0.0001$).

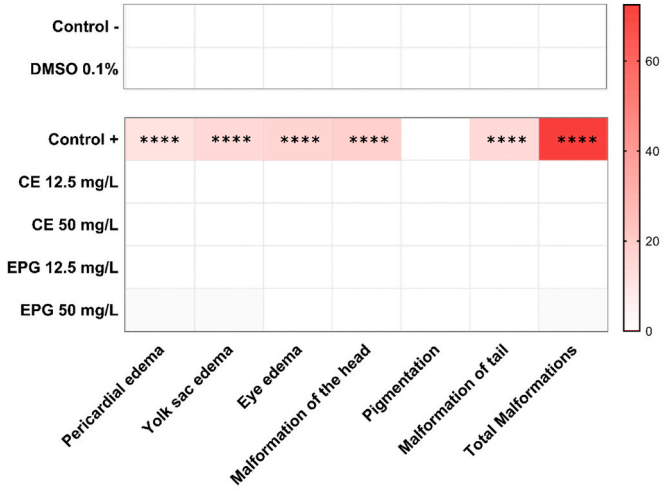

Fig. 3. Heat map summarizing phenotypic abnormalities observed during 120 h of zebrafish development for all treatments. Two-way ANOVA followed by Dunnett's test was conducted to assess statistical significance. Asterisks (*) indicate statistically significant differences between groups compared to the negative control. The sample size (N) for each group was 80. ****$p < 0.0001$.

3.3. Optomotor response

Fig. 4a depicts the protocol used for the optomotor response test. A total of 1200 larvae positions were analyzed, with 200 larvae positions per experimental group. All groups exhibited a higher frequency of positive optomotor responses (ORM+) compared to negative optomotor responses (OMR-), [$\chi^2 = 90.7$; df = 5, $p = 0.001$] (Fig. 4b).

In general, 63% of the 200 larvae positions in the negative control group followed the stripe movement, while in the 0.1% DMSO-treated group, this value was 58.5%. Conversely, the proportion of positive optomotor response for the other groups was as follows: 90.5% for CE 12.5 mg/L, 74% for CE 50 mg/L, and 74% for EPG 12.5 mg/L, and 89% for EPG 50 mg/L. The analysis of variance (ANOVA) revealed statistically significant differences in the proportion of larvae following stripes movement compared to the negative control group [$F(5, 54) = 25.95$; $p = 0.0001$] (Fig. 4c). Both CE and EPG compounds significantly enhanced the optomotor response of the tested larvae.

3.4. Adult behavioural analysis

3.4.1. Novel tank test

The Novel Tank Test was employed to assess the potential neurotoxicity of CE and EPG compounds by evaluating zebrafish's swimming and anxiety-like behavior in a new environment (Fig. 1a). Vertical exploration remained unaltered in all groups compared to the control groups. Swimming profiles were assessed based on total distance traveled (Fig. 5b) and average mobile speed (Fig. 5c). Neither of these parameters showed statistically significant differences when compared to the control group [Total distance traveled $F(5, 53) = 1.860$, $p = 0.1172$; Average mobile speed $F(5, 53) = 1.801$, $p = 0.1286$].

The anxiety-related parameters did not exhibit statistically significant alterations compared to the control groups. Results suggest no changes in the anxiety state of the zebrafish [Average bottom distance $F(5, 51) = 1.994$, $p = 0.0953$ (Fig. 5d); Latency to enter the top area $F(5, 54) = 1.390$, $p = 0.2425$ (Fig. 5e); and Time in top area $H(6) = 4.568$, $p = 0.4708$ (Fig. 5f)]. Both tested concentrations of CE and EPG compounds (12.5 mg/L and 50 mg/L) did not affect the behavior of the tested zebrafish.

3.4.2. Light and dark preference test

The behavioral analysis conducted through the light-dark test (Fig. 6a) did not reveal significant differences in the comparisons between treatments with CE and EPG at concentrations of 12.5 mg/L and 50 mg/L regarding latency to enter the light area (Fig. 6b) and the number of transitions between light and dark areas (Fig. 6c) [Kruskal-Wallis test, $H(6) = 4.953$, $p = 0.4216$ and One-Way ANOVA; $F(5, 52) = 1.304$, $p = 0.2768$, respectively].

The time spent in the light area was slightly different among the groups. In the negative control group, zebrafish spent an average of 41% of the total time in the light area, while in the 0.1% DMSO group, it was 49%. For CE, at concentrations of 12.5 mg/L and 50 mg/L, the values were 37% and 45%, respectively. As for EPG, at concentrations of 12.5 mg/L and 50 mg/L, the times in the light area were 35% and 41%, respectively. It is important to highlight that, despite these differences, none of them were statistically significant, indicating that none of the behavioral parameters were significantly affected by the treatments [One-way ANOVA; $F(5, 53) = 0.673$, $p = 0.6457$], (Fig. 6d).

3.4.3. Sociability test

In the social behavior test (Fig. 7a), no significant behavioral changes were observed in the groups treated with CE and EPG at 12.5 mg/L and 50 mg/L compared to the control groups. There were no statistically significant differences in the latency to enter the social area (Fig. 7b) [Kruskal-Wallis test; $H(6) = 10.22$, $p = 0.0693$], the time spent in the social area (Fig. 7c) [One-way ANOVA test $F(5, 53) = 0.07323$, $p = 0.9960$], distance traveled within the social area (Fig. 7d) [One-way

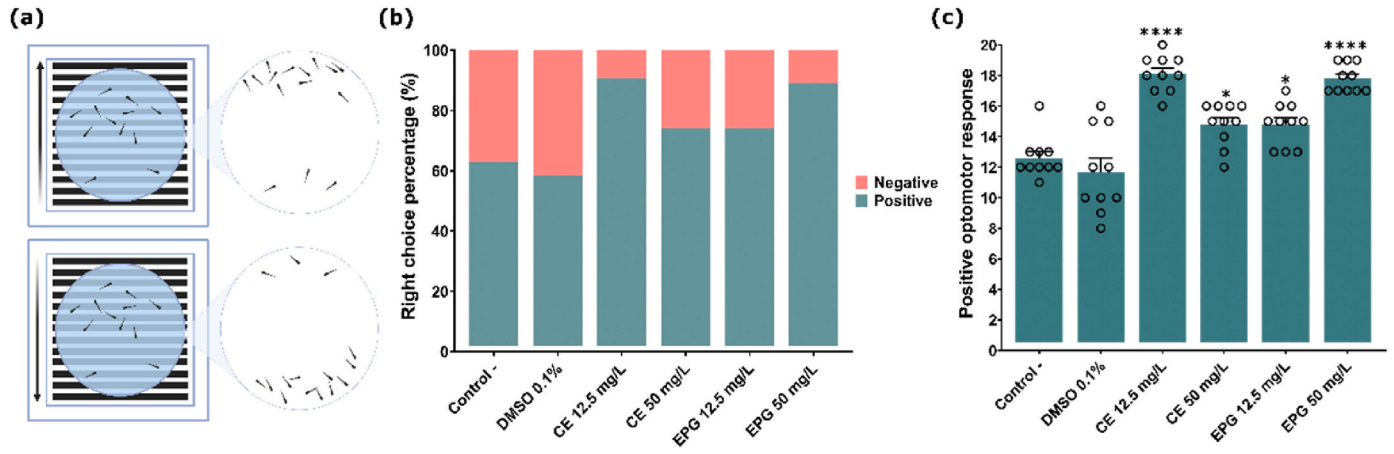

Fig. 4. Optomotor Response (ORM) in zebrafish larvae to track stripe movements, obtained from 7 days post-fertilization (dpf) larvae. (a) Schematic representation outlining the experimental design. **(b)** Percentage of correct responses (ORM+). **(c)** The proportion of larvae that followed the stripe movement showed significant differences between the groups [$F(5, 54) = 25.95$; $p = 0.0001$]. Each circle represents a data point for a specific interval (white screen) when larval positions were analyzed. Asterisks (*) indicate statistically significant differences between groups compared to the negative control. **** ($p < 0,0001$).

ANOVA test $F(5, 53) = 0.07323$, $p = 0.9960$], immobile time (Fig. 7e) [Kruskal-Wallis test $H(6) = 9.969$, $p = 0.0761$], and distance from the conspecific group (Fig. 7f) [Kruskal-Wallis test $H(6) = 5.546$, $p = 0.3529$].

4. Discussion

This research aimed to thoroughly analyze the toxicity of carotenoid-rich extracts from cantaloupe melons in zebrafish. The study involved encapsulating these extracts in porcine gelatin nanoparticles and assessing their safety and effectiveness. Zebrafish larvae were used to evaluate their impact, and neurotoxicity tests in adult zebrafish. The study's outcomes provide a comprehensive understanding of the compounds' safety profile, opening avenues for their potential use in diverse applications, including functional foods and pharmaceuticals.

The characterization of CE revealed significant concentrations of carotenoids, emphasizing β-carotene. β-carotene is one of the most studied carotenoids known for its antioxidant properties. These antioxidants play a crucial role in protecting cells against damage caused by free radicals, thus helping to maintain cellular integrity and overall health (Young and Lowe, 2018). Furthermore, carotenoids, including β-carotene, play a fundamental role as precursors to vitamin A, which is essential for various biological functions, such as visual and immunological functioning (Huang et al., 2018). Vitamin A deficiency is a significant health issue in many parts of the world and can lead to vision disorders, compromised immune systems, and other health problems (Huang et al., 2018). The presence of high concentrations of β-carotene in CE is particularly relevant since these carotenoids have the potential to improve overall health and organ function by providing antioxidant protection and contributing to vitamin A intake.

The efficient encapsulation of carotenoids in nanoparticles represents an important feature of this study. The spherical morphology of the EPG and its uniform size (with an average diameter of 88.7 nm) are crucial features that ensure the stability and effectiveness of these nanoparticles (Soukoulis and Bohn, 2018). Furthermore, the high encapsulation efficiency (94%) indicates substantial protection and efficient transport of carotenoids by nanoparticles, which is vital for the controlled delivery of these compounds.

FTIR spectroscopy provided valuable information about interactions between carotenoids and the gelatin matrix in EPG. Spectral changes suggest a potential interaction between carotenoid carbon chains and nonpolar amino acids in gelatin. This result suggests the successful incorporation of carotenoids into nanoparticles, confirming the effective encapsulation that can enhance the controlled release of these compounds. Thus, the findings obtained in this study align with those reported in other published studies (Medeiros et al., 2019, 2020; Queiroz et al., 2023; Gomes et al., 2023).

The safety assessment of CE and EPG carotenoids was conducted during a critical developmental phase, the gastrulation, using zebrafish embryos. Notably, 12.5 and 50 mg/L of these compounds showed no adverse effects on larval survival or hatch rates. Maintaining survival and hatch rates is crucial, as reductions in these parameters might indicate potential toxicity (Shaw et al., 2016). Furthermore, no morphological defects were observed in larvae exposed to these concentrations, which is a significant finding. Teratogenicity is a primary concern when assessing compound biosafety during embryonic development (Augustine-Rauch et al., 2010). The study results indicate the absence of teratogenic effects from CE and EPG carotenoids, even when exposed during the first 3 h of zebrafish embryo development.

The concentrations used in this study (12.5 mg/L and 50 mg/L) were consistent with those employed in previous studies involving rodents (Medeiros et al., 2020; Gomes et al., 2023; Queiroz et al., 2023). This allows valuable cross-species comparisons, suggesting that the safety profiles observed in zebrafish embryos align with results from rodent models.

The outcomes underscore the tested concentrations' safety and emphasize the potential translational relevance of these carotenoids for various health and nutrition applications, including their use as supplements or functional ingredients. Medeiros et al. (2020), when investigating the safety and bioactive potential of EPG in adult Wistar rats with chronic inflammation, observed that the general signs of toxicity, considering body weight, food consumption, hematological and biochemical parameters, relative weight, morphology, and histopathology of organs, demonstrated the absence of toxicity and that the bioactive effect of EPG was possibly related to its anti-inflammatory potential. However, the results presented in this study are of great importance considering the two life stages evaluated and the possibility of further understanding the effect of EPG on aspects such as embryonic development and observing malformations related to cardio or neurotoxicity. Besides, the concentrations employed in embryo studies revealed additional insights compared to rodent studies.

To improve the detection of visual and neurological damage, it is crucial to consider behavioral data related to the vision and movements of zebrafish larvae and traditional embryotoxicity tests (Jarema et al., 2022). As presented here, CE and EPG concentrations were not toxic or caused visual and behavioral impairment to zebrafish larvae. On the contrary, all larvae showed a high positive optomotor response (ORM+), indicating good performance of the treated fish. This test provides a

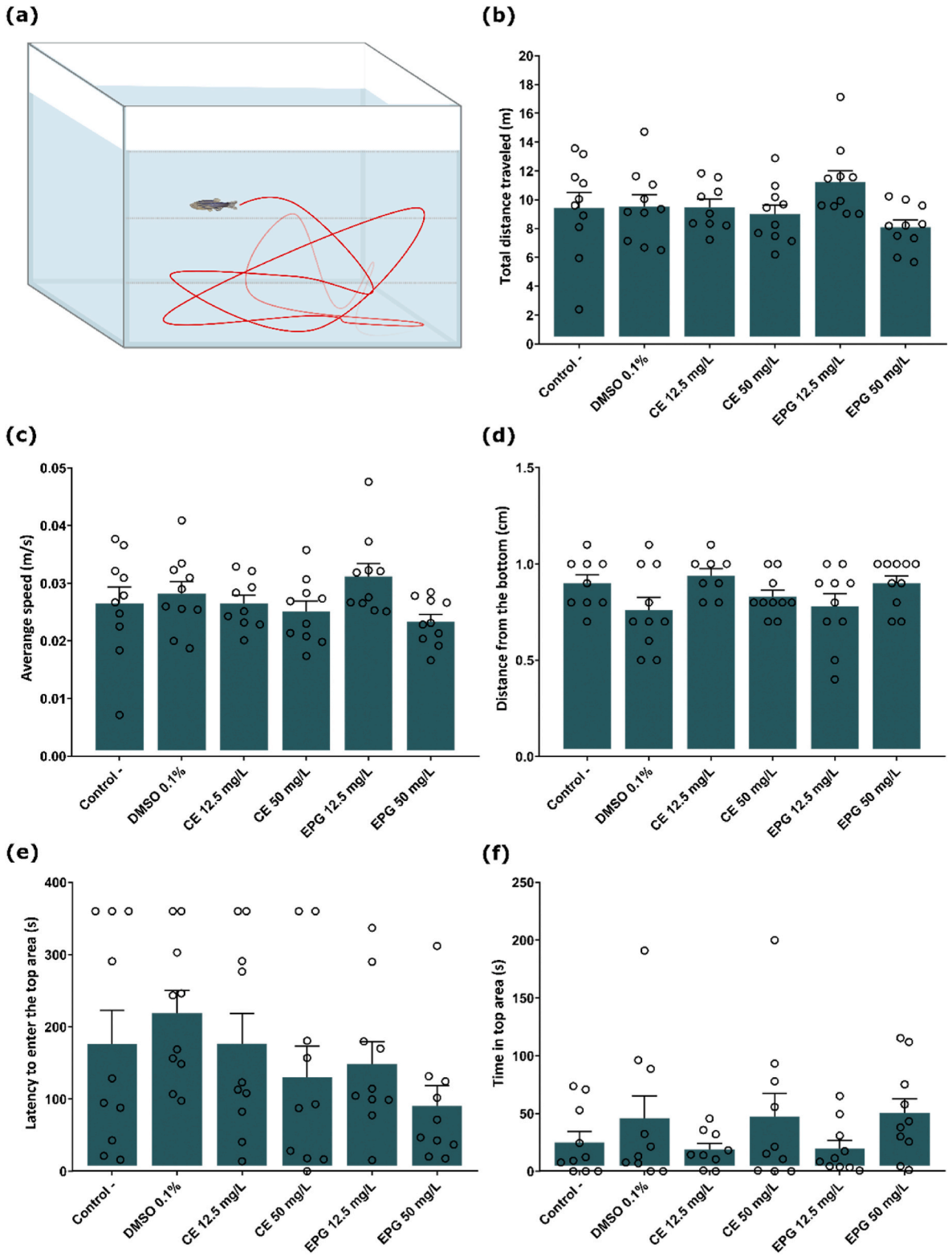

Fig. 5. Novel Environment Exploration Behavior (Novel Tank Test). Schematic representation outlining the experimental design **(a)**, total distance traveled **(b)**, average mobile speed **(c)**, average bottom distance **(d)**, latency to enter the top area **(e)**, and time spent in the top area **(f)**. Data are expressed as means ± SEM and were analyzed by One-way ANOVA with Dunnett's post hoc correction or Kruskal-Wallis test with Dunn's post hoc correction. Each circle represents an animal.

more complete view of the impacts of EPG and CE on the development of the fish. Analysis of the larvae's visual and motor behavior offers precise insights into how compounds can affect sensory and motor systems (Colwill and Creton, 2011; LeFauve et al., 2021). This approach goes beyond purely embryotoxic assessment, capturing subtle nuances of interactions between compounds and the developing nervous system.

Beta carotene is a precursor of vitamin A, in addition to having antioxidant properties capable of eliminating free radicals, increasing immunity, reducing symptoms of anxiety and stress, and promoting an improvement in cognitive function, among many other functions (Jalali-

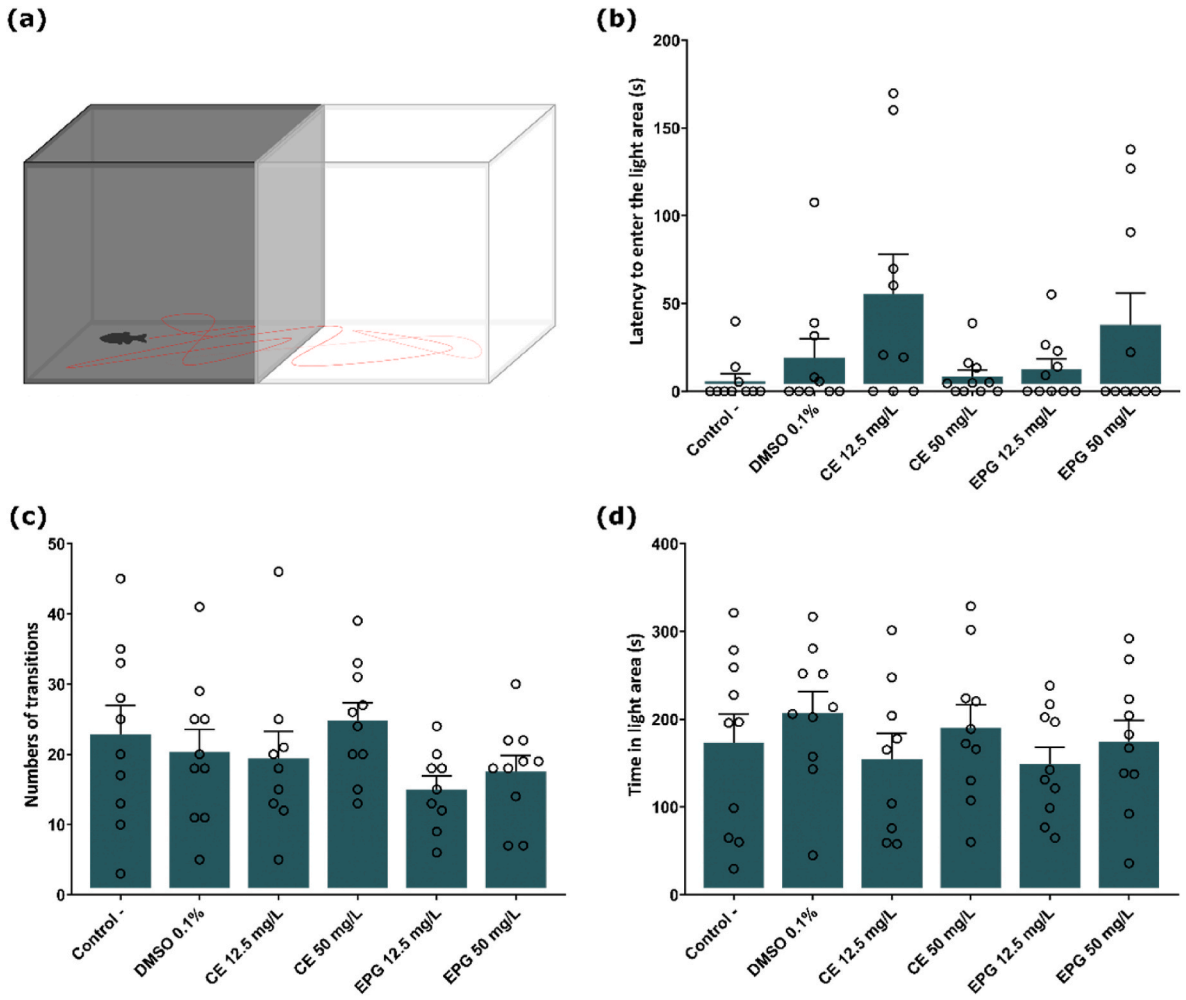

Fig. 6. Light-Dark Preference Behavior. Schematic representation of the apparatus (a). Latency to enter the light area (b), number of transitions between light and dark areas (c), and time spent in the light area (d). Data is presented as means ± SEM and was analyzed using the Kruskal-Wallis test with Dunn's post hoc correction. Each circle represents an individual animal.

Jivan et al., 2022; Liao et al., 2021). It acts as an antioxidant and precursor of vitamin A, which may have improved the sensory response of the larvae tested, mainly because vitamin A is responsible for improving visual acuity, brain function, immunity, and cell proliferation (Melén-dez-Martínez, 2019). Factors may be related to the cognitive improvement and visual stimulation observed in animals exposed to CE and EPG.

It is essential to point out that even the animals exposed to CE (12.5 mg/L) and EPG (50 mg/L) presented very similar responses. EPG contains only 0.62 mg/mL of CE, a dose 20 times lower than that used for non-encapsulated CE but demonstrating the same effect. Thus, this supports the hypothesis that the CE encapsulation protects these compounds and enhances the impact promoted.

Including this behavioral data strengthens the foundation for making informed decisions about the safe and effective use of EPG and CE, providing valuable insights for future research and the refinement of the carotenoid extracts. While the present study provides valuable insights into the influence of CE and EPG on optomotor responses, more research is needed to understand the underlying molecular, neural, and behavioral processes in greater detail.

In the second part of this study, we sought to investigate the toxicological impacts of a crude extract rich in carotenoids from Cantaloupe melon and nanoencapsulated in adult fish, which presents the nervous system with a completely developed and richer behavioral repertoire. The duration of exposure to these compounds was set at 96 h, during which we meticulously assessed the subsequent behavioral responses of the zebrafish using a battery of tests, including the novel tank test, light-dark test, and social test.

Remarkably, our findings revealed that the administration of the extracts did not induce any discernible alterations in behavior within the context of the novel tank test. This test is specifically designed to gauge anxiety levels and locomotor activity (Fontana et al., 2022), and the lack of behavioral changes in this assessment suggests the safety of the extract's exposure. Other studies approaching the effects of several compounds in zebrafish have used the novel tank test, which is a reliable test to access the brain-behavior relationship. For instance, two classical drugs well known for their effects are caffeine, which increases anxiety-like behavior in the novel tank test (De Carvalho et al., 2019), and alcohol, a depressive drug that decreases anxiety response in adult zebrafish tested for the novel tank (Agues-Barbosa et al., 2022). In this sense, plant extracts are usually tested using this same paradigm, and examples can be traced as Areca palm nut (*Areca catechu*) (Siregar et al., 2022), fruits of *Angelica archangelica* L. (Maciąg et al., 2020), lemongrass (*Cymbopogon citratus*) (Mendes Hacke et al., 2020).

Similarly, our observations during the light-dark test showed no significant shifts in behavioral patterns post-exposure. This test, functioning as another indicator of anxiety and exploratory behavior, further strengthens the notion that the applied extracts do not substantially disrupt the baseline behaviors of adult zebrafish. Although both tests are related to anxiety-like behavior, there are context-dependence effects (Moreira and Luchiari, 2022) that should be considered when testing a

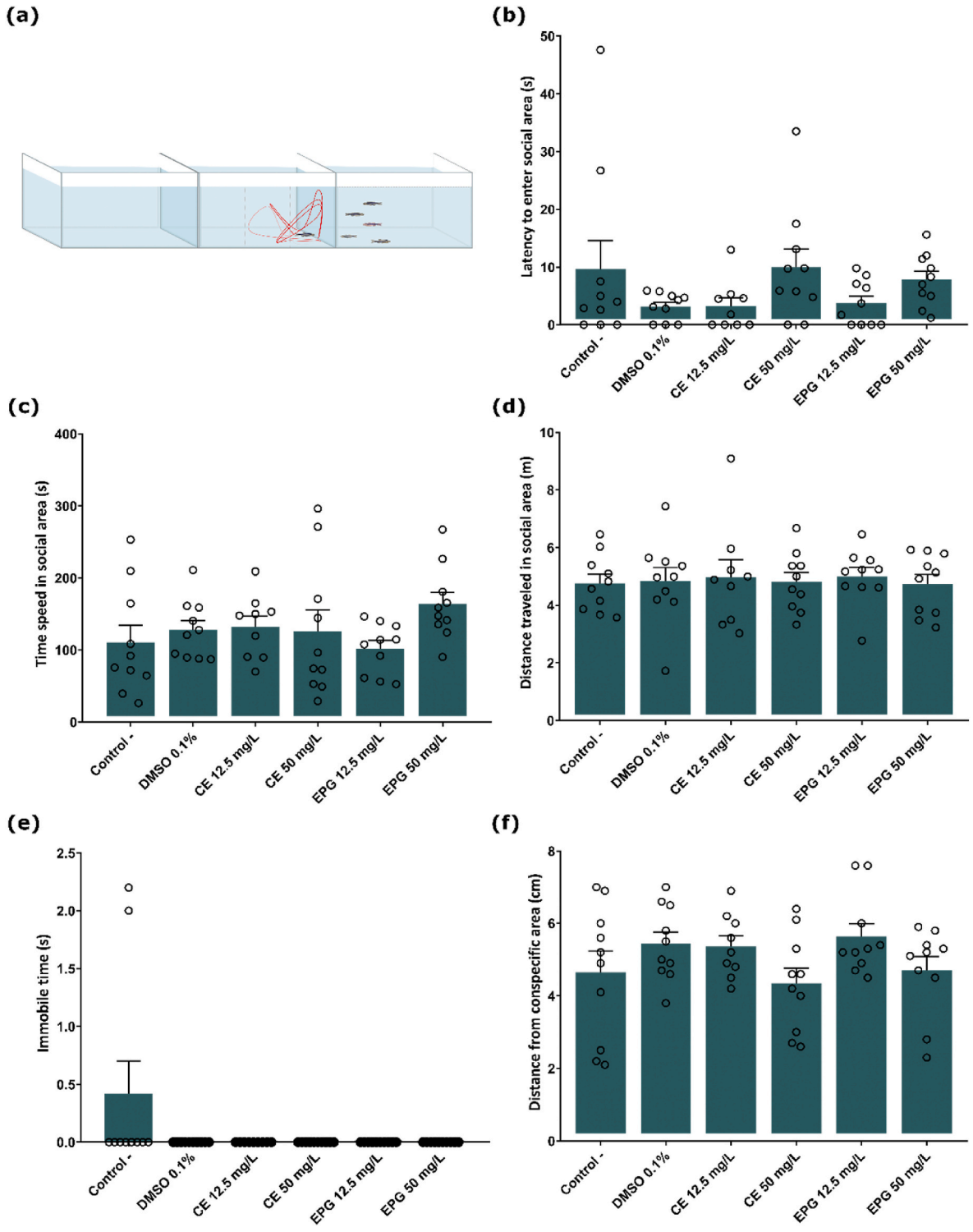

Fig. 7. Social Preference Behavior: Schematic representation of the testing apparatus **(a)**, latency to enter the conspecific area **(b)**, time spent in the social area (c), distance traveled in the social area **(d)**, immobile time **(e)**, and distance from the conspecific group **(f)**. Data are expressed as means ± SEM and were analyzed using One-way ANOVA (with Dunnett's post hoc) or Kruskal-Wallis test (with Dunn's post hoc). Each circle represents an individual animal.

novel compound. For instance, a study by Abidar et al. (2020) found that certain plant extracts, such as those containing flavonoids, polyphenols, terpenes, lignans, and tannins, may activate γ-aminobutyric acid-A (GABA A) receptors and interact with serotonergic and monoaminergic systems, which are implicated in the neurobiology of anxiety. Thus, we reinforce the Cantaloupe melon extracts' safety for pharmacological or nutritional preparations.

In the same way, we tested the social behavior of zebrafish, as the species is highly social, and any disturbance to the fish's welfare affects its social interaction (Canzian et al., 2017). Again, no changes in behavior emerged from the social test. Zebrafish, known for their sociable nature, display increased social attachment following exposure to anxiogenic substances, such as caffeine or alarm substance, while the fish shows reduced social proximity when exposed to anxiolytic

substances as diazepam (Cueto-Escobedo et al., 2022), The social behavior, typically considered a positive sign of welfare, was not altered by Cantaloupe melon extract, both crude and nanoencapsulated, indicating that the extract is free from risks to innate behaviors. While social interaction generally implies well-being, abnormal social behavior may suggest heightened health disturbances (Washburn et al., 2016).

The improvement in the optomotor response of larvae exposed to CE and EPG and the behavioral responses in adult fish presented in this study is an important finding to be highlighted. However, further research is needed to understand better the mechanisms that carotenoids may act in this modulation and, consequently, in cognitive and behavioral processes.

Finally, it is worth highlighting that this is the first study related to EPG exposure in a zebrafish model to investigate acute toxicity. So far, all the results obtained are very promising. Some limitations of the present study related to the genre of the model evaluated and the time of investigation. It is known that gender can influence the evaluation of the toxicity of new molecules. However, we chose to assess animals of both genders in different life cycles since it is impossible to distinguish them in the initial development phase. Therefore, in the second phase of the study, we chose to maintain adult males and females to ensure the standardization of the study in the different stages of the life cycle.

Furthermore, we recognize the importance of chronic toxicity assessment, which offers valuable information about the effects of the studied molecule on the whole organism and the specific impact on individual organs. However, in the present study, we chose to evaluate acute toxicity, which, according to OECD (Organization for Economic Cooperation and Development) guidelines, typically consists of an exposure time of 96 h. Both approaches are promising in advancing our understanding of the safety of carotenoid-containing nanoparticles.

Besides, several markers can be utilized to investigate the potentially harmful effects of a substance on the nervous system, such as neural death or the presence of macrophages, and morphometrics, observed by the number of cells or arborization. However, in a macroscopic approach, neurotoxicity can manifest as changes in behavior, which would further indicate the best molecular biomarker to be studied. Conducting a battery of behavioral tests in animal models can be informative, and it is usually used as a first approach. Therefore, new studies must be conducted to ensure a comprehensive assessment of potential risks and adverse effects.

5. Conclusion

Our study reveals intriguing insights into the toxicological effects of carotenoid-rich crude extracts from Cantaloupe melon and nanoencapsulated in larval and adult zebrafish analyses. The lack of harmful behavioral changes in optomotor response, anxiety-like behavior, locomotion, and sociability highlights the safety of these compounds for the nervous system. An important finding was the improvement in the optomotor response of the larvae for the groups exposed to CE and EPG and the potentiation of this effect in the encapsulated that acted the same way as the EB, even with a crude extract concentration 20 times lower. Our findings underscore the intricate interplay between compound exposure and behavioral responses in zebrafish, shedding light on the multifaceted nature of their behavior. More research is needed to unravel the underlying mechanisms driving these observed behaviors and their implications.

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CRediT authorship contribution statement

Tatiana dos Santos Pais: Methodology, Validation, Formal analysis, Investigation, Writing – original draft, Writing – review & editing. **Ana Carolina Luchiari:** Validation, Investigation, Writing – review & editing. **Augusto Monteiro de Souza:** Methodology, Validation, Formal analysis, Investigation, Writing – review & editing. **Isaiane Medeiros:** Methodology, Validation, Formal analysis, Investigation, Writing – review & editing. **Maria Gabriela Ferreira Rocha Silva:** Methodology, Validation, Formal analysis, Investigation, Writing – review & editing. **Yohanna Layssa dos Santos:** Methodology, Validation, Formal analysis, Investigation, Writing – review & editing. **Juliana Kelly Silva-Maia:** Validation, Investigation, Writing – review & editing. **Thaís Souza Passos:** Conceptualization, Validation, Investigation, Writing – review & editing, Supervision. **Ana Heloneida de Araújo Morais:** Conceptualization, Validation, Investigation, Writing – review & editing, Supervision.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

No data was used for the research described in the article.

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