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Article

Nanoparticles Loaded with a Carotenoid-Rich Extract from Cantaloupe Melon Improved Hepatic Retinol Levels in a Diet-Induced Obesity Preclinical Model

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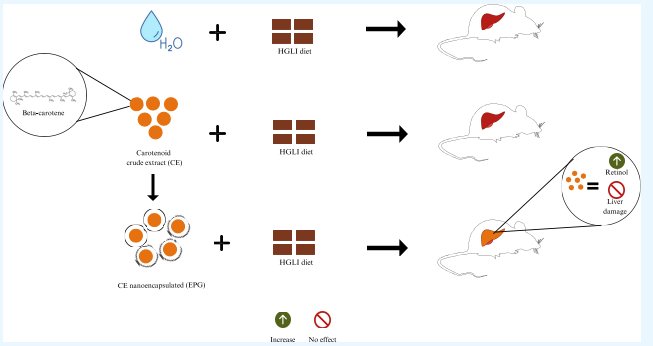
ABSTRACT: The study evaluated the effect of the carotenoid-rich extract from cantaloupe melon (CE) nanoencapsulated in porcine gelatin (EPG) on hepatic retinol concentration and liver damage scores in Wistar rats with obesity induced by high glycemic index and high glycemic load diet (HGLI diet). For 17 days, animals were fed the HGLI diet. They were divided into three groups and treated for 10 days [HGLI diet + water, HGLI diet + CE (12.5 mg/kg), and HGLI diet + EPG (50 mg/kg)]. The groups were evaluated for dietary intake, retinol, weight variation, hematological parameters, fasting glucose, lipid profile, hepatic retinol concentration, AST/ALT ratio, FIB-4 (Fibrosis-4 Index for Liver Fibrosis), and APRI (AST to Platelet Ratio Index) scores to evaluate the effects on the liver. Animals treated with EPG showed a lower dietary intake ($p < 0.05$). No significant weight change was detected in the evaluated groups ($p > 0.05$). The EPG-treated group had significantly higher concentrations ($p < 0.05$) of hepatic retinol [266 (45) μg/g] than the untreated group [186 (23.8) μg/g] and the one treated with CE [175 (8.08) μg/g]. Liver damage assessment scores did not show significant differences, but the lowest means were observed in the group treated with EPG. The nanoencapsulation of the extract rich in beta-carotene promoted reduced food consumption and increased hepatic retinol without causing significant changes in liver damage scores. Thus, EPG is a candidate for future clinical studies to evaluate the beneficial effects of treating diseases involving vitamin A deficiencies.



INTRODUCTION

Vitamin A is an essential nutrient; is soluble in fat; has functions in growth, vision, and metabolism; and can be obtained from animal source foods (liver, egg, yolk, and fish oils) or by consuming the precursors present in vegetable source foods, such as yellow and orange fruits and vegetables (papaya, carrots, melon, mango, and pumpkin). This micronutrient circulates in the body mainly as retinol linked to retinol-binding protein 4 (RBP4). It is delivered to the tissues and converted into retinoic acid (RA), a ligand of several nuclear receptors.[1]

Beta-carotene is an important dietary source of vitamin A for humans.[2] Cleavage of symmetric beta-carotene (containing two unsubstituted beta-ionone rings) produces two retinol molecules (vitamin A). In contrast, cleavage of nonsymmetric provitamin A carotenoids (alpha-carotene, beta-cryptoxanthin, and gamma-carotene, each containing only one beta-ionone ring) results in a retinol molecule and a non-vitamin A cleavage product. For alpha-carotene, this non-vitamin A cleavage product is alpha-retinal3. Retinal is subsequently reduced to retinol and esterified to retinyl esters before being packaged into chylomicrons, released into the bloodstream, and sequestered by the liver, the primary storage organ of this nutrient.[3,4]

In recent years, aspects related to vitamin A metabolism have been investigated regarding the development of metabolic and lifestyle diseases, including cardiovascular disease (CVD), type 2 diabetes mellitus (DM2), overweight, and obesity1. Obesity has been associated with low-grade systemic inflammation and micronutrient deficiencies. Studies show an increase in the prevalence of obesity in the world population, especially at younger ages, due to unhealthy eating habits and nutritional imbalances in diets. Among them is the excessive consumption

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of ultraprocessed foods with a high glycemic index and/or high glycemic load.[5,6]

Faienza et al.[7] described that the increase in obesity worldwide is also associated with liver abnormalities, which cover the clinical spectrum of nonalcoholic fatty liver disease (NAFLD). According to the literature, NAFLD is currently considered one of the leading causes of chronic liver diseases, with a global prevalence of up to 90% in patients with morbid obesity. Thus, it is recognized as the main component of liver disease in metabolic syndrome.[8,9]

Individuals with obesity have lower concentrations of vitamin A and a lower intake than those with average weight.[10] Adipose tissue plays an essential role in the metabolism and homeostasis of vitamin A, absorbing retinol bound to the circulatory chylomicron and converting retinol into physiologically active metabolites.[11] Vitamin A is involved in the signaling pathways regulating gene expression and has been postulated as one of the primary antioxidants and anti-inflammatories in the diet.[12] Furthermore, vitamin A precursors and carotenoid conversion products (CCPs) also act as ligands responsive to interaction with other transcription factors [retinoid X receptor (RXR) and PPAR] directly related to adipogenesis.[13]

In this context, Jeyakuma et al.[14] demonstrated that obese Wnin rats consuming a vitamin A enriched diet (129 mg vitamin A/kg diet) for 3 months showed improved nutritional status and obesity-associated complications. These effects occur by regulating genes that act on signaling pathways in the liver, retroperitoneal white adipose tissue, brown adipose tissue, skeletal muscle, and retina. However, they did not evaluate the possible damage to the liver of these animals. The literature still has evidence about the effect of vitamin A supplements on obesity status, liver cells, and the risk of hypervitaminosis. It is known that the liver is a target organ for alterations caused by unhealthy eating habits, common in individuals with obesity, and by the high concentrations of vitamin A stored in the case of supplementation.[7,15]

Several published studies demonstrate the excellent benefits of vitamin A, its precursors, and carotenoid conversion products (CCPs) in health. However, these bioactive compounds are susceptible to ultraviolet radiation, the presence of light and oxygen, and temperature variations, factors that can rapidly promote their degradation.[16] Thus, encapsulation has emerged as an alternative in bioactive compound preservation, such as carotenoids and vitamin A.[17]

Gomes et al.[18] highlighted in their review study that recent scientific findings have shown that vitamin A or its precursor's encapsulation brings essential benefits. Improving stability and preserving and maintaining functionality may improve digestion, bioavailability, and absorption, besides favoring these compounds' action in target tissues and/or organs, thus allowing them to act in the treatment of noncommunicable chronic diseases such as obesity and its comorbidities.

Researchers from the Nutrition and Bioactive Substances (NutriSBioativoS) research group at the Federal University of Rio Grande do Norte (UFRN) in Brazil have been investigating the crude extract rich in carotenoids from cantaloupe melon (rich in beta-carotene) nanoencapsulated in porcine gelatin (EPG). Per the results obtained by Medeiros et al.,[19] EPG improved water dispersibility and stability, expanding the potential technological use of melon carotenoids in food. Because of these excellent results obtained, evaluating the safety related to EPG administration was necessary. Thus, Medeiros et al.[20] initially investigated general signs of toxicity in an experimental model. The results demonstrated that EPG had low toxicity and bioactive potential, observed in histopathological analyses that revealed an improvement in the histological aspects of the animal's liver and intestine. Concerning bioactivity investigation, Oliveira et al.[21] demonstrated by an in vitro study carried out that nanoencapsulation potentiated and stabilized the antioxidant potential of carotenoids.

Therefore, this study continues the experiment conducted by Medeiros et al.,[20] evaluating the effect of EPG on dietary intake, retinol intake, and body weight and the influence on the concentration of hepatic vitamin A and liver damage assessment scores in Wistar rats (male adults) with obesity induced by a high glycemic index and high glycemic load diet (HGLI diet).

MATERIALS AND METHODS

The *Cucumis melo L.* var. *cantalupensis* fruits were purchased from the local market in Natal (RN) and registered in the National Genetic Heritage Management System and Associated Traditional Knowledge (SisGen) under A5A85DF. The materials used to obtain the nanoformulation (EPG) were porcine gelatin (Type A, Sigma-Aldrich), Tween surfactant (Sigma-Aldrich), and soy oil (Lizza) acquired from local sources. The β-carotene standard used to characterize the cantaloupe melon extract was purchased from Sigma-Aldrich.

Obtainment of the Carotenoid-Rich Crude Extract from Cantaloupe (CE) Melon and the Carotenoid-Rich Crude Extract Nanoencapsulated in Porcine Gelatin (EPG). The melon pulp processing to obtain the crude extract containing the carotenoids (CE) and the production of nanoparticles based on porcine gelatin loaded with a crude extract rich in carotenoids (EPG) were based on the previous report by Medeiros et al.[19]

First, the melon pulp was dried in a ventilated oven (55 °C/24 h) to obtain the melon pulp flour, which was used to promote CE extraction through the maceration technique in 95% ethanol (v/v) (1:4 w/v) until the color runs out. Subsequently, the ethanolic extract was partitioned using hexane PA (1:1 v/v) and sodium chloride (NaCl) 10% (w/v) and subsequent concentration in a rotary evaporator (Buchi R-215) followed by lyophilization to obtain the solvent-free dry extract (−57 °C/43 mmHg).[19]

The nanoparticles loaded with CE based on porcine gelatin (EPG), with a ratio of 1:80 (w/w), were synthesized through the O/W emulsification technique using Tween 20 as a surfactant (1.5% w/w) followed by dispersion of the aqueous phase containing the encapsulating agent in the emulsion obtained using an ultradisperser (Ultra-Turrax, IKAT18 basic) at 17,000 rpm for 10 min and subsequent lyophilization (−57 °C/43 mmHg) of the resulting emulsion to obtain the powdered material.[19]

Characterization of CE and EPG. The CE characterization analyses were performed according to Medeiros et al. (2019).[19] Thus, total carotenoids were determined by UV−vis absorption spectrophotometry (450 nm), and beta-carotene content was evaluated by ultra-efficiency liquid chromatography (UPLC).

For the characterization of EPG, scanning electron microscopy (SEM) analysis was performed to assess the morphology of the particles, laser diffraction to determine the diameter of nanoparticles, and Fourier transform infrared spectroscopy (FTIR) to evaluate the chemical interactions present in the system obtained according to Medeiros et al.[20] In addition, the determination of the incorporation efficiency (IE) of carote-

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noids in EPG was performed based on Medeiros et al.[20] and Hu et al.[22]

Preclinical Study. This study evaluated the effect of CE and EPG on the concentration of retinol in the liver of Wistar rats (male adults) with obesity induced by the high glycemic index and high glycemic load (HGLI) diet.[23] It should be noted that obesity and nutritionally inadequate diets are risk factors for hypovitaminosis A. For this reason, the diet-induced obesity model was chosen.

This investigation was performed to assess if EPG treatment potentiated the provitamin A effect of CE. Based on this, the evaluation of provitamin A activity in these animals was carried out at the Animal Facility of the Potiguar University (UnP) following the Guiding Principles on the Care and Use of Animals and approved by the Ethics Committee on the Use of Animals (CEUA-UnP) under protocol 019/2017.

Assessment of the Retinol Amount in the HGLI Diet. The HGLI diet offered to the animals throughout the experiment was produced based on Luz et al.[23] The retinol content present in the HGLI diet was determined considering the amount of retinol declared in the label of the Labina Presence diet and the amount present in other ingredients used to formulate the HGLI diet (Table 1) based on the data described in the Brazilian Table of Food Composition (TACO).[24]

Table 1. Ingredients Used in the Formulation of the High Glycemic Index and High Glycemic Load (HGLI) Diet (100 g) Offered to the Animals[a]

ingredients	g
condensed milk	45.20
Labina diet	45.20
sugar	9.60

[a]Luz et al.[23]

Experimental Draw. Male adult rats of the species *Rattus norvegicus* Wistar strain, with age 31 weeks and obesity induced by the HGLI diet, were randomly and individually distributed in polypropylene cages and kept in standard light conditions (12/12 h light/dark), humidity (50−55%), temperature (23−25 °C), water, and food ad libitum throughout the experiment, according to Luz et al.[23]

Three groups containing five animals were formed, which underwent an adaptation period of 5 days before starting the experiment to establish a pattern of food consumption and were submitted to the same procedures that would occur during the investigation, even gavage. Posteriorly, the adaptation was established followed by the 10 days of the experiment.

During the experiment, all animal groups remained fed with the HGLI diet. The animals were weighed at the beginning and the end of treatment. The dietary intake and retinol consumption were also monitored.

The animals were treated with 12.5 mg/kg (1 mL/day) of CE and 50 mg/kg (1 mL/day) of EPG (Table 2). Both were diluted in water and administered by gavage according to Medeiros et al.[20] The CE concentration was established based on the previous evaluation of the amount capable of inhibiting 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) and 2,2-diphenyl-1-picrylhydrazyl (DPPH) radicals by 50% (IC50). For the EPG, it was based on Medeiros et al.[20] and Pizón-Garcia et al.[25] It is essential to highlight that EPG presents a 1 CE/80 porcine gelatin (w/w) ratio, so this ratio and encapsulation efficiency were also considered in the established EPG dose.

Table 2. Distribution of Groups of Wistar Rats and Their Respective Diets during the 10 Days of the Experiment[a]

groups	*n*	treatment
HGLI diet + water	5	HGLI diet + 1 mL of water by gavage
HGLI diet + CE	5	HGLI diet + 1 mL of CE (12.5 mg/kg) by gavage
HGLI diet + EPG	5	HGLI diet + 1 mL of EPG (50 mg/kg) by gavage

[a]HGLI diet: mixture composed of Labina diet, condensed milk, and sugar (1:1:0.21); HGLI: high glycemic index and high glycemic load; CE: crude carotenoid-rich extract from cantaloupe melon; EPG: crude carotenoid-rich extract from cantaloupe melon nanoencapsulated in porcine gelatin.

Based on this, it is possible to note that the EPG dose presented 20 times lower crude extract rich in carotenoids than the free CE dose.

On the 11th day of the experiment, after 8 h of fasting, the rats were anesthetized with 250 mg of tiletamine hydrochloride/kg and 250 mg of zolazepam hydrochloride/kg. Then, the animal's blood was collected by cardiac puncture for biochemical analysis. Subsequently, they were euthanized and had their livers removed to measure the concentration of hepatic retinol. Specialized veterinarians performed this procedure.

Assessment of Dietary Intake, Retinol Intake, and Body Weight. To assess dietary intake, retinol intake, and body weight, on the first and last day of the experiment, the animals fasted for 8 h before treatments.

The variation in dietary intake was assessed using the following equation:

$$\Delta \text{ Dietary intake (g)} = \text{feed offered (g)} - \text{leftover feed (g)} \quad (1)$$

For the variation in retinol intake, the amount of retinol in the feed offered and the leftover feed was initially obtained, as described previously based on the data described in the Brazilian Table of Food Composition (TACO),[24] and evaluated using the following equation:

$$\Delta\text{Retinol intake (g)} = \text{retinol in the feed offered (g)} - \text{retinol in the leftover feed (g)} \quad (2)$$

On these same days, the animals were also weighed using a semianalytical digital scale (Filizola, MF-3, São Paulo, Brazil) to assess the variation in the change in total weight using the equation below:

$$\Delta \text{ Weight (g)} = \text{weight on the last day (g)} - \text{weight on the first day (g)} \quad (3)$$

In Vivo Hepatic Retinol Concentration. For the collection of livers, the bench was sanitized using 70% ethyl alcohol, and each animal was placed in dorsal decubitus on the bench. With scissors, a longitudinal cut was made from the base of the abdomen to the external segment, showing the entire abdominal and thoracic cavities. Thus, each animal's liver was removed and sectioned per hepatic lobe. Subsequently, the livers were packaged and protected from light, identified separately, immersed in liquid nitrogen, and stored in a freezer at −80 °C. The livers were immediately thawed for analysis on the day of retinol quantification. Based on previous analysis, the portion of the liver to be evaluated was randomly selected. It became

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evident that the vitamin A concentration is similar in all liver lobes of the animals.

The hepatic retinol was determined with modifications by high-performance liquid chromatography (HPLC) according to Rüegg and Dimenstein.[28] The entire process was carried out under light protection to avoid the degradation of the bioactive compound. An amount of 0.2 g of the liver was used to quantify retinol, ground, resuspended in 300 μL ethanol (1.5× the liver volume), and vortexed for 1 min. Potassium hydroxide (KOH, 100 μL) 50% (0.5× the liver volume) was added to the homogenate. Then, the tubes were shaken for 1 min and kept in a water bath at 45 °C for 30 min. Every 15 min, the tubes were removed from the water bath and homogenized for 15 s by vortexing. After saponification, retinol was extracted with 400 μL of hexane (HPLC purity, 2× the volume) (three times).

Extraction proceeded as follows: after adding hexane, the contents were homogenized by vortexing for 30 s and centrifuged at 4000 rpm for 5 min to separate the phases. The less-dense phase, containing hexane, was transferred to a new tube, and this procedure was repeated twice more. After removing the supernatant in the three extractions, the tubes containing the livers with hexane were submitted to nitrogen flow for hexane evaporation and to obtain the dry extract of each liver.

For the separation and identification of retinol, a chromatograph was used consisting of a set of two LC-20 AT pumps coupled to an SPD-20A UV−vis Shimadzu detector and a 5μ C18(2) 100A Phenomenex reverse phase column (150 × 4.6 mm) and a computer with the LC solution software for data processing. The mobile phase comprised 100% methanol in an isocratic system with 0.5 mL/min flow. The wavelength (λ) adopted was 325 nm.

The extract was diluted in 1000 μL of ethanol (HPLC purity), homogenized by vortexing for 30 s, and centrifuged at 4000 rpm for 10 min. Ten microliters was used for the analyses performed in triplicate.

A standard curve was constructed for retinol quantification using synthetic all-trans-retinol standard (retinol ≥99%, HPLC, Sigma Aldrich) diluted in ethanol. The concentration of the previous solution was calculated from the absorbance obtained at 325 nm in a spectrophotometer using the $A1\%_{(325\ nm)} = 746$.

For male eutrophic male Wistar rats fed a standard age-appropriate diet, the reference value of liver retinol concentration considered was based on Blomhoff et al.[29] with adaptations for the measurement units (Table 3).

Liver Damage Assessment Scores. Liver damage scores [AST/ALT ratio, FIB-4 (Fibrosis-4 Index for Liver Fibrosis), and APRI (AST to Platelet Ratio Index)] were evaluated for all animals in the three groups investigated. The AST/ALT ratio was calculated from the ratio between the concentrations of AST and ALT, showing signs of severe fibrosis and liver disease progression when the ratio is greater than 1. These are secondary data from a previous study published by Medeiros et al.[20] (Table S1).

FIB-4 was calculated according to Sterling et al.[30] based on age, concentrations of AST and ALT, and platelet count, according to the following equation:

$$\text{FIB} - 4 = [(\text{age (years)} \times \text{AST (U/L)}] / \text{platelet count (109/L)} \times \sqrt{(\text{ALT (U/L)}]} \quad (4)$$

Notably, the age of the animals evaluated in this study was 31 weeks and thus equivalent to a human age of approximately 20 years.[31] In nonalcoholic fatty liver disease (NAFLD), fibrosis in stages 0 to 1 has FIB-4 values <1.30, and that in stages 3 and 4 has FIB-4 values >2.67.

APRI was calculated as proposed by Wei et al.[32] using the upper limit of normality of AST (67 UI/L) and the maximum value of AST of the eutrophic Wistar rats acclimated in the experimental conditions carried out with the other groups (Table 2), as follows:

$$\text{APRI} = [(\text{AST value/upper limit of AST normality}) \times 100]/\text{platelet count (109/L)} \quad (5)$$

For an APRI value <0.5, the probability of F0 to F1 fibrosis is 72.7%, and for an APRI value >1.5, the probability of F3 to F4 fibrosis is 54.2%.

The values of the biochemical parameters evaluated in eutrophic adult male Wistar rats (320−380 g) acclimated under the same conditions of the experiment in question and consuming a standard Labina diet were considered as a reference standard and normality[26,27] in January 2019 in the vivarium of UnP/Brasil (Table S2).

Statistical Analysis. The sample size was calculated using simple and random sampling[33] per the 3R principle (replacement, reduction, and refinement). The coefficient of variation was adopted, with a probability of error of less than 5% and a power of 90%. The result was an n of 4.36 animals, resulting in five per group.

The Kolmogorov−Smirnov test was used to assess the normality of the data. For nonparametric variables (Δ body weight, Δ dietary intake, Δ dietary retinol intake, hematocrit, LDL-c, ALT, APRI, and FIB-4), the Kruskal−Wallis test and Dunn's post hoc test were used to verify the difference among the studied groups. For parametric data (hemoglobin, leukocytes, platelets, blood glucose, triglycerides, total cholesterol, HDL-c, VLDL-c, AST, hepatic retinol concentration, and AST/ALT ratio), ANOVA and Tukey post hoc test were used to determine the significant differences. In both cases, values of $p \leq 0.05$ were considered statistically significant.

The GraphPad Prism software, version 5.0 (GraphPad Software, San Diego, CA), was used for the statistical analysis of the data obtained. All data represented at least three independent experiments and were expressed as mean (standard deviation) units except when explicitly stated otherwise.

Table 3. Reference Values of Total Retinol Concentration in the Liver of Eutrophic Male Wistar Rats According to Age and Dietary Retinol Intake

group	age (months)	diet retinol	μg of retinol/g liver, mean (SD)
1	3	adequate	154.94 (5.73)
2	12	adequate	504.45 (68.46)
3	3	excess[a]	1922.14 (31.22)

[a]Rats received a standard diet until 6 weeks of age and then a diet supplemented with 2.4 mg retinol/kg body weight per day for 6 weeks. Adapted from Blomhoff et al.[29]

RESULTS

Obtainment and Characterization of the Carotenoid-Rich Crude Extract from Cantaloupe (CE) Melon and the Crude Carotenoid-Rich Extract Nanoencapsulated in Porcine Gelatin (EPG). In the present study, UV−vis

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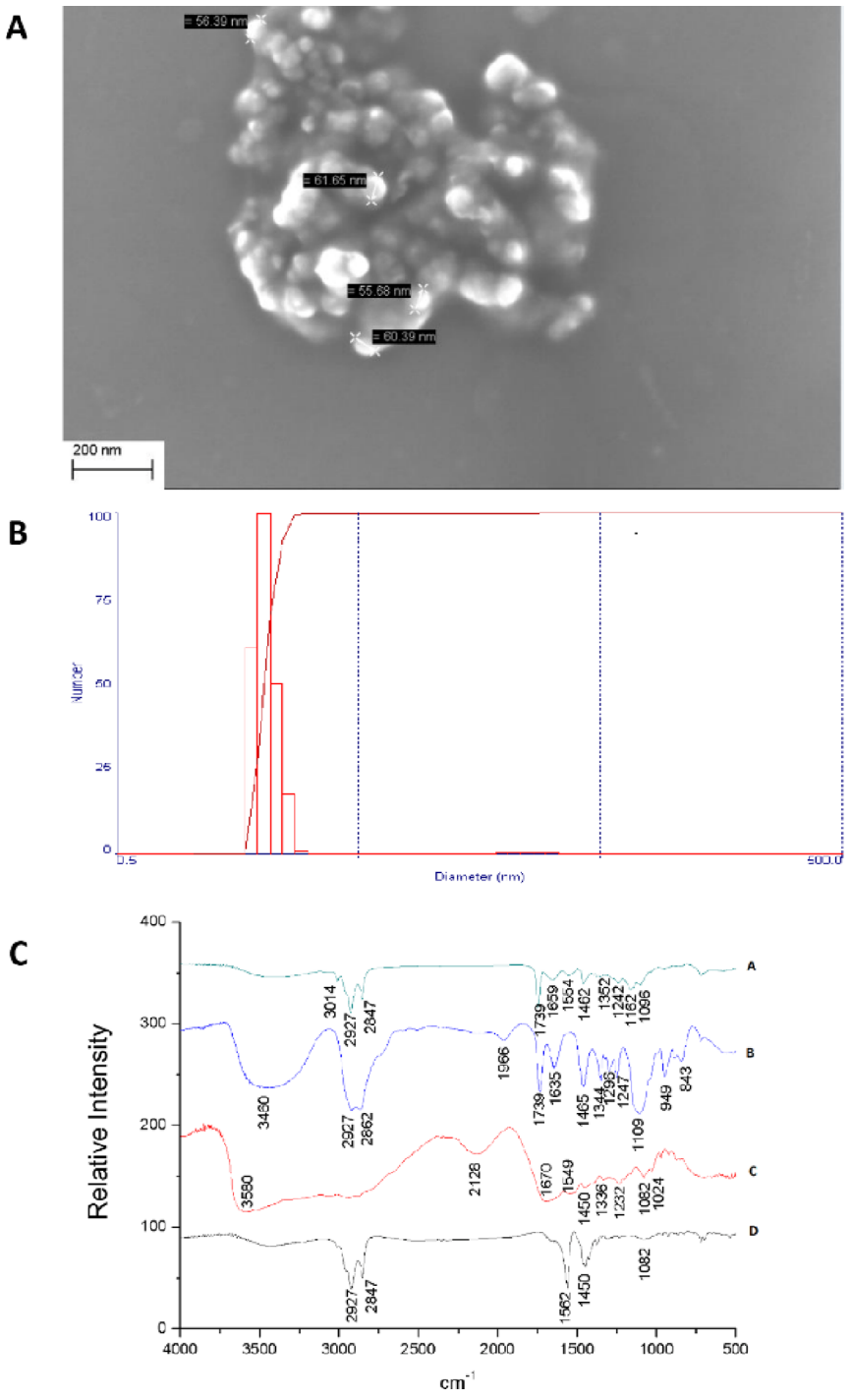

Figure 1. Characterization of gelatin-based nanoparticles containing carotenoid-rich extract from cantaloupe melon obtained by the O/W emulsification technique. (a) Scanning electron microscopy (SEM). (b) Laser diffraction. (c) Fourier transform infrared spectroscopy (FTIR): In panel c: (A) EPG: crude carotenoid-rich extract from cantaloupe melon nanoencapsulated in porcine gelatin; (B) Tween 20; (C) PG: porcine gelatin; and (D) CE: crude carotenoid-rich extract from cantaloupe melon. O/W: oil in water.

absorption spectrophotometry was performed to evaluate the total carotenoids in CE, obtaining a content equal to 51.1 (5.00) μg/g of melon pulp. In addition, ultra-efficiency liquid chromatography detected the presence of 30.1 (2.0) μg of beta-carotene/g of fresh cantaloupe melon pulp (Figure S1).

For the EPG formulation, by SEM, it was possible to observe the presence of spherical particles with a smooth surface, without cracks or depressions, and with homogeneous physical sizes on the nanometric scale (Figure 1a). Laser diffraction revealed that the nanoparticles had mean dimensions of 80 (8.59) nm and a polydispersion index of 0.47 (0.09). Thus, EPG presented the characteristics desired (Figure 1b).

By FTIR, the spectra of EPG, Tween 20, porcine gelatin, and CE were obtained, wherein it was possible to observe the formation of new vibrational bands in the EPG, which was not observed in the other materials (Figure 1c), such as in 3014, 1659, and 1554 cm^{-1} (C−H, C═N, and C═C, respectively). These results may indicate new chemical interactions between

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the constituents of the obtained system. The 2927 and 2847 cm^{-1} bands (CH binding) were attenuated and visualized in the CE with more intensity. Furthermore, the bands at 1450 and 1082 cm^{-1} (C−C and C−O, respectively) were not observed in CE, indicating that it was protected by porcine gelatin.

Finally, the incorporation efficiency obtained for EPG was equal to 95.20% (0.74).

Amount of Retinol in the HGLI Diet. According to the necessary amounts of each ingredient for producing 100 g of the HGLI diet (Table 1), the respective concentrations of retinol present in each component were estimated, obtaining 369.74 μg of retinol/100 g of HGLI diet (Table 4). Meanwhile, in 100 g of the Labina diet, 764.93 μg of retinol was estimated.

Table 4. Amount of Retinol Present in 100 g in the High Glycemic Index and High Glycemic Load (HGLI) Diet

ingredients	amount (g)	μg of retinol/100 g of HGLI diet
condensed milk	45.20 g	23.96[a]
Labina diet	45.20 g	345.78[b]
sugar	9.60 g	0.00
total	100 g	369.74

[a]Amount of retinol according to the Brazilian Table of Food Composition (TACO).[24] [b]Quantity of retinol according to the nutritional composition of Labina Presence diet for rats and mice.

Assessment of Dietary Intake, Retinol, and Body Weight. The evaluation of the variation in dietary intake during 10 days of the experiment indicated that there was a significantly lower intake ($p = 0.04$) in the EPG group (HGLI diet + EPG) compared to the untreated group (HGLI diet + water) (Figure 2A).

Consequently, regarding the variation in retinol consumption (Figure 2B), the data showed a statistically significant reduction ($p = 0.04$) among animals that received the nanoformulation compared to those without treatment.

The findings observed in the variation in dietary intake complement and reflect the data on variation in body weight (Figure 2C) despite not establishing a significant difference between the groups of animals analyzed ($p > 0.05$). It was possible to notice a greater loss in the average weight of the animals in the groups that received crude carotenoid-rich extract-free and nanoencapsulated (CE and EPG) after 10 days of treatment.

Hepatic Retinol Concentration in Obese Wistar Rats. The presence of retinol in the livers of the evaluated groups was confirmed by comparing the peaks observed in the chromatograms of each experimental group with the peak obtained for the standard retinol chromatogram (Sigma) (Figure S2). The line equation obtained ($y = 8419.1x - 18{,}702/R^2 = 0.9982$) was used according to the retinol concentration and the peak area obtained in the chromatogram for each retinol standard (Sigma) curve point.

The superficial coloration of the livers of Wistar rats with obesity submitted to different types of treatment for 10 days was demonstrated, in addition to the concentration of hepatic retinol (Figure 3). Initially, when assessing the image for the livers, after immersion in formalin, it was possible to notice a difference between the groups evaluated, suggesting that there could be a higher concentration of vitamin A in the organs of those animals treated with EPG (Figure 3a−c).

This hypothesis was confirmed by the results of the concentrations of retinol present in the rats' livers. The data showed that animals without treatment (HGLI + water) and

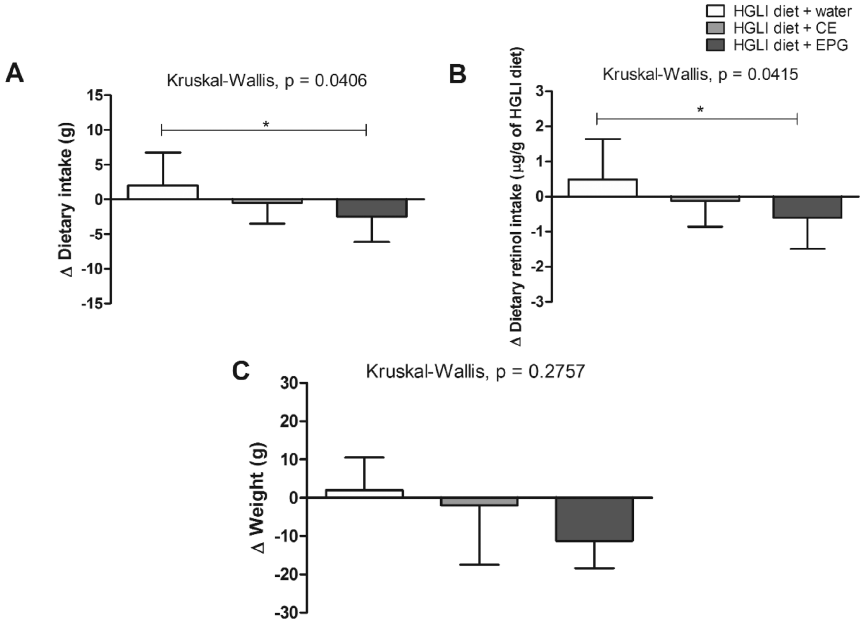

Figure 2. Variation in dietary intake, retinol, and body weight in obese rats. Adult Wistar rats (age 31 weeks) on the first and last day of the experiment were submitted to different treatments after 10 days of the investigation. (A) Variation of dietary intake. (B) Variation of dietary retinol intake. (C) Weight variation. HGLI diet + water: HGLI diet + 1 mL of water per gavage; HGLI diet + CE: HGLI diet + 1 mL of a crude extract rich in carotenoid at a concentration of 12.5 mg/kg per gavage; HGLI diet + EPG: HGLI diet + 1 mL of carotenoid-rich crude extract of cantaloupe melon nanoencapsulated in porcine gelatin at a concentration of 50 mg/kg by gavage; HGLI diet: mixture composed of Labina diet, condensed milk, and sugar (1:1:0.21 w/w/w); HGLI: high glycemic index and high glycemic load; CE: crude carotenoid-rich extract from cantaloupe melon; EPG: crude carotenoid-rich extract from cantaloupe melon nanoencapsulated in porcine gelatin. The significant difference between the groups was evaluated using the Kruskal−Wallis ($p < 0.05$) and subsequently using Dunn's post hoc test. *$p < 0.05$: statistical difference found compared to the EPG group.

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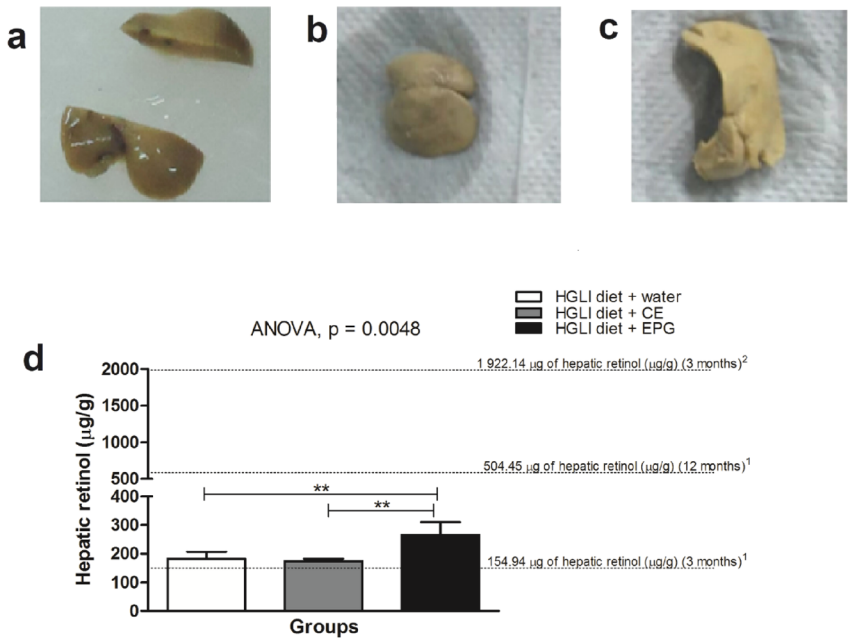

Figure 3. Surface color of livers and hepatic retinol concentration in obese Wistar rats. Adult Wistar rats (age 31 weeks) were submitted to different treatments after 10 days of the experiment. (A) Surface color of liver submitted to HGLI diet + water treatment (HGLI diet + 1 mL of water per gavage). (B) Surface color of liver submitted to the HGLI diet + CE treatment (HGLI diet + 1 mL of a crude extract rich in carotenoids at a concentration of 12.5 mg/kg per gavage). (C) Surface color of liver submitted to the treatment HGLI diet + EPG (HGLI diet + 1 mL of a crude extract rich in carotenoids nanoencapsulated in porcine gelatin at a concentration of 50 mg/kg per gavage). (D) Hepatic retinol concentration in rats submitted to different treatments. HGLI diet: mixture composed of Labina diet, condensed milk, and sugar (1:1:0.21 w/w/w). [1]Age-appropriate retinol concentration based on Blomhoff et al.[29] [2]Retinol excess for age based on Blomhoff et al.[29] Images of the livers were recorded after immersion in formalin (4%) to evaluate the superficial color. HGLI diet: mixture composed of Labina diet, condensed milk, and sugar (1:1:0.21 w/w/w); HGLI: high glycemic index and high glycemic load; CE: crude carotenoid-rich extract from cantaloupe melon; EPG: crude carotenoid-rich extract from cantaloupe melon nanoencapsulated in porcine gelatin. The significant difference between groups was assessed using ANOVA ($p < 0.05$) and Tukey's post-test. **$p < 0.01$: statistical difference found compared to the EPG group.

treated with CE (HGLI diet + CE) had lower concentrations of retinol compared to those treated with EPG (HGLI diet + EPG) (Figure 3d). These results evidenced a higher concentration of hepatic retinol ($p < 0.05$) compared to the other groups.

Liver Damage Assessment Scores. Noninvasive scores such as the AST/ALT ratio, FIB-4, and APRI were used to assess liver damage. The data obtained from the calculations performed for each animal's score reveal no significant differences concerning the parameters evaluated ($p > 0.05$). However, there is a lower mean of the FIB-4 and APRI scores in animals treated with CE and EPG than in the untreated group (Figure 4).

DISCUSSION

It was observed that obese individuals have a lower intake of vitamin A and lower concentrations of this vitamin in tissues. Thus, obesity is linked to a decreased bioavailability of this bioactive compound. On the other hand, the opposite is observed in healthy individuals with access to nutritionally adequate food.[34−36] Furthermore, Böhm et al.[15] described that the increase in fat mass also seemed to be related to a lower concentration of carotenoids in adipose tissue, correlated with serum concentrations and other concentrations in other tissues, such as the liver. Therefore, this supports using animals with obesity induced by a nutritionally inadequate diet in the present study.

Carotenoids from the most diverse sources are essential precursors of vitamin A (β-carotene, α-carotene, and β-cryptoxanthin),[13] which are determined by absorption (bioavailability) and subsequent conversion to retinol (bioconversion).[37] Medeiros et al.,[15] Medeiros et al.,[20] and Oliveira et al.[21] demonstrated in their studies that the extract of the cantaloupe melon pulp is an abundant source of beta-carotene. The present study verified the content of total carotenoids of 51.1 μg/g of fresh cantaloupe melon pulp. The presence of 30.1 μg of beta-carotene/g of melon pulp was observed. The evaluated contents may vary depending on the degree of maturation, soil type, growing conditions, climatic conditions, plant variety, and exposure to sunlight.[38] Therefore, the results were observed to approximate the levels found in previous studies,[19,21] reinforcing that CE from cantaloupe melon pulp is an essential plant source of carotenoids, emphasizing beta-carotene as a precursor of vitamin A.[39]

However, these compounds have low water solubility, stability, and bioavailability, significantly reducing their effectiveness as disease-prevention agents.[40,41] An effective way to improve these barriers has been to use nanotechnology to promote the encapsulation of these compounds, obtaining engineered nanoparticles based on delivery systems.[42]

Nanoparticles containing the crude extract rich in carotenoids from the cantaloupe melon pulp in porcine gelatin (EPG) have already been produced by Medeiros et al.[20] and Oliveira et al.[21] In this study, EPG showed the same physicochemical characteristics and incorporation efficiency of carotenoids demonstrated by Medeiros et al.,[19] Medeiros et al.,[20] and Oliveira et al.[21] even with the obtainment of new batches, which effectively contributed to the investigations in the present study. These findings showed that the replicated method used in this study

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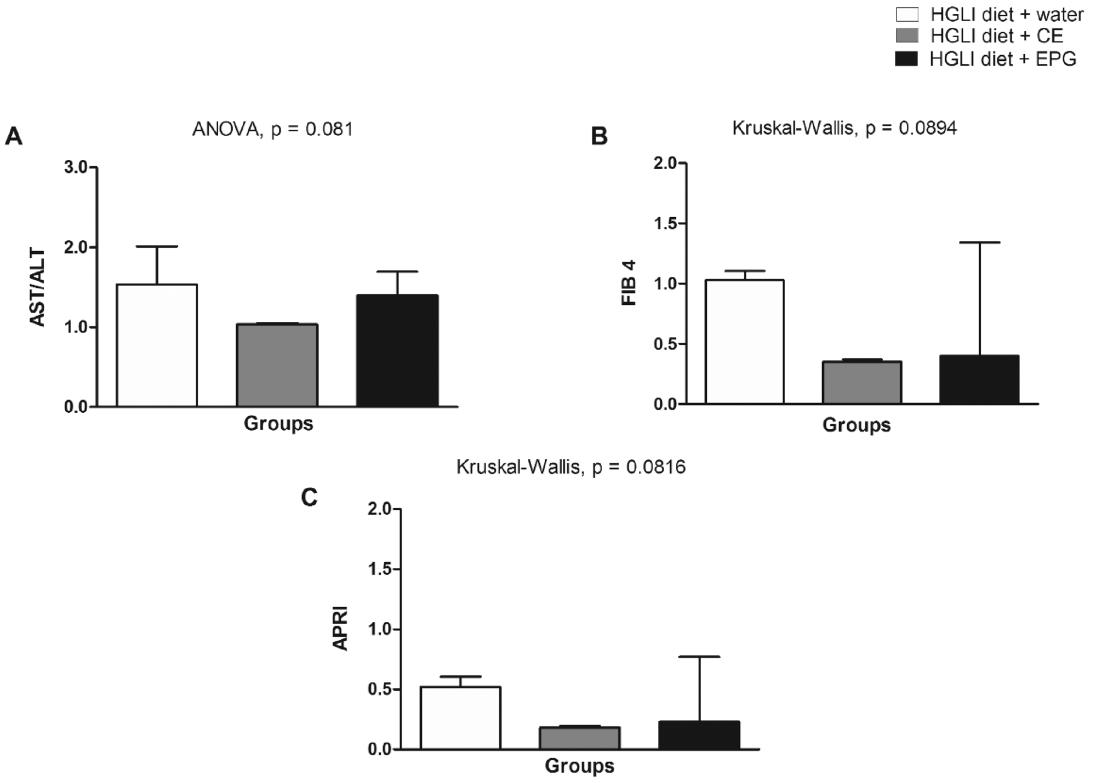

Figure 4. Assessment scores of liver damage in obese Wistar rats. Adult Wistar rats (age 31 weeks) were submitted to different treatments after 10 days of the experiment. (A) AST/ALT ratio. (B) FIB-4. (C) APRI. Experimental groups: HGLI diet + water, HGLI diet + CE, and HGLI diet + EPG. Values are expressed as mean ± standard deviation. The Kolmogorov−Smirnov test was applied to assess the normality of the data. FIB-4 and APRI showed nonparametric distribution, so the Kruskal−Wallis test and Dunn's post hoc test were used to detect significant differences between the evaluated groups. The AST/ALT ratio showed parametric distribution. ANOVA and Tukey's post hoc tests were used to determine significant differences. Values of $p \leq 0.05$ are considered statistically significant. AST: aspartate transaminase; ALT: alanine transaminase; FIB-4: Fibrosis-4 Index for Liver Fibrosis; APRI: AST to Platelet Ratio Index; HGLI diet: mixture composed of Labina diet, condensed milk, and sugar (1:1:0.21 w/w/w); HGLI: high glycemic index and high glycemic load; CE: crude carotenoid-rich extract from cantaloupe melon; EPG: crude carotenoid-rich extract from cantaloupe melon nanoencapsulated in porcine gelatin.

achieved the desired characteristics, overcoming the difficulties related to reproducibility in obtaining nanoparticles.

Because of the low toxicity and bioactive potential of EPG promoted in the liver,[20] the present study investigated the effect of EPG on dietary intake, retinol, and weight in Wistar rats with diet-induced obesity. Besides, retinol concentrations in the liver and liver function were analyzed by determining liver scores to evaluate the changes caused by the ingestion of these nanoparticles containing CE under the liver of animals with obesity. One of the study's limitations was the absence of groups with healthy animals (without obesity) feeding a nutritionally adequate diet, although these groups are not targets of vitamin A supplementation.

Besides, the present study only evaluated the nanoparticles containing CE (EPG) due to the evidence found in previous studies by our research group. Oliveira et al.[21] investigated the antioxidant activity of nanoparticles without carotenoids (control) and observed that the material did not show antioxidant activity. On the other hand, EPG also did not show antioxidant activity, a result attributed to the difficulty of interaction between free radicals in the reaction medium and carotenoids trapped inside the nanoparticles. Thus, to promote such action, it was necessary to extract CE from the interior of EPG, and the potentiation of antioxidant activity was verified, which, based on the literature, was attributed to the chemical interaction between CE and the amino acids in porcine gelatin. Therefore, this study proved that the encapsulating agent was not responsible for the bioactive effect but for the CE nanoencapsulation with porcine gelatin.

Regarding the variation in dietary and retinol consumption, the data showed a significantly lower consumption of the diet and, consequently, retinol among animals in the EPG-treated group compared to the untreated group ($p < 0.05$). This result showed that the nanoencapsulation of the carotenoid-rich extract from cantaloupe melon pulp promoted lower dietary intake. Meanwhile, in the animals treated with EPG, despite having a lower mean body weight than other animals in the other groups, this difference was not significant, which may be a reflection and consequence of the lower dietary intake. Thus, it is believed that a significant difference could be achieved in a more extended period of EPG treatment. These findings are relevant considering the severity of obesity and the need for further studies and possible therapies that can benefit this condition.

Through a meta-analysis, Yao et al.[10] evaluated the effects of carotenoids in overweight or obese individuals using available evidence. They reinforced that carotenoid supplementation was significantly associated with reductions in body weight, decreased body mass index (BMI), waist circumference losses, and reduced total cholesterol. However, they highlighted that the influence of carotenoids on people with excess body weight

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is not yet evident and that additional data from clinical trials are needed to elucidate these questions.

Retinol liver reserve is considered the standard gold method for assessing vitamin A status in the body.[43] Based on the studies' experimental model, it was impossible to investigate the gene or enzyme involved in retinol metabolism, serum retinol, and beta-carotene concentrations before and after treatment with EPG. This study limitation was overcome by assessing hepatic retinol, the gold standard for assessing vitamin A status.

The results regarding the concentration of retinol in the liver showed that animals treated with EPG had higher liver concentrations (45%) than those without treatment. Besides, animals treated with EPG had significantly higher concentrations of hepatic retinol at 52% compared to animals treated with CE. Thus, it is possible to infer that nanoencapsulation protected the bioactive compound against digestive processes and favored the largest reserves of vitamin A in the liver even though animals treated with EPG consumed smaller amounts considering the variation in dietary consumption of retinol.

Kunzler et al.[12] supplemented Wistar rats with retinol for 28 days (3000 IU/kg/day). They reported that the animals exhibited a higher retinol content in the liver reserves. The present study used a concentration of 12 mg of CE/kg and 50 mg of EPG/kg. According to Medeiros et al.,[20] it is essential to remember that the amount of CE present in EPG is 20 times lower than CE according to the evaluated method, with 50 mg of EPG having the presence of 0.620 mg of CE. The concentrations of hepatic retinol in this study confirm what researchers claim about using nanoformulations with beneficial effects on the protection, stability, solubility, and transport of carotenoids in the body. These benefits can increase the bioaccessibility, bioavailability, and bioconversion of carotenoids in retinol.[44,45]

Nevertheless, in the present study, it has not been possible to assess in more detail how nanoencapsulation acted to promote an improvement in the concentration of retinol in the livers of animals treated with EPG. Still, it is believed that the increase in the solubility and stability of carotenoids demonstrated in previous studies[19,21] may have provided increased absorption in the intestine and, consequently, greater bioconversion to retinol in the liver.

It is known that vitamin A precursors need to be incorporated into mixed micelles to be absorbed mainly by the enterocyte through uptake by membrane transport proteins necrophage receptor class B type 1 (SR-B1), cluster of differentiation 36 (CD36), and Niemann-Pick C1-like 1 transporter 1 (NPC1L1). Once inside the cell, they are converted into retinal and later reduced to retinol (ROL), converted to retinyl esters, and packaged in chylomicrons to be transported through the lymphatic system and deposited in the liver.[2]

Retinyl esters are reconverted to ROL in the liver, which binds to RBP4 to form holo-RBP4. Once the release of holo-RBP4 from the liver to the circulation is necessary, this molecule binds to transthyretin (TTR), forming a complex that promotes stabilization of this molecule (RBP-TTR) being transported from the hepatocyte mediated by retinoic acid stimulated membrane protein 6 (STRA6). TTR dissociates from the complex in the bloodstream for uptake of holo-RBP4 by the target cell, and RPB4 binds to STRA6 present in the cell membrane, promoting ROL absorption.[11]

Furthermore, in the context of obesity, it is observed that the holo- and apo-RBP4 ratio is affected by vitamin A deficiency. Thus, holo-RBP4 acts by suppressing the adipogenesis of precursor cells. Moreover, SRAT6 expression in preadipocytes can mediate holo-RBP4 ROL influx and consequently block adipocyte differentiation by activating retinoic acid receptors (RAR). Also, vitamin A precursors and CCPs act by suppressing the expression of transcription factors (RXR and PPAR) involved in regulating adipose tissue metabolism, adipogenesis, and obesity.[11,13]

Vulić et al.[46] encapsulated red pepper residues rich in carotenoids. They demonstrated that it promoted a protective effect against changes in pH and enzymatic activities throughout digestion, contributing to the increased bioaccessibility of these natural pigments in the intestine. According to Yi et al.,[47] the bioaccessibility of beta-carotene was inversely related to particle diameter, reinforcing the idea that nanoencapsulation can increase the bioaccessibility and bioavailability of lipophilic encapsulated bioactive compounds.

Jeyakumar et al.[14] fed obese rats with a diet enriched with vitamin A (129 mg/kg of diet) for 3 months, concluding that the early intervention reduced visceral adiposity. In the present study, it was impossible to carry out this association. However, considering the concentration and study period, the EPG is an interesting nanoformulation for future studies investigating these effects.

Based on the present study, it is noteworthy that even after carotenoid supplementation through EPG, the highest concentration of hepatic retinol observed among the animals in the studied groups significantly did not reach the reference values that indicate excess retinol in the liver (Table 3). Furthermore, the data on hepatic retinol for animals treated with EPG remained adequate and within the reference values established according to Blomhoff et al.[29]

However, considering the age of the animals (31 weeks) and the adequacy concentrations of hepatic retinol for age,[29] it is believed that the animals in this study presented vitamin A deficiency. Therefore, when offering a diet with less retinol content (HGLI diet) than the nutritionally adequate diet (Labina), all the animals at the beginning of the treatment did not present recommended hepatic retinol concentrations for their age.[29] However, when consuming that same HGLI diet, the animals treated with EPG had higher retinol concentrations in the liver. It may be that, at the age of the animals during the experiment, the treatment did not lead to adequacy. Still, it was evident that it promoted an enhancement of functionality, which could probably be related to a greater bioavailability of carotenoids and bioconversion to hepatic retinol. Therefore, further studies are needed to determine the increased uptake of carotenoids present in EPG.

Still, given the increase in hepatic retinol, it was necessary to assess whether this could cause any damage to the liver, considering a possible hypervitaminosis A. It is important to remember that the HGLI diet used to induce obesity in this study affected some biochemical parameters and liver enzymes related to the liver's health. However, Medeiros et al.[20] had already revealed that animals treated with EPG did not show any of the classic signs of hypervitaminosis A, such as enlarged spleen and liver, high serum concentrations of liver enzymes, lymphocytic infiltrate, and steatosis.

This study also evaluated liver damage through the non-invasive scores proposed by the AST/ALT, FIB-4, and APRI ratio. Although the data did not show a significant difference between the groups evaluated, the smallest mean of the FIB-4 and APRI scores was observed in the animals treated with EPG and CE in the period analyzed. It is essential to mention that no studies were found on the evaluation of scores analyzed under

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the conditions in this study, nor were there similar studies that addressed a similar theme, mainly nanoparticles containing carotenoids and their possible effects on liver damage parameters.

The data obtained in the present study showed the potential effect EPG promoted on the extract rich in carotenoids from cantaloupe melon due to nanoencapsulation, making it possible to use a smaller amount of CE to promote biological effects in retinol hepatic. The findings revealed promising evidence in the use of nanoparticles containing carotenoids, mainly because these bioactive compounds reached the liver more easily, as observed by the color and concentration of hepatic retinol, adding to the effect on the variation in dietary intake that was smaller after treatment with EPG.

It should be noted that this is a preliminary and innovative study, which introduces a new line of investigations in the NutriSBioativoS research group. Based on this, the treatment time and dosage of a crude extract rich in carotenoids used in this study should be considered for future investigations. In addition, other experiments can be planned (for example, immunohistochemistry, Western blot, and real-time PCR) to evaluate the effects of CE and EPG over a more extended treatment period. Finally, there is a possibility that using higher concentrations of the bioactive compound can generate even more promising results.

Gomes et al.[18] highlighted in their review study that recent clinical research demonstrates that vitamin A and its precursors affect adipogenesis, lipid catabolism, biosynthesis, and release of bioactive factors, thus interfering with obesity. Pereira et al.[48] evaluated the concentration of vitamin A through retinol reserves in the liver of individuals with obesity. They observed a reduction in the concentration of retinol in these patients. Besides, when associated with other clinical conditions affected by obesity, for example, xerophthalmia, the reserves had a higher percentage of inadequacy. Botella-Carretero et al.[49] mentioned a negative association between retinol concentration and BMI in morbidly obese patients.

Overall, EPG proved to be a candidate nanoparticle for clinical studies. It could become a possible adjuvant with a potential effect, together with dietary therapy, in treating diseases involving vitamin A deficiencies and obesity, and related comorbidities.

CONCLUSIONS

The animals treated with EPG had lower dietary intake and retinol consumption and significantly higher retinol concentrations in the liver than animals in the other groups without causing changes in liver parameters. Thus, given the above and the increasing prevalence of obesity, EPG proved to be a nanoformulation with great potential because the nanoencapsulation of CE promoted protection and ensured the absorption of the bioactive compound, observed in the data obtained in the experimental model.

INSTITUTIONAL REVIEW BOARD STATEMENT

This investigation was carried out at the Animal Facility of the Potiguar University (UnP) following the Guiding Principles on the Care and Use of Animals, approved by the Ethics Committee on the Use of Animals (CEUA-UnP), under protocol 019/2017.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.3c02750.

Chromatograms obtained by ultra-efficiency liquid chromatography (Figure S1); chromatograms obtained by high-performance liquid chromatography (HPLC) (Figure S2); evaluation values of platelet and liver enzymes in Wistar rats (Table S1); and reference values for platelet and liver enzymes in adult, eutrophic male Wistar rats (Table S2) (PDF)

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Camila de Carvalho Gomes: data curation; formal analysis; methodology; investigation; validation; writing-original draft and writing-review editing. Mayara Santa Rosa Lima: methodology and investigation. Grazielle Louise Ribeiro de Oliveira: methodology and investigation. Isaiane Medeiros: methodology and investigation. Hanna Stephany Trajano Xavier: methodology and investigation. Tatiana dos Santos Pais: methodology and investigation. Izael Sousa Costa: methodology and investigation. Fabiana Maria Coimbra de Carvalho: formal analysis; supervision and validation. Alexandre Coelho Serquiz: funding acquisition and supervision. Maíra Conceição Jerônimo de Souza Lima: formal analysis; supervision and validation. Ana Heloneida de Araújo Morais: conceptualization; funding acquisition; data curation; formal analysis; methodology; investigation; validation; writing-original draft; writing-review editing; and supervision. Thais Sousa Passos: conceptualization; funding acquisition; data curation; formal analysis; methodology; investigation; validation; writing-original draft; writing-review editing; and supervision.

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Notes

The authors declare no competing financial interest.

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