Journal of Food Quality

Natural Products and Nanomaterials for the Improvement of Aquatic Products

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Research Article

Effects of Dietary Inclusion of Canthaxanthin- and α-Tocopherol-Loaded Liposomes on Growth and Muscle Pigmentation of Rainbow Trout (*Oncorhynchus mykiss*)

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Dietary inclusion of canthaxanthin, a common carotenoid pigment, has been long practiced in aquaculture to give the favorable flesh color in farmed salmonids. However, carotenoids are associated with limited solubility and poor physicochemical stability, and their dose in fish feed is widely regulated. In this study, we included canthaxanthin- and α-tocopherol-loaded liposomes into fish diets and evaluated the effects of supplemented fish feed on fish growth, color, nutrition, and canthaxanthin deposition in fillets of cultured rainbow trout (*Oncorhynchus mykiss*). The liposomes were fabricated using lecithin as phospholipids with the initial concentrations ($IC = m_{canthaxanthin}/m_{lipids}$, % wt/wt) of canthaxanthin at 0.1%, 0.5%, and 1.0%. Particle size characterization showed that liposome mean sizes were 109.70 ± 6.36, 105.10 ± 8.41, and 109.20 ± 5.66 nm (mean ± SD; $n = 3$), respectively, corresponding with liposomes synthesized at canthaxanthin IC = 0.1%, IC = 0.5%, and IC = 1%. The polydispersity index (PDI) of all samples remained lower than 0.2. There were no significant differences in the mean size and PDI between blank lecithin liposome and canthaxanthin- and α-tocopherol-loaded liposomes. The encapsulation efficiency of canthaxanthin- and α-tocopherol-loaded liposomes decreased when increasing the concentration of canthaxanthin in lecithin liposomes, with EE% values of IC = 0.1%, IC = 0.5%, and IC = 1% being 85.3 ± 2.1, 72.9 ± 1.8, and 55.3 ± 2.6, respectively. For fish growth, at the end of the experiment, final weight was significantly higher in fish fed with diet supplemented with 1 g/kg canthaxanthin- and α-tocopherol-loaded liposomes (IC = 0.5%) in comparison to other experimental control groups. The difference in color of the salmon muscle was most apparent after two months of feeding. However, after three months, there was no noticeable change in the color score of the fish muscle, indicating saturation of color of the fish muscle. The above results suggest the potential of canthaxanthin- and *a*-tocopherol-loaded liposomes as the red pigment in fish aquaculture.

1. Introduction

In the market of farmed aquatics, reddish color is often perceived as indication of high quality fish flesh and is associated with consumer acceptance [1]. However, pigments responsible for the red coloration of fish flesh, comprising mostly carotenoids such as canthaxanthin and astaxanthin, can only be naturally synthesized in bacteria, algae, yeasts, molds, and some higher plants with *de novo* synthesis capacity [2, 3]. Therefore, supplementation of carotenoids in feeds has been commonly exercised in commercial farming of salmonids, crustaceans, and other aquatic organisms to give desirable coloration to the resulting products [4–6]. Carotenoids are a pigment class that is responsible for a wide range of colors in plant and animal species and figure prominently among other pigments as an important antioxidant primarily derivable from marine species [7]. It has been shown that dietary supplementation of canthaxanthin, a common carotenoid, in aquaculture salmon and trout could color the fillet with red-pink color [8, 9] and that pigmentation intensity of rainbow trout flesh was positively correlated with added canthaxanthin in fish feed [10].

Carotenoid feeding in aquaculture is associated with several issues. First, production of carotenoids largely depends on chemical processes and thus presents certain disadvantages including strict requirements on process control and generation of undesirable by-products that might be allergic to some parts of consumers [11]. In addition, high-dose consumption of canthaxanthin has been reported to result in health issues, most notably the development of retinal crystals in human eye [12] due to canthaxanthin-induced damage in lipid membranes of macula blood vessels [13]. As a result, canthaxanthin addition in commercial fish feed is widely regulated, and the allowable dose per kilogram of salmonid feed is 25 mg according to the regulation of European Union [14]. The second obstacle is that carotenoids such as astaxanthin and canthaxanthin, due to their unsaturated chemical structures, have low aqueous solubility and are sensitive to physicochemical conditions such as heat, oxygen, and light, resulting in low bioavailability of the substances when being used as nutraceutical ingredients [15, 16]. Indeed, Torrissen et al. [17] showed strong, inverse relationship between canthaxanthin dose in fish feed and apparent digestibility in rainbow trout, while increased dietary lipid levels increased its apparent digestibility [17]. Therefore, further measures in aiding the distribution of drugs into target sites and prevention of impacts of negative environmental factors on the carotenoids are essential.

In this study, we approached the aforementioned concerns by first formulating liposomes loaded with active substances derived from natural sources, followed by evaluation of the effect of dietary supplementation of liposomal canthaxanthin on growth, fillet color, muscle deposition, and nutrient composition of fish flesh of cultured rainbow trout (*Oncorhynchus mykiss*). The use of liposome has been regarded as an efficient strategy for delivery of drugs with low bioavailability since it allows better oral adsorption of lipid insoluble polar compounds, in turn resulting in improved drug efficacy. Canthaxanthin was extracted from bacterium *Paracoccus carotinifaciens*, an emerging natural source that is known for accumulation of canthaxanthin at high yield, at around 0.4% [18]. In liposome formation, α-tocopherol was coloaded along with canthaxanthin to maintain the quality and color of the fish flesh [19–22], and soybean lecithins were used as phospholipids owing to their continuous availability and advantages in terms of cost, emulsifying behavior, and sensorial characteristics [23, 24]. The inclusion of α-tocopherol is justified by its role in filling the gaps left by the imperfect packing of phospholipids in liposomal formation and in maintaining fish flesh quality, including the color of salmon, by affecting the oxidative stability of lipids [25]. Current findings are expected to justify the use of liposome containing natural-derived colorants in intensive aquaculture practices and greatly enhance the value of natural canthaxanthin given that consumers are increasingly aware of negative health effects that synthetic colorants may bring.

2. Materials and Methods

2.1. Materials. Bacterium *Paracoccus carotinifaciens* VTP 20181 strain was isolated from a salt field land in Diem Dien town, Thai Binh province, Vietnam, and fermented for 64 hours. Methanol (MeOH), acetone, n-hexane, dichloromethane, tetrahydrofuran (THF), 2-propanol, and acetonitrile solvents were obtained from Sigma Aldrich (St. Louis, MO, USA). Canthaxanthin standard was purchased from TRC Canada. A commercial preparation of soybean lecithin phospholipids was used to synthesize liposomes.

2.2. Extraction of Canthaxanthin. Biomass of the bacterium *Paracoccus carotinifaciens* VTP20181 obtained after fermentation was provided by the Food Industry Research Institute (Hanoi, Vietnam). Biomass was refrigerated at -40 °C prior to extraction process. Wet biomass was collected by centrifugation and washed with water, followed by drying. Then dried, canthaxanthin-rich biomass was extracted with solvent (ethanol 96% + 0.5% glycerol monostearate) under ultrasound assistance. Afterwards, the solvent in the mixture was removed by rotary evaporation to afford the canthaxanthin extract. The extract is crystallized with urea to remove saturated fatty acids. Subsequently, column chromatography was used to purify the extract of canthaxanthin. Finally, emulsification was carried out from the liquid, which was then dried to collect canthaxanthin powder [26]. The final yield was approximately 1.5 mg of canthaxanthin, accounting for 0.15 % of dry biomass weight. The compounds were elucidated using spectroscopic methods (IR, 2D-NMR and MS).

Canthaxanthin (1.5 mg): orange powder, $C_{40}H_{52}O_2$, melting point 211°C (literature 211-212 °C [25]). ^{1}H-NMR d($CDCl_3$): 1.20 (s, ca. 12H, 1,1′-gem-Me), 1.85 (m, J 6.7, 4H, 2,2′-H2), 1.87 (s, 6H, 18,18′-Me), 1.99 (s, 6H, 19,19′-Me),

2.00 (s, 6H, 20,20′-Me), 2.51 (mm, *J* 6.7, 4H, 3,3′-H2), 6.25 (mm, *J* 16, 2H, 7,7′-H), 6.27 (m, *J* 12, 2H, 10,10′-H), ca. 6.29 (m, 2H, 14,14′-H), 6.36 (d, *J* 16, 2H, 8,8′-H), 6.40 (d, *J* 14.8, 2H, 12,12′-H), ca. 6.65 (m, 2H, 15,15′-H), 6.68 (m, 4H, 11,11′ -H). ^{13}C-NMR d($CDCl_3$): 35.8 (1, 1′), 37.5 (2, 2′), 34.3 (3, 3′), 203.9 (4, 4′), 129.9 (5, 5′), 160.9 (6, 6′), 124.2 (7, 7′), 141.3 (8, 8′), 134.5 (9, 9′), 134.3 (10, 10′), 124.7 (11, 11′), 139.3 (12, 12′), 136.6 (13, 13′), 133.6 (14, 14′), 130.5 (15, 15′), 27.7 (1, 1′-gem-Me), 14.1 (5, 5′-Me), 12.5 (9, 9′-Me), 13.9 (13, 13′-Me). m/*z*: 564 (M, 100%), 549 (M-15, 20.6%), 508 (M-56, 8.4%), 484 (M-80, 9.4%), 472 (M-92, 44.9%), 458 (M-106, 12.2%).

2.3. Liposome Synthesis and Characterization

2.3.1. Synthesis of Liposome. Canthaxanthin- and α-tocopherol-loaded liposomes were prepared by thin-film evaporation method as described previously [27]. Briefly, a mixture of canthaxanthin and α-tocopherol at the fixed mass ratio of 1 : 9 (mixture A) was dissolved in 2 mL of dichloromethane together with soybean lecithin. The initial concentrations ($IC = m_{canthaxanthin}/m_{lipids}$, % wt/wt) of canthaxanthin were selected at 0.1%, 0.5%, and 1.0%, respectively. After dissolution, the thin membrane was obtained by removing the organic solvent in the water bath at 30 °C under flask pressure of 250 mbar and rotational speed of level 2. Distilled water (4 mL) was added to peel off the membrane, forming canthaxanthin-loaded liposomes. In order to get a fixed size, liposome was passed through a 100 nm polycarbonate membrane in a mini extruder 50 times. The final sample was sealed in a new eppendorf and kept in the refrigerator (about 4°C in the dark). All other samples (blank liposomes, canthaxanthin-loaded liposomes, and α-tocopherol-loaded liposomes) were prepared with the same procedure and respective ratios of materials. The structure of canthaxanthin- and α-tocopherol-loaded liposomes is shown in Figure 1.

2.3.2. Size Measurement. The z-average, mean size, and polydispersity index (PDI) of the liposome samples were monitored by using a SZ-100 nanoparticle size and zeta potential analyzer (Horiba, Japan) with a He/Ne laser ($\lambda = 633$ nm) and scattering angle 90°. The obtained results are the average of at least three measurements.

2.3.3. Canthaxanthin Loading. The encapsulation efficiency (EE%) and drug loading content (DL%) were determined by UV-Vis spectrophotometer. Unencapsulated canthaxanthin was removed by dialysis (14000 Da, 10 cm × 10 cm). The suspension was dialyzed for 24 hours against 1 L of distilled water. The distilled water was changed every 2 hours from the start 4 times. The liposomal system was dissolved in 3.5 mL of hexane and sonicated in a bath for 2 min to destroy the liposomal suspensions. After that, 100 μL of liposomes mixture was combined with 2900 μL of hexane and vortexed for 30 seconds. The free amount of canthaxanthin was quantified spectrophotometrically by Hitachi U-2900 spectrophotometer (Hitachi, Tokyo, Japan) at 474 nm with hexane as a blank. The EE % and DL % were determined, respectively, by the following equations:

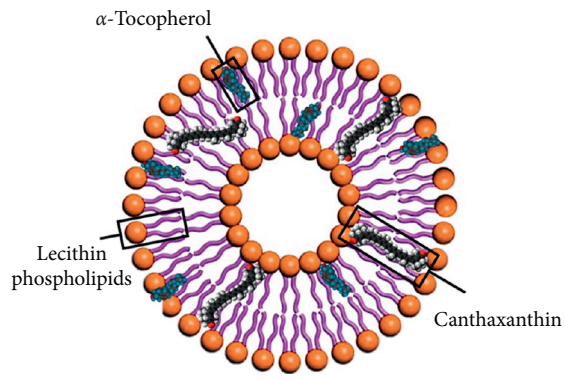

Figure 1: The structure of canthaxanthin- and α-tocopherol-loaded liposome.

$$EE\% = \frac{\text{the amount of canthaxanthin in the liposomes}}{\text{the total amount of canthanxanthin}} \times 100,$$

$$DL\% = \frac{\text{mass of canthaxanthin in the liposomes}}{\text{mass of canthaxanthin/liposomes}} \times 100. \quad (1)$$

2.3.4. In Vitro Drug Release. First, 1 mL of canthaxanthin- and α-tocopherol-loaded liposomes was placed into dialysis bags (molecular weight cutoff = 14000 Da), followed by incubation in 50 mL of phosphate buffered solution (PBS) (pH = 7.4) containing Tween 80 (0.5% wt) at 37°C with gentle shaking (100 rpm). After certain time period, 1 mL of sample was taken from the bag, and an identical volume of fresh buffer was added. Withdrawn media were extracted and analyzed by using UV-Vis spectrophotometer to calculate the cumulative amount of released canthaxanthin. Each experiment was carried out in triplicate and shown as mean ± standard deviation (SD).

2.4. In Vivo Experiments

2.4.1. Fish and Diet. The effects of canthaxanthin- and α-tocopherol-loaded liposomes as diet supplemented for feeding rainbow trout were investigated at Sapa Research Center for Coldwater Aquaculture, Research Institute for Aquaculture (Lao Cai Province, Vietnam). A total of 900 fish were individually weighed and randomly distributed into 30 tanks (280 L each). Each tank contained 30 fish, and each replication comprised 10 tanks. One month prior to dietary supplementation, fish were raised on noncolored feeds to have their muscle color returned to original color. Afterwards, each tank in each replication group was fed with one of the ten experimental diets for another 3 months (Table 1). The dietary feeds used in the experiment were produced by Kinh Bac Feed Mill, Bac Ninh province, Vietnam, with the basic nutrient content as follows: total protein 40%, total fat 18%, crude fiber 5%, and energy of 3800 kcal/kg. Fish were

Table 1: Composition of artificially composed fish feeds used in the feeding trial.

Diet	Dietary treatments	Weight in diets (mg)		
		Lecithin	α-Tocopherol	Canthaxanthin
I	Commercial diet	—	—	—
II	Commercial diet supplemented with 1 g/kg lecithin	1000	—	—
III	Commercial diet supplemented with 100 mg/kg α-tocopherol	—	100	—
IV	Commercial diet supplemented with 20 mg/kg canthaxanthin	—	—	20
V	Commercial diet supplemented with 100 mg/kg α-tocopherol and 20 mg/kg canthaxanthin	—	100	20
VI	Commercial diet supplemented with 1 g/kg α-tocopherol-loaded liposomes	990	10	—
VII	Commercial diet supplemented with 1 g/kg canthaxanthin-loaded liposomes (IC = 1%)	990	—	10
VIII	Commercial diet supplemented with 1 g/kg canthaxanthin- and α-tocopherol-loaded liposomes (IC = 0.1%)	990	9	1
IX	Commercial diet supplemented with 1 g/kg canthaxanthin- and α-tocopherol-loaded liposomes (IC = 0.5%)	950	45	5
X	Commercial diet supplemented with 1 g/kg canthaxanthin- and α-tocopherol-loaded liposomes (IC = 1%)	900	90	10

fed at 6 am, 10 am, 2 pm, and 6 pm every day until full. Tank water was continuously replaced with fresh water at the rate of 4 L/min. Water temperature and dissolved oxygen were maintained in the range from 6 to 17.5 °C and from 10 to 11 ppm, respectively. Air pumps were used for water aeration.

2.4.2. Weight Measurement. During the trial, fish were periodically weighed every month by taking the mean weight of three randomly caught fish from each treatment group. Growth indicators of fish were determined as follows:

$$\text{Weight gain (WG, \%)} = (W_f - W_i)/W_i) \times 100$$

$$\text{Specific growth rate (SGR) } g \text{ day}^{-1} = 100 \ (\ln W_f - \ln W_i)/t$$

Here, W_f is the final body weight (g), W_i is the initial body weight (g), and t is the experimental duration (days).

2.4.3. Muscle Canthaxanthin Extraction and Determination. After three months of feeding trial, fish fillets were collected and measured for color using SalmoFan Lineal colorimeter (DSM Nutritional Products, Kaiseraugst, Switzerland) on a scale of 20 to 34, which is the accepted international benchmark for salmon color (Figure 2). Scores recorded for each animal were made in agreement with three researchers.

Canthaxanthin disposition in fish muscle tissue was determined as follows. First, muscle samples were collected on the fish dorsal and stored at −20°C. Then, canthaxanthin was extracted following a previously described procedure [16]. Briefly, after crushing with an IKA basic ULTRA-TURRAX® homogenizer, the samples were extracted with organic solvents, then cleaned, and analyzed by a HPLC chromatograph with the standard canthaxanthin of Sigma Aldrich. The liquid gas chromatography system used the C-18 Hypersil Gold analysis column (5 μm, 150 mm × 4.6 mm), with flow rate set to 1 mL/min. The retention time was 6 minutes. The process was carried out at the analysis room of the Institute of Chemistry and Natural Compounds.

2.5. Statistical Analysis. Experimental data was calculated with mean and standard error. Average data of the treatments was processed by ANOVA on Minitab 16. Duncan comparison was used to differentiate each treatment. The difference is considered significant when $p < 0.05$.

3. Results and Discussion

3.1. Extraction of Canthaxanthin. Canthaxanthin was isolated from extract as an orange powder with melting point 212-213°C. A total of 40 carbons were observed in the ^{13}C NMR spectrum, and there is a signal of C=O group (C-4/4′) at δ 203.9. The DEPT spectrum indicated ten methyls (δ 27.7 (C-16, 16′), 27.7 (C-17, 17′), 13.9 (C-20, 20′), 14.1 (C-18, 18′), 12.5 (C-19, 19′)), four methylenes (δ 37.5 (C-2, 2′), 34.3 (C-3, 3′)), fourteen methines (δ 124.2 (C-7, 7′), 141.3 (C-8, 8′), 134.3 (C-10, 10′), 124.7 (C-11, 11′), 139.3 (C-12, 12′)), and twelve quaternary carbons (δ 203.9 (C-4, 4′), 160.9 (C-6, 6′), 136.6 (C-13,13′), 134.5 (C-9, 9′), 129.9 (C-5, 5′), 35.7 (C-1, 1′)) in the molecule. Further, a molecular ion peak was detected in the mass spectrum result at m/z 564, confirming the molecular formula $C_{40}H_{52}O_2$.

From ^1H NMR spectrum, five singlet signals at d 1.20, d 1.20, d 1.87, d 1.99, and d 2.00 were assigned to H-16/16′, H-17/17′, H-18/18′, H-19/19′, and H-20/20′, respectively. The canthaxanthin spectrum data are given in Table 2. Total amount of canthaxanthin from 1 g dry biomass was 1.5 mg. Yield = 1.5/1000 × 100 = 0.15%.

3.2. Synthesis and Characterization of Canthaxanthin- and a-Tocopherol-Loaded Liposomes. Liposomes were made by the established thin-film hydration method followed by extrusion. The physicochemical properties of the optimal blank lecithin liposome, canthaxanthin-loaded liposomes, α-tocopherol-loaded liposomes, and canthaxanthin- and α-tocopherol-loaded liposomes formulation were studied. As shown in Figure 3, the mean size of blank lecithin liposome was 105.53 ± 9.02 (mean ± SD; $n = 3$), while the mean sizes of canthaxanthin-loaded liposomes and α-tocopherol-loaded liposomes were 97.33 ± 4.64 and

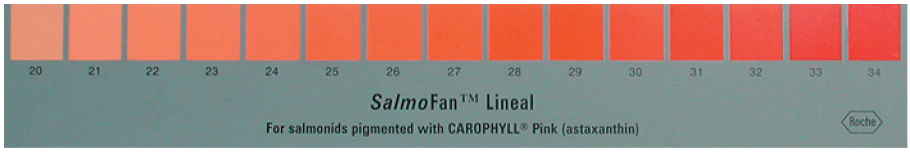

Figure 2: DSM SalmoFan Lineal colorimeter.

Table 2: NMR data for canthaxanthin in $CDCl_3$; 500 MHz.

	^{1}H (δ) (500 MHz, $CDCl_3$)	^{13}C (δ) (500 MHz, $CDCl_3$)
1/1′	—	35.8
2/2′	1.85 (m)	37.5
3/3′	2.51 (m)	34.3
4/4′	—	203.9
5/5′	—	129.9
6/6′	—	160.9
7/7′	6.25 m	124.2
8/8′	6.36 d	141.3
9/9′	—	134.5
10/10′	6.27 m	134.3
11/11′	6.68 m	124.7
12/12′	6.40 d	139.3
13/13′	—	136.6
14/14′	6.29 m	133.6
15/15′	6.65 m	130.5
16/16′	1.20 (s)	27.7
17/17′	1.20 (s)	27.7
18/18′	1.87 (s)	14.1
19/19′	1.99 (s)	12.5
20/20′	2.00 (s)	13.9

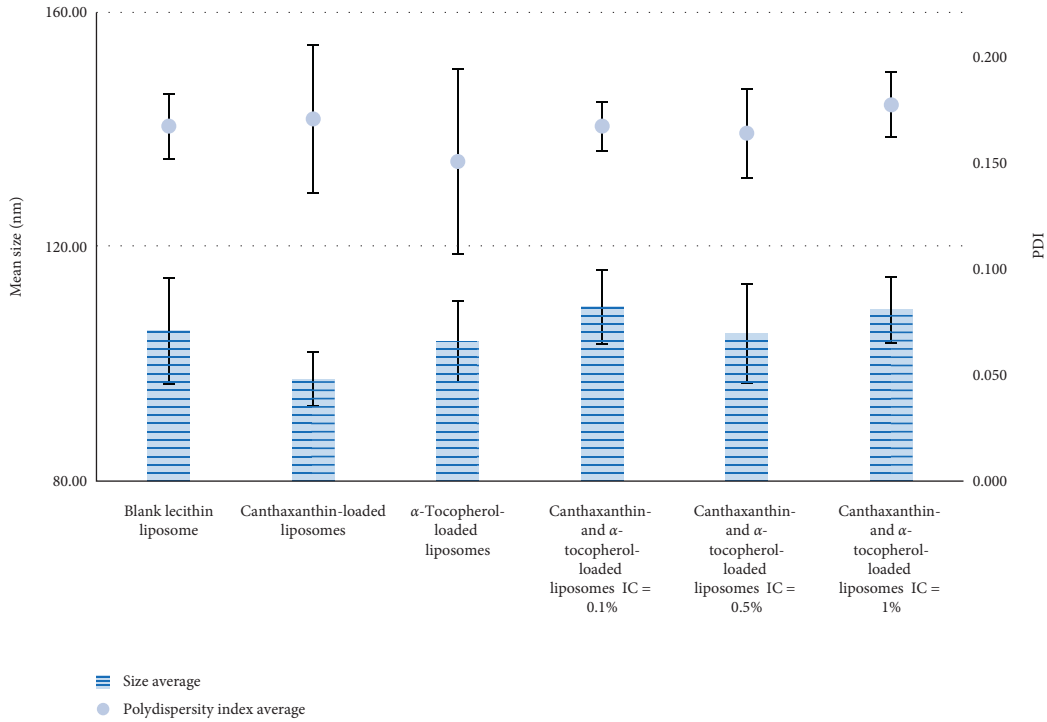

Figure 3: Mean size and PDI of blank lecithin liposome, canthaxanthin-loaded liposomes, α-tocopherol-loaded liposomes, canthaxanthin- and α-tocopherol-loaded liposomes with IC = 0.1%, canthaxanthin- and α-tocopherol-loaded liposomes with IC = 0.5%, and canthaxanthin- and α-tocopherol-loaded liposomes with IC = 1%.

103.80 ± 6.95 (mean ± SD; $n = 3$), respectively. In case of canthaxanthin- and α-tocopherol-loaded liposomes, the mean sizes of liposomes with canthaxanthin IC = 0.1%, IC = 0.5%, and IC = 1% were 109.70 ± 6.36, 105.10 ± 8.41, and 109.20 ± 5.66 (mean ± SD; $n = 3$). Polydispersity index (PDI), an indicator of dispersion homogeneity, of all formulated liposomes ranged from 0 and 0.2, suggesting that active ingredients were homogenously dispersed on the synthesized liposomes. PDI value of α-tocopherol-loaded liposomes was lowest at 0.150 ± 0.044 (mean ± SD; $n = 3$). All other liposomes have higher PDI values but remain lower than 0.2. There were no significant differences of the mean size and PDI between blank lecithin liposome and canthaxanthin- and α-tocopherol-loaded liposomes.

The encapsulation efficiency (EE%) and drug loading content (DL %) of canthaxanthin-loaded liposomes were 59.6 ± 2.3% (mean ± SD; $n = 3$) and 2.39 ± 0.23% (mean ± SD; $n = 3$), respectively (Table 3). The encapsulation efficiency of canthaxanthin- and α-tocopherol-loaded liposomes decreased when increasing the concentration of canthaxanthin in lecithin liposome. EE% values of liposomes loaded with canthaxanthin at IC = 0.1%, IC = 0.5%, and IC = 1% were 85.3 ± 2.1, 72.9 ± 1.8, and 55.3 ± 2.6, respectively.

The role of lipid layer in protecting core materials of the liposome is accentuated by the fact that active ingredients such as carotenoids easily degrade under acidic microenvironments and by the influence of enzymes. *In vitro* release profiles of canthaxanthin from canthaxanthin-loaded liposomes, canthaxanthin- and α-tocopherol-loaded liposomes with IC = 0.1%, canthaxanthin- and α-tocopherol-loaded liposomes with IC = 0.5%, and canthaxanthin- and α-tocopherol-loaded liposomes with IC = 1% in PBS at pH 7.4 are shown in Figure 4. Within the first 4 hours, release of canthaxanthin from liposomes formed without α-tocopherol seemed to be more rapid than other liposomes. By contrast, liposomes loaded with canthaxanthin at IC = 0.1% showed slower, sustained release rate, which reached 0.46 after 4 h. In addition, increasing the loaded canthaxanthin concentration apparently induced faster canthaxanthin release within the first 1 h. After 4 h of incubation, approximately 68% of the encapsulated canthaxanthin was released from the liposome containing 1% canthaxanthin. These data suggest that canthaxanthin can be well encapsulated in liposomes and released in an extended period.

3.3. Fish Growth. The culture of rainbow trout was carried out in the winter to ensure suitable growth conditions and water temperature [8]. Dissolved oxygen in experiment was controlled and ranged from 7.1 to 10.9 mg/l, with an average of 8.2 mg/L [28, 29].

Initially, weight of the fish was 309.43 ± 12.98 g before being raised with feeds, and there was no difference between groups. Mean wet weights of fish were recorded monthly during feeding trial and once before filleting. During the experiment, the fish remained in good health, and the mortality rate was 0%. At the end of the feeding trial, fish fed with diet supplemented with 1 g/kg canthaxanthin- and α-tocopherol-loaded liposomes (IC = 0.5%) showed significantly higher average weight than those of other experimental control groups ($p < 0.05$) (Figure 5).

Rainbow trout fed with diet supplemented with canthaxanthin at 20 mg/kg (diet IV) showed significantly lower wet weights ($p < 0.05$) after two, three, and four months compared to other groups. This finding is in line with that reported by Torrissen et al. [17] where increased dose of fed canthaxanthin resulted in the lower weight gain in cultured rainbow trout. Final weights of fish in groups fed with diets supplemented with 1 g/kg lecithin or 100 mg/kg α-tocopherol were higher, albeit not statistically significant ($p > 0.05$), than those fed with unsupplemented commercial diet. In case of groups fed with diets supplemented with canthaxanthin- and α-tocopherol-loaded liposomes (diets VII, IX, and X), their weight gain was higher when increasing the concentration of canthaxanthin from 0.1% to 0.5% but was stagnant when further increasing concentration of canthaxanthin from 0.5 to 1% (not significant difference).

Current results are similar to those of previous reports pointing out that inclusion of carotenoids such as canthaxanthin or astaxanthin in fish feed affected the growth rate and efficiency of food usage of freshwater salmon or parrot fish [30, 31]. In addition, several studies also reported that carotenoids could enhance growth by acting as fertilization hormones, but, up to date, biological functions of the carotenoids in fish have not been unconfirmed [32, 33].

Table 4 shows the results of the muscle proximate composition of rainbow trout after 3 months of feeding with various experimental diets. No significant differences were recognized among 10 treatment groups in all indicators (crude protein, crude lipid, ash, and moisture). These findings are not consistent with previous reports demonstrating that dietary canthaxanthin deficiency can affect digestion and absorption of lipid. To be specific, it was found that dietary supplementation of carotenoids did affect digestion and absorption of lipid in rats [34]. Similarly, for aquatic animals, Brizio et al. noted that canthaxanthin affected the digestion of lipid in fish [35]. Nevertheless, the effects of canthaxanthin and α-tocopherol on fish muscle composition are still controversial and complex, and further studies are needed to reveal their mechanisms.

3.4. Muscle Canthaxanthin. At the beginning of the experiment, the color of fish muscle was uniform among experimental groups with the color score ranging from 20.1 to 20.9 (Table 5). After 1 month of feeding, the color began to show differences between treatments ($p < 0.05$). The highest score (27.0) was recorded in fillets of fish fed with commercial diet supplemented with 100 mg/kg α-tocopherol and 20 mg/kg canthaxanthin (diet V), closely followed by that of diet IV and diet IX (26.9). The lowest score of 23.1 was recorded in diet I, which is in absence of canthaxanthin. After 2 months, the difference in color of the muscle became more apparent (Figure 6). However, towards the end of the experiment, improvements in color score were less noticeable, which is consistent with previous studies. This result is similar to previous research

Table 3: EE and DL of blank lecithin liposome, canthaxanthin-loaded liposomes, α-tocopherol-loaded liposomes, canthaxanthin- and α-tocopherol-loaded liposomes with IC = 0.1%, canthaxanthin- and α-tocopherol-loaded liposomes with IC = 0.5%, and canthaxanthin- and α-tocopherol-loaded liposomes with IC = 1%.

Liposomes	EE (%)	DL (%)
Blank lecithin liposome	—	—
Canthaxanthin-loaded liposomes	59.6 ± 2.3	2.39 ± 0.23
α-Tocopherol-loaded liposomes	—	—
Canthaxanthin- and α-tocopherol-loaded liposomes, IC = 0.1%	85.3 ± 2.1	1.87 ± 0.35
Canthaxanthin- and α-tocopherol-loaded liposomes, IC = 0.5%	72.9 ± 1.8	2.09 ± 0.13
Canthaxanthin- and α-tocopherol-loaded liposomes, IC = 1%	55.3 ± 2.6	2.23 ± 0.21

Numbers resulted from triplicate measurements and are presented as mean value ± standard deviation.

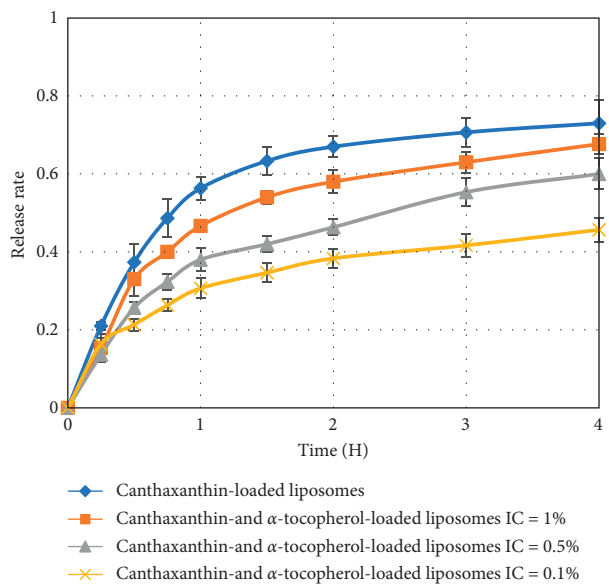

Figure 4: *In vitro* release profile of canthaxanthin from canthaxanthin-loaded liposomes, canthaxanthin- and α-tocopherol-loaded liposomes with IC = 0.1%, canthaxanthin- and α-tocopherol-loaded liposomes with IC = 0.5%, and canthaxanthin- and α-tocopherol-loaded liposomes with IC = 1% in PBS at pH 7.4.

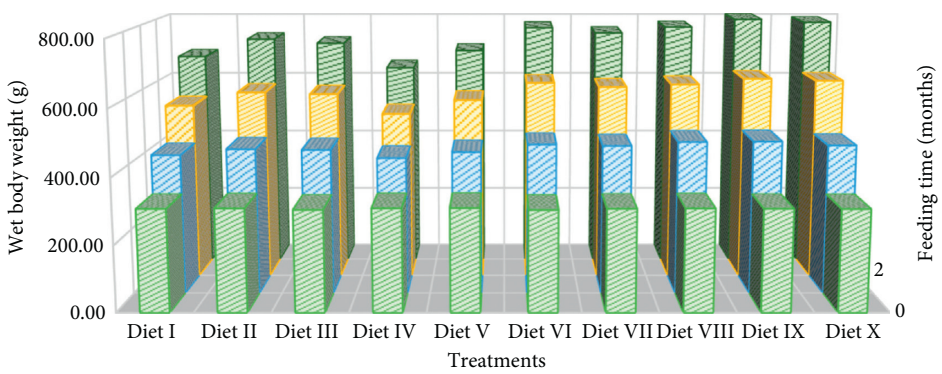

Figure 5: Weight variations of rainbow trout groups fed with different diet formulations. Feeding time of 0 indicates the initial time point where experimental feeding began.

with the astaxanthin and canthaxanthin supplementation at the ratio of 40 : 40 mg/kg of food [36]. In addition, Choubert et al. and Torrissen et al. also reported that the color of the fish muscle would gradually not increase and reach saturation, even with increased concentration or longer feeding [10, 17].

The concentration of canthaxanthin accumulated in fish muscle in different treatments after 90 days of culturing is shown in Figure 7. After 90 days of feeding, fish fed with different experimental diets showed varying concentrations of canthaxanthin accumulated in the fish muscle ($p < 0.05$). Canthaxanthin content in muscle was highest (2.91 mg/kg)

Table 4: Proximate composition of the muscle of rainbow trout after 3 months of feeding with experimental diets ($n = 3$, unit: % wet weight).

Diet	Experimental diets	Crude protein	Crude lipid	Ash	Moisture
I	Commercial diet	20.19 ± 1.15	6.79 ± 1.10	1.83 ± 0.12	73.79 ± 1.73
II	Commercial diet supplemented with 1 g/kg lecithin	18.89 ± 1.12	7.43 ± 1.09	1.75 ± 0.37	73.25 ± 0.81
III	Commercial diet supplemented with 100 mg/kg α-tocopherol	19.22 ± 1.32	7.42 ± 1.04	1.57 ± 0.22	72.21 ± 0.64
IV	Commercial diet supplemented with 20 mg/kg canthaxanthin	20.07 ± 2.37	8.67 ± 2.33	1.72 ± 0.17	71.57 ± 1.12
V	Commercial diet supplemented with 100 mg/kg α-tocopherol and 20 mg/kg canthaxanthin	19.86 ± 1.86	7.51 ± 1.56	1.68 ± 0.34	73.13 ± 0.79
VI	Commercial diet supplemented with 1 g/kg α-tocopherol-loaded liposomes	20.25 ± 1.06	6.32 ± 1.18	1.23 ± 0.23	72.13 ± 1.46
VII	Commercial diet supplemented with 1 g/kg canthaxanthin-loaded liposomes (IC = 1%)	19.29 ± 1.19	7.29 ± 1.24	1.97 ± 0.28	70.22 ± 0.91
VIII	Commercial diet supplemented with 1 g/kg canthaxanthin- and α-tocopherol-loaded liposomes (IC = 0.1%)	19.57 ± 1.28	7.12 ± 1.06	1.72 ± 0.21	73.15 ± 0.94
IX	Commercial diet supplemented with 1 g/kg canthaxanthin- and α-tocopherol-loaded liposomes (IC = 0.5%)	20.25 ± 2.17	8.13 ± 2.43	1.65 ± 0.12	74.75 ± 1.02
X	Commercial diet supplemented with 1 g/kg canthaxanthin- and α-tocopherol-loaded liposomes (IC = 1%)	19.26 ± 1.69	7.91 ± 1.96	1.83 ± 0.24	71.03 ± 0.94

Figures resulted from triplicate measurements and are presented as mean ± SE.

Table 5: Color scores of fillets taken from rainbow trout treated with different formulated diets at the initial time point and after one, two, and three months of experimental feeding ($n = 3$).

Diet	Experimental diets	0 th month	1st month	2nd month	3rd month
I	Commercial diet	20.56 ± 0.88	23.11 ± 1.10	24.67 ± 1.10	25.00 ± 1.41
II	Commercial diet supplemented with 1 g/kg lecithin	20.33 ± 0.71	23.22 ± 1.20	24.78 ± 1.20	25.22 ± 1.39
III	Commercial diet supplemented with 100 mg/kg α-tocopherol	20.56 ± 1.13	23.22 ± 1.00	24.44 ± 1.00	25.11 ± 1.27
IV	Commercial diet supplemented with 20 mg/kg canthaxanthin	20.89 ± 0.78	26.67 ± 1.00	28.33 ± 1.00	29.78 ± 1.20
V	Commercial diet supplemented with 100 mg/kg α-tocopherol and 20 mg/kg canthaxanthin	20.11 ± 0.78	27.00 ± 1.1	29.11 ± 0.80	30.56 ± 1.01
VI	Commercial diet supplemented with 1 g/kg α-tocopherol-loaded liposomes	20.44 ± 1.01	23.33 ± 0.70	24.89 ± 1.10	25.11 ± 1.54
VII	Commercial diet supplemented with 1 g/kg canthaxanthin-loaded liposomes (IC = 1%)	20.33 ± 1.00	25.89 ± 0.80	28.11 ± 0.80	29.11 ± 0.78
VIII	Commercial diet supplemented with 1 g/kg canthaxanthin- and α-tocopherol-loaded liposomes (IC = 0.1%)	20.33 ± 1.00	25.00 ± 1.40	27.11 ± 1.30	28.78 ± 1.30
IX	Commercial diet supplemented with 1 g/kg canthaxanthin- and α-tocopherol-loaded liposomes (IC = 0.5%)	20.67 ± 1.00	26.67 ± 0.70	28.89 ± 0.80	30.00 ± 0.71
X	Commercial diet supplemented with 1 g/kg canthaxanthin- and α-tocopherol-loaded liposomes (IC = 1%)	20.44 ± 1.13	26.89 ± 0.80	29.33 ± 1.10	30.22 ± 1.39

Color values for each muscle were made in agreement with three researchers. Figures resulted from triplicate measurements and are presented as mean ± SE.

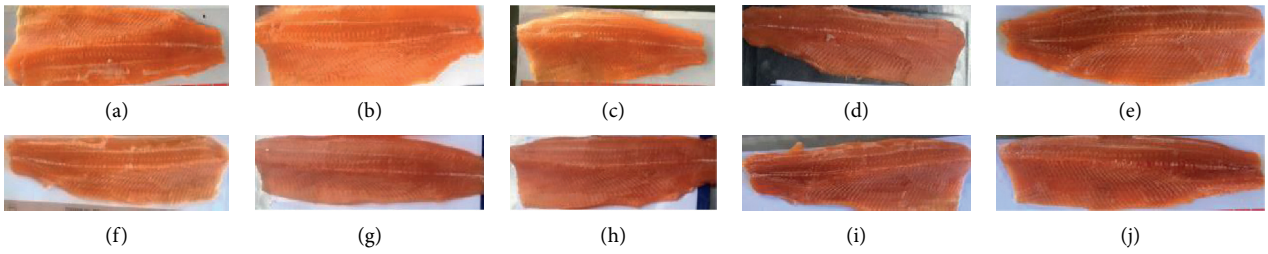

Figure 6: Muscle coloration of flesh at the end of experiments: (a) diet I; (b) diet II; (c) diet III; (d) diet IV; (e) diet V; (f) diet VI; (g) diet VII; (h) diet VIII; (i) diet IX; (j) diet X.

in the group fed with diet supplemented with 1 g/kg canthaxanthin- and α-tocopherol-loaded liposomes (IC = 0.5%), followed by diet supplemented with 1 g/kg canthaxanthin- and α-tocopherol-loaded liposomes (IC = 1%) (2.90 mg/kg) and commercial diet supplemented with 100 mg/kg α-tocopherol and 20 mg/kg canthaxanthin (2.75 mg/kg). The lowest canthaxanthin content in muscle was found in the group fed with commercial diet supplemented with 1 g/kg lecithin (0.99 mg/kg). However, between diet IX and diet V, there is no statistically significant difference in canthaxanthin disposition.

Economic viability of diet IX (1 g/kg canthaxanthin- and α-tocopherol-loaded liposomes, IC = 0.5%) is also justified by the prices of canthaxanthin and α-tocopherol. To be

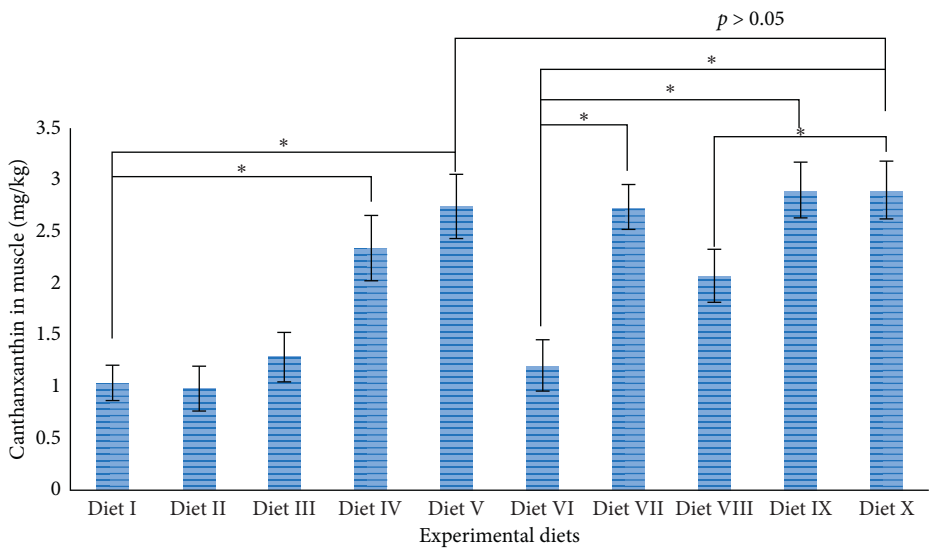

Figure 7: Canthaxanthin concentrations in muscle samples.

specific, while the price for canthaxanthin ranges between € 528 and € 4,278 per kilogram (based on commercial suppliers), the average global price of Vitamin E50 is negligible, at around $ 8.5 per kilogram [37]. This suggests that the cost of canthaxanthin in the composition of diet IX is approximately four times lower than those of commercial fish feed supplemented with 20 mg/kg of unencapsulated canthaxanthin and that formulation of liposomal fish diet carries important implications for reducing carotenoid feeding while still meeting international standards.

According to Torrissen et al., canthaxanthin disposition in rainbow trout muscle was proportional to the ratio of additional canthaxanthin in food [17]. However, with the inclusion of canthaxanthin at a concentrations higher than 50 mg/kg, the accumulation of canthaxanthin in the rainbow trout muscle decreased gradually and stopped when reaching saturation.

Interestingly, canthaxanthin deposition in fish flesh increased in the presence of α-tocopherol in the feed (diet V), which is in line with findings reported by Choubert et al. [10] proposing that, compared to feeding using bare canthaxanthin, the use of liposomes formed from lecithin and α-tocopherol could reduce the required amount of canthaxanthin needed to achieve the same color score. Further experiments are needed to clarify this assertion.

4. Conclusions

In the present study, liposomes containing α-tocopherol and canthaxanthin as active ingredients were fabricated and included in fish feeds of cultured rainbow trout. There was no difference in growth rate, survival rate, or flesh nutritional composition between cultured rainbow trout fed with diets supplemented with bare lecithin, α-tocopherol, and canthaxanthin and those fed with liposomes formed from lecithin, α-tocopherol, and canthaxanthin. However, the color and canthaxanthin disposition in fish muscle of fish fed with diets containing 1 g/kg canthaxanthin- and α-tocopherol-loaded liposomes (IC = 0.5%) were similar to those of fish fed with commercial diet supplemented with 20 mg/kg of unencapsulated canthaxanthin. However, the experimental diet containing liposomal canthaxanthin and α-tocopherol only required half of the amount of canthaxanthin compared to the diet supplemented with bare canthaxanthin. These results suggest the feasibility of using liposome formation techniques in manufacture of fish diet supplements. Further studies should focus on mechanisms of canthaxanthin pigmentation on fish flesh and evaluate industrial-scale utilization of supplemented fish diets.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest

The authors declare no conflicts of interest.

Authors' Contributions

Quoc Toan Tran, Viet Anh Dang, Nguyen Thanh Duong, Phi Hung Nguyen, Thu Huong Trinh Thi, Thuy Ha Tran, Van Thinh Do, Van Khoi Le, Xuan Luong Ngo, Pham Tri Nhut, Hai Ha Pham Thi, and Manh Van Do carried out investigation; Pham Quoc Long supervised the work; and Quoc Toan Tran wrote the original draft.

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Research Article

Biopreservation and Quality Enhancement of Fish Surimi Using Colorant Plant Extracts

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The biopreservation, flavoring, and coloration of foodstuffs, e.g., seafoods, with natural plant derivatives are major demands for consumers and overseers. Different colored plant parts, i.e., *Hibiscus sabdariffa* calyces, *Curcuma longa* rhizomes, and *Rhus coriaria* fruits, were extracted and evaluated as biopreservatives, antimicrobial and colorant agents for fish surimi from *Oreochromis niloticus*. All colorant plant extracts (CPEs) exhibited strong antibacterial activities against screened pathogens, *Escherichia coli*, *Salmonella typhimurium*, *Staphylococcus aureus*, and *Pseudomonas aeruginosa*. *H. sabdariffa* extract (HCE) was the most effectual antimicrobial CPEs. *S. aureus* was the most sensitive strain to CPEs, whereas *S. typhimurium* and *P. aeruginosa* were the most resistant strains. The exterior coloration of tilapia surimi with CPEs resulted in great bacterial count reduction in colored products; stored CPEs-colored surimi had enhanced sensorial attributes. HCE-exposed *S. aureus* indicated bacterial cell lyses in time-dependent manner. CPEs application as colorants and antibacterial and quality enhancing agents is recommended for seafoods' biopreservation.

1. Introduction

Fisheries products, e.g., whole fish and seafoods, are extremely susceptible to biological decomposition due to their perishable nature and nutritional composition [1, 2]. The microbial spoilage/contamination is the leading cause from the diverse occurred deterioration types, in stored fish products, which accompanied with severe quality reduction [3, 4]. Seafood spoilage, with food-borne bacteria, could dangerously influence the shelf-life of the products and could be a threatening risk factor for food-borne diseases transmission, via pathogens' contamination, e.g., *E. coli*, *Salmonella* sp., *Shigella* sp., *Staphylococcus* sp., *Listeria* sp., and *Clostridium* sp. [1, 5].

Man continuously depended on plant kingdom to supply him with most of his needs to attain healthier life, higher nutritional beliefs [6]. Natural plant derivatives, e.g., extracts, essential oils, powders, or bioactive compounds, were continually applied effectively for biopreserving of foodstuffs, maintaining their sensorial and microbiological qualities and extending their shelf lives [4, 7].

The lessening of antimicrobial agents' usage, especially in food and health disciplines, was always regarded as the inspiring challenge for overseers and researchers [8, 9]; the exploration of safe, effectual, and environmentally-innocent alternatives, from biological sources, was always endorsed for application in food preservation and decontamination. Plant-derived antimicrobial compounds were efficaciously employed to preserve numerous foodstuffs, but their usage in seafoods' preservation had, somewhat, a limited success [10–12].

Natural food-grade pigments/colorants were increasingly utilized in food sectors as safe substitutes to synthetic counterparts; this was mostly due to the assumed side-effects and environmental impacts from the chemical-based food colorants and the rising consumers' awareness about that [13, 14]; they demand fully natural food/beverages that are free from any synthetic substances.

Many types of food colorants were approved, including carotenoids, chlorophylls, anthocyanins, and betalains, for safe applications in food processing; besides to their sensory enhancing attributes, it was evidenced that consumption of naturally-colored foodstuffs was interrelated with increasing immunity and cutback of hazardous diseases, e.g., diabetes, obesity, and cancer [14, 15].

It could be more beneficial to apply plant derivatives to achieve many bioactive advantages, e.g., coloration, antioxidation, antimicrobial, quality enhancement, and health promotion [6, 8, 11, 12].

Accordingly, current research was designed for evaluating some colored plant extracts as food colorants, antimicrobial, and biopreservatives for fish surimi toward the enhancement of microbiological and sensorial features of the product.

2. Materials and Methods

2.1. Plant Extraction. Diverse plant parts were employed for obtaining their crude extracts, i.e., extracts of *Hibiscus sabdariffa* calyces (HCE), *Curcuma longa* rhizomes (CRE), and *Rhus coriaria* fruits (RFE); plant materials were obtained from El-Kaptin Herbal Company, Egypt. Dried herbal parts, using hot air (45°C), were powdered to 70 mesh particles size. 200 g from each ground material were immersed into 1 L of aqueous ethanol solution (70%), stirred for 8 h at 220 xg, and then filtered using filter paper (Whatman no. 2). Vacuum evaporation (40°C) was applied for omitting ~90% of extraction solvent, followed by placing in desiccator to almost complete dryness. Extracts, after weighing, were dissolved in distilled water (to have concentration of 10%, w/v) and then sterilized using syringe filter (Millipore, 0.22 μm).

2.2. Microbial Strains. Four standard microbial strains were used in the experiments, *Escherichia coli* (ATCC 25922), *Salmonella typhimurium* (ATCC 14028), *Staphylococcus aureus* (ATCC 25923), and *Pseudomonas aeruginosa* (ATCC 27853). The bacterial cultures were propagated and screened in Trypticase soy agar (TSA) and Trypticase soy broth (TSB) media (Difco, Sparks, MD).

2.3. Evaluation of In Vitro Antibacterial Capability. The potential antibacterial capabilities of color plant extracts (CPEs), against examined bacteria, were screened, qualitatively, using agar/disc diffusion and quantitatively through appraising of minimal concentrations of bactericidal activity (MBC). Screened bacterial cultures, after refreshment in TSB for 18 h, were centrifuged for harvesting and washed twice with phosphate buffered solution (PBS, pH 7). The number of each culture cells was adjusted to $\sim 2 \times 10^7$ CFU (colony forming units)/ml, using PBS for dilution. Cell suspension portions (100 μL) were spread onto TSA, to assess disc diffusion, and then discs of filter paper (Whatman No. 41, 6 mm diameter), impregnated with 25 μL from each CPE, were placed to contact the surface of inoculated agar. After incubation of inoculated plates, at 37 ± 1°C for one day, the ZOI (diameter of clear zones from grown bacteria) were precisely measured, in triplicates, and the means of them were calculated.

The MBCs of each CPE were assessed via the method of broth microdilution [9]; using a tissue culture microplate (24-well), bacterial cultures were exposed to gradual CPEs concentrations in TSB. After incubation, as mentioned above, 100 μL from each trial were spread, onto TSA plates, incubated, and examined for grown colonies' appearance. MBC was designated as the least CPEs concentration that caused complete bacterial inhibition, after these steps.

2.4. Application of Colored Extracts onto Fish Surimi. Fish fillets from *Oreochromis niloticus* (Nile tilapia), weighed 100–120 g each, were offered from the Fish Processing Plant, Kafrelsheikh University, Egypt. Minced fillets were utilized for preparing fish surimi gel [16]. The inoculation of surimi gels, with bacterial cultures, was conducted through blending of individual cultures with raw products to obtain a final count of $\sim 15 \times 10^5$ CFU/g. The inoculated, and control, surimi samples were treated by immersion in the individual CPEs, at concentrations of 2.5 mg/ml from each [17]. Produced colored samples were then packaged aseptically into polyethylene bags and stored at 4°C for one day before subjecting to microbiological and sensorial attributes evaluation.

2.5. Microbiological Examination. Treated fish surimi samples were microbiologically examined to assess the plant colorants' effectiveness in reducing bacterial counts. Samples (10 g) were aseptically taken from the surface of surimi fingers (2 mm thickness) and from the middle inner parts of fingers, homogenized in buffered peptone water, and subjected to microbial examination. Different microbiological analyses standards were followed to judge the usefulness of

colorant extracts' treatment, on the elimination of bacterial growth in fish products, as follows:

Escherichia coli enumeration (β-glucuronidase-positive) (ISO 16649–2 : 2001)

Detection of *Salmonella* spp. (ISO 6579: 2002)

Staphylococcus aureus enumeration (ISO 6888–1:1999)

Detection and enumeration of *Pseudomonas aeruginosa* (ISO 16266 : 2006)

2.6. Sensory Attributes Evaluation. A trained team of panelists (14 members, 5 males and 9 females) at Kafrelsheikh University, experienced in seafood evaluation, accomplished the sensory evaluation of CPE-colored fish surimi attributes, i.e., odor, appearance, color, and overall quality, after cold storage (4°C) for 7 days. The used hedonic scale, for evaluation, ranged from excellent (5) to extremely bad (1) [18].

2.7. Electron Microscopic Imaging. For illustrating potential morphological variations in bacterial cells, after exposure to *H. sabdariffa* calyces extract, for 0, 6, and 12 h, grown *S. aureus* cells (18 h old) were exposed to concentration of 2.5 mg/ml from extract and microscopically photographed using electron scanning microscope (SEM; Hitachi S-500, Tokyo, Japan) according to Marrie and Costerton (1984). The electron microscopic captures were taken at 20 kV and 20,000 X, based on the morphological alterations in treated cells after exposure.

2.8. Statistical Analysis. The triplicated trials' means and standard deviations were computed using Excel sheets software 2013, Microsoft office™; the significance of variance analysis between individual groups was predicted using the statistical software (MedCalc-V. 11.6.1) with CI of ≥95%.

3. Result and Discussion

Various colored plant extract (CPE) parts were assessed as potential antibacterial agents against screened food-borne pathogens (Table 1). Generally, the entire CPEs exhibited significantly stronger antibacterial activities against screened pathogens, as evidenced from the clear ZOIs (Figure 1) and the recorded MBCs. The bacterial inhibition potentialities, from CPEs, varied toward examined microorganisms; *H. sabdariffa* calyces extract (HCE) could be relatively specified as the most significant powerful examined CPE. The subsequent relatively powerful CPE was *R. coriaria* fruits extract (RFE) and then the extract of *C. longa* rhizomes (CRE).

Conversely, *S. aureus* was the most sensitive strain to HCE and both *S. typhimurium* and *P. aeruginosa* were significantly more resistant against this extract. *S. typhimurium* had the highest recorded resistance toward CRE and *P. aeruginosa* was the most sensitive against the same extract (Table 1).

Natural food colorants were anciently applied worldwide to increase consumers' ability to diverse foodstuffs. Moreover, these applications of food colorants were recurrently stated to enhance food shelf life and nutritional and therapeutic outcomes due to their potential antimicrobial, antioxidant, anti-inflammatory, and health protection characteristics [2, 19]. Therefore, the current trials aimed to apply natural colored plant extracts as quality enhancers and antimicrobial biopreservatives in processed fish products. The used concentration from CPEs was 2.5 mg/ml, which was higher than the recorded MBCs from each colorant extract; this was because of the fish products content from protein, lipids, and other food constituents. The components of food, e.g., water, fat, protein, and salt, could increase microbial resistance to microbicides; higher levels from spices or other antimicrobial agents are required to inhibit microbial growth in food systems than when culture media are used [4, 6, 20]. Additionally, the biopreservatives concentrations, to exert the desirable antimicrobial action, were suggested to be higher when examining food products than to study them *in vitro*, although that if they coupled with further agents, they may support the efficacy to control bacterial pathogens in foods [21, 22].

Tilapia fish surimi were supplemented with the extracted CPEs, i.e., HCE, RFE, and CRE, at CPE concentrations of 2.5 mg/ml. The impact of product supplementation with CPEs, on the survival of inoculated food-borne bacterial pathogens, is illustrated in Table 2.

The microbial counts in control (uncolored) group tended to increase during storage, for all screened strains; the counts in surface parts exceeded those in inner samples for all microbes. The contrary was evidenced in CPEs-colored products, where the bacterial counts in product surface samples were much less than the recorded counts from the inner parts. The treatment impact was very remarkable in the count of *S. aureus*, in the surface of HCE-colored surimi, and then in *P. aeruginosa*, in the surface of RFE-colored samples.

The impacts of fish surimi coloration, with CPEs, on the sensorial attributes of products, are indicated in Figure 2. All treatments significantly increased the sensorial characteristics of colored surimi, compared to the control, after cold storage at 4°C for 7 days; the coloration with CRE was the most favorable, from panelist team, to enhance the color, appearance, and overall quality.

RFE was the best to improve odor characters of colored products, but it was less effective than the other CPEs for the rest of the examined attributes. Generally, the color and appearance of CPE-colored tilapia surimi were notably improved as indicated by panelists' preferences (Figure 3).

The variations in antibacterial activity, from CPEs toward examined microorganisms, could be explained by the diverse bioactive compounds in each CPE that could have different antibacterial actions against each microbe [6, 12]. All of the screened plants, herein, were traditionally accustomed for human usage; this guarantees their biosafety and applicability in various food sectors/applications. *R. coriaria* (sumac) is frequently utilized in the

Table 1: Antibacterial activity of plant extracts against food-borne pathogens, measured as the diameter of inhibition zones (ZOI, mm) and minimal bactericidal concentration (MBC, mg/ml)*.

Extracted plants	Part	*Salmonella typhimurium*		*Staphylococcus aureus*		*E. coli*		*Pseudomonas aeruginosa*	
		ZOI	MBC	ZOI	MBC	ZOI	MBC	ZOI	MBC
Hibiscus sabdariffa	Calyces	19.7 ± 0.9^{a1}	1.6	24.5 ± 1.2^{a2}	1.0	22.6 ± 1.2^{a2}	1.2	20.2 ± 0.8^{a1}	1.6
Curcuma longa	Rhizomes	16.8 ± 0.8^{b1}	2.2	18.4 ± 0.8^{b1}	1.8	18.2 ± 0.7^{b1}	1.8	22.4 ± 1.2^{a2}	1.2
Rhus coriaria	Fruits	19.3 ± 1.1^{a1}	2.0	21.3 ± 1.2^{c12}	1.4	23.4 ± 1.3^{a2}	1.2	17.1 ± 0.7^{b3}	2.0

*Dissimilar superscript letters within a column or superscript numbers within a row indicate difference significance at CI = 95%.

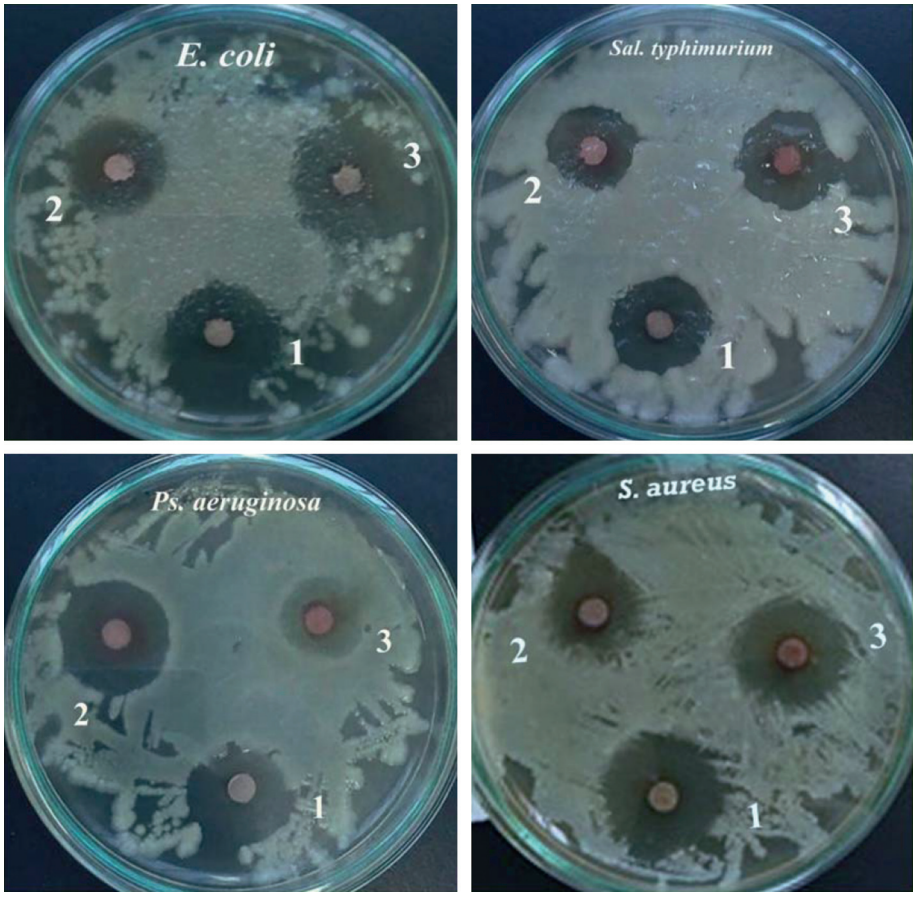

Figure 1: Appeared growth inhibition zones after challenging food-borne bacteria with colored extract of *Hibiscus sabdariffa* calyces (1), *Curcuma longa* rhizomes, (2) and *Rhus coriaria* fruits (3), using disc diffusion assay.

Table 2: Effect of fish surimi coloration with plant extracts, at their MICs∗, on the count of food-borne pathogens in the surface (S) and inner (I) parts, after 24 h of treatment and storage at 4°C.

Colorant plant extract	Examined part in product	Food-borne pathogens			
		Salmonella typhimurium	*Staphylococcus aureus*	*E. coli*	*Pseudomonas aeruginosa*
Control	S	4.3×10^7	7.2×10^6	7.6×10^7	2.2×10^7
	I	5.8×10^6	3.4×10^6	2.3×10^7	7.7×10^6
Hibiscus sabdariffa	S	5.2×10^3	1.8×10^2	6.1×10^2	7.3×10^3
	I	7.9×10^4	3.2×10^3	4.8×10^3	5.6×10^4
Curcuma longa	S	5.4×10^4	7.8×10^3	5.7×10^3	5.1×10^2
	I	2.3×10^5	8.1×10^4	7.2×10^4	2.8×10^4
Rhus coriaria	S	7.5×10^3	4.4×10^3	6.3×10^2	2.2×10^4
	I	6.2×10^4	4.9×10^4	1.9×10^4	7.5×10^4

Plant extract was applied at a concentration of 2.5 mg/ml. Initial microbial addition was $\sim 4 \times 10^5$ CFU/g.

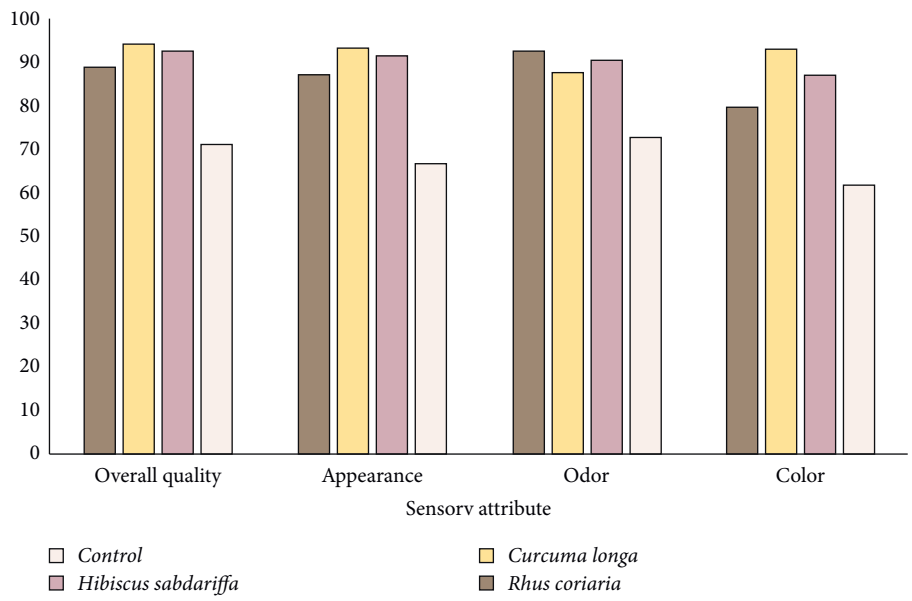

Figure 2: Effect of fish surimi coloration with plant extracts on the sensorial attributes of products after 7 days of treatment and storage at 4°C Results are means of 14 panelists' scores. Plant extract was applied at concentration of 2.5 mg/ml.

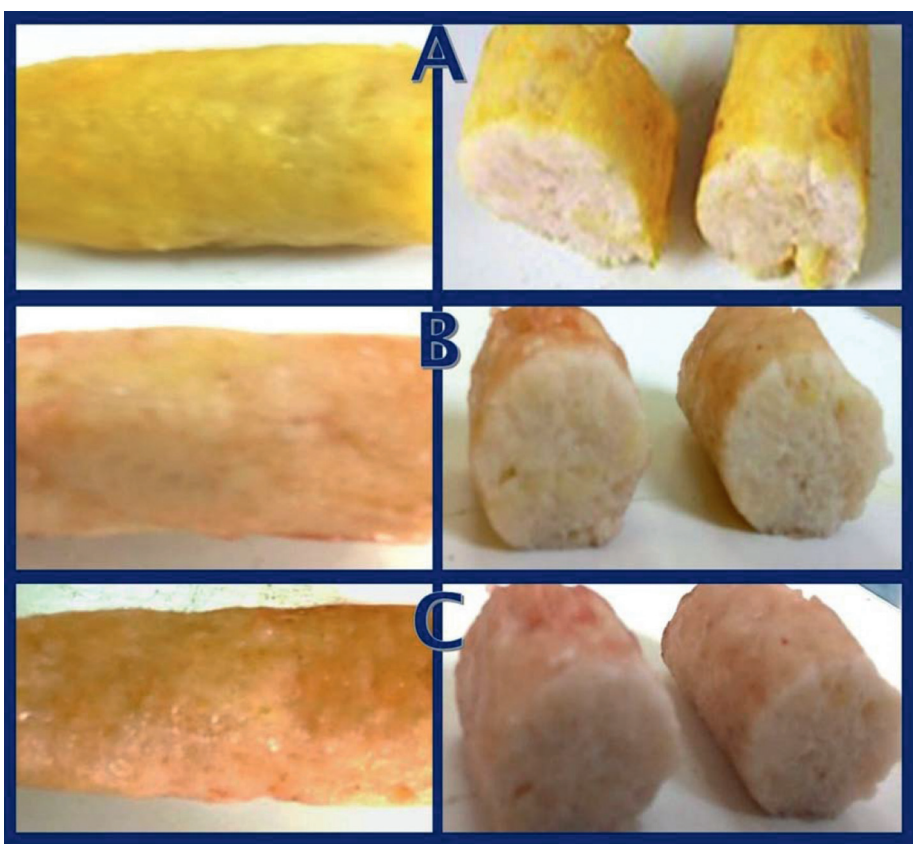

Figure 3: Appearance and visual attributes of colored tilapia fish surimi with extracts of *Curcuma longa* rhizomes (a), *Rhus coriaria* fruits, (b) and *Hibiscus sabdariffa* calyces (c), after 7 days of storage at 4°C.

Mediterranean area as spice, particularly in seafood and meat dishes. The antimicrobial potentiality from sumac was believed to be generated from its bioactive compounds contents [23]; over 120 aromatic constituents were identified in sumac varieties using chromatography and spectroscopy, most of them belonged to terpenoids and aliphatic compounds [24]. Some studies were published about specific antimicrobial substances in *R. coriaria*, which indicated the presence of 3 potentially antibacterial compounds, i.e., 4-methoxy-3,5-di-hydroxy-benzoic acid, gallic acid and methyl ester of 3,4,5-tri-hydroxy-benzoic acid (methyl gallate), in the methanolic extract of *R. glabra* L. These compounds could also represent the responsible bioactive substances in *R. coriaria*, as they belong to the same genus and family [23, 25, 26].

Turmeric (*C. longa*) extract also contains many groups from bioactive substances, e.g., alkaloids, glycosides, flavonoids, tannins, and carbohydrates; numerous reports displayed that flavonoids and alkaloids are from most bioeffective compounds, as antimicrobials, in plant kingdom [27]. The entire fractions of *C. longa* extract were highly effectual against numerous isolates of pathogenic bacteria and the inhibitory effects especially increased in ethanol and hexane extracts of turmeric [28, 29]. As evidenced from microscopically examined bacterial pathogens, exposed microbes to *C. longa* extract appeared with deforming morphology, cell disruption, and lyses of cytoplasmic membrane; this suggested the broad antimicrobial spectrum of the extract and recommended its usage for microbial infections management [22, 27].

The main detected bioactive compounds in *C. longa* extract were reported as curcumin or diferuloylmethane (1,7-bis(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione) oleoresin, curcuminoids, and essential oils [30, 31]. The powerful antimicrobial of curcumin and *C. longa* extract, was proved against several types from viruses, bacteria, fungi, and parasites; their biocidal effects were reported to increase through synergism with ordinary antimicrobial agents; many attempts were also conducted to enhance biochemical attributes of curcumin [32–35].

The roselle (*H. sabdariffa*) calyx was traditionally applied in herbal medicine as herbal drinks, beverages, and flavoring agents in food processing. Most research and clinical investigations revealed that *H. sabdariffa* extract had potent antioxidant, antibacterial, antihypertensive, hepato- and nephro-protective, diuretic/renal effect, and anticholesterol and antidiabetic effects [36, 37]. These effects were suggested to associate with *H. sabdariffa* strong antioxidant activities, suppression of angiotensin-conversion enzymes (ACE), suppression of α-amylase and α-glucosidase, and vaso-relaxant effect. Also, other phytochemical compounds, in *H. sabdariffa* extract, such as organic acids (hibiscus acid and hydroxy-citric acid), phenolic acids (protocatechuic acid), and anthocyanins (cyanidin-3-sambubioside and delphinidin-3-sambubioside) were contributed in these therapeutic effects of the extract [36].

The methanolic/aqueous extract of *H. sabdariffa* proved to have *in vitro* inhibitory potentialities against numerous bacterial species [38]; this biocidal activity was persistent through heat treatment, the alcoholic extract was stronger than aqueous extract, which recommended the application of these extracts for bio-prevention from food contaminants [37, 39]. The coloring potentialities and antimicrobial and antioxidant activities of used CPEs, especially *H. sabdariffa* extract, could improve the sensorial quality and consumers' ability to the produced fish surimi [40].

The impact of HCE exposure, on the cells morphology, viability, and features of *S. aureus*, is verified from Figure 4. The captured SEM micrographs of zero time-treated (control) *S. aureus* demonstrated that most cells had a natural, smooth, and unified structure (Figure 4(a)). Following the exposure to HCE, for 6 h, remarkable vigorous effects were observed on the bacterial cells morphology (Figure 4(b)); most exposed cells had lyses signs and their inner contents were released, the residual semiintact cells were enlarged and notable lyses initiation was seen. By the exposure period completion, to HCE (after 12 h), all exposed bacteria had completely ruptured and lysed; only the cell wall residues and liberated interior cellular contents were detected in this stage (Figure 4(c)).

The bacterial pathogen, *S. aureus,* was chosen for SEM examination after exposure to *H. sabdariffa* extract because it was the most sensitive strain toward examined CPEs, and *H. sabdariffa* extract was the most effectual to inhibit this pathogen. This is expected to provide some clear mode(s) of action from the application of CPEs toward bacterial pathogens.

The SEM micrographs of *S. aureus*, exposed to *H. sabdariffa* extract, could demonstrate that many potentially bioactive substance(s), in the extract, may possess some kinds of metabolic interference with microbial proliferation, development, or functioning. The extract could, also, be suggested to possess time-dependent biocidal potentiality, as evidenced from the vigorous alteration/damage in treated cells' morphology that increased with the prolongation of exposure period. The supposed antimicrobial mode(s) of action of *H. sabdariffa* extract, from SEM micrographs (Figure 4), could be through the destruction/lyses of bacterial cell membranes, cytoplasm coagulation, alteration in proteins of cytoplasmic

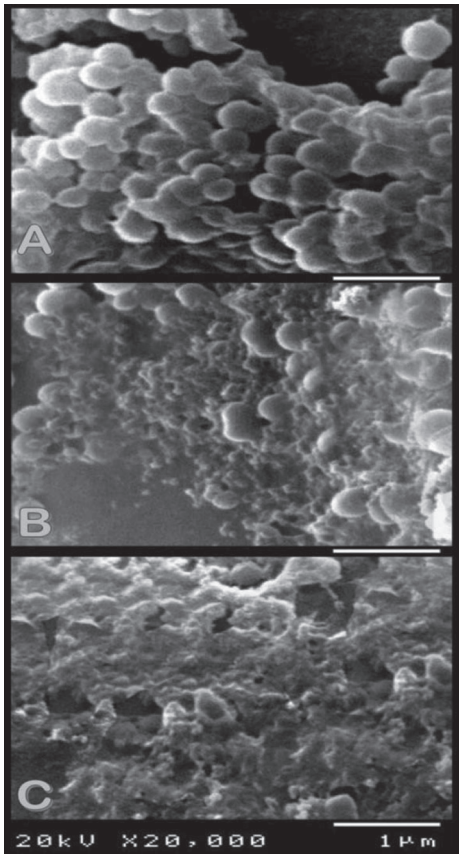

Figure 4: Scanning micrographs of treated *Staphylococcus aureus* with *Hibiscus sabdariffa* calyces extract after exposure for 0 h (a), 6 h (b), and 12 h (c).

membrane, seepage of cell contents, interaction with protein synthesis enzymes, or reducing the motive force of protons [17, 36, 41].

4. Conclusion

The colored extracts of screened plants (i.e., *H. sabdariffa* calyces, *R. coriaria* fruits, and *C. longa* rhizomes) were effectual for inhibiting food-borne bacterial pathogens and augmenting the sensorial attributes of tilapia fish surimi; *H. sabdariffa* extract was the most effectual antimicrobial and *C. longa* extract was the most favorable colorant. The application of CPEs as colorants and antibacterial and quality enhancing agents could be strongly recommended as powerful alternatives to synthetic and chemical agents for the preservation of tilapia fish surimi.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest

The authors declare no conflicts of interest.

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Research Article

Biopreservation of Shrimps Using Composed Edible Coatings from Chitosan Nanoparticles and Cloves Extract

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Shrimps are highly valuable and perishable foodstuff that could be rapidly spoiled. Chitosan (Cht) was extracted and transformed into nanoparticles (NPs) via ionic gelation and fortified with cloves (*Syzygium aromaticum*) buds extract (CLE) for usage as antimicrobial composites against food-borne bacterial pathogens (*Escherichia coli*, *Salmonella typhimurium,* and *Staphylococcus aureus*) and as edible coating (EC) for shrimp (*Fenneropenaeus indicus*) biopreservation throughout refrigerated storage. The synthesized CLE/Cht-NPs were electrostatically cross-linked and appeared with spherical shapes and homogenized distribution, with 159.4 nm mean size diameter and positive charge of 17.4 mV. The entire agents (Cht-NPs, CLE, and CLE/Cht-NPs) exhibited remarkable antibacterial activities toward all food-borne pathogens; CLE/Cht-NPs were significantly the most forceful. The scanning micrographs of treated *S. typhimurium* with CLE/Cht-NPs displayed NPs ability to attach and destroy bacterial cells. The ECs-treated shrimps exhibited sharp decrease in microbial groups load (aerobic microorganisms, *E. coli*, Enterobacteriaceae, and staphylococci) during refrigerated storage ($4 \pm 1^{\circ}$C) for 10 days. Additionally, the sensorial attributes (appearance, odor, color, and texture) of EC-treated samples preserved their elevated qualities for storage duration. The most effective EC blend contained 1.5% from Cht/NPs and 1.0% from CLE. The CLE/Cht-NPs could be impressively recommended as effectual natural composites for shrimps' biopreservation during cold storage.

1. Introduction

Chitosan (Cht) is the acquired polysaccharide from deacetylated chitin, which is the major constituent of crustacean exoskeleton [1]. Both of chitin and Cht can also exist in fungi mycelia and some insects' exoskeleton [2–4]. Cht has numerous astonishing bioactivities including antioxidation, metal chelation, and inhibition of cancerous cells and microbial pathogens (fungi, yeast, and bacteria) [2, 3, 5]. Cht was stated as effectual antibacterial materials (toward both Gram positive and negative bacteria) with approved GRAS (general recognition as safe) nature [1, 3].

Nanoparticles (NPs), synthesized from natural or synthetic polymers, are very effectual with their particles' size range from 10 to 1000 nm [6]. Polymers NPs show astonishing chemical and physical features, resulting from their effects, e.g., the macroquantum tunnel, quantum size, mini size, and surface effects. Chitosan nanoparticles (Cht-NPs) were proved as natural materials with excellent physicochemical, biological, and structural properties, along with their eco-friendly and bioactive nature [6, 7].

Cht-NPs have the nanoparticles properties such as surface and interface effect, quantum size effects, and small size, along with the original Cht bioactive characteristics [4].

Cht-NPs can be prepared by multiple methods; the most frequent is the ionotropic gelation between Cht and sodium tripolyphosphate (TPP). Cht/TPP-NPs were frequently used as controlled-release drug carrier and for the effectual delivery of bioactive compounds [8–11].

The most important group of Crustacea (crustaceans) is shrimps; they belong to phylum Arthropoda and order Decapoda. They are distributed in the whole world and live in aquatic environments [12]. Due to shrimp's high water activity value, it is a highly perishable food and susceptible to fast spoilage [13]. The quality and shelf life of shrimp during storage are influenced by oxidation, microbiological activities, and enzymatic changes, which mostly correlated with the activity and growth of Gram negative aerobic bacteria [14].

Cloves (*Syzygium aromaticum*, Fam. Myrtaceae) are commonly used spices worldwide. The cloves oil main properties are the antifungal, antioxidant, antibacterial, and insecticidal effects; clove is also widely used for improving flavor and as antimicrobial substance in food preservation [15, 16]. Cloves are supposed to be excellent sources of minerals (manganese, calcium, and magnesium), vitamin C, dietary fiber, Ω-3 fatty acids, and vitamin K [17]. *S. aromaticum* extract and oil contain a significant amount of carbohydrates, proteins, calcium, iron, phosphorus, sodium, potassium, and also rich in vitamins C and A [18, 19]. Cloves buds extract and oil were additionally verified as potent antimicrobial agents that could hinder growth of numerous microbial pathogens including *Candida albicans*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Shigella flexneri*, *Escherichia coli*, many *Salmonella* spp., dermatophytic fungi, and Enterobacteriaceae [15, 16, 19–22]. Additionally, *S. aromaticum* extract was suggested for natural preservation of fisheries products (fish fingers), alone or combining with other plant extracts [23].

Edible coatings (EC) were defined as "the thin layers of materials that cover food surfaces and can be eaten and considered as a part of the whole food product" [24]; the major benefits of ECs are that they can provide additional nutrients, be consumed with the food, contain quality-enhancing antimicrobials, and enhance sensory characteristics. Edible coatings also act as barrier to external elements such as oil, moisture, and vapors, to protect food, prevent dehydration, and extend the shelf-life [24–26].

Although various investigations evaluated the incorporation of Cht and Cht-NPs with *S. aromaticum* oil, for applying as ECs to preserve foodstuffs including shrimps and pork meat [26–29], it is presumed that literature could not provide sufficient researches concerning the incorporation of cloves' whole crude extract with chitosan nanoparticles and their usage as edible coatings for shrimp biopreservation. The fortification of Cht and Cht-NPs based ECs with plant extracts was recommended to augment the antimicrobial and antioxidant attributes of the composited EC [28–30], but cloves extract was not sufficiently investigated as a fortifier in the Cht-NPs based ECs.

Accordingly, the synthesis of Cht-NPs and their fortification with clove extract were planned for this study. The evaluation of these agents as antibacterial blends, *in vitro,* and as edible coatings, and their applications for biopreserving shrimps and upholding their qualities was also planned.

2. Materials and Methods

2.1. Chitosan Preparation. The extraction of Cht based on shells wastes of white prawn (*Fenneropenaeus indicus*), farmed in Aquaculture farm, Kafrelsheikh University. After shells cleaning and drying, they were immersed in 30 folds from 2.0 N NaOH solution (w/v, Sigma-Aldrich, MO) for 24 h; then after extensive washing with deionized water (DW), neutral materials were reimmersed in 30 folds from 2 N HCl solution (w/v, Sigma-Aldrich, MO) for 24 h, followed by extensive washing with DW. The resulting chitin was dried and immersed in 50% NaOH solution (with ratio of 1 g of chitin powder/13 ml of NaOH) and then heated in an oil bath at 125°C for 5 hours for obtaining chitosan. The molecular Cht weight was determined via CGP (chromatographic gel permeation), whereas its DD (deacetylation degree) measurement was based on Cht spectra using FTIR spectroscopic (Fourier transform infrared spectroscopy, FTS 45, Biorad, Germany).

2.2. Clove Extract Preparation. Dried cloves buds (*Syzygium aromaticum*) were obtained from ARC (Agricultural Research Centre, Giza, Egypt). The pulverized plant materials (60 mesh size) were immersed in 8 folds (w/v) from ethanol (70%, Sigma-Aldrich, MO) and agitated for 22 h at 160 x g speed in room temperature (25 ± 2°C). The plant residues were excluded via filtration and the result cloves extract (CLE) was rotary evaporated (IKA, RV 10, Germany) at 44°C until dryness [31]. The dried CLE was dissolved in 2% Tween 80 solution (in DW) to concentration of 1 mg/ml, and this solution was applied in further experiments.

2.3. Chitosan/Clove Extract Nanoparticles Preparation. For the synthesis of Cht-NPs and loading them with CLE, the following solutions were prepared according to Almutairi et al. [11]: Cht (1 mg/ml in 1% acetic acid solution), TPP, Sigma-Aldrich, MO (0.5 mg/ml in DW), and CLE (1 mg/ml of 2% Tween 80 solution in DW). The pH of Cht solution was adjusted to 5.2; then the TPP solution was slowly dropped onto Cht solution (while vigorously stirred at 620 x g) using a syringe needle at rate of 0.35 ml/min, until reaching equal volume from both solutions. For CLE/Cht-NPs formation, the previous steps were conducted with the addition of equal volume from CLE to Cht before TPP dropping. The formation of opalescent suspensions, after TPP dropping and persistent stirring for 110 min, indicated NPs formation. The formed NPs pellet was attained via centrifugation (SIGMA, 2–16 KL, Germany) at 11500 x g for 35 min, washing with DW and recentrifugation. The obtained NPs pellets were lyophilized and subjected to analysis.

2.4. Analysis of CLE/Cht-NPs Physiognomies

2.4.1. Structural Analysis. The morphological and organizational features of CLE/Cht-NPs (shape, size, and distribution) were appraised via spectroscopy (photon

correlation, PCS, Malvern™ Zetasizer, Malvern, UK), for determining the size, charge, and distribution of NPs, whereas the electron scanning microscopy (SEM, JEOL JEM IT-100, Tokyo, Japan) designated the CLE/Cht-NPs morphology and dispersion; the SEM imaging was conducted at 20 kV and x15000.

2.4.2. FTIR Spectral Analysis. The infrared analysis using FTIR (Fourier transform infrared spectroscopy, Perkin Elmer™ FTIR-V. 10.03.08, Germany) of synthesized Cht-NPs and CLE/Cht-NPs was perceived after samples' integration with 1% KBr, in transmission mode (at wavenumber range of 450–4000 cm^{-1}).

2.5. Antibacterial Evaluation of Natural Products

2.5.1. Bacteria Cultures. Three standard food-borne pathogens were employed for antimicrobial screening, that is, *Escherichia coli* ATCC-25922, *Salmonella typhimurium* ATCC-14028, and *Staphylococcus aureus* ATCC-25923. The entire bacterial strains were maintained and subcultured onto the nutrient agar and broth (NA and NB, Difco Laboratories, Detroit, MI), aerobically at 37°C.

2.5.2. Qualitative Antimicrobial Assay. The inhibitions zones (IZ), which appeared after disc diffusion test, were considered as indicators for antibacterial bioactivity of produced agents. Sterile Whatman No. 4 paper discs (6 mm diameter) were impregnated with 25 μL from 2% solutions of Cht-NPs, CLE, or CLE/Cht-NPs and placed onto freshly inoculated NA plate with each bacterial culture. The plates were then upturned incubated at 37°C for 18–24 h and the IZs diameters were measured using a precise caliper; then their triplicates means were calculated as ± standard deviation [3].

2.5.3. SEM Imaging of Treated Bacteria. The morphological variations in *S. typhimurium* cell surfaces, after exposure to CLE/Cht-NPs solution (2.0%, w/v), were screened via SEM imaging, after 0, 5, and 10 h of exposure and incubation at 37°C, as formerly described [32]. The SEM micrographs were captured at 20 kV and x10000, depending on the appeared distortions in bacterial cells layouts.

2.6. Treatment of Shrimp with Cht-NPs-Based Edible Coating

2.6.1. Edible Coating (EC) Preparation. For the EC solutions preparation, 1.5% Cht-NPs (w/v) was slowly dissolved in DW (at 100°C for 45 min, with stirring at 450 x g); the solution was cooled to 45°C during stirring; then 1% (v/v) acetic acid and 0.25 mL from the plasticizer glycerol per gram of Cht-NPs were added [33]. After stirring for additional 30 minute (during which the temperature of the coating solution decreased to approximately 37°C), clove extract dispersed in 2% Tween 80 solution was added to the EC solution by gradual concentrations (w/v).

2.6.2. Application of Coatings on Peeled Shrimp. Freshly harvested shrimps (*F. indicus*), after manual deheading, deshelling, and cleaning with DW, were divided into five groups (each of them consisted of 25 shrimps with weight of~10 ± 1 g/shrimp). The first group was the uncoated control (C), and the other 4 groups of shrimp were immersed into Cht-NPs based EC solutions incorporated with clove extract (CLE) with the following order: group (Ch) contained Cht-NPs only and groups Ch-Cl 0.5, Ch-Cl 1.0, and Ch-Cl 1.5 were fortified with 0.5, 1.0, and 1.5% (w/v) from CLE, respectively. The shrimps coating was performed through dipping of samples in EC solutions (for 30 min at 4°C), at 1 : 2 ratio (w/v) from shrimp/EC solution; then coated shrimps were drained for 5 min at 25 ± 2°C. Samples were then packaged in polystyrene trays and each tray was wrapped with plastic films and held at 4 ± 1°C for 10 days and at RH (relative humidity) of 65 ± 3% and sampled at 2 days intervals for further analyses [34, 35].

2.6.3. Microbiological Examination. The EC-treated and control shrimps were sampled aseptically (15 g/sample), immersed in 135 mL of buffered peptone solution (0.1%, LAB M, Lancashire, UK) in a stomacher sack, and then homogenized for 3 min using a Seward Stomacher 400 (Norfolk, UK). Serial dilutions from shrimp homogenization were made in NB and screened for the counts from different microbial groups via plating onto recommended agar media as illustrated by the standard microbiological protocols:

Total aerobic microorganisms enumeration of colony count at 30°C (**ISO 4833-1: 2013**) [36].

Enumeration of *Escherichia coli* (β-glucuronidase-positive) **(ISO 16649 -1: 2018)** [37].

Enterobacteriaceae detection and enumeration **(ISO 21528 -2: 2017)** [38].

Coagulase-positive staphylococci enumeration **(6888 -1: 2018)** [39].

2.6.4. Sensory Analysis. A well-trained panelist team (13 members; 8 females and 5 males), with experience in seafood judgments, was involved in evaluating the sensory attributes of EC-treated shrimps. The panelists were inquired to assess the samples appearance, odor, color, and texture, using a ranged hedonic scale from 1 (extremely poor) to 9 (extremely good) [31].

2.7. Statistical Analysis. The entire experiments were triplicated, with presented data as means ± SD. The SPSS package (SPSS V-11.5, Chicago, IL, USA) was applied for statistically analyzed data using *t*-test and one-way ANOVA at $p < 0.05$.

3. Results and Discussion

Chitosan was efficaciously extracted from shrimp wastes; the produced Cht powder had a creamy-white color, a 38.3 kDa molecular weight (MW), and a DD of 89.4%. The DD of

≥70% and the MW of produced Cht confirmed the transformation of shrimp chitin to low MW chitosan [40].

3.1. Physiognomies of Synthesized Clove Extract/Chitosan Nanoparticles

3.1.1. NPs Structural Attributes. Size (including size distribution) is an important characteristic parameter for nanosuspensions [41]. Particles size distribution of synthesized clove extract/chitosan nanoparticles is shown in Figure 1(a). The CLE/Cht-NPs had a mean diameter of 159.4 and median of 165 nm and their particles' size ranged between 142.3 and 179.1 nm. The charges of synthesized NPs were 24.3 mV for Cht-NPs and 17.4 mV for the composited CLE/Cht-NPs.

Morphology and microstructure of CLE/Cht-NPs were evaluated by SEM (Figure 1(b)). Electron micrograph of the sample demonstrates presence of spherical structures, absence of cracks, and creation of a continuous layer onto NPs surfaces. The SEM image for clove extract chitosan nanoparticles illustrates spherical shape and regular distribution, which seems separated and well stable during the preparation process [42, 43].

3.1.2. Fourier Transform Infrared Spectroscopy. The FTIR spectra of Cht-NPs and composited CLE/Cht-NPs are given in Figure 2. The most significant peaks of plain Cht-NPs spectrum were as follows: 3428.59 cm^{-1} (stretching vibrations of O–H and N–H), 2927.26 cm^{-1} (aliphatic C–H stretching vibration), 3068.88 cm^{-1} (CH_2 stretching vibration), 3027.81 cm^{-1} (C–H_3 stretching vibration), 1702.25 cm^{-1} (C=O stretching of amide I), 1664.02 cm^{-1} (NH of amide II stretching vibration), 1110.29 cm^{-1} (C3–OH stretching vibration), and 1039.48 cm^{-1} (C6–OH stretching vibration) [7, 44]. Also, in Cht-NPs spectrum, the band at 3450 cm^{-1}, which has lower wideness than bulk Cht indicates reduced hydrogen bonding. The reduced intensity of hydrogen bonding in the cross-linked NPs complexes is owing to more open structure caused from cross-linking with TPP [44].

A new sharp peak at 1625 cm^{-1} appeared, the amine bending intensity at 1625 cm^{-1} goes down, which assumingly attributed to linkage between TPP and the ammonium group of Cht-NPs [9, 10]. FTIR analysis of combined CLE with Cht-NPs showed a band at 3450 cm^{-1}, which could indicate the reduced hydrogen bonding in Cht-NPs and/or the O–H groups of alcoholic CLE. Another band at 1687.5 cm^{-1} represented ester group frequency patterns C–O or the aromatic ketone group C=O that combines with more than ring. At the wavenumber of 1562.5 cm^{-1}, the detected band designates matched patterns to the aromatic carbonyl group belonging to quinine [45], which is close to the aromatic group (C=C). The bands at 780 cm^{-1} and 1330 cm^{-1} could represent the CH_2 group frequency pattern [46].

3.2. Antibacterial Activity of CLE/Cht-NPs against Food-Borne Pathogens

3.2.1. Qualitative Assay. The antibacterial activities of produced natural products, i.e., clove extract (CLE), nanochitosan (Cht-NPs), and clove extract with nanochitosan (CLE/Cht-NPs), expressed as IZ diameters, are shown in (Table 1). The antibacterial activities, toward the entire food-borne pathogens, were significantly more forceful from CLE/Cht-NPs and then from Cht-NPs and CLE, respectively. *S. typhimurium* was the most susceptible strain to screened compounds compared to *E. coli* and *S. aureus*, as evidenced from the wider IZs. Larger IZ typically indicates higher antibacterial activity of tested solutions against challenged microbial species [20].

The Cht-NPs antimicrobial potentialities were recurrently confirmed from many investigations [4, 6, 9, 44]; they indicated that Cht-NPs had more forceful action than native Cht for inhibiting numerous pathogenic microorganisms. The Cht-NPs antibacterial action was principally attributed to their increased positive charges, which enable them to interact with microbial cells' surface and interior vital components (DNA, RNA, enzymes, etc.) and thus prohibit microbial growth and survival.

The alcoholic CLE contains plenty of bioactive compounds belonging to revealed existence of flavonoids, tannins, alkaloids, glycoside, steroids, terpenoids, ketones, aldehydes, and phenolics; more than 46 phenolic constituents were detected and quantified in CLE [22, 23], several compounds from these phytochemicals possess antimicrobial potentiality. Additionally, the high CLE content from tannins (10–19%) was also verified to augment its antimicrobial activity [21]. The major detected phytoconstituents in CLE and oil are eugenol, *a*- and *ß*-caryophyllene, eugenyl acetate, limonene, *a*-copaene, and *a*-terpinolene [47]. Eugenol and caryophyllene (the highest occurring constituents in CLE and oil) were verified to possess strong antimicrobial (antibacterial, antimycotic, and antifungal) properties toward numerous pathogenic species [17]; the crude ethanolic CLE exhibited also comparable antimicrobial powers against several food-borne strains as the essential oil [16].

The synergistic action of the composited Cht-NPs and CLE was stronger than each of the individual agents; this is because the microbial pathogen cannot resist a composite from different antimicrobial agents with diverse action modes [9, 31].

3.2.2. SEM Detection of Morphological Alterations in CLE/Cht-NPs-Treated Bacteria. The SEM image shows how bacterial pathogen (*S.* typhimurium) was influenced by CLE/Cht-NPs treatment (Figure 3). At zero time of treatment, there is no apparent change in bacterial cells wall/membrane; cells appeared with regular shapes, smooth and uniformed surfaces (Figure 3(c)). After 5 h of CLE/Cht-NPs exposure, *S. typhimurium* cell appeared with swollen and partially lyses manifestation (Figure 3, 5 h), many NPs were appeared to attach/interact with exposed bacterial cells. With prolongation of CLE/Cht-NPs exposure to10 h, most of bacterial cells were severely damaged; the lyses/explosion signs in their walls became obvious, the interior cellular components were released and composited with NPs (Figure 3, 10 h).

The CLE/Cht-NPs composite had powerful activity to deteriorate *S. typhimurium* cells; this is assumed to result

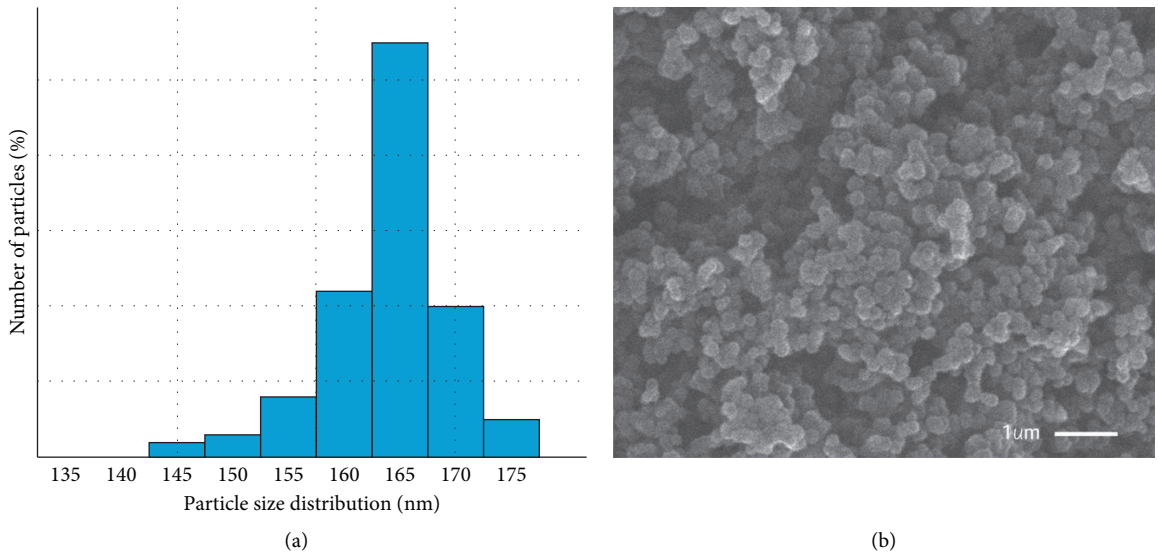

Figure 1: Structural features of synthesized clove extract/chitosan nanoparticles including their particles size distribution (a) and morphological attributes using scanning micrographs (b).

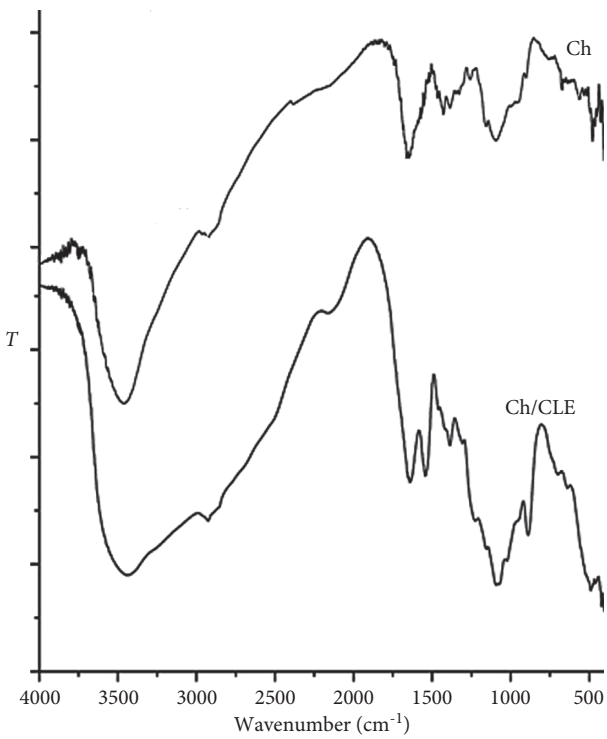

Figure 2: FTIR spectra of synthesized nanochitosan (Ch) and combined clove extract with nanochitosan (Ch/CLE).

from the combined bioactivities of CLE and Cht-NPs. The bioactivity of eugenol (the major component of CLE) was stated to enable it for deteriorating bacterial membrane through serial mechanisms that alter cell morphology, prompt leakage of cellular constituents, and increase permeability [48].

In addition, the electrostatic interaction between the positively charged Cht and negatively charged *S. typhimurium* walls/membranes could increase Cht-NPs attachment to bacteria and their penetration into their interior organelles; thus they can severely alter the negative-charged bacterial cell walls/membranes (generated from their constituents of lipopolysaccharide and teichoic acid) [49], and interfere with cells' vital functions and components to stop them, hinder cells progress, and disrupt their outer membranes [1, 3, 6, 50].

The bactericidal actions of Cht, resulting from its adsorption onto microbial surfaces and induction of cell walls' lyses, were described and stated [3, 9, 31, 51]; the intensity of absorbed particles depended on Cht concentration, its MW, contact time, challenged microorganisms, and the charges on their cells. Many of preceding investigations stated the disruption/explosion of microbial cells as a consequence after their exposure to Cht [1–3].

The wide antimicrobial spectrum (antibacterial, antimycotic, and antifungal) from *S. aromaticum* oil, crude extract, and their constituents, was indicated [15, 52]; they suggested that *S. aromaticum* antimicrobial actions could include the interaction with microbial cells, proteins' denaturation and reactions with phospholipids in cell membranes, which suggested to affect microbial membranes' permeability.

The treated cells of pathogenic Gram-negative bacteria (*S. enteritidis* and *E. coli*) with ethanolic CLE appeared with uniform, coarse surfaces, and withered and irregular morphology, with formed adhesions and aggregations, which suggest that CLE led to severe damages in bacterial cell membranes [53].

3.3. Biopreservation of Shrimp through CLE/Cht-NPs Edible Coatings

3.3.1. Impact on Microbiological Quality of Stored Shrimps. The influences of shrimps' coating with composed EC contained 1.5% w/v from Cht-NPs and its fortified blends

Table 1: Antibacterial activity of produced natural products; clove extract (CLE), nanochitosan (Cht-NPs), and their composites (CLE/Cht-NPs) against examined food-borne pathogens.

Antibacterial agent	Inhibition zone (mm)*		
	Salmonella typhimurium	*Staphylococcus aureus*	*E. coli*
CLE	8.9 ± 0.4^{a}	8.4 ± 0.6^{a}	8.7 ± 0.7^{a}
Cht-NPs	9.3 ± 0.3^{a}	8.8 ± 0.5^{a}	9.0 ± 0.6^{a}
CLE/Cht-NPs	12.7 ± 0.9^{b}	12.1 ± 0.8^{b}	11.4 ± 1.1^{b}

*Results represent means of triplicates ± SD. **Different superscript letters in the same column indicate significant difference at $p < 0.05$.

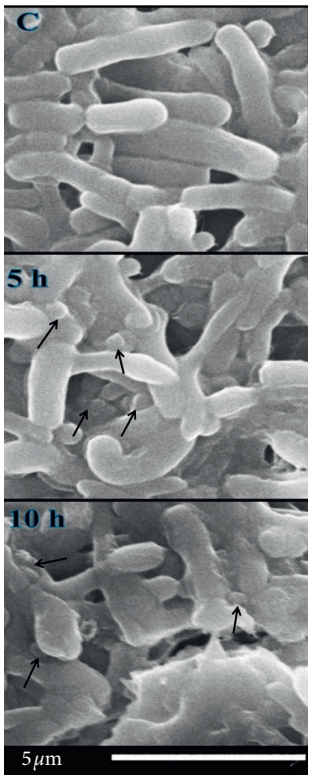

Figure 3: Scanning micrographs of treated *Salmonella typhimurium* cells with clove extract/chitosan nanoparticles* for 0, 5, and 10 (h) *The arrows in micrographs indicate selected examples of interacted NPs.

with CLE at percentages of 0.5, 1.0, and 1.5% (w/v), on the microbial load during refrigerated storage at 4 ± 1°C, are illustrated (Figure 4). While all examined microbial groups (Total aerobic microorganisms, *E. coli*, Enterobacteriaceae and coagulase-positive staphylococci) tended to sharply increase, in control (uncoated) groups, with storage time prolongation, the inhibitory effects of ECs on these microbial groups were very remarkable.

The ECs formulation could significantly reduce the microbial loads in treated shrimps, compared with uncoated control samples. Generally, the EC antimicrobial efficacy increased with CLE percentage increment; the most effectual EC mixture contained 1.5% w/v from Cht-NPs with 1.0 or 1.5% from CLE. These combinations could reduce the counts of *E. coli* and coagulase-positive staphylococci to zero after 4 and 6 days of cold storage, respectively. The Cht-NPs based EC was remarkably effective in hindering microbial growth, and its efficacy notably increased with CLE fortification.

The strong antimicrobial potentialities (of both Cht and Cht-NPs) were proved and confirmed against numerous species from bacteria (Gram positives and negatives), fungi, and yeasts [1–3, 6, 9, 49, 51], either using *in vitro* evaluation or in food models [5, 24, 32, 33, 54]. These antimicrobial potentialities of Cht were attributed to its superficial bioactive positive charges, high chelation, and antioxidant capabilities, and its strong interactions with microbial cells components. The Cht-NPs were reported to have extra bioactivities and powers than bulk Cht due to their interacting surface area and their minute size that lead to more effectual interactions with microbial cells [4, 6, 8, 43]. The microbial inhibitory action of Cht-NPs coating, especially toward aerobic microorganisms, is assumingly attributed to oxygen parrying capability of Cht coating films that prevents the essential O_2 penetration for microbial breathing [23, 24, 33].

The antimicrobial consequences generated from *S. aromaticum* extract and oil were recurrently stated toward wide pathogenic microorganisms varieties, especially food-borne species [15, 16, 19, 22, 47]; they mainly attributed these microbicidal impacts to CLE and oil contents from bioactive constituents, particularly eugenol, caryophyllene, and other phenolics. The high amounts from TPC (total phenolic compounds) in CLE were suggested as the main reason for its influential antimicrobial activity [47, 53]. The combined synergistic antimicrobial effect of CLE and Cht-NPs persisted for the duration of storage time, and that was illustrated for many formulated EC from Cht and other plant extracts [24, 28, 34, 35, 55].

3.3.2. Impact on Sensorial Quality of Stored Shrimps. The influences of shrimp coating with 1.5% solution from Cht-NPs and their fortified blends with CLE (at 0.5, 1.0 and 1.5% concentrations), on the sensorial attributes after refrigerated storage (4 ± 1°C) for 7 days, are presented in Figure 5.

Whereas, in the beginning of the EC experiment, no significant differences were observed for the examined sensory attributes of all samples (data not included), the panelists scores for the examined attributes (appearance, odor, color, and texture) revealed that the control (uncoated) sampled became inconsumable after this time, regarding the acceptance level of 5/9 as the limiting value. The entire sensorial scores for the control group were significantly much lower than EC treated samples. The best composition of EC blends, to preserve appearance, odor, and color of stored shrimps, contained 1.5% from Cht/NPs and 1.0% from CLE, whereas the best for texture attribute contained 1.5% from both agents.

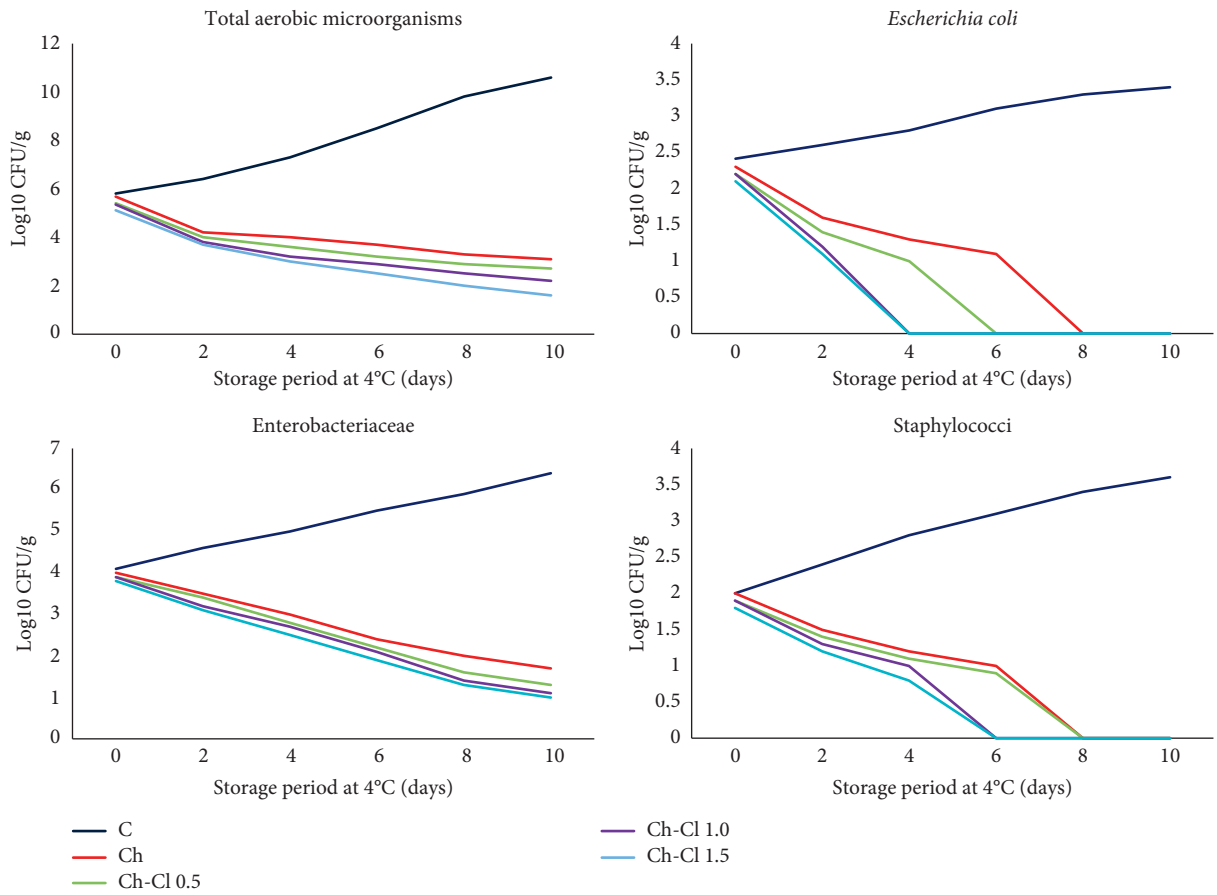

Figure 4: Influence of shrimp coating with 1.5% solution from nanochitosan (Ch) and fortified nanochitosan solution with 0.5, 1.0, and 1.5% (w/v) from clove extract (Ch-Cl 0.5, Ch-Cl 1.0, and Ch-Cl 1.5, respectively) on the microbial load during refrigerated storage (4 ± 1°C) for 10 days, compared to control (C)*. *Results represent means of triplicates.

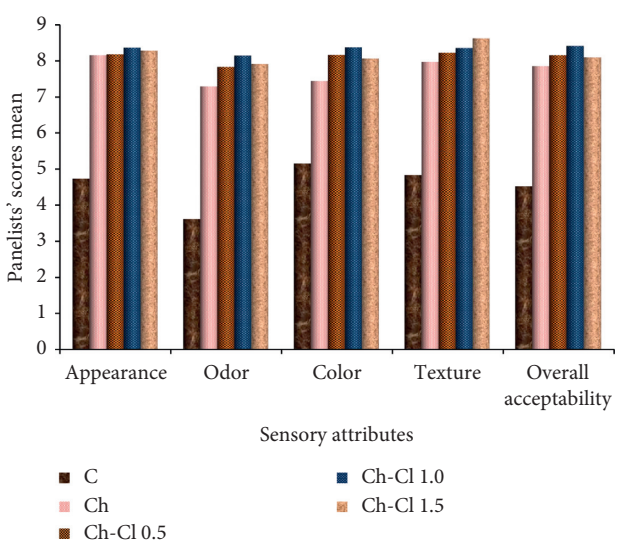

Figure 5: Influence of shrimp coating with 1.5% solution from nanochitosan (Ch) and fortified nanochitosan solution with 0.5, 1.0, and 1.5% (w/v) from clove extract (Ch-Cl 0.5, Ch-Cl 1.0, and Ch-Cl 1.5, respectively) on the sensory attributes after refrigerated storage (4 ± 1°C) for 7 days, compared to control (C)*. *Results represent means of panelists' sores ($N = 13$).

The quality upholding of appearance, color, and texture attributes, in EC treated shrimps, was notably evidenced, compared with non-EC (control) shrimps (Figure 6). Many health benefits from seafood are principally attributed to their elevated contents from beneficial lipids, especially omega 3 and long-chain PUFA (poly unsaturated fatty acids) [56]. But, these valued constituents in seafood are extremely susceptible to oxidation (resulting from autoxidation, photosensitized oxidation, peroxidation, lipoxygenase, or microsomal enzymes), which lead to off-flavors emergence, at any storage conditions.

The application of Cht-NPs and CLE-Cht-NPs based ECs for shrimps biopreservation led to sensory quality maintenance through the protection from oxidation. Cht was reported as powerful antioxidant material and chelating agent; this is assumed to make it able to bind with oxidation enzymes and inhibit their effects [32]. The oxygen parrying effect of Cht-NPs-based EC also protected shrimps from oxidative reactions that regularly happened [23, 24, 32, 35, 55]. Additionally, the efficacy of Cht to control lipids oxidation in cooked cod (*Gadus morhua*) was attributed to the influential capability of it for metal chelation [57].

The sensorial quality maintenance, in CLE/Cht-NPs coated shrimps, is noticeably correlated with their lowered microbial load after EC treatments, which is assumed to

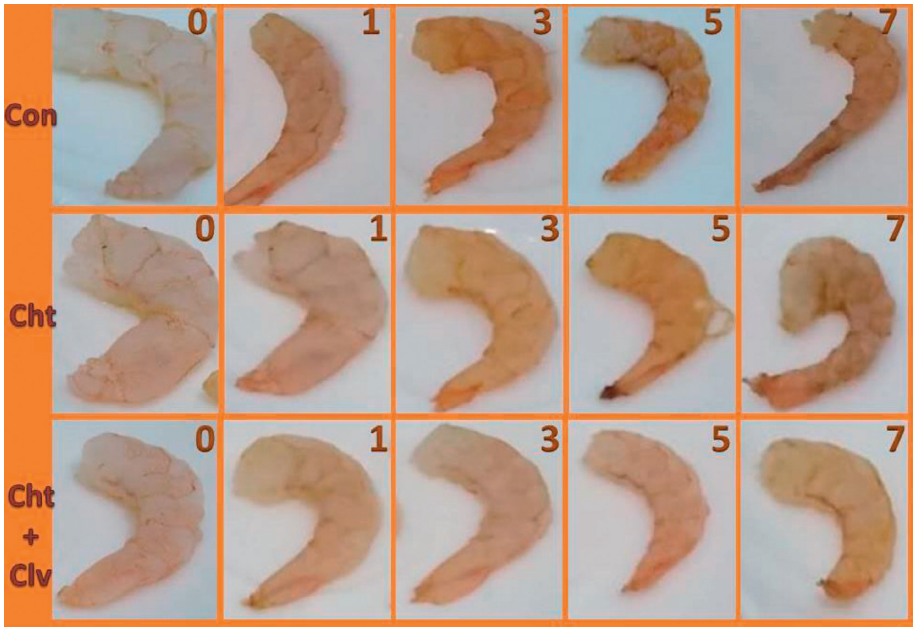

Figure 6: Influence of shrimp coating with 1.5% solution from nanochitosan (Cht) and fortified nanochitosan with 1% clove extract (Cht + Clv) on the appearance and texture of samples compared to control (Con) after refrigerated storage (4 ± 1°C) for 0, 1, 3, 5, and 7 days.

retard the microbial spoilage, melanosis formation and biochemical decompositions [35].

Many investigations recommended the application of Cht coatings and their blends with plant extracts/oils for shrimp biopreservation [24, 28, 34, 35, 55]. The achieved results here, for the microbiological and sensorial qualities, auspiciously exceeded those reported in these studies, which may be because of the usage of Cht-NPs (with higher antimicrobial and antioxidant activity than bulk Cht) in EC blends and the effectiveness of CLE for strengthen the NPs bioactivities. The CLE and oils convincingly stated to possess numerous phytoconstituents with high antimicrobial and antioxidant potentialities [16, 17, 19, 22, 47, 53]; these bioactivities are proved here to protect stored shrimps from spoilage signs and to augment the activity of Cht-NPs based coatings to uphold the shrimps' qualities during cold storage.

4. Conclusion

Toward the elimination of pathogenic food-borne bacterial pathogens and preserving the microbiological and sensorial qualities of stored shrimps, the bioactive formulations from nanochitosan (Cht-NPs) and cloves buds extract (CLE) were innovatively composited. The structural physiognomies of CLE/Cht-NPs were proved and their remarkable antimicrobial potentialities against bacterial pathogens were confirmed, along with their potential action modes. The coating treatment of unpeeled shrimps with CLE/Cht-NPs solutions resulted in sharp decrease in the microbial loads and the upholding of stored shrimps sensorial qualities during cold storage for 7 days. The CLE/Cht-NPs could be impressively recommended as effectual natural composites for the biopreservation of shrimp during cold storage. Future investigations are suggested for further appraisal of CLE/Cht-NPs biopreservation actions in different foodstuffs and their biomedical consequences.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest

The authors declare that there are no conflicts of interest.

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