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Natural Alternative Antimicrobial Compounds to Improve Food Safety and Quality

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Research Article

Antimicrobial Activity of Quinoa Protein Hydrolysate against *Streptococcus pyogenes* and *Escherichia coli*

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Quinoa seed, as a rich source of protein with strong antioxidant properties, plays an important role in improving consumers' nutrition. This study was aimed at comparing the antimicrobial activity of peptides from quinoa hydrolysed proteins (QHP) on *Streptococcus pyogenes* as a Gram-positive and *Escherichia coli* as a Gram-negative bacterium with gentamicin antibiotic as a positive control. Different enzymatic ratios of pepsin and alcalase (30–90 AU/kg protein) at different temperatures (50–55°C) and times (150–210 min) were used to determine the optimal conditions for peptide hydrolysis with the highest antimicrobial properties. Similar to gentamicin, the maximum growth inhibition zones were 11.88 ± 0.37 mm and 12.49 ± 0.58 mm for *S. pyogenes* and *E. coli*, respectively, with an enzyme/substrate ratio as 60 AU/kg protein, a peptides concentration of 800 μg/ml, and at 50°C for 150 min of hydrolysis. The results showed that QHP has a good inhibitory effect on the bacteria mentioned and can be used as a food preservative.

1. Introduction

The health adverse effects of chemical preservatives including their carcinogenic and teratogenic properties as well as their toxic residues are being proven every day, while the demand for long shelf-life foods is rising [1]. Thus, providing natural alternatives is of utmost importance. In recent years, characterizing natural antioxidants has received special attention, which leads to studies on antioxidant and antibacterial capacity of peptides derived from hydrolysed proteins of a variety of food resources, e.g., soy protein [2], casein [3], egg white protein [4], seeds of river tamarind [5], sesame seed [6], cowpea [7], Okra seed meal [8], and fish proteins [9].

Protein hydrolysis is a beneficial technology for providing high value-added products with antioxidant and antimicrobial activities known as bioactive peptides [10–12]. Bioactive peptides are defined as protein components that are inactive in the core protein structure and exhibit various physicochemical functions after being released by enzymatic hydrolysis [13, 14]. They have a positive impact on body functions and ultimately promote health quality [15–17].

Enzymatic hydrolysis (especially by pepsin and trypsin enzymes) is the most common path to produce potent bioactive peptides [18]. They are known to exert functional operations, i.e., antimicrobial, antioxidant, antithrombosis, and antihypertension properties as well as immune system regulation and mineral binding [13, 19, 20]. Among plant food sources rich in protein are quinoa seeds that are originated from South America and consumed for more than 5,000 years. Due to its botanical characteristics, quinoa is considered as a pseudo-cereal [21, 22].

It is known to be more digestible compared to many grains such as rice probably due to its high fiber content [22, 23]. This plant is well known for adapting to various climates and soils. In recent years, its cultivation in Iran has been started as well [24].

According to studies, quinoa cultivation is beneficial compared to wheat and rice owing to its lower water requirement and salinity tolerance [25]. Wheat, rice, and similar cereals can be replaced by quinoa for children, those who suffer from diabetes, celiac, and also those with special diets. Moreover, due to the fact that quinoa is rich in protein, magnesium, fiber, phosphorus, vitamin B_2, potassium and mineral (e.g., iron) contents and contains essential amino acids lysine and methionine, it can provide the body with complete protein and alleviate malnutrition [10, 21, 26–30]. Quinoa protein has been successfully used in antimicrobial edible coatings as a bio-preservative in food product packages [31, 32]. In addition, a recent study reported that the antimicrobial and antioxidant attributes of fresh burgers incorporated by quinoa peptide-loaded nano-liposomes were significantly improved [33]. Although the antibacterial and antioxidant properties of many local traditional seeds and plants in Iran have been extensively assessed [24, 34]; no much research on the antimicrobial activity of quinoa has been conducted.

Considering the high percentage of protein content in quinoa compared to other cereals (25% more), its hydrolysed protein and resulting bioactive peptides can exhibit high antibacterial activity against the Gram-positive and Gram-negative bacteria.

Streptococcus pyogenes is known as one of the major pathogens, associated with pharyngitis and deep tissue infections. Although it is not generally considered a food-borne pathogen [35, 36], outbreaks of food-borne pharyngitis have been rarely reported due to poor personal and hand hygiene [36–39]. This Gram-positive bacterium is known to have a significantly long-term survival, i.e., 2 to 88 h depending on the surface type [40, 41].

On the other hand, as a Gram-negative model bacterium, *Escherichia coli* is a well-studied food-borne pathogen, and its survival on surfaces is 2–36 days depending on the surface type [41]. Hence, the present study aimed to prepare quinoa protein hydrolysate and investigate its antimicrobial effect on bacterial strains of *Streptococcus pyogenes* (*S. pyogenes*) and *Escherichia coli (E. coli)* compared to that of gentamicin as the positive control.

2. Materials and Methods

2.1. Chemicals and Reagents. All chemicals and reagents in the analytical grade (purity >99%) were purchased from Merck (Darmstadt, Germany). Quinoa (*Chenopodium quinoa Willd*) seeds (*Santamaria* cultivar) were purchased from the Seed and Plant Improvement Institute (Karaj, Iran).

2.2. Preparation of Bacterial Strains. To evaluate the antimicrobial effect of an active peptide derived from quinoa seeds, standard strains of *S. pyogenes* (PTCC-1447) and *E. coli* (PTCC-1335) were provided by the Pasteur Institute of Iran.

2.3. Sample Preparation. The impurities of quinoa seeds were removed manually, and seeds were ground using a mill (MB 1001B, Magic Bullet Blender, China) in order to obtain the whole quinoa flour (degree of extraction: 96%). The flour was defatted by hexane solvent (1 : 5 ratio) in three stages during 24 h by using an orbital shaker (TM 52E, Fan Azma Gostar, Iran). Thereafter, the suspension was placed in an oven (40°C, 24 h) to separate the solvent residue. The obtained flour was passed through a 0.25 mm mesh sieve and stored in polyethylene bags and kept at −18°C prior to use [12].

2.4. Extraction of Protein. In order to extract proteins, the method described by Chauhan et al. was used with some modifications [42]. Briefly, the defatted quinoa flour was dispersed in a 0.015 M sodium hydroxide solution. The resulting slurry was kept for 24 h at 4°C for a clearer supernatant, and then was centrifuged (Sigma, 6k15, Germany) at 10,000 g, 10°C for 30 min. The supernatant was then filtered (Whatman No. 1), and the filtrate pH value was adjusted to 4.5 by addition of 0.1 N HCl in order to precipitate the proteins. The precipitated proteins were completely isolated by a 30 min centrifugation (Sigma, 6k15) at 10000 g, 10°C.

Thereafter, they were washed with distilled water and lyophilised (freeze dryer, alpha 2, Christ-Germany) to produce quinoa protein concentrate [18, 43–45].

2.5. Protein Hydrolysis. Extracted proteins were digested using pepsin and alcalase enzymes inside a glass container while being mixed by a magnetic stirrer (IKA BH B2, Germany). Protein extract was diluted 5 folds in a sodium phosphate buffer solution while pH was adjusted at 8.0 by a 2 M NaOH solution. Alcalase enzyme was added to the diluted protein samples at pH 8 and the proteins were allowed to be hydrolysed by keeping the condition (pH and temperature) constant. Subsequently, the pH was adjusted to 2.5, and pepsin enzyme was added for proteins to be digested. Afterwards, samples were incubated in boiling water in order to halt the enzyme activity. They were centrifuged after cooling down to room temperature for 15 min, at 8000 g and 10°C. The supernatant was isolated as the protein hydrolysate and passed through a stirred cell ultrafiltration setup (Amicon, U.S.) with the aid of a membrane (molecular weight cutoff of 3000 Da). The obtained permeate from each membrane was lyophilised and kept at −20°C until use [7, 46].

A set of pretreatments were carried out in order to detect the optimum hydrolysis conditions comprising of temperature: 50°C, 55°C; time: 150, 180, 210 min and enzyme/substrate ratio: 30, 60, and 90 Anson unit (AU)/kg protein [47]. The obtained permeate of membranes were lyophilised and maintained at −20°C [12, 46]. Detailed conditions of the hydrolysis process for each treatment are presented in Table 1.

2.6. SDS-Page Analysis. Protein electrophoresis was performed using the SDS-Page technique according to the Laemmli method [48, 49].

An aliquot of 25 μl extracted protein was transferred to a gel electrophoresis system (MSCHOICETRIO, England)

Table 1: Hydrolysis condition of quinoa peptides.

Treatments	Enzyme ratio (AU/kg protein)[B]	Peptide concentration (μg/ml)	Temperature (°C)	Time period (min)
T0[A]	—	—	—	—
T1	30	200	55	210
T2	30	400	55	210
T3	30	800	55	210
T4	60	200	50	150
T5	60	400	50	150
T6	60	800	50	150
T7	60	200	55	180
T8	60	400	55	180
T9	60	800	55	180
T10	90	200	50	150
T11	90	400	50	150
T12	90	800	50	150
T13	90	200	50	180
T14	90	400	50	180
T15	90	800	50	180

[A]T0: control (gentamicin). [B]AU/kg: anson unit per kg.

with the upper gel containing 3.75% acrylamide-bisacrylamide and the lower gel containing 12% acrylamide-bisacrylamide. The gel dimensions were $140 \times 110 \times 1$ mm. The electrophoresis process time was about 3 h (until the bromophenol blue dye reached the lower edge of the gel). The current intensity was 30 mA in a PROTEIN II xi cell (BIO-RAD, USA). After removing gels from the electrophoresis, they were stained according to the Coomassie blue staining protocol.

In the SDS-PAGE technique, the larger the protein, the shorter the distance traveled [46]. Normally, a protein marker, which consists of several peptides with specific molecular weights, is added when loading samples into one of the wells. By comparing the protein sample's movement through the gel with that of protein markers, the weight of the target molecule will be estimated. In the present study, the BIO-RAD protein ladder was used to determine the molecular weight of the subunits of the tested samples [49]. For this section treatments descriptions were as follow: 1: Protein marker, 2: nonhydrolysed protein, 3: [enzyme/substrate ratio (AU/kg protein), temperature (°C); respectively] [30, 50]; 4: [30, 55]; 5: [60, 45]; 6: [60, 55]; 7: [90, 40]; 8: [90, 55].

2.7. Assessment of Antimicrobial Activity (Agar Well Diffusion Method). Bacterial suspensions were prepared from 24 h aged inocula, *i.e.,* Gram-positive *S. pyogenes* and Gram-negative *E. coli*. They were cultured 24 h before the test in Müller–Hinton agar (MHA, Merck, Germany) by using spread plate technique. After creating a well in all samples under sterile conditions, 40 μL from peptide solution was poured in each well by using a 50 μL sampler with 200, 400, and 800 μg/ml peptide concentrations.

For the control sample, gentamicin (5 mg) was poured, and all the cultured samples were incubated at 37°C for 24 h to subsequently determine the diameter of the nongrowth halos by a Vernier Caliper (VWR International Inc., USA) [50]. For each bacterium two replications were performed. In total, six treatments were designated for each bacterium, comprising A: enzyme ratio of 30 AU/kg protein, 55°C and 210 min; B: enzyme ratio of 60 AU/kg protein, 50°C and 150 min; C: enzyme ratio of 60 AU/kg protein, 55°C and 180 min; D: enzyme ratio of 90 AU/kg protein, 50°C and 150 min; E: enzyme ratio of 90 AU/kg protein, 50°C for 180 min and Control: that represented gentamicin as the control treatment.

2.8. Statistical Analysis. The results were imported into version 20 of SPSS software. Mean and standard deviation were calculated, and one-way analysis of variance (ANOVA) was used to compare the means, and a *t*-test at the level of $P < 0.05$ was performed to detect significant differences between experiments.

3. Results and Discussion

3.1. SDS-Page Analysis. The SDS-Page profiles of quinoa proteins and peptides with different treatments showed different protein bands ranged from less than 6.5 to 100 kDa in the protein profile (Figure 1), which was in accordance with studies conducted by Valenzuela et al. [51]. Brinegar and Goundan showed that all extractable quinoa proteins were in the range of 8 to 100 kDa at pH 8 and assumed that the extracted proteins included all major quinoa proteins [52]. Polypeptide bands with 8 to 10 kDa have been reported to be commonly found in all seeds. Chenopedins are known as major proteins of quinoa, which are among the seed storage proteins belonging to the globulin family. Peptides with 22-23 and 32–39 kDa have been known as basic and acidic subunits of chenopedins, respectively. Polypeptides with a molecular weight of 15 kDa belong to the 2S albumin [48].

During the hydrolysis of quinoa protein, as shown in Figure 1, the bands corresponding to the higher molecular

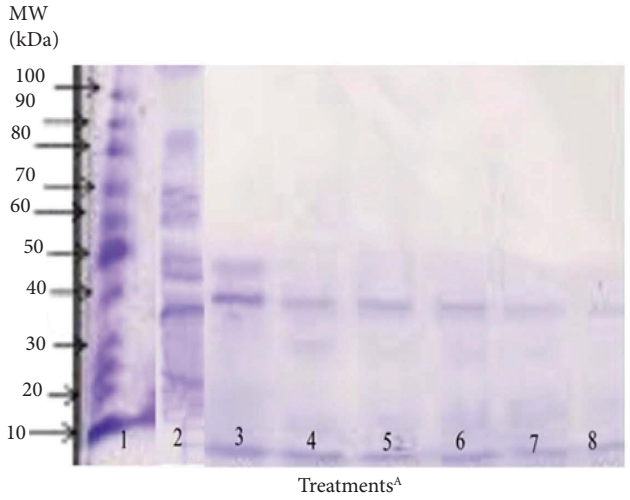

Figure 1: Quinoa protein profile of treatments hydrolysed at different conditions analysed by SDS-PAGE. [A]Treatments descriptions are as follows: 1: protein marker, 2: nonhydrolysed protein, 3 (enzyme/substrate ratio (AU/kg protein) and temperature (°C), respectively): [30, 50]; 4: [30, 55]; 5: [60, 45]; 6: [60, 55]; 7: [90, 40]; 8: [90, 55].

weight peptides were destroyed and solely low molecular weight peptides were observed [51, 53].

According to Figure 1, the molecular weight of T2, *i.e.*, nonhydrolysed protein, was the highest and T8 was the lowest. In the gels used for electrophoresis, smaller molecules move faster and travel longer distances than larger molecules, so that in Figure 1, the more hydrolysis, the lighter the blue colour, indicating more protein hydrolysis. The presence of high molecular weight proteins in this form may be due to incomplete hydrolysis or lack of hydrolysis by the enzymes [18, 54].

3.2. Assessment of Antibacterial Activity. The test results of the growth inhibition zone (mm) against *S. pyogenes* and *E. coli* in different conditions of hydrolysis are shown in Figures 2 and 3, respectively. From the results, it can be deduced that the peptide concentration had a significant influence on the inhibition of bacterial growth. In other words, the mean diameter of growth inhibition zone at 800 μg/ml peptide concentration was significantly greater among all the treatments. As such, the highest inhibitory effect belonged to treatment with the highest concentration (800 μg/ml) against *S. pyogenes* (Figure 2).

For a clearer perception of the influence of the studied treatments (temperature, time, enzyme/substrate ratio, and the concentration of peptides derived from quinoa protein), their role was assessed in individual groups (in-groups) and also between different groups (intergroups). The results of the in-group comparison of treatments showed that only the concentration of peptides had a significant effect on the diameter of the growth halo of pathogens ($P < 0.05$). In other words, under the same conditions of enzyme/substrate ratio, temperature, and time, solely the peptides concentration regulated the degree of inhibition of pathogen growth.

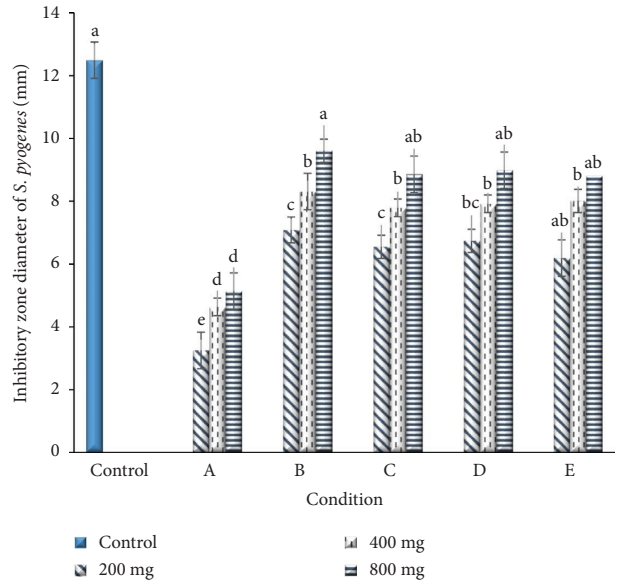

Figure 2: Growth inhibition zone of *S. pyogenes* in different conditions. Control: gentamicin, A: enzyme ratio of 30 AU/kg protein, 55°C and 210 min; B: enzyme ratio of 60 AU/kg protein, 50°C and 150 min; C: enzyme ratio of 60 AU/kg protein, 55°C and 180 min; D: enzyme ratio of 90 AU/kg protein, 50°C and 150 min; E: enzyme ratio of 90 AU/kg protein, 50°C for 180 min and control: represented gentamicin as the control treatment.

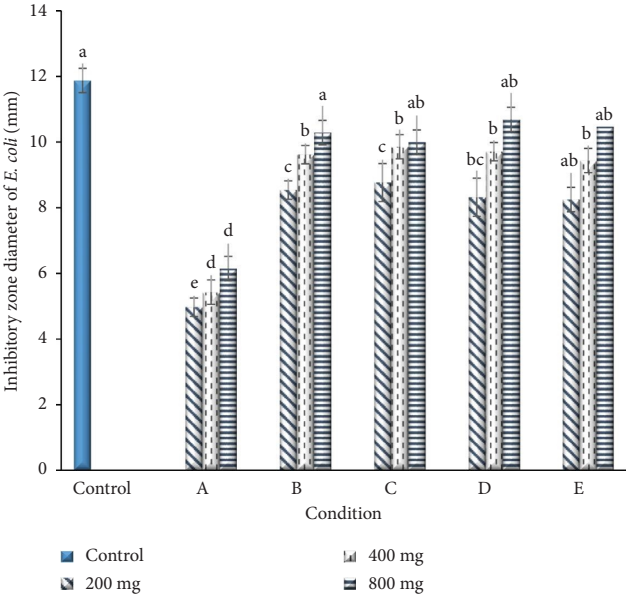

Figure 3: Growth inhibition zone of *E. coli* in different conditions. Control: gentamicin, A: enzyme ratio of 30 AU/kg protein, 55°C and 210 min; B: enzyme ratio of 60 AU/kg protein, 50°C and 150 min; C: enzyme ratio of 60 AU/kg protein, 55°C and 180 min; D: enzyme ratio of 90 AU/kg protein, 50°C and 150 min; E: enzyme ratio of 90 AU/kg protein, 50°C for 180 min and control: represented gentamicin as the control treatment.

Using intergroup comparison of treatments, the effect of time and temperature and enzyme/substrate ratio on inhibition of bacterial growth was detected, and the results

indicated that an increase in enzyme ratio and temperature can lead to a higher rate of pathogen growth inhibition. Furthermore, in A, B, C, and D treatments (Figure 2), with increasing the peptide concentration, the diameter of the growth inhibition zone against *S. pyogenes* gradually increased, so that in the stated conditions, at a concentration of 800 μg/ml, the highest and in concentration of 200 μg/ml the lowest diameter of growth inhibition zone were observed. In general, treatments B, C, and D at concentrations of 800 μg/ml and treatment E, at concentrations of 200 μg/ml and 800 μg/ml exhibited the greatest effect against in diameter of growth inhibition zone against *S. pyogenes* being equal to that of control (gentamicin antibiotic). The lowest antimicrobial activity was attributed to treatment A at 200 μg/ml concentration.

As illustrated in Figure 3 in each of the studied conditions, by increasing the peptide concentration, the diameter of the growth inhibition zone against *E. coli* increased. As such, the highest diameter of the growth inhibition zone against *E. coli* was at 800 μg/ml of concentration, and the lowest diameter of growth inhibition zone was at 200 μg/ml. However, this increase in antimicrobial activity due to higher peptide concentration was not statistically significant for treatments D and E. Generally, E and D treatments at all the three concentrations and B treatment at 800 μg/ml concentration showed the greatest effect on the diameter of the growth inhibition zone against *E. coli*, so that there was no significant difference between these samples and our positive control (gentamicin antibiotic). Nevertheless, the lowest antimicrobial activity was observed in treatment A at concentrations of 200 and 400 μg/ml (Figure 3).

The correlation between inhibitory effect and peptide concentration is unambiguously explained by bioactivity attributes of peptides as discussed earlier [10–12, 31–33]. Electrostatic interaction of peptides with the negatively charged molecules on the microbial cell membrane is known as the key mechanism of action of antimicrobial activity in peptides [55].

These results are consistent with those of Salehi et al., which investigated the antibacterial properties of the synthetic peptide D28 on *Staphylococcus aureus* and *Pseudomonas aeruginosa* strains [56]. Their results indicated that the synthesized peptide was only effective against *S. aureus*. In addition, according to their report, enhancing the antibacterial activity of peptides through dimerization depends highly on the methods of dimerization and the bacterial strain [56]. In another study, the antioxidant and antimicrobial properties of quinoa seeds in Korea was compared to those of quinoa seeds cultivated in the United States and Peru [57], and the highest antioxidant activity and total phenolic compounds belonged to the quinoa seeds cultivated in South Korea. In addition, quinoa seed extract showed high potency in DPPH free radical scavenging which tallies with the report by Mahdavi-Yekta et al. [44]. They also investigated the antimicrobial properties of quinoa seed extract by disk diffusion method, and in contrast to our study, reported a very low influence against food-borne pathogens [57]. The positive inhibitory effect of quinoa seed in the present study is mainly attributed to its bioactive peptides [58–60].

4. Conclusions

Results of the present study showed that quinoa is a good source to produce bioactive peptides with antimicrobial properties through enzymatic hydrolysis and that time, temperature, and enzyme/substrate ratio are indeed effective parameters in optimal production of peptides. The results also indicated that QHP had the ability to compete with gentamicin as a control treatment in terms of growth inhibition of *E. coli* and *S. pyogenes*. The highest growth inhibitions against *E. coli* and *S. pyogenes* were obtained at a concentration of 800 μg quinoa peptide per ml. As such, antimicrobial compounds of QHP can be exploited in formulating food products and packages in order to enhance the product's shelf life and maintain its quality during preservation. Nevertheless, further assessment is needed to evaluate the extent of the inhibitory effect of QHP either as an incorporated ingredient or a coating component for food packages against major food-borne pathogens, e.g., *Staphylococcus aureus, Salmonella* Typhimurium*, Campylobacter jejuni, Listeria monocytogenes, and Bacillus cereus* in different food products.

Data Availability

The data used to support the findings of this study are included within the article.

Disclosure

The research was performed as part of the employment of the authors.

Conflicts of Interest

The authors declare that there are no conflicts of interest.

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Research Article

Potential of *Citrullus colocynthis* L. Schrad. Immature Seed Extracts as Food Preservative against a Fungal Mycotoxigenic Contaminant

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The world of plant extracts and natural compounds have long been regarded as a promise land for the individuation of healthy alternatives to chemical preservatives, against microbial contamination, in food and feed commodities. A plethora of aromatic and medicinal plant species have been studied from decades to explore their antimicrobial and antioxidant properties, in order to both validate their ethnobotanical use for healing microbial illnesses and assess their suitability as food preservation agents. In fact, after terrestrialization and during the following evolutionary pathway, plants had to develop chemical compounds—constitutive and/or induced—for defence against specific pathogens, therefore becoming a potential source of new natural products usable with antimicrobial purposes. Aside from the most common contaminants that could occur in foodstuff, mycotoxigenic fungal species represent a big concern, mainly in cereals and derived products: aflatoxins in particular are the most dreaded among such toxic and cancerogenic secondary metabolites, and the control of the main producer *Aspergillus flavus* is currently one of the most pursued goals in the field of food safety. As aromatic and medicinal plants have a long history of use in the Mediterranean basin for both food preservation and pest control in crops, the exploitation of native species for the control of mycotoxigenic phytopathogens is almost rationale. The present work provides novel insights into the possible use of *C. colocynthis* seed organic extracts as antimycotoxigenic additives, demonstrating, for some of them, a feasible application as crop and food protectants with specific regard to aflatoxin contamination. Additionally, the evaluation of their cytotoxic potential and nitric oxide production on human cell lines has been reported for the first time.

1. Introduction

In the field of the pathogenic microorganism control, the problem of food-borne outbreaks has represented a true challenge for health regulatory authorities from decades. Indeed, the recent drift toward the use of natural preservatives—and mainly those obtained from plants—in the food chain has increasingly intensified the interest on the most various plant species [1–4]. Being exploited since the ancient times not only for coloring and flavoring, but also for healing purposes, spices and herbs in particular were thought to be the most promising source for the individuation of new preservation agents: in fact, over years, plant-derived extracts, essential oils, and peptides exhibited broad-

spectrum activities, mainly ascribed to relevant antioxidant properties relying on secondary metabolites such as phenylpropanoids, terpenes, flavonoids, and anthocyanins [5–7].

However, despite the demonstrated antimicrobial effect of countless plant extracts, and the advantages that the use of these products as an alternative of chemical additives can bring in terms of chemical residues and microbial resistance, some drawbacks still deter a large use in food industry; for example, the high variability in effectiveness of these compounds (pure or in mixture) against microorganisms in the laboratory system and in real food systems, the costs associated with the processes for their procurement and/or purification, and the concentration required for the achievement of the most effective containment activity are sometimes discouraging. Even if both the European Commission (EC) and the US Food and Drug Administration (FDA) approved few essential oils as food preservatives, the problematic reproducibility of their activity represents a main barrier not overcome yet. Additionally, although some plant compounds enjoy the status of "generally recognized as safe" (GRAS), toxicological assessment of their use in food is scarce or poorly available. Thus, the improvement in cost-effective producing and toxicological information of these compounds is highly desirable, as well as helpful, in the promotion of their use as food biopreservatives. On the other hand, commonly used botanical derivatives have become popular as pesticides in organic farming, as organically produced food raises premium prices; their popularity in agriculture is also due to the perception that they are safe to use on crops for human consumption if compared to chemical treatments [8].

Since the biological properties of plants extracts mainly derive from secondary metabolites, even at different extent, and molecules with antimicrobial function are synthesized in response to biotic stressors [9] that characterize almost less controlled environments, it could be suggested that the highest yield of such compounds should be found preferentially in wild or not completely domesticated species [10]. In this sense, the Mediterranean basin is undoubtedly an exceptional reservoir of genetic resources of both wild and cultivated aromatic and medicinal plants that can be addressed to innovative applications as antimicrobials in several fields related to crop management, from food and feed production to safety and quality. For example, several studies focused on the use of essential oils and crude extracts from Mediterranean species for the control of phytopathogenic fungi affecting the quality and safety of crops have been recently reported [11, 12]. Mycotoxigenic fungal species in particular have been widely considered, because of the big threat that their diffusion on cereal crops and derived commodities poses on human and animal health [13]; among them, *Aspergillus flavus* is probably the most studied.

Citrullus colocynthis L. Schrad. is an annual plant, belonging to the cucurbit family, which grows in arid and semiarid areas. Native to Asia and tropical Africa, it is now widely distributed also in the Arab-Saharan geographic region, and in the desert areas of the Mediterranean basin [14]. Due to its content in glucosides, such as colocynthis, the pulp of the fruit is an effective laxative and an excellent depurative and has been used in the Berber traditional medicine from centuries [15]. Most remarkably, aside from a number of pharmacological properties—ranging from anti-inflammatory, antidiabetic, analgesic, antiepileptic, even to abortive properties—important antimicrobial activities of organic extracts from different parts of the plant have been recognized. For example, aqueous and acetone extracts from roots, stems, leaves, and three maturation stages of fruit and seeds that were screened for their antimicrobial potential against Gram-negative and Gram-positive bacteria and *Candida* spp. demonstrated a broad-spectrum effectiveness [16]. Antimycotic properties have been assessed in plant pathogens also (such as *Alternaria alternata*, *Rhizoctonia solani*, *Fusarium oxysporum*, and *Fusarium solani*) that impact the quality and safety of a number of food feed and commodities [17]. Some aqueous and acetone extracts from *C. colocynthis* plants collected in southern Tunisia (Mednine) showed marked broad-spectrum antiradical properties *in vitro* antioxidant activity tests [18]; but, interestingly, the scavenging potential as assessed in organic extract from leaves, stems, and roots that proved a high effectiveness in inhibiting aflatoxin biosynthesis in *A. flavus* was demonstrated to be uncoupled from the antitoxigenic activity that commonly is thought to rely on the unbalancing of secondary metabolism toward a reduced state [19].

The investigation of traditional medicinal plants and the characterization of their potential as a source of natural preservatives agents are the first step for an optimal utilization of plants extracts as nontoxic-to-humans, sustainable food additives. With this goal, this work seeks to compare the antimycotoxigenic activity of 5 organic extracts from *C. colocynthis* immature seeds, already characterized for their antimicrobial potential on some human pathogens, and to assess their toxicological profile on human cell lines, in order to provide an almost complete information useful for their possible application as cereals derived-products preservatives.

2. Materials and Methods

2.1. C. colocynthis L. Schrad. Immature Seed Organic Extracts. For this work, immature seed organic extracts of *C. colocynthis* collected near Medenine (Tunisia), previously obtained and discussed, were used [20], namely, petroleum ether (PE), chloroform (CHL), ethyl acetate (EA), acetone (AC), and methanol (MET), in ascending polarity, that were obtained through Soxhlet extraction. The CHL extract subfraction F19 [21] was also included.

For biological assays, the crude extracts were redissolved in dimethyl sulfoxide (DMSO) and tested at increasing concentrations.

2.2. Fungal Strains, Media, and Culture Condition. *Aspergillus flavus* strains used in this study were previously isolated from corn fields of the Po Valley and reported [22]. Conidia suspensions were obtained from 10 day YES-agar

[2% (*w*/*v*) yeast extract (Difco, Detroit, MI, USA), 5% (*w*/*v*) sucrose (Sigma, St. Louis, MO, USA), and 2% (*w*/*v*) agar (Difco)] cultures incubated at 28°C; conidia concentration (quantified by OD600) and viability (>90%) were determined according to Degola et al. [23]. Coconut milk-derived medium (CCM) used for microplate assays was obtained as described in Degola et al. [23].

2.3. *Aspergillus flavus* Bioassays

2.3.1. Aflatoxin Production. The effects of seed extracts on aflatoxin biosynthesis were assessed by the microplate fluorescence-based procedure described in Bisceglie et al. [24]. Standard flat-bottom 96-well microplates (Sarstedt, Newton, NC, USA) were used. Suspensions of conidia were diluted to the appropriate concentrations and brought to the final concentration of 5×10^2 conidia/well and inoculated in a final volume of 200 μL/well of CCM medium. Extracts were DMSO resuspended and added to the culture medium. The plates were incubated in the dark under stationary conditions for up to 6 days at 25°C. Total aflatoxin accumulation was monitored by fluorescence emission determination; readings were performed directly from the wells bottom of the culture plate with a microplate reader (TECAN SpectraFluor Plus, Männedorf, Switzerland) using the following parameters: λex = 360 nm, λem = 465 nm, manual gain = 83, lag time = 0 μs, number of flashes = 3, integration time = 200 μs). Inocula were performed in quadruplicate, and experiments were performed in triplicate.

2.3.2. Biomass Production. Mycelium development was assessed in the same microwell cultures used for the toxin biosynthesis evaluation. After aflatoxin measurement, mycelia were manually recovered from the culture wells, slightly paper-dried, and weighted. Inocula were performed in quadruplicate, and experiments were performed in triplicate.

2.4. Human Cell Line, Media, and Culture Condition.

All cell lines were obtained from the American Type Culture Collection (ATCC, Rockville, MD).

HT29 (ATCC, HTB-3), human colon adenocarcinoma cell line, was maintained in 25 cm^2 flasks with 5 mL of Dulbecco's Modified Eagle Media (DMEM) supplemented with 10% (*v*/*v*) fetal bovine serum (FBS), 1% penicillin (100 U/ml)/streptomycin (100 μg/mL), and 1% L-glutamine (2 mM).

A549 (ATCC CCL-185), human lung carcinoma cell line, was cultured in Roswell Park Memorial Institute (RPMI-1640) medium, supplemented with 10% (*v*/*v*) fetal bovine serum (FBS), 1% penicillin (100 U/mL)/streptomycin (100 μg/mL), and 1% L-glutamine (2 mM).

HFL1(ATCC, CCL-153), human lung normal fibroblast, was cultured in Ham's Nutrient Mixture F-12 with L-glutamine supplemented with 10% (*v*/*v*) fetal bovine serum (FBS) and 1% penicillin (100 U/mL)/streptomycin (100 μg/mL). HFL1 cells were used between passage numbers 5 and 20.

Cell lines were maintained at 37°C in a 5% CO_2 humidified incubator and subcultured twice a week.

2.5. Cytotoxicity on Human Cell Lines. The cytotoxic activities of seed extracts on human cells were measured by MTS assay (Promega, Madison, WI) [25]. Cells were seeded (5×10^4 cell/mL) in 96-well flat-bottom plates. After 24 h of incubation, HT29 cells were treated, in quadruplicate, with increasing concentrations of seed extracts or vehicle control and incubated for 24 h. A volume of 20 μL of MTS solution was added directly to culture wells and after 4 h of incubation the absorbance at 450 nm with a 96-well plate reader (MULTISKAN EX, Thermo Electron Corporation, Vantaa, Finland) was recorded.

2.6. Nitric Oxide Production. The Griess Reagent System (Promega Corporation, Madison, WI, U.S.A) was used to quantify the production of nitric oxide (NO) through the evaluation of nitrite (NO_2^-), one of the NO primary breakdown product, stable and nonvolatile. Briefly, 5×10^3 cells/well were seeded in complete medium (DMEM + 10% FBS), after 24 h of incubation (37°C, 5% CO_2), the medium was discarded, and cells were supplemented with fresh medium without FBS and treated with samples for 24 h. After treatment, Griess reaction was performed as reported in the producer instructions. The absorbance of the reaction mixtures was measured at 570 nm using a microplate reader (TECAN SpectraFluor Plus, Männedorf, Switzerland). The NO concentration was determined by comparison to the nitrite standard reference curve.

2.7. Statistical Analysis. Antifungal and antiaflatoxigenic activity data were analyzed with Past 3.x software (https://past.en.lo4d.com/windows). One-way analysis of variance (ANOVA) was performed. Results of biomass production and AF accumulation were analyzed by Tukey's test; differences were considered significant at $p \leq 0.01$.

Statistical analysis of cytotoxicity assay on human cells was performed by the statistical and graphical function of IBM SPSS Statistics 27 (SPSS Inc., Chicago, IL, USA). The mean values from the repeated experiments were used in ANOVA. If significant F values ($p \leq 0.05$) were obtained, Student's *t*-test (Bonferroni's version) was performed.

3. Results

3.1. Biological Effect of C. colocynthis Immature Seed Organic Extracts on A. flavus. The evaluation of the antifungal potential of *C. colocynthis* immature seed extracts has been conducted on the biomass production in an aflatoxigenic *A. flavus* strain. Increasing concentrations ranging from 10 to 500 mg/mL were tested. As shown in Figure 1, a dose-dependent effect was observed for PE, CHL, EA, and MET extracts; on the contrary, AC extracts did not prove to exert any inhibition on fungal growth. The maximum of antifungal effect (47.5%) was achieved through the treatment with EA extract at the highest concentration (500 μg/mL), followed by PE (42.4%) administrated at the same concentration. Amongst all the organic extracts, both CHL and

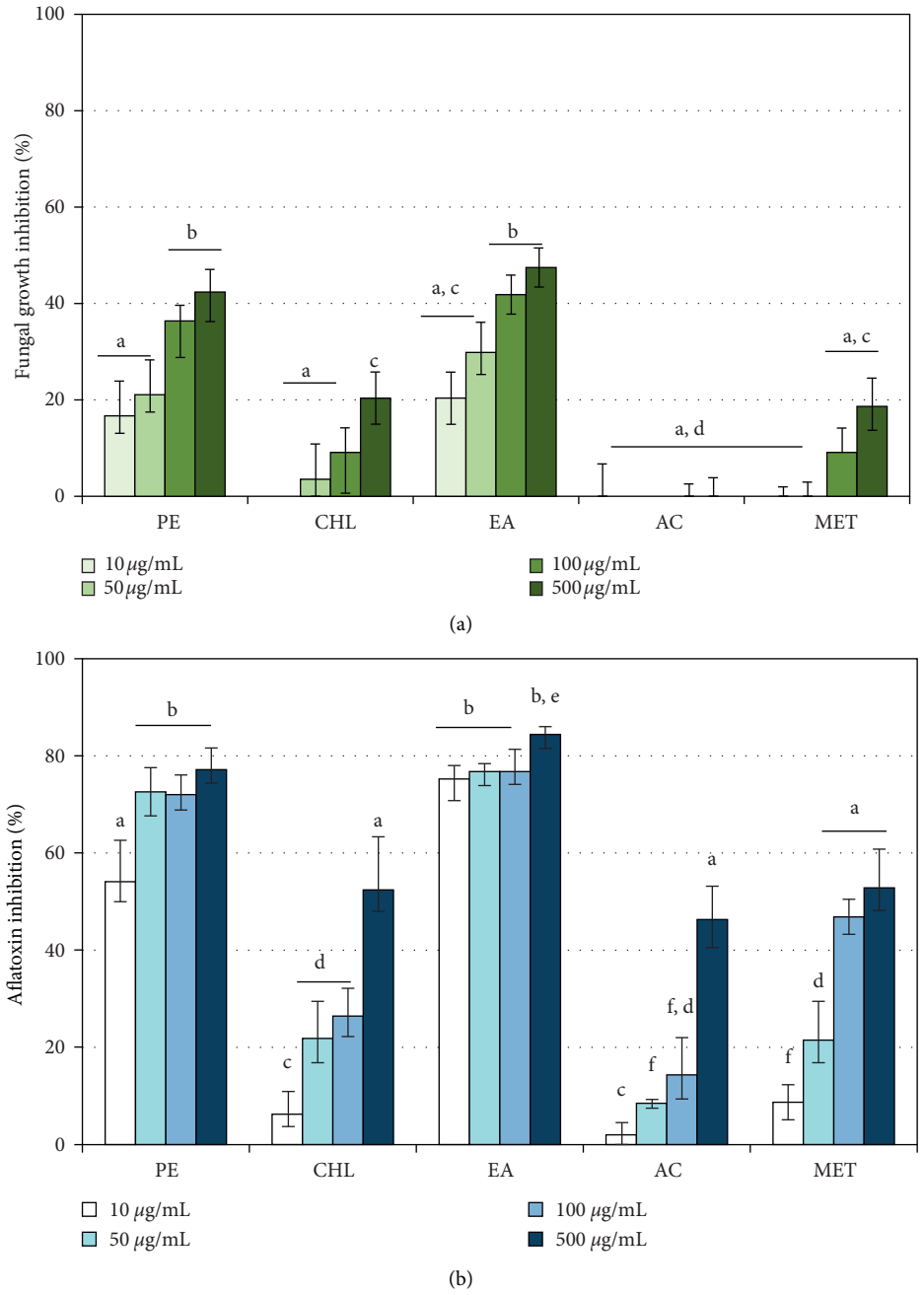


Figure 1: Activity on *A. flavus* growth and aflatoxin biosynthesis. Biomass production (a) and toxin accumulation (b) in CCM cultures after six days of growth with petroleum ether (PE), chloroform (CHL), ethyl-acetate (EA), acetone (AC), methanol (MET) *C. colocynthis* immature seed extracts. Results are expressed as percentage in respect to control (equivalent DMSO amended cultures). Error bars refer to mean values of four replicates S.D. Different letters indicate statistically significant differences at $p \leq 0.01$.

MET were the least effective in lowering the biomass production (and thus the mycelium growth) of *A. flavus*.

The same extracts have been then investigated for their possible effects on aflatoxin biosynthesis (Figure 2). As a general observation, a higher inhibitory potential was globally assessed with respect to the antifungal effect. PE and EA extracts showed the highest antiaflatoxigenic activity, exceeding the 50% inhibition to the PE lowest concentration, and even surpassing about the 70% at the intermediate ones. On the contrary (but according to the effect on the fungal growth), 10, 50, and 100 μg/mL concentration of CHL and AC and 10 and 50 μg/mL concentration of MET extracts resulted in lesser than about 30% aflatoxin containment. For these, the highest concentration was instead quite effective in lowering the toxin accumulation (about 52.4%).

The MET extract was compared, in its biological activity on *A. flavus*, with the derived F19 fraction. In Figure 3, this comparison is reported. As can be observed, F19 behaved as CHL for the efficacy on mycelium inhibition; on the

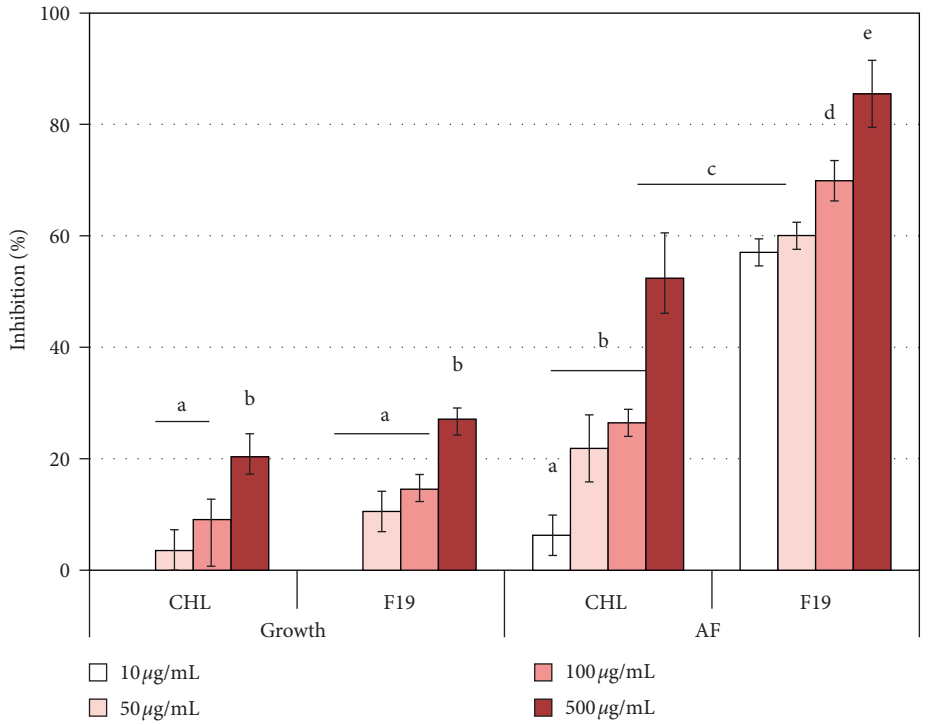
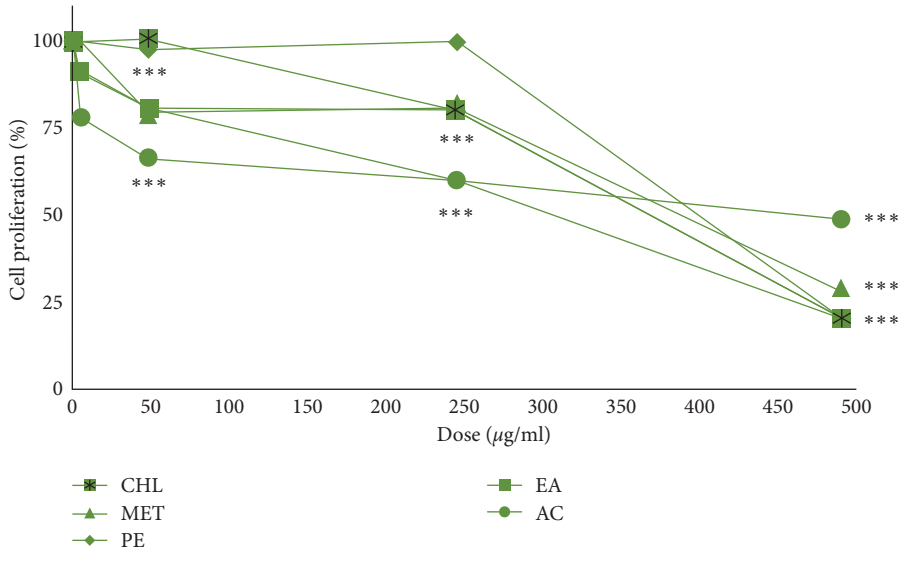


Figure 2: Antifungal and antiaflatoxigenic activity of *C. colocynthis* immature seed chloroform (CHL) extract and its fraction F19. Results are expressed as percentage in respect to control (equivalent DMSO amended cultures). Error bars refer to mean values of four replicates SD. Different letters indicate statistically significant differences at $p \leq 0.01$.



(a)

Figure 3: Continued.

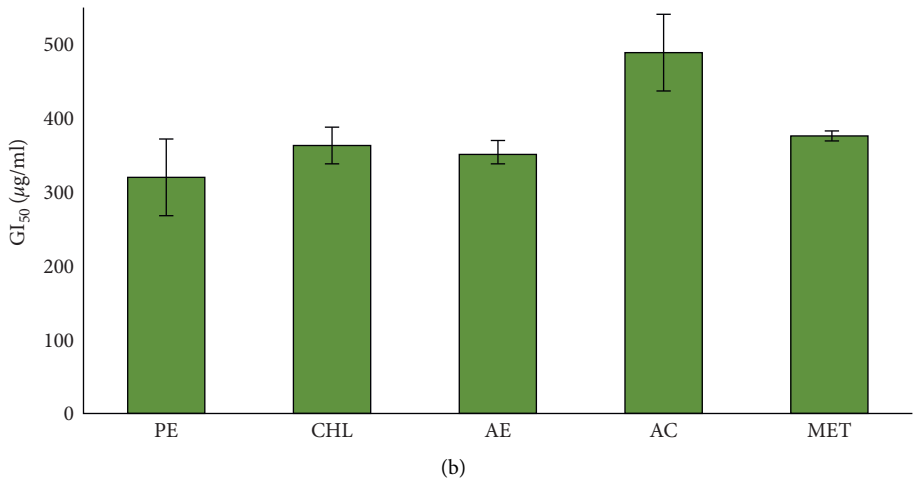


Figure 3: Antiproliferative activity of organic extracts from *C. colocynthis* seed extracts on HT29. (a) Dose-response curve. $^{***}p \leq 0.001$. (b) Concentration able to inhibit of 50% the cell growth (GI_{50}). PE: petroleum ether; CHL: chloroform; EA: ethyl acetate; AC: acetone; MET: methanol.

contrary, it proved to possess a significantly higher potential in aflatoxin containment than CHL.

3.2. Cytotoxicity Assessment on Human Cell Lines. Seed extracts and fraction F19 were evaluated for their cytotoxic activity on human cell lines.

The ability to affect cell proliferation was assessed through the MTS assay, a colorimetric method for determining the number of viable, metabolically active cells in proliferation.

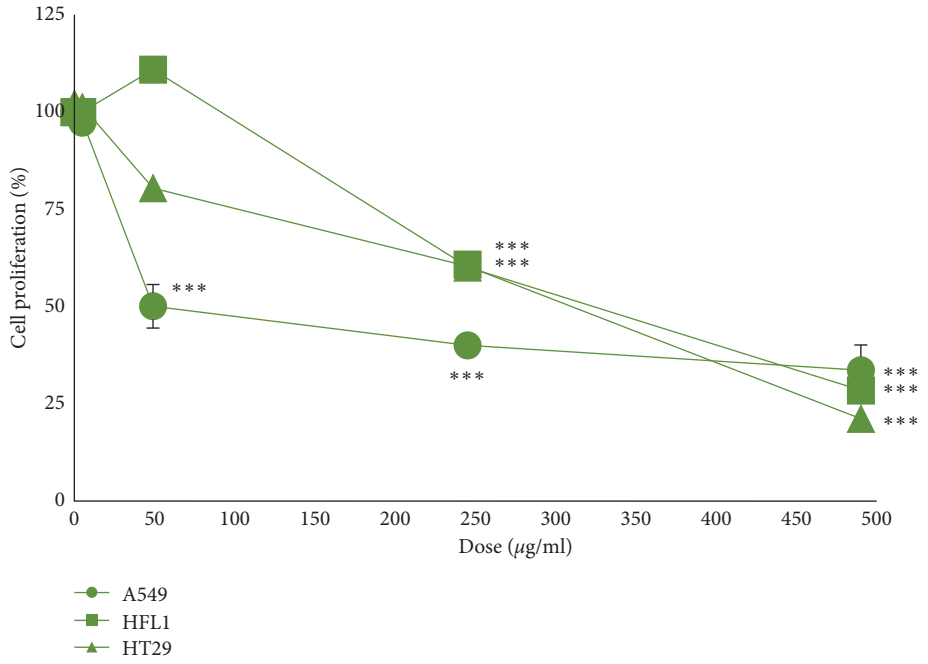
All the seed extracts induced a dose-dependent inhibition on the proliferation of human cell line HT29 (Figure 4(a)). The concentration able to inhibit 50% of the cell growth (GI_{50}) was calculated from the dose-response curve. Based on the GI_{50} values, the inhibitory effects of the seed extracts on HT29 cell proliferation were in decreasing order, as follows: AC (489 μg/mL) > MET (374 μg/mL) > CHL (363 μg/mL) > EA (351 μg/ml) > PE (321 μg/mL) (Figure 4(b)).

The chloroform extract and its F19 fraction, which presented an interesting antiaflatoxigenic activity, were tested on cell lines deriving from different tissues: HT29, A549, and HFL1 (Figure 5(a)). The chloroform extract showed a high, comparable, cytotoxic activity on the three cell lines. F19 induced a dose-dependent cytotoxicity on the cell lines HT29 and HFL1, while the A549 cell line did not show any sensitivity to F19 (Figure 5(b)). Interestingly, F19 was not able to induce an inhibition of 50% of cell proliferation on the different cell lines used. In comparison with the total chloroform extract, the fraction F19 shows a strong reduction in cytotoxic activity.

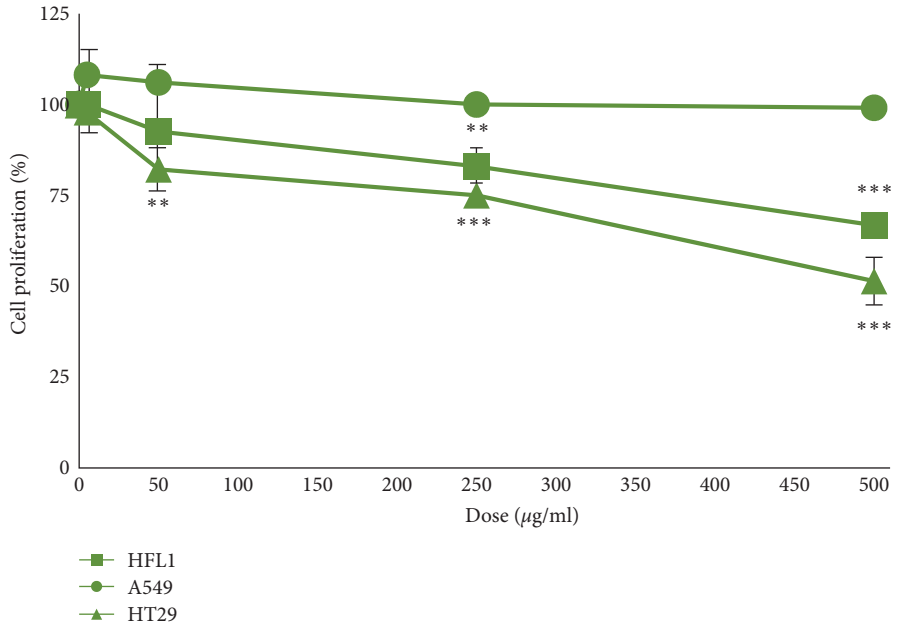
3.3. Evaluation of NO Production. Nitric oxide (NO) has modulating effects on several cellular processes, in particular on inflammation. High levels of NO are produced in response to inflammatory stimuli and mediate proinflammatory and destructive effects. NO production was determined after the treatment of HT29 cells for 24 h with *C. colocynthis* seed chloroform extract and its F19 fraction, using the Griess reaction that evidences nitrite, a stable breakdown product of NO. No significant induction of NO was detected

4. Discussion

Citrullus colocynthis L. Schrad. belongs to the family Cucurbitacea that grow in the arid areas of Mediterranean basin [14] and belongs to the ethnomedicinal arsenal of folk medicine of various Countries, being widely—and long-time—used for many diseases including dermatological, gynaecological, urinary, and pulmonary infections [26, 27]. The most used plant parts addressed to medicinal purposes are mainly fruits and seeds, even if roots and leaves infuses are used for the treatment of urinary infections [28, 29]. However, more recently, organic extracts obtained from leaf, stem, and root material have been successfully applied *in vitro* against some phytopathogenic fungi, and in particular against *A. flavus* and aflatoxin biosynthesis, that is relevant to cereal crops production [19]: in this study, an interesting dependence of the biological activity not only on the plant tissue, but also on the organic solvent utilized for the extraction, was showed. Results obtained with the immature seeds extracts are in accordance with the following: in fact, AC extracts did not exert any inhibitory effect on fungal growth, while others showed a variable extent. On the other hand, aflatoxin containment activity only partially showed a similar behavior, since the organics can be divided for their inhibition rate; indeed, PE and EA from one side, and CHL, AC and MET from the other shared similar antiaflatoxigenic potential. However, the antifungal activity of the same AC extracts determined against some human fungal pathogens [16] proved to be highly dissimilar from results obtained on *A. flavus* biomass production. This observation underlines the necessity, if interested in the specific potential of



(a)



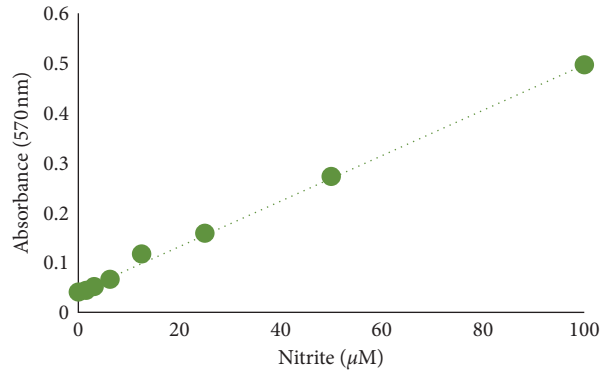
(b)

Figure 4: Antiproliferative activity of *C. colocynthis* immature seed chloroform extract (a) and its fraction F19 (b) on A549, HFL1, and HT29 human cell lines. $^{**}p \leq 0.01$ and $^{***}p \leq 0.001$.

botanicals as crop and/or food protective additives, to directly assess the antifungal activity on the fungal target species, because the inhibitory potential dramatically varies amongst genera—even at the species level.

Despite the demonstrated effectiveness of the most various botanicals for antimicrobial purposes, a deepen and accurate evaluation of their toxicological properties is still required, at least on human cell line models. Hence, we studied the abilities of the different seed extracts in altering human cell proliferation as a parameter of cytotoxicity. Taking in consideration the polarity of the solvent used, the highest activities on HT29 cell line were found with the most polar extracts, such as AC and MET that, on the other hand, were not the most effective as antiaflatoxigenic agents.

When compared to the CHL extract, its F19 subfraction showed not only an increased aflatoxin-containing activity (even at the lower concentration), but also an important



(a)

	DMSO	CHL	F19
NO (μM)	<1	<1	<1

(b)

Figure 5: Nitrite standard curve (a) and NO concentration measured in supernatants from HT29 cells treated with DMSO (control) and *C. colocynthis* immature seed chloroform extract (CHL) and its fraction F19. (b) Evaluation performed after 24 h.

reduction of the cytotoxic effect: in fact, an absence of a GI_{50} value on all the different human cell lines was observed. Furthermore, human cell treatment did not highlight any proinflammatory response, as revealed through NO production. These data are in accordance with a characterization study in which the seed chloroform extract, the F19 subfraction, and the purified compound 11-deoxocucurbitacin-I-2-O-β-d-glucoside (that was thought of as a possible effector present in the F19 subfraction) induced anti-inflammatory effects *in vivo* in rats [20].

5. Conclusions

The present work provides novel insights about the possible use of *C. colocynthis* seed organic extracts as antimycotoxigenic additives, suggesting, for some of them (namely, PE and EA extracts, as well as the F19 subfraction), a possible application in the crop protection and food and feed preservation, with specific regard to aflatoxin contamination. The assessed cytotoxicity on human cells of these extracts, however, seems to not represent a health concern, since their antiaflatoxigenic potential was high starting from the lowest concentration (10 μg/mL), while the relevant GI_{50} values have been assessed to be about 300 μg/mL for PE and EA, and >500 μg/mL for F19. As a final consideration, it should be reminded of that the use of natural compounds/extracts effective in the containment of mycotoxins, whereas not impacting on fungal viability, could represent, in some cases as the crop protection and agricultural purposes, a more ecological strategy, in order to prevent the loss of environmental biodiversity.

Data Availability

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

Disclosure

F. Degola and A. Buschini must be considered the co-last authors.

Conflicts of Interest

The authors declare no conflicts of interest.

Authors' Contributions

B. Marzouk, F. Degola, and A. Buschini designed the study. F. Mussi, S. Montalbano, M. Refifa, J. Kraiem, and B. Marzouk conducted the experiments. S. Montalbano wrote the original draft. Z. Marzouk, L. Arru, F. Degola, and A. Buschini edited the manuscript. F. Degola and A. Buschini equally contributed as senior.

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Research Article

Antibacterial Effect of Dihydromyricetin on Specific Spoilage Organisms of Hybrid Grouper

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This study aimed to investigate the mechanism of antibacterial activity level inhibition of dihydromyricetin (DMY) against specific spoilage bacteria of grouper. Firstly, the specific spoilage bacteria of grouper in the cold storage process are *Pseudomonas antarctica* (*P. antarctica*), which are selected by calculating the spoilage metabolite yield factor. It was determined that the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of DMY against grouper spoilage bacteria were 2.0 mg/mL and 6.4 mg/mL, respectively. DMY was added to the matrix of chitosan and sodium alginate, and DMY emulsions of different concentrations (0 MIC, 1 MIC, 2 MIC, 4 MIC) were prepared and characterized by differential calorimetry methods. Through analyzing cell permeability, enzyme activity, and images of the confocal laser scanning microscope (CLSM), we further studied the antibacterial mechanism of DMY emulsion on specific spoilage bacteria. The results showed that, with the increase of DMY concentration in the treatment group, the leakage of nucleic acid and protein increased significantly, the activity of ATPase and three critical enzymes in the Embden-Meyerhof-Parnas (EMP) pathway decreased significantly, and the activity of AKPase did not decrease significantly, . The metabolic activity and viability are reduced considerably. Analysis of the above results shows that DMY inhibits the growth and reproduction of *P. antarctica* by interfering with the metabolic activity of bacteria and destroying the function of bacterial cell membranes but has no inhibitory effect on the activity of AKPase. This study proves that DMY could be an effective and natural antibacterial agent against specific spoilage bacteria in aquatic products.

1. Introduction

It is well known that aquatic products are very susceptible to spoilage caused by microorganisms. Therefore, it is necessary to develop effective antibacterial measures to maintain the freshness and quality of fish after slaughter [1]. Considering the hazards to health, chemical or synthetic additives are preferred to be substituted by natural preservatives. Recently, edible plant extracts have become increasingly popular ingredients in food processing research [2]. Polyphenolic compounds are the main components in plant extracts and are considered the most potent antioxidants in the human diet, and flavonoids account for the main proportion of phenolic compounds. Moreover, flavonoids have been reported to have the potential of reducing the risk of many chronic diseases [3]. Therefore, they are combined into the nutritional and pharmaceutical products, and food to enhance products' health function [4].

Dihydromyricetin (DMY) is a flavonoid with high biological activity, and its content in vine tea can reach more than 30%, which is very rare and unique in the plant kingdom [5]. Some scholars have proved through in vitro experiments that DMY has anti-inflammatory effects [6, 7], antioxidant effects [8], and broad-spectrum antibacterial effects [9]. It can be seen that DMY has broad development prospects in the food industry as a bacteriostatic agent and

antioxidant. However, the antibacterial mechanism of DMY on spoilage bacteria has not been sufficiently studied.

Pseudomonas spp. is a common spoilage bacterium in aquatic products [10–12]. It is necessary to inhibit its growth and reproduction during storage and transportation for food safety. Although there are many antibacterial agents in applying fresh-keeping aquatic products, the research on DMY has not been involved. Therefore, this experiment studied the antibacterial activity of DMY against specific spoilage bacteria of grouper and developed DMY emulsion. Specifically, an emulsion was prepared to increase the availability of DMY during the preservation due to the low solubility of DMY. And to evaluate the efficiency of DMY emulsion, we measured the antimicrobial activity of dihydromyricetin (DMY) against specific spoilage bacteria of grouper and explored the mechanism of its antimicrobial function.

2. Materials and Methods

2.1. Activation and Inoculation of Strains. Through previous experiments [13], six strains were isolated. They screened from grouper under 4°C storage conditions, including *Shewanella putrefaciens* (*S. putrefaciens*), *Staphylococcus saprophyticus* (*S. saprophyticus*), *Pseudomonas azotoformans* (*P. azotoformans*), *Pseudomonas psychrophila* (*P. psychrophila*), *Pseudomonas antarctica* (*P. antarctica*), and *Pseudomonas koreensis* (*P. koreensis*). These strains were thawed at 4°C from −80°C and then added to 9 mL tryptic soy broth medium (TSB) liquid medium. In order to achieve an initial bacterial inoculum of about 10^6 CFU/mL, they were shocked and stunned again after 1% transfer to make a bacterial suspension with OD of 0.3 and set aside.

2.2. Analysis of Spoilage Capacity of Spoilage Bacteria. Taking the spoilage metabolites (total volatile base nitrogen, TVB-N) produced at the end of the shelf life as an indicator, the research was conducted by calculating spoilage bacteria's spoilage ability. The spoilage metabolite yield factor $Y_{\mathrm{TVB-}N/\mathrm{CFU}}$ was calculated as shown in

$$Y_{\mathrm{TVB-N/CFU}} = \frac{\mathrm{TVB} - N_s - \mathrm{TVB} - N_0}{\mathrm{CFU}_s - \mathrm{CFU}_0}. \quad (1)$$

TVB−N_0 is the TVB−N content of grouper on day 0 after injection of the strain; the unit is mg N/100 g; TVB−Ns is the TVB−N content of grouper at the end of storage after the injection of the strain; the unit is mg N/100 g; CFU_0 is the total number of colonies in grouper on day 0 after inoculation, in log10 CFU/g; CFU_S is the number of colonies in grouper at the end of storage after injection, in log10 CFU/g.

2.3. Measurement of Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC). The MIC and MBC analysis of the bacterial inhibitory capacity of DMY was performed using the broth microdilution method according to CLSI guidelines [14]. It was dissolved with 3% DMSO to obtain a 1.28 mg/ml DMY stock solution, and then serial two-fold dilution was performed in a 96-well microtiter plate. The spare bacterial suspension was added and incubated at 30°C for 24 h. DMY's TSB medium served as a control group. Each 1 ml sample was diluted by decimal in a 0.85% (w/v) sodium chloride solution used for colony counting, and each concentration was repeated three times. The minimum concentration of DMY that inhibits the growth of visible bacteria was defined as MIC. The test group's bacterial suspension with no visible bacterial growth was also cultured on nutrient agar. The minimum concentration of DMY that resulted in no colony growth was defined as MBC [15].

2.4. Preparation of DMY Emulsion. According to the characteristics of dihydromyricetin, which is soluble in hot water but not in cold water, the dihydromyricetin emulsion was prepared by ultrasonic wave with chitosan, referring to the modified method of Woranuch and Yoksan [16] and Yang et al. [17], and the operation steps are shown in Figure 1. The dihydromyricetin with a concentration of 0 MIC, 2 MIC, 4 MIC, and 8 MIC was mixed with 2% Tween 80, and water was added. The mixture was homogenized at a speed of 15000 rpm using a homogenizer for 2 min and then sonicated to obtain a coarse emulsion. At room temperature, chitosan (Ch) (2%, w/v) was added to the acetic acid solution (1%, v/v) at 40°C and stirred for 4 h and filtered through a 1 μm pore filter, to remove the insoluble chitosan from the solution. Sodium alginate (SA) (1%, w/v) was added to $CaCl_2$ solution (0.1%, w/v), and Ch-SA was made by mixing 1.5 g glycerol, 170 ml Ch solution, and 300 ml SA solution mix the solution, then an equal volume of dihydromyricetin crude emulsion was added, and the mixture was homogenized with an ultrasonic homogenizer at 13000 rpm under ice bath conditions for 10 minutes to obtain 0 MIC, 1 MIC, 2 MIC, and 4 MIC dihydromyricetin emulsions.

2.5. Characterization of DMY Emulsion

2.5.1. pH. The pH of the emulsion was measured using a pH meter at room temperature. All measurements were repeated three times.

2.5.2. Differential Scanning Calorimetry (DSC). The method of Phunpee et al. [18] was adapted. The samples were weighed and placed in an aluminum pot with a lid and measured using nitrogen gas at a constant flow rate of 60 ml/min. A blank aluminum pot with a lid was used as a control and heated by a differential scanning calorimeter (Mettler Toledo DSC823e, USA) at a temperature range of 30 to 300°C by 10°C/min. The DSC was recorded for all sample curves. All measurements were repeated three times.

2.6. Microbial Cell Integrity. The cell membrane integrity can be assessed by detecting DNA, RNA, and protein leakage in the cell. After treatment with 0 MIC, 1 MIC, 2 MIC, and 4 MIC DMY at 30°C for 4 hours, the cells were collected by centrifugation (3000 r/min, 4°C, 10 min) and washed with 0.1 m PBS for three times, re-suspended in 0.1 m PBS

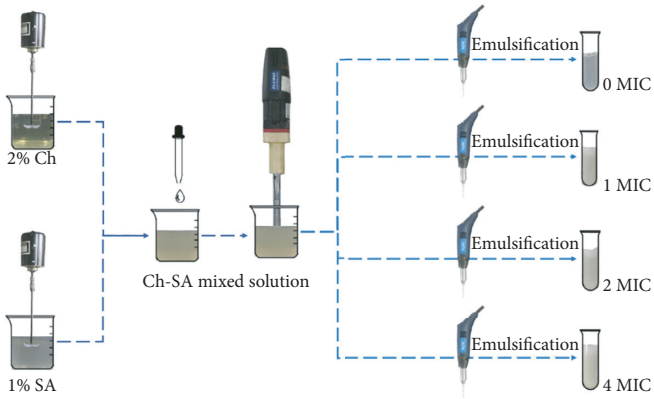


Figure 1: Flow chart of the preparation of Ch-SA stabilized DMY pickering emulsion.

solution, and immediately filtered the sample with 0.22 μm microporous filter. Absorbance readings were measured at 260 nm and 595 nm, and the number of nucleic acids and proteins released from the cytoplasm was determined by a UV spectrophotometer (UNICO UV-2100, USA) [19]. According to equations (2) and (3), the formula for nucleic acid is shown as follows:

$$\text{DNA concentration} = A_{260} \times 50\mu\text{g/mL}, \quad (2)$$

$$\text{RNA concentration} = A_{260} \times 40\,\mu\text{g/mL}, \quad (3)$$

where A_{260} is the absorbance value of the sample solution measured at 260 nm.

2.7. Using the Bicinchoninic Acid (BCA) Method to Measure Cell Permeability. The BCA protein determination kit got the protein leakage through the bacterial cell membrane. The OD value was measured at 562 nm using a UV spectrophotometer [20].

2.8. DMY Blocks the Three Metabolic Pathways of EMP. The activities of hexokinase (HK), phosphofructokinase (PFK), and pyruvate kinase (PK) in the EMP pathway of specific spoilage bacteria were tested with HK kit, PFK kit, and PK kit (Solarbio, Beijing Solarbio Science & Technology Co., Ltd.).

2.9. Cell Adenosine Triphosphate (ATPase) Activity Determination. ATPase assay kit (Jiangsu Jiancheng Institute of Biological Engineering) was used to test the inhibition of ATPase of specific spoilage bacteria by DMY emulsion, and the absorbance value of the extract at 636 nm was obtained and analyzed using a UV spectrophotometer [15, 20].

2.10. Alkaline Phosphatase (AKPase) Activity. According to the manufacturer's instructions, the AKP activity was determined using the AKP kit (Nanjing Jiancheng Institute of Bioengineering, Nanjing, China). *P. antarctica* was cultured in broth to a bacterial suspension with an OD of 0.3 and then mixed with DMY nanoemulsions with concentrations of 0 MIC, 1 MIC, 2 MIC, and 4 MIC. The mixture was incubated in a shaking incubator at 30°C and 120 r/min for 4 hours. The sample was then centrifuged at 4°C, 3500 r/min for 10 minutes, and the supernatant was used to detect AKP activity [21].

2.11. Confocal Laser Scanning Microscope (CLSM). To evaluate the damage of bacterial cell membrane by DMY treatment, CLSM (LEICA TCS SP5 II, Leica Microsystems, Germany) was used with dual fluorescence staining to analyze acridine orange/propidium iodide (AO/PI). AO emits green fluorescence and is used to stain live cells, while PI emits red fluorescence and is used to stain dead cells. The fluorescent dye was prepared by mixing 5 mg AO and 10 mg PI in 10 mL PBS (0.01 m, pH 7.2). In short, in the logarithmic growth phase, bacterial cells were treated with different concentrations of DMY for 4 hours. Then, the bacteria with 100 μL of fluorescent dye in the dark were stained for 15 minutes while shaking gently. After washing with PBS and centrifugation, the cells were examined in an argon laser at 515/488 nm using CLSM. A control assay was performed without DMY treatment [22].

2.12. Statistics and Analysis. All the results of physicochemical determinations were presented as mean ± standard deviation. Statistical analyses were performed using SPSS software (version 22.0; IBM Corp., Armonk, NY). One-way or multi-way analysis of variance (ANOVA) and Bonferroni statistical tests were used to determine the level of significance.

3. Results and Discussion

3.1. Analysis of the Spoilage Causing Ability of Spoilage Bacteria in Grouper. The spoilage potential of putrefying bacteria was expressed by the production of metabolites of spoilage bacteria per unit quantity (spoilage metabolite yield factor), as shown in Table 1. The total number of colonies of *P. psychrophila* and *S. putrefaciens* was 8.63 log10 CFU/g and 8.23 log10 CFU/g at the end of storage, respectively, which were significantly higher than those of the other four groups of spoilage bacteria. However, *P. antarctica* and *P. psychrophila* had the highest spoilage potential, while *P. koreensis* had the weakest. Moreover, the number of spoilage metabolites produced per unit number of *P. psychrophila* and *S. putrefaciens* was lower than *P. antarctica*. So, the metabolite production could be an indicator to reflect the spoilage potential of spoilage bacteria, and *P. antarctica* was selected as the target of the DMY inhibition in this experiment.

3.2. MIC and MBC. Six dominant spoilage bacteria strains obtained from the screening of hybrid grouper were added to the DMY dilutions. The TSB medium used as a control

Table 1: Analysis of the corrosion ability of grouper inoculated with specific spoilage bacteria at 4°C.

Strain name	Spoilage colony count log10 CFU/g		Decay product content mg N/100g		Spoilage metabolite yield factor
	CFU_0	CFU_S	TVB–N_0	TVB–N_S	$Y_{TVB-N/CFU}$
Shewanella putrefaciens	5.43	8.23	12.37	17.36	1.78
Staphylococcus saprophyticus	5.75	7.56	11.90	15.06	1.75
Pseudomonas azotoformans	5.71	7.28	11.56	14.37	1.79
Pseudomonas psychrophila	6.66	8.63	13.46	17.73	2.17
Pseudomonas antarctica	6.60	8.02	15.04	18.31	2.31
Pseudomonas koreensis	5.97	7.90	13.86	16.96	1.60

remained limpid, indicating that DMY was not contaminated. The results of MIC and MBC are shown in Table 2.

The results showed that DMY had a general inhibitory effect on all wild strains but a higher inhibition against *P. antarctica* with the MIC of 2.0 mg/mL and MBC of 6.4 mg/mL. The results indicated that DMY had a significant inhibitory effect on bacteria, which was consistent with the results of other researches [23, 24].

Table 2: MIC and MBC of DMY against specific putrefactive bacteria of hybrid grouper.

Strain name	MIC (mg/mL)	MBC (mg/mL)
Shewanella putrefaciens	3.2	>25.0
Staphylococcus saprophyticus	0.4	>25.0
Pseudomonas azotoformans	>25.0	>25.0
Pseudomonas psychrophila	3.2	8
Pseudomonas antarctica	2.0	6.4
Pseudomonas koreensis	6.4	16

3.3. Characterization of DMY Emulsions

3.3.1. pH Variation of DMY Emulsions at Room Temperature Storage. As shown in Table 3, DMY emulsions had weak acidity, and with the increase of concentration, the pH value decreased. The pH values of 1 MIC and 2 MIC were observed to have a non-significant decrease throughout the storage, while the value of 4 MIC decreased significantly ($p < 0.05$).

3.3.2. Thermal Properties. DSC is an efficient thermal analysis technique that can characterize the samples' thermal properties and the formation of the embedding material [25, 26]. The DSC thermal spectra of CH, SA, CH-SA, 1 MIC DMY, 2 MIC DMY, and 4 MIC DMY are shown in Figure 2. In this experiment, except for DMY, which was in a dry powder state, all other samples were in solution or emulsion, so there was a downward exothermic trend initially, which may be related to the evaporation of water [27]. Ch and SA had more profound heat absorption peaks at 162°C and 169°C, respectively. At the same time, the temperature range of the CH-SA thermal transition curve was 107–141°C, followed immediately by a small heat absorption peak. A heat absorption peak at 260°C can be found in the DSC thermal spectra of DMY, indicating its melting point. Comparison of the 1 MIC and 4 MIC emulsions of DMY mixed with CH-SA showed no heat absorption peak for dihydromyricetin, indicating that dihydromyricetin was encapsulated CH-SA.

In contrast, the emulsions of 1 MIC DMY, 2 MIC DMY, and 4 MIC DMY have independent endothermic peaks, and their melting points are significantly lower. The decrease in melting point indicates a reduction in sample stability and structural integrity [28]. However, the lack of independent endothermic peaks of DMY in the range of 263–285°C means that DMY and CH-SA have an interaction, confirming that DMY has been encapsulated or uniformly dispersed in the polymer mechanism in an amorphous state. Many scholars have found that the bound water in chitosan particles will reduce the endothermic peak [28–30]. Therefore, in this study, the endothermic peak changes of 1 MIC DMY, 2 MIC DMY, and 4 MIC DMY emulsions are due to the dispersion and interaction of DMY in chitosan particles.

3.4. Effect of DMY on Nucleic Acids and Proteins of Pseudomonas antarctica. The activities of bacteria are closely related to RNA, DNA, and proteins, so the integrity of cell membranes can be assessed by detecting the leakage of nucleic acids and proteins from the cells [19]. The leakage of nucleic acids and proteins from *P. antarctica* in 4 DMY treatments is shown in Figure 3. The amount of nucleic acid leakage in *P. antarctica* increased from 20.7 μg/mL to 78.3 μg/mL after 12 h of DMY treatment at 2 MIC concentrations. At the end of storage, the amount of protein leakage all increased to different degrees. It can be seen from Figure 3(b) that the protein leakage increased with increasing DMY concentration in the past. On day 12, the leakage of protein was only 48.2 μg/mL at 0 MIC concentration, while it was as high as 181.2 μg/mL in the 4 MIC group. And the protein leakage increased to 132.9 μg/mL and 152.8 μg/mL at 1 MIC and 2 MIC concentrations, respectively.

These results suggested that dihydromyricetin disrupted the cell membrane of *P. antarctica*, which led to leakage of proteins and nucleic acids. Di Pasqua et al. [31] explained the phenomenon that there were three hydroxyl groups in the chemical structure of DMY. The hydroxyl groups can bind to the cell membrane of bacteria through hydrogen bonding. And this interaction disrupted the cell membrane structure, which led to leakage of intracellular components. In these studies [9, 32], DMY showed its ability to significantly reduce membrane fluidity and act as an inhibitor of biofilm

Table 3: pH values at the beginning and end of 15 days of storage at room temperature for different DMY emulsions concentrations.

	Initial				15 days			
	0 MIC	1 MIC	2 MIC	4 MIC	0 MIC	1 MIC	2 MIC	4 MIC
pH	6.27 ± 0.67	5.42 ± 0.28	4.90 ± 0.11	3.16 ± 0.27	6.30 ± 0.17	5.29 ± 0.07	4.82 ± 0.09	2.73 ± 0.05

The symbol "±" indicates standard deviation.

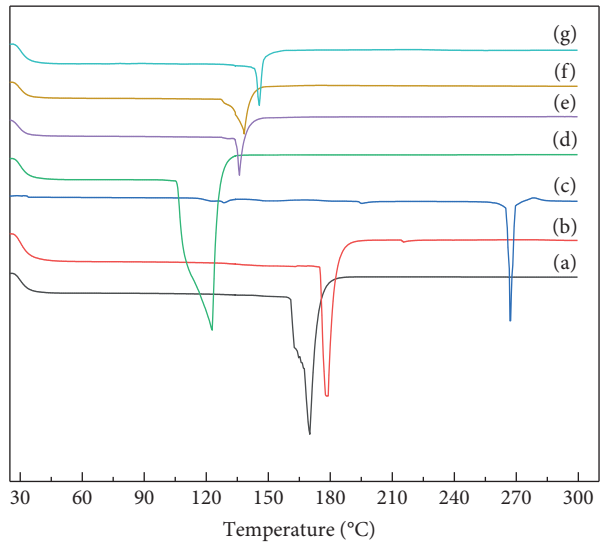


Figure 2: DSC analysis of the thermal properties of each component and the mixed emulsions. DSC thermograms of (a) Ch, (b) SA, (c) DMY, (d) Ch-SA, (e) 1 MIC DMY, (f) 1 MIC DMY, and (g) 1 MIC DMY.

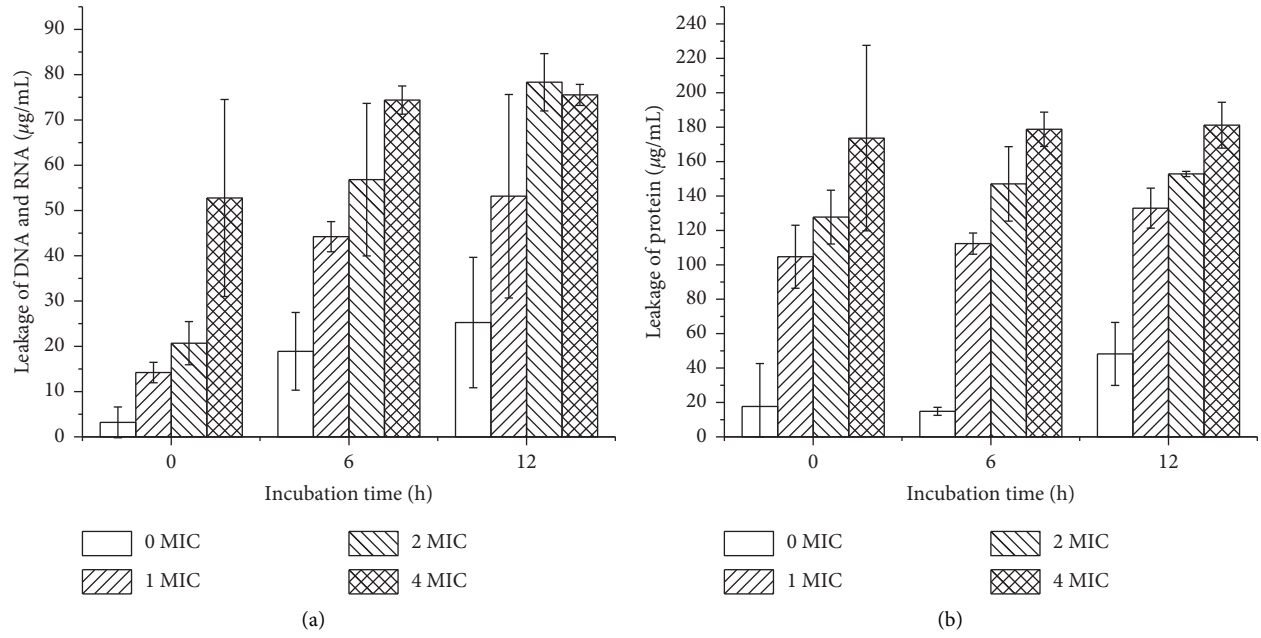


Figure 3: Leakage of DNA\RNA (a) and protein (b) from *P. antarctica* treated with DMY.

formation by interacting with phospholipids in the cell membrane. After breaking the first barrier, DMY caused leakage of intracellular nucleic acids and proteins by groove binding to intracellular DNA, further disrupting bacteria's normal function.

3.5. Effect of DMY on the Energy Metabolism of Pseudomonas antarctica. The energy metabolism of bacteria is closely related to the activity of ATPase [33, 34]. The results are shown in Figure 4. With different concentrations of DMY treatments, the ATPase activity decreased from 0.96 U/mg

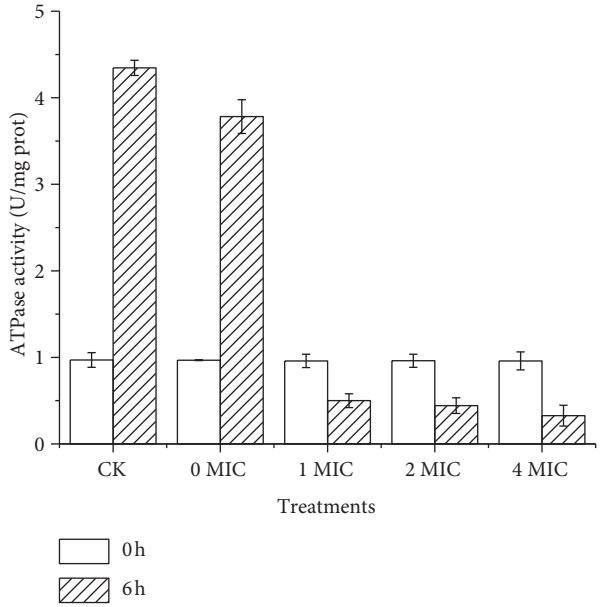


Figure 4: Effect of DMY on the ATPase activity of *Pseudomonas antarctica*.

prot to 0.50 U/mg prot, 0.44 U/mg prot, and 0.33 U/mg prot in the three treatment groups compared to the control group, with the most significant change in enzyme activity after DMY treatment at 4 MIC, decreasing by 65.6%. This result indicated that DMY had a significant inhibitory effect on energy metabolism in *P. antarctica* and demonstrated that DMY disrupted the bacterial cell membrane leading to ATP leak.

3.6. Effect of DMY on AKPase Activity of Pseudomonas antarctica. AKPase, which is present on the membrane between tissue cells and organelles, is a protease on biological membranes, and its action cannot be detected outside normal cells. Therefore, it can be used to measure the integrity of cell membranes [33]. As shown in Figure 5, there was no significant downward trend of AKPase activity in *P. antarctica* after DMY was treated in this experiment. It was indicating that there was no inhibitory effect on AKP activity despite the disruption of the bacterial cell membrane by DMY.

3.7. Effect of DMY on Pseudomonas antarctica EMP Pathway Enzymes. The glycolytic pathway (EMP) is an essential pathway for the production of ATP. It contains three irreversible reactions controlled by three vital regulatory enzymes (hexokinase, phosphofructokinase, and pyruvate kinase), so we can use the enzyme activities to study the mechanism of DMY inhibition on *Pseudomonas* spp. [33]. In the present study, DMY emulsions with 0 MIC, 1 MIC, 2 MIC, and 4 MIC concentrations were used to treat *P. antarctica* for 6 h. According to Figure 6, the three enzymes' contents were consistent among the groups at the initial point (0 h). As the time of DMY treatment increased, the three enzymes' activities became lower in all three treatment groups than that of the control group, indicating that DMY can effectively inhibit *P. antarctica* by controlling the TCA pathway of respiratory oxidative metabolism.

3.8. Analysis of the Effect of DMY on the Metabolic Activity and Bacterial Viability of P. antarctica. A fluorescence microscope was used to verify whether DMY affected the growth and reproduction of *P. antarctica*. AO can penetrate the intact cell membrane and stain the nucleus of living cells with uniform green fluorescence; the nucleic acid in apoptotic cells can be spoiled with red fluorescence by binding to PI. Figure 7 shows the CLSM images at 1000x. In the control group without DMY treatment, the bacteria were almost all green, which indicated that the untreated bacteria were active. However, as the DMY concentration increased, the red fluorescence observed on the DMY-treated slides intensified, implying a gradual decrease in the number of live cells. The maximum red fluorescence intensity was observed in the DMY-treated group after 4 MIC. It was concluded from the image analysis that DMY inhibited the growth and reproduction of *P. antarctica*.

As shown in Figure 7, the results indicated that the metabolic activity of *P. antarctica* after treatment with DMY was significantly reduced. The metabolic activity of cells was reported to play a vital role in forming biofilms [35, 36]. Similar reports have been made for tea polyphenols and mangiferin acid [37, 38], and these antibacterial substances achieved inhibition by inhibiting biofilm formation. At the same time, a significant reduction in the metabolic activity of the cells was observed. Therefore, in combination with the CLSM image results, DMY inhibited the viability and metabolic activity of *P. antarctica* and significantly inhibited the formation of bacterial biofilms.

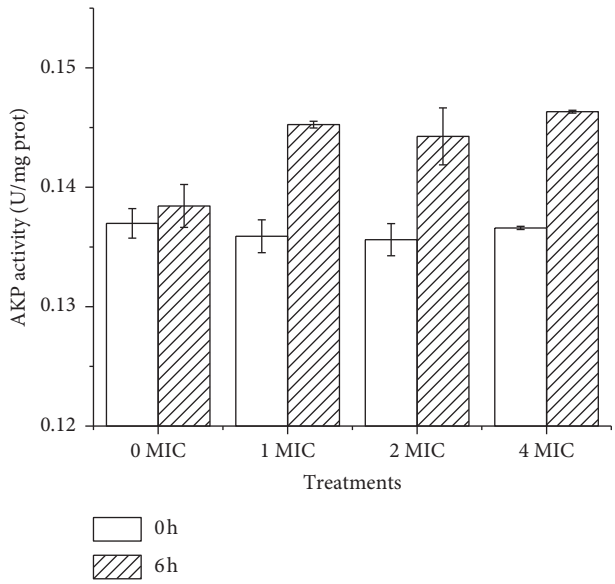


Figure 5: Effect of DMY on AKPase activity of *Pseudomonas antarctica*.

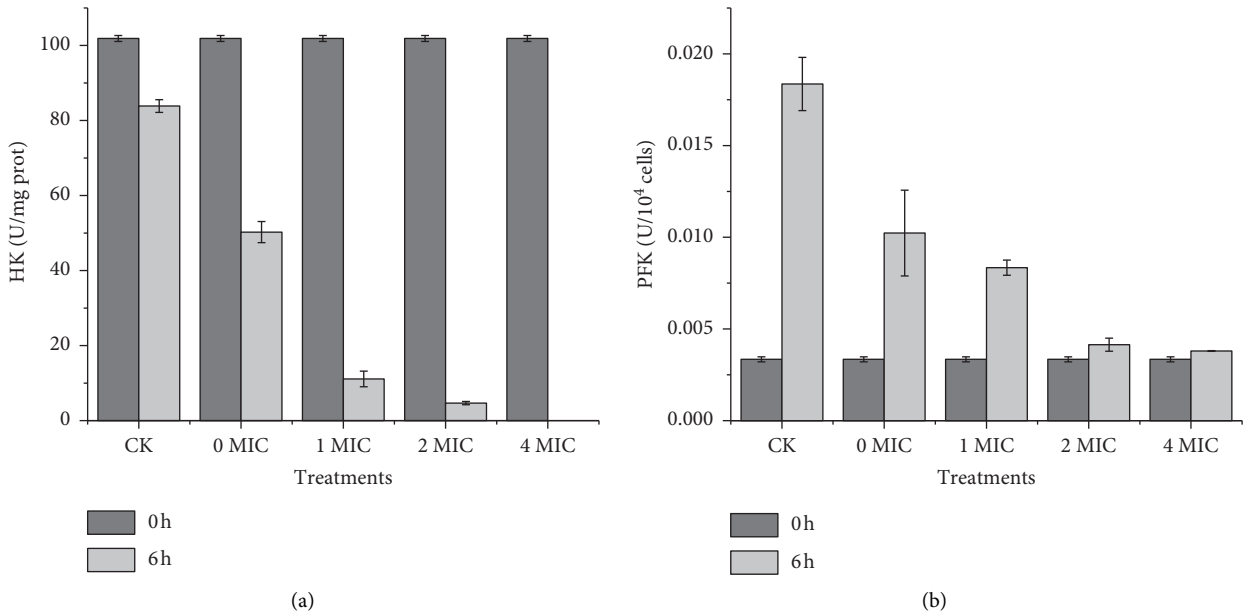
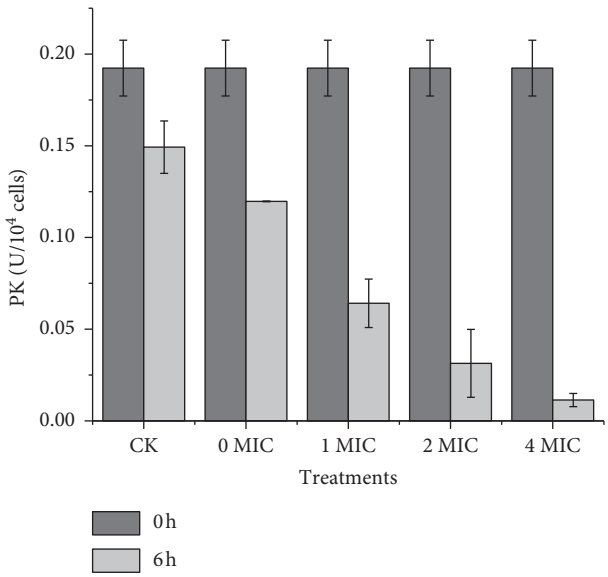
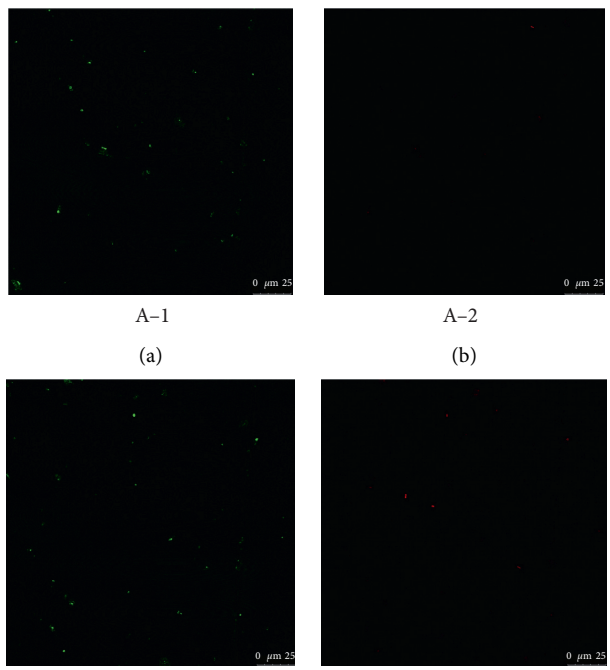


Figure 6: Continued.



(c)

Figure 6: Effect of different concentrations of DMY in the EMP pathway on hexokinase (a), phosphofructokinase (b), and pyruvate kinase (c) in *Pseudomonas antarctica.*



A–1 A–2

(a) (b)

B–1 B–2

(c) (d)

Figure 7: Continued.

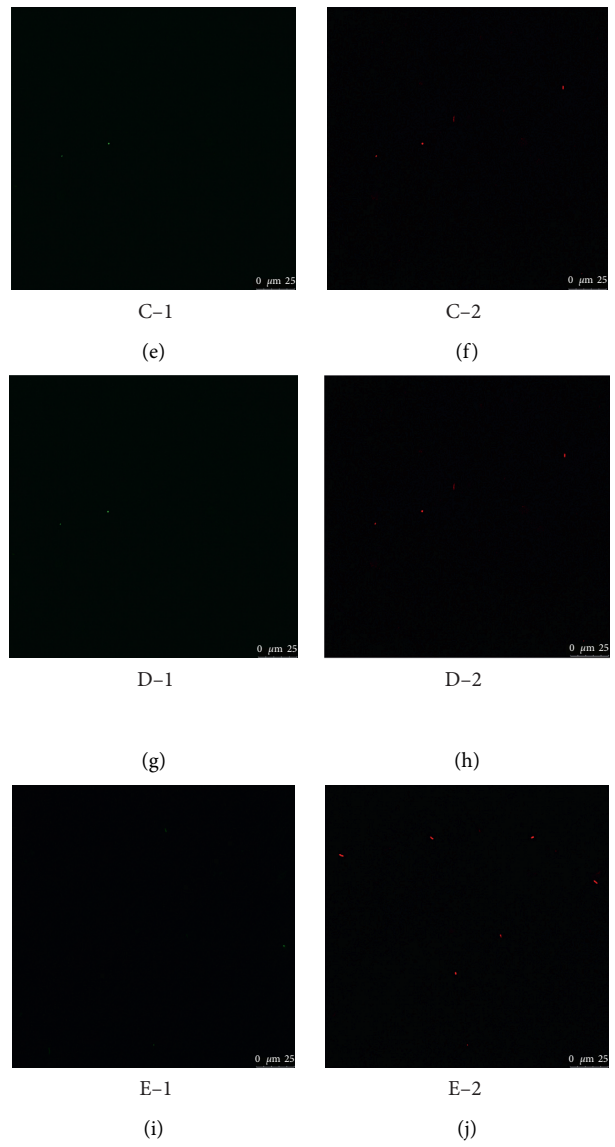


Figure 7: Confocal laser scanning microscope (CLSM) of *P. antarctica* at 1000 magnification. (a–e) CLSM images stained with AO, which are images of control, treatment by DMY at 0 MIC, 1 MIC, 2 MIC, and 4 MIC. (f–j) CLSM images stained with PI, which are images of control, treatment by DMY at 0 MIC, 1 MIC, 2 MIC, and 4 MIC. Nuclei of living cells show green fluorescence. Nucleic acids in apoptotic cells show red fluorescence.

4. Conclusions

The *P. antarctica* was the dominant spoilage bacterium in grouper. The emulsion is prepared by mixing with chitosan and sodium alginate to solve the problem that DMY is insoluble in water. CLSM images visually confirmed the inhibition effect of DMY against *P. antarctica*. Besides, DMY disrupted the biofilm structure of *P. antarctica* as evidenced by protein and nucleic acid leakage, AKPase leakage, and reduced ATPase activity. DMY also affected the respiratory metabolic pathway of *P. aeruginosa* by inhibiting key enzymes and thereby decreased the metabolic activity and viability of the bacteria. This work proved that DMY had potential as an effective and natural antibacterial in marine products.

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 regarding the publication of this paper.

Acknowledgments

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Research Article

Essential Oils from *Thymus capitatus* and *Thymus algeriensis* as Antimicrobial Agents to Control Pathogenic and Spoilage Bacteria in Ground Meat

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The antibacterial effects of essential oils (EOs) extracted from *Thymus capitatus* and *Thymus algeriensis* were assessed and evaluated against four pathogenic bacteria (*Escherichia coli* (ATCC 25922), *Listeria monocytogenes* (ATCC 19118), *Staphylococcus aureus* (ATCC 25923), and *Salmonella typhimurium* (ATCC 1402)) and one spoilage bacterium (*Pseudomonas aeruginosa* (ATCC 27853)). Both investigated EOs presented significant antimicrobial activities against all tested bacteria with a greater antibacterial effect of *T. capitatus* EO. In fact, the results indicated that the minimum inhibitory concentrations (MICs) and the minimum bactericidal concentrations (MBCs) of *T. capitatus* EO are in the range of 0.006–0.012% and 0.012–0.025%, respectively, while those of *T. algeriensis* EO ranged between 0.012 and 0.025% and 0.05%, respectively. Furthermore, the inhibitory effects of both EOs were appraised against the spoilage bacterium *P. aeruginosa*, inoculated in minced beef meat, at two different loads (10^5 and 10^8 CFU) mixed with different concentrations of EOs (0.01, 0.05, 1, and 3%) and stored at 4°C for 15 days. The obtained data demonstrated that the antibacterial effect of tested EOs varies significantly in regard to the levels of meat contamination and the concentrations of EOs. In fact, in the presence of 0.01 and 0.05% of oils, a decrease in bacterial growth ($p < 0.01$) was observed; but, such an effect was more pronounced in the presence of higher concentrations of EOs (1 and 3%), regardless the level of meat contamination. Besides, at the low contamination level, both EOs exerted a rapid and a more pronounced antibacterial effect, as compared to the high contamination level. The results illustrated the efficacy of both EOs as preservatives in food against well-known pathogens of food-borne diseases and food spoilage, particularly in *P. aeruginosa* in beef meat. As regards sensory evaluation, the presence of *T. capitatus* EO proved to improve the sensory quality of minced beef meat.

1. Introduction

Meat and meat products represent one of the most perishable foodstuffs [1] due to their complex composition which consists of proteins, saturated and unsaturated lipids, carbohydrates, vitamins, pigments, high water content, and moderate pH [2, 3]. A large amount of spoiled meat has to be discarded engendering significant economic losses. According to the European Regulation (EC) No 178/2002 [4], spoiled meat is considered as unsafe, unsuitable food for human consumption and forbidden by the law.

The mechanisms responsible for meat spoilage are related to microbial growth, lipid oxidation, and enzymatic autolysis. The breakdown of fats, proteins, and carbohydrates in meat leads to the formation of off-odors, off-flavors, and slime formation, rendering meat unacceptable for

the consumer [5–7]. The presence of pathogenic bacteria such as *E. coli*, *Salmonella*, and *S. aureus* in minced meat and contact surface samples can cause serious health risks [8]. Microorganisms which are generally responsible for meat spoilage are *Pseudomonas* spp., Enterobacteriaceae, and *Brochothrix thermosphacta* [9]. *Pseudomonas* causes meat and meat product spoilage and develops repulsive characteristics as putrefaction of proteins and lipids with changes in pH continue [10, 11].

Additionally, grinding has several detrimental effects on meat by increasing the surface area exposed to air and bacterial contaminations [12]. It increases losses of intracellular reductants as well as polyunsaturated fat, leading to deterioration of meat and the warmed-over flavors [13, 14].

To extend the shelf life and decrease bacterial growth on meat, the common method used is refrigeration. However, lower temperatures might also modify the composition of the microbiota present on meat, such as psychrotrophic bacteria like *Pseudomonas* spp., which could grow at low temperature [1]. For this, it is an important challenge to find a solution to prevent bacterial contamination of meat.

Many strategies are being used to control the growth of pathogenic and spoilage bacteria and prolong the shelf life of meat. Since ancient times, plants and plant extracts have been used as flavoring agents in the food processing industry; they also exhibit some antibacterial, antifungal, and antioxidant properties [15]. Several aromatic plants, essentially rosemary, garlic, lavender, leek, olive leaf, onion, oregano, pepper, peppermint, sage, and *Satureja montana*, are being added to meat and meat products [16].

Thyme oil is one of the top 10 EOs used as a natural preservative in food [17]. The genus *Thymus* L. is a member of the Lamiaceae family and contains about 215 species, particularly prevalent in the Mediterranean area [18]. Thyme species are aromatic plants widely used in Tunisia and they are well known for their antispasmodic, antimicrobial, expectorant, and antioxidant activities.

Several *in vitro* studies have reported the efficiency of plant EOs against food-borne pathogens, whilst few published papers have studied the antibacterial effect of *Thymus* EOs on pathogen growth, in meat.

Thus, the purpose of the current work is to evaluate the antioxidant and the antimicrobial activities of *Thymus capitatus* (*T. capitatus*) and *Thymus algeriensis* (*T. algeriensis*) EOs against pathogenic bacteria and the impact of different concentrations of such EOs on the proliferation of spoilage bacterium *P. aeruginosa* at two contamination levels of 10^5 CFU/g and 10^8 CFU/g at 4°C.

2. Materials and Methods

2.1. Extraction of the Essential Oils. The aerial parts of *T. capitatus* and *T. algeriensis* were collected from Zaghouan region (north of Tunisia) in June 2018. The freshly cut plants were dried for two weeks, in the shade, at room temperature. They were grounded into powder, followed by hydrodistillation in a Clevenger-type apparatus for 3 hours. The EOs were extracted, dried over anhydrous sodium sulphate (Na_2SO_4), filtered, and then stored in the dark at 4°C.

2.2. Free Radical Scavenging Assay. The DPPH (2, 20-diphenyl-1-picryl hydrazyl) radical scavenging capacity was measured according to the method described by Boulanouar et al. [19]. One ml of each concentration of the EO extract (200, 300, 400, and 500 μg/mL) was mixed with 250 μl of 0.2 mM methanolic DPPH solution. A negative control was prepared by mixing the same amounts of methanol and DPPH solution. The mixture was shaken vigorously and incubated for 30 min, in the dark, at room temperature. The absorbance was then measured at 517 nm using a UV spectrophotometer, and the percentage of activity inhibition (I%) was calculated by the following formula: $(I\%) = [(A0 - At/A0) \times 100]$, where A0 is the absorbance of the control sample (without EO) and At is the absorbance of the EO with DPPH at 30 min.

The EO concentration providing an I% of 50 (IC_{50}) is calculated from the regression equation prepared from the concentrations of the EO and the inhibition percentages. The experiment was carried out in triplicate.

2.3. Microorganisms and Growth Conditions. The bacteria used in the present study were obtained from the culture collections of ATCC and Institute Pasteur of Tunis. The strains of *L. monocytogenes* (ATCC 19118) were cultivated in PALCAM *Listeria* agar (Biokar Diagnostics), *S. aureus* (ATCC 25923) in Baird-Parker (Biokar Diagnostics), *E. coli* (ATCC 25922) in Mac Conkey Sorbitol (Biolife), *S. typhimurium* (ATCC 1402) in Hektoen (Biolife), and *P. aeruginosa* (ATCC 27853) in *Pseudomonas* agar F (King's Medium B) (Biolife), at 37°C. Working cultures were prepared by adding a loopful of each test bacterium to 5 ml of Luria–Bertani Medium (LB) (Oxoid Ltd., UK) and then incubated at 37°C for 18 h [20].

2.4. Determination of MIC and MBC. The minimum inhibitory concentrations (MICs) and the minimum bactericidal concentrations (MBCs) of *T. capitatus* and *T. algeriensis* were determined using the medium dilution method with minor modifications (NCCLS). The tested microorganisms were cultured at 37°C and diluted to approximately 10^6 CFU/ml, the negative control containing only the tested bacteria.

The inoculated plates were inverted and incubated at 37°C for 24 h. The MIC was defined as the lowest concentration of EO at which no visible growth of bacteria is shown. The plates showing a concentration of EO greater than or equal to MIC were incubated at 37°C for further 24 h. The concentration at which no visible growth is noticed was defined as the MBC. The experiment was carried out in triplicate.

2.5. Inhibitory Effect of EO against Pseudomonas aeruginosa Inoculated in Minced Beef Meat. The procedure reported by Careaga et al. [21] was followed with some slight modifications to study the inhibitory effect of EOs.

2.6. Preparation of the Meat Model. Four kilos and 500 g of fresh beefsteaks were obtained from a local meat supermarket. Meat samples were collected and transported for analysis in an insulated cooler. Each piece of meat was plunged in boiling water for 5 min to reduce the number of microorganisms attached to the beef muscle surface, which was eliminated with a sterile knife under aseptic conditions.

2.7. Treatment of Minced Beef Meat. To evaluate the antimicrobial activity of *T. capitatus* EO against bacteria in meat samples, pieces of meat were minced in a sterile grinder with 19 cm in diameter, and portions of 22 ± 0.1 g were put into a high-density polyethylene bag.

Decimal dilutions were prepared from a fresh culture of 24 h. For each dilution, the optical density at 620 nm and the CFU were determined by subculturing on agar. The data obtained were used to make a calibration. Thus, the initial inocula (10^5 CFU *P. aeruginosa* and 10^8 CFU *P. aeruginosa*) were obtained based on a spectrophotometer reading.

Halves of the meat samples were inoculated with 10^5 CFU *P. aeruginosa*/g of beef and the remaining halves with 10^8 CFU *P. aeruginosa*/g of beef. Then, the samples were treated with different concentrations (0.01, 0.05, 1, and 3%) of *T. capitatus* or *T. algeriensis* EO, dissolved in 10% DMSO and homogenized in a stomacher for 5 min. For the control sample, the EO extract was replaced by DMSO. Finally, all bags containing the meat samples were stored at 4°C and examined every three days, during 15 days of storage [20]. The experiment was carried out in triplicate.

2.8. Bacterial Enumeration. *P. aeruginosa* count was done by adding 9 ml of BHI broth to 1 g meat sample placed in a polyethylene bag. Bacterial strain enumeration was determined by the plate colony count technique. For this, a series of dilutions was performed with physiological saline solution, and 100 μL of each sample dilution was spread onto the surface of *Pseudomonas agar* F "King's Medium B" plates, followed by incubation at 37°C for 24 hours. The obtained results were expressed as $\log_{10}$ CFU/g of meat.

2.9. Sensory Analysis. The sensory test was carried out at the Laboratory of Epidemiology and Veterinary Microbiology, Institute Pasteur of Tunis, Tunisia. The day before the event tasting. meat samples were thawed in a refrigerator at 4°C. Minced beef meat samples were cooked with no added salt and divided into samples of 10 g. The samples should be of uniform size. These were placed in aluminum trays covered with aluminum foil identified and put in a conventional oven. The beef meat samples were warmed before the evaluation, covered with aluminum foil. and presented to the panelists. Twelve trained panelists, comprised student and employees of the Laboratory of Epidemiology and Veterinary Microbiology, Institute Pasteur of Tunis, were served five meat samples: 1, control; 2, treated with 1% *T. capitatus*; 3, treated with 3% *T. capitatus*; 4, treated with 1% *T. algeriensis*; 5, treated with 3% *T. algeriensis*, with water and an unsalted snack in between to remove the remaining flavor. Coffee was also served to neutralize their noses between samples. The panel evaluated each sample in triplicate. Judges were requested to evaluate the cooked beef meat (offered in a randomized order) with a 3-digit code. Each attribute was scored on a scale of 10 cm for each characteristic: taste, color, tenderness, flavor, juiciness, and odor. The attributes were ranged from the lowest intensity of each trait to the highest. They measured overall acceptability in beef meat samples using the 9-point hedonic scale (1: dislike extremely, 2: dislike very much, 3: dislike moderately, 4: dislike slightly, 5: neither like nor dislike, 6: like slightly, 7: like moderately, 8: like very much, and 9: like extremely) [22].

2.10. Statistical Analysis. For each test, the results were presented as mean ± SD of three independent samples. The inhibitory concentration 50% (IC_{50} values) for antioxidant activities was calculated by a nonlinear regression analysis using GraphPad Prism, version 5.0. The *in situ* antibacterial activity was also performed using GraphPad Prism, version 5.0. The results were analyzed by two-way analysis of variance (ANOVA) to evaluate different antimicrobial treatment effects during the time of storage of 0, 3, 6, 9, 12, and 15 days. The statistical data analysis processed by ANOVA was calculated at a significance level of $p < 0.05$ using Bonferroni's multiple comparison tests.

CMI, CMB, and sensory data were analyzed by one-way ANOVA with the general linear model procedure of SAS (9.1). The residual mean square error was used as the error term. Means were separated using Duncan's test with a significance level of $p < 0.05$ (SAS, 9.1).

3. Results and Discussion

3.1. Antioxidant Activity: Free Radical Scavenging Assay. The EOs of herbs possess antioxidant properties that improve the shelf life of food. Thus, incorporation of EOs directly into food helps preserving it from oxidation phenomena [23]. In this context, it was shown that the antioxidant activity of EO of *T. capitatus* exhibits higher antiradical activity with an IC_{50} value of 213.53 μg/ml than that of *T. algeriensis* showing an IC_{50} value of 861.12 μg/ml, butylated hydroxytoluene (BHT) presenting an IC_{50} value of 30 ± 0.01 μg/ml (Table 1). These results agreed with those of Amarti et al. [26], who showed that *T. capitatus* EOs possess strong antioxidant activities with IC_{50} equal to 69.04 μg/ml. However, the used *T. algeriensis* EO demonstrated weaker antioxidant effect with IC_{50} equal to 745 μg/ml.

The antiradical activity of *T. capitatus* EO could be attributed to its high content of carvacrol (88.89%). On the contrary, *T. algeriensis* EO presented a weaker activity because of its poor content in phenolic compounds. In fact, a highly positive link between phenolic compounds and antioxidant activity was provided in this study, which is in agreement with other reported findings [27–29]. Based on these results, *T. capitatus* can be used as a natural antioxidant in food or for pharmaceutical applications.

Table 1: Antioxidant and antimicrobial activities of *Thymus capitatus* and *Thymus algeriensis* essential oils.

	Thymus capitatus essential oils		*Thymus algeriensis* essential oils	
Main essential oil compounds*	Carvacrol (88.98%), thymol (0.51%), *p*-cymene (1.14%), and α-terpinene (0.40%)		Linalool (17.62%), camphor (13.82%), terpinen-4-ol (6.80%), α-terpineol (6.41%), and α-terpenyl acetate (6.27%)	
Antioxidant activities IC_{50} (μg/ml)	213.53		861.12	
Antimicrobial activities (%)	MIC	MBC	MIC	MBC
E. coli	0.006^{Ba}	0.012^{Ba}	0.025^{Aa}	0.05^{Aa}
S. aureus	0.006^{Aa}	0.012^{Ba}	0.020^{Ba}	0.05^{Aa}
L. monocytogenes	0.012^{Bb}	0.025^{Bb}	0.025^{Aa}	0.05^{Aa}
P. aeruginosa	0.012^{Bb}	0.025^{Bb}	0.025^{Aa}	0.05^{Aa}
S. typhimurium	0.006^{Aa}	0.012^{Ba}	0.025^{Ba}	0.05^{Aa}

*The detailed results were reported by El Abed et al. and Ahmed et al. [24, 25]. IC_{50}: concentration of essential oil required for 50% of inhibition. MIC: minimal inhibitory concentration. MBC: minimal bactericidal concentration. A and B: different letters in the same row indicate significant differences ($p < 0.05$). a and b: different letters in the same column indicate significant differences ($p < 0.05$).

3.2. In Vitro Antibacterial Effect of Thyme Essential Oil. According to the results of MIC and MBC, illustrated in Table 1, both investigated EOs' activities presented an antimicrobial activity against all tested bacteria with a greater antibacterial effect of *T. capitatus* EO. As illustrated in a previous study of El Abed et al. [24], the EOs of *T. capitatus*, harvested from Zaghouan region, presented 19 compounds with the presence of several bioactive compounds, including carvacrol (88.98%), thymol (0.51%), *p*-cymene (1.14%), and α-terpinene (0.40%). As reported by Ben Hadj Ahmed et al. [25], the EOs of *T. algeriensis* from Zaghouan region presented 39 compounds, with linalool as a major compound (17.62%), followed by camphor (13.82%), terpinen-4-ol (6.80%), α-terpineol (6.41%), and α-terpinyl acetate (6.27%).

In addition, the results indicated that the MICs and MBCs of *T. capitatus* EO are in the range of 0.006–0.012% and 0.012–0.025%, respectively, while those of *T. algeriensis* EO ranged between 0.020 and 0.025% and 0.05%, respectively. These results are similar to those presented by Amarti et al. [30], which reported that *T. capitatus* EO from Morocco, mainly composed of carvacrol (70.92%), inhibits the growth of *E. coli* and *S. aureus* at a concentration of 1/2000 (v/v). A previous study, carried out in Tunisia by Aouadhi et al. [27], showed that the *T. capitatus* plant from Bizerte, containing thymol (81.49%), exhibits significantly higher antibacterial activity than *T. capitatus* from Sousse that contains thymol (69.95%), with MIC values ranging between 0.025 and 0.8%. Our results indicated that the used *T. capitatus* EO, collected from Zaghouan, exhibits the strongest antibacterial effect due to its high content of carvacrol (88.98%) [24, 31, 32].

Findings from the present study indicated that *T. capitatus* EO has a very significant ($p < 0.0001$) antibacterial action against *E. coli*, *S. typhimurium*, and *S. aureus,* whereas *L. monocytogenes* and *P. aeruginosa* seem to be the least sensitive. These results are in accordance with previous studies revealing that the weakest activity of *T. pectinatus* EO is observed against *P. aeruginosa* [31]. The EO extracted from the *T. algeriensis* plant exhibited a moderate antimicrobial effect against most tested bacteria, without any significant difference ($P > 0.05$) between them. Our results showed that both EOs did not have selective antibacterial activity on the basis of the cell wall differences of bacterial microorganisms. These findings are in agreement with previous works carried out with several *Thymus* species [33].

The mechanism of action of EOs and phenolic compounds on microorganisms has not been elucidated; it is generally proved that these not only attack the cytoplasmic membrane, thus destroying its permeability and releasing intracellular constituents, but also could cause membrane dysfunction with respect to electron transport, nutrient absorption, nucleic acid synthesis, and ATPase activity. This could be the result of the alteration of various enzymatic systems, including those involved in the production of energy and the synthesis of structural components [34].

Besides, our results showed that the antibacterial properties could be attributed to the high percentages of linalool (17.62%) and camphor (13.82%) of *T. algeriensis* EO [25, 35]. In this regard, the research of Liu et al. [36] showed a good antibacterial activity of linalool against *P. aeruginosa* with MIC and MBC values in the range of 431 and 832 μg/ml, respectively. Likewise, the study carried out by Rezzoug et al. [37] showed that the EO of *T. algeriensis* grown in the Atlas Algerian Sahara and composed of linalool (1.2%) inhibits *P. aeruginosa* growth with a MIC value of 512 μg/ml, while that of Moroccan *T. algeriensis*, composed of camphor (27,7%), showed a weak antibacterial effect against *E. coli* and *S. aureus*, with a MIC value of 1/100 [38]. It is worth noting that the chemical compositions of the EOs of *T. algeriensis* from Algeria and Morocco are completely distinct from that of Zaghouan, used in the present work.

Our study revealed that the antimicrobial activity of *P. aeruginosa* is significantly ($p < 0.0001$) more sensitive to EO of *T. capitatus* than that of *T. algeriensis*. These results are consistent with the previous report [39].

3.3. Antibacterial Efficacy of T. capitatus Essential Oil against Pseudomonas aeruginosa in Minced Beef Meat. To study the antibacterial effect of *T. capitatus* EO, depending on the inoculum concentrations, two different loads of *P. aeruginosa* (10^5 CFU/g and 10^8 CFU/g) were used before treating minced beef meat with EOs. As shown in Figure 1,

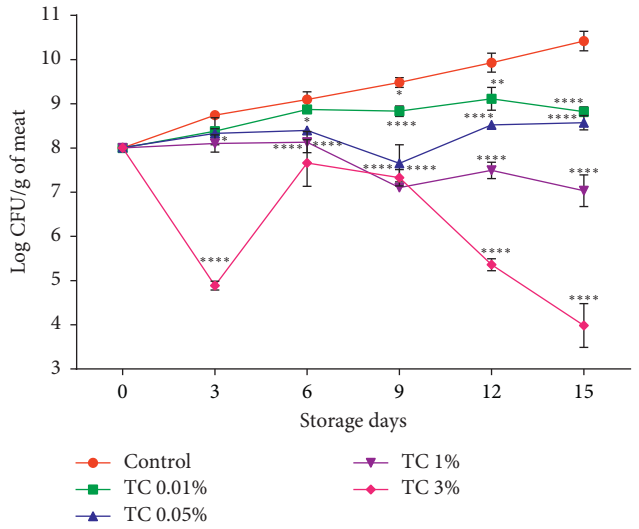

Figure 1: Time-related survival, at 4°C, of the high meat contamination level (10^8 CFU/g) of *P. aeruginosa*, following treatment with increasing concentrations of *T. capitatus* EO. The results represent the means of three replicate experiments, and error bars represent the standard error of the mean. Statistical significance differences: $^{*}p < 0.05$ (significant), $^{**}p < 0.01$ (very significant), and $^{***}p < 0.001$ and $^{****}p < 0.0001$ (extremely significant). CFU: colony-forming unit; TC: *Thymus capitatus*.

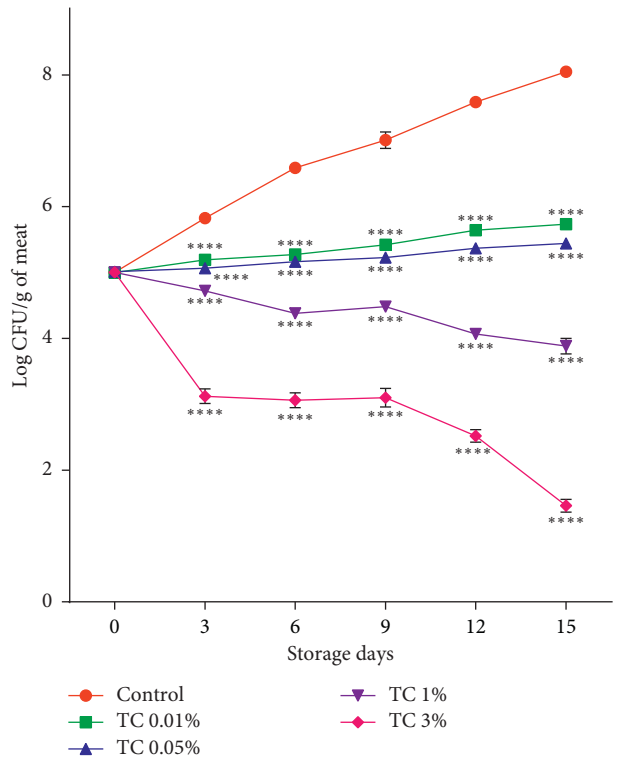

Figure 2: Time-related survival, at 4°C, of the low meat contamination level (10^5 CFU/g) of *P. aeruginosa*, following treatment with increasing concentrations of *T. capitatus* EO. The results represent the means of three replicate experiments, and error bars represent the standard error of the mean. Statistical significance differences: $^{*}p < 0.05$ (significant), $^{**}p < 0.01$ (very significant), and $^{***}p < 0.001$ and $^{****}p < 0.0001$ (extremely significant). CFU: colony-forming unit; TC: *Thymus capitatus*.

an increase in the *Pseudomonas* count was detected, from the first day of incubation, when a high inoculum (10^8 CFU/g) was used. *P. aeruginosa* count then increased by 2.41 $\log_{10}$ CFU/g and reached 10.42 $\log_{10}$ CFU/g, fifteen days later. In contrast and according to the results shown in Figure 2, lower inoculum (10^5 CFU/g) induced an exponential increase of *P. aeruginosa* growth by 3.04 $\log_{10}$, reaching 8.05 $\log_{10}$ CFU/g at the end of the incubation period. Thus, it can be assumed that, at high inoculum concentration, bacteria growth is limited due to a deficiency in nutrients, as described by Mytle et al. [40].

In this study, we have tested the antimicrobial effect of both *T. capitatus* and *T. algeriensis* EOs against one of meat food-borne pathogens (*P. aeruginosa*), inoculated in minced beef meat, at different concentrations (0.01, 0.05, 1, and 3%). As demonstrated in Figure 1 and Table 1, the concentrations employed for the *in situ* tests are higher than those used for the MIC and MBC tests. This could be due to intrinsic and extrinsic factors (proteins, fat, temperature, and oxygen limitation) which may influence the behavior of bacteria in food ecosystems and their interactions with the antimicrobial agents [21]. In fact, high protein and fat contents in meat are known to solubilize phenolic compounds, decreasing their sensitivity to the antimicrobial action. It is worth noting that the antimicrobial effects of the spices are lower in food systems than in microbiological media [41]. In addition, some studies have reported that many plant extracts and EOs are used to decrease food pathogens in meat products [42].

On the contrary, meat samples inoculated with a low bacterial contamination level (10^5 CFU/g), in the presence of low concentrations of *T. capitatus* EOs (0.01% and 0.05%), showed approximately 5.73 and 5.44 $\log_{10}$ CFU/g of *P. aeruginosa*, respectively, as compared to 8.05 $\log_{10}$ CFU/g shown for the control untreated samples, at the end of the storage period (15 days). Moreover, meat samples inoculated with a high level of bacterial contamination (10^8 CFU/g) presented the same trend of growth with a reduction of 2 $\log_{10}$ CFU/g. In fact, *Pseudomonas* growth titers achieved 8.82 and 8.57 $\log_{10}$ CFU/g in the presence of 0.01% and 0.05% of *T. capitatus* EO, respectively, as compared to 10.42 $\log_{10}$ CFU/g of the control untreated sample. Treatment based on low bacterial inoculum in the presence of a concentration of 1% of *T. capitatus* EO was able to significantly decrease *P. aeruginosa* growth by 4.17 $\log_{10}$ CFU/g, reaching 3.88 $\log_{10}$ CFU/g after 15 days of storage at 4°C.

When beef meat was inoculated with a high concentration of *P. aeruginosa*, in the presence of 1% of *T. capitatus* EO, a reduction in the bacterial load, from 7.03 to 3.39 $\log_{10}$ CFU/g, was obtained. Treatment with 3% of *T. capitatus* EO showed a greater antibacterial effect than in the presence of 1, 0.05, and 0.01%. Moreover, a 3% concentration of *T. capitatus* EO induced a bacteriostatic effect, leading to a very significant ($p < 0.0001$) reduction of bacterial growth, from 6.59 and 6.44 $\log_{10}$ CFU/g to 1.46 and 3.98 $\log_{10}$ CFU/g, for low and high inoculum loads, respectively, after a 15-day storage period.

3.4. Antibacterial Efficacy of T. algeriensis Essential Oil against Pseudomonas aeruginosa in Minced Beef Meat. As shown in Figures 3 and 4, meat samples contaminated with two different concentration levels of *P. aeruginosa* and then treated with EOs of *T. algeriensis*, at 0.01 and 0.05%, displayed a bacterial growth significantly lower than that of untreated meat samples ($p < 0.0001$), by the end of the storage period. Based on the data shown in Figure 4, applying an initial inoculum of 10^5 CFU/g and 0.01 and 0.05% of *T. algeriensis* EO induced a reduction of 2.22 and 2.46 $\log_{10}$ CFU/g in bacterial growth, respectively. As shown in Figure 3, practically, the same evolution was observed; using the higher inoculum of 10^8 CFU/g, a reduction of 1.56 and 1.68 $\log_{10}$ CFU/g, respectively, was obtained after 15 days of storage.

On the contrary, a concentration of 1% of *T. algeriensis* EO allowed a high bacteriostatic effect, leading to a significant decrease in bacterial titers ($p < 0.0001$) of 3.67 $\log_{10}$ CFU/g for the low contamination level as compared to 2.86 $\log_{10}$ CFU/g for the high contamination level, by the end of the storage days. An identical trend was observed after the addition of 3% of *T. algeriensis* EO, leading to a significant reduction in bacterial titers ($p < 0.0001$) of 4.75 $\log_{10}$ CFU/g for low initial inoculum and 3.76 $\log_{10}$ CFU/for high initial inoculum.

Therefore, it is important to note that both used EOs are effective and able to inhibit the growth of *P. aeruginosa*. Hence, increasing the concentrations of EOs to the treated samples allowed a gradual decrease in bacteria counts. This is in accordance with the findings of Emiroglu et al. [43], which revealed that *Pseudomonas* spp. was reduced in the ground beef patties when coated with thyme and oregano EOs. In contrast, the report of Ouattara et al. [44] did not show any significant effect of thyme oil on the growth of meat spoilage microorganisms such as *Pseudomonas fluorescens*.

The antibacterial effect of *T. capitatus* EO reported in the present study was significantly stronger than that of *T. algeriensis* EO, for both meat contamination levels.

We have thus examined the impact of the initial inoculum on the antibacterial effects of *T. capitatus* and *T. algeriensis* EOs on the growth of *P. aeruginosa*. It was shown that both *T. capitatus* and *T. algeriensis* EOs induce a rapid antibacterial activity against a low inoculum (10^5 CFU/g) of *P. aeruginosa*. In fact, our results demonstrated that the antibacterial effect of both EOs appears to be significantly weak when used at low concentrations but becomes more pronounced at higher EO concentrations, even in the presence of high inoculum. These findings are in accord with those reported by Udekwu et al. [45] who stated that bacteria may appear susceptible to bioactive molecules when the inoculum is of a standard level (10^5 CFU/ml) but resistant if the inoculum is increased. Accordingly, Bulitta et al. [46] proved that killing *P. aeruginosa* is 23-fold slower at a concentration of 10^9 CFU/ml and 6-fold slower at 10^8 CFU/ml than at 10^6 CFU/ml. Besides, our study did not show any immediate lethal (bactericidal) effect against the *Pseudomonas* population when *T. capitatus* and *T. algeriensis* EO are applied. In addition, *T. capitatus* EO did exhibit more pronounced antimicrobial activity than *T. algeriensis* EO.

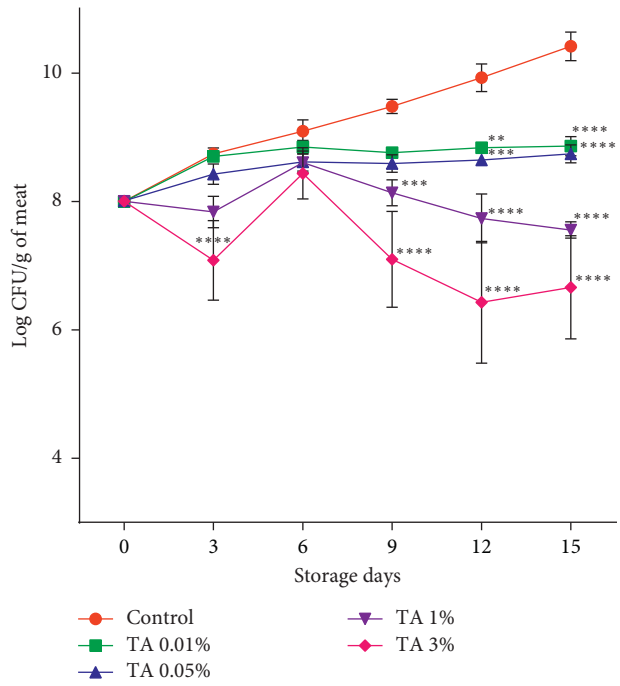

Figure 3: Time-related survival, at 4°C, of the high meat contamination level (10^8 CFU/g) of *P. aeruginosa*, following treatment with increasing concentrations of *T. algeriensis* EO. The results represent the means of three replicate experiments, and error bars represent the standard error of the mean. Statistical significance differences: $^{*}p < 0.05$ (significant), $^{**}p < 0.01$ (very significant), and $^{***}p < 0.001$ and $^{****}p < 0.0001$ (extremely significant). CFU: colony-forming unit; TC: *Thymus algeriensis*.

Both EOs showed rapid antibacterial activities against a low initial inoculum of 10^5 CFU/g of *P. aeruginosa* and weak and delayed antibacterial activities at a high concentration of initial inoculum of 10^8 CFU/g. This may be explained by the fact that the EO activities depend on the type, the composition, and the concentration of used EO, as well as the dose of targeted microorganisms present in meat.

3.5. Sensory Analysis. The sensory evaluation results are reported in Figures 5 and 6 and Table 2. Panelists ranked the samples treated with 3% of *T. capitatus* EO as superior ($p < 0.05$) to the other samples and to the control for smell. Notably, taste score showed that samples treated with 3% of *T. capitatus* EO were significantly higher ($p < 0.05$) compared to the samples treated with 1% of *T. capitatus* EO and the samples treated with 3 and 1% of *T. algeriensis* EO. Moreover, the samples treated with 1% of *T. capitatus* EO and the samples treated with 3 and 1% of *T. algeriensis* EO were scored significantly higher ($p < 0.05$) compared with the control. For the flavor, the treated samples with 3% of *T. capitatus* EO were ranked as superior ($p < 0.05$) to the treated samples with 1% of *T. capitatus* EO followed by treated samples with 1% of *T. algeriensis* EO and treated samples with 3% of *T. algeriensis* EO. For the tenderness attribute, samples treated with *T. capitatus* EO and *T. algeriensis* EO showed that the application of EO made

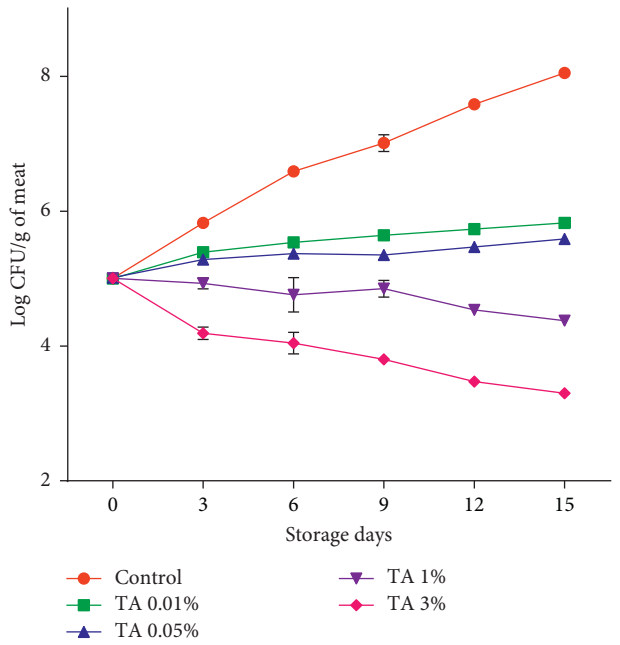

Figure 4: Time-related survival, at 4°C, of the low meat contamination level (10^5 CFU/g) of *P. aeruginosa*, following treatment with increasing concentrations of *T. algeriensis* EO. The results represent the means of three replicate experiments, and error bars represent the standard error of the mean. Statistical significance differences: $^{*}p < 0.05$ (significant), $^{**}p < 0.01$ (very significant), and $^{***}p < 0.001$ and $^{****}p < 0.0001$ (extremely significant). CFU: colony-forming unit; TC: *Thymus algeriensis*.

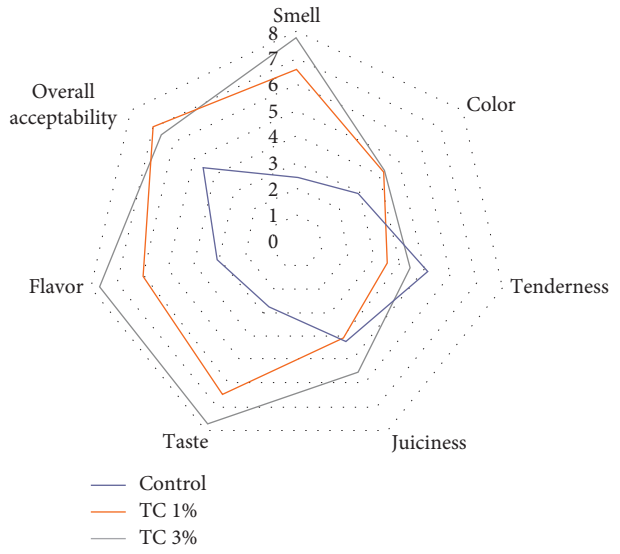

Figure 5: Sensory assay of meat samples treated with *T. capitatus* EO.

meat less tender. In terms of juiciness and color, there were no significant differences among samples treated with *T. capitatus* EO and samples treated with *T. algeriensis* EO and the control. The overall acceptability indicated that samples treated with *T. capitatus* EO were more acceptable ($p < 0.05$) than samples treated with *T. algeriensis* EO and

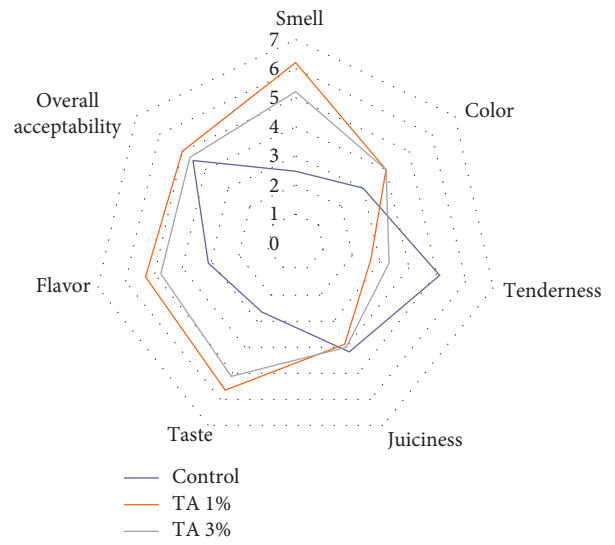

Figure 6: Sensory assay of meat samples treated with *T. algeriensis* EO.

Table 2: Mean (standard deviation) of sensory attributes of minced beef meat samples.

Sensory attribute	Control	TC1	TC3	TA1	TA3
Odor	2.44^{c}	6.55^{ab}	7.77^{a}	6.22^{ab}	5.22^{b}
Flavor	3.11^{c}	6^{ab}	7.66^{a}	5.33^{b}	4.77^{bc}
Taste	2.66^{c}	6.44^{ab}	7.66^{a}	5.66^{b}	5.11^{b}
Juiciness	4.22^{a}	4.11^{a}	5.44^{a}	3.88^{a}	4^{a}
Tenderness	5.11^{a}	3.55^{abc}	4.44^{ab}	2.66^{c}	3.33^{bc}
Color	3^{a}	4.22^{a}	4.33^{a}	4^{a}	4^{a}
Overall acceptability	4.55^{b}	7^{a}	6.55^{a}	5^{b}	4.66^{b}

Means in the same row followed by the same letter are not significantly different ($p > 0.05$). Acceptance was evaluated using a 9-point scale, where 1 = extremely dislike and 9 = extremely like.

the control. Generally, the present findings asserted that the application of *T. capitatus* EO has an important place in the improvement of the characteristic odor, taste, and flavor of minced beef meat. These results are in agreement with those obtained by Shalaby et al. [47], which reported that using olive leaf extracts as a natural preservative on minced beef improves the sensory attributes.

4. Conclusion

This study showed an interesting antioxidant effect using the DPPH assay and an interesting antimicrobial profile shown by MIC and MBC of *T. capitatus* EO. The results of "*in situ*" antibacterial activity confirmed those obtained by "*in vitro*" tests. It was also shown that *T. capitatus* EO is more effective than *T. algeriensis* EO, inhibiting *Pseudomonas* growth in inoculated minced beef meat at high concentrations (1% and 3%). In addition, at the low level of contamination, both EOs exerted a rapid and a more pronounced antibacterial effect, as compared to the high level of contamination. However, based on the sensory data, minced beef meat treated with *T. capitatus* EO was most acceptable to the panelists.

Therefore, *T. capitatus* EO could be used as a safe and a natural biopreservative for the improvement of microbiological and sensory quality of beef meat.

Data Availability

All the data used to support the findings of this study are approved and included within this article.

Conflicts of Interest

The authors declare that there are no conflicts of interest regarding the publication of this paper.

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Research Article

Evaluation of the Physicochemical, Antioxidant, and Antibacterial Properties of Tunichrome Released from *Phallusia nigra* Persian Gulf Marine Tunicate

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The aim of this study was to evaluate the physicochemical, nutraceutical, antioxidant, and antibacterial properties of tunichrome released from Persian Gulf tunicate (*Phallusia nigra*). For this purpose, molecular weight (SDS-PAGE), amino acid profile, chemical composition (GC-MS), mineral composition, functional groups (FTIR), total phenol content (TPC), total flavonoid content (TFC), antioxidant activity, and antimicrobial properties were investigated. The results showed that tunichrome contained a high amount of essential amino acids (i.e., Lys = 32.24 mg/100 g) and essential minerals. According to GC-MS results, tunichrome had different antioxidant and antimicrobial components. The TPC and TFC of tunichrome were 0.55 mg GA/g and 0.21 mg quercetin/100 g, respectively. Tunichrome showed higher antioxidant activity than ascorbic acid, and its radical scavenging activity values were increased from 30.28 to 82.08% by increasing concentration from 50 to 200 ppm. Inhibition zones of *Staphylococcus aureus, Bacillus cereus, Salmonella enterica,* and *Escherichia coli* O157:H7 were 14, 18, 17, and 15 mm, respectively. Moreover, the minimum inhibitory concentration values of tunichrome for *S. aureus, Bacillus cereus, S. enterica,* and *E. coli* O157:H7 were 1.17, 0.59, 0.59, and 1.17 mg/ml, respectively. The minimum bacterial concentrations were 2.34, 1.17, 1.17, and 2.34 mg/ml for *S. aureus, Bacillus cereus, S. enterica,* and *E. coli* O157:H7, respectively. These results showed that tunichrome of *Phallusia nigra* has excellent biological effects as a bioactive compound for food fortification.

1. Introduction

Since the synthetic antioxidants and antimicrobial components could exert several side effects, the interest of consumers and producers for safe and natural ingredients is growing [1]. Bioactive compounds are the most attractive ingredients in the design and development of functional foods. The tunicates are a wide marine animal group whose bodies are covered by the cellulose-containing tunic. They are marine filter invertebrates that exhibit the properties of the vertebrates. The accumulation of a high level of metals (i.e., either vanadium or iron) in seawater can be possible by intracellular polymer matrices [2, 3]. Thus, in the presence of metal chelators such as catechol and pyrogallol groups, tunichromes are associated with metal reduction. Moreover, tunichrome can form covalent crosslinking interactions or complexes with different types of multivalent ions in seawater. This ability can affect its functional properties such as swelling, solubilization, coagulation, and precipitation behavior.

The ascidians, thaliaceans, and appendicularians are the three main groups of tunicates [4]. These animals are consumed in Asia, Chile, and the Mediterranean in the past. These products come from sea wild and cultured

populations when the demand is high, especially for *Halocynthia* and *Styela* species. Edible species are usually from solitary stolidobranchs. *Halocynthia aurantium, H. roretzi, Microcosmus hartmeyeri, M. sabatieri*, *M. vulgaris, Polycarpa pomaria*, *Pyura chilensis, Styela clava,* and *S. plicata* are the important species eaten. These groups are presented in fresh and dried forms in the markets. High-quality processes of *H. aurantium, H. roretzi, P. chilensis*, *S. clava,* and *S. plicata* are the main seafood exports to Europe and America [5].

Generally, tunicates have a high nutritional value due to the presence of a high level of bioactive components and protein and low calories [6–10]. Moreover, some of them contain different vitamins (i.e., vitamin E, vitamin B12, and vitamin C), minerals (i.e., Na, K, Ca, Mg, P, Fe, Zn, and Cu), amino acids, folic acid, fatty acid, and pantothenic acid [11]. Some groups of tunicates have different low molecular weight peptides in their bodies such as styelins, plicatamide, halocyamines, lamellarins, and ferreascidin from *Styela clava*, *Styela plicata*, *Halocynthia roretzi*, *Didemnum chartaceum*, and *Pyura stolonifera*, respectively, as well as tunichromes from different tunicates species [5, 12, 13].

Some tunicates species can accumulate low molecular weight oligopeptides in their blood cell which are known as tunichromes. Tunichromes could play an important role in defense mechanism, mainly due to the phenoloxidase which can attack easily tunichrome. It is considered as a key component for both tunic formation and tunic wound healing which use the same primary biochemical mechanisms [13]. Tunichromes have different colors such as pink, red, and blue [14]. These pigments are ingredients in the blood cells of tunicates. Tunichromes were identified in 11 groups of different tunicates [5]. The broad biological activity of tunichromes remains mainly unknown. However, some researches offer unique functionalities for tunichrome in terms of antioxidant [15–17], antimicrobial [5, 18], and anticancer activities [19].

To the best of our knowledge, there is no information on the biological activities of the amazing tunichrome released from the *Phallusia nigra* Persian Gulf marine tunicate. Therefore, this research is a first attempt to evaluate the physicochemical, nutraceutical, antioxidant, and antibacterial properties of tunichrome released from *Phallusia nigra* Persian Gulf marine tunicate.

2. Materials and Methods

2.1. Materials. Phallusia nigra tunicate was randomly collected from Nayband Bay situated in the north (27° 30′S, 52° 35′E) of Bushehr, Iran, using SCUBA diving at the depth ranging from 0.5 to 1.5 m in spring of 2019. Media cultures and all other chemical materials were prepared in analytical grade from Merck and Sigma-Aldrich.

2.2. Methods

2.2.1. Sampling and Sample Preparation. 50 samples of *Phallusia nigra* tunicates were washed by double distilled water (DDW) to remove contaminants. They were immediately transported to the laboratory in a cool box (in ice-cold condition) and kept at room temperature for 30 min to release a pink color solution (tunichrome). The obtained suspension was centrifuged (4000 × g for 5 min) and then filtrated by Whatman No. 1 filter paper. The final solution was freeze-dried and powdered for further studies.

2.2.2. Total Protein Content. Total protein content was measured based on the AOAC method by the Kjeldahl device (Buchi, Switzerland).

2.2.3. Molecular Weight Measurement (SDS-PAGE). For the molecular weight determination, the tunichrome (5 mg/mL) was dissolved in 1% SDS solution prepared by phosphate buffer (pH 7.0) and stirred for 24 h followed by centrifugation at 5000 × g for 6 min at 20°C [20]. The supernatant (20 μl) was added to 10 μL Tris-HCl buffer (10 mM Tris-HCl, 10% (w/v) SDS, 0.1% (w/v) bromophenol blue, and 10% (v/v) glycerol) with or without 2% (v/v) β-mercaptoethanol. The suspension was then heated at 95°C for 11 min, and 20 μL of the suspension was developed on the gel slab which was contained resolving gel (12.5%, pH 8.8) and stacking gel (6%, pH 6.8). PowerPac 1000 (Bio-Rad, USA) was used for running the electrophoresis. The running buffer was made by diluting 100 mL 10 X Tris/Glycine/SDS buffer with the DDW at the constant voltage 220 V. The Coomassie Brilliant Blue was used for staining the gel for 60 min, and water was used for destaining for 24 h. The molecular weights were evaluated by comparison to Sinaclon markers (PR901641, CinnaGen Co., Tehran, Iran) at the ranges of 245–11 kDa.

2.2.4. Amino Acids' Profile. To evaluate amino acid profile in tunichrome, peptide bonds of protein were broken by hydrolysis. The hydrolysis process was conducted by heating the sample in an oxygen-free condition containing 6 M HCl and 0.1% phenolphthalein at 110°C overnight. Amino acids were measured by a Varian chromatographic system, containing a 1525 pump, a 9100 autoinjector, and a UV-vis detector. The hydrolyze suspension was injected into an automatic precolumn reaction by 0.1 mL of derivatizing reagent. This chromatographic process contained a solvent mixture (PBS buffer (10 mM, pH 4): acetonitrile (25 : 75) at a flow rate of 1 mL/min. In this system, a C18 Waters Nova-Pack reverse phase column (particle size 5 μm, 250 × 4.6 mm internal diameter) was applied. All the chromatographic data were processed in a V. 4.5 Star workstation supplied by Varian [21].

2.2.5. Na, Ca, K, Mg, Mn, Cu, Fe, and Zn Measurements. The concentrations of Na, Ca, K, Mg, Mn, Cu, Fe, and Zn of tunichrome were analyzed by a Selectra 2 auto-analyzer (Vital Scientific, Spankeren, Netherlands). To this end, 1 g of lyophilized powder was dissolved in the 5 g of DDW for 24 h and then centrifuged.

2.2.6. Heavy Metal (Cd, As, Pb, and Hg) Measurements. The lyophilized powder of tunichrome (0.2 g) was weighed and mixed with concentrated HNO_3 (7 mL, 65% v/v) and

H_2O_2 (1 mL, 30% v/v) in the polytetrafluoroethylene vessel. The vessel temperature was raised to 160°C for 21 min by mixing at 1500 W in the magnetron (ETHOS One, Milestone, Italy). After this digestion process, the sample was cooled for 120 min, and then, the final volume of the sample reached 50 mL with DDW. The determination of heavy metals was conducted by an atomic absorption spectrometry (Atomic Absorption Spectrophotometer, Varian AA240 FS) system which contained an electrode discharge lamp for evaluating volatile and nonvolatile toxic heavy metals by using argon gas. A graphite furnace was applied to determine nonvolatile compounds (Pb and Cd), and a flow injection-mercury hydride system was applied to calculate volatile (As and Hg) compounds.

2.2.7. Fluoride, Chloride, Bromide, Nitrite, Nitrate, Phosphate, and Sulfate Content Measurement. This measurement was done by an ion chromatograph 761 Compact IC (Metrohm, Herisau, Switzerland) with anion self-regenerating suppressor Metrohm Suppressor Module MSM and conductivity detector. Anion separation was performed by a "Star-Ion-A300" column (100 mm × 4.60 mm, Phenomenex, Torrance, USA). A "Metrosep A PCC 1 HC" column (12.5 mm × 4.0 mm, Metrohm, Herisau, Switzerland) was applied for preconcentration. The volume sample loop was 20 μl. Sample injection to the ion chromatography was done by a 5 ml Becton Dickinson syringe (Fraga, Spain) [22].

2.2.8. FTIR Spectroscopy. FTIR spectroscopy (WQF-510 FTIR Rayleigh, Beijing Rayleigh, China) was used for the evaluation of the peak absorbance intensity of the functional groups in the structure of tunichrome. To this end, dried powder of tunichrome was mixed with KBr to form a compact plate. The FTIR spectra were recorded in the wavenumber range of 4000–400 cm^{-1}.

2.2.9. Determination of Chemical Composition by GC-MS. The chemical composition of tunichrome was investigated by a GC (7890B, Agilent Technologies, Santa Clara, CA, United States) system containing MS (5977ANetwork, Agilent Technologies). Briefly, 200 mg of tunichrome was homogenized with 200 ml of methanol: chloroform: hexane (1 : 1 : 1), for 24 h in 200 rpm and then centrifuged for 20 min at 4000 rpm. The final supernatant was used for evaluating the chemical composition. The GC system was equipped with an HP5 MS column (nonpolar column, Agilent Technologies, internal diameter: 30 m × 250 μM, film thickness: 0.25 μm). The flow rate of carrier gas (Helium) was 1 mL/min. The temperature of the injector was set at 120°C. The oven temperature program included three steps: (1) 50°C for 1 min, (2) temperature raising to 300°C at a rate of 15°C/min, and (3) holding at 300°C for 20 min. The total processing time was 37.66 min. The scanning range of mass spectra was 50–550 m/z in the EI mode at 70 eV. Components were identified based on mass spectra in comparison with those deposited in the database of NIST11 (U.S. Department of Commerce, Gaithersburg, MD, United States) and literature data [23].

2.2.10. Antioxidant Properties

Antioxidant Activity. The radical scavenging activity (RSA) (%) was determined by the DPPH° radical scavenging method according to the technique of Ruengdech and Siripatrawan [24]. First, 200 mg of tunichrome with different concentrations within the range of 50, 100, 150, and 200 μg/mL was mixed with 800 μL methanol. Then, 400 μL of each diluted tunichrome was homogenized with 1.6 mL of DPPH° solution (0.1 mM). The suspension was kept at 25°C for 1 h under dark conditions, and finally, the absorbance of the suspension was recorded at 517 nm. A sample without tunichrome was used as a control. The RSA (%) was determined based on the following equation:

$$\text{RSA}(\%) = \left[\frac{(A_c - A_s)}{A_c}\right] \times 100, \quad (1)$$

where A_c and A_s express the absorbances of control and test samples, respectively.

Total Phenol Content (TPC). The TPC was determined by Folin-Ciocalteu reagent assay based on the method of Majzoobi et al. [25]. In brief, samples were mixed with 750 μL of 10% w/w Folin-Ciocalteu (diluted in DDW) and then hold at 20°C for 15 min. Afterward, 750 μL of 0.2% sodium carbonate was mixed with the suspension, and the absorbance of the sample was determined at 765 nm after 1 h incubation in a dark place. The TPC was calculated via a calibration curve of different concentrations of a gallic acid solution (0 to 150 μg/mL) and reported as mg gallic acid/g of tunichrome weight.

Total Flavonoid Content (TFC). The method developed by Bagheri et al. [26] was used to measure TFC. For this purpose, 0.5 g of samples and 500 μL of methanol were homogenized with 100 μL of $AlCl_3$ (10% w/v), 100 μL of CH_3CO_2K (1 M), and 2800 μL of DDW. Absorbance at 415 nm was measured after incubation at 23°C for 35 min. The standard calibration curve was plotted at the same wavelength for various quercetin concentrations (5 to 30 μg/mL methanol). The TFC was reported as mg quercetin/g of the sample weight.

2.2.11. Antimicrobial Properties

Well Diffusion Agar Method. The tunichrome sample antimicrobial activity was measured against selected Gram-positive and Gram-negative bacteria including *Bacillus cereus* (ATCC 11778), *Staphylococcus aureus* (ATCC 6538), *Salmonella enterica* (ATCC14028), and *Escherichia coli* (ATCC 35218) by well diffusion agar method. Muller Hinton agar plates were cultured using 0.1 mL of bacterial suspension with a cell density of 0.5 Mc-Farland standard ($\approx 1.5 \times 10^8$ CFU/ml). Wells with 4 mm diameter were

created by a sterile punch and filled with 50 μl of the sample (150 mg/ml). After 24 h incubation at 37°C, the diameter inhibition zone (DIZ) was determined in mm.

Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) Method. The broth microdilution technique was used for the evaluation of MIC. First, broth subcultures were produced by inoculating one colony of each bacteria grown 24 h in the 50 ml flask with 20 ml Mueller Hinton Broth in a shaker incubator (Jal, Tehran, Iran) at 150 rpm. Then, the final concentration of bacterium was set at 1.5×10^6 CFU/ml followed by separating the cells using centrifugation at $6000 \times g$ for 5 min. Then, the pellet was dispersed in sterile saline (0.9% NaCl) and was applied for inoculation by 96-well microplates with tunichrome serial dilutions from 0 to 150 mg/ml. The microplates were held at 37°C overnight. After incubation, bacterial growth was determined by turbidity methods. The concentration around MIC was cultured on the MRS agar for determining the MBC.

2.2.12. Statistical Analysis. All data were determined in triplicate. Analysis of variance (ANOVA), and the Duncan multiple range tests were done to evaluate the significant differences ($P < 0.05$) among the average values (SAS ver. 9.1, 2002–2003 by SAS Institute Inc., Cary, NC, USA).

3. Results and Discussion

3.1. Molecular Weight Measurement. SDS-PAGE of tunichrome released from Persian Gulf marine tunicate is shown in Figure 1. The bands at 245, 180, 135, 100, and 75, between 75 and 63, between 63 and 48, 48, 35, between 35 and 25, 25, 20, between 20 and 17, 17, between 17 and 11, and 11 kD were identified in the sample. Two main bands were between 63 and 48 and 20 kD. This result showed that there were different proteins and peptides with antioxidant and antimicrobial properties in the tunichrome.

3.2. Amino Acids' Profile. The protein content of tunichrome released from *Phallusia nigra* Persian Gulf marine tunicate was $0.7 \pm 0.02\%$. Amino acids' profile of tunichrome by HPLC is reported in Table 1. The main amino acids were lysine, arginine, glycine, aspartic acid, and proline with concentrations of 32.24, 14.34, 12.44, 10.62, and 9.47 mg/100 g, respectively. Therefore, tunichrome can be considered as a suitable source of lysine essential amino acid (EAA). Kumaran and Bragadeeswaran [27] reported that the release of *E. viride* is containing leucine (582.3 μg/g), arginine (365.4 μg/g), lysine (344.5 μg/g), threonine (295.6 μg/g), and isoleucine (231.2 μg/g) and the release of *D. psammathodes* contains leucine (540.9 μg/g), arginine (401.2 μg/g), lysine (385.4 μg/g), threonine (312.5 μg/g), and isoleucine (254.1 μg/g). Karthikeyan et al. [28] evaluated the amino acid profile of solitary ascidian *Microcosmus exasperatus* and reported the presence of a total of seventeen essential and nonessential amino acids (nEAA). Among them, ten essential and seven nonessential amino acids were reported in ascidians mussel. The maximum recorded level of the essential amino acid (567.3 mg) was leucine, and the least level of nonessential amino acids (0.212 mg) was aspartic acid. Tabakaeva and Tabakaev [29] also evaluated the different amino acids of the ascidian *Halocynthia aurantium* in the Japan Sea. Among eighteen identified amino acids, eight of them were essential and the others were nonessential. Internal organs had the highest essential amino acid (50.61%) content and tunic had the lowest (35.01%) content. The common nonessential amino acid in all parts (5.84–10.16%) was aspartic acid.

The contents of EAA, semiessential amino acids (SEAA), and nEAA of tunichrome released from Persian Gulf marine tunicate are reported in Figure 2. It contained 44.4, 21.3, and 34% of EAA, SEAA, and NEAA, respectively. Cho et al. [7] and Kang et al. [10] reported that the protein content and amino acid type are related to the age and type of tunicate, environmental condition, pH of water, and the salt type and concentration.

3.3. Mineral Content. Table 2 shows the Na, K, Mg, Ca, Mn, Zn, Fe, and Cu contents of tunichrome released from *Phallusia nigra* Persian Gulf marine tunicate. The main cation ions of tunichrome were sodium, potassium, and magnesium with concentrations of 858.4, 778.5, and 94.7 mg/100 g, respectively. Based on these results, this sample is a good source of essential minerals for the human body. Lee et al. [30] also reported that the internal part of tunicate from Korea was containing Na, K, Mg, Ca, Mn, Zn, Fe, and Cu, in the range of 1471.1–1257.9, 39–98.3, 42.8–78, 129.1–273.3, 5.2–0.9, 0.2–0.4, 2.5–0.9, and 0–0.2 mg/100 g, respectively. Papadopoulou and Kanias [31] reported that Zn and Fe of tunic of *Ciona intestinalis* were 110 and 610 mg/kg. Cho et al. [7] reported that the mineral type is related to the age and type of tunicate, environmental condition, and pollution.

Table 3 shows the heavy metals of tunichrome released from *Phallusia nigra* Persian Gulf marine tunicate. This source contained high amounts of heavy metals such as Pb and Hg with concentrations of 36.35 and 8 μg/g, respectively. The main idea is culturing this tunicate in the pure water instead of the sea. Lee et al. [30] evaluated the internal part of tunicate from Korea and reported a trace content of chromium, lead, silver, and arsenic heavy metals. Papadopoulou and Kanias [31] reported 1, 1.9, 0.011, 0.021, 0.041, 3.7, 0.44, and 0.15 ppm of Se, Cr, Ag, Cs, Sc, Rb, Co, and Sb for the tunic of *Ciona intestinalis*, respectively.

Table 4 shows the concentrations of fluoride, chloride, bromide, nitrite, nitrate, phosphate, and sulfate of tunichrome released from *Phallusia nigra* Persian Gulf marine tunicate. Chloride (1750 μg/g), nitrate (850 μg/g), and fluoride (17.50 μg/g) were the main anions of tunichrome. Since a high amount of nitrite and nitrate in the processed food can lead to different cancers, the low content of nitrite and nitrate in tunichrome showed its beneficial health effects [32].

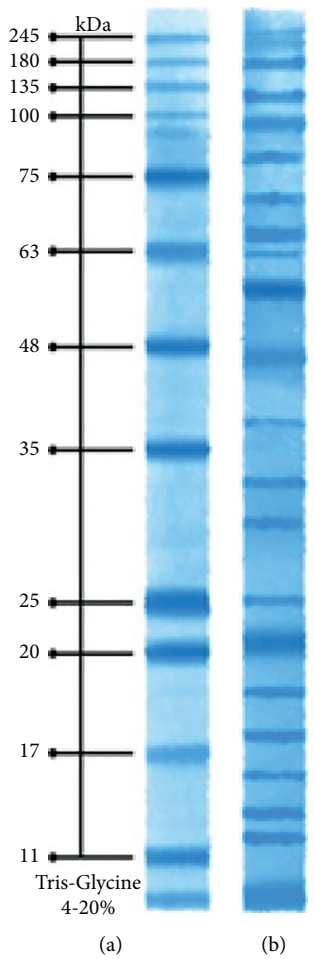

Figure 1: SDS-PAGE: (a) sinaclon marker (Tris-Glycine) and (b) tunichrome released from *Phallusia nigra* Persian Gulf marine tunicate.

Table 1: Amino acids' profile of tunichrome released from *Phallusia nigra* Persian Gulf marine tunicate by HPLC.

	Amino acid	Retention time (min)	Concentration (mg/100 g)	Content (%)
1	Asp	5.56	10.62 ± 0.004	9.2 ± 0.04
2	Glu	6.63	6.55 ± 0.003	6.7 ± 0.03
3	H-Pro	10.79	0.29 ± 0.002	0.5 ± 0.02
4	Ser	14.40	2.79 ± 0.003	7.7 ± 0.03
5	Gly	15.55	12.44 ± 0.003	9.7 ± 0.02
6	His	17.19	0.87 ± 0.002	3.2 ± 0.02
7	Arg	19.11	14.34 ± 0.002	7.8 ± 0.02
8	Thr	19.62	4.45 ± 0.002	4.6 ± 0.02
9	Ala	19.83	5.83 ± 0.004	5.4 ± 0.04
10	Pro	20.38	9.47 ± 0.002	6.6 ± 0.02
11	Tyr	26.00	0.72 ± 0.003	2.2 ± 0.03
12	Val	27.22	1.97 ± 0.002	3.5 ± 0.02
13	Met	28.43	5.32 ± 0.003	5.6 ± 0.03
14	Ile	31.17	4.02 ± 0.035	2.3 ± 0.04
15	Leu	31.55	7.72 ± 0.002	5.1 ± 0.02
16	Phe	33.92	5.87 ± 0.002	3.1 ± 0.02
17	Lys	34.72	32.24 ± 0.003	17.0 ± 0.03

Data represent mean ± standard deviation of three independent repeats.

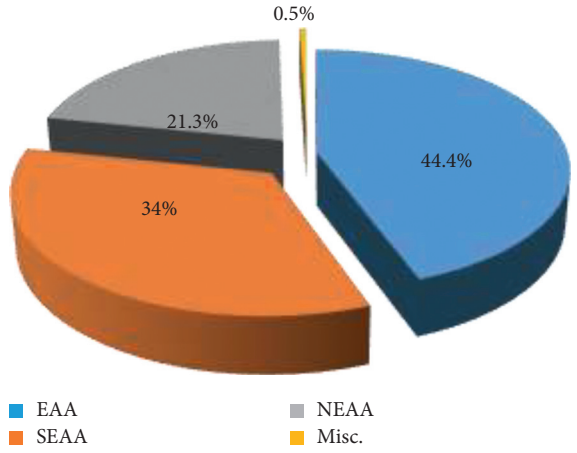

Figure 2: Essential amino acids (EAA), semiessential amino acids (SEAA), and nonessential amino acids (nEAA) of tunichrome released from *Phallusia nigra* Persian Gulf marine tunicate.

Table 2: Na, K, Mg, Ca, Mn, Zn, Fe, and Cu contents of tunichrome released from *Phallusia nigra* Persian Gulf marine tunicate.

Name	Concentration (mg/100 g)
Na	858.4 ± 0.02
K	778.5 ± 0.03
Mg	94.7 ± 0.02
Ca	2.1 ± 0.02
Mn	0.3 ± 0.03
Zn	0.6 ± 0.02
Fe	0.3 ± 0.03
Cu	0.1 ± 0.04

Data represent mean ± standard deviation of three independent repeats.

Table 3: Heavy metals of tunichrome released from *Phallusia nigra* Persian Gulf marine tunicate.

Name	LOQ	Concentration (μg/g)
Cd	0.3	0.013 ± 0.003
As	0.025	0.013 ± 0.002
Pb	4.2	36.35 ± 0.010
Hg	3	8.00 ± 0.001

Data represent mean ± standard deviation of three independent repeats.

3.4. FTIR. Infrared spectroscopy is a valuable analytical technique for obtaining rapid information about the structure and chemical changes of compounds during the different processes. The FTIR spectra of tunichrome released from *Phallusia nigra* Persian Gulf marine tunicate are presented in Figure 3. The spectrum had two low-intensity peaks at 1624 and 1728 cm^{-1}. The signal around 1624 cm^{-1} was due to the symmetric deformation vibration of adsorbed water molecules [33]. The peak at 1728 cm^{-1} was attributed to the C=O stretching vibration of formate ester moieties. The low intensity of this band suggested that only small amounts of this specific band are formed [34]. Tunichrome showed a strong O–H stretching absorption band around 3300 cm^{-1} and C–H stretching absorption band around 2962 cm^{-1} (Table 5). Similar results were reported by Núñez-Pons et al. [35] and Song et al. [36]. Moreover, these results were in agreement with those obtained by GC-MS for the detection of carbocyclic, acidic, and steric groups.

Table 4: Fluoride, chloride, bromide, nitrite, nitrate, phosphate, and sulfate contents of tunichrome released from *Phallusia nigra* Persian Gulf marine tunicate.

Name	RT (min)	Concentration (μg/g)
Fluoride	3.75	17.50 ± 0.2
Chloride	5.68	1750 ± 0.3
Bromide	8.59	15 ± 0.2
Nitrite	7.33	5 ± 0.1
Nitrate	10.39	850 ± 0.2
Phosphate	11.72	5 ± 0.1
Sulfate	14.41	15 ± 0.1

Data represent mean ± standard deviation of three independent repeats.

3.5. Chemical Composition. The chemical composition of tunichrome released from *Phallusia nigra* Persian Gulf marine tunicate by GC-MS is reported in Table 6. Octadecanoic acid methyl ester (25.74%), nonanal (14.08%), hydrazinecarbothioamide (12.38%), octadecanoic acid (9.67%), and hexadecanoic acid (9.06%) were the main components extracted by methanol: hexane: chloroform (1 : 1 : 1). Similar components in tunichrome of a marine ascidian, *Lissoclinum bistratum*, were reported by Karthi et al. [37] after analysis by the GC-MS method.

This important analysis showed that tunichrome released from *Phallusia nigra* Persian Gulf marine tunicate contained a large number of bioactive compounds which are known as antioxidant and antimicrobial natural products. Several amazing medicinal and pharmacological activities were also reported for tunichrome. According to the previous studies, some identified components such as 3-methyl-2-[4-(3-methyl-butoxy)-benzoylamino]-butyric acid [38], hexadecanoic acid [39], and phenol, 2,4-bis(1,1-dimethylethyl)- [40] exhibit high antioxidant activity.

The antimicrobial activities of oxazine [41], 1,3-oxazine [42], nonanal [43], hydrazinecarbothioamide [44], triazene [45], butyric acid [46, 47], (+)-trans-3,4-Dimethyl-2-phenyltetrahydro-1,4-thiazine [48–50], hexadecanoic acid [47], octadecanoic acid [51], spirost-8-en-11-one,3-hydroxy-, (3β, 5α, 14β.20β, 22β, 25R)- [52, 53], and phenol, 2,4-bis(1,1-dimethylethyl)- [40] were also well documented.

In addition, other pharmaceutical properties such as sedative, analgesic, antipyretic, anticonvulsant, antitubercular, antitumor, and antimalarial properties of 4-phenyltetrahydro-1,3-oxazine-2-thione [41], antitumor and anti-inflammatory properties of (+)-trans-3,4-dimethyl-2-phenyltetrahydro-1,4-thiazine [54], antitrypanosomal properties of hydrazinecarbothioamide, 2-[1-(4-nitrophenyl)ethylidene]- [55], and anticancer, antiproliferative, and anti-inflammatory properties of spirost-8-en-11-one,3-hydroxy-,(3β, 5α, 14β.20β, 22β, 25R)- [56, 57] were reported previously.

3.6. Antioxidant Properties. DPPH is a stable radical with an absorbance at 517 nm which can react with any antioxidant. This interaction can decrease the absorbance due to the color

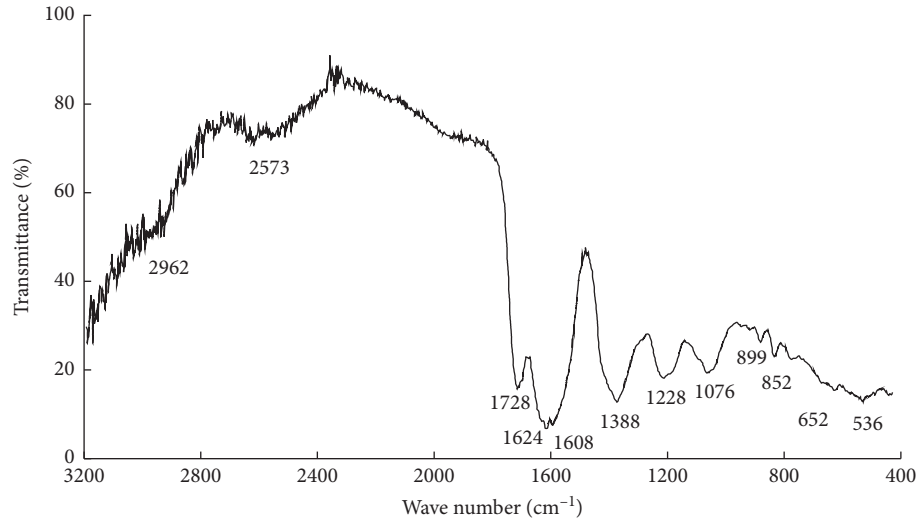

Figure 3: FTIR spectrum of tunichrome released from *Phallusia nigra* Persian Gulf marine tunicate.

Table 5: FTIR of tunichrome released from *Phallusia nigra* Persian Gulf marine tunicate.

	Wavenumber (cm^{-1})	
1	2962	C-H stretching
2	2573	O-H stretch vibration
3	1728	C=O stretch vibration
4	1624	C=C symmetric deformation vibration
5	1608	N-O stretch vibration
6	1388	
7	1228	
8	1076	
9	899	
10	852	
11	652	
12	536	C-Br stretch vibration

change from purple to white or yellow [58, 59]. TPC and TFC of the sample were 0.55 mg GA/g and 0.21 mg quercetin/100 g, respectively. The RSA (%) values of tunichrome released from *Phallusia nigra* Persian Gulf marine tunicate and ascorbic acid are reported in Table 7. Tunichrome showed higher antioxidant activity than ascorbic acid, and RSA values were increased from 30.28 to 82.08% by increasing the concentration from 50 to 200 ppm, indicating its dose-dependent activity. These results showed that tunichrome released from *Phallusia nigra* Persian Gulf marine tunicate can be an appropriate source of natural antioxidants for food and pharmaceutical purposes.

The antioxidant activities of 3-methyl-2-[4-(3-methyl-butoxy)-benzoylamino]-butyric acid [38] and hexadecanoic acid [39] and phenol, 2,4-bis(1,1-dimethylethyl)- [40] were also reported previously. Lee et al. [15] evaluated the antioxidant activity of extracts of Stalked sea squirt *Styela clava* tunic. They reported that this extract showed high dose-dependent antioxidant activity, and water extract had 0.192 mg/ml. Also, Lee et al. [60] showed that the RSA of the water and ethanol *Styela clava* tunic extracts was 31 and 48.6% at 10 mg/ml, respectively. Lee et al. [61] also reported high scavenging activities (50%) of starfish *Acanthaster planci* extracts at concentrations of 1.62 mg/ml, >10 mg/ml, and 4.03 mg/ml for the ethanol, ethyl acetate, and n-butanol extracts, respectively. Kim [14] reported that hydrolysates of solitary tunicate (*Styela clava*) had high antioxidant activity.

3.7. Antimicrobial Content

3.7.1. Well Diffusion Agar. Antimicrobial activity of tunichrome was reported by Sugumaran and Robinson [62] previously. Inhibition zones (mm) of tunichrome released from *Phallusia nigra* Persian Gulf marine tunicate (150 μg/ml) and gentamicin (10 μg/ml) are reported in Table 8. Inhibition zones of *Staphylococcus aureus, Staphylococcus aureus, Salmonella enterica*, and *Escherichia coli* O157:H7 were 14, 18, 17, and 15 mm, respectively. This sample had the highest antimicrobial activity against *Bacillus cereus*. Independent of bacterial strain, the antimicrobial activity of tunichrome was lower than gentamicin, as shown in Table 8. These results showed the significant antimicrobial activity of tunichrome released from *Phallusia nigra* Persian Gulf marine tunicate as a natural compound in food application.

The antimicrobial activities of oxazine and its derivatives were reported by Sindhu et al. [41] against *S. aureus* and *E. coli*. The antimicrobial activities of 1,3-oxazine against *Enterococcus faecalis* and *Listeria monocytogenes* were also reported by Hamza et al. [42]. The antimicrobial activity of nonanal was reported by Zhang et al. [43]. The antimicrobial potential of hydrazinecarbothioamide against *Bacillus subtilis*, *E. coli*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Paenibacillus macerans*, and *Salmonella typhimurium* was reported by Shim et al. [44]. Mohammadi [45] studied the antimicrobial activities of triazene against *Bacillus subtilis*, *Pseudomonas aeruginosa*, *Salmonella enterica,* and *Micrococcus luteus*. The butyric acid activities against *Salmonella enterica*, *E. coli* and *Campylobacter jejuni* [46], *Candida albicans*, *Streptococcus mutans*, and *Streptococcus sanguinis*

Table 6: Chemical composition of tunichrome released from *Phallusia nigra* Persian Gulf marine tunicate by GC-MS.

	RT (min)	Name	Formula	MW (g/mol)	Content (%)
1	3.22	4-Phenyltetrahydro-1,3-oxazine-2-thione	$C_{10}H_{11}NOS$	193.27	0.71
2	5.92	Nonanal	$C_9H_{18}O$	142.24	14.08
3	8.39	3,3-Dimethyl-1-(2-carboxyphenyl)triazene	$C_9H_{11}N_3O_2$	193.20	0.87
4	8.48	3-Methyl-2-[4-(3-methyl-butoxy)-benzoylamino]-butyric acid	$C_{17}H_{25}NO_4$	307.40	0.87
5	8.68	p-Cynophenyl p-(2-propoxyethoxy)benzoat	$C_{19}H_{19}NO_4$	325.40	0.87
6	8.88	(+)-*trans*-3,4-Dimethyl-2-phenyltetrahydro-1,4-thiazine	$C_{12}H_{17}NS$	207.30	0.87
7	17.06	Phenol, 2,4-bis(1,1-dimethylethyl)-	$C_{14}H_{22}O$	206.32	0.73
8	17.68	Hexadecanoic acid, methyl ester	$C_{17}H_{34}O_2$	270.50	9.06
9	18.77	n-Hexadecanoic acid	$C_{16}H_{32}O_2$	256.42	5.84
10	21.96	Hydrazinecarbothioamide, 2-[1-(4-nitrophenyl)ethylidene]-	$C_9\ H_{10}N_4O_2S$	238.27	12.38
11	23.02	Octadecanoic acid, methyl ester	$C_{19}H_{38}O_2$	298.50	25.74
12	24.02	Octadecanoic acid	$C_{18}H_{36}O_2$	284.50	9.67
13	27.32	Spirost-8-en-11-one,3-hydroxy-,(3β, 5α, 14β.20β, 22β, 25R)-	$C_{27}H_{40}O_4$	428.00	0.87
14	29.37	Hexadecanoic acid, 2-hydroxy-1-(hydroxymethyl)ethyl ester	$C_{19}H_{38}O_4$	330.50	17.43

Table 7: RSA (%) of tunichrome released from *Phallusia nigra* Persian Gulf marine tunicate and ascorbic acid.

Concentration (ppm)	Tunichrome	Ascorbic acid
50	30.28 ± 0.2Ca	17.86 ± 0.1Db
100	47.59 ± 0.1Ca	22.04 ± 0.2Cb
150	66.28 ± 0.2Ba	42.47 ± 0.1Bb
200	82.08 ± 0.1Aa	53.83 ± 0.1Ab

Data represent mean ± standard deviation of three independent repeats. Different capital letters in each column and lowercase ones in each row indicate significant differences ($P < 0.05$).

Table 8: Inhibition zones (mm) of tunichrome released from *Phallusia nigra* Persian Gulf marine tunicate (150 μg/ml) and gentamicin (10 μg/ml).

Bacteria	Tunichrome (mm)	Gentamicin (mm)
Staphylococcus aureus	14 ± 0.3Db	16 ± 0.2Da
Bacillus cereus	18 ± 0.3Ab	19 ± 0.2Aa
Salmonella enterica	17 ± 0.2Bb	18 ± 0.3Ba
Escherichia coli O157:H7	15 ± 0.2Cb	17 ± 0.3Ca

Data represent mean ± standard deviation of three independent repeats. Different capital letters in each column and lowercase ones in each row indicate significant differences ($P < 0.05$).

Table 9: MIC and MBC values of tunichrome released from *Phallusia nigra* Persian Gulf marine tunicate.

Bacteria	MIC (mg/ml)	MBC (mg/ml)
Staphylococcus aureus	1.17 ± 0.00Ab	2.34 ± 0.00Aa
Bacillus cereus	0.59 ± 0.00Bb	1.17 ± 0.00Ba
Salmonella enterica	0.59 ± 0.00Bb	1.17 ± 0.00Ba
Escherichia coli O157:H7	1.17 ± 0.00Ab	2.34 ± 0.00Aa

Data represent mean ± standard deviation of three independent repeats. Different capital letters in each column and lowercase ones in each row indicate significant differences ($P < 0.05$).

[47] were also documented. Moreover, the antimicrobial activities of (+)-trans-3,4-dimethyl-2-phenyltetrahydro-1,4-thiazine against *Mycobacterium tuberculosis* [48], *Staphylococcus aureus* and *E. coli* [49], *Staphylococcus aureus*, *E. coli*, *Bacillus subtilis*, and *Pseudomonas aeruginosa* were reported previously [50].

The antimicrobial activities of hexadecanoic acid against *Candida albicans*, *Streptococcus mutans*, and *Streptococcus sanguinis* were shown by Huang et al. [47]. According to da Silva et al. [51], octadecanoic acid had significant antimicrobial activities against *Bacillus subtilis* and *Bacillus cereus*. The antimicrobial activities of spirost-8-en-11-one,3-hydroxy-,(3β, 5α, 14β.20β, 22β, 25R)- against *E. coli, Bacillus subtilis, Staphylococcus aureus,* and *Pseudomonas aeruginosa* were reported in [52, 53]. The antimicrobial activities of phenol, 2,4-bis(1,1-dimethylethyl)- against *Aspergillus niger*, *Fusarium oxysporum*, and *Penicillium chrysogenum* were reported by Varsha et al. [40].

Galinier et al. [18] evaluated the two antimicrobial peptides from hemocytes of the *Halocynthia papillosa* tunicate. They reported that these components had high antimicrobial activities against *S. aureus and E. coli.* Cai et al. [5] reported that tunichromes showed antimicrobial activity against *E. coli* and *Photobacterium phosphoreum*.

3.7.2. MIC and MBC. Table 9 shows the MIC and MBC of tunichrome released from *Phallusia nigra* Persian Gulf marine tunicate. MIC values of tunichrome for *Staphylococcus aureus, Staphylococcus aureus, Salmonella enterica*, and *Escherichia coli* O157:H7 were 1.17, 0.59, 0.59, and 1.17 mg/ml, respectively. Moreover, the values of MBC for the *Staphylococcus aureus, Staphylococcus aureus, Salmonella enterica*, and *Escherichia coli* O157:H7 were 2.34, 1.17, 1.17, and 2.34 mg/ml, respectively. It was reported that styelins, clavanins, halocyamine, and plicatamide of tunichrome separated from ascidian blood cells exhibit antimicrobial properties [62, 63]. Cai et al. [5] also reported that there are different components in the tunichrome with *in vitro* antibiotic properties.

4. Conclusion

The aim of this study was to evaluate the physicochemical, nutraceutical, antioxidant, and antibacterial properties of tunichrome released from *Phallusia nigra* Persian Gulf marine tunicate. The results showed that this release contained a high amount of essential amino acids, especially

lysine. Also, the GC-MS profile showed that this compound included the functional, antioxidant, and antimicrobial components. Tunichrome had high antioxidant activity, TPC and TFC. Also, the strong antimicrobial activities against *Staphylococcus aureus, Staphylococcus aureus, Salmonella enterica,* and *Escherichia coli* O157:H7 were observed. Therefore, tunichrome can be considered as a good source of natural antioxidants and antimicrobials for future food and pharmaceutical applications.

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 they have no conflicts of interest.

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Research Article

Green Biosynthesized Selenium Nanoparticles by Cinnamon Extract and Their Antimicrobial Activity and Application as Edible Coatings with Nano-Chitosan

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Bioactive nanocomposites were constructed, containing chitosan (Cht), extracted from shrimps' wastes, and transformed into nanoparticles (NPs) using ionic-gelation. Selenium NPs (Se-NPs) were phytosynthesized using cinnamon (*Cinnamomum zeylanicum*) bark extract (CIE), characterized and evaluated with Cht-NPs as antimicrobial composites against bacterial food-borne pathogens "*Escherichia coli*, *Salmonella typhimurium*, *Staphylococcus aureus*, and *Listeria monocytogenes*" and as potential edible coating (EC) basements. The CIE-phytosynthesized Se-NPs had well-distributed and spherical shapes with 23.2 nm mean diameter. The CIE, CIE/Se-NPs, and innovative CIE/Se/Cht-NP composites exhibited distinguished antibacterial actions toward the entire screened pathogens; CIE/Se/Cht-NP composite was significantly the most potent. The formulated ECs from CIE/Se/Cht-NP nanocomposites had matching antibacterial manner, which was strengthened with CIE/Se-NP percentage increments. Scanning micrographs indicated the attachment of CIE/Se-NPs to bacterial cells to cause their complete lysis and death after 10 h of exposure. CIE/Se/Cht-NP composites are proposed as effectual control agents toward food-borne pathogens using efficient biological carriers and eco-friendly phytosynthesis protocol.

1. Introduction

Chitosan (Cht) is a derivative polysaccharide from deacetylated chitin (Ct), which is the principal constituent of crustacean exoskeleton [1]. Besides, Cht and Ct could present in many insects and fungal cell wall [2]. Cht has reported antimicrobial potentialities toward various microorganisms, e.g., bacteria, fungi, and yeast [3–5], with suggested higher activity against Gram-positive bacteria than Gram-negative species [1]. Cht is an extraordinary cost-effective biopolymer with numerous biological and environmental advantages, e.g., its elevated biocompatibility, biodegradability, bioactivity, and biosafety attributes, which advocate wide applications, Cht and their nanoparticles (NPs), in biomedical, nutritional, environmental, and therapeutic aspects [6–8]. The polymer NP formation, e.g., Cht-NPs, was proved to augment the biopolymer bioactivities (such as anticancer, antimicrobial, toxicant

adsorption, bioremediation, drugs' carrying, nanometals conjugation, and antioxidant and anti-inflammatory attributes, comparing with bulk materials [6, 7, 9–12].

Selenium (Se), the vital element in biological bodies (as antioxidative and prooxidative agent) has vast significance in nutrition and medicine [13], with narrow ranges between toxic levels and dietary deficiency (400 μg and 40 μg/day, respectively).

Se has vital functions in cellular metabolism, enzymes' activation, body protection from free radical species, human fertility, thyroid metabolism, and several additional energetic functions. The Se biological applications were succeeded in diverse fields, e.g., health, biochemistry, genetics, and molecular biology, including its usages in antioxidants, antitumors, enzyme inhibitors, anti-infective, cytokine inducers, and immunomodulator formulations [14]. Se nanoparticles (Se-NPs) have surplus bioactivities than bulk Se as low-toxicity chemopreventive and bioactive agents [15–19]. Accordingly, Se-NP synthesis via different protocols was extensively investigated for their potential bio-applications [15, 18].

Spices were historically employed as effectual natural food-antimicrobial materials and for improving foods' aroma and flavors [20]. With elevated concerns regarding the chemical additives' safety, consumers and researchers are always searching for natural alternatives to preserve and enhance food quality [21]. Herbs, spices, and plants' derivatives were always the perfect candidates to replace synthetic antimicrobial and antioxidant materials [22].

Cinnamon spices are gathered from the bark of *Cinnamomum* genus trees, which contain ~250 species, for global utilization in cooking and flavoring and in ethnic and modern medicines [23]. *Cinnamomum zeylanicum* barks are historical herbal medicines that have numerous curative and food-flavoring attributes [24].

Edible coating (EC) was emerged as promising tool for food preservation and is 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" [25]. The key rationales of EC are to provide supplementary nutrients, quality and sensory enhancers, antimicrobial agents, etc., while consumed on food materials. ECs could additionally act as barriers to exterior threats that endanger food quality (e.g., oxygen, vapors, moisture, and oil) to protect, prevent dehydration, and extend shelf-life of coated foods [25, 26]. The fabrication of coated metals' nanoparticles with polymer possessed elevated potentialities for application in pharmaceutical, biomedical, environmental, and food-related fields [11, 27–29].

Accordingly, this research intended the extraction and synthesis of Cht-NPs (as bioactive, eco-friendly, and cost-effective nanopolymers), phytosynthesis of Se-NPs using cinnamon extract (as a natural, biosafe, and bioactive method), and the innovative amalgamation of Cht-NPs with CIE/Se-NPs, evaluating their antimicrobial activities and potentiality for formulating bioactive ECs.

2. Materials and Methods

2.1. Chitosan Preparation. Cht was extracted from white prawn (*Fenneropenaeus indicus*) shell waste farmed in Kafrelsheikh University aquaculture farm, Egypt. Manually peeled shells were cleansed, dried, and pulverized. Shrimp shells were soaked in 2.0 N NaOH and then in 2.0 N HCl (at 1:20 w/v ratios), for 4 h each at 25°C [30], followed each by extensive washing with deionized water (DIW) and drying at 45°C for 12 h. Dry powdered Ct was immersed in 50% NaOH solution (1 g Ct powder/25 mL NaOH) and put in an oil bath at 125°C for 130 min to obtain Cht [31]. The molecular Cht weight was assessed via GPC "gel permeation chromatography, Water Breeze, Waters, USA," whereas the DD "deacetylation degree" was calculated from Cht IR spectra using FTIR "Fourier transform infrared spectroscopy, FTIR-V, 10.03.08; Perkin Elmer, Rodgau, Germany."

2.2. Nano-Chitosan Preparation. Sodium tripolyphosphate "TPP; Sigma-Aldrich, St. Louis, MO, USA" was employed as a cross-linker for Cht-NP synthesis. Cht stock solution of 0.1%, w/v (in acetic acidified solution), and TPP solution of 0.5%, w/v (in DIW), were prepared. The solutions' pH values were adjusted to 5.2, after their paper filtration. While Cht solution was vigorously stirred, the solution of TPP was slowly dripped into it (at 0.3 mL/min rate) using a syringe needle. The stirring of formed Cht-NPs opalescent suspension was sustained for additional 115 min, and then the formed NP pellet was harvested via 10,500 × g speed centrifugation for 30 min and repeated washing with DIW [32].

2.3. Cinnamon Extract Preparation. Dry identified cinnamon (*Cinnamomum zeylanicum*) bark was obtained from the ARC "Agricultural Research Centre, Giza, Egypt." Pulverized and sieved bark powder (60 mesh) was dipped and rotated in 10-folds (w/v) from 70% ethanol for 25 h at 165 × g and 25°C. After filtration and discarding of bark residues, the resulting cinnamon extract (CIE) was vacuum-evaporated at 44°C until dryness [33].

2.4. Phytosynthesis of Selenium Nanoparticles. The Se-NP phytosynthesis involved preparation of 10 mM sodium selenite solution (Na_2SeO_3; Sigma-Aldrich) and incorporation with equal volumes of CIE aqua solution to have overall CIE concentrations of 0.5%, 1.0%, and 1.5% to preliminarily evaluate the CIE potentiality for Se-NP phytosynthesis. The biosynthesis conditions were the composited solutions stirring in dark at 210 × g for 6 h and 25°C. The solution color changing to brownish-orange (due to Se-NP synthesis) was visually observed. The CIE-phytosynthesized Se-NPs (CIE/Se-NPs) were centrifuged at 12,500 × g for 28 min (Sigma 2–16 KL centrifuge; Sigma Lab. GmbH, Germany) at 15°C, washed with DIW three times, recentrifuged, and subjected to analysis and characterization [34].

For nanocomposite formation from CIE/Se-NPs and Cht-NPs (mentioned thereafter as CIE/Se/Cht–NPs), prepared powders from Cht-NPs and CIE/Se-NPs were dissolved (0.1%, w/v) in 1% acetic solution and DIW, respectively, via vigorous stirring that was followed by sonication. NP solutions (equal volumes) were mixed and stirred for 90 min, and then formed nanocomposites were precipitated via centrifugation, washing with DIW, recentrifugation, and then freeze-drying.

2.5. Analysis of CIE/Se/Cht-NP Physiognomies

2.5.1. FTIR Spectral Analysis. Infrared spectroscopic examinations of CIE, CIE/Se-NPs, and CIE/Se/Cht-NPs were conducted using the transmission mode of Perkin Elmer FTIR, Germany, after integrating samples with 1% KBr "at wavenumbers ranging from 400–4000 cm^{-1}."

2.5.2. Structural Analysis. The TEM imaging "transmission electron microscopy, Leo 0430; Leica, Cambridge, UK" was applied to assess the structural features "size, shape, morphology, and distribution" of phytosynthesized CIE/Se.

2.6. The Particles' Size (Ps) Distribution and the Zeta Potential. The Ps distribution and their zeta potential for synthesized Cht-NPs, CIE/Se-NPs, and CIE/Se/Cht-NPs were estimated via Zetasizer "Malvern Nano ZS instrument, Southborough, MA".

2.7. Antibacterial Evaluation of Natural Products

2.7.1. Bacteria Cultures. Standard strains of pathogenic food-borne bacterial "*Escherichia coli* ATCC-25922, *Salmonella typhimurium* ATCC-14028, *Staphylococcus aureus* ATCC-25923, and *Listeria monocytogenes* ATCC-19116" were employed for antibacterial screening. The microorganisms were maintained by subculturing on NA and NB "nutrient agar and nutrient broth, Difco Laboratories, Detroit, MI, USA" aerobically at 37°C.

2.7.2. Qualitative Antimicrobial Assay of Nanocomposites. The ZOI "zones of growth inhibition," after treatment of each screened bacterium with nanocomposites, were appraised via disc diffusion test, as indicators of their antibacterial bioactivities. Sterile paper discs "Whatman no. 4, with 6 mm diameter" were impregnated with 25 μL from 2% solutions of CIE, CIE/Se-NPs, or CIE/Se/Cht-NPs and sited onto a freshly inoculated NA plate with individual bacterial cultures. After upside plates' incubation for 18–24 h at 37°C, the visualized ZOI diameters were precisely measured and their triplicate means ± SDs (standard deviations) were calculated [5].

2.7.3. Quantitative Antimicrobial Assay of Nanocomposites. The MIC "minimal inhibitory concentration" of CIE, CIE/Se-NPs, or CIE/Se/Cht-NPs was appraised using microdilution method [4]. The exposed bacteria (2×10^7 cell/mL in NB medium) to gradual concentrations from each agent (ranged from 10 to 75 μg/mL in NB) were incubated for 16 h at 37°C and screened for turbidity. Subsequently, exposed wells were treated with TTC indicator solution "triphenyl tetrazolium chloride, Sigma-Aldrich" to confirm the bactericidal action, as viable cells transform TTC to violet-red color.

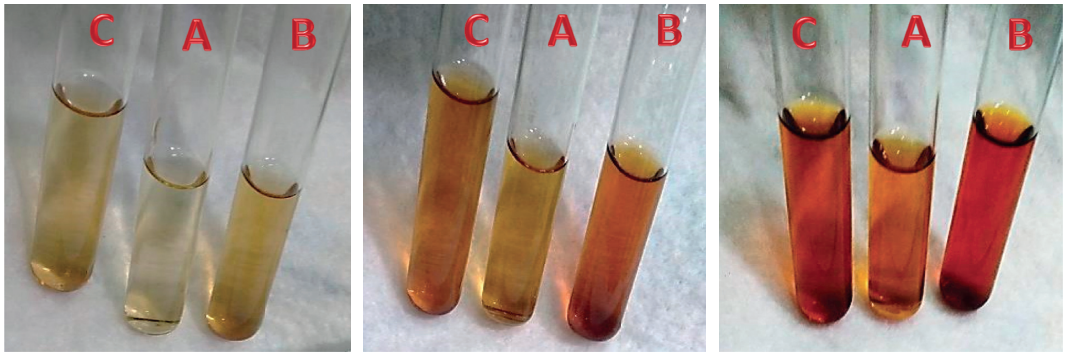
2.8. SEM "Scanning Electron Microscopy" Imaging of Nanocomposite-Treated Bacteria. SEM micrographs "JSM IT100; JEOL, Tokyo, Japan" were captured for determining the morphological and organizational alterations in *S. typhimurium* and *E. coli* cells after exposure to CIE/Se-NPs to elucidate the potential action mode of NPs. Bacterial SEM imaging was implemented after cells' exposure to 25 μg/mL CIE/Se-NPs (in tryptic soy broth) for 0 (control), 5, and 10 h and incubation at 37°C. The treated cells were collected, washed with DIW, centrifuged at 4600 × g for 30 min, and subjected to SEM preparation and imaging. The SEM micrographs' capturing was based on cell morphologies' modifications after nanocomposite exposure [35].

2.9. Coating Films Preparation and Evaluation. To prepare EC solutions, Cht-NP powder was gently dissolved (1.5%, w/v) in boiling DIW for 15 min with stirring (140 × g). The solution's temperature was reduced to ~45°C while stirring, and then 1% (v/v) acetic acid and 0.25 mL glycerol/g Cht-NPs were added as plasticizer [36]. After additional 30 min of stirring, the solution temperature was kept at ~37°C and then dispersed CIE/Se-NP composites in Tween 80 solution (2% v/v) were mixed with EC solutions to attain various CIE/Se-NP concentrations in EC (i.e., 25, 50, and 75 μg/mL). The achieved EC solution was poured into 15 cm plastic Petri dishes to create a film with ~1.0–1.5 mm thickness and dried with warmed air at 43 ± 2 °C in an incubator. The dried EC films were peeled and cut to ~1 cm^2 squares for use in the qualitative antibacterial assay, using EC squares instead of filter paper disks, as described previously.

2.10. Statistical Analysis. Triplicated experiments were performed; data were provided as means ± SD. The SPSS package (SPSS version 11.5; SPSS, Chicago, IL, USA) was applied for statistical analysis.

3. Results and Discussion

3.1. Se-NP Phytosynthesis. The bioreduction of Na_2SeO_3 to Se-NPs was accomplished via CIE, as visually evinced by color changes of NP solution from whitish-yellow to brownish-orange (Figure 1). The optimal concentration from CIE to generate Se-NPs was 1.0%, followed by 1.5% and 0.5%, respectively, as noticed from the colors deepness of the phytosynthesized Se-NPs. The development of brownish-orange color deepness was observed for all treatments with incubation prolongation, no additional color changings were observed after 4 h of incubation at 25°C.



After 0.5 h After 2 h After 4 h

Figure 1: Visual color change of biosynthesized Se-NPs using cinnamon extract after incubation of 10 mM of Na_2SeO_3 with 0.5% (A), 1.0% (B), and 1.5% (C) from the extract for 0.5, 2.0, and 4.0 h at 25°C.

The illustrated Se-NP phytosynthesis protocol is eco-friendly, simple, and cheap; the resultant NPs are assumed to be innocent, nontoxic, and highly stable [19, 37]. The color changes of Se-NPs, during synthesis, and their correlation with Ps were stated [38].

3.2. FTIR Analysis. The biochemical groups, bonds and their interactions in the generated molecules are appointed through their FTIR spectra (Figure 2).

The pure Cht-NP spectrum (Figure 2, black) represented the FTIR spectra of pure powder, where typical bands could be observed at wavenumbers ~3456.7 cm^{-1} (O–H stretch), 2883.4 cm^{-1} (C–H stretch), 1621.2 cm^{-1} (N–H bend), 1378.3 cm^{-1} (bridge O stretch), and 1134.8 cm^{-1} (C–O–C bonds). The bands located at wavenumber ~1062.5 cm^{-1} were assigned to the C–O stretch of glycosidic bonds [39, 40].

The Cht absorption band was similar to that of Ct. The differences occurred after the deacetylation step, wherein there were changes in the absorption spectrum at 1688.7 cm^{-1} from the $C=O$ stretch [31].

The characteristic fingerprints of CIE were mostly present between 1650–600 cm^{-1} range (Figure 2, violet). The peak at ~1604.3 cm^{-1} corresponded to the stretching vibration of aldehyde carbonyl $C=O$. The peak at 1445.7 cm^{-1} was typical for alcohol C–OH. The cinnamon peaks at ~987.4 and 1071.3 cm^{-1} were attributed to the stretching vibrations of C–O and C–OH deformation. The peak at 1281.9 cm^{-1} was attributed to $C–H_2$ alkanes that face the swing and the aromatic ring C–H for in-plane bending absorption [41, 42].

For CIE/Se-NPs, the possible biomolecules responsible for the reduction of Se^+ ions and capping of bioreduced Se-NPs phytosynthesized using CIE were identified [43]. FTIR spectra were used to identify the capping reagent and stability of the metal NPs present in cinnamon. The observed peak denoted the O–H stretching group of phenols and alcohols at 3418.4 cm^{-1}; it also denoted the carbonyl group at 1629.7 cm^{-1} [44].

The broad absorption band at ~3410 cm^{-1} appeared due to O–H stretching. The shifted band from 1604.3 cm^{-1} (in CIE) to 1629.7 cm^{-1} (in CIE/Se-NP spectrum) indicated the involvement of the $C=O$ bond in cinnamon aldehyde in Se-NP synthesis. The band at 1336.2 cm^{-1} corresponded to nitro compounds. This band was broader than the normal cases of aldehyde compounds due to the influence of conjugation and aromatic ring. The band at 1407.3 cm^{-1} was due to the aromatic $C=C$ bending, and the band at 1087.6 cm^{-1} was due to C–O stretching [45, 46].

The FTIR spectrum of the CIE/Se/Cht nanocomposite had the main distinctive peaks from each combined agent,

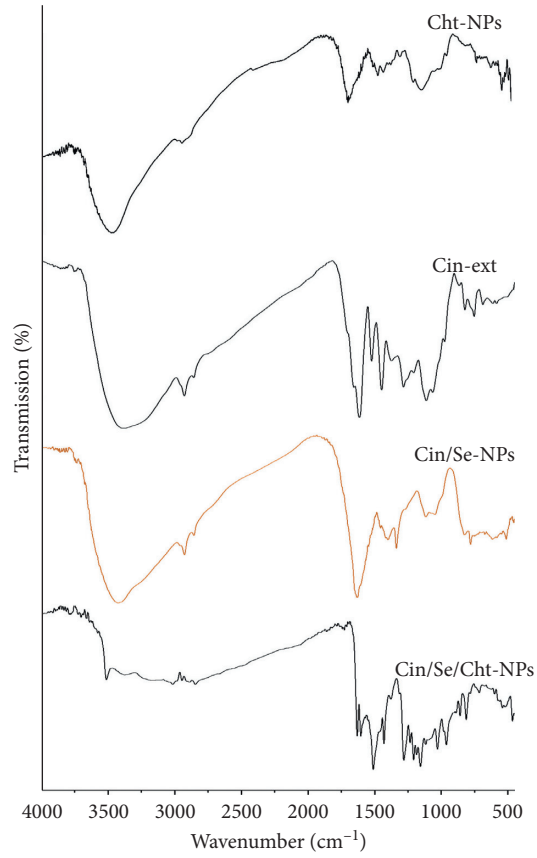


Figure 2: FTIR spectra of plain chitosan nanoparticles (Cht-NPs), cinnamon bark extract (Cin-ext), synthesized selenium nanoparticles with cinnamon extract (Cin/Se-NPs), and their nanocomposites with nano-chitosan (Cin/Se/Cht-NPs).

indicating the physiochemical reactions between these components, as evidenced by the shifts and differences in the transmission intensities of the characteristic bands.

3.3. Ps Distribution and NP Charges. The Ps distribution and their zeta potential for the synthesized Cht-NPs, CIE/Se-NPs, and CIE/Se/Cht-NPs are appraised in Table 1. The successfulness of CIE to generate Se-NPs with minute Ps range and mean diameter was proved. The phytosynthesized Se-NPs had negative Z-potential (−28.6 mV), whereas the Cht-NPs (with mean Ps of 42.1 nm) had strong positive surface charges (+39.4 mV). The nanocomposites of both NP types (CIE/Se/Cht-NPs) had slightly larger Ps range and mean diameters, which indicates their conjugation and integrations. The recorded Z-potential for investigated NPs indicated their high stability in solutions. These findings matched former stated results that suggested the formation of Cht and Se-NP nanocomposites with elevated stabilities and minute Ps [29, 47].

3.4. TEM Analysis of Se-NPs with CIE. The Ps investigation of CIE/Se-NPs via TEM micrographs showed that the size of NP ranged from 6.8 to 58.2 nm, with mean Ps diameters of ~23.2 nm.

The TEM images of phytosynthesized CIE/Se-NPs verified the homogenous NP distribution and their stabilization with CIE during phytosynthesis. Se-NPs were spherical in shapes with nearly no aggregation (Figure 3). Little CIE particles were appeared in combination with Se-NPs in the CIE/Se-NP matrix, as was formerly indicated using other plant extracts [48]. Previous studies investigated plant derivatives employment for achieving different Se-NP particle shapes and sizes based on the employed phytochemical reducing agents. For example, fenugreek seeds extraction generated Se-NPs with smoothly oval shaped and Ps of 50–150 nm [49], dried raisin extract with 3–18 nm Se nanoballs [50], *Bougainvillea spectabilis* flower with spherical shape of Se-NPs with Ps ranges between 18 and 35 nm [51], and spherical NPs with Ps 102–170 nm were achieved by microbial phytosynthesis [52]. The biomolecules and several organic compounds found in the plant extract (e.g., CIE) could lead to NP reduction and stabilization and stopping their aggregation [34].

3.5. Synthesis of Chitosan Nanoparticles. Cht-NP synthesis was efficaciously attained using the TPP gelation method, as evidenced by NP SEM imaging (Figure 4).

The Cht-NPs' appearance was semispherical and well-distributed, with PS of ~17.3–73.9 nm and a mean diameter of ~42.1 nm (Table 1). TPP cross-linkages were formerly verified as effective protocols for Cht-NP synthesis using the ionic-gelation interaction [32, 39]. The current synthesized Cht-NPs with this protocol had extraordinary properties, compared to bulk Cht, for applications as plain antibacterial agents, nanocarriers for bioactive constituents, and bases for functional ECs [10, 25].

3.6. Antibacterial Activity of Cinnamon Phytosynthesized Se-NPs. The antibacterial actions of CIE, CIE/Se-NP, and CIE/Se/Cht-NP composites were experimentally quantified with different assessments against four food-borne bacteria (Table 2). The CIE/Se/Cht-NP composite had the highest effectiveness and exhibited superior antibacterial activity. The qualitative ZOI and quantitative MIC assays revealed significant antibacterial action of the prepared antibacterial agents, as follows: CIE < CIE/Se-NPs < CIE/Se/Cht-NPs. *S. typhimurium* was significantly higher sensitive to CIE than the other species. In contrast, the most CIE/Se/Cht-NP resistant bacterium was *L. monocytogenes*. Generally, the sensitivity of tested bacteria to examined agents was: Gram-negative < Gram-positive
(*S. typhimurium* < *E. coli* < *S. aureus* < *L. monocytogenes*).

The CIE antimicrobial potentialities (including antibacterial, antifungal, and antiviral activities) were stated in many reports and attributed to its precious contents from active phytochemicals, e.g., cinnamaldehyde, eugenol, ß-caryophyllene, ethyl cinnamate, and terpenes [24, 41, 53, 54]. Conjugation of CIE with its green synthesized metals' NPs was validated to reinforce their combined antimicrobial performance, mostly because the synergistic actions of NPs and CIE phytochemicals can attack the microbial cells via diverse mechanisms, which are assumingly very hard to gain resistance toward them all [43, 45, 55–57].

3.7. SEM Analysis of Treated Bacteria. The effect of CIE/Se-NP exposure on the cellular morphology and cell wall deformation of bacterial strains (*S. typhimurium* and *E. coli*) are shown in Figure 5. The selection of screened strains was based on their higher sensitivity to nanocomposites; therefore, they were expected to provide more evidences for the antimicrobial action. The bacterial cells had healthy appearance with normal, smooth, and contracted shapes at the beginning of exposure time (control; Figure 5, 0 h)). Apparent morphological changes in the bacterial cell were occurred after 5 h exposure to CIE/Se-NPs; bacterial walls converted to puffy walls, and many NPs attached the cell membranes, disrupt them and enter the cells at this stage. The bacterial cell viability decreased and many cells were lysed after exposure to CIE/Se-NPs (Figure 5, 5 h).

With prolonged CIE/Se-NP exposure to 10 h, the compromised NPs with bacterial cells became more apparent with higher numbers. The bacterial cells were mostly lysed at this time; their released interior components apparently attached with CIE/Se-NPs. The CIE antibacterial activity was supposed to involve complex mechanisms, including suppression of nucleic acid metabolism and activity, restraint of cell wall/membrane synthesis, and deactivation of intracellular components and proteins [58, 59]. Furthermore, Se polymeric coatings were proposed as innovative antibacterial agents via the reduction of biological functions of microbes [60]. The shape and size of phytosynthesized CIE/Se-NPs augmented their antibacterial action. It was recently proposed that spherical and

Table 1: The Ps distribution and their zeta potential for synthesized Cht-NPs, CIE/Se-NPs, and CIE/Se/Cht-NPs.

NPs	Size range (nm)	Mean diameter (nm)	Zeta potential (mV)
CIE/Se-NPs	6.8–58.2	23.2	−28.6
Cht-NPs	17.3–73.9	42.1	+39.4
CIE/Se/Cht-NPs	19.6–84.1	51.9	+32.3

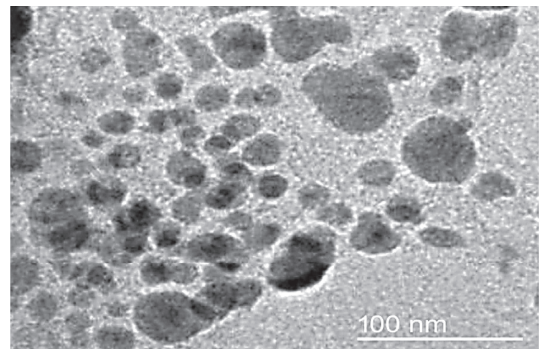


Figure 3: TEM micrograph of phytosynthesized Se-NPs with cinnamon bark extract.

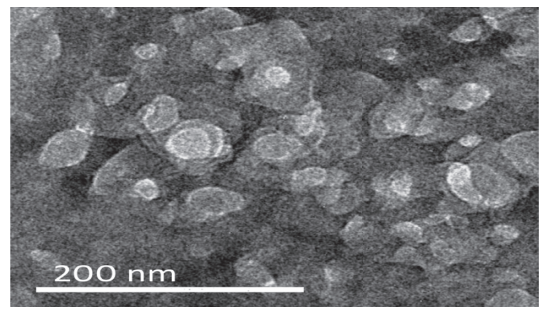


Figure 4: Scanning micrographs of chitosan nanoparticles synthesized using ionic-gelation method.

Table 2: Antimicrobial capacities of phycosynthesized Se nanoparticles with cinnamon extract against food-borne bacteria.

Examined agents	Antibacterial activity**							
	E. coli		*Salmonella typhimurium*		*Staphylococcus aureus*		*Listeria monocytogenes*	
	ZOI (mm)*	MIC (μg/ml)	ZOI (mm)	MIC (μg/ml)	ZOI (mm)	MIC (μg/ml)	ZOI (mm)	MIC (μg/ml)
Cinnamon Ext.	12.1 ± 0.7^{a}	40.0	12.8 ± 0.8^{a}	37.5	9.9 ± 0.7^{a}	47.5	8.7 ± 0.5^{a}	50.0
CIE/Se-NPs	15.3 ± 1.2^{b}	32.5	16.2 ± 1.2^{b}	30.0	12.7 ± 1.1^{b}	35.0	11.7 ± 1.0^{b}	37.5
CIE/Se/Cht-NPs	18.4 ± 1.4^{c}	22.5	20.9 ± 1.6^{c}	20.0	17.8 ± 1.2^{c}	27.5	17.2 ± 1.1^{c}	27.5

*Inhibition zones impart triplicates' diameter means ± SD, assay discs (diameter 6 mm) carrying 50 μg from cinnamon extract (CIE), phytosynthesized Se-NPs with cinnamon extract (CIE/Se-NPs) or their blend with nano-chitosan (CIE/Se/Cht-NPs).**"Dissimilar superscript letters within the same column indicate significant difference at $p < 0.05$."

smaller-sized Se-NPs could easily access the bacterial cell wall/membrane and hinder their biological activities [34].

Se-NPs exhibited more inhibitory actions against Gram + ve bacterial species in the current and previous investigations (including *Proteus* sp. and *Serratia* sp.), as explained by the lesser surface charges of NPs that effectively enabled them to bind to the bacterial cell membrane [37]. The antibacterial action of Se ions also depended on their absorption and accumulation onto microbial cells, leading to cytoplasm membrane shrinkage and cell bioactivity inhibition [35].

The definite mechanisms of Se-NPs as antimicrobial substances are still ambiguous, but former studies claimed that generating ROS "reactive oxygen species" and free radicals are major causes of bacterial cells' devastation by Se organic compounds [61, 62]. The metallic NP antimicrobial

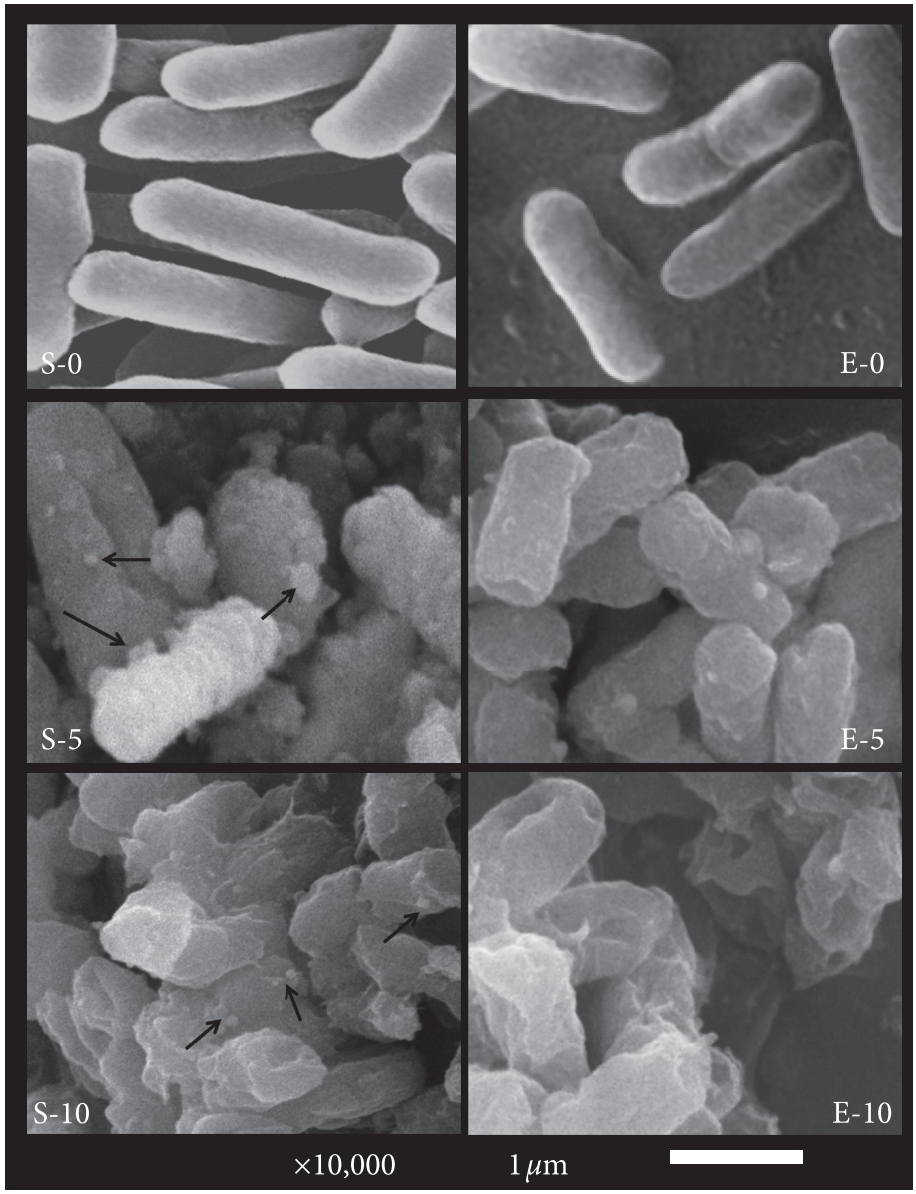


Figure 5: SEM micrographs of exposed *Salmonella typhimurium* (S) and *E. coli* (E) to phytosynthesized Se-NPs with cinnamon bark extract after 0, 5, and 10 h of treatment. Arrows indicate the some attached Se-NPs to compromised bacterial cells.

activities were attributed to their interactions with intracellular vital components (DNA, ribosomes, and RNA) to alter and deactivate their bioactive processes [63]. From former investigations, SEM and TEM imaging of exposed bacteria, *S. aureus* and *E. coli*, to Se-NPs indicated cells' wall shrinking, deformation, and damage [64], suggesting that Se-NPs can destroy bacteria via penetrating their cell membrane with increased ROS production.

3.8. Antimicrobial Capacities of Synthesized Edible Coating. The antibacterial capacities of Cht-NP (1.5%), Cht-NP + CIE/Se-NP (25 μg/mL), Cht-NP + CIE/Se-NP (50 μg/mL), and Cht-NP + CIE/Se-NP (75 μg/mL) ECs were validated against the four challenged food-borne pathogens (Table 3). The Cht-NP + CIE/Se-NP (75 μg/mL) EC was significantly the most forceful. Qualitative ZOI assay exhibited remarkable antibacterial actions of the prepared ECs, which augmented with CIE/Se-NP concentrations in Cht-NP- based ECs.

The composed ECs and films from Cht and Cht-NPs, after conjugation with bioactive phytochemicals, had evidenced extra activities and applicability for usage in foodstuff protection and preservation [10, 12, 36, 65]; the antioxidant, antimicrobial, and polymeric nature of Cht-NPs could increase the actions of conjugated materials to prevent food spoilage factors. From the documented advantageous attributes of the components of produced EC nanocomposites, i.e., Cht-NPs (antioxidant, surface barring, and antimicrobial activities), CIE (antimicrobial and antioxidant activities), and Se-NPs (powerful microbicidal action); these nanocomposited ECs are supposed to have

Table 3: Antimicrobial capacities of formulated edible coating from chitosan nanoparticles (Cht-NPs) and phycosynthesized Se nanoparticles with cinnamon extract (CIE/Se-NPs) against food-borne bacteria.

Edible coating	Antibacterial activity (IZ: mm)*			
	E. coli	*Salmonella typhimurium*	*Staphylococcus aureus*	*Listeria monocytogenes*
Cht-NPs (1.5%)	5.5 ± 0.5	6.1 ± 0.4	5.1 ± 0.3	4.6 ± 0.4
Cht-NPs + CIE/Se-NPs (25 μg/ml)	8.6 ± 0.7	10.1 ± 0.9	7.5 ± 0.6	7.2 ± 0.7
Cht-NPs + CIE/Se-NPs (50 μg/ml)	10.8 ± 0.9	12.2 ± 1.2	8.9 ± 0.8	8.4 ± 1.0
Cht-NPs + CIE/Se-NPs (75 μg/ml)	13.2 ± 1.2	14.8 ± 1.4	10.9 ± I0.9	10.1 ± 1.1

*Inhibition zones impart triplicates' diameter means ± SD, Inhibition zones impart triplicates' diameter means ± SD, without coating films diameter $p| < |0.05$.

elevated capability for protecting foodstuff from external spoilage factors (e.g., oxygen and free radicals attack and cross contamination) besides the internal factors (microbial load, lipid oxidation, and enzymatic actions), using these biosafe and natural components [7, 12, 37, 66].

4. Conclusion

The synthesis of Cht-NPs and green synthesized Se-NPs with CIE was successfully achieved. The phytosynthesized Se-NP had mean diameter of 23.2 nm, spherical shape, and high stability. The CIE/Se/Cht-NP composite exhibited potent antibacterial action against different food-borne bacterial pathogens. The bactericidal action was confirmed by imaging and antimicrobial assays, and the efficiency of phytosynthesized NPs was validated. The CIE/Se/Cht-NP bioactive edible coatings were additionally formulated and their capability to prohibit food-borne bacterial growth was evidenced. The based coating on CIE/Se/Cht-NP nanocomposites could be outstandingly recommended for the prospective applications in foodstuff preservation.

Data Availability

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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Research Article

Proximate Analysis, Antioxidant Activity, and Antibacterial Activity of Fish Sausages Fortified with Bee Bread Extract

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Bee bread is rich in phenolic compounds and recently has gained attention as a food additive, which provides an alternative use to synthetic preservatives. Therefore, this study aims to analyze the proximate composition and antioxidant and antibacterial activities of fish sausages fortified with *Heterotrigona itama* bee bread ethanolic (BBE) extract at 0.25, 0.5, and 0.75% concentrations. Incorporation of BBE in fish sausages significantly increased the carbohydrate level and lowered the moisture content. The highest phenolic and flavonoid content were observed in fish sausages with 0.75% BBE with 23.46 ± 1.60 mg GAE/g sample and 8.05 ± 0.24 mg rutin/g sample, respectively. The antioxidant activity revealed the highest DPPH scavenging activity for 0.75% BBE fish sausage compared to synthetic additive BHT. After 28 days of frozen storage, lipid oxidation activity of fish sausages with 0.75% BBE had lower thiobarbituric acid reactive substances (TBARS) value than the negative control, but similar ($p > 0.05$) to BHT. The phenolic compounds and antioxidant activities were significantly reduced ($p < 0.05$) after 28 days of storage. Based on the antibacterial activity, BBE was able to inhibit tested foodborne pathogens, and the addition of BBE in fish sausage showed total plate count below 6 $\log_{10}$ CFU/g within 6 days of frozen storage. This research demonstrated the BBE efficiency as a natural antioxidant with antibacterial properties in fish sausages.

1. Introduction

Fish sausage, or locally known as "*keropok lekor*" is one of the popular fish-based products in Malaysia. It is a traditional snack originated from East Coast Malaysia, highly commercialized among locals to be sold at night stalls, school canteen, and hawker stall because of its appetizing taste and cheap price [1, 2]. It is made from fish, sago flour, and salt which are kneaded and rolled into cylindrical shape before being boiled. Just like any other fish products, fish sausages are prone to spoilage and microbial contamination such as *Pseudomonas, Flavobacterium, Corynebacterium, Lactobacillus, Bacillus*, and *Micrococcus* [1]. Therefore, it is perishable with short shelf life storage [3].

Lipid oxidation is one of the major causes of food spoilage in fish products because fish, such as Indian mackerel (commonly used in making "*keropok lekor*"), contains high polyunsaturated fatty acids (PUFAs) which are susceptible to oxidation [4]. Fish oxidized during processing, cooking, and storage consequently leads to an end product with a foul smell, unpleasant taste, discoloration with slimy texture, development of toxic substances, and also nutrient loss [5]. To sustain the consumption of fish sausage, especially to increase its global commercialization and marketability, its quality needs to be enhanced [2]. In the food industry, the addition of additives is commonly applied to maintain food quality and prolong its shelf life. However, the uses of synthetic preservatives such as

Table 1: Formulation of fish sausages incorporated with BBE at different concentrations and with synthetic additives.

Ingredient (g)	0% (negative control)	0.25%	0.50%	0.75%	Positive control
Sago starch	197.00	195.75	194.50	193.25	196.00
Mackerel fish (*Rastrelliger kanagurta*)	280	280	280	280	280
Salt	10	10	10	10	10
Sugar	8	8	8	8	8
Ice water	5	5	5	5	5
BBE[1]	—	1.25	2.50	3.75	—
Synthetic additive[2]	—	—	—	—	1
Total weight	500	500	500	500	500

[1]Percentage of BBE was based on the substitution with sago starch. [2]All formulations used potassium sorbate except for antioxidant analysis which used BHT.

butylhydroxyanisole (BHA), butylhydroxytoluene (BHT), *tert*-butylhydroquinone (TBHQ), and propyl gallate (PG) are strictly controlled due to their potential health hazard. Not to mention, these ingredients are poorly perceived by consumers who opt for a safer natural approach to food preservation. The application of plant extracts as natural antioxidants has become a preferable choice in these recent years to preserve fish products [6]. An example of a natural product which was the main interest of this research was bee bread.

In recent years, bee pollen has been added to various feed and food ingredients such as bakery products, beverages, and meat to improve the nutritional, functional, and sensory values of these newly formulated products [7]. Bee bread is made from pollen, added with bee salivary enzymes and nectar. It is stored and fermented in the beehives [8]. Recently, bee bread from Malaysian stingless bee, *Heterotrigona itama*, is characterized with high protein, carbohydrate, minerals content, and traces of vitamin C [9]. It is also rich in phenolic compounds, which have been shown to attribute toward its antioxidant and antibacterial properties *in vitro* [10–12]. For these reasons, it is intriguing to analyze bee bread extract, not only as a potential natural preservative in fish sausage during storage but also to enhance its nutritional values for consumer consumption.

Thus, the objectives of this study were to fortify fish sausages with bee bread extracts at different concentrations. Then, their nutritional composition, total phenolic and flavonoid content, antioxidant and lipid peroxidation activity, antibacterial activity, and microbial analysis were investigated during storage period.

2. Materials and Methods

2.1. Sample Preparation

2.1.1. Bee Bread Extract. Fresh bee bread sample was collected from Ladang 10, Faculty of Agriculture, Universiti Putra Malaysia from stingless bee *Heterotrigona itama* colony in 2018. Samples were obtained using sterile spatula and placed inside sterile falcon tubes before storing in a chiller at 4°C.

Bee bread extract (BBE) was prepared by modifying the method of Urcan et al. [13] using 70% ethanol as solvent. For every 25 g of fresh bee bread, 250 g of 70% ethanol was added. The mixture was boiled using Soxhlet apparatus for 2 h. The obtained solution was then filtered using a filter paper and the filtrate was evaporated using a rotary evaporator and then evaporated in a water bath at 50°C until the solvent was fully evaporated when the constant weight was achieved. BBEs were stored in a Falcon tube in 4°C chiller before use.

2.1.2. Fish Sausages. Fish sausages were prepared and modified from a formulation provided by the Department of Fisheries, Malaysia (2014), at concentrations of 0% (negative control), 0.25%, and 0.5% of BBE and with preservative (positive control) as shown in Table 1.

The ingredients were bought from a local store in Seri Kembangan, Selangor, Malaysia. The fish sausages were prepared according to a method by Nor-Khaizura et al. [1]. The fish were cleaned, deboned, and minced before mixing with other ingredients. The mixture was mixed until a soft dough was formed. The dough was rolled into a cylindrical shape with a length 10 cm each and boiled at 100°C until the fish sausage float. It was then cooled down at room temperature before being sealed in a transparent plastic bag and kept stored in a chiller at 4°C.

2.1.3. Fish Sausage Extract (FSE). Fish sausage extract (FSE) was prepared using the method of Ismail et al. [14] with few modifications. The raw fish sausages were defatted before being extracted. Three grams (3 g) of finely chopped raw fish sausages was mixed with 30 mL of *n*-hexane (Merck, Germany) and sonicated in a sonicator (PowerSonic 505, Hwashin Technology Co., Seoul, Korea) for 1h. The fish sausages were centrifuged for 10 min and the supernatant was removed. Then, the defatted fish sausages were added with 30 mL of methanol. The mixture was homogenized (Homogenizer Multi-Gen 7, USA) for 10 min at 2000 rpm before being sonicated for 2 h at 30°C. The sample was centrifuged at 7500 rpm for 10 min at 25°C. FSE was transferred into tubes for 1 mL with a concentration of 1000 mg/L each and stored at −80°C for antioxidant analysis usage.

2.2. Proximate Analysis. The proximate analysis was performed for fish sausages added with 0%, 0.25% 0.50%, and 0.75% of BBE and with synthetic additives. Each sample was first homogenized using quartering technique by cutting them into small pieces and homogenously mixed before performing analysis using method from AOAC [15].

The crude protein was determined using the Kjeldahl method and calculated using the conversion factor of 6.25 ($N \times 6.25$) (AOAC 981.10). The crude fat content was determined using the Soxhlet method (Soxtec™ 2050 Auto Fat Extraction System, FOSS Analytical, Denmark) (AOAC 991.36). The moisture content was determined by using oven drying method (Memmert GmbH + Co. KG, Germany) at 105°C until a constant weight was achieved (AOAC 950.46). The ash content was determined using a muffle furnace at temperature 600°C for at least 4 hours until there was no presence of black ash (AOAC 923.03). Dietary fibre was determined using chemical digestion in sulphuric acid and sodium hydroxide. The total carbohydrate was obtained by difference as follows:

$$g \text{ carbohydrates} = 100\,g - (g \text{ moisture} + g \text{ lipid} + g \text{ protein} + g \text{ ash}). \quad (1)$$

2.3. Determination of Total Phenolic Content (TPC). The TPC was determined by the Folin–Ciocalteu method according to Roy et al. [16] with minor modifications. The FSE samples were prepared in diluted concentration of 500 mg/L. Briefly, 100 μL of sample was added with 500 μL of diluted Folin–Ciocalteu's reagent and then vortexed. Then, 400 μL of sodium carbonate was added and vortexed again. The mixture was incubated for 1 hour at 40°C in the dark. Then, 200 μL of the mixture was loaded into 96-well plates and the absorbance was measured at 765 nm using microplate readers (Synergy™ H1 microplate reader, BioTek Instruments, Inc., USA). Gallic acid and 1000 μL methanol were used as the standard and blank, respectively. The results were expressed as mg gallic acid equivalent (GAE)/g fish sausage.

2.4. Determination of Total Flavonoid Content (TFC). The TFC was determined by the aluminium calorimetric method according to Chakraborty et al. [17] with minor modifications. The FSE samples were prepared in diluted concentration of 500 mg/L. The samples (100 μL) were mixed with 100 μL of 2% $AlCl_3$ solution. The mixtures were incubated for 15 minutes at room temperature in the dark condition. Then, 200 μL of the mixture was loaded into 96-well plates, and the absorbance was measured at 435 nm using a microplate reader (Synergy™ H1 microplate reader, BioTek Instruments, Inc., USA). Rutin and 200 μL methanol were used as the standard and blank, respectively. The results of the scavenging activity were expressed as mg rutin/g fish sausage.

2.5. Determination of 2,2-Diphenyl-1-picrylhydrazyl (DPPH) Radical Scavenging Activity. The antioxidant activity was evaluated by DPPH radical scavenging assay according to the method described by Ismail et al. [14] with some modifications. The FSE samples were prepared in diluted concentration of 500 mg/L. The samples (50 μL) were mixed with 195 μL of diluted DPPH stock solution in 96-well plate. The plate was incubated at room temperature in dark condition for 1 h. The absorbance was measured at 540 nm using a microplate reader (Synergy™ H1 microplate reader, BioTek Instruments, Inc., USA). Gallic acid and 245 μL of methanol were used as the standard and blank, respectively. DPPH radical scavenging activity was expressed as mg GAE/g fish sausages. The antioxidant activity (%) of the sample was calculated according to the following equation:

$$\%\ \text{scavenging activity} = \frac{A_{\text{control}} - A_{\text{sample}}}{A_{\text{control}}}. \quad (2)$$

2.6. Thiobarbituric Acid Reactive Substances (TBARS) Assay. The lipid peroxidation of the fish sausages was determined by TBARS assay described by Chan et al. [18] with few modifications. Firstly, 50 mg of fish sausage sample was added with 50 μL of distilled water. Then, 50 μL of the diluted sample was mixed with 250 μL of 0.25 N HCl, 250 μL of trichloroacetic acid (15%, w/v), and 250 μL of thiobarbituric acid solution (0.375%, w/v). The mixtures were vortexed before incubation in a water bath at 100°C for 10 min. The mixture was cooled down and centrifuged at 3000 rpm for 15 min. Then, 100 μL of the supernatant was loaded in a 96-well microplate. The absorbance was measured at 540 nm (Synergy™ H1 microplate reader, BioTek Instruments, Inc., USA). 1,1,3,3-tetramethoxypropane (TMP) and 800 μL of the distilled water were used as the standard and blank, respectively. The result was expressed in mg MDA/kg.

2.7. Antibacterial Activity of Bee Bread Extract (BBE). The antibacterial activity of the BBE against foodborne pathogens was determined using disc diffusion method according to the method of Akhir et al. [10]. The extracts were tested against *Staphylococcus aureus, Escherichia coli, Bacillus cereus*, and *Salmonella typhimurium*, which were obtained from the Institute of Bioscience, UPM. The overnight bacterial suspension was grown at 37°C, and the concentration was adjusted to 0.5 McFarland. Then, 100 μL of the bacterial solution was spread on Mueller–Hinton Agar (MHA) (Oxoid, UK). Meanwhile, 6 mm sterile disc was impregnated with 20 μL of BBE at 1 g/mL and left to dry before placing it onto the agar. Tetracycline (30 mg/ml) was used as a positive control. The agar plate was incubated for 24 h at 37°C. The zone of inhibition was measured in mm.

2.8. Microbial Quality. The microbial analysis of fish sausage during shelf life was analyzed according to Mohammad et al. [19] with slight modifications. The fish sausages were stored in a freezer at 0°C and the microbial count was calculated after 0, 2, 4, and 6 d of storage. After the designated time, 10 g of the fish sausages was added with 90 mL of 0.1% buffered peptone water and digested in a Stomacher bag using a Stomacher for 2 min. Four 10-serial dilutions were prepared using peptone water and 1 mL of diluent was spread onto

Table 2: Proximate composition of fish sausages fortified with different percentage of bee bread extract (BBE) and positive control.

	Proximate composition (%)					
Fish sausage	Crude protein	Crude fat	Moisture	Ash	Fibre	Carbohydrate
0.0% BBE	12.74 ± 1.03	1.60 ± 1.25	47.79 ± 0.71[a]	8.11 ± 0.19	0.081 ± 0.001[a]	28.12 ± 1.40[b]
0.25% BBE	12.24 ± 0.63	1.20 ± 0.92	47.33 ± 0.58[a]	8.17 ± 0.09	0.078 ± 0.001[b]	29.50 ± 0.89[b]
0.5% BBE	14.47 ± 2.58	1.33 ± 0.12	48.33 ± 0.57[a]	8.22 ± 0.01	0.082 ± 0.001[a]	26.01 ± 2.79[b]
0.75% BBE	13.00 ± 1.97	0.47 ± 1.97	40.33 ± 0.58[b]	7.98 ± 0.11	0.082 ± 0.001[a]	36.59 ± 1.26[a]
Synthetic∗	11.36 ± 0.89	1.40 ± 0.40	41.30 ± 0.58[b]	8.18 ± 0.03	0.077 ± 0.001[b]	36.18 ± 1.77[a]

Values of means ± standard deviation (SD) of triplicate independent experiments. Different letters in the same column mean significant difference ($p < 0.05$). ∗Potassium sorbate.

plate count agar (Oxoid, UK) before being incubated aerobically at 37°C for 24 h. The viable colonies were enumerated and expressed as $\log_{10}$ CFU/mL.

2.9. Statistical Analysis. All of the data were obtained in triplicate ($n = 3$) and the results were expressed as means ± standard deviations. Data were analyzed using Minitab version 17 using one-way ANOVA tests. If ANOVA test indicated a significant result ($p < 0.05$), then the significant means were separated using Tukey's test.

3. Results and Discussion

3.1. Proximate Composition. The proximate composition of the fish sausages fortified with BBE and synthetic additive is shown in Table 2. Higher BBE concentration in fish sausages significantly ($p < 0.05$) reduced the moisture level and increased the carbohydrate content.

Moisture above 40% is not favorable as it could promote the microbial growth in fish sausages [20]. By increasing the incorporation of BBE from 0 to 0.75%, the moisture level decreased from 47.79 to 40.33%. Increment in pollen concentration also decreased the moisture content when added to meatballs [21] and gluten-free bread [22]. One of the possible factors of decreasing moisture content is due to the substitution of sago starch to make fish sausage with BBE. Sago starch contains high amylopectin, which has high water absorbing capacity [23]. Hence, lowering sago starch content reduces fish sausages' moisture level.

Carbohydrate content significantly increased ($p < 0.05$) from 28.93 to 37.41% in 0.75% BBE fish sausage compared to the negative control. *H. itama* bee bread and its extract contain about 55 to 58% carbohydrate [9, 24]. This could compensate for the loss of carbohydrate content contributed by sago starch.

However, there was no significant difference ($p > 0.05$) for protein, fat, and ash content between fish sausages at different BBE concentrations. BBE did not affect these nutritional parameters. Protein was the second-largest macronutrient found contributed by the Indian mackerel fish, which is the primary protein source for all the samples. The fish mass used in the formulations was constant; thus, it had no effect on the samples' protein level. Similarly, protein content in the fish sausage was also not affected when incorporated with seaweed [19]. Although the *H. itama* bee bread contains an average of 22.26% protein (Mohammad et al., 2020), it showed an insignificant effect towards fish sausage protein when added at low concentration. The samples' protein level could be increased when a higher bee bread concentration, minimum at 1%, is added to the food system [21, 22].

The fat and ash content were also constant in all sample concentrations because *H. itama* bee bread contains a minute amount of these nutrients [9]. Even at high concentrations, bee bread had shown to not affect these nutrients' values [21].

3.2. Total Phenolic Content and Total Flavonoid Content. Figure 1 shows the total phenolic content (TPC) for fish sausage samples fortified with BBE and synthetic additive BHT. The TPC in fish sausages significantly increased ($p < 0.05$) when the concentration of BBE in the samples increased. On day 1, the TPC varied among samples from 14.87 to 23.46 mg GAE/g sample. Fish sausage fortified with 0.75% BBE had the highest TPC content (23.46 mg GAE/g sample) compared to other concentrations and sample with BHT.

This result was higher than other reported studies of food fortified with bee pollen. For example, phenolic content in sheep milk yoghurt enriched with bee pollen was 8.78 mg GAE/g [25]. In another study, Krystyjan et al. [26] reported 4.84 mg GAE/g in 10% bee pollen biscuits while Conte et al. [22] only reported a maximum of 4.42 mg GAE/g in 5% bee pollen bread. According to Othman et al. [27], Malaysia *H. itama* bee bread is rich in phenolic compounds as it was reported to contain up to 22.4 mg GAE/g.

However, the results showed a decreasing trend ($p < 0.05$) after 14 and 28 days (Figure 1). On day 14, fish sausages with 0.25 to 0.75% BBE had significantly higher phenolic content ($p < 0.05$) than control and BHT. But after 28 days, the phenolic content in all samples was not statistically different ($p > 0.05$) from each other. Loss of phenolic compounds can happen at low-temperature storage between 4 and 8°C [28] as the oxidation process gradually degrades these compounds.

Flavonoid is the most prevalent phenolic compounds group. Figure 2 displays the total flavonoid content (TFC) for fish sausage samples fortified with BBE and with BHT. The TFC in fish sausages significantly increased ($p < 0.05$) when the concentration of BBE in the samples increased. On day 1, the TFC varied from 2.90 to 8.05 mg rutin/g sample. The highest TFC content was recorded for fish sausage fortified with 0.75% BBE (8.05 mg rutin/g sample).

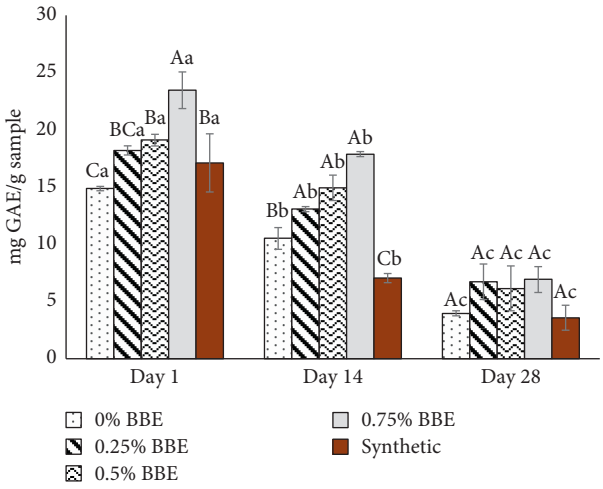

Figure 1: The total phenolic content (mg GAE/mg sample) of fish sausages samples fortified with 0%, 0.25%, and 0.5% BBE and synthetic additives BHT. Values of means ± standard deviation (SD) of triplicate independent experiments. Different capital letters indicate significant differences ($p < 0.05$) within the samples in the same day. Different small letters indicate significant differences ($p < 0.05$) of the same sample across the storage days 1, 14, and 28 at 4°C.

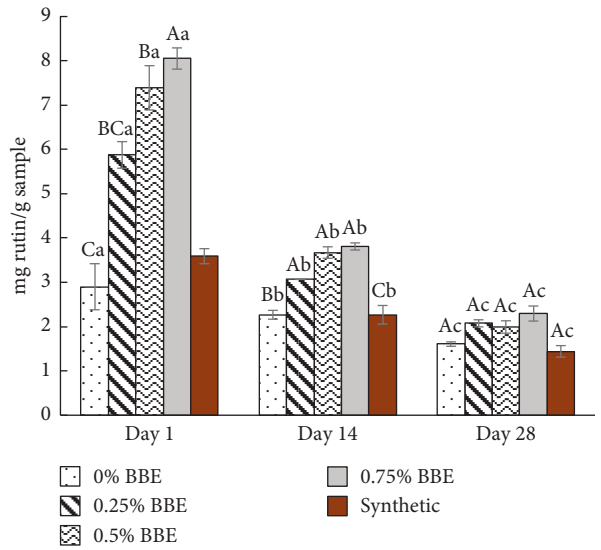

Figure 2: The total flavonoid content (mg rutin/g sample) of fish sausage samples fortified with 0%, 0.25%, and 0.5% BBE and synthetic additive BHT. Values of means ± standard deviation (SD) of triplicate independent experiments. Different capital letters indicate significant differences ($p < 0.05$) within the samples in same day. Different small letters indicate significant differences ($p < 0.05$) of the same sample across the storage days 1, 14, and 28 at 4°C.

Studies on flavonoid content of food incorporated with bee bread or bee pollen are scarce even though the commonly found phenolic compounds in bee bread were flavonoids such as kaempferol, quercetin, and luteolin [29]. According to Othman et al. [30], TFC in *H. itama* BBE was in the range of 16.71 to 26.57 mg QE/g extract. Nevertheless, in this study, the TFC range for BBE fish sausages was

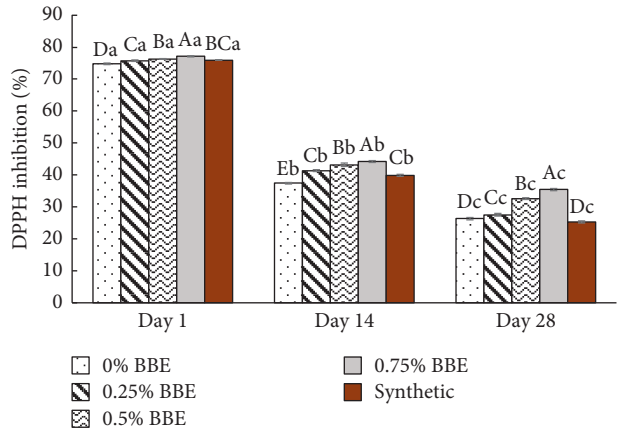

Figure 3: The DPPH radical scavenging activity (%) of fish sausages samples fortified with 0%, 0.25%, and 0.5% BBE and synthetic additives. Values of means ± standard deviation (SD) of triplicate independent experiments. Different capital letters indicate significant differences ($p < 0.05$) within the samples in the same day. Different small letters indicate significant differences ($p < 0.05$) of the same sample across the storage days 1, 14, and 28 at 4°C.

incredibly low (1.99 to 8.05 mg rutin/sample). Higher temperature imposed on fish sausages during the boiling stage could degrade some of the flavonoid compounds.

Variation of phenolic and flavonoid content could also be attributed to different extraction methods. In addition, bee bread phenolic compounds are also influenced by the botanical origin and geographical origin of the pollen.

3.3. DPPH Activity and TBARS Activity. DPPH activity measures the extract ability to scavenge the free radical molecules (DPPH). Figure 3 shows the DPPH radical scavenging activity for fish sausage samples fortified with BBE and synthetic additive BHT. On day 1, the fish sausage fortified with 0.75% BBE recorded the highest DPPH activity (77.15%), higher than fish sausages with BHT ($p < 0.05$). Meanwhile, fish sausage without BBE recorded the lowest (74.80%).

This study reported higher DPPH inhibition activity than gluten-free bread fortified with bee pollen [22]. The antioxidant activity of fish sausage bee bread was partly due to the presence of phenolic compounds such as isorhamnetin, kaempferol, and apigenin and also vitamin C found in *H. itama* bee bread [9, 27]. Incorporation of bee bread or bee pollen has also shown to increase the DPPH radical scavenging activity in biscuit [31], gluten-free bread [22], and yoghurt milk [25]. Similar to this study, the antioxidant activities of these bee pollen products were shown to be positively correlated to the bee pollen concentrations added to the product [22].

However, storage until 28 days significantly reduced ($p < 0.05$) the DPPH activity by the samples. Yet, 0.75% BBE fish sausage had the highest DPPH inhibition values ($p < 0.05$) compared to fish sausages with other concentrations and with BHT. When the storage period increased, the available phenolic compounds to oxidize free radicals in

Table 3: The thiobarbituric acid reactive substance (TBARS) inhibitory activity (mg MDA/kg) of fish sausage samples fortified with 0%, 0.25%, and 0.5% BBE and synthetic additives BHT after storage for 1, 14, and 28 days at 4°C.

	TBARS values (mg MDA/kg)				
	0% BBE	0.25% BBE	0.5% BBE	0.75% BBE	Synthetic
Day 1	3.30 ± 0.22^{Aa}	3.10 ± 0.06^{Aa}	1.16 ± 0.01^{Ca}	0.59 ± 0.11^{Da}	2.19 ± 0.23^{Ba}
Day 14	5.50 ± 0.18^{Ab}	5.41 ± 0.90^{Ab}	3.89 ± 0.07^{Bb}	3.61 ± 0.25^{Bb}	3.07 ± 0.51^{Ba}
Day 28	24.14 ± 0.96^{Ac}	14.40 ± 0.69^{Bc}	14.29 ± 14.29^{Bc}	12.84 ± 0.70^{BCc}	11.92 ± 0.59^{Cb}

Values of means ± standard deviation (SD) of triplicate independent experiments. Different capital letters in the same row indicate significant differences ($p < 0.05$). Different small letters in the same column indicate significant differences ($p < 0.05$).

fish sausages were reduced (Figure 1). Consequently, the antioxidant activities became lowered.

Malonyldialdehyde (MDA) is one of the end products of lipid oxidation and can be considered as a biomarker for oxidative stress [32]. The determination of lipid peroxidation in the fish sausages was conducted using TBARS assay that measured the amount of MDA-TBA complex in the sample. The effectiveness of the natural antioxidants, which was BBE, in reducing the lipid oxidation in the fish sausage was demonstrated after 14 and 28 days of refrigeration at 4°C.

Table 3 illustrates the TBARS activity for the fish sausage samples. After 1 day of storage, fish sausages fortified with 0.75% had the lowest TBARS activity (0.59 mg MDA/kg) than other samples, including samples with BHT. Meanwhile, sample without BBE showed the highest TBARS value and significantly no difference ($p > 0.05$) compared to the sample with 0.25% BBE.

After 28 days of refrigeration, the TBARS activities for fish sausages with 0.75% BBE were similar ($p > 0.05$) to fish sausage with BHT. Using a higher concentration of BBE was able to protect and delay lipid oxidation, which was indicated by low TBARS values. This result is in agreement with Almeida et al. [33], which demonstrated that 0.2 g/kg lyophilized bee pollen in pork sausages lowered the TBARS value compared to control after 30 days refrigeration. Turhan et al. [34] also reported the lowest TBARS values in 4.5 and 6.0% meatballs bee pollen after 30 days of storage.

However, this research observed an increasing trend ($p < 0.05$) in the TBARS value after 28 days of storage, indicating an increase in lipid oxidation in fish sausages. This result was similar to other meat products added with bee pollen [33, 34]. At low-temperature storage, lipid inhibition still occurs but at a slower rate [33]. Some of the phenolic compounds could be degraded and could not compensate for the increase of lipid oxidation throughout storage. This was observed by the gradual loss of phenolic compounds reported in the previous section (Figures 1 and 2). TBARS could also be increased because of the partial dehydration and oxidation of unsaturated fatty acids [35], especially when Malaysian Indian mackerel fish contain high unsaturated fatty acids [4].

TBARS values also provide insight towards meat odor and consumer acceptance. Consumers more likely to detect rancid odors at MDA concentration above 0.5 mg MDA/kg [36]. Besides, meat products are suggested to be favourable with less than 3 mg MDA/kg sample [37]. In this study, after 14 days of storage, all fish sausage samples recorded TBARS values above 3 mg MDA/kg. Fish sausages have an approximate 2 weeks of shelf life in the chilled refrigerator.

Based on the result from DPPH and TBARS activity, this shows BBE ability as a natural antioxidant to inhibit lipid oxidation in fish sausage at a high concentration similar to BHT. The lipid oxidation is dependent on the BBE concentration and storage period.

3.4. Antibacterial Activity of Bee Bread Extract (BBE). Table 4 displays the antibacterial activity of BBE against foodborne pathogens. BBE showed the highest antibacterial activity against *S. aureus* followed by *B. cereus, S. typhimurium,* and *E. coli*. BBE was more effective ($p < 0.05$) against the tested pathogens compared to tetracycline (positive control).

This result is in agreement with those of Akhir et al. [10], which showed the ability of bee bread ethanolic extract to inhibit *S. aureus, B. subtilis, E. coli*, and *Salmonella* sp. growth. However, there was variation in the degree of inhibition as it was influenced by bacteria type. Gram-negative bacteria such as *E. coli* and *S. typhimurium* are more resistant to the antimicrobial agent because they have thicker peptidoglycan cell walls, which acts as a barrier to hydrophobic compounds penetration [38]. According to Sulbarán-Mora et al. [39], bee bread antibacterial activity was correlated to its phenolic content.

3.5. Microbial Quality. Table 5 shows the plate count of fish sausages fortified with BBE and synthetic additive during shelf life at days 0, 2, 4, and 6. The total plate count ($\log_{10}$ CFU/g) increased across the sample from day 0 until day 6. After 6 days, fish sausages without BBE had the highest microbial count with 5.21 ± 0.86 $\log_{10}$ CFU/g while fish sausages with synthetic additive recorded the lowest with 3.50 ± 1.29 $\log_{10}$ CFU/g. However, these differences were not statistically different ($p > 0.05$).

Fish sausages were boiled beforehand, which reduced the microbial count significantly [1, 40]. Adequate boiling was able to fully eliminate the growth of *S. aureus* and *Vibrio* sp. in fish sausages [40]. Thus, no presence of microbial growth was observed at the beginning of day 0. Still, boiled fish sausages can be contaminated by microorganisms in the postboiling process (cooling stage) as observed by Nor-Khaizura et al. [1]. They reported a microbial growth increase to 6.44 $\log_{10}$ CFU/g during the cooling stage. Some of the assumptions for these different observations were probably due to the differences in boiling and

Table 4: The antibacterial activity of bee bread extract (BBE) against foodborne pathogens.

	Zone of inhibition (mm)			
Fish sausage	*Escherichia coli*	*Salmonella typhimurium*	*Staphylococcus aureus*	*Bacillus cereus*
BBE (1 g/ml)	23.00 ± 1.41^{a}	31.00 ± 1.41^{a}	43.50 ± 2.12^{a}	31.00 ± 1.41^{a}
Tetracycline (30 mg/ml)	17.50 ± 0.70^{b}	20.5 ± 0.70^{b}	3.95 ± 0.07^{b}	18.00 ± 0.70^{b}

Tetracycline is the positive control. Values of means ± standard deviation (SD) of triplicate independent experiments. Different letters in the same column mean significant difference ($p < 0.05$).

Table 5: Total plate count ($\log_{10}$ CFU/g) of fish sausages fortified with different concentrations of bee bread extract (BBE) and synthetic additive potassium sorbate after 0, 2, 4, and 6 days of shelf life at 0°C.

	Total plate count ($\log_{10}$ CFU/g)			
Fish sausage	Day 0	Day 2	Day 4	Day 6
0% BBE	0.00^{a}	3.55 ± 2.63^{bA}	4.86 ± 0.84^{b}	5.21 ± 0.86^{b}
0.25% BBE	0.00^{a}	0.00^{aB}	3.90 ± 0.84^{b}	3.84 ± 0.87^{b}
0.50% BBE	0.00^{a}	3.75 ± 0.95^{bA}	4.15 ± 0.59^{b}	3.32 ± 0.47^{b}
0.75% BBE	0.00^{a}	3.61 ± 1.87^{bA}	3.50 ± 1.29^{b}	4.86 ± 1.64^{b}
Synthetic[1]	0.00^{a}	3.32 ± 0.47^{bA}	3.50 ± 1.29^{b}	3.50 ± 1.29^{b}

[1]Synthetic potassium sorbate. Values of means ± standard deviation (SD) of triplicate independent experiments. Different capital letters in same column mean significant difference ($p < 0.05$). Different small letters in same row mean significant difference ($p < 0.05$).

sterile handling techniques in. these studies. However, cross-contamination is more likely to happen from food handlers and food contact surfaces on the scene [40].

The fish sausages were later stored in the freezer at 0°C, whereas the microbial counts were shown to gradually increase until day 6. Still, all of the samples showed a microbial count lower than 6 $\log_{10}$ CFU/g, which is the standard limit set for fish products ready for consumption according to Malaysia Food and Agricultural Organization (2017). Increment of microbial loads also suggests the growth of psychrophile bacteria that thrive in low temperature in fish-based products [41]. On day 6, fish sausage with BBE showed lower microbial count than control. This is in conjunction with the finding from the previous section which demonstrated the efficiency of the BBE against the tested microbes *in vitro*.

Thus, the result indicated that BBE was as efficient as a synthetic preservative in reducing the microbial load in fish sausages during 6 days of frozen storage compared to without preservative.

4. Conclusions

The effect of BBE at different concentrations on the proximate, phenolic compounds, and antioxidant and antibacterial activity in fish sausages has been studied. It demonstrated the BBE has a potential as a natural antioxidant and antibacterial agent in fish sausages. However, its efficacy relatively depends on the BBE concentration and storage period. In the future, more study should be conducted to determine BBE effects on the fish sausage physical properties and the final product acceptance among consumers.

Data Availability

The data used to support the findings of this study are included within the article.

Conflicts of Interest

The authors declare that they have no conflicts of interest regarding the publication of this paper.

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Research Article

Benzothiazole (BTH) Induced Resistance of Navel Orange Fruit and Maintained Fruit Quality during Storage

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Current research aimed at studying the effect of benzothiazole (BTH) on the fruit quality and resistance against *Penicillium italicum* (*P. italicum*). Recently, a synthetically prepared novel BTH was introduced that elicits the induction of resistance against various diseases of fruits. However, little was reported on the effect of BTH on the disease resistance and fruit quality of postharvest navel orange fruit. In this study, 50 mg·L^{-1} BTH significantly reduced the decay rate of fruits during 36 days of storage at 20 ± 0.5°C ($P < 0.05$). BTH markedly inhibited the weight loss rate in fruits ($P < 0.05$) and effectively maintained higher soluble solid content (SSC), titratable acid (TA), and vitamin C (VC) content compared with control navel orange fruits. Further, BTH significantly suppressed the increase of disease incidence and lesion area of orange fruits challenged with *P. italicum* ($P < 0.05$). BTH treatment significantly enhanced antioxidant capacity (DPPH, ABTS radical scavenging activity, and reducing power), and superoxide dismutase (SOD) and peroxidase (POD) activities were significantly increased, while the activity of catalase (CAT) was opposite to the former ($P < 0.05$). The activities of β-1,3-glucanase (GLU), phenylalanine ammonia-lyase (PAL), and chalcone isomerase (CHT) were significantly higher in BTH-treated navel orange fruits ($P < 0.05$). Our results suggested that BTH treatment may be a promising treatment for maintaining the quality and inhibiting blue mold of postharvest navel orange in the future.

1. Introduction

Navel orange (*Citrus sinensis* L., Osbeck) is pretty popular among the consumers worldwide and is a favorite fruit due to its unique taste and high nutritional value. However, navel orange is susceptible to pathogenic fungi. *Penicillium italicum* and *P. digitatum*, which cause blue mold and green mold, are the major fungi [1, 2] and account for up to 60–80% of the total fungal decay during citrus fruit storage [3, 4]. Postharvest diseases are being controlled through multiple approaches specifically by applying synthetic fungicides. However, the excessive and misuse of fungicides caused resistance of pathogens against synthetic fungicides and caused environmental degradation and human health issues which led to a worldwide trend for exploring novel natural alternatives with reduced or no side effects. The induction of the resistance using various biological, chemical, or physical means may become potential strategies for controlling postharvest decaying of fruits [5]. Some exogenous compounds such as chitosan [6], salicylic acid [7],

terpene limonene [4], and nitric oxide [8] have been used to induce disease resistance of postharvest orange fruits. BTH is a newly reported analogue of naturally occurring salicylic acid (SA) reported in plants and is highly effective for induction of systemic-acquired resistance (SAR) in plants to protect from various microbial diseases. Moreover, it has been reported as nontoxic to plants without any unpleasant environmental impacts [9]. BTH exposure can induce resistance for diseases and even wound-mediated suberization in fruits and vegetables such as muskmelon [10], banana [11], strawberry [12], tomato [13], and potato [14, 15] and effectively reduce the occurrence of disease. However, according to the authors' knowledge, no study explained the efficacy of BTH for the control of blue mold in navel orange.

The current study aimed to examine how BTH affects the growth of postharvest green mold caused by *P. italicum* and quality of navel orange fruits under storage. Decay rate and fruits' quality for 36 days of storage at $20 \pm 0.5^{\circ}$C were investigated, and antioxidant capacity, antioxidant enzymes activities, and disease resistance related enzymes activities in navel orange fruits following inoculation with *P. italicum* were also determined.

2. Materials and Methods

2.1. Navel Fruits and BTH Treatment. Navel orange fruits (*Citrus sinensis* L. Osbeck *cv.* Newhall) were harvested from the orchard in Ganzhou City, Jiangxi Province, China, at a commercially mature period with a mean soluble solid content (SSC) of 12 °Brix. Fruits of uniform size and free of wound were picked out for the experiments. The navel orange fruits were randomly classified in two groups (each group comprised of over 300 fruits) and exposed with $50\,\text{mg}\cdot\text{L}^{-1}$ of BTH (containing 0.05% Tween-80) and deionized H_2O (control, containing 0.05% Tween-80) for 10 min. After that, the fruits were air-dried for further 2 h at room temperature, and all navel oranges were packed in PE bags (1 orange per bag) and were kept at $20 \pm 0.5^{\circ}$C, 85% relative humidity (RH) for 36 days.

Certain quality parameters were measured at 6, 12, 18, 24, 30, and 36 days after storage at $20 \pm 0.5^{\circ}$C using 10 fruits of each replicate. This measurement of newly picked navel orange fruits prior to experimental studies was named as 0 day, and each treatment was replicated three times.

2.2. Decay Rate and Fruit Quality Parameters Assay. The fruits decay process under room temperature storage ($20 \pm 0.5^{\circ}$C, RH 85%–95%) was visually examined for 60 fruits in each treatment group. If there were visible decay symptoms, fruits were considered to be decayed. The percentage decaying of the fruits was counted on every 6th day of storage, and the decay rate of fruits was tabulated by taking the percentage of the total number of fruits with decayed fruits accounting the total number investigated.

The weight loss was defined as the percentage of the reduced weight to the initial weight of navel orange fruit during storage. SSC of fruit juice was assayed by a hand-held refractometer (ATAGO PAL-1, Tokyo, Japan) and expressed as °Brix. TA in fruit juice was assayed using the titration method and expressed as a percentage of citric acid. Vitamin C content was determined by spectrophotometric method [16] and expressed as mg $100\,\text{g}^{-1}$ FW.

2.3. Pathogen Preparation and Inoculation. A second experiment was then conducted to determine the lesion area and inoculation infection rate. For this reason, fruits treated with BTH or deionized water (as control) were inoculated with *P. italicum*. *P. italicum* was isolated and purified from infected navel orange fruits. The *P. italicum* was cultivated in PDA medium at 25°C for a week, and then, spore suspension (1×10^5 spores per milliliter) was prepared with sterile water. Navel orange fruits were pierced at two opposite points (3 mm deep × 3 mm wide) in the middle, and then, 10 μL of spore suspension was injected in each wounded area. During storage, the number of fruits with obvious disease symptoms was recorded. Three replicates of 20 fruits in each group were used for inoculation infection rate and lesion area determination. The pericarp tissue around the lesion of 10 fruits per replicate was sampled, dipped into liquid nitrogen, and stored at −80°C for further analysis. There were three replicates in each treatment.

2.4. The Decay Rate, Disease Incidence, and Lesion Area. The disease incidence of navel orange challenged with *P. italicum* was done by the percentage of the number of infected wounds with decay accounting the total wounds per replicate. The lesion diameter of the inoculated fruits was measured by vernier caliper. The lesion area was calculated as follows: lesion area $= (\text{mm}^2) = \pi \times (d/2)^2$.

2.5. Determination of Antioxidant Capacity. Navel orange samples (5 g) were added to 30 ml methanol solution and extracted by ultrasonic assist for 40 min and then centrifuged at 13,000xg for 15 min. The supernatant was collected to determine antioxidant capacity of navel orange fruit. 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging capacity, 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid (ABTS) radical scavenging capacity, and reducing power were determined by Sun et al. [17]. DPPH radical scavenging capacity was measured at 517 nm. ABTS radical scavenging capacity was measured at 734 nm. For determination of reducing power, the absorbance was measured at 700 nm.

2.6. Assays of Enzyme Activities. 1.0 g of navel orange was homogenized in 8 mL of 50 mM pH 7.8 PBS containing $0.8\,\text{g}\cdot\text{L}^{-1}$ polyvinylpyrrolidone (PVP) and 1 mM ethylenediaminetetraacetic acid (EDTA) and then centrifuged at 12,000xg for 15 min at 4°C. The supernatants were used for the SOD, POD, and PPO activity assays. Frozen pericarp tissue (1 g) was ground with 8 mL of 50 mM PBS, containing 2% PVP and 5 mM (dithiothreitol) DTT for CAT extraction. SOD activity was assayed following the steps of Prochazkova et al. [18]. CAT and POD activities were assayed according to previous report [17]. PPO activity was assessed following Liu

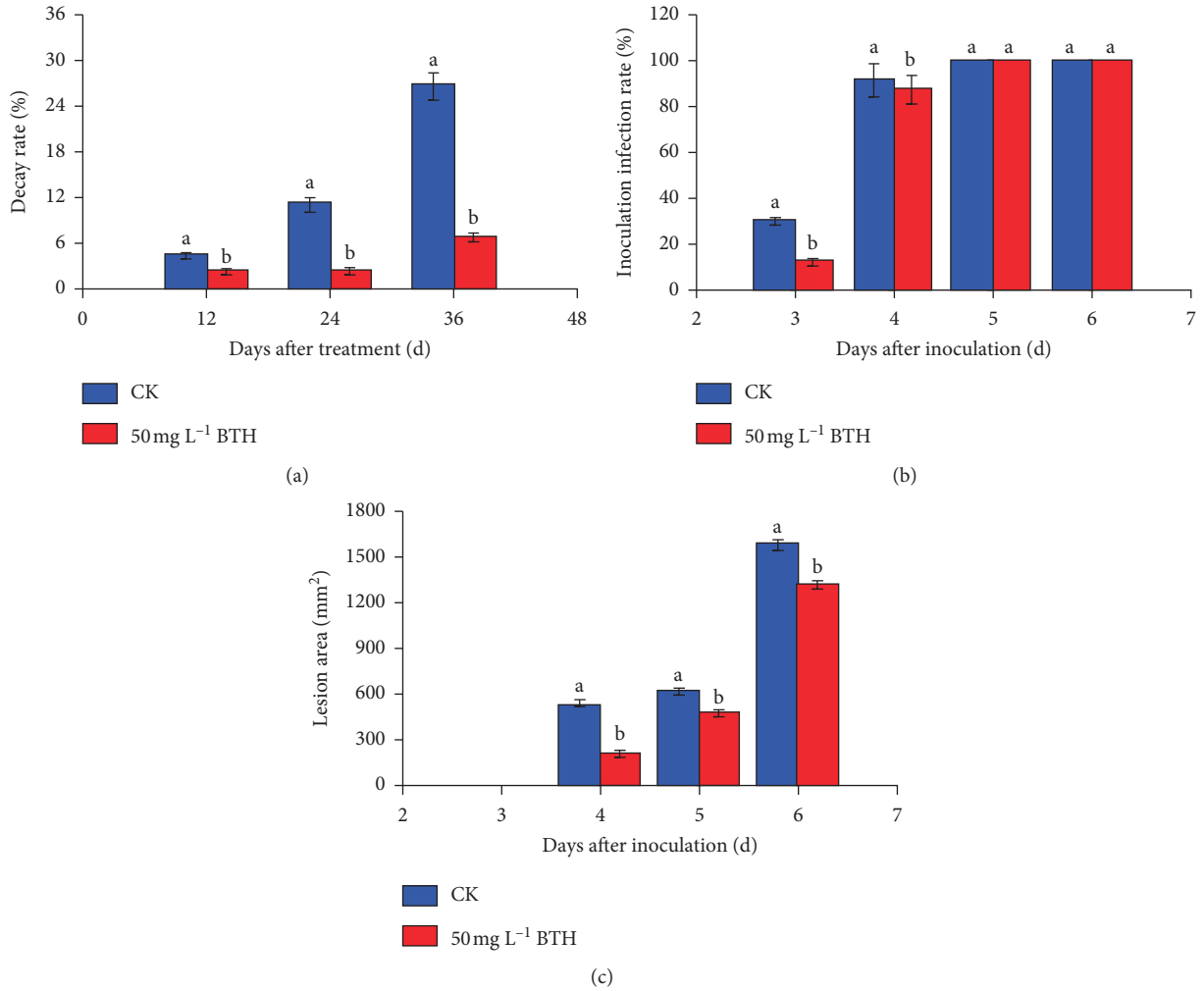

Figure 1: Effects of BTH on the decay rate during storage and disease incidence and lesion area of navel orange fruits challenged with *P. italicum* at 20°C.

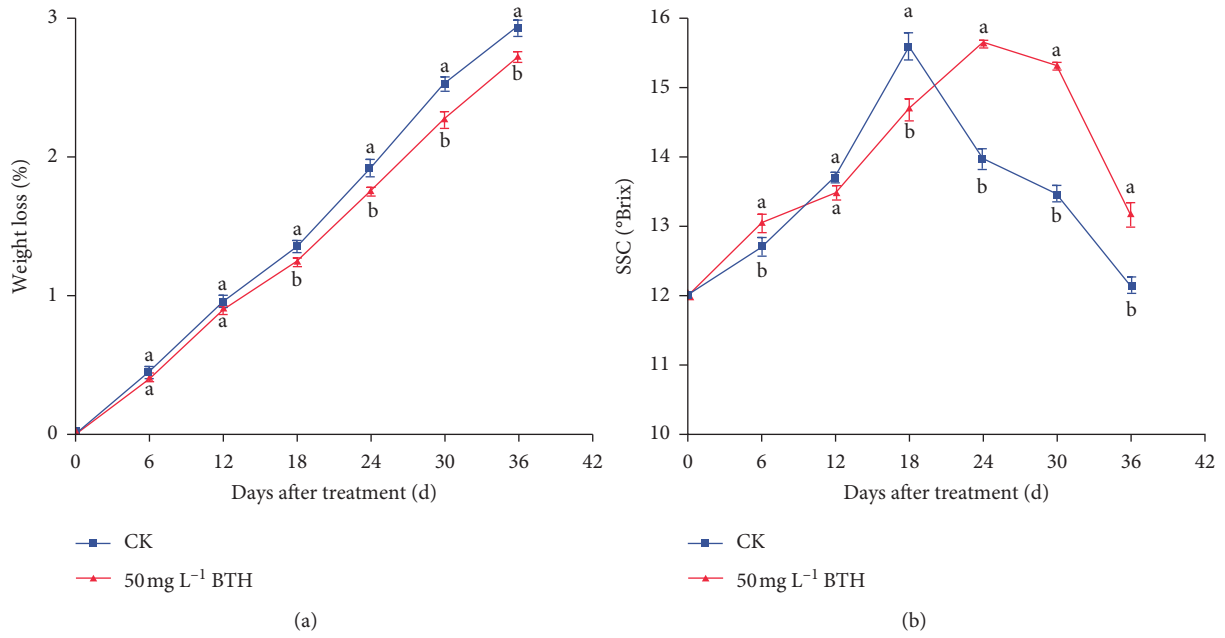

Figure 2: Continued.

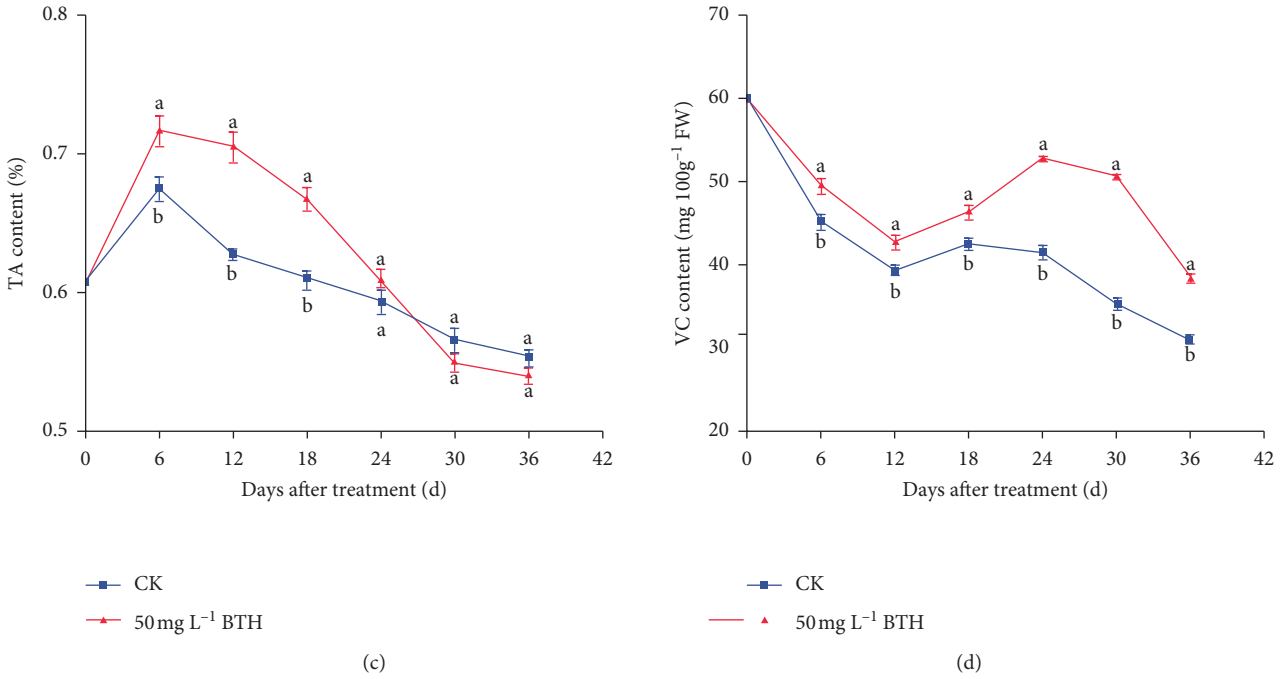

Figure 2: Effects of BTH on weight loss, SSC, VC, and TA contents of navel orange fruits under storage at 20°C.

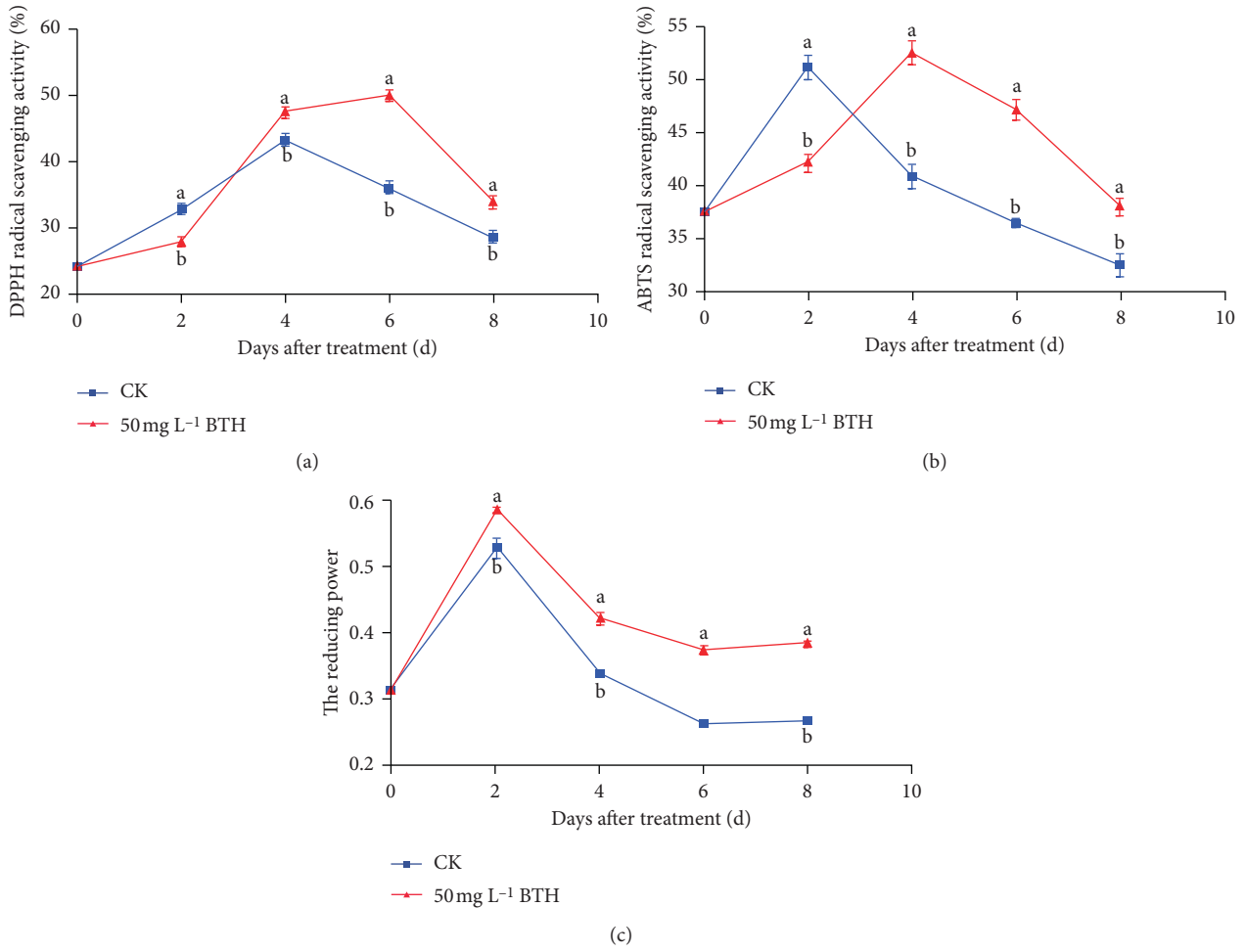

Figure 3: Effects of BTH on antioxidant capacity of navel orange fruits challenged with *P. italicum*.

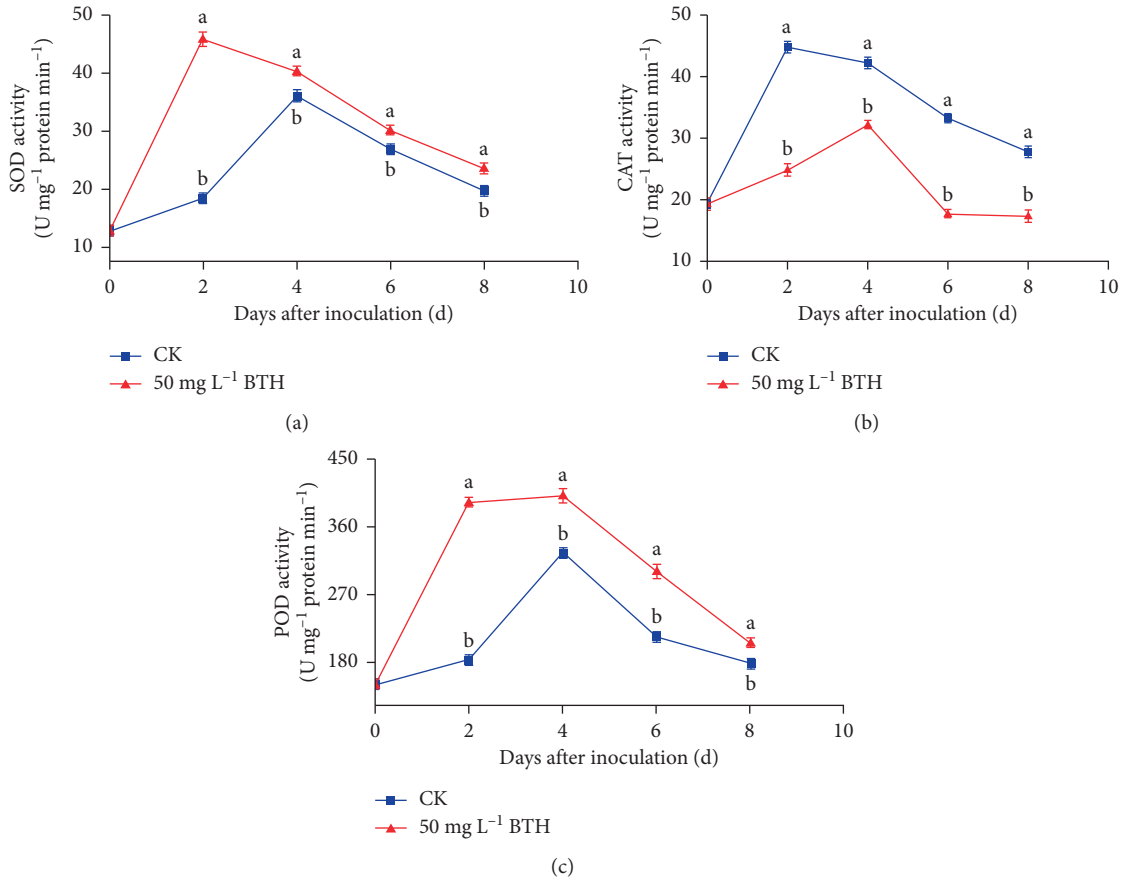

Figure 4: Effects of BTH on the activities of SOD, CAT, and POD of navel orange fruits challenged with *P. italicum*.

et al. [19]. SOD, CAT, POD, and PPO activity was expressed as U·mg^{-1} protein min^{-1}.

Frozen pericarp tissues were ground to finally powder form in 8 mL of 50 mM precooled boric acid buffer (pH 8.8) containing 0.5 g PVP, 5 mM β-mercaptoethanol, and 2 mM EDTA for PAL extraction. PAL activity was assayed following the steps of Lu et al. [20]. Frozen pericarp tissue was ground in 8 mL of 100 mM acetic acid buffer (pH 5.0) containing 1 mM EDTA and 5 mM β-mercaptoethanol for GLU and CHT extraction. GLU activity and CHT activity were assayed as described by Chen et al. [2]. PAL activity was expressed as U·mg^{-1} protein h^{-1}. GLU and CHT are expressed as U·mg^{-1} protein. Soluble protein content in all enzyme extracts was determined following Bradford method [21], using bovine serum albumin as standard.

2.7. Statistical Analysis. Data were analyzed with SPSS (18.0 version). The significance of difference between the data was determined by Duncan's multiple range test.

3. Results

3.1. Effect of BTH on Decay Rate of Navel Orange. The decay rate of navel orange fruit increased with the extension of storage time at room temperature storage (Figure 1(a)). BTH treatment significantly inhibited the decay rate of navel oranges stored at 20°C ($P < 0.05$). At the 36th day of storage, the decay rate of BTH treated orange fruit was only 6.67%, which was a quarter of the control group.

3.2. Effect of BTH on Disease Incidence and Lesion Area. The lesion diameter had a gradual enlargement starting at the third day after exposure to *P. italicum*. BTH significantly reduced the disease incidence (Figure 1(b)) and lesion diameters (Figure 1(c)) in navel orange fruit inoculated with *P. italicum* compared with its respective controls ($P < 0.05$).

3.3. Effect of BTH on Fruit Quality of Navel Orange. During storage, weight loss increased in all groups, and weight loss in BTH treated navel orange fruits was significantly lower than its control group after 12 days of storage ($P < 0.05$) (Figure 2(a)). The SSC in all samples was initially increased and then declined. Compared with the control, the navel orange fruit treated with BTH showed significantly higher SSC after 18 days of storage ($P < 0.05$) (Figure 2(b)). TA content increased slightly at first and then decreased. The TA content in the BTH-treated navel orange fruits was significantly higher than that in the control group ($P < 0.05$) at the early stage, and there was no significant difference after 24 days of storage (Figure 2(c)). VC content of the navel orange fruits in control gradually decreased. There was an increase in BTH treated navel orange fruits on day 18 and VC content of

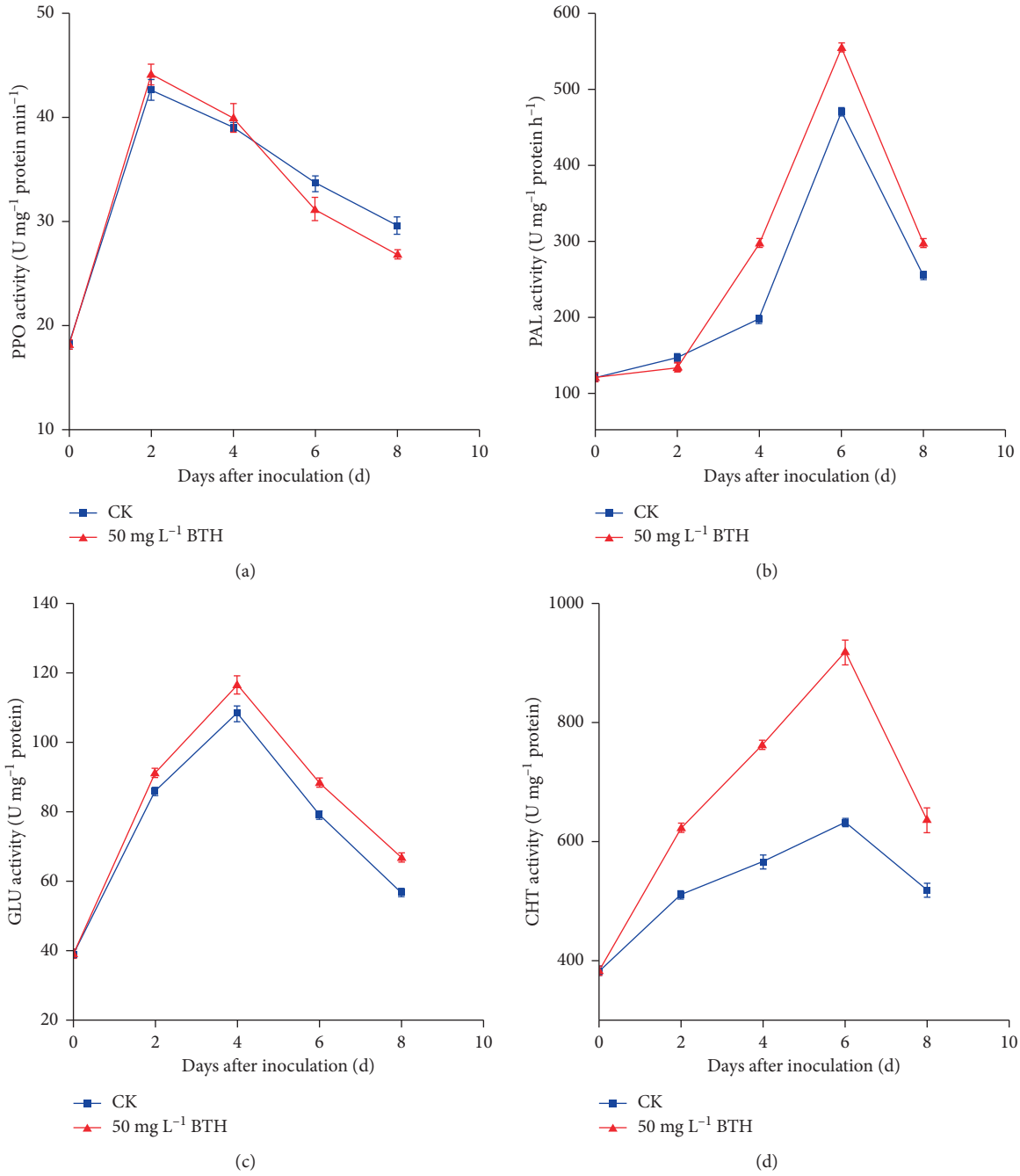

Figure 5: Effects of BTH on PPO, PAL, GLU, and CHT activities in navel orange fruits challenged with *P. italicum*.

the BTH treatment maintained significantly higher VC content after 18 days of storage (Figure 2(d)).

3.4. Effect of BTH on Antioxidant Capacity. DPPH, ABTS radical scavenging activity, and reducing power exhibited a similar trend after inoculation and showed a sharp increase initially and followed by a decrease (Figures 3(a)–3(c)). The fruits exposed to BTH depicted significantly higher DPPH radical scavenging potential, ABTS radical scavenging activity, and reducing power during storage ($P < 0.05$).

3.5. Effect of BTH on Antioxidant Enzymes Activities. The changes of the activity of SOD, CAT, and POD activities in navel orange fruits challenge with *P. italicum* are shown in Figure 4. SOD, CAT, and POD activities in navel orange initially arose in the storage period and then gradually declined at later storage periods (Figure 4). The SOD and POD activities reached the peak on day 4 after storage, but BTH treated navel fruits reached the peak on day 2 and were significantly higher than those of control navel orange fruits ($P < 0.05$). The activity of CAT in BTH treated navel orange fruits was significantly lower than that of control fruits ($P < 0.05$).

3.6. Effect of BTH on Defense-Related Enzymes Activities. The activities of PPO, PAL, GLU, and CHT in the navel orange fruits challenge with *P. italicum* were initially increased and later were decreased (Figure 5). PPO activity in BTH treated fruits was significantly lower than its control on the sixth day in storage ($P < 0.05$). The activity of PAL in the BTH treatment significantly higher than that of control navel orange after four days of storage ($P < 0.05$), while GLU and CHT activities were significantly higher compared with respective control after 48 hours of storage ($P < 0.05$).

4. Discussion and Conclusion

BTH was one of the inducers that were known to have potential for application to induce SAR production in fruits and vegetables [9]. In this current study, 50 mg·L^{-1} BTH significantly reduced the decaying rate of navel fruits during 20°C storage ($P < 0.05$). BTH significantly inhibited weight loss of navel fruits ($P < 0.05$) and effectively maintained high SSC, VC, and TA contents at the later storage. So, BTH can reduce the decay rate and maintain quality of navel orange during storage. Further, we used *P. italicum*-inoculated navel orange fruits to investigate how BTH reduced the decay rate of navel orange fruits.

ROS is a signaling molecule regulating plant disease resistance against pathogens, and ROS accumulation can inhibit pathogen infection by inducing hypersensitive responses (HR) [22]. However, high level of ROS can cause oxidative damage, which might make tissues more susceptible to pathogens. Therefore, the existence of antioxidant enzymes and nonenzymatic antioxidants plays an important role in maintaining ROS at a nontoxic level. SOD, CAT, and POD are crucial antioxidant enzymes that efficiently scavenge ROS [23]. BTH remarkably raised the SOD and POD activities but had inhibitory effects on CAT level (Figure 4). Similar phenomena were also observed in chitosan treated navel oranges [24]. Increasing of SOD and POD activities by BTH may protect navel orange fruits cells against oxidative damage. Herein, BTH depicted significantly higher DPPH, ABTS scavenging activity, and reducing power (Figure 3), which is being considered as an additional mechanism involved in inhibiting the increase of disease incidence (Figure 1). Therefore, increasing of SOD and POD activities and the higher antioxidant capacity in BTH treated navel orange fruits help improve disease resistance capability.

The metabolic pathway of phenylpropanoid is quite essential and provides multiple substances especially phenolics, lignin, and hundreds of flavonoids directly with disease resistance. POD, PAL, and PPO are crucial enzymes taking part in phenylpropanoid pathway that leads to the biological synthesis of lignin [25]. Anam et al. [26] had found that an increase in POD and PPO activities in SA treated citrus fruits, and there was a clear relationship between increased POD and PPO activities and disease resistance against blue mould in citrus species. BTH significantly enhanced PAL activity and POD activity (Figures 3(a) and 2(d)), and it indicated that BTH induces navel orange fruits resistance to diseases via improving the phenylpropanoid metabolism pathway in this study. β -1, 3-glucanase (GLU) and chitinase (CHT) are two important plant pathogenesis-related proteins [27] which can hydrolyze alone or synergistically to destroy fungal cell wall structures and thus have direct antibacterial effects. In this study, BTH treatment significantly increased the GLU activity and CHT activity in the navel orange fruits challenge with *P. italicum*, and it indicated that enhancing disease resistance related enzyme activities was also one of the important mechanisms of BTH induced disease resistance in navel orange fruits.

In summary, our results demonstrated that BTH had promising effects on improving resistance against postharvest blue mold disease in navel orange. The elevated disease resistance in BTH treated orange fruits may be attributed to enhanced antioxidant capacity and antioxidant enzyme activities to the ROS homeostasis, and BTH induced the key enzymes activities in defense response. BTH may be a promising treatment for maintaining the quality and inhibiting blue mold of postharvest navel orange in the future.

Data Availability

All data used to support the findings of this study are included within the paper.

Conflicts of Interest

The authors declare that there are no conflicts of interest.

Acknowledgments

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Research Article

Citral Delays Postharvest Senescence of Kiwifruit by Enhancing Antioxidant Capacity under Cold Storage

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Citral is an aliphatic aldehyde extracted from citrus essential oil. The aim of the study was to examine how citral treatment affects the weight loss, firmness, respiration, and ripening index, as well as the antioxidant capacity of kiwifruit (*Actinidia chinensis cv.* 'Jinkui'). The citral treatment was seen to reduce the weight loss, softening, and fruit respiration compared to control fruits. Citral treatment also had an inhibitory effect on ripening index, $O_2^{\bullet-}$ production rate, and malondialdehyde (MDA) accumulation. The degradations of ascorbic acid (AsA) content, total flavonoids content (TFC), and total phenolics content (TPC) were also suppressed by citral. In contrast, citral treatment induces the activation of antioxidant enzyme system such as superoxide dismutase (SOD), peroxidase (POD), and catalase (CAT). Collectively, the results indicated that citral treatment delays postharvest senescence and prolongs storage life by enhancing antioxidant capacity in harvested kiwifruits. These findings suggest that citral has the potential to be used as a promising natural preservative for the extension of postharvest quality in harvested kiwifruit.

1. Introduction

Kiwifruit (*Actinidia deliciosa*, Actinidiaceae) is quite popular because of its taste and having high vitamin C and antioxidant capacities [1]. Kiwifruit is highly perishable and susceptible to pathogenic fungi causing significant economic losses due to the rotting of fruits during storage [2, 3]. Till date, various postharvest approaches such as edible coatings (alginate oligosaccharide, chitosan, and lacquer wax) [2, 4, 5], 24-epibrassinolide [6], oxalic acid [7, 8], aminoethoxyvinylglycine [9], methyl jasmonate [10], hydrogen sulfide [11, 12], and ozone treatment [13] have been implied and found to prolong the postharvest storability of kiwifruit. Postharvest application of natural phytochemicals, particularly plant essential oils, has been proved to be effective in controlling diseases and preservation of fruit quality [14, 15].

Recently, essential oils (EO) and their various constituents are used as natural fungicides for controlling postharvest fruits diseases and have been extensively studied. Essential oils from *Artemisia herba-alba*, *Mentha pulegium*, and *Syzygium aromaticum* had certain antimicrobial effects on *Penicillium expansum* population and blue mold development of apples [15]. Moreover, cinnamon essential oil displayed a strong antifungal inhibition on *Colletotrichum acutatum* of 'Hongyang' kiwifruit [16], and cassia oil has been used against *Alternaria alternata* (black spot rot) in tomatoes [17]. It is also shown that some essential oils could enhance antioxidant capacities of many horticultural products such as strawberries and blueberries [18, 19]. Citral, a terpenoid isolated from *Litsea cubeba* and *Cymbopogon citratus* (lemongrass), is widely used for food, beverages, and cosmetics industry [20]. Recently, citral has been reported to have significant antifungal effects on postharvest pathogens such as *P. italicum*, *P. digitatum*, and *Geotrichum citri aurantii* [21, 22], which effectively enhances the antioxidant capacities and disease resistance in some fruits [23, 24]. In

addition to these, citral has been used a promising plant-derived pesticide, which seems to be a surprising application to protect horticultural products from fungal attacks [21, 24]. Therefore, it remains a mighty possibility that citral could be used commercially for controlling postharvest fungal decay and enhancing disease resistance of horticultural products.

To develop a postharvest preservation using more applicable form of citral for kiwifruit, the effects of citral on the postharvest quality and on the response of reactive oxygen species (ROS) and antioxidant capacity shall also be studied in the present study.

2. Materials and Methods

2.1. Materials and Treatment. Healthy "Jinkui" kiwifruits (*Actinidia chinensis*) were harvested from a commercial orchard in Fengxin County (Jiangxi Province, China) with a commercial maturity [mean flesh firmness: 7.19 N; soluble solids content (SSC): 7.61 ± 0.16%]. The picked fruits with uniform size, color, and shape, free from any mechanical damage or pests were randomly divided into 39 groups comprising 10 fruits each. Eighteen groups were dipped in 0.6 μLmL^{-1} citral (prepared in 0.1% Tween 80 solution) for 15 min, and another eighteen groups immersed in 0.1% Tween 80 solution only, to serve as the controls. The citral concentration of 0.6 μLmL^{-1} was chosen based on the evaluation in a preliminary experiment using 0, 0.2, 0.4, 0.6, and 0.8 μLmL^{-1} citral solutions (data not shown). The remaining three groups were also sampled before treatment to determine physicochemical quality parameters at harvest time (0 day). Both citral-treated and control kiwifruits were air-dried (at 20 ± 1°C and 90 ± 5% relative humidity), packed in low-density polyethylene bags (39 × 30 cm, 0.03 mm thickness), and then stored at 0-1°C and 90 ± 5% RH for 90 d. Three replicates of 30 fruits for each treatment were taken at intervals of 15, 30, 45, 60, 75, and 90 d and sampled the flesh tissue for determination of flesh firmness, quality parameters, membrane permeability, and antioxidant enzyme activities.

2.2. Evaluation of Weight Loss, Fresh Firmness, Total Soluble Solids (TSS) and Titratable Acid (TA) Content, and Ripening Index. The weight of the same 10 kiwifruits from the control and citral-treated groups was measured immediately after days 0 and at the different sampling times (15, 30, 45, 60, 75, and 90 d). Weight loss is defined as the ratio of final sample weights to the harvested sample weights and expressed as percentage (%).

Fresh firmness of kiwifruit was evaluated on two opposite positions around the equator of 10 fruits (skin removed) using a texture analyzer (TAXT Plus, Stable Micro Systems, Surrey, UK) equipped with a 2 mm diameter probe and expressed as N.

The SSC in kiwifruit juice was recorded on a digital Brix-meter (RA-250WE, KYOTO, Tokyo, Japan) and specified in °Brix. TA content was analyzed by the titration with 0.1 M NaOH and expressed in terms of the percentage of citric acid following the method described by Horak et al. [25].

Ripening index was calculated by simply taking the ratio of SSC and TA (SSC : TA).

2.3. Respiration Rate, $O_2^{\bullet-}$ Production Rate, and Malondialdehyde (MDA) Content. The respiration rate was determined according to a method described by Hu et al. [5]. The respiration rate was analyzed by an infrared CO_2 fruit breathing instrument (GHX-3051H, Shanghai, China) and recorded as mg CO_2 kg^{-1} h^{-1}.

For $O_2^{\bullet-}$ production rate assay, 2.0 g frozen tissue powder was homogenized with 5 mL ice-cooled 50 mM phosphate buffer (pH 7.8) containing 1 mM EDTA, 0.3% (v/v) Triton X-100, and 2% (w/v) polyvinyl pyrrolidone (PVP) and then centrifuged at 10 000 × g for 20 min at 4°C. 1 mL of the supernatant was mixed with 1 mL of 50 mM phosphate buffer (pH 7.8) and 1 mL of 1 mM hydroxylammonium hydrochloride and incubated for 1 h at 25°C. 2 mL of the mixed solution comprised of 17 mM 4-amino-benzenesulfonic acid and 7 mM naphthylamine (1 : 1) was then added into the mixture, followed by incubation for 20 min at 25°C. The absorbance of the mixture was recorded at 530 nm. The unit of $O_2^{\bullet-}$ production rate was expressed as nmo1 g^{-1} min^{-1}.

MDA content was measured using the thiobarbituric acid colorimetric method according to the procedure of Nie et al. [26]. Frozen flesh tissue was milled to a powder with liquid nitrogen, and 1.0 g of powder was homogenized in 5 mL 10% (*m/v*) trichloroacetic acid (TCA) and then centrifuged at 10 000 × g for 15 min. 2 mL of the obtained supernatant was mixed with 2 mL of 0.67% thiobarbituric acid, followed by boiling water bath for 15 min. The mixture was then cooled and the absorbance of the supernatant was determined at 450, 532, and 600 nm. Each treatment was conducted in triplicate.

2.4. Assays for Ascorbic Acid (AsA) Content, Total Phenolics Content (TPC), and Total Flavonoids Content (TFC). Ascorbic acid (AsA) content of kiwifruit flesh was acquired by the titration with a standard solution of 2,6-dichlorophenol indophenol [26] and expressed in terms of milligram on a fresh weight basis (g kg^{-1}).

The levels of TPC and TFC were measured following the method described by Jiang et al. [27]. Both TPC and TFC were represented as milligram of gallic acid and rutin equivalent per gram of frozen sample (mg kg^{-1}), respectively.

2.5. Assay of 2,2-Diphenyl-1-picrylhydrazyl (DPPH) Scavenging Capacity and Ferric Reducing Antioxidant Power (FRAP). The DPPH scavenging capacity was carried out according to a previous method described by Horak et al. [25]. 100 μL of extracted juice was added to 1.9 mL of 0.1 mM DPPH solution (dissolved in ethanol) and then left in the dark at 25°C for 30 min. The absorbance of the tested sample was monitored at 515 nm. DPPH free radical scavenging capacity was calculated and expressed as a percentage using the following equation:

$$\text{Scavenging capacity } (\%) = \frac{A_0 - A_t}{A_0} \times 100, \quad (1)$$

where A_0 and A_t are the absorbance of the control (without extracted juice) and kiwifruit juice added after 30 min of reaction incubation, respectively. The unit of DPPH scavenging capacity was expressed as %.

The FRAP assay was carried out according to the method of Benzie and Strain [28] with a slight modification. 0.5 mL of extracted juice was added to 3 mL of fresh FRAP reagent [0.3 M acetate buffer (pH 3.6), 10 mM 2, 4, 6-tripyridyl-s-triazine (dissolved in 40 mM HCl), and 20 mM $FeCl_3$ $6H_2O$ (10 : 1 : 1, v/v/v)] and then left in the dark at 37°C for 30 min. The absorbance of the mixture was determined at 700 nm using distilled H_2O as the blank solution.

2.6. Measurement of Antioxidant Enzyme Activities. Fresh tissue (2.0 g) from 10 fruits was homogenized with 8 mL of ice-cold 50 mM phosphate buffer solution (pH 7.0) containing 2% polyvinyl pyrrolidone and 5 mM dithiothreitol and then centrifuged at 12,000 × g for 20 min at 4°C. The obtained supernatant was used to determine the activity of the antioxidant enzymes.

Superoxide dismutase (SOD, EC 1.15.1.1) activity was measured using a SOD test kit (No : A001-1-2; Nanjing Jiancheng Bioengineering Institute, Nanjing, China) with the hydroxylamine method. The absorbance of the tested sample was monitored at 550 nm. The quantity of enzyme that controlled 50% of nitroblue tetrazolium (NBT) photoreduction per hour was defined as one unit of SOD activity.

Catalase (CAT, EC 1.11.1.6) activity was determined by measuring the decline of absorbance at 240 nm (Shimadzu UV-2600, Tokyo, Japan) due to the decomposition of H_2O_2. The collected supernatant (200 μL) was mixed with 2.8 mL of 20 mM H_2O_2 (prepared in 50 mM phosphate buffer solution). The quantity of CAT that changed the absorbance of the reaction solution by 0.01 at 240 nm per minute was defined as one unit of CAT activity.

Peroxidase (POD, EC 1.11.1.7) activity was assayed using the guaiacol method reported by our previous studies [3, 26]. The reaction system consisted of 50 μL of enzyme solution, 3.0 mL of 25 mM guaiacol (prepared in 50 mM phosphate buffer solution), and 200 μL of 0.5 mM H_2O_2. The increase in absorbance at 470 nm was determined at 25°C for 5 min. The quantity of POD that changed the absorbance of the reaction solution by 1 at 470 nm per minute was defined as one unit of POD activity.

2.7. Statistical Analysis. All data were described as the means of three replicated samples ± standard error (S.E.), and analyses were carried out by using SPSS version 20.0 (SPSS Inc., Chicago, IL, USA). *P* value less than 0.05 ($p < 0.05$) was considered as significant. Origin version 8.5 (Microcal Software, Northampton, MA) was applied to create figures.

3. Results

3.1. Change of Fruit Weight Loss and Firmness after Citral Treatment. Fruit weight loss in both control and citral-treated fruits depicted a clear increase during storage (Figure 1). However, the weight loss in the fruit treated with citral maintained a low level compared with the control fruits. By the end of cold storage period of 90 d, the weight loss in the citral-treated kiwifruit was lower (18.9%, $p < 0.05$) than that in the control fruits.

Flesh firmness in the control and citral-treated kiwifruit decreased throughout the storage period (Figure 2). Further comparison indicated that there was a higher level of flesh firmness in the citral-treated kiwifruit than that in the control kiwifruit under storage, with a remarkable ($p < 0.05$) difference during the entire storage period.

3.2. Effects of Citral Treatment on Postharvest Senescence Related Parameters of Harvested Kiwifruit. The rate of fruit respiration in both control and citral-treated fruits sharply increased in the first 15 d after treatment, followed by a sharp drop during the rest of storage periods (Figure 3(a)). The peak of respiration rate in the control kiwifruit was 1.56-folds higher than that of the citral-treated kiwifruits. Respiration rate in the citral-treated kiwifruit was significantly ($p < 0.05$) lower than that in the controls during the entire storage periods (except for 75 d).

Ripening index of the control and citral-treated kiwifruits increased over the period of cold storage (Figure 3(b)). Citral treatment delayed the increase of ripening index in kiwifruits. There was a lower level of ripening index in the citral-treated kiwifruit than that in the control fruits during storage period. The ripening index in the citral-treated kiwifruit was lower (12.7%) than that of the control fruit at day 90.

The alterations of $O_2^{\bullet-}$ production rate in the control and citral-treated kiwifruit flesh were increased at the early storage (0 d to 45 d) but afterwards declined (Figure 3(c)). Compared to the control fruits, the $O_2^{\bullet-}$ production rate in the citral-treated kiwifruit retained lower levels during storage, with a significant ($p < 0.05$) difference during the middle and later periods of postharvest storage (45 d to 90 d).

MDA is the final product in the peroxidation of membrane lipids, which can produce the Schiff alkali, destroying membrane integrity to promote membrane leakage. The MDA content in both control and citral-treated fruits increased with the prolongation of the storage time (Figure 3(d)). Citral treatment significantly ($p < 0.05$) delayed the accumulation of MDA content. The MDA content in the citral-treated kiwifruits was lower than that in the control fruits. There was a decrease (15.7%) in the citral-treated kiwifruit at the end of storage period. It is worth mentioning that there was a prominent difference ($p < 0.05$ or $p < 0.01$) between the control and citral-treated kiwifruits during the entire storage period (Figure 3(d)).

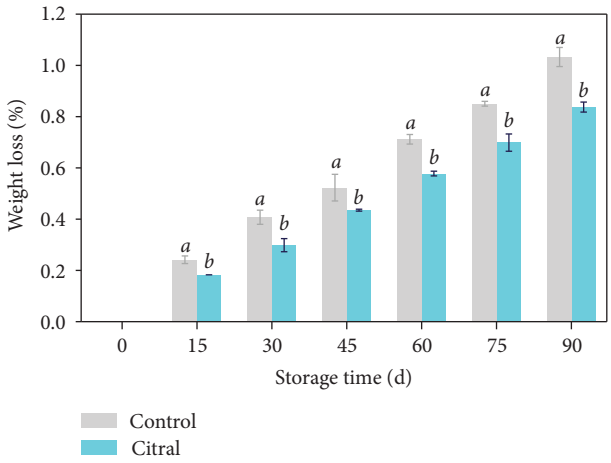

Figure 1: Effect of citral treatment on weight loss of harvested kiwifruit stored at 0-1°C for 90 d. Vertical bars represent the mean ± standard error (S.E., $n = 3$). Letters indicate the statistical differences according to the independent samples t-test ($p < 0.05$) on each storage day.

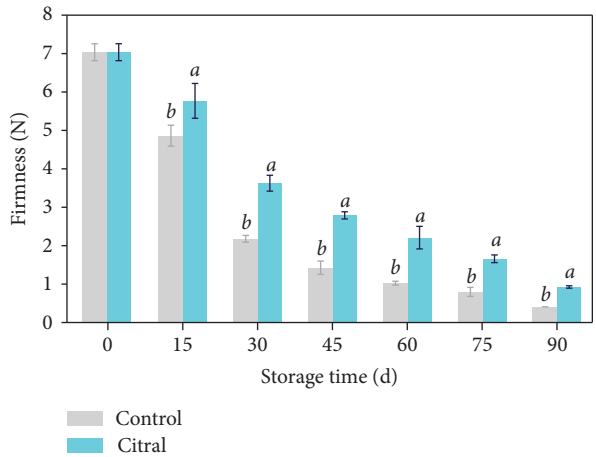

Figure 2: Effect of citral treatment on fresh firmness of harvested kiwifruit stored at 0-1°C for 90 d. Vertical bars represent the mean ± standard error (S.E., $n = 3$). Letters indicate the statistical differences according to the independent samples t-test ($p < 0.05$) on each storage day.

3.3. Effects of Citral Treatment on Antioxidant Capacity of Harvested Kiwifruit. The AsA content in the flesh decreased gradually as storage time prolonged in both control and citral-treated kiwifruits (Figure 4(a)), with the citral-treated fruit exhibiting a slower decrease of AsA content. Statistical analysis showed that AsA content in the citral-treated fruits was significantly ($p < 0.05$) higher than that of the control fruits throughout the cold storage period.

By increasing in storage time, the TPC was reduced in both control and citral-treated kiwifruits. However, this decline of TPC was less prominent in kiwifruits subjected to the postharvest application of citral (Figure 4(b)). Overall results suggested that citral treatment exerted a significant inhibition on the degradation of TFC compared to its control fruits after 30 d of storage.

The TFC depicted the same trend as TPC, where the former in both control and citral-treated kiwifruits decreased over the period of cold storage (Figure 4(c)). Citral treatment prevented the decline of TFC in kiwifruit flesh, which maintained higher level of TFC in comparison with the control sample (Figure 4(c)). The TFC in the citral-treated fruits was significantly ($p < 0.05$) higher than that of the control fruits during the whole of storage period.

The DPPH scavenging capacity was decreased gradually during storage time in both control and citral-treated fruits (Figure 4(d)). Citral treatment significantly ($p < 0.05$) inhibited the decrease of DPPH scavenging capacity during the middle and later period of storage (45 d to 90 d). Kiwifruits treated with citral exhibited a remarkable higher levels of DPPH scavenging capacity than the control fruits.

The FRAP showed a similar pattern with DPPH scavenging capacity in both control and citral-treated fruits during storage time (Figure 4(e)). In addition, the FRAP in the citral-treated fruits was significantly higher ($p < 0.05$) than that in the control fruits. Moreover, there was a prominent difference between the control and citral-treated kiwifruits during the entire storage period (except for 60 d).

3.4. Effects of Citral Treatment on SOD, CAT, and POD Activities of Harvested Kiwifruit. Antioxidant enzymes such as SOD, CAT, and POD serve an indispensable role in scavenging ROS in horticultural fruits. Both SOD and CAT activities exhibited similar tendency; i.e., they gradually increased during the initial storage of 45 d and then dropped rapidly. The activities of SOD and CAT in the citral-treated fruits were consistently higher ($p < 0.05$) than those in the control fruits, and there was a prominent difference between the control and citral-treated kiwifruits during the entire storage period (Figures 5(a) and 5(b)). The POD activity in both control and citral-treated fruits increased for 0–75 d and then declined in the following storage period. The POD activity significantly increased by citral treatment after treatment, and even at 75 d the POD levels still remained 1.15 times higher than those in the control fruits (Figure 5(c)). It is worth mentioning that an obvious significance was detected between the control and citral-treated groups during the whole storage period up to 90 d (except for 45 d).

4. Discussion

The application of plant essential oils could effectively control postharvest fruit diseases by enhancing the antioxidant ability of many horticultural products that have extensively been reported recently [29, 30]. It is widely accepted that the important fruit quality parameters evaluating kiwifruit acceptability by consumers include fruit weight, firmness, color, and fragrance [31, 32]. However, fruit quality of kiwifruit declined rapidly in both storage period and shelf life. Consequently, help to develop an effective preservations method for maintaining postharvest fruit quality of kiwifruits is of great interest [6, 32]. However, there is hardly any study that describes the effect of citral

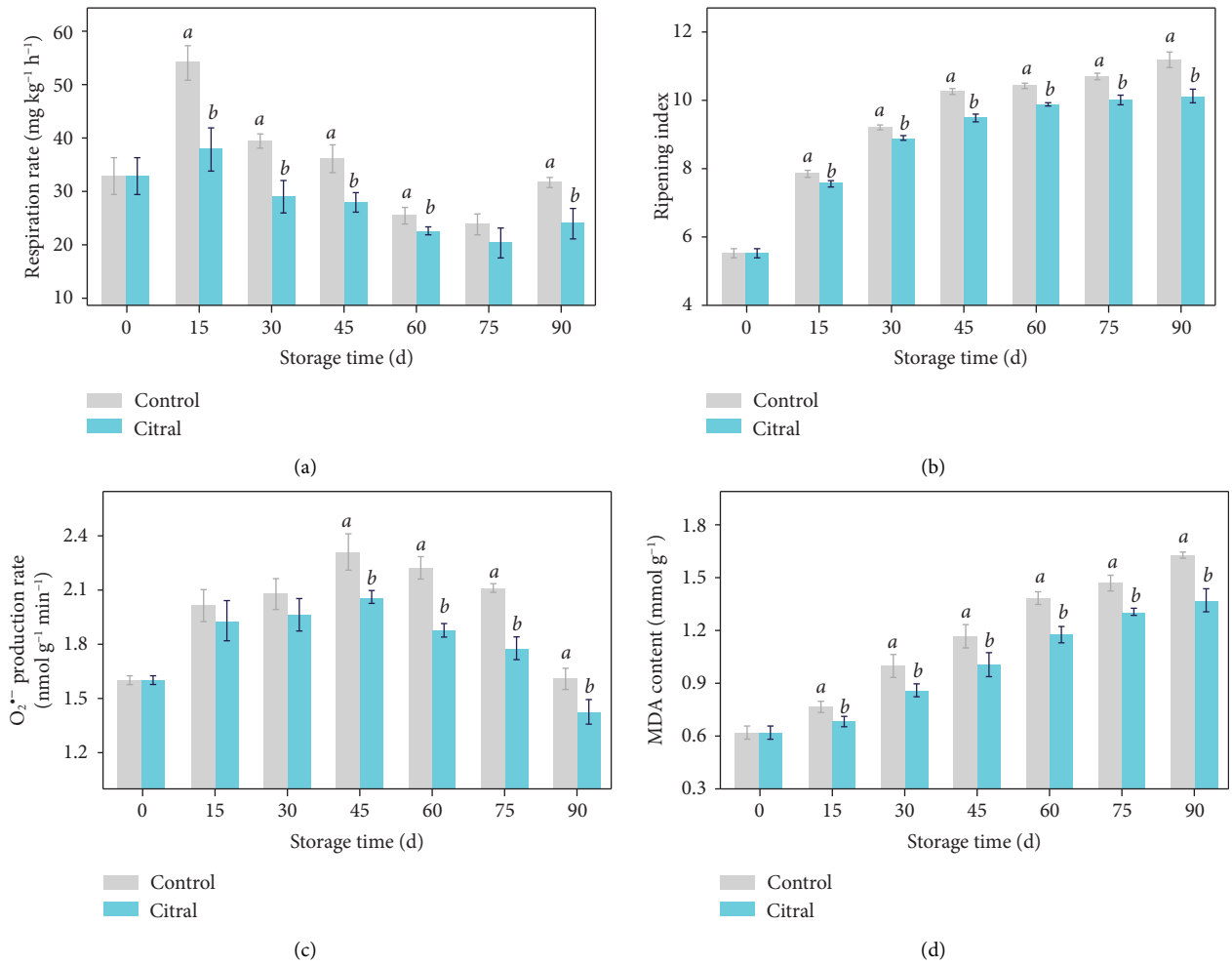

FIGURE 3: Effect of citral treatment on respiration rate, (a) ripening index, (b) $O_2^{\bullet-}$ production rate, (c) and MDA content (d) of harvested kiwifruit stored at 0-1°C for 90 d. Vertical bars represent the mean ± standard error (S.E., $n = 3$). Letters indicate the statistical differences according to the independent samples t-test ($p < 0.05$) on each storage day.

treatment on kiwifruit postharvest quality during cold storage or analyzes different quality parameters related to senescence stress. The current study examined that kiwifruits treated with citral exhibited lower ripening index while maintaining the antioxidant capacity in comparison with the control fruits.

For the senescence related parameters, citral treatment reduced changes on weight loss, firmness, respiration rate, and ripening index throughout the storage period of kiwifruits. In the current study, an increase in ripening index showed the process of kiwifruit ripening and senescence after harvest, which was significantly delayed in the citral-treated kiwifruits (Figure 3). A similar response has been reported following 5 μmol L^{-1} 24-epibrassinolide treatment during storage in kiwifruit, by Wang et al. [6].

Ascorbic acid (AsA) could protect kiwifruits from the oxidative damage due to higher scavenging capacity for various ROS through the ascorbate peroxidase reaction [26]. A huge loss of AsA content occurred in the control treatment followed by citral treatment quite similar to a recent study where citral was applied during postharvest storage that declined AsA content in tomato during cold storage [24]. The results in the present study indicated that citral treatment could delay the oxidation process in kiwifruits by maintaining higher levels of AsA content than the control fruits after the harvest.

Both phenolics and flavonoids compounds are effective antioxidants that contribute to eliminating ROS. The current study revealed that citral treatment maintained the higher levels of both TPC and TFC in comparison with control kiwifruits. This is further accompanied with higher levels of DPPH scavenging capacity and FRAP, thereby reducing the damage caused by the ROS. These results indicated that both TPC and TFC were positively correlated with DPPH scavenging capacity and FRAP and negatively correlated with the ROS damage in kiwifruits. Several studies have also reported the inalienable relationship between phenolics content and their antioxidant capacity in postharvest fruits. Jin et al. [33] demonstrated that higher antioxidant capacity in Chinese bayberries treated with linalool and cinnamaldehyde was mainly linked to the higher phenolics contents. The high levels of antioxidant contents may delay

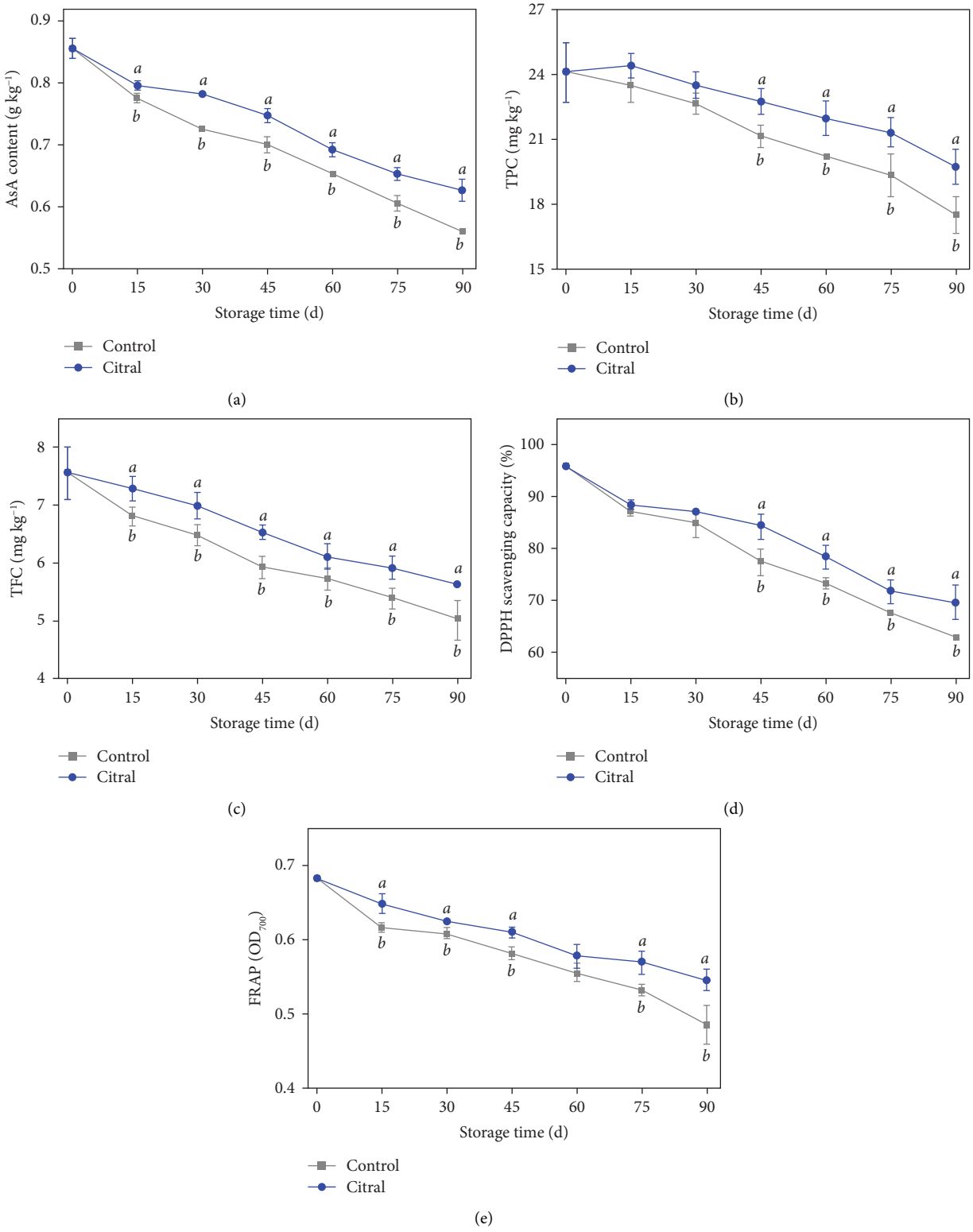

Figure 4: Effect of citral treatment on AsA content, (a) total phenolics content (TPC), (b) total flavonoids content (TFC), (c) 2,2-diphenyl-1-picrylhydrazyl (DPPH) scavenging capacity, (d) and ferric reducing antioxidant power (FRAP) (e) of harvested kiwifruit stored at 0-1°C for 90 d. Vertical bars represent the mean ± standard error (S.E., $n = 3$). Letters indicate the statistical differences according to the independent samples t-test ($p < 0.05$) on each storage day.

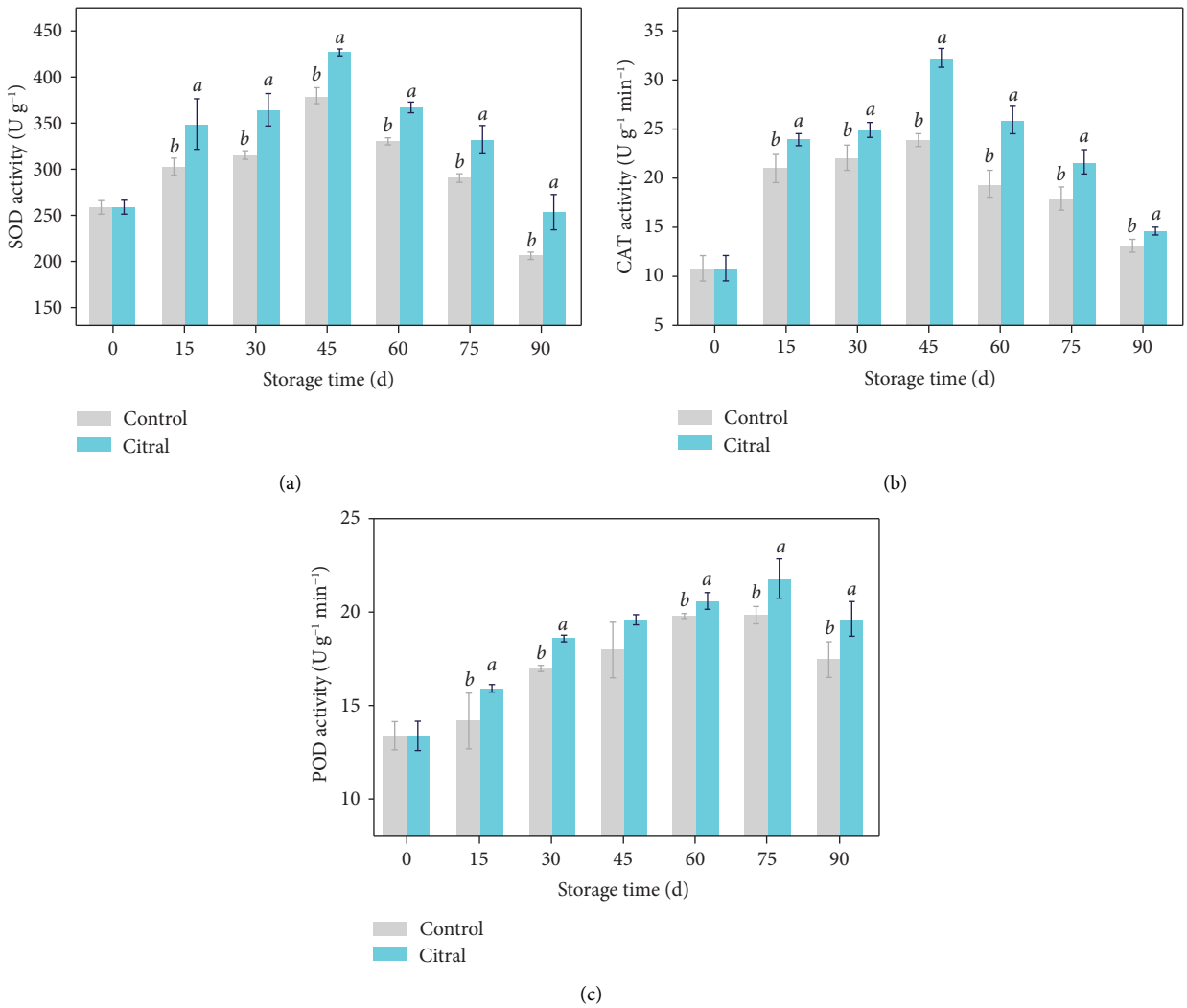

Figure 5: Effect of citral treatment on the activities of superoxide dismutase (SOD), (a) catalase (CAT), (b) and peroxidase (POD) (c) of harvested kiwifruit stored at 0-1°C for 90 d. Vertical bars represent the mean ± standard error (S.E., $n = 3$). Letters indicate the statistical differences according to the independent samples t-test ($p < 0.05$) on each storage day.

the physiological deterioration and hence postpone the fruit senescence.

Respiration rate is an important parameter for postharvest fruit metabolic activity and quality assessment. The findings herein showed that citral-treated fruits exhibit lower respiration rates and hence offer better storage quality. In addition, MDA content is another indicator for cellular oxidative damages. Lipid peroxidation is a major reason for membrane integrity damages during fruits senescence [34]. High MDA content could cause the damage in cellular membrane systems, which further led to the softening of kiwifruits. The current results indicated that citral treatment could reduce the MDA accumulation in kiwifruits to resist the senescence induced ROS damage, thereby delaying postharvest senescence in kiwifruits. Furthermore, similar findings in kiwifruits have been observed when treated with 24-epibrassinolide [6].

The ripening process in plant has been seen as an oxidative phenomenon accompanied by burdens of ROS (H_2O_2 and $O_2^{\bullet-}$) [35]. The ROS burst may result in oxidative stress mediated damages to the plant tissue specifically the membranes. Herein, the citral-treated kiwifruits had significantly lower level of $O_2^{\bullet-}$ production rate, which suggested that citral treatment was better for protecting kiwifruit against the ROS-mediated oxidative damages. The elimination of ROS is linked with the activities of antioxidant enzymes such as SOD, CAT, and POD in fruits and vegetables [6, 13, 36]. SOD plays a key role in the dismutation of $O_2^{\bullet-}$ into H_2O_2, while H_2O_2 is obliterated by CAT which is indispensable for the detoxification of ROS [11]. POD is the key enzyme in the biosynthesis and oxidation of phenolics compounds [37]. Therefore, higher antioxidant enzyme activities and their coordinated actions might be the underlying mechanism for reducing lipid peroxidation and delaying senescence in horticultural fruits [35, 36]. The exogenous treatment of methyl jasmonate (MeJA) help induce the increased activities of POD and SOD, thereby reducing oxidative damage and enhancing disease resistance in kiwifruit [3].

Meanwhile, alginate oligosaccharide (AOS) treatment elevated SOD and CAT activities, which contributed to enhancing abiotic stress resistance and delaying postharvest senescence in kiwifruits [2]. Herein, citral treatment induces higher levels of SOD, CAT, and POD activities compared with the respective controls. Thus, the findings suggested that citral treatment effectively induces the increases in antioxidant enzymes activities and helps coordinate the balance of ROS level in kiwifruits. The link between these antioxidant enzymes might be the major factor that triggers the oxidation resistance, thereby maintaining postharvest quality and prolonging storage time of kiwifruits.

5. Conclusions

The beneficial effects of citral treatment on postharvest quality and antioxidant capacity of 'Jinkui' kiwifruit were investigated in the present study. The study revealed that citral treatment has significantly decreased fruit weight loss, delayed softening, alleviated senescence progress, and maintained higher levels of antioxidant contents that help boost antioxidant enzyme activities in comparison with the respective control fruits during storage. The alleviation of postharvest senescence in kiwifruits treated with citral might be attributed to maintaining antioxidant capacity, higher levels of AsA content, TPC, and TFC, contributing to enhanced DPPH scavenging capacity and FRAP. Furthermore, weight loss, respiration rate, and ripening index were lower in the citral-treated kiwifruits than those in the control fruits, which have been dedicated to less consumptions of nutrients and have got extended storage time. Therefore, it is concluded that citral treatment could serve as a potential and feasible preservation method for delaying postharvest senescence decay and maintaining antioxidant capacity in 'Jinkui' kiwifruit.

Data Availability

All the statistical data used to support the findings of this study are included within the paper.

Conflicts of Interest

The authors declare no conflicts of interest.

Acknowledgments

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