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Phenolic Extracts from Myrtaceae Leaves Improve the Quality and Shelf-life of Pacific Whiteleg Shrimp

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Abstract

This study aimed to quantify the phenolic contents and the antioxidant capacities of leaf extract powders from three Myrtaceae plants, namely *Cleistocalyx operculatus* (Vietnamese name: Vối), *Psidium guajava* (Ổi), and *Rhodomyrtus tomentosa* (Sim), investigate their inhibitive activities on the growth of bacteria isolated from spoiled shrimps, and evaluate their values as preservative agents in the cold storage of Pacific whiteleg shrimps (*Penaeus vannamei*). The extract leaf powders from *R. tomentosa*, *C. operculatus*, and *P. guajava* had high phenolic contents of 281.25, 282.36, and 349.51 mg gallic acid equivalent/g leaf extract, respectively. Among the three plants, the extract powders of *R. tomentosa* and *P. guajava* leaves had the highest antioxidant capacities (about 4 mmol Trolox equivalent (TE)/g) followed by the one of *C. operculatus* (2.85 mmol TE/g). These three extract powders showed significant antibacterial activities against the seven bacteria isolated from cold-stored spoiled whiteleg shrimps with the inhibition zones ranging from 0.33 to 19.67mm depending on the extract concentration. Among the three leaf extracts, the one from *P. guajava* leaves showed the highest inhibitive activity. *Aeromonas* sp2 was the most sensible to Myrtaceae leaf extracts while *Aeromonas* sp4 was the least affected strain. All the extracts showed high inhibitive activities against melanosis, volatile nitrogen-containing compounds formation, lipid oxidation, and microbial growth in stored shrimps, thereby prolonging the shelf-life of the shrimps. The results suggested the potential application of these three Myrtaceae plants as sources of antioxidant and antimicrobial agents in the cold storage of shrimps.

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Keywords

Phenolic compounds, antioxidant capacity, antimicrobial activity, cold storage.

Introduction

Pacific whiteleg shrimp (*Penaeus vannamei*) is an economically important crustacean in Vietnam thanks to its high and increasing production (480.0 and 632.3 thousand tons in 2019 and 2020, respectively) (Vietnamese Directorate of Fisheries, 2019; 2020). This high-value crustacean is very perishable, and their quality and freshness rapidly decrease during post-mortem handling and storage due to spoilage bacteria contamination, hydrolysis catalyzed by endogenous/exogenous protease, and melanosis (Manheem *et al.*, 2013). Indeed, the number of spoilage bacteria increases rapidly and this leads to the acceleration of protein decomposition and off-odour of shrimps (Qian *et al.*, 2013). According to da Silva *et al.* (2015), *Pseudomonas* sp. and *Aeromonas* sp. have been found as the main strains of spoiling shrimps in cold conditions. Besides the negative impacts of bacteria, melanosis also reduces the quality of shrimps during storage. This biochemical reaction negatively affects the sensorial quality of shrimps and results in reducing their shelf-life.

In order to inhibit melanosis and bacterial growth in stored shrimps, chemical agents such as sulfite derivatives and phosphates have been used (Gómez-Guillén *et al.*, 2005). However, the use of synthetic compounds is often limited due to their side effects. For example, metabisulphite (E223) has been considered to be responsible for asthma attacks and the cause of serious allergic reactions in people who are exposed to it (Collins-Williams *et al.*, 1983). Because of the potential health hazards of chemical additives, natural products, especially natural antioxidants and antimicrobial agents, have been intensively examined as safe alternatives to synthetic compounds.

Phenolic compounds are known as the most common secondary plant metabolites (Alara *et al.*, 2021) with about 10,000 phenolic structures currently known (Kennedy & Wightman, 2011). In food technology, phenolic compounds can be considered as natural preservatives resulting from their important biological properties including antioxidant and antimicrobial activities.

Research has been done on the potential application of natural extracts to extend the shelf-life of seafood. According to Miraglia *et al.* (2020), a phenolic extract derived from olive vegetation water at a concentration of 2 g L^{-1} led to a significant reduction in total volatile basic nitrogen, thiobarbituric reactive substances values, bacterial growth, and black spot formation on shells in deep-water rose shrimps (*Parapenaeus longirostris*) stored at 2^{o}C. In another study, treating Indian white prawns (*Fenneropenaeus indicus*) with green tea and amla (*Phyllanthus emblica* Linn) extracts significantly lowered some biochemical indices, aerobic bacterial counts, and polyphenol oxidase activity during 28 days of chilled storage compared to untreated samples (Firdous *et al.*, 2020).

Although the use of phenolic extracts as natural preservatives for shrimp storage have been reported for several plants around the world, the benefits of phenolic extracts from Vietnamese plants is still limited. Our recent screening study indicated that among 12 potential Vietnamese medicinal plants, three Myrtaceae plants, namely *Cleistocalyx operculatus* (Vối), *Psidium guajava* (Ổi), and *Rhodomyrtus tomentosa* (Sim), had the highest antioxidant and antimicrobial activities (data not published). Hence, in this study, we aimed to evaluate the preservative potential of leaf extract powders from these three Myrtaceae plants during the cold storage of Pacific whiteleg shrimps. In order to achieve this goal, firstly, the antibacterial activities of the leaf extracts against seven bacteria isolated from cold-stored spoiled whiteleg shrimps were determined. Then, shrimps were treated with different concentrations of the extracts and stored under cold conditions. During storage, biochemical and microorganism changes in the shrimps were followed.

Methodology

Chemicals

Gallic acid, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylicacid (Trolox), diphenyl-1-picrylhydrazyl (DPPH) radical,

thiobarbituric acid (TBA), and antifoam 204 were purchased from Sigma-Aldrich (St. Louis, MO). Malondialdehyde bis was produced by Chemservice (West Chester, PA). Ethanol, methanol, glucose, boric acid, sodium hydroxide, sodium metabisulfite (SMS), potassium chloride, chlorhydric acid, calcium chloride, sodium carbonate, sodium chloride, dimethyl sulfoxide (DMSO), and sodium hypochlorite were of analytical grade and were produced by the Xilong Company (Guangdong, China). Agar, yeast extract and peptone, and perchloric acid were made by the Hai Long Company (Hai Duong, Vietnam), Titan Biotech Limited (Delhi, India), and Guangzhou Fischer Chemical (Guangzhou, China), respectively. Tryptic Soya Agar medium and tryptone were purchased from Scharlau (Spain).

Leaf collection and preparation of the extract powders

Leaves of three Myrtaceae plants, namely *C. operculatus*, *P. guajava*, and *R. tomentosa*, were harvested in Hanoi and Quang Ninh provinces in August 2016. Plants were identified at the species level as *R. tomentosa*, *C. operculatus*, and *P. guajava* by morphologic comparisons of leaves, buds, flowers, and fruits based on the descriptions of Pham Hoang Ho (2003). All leaves were first washed with tap water and rinsed in distilled water. They were then dried in an oven at 50^{o}C for 15h and ground into fine powders with particle sizes less than 0.3mm by using a Tecator Cyclotec 1093 Sample Mill (Foss A/S, Denmark). The leaf powders were stored in airtight containers and kept at 4^{o}C until further use.

Extraction was done with an aqueous ethanol solution and a solid/liquid ratio of 1/10. For *C. operculatus* and *P. guajava* leaves, 100g leaf powder was added to 1L of ethanol 70% (*v/v*). Extraction was carried out for 30min at room temperature. For *R. tomentosa* leaves, the extraction was done by using the conditions optimised by Ha *et al.* (2016) (ethanol 40% (*v/v*) at 90^{o}C for 15min). After extraction, the mixtures were centrifuged at 3642x*g* for 10min at 4^{o}C. The supernatants were then concentrated by using a R210 rotary evaporator (Buchi, Switzerland) under reduced pressure at 40^{o}C and dried by using a Modulyo freeze dryer (Thermo Fisher Scientific, MA). The extract powders were stored at 4^{o}C in plastic bags for further studies.

Bacteria

Seven bacterial strains were isolated from cold-stored spoiled whiteleg shrimps at the Faculty of Aquaculture (Vietnam National University of Agriculture - VNUA). They were characterized as members of the *Aeromonas* (six strains named *Aeromonas* sp1 - 6) and *Pseudomonas* (one strain named *Pseudomonas* sp) genera basing on morphological observations (cell form, colony form, and color), and physiological (mobility) and biochemical tests (Gram staining; oxidase, catalase, amylase tests; fermentation of glucose, maltose, and lactose; H_2S production; growth on Nutrient Agar and Thiosulfate-Citrate-Bile Salts-Sucrose media).

Determination of the total phenolic content

The total phenolic contents of the extract powders were determined by using the Folin-Ciocalteu reagent as described by Singleton & Rossi (1965). First, the extract powder was dissolved in ethanol 70% (*v/v*) to obtain a concentration of 50 mg mL^{-1}. An aliquot of 0.5mL of the diluted extract was mixed with 0.25µL of 1 N Folin-Ciocalteu reagent. The mixture was shaken and allowed to react over a period of 5min. Then, 1.25mL of 7.5% Na_2CO_3 was added and the mixture was mixed thoroughly. After incubation for 30min, the absorbance of the reaction solution was measured at 755nm by using a UV-1800 UV-VIS spectrophotometer (Shimadzu, Japan). Gallic acid was used as the standard for building the calibration curve. The total phenolic content of each extract powder was expressed as mg of gallic acid equivalents per gram extract (mg GAE/g).

Determination of the antioxidant capacity

The antioxidant capacities of the extract powders were measured by the DPPH radical scavenging test according to Duan *et al.* (2007) with minor modifications. Briefly, 100µL of the diluted extract was added to 2,900µL of 0.1mM

free radical DPPH in methanol. The mixture was shaken vigorously and incubated at room temperature for 30min in the dark. The absorbance of the resulting solution was then measured at 517nm. The control contained methanol instead of the sample solution. The inhibition of the DPPH radical by the sample was calculated according to the following equation:

DPPH-scavenging activity (%) = $100*(A_{control} - A_{sample})/ A_{control}$.

Trolox was used as the standard. The antioxidant capacity was expressed in mmol Trolox equivalents per gram extract (mmol TE/g).

In vitro antimicrobial activity test

The antimicrobial activities of the three extract powders were measured by using the agar well diffusion method designed by Dang *et al.* (2019) with small modifications. Firstly, seven bacterial strains isolated from cold-stored spoiled whiteleg shrimps were taken from stocks and activated by being streaked on Petri dishes containing Tryptic Soya Agar (for *Aeromonas* sp.) or Luria-Bertani (for *Pseudomonas* sp.) media. The dishes were incubated at 37°C in an incubator for 24h. After incubation, single colonies of each strain were selected, individually transferred to corresponding liquid media, and then incubated at 37°C in a shaking incubator (200rpm) for 15 hours to reach the stationary growth phase.

One hundred microliters of each bacterial culture, adjusted to a microorganism concentration of 10^8 colony-forming units per mL (CFU/mL), was spread on a Petri dish containing 25mL of their specific media. The leaf extract powders were dissolved in 10% DMSO to make concentrations of 10, 20, 30, 40, 50, and 75 mg mL^{-1} for the antibacterial activity test. One hundred microliters of each extract solution at different concentrations were added to the test wells, while the control well consisted of 100µL of 10% DMSO. The Petri dishes were kept at 4°C for 2 hours and then incubated at 37°C for 18 hours. After incubation, the zone of inhibition was measured as the difference between the diameter of the inhibition zone surrounding the well and the diameter of the well (8mm).

Shrimp treatments

About 20kg of fresh Pacific whiteleg shrimps, 35-45 shrimps kg^{-1} in size, were purchased from a supermarket in Hanoi. The shrimps were kept in ice with a shrimps/ice ratio of 1:2 (*w/w*) and transported to the Faculty of Food Science and Technology, VNUA. Upon arrival, the shrimps were washed in cold, sterile water before treatment.

The three leaf extract powders were dissolved in distilled water. Whiteleg shrimps were treated with one of three treatments: 0% (control), 0.25%, and 0.5% (w/v). Briefly, whiteleg shrimps were immersed into one of the plant extract solutions with a shrimps/solution ratio of 1:2 (*w/v*) at 4°C for 30min. For the controls, the whiteleg shrimps were soaked in 1.25% SMS (positive control) or were treated with distilled water (negative control). After immersion, the whiteleg shrimps were drained at ambient temperature for 3min. Five shrimps were placed in a tray with plastic cover. All the trays were placed in a refrigerator at a temperature of 1-4°C. Samples were taken every two days over a 12-day period. At the sampling time, three trays from each treatment were taken and the shrimps were analyzed for firmness, melanosis assessment, pH, total volatile bases nitrogen (TVB-N) value, thiobarbituric acid reactive substances (TBARS) value, and total microbial count. A total of 168 trays (= 8 treatments*7 sampling times*3 biological replications) were prepared and stored.

Shrimp quality analysis

Melanosis analysis: Melanosis of whiteleg shrimps was evaluated through visual inspection by six trained panelists using the 10-point scoring method described by Nirmal & Benjakul (2011). Samples were analyzed once.

Texture analysis: The texture analysis of shrimp muscle was performed by using an Agrosta 14 Digital Firmness Tester (Agro Technologies, France). An eight-mm diameter flattened cylinder was used. A constant penetration depth of 2mm was applied on the whiteleg shrimp muscle and the force was recorded. Measurements were taken on

three different parts of three individual shrimps per sample.

pH measurement: The pH of shrimp muscle was determined using the protocol described by Tsironi *et al.* (2009) with small modifications. Briefly, 5g of shrimp flesh was transferred to a plastic tube and 45mL of Ringer solution was added. The mixture was homogenized for 60s. The pH of this homogenized sample was measured by using a pH meter (Thermo Scientific, Singapore).

TVB-N value: The TVB-N value of the shrimp flesh was determined by the protocol described in the European Commission guidelines (Commission Decision 95/149/EC, 1995), which fixes the TVB-N limit values for certain categories of fishery products and specifies the analytical methods to be used. Analyses were conducted by distillation in a Vapodest 30s machine (Gerhardt, Germany) and titration with HCl 0.01 N. Malondialdehyde (MDA) content was determined by using the method described by Tsironi *et al*. (2009). TBARS values were calculated from the standard curve of malondialdehyde (0-10 µmol L^{-1}) and expressed as mg malondialdehyde/kg shrimp flesh.

Bacteria count: The total plate count of shrimps was determined by using the protocol described by Kalleda *et al.* (2013) with minor modifications. Briefly, two whole whiteleg shrimps were aseptically collected and used as the composite sample. A representative ground sample (25g) was transferred to a sterile conical flash with 225mL of sterile water containing 1% peptone and 0.85% NaCl, and homogenized for 1min with a T10 basic Ultra-turrax (IKA, Germany). Samples (0.1mL) of 10-fold appropriate dilutions of the shrimp homogenates were spread on the surface of the Plate Count Agar media in Petri dishes. The dishes were incubated at 37^{o}C for 48h.

Statistical analysis

Data were analyzed using the statistical software SAS 9.4 (SAS Institute, Cary, NC). The zone of inhibition was expressed as the mean ± standard deviation of three experimental replications. Analysis of variance was carried out using a generalised linear model (GLM) procedure to determine (1) the effects of the plant source, extract concentration, and bacterial strain as well as their interactions on the zone of inhibition, and (2) the effects of the type of treatment and storage time as well as their interaction on the index of quality of shrimps.

Results and Discussion

Total phenolic content and antioxidant capacity of the extract powders

The polyphenol contents of the extract powders were relatively high and ranged from 281.25 ± 0.75 to 349.41 ± 0.72 mg GAE/g (**Table 1**). The extract of *P. guajava* had the highest phenolic content (349.41 ± 0.72 mg GAE/g) followed by the extracts of *C. operculatus* (282.36 ± 1.17 mg GAE/g) and *R. tomentosa* (281.25 ± 0.75 mg GAE/g). These figures were similar to the phenolic contents of extracts made from lyophilised leaves, bark, and whole plants of four medicinal plants from Burkina Faso, namely *Combretum micranthum*, *Khaya senegalensis*, *Pterocarpus erinaceus*, and *Sida acuta* (284.2-378.0 mg/g extract) (Karou *et al.*, 2005).

In comparison with other medicinal plants, the three Myrtaceae plants in the present study had higher phenolic contents in the extracts.

Table 1. Total polyphenol contents and antioxidant capacities of the three extract powders

Leaf extract powder	Polyphenol content (mg GAE/g)	Antioxidant capacity (mmol TE/g)
R. tomentosa	281.25 ± 0.75	4.00 ± 0.07
P. guajava	349.41 ± 0.72	3.99 ± 0.19
C. operculatus	282.36 ± 1.17	2.85 ± 0.17

Note: n = 3 analysis replications.

Indeed, Aryal *et al.* (2019) reported the phenolic contents of extracts from eight wild medicinal plants from Nepal ranged from 72.66 to 292.65 mg GAE/g with an average value of 161.71 mg GAE/g. In the study of Sing *et al.* (2016), twelve Indian traditional medicinal plants were selected based on their reported pharmacological activities including antioxidant, anticancer, antimicrobial, anti-arthritis, anti-inflammatory, and antidiabetic abilities, and the phenolic content in their extract powders were analyzed. The highest total phenolic content was recorded in the extract of *Bombax ceiba* (Linn.) with a value of 181.91mg of GAE/g.

Regarding the antioxidant capacities, the extract of *R. tomentosa* had the highest value (4.00 ± 0.07 mmol TE/g) while the one of *C. operculatus* had the lowest (2.85 ± 0.17 mmol TE/g). These values were two times higher than the one of extract powders from four medicinal plants in Burkina Faso, which ranged from 1.20 ± 0.04 to 2.21 ± 0.04 mmol Trolox/g (Karou *et al.*, 2005), although the extracts had comparable phenolic content as mentioned above. As the antioxidant capacity of phenolic compounds strongly depends on their chemical structure (Lai, 2016), the higher antioxidant capacities of the three Myrtaceae leaf extract powders compared to one of the extract powders from the medicinal plants in Burkina Faso could be explained by differences of the phenolic compositions of the extracts. Another possible reason could be the presence of compounds other than phenolics and their involvement in the antioxidant activities of the extract powders from the three Myrtaceae plants. In the study of Karou *et al.* (2005), the total phenolic compound contents were highly correlated with the antioxidant activities (r = 0.94) showing that the antioxidant activities present in the extracts were essentially due to polyphenols, while no significant positive correlation between the phenolic contents and antioxidant capacities of the extract powders was found (r = 0.48, p = 0.6805) for the three Myrtaceae extracts in the present study. This means that non-phenolic antioxidants should be present in the Myrtaceae leaf extracts. Indeed, some other antioxidants besides polyphenols, such as triterpenoids, have been reported to be present in the three Myrtaceae plants (Flores *et al.*, 2015; Díaz-de-Cerio *et al.*, 2016; Wang *et al.*, 2016; Hamid *et al.*, 2017).

In vitro antimicrobial activity against bacteria isolated from cold-stored spoiled whiteleg shrimp of the three Myrtaceae leaf extract powders

Figures 1 and **2** show the antibacterial activities of the three Myrtaceae extract powders against shrimp-spoiling bacteria measured by the agar well diffusion method. The variance analysis for the effects of the extract, bacterial strain, and extract concentration on the inhibition zone are shown in **Table 2**. According to the data, all three factors, the plant extract, bacterial strain, and extract concentration, as well as their interactions, significantly affected the inhibition zone.

The extracts showed different inhibitions to the seven isolated bacteria. Among the three plant extracts, the one from *P. guajava* leaves had the highest antibacterial activity with an average zone of inhibition for all seven bacteria of 8.70mm, followed by the one of *C. operculatus* leaves and then *R. tomentosa* leaves (8.27mm and 7.19mm, respectively). This reflected, on one hand, the differences in the composition of the extract powders, and on other hand, the differences in the susceptibility of bacteria to antimicrobial compounds in the extracts.

The inhibition zones for all treated bacteria ranged from 0.33 to 19.67mm and depended significantly on the bacterial strain. Among the seven bacteria isolated from spoiled shrimp, *Aeromonas* sp2 and sp3 were the most susceptible to the extract powders with the average inhibition zones being 15.67 and 14.54mm, respectively (**Figures 1** and **2**) while *Aeromonas* sp4, which had an average inhibition zone of 2.80mm, was the least susceptible. The sensitivity of the bacteria to the extracts varied depending on the type of extract. For the *P. guajava* leaf extract, the ranking of bacterial sensitivity was as follows:

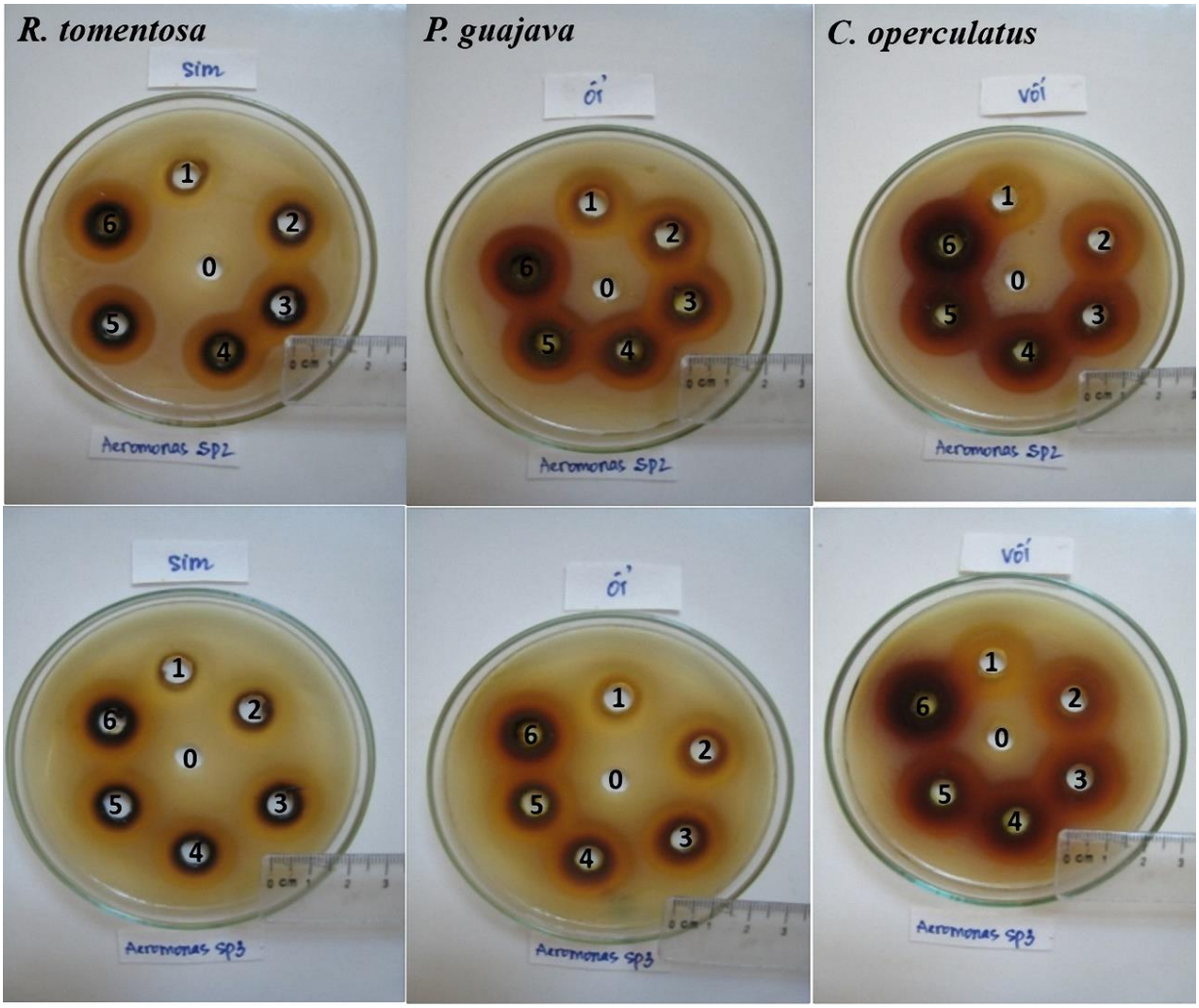


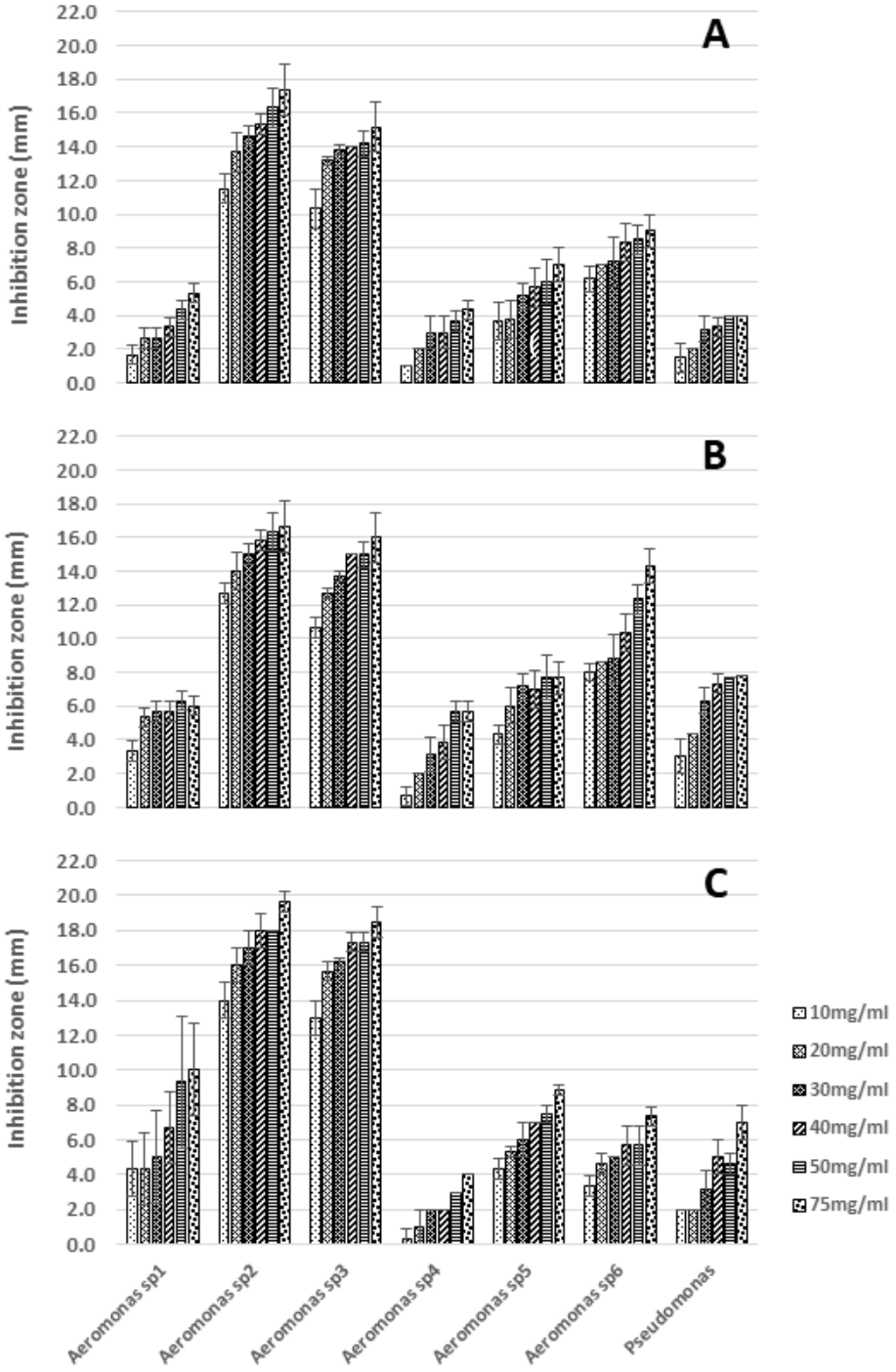
Figure 1. Inhibition zones of the three Myrtaceae leaf extract powders against *Aeromonas* sp2 and *Aeromonas* sp3 isolated from cold-stored spoiled whiteleg shrimp (0: control, 1: 10 mg/mL, 2: 20 mg/mL, 3: 30 mg/mL, 4: 40 mg/mL, 5: 50 mg/mL, 6: 75 mg/mL)

Table 2. Effects of the plant extract, extract concentration, and bacterial strain on the antibacterial activities of the three Myrtaceae extract powders against shrimp-spoiling bacteria

Term	Degrees of freedom	Pr > F
Plant extract	2	<.0001
Bacterial strain	6	<.0001
Plant extract*Bacterial strain	12	<.0001
Extract concentration	5	<.0001
Plant extract*Extract concentration	10	0.0482
Bacterial strain*Extract concentration	30	0.1732
Plant extract*Bacterial strain*Extract concentration	60	0.0767

Aeromonas sp2 > *Aeromonas* sp3 > *Aeromonas* sp6 > *Aeromonas* sp5 > *Pseudomonas* sp. > *Aeromonas* sp1 > *Aeromonas* sp4. For the *R. tomentosa* and *C. operculatus* leaf extracts, the decreasing sensitivity orders were *Aeromonas* sp2 > *Aeromonas* sp3 > *Aeromonas* sp6 > *Aeromonas* sp5 > *Aeromonas* sp1 = *Pseudomonas* sp. = *Aeromonas* sp4, and *Aeromonas* sp2 > *Aeromonas* sp3 > *Aeromonas* sp1 = *Aeromonas* sp5 > *Aeromonas* sp6 >

Pseudomonas sp. > *Aeromonas* sp4, respectively. Among the three tested plants, the extract from *C. operculatus* leaves was more effective against *Aeromonas* sp1, sp2, and sp3 as compared to the ones from *R. tomentosa* and *P. guajava* leaves, while the extract from *P. guajava* leaves had the highest inhibitive activity against *Aeromonas* sp4 and sp6, and *Pseudomonas* sp. (**Figure 2**).



Note: n = 3 experimental replications; values are expressed as mean ± standard deviation.

Figure 2. Inhibition zones (mm) of *R. tomentosa* (A), *P. guajava* (B), and *C. operculatus* (C) leaf extract powders against bacteria isolated from cold-stored spoiled whiteleg shrimp

Extract concentration significantly affected the antimicrobial activity (P <0.0001). In general, higher concentrations of leaf extract had larger inhibition zones. The inhibition zones remarkably increased when the extract concentration increased from 10 to 30 mg/mL. A slight increase was observed when the extract concentration increased from 30 to 75 mg/mL.

The antimicrobial activities of extract powders made from the three leaves used in this study have been reported in other papers. Biswas *et al.* (2013) reported that the methanol extract of *P. guajava* leaves had antibacterial activity with mean zones of inhibition of 8.27 and 12.3mm, and the ethanol extract had mean zones of inhibition of 6.11 and 11.0 mm against the food-borne and spoilage bacteria *B. cereus* and *S. aureus*, respectively. By using qualitative chemical tests for the screening and identification of the bioactive chemical constituents in the extracts, the authors found that phenolic compounds including tannins and flavonoids in the methanol and ethanol extracts contributed to the antimicrobial activity.

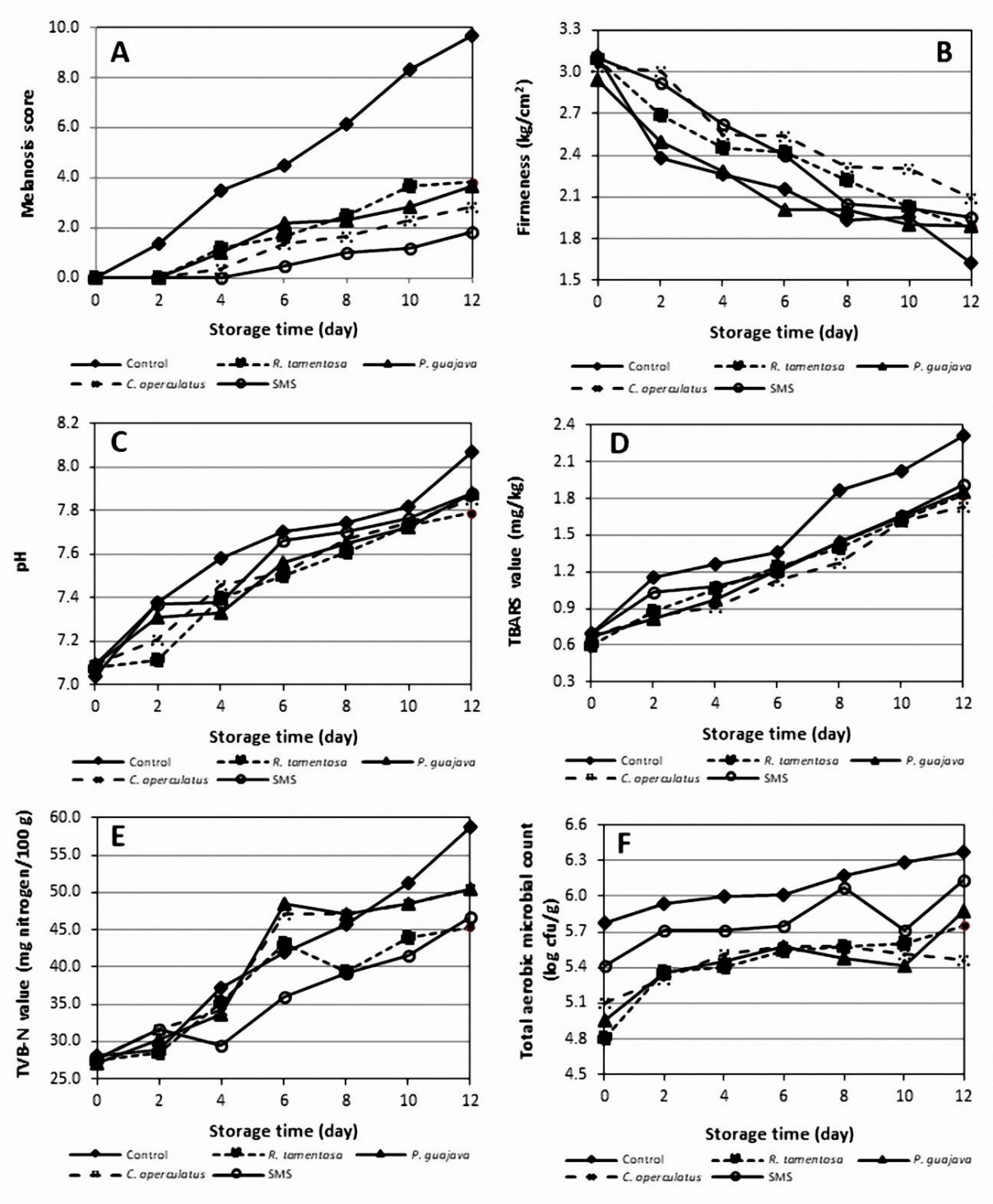
The antibacterial activities of *C. operculatus* leaf extracts have been reported in several works. In the study of Nguyen *et al.* (2017), the methanolic extract of *C. operculatus* leaves inhibited bacterial activity against Gram-positive bacteria (*Staphylococcus aureus*, *Bacillus subtilis*, and *Streptococcus mutans* GS-5) and three multi-resistant bacteria (*Staphylococcus epidermidis* 847, *Staphylococcus haemolyticus* 535, and *Staphylococcus aureus* North German epidemic strain) with the inhibition zone diameters ranging from 7 to 16mm. The phytochemical screening of the extract using thin layer chromatography showed positive results for flavonoids and triterpenes in the extract. Moreover, by using a variation of a flavonoid-aluminum complex test, flavonoids were determined as the major constituents of the extract with a concentration of 6.8 mg/g dry matter. This phenolic group hence surely contributed to the antibacterial activity of the *C. operculatus* leaf extract.

The antibacterial activity of *R. tomentosa* leaves has also been reported in several publications. An ethanolic extract *of R. tomentosa* leaves had antibacterial activity against *Listeria monocytogenes* in a cooked chicken meat model system and was considered as a potent bio-additive agent to control contaminations from *L. monocytogenes* in ready-to-eat meat (Odedina et al., 2016). The antimicrobial activity of *R. tomentosa* leaves might be due to rhodomyrtone, a natural antibiotic, which has been found in the leaves (Limsuwana *et al.*, 2009) and has displayed significant antibacterial activities against Gram-positive bacteria including pathogen and antibiotic-resistant strains (Saising *et al.*, 2008, Limsuwan *et al.*, 2009). However, it is important to note that besides rhodomyrtone, phenolic compounds and triterpenoids in *R. tomentosa leaves* (Hamid et al., 2017) could also contribute to the antibacterial action of *R. tomentosa leaf extracts*.

In this study, the antibacterial activity of the three Myrtaceae leaf extract powders against the seven bacteria isolated from cold-stored spoiled whiteleg shrimp could be partly explained by the presence of a high quantity of phenolic compounds in the extract powders as shown in **Table 1**. The inhibitive mechanisms of phenolic compounds on microbial growth are relatively well understood as shown in the review of Lai (2016). It is interesting to note that all the isolated bacteria used in this study were Gram (-) bacteria whose surfaces are coated with lipopolysaccharides and hence are generally more resistant to phenolic compounds than Gram (+) bacteria (Tong & McIntosh, 2004). This could expand the application of the three Myrtaceae leaf extracts not only in shrimp preservation but also in the bio-control of other microorganism contaminations in food products.

Effects of the Myrtaceae leaf extract treatments on the quality of whiteleg shrimps during cold storage

The effects of the three leaf extract powders on the biochemical and microbiological quality changes of whiteleg shrimps during cold storage are presented in **Figure 3**.



Note: n = 3 experimental replications.

Figure 3. Melanosis score (A), firmness (B), pH (C), TBARS value (D), TVB-N value (E), and total aerobic microbial count (F) of whiteleg shrimps with and without the treatment of 0.5% Myrtaceae leaf extract powders or 1.25% SMS during 12 days of cold storage

Melanosis

Changes in the melanosis scores of the stored shrimps in this study are shown in **Figure 3A**. In general, when the storage time increased, the melanosis scores significantly (P <0.0001) increased. However, the increases in the melanosis scores were lower in the samples treated with 1.25% SMS and with the Myrtaceae

extract powders. On the first day of storage, there was no melanosis formation in any of the samples. After only two days of storage, in the control, black spots appeared on 13% of the shrimps' surface and the surface of almost all shrimps (96.7%) changed to black after 12 days of storage. In samples treated with 1.25% SMS, black spot formation was first observed on the 6th day of storage and evaluated as "slight" on the 12th day with a melanosis score of 1.83. SMS is a legal antioxidant and preservative agent that might inhibit melanosis by reacting with intermediate quinone and forming sulphoquinone, hence preventing the formation of black spots (Gómez-Guillén *et al.*, 2005). Regarding the samples treated with the Myrtaceae extracts, during storage, their melanosis scores were always lower than that of the control. Black spot formations were observed only from the 4th day of storage with melanosis scores of 0.33-2.00, which increased to 2.83-4.50 at the end of storage. The inhibition of black spot formation by plant extracts was indicated in the study of Nirmal & Benjakul (2011). These authors found that shrimps treated with lead seed extract powder (0.5%) had lower melanosis scores throughout 12 days of storage compared with the control (without treatment). According to the authors, this inhibitory action was due to the presence of mimosine (8.8g/100g) and polyphenols (17.4g/100g) in the lead seed extract. Similarly, in the study of Firdous *et al.* (2020), white prawns (*Fenneropenaeus indicus*) treated with green tea and *Phyllanthus emblica* Linn extracts at a concentration of 50 g L^{-1} remained appealing until the 18th to 19th days and the 16th to 18th days, respectively, because their obtained scores were within the 4-6 range while the control reached a score above 8 from the 14th day of chilled storage. Some pure polyphenols including ferulic acid and catechin have been shown to inhibit polyphenoloxidase from Pacific whiteleg shrimps in a dose-dependent manner and could be used as effective additives to retard melanosis and maintain the quality of Pacific whiteleg shrimps when they are stored in ice (Nirmal & Benjakul, 2010).

The melanosis inhibition activities of the three Myrtaceae leaf extracts were dose-dependent. The increasing melanosis score rate was lower in shrimps treated with 0.5% of an extract compared with those treated with 0.25% (P <0.0001 for all three leaf extracts). The difference between scores was clearly observed from the 4th day when samples treated with the 0.25% and 0.5% extract powders had average melanosis scores of 1.7 and 0.8, respectively. The *C. operculatus* leaf extract showed the highest melanosis inhibitive activity as compared to its Myrtaceae counterparts. After 12 days of storage, shrimps treated with the *C. operculatus, P. guajava*, and *R. tomentosa* leaf extracts had melanosis scores of 2.83, 3.67, and 4.50, respectively. However, in comparison with 1.25% SMS, the *C. operculatus* leaf extract powder was less effective in preventing black spot formations in cold-stored whiteleg shrimps. Phenolic compound extracts from the three Myrtaceae leaves could have inhibited the polyphenol oxidase activity in the shrimps and thus limited the formation of black spots on the shrimps' heads and shells.

Firmness of shrimp flesh

The most important quality phenomena occurring during shrimp storage are color fading, lipid oxidation, and decomposition of proteins. Among these changes, protein hydrolysis can result in texture change (Yamagata & Low, 1995). The changes in shrimp flesh firmness during cold storage are shown in **Figure 3B**. The flesh firmness decreased in all the shrimp samples as the storage time increased (P <0.0001). On the first day of storage, the shrimp flesh was firm and elastic. The force needed to have a penetration depth of 2mm was around 5.5 kg/cm^2 for all the samples. At the end of storage, the firmness decreased to 3.22-4.17 kg/cm^2, depending on the treatment applied on the shrimps.

There were significant differences in the rate of firmness decrease between the control shrimps and shrimps treated with 1.25% SMS or Myrtaceae leaf extracts (P <0.0001). Among the eight samples, shrimps treated with the 0.5% *C. operculatus* leaf extract showed the lowest rate of firmness decrease. After 12 days of storage, the texture value was 4.17 kg/cm^2, similar to the

one of shrimps treated with 1.25% SMS on the 8th day. By contrast, the control shrimps showed the fastest decrease in firmness (from 6.20 on the 1st day to 3.22 kg/cm^2 on the last day of storage).

pH of shrimp flesh

The pH of all the samples increased when the storage time increased (P <0.0001) (**Figure 3C**). An increase of pH was also reported for deep-water rose shrimps (*Parapenaeus longirostris*) during refrigerated storage (Miraglia *et al.*, 2020). The increase in the pH values of shrimp flesh during cold storage was the result of the accumulation of basic compounds, such as amines and trimethylamines, generated from both autolytic processes by endogenous enzymes and microbial enzymatic actions (Nirmal & Benjakul, 2009). The initial pH of fresh shrimps was about seven, similar to those reported by Shamshad *et al.* (1990), Tsironi *et al.* (2009), and Miraglia *et al.* (2020). After 12 days of storage, the control shrimps showed the highest pH, which increased up to 8.07. There were no significant differences in pH change between shrimps treated with Myrtaceae leaf extract powders and the those treated with 1.25% SMS (P >0.05). However, in comparison with the control, most of the shrimps dipped in Myrtaceae leaf extracts showed a lower rate of pH increase (P <0.05). Shamshad *et al.* (1990) reported that shrimps (*Penaeus merguiensis*) were not acceptable or were considered spoiled when the pH was greater than 7.6 from a sensory point of view (odor, texture, and appearance). Based on the pH values of the shrimp flesh in this study, the control samples could have been consumed until the 4th day whereas the treated shrimps could have been used until the 6th or 8th days of storage.

TBARS value

Lipid oxidation is one of the deteriorative reactions causing the unacceptability of fish and shrimp products. When lipid oxidation occurs, unstable hydroperoxide is formed which rapidly decomposes into secondary oxidative products, including MDA and other carbonyl compounds during storage (Takeungwongtrakul & Benjakul, 2016). Malondialdehyde can be detected and quantified by using TBA (Nirmal & Benjakul, 2009). Changes of the TBARS values of whiteleg shrimps during the 12-day cold storage period are presented in **Figure 3D**. At the beginning of storage, the TBARS values were 0.6-0.7 mg MDA/kg shrimp flesh and then significantly increased in all the samples during storage (P <0.0001). A similar trend was indicated in deep-water rose shrimps during chilled storage (Miraglia *et al.*, 2020).

The treatments had significant effects on the changes of the TBARS values of the shrimps (P <0.0001) during storage. The control had the largest increase in the TBARS value (from 0.69 to 2.31 mg MDA/kg shrimp flesh) while shrimps treated with the 0.5% *C. operculatus* leaf extract showed the smallest one (from 0.67 to 1.73 mg MDA/kg shrimp flesh). SMS 1.25% also inhibited lipid oxidation in the shrimps (P = 0.0007). There were no significant differences in the changes of the TBARS values in the shrimps dipped in 0.25% *C. operculatus*, *P. guajava*, and *R. tomentosa* leaf extracts or in 1.25% SMS (P = 0.1404, 0.8347, and 0.8505, respectively). However, shrimps immersed in 0.5% *C. operculatus* or *R. tomentosa* leaf extracts had a lower increase rate of their TBARS values than those treated with 1.25% SMS (P = 0.0002 and 0.002, respectively).

TVB-N value

The TVB-N is one of the important indicators of chemical spoilage of shrimps. The changes in the TVB-N content of whiteleg shrimps are shown in **Figure 3E**. In general, the TVB-N content in shrimp flesh increased continuously in all the shrimps during storage (P <0.0001). Increases in TVB-N values have been reported for Pacific white shrimps (Nirmal & Benjakul, 2010), Indian white prawns (Firdous *et al.*, 2020), and deep-water rose shrimps (Miraglia *et al.*, 2020). The initial TVB-N values of the shrimps in this study were about 27mg of nitrogen/100g shrimp flesh in all the samples. This value was higher than the ones found by Yamagata & Low (1995) (3.08mg of nitrogen/100g flesh for banana shrimps), by Nirmal & Benjakul (2010) (4.1mg of nitrogen/100g shrimp flesh for Pacific whiteleg

shrimps), and by Miraglia *et al.* (2020) (16.3mg of nitrogen/100g shrimp flesh for deep-water rose shrimps). However, they were similar to those obtained by Leitão & Rios (2000) (20.09-24.00mg of nitrogen/100g shrimp flesh) but lower than the TVB-N content (33.5mg/100g) in *Pandalus borealis* shrimps as reported by Zeng *et al.* (2005). The differences in the initial TVB-N values in the shrimps might be due to the analysis method, the type of shrimp, and the degree of freshness at the beginning of storage. In this study, the high initial TVB-N was in agreement with the high total plate count (**Figure 3F**) detected on the first day of storage.

The shrimp treatment significantly affected the increase of the TVB-N values of the stored shrimps (P <0.0001). The control shrimps had the highest increase in TVB-N values (from 28 mg of nitrogen/100 g shrimp flesh at the beginning to 58.80mg of nitrogen/100g at the end of storage) while shrimps dipped in the 0.5% *R. tomentosa* leaf extract and 1.25% SMS showed the lowest changes (from 27.53 to 45.40 for 0.5% *R. tomentosa* leaf extract and from 27.77 to 46.67 for 1.25% SMS). The *C. operculatus* and *P. guajava* leaf extracts at concentrations of 0.25% and 0.5% also inhibited the formation of volatile nitrogen-containing compounds in the shrimps. Concerning the effects of extract concentration, the higher extract concentration lowered the increase rate of the TVB-N values in the shrimps treated with the *C. operculatus* or *R. tomentosa* leaf extracts (P <0.0001 for these two leaf extracts). However, no significant differences were observed in the TVB-N values of shrimps treated with 0.25% or 0.5% *P. guajava* leaf extract (P = 0.1907).

Total plate count

Changes of the total plate counts of whiteleg shrimps during 12 days of storage are shown in **Figure 3F.** Fresh shrimps showed an initial total viable count of 5.78 log cfu/g. SMS and the extract powders from the three Myrtaceae plants exhibited their antimicrobial activity immediately after treatment, as illustrated by the lower total plate count in comparison with the control sample. On the first day, just after treatment, the total plate counts were 4.8, 4.96, 5.11, and 5.41 log cfu/g in shrimps dipped in 0.5% *R. tomentosa*, *P. guajava*, and *C. operculatus* leaf extracts and 1.25% SMS, respectively. This observation could be due to the antibacterial activities of the three Myrtaceae extract powders, which were reported in a previous section. The total plate count of the stored shrimps increased during storage (P <0.0001). The control had the highest rate of increase, followed by shrimps treated with 1.25% SMS, while shrimps immersed in the 0.5% Myrtaceae extract powders showed the lowest rate (P <0.0001).

Conclusions

The study indicated that the three Myrtacetae leaf extract powders had high polyphenol contents and antioxidant activities. They also exhibited potent antibacterial activity against seven bacteria isolated from cold-stored spoiled whiteleg shrimps (*Penaeus vannamei*). The inhibitive action was dependent on the plant, the extract concentration, and the type of bacterium. These three extracts decelerated biochemical changes including melanosis, volatile nitrogen-containing compounds formation, lipid oxidation, and microbial growth, hence retarding quality loss and prolonging the shelf-life of whiteleg shrimps. Taken together, the results of this study showed that extract powders from the three Myrtaceae plants could be used as sources of natural preservative agents in the cold storage of shrimps.

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