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Antimicrobial Activity of Phenolic-Rich Extracts from Mango Seed Kernel on Microorganisms

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Replacing synthetic additives to preserve food products with natural antimicrobial compounds needs to be considered due to public health. In the present study, the phenolic extract from mango seed kernel (MSK) with the total phenolic content (TPC) value of 5300 ± 380 mg gallic acid equivalent (GAE)/L was used for evaluating the antimicrobial properties against five types of bacterial strains, including *Listeria monocytogenes*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, and *Salmonella typhimurium* in vitro. This extract was also used for *in situ* method on the storage of *Pangasianodon hypophthalmus* fish fillets. The minimum inhibitory concentration (MIC) values for all tested strains were determined at an average concentration of 1325 mg GAE/L. Furthermore, the minimum bactericidal concentration (MBC) values of *E. coli*, *S. aureus*, and *S. typhimurium* were seen at 5300 mg GAE/L, while the extract did not show eliminations in the growth of the remaining strains. The bacterial inhibition speed of the extract illustrated that the concentration equal MIC value eliminated *S. typhimurium* growth after 24 h, the 4 times MIC value had remarkable effects on *S. aureus* growth after the 9 h of incubation, and 24 h of incubation for *E. coli, L. monocytogenes*, *P. aeruginosa*. Additionally, the MSK extract could inhibit the growth of *P. aeruginosa* on fish fillets in 4 days of storage. These results provide important evidence for the utilization of MSK as a natural source of antimicrobial agents in food products.

Keywords: **Antimicrobial activity, mango seed kernel, minimum inhibitory concentration, minimum bactericidal concentration, *Pangasianodon hypophthalmus***

Introduction

Mango is one of the wide plants cultivated in many countries in the world, representing production of about 56.5 million tons (MT) in the year 2019 [1], most of which is mainly moved to processing and exported to global markets. The large amounts of by-products such as peel, seed husk, and kernels which are estimated to be approximately 50% of the fruit mass combined, are left as waste materials and simply disposed of in landfill [2]. Based on the 2019 production [1], more than 350,000 MT of these waste materials are generated, while these by-products are rich sources of phenolic compounds. The four most abundant phenolic compounds in mango are ellagic acid, gallic acid, mangiferin, and quercetin, and their derivatives are either in the form of tannins or glucosides (penta-galloylglucose, gallotannins, homomangiferin, methyl-gallate, quercetin, dimethylmangiferin among others) [3]. These compounds keep the roles of anti-inflammatory, antioxidant, antimicrobial, and anticancer activities [4].

Among the numerous applications of phenolic compounds, antimicrobial use is one of the major concerns. Up to now, food spoilage by microorganisms is a main problem related directly to the consumer and food industry and difficult to control in developing countries [5]. More and more synthetic additives or toxic chemicals are being used for preserving food products that directly

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affect human health, so extracting natural additives from by-products of fruit processing is extremely necessary. Therefore, the key objectives of this study were: (1) to test the antimicrobial activities of phenolic-rich extracts from mango seed kernel (MSK) against Gram-positive and Gram-negative bacteria such as *Listeria monocytogenes*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, and *Salmonella typhimurium in vitro* by determining minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC); (2) evaluating the time to inhibit the growth of tested bacteria by using Time-kill kinetics assay; (3) applying mango seed kernel extract (MSKE) to study the shelf-life of *Pangasianodon hypophthalmus* fish fillets (*in situ* study).

Materials and Methods

Media and Chemicals

Sodium carbonate and ethanol were from Guangdong Guanghua Sci-Tech (China); Folin-Ciocalteu reagent was bought from Merck (Germany), and gallic acid was from Spectrum Chemical (USA). Malt extract agar (MEA) was from Titan Biotech Limited (India). Plate count agar (PCA) and Dichloran-rose bengal chloramphenicol (DRBC) were obtained from Oxoid (England). Brain heart infusion (BHI), Nutrition broth (NB), De Man-Rogosa-Sharpe (MRS), and Pseudomonas CFC/CN agar were from Merck (Germany).

Mango Seed Kernel Extracts

Extract preparation

Mango seeds were collected from WESTFOOD company (Vietnam) and then was prepared following the procedure of Ribeiro *et al.* [6]. The mango seed kernel (MSK) were carefully separated by hand and then oven-dried at 65℃ to reach the final moisture content is about 6–8%. Next, the dried materials were packed in vacuum-sealed polyethylene (PE) and aluminum bags, after that, it was stored at -20℃ [7]. Before extracting, the dried materials were ground into powder using a kitchen grinder and passed through a 1–mm sieve mesh.

According to the method of Huynh *et al.* [8] with some modifications, soluble phenolic compounds were extracted using 50% ethanol. An aliquot of 1 g powdery sample was homogenized in 12 ml of 50% ethanol using an ultra-turrax homogenizer (DLAB D-500, China) for 1 min. The homogenate is kept in a water bath at 50℃ for 15 min and then centrifuged at 6000 *g* for 6 min at 4℃ using a centrifuge (HERMLE D-78564, Germany). The supernatant was collected in a volumetric flask. Similarly, the pellet was re-extracted with 12 ml of 50% ethanol and followed by the same steps. A vacuum evaporator (Heidolph, Germany) was used to evaporate ethanol in the extract at 50℃ (0.008 mbar). Then the liquid phase was decanted and stored in vials and kept in a freezer prior to sample preparation for subsequent analyses [9]. The extract was prepared one time and used for all experiments.

Total phenolic content

The total phenolic content (TPC) of the extract was determined using the modified Folin-Ciocalteu method [10, 11]. One ml of distilled water and 1 ml of sample known dilution concentrations were put into a test tube. Folin-Ciocalteu reagent (0.5 ml, 10%) was added to the solution and permitted to react for 6 min. Next, 1.5 ml Na_2CO_3 (20%) was withdrawn and transferred to the test tube, and this tube was titrated to 5 ml with distilled water. This tube then was put in the dark for color development for 2 h. After that, it was measured by a GENESYS 30 Spectrophotometer (Thermofisher Scientific, USA) and read at 760 nm. TPC was expressed as mg of gallic acid equivalent per liter of MSKE [12].

Microbiological Experiments

Tested microorganisms

The *E. coli* ATCC 25922, *S. aureus* ATCC 25923, *P. aeruginosa* ATCC 9027, and *L. monocytogenes* ATCC 13932 bacterial strains were grown on BHI medium at 37℃ for 24 h.

S. typhimurium ATCC 14028 was grown on NB medium at 37℃ for 24 h [13–16]. The viability of bacteria was estimated using the Pour-Plate technique with the specific medium, confirming populations at concentrations higher than 10^7 CFU/ml [17].

Minimum inhibitory concentration and minimum bactericidal concentration determination

Twelve numbered screw tubes were prepared (Fig. 1), and then 1 ml of suitable medium was added into the tubes, except for tube number 1. Next, the tubes were

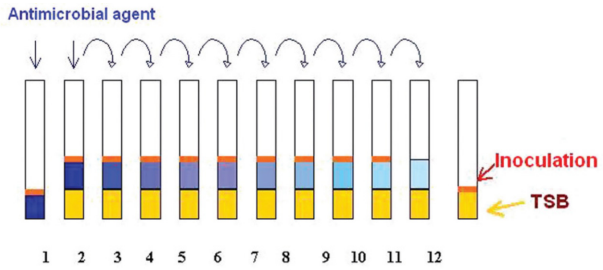

Fig. 1. Procedure for determining minimal inhibitory concentration [12].

sterilized at constant pressure and temperature of 121℃. For the first and second tubes of the series, 1 ml of tested MSKE was distributed; tube 2 was stirred and 1 ml was then withdrawn and transferred to tube 3. This successive transference was repeated until tube 11. Zero-point one milliliter of inoculation (tested microorganisms) at a known concentration (10^2 CFU/ml) was added to all tubes, except for tube number 11. Incubation at optimal temperature (37℃) was developed for 24 h (for MIC) and 48 h (for MBC). Tube 11 and 12 are negative (medium + antimicrobial) and positive (medium + inoculation) controls [17]. Besides, MBC was determined following the method reported by Rodríguez Vaquero *et al.* [18]. Briefly, one hundred microliters of each sample suspension without microbial growth in the MIC test is sucked out by using a pipette and then subcultured on the specific medium for 24 h at 37℃.

Time-kill kinetic assay

According to the procedure described by Tsuji *et al.* [19], the time-killing kinetics of the MSKE was carried out. Concentrations of equal the MIC, twice the MIC, and four times the MIC of the MSKE were prepared. One hundred microliter of inoculum (10^2 CFU/ml) was added and incubated at 37℃. One hundred microliter of the medium was taken at different set-up times, inoculated aseptically into 20 ml specific medium, and incubated at 37℃ for 24 h. A control sample was performed for the microorganisms without the MSKE. The colony-forming unit (CFU) of the microorganisms was determined.

In situ antimicrobial activity of mango seed kernel extract

Each piece of 10-gram *Pangasianodon hypophthalmus* fish fillets sample was cut, then put into plastic bags and stored at 4℃ for 3 h. And then, 100 µl of *P. aeruginosa* (10^2 CFU/ml) was spread on each sample. The inoculated samples were put in other plastic bags, and placed in a fridge at 4℃, for 24 h to evaporate excess moisture from inoculation and permit adhesion of the cells. In sterile falcon tubes, the inoculated samples were dipped in the MSKE with a specific concentration for 5 min. Each piece of the sample was gently pressed against the inside wall of the falcon tube to remove excess solution. One piece of fish was sealed in a separate plastic bag and stored at 4℃ for 14 days. The samples prepared under the same conditions without dipping in the extract were seen as controls. At 0, 1, 4, 7, 10, and 14 days samples were taken for *P. aeruginosa* using selective medium (Pseudomonas CFC/CN agar), yeasts and molds (Y&M) using Dichloran-rose bengal chloramphenicol, lactic acid bacteria (LAB) using De Man-Rogosa-Sharpe, and total aerobic count (TAC) using Plate count agar. The number of colonies was examined by the pour plate technique [20]. The microorganisms were incubated for 24–48 h. All steps were performed under aseptic conditions. The results were reported as log CFU/g.

Statistical Analysis

All the experiments were performed in two biological independent replicates and each replicate was analyzed twice. Results are presented as mean ± standard deviation. The one-way ANOVA and LSD test were conducted using the STATGRAPHIC CENTURION XIX (Statgraphics Technologies, Inc., USA). Differences between the mean levels of treatments in the different experiments were taken to be statistically significant at $p < 0.05$.

Results and Discussion

Antimicrobial activity of phenolic-rich extracts of mango seed kernel

Table 1 shows MIC of the MSKE showing 5300 ± 380 mg GAE/L of TPC value for tested bacteria. In detail, four different types of bacteria including *L. monocytogenes, E. coli, S. aureus*, and *S. typhimurium* presented turbidity in broth at the concentration of 330 mg/l, but not at higher concentrations. This can be concluded that the concentration of 660 mg/l is the MIC for

Table 1. Minimum inhibitory concentration of the MSK extract for tested strains.

Concentration of MSK extract (mg/l)	Turbidity in broth (MIC)				
	L. monocytogenes ATTC 13932	*E. coli* ATCC 25922	*S. aureus* ATCC 25923	*P. aeruginosa* ATCC 9027	*S. typhimurium* ATCC 14028
5300	-	-	-	-	-
2650	-	-	-	-	-
1325	-	-	-	-	-
660	-	-	-	+	-
330	+	+	+	+	+
165	+	+	+	+	+
80	+	+	+	+	+
40	+	+	+	+	+
20	+	+	+	+	+
10	+	+	+	+	+
Negative control	-	-	-	-	-
Positive control	+	+	+	+	+

(-): No growth
(+): Growth
MSK: Mango seed kernel

these tested microorganisms. Additionally, it is not turbid in broth for the tube containing *P. aeruginosa* at a concentration higher than the concentrations of other bacteria, 1325 mg/l. Therefore, this is suggested that the concentration of 1325 mg/l is *P. aeruginosa*'s MIC value. These results were not in line with the previous report by Taguri *et al.* [21], which indicated that *S. aureus* was more susceptible to phenolics than the genus *Salmonella* and *E. coli*, because *S. aureus* is Gram-positive and showed a higher sensitivity to phenolics. The reason might be that the phenolics used in the previous study were pure compounds. Also, at the same MSKE concentrations, the inhibition time was different between Gram-negative and Gram-positive bacteria, which was confirmed by Bernal-Mercado *et al.* [22]. Many studies indicated that the crucial point that is responsible for the variant resistance to antimicrobial agents is the difference in cell membrane structure of Gram-negative and Gram-positive bacteria [23]. Due to the fact that Gram-positive bacteria have only one lipid layer, while the Gram-negative ones are coated with lipopolysaccharide which slows down the passage of phytochemicals [24]. In addition, the shape of the microorganisms is sustained by cell membrane and it allows the permeating of biochemicals and ATP synthesis. The bacterium will die by leaking of cell cytoplasm, nucleic acids, proteins, and some granules into the environment because of cell membrane disruption [25].

Moreover, Table 2 displays the extract's minimum bactericidal concentration (MBC) for tested microorganisms. Specifically, there is still the growth of *L. monocytogenes* and *P. aeruginosa* onto agar plates at the concentration of 5300 mg/l (original extract), so it can be concluded that the original extract has not significant effect on the inactivation of these types of bacteria. However, for *E. coli* and *S. aureus*, the MBC is 5300 mg/l since there was no growth onto agar plates after 24 h incubation at 37℃. This result was confirmed by the conclusions of previous reports showing that the most sensitive strain of ethanolic mango peel and seed extracts was *S. aureus* [26, 27]. However, these results did not match with this present study for *L. monocytogenes*. The reason might be that the extracts used in previous studies were different in phenolic composition due to extraction methods. Besides, *S. typhimurium* showed growth onto plates at a lower concentration compared to the values of the other tested microorganisms, 2650 mg/l. This is determined that the concentration of 2650 mg/l is the MBC value of *S. typhimurium*.

In comparison to other antimicrobial agents, following

Table 2. Minimum bactericidal concentration of the MSK extract for tested strains.

Concentration of MSK extract (mg/l)	Growth on agar (MBC)				
	L. monocytogenes ATTC 13932	*E. coli* ATCC 25922	*S. aureus* ATCC 25923	*P. aeruginosa* ATCC 9027	*S. typhimurium* ATCC 14028
5300	+	-	-	+	-
2650	+	+	+	+	-
1325	+	+	+	+	+
660	+	+	+	+	+
330	+	+	+	+	+
165	+	+	+	+	+
80	+	+	+	+	+
40	+	+	+	+	+
20	+	+	+	+	+
10	+	+	+	+	+
Negative control	-	-	-	-	-
Positive control	+	+	+	+	+

(-): No growth
(+): Growth
MSK: Mango seed kernel

the results given by Abkhoo *et al.* [28] it could be seen that *Mentha spicata* extracts showed positive effects on the growth of *E. coli* (MIC = 25 mg/l, MBC = 50 mg/l) and *S. aureus* (MIC = 100 mg/l, MBC = 200 mg/l) compared with the MSKE which gave negative ones was MIC = 660 mg/l and MBC = 5300 mg/l for these bacteria. By contrast, the MSKE played a positive impact on the growth of *E. coli* (MIC = 660 mg/l), while that was not true for essential oil from *Cinnamomum zeylanicum* Blume (*Lauraceae*) showing MIC = 1120 mg/l for this microorganism [29].

Time-kill kinetics of mango seed kernel

The antimicrobial activity of MSKE against *L. monocytogenes* strain (Fig. 2) was different among three observed concentrations (MIC, 2-MIC, and 4-MIC). Specifically, the number of viable cells was kept unchanged after 6 h and 9 h of incubation at the extract treatments of 660 (MIC) and 1325 mg/l (2-MIC) compared to 0 h ($p > 0.05$). Additionally, the concentration of 2650 mg/l (4-MIC) has no effect on the number of *L. monocytogenes* viable cells after 24 h of incubation (2.29 ± 0.09 – 2.0 ± 0.09 log CFU/ml) ($p > 0.05$).

The extract at the concentration of 2650 mg/l inhibited the growth of *E. coli* at 24 h of incubation (Fig. 3).

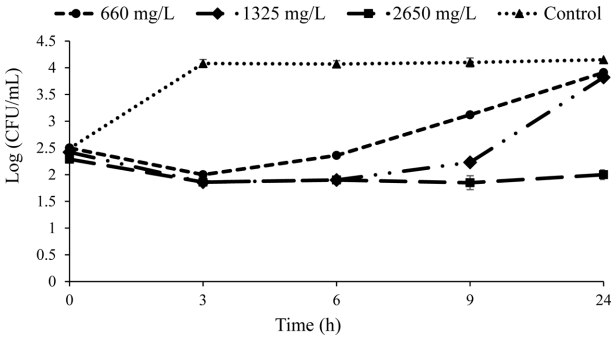

Fig. 2. Time-kill kinetics curve of *L. monocytogenes* (ATTC 13932). Results expressed as mean ± STDEV (n = 4); control referred as treatment without MSKE.

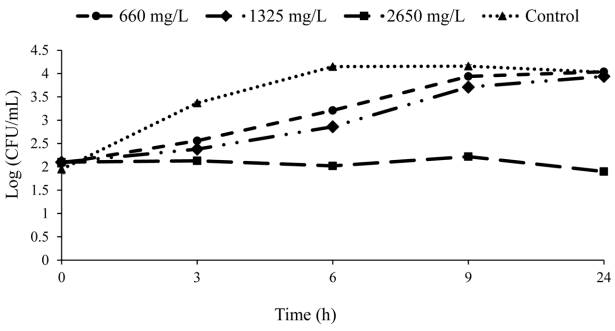

Fig. 3. Time-kill kinetics curve of *E. coli* (ATCC 25922). Results expressed as mean ± STDEV (n = 4); control referred as treatment without MSKE.

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Indeed, the CFU at 24 h was about 1.90 ± 0.06 log CFU/ml and 4.03 ± 0.05 log CFU/ml for the control sample ($p < 0.05$). By contrast, the remaining concentrations of MSKE showed the growth of *E. coli*, but the growth levels at 660 and 1325 mg/l were lower than that of control treatment at 6 h of incubation (3.21 ± 0.015 ($p > 0.05$), 2.86 ± 0.003 ($p < 0.05$), and 4.15 ± 0.008, respectively). After that, an increase in the CFU/ml to the same point as the control sample was observed at the end of the period.

For *S. typhimurium*, all three concentrations tested showed the same inhibition results in the growth of this bacterium (Fig. 4). Indeed, the number of *S. typhimurium* viable cells kept unchanged during 24 h of incubation, 1.40 ± 0.001, 1.70 ± 0.001, and 1.81 – 1.95 ± 0.048 log CFU/ml for 660, 1325, and 2650 mg/l, correspondingly ($p > 0.05$). Besides, there was the same growth inhibition among the three tested concentrations for *S. aureus* (Fig. 5). Specifically, the inhibition of all the MSKE treatments against *S. aureus* was only found at 9 h of incubation, and then the number of *S. aureus* viable cells increased rapidly to reach the highest point after the 24th incubation. This could be explained that at the first 9 h, the amount of phenolic in MSK extract still eliminated bacteria, but after 9 h these microorganisms adapted to the growth environment and had a chance to grow.

Another Gram-negative bacterium, *P. aeruginosa* (Fig. 6) was also affected by the MSKE. Indeed, the extracts of 1325 and 2650 mg/l (MIC and 2-MIC) inhibited the growth of viable cells of *P. aeruginosa* during 9 h of incubation, from 2.16 ± 0.0015 at 0 hour to 2.19 ± 0.04 log CFU/ml at 9 h ($p > 0.05$), and from 2.11 ± 0.04 at 0 h to 1.70 ± 0.001 log CFU/ml at 9 h ($p < 0.05$), respectively. Moreover, there was inhibition in the growth of this bacterium at the concentration of 5300 mg/l (4-MIC) after 24 h of incubation of about 2.2 log CFU/ml compared to the control treatment ($p < 0.05$).

Additionally, the findings in this study give the same result as a report of Appiah *et al.* [30] who showed a reduction in the number of viable cells by phenolic-rich extract from mushrooms over 4, 6 h for *S. aureus*, *E. coli*, respectively, and followed by a gradual increase to the highest point after 24 h. However, the inhibition time of mushroom extract was shorter than that of MSKE. This could be explained depending on the amount and type of phenolic compounds contained in the extract that affect the antimicrobial activity. It could be concluded that the inhibition or reduction in the number of viable cells of bacteria, in some cases, by MSKE might occur in the first hours of incubation (*i.e.* 6, 9 h), but some bacteria were inhibited during 24 h of incubation.

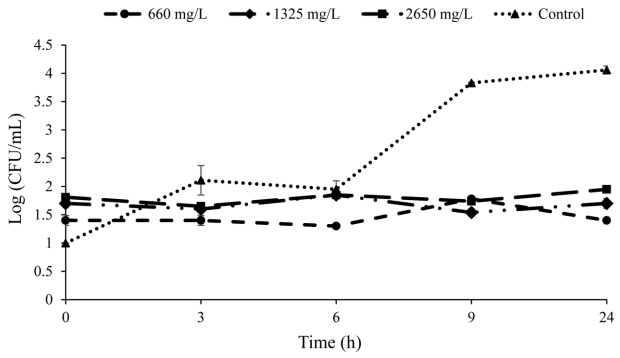

Fig. 4. Time-kill kinetics curve of *S. typhimurium* (ATCC 14028). Results expressed as mean ± STDEV (n = 4); control referred as treatment without MSKE.

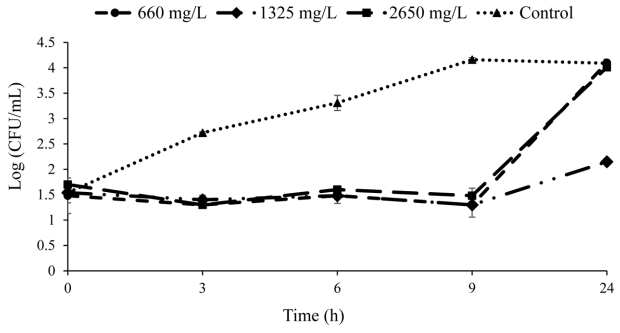

Fig. 5. Time-kill kinetics curve of *S. aureus* (ATCC 25923). Results expressed as mean ± STDEV (n = 4); control referred as treatment without MSKE.

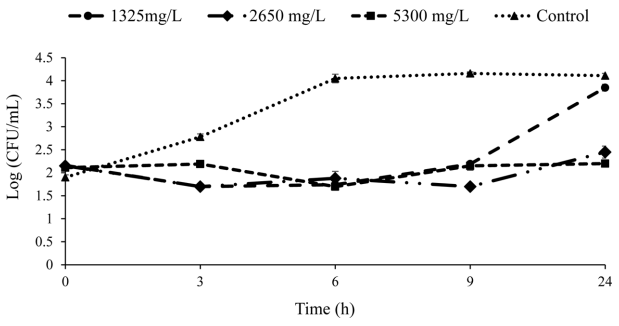

Fig. 6. Time-kill kinetics curve of *P. aeruginosa* (ATCC 9027). Results expressed as mean ± STDEV (n = 4); control referred as treatment without MSKE.

In situ antimicrobial activity

Mango seed kernel extract of 5300 mg/l inhibited the growth of *P. aeruginosa* after 4 days of storage (Fig. 7). The population of *P. aeruginosa* was unchanged during this period, from 3.48 to 3.64 ± 0.06 log CFU/g, while it was recorded the remarkable increase in the population of *P. aeruginosa* in control treatment by 1.23 ± 0.03 log CFU/g (from 3.48 to 4.71 CFU/g). In addition, the growth of bacteria could be affected by MSKE on the 7th day. Although there was a rise in the number of *P. aeruginosa* in the sample treated by the extract (4.63 ± 0.02 log CFU/g), this was lower than that of the control (5.98 ± 0.01 log CFU/g) ($p < 0.05$). This could be concluded that the MSKE inhibited the growth of *P. aeruginosa* until the 7th day, compared to the control treatment before reaching the highest point at the end of the period.

The number of total aerobic count (TAC) (Fig. 8) was affected by the MSKE. There was no significant difference in the number of TAC during 10 days of storage, from 4.99 ± 0.16 to 5.14 ± 0.09 log CFU/g, whilst the control treatment illustrated the upward trend in the CFU of TAC from 4.9 ± 0.02 to 6.6 ± 0.001 log CFU/g. This could be seen that MSKE inhibited the growth of aerobic bacteria by comparison with the control ($p < 0.05$), before getting the same point on the 14th day.

The MSKE had no effect on the growth of lactic acid bacteria (LAB) compared to the control treatment. Indeed, it was seen the reduction of 1 log CFU/g in both treatments. There was no significant difference between the extract and control ($p > 0.05$). This could be concluded that MSKE could not given any effect on LAB (Fig. 9). Engels *et al.* [31] also reported that gallotannins extracted from mango kernels (acetone MSKE) could not inhibit lactic acid bacteria growth.

The graph (Fig. 10) demonstrated the effect of MSKE on the growth of molds and yeasts in comparison to the control. It could be seen that MSKE eliminated the growth of yeast and molds during 10 days of storage

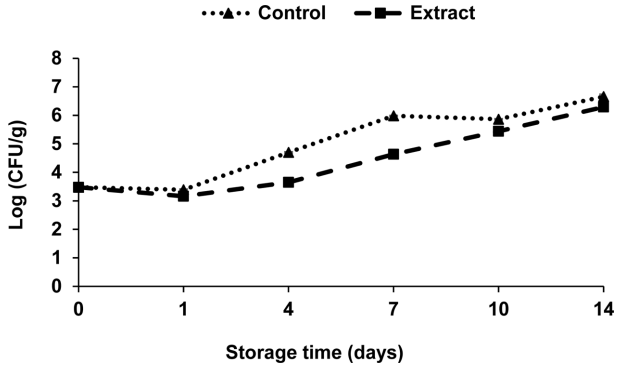

Fig. 7. Cell growth of *P. aeruginosa* (ATCC 9027) during storage time. Results expressed as mean ± STDEV (n = 4); control referred as treatment without MSKE.

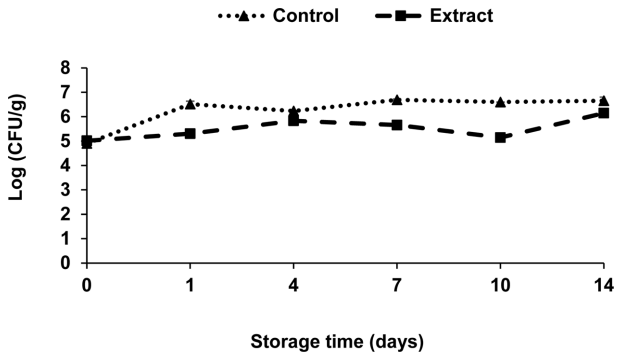

Fig. 8. The number of total aerobic bacteria during storage time. Results expressed as mean ± STDEV (n = 4); control referred as treatment without MSKE.

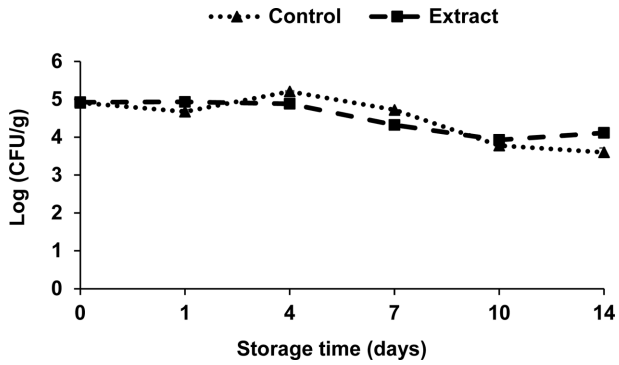

Fig. 9. Cell growth of Lactic acid bacteria during storage time. Results expressed as mean ± STDEV (n = 4); control referred as treatment without MSKE.

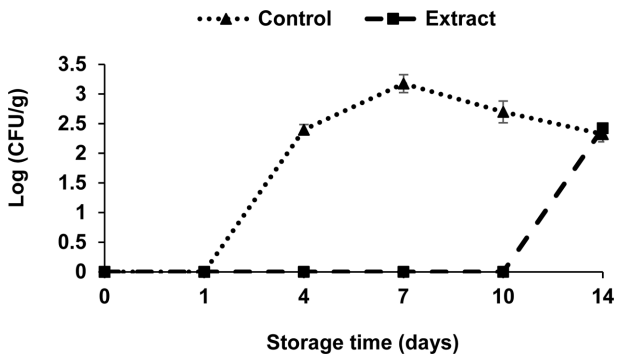

Fig. 10. The number of molds and yeasts during storage time. Results expressed as mean ± STDEV (n = 4); control referred as treatment without MSKE.

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before reaching the same point compared to the control. On the other hand, the appearance of molds and yeasts increased in the control treatment to get the highest point on the 4th day of storage before witnessing a fall down by approximately 1 log at the end of the period ($p < 0.05$).

In conclusion, ethanolic MSK extract showed antimicrobial activity at different concentrations (660–5300 mg/l) and incubation duration (0–24 h) against Gram-positive (*L. monocytogenes* and *S. aureus*) and Gram-negative (*E. coli*, *S. typhimurium*, and *P. aeruginosa*) microorganisms *in vitro*. The application of phenolic-rich extract from MSK on food matrices (*Pangasianodon hypophthalmus* fish fillets) during cold storage was effective against *P. aeruginosa*. Therefore, mango seed kernel by-products could be used as natural antibacterial substances source for extracting and applying for food/feed. However, further recommendations have been made regarding the effects of the antimicrobial of pure phenolic compounds on other aspects of the food as well, *i.e.* impact on oxidation, and sensorial evaluation.

Author Contributions

Dang Thi Thu Tam contributed to writing and original draft preparation and formal analysis. Ly Nguyen Binh contributed to conceptualization. Tran Chi Nhan and Nguyen Bao Loc contributed to the methodology. Nguyen Nhat Minh Phuong was responsible for writing, reviewing and editing as well as supervision, project administration, and funding acquisition.

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Conflict of Interest

The authors have no financial conflicts of interest to declare.

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