

Effect of Ethanolic Extracts from Fruit By-Products on Inhibition of Melanosis in Iced White-Leg Shrimps (*Litopenaeus vannamei*)

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Abstract

The purpose of this study was to investigate possibility of using natural extract from plant materials instead of synthetic chemicals for control of melanosis in iced white-leg shrimps (*Litopenaeus vannamei*). Shrimp is a highly perishable product with limited shelf life due to development of melanosis during cold storage. Extracts from mangosteen peel (*Garcinia mangostana L.*), grape seed (*Vitis vinifera*), mango seed (*Mangifera indica*), avocado seed (*Persea americana Mill*) and pomegranate peel (*Punica granatum L.*) were obtained by using ethanol 70%. The extraction yield, total phenolic content and antioxidant capacity, PPO inhibition capacity of the extracts were determined and discussed. Also, the effect of the extracts on preserving color in white-leg shrimps stored on ice was studied at a dose 7.5 and 15 g/L. All the extracts contained significant quantity of phenolic compounds, of which the mango seed extract was highest (419.9 mgGAE/g DM). The higher TPC, the higher DPPH inhibition capacity was observed for the extracts. In general, there was a correlation between antioxidant capacity and PPO inhibition capacity in the extracts, except for the case of avocado seed. The avocado seed extract showed lowest antioxidant capacity (25.85%) among the five, but highest PPO inhibition capacity (83.38%). All the extracts were effective for preventing color change in iced white-leg shrimps at a concentration of 15 g/L, comparably to that of sodium metabisulfite at a dose of 12.5 g/L in a 10-day storage period. It suggested that the extracts could be used as an effective alternative to SMS.

Keywords: Antioxidant; Fruit By-Product; Melanosis; Polyphenol Oxidase; White-Leg Shrimp

Introduction

Shrimp is the leading item in seafood production and export in some Asian countries and in the world. Some major species are white-leg shrimp (WLS), giant tiger prawn, and ocean shrimp. Shrimps are among the most perishable agricultural commodities. The shelf life is limited due to melanosis and microbiological deterioration. Melanosis is the development of "black spot" in certain crustaceans during post-harvest storage [1]. This process not only creates undesirable colors but also results in loss of nutrients, shelf life and market values of the shrimp products. In the US, each year there are 10% of the total export shrimps were sent back due to melanosis [2,3]. The cause of "black spot" development is the effect of the enzyme polyphenol oxidase (PPO), also known as tyrosinase, catechol oxidase, o-diphenol oxidase, monophenol oxidase [4]. It is present in and under the shell of shrimp, acting as a catalyst in the synthesis of melanin, a form of high-molecular-weight brown pigments. In general, in the presence of oxygen, PPO catalyzes the hydroxylation of monophenols to diphenols (monophenolase activity), which is then converted to highly colored quinones. Finally, quinones will react with amino acids to form complex brown polymers. On the other hand, catecholoxidases only catalyze the oxidation of o-dihydroxyphenols. The final product of these activities, the o-quinones react to O₂ undergoing further oxidation, this time without the presence of enzymes, and gives place to a high-molecular-weight insoluble polymer called melanin [5].

Many melanosis-controlling factors have been investigated, such as low temperature, cooking, high pressure, level of oxygen, and anti-melanosis agents such as chemicals or substrates rich in phenolics compounds [5]. The anti-melanosis agents, such as 4-hexyl-1,3 benzenediol (4-hexylresorcinol or 4-HR), sulphite-based compounds (such as sodium metabisulfite), and phosphates (such as sodium tripolyphosphate and sodium hexametaphosphate), have been intensively studied and proved to be effective to inhibit melanosis [6,7]. However, the chemicals mentioned above all have limited application essentially due to their toxicity with additional consequences on sensory characteristics. Sulfiting agents have several adverse effects such as allergic reaction and disturbances in asthmatic subjects [8]. Even though 4-HR does not present clear health risks, its use may negatively alter the taste of shrimp [9]. Along these lines, strict food safety regulations and consumer awareness has led to the search for natural alternatives for anti-melanosis [10].

Plant phenolics have been considered as potential natural additives, for the fact that they possess antioxidant and antimicrobial activities [11]. They are present naturally in all parts of plants, for example, grape, green tea and others, and render their inhibitory activity against PPO [9]. Several previous studies showed that mangosteen (*Garcinia mangostana L.*) peel, avocado (*Persea americana Mill*) seed, mango (*Mangifera indica*) seed, pomegranate (*Punica granatum L.*) peel and grape (*Vitis vinifera*) seed are rich in phenolic compounds such as flavonoids, carotenoids, tocopherols and sterols [12] and they are materials exerting anti-melanosis activity. In addition, all these materials are available in abundant as agri-industrial by-products [13]. Efforts of researchers have been attempted for utilization of natural alternatives in contrast to the conventional anti-melanosis techniques. Along these lines, the extract of mangosteen peel [1], *Garcinia cowa* leaf [14], grape seed [10], green tea [15], enokitake mushroom [16] and others have been studied in anti-melanosis research with a certain degree of success.

So far, data on control of melanosis by using plant extracts are still scarce. This study was aimed at investigation on possibility for utilizing the extracts from several common fruit by-products to inhibit the melanosis in iced WLS. The use of such agri-industrial by-products can greatly reduce the environmental burden.

Materials and Methods

Materials and chemicals

Materials such as mangosteen, grape, pomegranate, avocado and mango were bought from the local markets. White-leg shrimps with the size of 30 - 40 shrimps/kg were purchased from the local market. The shrimps were kept alive and transported to laboratory. Upon arrival, shrimps were washed in cold water and stored in ice until being treated (no more than 2h).

L-β-(3, 4 dihydroxyphenyl) alanine (L-DOPA), enzyme Tyrosinase (EC number 1.14.18.1) were purchased from Sigma Chem. Co. Chemicals and solvents for analytical tests such as 2, 2 - Diphenyl-1-picrylhydrazyl (DPPH) (Sigma, St Luis, MO, USA), Gallic acid (Merck), Folin-Ciocalteu (Merck), and ethanol (AR) were purchased from local agents. The solutions were prepared freshly before testing.

Material preparation and extraction

Procured fruits were washed, peeled, and separated for selecting the parts of interest such as seed or peel. The fresh fruit by-product samples (mangosteen peel, grape seed, pomegranate peel, mango and avocado seed) were sliced and spread on a tray in a thin layer and oven-dried at 50°C until the moisture content was below 10%. After that, the dried materials were ground in a Lab grinder (Phillips HR2115), sieved through a sieve with mesh No. 35 (0.5 mm). Extraction was carried out in a 250 mL beaker covered with aluminum foil. A sample of 10g of each powder was blended with 100 mL of ethanol 70% solvent, kept on continuous shaking in water bath at 40°C for 4h and let it sit at room temperature overnight. After extraction, the supernatant was filtered through Whatman paper No.2 by using a vacuum pump and adjusted to a volume of 100 mL. Then the total soluble solid (hence the extraction yield) was measured by evaporating

solvent from a volume of supernatant. The extracts were stored at 0°C until being used for chemical analysis.

Shrimp treatment

The treating solutions were prepared from the fruit by-product extract (FBE) at concentrations 7.5 and 15 g/L in water, and then cooled down to 4°C. Whole WLS was immersed in the FBE solutions (0, 7.5 and 15 g/L) at a shrimp/solution ratio of (1:2, w/v) at 4°C for 15 minutes. Treated shrimps were drained on the screen for 3 minutes. Shrimps treated in a solution without FBE were used as the blank and shrimps dipped in a solution with 12.5 g/L of sodium metabisulfite (SMS) were used as the control sample. All samples were stored in polystyrene box containing ice using a shrimp/ice ratio of 1:2 (w/w). To maintain shrimp/ice ratio, the molten ice was removed, and the same amount of ice was added daily [17]. Samples (4 shrimps) were taken for each treatment every 2 days up to 10 days for color measurement. The results of color measurement were used to illustrate the effect of FBE on the color of iced storage shrimp.

Physico-chemical analysis

Determination of total phenolic content

The method was taken from Sihrazi, *et al.* [18] with a slight modification. 1 mL of each FBE solutions were mixed with 5 mL of Folin-Ciocalteu reagent (diluted ten-fold) and allowed to stand for 3 minutes. Then 4 mL of sodium carbonate solution (75 g/L) was added to the reaction mixture. The absorbance was recorded after 30 minutes at 765 nm using US-VIS spectrophotometer. A standard Gallic acid curve was constructed by preparing the dilutions of (0, 20, 40, 60, 80, 100, 120, and 140 ppm) in 70% ethanol solvent. The total phenolic content of the extract was calculated by formula:

$$\text{TPC} = \frac{C \times V}{m}$$

Where TPC: Total phenolic content in mg GAE/g dry mass; C: Concentration of gallic acid established from the calibration curve in mg/g; V: Volume of extract in mL; m: Weight of the FBE in g.

Determination of DPPH free radical scavenging assay

A method in the literature [18] was used to determine antioxidant capacity of the FBEs by DPPH free radical scavenging assay with a slight modification. Briefly, a 0.2 mM solution of DPPH in 90% ethanol are prepared and then 3.9 mL of this solution are mixed with 0.1 mL of each extract solution (prepared at concentrations of 0.1 mg/mL in 70% ethanol). After 30 minutes incubation in the dark, the decrease in the solution absorbance were measured at 517 nm using US-VIS spectrophotometer. The percentage inhibition of DPPH by the extracts was calculated by using the following formula:

$$\text{Analysis: Inhibition (\%)} = \frac{A-B}{A} \times 100$$

Where A, the absorbance of pure DPPH; B, the absorbance of sample taken after 30 minutes of reaction with DPPH.

Determination of PPO inhibition effect of fruit by-product extracts

PPO activity was assayed using L-DOPA as a substrate according to the method of Nirmal and Benjakul [19]. The FBE was dissolved in distilled water at different concentrations (0.05, 0.1, 0.25, 0.5 and 1.0%, w/v). A volume of 100 µL of the FBE solutions were mixed with 100 µL of PPO solution, and the mixture was left for 30 minutes at room temperature. The assay buffer (400 µL, 0.05 M phosphate buffer, pH 6.0) was then added. To initiate the reaction, 600 µL of pre-incubated 15 mM L-DOPA (45°C) were added. The reaction was occurred at 45°C and the absorbance at 475 nm was monitored for 3 minutes. The control was run in the same manner, except the deionized water was used instead of FBE solution. One unit of PPO activity was defined as an increase in the absorbance by 0.001 per min/mL [20]. The

residual activity was calculated, and the inhibitory activity was expressed as the percentage inhibition which was used to determine the best concentration of FBE for PPO inhibition.

$$\text{Analysis: Inhibition (\%)} = \frac{A-B}{A} \times 100$$

Where A: PPO activity of control; B: PPO activity in the presence of FBE.

Color measurement of shrimp

Shrimp samples (treated by FBEs, SMS and pure water) were evaluated for color change in shrimp during storage. The color of the samples was measured by using a color analyzer probe meter (Lutron RGB-1002, Taiwan) and expressed in the CIE L*ab color scale. The total color difference was calculated and compared to that of the control and blank samples at the same storage conditions to evaluate the effect of FBE against melanosis.

The total color difference (TCD) was calculated as ΔE value, by the formula:

$$\Delta E = \sqrt{(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2}$$

Statistical analysis

Each treatment was done in triplicate and the obtained results were subjected to one-way analysis of variance (ANOVA), followed by Tukey's HSD multiple comparison test, using standard software SPSS, version 22. The significant difference in means was evaluated at $p < 0.05$.

Results and Discussions

Material preparation and extraction yield

The moisture content of the fresh fruit by-product samples was (in the decreasing order) 83.16, 64.84, 62.76, 58.62, and 52.38%, for pomegranate peel, avocado seed, grape seed, mangosteen peel, and mango seed, respectively. After drying in the oven at 50°C for 72h, all the moisture content values dropped down to below 10% (9.98, 9.17, 8.67, 4.96, and 4.14% for mango seed, grape seed, mangosteen peel, pomegranate peel, and avocado seed, respectively). The avocado seed gave the highest extraction yield, which was 39.17% (w/w). The lowest yield was of the mango seed 11.97%, which was higher than the yield reported elsewhere [21]. The extraction yield of the mangosteen peel was 20.83%, of grape seed 37.00% and of pomegranate peel 28.17%. The yield of extract from grape seed and pomegranate peel were higher than that from other studies in the literature [22,23]. The difference in the extraction yields may be due to the different solvent, source of materials and extraction procedure.

Total phenolic content

The TPC values in the five FBEs were significantly different (Figure 1). The mango seed gave the highest TPC which was 419.9 mg GAE/g DM. The TPC of avocado seed was 126.82 mg GAE/g DM which was the lowest TPC value. The TPC of mangosteen peel was 245.78 mg GAE/g DM and the TPC of pomegranate peel was 225.07 mg GAE/g DM. Lastly, the TPC of grape seed was 324.6 mg GAE/g DM. The results are well comparable well with data in the literature. For instance, The TPC value of the mango seed extract was higher than that reported elsewhere [21], which was 53.5 mg GAE/g DM. The TPC of mangosteen peel was comparable to that in Cheok, *et al.* [24], which was 245.78 mg GAE/g DM and the pomegranate peel extract had similar TPC value to the result by Fawole, *et al.* [25], which was in a range 179 - 295 mg GAE/g DM. The avocado seed extract TPC value was 126.82 mg GAE/g DM, higher than that reported in Gómez, *et al* [26].

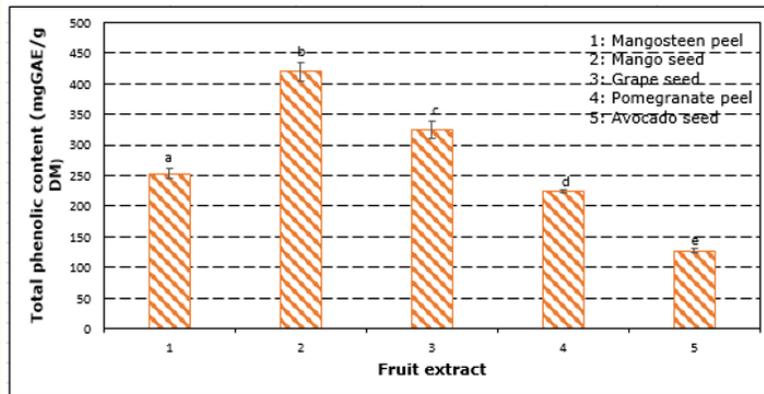


Figure 1: Total phenolic content of fruit by-product extracts.

DPPH free radical scavenging activity

The five FBEs were significantly different in antioxidant capacity (Figure 2). The mango seed extract expressed the highest scavenging capacity (71.58%), as compared to 62.51% as reported in the literature [27]. The avocado seed extract showed the lowest scavenging capacity, 25.86% which was comparable to 27.3% as reported by other authors [28]. In the decreasing order, the grape seed, mangosteen peel and pomegranate peel expressed the value of DPPH scavenging capacity of 45.62, 41.38 and 35.82%, respectively. For comparison, Fawole, *et al.* [25] reported that the DPPH scavenging capacity of methanolic extract from pomegranate peel was 12.34%.

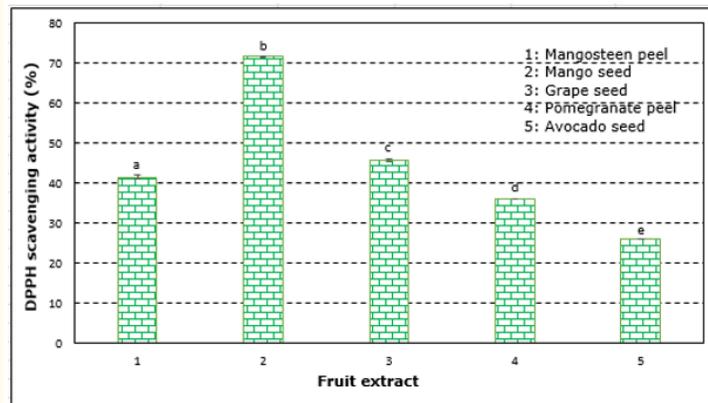


Figure 2: Antioxidant capacity of fruit by-product extracts.

Effect of the fruit extract on the inhibition of PPO

The Polyphenol oxidase (PPO) inhibition effects of the five FBEs were monitored at five different concentrations as showed in figure 3. In the absence of inhibitor, PPO catalyzes oxidation of DOPA to DOPA-quinone which subsequently undergoes polymerization to produce melanin [20]. Increase in the concentration of fruit extracts resulted in higher level of PPO inhibition. The highest % PPO inhibition

belongs to the avocado seed extract, which was 83.38% and the lowest is the pomegranate peel extract, which was 28.61%. The % PPO inhibition of pomegranate peel extract are comparable to the previous study [29]. Polyphenols are considered as the primary category of inhibitors of polyphenol oxidase [25]. Phenolic compounds inhibit PPO activity by interacting with the active site of the enzyme. Some phenolics might be able to chelate a copper ion at the active site of the PPO [19]. Furthermore, compounds like tannins in the extract could precipitate PPO (proteins), thus inhibiting PPO activity in the solution [25]. The strong antioxidant phenolics could also act as a strong reducing agent and oxygen scavenger, hence lowering the oxygen concentration, which is needed for pigment development in melanosis. These activities could be associated with the lowered blackening caused by PPO in shrimp during extended refrigerated storage [15]. Thus, all the five FBEs exhibited significant polyphenol oxidase inhibition and showed potential for being used as a preservative to inhibit the melanosis in WLS.

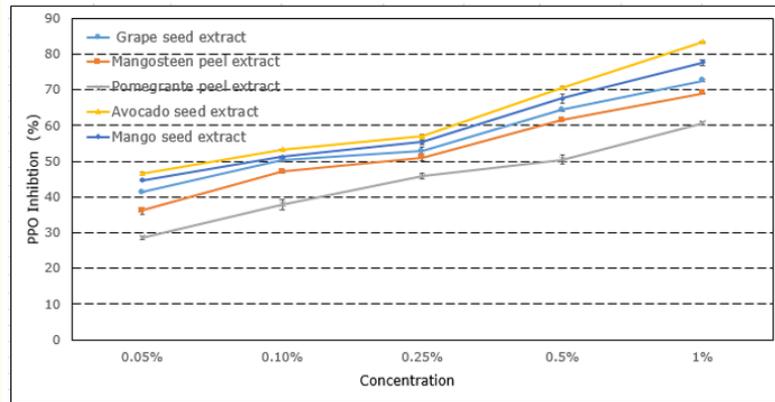


Figure 3: PPO inhibition capacity of fruit by-product extracts.

The PPO inhibition capacity among the five FBEs was observed in decreasing order as follows, avocado seed > mango seed > grape seed > mango peel > pomegranate peel. There was a trend for correlation between antioxidant capacity and the PPO inhibition capacity, the extract with high antioxidant capacity will result in high rate of PPO inhibition, similarly to findings in the literature [17,30]. The antioxidant capacity, in turn is correlated to the presence of phenolic compounds in the extracts. However, there is a particular case, that the avocado seed extract showed very high % PPO inhibition capacity despite of low antioxidant capacity. This may be due to substances other than phenolics in avocado seed, suggesting a good potential of this kind of material for use in controlling melanosis.

Color change in white-leg shrimps during iced storage

The color change in the shrimps was measured as total color difference (ΔE) at different storage periods, compared to the color values at zero time of treatment (Figure 4a and 4b). At beginning, the shell appearance of shrimps was quite dark, showing low value of L^* (about 18.0). After 2 days of storage on ice, the appearance of the shrimps became lighter than at day 0 (L^* value around 45.0), which lead to high TCD of all samples at day 2 except from the blank. The TCD value of the blank was low due to early development of melanosis on the shrimp shelves at beginning of storage [31]. The overall trend was that the TCD values in all samples treated with the FBEs and SMS decreased to the end of storage period (10 days), meanwhile, the TCD value of the blank sample increased significantly at the end, as the melanosis developed. At the end of iced storage period (day 10), all shrimp samples treated with FEWs at a ratio 7.5 g/L showed small change in color (low values of TCD), in a range of 3.0 - 6.0, lower than that in the control sample (SMS-treated). On the course of storage time, avocado seed seemed to be most effective, and pomegranate peel least, meanwhile the effects of mango seed, grape seed, mangosteen peel were comparable. However, at the end, their effects were not significantly different, but better than the control sample. The order of effectiveness of the extracts was in consistency with the results of their effect on PPO inhibition capacity.

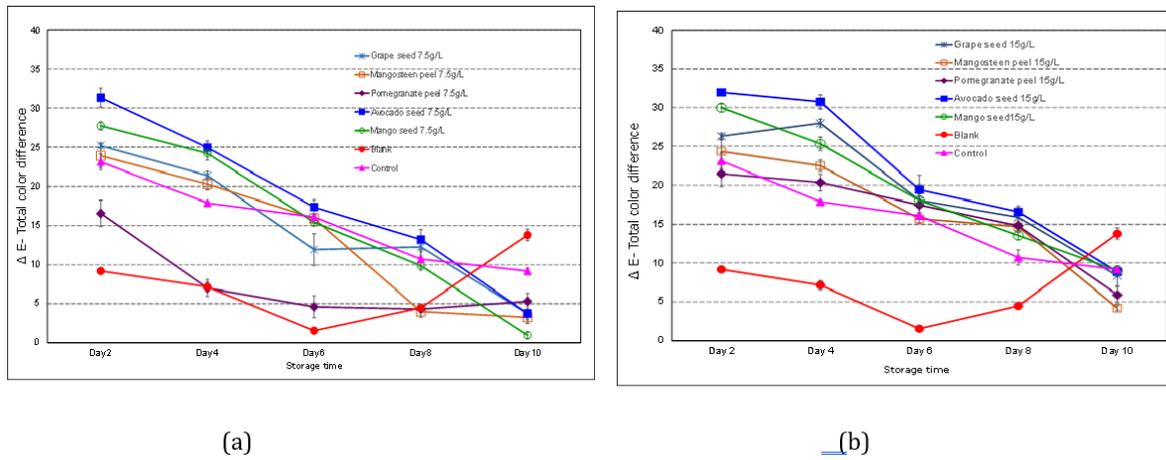


Figure 4: Color change in shrimps treated in 7.5 g/L (a) and 15 g/L (b) FBE solutions.

Similar trend was observed for treatments at the extract concentration of 15 g/L (Figure 4b). The TCD values of all samples including the control one was decreasing and converging at day 10 to a range of insignificant difference. Meanwhile, the TCD value of the black sample was increasing to a significant higher value, indicating the cause of melanosis development in shrimps, and hence the color change. As mentioned above, shrimps become light pale on day 2 of storage, obviously because of antioxidants in the extracts on the shrimp shells. This effect was higher at higher extract concentration, as the TCD values at concentration of 15 g/L was higher than that at 7.5 g/L. It was decreasing towards the end of day 10. As for comparison, Phan and Do [1] reported mango seed and mangos teen peel extracts were effective in melanosis control, but at quite higher dose (about 67 g/L or ratio of extract-to-water as 1:15). On the other side, Nirmal and Benjakul [19] concluded that *Leucaena leucocephala* seed extract was effective against melanosis at a dose of 5 g/L for a period of 12 days storing on ice. Fang, *et al.* [30] also found that the pomegranate peel extract was effective at concentration of 7.5 - 15 g/L. It is necessary to note that the degree of melanosis was evaluated in this study by measuring color indices only. It is recommended that the color measurement would be done in combination with evaluation of melanosis score by a sensory panel, as conducted elsewhere [17, 30]. It was possible to conclude that, all the FBEs in this study were effective to control melanosis at concentration 7.5 - 15 g/L (except for the pomegranate peel extract, it should be used at 15g/L) as an alternative to replace SMS at 12.5 g/L. Among them, avocado seed could be a material of choice since it gave high extraction yield and showed high PPO inhibition capacity at the same time.

Conclusion

Extraction of several fruit by-products, namely avocado, grape, an mango seed; pomegranate and mangosteen peel, using ethanol 70% as a solvent resulted in considerably high yield of extracts, ranking in decreasing order as avocado seed > grape seed > pomegranate peel > mangosteen peel > mango seed.

All the extracts contained significant amount of phenolics. The TPC was observed in decreasing order as the mango seed > grape seed > mangosteen peel > pomegranate peel > avocado seed extracts. Furthermore, there was a certain degree of correlation between the antioxidant activity and total phenolic content. The decreasing order in antioxidant capacity of the extracts was the mango seed > grape seed > mangosteen peel > pomegranate peel > avocado seed, like the order in TPC.

It was a correlation between antioxidant capacity and the PPO inhibition effectiveness. The extract with higher antioxidant capacity resulted in higher PPO inhibition capacity and lower in total color difference, except for the case of avocado seed. In the avocado seed extract, there may exist substances other than the antioxidants, which have positive effect in PPO inhibition capacity.

All the fruit by-product extracts showed positive effect in control of color change in WLSs stored on ice, treated at a dose of 7.5 and 15 g/L. The effect was similar and comparable to that of sodium metabisulfite (SMS) at a dose of 12.5 g/L. All the extracts could be used as a natural alternative anti-melanosis agent.

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