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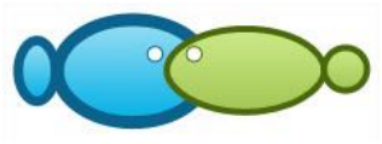

Anthocyanin extract of *Rosa* sp. as a natural preservative in *Euthynnus affinis*

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Abstract. In southeast Asia, the red rose (*Rosa* sp.) is among the flowers that can be found in abundance. The rose is primarily utilized for decoration and aroma, meanwhile, it has a lot of medicinal functions. Extract of rose petals has been reported to show antibacterial and antioxidant activity. Red rose as an antioxidant and antibacterial should be equal with its potential as a natural food preservative. This study aimed to develop anthocyanin's antibacterial and antioxidant activity from red rose as a natural preservative. The results of LC-MS (Liquid Chromatography-Mass Spectrometry) analyzes showed the presence of pelargonidin 3-O-(6-O-malonyl-β-D-glucoside) and cyanidin 3-(6"-malonylglucoside). *Rosa* sp.extract, when tested on *Pseudomonas* sp., is a good bactericide. The MBC analysis showed a bacterial density of 0 CFU mL^{-1} with all red rose concentrate concentrations (100, 50, 25 and 12.5%). The results of the variance analysis showed that the red rose concentrate had a significant effect on the moisture content, but no significant effect on the protein and fat content of tuna for both 0 and 12 hours of fish storage. In fish treated with rose extract with 2 days shelf life and rose extract with 4 days shelf life there were no bacteria grown for 12 hours, and for the fresh rose extract treatment bacterial colony grew starting from the 6th hour.
Key Words: anthocyanin, pigment, red rose, natural preservative, tuna.

Introduction. Tuna (*Euthynnus affinis*), a common seafood, is mainly consumed fresh. Therefore, redness, aroma, microbial composition and consistency are significant factors for illustrating its quality (Jiang et al 2019). Moreover, tuna quickly deteriorates because of its chemical compositions, handling and microorganisms (Ghaly et al 2010; Hizbullah et al 2019; Semeano et al 2018), which changes taste, odor and texture. The high moisture content, nutrients and pH are believed to cause the deterioration process (Suyani et al 2020). Consequently, many studies have been conducted to find preservation agents for inhibiting the microorganism's growth (Gómez-Estaca et al 2010; Rajesh et al 2002; Suyani et al 2019). A food preservative is a natural or artificial substance that prevents food degradation caused by oxidation, microbial growth, and enzyme activity (García-García & Searle 2016; Kalpana & Rajeswari 2019; Wedzicha et al 1991). Lately, many researchers have been more concerned about utilizing natural resources to preserve matter than synthetic preservatives for dealing with the microbial and chemical processes leading to spoilage in fish (Abdel-Wahab et al 2020; Mei et al 2019; Suyani et al 2020). Artificial preservatives use generates severe diseases, such as hypersensitivity, asthma, cancer and other diverse effects (Anand & Sati 2013; Linke et al 2018; Mir & Razvi 2016). Therefore, natural matters, such as plant extracts and essential oils, become a considerable ingredient used as a preservative agent to replace chemical preservatives (Olatunde & Benjakul 2018). According to Hassoun & Emir Çoban (2017) and Sofi et al (2016), they have suitable substances, including antioxidant and antibacterial properties that inhibit deterioration of fish products. For instance, a 3% tulsi extract could keep *Thunnus obesus* chunk quality during the chilling process (Suyani et al 2020). The combination of clove oil and chitosan, studied by Vieira et al (2019), could decrease lipid oxidation and inhibit microbial growth in *Colossoma macropomum* fillets during the frozen storage.

Based on this evidence, red rose (*Rose* sp.) might potentially be the right preservative agent for keeping the fish product's quality. *Rosa* sp. is one of the flowers that was abundantly found in South East Asia (Takahashi et al 2020). They are mostly used as decoration, fragrance, food ingredients and even considered as a medical treatment (Naveena & Thamaraiselvi 2020). Some studies have reported that the rose extract has antibacterial and antioxidant Activity (Abdel-Hameed et al 2013; Khoo et al 2017; Yi et al 2007). In addition, *Rose* sp. contains more antioxidants than vitamin A and vitamin C combined. (Roman et al 2013). Meanwhile, its antibacterial activity has been studied in some pathogens, including bacterial peritonitis (El-Shouny et al 2016), *Bacillus subtillis*, and *Escherichia coli* (Ruban & Gajalakshmi 2012), *Pseudomonas aeruginosa*, *Chromobacterium violaceum*, *Staphylococcus aureus* and *Erwinia carotovora* (Ulusoy et al 2009). Moreover, according to Tatke et al (2015), the presence of flavonoid and phenolic compounds in 40 mg of the *Rosa damascena* flower extracts possess antioxidant, antibacterial and antifungal activities to *Streptococcus pyrogens*, *S. aureus*, *E. coli*, *Clostridium perfringens*, *Aspergillus niger*, *P. aeroginosa*, *Klebsiella aerogens*, *K. pneumonia and Candida albicans*. According to Ma et al (2019), it can scavenge oxygen free radicals in vitro and sequester oxygen and hydroxyl radicals, acting as an antioxidant to eliminate reactive oxygen species (ROS) following infections with *Escherichia coli* and *Salmonella*. Furthermore, it works better at higher concentrations to reduce the value of SGOT (serum glutamic oxaloacetic transaminase) on rats, when administered with CCl_4 (Saati et al 2018). Besides, it contains precious compounds, such as: terpenoids (Ragasa et al 2007; Saaby et al 2011), glycosides, flavonoids (Wan et al 2019) and anthocyanins (Knapp et al 1998).

Anthocyanins, known as flavonoid classes, are water-soluble and have a diverse spectrum, including blue, purple and red coloring, found in the different plant parts (Cisowska et al 2011; Katsumoto et al 2007). Anthocyanin synthesis (like other flavonoids) develops in the reticulum endoplasma and is accumulated in the large central vacuole (Grotewold & Davies 2008). Moreover, they have been studied to have preservation ability in different food products (Sun et al 2018; Thach-Nguyen et al 2020; Wen et al 2016). Therefore, a red rose might be a potential natural food preservative agent based on its antimicrobial and antioxidant properties. This study aimed to investigate whether *Rose* sp. could preserve *E. affinis*.

Material and Method

Fish and plant extraction. All preparations were conducted in the Food science laboratory, University of Muhammadiyah Malang. First, 1 kg of *E. affinis* was obtained from Merjosari market, Malang, East Java, Indonesia. Afterward, it was cleaned and slashed into a cube (around 5 g) before washing it with tap water several times. In the next step, the chunks were divided into four groups containing 5±0.1 g of pieces and promptly cooled before being treated in red rose extract solution. Meanwhile, the red rose was prepared with red roses extract treatments of a different shelf life, of: 0 hours (T1), 48 hours (T2) and 96 hours (T3). Furthermore, anthocyanin extraction of red rose petals was used to prepare the three life shelves food preservative treatmentss. In the first step, 45 g of the rose petals were crushed with a blender and macerated using 300 mL aquadest mixed with 1% citric acid at room temperature. After 24 hours, the anthocyanin extract was strained with filter paper and the filtrate was poured to a glass container. Finally, the filtrate was evaporated 3 to 4 times using a rotary evaporator at 50 to 60ºC.

Antibacterial analysis. This study used *Pseudomonas* sp. grown on MHA (Mueller Hinton Agar) in an incubator (37°C) for 24 hours. Afterward, sterile water was used to dilute the bacteria, in reference to the McFarland standard (10^8 CFU mL^{-1}), and then it was dissolved again on MHB at 10^6 CFU mL^{-1}. Following the next step, the anthocyanin extract was applied to the test tube containing the bacterial sample, incubated at 37ºC for 18 hours to 24 hours and the colony was counted. Furthermore, MBC analysis used four differents red rose extract concentrations: 100, 50, 25 and 12.5%.

Antioxidant activity analysis. The analysis followed Kedare & Singh (2011) and Fatiha & Abdelkader (2019). One of the most commonly used methods for testing the red rose antioxidant activity is the DPPH (2,2-diphenyl-2-picrylhydrazyl). The first step was to dilute 1 mL of red rose extract in 9 mL of 96% ethanol. On methanol 0.20 M, 4 mL of the sample was added to 1 mL of DPPH, and the mixture was then run. Finally, after 10 minutes, a spectrophotometer at 517 nm wavelength measured the absorbance of the sample (Fatiha & Abdelkader 2019).

$$\text{Antioxidant activity (\%)} = 100 \times \left(1 - \frac{Absorbance\ sample}{Absorbance\ control}\right)$$

Anthocyanin identification. The identification used LC-MS (Liquid Chromatography-Mass Spectrophotometry) method, demonstrated by Vagula et al (2018), with some modifications. Briefly, it was performed on a Shim Pack FC-ODS (2 mmD x 150 mm, 3 µm) using a CH_3CN (0.1% TFA)/H_2O (0.1% TFA) solvent.

Euthynnus affinis preservation. The fish meat was marinated in red rose extract for 10 min and rinsed. Furthermore, the fish meat was stored in a clean and dry container for 12 hours. Finally, the marinated fish meat was analyzed after 0 h, 6 hours and 12 hours storage time for determining the moisture, protein, fat content and total plate count (TPC).

Data collections. Moisture content was determined following the AOAC (1995) method. Firstly, 1 to 2 g sample was dried in the oven from 100 to 105ºC for 3 to 5 hours. After that, dried samples were cooled using a desiccator and then it was weighted. Furthermore, the samples were heated again in the oven for 30 min and weighed to constant weight. Finally, the moisture level was calculated using the formula (AOA 1995):

$$\text{Moisture (\%)} = \left(\frac{\text{Water content}}{\text{Sample weight}}\right) \text{x } 100$$

The protein content analysis followed the standard operation of the Biotechnology Laboratory, University of Muhammadiyah Malang. Briefly, 1 g sample of fish meat was placed in a tube and then 9 mL ddH_2O were added. Afterwards, the sample was shaken several times before it was filtered and carefully moved to another tube. Following the next step, 1 mL sample was diluted in 9 mL ddH_2O and 5.5 mL of Lowry B solution were added. After being gently homogenized for several minutes, the mixture was kept at room temperature for 10 to 15 minutes. Furthermore, the mixture was reacted with 0.5 mL of Lowry A solution and quickly shaken. The mixture was left for 30 min until the color changed to blue. In the last step, the protein content was measured using the spectrophotometry at 660 nm wavelength.

Moreover, the fat content analysis used a method from AOAC (1995), with some modifications. Briefly, 2 g of sample were powdered and transferred to a tube before being reacted with 4 mL ethanol 96% and 2 mL HCl (1:4), respectively. Afterward, the mixture was heated in the water bath at 70 ºC for 30 min and cooled naturally. Furthermore, the solution was poured into a separating funnel at the top of which a funnel and filter paper were placed to filter the sample. Following the next step, 20 mL petroleum ether and 20 mL aquadest were added to the sample and filtered until the solution was no longer dripping. The mixture was then shaken for 1 min and let separated to be two layers. The bottom layer was removed until only remained the top layer (fat ether phase). After that, the top layer was placed into a weighing glass before it was dried in the oven. Fat content (%) was calculated using a formulation (AOA 1995):

$$\text{Fat percentage } (\%) = \left(\frac{c-b}{a} \times 100\%\right)$$

Total bacteria determination or total plate count (TPC) followed a SOP of the Biomedical Lab, Faculty of Medicine, University of Muhammadiyah Malang.

Statistical analysis. All data were analyzed using the SPPS program's statistical analysis (ANOVA). In addition, the Duncan's Multiple Range Test was used to examine the differences between treatments.

Results

Identification with LC-MS. This study attempted to analyze red rose anthocyanin using the liquid chromatography–mass spectrometry (LC–MS) method. Moreover, the LC-MS data consisted of the peak figure, retention time, molecular weight and the compound name identified with the LC-MS. Furthermore, the present study could only observe two different anthocyanin types in the rose petals (Table 1).

Table 1

Chromatogram resume report

Peak	*RT (min)*	*Formula*	*Molecule weight (m/z)*	*Name*
1	12.387	$C_{24}H_{23}O_{14}$	519,43	Pelargonidin 3-O-(6-O-malonyl-β-D-glucoside)
2	17.833	$C_{24}H_{23}O_{14}$	535,43	Cyanidin 3-(6-malonylglucoside)

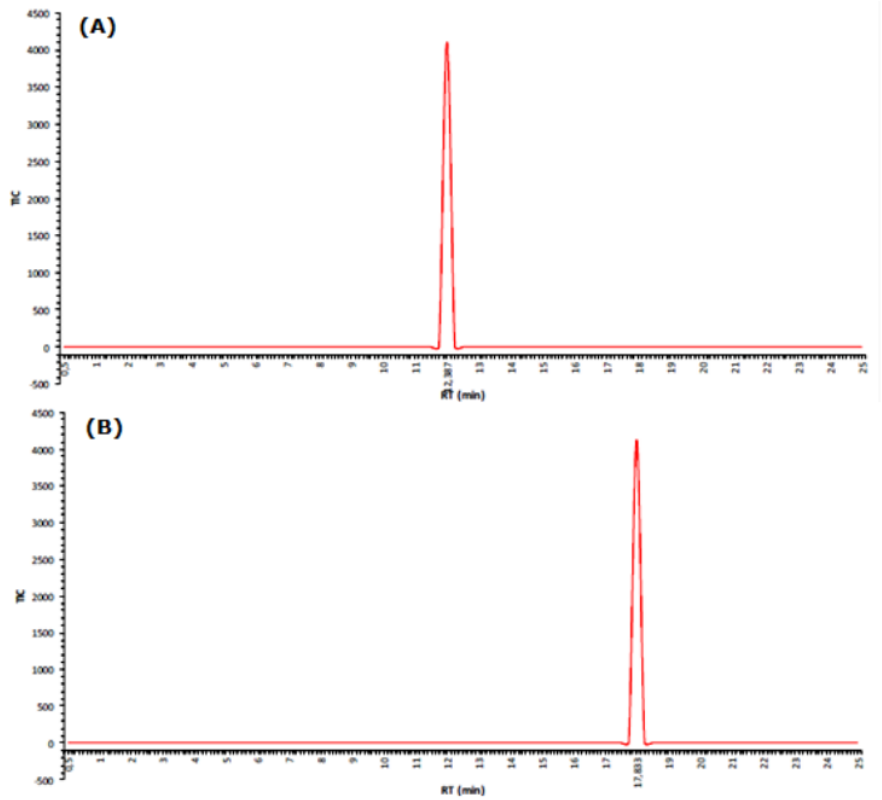

Figure 1. Chromatogram of anthocyanin pigment of rose (A) Pelargonidin 3-O-(6-O-malonyl-β-D-glucoside; (B) Cyanidin 3-(6-malonylglucoside).

Figure 1 revealed that anthocyanin pigment's retention times (RT) were 12.387 and 17.833 min (Table 1). Meanwhile, the peaks showed the presence of pelargonidin 3-O-(6-O-malonyl-β-D-glucoside) with a mass divided by charge number (m/z) of 519.43 and cyanidin 3-(6"-malonylglucoside) with an m/z of 535.43. They are compounds of the

anthocyanin group that were successfully extracted by the acid addition, so that they were stable. The rose extract was tested for antioxidant and antibacterial activity.

Red rose extract's minimum bactericidal (MBC) analysis. The red rose antibacterial properties were analyzed through the minimum bactericidal concentration (MBC) method against the *Pseudomonas* sp. According to the regulations of the Biomedical Laboratory of the Faculty of Medicine, University of Muhammadiyah Malang, the red rose extract can still be declared as an antibacterial when the MBCs value of 1% and the minimum MBC value of *Pseudomonas* sp. is 2.41 ± 10^8 CFU mL^{-1}. As a result, all concentrations of red rose (100, 50, 25 and 12.5%) were able to inhibit *Pseudomonas* sp growth down to 0 CFU mL^{-1}. Interestingly, the MBC value decreased as the red rose extract concentration increased (Figure 2).

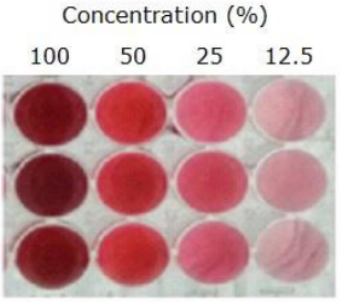

Figure 2. Red rose extract MBC analysis on *Pseudomonas* sp.

The TPC value presented in Table 2 provides information on the differences among the treatments, with their replications. All data are measured in CFU mL^{-1}. Overall, all treatments have a better result than the control group (T0), inhibiting the growth of *Pseudomonas* sp. Furthermore, T2 and T3 are determined as great antibacterial materials, through MBC analysis.

In the first observation, 0 h after preservation, the data are significantly different among treatments. T1, T2, and T3 could inhibit *Pseudomonas* sp. replication directly to 0 CFU mL^{-1}. Meanwhile, the bacteria started to grow at 5.4 ± 10^7 CFU mL^{-1} in T0, in the first record. Following the next sampling time, T2 and T3 were superior to others, with 0 CFU mL^{-1} of bacterial concentration, while T1 could not inhibit *Pseudomonas* sp growth (6.1×10^7 CFU mL^{-1}). It was believed that the antibacterial compound of T1 worked only during the first hour after the preservation treatment application. After 12 h, there is a surge in the TPC of T1 by over 100 fold. In contrast, T2 and T3 could completely inhibit the *Pseudomonas* sp development.

Table 2

TPC post-treatment

Treatment	*TPC (CFU mL^{-1})*		
	0 hour	*6 hour*	*12 hour*
T0	5.4×10^7	8.3×10^9	5.2×10^{13}
T1	0	6.1×10^7	1.2×10^9
T2	0	0	0
T3	0	0	0

Euthynnus affinis product preservation. Each red rose extract was applied in *E. affinis* meat for 0 hours, 6 hours and 12 hours in four different treatments, including T0 (without red rose extract), T1, T2 and T3. The variance analysis showed that the red rose concentrate has a considerable effect ($p<0.05$) on the moisture content (Figure 3), protein (Figure 4) and TPC (Table 2). In contrast, only the fat content was identified as

not significant. The data mainly fluctuates from 0 hours to 12 hours for the four treatments using red rose extract. Overall, T2 and T3 were decided as recommendable treatments for keeping fish quality and killing microorganisms.

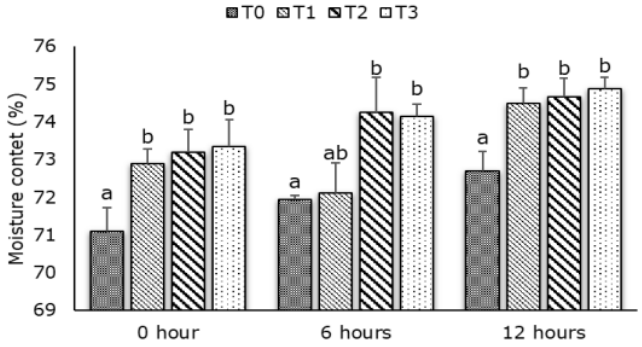

Figure 3. The moisture content of *Euthynnus affinis* post-treatment.

Figure 3 reveals the moisture content of *E. affinis* meat after the preservation process using different red rose extracts. To sum up, T2 and T3 were the best treatments that accelerated the moisture content of *E. affinis* meat after storage, and T0 is the worse option. After 0 hours, T1, T2 and T3 showed no difference (around 72.6% moisture), but they were significantly differentcompared with the control group (T0) (by 1.26%). Furthermore, the moisture content of *E. affinis* meat increased in T2 and T3 after 6 hours of preservation (74.26% versus 74.16%), while there was a moderate decrease to 72.16% for T1. Finally, 12 hours of observation presented a positive trend for T0, T1, T2 and T3, accounting for around 72.27, 74.5, 74.66 and 74.88%, respectively. Moreover, T3, T2 and T1 took the first places, while T0 was the last.

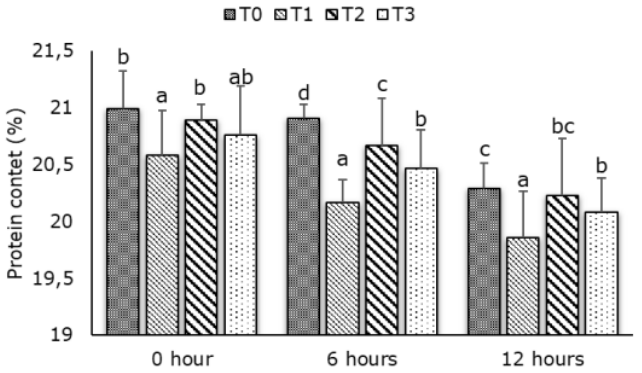

Figure 4. The protein content of *Euthynnus affinis* post-treatment.

All treatment's protein percentage is displayed in Figure 4, revealing a fluctuating trend, with no pattern between treatments. Overall, however, all data had a drop pattern until the end of observation. Besides, T0 remains the most considerable method of preservation for keeping the fish product quality, while T1 was unrecommended. Treatment 0, the highest level, decreases slightly from 0 hours to 6 hours of observation by 0.8%, before a dramatic decline to 20.29%. In the median position, T2 and T3 have statistically similar protein contents between 0 and 12 h hours of preservation. However, the T2 value exceeds T3's by 0.2% in the 6 hours after applying the red rose extract as a preservation material. Furthermore, there is a moderate decline in T1 protein percentage at the lowest level, accounting for 19.68% at the end of the period.

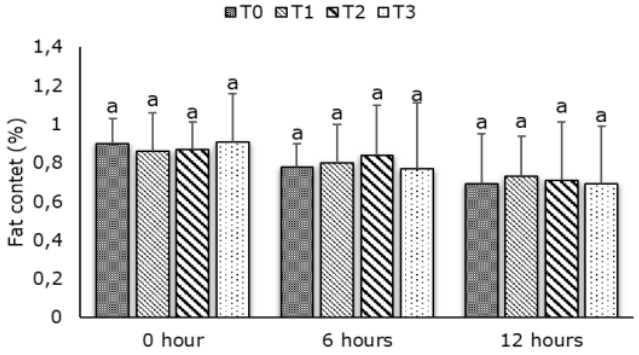

Figure 5. The fat content of *Euthynnus affinis* post-treatment.

On the other hand, fat results in all treatments patterns could not be inferred because for all data there were found "no significant differences" ($p>0.05$) between treatments, over time (Figure 5). Therefore, the application of red rose extract from various red rose life shelves did not affect the level of *E. affinis* fat during preservation.

Discussion

Anthocyanin is one of the flavonoid groups obtained from anthocyanidins by adding a sugar material (Cisowska et al 2011). They are water-soluble colors located on the cell vacuole that change of color depending on the concentration of the surrounding environment. They can be found in various plants' flowers, leaves, fruits, stems, and roots. (Santos-Buelga & González-Paramás 2019). Moreover, their chemical structure consists of an oxygenated heterocycle and two benzene rings (Bueno et al 2012). Furthermore, using LC-MS analysis, the present study found that pelargonidin-3-O-malonylglucoside and cyanidin-3-O-glucoside make up most of the overall anthocyanin content in red roses (Figure 1). They are found in 80% of pigmented leaves, 69% of fruits and 50% of flowers (Giuliani et al 2016). According to a study in the 1970s, paper chromatography of tetraploid rose cultivars showed variations in anthocyanidin quantities (pelargonidin and cyanidin) (Debener 2017). On another review, most wild-growing Rosa species are composed of two types of glycosides, such as cyanidin and peonidin, which are the major components (Mikanagi et al 2000). Besides, a few wildflower varieties also make up commercial cultivars for these components, like 3,5-di-diglycoside and pelargonidin 3,5-diglycoside (Tanaka et al 2017).

In the case of TPC, all extracted red rose with various life shelves worked for killing *Pseudomonas* sp., which is the key organism responsible for the degradation of food proteins. They are often separated from spoiled seafood and cause slime and odors (Tsironi et al 2020). Cendrowski et al (2020) studied the aqueous *Rosa rugosa* extract containing anthocyanin, which inhibited *Pseudomonas aeroginosa* replication by applying around 30 mg mL^{-1} extract. In vitro, anthocyanins can eliminate reactive oxygen species and sequester free radicals, such as hydroxyl radicals (Ma et al 2019). Moreover, anthocyanins also had effective anti-toxic properties that inhibited the Vtx1 and Vtx2 genes of bacteria. At the same time, they worked in conjunction with hydrophobic agents to break down the cell membranes by hydrophobic interactions (Doughari et al 2012). Finally, all aqueous extract of red rose was tested on *E. affinis* fillet as a preservative material. As a result, all treatments reveal no significant difference in the fat content, but show significant protein and moisture differences, compared to the control group (T0). Interestingly, the moisture content of *E. affinis* fillet increased moderately in all treatments during the 12 h evaluation after introducing the red rose extract, which leads to microorganisms replication (Figure 3). According to Sofos (2014), most microbial organisms are significantly and specifically dependent on the water availability for their survival. Moreover, water activity refers to the free water accessible in the matrix of

foods, favoring the microorganisms' growth. However, T2 and T3 could still overcome *Pseudomonas* sp. compared to T0 and T1, although they had more water or moisture. Therefore, it was believed that the red rose extract could inhibit the *Pseudomonas* sp. replication through a killing mechanism.

It is necessary to understand the lipid and protein oxidation systems thoroughly. Lipid oxidation is a major factor that causes food quality to deteriorate, particularly in foods that consist mainly of unsaturated fatty acids triggering protein oxidation (Frankel 2005). The present study found that the protein content decreased during 12 h observation after introducing red rose extract, while it did not happen for the fat content. Moreover, the decrease of protein could be considered that *E. affinis* fillet began to deteriorate. According to Hematyar et al (2019), there are two primary routes to the target amino acids: protein and lipid oxidation. The first pathway is the secondary lipid oxidation products, such as malonaldehyde, further reacting with the amino groups, or the unsaturated lipid aldehydes binding with the cysteinyl-lysyl-histidine (His-Cys-Lys) (Jové et al 2020). Afterward, some lipid oxidation products (lipid free radicals, hydroperoxides and volatile secondary oxidation products) combine with proteins to form protein-centered free radicals. As a result, there is a drop in myosin solubility, sulfhydryl and free amino acid groups (Hematyar et al 2019). The second pathway is when aldehydes, as a byproduct of metal-catalyzed lipid oxidation, enable a covalent bond to bind promptly to amino in proteins. Those activities could decline the water holding capacity (WHC) and the protein solubility in the fish fillet (Nielsen & Jørgensen 2004). To sum up, based on those reviews, the application of red rose extract containing anthocyanins did not affect the nutrition value of the *E. affinis* fillet because it could not inhibit protein oxidation. In contrast with a review carried out by Li et al (2020), the association between anthocyanins and proteins can increase the overall nutritional and functional properties of the food. Meanwhile, our results showed that the anthocyanins of *Rose* sp. did not affect the fat content and even dropped the protein level of *E. affinis.*

Conclusions. The results of the present study demonstrated that the red rose extract improved the moisture of *E. affinis* fillet and could also effectively inhibit the *Pseudomonas* sp. growth. However, there were negative impacts on the amount of protein quantity of *E. affinis* fillet after applying the extract. Therefore, further research needs to be conducted to evaluate the decline of protein content and compare it with commercial preservation.

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Conflict of interest. The authors declare no conflict of interest.

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