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Antioxidant Effects of Hutgae (*Hovenia dulcis* Thunb.) Fruit Extracts on Peroxidation of Refrigerated Eels

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Abstract

The antioxidant effects by pre-treatment of Hutgae fruit water and ethanol (30°, Soju) extract on refrigerated eels were analyzed. The antioxidant activities were measured through DPPH and ABTS scavenging effect, values of acidity, peroxide, carbonyl, and TBA. The peroxide prevention effects of linoleic acid and eel oil were also assessed. Regarding DPPH radical scavenging, Hutgae ethanol extract presented higher scavenging effects than vitamin C 5 mM solution (p<0.05). The eel's peroxidation degree was measured through 21 days of refrigeration after cleaning and immersion into the extract solution for one hour. Upon measuring the values of four different peroxide indicators, those of eels pre-treated with Hutgae extracts were lower than those of eels untreated. The POV of Hutgae ethanol extract, vitamin C 5 mM, and the control was 11.1, 11.3, 15.5 meq/kg, respectively. Hutgae ethanol extract showed higher antioxidant activities in TBA value, and carbonyl value than other samples. In linoleic acid or eel oil, Hutgae extract was as superiorly effective in preventing peroxide generation of refrigerated eels as vitamin C 10 mM solution. In conclusion, pre-application of Hutgae water and ethanol (30°, Soju) extract on eels was proved to be competent in stopping peroxidation of eel in refrigeration.

Key words: Hutgae (*Hovenia dulcis* Thunb.), DPPH, acid value, peroxide value, TBA

Introduction

Among various factors making food qualities inferior, oxidation of lipid components is one of them. The oxidation occurs in the forms of either rancid flavor or property changes in material when oil in food gets oxidized by oxygen, moisture, heat, etc. Unsaturated fatty acid, for instance, is easily oxidized by radicals or oxygen, and the 2nd and the 3rd subsequent oxidation follow by superoxide radical (Cho et al. 2011). The topic of peroxide prevention in fishes known for high percentage of unsaturated fatty acid, consequently easy rancidity has been widely investigated (Cho et al. 1998; Yang et al. 1999; Kang et al. 2007; Nam et al. 2011). As the skills of eel farming have advanced, the supplies of eels also increased to meet the consumers' demands. Moreover, mass production of eels has promoted the need not only for product development and processing (Kim et al. 2000; Choi et al. 2006; Ahn et al. 2015; Moon & Yoo 2016; Song HS 2019), but for their quality control and their improvement of storage and distribution through the peroxide prevention. Because Hutgae, known for its function on ethanol oxidation and hangover, has been reported to have the antioxidant effects in several researches (Ahn et al. 2010; Jung et al. 2012; Won & Song 2013; Song HS 2018), the aim of this research was to investigate the antioxidant effect of Hutgae extracts when they were pre-applied to refrigerated eels.

Materials and Methods

1. Samples

Eels (Anguilla *bicolor pacifica*) used in the research were fed and farmed in the city of Naju, weighed 250~300 g, gutted, and washed before the use. Hutgae fruit used to obtain solutions to be administered to eels beforehand in order to hinder eel's peroxide was procured at Jangheung Hutgae Farming Cooperative in Korea. 50 g of Hutgae fruit was poured to distilled water or 2 L alcohol (30°, Damgeumju,

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Hitejinro, Naju, Korea). Using an electronic dual pressure boiling pot (OC-2300R, OCOO Co., Ltd., Boryoung, Korea), it was drawn out for 2 h at 112℃. Once the first extraction was over, the solutions were let flow into separated cups. The second solutions were prepared using the same steps as in the first. When the first and the second solutions were set, two were put together, and filtrated using gauze at first and paper filter (No.1, Whatman, GE Healthcare, London, UK) secondly. Water and ethanol extracts were acquired as such. The yield value of extract was about 70%.

2. Pre-application of eels and eel oil extraction

Eel (80 g) and 150 mL of obtained Hutgae extract solution were included in a clean vinyl bag, and got rested for one hour in a tied-up bag. After 1 h, eel was drawn out for washing. Once cleaned and soaked up with paper towel, eel was vacuumed and refrigerated for 21 days (Song HS 2018). On the zeroth, seventh, and twenty first day, eel oil was extracted to measure peroxide value, carbonyl value, and TBA value.

Eel oil was obtained by following the method in Folch et al. (1957). 80 g of cut eel was combined with 300 mL of mixed solution of chloroform and methanol (2:1, v/v), then they were squeezed out via a homogenizer (SMG-G, Shinsang Co., Ltd., Hwasung, Korea), and filtrated utilizing a paper filter (No.1, Whatman, GE Healthcare, London, UK). Another 250 mL of chloroform and methanol- blended-solution was blended with the remainder. By using a homogenizer, mixed solution was extracted again, which consisted of the second screening. All acquired solutions were flown into a separatory funnel and joined with distilled water. The layer of chloroform was separated after 15 to 20 h. And it was dehydrated by Na_2SO_4 and filtered (No.1, Whatman, GE Healthcare, London, UK). All filtered solutions were concentrated using a rotary evaporator (Rotavaor R-215, Büchi, Germany) under reduced pressure at 40℃ to produce eel oil.

3. DPPH radical scavenging effects

For measurement of DPPH radical scavenging effects, Hugae extracts were filtrated with a paper (No.1, Whatman, GE Healthcare, London, UK) once. After obtaining 0.1 mL, 0.2 mL, 0.4 mL, and 0.8 mL of Hutgae fruit extract, they were diluted with water to be 1 mL. 1 mL of Hutgae extract diluted by distilled water was combined with 2 mL of ethanol and 0.5 mL of 700 μM DPPH (2,2-diphenyl-1-picrylhydrazyl, Sigma Co., Missouri, USA) solution. DPPH radical scavenging effects were calculated with UV-spectrophotometer (UV-1650, Shimadzu, Tokyo, Japan) at 517 nm absorbance (Song et al. 2007).

4. ABTS scavenging effects

7 mM ABTS (Sigma Co., Missouri, USA) and 2.45 mM potasium persulfate (Sigma Co., Missouri, USA) were mixed at the rate of 1:1, and then the solution was refrigerated for 18 h. The solution was diluted in order to yield the absorbance of 1±0.1 at 734 nm in advance. After obtaining 10 μL, 25 μL, 50 μL, and 100 μL of Hutgae fruit extract, they were diluted with water to be 0.3 mL. 2.7 mL of diluted ABTS solution was combined with 0.3 mL of the extract, and sat for 20 minutes at the room temperature. After 20 minutes, the absorbance was computed at 734 nm (Kim et al. 2015a). The relative free radical scavenging effects of extract in relation to DPPH and ABTS were determined based on the formula below (Song HS 2018).

$$\text{Relative radical scavenging activity (\%)} = \left(1 - \frac{\text{Extract absorbance}}{\text{Control absorbance}}\right) \times 100$$

5. The measurement of peroxide value

When 25 mL blended solution of acetic acid and chloroform (3:2, v/v) was placed into a flask containing 1 to 2 gram of lipid recovered according to Korean food code. 1 mL of KI saturated solution was also put together, shaken for a minute, and rested in the dark place for 10 minutes. When starch indicator solution and 30 mL of distilled water were poured, the non-colored point was chosen as the end point through titration by 0.01 N $Na_2S_2O_3$ (Kim et al. 2015b; Ministry of Food and Drug Safety 2017).

6. The measurement of acid value

1 to 2 gram of eel oil gained in the manner found in Korean food code (Ministry of Food and Drug Safety 2017) was taken into a flask. After 100 mL combined solution of methanol and ether (2:1) and 1~2 drop of phenolphthalein indicator were flown into the flask, the pink colored point was chosen as the end point through titration by 0.1 N KOH

(Kim et al. 2015b; Ministry of Food and Drug Safety 2017).

7. The assessment of carbonyl value

Eel oil 0.05g, benzene 5 mL, 0.05% 2,4-DNPH (dinitrophenyl hydrazine) benzene 5 mL, and 4.3% trichloroacetic acid 3 mL were placed into 100 mL glass bottle with a cap. The blend got warmed up in 60℃ water bath for 30 minutes. On cooling it off at room temperature and popping the color with 10 mL of 4% KOH-ethanol, the absorbance was assessed at 440 nm (Choi et al. 2006; Song HS 2018).

8. The valuation of TBA value

Based on the method in Korean food code (Ministry of Food and Drug Safety 2017), 200 mg of TBA and 100 mL of 95% butanol were blended and sonicated at 60℃ in sonicator (Ultrasonic, JAC 4020, KODO, Hwasung, Korea) for 30 minutes. TBA (thiobarbituric acid) reagent was formed with a direct input of glacial acetic acid at the rate of 1:1. TBA value was calculated through the absorbance measurement at 530 nm after cooling off the solution, a blend of 0.05 g eel oil, 10 mL benzene, and 10 mL of TBA reagent kept for 2 h in 95℃ water bath in the flowing water (Ministry of Food and Drug Safety 2017; Song HS 2018).

9. The peroxide prevention effects of linoleic acid and eel oil

Eel oil 0.2 mL (or linoleic acid 0.13 mL), 100% ethanol 10 mL, phosphate buffer solution (pH 7.4) 50 mM, Hutgae extract 1 mL, and distilled water were placed together in a 50 mL conical tube to produce the final solution of 25 mL. The peroxide was stimulated during 20 reaction days in 40℃ water bath. The thiocyanate method was used to obtain the peroxide value at 500 nm absorbance. The combination of 0.1 mL reactant, 4.7 mL of 70% ethanol, 0.1 mL of 30% NH_4SCN, and 0.1 mL of 0.02 M $FeCl_2$ in 3.5% hydrochloric acid was placed and set for 3 minutes. In order to find out the density of peroxide in linoleic acid and eel oil, the reactions to absorbance on the zeroth and twentieth day of each reactant were measured, calculated in percentage, and named as the peroxide percentage.

10. Statistical analysis

Using IBM SPSS Statistics 20, ANOVA between the experiment group and the control group was undertaken at first, and then Duncan's multiple range test was also performed with 5% confidence level (α=0.05).

Results and Discussion

1. DPPH and ABTS scavenging effect of Hutgae fruit extracts

The DPPH and ABTS scavenging effects of water and ethanol extract from Hutgae fruit were compared to those of vitamin C 5 mM solution, a positive control (Fig. 1, Fig. 2). The correlation coefficient between concentration and absorbance was more than 0.9 (r>0.9). Hutgae extracts were useful in DPPH and ABTS radical scavenging subjective to the amount of extracts. Ethanol extract from Hutgae had higher effects than water extract, which was supported by previous researches (Shon et al. 2001; Son et al. 2005; Song HS 2018). The DPPH radical scavenging effects of ethanol extract from Hutgae were meaningfully higher than those from vitamin C 5 mM (p<0.05). Song & Kim (2018) also reported the ethanol extract from Hutgae, just like the one from green-tea extract, had higher scavenging effects than those from vitamin C 5 mM. As for the DPPH radical scavenging effects, water extracts from Hutgae had low scavenging effects. But, unlike DPPH radical, both water and ethanol extracts from Hutgae had lower scavenging effects on ABTS radicals than vitamin C 5 mM solution (p<0.05). The fact that Hutgae extracts were not so much effective in DPPH radical scavenging, but were effective in ABTS radical was agreed to earlier researches, and the radical scavenging effects are influenced by various anti-oxidant materials in each extract (Kim et al. 2015a; Park & Han 2015; Song HS 2018).

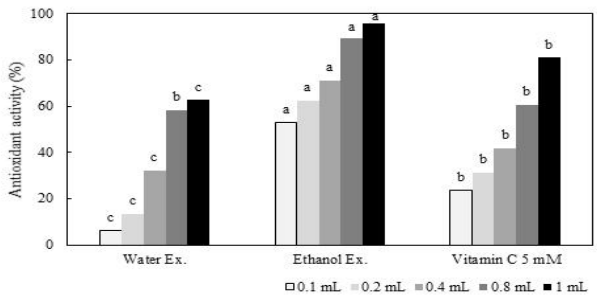

Fig. 1. Antioxidant effect of Hutgae fruit extract on DPPH radical. [a-c]Values are significantly different at p<0.05 within the same content.

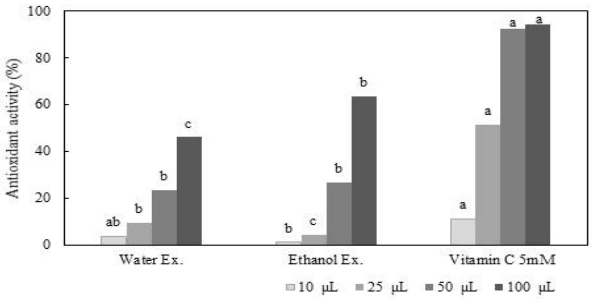

Fig. 2. Antioxidant effect of Hutgae fruit extract on ABTs radical. [a-c]Values are significantly different at $p<0.05$ within the same content.

2. Acid value of eel oil

When lipid in food gets acidified, free fatty acid increased. Acid value is the way to find out the degree of rancidity of lipid by measuring the amount of free fatty acid in food (Cho et al. 2011). The pre-refrigeration acid value of eels, whether they were pre-treated with ethanol Hutgae extract, water Hutgae extract, vitamin C, or in the control, were between 2.57~2.69 mg KOH/g (Table 1). There was no meaningful difference in samples ($p<0.05$). The acid value of mackerel treated with green-tea extract in advance was claimed to be 2.3 mg KOH/g in the prior stage of refrigeration, and that of eel pre-treated with green-tea extract was 2.7 mg KOH/g (Choi et al. 2015; Song HS 2018). On the 7th day of refrigeration, the amount of free fatty acid in the control increased more, but there was no meaningful difference in acid values ($p<0.05$). On the 21st refrigeration day, the control's acid value was 4.36 mg KOH/g, which was relatively higher than that of Hutgae fruit extracts and vitamin C 5 mM solution. While there were 62% and 50% increases of the acid value in the control group and in the group pre-treated with Hutgae, respectively, the acid value of eels pre-treated with vitamin C solution increased by 10%. Compared to the control group, the pre-treatment of Hutgae fruit extracts proved to be effective in antioxidation by lowering the acid value ($p<0.05$).

3. Peroxide value of eel oil

The values primarily were obtained by measuring peroxide value of eels refrigerated for 21 days (Table 2). Prior to refrigeration, the peroxide values lie between 7.21 and 7.35 meq/kg, and there was no meaningful difference in samples ($p<0.05$). According to the earlier researches the peroxide value of eels pre-treated with green-tea extract prior to refrigeration was 7.22 meq/kg (Song HS 2018), and the peroxide value of half-dried eels was 13~18 meq/kg (Song HS 2019). On the 7th day of refrigeration, the peroxide value of the control was 13.97 meq/kg, 93% increase from the zeroth day. Until the 7th day of refrigeration, the peroxide value of eels pre-treated with Hutgae fruit extracts and vitamin C solution increases up to 27~38%. On the 21st day of refrigeration, the peroxide value of the control was 15.45 meq/kg, which was a huge increase of 114%. The peroxide value of eels pre-treated with Hutgae fruit ethanol extracts and water extracts was 11.08 (52% increase), and 11.23 meq/kg (56% increase), respectively. On the 21st day of refrigeration, the peroxide value of eels pre-treated with vitamin C solution was 11.34 meq/kg. Upon reviewing the results, the Hutgae fruit extracts were deduced to have comparable level of effect in hindering the peroxide as in the case of vitamin C 5 mM solution ($p<0.05$).

Table 1. Acid value of eel treated with Hutgae fruit extracts

Extracts \ Storage periods[1)]	Acid value (mg KOH/g[2)])		
	0 day	7 days	21 days
Control	2.69±0.17^{Aab}	3.22±0.53^{Ac}	4.36±0.25^{Ce}
Hutgae water extracts	2.69±0.21^{Aab}	3.07±0.33^{Abc}	4.03±0.14^{Bde}
Hutgae ethanol extracts	2.57±0.25^{Aa}	3.07±0.21^{Abc}	3.85±0.14^{Bd}
Vitamin C 5 mM	2.66±0.28^{Aab}	2.83±0.46^{Aabc}	2.93±0.28^{Aabc}

[1)] Storage at refrigerator (4~8℃).

[2)] 1 g of oil from eel treated with Hutgae fruit extracts.

[A-C]Values are significantly different at $p<0.05$ within the same storage period. [a-e]Values are significantly different at $p<0.05$ within all acid values.

Table 2. Peroxide value of eel treated with Hutgae fruit extracts

Extracts \ Storage periods[1)]	Peroxide value (meq/kg[2)])		
	0 day	7 days	21 days
Control	7.23±0.74^{Aa}	13.94±0.03^{Bd}	15.45±0.63^{Be}
Hutgae water extracts	7.21±0.48^{Aa}	9.95±0.79^{Ab}	11.23±0.64^{Ac}
Hutgae ethanol extracts	7.30±0.48^{Aa}	9.26±0.35^{Ab}	11.08±0.45^{Ac}
Vitamin C 5 mM	7.35±9.71^{Aa}	9.71±0.49^{Ab}	11.34±0.95^{Ac}

[1)] Storage at refrigerator (4~8℃).

[2)] 1 kg of oil from eel treated with Hutgae fruit extracts.

[A,B]Values are significantly different at $p<0.05$ within the same storage period. [a-c]Values are significantly different at $p<0.05$ within all acid values.

4. TBA value of eel oil

When lipid gets oxidated, various oxidation products are produced, including malonaldehyde (Kim et al. 2015b). Malonaldehyde produces harmful oxidation products by being combined with carbonyl products (Berlett & Stadtman 1997). Therefore, it is important to check out the amount of malonaldehyde produced by measuring TBA value. TBA value of eels on the zeroth day of refrigeration was 2.11~2.16 (Table 3). TBA value of untreated eels on the 7th day of storage was 3.60, which was about 70% increase. However, TBA value got lowered in the order of vitamin C 5 mM solution, Hutgae ethanol extract, Hutgae water extract. While TBA value of untreated eels on the 21st day of refrigeration increased to 37.52, TBA value of Hutgae fruit ethanol extract, Hutgae water extract, and vitamin C 5 mM solution was 4.46, 5.08, and 5.58, respectively, which proved to be inhibitory for malonaldehyde products ($p<0.05$). Hutgae ethanol extract was the most effective in preventing the rise of malonaldehyde products.

5. Carbonyl value of eel oil

Carbonyl compound is reported to be generated not only by the oxidation of lipid, but also by the reactions to amino acid and fatty acid compound (Berlett & Stadtman 1997). According to Kim et al. (2015b), carbonyl compound of meat massively increases at the point when proteolysis increases greatly. On the zeroth day of storage, carbonyl value of eel was 2.01~2.07 meq/kg (Table 4). On the 7th day of refrigeration, carbonyl value of untreated eel was 2.51 meq/kg, and it was 21.7% increase compared to the value prior to refrigeration. However, carbonyl values of eels pre-treated with Hutgae fruit extracts or vitamin C 5 mM was 2.14 and 2.23 meq/kg, respectively, and it was 6.3% and 10.4% increase of carbonyl compound. Choi et al. (2006) said that carbonyl value of eel oil kept at 37℃ for one week was 9 meq/kg, and Song HS (2018) also said the speed of carbonyl compound generation in vacuum-packed and refrigerated eel was not very fast. Considering the prior researches, the antioxidant effects of Hutgae extratcts on the 7th day of refrigeration was deemed as effective as vitamin

Table 3. TBA value of eel treated with Hutgae fruit extracts

Extracts \ Storage periods[1)]	TBA value		
	0 day	7 days	21 days
Control	2.11±0.31^{Aa}	3.60±0.84^{Bb}	7.52±1.80^{Cd}
Hutgae water extracts	2.16±0.40^{Aa}	3.45±0.45^{Bb}	5.08±0.63^{Bc}
Hutgae ethanol extracts	2.13±0.18^{Aa}	2.85±0.36^{Aa}	4.46±0.86^{Abc}
Vitamin C 5 mM	2.11±0.60^{Aa}	2.37±0.25^{Aa}	5.58±0.96^{Bc}

[1)] Storage at refrigerator (4~8℃).

[A-C]Values are significantly different at $p<0.05$ within the same storage period. [a-d]Values are significantly different at $p<0.05$ within all TBA values.

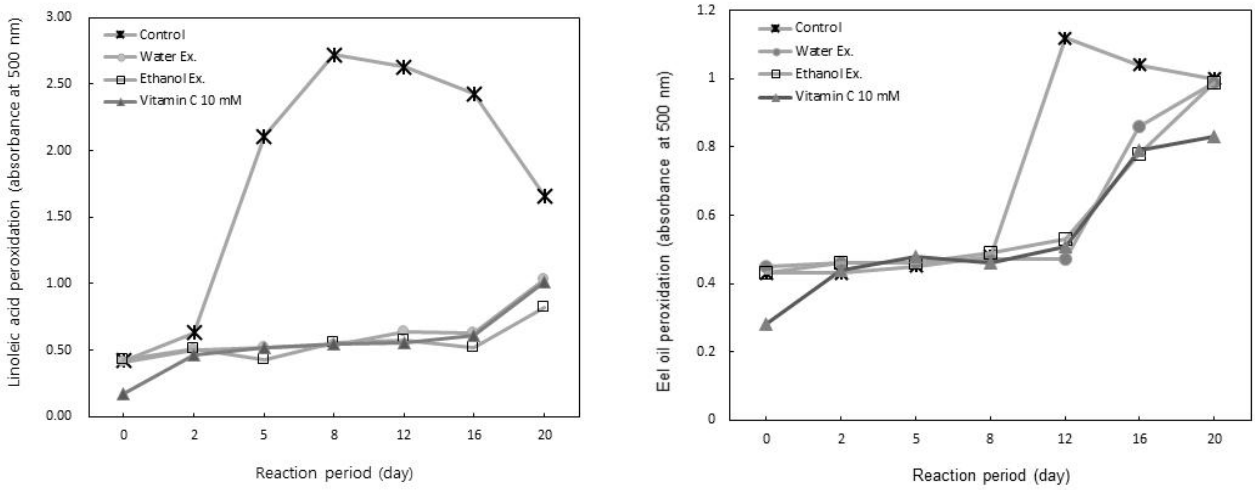

Fig. 3. Effects of peroxide delay reaction of Hutgae fruit extracts on linoleic acid and eel oil.

C 5 mM solution (p<0.05). On the 21st day of refrigeration, carbonyl value of untreated eel was 2.90 meq/kg, about 40% increase of carbonyl compound. Carbonyl value of eel pre-treated with vitamin C was 2.58 meq/kg, which was 24.6% increase of carbonyl compound. Carbonyl value of eel pre-treated with Hutgae ethanol or water extract was 2.38 and 2.20 meq/kg, respectively, and it was 17.8% and 9.5% increase of carbonyl compound each. Carbonyl compound of eel pre-treated with Hutgae ethanol or water extract on the 21st day of refrigeration generated the lower amount than that of eel pre-treated with vitamin C 5 mM solution (p<0.05). According to prior research, carbonyl compound increased less than 10% when pre-treated with green-tea extracts (Song HS 2018). Based on that, Hutgae fruit ethanol extract was deemed to be as effective as reported previously in preventing carbonyl compound generation.

6. Antioxidant effect on linoleic acid and eel oil

To determine the peroxide prevention effects of Hutgae fruit extracts, they were combined with linoleic acid or eel oil extract, and brought about oxidative responses at 40℃ for 20 days (Song & Kim 2018). Even though the peroxide of linoleic acid grew sharply on the 5th reaction day and resulted in the largest amount thru 12th reaction day (Fig. 3), the peroxide value, in the case that vitamin C solution and Hutgae extracts were added, increased slowly and went up very high on the 20th reaction day.

As for eel oil, on the 12th reaction day, the amount of peroxide increased sharply and ran thru the 20th reaction day. In the cases that vitamin C solution and Hutgae extracts were added, the peroxide value increased in a slow pace but went up sharply on the 16th reaction day. When vitamin C solution was added, the peroxide value on the

Table 4. Carbonyl value of eel treated with Hutgae fruit extracts

Extracts \ Storage periods[1)]	Carbonyl value (meq/kg[2)])		
	0 day	7 days	21 days
Control	2.07±0.13^{Aab}	2.51±0.13^{Bde}	2.90±0.12^{Cf}
Hutgae water extracts	2.02±0.07^{Aa}	2.23±0.18^{Abc}	2.38±0.30^{ABcd}
Hutgae ethanol extracts	2.01±0.09^{A}	2.14±0.11^{Aab}	2.20±0.35^{Aabc}
Vitamin C 5 mM	2.07±0.13^{Aab}	2.20±0.34^{Aabc}	2.58±0.10^{Be}

1) Storage at refrigerator (4~8℃).

2) 1 kg of oil from eel treated with Hutgae fruit extracts.

$^{A-C}$Values are significantly different at p<0.05 within the same storage period. $^{a-f}$Values are significantly different at p<0.05 within all acid values.

16th reaction day was 186% higher than that of the zeroth reaction day. However, the increase rate was 82~93% when Hutgae extracts were added. Therefore, it was effective in preventing the generation of peroxide. The findings agree to Song HS (2018) which reported that the addition of green-tea extracts which was known for their high antioxidant activities was as effective in preventing the generation of peroxide as vitamin C solution. Song & Kim (2018) also pointed out that Hutgae ethanol extract and green-tea ethanol extract prevented the peroxide of half-dried eel oil, and Hutgae extract delayed peroxide induction period longer than green-tea extract. Considering the findings regarding acid value, peroxide value, TBA value, and carbonyl value, it is believed that Hutgae fruit ethanol or water extract is as comparatively or superiorly effective in preventing peroxide generation of refrigerated eels as vitamin C 5 mM solution.

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