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Effect of Extract of Perilla Leaves on the Quality Characteristics and Polycyclic Aromatic Hydrocarbons of Charcoal Barbecued Pork Patty

Abstract

This study aimed to investigate the effect of ethanolic extracts from perilla leaves (PLE) on the quality attributes and polycyclic aromatic hydrocarbons (PAHs) in charcoal-barbecued pork patties. The PLE addition and doneness had no significant effect on the pH of pork patties ($P > 0.05$). Regardless of the concentration, the PLE significantly lower malondialdehyde concentrations and reduced the CIE L*, a*, and b* values when compared to control. The addition of 0.2% of PLE did not adversely affect the organoleptic properties of doness of medium and well-done pork patties. Addition of PLE at 0.4% to medium-cooked pork patties had stronger suppressing effect on the formation of light PAHs compare to control ($P < 0.05$), also adding it to well-done pork patties had the lowest concentration of 4 PAH and 8 PAH, and a total of 16 PAHs ($P < 0.05$). Therefore, PLE at 0.40% can be used for suppressing the formation of PAHs and lipid oxidation in well-cooked pork patty.

Keywords polycyclic aromatic hydrocarbons, perilla leaves, ethanol extracts, pork patty, charcoal

Introduction

Along with the increasing interest of consumers toward functional foods, the meat industry has been focusing on developing products with additional health benefits, in addition to fulfilling the basic demand for pleasant sensory characteristics, long shelf-life, and high nutritional value (Fadda et al., 2010). To achieve these goals, phenolic acids and bioactive compounds derived from natural plants have gained interest in recent years because they increase functional quality and reduce the potentially harmful substances that may be formed during the processing of meat products (Barido et al., 2022a; Park et al., 2017). Although it is exceptional in improving organoleptic properties and safety and providing unique flavor characteristics, cooking meat, mainly at high temperatures and open flame conditions, has been reported to result in the highest formation of substances with adverse health effects, especially polycyclic aromatic hydrocarbons (PAHs) (Chung et al., 2011; Cordeiro et al., 2020; Viegas et al., 2014). PAHs are organic compounds with two or more fused aromatic rings that potentially form during high temperature cooking of meat (>200℃), especially during grilling and barbecuing.

According to a report by the European Food Safety Authority (EFSA, 2008), PAHs are categorized as a group of compounds suspected to be the predominant carcinogenic substances in meat and meat products. Through an epidemiological study, Sinha et al. (2005) suggested that dietary intake of the PAH benzo[a]pyrene could increase the risk of colorectal cancer. Therefore, the presence of 4 PAH such as naphthalene, phenanthrene, anthracene, and pyrene in meat and meat products is limited to only 30 ng/g (Cordeiro et al., 2020; EFSA, 2008). Viegas et al. (2012) mentioned three possible mechanisms of PAH formation during open flame cooking: (i) the occurrence of pyrolysis and pyrosynthesis on the surface of the meat, (ii) continuous contamination of meat with charcoal or

propellant smoke, and (iii) reaction of the dripped fat with a heat source attached to the meat. To date, among 16 individual PAHs, namely naphthalene, acenaphthene, acenaphthylene, fluorene, anthracene, phenanthrene, fluoranthene, pyrene, benzo[a]anthracene (BaA), chrysene (Ch), benzo[b]fluoranthene (BbF), benzo[k]fluoranthene, benzo[a]pyrene (BaP), dibenzo[a,h]anthracene, benzo[g,h,i]perylene, and indeno[1,2,3-cd]pyrene (IP), the sum of the last 8 aforementioned PAHs (8 PAHs) is a potent indicator of the carcinogenic potential of certain meat and food products (EFSA, 2008). In addition, studies have mentioned that the sum of BaA, BaP, BbF, and Ch (4 PAHs) could increase the level of accuracy of carcinogenic potential measurements (Cordeiro et al., 2020; Viegas et al., 2014; EFSA, 2008). Therefore, experiments have been conducted to reduce the levels of 4 PAHs and 8 PAHs.

Studies have demonstrated that fermentation products such as vinegar and beer can mitigate PAH levels in grilled pork (Cordeiro et al., 2020; Viegas et al., 2014). Moreover, some plant extracts, including bamboo, tea, and rosemary extracts, have been reported to exert potent inhibitory effects against oxygenated PAHs and PAHs in Chinese “youtiao” (Gong et al., 2018). The quenching ability of antioxidant compounds toward free radicals involved in the formation of PAHs was assumed to be the main mechanism for the reduction of their concentration, indicating a possible protective effect from other natural compounds rich in antioxidants (Farhadian et al., 2012; Kim et al., 2021b). The lipophilic extract from perilla leaves (*Perilla frutescens*) was found to contain an appreciable 12.14 mg gallic acid equivalent (GAE)/g of total polyphenol and exhibit a 14.90 μmol Fe (II)/g ferric reducing antioxidant power (FRAP) assay at fresh weight (Cross et al., 1976). However, studies examining the effect of perilla leaf extracts (PLE) on PAH formation are

scarce.

In addition to providing safety against potential pathogenic bacteria, cooking pork patties until reaching a different range of final internal temperatures (medium- or well-cooked) significantly affects consumer palatability due to the different effects on quality attributes, including tenderness, juiciness, and flavor intensities (Cross et al., 1976). With respect to the essential factor of sensory acceptance of pork patties cooked at different levels, together with the potential inclusion of natural antioxidants, this study aimed to evaluate the inhibitory effects of ethanolic extracts from perilla leaves on the formation of PAHs in charcoal-barbecued pork patties at different final internal temperatures (medium- and well-cooked).

Material and methods

Preparation of ethanolic extracts

Perilla leaves (*Perilla frutescens*) were purchased from a local market (Chuncheon, South Korea). All samples were washed under running tap water prior to extraction. The samples were lyophilized, ground, passed through a 20-mesh sieve, and stored at -20℃ until extraction. The sample powders were macerated with 50%, 70%, or 90% ethanol (1:50 w/v) for 3 or 6 d at 25℃. The obtained extracts were filtered through Whatman No. 4 paper (Clifton, NJ, USA), and the filtrates were collected and concentrated using a rotary evaporator at 40℃. The concentrated extracts were lyophilized and stored at -20℃ until analysis.

1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity

DPPH radical scavenging activity was analyzed following the method of Cho et al. (2021), with slight modifications. The extract solution (100 μL) was placed in 100 μL of methanolic solution containing DPPH radicals (0.2 mM) in a 96-well microplate. The mixture was allowed to react for 30 min at 25℃ in the dark. The absorbance of each extract solution was measured at 517 nm using a spectrophotometer (SpectraMax M2, Molecular Devices, San Jose, California, USA). The standard curve was established using Trolox, and the DPPH values were expressed as mmol Trolox equivalent (TE)/g dry matter (DM).

FRAP activity

The FRAP assay was performed as described by Kim et al. (2019), with slight modifications. The FRAP working solution was prepared with 300 mM acetate buffer, 10 mM 2,4,6-tripyridyl-S-triazine in 40 mM HCl, and 20 mM $FeCl_3 \cdot 6H_2O$ solution mixed at a ratio of 10:1:1 (v/v/v). Twenty-five microliters of the extract sample were reacted with 175 μL of FRAP working solution for 30 min at 37°C in the dark. The absorbance of the reacted solution was determined at 590 nm using a spectrophotometer (Spectra Max M2, Molecular Devices). The FRAP activity was expressed as mmol TE/g DM.

Oxygen radical absorption capacity (ORAC)

The ORAC assay was performed as described by Kim et al. (2019), with slight modifications. To measure ORAC, a mixture composed of 25 μL PLE and 150 μL fluorescein (80 nM) was mixed and incubated for 15 min at 37℃. After incubation, 25 μL 2,2′-azobis (2-amidinopropane) hydrochloride (150 mM) was added to generate peroxyl radicals, and each well contained a final volume of 200 μL. The change in the absorbance of the reacted extract sample was recorded every minute at an excitation and emission

wavelength of 480 and 520 nm, respectively, at 37℃. The ORAC assay was performed using a spectrophotometer (Spectra Max M2; Molecular Devices). Trolox was used as the standard, and the results are expressed as mmol TE/g.

Total phenolic content (TPC)

TPC was measured using the Folin-Ciocalteu colorimetric method as described by Cho et al. (2021), with slight modifications. The 50%, 70%, and 90% ethanol extracts were dissolved in 50%, 70%, and 90% ethanol, respectively. Each extract solution was diluted in ethanol. The diluted extract solution (0.5 mL) was mixed with 5 mL distilled water and Folin-Ciocalteu phenol reagent (Sigma, St. Louis, Missouri, USA) and kept for 3 min, after which 1 N Na_2CO_3 was added, and the mixture reacted for 90 min at 25℃ in the dark. The absorbance of the reacted samples was measured at 760 nm using a spectrophotometer (Spectra Max M2). A standard curve was established using gallic acid, and the TPC was expressed as mg GAE/g.

Preparation of pork patty

Frozen lean pork leg and pork back fat were purchased from a local market in Chuncheon, South Korea. The visible fat on the pork legs was trimmed. The defatted pork leg and fat were minced through the first 8 mm plate and then through the second 4 mm plate using a meat chopper (M-12S, Fujee, Siheung, Gyeonggi, Korea). After mincing, the defatted pork leg and fat were mixed with salt, water, and ethanol or perilla leaves ethanol extract using a mixer (5 KPM50, Kitchen Aid, Benton Harbor, Michigan, USA). The formulations of the pork patties are presented in Table 1. Approximately 80 g of the mixture was formed into pork patties using a Petri dish (15 mm thick × 90 mm diameter).

149 The patties were covered with polyethylene film and stored under refrigeration (4℃) for
150 24 h before charcoal barbecue.
151
152 **Charcoal barbecue condition**
153 The charcoal barbecue condition was performed according to Kim et al. (2021a), with
154 slight modifications. Black charcoal with extruded charcoal (for ignition) was placed into
155 a garden-type grill (55 cm wide, 34 cm long, and 14 cm high: Allcook, Chilgok,
156 Gyeongsangbuk, Korea). The temperature of the charcoal fire was measured using a laser
157 thermometer (IR-302; Custom, Koto-ku, Tokyo, Japan). When the temperature of the
158 charcoal fire reached 550 to 600℃, the barbecue started; the distance from the charcoal
159 was 8 cm for medium-cooked patties, the barbecue time was 9 min (5 min, front side; 4
160 min, back side), and the internal temperature of the patty was approximately 71℃. For
161 well-cooked patties, the barbecue time was 16 min (8 min on each side), and the internal
162 temperature of the patties was approximately 80℃. The patty was turned once during the
163 barbecue period.
164
165 **Proximate composition**
166 The proximate composition was measured according to the methods of the
167 Association of Official Agricultural Chemist (AOAC, 2012). The moisture content of the
168 barbecued pork patties was measured by weight loss after oven-drying at 105℃ for 12 h.
169 The crude protein content was measured using the Kjeldahl method. Crude fat content was
170 measured by solvent extraction using ether. The burned pork patties in the furnace at
171 550℃ were analyzed for crude ash.
172

Cooking loss

Cooking loss was calculated as the difference in the weight of pork patties before and after cooking. The equation for cooking loss of barbecued pork patties is as follows:

Cooking loss (%) = (raw pork patty weight (g) – barbecued pork patty weight (g))/raw pork patty weight (g) × 100.

pH value

The pH was determined using a pH meter (Orion 230A, Thermo Fisher Scientific, Waltham, MA, USA). Ten grams of pork patty were homogenized with 90 mL distilled water using a homogenizer (PolyTron ® PT-2500E, Kinematica, Malters, Luzern, Switzerland).

Instrumental color

The instrumental color of raw and barbecued (medium- and well-cooked) pork patty was determined using colorimeter (CR-400 Minolta colorimeter, Minolta, Osaka, Japan) with an aperture of 8 mm size and an illuminant-C. The color values of lightness (L*), redness (a*), and yellowness (b*) of the raw pork patties were measured after removing the polyethylene films for 10 min. The color values of the barbecued pork patties were measured after barbecue.

2-thiobarbituric acid reactive substances (TBARS) assay

TBARS was analyzed using the method described by Kim et al. (2022). The pork patties of 5 g were added in 50 μL of 7.2% *tert*-butyl-4-hydroxyanisole and 15 mL distilled water and then homogenized for 30 s using a homogenizer (Polytron PT-2500E,

Kinematica, Lucerne, Switzerland). A 1 mL homogenate was transferred to a test tube, and 2 mL of thiobarbituric acid (TBA)/trichloroacetic acid (TCA) solution (20 mM TBA/15% TCA) was added. For a blank sample, 2 mL of each patty homogenate was added to 2 mL of 15% TCA solution. The sample mixture was incubated in a water bath at 90℃ for 15 min to develop color. After incubation, the samples were cooled in ice water for 10 min and centrifuged at 2,000 × g at 4℃ for 15 min. The absorbance of the supernatant solution was measured at 531 nm using a spectrophotometer (Spectra Max M2, Molecular Devices). The TBARS content was expressed as mg of malondialdehyde (MDA)/kg of patty, as follows:

TBARS (mg MDA/kg of patty) = (absorbance of sample – absorbance of blank sample) × 5.88.

Sensory evaluation

Sensory evaluation of barbecued pork patties was performed by 15 panelists from the College of Animal Life Sciences, Kangwon National University. The sensory properties were evaluated for medium- and well-cooked pork patties. Barbecued pork patties were evaluated for color, aroma, flavor, taste, juiciness, texture, and overall acceptability using a 9-point scale as follows: color, aroma, flavor, taste, texture, and overall acceptability (1=extremely undesirable, 9=extremely desirable) and juiciness (1=extremely low, 9=extremely high). Sensory evaluation was approved by the Kangwon National University Institutional Review Board (KWNUIRB-2020-09-005-002).

PAHs content

The PAHs in barbecued pork patties were analyzed using the methods of Kim et al.

(2021a), with slight modifications. The sample (2.5 g) was placed in a 50 mL conical tube, and 5 mL ethyl acetate/acetonitrile (20:80, v/v) and 1 mL ISTD mix (naphthalene-d_8, acenaphthene-d_{10}, phenanthrene-d_{10}, chrysene-d_{12}, and perylene-d_{12}, 400 ng/mL) was added. The mixture was ultrasonicated for 20 min and centrifuged (1968 × *g* for 7 min at 15℃). The supernatant was transferred to a 15 mL conical tube. The remaining pellet was re-extracted using 5 mL ethyl acetate/acetonitrile (20:80, v/v) and the supernatants were combined. The combined supernatants were evaporated under vacuum conditions using a rotary evaporator (Scilab Korea, Seoul, Korea) until a volume of 2 mL was reached, and distilled water (0.5 mL) was added. Subsequently, the mixture was purified using a Captiva EMR-Lipid cartridge (Agilent Technologies, Santa Clara, CA, USA) and 0.625 mL of ethyl acetate/acetonitrile/water (16:64:20, v/v/v) was eluted through the cartridge. The 1.875 mL eluent was mixed with 2.625 mL distilled water and 1.2 mL isooctane in a new 15 mL conical tube, followed by vigorous shaking. Subsequently, the mixed samples were centrifuged (1968 × *g*, 7 min, 15℃), and the supernatant was transferred to a glass tube, concentrated by nitrogen gas, and analyzed by GC/MS (Agilent 8890 GC with an Agilent 5977 B GC/MSD: Agilent Technologies).

The PAHs were separated using a DB-EUPAH capillary column (20 m × 0.18 mm inner diameter, 0.14 μm film thickness: Agilent Technologies). The carrier gas used pure helium (99.99%) at a constant flow rate (1.2 mL/min). The extracted samples were injected in the splitless mode (1 μL) and maintained at an initial temperature of 300°C. The source temperature was 290℃, and the mass selective detector temperature was 310℃. The initial temperature of the oven was 70℃, increased to 190℃ at a rate of 30℃/min, increased to 290℃ at a rate of 10℃/min, and maintained for 5 min; it was then increased to a final temperature of 320℃ at a rate of 30℃/min and maintained for 1 min. The

electron ionization of the mass spectrometer was operated at 70 eV, and data acquisition was conducted in the selective ion monitoring mode for the characteristic molecular ions of each PAH. The ISTD mix and 16 PAH standards (naphthalene, acenaphthylene, acenaphthene, fluorene, phenanthrene, anthracene, fluoranthene, pyrene, benzo[*a*]anthracene, chrysene, benzo[*b*]fluoranthene, benzo[*k*]fluoranthene, benzo[*a*]pyrene, indeno[1,2,3-*cd*] pyrene, dibenzo[*a*,*h*]anthracene, and benzo[*g*,*h*,*i*]perylene) were used. Nine-point calibration curves from 9 to 2,400 ng/mL were used to quantify the contents of the 16 PAHs in the samples. All PAHs were quantified by the relative response factors related to the list, in which the individual characterization of light PAHs, heavy PAHs, PAH4, PAH8, and a total of 16 PAHs are shown in Table 2.

Statistical analysis

All determinations were carried out in triplicate. All data were analyzed using the general linear model procedure of the SAS program (ver. 9.2; SAS Institute, Cary, NC, USA) using one-way analysis. Tukey's test was used to determine the significance of the differences in the mean values for the different extract samples. Differences were considered significant at $p<0.05$.

Results and discussion

Antioxidant activities

Three different sets of antioxidant activity measurements were performed in this study, and the results are shown in Table 3. Based on these results, both ethanol concentration and

extraction period significantly influenced the antioxidant activities of PLE ($p<0.05$). However, exceptionally, the DPPH assay showed that the highest activities were obtained when perilla leaves were extracted using 90% ethanol solution for 3 d (593.27 μmol TE/g DM), with no notable effect following extension of the extraction period (day 6; 521.92 μmol TE/g DM; $p<0.05$). In addition, the FRAP assay yielded similar results, wherein the highest activities were observed when perilla leaves were extracted using 90% ethanol in comparison to that by 70 and 50% ($p<0.05$). The extension of extraction period, however, significantly increased the antioxidant activities by 6% from 0.91 mmol TE/g DM on day 3 to 0.97 mmol TE/g DM on day 6 ($p<0.05$). Furthermore, the ORAC assay indicated that PLE produced with 70 and 90% ethanolic solution shared similar antioxidant activities, but notably than those of 50% ($p<0.05$). No remarkable effects were observed when the extraction period was extended.

Studies have shown that the antioxidant activities of certain compounds are strongly correlated with the concentration of phenolic compounds (Gong et al., 2018; Singleton, 1966). Antioxidant activity may have different correlations with TPC because there are different principles depending on the different methods of antioxidant activity. The method of FRAP is based on the ferroin analog reduction and can determine the total reducing capacity (Antolovich et al., 2002). The method of ORAC is used to determine the antioxidant activity against peroxyl radicals produced by AAPH and is the most biologically relevant method for analyzing antioxidant activity (Ou et al., 2002). The assay of DPPH is based on the principle that DPPH$^{\bullet}$ is reduced to DPPH2 when it accepts a hydorgen (H) atom a scavenger molecule (Mishra et al., 2012). Additionally, each antioxidant activity may have a different level of correlation with TPC, and thus may not always have a positive correlation with TPC. A previous study (Zheng et al., 2018) showed

that the linear regression (R2) correlation coefficients of the three antioxidants (FRAP, ABTS, and DPPH) and TPC differed by 0.8588, 0.7587, and 0.6344, respectively. The efficacy of solutions in extracting phenolic compounds is one of the most determining factors. Compared to aqueous solutions, organic solutions have been reported to exhibit a stronger capacity to extract major phenolic compounds, including sinapic, chlorogenic, isovanillic, caffeic, and gallic acid (Gong et al., 2018). Consistent with the results of this study on TPC, the order of phenolic compound concentration on day 3 from the highest to the lowest for 90, 70, and 50% ethanolic solution was 102.39, 81.97, and 79.15 mg GAE/g DM, respectively ($p<0.05$). Similarly, on day 6, the TPC for PLE produced using 90, 70, and 50% ethanolic solution were 114.93, 91.48, and 81.91 mg GAE/g DM, respectively ($p<0.05$), indicating a stronger capability of high ethanol concentration in extracting antioxidant compounds from perilla leaves. According to previous study (Kang and Lee, 2011), cyanidin-3-O-(6-O-coumaroyl)-glucoside5-O-glucoside and rosmarinic acid were major phenolic compounds in the perilla leaves, and their contents showed more than 15% and 60% in total content, respectively. Additionally, regarding the essential role of TPC concentration in dictating the functional properties of foods, extraction of perilla leaves for 6 d resulted in a notably higher TPC compared to that for 3 d ($p<0.05$). Therefore, in this study, extraction of perilla leaves was performed using 90% ethanolic solution for 6 d.

Proximate composition

The proximate composition of pork patties after barbecue cooking and treatment with PLE is shown in Table 4. The crude protein and crude ash compositions of barbecued pork patties were not greatly influenced by treatment with PLE. The crude fat percentage was significantly higher only when PLE were added in medium patties ($p<0.05$). In

317 contrast, the moisture percentage of medium patties was lower when PLE was added in
318 comparison to the control group ($p<0.05$). Its percentage was significantly higher in
319 medium-cooked than in well-cooked patties, irrespective of PLE addition percentage
320 ($p<0.05$). No significant effect was recorded with respect to the different percentages of
321 PLE addition on the proximate composition of pork patties. Furthermore, the significant
322 effect of PLE addition on the proximate composition of the pork patties was only seen in
323 the medium-cooked patties, with no notable effect in the well-cooked patties. Regarding
324 the different cooking levels, well-cooked patties had significantly higher crude protein
325 and crude ash percentage than those of medium-cooked patties. ($p<0.05$). In contrast, in
326 crude fat, the medium-cooked patties were significantly higher than the well-cooked
327 patties only when PLE was added ($p<0.05$). In the case of moisture content, it was
328 observed that well-cooked patties were lower than medium-cooked patties regardless of
329 with or without PLE. Notable differences in some of the proximate attributes were mainly
330 caused by the acceleration of moisture loss, which consequently changed the state of other
331 variables (Berry, 1994). Cooking at a higher final internal temperature resulted in a lower
332 moisture percentage but higher moisture content in the retained protein, which agrees
333 with previous reports (Barido et al., 2022b; Berry, 1994).

334

335 **Cooking loss and pH value**

336 The inclusion of PLE in pork patties did not significantly affect to the percentage of
337 cooking loss, as different cooking levels did. As seen in Table 5, a markedly higher
338 cooking loss percentage was observed in well-cooked patties than in medium-cooked
339 patties at any given PLE treatment ($p<0.05$). Apart from the excessive loss of moisture at
340 a high final internal temperature, poor fat retention in high-fat formulated patties is

another important factor that causes a remarkable yield loss in a well-cooked patties. This study revealed that the fat retention ability of patties formulated with low fat at 4% was remarkably higher than that of patties formulated with 20% of fat content at high temperature cooking (Berry, 1994); the pork patty in this study formulated with 20% of fat might confirm a similar trend. Furthermore, the addition of PLE to pork patties and different final internal temperatures had no significant effect on pH. This finding agrees with a previous study, wherein no significant effect on pH value was observed when 0.4% of perilla leaf powder was added to emulsion-type pork sausage (Kim et al., 2005).

Instrumental color

Instrumental color is critically important in influencing the organoleptic perception of consumers toward meat products; therefore, the documentation of color changes after cooking at different final internal temperatures together with the addition of PLE is of critical importance. The effects of different final internal temperatures and PLE treatments on the instrumental color of pork patties are shown in Table 6. The addition of PLE in a concentration-dependent manner significantly reduced the CIE L* value of medium- and well-cooked pork patties compared to the control ($p<0.05$). Similarly, the CIE a* and b* values of the pork patties were significantly reduced following treatment with PLE at any final internal temperature, wherein the highest reduction was shown in groups treated with the highest percentage of PLE at 0.4% compared to other treatments ($p<0.05$). Previous studies have shown that the primary color of plant-derived phenolic extracts might be absorbed by meat during processing (Barido and Lee, 2021; Smaoui et al., 2019; Maqsood et al., 2015). According to research of Boles and Pegg (2010), during the cooking process, myoglobin in meat is denatured. Because it is not affected by

365 cooking at the same temperature and intensity, it shows a red color at the end temperature,
366 and the brown color seen in cooked meat products is due to metmyoglobin. The results
367 on the effect of treatment with PLE on the instrumental color of pork patties are in
368 agreement with those of a previous study by Kim et al. (2005), who found a decrease in
369 both CIE L* and CIE a*, while maintaining a significant increase in CIE b* value
370 following inclusion of 0.4% perilla leaf powder in emulsion-type pork sausage.
371

372 **TBARS assay**

373 The TBARS assay is one of the prominent analyses to measure the degree of lipid
374 oxidation in meat and meat products; it is based on the concentration of MDA free radicals
375 (Kim et al., 2020). The changes in TBARS score following treatment are depicted in Table
376 7. The TBARS value in the control samples was 5.88 mg MDA/kg in medium-cooked
377 patties, while it was significantly lower at 3.39 in well-cooked patties. Moreover, this
378 study showed a significant inhibitory effect of PLE addition, wherein at any final internal
379 temperature, the PLE groups exhibited a markedly lower MDA concentration compared
380 to that of the control ($p<0.05$). However, no further differences were observed with
381 respect to different PLE addition percentages ($p<0.05$). Efforts to reduce excessive lipid
382 oxidation in meat products using natural compounds have been carried out for decades,
383 and certain phenolic acids are the most effective (Zhao et al., 2019). In perilla leaves, the
384 abundant content of anthocyanin and rosmarinic acid is believed to provide strong
385 antioxidant activities (Li et al., 2016; Zhu et al., 2014). Furthermore, the potent inhibitory
386 effect of PLE on lipid oxidation agrees with a previous study on surimi fish balls (Zhao
387 et al., 2019).
388

Sensory evaluation

The effects of cooking level and addition of PLE on the sensory characteristics of pork patties are shown in Table 8. In terms of color perception, the addition of PLE at any final internal temperature promoted a lower perception compared to the control group ($p<0.05$). Furthermore, a higher addition percentage of PLE tended to further decrease the color perception score of the pork patties. Accordingly, the flavor perception displayed a notably lower score when well-cooked pork patties were supplemented with PLE at any percentage in comparison to the control treatment ($p<0.05$). However, no significant difference was observed in medium-cooked pork patties. No further significant differences were observed in the aroma, taste, juiciness, and texture perception of pork patties after treatment with PLE ($p>0.05$). Meanwhile, the higher final internal temperature (well-cooked) significantly lowered juiciness and texture perception compared to medium-cooked patties for all treatments ($p<0.05$). The acceleration of moisture loss along with excessive muscle shrinkage was assumed to be responsible for this effect (Barido et al., 2021; Viegas et al., 2012). In addition, a different trend was recorded between the control and PLE treatments regarding flavor and taste perception. In control treatment, the scores for flavor and taste were significantly higher when pork patties were well-cooked ($p<0.05$), whereas in the highest PLE addition group (0.04%), flavor perception received significantly higher score when pork patties were only medium-cooked. Eventually, for overall acceptability, the addition of PLE at 0.2% maintained a similar preference for pork patties compared to the control group at any cooking level, whereas the addition of PLE up to 0.4% tended to receive lower score compared to the 0.2% in medium-cooked patties and control group in well-cooked

412 samples ($p<0.05$). The lower overall acceptability of 0.4% PLE in this study may be due
413 to the low perception of color and flavor attributes.
414
415 **PAHs content**
416 Table 9 displays the effects of different final internal temperatures and the addition
417 of PLE on the 16 PAHs contents of the roasted pork patties. In control samples without
418 any PLE addition, the total concentrations of the 16 PAHs were 198.33 and 280.79 ng/g
419 for medium- and well-cooked patties, respectively, wherein cooking to a higher final
420 internal temperature promoted higher production of PAHs ($p<0.05$). However, a similar
421 trend was not observed in pork patties treated with PLE, in which the concentration of
422 total PAHs was significantly lower in well-cooked patties than medium-cooked patties
423 ($p<0.05$). In addition, this study showed a different inhibitory effect of PLE addition at
424 different cooking levels of pork patties. In medium-cooked patties, the addition of 0.2%
425 PLE was not found to significantly prevent the formation of PAHs in pork patties
426 compared to the control groups, except for light PAHs, such as phenanthrene ($p<0.05$).
427 Meanwhile, when the pork patty was added with the higher concentration of PLE at 0.4%,
428 the formation of the light PAHs (naphthalene, phenanthrene, anthracene, pyrene) was
429 significantly suppressed; no effect on the heavy PAHs (including 4 PAH and 8 PAH) was
430 observed compared to the control.
431 Furthermore, regarding the addition of PLE to pork patties under well-cooked patties,
432 the 0.2% PLE exhibited a significant inhibitory effect on most of the light PAHs ($p<0.05$),
433 except fluorene ($p<0.05$). Moreover, the suppressive activity of 0.2% PLE on heavy PAHs
434 was recorded for benzo[k]fluoranthene and dibenzo[a,h]anthracene compared to the
435 control ($p<0.05$). However, in well-cooked patties, the addition of 0.4% PLE showed the

436 strongest inhibitory effect on the formation of both light and heavy PAHs ($p<0.05$). In
437 addition, owing to the hazardous effect of benzo[a]pyrene in possibly increasing the risk
438 of tumors and cancers due to the mode of action and interference with DNA replication
439 in human cells (Park et al., 2017), the reduction of benzo[a]pyrene concentration in meat
440 and meat products is essential. In this study, the addition of 0.4% PLE to pork patties
441 exhibited the highest suppressive effect on the formation of the benzo[a]pyrene in well-
442 cooked patties among the various treatments ($p<0.05$). Furthermore, the production of the
443 highly toxic 4 PAH and 8 PAH in pork patties was remarkably inhibited by the inclusion
444 of the 0.4% PLE. In addition, this study indicated a stronger inhibitory effect of PLE
445 addition on the formation of both 4 PAH and 8 PAH in a percentage-dependent manner,
446 wherein 0.4% addition exhibited a stronger effect than 0.2%.

447 At high temperatures, small organic molecules in meat undergo pyrolysis, resulting
448 in the formation of more free radicals, and the stable polynuclear aromatic compounds in
449 cooked meat might be a consequence of this process (Viegas et al., 2014). Owing to the
450 involvement of free radicals in the production of PAHs, studies have inferred that the
451 reduction of free radicals by antioxidants strongly contributes to the reduction of PAH
452 concentration in meat products (Park et al., 2017; Cordeiro et al., 2020; Viegas et al., 2014;
453 Gong et al., 2018; Wang et al., 2019). While the continuous application of synthetic
454 antioxidants may detrimentally affect human health owing to their potential toxicity (Bera
455 et al., 2006), natural compounds containing abundant polyphenols have been shown to
456 possess potent antioxidative activities (Gong et al., 2018; Kim et al., 2021b).

457 In our study, the highest inhibitory effect of 0.4% PLE treatment on PAHs under
458 well-cooked conditions might be due to the higher antioxidant activity of the phenolic
459 compounds at high temperatures. These results are in accordance with a previous report

460 by Wang et al. (2019), who found a higher inhibitory effect of phenolic compounds on
461 PAHs at higher grilling temperatures (240℃ and 270℃) than at low temperatures (210℃).
462 In addition, well-cooked patties treated with 0.4% PLE showed a higher inhibitory effect
463 on benzo[a]pyrene and PAH4 than medium-cooked patties. As explained by Min et al.
464 (2018), the factors influencing PAH formation may include heating conditions, the
465 presence of water, and antioxidants. Also, this trend was correlated with previous study,
466 which the interaction effect between natural materials having antioxidant activity and
467 doneness was observed for carcinogenic PAHs (Kim et al., 2021).
468

469 ## Conclusion

470 In this study, the effects of different PLE addition percentages at different final
471 internal temperatures were studied. Significantly lower MDA concentrations were
472 observed in both 0.2PLE and 0.4PLE compared to control, indicating the potential
473 antioxidant activity of PLE. Instead, 0.4% PLE exhibited a remarkably stronger
474 suppressing effect on the formation of the 4 PAH, 8 PAH, and total 16 PAHs under well-
475 cooked conditions compared to 0.2% and CON groups. This study suggests the addition
476 of PLE to well-done pork patties at 0.4% to improve their functional properties by
477 suppressing the formation of PAHs (4 PAH, 8 PAH, and 16 PAHs) and lipid oxidation
478 while maintaining no adverse effect on its physicochemical properties. However, further
479 study should be done to improve sensory acceptance of barbequed pork patties containing
480 0.4% PLE for consumers.
481

482 ## Acknowledgements

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Table 1. Formulation of pork patties added with various concentration of perilla leaves ethanol extracts

Ingredients (%)	Treatment		
	CON	0.2PLE	0.4PLE
Lean pork leg	70.00	70.00	70.00
Pork back fat	20.00	20.00	20.00
Salt	0.50	0.50	0.50
Water	6.00	6.00	6.00
Ethanol	3.50	3.50	3.50
Plant extract	0.00	0.20	0.40

CON, control; 0.2PLE, pork patty made with the addition of 0.2% perilla leaves extract; 0.4PLE, pork patty made with the addition of 0.4% perilla leaves extract.

598 **Table 2. Identified polycyclic aromatic hydrocarbons (PAHs) groups**

Type	16 PAHs	8 PAH	4 PAH
Light PAHs			
Naphthalene	√		
Acenaphthylene	√		
Acenaphthene	√		
Fluorene	√		
Phenanthrene	√		
Anthracene	√		
Fluoranthene	√		
Pyrene	√		
Heavy PAHs			
Benzo[a]anthracene	√	√	√
Chrysene	√	√	√

Benzo[b]fluoranthene	√	√	√
Benzo[k]fluoranthene	√	√	
Benzo[a]pyrene	√	√	√
Indeno[1,2,3-cd]pyrene	√	√	
Dibenzo[a,h]anthracene	√	√	
Benzo[ghi]perylene	√	√	

599

600 **Table 3. Antioxidant activities of the perilla leaves ethanol extract measured by various antioxidant assays**

Ethanol concentration (%)	DPPH (μmol TE/g DM)		SEM	FRAP (mmol TE/g DM)		SEM	ORAC (mmol TE/g DM)		SEM	TPC (mg GAE/g DM)		SEM
	Day 3	Day 6		Day 3	Day 6		Day 3	Day 6		Day 3	Day 6	
50	454.80^{Cb}	509.12^{Aa}	1.26	0.65^{Ba}	0.63^{Ba}	0.02	2.41^{Bb}	2.68^{Ba}	0.04	79.15^{Ca}	81.91^{Ca}	1.08
70	502.68^{Ba}	451.74^{Bb}	11.96	0.71^{Ba}	0.71^{Ba}	0.02	2.82^{Aa}	2.90^{Aa}	0.04	81.97^{Bb}	91.48^{Ba}	0.35
90	593.27^{Aa}	521.92^{Ab}	11.34	0.91^{Ab}	0.97^{Aa}	0.01	2.91^{Aa}	2.95^{Aa}	0.03	102.39^{Ab}	114.93^{Aa}	1.45
SEM	10.95	7.88		0.02	0.02		0.05	0.02		0.44	1.44	

601 DPPH, 2,2 diphenyl 1 picrylhydrazyl; FRAP, fluorescence recovery after photobleaching; ORAC, oxygen radical absorbance capacity; TPC,

602 total phenolic content.

603 $^{A-C}$ Means within a column with different superscript differ significantly at $p<0.05$ (n=3).

604 $^{a-b}$ Means within a row with different superscript differ significantly at $p<0.05$ (n=3).

Table 4. Effect of perilla leaves ethanol extract on proximate composition of the barbecued pork patty

Proximate composition (%)	Cooking doneness	Treatment			SEM
		CON	0.2PLE	0.4PLE	
Moisture	Medium	57.92^{Aa}	55.35^{Ab}	55.70^{Ab}	0.17
	Well-done	52.54^{B}	52.31^{B}	52.32^{B}	0.11
	SEM	0.18	0.11	0.13	
Crude protein	Medium	25.26^{B}	25.27^{B}	24.93^{B}	0.47
	Well-done	29.00^{A}	28.20^{A}	27.88^{A}	0.32
	SEM	0.45	0.11	0.52	
Crude fat	Medium	16.06^{b}	17.45^{Aa}	17.36^{Aa}	0.24
	Well-done	15.58	16.45^{B}	16.57^{B}	0.33
	SEM	0.35	0.23	0.27	
Crude ash	Medium	1.61^{B}	1.64^{B}	1.67^{B}	0.01
	Well-done	1.82^{A}	1.90^{A}	1.84^{A}	0.02
	SEM	0.01	0.03	0.01	

CON, control; 0.2PLE, pork patty made with the addition of 0.2% perilla leaves extract; 0.4PLE, pork patty made with the addition of 0.4% perilla leaves extract; Medium, barbecued for 9 min until internal temperature reaches 71℃; Well-done, barbecued for 16 min until internal temperature reaches 80℃.

$^{A-B}$ Means within a column with different superscript differ significantly at $p<0.05$ (n=3).

$^{a-b}$ Means within a row with different superscript differ significantly at $p<0.05$ (n=3).

613 **Table 5. Effect of perilla leaves ethanol extracts on pH value and cooking loss of the**
614 **barbecued pork patty**

Traits	Cooking doneness	Treatment			SEM
		CON	0.2PLE	0.4PLE	
pH	Medium	6.15	6.16	6.14	0.00
	Well-done	6.16	6.15	6.15	0.00
	SEM	0.00	0.01	0.00	
Cooking loss (%)	Medium	37.96^{B}	37.99^{B}	39.79^{B}	0.62
	Well-done	48.57^{A}	47.75^{A}	48.71^{A}	0.78
	SEM	0.69	0.12	0.99	

615 CON, control; 0.2PLE, pork patty made with the addition of 0.2% perilla leaves extract;
616 0.4PLE, pork patty made with the addition of 0.4% perilla leaves extract; Medium, barbecued
617 for 9 min until internal temperature reaches 71℃; Well-done, barbecued for 16 min until
618 internal temperature reaches 80℃.

619 $^{A-B}$ Means within a column with different superscript differ significantly at $p<0.05$ (n=3).

620 **Table 6. Effect of perilla leaves ethanol extracts on instrumental color of barbecued pork**
621 **patty**

Color	Cooking doneness	Treatment			SEM
		CON	0.2PLE	0.4PLE	
CIE L*	Medium	53.37^{Aa}	27.92^{Ab}	24.77^{Ac}	0.34
	Well-done	30.98^{Ba}	23.47^{Bb}	22.18^{Bc}	0.22
	SEM	0.34	0.20	0.33	
CIE a*	Medium	10.32^{Ba}	5.36^{Bb}	4.49^{Bc}	0.17
	Well-done	16.42^{Aa}	8.09^{Ab}	5.81^{Ac}	0.22
	SEM	0.28	0.20	0.02	
CIE b*	Medium	26.57^{Aa}	14.96^{Ab}	11.89^{Ac}	0.23
	Well-done	23.15^{Ba}	10.86^{Bb}	8.13^{Bc}	0.19
	SEM	0.19	0.22	0.22	

622 CON, control; 0.2PLE, pork patty made with the addition of 0.2% perilla leaves extract;
623 0.4PLE, pork patty made with the addition of 0.4% perilla leaves extract; Medium, barbecued
624 for 9 min until internal temperature reaches 71℃; Well-done, barbecued for 16 min until
625 internal temperature reaches 80℃.
626 $^{A-B}$ Means within a column with different superscript differ significantly at $p<0.05$ (n=3).
627 $^{a-b}$ Means within a row with different superscript differ significantly at $p<0.05$ (n=3).
628

Table 7. Effect of perilla leaves ethanol extract on the TBARS value (mg MDA/kg) of the barbecued pork patty

Cooking doneness	Treatment			SEM
	CON	0.2PLE	0.4PLE	
Medium	5.88^{Aa}	0.51^{Ab}	0.53^{Ab}	0.02
Well-done	3.29^{Ba}	0.48^{Bb}	0.50^{Bb}	0.00
SEM	0.02	0.00	0.01	

CON, control; 0.2PLE, pork patty made with the addition of 0.2% perilla leaves extract; 0.4PLE, pork patty made with the addition of 0.4% perilla leaves extract; Medium, barbecued for 9 min until internal temperature reaches 71℃; Well-done, barbecued for 16 min until internal temperature reaches 80℃.

$^{A-B}$ Means within a column with different superscript differ significantly at $p<0.05$ (n=3).

$^{a-b}$ Means within a row with different superscript differ significantly at $p<0.05$ (n=3).

637 **Table 8. Effect of perilla leaves ethanol extracts on organoleptic properties of the**
638 **barbecued pork patty**

Sensory traits	Cooking doneness	Treatment			SEM
		CON	0.2PLE	0.4PLE	
Color	Medium	8.40^{a}	7.13^{b}	6.27^{c}	0.23
	Well-done	8.13^{a}	6.87^{b}	5.73^{c}	0.21
	SEM	0.11	0.22	0.30	
Aroma	Medium	7.47	7.47	8.00	0.19
	Well-done	7.93	7.40	7.67	0.27
	SEM	0.19	0.26	0.26	
Flavor	Medium	7.13^{B}	7.20	7.70^{A}	0.26
	Well-done	7.83^{Aa}	6.53^{b}	6.67^{Bb}	0.22
	SEM	0.15	0.30	0.25	
Taste	Medium	6.87^{B}	7.33	7.57	0.24
	Well-done	7.53^{A}	7.00	7.33	0.22
	SEM	0.22	0.25	0.22	
Juiciness	Medium	6.73^{A}	7.20^{A}	6.63^{A}	0.29
	Well-done	5.33^{B}	5.13^{B}	4.87^{B}	0.29
	SEM	0.29	0.30	0.27	
Texture	Medium	6.73^{A}	7.40^{A}	7.30^{A}	0.26
	Well-done	5.93^{B}	6.00^{B}	5.87^{B}	0.31
	SEM	0.26	0.30	0.29	
Overall acceptability	Medium	7.23^{ab}	7.50^{a}	6.77^{b}	0.20
	Well-done	7.13^{a}	6.87^{ab}	6.30^{b}	0.23
	SEM	0.16	0.23	0.26	

639 CON, control; 0.2PLE, pork patty made with the addition of 0.2% perilla leaves extract;
640 0.4PLE, pork patty made with the addition of 0.4% perilla leaves extract; Medium, barbecued
641 for 9 min until internal temperature reaches 71℃; Well-done, barbecued for 16 min until
642 internal temperature reaches 80℃.
643 $^{A-B}$ Means within a column with different superscript differ significantly at $p<0.05$ (n=3).
644 $^{a-c}$ Means within a row with different superscript differ significantly at $p<0.05$ (n=3).

645 **Table 9. Effect of perilla leaves extracts on the formation of the polycyclic aromatic**
646 **hydrocabons (PAHs) in barbecued pork patty**

PAHs (ng/g)	Cooking doneness	Treatment			SEM
		CON	0.2PLE	0.4PLE	
Light PAHs					
	Medium	19.77^{Ba}	19.36^{Aa}	16.94^{Ab}	0.11
Naphthalene	Well-done	31.14^{Aa}	15.51^{Bb}	15.87^{Bb}	0.69
	SEM	0.65	0.43	0.36	
	Medium	6.57^{Bb}	7.97^{Aa}	7.94^{Aa}	0.12
Acenaphthylene	Well-done	8.73^{Aa}	6.83^{Bb}	7.11^{Bb}	0.25
	SEM	0.30	0.08	0.13	
	Medium	4.61^{Bb}	5.79^{Aa}	5.50^{Aa}	0.153
Acenaphthene	Well-done	6.00^{Aa}	4.95^{Bb}	4.37^{Bc}	0.121
	SEM	0.13	0.18	0.08	
	Medium	19.00^{Bc}	26.18^{Aa}	21.11^{Ab}	0.31
Fluorene	Well-done	27.09^{Aa}	23.55^{Ba}	16.75^{Bb}	0.95
	SEM	1.04	0.37	0.54	
	Medium	98.20^{Ba}	68.66^{Bb}	63.50^{Ab}	2.07
Phenanthrene	Well-done	125.85^{Aa}	70.94^{Ab}	61.44^{Ac}	1.38
	SEM	2.80	0.56	1.05	
	Medium	20.65^{Bb}	28.43^{Aa}	14.28^{Ac}	0.76
Anthracene	Well-done	28.72^{Aa}	15.34^{Bb}	14.56^{Ab}	0.70
	SEM	1.07	0.64	0.23	
	Medium	ND	ND	ND	-
Fluoranthene	Well-done	ND	ND	ND	-
	SEM	-	-	-	
Pyrene	Medium	19.69^{Bb}	28.16^{Aa}	15.58^{Bc}	0.34

	Well-done	35.84^{Aa}	19.04^{Bb}	18.33^{Ab}	0.45
	SEM	0.36	0.52	0.27	
Heavy PAHs					
	Medium	2.02^{Bb}	3.04^{Ba}	3.12^{Aa}	0.03
Benzo[a]anthracene	Well-done	3.62^{Aa}	3.55^{Aa}	2.49^{Bb}	0.02
	SEM	0.03	0.06	0.03	
	Medium	3.19^{Bb}	5.20^{Ba}	4.93^{Aa}	0.08
Chrysene	Well-done	6.11^{Aa}	6.39^{Aa}	4.20^{Bb}	0.12
	SEM	0.11	0.09	0.10	
	Medium	1.28^{Bb}	1.91^{Ba}	1.97^{Aa}	0.04
Benzo[b]fluoranthene	Well-done	2.51^{Aa}	2.45^{Aa}	1.80^{Ab}	0.08
	SEM	0.03	0.07	0.08	
	Medium	0.67^{Bc}	0.80^{Bb}	0.92^{Aa}	0.01
Benzo[k]fluoranthene	Well-done	1.10^{Aa}	0.91^{Ab}	0.79^{Bb}	0.03
	SEM	0.04	0.01	0.01	
	Medium	1.08^{Bc}	1.59^{Bb}	2.16^{Aa}	0.03
Benzo[a]pyrene	Well-done	1.81^{Ab}	1.93^{Aa}	1.59^{Bc}	0.02
	SEM	0.03	0.01	0.04	
	Medium	ND	ND	ND	-
Indeno[1,2,3-cd]pyrene	Well-done	ND	ND	ND	-
	SEM	-	-	-	
	Medium	0.69^{Bab}	0.71^{Ba}	0.69^{Bb}	0.01
Dibenzo[a,h]anthracene	Well-done	0.98^{Aa}	0.81^{Ac}	0.91^{Ab}	0.01
	SEM	0.00	0.01	0.00	

	Medium	0.91^{Bb}	1.09^{Bb}	1.36^{Aa}	0.05
Benzo[ghi]perylene	Well-done	1.30^{Ab}	1.50^{Aa}	1.11^{Ac}	0.03
	SEM	0.02	0.01	0.07	
	Medium	7.56^{Bb}	11.73^{Ba}	12.18^{Aa}	0.15
4 PAH	Well-done	14.05^{Aa}	14.32^{Aa}	10.09^{Bb}	0.13
	SEM	0.12	0.07	0.20	
	Medium	9.84^{Bb}	14.33^{Ba}	15.16^{Aa}	0.20
8 PAH	Well-done	17.43^{Aa}	17.54^{Aa}	12.90^{Bb}	0.13
	SEM	0.15	0.07	0.24	
	Medium	198.33^{Ba}	198.88^{Aa}	160.02^{Ab}	2.30
Total 16 PAHs	Well-done	280.79^{Aa}	173.69^{Bb}	151.35^{Bc}	1.06
	SEM	2.54	1.00	1.47	

CON, control; 0.2PLE, pork patty made with the addition of 0.2% perilla leaves extract; 0.4PLE, pork patty made with the addition of 0.4% perilla leaves extract; Medium, barbecued for 9 min until internal temperature reaches 71℃; Well-done, barbecued for 16 min until internal temperature reaches 80℃.

4 PAHs: Benzo[a]anthracene, Chrysene, Benzo[b]fluoranthene, Benzo[a]pyrene; 8 PAHs: Benzo[a]anthracene, Chrysene, Benzo[b]fluoranthene, Benzo[k]fluoranthene, Benzo[a]pyrene,Indeno[1,2,3-cd]pyrene, Dibenzo[a,h]anthracene, Benzo[ghi]perylene; ND, not detected.

$^{A-B}$ Means within a column with different superscript differ significantly at $p<0.05$ (n=3).

$^{a-c}$ Means within a row with different superscript differ significantly at $p<0.05$ (n=3).