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RESEARCH ARTICLE

Antioxidative Activities of *Mao Feng* Tea (*Camellia* spp.) and *Kamtae* (*Ecklonia cava*) Extracts and Their Effects on Structured Lipid from Corn and Perilla Oil

Kanika Mitra, Jung-Ah Shin, Jeung-Hee Lee, Seong-Ai Kim, Soon-Taek Hong, Chang-Keun Sung, Dan Yang, Lu-Jing Gan, and Ki-Teak Lee

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Abstract In the study, solvent extracts of *kamtae* (*Ecklonia cava*) and *mao feng* tea (*Camillia sinensis*) were used for obtaining different fractions of organic solvents (diethyl ether, butanol, and ethyl acetate) and the extracted fractions were studied for their antioxidative activities. The total phenolic contents of the *mao feng* tea ranged from 1.44 to 5.97 mM GAE/g while *kamtae* ranged from 1.13 to 4.41 mM GAE/g, respectively. Among them, ethyl acetate fraction showed the highest content of phenolic compounds, resulting in Trolox equivalent antioxidant capacity (TEAC) values as 1,554.54 (from *mao feng* tea) and 1,097.63 mM Trolox E/g (from *kamtae*). Also, ethyl acetate fractions from *mao feng* tea showed the highest DPPH (89.27 RSC%), superoxide anion scavenging activity (46.58%), and ferric reducing antioxidant power (FRAP) (242.2 mg GAE/g) while ethyl acetate fractions from *kamtae* (K-EA) showed the highest DPPH (82.23 RSC%), superoxide anion scavenging activity (28.82%), and FRAP (162.43 mg GAE/g) among the obtained fractions.

Keywords: *kamtae* (*Ecklonia cava*), *mao feng* tea (*Camillia sinensis*), antioxidant activity, structured lipid

Jung-Ah Shin, Soon-Taek Hong, Chang-Keun Sung, Dan Yang, Lu-Jing Gan, Ki-Teak Lee (✉)
Department of Food Science and Technology, Chungnam National University, Daejeon 305-764, Korea
Tel: +82-42-821-7873; Fax: +82-42-822-6729
E-mail: ktlee@cnu.ac.kr

Kanika Mitra, Seong-Ai Kim
Department of Food and Nutrition, Chungnam National University, Daejeon 305-764, Korea

Jeung-Hee Lee
Institute of Agricultural Science, Chungnam National University, Daejeon 305-764, Korea. At present: US Department of Agriculture, Agricultural Research Service, Eastern Regional Research Center, Wyndmoor, PA19038, USA

Introduction

Synthetic and natural antioxidants are used for preventing the oxidative deterioration of constituent of lipids in food industry. To preserving food quality, the most commonly used antioxidants are butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), propyl gallate (PG) (1), and tocopherols (2). BHA, BHT, and PG are synthetic antioxidants, which are suspected to show potential adverse health effects (3,4). Therefore, much attention has been focused on dietary natural antioxidants due to the growing demands from the functional food industry. Tocopherols, as natural antioxidants, are regarded as safe food additives. However, they may lead to pro-oxidant effects under excessive circumstance (2). Furthermore, the maximum addition of tocopherols is regulated in the UK and some other European countries (5). Hence, other natural plant sources and their extracts, especially those contained phenolic compounds are interested. *Ecklonia cava*, known as *kamtae* in Korea, is brown algae that could be found in the sub-tidal region of East Asia. *Kamtae* contains more phenolic compounds, phlorotannins, than other brown seaweeds (6). Recent studies have demonstrated that *kamtae* shows strong antioxidant properties (7) and radical scavenging effects (8). Almost photosynthesizing plants including seaweeds are exposed to a combination of light and high oxygen concentrations, which lead to the formation of free radicals and other strong oxidizing agents. But they seldom suffer any serious photodynamic damage during metabolism, implying that their cells have some protective antioxidative mechanisms and compounds (9). On the other hand, green tea (*Camillia sinensis*) as the most popular beverage in the East Asia has great interest due to its beneficial health effects. For example, consumption

of tea flavonoids has been linked to lower incidences of chronic diseases such as cardiovascular disease and cancer (10). Among many compounds, catechins and other polyphenols in tea exhibited strong antioxidant activities (11). These compounds act as an antioxidant by sequestering metal ions or by scavenging reactive oxygen and nitrogen species (12).

Lipids that have been modified to change the composition of fatty acids and /or the location of the glycerol backbone through chemical or enzymatic process is called structured lipid (SL). SL is not available in the nature but can be produced as tailor-made lipids, considering as nutraceuticals since it can prove several health benefits (13). Until now, there are several fats and oils used for the synthesis of SL (14). In some cases, SL is more susceptible to oxidation due to the removal of natural antioxidants, such as tocopherols, during the reaction (15). This leads to the necessary of adding antioxidants into SLs. The present work focused on investigating the antioxidant efficacy of *kamtae* (*Ecklonia cava*) and *mao feng* tea (*Camillia sinensis*) on oxidative stability of SL under accelerated oxidation conditions.

In the study, solvent extracts of *kamtae* and *mao feng* tea were studied for their antioxidative activities. To clarify the presence of the antioxidants in *kamtae* and *mao feng* tea, different fractions of organic solvents were investigated for the amount of total phenolic compounds, free radical scavenging activity, superoxide anion scavenging activity, Trolox equivalent antioxidant capacity (TEAC), and ferric reducing antioxidant power (FRAP) were evaluated. Also, the fractions were used as antioxidant on SL prepared from corn oil and perilla oil through lipase-catalyzed reaction. During 30-day oxidation of SL, antioxidative activities of the extracts (200 and 500 ppm) were evaluated by peroxide value, ρ-anisidine and 2-thiobabituric acid-reactive substances (TBARS). Besides, such values with same amount of BHT and α-tocopherol were obtained to compare the antioxidant activities of the extracts from *kamtae* and *mao feng* tea.

Materials and Methods

Materials *Kamtae* (*Ecklonia cava*) was obtained from Seosan Maru Inc. (Seosan, Korea). *Mao feng* tea (*Camillia sinensis*) was purchased from Anhui province in China. ρ-Anisidine reagent (*p*-methoxyaniline), 4,6-dihydroxy-2-mercaptopyrimidine (TBA) reagent, Trolox reagent, ABTS reagent, potassium persulfate, nitroblue tetrazolium (NBT), NADH, phenazine methosulphate (PMS), TPTZ, ferrous chloride, sodium acetate trihydrate, gallic acid, ascorbic acid, folin-ciocalteu's phenol, DPPH, and BHT were purchased from Sigma-Aldrich (St. Louis, MO, USA). All chemicals and solvents were HPLC grade, and obtained from Fisher Scientific (Norcross, GA, USA). Lipozyme RMIM was purchased from Novozyme A/S (Bagsvaerd, Denmark).

Extraction for fractions Extraction was based on the method described by Tsaknis and Lalas (16) with slight modification. One-hundred g of each sample (*mao feng* tea and *kamtae*) was defatted with *n*-hexane (400 mL×3) and each defatted material was then extracted by methanol. After evaporating solvent by vacuum rotary evaporator, 19.67 g of light green gum residue of from tea and 10.5 g from *kamtae* were obtained. The methanol remains was dissolved in distilled water (200 mL) and then extracted with diethyl ether (200 mL×3) using a separating funnel. The lipophilic part was collected and solvent was evaporated using the vacuum rotary evaporator. The remaining gum was held in reserve overnight at room temperature, yielding a 1.87 g of green residue from *mao feng* tea (fraction T-DE) and 3.5 g from *kamtae* (fraction K-DE). The hydrophilic layer of the previous action was extracted with ethyl acetate (100 mL×3). The ethyl acetate layer was collected, and after evaporated the solvent with evaporator, the residue was dried in an oven at 60°C for overnight. The residue (fraction T-EA) was weighted from *mao feng* tea (2.85 g) and from *kamtae* (3.2 g, fraction K-EA). The remaining hydrophilic layer was extracted with saturated *n*-butanol (100 mL×3). The butanol layer was collected, evaporated, and dried in the oven (60°C, over night). Then, a light orange color residue from *mao feng* tea (fraction T-BU, 1.89 g) and from *kamtae* (fraction K-BU, 1.9 g) were obtained, respectively.

Determination of phenolic compounds Total phenolic compounds in the extracts were determined using Folin-Ciocalteu reagent by Netzel *et al*. (17) with slight modification. Gallic acid was used as a standard compound. In a test tube, 0.05 mL of sample (1 mg/mL) was diluted with distilled water (5 mL). Then, 0.5 mL of Folin-Ciocalteu reagent was added and mixed thoroughly. After 3 min, 1 mL of 1 N Na_2CO_3 was added and the mixture was allowed to stand in a dark place for 1 h. The absorbance was measured at 750 nm by spectrophotometer. The concentration of total phenolic compounds in the extracts was expressed as mM of gallic acid equivalent (GAE)/g of extraction by obtained standard curve (R^2= 0.98).

Free radical scavenging activity (DPPH assay) The free radical scavenging activity of the extracts were measured by the method of Mazor *et al*. (18) using DPPH with modification. Briefly, 100 μL sample solution was diluted with ethanol and then 2.5 mL of DPPH solution (1.5×10^{-4} M) was added. This mixture was shaken vigorously, and

allowed to stand for 30 min in the dark. Then, the absorbance was measured at 517 nm by spectrophotometer against a blank. The percentage inhibition of free radical scavenging activity was calculated using the following formula:

$$\text{Radical scavenging capacity (RSC) (\%)} = [(A_0 - A_1)/A_0] \times 100$$

where, A_0 was the absorbance of the control, and A_1 was the absorbance of the extracts.

Superoxide anion scavenging activity (SOD assay) Measurements of superoxide anion scavenging activity of the extracts were performed by the method described by Gülcin *et al*. (1) with modification. Superoxide radicals are generated in PMS NADH system by the oxidation of NADH, and assayed by the reduction of NBT. Working solution was prepared by 1 mL of NBT (156 μmol/L NBT in 100 mmol/L Tris-HCl buffer, pH 8) solution, 1 mL of NADH (468 μmol/L in 100 mmol/L Tris-HCl buffer, pH 8) solution, and 0.1 mL of sample solution. Then, 100 μL of PMS (60 μmol/L in 100 mmol/L Tris-HCl buffer, pH 8) was added thereafter the reaction mixture was incubated at 25°C for 5 min. Absorbance at 560 nm was measured against blank samples. The percentage inhibition of superoxide anion generation was calculated using the following formula:

$$\text{Inhibition ratio (\%)} = [(A_0 - A_1)/A_0] \times 100$$

where, A_0 was the absorbance of the control and A_1 was the absorbance of the extracts.

Trolox equivalent antioxidant capacity (TEAC) assay The method is based on the ability of antioxidant molecules to quench the long-lived ABTS radical, a blue green chromophore with absorption at 734 nm, compared with that of Trolox, a water-soluble analog of vitamin E (19,20). The ABTS radical was generated by chemical reaction with potassium persulfate. For this purpose, 7 mM ABTS was dissolved in water. Then 3.5 mM potassium persulfate was added and allowed to stand in dark place at room temperature for 12-16 h. Subsequently, absorbance of the working solution (5 mL) was diluted with ethanol until an absorbance of 0.7 (±0.2) and then the working solution with 250 μL of sample or standard was measured. A standard curve was prepared using different concentrations of Trolox. The antioxidant capacity of the extracts was expressed as mM of Trolox equivalent/g of extraction by obtained standard curve.

Ferric reducing-antioxidant power (FRAP) assay The FRAP assay was conducted according to the method of Benzie and Strain (21) followed by some modification (18). The method is based on the reduction of the Fe^{3+} TPTZ complex to the ferrous form at low pH. This reduction is monitored by measuring the absorption change at 593 nm. Briefly, the absorbance of working FRAP solution (10 mM TPTZ and 20 mM $FeCl_3 \cdot 6H_2O$ in 0.25 M acetate buffer, pH 3.6) was measured. Then, 200 μL sample or gallic acid was mixed with working solution. The absorption of diluted sample was measured after 10 min incubation at room temperature. A standard curve was prepared using different concentrations of gallic acid. The antioxidant capacity of the extracts was expressed as mg GAE/g of extraction by obtained standard curve.

Synthesis of structured lipid (SL) Corn oil (100 g, CO) and perilla oil (100 g, PO) were mixed (1:1, w/w) in a 250-mL flask with a cap for synthesis of SL. Lipozyme RMIM (10% by weight of total mixture) was added to the mixture. No additional solvent was added into reaction system. The combined mixtures were incubated in an orbital-shaking (200 rpm) water bath for 24 h at 65°C. After incubation, the reaction product was passed through anhydrous sodium sulfate column.

Oxidation studies SL was uniformly mixed with fractions in 2 different concentrations (200 and 500 ppm) by an ultra sonicator and stored in an oven at 60°C for 30 days. For the evaluation of oxidative stability, their peroxide value (PV for measuring hydroperoxide), ρ-anisidine (AV for aldehydes, principally 2-alkanal, and 2, 4-dienal) and 2-thiobabituric acid-reactive substances (TBARS value for substances like malonaldehyde) were measured at every 5-day interval (0, 5, 10, 15, 20, 25, and 30 days) according to the AOCS official methods (22).

Results and Discussion

Total phenolic content and TEAC assay Under alkaline conditions, Folin-Ciocalteu's phenol reagent (yellow color) reacts with phenolic compounds and a phenolate anion is consequently formed by dissociation of a phenolic hydrogen atom. This sequence of reversible 1 or 2-electron reductions leads to blue-coloured chromophores being formed between phenolate and Folin-Ciocalteu's reagent (23). Based on the reaction mechanism, total phenolic assay actually measures the reducing capacity of a sample. The total phenolic assay is routinely used because it is simple, sensitive and precise (24). The total phenolic contents of the *mao feng* tea used in this study ranged from 1.44 to 5.97 mM GAE/g (Table 1) and *kamtae* ranged from 1.13 to 4.41 mM GAE/g, respectively (Table 2). Hence, it may be concluded that ethyl acetate fraction showed the highest content of phenolic compounds among the other fractions in this study.

Table 1. Antioxidative activities and phenolic content of different solvent extracts of *mao feng* tea, BHT, and α-tocopherol

Fractions	DPPH-RSC (%)	O_2^--SC (%)	FRAP (mg GAE/g)	TEAC (mM Trolox E/g)	Phenolic (mM GAE/g)
Tea-Diethyl (T-DE)	57.89±0.15d[1)]	35.76±1.66c	137.90±0.33e	972.82±1.14e	1.44c
Tea-Ethyl acetate (T-EA)	89.27±0.54a	46.58±0.99a	242.20±0.21a	1,554.54±1.08a	5.97a
Tea-Butanol (T-BU)	81.97±0.23b	41.29±0.50b	227.90±0.32b	1,256.91±2.12b	4.92b
BHT	81.59±0.14b	26.41±1.83d	155.65±0.61c	1,067.68±1.11c	ND
α-Tocopherol	79.63±0.28c	24.47±0.99e	133.69±0.31d	1,018.59±1.09d	ND

[1)]Different letters (a-e) within column are significantly different ($p<0.05$); ND, not detected

Table 2. Antioxidative activities and phenolic content of different solvent extracts of *kamtae*, BHT, and α-tocopherol

Fractions	DPPH-RSC (%)	O_2^--SC (%)	FRAP (mg GAE/g)	TEAC (mM Trolox E/g)	Phenolic (mM GAE/g)
Kamtae-Diethyl (K-DE)	65.40±0.48d[1)]	20.82±0.83d	83.78±0.63d	420.63±2.09e	1.13c
Kamtae-Ethyl acetate (K-EA)	82.23±0.07a	28.82±0.50a	162.43±0.32a	1,097.63±1.11a	4.41a
Kamtae-Butanol (K-BU)	79.21±0.34c	27.06±0.33b	156.32±0.31b	1,071.86±2.11b	3.40b
BHT	81.59±0.14b	26.41±1.83b	155.65±0.61b	1,067.68±1.11c	ND
α-Tocopherol	79.63±0.28c	24.47±0.99c	133.69±0.31c	1,018.59±1.09d	ND

[1)]Different letters (a-e) within column are significantly different ($p<0.05$); ND, not detected

The TEAC assay has been used frequently to determine the total antioxidant capacities of many food samples such as fruits, vegetables, and spices (1,17,23). In this assay, peroxyl radicals or other oxidants (e.g., potassium persulfate) oxidize ABTS to its radical cation, $ABTS^{\bullet+}$ (intense blue color). Basically, the antioxidant capacities of test compounds are determined by measuring decreased in the intensity of the blue color as a result of reaction between the $ABTS^{\bullet+}$ and the antioxidant compounds in the sample (19). The TEAC values of different extract fractions from *mao feng* tea and *kamtae* are presented in Table 1 and 2, respectively. On a mM Trolox E/g basis, ethyl acetate extracts from the *mao feng* tea (T-EA) and *kamtae* (K-EA) demonstrated the highest antioxidant capacities (1,554.54 and 1,097.63 mM Trolox E/g). Comparing α-tocopherol and BHT, ethyl acetate, and butanol fraction of *mao feng* tea (T-EA and T-BU) and *kamtae* (K-EA and K-BU) showed significantly higher antioxidative capacity ($p<0.05$).

The TEAC values of each fraction followed the same order as did result of total phenolic content of *mao feng* tea and *kamtae*. There is controversy over what antioxidant capacity assays measure-only phenols or phenols and reducing agents or metal chelators (23). Moreover, Amarowicz *et al*. (25) stated that total phenolic results cannot be expressed as the antioxidant capacity of extracts. However, in this study, correlation between total phenolic contents and TEAC assay results (data not shown) showed that phenolic compounds may main compounds to exhibit antioxidative capacity in the extracts.

FRAP, DPPH, and SOD assay Basically, iron can stimulate lipid peroxidation by the Fenton reaction, and also accelerates peroxidation by decomposing lipid hydroperoxides into peroxyl and alkoxyl radicals that can themselves abstract hydrogen and perpetuate the chain reaction of lipid peroxidation (26). In FRAP assay, a FRAP method is based on the ability of antioxidant to reduce (electron transfer) Fe^{3+} to Fe^{2+} ions in the presence of TPTZ forming an intense blue Fe^{2+}-TPTZ complex with an absorption maximum at 593 nm. An absorbance exponentially increased due to the formation of absorbing product of the reaction of antioxidants with Fe^{2+}-TPTZ complex. As shown in Table 1 and 2, The T-EA, T-BU, and K-EA showed significantly higher FARP values than both BHT and α-tocopherol ($p<0.05$). Also, the fractions from *mao feng* tea showed 137.90-242.20 mg GAE/g while the fractions from *kamtae* showed 83.78-162.43 mg GAE/g, indicating that ferric ion reducing antioxidant power effect of both fractions from *mao feng* tea and *kamtae* increased when those were extracted with ethyl acetate and butanol. Fractions from diethyl ether from both materials showed a little lower value than the other fractions.

$DPPH^{\bullet}$ assay is a widely used method for evaluating antioxidant activates in a short time compared with other methods. The effect of antioxidants on DPPH radical scavenging was thought to because of their hydrogen donating ability. DPPH is a stable free radical, when it accepts an electron or hydrogen radical, it will become a stable diamagnetic molecule (27). The decrease in absorbance at 517 nm was used to determine the reduction capacity of DPPH radicals. Ethyl acetate extracts of both materials showed stronger DPPH radical scavenging capacity than the other fractions with significant difference

Table 3. Fatty acid compositions (mol %) of corn oil, perilla oil, and structured lipid (SL)

Fatty acids	Perilla oil	Corn oil	SL
C16:0 (palmitic acid)	6.4±0.1	12.3±0.1	9.7±0.2
C18:0 (stearic acid)	1.9±0.1	2.1±0.1	2.1±0.1
C18:1 (oleic acid)	18.8±0.1	30.2±0.3	25.5±0.3
C18:2 (linoleic acid)	11.8±0.1	54.8±0.4	35.5±0.2
C18:3 (alpha-linolenic acid)	61.1±0.6	0.7±0.1	30.5±0.3
ω3 fatty acids	11.8±0.1	54.8±0.4	35.5±0.2
ω6 fatty acids	61.1±0.6	0.7±0.1	30.5±0.3

(p<0.05). Further, DPPH scavenging effects of both extracts were reduced in the order of ethyl acetate > butanol > diethyl ether fractions. In addition, DPPH scavenging capacity of ethyl acetate fractions of both materials were higher than that of α-tocopherol and BHT, showing the noticeable effect on scavenging free radicals.

Superoxide anion scavenging activity derived from dissolved oxygen by PMS/NADH coupling reaction reduces NBT in the PMS/NADH-NBT system. Thus, a decrease of absorbance at 560 nm with certain antioxidants indicates the consumption of superoxide anion. The ethyl acetate fractions of both materials have strong superoxide radical scavenging activity, exhibiting higher superoxide radical scavenging activity than BHT and α-tocopherol with significant difference (p<0.05). The anion scavenging results of all fractions, BHT, and α-tocopherol followed the order: T-EA > T-BU > K-EA > K-BU > BHT > α-tocopherol > T-DE > K-DE. This result indicates that fractions by ethyl acetate and butanol have higher superoxide radical scavenging activity than BHT and α-tocopherol. Our

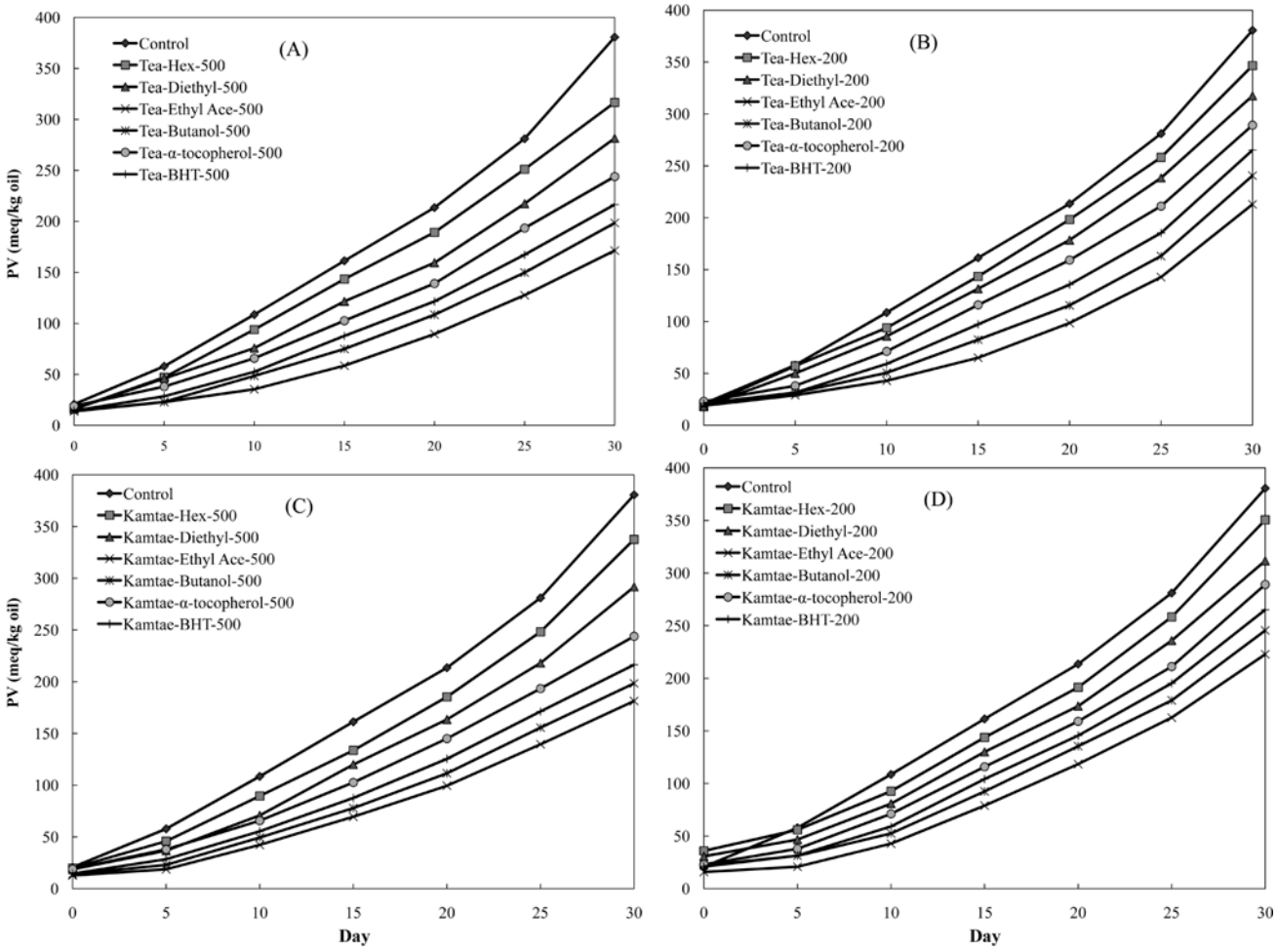

Fig. 1. Effect of different tea fractions on the formation of peroxide value (PV) in structured lipid (SL). (A) *mao feng* tea 500 ppm, (B) *mao feng* tea 200 ppm, (C) *kamtae* 500 ppm, (D) *kamtae* 200 ppm

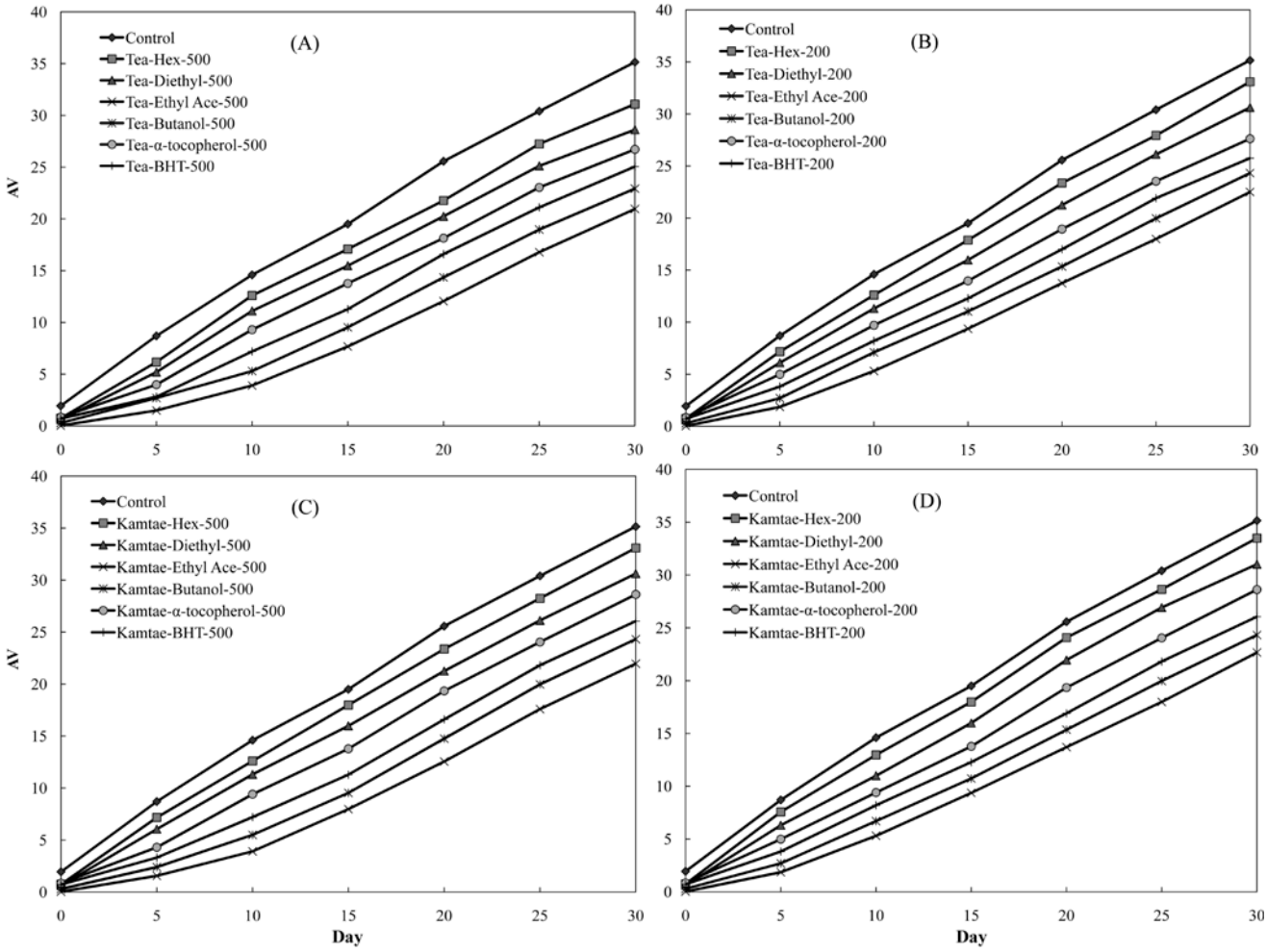

Fig. 2. Effect of different tea fractions on the formation of ρ-anisidine value (AV) in structured lipid (SL). (A) *mao feng* tea 500 ppm, (B) *mao feng* tea 200 ppm, (C) *kamtae* 500 ppm, (D) *kamtae* 200 ppm

findings is partially comparable with previous studies by Heo *et al*. (6) and Shin *et al*. (28), in which some brown seaweed extracts showed high antioxidative activities.

Oxidation study on SL α-Linolenic acid is ω3 unsaturated fatty acid that may further convert to other important biological metabolite and perilla oil is one of the rich sources of α-linolenic acid. However, dietary inclination to certain oil such as perilla oil would not be beneficial because consumption of the balanced ratio of ω3 and ω6 fatty acids is not desirable in the body. One possible way for such balanced ratio of ω3 and ω6 fatty acid is lipase-catalyzed modification, in which each fatty acid composition of corn oil, perilla oil, and SL was presented (Table 3). In this study, SL from perilla oil and corn oil was produced by lipase-catalyzed interesterification and the obtained SL was used to evaluate the antioxidant capacity of the fractions from *mao feng* tea and *kamtae*. Different concentrations (200 and 500 ppm) of the fractions were studied for their antioxidative activity on SL, comparing to commercial antioxidants (BHT and α-tocopherol). To measure the extent of lipid oxidation of SL, peroxide value (PV) for primary oxidation and ρ-anisidine value (AV), and TBARS value for secondary oxidation stage were measured. The results of PV showed that 200 and 500 ppm addition of any fractions from *mao feng* tea and *kamtae* are effective to retard oxidation of SL, in which 500 ppm addition was more effective than 200 ppm addition (Fig. 1). Any amount (200 and 500 ppm) of T-BU, T-EA, K-BU, and K-EA was more antioxidative effect on SL than BHT and α-tocopherol, showing lower PVs for 30 days. After 30 days, PV of control (SL without any antioxidant) was about 380 meq/kg oil, while PVs of T-EA (500 ppm) and K-EA (500 ppm) were 153 and 165 meq/kg oil, respectively. Among fractions, ethyl acetate extracts from the materials with addition of 500 ppm were the most effective to retard the oxidation of SL. Similar trends were shown from AV, in which 500 ppm of T-EA and K-EA addition showed the most effective antioxidant activity on SL (Fig. 2). Also, similar results were observed in TBARS analysis. Addition

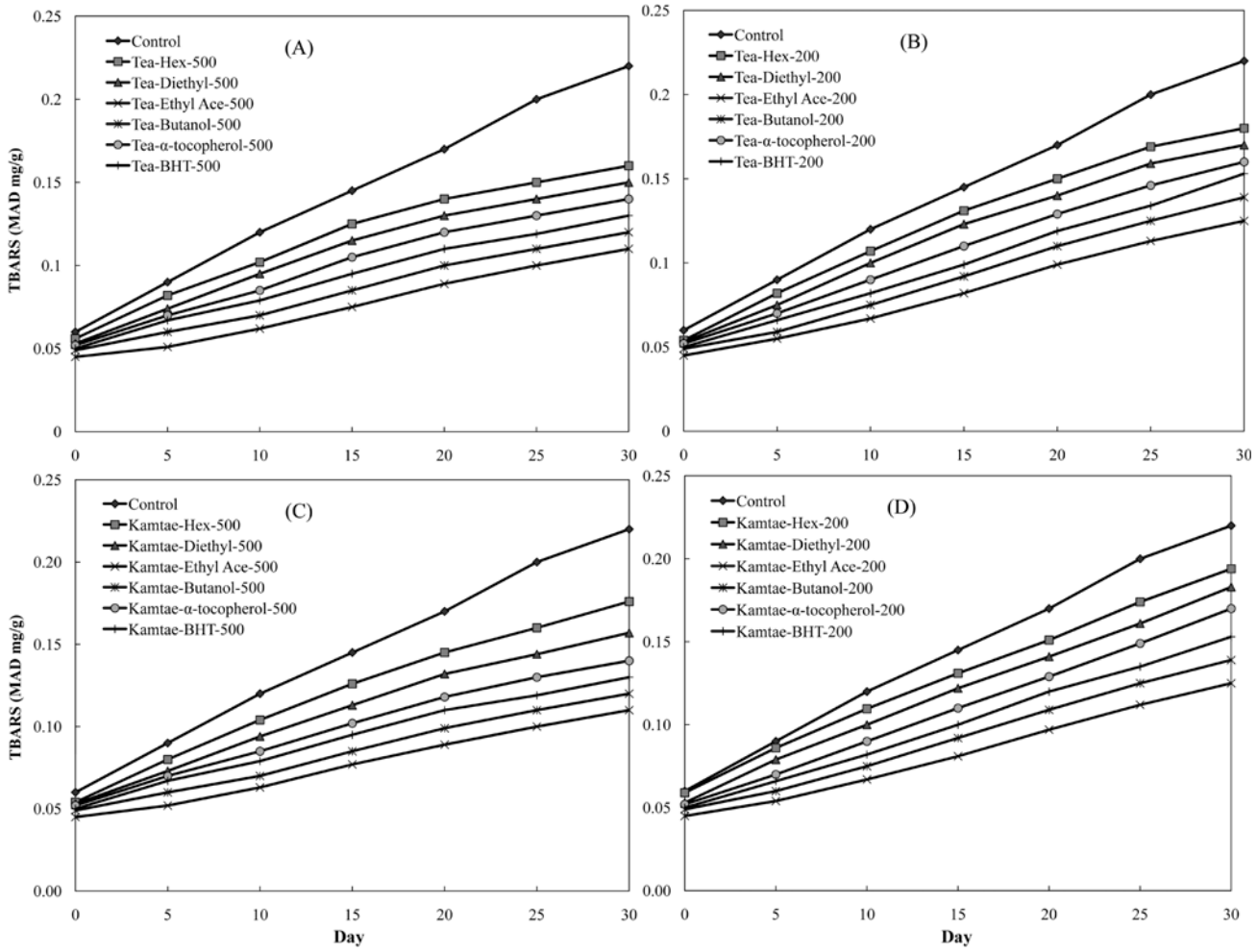

Fig. 3. Effect of different tea fractions on the formation of TBARS in structured lipid (SL). (A) *mao feng* tea 500 ppm, (B) *mao feng* tea 200 ppm, (C) *kamtae* 500 ppm, (D) *kamtae* 200 ppm

of any fractions of 2 materials with any 200 and 500 ppm showed lower TBARS values than control SL (Fig. 3).

Therefore, it could be expected that the fractions from *mao feng* tea and *kamtae*, especially extract of ethyl acetate, would be significantly considered to assembled large amount of antioxidants. This result can be supported in part by the result from Senevirathne *et al*. (29), in which fraction of ethyl acetate from *kamtae* was superior to those of other fractions, BHT, and tocopherols in fish oil. Therefore, the results indicated that SL containing K-EA and T-EA showed low oxidation degree, suggesting that these 2 fractions showed excellent antioxidant activities at a level of 200 and 500 ppm in this study.

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