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Comparative studies of four different phenolic compounds on *in vitro* antioxidative activity and the preventive effect on lipid oxidation of fish oil emulsion and fish mince

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ABSTRACT

Antioxidative activities of different phenolic compounds (catechin, caffeic acid, ferulic acid and tannic acid) at various levels were determined by different assays. Among all the phenolic compounds tested, tannic acid exhibited the highest 2,2-diphenyl-1-picryl hydrazyl (DPPH) and 2,2-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radical scavenging activities and ferric reducing antioxidant power (FRAP). Nevertheless, catechin showed the highest metal chelating activity ($P < 0.05$), whereas caffeic acid had the highest lipoxygenase (LOX) inhibitory activity ($P < 0.05$). The impact of different phenolic compounds at a level of 100 mg/l on lipid oxidation of menhaden oil-in-water emulsion and mackerel mince was investigated. Tannic acid showed the highest efficacy in retardation of lipid oxidation for both model systems as evidenced by the lower peroxide value (PV), conjugated diene (CD) and thiobarbituric acid-reactive substances (TBARS) values. This was also related with the lower non-heme iron content in tannic acid treated samples. Tannic acid was therefore considered as the most potential natural antioxidant for controlling oxidation of fish oil-in-water emulsion and fish mince, whereas ferulic acid seemed to possess the lowest preventive effect on lipid oxidation.

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1. Introduction

Fatty fish species are considered to be of great nutritional importance. This is mainly due to their naturally high content of essential n-3 polyunsaturated fatty acids (PUFAs) such as eicosapentaenoic acid (20:5) and docosahexaenoic acid (22:6). These fatty acids have been shown to have potential benefits for human health (Lee & Lip, 2003). Nevertheless, they are susceptible to oxidation, which is associated with their rancidity and loss in nutritive value (Frankel, 1998a; Hsieh & Kinsella, 1989). Apart from high amounts of PUFAs, the presence of heme pigments and trace amounts of metallic ions makes the fish, especially dark flesh fatty fish, prone to lipid oxidation (Hsieh & Kinsella, 1989). To retard such a quality loss, synthetic antioxidants have been used to decrease lipid oxidation during the processing and storage of fish and fish products (Boyd, Green, Giesbrecht, & King, 1993). However, the use of synthetic antioxidants has raised questions regarding food safety and toxicity (Chang, Ostric-Matijasevic, Hsieh, & Chang, 1977). The use of natural antioxidants is emerging as an effective methodology for controlling rancidity and limiting its deleterious consequences. Natural phenolic compounds with antioxidant activity such as rosemary extract, tea catechin, tannins, etc. have been gaining increasing attention due to their safety (Frankel, 1998b).

Phenolic compounds are bioactive substances widely distributed in plants and are important constituents of the human diet. Plant phenolics comprise a great diversity of compounds, such as flavonoids (anthocyanins, flavonols, flavones, etc.) and several classes of non-flavonoids (phenolic acids, lignins, stilbenes) (Harborne, 1989). Phenolic compounds vary in structure and the number of hydroxyl groups (Fig. 1), leading to the variation in their antioxidative activity. In general, phenolic compounds play a role as antioxidants through different mechanisms of action, such as scavenging of free radicals (Antolovich, Prenzler, Patsalides, McDonald, & Robards, 2002), quenching of reactive oxygen species, inhibition of oxidative enzymes (Edenharder & Grunhage, 2003), chelation of transition metals or through interaction with biomembranes (Liao & Yin, 2000). Therefore, these compounds have been considered as promising candidates as potential protectors against lipid oxidation and biological ageing of tissues.

Although the single phenolic compound has been proved as antioxidant, no comparative studies have been conducted among those phenolic compounds, which possess different molecular properties, mode of action, stability etc. Additionally, different phenolic compounds may act as antioxidants at varying degrees in different food systems, depending on the polarity and molecular characteristics. Hydrophilic antioxidants could prevent the oxida-

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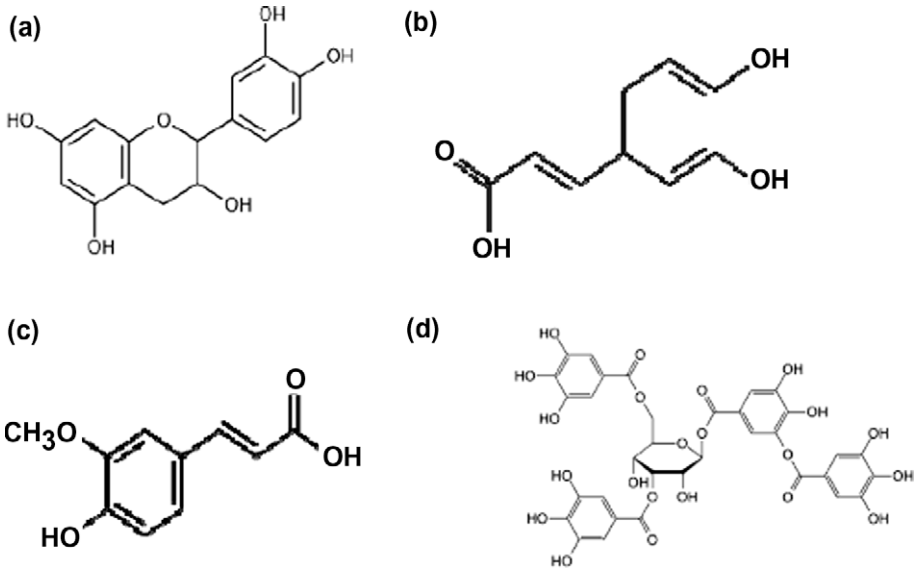

Fig. 1. Structures of four phenolic compounds (a) catechin, (b) caffeic acid, (c) ferulic acid and (d) tannic acid.

tion of bulk oil, while its hydrophobic counterpart effectively retards lipid oxidation in oil-in-water emulsion (Frankel, 1998b). Thus, the present study aimed to determine *in vitro* antioxidative activities of different phenolic compounds (catechin, caffeic acid, ferulic acid and tannic acid) and to investigate their preventive effect on lipid oxidation of menhaden oil-in-water emulsion and mackerel mince during extended storage.

2. Materials and methods

2.1. Chemicals and fish oil

Catechin, tannic acid, 2,2-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), 2,2-diphenyl-1-picryl hydrazyl (DPPH), linoleic acid, soybean lipoxygenase-1 (EC 1.13.11.12, type 1), thioglycolic acid, bathophenanthroline disulfonic acid, ferrozine and menhaden oil were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Sodium chloride, anhydrous sodium sulfate, potassium iodide, trichloroacetic acid, iron standard solution, ethanol and methanol were obtained from Merck (Damstadt, Germany). Caffeic acid, ferulic acid, disodium hydrogen phosphate, 2-thiobarbituric acid, 2,4,6-tripyridyl-s-triazine (TPTZ), ferric chloride hexahydrate and potassium persulfate were procured from Fluka Chemical Co. (Buchs, Switzerland). Chloroform was purchased from Lab-Scan (Bangkok, Thailand).

2.2. Comparative study on in vitro antioxidative activity of different phenolic compounds

Four phenolic compounds including catechin, caffeic acid, ferulic acid and tannic acid were comparatively determined for their antioxidative activities by different *in vitro* assays. Prior to the assay, phenolic compounds were added to distilled water and the mixtures were adjusted to pH 8–9 using 2 M NaOH until the compounds were completely dissolved. Thereafter, the obtained solutions were adjusted to pH 7 using 2 N HCl and subjected to the assays for antioxidative activities.

2.2.1. DPPH radical scavenging activity

DPPH radical scavenging activity was determined as described by Wu, Chen, and Shiau (2003) with a slight modification. Samples (1.5 ml) with a concentration range of 0.5–10 mg/l were added to 1.5 ml of 0.15 mM 2,2-diphenyl-1-picryl hydrazyl (DPPH) in 95% ethanol. The mixture was mixed vigorously and allowed to stand at room temperature in the dark for 30 min. The absorbance of the resulting solution was measured at 517 nm using a UV-1601 spectrophotometer (Shimadzu, Kyoto, Japan). The sample blank at each concentration was prepared in the same manner except that ethanol was used instead of DPPH solution. A standard curve was prepared using Trolox in the range of 10–60 μM. The activity was calculated after the sample blank substraction and expressed as μmol Trolox equivalents (TE)/ml of phenolic compound.

2.2.2. ABTS radical scavenging activity

ABTS radical scavenging activity was assayed as per the method of Arnao, Cano, and Acosta (2001) with a slight modification. The stock solutions included 7.4 mM ABTS solution and 2.6 mM potassium persulphate solution. The working solution was prepared by mixing the two stock solutions in equal quantities and allowing them to react for 12 h at room temperature in the dark. The solution was then diluted by mixing 1 ml ABTS solution with 50 ml methanol in order to obtain an absorbance of 1.1 ± 0.02 units at 734 nm using a UV-1601 spectrophotometer (Shimadzu, Kyoto, Japan). A fresh ABTS solution was prepared for each assay. Samples (150 μl) with a concentration range of 0.5–10 mg/l were mixed with 2850 μl of ABTS solution and the mixture was left at room temperature for 2 h in dark. The absorbance was then measured at 734 nm using the spectrophotometer. A sample blank at each concentration was prepared in the same manner except that methanol was used instead of ABTS solution. A standard curve of Trolox ranging from 50 to 600 μM was prepared. The activity was expressed as μmol Trolox equivalents (TE)/ml of phenolic compound.

2.2.3. FRAP (ferric reducing antioxidant power)

FRAP was assayed according to Benzie and Strain (1996). Stock solutions included 300 mM acetate buffer (pH 3.6), 10 mM TPTZ (2,4,6-tripyridyl-*s*-triazine) solution in 40 mM HCl, and 20 mM $FeCl_3 \cdot 6H_2O$ solution. A working solution was prepared freshly by mixing 25 ml of acetate buffer, 2.5 ml of TPTZ solution and 2.5 ml of $FeCl_3 \cdot 6H_2O$ solution. The mixed solution was incubated at 37 °C for 30 min in a water bath (Memmert, D-91126, Schwabach, Germany) and was referred to as FRAP solution. A sample (150 μl) with the concentration range of 0.5–10 mg/l was mixed with

2850 μl of FRAP solution and kept for 30 min in the dark at room temperature. The ferrous tripyridyltriazine complex (coloured product) was measured by reading the absorbance at 593 nm. A sample blank at each concentration was prepared by omitting $FeCl_3$ from the FRAP solution and distilled water was used instead. The standard curve was prepared using Trolox ranging from 50 to 600 μM. The activity was expressed as μmol Trolox equivalents (TE)/ml of phenolic compound.

2.2.4. Metal chelating activity

The chelating activity towards Fe^{2+} was measured by the method of Boyer and McCleary (1987) with a slight modification. Samples (4.7 ml) with a concentration ranging from 100 to 200 mg/l were mixed with 0.1 ml of 2 mM $FeCl_2$ and 0.2 ml of 5 mM ferrozine. The reaction mixture was allowed to stand for 20 min at room temperature. The absorbance was then read at 562 nm. The blank was prepared in the same manner except that distilled water was used instead of the sample. For the sample blanks at each concentration, $FeCl_2$ solution was excluded and distilled water was used instead. The chelating activity after the sample blank substraction was calculated as follows:

$$\text{Metal chelating activity (\%)} = \{(A_{blank} - A_{sample})/A_{blank}\} \times 100$$

where, A_{sample} is the absorbance of the sample and A_{blank} is the absorbance of blank.

2.3. Comparative study on lipoxygenase inhibitory activity of different phenolic compounds

Lipoxygenase assay was performed as previously reported by Ha and Kubo (2005) with a slight modification. To study the inhibitory activity towards soybean lipoxygenase, 10 μl of different phenolic compounds at various concentrations (10, 25, 50 and 100 mg/l) were mixed with 20 μl of 0.1 M sodium borate buffer solution (pH 9.0) containing lipoxygenase (0.52 μM). The mixtures were allowed to stand at 25 °C for 10 min, followed by the addition of 2.97 ml of 0.1 M sodium borate buffer (pH 9.0). To initiate the reaction, 30 μl of 3 mM linoleic acid were added. The resultant solution was mixed well, and the linear increase of absorbance at 234 nm was measured after 5 min. One unit of lipoxygenase was defined as the increase in 0.1 unit of absorbance at 234 nm/min. The percentage inhibition was calculated as follows:

$$\text{Lipoxygenase inhibition (\%)} = \{(A_0 - A_1)/A_0\} \times 100$$

where, A_0 is the activity without inhibitor and A_1 is the activity in the presence of inhibitor (Banerjee, Khokhar, & Owusu Apenten, 2002).

2.4. Comparative study of different phenolic compounds in prevention of lipid oxidation in various food model systems

2.4.1. Preparation of oil-in-water emulsion

Oil-in-water emulsions (250 ml) were prepared by homogenising 25 ml of menhaden oil with 225 ml 0.1 M acetate buffer (pH 5.4) containing different phenolic compounds (100 mg/l of emulsion) and 1% lecithin as an emulsifier. The mixture was kept in an ice bath during homogenisation with an Ultra-Turrax T25 high speed homogeniser (Janke & Kunkel, Staufen, Germany) at a speed of 13,500 rpm for 5 min. The emulsion was then sonicated with an Elma (S 30 H) sonicator (Kolpingstr, Singen, Germany) in an ice bath for 5 min. The prepared oil-in-water emulsion (225 ml) was transferred to a 250 ml-Erlenmeyer flask and kept at 30 °C in the dark. The control oil-in-water emulsion was prepared in the same manner except the distilled water was added instead of the solution of phenolic compounds. Samples were taken every 24 h for the determination of peroxide value (PV), conjugated dienes (CD) and thiobarbituric acid-reactive substances (TBARS).

2.4.2. Preparation of fish mince

Fish mince was prepared according to the method of Kamil, Jeon, and Shahidi (2002) with a slight modification. Mackerel (*Rastrelliger kanagurta*) with an average weight of 100–150 g off-loaded 24 h after capture were purchased from the local market in Hat Yai, Thailand. The fish were kept in ice during the transportation. Upon arrival, the fish were washed, filleted, de-skinned and minced using a mincer with a whole diameter of 5 mm. The fish mince obtained was divided into five portions (750 g each). One portion, without the addition of phenolic compounds, was used as the control and 25 ml of distilled water was added instead. The other four portions were mixed with 25 ml of the different phenolic compounds (pH 7) to obtain a final concentration of 100 mg/kg of mince. The mince was then thoroughly mixed in order to ensure the homogeneous distribution of phenolic solution in the mince. Different mince samples were packed in polyethylene bags, sealed and kept in ice using a mince/ice ratio of 1:2 (w/w). Molten ice was removed every day and the same quantity of ice was replaced. After the designated storage time (0, 3, 6, 9, 12 and 15 days), the samples were taken for analyses of peroxide value (PV), conjugated diene (CD), thiobarbituric acid-reactive substances (TBARS), heme iron and non-heme iron contents.

2.4.3. Lipid extraction and analysis

The lipid was extracted by the method of Bligh and Dyer (1959). Fish mince (25 g) was homogenised with 200 ml of a chloroform:methanol:distilled water mixture (1:2:1) at a speed of 9500 rpm for 2 min at 4 °C using an Ultra-Turrax T25 homogeniser (Janke & Kunkel, Staufen, Germany). The homogenate was then added to 50 ml of chloroform and homogenised at 9500 rpm for 1 min. Then, 25 ml of distilled water was added and the mixture was homogenised again for 30 s. The homogenate was centrifuged at 14,500×*g* at 4 °C for 15 min using a refrigerated centrifuge (model J-E Avanti, Beckman Coulter, Inc., Palo Alto, CA, USA), and transferred into a separating flask. The chloroform phase was drained off into a 125 ml-Erlenmeyer flask containing about 2–5 g of anhydrous sodium sulfate, shaken well and decanted into a round-bottom flask through Whatman No. 4 filter paper (Whatman International, Ltd., Maidstone, England). The solvent was evaporated at 25 °C, using an EYELA rotary evaporator N-100 (Tokyo, Japan), and the residual solvent was removed by flushing with nitrogen.

2.4.3.1. Peroxide value (PV). Peroxide value was determined according to the method of Sakanaka, Tachibana, Ishihara, and Raj Juneja (2004). To 50 μl of the oil extracted from mince or 50 μl of oil-in-water emulsion sample, 2.35 ml of 75% ethanol, 50 μl of 30% ammonium thiocyanate and 50 μl of 20 mM ferrous chloride solution in 3.5% HCl were added and mixed thoroughly. After 3 min, the absorbance of the coloured solution was measured at 500 nm using a UV-1601 spectrophotometer (Shimadzu, Kyoto, Japan). An increase in absorbance at 500 nm indicated the formation of peroxide (Yen & Hsieh, 1998).

2.4.3.2. Conjugated diene (CD). The conjugated diene was measured according to the method of Frankel, Huang, Aeschbach, and Prior (1996). The extracted oil or oil-in-water emulsion (0.1 ml) was dissolved in 5.0 ml of methanol and the absorbance was measured at 234 nm using a UV-1601 spectrophotometer (Shimadzu, Kyoto, Japan). The conjugated diene was measured as the increase in absorbance at 234 nm.

2.4.3.3. Thiobarbituric acid-reactive substances (TBARS). The thiobarbituric acid-reactive substances (TBARS) assay was performed as described by Buege and Aust (1978). Fish mince (0.5 g) or 0.5 ml of oil-in-water emulsion sample were mixed with 2.5 ml of a TBA solution containing 0.375% thiobarbituric acid, 15% trichloroacetic

acid and 0.25 N HCl. The mixture was heated in boiling water for 10 min to develop a pink colour, cooled with running tap water and then sonicated for 30 min followed by centrifugation at 5000×*g* at 25 °C for 10 min. The absorbance of the supernatant was measured at 532 nm. A standard curve was prepared using 1,1,3,3-tetramethoxypropane (malonaldehyde; MAD) at a concentration ranging from 0 to 10 ppm and TBARS was expressed as mg of MAD equivalents/kg sample.

2.4.3.4. Determination of heme iron content. Heme iron content was determined according to the method of Gomez-Basauri and Regenstein (1992) with a slight modification. A fish mince sample (2 g) was weighed into a 50-ml polypropylene centrifuge tube and 20 ml of cold 40 mM phosphate buffer (pH 6.8) were added. The mixture was homogenised with an Ultra-Turrax T25 homogeniser (Janke & Kunkel, Staufen, Germany) at 13,500 rpm for 10 s. The homogenate was centrifuged at 3000×*g* for 30 min at 4 °C using a Sorvall RC 26 Plus refrigerated centrifuge (Sorvall, Norwalk, CT, USA). The supernatant was filtered with Whatman No. 1 filter paper (Whatman International, Ltd., Maidstone, England). Total heme pigment was determined by direct spectrophotometric measurement at 525 nm using a UV-1601 spectrophotometer (Shimadzu, Kyoto, Japan). Myoglobin content was calculated from the millimolar extinction coefficient of 7.6 and a molecular weight of 16,110. Heme iron content was calculated based on myoglobin, which contains 0.35% iron. The heme iron content was expressed as mg/100 g sample.

2.4.3.5. Determination of non-heme iron content. Non-heme iron content was determined as described by Schricker, Miller, and Stouffer (1982) with a slight modification. The fish mince sample (1.0 g) was weighed into a screw cap test tube and 50 μl of 0.39% (w/v) sodium nitrite were added. A mixture (4 ml) of 40% trichloroacetic acid and 6 N HCl (ratio of 1:1 [v/v], prepared freshly) was added. The tightly capped tubes were placed in an incubator shaker at 65 °C (Memmert, D-91126, Schwabach, Germany) for 22 h and then cooled down to room temperature (28–30 °C) for 2 h. The supernatant (400 μl) was mixed with 2 ml of the non-heme iron colour reagent (prepared freshly). After vortexing and standing for 10 min, the absorbance was measured at 540 nm. The colour reagent was prepared by mixing a 1:20:20 ratio (w/v/v) of: (1) bathophenanthroline (0.162 g, dissolved in 100 ml of double-deionised water with 2 ml thioglycolic acid [96–99%]); (2) double-deionised water; and (3) saturated sodium acetate solution.

The non-heme iron content was calculated from an iron standard curve. The iron standard solution, ranging from 0 to 2 ppm (400 μl), was mixed with 2 ml of the non-heme iron colour reagent. The concentration of non-heme iron was expressed as mg/100 g sample.

2.5. Statistical analysis

All experiments were run in triplicate. The experimental data were subjected to Analysis of Variance (ANOVA) and the differences between means were evaluated by Duncan's Multiple Range Test (Steel & Torrie, 1980). Data analysis was performed using a SPSS package (SPSS 14.0 for Windows, SPSS Inc., Chicago, IL, USA).

3. Results and discussion

3.1. In vitro antioxidant activity of different phenolic compounds

3.1.1. DPPH radical scavenging activity

DPPH radical scavenging activity of catechin, caffeic acid, ferulic acid and tannic acid at different concentrations is shown in Fig. 2a. The activity of all phenolic compounds increased with increasing concentration ($P < 0.05$). At the same concentration used, the descending order of DPPH radical scavenging activity of the phenolic compounds tested was as follows: tannic acid > catechin > caffeic acid > ferulic acid ($P < 0.05$). The high radical scavenging activity of tannic acid and catechin was probably attributed to the higher degree of hydroxylation in their structure. As a result, their capability to donate hydrogen to the free radical was more pronounced (Scherer & Godoy, 2009). The effect of antioxidants on DPPH radical scavenging is generally due to their hydrogen-donating ability (Siddhuraju & Becker, 2007). Tannins extracted from stem bark of *Cassia fistula* or from canola and rapeseed hulls possessed DPPH radical quenching capacity (Amarowicz, Naczk, & Shahidi, 2000; Siddhuraju, Mohan, & Becker, 2002).

The lower DPPH radical scavenging activity of ferulic acid was observed compared with other phenolic compounds tested ($P < 0.05$). This might be attributed to the presence of an adjacent substituted methoxyl group of a hydroxyl group in the aromatic ring, which reduced free radical scavenging capacity of ferulic acid (Rice-Evans, Miller, & Paganga, 1996; Siddhuraju & Becker, 2007). Additionally, the carboxyl group in ferulic acid might explain the negative effect on its antioxidative activity. The carboxyl group is an electron-withdrawing group, which does not benefit the radical scavenging activity of the compound (Thiago Inacio, Roberta, Nidia, & Neli, 2008). A higher radical scavenging activity of caffeic acid has been reported in comparison with ferulic acid (Bratt et al., 2003; Scherer & Godoy, 2009). The presence of a second hydroxyl group in the ortho or para position is known to increase antioxidative activity due to the additional resonance stabilisation and *o*-quinone or *p*-quinone formation (Graf, 1992). Increasing the number of donated electrons helps caffeic acid in scavenging the DPPH radical more efficiently than ferulic acid (Medina, Gallardo, Gonzaalez, Lois, & Hedges, 2007). Thus, the presence of higher numbers of hydroxyl groups in catechin and tannic acid was most likely associated with the increased DPPH radical scavenging activity.

3.1.2. ABTS radical scavenging activity

ABTS radical scavenging activity of different phenolic compounds at various concentrations is presented in Fig. 2b. ABTS radical scavenging activity of all phenolic compounds increased as the concentration increased ($P < 0.05$). However, the activity varied with the types of phenolic compounds tested. Similar to the results of the DPPH radical scavenging activity, tannic acid and catechin were more effective in scavenging ABTS radical, compared with caffeic acid and ferulic acid ($P < 0.05$). Hagerman et al. (1998) reported that the high molecular weight phenolics such as tannic acid have more ability to quench the ABTS radical and the effectiveness depends on the molecular weight, the number of aromatic rings and nature of hydroxyl groups' substitution than the specific functional groups. Phenolic compounds capable of donating a hydrogen atom were more effective in scavenging ABTS radical (Leong & Shui, 2002). It was noted that ferulic acid exhibited the higher ABTS radical scavenging activity than caffeic acid did ($P < 0.05$), while the later showed the higher DPPH radical scavenging activity than the former did (Fig. 2a and b). Though caffeic acid contains the higher number of hydroxyl groups, it exhibited the lower ABTS radical scavenging activity, in comparison with ferulic acid. Thus, it was more likely that the structure and side groups of phenolic compounds determined their ability in scavenging ABTS radical. The results suggested that different phenolic compounds had the capacity to scavenge different radicals. As a consequence, different assays should be conducted to verify the antioxidant activity of various compounds, in which the mode of action could be different.

3.1.3. FRAP

Antioxidant potential of different phenolic compounds was estimated from their ability to reduce TPTZ–Fe(III) complex to TPTZ–Fe(II) complex as shown in Fig. 2c. Among all phenolic compounds tested, tannic acid showed the highest FRAP ($P < 0.05$), indicating that tannic acid could easily donate the electron to Fe(III), thus reducing it to Fe(II). The reducing capacity measures the ease of the compounds in donating electrons (Medina et al., 2007). The results were in agreement with the highest DPPH and ABTS radical scavenging of tannic acid (Fig. 2a and b). Lopes, Schulman, and Hermes-Lima (1999), Andrade, Ginani, Lopes, Dutra, and Hermes-Lima (2006) also reported that tannic acid was able to reduce Fe(III) to Fe(II).

Caffeic acid and ferulic acid showed the higher FRAP than did catechin, which had the higher DPPH and ABTS radical scavenging activities (Fig. 2a and b). Caffeic acid can donate the highest number of electron (2.2 mol electron/mol phenolic), followed by ferulic acid (1.9 mol electrons/mol phenolic) and chlorogenic acid (1.6 mol electrons/mol phenolic) (Medina et al., 2007). Gulcin (2006) reported that the reducing power of caffeic acid was higher than that of BHA, BHT, α-tocopherol and Trolox.

In the present study, catechin possessed a higher number of hydroxyl groups than ferulic acid and caffeic acid, but showed the lowest FRAP ($P < 0.05$). This result was in accordance with Medina et al. (2007) who recently discussed that the absolute antioxidant capacity cannot be predicted simply by determining the number of hydroxyl groups. The higher number of hydroxyl groups in the chlorogenic acid did not increase its reducing power (Medina et al., 2007).

3.1.4. Metal chelating activity

Metal chelating activity of different phenolic compounds at various concentrations is depicted in Fig. 2d. Catechin showed the highest metal chelating activity, followed by caffeic acid, tannic acid and ferulic acid at all concentrations tested ($P < 0.05$) except at a level of 100 mg/l, where catechin had an activity similar to caffeic acid ($P > 0.05$). Metal chelating activity of catechin was also reported by Morel et al. (1993). Owing to polyhydroxylated structure, catechin could act as antioxidant either through the chelation of metals with redox property or by acting as scavenger of free radical (Rice-Evans et al., 1996). The capacity of antioxidant for chelating metals is strongly dependent on the number of hydroxylic groups in ortho-position. Caffeic acid was reported to have ferrous chelating activity (Gulcin, 2006). It was noted that tannic acid possessed the poorest metal chelating property, while it had the superior activity for the other assays (Fig. 2a–c). Lopes et al. (1999)

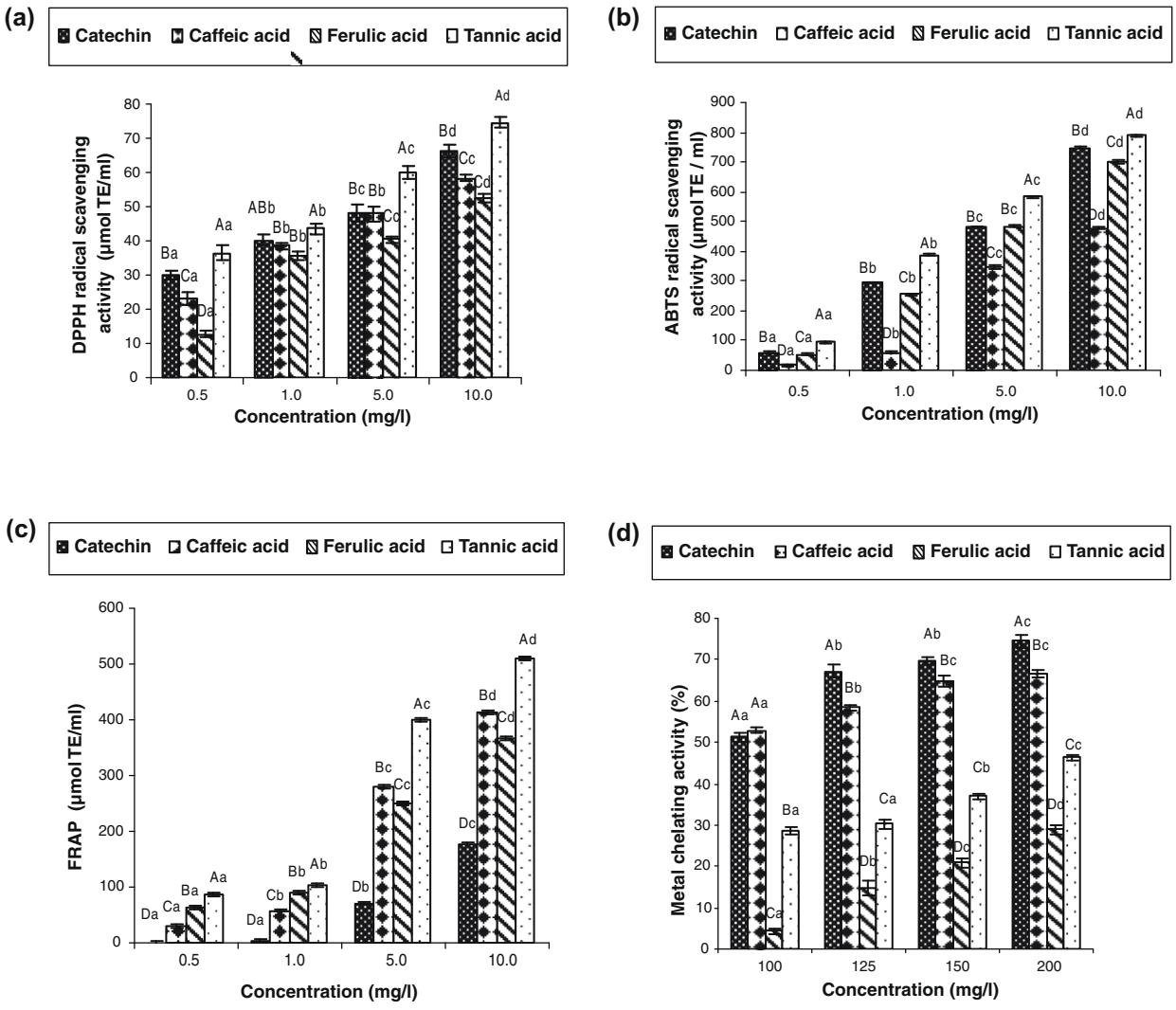

Fig. 2. Antioxidative activity of different phenolic compounds at various levels as determined by DPPH radical scavenging (a), ABTS radical scavenging (b), FRAP (c) and metal chelating (d) assays. Bars represent the standard deviation ($n = 3$). Different capital letters within the same concentration denote the significant differences ($P < 0.05$). Different small letters within the same type of phenolic compound denote significant differences ($P < 0.05$).

found that antioxidant mechanism of tannic acid against OH· formation *via* a Fenton reaction was mainly due to its iron chelating effect rather than OH· scavenging activity.

The low metal chelating activity of ferulic acid was possibly due to the presence of only one hydroxyl group in its structure. The presence of a methoxy group, which hindered their chelating capacity, was also postulated. Methoxy groups could stabilise phenoxyl radicals owing to its electron-donating abilities but did not contribute to the chelating abilities (Danilewicz, 2003). Also, ferulic acid does not have a galloyl moiety, thus it cannot bind iron. Gulcin (2006) reported that ferulic acid possessed very little chelating activity against Fe(II). Thus, the efficiency in metal chelation varied with the type of phenolic compounds and did not relate with radical scavenging activity and reducing power.

3.2. Lipoxygenase inhibitory activity of different phenolic compounds

The percentage inhibition of lipoxygenase (LOX) by different phenolic compounds at various concentrations is shown in Fig. 3. For all the phenolic compounds tested, inhibitory activity increased with increasing concentrations used ($P < 0.05$). At the same concentration tested, caffeic acid showed the highest inhibitory activity towards LOX ($P < 0.05$). Koshihara et al. (1984), Vob, Sepulveda-Boza, and Zilliken (1992) reported that caffeic acid inhibited LOX-5 with the IC_{50} value of 3.7 μM. Caffeic acid and its phenethyl ester are well recognised as natural inhibitors of LOX (Sudina et al., 1993). No differences in LOX inhibitory activity were observed among catechin, ferulic acid and tannic acid at all concentrations used ($P > 0.05$). In general, the inhibitory activity of phenolic compounds towards LOX increases with the number of hydroxyl substituents (Laughton, Evans, Moroney, Hoult, & Halliwell, 1991; Sadik, Sies, & Schewe, 2003). Though tannic acid contains a larger number of hydroxyl groups in its structure, its LOX inhibitory activity was lower than caffeic acid ($P < 0.05$). Due to the large molecular structure, tannic acid might not be able to bind to the active site of LOX effectively. The mechanism of LOX inhibition by phenolic compounds is most likely a combination of radical scavenging and binding to the hydrophobic active site of LOX and/or an interaction with the hydrophobic fatty acid substrate (Schurink, 2007).

Catechin and ferulic acid also showed LOX inhibitory activity in a concentration dependent manner. Green tea catechin displayed the inhibitory effects on both LOX-catalysed and Hb-catalysed oxidation of arachidonic acid and linoleic acid (Liu & Bonnie, 2004). Catechin mixtures prepared from tea effectively prevented the pro-oxidant activity of lipoxygenase (LOX) in fish skin extract (Mohri, Tokuori, Endo, & Fujimoto, 1999). The mechanism of LOX-catalysed lipid oxidation is radical-based, where the fatty acid is oxidised by the ferric iron within the active site of the enzyme to form a fatty acid radical and a ferrous iron. This radical intermediate is then attacked by dioxygen to form the fatty acid hydroperoxide (Schurink, 2007). Therefore, the phenolic compounds like caffeic acid and tannic acid having higher reducing power and metal chelating activity showed the highest inhibitory effect towards LOX. The results revealed that all phenolic compounds tested could inhibit LOX at different degrees. Phenolic compounds might bind with LOX *via* hydrophobic interactions or hydrogen bonds, leading to the conformational changes of LOX. This resulted in the loss in LOX activity, particularly when the higher levels of phenolic compounds were used.

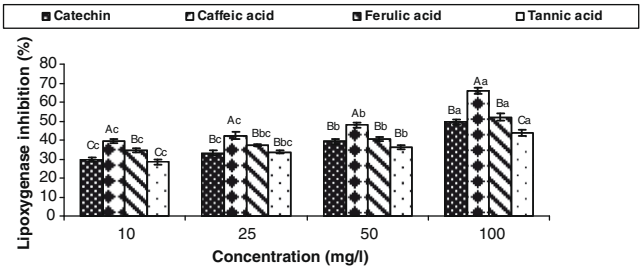

Fig. 3. Lipoxygenase inhibitory activity of different phenolic compounds at various levels. Bars represent the standard deviation ($n = 3$). Different capital letters within the same concentration denote the significant differences ($P < 0.05$). Different small letters within the same type of phenolic compound denote significant differences ($P < 0.05$).

3.3. Prevention of lipid oxidation in menhaden oil-in-water emulsion by different phenolic compounds

3.3.1. Changes in lipid oxidation products

The PV of menhaden oil-in-water emulsion added without and with different phenolic compounds at a level of 100 mg/kg during the storage is presented in Fig. 4a. The gradual increases in PV were observed in all samples during the first 48 h of storage. Thereafter, PV increased at a higher rate up to 96 h of storage ($P < 0.05$). No marked change in PV was noticed during 96–168 h of storage ($P > 0.05$). When comparing PV of all samples, it was found that the control sample contained the higher PV than the samples added with phenolic compounds throughout the storage, particularly after 12 h of storage.

Tannic acid was more effective in lowering the increase in PV in menhaden oil-in-water emulsion than the other three phenolic compounds used in the present study ($P < 0.05$). The higher efficiency of tannic acid in prevention of the hydroperoxide formation correlated well with the higher DPPH and ABTS radical scavenging activities and reducing power (Fig. 2a–c). Tannic acid contained a large number of hydrophobic portions, which could align themselves at the oil–water interface and functioned as a hydrogen donor or radical scavenger. This resulted in the retardation of the initiation and propagation stages, as evidenced by the lower PV.

Caffeic acid and ferulic acid showed a similar effect on retarding the formation of PV in menhaden oil-in-water emulsion ($P > 0.05$) (Fig. 4a) but their efficiency was lower than that of tannic acid ($P < 0.05$). Nevertheless, caffeic acid and ferulic acid exhibited the higher ability in prevention of PV formation than catechin did ($P < 0.05$). De Leonardis and Macciola (2003) reported that the antioxidant effectiveness of caffeic acid was better than that of BHA in hydrophobic phases such as cod liver oil.

The differences in ability in inhibiting PV formation among all phenolic compounds were more likely governed by their hydrophobicity/hydrophilicity balance as well as their localisation in emulsion system. Catechin is the more polar antioxidant than tannic acid and caffeic acid (Medina et al., 2007). Therefore, catechin was mostly found in the aqueous phase in close proximity to the transition metal ions present in the aqueous phase. Furthermore, phenolic compounds including, catechin could act as a pro-oxidants rather than an antioxidant in the presence of transition metal ions, particularly Fe^{3+}. Catechin might reduce transition metal ions ($Fe^{3+} \rightarrow Fe^{2+}$) and generated hydrogen peroxide through autoxidation, which drives the production of hydroxyl radicals *via* the Fenton reaction (Retsky, Freeman, & Frei, 1993). Sorensen et al. (2008) also reported that the caffeic acid was found to be pro-oxidative in the omega-3-enriched oil-in-water emulsion irrespective of pH, emulsifier type and presence of iron. The formation of H-bonded complexes between catechin and water molecule at the water–oil interface or in the aqueous phase resulted in the availability of less catechin to act as antioxidant. Catechin showed a low inhibition on the formation of peroxides and TBARS in chilled horse mackerel (Medina et al., 2007).

The impact of phenolic compounds on conjugated diene (CD) and thiobarbituric acid-reactive substances (TBARS) formation in

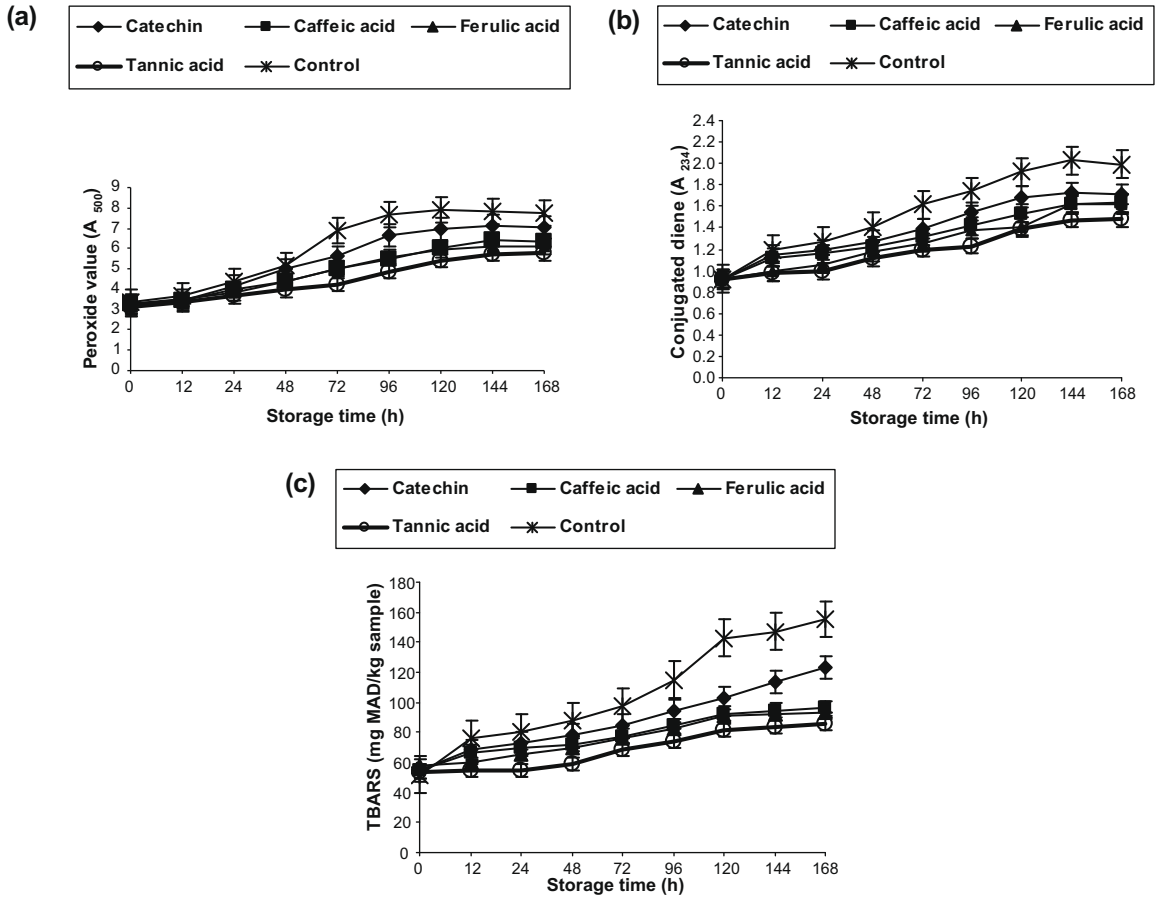

Fig. 4. Effect of different phenolic compounds on the formation of lipid oxidation products in menhaden oil-in-water emulsion stored at 30 °C for a period of 168 h. Peroxide value (a), conjugated diene (b) and thiobarbituric acid-reactive substances (TBARS) values (c). Bars represent the standard deviation ($n = 3$). For all phenolic compounds, a final concentration of 100 mg/l was used in the system.

menhaden oil-in-water emulsion during the storage is shown in Fig. 4b and c, respectively. After the initiation stage, oxidation is propagated *via* hydrogen subtraction in the vicinity of double bonds. This propagation step implies the formation of isomeric hydroperoxides that frequently carry conjugated diene groups. This is the mechanism of formation of CD. CD and TBARS levels increased continuously in all samples throughout the 168 h of storage ($P < 0.05$). Similar patterns in changes of CD and TBARS were noticeable in comparison with that of PV (Fig. 2a), in which tannic acid exhibited the highest efficacy in preventing the increase in PV. The increase in CD was coincidental with the increase in PV. All samples added with phenolic compounds showed lower CD formation, compared with the control (Fig. 4b) ($P < 0.05$). The inhibitory effect of tannic acid on CD formation was slightly higher than other phenolic compounds tested. This was due to the higher radical scavenging activity of tannic acid, thereby lowering the subsequent generation of reactive lipid radicals, which can undergo further chain reactions.

The increase in TBARS of emulsion indicated the formation of lipid oxidation products. This was dominant in the control samples and was retarded in the samples added with phenolic compounds, particularly tannic acid. The increases in TBARS values after 96 h of storage in the control samples were coincidental with the decrease in PV. A decrease in the level of primary oxidation products is related to hydroperoxide degradation, producing secondary lipid peroxidation products (Undeland, Ekstrand, & Lingnert, 1998).

Thus, in menhaden oil-in-water emulsion, tannic acid acted as an efficient antioxidant while catechin showed the lowest preventive effect on lipid oxidation. The result confirmed the role of phenolic compounds in lowering the lipid oxidation in menhaden oil-in-water emulsion.

3.4. Prevention of lipid oxidation in mackerel mince by different phenolic compounds

3.4.1. Changes in lipid oxidation products

The formation of PV in mackerel mince added without and with different phenolic compounds at a level of 100 mg/kg during iced storage is depicted in Fig. 5a. PV in the control sample increased drastically from day 0 to day 6 ($P < 0.05$). Thereafter, no marked change in PV was found until the end of the storage period ($P > 0.05$). PV of the control sample was higher than that of samples added with phenolic compounds throughout the storage ($P < 0.05$). Samples added with tannic acid contained a lower PV than the control and those added with other phenolic compounds used. The results indicated the efficient antioxidative activity of tannic acid in the fish mince system. Tannic acid also showed higher antioxidant activity in most *in vitro* antioxidant assays (Fig. 2) as well as in menhaden oil-in-water emulsion (Fig. 4) ($P < 0.05$). Free radical scavenging antioxidants including tannic acid interfere with the initiation or propagation steps of lipid oxidation reactions by scavenging lipid radicals and forming low-energy antioxidant radicals that do not readily promote oxidation of unsaturated fatty acids (Frankel, 1998b). The reducing capacity of phenolic antioxidants was realised as a key function for retarding and inhibiting lipid oxidation in fish tissues (Medina et al., 2007).

PV of samples added with caffeic acid, ferulic acid and catechin was lower than that of the control samples ($P < 0.05$), but was

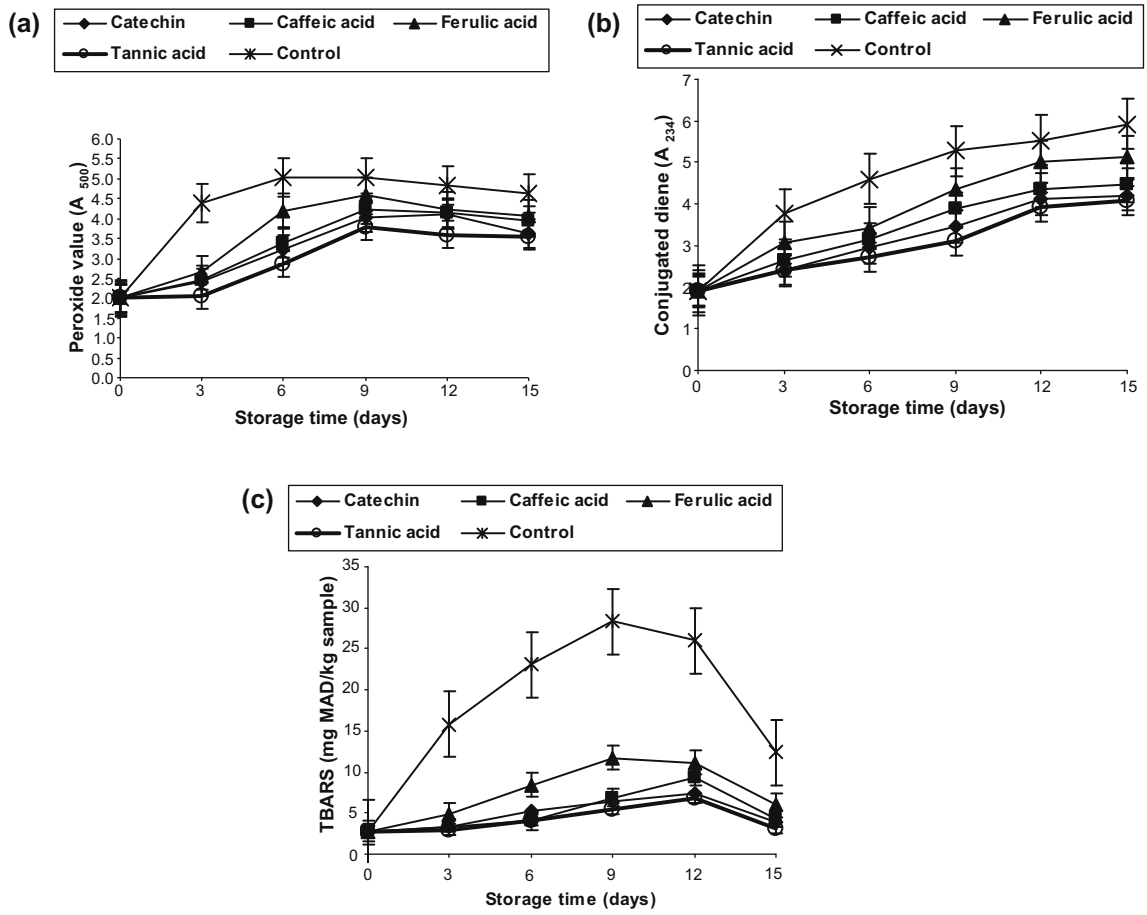

Fig. 5. Effect of different phenolic compounds on the formation of lipid oxidation products in mackerel mince during iced storage for a period of 15 days. Peroxide value (a), conjugated diene (b) and thiobarbituric acid-reactive substances (TBARS) values (c). Bars represent the standard deviation ($n = 3$). For all phenolic compounds, a final concentration of 100 mg/kg was used in the system.

higher than that of samples added with tannic acid ($P < 0.05$). Both caffeic acid and catechin showed similar efficiency in preventing the lipid oxidation in the mackerel mince, but the tannic acid was a more effective antioxidant in the fish mince system. The results were in agreement with Alghazeer, Saeed, and Howell (2008), Tang, Sheehan, Buckley, Morrissey, and Kerry (2001b) who reported that the incorporation of green tea catechin in frozen mackerel was effective in lowering PV and TBARS values as compared to the control during frozen storage. Tea catechin addition (300 mg/kg meat) effectively reduced lipid oxidation in cooked beef and chicken meat (Tang, Kerry, Sheehan, & Buckley, 2001a) and in raw red meat, poultry and fish muscle (Tang et al., 2001b). Medina et al. (2007) found that caffeic acid employed at 10 ppm showed similar antioxidative effectiveness in chilled horse mackerel muscle to propyl gallate at the same concentration.

CD values of all samples increased gradually during 15 days of iced storage (Fig. 5b) ($P < 0.05$). Among all phenolic compounds tested, ferulic acid showed the lowest activity in prevention of CD formation and tannic acid tended to exhibit the highest preventive effect. The PV of sample added with ferulic acid started to decline after day 9 of iced storage (Fig. 5a). These results indicated that ferulic acid was the least effective phenolic compound for the prevention of lipid oxidation in mackerel fish mince. This was coincidental with the lower ability of ferulic acid to chelate Fe^{2+} (Fig. 2d). Fe^{2+} has been known as the most important pro-oxidant in the fish muscle (Love, 1983). For the other phenolic compounds tested, the preventive effect on lipid oxidation might be associated with the capacity of metal chelating in fish mince.

For TBARS, samples added with phenolic compounds as well as the control samples had increased values as the storage time increased up to 12 days (Fig. 5c) ($P < 0.05$). The decreased TBARS value was found at day 15 of storage ($P < 0.05$). This was probably due to the losses in oxidation products formed, particularly the volatile counterparts. Malonaldehyde and other short-chain carbon products of lipid oxidation are not stable and are decomposed to organic alcohols and acids, which are not determined by the TBA test (Fernandez, Perez-Alvarez, & Fernandèz-Lopez, 1997). Alghazeer et al. (2008) reported the gradual increase of PV and TBARS in frozen mackerel and an instant decrease in both PV and TBARS after 10 weeks of storage. The decrease in TBARS is also probably due to thiobarbituric acid (TBA)-reactive substances interacting with proteins (Saeed, Fawthrop, & Howell, 1999).

When comparing TBARS values among the samples, the control sample contained the higher TBARS value than the other samples during the 15 days of iced storage ($P < 0.05$). During the first 9 days of storage, the samples added with ferulic acid had the highest TBARS values, compared with those added with other phenolic compounds ($P < 0.05$). In general, no difference in TBARS values were obtained among samples added with tannic acid, catechin and caffeic acid throughout the storage ($P > 0.05$).

Therefore, the ability of phenolic compounds in prevention of lipid oxidation in fish mince varied with the type of phenolic compounds used. This was governed by the varying ability in metal chelating, radical scavenging and LOX inhibitory activities.

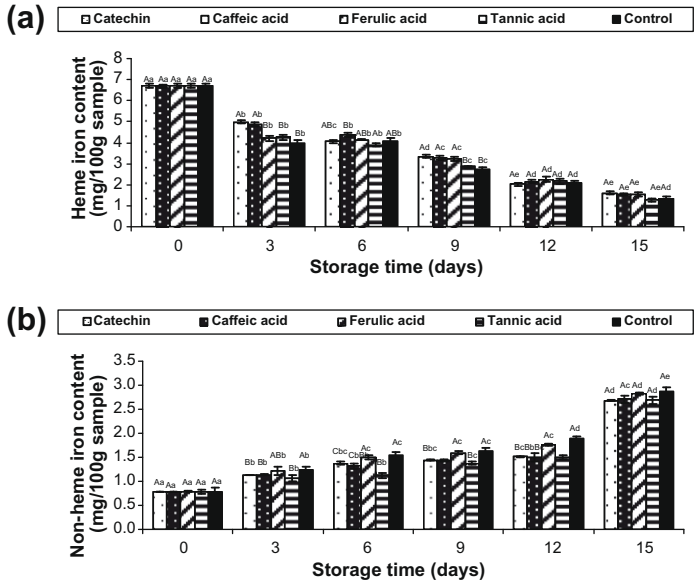

Fig. 6. Changes in heme iron content (a) and non-heme iron content (b) in mackerel mince treated without and with different phenolic compounds during iced storage for a period of 15 days. Bars represent the standard deviation ($n = 3$). Different capital letters within the same concentration denote the significant differences ($P < 0.05$). Different small letters within the same type of phenolic compound denote significant differences ($P < 0.05$). For all phenolic compounds, a final concentration of 100 mg/kg was used in the system.

3.4.2. Changes in heme and non-heme iron contents

Changes in heme and non-heme iron contents in mackerel mince with and without the addition of different phenolic compounds during ice storage are presented in Fig. 6a and b, respectively. Heme iron content of all samples decreased with increasing storage time up to 15 days of iced storage ($P < 0.05$). There was no difference in heme iron content of samples added with different phenolic compounds and the control sample throughout the storage, except at day 3 and 9, when heme iron content of samples added with catechin and caffeic acid was higher than the control sample ($P < 0.05$). Heme iron content decreased due to the heme breakdown, which resulted in the increase of non-heme iron content (Benjakul & Bauer, 2001). Additionally, the lowered heme pigment extractability with increasing storage time also resulted in the lower iron content of the heme extracted (Chaijan, Benjakul, Wonnop, & Cameron, 2005). This is possibly associated with the higher lipid oxidation in dark muscle, which had a high fat content (Chaijan et al., 2005). Benjakul and Bauer (2001), Gomez-Basauri and Regenstein (1992) reported that the decrease in heme iron content in the muscle was inversely related to non-heme iron content.

Non-heme iron content of all mackerel mince samples increased during the iced storage ($P < 0.05$) (Fig. 6b). During 6–12 days of storage, the control sample and those added with ferulic acid showed the higher non-heme iron content than others ($P < 0.05$). The lower metal chelating ability of ferulic acid might contribute to the high non-heme iron remaining in the mince. The continuous increase in non-heme iron content with increasing storage period was in agreement with the release of free iron from the muscle, which continued to degrade with the extended storage. Decker and Hultin (1990a, 1990b) suggested that the heme pigment or other iron-containing proteins are possibly denatured with increasing storage time, resulting in the release of iron. Deterioration of sub-cellular, organelles, e.g. mitochondria, and the release of cytochrome-C, could also be responsible for the increase in non-heme iron content. As non-heme iron content of the control and sample added with ferulic acid was slightly higher than other samples, this could be related with the higher degree of oxidation in both samples. Brune, Rossander, and Hallberg (1989) found that the absorption effects of tannin and catechin on non-heme iron content were more pronounced when there were 3 hydroxyl groups (galloyl) on the phenolic structure than when there were 2 hydroxyl groups (catechol). Therefore, the ability in binding non-heme iron was governed by the structure and hydroxyl group of phenolic compounds and more likely affected the efficacy in inhibiting or preventing lipid oxidation mediated by free iron in the fish mince during iced storage.

4. Conclusions

Antioxidant activity of phenolic compounds varied with the types and molecular structure. Tannic acid exhibited the highest antioxidative activity and could prevent lipid oxidation effectively in menhaden oil-in-water emulsion as well as in fish mince. This was most likely caused by its radical scavenging activity, LOX inhibitory activity as well as metal chelating activity, especially towards non-heme iron. Thus, the selection of suitable phenolic compound as an antioxidant is of great importance to maximise the prevention of lipid oxidation in different food model systems.

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