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Antioxidant activity of *Terminalia nigrovenulosa* and *Premna integrifolia* extracts in soybean oil

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Abstract

This study was to investigate the inhibition of linoleic acid peroxidation and the protective effects in stabilizing soybean oil of methanol extracts of *Terminalia nigrovenulosa* leaf (TnL), bark (TnB) and *Premna integrifolia* leaf (Pi). The parameters investigated were the inhibition percentage of a linoleic acid emulsion, peroxide values (PV), acid values (AV), TBA (thiobarbituric acid) values, p-anisidine values (AnV), and hexanal formation in soybean oil during storage. The results showed that TnL most effectively inhibited linoleic acid peroxidation with a inhibition percentage that varied from 13.45–82.59%, followed by TnB and BHA (butylated hydroxyanisole). Moreover, these plant extracts also showed an antioxidative effect for reducing the oxidation rate of soybean oil. The TnL extract was the most effective antioxidant and had higher activity than that of BHA for lowering the formation of primary (PV and AV) and secondary (AnV, TBA value, and hexanal concentration) products. The TnB and Pi extracts also possessed a highly effective antioxidant effect similar to BHA. These results indicate that the extracts of these plants have significant potential to be used as natural antioxidants to retard oil oxidation.

Introduction

Vegetable oils and fats are important components in our diet. Lipid oxidation promotes deterioration of vegetable oil, fats, and other food systems (Che and Tan, 1999) and results in a loss of nutritional value of the food and diminishes the flavor and taste of food as well as other physiological properties (Kazuhisa, 2001). Lipid oxidation also causes premature aging, heart disease, stroke, emphysema, mutagenesis, and carcinogenesis (Barlow, 1990). Therefore, it is necessary to suppress lipid oxidation to preserve the safety and value of food. Synthetic antioxidants, such as butylated hydroxyanisole (BHA), butylated hydroxytoluene, and ter-butyl hydroquinone have been used to preserve oils. However, these antioxidants are also suspected of being responsible for liver damage, carcinogenesis (Whysner *et al*., 1994), and toxicity (Wanasundara and Shahidi, 1998; Moure *et al*., 2001). Thus, there is an increasing need to replace synthetic antioxidants with natural, safer compounds. Natural antioxidants protect the human body from radicals and retard the progress of many chronic diseases. They also prevent oxidative deterioration of vegetable oils and fats during processing, distribution, and storage (Vagi *et al*., 2005). The effect of different natural antioxidants in stabilizing vegetable oils has been reported previously (Jung *et al*., 2001; Anwar *et al*., 2003; Siddiq *et al*., 2005; Shaker, 2006). Various plant materials containing phenolic compounds have been demonstrated to be effective antioxidants in model systems (Pan *et al*., 2007). Several herbs and spices such as black papper, propolis (Dessouki *et al*., 1980), oriental herbs (Kim *et al*., 1994), *Cratoxylum formosum* extract (Pitchaon and Suphan, 2007), blackcurrant seeds (Urszula and Maria, 2007) have been reported to provide significant retarding lipid oxidation.

In addition, a great number of medicinal plants containing chemical compounds that exhibit strong activity against lipid substrates such as rosemary, sage, thyme, marjoram, oregano, mint, lavender, and basil have been investigated. However, there are still many plants that remain unstudied regarding their antioxidative properties. Therefore, an assessment of such properties is an interesting and useful topic, particularly to find new sources of natural antioxidants, functional foods, and neutraceuticals.

Vietnam is considered to be the sixteenth most biodiverse country in the world. There are important sources of medicinal and aromatic plants, which have been used in the preparation of various kinds of

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traditional medicine for long ages. Vietnam has about 4,000 plant species used as medicinal sources (Handa *et al.*, 2006). About 700 species are often used in oriental medicines, around 150 to 180 medicinal substances derived from medicinal plants used by various traditional medicine used in hospitals or local physicians' and 120 medicinal plant species used frequently local people, particularly, those living in rural and mountainous areas. Medicinal and aromatic plants constitute the basis of primary health care for the majority of the population in Vietnam and are a critical source of income for rural populations (Handa *et al.*, 2006).

T. nigrovenulosa was used as an anti-diarrhea in the treatment of chronic dysentery, sore throat, laryngitis, and hemorrhoids. There are many researches showed that the extracts of *Terminalia* species possessed a variety of biological activities. For instances, the solvent extracts of *T. nigrovenulosa* bark and leaves showed strongly antioxidant activity (Nguyen and Eun, 2011) and antimicrobial activity (Nguyen and Eun, 2013).

Premna integrifolia L. is a garden shrub, belonging to the *Verbenaceae* family, widely spreads in tropical and subtropical regions throughout the world. In Vietnam, it is cultivated as an ornamental and shade tree. It is one of the important constituents among ten herb formulations called ''Dashmula'', a favorite decoction of ten plants used in India. The plant possessed antirheumatic, carminative, galactogenic, bechic, febrifuge, stomachic and anti-inflammatory activities. The solvent extracts of *Premna integrifolia* L. leaf showed antioxidant activity (Nguyen and Eun, 2011) and antimicrobial activity (Nguyen and Eun, 2013). Previous data showed that root of *P. integrifolia* contained bioactive compounds including alkaloids, flavonoids, glycosides, tannins, phenolic compounds and diterpenoids (Mali and Bhadane, 2010; Yadav *et al*., 2010, 2011). Extracts of the bark and leaves of *Terminalia nigrovenulosa* Pierre ex Laness and *Premna integrifolia* grown in Vietnam were selected for this investigation. Few data are available on the antioxidative properties of these plants as applied to oil preservation. The objective of this study was to examine the oxidative inhibition of soybean oil using methanol extracts of *T. nigrovenulosa* and *P. integrifolia* during accelerated storage conditions and compare the result to those of BHA.

Materials and Methods

Chemicals

Hexanal, p-anisidine, and thiobarbituric acid were obtained from Sigma-Aldrich (St Louis, MO, USA). All other chemicals and reagents were of analytical grade.

Materials

T. nigrovenulosa Pierre ex Laness leaves (TnL) and trunk-bark (TnB) and *P. integrifolia* leaves (Pi) were gathered in Daklak province Vietnam in August 2010. The different parts of the fresh plant were cut and dried in an ambient temperature room with active ventilation. They were then packed in PE bags and stored at -80°C before use. Soybean oil made without the incorporation of any synthetic antioxidants was obtained from Cheiljedang (Seoul, South Korea).

Extraction

Plant parts were powdered (10 g) and extracted for 24 hours with 100 mL methanol in a glass conical flask using a shaker at room temperature and then filtered through filter paper (No. 1, Whatman International LTD, Maidstone, England). The residues were extracted twice with 200 mL methanol, as described above. The combined methanol extracts were concentrated in a rotary evaporator (Heidolph VV 2011-Antrieb, Heidolph Instruments GmbH & Co. KG, Schwabach, Germany) at 40°C under vacuum. The extracts were stored at -80°C until use.

Linoleic acid emulsion system–thiocyanate assay

The inhibition of linoleic acid peroxidation activity of the TnL, TnB and Pi extracts was assayed using the slightly modified method of Pan *et al*. (2007). Briefly, 0.1 ml (0.4 mg/ml ethanol) of the TnB, TnL and Pi extracts was mixed with 2.5 ml of linoleic acid emulsion (0.2M, pH 7.0) and 2 ml of phosphate buffer (0.2 M, pH 7.0). The linoleic acid emulsion was prepared by mixing 0.2 g linoleic acid, 0.2 g Tween-20 as an emulsifier and 50 ml phosphate buffer pH 7, and the mixture was homogenized. The reaction mixture was incubated at 37°C to accelerate the oxidation process. Samples were taken each 24 h to evaluate antioxidative activity. The mixture without added extract was used as the control. The mixture (0.1 ml) was mixed with 5.0 ml 75% ethanol, 0.1 ml 30% ammonium thiocyanate and 0.1 ml 20 mM ferrous chloride in 3.5% HCl and kept at room temperature. After 3 min, the absorbance of the reaction mixture was measured at 500 nm with a spectrophotometer (Optizen, Mecasys, Daejeon, Korea) The percentage of inhibited peroxidation (IP%) : IP% = [1- (absorbance of sample at 500 nm)/(absorbance of control at 500 nm)] × 100. The antioxidant activity of BHA (0.4 mg/m) was also assayed for comparison. All tests were performed in triplicate.

Stable oxidative measurements

Sample preparation

The plant extracts and reference (BHA) were added to soybean oil at 200 mg/kg. All samples (25 g) were placed in a closed 1000 ml glass conical flask, covered with aluminum foil, and mixed on a magnetic mixer for 30 min at 60°C. Then all samples (25 g) were stored in an incubator at a fixed temperature of 60°C. Control samples without antioxidants were also maintained under the same storage conditions. The oxidative stability was evaluated by measuring the peroxide (PV), acid (AV), thiobarbituric acid (TBA), p-anisidine (AnV) and volatile compounds during 15 days of storage.

Determination of oxidative parameters

American Oil Chemist's Society methods were used to determine the AV (Method Cd 3d–63), PV (method Cd 8-53), TBA (Cd 19-90), and AnV values (method Cd 18-90). All methods are described in detail in AOCS, 2006.

Hexanal formation was observed by static headspace gas chromatography (HS- GC) analysis. Analyses were carried on an HP 6890 series gas chromatograph equipped with an FID detector. A BD-5 column (30 m in length × 0.25 mm i.d × 0.25 µm in thickness) (BD J&W Scientific, Folsom, CA, USA) (Agilent Technologies, Santa Clara, CA, USA) and headspace SPME silica fiber coated with a 50/30 µm layer of divinylbenzene-caboxen-polydimethylsiloxane (Supelco, Belafonte, PA, USA) were employed. Helium was used as the carrier gas at a head pressure of 70 kPa.

The static HS-GC analysis consisted of two steps. In the first step, the HS was exposed to a closed vial containing sample heated for 20 min at 60°C. Then the HS is introduced to the carrier gas stream and was carried into the column. Oven temperature was as follows: 40°C for 1 min, increased to 120°C at 20°C/min, held for 8 min and then increased to 260°C at 20°C/min and finally held for 2 min. The injector temperature was 260°C.

Hexanal retention time was determined by comparison with that of a known hexanal standard (Sigma-Aldrich) and quantified from a standard curve. The standard was added to the matrix prepared with soybean oil.

Data analysis

All experiments were performed in triplicate and an analysis of variance (using STATGRAPHICS Centurion XV statistical software) was used to compare the mean values of each treatment. Significant differences between the means of parameters were determined by the least significant difference test ($p<0.05$). Results are presented as mean ± standard deviation of three replicates.

Results and Discussion

Effect of extracts on peroxidation of linoleic acid emulsion

Figure 1 shows the effect of the Pi, TnB and TnL extracts and BHA on peroxidation of the linoleic acid emulsion. The results indicate that all extracts and BHA inhibited linoleic acid peroxidation. Of these extracts, the TnL extract possessed the highest inhibition of linoleic emulsion peroxidation. The antioxidative activity of TnL shown by percent inhibition of peroxidation after incubation at 37°C for 24 to 96 h varied from 13.45 to 82.59% compared to that of BHA and the TnB extract (12.10–77.35% and 12.73–78.26%, respectively. The TnB, TnL and Pi extracts have total phenolic contents of 833.802, 453.608, and 246.485 (mg gallic acid equivalent/g extract), respectively (Nguyen and Eun, 2011). Therefore, the ability of these extracts to inhibit linoleic acid peroxidation may be attributed to the presence of phenolic compounds. Moreover, the greater effect of the TnL extract on inhibiting linoleic acid peroxidation may be because the TnL extract possesses relatively high DPPH radical scavenging and chelating activities among these extracts (Nguyen and Eun, 2011). The ability of plant extracts to inhibit peroxidation of the linoleic acid emulsion due to phenolic content has been reported (Mathew and Abraham, 2006; Pan *et al*., 2007; Gulcin *et al*., 2010). The results indicate that the components present in the Pi, TnL and TnB extracts possessed a high inhibitory activity against linoleic acid peroxidation.

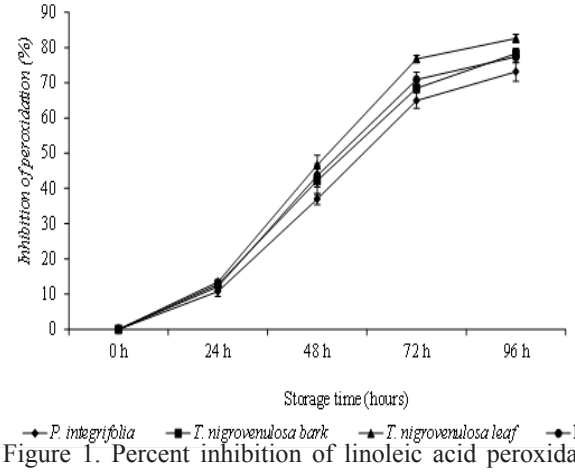

Figure 1. Percent inhibition of linoleic acid peroxidation due to *Terminalia nigrovenulosa* (leaf and bark) and *Premna integrifolia* extracts and BHA using the thiocyanate method. Results are presented as mean ± standard deviation (STD) of three replicate determinations

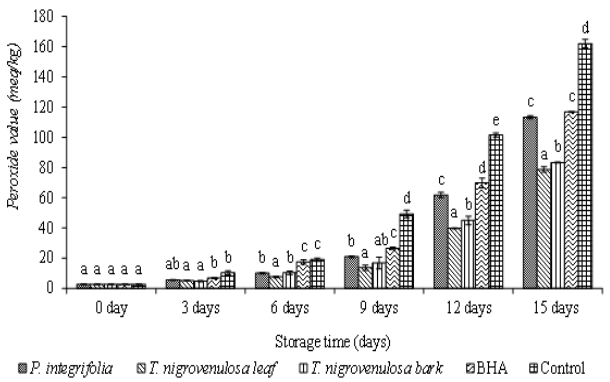

Figure 2. Effect of *Terminalia nigrovenulosa* (leaf and bark) and *Premna integrifolia* extracts and BHA at a concentration of 0.02 % (w/w) on the formation of hydroperoxides in soybean oil stored under accelerated oxidation at 60°C. Different labels (a–e) above the bars for the same storage day indicate a significant difference at $P < 0.05$

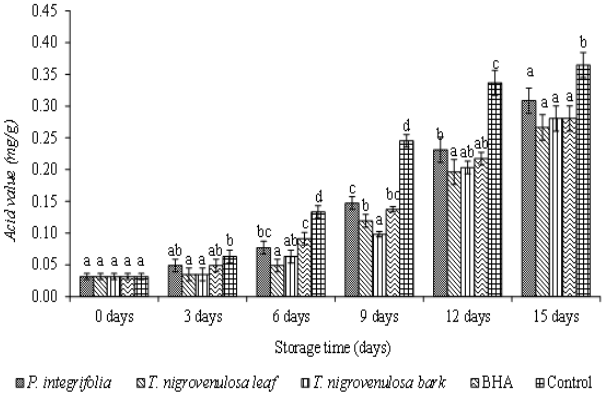

Figure 3. Effect of *Terminalia nigrovenulosa* (leaf and bark) and *Premna integrifolia* extracts and BHA at a concentration of 0.02 % (w/w) on acid values in soybean oil stored under accelerated oxidation at 60°C. Different labels (a–d) above the bars for the same storage day indicate a significant difference at $P < 0.05$

Effects of the extracts on soybean oil oxidation

Effect of the extracts on PV and AV

The PV represents the concentration of peroxides and hydroperoxides formed in the initial stages of lipid oxidation. It is a way to measure the amount of primary oxidative products in oils. The influence of the extracts and BHA on PVs in soybean oil is shown in Figure 2. PVs increased gradually during the initial storage period and then increased rapidly after the ninth day of storage in the control (sample without antioxidant) and the twelfth day in the samples with added TnB, TnL and Pi extracts, and BHA. The PVs measured in the soybean oil samples with the extracts and BHA were significantly lower than that of the control, indicating that the TnB, TnL and Pi extracts, and BHA inhibited the formation of hydroperoxides and peroxides, which are primary oil oxidation products. The TnB and TnL extracts had a greater effect on retarding lipid oxidation in soybean oil than that of BHA and the Pi extract. After 15 days under accelerated storage conditions, the PVs of the samples with the TnL and TnB extracts were 78.748 and 83.248, meq/kg, respectively, compared with 161.995 meq/kg in the control and 116.747 meq/kg in the sample containing BHA at the same concentration of 0.2 g/kg. These results show that the TnL and TnB extracts inhibited primary oxidative changes in soybean oil more effectively than that of BHA. This result is in accordance with a previous study showing that a Cortex fraxini extract inhibited linoleic acid peroxidation to a greater degree being (Pan *et al.*, 2007). This results may have occurred because linoleic acid is a predominant unsaturated fatty acid in soybean oil (Frank, 2005).

Formation of free fatty acids (FFAs) is an important parameter to measure food rancidity. FFAs are formed due to hydrolysis of triglycerides, which is accelerated by the reaction of oil with moisture (Frega *et al*., 1999). FFA content increased with increasing storage time for all samples. However, no regular pattern of increase was observed (Figure 3). A significant difference ($p < 0.05$) in FFA content was observed between the control and samples with the TnL, TnB and Pi extracts, and BHA.; Nevertheless, no significant difference ($p < 0.05$) was observed between samples with the TnL and TnB extracts and BHA. The FFA contents in the samples with antioxidants were lower than that in the control indicating that these plant extracts and BHA can retard soybean oil rancidity. The high amount of total phenolic compounds in the TnB, TnL and Pi extracts (Nguyen and Eun, 2011) may be responsible for the decreased PVs and AVs in soybean oil during storage. This result was in agreement with those of Mariod *et al.* (2011) and Poiana (2012) who showed that phenolic compounds have a significant effect on lipid oxidative stability. Phenolic compounds donate hydrogen atoms to scavenge and stabilize lipid radicals (Eskin and Przybylski, 2001; Jayaprakash *et al*., 2001). The contribution of plant extracts to inhibit lipid oxidation due to their phenolic content has been shown previously (Wanasundara and Shaidi, 1998; Bandoniene *et al*., 2000; Morteza-Semnani *et al.*, 2006; Sikwese and Doudo, 2007). In general, the inhibitory effect of the TnL and TnB extracts on the PV was higher than that of the Pi extracts, which could be due to the higher radical scavenging activity and reducing capacity of the TnL and TnB extracts (Nguyen and Eun, 2011), thereby preventing subsequent generation of reactive lipid radicals, which can undergo further chain reactions. These data were similar to the results of Zhang *et al.* (2010) who

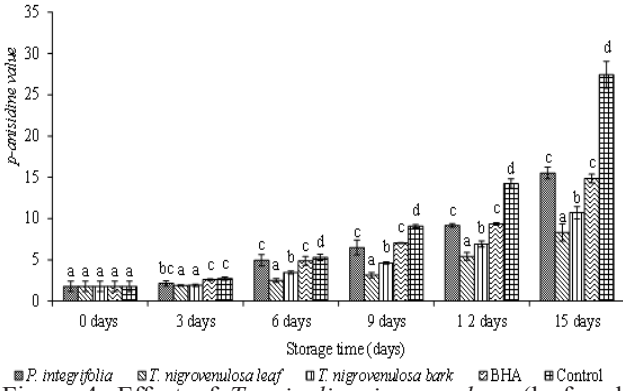

Figure 4. Effect of *Terminalia nigrovenulosa* (leaf and bark) and *Premna integrifolia* extracts and BHA at a concentration of 0.02 % (w/w) on *P*-anisidine values in soybean oil stored under accelerated oxidation at 60°C. Different labels (a–e) above the bars for the same storage day indicate a significant difference at $P < 0.05$

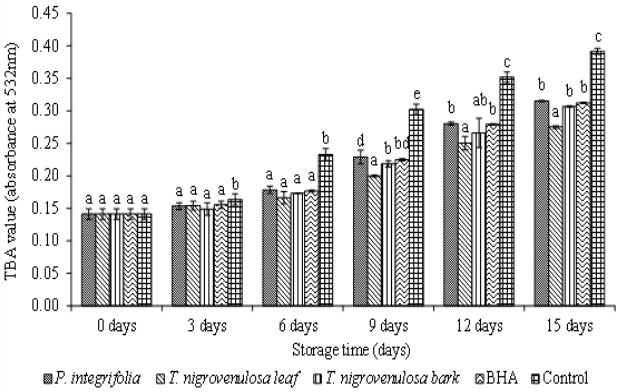

Figure 5. Effect of *Terminalia nigrovenulosa* (leaf and bark) and *Premna integrifolia* extracts and BHA at a concentration of 0.02 % (w/w) on TBA values in soybean oil stored under accelerated oxidation at 60°C. Different labels (a–e) above the bars for the same storage day indicate a significant difference at $P < 0.05$

showed that a rosemary extract with higher DPPH radical scavenging activity and reducing power exhibit a greater effect on lipid oxidative stability.

Effect of the extracts on the secondary oxidation stage; P-anisidine value, TBA value, and hexanal content

Peroxides are characteristic products of the first stage of oxidation, as they are unstable, and easily undergo decomposition. Therefore, measuring peroxide only provides information about the initial oxidation potential of the oil. To evaluate the antioxidant effect of these extracts on other stages of lipid oxidation, it is necessary to consider whether they have an inhibitory effect at the later stage of peroxidation. P-anisidine, TBA values, and hexanal formation are used to measure secondary oxidation products (carbonyls) formed during lipid oxidative degradation.

Figure 4 shows the p-anisidine values of soybean oil stabilized with the plant extracts and BHA. The p-anisidine values showed the same trend as the PV values with increasing storage time. The p-anisidine values increased slowly during the first 3 days; however, they sharply increased after 6 days in the control and 9 days in the samples treated with the extracts and BHA. The rate of carbonyl formation was higher in the control than that in samples with the extracts and BHA during storage. Moreover, a significant difference ($p < 0.05$) in p-anisidine values was observed between the samples treated with the extracts and BHA. The TnL and TnB extracts showed higher efficiency for inhibiting secondary oxidation of soybean oil than that of BHA and the Pi extract. The p-anisidine values after 15 days of storage in the TnL TnB and Pi extracts, and BHA at the same concentration of 0.02% were 8.296, 10.7, 14.85 and 15.517, respectively, compared to 27.433 for the control. The greater the p-anisidine value obtained, the lower the oxidative stability of the oil (Anwar *et al*., 2004). This result indicates that the TnL and TnB extracts possessed higher antioxidant capacity than that of BHA for retarding oil oxidation.

TBA represents the level of malondialdehyde formed during lipid oxidation and is responsible for the development of rancid odors and off-flavor of oils. Our results showed that TBA values of all soybean oil samples increased progressively with storage time under accelerated oxidation at 60°C (Figure 5). The TBA values of samples supplemented with the plant extracts and BHA were significantly lower ($p < 0.05$) than that of the control during storage. The TBA value of the control increased suddenly after the third day of storage and reached a maximum of 0.391 ± 0.005 after 15 days, which was 1.42-fold, 1.3-fold, and 1.25-fold higher than that of samples treated with the TnL and TnB extracts and BHA (Figure. 5), respectively.

Hexanal is an important volatile compound product and has proved useful as an analytical indicator for oxidative decomposition of n-6 polyunsaturated fatty acids (Frankel, 1982). In this study, the concentration of hexanal increased gradually and then increased sharply at the eighth day of storage in the control and at the twelfth day in samples with the plant extracts and BHA (Figure 6). These results indicate that the antioxidant activity of the extracts and BHA were maintained during the second stage of oxidation. The p-anisidine, TBA values, and hexanal formation were significantly lower compared to those of the control during storage. No significant difference in hexanal content was observed between the soybean oil samples treated with the extracts and BHA after 8 days of storage, but it was significantly lower than

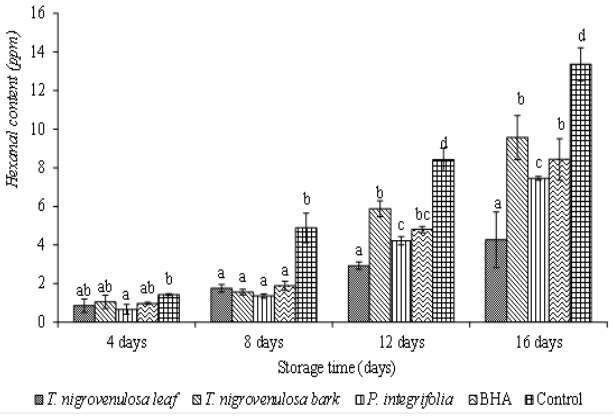

Figure 6. Effect of T*erminalia nigrovenulosa* (leaf and bark) and *Premna integrifolia* extracts and BHA at a concentration of 0.02 % (w/w) on the formation of hexanal in soybean oil stored under accelerated oxidation at 60°C. Different labels (a–e) above the bars for the same storage day indicate a significant difference at $P < 0.05$

that of the control. However, hexanal content was markedly different at the twelfth to sixteenth day of storage. Of these samples, the soybean oil treated with the TnL extract showed the lowest hexanal content followed by the Pi extract, BHA, and the TnB extract, indicating that the TnL extract was the most effective for retarding secondary oxidation products by lowering the p-anisidine and TBA values and hexanal content in soybean oil during storage. The total phenolic and flavonoid contents in the TnL extract has been reported to be lower than those of a TnB extract, but higher than those of a Pi extract (Nguyen and Eun, 2011). This result means that the antioxidative activity of the extracts depends not only on total phenolic and flavonoid contents but also on the structure of the compounds present in the extracts. This result agrees with those of Bandoniene *et al.* (2000), Tovar *et al*. (2001) and Mariod *et al*. (2011) who showed that the oxidative stability of oils does not correlate directly with the amount of phenolics, but is significantly affected by the type of phenolic compound. The different efficiencies of variuos phenolic compounds and extracts on oxidative stability of oils has been reported (Rehman *et al*., 2004; Morteza-Semnani *et al.*, 2006; Maqsood and Benjakul, 2010; Samotyja and Małecka, 2010; Yalcin, 2011). The results also indicate that the TnB extract, possessing the lowest ability to metal chelate (Nguyen and Eun, 2011) was the least effective extract for preventing lipid oxidation in soybean oil. These results were in agreement with those of Maqsood and Benjakul (2010) who reported that the ability of phenolic compounds to prevent lipid oxidation of minced fish is governed by the metal chelating,radical scavenging, and lipooxygenase inhibitory activities of the phenolic compounds used. This study is the first on the effect of TnL, TnB and Pi extracts to delay the primary and secondary soybean oil oxidation stages.

Conclusion

Our results demonstrated that the *T. nigrovenulosa* leaf extract possessed the greatest inhibitory effect on linoleic acid peroxidation. In particular, the suitability of these plant extracts as antioxidants was investigated in soybean oil. Soybean oil treated with the tested plant extracts was more stable than that of the control. The formations of primary oxidative products (PV and AV) as well as secondary oxidative products (AnV, TBA value, and hexanal formation) of samples treated with the plant extracts and BHA were significantly lower than that of the control during accelerated storage at 60°. Among these extracts, the most efficient soybean oil antioxidant was the *T. nigrovenulosa* leaf extract, which was more effective than BHA for inhibiting the formation of primary and secondary oxidative products at the same concentration of 0.2 g/kg. The other extracts also had highly efficient antioxidant activity to stabilize the oil, which was similar to BHA. Further studies are required to isolate and identify the individual active compounds and determine their *in vivo* antioxidant activities and mechanisms.

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