ชื่อเรื่อง **ผลของสารสกัดจากเมล็ดมะเกี๋ยง (*Cleistocalyx nervosum* var paniala) ในการยับยั้งการหืนในต้นแบบน้ำมันถั่วเหลือง**

Title **EFFECT OF MAKIANG (*Cleistocalyx nervosum* var paniala) SEED ON RANCIDITY INHIBITION IN SOYBEAN OIL MODEL**

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บทคัดย่อ

Cleistocalyx nervosum var paniala หรือที่รู้จักกันทั่วไปว่า มะเกี๋ยง เป็นพืชที่พบมากในภาคเหนือของประเทศไทย เมล็ดมะเกี๋ยงถูกนำมาสกัดเป็นสารให้กลิ่นในอาหาร ซึ่งเป็นสารในกลุ่ม terpene alcohol แต่ข้อมูลเกี่ยวกับสมบัติแอนติออกซิแดนท์ของเมล็ดมะเกี๋ยงมีน้อยมาก ดังนั้นงานวิจัยนี้จึงมีวัตถุประสงค์เพื่อ ศึกษากิจกรรมแอนติออกซิแดนท์ของสารสกัดของเมล็ดมะเกี๋ยงด้วยเอทธานอลเปรียบเทียบกับแอลฟา - โทโคเฟอรอล และ BHT โดยทำการวัดค่ากิจกรรมต้านอนุมูลอิสระด้วยอนุมูลของ 2,2'-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) cation ($ABTS^{\bullet+}$) ค่ากิจกรรมแอนติออกซิแดนท์ของตัวอย่างในน้ำมันที่ผ่านการ strip โดยทำการวิเคราะห์ค่าเปอร์ออกไซด์ (PV), Conjugated diene (CD) hydroperoxide value, Thiobarbituric acid reactive substances (TBARS) และ *p*-anisidine value (*p*-AV) พบว่า สารสกัดเมล็ดมะเกี๋ยงมีความสามารถในการต้านอนุมูลอิสระได้ดีกว่า แอลฟา - โทโคเฟอรอล และ ค่ากิจกรรมแอนติออกซิแดนท์ในน้ำมันมีแนวโน้มเช่นเดียวกับค่ากิจกรรมการต้านอนุมูลอิสระ จากผลการศึกษานี้ชี้ให้เห็นว่า สารสกัดจากเมล็ดมะเกี๋ยงสามารถเป็นแหล่งของสารแอนติออกซิแดนท์ตามธรรมชาติได้

คำสำคัญ : แอนติออกซิแดนท์, อนุมูลอิสระ, เมล็ดมะเกี๋ยง, *Cleistocalyx nervosum*

Abstract

Cleistocalyx nervosum var paniala, which is commonly known in Thailand as Makiang, is mostly found in the North of Thailand. Makiang seed is extracted for food flavors. The contribution of plant seed to a flavor can be related to terpene alcohol. Data on the antioxidant properties of this plant is relatively scarce. This study was aimed at evaluating the antioxidant activity of the ethanolic extract of Makiang seed comparing to α-tocopherol and BHT. The antiradical activity of the extract was measured using the radical scavenging ability against 2,2'-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) cation ($ABTS^{\bullet+}$) radicals. The antioxidant activity of the extract or reference antioxidants including α-tocopherol and BHT was studied in stripped soybean oil. Samples were analyzed periodically for peroxide value (PV), conjugated diene (CD) hydroperoxide value, thiobarbituric acid reactive substances (TBARS), and *p*-anisidine value (*p*-AV). The Makiang seed extract scavenged both free radicals more than α-tocopherol. Comparing to $ABTS^{\bullet+}$ scavenging activity, the relative ranking of activity of different antioxidants

assessed by radical scavenging methods and lipid oxidation in oil models showed the same order. These results suggest that the Makiang seed extract is a promising source of a natural antioxidant.

Keywords : Antioxidant, free radical, Makiang seed, *Cleistocalyx nervosum*

Introduction

In recent years there has been a remarkable increment in scientific articles dealing with plant phenolic compound. Tea catechins and rosemary, olive oil, and ginger extracts have successfully inhibited rancidity of different food (Pazos et al., 2006). Lipid peroxidation leads to the development of food rancidity and off-flavors.

Soybean oil is widely used and is an important foodstuff because of its high quality and low cost. It has a unique fatty acid composition with a substantial amount (8%) of linolenic acid (C18: 3) compared to other vegetable oils, such as sunflower, olive and corn, which contain 0.2, 0.8 and 0.7% C18:3 respectively. Furthermore, soybean oil enriched of linoleic acid (C18: 2), which is the essential fatty acid for human, is easy to oxidize. The high content of unsaturated fatty acids influences its stability and keeping quality. These unsaturated fatty acids undergo rapid autoxidatiion and produce undesirable off-flavors and off-odors during storage and heating (Ganthavorn and Huhes, 1997).

Cleistocalyx nervosum var paniala (*C. nervosum*) has a synonym as *Eugenia panialla* Roxb. (Food and Agriculture Organization of the United Nations Regional Office for Asia and the Pacific, 2001). It is one of the important sources of plant-derived medicinal herbs according to their nutritional facts and medicinal properties. Because of the unique color, flavor and high range of nutritional values of Makiang flesh, it has been used for wine making that give a special and unique taste, enhancing the color of food and is now popular for health drinks. Moreover, the high polyphenols and flavanoids contents in Makiang flesh have been shown to have antioxidant and anticarcinogenic properties (Falk *et al*., 2000). Cyanidin-3-glucoside, cyaniding-3-sophoroside, pelarginidin-3-glucoside were found in Makiang. Most of phenolic compound of Makiang flesh is anthocyanin which is less than mulberry (Lohachoompol, 2007). However, there is no report on antiradical activity and lipid oxidation inhibition of the *C. nervosum* seed.

The objective of this research was to determine the antioxidant effect of *C. nervosum* seed extract upon storage and stability of refined soybean oil to oxidative rancidity.

Materials and Methods

Materials. α-Tocopherol was purchased from Fluka Co. (Buchs, Switzerland). Ethanol, and potassium persulfate were purchased from Sigma (Milwaukee, USA). Butylated hydroxytoluene (BHT) was purchased from BDH (Poole, United Kingdom). 2′,2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) and other chemicals and solvents used in this experiment were reagent - grade quality and purchased from Sigma-Aldrich Company Ltd. (Gillingham, UK).

Three batches of *C. nervosum* seed were donated from a factory at Chiengmai province, Thailand, during 2006. Immediately on receipt, the seeds were washed and selected for extraction. Yield (%, db) of the *C. nervosum* seed extract was calculated from three replications.

Soybean oil known to lack added antioxidants was provided by Thai Oil Industry, Bangkok, Thailand. The oil contained 115.7 mg kg^{-1} of α-tocopherol, 918.7 mg kg^{-1} of γ-tocopherol, 228.1 mg kg^{-1} of δ-tocopherol, and the peroxide value determined by AOCS official method cd 8-53 (1990) was 0.70 ($\pm$0.12) meq. kg^{-1} oil. The thiobarbituric acid reactive substances value (TBARS), determined according to McDonald and Hultin (1987) was 0.0001 mmol.kg^{-1} oil. Refined soybean oil is selected to study in order to eliminate the possibility of pigments and other compound interfering with the oxidation process.

Preparation of Plant Extracts. *C. nervosum* seeds (80 g) were blended for 1 min with ethanol at -20°C and the containers were then flushed with nitrogen and shaken at 250 rpm for 4.5 hours in the dark at 25°C. The solvent was evaporated under vacuum. The residue was dried in a freeze dryer and stored in aluminum foil after flushing with nitrogen at -20°C.

Determination of Free Radical-Scavenging Activity. The $ABTS^{+}$• scavenging activity was determined according to Re *et al*. (1999). Briefly, a mixture of 7 mM ABTS and 2.45 mM potassium persulfate was prepared and allowed to stand at 25 °C for 12-16 h in the dark. The $ABTS^{+}$• solution was diluted to an absorbance of 0.70 ($\pm$0.02) at 734 nm in ethanol before use. $ABTS^{+}$• solution (2 mL) was added to 20 µL aliquots of Trolox or sample in water with different concentrations. The activity of each antioxidant was determined within the range of the dose-response curve of Trolox, and the radical-scavenging activity was expressed as the Trolox equivalent antioxidant capacity (TEAC), defined as mmol of Trolox per gram of sample.

Preparation of Stripped Soybean Oil. The tocopherols were removed from soybean oil by column chromatography using alumina with some modifications to the method described by Yoshida (1993) (Maisuthisakul *et al*., 2006).

Antioxidants were added to stripped soybean oil in the following quantities: 100 mg.kg^{-1} of α-tocopherol, *C. nervosum* seed extract and BHT.

Oxidation and Analysis. Stripped soybean oil samples with or without added antioxidant were transferred to screw-capped sample vials with aluminium foil wrapping and held in an oven at 60°C for 15 days. The lids were only screwed loosely on the vials, therefore the air could pass in and out of the headspace above the samples. Aliquots (10 g) were removed at 0, 1, 3, 5, 7, 9, 11, 13 and 15 days for analysis. The oxidative state of sample was monitored by analysis of PV, CD, TBARS and *p*-AV.

Peroxide value (PV) of soybean oil samples was determined according to AOCS official method Cd 8-53 (7) with an automatic titrator (Mettler-Toledo model DL 5X, Switzerland) equipped with stirrer and redox electrode. The solution was titrated against standard sodium thiosulfate (0.01 M). PV was calculated and expressed as milliequivalent peroxide per kg of oil sample (Meq•kg^{-1} oil) (Maisuthisakul et al., 2006).

Conjugated Diene (CD) hydroperoxide values were determined spectrophotometrically by using an absorptivity of 26,000 (λmax = 234 nm), as previously reported by Khallouki *et al*. (2008). Specifically, the oil samples were diluted in *iso*-octane and the absorbance of the resulting solutions were measured. Conjugated diene (CD) values are expressed in conjugated dienoic acid (%).

TBARS of stripped and non-stripped soybean oils was determined according to Mcdonald and Hultin (1987). Oil (0.1 mL) was mixed with water (0.9 mL) and TBA reagent (2.0 mL, 15% w/v trichloroacetic acid and 0.375% w/v thiobarbituric acid in 0.25 M HCl) in test tubes and placed in a boiling water bath for 15 min. The tubes were cooled to room temperature for 10 min and then centrifuged (1000 × g) for 15 min. The absorbance was measured at 532 nm. Concentrations of TBARS were determined from a standard curve prepared using 1, 1, 3, 3-tetraethoxypropane.

p-anisidine value (*p*-AV) of soybean oil samples was determined according to AOCS official method cd-18-90 (AOCS, 1998). The sample (0.5–4.0 g) was dissolved and diluted to volume with *iso*-octane in a 25 ml volumetric flask. The absorbance (A_b) of the solution was measured at 350 nm. Exactly 5 ml of the fat solution were transferred to a test tube and 5 ml of only the solvent were added to another test tube. One millilitre of *p*-anisidine reagent (2.5 g/l solution in glacial acetic acid) was added to each tube, and shaken. After exactly 10 min, the absorbance (A_s) of the solution in the first test tube was measured at 350 nm, using the solution in the second test tube as blank.

$$p\text{-AV} = [25\times(1.2A_s - A_b)]/m$$

A_s is absorbance of the fat solution after reaction with the *p*-anisidine reagent, A_b is absorbance of the fat solution, m mass of the test portion in g.

***Statistical Analysis*.** Each experiment, from sample preparation to analysis, was repeated in triplicate, and the data were then analyzed by SPSS software program (SPSS Inc., Chicago, IL, USA). The general linear model procedure was applied and Duncan's New Multiple Range Test was used to compare the mean values at $P < 0.05$. Mean values and pooled standard error of the mean (SEM) were calculated.

Results and Discussion

***Free Radical Scavenging Activity of Phenolic Antioxidants*.** Free radical scavenging is the main mechanism by which antioxidants inhibit lipid oxidation. ABTS radical scavenging was used to assess the antioxidant activity of the *C. nervosum* seed extract compared to that of α-tocopherol and BHT as reference standards. The advantage of this radical is less susceptible to stearic hindrance when bulky antioxidants are studied. The ABTS radical scavenging activity of the extract was higher than α-tocopherol and BHT (Table 1). It was observed here that α-tocopherol and BHT is not water soluble contributes to its low value in the ABTS assay.

The yield of extractable compound was 2.65±0.12% (db) (Table 1) which was high comparing to other plant seed extracts (Maisuthisakul *et al*., 2008).

Table 1 Antioxidant activity determined by the ABTS decolorization assay and yield of *C. nervosum* seed extract [A]

Compound	TEAC (mmol of Trolox/g sample)	Yield (%, db)
C. nervosum seed extract	3.67 [a] ±0.03	2.65±0.12
α- tocopherol	2.30 [b] ±0.03	-
BHT	0.73 [c] ±0.01	-

Note : [A] Data followed by different letters within each column are significantly different according to Duncan's multiple range test at $P < 0.05$. Data obtained from at least three replicates.

Effect of C. nervosum* seed *extract on stripped soybean oil stability. Stored samples were analyzed periodically for PV, CD, TBARS and *p*-AV to allow both hydroperoxides and hydroperoxide degradation products to be monitored. The degradation products contribute oxidative off-flavours to foods, and consequently, it is important to monitor both precursors of these off-flavours as well as the degradation products themselves.

Figure 1 showed PV and CD of stripped soybean oil with and without studied antioxidants. Antioxidant efficiency for 100 mg kg^{-1} addition of *C. nervosum* seed extract was higher than that of α-tocopherol. The result was consistent to antiradical activity, the *C. nervosum* seed extract has been shown higher antiradical activity than α-tocopherol (Table 1). It was found that the PV and CD value of control at 15 day storage was less that 13 day storage because the rate of peroxide degradation was more than the rate of increasing hydroperoxides at 15 day storage. Normally, peroxide value (PV) and conjugated diene (CD) measurements are both well-established methods for the determination of primary oxidation products in fats and oils. Peroxidation of unsaturated fatty acids is accompanied by a shift in the position of double bonds to form conjugated hydroperoxides, and the conjugated structure absorbs strongly at 232-234 nm. For assessing primary oxidation, the CD method is faster and simpler than the PV method and requires very little sample. It is notice here that the PV and CD curves were similar, moreover, the differences of CD values of each sample were less than those of the PV value from same samples.

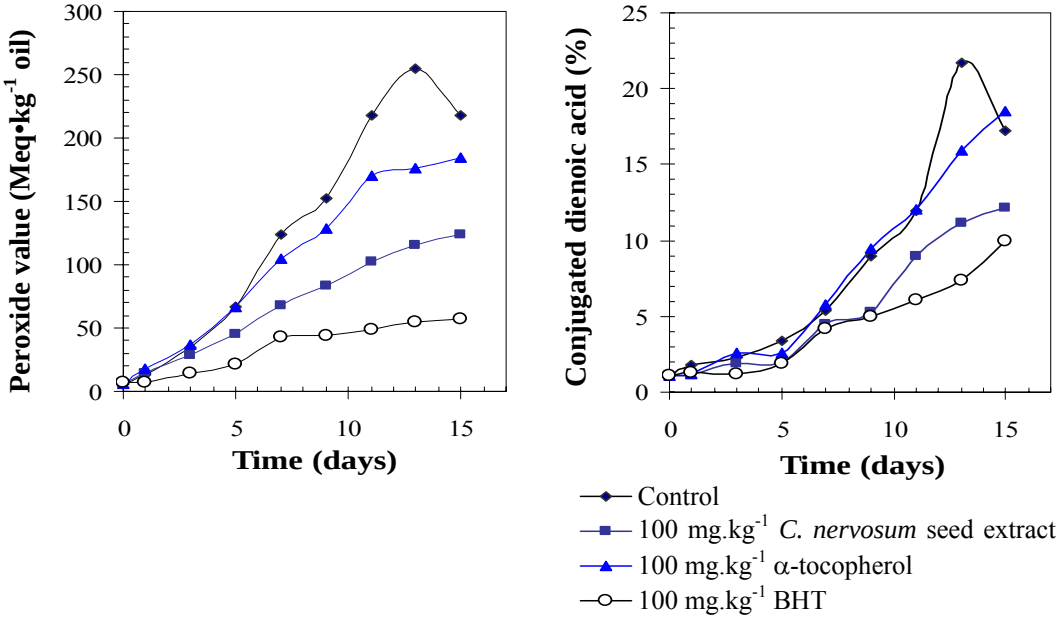

Figure 1 Relative increase in peroxide value (PV) and conjugated diene (CD) of stripped soybean oil samples during storage at 60°C. Data points represent mean (n=3) ± standard deviation.

The TBARS and *p*-AV determinations (Figure 2) confirmed that α-tocopherol was weaker antioxidant in oil than *C. nervosum* seed extract. The highest contents were observed for control, indicating greater intensity of oxidation. The determination of TBARS and *p*-AV is a good measure of the oxidative state of oils and thus a good indicator of effectiveness of antioxidants. TBARS and *p*-AV measures the formation of secondary oxidation products such as aldehydes or carbonyls, which may contribute to off-flavor of oxidized oil.

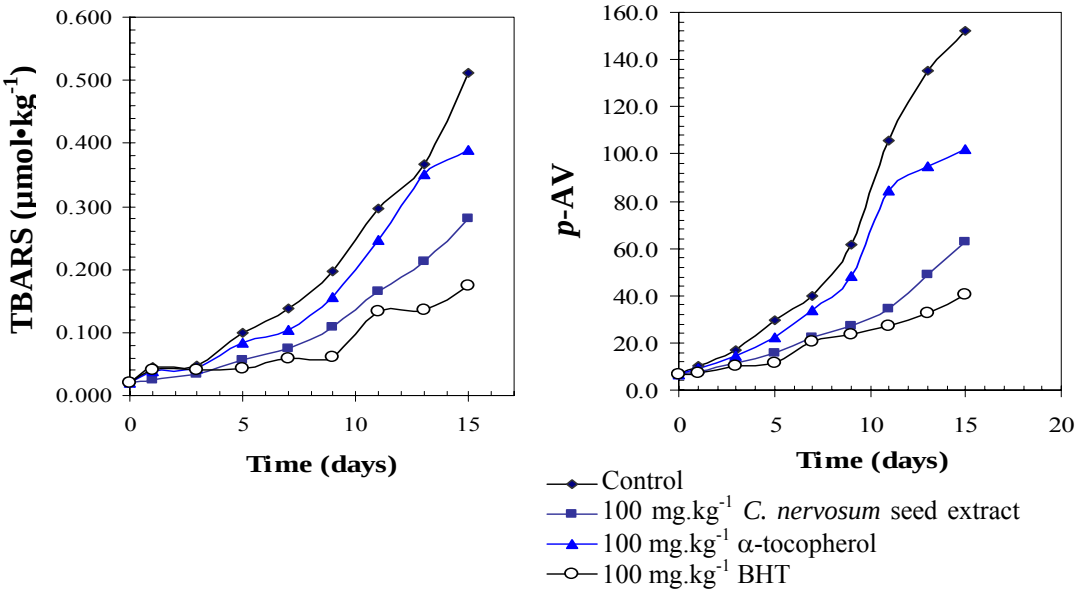

Figure 2 Relative increase in Thiobarbituric acid reactive substances (TBARS) and *p*-anisidine value (*p*-AV) of stripped soybean oil samples during storage at 60°C. Data points represent mean (n=3) ± standard deviation.

The ratio of PV: TBARS and PV: *p*-AV for the different samples were shown in Figure 3. The ratio shape during storage showed hyperbola curve. These could be explained that hydroperoxide degradation was occured after high content of hydroperoxide formation, therefore at the first period of oxidation the ratio increased gradually and began to decrease at the middle stage of studied storage time. α-Tocopherol curve showed similarity to *C. nervosum* seed extract which both ratio values were higher than for the samples with BHT and control throughout the storage period. Satue *et al*. (1995) reported that antioxidants showed different activities toward hydroperoxide decomposition. For the antioxidants used in this study, the differences in the relative values for PV, CD and TBARS including *p*-AV values might relate to the metal chelating ability of the antioxidant. Metals are known to catalyse hydroperoxide decomposition, which leads to the formation of the aldehydes and related compounds that are determined in the TBARS and *p*-AV assay. α-Tocopherol and some plant phenolics are known as a metal chelator (Hras *et al*., 2001), and the *C. nervosum* seed extract contained some phenolic compound (Treephrom and Deming, 2004). Consequently, it is consistent with the known metal chelating activity of α-Tocopherol and *C. nervosum* seed extract that the PV: TBARS and PV: *p*-AV ratio for oil samples containing α-Tocopherol should be higher than for BHT, which do not have the o-diphenol structure necessary for molecules to chelate metal ions.

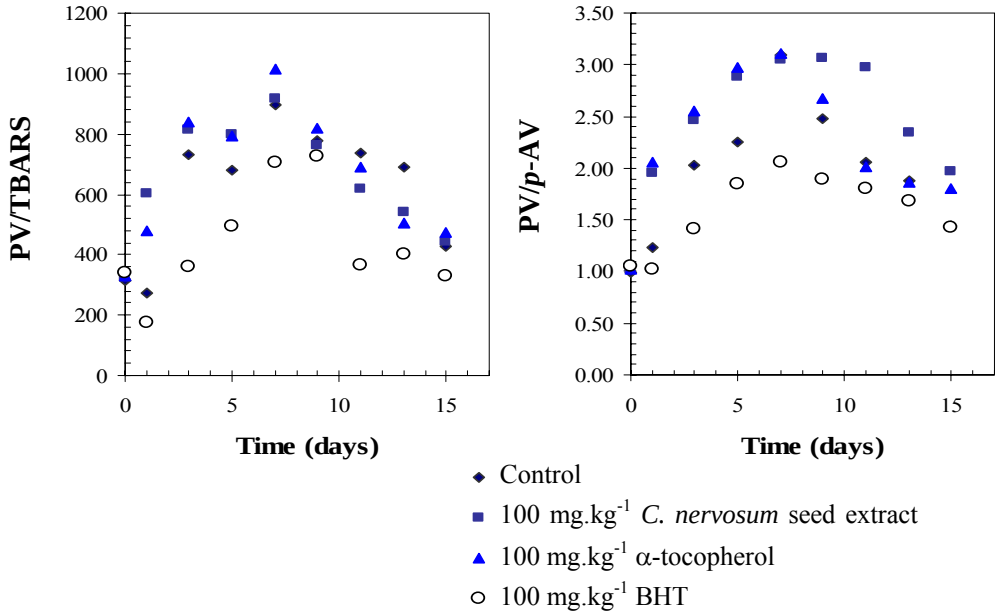

Figure 3 The ratio of peroxide and TBARS values (PV/TBARS) and peroxide and *p*-anisidine value (PV/*p*-AV) of stripped soybean oil containing antioxidants during storage at 60°C. Data points represent mean (n=3) ± standard deviation.

The changes in the ratio of the CD: TBARS and CD: *p*-AV values with time were shown in Figure 4. The ratios were not showing any specific curve. Moreover, the ratio values of studied antioxidant were scattering. It is notice here that CD value is not appropriate for antioxidative mechanism evaluation. Although measurement of lipid oxidation using conjugated dienes absorption at 234 nm has been extensively used during the last decades, but the CD values measured during oxidation of fats and oils have been found to correlate moderately with PV (Shahidi *et al*., 1994; Birch *et al*., 2001). In food lipid systems, measuring CD value is not appropriate due to matrix interferences (Baron *et al*., 1997) and increasing pH affects to the reduce formation of peroxides and higher content of conjugated dienes (Lesniak *et al*., 2005). Typically, PV is a direct measurement of peroxides while it appears likely that oxidation products of other origins with conjugated diene structures, e.g. fatty acid hydroxyl compounds, contribute to the CD value. Moreover, homolytic cleavage of only hydroperoxide group is generally regarded as the most important pathway for decomposition to produce volatile secondary compounds which response for rancidity and is measured by TBARS and *p*-AV (Kulas *et al*., 2001).

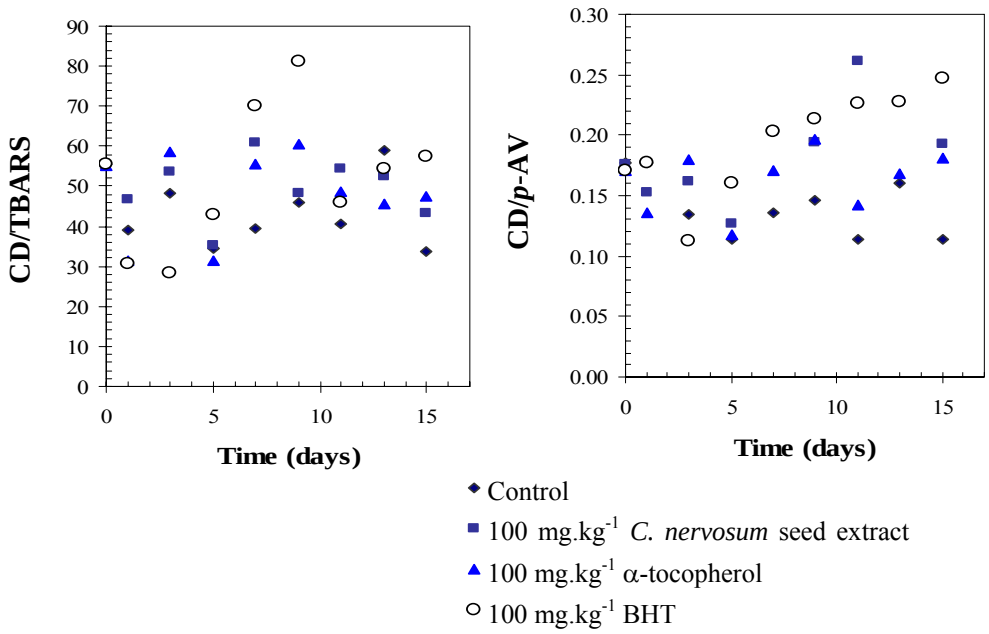

Figure 4 The ratio of CD and TBARS values (CD/TBARS) and CD and *p*-anisidine value (CD/*p*-AV) of stripped soybean oil containing antioxidants during storage at 60°C. Data points represent mean (n=3) ± standard deviation.

Conclusion

C. nervosum seed extract can stabilize soybean oil upto a grater extent than commonly natural antioxidant, α-tocopherol. It inhibits double bond conjugation and reducing the secondary oxidation products.

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