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Protection of polyunsaturated fatty acids of fish oil from common Kilka (*Clupeonella cultriventris caspia*) using holy basil (*Ocimum sanctum*) essential oil

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Abstract

The objective of this study was to investigate the effects of different concentrations of holy basil (*Ocimum sanctum*) essential oil (HBEO) on the oxidative stability and fatty acid profile of common Kilka (*Clupeonella cultriventris caspia*) oil (CKO) at different temperatures. HBEO were extracted using microwave-assisted hydrodistillation method and its main components were identified as methyl chavicol (17.97%), β-bisabolene (15.89%), eugenol (13.82%), and 1, 8-cineole (12.10%). When used at concentrations of 500 and 750 ppm, the HBEO significantly reduced the peroxide value and anisidine value of CKO during storage at 45 and 60 °C. By comparison, 100 ppm BHA and 250 ppm HBEO were less effective in reducing the peroxide value and anisidine value of CKO during storage at 45 and 60 °C. Furthermore, HBEO could preserve polyunsaturated fatty acids of CKO during storage at both temperatures. Based on the results of this research, HBEO can be applied to increase the oxidative stability of CKO, and can protect the polyunsaturated fatty acids in CKO.

Keywords: Essential oil, Holy basil, Kilka oil, Oxidative stability, Polyunsaturated fatty acid

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Introduction

Fish oil is considered as one of the most healthy and functional oils. It is highly rich in polyunsaturated ω-3 fatty acids such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). EPA and DHA are two compounds that benefit human health due to their ability to lower the risk of cardiovascular diseases (Arkhipenko and Sazontova, 1995).

Common Kilka has been identified as one of the most important industrial and commercial fish in the Caspian Sea. The amount of ω-3 and polyunsaturated fatty acids (PUFA) of common Kilka oil (CKO) are determined to be 13.40-16.81% and 19.43-21.77%, respectively (Fazel *et al*., 2008; Pirestani *et al*., 2010; Motalebi Moghanjoghi *et al*., 2015; Pazhouhanmehr *et al*., 2015). CKO is known to have the highest level of ω-3/ω-6 ratio and polyene index (EPA+DHA/C16:0 ratio) among the oils from the most important fish species in the southern regions of the Caspian Sea. Since polyene index is a good indicator of lipid oxidation potential in fish oils, CKO is expected to be highly susceptible to oxidation. Oxidations of PUFAs such as EPA and DHA result in a number of oxidation products that have negative impacts on the flavor and odor of fish oil, and also can affect the amount of these fatty acids that are made available to the body. In order to preserve PUFAs of fish oil from oxidative degradation, it is considered to be more optimal to use novel and effective antioxidant systems (Fhaner *et al*., 2015).

Natural or synthetic antioxidants such as α-tocopherol, tert-butylhydroquinone (TBHQ), butylated hydroxyanisole (BHA), and butylated hydroxytoluene (BHT) can be effective in stabilizing fish oil against autoxidation (Kulås and Ackman, 2001). However, the application of synthetic antioxidants to fish oils has been restricted by law enforcements because of concerns over their toxic and carcinogenic effects. Therefore, there is a growing interest in using natural and safer antioxidants for the purpose of preserving foods and for furthering pharmaceutical applications (Pazhouhanmehr *et al*., 2014).

Holy basil (*Ocimum sanctum*) is one of the species of the genus *Ocimum* which is native to tropical and subtropical regions. The essential oil (EO) is predominantly composed of methyl chavicol, eugenol, and methyl eugenol (Suanarunsawat *et al*., 2009). Hakkim *et al*. (2007) reported that holy basil exhibits strong radical scavenging activity (RSA), a strong reducing power, and good chelating activity. Salles Trevisan *et al.* (2006) showed that EOs from different species of the genus *Ocimum* exhibited strong antioxidant activities. Furthermore, reports claim that eugenol is more effective in inhibiting lipid peroxidation of α-linoleic acid emulsion than the action of BHA, BHT, α-tocopherol, and Trolox at the same concentration (Gülçin, 2011).

The objective of this study was to investigate the effect of different concentrations of holy basil essential oil (HBEO) when applied to CKO. The formation of primary and secondary

oxidation products are also studied herein, along with the different levels of PUFA protection during storage at different temperatures.

Materials and methods

Chemical materials

The practice of this study was based on chemicals such as acetic acid, chloroform, sodium iodide, sodium thiosulphate, iso-octane, methanol, *n*-hexane, and *p*-anisidine. These chemicals were purchased from Merck (Darmstadt, Germany). Other chemicals such as 2, 2-diphenyl-1-picrylhydrazyl free radical (DPPH°) and BHA were purchased from Sigma-Aldrich (St. Louis, MO).

Holy basil

During the month of September, fresh plants of holy basil were harvested from cultivated fields. The best branches were selected, and damaged leaves were removed. The branches were dried on large trays under ambient conditions at 30 °C for 3 days. The genus and species were verified by a plant taxonomy expert. The moisture content of holy basil was measured when the plants were dried in a laboratory oven at 105 °C until constant weight was reached. Prior to EO extraction, the samples were stored in a cold environment and were protected from light.

EO extraction

HBEO was extracted by the method of microwave-assisted hydrodistillation for 20 min at 300 W using a domestic microwave oven (ME3410W, Samsung, Malaysia, 2.45 GHz). Thirty grams of holy basil leaves were added to 450 mL distilled water (solid: liquid ratio of 1: 15). The extracted HBEO was collected and dried with anhydrous sodium sulphate and was stored in amber vials at 4 °C until further analysis (Golmakani and Moayyedi, 2016).

EO analysis

The chemical composition of HBEO was determined by a gas chromatography system (Agilent technologies 7890A, USA) coupled to a mass spectrometer (Agilent Technologies 5975C, USA), operating at 70 eV ionization energy, 0.5 s $scan^{-1}$, and at the mass range of 35–400 amu. The column was BPX5 (phenyl methyl siloxane, 30 m×0.25 mm internal diameter with 0.25 μm film thickness). One μl of the HBEO sample was injected into the gas chromatography system in split mode (split ratio of 1:50). Helium was used as the carrier gas at a flow rate of 1 ml min^{-1}. Oven temperature was programmed to reach 210 °C at the rate of 3 °C min^{-1} starting from 60 °C. Then, the temperature was increased to 240 °C at the rate of 20 °C min^{-1}, and was kept constant at 240 °C for 8.5 min. Injector and detector temperatures were 280 and 290 °C, respectively. The MSD ChemStation (G1701EA, E.02.01.1177, USA) software was used in order to analyze mass spectra and chromatograms. Relative percentage data were obtained from the electronic integration of peak areas. Retention indices were calculated using retention times of *n*-alkanes (C5-C25) injected under the same

chromatographic conditions. The compounds were identified by comparing their patterns of mass spectral fragmentation with those stored in the data bank (Wiley/NBS) and with mass spectral data derived from the relevant literature (Adams, 2007; Khair-ul-Bariyah, 2013; Keramat *et al*., 2016a).

Physical properties of EO

Specific gravity of HBEO was measured at 25 °C, and the refractive index was measured at 20 °C according to the Official Methods of Food Chemical Codex (FCC, 2003).

RSA

The RSA of HBEO sample was quantified using the DPPH° measure as described by Golmakani and Moayyedi (2016). The IC_{50} value is defined as the concentration of an antioxidant which is required to inhibit 50% of the DPPH° activity. Here, the IC_{50} value was determined by graph plotting, and by considering the percentage of the remaining DPPH° against different concentrations of HBEO.

Determination of oxidative stability of CKO during accelerated storage

CKO preparation

Virgin CKO containing no additive was purchased from a certified company (Rasht, Iran). Different concentrations of HBEO (250, 500, and 750 ppm) and BHA (200 ppm) were added to CKO which were then stored at 45 °C (for a duration of 14 days) and at 60 °C (for a duration of 5 days) in a dark environment. Samples stored at 45 °C were evaluated every alternate day, while samples stored at 60 °C were evaluated daily.

Chemical analysis of CKO samples

The PV was determined according to the Official Method of American Oil Chemists' Society (AOCS) (Cd 8-53) and was expressed as meq O_2 per kg oil. The AV was also determined using the Official Method (Cd 8-53) and was expressed as mg per kg oil (AOCS, 1998). The Totox value (TV) indicates total oxidation value (Frankel, 2012). The TV was determined according to eq. (1):

$$TV = 2\,(PV) + AV \quad \text{eq. (1)}$$

Kinetic measurement

The protection factor (PF) was calculated according to eq. (2).

$$PF = IP_a/IP_c \quad \text{eq. (2)}$$

where IP_a is the induction period (IP) of the CKO samples containing antioxidants (BHA and different concentrations of HBEO), the IP_c is the IP of the control sample (Hraš *et al*., 2000). IP is commonly considered as the number of days required for a sample to reach a PV of 20 meq O_2 kg^{-1} (Hashemi *et al*., 2011).

Antioxidant activity (AA) is a function of an antioxidant concentration. AA was calculated according to eq. (3).

$$AA = (IP_a\text{-}IP_c)/[AH] * IP_c \quad \text{eq. (3)}$$

where [AH] is the concentration of the antioxidant in proper units (Antolovich *et al*., 2002).

Antioxidant power (AOP) was calculated according to eq. (4) (Silva *et al*., 2001).

$$AOP = 100 - ((IP_c/IP_s) * 100) \quad \text{eq. (4)}$$

Fatty acid analysis

Fatty acid analysis was carried out according to the method described by Golmakani *et al.* (2012) with some modifications. Gas chromatography was performed using a Beifen system (3420A, China) with a split/splitless injector, a flame ionization detector, and a BPX70 capillary column (Bis–cyanopropylsiloxane-silphenylene, 30 m×0.25 mm internal diameter with 0.25 µm film thickness). The column temperature program started at 140 °C and was maintained at that temperature for 5 min. It was then increased to 180 °C at a rate of 20 °C min^{-1}. It was maintained at 180 °C for 9 min and then was increased to 200 °C at a rate of 20 °C min^{-1}. It was finally held at 200 °C for 3 min. The temperatures of the injector and detector were 250 and 300 °C, respectively. Nitrogen was used as the carrier gas. The samples were injected into the column with the volume of 1 µl (split ratio of 1:10). The CKO fatty acids were identified according to their retention times in comparison with the standard. Quantitative determination of fatty acids was performed by calculating their relative peak areas.

Statistical analysis

All experiments were performed in triplicates. A general linear model (GLM) procedure was applied using the Statistical Analysis Software (SAS, version 9.1; SAS Institute Inc. Cary, NC) in order to compare the mean values of measurements with each other.

Results

Chemical composition of HBEO

HBEO extraction yield was 0.50±0.01% v/w. Sesquiterpene hydrocarbons and benzene derivatives were found to be the major class of substances in the HBEO, thereby making up 38.12% and 35.26%, respectively. The oxygenated monoterpenes made up 18.00% of the HBEO, while oxygenated sesquiterpenes and monoterpene hydrocarbons comprised 3.96% and 3.33%, respectively. The main components of HBEO were methyl chavicol (17.97%), β-bisabolene (15.89%), eugenol (13.82%), and 1,8-cineole (12.10%).

Antioxidant activity and physical properties of HBEO

The HBEO reduced the concentration of DPPHº substantially, allowing IC_{50} value to reach 29.17±0.62 µg ml^{-1}. The specific gravity and refractive index of HBEO were 0.860±0.001 and 1.530±0.005, respectively.

Effect of HBEO on CKO oxidation

PV determination

Changes in PVs of CKO samples during storage at 45 and 60 °C are illustrated in Fig. 1A and Fig. 1D, respectively. HBEO and BHA significantly inhibited the formation of primary oxidation products of CKO, in comparison with the control, during storage at both temperaturas of 45 and 60 °C. By the end of the storage period at 45 °C, the corresponding percentage of inhibition by the BHA was 63.83%, while the percentages of inhibition by

the HBEO at 250, 500, and 750 ppm were 61.20%, 79.69%, and 85.32%, respectively, compared with the control. In the case of samples stored at 60 °C, the corresponding percentage of inhibition performed by BHA was 36.02%, while the percentages of inhibition by the EO at 250, 500, and 750 ppm were 33.96%, 53.49%, and 60.07%, respectively, compared with the control.

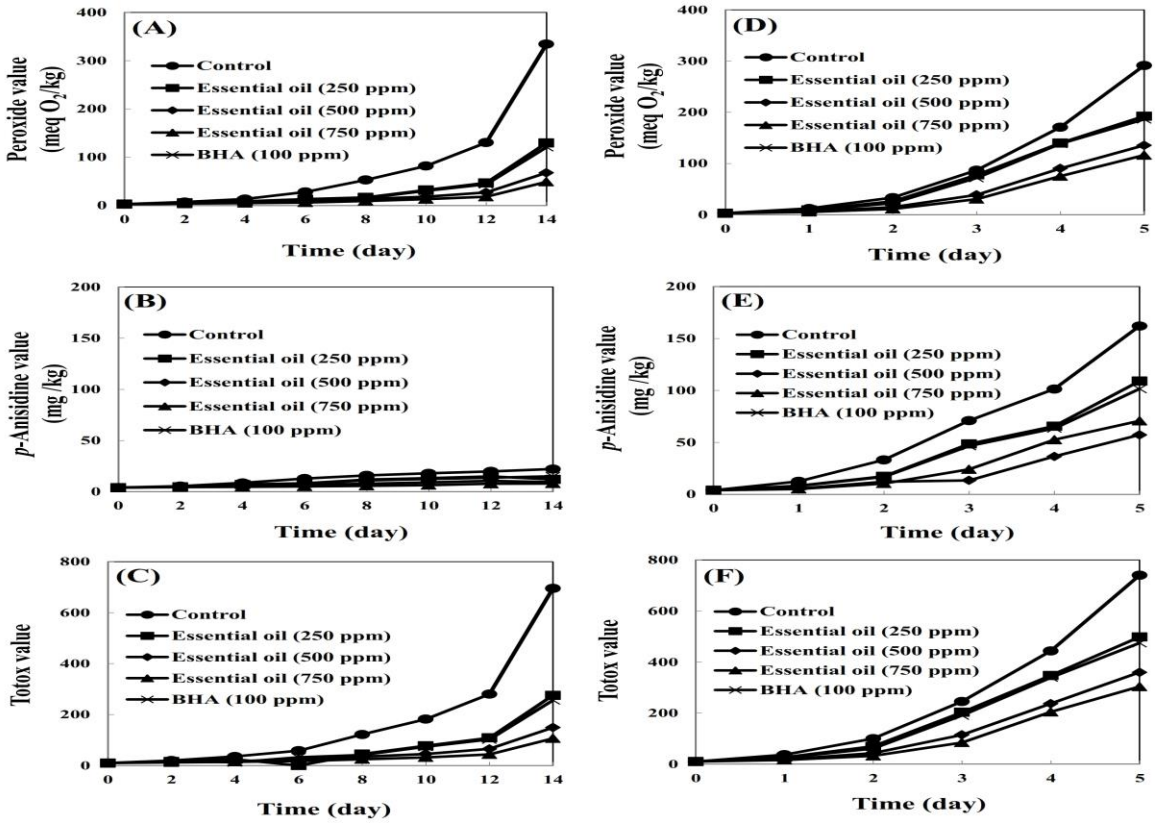

Figure 1: Changes in peroxide, *p*-anisidine, and Totox values of common Kilka oil containing different concentrations of holy basil essential oil at 45 (A-C) and 60 °C (D-F).

AV determination

The AVs of CKO samples during storage at 45 and 60 °C are shown in Fig. 1B and Fig. 1E, respectively. There was a smaller increase in AVs of CKO samples stored at 45 °C than the increase of those stored at 60 °C. By the end of storage at 45 °C, the AVs of samples of the control group, samples of the BHA treatment, and the samples containing 250, 500, and 750 ppm HBEO increased to 25.50, 15.62, 15.95, 12.65, and 8.51 mg kg^{-1}, respectively. However, by the end of storage at 60 °C, the AVs of samples of the control group, samples of the BHA treatment, and the samples containing 250, 500, and 750 ppm HBEO increased to 162.00, 101.50, 109.00, 57.25, and 7.70 mg kg^{-1}, respectively. Similar to the PV results, the effect of HBEO on CKO oxidation was concentration-dependent at both temperatures. The intensity of this effect increased parallel to higher concentrations of HBEO. The AV of samples treated with 250 ppm HBEO

was similar to the value of samples treated with BHA.

TV determination

TVs of CKO samples during storage at 45 and 60 °C are shown in Fig. 1C and Fig. 1F, respectively. CKO containing BHA and different concentrations of HBEO (250, 500, and 750 ppm) exhibited lower TVs in comparison with that of the control during storage at both temperatures of 45 and 60 °C. The effect of 250 ppm HBEO was similar to that of BHA on inhibiting the oxidation of CKO, but higher concentrations of HBEO (i.e. 500 and 750 ppm) protected the oil against oxidation more efficiently in comparison with the protective action of BHA.

Changes in TVs of CKO samples stored at 45 °C showed a pattern similar to that of 60 °C. However, the time required for reaching a specific TV was shorter at a higher temperature. The control sample stored at 60 °C reached a TV of 740.50 after 5 days of storage, while the control sample stored at 45 °C reached a TV of 695.50 after 14 days of storage.

Kinetic measurement

IP, PF, AA and AOP of CKO samples are presented in Table 2. Samples containing 750 ppm HBEO showed the highest stability by having IPs of 6.07 and 0.88 days during storage at 45 and 60 °C, respectively. IPs of samples containing BHA were similar to those of samples containing 250 ppm HBEO at both temperatures.

Table 1: kinetic measurements of common Kilka oil containing different concentrations of holy basil essential oil (HBEO) stored at different temperatures.

Parameter	Control	HBEO (250 ppm)	HBEO (500 ppm)	HBEO (750 ppm)	BHA (100 ppm)
45 °C					
Induction period (day)	0.84^{d*}	2.21^{c}	4.31^{b}	6.07^{a}	2.37^{c}
Protection factor	-	2.63^{c}	5.13^{b}	7.23^{a}	2.82^{c}
Antioxidant power	-	61.97^{c}	80.52^{b}	86.17^{a}	64.40^{c}
Antioxidant activity	-	6517.74^{c}	8264.31^{b}	8307.54^{b}	9124.29^{a}
60 °C					
Induction period (day)	0.35^{d}	0.53^{c}	0.75^{b}	0.88^{a}	0.55^{c}
Protection factor	-	1.51^{c}	2.16^{b}	2.52^{a}	1.56^{c}
Antioxidant power	-	33.69^{c}	53.61^{b}	60.33^{a}	35.79^{c}
Antioxidant activity	-	2032.50^{c}	2311.69^{b}	2027.81^{c}	2787.25^{a}

*In each row, means with different letters are significantly different (p<0.05).

HBEO significantly increased the PF of CKO during storage at both temperatures. In CKO samples stored at 45 °C, PFs of 500 and 750 ppm HBEO were 2.56 and 1.82 times higher than that of BHA, respectively. In the case of CKO samples stored at 60 °C, PFs of 500 and 750 ppm HBEO were 1.38 and 1.61 times higher than that of BHA, respectively. At both temperatures of 45 and 60 °C, PFs of 250 ppm HBEO were similar to those of BHA.

AAs of different concentrations of HBEO were lower than that of BHA during storage at both temperatures of 45 and 60 °C. This may be due to the fact that AA is a function of antioxidant concentration. The AA of HBEO increased by increasing its concentration at both temperatures of 45 and 60 °C, but the efficiency of AA was higher by the HBEO at 45 °C than at 60 °C.

Similar increasing trends of PF values were observed by increasing the concentration of HBEO in AOP. HBEO at the concentration of 750 ppm showed the highest AOP values at both temperatures of 45 and 60 °C. It was also observed that the different concentrations of HBEO had higher AOP values and better efficiency at 45°C than those at 60 °C.

Fatty acid analysis

Changes in the fatty acid profile of CKO samples are presented in Table 3. Fatty acid composition of the fresh CKO was mainly comprised of monounsaturated fatty acids (MUFA), followed by PUFA, and saturated fatty acids (SFA). Moreover, CKO has a high polyene index (EPA+DHA/C16:0 ratio). Thus, CKO is intrinsically expected to exhibit a high level of susceptibility to oxidation. The PUFA content of all CKO samples markedly decreased at the end of the storage period. A very strong and significant correlation was found between the PUFA reduction percentage and the TV at the end of the storage period (R^2 of 0.929 and 0.965 at 45 and 60 °C, respectively).

Table 2: fatty acid profile (%) of common Kilka oil containing different concentrations of holy basil essential oil (HBEO) stored at different temperatures.

Fatty acid	Virgin oil	45 °C (14 days)					60 °C (5 days)				
		Control	HBEO (250 ppm)	HBEO (500 ppm)	HBEO (750 ppm)	BHA (100 ppm)	Control	HBEO (250 ppm)	HBEO (500 ppm)	HBEO (750 ppm)	BHA (100 ppm)
Myristic acid	4.69	6.06	5.26	5.18	5.04	5.57	6.18	5.15	5.00	5.47	5.44
Mtristoleic acid	0.36	0.29	0.23	0.29	0.27	0.35	0.08	0.23	0.26	0.28	0.17
Pentadecylic acid	0.44	0.77	0.85	0.71	0.62	0.72	0.45	0.19	0.32	0.29	0.20
Palmitic acid	11.14	15.88	13.68	13.68	13.39	14.47	18.70	16.66	15.14	14.29	16.14
Palmitoleic acid	3.06	2.20	2.30	2.20	2.45	2.23	1.40	2.24	2.33	2.32	2.27
Stearic acid	2.38	3.19	2.68	2.59	2.55	2.60	1.06	2.52	2.13	2.25	2.11
Oleic acid	37.19	40.39	39.53	38.40	38.56	39.88	44.78	42.63	41.60	41.14	43.13
Vaccenic acid	1.50	0.67	0.94	1.07	1.03	0.83	0.58	0.87	0.98	1.06	0.96
Linoleic acid	2.04	1.77	1.82	1.95	1.88	1.79	1.28	1.40	1.54	1.69	1.54
Linoleic acid	2.03	1.69	1.86	1.94	2.00	1.75	1.48	1.56	1.88	1.88	1.58
Arachidonic acid	0.59	0.44	0.55	0.49	0.54	0.47	0.32	0.31	0.46	0.47	0.36
Eicosapentaenoic acid (EPA)	8.88	6.41	7.71	7.91	7.97	7.14	5.81	7.04	7.70	7.93	6.56
Docosatetraenoic acid	0.39	0.27	0.35	0.35	0.34	0.32	0.29	0.33	0.31	0.33	0.32
Docosapentaenoic acid	0.52	0.32	0.41	0.43	0.42	0.39	0.39	0.42	0.45	0.50	0.40
Docosahexaenoic acid (DHA)	24.79	19.67	21.86	22.80	22.95	21.50	17.21	18.49	19.91	20.12	18.83
(EPA+DHA)/C16:0	3.02	1.64	2.16	2.24	2.31	1.98	1.23	1.53	1.82	1.96	1.57
SFA[1]	18.64	25.90	22.46	22.17	21.60	23.36	26.39	24.51	22.60	22.30	23.89
UFA[2]	81.36	74.10	77.54	77.83	78.40	76.64	73.61	75.49	77.40	77.70	76.11
MUFA[3]	42.11	43.54	43.00	41.96	42.31	43.29	46.84	45.96	45.16	44.79	46.53
PUFA[4]	39.25	30.56	34.53	35.87	36.10	33.35	26.78	29.53	32.24	32.91	29.59

[1] Saturated fatty acid
[2] Unsaturated fatty acid
[3] Monounsaturated fatty acid
[4] Polyunsaturated fatty acid

HBEO significantly inhibited the loss of PUFA during storage at both temperatures. At the end of the storage period at 45 °C, the BHA and different concentrations of HBEO exhibited various percentages of inhibition which were in the range of 9.44% - 18.54% in comparison with the control. In samples stored at 60 °C, BHA and different concentrations of HBEO inhibited the loss of PUFAs by amounts in the range of 10.27% - 22.89%. The HBEO at a concentration of 750 ppm protected the PUFA most strongly against oxidation during storage at both temperatures.

Discussion

Chemical composition of HBEO

The main components of HBEO were phenylpropenes (such as methyl chavicol and eugenol), sesquiterpene hydrocarbons (such as β-bisabolene), and oxygenated monoterpenes (such as 1,8-cineole). Methyl chavicol was the predominant component in the present study, which was also considered as one of the main components of HBEO by Amber *et al*. (2010). Suanarunsawat *et al*. (2009) reported that HBEO is predominantly composed of eugenol and methyl eugenol. According to Ruberto and Baratta (2000), the intensity of antioxidant activities by different EO compounds of the genus *Ocimum* can be rated according to the following: eugenol > methyl chavicol > 1,8-cineole. Therefore, the antioxidant activity of *O. sanctum* EO may be attributed to its eugenol content.

Antioxidant activity and physical properties of HBEO

DPPH° is commonly used in preliminary stages of screening for compounds capable of scavenging activated oxygen species. It is much more stable and easier to handle than oxygen free radicals (Tominaga *et al*., 2005). The RSA of HBEO in the present study (IC_{50} value of 29.17±0.62 µg ml^{-1}) was higher than those extracted by hydrodistillation method (IC_{50} value of 40.31±3.5 µg ml^{-1}) (Chaudhary *et al*., 2014). Therefore, the microwave-assisted hydrodistillation method can increase the RSA of the extracted HBEO. Also, Chaudhary *et al*. (2014) reported that EO, methanolic extract, and the ethyl acetate fraction of holy basil showed IC_{50} values of 40.31± 3.5, 105.62±4.6, and 145.31±5.8 µg ml^{-1}, respectively. These results are indicative of the fact that HBEO exhibits higher RSA compared to its methanolic extract and ethyl acetate fraction. Bunrathep *et al*. (2007) showed that the IC_{50} value of *O. basilicum* EO containing high amounts of methyl chavicol (92.48%) and zero amounts of eugenol was 470.57 mg ml^{-1}, whereas the IC_{50} value of *O. gratissimum* EO containing high amounts of eugenol (25.02%) was 0.03 mg ml^{-1}. Also, Dawidowicz and Olszowy (2014) showed that eugenol exhibited higher RSA than methyl chavicol. These results indicate that the RSA of HBEO is mainly due to the presence of eugenol.

EO quality depends on a number of physical parameters such as specific gravity and refractive index (Tripathi *et*

al., 2008). The measured values of specific gravity and refractive index of HBEO in the present study were similar to the values of HBEO extracted by hydrodistillation method (Tripathi *et al*., 2008; Khair-ul-Bariyah, 2013). Therefore, no negative impacts were observed on the physicochemical properties of HBEO because of the microwave-assisted hydrodistillation method.

Effect of HBEO on CKO oxidation

PV determination

Determination of the changes in the hydroperoxide content is known as PV, which is applied over time and can be used as a suitable chemical method to report the level of oxidation and antioxidant activity (Frankel, 2012). By adding the HBEO and BHA to the samples, the PV of samples decreased significantly in comparison with the control during storage at temperatures of both 45 and 60 °C. The concentration of 250 ppm HBEO inhibited the oxidation of CKO in a manner that was similar to the action of BHA on inhibiting the oxidation, but higher concentrations of HBEO (i.e. 500 and 750 ppm) inhibited oxidation more powerfully in comparison with the action of BHA. Also, Pazhouhanmehr *et al*. (2014) reported that bene kernel oil reduced the PV of CKO in a manner similar to the action of BHT.

Also, the HBEO had a more prolonged inhibitory effect on the formation of primary oxidation products at lower temperatures. Also, Guiotto *et al*. (2014) reported that by increasing the temperature, the time needed for reaching a specific PV is reduced when applying the sunflower–chia oil blends (90:10 and 80:20) stored at 4, 20, and 45 °C.

AV determination

The anisidine test is designed and set up to measure heavy molecular weights of saturated and unsaturated carbonyl compounds (Frankel, 2012). CKO samples containing HBEO at 750 ppm concentration exhibited the highest oxidative stability during storage at both temperatures. Similarly, Hashemi *et al*. (2011) reported that samples containing 0.075% *Zataria multiflora* EO were more stable than samples containing BHA and BHT (both at 200 ppm) during storage at 37 and 47 °C.

Samples stored at 60 °C exhibited higher AVs than those stored at 45 °C because their primary oxidation products have less stability at higher temperature. Those primary oxidation products further degrade to form secondary oxidation products, such as aldehydes and ketones (Malheiro *et al*., 2013).

TV determination

The TV is an indicator of primary and secondary oxidation products. HBEO significantly inhibited the formation of primary and secondary oxidation products in comparison with the control sample during storage at both temperatures of 45 and 60 °C. The inhibitory effect of HBEO on CKO oxidation was concentration-dependent at both temperatures and it increased by increasing the HBEO concentration. Also, Tomaino *et al*. (2005) reported

that the antioxidant activity of *O. basilicum* was significantly higher than oregano EO in retarding lipid oxidation.

By increasing the temperature, the time needed for reaching a specific TV decreased, but the samples stored at 45 °C exhibited a declining trend regarding the formation of primary and secondary oxidation products that was similar to samples stored at 60 °C. Similarly, Hashemi *et al*. (2014) reported that the efficiency of *Carum copticum* EO does not show significant differences in retarding the oxidation of sunflower oil at 37 °C and 47 °C, but the oxidation rates of sunflower oil samples stored at 47 °C were higher than samples stored at 37 °C. In contrast, Erkan *et al*. (2012) concluded that *Nigella sativa* EO can become more efficient in inhibiting lipid oxidation when the temperature increases from 40 to 80 °C. Yanishlieva and Marinova (2001) found that the antioxidant activity of some antioxidants like fraxetin can be increased by increasing the temperature. This phenomenon can be explained by the fact that the degraded products of antioxidants also have antioxidative effects.

Kinetic measurement

By adding HBEO and BHA, the result can be a dramatic increase in the IP of CKO. Accordingly, BHA as a synthetic antioxidant can be replaced by its natural counterpart, HBEO. Also, Keramat *et al*. (2016a) reported that the IP of virgin olive oil samples containing *Bunium persicum* and *Rosmarinus officinalis* EOs was similar to that of BHT. Furthermore, Rupasinghe et al. (2010) showed that incorporating apple skin extract into fish oil made its IP become (400 μg ml^{-1}) similar to that of BHT (200 μg ml^{-1}).

The effectiveness of different HBEO concentrations was compared to that of BHA based on their PF. The following ranges can be claimed for the PF values: 1.0-1.5 (very low), 1.5-2.0 (low), 2.0-2.5 (medium), 2.5-3.0 (high), and >3.0 (very high) (Ahmad *et al*., 1983). In samples stored at 45 °C, the PF values of HBEO at 500 and 750 ppm were higher than 3, indicating a "very high" antioxidant activity in CKO. Also, 250 ppm HBEO exhibited "high" antioxidant activity which is a magnitude similar to the activity exhibited by BHA. Samples stored at 60 °C indicated a "medium-to-high" antioxidant activity in CKO. Furthermore, samples containing 250 ppm HBEO exhibited a "low" antioxidant activity which was similar to samples containing BHA. Accordingly, the PF of antioxidants decreased parallel to the increase in storage temperature from 45 to 60 °C. Similarly, Yagcı *et al*. (2012) reported that the effectiveness of corn and palm oils containing *Thymbra spicata* EO increased by lowering the storage temperature from 120 to 90 °C. Also, Farhoosh *et al*. (2016) showed that the oxidative stability of fish oil containing phenolic acids decreased when the temperature increased from 35 to 55 °C. Unlike IP and PF, the value of AA tends to change as a function of antioxidant concentration (Antolovich *et al*., 2002). Since HBEO retarded the

oxidation of CKO at higher concentrations, AAs of different concentrations of HBEO were significantly lower than that of BHA. Similarly, Keramat *et al*. (2016b) reported that AAs of *Z. multiflora* and *R. officinalis* EOs were significantly lower than those of BHT. Higher concentrations of natural antioxidants could be added, whereas synthetic antioxidants cannot be applied in high concentrations because of potential health risks. Therefore, natural antioxidants have better potential in increasing the oxidative stability of CKO.

AOP is expressed as a value between 0 and 100; the greater the AOP, the stronger the antioxidant capacity. HBEO at 500 and 750 ppm concentrations increased the AOP of CKO to a greater extent than BHT. Similarly, Hashemi *et al.* (2011) reported that sunflower oil samples containing 750 ppm *Z. multiflora* EO exhibited higher AOP than samples containing 200 ppm BHA and BHT during storage at both temperatures of 37 and 47 °C.

Fatty acid analysis

The PUFA amount of CKO in the present study was significantly higher than that of red toothed trigger fish (*Odonus niger*) (Immanuel and Palavesam, 2010). The PUFA content of all CKO samples markedly decreased in response to extended storage periods, indicating that the PUFAs underwent oxidative deterioration. Different concentrations of HBEO showed various strengths of inhibitory effects on PUFA oxidation. The intensity of this effect increased parallel to higher concentrations of HBEO. Also, the fatty acid profile of CKO containing 250 ppm EO was similar to that of CKO containing BHA at both temperatures. Similarly, Wang *et al*. (2011) reported that the PUFA content of the fish oil sample containing 0.3 mg g^{-1} carnosic acid was higher than that of the sample containing 0.2 mg g^{-1} TBHQ. Furthermore, Fhaner *et al*. (2015) showed that sesamol can significantly reduce the loss of EPA and DHA during storage at 30 and 50 °C.

The objective of this study was to evaluate the action of different concentrations of HBEO on the oxidative stability and fatty acid profile of CKO in comparison with the action of BHA on the oil. The evaluations were done during storage at different temperatures of 45 and 60°C. The results showed that HBEO significantly retarded the oxidation of CKO, increased the IP value and protected the PUFAs during storage at both temperatures of 45 and 60 °C. The effect of HBEO on CKO oxidation was concentration-dependent at both temperatures and increased along with the increase in the HBEO concentration. The effect of 250 ppm HBEO on the oxidative stability of CKO was similar to the effect of BHA on the oil, but higher concentrations of HBEO (i.e. 500 and 750 ppm) resulted in higher oxidative stability. Furthermore, the oxidation rate of CKO increased when the storage temperature increased from 45 to 60 °C, but their trends of oxidation rate appeared to be

similar regarding the formation of primary and secondary oxidation products. It can be concluded that HBEO can be used instead of synthetic antioxidants. HBEO is an efficient natural antioxidant that can be applied to CKO for the purpose of increasing its oxidative stability and protecting its PUFA content.

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