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Antioxidant activity and phenolic content of phenolic rich fractions obtained from black cumin (*Nigella sativa*) seedcake

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ABSTRACT

The antioxidant activities of crude methanolic extract (CME) and its fractions using ethyl acetate (EAF), hexane (HF) and water (WF) of black cumin seedcake were investigated. DPPH radical scavenging activity, β-carotene–linoleate bleaching, and inhibition of corn oil oxidation were used to evaluate the antioxidant capacity. The total phenolics were found to be 78.8, 27.8, 32.1 and 12.1 mg gallic acid equivalents (GAE)/g in EAF, CME, WF and HF, respectively. The CME and EAF exhibited the highest DPPH followed by WF and HF. The extract/fractions showed high effect on reducing the oxidation of β-carotene. The effect of extract/fractions on the oxidative stability of corn oil at 70 °C was tested in the dark and compared with butylated hydroxyanisole (BHA). The oil peroxide and anisidine values were generally lower with addition of PRFs in comparison to a control. The predominant phenolic compounds identified by HPLC–DAD in CME and WF of black cumin seedcake were hydroxybenzoic, syringic and *p*-cumaric acids.

1. Introduction

Epidemiological and *in vitro* studies strongly suggest that foods containing phytochemicals such as phenolic compounds have potential protective effects against many diseases. Therefore, they may be used as antimutagenic, antibacterial, antiviral and anti-inflammatory agents (Senevirathne et al., 2006). There is increasing evidence that consumption of a variety of phenolic compounds present in natural foods may lower the risk of serious health disorders because of the antioxidant activity of these compounds (Hertog, Feskens, Hollman, Katan, & Kromhout, 1993; Surh, 2002; Surh et al., 1999). When added to foods, antioxidants minimize rancidity, retard the formation of toxic oxidation products, maintain nutritional quality and increase shelf life (Jadhav, Nimbalkar, Kulkarni, & Madhavi, 1996). The antioxidant activity of extracts of several plants including their leaves, bark and roots (Kubola & Siriamornpun, 2008; Mariod, Matthaus, & Hussein, 2008), fruits and seeds (Liyana-Pathirana, Shahidi, & Alasalvar, 2006; Malencic, Maksimovic, Popovic, & Miladinovic, 2008), tree nuts oils (Miraliakbari & Shahidi, 2008) and seedcake (Mariod, Matthaüs, Eichner, & Hussein, 2006; Matthaüs, 2002) has been extensively studied.

Seeds of black cumin (*Nigella sativa* L.) are used as a spice in cooking and in a wide traditional medicinal uses, the seed volatile oil and its main active constituent, thymoquinone, are extensively reported to exhibited protective effect against many diseases depending on its high antioxidant activity (El-Dakhakhny, Barakat, El-Halim, & Aly, 2000; Nagi & Mansour, 2000). Peroxide value (PV) is often used as an indicator for the initial stages of oxidation (Gray, 1978). The anisidine value is a more meaningful test for the assessment of the oils quality during heating than the peroxide value. Measurement of the content of conjugated dienes (at 234 nm) and trienes (at 268 nm) is employed to assess the oxidative stability of vegetable oils (St Angelo, Ory, & Brown, 1975).

High performance liquid chromatography (HPLC) with diode array detection (DAD) is an indispensable tool for the provisional identification of the main phenolic structures present in foods (Chirinos et al., 2009).

Several studies on black cumin seeds (Nagi & Mansour, 2000) and shoots and roots (Bourgou et al., 2008) have been reported recently but there are no relevant studies on antioxidant activity of black cumin seedcake. Therefore, the purpose of the present study was to investigate phenolic compounds of black cumin seedcake extract/fractions and to evaluate their antioxidant activity (AOA) by using different *in vitro* methods. The different extracts were dissolved in few amount of methanol and applied to corn oil at levels of 0.25% and 0.5% to examine their antioxidative activity; the development of the peroxide value (PV), anisidine value (AV) during oxidation of corn oil was evaluated at 70 °C for 72 h.

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2. Materials and methods

2.1. Materials

All solvents used were of analytical grade. Methanol, ethyl acetate, hexane, chloroform, butylated hydroxyanisole (BHA), β-carotene, linoleic acid and Folin–Ciocalteau reagent as well as polyoxyethylene sorbitan monopalmitate (Tween 40) were obtained from Merck (Merck, Darmstadt, Germany).

Black cumin (*N. sativa)* seeds, a product of Iran, were purchased from a local herbal medicine store in Kuala Lumpur, Malaysia. Black cumin seeds were cleaned under running tap water for 10 min, rinsed twice with distilled water and air-dried in an oven at 40 °C overnight. The seeds were ground to a powder using an electric grinder (National, Model MX-915, Kadoma, Osaka, Japan) for 10 min and then passed through a 35 mm (42 mesh) sieve. The oil was extracted from the ground seeds by extraction with *n*-hexane (b.p. 50–60 °C) using Soxhlet apparatus for 6 h. following the AOCS (1993) method Aa 4-38. Drying the residue under vacuum at 40 °C for 30 min led to a fine and homogenous fat-free powder which was weighed and was used to extract phenolic compounds.

2.1.1. Corn oil

The edible corn oil which was produced by Lam Soon edible oil Co. Ltd. Shah Alam, Selangor, Malaysia was obtained from local store Serdang Malaysia; the oil was free of any synthetic antioxidant.

2.2. Extraction of phenolic compounds

Twenty grams of the dried ground black cumin seedcake were extracted successively with 80% methanol (3 × 200 ml) by sonication (Hwasin Technology, Seoul, Korea) to obtain crude methanolic extract (CME) with solid to solvent ratio of 1:10 (w/v) at room temperature for 1 h; then combined and concentrated by removing methanol by rotary evaporator (Buchi, Flawil, Switzerland). The obtained CME (4.4 g) was fractionated by using hexane, ethyl acetate and water (3 × 100 ml) individually, where the residue from each fractionation step was used to obtain the subsequent fraction. Each extraction process involved homogenisation of CME and its fractions and solvent at 13,000 rpm for 15 min followed by sonication at constant temperature of 30 °C for 1 h. The CME and its fractions (hexane fraction HF, ethyl acetate fraction EAF, water fraction WF) were filtered through filter paper Whatman no. 1. Then solvents were removed by using rotary evaporator (Buchi, Flawil, Switzerland). The yield of each extract and its fractions was measured before kept in −80 °C for further analysis.

2.3. Determination of total phenolics in black cumin seedcake

The total phenolic (TPC) in the CME and its fractions was determined with the Folin–Ciocalteu reagent following the method of Kaur, Arora, and Singh (2008) using gallic acid as standard. Absorbance was measured at 760 nm using spectrophotometer (Shimazu, Co., Ltd., Kyoto, Japan). TPC of sample was expressed as gallic acid in terms of mg gallic acid equivalents (GAE)/100 g dried samples.

2.4. Antioxidant activities (AOA) measurement

2.4.1. 1,1-Diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity test

The antioxidant activity of phenolic extract/fractions from black cumin seedcake was measured following Gordon, Paiva-Martins, and Almeida (2001) using the stable radical 1,1-diphenyl-2-picrylhydrazyl (DPPH). A methanolic solution (100 μL) of the phenolic compounds extracted from the seedcakes was placed in a cuvette and 0.5 mL of a methanolic solution of DPPH (50 mg DPPH/100 mL MeOH) was added. After 30 min incubation in darkness and at ambient temperature (23 °C), the resultant absorbance was recorded at 515 nm. The decrease in absorbance at 515 nm was determined using a spectrophotometer (Shimadzu Co., Ltd., Kyoto, Japan). The absorbance of the DPPH radical without antioxidant, i.e. the control was measured. The data is commonly reported as IC_{50}, which is the concentration of antioxidant required for 50% scavenging of DPPH radicals in the specified time period. All determinations were performed in triplicate.

2.4.2. The β-carotene–linoleic acid assay

The antioxidant activity (AOA) of the different extract/fractions was evaluated using the β-carotene–linoleic acid assay following the method of Amarowicz, Karamac, and Shahidi (2003). In brief a solution of *β*-carotene was prepared by dissolving 2 mg of β-carotene in 10 ml of chloroform. Two millilitres of this solution were pipetted into a 100 ml round-bottom flask. After chloroform was removed under vacuum, using a rotary evaporator at 40 °C, 40 mg of purified linoleic acid, 400 mg of Tween 40 as an emulsifier and 100 ml of aerated distilled water were added to the flask with vigorous shaking. Aliquots (4.8 ml) of this emulsion were transferred into a series of tubes containing 200 μl of the extract (200 ppm in methanol). The total volume of the systems was adjusted to 5 ml with methanol. As soon as the emulsion was added to each tube, the zero time absorbance was measured at 470 nm with a Shimadzu spectrophotometer (Shimadzu Co., Ltd., Kyoto, Japan). Sub-sequent absorbance readings were recorded over a 2 h period at 20 min intervals by keeping the samples in a water bath at 50 °C. Blank samples, devoid of β-carotene, were prepared for background subtraction.

2.4.3. Stability of corn oil as affected by the addition of black cumin phenolic rich fractions (PRFs)

The collected different black cumin seedcake PRFs were applied to 100 g commercial edible corn oil obtained from local market (free of any antioxidant) at levels of 0.25% and 0.5% to examine their antioxidative activity. BHA at a level of 0.02% was used as a standard. A control sample was prepared by using the same amount of methanol used to dissolve the antioxidant and the extracts (Moure et al., 2000). The corn oil with added antioxidants were heated at 70 °C for 72 h. Samples (5 g) were removed periodically every 4, 8, 24, 32, 48 and 72 h for analysis. The absorbance at 234 and 270 nm, peroxide value (PV) and *p*-anisidine value (AV) were determined by the AOCS (1993) methods.

2.5. HPLC–DAD system for analysis of phenolic compounds

HPLC analysis was performed using Agilent G1310A pumps (Agilent, Stevens Creek Blvd Santa Clara, USA), with diode array detector and chromatographic separations were performed on a LUNA C-18 column (5 μm, 250 × 4.6 mm) (Phenomenex, Torrance, CA, USA). The composition of solvents and used gradient elution conditions were described previously by Chirinos et al. (2009) with some modifications. The mobile phase was composed of solvent (A) water–acetic acid (94:6, v/v, pH 2.27) and solvent (B) acetonitrile. The solvent gradient was as follows: 0–15% B in 40 min, 15–45% B in 40 min and 45–100% B in 10 min. A flow rate of 0.5 ml/min was used and 20 μl of sample were injected. Samples and mobile phases were filtered through a 0.22 μm Millipore filter, type GV (Millipore, Bedford, MA) prior to HPLC injection. Each fraction was analyzed in duplicate. Phenolic compounds were identified and quantified by comparing their retention time and UV–Vis spec-

tral data to known previously injected standards (Chirinos et al., 2009).

2.6. Statistical analyses

Statistical analyses were conducted using SPSS (Statistical Program for Social Sciences, SPSS Corporation, Chicago, IL) version 12.0 for Windows. Analysis of variance (ANOVA) and Pearson's correlation coefficients were performed to compare the data. All determinations were done at least in triplicate and all were averaged. The confidence limits used in this study were based on 95% ($p < 0.05$).

3. Results and discussion

3.1. Amount of extractable compounds vs extractable phenolic compounds

The results of using different solvents for the extraction/fractionation of phenolic compounds are given in Table 1. From this table it was evident that black cumin seedcake contained noticeable amounts of extractable compounds. It is clear that the different solvents used for the extraction and fractionation of black cumin seedcake, had different abilities to extract substances from this seedcake. In general, the amount of total extractable compounds decreased with decreasing polarity of the solvent in the order of water, ethyl acetate, methanol and hexane.

The extraction of extractable substances from black cumin seedcake with water was found most effective. With this solvent, the highest amount of total extractable compounds (TEC) was extracted and found to be 642.0 mg/g followed by EAF 229.0 and CME 218.0 mg/g in black cumin seedcake, respectively. These findings are in good agreement with that of Matthaüs (2002) who studied the antioxidant activity of extracts obtained from seedcake of different oilseeds and he found that extraction with water gave the highest total extractable compounds. So the extraction of black cumin seedcake with water resulted in the highest amount of TEC. The extraction with hexane showed the lowest TEC (125.0 mg/g) in comparison with that of the other solvents.

3.2. Total phenolic compounds (TPC)

The content of the total phenolic compounds of CME and its different fractions (HF, EAF and WF) from black cumin seedcake determined using Folin–Ciocalteau method expressed as gallic acid equivalents is shown in Table 1. Results in this table show that EAF in black cumin samples contained the highest amount of total phenolic compounds followed by WF, CME and HF, respectively. The estimation of phenolic content amongst different fractions of black cumin seedcake, surprisingly revealed that the ethyl acetate fraction exhibited higher phenol content of 78.8 ± 0.08 mg/g GAE followed by the water fraction (32.1 ± 0.003 mg/g) > crude methanolic extract (27.8 ± 0.011 mg/g) > hexane fraction (12.1 ± 0.003 mg/g GAE). Ethyl acetate is often used as an extraction solvent with a significant selectivity in the extraction of low-molecular-weight phenolic compounds and high-molecular-weight polyphenols (Scholz & Rimpler, 1989). It was observed that the crude extract had a lower phenolic content as compared to the water and ethyl acetate fractions.

The relationship between the total extractable materials and its content of phenolic compounds was represented in percentages of TPC/TEC. The total phenolics found in the total extractable compounds was low in all fractions. The ratio of total phenolic compounds to the total extractable compounds ranged from 5.0% to 34.4% (Table 1). From these results it can be understood that in CME, HF, EAF and WF of black cumin seedcake more than 87.3%, 90.3%, 65.6% and 95.0% of the extractable materials, respectively, were compounds other than phenolic compounds (Table 1).

Table 1
Total extractable compounds (TEC), Total phenolic compounds (TPC), DPPH IC_{50} (mg/ml) of different extract/fractions obtained from black cumin seedcake.

Extraction	TEC (mg/g)[a]	TPC[a]	TPC/TEC (%)	DPPH IC_{50} (mg/ml)
CME	218.0	27.8 ± 0.11	12.7	2.26 ± 0.21
HF	125.0	12.1 ± 0.03	9.7	2.65 ± 0.32
EAF	229.0	78.8 ± 0.08	34.4	1.89 ± 0.12
WF	642.0	32.1 ± 0.03	5.0	2.17 ± 0.41

[a] Results are mean ± SD ($n = 3$), results are given in mg/g extract.

3.3. DPPH scavenging activity test

DPPH and β-carotene/linoleic acid methods were used to evaluate the antioxidant activity of black cumin seedcake. The antioxidant activity of the extracts in corn oil was assessed using peroxide and anisidine values in comparison with a synthetic antioxidant. The DPPH radical has been widely used to test the free radical scavenging ability of different seedcakes and fat-free residues of the oilseeds (Mariod et al., 2006; Matthaüs, 2002; Peschel, Diekmann, Sonnenschein, & Plescher, 2007). The DPPH scavenging activities of different extract/fractions of seedcakes of black cumin are shown in Table 1.

The DPPH values for investigated extract/fractions were expressed as IC_{50}; the IC_{50} values for different PRFs from black cumin seedcake were 2.26, 2.65, 1.89 and 2.17 for CME, HF, EAF and WF, respectively. From this table the CME and different fractions of black cumin seedcake showed potent free radical scavenging activity on DPPH. The EAF showed the highest DPPH radical scavenging activity followed by WF, CME and HF. From Table 1, a correlation was found between the TPC and IC_{50} when the TPC level was high; the IC_{50} was low which indicates high antioxidant activity. This is due to the high amount of polyphenolic constituents present in the PRFs from black cumin seedcake that act as free radical scavengers.

It was clear that the antioxidant potential of CME and its fractions in DPPH assay was linearly correlated to its total phenolic compounds. The antioxidant activity increased proportionally to the polyphenol content, and a positive linear relationship between IC_{50} values and total phenolic compounds was found. Malencic et al. (2008) found that antioxidant activity of soya bean seed extracts increased proportionally to the polyphenol content with a linear relationship between DPPH values and total polyphenols. In the same manner Chew, Lima, Omara, and Khoob (2008) found a correlation between the TPC and IC_{50} in edible seaweeds extracts and they mentioned that, high level TPC gives low IC_{50} and results in high level of antioxidant capacity due to the high amount of polyphenolic constituents

3.4. β-carotene bleaching (BCB) assay

In the BCB assay, the oxidation of linoleic acid generates peroxyl free radicals due to the abstraction of hydrogen atom from diallylic methylene groups of linoleic acid (Kumaran & Karunakaran, 2006). The free radical then will oxidize the highly unsaturated β-carotene. The presence of antioxidants in the extract will minimize the oxidation of β-carotene by hydroperoxides. Hydroperoxides formed in this system will be neutralized by the antioxidants from the extracts. Thus, the degradation rate of β-carotene depends on the antioxidant activity of the extracts.

Effect of black cumin seedcake extract/fractions (CME, HF, WF and EAF) on oxidation of β-carotene/linoleic acid at 50 °C is shown in Fig. 1. It was clear that the presence of antioxidants in the black

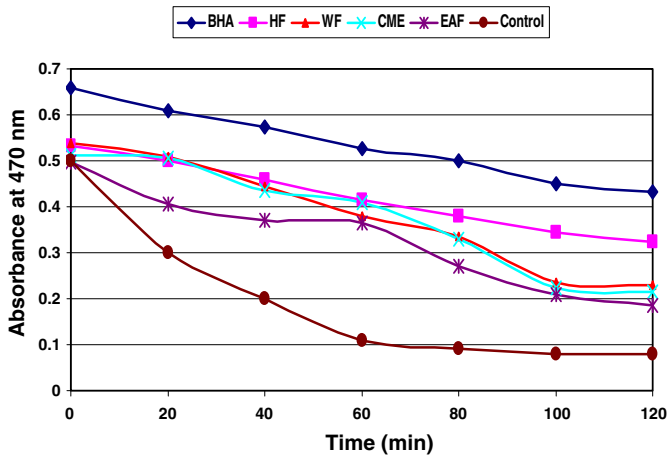

Fig. 1. Effect of *Nigella sativa* extract/fractions (HF, WF, CME and EAF) on oxidation of β-carotene/linoleic acid at 50 °C.

cumin seedcake extract/fractions reduced the oxidation of β-carotene by hydroperoxides from these extract/fractions. The control sample, without addition of extract solution, oxidized most rapidly. There were significant differences ($p < 0.05$) between the different extract/fractions, control and BHA. Thus, the degradation rate of β-carotene depends on the antioxidant activity of the extracts. The phenolic compound rich fractions of black cumin seedcake exhibited antioxidant activity in a β-carotene–linoleate model system. From Fig. 1 the effect of hexane and water fractions on the coupled oxidation of linoleic acid and β-carotene was the highest, and that, the antioxidant activity of *N. sativa* PRFs followed the order: hexane > water > crude methanol extract > ethyl acetate, and there was a significant difference ($p < 0.05$) between the antioxidant activities of these fractions.

It is clear that HF, WF fractions performed better in their effect on reducing the oxidation of β-carotene than CME and EAF fractions, and that their degradation rate of β-carotene dose not depends on their antioxidant activity. There was no correlation between the degradation rate and the bleaching of β-carotene, by other words, no correlation between TPC, and BCB. This is due to the different types of antioxidants that are assayed by the two methods, where TPC gives an indication of the levels of both lipophilic and hydrophilic compounds. BCB in contrast, only gives an indication of the levels of lipohilic compounds (Chew et al., 2008). Most studies showed there was no correlation between TPC and BCB (Mariod et al., 2006; Matthaüs, 2002).

3.5. Stability of corn oil as affected by the addition of black cumin phenolic rich fractions (PRF)

From the above results of TPC, DPPH and BCB it is clear that the phenolic compounds of seedcakes from black cumin contains effective antioxidants, as phenolics are present in seeds mainly within the hulls to protect the seeds from invasive diseases development and from consumption by insects (Liyana-Pathirana et al., 2006). Synthetic antioxidants e.g. tartery-butylhydroquinone (TBHQ), butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA) are added to fats and oils to retard oxidation of unsaturated fatty acids and to decrease the development of rancidity, natural phenolic antioxidants inhibit oxidation reactions when added to oils by acting as a hydrogen donor and afford relatively stable free radicals and/or non-radical products (Wanasundara & Shahidi, 1994).

The effect of black cumin phenolic rich fractions (PRFs) and BHA on corn oil oxidation at 250 and 500 mg/100 g oil is shown in Figs. 2 and 3, respectively. The development of PV during the oxidation of corn oil was evaluated at 70 °C. This temperature was ideal, because at higher temperatures the peroxides will decompose very fast (Duh & Yen, 1997). In Fig. 2 the PV of corn oil (control) with and without black cumin extract/fractions (CME, HF, WF and EAF) or BHA showed a gradual increase. As demonstrated in this figure, a maximum PV of 17.2 meq O_2/kg was reached after 72 h of storage in the control without addition of extract or BHA. Significant differences ($p < 0.05$) were found between the control and different PRFs or BHA, which decreased and slowed down the rate of peroxide formation, resulting in lower PVs after 72 h of storage at 70 °C. The PVs of corn oil containing CME, and EAF fractions were found to be more effective than BHA while WF and HF fractions were found to be less effective than the synthetic antioxidant.

In Fig. 3. The PV of corn oil (control) with and without black cumin extract/fractions (CME, HF, WF and EAF) or BHA showed a gradual increase. As demonstrated in this figure, a maximum PV of 17.5 meq O_2/kg was reached after 72 h of storage in the control without addition of extract or BHA. Significant differences ($p < 0.05$) were found between the control and different PRFs or BHA, which decreased and slowed down the rate of peroxide formation, resulting in lower PVs after 72 h of storage at 70 °C. The

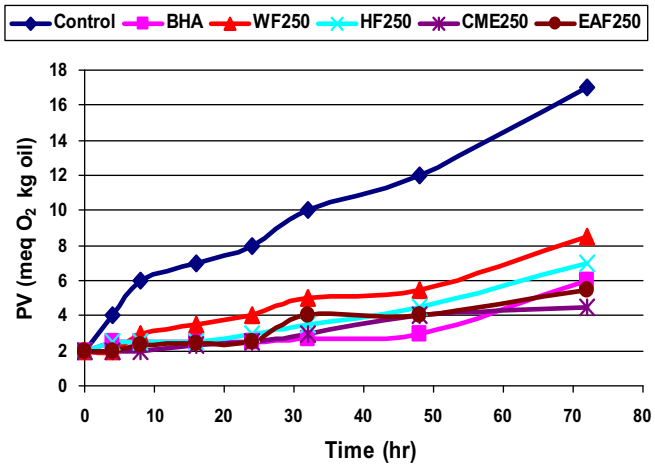

Fig. 2. Oxidation of corn oil treated with *Nigella sativa* CME and its fractions 250 mg/100 g oil during storage at 70 °C.

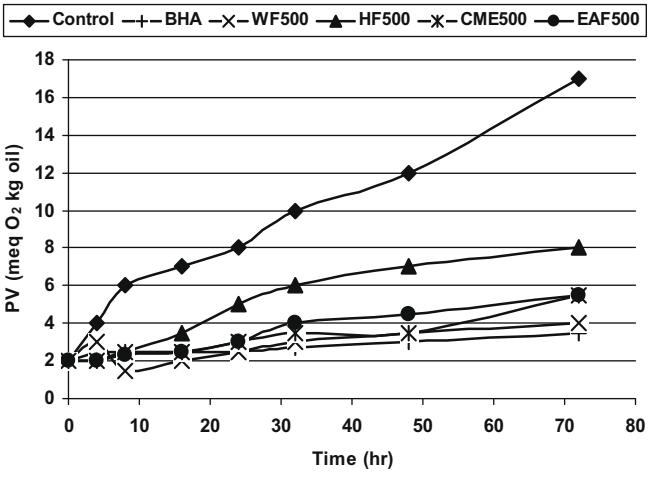

Fig. 3. Oxidation of corn oil treated with *Nigella sativa* CME and its fractions 500 mg/100 g oil during storage at 70 °C.

PVs of corn oil containing CME, and WF fractions were found to be more effective than EAF and HF.

It can be concluded that black cumin seedcake PRFs at concentrations of 0.25% and 0.5% were effective in stabilizing corn oil during storage at 70 °C, and addition of 0.25% PRFs from black cumin as natural antioxidant was found to be better in inhibition corn oil oxidation than using 0.5% and this concentration will be preferable from economic point of view. The PRFs from black cumin seed cake possessed good antioxidant activity and extended the induction period and decreased the formation of peroxides in corn oil more effectively than BHA at rate of 250 mg/100 g oil.

The effect of black cumin PRFs and BHA on corn oil oxidation (measured by *p*-anisidine value) is shown in Table 2. The successive heating of corn oil (at 70 °C for 72 h) mixed with PRFs leads to autoxidation and formation of primary products that decomposed readily and formed aldehydes, ketones and alcohols as secondary products. As anisidine value is a more meaningful test for the assessment of the heating oils quality during heating than the peroxide value, so it was used here because it measures the secondary products of oxidation reactions. Using PRFs extracted from black cumin seedcake as natural antioxidant, at the ratio of 250, 500 mg/kg oil, inhibited the formation of the secondary products in comparison with the control, the amount of secondary products formed seem to be less than that formed in the control samples. These results indicated that almost addition of CME and WF gave better effect than HF and EAF as shown in Table 2. The

Table 2
Comparative inhibition of edible corn oil oxidation by *Nigella sativa* seedcake phenolic rich fractions at 250 and 500 mg/100 g oil measured by *p*-anisidine value.

Time (h)	Control	BHA	HF250	HF500	EAF250	EAF500	CME250	CME500	WF250	WF500
0	0.11 ± 0.1^{a}	0.11 ± 0.1^{a}	0.12 ± 0.1^{a}	0.12 ± 0.1^{a}	0.12 ± 0.1^{a}	0.12 ± 0.1^{a}	0.11 ± 0.1^{a}	0.12 ± 0.1^{a}	0.11 ± 0.1^{a}	0.13 ± 0.1^{a}
4	3.86 ± 0.2^{a}	1.80 ± 0.2^{b}	2.95 ± 0.1^{c}	1.49 ± 0.1^{b}	2.76 ± 0.2^{c}	2.02 ± 0.2^{d}	2.80 ± 0.3^{c}	2.62 ± 0.2^{c}	2.39 ± 0.2^{d}	2.57 ± 0.3^{c}
8	4.47 ± 0.3^{a}	2.71 ± 0.1^{b}	3.51 ± 0.2^{c}	3.37 ± 0.2^{c}	3.96 ± 0.1^{d}	3.81 ± 0.2^{d}	3.21 ± 0.1^{c}	2.92 ± 0.1^{b}	3.34 ± 0.2^{c}	3.50 ± 0.2^{c}
24	4.91 ± 0.2^{a}	3.14 ± 0.1^{b}	4.50 ± 0.2^{c}	3.99 ± 0.1^{d}	4.51 ± 0.3^{c}	3.95 ± 0.2^{d}	3.52 ± 0.1^{e}	3.50 ± 0.1^{e}	3.90 ± 0.2^{d}	4.50 ± 0.1^{c}
32	5.32 ± 0.3^{a}	3.52 ± 0.1^{b}	4.91 ± 0.2^{c}	4.58 ± 0.2^{d}	4.81 ± 0.3^{c}	4.41 ± 0.2^{d}	3.90 ± 0.1^{b}	3.80 ± 0.1^{b}	4.52 ± 0.2^{d}	4.50 ± 0.1^{d}
48	5.79 ± 0.2^{a}	3.87 ± 0.1^{b}	5.52 ± 0.2^{c}	5.42 ± 0.1^{c}	5.40 ± 0.2^{c}	5.01 ± 0.1^{d}	4.40 ± 0.1^{e}	4.20 ± 0.1^{e}	4.80 ± 0.1^{d}	4.80 ± 0.1^{d}
72	6.94 ± 0.2^{a}	4.10 ± 0.1^{b}	6.20 ± 0.2^{c}	6.40 ± 0.2^{d}	5.70 ± 0.1^{e}	6.02 ± 0.1^{e}	4.80 ± 0.1^{f}	4.60 ± 0.1^{f}	5.10 ± 0.1^{e}	5.20 ± 0.1^{e}

Means in every row without a common superscript differ significantly at $p < 0.05$. BHA, butylated hydroxyanisol; HF, hexane fraction; WF, water fraction; CME, crude methanolic extract; EAF, ethyl acetate fraction.

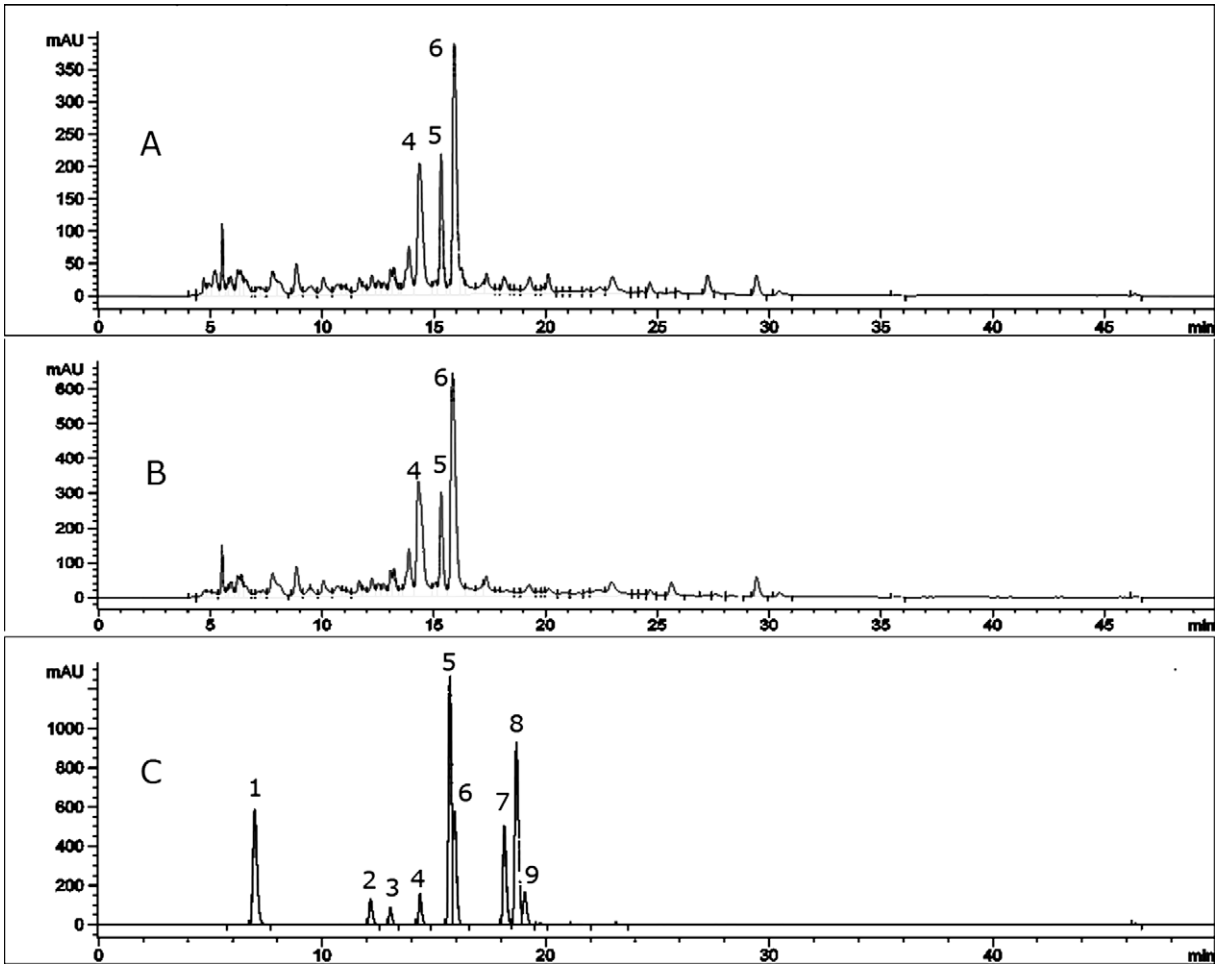

Fig. 4. HPLC/DAD chromatogram of phenoilc compounds in black cumin (A) CME. Detection was at 280 nm. Peak: (4) hydroxybenzoic acid, (5) syringic acid, (6) *p*-cumeric (B) WF. Detection was at 280 nm. Peak: (4) hydroxybenzoic acid, (5) syringic acid, (6) *p*-cumeric (C) Standards of phenolic acids recorded at 280 nm. Peak: (1) gallic acid, (2) (+)-catechin, (3) chlorogenic acid, (4) hydroxybenzoic acid, (5) syringic acid, (6) *p*-cumaric, (7) vanillin, (8) ferulic acid, (9) quercetin.

phenolic rich fractions (PRFs) obtained from black cumin seedcake, seem to be less effective in inhibition of secondary products than BHA. From Table 2 it was clear that using 250 mg from these natural antioxidants gave the same effect of using 500 mg so using 250 mg will be more preferable from economical point of view.

3.6. Identification of phenolic compounds using HPLC–DAD

To know what is/are the responsible active ingredient(s) in black cumin seedcake PRFs, HPLC–DAD was used. CME and WF fractions were used to identify their important phenolic compounds. Fig. 4 shows a representative chromatogram of the (A) CME, (B) WF of black cumin seedcake PRFs and standard (C) monitored at 280 nm.

It shows that the crude methanolic extracts and water fraction of black cumin seedcake contains hydroxybenzoic, syringic and *p*-cumaric acids, with high area in *p*-cumaric acid in both two fractions. These compounds have been identified according to their retention time and the spectral characteristics of their peaks compared to those of standards in Fig. 4C, as well as by spiking the sample with standards. *p-cumaric* acid was detected to be the major phenolic component in the two fractions (CME and WF), contributing about 66.8% and 72.1% to the total amount, respectively, and showing the levels of 0.631 and 3.83 mg/100 g dry weight (DW) in CME and WF, respectively (Table 3). Hydroxybenzoic and syringic acids were also predominant, but slightly higher in WF (0.989 and 0.496 mg/100 g DW) than in CME (0.188 and 0.125 mg/100 g DW), respectively. Results demonstrated that differences in CME and WF phenolic composition were significantly more quantitative than qualitative, where water fraction showed higher amount than methanol crude extract. Black cumin seedcake CME and WF possess similar composition. The amounts of the detected *p*-cumaric acid in both fractions is much higher than (0.36 mg/100 g dry sample) that reported by Bourgou et al. (2008) in black cumin roots methanolic extract. While these authors reported higher amount of hydroxybenzoic acid (1.73 mg/100 g) in black cumin roots methanolic extract. Surprisingly these authors did not identify any amount of syringic acid in black cumin shoots or roots extracts. The levels of total phenolic compounds in black cumin CME and WF determined by HPLC were 0.0094 and 0.053 mg/g DW, respectively, and thus lesser than (36.1 and 31.3 mg/g) the ones obtained by the Folin–Ciocalteu method. This result is predictable due to the weak selectivity of the Folin–Ciocalteu reagent, as it reacts positively with different antioxidant compounds (phenolic and non-phenolic substances).

The EAF and HF fractions of black cumin seedcake also presented some phenolic compounds with less area (data not shown). Previous studies reported that phenolic compounds such as hydroxybenzoic, syringic, *p*-cumaric and vanillin containing significant antioxidant activities (Zhang, Liao, Moore, Wu, & Wang, 2009). The above mentioned HPLC–DAD results indicate that such phenolic rich fractions from black cumin seedcake may inhibit the oxidation of corn oil. Isolation and characterisation of such PRFs may be useful in developing natural antioxidants.

Table 3
Phenolic compound content mg/100 g in black cumin methanolic extract and water fraction.

Compounds	Crude methanolic extract (CME)	Water fraction (WF)
p-Cumeric acid	0.631 ± 0.32	3.83 ± 0.35
Hydroxybenzoic acid	0.188 ± 0.21	0.989 ± 0.42
Syringic acid	0.125 ± 0.12	0.496 ± 0.15

Values are means ± SD ($n = 3$), and they are given as mg/100 g dry weight of investigated black cumin extract/fractions.

4. Conclusions

This investigation indicated the presence of compounds possessing antioxidant activity in PRFs of black cumin seedcake. The total phenolic content of the PRFs revealed that the EAF has a higher phenolic content 78.8 mg/g followed by the other fractions. The PRFs showed a potential value as natural antioxidants and possibly can be used to improve oxidative stability of corn oil. Some of the black cumin PRFs were more effective than BHA in retarding the formation of primary and secondary oxidation products of corn oil. These PRFs act as free-radical terminators and chelating agents, so the application of these natural antioxidants to stabilize edible oils may be considered. Three phenolic compounds were identified in CME and WF as hydroxybenzoic, syringic and *p*-cumaric acids and quantified. Further more studies in isolation and quantification of individual phenolic compounds, to elucidate their different antioxidant mechanisms and the existence of possible synergism, if any, amongst the compounds in black cumin seedcake, and effects of these phenolics on antioxidant status in animal models are needed to evaluate their potential benefits.

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