

Phenolic Composition, Antiradical Activity and Effect on Hexanal of Olive Mill Wastewater

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Abstract: Olive mill wastewater (OMWW) contains a substantial amount of valuable antioxidant phenols that can be recovered for industrial applications as food additives and pharmaceuticals. The present study aimed to extracting different phenolic OMW fractions and determining their antiradical potential as well as their effect on hexanal. Total phenolic content was measured according to the Folin-Ciocalteu method of OMWW either crude or acidified samples while the antiradical activities were assessed using 1, 1'-diphenyl-2-picryl-hydrazyl radical (DPPH⁰) and β -carotene bleaching tests. HPLC analysis showed that eight compounds were identified: phenyl acids (gallic acid, vanillic acid, *p*-qumaric acid, caffeic acid, ferulic acid), phenyl alcohols (hydroxytyrosol, tyrosol), as well as rutin. Rancimat method using sunflower oil (SFO) as model system showed that antioxidant activity increased by 27.45% with SFO treated with 175ppm extracted phenolic compound compared with control sample. Prooxidant effect has obtained when increase concentrations to 350 and 525 ppm of phenolic compounds extracted from OMWW. The OMWW extract inhibited significantly ($P=0.05$) hexanal formation in SFO oil. (81.82% inhibition).

Key words: Olive Mill Wastewater • Phenolic Compounds • Antiradical Activity • Hexanal

INTRODUCTION

Recently, there is a rising interest in natural antioxidants as currently used synthetic antioxidants have been suspected to cause or promote undesirable effects on human health [1]. The manufacturing process of olive oil yields a liquid waste called 'olive mill wastewater' (OMWW). This waste arises from the naturally present water as well as the process water and soft tissues from the olive pulp and forms a very stable emulsion. It is an excellent source of natural antioxidants. Phenolic compounds, which are considered to be the main antioxidant compounds in olive oil mill waste, are able to donate a hydrogen atom to the lipid radical formed during the propagation phase of lipid oxidation. So, they could be added to fatty foods to prevent the formation of off-flavour and toxic compounds resulting from lipid oxidation. The most abundant phenolic compounds in olive oil mill waste are mainly secoiridoid aglycones [2].

Olive oil is extracted primarily by the traditional discontinuous press process or by the continuous centrifugation of a mixture of milled olives and water.

In both systems, three phases are produced: (i) olive oil; (ii) solid residue; and (iii) liquid phase called OMWW. In the Mediterranean Basin alone, OMWW accounts for 10–12 X10⁶ m³ of pollution each year [3]. Roughly, one ton of olives yields one to two tons of OMWW. The OMWW waste is claimed to be one of the most polluting effluents produced by the agro-food industries because of its high polluting load. It also exhibits high toxicity to plants, bacteria and aquatic organisms, due to its composition of organic substances (14–15%) and phenols (up to 10 g/l) [4]. These latter compounds, characterized by high specific chemical oxygen demand (COD) and resistance to biodegradation, are responsible for its black colour, depending on their state of degradation and the olives they come from and its phytotoxic and antibacterial properties [5].

Under mechanical processing, about 1% of the total phenols present in olives is in the oil. Most part of the olive phenols, in fact, remains in the wastewater and also in solid wastes [6]. Several researches have evaluated the feasibility and economic processes for recovering olive phenols from olive oil mill wastewater or solid wastes [7].

Antioxidants are used to preserve food quality mainly by prevention of oxidative deterioration of constituents of lipids. The most commonly used antioxidants at the present time are butylated hydroxyanisole (BHA), butylated hydroxyl-toluene (BHT), propyl gallate (PG) and *tert*-butylhydroquinone (TBHQ). However, BHA and BHT have suspected of being responsible for liver damage and carcinogenesis. Therefore, the development and utilization of more effective antioxidants of natural origin are desired [8].

Natural antioxidant can protect the human body from free radicals and retard the progress of many chronic diseases as well as lipid oxidative rancidity in foods [9]. The auto-oxidation of fats is a big problem because of the deterioration in the quality of the foods in which they are contained and the reduction in their nutritional value. In addition, the oxidation of polyunsaturated fatty acids in biological membranes leads to serious damage such as coronary atherosclerosis, emphysemas, cancer and cirrhosis, safe guarding fats against oxidation is normally done by restricting the access of oxygen or adding antioxidants.

The present work attempted to develop effective procedures to recover the potentially high-added-value phenolic compounds contained in OMWW, to produce antioxidant additives extracts, determine phenolic composition using HPLC, antiradical activity and evaluate their effect on hexanal.

MATERIALS AND METHODS

Materials: Olive mill wastewater (OMWW) was obtained from traditional olive mill press in Cairo region (Egypt) and stored during the experimental period at 4°C until use within few days.

Sunflower Oil (Sfo): Completely refined sunflower oil without addition of antioxidants was obtained from ARMA Factory, 10th of Ramadan City, Egypt and used as a model system to evaluate the antioxidant activity of phenolic compounds.

Chemicals: Butylated hydroxyanisole (BHA), *tert*-Butylated hydroxyl quinine (TBHQ), linoleic acid, Folin–Ciocalteu and Tween 20 were purchased from Sigma Aldrich Chemical (Germany). Tyrosol, Hydroxy tyrosol, Caffeic acid, Vallinic acid, *P*-Qumaric, Rutin and ferulic acid; 1,1'-diphenyl-2-picrylhydrazyl (DPPH⁰) and β -carotene were purchased from Fluka (Switzerland). Methanol, acetonitrile, ethyl acetate and orthophosphoric acid were HPLC-grade.

Extraction of Phenolic Compounds: Phenolic compounds were extracted according to the method of Elena *et al.* [10].

Total Phenol Content Determination: The total phenol (TP) content of the crude and acidified extracts was determined colourimetrically at 725 nm using the Folin-Ciocalteu reagent according to the method of Gutfinger [11]. The standard curve was prepared using 20, 40, 80, 120, 160 and 200 mg/l solution of caffeic acid in methanol: water. Total phenol values were expressed as caffeic acid equivalents (CAE ug/ml).

Hplc Analysis Phenolic Compounds from Omww: The phenolic compounds characteristic of OMWW extracts were evaluated using the HPLC equipment and methods developed and described in Soler-Rivas *et al.* [12]. Briefly, samples were injected onto a reversed phase HPLC column Zorbax 300SB C₁₈ column (4.5 X 250 mm) (Agilent 1100 Technologies, USA) and eluted with a flow rate of 1 ml min⁻¹ and a mobile phase containing: (A) acetic acid/water (2.5%) and (B) acetonitrile following a gradient: from 0 to 10 min, 0% B, from 10 to 40, 10% B, from 40 to 70, 40% B, up to 72, 100% B. Peaks were monitorized using variable wave length detector (G1314A), identified on the basis of their retention times and spectra compared to standards and quantified at 280 nm.

Antiradical Assays

1,1'-diphenyl-2-picryl-hydrazyl Radical (Dpph⁰) Radical Scavenging: The antioxidant activity of the phenol extracts at 50, 100 and 150 ppm was evaluated by using the stable 1,1'-diphenyl-2-picryl-hydrazyl radical (DPPH⁰) according to the method of Bandoniene *et al.* [13].

The radical scavenging activities of the tested samples, expressed as percentage inhibition of DPPH⁰, were calculated according to the following formula proposed by Yen and Duh [14]:

$$\% \text{ Inhibition} = 100 \times (A - A_0) / A_0$$

where,

A₀ is the absorbance at 515 nm of the blank sample and A is the absorbance of the tested sample at 515 nm.

β -carotene Bleaching Method: The antioxidant activity in aqueous media was evaluated using- β -carotene according to Farag *et al.* [15].

Gas Chromatography/flame Ionization Detector Analysis of Omww Effect on Hexanal:

The GC analyses were carried out on a Varian 3400 equipped with an DB-5 fused bonded column (60 m × 0.25 mm × 0.25 μ m) (Ohio Valley, Marietta, USA) and FID detector; carrier gas was helium (1 ml/min); the operating conditions were: initial temperature 45°C, 2 min isothermal, 300°C, 4°C/min 300°C, then 20 min isothermal. Detector and injector temperatures were 300 and 250°C, respectively. The split ratio was 1: 20.

Characteristics of SFO: Refractive index, acid value, peroxide value and unsaponifiable matter were measured according to methods described by A.O.A.C [16]. While Fatty acids compositions were determined using GC as follows:

Gas Chromatography Analysis

Methylation of Fatty Acids: An aliquot of fatty acids, about 10 mg, was dissolved in 2ml hexane and then 0.4 ml of 2N KOH in anhydrous methanol was added [17], after 3 min, 3 ml water was added. The organic layer, separated by centrifugation, was dried over anhydrous sodium sulfate and then concentrated, with a N₂ stream to around 0.5 ml for GC analysis of fatty acids methyl esters (FAME) as described below.

Gc Analysis of Fame: Agilent 6890 series GC apparatus provided with a DB-5 column (60 m × 0.32 mm × 0.25 μ m) was used. Oven temperatures were 150°C ramped to 195°C at 5°C min⁻¹, ramped to 220°C at 10°C min⁻¹ and flow rate was 1.5 min⁻¹. Fatty acids results after the previous procedures steps were transformed into methyl esters and directly injected into the GC.

Evaluation of Antioxidant Activity of Acidified Phenolic Compounds by SFO:

Different concentrations (175, 350 and 525 ppm) of phenolic compounds recovered from acidified OMWW, BHA and TBHQ using 175 and 120 ppm as commercial synthetic antioxidant were individually added to sunflower oil to study their antioxidant efficiency. The oxidative stability of SFO was evaluated by the Rancimat method [18]. Stability was expressed as the induction time (hrs), measured with the Rancimat 679 apparatus (Metrohm Co., Switzerland), using an oil sample of 5 g heated to 100 °C and air flow of 20 L/h.

Volatile Components Extraction: Rancimat 679 apparatus (Metrohm Co., Switzerland) was equipped to extract rancidity (off-flavour) volatile components from SFO treated with phenolic compound extracted from acidified

OMWW. Five gram of SFO containing 175, 350 and 525 ppm phenolic compounds separately weighed into Rancimat vessels, the temperature adjusted to 100 °C, air flow of 20L/h were carried out. Diethyl ether (20 ml) was added to the distilled water vessel to capture the extracted volatile components. After 3 hrs (the rancidity smell was appeared) another 10 ml of diethyl ether was added to the distilled water vessel. Using separatory funnel the ether layers were separated from distilled water vessel for each concentration. The volatile component from fresh SFO as a control was extracted using the same method at temperature 45 °C instead of 100°C for only 20 min (un-rancid volatile components). All ether layers were dried over sodium sulfate anhydrous. Then the volatile components were analyzed using GC-MS.

Statistical Analysis: All measurements were obtained (at least) in triplicate and the values were averaged and reported along with the standard deviation (SD). Data were analysed with One-Way ANOVA Test. All the data were analysed with the SPSS 16.0 statistical software.

RESULTS AND DISCUSSION

Phenoilc Compounds: Acidification determines the protein precipitation, the release of biophenols bounded to cell wall components and the increase of solubility of phenolic compounds in organic solvents; these effects were confirmed by the higher total phenol content (determined spectrophotometrically by the Folin-Ciocalteu method) we found in the extract from an acidified OMWW: 123.5 mg /L versus 2.8 g /L OMWW in the extract from a crude OMWW. These results are in agreement with Obied *et al.* [19].

As solvent for the extraction, ethyl acetate was chosen which is frequently used to extract biophenols from aqueous matrices such OMWW. Allouche *et al.* [20] demonstrated that ethyl acetate exhibits a higher extraction power respect to other solvents, such as methyl isobutyl ketone, methyl ethyl ketone, diethyl ether, even though it is somewhat selective towards low (about 180 Da) and medium (about 13 kDa) molecular mass phenolic compounds [21]. The effect of OMWW acidification on biophenol extraction was tested; extracts from crude and acidified OMWW were compared.

HPLC of Phenoilc Compounds: Identification of phenolic compounds in the OMWW extracts was preliminary performed by HPLC-UV, comparing the relative retention times and UV spectra with those of standard solutions.

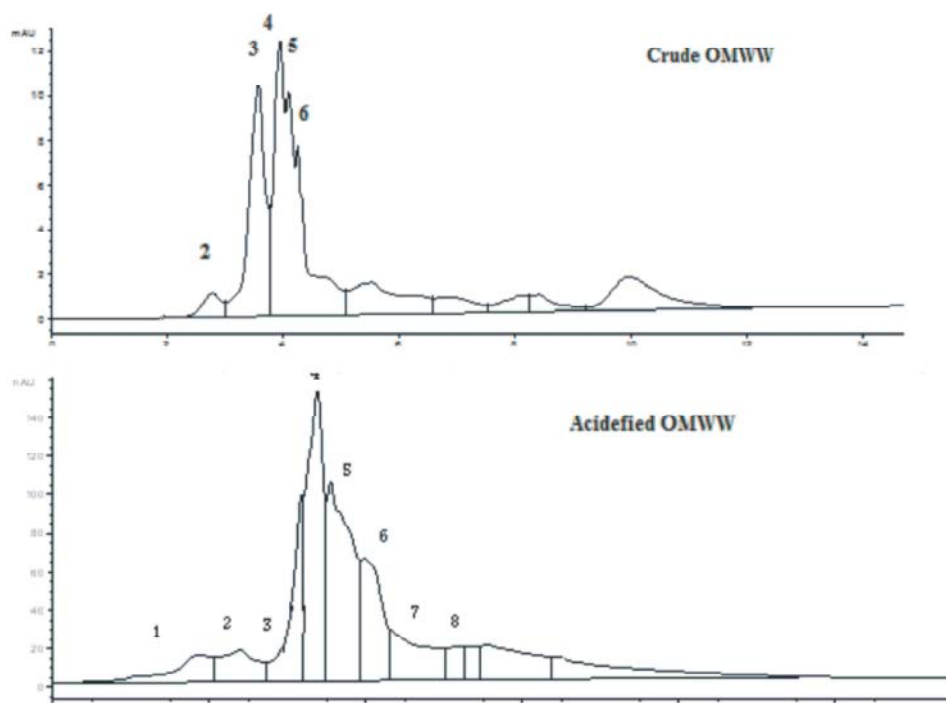


Fig. 1: Typical HPLC chromatogram of phenolic extracts from crude (a) and (b) acidified OMWW (pH 2 with HCL). Key to peak identities: 1-gallic acid; 2-hydroxytyrosol; 3-tyrosol; 4-caffeic acid; 5-vanillic acid; 6-ferulic acid; 7-p-qoumaric acid.; 8-rutin.

A representative chromatogram of either crude or acidified OMWW extracts phenolic and acidified extracts were reported in Fig. 1. The HPLC profile showed several peaks corresponding to different biophenols, among which eight compounds were identified: phenyl acids (gallic acid, vanillic acid, *p*-qumaric acid, caffeic acid, ferulic acid), phenyl alcohols (hydroxytyrosol, tyrosol), as well as rutin.

Concentration of phenolic compounds in the extracts was calculated comparing HPLC peak areas with those of external standard and the obtained data are reported in Table (1).

In addition, acidification causes the hydrolysis of complex phenolic compounds with consequent release of phenolic monomers, as confirmed by the chromatograms of extracts from crude and acidified OMWW shown in Fig. 1, where it can be observed an increase of gallic acid, hydroxytyrosol, tyrosol and caffeic acid. On the other hand, decrease in vanillic, ferulic acid and *p*-qumaric as shown in Table (1).

Many other phenolic compounds were found in the phenolic extracts, though not all were identified, due to the complex nature of the OMWW phenolic fraction [22], which has not been completely elucidated yet. This complexity is due to the high number of factors that

Table 1: Concentration (mg/L) of identified phenolic compounds in the extracts from an acidified and from crude OMWW

Phenolic	Crude	Acidified
Gallic	0.89	1.4
Hydroxy tyrosol	45.3	95.8
Tyrosol	61.4	124.6
Caffeic acid	34.7	44.8
Vanillic acid	3.8	2.4
Ferulic acid	4.2	3.7
<i>P</i> -Qumaric	3.9	2.9
Rutin	0.59	0.61

influence the occurrence of specific biophenols in OMWW, such as olive cultivar, ripeness degree of the fruit, climatic and agronomic conditions, storage conditions prior to extraction and processing technique [23]. Further investigation is required to better understand the phenolic composition of OMWW, as regards in particular the complex compounds, which are reported to be aglycons, glycosides or other derivatives of simple phenols [24].

According to previous investigations [25], the most abundant phenolic compound present in the OMWW extracts resulted to be hydroxytyrosol, which is formed as

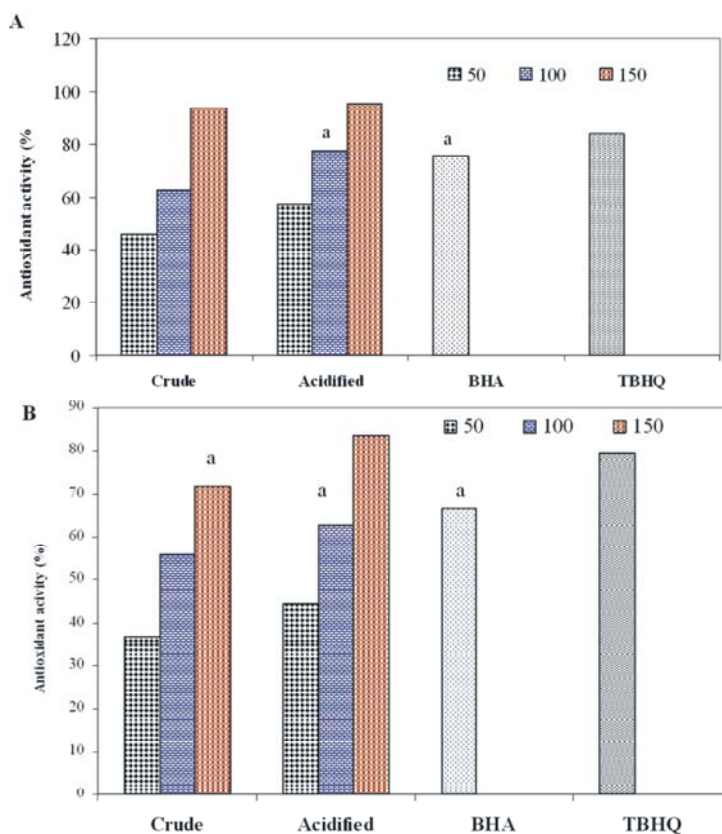


Fig. 2: Antiradical activities of crude and acidified extracts of OMWW measured as DPPH° scavenging (A) and β -carotene assay (B). Values with the same letter are not significant ($P < 0.05$)

a result both of hydrolysis of oleuropein during oil extraction [26] and of acid hydrolysis of secoiridoid derivatives caused by the addition of HCl to the OMWW. This biophenol is object of great attention and of numerous studies as it shows antioxidant, cardioprotective and antiatherogenic [21], antimicrobial [27] and anti-inflammatory [8] activities.

Olive mill waste (OMWW) contains substantial amounts of valuable antioxidant biophenols that can be recovered for possible applications in food, pharmaceutical and cosmetic industries. Bioactivity-guided fractionation combines the use of bioassay and chromatographic separation for isolation of potent bioactive compounds from highly complex plant extracts, such as OMWW and avoids tedious purification and identification of inactive phytochemicals [28].

Antiradical Activity: The antioxidant activities of different concentrations of crude and acidified OMWW were tested using 2 tests; DPPH° and β -carotene and results are given in Fig. 2. Although there are some commonly used methods for determining antioxidant activities of

functional foods, the results are sometimes inconsistent and vary depending on the method used even on the same commodity of food. Therefore, we determined antioxidant capacity of our OMWW fractions using DPPH radical scavenging activity and β -carotene bleaching tests. It showed very important antioxidant activity which was mainly attributed to total phenol content and hydroxytyrosol level. Therefore, the OMWW is a low-cost, renewable and abundant source of phenolic antioxidants.

Antiradical activities (%) were compared with BHA and THBQ as positive control. Several *in vitro* and *in vivo* studies have shown that phenols found in olives, olive oil and OMWW exert potent biological activities including, but not limited to, antioxidant and free radical scavenging actions and as such are potentially capable of preventing oxidative stress [29, 30].

Bleaching of β -carotene is a free-radical-mediated phenomenon resulting from the hydroperoxides formed by air oxidation. In the absence of antioxidants, the β -carotene molecules lose their double bonds by oxidation as well as the characteristic orange colour,

which can be monitored spectrophotometrically. The presence of different antioxidants can hinder the extent of β -?carotene bleaching by neutralizing the free radicals formed in the system [31]. This forms the basis by which OMWW fractions can be screened for their antioxidant potential.

Other studies showed that OMW polyphenols have an antioxidant effect on intestinal human epithelial cells [32] and a cytostatic action on some tumour cells [33]. Fki *et al.* [34] showed that OMW extract can be used as alternative natural antioxidant to stabilize edible oils, while at the same time appeasing a major concern of consumers over the use of synthetic antioxidants in food products.

Effect of OMWW on Hexanal: Hexanal, which is considered as a typical oxidation indicator derived from linoleic acid degradation and in general, a good indicator of the oxidation level [35], was significantly lower with increase the level of OMWW phenolic compounds and reach the lowest value (8.37 mg/L) when 525 ppm of OMWW was added to the fresh control sample (Table 2). The OMWW extract inhibited ($P=0.05$) hexanal formation in SFO oil (81.82% inhibition).

SFO Characterization: Results in Table (3) showed that the refractive index of SFO was 1.4733 (at 25°C). Also, it could be noticed that the acid value (mg KOH/g oil) and free fatty acid percent (FFA) were 0.17 and 0.085, while the peroxide value was 2.88 (meq/kg oil). From results in Table (3) the unsaponifiable matter content of sunflower oil was 0.38%, these results indicating that SFO used as model system for evaluation of the antioxidant activity under investigation had good quality. Also, data at the same Table showed that, linoleic acid was the major unsaturated fatty acid (59.23%). Meanwhile, palmitic acid was the major saturated fatty acid in the investigated oil (6.31%). In addition, stearic acid represented only a low percentage since, it represented only 3.30%. Moreover, oleic acid was found to represent 28.23% while, linolenic acid $C_{18:3}$ ($n-3$) was present at a very low level < 1 % (0.38%).

Results also revealed that, the total saturated fatty acids (SFA) and unsaturated fatty acids (USFA) were 10.09 and 88.65%, respectively and the ratio of USFA / SFA was 8.78: 1.

The oxidative stability is related not only to fatty-acid composition, but also to several other factors; pro- and/or anti-oxidant substances and concentration of natural antioxidants, such as polyphenols, in the oil [36].

Table 2: Hexanal remaining concentration as OMWW treatment

Sample	Hexanal concentration (mg/L)
Fresh SFO (C)	10.23
C + (175 ppm of OMWW phenolic compounds)	9.36
C+ (350 ppm)	8.41
C+ (525 ppm)	8.37

Table 3: Characteristics of fresh refined, bleached and deodorized (RBD) sunflower oil (SFO).

Parameters	Values
Refractive index (25°C)	1.4733
Acid value (mg KOH/g oil)	0.17
Acidity (as % oleic acid)	0.085
Peroxide value (meq/kg oil)	2.88
Unsaponifiable matter (%)	0.38
Fatty acids (%)	
$C_{16:0}$	6.31
$C_{16:1}$	0.12
$C_{17:0}$	0.04
$C_{17:1}$	0.03
$C_{18:0}$	3.30
$C_{18:1}$	28.23
$C_{18:2}$	59.23
$C_{18:3}$	0.38
$C_{20:0}$	0.41
$C_{20:1}$	0.66
$C_{22:0}$	1.04
$C_{24:0}$	0.21
SFA	10.09
USFA	88.65
USFA/SFA	8.78

Table 4: Antioxidant activity of phenolic compounds extracted from acidified OMWW.

System	Induction period (hrs).	Antioxidant activity (AA) (%)
Sunflower oil (C)	10.2	0.00
C + BHA (175 ppm)	12.4	21.56
C + TBHQ (120 ppm)	29.2	186.27
C + (175 ppm OMWW phenolic compounds)	13	27.45
C+ (350 ppm)	10	- 1.96
C+ (525 ppm)	9.68	- 5.09

(AA): The percentage of antioxidant activity (AA %) = $\frac{IP \text{ of sample} - IP \text{ of control}}{IP \text{ of control}} \times 100$

From the abovementioned results it could be noticed the freshness of SFO was used as model system for evaluation of the antioxidant activity under investigation. These results are in agreement with those reported by CODEX-STAN 210 [37].

The antioxidant activity (AA) of phenolic compounds extracted from OMWW compared with BHA and TBHQ as the most commonly used synthetic antioxidants was assessed by the Rancimat method using SFO as model system. This method assigned the induction period for the oxidative stability in SFO. The longer induction period indicates the stronger antioxidant activity. Table (4) shows that TBHQ had the superior AA (186.27%). The AA increased by 27.45% with SFO treated with 175ppm extracted phenolic compound compared with control sample whereas BHA increased the AA by 21.56%. Results also, indicated that prooxidant effect has obtained when increase concentrations to 350 and 525 ppm of phenolic compounds extracted from OMWW. Where, the AA decreased to 1.96 and 5.09% compared with control sample, respectively.

CONCLUSION

The high antioxidant, radical scavenging activity and phenolic content of the OMWW extract suggest potential applications in the food and pharmaceutical industries and may be used as alternatives in the search for natural replacement of synthetic antioxidant food additives. Phenolic extracts from OMWW can be used as alternatives to synthetic antioxidants in order to increase the stability of foods by preventing lipid peroxidation and protect living systems from oxidative damage by scavenging oxygen radicals. A profitable use of OMWW emerges as food additive. Food enriched with OMWW has two advantages: improved oxidative stability and increased nutritional value.

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