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# Investigation of Antioxidant Activity of Thymoquinone and Its Protective Effect on Edible Oils

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**Abstract**: In present study, the oxidation protection efficiency of TQ (Thymoquinone) on two different types of oils was evaluated. In addition, antioxidant capacity and hydroxyl radical scavenging (HRS) activity of the TQ were investigated according to the CUPric reducing antioxidant capacity (CUPRAC) and ferric reducing antioxidant potential (FRAP) methods. The FRAP and CUPRAC methods revealed that antioxidant and hydroxyl radical scavenging activity of TQ was remarkably effective. It was determined that TQ had a high HRS potential (80.36 ± 0.92%) even at very low concentrations (1.6 µg/mL). Research findings revealed that, compared with synthetic antioxidant (BHT), TQ was more effective in retarding the oxidation of the two types of oil. The oils with TQ incorporated exhibited much better chemical stability and lower peroxide value. As an alternative to synthetic antioxidants, TQ could be recommended as an effective natural antioxidant to improve the stabilization of edible vegetable oils.

**Keywords**: Antioxidant, thymoquinone, lipid oxidation, CUPRAC assay, FRAP.

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## 1. INTRODUCTION

Lipid oxidation is an extremely versatile process involving various reactions that cause various physical and chemical changes in lipid-rich foodstuffs (Maqsood et al. 2014). During the period from processing to storage of edible oils, compounds such as peroxides, aldehydes, ketones and other small molecules, which represent lipid oxidation, are formed. Many of these compounds formed during lipid oxidation affect food quality parameters including flavor, texture, nutritional value, odor and color. Moreover, some of these oxidizing agents have toxic effects on human health. (Karami et al. 2020). Synthetic and natural antioxidants are used to increase the storage stability of lipids. Butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) are the most commonly used synthetic antioxidants in the vegetable oil industry. However, some synthetic antioxidants used in food processing, such as (BHT) and (BHA), have proven to have side effects. These substances have been reported to have carcinogenic effects on living organisms (Ames 1983; Baardseth 1989). Therefore, government officials and consumers are concerned about the safety of foods, as well as the potential health effects of synthetic additives. At present, the research is directed towards replacing synthetic additives such as BHA and BHT with natural substances which have more antioxidant activity and stability in different edible oils and meet the increasing interest of consumers on safety. The benefits of adding natural, plant-based antioxidants to lipids in order to prevent lipid oxidation have been highlighted in recent years. Natural antioxidants retard oxidative rancidity via the following pathways: (1) capturing of free radicals; (2) decomposing/deoxidizing peroxides; and (3) scavenging oxygen (Sun et al. 2010; Lianhe et al. 2012).

Thymoquinone (TQ), a biologically active compound in black cumin (*Nigella sativa*) seeds, is responsible for its health beneficial effects (Soleimanifar et al. 2019). TQ is a natural compound which possesses potent antioxidant activity without having any phenolic hydroxyl group which is responsible for antioxidant activity (Hossen et al. 2021). The antioxidant activity of TQ is well known and is reported by many authors (Kassab et al. 2017; Mahmoud and Abdelrazek 2019).

In this study, antioxidant capacity and hydroxyl radical scavenging (HRS) potential of TQ were evaluated according to the CUPric reducing antioxidant capacity (CUPRAC) and

ferric reducing antioxidant potential (FRAP) methods. Moreover, the effects of TQ on the oxidative stability of extra virgin olive (EVOO) and safflower oils (SFO) were investigated using tests performed under accelerated thermal storage conditions.

## 2. MATERIAL AND METHODS

### 2.1. Chemicals

Copper(II) chloride dihydrate ($CuCl_2 \cdot 2H_2O$), catalase from bovine liver (2000-5000 U $mg^{-1}$ solid), Neocuproine ($C_{14}H_{12}N_2$), and thymoquinone were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Absolute ethanol (EtOH) was purchased from ISOLAB Laborgeräte GmbH (Eschau, GERMANY). Ammonium acetate ($NH_4Ac$), iron(II) chloride tetrahydrate ($FeCl_2 \cdot 4H_2O$),sodium salicylate ($C_7H_5NaO_3$), sodium thiosulfate pentahydrate ($Na_2S_2O_3 \cdot 5H_2O$), Potassium hexacyanoferrate(III) ($K_3[Fe(CN)_6]$), hydrogen peroxide ($H_2O_2$, 30 wt.%), di-Sodium hydrogen phosphate dihydrate ($Na_2HPO_4 \cdot 2H_2O$), Sodium dihydrogen phosphate dihydrate ($NaH_2PO_4 \cdot 2H_2O$), Iron(III) chloride hexahydrate ($FeCl_3 \cdot 6H_2O$), trichloroacetic acid (TCA), were purchased from Merck (Darmstadt, Germany). All other chemicals used were analytical grade and obtained from either Sigma–Aldrich or Merck.

### 2.2. Plant materials

Extra virgin olive oil (EVEO) were supplied by TARIS Figs, Raisins, Cotton and Oilseed Agricultural Sales Cooperatives Union (Aydın-Turkey). Safflower seeds were obtained from Isparta University of Applied Sciences. Later, SFO was obtained by cold pressed extraction method.

### 2.3. Preparation of solutions

$CuCl_2$ solution,10 mmol $L^{-1}$, was prepared by dissolving 0.4262 g $CuCl_2 \cdot 2H_2O$ in water, and diluting to 250 mL. Ammonium acetate buffer at pH= 7.0, 1.0 mol $L^{-1}$, was prepared by dissolving 19.27 g $NH_4Ac$ in water and diluting to 250 mL. Neocuproine (Nc) solution, 7.5 mmol $L^{-1}$, was prepared daily by dissolving 0.078 g Nc in absolute ethanol, and diluting to 50 mL with ethanol. TQ solution was prepared in absolute ethanol at 1x $10^{-2}$ mol $L^{-1}$ concentration. The salicylate buffer (at 10 mmol $L^{-1}$ concentration) was prepared by dissolving 0.160 g of sodium salicylate in double-distilled water ($DDH_2O$). Fe(II) at 20 mmol $L^{-1}$ concentration was prepared by dissolving 0.1988 g $FeCl_2 \cdot 4H_2O$ with 2mL of 1 mol $L^{-1}$ HCl, and diluting to 50 mL with $DDH_2O$. $Na_2$–EDTA at 20 mmol $L^{-1}$ concentration was prepared by dissolving 0.372 g of the salt in $DDH_2O$ and diluting to 50 mL. Hydrogen peroxide at 10 mmol $L^{-1}$ concentration was prepared from a 0.5 mol $L^{-1}$ intermediary stock solution, the latter being prepared from 30% $H_2O_2$. The $NaH_2PO_4$–$Na_2HPO_4$ buffer solution (pH 7.4) at 200 mmol $L^{-1}$ was prepared in $DDH_2O$. The original catalase solution of initial activity 2000-5000U $mg^{-1}$ solid was diluted with 0.2 mmol $L^{-1}$ phosphate buffer (pH 7.4) to a concentration of 400-1000 U $mL^{-1}$. The dilution ratio of TQ was selected so as not to give an initial CUPRAC absorbance.

The FRAP reagents were prepared as follows: To prepare 0.2 mol $L^{-1}$ phosphate buffer at pH 6.6, 7.80 g of $NaH_2PO_4 \cdot 2H_2O$ was dissolved in water and diluted to 250 mL with $H_2O$ such that its final concn. would be 0.2 mol $L^{-1}$; 8.90 g of $Na_2HPO_4 \cdot 2H_2O$ was dissolved in water and diluted to 250 mL such that its final concn. would be 0.2 mol $L^{-1}$. To prepare 0.2 mol $L^{-1}$ phosphate pH 6.6 buffer, 62.5 mL of $NaH_2PO_4 \cdot 2H_2O$ solution was mixed with 37.5 mL of $Na_2HP0_4 \cdot 2H_2O$ and diluted to a total of 200 mL with $H_2O$ (Stoll and Blanchard 2009). Potassium ferricyanide solution (1%, w/v) was prepared daily by dissolving 1 g $K_3Fe(CN)_6$ in 1 mL of 1 mol $L^{-1}$ HCl and some water and diluting to 100 mL with water. Ferric chloride solution (0.1%, w/v) was prepared daily by dissolving 0.1 g of $FeCl_3 \cdot 6H_2O$ in 1 mL of 1 mol $L^{-1}$ HCl and some water and diluting to 100 mL with water. Trichloroacetic acid (TCA) solution (10%, w/v) was prepared by dissolving 10 g of TCA in water and diluting it to 100 mL with $H_2O$ (Berker et al. 2007).

### 2.5. Ferriccyanide ($Fe^{3+}$) reducing antioxidant power (FRAP) assay

**Procedure.** The reducing capacity (RP) of samples was assessed as described by Oyaizu (1986). 2.5 mL of 0.2 mol $L^{-1}$ phosphate buffer (pH 6.6) and 2.5 mL of $K_3Fe(CN)_6$ solution (1%) were added to 1 mL of sample solution at different concentrations (50-100 µg/mL) in ethanol); the mixture was incubated at 50 ◦C on a water bath for 20 min. The incubated mixture was let to cool to room temperature, and 2.5 mL of TCA (10%) was added. The solution was thoroughly mixed by vortexing for 30 s. an aliquot of 2.5 mL was withdrawn from the supernatant, and 2.5 mL water was followed by 0.5 mL of $FeCl_3 \cdot 6H_2O$ solution (0.1%) added so that the final volume was 5.5 mL. The colored solution was read at 700 nm against the blank regarding standard using UV Spectrophotometer (SHIMADZU UV-1280 UV-Vis Spectrophotometer). BHA and BHT were used as standard references.

### 2.6. GC analysis of fatty acids

Fatty acid methyl esters (FAMEs) were prepared from the oil samples according to the method described by Seyhan et al. (2007). Briefly, 2 mL of 0.01 mol $L^{-1}$ NaOH in methanol was added to a tube containing the oil sample (ca. 20 mg) dissolved in 0.5 mL hexane and then held in a water bath at 60 °C for 10 min. Thereafter, 0.5 mL boron trifluoride in methanol (20% of $BF_3$ in methanol) was added and the samples held an additional 10 min in a water bath at 60 °C. The sample was cooled under running water and 2 ml of 20% (w/v) of sodium chloride and 1 ml hexane was added. After mixing completely, the hexane layer that contained the FAMEs was separated by centrifugation and then analyzed by GC. Peak identification was performed by comparing the relative retention times with those of a commercial standard mixture of FAME (Sigma, Supelco 37 Component FAME Mix). The yields of the independent ingredients on oil content and palmitic, stearic, oleic, linoleic, and linolenic acid concentrations of the oil were examined on a percentage basis (Erdoğan and Gökçe 2021).

### 2.7. Incorporation of EVOO and SFO with BHT and TQ

Three EVOO and three SFO (triglyceride) experimental sets were prepared as follows.

- ✓ Extra virgin olive oil (EVOO)
- ✓ EVOO incorporated with 200 ppm of BHT
- ✓ EVOO incorporated with 200 ppm of TQ.
- ✓ Safflower oil (SFO)
- ✓ SFO incorporated with 200 ppm of BHT
- ✓ SFO incorporated with 200 ppm of TQ.
- ✓ The BHT concentration in the oil samples was 0.02% (w/w) corresponding to the level determined by Codex Alimentarius Commission (CAC), (2019).

### 2.8. Schaal oven test

The schaal oven test, which is aimed to determine the effect on the oxidative stability of oils under accelerated storage conditions, was performed as described by Tinello and Lante (2020). In detail, the oil samples were precisely weighed (30 g ± 0.01 g) into sealed bottles covered with aluminum foil, without headspace, and stored in an oven at a constant temperature of 60 ± 1 °C for 14 days SFO and 28 days for EVOO. Samples were taken at 7-day intervals and subjected to peroxide value (PV) and total tocopherol analysis.

### 2.9. Determination of PV

Determination of PV was accomplished according to the official methods AOAC Association of Official Analytical Chemists (2000b). The oil sample (1 g ± 0.01 g) was dissolved in 25 mL mixture of acetic acid-chloroform (3:2 v/v) and then 0.5 mL saturated KI solution was added. The reaction solution was shaken vigorously for 1 min and kept at room temperature for 5 min in the dark. After adding 75 mL of distilled water and 1 mL of 1% starch indicator, the reaction solution was titrated with 0.001 N for 30 s until the colorless endpoint was reached. PV was calculated according to the following equation (2).

$$PV\ (meq\ O_2/kg\ oil) = (VxNx1000)/m \quad \textbf{(2)}$$

where V is the volume of sodium thiosulfate added to the oil sample (mL); N is the normality of sodium thiosulfate; m is the mass of oil sample (g).

### 2.10. Statistical analysis

The presented data (mean ± standard deviation) resulted from at least three independent experiments and analyzed by SPSS (version 23 for Windows 10 pro, SPSS Inc.). The values were analyzed by one-way analysis of variance (ANOVA) and the post hoc Tukey's test, with significance set at $p < 0.05$.

## 3. RESULTS AND DISCUSSION

### 3.1. Hydroxyl radical scavenging (HRS) activity

Among the reactive oxygen species, the $OH^{\cdot}$ radical exhibits the strongest oxidative activity. Since the $OH^{\cdot}$ radical oxidizes all classes of biological macromolecules, including lipids, proteins, and nucleic acids, it can cause oxidative damage leading to many diseases, such as arthritis, atherosclerosis, cirrhosis, diabetes, cancer, Alzheimer's disease, emphysema, and aging (Özyürek et al. 2008). The HRS activity of the TQ was evaluated according to the modified CUPRAC method. Figure 1 indicates that the TQ exhibited very a potent inhibition of $OH^{\cdot}$ , even at very low concentrations. The scavenging activities of the TQ on the $OH^{\cdot}$ radicals were 65 ± 0.49% and 80.36 ± 0.92% at concentrations of 0.8 µg/mL and 1.6 µg/mL, respectively. However, it was observed that the HRS activity increased with the increasing TQ concentration**.**

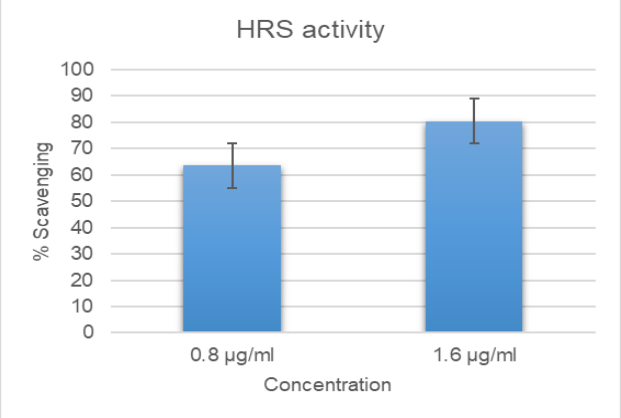


**Figure 1**. Hydroxyl radical scavenging activity of thymoquinone

### 3.2. Frap assay of total antioxidant capacity

In the FRAP method, the reducing capacity of TQ was accomplished using $Fe^{3+}$ to $Fe^{2+}$ reduction assay. In this analysis, the yellow color of the frap test solution changed to shades of green and Prussian blue depending on the concentration of the reducing agent. The presence of reducing agents acting as antioxidants in the samples causes the $Fe^{3+}$/ferricyanide complex to be reduced to the ferric form. Measuring the formation of Prussian blue of Perl at 700 nm (Gülçin et al. 2006). The absorbance values of samples and reference antioxidant substances at different concentrations at 700 nm were presented in Table 1. The higher the absorbance measured at 700 nm, the higher the reducing power. The data in Table 1 revealed that BHA had the highest FRAP value at 100 µg/mL concentration, followed by BHT, and TQ respectively. However, the absorbance value measured at 700 nm increased depending on the concentration.

**Table 1.** Total reducing power of different concentrations (50–100 µg/mL) of TQ, BHA and BHT determined by Ferriccyanide method of the $Fe^{3+}$–$Fe^{2+}$ transformation.

| Sample | FRAP value ( at 700 nm) | |
|---|---|---|
| | 50 µg/mL | 100 µg/mL |
| **BHA** | 0.548 ± 0.018* | 0.822 ± 0.018 |
| **BHT** | 0.429 ± 0.015 | 0.653 ± 0.019 |
| **TQ** | 0.143 ± 0.01 | 0.286 ± 0.009 |

* Data expressed as mean ± S.D (n=3). The higher the absorbance measured at 700 nm, the higher the reducing power.

### 3.3. Fatty acid composition analysis

The composition of EVOO and SFO fatty acids determined according to the results of GC-FID analysis were presented in Table 2. Both EVOO and SFO consisted of varying amounts of fatty acids as well as saturated fatty acids(SFAs), monounsaturated fatty acids (MUFAs), and polyunsaturated fatty acids (PUFAs). Oleic acid (18:1) contained 66.29% of the major fatty acids of olive oil. The other fatty acids of olive oil were as follows; palmitic acid (C16:0, 13.45%), linoleic acid (C18:2, 13.16%), and stearic acid (18:0, 2.3%), respectively. On the other hand, linoleic acid (18:2) was dominant in the oil obtained from safflower seeds. Oleic acid (C18:1) contained 27.12% of the fatty acids profile of safflower seeds. Oleic acid was followed by palmitic acid (C16:0, 6.28%). While the main fatty acids of the two oils were similar, their percentage amounts were different.

**Table 2.** Fatty acid composition of EVOO and SFO

| Fatty acids | EVOO | SFO |
|---|---|---|
| C16:0 | 13.45 ± 0.24 | 6.28 ± 0.11 |
| C18:0 | 2.3 ± 0.04 | 2.42 ± 0.03 |
| C18:1 cis-9 (n-9) | 66.29 ± 0.28 | 27.12 ± 0.64 |
| C18:2 cis9,12 | 13.16 ± 0.15 | 59.40 ± 0.56 |
| Σ SFA | 15.75 ± 0.28 | 8.70 ± 0.14 |
| Σ MUFA | 66.29 ± 0.28 | 27.12 ± 0.64 |
| Σ PUFA | 13.16 ± 0.15 | 59.40 ± 0.56 |
| Σ UFA | 79.45 ± 0.43 | 86.52 ± 1.20 |

C16:0, palmitic acid; C18:0, stearic acid; C18:1, oleic acid; C18:2, linoleic acid; EVOO, Extra virgin olive oil; SFO, Safflower oil. Data obtained by GC FID are expressed as relative values (%): mean % of total FAs ± SD; Σ SFA, sum of saturated fatty acids; Σ MUFA, sum of monounsaturated fatty acids; Σ PUFA, sum of polyunsaturated fatty acids; Σ UFA, sum of unsaturated fatty acids

### 3.4. Evaluation of oxidative stability

The peroxide value (PV) is used to measure the initial level of oxidation in edible oils (Iqbal and Bhanger 2007) and are the primary oxidation products. In the initial phase, molecular oxygen combines with polyunsaturated fatty acids to form peroxide and free radicals. In the presence of active methylene groups, peroxides are also unstable and turn into dihydroperoxides and secondary oxidation products. Hydroperoxides are the primary products of lipid oxidation without undesirable flavor, whereas their secondary oxidation products are mostly responsible for rancid off-flavor (Choe and Min 2007). A higher PV is attributed to a lower oxidative stability (Naghshineh et al. 2010). Changes in PV (meq $O_2$/kg oil) of EVOO and SFO incorporated with BHT and TQ during storage at 60 °C for different lengths of time were shown in Table 3. During the 28-day storage period, the PV values of olive oils with and without antioxidants increased significantly ($p < 0.05$), except for the TQ. On day 28, blank EVOO had higher PV (59.83 meq $O_2$/kg oil), while olive oil incorporated with TQ had lower PV (17.39 meq $O_2$/kg oil). At the end of storage, the PV value of olive oil (30.69 meq $O_2$/kg oil) incorporated with synthetic antioxidant (BHT) was lower than the PV value of blank olive oil, but higher than the PV value of olive oil incorporated with TQ. As a result, TQ exhibited a greater ability to delay the PV values of the oils, i.e. oils with TQ showed significantly lower PV values than oils with incorporated synthetic antioxidants. More specifically, the peroxide values of olive oil (17.39 meq $O_2$/kg oil) combined with TQ were consistent with the maximum Codex standard peroxide value (≤ 20.0 meq $O_2$/kg) for vegetable oil degradation (CAC 2019). On the other hand, peroxide values of SFO with and without antioxidants increased sharply during storage when compared to those of EVOO. This is because the degree of unsaturation of fatty acids of SFO is greater than that of fatty acids of EVOO. That is, the more double bonds the oil contains, the more it is prone to oxidation. During the storage period, PV values of SFO with and without antioxidants increased dramatically. However, the rate of increase in peroxide value of TQ-enriched SFO was lower compared to the rate of increase in peroxide value of SFO combined with Blank and BHT. However, TQ retarded the oxidation process of safflower oil after 7 days of storage (18.19 meq $O_2$/kg oil). On Day 14, SFO combined with TQ had significantly ($p < 0.05$) lower PV values than their corresponding oils with included blank and synthetic antioxidant (BHT).

**Table 3.** Changes in PV (meq O2/kg oil) of EVOO and SFO incorporated with BHT and TQ during storage at 60 °C for different lengths of time.

| Oil sample | Storagetime (days) | PV meq $O_2$/kg-oil) | | |
|---|---|---|---|---|
| | | Blank | BHT | TQ |
| **EVOO** | 0 | $7.59 \pm 0.29^a$ | $6.96 \pm 0.06^a$ | $7.50 \pm 0.32^a$ |
| | 7 | $20.04 \pm 0.37^a$ | $9.44 \pm 0.24^b$ | $9.24 \pm 0.11^b$ |
| | 14 | $35.91 \pm 1.10^a$ | $14.96 \pm 0.12^b$ | $10.85 \pm 0.26^c$ |
| | 21 | $45.36 \pm 0.50^a$ | $20.00 \pm 0.87^b$ | $11.92 \pm 0.83^c$ |
| | 28 | $59.83 \pm 0.25^a$ | $30.69 \pm 0.46^b$ | $17.39 \pm 0.99^c$ |
| **SFO** | 0 | $6.02 \pm 0.05^a$ | $4.81 \pm 0.03^c$ | $5.81 \pm 0.04^b$ |
| | 7 | $83.47 \pm 1.38^a$ | $57.36 \pm 1.13^b$ | $18.19 \pm 0.31^c$ |
| | 14 | $633.61 \pm 11.53^a$ | $114.17 \pm 3.11^b$ | $62.44 \pm 2.31^c$ |

Mean ± standard deviation. Mean values (n=3) in the same row for each oil followed by the same superscript letters are not significantly different ($p < 0.05$). Abbreviations: EVOO, extra virgin olive oil; SFO, safflower oil; PV, peroxide value; BHT, butylated hydroxytoluene; TQ, thymoquinone.

## 4. CONLUSION

Analysis of the TQ according to the CUPRAC and FRAP method showed that the total antioxidant and hydroxyl radical scavenging activity was quite effective. The oxidation protection efficiency of TQ on two different types of oils was evaluated. Research findings revealed that, compared with synthetic antioxidant (BHT), TQ was more effective in retarding the oxidation of the two types of oil. The oils with TQ incorporated exhibited much better chemical stability, including lower peroxide value. As an alternative to synthetic antioxidants, TQ could be recommended as an effective natural antioxidant to improve the stabilization of edible vegetable oils.

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No conflict of interest was reported and the authors were responsible for the content of this article. The authors alone are responsible for the content and writing of the article.

**Ethics Committee Approval**
N/A

**Peer-review**
Externally peer-reviewed.

**Author Contributions**
Conceptualization: Ü.E.; Investigation: Ü.E.; Material and Methodology: Ü.E.; Supervision: Ü.E.; Visualization: Ü.E.; Writing-Original Draft: Ü.E.; Writing-review & Editing: Ü.E.; Other: All authors have read and agreed to the published version of manuscript.

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