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**EFFECT OF ROSEMARY ESSENTIAL OIL ON LIPID PEROXIDATION
IN THE WALNUTS OIL
ВПЛИВ ЕФІРНОЇ ОЛІЇ РОЗМАРИНУ НА ПЕРЕКИСНЕ ОКИСЛЕННЯ ЛІПІДІВ
В ОЛІЇ ВОЛОСЬКОГО ГОРИХА**

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ABSTRACT

Purpose. To investigate the content of 2-thiobarbituric acid reactive substances (TBARS) in the walnuts oil with the use of rosemary essential oil (Etja, Elblag, Poland) as an antioxidant agent by monitoring the lipid peroxidation during 40 days storage period.

Methodology. Lipid oxidation was evaluated by TBARS according to the method described by Kamyshnikov with some modifications. All variables were tested for normal distribution using the Kolmogorov-Smirnov test ($P > 0.05$). Significance of differences in the lipid peroxidation biomarker in the samples (significance level at $p < 0.05$) was examined using the Mann-Whitney test according to Zar. All statistical calculations were performed on separate data from each sample with STATISTICA 8.0 software (StatSoft, Krakow, Poland).

Scientific novelty. Investigated the content of 2-thiobarbituric acid reactive substances (TBARS) in the walnuts oil with the use of rosemary essential oil Etja, Elblag, Poland.

Conclusions. This study evaluated the effects of rosemary essential oil (REO) as an antioxidant agent by monitoring the lipid peroxidation in the walnuts oil during 40 days storage period by the assessment of the content of TBARS as a biomarker of lipid peroxidation.

The effect of the REO on oxidative stability of the walnuts oil was evaluated throughout 40 days of storage. The inclusion of the REO in plant oil and storage time significantly affected TBARS values at 8 days. Lipid oxidation decreased significantly ($p < 0.05$) during storage, particularly in the control sample, which showed the highest decrease at 15 days (by 57.14 %, $p < 0.05$) and at 8 days (by 3.6 %, $p > 0.05$). The REO decreased lipid oxidation compared to the control sample by 13.6% ($p < 0.05$) at 8 days and increase by 10.9 % ($p > 0.05$) at 40 days. At 15 days, the TBARS values reached approximately $73.85 \mu\text{mols} \times \text{L}^{-1}$, corresponding to a lipid oxidation increase of approximately 19 % ($p > 0.05$) for samples enriched by REO. The present results demonstrate that the administration of REO, exhibiting free radical scavenging activity determined by TBARS assay, exerts beneficial effects on preventing lipid peroxidation in walnuts oil by limiting the TBARS level at 8 days of storage.

Key words: walnuts oil, rosemary essential oil, 2-thiobarbituric acid reactive substances, lipid peroxidation.

АНОТАЦІЯ

Мета роботи. Дослідити вміст реагуючих речовин 2-тіобарбітурової кислоти (РРТБК) в олії волоського горіха з використанням ефірної олії розмарину (Etja, Elblag, Poland) як антиоксиданту шляхом моніторингу перекисного окислення ліпідів протягом 40 днів зберігання.

Методологія. Окислення ліпідів оцінювали РРТБК за методикою, описаною Камишиниковим з деякими модифікаціями. Усі змінні були перевірені на нормальний розподіл за допомогою тесту Колмогорова-Смірнова ($P > 0,05$). Достовірність відмінностей біомаркера

перекисного окислення ліпідів у зразках (рівень значущості при $p < 0,05$) досліджували за допомогою тесту Манна-Уїтні за Zar. Усі статистичні розрахунки здійснювали за окремими даними з кожної вибірки за допомогою програмного забезпечення STATISTICA 8.0 (StatSoft, Краків, Польща).

Наукова новизна. Досліджено вміст РРТБК в олії волоського горіха з використання ефірної олії розмарину Etja, Elblag, Poland.

Висновки. У цьому дослідженні оцінювали вплив ефірної олії розмарину (EOP) як антиоксидантного агента шляхом моніторингу перекисного окислення ліпідів у олії волоських горіхів протягом 40 днів зберігання шляхом оцінки вмісту РРТБК як біомаркера перекисне окислення ліпідів.

Вплив EOP на окислювальну стабільність олії волоських горіхів оцінювали протягом 40 днів зберігання. Включення EOP до рослинної олії та час зберігання суттєво вплинули на значення РРТБК через 8 днів. Окислення ліпідів значно зменшилося ($p < 0,05$) під час зберігання, особливо в контрольній пробі, яка показала найбільше зниження на 15 добу (на 57,14 %, $p < 0,05$) та на 8 добу (на 3,6 %, $p > 0,05$). EOP зменшив окислення ліпідів порівняно з контрольним зразком на 13,6 % ($p < 0,05$) через 8 днів і збільшив на 10,9 % ($p > 0,05$) через 40 днів. Через 15 днів значення TBARS досягли приблизно $73,85 \text{ мкмоль} \times \text{л}^{-1}$, що відповідає зростанню окислення ліпідів приблизно на 19 % ($p > 0,05$) для зразків, збагачених EOP. Ці результати свідчать, що введення EOP, що демонструє активність поглинання вільних радикалів, визначену за допомогою аналізу РРТБК, сприятливо впливає на запобігання перекисного окислення ліпідів у олії волоських горіхів, обмежуючи рівень РРТБК протягом 8 днів зберігання.

Ключові слова: олія волоського горіха, ефірна олія розмарину, реактивні речовини 2-тіобарбітурової кислоти, перекисне окислення ліпідів.

Introduction

Rosemary (*Rosmarinus officinalis* Linn.) is a common household plant grown in many parts of the world used as spices in a variety of foods and employed in traditional medicine for its healing properties [2, 12]. It is used for flavoring food, a beverage drink, as well as in cosmetics; in folk medicine, it is used as an antispasmodic in renal colic and dysmenorrhoea, in relieving respiratory disorders and to stimulate the growth of hair. Extract of rosemary relaxes smooth muscles of trachea and intestine and has choleric, hepatoprotective and antitumorigenic activity [2]. Rosemary is a rich source of active antioxidant constituents such as phenolic diterpenes, flavonoids, and phenolic acids. Caffeic acid and rosmarinic acid are the most important bioactive constituents. Rosmarinic acid is the ester of caffeic acid and 3,4-dihydroxy phenyl lactic acid and is widely identified in different plant species. Chemical structure of rosmarinic acid contains two phenolic rings: one of them is derived from phenylalanine via caffeic acid

and the other from tyrosine *via* dihydroxy phenyl lactic acid [12]. The rosemary and its constituents especially caffeic acid derivatives such as rosmarinic acid have a therapeutic potential in treatment or prevention of bronchial asthma, spasmogenic disorders, peptic ulcer, inflammatory diseases, hepatotoxicity, atherosclerosis, ischaemic heart disease, cataract, cancer, and poor sperm motility [2]. Besides the therapeutic purpose, it is commonly used as a condiment and food preservative. *R. officinalis* is constituted by bioactive molecules, the phytochemicals, responsible for implementing several pharmacological activities, such as anti-inflammatory, antioxidant, antimicrobial, antiproliferative, antitumor and protective, inhibitory and attenuating activities [6].

The antioxidants, as well as plants containing metabolites with antioxidant properties, are added to maintain the quality and shelf-life of lipid-rich products. Antioxidants are capable of stabilizing free radicals by donating hydrogen (H) to free radicals or

accepting electrons from free radicals to form a complex [10, 21]. The adding of antioxidants to the final products is one strategy used to minimize deterioration during storage and consequently increase the shelf-life of the products [9, 21]. Synthetic antioxidants have been used extensively to minimize lipid oxidation in foods. However, due to an increasing concern about the safety of synthetic chemicals, the use of natural and bioactive antioxidants are preferred and have attracted the attention of researchers [3, 4, 21].

Essential oils are mixtures of volatile compounds obtained, mainly by steam distillation, from medicinal and aromatic plants. They are an alternative to synthetic additives for the food industry, and they have gained attention as potential sources for natural food preservatives due to the growing interest in the development of safe, effective, natural food preservation [13].

Therefore, it is interesting to study the progress of lipid oxidation in plant oils under the same conditions with and without the addition of rosemary extract as antioxidant. We hypothesized that rosemary essential oil would inhibit or reduce lipid oxidation in plant oils due to the antioxidative properties of the essential oil. In addition, contents of the lipid peroxidation marker in the plant oils were monitored during the storage period to investigate if lipid oxidation or food composition can have effects on the fate of bioactive compounds in the plant oils during storage. The goal of this study was to investigate the content of 2- thiobarbituric acid reactive substances (TBARS) in the walnuts oil with the use of rosemary essential oil (Etja, Elblag, Poland) as an antioxidant agent by monitoring the lipid peroxidation during 40 days storage period.

Preparation of samples. The walnuts oil was obtained from local shops. The plant oil sample (5 mL) was incubated with 0.1 mL of rosemary essential oil (REO) (Etja, Elblag, Poland) (final concentration was 20 µg/mL) at 25 °C for 40 days.

This reaction mixture was shaken gently while being incubated for a fixed interval at 25 °C. Samples were removed at 0, 8, 15, and 40 days of storage for analysis. The walnuts oil was used as the control sample.

Assay of 2-thiobarbituric acid reactive substances (TBARS). Lipid oxidation was evaluated by 2-thiobarbituric acid reactive substances (TBARS) according to the method described by Kamyshnikov (2004) with some modifications. Briefly, 0.1 mL of sample was added with 2 mL of distilled water, 1 mL of 20 % trichloroacetic acid and 1 mL of 2-thiobarbituric acid in a test tube and, the tube content was immediately vortexed. Following water bath treatment at 100 °C for 15 min, the tube content was cooled rapidly down to room temperature and centrifuged at 1000 × g for 10 min. Then, absorbance was measured at 540 nm with a spectrophotometer (Specol 11, Carl Zeiss Jena, Germany) against blind (2.1 mL distilled water and 2 mL TCA-TBA solution). TBARS were calculated as µmoles malonic dialdehyde (MDA) per L of the sample [7].

Statistical analysis. Results are expressed as the mean. All variables were tested for normal distribution using the Kolmogorov-Smirnov test ($P > 0.05$). Significance of differences in the lipid peroxidation biomarker in the samples (significance level at $p < 0.05$) was examined using the Mann-Whitney test according to Zar (1999) [22]. All statistical calculations were performed on separate data from each sample with STATISTICA 8.0 software (StatSoft, Krakow, Poland).

Results of the research

Lipid oxidation is a very complex process initiated by peroxidation of the unsaturated fatty acid in phospholipid membranes to form primary oxidation products, hydroperoxides. The hydroperoxides decompose into further secondary oxidation products, such as aldehydes, ketones, alkenes and alcohols that cause off-flavors and odors in food products [9, 21].

The effect of the REO on oxidative stability of the walnuts oil was evaluated throughout 40 days of storage. The inclusion of the REO in plant oil and storage time significantly affected TBARS values at 8 days (Fig. 1).

The effect of the interaction of the addition of REO and storage time on TBARS value in the walnuts oil was presented in Fig. 1.

Lipid oxidation decreased significantly ($p < 0.05$) during storage, particularly in the control sample, which showed the highest decrease at 15 days (by 57.14 %, $p < 0.05$) and at 8 days (by 3.6 %, $p > 0.05$). The REO decreased lipid oxidation compared to the control sample by 13.6 % ($p < 0.05$) at 8 days and increase by 10.9 % ($p > 0.05$) at 40 days. At 15 days, the TBARS values reached approximately $73.85 \mu\text{mols} \times \text{L}^{-1}$, corresponding to a lipid oxidation increase of approximately 19 % ($p > 0.05$) for samples enriched by REO (Fig. 1).

In our previous study [5], the content of 2-thiobarbituric acid reactive substances (TBARS) in the various plant oils (rapeseed oil, olive oil, rice oil) with the use of rosemary essential oil as an antioxidant agent by monitoring the lipid peroxidation during one month storage period were evaluated. Lipid peroxidation retarding capacity of rosemary essential oil was found obviously promising. These antioxidant activities seem to be attributed to antioxidant compounds present in the rosemary essential oil. The rosemary essential oil decreased lipid oxidation in the rapeseed oil compared to the control sample by 23.9 % ($p < 0.05$) at 8 days and by 9.4 % ($p > 0.05$) at 40 days. The addition of rosemary essential oil to olive oil increased significantly TBARS values only at 8 days of the storage. The reduction of the lipid oxidation was the highest at 40 days as compared to the start of the study. Rosemary essential oil added to rice oil-induced the increase of TBARS level at 8 days (by 23.7 %, $p < 0.05$) and 0 days (by 64.4 %, $p < 0.05$), respectively.

Consequently, rosemary essential oil could be successfully added to various plant oils as a natural antioxidant source [5].

Herbs and herbal extracts which contain antioxidants and aromatic substances with antimicrobial effects are commonly used in foods. In a study of Karaton Kuzgun and Gürel İnanlı (2018), the chemical, microbiological and sensory changes during storage at $2 \pm 1 \text{ }^\circ\text{C}$ of *Luciobarbus esocinus* fillets coated with edible films prepared with chitosan incorporation of thyme, clove, rosemary essential oils were examined. To create the experimental samples, a total of six groups of *L. esocinus* fillets coated with different edible films (normal, vacuum-packed, chitosan, chitosan with added thyme oil, chitosan with added clove oil, and chitosan with added rosemary) were used. The food composition of the fillets and experimental samples was determined after they had been coated with edible films. The results of the analysis showed that the preservation period of fresh fillets ended on day 12th, that of vacuum-packed fillets on day 15th, that of fillets coated with chitosan incorporation of rosemary on day 27th, that of fillets coated with chitosan incorporation of thyme and chitosan incorporation of cloves on day 30th. In comparison with the control group, fish spoilage was significantly delayed in samples coated with chitosan incorporation of thyme and chitosan incorporation of cloves ($p < 0.05$). The lowest bacterial growth, values of PV, TBA and TVB-N were obtained in fish samples coated with thyme + chitosan and cloves + chitosan. In terms of the general acceptability of the fish, as determined by qualified panelists, it was determined that the highest score was given to the experimental group to which essential oil of clove had been applied [8].

Mezza and co-workers (2018) have evaluated the antioxidant activity of rosemary essential oil fractions obtained by molecular distillation (MD) and investigate their effect on the oxidative stability of sunflower oil [11]. MD fractions were prepared in a series of low-pressure stages where rosemary essential oil was the first feed. Subsequently, a distillate (D1) and residue (R1) were obtained and the residue fraction from the previous stage used as the feed for the next. The residue fractions had the largest capacity to capture free radicals, and the lowest peroxide values, conjugated dienes, and conjugated trienes. The antioxidant activity of the fractions was due to oxygenated monoterpenes, specifically α -terpineol and cis-sabinene hydrate. Oxidative stability results showed the residues (R1 and R4) and butylated hydroxytoluene had greater antioxidant activity than either the distillate fractions or original rosemary essential oil. The residue fractions obtained by the short path MD of rosemary essential oil could be used as a natural antioxidant by the food industry [11].

Upadhyay and Mishra (2015) have classified of sunflower oil blends stabilized by oleoresin rosemary (*Rosmarinus officinalis* L.) using the multivariate kinetic approach. The sunflower oil-oleoresin rosemary blends (SORB) at 9 different concentrations (200 to 2000 mg/kg), sunflower oil-tertiary butyl hydroquinone (SOTBHQ) at 200 mg/kg and control (without preservatives) (SO control) were oxidized using Rancimat (temperature: 100 to 130 °C; airflow rate: 20 L/h). The oxidative stability of blends was expressed using induction period (IP), oil stability index and photochemiluminescence assay. The linear regression models were generated by plotting \ln IP with temperature to estimate the shelf life at 20 °C (SL₂₀; $R^2 > 0.90$). Principal component analysis (PCA) and hierarchical cluster analysis (HCA) was used to classify the oil blends depending upon the oxidative stability and kinetic parameters. The

multivariate kinetic approach effectively screened SORB1500 as the best blend conferring the highest oxidative stability to sunflower oil. This approach can be adapted for quick and reliable estimation of the oxidative stability of oil samples [20].

Sirocchi and co-workers (2017) have investigated the effect of Rosemary essential oil (REO) combined with modified atmosphere packaging conditions (MAP), i.e., aerobic, vacuum or high O₂, to extend the shelf life of beef. Beef slices were wrapped in special three-layer sheets of packaging material, some with a coating of REO (active packaging, AP), and some without REO (non-active packaging, NAP), and stored at 4°C for 20 days. The use of REO proved efficacious in every storage condition, as seen in the lower counts of psychrotrophics, *Brochothrix thermosphacta*, *Pseudomonas* spp., and *Enterobacteriaceae* in AP meat compared to NAP meat. Sensory and colorimetric analyses showed that the best packaging conditions were the high-O₂ atmosphere in combination with REO. Based on microbiological data, the shelf life of beef was 5-6 days for AP samples packaged under aerobic conditions and 14-15 days for AP samples in high-O₂ conditions [18].

Anisakidosis is caused by the ingestion of raw or undercooked fish or cephalopods containing viable *Anisakis* larvae. Several natural extracts, oils, essential oils, and their compounds have been tested against *Anisakis*. In a study of Trabelsi and co-workers (2019), the effectiveness of Tunisian olive oil with different spices or plants (cardamom, cinnamon, ginger, laurel, and rosemary) was tested against *Anisakis* larvae type 1. For the in vitro test, larvae were submerged separately in the oils mentioned above and observed to check viability. Cinnamon oil was the most effective against parasites with lethal time (LT) scores being LT₅₀ = 1.5 days and LT₁₀₀ = 3 days, followed by rosemary. Laurel, cardamom, and ginger oils were less effective.

For the *ex vivo* experiment, cinnamon, and rosemary oils were tested in anchovy fillets, previously artificially parasitized. Cinnamon was the most effective against parasites (dead after 4 days) as compared to rosemary (7 days). Therefore, the uses of cinnamon and rosemary-flavored olive oil in the industrial marinating process can be considered as an efficient alternative to the freezing process required by European Regulation EC No 853/2004 to devitalize *Anisakis* [19].

The application of rosemary essential oils as natural preservatives is recommended in meat products, especially in chicken meats. Raeisi and co-workers (2016) have conducted the study aimed to preserve the microbial quality of chicken meat fillets during storage time by using sodium alginate active coating solutions incorporated with different natural antimicrobials including nisin, *Cinnamomum zeylanicum* (cinnamon), and rosemary essential oils (EOs) which were added individually and in combination. The samples were stored in refrigeration condition for 15 days and were analyzed for the total viable count, *Enterobacteriaceae* count, lactic acid bacteria count, *Pseudomonas* spp. count, psychrotrophic count, and yeast and mold count, as well as the fate of inoculated *Listeria monocytogenes* at 3-day intervals. Results indicated that values of tested microbial indicators in all samples increased during storage. Antimicrobial agents, when used in combination, had a stronger effect in preserving the microbial quality of chicken meat samples rather than their individual use and the strongest effect was observed in samples coated with alginate solution containing both cinnamon and rosemary EOs (CEO+REO). However, all treatments significantly inhibited microbial growth when compared to the control ($P < 0.05$). Therefore, based on the results of this study, the application of alginate coating solutions containing nisin, cinnamon, and rosemary EOs as natural preservatives is

recommended in meat products, especially in chicken meats [16].

The use of dietary rosemary extract (DRE) at low doses is proposed as a nutritional strategy to improve meat preservation in the study of Ortuño and co-workers (2014). Lamb diet was supplemented with 0, 200 or 400 mg DRE (containing carnosic acid and carnosol at 1:1 w:w) per kg feed during the fattening stage. Meat quality was evaluated in lamb fillets packed under a protective atmosphere and kept in retail conditions for up to 14 days. The effects of diet and storage time were determined on different physical-chemical ($L^*a^*b^*$ color, pH, TBARS, protein oxidation and volatiles from lipid oxidation), microbial (total viable and psychrophilic bacteria, *Enterobacteriaceae*, molds, and yeasts) and sensory (appearance and odor) characteristics of the meat. The antioxidant and antimicrobial effects of DRE on meat were demonstrated. DRE delayed lean and fat discoloration, lipid oxidation, odor deterioration, and microbial spoilage, extending the shelf life of fillets from around 9 to 13 days. Both DRE doses provided similar shelf life extension [15].

The rosemary aqueous extract was used as a functional ingredient for cottage cheese in the study of Ribeiro and co-workers (2016), after proving that it possesses both higher content of phenolic compounds and antioxidant activity, compared with the corresponding hydroethanolic extract. However, a decrease of bioactivity was observed for the cheese samples enriched with the extracts in free form after seven days under storage. Therefore, in order to preserve the antioxidant activity, the rosemary aqueous extract was efficiently microencapsulated by using an atomization/coagulation technique. Overall, the introduction of both free and microencapsulated extracts provided bioactivity that was better preserved with microencapsulated extracts without changing the nutritional value of cottage cheese [17].

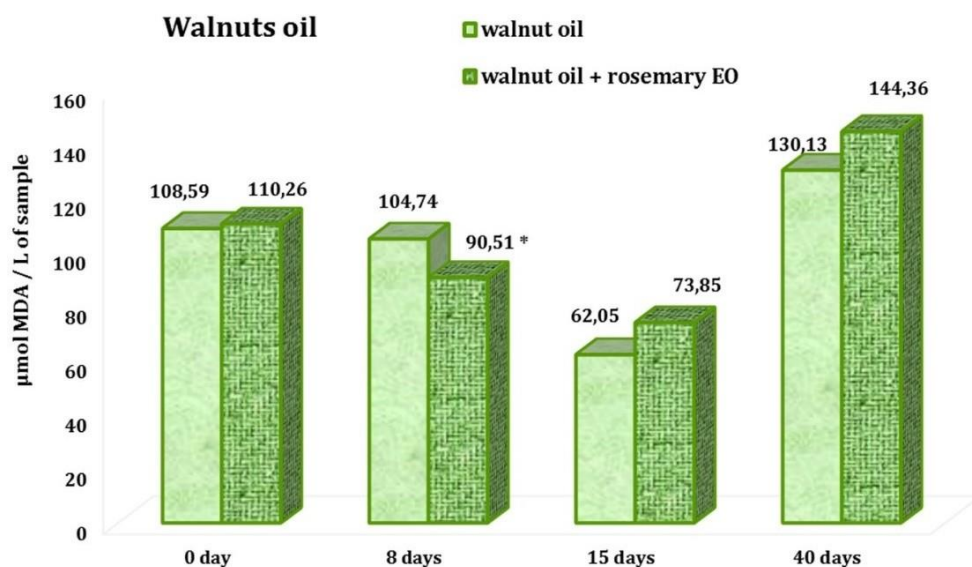


Fig. 1. The effect of the addition of rosemary essential oil and storage time on TBARS value (a biomarker of lipid peroxidation) in the walnuts oil

* means with different superscripts are significantly different ($P < 0.05$)

The effect of two levels (0.05 % and 0.4 %) of essential oil of rosemary, oregano, or garlic on protein oxidation in pork patties was studied by Nieto and co-workers (2013) during storage under modified atmosphere (MAP: 70 % O_2 : 20 % CO_2 : 10 % N_2) or under aerobic conditions (AE) at 4°C. The oxidative stability of the meat proteins was evaluated as loss of thiols for up to 9 days of storage, and as the formation of myosin cross-links analyzed by SDS-PAGE after 12 days of storage. Protein thiols were lost during storage to yield myosin disulfide cross-links. Essential oils of rosemary and oregano were found to retard the loss of thiols otherwise resulting in myosin cross-links. Garlic essential oil, on the contrary, was found to promote protein oxidation, as seen by an extreme loss in thiol groups, and elevated myosin cross-link formation compared to control [14].

Microencapsulated rosemary oil has the potential to improve the quality of button mushrooms and extend shelf-life. Effects of microencapsulated thyme

(*Thymus vulgaris* L.) and rosemary (*Rosmarinus officinalis* L.) on quality of fresh button mushroom were compared in the study of Alikhani-Koupaei and co-workers (2014). Fresh button mushrooms (*Agaricus bisporus* L.) are sensitive to browning, water loss, and microbial attack. The short shelf-life of mushrooms is an impediment to the distribution and marketing of the fresh product. Essential oils outstand as an alternative to chemical preservatives and their use in foods meets the demands of consumers for natural products. To resolve the controlled release of oil and an increase in antioxidant and antimicrobial activities, the oil was incorporated into microcapsules. Physicochemical qualities were evaluated during 15 days of storage at $4 \pm 0.5^\circ C$. All treatments prevented product weight loss and a decrease in polyphenol oxidase and peroxidase activities during storage.

Color and firmness, microbiological analysis, and total phenolic content caused the least change. With the use of microencapsulated oils, mushrooms were within acceptable limits during 10 days of storage [1].

Conclusions

In summary, the present results demonstrate that the administration of REO, exhibiting free radical scavenging activity determined by TBARS assay, exerts beneficial effects on preventing lipid peroxidation in walnuts oil by limiting the TBARS

level at 8 days of storage. At other periods of storage (15 and 40 days), the TBARS level non-significantly differed from control samples. Thus, edible adding containing essential oils have potential application in the plant oils to maintain/improve their characteristics during the shelf-life.

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