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Antioxidant Properties of Individual *vs.* Combined Extracts of Rosemary Leaves and Oak Fruit

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ABSTRACT

The aim followed in the present study was to evaluate the antioxidant activity of individual *vs.* combined extracts of rosemary leaves and oak fruit to detect the possible interactions in their antioxidant activity following combination and in order to find a way to use oak fruit natural antioxidants as an available massive source in Iran. Towards this end, methanolic extracts of rosemary and oak fruit were prepared and antioxidant activity of individual *vs.* combined extracts evaluated through 2, 2-Di Phenyl-1-Picryl Hydrazyl (DPPH) free radical scavenging activity, Total Antioxidant Capacity (TAOC), reducing power assays as well as peroxide value measurement in soybean oil. Rosemary extract revealed a significantly (P< 0.05) higher antioxidant activity than the extract of oak and than the synthetic antioxidant [butylated hydroxytoluene (BHT)] taken as standard. In combined samples, all the three kinds of interactions were observed namely: as additive effect at 50 and 150 µg ml^{-1} in DPPH assay, synergistic effect at 150 and 200 µg ml^{-1} in total antioxidant capacity assay and antagonistic effect in the process of peroxide value measurement in soybean oil. In the peroxide value measurement assay, antioxidant activity of the combined extract was significantly (P< 0.05) higher than those of the individual extracts and that of BHT. Thus, the combined extracts of oak and rosemary can be used as natural sources' to replace such synthetic antioxidants as BHT to either alleviate or prevent the oxidation process in vegetable oils.

Keywords: Antioxidant activity, Oak, Rosemary, Synergistic effect.

INTRODUCTION

Oils, fats and fat-rich foods are subjected to oxidative processes during storage and throughout the process of their production. Food oxidation results in undesirable changes in flavor, decreased nutritive value, production of anti-nutritional compounds, etc. (Gramza *et al*., 2006). Antioxidants, delay the production of free radicals and thus increase the oxidative stability of the product (Gramza *et al.,* 2006). Due to harmful effects of synthetic antioxidants on human health, interests in finding natural antioxidants with no pernicious effects are increasing (Sheng *et al.,* 2011).

Rosemary (*Rosmarinus officinalis*) is well known as a plant with antioxidant properties. It is used as a flavoring agent and as an agent of medicinal purposes in foods and beverages (Shylaja *et al.,* 2004). Major components in rosemary extract are carnosic acid, carnosol and rosmarinic acid (Cuvelier *et al*., 1994) which by affecting the activity of each other, lead to antioxidant property and free radical removal (Romano *et al*., 2009).

Oak (*Quercus branti*) contains substantial quantities of such biologically active compounds as tanins, gallic and elagic acids, as well as galoil or hexadihydroxydiphenoyl derivatives that bear antioxidant properties (Rakicet *et al*., 2007). *Quercus* is one of the

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predominant genera in northern and central parts of Iran. People in these areas have been well using the fruits of the tree in their daily traditional foods (Ghaderi *et al*., 2012).

When compounds with antioxidant properties are combined, they will have various interactions towards each other, thus possible varying effects often different from the formers could occur. The observed effect can be either synergism, antagonistic or additive.

Naturally produced antioxidants are safe and secure, within their allowed small amounts in plant materials, and if added to food, the safety aspects should be carefully noted. With synergistic effects in antioxidant activity of plant extracts, smaller quantities of each extract could be used with the pernicious effects caused by the use of extra amounts of a single extract prevented (Jain *et al*., 2011). Finally, obtaining synergistic effect by combining extracts means using safe, low cost and more useful antioxidants.

The aim of followed in the present study was a comparison of the antioxidant properties of rosemary and oak extract with those of the synthetic antioxidant BHT, and an evaluation of the possible interactions occurring in rosemary plus oak extract intermixture, and as well the possibility of using these extracts, either individually or in combined from, in preventing the oxidation of soybean oil.

MATERIALS AND METHODS

Preparation of Extracts

Rosemary (*Rosmarinus officinalis*) leaves and oak fruits (*Quercus branti)* were collected from botanical garden, Gorgan University of Agricultural Sciences and Natural Resources, Golestan, Iran and dried at room temperature, then ground to fine powder and passed through a 60-mesh sieve. Refined soybean oil with no added antioxidants was purchased from Alia Kordkuy Factory, Kordkuy, Iran. The chemicals and solvents used in the study were supplied by Sigma and Merck Companies.

Extraction

The aim of the present study being to evaluate the possible interactions between rosemary and oak extracts; thus, those available extraction methods that yielded the highest antioxidant compounds were taken from the available literatures. Methanol extract of rosemary leaves was prepared after 5 hours of extraction using Soxhlet Apparatus with pure methanol in a powder: solvent ratio of 1:22 (Tavassoli and Emam Jomeh, 2011) and while methanol extract of oak fruit being prepared after a duration of 4 hours being soaked in methanol (Ghaderi *et al*., 2012). Extracts were then filtered and the excess solvent removed through a rotary evaporator (IKA, RV05 Basic). Samples were finally dried, using freeze dryer (Operun, FDB550). Freeze dried extracts were kept in a freezer (Whirlpool WVG301) at -18°C until use.

Assessment of Total Phenols

The total phenol content of extracts was estimated through Folin-Ciocalteu (Arabshahi and Urooj, 2007). Some 20 µg of extract was combined with 1.16 ml distilled water and 100 µl Folin-Ciocalteu reagent. With a passage of 1 through 8 minutes, 300 µl sodium carbonate (20%) was added. The mixture was incubated at 40°C for 30 minutes. The absorbance of the mixture was recorded at 760 nm through UV-visible spectrophotometer (PG instrument Ltd T80+). Total phenols were expressed as gallic acid equivalents using the following linear equation, based on the calibration curve (concentrations 50 to 750 µg ml^{-1} of gallic acid):

$$A= 0.023\ C+0.109,\ R^2= 0.997, \quad (1)$$

Where, *A* stands for the absorbance at 760 nm and *C* denotes concentrations as gallic acid equivalents (µg ml^{-1}).

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DPPH Radical Scavenging Activity

The capability of the extracts to scavenge DPPH free radical was evaluated according to the method of Arabshahi and Urooj (2007). Some 3 ml of sample (freeze dried sample dissolved in methanol with concentrations of 50 to 250 µg ml^{-1}) was added to 1 ml of a 1 mM methanolic solution of DPPH. Samples were vortex shaken and left in dark for 30 minutes. The absorbance of the samples was recorded at 517 nm with the percentage of scavenging activity found out from the following equation:

$$DPPH\ S.A.(\%) = \frac{AC - AS}{AC} \qquad (2)$$

DPPH S. A. is DPPH scavenging activity and AC is Absorbance of control and AS is Absorbance of Sampel.

Total Antioxidant Capacity

The basis for this assay was the reduction of Mo (VI) to Mo (V) and formation of a green phosphate/Mo (V) complex in acidic conditions (Prieto *et al*., 1999). Some 0.1 ml of the sample was mixed with 1 ml of reaction solution (0.6M sulphuric acid, 28 mM sodium phosphate, and 4 mM ammonium molybdate) and incubated for 90 minutes at 95°C. The absorbance was recorded at 695 nm. The total antioxidant activity was expressed as absorbance of the sample. A higher absorbance value would indicate a higher antioxidant activity (Li and Wang, 2011).

Reducing Power

This assay was performed to evaluate the capability of the extracts to reduce iron (III) by the method of Yildirim *et al*. (2001). A 1 ml of the sample was mixed with 2.5 ml of phosphate buffer (0.2M, pH 6.6) and 2.5 ml of potassium ferricyanide ($K_3Fe(CN)_6$; 10 g l^{-1}) and incubated for 30 minutes at 50°C. Some 2.5 ml of trichloroacetic acid (100 g l^{-1}) was added to the incubated solution and centrifuged with a centrifuge (Centurion K2042) for 10 minutes. Finally, 2.5 ml of the supernatant was mixed with 2.5 ml of distilled water and 0.5 ml $FeCl_3$ (1 g l^{-1}). The absorbance was then recorded at 700 nm. Reducing power was then expressed as the absorbance of the sample. Higher absorbance means higher reducing power (Arabshah and Urooj, 2007).

Calculation of Synergistic Effects (SEs) of Antioxidant Mixtures

To compare the antioxidant activity trait of individual *vs.* combined extracts, the *SE* (Synergistic Effect) was calculated from the following equation:

$$SE = \frac{Experiment\ value}{Theoretical\ value} \qquad (3)$$

According to Queirós *et al*. (2009) and Viera *et al*. (2012), the theoretical values were found out as the average of individually observed quantities for each one of the two combined extracts, and while the experimental values coming from the observed figures for the combined extracts. *SE>1* is indicative of synergistic effect, *SE= 1* represents the additive effect and while *SE<1* standing for an antagonistic effect (Fuhrman *et al*., 2000).

GC Analysis of Soybean Oil

To determine the fatty acid profile of soybean oil for the study, a GC system (GC Agilent Technology 6890N) equipped with capillary column (Agilent Technology DB-23, 60 m×0.25 mm×0.25 µm) was made use of.

Antioxidant Activity, Soybean Oil

According to the results obtained from former assays, a mixture with minimum amount of individual extracts that had

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exhibited synergistic effects along with the related individual extracts, control sample (without antioxidant) and BHT were used for this assay. Samples were stored in an oven of 60°C temperature. Oxidative stability was determined through an assessment of Peroxide Value (PV) and in 5-day intervals up to 25 days (Azizkhani and Zandi, 2009). The Peroxide Value (PV) was determined according to AOCS (2003) method and considered as the number of days needed for *PV* of the samples to reach 20 (meq O_2 kg^{-1}) of fat. This is in line with the general agreement that oils become rancid at *PVs* higher than 20 (Economou *et al*., 1991; Hras *et al*., 2000).

Synergism in Oil System

Percentage synergism was evaluated from the following equation (Bishov *et al*., 1977):

$$Syn\% = \frac{(IP_m - IP_c) - (IP_1 - IP_c) - (IP_2 - IP_c)}{IP_m - IP_c} \times 100 \quad (4)$$

Where, IP_m and IP_c respectively stand for the induction periods of oil with antioxidant, and control sample containing no antioxidant. The IP_1 and IP_2 sybols stand for the induction periods of oil sample containing an antioxidant. Positive figures would indicate synergism, and while negative ones antagonism.

Statistical Analysis

The data are all reported as mean±standard deviations of three replicates. One-way Analysis Of Variance (ANOVA) was employed to compare the means related to the evaluated parameters. Differences were considered as significant at $P< 0.05$.

RESULTS AND DISCUSSION

Total Phenols

Because of the capability to scavenge free radicals through their hydroxyl groups, phenols are considered as among the important plant components (Hatano *et al*., 1989). Varying levels of total phenols in different study cases are related to the type of plant, its origin and as well the extraction conditions. Within the present study, a total phenol content of rosemary extracts (45.71±1.84 mg gallic acid g^{-1} of dried sample) was significantly higher than the total oak phenol content (22.64±3.6 mg gallic acid g^{-1} of dried sample). Tavassoli and Emamjome (2011) reported the level of total phenols in methanol extract of rosemary as 49.9 mg gallic acid g^{-1} of dried sample.

The type and volume of solvent, temperature and state of material are some of the effective parameters in the efficiency of extraction. There are also such other factors (affecting the efficiency) as selection and saturation of the solvent. Rhim *et al*. (2009) studied the effect of drying methods on plant Jiwhang and demonstrated that most of the drying methods (hot air, solar) have undesired effects on antioxidant activity. Thus, a suitable drying method before the extraction can have a substantial effect on antioxidant components (Anwar *et al*., 2013). Throughout the present study, room temperature together with natural air flow was employed the processes of drying.

Several studies have reported direct relationships between total phenol content and antioxidant activity, but on the other hand, some scientists like Kahkonen *et al*. (1999) and Mata *et al*. (2007) have found that the antioxidant activity of plant extracts was not necessarily related to total phenol content. Most of the plant components may get dissolved in the solvent but some of them may exhibit weak antioxidant activity or not bear any antioxidant activity at all (Anwar *et al*., 2013).

DPPH Radical Scavenging Activity

The radical scavenging activity of extracts is presented in Figure 1. For all the concentrations, rosemary extract was of a significantly ($P< 0.05$) higher activity than

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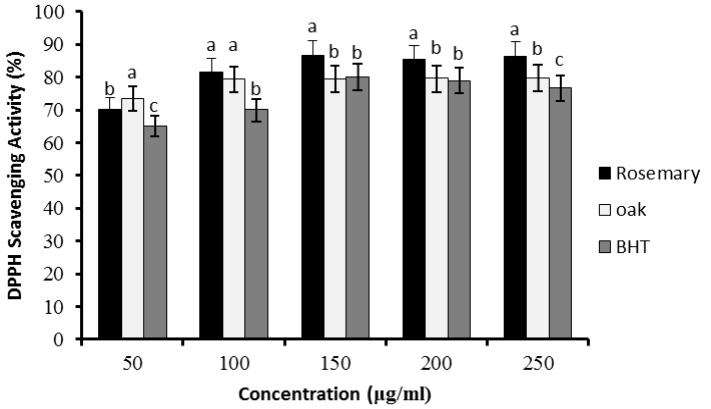

Figure 1. 2-Di Phenyl-1-Picryl Hydrazyl (DPPH) radical scavenging activities of methanol extracts of rosemary, oak and butylated hydroxytoluene (BHT).

BHT and, except for 50 µg ml^{-1}, rosemary extract was a more efficient scavenger than the oak extract. This can naturally be attributed to the content and more possibly to the composition of endogenous antioxidant components of extracts (Farhoosh *et al*., 2013). There was a direct relationship found between an increase in concentration and scavenging activity, up to 150 (µg ml^{-1}), but higher concentrations had no effect in increasing the activity. The free radical scavenging activity is due to hydrogen and/or electron donation (Bidchol *et al*., 2011) which might prevent reactive radical species from reaching such biomolecules as lipoproteins, polyunsaturated fatty acids (PUFA), DNA, amino acids, proteins and sugars in susceptible biological and food systems (Arabshahi and Urooj, 2007). As regards the present study, a proper relationship was found between the total phenol content and free radical scavenging activity.

A combination of the two extracts exhibited additive effect (SE> 1) in two concentrations (50 and 150 µg ml^{-1}) and in other concentrations, the observed effect was of an antagonism one (Figure 2). According to previous studies (Liu *et al*., 2008), results

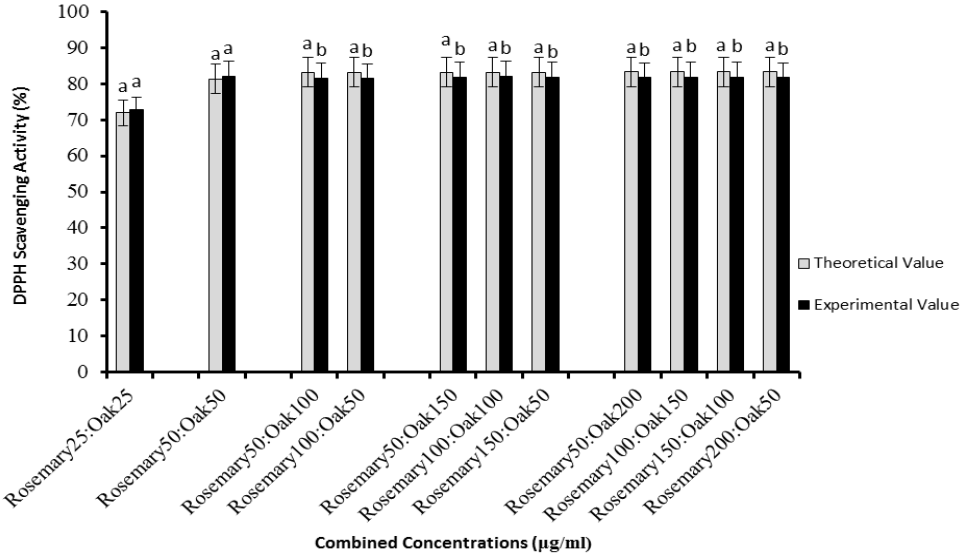

Figure 2. Experimental and theoretical scavenging capacity values of rosemary/oak extract at their different combinations.

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showed a relationship between the concentration of indivitual extracts and *SE*, although, there was no linear relation established between an increase in concentration of one extract in combination, and synergism effect.

Total Antioxidant Capacity

Total antioxidant capacity assay is a method to quantify antioxidant capacity of water and fat soluble compounds. Comparing Total AntiOxidant Capacity (TAOC) of extracts showed that, TAOC of rosemary in two concentrations of 50 and 100 µg ml^{-1} was significantly higher than that of BHT, but with an increase in the concentration up to 150 µg ml^{-1}, there was no significant difference found between rosemary *vs.* BHT and in higher concentrations, BHT was of a higher TAOC (Figure 3), although, TOAC for rosemary increased with an increase in concentration of the extract. Oak in none of the tested concentrations could compete with BHT. TAOC of rosemary was significantly higher than that of oak in all the tested concentrations. Therefore, rosemary extract is proved to have a higher ability in electron donation and can be used as a terminator in electron chain and turn active free radicals into more stable non-radical ones. Raghu *et al*. (2011) suggested that antioxidant activities of different plants are different and this variation can be due to the difference in the structure of their phenolic compounds, or to their hydroxylation and methylation patterns. Plant extracts may have other such antioxidant components as proteins, ascorbate, β-carotene, α-carotene, etc. that are effective in increasing TAOC.

As shown in Figure 4, after being combined, rosemary and oak exhibited all the three kinds of interactions in TAOC assay, synergistic effect at concentrations of 150 and 200 µg ml^{-1}, additive effect at 200 and 250 µg ml^{-1}, as well as antagonistic effect. Synergism occurred when the concentration of oak was 2 to 3 times of rosemary, but increases in concentration and change in the ratio, led to additive effect. Heo *et al*. (2007) evaluated TAOC of individual *vs.* combined phenolic compounds in model systems. The phenolic compounds they used were from among such major types prevalent in fruits and vegetables as, catechins, chlorogenic acid, cyanidine, etc. These compounds benefited from a significant TAOC individually, whereas after being combined two or three, no synergism was observed with the only effect being of an additive one. They suggested that to evaluate TAOC in complex plant extracts, the type and concentration of phenolic compounds existing in the extract, and as well the TAOC for each one of them must be investigated and taken into

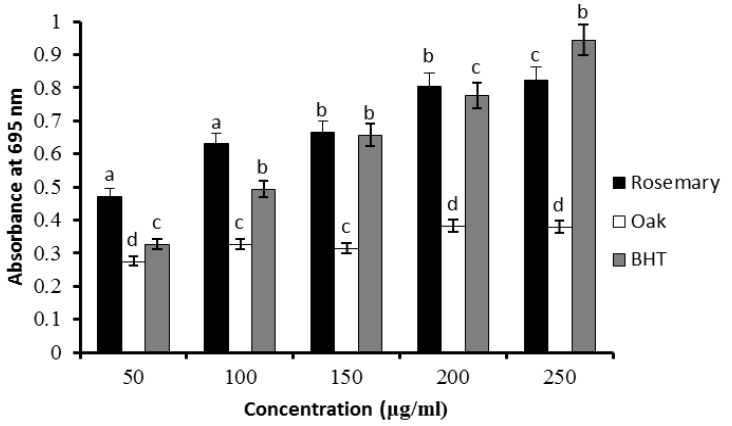

Figure 3. Total antioxidant activities of methanol extracts of rosemary, oak and BHT.

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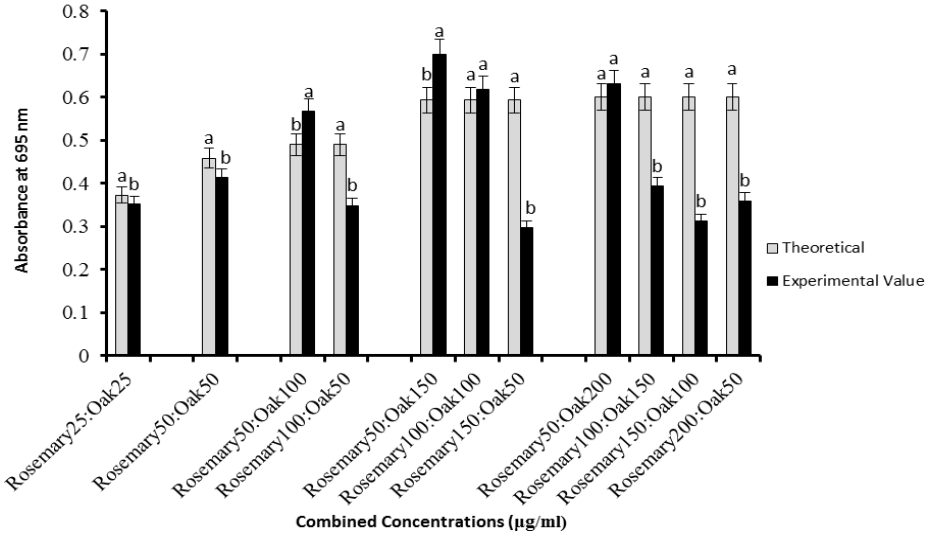

Figure 4. Experimental *vs.* theoretical TAOC values of rosemary/oak extract at their different combinations.

consideration.

Reducing properties are generally related to the presence of reducing compounds (Pin 1998) that with hydrogen donating, lead to free radical chain breaking (Gordon 1990), reductones are also reported to react with certain precursors of peroxide, thus preventing peroxide formation (Bidchol *et al*., 2011). As shown in Figure 5, the reducing power of rosemary extract is in competence with BHT (except in concentration of 100 µg ml^{-1}). The reducing power of oak extract was significantly lower than those of both rosemary extract and BHT.

A combination of rosemary and oak extracts showed a significant synergistic effect in all the combined concentrations (Figure 6). The suggested mechanism for synergistic effect is electron transferring from the component of low antioxidant activity to the component with a higher antioxidant activity and a retrieval of this component so that it can give its hydrogen group to another free radical and continue the process. An increase or decrease in the concentration of one or more compounds may influence these interactions and decrease the antioxidant activity (Young and Lowe, 2001). Hidalgo *et al*. (2010)

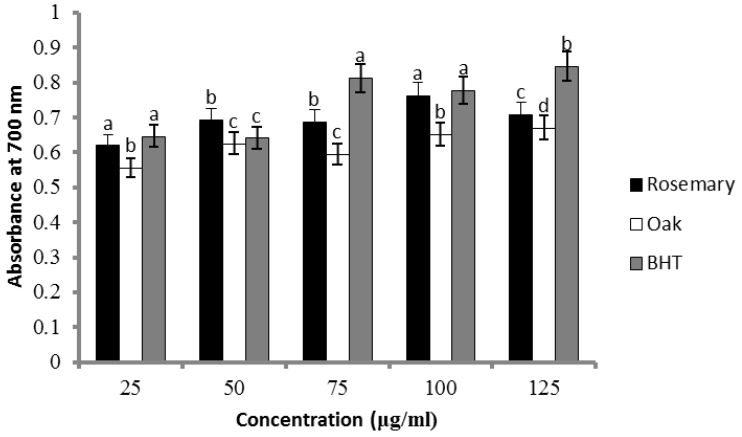

Figure 5. Reducing power of methanolic extracts of rosemary, oak and BHT.

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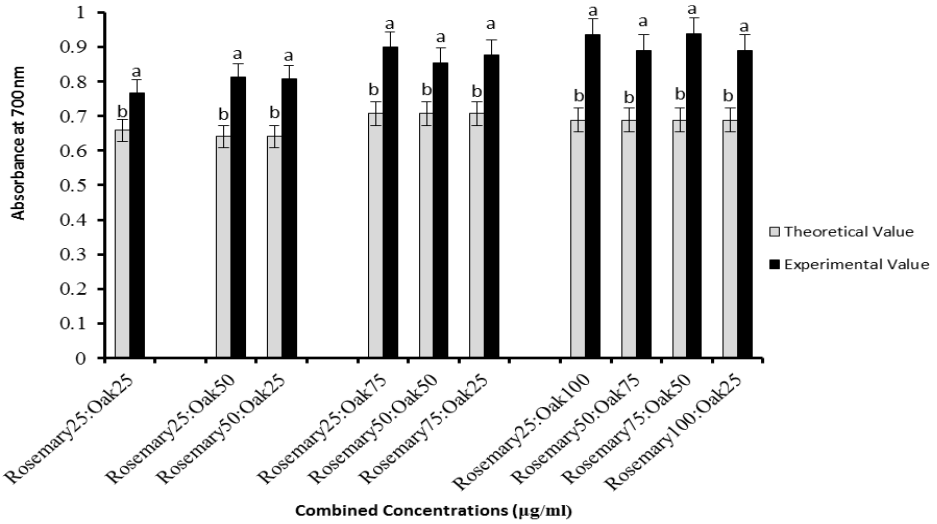

Figure 6. Experimental *vs*. theoretical reducing power values of rosemary/oak extract at their different combinations.

evaluated the interactions of flavonoids. They demonstrated that although results from different assays were variant, this variation could be explained by the difference in chemical nature and reactivity of compounds and as well the nature of solvents. In general, the antioxidant potential of a given antioxidant depends on its structure, for example, the site of hydroxyl groups and double bonds. All these mechanisms are affected by the presence of glycosides, number and situation of hydroxyl and methoxyl groups and as well, the interactions that change the structure. Consequently, the final antioxidant activity is determined from a combination of these factors. In total, all these factors influence the type of interaction between antioxidants and antioxidant mechanisms of the extracts.

GC Analysis of Soybean Oil

Results of fatty acid profile of soybean oil showed that it contained 10.86% Palmitic, 2.1% Stearic, 22.35% Oleic, 53.11% Linoleic, and 6.73% Linolenic acids, and as well 4.85% of other fatty acids.

Antioxidant Activity in Soybean Oil

Results related to a measurement of peroxide value during a 20 day period of 5 day intervals are presented in Figure 7. There were significant differences observed between antioxidant ability of different extracts and BHT indicating the capability of these extracts to be used as a substitutes for BHT when even in their small amounts. The combined extract of rosemary and oak showed the lowest *PV* with its Induction Period (IP) of 12.5 days, although there was no synergistic effect in this sample, it increased the *IP* of oil to a further extent than BHT. The control sample with no antioxidant bore the lowest *IP* (10.9). Consequently, it can be said that, use of extracts in complex situation of oil system influences its activity and type of interaction and antioxidant activity of individual and combined extracts in oil systems should be evaluated as a separate assay along with other assays.

Marinova *et al*. (2008) suggested that, as lipid oxidation is a complex and multi-stage process, antioxidant activity in preventing each one of these stages should be studied; because, hydroperoxide production and

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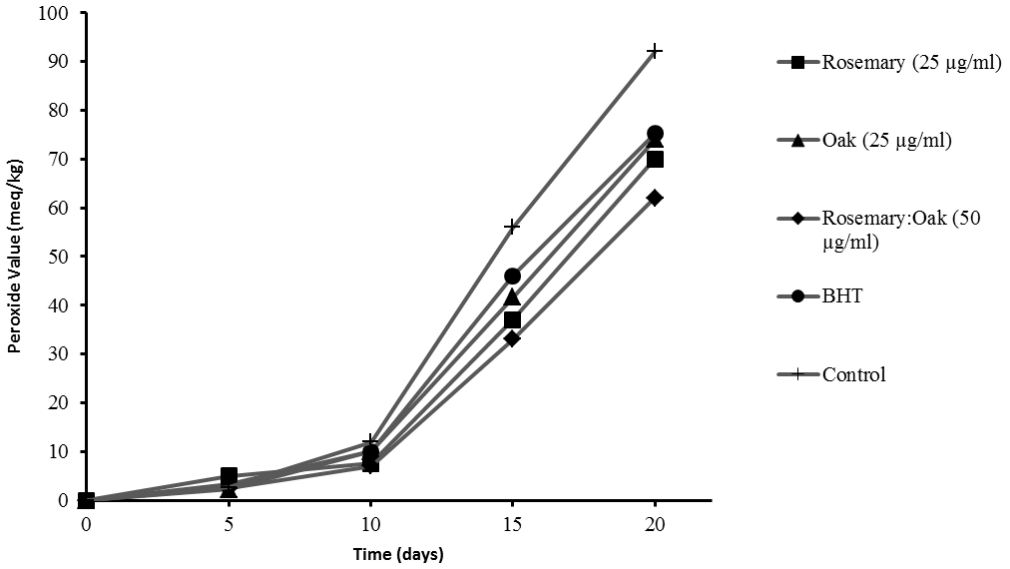

Figure 7. Peroxide values of samples containing different levels of antioxidants during their storage at 60°C.

destruction are separable stages that can be affected by antioxidants in some completely differing mechanisms.

CONCLUSIONS

There was substantial relationship observed between total phenols and antioxidant activity. Rosemary extract, with a higher total phenol content, exhibited a higher antioxidant activity than oak extract. Combined extracts (in different assays) showed different behaviors with all the three kinds of interactions (synergistic, antagonistic and additive effect) observed. Different behavior of the combined extracts can be attributed to the chemical properties, nature and reactivity of the components of the extracts. Individual compounds may suffer polymerization, leading to structural changes, causing different antioxidant activities. In the present study, oak fruit exhibited a weak antioxidant activity, but results indicated that a combination of oak extract with rosemary extract can produce an extra considerable antioxidant activity even when mixed in very low concentrations (50 µg ml^{-1}). Consequently, a combined extract of oak and rosemary can be utilized as an effective natural source to replace such synthetic antioxidants as BHT to alleviate or prevent oxidation in vegetable oils.

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ویژگی‌های آنتی‌اکسیدانی عصاره‌های برگ رزماری و میوه بلوط به صورت انفرادی و ترکیبی

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چکیده

هدف از مطالعه حاضر بررسی فعالیت عصاره‌های رزماری و بلوط به صورت انفرادی و ترکیبی به منظور تشخیص برهم‌کنش‌های احتمالی در فعالیت آنتی‌اکسیدانی آنها پس از ترکیب شدن بود تا بتوان روشی مناسب برای استفاده از آنتی‌اکسیدان‌های بلوط به عنوان یک منبع در دسترس و فراوان در ایران یافت. به این منظور، فعالیت آنتی‌اکسیدانی عصاره متانولی رزماری و بلوط به صورت انفرادی و ترکیبی توسط آزمون مهار رادیکال آزاد DPPH، ظرفیت آنتی‌اکسیدانی کل، قدرت احیاکنندگی و آزمون

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پراکسید در روغن سویا بررسی شد. فعالیت آنتی‌اکسیدانی رزماری به شکل معنی‌داری (P<۰/۰۵) بیشتر از بلوط و آنتی‌اکسیدان سنتزی BHT (به عنوان استاندارد) بود. در نمونه‌های ترکیبی، هر سه نوع برهم‌کنش شامل جمع‌پذیری در غلظت‌های ۵۰ و ۱۵۰ میکروگرم/ میلی‌لیتر در آزمون مهار رادیکال DPPH، سینرژیسم در غلظت‌های ۱۵۰ و ۲۰۰ میکروگرم/ میلی‌لیتر در آزمون ظرفیت آنتی‌اکسیدانی کل و آنتاگونیسم در آزمون اندازه‌گیری عدد پراکسید در روغن سویا، مشاهده شد. در آزمون پراکسید، فعالیت آنتی‌اکسیدانی عصاره ترکیبی به شکل معنی‌داری (P<۰/۰۵) بیشتر از عصاره‌های انفرادی و BHT بود. بنابراین عصاره‌های ترکیبی رزماری و بلوط را می‌توان به عنوان منبعی طبیعی برای جایگزینی آنتی‌اکسیدان‌های سنتزی در جلوگیری از اکسیداسیون روغن‌های گیاهی معرفی نمود.

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