Evaluation of antioxidant interactions in combined extracts of green tea (*Camellia sinensis*), rosemary (*Rosmarinus officinalis*) and oak fruit (*Quercus branti*)

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Abstract Green tea (*Camellia sinensis*), rosemary (*Rosmarinus officinalis*) and oak fruit (*Quercus branti*) are of known medicinal plants used in traditional medicine. They provide substantial antioxidant activities but the possible antioxidant interaction between them has not been studied. In the present study first the bioactive compounds from these three plants were first extracted and thereafter assayed for total phenols, 2, 2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity, total antioxidant capacity (TAOC) and reducing power. In addition, the antioxidant properties of the extracts individually and in combinations were evaluated in soy bean oil as food system. There was a direct relation between total phenolics and antioxidant activities of extracts. Green tea and oak fruit extracts had the highest and least activity, respectively. All three kinds of interactions (synergistic, antagonistic and additive) were observed. In soy bean oil, the only effect was antagonism but even with this effect, combined extract was significantly ($P<0.05$) better than butylated hydroxytoluene (BHT) and control sample. Results showed that these three natural extracts and their combination can be effectively used as a substituent of synthetic antioxidant BHT.

Keywords Green tea · Rosemary · Oak fruit · Synergism · Antagonism

Introduction

Antioxidants are widely used to reduce or prevent oxidation in natural food systems (Kumar 2006). Synthetic antioxidants that are commonly used have some toxic and carcinogenic effects, thus inclination to natural antioxidants is increasing (Sheng et al. 2011). Natural antioxidants like green tea (*Camellia sinensis*), rosemary (*Rosmarinus officinalis*) and oak fruit (*Quercus branti*) are rich source of catechins, terpenoids and organic acids with potential antioxidant properties (Bhagwat et al. 2003; Aguilar et al 2008; Cuvelier et al. 1994; Romano et al. 2009; Ghaderi et al. 2012).

In the present study, antioxidant free soy bean oil was used as oxidative food system. Soy bean oil is widely used in Iran and it is highly prone to oxidative reactions because of its high content of unsaturated fatty acids.

Plant antioxidants are natural and safe when used in small amounts from their natural source, but when use in larger amounts to prevent oxidation of food systems, the safety effects are unknown.

When some compounds with antioxidant properties are combined, different interactions may occur showing various effects that may be synergistic, antagonistic or additive. These effects are not definite properties for plant extracts and drugs, but they are widespread properties related to the way they are explained and their ratios (Borgert et al. 2005). Jain et al. (2011) showed that a synergistic effect would enhance the antioxidant activity and thus make it possible to use lower doses of each extract and prevent the side effects of using large amounts of individual plant extracts.

There are some studies about antioxidant properties of green tea (Frankel et al. 1977; Gramza et al. 2006; Bozkurt 2006; Shalini and Sudha 2010; Ziaedini et al. 2010), rosemary

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(Lalas and Dourtoglou 2003; Marutti et al. 2008; Nasiri 2012) and oak fruit (Ghaderi et al. 2012) that show the antioxidant activity of their extracts. Also, possible interactions for combined extracts of these natural antioxidants that show the ability of using very low doses of each individual extract in preventing oxidation in food systems which is the main purpose of present study has still not been evaluated even though some other studies has evaluated interactions between some antioxidants compounds (Romano et al. 2009; Hidalgo et al. 2010; Jain et al. 2011; Li et al. 2011; Yin et al. 2012; Chanda et al. 2013). The aim of the present study was to evaluate antioxidant properties of green tea, rosemary and oak fruit extracts individually and in combination and try to find specific combinations with least concentrations that exhibit synergistic effect which may help to use natural and safe antioxidants in very low doses instead of synthetic antioxidants.

Materials and methods

Materials

Rosemary (*Rosmarinus officinalis*) leaves and oak fruits (*Quercus branti*) were collected from the botanical garden of Gorgan University of agricultural Sciences and natural resources, Golestan, Iran and green tea (*Camellia sinensis*) leaves were collected from the tea fields of Ramsar, Mazandaran province, Iran. All the plants were harvested three times and final weight for each plant bulk sample was 5 kg. The leaves and fruits were dried at room temperature for 4 days to save components from heat damage (Rhim et al. 2009), then ground to fine powder and passed through a 60-mesh sieve. The refined soy bean oil without added antioxidants was purchased from Alia Kordkoy Factory, Kordkoy, Iran. All the chemicals and solvents used in this study were supplied by Sigma, Merck and Aplichem.

Extraction

Ethanol extract of green tea leaves were obtained after 24 h soaking of 1 g powder from the crushed leaves in 10 ml ethanol 95 % (Gramza et al. 2006). Methanol extract of rosemary leaves was prepared after 5 h of soxhlet extraction with pure methanol in a powder to solvent ratio of 1: 20 (Tavassoli and Emam Jomeh 2011). Methanol extract of oak fruit was prepared after soaking of 1 g of the crushed powder in 10 ml methanol for 4 h (Ghaderi et al. 2012). Extracts were then filtered and excess solvents removed with a rotary evaporator (IKA, RV05 Basic). Finally, samples were freeze-dried (Operun, FDB550 freeze drier). Freeze dried extracts were kept at -18 °C until use.

Sample preparation

Samples were prepared and used for the different experiments as follows.

Individual extracts: green tea, rosemary, oak and BHT were used in concentrations of 50, 100, 150, 200 and 250 µg/ml (freeze dried powder in the same solvent used for extraction).

Combined extracts: combinations of the three individual herbal extracts were made in concentrations of 150, 200 and 250 µg/ml of methanol.

Antioxidants prevent oxidations through different mechanisms, thus for the present study, different methods are used to perform a proper comparison.

Total phenol content

The total phenol content of extracts was estimated by Folin-Ciocalteu (Arabshahi and Urooj 2007) and expressed as gallic acid equivalents using the following linear equation based on the calibration curve:

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

Where A is absorbance at 760 nm and C is concentrations of gallic acid equivalents (µg/ml).

DPPH radical scavenging activity

The ability of extracts to scavenge DPPH free radical was evaluated according to the method of Arabshahi and Urooj (2007). Three milliliters of samples was added to 1 ml of 1 mM methanolic solution of DPPH. Samples were vortexed and left in dark for 30 min. The absorbance of each sample was measured at 517 nm and the percentage of scavenging activity calculated from the following equation:

DPPH scavenging activity (%)

$$= \frac{\text{Absorbance of control} - \text{Absorbance of sample}}{\text{Absorbance of control}} \quad (2)$$

Total antioxidant capacity

This assay is based on the reduction of Mo (VI) to Mo (V) by the sample and the subsequent formation of a green phosphate/Mo (V) complex at acidic pH (Prieto et al. 1999). The assay was done using the method of Prieto et al. (1999). 0.1 ml of sample was mixed with 1 ml of reaction solution (0.6 M sulphuric acid, 28 mM sodium phosphate, and 4 mM

ammonium molybdate) and incubated for 90 min at 95 °C. and the absorbance of samples was measured at 695 nm.

Reducing power

The ability of extracts to reduce iron (III) was evaluated using the method of Yildirim et al. (2001). Samples (1 ml) were mixed with 2.5 ml of phosphate buffer (0.2 M, pH 6.6) and 2.5 ml of potassium ferricyanide ($K_3Fe(CN)_6$; 10 g l⁻¹) and incubated for 30 min at 50 °C. Then 2.5 ml of trichloroacetic acid (100 g l⁻¹) were added to the solution and centrifuged for 10 min. Finally, 2.5 ml of supernatant was combined with 2.5 ml of distilled water and 0.5 ml $FeCl_3$ (1 g l⁻¹). The absorbance of samples was measured at 700 nm. Higher absorbance means higher reducing power.

Calculation of synergistic effects (SEs) of antioxidant mixtures

To compare the antioxidant activity of individual and combined extracts, the SE (synergistic effect) was calculated from the following equation:

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

Theoretical values were calculated as the average of individual observed amounts for each one of two combined extracts, and experimental values came through the observed amounts for combined extracts (Queirós et al. 2009; Viera et al. 2012). $SE > 1$ is synergistic effect, $SE = 1$ is additive effect and $SE < 1$ is antagonistic effect (Fuhrman et al. 2003).

GC analysis of soy bean oil

To determine the fatty acid profile of soy bean oil for this study and make sure that the oil is pure and it is not mixed with other vegetable oils, a GC system (GC Agilent Technology 6890N) equipped with capillary column (Agilent Technology DB-23, 60 m×0.25 mm×0.25 μm) was applied.

Antioxidant activity in soy bean oil

The appropriate sample that met the necessary conditions and showed synergism in lowest combined concentration was selected for this assay, with control sample (without antioxidant) and BHT. Samples were stored in a 60 °C oven. Peroxide value (PV) was measured in 5-days intervals up to 25 days (Azizkhani and Zandi 2009) and determined according to AOCS (2003) method. Induction period was measured as days needed to

Table 1 Total phenolic content of green tea, rosemary and oak

Sample	Total phenol content*
Green tea	76.15±4.2 ^a
Rosemary	45.71±1.84 ^b
Oak	22.64±3.6 ^c

Values in the same column followed by different letters are significantly different ($P < 0.05$)

* g of gallic acid per 100 g (dry weight) of extract

reach PV 20 (meqO₂/kg of fat). This is in agreement with general consideration that oils become rancid at PV higher than 20 (Economou et al. 1991; Hras et al. 2000).

Synergism in oil system

The percentage of synergism was calculated from the following equation (Bishov et al. 1977):

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

Where IP_m and IP_c are induction period of oil with antioxidant, and induction period of control sample without antioxidant, respectively, and IP_1 and IP_2 are induction period of oil sample containing an antioxidant. Positive amounts indicate synergism and negative amounts indicate antagonism.

Statistical analysis

All data are reported as mean±standard deviation of three replicates. One-way analysis of variance (ANOVA) was used to compare the means of all evaluated parameters. Differences were considered significant at $P < 0.05$. Calculations were done by SAS 9.1.3 Portable software.

Fig. 1 DPPH radical scavenging activities of methanol extracts of green tea, rosemary, oak and BHT

Fig. 2 Experimental and theoretical scavenging capacity values of green tea/rosemary/oak extract at different combinations

Results and discussion

Total phenol content

Phenols are important components due to their hydroxyl groups and scavenging properties and may have a direct relation with antioxidant activity (Bidchol et al. 2011). Total phenol content of green tea, rosemary and oak are presented in Table 1. A significant ($P < 0.05$) difference between total phenol contents of these three extracts were observed. The highest amount of total phenols was given by green tea, while oak had the least total phenolic content. The observed difference is due to the type of plant, extraction conditions, solvent polarity and solubility of components. A proper drying method before the extraction can have a remarkable effect on total phenols extraction (Anwar et al. 2013). Rhim et al. (2009) reported that most drying methods have an undesired effect on antioxidant activity. In the present study, drying conditions were room temperature and air convection that are not destructive.

DPPH radical scavenging activity

DPPH scavenging activity assay is widely used to evaluate the ability of compounds to scavenge free radicals or donate hydrogen, and determine the antioxidant activity in foods (Bidchol et al. 2011). Figure 1 shows the DPPH scavenging activity of green tea, rosemary and oak extract compared to BHT. The scavenging activity of all concentrations of green tea and rosemary extracts used were significantly ($P < 0.05$) higher than that of BHT. Most concentrations of oak extract used equally showed higher activity than BHT except the concentrations 150 and 200 μg/ml which had equal scavenging activity with BHT which means that increasing the concentration of oak extract more than 100 μg/ml cannot increase its scavenging activity. Scavenging activity of green tea and

rosemary extract were concentration dependent up to 150 μg/ml while oak extract showed no dependency on extract concentration and increasing the oak fruit extract concentration did not increased the radical scavenging activity.

Radical scavenging activity of extracts is attributed to their ability to donate hydrogen group (Bidchol et al. 2011). In the present study, green tea extract which had the highest total phenol contents showed the highest radical scavenging activity while oak fruit extract gave the least DPPH and total phenolic content.

Combining extracts can lead to various interactions and different effects. The amount of antioxidant components do not necessarily reflect the total antioxidant content of extracts because a synergistic effect in the extract is possible. After combining green tea, rosemary and oak extracts in different ratios, for radical scavenging assay, an additive effect was only observed in concentration 150 μg/ml while other combinations showed antagonism (Fig. 2). Shi and Qu (2004) reported that there is no direct relationship between lycopene concentration and synergistic effect. In the present study, there

Fig. 3 Total antioxidant activities of methanol extracts of green tea, rosemary, oak and BHT

were equally no relationship between natural extracts concentrations and synergistic effect and even increasing concentrations had no effects.

Total antioxidant capacity

Extracts with higher electron donating activity can terminate the radical chain and turn free radicals into more stable products (Pan et al. 2011). The extracts in the present study were shown to have considerable amounts of phenolic compounds that can donate electrons. However, each extract behaved differently from the others, no doubt due to differences in plants types and concentrations. Results (Fig. 3) showed that at concentrations 50 to 250 $\mu\text{g/ml}$, green tea extract had significantly ($p<0.05$) higher antioxidant activity than BHT and oak, while rosemary extract showed comparable activity to BHT. Oak extract, had the least antioxidant capacity. By increasing their concentrations, the total antioxidant activities of green tea and rosemary increased but increasing concentration had no effect on oak fruit extract. At lower concentrations rosemary extract had higher antioxidant capacity than green tea, but at higher concentrations, this effect was reversed.

Combinations of the three extracts (green tea, rosemary and oak), at all ratios, exhibited synergistic effect with respect to antioxidant capacity (Fig. 4). Different foods have various bioactive compounds and antioxidant activities. When these foods are consumed together, total antioxidant capacity may be affected by synergistic, antagonistic or additive effects and produce new physiological properties (Wang et al. 2011).

Reducing power

Reducing properties are generally related to the ability of reductants to donate a hydrogen atom and thereby break a radical chain. Furthermore, reductants react with peroxide

Fig. 4 Experimental and theoretical TAOC values of green tea/rosemary/oak extract at different combinations

Fig. 5 Reducing power of methanolic extracts of green tea, rosemary, oak and BHT

precursors and prevent the formation of peroxides (Bidchol et al. 2011). Thus, samples with higher reducing powers are more able to donate electrons. The reducing powers of different samples are shown in Fig. 5. Only lower concentrations of green tea and rosemary extract showed significantly ($P<0.05$) higher reducing power than BHT. In low concentrations, the reducing power of green tea extract was higher than that of rosemary extract, and this fact was also observed by Gramza-Michałowska (2007). Therefore, increasing concentrations did not lead to increase in the reducing power of extracts.

All ratios of combinations of the three extracts, showed synergistic effect (Fig. 6). Queirós et al. (2009) evaluated the reducing power of combined edible mushrooms and the most observed effect was synergism (70 %).

GC analysis of soy bean oil

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

Fig. 6 Experimental and theoretical reducing power values of green tea/rosemary/oak extract at different combinations

Antioxidant activity in soy bean oil

The appropriate sample with the least combined concentration that showed synergistic effect was selected and examined in soy bean oil along with related individual extracts, BHT and the control sample without added antioxidant (Fig. 7). There was a significant difference ($P < 0.05$) between the induction period of extracts, and extracts with BHT and control sample which indicates the ability of these extracts to serve as substitutes to BHT, even in small amounts.

Rosemary extract (50 μg/ml) had the longest induction period (IP) at 15.4 days while the control sample with an induction period of 10.9 days had the least IP. There was no synergistic effect when combinations of extracts were used for this assay, but even with the antagonism observed; this sample still had longer IP than both BHT and the control sample. This could be because of the complex nature of oil which may have influenced the activity of extracts and the interaction types. Therefore, the effect of combinations of natural extracts in oil in the prevention of peroxide formation must be evaluated as

separate and independent assays along with other assays. Lipid oxidation is a complicated and multistage process, and the ability of antioxidants to control each one of these steps must be studied because formation and decomposition of hydroperoxides are separate stages and antioxidants influence them through various procedures (Marinova et al. 2008).

Conclusions

Antioxidant activities of natural antioxidants in this study were related to their total phenols. Green tea had the highest while oak had the least such activity. After combinations, extracts showed different behaviours and all three kind of interactions were observed. Although in peroxide value evaluation the only effect observed was antagonism, yet the ability of the combined extracts was still higher than that of BHT. These differences in behaviour may be due to chemical composition of extracts, reactivity or polymerization, and also conformation of components (Queirós et al. 2009). Results

Fig. 7 Peroxide value of samples contained different extract during storage at 60 °C

from present study indicate that green tea, rosemary and oak and their combination can be used as appropriate substitutes for the synthetic antioxidant BHT.

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