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MYRTUS COMMUNIS L. LEAVES AND TEAS AS POTENTIAL ANTIOXIDANTS AND PROTECTORS AGAINST *IN VITRO* LDL–OXIDATION

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Abstract

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Myrtus communis L. plant contained phenolic compounds, having important biological activities. In this study, using berries and leaves of *Myrtus communıs* L. were produced teas by different production techniques (whole grains, fragmented state, berry seeds, pulverized state with different periods of infusion: 3 min, 5 min, 10 min) and their antioxidant potential were determined. The total phenolic content was determined as gallic acid equivalent (GAE), antioxidant capacity as Trolox equivalent (TEAC), the free radical scavenger capacity by DPPH method, antioxidant reducing power by FRAP analysis and low-density lipoproteins (LDL) oxidation inhibition analyzes were carried out, based on the evaluation of parameters, as the product of the highest antioxidant activity was determined pulverized tea. Principal component analysis (PCA) results supported these findings.

Key words: Myrtus communıs L., tea, antioxidant activity, LDL – oxidation

Abbreviations: GAE: gallic acid equivalents; TEAC: trolox equivalent antioxidant capacity; DPPH: 1,1diphenyl-2-picrylhydrazyl; FRAP: ferric reducing antioxidant power; LDL: low-density lipoprotein; DMSO: dimethyl sulfoxide; ABTS: 2,2'-Azino-bis-3ethylbenzthiazoline-6-sulfonic acid; TPTZ: 2, 4, 6 Tripyridyl-S-triazine; MDA: malondialdehyde; TBARS: thiobarbutric acid reactive substances; TBA: thiobarbituric acid; PBS: phosphate buffered saline; LSD: least significant differences; PCA: principal component analysis; TAO: total antioxidant activity

Introduction

Myrtle (*Myrtus communis* L.*)* is an aromatic plant whose leaves (or berries) are used for the production of the Sardinian typical liqueur and are an interesting source of antioxidant compounds with medicinal properties (Tuberoso et al., 2010). The blue-blackish berries, that are astringent but sweetish and edible, contain flavonoids and anthocyanins (mainly myricetin glycosides), having strong antioxidant properties (Barboni et al., 2010).

The black *Myrtus communis* L. fruit was determined as one of the richest sources of antioxidants and fruit exhibiting one of the highest in vitro antioxidant capacities among various studied fruits and vegetables (Romani et al., 2004; Faria et al., 2005). *Myrtus communis* leaves not only posses specific phenolic compounds (Yoshimura et al., 2008; Wannes et al., 2010) but also have higher antioxidant activity (Alamanni and Cossu, 2004).

Phenolic compounds found in such foods can act as antioxidants by many potential pathways such as free radical-scavenging, oxygen radical absorbance and chelating of the metal ions (Bagchi et al., 2004; 2006). Due to the nature and quantities of phenolic compounds present in *Myrtus communis* L. leaves and fruits, plant with this origin have special medical importance (Tuberoso et al., 2010).

Myrtus communis L. teas obtained with different infusion methods were evaluated for their phenolic profile,

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aroma compounds and antimicrobial and antioxidant activities. During the infusion process in six of phenolic compounds have been determined significant changes. Additionally studied examples demonstrated antimicrobial effects of teas against six bacteria (Messaoud et al., 2012).

In this study, *Myrtus communıs* L. berries and leaves were evaluated. Teas produced by different production techniques: whole grains, fragmented state, and berry seeds, pulverized state and different infusions: 3 min, 5 min, 10 min were studied for their antioxidant activities by different methods and LDL oxidation inhibition potential.

This is the first study evaluating the antioxidant potential of *Myrtus communıs* L. berry and leaves teas produced by different techniques with materials obtained from Menders region of Izmir (Turkey) by multivariate techniques.

Materials and Methods

Black *Myrtus communis* L. berries were harvested from Menderes region near to Izmir. Hand harvested fruits were destemmed and calibrated on the basis of equal sizes. Samples were stored at -20ºC for a week before analyses.

Production of Myrtus communis L. leaves and teas

Myrtus communis L. plants were harvested from random samples of plants growing wild in Menders region of Izmir (Turkey) in October 2012. Leaves (L) were separated manually from aerial parts in Biotechnology Laboratory of Ege University and dried at room temperature for 2 weeks and finely ground with small home blender. The Fruit seed (Fs) were isolated from fruits and analyzed. Whole fruit old (Wo) were obtained after storage at 4˚C for a year. The Whole fruit new (Wn) were used immediately after harvest. Fragmented fruits (Ff) were obtained by crushing and pressing (home blender: Joyce home electrical accessory Co. Ltd). The Pulverized fruit (Pf) were processed in similar way to leaves, dried and finely ground with small home blender. Teas components were given in Table 1.

Obtained samples (leaves powder/fruit seeds/whole fruit - old, whole fruit - new, fragmented fruit, pulverized fruit) were used for tea preparation with ratio: dry powder/water (1:1). The infusion time was 3 min, 5 min and 10 min.

Analyses of *Myrtus communis* L. leaves and teas

Evaluation of the total polyphenol content by gallic acid equivalents (GAEs)

Total phenolic content was determined by Folin–Ciocalteu method (Singleton and Rossi, 1965; Stratil et al., 2006) by carried out following modification. At the beginning, 0.200 ml of sample and 1.0 ml of Folin – Ciocalteu reagent diluted with water (1/10) were mixed. The following procedure was the addition of 0.8 ml of saturated sodium carbonate (20g of Na_2CO_3 in 100 ml of H_2O). After 2 min mixing on a shaker and heating at 5˚C for 5 min the absorbances were determined at 760 nm in a spectrophotometer against blank. The results were expressed as gallic acid equivalents (GAE) using calibration curve. Gallic acid was supplied from Merck (Darmstadt/Germany).

Evaluation of free radical scavenging capacity by 1,1-diphenyl-2-picrylhydrazyl method

DPPH is a radical generating substance that is widely used to monitor the free radical scavenging abilities (the ability of a compound to donate an electron) of various antioxidants. The solution of 0.1 mM DPPH (1,1-diphenyl-2-picrylhydrazyl - DPPH) was rapidly mixed with the sample (1/100; v/v). The decline in absorbance was recorded at 550 nm against ethanol blank over a period of 20 min in 5 min intervals in micro plate reader. The decreases of absorbance corresponding to 100% radical scavenging was determined with a solution of pyrogallol in DMSO (ca. 0.5%) which caused complete scavenging within seconds (Yildirim et al., 2013).

Table 1
Tea components at different infusion times

COD	Tea components	Infusion times, min
L	Leaf	L3, L5, L10
Fs	Fruit seed	Fs3, Fs5, Fs10
Wo	Whole fruit old	Wo3, Wo5, Wo10
Wn	Whole fruit new	Wn3, Wn5, Wn10
Ff	Fragmented fruit	Ef3, Ef5, Ef10
Pf	Pulverized fruit	Pf3, Pf5, Pf10

Evaluation of antioxidant potency by the ABTS/TEAC method

ABTS (2,2'-Azino-bis-3-ethylbenzthiazoline-6-sulfonic acid) (7 mM.l-1) and potassium persulphate (4.95 mM.l-1) were mixed (1/1:v/v) and stored in room temperature at least for 12 h before using. The reactive was diluted by phosphate buffer (1/25:v/v) until absorbance value reached up 1.0 -1.5. The part of working solution (975 µl) was mixed with 5-25 µl sample and absorbances were read at 734 nm wavelengths in a spectrophotometer. As control and standard, were used phosphate buffer and trolox, respectively (Re et al., 1999).

Evaluation of the ferric reducing antioxidant power

Mixing solution (10:1:1, v/v/v) of acetate buffer (10 mM, pH = 3.6), TPTZ (2,4,6-Tripyridyl-S-triazine) (10 mM) and $FeCl_3$ (20 mM) were added into sample and stored at room temperature for 30 min. Readings was done at 620 nm by using micro plate reader. Distilled water and $FeSO_4$ (1mM) were used as control and reference standard respectively (Pulido et al., 2000).

Evaluation of inhibition of low-density lipoprotein (LDL) oxidation. Isolation of low-density lipoprotein

The LDL isolation was done according to the method described by Taus et al. (1994). LDL precipitate solution (Merck/Darmstadt/Germany) was mixed in equal amounts with plasma and kept for 30 min at room temperature before centrifugation (3000 xg/10 min). Obtained LDL pellet was dissolved in 15 mM NaCl. Protein analysis was performed by Lowry method after dilution (1/10) procedure (Lowry et al., 1951). In alkaline solution the peptide bounds formed the colored complexes and at the same time the amino acids such as tyrosine and tryptophan are reduced by phosphomolibdate – phosphotungstate solution (Folin-Ciocalteu). The samples (0.1 ml) were mixed with 0.9 ml distilled water, 5 ml of $CuSO_4$ and kept for 10 min at room temperature. Folin-Ciocalteu solution (0.5 ml) diluted with 1N HCl at 1:1 proportion was added to the samples and absorbances are read at 750 nm. The samples mixtures were kept at room temperature for 30 min. Bovine serum albumin (200 mg/100 ml) diluted with distilled water at concentrations of 25 - 200 $mg.dl^{-1}$ was used as a protein standard.

Thiobarbutric acid reactive substances (TBARS) analysis

The TBARS analysis was done according to the method described by Sozmen et al. (1999). The main principle of this method is based on the detection of the light red color of TBA-MDA complexes obtained as a result of the reaction between MDA (Malondialdehyde) found in samples and TBA (Thiobarbituric acid). The serum samples was diluted with PBS (Phosphate buffered saline) and mixed in equal amounts with TBA (0.12M TBA, pH=7.0) before heating (90ºC) for 45 min. After cooling and centrifugation, the supernatant's absorbances were read at 532 nm and the results were expressed as mM MDA/mg LDL protein. In order to determine the inhibition of LDL oxidation by the blueberry products, 20µl of blueberry product was added into serum samples before incubating with 5 mM $CuSO_4$. The reaction mixtures were incubated for 2 h and TBARS were determined in all samples (with and without $CuSO_4$). Differences between the blank and samples with $CuSO_4$ were evaluated as the inhibition of serum oxidation.

Statistical analysis

Significant differences between averages were determined at a 95% significance level. By using a Post-Hoc test, the least significant differences (LSD) tests were performed.

Using multivariate exploratory techniques, principal component analysis (PCA) was performed. Principal component analysis permits the visualization of the original arrangement of samples in an n-dimensional space, by identifying the directions in which most of the information is retained. It is therefore possible to explain differences in the various wines by means of these factors obtained from the generalized correlation matrix of the data sets and at the same time to determine which variables contribute most to such differentiation.

Results and Discussion

Evaluation of total phenolic contents of Myrtus communis L. teas

Considering the knowledge that black *Myrtus communis* L. are one of the richest sources of phenolic compounds among various fruits and vegetables studied (Martin-Aragon et al., 1998; Kahkönen et al., 2001; Stratil et al., 2006) it's beery and leaves teas were produced by different techniques and evaluated for their phenolic contents.

The total phenol contents of different teas were found statistically significant which were conformed by LSD test ($P < 0.05$). The total phenol values of pulverized tea were determined to have positive correlation with all tea types. The order determined on the bases of total phenols results was as: pulverized fruit > fragmented fruit > fruit seeds > leaves > whole fruit – new > whole fruit – old.

Different products including teas produced from black *Myrtus communis* L. showed remarkable differences in polyphenol content with values ranging from 261 mg.l^{-1} GAE to 2905 mg.l^{-1} GAE (Yildirim et al., 2013). In study done by Messaoud et al. (2012) the total phenols of tea leaves obtained with infusion at 5, 10 and 15 min were determined. The highest amounts of total phenols were found in myrtle leaves with infusion of 15 min. There are no data available regarding the total phenols of myrtle teas obtained by processing with different forms.

In literature there are some different results confirming the variations of such order due to applications of different production procedures (Yang and Liu, 2013). Polyphenolic compounds including anthocyanins and proanthocyanidins found in plant are not completely stable. After harvest these compounds could be changed during food processing and storage. In a study of Skrede et al. (2004) the effect of different environmental conditions were evaluated as factors affecting pigment stability and especially the color stability of berry. Kalt et al. (1999) emphasized the effects of heat, pH and oxygen concentration on antioxidant stability.

There are some studies confirming the fact that infusion time favor extraction enhancing both the solubility of solute and diffusion coefficient (Komes et al., 2010; Harbourne et al., 2009).

Evaluation of antioxidants potential and protector's activities against in vitro LDL–oxidation of studied Myrtus communis L. leaves and teas

Evaluating the mean values of LDL-MDA, FRAP, TEAC and TAO (total antioxidant activity) results demonstrated the importance of black *Myrtus communis* L. teas. (Yıldırım et al., 2013) The total phenol of teas was positively correlated with all performed antioxidant methods at $P < 0.05$ level. Positive correlations were determined also among different methods (at $P < 0.05$) except between TAO and LDL-MDA. This emphasized the presence of bioactive compounds present in black *Myrtus communis* L. teas that are responsible for antioxidant potency, free radical scavenging capacity and ferric reducing antioxidant power of used products.

Significant differences were determined among different *Myrtus communis* L. teas types. These results were confirmed by LSD test. Evaluations concerning antioxidant activities of different *Myrtus communis* L. teas demonstrated the differences in total phenols by quality and quantities. This could be attributed to heterogonous distribution of these compounds in plants.

The result of LSD test concerning LDL-MDA demonstrated the significance differences between leaf tea and fruit seed tea ($r = 0.035$) and leaf tea and pulverized fruit tea ($r = 0.008$); whole fruit – new and whole fruit – old ($r = 0.013$); whole fruit – old and pulverized fruit ($r = 0.019$); fragmented fruit and whole fruit-new year ($r = 0.010$); fragmented fruit and pulverized fruit ($r = 0.006$); fruit seed and leaf ($r = 0.035$); fruit seed and whole fruit – new ($r = 0.005$); fruit seed and pulverized fruit ($r = 0.004$). Except these relations, the pulverized form of the *Myrtus communis* L. tea was determined as significantly different from all other type teas ($P < 0.05$).

The results of FRAP analyses demonstrated the significant differences among all groups except between some combination (leaf tea and whole fruit – old; leaf and whole fruit – new; whole fruit – new and whole fruit – old). Results concerning analyses done as TAO and TEAC lead to importance of pulverized fruit teas since they have positive correlation with all other tea types.

In a study done by Messaoud et al. (2012) the radical scavenging activities demonstrated concentration-dependent manner relations with all myrtle infusion times. Figure 1 demonstrated the standardized antioxidant values of black *Myrtus communis* L. teas evaluated by different methods (LDL-MDA, FRAP, TAO, TEAC and total phenols). For all parameters, pulverized tea was found to have the highest value, followed by fragmented fruit, fruit seed, leaf, whole fruit-old and whole fruit- new. For LDL-MDA the order of classification of the teas from the highest to the lowest value was as: pulverized fruit > fruit seed > whole fruit-old > fragmented fruit > leaf > whole fruit-new. The order of teas determined for FRAP analysis were determined as: pulverized fruit > fragmented fruit > fruit seed > whole fruit- new = leaf = whole fruit-old. Different teas were ordered as followed pulverized fruit > fragmented fruit > fruit seed > leaf > whole fruit-old > whole fruit- new concerning TAO results. The order based on TEAC results was determined as: pulverized fruit > fragmented fruit > fruit seed > whole fruit-old > whole fruit- new > leaf.

For all parameters the tea prepared from pulverized fruit of black *Myrtus communis* L. have the highest value. This could be connected to the applied procedure of pulverized powder for tea preparation enabling more extraction of phenolic compounds.

Jang and Liu, 2012 demonstrated that the time kinetics of soluble compounds dissolved in hot water from bag teas and leaf teas were different. In the same study was determined that the extraction of total phenols–soluble solid compounds was faster in loose teas than in bag teas. Astill et al. (2001) emphasized the importance of preparation methods (particle size and extraction methods) on phenolic content and caffeine content in black tea.

Figure 2 demonstrated the standardized values of black *Myrtus communis* L. teas produced with different infusion times (3 min, 5 min, and 10 min) evaluated by different methods (LDL-MDA, FRAP, TAO, TEAC and total phenols). The best results were determined with infusion time of 5 min. Jang and Liu, 2012 demonstrated that the optimal brewing time for green tea in the tea bag as 5 min, yielding maximum total matter soluble solids. In other studies (Harbourne et al., 2009; Komes et al., 2010; Messaoud et al., 2012) was stated that longer extraction time may cause oxidation of phenols.

Overall evaluation of Myrtus communis L. products by cluster analysis and PCA

Principal component analysis was run on the set of data to examine attribute relationship and to demonstrate the differences among products and analyzed parameters. Loading plots for the first two factors (92.69% x 5.75%) were accepted to account for more than 98.44% as a summation of the first two principal components.

As could be observed from the Figure 3, the majority of analyzed parameters were found in the left side of the coordinate. Two main groups were obtained. In the first one was

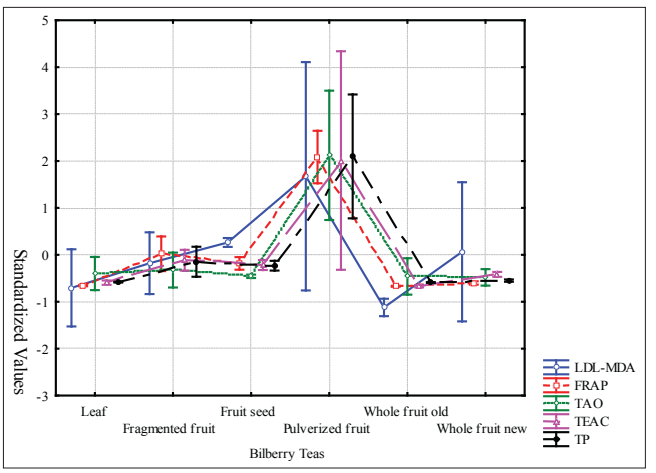

Fig. 1. The standardized values of produced *Myrtus communis* L. teas with different form concerning their antioxidant parameters

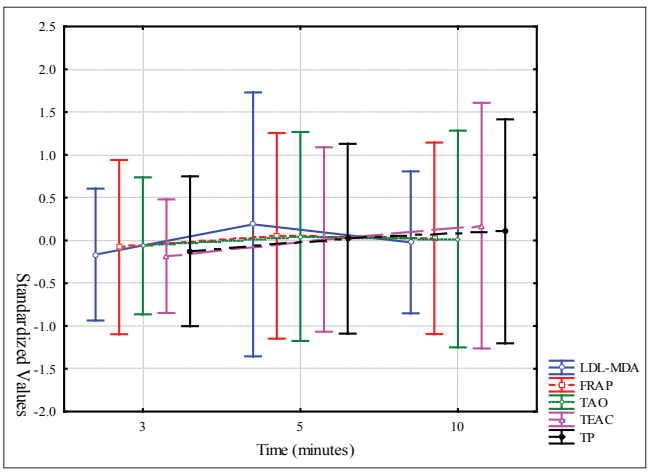

Fig. 2. The standardized values of produced *Myrtus communis* L. teas with different infusion time concerning their antioxidant parameters

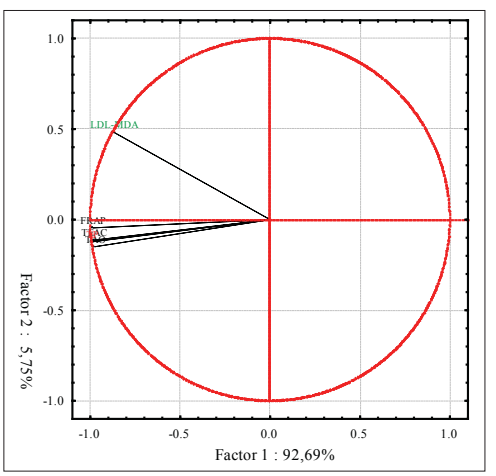

Fig. 3. PCA results performed for analyzed parameters

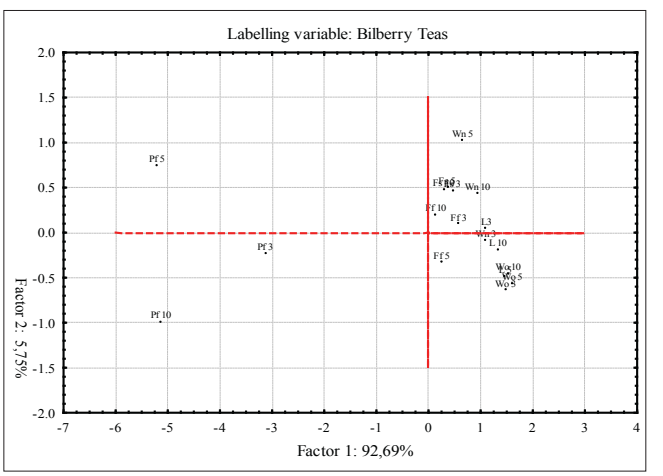

Fig. 4. PCA results performed for teas (different form and different infusion times)

located LDL-MDA and in the second one all others parameters. The distribution of teas produced from various forms of black *Myrtus communis* L. in an n-dimensional space demonstrated the differences in composition of bioactive compounds and their bioactivities (Figure 4). As can be observed, all teas produced from pulverized form (with 3 min, 5 min, 10 min infusion) were situated in the left part of the coordinate. Since all other processed teas were located in the right side of the coordinate, it could be concluded that *Myrtus communis* L. teas obtained by pulverized form have developed different profiles.

By plotting Figure 3, 4 as figures with the same coordinate values (%92.69 x 5.75%), interesting results were obtained. LDL-MDA was fitted with pulverized teas prepared with 5 min infusion. The other pulverized form but with 3min and 10 min infusions were fitted with all methods of antioxidant activities.

The study is the first attempt to determine the antioxidant properties of *Myrtus communis* L. teas produced from plants with different form and processed with different infusion times.

Conclusion

The analyses of data from our study demonstrated the importance of black *Myrtus communis* L. tea produced from pulverized fruits, as having the highest antioxidant/bioactivity potential power. Grouping of different parameters in n-dimensional space with different teas confirmed the significant differences of this type product.

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