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EVALUATION OF ANTIOXIDANT/ BIOACTIVITY POTENTIAL OF *MYRTUS COMMUNIS* L. PRODUCTS USING MULTIVARIATE STATISTICAL TECHNIQUES

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Abstract

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Myrtus communis L. plant contains phenolic compounds that have some significant biological activities. The objective of this study was to produce and determine the antioxidant/bioactivity potential of black *Myrtus communis* L. products: juice (J), stewed berry(S), sugar added stewed berry (SS), wine (W), jam (JM), compote (C) and tea (T) which may act as antioxidants and protectors against in vitro LDL oxidation. Products were evaluated for their total phenolic content as gallic acid equivalents – GAE's; for their antioxidant potency by the TEAC; the free radical scavenging capacity by DPPH and the ferric reducing antioxidant power (FRAP) assays. The antioxidant functionality, that is, inhibition of LDL oxidation was evaluated by malondialdehyde method. The result of grouping of different parameters in n-dimensional space with different bilberry products demonstrated the importance of tea as a product with natural antioxidant properties.

Key words: black *Myrtus communis* L. products, processing, antioxidant activities, LDL-oxidation, phenolic content

Abbreviations: J: juice; S: stewed berr; SS: sugar added stewed berry; W: wine; JM: jam; C: compote; T: tea; GAE: gallic acid equivalents; TEAC: trolox equivalent antioxidant capacity; DPPH: 1,1-diphenyl-2-picrylhydrazyl; FRAP: ferric reducing antioxidant power; LDL: low-density lipoprotein; DMSO: dimethyl sulfoxide; ABTS: 2,2'-Azino-bis-3-ethylbenzthiazoline-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

There are intense researches on antioxidant activities of fruits and vegetables since consumers preferred more health promoting foods. As the most health, related foods could be seen those including phenolic compounds in different form: fresh, semi-processed or completed form. 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 (Faria et al., 2005). Phenolic compounds found in these 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). Antioxidant activities of fruit products could be impacted by many factors such as maturity processing and storage (Yildirim et al., 2005).

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).

Many studies showed that essential oils and extracts from different *M. communis* L. organs have appreciable antioxidant activity. The maximum value of antioxidant efficiency

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after 40 days of maceration was observed in ethanolic extracts 80% (87.5%), whereas the minimum was found in ethanolic extracts 60% (65.0%) (Snoussi et al., 2012).

In a study concerning production of milk products enriched with different fruits, the milk extracts obtained by addition of berries have been found to contain the highest antioxidant activities (Talavera et al., 2006). In another study the effects of some procedures (quick freezing, freeze-drying, spray drying) on berry's antioxidant activities have been investigated and the highest value has been determined for fresh fruits, followed by products obtained by freeze-drying (Schmidt et al., 2005).

Most of the studies concerning *Myrtus communis* L. are done with their leaves, berries or liqueur. The purpose of the present study was to process black *Myrtus communis* L. berries into different products: juice (J), stewed berry(S), sugar added stewed berry (SS), wine (W), jam (JM), compote (C) and tea (T) and evaluate their antioxidant / bioactivity potential by different methods. To the best of our knowledge, this is the first study concerning the production of different products from black *Myrtus communis* L. and their evaluation for antioxidant / bioactivity potential by using multivariate statistical techniques.

Materials and Methods

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

Production of bilberry products

Samples obtained from different products were taken and filtered through a 0.45 µm membrane before analysis. Each experiment was carried out in triplicate, and results were stated in means.

Supplied berries were crushed, pressed by home blender (Joyce Home Electrical Accessory Co. Ltd) diluted with water (1:1) and stored at 4ºC for a day. Obtained berry juice (J) was used for the next stages of other products. Stewed berry (S) was produced by keeping the fruits in previously boiled water (fruit / water ratio 1:1) for 10 min. As the third product was evaluated stewed berry by addition of glucose (up to 20 brix), so named sugar added stewed berry (SS). Sugar and fruits were added before boiling into previously boiled water (fruit / water ratio 1:1). The duration before taking the sample for analysis was adjusted for 10 min. The forth product was berry wine (W). The production of berry wine was performed by following the steps of fruit wine productions (Aktan and Kalkan, 2000). After crushing the brix value of must was adjusted to 20-brix value with glucose (Merck/ Darmstadt/ Germany) The fruit / water ratio was kept as 1:1. Fermirouge®, *S. cerevisiae* (INRA, Gist - Brocades Co/Delft /The Netherlands) was used as a starter culture. Alcoholic fermentation was performed at 25ºC until 1% glucose residue. After settlement of dead cells, first racking was performed by siphon. Finished wine was filtered through 0.45µm filters before analysis. The production of the jam (JM) was done according to the traditional method by adding sugar (up to 20 brix) and water with ratio 1:1. Boiling time was determined by dry matter content (60%) of product. The compote (C) which was produced similarly to stewed berry with the main difference that boiling of water and fruits (1:1) were performed simultaneously for 10 min. Preparation of tea (T) was performed by drying of 1kg fresh fruit (traditional air-drying) and milling with small home blender. Obtained powder was used for tea preparation with ratio: dry powder / water (1:1). The boiling time was 10 min.

Analyses of bilberry product

All analyses were performed in triplicate. All products were diluted 1/20 (v/v) before measurement.

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 5ºC 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 from Merck (Darmstadt/Germany).

Antioxidant activities

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 microplate 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.

Evaluation of antioxidant potency by the ABTS/TEAC method. ABTS (2,2'-Azino-bis-3-ethylbenzthiazoline-6-sul-

fonic 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 minutes 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 (0.1ml) were mixed with 0.9 ml distilled water, 5ml 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 between 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 5mM $CuSO_4$. The reaction mixtures were incubated for 2h 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.

Multivariate data analysis techniques were performed to analyze all data by using standardized values of parameters. The cluster analysis (CA) was performed as joining type (tree cluster) by using raw data. Furthest neighbor shape was selected as linkage and 1-Pearson r as a distance measure. Scale plot was demonstrated as (dlink/dmax) x 100.

Using multivariate exploratory techniques, principal component analysis (PCA) was performed. The cluster analysis and PCA were performed by using Statistica software. Principal component analysis permits the visualization of the original arrangement of wines 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 bilberry products

Black *Myrtus communis* L. are one of the richest sources of anthocyanins and exhibit one of the highest in vitro antioxidant capacities of various fruits and vegetables studied (Martin-Aragon et al., 1998; Kahkönen et al., 2001). Considering this knowledge different black *Myrtus communis* L. products were evaluated for their potential as antioxidants and as protectors against in vitro LDL – oxidation. The mean value of total phenols in different black *Myrtus communis* L. products were determined as followed: J (1498 mg.l-1 GAE); S (261 mg.l-1 GAE); SS (967 mg.l-1 GAE); W (529 mg.l-1 GAE); JM (1425 mg.l-1 GAE); C (282 mg.l-1 GAE); T (2905 mg.l-1 GAE). The order of products from the highest to the lowest number evaluated based on total phenols was determined as: tea > juice > jam > sugar added stewed berry > wine > compote > stewed berry.

Different products produced from black *Myrtus communis* L. showed remarkable differences in polyphenol content with values ranging from 261mg.l-1 GAE to 2905 mg.l-1 GAE.

In literature there are some different results confirming the variations of such order due to applications of different production procedures. The total phenolic content of some blue berry products (without skin contact fermentation – BW1 and wines made with – BW2) were obtained in ranges of 85.8 (BW1) to 115 (BW2) mg/100 ml GAE (Su and Chien, 2007).

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 by Skerde 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.

In a study concerning the effects of different treatments including using of freeze-drying, canning and concentration procedures the total phenols were determined to have been decreased in the heat-treated products (Schmidt et al., 2005).

Evaluation of antioxidants potential and protector's activities against in vitro LDL–oxidation of studied bilberry products

Evaluating the mean values of LDL-TBARS, FRAP, TEAC and TAO (Total antioxidant activity) results demonstrated the importance of black *Myrtus communis* L. teas. The high value of total phenols in teas was positively correlated with all performed antioxidant procedure at $P<0.01$ level. Positive correlations were determined also between different methods (at $P<0.01$): FRAP and TAO ($r = 0.928$); FRAP and TEAC ($r = 0.964$); TAO and total phenols ($r = 0.964$); TEAC and total phenols ($r = 0.892$). This emphasized the importance of bioactive compounds present in black *Myrtus communis* L. that, which are responsible for antioxidant potency, free radical scavenging capacity and ferric reducing antioxidant power of used products.

The results concerning LDL-TBARS analysis demonstrated significant differences between juice and compote ($r = 0.738$); juice and stewed berry ($r= 0.721$); stewed berry without sugar and wine ($r = 0.024$); stewed berry and compote ($r= 0.981$) at $P<0.01$. The results of FRAP analyses demonstrated the significant differences among groups except between wine and juice ($r =0.738$); stewed berry and compote ($r = 0.213$) at $P<0.01$. The TAO analyses results demonstrated the significant differences among groups except between wine and compote ($r = 0.034$); stewed berry without sugar and wine ($r = 0.046$); stewed berry and compote ($r = 0.309$) at $P<0.01$. The results of TEAC analyses showed significant differences among all groups except between wine and juice ($r = 0.049$); wine and compote ($r = 0.059$) at $P<0.01$ level. The differences among groups demonstrated the differences between product groups concerning antioxidant potency, free radical scavenging capacity and ferric reducing antioxidant power. Differences in bioactivities among myrtle products could be attributed to their differences in phenolic compound amounts. The antioxidative properties of plant extracts are related not only with the total amount of antioxidants but also with the presence of selected compounds.

Figure 1 showed the standardized antioxidant values of black *Myrtus communis* L. products evaluated by different methods (LDL TBARS, FRAP, TAO, TEAC and total phenols). For all parameters, tea was found to have the highest value, followed by jam, wine, stewed berry without sugar and stewed berry. For LDL-TBARS the order of classification of the products from the highest to the lowest value was: tea> jam> wine> stewed berry without sugar> juice= compote=stewed berry. The order of products determined for FRAP analysis were determined as: tea > jam > stewed berry without sugar > wine > juice > compote. Products were ordered as followed tea > jam > juice > stewed berry without sugar > wine > compote = stewed berry, concerning TAO results. The order based on TEAC results was as: tea > jam > stewed berry without sugar > wine = juice = compote = stewed berry. Evaluating the products based on their contents the order was: tea > juice > jam > stewed berry without sugar > wine > compote > stewed berry.

For all parameters, the black *Myrtus communis* L. tea values were the highest one. This could be connected to the applied procedure including production of pulverized powder for tea preparation enabling more extraction of phenolic compounds. The other products: fruit juice and stewed fruit with and without sugar and compote have almost similar phenolic content and similar related antioxidant activities.

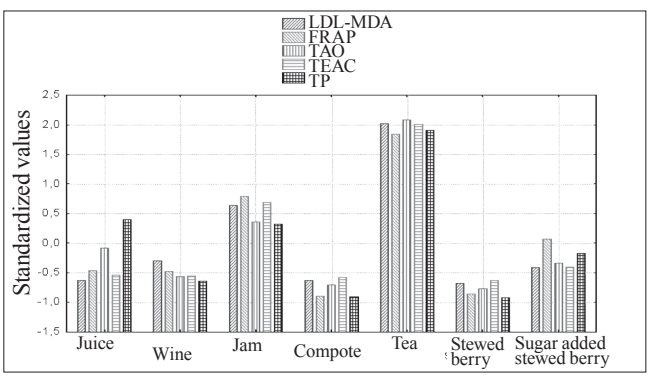

Fig. 1. The standardized values of obtained black *Myrtus communis* L. products of different analysis

Overall evaluation of bilberry products by cluster analysis and PCA

The results of cluster analyses performed for analyzed parameters (Figure 2) and used products (Figure 3) demonstrated the differences which enable for grouping. Evaluating the performed analysis (Figure 2), two main groups were obtained. The first one includes LDL-TBARS, TEAC and FRAP analyses and the second one include TAO and total phenols. Considering only the products four main groups were obtained (Figure 3). The first group is made of juice and stewed berry without sugar. In the second one are found stewed berry and compote. The tea was found in separated from having indirect relation with the second group. In the last group are located wine and jam.

The distribution of the black *Myrtus communis* L. products in an n- dimensional space demonstrated the differences in composition of bioactive compounds and their bioactivities. In the first two groups are found products processed by heating, differentiating in sugar contents. The division of tea in separate group could be attributed to its production procedure, drying. The location of wine and jam could be explained by presence of alcohol fermentation in wines and concentration procedure in jams.

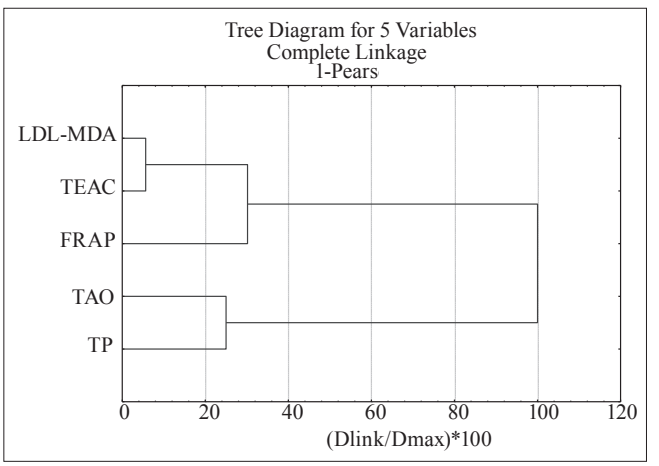

Fig. 2. The cluster analyses performed for analyzed parameters

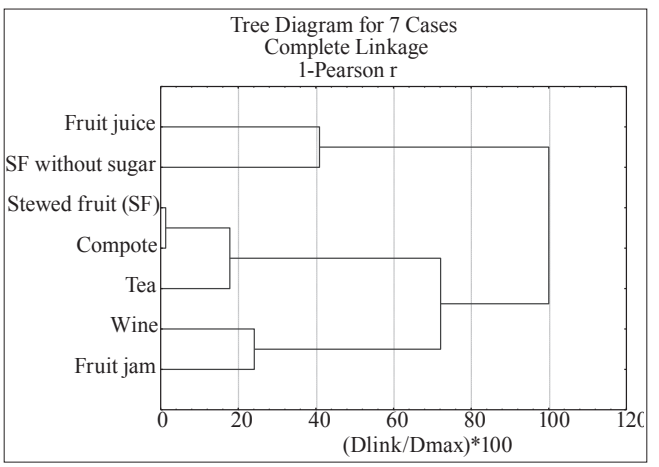

Fig. 3. The cluster analyses performed for used products

High temperatures and oxidative conditions have been shown to significantly reduce monomeric anthocyanins, total phenolic content and antioxidant activity of lowbush blueberries (Kalt et al., 2000) and diminish levels of the stilbene resveratrol in baked or heat processed blueberries and bilberries (Lyons et al., 2003).

In another study, the antioxidant activity was evaluated in different way. Some bilberry products that were heat-processed retained most of their antioxidant activity and total phenolics as found in unprocessed whole fruit. However, the heat-treated products lacked or had diminished anti-proliferation activity, suggesting that although products may be high in phenolic compounds and antioxidant activity, some forms of bioactivity may be compromised by harsh processing methods (Schmidt et al., 2005).

The differences of heat processing on berries antioxidant activity are possible related to chosen heating parameters and times.

In another study analyzed the effects of air-drying and subsequent storage of dried products on the content of polyphenols, anthocyanins and the antioxidant properties of selected berry fruits. It was demonstrated that in particular, bilberry maintained a high polyphenol and anthocyanin content and high antioxidant potential despite the greatest losses of these compounds (Michalczyk et al., 2009).

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 (95.7% x 3.19%) were accepted to account for more than 98.89% as a summation of the first two principal components. As could be observed from the Figure 4, the majority of analyzed parameters were found in the left side of the coordinate which demonstrated the close relation between tea and LDL-MDA, FRAP and TEAC parameters.

Considering the black *Myrtus communis* L. products (Figure 5) three main groups were obtained. In the first group were jam, stewed berry with and without sugar, compote and wine. In the second group, on the left side of the upper part was located tea. In the third group found in the right side of the lower part of the coordinate was juice.

In the first two groups are found products processed by heating and alcohol fermentation. The separate location of tea as in cluster distribution demonstrated the strong differences of this product from all other. The juice as a product with the lowest production procedure is located in different place from tea and other products. By plotting Figure 4 and Figure 5 having the same coordinate factor (95.67% x 3.19%) the tea location is fitted with coordinates of analyses parameters.

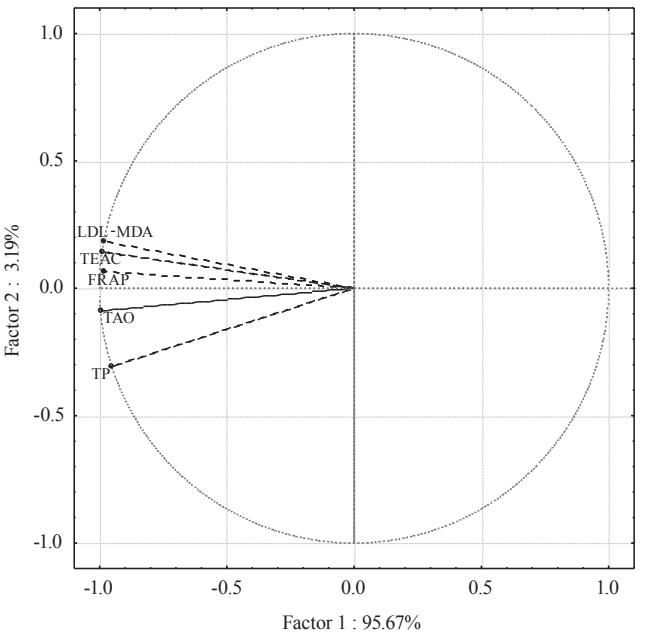

Fig. 4. The PCA analyses performed for analyzed parameters

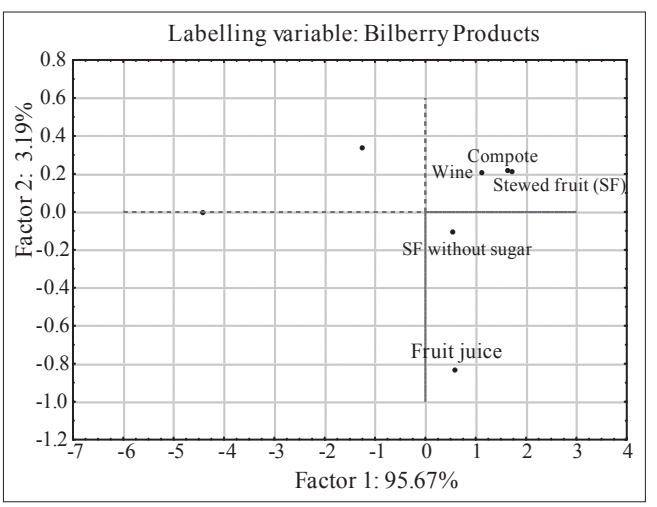

Fig. 5. The PCA analyses performed for used products

Conclusions

The analyses of data from our study demonstrated the importance of black *Myrtus communis* L. tea as having the highest antioxidant/bioactivity potential power. Grouping of different parameters in n-dimensional space with different products demonstrated the potential of tea to be recommended as natural product with antioxidant properties.

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