



# Effect of the spray and freeze dryers on the bioactive compounds of olive leaf aqueous extract by chemometrics of HCA and PCA

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Received: 23 April 2019 / Accepted: 16 June 2019 / Published online: 21 June 2019  
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## Abstract

Olive leaf extract contains significant amounts of valuable compounds such as Oleuropein and phenolic compounds. The study aimed to introduce a proper method for drying olive leaf extract to increase its value in terms of valuable compounds with antioxidant properties and the correlation among all parameters. Extraction of olive leaf (Mary cultivar, Fars province) was performed using water and ultrasound device and the extract was condensed with two freeze-drying and spray drying methods, and some features were measured as follows: total tannin content, antioxidant activity and inhibitory strength of 50% using the DPPH method, total phenol content using Folin Ciocalteu colorimetric method, total flavonoid by aluminum chloride colorimetric method, and polyphenol compounds using HPLC in olive leaf extract samples. The results showed that the dried extract with freeze-drying had the highest total phenolic content  $446.63 \pm 7.6$  (mg gallic acid per gram dry matter) and the highest antioxidant activity of  $96.57 \pm 0.19\%$ , inhibitory effect of  $0.44 \pm 0.32$   $\mu\text{g/ml}$ , tannin content of  $128.71 \pm 0.26$  mg of gallic acid per g of dry matter and total flavonoid  $396.43 \pm 0.25$  mg of quercetin per gram of dry matter were seen in the dried extract with spray drying. Evaluation of the type and amount of bioactive compounds in three types of extracts (solution and condensate extracts) by principal components analysis and agglomerative hierarchical clustering (AHC) showed that the use of spray drying has been effective in maintaining the amount of unstable polyphenolic and bioactive compounds.

**Keywords** Bioactive compounds · PCA · HCA · Olive leaf aqueous extract

## Introduction

Olive tree (*Olea europaea* L.) is one of the most important fruit trees in the Mediterranean region, which has various applications due to its proven properties [1]. Many reports have been published about the olive leaf extract

and its effect on lowering blood pressure, increasing blood flow in coronary arteries and preventing muscle cramps in animals [2]. Olive leaf is one of the major by-products of olive oil factories, which always exists in large amounts in olive oil industry and related factories [1]. Olive leaf has a low crude protein content of about 6.31–10.9 g (per

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100 g of dry matter). Its organic matter content is between 76.4 and 92.7 g (per 100 g of dry matter) and crude fat in it is between 2.28 and 9.57 g (per 100 g of dry matter), and 89% of its nitrogen is from protein nitrogen [3].

Oleuropein is a heterocyclic ester of elenolic acid and 3,4-dihydroxyphenyl ethanol, which causes bitter taste in olive leaf. It exists in unprocessed olive leaf and fruit in large amounts (60–90 mg/g of olive leaves) and has anti-inflammatory properties in addition to its anti-oxidant and antimicrobial properties [3]. Another important phenolic compounds in the olive leaf is the hydroxytyrosol compound that has a high antioxidant and antimicrobial properties. After oleuropein and hydroxytyrosol, flavone, luteolin 7-glucoside, apigenin and verbascosides are the most frequent phenolic compounds found in olive leaves, respectively [2].

The desire to use olive leaves as a natural additive to artificial additives and nutrient compound in the food and medicine industry is increasing due to the presence of anti-oxidant compounds [4]. After extraction of the extract by various methods, the concentration and purification of the extract are necessary. Nowadays, with the advancement of the industry, the extraction of plant leaves and drying methods of extracts or condensation have become commonplace in order to preserve active bioactive compounds, and also has evolved over time [5]. Various studies have been done on the effect of drying process on leaves and on the olive leaf extract. For example, the olive oil drying kinetic with solar drying convective [6] to evaluate the effect of solar drying convective on color, polyphenols and antioxidant activity of olive leaves [7]. The effect of drying with infrared radiation on polyphenols and olive leaf color [8], the effect of using thin film dryer at different temperatures and velocity ranges in the drying of olive leaves [9], the effect of using hot air and freezing dryer along the maintenance period on the bioactive compounds of olive leaf extract and their shelf life [10].

Considering the therapeutic properties and antioxidant properties of olive leaves and the availability of olive leaves in all seasons and the low cost of this raw material rich in phenolic compounds, the purpose of this study was to identify and determine the amounts of bioactive compounds of olive oil, Mari Fars cultivar as well as extracting aqueous extract from olive leaves and identifying the appropriate drying method to better maintain bioactive compounds. To this end, the effect of drying with two methods of spraying and freezing on antioxidant properties and phenolic compounds of olive leaf extract was evaluated before and after drying. The correlation between all parameters analyzed using principal components analysis (PCA) was also evaluated. Finally, the difference of bioactive compounds of the olive leaf extract of the two methods is introduced to be used in the industry.

## Materials and methods

### Materials

Acetonitrile (ACN), ortho phosphoric acid, pure water (HPLC Grade), hexane, ethanol, and methanol (HPLC Grade), folicum, covalent, sodium carbonate, aluminum chloride were prepared from Merck, Germany).

2,2-Diphenyl-1-picrylhydrazyl (DPPH) and standard reference materials for glycine, quercetin, tyresol, hydroxy triazole and oleuropein, vanillin, vanillic acid, fumaric acid and caffeic acid were purchased from Sigma-Aldrich (USA).

### Preparing of samples

Mari olive tree leaves of Fasa city in Fars province (35°19′–54°15′ east longitude and 28°31′–29°24′ north latitude) from young and fresh olive trees were cut manually. The physicochemical properties of the olive leaf were identified in accordance with AOAC methods, 1995. Ash, moisture, protein, and lipid percentage were measured through muffle method at 550 °C (4 h), gravimetric method at 105 °C (24 h), macrochondal method, and Soxhlet method and hexane as solvent, respectively. Raw fiber was carried out according to the ISO 5498: 1981 method [11]. Fatty acid analysis of olive leaf was performed with the methylation of fatty acids according to ISO 12966-2 [12]. The leaves were washed in the shade and room temperature for 48 h after washing with water; they were kept in a dark glass container in a cool place after being powdered with a mill, and passing through a grid of mesh number 80.

### Extraction of olive leaves

First, 100 g of olive leaf powder, 800 ml of deionized water (60 to 40 °C) was added and the mixture was placed in an electric stirrer at 200 rpm and 37 °C for 3 h. Then the mix was put in the ultrasound bath for 15 min and finally the suspension was smooth. The transparent extract was kept in a freezer at -20 °C.

### Freeze dryer (FD)

In order to prepare olive leaf extract, a freeze dryer was used with the brand (CHRIST ALPHA 1–4 LD). For this purpose, disposable plates containing olive leaf extract were placed in a freezer (frozen by liquid nitrogen

at  $-57\text{ }^{\circ}\text{C}$ , low pressure (0.05 bar) for 24 h). The powder was placed in non-air permeable plastic containers containing silica gel and stored until refrigerated.

### Spray dryer (SD)

In the spray drying method, the extract was turned into powder after defrosting by spray dryer. Operating conditions SD was set at  $160\text{ }^{\circ}\text{C}$ , operating temperature of  $162\text{ }^{\circ}\text{C}$  and output temperature of  $68\text{ }^{\circ}\text{C}$  (65% The powder from the spray dryer was placed in). The powder from the spray dryer was placed in non-air permeable plastic containers containing and kept at refrigeration temperature until the tests were carried out.

### Measurement of total phenol content (TPC)

Total phenol content of aqueous extract and dried extracts using two methods of SD and FD were measured at a wavelength of  $745\text{ nm}$  using the Folin-Syclatoy method and by a spectrophotometer (Perkin Elmer, manufactured by the United States). For this purpose, the total amount of phenolic compounds found in the diluted samples was determined by Folin-Siocatello method. Therefore, about  $1.5\text{ ml}$  of the folicum Seawalto (10%) was added to  $300\mu\text{L}$  of diluted specimens and then  $1.2\text{ ml}$  of sodium carbonate solution (7.5%) was added. The mixture was stirred with Vertex for a few seconds, laced in the dark for 90 min, and then, the absorbance of the samples was read using a UV-Vis spectrophotometer at a wavelength of  $760\text{ nm}$ . The total amount of phenol was reported in milligrams per gallon of gallic acid (standard gallic acid curve) [13].

### Antioxidant activity percentage (AA%)

Antioxidant activity of the samples (olive oil extract and olive extract powder obtained by two methods of drying) was measured in equilibrium concentration.  $1\text{ ml}$  of each sample was mixed with  $1\text{ ml}$  of methanolic solution 2,2-diphenyl-1-picrylhydrazyl (DPPH) ( $0.2\text{ mM}$ ) and the samples were placed in a dark environment for 30 min. The absorption of each sample was compared with control (DPPH methanol solution) at  $517\text{ nm}$  [14]. The antioxidant activity was then calculated using the formula below.

$$\text{AA}\% = \left( \frac{A_{\text{con}} - A_{\text{test}}}{A_{\text{con}}} \right) \times 100$$

AA = is antioxidant activity,  $A_{\text{con}}$  = is the absorbance of the control reaction,  $A_{\text{test}}$  = is the absorbance in the presence of the sample of the extracts.

### Inhibitory concentration of 50% (IC50)

The inhibitory concentration of 50% is a measure for antioxidant compounds. The smaller this amount is for a compound, the greater the power of that compound in inhibiting free radicals. In order to determine the IC50 for each sample, solutions were first prepared at concentrations of  $0.5$  to  $5\text{ }\mu\text{g./ml}$ . For each of the concentrations, an antioxidant activity was measured according to the method described, and the antioxidant activity of the samples was determined by DPPH. After plotting, 50% free radical inhibitory was calculated [15].

### Total tannin content (TT)

The amount of tannin was measured by Folin-Cyclototyping method. In this method,  $1\text{ ml}$  of diluted specimens (soluble extract and dilute powder diluted solvent extracts) were placed in  $25\text{ ml}$  balloons with 96% ethanol and after 30 min,  $0.2\text{ ml}$  of each diluted specimen was added to the  $5\text{ ml}$  vial containing  $2\text{ ml}$  of pure distilled water. Then, a value of  $0.25\text{ ml}$  of colecular folin reagent was added to them. After 5 min,  $0.5\text{ ml}$  of sodium carbonate solution (7%w/v) was added to each of the specimens, and each of the samples was distilled twice with pure water. The mixture was placed in a dark environment for 90 min, and then the samples were absorbed over a wave of  $745\text{ nm}$  compared to the control. Results were reported in milligrams per gallon of gallic acid and using standard gallic acid curves [16].

### Determination of total flavonoids (TF)

The total amount of flavonoids was measured by chromatography of aluminum chloride. In this method,  $0.5\text{ ml}$  of dilute specimens (Soluble Extract and Powdered Extracts diluted with water) was dissolved in  $1.5\text{ ml}$  of methanol. Then,  $0.1\text{ ml}$  of 10% aluminum chloride,  $0.1\text{ ml}$  of  $1\text{ mol}$  of potassium acetate and, at the end,  $2.8\text{ ml}$  of distilled water was added and stirred with vortex. The samples were placed at room temperature for 30 min and then read at  $415\text{ nm}$ . Quercetin was used to draw the standard chart. The results were reported in  $\text{mg./ml}$  Quercetin, based on the quercetin standard curve [17].

### Polyphenolic compounds (TPP)

Determination of the amount and type of polyphenolic compounds in soluble olive leaf extract and powdered extract samples was performed using HPLC according to the method of COI/T.20/Doc No 29: 2009 [18]. Polyphenolic compounds were prepared using a high performance chromatography apparatus (Young Lin Model 9100, South Korea), and column 18 RP-C (column characteristics:

particle size 5  $\mu\text{m}$ , length 25 cm, internal diameter 0.46 mm) was determined. In this test, the mobile phase A was deionized water + 5% acetic acid and the mobile phase B was acetonitrile with acetic acid 5%. In order to determine the type and amount of polyphenolic compounds, diluted specimens (1%) were filtered through 0.2-micron syringes. The pomegranate juice was injected into a HPLC device at a rate of 20  $\mu\text{l}$  with 100  $\mu\text{l}$  syringe. It should be noted that a concentration of 1 mg/ml was prepared from each of the polyphenolic compounds and injected into the device to determine the location of each peak of each antioxidant index.

### Statistical analysis

All tests were performed in three replications. The analyses were performed using SPSS 22 software (Version 18.0; SPSS Inc., Chicago, Illinois, USA). Data were analyzed using a one-way ANOVA (group effects with  $p$ -values  $< 0.05$ ), followed by post-hoc analysis (Duncan test).

### Principal components analysis (PCA)

A principal components analysis (PCA) and agglomerative hierarchical clustering (AHC) was performed to establish which variables were more correlated to oxidative stability and if they permit to establish a more precise distinction between oxidative stability and minor constituents of virgin olive oil [19]. The statistical analyses were carried out using SPSS 22 software.

## Results and discussions

### Physico-chemical tests of fresh olive leaf

The results of the physicochemical properties of the olive tree leaves are presented in Table 1. The highest concentration of olive leaves tested after water (humidity) was fiber, then protein, ash, and lipid respectively. Lafka et al. Reported moisture, lipid, and ash content of olive leaves of Agrielia variety,  $50.5 \pm 1.9$ ,  $1.2 \pm 0.2$  and  $3.7 \pm 0.9\%$  w/w, respectively, which was consistent with moisture, lipid, and ash content obtained results in this study [20]. The protein content in this study was consistent with the protein level (6.61 w/w) reported by Boudhrioua et al. According to these results, it can be stated that olive leaf is rich in compounds that are found in animal sources and hence can be introduced as a source of nutrients [8]

The results of the measurement of the compound of Mari olive oil are shown in Table 2. The predominant fatty acids found in the tested specimen included linolenic ( $38.41 \pm 3.13\%$ ), palmitic (23.27%), linoleic (13.39%) and then oleic acid (10.06%), respectively. Based on the results

of the measurements, the fatty acid compounds of Mari olive oil are shown in Table 2. Previous research results confirm this difference. According to Bahloul et al. the fatty acid compounds of four varieties of olive leaf, including linolenic acid, oleic acid and palmitic acid, were in the range of (30.02–42.16%) (26.36–18.28%), and (22.42–18.28%) [21].

### Total phenol content

The effect of SD and FD methods on the phytochemical compounds of olive leaves revealed variations in their compounds. The results of evaluating the content of TFC, TT, TPP, in the soluble extract and dried olive leaves extracts are presented in Table 3. The results showed that the highest total phenol content in dry extract powder with FD  $644.6 \pm 7.6$  mg gallic acid per gram dry matter and lowest total phenol content in leaf extract solution was  $87.42 \pm 0.23$  mg gallic acid per gram dry matter. The amount of total phenol content measured in the aqueous extract of dried olive leaves was not significantly different with SD and FD methods ( $p < 0.05$ ). Drying in the air and the sun is one of the most commonly used methods that are often used in many parts of the world to maintain the quality of fruits and vegetables while FD and spray drying are used only for sensitive or high-value products [10]. However, these drying techniques may significantly affect the concentration and bioavailability of some of the basic ingredients of the food. The phytochemical analysis of olive leaves showed that not only alterations in phenols, flavonoids, tannins, antioxidant activity, but also effect on their functional properties by drying processes [10]. Plant phenolic compounds are one of the most important secondary metabolites and studies have shown that the antioxidant properties of olive leaves are directly proportional to their phenolic content. In addition, the concentration of polyphenol compounds in olive leaves varies depending on the quality, origin, and diversity of plant materials and processing methods [22]. In addition, due to the lack of significant difference in antioxidant activity between extracts powder from both dryers, it was found that both dryers with the least damage to phenolic compounds caused the change of the extract from soluble to solid. The low stability of phenolic compounds in aquatic environments is one of the main reasons for the necessity of using powdered extracts in the industry and justifies the use of powdered extracts in comparison with soluble extracts in the industry [23]. Freezing drying because of the lack of use of high temperature and SD due to the use of high temperature over a very short time can maintain the quality of phenolic compounds in addition to concentrating and purifying the compounds in the extract (by evaporation of water). In addition, increasing the rate of freezing has a significant effect on the amount of total phenol [24].

Some researchers attributed the decrease in phenolic compounds during drying to traditional methods which produce irreversible oxidative and thermal degradation during high temperature heating and storage. Reducing total antioxidant capacity of olive leaf extracts could be explained by thermal oxidation and decomposition of phenolic compounds [25]. The results of Salah et al. in the study of olive leaf extract of three Tunisian cultivars showed that total phenol content in different cultivars ranged from  $73.05 \pm 15.52$  to  $144.19 \pm 10.27$  mg gallic acid to dry matter. Different methods of extraction can be one of the main reasons for the difference in results [13].

## Total tannin content

According to the results of the tannin content of the extracts in Table 3, it was determined that the highest content of total tannin is for dried extract with SD with  $128.71 \pm 0.26$  mg galactic acid per gram of dry matter and the lowest content of tannin is for  $44.54 \pm 0.03$  mg of gallic acid per gram of dry matter aqueous extract. The amount of total tannin content in extract powder was significantly higher than tannin content in aqueous extract. Total content of total tannin in extract powders was significantly higher than tannin content in aqueous extract. The total content of tannin in extract powders was also significantly different ( $p < 0.05$ ).

Tannins are high molecular weight phytochemicals that contain many hydroxyl and carboxyl groups in their structure. These antioxidants known in some vegetables and fruits have multifunctional properties beneficial to human health, which can effectively form strong compounds with proteins and other macromolecules. This may be due to the effect of drying methods on the failure condition or binding to other macromolecules and cellular components, thereby increasing the content of tannin. In addition, changing the amount of tannins in the drying can affect the amount of other compounds due to their polymerization or binding to other compounds at drying temperatures [26]. It has been reported that plant tannins have antioxidant and anti-diabetic effects, and this may be due to binding to proteins and carbohydrates and their physiological potential [27].

## Total flavonoid content

Olive leaves, like many green leafy vegetables, contain phytochemicals such as phenolic compounds and flavonoids, whose effects on health have been proven. HPLC was used to prove the effect of different drying methods on the phenolic components of olive leaves. A review of the data from the total flavonoid content in Table 3 showed that the flavonoids content of all extracts of SD and vitamins

was significantly higher than the aqueous extract. In addition, the total flavonoid content of dried extract with SD ( $396.43 \pm 0.25$  mg quercetin per gram of dry matter) was significantly higher than the total flavonoid content of dried FD extract ( $p < 0.05$ ). Total flavonoid content for extract powder was significantly increased compared to the aqueous extract of olive leaf ( $p < 0.05$ ).

The results of some reports have shown that processing has a significant impact on the content and bioavailability of this important phytochemical category in olive leaves. Changes in the content of flavonoids by drying may be due to their binding to other compounds, such as proteins, or due to the alteration in the structure of these compounds, which is not possible with existing methods, extraction, and measurement [25, 28].

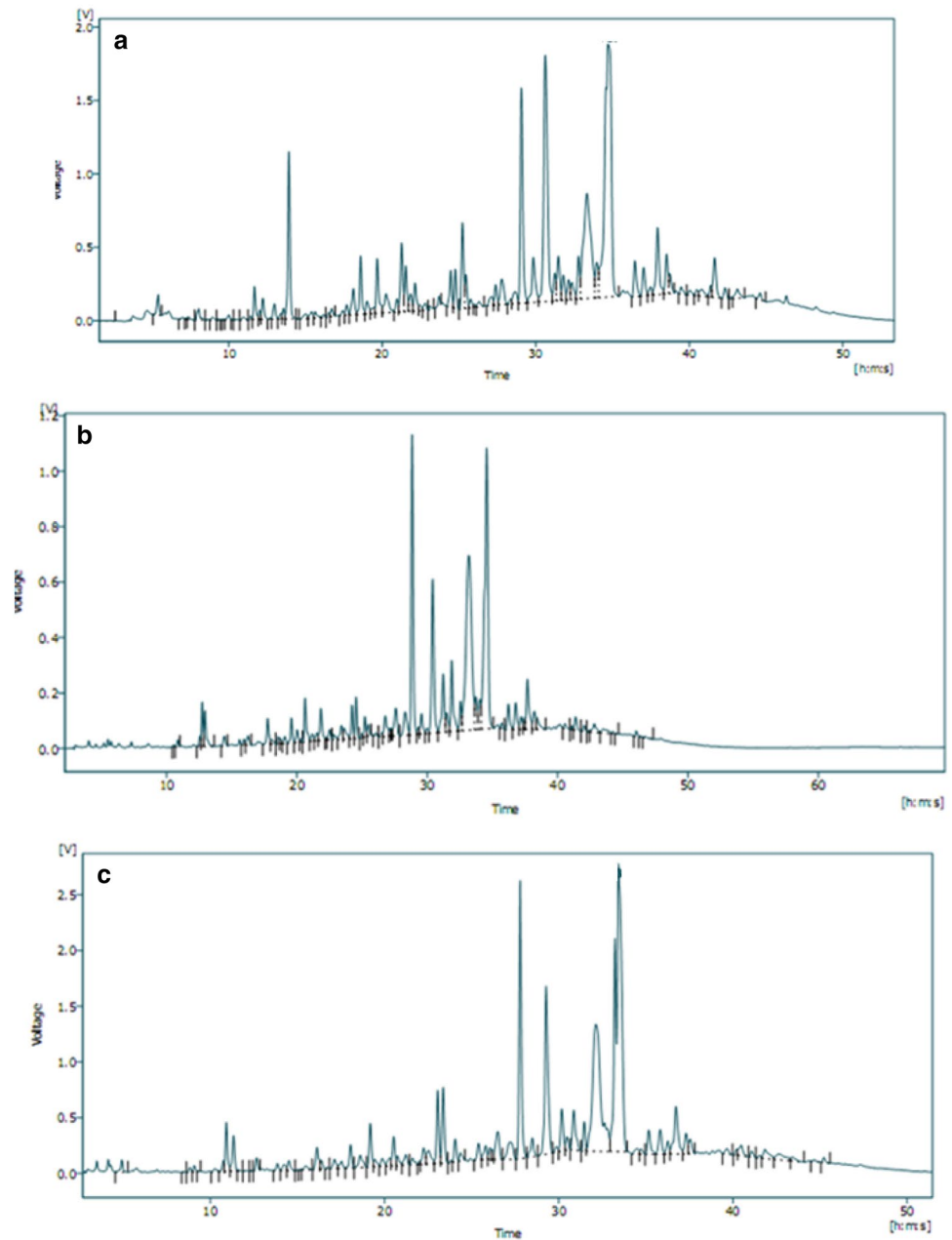
The results of the present research were higher than results reported by Salah et al. with the total flavonoid content of the aqueous-alcoholic extract of olive leaf ( $125.64 \pm 3.36$  to  $15.75 \pm 1.3$  mg quercetin per gram of dry matter). This difference in total flavonoid content can depend on several factors such as geolocation of plant growth site, extraction method, solvent type used, extraction time, post-extraction process types such as drying of extracts and test variety, the time and season for leaf placement and test methods [13].

## Polyphenolic compounds

According to the results of the evaluation of the phenolic compounds of the samples in Table 4, 17 phenolic compounds were identified in three samples of the extract in which the amounts of phenolic compounds were different according to the processes performed on the solvent extract (Fig. 1). The most important phenolic compounds in the extracts, respectively, are oleuropein, oleuropein-gaglycone, tyrozole, hydroxy tyrozole, and pcomaric acid. Oleuropein is the most abundant compound identified in the aqueous extract of olive leaves. This compound was observed in all three samples, the highest amount was found in dry extract with spray dryer  $25.6 \pm 20.9\%$  and its lowest in soluble extract  $20.9 \pm 1.21\%$ . The results showed that the level of oleuropein in extract powder was significantly higher than soluble extract and there was a significant difference between the amounts of oleuropein in dry extracts in both dryers. Although there was a higher amount of oleuropein in FD dried powder compared to spray dryer, there was no significant difference in the amount of oleuropein for samples after the statistical analysis.

The amount of oleuropein aglycone in the solvent extract was significantly higher than powdered dried extracts using two freeze drying and spray drying methods. This compound powdered dried extract with SD was significantly less than

**Fig. 1** Chromatogram relative to the polyphenolic components of olive leaves **a** (FD), **b** (Liquid), **c** (SD)



**Table 1** Physicochemical properties of fresh olive leaves

Moisture (%)	Fibre (%)	Fat (%)	Protein (%)	Soluble carbohydrates (%)	Ash (%)
47.75 ± 0.34	37.24 ± 0.13	1.32 ± 0.18	6.32 ± 0.16	4.43 ± 0.15	2.94 ± 0.2

freeze drying extract ( $p < 0.05$ ). The amount of combinations of tyrosol and hydroxytyrosol in extracts powder was significantly higher than soluble extract after drying by freeze drying and spray drying methods ( $p < 0.05$ ).

The amount of tyrosol and hydroxytyrosol in extract powder of SD was significantly higher than freeze drying extract. The combination of pecomaric acid in powdered dried extract with spray dryer was significantly increased

compared to dried extract with FD and soluble extract ( $p < 0.05$ ).

The effect of drying methods on phenolic compounds of the leaf with antioxidant properties does not have the same trend, since the pattern of observed changes is not the same. The amount of phenolic compounds and flavonoids with the different drying methods used in this study were significant. Chlorogenic acid, glycolic acids, caffeic acid and ruthenium

**Table 2** Compounds of olive leaves fatty acids

Fatty acids compound	(%)
C14:0	1.3 ± 0.03
C14:1c	0.37 ± 0.003
C16:0	23.27 ± 4.67
C16:1c	0.84 ± 0.01
C17:0	0.34 ± 0.05
C17:1c	0.59 ± 0.93
C18:0	2.25 ± 0.13
C18:1c	1.23 ± 10.06
C18:2c	13.39 ± 1.97
C18:3c	38.14 ± 3.52
C20:0	1.1 ± 0.04
C20:1c	1.12 ± 0.008
C22:0	0.006 ± 1.18
C24:0	3.5 ± 0.09

had the highest concentrations. This was the most abundant phenolic compounds and flavonoids in spray dried leaves. The dried freezing sample had the highest concentrations of chlorogenic acid, caffeic acid and rutin. High concentrations of phenolic compounds can inhibit more free radicals; prevent lipid peroxidation, and antioxidant properties of olive leaf extract powder dried by freezing method.

### Antioxidant activity and inhibitory concentration of 50% (IC50)

In addition, the antioxidant properties of DPPH of olive leaves are also studied and presented in Table 2. The results of the calculated antioxidant activity of the samples showed that the antioxidant activity of the samples was significantly higher than the solution after drying by freeze drying and spray drying methods. So that the dried samples with SD had the highest percentage of antioxidant activity  $96.57 \pm 0.19$  and the water sample had the least antioxidant activity  $62.05 \pm 0.028$ . There was no

significant difference between the antioxidant activity levels of powdered extracts. The results show that there is a direct correlation between total phenolic and antioxidant activity in each sample. Thus, a sample with the lowest phenol content showed the least antioxidant activity. The amount of 50% inhibition (IC50) for dried extract with spray dryer, broom and aqueous extract was obtained as much as  $0.44 \pm 0.32$ ,  $0.48 \pm 0.12$  and  $4.4 \pm 0.034$  µg/ml, respectively. The amount of inhibition for the dried extract with SD was slightly higher than dried extract with FD.

The single-electron nitrogen atom in DPPH reduces the amount of hydrogen from the antioxidant to the corresponding hydrazine and converts to a stable diamagnetic molecule. The desire to perform this reaction is an important factor in evaluating the antioxidant activity of the various compounds.

The results showed that the extract powder produced with spray dryer in lower amount shows an inhibitory effect against 50% of free radicals. Thus, it has a higher antioxidant effect than a solvent extract and powdered extract produced by freeze drying. Meanwhile, 50% of inhibitors for synthesized antioxidants of BHT, BHA, and quercetin were reported as much as 17.02 µg/ml, 0.55, and 0.54 µg/ml, respectively [29]. This indicates the antioxidant concentration of the spray dried extracted olive leaf can have more antioxidant activity than some synthetic antioxidants. Salah et al. reported a 50% inhibitory effect of DPPH on the extract of aqueous-alcohol solution of soluble olive leaves in the range of  $0.56 \pm 7.9$  to  $7.43 \pm 18.64$  µg/ml. The values obtained in this study were higher and larger numbers indicate a lower inhibitory concentration of that compound because it should exhibit intrusive properties in greater quantities. This difference can be due to the variability of the cultivars studied, as well as the chaptering of leaves from the tree. Also in this study, there was a negative correlation between 50% inhibitory and the amount of oleuropein as in the study of Salah et al. (2012) [13]. Luo et al. [30] indicated that water extract of olive leaves had IC50 = 400 µg/ml which near to our results.

**Table 3** Total phenol content, total antioxidant activity, tannin and total flavonoid in aqueous extract and extracts of olive leaf powder

Treatments	Total flavonoids content (Mg quercetin per gram of dry matter)	Tannin content (Mg of gallic acid per gram of dry matter)	Total phenol content (Mg gallic acid per gram dry matter)	Antioxidant activity (%)
Aqueous solution of olive leaves	158.34 ± 0.23c	44.54 ± 0.03 c	87.42 ± 0.23b	62.05 ± 0.28b
Powder of aqueous extract of olive leaves from a spray dryer	396.4 ± 0.25a	128.71 ± 0.26 c	442.84 ± 0.34a	96.05 ± 0.19a
Olive leaf powder extracted from freeze-dried extract	298.16 ± 0.26b	117.32 ± 0.08b	446.63 ± 7.6a	96.1 ± 0.3a

**Table 4** The amount of phenolic compounds in soluble and powdered olive leaves

Phenolic compound	Powdered aqueous extract of olive leaf resulting from freeze dryer (%)	Aqueous extract of olive leaf from spray dryer (%)	Aqueous solution of olive leaf solution (%)
Hydroxy tyrosol	0.2±0.13 <sup>b</sup>	0.6±0.14 <sup>a</sup>	2.2±0.01 <sup>c</sup>
Tyrosol	3.4±0.07 <sup>b</sup>	3.8±0.01 <sup>a</sup>	3.0±0.07 <sup>c</sup>
Gallic acid	3.5±0.02 <sup>a</sup>	2.3±0.1 <sup>c</sup>	0.3±0.12 <sup>b</sup>
Catechin	2.5±0.34 <sup>b</sup>	3.5±0.11 <sup>a</sup>	2.7±0.24 <sup>b</sup>
Vanillic acid	3.4±0.064 <sup>a</sup>	1.2±0.28 <sup>c</sup>	2.7±0.02 <sup>b</sup>
Vanillin	2.5±0.11 <sup>a</sup>	2.5±0.17 <sup>a</sup>	0.7±0.02 <sup>b</sup>
P. cumaric acid	9.7±0.02 <sup>c</sup>	11.8±0.02 <sup>a</sup>	3.1±0.14 <sup>b</sup>
Ferulic acid	10.01±0.1 <sup>b</sup>	9.9±0.01 <sup>c</sup>	13.7±1.14 <sup>a</sup>
Caffeic acid	2.4±0.23 <sup>b</sup>	2.9±0.01 <sup>c</sup>	3.6±0.17 <sup>a</sup>
Quercetin	0.7±0.01 <sup>c</sup>	2.1±0.25 <sup>a</sup>	1.01±0.01 <sup>b</sup>
Epinephrine	0.8±0.43 <sup>b</sup>	0.2±0.09 <sup>c</sup>	4.2±1.02 <sup>a</sup>
Routine	2.5±0.09 <sup>c</sup>	2.2±0.34 <sup>b</sup>	7.1±0.15 <sup>a</sup>
Cinnamic acid	2.5±0.07 <sup>b</sup>	1.9±0.51 <sup>c</sup>	4.2±0.03 <sup>a</sup>
Oleuropein	3.3±0.85 <sup>c</sup>	2.8±0.03 <sup>b</sup>	22.5±0.07 <sup>a</sup>
Aglycone	13.3±0.85 <sup>c</sup>	25.6±0.4 <sup>a</sup>	20.9±0.01 <sup>b</sup>
Oleuropein	1.9±1.1 <sup>a</sup>	0.8±0.02 <sup>b</sup>	0.5±0.04 <sup>b</sup>
Syringic acid	–	0.4±0.18 <sup>a</sup>	1.1±0.02 <sup>a</sup>
Luteolin			
Verbascoside	12.1±1.1 <sup>b</sup>	11.9±2.5 <sup>b</sup>	7.1±0.25 <sup>a</sup>
Others	25.2±0.35 <sup>a</sup>	13.6±0.25 <sup>b</sup>	9.39±0.54 <sup>c</sup>

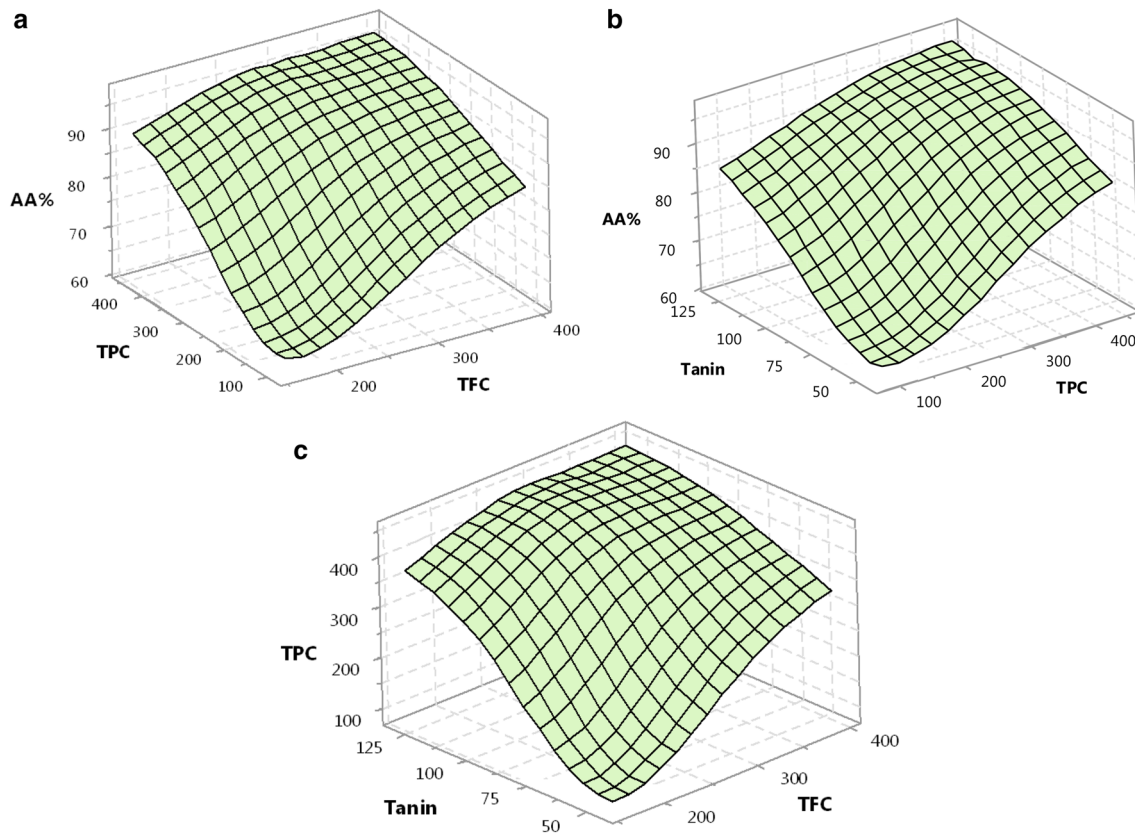
Increasing the regeneration potential observed in dried samples by freezing of olive leaves compared to other cases may be due to high phytoconstituents. The obtained pattern of antioxidant activity in this study is consistent with the phytochemical distribution of olive leaf extract and previous studies [22].

The interactions of the drying process with the AA, TFC, TT, oleuropein and TPP content are shown in three-dimensional curves (Fig. 2). As shown in Fig. 2a, three TPC, TFC and TT responses of oleuropein to drying were similar with FD and SD methods. In fixed TFC content, the antioxidant activity of olive leaf extract was increased with increasing TT. As already mentioned, increasing the concentration of TFC, TT, TPP, oleuropein alone increases the antioxidant properties. In this figure, it can be seen that even low concentrations in high TPPs tend to increase the antioxidant property by a higher concentration than the high TFC concentration. The best antioxidant property is obtained at the highest concentrations of TFC and TPP. Following the changes in TPP and TFC, the gradient of the curve was converted to a horizontal position after an upward trend, which indicates a significant relationship between these variables and the antioxidant property of olive leaf powder.

The amount of AA, TFC, TT, oleuropein and TPP compounds in dried olive leaf powder is influenced by several factors, among which drying method is one of the most important factors affecting the preservation or degradation

of these compounds. Maintaining more phenolic compounds of olive leaves in this method may be due to less time and lower temperature in the SD method, as well as reducing the activity time of the oxidative and hydrolytic enzymes due to the short drying time, can also play an important role in this phenomenon. Various studies have shown that the effect of the drying method of leaves, vegetables, and fruits is different in terms of changing the amount of bioactive and phytochemical compounds, such as compounds of polyphenolic compounds, catechins, tocopherols, carotenoids and anthocyanins, and antioxidant properties [22]. In this regard, some studies have confirmed a significant relationship between the amount of TFC, TT, TPP, and antioxidant properties. Due to the complexity of phenolic compounds decomposition paths during processing, recognition of the governing relationships between the variables in TFC, TT, TPP and antioxidant properties is essential and can help maintaining the amount of bioactive compounds and nutritional value. The relationship between the type of drying method, the amount of TFC, TT, TPP, oleuropein and antioxidant properties can be used to maintain the super beneficial properties of food [22]. The interaction of high temperature and time can greatly reduce the amount of phenolic and antioxidant compounds by reducing the mass of free radicals developed in the olive leaf extract and increasing the speed of the oxidation chain reactions. The results of previous studies have shown that the occurrence of irreversible oxidative reactions





**Fig. 2** Three dimensional curves showing the interactive effects of drying process on the the TFC, TT, and TPP content

and thermal decomposition is one of the main factors affecting the concentration of phenolic compounds obtained from olive leaves during processing, in which the thermal decomposition of these compounds continues over time and temperature. However, the degradation rate increases with increasing drying temperatures. Shahin et al. reported that the content of phenolic and flavonoids in freeze drying (FD), vacuum drying (VD) is better than traditional drying methods [31].

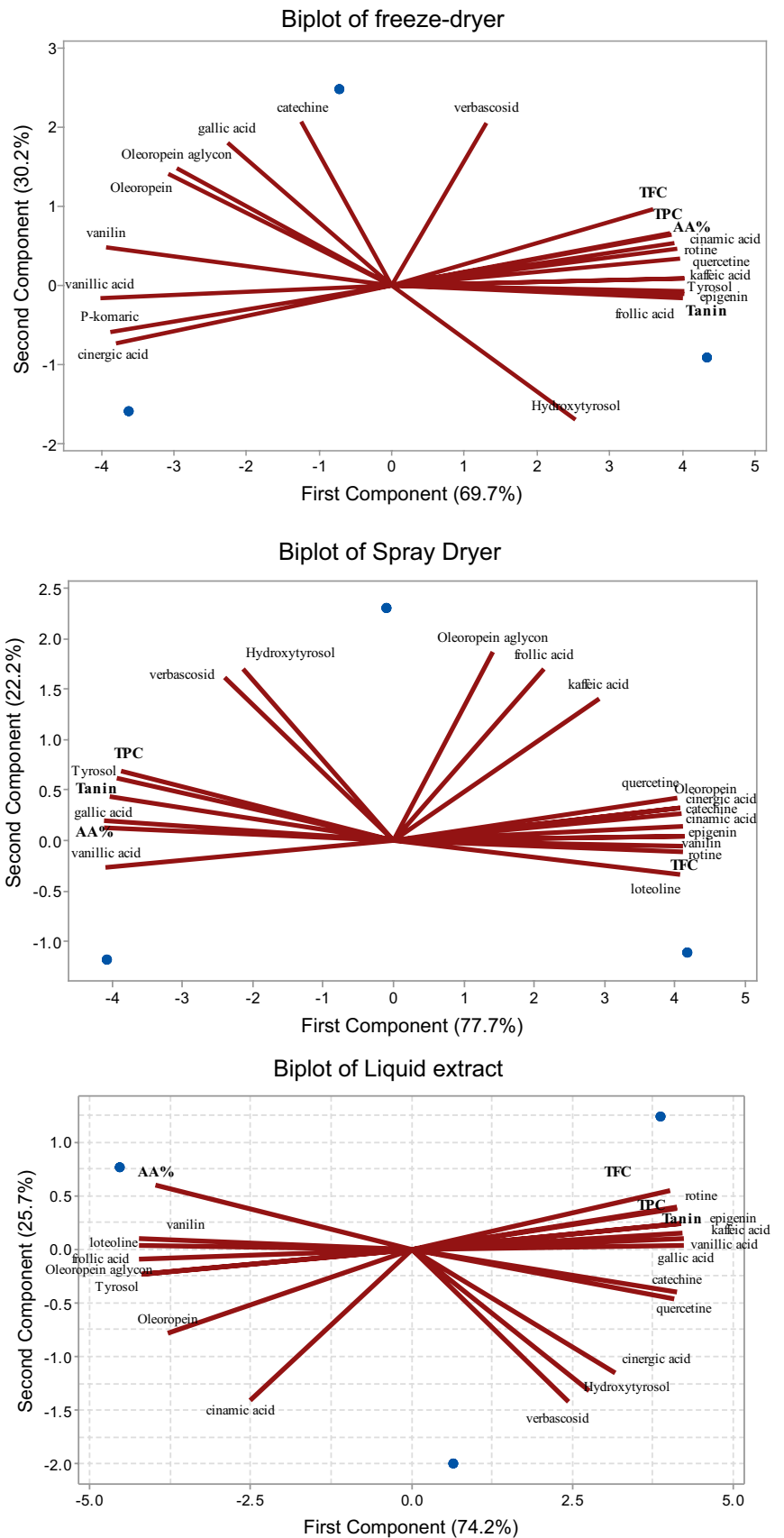
### Principal component analysis (PCA)

The data of TA, TFC, TCA, TT, TPC, and antioxidant activity mean values were obtained from both SD and freezing and aqueous extracts using principal components analysis. In the samples for freeze drying, the first component and the second component compute 79.678% and 20.322% of the total variability. The contribution of each of the parameters in two factors and distribution of cultivars in Biplot are shown in Fig. 3. The amount of antioxidant activity, total phenol and biphenol compounds, total tannin content and flavonoids had a positive, high and significant correlation with PC1, but they had a negative

and significant correlation coefficient with other factors. The results show that oleuropein, Oleuropein aglycon, gallic acid, catechin, vanillic were positively correlated with PC2, and P-komarcic, acid vanilin cinamic acid had negative correlation coefficients. They had low correlation coefficients with other factors. The results obtained from the correlation coefficients confirm the results of the analysis of the main components.

In SD samples, the first component and the second component compute 77.7% and 22.24% of the total variability. The contribution of each of the parameters in two factors and distribution of cultivars in Biplot is shown in Fig. 3. Total flavonoids and catechin, vanillic acid, vanillin, kaffeic acid, frollic acid, quercetine, epigenin, rotine, cinamic acid, cinergic acid, loteoline had a very positive and significant correlation with PC1, but they had a negative and low coefficients with other factors. The results show that the total phenol and biphenol compounds, total tannin content and antioxidant activity of PC2 have a positive correlation, which have negative correlation coefficients with P-komarcic, acid vanilin cinamic acid. They had low correlation coefficients with other factors. The results obtained from the correlation coefficients confirm the results of the analysis of the main components.

**Fig. 3** Biplot of the PCA for the three types of extracts (freeze-drying and spray drying and the aqueous extract of olive leaf)



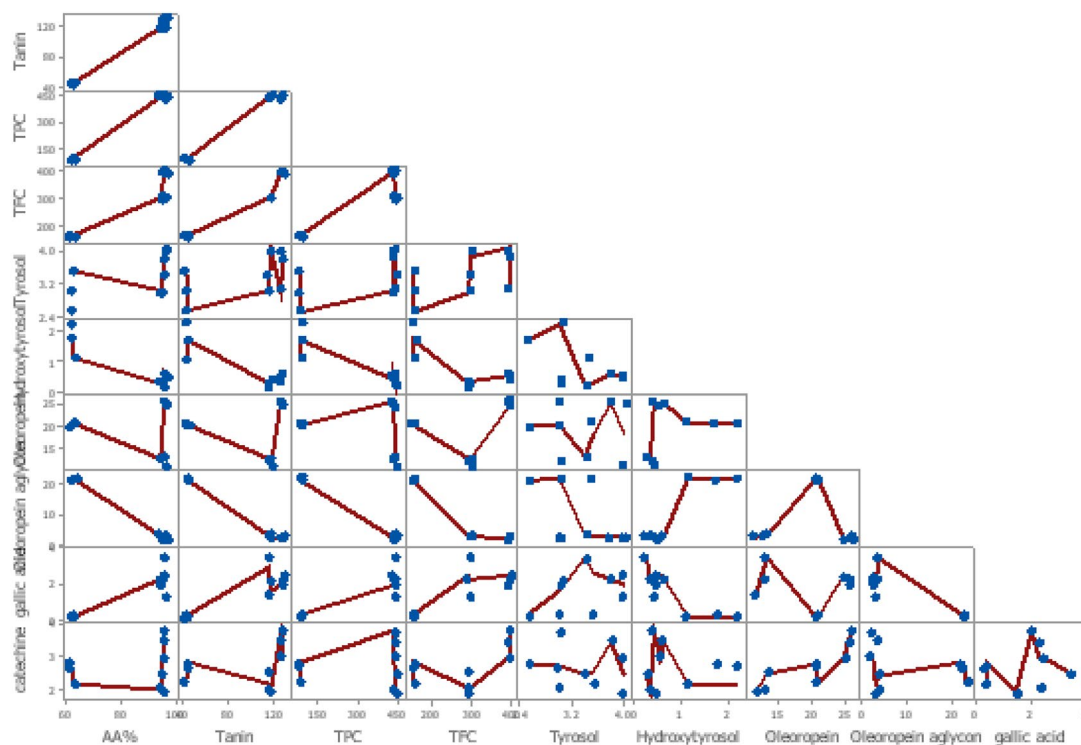
In the aqueous extracts, the first component and the second component compute 74.20% and 25.79% of the total variability. The contribution of each of the parameters in two factors and distribution of cultivars in Biplot is shown in Fig. 3. The levels of antioxidant activity, total phenol and bovine phenolic compounds, total tannin content and total flavonoid and catechin, vanillic acid, kaffeic acid, quercetine, epigenin, and rotine had a very positive and significant correlation with PC1, but they had a negative and significant correlation coefficient with other factors. The results show that antioxidant activity with vanillin, luteoline PC2 has a positive correlation with frollic acid, cinamic acid, Oleuropein aglycon, Tyrosol has negative correlation coefficients. They had low correlation coefficients with other factors (Fig. 4). The results obtained from the correlation coefficients confirm the results of the analysis of the main components. Similar effect was observed by Şahin et al. [31] who evaluated the effect of freeze-dryer and microwave to bioactive compounds such as total phenolic and flavonoid contents, oleuropein, and antioxidant activity of the olive leaves.

The destruction and loss of bioactive compounds, such as phenolic compounds, tannin, as well as total flavonoids, flavonols, flavones, catechins was further found to be underestimated in freeze-dried method in green leafy vegetables [32]. Also, some authors have even mentioned an increase in the

antioxidant activity by at least 13% in several freeze-dryer products [33, 34]. Moreover, freeze-dried and spray-dried materials displayed less total phenolic contents deterioration than conventionally dried olive leaf extracts [10, 31, 35]. Furthermore, freeze-dryer and spray-dryer techniques seems to be appropriate methods for protecting total phenolic contents (high retention level of 44%) [36, 37], total flavonoid contents (retention up to 25.1%) [38].

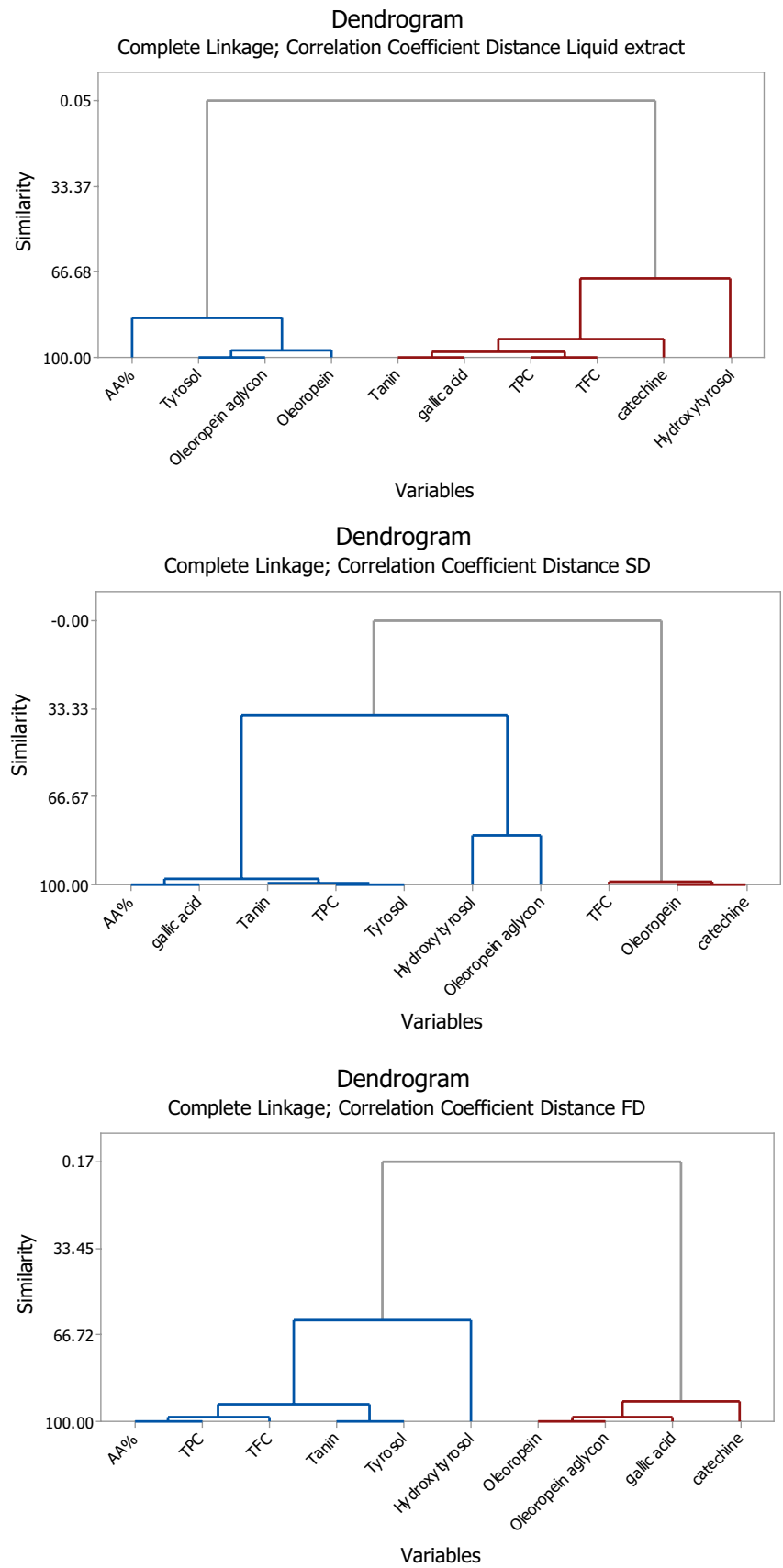
## Agglomerative hierarchical clustering (AHC)

In our study, an overall cluster analysis based on the bioactive compounds contents of powder of olive leaf was performed to study the general differences among samples. To display the similarities between powders of olive leaf samples from different methods, a dendrogram was produced by using a hierarchical clustering algorithm. The resulting dendrogram (Fig. 5) showed two main clusters. In liquid extract, one cluster contains two sub-groups: one with antioxidant activity of liquid extract, while the second sub-group includes tyrosol, oleuropein aglycon and oleuropein. The second cluster contains also three sub-groups: one with tannin, gallic acid, total phenolic content, total flavonoid contents, the second sub-group includes catechin, while the second cluster only constituted of hydroxy tyrosol. In spray



**Fig. 4** The degree of internal adaptation of factors affecting the bioactive compounds, oleuropein, phenolic compounds, tocopherols, flavonoid, tannin content and antioxidant activity in three types of extracts (freeze-drying and spray drying and the aqueous extract of olive leaf)

**Fig. 5** Dendrogram showing the hierarchical clustering results for the three types of extracts (freeze-drying and spray drying and the aqueous extract of olive leaf)



dryer samples, one cluster contains two sub-groups: one with liquid extract antioxidant activity, gallic acid, tannin, total phenolic content and tyrosol, and the last sub-group includes hydroxy tyrosol and oleuropein aglycon. While the second cluster constituted of total flavonoid, oleuropein and catechin. In freeze dryer samples, one cluster contains two sub-groups: one with antioxidant activity of liquid extract, total phenolic content, total flavonoid contents, tannin and tyrosol, while the second sub-group includes hydroxy tyrosol, the second cluster contains oleuropein, oleuropein aglycon catechin and gallic acid.

## Conclusion

In this study, chromatographic, coupled with chemometrics analysis, including similarity assessment, PCA and HCA, were successfully employed. 19 characteristic of bioactive compounds, oleuropein, phenolic compounds, tocopherols, flavonoid, tannin content and antioxidant activity were chosen and proved effective in evaluating the quality of olive leaf extract. The results showed that the freezing and spray drying method had no significant effect on phenolic compounds and tocopherols and tannins and antioxidant activity of olive leaves compared to fresh extract. The FD method preserves most flavonoids, while the SD method maintains more phenolic compounds. The antioxidant activity of both FD and SD were significantly higher than the fresh extract of olive leaf. The evaluation of the type and amount of phenolic compounds in the three types of aqueous extracts and dried extracts showed that the use of SD has been effective in maintaining the amount of unstable polyphenols and active bioactive compounds.

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