Polyphenol profile and antioxidant activity of extracts from olive leaves

Polifenolen profil i antioksidantna aktivnost na ekstrakti ot maslinovi lista

Svetla YANCHEVA, Petros MAVROMATIS and Lidiya GEORGIEVA*

Agricultural University - Plovdiv, Department of Genetics and Plant Breeding, 12 Mendeleev Blvd., 4000 Plovdiv, Bulgaria, *correspondence: lid_georgieva@abv.bg

Abstract

The compounds, derivatives of olive leaves have a high antioxidant activity. The content of the total phenolic compounds (TPC), antioxidant activity (AOA) and HPLC polyphenol profile of methanol extracts from the leaves of the olive cultivars Chondrolia Halkidiki, Kalamon, Koroneiki grown in the nursery (*in vivo*) and *in vitro* plants of Chondrolia Halkidiki were compared. The results obtained for TPC varied between 9.2±0.5 mgGAE*gDW^{-1} and 16.4±0.5 mgGAE*gDW^{-1} . Antioxidant capacity was determined by four methods DPPH, ABTS, FRAP and CUPRAC. The highest results for TPC and AOA were achieved for the leaves of Chondrolia Halkidiki grown *in vitro*. A high correlation between the results gained from the TPC and AOA was established. Conducted HPLC analysis revealed the presence of 3,4-dihydroxybenzoic, caffeic, sinapic and ferulic acids and quercetin, hesperidin and luteolin and the quercetin glycosides rutin and hyperoside.

Keywords: antioxidants, *in vitro*, leaves, olive, polyphenols

Резюме

Съединенията, деривати от листата на маслиновото дърво притежават висок антиоксидантен капацитет. Изследвано е съдържанието на общи фенолни съединения (ОФС), антиоксидантна активност (АОА) и HPLC полифенолния профил на метанолни екстракти от листа на маслиновите сортове Хондролия Халкидики, Каламон и Коронейки, отглеждани в разсадник (*in vivo*) и Хондролия Халкидики от *in vitro* култура. Получените резултати за ОФС варират от 9.2±0.5 mgGAE*gDW^{-1} до 16.4±0.5 mgGAE*gDW^{-1} . Антиоксидантната способност е определена по четири метода DPPH, ABTS, FRAP и CUPRAC, като най-високи резултати са установени за листа на сорта Хондролия Халкидики от *in vitro*

растения. Отчетена е висока корелационна зависимост между резултатите получени за съдържание на ОФС и АОА. Проведените HPLC анализи установиха присъствието на 3,4-дихидроксибензоена, кафеена, синапена и ферулова киселини и кверцетин, хесперидин и лутеолин, и гликозидите рутин и хиперозид.

Ключови думи: антиоксиданти, ин витро, листа, маслина, полифеноли

Introduction

The olive tree (*Olea europaea* L.) is one of the most ancient fruit trees of the Old world cultivated for its edible fruits as table olives and for preparation of olive oil (Fabbri et al., 2004). The famous “Mediterranean diet” is determined as one of most healthy diets and the olives are a significant part of the traditional culinary culture of many nations (El-Kholi et al., 2012).

Health properties of polyphenolic antioxidant plant components and their potential use as natural food additives have been subject to high scientific and commercial interest (Kim et al., 2009; Omar, 2010; Lafka et al., 2013). It is known that free radicals cause oxidative stress and therefore they can provoke damaging of DNA molecules, proteins and lipids in biological systems, leading to different diseases as atherosclerosis, rheumatoid arthritis and inflammatory bowel diseases. Antioxidants can considerably delay or prevent oxidation of target substrates via scavenging of free radicals (Sanchez-Moreno, 2002; Chang et al., 2007).

Olive leaves have been used in traditional medicine in some regions around the Mediterranean Sea (Soler-Rivas et al., 2000; Mitsopoulos et al., 2010; El-Kholi et al., 2012). Recent studies have shown that compounds, derivatives from the leaves and fruits of the olive tree are strong antioxidants (Kontogianni and Gerothanassis, 2012). For the leaf extracts is proved antimalarial, anti-HIV action, vasomotor and hypoglycemic effects (Yang and Ouyang, 2012; Lafka et al., 2013). Many reports indicate that olive leaves contain significant amounts of oleuropein and phenols possessing high antioxidant capacity (Mourtzinos et al., 2007; Lee et al., 2009; Abaza et al., 2011).

Olive leaves are a rich source of bioactive phenolic compounds, whose concentrations are comparable to those of olive oil and fruit. Oleuropein, the most abundant biological active ingredient in olive leaves may protect against cardiovascular diseases (Khayyal et. al., 2002; Somova et al., 2003; Japon-Lujan et al., 2006).

This study presents the evaluation of antioxidant activity and polyphenol profile in leaf extracts of olive cultivars grown *in vivo* and *in vitro*. Moreover, this investigation demonstrates that micropropagation of olive plants *in vitro* could be a method for production of biomass for extraction of valuable biological substances. It could be of interest to a number of countries in which the olive is not a traditional culture or can not be cultivated due to temperature requirements and special features of the species.

Materials and methods

Plant material

Potted plants of the cultivars Chondrolia Halkidiki, Kalamon, Koroneiki grown in the nursery and *in vitro* plants of Chondrolia Halkidiki were used for the study. Leaf materal was lyophilized and ground before extraction.

Extraction procedure

Each plant sample (1.0 g) was extracted three times with 70% methanol at 70 ºC in water bath for 15 min. The biomass was removed through filter paper filtration, and the combined methanol extracts were evaporated to dryness by rotary vacuum evaporator at 60 ºC. The dry extract was diluted in 1 mL 70% methanol before analysis.

Total phenolic content

TPC content was measured using a Folin-Ciocalteu assay according to the procedure described by Stintzing et al. (2005) with some modifications. Folin-Ciocalteu reagent (1mL) (Sigma) diluted five times was mixed with 0.2 mL of sample and 0.8 mL 7.5% Na_2CO_3 (Sigma). The reaction was performed for 20 min at room temperature in darkness. After reaction time, the absorption of the sample was recorded at 765 nm against control sample, developed the same way but without extract added. The results were expressed as mg equivalent of gallic acid (GAE) per gram dry weight (DW), according to calibration curve, build on the range of 0.02 - 0.10 mg gallic acid (Sigma) used as a standard.

Antioxidant activity

DPPH assay

The assay was performed according to the method described by Kivrak et al. (2009) with some modifications. Each analyzed extract (0.15 mL) was mixed with 2.85 mL freshly prepared 0.1mM solution of 1,1-diphenyl-2- picrylhydrazyl radical (DPPH, Sigma) in methanol (Merck). The reaction was performed at 37 °C in darkness and the absorptions at 517 nm were recorded after 15 min against methanol (control). The antioxidant activity was expressed as mM Trolox equivalents (TE) per gram dry weight (DW) by using calibration curve, build by 0.05, 0.1, 0.2, 0.3, 0.4 and 0.5 mM 6-hydroxy- 2,5,7,8-tetramethylchroman- 2- carboxylic acid (Trolox, Fluka) dissolved in methanol (Sigma).

ABTS assay

The method described by Thaipong et al. (2006) was used with some modifications. ABTS radical was generated by mixing aliquot parts of 7.0 mM 2, 2`azinobis (3)-ethylbenzthiazoline-6-sulfonic acid (ABTS, Sigma) in dd H_2O and 2.45 mM potassium persulfate (Merck) in dd H_2O. The reaction was performed for 16 h at ambient

temperature in darkness and the generated ABTS radical ($ABTS^{*+}$) is stable for several days. Before analyses 2.0 mL of generated $ABTS^{*+}$ solution was diluted with methanol at proportions 1:30 (v/v), so the obtained final absorbance of the working solution was about 1.0÷1.1 at 734 nm. For the assay 2.85 mL of this $ABTS^{*+}$ solution was mixed with 0.15 mL of tested extracts. After 15 min at 37 °C in darkness the absorbance was measured at 734 nm against methanol. The antioxidant activity was expressed as mM (TE)*gDW^{-1} by using calibration curve, build in the range of 0.05-0.5 mM Trolox (Fluka) dissolved in methanol (Merck).

Ferric reducing antioxidant power (FRAP) assay

The assay was performed according to method, described by Benzie and Strain (1996) slightly modified as follow: the FRAP reagent was freshly prepared before analyzes by mixing 10 parts 0.3 M acetate buffer (pH 3.6), 1 part 10 mM 2,4,6-tripyridyl-s-triazine (TPTZ, Fluka) in 40 mM HCl (Merck) and 1 part 20 mM $FeCl_3*6H_2O$ (Merck) in dd H_2O. The reaction was started by mixing 3.0 mL FRAP reagent with 0.1 mL of investigated extracts. Control sample prepared with methanol instead of the extract was used. The reaction time was 10 min at 37 °C in darkness and absorbance at 593 nm. Antioxidant activity was expressed as mM TE*gDW^{-1} by using calibration curve, build in the range of 0.05-0.5 mM Trolox (Fluka) dissolved in methanol (Merck).

Cupric reducing antioxidant capacity (CUPRAC) assay

The assay was performed according to Apak et al. (2006) with some modifications. The reaction was started by mixing 1.0 mL 10 mM $CuCl_2*2H_2O$ (Sigma) in dd H_2O, 1.0 mL 7.5 mM Neocuproine (Sigma) in methanol, 1.0 mL 0.1 M ammonium acetate buffer (pH 7.0), 0.1 mL of investigated extract and 1.0 mL dd H_2O. Control sample was with methanol only. The reaction was carried out for 20 min at 50 °C in darkness and the sample absorption 450 nm. Antioxidant activity was expressed as mM (TE)*gDW^{-1} by using calibration curve, build in the range of 0.05-0.5 mM Trolox (Fluka) dissolved in methanol (Merck).

HPLC assays

Qualitative and quantitative determination of phenolic acids and flavonoids were performed by using Waters 1525 Binary Pump HPLC systems (Waters, Milford, MA, USA), equipped with Waters 2484 dual Absorbance Detector (Waters, Milford, MA, USA) and Supelco Discovery HS C18 column (5 µm, 25 cm×4.6 mm), operated under control of Breeze 3.30 software. For separation of the phenolic acids a mobile phase of 2% (v/v) acetic acid (solvent A) and 0.5% (v/v) acetic acid:acetonitrile (1:1 v/v) (solvent B) was used. For separation of flavonoids a mobile phase consists of 2.0% (v/v) acetic acid (solvent A) and methanol (solvent B). For separation of quercetin glycosides rutin and hyperoside a mobile phase of 2% (v/v) acetic acid (solvent A) and acetonitrile (solvent B) was used. The elution program for separation of phenolic acids, flavonoids and quercetin glycosides was described previously by Marchev et al. (2011).

Results and discussion

Olive leaves are a rich source of biological active substances which concentration is comparable with those of olive oil and fruits. The aim of the present study was to investigate and compare total phenol content, antioxidant capacity and polyphenol profile of olive leaf methanol extracts from plants grown *in vivo* and *in vitro*.

TPC and AOA of leaf extracts from Chondrolia Halkidiki, Kalamon, Koroneiki grown *in vivo* and *in vitro* plants of Chondrolia Halkidiki are present in Table 1.

Table 1. Total phenol content and antioxidant activity in olive leaf methanol extracts

Таблица 1. Съдържание на общи фенолни съединения и антиоксидантна активност в метанолни екстракти на маслинови листа

Sample	TPC, mgGAE*gDW^{-1}	DPPH, mMTE*gDW^{-1}	ABTS, mMTE*gDW^{-1}	FRAP, mMTE*gDW^{-1}	CUPRAC, mMTE*gDW^{-1}
Chondrolia Halkidiki	15.6±0.5	110.8±0.2	106.8±2.7	88.5±1.3	398.0±0.9
Kalamon	14.7±0.5	98.7±0.5	102.9±0.3	85.1±1.1	382.2±1.3
Koroneiki	9.2±0.5	69.7±0.2	76.2±0.9	51.3±0.6	271.3±2.7
Chondrolia Halkidiki – *in vitro*	16.4±0.5	111.3±0.2	109.8±0.8	91.14±0.5	420.6±4.5

The results for TPC determined by the Folin Ciocalteu method varied from 9.2±0.5 mgGAE*gDW^{-1} to 16.4±0.5 mgGAE*gDW^{-1} . The highest content was observed for *in vitro* plants of Chondrolia Halkidiki. In the case with *in vivo* grown cultivars the highest result was counted for Chondrolia Halkidiki and the lowest for Koroneiki (Table 1).

Investigation of TPC in extracts from Koroneiki and Kalamon by Mitsopoulos et al. (2010) showed results significantly lower than those established by us.

In the resent years TPC in extracts of olive leaves has been interested as a potential of antioxidant activity (Abaza et al., 2011; Kontogianni and Gerothanassis, 2012; Lafka et al., 2013).

Obtained results about antioxidant activity determined by DPPH, ABTS, FRAP and CUPRAC methods (Table 1) showed a high comparability with the results for the total phenol content. Comparing data for AOA the highest values were observed for cultivar Chondrolia Halkidiki grown *in vitro* and *in vivo*. Presented results are in accordance with Yang and Ouyang (2012) and Lafka et al. (2013) confirming the highest antioxidant potential of olive leaf extracts.

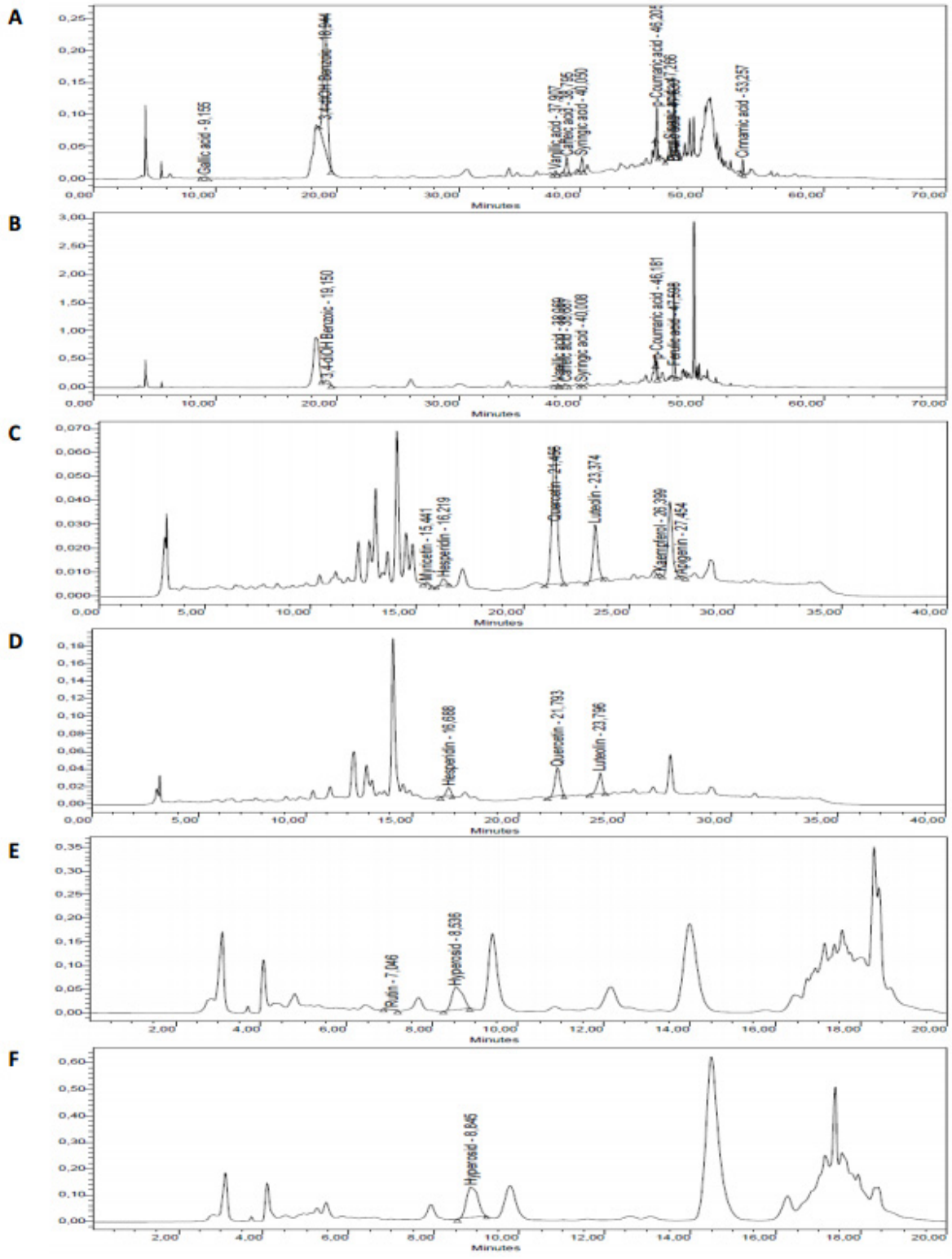

Figure 1. HPLC profile of olive leaf extracts from cultivar Chondrolia Halkidiki *in vivo* (A, C, E) and *in vitro* plants (B, D, F)

Фигура 1. HPLC профил на екстракти от маслинови листа на сорт Хондролия Халкидики *in vivo* (A, C, E) и *in vitro* растения (B, D, F)

Present investigation demonstrated that *in vitro* plants possess higher content of TPC and AOA in comparison with *in vivo* cultivated olive plants. The differences in the reported results can be explained by genotypic characteristics of the cultivars and different age, nutritional supplement, the effect of growth regulators and culture conditions *in vitro*. This dependence has been observed by Zayova et al. (2012) and Dakah et al. (2014) studying the antioxidant ability of the *in vitro* and *in vivo* cultures of medicinal plants.

Polyphenol profile of investigated extracts was determined by HPLC assays (Figure 1) and the results were expressed as μg*gDW^{-1} (Table 2). It was established that the dominant phenolic acids in the tested olive cultivars were 3,4-dihydroxybenzoic, caffeic, sinapic and ferulic acids. Among the representatives of the flavonoids, the most common were quercetin, hesperidin, luteolin and quercetin glycosides rutin and hyperoside. The highest content of 3,4-dihydroxybenzoic acid was measured in the extract of Chondrolia Halkidiki (1699.4 μg*gDW^{-1}) and the lowest concentration was determined for the *in vitro* plants of the same cultivar.

Table 2. Polyphenol profile of olive leaf metanolic extracts

Таблица 2. Полифенолен профил на метанолни екстракти от маслинови листа

Content (μg*gDW^{-1})	Chondrolia Halkidiki	Kalamon	Koroneiki	Chondrolia Halkidiki – *in vitro*
		Phenolic acids		
3,4-Dihydroxybenzoic acid	1699.4	976.6	434.1	104.5
Caffeic acid	trace	49.3	trace	trace
Syringic acid	trace	31.4	trace	trace
p-Coumaric acid	trace	64.0	trace	trace
Sinapic acid	trace	155.9	37.8	trace
Ferulic acid	216.9	77.1	59.5	555.3
		Flavonoids		
Quercetin	205.2	104.5	130.8	26.4
Hesperidin	113.7	139.8	50.2	51.9
Luteolin	439.1	335.8	300.6	88.0
Rutin	trace	19.1	18.1	not found
Hyperoside	462.9	284.9	210.5	230.6

In contrast, the content of ferulic acid was the highest in the extracts of Chondrolia Halkidiki *in vitro* (555.3 μg*gDW^{-1}) comparing to Chondrolia Halkidiki *in vivo* (216.9 μg*gDW^{-1}) and more than three times less in Kalamon (77.1 μg*gDW^{-1}) and Koroneiki (59.5 μg*gDW^{-1}). It is important to mention that only in the extract of Kalamon caffeic (49.3 μg*gDW^{-1}), syringic (31.4 μg*gDW^{-1}) and p-coumaric (64.0 μg*gDW^{-1}) acids were found. The presence of sinapic acid was detected in extracts from Kalamon (155.9 μg*gDW^{-1}) and Koroneiki (37.8 μg*gDW^{-1}).

The analysis of flavonoid profile (Table 2) showed that for most polyphenol compounds the highest values were determined for Chondrolia Halkidiki, and the lowest for the same cultivar grown *in vitro*. Furthermore, there were a large number of unidentified peaks corresponding to other compounds in the HPLC profiles of the tested extracts. Presented results are in accordance with previously established by Dekanski et al. (2009) detection of quercetin, luteolin-7-O-glicoside and apigenin-Oglicoside and Mitsopoulos et al. (2010) of rutin and oleuropein.

Conclusion

The aim of this study was to examine and compare the total phenol content, antioxidant capacity and polyphenol profile of extracts from olive leaves. The highest content of TPC and AOA was detected in the leaves of Chondrolia Halkidiki grown *in vitro* and decreasing in the order cultivars Chondrolia Halkidiki, Kalamon and Koroneiki *in vivo*. After examination of the HPLC polyphenol profile of the investigated samples was determined the presence of different phenolic substances with important biological activities, which could be of interest in further experiments. Moreover, this investigation demonstrates the potential of micropropagated olive plants *in vitro* as a source for production of biomass for extraction of valuable biological substances.

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