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Effect of genotype and extraction method on polyphenols content, phenolic acids, and flavonoids of olive leaves (*Olea europaea* L. subsp. *europaea*)

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ABSTRACT

Polyphenol's extraction varied according to various factors. In this study, the effect of genotype and method of polyphenols extraction were investigated using leaves of two cultivated and two wild olive varieties and four hydromethanolic extraction methods. Quantitatively, significant differences were observed according to the extraction method, the genotype, and the interaction genotype-method of extraction. The heat reflux extraction showed the highest polyphenols content in wild olive leaves having an amount of 841.17 mg GAE/100 g DM. The qualitative phytochemical examination using high performance liquid chromatography (HPLC) of olive leaves showed some significant differences of phenolic compounds between genotypes. For the same oleaster genotype, the extraction method seemed to influence qualitatively the polyphenols profiles. The quinic acid was the dominant phenolic acid and the luteolin-7-*O*-glucoside was the major flavonoid observed in wild olive leaves having, respectively, 618.24 and 3211.44 mg/kg DM. The quinic acid has an amount of 400.15 and 275.39 mg/kg and the luteolin-7-*O*-glucoside has an amount of 2059.62 and 1214.49 mg/kg in cultivars leaves. The extraction by Soxhlet of wild olive leaves showed the highest quinic acid (1085.80 mg/kg DM) and luteolin-7-*O*-glucoside (3720.15 mg/kg DM) amounts. The hydromethanolic extraction assisted by Soxhlet of wild olive leaves constituted the optimal method to obtain high polyphenols contents enriched with phenolic acids and flavonoids.

1. Introduction

Plants are natural source of molecules used in therapeutic, pharmaceutic and cosmetology fields. Currently, about 25-30% of all drugs are derived from natural products (Boldi, 2004). Phenolic compounds are known as secondary plant metabolites and are the most important phytochemicals due to their bioactive functions.

Olive tree (*Olea europaea* L. subsp. *europaea*) includes two botanical varieties, the cultivated olive tree (variety *europaea*) grouped olive cultivars and wild olive trees or oleasters (variety *sylvestris*) including natural oleasters and feral forms (Besnard et al., 2001; Hannachi et al., 2008). *Olea europaea* L. is an exceptional species and was widely studied for their organs benefits that are rich in phenolic compounds (Ben Salah et al., 2012; Hannachi et al., 2013; Hannachi et al., 2019). The olive leaves have an important antioxidant activity and are richer in bioactive compounds compared to other parts of olive tree (Hannachi et al., 2020). In addition, phenolic compounds of olive leaves have beneficial effects on health such as antihypertensive, hypoglobulin, hypocholesterolemic, cardioprotective and anti-inflammatory properties. The olive leaves properties are mostly attributed to their polyphenols (Ryan et al., 2002; Vermerris and Nicholson, 2006).

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The phenolic compounds extraction is the first and the important step in the isolation and identification of phenolic compounds (Bucić-Kojić et al., 2007). Several extraction methods were used as solid-liquid extraction that is an old operation by using water or an organic solvent. Many studies have shown that the highest yield was obtained using a co-solvent as mixture of ethanol or methanol with water (Tsakona et al., 2012; Miguel et al., 2010). Olive is one of the most investigated plant all around the world as well as this topic about extraction influence applied on different plant materials (Hannachi et al., 2019). Conventional and modern extraction methods have been used to extract biomolecules from plant material (Liazid et al., 2007; Hannachi et al., 2019; Yahia et al., 2020). Several factors can affect the phenolic compounds profile such geographical origin, genotype, and extraction process (Vinhaet al., 2005; Papoti et al., 2009). The chemotaxonomy is a plant classification based on chemical constituents (Singh, 2016). The plant classification based on secondary metabolites were used for several species as the genus *Aquilaria* (Andary et al., 2019), *Propolus* (Abdellatif et al., 2019), *Solanum lycopersicum* L. (Siracusa et al., 2012) and Olive cultivars (Ben Mohamed et al., 2018).

The aim of this study was to compare four extraction methods on olive leaves using two cultivars and two oleasters, highlighting the effect of genotype, the extraction method, and the method-genotype interaction on phenolics contents and profile using HPLC analysis.

2. Materials and methods

2.1. Chemicals and reagents

HPLC grade methanol, methanol solvents and hydrochloric acid were obtained from Lab-Scan. Folin–Ciocalteu reagent and aluminum chloride were obtained from Loba Chemie. Gallic acid, Rutin, Trolox (6-hydroxy2,5,7,8-tetramethylchroman-2-carboxylic acid), DDPH (2,2-diphényl-1-picrylhydrazyl), ABTS [2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)] and catechin were supplied by Sigma Chemical Co. (USA). Vanillin and sodium carbonate were purchased from Chemi-Pharama.

2.2. Plant materials

Leaves from four Tunisian genotypes of olive (*Olea europaea* L.) including two cultivated olive trees called 'Chemlali' and 'Zarrazi' cultivated in the Gabes region (variety *europaea*) and two wild olive trees or oleasters (variety *sylvestris*) originating from natural ecosystems at Tunisia southern (Tounine and Toujene) were harvested and collected. The olive leaves were washed, and air-dried, then grounded to a fine powder using a crusher (type FW135, 200Mic).

2.3. Methods of extraction

Polyphenols extraction from olive leaves was conducted with 70% methanol using four techniques of extraction: Soxhlet extraction (SOE), heat reflux extraction (HRE), maceration (MAC) and ultrasonic assisted extraction (UAE). The extraction procedures were repeated three times. All obtained extracts were filtered. Then, the filtrates were centrifuged at 4000 tr/min for 20 min. The extracts were recovered and stored at 4°C in the dark for analyses. Dried powdered olive leaves (5 g) were extracted with 100 mL of 70% methanol: i) by maceration (MAC) for 24 h at 25°C under agitation, ii) by Soxhlet (SOE) for 4h at 70°C, iii) by heat reflux extraction (HRE) for 1h at 70°C, and iv) by ultrasonic assisted extraction (UAE) for 40 min at 25°C (Hannachi et al., 20019; Yahia et al., 2020).

2.4. Total polyphenols content (TPC)

TPC was determined by Folin Ciocalteu method. Folin reagent (0.5 mL) was added to 0.1 ml of olive leaves extract. After 5 min, 4 ml of Na_2CO_3 (1M) was added. The mixture was then leaved for 90 min in the dark at room temperature. The absorbance was recorded at 765 nm using a T60 UV-Spectrophotometer. A calibration curve was prepared with a gallic acid solution having a concentration ranged between 0 and 500 µg/ml. Results were expressed as milligram of gallic acid equivalent (GAE) per 100 grams of dry matter (mg GAE/100g DM) (Hannachi et al., 20019; Yahia et al., 2020).

2.5. Total flavonoids content (TFC)

Olive leaves extract (1 ml) was added to 1 ml of aluminum solution $AlCl_3$ (10%). The mixture was incubated at room temperature for 30 min and then, the absorbance was measured at 430 nm using a T60 UV-Spectrophotometer. The average data were interpolated in a rutin calibration curve with a concentration ranging between 0 and 250 µg/ml. The TFC was expressed as milligram of rutin equivalent per 100 grams of dry matter (mg RE/100g DM) (Elfalleh et al., 2009).

2.6. Condensed tannins content (CTC)

The condensed tannins content was determined by the vanillin method in acid medium. Olive leaves extract (250 µl) was added to 1500 µl of vanillin/methanol solution (4%). Then, 750 µl of 37% hydrochloric acid was added. The mixture was incubated at room temperature for 15 min and the absorbance was measured at 500 nm using a T60 UV-Spectrophotometer. The average data were interpolated in a catechin calibration curve with a concentration ranging from 0 to1000 µg/ml. Result was expressed as milligram of catechin equivalent per 100 grams of dry matter (mg CE/100g DM) (Elfalleh et al., 2009).

2.7. High performance liquid chromatography analysis

The olive leaves of oleaster from Tounine natural ecosystem were used to extract phenolic compounds by four different extraction methods to determine the effect of the extraction methods on the quality of phenolic compounds. For other parts, phenolic compounds of the four studied olive leaves were extracted by maceration process to evaluate the effect of genotype on the quality of phenolic compounds.

An Agilent HPLC (high performance liquid chromatography) system with DiscoVery BIO Wide Pore C18-5 binary pump with LC-20ADXR type pump A and LC-20ADXR type B pump was used to identify phenolic compounds. The column has a length of 250 mm and an internal diameter of 4.0 mm (Vinha et al., 2002). The total flow of the mobile phase was 0.4000 ml/min and the pressure varied from 0% to 10%. Elution was carried out at a maximum temperature of 75°C for 35 min. The mass spectrometer (MS) was coupled with a high-speed liquid chromatography system to identify the phenolic compounds. The peaks of phenolic compounds were identified by comparing their retention time with those of the standards and verifying their characteristic spectrum (λ = 163 to 717 nm). Results were expressed as milligram per kg dry matter (mg/kg DM).

2.8. Antioxidant activities

2.8.1. DPPH· radical scavenging activity

Olive leaves extract (20 µl) was mixed with 180 µl of 1,1-diphenyl-2-picrylhydrazyl (DPPH·) methanolic solution (0.2 mM). The mixture was shaken and left for 30 min in darkness at 25°C. The presence of an antioxidant donator of hydrogen, the DPPH. Radical was reduced in 2,2-diphényl-1-picrylhydrazine (DPPH-H). The absorbance of the solution was measured at 517 nm (Elfalleh et al., 2009). A calibration curve was done using Trolox. The antioxidant activity was recorded as equivalent antioxidant capacity expressed as mg equivalent Trolox per 100 g dry matter (mg ET/100 g DM).

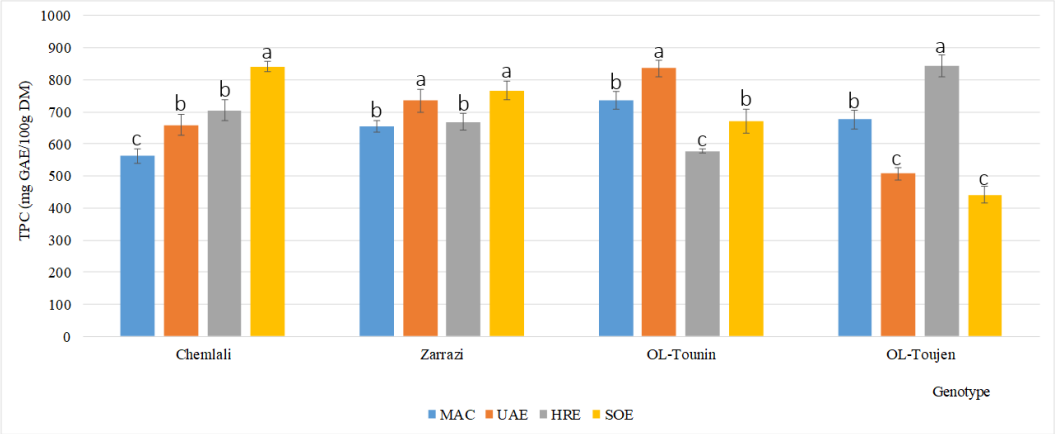

Figure 1. Total polyphenols content (TPC) of olive leaves according to genotype and extraction method (GAE: gallic acid equivalent, DM: dry matter; MAC: maceration extraction; UAE: ultrasonic assisted extraction; HRE: heat reflux extraction; SOE: Soxhlet extraction, different letters for each genotype showed significant difference at $p < 0.05$)

2.8.2. ABTS⁺ radical scavenging activity

Le radical monocation ABTS⁺· [2,2′-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid)] was generated by oxidation of ABTS with potassium persulfate and is reduced in the presence of hydrogen-donating from an antioxidant (Re et al., 1999). The ABTS⁺· radical generated by mixing 7 mM 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) ABTS solution with 2.45 mM potassium persulfate ($K_2S_2O_8$) and allowing the mixture in the dark at room temperature for 16 h. Before usage, the ABTS⁺· solution was diluted with ethanol to get an absorbance of 0.700 ± 0.02 at 734 nm. Then, a quantity of 20 µl of the olive leaves extract was added to a 180 µl of ABTS⁺· solution. The absorbance was measured at 734 nm after 5 min of incubation (Elfalleh et al., 2011). The curve absorbance reduction at 734 nm function of Trolox concentration (mM) were used to determine the ABTS radical scavenging activity. Results were expressed as mg ET/100 g DM.

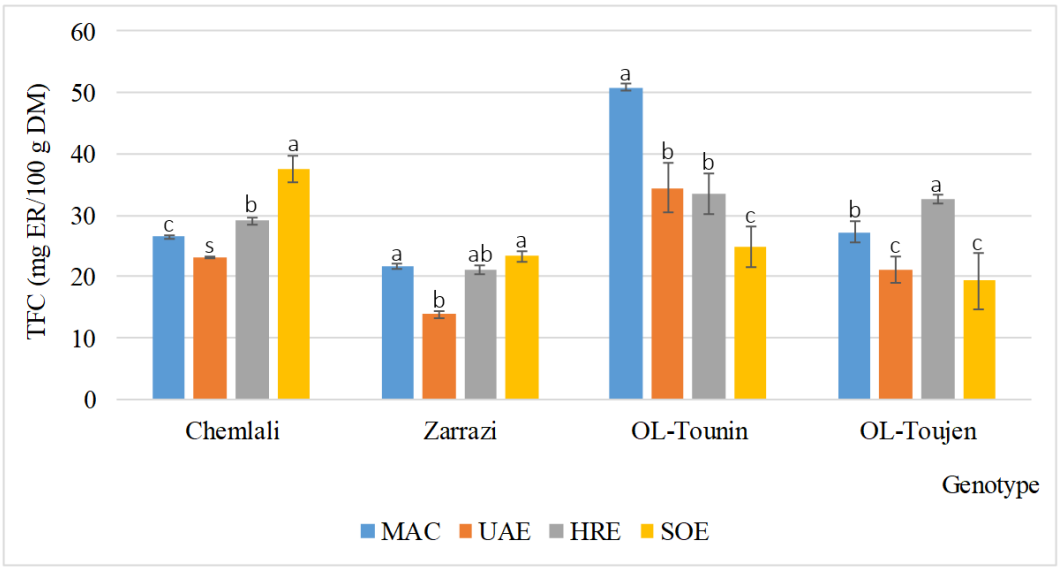

Figure 2. Total flavonoids content (TFC) of olive leaves according to genotype and extraction method (RE: rutin quivalent, DM: dry matter, MAC: maceration extraction, UAE: ultrasonic assisted extraction, HRE: heat reflux extraction, SOE: Soxhlet extraction, different letters for each genotype showed significant difference at $p < 0.05$)

2.9. Statistical analyses

Statistical analyses were performed using the XLSTAT software (www.xlstat.com). All analyzes were conducted in triplicate and the data were presented as an average ± standard deviation. The influence of the genotype, the extraction method and their possible interactions were evaluated by analysis of the variance (ANOVA) and Duncan's multiple range. An effect was considered significant when $p < 0.05$. Principal component analysis (PCA) was conducted on obtained data to show the distribution of genotypes and extraction methods based on polyphenols, flavonoids, condensed tannins, phenolic acids content and antioxidant activities.

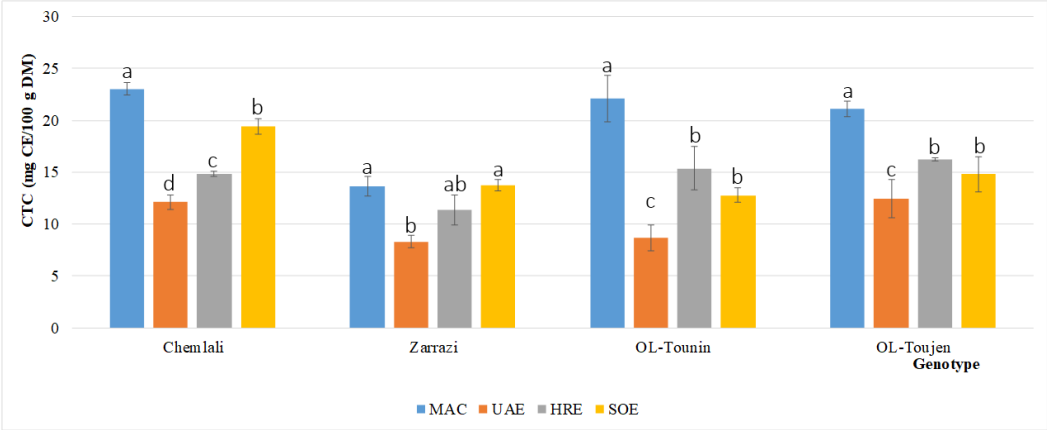

Figure 3. Condensed tannin content (CTC) of olive leaves according to genotype and extraction method (CE: catechin equivalent, DM: dry matter, MAC: maceration extraction, UAE: ultrasonic assisted extraction, HRE: heat reflux extraction, SOE: Soxhlet extraction, different letters for each genotype showed significant difference at $p < 0.05$)

3. Results and discussion

3.1. Total polyphenols contents (TPC)

The oleaster OL-Toujene showed the highest TPC obtained by heat reflux extraction (843.17 mg GAE/100 g DM) and the lowest one (441.01 mg GAE/100 g DM) obtained by Soxhlet. The TPC varied from 653.42 to 841.17 mg GAE/100 g DM and from 441.01 to 843.17 mg GAE/100 g DM of leaves from cultivars and oleasters, respectively. ANOVA showed that genotype, method of extraction and genotype-method interaction have a significant effect on TPC (Figure 1).

Extraction by maceration of oleaster OL-Tounine leaves showed the highest TFC (50.85 mg RE/100g DM), followed by a Soxhlet extraction of Chemlali leaves (37.48 mg RE/100g DM). Ultrasonic assisted extraction of Zarrazi olive leaves yielded the lowest TFC (13.87 mg RE/100g DM). ANOVA showed significant effect of genotype, extraction method and genotype-extraction method interaction (Figure 2).

The CTC obtained from Chemlali leaves (23.03 mg CE/100g DM) using maceration were higher than other extraction methods. The condensed tannins content obtained by ultrasonic assisted extraction (Zarrazi leaves) showed the lowest content (8.32 mg CE/100g DM). ANOVA showed a significant effect of genotype, extraction method and genotype-extraction method interaction on the condensed tannins contents (Figure 3).

Results showed that the genotype, method of extraction and the genotype-method of extraction interaction influenced the polyphenols, flavonoids, and condensed tannins contents.

It has been reported that the extractions method affected the polyphenols yields extracted from plants (Yahia et al., 2020). Results showed that polyphenols content increased at high temperature. Indeed, the highest TPC were obtained by using Soxhlet and heat reflux extractions. High temperature during promotes the diffusion and solubility of the extracted substances. It has been noted that Soxhlet extraction provided high yields of polyphenols among conventional techniques (Tsakona et al., 2012; Al-Bandak and Oreopoulou, 2007; Horžić et al., 2009). Conventional method of extraction is usually conducted at high temperature such as Soxhlet and reflux or under longer time of extraction as maceration. New extraction method has been developed as ultrasonic and microwave assisted extractions to avoid energy and solvent consumption (Hannachi et al., 20019; Yahia et al., 2020). However, in this study the ultrasonic assisted extraction showed lower polyphenols, flavonoids and condensed tannins contents as noted previously (Da Porto et al., 2013). Based on literature, the ultrasonic assisted polyphenols extraction is more efficient for polyphenols extraction (Nayak et al., 2015; Yahia et al., 2020). These differences would be explained by genetic factors.

3.2. Influence of the genotype on phenolic profile

The phenolic compounds identification of HPLC analysis were conducted by using 33 available standards.

Quinic acid is the major phenolic acid, varying from 275.39 to 618.24 mg/kg DM, followed by 4,5-di-*O*-cafeoylquinic acid varying from 7.39 to 32.26 mg/kg DM. Gallic acid was detected only in leaves extract of Zarrazi cultivar. In contrast, caffeic acid was absent only in Chemlali leaves extract. 1,3-di-*O*-caffeoyquinic acid was detected only in oleaster OL-Tounine only and *trans*-ferulic acid was detected in the both Zarrazi and oleaster OL-Toujene leaves extracts.

Luteolin-7-*O*-glucoside was the predominant flavonoids (from 1214.49 to 3211.44 mg/kg DM), followed by quercetrin (quercetin-3-*O*-rhamnose) (from 767.45 to 1574.63 mg/kg DM). The *(+)*-catechin was identified only in the Chemlali leave extract. The genotype significantly influenced the polyphenols compounds

reflecting by analysis of variance followed by Duncan's multiple range (Table 1).

The PCA analysis showed that the oleaster Tounine was distinguishable for other genotype and was closely with SOX extraction method (Figure 4).

Table 1. Identification of phenolic compounds by HPLC of olive leaves according to genotypes

Phenolic compounds (mg/kg extract)	Brut formula	[M-H] m/z	RT (min)	Cultivars leaves		Oleaster leaves	
				Chemlali	Zarrazi	Tounine	Toujene
Quinic acid	$C_7H_{12}O_6$	191	2.048	400.15 ± 4.31^b	275.39 ± 27.73^c	618.24 ± 6.22^a	394.71 ± 18.26^b
Gallic acid	$C_7H_6O_5$	169	3.926	nd	7.43 ± 0.07	nd	nd
Chlorogenic acid	$C_{16}H_{18}O_9$	353	9.882	12.02 ± 0.99^b	6.75 ± 0.41^b	28.42 ± 0.48^a	11.35 ± 3.55^b
Catechin (+)	$C_{15}H_{14}O_6$	289	11.059	5.54 ± 0.03	nd	nd	nd
4-*O*-caffeoylquinic acid	$C_{16}H_{18}O_9$	353	12.533	12.6 ± 0.04^b	7.39 ± 0.52^c	32.26 ± 1.05^a	12.83 ± 0.98^b
Caffeic acid	$C_9H_8O_4$	179	14.528	nd	14.2 ± 2.52	3.28 ± 0.23	8.60 ± 1.64
1,3-di-*O*-caffeoylquinic acid	$C_{25}H_{24}O_{12}$	515	20.367	nd	nd	10.44 ± 0.42	nd
p-Coumaric acid	$C_9H_8O_3$	163	20.90	5.12 ± 0.65^c	115.14 ± 3.11^a	22.15 ± 1.77^b	29.66 ± 7.16^b
Salviolinic acid	$C_{36}H_{30}O_{16}$	717	28.946	11.8 ± 2.12	nd	nd	nd
trans-Ferulic acid	$C_{10}H_{10}O_4$	193	23.142	nd	17.14 ± 8.82	nd	32.68 ± 6.99
Rutin	$C_{27}H_{30}O_{16}$	609	23.798	825.77 ± 0.81^c	531.24 ± 17.17^d	2612.59 ± 30.5^a	1342.01 ± 0.25^b
Luteolin-7-*O*-glucoside	$C_{21}H_{20}O_{11}$	447	24.440	2059.62 ± 3.22^c	1214.49 ± 13.99^d	2905.97 ± 16.76^b	3211.44 ± 4.02^a
Naringin	$C_{27}H_{32}O_{14}$	579	25.855	104.07 ± 1.71^a	27.95 ± 1.06^b	94.95 ± 0.52^a	100.10 ± 9.25^a
Apigenin-7-*O*-glucoside	$C_{21}H_{20}O_{10}$	431	26.711	135.95 ± 1.15^b	28.54 ± 0.91^d	123.98 ± 0.4^c	315.67 ± 0.27^a
Quercetin (quercetin-3-*O*-rhamnose)	$C_{21}H_{20}O_{11}$	447	26.806	1108.18 ± 22.5^b	767.45 ± 15.68^c	1536.5 ± 2.12^a	1574.63 ± 15.68^a
4,5-di-*O*-caffeoylquinic acid	$C_{25}H_{24}O_{12}$	515	26.811	122.33 ± 0.07^b	40.08 ± 0.06^d	139.94 ± 1.81^a	104.93 ± 0.78^c
Quercetin	$C_{15}H_{10}O_7$	301	31.714	33.96 ± 1.75^b	4.29 ± 0.01^c	168.12 ± 3.25^a	38.03 ± 1.00^b
Kaempferol	$C_{15}H_{10}O_6$	285	31.762	34.27 ± 0.66^c	33.79 ± 0.21^c	53.44 ± 0.82^b	90.01 ± 0.04^a
Naringenin	$C_{15}H_{12}O_5$	271	33.730	2.71 ± 0.38^b	1.00 ± 0.04^c	2.7 ± 0.01^b	4.54 ± 0.31^a
Apigenin	$C_{15}H_{10}O_5$	269	34.364	1.78 ± 0.37^c	0.61 ± 0.07^d	6.04 ± 0.14^b	23.09 ± 0.01^a

RT: retention time, nd: not detected, different letters in the same line showed significant difference at $p < 0.05$

The genotype influenced the phenolic composition of olive leaves extracts. It has been reported that quantitative differences in the phenolic profile were observed between the two Tunisian cultivars Chamlali and Neb Jmel (Brahmi et al., 2013). Results showed differences between the studied cultivars and oleasters. Furthermore, the phenolic profiles depended to the varieties sylvestris and europaea of *Olea europaea* subsp. *europaea*. The variations in phenolic profile can be related to the olive-growing geographical area. It was noted also that the levels of polyphenols in fennel seeds (*Foeniculum vulgarae* Mill.) were affected by their provenances (Bettaieb-Rebey et al., 2011). Moreover, the phenolic composition can vary between the organs of the same plant.

Table 2. Identification of phenolic compounds by HPLC of wild olive leaves (OL-Tounine) according to the extraction methods

Phenolic compounds (mg/kg extract)	Brut formula	[M-H] m/z	RT (min)	Maceration Extraction	Soxhlet extraction	Heat reflux extraction	Ultrasonic extraction
Quinic acid	$C_7H_{12}O_6$	191	2.101	633.61 ± 1.35^a	1085.80 ± 0.42^c	745.65 ± 0.49^d	778.96 ± 0.06^b
Chlorogenic acid	$C_{16}H_{18}O_9$	353	9.817	28.27 ± 0.64	nd	nd	nd
4-*O*- Caffeoylquinic acid	$C_{16}H_{18}O_9$	353	3.630	28.11 ± 0.39^a	71.83 ± 1.09^b	49.55 ± 0.64^d	40.82 ± 0.25^c
Caffeic acid	$C_9H_8O_4$	179	14.959	6.05 ± 0.58^a	13.75 ± 0.35^{ab}	12.66 ± 0.48^c	10.99 ± 0.01^b
p-Coumaric acid	$C_9H_8O_3$	163	21.312	25.94 ± 0.45^a	50.83 ± 0.83^b	43.52 ± 0.68^d	39.79 ± 0.3^c
trans-Ferulic acid	$C_{10}H_{10}O_4$	193	23.537	nd	25.61 ± 0.80^a	20.78 ± 0.31^b	18.90 ± 0.14^c
Rutin	$C_{27}H_{30}O_{16}$	609	24.327	2261.39 ± 1.10^a	2582.89 ± 0.90^c	2178.54 ± 2.06^b	1381.96 ± 0.06^d
Luteolin-7- *O*-glucoside	$C_{21}H_{20}O_{11}$	447	25.095	2459.50 ± 1.47^a	3720.15 ± 0.69^a	3499.67 ± 0.47^a	2769.50 ± 0.71^a
Quercetin (quercetin-3- *O*-rhamnose)	$C_{21}H_{20}O_{11}$	447	27.402	1581.39 ± 1.48^a	1800.05 ± 0.33^c	1525.67 ± 0.64^b	882.11 ± 1.15^d
Naringin	$C_{27}H_{32}O_{14}$	579	26.431	93.83 ± 0.83^b	82.83 ± 0.30^c	76.89 ± 0.52^a	58.16 ± 0.45^d
Apigenin -7- *O*-glucoside	$C_{21}H_{20}O_{10}$	431	27.332	123.00 ± 0.39^a	170.78 ± 0.90^{ab}	169.05 ± 0.58^c	167.25 ± 0.64^b
4,5-di-*O*- caffeoylquinic acid	$C_{25}H_{24}O_{12}$	515	26.803	143.42 ± 0.71	nd	nd	nd
trans Cinnamic	$C_9H_8O_2$	147	32.214	nd	2.46 ± 0.57	1.31 ± 0.36	2.26 ± 0.31
Quercetin	$C_{15}H_{10}O_7$	301	32.538	154.05 ± 0.83^a	324.28 ± 0.34^c	102.83 ± 0.83^b	63.94 ± 0.33^d
Kaempferol	$C_{15}H_{10}O_6$	285	32.460	56.00 ± 0.64^a	137.89 ± 0.65^b	104.94 ± 0.96^d	76.70 ± 0.71^c
Naringenin	$C_{15}H_{12}O_5$	271	34.239	2.55 ± 0.38^a	2.65 ± 0.21^a	2.33 ± 0.35^a	2.26 ± 0.28^a
Apigenin	$C_{15}H_{10}O_5$	269	34.975	6.66 ± 0.25^a	19.48 ± 0.40^{ab}	18.00 ± 1.15^c	15.34 ± 0.45^d
Cirsiliol	$C_{17}H_{14}O_7$	329	35.793	nd	14.47 ± 0.49^a	11.14 ± 0.17^b	9.03 ± 0.19^c
Cirsilineol	$C_{18}H_{16}O_7$	283	38.949	nd	1.92 ± 0.03^a	1.19 ± 0.01^b	0.82 ± 0.01^c

RT: retention time; nd: not detected, ET: Equivalent Trolox, DM: dry matter, different letters showed significant difference at $p < 0.05$

3.3. Influence of the extraction method on phenolic profile

Four extraction methods were used to extract polyphenols from oleaster OL-Tounine leaves (Table 2). 15 phenolic compounds were identified in extract prepared by maceration and 17 phenolic compounds were identified using Soxhlet, heat reflux and UAE. Ferulic and *trans*-cinnamic acids were not obtained by maceration. However, the 4,5-di-*O*-caffeoylquinic and chlorogenic acids were obtained only in the extract obtained by maceration. Quinic acid was the major phenolic acid of the oleaster OL-Tounine leaves having an amount of 1085.80 mg/kg DM (Soxhlet), followed by 4,5-di-*O*-caffeoylquinic (143.42 mg/kg DM) (maceration). *trans*-Cinnamic acid was the minor phenolic acid detected by all extraction methods excepting maceration.

For flavonoids, the same compounds profile was obtained by four extraction methods, except cirsiliol and cirsilineol, which were not obtained by maceration extraction. Luteolin-7-*O*-glucoside was the major flavonoid followed by rutin having an amount of 3720.15 and 2582.80 mg/kg DM, respectively, obtained by Soxhlet (Table 2).

The PCA showed that the UAE was more related to the composition of cultivars Zarrazi and Chemlali et the oleaster OL-Toujène. However, the oleater OL-Tounine was more related to the Soxhlet extraction (Figure 4).

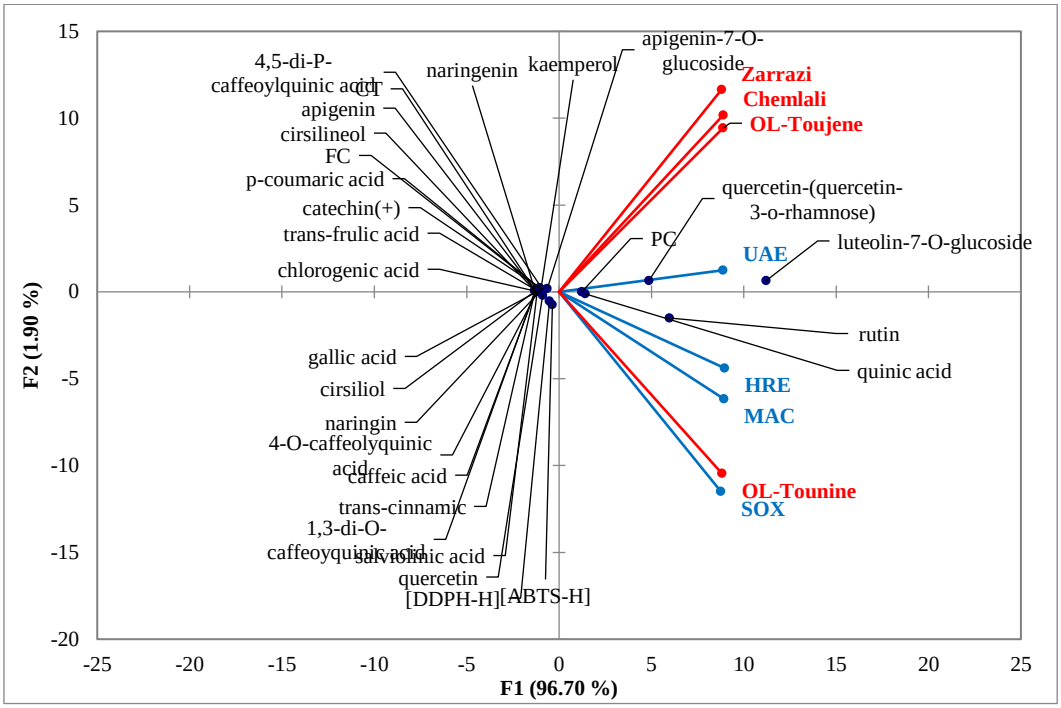

Figure 4. Principal Component analysis based on phenolics compounds and antioxidant activities of olive genotypes using four extraction methods (UAE: ultrasound asisted extraction, HRE: heat reflux extraction, MAC: maceration method, SOX: Soxhlet extraction: PC: polyphenols content, FC: flavonoids content, CT: condensed tannins content)

Extraction by Soxhlet provided extracts richer in phenolic compounds compared to extracts obtained by heat reflux, ultrasonic assisted extraction, and maceration. The effect of extraction methods on flavonoids stability form plants has been previously studied (Trusheva et al., 2007; Biesaga et al., 2011). Significant decomposition of myrcetin, kaempferol and quercetin was noted by using ultrasonic and microwave assisted extraction from maize (Cui et al., 2008). Gourguillon et al. (2016) indicated that the choice of extraction method influenced the extraction of dicafeoylquinic acids in halophytes.

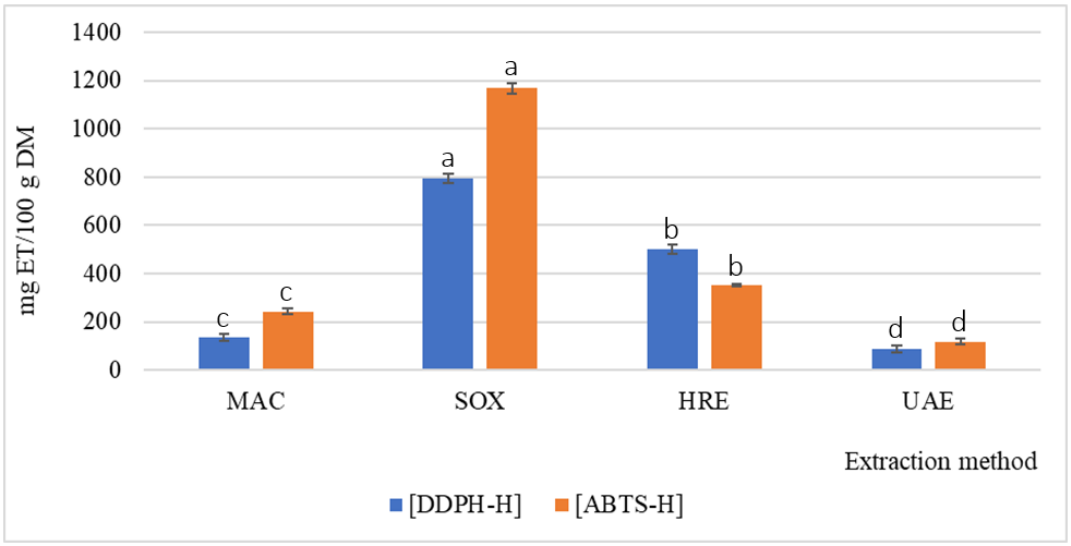

Figure 5. Antioxidant activities of oleaster leave extracts according extraction method (different letters for extraction method showed significant difference at $p < 0.05$; ET: equivalent trolox, UAE: ultrasound asisted extraction, HRE: heat reflux extraction, MAC: maceration method, SOX: Soxhlet extraction)

The qualitatively analysis by HPLC showed the identification of 9 phenolic acids and 11 flavonoids according to the availability of standards. Some phenolic compounds were showed high levels. Although, olive leaves were considered richer in phenolic

compounds compared to olive oil and fruits (Lalas et al., 2011; Hannachi et al., 2020).

The extraction method of olive leaves has a quantitative and qualitative influences on phenolic compounds. Results of HPLC analysis confirm the presence of phenolic acids and flavonoids in the various extracts of olive leaves recording the effect of the extraction method used and the genotype on certain compounds.

3.4. Influence of the extraction methods on antioxidant activities

Results showed that all olive extracts have an antioxidant activity using the DPPH and ABTS assays. The extraction method influent significantly the antioxidant activities. The extract obtained by SOX method showed the highest antioxidant activity by DPPH (793.60 mg ET/100 g DM) and ABTS (1168.00 mg ET/100 g DM) followed by the extracts obtained by HRE, and by MAC (Figure 5).

4. Conclusions

Olive leaves are considered as sustainable source of natural antioxidants and phenolic compounds. Four extraction methods were compared to evaluate quantitatively and qualitatively phenolic compounds of olive leaves (*Olea europaea* var. *sylvestris* and *Olea europaea* var. *europaea*). The polyphenols content varied according to the genotype, the extraction method, and their interaction. However, extracts of oleasters leaves obtained by Soxhlet extraction and by heat reflux extraction gave high polyphenols content. Qualitatively, genotype and extraction method influenced the phenolic compounds profile. Results showed the richness of *Olea europea* var. *sylvestris* in phenolics representing a new potential source of bioactive molecules.

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Conflict of interest

The authors declare no conflict of interest.

CRediT authorship contribution statement

Salma Guebebia: Experimental analyses, Writing original draft
Khadija Ben Othman: Supervision on experimental analyses, Methodology, Investigation
Yassine Yahia: Supervision on high performance liquid chromatography analyses
Mehrez Romdhane: Conceptualization, Methodology, Supervision
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Supplementary File

None.

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