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Characterization of Phenolic Profile of Moroccan Picholine Olive Variety

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Abstract
Phenolic compounds were extracted from olive leaves of Moroccan Picholine variety with acetone-water mixture. The extract was fractioned with diethyl ether and ethyl acetate solvents. The three fractions obtained (diethyl ether, ethyl acetate and aqueous fractions) were characterized by HPLC/MS analyses. The results obtained showed that the diethyl ether fraction is characterized by ligstroside acid. Hydroxybezoic, hydroxyphenyl acetic, ferrulic acids and the flavonoids aglycon such as quercetin and hesperitin were also detected. This fraction contained also methylated and/or hydroxylated secoiridoids derivatives. The ethyl acetate fraction is dominated by hydroxyoleoside dimethyl ester and methoxyligstroside. The other phenolics found in this fraction were oleuropein, oleoside, ligstroside, ligstroside derivatives and verbascoside. The oleuropein was found under two isomers and four derivatives. The aqueous fraction is characterized by the oleuropein pentose. It contains secoiridoids, such as oleoside, ligstroside, oleuropein and oleuropein derivatives, and flavonoids mainly quercetin and cyanidin glucose. According to these results, the Moroccan Picholine olive variety may be characterized by ligstroside acid and oleuropein pentose, which may be used as markers for olive oil and table olives labeling.

Keywords: phenolic profile, olive leaves, Moroccan Picholine, HPLC/MS, ligstroside acid, oleuropein pentose.

1. Introduction
Moroccan Picholine is the main local olive variety cultivated in Morocco. It represents about 96% of the national orchard, with 50 million olive trees covering almost 580,000 ha across the country, from mountains to arid areas [1]. To extend the Moroccan olive orchard, new olive varieties have been introduced, mainly Arbequina, Hojiblanca, Languedoc Picholine, Dahbia, Haouzia, Menara, Gordal, Sevillana, Manzanilla and Picual. The introduction of new varieties influences the quality control of their end products (table olives and olive oil), due mainly to their phenolic profiles.
Several olive varieties studied exhibited quantitative and qualitative differences in their phenolic profiles [2-4], leading to their discrimination by the presence or absence of some phenolic compounds. Thereby, some Tunisian varieties, including Oueslati, Chetoui, Chemlali, El Hor, are characterized by the absence of hydroxytyrosol glucoside [5]. The Greek olive varieties are characterized by oleuropein as the major phenolic component, and can be discriminated from Tunisian varieties by the presence of demethyloleuropein [6]. In Italian varieties, the oleuropein is the major phenolic component followed by verbascoside [7]. The French Picholine variety can be characterized by the presence of diosmetin aglycon [8]. However, to our knowledge no studies were conducted on the characterization of the phenolic profile of the Moroccan Picholine variety.
The phenolic compounds, mainly oleuropein and its derivatives, such as oleuropein aglycone, hydroxytyrosol, elenolic acid glucoside and elenolic acid, are highly desired in foods as antioxidants, anti-tumour and antimicrobial agents [9-11]. However, high concentrations of phenolics, mainly oleuropein, lead to bitterness in end product (table olives and olives oil), and may influence the fermentation process of table olives, because of their inhibitory effect on lactic acid bacteria highly desired in this process [12-13]. Hence, the characterization of phenolic profile of each variety is necessary to better control the industrial processes of table olives and olive oil.

The main objective of this work is to characterize the phenolic profile of the Moroccan Picholine olive variety, basing the extraction with acetone-water and separation with diethyl ether, ethyl acetate and water solvents and the HPLC/MS analysis.

2. Materials and methods

2.1. Chemical reagents

The phenolic compounds used as standards in this work were 4-hydroxybezoic acid, vanilic acid, caffeic acid, ferilic acid, salicylic acid, coumarin, rutin, sinapic acid, naringin, hesperidin, cinnamic acid, quercetin, epicatechin, hesperitin, 6-hydroxyflavone and methoxyflavone. They were purchased from Sigma Aldrich. The LC solvents (methanol, acetone, ethyl acetate, diethyl ether, chloroform) ware LC grade and purchased from Sigma Aldrich. The oleuropein was purchased from Extrasynthese (Genay, France).

2.2. LC-MS system

LC system used in this study was equipped with photodiode array detector and column BDS Hypersil C18 (150 mm x 4.5 µm). It was coupled to Mass Spectrometry (MS) detector Thermo Finnigan surveyer electron France. The MS detector was composed of electrospray ionization interface.

2.3. Samples preparation

Samples of olive leaves of the Moroccan Picholine variety were collected from Taza region (east of Morocco) during June (spring period). The samples were dried at 40°C for five days, and then milled in a mixer.

2.4. Phenolic compounds extraction

Samples of 20 g of milled dried olive leaves were defatted with chloroform during one night in dark at room temperature. Extraction of phenolic compounds was then carried out twice with distillated water-acetone (13:9, v:v), in dark for 10 hours. The mixture was filtered using Whatman filters. The filtrate was evaporated in Rotavapor to remove acetone. After a second wash with chloroform, the aqueous fraction was extracted with diethyl ether (3 x100ml). After separation, the aqueous phase was further extracted with ethyl acetate (3 x 100 ml). The diethyl ether, ethyl acetate and aqueous fractions were evaporated to dried extracts in Rotavapor.

2.5. LC/MS analyses

An aliquot of 0.3 mg of diethyl ether and ethyl acetate fractions were dissolved in 1 ml of methanol. 0.3 mg of aqueous fraction was dissolved in 1 ml of ultra pure water for LC. The three fractions were filtered through 0.2 µm filters, and 20 µl of each fraction were automatically injected in LC system. The flow rate used was 1ml/min, and the absorbance changes were monitored at 280 nm. Phenolic compounds were separated using flow elution gradient described by Mateos et *al*. (2001) [14]. The MS analysis were performed in positive and negative modes with the following conditions: Helium gas was used as stealth gas at flow rate of 1.5 l/min, the electrospray voltage was 3.7 Kv, the capillary temperature and voltage were maintained at 250°C and -37v, respectively, for negative mode. For positive mode scanning, the electrospray voltage was 4.7 Kv, and the capillary temperature and voltage were maintained at 250°C and 15v, respectively. The molecular ions were scanned from 100 to 1500 m/z. The standards were monitored under the same conditions as described for the three fractions.

2.6. Peaks identification

The main phenolic compounds were identified by comparing their retention time and mass spectra with those of authentic standards when available, or by comparing their mass spectra with those reported in literature.

3. Results and discussion

3.1. LC/MS analyses of standards

A mixture of 14 standards was successfully separated as indicated on Figure 1. Most of standards were better ionized in negative mode than in positive mode, but some of them did not ionized in any mode. Most of standards gave fragment ions such as $(M\text{-}H\text{-}CH_3COOH)^-$, $(M\text{-}H+Cl)^-$, $(2M\text{-}H)^-$, $(2M\text{-}H+CH_3COOH)^-$ and $(2M\text{-}H+Cl)^-$ (Figure 2). Oleuropein standard from (Extrasynthese) generated three main peaks in LC/MS (figure 3). The first peak appeared at 30.48 min, the second and major peak appeared at 33.36 min and the later peak appeared at 37.40 min. The three peaks exhibited the same molecular weight and the same fragment profile (figures 2, 4 and 5). These results may be explained by the fact that the oleuropein standard exists in three isomer forms.

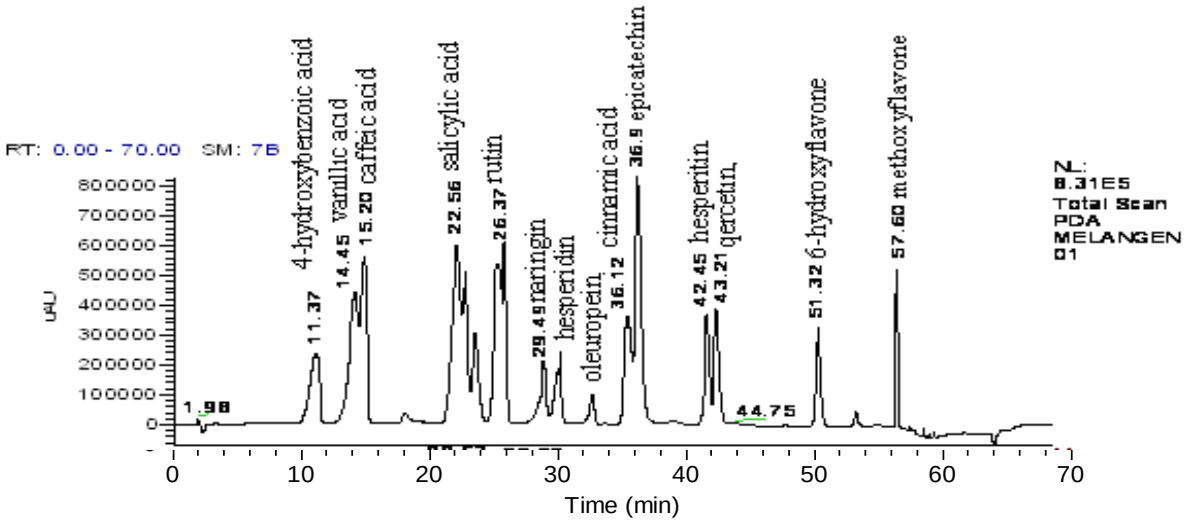

Figure 1: LC Chromatogram of the phenolic standards (4-hydroxybenzoic acid, vanillic acid, caffeic acid, salicylic acid, rutin, hesperidin naringin, oleuropein, cinnamic acid, epicatechin, hesperitin, quercetin, 6-hydroxyflavone and methoxyflavone)

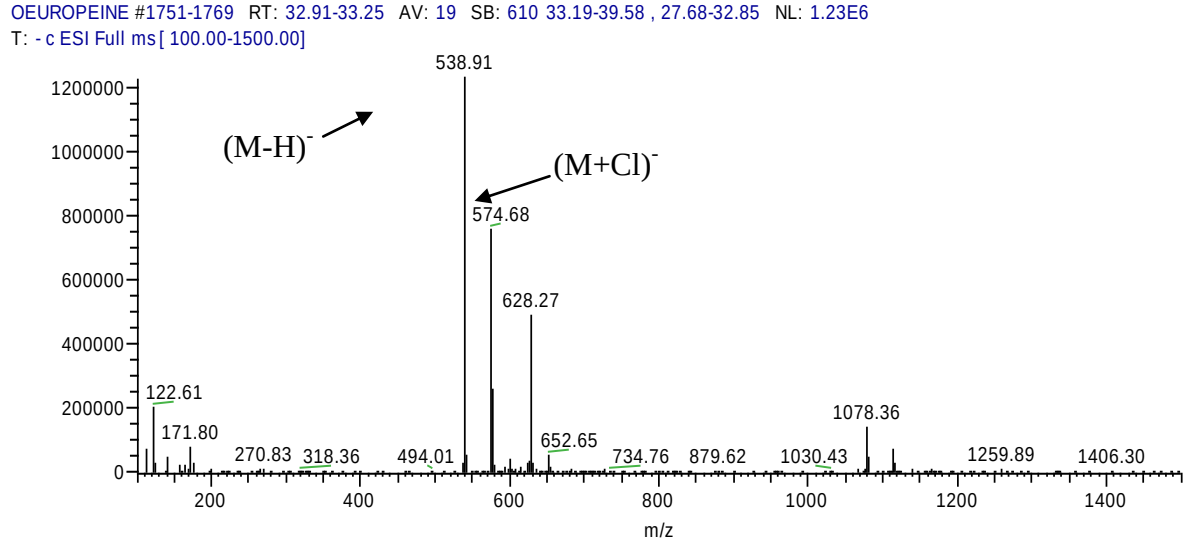

Figure 2: Mass spectra of oleuropein standard in negative mode eluted at 33.25 min.

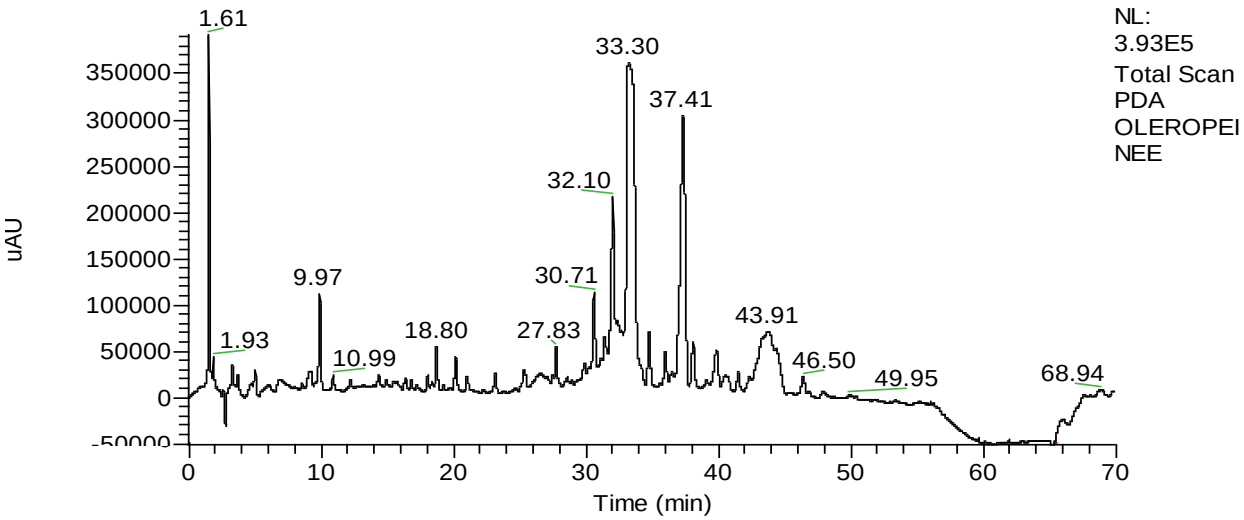

Figure 3: LC Chromatogram of oleuropein standard

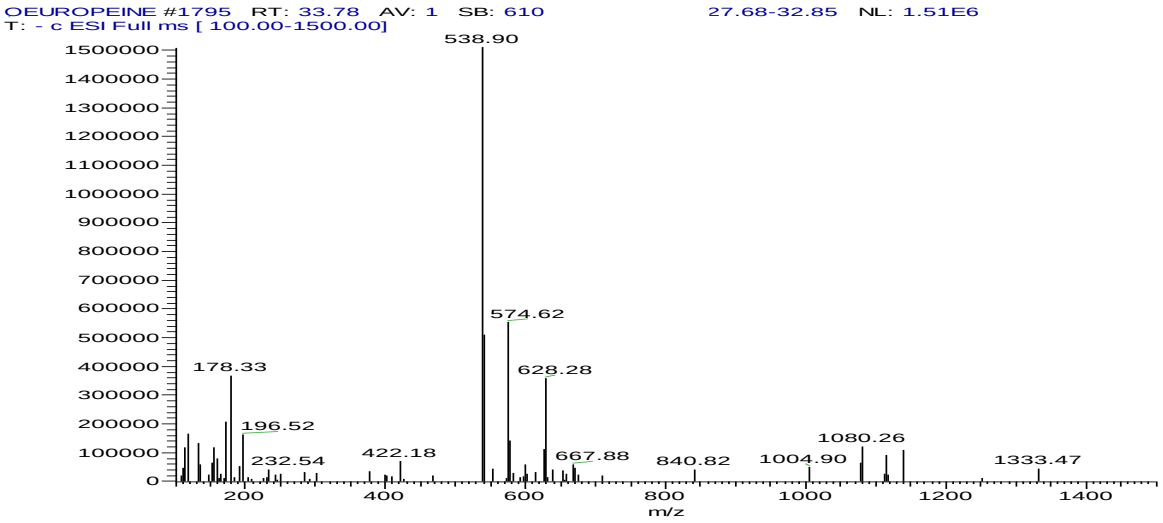

Figure 4: Mass spectra of oleuropein standard in negative mode eluted at 33.78 min.

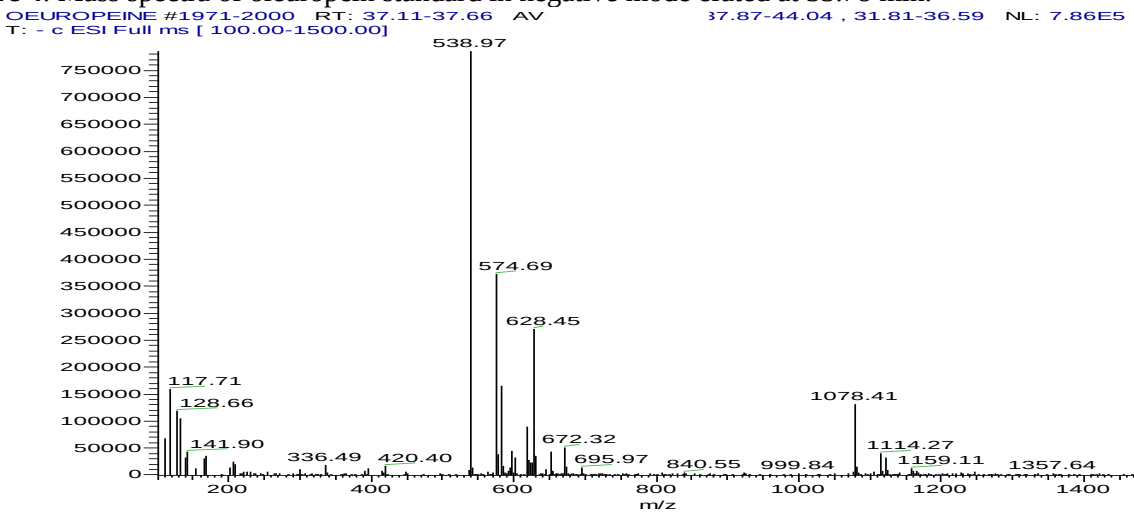

Figure 5: Mass spectra of oleuropein standard in negative mode eluted at 37.66 min.

3.2. LC/MS analyses of diethyl ether fraction (DEF)

The diethyl ether fraction LC profile showed many peaks, corresponding to different phenolic compounds (Figure 6). 14 of them were identified (Table 1). This fraction is rich of phenolic acids like ligstroside acid, hydroxybezoic acid, hydroxyphenyl acetic acid and ferrulic acid. Ligstroside acid represented about 40% of the total phenolic compounds (figure 7).

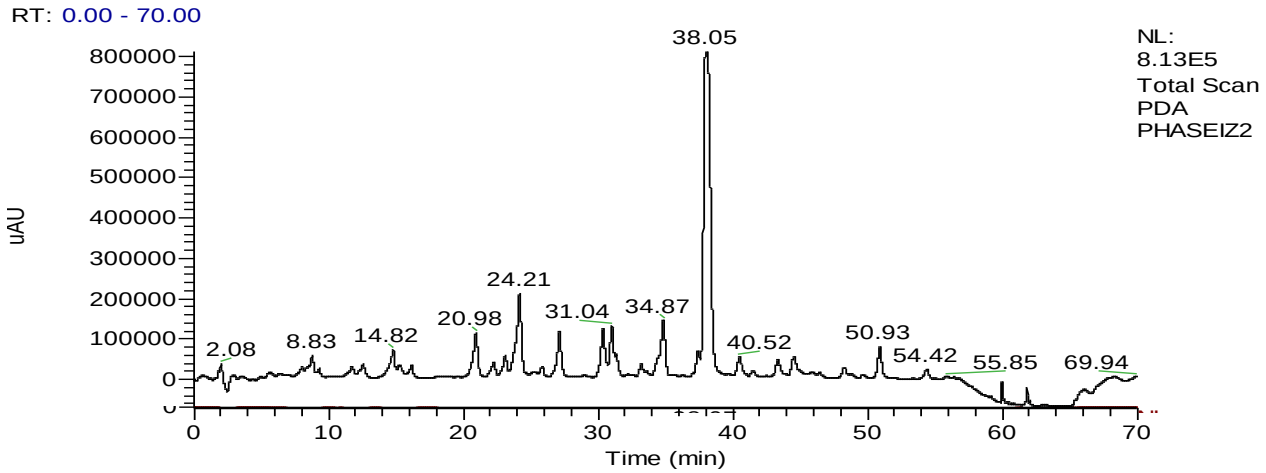

Figure 6: LC Chromatogram of diethyl ether fraction (DEF) obtained from olive leaves.

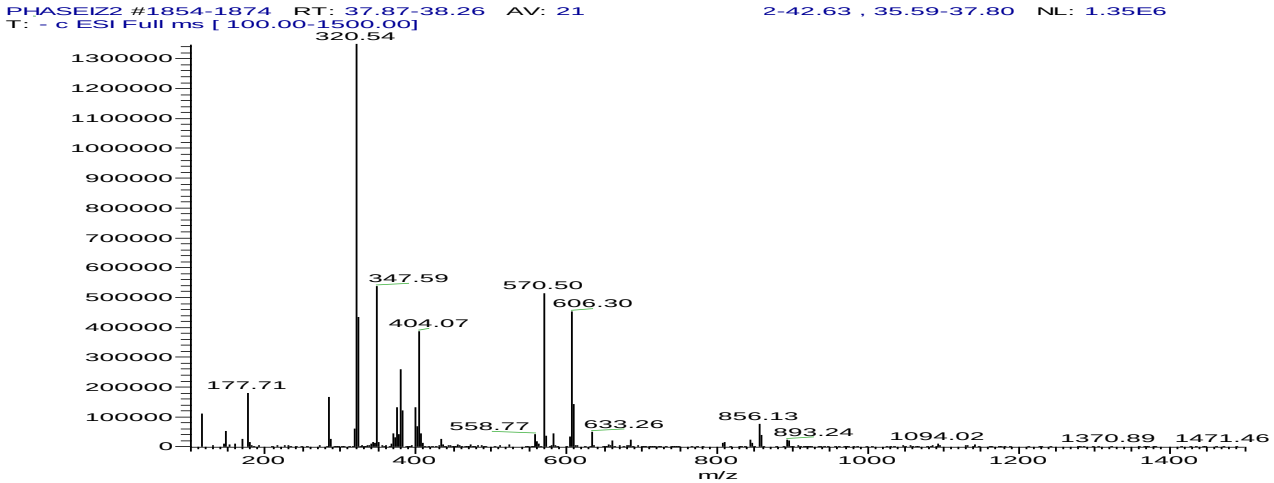

Figure 7: Mass spectra of ligstroside acid found in diethyl ether fraction (DEF) in negative mode.

The ligstroside acid was not detected in several other olive varieties, such as French Picholine, Koroneiki, Megaritiki, Kalamon, Oueslati, Chetoui, Chemlali, El Hor, Jarboui and Chemchali [5, 6, 8, 15, 16]. The presence of this compound may be due to several factors, including variety, geography, agronomy and environmental factors and experimental process used for the extraction, as reported in literature [4-7, 17, 18].
Other acids were detected in methanol leaves extract, such as caffeic, vanillic and cinnamic acids [19]. In our DEF, these three phenolic acids were absent or present at no detectable level, because they were ionisable under the experimental conditions used in our study. The DEF contains some flavonoides and secoiridoids aglycon such as deacetoxyligstroside aglycon, quercetin and hesperitin. Quercetin was detected in methanol-water olive leaves extract of some Tunisian varieties [5]. The oleuropein, the major phenolic compound found in olives, was not detected in this fraction. However, some derivatives of oleuropein, such as methyl oleuropein and hydroxyl derivatives of oleuropein, were detected at low concentrations; the absence of oleuropein in this fraction can be explained by its less solubility in the diethyl ether solvent. Hence, diethyl ether, the lower polar solvent used in this study, can be use to dissolve low-polar phenolic. To extract high-polar phenolic compounds from water-acetone extract, we had used ethyl acetate known as high-polar solvent.

3.3. LC/MS analyses of ethyl acetate fraction (EAF)

The ethyl acetate fraction LC profile showed 14 peaks corresponding to different phenolic compounds (Figure 8 and Table 1).

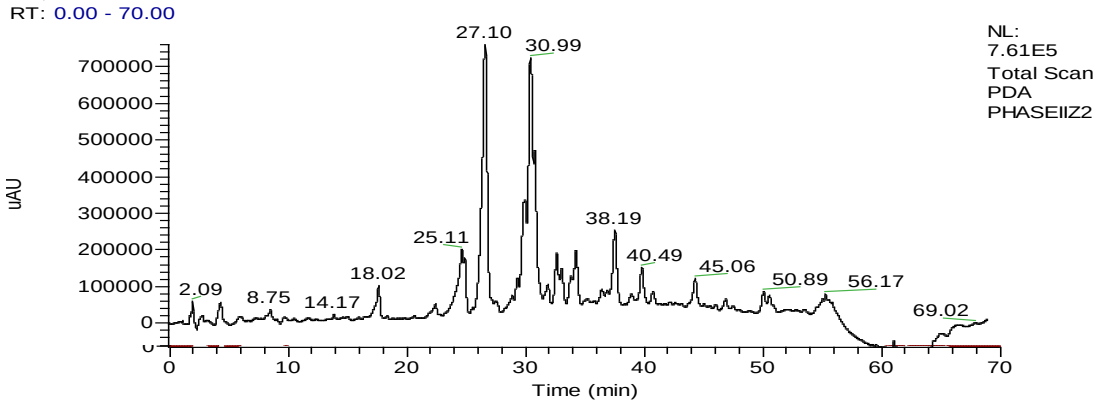

Figure 8: LC Chromatogram of ethyl acetate fraction (EAF) obtained from olive leaves.

This fraction is rich of secoiridoids glucoside, soluble in ethyl acetate as a more polar solvent than diethyl ether used in the first fraction. The EAF is dominated by oleuropein, ligstroside, oleoside and their derivatives, mainly dimethyl oleuropein methoxyligstroside and hydroxyoleoside dimethyl ester and verbascoside. Oleuropein and ligstroside are detected in Greek and French varieties [6, 8]. However, ligstroside is not expected in Tunisian and Italian varieties [5, 7].
The EAF contains oleuropein under two isomer forms and four derivatives. Some authors have also reported the presence of two isomers of oleuropein, identified as oleuropein and oleuroside [5, 8, 16]. In our study the high

contents of oleuropein may be explained by the multiple forms of oleuropein found in the three fractions. Two other compounds, diosmetin aglycone and luteolin-glucoside, were detected in French Picholine variety, using ethyl acetate as solvent, high pressure and high temperature [8].

Table 1: Mass spectrometry data and retention times of phenolics found in diethyl ether (DEF), ethyl acetate (EAF) and aqueous fractions (AF) obtained by LC/MS (Legends: 1: Diethyl ether fraction (DEF), 2: Ethyl acetate fraction (EAF), 3: Aqueous fraction (AF), Rt: Retention time (min) and $(M-H)^-$: pseudomolecular ion, +: presence, -: absence).

Rt	$(M-H)^-$	Other ions (m/z)	1	2	3	Compound
2.92	374	205 $(CH_2CHphOH)^-$, $(CH_3OH-H_2O)^-$, 229 $(M-H-C_4H_6O-CH_3)^-$, $(CH_2COOH)^-$, 339 $(M-H-CH_3OH-3H)^-$	-	-	+	methyl ligstoside aglycon
3.92	391	119 $(M-H-COOH-(C_5H_3O-OH)-CH_2CHO-CH_2-CH_2CH_2-(OH)_2)^-$, 153 $(M-H-COOH-(C_5H_3O-OH)-CH_2CHO-CH_2-CH_2CH_2)^-$, 175	-	-	+	methyl oleuropien aglycon
8.49	448	150 $(M-H-CH_2-CH-OH-C_6H_3OH-Glu)$, 177, 283 (M-3H-Glu), 318 $(M-H+OCH_3-COOH-Glu)^-$, 434$(M-H-CH_3)^-$	-	-	+	Decarboxymethoxyligstroside
9.38	153	188$(M-H+Cl)^-$, 212 $(M-H+CH_3COOH)^-$.	+	-	-	Hydroxytyrosol
9.9	690	149 $(M-H-CH_2CHph(OH)_2-CH_3COOH-CH_3OH)^-$, 177 $(M-H-CH_2CHph(OH)_2-CH_3COOH,3H)^-$, 212 $(M-H-CH_2CHph(OH)_2-CH_2O)^-$, 259 $(M-H-CH_2CHphOH)^-$, 377 $(M-H-Glu-Pent)^-$, 539 $(M-H-pent)^-$.	-	-	+	oleuropien pentose
10.24	389	424 $(M-H+Cl)^-$, 779 $(2M-H)^-$	-	-	+	Oleoside
11.83	138	172 $(M-H+Cl)^-$, 196 $(M-H+CH_3-COOH)^-$, 275 $(2M-H)^-$	+	-	-	hydroxybezoic acid
14.81	389	779 $(2M-H)^-$	-	-	+	oleoside isomer.
14.82	151	186 $(M-H+Cl)^-$, 210 $(M-H+CH_3-COOH)^-$	+	-	-	hydroxyphenyl acetic acid
16.06	625	603, 659 $(M+H+Cl)^-$.	-	-	+	Verbascoside isomer
18.02	403	171 $(M-H-Glu-CH_2COO-OCH_2)^-$, 154 $(M-H-Glu-CH_2COO-CH_2)^-$,438 $(M-H+Cl)^-$, 462 $(M-H-CH_2COOH)^-$, 807 $(2M-H)^-$	-	+	+	oleoside methyl ester
18.69	445	287 $(M-H-Glu)^-$	-	-	+	cyanidin glucoside
20.98	193	198, 253 $(M-H+CH_3-COOH)^-$	+	-	-	ferrulic acid
23	403	177, 154 $(M-H-Glu-CH_2COO-CH_2)^-$,438 $(M-H+Cl)^-$, 462$(M-H-CH_2COOH)^-$, 807 $(2M-H)^-$	-	-	+	oleoside methyl ester isomer
24.21	303	338 $(M-H+Cl)^-$, 362 $(M-H-CH_3COOH)^-$, 606 $(2M-H)^-$.	+	-	-	deacetoxyligstroside aglycon
25.23	555	590 $(M-H+Cl)^-$,1110 $(2M-H)^-$	+	+	+	hydroxyoleuropein
25.8	301	338 $(M-H+Cl)^-$, 362 $(M-H-CH_3COOH)^-$	+	-	-	deacetoxyligstroside aglycon isomer
27.10	447	390 $(M-3(CH_3)-OH)^-$, 404 $(M-2(CH_3)-OH)^-$, 433 $(M-CH_3-OH)^-$, 482 $(M-H+Cl)^-$, 507 $(M-H+CH_3COOH)^-$, 895 $(2M-H)^-$	+	+	-	hydroxyoleoside trimethyl ester.
28.32	555	590 $(M-H+Cl)^-$, 1111 $(2M-H)^-$.	-	-	+	hydroxyoleuropein isomer
30.46	447	390 $(M-3(CH_3)-OH)^-$, 404 $(M-2(CH_3)-OH)^-$, 433 $(M-CH_3-OH)^-$, 482 $(M-H+Cl)^-$, 507 $(M-H+CH_3COOH)^-$, 895 $(2M-H)^-$	+	+	-	hydroxyoleoside trimethyl ester isomer
30.99	553	301 $(M-H-Glu-CH_3COO-CH_2O)^-$, 523 $(M-H-OCH_3)^-$, 588 $(M-H+Cl)^-$, 615 $(M-H+CH_3COOH)^-$.	-	+	-	methoxyligstroside
31.22	301	130, 177, 603 $(2M-H)^-$	-	-	+	quercetin isomer
31.76	567	551 $(M-H-CH_3)^-$, 602 $(M-H+Cl)^-$, 629 $(M-H+CH_3COOH)^-$	-	+	-	dimethyl oleuropein
33.65	539	574$(M-H+Cl)^-$, 599 $(M-H+CH_3COOH)^-$	-	+	-	oleuropein
33.97	539	575 $(M-H+Cl)^-$	-	-	+	oleuropein isomer 1
34.88	537	275 $(M-H-C_4H_6O-CH_3)^-$, 377 $(M+OH-H-Glu)^-$, 555 $(M+CH_3-H-Glu-OH)^-$, 572 $(M-H+Cl)^-$, 596 $(M-H+CH_3COOH)^-$	-	+	-	methyl ligstroside
37.45	301	336$(M-H+Cl)^-$, 363 $(M+H_2+CH_3COOH)^-$, 396, 420, 603 $(2M-H)^-$	+	-	-	Quercetin
37.74	539	575 $(M-H+Cl)^-$, 1178 $(2M-H)^-$	-	-	+	oleuropein isomer.
37.90	537	275 $(M-H-C_4H_6O-CH_3)^-$, 377 $(M+OH-H-Glu)^-$, 555 $(M+CH_3-H-Glu-OH)^-$, 572 $(M-H+Cl)^-$, 596 $(M-H+CH_3COOH)^-$	-	+	-	methyl ligstroside isomer
38.05	570	606 $(M-H+Cl)^-$, 404 $(M-H-Glu)^-$, 347 $(M-H-Glu-CH_3COOH)^-$ 321 $(M-H-Glu-CH_3COO-CH_2O)^-$, 807 $(2M-H)^-$	+	-	-	ligstroside acid.
38.19	523	321 $(M-H-Glu-C_2H_2O)^-$, 347 $(M-H-Glu-CH_3)^-$, 404 $(M-H-CH_2CHphOH)^-$, 558 $(M-H+Cl)^-$ 582 $(M-H+CH_3COOH)^-$	-	+	-	Ligstroside
38.36	523	321 $(M-H-Glu-C2H2O)^-$, 347 $(M-H-Glu-CH3)^-$, 404 $(M-H-CH2CHphOH)^-$	-	-	+	ligstroside isomer
40.52	623	658 $(M-H+Cl)^-$, 685 $(M-H+CH_3COOH)^-$	+	+	+	verbascoside
43.38	304	126, 172, 193	+	-	-	Hesperitin
45.10	557	592 $(M+2H+Cl)^-$, 617 $(M+2H+CH3COOH)^-$, 275 $(M+H-Glu-C4H6O-CH3OH-OH)^-$, 342 $(M+H-Glu-CH3-OH-OH)^-$, 378 $(M+H-Glu-OH)^-$, 396 $(M+H-Glu)^-$	-	+	-	hydroxyoleuropein derivative.
51.3	615	539 $(M-H-OCH_3-OCH_3)^-$, 569 $(M-H-OCH_3)^-$, 650$(M-H+Cl)^-$, 674 $(M-H+CH_3COOH)^-$	+	+	+	dimethoxyhydroxyoleuropein isomer

3.4. LC/MS analyses of aqueous fraction (AF)

The aqueous fraction LC profile showed 17 peaks corresponding to different phenolic compounds (figure 9 and table 1). This fraction contains secoiridoids which were detected in ethyl acetate fraction as oleoside, ligstroside, oleuropein and their derivatives (methyl ligstoside aglycon, methyl oleuropien aglycon, decarboxymethoxyligstroside, oleoside methyl ester, hydroxyoleuropein) and verbascoside. It also contains some flavonoids as quercetin and cyanidin glucose. In addition to these phenolics, it contains a novel compound which is oleuropein pentose (figure 10). This compound was detected neither in diethyl ether fraction nor in ethyl acetate fraction. The oleuropein pentose was not detected in several olive varieties, such as French Picholine, Koroneiki, Megaritiki, Kalamon, Oueslati, Chetoui, Chemlali, El Hor, Jarboui and Chemchali, [5, 6, 8, 15, 16]. This finding may lead us to consider the oleuropein pentose as a main phenolic compound to characterize the Moroccan Picholine olive variety.

In this study, we have identified for the first time in the Moroccan Picholine variety, two phenolic compounds, ligstroside acid and oleuropein pentose. Both of them contain phenolic cycle and glucosidic part, known for their nutritional and health benefices, and easily extracted in water. This finding may lead to their potential use as infusion for medicinal purpose. Furthermore, ligstroside acid, which is soluble in oil, may be used as a marker for retailed olive oil of Moroccan Picholine variety. The oleuropein pentose may be used as a marker of fresh or processed table olives.

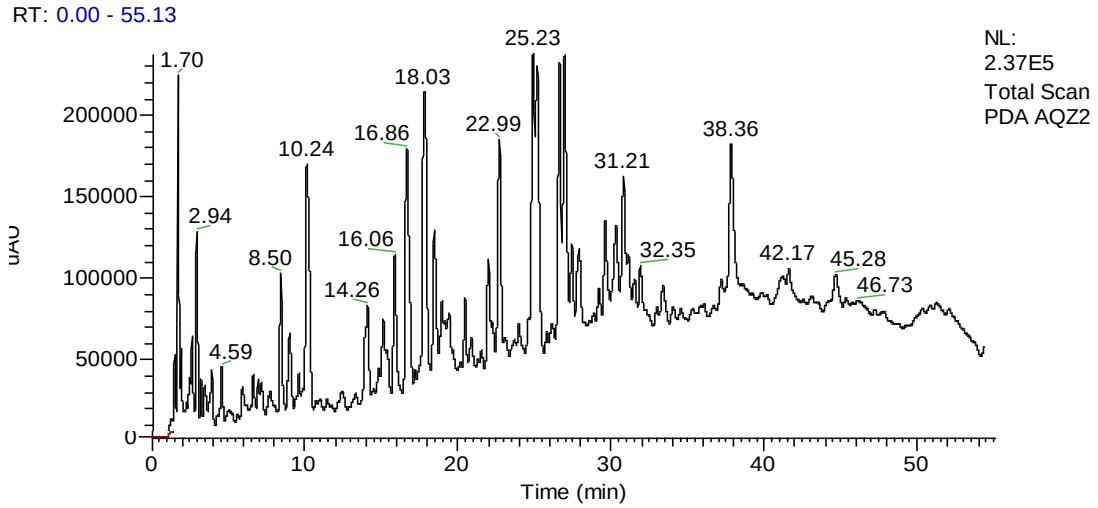

Figure 9: LC chromatogram of aqueous fraction (AF) obtained from olive leaves.

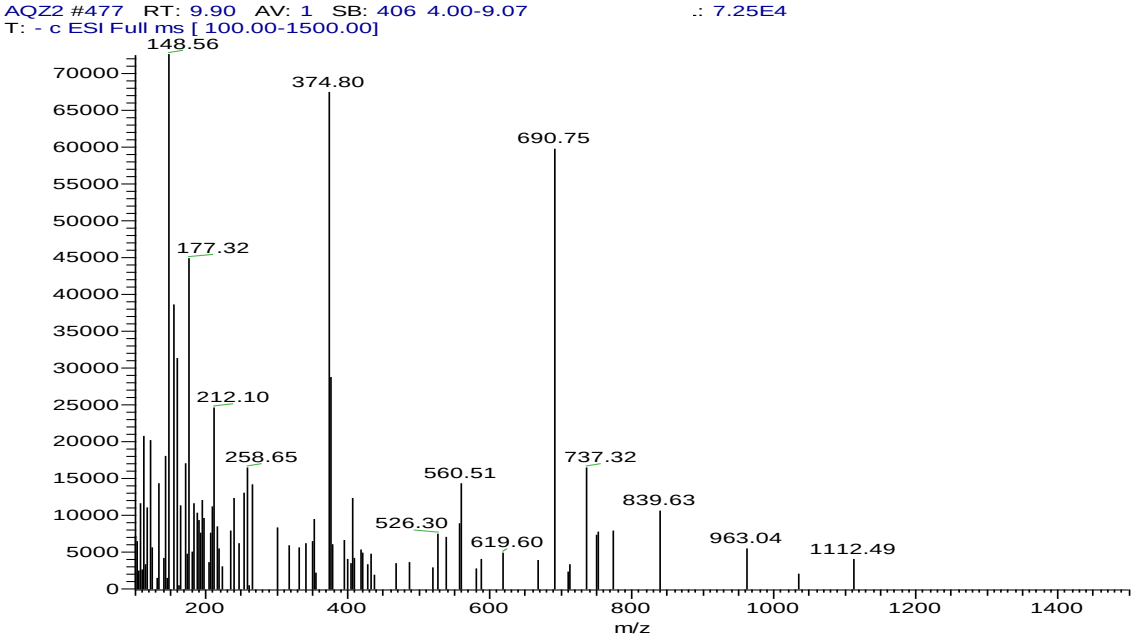

Figure 10: Mass spectra of oleuropein pentose found in aqueous fraction (AF) in negative mode.

Conclusion

To the best of our knowledge, this work is the first report on characterization of phenolic profile of the Moroccan Picholine olive variety. The use of water-acetone (13v /9v) as solvent allowed the extraction of a wide range of polyphenols. The splitting of water-acetone extract by diethyl ether and ethyl acetate allowed a successful separation and clear LC chromatograms. The diethyl ether fraction was rich of phenolic acids, mainly ligstroside acid. It contains some flavonoids and secoiridoids aglycon. The ethyl acetate fraction showed high contents of secoiridoids and their derivatives, mainly hydroxyoleoside dimethyl ester and methoxyligstroside. It contained oleuropein under two isomer forms and four derivatives. It contained also oleoside, ligstriside and their derivatives and verbascoside. The aqueous fraction contained secoiridoids like oleoside, ligstroside and oleuropein and its derivatives. It contained oleuropein pentose, and some flavonoids as quercetin and cyanidin glucose. These finding may lead us to the possibility of characterization of the Moroccan Picholine variety by ligstroside acid and oleuropein pentose.

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