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UPLC-Q-TOF/MS Screening of Bio-Active Compounds Extracted From Olive Mill Solid Wastes and their Effect on Oxidative Stability of Purslane Seed Oil

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ABSTRACT

As purslane seed oil consists of desirable fatty acid, it could be considered as a new nutritious table oil. This work is aimed to study the effect of bio-active compounds of olive mill solid wastes (OMSWs) on oxidative stability of purslane seed oil, and the influence of solvent type on the extraction of polyphenols. Extraction was conducted using 80% methyl and ethyl alcohols "in water". Total phenolic content, antioxidant activity was investigated. Identification of bio-active compounds was achieved using ultra-performance liquid chromatography/quadrupole-time-of-flight mass-spectrometry (UPLC/Q-TOF-MS) method. Forty two phenolic compounds were identified. The highest antioxidant activity by DPPH was determined in olive leaves (OL) in both methanol and ethanol extract (89.32 and 84.18 %, respectively). The obtained results revealed that type of extracting solvent has a significant influence on total phenolic concentration of OMSWs in which methanol is the most efficient extraction solvent. Furthermore, the oxidative stability of the control oil was found to be the lowest (5.33 h). While addition of olive extracts improved the oil oxidative stability, so that the highest oxidative stability (13.17 h) was found in oil treated by OL methanol extract.

Key words: UPLC-MS, Bio-active compounds, Olive mill wastes, Olive leaves, Oxidative stability, Purslane seed oil

Introduction

International production of olive oil reached about 3 million tons during the year 2016/2017. Egypt's production of olive oil reached to about 27 thousand tons during 2016/2017 season. The amount of solid waste (leaves and cake) produced from one ton of olive fruits is about 600 kg for three-phase processing system and about 900 kg for two-phase processing system. In other meaning, the olive oil industry in Egypt produces more than 80 to 90 thousand tons of solid waste (IOC, 2017).

The two systems adopted for extraction of olive oil from its fruits are the two phases and the three phase systems. The amount of solid and liquid wastes from both systems is not less than 30% of input material. The utilization of these wastes to extract its content of valuable phenolic compounds is thus an economic target that should be considered in olive oil mills. Moreover, such extraction will have an additional beneficial impact on the environment by reducing the biological and chemical load of the disposed waste streams (Alu'datt *et al.,* 2010).The solid wastes resulting from olive oil processing generates a great environmental problem in the Mediterranean countries (Lesage-Meessen *et al.,* 2001). For that reason, the appropriate use of these olive oil wastes could not only impact the economic level of olive oil producers but could also decrease the environmental pollution.

Olive leaves is a good source for so many bio-active compounds, *oleuropein* is the predominant one, and it was recorded as a highly antimicrobial agent (Bisignano *et al.,* 2001). Other pharmaceuticals researchers' identified the active agent in *oleuropein*. Then a process for extracting *d*-elenolic acid was formulated and patented by a pharmaceuticals research agency. This component becoming widely accepted and marketed under different trade names due to its high power to retard so many disease-causing pathogens and to enhance the immune system (Wellman, 2001).

Within olive oil extraction from its fruit, only 2% of its phenolic contents are produced in the oil while the remaining is left in the pomace in general. Therefore, the latter is considered as a rich source

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of bio-active ingredients that can be used as food supplement or used in some pharmaceutical applications (Suárez *et al.,* 2009)

It is well known that oil from Purslane seeds and leaf is very richest with omega-3 fatty acids (Simopoulos and Salem, 1986). And as it posses high concentration of unsaturated fatty acids it could be used for cholesterol reduction, on the other hand, this oil is widely used for arthritis therapy, burns healing, cancer inhibition, treatment of mental depression, and autoxidation (De Lorgeril *et al.,* 2001, Mazza *et al.,* 2007).

In this study, we aim to investigate the effect of type of extraction solvent and OMSWs on oxidative stability of purslane seed oil as a reached source of omega-3 fatty acids.

Materials and Methods

Chemicals

Methanol, ethanol, acetonitrile and acetic acid were purchased from Merck (Darmstadt, Germany). The phenolic standards were of purity of 98–99%, and were purchased from Extrasynthese (Lyon, France).

Materials

OMSWs were provided by an olive oil industry (two-phase and three-phase decanter system). The olive wastes were dried under vacuum oven at 40-50°C for 48 h to reduce the moisture content, then the dried wastes were grinded and packed in dark polyethylene bags and kept in refrigerator for further experimentation. Purslane oil was extracted by hydraulic cold pressing machine at room temperature using pressure at 10 MP for 10 min. Then, extracted oil was filtered and afterward was centrifuged at 3500 rpm for 20 min in order to separate those components settled during storage.

Analysis of purslane seed oils

Fatty acid composition

The fatty acid methyl esters of purslane seed oil was prepared with BF_3 in methanol as the methylating agent according to the method described by Aniołowska *et al.* (2016). The obtained methyl esters of fatty acids were separated with a PU 4410 gas chromatograph (Philips, UK), using a capillary column RTX-2330 (Restek, USA), 105 m length, diameter 0.25 mm i.d. and film thickness 0,2 µm. Detector (FID) and injection temperature was 260 °C. Column temperature was 160 °C (30 min.) to 180 °C (17 min.) at 3 °C/min. and to 220 °C (15 min.) at 5 °C/min. The carrier gas was helium.

Determination of physicochemical properties

Physicochemical analyses of oil samples as refractive index, peroxide value, acid value, saponification value and iodine value were accomplished based on official methods of AOAC (2000).

Extraction of phenolic compounds

The extraction of phenolic compounds was carried as follows: five gram from each olive wastes was weighted in conical flask 250 ml with stopper, and added 50 ml of solvent (80% EtOH and 80% MeOH individually). Then, extraction was running using ultrasonic water bath for 30 min and shaking for 90 min, after that the samples were storage at -22°C for 20 h. Then, the flasks were shaking for 90 min before centrifugation at 10000 rpm for 10 min, finally, solvent was evaporated at 30°C and vacuum 100 mbar. The samples were freeze at -22°C, and freeze-dried for 24 h. After that the samples were stored at -22°C until analyses.

Total phenol content determination (TPC)

TPC for OMSWs Extracts

The total phenol content of the OMSWs extracts was determined colorimetrically at 725 nm using the Folin–Ciocalteau reagent according to a modification of the Gutfinger (1981) method. The methanolic solution of OMSWs extract (0.1– 0.3 ml), 20 ml of deionized water and 0.625 ml of the Folin–Ciocalteau reagent were added in a 25 ml volumetric flask. After 3 min, 2.5 ml of saturated solution of Na_2CO_3 (35%) were added. The content was mixed and diluted to volume with deionized water. After 1 h, the absorbance of the sample was measured at 725 nm against a blank using a double-beam ultraviolet–visible spectrophotometer Hitachi U-3210 (Hitachi, Ltd., Tokyo, Japan). Gallic acid served as a standard for preparing the calibration curve, and ranged from 60 to 140 mg ·25 mL^{-1} of assay solution.

TPC for purslane seed oil

Phenolic compounds of purslane seed were extracted by methanol-water solution and determined by Folin-Ciocalteu method described by Liu *et al.,* (2012). 2.5 g of oil was dissolved in 5 ml hexane and extraction was carried out by methanol-water solution (80:20 %v/v). The aqueous phase was collected by centrifugation at 3500 rpm for 5 min, followed by vacuum drying at room temperature. Dried sample was dehydrated in 5 ml of methanol solution and was mixed with 2.5 mL of Folin reagent and 10 mL of sodium carbonate solution in 50 ml volumetric flask and was adjusted to volume with deionized water. The absorbance was evaluated at 765 nm after 30 min. Gallic acid was used for calibration and the results were expressed as mg gallic acid equivalent per 100 g of oil samples. Triplicate test were performed for each sample.

Antioxidant activity determination

Antioxidant activity of OMSWs and purslane seed oil

Stable 2,2-diphenyl-1-picryl- hydrazyl radical (DPPH) was used to evaluate The antioxidant activity of the phenol extracts of OMSWs according to a modification method of Zahran *et al.,* (2015) for preparing extract. Methanolic solutions of both phenol extracts (0.1 ml) and DPPH (3.9 ml) (0.0025 g/100 ml CH3OH) were placed individually in dark and at room temperature for 30 min, by means of a double-beam ultraviolet–visible spectrophotometer Hitachi U-3210 (Hitachi, Ltd., Tokyo, Japan) the absorbance at 517 nm was measured against methanol. Also, blank sample (0.1 ml methanol + 3.9 ml methanolic solution of DPPH) was measured against methanol at 517 nm. Thus the DPPH radical scavenging activity of the samples were calculated as follow:

$$DPPH\ scavenging\ activity\ (\%) = [(A_{control} - A_{sample}) / A_{control}] * 100$$

Antioxidant activity of the purslane oil samples was determined by the utilization of DPPH (2,2-diphenyl-1-picryhydrazyl) radical scavenging activity (Hanaa M.Soliman *et al.,* 2017). Where purslane seed oil (0.1 ml) was diluted and mixed with 3.9 ml methanolic solution of DPPH (0.1 mM), after incubation in darkness at room temperature for 30 min, the absorbance at 517 nm was decrease. A sample free DPPH solution was used as a control. The following equation was used to determine the DPPH radical scavenging activity (%):

$$DPPH\ scavenging\ activity\ (\%) = [(A_{control} - A_{sample}) / A_{control}] * 100$$

Oxidative stability determination

Based on the method of Gutierrez（1989）, Rancimat (Metrohm 743 Rancimat; Metrohm, Riverview, FL, USA) was employed to determine the oxidative stability of purslane seed oils at 110 °C and 20 L/h air flow rate. The oxidative stability of oil was expressed as the induction time which refer to the time (h) required for a rapid change in the rate of oxidation.

UPLC-MS/MS analysis of polyphenols

Identification of OMSW and olive leaves polyphenols was carried out using an ACQUITY Ultra Performance LC™ system (UPLC™) with binary solvent manager (Waters Corporation, Milford, USA) and a Micromass Q-Tof Micro mass spectrometer (Waters, Manchester, U.K.) equipped with an electrospray ionization (ESI) source operating in negative mode. By means of UPLC BEH C18 column (1.7 mm, 2.1 mm × 50 mm, Waters Corporation, Milford, USA) and at 30°C, polyphenols were separated individually. Aqueous 0.1% formic acid (A) and 100% acetonitrile (B) were used as elution solvents. According to the linear gradient described by Kolniak-Ostek *et al.* (2013a), samples (10 µL) were eluted. Full scan, data-dependent MS scanning from m/z 100 to 2500 was applied for data analysis. The effluent was led directly to an electrospray source with a source block temperature of 130°C, desolvation temperature of 350°C, capillary voltage of 2.5 kV and cone voltage of 30 V. Nitrogen was used as a desolvation gas at flow rate of 300 $L \cdot h^{-1}$.

Statistical analysis

Each sample were performed for three replications. Statistical analysis using ANOVA program version 9.1 was applied to get average values and standard deviation.

Results and Discussion

Chemical characteristics of purslane seed oil

Refractive index, the initial allusion for polymer formation and conjugation degree that created by broken of the unsaturated center was 1.4803 for purslane seed oil at 25ºC (table 1). Acidity (% as oleic acid) of purslane seed oil was recorded as 1.14 %, and as it is known, acidity is mainly attributed to hydrolysis of triglyceride or oxidation of the unsaturated center, and this little rise in acidity is accepted due to presence of so many unsaturated center. On the other hand, peroxide value of this highly unsaturated oil was shown to be 13.42 mEq.O2 /kg oil, refer to the double bonds primary oxidation. More over iodine value, which is an indicator for the level of unsaturation, was found to be 158.39 g I2 /100 g oil, it's clear that this high value is imputed to the presence of more than 82% unsaturated fatty acid. Saponification value which is a sign for the average molecular weight was exposed as 185.55 mg K/g oil. Finally, the total phenolic contents extracted from this oil and that was responsible for its stability against oxidation was 62.12 mg GAE/kg. And this oil showed antioxidant activity equal to 57.11% when evaluated by 2,2-diphenyl-1-picryl- hydrazyl radical (DPPH•).

Table 1: Physicochemical characteristics of purslane seed oil

Parameters	Value
Refractive index	1.4803 ± 0.0001
Acidity (%) as oleic acid	1.14 ± 0.16
Peroxide value (mEq/kg)	0.89 ± 0.07
Iodine value (g/100g)	158.39 ± 1.13
Saponification value (mg/g)	185.55 ± 2.07
Total phenolic content (mg GAE/kg)	62.12 ±3.43
Antioxidant activity (%)	57.11 ±1.47
Fatty acid composition:	
$C_{14:0}$	0.13 ± 0.01
$C_{16:0}$	12.02 ± 0.21
$C_{18:0}$	4.62 ± 0.71
$C_{18:1}$	16.56 ± 0.42
$C_{18:2}$	25.66 ± 0.33
$C_{18:3}$	40.29 ± 0.23
$C_{20:0}$	0.74 ± 0.02
Saturated fatty acids	17.50 ± 0.52
Unsaturated fatty acids	82.51 ± 0.52
Polyunsaturated fatty acids (PUFA)	65.95 ± 0.09
Monounsaturated Fatty acids (MUFA)	16.56 ± 0.42

Data recorded are mean values ± standard deviations

Fatty acid composition of purslane seed oil

Fatty acids composition of purslane seed oil is illustrated in Table (1). And as it shown, palmitic acid ($C_{16:0}$) which represent 12.02% was the dominant saturated fatty acids. On the other hand oleic acid ($C_{18:1}$) which is associated with decreased low-density lipoprotein (LDL) was found to be the major monounsaturated fatty acid as it presented 16.56% (Ooi *et al.* 2015)
Moreover, linoleic acid ($C_{18:2}$) that used in the biosynthesis of arachidonic acid and thus some prostaglandins, leukotrienes and thromboxane was recorded 25.66%. in addition to linolenic acid($C_{18:3}$) which strongly affect the function of the cell receptors in cell membranes, acts as a precursor for synthesis of hormones that regulate blood clotting, has a vital role in artery walls contraction and relaxation, and helps to prevent heart disease and stroke, was represented by 40.29% (Holub, 2002). From Table (1) other fatty acids myristic ($C_{14:0}$), stearic ($C_{18:0}$) and arachidic acids ($C_{20:0}$) were found in small amount 0.13%, 4.62% and 0.74%, respectively. Regarding the total saturated and unsaturated fatty acids they were 17.50% and 82.51%, respectively.

Analysis of OMSWs

Total phenols content (TPC)

The amount of total phenolic substances in the annualized mill wastes, varies significantly *($p \leq 0.05$)* among the different materials (Table 2). The highest concentration of phenolic substances was determined in leaves (255.32 mg · g^{-1} in methanol and 213.11 mg · g^{-1} in ethanol solution), whereas the lowest concentration was found in 2-phase samples (23.57 mg · g^{-1} in methanol and 19.22 mg · g^{-1} in ethanol). In addition analysis revealed significant differences *($p \leq 0.05$)* of TPC in samples obtained with use of different solvents. Samples extracted with 70% methanol had about 10% higher concentration of phenolic substances in comparison with ethanol samples (Table 2). Also Kalogerakis *et al.,* (2013) in their studies on olive mills wastewaters revealed significant differences in content of antioxidant substances, in samples extracted with use of different solvents.

Concentration of total phenolic substances was in agreement with others. Where Lafka *et al.,* (2001) presented that olive leaves had polyphenolic content, varying from 206 mg · g^{-1} to 273 mg · g^{-1} DW, while Borja *et al.,* (2006) found that total phenolics in liquid and solid wastes from two-phase olive oil mills was ranging from 0.08 mg · g^{-1} to 0.4 mg · g^{-1}, which was 100- times lower than our results. Research conducted by Wang and Lin (2000) who reported that total phenolic content in the leaves from thornless, black raspberry, red raspberry, and strawberry plants was higher than those of their fruit tissues. Also Kolniak-Ostek *et al.,* (2013b) in their studies on apple beverages found that the total phenolic content and antioxidant capacity was increased by addition of apple leaves.

Table 2: Characterization and total phenol content of OMSWs*

Parameters	Olive leaves	2-ph, olive mill waste	3-ph, olive mill waste
Total phenol content (mg.g^{-1}, dry weight) £			
Ethanol 80%	213.11 ± 0.088	19.22 ± 0.033	28.86 ± 0.036
Methanol 80%	255.32 ± 0.037	23.57 ± 0.035	30.17 ± 0.465
Antioxidant activity (%) by DPPH			
Ethanol 80%	84.18±3.51	57.13±1.88	65.11±0.97
Methanol 80%	89.32±2.04	58.22±1.52	67.82±2.48

* Data recorded are mean values ± standard deviations; £Values are presented as mg of GAE.g^{-1}

Antioxidant activity (%) of OMSWs extracts

Antioxidant activity of OMSWs extracts were measured using DPPH radical and ranged between 57.13 to 84.18 % for ethanol extracts and between 58.22 to 89.32 % for methanol extracts, as shown in Table (2). Extract of olive leaves showed the highest DPPH radical scavenging activity other than

extracts from 2-phase and 3-phase olive mill wastes. In general, samples extracted with 80% methanol, were characterized by significantly higher ($p \leq 0.05$) antioxidant capacities, compared to samples with ethanol (Table 2). Abaza *et al.,* (2011) in their studies on different extraction solvents obtained similar results. DPPH activity in samples extracted with 70% methanol was about 20% higher in comparison with ethanol extracts. The data presented by Rice-Evans *et al.*, (1996) shows that, strong antioxidant activity was caused by flavan-3-ol derivatives, while Houhoula *et al.,* (2003) stated that quercetin and tannins, have much more antioxidant activity than ascorbic acid.

Table 3: Oxidative stability of purslane seed oil affected by addition OMSWs extracts

Treated oil samples	Induction period (h)	Protection factor	Calculated periods at ambient temperature (25±3 °C)	
			Shelf life (m)	Expired (m)
Control (PSO)	5.33^{d}	---	5.44^{d}	7.89^{d}
PSO + 200 ppm BHT	9.84^{c}	1.85^{c}	10.04^{c}	14.56^{c}
PSO + 600 ppm OL extract	13.17^{a}	2.47^{a}	13.43^{a}	19.49^{a}
PSO + 600 ppm 2-ph extract	9.16^{c}	1.72^{c}	9.34^{c}	13.56^{c}
PSO + 600 ppm 3-ph extract	11.51^{b}	2.16^{b}	11.74^{b}	17.03^{b}

Values inside each column accompanied by different letters are significantly different ($P < 0.05$); PSO= purslane seed oil; BHT= Butylated hydroxytoluene; h= hour; m= month.

Qualitative analysis of polyphenols in general

Table (4) shows the list of forty two compounds identified through UPLC-MS/MS experiments as company with their retention times (tR), UV-Vis spectral profiles at 200-600 nm and by comparison with standards reference compounds, when available. Molecules that were obviously identified in negative ion mode belong to the compound groups of simple phenols, cinnamic acids, flavonoids, lignans and secoirydoids.

As shown in table (4): Olive oil mill waste contains two simple phenols (peaks 1 and 16), five cinnamic acid derivatives (3, 5, 12, 24 and 31), eleven flavonoids (8, 9, 10, 17, 22, 25, 28, 33, 36, 40 and 42), four lignans (13, 14, 19 and 35), and twenty secoirydoids (peaks 2, 4, 6, 7, 11, 15, 18, 20, 21, 23, 26, 27, 29, 30, 32, 34, 37, 38, 39 and 41). While in the leaves of olives, twenty compounds mainly from the group of flavonoids and secoirydoids, had been identified. In addition compounds 2, 23, 33 and 42 were found only in those samples. In samples obtained by 2-phase extraction, twenty nine compounds, mainly from the group of secoirydoids were identified. Peaks 12, 15, 16, 31 and 39, were identified only in 2-phase samples. Twenty seven compounds, mainly from the group of secoirydoids, were identified in samples obtained during 3-phase extraction. Compounds 4, 6, 11, 13 and 19 were identified only in these samples.

Analysis revealed that samples extracted with methanol were distinguished by a greater variety of polyphenolic compounds in comparison to samples with ethanol (Table 4).

Simple phenols

The presence of two simple phenols was revealed in the investigated olive mill waste through the analysis in the TOF-MS, with negative ionization mode (Table 4). The identity of simple phenols was carried out by means of the compatible information of chromatographic actions and mass fragmentation, simultaneously with retention time and UV-Vis profile. The examination of the UPLC chromatograms in TOF-MS mode of OMSWs revealed the presence of two hydroxytyrosol derivatives (peaks 1 and 16). Peak 1 has a pseudomolecular ion at *m/z* 481.2 and fragments at *m/z* 265 and 163, and was tentatively identified as hydroxytyrosolrhamnoside. Peak 16 has a pseudomolecular ion at *m/z* 315.1 and MS/MS fragments at *m/z* 153 and 123, and was identified as hydroxytyrosolhexoside (Obied *et al.,* 2007).

Cinnamic acid derivatives

The data obtained by UPLC chromatograms in TOF-MS mode of OMSWs detected the four cinnamic acid derivatives. Hydroxycinnamic acid (peak 3) yielded $[M-H]^-$ at *m/z* 341.1, which was fragmented to generate *m/z* at 179 (caffeine ion). This component was identified as caffeic acid hexoside by comparing it with retention times and UV-Vis profile of authentic standards of caffeic, chlorogenic and *p*-coumaric acids. The MS/MS fragmentation demonstrated that the pseudomolecular anions of identified derivatives (peaks 5, 12 and 24) were the precursors of verbascoside ($[M-H]^-$ at *m/z* 623.2) (Innocenti *et al.,* 2006) Peak 31 had pseudomolecular ion at *m/z* 461.2 that fragmented in *m/z* 315, owing the loss of rhamnose (146 Da). This compound was therefore tentatively identified as verbasoside (Sanz *et al*., 2012).

Flavonoids

The mentioned of the UPLC chromatograms in TOF-MS mode of OMSWs exposed the presence of free luteolin (peak 40, *m/z* 285.1; identified by comparing it with retention times and UV-Vis profile of authentic standard) and 5 luteolin derivatives (peaks 8, 10, 17, 24 and 36). Peak 8 has a pseudomolecular ion at *m/z* 593.2 and MS/MS fragment at *m/z* 285; and was tentatively identified as luteolinrutinoside [21]. The analysis in the TOF-MS revealed the presence of luteolindihexoside (peak 10, *m/z* 609.1). The MS/MS mass spectrum showed peaks at *m/z* 447 and 285 corresponding to the loss of two hexosides (162 and 162 Da) (Herrero *et al.,* 2011). Peaks 17, 22 and 36, which showed $[M-H]^-$ at *m/z* 447.1 and fragmentation with MS/MS ions at *m/z* 285 owing the loss of hexose residue (162 Da), based on retention times and UV-Vis profile of authentic standard and literature (Yorulmaz *et al.,* 2011), were identified as luteolinhexoside isomers. The analysis in the TOF-MS revealed the presence of free apigenin (peak 42, *m/z* 269.1; identified by comparing it with retention times and UV-Vis profile of authentic standard) and one apigenin derivative. Peak 25 had a $[M-H]^-$ at *m/z* 577.2 that fragmented in the MS/MS at *m/z* 269 corresponding to the loss of rutinose residue (308 Da). This compound was identified as apigeninrutinoside (Romero *et al.,* 2002). The analysis in the TOF-MS revealed the presence of three quercetin derivatives (peaks 9 with *m/z* at 435.1, 28 with *m/z* at 607.2 and 33 with *m/z* at 463.2). Those compounds were identified as dihydroquercetinxyloside, quercetin glucoside-piranoside and quercetin hexoside, respectively (Bouaziz *et al.,* 2005).

Lignans

The illustrated data of the UPLC chromatograms in TOF-MS mode of OMSWs detect the presence of two fraxiresinoldrivatives. Peaks 13 and 35 have a pseudomolecular ion at *m/z* 565.2 and MS/MS fragments at *m/z* 403, corresponding to the loss of hexose residue. Those peaks were identified as fraxiresinolhexosides. Peak 14, which showed $[M-H]^-$ at *m/z* 579.2 and fragmentation with MS/MS ions at *m/z* 417 owing the loss of hexose residue (162 Da), was tentatively identified as syringaresinolhexoside (Sanz *et al.,* 2012). Peak 21 had a $[M-H]^-$ at *m/z* 415.2. This compound was identified as acetoxypinoresinol (Suárez *et al.,* 2010).

Secoirydoids

The analysis revealed the presence of elenolic acid diglucose (peak 37, *m/z* 565.2). The MS/MS mass spectrum showed peaks at *m/z* 403 and 241 corresponding to the loss of two hexosides (162 and 162 Da) (Obied *et al.,* 2008). Peaks 38 and 41 had a pseudomolecular ion at *m/z* 403.1 and MS/MS fragment at *m/z* 241, corresponding to the loss of hexose residue. Those peaks were identified as elenolic acid hexosides (Kanakis *et al.,* 2013). Peaks 2, 7, 15, 18, 19 and 20 were identified as oleuropein derivatives. Peak 2 has a pseudomolecular ion at *m/z* 701.2 and MS/MS fragments at *m/z* 539 and 377, corresponding to the loss of two hexosides. This peak was identified as oleuropeindihexoside (Silva *et al.,* 2006). The examination of the UPLC chromatograms in TOF-MS mode of OMSWs exposed the presence of three oleuropein isomers (peaks 19 and 20). Those peaks have a $[M-H]^-$ at *m/z* 539.1 which fragmented at *m/z* 377, owing the loss of hexose residue (162 Da) (Suárez *et al.,* 2010). Peak 7 had a

pseudomolecular ion at *m/z* 555.2 and fragmented at *m/z* 403. This peak was characterized as 10-hydroxy-oleuropein (Peralbo-Molina *et al.,* 2012). Peak 15 with *m/z* at 543.2 and fragment at *m/z* 539 was identified as dihydro-oleuropein. The data showed the presence of free oleoside (peak 39, *m/z* at 389.1) and three oleoside derivatives (peaks 11, 32 and 34) (Silva *et al.,* 2006, Peralbo-Molina *et al.,* 2012, Obied *et al.,* 2008). Peaks 11 and 34 had a pseudomolecular ion at *m/z* 551.3 and fragmented at *m/z* 389, owing the loss of glucose residue. Those peaks were identified as oleosidehexosides. Peak 32 had a pseudomolecular ion at *m/z* 565.1. This peak was tentatively identified as 7-β-1-D-glucopyranosyl-11-methyloleoside. The examination of the UPLC chromatograms in TOF-MS mode revealed the presence of three comselogoside isomers (peaks 20, 27 and 30) with $[M-H]^-$ at *m/z* 535.1 (Obied *et al.,* 2008). The analysis in the TOF-MS revealed also the presence of nüzhenide (peak 4, *m/z* at 685.2) (Romero *et al.,* 2002), loganinhexoside (peak 6, *m/z* at 569.2) (Peralbo-Molina *et al.,* 2012) and ligstroside (peak 29, *m/z* at 523.2) (De Marco *et al.,* 2007).

Effect of OMSWs on oxidative stability of purslane seed oil

Antioxidant compound can capture electrons, hydrogen and free radicals of other substance to inhibit the oxidation process which is the main source of free radicals and consequently the chain reactions. Thus, antioxidants are usually reducing agents where they used to oxidize themselves. Capture of an electrons or hydrogen takes place via delocalization of the captured radical through a resonating system and /or by hydroxyl group, consequently, antioxidant strength is attributed to number of hydroxyl groups and strength of the resonating system and them Stereochemistry. Table (3) illustrated that Oxidative stability of purslane seed oil which treated with extracts of the three-phase is higher than two phase, while that of olive mill solid wastes was the highest, thus, extracts of the three-phase is stronger antioxidant than two phase, while that of olive mill solid wastes was the strongest. From table (4) olive leaves and mills contain so many phenolic antioxidant, but, oleuropein and apigenin rutinosidec (a high resonating poly phenol with a spatial steriochemitry of hydroxyl and phenolic groups, as in fig (1 and 2), that facilitate the radical cupture) concentration in olive leaves was found to be more than 60 and 9.5 multiples, respectively, of those in two phase either-or three-phase extract, and consequently they could be considered as the main cause for that high rise in oxidative stability of purslane seed oil which treated with OL extract. On the other hand Oleoside glucoside (other resonating poly phenolic compound) was only foundin the extract of the three-phase, thus, it may be the cause of excellence of the three-phase than two phase.

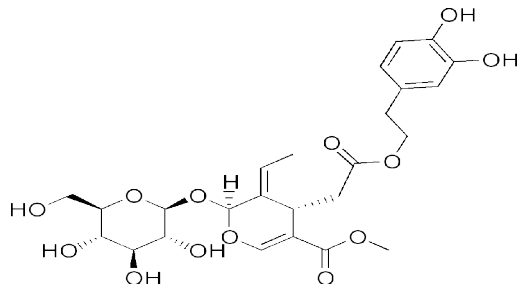

Fig. 1: Oleuropein structure

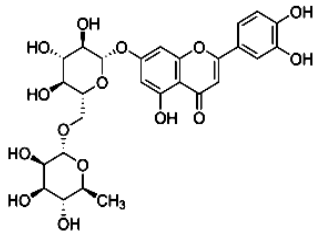

Fig. 2: Apigenin rutinosidec structure

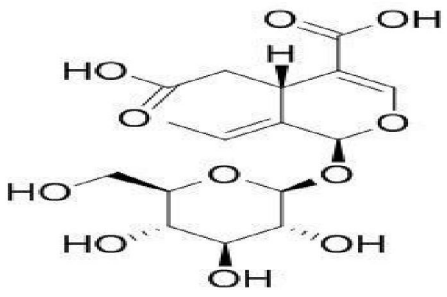

Fig. 3: Oleoside glucoside structure

Table 4: Identification of phenolic compounds in olive leaves and mills wastes

No.	t_R (min)	Λ_{max} (nm)	MS/MS (m/z)[b]	Tentative identification	Peak area (mm^2)					
					Leaves		2-phase mill waste		3-phase mill waste	
					EtOH	MeOH	EtOH	MeOH	EtOH	MeOH
1.	2.45	236/279	265 / 163	Hyroxytyrosol rhamnoside	nd	nd	42.1	nd	76.5	nd
2.	3.32	236/280	539/377/275/3	Oleuropein dihexoside	57.1	63.8	nd	nd	nd	nd
3.	3.69	324	179 / 161 /	Caffeic acid hexoside[c]	nd	nd	77.3	45.4	nd	53.1
4.	4.36	250	523/ 421/ 453	Nüzhenide	nd	nd	nd	nd	47.3	67.2
5.	4.46	247/331	621	β-OH-verbascoside	nd	nd	102.	88.9	107.2	111.9
6.	4.58	235	389	Loganin hexoside isomer	nd	nd	nd	nd	33.1	nd
7.	4.96	238/280	403/393	10-hydroxy-oleuropein	320.5	289.1	nd	303.7	nd	282.4
8.	5.09	254/343	285	Luteolin rutinoside	27.8	43.5	nd	36.1	nd	nd
9.	5.24	340	303/301	Dihydroquercetin xyloside	nd	nd	33.1	52.3	35.8	37.3
10.	5.39	269/338	285/447	Luteolin dihexoside	52.1	55.9	nd	nd	159.7	nd
11.	5.48	220/266	507/389	Oleoside glucoside	nd	nd	nd	nd	420.4	413.5
12.	6.14	246/329	667/621/ 487	β-ethyl-OH-verbascoside	nd	nd	178.	nd	nd	nd
13.	6.42	236/279	505 / 439	Fraxiresinol hexoside	nd	nd	nd	nd	nd	64.5
14.	6.50	276	417	Syringaresinol hexoside	85.1	nd	nd	nd	nd	73.2
15.	6.53	240/280	539	Dihydrooleuropein	nd	nd	nd	28.9	nd	nd
16.	7.02	235/276	153 / 123	Hydroxytyrosol hexoside	nd	nd	38.7	31.6	nd	nd
17.	7.18	254/349	285	Luteolin hexoside[c]	415.3	425.8	155.	nd	146.7	166.1
18.	7.39	236/280	377/275/307	Oleuropein	11582.	10762.2	nd	122.1	154.6	136.8
19.	7.39	236/280	377/275/307	Oleuropein isomer	nd	nd	125.	nd	nd	nd
20.	7.40	236/280	377/275/307	Oleuropein isomer	288.6	301.5	nd	nd	144.8	135.4
21.	7.56	238/279	461	Acetoxypinoresinol	nd	nd	nd	nd	106.2	98.3

22.	7.92	238/314	535/ 491/ 389	Comselogoside isomer	nd	nd	207.	189.2	111.7	122.8
23.	7.93	238/314	535/ 491/ 389	Comselogoside isomer	nd	nd	420.	718.9	723.4	520.2
24	8.62	268/339	285	Luteolin hexoside[c]	411.5	388.3	nd	88.9	174.3	101.1
25	8.63	236/280	539/371/307	Unidentified oleuropein derivative	152.0	123.1	nd	nd	nd	nd
26	8.80	247/330	461/315	Verbascoside	144.2	130.5	nd	15.3	55.6	nd
27	8.81	335	269	Apigenin rutinosidec	7380.4	6979.4	720.	655.3	612.7	841.7
28	9.05	340	463/301/ 178	Quercetin glucoside-piranoside	111.8	109.7	nd	nd	nd	nd
29	9.20	236/280	361/291/ 259	Ligstroside	52.1	54.2	nd	nd	nd	nd
30	9.36	238/314	535/ 491/ 389	Comselogoside	388.9	356.5	nd	nd	nd	nd
31	9.42	222/280	315 / 297 /	Verbasoside	nd	nd	nd	4288.6	4102.	2033.7
32	9.53	240	539	7-β-1-D-glucopyranosyl-11-	213.2	210.5	nd	nd	nd	nd
33	9.83	340	301/ 178/ 151	Quercetin hexoside	108.4	110.8	nd	89.5	nd	nd
34	10.16	220/266	507/389	Oleoside glucoside	nd	nd	nd	nd	55.4	64.8
35	10.35	236/270	461	Fraxiresinol hexoside	nd	nd	nd	43.2	33.2	32.7
36	10.60	269/340	285	Luteolin hexosidec	nd	nd	622.	914.6	955.3	413.7
37	11.00	340	403 / 241	Elenolic acid diglucoside	nd	nd	66.4	78.8	77.3	55.1
38	11.21	345	241/179	Elenolic acid hexoside	130.2	135.8	715.	2088.2	2164.	955.7
39	11.39	240	226 /182 / 121	Oleoside	86.1	79.5	nd	nd	nd	nd
40	11.41	254/350	285	Luteolinc	nd	nd	150.	166.4	42.1	111.6
41	11.68	345	241/179	Elenolic acid hexoside	108.3	104.5	255.	222.7	234.6	264.2
42	12.00	266/337	269	Apigeninc	18035.	29038.5	229	3349.1	1343.	1198.0

aAbbreviations: tR = retention time; EtOH = 80% Ethanol; MeOH = 80% Methanol; bExperimental data; cIdentification confirmed by commercial standards; nd= not detected

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Conclusions

The low-cost, renewable and abundant OMSWs and olive leaves could be used as sources of phenolic compounds. Thus they can be regard as natural antioxidant that enhance the stability of fatty foods riches in omega-3 fatty acids by preventing lipid oxidation, and protect the bio-systems. Also methanol was found to be the most efficient extraction solvent for those phenolic compounds.

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