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Antioxidant and anticholinesterase activities of Algerian pomaceolive oil

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ABSTRACT

In this study, pomace olives coming from different mills (Press process, continuous process two-phases and three-phases) were used for extraction of oils. The highest oil yield (12.92%) was obtained with pomace olives coming from press process. Total contents of phenolics (13.47 - 21.25 mg GAE/100 g oil) and flavonoids (5.90 - 12.52mg QE/100 g oil)were determined spectrophotometrically. The pomace olive oil "POO3" (Pomace olive coming from 3-phases system) presented the highest phenolic, flavonoid contents and showed the highest DPPH, ABTS scavenging, metal chelating activity. In vitro anticholinesterase activity, the olive pomace oils showed moderate inhibition against AChE and BChE which are the key enzymes taking place in pathogenesis of Alzheimer's disease. These results showed that the tested oils can be considered as sources of natural antioxidant, as well as moderate anticholinesterase agents.

Keywords: Antioxidant, anticholinesterase, flavonoid, phenolic content, oil,pomace olive.

INTRODUCTION

Olive (*Olea europaea* L.) cultivation and olive oil production have been with humankind since ancient times. The olive tree played an integral part in the life of Mediterranean basin's people, since the third millennium B.C., and olive oil has always been central to the economy of this region[1]. Over the centuries, the benefits of olive oil have been documented and the consumption of olive oil has increased throughout the world. In recent years, the increasing popularity of olive oil has been mainly attributed to its high content of oleic acid, which may affect the plasma lipid/lipoprotein profiles and its richness in phenolic compounds acting as natural antioxidants, which may contribute to the prevention of human disease[2].

The olive oil industry is one of the agro-industrial activities that produce a significant amount of by-products. As regards the olive pomace, today two kinds of process are mainly used to separate oil from olive pastes: the three-phase centrifugation system, which produces a relatively dry solid waste named three-phase pomace and a large volume of olive mill waste waters, and the two-phase system in which the extraction water injection is carried out only in the final vertical centrifugation step, reducing by one-third on average the volume of liquid effluent[3].

Pomace olive can reach up to 30% of olive oil manufacturing, depending on the milling process which, after oil extraction, is generally distributed by means of controlled spreading on agricultural soil. However, a large quantity of olive mill solid residue remains without actual application because only small amounts are used as natural

fertilizers, combustible biomass and additives in animal feeding. Many researchers have also studied the use of olive pomace in direct combustion and in the production of chemical compounds, as animal feed or soil conditioner, and activated carbon[4].This solid waste is of heterogeneous nature and can be found along with many chemical compounds, such as alkaline (potassium) and alkaline-earth (calcium and magnesium metals), sugars and polyphenols, which come from the vegetation water [5]. Since, only 2% of the phenolic compounds are transferred to the oil and as much as 98% retained in the cake, olive pomace has been considered to be an interesting source of phenolic compounds [6].

Until now, only a few papers in the literature have focused on the evaluation of the phenolic content of solid olive oil residues from different milling processes. In the past decade, several researchers have studied the replacement of Cocoa Butter with refined pomace olive oil to a certain level may reduce the costs of confectionary manufacture[7], the presence of contaminants (pesticides) in the pomace and the detoxification of this residue by the use of microorganisms or the reuse of olive pomace as metal ion adsorbent[4].

Oxidation processes are considered as major contributors to the induction and/or progress of many diseases such as cancer, Alzheimer's, Parkinson's, and heart diseases. Antioxidants can interfere with oxidative processes by reacting with free radicals, chelating catalytic metals, and also by acting as O_2 scavengers. Among the synthetic antioxidants, the most frequently used to preserve food are butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA). The use of these antioxidants is restricted due to their carcinogenicity. Thus, research efforts to identify alternative, natural, and probably safer sources of food antioxidants are an important issue. In this case, many plants can play an important role in adsorbing and neutralizing free radicals due to their high content of antioxidants such as polyphenols[8].

The objectives of this study were: (i) to determine total phenolics and flavonoids of pomace olive oils coming from different system mills, using spectrophotometric methods, (ii) to determine their antioxidant activity on DPPH free radical, ABTS cation radical decolorization and ferrous ions chelating, (iii) to determine the anti-Alzheimer(Anticholinesterase)activity.

MATERIALS AND METHODS

Plant material

The raw material used in this work was pomace olive from a different process for extraction of olive oil (Press process, continuous process two-phases and three-phases), provided by an oil factories located in Batna (Estern Algeria). The pomace was collected just after the pressing operation. The initial moisture content was determined by drying in a vacuum chamber at 70°C until reaching constant weight[9].

Spectral measurements and chemicals

The antioxidant and anticholinesterase activity measurements were carried out on a 96-well microplate reader, SpectraMax 340PC384, Molecular Devices (USA); at the Department of Chemistry, Mugla University. The measurements and calculations were evaluated by Softmax PRO v5.2 software.

Potassium persulfate, n-hexane, ferrous chloride, ferric chloride, copper (II) chloride and ethylenediaminetetraacetic acid (EDTA) were obtained from E. Merck (Darms-tadt, Germany), quercetin, Folin–Ciocalteu's reagent (FCR), 3-(2-pyridyl)-5,6-di(2-furyl)-1,2,4-tri-azine-5',5''-disulfonic acid disodium salt (Ferene), neocuproine and ammonium acetate butylated hydroxytoluene (BHT), 1,1-diphenyl-2-picrylhydrazyl (DPPH), Electric eel acetylcholinesterase (AChE, Type-VI-S, EC 3.1.1.7, 425.84 U/mg), horse serum butyrylcholinesterase (BChE, EC 3.1.1.8, 11.4 U/mg), 5,50-dithiobis (2-nitro-benzoic) acid (DTNB), acetylthiocholine iodide and butyrylthiocholine chloride, galantamine were obtained from Sigma Chemical Co. (Sigma–Aldrich GmbH, Stern-heim, Germany). 2.20-Azinobis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) was obtained from Fluka Chemie (Sternheim, Germany). Hexane was purchased from "Biochem-Chemopharma". All other chemicals and solvents were in analytical grade.

Extraction oil

The oil extraction is carried out by "Soxhlet" method for the determination of fat in dried solid foods[10]with a slight modification. 20 g of dry pomace olive was put into cellulose extraction thimbles which covered with cotton and then transferred into a Soxhlet apparatus "Gerhardt Soxtherm 2000". 150 ml of hexane was added to each flask, which was connected to the extractor. Each extraction was performed in triplicate during 3 hours. The temperature of extraction was 180°C. After extraction was completed, the excess solvent was eliminated by using drying procedure at 40ºC until reaching constant weight.

Preparation of methanol extract

The liquid/liquid extraction was performed according to the procedure described by Ollivier *et al*. [11]. 1 g of pomace olive oil was weighed into a centrifuge tube, to which 1 ml of methanol/water (80/20, v/v) was added. The mixture was stirred for 10 min in a vortex apparatus, and the tube was centrifuged at 3800 rpm for 15 min. The methanol layer was then separated and the extraction repeated twice. The methanolic extracts were combined to be used for colorimetric determination of total phenols and flavonoids.

Determination of total phenolic content (TPC)

Total phenolic constituent of the methanol extracts was determined by employing the methods given in the literature [11] involving Folin–Ciocalteu reagent and gallic acid as standard. 0.5 ml of methanolic extract solution was added to a volumetric flask. 5 ml distilled water and 1 ml Folin–Ciocalteu reagent was added and flask was shaken vigorously. After 4 min, a 0.8 ml of Na_2CO_3 (7.5%) solution was added and the mixture was allowed to stand for 2 h by intermittent shaking. Absorbance was measured at 640 nm. The concentrations of phenolic compounds were determined using the calibration curve of gallic acid standard.

Determination of total flavonoids content (TFC)

Total flavonoid content was determined using the method as adapted by Bahorun*et al.*[12]. Briefly, 1ml of 2% aluminum trichloride ($AlCl_3$) in methanol was mixed with the same volume of the methanolic extracts. Absorption readings at 430 nm were taken after 10 min against a blank sample consisting of a 1 ml extract solution with 1 ml methanol without $AlCl_3$. The total flavonoid content was determined using the calibration curve of quercetin standard.

Determination of antioxidant activity

DPPH free radical-scavenging assay

The free radical scavenging activity was determined spectrophotometrically by the DPPHassay [13]with slight modification. In its radical form, DPPHabsorbs at 517 nm, but upon reduction by an antioxidant or a radical species, its absorption decreases. Briefly, 0.1 mM solution of DPPH in methanol was prepared and 4 ml of this solution was added to 1ml of sample solutions in methanol at different concentrations. Thirty minutes later, the absorbance was measured at 517 nm by using a 96-well microplate reader. Lower absorbance of the reaction mixture indicates higher free radical scavenging activity. The capability to scavenge the DPPHradical was calculated using the following equation [14].

$$DPPH\ radical\ scavenging\ effect\ (\%) = \frac{A_{Control} - A_{Sample}}{A_{Control}} \times 100$$

Where: $A_{Control}$ is the initial concentration of the DPPH and A_{Sample} is the absorbance of the remaining concentration of DPPH in the presence of the extract and positive controls. The extract concentration providing 50% radical scavenging activity (EC 50) was calculated from the graph of DPPH radical scavenging effect percentage against extract concentration. BHA, α-tocopherol, (+)– catechin and quercetin were used as antioxidant standards for comparison of the activity.

ABTS cation radical decolorization assay

The spectrophotometric analysis of ABTS scavenging activity was determined according to the method of Re *et al.* [15], with slight modifications. The ABTS was produced by the reaction between 7 mM ABTS in water and 2.45 mM potassium persulfate, stored in the dark at room temperature for 12 h. Oxidation of ABTS commenced immediately, but the absorbance was not maximal and stable until more than 6 h had elapsed. The radical cation was stable in this form for more than 2 days in storage in the dark at room temperature. Before usage, the ABTS solution was diluted to get an absorbance of 0.708 ± 0.025 at 734 nm with ethanol. Then, 160 µl of ABTS solution was added to 40 µl of sample solution in ethanol at different concentrations. After 10 min the absorbance was measured at 734 nm by using a 96-well microplate reader. The percentage inhibitions were calculated for each concentration relative to a blank absorbance (ethanol). The scavenging capability of ABTS was calculated using the following equation:

$$ABTS\ scavenging\ effet\ (\%) = \frac{A_{Control} - A_{Sample}}{A_{Control}} \times 100$$

Where: $A_{Control}$ is the initial concentration of the ABTS and A_{Sample} is the absorbance of the remaining concentration of ABTS in the presence of sample. The extract concentration providing 50% radical scavenging activity (EC_{50}) was

calculated from the graph of ABTS radical scavenging effect percentage against extract concentration. BHA, α-tocopherol, (+)– catechin and quercetin were used as antioxidant standards for comparison of the activity.

Ferrous ions chelating activity

The chelating activity of the extracts on Fe^{2+} was measured by using Ferrin [16] with slight modifications. The extracts solution (80 µl dissolved in ethanol in different concentrations) was added to 40 µl 0.2 mM $FeCl_2$. The reaction was initiated by the addition of 80 µl 0.5 mM ferene. The mixture was shaken vigorously and left at room temperature for 10 min. After the mixture reached equilibrium, the absorbance was measured at 562 nm. The metal chelation activity was calculated using the following equation:

$$Metal\ chelating\ activity\ (\%) = \frac{A_{Control} - A_{Sample}}{A_{Control}} \times 100$$

Where: $A_{Control}$ is the absorbance of control devoid of sample and A_{Sample} is the absorbance of sample in the presence of the chelator. The extract concentration providing 50% metal chelating activity (EC_{50}) was calculated from the graph of Fe^{2+} chelating effects percentage against extract concentration. EDTA was used as antioxidant standards for comparison of the activity.

Determination of anticholinesterase activity

Acetylcholinesterase and butyrylcholinesterase inhibitory activities were measured by slightly modifying the spectrophotometric method of Ellman *et al.*[17]. AChE from electric eel and BChE from horse serum were used, while acetylthiocholine iodide and butyrylthiocholine chloride were employed as substrates of the reaction. DTNB (5.5'-Dithio-bis (2-nitrobenzoic) acid was used for the measurement of the cholinesterase activity. Briefly, 130 µl of 100 mM sodium phosphate buffer (pH 8.0), 10 µl of sample solution dissolved in ethanol at different concentrations and 20 µl AChE (5.32×10^{-3} U) or BChE (6.85×10^{-3} U) solution were mixed and incubated for 15 min at 25°C, and then 10 µl of DTNB (0.5 mM) was added. The reaction was then initiated by the addition of 20 µl of acetylthiocholine iodide (0.71 mM) or 20 µl of butyrylthiocholine chloride (0.2 mM). The hydrolysis of these substrates was monitored spectrophotometrically by the formation of yellow 5-thio-2-nitrobenzoate anion as the result of the reaction of DTNB with thiocholine, released by the enzymatic hydrolysis of acetylthiocholine iodide or butyrylthiocholine chloride, respectively, at a wavelength of 412 nm utilizing a 96-well micro-plate reader. Percentage of inhibition of AChE or BChE enzymes was determined by comparison of reaction rates of samples relative to blank sample (Ethanol in phosphate buffer pH 8) using the formula *(E-S)/ E×100*, where *E* is the activity of enzyme without test sample, and *S* is the activity of enzyme with test sample. The experiments were carried out in triplicate. Galantamine was used as a reference compound.

Statistical analysis

The experimental results were performed in triplicate. The data were recorded as mean ± standard deviation and analyzed by SPSS statistical software (Version 20.0. Armonk, NY: IBM Corp.). The data obtained are treated statistically by analysis of variances, multiple comparisons of Duncan test and $p<0.05$ was regarded as significant.

RESULTS AND DISCUSSION

Ash (Humidity) and oil yield

The olive pomace is produced in miller (oil mills) processing of olives for receiving the olive oil. Removal of olive oil, the vast majority of mills, made by centrifugation in a centrifugal separators two or three phases. Thus, we determined humidity and oil yield of olive pomace coming from three different process trituration of olive oil. According to the results shown in Table 1, humidity ranged from 34.92 ± 0.86 to 61.72 ± 0.98 %. Oil yield ranged from 10.22 ± 0.33 to 12.97 ± 0.70 g oil/100 g pomace olive.

Pomace produced from 2-phases system were characterised by higher humidity (61.96 ± 0.70%) comparing to that of 3-phases (48.60 ± 0.82%) and hydraulic pressing system (34.98 ± 0.14%). This was explained that two-phases system (Named ecological system) did not produce wastewater during oil extraction and generated margines with pomace coming out of decanter. However, it creates a high humidity pomace, which is difficult to handle [18].

In contrary, in the 3-phases system water with specific heat is added to decanter during pressing. Pomace humidity is two times lower than the two-phases pomace, because wastewater and pomace comes out of the decanter separately 18].

This observation was in agreement with Valta *et al.*[19]and Chimi[20], who reported that the highest humidity of Greek and Moroccan olive pomace obtained from two-phases system (62%) and (60%) respectively, followed by those obtained from three-phases system (55%) and (45-55%) respectively. Also, these results were in agreement with those previously found by Sánchez Moral & Ruiz Méndez[5] who cited the humidity of Spanish olive pomace coming from two-phases system (70%), three-phases system (45%) and pressing system (25-30%).

In all these cases the olive pomace still contains oil the range 10-13% (dry) not received by centrifugation: two-phases (10.22 ± 0.33%) three-phases (11.61 ± 0.18%) pressing system (12.97 ± 0.43%). These finding results were highest than oil yield for Moroccan olive pomace coming from two-phases system (3.5%), three-phases system (3.6%) and pressing system (6.8%) [20].

Amount of total phenolics and flavonoids

Table 2 presents total phenolic and flavonoid contents of the olive pomace oil determined as gallic acid and quercetin equivalent, respectively. The POO3 afforded rich phenolic and flavonoid contents, exhibiting 21.25 ± 0.22 mg GAE /100 g oil and 12.52 ± 0.63 mg QE /100 g oil, respectively. While the most poor phenolic and flavonoid contents was found to be the POO2 pomace olive oil from (13.47 ± 0.35 mg GAE, 5.9 ± 0.29 mg QE/100g oil respectively).

The Folin-Ciocalteu method was commonly used to determine the total phenolics in the substrate and usually incorporates the usage of gallic acids as the standard[21].

The color of Folin-Ciocalteu reagent changes from yellow to blue upon the detection of phenolics in the extracts which is normally due to the chemical reduction of tungsten and molybdenum oxides mixture in the reagent. In this study, methanol was used to dilute the gallic acid standard because gallic acid showed higher solubility in methanol compared to water and other solvents (eg. ethanol) [22].

Previous study on the TPC in some Australian POO generated by traditional press exhibited 0.25 mg GAE/g oil [23]. Chimi[20], investigated the effects of malaxation temperature on the phenolic composition of VOO reported that the concentration in total phenols of the oils increased with increasing levels of olive paste kneading temperature (20-25°C).

The findings of Ranalli *et al.*[24] who investigated the effects of malaxation temperature on the phenolic composition of VOO reported that the concentration in total phenols of the oils increased with increasing levels of olive paste kneading temperature. The increase in phenol concentration was more significant when the paste temperature increased from 25 to 30°C whereas phenol content did not increase when the paste temperature increased from 30 to 35°C. The method of oil extraction has a significant effect on the content of phenols. The physical forces used for oil separation and the amount of water added to the olive paste during extraction are important parameters.

The study of Di Giovacchino *et al.*[25]mentioned that addition of water to the olive paste effectively reduced the phenolic content of the oil. It was also shown that the total phenol and *o*-diphenol content of oils obtained by pressing and percolation were significantly greater than that of the centrifugally extracted oils. However, phenolic concentration of olive oil obtained by the pressure system was higher than one obtained by the traditional centrifugation process because of the low addition of water to the olive paste in pressure system.

Chimi [20] affirmed that VOO obtained by centrifugation (two-phases) was TPC more rich than 3 phase and hydraulic press VOO. In opposition to this, the concentration of TPC from POO, remained.

Antioxidant and anticholinesterase activities

Antioxidant activity tests are performed using several methods in the literature. Because of the chemical complexity of extracts, often, a mixture of dozens of compounds with different functional groups, polarity and chemical behavior could lead to scattered results, depending on the test employed. Therefore, an approach with multiple assays for evaluating the antioxidant potential of extracts would be more informative and even necessary[26].

The olive pomace oils extracted were screened for their possible antioxidant activity using four complementary test systems, namely DPPH scavenging, ABTS scavenging and metal chelating assays. The total antioxidant activity of these oils, compared with α-tocopherol, BHA, (+)- catechin, quercetin and EDTA were shown in Table 3. The results were found to be statistically significant ($p<0.05$) when compared with those of controls in each test.

In general, these oils exhibited good radical scavenging activity. In DPPH assay, the highest activity was observed in POO3 (IC_{50}: 24.53 ± 0.7 µg/ml), followed by POO1 (IC_{50}: 31.57 ± 0.62 µg/ml) and POO2 (IC_{50}: 47.64 ± 0.37 µg/ml). In ABTS assay, however, POO3 (IC_{50}: 15.22 ± 1.03 µg/ml) also showed higher radical scavenging activity, followed by POO1 (IC_{50}: 16.97 ± 0.36 µg/ml) and POO2 (IC_{50}: 22.91 ± 1.57 µg/ml). For these extracts, the ABTS assay supported the DPPH assay almost in all oils of pomace olive. The difference between the tested oils and control was statistically significant ($p<0.05$) in both antiradical assays. The scavenging effects of the olive pomace oils and standards on the DPPH radical decreased in the following order: quercetin > (+)- catechin > α-tocopherol > POO3 > POO1 > BHA > POO2. As shown, in Table 3, the scavenging effect on the ABTS cation radical, however, decreased in the following order: (+)- catechin > quercetin > BHA> α-tocopherol > POO3 > POO1 > POO2

Ferrous ions are considered as one of the effective pro-oxidants because the ferrous state of iron accelerates lipid oxidation by breaking down hydrogen and lipid peroxides to reactive free radicals through the Fenton reaction[27].

In fact, the reaction is very slow, and peroxidation accelerates when catalyzed by ferrous state iron. Table 3 shows the chelating effects of the olive pomace oils compared with EDTA on ferrous ions. The POO3 showed the highest metal-chelating activity among the other oils studied. However, none of the extracts have comparable results with that of EDTA. As seen in Table 3, the metal-chelating effect of the oils and EDTA decreased in the following order: POO3 > POO1 > EDTA > POO2

Table 3 shows the AChE and BChE inhibitory activities of the oils compared with that of galantamine. Against AChE and BChE enzyme, POO3 exhibited significant activity, followed by POO1 and POO2. At the same conditions, the IC_{50} values of galantamine were 5.0 ± 0.1 and 11.6 ± 0.9 against AChE and BChE, respectively

CONCLUSION

In conclusion, the oils extracted of olive pomace coming from different mills (Press process, continuous process two-phases and three-phases) were effective antioxidant activity depending on the process of mills. High total phenolic and total flavonoid content of POO3 was moderately to highly associate with the antioxidant properties. The antioxidant activity of phenolics is mainly due to their redox properties, which allow them to act as reducing agents. Among the four used antioxidant methods, the highest activity was observed for metal-chelating effect. These oils also demonstrated mild acetylcholinesterase inhibitory activity as well as butyrylcholinesterase inhibitory activity. Therefore, these oils may be useful as a moderate anticholinesterase agent, particularly against AChE and they could be a good source of natural antioxidants for medicinal and food industry.

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