# Olive stones as a source of antioxidants for food industry

**UMILE GIANFRANCO SPIZZIRRI – DONATELLA RESTUCCIA – SALVATORE CHIRICOSTA – ORTENSIA I. PARISI – GIUSEPPE CIRILLO – MANUELA CURCIO – FRANCESCA IEMMA – FRANCESCO PUOCI – NEVIO PICCI**

**Summary**

The aim of this work was to produce a polar extract with antioxidant properties to be used in the food and cosmetic industries, as functional foods and nutraceutical additives, starting from the olive stones, a by-product in the destoned Extra Virgin Olive Oil production. The multistep extraction procedure to produce the ethanolic extract of olive stones significantly increases the economic value of a cheap, renewable and potentially rich source of antioxidant compounds. The impact of extraction method and the efficiency of the solvent extraction were evaluated by running the same extraction protocol using solvents with different polarity. For each solvent, the recovery yield and the total phenol compounds were determined. The evaluation of the antioxidant activity of the alcoholic fraction was performed by measuring the total antioxidant activity and the scavenging properties against 2,2'-diphenyl-1-picrylhydrazyl radical, which show the efficiency of the polar extract in preventing the damages induced by free radicals, with low inhibitory concentration $IC_{30}$ (concentration required to reduce initial DPPH concentration by 30%) value (0.060 mg·ml$^{-1}$). In addition, the concentrations of total phenols and flavonoids in the extract were determined, showing that flavonoids represent about the 60% of antioxidants with phenolic groups. Finally, the extract showed a relevant ability ($IC_{30}$ of 1.30 mg·ml$^{-1}$) to preserve β-carotene from lipidic peroxidation.

**Keywords**

antioxidant; olive stones; multi-step extraction; nutraceuticals

Olive oil extraction represents an industrial activity of a high economic and social relevance to Mediterranean countries. The extraction process has a large environmental impact due to the production of highly polluted wastewater and/or solid residue, depending on the olive oil extraction (destoning process in particular) or table olive process [1]. It follows that large amounts of lignocellulosic by-products are generated by the industrial processing of the olive fruit. Olive stone is such a material, with hemicellulose, cellulose and lignin as main components. Many current studies aim to develop methods of recovering the lignocellulosic material or biomass in order to produce solid, liquid or gas biofuel [2, 3]. A widespread use of olive stones is directed towards solid fuels or their derivatives as renewable source of energy. Nevertheless, despite the environmental benefits, some problems remain, such as air pollution (by carbon monoxide, nitrogen oxides and particulates, such as soot and ash produced by combustion). On the other hand, lignocellulosic materials, particularly those of residual origin coming from agro-industrial and forest activities, are rich in antioxidant compounds and, for this reason, they could be considered for possible application as food additives and as sources of these phenolics. [4, 5].

At the moment, indeed, there is a great interest in cheap, renewable and abundant sources of natural antioxidants due to safety concerns, contradictory toxicological data on synthetic antioxidants and consumers' preference for natural additives [6].

Olive fruits contain high concentrations of

**U. Gianfranco Spizzirri, Donatella Restuccia, Ortensia I. Parisi, Giuseppe Cirillo, Manuela Curcio, Francesca Iemma, Francesco Puoci, Nevio Picci**, Department of Pharmaceutical Sciences, Faculty of Pharmacy, Nutritional and Health Sciences, University of Calabria, Via P. Bucci Edificio Polifunzionale, 87036 Arcavacata di Rende (CS), Italy.
**Salvatore Chiricosta**, Department of Business-Economics and Environmental Studies and Research, Faculty of Economy, University of Messina, Via dei Verdi 75, 98121 Messina (ME), Italy.

*Correspondence author:*
U. Gianfranco Spizzirri, tel: 0039 0984493296, fax :0039 0984493298, e-mail: g.spizzirri@unical.it

 

phenolic compounds ranging from 1% to 3% of the fresh pulp weight, comprising phenolic acids, phenolic alcohols, flavonoids and secoiridoids [7]. While phenolic acids, phenolic alcohols and flavonoids occur in many fruits and vegetables belonging to various botanical families, secoiridoids (oleuropein, demethyloleuropein, ligstroside and nuzhenide) are present exclusively in plants belonging to the *Oleaceae* family, which includes *Olea europaea* L. [8–10]. They are characterized by the presence of either elenolic acid or its derivatives in their molecular structures. There are differences in levels and type of phenolic compounds in *Olea europaea* L. leaves, fruits and seeds [11] and, in particular, the distribution of phenolic compounds in olive fruits depends on several factors such as olive cultivar [12], degree of maturation [13] and agronomic aspects, while technological aspects should also be taken into account considering the phenolic fraction present in virgin olive oil [9, 14–18].

While in recent years many studies have been conducted regarding phenolic compounds of olive oil in relation to their antioxidant activity [19–22], much less attention has been paid to the study of phenolic molecules present in olive processing by-products [23]. Nevertheless, it has been found that the whole olive stone is a rich source of valuable components due its chemicals and physical properties [24] in addition to its combustion heat [25]. The obtained results showed that nuzenide and lignans are detectable mostly in the seed as well as tocopherols [26, 27], while secoiridoid compounds are concentrated mainly in the pulp [28].

In order to increase the value of the olive stones, the aim of this work was the application of an extraction/purification method for obtaining the polar fraction from stones coming from an Italian organic destoned olive oil producer. Different solvents have been considered for extraction in relation to their polarity. The evaluation of the antioxidant activity of the polar fractions produced from the olive stones was performed by determination of total antioxidant activity, polyphenol and flavonoid concentrations. In addition, the scavenging activity of the hydrophilic parts and their ability to preserve β-carotene from linoleic acid lipidic peroxidation products were also studied.

## MATERIALS AND METHODS

### Materials and instrumentation

(+)-Catechin hydrate (CT), gallic acid (GA), 2,2'-diphenyl-1-picrylhydrazyl radical (DPPH), Folin-Ciocalteu reagent, sodium carbonate, sulphuric acid (96% w/w), trisodium phosphate, ammonium molybdate, β-Carotene, linoleic acid and Tween 20, were obtained from Sigma-Aldrich (Sigma Chemical, St Louis, Missouri, USA). Ethanol, chloroform and methanol were HPLC-grade and provided by Fluka Chemika-Biochemika (Buchs,

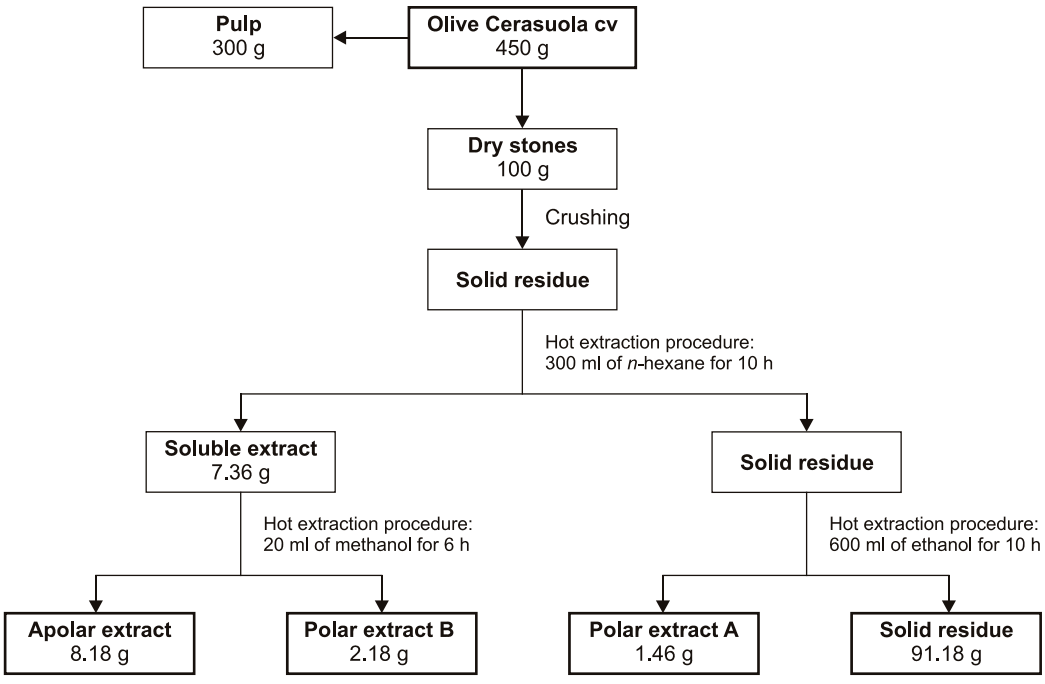


**Fig. 1.** Extraction procedure of polar extract from olive stones.

Switzerland). UV-Vis absorption spectra were recorded with a Jasco V-530 UV/Vis spectrometer (Jasco, Tokio, Japan).

### Extraction procedures

Olive fruits (Cerasuola cultivar) were manually picked on M. Layla Fatta's farm at the beginning of October in the olive groves located in the countryside near Palermo (Sicily, Italy). The stones represent a relevant part of the total weight of the fruit and starting from the olive fruit, they were subjected to a separation of the stone from the pulp by a Toscana Enologica Mori (Firenze, Italy) destoner, without significant pulp modifications. To inhibit enzymatic activities, stones were immediately frozen in liquid nitrogen and freeze-dried. An amount of 150 g of dried stones was grounded to a fine powder using a knife mill (Grindomix GM 300; Retsch, Düsseldorf, Germany) and then sieved through a 63 nm stainless steel sieve. A portion of the sample was extracted applying the procedures reported in Fig. 1. In a standard procedure, to remove all the lipophilic molecules, 100 g of grounded and sieved stones were Soxhlet extracted with hot *n*-hexane (300 ml, 10 h). Then, the solid residue was Soxhlet extracted with hot ethanol (600 ml, 10 h) to extract the polar compounds, potentially useful as antioxidants (extract A). At the same time, to recover the amphiphilic compounds in the lipophilic extract, this, after drying, was treated with hot ethanol (20 ml, 10 h) providing a second polar extract, labelled B. The fractions were evaporated under reduced pressure, re-dissolved in a known volume of ethanol and the weight of each extract was determined gravimetrically.

In order to deeper understand the impact of method extraction and to compare solvent extraction efficiencies, the same extraction protocol was performed using different extraction solvents (water, acetone, ethyl acetate). The recovery yields of the extraction procedure were also considered and total phenolic compounds were determined for extracts obtained using all solvents. All fractions were evaporated under reduced pressure and re-dissolved in a known volume of the solvent originally used for their extraction. The weight of each extract was determined gravimetrically.

### Measurement of antioxidant activity

#### Evaluation of disposable phenolic equivalents by Folin-Ciocalteu procedure

Total phenolic equivalents were determined using Folin-Ciocalteu reagent procedure, according to the literature with some modifications [29]. A 2 ml aliquot of four different extracts was mixed thoroughly with 1 ml of Folin-Ciocalteu reagent in a volumetric flask. After 3 min, 1 ml of $Na_2CO_3$ (7.5% w/w) were added, and then the mixture was allowed to stand for 2 h with intermittent shaking. The final concentrations of extracts in the samples were 0.02, 0.05, 0.08, 0.20 $mg \cdot ml^{-1}$, respectively. The absorbance of the solutions was measured at 760 nm and a calibration curve was constructed, the correlation coefficient ($R^2$), slope and intercept of the regression equation being calculated by the method of least squares.

The value of total phenolic compounds in the extracts was expressed as mean (micromoles of gallic acid equivalents per gram of polar extract) ± standard deviation (*SD*) for five replicates, by using the equation obtained from the calibration curve ($R^2 = 0.996$) for the antioxidant. This was recorded by employing five different gallic acid standard solutions in the same conditions as reported for the olive stone extracts. The final concentrations of gallic acid were 0.8, 2.4, 3.2, 4.0 $\mu mol \cdot l^{-1}$, respectively.

#### Determination of scavenging effect on DPPH radicals

In a standard procedure, alcoholic extracts were allowed to react with a stable free radical, 2,2'-diphenyl-1-picrylhydrazyl radical (DPPH), with the aim of evaluating the free radical-scavenging properties [30]. A solution of DPPH in ethanol (200 $\mu mol \cdot l^{-1}$) was freshly prepared. An 8.0 ml aliquot of this solution was mixed with 12.0 ml of six different extract solutions to obtain the final concentrations of 2.0, 4.0, 10.0, 20.0, 30.0, 40.0 $mg \cdot ml^{-1}$, respectively. The solutions in the test tubes were shaken well, incubated in the dark for 30 min at room temperature and the absorbance of the remaining DPPH was determined colorimetrically at 517 nm. The scavenging activity of the extracts was measured as the decrease in absorbance of the DPPH and it was expressed as percent inhibition of DPPH radicals calculated according to the following equation:

$$Inhibition\ [\%] = \frac{A_0 - A_1}{A_0} \times 100 \qquad (1)$$

where $A_0$ is the absorbance of a standard that was prepared in the same conditions, but without extracts, and $A_1$ is the absorbance of extract samples.

#### Determination of total antioxidant activity

The total antioxidant activity of alcoholic extracts was evaluated according to the method reported in literature [31]. Briefly, in five test tubes, 0.3 ml of five different extract solutions were

mixed with 1.2 ml of reagent solution (0.6 mol·l$^{-1}$ $H_2SO_4$, 28.0 mol·l$^{-1}$ $Na_3PO_4$, and 4.0 mol·l$^{-1}$ $(NH_4)_2MoO_4$) to increase the final concentrations of 0.02, 0.05, 0.08, 0,11, 0,16 mg·ml$^{-1}$, respectively. Then the reaction mixture was incubated at 95 °C for 150 min and, after cooling to room temperature, the absorbance of the mixture was measured at 695 nm to record data for the calibration curve. The total antioxidant activity of the extracts was expressed as mean (micromoles of gallic acid equivalents per gram of polar extract) ± *SD* for five replicates, by using the equation obtained from the calibration curve of the antioxidant. This was obtained by employing five different gallic acid standard solutions with the same procedure ($R^2$). The final concentrations of gallic acid were 8.0, 16.0, 24.0, 32.0, and 40.0 μmol·l$^{-1}$, respectively.

#### β-Carotene-linoleic acid assay

Antioxidant properties of the alcoholic extract were evaluated through measurement of percent inhibition of peroxidation in linoleic acid system by using the β-carotene bleaching test [32]. Briefly, 1.0 ml of β-carotene solution (3.0 mg·ml$^{-1}$ in chloroform) was added to 7 μl of linoleic acid and 70 μl of Tween 20. The mixture was then evaporated at 40 °C for 10 min in a rotary evaporator to remove chloroform. After evaporation, the mixture was immediately diluted with 35 ml of distilled water. The water was added slowly to the mixture and agitated vigorously to form an emulsion. The emulsion (5 ml) was transferred to five different test tubes and 1.5 ml of extract were added to increase the final concentrations of 0.015, 0.050, 0.090, 0.130, 0.180 mg·ml$^{-1}$, respectively. The tubes were then gently shaken and placed in a water bath at 45 °C for 60 min. The absorbance of the filtered samples and control was measured at 470 nm against a blank, consisting of an emulsion without β-carotene. The measurement was carried out at the initial time ($t$ = 0) and successively at 60 min. The antioxidant activity (*AA*) was obtained in terms of successful bleaching of β-carotene using the following equation:

$$AA = \left(1 - \frac{A_0 - A_{60}}{A_0^\circ - A_{60}^\circ}\right) \quad (2)$$

where $A_0$ and $A_0^\circ$ are the absorbance values measured at the initial incubation time for samples and control, respectively, while $A_{60}$ and $A_{60}^\circ$ are the absorbance values measured in the samples and in the control, respectively, at $t$ = 60 min.

#### Determination of flavonoids

A slightly modified version of the spectrophotometric method was used to determine the flavonoid concentrations of samples [33]. Briefly, in a graduated flask, 0.7 ml of five different alcoholic extract solutions were mixed with 2.0 ml of distilled water followed by addition of 0.3 ml of a $NaNO_2$ solution (5% w/w). After 6 min, 600 μl of a $AlCl_3 \cdot 6H_2O$ solution (10% w/w) were added and allowed to stand for another 5 min before 2.0 ml of 1 mol·l$^{-1}$ NaOH was added. The mixture was brought to 10 ml with distilled water and mixed well. The final concentrations of extract in the samples were 0.10, 0.25, 0,030, 0,045, 0,060 mg·ml$^{-1}$, respectively. The absorbance was measured immediately against the blank at 510 nm to record data for the calibration curve. The amount of total flavonoids in the starting material was expressed as mean (micromoles of catechin equivalents per gram of initial material) ± *SD* for five replications, by using the equation obtained from the calibration curve of the antioxidant ($R^2$). This was obtained by employing five different catechin standard solutions with the same procedure. The final concentrations of catechin in the test tubes were 10, 25, 50, 75, 100 μmol·l$^{-1}$, respectively.

#### Statistical analysis

The results are presented as the average of five experiments and standard deviation (± *SD*). Data were analysed using one-way analysis of variance (ANOVA), and differences were considered significant at $p < 0.05$. The inhibitory concentrations $IC_{30}$, which is the efficient concentration required to reduce initial DPPH concentration by 30%, were obtained by interpolation from linear regression analysis.

## RESULTS AND DISCUSSION

#### Extract

Olives contain complex phenols, as glycosides, present in a rather polar and hydrophilic form, whereas the oil contains their aglyconic form, which is the most lipid-soluble part of the molecule [34]. In order to increase the value of the olive stones, which are a by-product obtained in the production of extra virgin olive oil from destoned olives, the aim of this work was focussed on the suggestion of a possible exploitation of this by-product as a source of antioxidant compounds.

Molecules with a phenolic structure isolated from olive stones are, in particular, three glucosides including salidroside (tirosol–glucose), nuzhenide (glucose–elenolic acid–glucose–tyrosol) and nuzhenide-oleoside, and two secoroiridoid glucosides with tirosol, elenolic acid and glucose moieties with differences in sequence [35]. The

isolated glucosides were similar to others present in different oleaceae. They are supposed to be involved in the germination of the seed. Nuzhenide is found only in the seed, as a predominant phenol, while verbascoside only appears in significant quantities in the seed and pulp [36]. Tyrosol, hydroxytyrosol, oleuropein and dialdehydic form of decarboxymethyl oleuropein were present in olive tissues including the pulp, leaves, seed and stone. Tyrosol and hydroxytyrosol were identified for the first time in the olive stone by Fernández-Bolaños et al. and their presence as a structural component was suggested [37].

In order to separate these minor polar compounds from the stone matrix, a multistep extraction procedure has been proposed (Fig. 1). This was necessary for the complex composition of the stone accounting cellulose, hemicellulose and lignin as the main components, although lipids and proteins are also present in considerable quantities. Isolation of phenolic compounds from olive fruit is more difficult than from olive oil. This can be attributed to the greater homogeneity and reduced enzyme content of the oil compared with the fruit or stone. Hence, extraction of phenolics from olive tissues (fruit, stone, seed, etc.) requires more handling [26, 27, 38].

In the case of olive stones, the most common solvent used for extraction of antioxidant molecules is methanol although the use of ethanol has been also considered [26, 39]. The choice of ethanol as extraction solvent in our experiments has been based on its good extraction power for minor polar compounds and high biocompatibility as well as its lower environmental impact, in comparison with methanol. The optimization of the extraction protocol was performed by modulating the extraction temperature and the liquid/solid ratio at each step. In particular, as reported on Fig. 1, the grounded and sieved stones were first Soxhlet extracted by using *n*-hexane with a solid/liquid ratio equal to 0.33 $g \cdot ml^{-1}$ to remove amphiphilic compounds, and then the solid was Soxhlet extracted by using a hydrophilic solvent, such as ethanol (solid/liquid ratio equal to 0.17 $g \cdot ml^{-1}$), in order to obtain a polar extract containing compounds with antioxidant properties (Extract A). At the same time, to recover the lipophilic compounds in the hydrocarbon extract, this was treated, after the drying process, with hot ethanol (solid/liquid ratio equal to 0.17 $g \cdot ml^{-1}$) providing a second polar extract, labelled B. The masses of polar extracts obtained by using this extraction protocol represented about 3.7% (w/w) of the stone and 0.9% (w/w) of the fruit, while the fractions A and B constituted 1.5% (w/w) and 2.2% (w/w) of the stone, respectively.

**Tab. 1.** Recovery yield and disposable phenolic groups of the extracts from olive stones

| Extracts | Recovery yield* [%] | Disposable phenolic groups** [$\mu mol \cdot g^{-1}$] |
|---|---|---|
| Ethanol (Fraction A) | 1.5 ± 0.1 | 0.15 ± 0.02 |
| Ethanol (Fraction B) | 2.2 ± 0.2 | 0.023 ± 0.003 |
| Acetone | 22.3 ± 0.1 | 0.010 ± 0.002 |
| Ethyl acetate | 7.9 ± 0.1 | < 0.001 |
| Water | 4.9 ± 0.2 | 0.05 ± 0.01 |

* – weight of extract per weight of stone, ** – $\mu$mol of gallic acid equivalent per 1 g of polar extract.

Finally, the impact of the proposed extraction method was investigated by evaluating several extraction solvents of different polarity (water, acetone, ethyl acetate). In particular, the grounded and sieved stones were first Soxhlet extracted by using *n*-hexane to remove lipophilic compounds and then the solid was Soxhlet extracted by using solvents with different polarity. The same solid/liquid ratio was adopted when using ethanol or other solvents. The extraction efficiency of the protocols was evaluated and determinations are reported for all solvents in Tab. 1. In particular, the majority (22.3% w/w) of the extractable compounds in the olive stone sample consisted of acetone soluble constituents. Polar compounds soluble in water constituted 4.9% (w/w), whereas non-polar constituents (ethyl acetate fraction) constituted 7.9% (w/w).

### Measurement of antioxidant activity

#### Concentration of total phenolics

The total phenolic concentration was determined in each extract since it is considered as a major determinant of the antioxidant activity of nuts and plants [6]. The Folin-Ciocalteu reagent is used to obtain a crude estimate of the amount of total phenolic compounds present in the sample. They undergo a complex redox reaction with phosphotungstic and phosphomolybdic acids present in the Folin-Ciocalteu reactant. The colour development is due to the transfer of electrons at basic pH to reduce the phosphomolybdic/phosphotungstic acid complexes to form chromogens in which the metals have a lower valence. By comparing the data with the GA calibration curve, the amounts of GA equivalent were determined to be 0.15 $\mu mol \cdot g^{-1}$ and 0.023 $\mu mol \cdot g^{-1}$ for extracts A and B, respectively (Tab. 1). The concentration of phenolics in the water extract was 0.05 $\mu$mol of GA

equivalent per 1 g of the polar extract. This can be attributed to the presence of proteins and other water soluble constituents that contain phenolic rings. Among the other fractions, the phenolic content of the acetone and ethyl acetate fractions is negligible. These findings consequently suggested to perform further antioxidant assays only with ethanolic extracts (A and B).

XANTHOPOULOU et al. [40] determined total phenolic contents of four commercially available pumpkin seeds treated with two extraction methodologies in order to analyse fractions with different concentration levels. Obtained results showed that, in relation to the solvent used, the water extracts were the richest in phenolic constituents. As reported, the high concentration of phenolics in the water extract has been attributed to the presence of proteins and other water soluble constituents that contain phenolic rings enhancing the response of Folin-Ciocalteu assay. To this regard, in the olive stone, the level of proteins is higher than in the rest of the olive fruit. Protein extraction was studied using assays of solubility and precipitation, with concentrates reaching 75% proteins by weight [41, 42].

Among the other three fractions, methanol extracts contained higher amounts of phenolics, while the phenolic concentration of the acetone and ethyl acetate fractions was negligible. These results are in agreement with those already reported by MATTHÄUS for oil seeds [43]. The dependence of the total phenolic compounds on the extraction solvent depicted that the smallest absolute level was found for ethyl acetate extracts, while the highest concentration was found in water extracts. Methanol extracts also contained high absolute levels, whereas the concentrations obtained with acetone were much smaller.

PARRY et al. reported the total phenolic concentration of acetone/water fraction of flour [29] and methanol fraction of oil of cold-pressed pumpkin seeds [44]. The reported values (1.58 mg and 0.98 mg GA equivalents per g of flour or oil, respectively) are in agreement with value obtained for olive stones (0.39 mg GA equivalents per g stone for fraction A and 0.09 mg GA equivalents per g of stone for fraction B), although direct comparisons are difficult to be made since the initial sample and the extraction procedures were different from ours.

SILVA et al. analysed extracts of leaves, fruits and seeds of olive tree cultivars of Trás-os-Montes e Alto Douro (Portugal) [45]. Total phenolic concentration expressed as tannic acid, for extracts and infusions, were in the range of 0.09 $g \cdot l^{-1}$ to 0.80 $g \cdot l^{-1}$. The highest amount was found in fruits (paste and pulp extracts) and the lowest in the infusion of the commercial product. In the case of seeds, total phenolic concentration was in the range 0.22–0.34 $mg \cdot l^{-1}$. Also in this case, the direct comparison is difficult to be made for many differences between experimental protocols as well as for results expression.

### Determination of scavenging effect on DPPH radicals

The antioxidant activity of phenolics from different sources is well known [46]. Model compounds such as benzoic acids and hydroxycinnamic acids were assayed for antioxidant activity using different tests, but none of the available methods provided an absolute measurement of the phenomena involved [47]. Different behaviour was observed depending on the chemical structure and type of assay considered. Lignin monomers and dimers are effective antioxidants [48, 49]. Simple phenolic acids (related to benzoic and cinnamic acids) are absorbed and have a role in the antioxidant defence [50]. Esters of phenolic acids are more active than phenolic acids [51, 52], whereas oligomers and condensed tannins are more active than monomeric phenols [53].

The scavenging of hydrogen radicals is one of the important mechanisms of antioxidation. In this study, DPPH was used to determine the free radical-scavenging activity of the alcoholic extracts obtained by olive stone. The DPPH radical is a stable organic free radical with an absorption maximum band around 515–528 nm and, thus, it is a useful reagent for evaluation of the antioxidant activity of various compounds. In the DPPH test, the antioxidants reduce the DPPH radical to a yellow compound, diphenylpicrylhydrazine, and the extent of the reaction depends on the hydrogen-donating ability of the antioxidants. It has been documented that cysteine, glutathione, ascorbic acid, tocopherols and polyhydroxy aromatic compounds (e.g. ferulic acid, hydroquinone, catechin, gallic acid) reduce and decolorize 1,1-diphenyl-2-picrylhydrazine (DPPH) by their hydrogen-donating capabilities. Olive stone extracts (A and B) radical-scavenging ability was evaluated in terms of DPPH reduction at different alcoholic extract concentrations, and data were expressed as inhibition (percent; Fig. 2).

The value $IC_{30}$ of products was also calculated and was found to be 0.060 mg and 1.40 mg polar extract per 1 ml for extracts A and B, respectively (Tab. 2).

These data clearly show as the scavenging activity of the chemical compounds in the polar extracts A was about 25 times higher than the com-

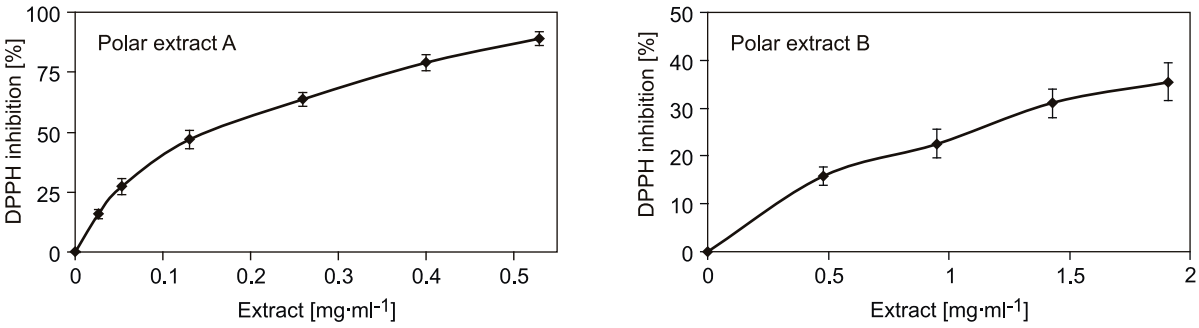


**Fig. 2.** Scavenging activity of polar extract A and B.

**Tab. 2.** Antioxidant activities of ethanolic extracts from olive stones.

| Ethanolic extracts | Antioxidant activity | | | |
|---|---|---|---|---|
| | Total antioxidant activity* [$\mu$mol·g$^{-1}$] | Total flavonoids** [$\mu$mol·g$^{-1}$] | Scavenging effect on DPPH radical $IC_{30}$ [mg·ml$^{-1}$] | β-Carotene-linoleic acid assay $IC_{30}$ [mg·ml$^{-1}$] |
| Fraction A | 0.20 ± 0.03 | 0.09 ± 0.01 | 0.060 ± 0.004 | 1.30 ± 0.05 |
| Fraction B | 0.11 ± 0.02 | < 0.001 | 1.40 ± 0.02 | 6.10 ± 0.02 |

* – $\mu$mol of gallic acid equivalent per 1 g of polar extract, ** – $\mu$mol of (+)-catechin hydrate equivalent per 1 g of polar extract.

pounds in the B fraction. The results indicate that extracts possessed phenol concentration-dependent antiradical activity. It is worth commenting on the relationship between the total phenolic concentration and scavenging activity against DPPH, since phenolics contribute to the antiradical activity. Fractions rich in total phenolics are more effective scavengers of DPPH radicals than fractions poor in phenolics. However, the inhibition of DPPH radical scavenging by different extracts is not strictly proportional to the concentration of total phenolics, since it has been reported that fractions possessing similar antioxidant properties show different concentration of phenolics [40]. This may be attributed to the different quality of phenolics they contain and, consequently, to the different antioxidant activity they possess, but also to the different contents of other constituents (saccharides, phospholipids, fatty acids) that may contribute to the antioxidant activity as well.

#### Determination of total antioxidant activity

The assay was based on the reduction of Mo(VI) to Mo(V) by antioxidant compounds and subsequent formation of a green phosphate/Mo(V) complex at acid pH. As can be seen from the data reported in Tab. 2, total antioxidant activity of extract A was about 2 times higher than of extract B. Comparing this data with total phenolic concentration of the extracts, it clearly appears that the antioxidant properties of extracts A are mainly due to phenolic compounds (75% of the components showing antioxidant efficiency), while the contribution of these compounds to the total antioxidant activity was significantly lower in extract B (21% of the components showing antioxidant efficiency). It follows that, as already reported for DPPH assay, also the total antioxidant activity was dependent on the concentration of phenolics.

Silva et al. reported the total antioxidant activities evaluated using ABTS$^+$ method in olive fruits and leaves were higher in fruit extracts and lower in infusions and in the tegument extracts [39]. In particular, the seed extracts had high total antioxidant activities despite low total phenolics concentrations. A possible explanation has been attributed to the existence of several compounds containing a tyrosol unit, like nuzhenide, as shown by HPLC-APCI-MS. Compounds with this type of phenolic structure have lower contributions to the total phenolic content measured with the Folin Denis reagent [45].

#### Results of β-carotene-linoleic acid assay

In this model system, β-carotene undergoes rapid discoloration in the absence of an antioxidant, which results in a reduction in absorbance of the test solution with reaction time. This is due to the oxidation of linoleic acid that generates free

radicals (lipid hydroperoxides, conjugated dienes and volatile byproducts) that attack the highly unsaturated β-carotene molecules in an effort to reacquire a hydrogen atom. When this reaction occurs, the β-carotene molecule loses its conjugation and, as a consequence, the characteristic orange colour disappears. The presence of an antioxidant avoids the destruction of the β-carotene conjugated system and the orange colour is maintained [54]. The inhibition percentages of lipidic peroxidation by alcoholic extracts were evaluated at different concentration as shown in Fig. 3. The $IC_{30}$ values were found to be 1.30 mg and 6.10 mg of extract per 1 ml, for extracts A and B, respectively (Tab. 2).

MATTHÄUS reported the data expressed as percentage of the initial available β-carotene after an incubation of 60 min at 40 °C [43]. Under these circumstances, the percentage of β-carotene in a control sample without extract was 10%. The highest effect resulted from addition of extracts obtained by extraction with ethyl acetate. After 60 min, only about 20% of β-carotene was decolorized. The extracts of acetone showed the weakest antioxidant activity in the β-carotene-linoleic acid system, while when using methanol as the extraction solvent, about 40–60% of the initial concentration of β-carotene was preserved. Apart from some exceptions, the antioxidant activity of the extracts from residues of different oilseeds decreased in the order ethyl acetate extract > water extract > methanol extract > acetone extract. As a rule, extracts obtained from *Brassica carinata* and sunflower with different solvents were the most effective in inhibiting the oxidation of β-carotene. In our case, after one hour of incubation with 1.5 ml of the ethanolic extract, the percentage of the initial available β-carotene was 47%, although it should be underlined that the extraction protocol and the analysed samples were different from those used in the previous studies.

**Total flavonoids concentration**

Flavonoids (specifically flavanoids such as the catechins) are the most common group of polyphenol compounds in the human diet, being found ubiquitously in plants. Flavonols, the original bioflavonoids such as quercetin, are also found ubiquitously, but in lesser quantities. The widespread distribution of flavonoids, their variety and their relatively low toxicity compared to other active plant compounds (e.g. alkaloids) mean that many animals, including humans, ingest significant quantities in their diet. Flavonoids are most commonly known for their antioxidant activity in vitro [33]. At high experimental concentrations, which would not exist in vivo, the antioxidant abilities of flavonoids in vitro are stronger than those of vitamin C and E. Consumers and food manufacturers have become interested in flavonoids for their possible medicinal properties, especially their putative role in prevention of cancer and cardiovascular diseases. Although physiological evidence is not yet established, the beneficial effects of fruits, vegetables, tea and red wine have sometimes been attributed to flavonoid compounds. RYAN et al. detected compouds with flavonoic structure in the stone of different olive cultivars by HPLC with UV and ESI MS detection. [38].

$AlCl_3$ assay was employed to achieve direct determination of the total flavonoid concentration of the product expressed as $\mu$mol catechin equivalent per g of polar extract. In particular, this assay allowed to estimate for extract A a flavonoid amount of 0.09 $\mu$mol of CT per 1 g of polar extract, while in the extract B, the flavonoid compounds were below the detection limits of the method (Tab. 2). In addition, it is important to observe that in extract A, flavonoids represented about 60% of antioxidant components with phenolic groups. It could be inferred from our results that there was a positive correlation between flavonoid concentration and antioxidant activity, as the higher ac-

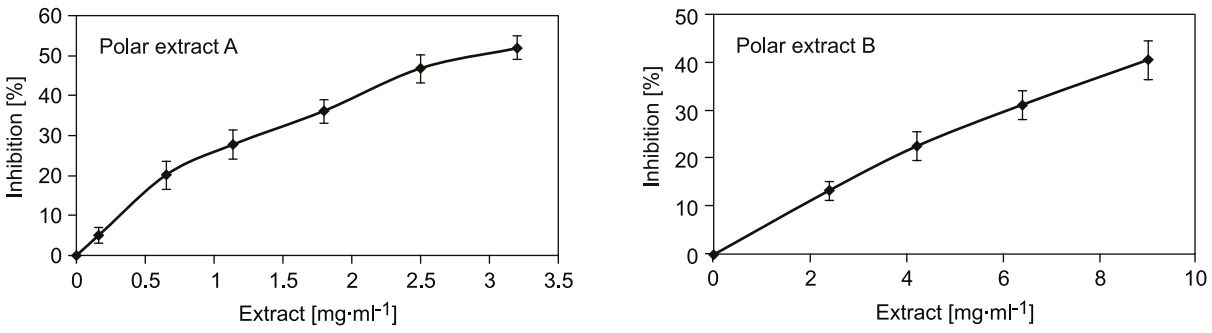


**Fig. 3.** Results of linoleic acid-β-carotene assay of polar extract A and B.

tivity of the ethanolic fraction could be attributed to the higher concentration of flavonoids. The relationship between the chemical structure of flavonoids and their antioxidant activities was analysed by VAN ACKER et al. [55]. According to the results of this study, a catechol or a pyrogallol type moiety substitution on B ring appeared to be essential for antioxidant activities of flavonoids. The presence of hydroxyl substituents on the flavonoid skeleton boosted the antioxidant activity, whereas methoxyl substitution suppressed the activity. The most potent activity of ethanolic fraction in our case might have resulted from the presence of compounds with such structures possessing the strongest antioxidant activity.

## CONCLUSIONS

In this work, stones of olive (Cerasuola cv), which are normally discarded when the fruit is processed to produce destoned Extra Virgin Olive Oil, were investigated as a potential source of antioxidants. In order to separate the antioxidant components from olive stones, a multistep extraction procedure was utilized, using alcoholic solvent (ethanol), which produced two fractions containing compounds with different polarity. Furthermore, to understand the impact of the extraction method, several extraction solvents (water, acetone, ethyl acetate) with different polarity were also evaluated. The extraction efficiency of the solvents was gravimetrically determined and the order was found to be acetone > ethyl acetate > water > ethanol. Preliminary data about the antioxidant properties of the extracts were achieved by determining the total phenolics concentration by Folin-Ciocalteu assay. The results clearly showed that the ethanolic extracts had the highest concentrations of disposable phenolic groups, while the phenolic contents of the acetone and ethyl acetate extracts was negligible. The antioxidant activity of the ethanolic extracts was assessed in homogeneous solution by the 2,2-diphenyl-1-picrylhydrazyl radical-scavenging assay, by determination of flavonoid contents and by determination of the capacity to preserve β-carotene from lipid peroxidation. The results showed that extract A may be suitable for use as an antioxidant in food, cosmetic, nutraceutical and pharmaceutical applications, reducing at the same time the amount of disposed by-products and the environmental impact of the elaiotechnical industry.

**ACKNOWLEDGEMENTS**

This work was financially supported by MIUR (Programma di ricerca di rilevante interesse nazionale) and University of Calabria funds. Authors thank Dr. Alessandra Cuscinà (Azienda Agricola Fatta Maria Layla, Palermo, Italy) for providing olive stones.

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Received 6 December 2010, revised 4 February 2011; accepted 3 March 2011.