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Optimization of ultrasound-assisted extraction of phenolic compounds: Oleuropein, phenolic acids, phenolic alcohols and flavonoids from olive leaves and evaluation of its antioxidant activities

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ABSTRACT

Olive leaves are well known for many useful pharmacological effects. Some of the health benefits are related to phenolic composition, especially to oleuropein (OLE) and flavonoids (FLs) content. This study aimed to investigate the influence of ultrasound-assisted extraction conditions (solvent type, solvent concentration, extraction time and temperature) on the extract yield of OLE, hydroxytyrosol (HTR), FLs (rutin, luteolin, luteolin-7-*O*-glucoside and apigenin) and phenolic acids (protocatechuic, 4-hydroxybenzoic, vanillic, *p*-coumaric and ferulic acids) from olive leaves using single factor experiments approach. The total phenolic compounds (TPC) and their antioxidant activity based on 2,2-diphenyl-1-picrylhydrazyl radical scavenging activity (RSA) and ferric reducing antioxidant power (FRAP) were also evaluated. The highest yields of OLE and FLs, the major phenolics in olive leaves, were obtained with 50% acetone, at 60 °C for 10 min extraction. However, the yields of HTR and PAs increased when water was used as extraction solvent. Good, positive, correlation coefficients were found between OLE, TPC, RSA and FRAP in olive leaves, especially under the influence of solvent type and solvent concentration.

1. Introduction

The olive tree *(Olea europaea* L.*)* is one of the most important fruit trees in Mediterranean countries such as Italy, Spain, Greece and Tunisia. Although olive leaves are always used as animal feed, there is a rising interest in their application as a valuable material in various fields. They are regarded as a cheap raw material which can be used as a good source of bioactives and they are also one of the by-products in olive-oil production, representing 10% of the weight of olives collected. Furthermore, they also accumulate in large volumes on olive groves during the pruning of the trees (Herrero et al., 2011).

Olive leaves are rich in a wide variety of phenolic compounds, such as secoiridoids (oleuropein, ligstroside, dimethyloleuropein) and flavonoids (apigenin, luteolin, luteolin-7-O-glucoside etc), along with other phenolic compounds (hydroxytyrosol, tyrosol, caffeic acid, ferulic acid etc) (Quirantes-Piné et al., 2013), that are responsible for several biological properties, including antioxidant and anti-inflammatory, antimicrobial, antiviral, anti-carcinogenic, as well as beneficial cardiovascular effects (El and Karakaya, 2009).

OLE is the most representative polyphenolic constituent of olive leaves, responsible for the bitterness of both table olives and extra-virgin olive oil. The majority of studies attribute the biological activities of olive leaves to the total or individual phenolic compounds such as OLE (Al-Azzawie and Alhamdani, 2006), HTR (Bouallagui et al., 2011), and FLs (Goulas et al., 2010). However, the phenolic profile in olive leaves varies depending on the origin and variety of the plant material, the geographical location and the agro-ecological conditions, and especially the seasons (Ranalli et al., 2006).

Various methods have been used to isolate the bioactive molecules present in olive leaves, from the most common techniques to the more sophisticated including microwave-assisted extraction (Rafiee et al., 2011; Habibi et al., 2018), pressurized liquid extraction (Xynos et al., 2014) and supercritical fluid extraction (Sahin and Bilgin, 2012). It should be pointed out that most of these techniques suffer from high energy costs as they operate under high pressure. Therefore, the development of an effective, suitable and low-cost extraction method is of major importance. In this sense, ultrasound-assisted extraction (UAE) has the potential to reduce extraction times and extraction solvent volumes, as well as to increase the recoveries of active compounds. It has become a well-established technique both, in laboratories and in

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industrial scale as well.

Several authors found that UAE method is faster, simpler and more efficient than maceration/stirring for the extraction of OLE from olive leaves (Ahmad-Qasem et al., 2013; Xie et al., 2015; Cifá et al., 2018). A recent review highlighted the extraction methods and potential application of the bioactive components of olive leaves (Rahmanian et al., 2015). Although the effect of extraction conditions on the extraction yield of OLE, has been extensively investigated, few studies reporting the effect of extracting parameters on the recovery of other class of phenolic compounds from olive leaves (Delgado-Povedano et al., 2017).

The aim of the present study was to maximize the extraction yields of OLE, HTR, FLs and PAs from olive leaves in respect to decrease operating costs with the possibility of lower volumes of solvent and lower extraction times and temperatures. We investigated the application of UAE and the optimization of extraction parameters such as solvent type, solvent concentration, extraction time and temperature in order to obtain extract rich in bioactive compounds with high antioxidant activities as evaluated by RSA and FRAP tests using single factor experiments approach.

2. Materials and methods

2.1. Chemicals

OLE, TYR, HTR, luteolin-7-*O*-glucoside (LUTG), protocatechuic acid (PRCA), *p*-coumaric acid (*p*CA), ferulic acid (FA), vanillic acid (VA) and *p*-hydroxy-benzoic acid (*p*HBA) were supplied by Sigma-Aldrich (Steinheim, Germany). Apigenin (API), rutin (RUT), luteolin (LUT) and gallic acid (GA) were obtained from Extrasynthese (Genay Cedex, France). Analytical grade of Folin-Ciocalteu, 1,1-diphenyl-2-picrylhydrazyl (DPPH), 6-hydroxyl-2,5,7,8- tetramethychromane-2-carboxylic acid (Trolox) and 2, 4, 6-tripyridyl-s-triazine (TPTZ) were from Sigma-Aldrich (Steinheim, Germany). All other solvents/chemicals obtained from Chem-Lab (Zedelgem, Belgium) were of analytical grade or high-performance liquid chromatography (HPLC) grade.

2.2. Plant material

Fresh green olive leaves (*Olea europaea* L., variety Chalkidiki), were collected from the trees grown in north of Greece. Collected leaves were air dried in oven at 40 °C and then were milled using a laboratory mill equipped with a 0.5 mm sieve and finally stored at 4 °C prior to extraction.

2.3. Ultrasound-assisted extraction of phenolic compounds

Powdered and dried olive leaves (250 mg) were extracted with solvent (20 mL) in an ultrasound bath (frequency 37 kHz, model FB 15051, Thermo Fisher Scientific Inc. Loughborough, England) for the different times and temperatures. Then, the crude extracts were centrifuged at 1500 × *g* for 10 min (Universal 320R, Hettich, Germany), the supernatants were filtered using 0.45-μm syringe filters and used directly for estimation of TPC, assessment of antioxidant capacities and HPLC analysis of phenolic compounds. Each extraction was triplicated and all analysis were performed in three replications.

2.4. Experimental design

In the present study, single factor experiments was used to determine the optimum conditions for extracting phenolic compounds from olive leaves. Four extraction solvents were used: ethanol, methanol, acetone and water. Three independent variables were studied, namely organic solvent concentration, extraction time and extraction temperature in order to optimize the extraction conditions. The level for each independent variable was chosen based on the process responses, OLE, HTR, FLs and PAs as well as TPC, RSA and FRAP. Initially, samples were extracted with different concentrations of three organic solvents tested ranged from 0 to 90% v/v, fixing extraction time and extraction temperature constant at 30 °C for 10 min. Subsequently, the effect of extraction time was investigated by varying the extraction time from 10 to 120 min using the best organic solvent concentration chosen in the initial step and kept the extraction temperature constant at 30 °C. Lastly, the effect of extraction temperature was investigated using the best organic solvent concentration and extraction time determined in the previous stage with extraction temperature ranged from 30 to 65 °C.

2.5. Determination of total phenolic content (TPC)

The amount of TPC in olive leaves extracts was determined according to the Folin-Ciocalteu method (Singleton et al., 1999) with minor modifications. Briefly, 200 μL of each extract was reacted with 800 μL of Folin-Ciocalteu reagent (diluted 10-fold) for 2 min. Then, 2 mL of sodium carbonate (7.5% w/v) was added and the volume was adjusted to 10 mL with distilled water. The mixture was allowed to stand for 1 h at room temperature in the dark and the absorbance was measured at 765 nm against blank. The results were expressed as mg of GA equivalents per g of the dried sample (mg GAE/ g dw).

2.6. Antioxidant activity

2.6.1. DPPH free radical scavenging activity (RSA)

The RSA was based on the protocol described by Yen and Chen (1995) with some modifications. Aliquots (150 μL) of extracts were reacted with 2.85 mL DPPH solution in methanol (0.1 mM). After agitation, the reaction mixture was incubated in the dark at room temperature for 5 min and the absorbance was measured at 516 nm. The free radical scavenging capacity (in percentage) was calculated by using the following equation:

$$RSA\ (\%) = (Ao - As)\ /Ao \times 100$$

where Ao is the absorbance of the blank (methanol) and As is the absorbance of the sample. Results were expressed as mg Trolox equivalents per g of dried sample (mg TE/g dw).

2.6.2. Ferric reducing antioxidant power assay (FRAP)

The FRAP assay was performed according to Benzie and Strain (1999) with some modifications. Briefly, the fresh FRAP reagent included 10 mL of 10 mM TPTZ solution in 40 mM HCl plus 10 mL of 20 mM $FeCl_3.6H_2O$ and 100 mL of 300 mM acetate buffer pH 3.6. Aliquot of extract (100 μL) was reacted with the FRAP solution (3 mL) at 37 °C for exactly 4 min under dark conditions. Readings of the colored product were then taken at 593 nm against blank and the results were expressed as mg Trolox equivalents per g of dried sample (mg TE/g dw).

2.7. HPLC profile of phenolic compounds

The analyses were performed on an HPLC Agilent 1200 system (Agilent Technology, Urdorf, Switzerland) equipped with a 250 × 4.6 mm i.d., 5 μm Nucleosil 100 C_{18} column (MZ, Mainz, Germany) maintained at 30 °C, a 20 μL loop and diode-array detector (DAD). Mobile phase consists of three solvents: (A) 1% acetic acid in water, (B) acetonitrile and (C) methanol and the following gradient program was performed: 0 min, 90% A-0% B; 10 min, 80% A-4% B; 25 min, 75% A-5% B; 30 min, 65% A-5% B; 31 min, 40% A-0% B; 37 min, 35% A-20% B; 50 min, 20% A-80% B. The flow rate of mobile phase was 1.3 mL/min. The DAD recorded the spectra at 260, 280, 320, and 360 nm and the chromatograms were analysed using the Agilent Chemstation software (version B.04.01, Agilent Technologies). Identification of phenolics in olive leaves extract was obtained by comparison of retention times and UV/VIS spectra with those of