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Chemical characterization and effects on *Lepidium sativum* of the native and bioremediated components of dry olive mill residue

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Abstract

Dry olive mill residue (DOR) from the olive oil production by two phase centrifugation system was fractionated by a consecutive continuous solid–liquid extraction obtaining the EAF, PF, MF and WF fractions with ethyl acetate, *n*-propanol, methanol and water, respectively. The chemical, chromatographic and mass spectrometric analyses showed EAF, PF and MF to be mainly composed of simple phenols, phenolic acids, flavonoids and glycosilated phenols (glycosides of phenols, secoiridoids and flavonoids), whereas WF was mainly consisting of polymerin, the metal organic polymeric mixture previously identified in olive oil mill waste waters and composed of carbohydrates, melanin, proteins and metals (K, Na, Ca, Mg and Fe). The identification in DOR of oleoside, 6′-β-glucopyranosyl-oleoside and 6′-β-rhamnopyranosyl-oleoside, and of its organic polymeric component, known as polymerin, are reported for the first time in this paper. The inoculation of the previously mentioned fractions with saprobe fungi *Coriolopsis rigida*, *Pycnoporus cynnabarinus* or *Trametes versicolor* indicated these fungi to be able to metabolize both the phenols and glycosilated phenols, but not polymerin. In correspondence, EAF, PF, MF and WF, which proved to be toxic on *Lepidium sativum*, decreased their toxicity after incubation with the selected fungi, WF showing to be also able to stimulate the growth of the selected seeds. The phytotoxicity appeared mainly correlated to the monomeric phenols and, to a lesser extent, to the glycosilated phenols, whereas polymerin proved to be non toxic. However, the laccase activity was not associated with the decrease of phytotoxicity. The valorization of DOR as a producer of high added value substances of industrial and agricultural interest in native form and after their bioremediation for a final objective of the total DOR recycling is also discussed.

Keywords: Dry olive mill residue; Phenols; Polymerin; Mass spectrometry; Saprobe fungi; Phytotoxicity; Antioxidants; Biofertilizer

1. Introduction

Olive oil extraction is one of the most traditional agricultural industries with a great economical importance in most of the Mediterranean countries (Owen et al., 2000). However, the manufacture of olive oil yields large quantities of solid and liquid wastes and by-products during a short period of time. This may have a great impact on land and water environment because of their high phytotoxicity (Roig et al., 2006). Therefore, there is a need for guidelines to manage these wastes through technologies that minimize their environmental impact and lead to a sustainable use of resources. Different treatment methods such as biological, chemical, physical, physico-chemical have been used for elimination or transformation of olive oil residues. Nevertheless, none of these approaches appears as a general solution and therefore the scientific community is still

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in search of effective processes for reducing these contaminants (Cermola et al., 2004).

The two-phase centrifugation system for olive oil extraction produces a liquid phase (olive oil) and a solid and very humid by-product called "alpeorujo" (AL). AL is usually treated with a second centrifugation to extract the residual oil. The resulting by-product of this second extraction is dried, then subjected to chemical extraction with hexane with generation of a new final solid waste (dry olive mill residue or DOR). DOR contains large amount of mineral and organic matter, including sugars, tannins, phenolic compounds, polyalcohols, pectins and lipids (Ayed et al., 2005).

The management of olive residues (alpeorujo) has been objective of several investigations, but a satisfactory solution of their disposal problem has been not found until now (Fernandez-Bolaños et al., 2002; Yañez-Ruiz et al., 2004; Benitez et al., 2005; Alburquerque et al., 2006). DOR is also a potentially rich source of a large range of phenols with a wide array of biological activities. Recently, a detailed review on bioactivity and analysis of bio-phenols from olive mill waste has been published (Obied et al., 2005). It has been described that olive mill waste is rich in hydroxytyrosol, tyrosol, oleuropein and caffeic acid. A wide number of scientific articles proved the antioxidant, cardioprotective, antimicrobial, antihypertensive, and anticarcenogenic activities of these compounds, which could be used in pharmaceutical, cosmetic and food industries. Physical technologies were developed for the improvement of their extraction methods but little information has been provided for the biological methods to obtain hydroxytyrosol (Fernandez-Bolaños et al., 2002; Bouzid et al., 2005).

On the other hand, DOR might be used as fertilizer due to its high organic and mineral content, but like the majority of plant by-products, DOR is phytotoxic (Sampedro et al., 2004). One of the most promising studies on DOR treatment technologies is the biological degradation with white rot fungi, however, the effectiveness of this treatment is not always satisfactory, in particular with respect to the time consuming (Sampedro et al., 2004, 2005). It has been described the possibility of enhancing the removal of bio-reclacitrant phenols of olive mill waste waters by pre-treating; however to date no related studies have been carried out in DOR (Beccari et al., 1999; Di Gioia et al., 2001).

The treatment of DOR is a complex problem that has not been satisfactorily resolved mainly due to socio-economic and, to a lesser extent, technological reasons. A single-stage biological or chemical treatment is unlikely to achieve complete mineralization at reasonable cost due to the complexity and heavy polluting load of olive oil residue. On the other hand, a well-designed sequential treatment consisting of various chemical, physical and biological processes with well-defined treatment objectives may be the optimal solution (Mantzavinos and Kalogerakis, 2005).

The aim of the present work was the fractionation and identification of the compounds natively occurring in DOR and after the biological treatment. In particular, this paper reports the partition of DOR in four fractions, EAF (ethyl acetate), PF (propanol), MF (methanol) and WF (water) obtained by a consecutive continuous solid–liquid extraction in a soxhlet apparatus with increased polarity solvents such as ethyl acetate, *n*-propanol, methanol and water, respectively. These fractions were characterized by combined chemical, chromatographic and mass spectrometric analyses in order to identify the nature of its native chemical components and after their treatment with the saprobe fungi *Coriolopsis rigida*, *Pycnoporus cynnabarinus* or *Trametes versicolor*. More specifically, they were analysed by HPLC with UV detector (HPLC–UV) and by HPLC coupled with electrospray ionization mass spectrometry (HPLC–ESI/MS). The involvement of the identified substances in the toxicity of DOR was also investigated, by analysing their effects on *Lepidium sativum* seeds after incubation with the saprobe fungi above mentioned.

The potential exploitation of the fractionation methodology for the recycling of DOR is briefly discussed.

2. Materials and methods

2.1. Chemicals

Ethyl acetate, *n*-propanol, methanol for the soxhlet extraction were obtained from Panreac Quimica SA (Spain). Standards compounds were purchased from Sigma. *p*-Tyrosol was purchased from Fluka. Hydroxytyrosol was obtained as described by Capasso et al. (1999). HPLC grade methanol and acetic acid were purchased from Carlo Erba (Milan, Italy). HPLC grade water (18 mΩ cm) was prepared using a Millipore Milli-Q purification system (Millipore Corp., Bedford, MA, USA). Polymerin was recovered from olive oil mill wastewaters as previously reported (Capasso et al., 2004).

2.2. Sample preparation

DOR was collected from an orujo manufacturer (Aceites Sierra Sur, Granada, Spain). It was subjected to consecutive soxhlet extractions with organics solvents. Samples of dry DOR (100 g) were placed in extraction thimbles and loaded into the soxhlet apparatus. A volume of 400 ml of ethyl acetate was added to a round-bottomed flask (500 ml capacity). The flask was connected to the soxhlet extractor (Pobel, Barcelona, Spain) and the ethyl acetate was heated with an electrical heater to boil the solution. After eight h of continuous extraction and cooling, the resulting ethyl acetate extract was concentrated by rotary evaporation (Buchi R, Switzerland). Subsequently the same DOR thimble with the remained exhausted DOR was extracted by soxhlet with *n*-propanol following the same procedure to give an *n*-propanol extract. The remained exhausted DOR after ethyl acetate and *n*-propanol extractions was also extracted by soxhlet with methanol for eight h obtaining a methanol