



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 glycosylated 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 *Corioloropsis rigida*, *Pycnoporus cinnabarinus* or *Trametes versicolor* indicated these fungi to be able to metabolize both the phenols and glycosylated 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 glycosylated 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.

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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 anticarcinogenic 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 *Corioloopsis rigida*, *Pycnoporus cinnabarinus* 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

extract. Finally, the exhausted DOR after ethyl acetate, *n*-propanol and methanol extraction was extracted with distilled water for 16 h. The DOR ethyl acetate, *n*-propanol, methanol and water extract were evaporated to dryness and the residue was resuspended in water in the same initial proportion.

2.3. Treatment of liquid extracts from DOR with saprobe fungi

All the DOR extracts resuspended in water were used as growth medium for the saprobe fungi except for the DOR ethyl acetate extract whose pH was adjusted to 5.5 and additionated with nutrients from Czapek medium. The saprobe fungi *C. rigida*, *P. cinnabarinus* and *T. versicolor* were maintained on Malt Extract Agar (MEA) plates at 4 °C and periodically subcultured. The inoculum was produced by growing the fungus under orbital shaking at 125 rpm and 28 °C on Czapek in the presence of 50% of aqueous DOR extracts for 15 days. The mycelium was collected by filtration, extensively washed with distilled water and homogenized. The fungi were grown in Erlenmeyer flask (250 ml) containing 70 ml of each DOR extract for 15 days at 28 °C with orbital-shaking at 125 rpm. Each flask was inoculated with 0.45 g l⁻¹ of each inoculum. The culture liquid was separated from the mycelium by centrifugation (8000 rpm for 15 min) and the supernatants were analysed. Appropriate controls were prepared. Experiments were performed in quadruplicate.

2.4. Chemical analysis

The total phenolic content of DOR extract was estimated according to Ribereau-Gayon (1968), using tannic acid as standard and was expressed as g kg⁻¹ of DOR.

Laccase (EC 1.10.3.2) activity was determined spectrophotometrically by measuring the oxidation of 5 mM 2,6-dimetoxiphenol in 100 mM acetate buffer, pH 5.0. The formation of the cation radical was followed at 469 nm ($\epsilon_{469} = 27,500 \text{ M}^{-1} \text{ cm}^{-1}$) (Saparrat et al., 2000). The results were expressed as IU (g DOR⁻¹).

2.5. Chromatographic analysis

2.5.1. HPLC–UV analysis

EAF, PF, MF and WF fractions were analysed by HPLC using a SURVEYOR MS micro HPLC, (Thermo Finnigan, San José, CA, USA). Individual phenols were separated on a Hypersil BDS C18 column (250 mm × 4.6 mm, 5 µm) (Thermo, Bellefonte, PA, USA) at a flow rate of 1 ml min⁻¹; solvent A was 0.05% acetic acid and solvent B was methanol. After a 2 min hold at 5% solvent B, elution was performed according to the following conditions: from 5% (B) to 10% (B) in 3 min; to 20% (B) in 13 min; to 30% (B) in 25 min; to 50% (B) in 10 min; and to 95% (B) in 17 min, followed by 5 min of maintenance. Detection was performed at 280 and 320 nm.

2.5.2. HPLC–ESI/MS analysis

The HPLC conditions were as described for the HPLC–UV system. The column effluent was analysed by ESI/MS in negative ion mode using a Finnigan LCQ DECA XP Max ion trap instrument (Thermo Finnigan, San José, CA, USA) equipped with Xcalibur (Thermo Finnigan, San José, CA, USA) version 1.3 software. The capillary voltage was set at –10 V, the spray voltage was at 3 kV and the tube lens offset was at –10 V. The capillary temperature was 270 °C. Data were acquired in MS and MS/MS scanning mode and recorded in the 50–1500 *m/z* region. Total ion current (TIC) profile was produced by monitoring during the chromatographic run the intensity of all the ions produced and acquired in every scan.

2.5.3. Phytotoxicity bioassay

The phytotoxicity bioassay was a slight modification of the method described by Zucchini et al. (1981). DOR extracts, colonized or not with the fungi, were incubated (25 °C) in the dark for 48 h with *L. sativum* seeds. Distilled water was used as a control. Twenty seeds were placed, in quadruplicate, in 9 cm diameter Petri dished lined with filter paper containing 2 ml of each extract undiluted and variably diluted with distilled water. Germinated seeds were counted and radicle growth measured. The germination index (GI) was calculated according to the formula $GI = (G/G_0) \times (L/L_0) \times 100$, where G_0 and L_0 are, respectively the germination percentage and radicle growth of the control.

2.5.4. Statistical treatment of data

The data obtained were subjected to ANOVA. The mean values of four replicates were compared using Tukey test.

3. Results and discussion

3.1. Identification of phenol compounds present in the different fractions

The continuous solid–liquid soxhlet extraction with ethyl acetate, propanol, methanol and water led to the obtainment of the corresponding fractions, EAF, PF, MF and WF, respectively, which contained a high quantity of phenols detected by chemical analysis and identified (Fig. 1) by combined HPLC–UV and HPLC–ESI/MS analyses.

More specifically, the extracts were first analysed by HPLC–UV which allowed the identification of the corresponding compounds by comparison with the retention time of their standards. Moreover, on-line HPLC–ESI/MS experiments were carried out in order to characterize the compounds not revealable at the two selected wavelength (280 and 320 nm). In some cases, the structure of the compounds was hypothesized on the basis of MS/MS experiments (data not shown). It is important to underline

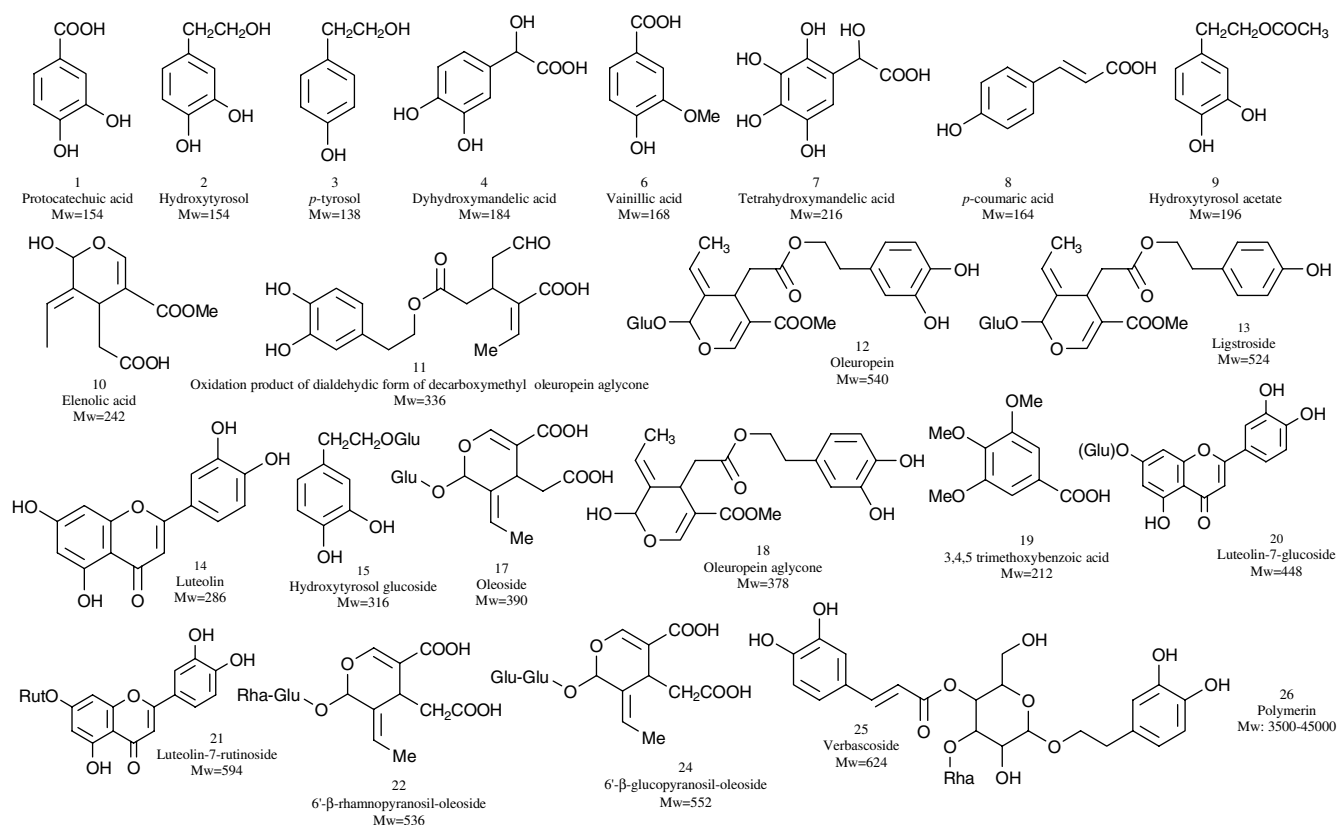


Fig. 1. Phenols and related compounds identified in EAF, PF, MF and WF fractions from DOR by HPLC–UV and HPLC–ESI/MS analyses.

that the HPLC–ESI/MS analyses of all the DOR fractions confirmed the data obtained by HPLC–UV.

The HPLC–UV analysis of EAF (Fig. 2a) showed in this fraction the presence of not only simple phenols (2, 3, 9) and phenolic acids (1, 4, 6–8, 11) as the major constituents (Fig. 1), but also a flavonoid (14) and glycosides of seco-iridoids (12, 13). Moreover, the HPLC–ESI/MS analysis (chromatogram not shown) revealed the presence of elenolic acid (10) (Fig. 1). In particular, at the retention time (t_R) of 19.95 min in the HPLC–UV profile there was a co-elution of two compounds. The first one (6) corresponded to the vanillic acid, while for the second compound (5), which displayed on the mass spectrum a peak at m/z 241, was not possible to define its structure from the study of the MS/MS spectrum. In the HPLC–UV profile a peak was eluted at t_R 24.35 min. Based on its MS/MS spectrum exhibiting a pseudo-molecular ion at m/z 215, we can speculate that this peak could correspond to the tetrahydroxymandelic acid (7). It should be noted that a peak at similar t_R and with the same MS/MS spectrum has been already observed in DOR, as reported in Aranda et al. (2007). On the basis of this hypothesis, of the molecular weight and the of MS/MS spectrum, the peak at t_R 17.48 min in the HPLC–UV profile was attributed to the dihydroxymandelic acid (4).

A peak with t_R of 40.75 min was observed in the TIC chromatogram but not in the HPLC–UV profile. This peak was originated from elenolic acid (10), as confirmed by

mass spectrometric data, and was not revealable at the wavelength (280 nm) selected for the HPLC–UV analysis. In the HPLC–UV chromatogram (Fig. 2a), there was a peak at t_R 50.81 min that could correspond to the oxidation product of dialdehydic form of decarboxymethyl oleuropein aglycon (11), an oxidation product of oleuropein, found in oxidized virgin olive oil (Rios et al., 2005). This hypothesis derived from the study of the corresponding mass spectrum and was strongly supported by the MS/MS spectrum data.

The HPLC–UV analysis of PF (Fig. 2e) showed with respect to EAF the abatement of the phenols 1–9 (more specifically, 1 and 2 decreased, whereas the other disappeared) and ligstroside (13), and the appearance of an unidentified substance (16), 3,4,5-trimethoxybenzoic acid (19), and of the glycosilated phenols 17, 18, 20, 21 and 22 (Fig. 1). Oleuropein (12) and luteolin (14) (Fig. 1) maintained a similar amount. Moreover, the HPLC–ESI/MS analysis of PF showed the disappearance of elenolic acid (10). In particular, the peak with the t_R of 16.69 min (peak 16) observed in the HPLC–UV profile displayed on the mass spectrum a peak at m/z 359; unfortunately, it was not possible to define its structure from the study of the MS/MS spectrum. On the contrary the peak with t_R of 25.80 min in the HPLC–UV profile displayed in the mass spectrum a peak at m/z 389; the MS/MS spectrum suggested the presence of oleoside (17), a compound found for the first time in olive fruit by Cardoso et al. (2005).

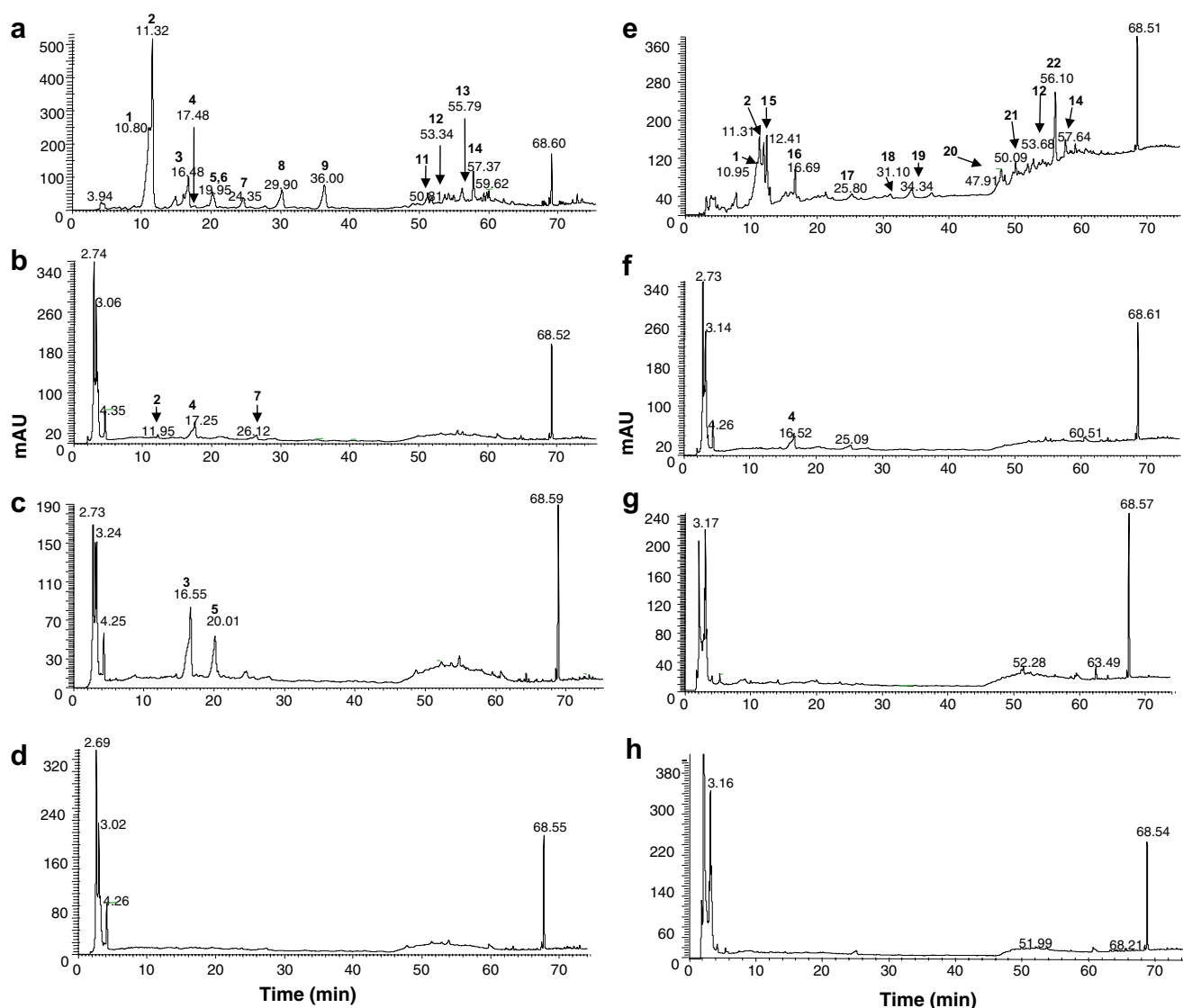


Fig. 2. HPLC–UV analysis of phenolic compounds of EAF and PF fractions before (panels a and e) and after incubation with *Corioliopsis rigida* (panels b and f), *Pycnoporus cinnabarinus* (panels c and g) and *Trametes versicolor* (panel d and h) (detection at $\lambda = 280$ nm). Peaks: (1) protocatechuic acid, (2) hydroxytyrosol, (3) *p*-tyrosol, (4) dihydroxymandelic acid, (5) unknown, (6) vanillic acid, (7) tetrahydroxymandelic acid, (8) *p*-coumaric acid, (9) hydroxytyrosol acetate, (11) oxidation product of the dialdehydic form of decarboxymethyl oleuropein aglycone, (12) oleuropein, (13) ligstroside, (14) luteolin, (15) hydroxytyrosol glucoside, (16) unknown, (17) oleoside, (18) oleuropein aglycon, (19) 3,4,5 trimethoxybenzoic acid, (20) luteolin-glucoside, (21) luteolin-rutinoside, (22) 6'- β -rhamnopyranosil-oleoside.

Moreover, another peak in the same HPLC–UV profile at t_R of 56.10 min was attributed to the 6'- β -rhamnopyranosyl-oleoside (22), a derivative of oleoside detected in olive fruit in the same study above cited (Cardoso et al., 2005). The identification was based on the peak arising at m/z 535 in the mass spectrum that corresponded to the deprotonated molecular ion and on the fragmentation pattern in agreement with the fragmentation profile of 6'- β -rhamnopyranosyl-oleoside described by Cardoso et al. (2005). It is important to underline that the presence of oleoside (17) and its 6- β -rhamnopyranosyl derivative (22) in DOR is here demonstrated for the first time.

The compounds present in the MF were substantially those occurring in that of PF, even if to a lesser extent,

except that 19 disappeared, whereas the new compounds 23 and 24 (Fig. 1) were detected in MF. In particular, at t_R 25.27 min in the HPLC–UV profile (Fig. 3a) there was a co-elution of two compounds. The first one corresponded to the oleoside (17) while the second one displayed on the mass spectrum a peak at m/z 481; also in this case it was not possible to define its structure from the study of the MS/MS spectrum. The peak at t_R 54.17 min in the HPLC–UV profile was attributed to 6'- β -glucopyranosyl-oleoside (24) (Fig. 1), another derivative of oleoside, also detected in olive fruit by Cardoso et al. (2005) and found in DOR for the first time in the present study. The identification of this compound was based on the molecular weight determination and on the MS/MS spectrum, in

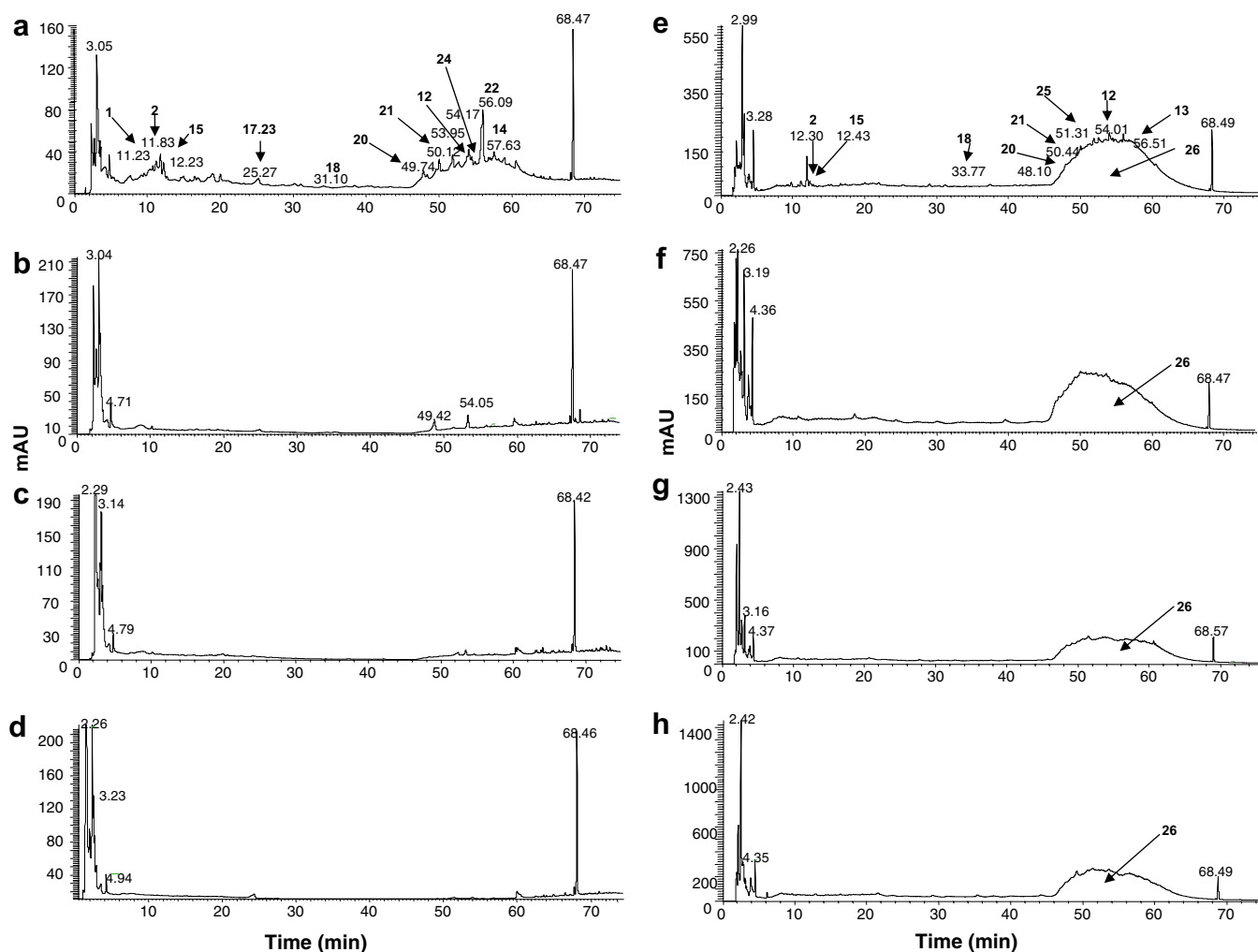


Fig. 3. HPLC–UV analysis of phenolic compounds of MF and WF fractions before (panels a and e) and after incubation with *Corioloropsis rigida* (panels b and f), *Pycnoporus cinnabarinus* (panels c and g) and *Trametes versicolor* (panels d and h) (detection at $\lambda = 280$ nm). Peaks: (1) protocatechuic acid, (2) hydroxytyrosol, (12) oleuropein, (13) ligstroside, (14) luteolin, (15) hydroxytyrosol glucoside, (18) oleuropein aglycon, (20) luteolin-glucoside, (21) luteolin-rutinoside, (23) unknown, (24) 6'- β -glucopyranosil-oleoside, (25) verbascoside, (26) polymerin.

agreement with the spectra already reported by Cardoso et al. (2005).

The HPLC–UV and HPLC–ESI/MS analyses of WF revealed the presence of a little amount of hydroxytyrosol (2), and secoiridoid and flavonoid glycosides previously described. In addition, the verbascoside (25) and, mainly, a high amount of a water soluble complex and unseparated mixture (26) were also detected at 280 nm as a very broad peak in the range between 46 and 68 min, co-eluting with the previously mentioned secoiridoids, and flavonoid glycosides as showed in Fig. 3e. We strongly hypothesize that this mixture is the so-called polymerin, a dark and complex metal polymeric organic mixture that was previously recovered from olive oil mill waste waters and proved to be composed of polysaccharides, melanin, proteins and metals (Capasso et al., 2004).

In order to support this hypothesis, an aliquot of polymerin was analysed by HPLC–UV in the same chromatographic

conditions used to separate WF and the HPLC profile of the polymerin standard (Fig. 4) showed to be very similar to that of WF (Fig. 3e). In addition, in this fraction proteins, melanin, carbohydrates and metals were detected (data not shown), these components being the same as revealed by polymerin (Capasso et al., 2004). These findings strongly indicate that the considered mixture is in fact polymerin and we report here for the first time its occurrence in DOR.

It is important to underline that the broad peak of polymerin was not only detected in the WF chromatogram (Fig. 3e) as a very intense one, but also in the chromatograms of EAF, PF and MF (Figs. 2a,e and 3a, respectively) in a very small amount, because it was extracted by the selected organic solvents in the soxhlet apparatus, by the presence in them of small quantities of water. Moreover, we point out that the peak mixtures detected at the front of the chromatograms of MF and WF (Fig. 3a and e)

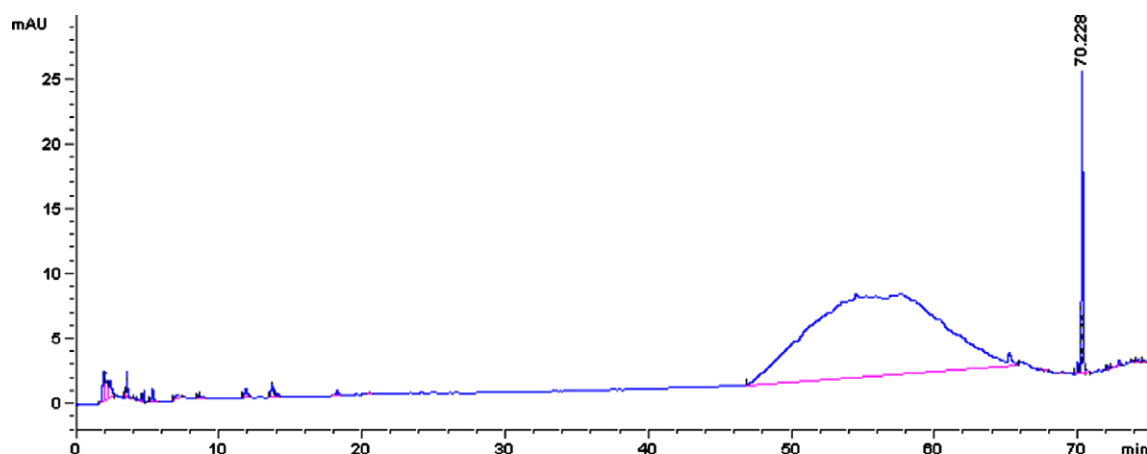


Fig. 4. HPLC–UV analysis of polymerin.

and of all other corresponding inoculated fractions (Fig. 3b–d and f–h), proved to be neither phenol substances or phytotoxic ones after their isolation (data not shown).

Therefore, DOR is substantially composed of phenols (distributed among the simple, acid and glycosilated phenols, melanin and lignin), carbohydrates (distributed between glycosylated phenols, polysaccharides, cellulose and hemicellulose), proteins and metals (K, Na, Ca, Mg and Fe) (Aranda et al., 2007). The higher total phenol content (16.0 g) detected in WF, compared with that of EAF, PF and MF (Table 1), should be attributed to polymerin, and, to the other hydrosoluble identified and unidentified phenol components occurring in this fraction. Instead, the total phenol content revealed by the other fractions (Table 1) should be mainly attributed to the monomeric and glycosilated phenols.

3.2. Qualitative and quantitative evaluation of phenols after fungi treatment

In our previous paper (Aranda et al., 2007), we found a relationship between phenol compounds occurring in DOR and the phytotoxicity of this material, as well as that some saprobe fungi were able to metabolize the above mentioned phenols with the consequent decrease of the phytotoxicity of DOR. In order to develop further knowledge on these aspects, three saprobe fungi *C. rigida*, *P. cinnabarinus* and *T. versicolor* were individually inoculated with EAF, PF, MF and WF. The control and inoculated fractions were then tested for their effects on the *L. sativum* seeds.

Coriopsis rigida, *P. cinnabarinus* and *T. versicolor* were able to decrease the total phenol content of EAF, PF, MF and WF from 70% up to 93% (Table 1). This abatement was confirmed by the disappearance of the peaks of phenols and phenol glycosides described above by HPLC analysis (see Figs. 2b–d, f–h and 3b–d, f–h). However, the residual occurrence of total phenol content, from 30% up to 7% (as complementary data of those above reported, 70% and 93%, respectively), can be very likely attributed in part, to: (i) the formation of polymeric polar phenols, originated from the spontaneous polymerization of the monomeric and glycosilated phenols activated by the selected fungi (D'Annibale et al., 2004); in a second part, to (ii) the unaffected polymerin, very likely co-eluted with the above described polymers, and, in the remaining part, to (iii) the incomplete transformation by the selected fungi of the previously mentioned monomeric and glycosilated phenols, that were not detected in the cited chromatograms in the experimental conditions used.

Furthermore, the residual phenol content (Table 1) detected in bioremediated WF could derive from the polymerization of the hydrosoluble phenols naturally occurring in the native WF and the unaffected polymerin.

3.3. Evaluation of the effects on *L. sativum* of native and bioremediated phenol compounds

Correspondingly, this latter finding should also explain the residual toxic effects on *L. sativum* by the bioconverted

Table 1

Phenol content in EAF, PF, MF and WF inoculated or not (control) by saprobe fungi (the result are expressed as g kg⁻¹)^a

Fraction	Control	<i>Coriopsis rigida</i>	<i>Pycnoporus cinnabarinus</i>	<i>Trametes versicolor</i>
EAF	7.1 c	1.0 ab	1.3 b	0.5 a
PF	5.9 c	0.7 a	1.3 b	0.5 a
MF	4.1 c	0.5 a	1.2 b	0.7 a
WF	16.0 b	3.2 a	4.5 a	3.5 a

^a Data are the means of four determinations. Row followed by the same letter are not significant different as determined by one-way ANOVA followed by the Tukey test ($p = 0.05$).

fractions in the range of the high concentrations used for the considered test (Tables 3–5). A phytotoxicity contribution of polymerin was excluded because no toxicity was detected in its assay on *L. sativum*, as shown by the corresponding data in the Table 5. Moreover, no significant decrease of phytotoxicity was revealed by the WF extracts inoculated with *P. cinnabarinus* and *T. versicolor* at higher concentrations (Table 5) in correspondence of the strong decrease of their phenol content with respect to the WF control (Table 1), suggesting that, very probably, there were, in addition to phenolics, other phytotoxic chemically different compounds extractables with water, which were not detected in our present study.

More specifically, the inoculation of EAF with the selected fungi provided a strong change of the HPLC–UV profile (Fig. 2b–d). The inoculation of this fraction with *C. rigida* and *P. cinnabarinus* showed that the majority of phenol compounds disappeared, revealing a residual presence of hydroxytyrosol (2), dihydroxy- (4) and tetrahydroxymandelic acid (7) after *C. rigida* treatment and *p*-tyrosol (3) and one unidentified compound after *P. cinnabarinus* inoculation (Fig. 2b and c). A complete disappearance of phenol components was obtained after inoculation with *T. versicolor* (Fig. 2d). However, all the inoculated fractions (Fig. 2b–d) revealed the occurrence of small amounts of polymerin, indicating that this latter remained unaffected after the inoculation with the selected fungi. Polymerin was also identified in the other control and bioconverted fractions PF, MF and WF (Figs. 2e–h and 3a–d,e–h), the highest content being detected in this latter fraction on account of its high water solubility (Capasso et al., 2004).

All the above results were confirmed by HPLC–ESI/MS analysis of the EAF inoculated with the three selected fungi (data not shown). In addition, elenolic acid (10), detected only in the TIC chromatogram of EAF control, disappeared in that of all the inoculated EAF extracts (data not shown). PF inoculated with *C. rigida* revealed in the corresponding HPLC–UV chromatogram (Fig. 2f) only the presence of a little amount of dihydroxymandelic acid (4). Treatment with the other two saprobe fungi led to a total disappearance of phenol components (Fig. 2g and h). The inoculation of MF and WF with the three selected fungi led to a total disappearance of the phenol components except for polymerin, this latter found in WF to very high extent with respect to its occurrence in the other fractions (Fig. 3b–d and f–h). Therefore, the inoculation of EAF, PF, MF and WF with *C. rigida*, *P. cinnabarinus* and *T. versicolor* indicated that these fungi were able to metabolize either the monomeric phenols or the glycosylated phenols, but not polymerin.

As shown in Tables 2–6, the inoculation of the three selected fungi with EAF, PF, MF and WF produced a highly significant increase in germinability in comparison with the control at low concentrations (25–12.5 g l⁻¹). It is noteworthy that an increase of germinability was also observed at 50 g l⁻¹ relatively to the inoculation of PF and at 100 g l⁻¹, relatively to the inoculation of MF.

These findings suggest that the phytotoxicity of the considered extracts decreases in correspondence of the diminution of the monomeric phenols. The inoculation with only *C. rigida* was able to increase the WF germinability at high concentrations (250–50 g l⁻¹). It is important to underline that WF treated with *C. rigida* was also able to stimulate

Table 2
Germination index (%) of *L. sativum* in presence of EAF inoculated or not (control) with saprobe fungi^a

Concentration (g l ⁻¹)	Control	<i>Corioloopsis rigida</i>	<i>Pycnoporus cinnabarinus</i>	<i>Trametes versicolor</i>
250	0.4 a	1.1 b	0.9 b	0.7 ab
200	0.4 a	1.4 c	1.0 bc	0.6 ab
100	2.0 a	3.4 b	1.9 a	3.2 b
50	3.3 a	6.5 b	4.3 a	5.3 b
25	15.0 a	54.8 b	58.2 b	65.2 b
12.5	69.4 ab	86.7 c	85.5 bc	87.4 c

^a Data are the means of four determinations. Row followed by the same letter are not significant different as determined by one-way ANOVA followed by the Tukey test ($p = 0.05$).

Table 3
Germination index (%) of *L. sativum* in presence of PF inoculated or not (control) with saprobe fungi^a

Concentration (g l ⁻¹)	Control	<i>Corioloopsis rigida</i>	<i>Pycnoporus cinnabarinus</i>	<i>Trametes versicolor</i>
250	0.5 a	2.8 b	1.1 a	3.0 b
200	0.7 a	3.7 c	1.5 a	2.6 b
100	2.4 a	28.3 c	2.5 a	8.1 b
50	8.5 a	83.9 c	7.0 a	45.7 b
25	55.0 a	90.8 c	63.9 ab	75.4 bc
12.5	88.8 a	94.2 a	79.2 a	100.0 a

^a Data are the means of four determinations. Row followed by the same letter are not significant different as determined by one way ANOVA followed by the Tukey test ($p = 0.05$).

Table 4
Germination index (%) of *L. sativum* in presence of MF inoculated or not (control) with saprobe fungi^a

Concentration (g l ⁻¹)	Control	<i>Corioloopsis rigida</i>	<i>Pycnoporus cinnabarinus</i>	<i>Trametes versicolor</i>
250	1.2 a	4.2 b	2.6 ab	4.3 b
200	3.3 a	7.3 b	3.7 a	7.5 b
100	30.5 a	68.7 b	33.2 a	76.8 b
50	60.7 a	87.8 b	94.7 b	97.1 b
25	87.7 a	116.9 a	96.1 a	113.3 a
12.5	90.6 a	104.5 a	105.8 a	105.2 a

^a Data are the means of four determinations. Row followed by the same letter are not significant different as determined by one-way ANOVA followed by the Tukey test ($p = 0.05$).

Table 5
Germination index (%) of *L. sativum* in presence of WF inoculated or not (control) with saprobe fungi^a

Concentration (g l ⁻¹)	Control	<i>Corioloopsis rigida</i>	<i>Pycnoporus cinnabarinus</i>	<i>Trametes versicolor</i>	Polymerin
250	10.4 a	36.7 b	3.5 a	9.0 a	107.0 c
200	27.7 b	67.8 c	7.4 a	11.4 a	100.5 d
100	64.8 b	107.2 c	27.6 a	23.2 a	110.4 c
50	74.5 ab	109.1 c	52.9 a	77.8 b	160.3 d
25	78.3 a	131.3 c	104.3 ab	113.1 ab	140.1 c
12.5	95.7 a	126.2 b	101.3 a	106.4 ab	145.7 c

^a Data are the means of four determinations. Row followed by the same letter are not significant different as determined by one-way ANOVA followed by the Tukey test ($p = 0.05$).

Table 6
Laccase activity produced by different saprophytic fungi grown on EAF, PF, MF and WF (the results are expressed as IU g⁻¹)^a

Fraction	<i>Corioloopsis rigida</i>	<i>Pycnoporus cinnabarinus</i>	<i>Trametes versicolor</i>
EAF	29 a	33 a	35 a
PF	48 b	63 b	10 a
MF	37 a	401 b	21 a
WF	834 c	439 a	517 b

^a Data are the means of four determinations. Row followed by the same letter are not significant different as determined by one-way ANOVA followed by the Tukey test ($p = 0.05$).

the growth of the *L. sativum* seeds at concentrations ranging between 25 and 12.5 g l⁻¹ (Table 5).

Corioloopsis rigida and *T. versicolor* induced a strong decrease of the phenolic content of EAF, PF and both fungi also caused decrease of their phytotoxicity. However, our data indicated that the decrease of toxicity was not proportional to the phenolic removal in the DOR fractions. In fact all of the fungi tested were able to decrease the phenol content of WF but only *C. rigida* induced reduction of its phytotoxicity (Table 5). Similar results have been reported by Martirani et al. (1996) and Tsioulpas et al. (2002). It seems that there was a relationship between removal in the phenol concentration with phytotoxicity decrease in EAF, PF and MF but not in the WF fraction. These findings confirmed that phenols could be the main determinants of DOR germinability suppression in *L. sativum*, but not the only compounds (Capasso et al., 1992; Greco et al., 2006). The marked increase of germinability observed in saprobe fungi treated samples shows that DOR phytotoxicity can be significantly reduced by biological treatment able to remove DOR phenol components.

The results obtained in the present study showed that EAF, PF and MF exerted an almost complete suppression of the germinability at concentration of 250 and 200 g l⁻¹ (Table 2–5). This evident inhibition of germinability was diminished in WF (Table 5). WF had the lowest phytotoxicity among all the tested fractions. Each fraction simply reduced the concentration of all phytotoxic constituents. The olive residue is known to contain high concentration of phenol compounds (Lessage-Meessen et al., 2001; Aggelis et al., 2003). This concentration of phenol compounds would be the responsible for the high toxicity and antimicrobial activity of this waste (Pérez et al., 1992; Casa et al., 2003). In particular, the low molecular weight aromatic fraction of DOR has also been shown to be particularly phytotoxic (Fiorentino et al., 2003) and the occurrence of synergistic inhibitory effects between phenols has been shown (Capasso et al., 1992; Della Greca et al., 2001). In addition low molecular weight phenols like *trans*-cinnamic acid and *p*-coumaric acid inhibit plant growth regardless of their concentration (Li et al., 1993). In general, the phytotoxic effects of phenol compounds

have been reported to mainly involve alterations of water uptake and of the metabolism of phytohormones such as auxins and abscisic acid (Li et al., 1993; Aliotta et al., 2000).

The white rot fungi enzymatic system is reported to be activated in the presence of compounds such as polyphenols and aromatic amines in DOR, the biodegradation of which results in DOR detoxification (Martirani et al., 1996). The saprobe fungi remove these toxic compounds by synthesizing and excreting laccases into the growth media containing phenolic substances. Laccases are remarkably non-specific extracellular multicopper enzymes that use molecular oxygen as an electron acceptor and that can oxidize polyphenols as well as other compounds (Davis and Burns, 1990; Robles et al., 2000). In our study, we detected laccase activities in the DOR fraction degraded by the saprobe fungi (Table 6), as it has been observed in other studies involving phenolic-rich substrates (Tomati et al., 1991; Linares et al., 2003). This enzymatic activity implies that this enzyme can be produced during the primary metabolic growth of saprobe fungi and not only in other stages of the fungi (Tour et al., 1995). In fact, it would suggest, as mentioned by Tomati et al. (1991) and Tsioulpas et al. (2002) that the white-rot fungus benefits from the laccase reaction, which is involved in a detoxification procedure, preparing the environment for microbial growth.

The activity of laccase from the selected fungi proved to be much stronger on WF compared with the other fractions and at variable extent (Table 6), as expected by measuring the strong decrease of the phenol content of the inoculated WF extracts with respect to the WF control (Table 1). However, this enzymatic activity is not closely associated with the decrease of phytotoxicity as it has been described by Aranda et al. (2007). Other mechanisms a part of the laccase activity may be involved in this process (Tsioulpas et al., 2002; D'Annibale et al., 2004).

4. Conclusions

Three known phenolic substances, oleoside (17), 6'- β -rhamnopyranosyl-oleoside (22), 6'- β -glucopyranosyl-oleoside (24), and polymerin (26), the organic polymeric component, are reported here for the first time occurring in DOR. The monomeric and glycosilated phenols are the main phytotoxicity factors of EAF, PF, MF and WF, whereas polymerin, the main component of WF, showed no phytotoxicity. So, the DOR phytotoxicity confirms to be derived mainly from the monomeric (simple and acidic) phenols and, to a lesser extent, from phenol glycosides.

The selected saprobe fungi are able to transform the phytotoxic polyphenols into the corresponding lesser toxic polymeric ones, which, very likely, are co-eluted with polymerin, as shown in Figs. 2a–h and 3a–h. The residual phytotoxic activity found in the bioremediated EAF, PF, MF and WF, particularly at high concentrations, (Tables 3–5), could be attributed to the incomplete transformation of the above mentioned phenols, as proven by the residual

phenol content detected in the considered inoculated fractions (Table 1). Furthermore, the residual phytotoxic activity could be also attributed to new compounds formed during the industrial dried of DOR. Future investigations carried out on alperujo could better clarify this aspect. It is noteworthy that *C. rigida* is the most active fungus on WF, because of its ability to induce a stimulating effect on the growth of *L. sativum* in the range of concentrations of 25–12.5 g l⁻¹, very likely due to the presence of polymerin (Table 5).

As regards the perspectives of possible application of this study, the method here described for the fractionation of DOR could be utilized for the recovery from DOR of all the monomeric and glycosilated polyphenols (occurring mainly in EAF, MF and PF) separately from WF, mainly containing the humic acid-like polymerin (Capasso et al., 2004). The first products are characterized by well known antioxidant and pharmacological effects (Ranalli et al., 2003; Manna et al., 2004), whereas polymerin could be utilized as possible biofertilizer, particularly after the treatment with *C. rigida*. The residual insoluble DOR could be incinerated or used as component for compost. So, this latter process and the previously described fractionation method could represent a potential procedure for the total recycling of DOR, reducing drastically the environmental impact of this biomaterial.

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