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# Laccase-catalyzed oxidation of ferulic acid and ethyl ferulate in aqueous medium: a green procedure for the synthesis of new compounds

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## ABSTRACT

The enzymatic oxidation of ferulic acid (FA) and ethyl ferulate (EF) with *Myceliophthora thermophila* laccase, as biocatalyst, was performed in aqueous medium using an eco-friendly procedure to synthesize new active molecules. First, the commercial laccase was ultrafiltrated allowing for the elimination of phenolic contaminants and increasing the specific activity by a factor of 2. Then, kinetic parameters of this laccase were determined for both substrates (FA, EF), indicating a higher substrate affinity for ethyl ferulate. Additionally, enzymatic oxidation led to the synthesis of a FA-major product, exhibiting a molecular mass of 386 g/mol and a EF-major product with a molecular mass of 442 g/mol. Structural analyses by mass spectrometry allowed the identification of dimeric derivatives. The optical properties of the oxidation products showed the increase of red and yellow colours, with FA-products compared to EF-products. Additionally, enzymatic oxidation led to a decrease of antioxidant and cytotoxic activities compared to initial substrates. Consequently, this enzymatic procedure in aqueous medium could provide new compounds presenting optical, antioxidant and cytotoxic interest.

*Keywords:* Laccase; Enzymatic oxidation; Ferulic acid; Ethyl ferulate; dye; antioxidant; cytotoxicity.

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## 1. Introduction

The enzymatic oxidation of organic compounds, in order to produce new classes of molecules, has been widely investigated with a strong environmental concern (Burton, 1994). Oxidative enzymes can be subdivided into polyphenol oxidases (PPOs) using molecular oxygen as a co-substrate and peroxidases (POD) using hydrogen peroxide ($H_2O_2$) (Duran & Esposito, 2000). There has been increased interest concerning enzymatic oxidation, due to its potential for the synthesis of bioactive molecules from phenolic compounds, which exhibit novel structures, such as dimers and interesting properties, such as antioxidant activities (Adelakun, Kudanga, Parker, Green, le Roes-Hill & Burton, 2012).

Polyphenol oxidases (PPOs) are multi-copper enzymes which catalyze phenol oxidation to quinines, which are responsible for the brown pigments in wounded tissues of vegetables, with the concomitant reduction of oxygen to water (Nicolas, Richardforget, Goupy, Amiot & Aubert, 1994). These enzymes can be subdivided into tyrosinases and laccases (Walker & Ferrar, 1998). Recently, there have been a number of attempts to enzymatically modify phenolic molecules, to improve their properties, such as antioxidant activity (Adelakun et al., 2012; Yu, Han & Lou, 2007) and colour (Mustafa, Muniglia, Rovel & Girardin, 2005; Ridgway, Oreilly, West, Tucker & Wiseman, 1996). Laccases (p-diphenol oxidase, E.C. 1.10.3.2) are among the enzymes that are currently being investigated. They belong to a small group named the blue copper enzymes, containing a minimum of four copper atoms directly

involved per active protein (Claus, 2004; Solomon, Sundaram & Machonkin, 1996). They are capable of catalyzing the one-electron oxidation of phenols to produce phenoxy radicals as the primary oxidation products (semi-quinone) and concomitantly reduce molecular oxygen to water. The phenoxy radicals which are very reactive species and powerful electrophiles, which can undergo a number of non-enzymatic reactions including covalent coupling to form dimers, oligomers and polymers through C–C, C–O and C–N bonds (Claus, 2004).

Phenolic compounds, such as ferulic acid and ethyl ferulate, have already been oxidized by oxidative enzymes, such as laccases or peroxidases in organic medium and several dihydrodimers have been described. In fact, enzymatic oxidation of ferulic acid led to the formation of semi-quinone feruloyl radicals and then to the formation of oligomers (dimers, trimers) (Adelakun et al., 2012; Carunchio, Crescenzi, Girelli, Messina & Tarola, 2001; Mustafa et al., 2005). This oxidation can also be catalyzed by peroxidases using hydrogen peroxide ($H_2O_2$) (Derat & Shaik, 2006; El Agha, Makris & Kefalas, 2008; Ou, Kong, Zhang & Niwa, 2003; Yu et al., 2007) in a hydro-organic biphasic system. Additionally, previous studies have reported enzymatic oxidation of ferulic acid esters as model feruloyl esters *in vitro,* such as methyl ferulate (Wallace & Fry, 1995) or ethyl ferulate (Bunzel, Heuermann, Kim & Ralph, 2008; Ralph, Conesa & Williamson, 1998; Tufegdzic, Bogdanovic, Maksimovic & Vucinic, 2005) to determine the chemical structures of the products. These oxidative reactions leading to the formation of ferulate dimers was catalyzed by peroxidases using hydrogen peroxide ($H_2O_2$). However, no studies have been reported concerning enzymatic oxidation in an aqueous medium.

In the present study, a green procedure for the synthesis of nature identical stable compounds by laccase-catalyzed oxidation of ferulic acid (FA) and the ethyl ester of ferulic acid (ethyl ferulate, EF) in aqueous medium was investigated. Ferulic acid (FA), a widespread

phenolic acid that can be considered as a model substrate for laccase enzyme, and ethyl ferulate (EF), ethyl ester of ferulic acid, were chosen to compare the reactivity of phenolic substrates in the presence, or not, of free carboxylic acid groups. Laccase from *Myceliophthora thermophila* sp. was chosen due to its high reactivity in aqueous medium, at room temperature and neutral pH (Mustafa et al., 2005). Additionally, this work aimed to determine enzymatic oxidation parameters for both ferulic acid and its ethyl ester. Finally, colour, antioxidant and cytotoxic properties will be evaluated for the laccase-catalyzed oxidation products compared to the initial substrates (FA or EF).

## 2. Materials and methods

### *2.1. Chemicals and enzyme*

Ferulic acid and ethyl ferulate were obtained from Fluka (France) with purity ≥99%; trolox was obtained from Fluka (Germany); syringaldazine was obtained from Ega-chemie with purity of ≥ 99%. Methanol and acetonitrile (HPLC grade) were purchased from Carlo Erba (Milwaukee, WI, USA); trifluoroacetic acid (TFA) (98%) and other chemicals, of the highest quality available, were obtained from Sigma-Aldrich (France).

An industrial laccase, named Suberase® (Novo Nordisk A/S, Bagsvaerdt, Denmark), was purchased commercially from the Society Novozymes in liquid form. It was stored between 0 and 10°C, for suitable conservation. The Suberase® is a fungal laccase from *Myceliophthora thermophila* sp., which is a member of the polyphenol oxidases family, it was obtained by submerged fermentation of a genetically modified *Aspergillus oryzae*. The enzymatic preparation was supplied as a brown liquid with a density at approximately 1.15 $g.ml^{-1}$. It was completely miscible with water.

### *2.2. Laccase characterization*

94 To remove inactive peptides or proteins and to avoid the interaction between the
95 phenolic interesting substrate and contaminated phenols which were initially present with the
96 industrial laccase, partial purification of Suberase® (UF-laccase) was carried out at 4°C using
97 an ultrafiltration device Amicon-8200 cell (20 ml) equipped with a membrane of cellulose
98 acetate (cut off: 10 kDa) (SARTORUIS - 14539-63-G) under nitrogen gas to avoid oxidation.
99 The membrane was cleaned in an acetone: distilled water mixture (1:1) prior to
100 unltrafiltration. Additionally, the industrial laccase was diluted with phosphate buffer at pH
101 7.5.

102 The laccase purity was checked by sodium dodecyl sulphate-polyacrylamide gel
103 electrophoresis (SDS–PAGE) on a 16% (w/v) polyacrylamide gel (Laemmli, 1970). SDS-
104 PAGE was carried out using a mini-protein electrophoresis cell unit (Bio-Rad, USA). The
105 laccase sample dissolved in sample buffer (10% SDS, 7.5% b-mercaptoethanol, 0.25 M Tris-
106 HCl pH 6.8, 50% water, 50% glycerol and 0.1% bromophenol blue), then heated at 95°C for
107 (5-10 min). Electrophoresis was performed at constant current 30 V for 60 min and then 180
108 V for 45 min. The proteins sub-unit bands were stained with (6%) Coomassie Brilliant Blue
109 R-250 in acetic acid (10%), and detained in the same solvent system (but without dye). The
110 molecular weight of the enzyme protein was estimated using a Mark12™ Unstained Protein
111 Standard of molecular weights between 6 - 200 kDa (Bio-Rad, USA).

112 Protein concentration in Suberase® was determined by Bicinchoninic acid (BCA)
113 spectrophotometric method (Slocum & Deupree, 1991). Bovine serum albumin was used as a
114 standard in phosphate buffer (50 mM, pH 7.5).

115 The laccase activity at different pH (from 5 to 8) and different temperatures (from 20 to
116 50°C) was determined spectrophotometrically by measuring the increase of the absorbance at
117 525 nm caused by the oxidation of syringaldazine (2 µM, pH 7.5) in phosphate buffer (50

mM) using a spectrophotometer (Shimadzu UV- 1605). One unit of the enzyme (UI) was defined as the amount of syringaldazine (in µmole) oxidized per minute and per µg of enzymatic protein using an extinction coefficient $\varepsilon_{525}$ 65 $mM^{-1}.cm^{-1}$ (Mustafa et al., 2005).

To evaluate the purification efficacy, laccase decolorization was carried out according to the method by Smith & Montgomery, (1985) by measuring the absorbance at 420 nm toward raw laccase without purification (as a control). This wavelength (420 nm) was chosen because it gave a maximal absorbance corresponding to the UV/Visible spectra of the coloured initial enzymatic solution. The decolorization percent was calculated using the following equation (1):

$$\%\text{decolorization} = [Abs_{420}\,(\text{raw laccase}) - Abs_{420}\,(\text{UF-laccase}) / Abs_{420}\,(\text{raw laccase})] \times 100 \quad (1)$$

*2.3. Enzymatic oxidation of phenols*

The oxidation of the phenols (5 mM, final concentration) in a magnetic stirred batch reactor was carried out at 30°C in phosphate buffer (50 mM, pH 7.5). The reaction mixture was composed of 5 ml of methanol solution, 50 mM FA or EF and 45 ml of phosphate buffer (50 mM, pH 7.5). The reaction was started by adding 0.13 ml of UF-laccase (13.5 U/ml). For the control, laccase was not added. The reaction kinetics were monitored at various time intervals, by withdrawing samples (0.1 ml) from the reaction mixture. The enzymatic reaction was stopped by the addition of methanol (0.9 ml) containing 0.03% (v/v) of TFA (Lacki & Duvnjak, 1996).

*2.4. Enzyme kinetics and substrate specificity*

The $K_m$ value and maximum velocity ($V_{max}$) (Michaelis-Menten kinetic parameters) of UF-laccase were determined by a Lineweaver-Burk plot. For that, different concentrations

from 1 to 5 mM of the two substrates (ferulic acid or ethyl ferulate) were oxidized by the same quantity of enzyme at 30°C in phosphate buffer (50 mM, pH 7.5). Substrate specificity ($V_{max}/K_m$) which determine enzymatic affinity toward phenolic substrate, was calculated by using the data obtained from the above plot (Lineweaver & Burk, 1934).

*2.5. High performance liquid chromatography (HPLC)*

To monitor the oxidation of the substrate, reaction mixtures were analyzed by high performance liquid chromatography (HPLC), using a Shimadzu Class-VP HPLC system with a computer controlled system containing upgraded Class-VP 6.1 software. Separations were carried out on a reversed phase column LiChroCART RP-18 (Merck, 25 cm x 0.4 cm, particle size 5 µm). The detection was conducted between 190 and 700 nm on a multichannel photodiode- array detector (SPD-M10A VP). The elution was performed using a gradient of solvent A: water/ TFA (100:0.03; v/v) and solvent B: acetonitrile /water/ TFA (80:20:0.03; v/v/v) at a flow rate of 0.7 ml/min.

For ferulic acid**,** the steps of the gradient were as follow: linear gradient from 5% to 40% of B for 15 min, from 40% to 60% of B for 5 min and from 60% to 80% of B for 15 min. For ethyl ferulate, the steps of the gradient were as follow: linear gradient from 5% to 80% B for 10 min, constant at 80% of B for 10 min and from 80% to 100% for 5 min. Each analysis was performed in triplicate.

*2.6. Liquid chromatography–mass spectrometry (LC–MS)*

The mass spectra were obtained with a HPLC-MS system, which comprised of a binary solvent delivery pump and a linear ion trap mass spectrometer (LTQ-MS, Thermo Fisher Scientific, San Jose, CA, USA). The LTQ was equipped with an atmospheric pressure ionization interface operating in APCI positive mode (Atmospheric Pressure Chemical

Ionisation). Data were processed using Xcalibur software (version 2.0). The operational parameters of the mass spectrometer were as shown below. The spray voltage was 6.0 kV. The temperatures of the APCI vaporizer and of the heated capillary were set to 400°C and 225°C, respectively. The flow rates of sheath gas, auxiliary gas, and sweep gas were set (in arbitrary units/min) to 48, 5, and 5, respectively. Capillary voltage was 1 kV, tube lens was 50 V, split lens was 70 V, and the front lens was - 6.75 V. All parameters were optimized by infusing a standard solution of ferulic acid at 0.1 g/l in mobile phase [A=water + TFA (0.03%) / B=phase A (20%) + acetonitrile (80%) – (1:4)] at a flow rate of 5 $\mu l.min^{-1}$. In addition to a full scan between 50 m/z and 1000 m/z, the products were monitored through specific $MS^n$ scans ($MS_2$, $MS_3$) in order to obtain structural information.

*2.7. Recovery of laccase-catalyzed oxidation products*

After 3 h of the phenol oxidation reaction, to ensure a complete oxidation of substrate and to avoid the increasing the degree of polymerization, the products were recovered by a rotary evaporator under reduced pressure, then freeze-dried for 48 h. After that, the recovered products were stored in a desiccator until use.

*2.8. Colour measurement of laccase-catalyzed oxidation products*

The recovered oxidation products colour was described according to the CIE L*a*b* colour system, as a three-dimensional space based on opposite colours, where L*, a* and b* were the coordinates of the colour in the cylindrical colour space, based on the theory that colour was perceived by black-white (L* = lightness), red-green (a*) and yellow-blue (b*) sensations (Lathasree, Rao, SivaSankar, Sadasivam & Rengaraj, 2004). These parameters a*, b* and L* were measured directly in triplicate using a colorimeter (model 200) (Montreuil, France). The other optical parameter used was the Chroma (C) = $(a^{*2} + b^{*2})^{1/2}$, that

represented the perpendicular distance from lightness axis. This parameter was indicative of intensity.

*2.9. Antioxidant properties of laccase-catalyzed oxidation products by ABTS assay*

The ABTS radical scavenging activities of ferulic acid, ethyl ferulate and laccase-catalyzed oxidation products were determined according to the method described by Re et al. (Re, Pellegrini, Proteggente, Pannala, Yang & Rice-Evans, 1999). The $ABTS^{\cdot+}$ radical cation was produced by the reaction between 7 mM ABTS and 2.45 mM potassium persulfate in $H_2O$ and allowing the mixture to stand in the dark, at room temperature, for 12–16h before use. For this study, the $ABTS^{\cdot+}$ solution was diluted with ethanol at 30°C, in order to obtain an absorbance of 0.700 ± 0.025 at 734 nm. Then, 10 µl of each sample prepared in ethanol at different concentrations were mixed with 1 ml of the diluted $ABTS^{\cdot+}$ solution and then the absorbance of $ABTS^{\cdot+}$ was measured at 734 nm and at 30°C exactly 6 min after the initial mixing. Appropriate solvent blanks were run in each assay. The extent of decolorization is calculated as the percentage reduction of ABTS absorbance by following equation (2):

$$ABTS^{\cdot+} \text{ radical scavenging activity } (\%) = (1 - Abs_{sample}/Abs_{control}) \times 100 \quad (2)$$

$ABTS^{\cdot+}$ radical scavenging activity was expressed as the half-maximal inhibition concentrations ($IC_{50}$) and Trolox Equivalent Antioxidant Capacity (TEAC) values. The $IC_{50}$ value, which expressed the antioxidant concentration required to reduce the radicals by 50%, was calculated for each compound by linear regression analysis (Gulcin, 2010). The TEAC value was defined as the concentration of standard trolox, a water-soluble vitamin E analogue that exhibited the same antioxidant capacity as a 1 µg/ml solution concentration of the antioxidant compound under investigation (Maisuthisakul, Pongsawatmanit & Gordon, 2007).

All analyses were carried out in triplicate and results represented the mean values with standard deviation.

### *2.10. Cytotoxic properties of laccase-catalyzed oxidation products*

#### *2.10.1. Cells and cell culture*

Human umbilical vein endothelial cells (HUVEC) were obtained from the European Collection of Animal Cell Cultures (ECACC, UK) and cultivated in Endothelial Basal Medium (Sigma-Aldrich, France). The medium was prepared by the addition of growth supplement (3%) (Sigma-Aldrich, France). HUVEC cells (Sigma-Aldrich, France) were cultivated between passages 1 and 7. The cells were usually split when 80% confluence was reached (5–7 days). They were first rinsed with Dulbecco's phosphate-buffered saline without calcium (D-PBS) and then trypsinised with a solution containing 0.25% trypsin and 1 mM EDTA (GIBCO, USA) and counted by Thoma cell under an optical microscope. For maintenance of the cell line, the cells were seeded at $2.10^4$ cells/cm$^2$ in flasks. All experiments of cytotoxicity evaluation were performed between the passage 3 and 6.

#### *2.10.2. Cell viability evaluation by Neutral red uptake (NRU) assay*

To study the cytotoxic activity of ferulic and ethyl ferulate and their oxidation products, 96-well microplates were filled with 190 µl of diluted HUVEC cells at $5.10^4$ cells/well. After 24 h of incubation at 37°C, under 5% $CO_2$ atmosphere, the cells were exposed to 10 µl of the phenolic compounds solubilised in DMSO (final concentration of DMSO was 0.25%) at various concentrations and incubated for 48 h at 37°C, under 5% $CO_2$ atmosphere. Two controls were used in two columns of the microplate which contain 190 µl of diluted cells: one with 10 µl of D-PBS and another with 10 µl of DMSO solution (final concentration of DMSO was 0.25%).

The NRU (neutral red uptake) assay is a cell viability method, based on the ability of viable cells to bind a neutral red dye within the membranes of intracellular lysosomes (Borenfreund, 1992). After 48 h of incubation, the culture medium (200 µl) was removed from each well of the microplate. The attached cells were rinsed with equal volume of D-PBS at pH 7.2. Then, 200 µl of neutral red solution (50 µg/ml) freshly prepared in the culture medium was added to each well. The microplate was then incubated with the cells and the neutral red solution for 3 h at 37°C. After incubation, the neutral red solution was eliminated and the attached cells were rinsed with D-PBS. The attached cells were solubilised in 200 µl of an ethanol/acetic acid/water solution (50%, 1%, 49%, v/v/v) to release the neutral red remained within the lysosomal or cytoplasmic compartments. The plates were then shaken at room temperature for 10 min. The absorbance of this final solution was measured at 540 nm using a microplate reader (multiscan GO W382TA, Thermo Scientific MIB, France). The relative cell viability was calculated according to the following equation (3):

$$\text{Relative cell viability (\%)} = ((1- (Abs_{\text{treated cell}}/Abs_{\text{control}})) \times 100 \quad (3)$$

With $Abs_{\text{treated cells}}$ and $Abs_{\text{control}}$ being the absorbance values at 540 nm of sample with treated cells and control, respectively. Each test was carried out in six copies, and each experiment was repeated triplicate. The results were expressed as $IC_{50}$ mean values with the standard deviations. $IC_{50}$ was defined as the concentration of a molecule leading to 50% of cell mortality. The cytotoxicity was evaluated based on cell viability relative to controls as proposed by Kong (Kong, Jiang, Zhou & Fu, 2009).

## 3. Results and discussion

### *3.1. Partial purification of laccase*

Laccase was partially purified by ultrafiltration, with a yield 91% of total activity. The results demonstrated that this method could eliminate almost 2.5 fold of total proteins and the specific activity of the ultrafiltrated laccase (UF-laccase) increased by 2.1 fold, compared to raw laccase (Table 1). Additionally, the colour of the enzyme decreased by 90% after the ultrafiltration, due to the elimination of the phenolic molecules present in the raw laccase.

The optimal activities were determined to be pH 7.5 and a temperature of 30°C, using syringaldazine as a specific substrate (data not shown). The protein characterization showed that the UF-laccase presented a major protein band on the SDS-PAGE, with an apparent molecular mass close to 85 kDa as presented in Fig. 1, which is consistent with the molecular mass (82 kDa) of *Myceliophthora thermophila* laccase (Mustafa et al., 2005).

### *3.2. Kinetics of oxidation catalyzed by UF-laccase*

Kinetics showed differences between the laccase-catalyzed oxidation of FA and EF as presented in Fig. 2A. The oxidative rate of EF was higher than that of FA. Thus, the complete oxidation of FA was achieved after 150 min, while the complete consumption of EF was after 120 min. Furthermore, it was verified that without laccase, FA or EF were not chemically oxidized by auto-oxidation (data not shown). After 3 h of the oxidation reaction, the laccase was inactive, probably due to the interaction between the oxidation products and the active site of enzyme (Whitaker, 1994; Whitaker, 1995). In fact, the enzyme inactivation is due to the reaction between the intermediate products (free radicals from quinones or semi-quinones) and histidine groups of the enzyme active site linked to the binuclear copper. A previous study confirmed that the presence of macromolecules in the reaction medium, such as chitosan, quickly capture the oxidation products, which could protect the enzyme from inhibition during the oxidation reaction (Aljawish et al., 2012).

The initial oxidation rate of FA and EF obeys Michaelis-Menten kinetics. The results demonstrated that the Michaelis constant ($K_m$) of EF (0.95 ± 0.03 mM) was lower than that of FA (1.64 ± 0.01 mM) and the substrate specificity ($V_{max}/K_m$) of EF (0.072 ± 0.001) was 2 fold higher than that of FA (0.038 ± 0.002) while the maximum rates ($V_{max}$) of FA and EF were similar (0.062 ± 0.003 and 0.068 ± 0.002 mM/min, respectively). As the Km value reflects the affinity between the enzyme and substrate, the lower $K_m$ value of EF indicated a higher affinity for this substrate. Finally, the catalytic efficiency is higher for EF substrate than for FA due to a better $K_m$.

### *3.3. Analyses HPLC/LC-MS of oxidation products*

HPLC analyses of the reaction mixture, after 90 min of the oxidation reaction of ferulic acid (FA) and ethyl ferulate (EF) by UF-laccase, revealed the presence of several peaks corresponding to various products as shown in Fig. 2B. The identification of laccase-catalyzed oxidation products was focused only on the major products for FA and EF, because the other products are present in low concentrations and thus can be neglected compared to the major products. According to the retention time (Rt), these major products were called P (FA) corresponding to Rt of 26 min and P (EF) corresponding to Rt of 23 min.

The UV spectra of the initial substrates, and their major products obtained by HPLC are shown in Fig. 2B. After enzymatic oxidation, the absorbance at 322 nm decreased and increased at 230 nm, due to the presence of oxidation products (quinones). These changes in the spectra were explained by ascribing the violet-shift of the absorption range and the intensity decrease of the distinguishable long-wave bands to the dissociation of carboxylic groups belonging to a conjugated system (Carunchio et al., 2001).

The LC–PDA-MS analyses, in APCI positive ion mode, showed that the major FA-oxidation product observed in UV led to mass spectrometry peaks at m/z= 387 $[M+H]^+$, as shown in Fig. 3A. A fragmentation spectrum by $MS^2$ of the ion 387 allowed for characterisation of the structure (Fig. 3B). Fragments indicating losses of $H_2O$ (m/z=369) and $CO_2$ (m/z=343) from the parent ion were detected. Subsequent removal of $H_2O$ from the single dehydrated parent ion (m/z=369) could account for the fragment with a m/z=351, corresponding to double dehydrated parent ion. The ion with a m/z=327 was indicative of a stepwise loss of a methyl radical and a hydrogen radical from a decarboxylated dimer (m/z=343), leading to $[M+H-CO_2-CH_4]^+$. The formation of the ion at m/z=281, after the fragmentation of the ion at m/z= 343 by $MS^3$, could be attributed to decarboxylation of the decarboxylated dimer (m/z=343) with concomitant removal of $H_2O$ (Fig. 3-C). Moreover, further neutral loss of CH could be responsible for the ion at m/z=268. In addition, ion m/z=297, seen on the $MS^3$ fragmentation spectrum (m/z=387,343) could be explained by concomitant loss of CO and $H_2O$ from the decarboxylated ion at m/z=343 (Fig. 3-C). These results allowed for identification of the molecular mass of the major FA-oxidation product, with a molecular mass of 386 g/mol, corresponding to the molecular ions of the dimeric species of ferulic acid.

These observations were in accordance with those already described concerning FA-oxidation by *Pyricularia orysae* laccase (pH 6 at 37°C), in acetate buffer with 45% ethanol (Carunchio et al., 2001) and peroxydase from onion solid wastes (pH 4 at 25°C) (El Agha et al., 2008). Indeed, both studies described the formation of three dimers as major oxidation products and the corresponding molecular masses were always (whatever the bonding type) the same as the one found in the present study (M=386 g/mol). Daughter ions described above are extremely close to those seen in case of the dimer reported in the previous study (El Agha et al., 2008).

Moreover, The LC–PDA-MS analyses, in APCI positive ion mode, showed that the major EF-oxidation product observed in UV led to mass spectrometry peaks at m/z 443 as shown in Fig. 3-D. This observation was consistent with a dimer structure and was in agreement with data previously described concerning the oxidation of ethyl ferulate by peroxidase/$H_2O_2$ at pH 4 (Bunzel et al., 2008; Ralph et al., 1998).

This structure was confirmed by the $MS^2$ fragmentation spectrum of the ion 443 (Fig. 3E), which showed the most intense daughter ion with m/z= 397, indicating a stepwise loss of a $H_2O$ and CO from the parent ion (m/z=443), leading to the ion $[M+H-CO-H_2O]^+$. Subsequent removal of CO could account for the fragment with m/z=369, corresponding to the ion $[M+H-2CO-H_2O]^+$. A fragment indicating subsequent loss of $H_2O$ (m/z=351) was detected, corresponding to the ion $[M+H-2CO-2H_2O]^+$. Moreover, further neutral loss of two $CH_4$ could be explained by two concomitant removals of methyl radical and hydrogen radical leading to ion $[M+H-2CO-2H_2O-2CH_4]^+$ with m/z=319. The ion with a m/z=275 was indicative of subsequent decarboxylation ($CO_2$) and ion m/z=247 was probably obtained after further neutral loss of CO corresponding to structure $[M+H-3CO-2H_2O-2CH_4-CO_2]^+$. Finally the ion m/z=221 was detected after concomitant losses of 2CH, corresponding to structure $[M+H-3CO-2H_2O-2CH_4-CO_2-2CH]^+$ $[M_{FE}-2H+H]^+$.

### *3.4. Colour measurement of oxidation products*

The optical properties of the oxidation products were analyzed. During the oxidation reaction of ferulic acid and ethyl ferulate by laccase in aqueous medium, the colour of the reaction mixture changed. In fact, during the EF-oxidation reaction, the reaction mixture colour changed from colourless to white and remained white until the complete consumption of EF, while the FA-oxidation the reaction mixture colour changed from colourless to crystal light brown at the complete consumption of FA and after that, the FA-reaction mixture colour

347 became dark brown. To avoid brown oxidation products, the oxidation reactions were stopped
348 after 3 h, limiting the high degree of polymerization.

349 The values for the colour parameters (a*, b* and L*) and C* values of laccase-catalyzed
350 oxidation products recovered and freeze dried after 3 h of initial oxidation reaction, were
351 determined. FA-products were darker than EF-products, the L* value of FA-products (62.7 ±
352 0.16) was lower compared to the EF-products (82.3 ± 0.49). Simultaneously, FA-products
353 presented a higher colour saturation (C*) (27.4 ± 0.60) than EF-products (15.9 ± 0.28). In all
354 cases, the chromatic coordinates a* and b* for laccase-catalyzed oxidation products were
355 positive. In fact, the FA-products showed an increase in a*, b* values (12.7 ± 0.31 and 24.3 ±
356 0.39, respectively) indicating an increase in red and yellow colours, more than that of the EF-
357 products (5.28 ± 0.05 and 15.3 ± 0.26, respectively). Furthermore, the colour stability of the
358 FA-products was observed for two months under light and at room temperature (data not
359 shown). This colour varied from yellow to orange, depending on the concentration of the
360 products.

361 ***3.5. Antioxidant properties of laccase-catalyzed oxidation products***

362 The ABTS radical scavenging activity method is based on the ability of molecules to
363 quench the ABTS radical cation, in comparison with trolox, which was used as a reference
364 molecule. The ABTS radical scavenging activities of the initial phenols and their laccase-
365 catalyzed oxidation products were expressed as the half-maximal inhibition concentrations
366 ($IC_{50}$) and Trolox Equivalent Antioxidant Capacity TEAC. The comparative $IC_{50}$ and TEAC
367 values of the tested phenols were shown in Table 2.

368 The ABTS radical scavenging activity of the initial phenols and their laccase-catalyzed
369 oxidation products, showed that FA exhibited higher radical scavenging activity than trolox

(TEAC value higher than 1), corresponding to the results reported by other authors (Nilsson, Pillai, Onning, Persson, Nilsson & Akesson, 2005; Perez-Jimenez & Saura-Calixto, 2008). Additionally, the FA-scavenging activity was higher than the EF-scavenging activity due to the esterification of the carboxylate group of ferulic acid, which decreases its antioxidant effectiveness (Garcia-Conesa, Plumb, Kroon, Wallace & Williamson, 1997; Rice-Evans, Miller & Paganga, 1996). In fact, the scavenging activity of hydroxycinnamic acids, such as FA, against ABTS radical was dependent on the number of hydroxyl groups on the benzene ring and *ortho* substitution with the electron donor methoxy group, which increases the stability of the phenoxy radical as expected from the results reported by other authors (Chen & Ho, 1997; Garcia-Conesa et al., 1997; Pekkarinen, Stockmann, Schwarz, Heinonen & Hopia, 1999).

The laccase-catalyzed oxidation products of FA and EF exhibited similar scavenging activity, which was lower than that of initial substrates (FA or EF) (Table 2). The similar activity of the products are due to the oxidation and polymerization processes decreasing the number of substitutes groups on the aromatic ring, such as -COOH, -OH and $-OCH_3$, which are mainly responsible for radical scavenging activity, as well as the saturating of C=C bonds (Adelakun et al., 2012). Consequently, the esterification and polymerization decreased the antioxidant action in the order: ferulic acid > trolox > ethyl ferulate > EF- or FA- laccase-catalyzed oxidation products. The similar antioxidant activity of the oxidation products is mainly due to their similar chemical structures, as shown in Fig.3, except for the presence of ethyl groups in EF-dimers. In previous studies, it was shown that the $IC_{50}$ value of natural antioxidants, such as ascorbic acid (vitamin C) was almost 5.3 ± 0.3 µg/ml (Lin, Liu, Hu, Song & Zhao, 2012; Sheih, Wu & Fang, 2009) by the ABTS method. Consequently, oxidation products of FA and EF presented similar antioxidant properties to that of ascorbic acid and can be considered as promising antioxidant agents.

395 *3.6. Cytotoxic properties of laccase-catalyzed oxidation products*

396 The cytotoxic activity of ferulic acid, ethyl ferulate and their oxidation products were
397 evaluated toward HUVEC cells by the neutral red uptake (NRU) assay. Prior to the assay, the
398 effect of 0.25% (v/v) DMSO (the final concentration of DMSO in the assay) was investigated
399 and no cytotoxic effect was observed on the growth of HUVEC cells (data not shown).

400 The neutral red uptake (NRU) assay was used to evaluate the viability of the cells
401 treated with phenol compounds tested at several concentrations (50, 100, 150 and 200 μg/ml)
402 over 48 h. The results were expressed as $IC_{50}$ mean values with standard deviations. As
403 shown in Fig. 4, presenting cell viability of phenolic compounds, it was found that at 50
404 μg/ml, the oxidation products did not present any cytotoxic effect towards HUVEC cells,
405 while the initial phenols (FA, EF) presented a slight cytotoxic effect, as also shown by a
406 previous study (Kong et al., 2009). Consequently, the oxidation and the polymerization of FA
407 and EF decreased the cytotoxic effect of these phenolic compounds. Additionally, it was
408 found that ethyl ferulate ($IC_{50}$ = 147 ± 4.7 μg/ml) exhibited a higher cytotoxic effect towards
409 HUVEC cells, compared to ferulic acid ($IC_{50}$ =163.5 ± 6.8 μg/ml).

410 In previous studies, the cytotoxic activity of phenolic compounds was shown to increase
411 with increasing lipophilicity and consequently their ability to interact with the cell membrane
412 and their transfer through it (Hadj Salem, Chevalot, Harscoat-Schiavo, Paris, Fick & Humeau,
413 2011; Mellou, Loutrari, Stamatis, Roussos & Kolisis, 2006). In addition, methoxy groups (O-
414 $CH_3$) and alkyl chains through an ester or amide bond also increased the cytotoxic activity, by
415 increasing the hydrophobicity of the molecules and consequently improve their interaction
416 with cell membranes (Plochmann et al., 2007; Serafim et al., 2011). Thus, the cytotoxic
417 activity of ferulic acid and its ethyl ester (ethyl ferulate) is probably related to their
418 lipophilicity, which is due to methoxy groups and alkyl chains. As the lipophilicity of ethyl

ferulate was higher, due to the esterification of the carboxylate group, its cytotoxic activity was higher than that of ferulic acid against HUVEC cells. In previous studies, it was found that ester molecule presents a higher cytotoxic effect, than the initial molecule, with flavonoids, such as quercetin and isoquercitrin (Hadj Salem et al., 2011) or phenolic acids, such as ferulic acid and hexyl ferulate (Serafim et al., 2011). In another study, ferulic acid presented a cytotoxic effect toward HUVEC cells from a concentration of 80 µg/ml. This effect could be explained by the inhibition of adhesion protein synthesis of HUVEC cells by FA (Zhao, 2003).

Furthermore, FA or EF oxidation products ($IC_{50}$ = 198 ± 8.8 µg/ml and 173 ± 8.1 µg/ml, respectively) presented lower cytotoxic activity toward HUVEC cells, compared to their initial compounds ($IC_{50}$ =164 ± 6.8 µg/ml for FA and 147 ± 4.7 µg/ml for EF). The low cytotoxic activity could be attributed to the enzymatic oxidation and polymerization of the phenolic compounds. In fact, the oxidation procedure decreased the number of benzene ring substitutions, especially hydroxyl, with methoxy groups being responsible of cytotoxic effects (Plochmann et al., 2007; Serafim et al., 2011) and the saturation of double bond of the alkyl chain decreasing the hydrophobic character (Hadj Salem et al., 2011). Additionally, the cytotoxic activity of EF-products and FA-products were similar due to their similar chemical structure as presented in Fig. 3, except for the presence of ethyl groups in EF-dimers, which enhanced the hydrophobicity and hence the cytotoxic activity.

## 4. Conclusion

Enzymatic oxidation of ferulic acid and its ethyl ester was catalysed in aqueous medium by partially purified laccase from *Myceliophthora thermophila* in mild controlled temperature and pH (30°C and pH 7.5) as an eco-friendly procedure. This study demonstrated that the ultrafiltration method can increase the commercial laccase activity by a factor 2. This laccase

had a higher affinity toward ethyl ferulate, compared to ferulic acid. Furthermore, enzymatic oxidation led to two major dimers of FA and EF, which were identified by HPLC and LC-MS. Furthermore, after a 3 h reaction, the reaction mixture colour was transformed from colourless to white for FE-oxidation and from colourless to dark brown for FA-oxidation. Additionally, oxidation products presented lower antioxidant and cytotoxic properties compared to the initial phenols. Despite the lower antioxidant properties, these products can be considered as antioxidant agents, in comparison with natural antioxidant ascorbic acid. Consequently, the recovery of these laccase-catalyzed oxidation products provided new compounds with optical, antioxidant and cytotoxic interests.

These new compounds with double functionalities (colour and antioxidant) and presenting low cell cytotoxicity may find useful applications in biotechnological domains, such as food colorants for the replacement of synthetic colorants, or as antioxidant additives for food preservation. Furthermore, previous studies demonstrated that these products tend to bind to other macromolecules such as chitosan (Aljawish et al., 2012; Bozic, Gorgieva & Kokol, 2012; Sousa, Guebitz & Kokol, 2009) as a method to avoid their high polymerization degree leading to some advantages as for the colour stability in food products.

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Fig. 1

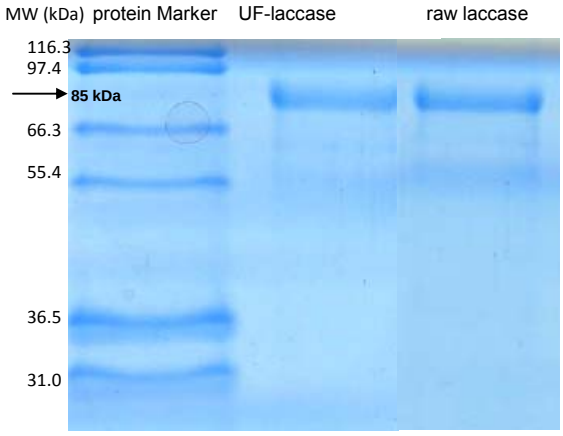
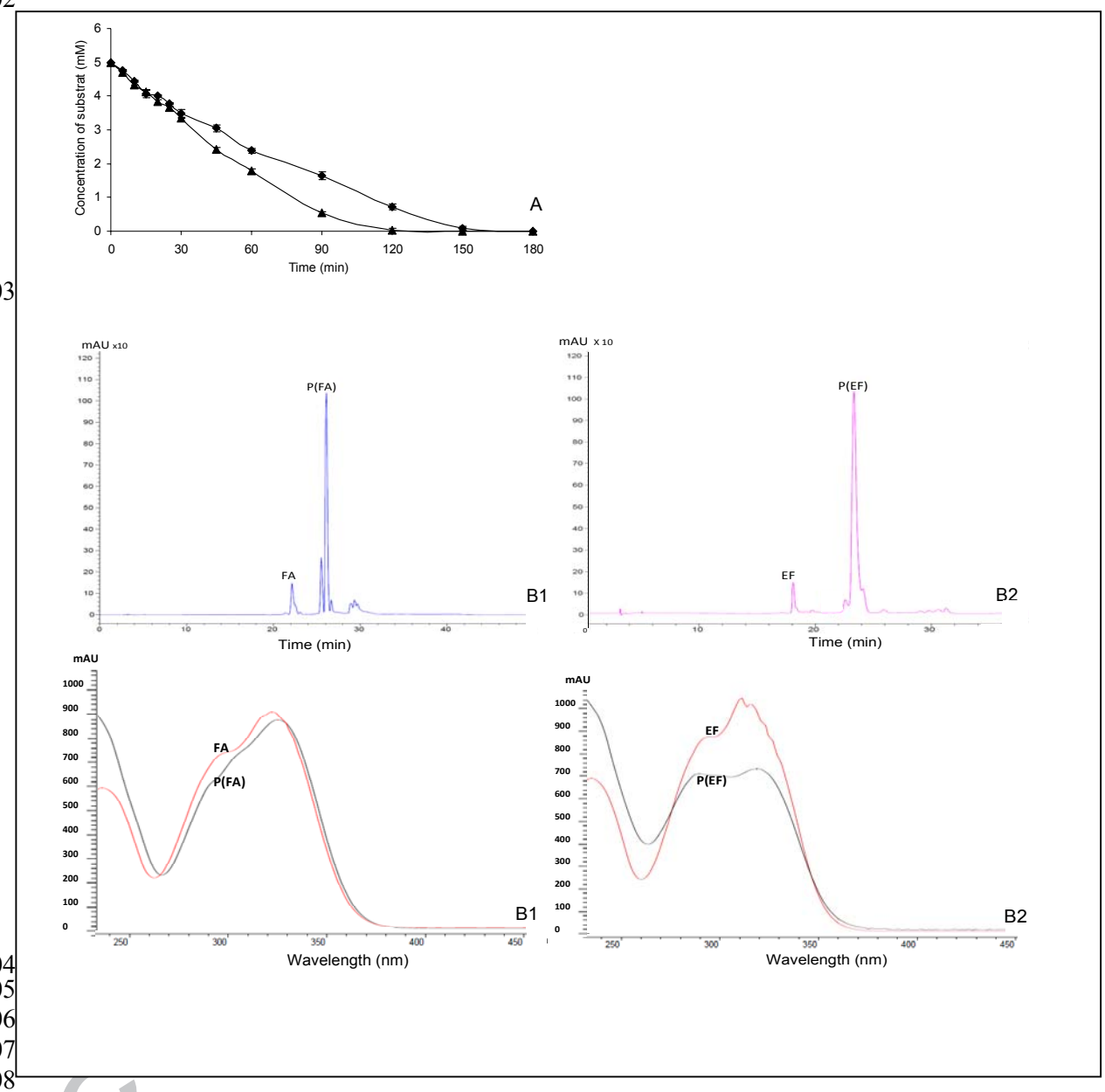
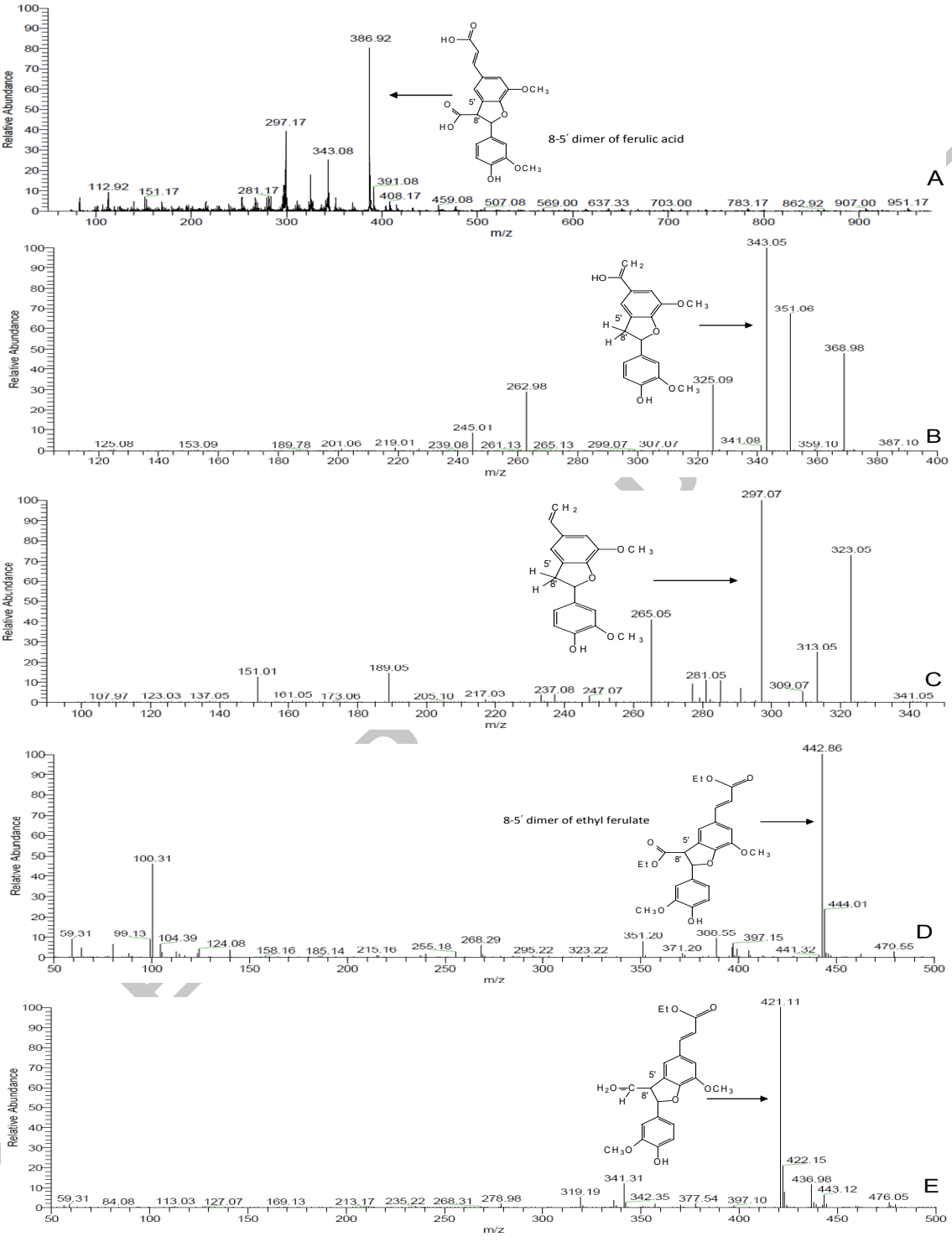


Fig. 2



614 Fig. 3

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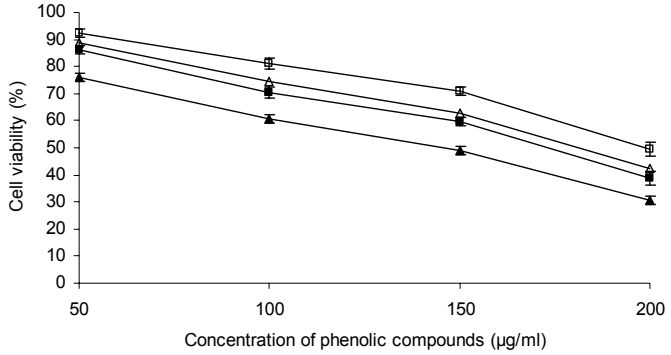
624 Fig. 4

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628 **Figures**

629 Fig. 1. SDS-PAGE of UF-laccase and raw laccase. MW, molecular weight

630 Fig. 2. (A) Kinetics of oxidation of ferulic acid (♦) and ethyl ferulate (▲) by *Myceliophthora*
631 *thermophila* laccase in aqueous medium (50 mM, pH 7.5). (B) Reaction mixture
632 chromatogram at 322 nm and UV-spectra collected by HPLC of FA and its major product (1)
633 and EF and its major product (2).

634 Fig. 3. LC-MS spectrums: (A) full scan MS spectrum of the major FA-oxidation product, (B)
635 $MS^2$ fragmentation spectrum of ion m/z= 387, (C) $MS^3$ fragmentation spectrum of ion m/z=
636 343, (D) full scan MS spectrum of the major EF-oxidation product, (E) $MS^2$ fragmentation
637 spectrum of ion m/z= 443

638 Fig. 4. Cell relative viability (%) of phenolic compounds tested toward HUVEC cells:
639 FA (■), EF (▲), FA-products (□) and EF-products (Δ)
640

641 Table 1: Characterization of *Myceliophthora thermophila* laccase before/after partial
642 purification by ultrafiltration

| | Raw laccase | UF-laccase |
|---|---|---|
| Protein (mg/ml) | 21.5 | 8.5 |
| Total activity (µmole/min/ml) | 5590 | 5100 |
| Specific activity (µmol/min/µg protein) | 0.26 | 0.57 |
| Purification (fold) | 1.0 | 2.1 |
| Yield of activity (%) | 100 | 91 |
| Yield of coloration (%) | 100 | 10 |

643

644 Table 2: $IC_{50}$ values and TEAC coefficients of laccase-catalyzed oxidation products and
645 initial substrates for ABTS method

| ABTS | FA | P(FA) | EF | P(EF) | Trolox |
|---|---|---|---|---|---|
| $IC_{50}$ (µg/ml) | 1.34 ± 0.02 | 8.16 ± 0.21 | 2.89 ± 0.03 | 7.80 ± 0.12 | 2.19 ± 0.02 |
| TEAC | 1.64 ± 0.03 | 0.27 ± 0.01 | 0.76 ± 0.02 | 0.28 ± 0.01 | 1 |

646

The enzymatic oxidation of ferulic acid (FA) and ethyl ferulate (EF) by *Myceliophthora thermophila* laccase. The enzymatic oxidation was performed in aqueous medium under an eco-friendly procedure to synthesize new active molecules. The enzymatic procedure in aqueous medium could provide new compounds presenting optical, antioxidant and cytotoxic interest.