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Synthesized tyrosyl hydroxyphenylacetate, a novel antioxidant, anti-stress and antibacterial compound

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ABSTRACT

New tyrosyl ester derivative, a naturally occurring phenol with interesting biological properties, has been synthesized in good yield by a direct esterification of tyrosol (Ty) with *p*-hydroxyphenylacetic acid (*p*-HPA) using *Candida antarctica* lipase as a catalyst. The response surface methodology was used to modulate the effects of the enzyme amount (10–50 mg), the tert-butanol/hexane (v/v) ratio (0.16–0.84), the temperature (35–55 °C) and the reaction time (15–45 h) on the tyrosyl hydroxyphenylacetate (Ty-HPA) conversion yield. Under the optimal predicted conditions (enzyme amount: 10 mg, solvents volume ratio 0.16, reaction temperature; 45 °C and 34 h of incubation), a high conversion yield of 79.33 ± 4% was reached. The obtained ester was purified and characterized by NMR, LC/MS and FT-IR methods. ABTS free radical quenching potency demonstrated that the esterified tyrosol (Ty-HPA) was more effective than the natural separated antioxidants: Ty and *p*-HPA. Furthermore, when used at a non-cytotoxic concentration (100 μM), tyrosyl ester showed significant effectiveness in preventing iron-induced oxidative stress in blood cells compared to the two separated compounds. The antibacterial activity of Ty, *p*-HPA, mixed solution of Ty + *p*-HPA and Ty-HPA was performed by determining the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) using a micro-well dilution method. Compared to the separated substrates, synthesized ester exhibits the most antibacterial effect mainly against Gram+ bacteria.

1. Introduction

Natural phenolic acid compounds are numerous and widely distributed in the plant kingdom [1]. These molecules can be present in considerable amounts in the human diet [2]. The phenolic acid derivatives were revealed with a broad spectrum of pharmacological properties including anti-oxidation [3], anti-thrombosis [4], anti-inflammatory [5], anti-viral and inhibition of human immunodeficiency virus (HIV) [6]. For example, it is well known that caffeic acid phenethyl ester (CAPE), naturally extracted from the propolis of honeybee hives [7], has a high antioxidant activity. This product presents also an anti-inflammatory [8], immunomodulatory, anti-oral and anti-cancer activities [9]. Turkoglu et al. [10] have reported that CAPE has a protective effect against neuronal damage in the hippocampus of rats. Furthermore, the aromatic esters of hydroxycinnamic acid derivatives such as phenylethyl- or tyrosyl-ferulate, present in natural sources, have been found to exhibit an anti-oxidant, anti-cancer, anti-HIVand anti-fungal/microbial activities [11]. In addition, tyrosyl gallate, a chemical derivative of tyrosol, could significantly suppress the cellular melanin formation without cytotoxicity of melana cells [12].

Synthesis of the phenolic acid esters can be performed using chemical or biological catalysts. The chemical synthesis is usually carried out with basic or acidic catalysts under reflux. However, these methods do not meet the requirements for food applications. CAPE was synthesized by direct esterification of caffeic acid with phenethyl alcohol using dicyclohexyl carbodiimide as catalyst [13]. A conversion yield of 38% was reached at the end of the reaction. Some disadvantages of the chemical synthesizing processes include the environmental pollution and the harm to the human body caused by the use of chemical reagents. Nevertheless, the use of enzyme synthesis offers the advantages of specificity, milder

Abbreviations: Ty, tyrosol; *p*-HPA, *p*-hydroxyphenylacetic acid; Ty-HPA, tyrosyl hydroxyphenylacetate; RSM, response surface methodology; LC/MS, liquid chromatography coupled to mass spectrometry; FT-IR, Fourier transformed infrared spectroscopy; NMR, nuclear magnetic resonance; ABT, S2,2-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid; TEAC, trolox equivalent antioxidant capacity; RBC, red blood cells; MDA, Malondialdehyde; ROS, reactive oxygen species; TBA, thiobarbituric acid; TCA, trichloroacetic acid; TEP, 1.1.3.3-tetraethoxypropane; MIC, minimum inhibitory concentration; MBC, minimum bactericidal concentration; MTT, 4,5-dimethyl-thiazol-2-yl-2,5-diphenyltetrazolium bromide.

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Table 1
Levels of the factors tested in the Box-Behnken design.

Factors	Symbol	Coded levels		
		−1	0	+1
Enzyme amount (mg)	X_1	10	30	50
Tert-butanol/hexane volumic ratio	X_2	0.16	0.5	0.84
Temperature (°C)	X_3	35	45	55
Reaction time (h)	X_4	15	30	45

reaction conditions and minimization of side effects. Recently, Chen et al. [14] have reported the use of Novozyme 435 for the synthesis of CAPE with a yield of 91.65% after 59 h at 69 °C. Same enzyme was used recently by Croitoru et al. [15] for the synthesis of aromatic diesters of different sugar alcohols with 3-(4-hydroxyphenyl) propionic acid.

In this study, a new phenethyl ester was synthesized by the esterification of tyrosol (Ty) with the *p*-hydroxyphenylacetic acid (*p*-HPA) using Novozyme 435 as a biocatalyst. A response surface study using a Box Behnken design was set up to model the relationship between the conversion yield of tyrosyl hydroxyphenylacetate (Ty-HPA) and the reaction factors (enzyme amount, tert-butanol/hexane (v/v) ratio, temperature and reaction time). In addition, chemico-biological activities of the newly synthesized esters were investigated.

2. Material and methods

2.1. Chemicals

The deuterated acetone, 4-hydroxyphenylacetic acid, 2,2-azino-bis-3ethylbenzothiazoline-6-sulfonic acid (ABTS) and 4,5-dimethyl-thiazol-2-yl-2,5-diphenyltetrazolium bromide (MTT) were purchased from Fluka (Suisse). The *n*-hexane and 2-methyl-2-propanol were purchased from Prolabo (Paris, France). Tyrosol, *Candida antarctica* lipase (Novozyme 435).

2.2. Experimental designs

2.2.1. Box-Behnken designs and response surface analysis

Box-Behnken designs of RSM were employed to optimize the four-selected factors (enzyme amount, tert-butanol/hexane volume ratio, temperature and reaction time) for enhancing the conversion yield of Ty-HPA. The four-independent factors were investigated at three different levels (−1, 0, +1) (Table 1) and the experimental design used for the study is shown in Table 2.

The conversion yield of Ty-HPA was fitted using a second-order polynomial equation and a multiple regression of the data was carried out to obtain an empirical model related to the factors [16]. The general form of the second-order polynomial equation is:

$$Y = \beta_0 + \sum \beta_i X_i + \sum \beta_{ii} X_i^2 + \sum \beta_{ij} X_i X_j$$

where Y is the predicted response, X_i and X_j are independent factors, β_0 is the intercept, β_i is the linear coefficient, β_{ii} is the quadratic coefficient and β_{ij} is the interaction coefficient.

2.2.2. Data analysis and software

Design-expert, version 7.0 (STAT-EASE Inc., Minneapolis, USA) was used for the experimental designs and statistical analysis of the experimental data. The analysis of variance (ANOVA) was used to estimate the statistical parameters.

2.3. Esterification reactions

Production of tyrosyl derivative was performed by direct esterification of tyrosol with *p*-HPA in screw-capped flasks. Equimolar of *p*-HPA to tyrosol ratio was dissolved in 6 mL of various tert-butanol/n-hexane volume ratios. The mixture was stirred at different temperatures in an orbital shaker at 200 rpm and in presence of *Candida antarctica* lipase (Novozyme 435). Controls were run in parallel, under the same conditions, without enzyme addition. Aliquots of the mixture reaction were withdrawn at different time of incubation and filtered to be used for HPLC analysis. The conversion yield of tyrosyl derivative was calculated as the ratio of number of mol of synthesized compound (determined by a standard range previously established) per total number of mol of tyrosol.

Table 2
The Box-Behnken design of RSM for optimization of the conversion yield of Ty-HPA.

Experimental conditions					Conversion yield (%)	
Run	X_1	X_2	X_3	X_4	Observed	Predicted
1	10	0.16	45	30	79.40	78.90
2	50	0.16	45	30	47.51	49.72
3	10	0.84	45	30	17.50	17.32
4	50	0.84	45	30	31.00	33.52
5	30	0.50	35	15	9.25	14.93
6	30	0.50	55	15	24.00	22.69
7	30	0.50	35	45	17.72	21.06
8	30	0.50	55	45	36.86	33.20
9	10	0.50	45	15	25.40	26.20
10	50	0.50	45	15	28.50	29.51
11	10	0.50	45	45	46.00	44.32
12	50	0.50	45	45	29.50	28.03
13	30	0.16	35	30	51.20	52.63
14	30	0.84	35	30	12.45	9.06
15	30	0.16	55	30	55.20	57.91
16	30	0.84	55	30	25.80	23.69
17	10	0.50	35	30	32.34	30.27
18	50	0.50	35	30	17.59	12.60
19	10	0.50	55	30	25.40	29.04
20	50	0.50	55	30	33.00	33.73
21	30	0.16	45	15	66.30	60.96
22	30	0.84	45	15	14.40	13.57
23	30	0.16	45	45	61.30	60.78
24	30	0.84	45	45	26.40	30.39
25	30	0.50	45	30	41.00	42.30
26	30	0.50	45	30	42.50	42.30
27	30	0.50	45	30	43.40	42.30

2.4. Chromatography conditions

The identification of tyrosol, *p*-HPA and their ester was carried out using HPLC system (Ultimate 3000, Dionex, Germany). The HPLC system was equipped with a pump (LPG-3400SD), column oven and diode-array UV–vis detector (DAD-3000RS). The output signal of the detector was recorded using Dionex Chromeleon™ chromatography Data System. The separation was executed on an Inertsil ODS-4 C18 colomn (5 μm, 4.6 mm × 150 mm) maintained at 35 °C. The flow rate was 1.5 mL/min, the injection volume was 20 μL and the detection UV wavelength was set at 280 nm. The used mobile phase was 1‰ acetic acid in water (A) versus 0.5‰ acetic acid in acetonitrile (B) for a total running time of 8 min and the following proportions of solvent B were used for the elution: 0–10% at 0–3 min; 10–30% at 3–5 min; 30–90% at 5–7 min and 90–10% at 7–8 min.

2.5. Purification of tyrosyl ester

The reaction mixture resulting from the esterification of *p*-HPA with tyrosol contains a mixture of tyrosyl ester and residual substrates. After the removal of the enzyme by centrifugation at 8000 rpm for 15 min, the mixture reaction was dried under nitrogen and 100 mg was taken up in 1 mL chloroforme. The purification of esters was achieved by chromatography on a silica gel 60 column (Merck) (25 cm × 2 cm), previously equilibrated in chloroforme. Elution was carried using chloroforme/methanol mixtures (90:10). The collected solvent fractions were analyzed by TLC using as the same mobile phase. The spots are revealed through evaporated iodine. Purified fractions were pooled and solvents were evaporated at 62 °C under vacuum.

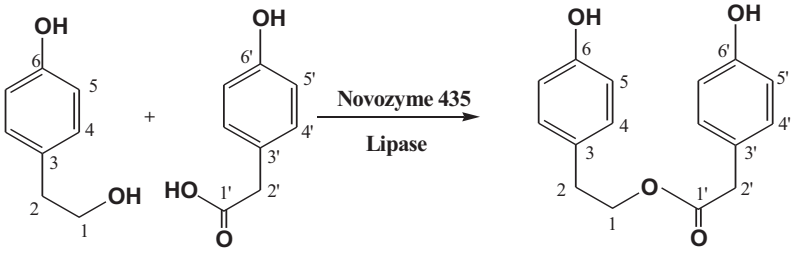
2.6. The LC/MS analysis

The LC/MS experiments were carried out with an Agilent 1100 LC system consisting of degasser, binary pump, auto sampler, and column heater. The column outlet was coupled to an Agilent MSD Ion Trap XCT mass spectrometer equipped with an ESI ion source. Data acquisition and mass spectrometric evaluation were carried out on a personal computer with Data Analysis software (Chemstations).

2.7. NMR and FT-IR experiments

^{1}H NMR and ^{13}C NMR spectra were recorded in deuterated acetone on a Bruker AV300 spectrometer operating at 300 MHz and 75 MHz respectively. IR spectra were recorded on FT/IR-410 (JASCO).

Ty-HPA. Colorless oil. ^{1}H NMR (300 MHz, Acetone-d_6): 8.16 (1H (**H$_{6'}$**), s); 6.90 (2H (**H$_5$**), d, J = 9 Hz); 6.93 (2H (**H$_{5'}$**), d, ^3J = 9 Hz); 6.62 (2H (**H$_4$**), d, ^3J = 8.5 Hz); 6.64 (2H (**H$_{4'}$**), d, ^3J = 8.5 Hz); 4.05 (2H (**H$_1$**), t, —**CH$_2$**—O—C—O); 3.36 (2H (**H$_{2'}$**), s, —CH$_2$—OCO**CH$_2$**); 2.67 (2H (H$_2$), t, —**CH$_2$**—CH$_2$—O—C—O). ^{13}C NMR (75 MHz, Acetone-d_6): 34.84 (**C$_2$**), 40.79 (**C$_{2'}$**), 66.05 (**C$_1$**), 116.01 (**C$_5$** + **C$_{5'}$**), 126.16 (**C$_3$**), 129.6



Scheme 1. Esterification reaction between Tyrosol and *p*-hydroxyphenyl acetic acid.

($\mathbf{C_{3'}}$), 130.75 ($\mathbf{C_4}+\mathbf{C_{4'}}$), 156.88 ($\mathbf{C_6}+\mathbf{C_{6'}}$); 172.13 ($\mathbf{C_{1'}}$, C—O). MS: mass calculated 272.31 for $C_{16}H_{16}O_4$ found m/z = 290 [M+NH_4^+], 272 [M^+], 191 [M−C_5H_5O] and 121[M−p-HPA-H_2O]. IR (liquid) cm^{-1}: 3256–3023(HO-Ø), 2952 (C—H), 1703(C—O), 1598 (C—C) and 1220 (C—O).

2.8. Measurement of the trolox equivalent antioxidant capacity (TEAC)

The ABTS radical-scavenging activity was determined according to Re et al. [17]. The ABTS radical cation was prepared by reacting an aqueous solution of ABTS (7 mM) with potassium persulfate (2.45 mM, final concentration) which was kept in the dark at 25 °C for 12–16 h. The obtained solution was diluted in ethanol to an absorbance of 0.70 (±0.020) at 734 nm before use. 10 μL of trolox or sample in ethanol were mixed with 990 μL of this diluted solution and the absorbance was determined at 734 nm and 30 °C, 6 min after initial mixing. Appropriate solvent blanks were run in each assay. The extent of decolorization was estimated by monitoring the reduction of the absorbance at 734 nm. The anti-oxidant activity was determined as a function of compounds and calculated relative to the equivalent trolox concentration. The activity of each antioxidant was determined at three concentrations, within the range of the dose–response curve of trolox. The radical-scavenging activity was expressed as the trolox equivalent antioxidant capacity (TEAC) defined as mM of trolox.

2.9. Prevention of oxidative stress in human blood

Blood was collected from healthy donors in centrifugal tubes containing 1 mL of citrate as anti-coagulant. After centrifugation 10 min at 1500 rpm, the sera were discarded and the red cells were collected and diluted to 1/4. 100 μL was added to 400 μL of water and subjected to different treatments (with or without the tested compounds). Oxidative stress was induced by adding 100 μmol of Fe^{2+} to cells suspension and incubated at 37 °C. After 1 h, an equal volume of TBA reagent (0.8% of TBA and 15% TCA in HCl 0.25 N) was added. The mixture was heated at 95 °C during 15 min. After centrifugation at 3000 rpm during 10 min, the optical density was recorded at 532 nm. Values were reported to a calibration curve of 1.1.3.3-tetraethoxypropane (1.1.3.3 TEP).

2.10. Determination of the minimum inhibitory concentration (MIC) and the minimum bactericidal concentration (MBC)

The antibacterial activities of Ty, p-HPA, Ty + p-HPA (taken together at the same concentration) and Ty-HPA were tested against *Bacillus* (*B.*) *cereus*, *B. subtilis*, *Enterococcus* (*E.*) *faecalis*, *Staphyloccocus* (*S.*) *aureus*, *S. xylosus*, *Enterobacter* (*E.*) *cloacae*, *Echerichia* (*E.*) *coli*, *Klebsielle* (*K.*) *pneumonia* and *Pseudomonas* (*P.*) *aerigenosa* using Luria-Bertani (LB) medium. The minimum inhibitory concentration (MIC) values, which correspond to the lowest compound concentration that completely inhibits the growth of microorganisms, were determined by a micro-well dilution method as previously described by Eloff [18] using 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT). The inoculum of each bacterium was prepared and the suspensions were adjusted to 10^6 CFU/mL. All the compounds were dissolved in 100% ethanol, and then dilutions series were prepared in a 96-well plate, ranging from 6.25 μg/mL to 4 mg/mL. Each well of the microplate contains 175 μL of the growth medium, 5 μL of inoculum and 20 μL of the diluted sample extract. Ethanol is used as a negative control. The plates were incubated at 37 °C for 24 h, then 40 μL of MTT, at a final concentration 0.5 mg/mL freshly prepared in sterile water, was added to each well and incubated for 30 min. The change to purple color indicated that the bacteria were biologically active. The MIC was taken where no change of colour of MTT was observed in the well.

For the determination of minimum bactericidal concentration (MBC), a portion of liquid from each well that showed no change in color will be placed on solid LB and incubated at 37 °C for 24 h. The lowest concentration that yielded no growth after this sub-culturing will be taken as the MBC [19]. All experiments were done in duplicate.

3. Results and discussion

A response surface design was performed to optimize the enzymatic synthesis of tyrosyl ester by a direct esterification of tyrosol with *p*-hyroxyphenylacetic acid using Novozyme 435 as a catalyst (Scheme 1). Several conditions were tested including various enzyme amounts, solvents' ratios, temperatures and reaction times. In a preliminary study, we have shown that the addition of hexane to the reaction medium was necessary to improve the conversion yield and the stability of the immobilized lipase. Also, we have found that neither the increase of reaction time up to 45 h, nor the addition of water or a crude molecular sieve 4 Å (up to 5%, w/w) at the beginning of reaction improved the conversion yield. In the light of these results, enzyme amount, tert-butanol/hexane (v/v) ratio, temperature and reaction time were chosen as the most effective operating variables on the response.

3.1. Analysis of variance and validation of the models

Based on this preliminary study, RSM using Box Behnken was applied to determine the optimal levels of the four-selected variables (enzyme amounts, tert-butanol/hexane volume ratio, temperature and reaction time) which significantly influenced the conversion yield. The respective low and high levels with the coded levels for the four variables are defined in Table 1. A total of 27 runs with different combination of enzyme amounts (X_1), solvent's ratio (X_2), temperature (X_3) and reaction time (X_4) was designed in Table 2.

The observed and predicted responses of the twenty-seven experiments are also presented in Table 2. The experimental results were analysed by standard ANOVA and the Box Behnken design was fitted with the second order polynomial equation:

$$\hat{y} = 42.3 - 3.25X_1 - 19.45X_2 + 4.98X_3 + 4.16X_4 - 3.43X_1^2 + 5.99X_2^2 - 12.47X_3^2 - 6.86X_4^2 + 11.35X_1X_2 + 5.59X_1X_3 - 4.9X_1X_4 + 2.34X_2X_3 + 4.25X_2X_4 + 1.1X_3X_4$$

where X_1, X_2, X_3 and X_4 are the coded factors of enzyme amount, solvent's ratio, temperature and reaction time respectively.

As shown in Table 3, the fit of the model was checked by the coefficient of determination R^2 which was equal to 0.9753 indicated that 97.53% of the variability in the response could be explained by the model. The statistical significance of the model equation was evaluated by the *F*-test for ANOVA. The model *F*-value of 33.84 implied the model was significant. There was only a 0.01% chance that the model *F*-value could occur due to noise. The *P*-value was also very low ($P < 0.0001$) indicating the significance of the model. The lack of fit *F*-value of 12.95 implied that there was a 7.38% chance that the lack of fit *F*-value could occur due to noise.

Table 3
Statistical analysis of the model (ANOVA).

Source	Sum of squares	Degrees of freedom	Mean square	*F* value	*p*-value Prob > *F*
Model	7630.48	14	545.03	33.84	<0.0001[a]
Residual	193.27	12	16.11		
Lack of fit	190.33	10	19.03	12.95	0.0738
Pure error	2.94	2	1.47		
Cor total	7823.75	26			
R-squared	0.975				

Estimated standard deviation is equal to Std. Dev. = $\sqrt{193.27/12} = 4.01$.
[a] Statistically significant at 95% of confidence level.

3.2. Graphical interpretation of the response surface model

The response surface curves are plotted to explain the interaction of the variables and to determine the optimum level of each variable to reach a maximum conversion yield. The response surfaces curves are shown in Figs. 1–3. Each figure demonstrates the effect of two factors while the others were fixed at zero level.

We can notice that the increasing of the solvent volume ratio at any given enzyme amount (Fig. 1) or at any given time (Fig. 2) leads to a decrease in the conversion yield. These results could be explained by the negative effect of the hydrophilic solvent on the enzyme activity. The changes in the enzyme microenvironment induced by the nature of the organic solvents could explain the low bioconversion yield in the polar medium [20]. In fact, hydrophilic solvents with log $P < 2.5$ might strip off even the essential water from the enzyme surface, leading to an insufficiently hydrated enzyme molecule and in consequently to a decrease of the enzymatic activity [21]. The use of solvent with a log $P > 3$ (hexane) shows better reaction rates. Therefore, the hydrophobic solvents preserve the catalytic activity without disturbing the micro-aqueous layer of the enzyme [22]. Furthermore, the variation in the bioconversion yield and enzyme activity with the organic solvents' ratio may be due to the substrate–solvent interactions which could affect the partition coefficients of substrates as well as products and subsequently the overall activity of the enzyme-catalyzed reaction [23].

Fig. 2 represents the effect of varying solvents' ratio and reaction time at 45 °C in presence of 30 mg enzyme amount. At low solvents volume ratio (0.16), the conversion yield passes from 66.3% to 61.3% at (15 h) and (45 h) reaction time respectively. Under these conditions, the maximum conversion yield was reached at 35 h

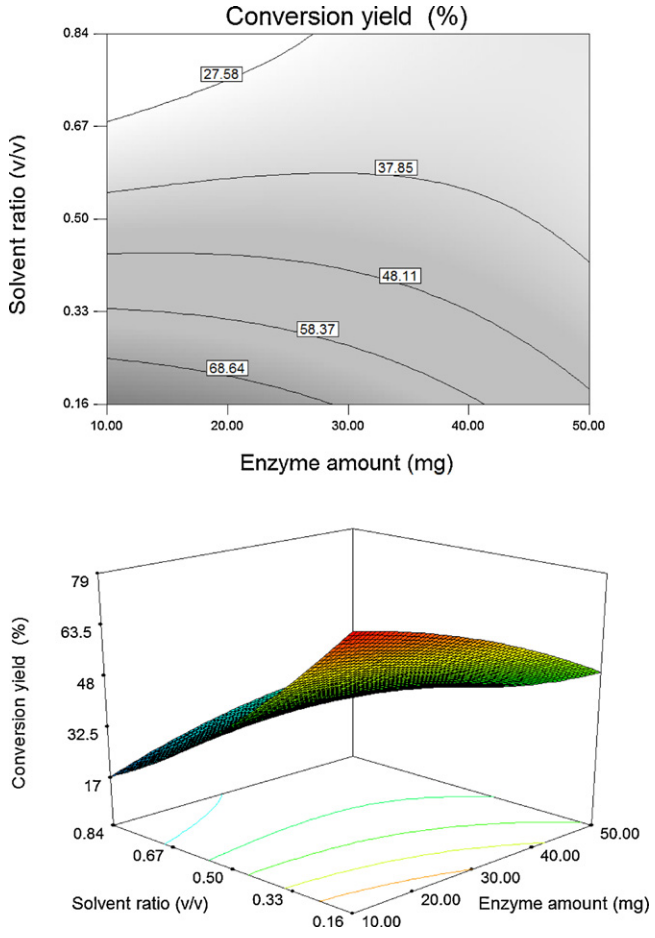


Fig. 1. Contour plots and response surface curve for the conversion yield of Ty-HPA showing the interaction between the enzyme amount and solvent's volume ratio. Temperature and time are equal to 45 °C and 30 h respectively.

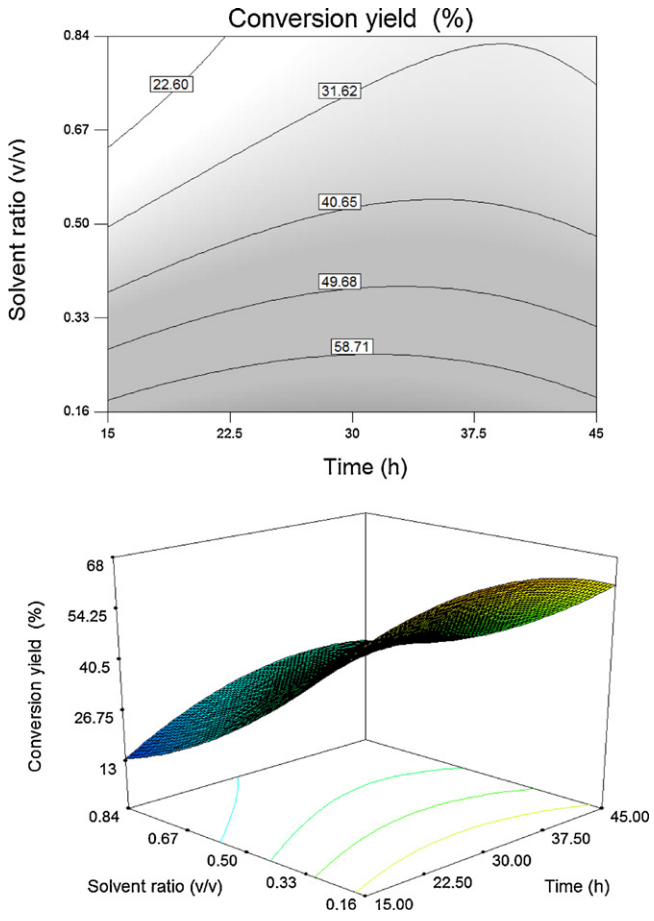


Fig. 2. Contour plots and response surface curve for the conversion yield of Ty-HPA showing the interaction between the solvent's volume ratio and the reaction time. Temperature and enzyme amount are equal to 45 °C and 30 mg respectively.

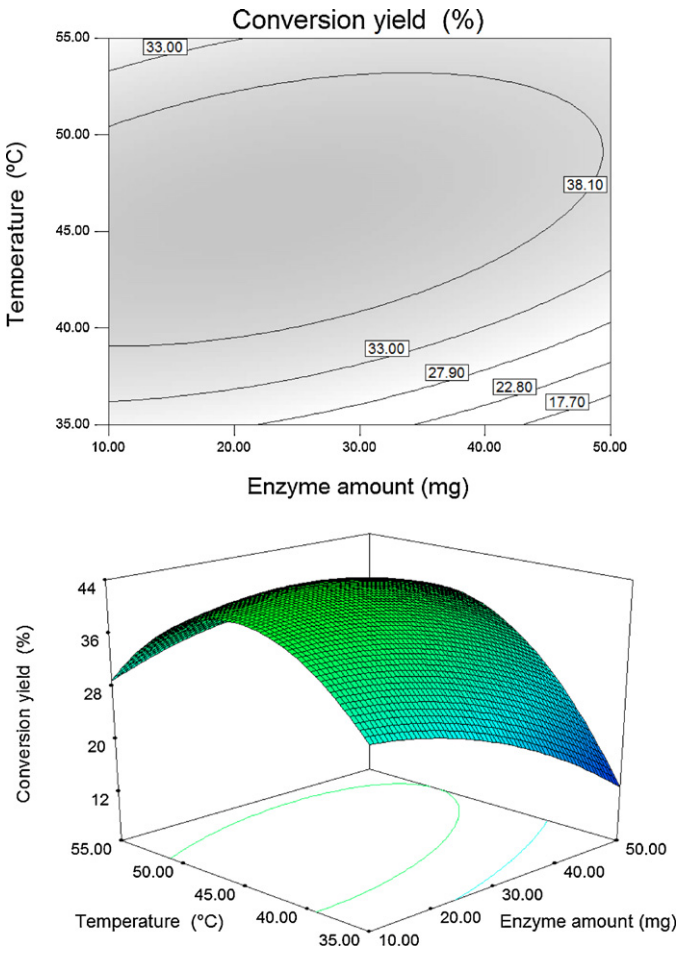


Fig. 3. Contour plots and response surface curve for the conversion yield of Ty-HPA showing the interaction between the enzyme amount and temperature. Solvent's volume ratio and reaction time are equal to 0.5 and 30 h respectively.

of incubation time. The same enzyme was used in the synthesis of aromatic esters of hydroxycinnamic acid derivatives, such as phenylethyl- or tyrosyl-ferulate by Stevenson et al. [11]. The authors found that the reaction rates reached the equilibrium after a long incubation time (200 h).

Fig. 3 shows the effect of varying enzyme amount and the reaction temperature on the esterification yield checked at a constant solvents' ratio of 0.5. It can be seen from Fig. 3 that at any given enzyme amount, the conversion yield increases when the temperature passes from 35 to 45 °C and decreases above 45 °C. Considering the fact that Novozym 435 possesses high thermostability in organic solvents [24], this result can be related to the decrease in equilibrium constants at higher reaction temperatures [25]. The same behavior was previously described by Rahman et al. [25] for the synthesis of dioleyl succinate ester by Novozyme 435. These authors showed that the maximum conversion yield was reached at 40.2 °C then the esterification yield decreased with an increase in the temperature from 40.2 to 65.0 °C.

We can also conclude, from Fig. 3, that the conducted reaction with a low amount of enzyme (10 mg) at 45 °C leads to an enhancement of the conversion yield. However, the increase of the enzyme concentration (50 mg) is accompanied by a significant decrease of the conversion yield. This phenomenon has also been reported by Zheng et al. [26] for the lipase-catalyzed transesterification of ethyl ferulate with triolein. These results may be explained by a mass transfer limitation at a high enzyme concentration, which could affect the diffusion of the substrate to the enzyme active site and

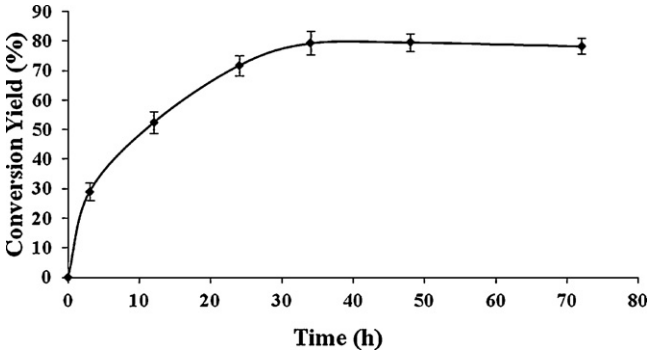


Fig. 4. Production of Ty-HPA during esterification reaction. Reaction conditions: enzyme amount of 10 mg, tert-butanol/hexane volume ratio of 0.16 and temperature of 45 °C. The tyrosol ester yield was estimated using HPLC system.

hence its availability for the reaction [27]. The presence of high protein-support interactions as a result of an excess of the enzyme could alter its active conformation and hence it's catalytic efficiency [28]. Steric hindrance of the enzyme active site may contribute to a decrease in the enzymatic activity at high enzyme concentrations [29].

3.3. Optimal reaction conditions

The optimal values of the four variables predicted by the model are $X_1 = 10.05$ mg, $X_2 = 0.16$, $X_3 = 44.76$ °C and $X_4 = 34.49$ h corresponding respectively to enzyme amount, solvent's ratio, temperature and reaction time. The maximum predicted conversion yield was $79.7 \pm 3.18\%$. Under these optimal conditions, the time course of the esterification reaction between the Ty and *p*-HPA was presented in Fig. 4. A good correlation was found between predicted and experimental values implying that empirical models derived from response surface methodology can adequately describe the relationship between the factors and their influence on the tyrosyl ester synthesis. The level of Ty-HPA increased rapidly to reach its maximal value at 34 h. The experimental conversion yield of Ty-HPA ($79.33 \pm 4\%$) was very close to the predicted estimated value ($79.7 \pm 3.18\%$) at a reaction time of 34 h.

3.4. Structure determination of Ty-HPA ester

Tyrosol contains two hydroxyl groups in its structure, and therefore two esterified derivatives with *p*-HPA were expected. HPLC analysis of the reaction mixture after 34 h (Fig. 5) showed that tyrosol and *p*-HPA were transformed by enzymatic reaction to a new product eluted at a retention time of 6.51 min.

On the basis of the NMR (Fig. 6), LC/MS (Fig. 7) and FT-IR data, it was possible to determine the structure of the new compound. With regard to ^{13}C NMR data, the spectrum of the esterified tyrosol showed signals at δ 172 ppm, which was attributed to a carbon ester function ($C_{1'}$) (Fig. 6A) was confirmed by ^1H NMR. Indeed, with comparison to the ^1H NMR spectrum of tyrosol [30], no significant change in chemical shift was observed, only a downfield at 4.05 ppm for the triplet (2H) observed at 3.8 ppm in ^1H NMR of tyrosol suggesting that ester function was linked to the methylene group C_1 (Fig. 6B). For the aromatic protons of the tyrosol and its esterified form, no modification of chemical shift was observed. It follows that by using Novozyme 435 as biocatalyst, tyrosol was esterified on the primary hydroxyl group.

The LC/MS analysis in positive mode exhibited a molecular ion at $m/z = 290$ [M+NH_4^+] with intermediate fragments at $m/z = 272$ [M^+], 191 [M−C_5H_5O] and 121[M−*p*HPA-H_2O]. The MS/MS experiments focusing on the fragment generated from the peak in $m/z = 290$ [M+NH_4^+] revealed a fragment at $m/z = 272$ corresponding to the

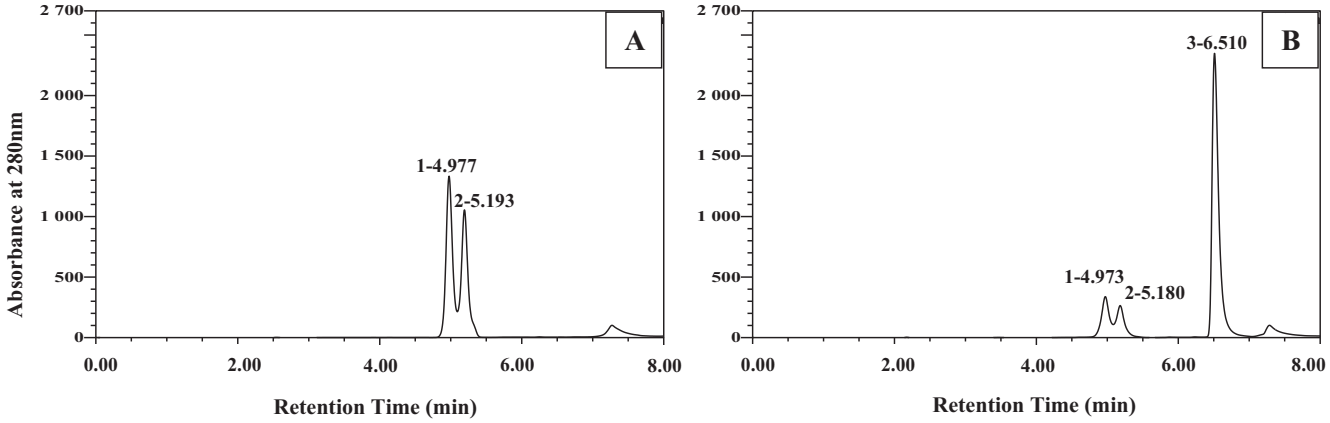


Fig. 5. HPLC chromatograms of the reaction medium before and after synthesis of Ty-HPA. The separation was made on C18 reverse-phase HPLC. Flow 1.5 mL/min, and UV detection was at 280 nm. (A) Time of esterification = 0 h. (B) Time of esterification = 34 h. 1: Tyrosol. 2: *p*-HPA. 3: Ty-HPA. Synthesis was performed under optimal conditions.

synthesized compound and the pseudo molecular ion at $m/z = 121$ attributed to the tyrosol ion.

The formation of Ty-HPA was confirmed by comparing the FT-IR spectra of tyrosol and its ester. The FT-IR spectra showed a peak at 1703 cm^{-1} attributed to a vibration of carbonyl group and peak at 2952 cm^{-1} attributed to C—H vibration of methylene groups. In addition, a large band around 3256–3023 cm^{-1} was attributed to the hydroxyl group linked to the aromatic ring. Other peaks were detected at 1598 and 1220 cm^{-1} attributed to C=C and C—O respectively. The chemical change in functional groups of the simples indicated the formation of Ty-HPA ester. To our knowledge, no reports on the chemical or enzymatic synthesis of such phenyl ethyl ester have been reported.

3.5. The ABTS assay

A widely used method for measuring the radical-scavenging activity of antioxidants is the ABTS assay, where the activity toward a stable free radical, 2,2-azino-bis-3ethylbenzothiazoline-6-sulfonic acid ($ABTS^{\bullet+}$), is evaluated. The radical-scavenging activities of the evaluated antioxidants Tyrosol, *p*-hydroxyphenylacetic acid and their corresponding ester and BHT are summarized in Table 4. Results are expressed as Trolox equivalent antioxidant capacity (TEAC, mM). The TEAC measured with synthesized compound is higher than the value obtained for each substrate tyrosol or phenolic acid. The substrates taken together in the same solution at a final concentration of 2 mg/mL presents a TEAC value lower than the synthesized ester (data not shown). Their scavenging activity of ABTS radicals decreased in the following order BHT > Ty-HPA > Ty > *p*-HPA with all values significantly different at $p < 0.05$. This sequence indicates that the ABTS radical scavenging activity of the tested compounds is to their hydrogen-donating ability. It is generally assumed that the ability to act as an hydrogen donor and the inhibition of oxidation are enhanced by increasing the number of hydroxyl groups in the phenol ring [31].

Table 4
Reducing antioxidant power of tyrosol, *p*-HPA and their derivative. Evaluation by ABTS assay.

Compounds	TEAC (mM)[a]
Tyrosol	1.22 ± 0.03
p-HPA	0.87 ± 0.015
Ty-HPA	1.56 ± 0.006
BHT	2.15 ± 0.001

[a] Each value is the mean of triplicate measurements ± standard deviations ($p < 0.05$). Results are expressed as Trolox equivalent antioxidant capacity (TEAC) in units of mmol Trolox/L.

3.6. Biological antioxidant activity in human cell cultures

The investigation of the biological antioxidant activity of Ty, *p*-HPA and Ty-HPA was carried out on freshly isolated human red blood cells (RBC). RBC were incubated with or without adding the compounds at different concentrations. Oxidative stress was induced by adding 100 μM Fe^{2+} solution (as Fe_2SO_4) in PBS for 1 h. Malondialdehyde (MDA) production, a lipid peroxidation marker, was evaluated and compared with control cells. As shown in Fig. 8, a significant protection against ROS (reactive oxygen species) inducing damage was obtained in the presence of tyrosyl hydroxyphenyacetate (Ty-HPA) at a concentration of 100 μg/mL. The addition of Ty and *p*-HPA at the same concentration failed to decrease the MDA level as compared to control cells. This suggests that the tyrosyl ester is more efficient than the two separated substrates used in synthesis. These results are in correlation with those found by Stevenson et al. [11]. In fact, the authors reported that the esterification of some phenolic acids can apparently increase the strength of protection of Jurkat cells from hydrogen peroxide mediated cytotoxicity. This could be a result of higher hydrophobicity increasing absorption of the esters into the cell membrane and resulting in greater bioavailability.

3.7. Determination of the minimum inhibitory concentration (MIC) and the minimum bactericidal concentration (MBC)

The antibacterial activity of Ty, *p*-HPA, Ty + *p*-HPA and the synthesized ester Ty-HPA was checked against Gram-positive (*B. cereus*, *B. subtilis*, *E. faecalis*, *S. xylosus*, *S. aureus*) and Gram-negative (*E. cloacae*, *K. pneumoniae*, *E. coli*, *P. aerigenosa*) bacteria by the determination of MIC and MBC values (Table 5). As it can be seen from Table 5, Ty has not a significant inhibition activity against all bacteria tested (MIC and MBC values are ≥ 4 mg/mL). Compared to Ty, *p*-HPA and mixed solution of Ty + *p*-HPA, the synthesized ester exhibits the most effect against the bacteria tested, in particular the Gram+ ones. With Gram+ bacteria, the MIC values are ranged from 0.5 to 0.8 mg/mL and the MBC values are between 0.5 and 2 mg/mL.

Gram− bacteria appeared to be less sensitive to the tested compounds. Previous studies [32] reported that Gram+ bacteria presented higher sensitivity than Gram− bacteria to various polyphenols. This higher resistance can be related to the composition of the cell-wall membrane [32]. As it can be seen from Table 5, MIC values showed that Ty-HPA exhibits a similar or a slight higher activity against Gram− bacteria than the separated substrates. Nevertheless, except *E. cloacae* strain, the obtained MBC values confirmed that the synthesized ester (Ty-HPA) has more bactericidal effect against Gram− bacteria than the separated

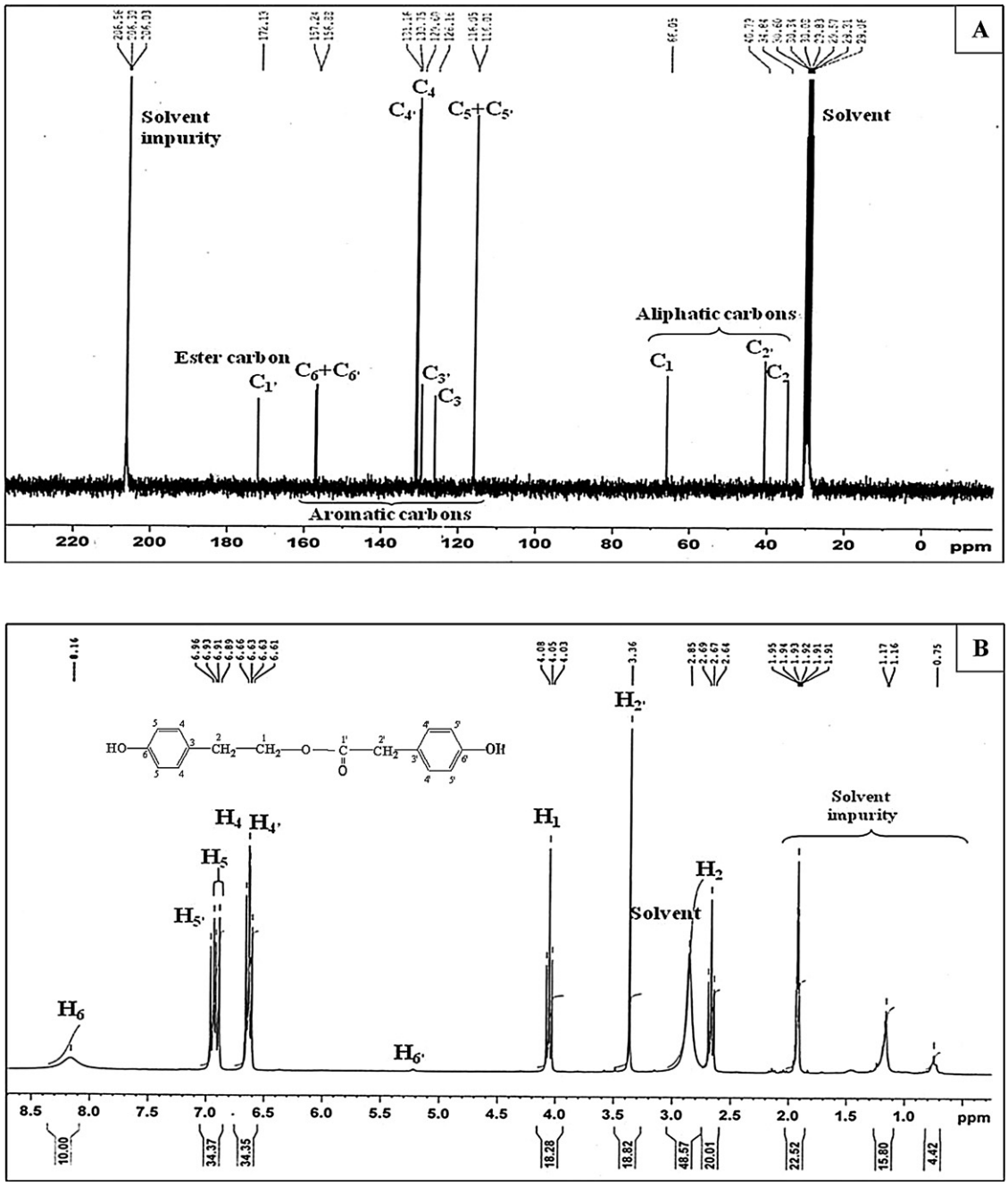


Fig. 6. The ^{1}H NMR (A) and ^{13}C NMR (B) spectra of synthesized ester.

Table 5
Antimicrobial activities of Ty, p-HPA, Ty + p-HPA and Ty-HPA.

Strain	Gram	CMI (mg/mL)				CMB (mg/mL)			
		Ty	*p*-HPA	(Ty + *p*-HPA)[a]	Ty-HPA	Ty	*p*-HPA	(Ty + *p*-HPA)[a]	Ty-HPA
Bacillus cereus	+	>4	2	2	0.8	>4	>4	>4	2
Bacillus subtilis	+	4	1	1	0.5	>4	>4	4	1
Enterococcus faecalis	+	4	1	2	0.8	>4	4	4	0.8
Staphylococcus aureus	+	>4	2	2	0.5	>4	4	2	0.5
Staphylococcus xylosus	+	>4	2	2	0.5	>4	4	2	0.8
Enterobacter cloacae	–	>4	2	2	1	>4	4	4	4
Klebsielle pneumoniae	–	>4	2	2	1	>4	4	4	2
Escherchia coli	–	>4	1	1	1	>4	>4	>4	2
Pseudomonas aerigenosa	–	>4	1	1	1	>4	>4	>4	2

[a] Taken together at the same concentration.

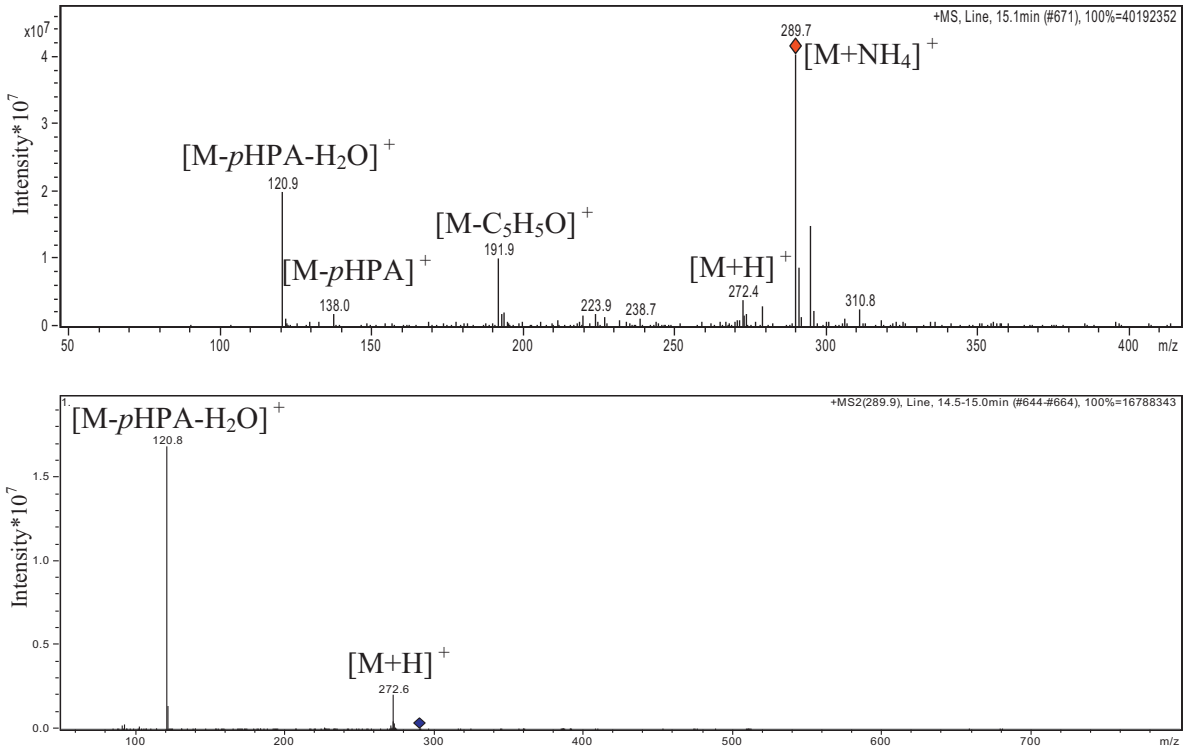


Fig. 7. LCMS (+ve mode) profile of peak identified as Ty-HPA.

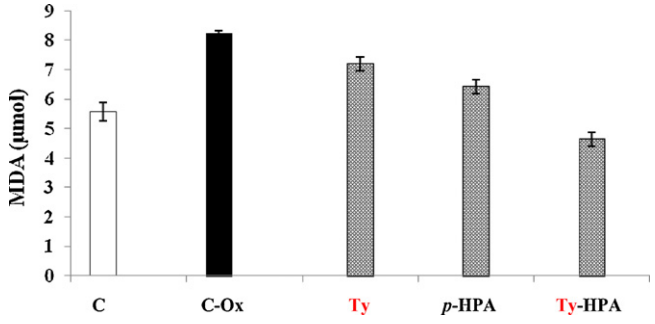


Fig. 8. Ability of hydroxytyrosol and acylated derivatives to reduce iron-induced oxidative stress within blood cells. C-untreated control cells, C-Ox iron-stressed or positive control cells, Ty, *p*-HPA and Ty-HPA refer to cells treated with both $FeSO_4$ and the Ty, *p*-HPA and Ty-HPA corresponding compound. Statistical significance at $p < 0.0001$ was calculated relatively to the positive control.

substrates. These results indicate that we have enhancing significantly the antimicrobial activity of the Tyrosol and *p*-HPA after esterification of these compounds. The potent antibacterial activity of the synthesized compound could be related to the presence of two hydroxyl group at the *p*-positions of the benzene rings of Ty-HPA ester. These results are in concordance with those reported by Fu et al. [33]. In fact, they have found that caffeic acid anilides with electron donating groups at *p*-position of benzene ring have better antibacterial activities.

4. Conclusion

In this study, the evaluation of the effect of the enzyme amount, solvents volume ratio, temperature and reaction time on tyrosyl derivative yield were studied. A Box-Behnken design of RSM was employed to optimize the synthesis conditions. A conversion yield of 79.33% was reached under the optimal conditions. The measure of its anti-oxidant and anti-radical activities showed that the ester was more efficient than the tyrosol or the phenolic acid. The synthesis of phenol derivatives via esterification of the hydroxyl functions with aliphatic phenols can be used as a tool to increase their antioxidant activity and to improve therefore their stability and biological function. Moreover, this aromatic analogue could be used in cosmetic and pharmaceutical fields.

Conflict of interest

The authors declare that they have no conflict of interest.

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