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Laccase mediated-synthesis of hydroxycinnamoyl-peptide from ferulic acid and carnosine

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Abstract

Carnosine (CAR) dipeptide was functionalized with ferulic acid (FA) as substrate using laccase from *Myceliophthora thermophila* as biocatalyst. The enzymatic reaction was performed in aqueous medium under mild conditions (pH 7.5, 30°C) as an eco-friendly procedure.

Results showed that this enzymatic process led to the synthesis of two new derivatives (P1, P2), from the coupling between CAR and FA derived products. Conditions allowing a high production of P1, P2 derivatives were determined with an optimal ratio of (FA: CAR) of (1:1.6) at optimal time reaction of 8 h. Under these optimal conditions, the coupling between CAR and FA- products was demonstrated, resulting in the decrease of -NH₂ groups (almost 50%) as quantified *via* derivatization. Due to the presence of FA in the structure of these new derivatives, they exhibited higher hydrophobic property than carnosine. Structural analyses by mass spectrometry showed that P1 and P2 (FA-CAR) derivatives exhibited the same molecular mass (MM 770 g/mol) containing one CAR-molecule and three FA-molecules but with different chemical structures. Furthermore, these derivatives presented improved antioxidant (almost 10 times) and anti-proliferative (almost 18 times) properties in comparison with CAR. Moreover, P1 derivative exhibited higher antioxidant and anti-proliferative activities than P2 derivative, which confirmed the different structures of P1 and P2. These results suggested that the oxidized phenols coupling with carnosine is a promising process to enhance the CAR-properties.

Keywords: Carnosine, ferulic acid, laccase, antioxidant activity, Caco2 cells.

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31 **1. Introduction**

32 Carnosine (CAR) is an endogenous dipeptide, composed of β -alanine-L-histidine. This
33 dipeptide is naturally found in the central nervous system, skeletal muscles and in the lens of
34 the eye in some vertebrates, including humans (Boldyrev and Severin, 1990; Budzen and
35 Rymaszewska, 2013). Substantial amounts of carnosine can be easily obtained from animal
36 protein such as chicken and fish (Chan and Decker, 1994; Gil-Agusti et al., 2008), beef (Park
37 et al., 2005). In humans, carnosine is synthesized from two constitutive amino acids by
38 carnosine synthase (EC 6.3.2.11) and is degraded by carnosinase (Song et al., 2014). This
39 dipeptide exhibits various biological functions as a physiological intracellular buffer against
40 lactic acid in muscles, a neurotransmitter, a regulator of glycogenolysis, an antiglycating agent
41 and a rejuvenating agent (Fontana et al., 2002; Kwak et al., 2009; Song et al., 2014). Many
42 biochemical studies have also demonstrated that carnosine possesses antioxidant and oxygen-
43 free radical-scavenging properties (Fontana et al., 2002). Due to its broad spectrum of activity,
44 carnosine have several pharmacological potentials as diabetes, neurodegenerative diseases, a
45 role against inflammation, cancer, ageing, cataracts and atherosclerosis (Budzen and
46 Rymaszewska, 2013; Hipkiss and Brownson, 2000). Carnosine can detoxify cytotoxic
47 reactive carbonyl groups by forming unreactive adducts and is able to reverse glycosylated protein
48 (Song et al., 2014).

49 However, the potentials of carnosine can be restricted by its polarity that can limit its
50 transport through biological membranes (Khandelia et al., 2008). The use of carnosine as a
51 potential drug can be also limited due to its fast metabolism by means of serum and kidney
52 carnosinases, which quickly reduces serum level of carnosine and then prevents its long-
53 lasting action (Pegova et al., 2000). Moreover, its short half-life due to its sensibility towards
54 endogenous proteases represents an additional limitation. One way to overtake these
55 constraints is to modify the chemical structure of carnosine by chemical or enzymatic process
56 such as the grafting of an acyl group onto this dipeptide (Husson et al., 2009). The chemical
57 acylation of carnosine with trolox (a vitamin E derivative), a hydrophobic molecule was
58 performed as a way to increase the stability and the antioxidant of carnosine (Stvolinsky et al.,
59 2010). The trolox-carnosine derivative showed higher resistance towards human serum
60 carnosinase action, higher antioxidant capacity than their precursors, carnosine and trolox.
61 Moreover, the acylation of carnosine was performed by lipase enzyme in organic media (2-
62 methyl-2-butanol) with activated fatty acids such as oleic acid or with fatty acid ester such as

63 ethyl oleate (Husson et al., 2010; Husson et al., 2008; Husson et al., 2011; Husson et al.,
64 2009). These acetyl derivatives of carnosine exhibited improved antioxidant properties in the
65 liposome peroxidation system and enhanced transfer capacity throughout biological
66 membranes due to its higher hydrophobicity. Other authors reported the chemical conjugation
67 of carnosine with hydroxycinnamic acids such as ferulic acid, caffeic acid and sinapic acid
68 (Kwak et al., 2009; Seo et al., 2010) and with hydroxybenzoic acids such as gallic acid (Kim
69 et al., 2014) in order to synthesize new derivatives with improved antioxidant properties. In
70 the human fibrosarcoma (HT1080) cell line, the carnosine-gallic acid conjugate showed high
71 inhibitory levels on Matrix metalloproteinases (MMPs) that degrade extracellular matrix
72 components and play important roles in several biological and pathological processes such as
73 invasion and malignant tumor metastasis. Furthermore, new carnosine derivatives were
74 produced by chemical conjugation of carnosine with small sugars such as lactose and glucose
75 (Lanza et al., 2011) or oligosaccharides such as β -cyclodextrin (Amorini et al., 2007; Bellia et
76 al., 2008; La Mendola et al., 2002). The glycoconjugation process protected the carnosine
77 moiety from carnosinase hydrolysis and enhanced scavenger hydroxyl radical activity making
78 them promising agents against harmful oxygen species.

79 Up to now, only one study was reported concerning the chemical conjugation between
80 the carnosine and ferulic acid (Kwak et al., 2009). This approach improved the lipid
81 peroxidation inhibition of FA-CAR derivative using lipid antioxidant test without improving
82 its antiradical activity using DPPH assay in comparison with FA and CAR. On the contrary,
83 the enzymatic conjugation between carnosine dipeptide and phenol compounds using
84 enzymatic catalysis in aqueous medium has never been described in the literature. In this
85 paper, the enzymatic conjugation between carnosine and ferulic acid (hydroxycinnamic acid)
86 was reported using *Myceliophthora thermophyla* laccase in mild conditions as an attractive
87 alternative to toxic, environmentally unfriendly and non-specific chemical approaches. In fact,
88 laccase enzyme oxidizes phenol derivatives into free radicals that can, in turn, either condense
89 together or react with nucleophilic function by different types of reaction to yield either
90 Schiff-bases or Michael-type adducts such as the free amino groups of chitosan (Aljawish et
91 al., 2012). Moreover, this enzyme is active in aqueous medium, at room temperature and
92 neutral pH (Aljawish et al., 2014). Ferulic acid (FA) (4-hydroxy-3-methoxycinnamic acid),
93 presents in a wide variety of grains, fruits and vegetables, is the most abundant
94 hydroxycinnamic acid in the plant kingdom (Zhao and Moghadasian, 2008). It is used in food,
95 cosmetics and pharmaceutical industries owing to its effective antimicrobial, antioxidant,

96 antithrombotic, anti-inflammatory and anticancer activities (Balasubashini et al., 2003; Ou
97 and Kwok, 2004).

98 The current work reports the enzymatic synthesis of hydroxycinnamoyl-peptide
99 derivatives using ferulic acid (FA) as a resource of electrophilic function and carnosine (CAR)
100 as a resource of nucleophilic function in mild conditions (pH 7.5 and 30°C). These derivatives
101 were identified in terms of structural characterization and in terms of physicochemical and
102 biological properties.

103 **2. Materials and methods**

104 **2.1. Chemicals and enzyme**

105 Ferulic acid (FA) and carnosine (CAR) were bought from Fluka (France) with purity
106 over than 99%; trolox was purchased from Fluka (Germany); syringaldazine was obtained
107 from Ega-chemie with purity over than 99%. Methanol and acetonitrile (HPLC grade) were
108 obtained from Carlo Erba (Milwaukee, WI, USA); 2,2'-azino-bis(3-ethyl-benzthiazoline-6-
109 sulfonic) acid (ABTS), trifluoroacetic acid (TFA) (98%) and other chemicals with highest
110 quality available were obtained from Sigma-Aldrich (France).

111 An industrial laccase named Suberase[®] (Novo Nordisk A/S, Bagsvaerd, Denmark) was
112 bought from the Society Novozymes under liquid form. The Suberase[®] is a fungal laccase
113 from *Myceliophthora thermophila* sp., which is considered as a member of family of
114 polyphenol oxidases, produced by submerged fermentation of a genetically modified
115 *Aspergillus oryzae*. The enzymatic preparation was supplied as a brown liquid with a density
116 of approximately 1.15 g.mL⁻¹. It was completely miscible with water. To remove inactive
117 peptides or proteins and to avoid the interaction between the interesting phenols and
118 contaminated phenols which were initially present with the industrial laccase, the Suberase[®] is
119 partially purified as already described (Aljawish et al., 2014)

120 **2.2. Laccase characterization**

121 Protein concentration in Suberase[®] was determined by Bicinchoninic acid (BCA)
122 spectrophotometric method (Slocum and Deupree, 1991). Bovine serum albumin was used as
123 standard in phosphate buffer (50 mM, pH 7.5).

124 The laccase activity at different pH (from 5 to 8) and different temperatures (from 20 to
125 50°C) was spectrophotometrically determined by measuring the increase of the absorbance at
126 525 nm due to the oxidation of syringaldazine (2 μ M, pH 7.5) in phosphate buffer (50 mM)
127 using a spectrophotometer (Shimadzu UV- 1605). One unit of enzyme (UI) was defined as the
128 amount (μ mole) of syringaldazine oxidized per minute and per μ g of enzymatic protein using
129 an extinction coefficient ϵ_{525} of 65 $\text{mM}^{-1}.\text{cm}^{-1}$ (Mustafa et al., 2005).

130 **2.3. Enzymatic reactions**

131 To evaluate the ability of enzymatic conjugation between CAR and the products from
132 laccase-catalyzed oxidation of FA in an aqueous medium, the reactions were carried out at
133 30°C in a magnetic stirred reactor (100 mL) under atmospheric conditions. In these
134 experimental conditions, the dissolved oxygen was not limited for the reaction. Two reactions,
135 with (I) and without (II) CAR, were performed: for the reaction (I) 5 mL of methanol solution
136 of FA (50 mM), 8 ml of aqueous solution of CAR (50 mM) and 37 mL of phosphate buffer
137 (50 mM, pH 7.5) were mixed in a magnetic stirred reactor at 300 rpm. For the reaction (II),
138 CAR was not added, but the reaction medium was composed of 5 mL of FA-solution (50
139 mM) and 45 mL of phosphate buffer (50 mM, pH 7.5). The reactions were started by adding
140 200 UI of Suberase[®] for 15 ml of reaction medium (13.5 U/mL of laccase). Additionally, a
141 control reaction without laccase (III) was performed in the same conditions. Reaction kinetics
142 were monitored at various interval times by withdrawing samples (0.1 mL) from the reaction
143 mixture. The enzymatic reaction was stopped by the addition (0.9 ml) of methanol containing
144 0.03% (v/v) of TFA (Lacki and Duvnjak, 1996).

145 **2.4. Kinetic analysis of substrate oxidation**

146 To monitor the oxidation of substrate, reaction mixtures were analyzed by high
147 performance liquid chromatography (HPLC) using a Shimadzu Class-VP HPLC system with
148 a computer controlled system containing upgraded Class-VP 6.1 software. Separations were
149 carried out on a reversed phase column LiChroCART RP-18 (Merck, 25 cm x 0.4 cm, particle
150 size 5 μ m). The detection was conducted between 190 and 800 nm on a multichannel
151 photodiode- array detector (SPD-M10A VP). The elution was performed using a gradient of
152 solvent A: water/ TFA (100:0.03; v/v) and solvent B: acetonitrile /water/ TFA (80:20:0.03;
153 v/v/v) at a flow rate of 0.7 ml/min. The steps of the gradient were as follow: linear gradient

154 from 5% to 40% of B during 15 min, from 40% to 60% of B during 5 min and from 60% to
155 80% of B during 15 min. Each analysis was performed in triplicate.

156 **2.5. Characterization of (FA-CAR) derivatives**

157 **2.5.1. Recovery of (FA-CAR) derivatives**

158 To ensure a complete oxidation of substrate and to avoid the degradation of new
159 products, recovery time was determined at the complete oxidation of FA substrate. The
160 products were recovered by a rotary evaporator under reduced pressure, and then freeze-dried
161 for 24 h. After that, the preservation of recovered products was carried out in a desiccator
162 until use.

163 **2.5.2. Purification of (FA-CAR) derivatives by semi-preparative HPLC**

164 The purification of FA-CAR derivatives was elaborated by a semi preparative high
165 performance liquid chromatography (HPLC) using a Gilson's Analytical to Semi-preparative
166 HPLC System with a computer controlled system containing Trilution[®] LC software.
167 Separations were carried out on a reversed phase column LiChroCART RP-18 (Merck, 25 • 3
168 cm, particle size 5 μ m). The detection was conducted at 280 nm on UV/Vis detector. The
169 elution was performed using a gradient of mobile phase consisting of water/ TFA (100:0.03;
170 v/v) (solvent A) and acetonitrile /water/ TFA (80:20:0.03; v/v/v) (solvent B) at a flow rate of
171 5 ml.min⁻¹. The injection volume was 1 mL of sample in concentration 10 mg mL⁻¹. The steps
172 of the gradient were the same used in HPLC analysis: linear gradient from 5% to 40% of B in
173 15 min, from 40% to 60% of B in 5 min and from 60% to 80% of B in 15 min, followed by
174 the washing and the reconditioning of the column (80% to 5% of B in 15 min).

175 **2.5.3. Liquid Chromatography–Mass Spectrometry (LC–MS) analysis**

176 The mass spectra were obtained using the HPLC-MS system which comprised the
177 binary solvent delivery pump and a linear ion trap mass spectrometer (LTQ-MS, Thermo
178 Fisher Scientific, San Jose, CA, USA). LTQ equipped with an atmospheric pressure ionization
179 interface operating in APCI positive mode (Atmospheric Pressure Chemical Ionisation). Data
180 were processed using Xcalibur software (version 2.0). The operational parameters of the mass
181 spectrometer were as shown below. The spray voltage was 6.0 kV. The temperatures of the
182 heated capillary and of the APCI vaporizer were set to 225°C and 400°C, respectively. The

183 flow rates of auxiliary gas, sheath gas and sweep gas were set (in arbitrary units/min) to 48, 5,
184 and 5, respectively. Tube lens was 50 V, capillary voltage was 1 kV, the front lens was - 6.75
185 V, split lens was 70 V. All parameters were optimized by infusing a standard solution of
186 ferulic acid at 0.1 g L⁻¹ in mobile phase [A: water + TFA (0.03%) / B: phase A (20%) +
187 acetonitrile (80%) – (1:4)] at a flow rate of 5 µl min⁻¹. In addition to full scan between 50 m/z
188 and 1000 m/z, the products were monitored through specific MSⁿ scans (MS², MS³) in order
189 to obtain structural data.

190 **2.5.4. Stability assay**

191 To verify the stability of (FA-CAR) derivatives, three samples of each derivative were
192 prepared in acetonitrile/water (80:20; v/v) (solvent B of HPLC) at concentration 1 mg mL⁻¹
193 and in different pH (2.5, 7, and 11.5). Each sample is analysed by HPLC and LC-MS as a
194 function of time at room temperature.

195 **2.6. Determination of the coupling efficiency by ninhydrin assay**

196 The ninhydrin (NHN) assay as described by Wu and his colleagues (Wu et al., 2005)
197 with some modifications was used to determine the amount of residual free amino groups
198 remaining in CAR after cross-linking. Four milliliters of reaction mixture at various interval
199 times was supplemented with 0.5 mL of 0.8% ninhydrin and 0.5 mL of 0.2 M phosphate
200 buffer (pH 6.0). For blank sample, CAR was replaced with laccase solution since laccase
201 enzyme also contains amino groups. Blank and samples were heated at 100 °C in a water bath
202 for 30 min. Reaction was stopped by placing the test tubes into ice bath and the color changes
203 were detected at 570 nm using a UV-visible spectrophotometer after they were held at room
204 temperature for 10 min. The content of amino groups is calculated by following equation (1):

$$205 \quad \text{Amino group content (\%)} = (\text{Abs}_{\text{TX}}/\text{Abs}_{\text{T0}}) \times 100 \quad (1)$$

206 where A_{T0} is the absorbance of reaction mixture at zero time and A_{TX} is the absorbance of
207 reaction mixture at different times. Each value represents the average ± standard deviation of
208 three independent experiments.

209 **2.7. Physical properties**

210 **2.7.1. logP Solubility measurement**

211 Dispersions (0.3 mM) of FA, CAR and FA-CAR derivative were prepared in 1-octanol
212 and were heated to 60 °C for 1 h to help to solubilise all of tested compounds. UV spectra for
213 each solution were measured and absorbance at the maximum was determined (A₀). Equal
214 volumes of the organic solution (2 mL) and a phosphate buffer (0.1 M, pH 7.4) were
215 vigorously mixed using a vortex mixer for 1 min. The mixture was allowed to stabilize and
216 separate for 30 min; then the organic phase was obtained and its absorbance determined (A_x).
217 Partition coefficient (logP) was determined according to following equation (2):

$$218 \quad P = A_x / (A_0 - A_x) \quad (2)$$

219 All tests were performed in triplicate and results represented the mean values with standard
220 deviation.

221 **2.7.2. Melting point measurement**

222 The melting point of FA, CAR and AF-CAR derivatives was performed using melting
223 device (M-565, BUCHI, Switzerland). The measurement was conducted in Volt 100-240
224 VAC, Frequency 50/60 Hz and power 150 W using capillary tube 10 µL (Ringcaps, Germany)
225 to take samples.

226 **2.8. Antiradical activity by ABTS radical cation decolorization assay**

227 The ABTS radical scavenging activities of FA, CAR and FA-CAR derivatives were
228 evaluated according to the method described by Re et al. (Re et al., 1999). The ABTS^{•+} radical
229 cation was produced by the reaction between 2.45 mM potassium persulfate and 7 mM ABTS
230 in H₂O and allowing the mixture to stand in the dark, at room temperature, for 12–16h before
231 use. For this study, the ABTS^{•+} solution was diluted with ethanol at 30°C, in order to obtain an
232 absorbance of 0.700 ± 0.025 at 734 nm. Then, 10 µL of each molecule sample prepared in
233 ethanol at different concentrations were mixed with 1 mL of diluted ABTS^{•+} solution. The
234 mixture was shaken vigorously for 15 s and then left to stand in the dark at room temperature
235 for 15 min. The absorbance of ABTS^{•+} was measured at 734 nm against an ethanol as a
236 blank. The extent of decolorization is calculated as the percentage reduction of ABTS
237 absorbance by following equation (3):

$$238 \quad \text{ABTS}^{\bullet+} \text{ radical scavenging activity (\%)} = (1 - \text{Abs}_{\text{sample}} / \text{Abs}_{\text{control}}) \times 100 \quad (3)$$

239 ABTS⁺ radical scavenging activity was expressed as Trolox Equivalent Antioxidant
240 Capacity (TEAC) values and the half-maximal inhibition concentrations (IC₅₀). All analyses
241 were carried out in triplicate and results represented the mean values with standard deviation.

242 **2.9. Anti-proliferative activity**

243 **2.9.1. Cells and cell culture**

244 Colonic endothelial tumor (Caco2) cells were kindly provided by the laboratory
245 URAFFPA (Nancy, France). These cells were used between passages 60 and 70, and were
246 cultivated in Dulbecco's modified eagle medium (DMEM) with high glucose (4.5 gL⁻¹)
247 (Sigma, Germany), and supplemented with 10% foetal calf serum (FCS) (EuroBio, France), 2
248 mM L-glutamine, and 1% nonessential amino acids (GIBCO, USA). The cells were usually
249 split when reaching confluence (2–3 days). They were first rinsed with Dulbecco's phosphate-
250 buffered saline without calcium (D-PBS) (Sigma, Germany) and then trypsinised with a
251 solution containing 0.25% trypsin and 1 mM EDTA (GIBCO, USA). For maintenance of the
252 cell line, the cells were seeded at 2 x 10⁴ cells cm⁻² in flasks.

253 **2.9.2. Cell viability evaluation by Neutral red uptake (NRU) assay**

254 To study the cytotoxic properties of FA, CAR and FA-CAR derivatives, Caco2 cells
255 were seeded into 96-well microplates at 5 x 10⁴ cells/well in 195 μL of appropriate culture
256 medium. After 24 h, the cells were exposed to 5 μL of the phenolic compounds solubilised in
257 DMSO (final concentration of DMSO did not exceed 1%) with various concentrations and
258 incubated for 48 h at 37°C, under 5% CO₂ atmosphere. Two controls were used in two
259 columns of the microplate which contain 195 μL of diluted cells: one with 5 μL of D-PBS and
260 another with 5 μL of DMSO solution (final concentration of DMSO was 1%).

261 The NRU (neutral red uptake) assay is a cell viability method, based on the ability of
262 viable cells to bind a neutral red dye within the membranes of intracellular lysosomes
263 (Borenfreund, 1992). After 48 h of incubation, the culture medium (200 μL) was removed
264 from each well of the microplate. The attached cells were rinsed with equal volume of D-PBS
265 at pH 7.2. Then, 200 μL of neutral red solution (50 μg/mL) freshly prepared in the culture
266 medium was added at each well. The microplate was then incubated with the cells and the
267 neutral red solution for 3 h at 37°C. After incubation, the neutral red solution was eliminated
268 and the attached cells were rinsed with D-PBS. The attached cells were solubilised in 200 μL

269 of an ethanol/acetic acid/water solution (50%, 1%, 49%, v/v/v) to release the neutral red
270 remained within the lysosomal or cytoplasmic compartments. The plates were then shaken at
271 room temperature for 10 min. The absorbance of this final solution was measured at 540 nm
272 using a microplate reader (multiscan GO W382TA, Thermo Scientific MIB, France). The
273 relative cell viability was calculated according to the following equation (4):

$$274 \quad \text{Relative cell viability (\%)} = ((1 - (\text{Abs}_{\text{treated cell}}/\text{Abs}_{\text{control}})) \times 100) \quad (4)$$

275 With $\text{Abs}_{\text{control}}$ and $\text{Abs}_{\text{treated cells}}$ are the absorbance values at 540 nm of control and sample
276 with treated cells, respectively. Each test was carried out in six copies, and each experiment
277 was repeated triplicate. The results were expressed as IC_{50} (IC_{50} was defined as the
278 concentration of a molecule leading to 50% of cell mortality) values with the standard
279 deviations. The cytotoxicity was evaluated based on cell viability relative to controls as
280 proposed by Kong (Kong et al., 2009).

281 **2.10. Statistical analysis**

282 The experimental results were performed in triplicate. The data were recorded as means
283 \pm standard deviation (SD) and analyzed by SPSS (version 11.5 for Windows 2000, SPSS
284 Inc.). One-way analysis of variance was performed by ANOVA procedures. Significant
285 differences between means were determined by Duncan's Multiple Range tests. Differences at
286 $p > 0.05$ were considered significant.

287 **3. Results and discussion**

288 **3.1. Laccase characterisation**

289 Protein assay showed that protein concentration in laccase was 8.5 mg mL^{-1} . The
290 optimal activities were determined to be at pH 7.5 and at temperature of $30 \text{ }^{\circ}\text{C}$, using
291 syringaldazine as specific substrate (data not shown). At pH 7.5 and $30 \text{ }^{\circ}\text{C}$, the specific
292 activity of laccase was $0.26 \text{ } \mu\text{mol min}^{-1} \text{ } \mu\text{g}^{-1}$ of enzymatic protein (UI).

293 **3.2. Kinetics of laccase-catalysed reaction**

294 Kinetics showed that the disappearance rate of FA increased in the presence of CAR in
295 comparison with that of FA in the absence of CAR. In fact, in the presence of CAR, the
296 complete disappearance of FA was achieved after 5 h while in the absence of CAR, the

297 complete disappearance of FA was after 6 h as shown in Fig. 1. This result can be explained
298 by the protection of laccase activity from the negative influence of oxidation products that
299 bound to CAR molecule before reaction with laccase protein. Without carnosine, FA oxidized
300 derivatives were produced as demonstrated in a previous study (Mustafa et al. 2005), that can
301 inhibit the laccase activity. In other studies, it was found that the disappearance rate of several
302 substrates including phenols using tyrosinase enzyme was accelerated in the presence of
303 chitosan (Sun, 1992; Wada et al., 1993).

304 Moreover, it was verified that if the laccase was not added to the reaction system, the
305 initial concentration of FA did not decrease in the presence or in the absence of CAR (data not
306 shown). This result indicated that FA could not chemically be oxidized by auto-oxidation and
307 no physico-chemical interactions between FA and CAR occurred. Additionally, in the absence
308 of FA and in the presence of laccase, the initial concentration of CAR did not decrease (data
309 not shown) exhibiting that laccase could not oxidize CAR.

310 In the absence of CAR and by the new addition of FA at reaction medium after 6 h of
311 oxidation reaction, it was found that FA was not oxidized as shown in Fig. 1. Thus, this result
312 indicated that laccase was inactive probably due to the interaction between oxidation products
313 and the active site of enzyme. While, in the presence of CAR, the initial concentration of FA
314 decreased indicating that the laccase is yet active after 6 h of reaction (Fig. 1). This result
315 appeared that CAR could protect the enzyme from the inhibited effect of oxidation products.
316 This could be explained by the possible influence of a suicide inactivation mechanism of
317 laccase through interaction with aqueous reaction products that were blocked by CAR. These
318 results are in accordance to another study that showed that the presence of chitosan led to
319 protect the enzyme from inhibition during the oxidation of FA (Ikehata and Nicell, 2000;
320 Sousa et al., 2009). Previous results have also been reported (Sun, 1992) with tyrosinase using
321 chitosan as sorbent that can trap quinonic intermediates preserving the activity of the enzyme.
322 Recent study demonstrated (Aljawish et al. 2012) that quinonic intermediates covalently react
323 with nucleophilic amine functions of chitosan. Consequently, quinonic compounds did not
324 react with nucleophilic part of enzymatic protein avoiding its inactivation. The same
325 phenomenon might occur with amine function of carnosine and can explain the preservation
326 of enzymatic activity.

327 **3.3. Characterization of (FA-CAR) derivatives**

328 3.3.1. HPLC analysis

329 HPLC analyses of the reaction mixture after 3h of oxidation reaction of FA in the
330 presence and in absence of CAR by laccase enzyme revealed the presence of two new peaks
331 before the FA peak and a major peak after the FA peak as shown in Fig. 2. The major product
332 called P (FA) presenting a retention time (RT) of 27.2 min was already identified as FA-dimer
333 with a molecular mass of 386 g/mol (Aljawish et al., 2014).

334 According to the RT, the first new product was called P1 (FA-CAR) corresponding to
335 RT of 22.5 min and the second new product was called P2 (FA-CAR) corresponding to RT of
336 22.8 min. These new products were released between CAR corresponding to RT of 5 min
337 using HPLC-DEDL (data not shown) and FA at RT of 23.6 min. This result indicated that
338 both products of (FA-CAR) are more hydrophobic than CAR and less hydrophobic than FA.
339 Moreover, these products of (FA-CAR) were not present in the reaction medium in the
340 absence of CAR. Consequently, these results probably indicated that these new products were
341 produced from the coupling between CAR and FA-oxidation products.

342 To determine the optimal time of reaction, the production kinetics of (FA-CAR)
343 derivatives were determined. It was found that the quantity of (FA-CAR) derivatives
344 increased until 4 h and then almost stabilized until 8h as shown in Fig. 3. Consequently, all
345 reactions were conducted during 8h to assure the complete oxidation of FA. After 8h, the
346 production rate of P2 (FA-CAR) relative to P1 (FA-CAR) was 1.6 ± 0.2 .

347 To determine the optimal ratio between FA and CAR, different concentrations of CAR
348 were used and the quantity of (FA-CAR) derivatives at 8h was evaluated. Results showed that
349 the optimal ratio of FA: CAR was 5:8 (mM) as shown in Fig. 4 ($p>0.05$).

350 The UV-spectra of initial substrates (FA and CAR) and new products obtained by
351 HPLC were presented in Fig. 5. P1 (AF-CAR) and P2 (AF-CAR) presented the same UV-
352 spectra. The UV-spectra showed that the maximal absorbance of FA is at 322 nm and no
353 absorbance was observed at 215 nm as reported in another study (Aljawish et al., 2014).
354 Moreover, the maximal absorbance of CAR is at 215 nm as described in another work (Kim et
355 al., 2014). Furthermore, new products presented maximal absorbance at 280 and 215 nm. In
356 fact, after the enzymatic oxidation of FA, the absorbance at 280 nm increased and the
357 absorbance at 322 nm decreased, which is due to FA-oxidation products (quinones) (Aljawish

358 et al., 2014). Moreover, the presence of maximal absorbance at 215 nm suggested that CAR is
359 present in the chemical structure of new products. These modifications in the spectra
360 confirmed that new products (FA-CAR) contain FA-oxidation products and CAR.

361 3.3.2. (FA-CAR) derivatives characterisation

362 The LC-PDA-MS analyses in APCI positive ion mode showed that both (FA-CAR)
363 derivatives observed in UV led to mass spectrometry peaks on full scan MS at $m/z=761$
364 $[M+H]^+$ as shown in Fig. 6. Fragmentation spectrum by MS² of the ion ($m/z=761$) for two
365 (FA-CAR) derivatives allowed the characterisation of the structure (Fig. 6 A-B).

366 For P1 (FA-CAR), fragments of FA- molecule (MM 194), CAR molecule (MM 226)
367 and of FA-dimer (MM 340) from the parent ion ($m/z=761$) were detected. In fact, the ion
368 with $m/z=341$ can be attributed to decarboxylation of dimer ($m/z=387$) leading to $[M+H-$
369 $CO_2-H_2]^+$. The ion with $m/z=327$ is indicative of a stepwise loss of a methyl radical from
370 decarboxylated dimer ($m/z=341$) leading to $[M+H-CO_2-CH_4]^+$. Consequently, the ion with
371 $m/z=567$ can be attributed to FA-dimer ($m/z=387$) after the loss of $[-CO_2-H_2]$ with CAR
372 molecule (MM=226) probably leading to formation of covalent bond. Moreover, the ion with
373 $m/z=374$ can be attributed to FA molecule ($m/z=194$) after the loss of $[-CO_2-H_2]$ with CAR
374 molecule (MM=226) probably leading to formation of covalent bond.

375 For P2 (FA-CAR), fragments of FA- molecule (MM 194), CAR molecule (MM 226)
376 and of FA-dimer (MM 340) from the parent ion ($m/z=761$) were detected. In fact, the ion
377 with $m/z=744$ can be attributed to dehydration of P2 (FA-CAR) ($m/z=761$) leading to $[M+H-$
378 $H_2O]^+$. The ion with $m/z=341$ can be attributed to decarboxylation of dimer ($m/z=387$)
379 leading to $[M+H-CO_2-H_2]^+$. The ion with $m/z=327$ is indicative of a stepwise loss of a methyl
380 radical from decarboxylated dimer ($m/z=341$) leading to $[M+H-CO_2-CH_4]^+$. Moreover, the ion
381 with $m/z=374$ can be attributed to FA molecule ($m/z=194$) after the loss of $[-CO_2-H_2]^+$ with
382 CAR molecule (MM=226).

383 The presence of these fragments suggested that P1 and P2 (FA-CAR) is composed by
384 FA-dimer (MM= 386 g mol⁻¹) and FA molecule (MM= 194 g mol⁻¹) after loss of $[-CO_2-H_2]^+$
385 and one of CAR molecule (MM= 226 g mol⁻¹) at a molecular mass of 760 g mol⁻¹. This result
386 can be probably explained by the oxidation of FA-dimer in the presence of laccase leading to
387 a decarboxylation of dimer and then the coupling with carnosine by the occurrence of a

388 cyclization step involving a dihydroxylation as reported in precedent study between
389 chlorogenic acid dimer and lysine (Prigent et al., 2008). To confirm this hypothesis, FA-dimer
390 (MM= 386 g mol⁻¹) was oxidized using *Myceliophthora thermophila* laccase. The oxidation
391 reaction led to a FA-dimer (MM= 340 g mol⁻¹) mainly due to a decarboxylation through a
392 cyclization step.

393 Finally, results showed that the molecular mass of both (FA-CAR) derivatives is 760
394 g mol⁻¹ corresponding to molecular ions of FA-dimer, FA-molecule and CAR-molecule. As
395 the fragments of both (FA-CAR) derivatives were not identical, each derivative probably
396 presents a different chemical structure. A previous study showed that the chemical
397 conjugation of FA and CAR resulted in FA-CAR derivative with molecular mass of 403.7
398 containing one molecule of FA with one molecule of CAR (Kwak et al., 2009).

399 **3.3.3. Recovery and purification of (FA-CAR) derivatives**

400 After 8 h of oxidation reaction, to ensure a complete oxidation of FA and to avoid the
401 degradation of new products, they were recovered by a rotary evaporator under reduced
402 pressure and then freeze-dried for 24 h. After that, the preservation of recovered products was
403 carried out in the desiccator until use.

404 The purification of two different FA-CAR-derivatives, named P1 and P2, by semi-
405 preparative HPLC allowed a purity of 99.5 % for P1 and 95.6 % for P2. Furthermore, the
406 yields of P1 and P2 are of 5.1% and of 9.3 % respectively compared with initial quantity of
407 FA and CAR. In the literature, the chemical grafting of FA on CAR led to the synthesis of a
408 single molecule with a reaction yield of 48% and a purity of 85% (Kwak et al., 2009).

409 **3.3.4. Stability of (FA-CAR) derivatives**

410 It is known that pKa of CAR and FA is about 9.5 and 4.6, respectively. To verify that
411 the attachment of FA molecules and CAR molecule did not occur by ionic bonds, the
412 influence of pH modification at 2.5 - 7 and 11.5 on the bond was performed. Results of HPLC
413 and LC-MS indicated that no change was detected in HPLC-chromatograms and in molecular
414 mass for each sample at tested different pH after 1- 7 and 14 days at room temperature. These
415 results indicated that the attachment between FA and CAR is not ionic.

416 Moreover, it was shown above (3.2.) that without laccase, ionic interactions were not
417 detected between FA and CAR during the reaction time. Consequently, the attachment
418 between FA and CAR was mainly achieved by covalent bonds.

419 **3.4. Determination of the coupling efficiency of FA and CAR**

420 Laccase-catalyzed oxidation of FA and its subsequent non-enzymatic reaction with the
421 primary amino groups of CAR was investigated. The quantification of -NH₂ groups onto CAR
422 was performed to determine the coupling efficiency between FA and CAR. As shown in Fig.
423 7, it was observed that the percentage of amino groups during enzymatic conjugation
424 decreased. Then, from 6 h to 8 h of reaction time, the amino group content was almost
425 constant. Consequently, about 50 % of free amino groups remained indicating that almost 50
426 % of amino groups were bound with FA-oxidation products during 8 h of reaction time.

427 **3.5. Physical properties**

428 ***3.5.1. Solubility of (FA-CAR) derivatives***

429 The LogP represents the partitioning of compound in an n-octanol/water system. The
430 results showed that logP was 0.05 ± 0.02 for CAR, 1.45 ± 0.05 for FA and 1.10 ± 0.03 for P1
431 (FA-CAR) and 1.21 ± 0.05 for P2 (FA-CAR). In fact, a higher logP value indicates a higher
432 hydrophobicity (Nenadis et al., 2003). In this work, the hydrophobicity of FA-CAR
433 derivatives is lower than FA-oxidation products and FA due to the presence of CAR in the
434 chemical structure of FA-CAR derivatives. Consequently, it was found that FA was soluble in
435 acetonitrile solvent and CAR was soluble in water whereas (FA-CAR) derivatives were
436 soluble in solvent mixture (acetonitrile/water, 4:1). This result is in accordance with a
437 previous result reported by chemical conjugation of FA and CAR (Kwak, et al., 2009). This
438 study showed that LogP of FA-CAR derivative (1.16) was lower than that of FA (1.42) and
439 higher than that of CAR (0.97).

440 ***3.5.2. Melting point***

441 The melting point of a solid is the temperature at which it changes state from solid to
442 liquid at atmospheric pressure. The melting point usually depends on the strength of the
443 intermolecular forces. The stronger the force of attraction between molecules or atoms, the

444 higher the melting point will be, because more energy is required to break these bonds and
445 change the state of the molecule.

446 Results showed that melting points of FA and CAR (173.4 ± 3.3 and 258.5 ± 4.1 ,
447 respectively) are higher than that of P1 and P2 (FA-CAR) derivatives (146.8 ± 3.02 and 134.1
448 ± 5.9 , respectively). These results indicate that the intermolecular forces between atoms of FA
449 or CAR are stronger than that of (FA-CAR) derivatives.

450 **3.6. Antiradical activity by ABTS assay**

451 The ABTS radical scavenging activity method is based on the ability of molecules to
452 quench the ABTS radical cation in comparison with trolox used as reference molecule. The
453 ABTS radical scavenging activities of FA, CAR and P1, P2 (FA-CAR) derivatives were
454 expressed as the half-maximal inhibition concentrations (IC_{50}) and Trolox Equivalent
455 Antioxidant Capacity TEAC (Table 1) ($p < 0.05$).

456 The evaluation of ABTS radical scavenging activity showed that FA exhibited higher
457 radical scavenging activity than trolox (TEAC value higher than 1) corresponding to the
458 results reported by other authors (Aljawish et al., 2014; Nilsson et al., 2005; Perez-Jimenez
459 and Saura-Calixto, 2008). Additionally, the scavenging activity of FA was higher than that of
460 CAR and its derivatives. Antioxidant activity of derivatives was higher than that of CAR.
461 Moreover, P1 (FA-CAR) presented a high radical scavenging activity in comparison with P2
462 (FA-CAR). Consequently, the ABTS radical scavenging activity of tested molecules was in
463 the order: FA > trolox > P1 (FA-CAR) > P2 (FA-CAR) > CAR.

464 In fact, the antioxidant activity of phenolic compounds depends on various structural
465 features such as the OH bond dissociation energy, resonance delocalisation of the phenoxyl
466 radical and the steric hindrance due to bulky groups substituting hydrogen in the aromatic ring
467 (Sanchez-Moreno et al., 1998). The increase in antioxidant capacity of derivatives could be
468 attributed to the presence of phenolic compounds grafted on CAR. Moreover, the difference
469 of antioxidant activity of derivatives can be mainly due to the difference of chemical structure
470 that affects the number of electron donating groups (-OH, -COOH). Finally, the enzymatic
471 coupling of CAR with laccase-catalyzed oxidation products of FA increased the scavenging
472 activity of CAR almost 10 times for P1 and 5 times for P2.

473 In the literature, the FA-CAR derivative obtained by chemical conjugation presented
474 lower free radical scavenging activity (RSA %) (23 ± 3.8) than that of FA (51 ± 1.0) and CAR
475 (91 ± 2.8) using DPPH assay (Kwak, et al., 2009). Whereas, in the lipid antioxidant test, the
476 FA-CAR derivative presented the highest percent lipid peroxidation inhibition (Pi %) ($81 \pm$
477 1.3) in comparison with FA (74 ± 3.0) and CAR (29 ± 3.3). The difference between Pi and
478 RSA is probably due to the hydrophilicity.

479 **3.7. Anti-proliferative activity by NRU assay**

480 The anti-proliferative activities of FA, CAR and their derivatives were evaluated at
481 several concentrations over 48 h against colonic endothelial tumor cell line Caco-2 by the
482 neutral red uptake (NRU) assay. The results were expressed as IC_{50} mean values with standard
483 deviations. Prior to the assay, the effect of 1% (v/v) DMSO (the final concentration of DMSO
484 in the assay) was investigated and no significant cytotoxic effect was observed on the growth
485 of Caco2 cells (data not shown). As shown in Fig. 8, IC_{50} values of anti-proliferative activities
486 for FA, CAR and their derivatives ($p > 0.05$) were determined. FA-CAR derivatives exhibited
487 higher cytotoxic effect against colon cells than the initial molecules (FA, CAR). Moreover, P1
488 (FA-CAR) presented higher anti-proliferative activity than P2 (FA-CAR).

489 In fact, the effect of phenolic compounds on tumor cells is mainly due to their
490 antioxidant effect by which the compound scavenges reactive oxygen species (ROS) and
491 related compounds. Normal cells are highly sensitive to ROS and FA has been demonstrated
492 to reduce ROS-mediated damage in neuronal cell systems (Kanski et al., 2002). Tumor cells
493 may, however, depend on a certain amount of hydrogen peroxide and deprivation of hydrogen
494 peroxide could cause cell cycle arrest or apoptosis of these cells (Loo, 2003). For example,
495 ferulic acid at 1.5 mM decreased the number of Caco2 cells to 43% of control after 2 days of
496 treatment (Janicke et al., 2005).

497 The inhibition effect of carnosine against the proliferation of human colorectal
498 carcinoma cells is mainly due to its effect on the ATP and Reactive Oxygen Species (ROS)
499 production and by inducing the cell cycle arrest (Iovine et al., 2014a). This peptide can also
500 reduce the HIF-1 α protein level affecting its stability and decrease the HIF-1 transcriptional
501 activity. HIF-1 α protein is the major cause of resistance to drugs and radiation in solid tumors
502 (Iovine et al., 2014b). For example, carnosine at 20 mM could reduce cell viability of Human

503 gastric cancer cell line SGC-7901 (SGC-7901) to 46% of control (without carnosine) after 2
504 days of treatment (Shen et al., 2012).

505 Consequently, the higher anti-proliferative activity of FA-CAR derivatives compared
506 with initial molecules (FA, CAR) could be attributed to their radical scavenging activity
507 related to FA and to their inhibition activity of HIF-1 α protein related to carnosine.

508 **4. Conclusion**

509 The present study demonstrated that the presence of carnosine during the course of
510 enzymatic oxidation of ferulic acid could accelerate the disappearance of FA and protect
511 laccase enzyme from the inhibition related to oxidation products. Additionally, this enzymatic
512 process led to the synthesis of two new derivatives from the coupling between carnosine and
513 ferulic acid derived oxidation products. These derivatives were more hydrophobic than
514 carnosine and less hydrophobic than ferulic acid. Both derivatives presented the same
515 molecular mass but not the same chemical structure. The different structures of these two
516 derivatives affected their properties, especially their melting point, antioxidant activity and
517 anti-proliferative activity. The enzymatic coupling between carnosine and ferulic acid
518 oxidation derivatives could improve the antioxidant and the anti-proliferative activities of
519 carnosine and anti-proliferative activity of ferulic acid.

520 These results are very promising as they showed that the new derivatives present
521 improved antioxidant and anti-proliferative properties compared with carnosine. Further
522 works are in progress to study the anti-proliferative effect of new derivatives towards different
523 cancer cell lines in comparison with known drugs used as anticancer agents to validate their
524 potential biomedical applications.

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667 **Table 1:** IC₅₀ values and TEAC coefficients of FA, CAR and FA-CAR derivatives for ABTS
668 radical scavenging activity.

ABTS	FA	CAR	P1 (FA-CAR)	P2 (FA-CAR)	Trolox
IC ₅₀ (mM)	0.007 ± 0.001 ^e	1.8 ± 0.2 ^a	0.18 ± 0.03 ^c	0.39 ± 0.02 ^c	0.009 ± 0.001 ^e
TEAC	1.26 ± 0.02 ^b	0.005 ± 0.003 ^e	0.05 ± 0.003 ^d	0.023 ± 0.001 ^d	1

669 Each value is expressed as mean ± standard deviation (n = 3). Values not followed by the same letter in each
670 column are significantly different at the 0.05% level (Duncan's test).
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Figures captions

675

676 **Fig. 1.** Kinetics of FA oxidation in the presence of CAR (◆) and in the absence of CAR (■) by
677 *Myceliophthora thermophila* laccase in aqueous medium (50 mM, pH 7.5). The arrow at 6h
678 corresponds to the addition of 5mM of FA.

679 **Fig. 2:** Reaction mixture chromatogram of FA (A) and FA-CAR (B) after 3h of oxidation
680 reaction. Column: LiChroCART RP-18, eluent: acetonitrile/water/ TFA (80:20:0.03; v/v/v),
681 flow rate: 0.7 ml min⁻¹. $\lambda=322$ nm.

682 **Fig.3:** Production rate of P1 (FA-CAR) (◆) and P2 (FA-CAR) (■) as a function of reaction
683 time

684 **Fig. 4:** Production rate of P1 (FA-CAR) (■) and P2 (FA-CAR) (■) as a function of FA-CAR
685 ratio at 8h. Values not followed by the same letter are significantly different at the 0.05%
686 level (Duncan's test)

687 **Fig. 5:** UV-spectra collected with HPLC–DAD of FA (A), of CAR (B) and of P1/P2 (FA-
688 CAR) (C)

689 **Fig. 6:** LC-MS spectra: (A) full scan MS spectrum and MS² fragmentation spectrum of P1
690 (FA-CAR) derivative, (B) full scan MS spectrum and MS² fragmentation spectrum of P2 (FA-
691 CAR) derivative

692 **Fig. 7:** Amino group content (%) of CAR during the coupling reaction between FA- oxidation
693 products and CAR.

694 **Fig. 8:** Anti-proliferative activity as IC₅₀ (mM) of FA, CAR, P1 (CAR-FA) and P2 (CAR-FA)
695 toward Caco2 cells. Values not followed by the same letter are significantly different at the
696 0.05% level (Duncan's test)

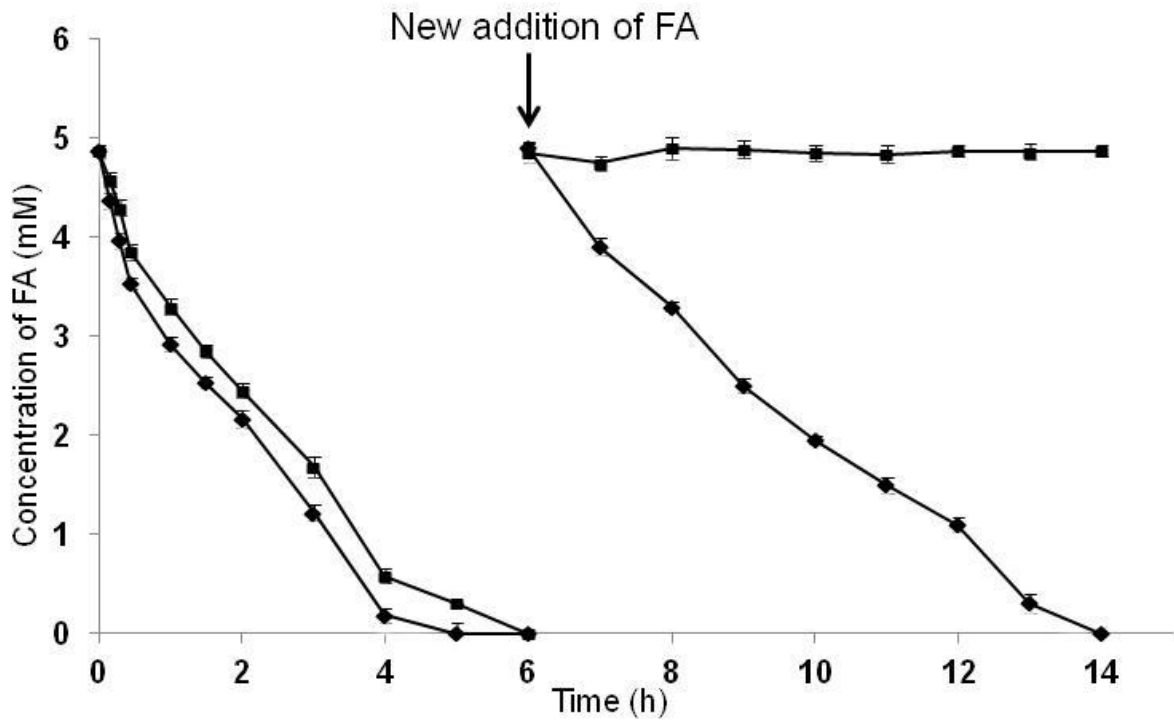
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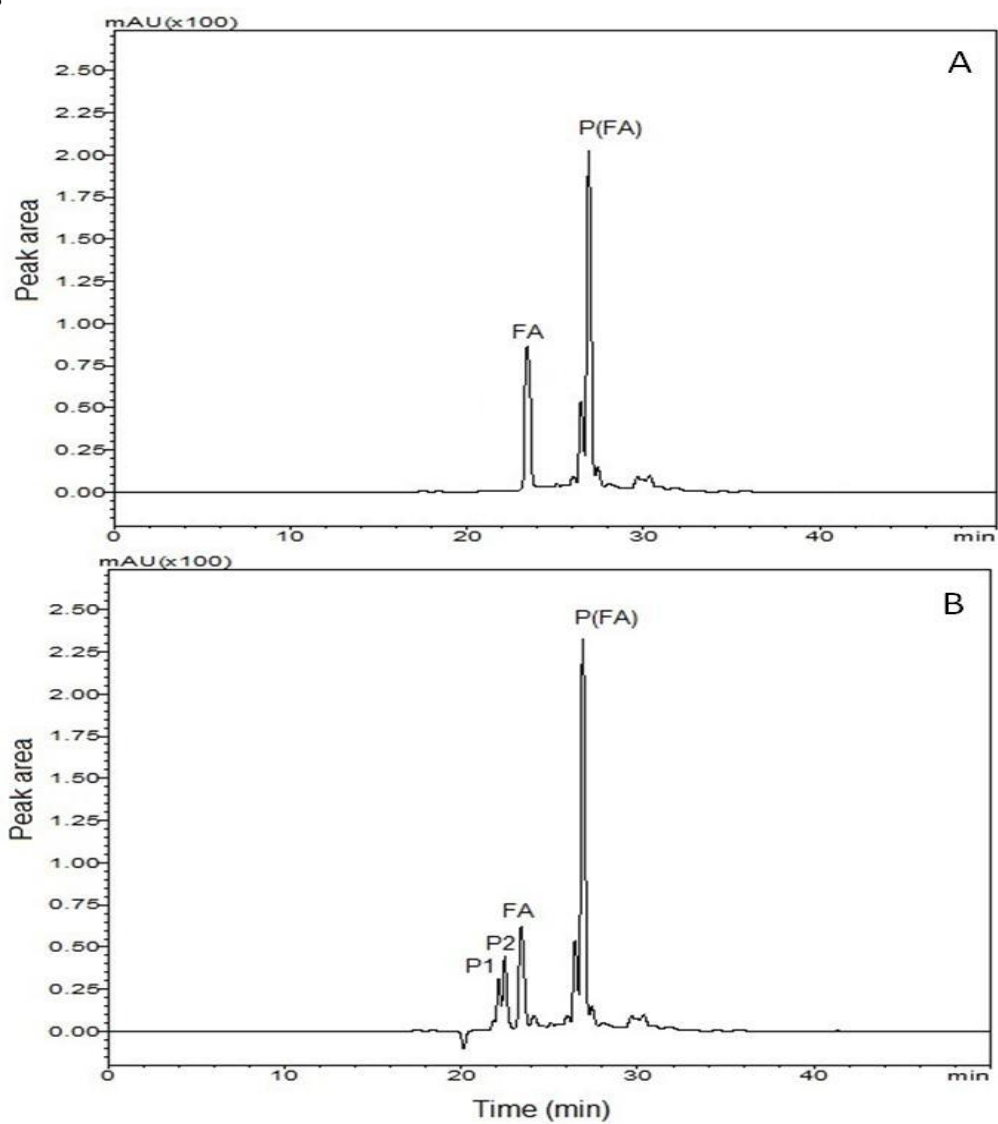
701 **Fig 1**



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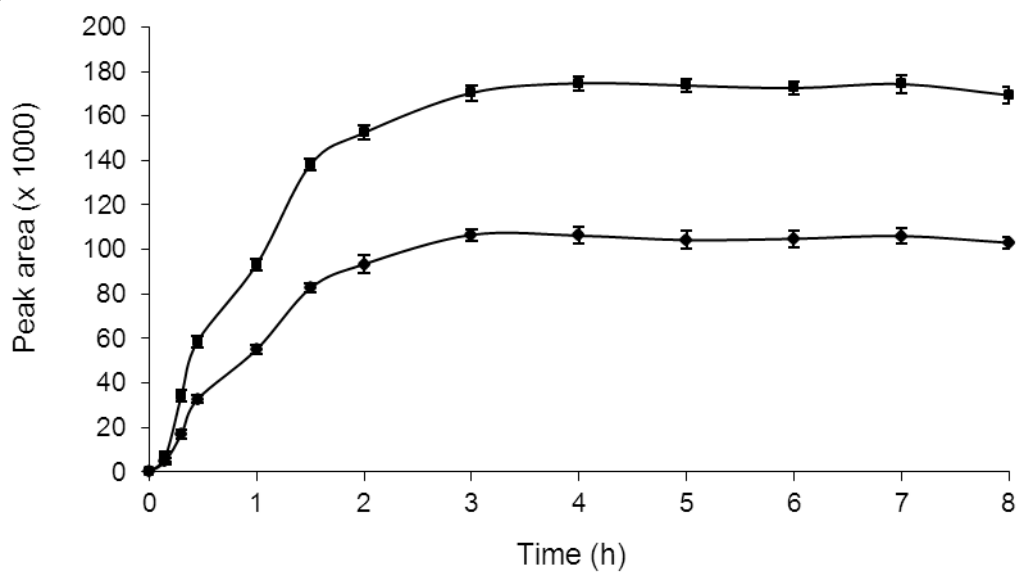
Fig 2



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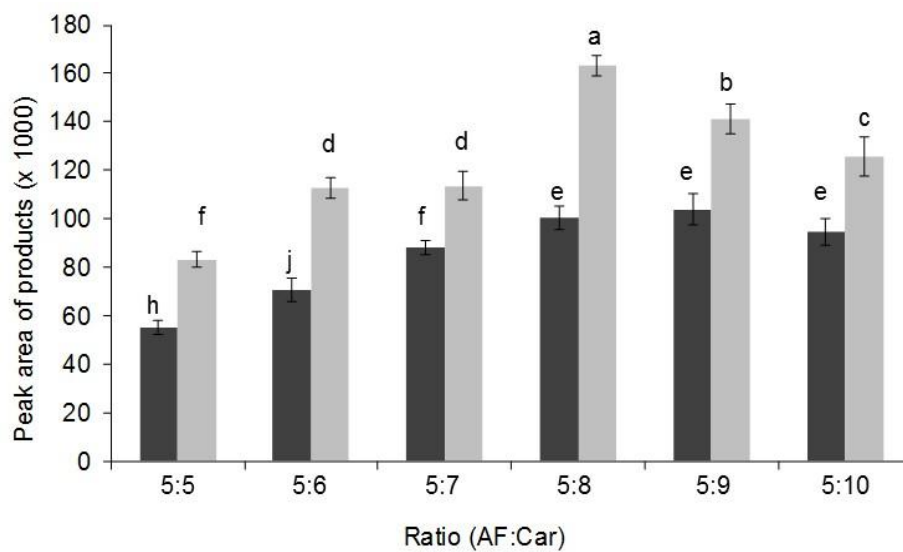
Fig 3



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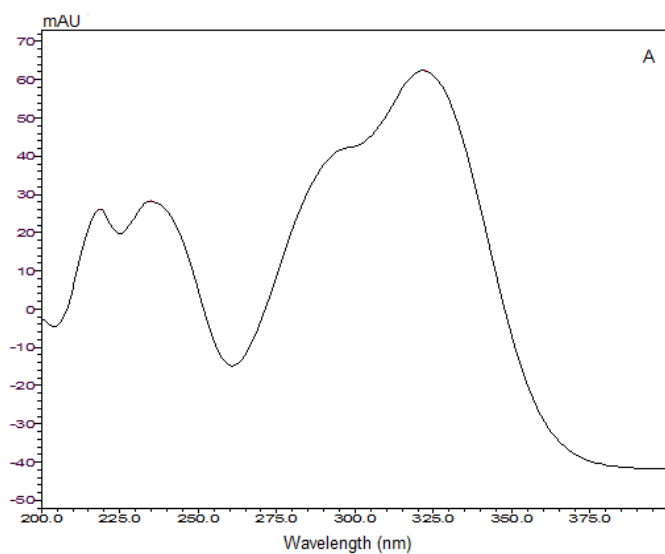
Fig 4



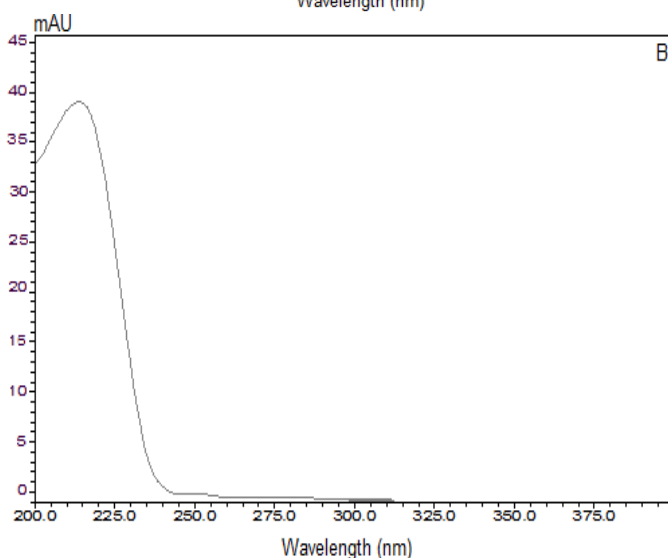
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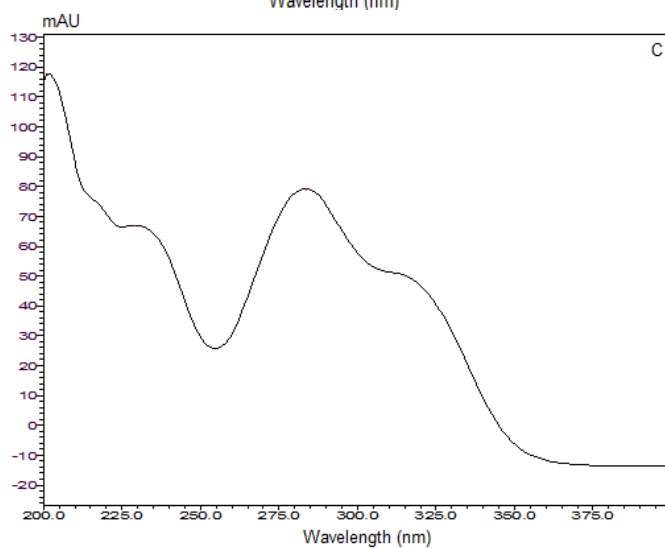
Fig 5



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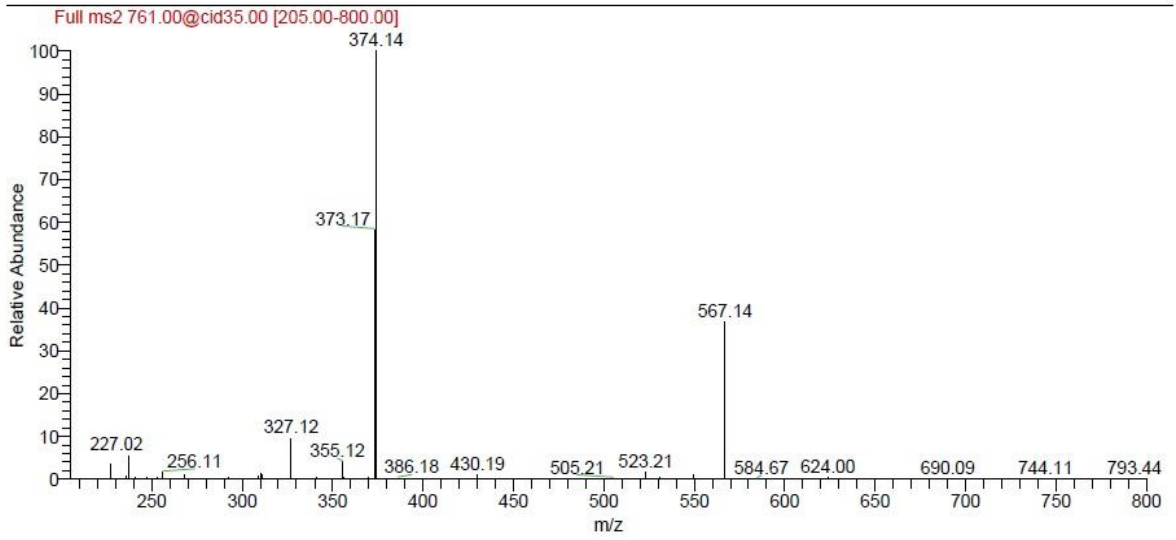
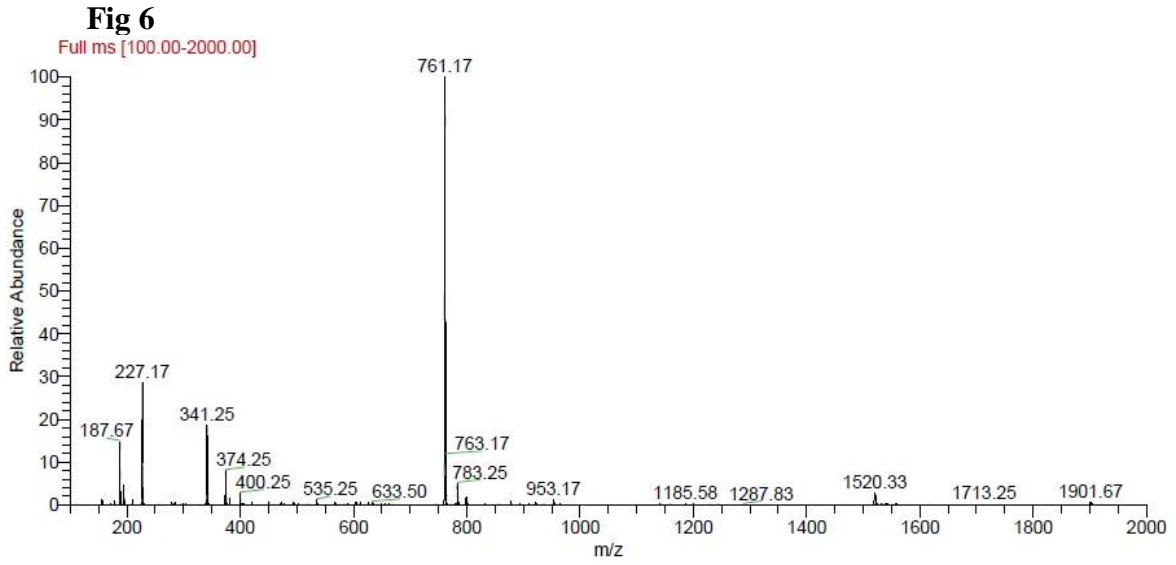


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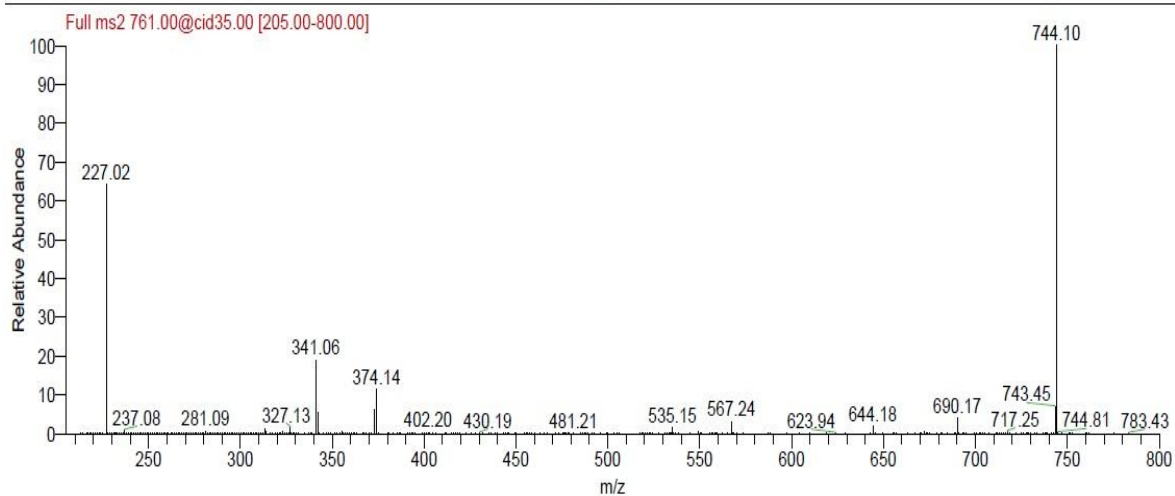
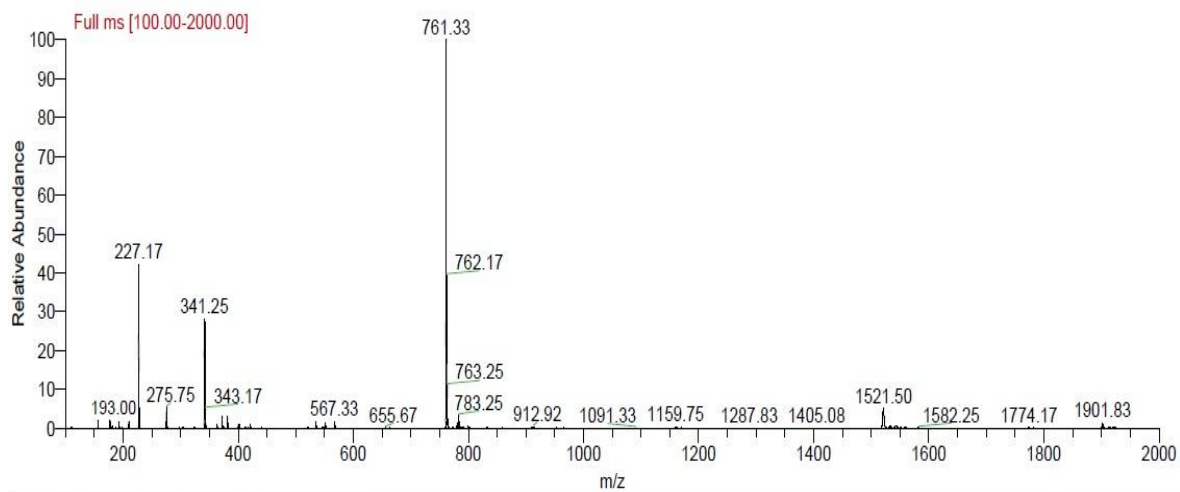
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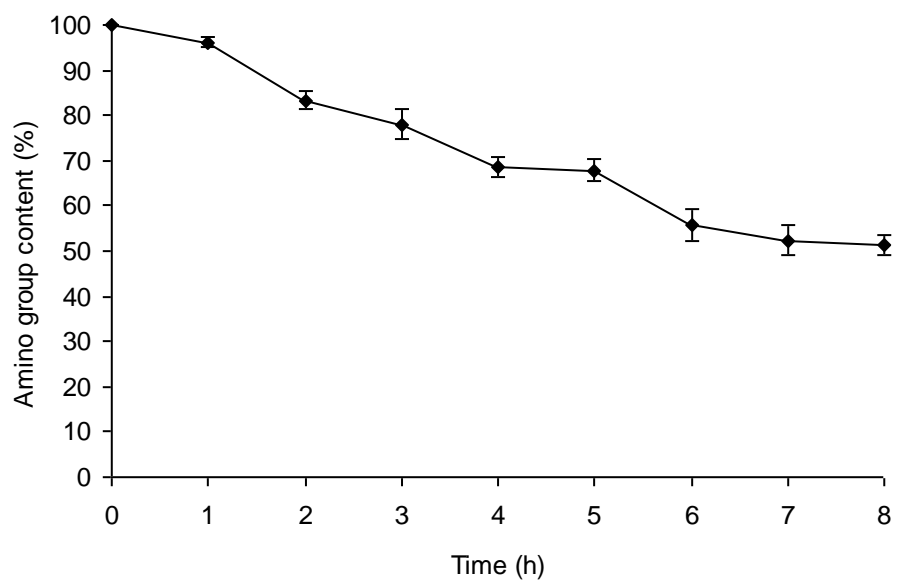


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727 **Fig 7**

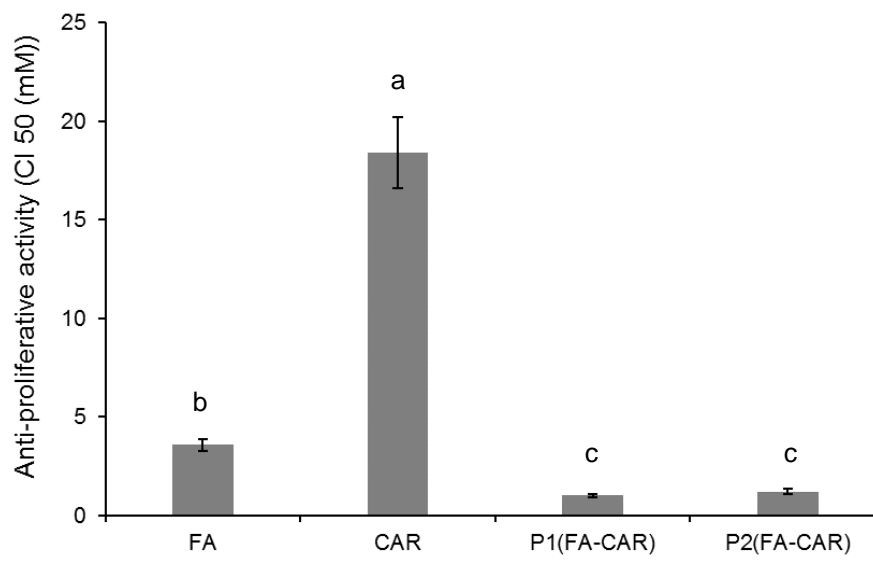


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731 **Fig 8**



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