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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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1. Introduction

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

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

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

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

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

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

2. Materials and methods

2.1. Chemicals and enzyme

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

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

2.2. Laccase characterization

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

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

2.3. Enzymatic reactions

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

2.4. Kinetic analysis of substrate oxidation

To monitor the oxidation of 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 μ m). The detection was conducted between 190 and 800 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. The steps of the gradient were as follow: linear gradient

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

2.5. Characterization of (FA-CAR) derivatives

2.5.1. Recovery of (FA-CAR) derivatives

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

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

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

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

The mass spectra were obtained using the HPLC-MS system which comprised the binary solvent delivery pump and a linear ion trap mass spectrometer (LTQ-MS, Thermo Fisher Scientific, San Jose, CA, USA). LTQ 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 heated capillary and of the APCI vaporizer were set to 225°C and 400°C, respectively. The

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

2.5.4. Stability assay

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

2.6. Determination of the coupling efficiency by ninhydrin assay

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

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

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

2.7. Physical properties

2.7.1. logP Solubility measurement

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

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

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

2.7.2. Melting point measurement

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

2.8. Antiradical activity by ABTS radical cation decolorization assay

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

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

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

2.9. Anti-proliferative activity

2.9.1. Cells and cell culture

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

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

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

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 at 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 (4):

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

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

2.10. Statistical analysis

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

3. Results and discussion

3.1. Laccase characterisation

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

3.2. Kinetics of laccase-catalysed reaction

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

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

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

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

3.3. Characterization of (FA-CAR) derivatives

3.3.1. HPLC analysis

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

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

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

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

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

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

3.3.2. (FA-CAR) derivatives characterisation

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

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

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

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

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

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

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

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

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

3.3.4. Stability of (FA-CAR) derivatives

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

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

3.4. Determination of the coupling efficiency of FA and CAR

Laccase-catalyzed oxidation of FA and its subsequent non-enzymatic reaction with the primary amino groups of CAR was investigated. The quantification of -NH_2 groups onto CAR was performed to determine the coupling efficiency between FA and CAR. As shown in Fig. 7, it was observed that the percentage of amino groups during enzymatic conjugation decreased. Then, from 6 h to 8 h of reaction time, the amino group content was almost constant. Consequently, about 50 % of free amino groups remained indicating that almost 50 % of amino groups were bound with FA-oxidation products during 8 h of reaction time.

3.5. Physical properties

3.5.1. Solubility of (FA-CAR) derivatives

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

3.5.2. Melting point

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

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

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

3.6. Antiradical activity by ABTS assay

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

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

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

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

3.7. Anti-proliferative activity by NRU assay

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

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

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

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

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

4. Conclusion

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

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

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

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

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

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

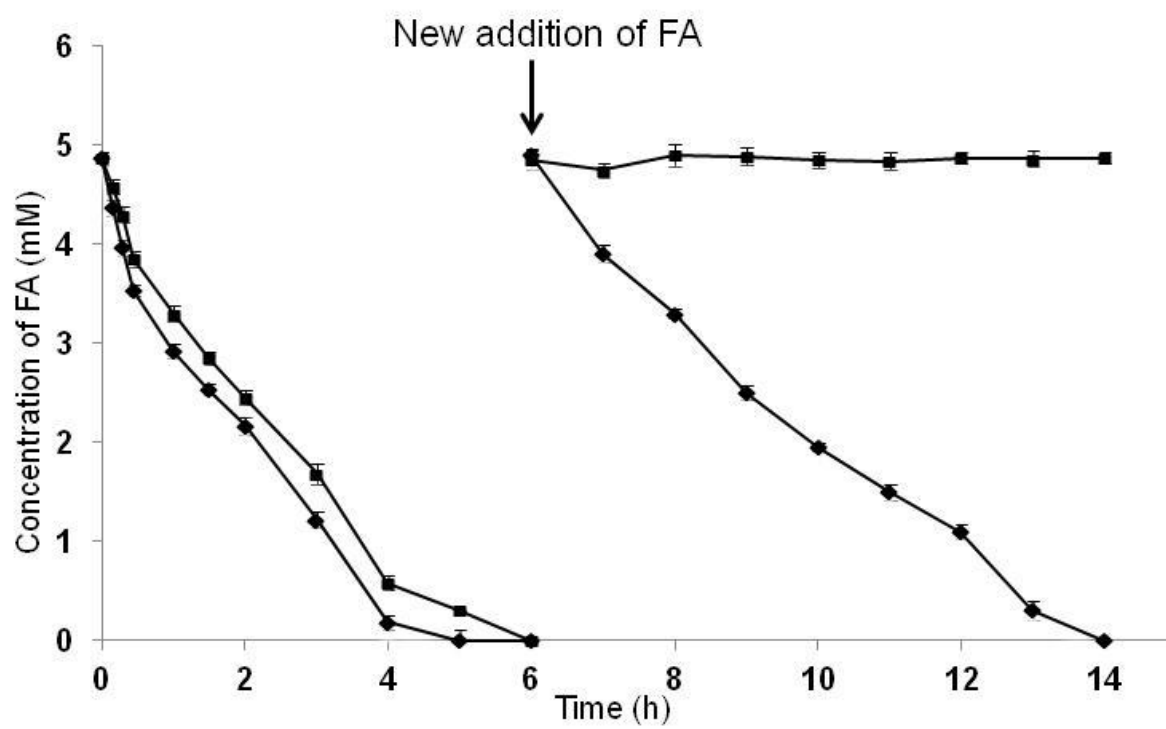
Fig. 5: UV-spectra collected with HPLC–DAD of FA (A), of CAR (B) and of P1/P2 (FA-CAR) (C)

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

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

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

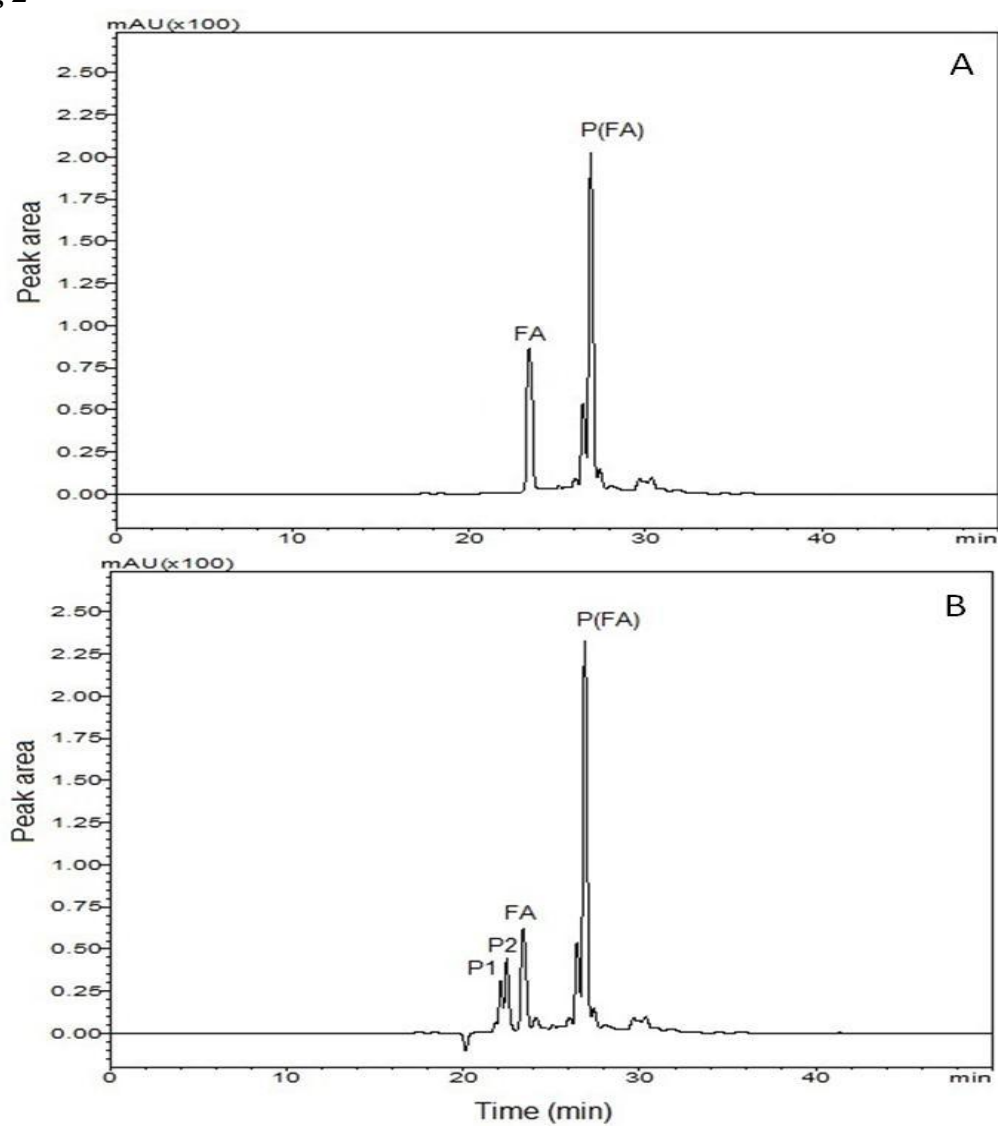
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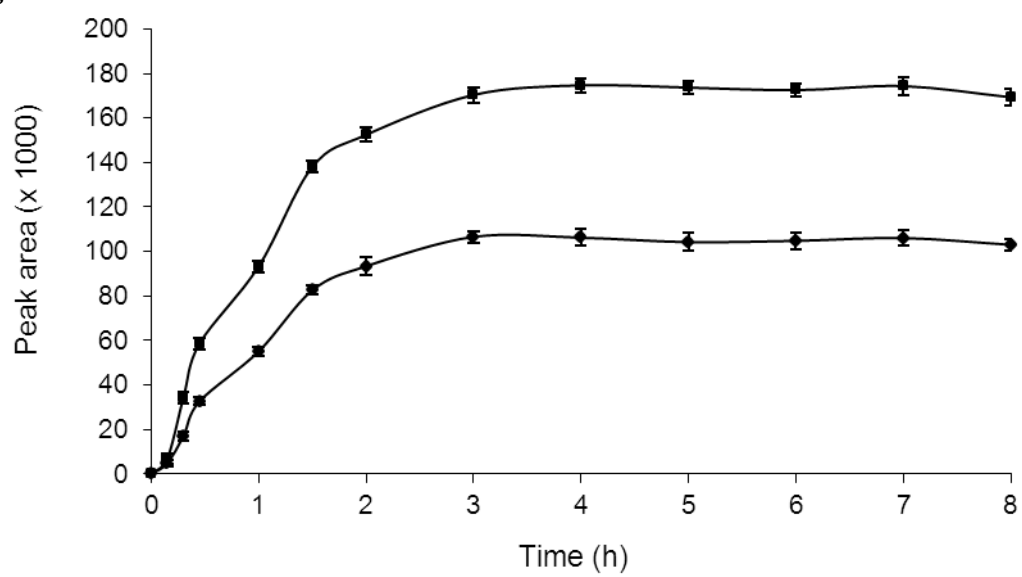
Fig 2



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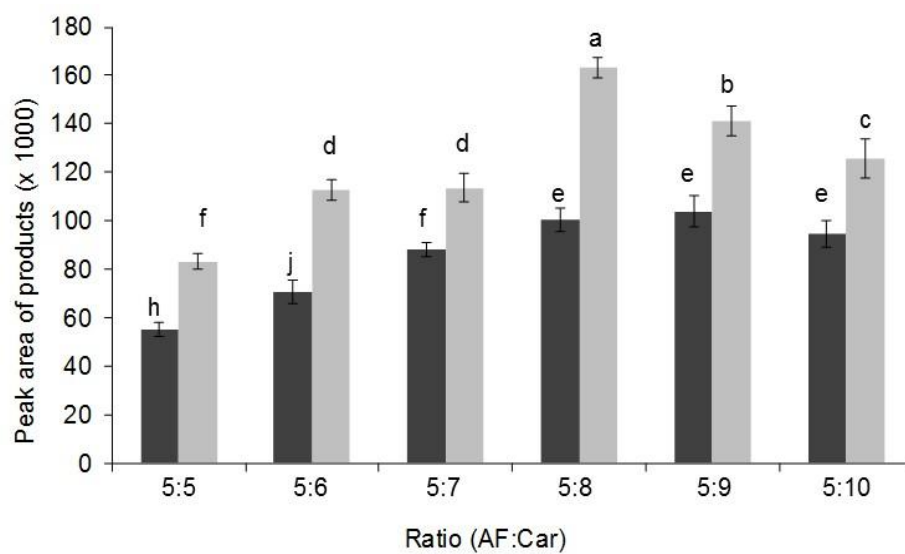
Fig 3



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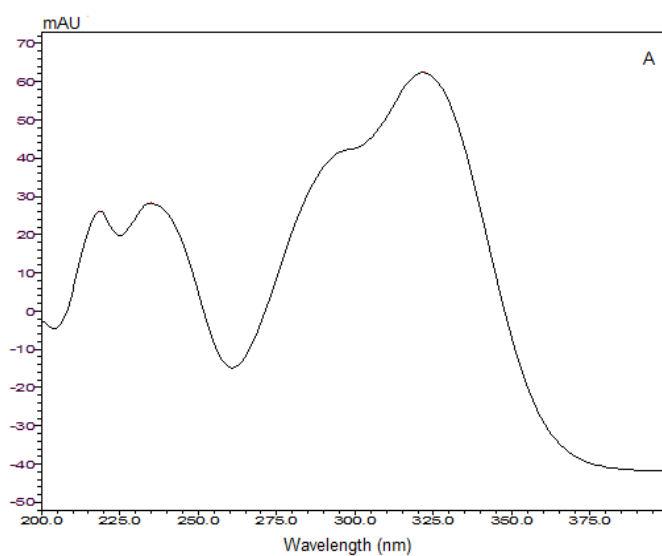
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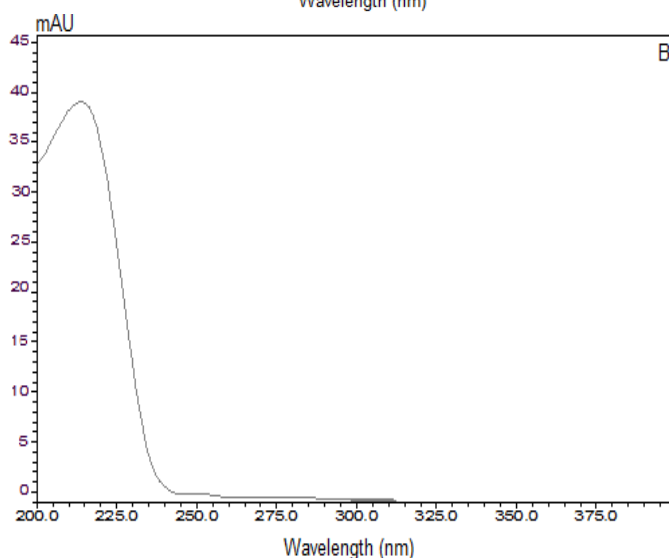
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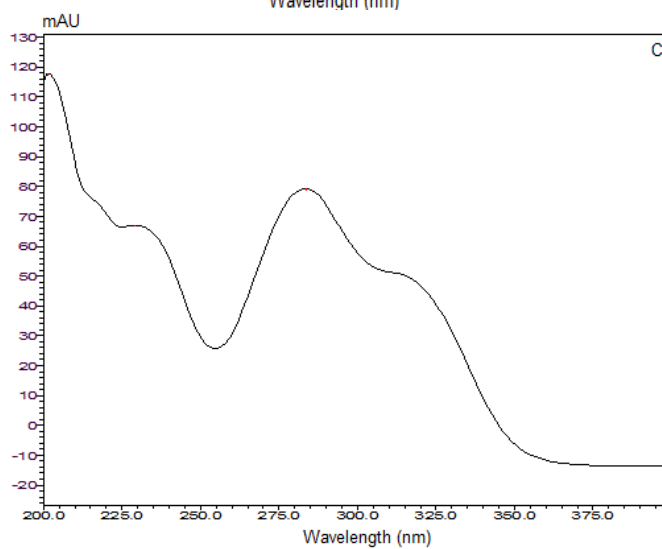
Fig 5



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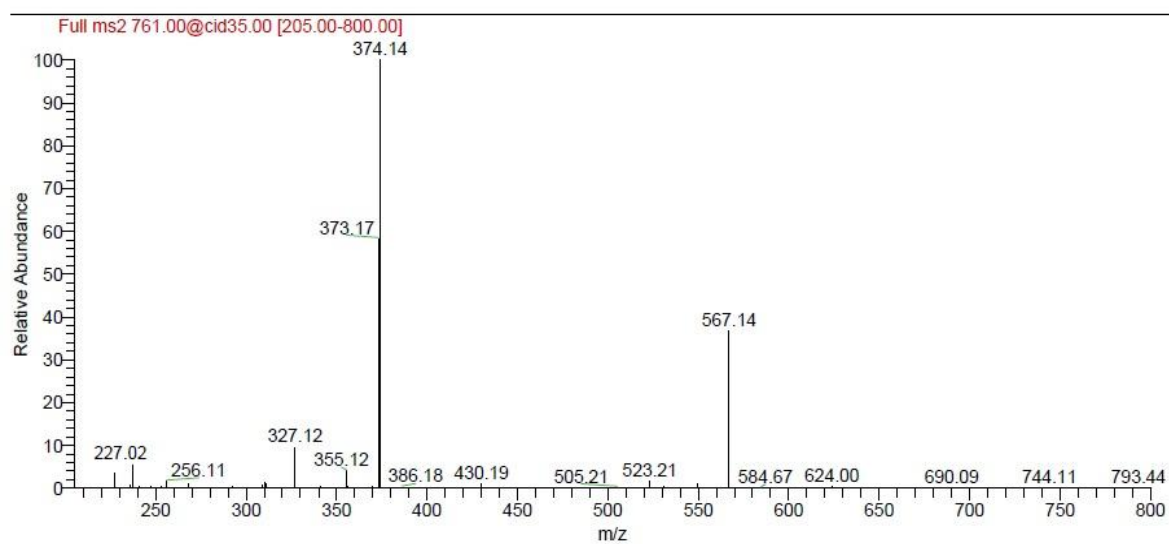
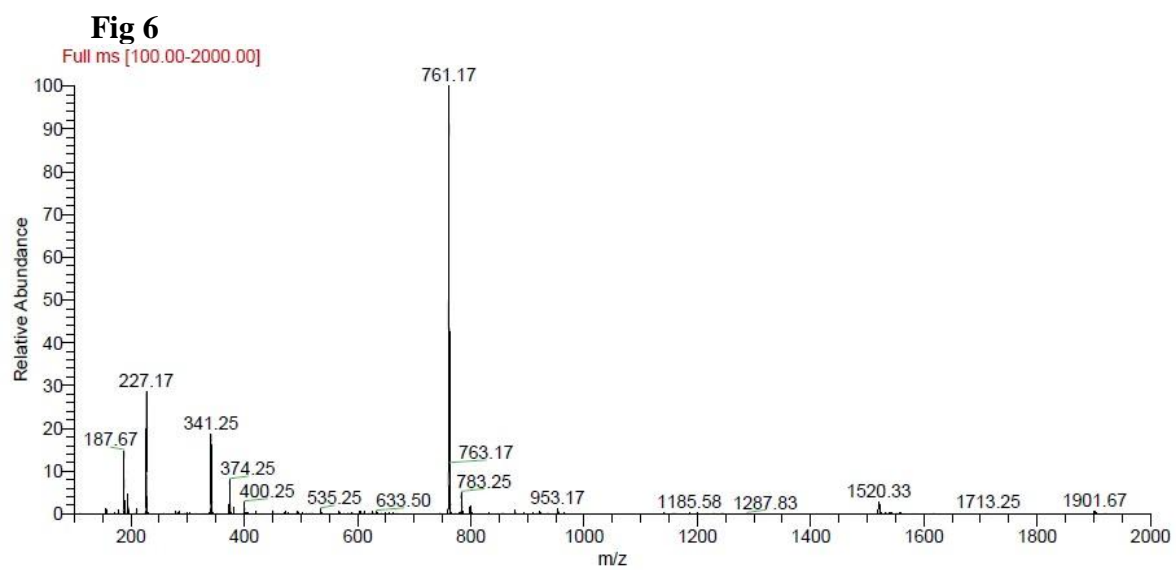


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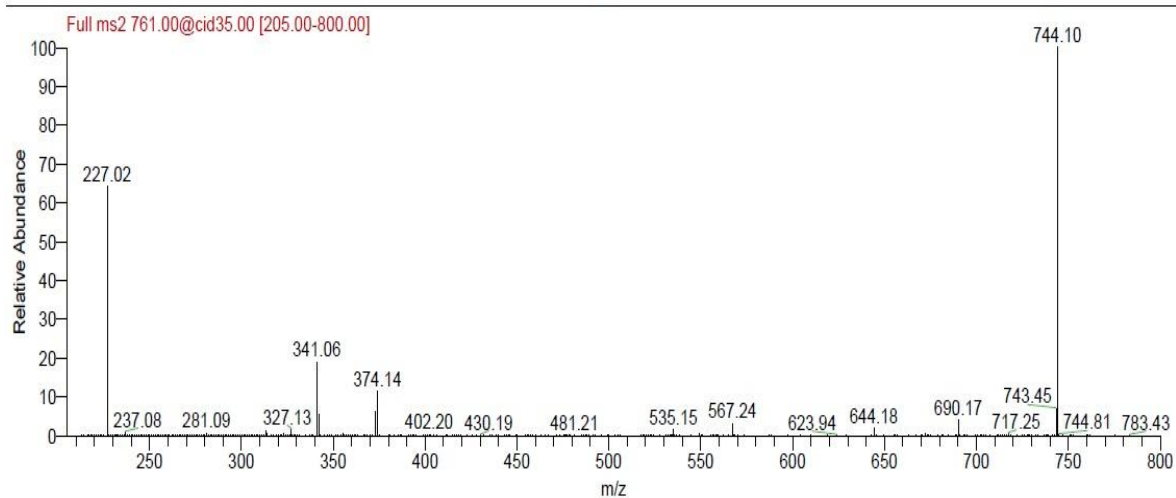
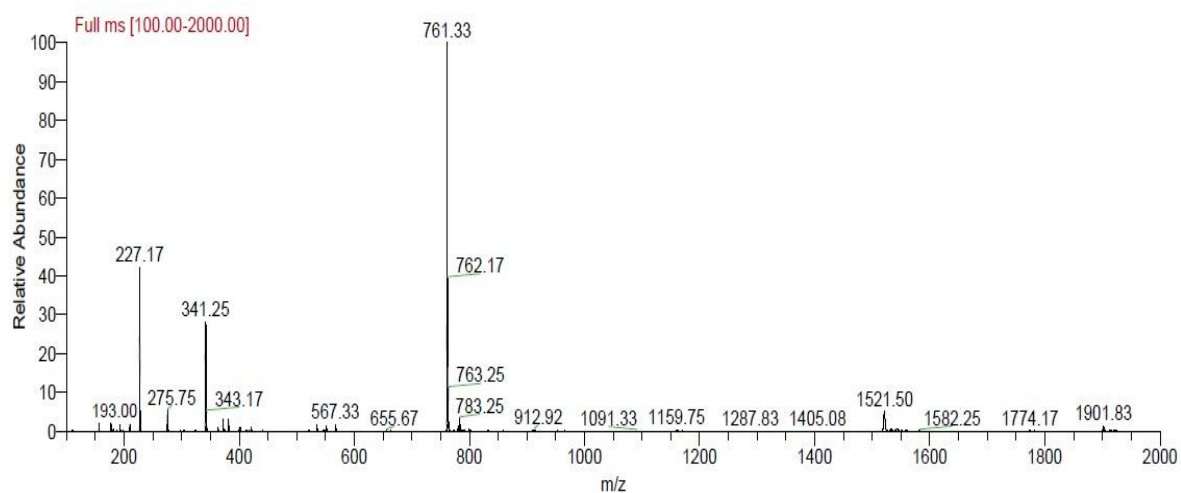
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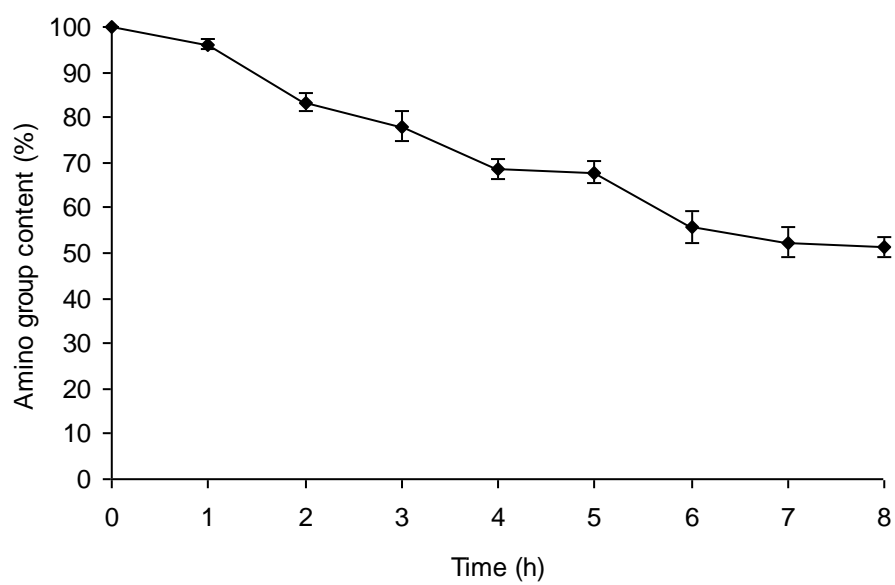
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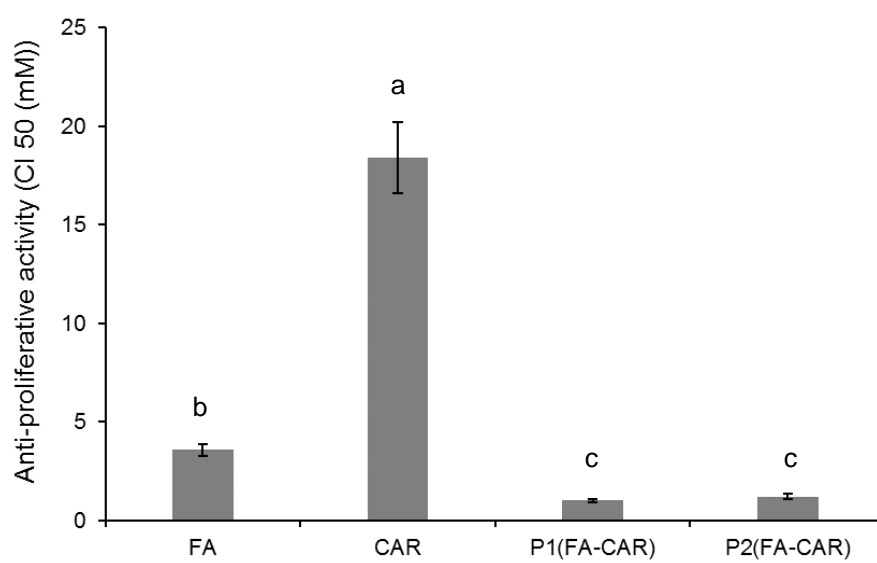
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727 **Fig 7**



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731 **Fig 8**



732
733