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Peptide identification in Alcalase hydrolysated pollen and comparison of its bioactivity with royal jelly

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23

24 **Abstract**

25 Peptides with a similar antioxidant and ACE-inhibitory activity of royal jelly (RJ) generated
26 from Alcalase hydrolysated pollen (AHP) were predicted by Response Surface Methodology
27 (RSM). Later, AHP was prepared and deproteinised to be further analysed using size-exclusion
28 chromatography (SEC). After SEC separation, fractions 49-57, 64-66 and 52-54 of AHP and
29 fractions 43-55 of RJ that showed the highest ACE-inhibitory, DPPH radical scavenging and
30 ferric-reducing power activities, were purified by RP-HPLC. After the separation of fractions 49-
31 57 of AHP, fractions eluting at 3, 4, 5, 37 and 60 min and fractions eluting at 12 to 33 min
32 showed ACE-inhibitory activity higher than 80% whereas fraction eluting at 34 min showed the
33 highest DPPH scavenging activity. 195 peptide sequences were identified by nano-liquid
34 chromatography and mass spectrometry in tandem (nLC-MS/MS). The origins of all identified
35 peptides were herbal proteins and certain similarities with previously described bioactive
36 sequences were discussed.

37 **Keywords**: pollen hydrolysate, RJ, ACE-inhibitory activity, Antioxidant activity, Mass
38 spectrometry, bioactive peptides.

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45 **1. Introduction**

46 Bioactive peptides are protein fragments that have a positive impact on conditions or functions of
47 body and health. Inactive peptides within the sequence of the parent protein can be released
48 through enzymatic hydrolysis, fermentation, pH changes during acidification, heat treatments,
49 and chemical treatments such as glycosylation, acylation, and phosphorylation (Ryder, Bekhit,
50 McConnell and Carne, 2016). Bioactive peptides have been described to usually contain 2–20
51 amino acids in length (Himaya, Ngo, Ryu and Kim, 2012). The beneficial health effects of
52 bioactive peptides include ACE-inhibitory, antioxidative, antimicrobial, antihypertensive,
53 antithrombotic and immunomodulatory activities (Toldrá, Reig, Aristoy and Mora, 2017). The
54 activity of peptides depends on their amino acid composition, sequence length, and molecular
55 mass (Alemán, Pérez-Santín, Bordenave-Juchereau, Arnaudin, GómezGuillén and Montero,
56 2011).

57 Honey bee-derived apicultural products such as pollen and RJ have been applied for centuries as
58 an alternative medicine as well as in food diets and supplementary nutrition due to their
59 nutritional and physiological properties. Nowadays, pollen and RJ are well-known as interesting
60 protein sources. Bee pollen, commonly referred as the ‘‘life-giving dust’’, results from the
61 agglutination of flower pollens with nectar and salivary substances of honey bees and is
62 consumed by honey bees during all their developmental stages (Feás, Vázquez-Tato, Estevinho,
63 Seijas and Iglesias, 2012). RJ is a secretion produced by the hypopharyngeal and mandibular
64 glands of worker honey bees *(Apis mellifera)* (Balkanska, Zhelyazkova and Ignatova, 2012).
65 This secretion is produced in the worker bees' stomach by the incomplete digestion of honeydew
66 (Melliou and Chinou, 2014). RJ and pollen composition include proteins, lipids, carbohydrates,
67 vitamins and minerals. Due to they also contain bioactive compounds, they are known as

68 functional and/or nutraceutical foods (Fatrcová-Šramková, Nôžková, Kačániová, Máriássyová,
69 Rovná and Stričík, 2013). Pollen and RJ contain 10 to 40% and 27 to 41% of proteins,
70 respectively (Bogdanov, 2014). Guo, Ekusa, Iwai, Yonekura, Takahata and Morimatsu (2008)
71 have reported that RJ's proteins and peptides inhibit lipid peroxidation *in vitro* and *in vivo*. RJ's
72 proteins have shown cholesterol-lowering effect and blood pressure lowering activity.

73 There are a considerable number of studies regarding the hydrolysis of foods and its relation with
74 the generation of bioactive peptides. For instance milk, meat, fish, eggshell membrane proteins
75 and plant by-products were hydrolysed using trypsin (Deng, Veer, Sforza, Gruppen and
76 Wierenga, 2017), pepsin (Xu, Cao, He and Yang, 2009), chymotrypsin (Wei and Chiang, 2009),
77 papain (Xu et al., 2009), Alcalase (Shi, Kovacs-Nolan, Jiang, Tsao and Mine, 2014), or Corolase
78 PP (Coscueta, Amorim, Voss, Nerli, Picó and Pintado, 2016; Guan, Diao, Jiang, Han and Kong,
79 2018). Wiriyaphan, Chitsomboon and Yongsawadigu (2012), Moayedi, Mora, Aristoy,
80 Hashemi, Safari and Toldrá (2016) and Lassoued, Mora, Barkia, Aristoy, Nasri and Toldrá
81 (2016) reported that the hydrolysis of food proteins by pepsin, trypsin, Alcalase and *Bacillus*
82 *subtilis* A26 proteases leads to the generation of antioxidant and ACE-inhibitory peptides. On the
83 other hand, an antioxidant enzymatic hydrolysate from honey bee-collected pollen showing 42–
84 46% of DPPH radical scavenging activity was prepared using food-grade proteinase and
85 aminopeptidases entirely of plant origin (Marinova and Tchorbanov, 2010). On the other hand,
86 Nagai, Inoue, Suzuki, Myoda and Nagashima (2005) prepared enzymatic hydrolysates from
87 pollen using pepsin, trypsin, and papain enzymes showing strong antioxidant and radical
88 scavenging abilities. Despite bee pollen is the only source of protein for producing RJ, there are
89 very little studies comparing proteins and peptides of pollen and RJ. Therefore, main objective of

90 this study was the generation and identification of bioactive peptides in AHP and its comparison
91 in terms of peptides profile and bioactive capacity with RJ.

92 ## 2. Material and methods

93 ### 2.1. Chemicals and reagents

94 1, 1-diphenyl-2-picrylhydrazyl (DPPH), potassium ferricyanide, trichloroacetic acid (TCA),
95 ferric chloride and angiotensin-converting enzyme (ACE, from rabbit lung) have been purchased
96 from Sigma (St. Louis, MO). Alcalase® 2.4L serine-protease from *Bacillus licheniformis*, was
97 purchased from Sigma Chemical Co. (St. Louis, MO, USA). Abz-Gly-p-nitro-phe-pro-OH
98 trifluoroacetate salt was obtained from Bachem (Bubendorf, Switzerland). Solvents and
99 chemicals used in the HPLC analysis were from Sigma (St. Louis, MO) and HPLC grade.
100 Solvents and chemicals used in the mass spectrometry analysis were from Sigma (St. Louis, MO)
101 and MS grade. All other chemicals were of analytical grade.

102 ### 2.2. Optimization of hydrolysis conditions using Response Surface Methodology
103 ### (RSM)

104 A factorial in randomized complete block design (RCBD) was used to obtain the combination of
105 values that optimized the response. To identify optimum levels of two variables, the response
106 surface methodology was applied. The two variables (X_1 and X_2) studied were hydrolysis time
107 (1, 2.5, 4 h) and enzyme concentration (1, 1.5, 2 % w/w)[1] respectively.

108 ### 2.3. Preparation of pollen hydrolysate

[1] - Per gram of protein dry weight in substrate

109 The bee pollen and RJ came from hives of *Apis mellifera* bees, collected during spring by local
110 beekeepers of Gorgan, Iran. The powder of pollen was defatted with hexane (1:3) for 24 h using
111 an orbital shaker (Fan, 52E TM Iran, Gostar). The defatted pollen was packed in Ziploc bags
112 after removing residual hexane in oven at 40 °C for 24 h.

113 In order to prepare the enzymatic hydrolysates, the defatted pollens (14.5% of protein) were
114 added, suspended in 5 volumes of 0.1 M potassium phosphate buffer (pH 8) and homogenized by
115 ultrasonic homogenizer (Hielscher, UP100H). The digestion started with the addition of Alcalase
116 at concentrations of 1, 1.5 and 2% to the defatted pollen suspensions. During the reaction,
117 temperature was set at 50 °C and pH was controlled to be kept at 8.0 after incubation in the
118 shaking incubator (Vision Scientific co, LTD). Aliquots of the hydrolysate were removed at time
119 intervals of 1, 2.5 and 4 h and immediately heated at 85°C for 15 min to stop the reaction and
120 then cooled down to room temperature. The hydrolysates were centrifuged at 4000 x g (Hanil,
121 Combi 514R manufactured in Korea) for 30 minutes and supernatants were lyophilized (Operon-
122 FDB5503 manufactured in Korea) and stored at -20°C until use.

123

124 **2.4. Determination of antioxidant activity**

125 **2.4.1. DPPH radical scavenging assay**

126 The DPPH radical-scavenging activity of the hydrolysates was determined as described by
127 Bersuder, Hole and Smith (1998) with minor modifications. Briefly, a volume of 100 μL of each
128 sample was mixed with 500 μL of ethanol and 125 μL of DPPH solution (0.02% in ethanol). The
129 mixture was shaken vigorously and incubated in the dark. After 60 min, the absorbance was
130 measured at 517 nm using a spectrophotometer. DPPH radical-scavenging activity was
131 calculated as:

DPPH radical-scavenging activity (%) =

((Absorbance of control- Absorbance of sample)/ (Absorbance control)) × 100

DPPH has an absorption band at 517 nm which disappears upon reduction by an antiradical compound. Lower absorbance of the reaction mixture indicated higher free radical-scavenging activity. The control was conducted in the same manner, except that distilled water was used instead of sample. BHT[2] was used as positive control. The reduction of DPPH with a hydrogen atom donor (AH) is given by the following reaction:

$AH + DPPH^{\bullet} \rightarrow A^{\bullet} + DPPH{-}H.$

2.4.2. Ferric-reducing antioxidant power assay

The Ferric reducing power assay uses the reducing potential of antioxidant compounds to react with a ferricyanide complex to produce a colored ferrous form. Since this complex has a maximum absorption at 700 nm, it is possible to determine the concentration of ferrous ions by measuring the absorbance of the solution (Yildirim, Mavi and Kara, 2001).

The reducing power of the hydrolysates was measured similar to the method described by Yildrim et al. (2001) with minor modifications. Briefly, 70 µL of sample was mixed with 70 µL of 0.2 M sodium phosphate buffer (pH 6.6) and 70 µL of potassium ferricyanide (10 mg/mL) and incubated for 20 min at 50 °C. Then, 70 µL of TCA (100 mg/mL) was added, mixed, and centrifuged at 2000 rpm for 10 min. After that, 140 µL of the supernatant were taken and 140 µL of distilled water and 28 µL of ferric chloride (1 mg/mL) were added. After standing the mixture

[2] - Butylated hydroxytoluene

152 at room temperature for 10 min, the absorbance was measured at 700 nm. The reduction of ferric
153 ions with a reducing antioxidant (AO) is given by the following reaction: Fe^{3+}+AO→Fe^{2+}+ ferric
154 ferrocyanide (intense blue at 690 nm).

155

156 ## 2.5. Determination of ACE-inhibitory activity

157 The ACE-inhibitory activities of hydrolysates were measured according to the fluorescence
158 based method previously described by Sentandreu and Toldrá (2006). In this assay, the internally
159 quenched fluorescent substrate o-aminobenzoylglycyl-p-nitro-L-phenylalanyl-L-proline (Abz-
160 GlyPhe-(NO2)-Pro) is hydrolysed by ACE to release the fluorescent product o-
161 aminobenzoylglycine (Abz-Gly). 50 microliters of 3 mU/mL ACE preparation in Tris-base
162 buffer (150 mM, pH 8.3) was added to 50µL of sample, and the mixture was pre-incubated at 37
163 °C for 10 min. Then, 200 µL of 150 mM Tris–HCl buffer (pH 8.3) containing 1.125 M NaCl and
164 10 mM Abz-GlyPhe-(NO2)-Pro was added and the reaction mixture was incubated for 60 min at
165 37 °C. The fluorescence intensity was measured using excitation and emission wavelengths of
166 355 and 405 nm, respectively. Bidistilled water was used as negative control whereas Captopril
167 (0.1 mg/mL) was used as positive control in the assay. ACE-inhibitory activity was expressed as
168 percentage.

169 ## 2.6. Size-exclusion chromatography

170 The hydrolysate showing the best ACE-inhibitory and antioxidant activity was deproteinised to
171 be further analysed using size-exclusion chromatography (SEC). In order to deproteinise the
172 hydrolysate, 4 g of lyophilized hydrolysate was dissolved in 20 ml of 0.01N HCL and mixed by
173 magnetic stirrer. The solution was mixed with 3 volume of ethanol and maintained in cold (4-

174 5°C) for 20 h. Then, the mixture was centrifuged (12000 g for 20 min at 4°C), and the supernatant
175 was lyophilized after removing the ethanol by using a rotary evaporator. In the deproteinised
176 hydrolysate, a Sephadex G-25 column (2.5 × 65 cm, Amersham Biosciences, Uppsala, Sweden)
177 was used to fractionate the peptides according to their molecular mass. 100 mg of the freeze-
178 dried deproteinised hydrolysate was dissolved in 5 mL of bidistilled water, filtered using a 0.2
179 µm filter and then eluted with filtered and degassed bidistilled water at a flow rate of 15 mL/h. 5
180 mL fractions were monitored at 214, 280 and 254 nm (Agilent Cary 60 UV–vis
181 spectrophotometer, Agilent Technologies, Palo Alto, CA, USA). All fractions were assayed for
182 antioxidant and ACE-inhibitory activities. Fractions showing the highest activities were pooled
183 together and freeze dried. 1 mg of RJ was dissolved in 5 mL of bidistilled water, filtered using
184 0.2 µm filter and then eluted with filtered and degassed bidistilled water at a flow rate of 15
185 mL/h. The collected 5 mL fractions were monitored at 214, 280 and 254 nm (Agilent Cary 60
186 UV–Vis spectrophotometer, Agilent Technologies, Palo Alto, CA, USA). Fractions showing the
187 highest activities were pooled together and freeze dried.

188

189 **2.7. Reversed-phase high performance liquid chromatography**

190 Fractions obtained from SEC of AHP and RJ were re-dissolved in 130 µL and 100 µL bidistilled
191 water, respectively, and 20 µL was injected into an Agilent 1100 HPLC system (Agilent
192 Technologies) using a Symmetry Prep™ C18 column (7.8 × 300 mm, 7µm) from Waters
193 (Milford, MA). Solvent A was 0.1% TFA in bidistilled water and solvent B consisted of
194 acetonitrile/ bidistilled water (60:40, v/v) containing 0.085% of TFA. The mobile phases were
195 filtered through a 0.45µm filter and degassed. The elution was 100% solvent A for 2 min,
196 followed by a linear gradient from 0% to 50% of solvent B in 50 min at a flow rate of 3.5

197 mL/min. Collected fractions (1 mL) were monitored at 214 nm and assayed for antioxidant and
198 ACE-inhibitory activities for AHP. Fractions showing remarkable activities were freeze dried
199 and further analysed.

200 **2.8. Peptide identification by mass spectrometry**

201 The nano LC–MS/MS analysis was done for peptide identification using an Eksigent Nano-LC
202 Ultra 1D Plus system (Eksigent of AB Sciex, CA) coupled to the quadrupole-time-of-flight (Q-
203 ToF) TripleTOF® 5600 system from AB Sciex Instruments (Framingham, MA) that is equipped
204 with a nanoelectrospray ionization source (nano-ESI). Systems parameters were adjusted as
205 previously reported in Mora, Escudero, Aristoy and Toldrá (2015). The AHP fractions and RJ
206 were re-suspended in double distilled water with 0.1% of TFA to obtain a final concentration of
207 0.2 mg/mL, and 5µl of the supernatant was injected. Preconcentration was done for 5 min, then
208 the trap column was automatically switched in-line onto a nano HPLC capillary column (3µm,
209 75µm×12.3cm,C18) (Nikkyo Technos Co., Ltd. Japan). Mobile phase A contained 0.1% v/v
210 formic acid in water, and solvent B, contained 0.1% v/v FA in 100% acetonitrile. Peptides were
211 first eluted with a linear gradient from 5% to 35% of solvent B over 90 min, and then from 35%
212 to 65% of solvent B for 10 min, at a flow rate of 0.3µL/min and running temperature of 30 °C.
213 Automated spectral analysis, the peak list generation, and the identification of the peptides by
214 database searching were performed using Mascot Distiller v2.4.2.0 software (Matrix Science,
215 Inc., Boston, MA) (hppt://www.matrixscience.com). BIOPEP database was used in the search of
216 similar sequences previously identified showing ACE-inhibitory and antioxidant activity.
217

218 **2.9. Statistical analysis**

219 Optimization of enzymatic hydrolysis was done by central composite design in Response Surface
220 Methodology (RSM) in Design expert 8.0.7.1 (Stat-Ease Company, Minneapolis, Minnesota,
221 United States) software. Also analysis of variance (ANOVA) between variables was conducted
222 by this software. A difference was considered statistically significant when $p < 0.05$.
223

224 3. Results and discussion

225 3.1. Optimisation of antioxidant and ACE-inhibitory activity in AHP and RJ using 226 response surface methodology (RSM)

227 The optimal hydrolysis conditions were predicted by using RSM. The results of the antioxidant
228 activity tests of AHP using the parameters determined with the central composite design in the
229 RSM are given in **Table 1**. Correlation coefficients and variance analysis results of reducing
230 power activity, DPPH free radical scavenging and ACE inhibition are shown in **Table 2**.

231 3.1.1. Ferric reducing power

232 According to results in **Table 2**, relation between reducing power and hydrolysis variables (time
233 and enzyme concentration) were quadratic and their correlation coefficients were $R^2= 0.99$. The
234 model equation obtained is shown as equation 1. X_1, X_2 and Y are time of hydrolysis, enzyme
235 concentration and reducing power, respectively.
236

237 Equation 1:

238 $$Y= 0.331195 + 0.06153 X_1+ 0.49957 X_2- 0.0229 X_1^2- 0.205X_2^2 + 0.03367 X_1X_2$$

239

240 This statistical model was suitable for predicting the effect of variables (time of hydrolysis and
241 enzyme concentration) on the ferric reducing power of AHP. Quadratic and interaction effects

242 were significant ($p< 0.05$). The model was fitted to the ferric reducing power data, because the
243 test of lack of fit hypothesis was not significant ($p> 0.05$) in model equations.
244 Three-dimension plots based on the proposed models (**Fig 1A of Supplementary material**)
245 showed that when increasing enzyme concentration (1.5%) and hydrolysis time (2.5h), the ferric
246 reducing power of AHP increased. However, when a longer hydrolysis time (4h) and higher
247 enzyme concentration (2%) were used, the antioxidant activity decreased. AHP obtained using
248 an enzyme concentration of 1.5% during 2.5 h showed the highest ferric reducing power capacity
249 with a value of absorbance of 0.756. As it is showed in **Table 1**, the highest values of ferric
250 reducing power activity obtained in AHPs were very close to of the observed in RJ.
251 Alcalase enzyme is a serine protease, which has a serine amino acid at its active site. Serine
252 amino acid, in active site of Alcalase enzyme, acts as a nucleophile and cleaves peptide bonds.
253 These characteristics affect the generation of peptides and the donation of electrons by reducing
254 iron ions. By increasing enzyme concentration and hydrolysis time, the degree of hydrolysis
255 increases and produces smaller peptides showing smaller molecular weight and shorter chains
256 with high antioxidant activity (Lassoued, Mora, Barkia, Aristoy, Nasri and Toldrá, 2015).
257 Enzyme activity and speed of hydrolysis decreased after increasing the time of hydrolysis
258 probably due to the reduction of the available substrate.
259

260 3.1.2. **DPPH scavenging capacity**

261 The relation between DPPH scavenging capacity and hydrolysis variables (time and enzyme
262 concentration) were quadratic and correlation coefficients were $R^2= 0.99$. The best model
263 equation for the DPPH radical scavenging obtained from AHP is shown as equation 2. X_1, X_2

264 and Y are time of hydrolysis, enzyme concentration and DPPH radical scavenging capacity,
265 respectively.
266 Equation 2:
267 $$Y=109.96 + 6.5022 X_1 -125.92X_2 + 2.98299X_1^2 + 47.3069X_2^2 - 6.8633 X_1X_2$$
268
269 This statistical model was suitable for predicting the effect of variables (time of hydrolysis and
270 enzyme concentration) on the DPPH radical scavenging activity of AHP. The effect of time was
271 significant ($p<0.05$) in all tested hydrolysates whereas the effect of enzyme concentration was
272 non-significant ($p>0.05$). Also quadratic and interaction effects were significant ($p<0.05$). The
273 test of lack of fit hypothesis was non-significant ($p> 0.05$) in model equations, so the model was
274 fitted to DPPH radical scavenging. Three-dimension plots based on the proposed models (**Fig.
275 1B of Supplementary material**) showed that the DPPH radical scavenging of AH increased by
276 increasing hydrolysis time. AHP prepared using 1% enzyme concentration during 4 h gave the
277 best DPPH radical scavenging activity (78.48%) which was less than that obtained in RJ
278 (95.27%). In previous researches, the antioxidant properties of RJ and pollen were described to
279 be related to their proteins, peptides and phenolic compounds (Bogdanove, 2014). Considering
280 that RJ is produced after the digestion of bee pollen by natural enzymes in honey bee, and that all
281 phenolic compounds of pollen are also found in RJ showing same antioxidant activity (Melliou
282 and Chinou, 2014), it can be concluded that the difference between the antioxidant activity of
283 pollen and RJ is related to their proteins and peptides. Marinova and Tchorbanov (2010)
284 demonstrated that DPPH radical scavenging of pollen increased a 46 % after hydrolysis by plant
285 proteases. In the present study, the highest DPPH radical scavenging activity value obtained after
286 4h of hydrolysis was 78.48%. Some studies concluded that increasing the hydrolysis time leads

287 to the release of antioxidant peptides from the protein chains (Lassoued et al., 2015; Castro,
288 Cason and Sato, 2017).

289

290 ### 3.1.3. ACE-inhibitory activity

291 According to the results of **Table 2**, the relation between ACE-inhibitory activity and variables
292 (time of hydrolysis and enzyme concentration) was quadratic with correlation coefficients of R^2=
293 0.99. The best model equation for the ACE-inhibitory activity obtained from AHP is shown as
294 equation 3. (X_1, X_2 and Y are time of hydrolysis, enzyme concentration and ACE-inhibitory
295 activity, respectively).

296

297 Equation 3:

298 $$Y=-169.981 - 16.3143X_1 + 334.058 X_2 + 2.05885X_1^2 - 116.29 X_2^2 + 8.4133 X_1X_2$$

299 This statistical model was suitable for predicting the effect of hydrolysis variables (time and
300 enzyme concentration) on ACE-inhibitory activity of AHP. The effect of time and enzyme
301 concentration on ACE-inhibitory activity was significant ($p < 0.05$). Also quadratic and
302 interaction effects were significant ($p < 0.05$). The model was fitted to the ACE-inhibitory data,
303 because the test of lack of fit hypothesis was not significant ($p > 0.05$) in model equations. Three-
304 dimension plots based on the proposed models (**Fig. 1C of Supplementary material**) showed
305 that increasing hydrolysis time, ACE-inhibitory activity of AHP also increases. These results are
306 similar to those described by Coscueta et al. (2016). They showed that increasing enzymatic
307 hydrolysis time of soybean flour with Corolase PP, peptides with a molecular weight smaller

308 than 3 kDa were generated. Coscueta et al. (2016) reported that ACE-inhibitory activity of
309 hydrolysates increased due to the formation of small peptides by increasing the hydrolysis time.
310 According to **Fig. 1C of Supplementary material**, increasing the enzyme concentration up to
311 1.5% increased the ACE-inhibitory activity of hydrolysates, although it decreased at higher
312 concentrations. AHP showed the highest ACE-inhibitory activity (87.07%) using 1.5% enzyme
313 concentration and 4h hydrolysis time. These results are similar to those obtained by Lassoued et
314 al. (2015). They reported an ACE-inhibitory activity of thornback ray muscle hydrolysed by
315 Alcalase of 84%. As it is evident in the results showed in **Table 1**, the ACE-inhibitory activity in
316 AHPs was higher than the obtained in RJ probably due to their differences in molecular weights
317 and amino acid sequences as well as peptides polarity. It has been shown that size and
318 hydrophilic–hydrophobic balance of peptides and type of amino acids in the three positions
319 closest to the C-terminal site are important factors in ACE-inhibitory activity (Ktari et al., 2014;
320 Lassoued, Mora, Barkia, Aristoy, Nasri and Toldrá, 2016).
321

322 **3.2. Optimisation of pollen enzymatic hydrolysis based on antioxidant and ACE-**
323 **inhibitory activity**

324 Validation tests to determine the adequacy of the obtained models for the ACE-inhibitory and
325 antioxidant activities of the AHP in predicted time of 4h and enzyme concentration of 1.5% were
326 performed. Predicted and experimental results are shown in **Table 3**. The results showed that it is
327 possible to predict the responses for ACE-inhibitory and antioxidant activities of AHP using the
328 Ferric reducing power and DPPH radical scavenging assays. Finally, AHP generated under
329 predicted condition were selected and used for comparison with RJ.
330

331 ## 3.3. Fractionation and purification of bioactive peptides from AHP and RJ

332 ### 3.3.1. Fractionation of ACE-inhibitory and antioxidant peptides from AHP and RJ
333 by SEC

334

335 In order to understand the role of molecular mass distribution affecting ACE-inhibitory and
336 antioxidant activity of AHPs and to compare RJ and AHP, both were fractionated using SEC.
337 The fractions were measured at two wavelengths: 214 nm and 280 nm.
338 SEC profiles of AHP and RJ were analysed using a Sephadex G-25 column as it is shown in
339 **Fig.1.** AHP showed three main peaks corresponding to proteins with molecular weight ranged
340 from 200 to 70,000 Da according to the chromatogram of standards proteins (see **Fig.1c**). The
341 first peak corresponds to proteins or peptides having a molecular mass between 13,000-70,000
342 Da and second peak between 1423-13,000Da. The third peak corresponds to low molecular
343 weight fragments since they are eluted later than the standard bacitracin (1423 Da). Regarding
344 RJ sample, SEC profile also showed three main peaks corresponding to molecular masses of
345 70,000Da, 13,000Da, and 200 Da, according to the standards. Comparing both SEC patterns it
346 can be concluded that hydrolysis of pollen by Alcalase led to the generation of peptides with a
347 similar molecular weight profile than those existing in RJ. Many researchers have shown
348 Alcalase as a very effective enzyme used in the preparation of protein hydrolysates obtaining
349 peptides covering a wide range of molecular weights and diverse biological activity (Silva,
350 Fonseca and Prentice, 2014).
351 The use of SEC as first purification step followed by RP-HPLC for the separation of enzymatic
352 hydrolysates is very common (Jemil et al., 2016; Lassued et al., 2015). Several researchers have
353 fractionated protein hydrolysates using SEC and then studied the effects of molecular size on

354 ACE-inhibitory and antioxidant activities of resulting fractions (Lassued et al., 2015; Moayedi
355 et al., 2016). As reported in **Fig.2B**, the highest ACE-inhibitory activity of AHP (100%) was
356 observed in fractions 49 to 57 which correspond to a molecular weight of 13000 kDa.
357 Furthermore, fractions 58 to 66 and 68 to 93 also showed ACE-inhibitory activity of 95% and
358 80-90%, respectively. These fractions correspond to molecular weights ranged from 200 to
359 1420 kDa, according to the standards. Generally, it is believed that low molecular weight
360 peptides display stronger ACE-inhibitory activity (Lassoued et al., 2016; Ruiz, Dávila-Ortíz,
361 Chel-Guerrero and Betancur-Ancona, 2013). In the present study, fractions 49 to 57 showed
362 higher ACE-inhibitory activity compared to fractions 58 to 93, indicating that lower molecular
363 weight peptides do not always show higher activities (Moayedi et al., 2016; Ruiz et al., 2013). It
364 has been shown that both, size and hydrophilic-hydrophobic balance of peptides are important
365 factors in ACE-inhibitory activity (Ktari et al., 2014). The highly hydrophilic peptides are
366 inaccessible to the active site of ACE. This fact could explain the lower activity detected in
367 smaller peptides obtained in AHP since it contains high amount of hydrophilic peptides;
368 however it may also be related to the inherent properties of the proteins and peptides of pollen.
369 Finally, the last fractions of SEC separation mainly contain free amino acids. Free amino acids
370 have been reported to show lower bioactivities than peptides (Lassoued et al., 2016). As
371 reported in **Fig.2E**, the highest ACE-inhibitory activity of RJ (78.77%) was observed in
372 fractions 43 to 55 which correspond to a molecular weight around 13000 kDa, according to the
373 standards. Furthermore, fractions 25 to 40 also showed ACE-inhibitory activity of 21 %
374 corresponding to a molecular weight around 70000 kDa, according to the standards.
375 Fractions from SEC were also assayed for their antioxidant activity using two *in vitro* assays:
376 DPPH radical-scavenging and ferric reducing power. As it is showed in **Fig. 2A**, the highest

DPPH radical scavenging activity of AHP (82.5%) was observed in fractions 64 to 66 which correspond to a molecular weight of 1420 Da. Furthermore, fractions 46 to 48 also showed DPPH radical scavenging activity of 69.1%. These fractions correspond to a molecular weight of 13000 Da. The highest peak value for ferric reducing power assay of AHP (1.65) was observed in fractions 52 to 54 (**Fig. 2C**) correspond to a molecular weight around 13000 Da, according to the standards. In this regard, it was reported that peptides with molecular weight less than 3000 Da showed most antioxidant activity (Kim, Je and Kim, 2007; Lassoued et al., 2015). As it is observed in **Fig. 2D** and **Fig. 2F**, the highest DPPH radical scavenging of RJ (52.58%) and the highest reducing power of RJ (0.99) were observed in fractions 43 to 55 which correspond to a molecular weight around 13000 kDa, according to the standards. In fact, the highest antioxidant and ACE-inhibitory activities of RJ were detected in those fractions showing the highest absorbance.

On the other hand, it has been proved that major proteins of RJ with molecular masses of 49-87 kDa, was accounted 90% of total RJ protein. These proteins are responsible for functional properties of RJ. The physiological functions of major RJ protein have been widely studied (Bíliková, Mirgorodskaya, Bukovská, Gobom, Lehrach and Simúth, 2009).

In this study, the most potent bioactive fractions in AHP were at similar retention times than in RJ. Pollen proteins are the main source of RJ proteins. RJ is produced in the worker bees' stomach after the incomplete digestion of pollen (Melliou and Chinou, 2014) and this suggests that main proteins of RJ have probably been obtained from the hydrolysis of pollen proteins (Bogdanove, 2014). AHPs showed higher antioxidant and ACE-inhibitory activity than RJ which was probably due to higher concentrations of these fractions in hydrolysates. Based on

399 these results, RJ and fractions 49 to 57 and 64 to 66 of AHP were selected for further
400 purification using RP-HPLC.

401

402 3.3.2. Fractionation and purification of ACE-inhibitory and antioxidant peptides 403 from AHP and RJ by RP-HPLC

404 RP-HPLC is the most used method to isolate peptides from protein hydrolysates based on their
405 hydrophobic/hydrophilic characteristics. Several researchers have used RP-HPLC analysis to
406 determine the hydrophilic/hydrophobic peptide ratio of the protein hydrolysates and have used
407 some assays to evaluate antioxidant and ACE-inhibitory activity. As an example Lassoued et al.
408 (2015) and Jemil et al. (2016), used this technique to study protein hydrolysates from bovine-
409 lactoglobulin, thornback ray (Raja clavata) muscle hydrolysates and zebra blenny (*Salaria*
410 *Basilisca*) muscle protein, respectively. Reversed-phase chromatographic separation of the
411 selected pooled fractions (49 to 57) and (64 to 66) obtained from size-exclusion chromatography
412 of AHP (**Fig. 3)** revealed a large number of peaks. Considering two aromatic amino acids,
413 Tyrosine (as a hydrophilic compound) and Tryptophan (as a hydrophobic compound), the area
414 under the chromatograms was divided in three zones. Zone 1 consisted the hydrophilic peptides
415 eluted before tyrosine (before 13 min). Zone 2 contained low hydrophobic peptides eluted
416 between tyrosine and tryptophan (between 13 and 26 min) and zone 3 comprised of high
417 hydrophobic peptides eluted after tryptophan (after 26 min) (Lassoued et al., 2015). According to
418 this, heterogeneous composition of hydrophobic and hydrophylic peptides of AHP fractions are
419 shown in **Fig. 3**. The fractions were automatically collected and assayed for their ACE-inhibitory
420 activity (**Fig. 3A**) and ferric reducing power (**Fig. 3B**) in fractions 49 to 57 and DPPH radical

421 scavenging capacity in fractions 64 to 66 (**Fig. 3C**). Regarding ACE-inhibitory activity, a total
422 of 26 fractions showed ACE-inhibitory activity with an average activity higher than 80% (**see**
423 **Fig. 3A**). Among them, fraction eluting at 3 min showed ACE-inhibitory activity of 100%,
424 whereas those at 4, 13, 16, 19, 25 and 60 min showed the high activity of 96%, 92%, 90%, 91%,
425 92% and 98%, respectively. ACE-inhibitory peptides may bind to active site of the ACE or to an
426 inhibitor site located on the ACE, hence modifying the protein confirmation and preventing the
427 Angiotensine from binding to the ACE active site (Ktari et al., 2014). The profile of ferric
428 reducing power in AHP (fractions 49 to 57) showed one peak at 3 min with high values of
429 activity (see **Fig. 3B**). Regarding DPPH scavenging capacity of fractions 64 to 66 in AHP,
430 fraction eluting at 34 min showed the highest activity (66.61%) whereas fraction eluting at 11
431 min showed DPPH scavenging activity of 20% (see **Fig. 3C**). In this case, low hydrophobic
432 peptides showed the highest DPPH radical scavenging. For fractions 49 to 57 obtained from
433 SEC, fractions eluted from 3 min to 4 min and 25 min in RP-HPLC were selected for
434 identification.

435 Reversed-phase chromatographic separation of the selected pooled fractions 25 to 40, 43 to 55,
436 78 to 80 and 117 to 125 obtained from size-exclusion chromatography of RJ are shown in **Fig. 2**
437 **of Supplementary material (A, B, C and D).**

438 The presence of proteins and peptides in RJ prove the influence of honey bee enzymes such as
439 endopeptidases and exopeptidases in the final composition of RJ. Honey bees use these enzymes
440 for the digestion of pollen proteins and changing the pollen proteins to major proteins of RJ
441 (Jamnik, Raspor and Javornik, 2012). The fact that previous studies reported the presence of
442 several phosphorylated, glycosylated, methylated and deamided proteins in RJ could explain the
443 heterogeneous composition of peptides in the RP-HPLC profile of RJ. When proteins undergo

444 some modifications, their charge and polarity change. Protein modifications may also increase
445 their functional properties (Bíliková, Mirgorodskaya, Bukovská, Gobom, Lehrach and Simúth,
446 2009). Zhang, Wei, Wu, Hu and Dietemann, (2012) reported that once a methylation
447 modification occurs, it covers up a negative charge and enhances the hydrophobicity of the
448 protein. Protein methylation modification plays key roles in changing the biochemical features of
449 proteins. Finally, RJ and fractions 49 to 57 obtained from SEC, which were eluted from 3 min to
450 4 min and 25 min were selected for identification.

451 **3.4. Identification of ACE-inhibitory and antioxidant peptides by mass spectrometry**
452 **in tandem**

453 Peptides from RJ and selected fractions from AHP were analysed by nano-liquid
454 chromatography and mass spectrometry in tandem (Q-ToF). A total of 195 peptide sequences
455 were identified in all samples as it is showed in **Table 1 of supplementary material.**

456 The protein of origin in all the identified peptides in RJ and AHP fractions were 27 and 50 herbal
457 proteins, respectively. The 50% of origin proteins for fractions of AHP and RJ were same
458 proteins. The amino acid sequences of the identified peptides were characterized as well as their
459 position in the protein, posttranslational modifications, observed molecular masses, theoretical
460 molecular masses and mass/charge ratio (*m/z*) are also shown in **Table 1 of supplementary**
461 **material**.

462 Major RJ proteins with a molecular weight higher than 49 kDa were not identified in this study.
463 In previous studies, it has been determined by proteomics that the major proteins of RJ are multi-
464 functional proteins attributed to honey bee. Also it has been previously reported that the major
465 RJ proteins genes are expressed in honey bee body, as these proteins can be made by honey bee

466 (Zhang et al., 2012). Schönleben, Sickmann, Mueller and Reinders (2007) achieved a high
467 coverage of the RJ proteome using a proteomic approach. They identified a total of 20 different
468 proteins, as well as demonstrate a very high degree of cleavage of different proteins of the major
469 RJ protein family and investigated protein phosphorylation of RJ proteins. Zhang et al. (2012)
470 reported potentially posttranslational modified (phosphorylated and glycosylated) RJ proteins.
471 Methylation and deamidation were also identified in most of the major RJ proteins.

472 Despite there are few studies about the mechanism of peptide generation in RJ, it is interesting to
473 note that none of the natural peptides of RJ identified in the current study have been attributed to
474 honey bee as they are originated from the source of the plant species which the pollen was
475 collected by honey bee (see **Table 1 of supplementary material**). Therefore, it can be
476 concluded that probably these peptides have not been produced in the honey bee body. It is
477 proposed that the honey bees do not have the gene involved in the production of these peptides.
478 Some of these peptides are naturally found in pollen and the honey bee receives them directly by
479 eating pollen (Schönleben et al., 2007). Also it is possible that some of the pollen proteins are
480 converted into peptides in RJ by some honey bee enzymes (Bíliková et al., 2009).

481 Biological activities of peptides are related to their composition and sequence of amino acids as
482 well as their size (Mora, Aristoy and Toldrá, 2016). ACE-inhibitory peptides have been
483 described to typically show less than 20 amino acids in length (Torkova, Kononikhin, Bugrova,
484 Khotchenkov, Tsentalovich and Medvedeva, 2016). In the current study, conditions of mass
485 spectrometry and data analysis tools did not allow the identification of dipeptides and tripeptides.
486 Many studies reported the molecular weight distribution of protein hydrolysates using mass
487 spectrometry technique (Mora et al., 2016; Zou, He, Li, Tang and Xia, 2016). Identified peptides
488 from AHP (fractions eluted from 3 min to 4 min and 25 min after RP-HPLC) showed peptides

489 with molecular weights from 690 to 2300 Da, whereas RJ peptides showed peptide sizes from
490 830 to 3253Da which is in accordance with the studies of Moayedi et al. (2016), Kim et al.
491 (2007) and Lassoued et al. (2015) which reported similar peptide sizes exerting ACE-inhibitory
492 and antioxidant activity. These peptides show a number of 5 to 20 amino acids in length (see
493 **Table 1 of supplementary material**). Zou et al. (2016) reported antioxidant sequences between
494 3 to 15 amino acids length whereas Balti, Bougatef, Sila, Guillochon, Dhulster and Nedjar-
495 Arroume (2015) and Torkova et al. (2016) reported same size range for ACE-inhibitory peptides.
496 On the other hand, Zou et al. (2016) studied the relationship between the antioxidant activity and
497 the structure of peptides generated from natural proteins, showing that antioxidant peptides use
498 to be more than three amino acid residues length. Liu et al. (2010) studied the antioxidant
499 activity of peptides from porcine plasma protein hydrolysated with Alcalase and the influence of
500 the molecular weight and reported that fractions with molecular weight less than 3 kDa exhibit
501 the highest reducing power and DPPH radical scavenging activity. Balti et al. (2015) identified
502 nine ACE-inhibitory peptides from the hydrolysed muscle of cuttlefish prepared with crude
503 enzymes from *B. mojavensis A21* and cuttlefish hepatopancreas. Amino acid sequences of
504 peptides were determined using ESI–MS/MS. The results suggested that all of the ACE-
505 inhibitory peptides were 5 to 8 amino acids length. Torkova et al. (2016) investigated the effect
506 of *in vitro* gastrointestinal digestion using Trypsin, Pepsin and α-Chymotrypsin on ACE-
507 inhibitory activity of poultry protein hydrolysate. Analysis of the ACE-inhibitory peptide profiles
508 revealed that all of the ACE-inhibitory peptides were 5 to 9 amino acids length.

509 Regarding the amino acid composition, Glycine and Proline were the two main hydrophobic
510 amino acids present in the peptide sequences identified in AHP (**Table 1 of supplementary**
511 **material**) which is in accordance with the study of Ktari et al. (2014) which reported that ACE-

512 inhibitory activity would be related with the number of hydrophobic amino acids present on the
513 peptide sequences probably due to active site of ACE is more accessible by hydrophobic
514 peptides (Lassoued et al., 2016). ACE-inhibitory peptides have also been described by Mora,
515 Escudero, Aristoy and Toldrá (2015) to contain Lysine, Proline or aromatic residues preferably
516 in the three positions closest to the C-terminal site. However, the most abundant amino acids
517 found in RJ peptides were Proline and Serine. In this respect, different peptides identified in both
518 AHP and RJ in **Table 1 of supplementary material** could be responsible for the detected ACE-
519 inhibitory activity determined in these fractions.

520 The antioxidant activity of peptides has been described to be closely related to their amino acid
521 composition, hydrophobicity, molecular mass and sequence length. In addition to hydrophobic
522 peptides, the presence of charged amino acids (Asparagine, Histidine, Arginine and Lysine)
523 could justify the ferric reducing power of AHP fractions by increasing the electron donation
524 capability of the peptides. Esteve, Marina and García, (2015) identified antioxidant dipeptides
525 and tripeptides showing the amino acids Asparagine, Histidine, Arginine and Lysine in their
526 sequences.

527 Peptides identified in this study were compared with previously identified bioactive peptides
528 included on Biopep database and main results are shown in **Tables 2, 3, and 4 of**
529 **Supplementary material**.

530 In fraction 25 from AHP, more than 100 peptides were found to share part of their sequence with
531 previously identified ACE-inhibitory and antioxidant peptides (**Table 2 of supplementary**
532 **material**). As an example, GYKDVNKAPFN and GYKDVNKAPFNSM contain GYK at the N-
533 terminal position. GYK is an ACE- inhibitory treipeptide with EC50 of 160 µM derived from

534 pea vicilin (Meisel, Walsh, Murray and Fitz Gerald, 2006). Further, SDG an ACE-inhibitory
535 tripeptide found at the N-terminal sequence of SDGGGPTYGY was also derived from bean
536 (*Phaseolus vulgaris*) (Mojica, Chen and Mejia, 2015). SPY**,** an ACE-inhibitory tripeptide found
537 at the C-terminal sequence of ARFQGGSPY, was also present at the N-terminal sequence of
538 SPYCYG, an ACE-inhibitory peptide derived from bean (*Phaseolus vulgaris*) (Mojica et al.,
539 2015). AGG**,** an ACE-inhibitor tripeptide found at the N-terminal sequence of
540 AGGGVEDVYGEDR, was also present at the C-terminal sequence of YAGG, an ACE-
541 inhibitory peptide derived from bean (*Phaseolus vulgaris*) (Mojica et al., 2015). AGA**,** a
542 tripeptide found at the C-terminal sequence of AGEGYGGGAGA, was also present at the C-
543 terminal sequence of LPAGA, an ACE-inhibitory peptide derived from olive seeds (*Olea*
544 *europaea*) (Esteve et al., 2015). KLPDHPKLPKGK contain KLP at the N-terminal position.
545 KLP is an antioxidant and ACE- inhibitory peptide with EC50 of 367.10 μM derived from
546 enzymatic hydrolysate of *Mactra veneriformis* (Liu et al., 2014). In another study Zou et al.
547 (2016) reported that the tripeptide GAA, obtained from Spotless smoothhound (*Mustelus*
548 *griseus*) muscle, exhibited high antioxidant activity. In present study, DTGKLAGAA derived
549 from AHP contain the tripeptide GAA at the C-terminal position. GFTAGSKVFK contain the
550 tripeptide VFK at the C-terminal position.

551 In fraction 3 and 4 from AHP, 7 peptides were found to share some homology with previously
552 identified ACE-inhibitory and antioxidant peptides (**Table 3 of supplementary material**). As an
553 example, VSP was found at the N-terminal sequence of VSIPESC. Miyoshi, Kaneko, Ishikawa,
554 Tanaka and Maruyama (1995) reported that the tripeptide VSP obtained from corn endosperm
555 proteins by some proteases, exhibited high ACE-inhibitory activity with EC50 of 10 μM. DGL

556 an ACE-inhibitory tripeptide with EC50 of 2.14 μM found at the C-terminal sequence of
557 CLGAGVDGL was also derived from shark meat hydrolysate (Wu, Aluko and Nakai, 2006).

558 In RJ, more than 40 peptides were found to share some homology with previously identified
559 ACE-inhibitory and antioxidant peptides (**Table 4 of supplementary material**). For example
560 SIEDPFDQDDWE, IVSIEDPFDQDDWE, SIEDPFDQDDW and IEDPFDQDDWE are
561 identified peptides of RJ that contain DDW (**Table 4 of supplementary material**). The
562 tripeptide DDW, an ACE-inhibitory peptide was also found at the C-terminal sequence of
563 GAAELPCSADDW derived from bullfrog (R.catesbeiana) (Qian, Jung, Lee, Byun and Kim,
564 2007). PQPNLLWYITT contain ITT at the C-terminal position. ITT is an ACE-inhibitory
565 tripeptide with EC50 of 678.50 μM derived from porcine myosin (306-308). IPTPAPSPSPLPPK
566 contain PPK at the C-terminal position. PPK is an ACE-inhibitory tripeptide with EC50 of 1001
567 μM derived from porcine myosin (981-983) (Meisel et al., 2006). FNFDSIGAFRIF contain FNF
568 at the N-terminal position. FNF is an ACE-inhibitory tripeptide with EC50 of 6.92 μM derived
569 from shark meat hydrolysate (Wu et al., 2006).

570 ## 4. Conclusion

571 The current research proved that the hydrolysis of pollen by Alcalase in predicted time and
572 enzyme concentration (4h and 1.5%, respectively) generated peptides showing a similar
573 molecular weight to those existing in RJ. In AHP, ACE-inhibitory activity and DPPH radical
574 scavenging and ferric reducing power capacities were higher than in RJ fractions. RP-HPLC
575 profiles of RJ and AHP most active fractions were obtained and a total of 195 peptides were
576 identified by nano-liquid chromatography and mass spectrometry in tandem (Q-ToF). The origin
577 of all peptides identified was herbal proteins and 50% of them corresponded to same proteins in

578 AHP and RJ. In addition, many of the identified peptides share part of their sequence with ACE-
579 inhibitory and antioxidant peptides previously described in BIOPEP database.

580

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587

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Table 1

Table 1. Antioxidant activity and ACE inhibitory tests of AHP in the points determined with the central composite design in RSM and royal jelly

Treatment	Enzyme concentration	Time (hr)	Ferric reducing power (Absorbance at 700 nm)	DPPH radical scavenging (%)	ACE-inhibitory activity (%)
1	2	1	0.614	41.93	35.84
2	1.5	1	0.708	27.86	67.28
3	1	1	0.698	34	42.23
4	2	2.5	0.688	49.49	45.59
5	1.5	2.5	0.755	36.34	72.72
6	1.5	2.5	0.756	37.35	73.72
7	1.5	2.5	0.755	36.34	71.72
8	1.5	2.5	0.756	37.35	74.72
9	1.5	2.5	0.756	35.34	73.27
10	1	2.5	0.721	48.23	40.36
11	2	4	0.658	78.46	49.41
12	1.5	4	0.701	59.63	87.08
13	1	4	0.641	78.46	68.26
royal jelly (1000 mg/ml)			0.77	95.27	64.3

Table 2

Table 2. Results of the analysis of variance (ANOVA) for antioxidant and ACE-inhibitory activity of AHP

	Degree of freedom			**Coefficients**			**P-value**		
	ACE inhibitory	**DPPH radical scavenging**	**Reducing power**	**ACE inhibitory**	**DPPH radical scavenging**	**Reducing power**	**ACE inhibitory**	**DPPH radical scavenging**	**Reducing power**
Model	5	5	5	-169.981	109.96	0.33195	<0.0001	<0.0001	<0.0001
X_1 *	1	1	1	-16.3143	6.50228	0.06153	<0.0001	<0.0001	<0.0001
X_2 **	1	1	1	334.068	-125.92	0.49947	0.0001	0.2709	<0.0001
X_1^2	1	1	1	2.05885	2.98299	-0.0229	<0.0001	<0.0001	<0.0001
X_2^2	1	1	1	-0.-116.29	47.3069	-0.2057	0.0001	<0.0001	<0.0001
X_1X_2	1	1	1	8.41333	-6.8633	0.03367	<0.0001	<0.0001	<0.0001
Lack of fitness	3	3	3				0.7295	0.1397	0.69
R^2- Pred				0.99	0.99	0.99			
R^2- Adj				0.99	0.97	0.99			

*Hydrolysis time ** Enzyme concentration

Table 3

Table 3. Predicted and experimental results of validation tests for AHP

	DPPH scavenging activity (%)	Ferric reducing Power (Absorbance)	ACE-inhibitory activity (%)
Predicted values by RSM	59.14 [a]	0.7[b]	87.08[c]
Observed values	57.76[a]	0.68[b]	86.793[c]

Those with different letters are significantly different, with $p < 0.05$. Comparisons were made between the observed and predicted values for each correspondent response.

Figure 1
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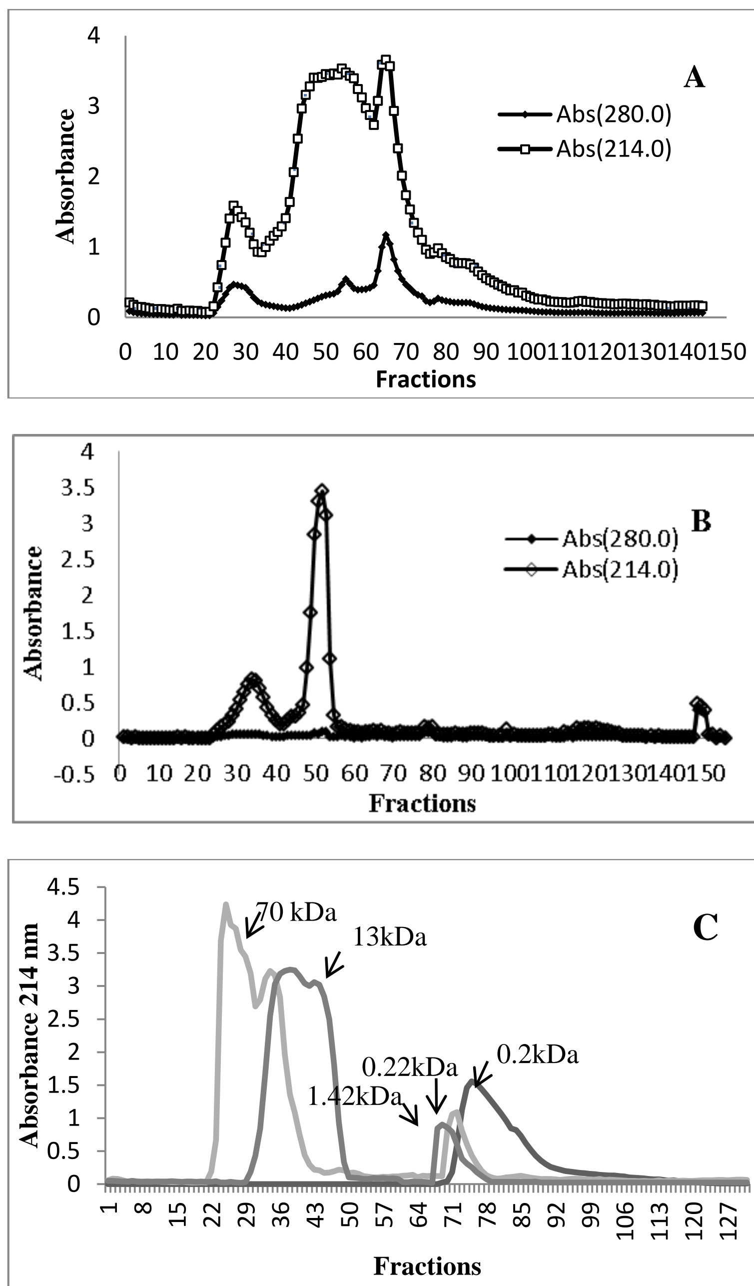

Fig 1. Size-exclusion chromatography (SEC) profile of AHP (a) Royal jelly (b) at a concentration of respectively 100 mg/mL and 1 mg/ml, in comparison to distribution of molecular weights of the standards on Sephadex G-25 column (c). Standard proteins used for column calibration include bovine serum albumin (70 kDa), cytochrome C (13 kDa), Bacitracin (1.42 kDa), Carnosin (0.22 kDa) and Tyrosine (0.2 kDa).

Figure 2
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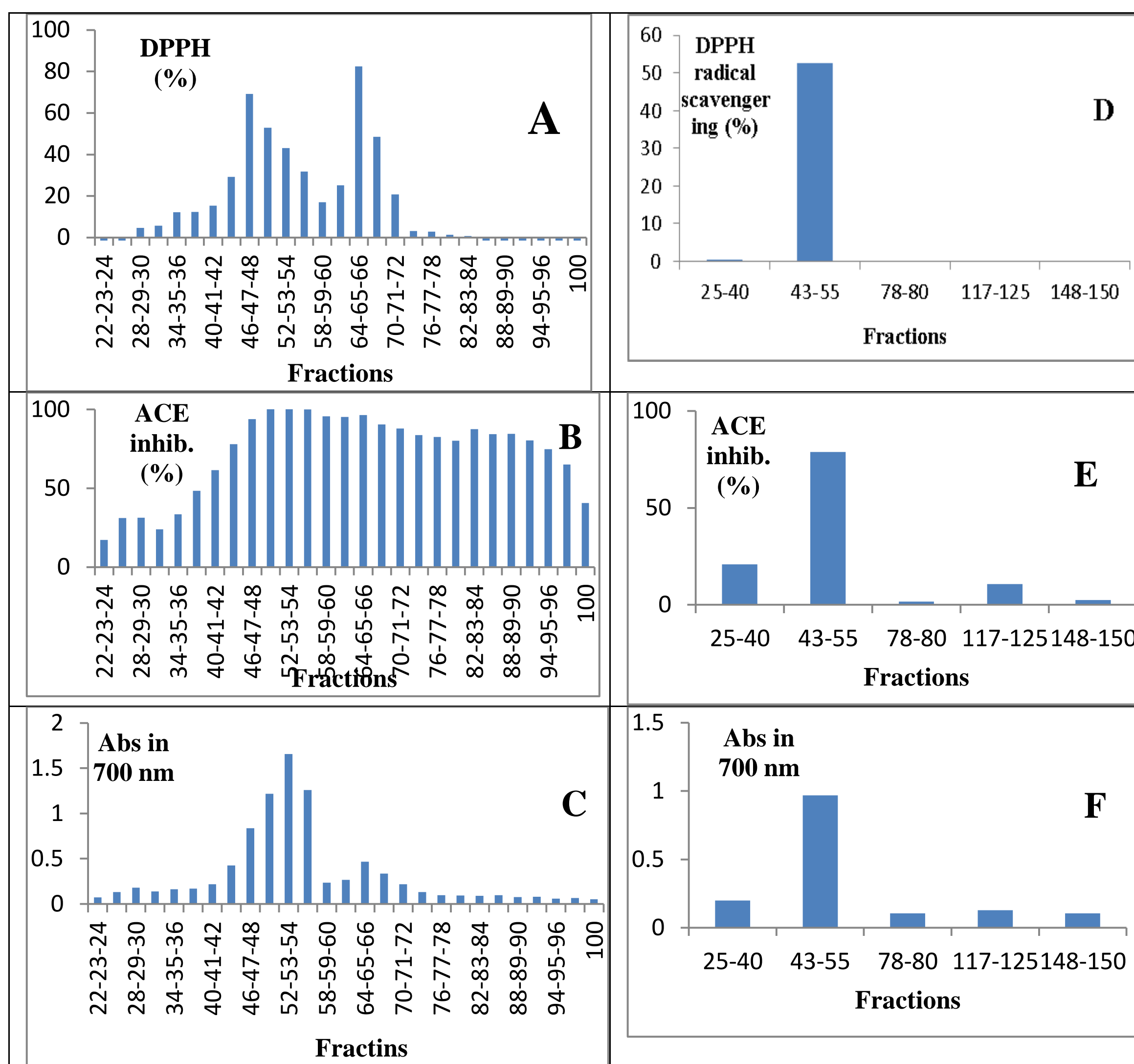

Fig.2. DPPH radical scavenging activity for AHP (A) and Royal jelly (D), ACE-inhibitory activity for AHP (B) and Royal jelly (E) and reducing power for AHP (C) and Royal jelly (F), in different fractions obtained from size exclusion chromatography. Fractions 49 to 57 and 64 to 66 selected for more purification.

Figure 3
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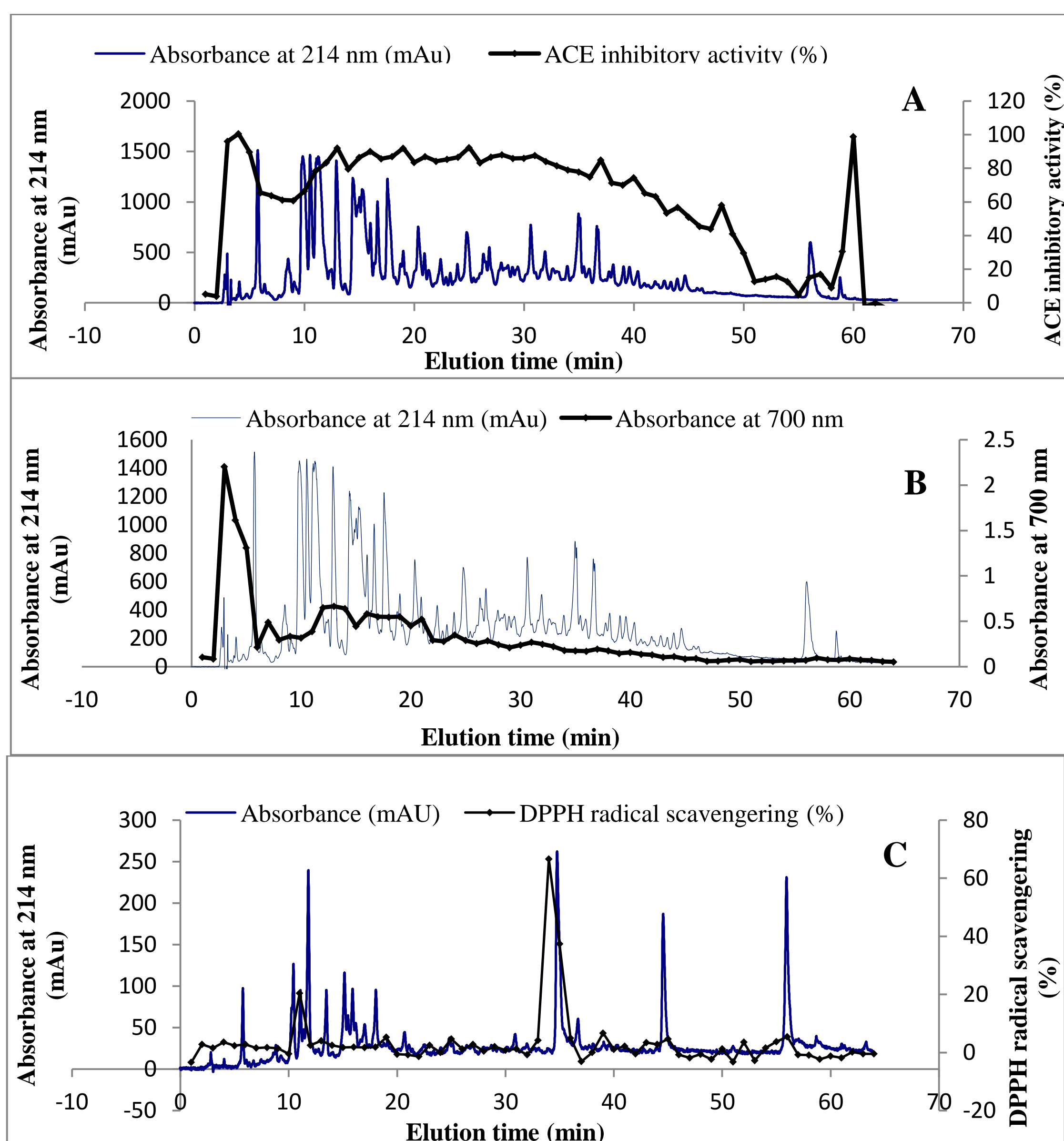

Fig 3. Reversed-phase chromatographic separation of the selected pooled fractions (49 to 57) and (64 to 66) obtained from size-exclusion chromatography of AHP. Fractions were automatically collected and assayed for their ACE inhibitory activity (A) and reducing power (B) for fractions (49 to 57) and DPPH radical scavengering (C) for fractions (64 to 66). For fractions (49 to 57) obtained from SEC, fractions eluted from 3 min to 4 min and 25 min were selected to identification.

Table 1_Supplementary Material

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