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## Chemical Composition, Antioxidant and Anti-AGEs Activities of a French Poplar Type Propolis

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1 **Chemical composition, antioxidant and anti-AGEs activities of a**  
2 **French poplar type propolis**

3

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9 **Abstract**

10 Accumulation in tissues and serum of advanced glycation end-products (AGEs) plays an  
11 important role in pathologies such as Alzheimer's disease or, in the event of complications of  
12 diabetes, atherosclerosis or renal failure. Therefore there is a potential therapeutic interest in  
13 compounds able to lower intra and extracellular levels of AGEs. Among them, natural  
14 antioxidants (AO) with true anti-AGEs capabilities would represent good candidates for  
15 development. The purpose of this study was to evaluate the AO and anti-AGEs potential of a  
16 propolis batch, then to identify the main compounds responsible for these effects. *In vivo*, protein  
17 glycation and oxidative stress are closely related. Thus AO and antiglycation activities were  
18 respectively evaluated using both DPPH and ORAC assays as well as a newly developed  
19 automated anti-AGEs test. Several propolis extracts exhibited very good AO and anti-AGEs  
20 activities and a bio-guided fractionation allowed us to identify pinobanksin-3-acetate as the most  
21 active component.

22

23 **Keywords**

24 Poplar type propolis, phenolic compounds, flavonoids, antioxidant, anti-AGEs

## 25 INTRODUCTION

26 Propolis is a resinous material, most commonly collected by honeybees from buds and exudates  
27 of various trees and plants. Propolis has been largely used in folk medicine since ancient times  
28 due to its pharmacological potential associated with antioxidant,<sup>1,2</sup> anti-inflammatory<sup>3</sup> as well as  
29 antimicrobial<sup>4,5</sup> properties.

30 Propolis is generally composed of 50% of resin and balm (including polyphenolic compounds),  
31 30% of wax and fatty acids, 10% of essential oils, 5% of pollen and 5% of various organic and  
32 inorganic compounds. The composition of propolis can be specified as it depends on the  
33 vegetation at the site of collection. Indeed, propolis from temperate climatic zones, like Europe,  
34 North America and non-tropical regions of Asia, originate mainly from the bud exudates of  
35 *Populus* species and are rich in flavonoids, phenolic acids and their esters<sup>6</sup> while tropical propolis  
36 are rich in prenylated derivatives of *p*-coumaric acids, benzophenons and terpenoids,<sup>1,7</sup> as no  
37 poplars or birches grow in this region.

38 During Maillard's reaction, a nucleophilic addition between a free amino group and a carbonyl  
39 group of a reducing sugar leads to the glycation of proteins. The resulting Schiff base rearranges  
40 to a more stable ketoamine, so-called Amadori product, which can undergo further reactions,  
41 involving dicarbonyl intermediates, giving advanced glycation end-products (AGEs) (Fig. 1).<sup>8</sup>  
42 Extra and intracellular accumulation of AGEs with time play an important role in the  
43 development of organ damage in such a way that AGEs are involved in many important  
44 pathologies *e. g.* Alzheimer's disease<sup>9</sup> and complications of diabetes<sup>8</sup> such as atherosclerosis<sup>10</sup> or  
45 or renal failure.<sup>11</sup> Therefore, numerous compounds have been investigated for their anti-AGEs  
46 activity and the synthetic hydrazine aminoguanidine has received the most efforts to be  
47 developed as a drug.<sup>8</sup> Protein glycation is a self-generated process. Thus, any potential inhibitor  
48 of AGEs formation should exhibit a long half-life while being virtually atoxic. Since reactive

49 oxygen species (ROS) are involved in AGEs formation, a food diet rich in antioxidants may  
50 protect the organism against AGEs accumulation as well as free radicals derived *via* glycation  
51 (Fig 1). There has also been a growing interest in natural products exhibiting both anti-AGEs and  
52 antioxidant properties. In that way, plant polyphenols such as quercetin have already been  
53 reported to significantly inhibit glycation *in vitro* and *in vivo*.<sup>12</sup> On the one hand poplar type (such  
54 as European type) propolis generally exhibit high total polyphenol contents (*ca.* 200-300 mg of  
55 gallic acid equivalent/g of extract) whereas, on the other hand, very few chemical studies on  
56 French-originated propolis were available in the literature.<sup>13</sup> Thus the purpose of this paper was  
57 to identify the polyphenol constituents of a French-originated propolis mixture and to evaluate  
58 both their antioxidant and anti-AGEs potential. Even collected in the same geographical region,  
59 propolis may differ qualitatively and quantitatively between apiaries, and even inside the same  
60 apiary from one hive to another one.<sup>14</sup> Keeping in mind any potential economic development, it  
61 then appeared more appropriate to study a mixture, *i.e.* some material exhibiting an average  
62 chemical composition, rather than a specific sample. Therefore 24 batches of propolis collected  
63 over two years (2010 and 2011) from different places in France, were homogeneously mixed to  
64 undergo this study.

65

## 66 MATERIAL AND METHOD

67 **Chemicals.** 1,1-Diphenyl-2-picrylhydrazyl (DPPH), Aluminium chloride hexahydrate, Folin-  
68 Ciocalteu reagent, potassium acetate, 2,4-dinitrophenylhydrazine, formic acid, gallic acid,  
69 quercetin, naringenin, p-coumaric acid, ferulic acid, isoferulic acid, 3,4-dimethoxycinnamic acid,  
70 prenyl caffeate, bovine serum albumin (BSA, fraction V), potassium phosphate monobasic,  
71 potassium phosphate dibasic trihydrate, sodium azide, aminoguanidine hydrochloride, all of

72 analytical grade, were purchased from Sigma-Aldrich (St Quentin Fallavier, France). 2,2'-Azobis  
73 (2-methylpropionamide) dihydrochloride (AAPH), fluorescein (FL), 6-Hydroxy-2,5,7,8-  
74 tetramethylchroman-2-carboxylic acid (Trolox®), 5'-caffeoylquinic acid (chlorogenic acid),  
75 caffeic acid and chrysin were obtained from Acros Organics (Geel, Belgium). Ribose was from  
76 Alfa Aesar (Schiltigheim, France). Galangin was purchased from Extrasynthese (Genay, France)  
77 and caffeic acid phenylethyl ester from Tocris biosciences (Bristol, United Kingdom).  
78 Pinocembrin, pinobanksin-3-acetate and pinostrobin were isolated from the DCM extract of  
79 propolis.

80 **Propolis batch.** A batch (240 g) corresponding to 24 propolis samples (10 g of each), collected  
81 in 2010 and 2011 in apiaries originating from different regions of France was used for this study.  
82 This batch was provided by the "Ballot-Flurin Apiculteurs" company, specialized in organic  
83 beekeeping. The different collection sites of propolis samples are given in figure 1.

84 **Instrumentation.** NMR spectra (1D and 2D) were recorded on a Bruker (Wissembourg France)  
85 Avance spectrometer at 500 MHz for  $^1\text{H}$  and 125 MHz for  $^{13}\text{C}$ . Absorbances were obtained from  
86 a Tecan (Lyon, France) Infinite M200 microplate spectrophotometer.

87 **Extractions.** The propolis batch was homogeneously pulverized in the presence of liquid  
88 nitrogen and divided into 1g samples. Four different extractions were then carried out on 1g  
89 samples with water (E1), 95% EtOH (E2), 70% EtOH (E3) and MeOH (E4). Then, two  
90 extractions, preceded by a cyclohexane wax elimination, were independently performed on 1g  
91 samples with DCM (E5) and a mixture of DCM, MeOH and  $\text{H}_2\text{O}$  (31/19/4) (E6). For E1, a  
92 decoction of 1 g of propolis powder was boiled in 20 mL  $\text{H}_2\text{O}$  at 100°C for 15 min. After  
93 cooling, the solidified wax and the residue were removed by filtration, and the filtrate was  
94 concentrated. For other solvents, 1 g of propolis powder (or of residue issued from a previous

95 extraction) was macerated in 3x20 mL of solvent. After stirring for 3x2h at room temperature, the  
96 mixture was filtered. The filtrates were gathered and evaporated under vacuum.

97 **Total polyphenol content.** Total polyphenol content was determined according to the Folin-  
98 Ciocalteu colorimetric method.<sup>15</sup> Briefly, 20  $\mu$ L of extract solution (2.5 mg/mL in MeOH) were  
99 mixed with 280  $\mu$ L of distilled water and 100  $\mu$ L of Folin-Ciocalteu's phenol reagent. After 3  
100 min, 1200  $\mu$ L of distilled water and 400  $\mu$ L of 20% aqueous sodium carbonate solution were  
101 added. 200  $\mu$ L of each solution were distributed in a 96-well microtiter plate. The absorbance  
102 was measured at 760 nm after 30 min in the dark at room temperature. A blank was prepared in  
103 the same way by using MeOH instead of the extract solution. Gallic acid was used to calculate  
104 the calibration curve (0.4-1.2 mg/mL;  $y=0.5800x$ ;  $r^2=0.9941$ ) and total polyphenol contents were  
105 expressed in terms of Gallic Acid Equivalent (mg) per gram of extract (mg GAE/g). All  
106 measurements were performed in triplicate.

107 **Flavone and flavonol content.** Flavone and flavanol content was determined according to the  
108 aluminium chloride colorimetric method described by Woisky and Salantino.<sup>16</sup> 300  $\mu$ L of 95%  
109 EtOH were mixed with 100  $\mu$ L of extract solution (1.5 mg/mL in EtOH 80%), 20  $\mu$ L of 10%  
110 aqueous aluminium chloride solution, 20  $\mu$ L of 1M potassium acetate aqueous solution and 560  
111  $\mu$ L of distilled water. 200  $\mu$ L of each solution were put into the 96-well microtiter plate. After  
112 incubation at room temperature for 30 min, the absorbance was measured at 415 nm. The amount  
113 of 10% aqueous aluminium chloride solution was replaced by the same amount of distilled water  
114 in blank for each extract solution. Quercetin, used as standard, was prepared at concentrations of  
115 25-200  $\mu$ g/mL to build the calibration curve ( $y=2.7677x$ ;  $r^2=0.9988$ ). Flavone and flavonol  
116 content was expressed as mg QE/g (Quercetin Equivalent per gram of extract). All assays were  
117 performed in triplicate.

118 **Flavanone and dihydroflavonol content.** The modified method described by Nagy and  
119 Grancai<sup>17</sup> was used to determine flavanone and dihydroflavonol content. Briefly, 500  $\mu$ L of  
120 extract solution (2.5 mg/mL in MeOH) were reacted with 250  $\mu$ L of MeOH and 500  $\mu$ L of 1%  
121 2,4-dinitrophenylhydrazine (DNP) solution (500 mg of DNP mixed with 1 mL of 96% sulfuric  
122 acid and diluted to 50 mL with MeOH) at 50°C for 50 min. After cooling, 500  $\mu$ L of the solution  
123 were mixed with 500  $\mu$ L of 20% potassium hydroxide in 70% EtOH and then centrifuged at 4000  
124 rpm for 10 min to remove the precipitate. 20  $\mu$ L of the supernatant were collected, put into the  
125 96-well microtiter plate and mixed with 180  $\mu$ L of 1% potassium hydroxide methanolic solution.  
126 Absorbance was measured at 495 nm. A blank was prepared using the same amount of MeOH  
127 instead of the extract solution. Standard solutions of ( $\pm$ )-naringenin (0.25-2.00 mg/mL in MeOH)  
128 were used to build the calibration curve ( $y=0.2053x$ ;  $r^2=0.9945$ ). Flavanone and dihydroflavonol  
129 content was calculated as mg NE/g (Naringenin Equivalent per gram of extract). All  
130 measurements were performed in triplicate.

131 **HPLC-DAD and HPLC-MS procedures.** 5 mg of propolis extracts dissolved in 1 mL of MeOH  
132 were centrifuged at 13000 rpm for 10 min and filtered through a 0.2  $\mu$ m nylon-membrane syringe  
133 filter prior to injection (10  $\mu$ L). Analytical HPLC was run on a 2695 Waters® (Guyancourt,  
134 France) separation module equipped with a diode array detector 2996 Waters®. Separation was  
135 achieved on a Phenomenex® (Le Pecq, France) Luna column 3 $\mu$ m C18 100A (150x4.6 mm i.d.,  
136 3 $\mu$ m) protected with a Phenomenex® SecurityGuard cartridge C18 (4x3 mm i.d.) at a flow rate of  
137 0.4 mL/min. The mobile phase consisted of 0.1% formic acid in water (solvent A) and 0.1%  
138 formic acid in MeOH (solvent B). The separation was performed using the following gradient:  
139 40%B (0-10 min), 40-50%B (10-25 min), 50-60%B (25-55 min), 60-90%B (55-70 min), 90%B  
140 (70-80 min). UV detection and quantification were achieved at two wavelengths: 254 and 280  
141 nm. The mass analyses were performed on a Bruker (Bremen, Germany) ESI/APCI Ion Trap

142 Esquire 3000+ in both, positive and negative modes, with the conditions as follows: collision gas,  
143 He; collision energy amplitude, 1.3 V; nebulizer and drying gas, N<sub>2</sub>, 7 L/min; pressure of  
144 nebulizer gas, 30 psi; dry temperature, 340°C; flow rate, 1.0 mL/min; solvent split ratio 1:9; scan  
145 range, m/z 100–1000.

146 **Quantification of 12 compounds by HPLC/DAD.** MeOH stock solutions of 12 major  
147 compounds chosen as markers for this quantification analysis, were prepared as follows: caffeic  
148 acid (0.5 mg/mL), p-coumaric acid (0.5 mg/mL), ferulic acid (0.3 mg/mL), isoferulic acid (0.3  
149 mg/mL), 3,4-dimethoxycinnamic acid (0.3 mg/mL), pinocembrin (0.8 mg/mL), pinobanksin-3-  
150 acetate (1.0 mg/mL), prenyl caffeate (0.4 mg/mL), chrysin (0.8 mg/mL), Caffeic Acid  
151 Phenylethyl Ester (CAPE) (0.3 mg/mL), galangin (0.6 mg/mL) and pinostrobin (0.2 mg/mL).  
152 Stock solutions were diluted (3/4, 1/2, 1/4, 1/8, 1/16) and used to determine the calibration curves  
153 of the 12 markers (n=2): caffeic acid ( $y=78,027x$ ;  $r^2=0.9999$ ), p-coumaric acid ( $y=113,20x$ ;  
154  $r^2=0.9999$ ), ferulic acid ( $y=78,364x$ ;  $r^2=0.9999$ ), isoferulic acid ( $y=103,68x$ ;  $r^2=0.9998$ ), 3,4-  
155 dimethoxycinnamic acid ( $y=77,748x$ ;  $r^2=0.9998$ ), pinocembrin ( $y=54,982x$ ;  $r^2=0.9998$ ),  
156 pinobanksin-3-acetate ( $y=49,022x$ ;  $r^2=0.9994$ ), prenyl caffeate ( $y=60,462x$ ;  $r^2=0.9987$ ), chrysin  
157 ( $y=100,4x$ ;  $r^2=0.9998$ ), CAPE ( $y=43,434x$ ;  $r^2=0.9996$ ), galangin ( $y=48,653x$ ;  $r^2=0.9984$ ) and  
158 pinostrobin ( $y=93,633x$ ;  $r^2=0.9995$ ). All propolis extracts (E1 to E6) were analyzed in triplicate.

159 **Fractionation by Flash Chromatography.** 50.0 g of the propolis mixture were extracted with  
160 cyclohexane (3x200 ml, 2h, room temperature) to remove beeswax. Then the residue was  
161 extracted with DCM (5x200ml, 2h, room temperature), filtered and concentrated to give 25,0 g of  
162 DCM extract (50% yield). 21 g of DCM extract was dissolved in 200 mL of DCM, mixed with  
163 42 g of silica gel (extract/silica gel: 1/2) and concentrated to obtain a dry thin powder.  
164 Fractionation was performed using a CombiFlash<sup>®</sup> Teledyne ISCO (Lincoln, NE, USA)  
165 apparatus, on a prepacked silica gel column (Interchim PF-50SI HC/300g, 50  $\mu$ m, 20 bars) at a

166 flow rate of 100 mL/min with the following gradient: 100% C<sub>6</sub>H<sub>12</sub> (2.0 L), C<sub>6</sub>H<sub>12</sub>:EtOAc 90:10  
167 (1.7 L), C<sub>6</sub>H<sub>12</sub>:EtOAc 90:10 to 80:20 (2.2 L), C<sub>6</sub>H<sub>12</sub>:EtOAc 80:20 to 70:30 (2.5 L), C<sub>6</sub>H<sub>12</sub>:EtOAc  
168 70:30 to 60:40 (2.2 L), C<sub>6</sub>H<sub>12</sub>:EtOAc 60:40 to 50:50 (3.0 L) and at last DCM:MeOH 96:4 (2.2 L).  
169 UV detection, achieved at 254 and 280 nm, allowed to separate 21 fractions.

170 **Scavenging of DPPH radicals.** The diphenyl-picrylhydrazyl (DPPH) radical scavenging  
171 evaluations of propolis extracts were carried out using a modified previously established  
172 methodology.<sup>18</sup> Tested compounds and standards were diluted in absolute EtOH at 0.02 mg/mL  
173 from stock solutions at 1 mg/mL in DMSO. Aliquots (100 µL) of these diluted solutions were  
174 placed in 96-well plates in triplicates. The reaction was initiated by adding 25 µL of freshly  
175 prepared DPPH solution (1mM) and 75 µL of absolute EtOH using the microplate reader's  
176 injector (Infinite<sup>®</sup> 200, Tecan, France) to obtain a final volume of 200 µL per well. After 30  
177 minutes in the dark and at room temperature, the absorbance was determined at 517 nm. EtOH  
178 was used as a blank, whereas 10, 25, 50, and 75 µM solutions of Trolox (hydrophilic α-  
179 tocopherol analog) were used for the calibration curve. A sample of chlorogenic acid ethanolic  
180 solution (0.02 mg/mL) was used as the quality control standard. Results were expressed in terms  
181 of Trolox Equivalents (micromoles of TE per gram of extract).

182 **Measurement of oxygen radical absorbance capacity (ORAC).** ORAC assays were carried out  
183 according to the method described by Huang et al.<sup>19</sup> with some modifications. The assay was  
184 performed in a 96-well plate. The reaction mixture contained 100 µL of 75 mM phosphate buffer  
185 (pH 7.4), 100 µL of freshly prepared fluorescein (FL) solution (0.1 µM in phosphate buffer), 50  
186 µL of freshly prepared 2,2'-azobis(2-methylpropionamide) dihydrochloride (AAPH) solution  
187 (51.6 mg/mL in phosphate buffer), and 20 µL of sample per well. Samples were analysed in  
188 triplicates and diluted in phosphate buffer at different concentrations (25, 12.5, 6.25 and 3.12  
189 µg/mL) from stock solutions at 1 mg/mL in DMSO. FL, phosphate buffer, and samples were

190 preincubated at 37 °C for 10 min. The reaction was started by the addition of AAPH using the  
191 microplate reader's injector (Infinite® 200, Tecan, France). Fluorescence was then measured and  
192 recorded during 40 minutes ( $\lambda_{exc}$  485 nm,  $\lambda_{em}$  520 nm). The 75 mM phosphate buffer was used as  
193 a blank, and 12.5, 25, 50, and 75  $\mu$ M solutions of Trolox were used as calibration solutions. A  
194 chlorogenic acid solution in phosphate buffer (8.8  $\mu$ M) was used as quality control standard. The  
195 final ORAC values were calculated using a regression equation between the Trolox concentration  
196 and the net area under the FL decay curve and were expressed as micromole of Trolox  
197 equivalents per gram of dry matter. Areas under curves were calculated using Magellan™ data  
198 analysis software (Tecan, France).

199 **Determination of sample concentrations inhibiting 50% of AGEs formation.** IC<sub>50</sub> were  
200 determined using a previously described method<sup>20</sup> with slight modifications. Briefly, BSA (10  
201 mg/mL) was incubated with D-ribose (0.5 M) together with the tested compound (3  $\mu$ M to 3  
202 mM) or extract (1  $\mu$ g to 1 mg) in 50 mM phosphate buffer at pH 7.4 (NaN<sub>3</sub>, 0.02%). Solutions  
203 were incubated in 96-well microtiter plates at 37°C for 24 h in a closed system before AGE  
204 fluorescence measurement. Fluorescence resulting from the incubation, under the same BSA (10  
205 mg/mL) and tested compound (3  $\mu$ M to 3 mM) or extract (1  $\mu$ g to 1 mg) conditions, was  
206 subtracted for each measurement. A control, i.e. no inhibition of AGE formation, consisted of  
207 wells with BSA (10 mg/mL) and D-ribose (0.5 M). A blank control, i.e. 100% inhibition of AGE  
208 formation, consisted of wells with only BSA. The final assay volume was 100  $\mu$ L. Pentosidine-  
209 like ( $\lambda_{exc}$  335 nm,  $\lambda_{em}$  385 nm) AGE fluorescence were measured using a microplate  
210 spectrofluorometer. In this type of automation, a single analysis is sufficient for an accurate IC<sub>50</sub>  
211 determination.<sup>20,21</sup> The percentage of AGE formation was calculated as follows for each  
212 compound/extract concentration:

$$AGEs (\%) = \frac{\text{fluorescence intensity (sample)} - \text{fluorescence intensity (blank of sample)}}{\text{fluorescence intensity (control)} - \text{fluorescence intensity (blank of control)}} \times 100$$

213 Dose-effect curves were best fitted with a sigmoidal dose-response equation using Sigma Plot  
214 12.0 software, which enabled calculation of the IC<sub>50</sub> values.

215

## 216 **RESULTS AND DISCUSSION**

217 **Extraction yield.** As shown in table 1, the extraction yield of E1 was very low (6.9 %) whereas  
218 those of the alcoholic extracts E2-4 were much higher (65-69 %). Those of E5 and E6 were  
219 respectively 50.3 and 58.6 % after a prior cyclohexanic extraction, to remove waxes, which  
220 exhibited a 33.0 % yield. Thus, yields of E2-4 were higher than those of EtOH extracts of  
221 propolis (EEP) collected in Greece and Cyprus (23.9 to 61.2 %).<sup>22</sup> These yields were also higher  
222 than those observed for 70% EtOH propolis extracts from Bulgaria (58%), Albania (41%), Egypt  
223 (18%), Brazil (12-55%)<sup>5</sup> and a MeOH propolis extract from Mexico (40%).<sup>23</sup>

224 **Total polyphenol content.** Total polyphenol (TP) contents (Table 1) were high for E1-6, in a  
225 range of 238.6 to 292.1 mg GAE/g. This is in accordance with values observed for European and  
226 Asian poplar-type propolis (*ca.* 200-300 mg GAE/g).<sup>1</sup> Best TP contents were observed for E1, E5  
227 and E6. E2 (253.6 mg GAE/g) had similar value to EEP from Ukraine (255 mg GAE/g) and  
228 United States (256 mg GAE/g) but higher than the ones found in Hungary (242 mg GAE/g),  
229 Bulgaria (220 mg GAE/g), Argentina (212 mg GAE/g), Uzbekistan (174 mg GAE/g), Brazil (120  
230 mg GAE/g), South Africa (100 mg GAE/g) and Thailand (31 mg GAE/g).<sup>1</sup>

231 **Flavone/flavonol and flavanone/dihydroflavonol contents.** As indicated in Table 1, E1 showed  
232 the lowest flavone/flavonol (FF) and flavanone/dihydroflavonol (FD) contents (respectively 21  
233 mg QE/g and 77 mg NE/g) whereas FF contents of the other extracts were in a range of 66-80 mg  
234 QE/g and FD contents in a range of 153-176 mg NE/g, E5 and E6 showing the best values. For

235 all extracts FD was superior to FF contents. Alcoholic extracts E2-4 had similar values (66-69 mg  
236 QE/g for FF and 153-159 mg NE/g for FD contents), that appeared superior to those of propolis  
237 from Northeastern Spain (*ca.* 48.2 mg/g for FF and 78.8 mg/g for FD).<sup>2</sup> They seem closer to  
238 those of EEP from Pueblo de Alamos in Northwestern Mexico (57.8 mg/g for FF and 150.6 mg/g  
239 for FD content).<sup>24</sup> Nevertheless, FF and FD contents of E2-4 differed from those of Italian and  
240 Swiss 70% EtOH propolis extracts where FF > FD.<sup>25</sup>

241 **Identification of components by HPLC analysis with DAD and MS detection.** Fig. 2 shows  
242 the HPLC chromatograms of E1 and E5, the profiles of E2-4 and E6 (not shown) being very  
243 similar to E5. As expected with differences in solvent polarities, we observed two profile-types:  
244 the aqueous type with retention times ranging from 6 to 33 min and the organic one, with  
245 retention times of most compounds ranging from 10 to 72 min. Chemical profiling were achieved  
246 by comparison with literature data (UV/MS), using pure standards or, when needed, after  
247 isolation through <sup>1</sup>H and <sup>13</sup>C (1D and 2D) NMR analysis. These studies allowed us to identify 40  
248 compounds as benzaldehyde, benzoic and cinnamic acid derivatives and their ester, glycerols and  
249 different classes of flavonoids (flavones, flavonols, flavanones and dihydroflavonols). E1  
250 contained benzaldehyde and benzoic acid derivatives (**1, 3-5, 9**), cinnamic acid derivatives (**2, 6-**  
251 **8, 10, 12, 13**) and some flavanones/dihydroflavonols (**11, 14, 15**). E5 exhibited the same  
252 compounds, in less quantities, added with cinnamylidene acetic acid **19**, cinnamic ester  
253 derivatives (**23, 24, 27, 29, 31, 32, 34, 35, 38-40**), glycerol derivatives (**16, 25**) and other  
254 flavonoids, as flavanones/dihydroflavonols (**21, 22, 26, 33, 36**), flavones/flavonols (**17, 18, 20,**  
255 **28, 30, 37**). So mainly exhibiting cinnamic acid derivatives (caffeic and p-coumaric acids...) and  
256 their esters (prenyl caffeate, CAPE...), and flavonoids (pinocembrin, pinobanksin-3-acetate,  
257 chrysin, galangin...), this French propolis clearly belongs to the poplar type with polyphenols  
258 originating from *Populus* spp. of section *Aigeiros* and especially *P. nigra* L.<sup>7,26</sup>

259 **Quantification of 12 major compounds.** Results of the quantitative analysis of E1-6 are given  
260 in Table 2. As already outlined in Figure 1a, E1 contained mainly phenolic acids with a majority  
261 of caffeic **2** and *p*-coumaric **6** acids with  $76.9 \pm 0.6$  and  $61.4 \pm 0.3$  mg/g respectively. In E2-6,  
262 pinobanksin-3-acetate **28** appeared as the major component, followed by pinocembrin **25**,  
263 chrysin **32**, galangin **34** and prenyl caffeate **29**. Taken as a whole, the higher cumulative amount  
264 of these 12 components was observed for E5 with  $271.6 \pm 3.5$  mg/g whereas the lowest indexes  
265 were associated with E4 and E1 (186 and 185.3 mg/g respectively). For the aqueous extract (E1),  
266 it can be noticed that this amount corresponded to only 5 detectable markers (phenolic acids).  
267 Therefore the quantification results were in accordance with our total polyphenol and flavonoid  
268 contents (Table 1). The same three major compounds were also observed in different proportions  
269 with poplar type propolis from China (chrysin >> pinocembrin > pinobanksin-3-acetate),  
270 Hungary (chrysin >> pinobanksin-3-acetate > pinocembrin) and Uruguay (pinobanksin-3-acetate  
271 > chrysin > pinocembrin).<sup>1</sup>

272 **Antioxidant and anti-AGEs activities.** AO and anti-AGEs activities observed for E1-6 are  
273 shown in Table 3. E1-6 are compared with specific references (extract or pure compounds) on the  
274 one hand and literature data on the other hand. E392 is an ethanolic rosemary extract used as an  
275 AO food additive in Europe (Official journal of European Union – Directive 2010/67/UE – L  
276 277/17). It should be noticed that, at least in our survey, E392 only showed very weak activities.  
277 As expected E1 exhibited good AO activities but, lacking flavonoids, no real anti-glycation  
278 potential. At the opposite E6 showed very good overall activities due to a comprehensive  
279 polyphenols extraction. However, as far as anti-AGEs activity was concerned, similar results  
280 were obtained with E3-5. Therefore, from a food additive point of view, a mother tincture such as  
281 E3, exhibiting fair AO activities associated with a true anti-AGEs potential, appears as quite  
282 interesting. According to its very good anti-AGEs activity ( $IC_{50}=0.03$  mg/mL), combined with

283 the best cumulative content of the 12 quantified markers (272 mg/g), E5 was chosen for a bio-  
284 guided fractionation by Flash chromatography.

285 **Anti-AGEs guided fractionation of E5.** Among 21 fractions (F) issued from the Flash  
286 chromatography, F1-5 didn't exhibit any activity ( $IC_{50} > 1$  mg/mL) whereas  $IC_{50}$  for F6-21 were  
287 in a range of 0.01 to 0.13 mg/mL. Eight fractions showing  $IC_{50} \leq 0.04$  mg/mL (namely F8-12 and  
288 F16-18 *i.e.* F8:0.02; F9:0.04; F10:0.01; F11:0.03; F12:0.03; F16:0.03; F17:0.03 and F18:0.04  
289 mg/mL) were then selected for a chemical profiling and the anti-AGEs activities of purified  
290 components were finally achieved (Table 4). The 16 analyzed compounds can be divided into 3  
291 groups. The first one contained the most active components in our assay, with  $IC_{50} \leq 0.10$  mM,  
292 *i.e.* in decreasing order, pinobanksin-3-acetate **26**, 2-acetyl-1,3-dicoumaroylglycerol **25**,  
293 pinobanksin **14**, prenyl caffeate **27** and pinobanksin-5-methyl ether **11**. The second group was  
294 composed of moderately anti-AGEs compounds ( $0.2 \leq IC_{50} \leq 1.6$  mM) like naringenin **15**,  
295 caffeates **24**, **38** and **29**, ferulic and isoferulic acids **7** and **8** and ester **34**. The last group exhibited  
296 non active molecules ( $IC_{50} > 3$  mM) like *p*-coumaric and cinnamic acids **6** and **12** as well as  
297 chrysin **28**. Thus, among the most active components, three of them were pinobanksin derivatives  
298 (flavanones **26**, **14** and **11**). Besides, a trisubstituted glycerol (**25**) and a prenylated caffeate (**27**)  
299 also appeared as good glycation inhibitors.

300  
301 There is nowadays a growing interest in compounds exhibiting anti-glycation combined with  
302 antioxidant properties. However, if a fair anti-AGEs activity is expected from any AO compound  
303 (Fig. 1) the measured effect may be deceptive, at least because glycation spontaneously occurs so  
304 that AO must exhibit long life-times. Indeed, whereas coincubation of methylglyoxal (MGO) and  
305 reduced glutathione or Trolox totally prevents ROS production, it only partially prevents the  
306 MGO-induced decrease of brain cell viability.<sup>27</sup> *A contrario*, an AO synthetic compound such as

307 aminosalicic acid proved *in vitro* more effective than aminoguanidine in reducing the  
308 antiproliferative effects of both high glucose and BSA-issued AGEs.<sup>28</sup> One may then turn to  
309 natural AO, especially in the search for compounds exhibiting low toxicities. As a food  
310 ingredient used from ancient times with very high AO activity, it was therefore of great interest to  
311 evaluate the anti-glycation potential of our propolis batch. Our study actually demonstrates that a  
312 mother tincture of propolis such as E3 represents a very good candidate for further food  
313 development.

314 Bioactive compound may also act by blocking the interaction between AGEs and their specific  
315 receptors, *i.e.* RAGEs. As far as neurodegeneration is concerned, it was recently demonstrated  
316 that, inhibiting the upregulation of RAGEs transcripts, pinocembrin (**22**), a major component in  
317 E2-6 extracts, truly improved cognitive function, preserved the ultrastructural neuropil and  
318 decreased the neurodegeneration of the cerebral cortex in amyloid- $\beta$  peptide treated mice.<sup>29</sup> All in  
319 all a poplar type propolis like this French batch have therefore the greater potential to be used as  
320 food additive to prevent glycooxidation.

321

## 322 **ABBREVIATION USED**

323 AGEs: Advanced Glycation End-products; AO: Antioxidant; APCI: Atmospheric Pressure  
324 Chemical Ionization; BSA: Bovin Serum Albumin; DCM: Dichloromethane; DPPH: Diphenyl-  
325 picrylhydrazyl; DNP: 2,4-dinitrophenylhydrazine; ESI: Electrospray Ionization; FD:  
326 Flavanone/Dihydroflavonol; FF: Flavone/Flavonol; HPLC/DAD: High Performance Liquid  
327 Chromatography coupled with Diode Array Detector; MGO: Methylglyoxal; NMR: Nuclear  
328 Magnetic Resonance; ORAC: Oxygen Radical Absorbance Capacity; RAGEs : Receptor for  
329 Advanced Glycation End-products; ROS: Reactive oxygen species; TP: Total Polyphenol

330

331 **ASSOCIATED CONTENT**

332 **Supporting information**

333 Chemical structures of the identified compounds with their UV/MS data and the anti-AGEs  
334 guided fractionation of E5. This material is available free of charge via the Internet at  
335 <http://pubs.acs.org>.

336

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346

347

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433

434 **FIGURE CAPTIONS**

435 Fig. 1. Collection sites of French propolis samples

436  
437 Fig. 2. Schematic formation of AGEs. AGE inhibitors may follow different inhibition pathways  
438 in order to prevent AGE formation. For exemple, they can prevent glycation *via* reaction with  
439 free amino groups in proteins or carbonyl groups on reducing sugars. In addition, compounds  
440 with antioxidant (AO) properties (free radical scavengers or transition metal chelators) may  
441 reduce reactive oxygen species (ROS). Finally, they can also scavenge  $\alpha,\beta$ -dicarbonyl  
442 intermediates or break them (AGE breakers) [adapted from Ahmed, 2005<sup>8</sup>].

443  
444 Fig. 3. HPLC chromatograms of E1 (a) and E5 (b): **1** 3,4-dihydroxybenzaldehyde, **2** caffeic acid,  
445 **3** 4-hydroxybenzaldehyde, **4** vanilline, **5** 4-hydroxyacetophenone, **6** *p*-coumaric acid, **7** ferulic  
446 acid, **8** isoferulic acid, **9** benzoic acid, **10** 3,4-dimethoxycinnamic acid, **11** pinobanksin-5-methyl  
447 ether, **12** cinnamic acid, **13** 4-methoxycinnamic acid, **14** pinobanksin, **15** naringenin, **16** 1,3-  
448 dicoumaroylglycerol, **17** kaempferol, **18** apigenin, **19** cinnamylidene acetic acid, **20** rhamnetin,  
449 **21** pinocembrin-5-methyl ether, **22** pinocembrin, **23** isopent-3-enyl caffeate, **24** benzyl caffeate,  
450 **25** 2-acetyl-1,3-dicoumaroylglycerol, **26** pinobanksin-3-acetate, **27** prenyl caffeate, **28** chrysin,  
451 **29** caffeic acid phenylethyl ester (CAPE), **30** galangin, **31** benzyl *p*-coumarate, **32** cinnamyl  
452 caffeate, **33** pinostrobin, **34** cinnamyl isoferulate, **35** cinnamyl *p*-coumarate, **36** tectochrysin, **37**  
453 alpinone-3-acetate, **38** benzyl cinnamate, **39** cinnamyl cinnamate, **40** cinnamyl cinnamylidene  
454 acetate

455

## TABLES

Table 1. Extraction yield, total polyphenol (TP), flavone/flavonols (FF) and flavanone/dihydroflavonols (FD) contents of French propolis extracts (E1-6)

Extract	Solvent	Extraction yield (%)	TP <sup>a</sup> (mg GAE/g)	FF <sup>b</sup> (mg QE/g)	FD <sup>c</sup> (mg NE/g)
E1	H <sub>2</sub> O	6.9 ± 0.4	292.1 ± 13.1	20.5 ± 1.2	76.6 ± 7.7
E2	EtOH	68.4 ± 0.8	253.6 ± 5.1	67.0 ± 2.6	153.1 ± 2.5
E3	70% EtOH	65.3 ± 1.5	246.3 ± 10.6	69.3 ± 3.7	158.6 ± 8.5
E4	MeOH	67.3 ± 0.4	238.6 ± 13.3	66.4 ± 4.9	156.8 ± 3.1
E5	DCM	50.3 ± 0.5	273.5 ± 6.8	78.6 ± 2.7	176.0 ± 9.9
E6	Mixed solvents	58.6 ± 0.7	281.0 ± 7.1	80.0 ± 1.2	156.0 ± 10.2

<sup>a</sup> Total polyphenol contents were determined by the Folin-Ciocalteu method. The results are expressed as milligram of gallic acid equivalent per gram of extract. <sup>b</sup> Flavone/flavonol contents were determined by AlCl<sub>3</sub> coloration. The results are expressed as mg of quercetin equivalent per gram of extract. <sup>c</sup> Flavanone/dihydroflavonol contents were determined by the DNP method. The results are expressed as mg of naringenin equivalent per gram of extract. For all, each value is the mean ± standard deviation (n=3).

Table 2. Contents of the 12 markers in propolis extracts (E1-6)

	Content <sup>a</sup> (mg/g of extract)					
	E1: H <sub>2</sub> O	E2: 95% EtOH	E3: 70% EtOH	E4: MeOH	E5: DCM	E6: Mixed solvents
Caffeic acid ( <b>2</b> )	76.9 ± 0.6	6.9 ± 0.1	6.0 ± 0.1	6.6 ± 0.1	5.0 ± 0.1	8.0 ± 0.1
p-coumaric acid ( <b>6</b> )	61.4 ± 0.3	10.7 ± 0.1	11.1 ± 0.1	10.2 ± 0.1	15.4 ± 0.1	12.0 ± 0.1
Ferulic acid ( <b>7</b> )	20.8 ± 0.4	4.1 ± 0.1	4.4 ± 0.1	3.9 ± 0.1	6.2 ± 0.1	4.2 ± 0.1
Isoferulic acid ( <b>8</b> )	16.2 ± 0.2	4.5 ± 0.1	4.4 ± 0.1	4.1 ± 0.1	6.5 ± 0.1	4.6 ± 0.1
3,4-dimethoxy- cinnamic acid ( <b>10</b> )	9.9 ± 0.1	7.7 ± 0.1	7.7 ± 0.1	7.1 ± 0.1	10.8 ± 0.1	7.7 ± 0.1
Pinocembrin ( <b>22</b> )	-	35.2 ± 0.2	33.4 ± 0.2	33.0 ± 0.2	49.6 ± 0.4	36.8 ± 0.3
Pinobanksin-3- acetate ( <b>26</b> )	-	40.2 ± 0.3	38.7 ± 0.5	38.1 ± 0.2	59.1 ± 0.5	42.8 ± 0.4
Prenyl caffeate ( <b>27</b> )	-	20.2 ± 0.5	20.2 ± 0.3	19.5 ± 0.2	27.4 ± 0.3	22.0 ± 0.4
Chrysin ( <b>28</b> )	-	24.7 ± 0.1	23.5 ± 0.2	23.1 ± 0.2	36.5 ± 0.4	27.6 ± 0.2
CAPE ( <b>29</b> )	-	11.2 ± 0.2	10.6 ± 0.2	10.4 ± 0.4	15.8 ± 0.7	11.8 ± 0.2
Galangin ( <b>30</b> )	-	21.9 ± 0.2	20.7 ± 0.3	20.4 ± 0.5	31.1 ± 0.8	23.5 ± 0.4
Pinostrobin ( <b>33</b> )	-	10.7 ± 0.1	12.9 ± 0.3	10.0 ± 0.2	8.1 ± 0.4	7.8 ± 0.3
Total	185.3 ± 1.5	195.3 ± 1.1	193.7 ± 1.8	186.5 ± 1.8	271.6 ± 3.5	208.9 ± 2.4

<sup>a</sup>: Each value is the mean of triplicate analyses ± standard deviation

-: not detected

Table 3. Antioxidant and anti-AGEs activities of propolis extracts (E1-6)

Extract/ Compound	Solvent	DPPH <sup>a</sup> ( $\mu\text{mol TE/g}$ )	ORAC <sup>a</sup> ( $\mu\text{mol TE/g}$ )	Anti-AGEs <sup>b</sup> IC <sub>50</sub> (mg/mL)
E1	H <sub>2</sub> O	1731 $\pm$ 28	9722 $\pm$ 273	0.34
E2	EtOH	1605 $\pm$ 26	8155 $\pm$ 114	0.05
E3	70% EtOH	1650 $\pm$ 149	9890 $\pm$ 480	0.03
E4	MeOH	1386 $\pm$ 171	7769 $\pm$ 360	0.03
E5	DCM	1437 $\pm$ 105	9242 $\pm$ 739	0.03
E6	Mixed solvents	1964 $\pm$ 124	11278 $\pm$ 11	0.04
Propolis (Greece) <sup>c</sup>	EtOH	1110 $\pm$ 70	-	-
Propolis (Colombia) <sup>d</sup>	EtOH	190	1965	-
E392 <sup>e</sup>	EtOH	591 $\pm$ 20	2433 $\pm$ 88	0.60
<i>S. japonicum</i> <sup>f</sup>	EtOH	1714 $\pm$ 68	7896 $\pm$ 711	0.09
Chlorogenic acid		2740 $\pm$ 64	11289 $\pm$ 531	0.04
Quercetin		6723 $\pm$ 66	13907 $\pm$ 662	0.06

<sup>a</sup> DPPH and ORAC results are expressed as micromoles of trolox equivalent per gram of extract.

For both, each value is the mean  $\pm$  standard deviation (n=3). <sup>b</sup> Anti-AGEs activity is evaluated by the half maximal inhibitory concentration (IC<sub>50</sub>) expressed as milligram per milliliter (mg/mL) (n=1). <sup>c</sup> (Kalogeropoulos et al., 2009). <sup>d</sup> (Rodríguez et al., 2012). <sup>e</sup> *Rosmarinus officinalis*. <sup>f</sup>

*Styphnolobium Japonicum*.

Table 4. Anti-AGEs activities of compounds presents in the 8 selected fractions (with  $IC_{50} \leq 0.04$  mg/mL)

Compound (n°)	Anti-AGEs <sup>a</sup> IC <sub>50</sub> (mM)	Fraction
Pinobanksin-3-acetate ( <b>26</b> )	0.06	F8
2-Acetyl-1,3-dicoumaroylglycerol ( <b>25</b> )	0.07	F17
Pinobanksin ( <b>14</b> )	0.08	F9
Prenyl caffeate ( <b>27</b> )	0.09	F10
Pinobanksin-5-methyl ether ( <b>11</b> )	0.10	F18
Naringenin ( <b>15</b> )	0.2	F11-12
Benzyl caffeate ( <b>24</b> )	0.4	F11
Cinnamyl caffeate ( <b>38</b> )	0.5	F11
Cinnamyl isoferulate ( <b>34</b> )	0.6	F8
Ferulic acid ( <b>7</b> )	0.7	F16
Isoferulic acid ( <b>8</b> )	0.9	F18
CAPE ( <b>29</b> )	1.6	F11
<i>p</i> -Coumaric acid ( <b>6</b> )	> 3	F16
Cinnamic acid ( <b>12</b> )	> 3	F9
Chrysin ( <b>28</b> )	> 3	F9

<sup>a</sup> The anti-AGEs activity was evaluated by the means of the half maximal inhibitory concentration (IC<sub>50</sub>) expressed as millimole of compound per liter (mM) (n=1). Reference: quercetin IC<sub>50</sub>=0.2 mM.

# FIGURE GRAPHICS

Fig. 1.

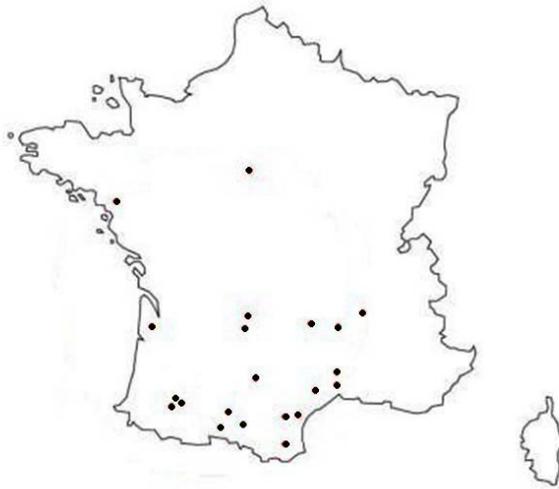


Fig. 2

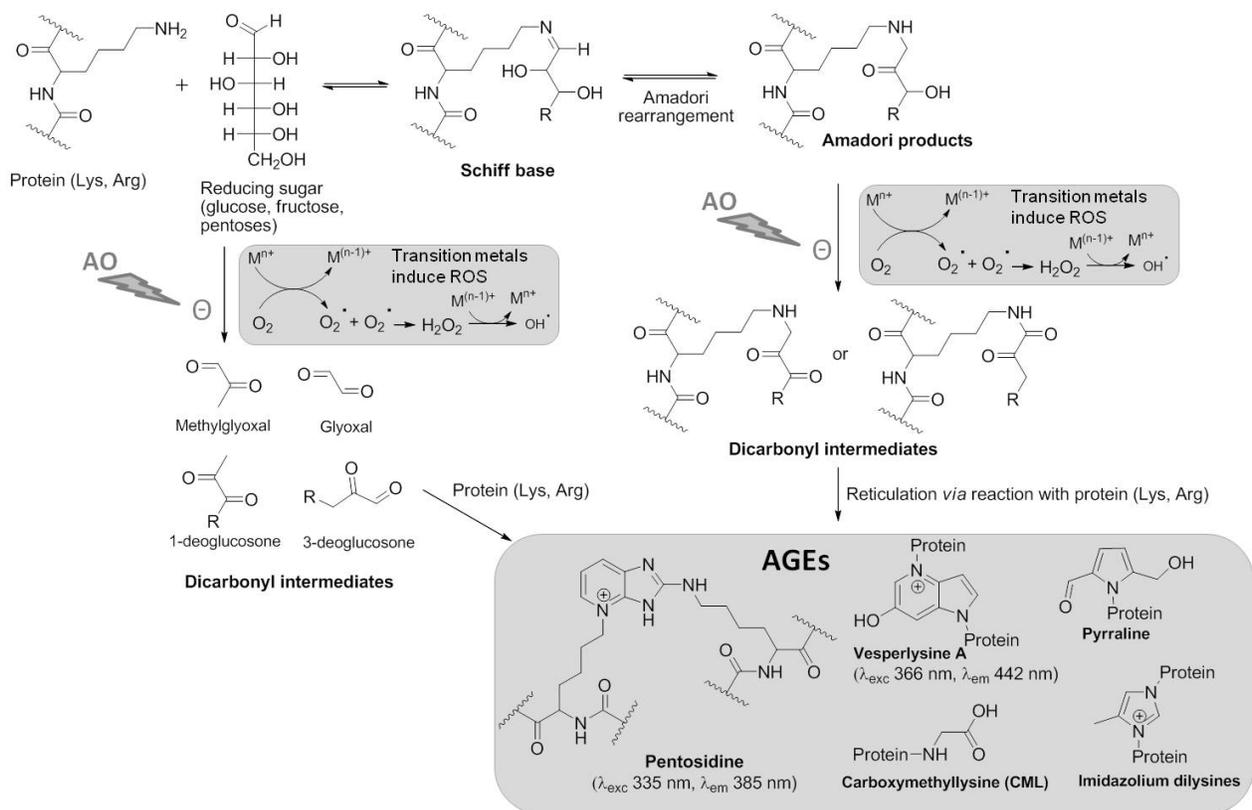
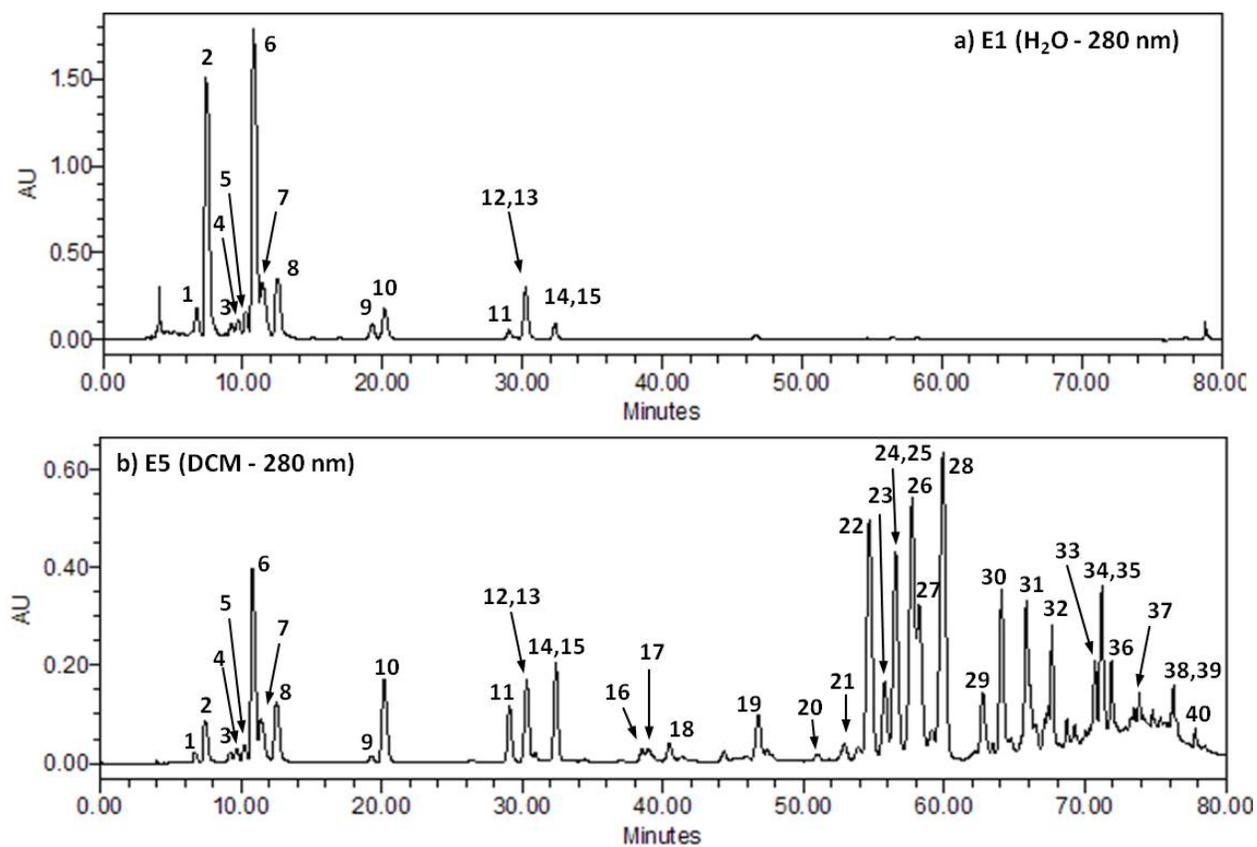


Fig. 3



GRAPHIC FOR TABLE OF CONTENT

