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1 **Chemical composition, antioxidant and anti-AGEs activities of a**

2 **French poplar type propolis**

3

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Abstract

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

Keywords

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,[1,2] anti-inflammatory[3] as well as
29 antimicrobial[4,5] 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[6] while tropical propolis
36 are rich in prenylated derivatives of *p*-coumaric acids, benzophenons and terpenoids,[1,7] 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).[8]
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[9] and complications of diabetes[8] such as atherosclerosis[10] or
45 or renal failure.[11] 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 developped as a drug.[8] 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*.[12] 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.[13] 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.[14] 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-methylpropionamidine) 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}H and 125 MHz for ^{13}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 H_2O (31/19/4) (E6). For E1, a
92 decoction of 1 g of propolis powder was boiled in 20 mL H_2O 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.[15] Briefly, 20 µL of extract solution (2.5 mg/mL in MeOH) were
99 mixed with 280 µL of distilled water and 100 µL of Folin-Ciocalteu's phenol reagent. After 3
100 min, 1200 µL of distilled water and 400 µL of 20% aqueous sodium carbonate solution were
101 added. 200 µL 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.[16] 300 µL of 95%
109 EtOH were mixed with 100 µL of extract solution (1.5 mg/mL in EtOH 80%), 20 µL of 10%
110 aqueous aluminium chloride solution, 20 µL of 1M potassium acetate aqueous solution and 560
111 µL of distilled water. 200 µL 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 µg/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[17] was used to determine flavanone and dihydroflavonol content. Briefly, 500 µL of
120 extract solution (2.5 mg/mL in MeOH) were reacted with 250 µL of MeOH and 500 µL 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 µL of the solution
123 were mixed with 500 µL of 20% potassium hydroxide in 70% EtOH and then centrifuged at 4000
124 rpm for 10 min to remove the precipitate. 20 µL of the supernatant were collected, put into the
125 96-well microtiter plate and mixed with 180 µL 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 (±)-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 µm nylon-membrane syringe
133 filter prior to injection (10 µL). 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 3µm C18 100A (150x4.6 mm i.d.,
136 3µm) 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_2, 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 beewax. 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® Teledyne ISCO (Lincoln, NE, USA) 165 apparatus, on a prepacked silica gel column (Interchim PF-50SI HC/300g, 50 µm, 20 bars) at a

166 flow rate of 100 mL/min with the following gradient: 100% C_6H_{12} (2.0 L), C_6H_{12}:EtOAc 90:10
167 (1.7 L), C_6H_{12}:EtOAc 90:10 to 80:20 (2.2 L), C_6H_{12}:EtOAc 80:20 to 70:30 (2.5 L), C_6H_{12}:EtOAc
168 70:30 to 60:40 (2.2 L), C_6H_{12}: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.[18] 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® 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.[19] 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-methylpropionamidine) 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 (λ_{exc} 485 nm, λ_{em} 520 nm). The 75 mM phosphate buffer was used as
193 a blank, and 12.5, 25, 50, and 75 μM solutions of Trolox were used as calibration solutions. A
194 chlorogenic acid solution in phosphate buffer (8.8 μM) 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_{50} were
200 determined using a previously described method[20] with slight modifications. Briefly, BSA (10
201 mg/mL) was incubated with D-ribose (0.5 M) together with the tested compound (3 μM to 3
202 mM) or extract (1 μg to 1 mg) in 50 mM phosphate buffer at pH 7.4 (NaN_3, 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 μM to 3 mM) or extract (1 μg 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 μL. Pentosidine-
209 like (λ_{exc} 335 nm, λ_{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_{50}
211 determination.[20,21] The percentage of AGE formation was calculated as follows for each
212 compound/extract concentration:

$$AGEs\ (\%) = \frac{\text{fluorescence intensity (sample)} - \text{fluorescence intensity (blank of sample)}}{\text{fluorescence intensity (control)} - \text{fluorescence intensity (blank of control)}} x100$$

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_{50} 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 %).[22] 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%)[5] and a MeOH propolis extract from Mexico (40%).[23]

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).[1] 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).[1]

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).[2] 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.[24] Nevertheless, FF and FD contents of E2-4 differed from those of Italian and
240 Swiss 70% EtOH propolis extracts where FF > FD.[25]
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 ^{1}H and ^{13}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.[7,26]

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 ± 0.6 and 61.4 ± 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 ± 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).[1]
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 exibited
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.[27] *A contrario*, an AO synthetic compound such as

307 aminosalicylic acid proved *in vitro* more effective than aminoguanidine in reducing the
308 antiproliferative effects of both high glucose and BSA-issued AGEs.[28] 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-β peptide treated mice.[29] 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 glycoxidation.
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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340 **Note**

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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 α,β-dicarbonyl
442 intermediates or break them (AGE breakers) [adapted from Ahmed, 2005[8]].

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[a] (mg GAE/g)	FF[b] (mg QE/g)	FD[c] (mg NE/g)
E1	H_2O	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

[a] Total polyphenol contents were determined by the Folin-Ciocalteu method. The results are expressed as milligram of gallic acid equivalent per gram of extract. [b] Flavone/flavonol contents were determined by $AlCl_3$ coloration. The results are expressed as mg of quercetin equivalent per gram of extract. [c] 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[a] (mg/g of extract)					
	E1: H_2O	E2: 95% EtOH	E3: 70% EtOH	E4: MeOH	E5: DCM	E6: Mixed solvents
Caffeic acid (**2**)	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 (**6**)	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 (**7**)	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 (**8**)	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 (**10**)	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 (**22**)	-	35.2 ± 0.2	33.4 ± 0.2	33.0 ± 0.2	49.6 ± 0.4	36.8 ± 0.3
Pinobanksin-3-acetate (**26**)	-	40.2 ± 0.3	38.7 ± 0.5	38.1 ± 0.2	59.1 ± 0.5	42.8 ± 0.4
Prenyl caffeate (**27**)	-	20.2 ± 0.5	20.2 ± 0.3	19.5 ± 0.2	27.4 ± 0.3	22.0 ± 0.4
Chrysin (**28**)	-	24.7 ± 0.1	23.5 ± 0.2	23.1 ± 0.2	36.5 ± 0.4	27.6 ± 0.2
CAPE (**29**)	-	11.2 ± 0.2	10.6 ± 0.2	10.4 ± 0.4	15.8 ± 0.7	11.8 ± 0.2
Galangin (**30**)	-	21.9 ± 0.2	20.7 ± 0.3	20.4 ± 0.5	31.1 ± 0.8	23.5 ± 0.4
Pinostrobin (**33**)	-	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

[a]: 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[a] (μmol TE/g)	ORAC[a] (μmol TE/g)	Anti-AGEs[b] IC_{50} (mg/mL)
E1	H_2O	1731 ± 28	9722 ± 273	0.34
E2	EtOH	1605 ± 26	8155 ± 114	0.05
E3	70% EtOH	1650 ± 149	9890 ± 480	0.03
E4	MeOH	1386 ± 171	7769 ± 360	0.03
E5	DCM	1437 ± 105	9242 ± 739	0.03
E6	Mixed solvents	1964 ± 124	11278 ± 11	0.04
Propolis (Greece)[c]	EtOH	1110 ± 70	-	-
Propolis (Colombia)[d]	EtOH	190	1965	-
E392[e]	EtOH	591 ± 20	2433 ± 88	0.60
S. japonicum[f]	EtOH	1714 ± 68	7896 ± 711	0.09
Chlorogenic acid		2740 ± 64	11289 ± 531	0.04
Quercetin		6723 ± 66	13907 ± 662	0.06

[a] DPPH and ORAC results are expressed as micromoles of trolox equivalent per gram of extract. For both, each value is the mean ± standard deviation (n=3). [b] Anti-AGEs activity is evaluated by the half maximal inhibitory concentration (IC_{50}) expressed as milligram per milliliter (mg/mL) (n=1). [c] (Kalogeropoulos et al., 2009). [d] (Rodríguez et al., 2012). [e] *Rosmarinus officinalis*. [f] *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[a] IC_{50} (mM)	Fraction
Pinobanksin-3-acetate (**26**)	0.06	F8
2-Acetyl-1,3-dicoumaroylglycerol (**25**)	0.07	F17
Pinobanksin (**14**)	0.08	F9
Prenyl caffeate (**27**)	0.09	F10
Pinobanksin-5-methyl ether (**11**)	0.10	F18
Naringenin (**15**)	0.2	F11-12
Benzyl caffeate (**24**)	0.4	F11
Cinnamyl caffeate (**38**)	0.5	F11
Cinnamyl isoferulate (**34**)	0.6	F8
Ferulic acid (**7**)	0.7	F16
Isoferulic acid (**8**)	0.9	F18
CAPE (**29**)	1.6	F11
p-Coumaric acid (**6**)	> 3	F16
Cinnamic acid (**12**)	> 3	F9
Chrysin (**28**)	> 3	F9

[a] The anti-AGEs activity was evaluated by the means of the half maximal inhibitory concentration (IC_{50}) expressed as millimole of compound per liter (mM) (n=1). Reference: quercetin IC_{50}=0.2 mM.

FIGURE GRAPHICS

Fig. 1.

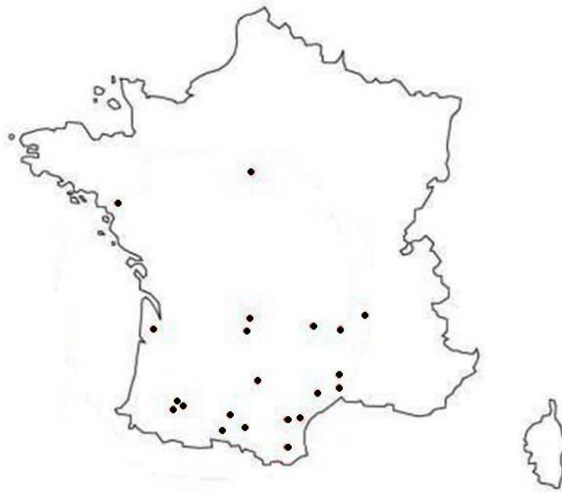


Fig. 2

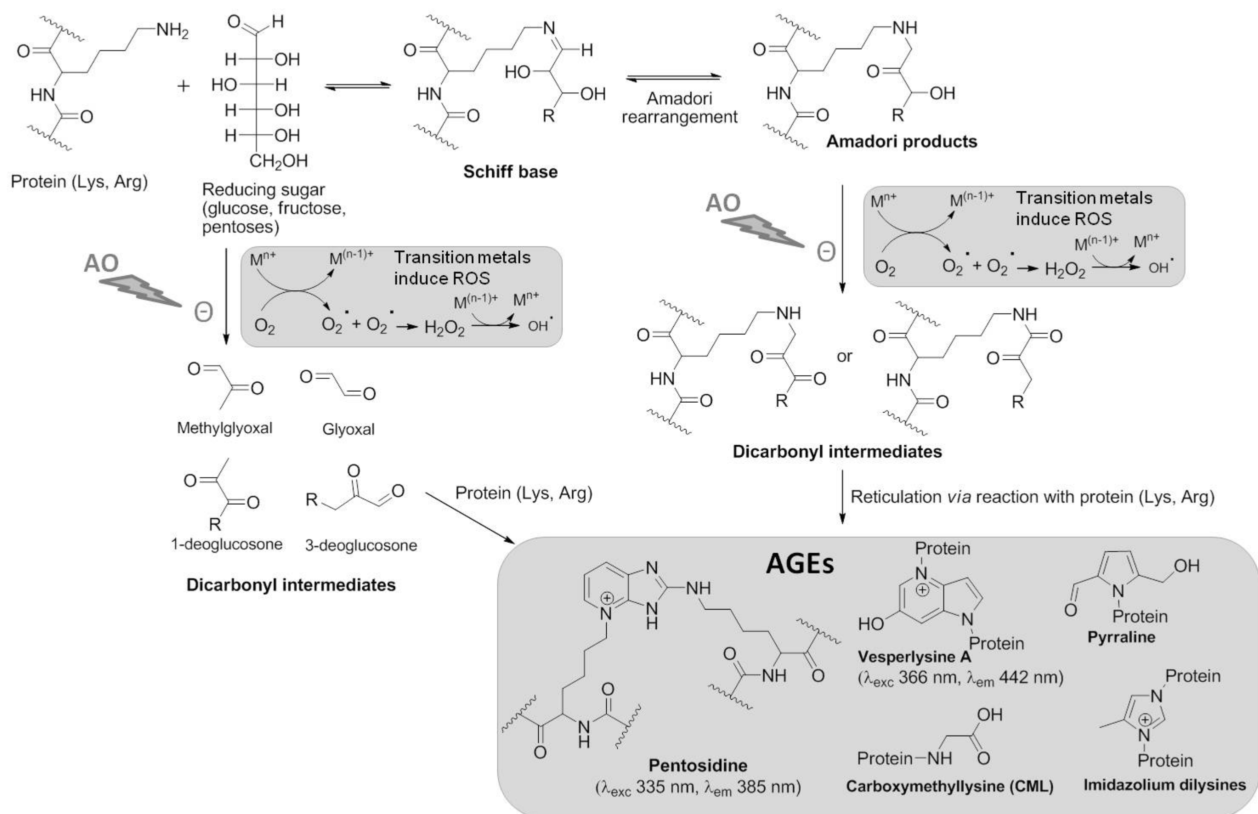
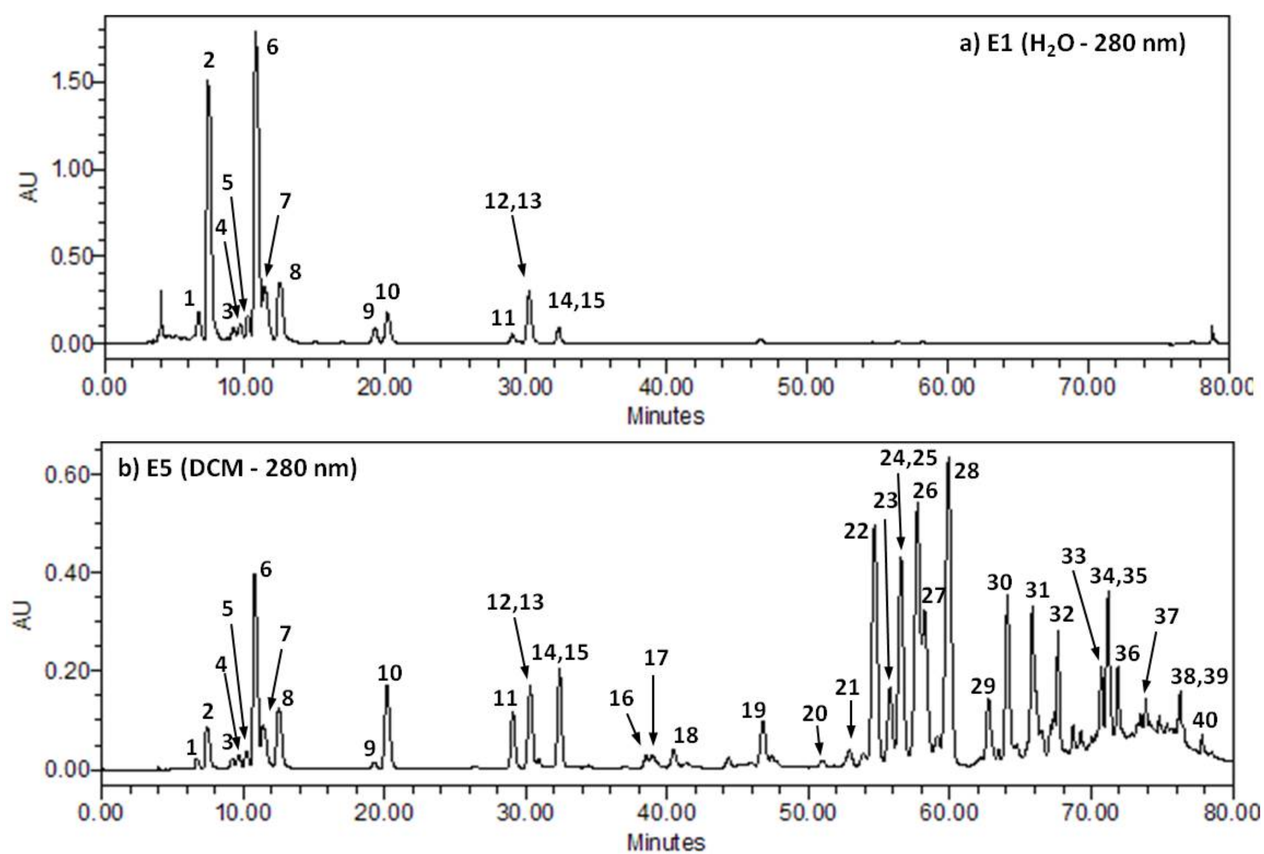


Fig. 3



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