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

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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

INTRODUCTION

Propolis is a resinous material, most commonly collected by honeybees from buds and exudates of various trees and plants. Propolis has been largely used in folk medicine since ancient times due to its pharmacological potential associated with antioxidant,^{1,2} anti-inflammatory³ as well as antimicrobial^{4,5} properties.

Propolis is generally composed of 50% of resin and balm (including polyphenolic compounds), 30% of wax and fatty acids, 10% of essential oils, 5% of pollen and 5% of various organic and inorganic compounds. The composition of propolis can be specified as it depends on the vegetation at the site of collection. Indeed, propolis from temperate climatic zones, like Europe, North America and non-tropical regions of Asia, originate mainly from the bud exudates of *Populus* species and are rich in flavonoids, phenolic acids and their esters⁶ while tropical propolis are rich in prenylated derivatives of *p*-coumaric acids, benzophenons and terpenoids,^{1,7} as no poplars or birches grow in this region.

During Maillard's reaction, a nucleophilic addition between a free amino group and a carbonyl group of a reducing sugar leads to the glycation of proteins. The resulting Schiff base rearranges to a more stable ketoamine, so-called Amadori product, which can undergo further reactions, involving dicarbonyl intermediates, giving advanced glycation end-products (AGEs) (Fig. 1).⁸ Extra and intracellular accumulation of AGEs with time play an important role in the development of organ damage in such a way that AGEs are involved in many important pathologies *e. g.* Alzheimer's disease⁹ and complications of diabetes⁸ such as atherosclerosis¹⁰ or renal failure.¹¹ Therefore, numerous compounds have been investigated for their anti-AGEs activity and the synthetic hydrazine aminoguanidine has received the most efforts to be developed as a drug.⁸ Protein glycation is a self-generated process. Thus, any potential inhibitor of AGEs formation should exhibit a long half-life while being virtually atoxic. Since reactive

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

MATERIAL AND METHOD

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

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

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

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

Extractions. The propolis batch was homogeneously pulverized in the presence of liquid nitrogen and divided into 1g samples. Four different extractions were then carried out on 1g samples with water (E1), 95% EtOH (E2), 70% EtOH (E3) and MeOH (E4). Then, two extractions, preceded by a cyclohexane wax elimination, were independently performed on 1g samples with DCM (E5) and a mixture of DCM, MeOH and H_2O (31/19/4) (E6). For E1, a decoction of 1 g of propolis powder was boiled in 20 mL H_2O at 100°C for 15 min. After cooling, the solidified wax and the residue were removed by filtration, and the filtrate was concentrated. For other solvents, 1 g of propolis powder (or of residue issued from a previous

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

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

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

Flavanone and dihydroflavonol content. The modified method described by Nagy and Grancai¹⁷ was used to determine flavanone and dihydroflavonol content. Briefly, 500 μ L of extract solution (2.5 mg/mL in MeOH) were reacted with 250 μ L of MeOH and 500 μ L of 1% 2,4-dinitrophenylhydrazine (DNP) solution (500 mg of DNP mixed with 1 mL of 96% sulfuric acid and diluted to 50 mL with MeOH) at 50°C for 50 min. After cooling, 500 μ L of the solution were mixed with 500 μ L of 20% potassium hydroxide in 70% EtOH and then centrifuged at 4000 rpm for 10 min to remove the precipitate. 20 μ L of the supernatant were collected, put into the 96-well microtiter plate and mixed with 180 μ L of 1% potassium hydroxide methanolic solution. Absorbance was measured at 495 nm. A blank was prepared using the same amount of MeOH instead of the extract solution. Standard solutions of (\pm)-naringenin (0.25-2.00 mg/mL in MeOH) were used to build the calibration curve ($y=0.2053x$; $r^2=0.9945$). Flavanone and dihydroflavonol content was calculated as mg NE/g (Naringenin Equivalent per gram of extract). All measurements were performed in triplicate.

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

Esquire 3000+ in both, positive and negative modes, with the conditions as follows: collision gas, He; collision energy amplitude, 1.3 V; nebulizer and drying gas, N₂, 7 L/min; pressure of nebulizer gas, 30 psi; dry temperature, 340°C; flow rate, 1.0 mL/min; solvent split ratio 1:9; scan range, m/z 100–1000.

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

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

flow rate of 100 mL/min with the following gradient: 100% C₆H₁₂ (2.0 L), C₆H₁₂:EtOAc 90:10 (1.7 L), C₆H₁₂:EtOAc 90:10 to 80:20 (2.2 L), C₆H₁₂:EtOAc 80:20 to 70:30 (2.5 L), C₆H₁₂:EtOAc 70:30 to 60:40 (2.2 L), C₆H₁₂:EtOAc 60:40 to 50:50 (3.0 L) and at last DCM:MeOH 96:4 (2.2 L). UV detection, achieved at 254 and 280 nm, allowed to separate 21 fractions.

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

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

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

Determination of sample concentrations inhibiting 50% of AGEs formation. IC₅₀ were determined using a previously described method²⁰ with slight modifications. Briefly, BSA (10 mg/mL) was incubated with D-ribose (0.5 M) together with the tested compound (3 μ M to 3 mM) or extract (1 μ g to 1 mg) in 50 mM phosphate buffer at pH 7.4 (NaN₃, 0.02%). Solutions were incubated in 96-well microtiter plates at 37°C for 24 h in a closed system before AGE fluorescence measurement. Fluorescence resulting from the incubation, under the same BSA (10 mg/mL) and tested compound (3 μ M to 3 mM) or extract (1 μ g to 1 mg) conditions, was subtracted for each measurement. A control, i.e. no inhibition of AGE formation, consisted of wells with BSA (10 mg/mL) and D-ribose (0.5 M). A blank control, i.e. 100% inhibition of AGE formation, consisted of wells with only BSA. The final assay volume was 100 μ L. Pentosidine-like (λ_{exc} 335 nm, λ_{em} 385 nm) AGE fluorescence were measured using a microplate spectrofluorometer. In this type of automation, a single analysis is sufficient for an accurate IC₅₀ determination.^{20,21} The percentage of AGE formation was calculated as follows for each compound/extract concentration:

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

Dose-effect curves were best fitted with a sigmoidal dose-response equation using Sigma Plot 12.0 software, which enabled calculation of the IC₅₀ values.

RESULTS AND DISCUSSION

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

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

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

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

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

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

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

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

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

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

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

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

ABBREVIATION USED

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

ASSOCIATED CONTENT

Supporting information

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

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FIGURE CAPTIONS

Fig. 1. Collection sites of French propolis samples

Fig. 2. Schematic formation of AGEs. AGE inhibitors may follow different inhibition pathways in order to prevent AGE formation. For exemple, they can prevent glycation *via* reaction with free amino groups in proteins or carbonyl groups on reducing sugars. In addition, compounds with antioxidant (AO) properties (free radical scavengers or transition metal chelators) may reduce reactive oxygen species (ROS). Finally, they can also scavenge α,β -dicarbonyl intermediates or break them (AGE breakers) [adapted from Ahmed, 2005⁸].

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

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 ₂ 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

^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 AlCl₃ 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 ₂ O	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 ($\mu\text{mol TE/g}$)	ORAC ^a ($\mu\text{mol TE/g}$)	Anti-AGEs ^b IC ₅₀ (mg/mL)
E1	H ₂ 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) ^c	EtOH	1110 \pm 70	-	-
Propolis (Colombia) ^d	EtOH	190	1965	-
E392 ^e	EtOH	591 \pm 20	2433 \pm 88	0.60
<i>S. japonicum</i> ^f	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

^a 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). ^b Anti-AGEs activity is evaluated by the half maximal inhibitory concentration (IC₅₀) 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 ^o)	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
<i>p</i> -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.

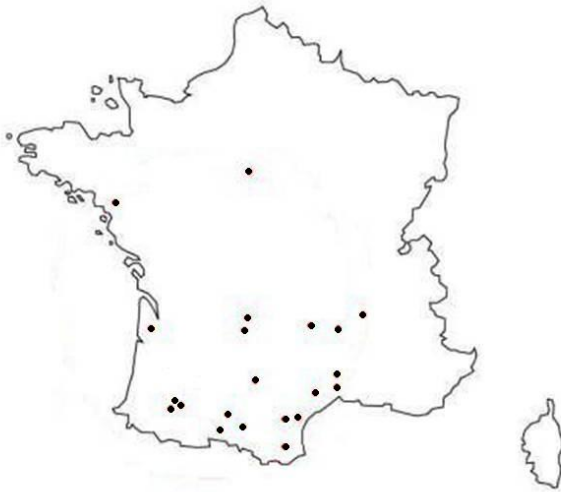


Fig. 2

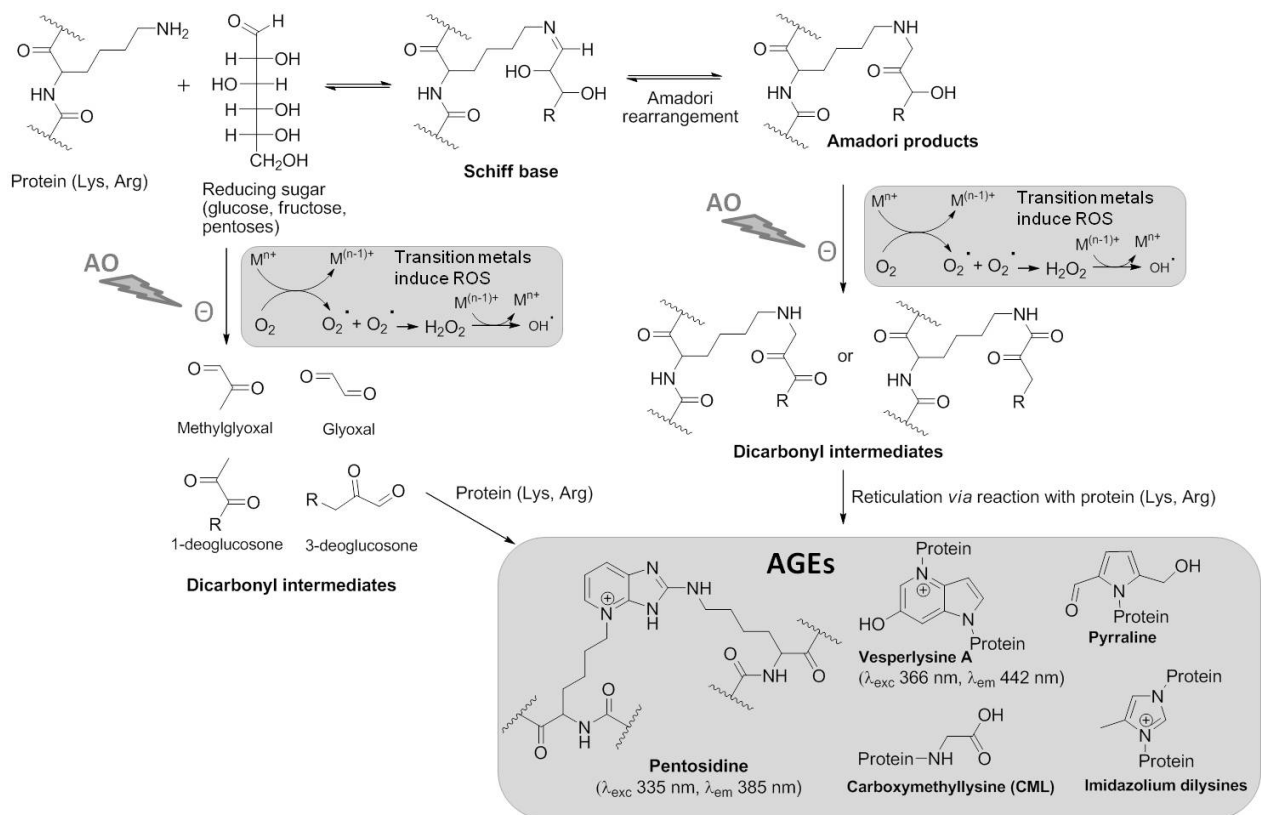
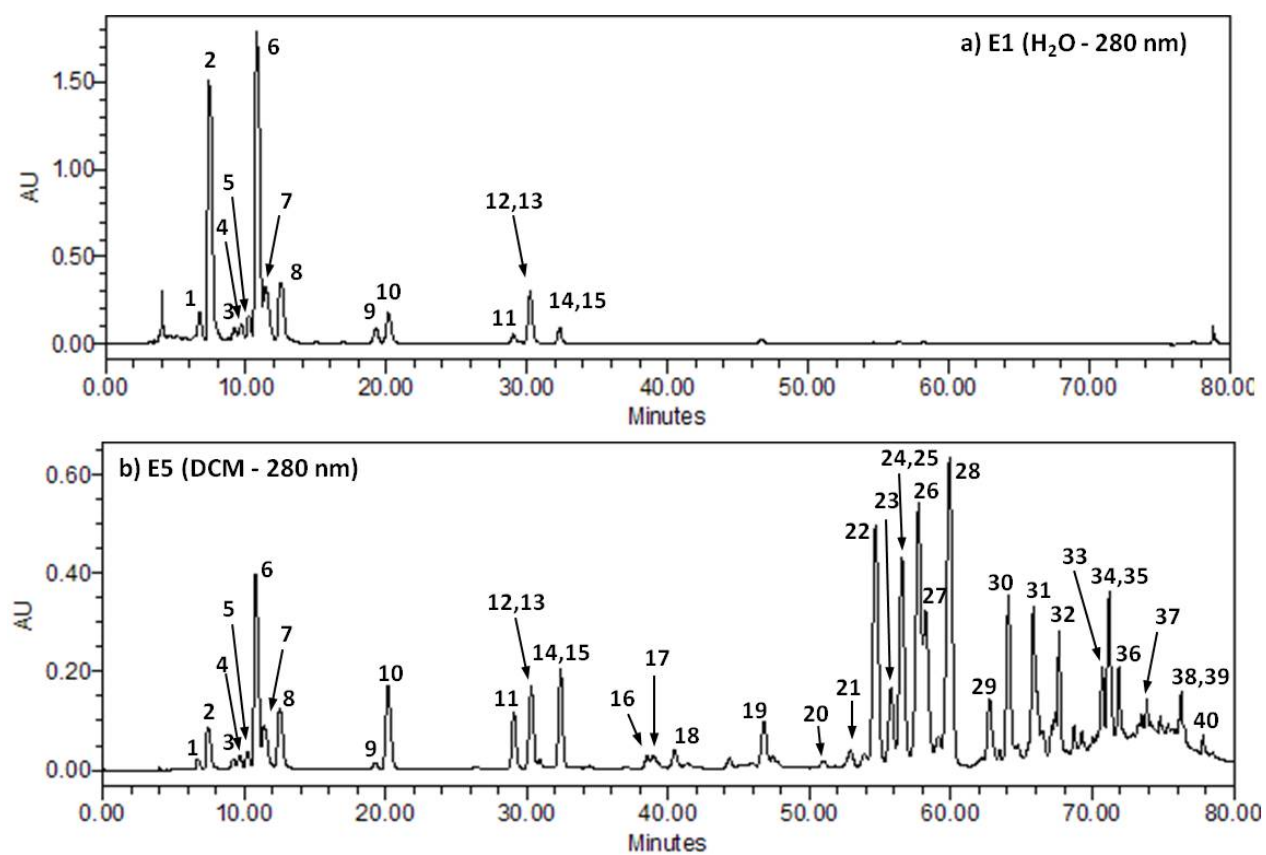


Fig. 3



GRAPHIC FOR TABLE OF CONTENT

