Chemical composition and antioxidant activity of French BFA propolis extracts

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Abstract

Introduction: Propolis is a resinous natural substance collected by honeybees from buds and exudates of various trees and plants, mixed with beewax and salivary enzymes. Bees generally use it as a sealer, to smooth out the internal walls of the hive as well as a protective barrier against intruders. Several pharmacological activities have been attributed to propolis extracts, mainly antibacterial, antiviral, antifungal, antitumoral but also antioxidant properties we will focus on here. **Research methods:** A mixture of 24 batches of French propolis, supplied by "Ballot-Flurin Apiculteurs" (BFA), a company located in the South-West of France, was extracted with five different solvents: water, 70% ethanol, methanol, methylene chloride and a mixture of solvents (methylene chloride/methanol/water). Their chemical composition was determined by High Performance Liquid Chromatography coupled with Diode Array Detector (HPLC/DAD) and with Mass Spectrometry (HPLC/MS) profilings followed, when necessary, by Nuclear Magnetic Resonance (NMR) 1D and 2D studies. Total polyphenol content and antioxidant activities were evaluated for these five BFA propolis extracts using respectively Folin-Ciocalteu, 2,2-diPhenyl-1-PicrylHydrazyl (DPPH) and Oxygen Radical Absorbance Capacity (ORAC) assays. **Results and discussion:** All extracts of BFA French propolis exhibited phenolic acids and esters as well as flavonoids, except for the aqueous one which predominantly contained phenolic acids. They also showed high antioxidant activities, about 2-5 times higher than an ethanolic rosemary extract which was recently approved as a food additive in Europe (E392). Therefore, among these extracts, the aqueous one offers the advantage of a strong antioxidant activity combined with a "green extraction", when toxic residual solvents are a real issue in pharmaceutical, cosmetic and food additive products .

1. Introduction

Propolis is a resinous natural substance collected by honeybees from buds and exudates of various trees and plants, mixed with beewax and salivary enzymes. Bees generally use it as a sealer, to smooth out the internal walls of the hive as well as a protective barrier against intruders. Propolis has been used in folk medicine since ancient times due to its pharmacological potential associated with antioxidant,[1–3] antifungal,[4,5] antibacterial[6,7] as well as anti-inflammatory[9] properties.

Propolis is generally composed of 50% resin and balm (including polyphenolic compounds), 30% wax and fatty acids, 10% essential oils, 5% pollen and 5% various organic and inorganic compounds. But the composition of propolis depends on the vegetation at the site of collection.[10] Thus, 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 esters[11] while propolis from tropical regions, where no poplars and birches exist, are rich in prenylated derivatives of *p*-coumaric acids and benzophenons, terpenoids.[12,13]

The chemical composition and biological activities of propolis of many countries all over the world,[13,14] and in Europe specially in Netherlands,[15] Greece,[16] Portugal,[3] Spain,[17] have been widely studied, but few articles related to the quality of French propolis have been reported.

In this study, we identified the chemical constituents of a French propolis representative mixture by high performance liquid chromatography (HPLC) coupled with a diode array detector (DAD) and with mass spectrometry (MS) and, when necessary, by nuclear magnetic resonance (NMR) 1 or 2 dimensions studies after isolation. Then, we determined the total polyphenol content and evaluated in vitro antioxidant activity, evaluated by 2,2-diPhenyl-1-PicrylHydrazyl (DPPH) and Oxygen Radical Absorbance Capacity (ORAC) assays, of five propolis extracts prepared with different solvents: water, 70% ethanol, methanol, methylene chloride, and a miscible mixture of solvents (methylene chloride/methanol/water).

2. Materials and methods

2.1. Reagents and chemicals

2,2-Diphenyl-1-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, caffeic acid 1,1-dimethylallyl ester , all of analytical grade, and cyclohexane, ethyl acetate and dichloromethane, laboratory reagent grade, were purchased from Sigma-Aldrich (L'Isle d'Abeau Chesnes, France). Methanol and ethanol,

pure grade, as well as gradient HPLC grade and methanol potassium hydroxide pellets were supplied by Carlo Erba (Val de Reuil, 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). Sulfuric acid and sodium carbonate from VWR Prolabo (Fontenay-sous-Bois, France).

2.2. Propolis sample

A mixture of 24 propolis collected from apiaries of different regions of France on 2010 and 2011 were used for this study. They were supplied by the company Ballot-Flurin Apiculteurs, located in the South-West of France, pioneer in organic beekeeping in France and expert in natural health. They locally develop food, cosmetic and hygiene preparations of high quality with bee products, including propolis, collected and manufactured by their own Apiculture Douce® method.

2.3. Instrumentation

NMR spectra (1D and 2D) were recorded on a Brüker spectrometer at 500 MHz for ^{1}H NMR and at 125 Hz for ^{13}C NMR. MS analysis was performed on an ESI/APCI Ion Trap Esquire 3000+ from Brüker. Absorbance was measured on a Tecan Infinite M200 microplate spectrophotometer.

2.4. Extractions

Propolis mixture was first pulverized in presence of liquid nitrogen into a homogenous powder. Three single extractions were first carried out with water, 70% ethanol and methanol. Then, two extractions were sequentially performed: cyclohexane extraction (to eliminate beewax) followed by dichloromethane DCM for the first one and cyclohexane extraction followed by the miscible solvent (DCM/MeOH/H_2O 31/19/4 called mixed solvents) for the last one. For aqueous extraction, 1 g of pulverized propolis was extracted with 20 mL at 100°C for 15 min. After beewax coat removal, the mixture was filtered and concentrated. For the other solvents (single or sequential) extractions, 1 g of pulverized propolis (or the residue of the previous extraction) were extracted three times with 20 mL of solvent. After stirring for 2h at room temperature, the mixture was filtered. The filtrates were collected and evaporated under vacuum. Yield of extracts: water 7%; 70% EtOH 65%; MeOH 68%; DCM 50%, mixed solvents 59%.

2.5. Total polyphenol content

Total polyphenol content was determined according to the Folin-Ciocalteu colorimetric method.[18] 20 µL of extract solution (2.5 mg/mL in methanol) were mixed with 280 µL of distilled water and 100 µL of Folin-Ciocalteu's phenol reagent. After 3 min, 1200 µL of distilled water and 400 µL of 20% aqueous sodium carbonate solution were added. 200 µL of each solution were put into the 96-well microtiter plate. The absorbance was measured at 760 nm after 30 min in the dark at room temperature. A blank was prepared the same way by using methanol 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 as milligram of Gallic Acid Equivalent per gram of extract (mg GAE/g). All determinations were performed in triplicate.

2.6. Fractionation by Flash Chromatography

Extraction of 50 g of propolis: 50.0 g of the propolis mixture were extracted with cyclohexane (3x200 ml, 2h, room temperature) to remove beewax. Then the residue was extracted with dichloromethane (5x200ml, 2h, room temperature), filtered and concentrated to give 25,0 g of extract 5 (R=50%).

Preparation of the sample: 21 g of DCM extract was dissolved in the minimum of dichloromethane (200 mL), mixed with 42 g of silica gel (DCM extract:silica gel 1:2) and concentrated to obtain a dry fine powder.

Fractionation by Flash Chromatography: The fractionation was performed by using a CombiFlash Teledyne ISCO apparatus, with a silica gel column (Interchim PF-50SI HC/300g, 50 µm, 20 bars) at a flow rate of 100 mL/min with the following gradient: 100% C_6H_{12} to C_6H_{12}:AcOEt 90:10 (1.7 L), C_6H_{12}:AcOEt 90:10 to 80:20 (2.2 L), C_6H_{12}:AcOEt 80:20 to 70:30 (2.5 L), C_6H_{12}:AcOEt 70:30 to 60:40 (2.2 L), C_6H_{12}:AcOEt 60:40 to 50:50 (3.0 L) and then CH_2Cl_2:MeOH 96:4 (2.2 L). UV detection, achieved at 254 and 280 nm, led to a separation into 21 fractions.

2.7. HPLC-DAD-MS procedure and quantification

Extracts and fractions were dissolved in methanol (5 mg/mL) centrifuged at 13000 rpm for 10 min and filtered through 0.2 µm nylon-membrane syringe filter prior to injection (10 µL) into the HPLC system. Analytical HPLC was run on a 2695 Waters® separation module equipped with a diode array detector 2996 Waters®. Separation was achieved on a Phenomenex® Luna column 3µm C18 100A (150x46 mm, 3µm) protected with a Phenomenex® SecuryGuard cartridge C18 (4 x 3 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 methanol (solvent B). The separation was performed by the following gradient: 40% B (0-10min), 40-50%B (10-25 min), 50-60%B (25-55min), 60-90%B (55-70 min), 90%B (70-80min). UV detection and quantification were achieved at 280 nm.

The mass analyses were performed with an ESI interface in both, positive and negative modes, with the conditions as follows: collision gas, He; collision energy amplitude, 1.3 V; nebulizer and drying gas, N_2, 7 L/min; pressure of nebulizer gas, 30 psi; dry temperature, 340 °C; flow rate, 0.4 ml/min; solvent split ratio 1:9; scan range, m/z 100–1000.

Stock solutions of the 12 markers 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), pinobanksine-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) and pinostrobin (0.6 mg/mL). Stock solutions and the diluted ¾, 1/2, ¼, 1/8, 1/16 ones were used to determine the calibration curves of the 12 markers (n=2). The five French propolis extracts (aqueous, 70% EtOH, MeOH, DCM and mixed solvents) were analysed at 5 mg/mL (n=3).

2.8. Scavenging Activity of diphenyl-picrylhydrazyl DPPH radicals

The DPPH radical scavenging effects of propolis extracts were carried out using a modified previously established methodology.[19] In its radical form, $DPPH^{\bullet}$ has an absorption band at 517 nm which disappears upon reduction by an antiradical compound. The tested compounds and standards were diluted in absolute ethanol at 0.02 mg/mL from stock solutions at 1 mg/mL in DMSO. 100 µL aliquots 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 ethanol 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. Ethanol was used as a blank, whereas 10, 25, 50, and 75 µM of Trolox (hydrophilic α-tocopherol analog) were used as calibration solutions. A sample of 0.02 mg/mL chlorogenic acid was used as a quality control. The DPPH-scavenging activity of tested compounds was compared with that of Trolox calibration curve. Results were expressed in terms of Trolox equivalent (micromoles of Trolox equivalents per gram of extract).

2.9. Measurement of oxygen radical absorbance capacity (ORAC)

ORAC assays were carried out according to the method of Huang et *al.*[20] with some modifications. This assay measures the ability of antioxidant compounds to inhibit the decline in fluorescein (FL) fluorescence that is induced by a peroxyl radical generator, 2,2'-azobis(2-methylpropionamidine) dihydrochloride (AAPH). 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 FL solution (0.1 µM in phosphate buffer), 50 µL of freshly prepared AAPH solution (51.6 mg/mL in phosphate buffer), and 20 µL of sample per well. Samples were analysed in triplicates and diluted at different concentrations (25 µg/mL, 12.5 µg/mL, 6.25 µg/mL 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 at the excitation of 485 nm and emission of 520 nm. The 75 mM phosphate buffer was used as a blank, and 12.5, 25, 50, and 75 µM of Trolox (hydrophilic α-tocopherol analog) were used as calibration solutions. A sample of 8.8 µM chlorogenic acid was used as quality control. The final ORAC values were calculated by 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. The area under curve was calculated using Magellan™ data analysis software (Tecan, France).

3. Result and discussion

Fig. 1 shows the HPLC chromatograms of the aqueous and the DCM extracts, the profiles of the two alcoholic and mixed solvents extracts being very similar from the DCM one (not shown). So there are two types of profile: the first one, the aqueous type with polar major components (retention time 6-30 min) and the second one, the organic solvent type with less polar major compounds (retention time 54-72 min). Their chemical composition were determined using HPLC/UV(DAD) and HPLC/MS(ESI) analyses on DCM extract and its fractions obtained by Flash chromatography, using ^{1}H and ^{13}C NMR (1 and 2 dimensions) after purification and by means of scientific literature and commercial standards when available.

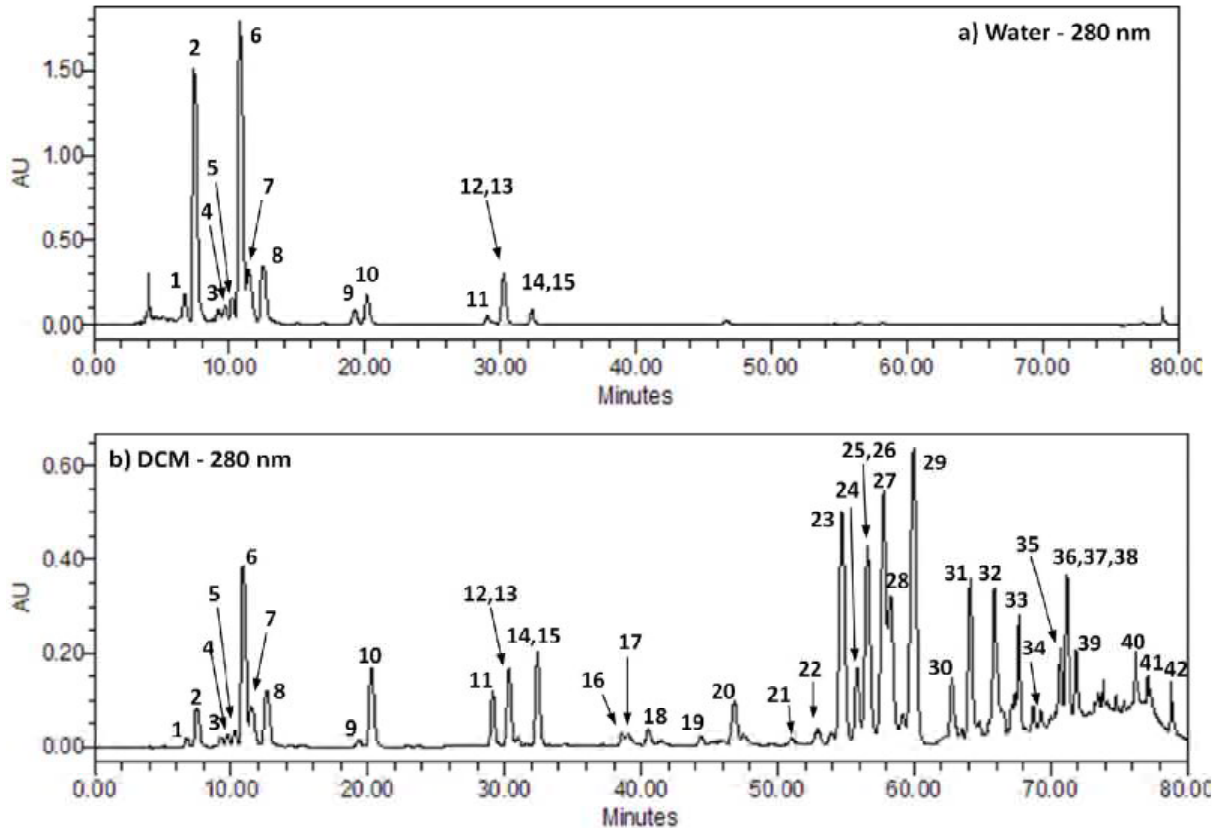

Fig. 1. HPLC chromatograms of aqueous extracts (a) and DCM (b) extracts of French propolis: **1** 3,4-dihydroxybenzaldehyde, **2** caffeic acid, **3** 4-hydroxybenzaldehyde, **4** vanilline, **5** 4-hydroxyacetophenone, **6** *p*-coumaric acid**, 7** ferulic acid, **8** isoferulic acid, **9** benzoic 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** luteolin methyl ether, **20** cinnamylidene acetic acid, **21** quercetin-7-methyl ether, **22** pinocembrin-5-methyl ether, **23** pinocembrin, **24** isopent-3-enyl caffeate, **25** benzyl caffeate**, 26** 2-acetyl-1,3-dicoumaroylglycerol**, 27** pinobanksin-3-acetate**, 28** prenyl caffeate, **29** chrysin, **30** caffeic acid phenylethyl ester (CAPE), **31** galangin**, 32** benzyl *p*-coumarate, **33** cinnamyl caffeate, **34** new flavan-3-ol, **35** pinostrobin, **36** cinnamyl isoferulate, **37** cinnamyl *p*-coumarate, **38** alpinone-3-acetate, **39** tectochrysin, **40** benzyl cinnamate, **41** cinnamyl cinnamate, **42** Cinnamyl cinnamylidene acetate

The aqueous extract is composed of benzaldehyde and benzoic acid derivatives (compounds **1**, **3**-**5**, **9**), cinnamic acid derivatives (**2**, **6**-**8**, **10**, **12**, **13**) and some flavanones/dihydroflavonols (**11**, **14**, **15**). The DCM extract possesses the same compounds as the aqueous one in the first part of the chromatogram in less quantities, but especially contains cinnamic ester derivatives (**24**, **25**, **28**, **30**, **32**, **33**, **36**, **37**, **40**-**42**), glycerol derivatives (**16**, **26**) and flavonoids, as flavanones/dihydroflavonols (**22**, **23**, **27**, **35**, **38**), flavones/flavonols (**17**-**19**, **21**, **29**, **31**, **39**) and a new flavan-3-ol (**34**) in the second part of the chromatogram.

So mainly exhibiting cinnamic acid derivatives (caffeic and p-coumaric acids…) and their esters (prenyl caffeate, CAPE…), but also flavonoids (pinocembrin, pinobanksin-3-acetate, chrysin,

galangin…), the French propolis from BFA belongs to the poplar-type, from *Populus* spp. of section *Aigeiros* and especially *P. nigra* L.[12]

The results of a quantitative study, carried out to determine the content of 12 major components in the five extracts of French propolis, are given in table 1.

Table 1. Contents of the 12 major compounds in the five extracts of French propolis

	Content[a] (mg/g of extract)				
	H_2O	**70% EtOH**	**MeOH**	**DCM**	**Mixed solvents**
Caffeic acid (2)	76.9 ± 0.6	6.0 ± 0.1	6.6 ± 0.1	5.0 ± 0.1	8.0 ± 0.1
***p*-coumaric acid (6)**	61.4 ± 0.3	11.1 ± 0.1	10.2 ± 0.1	15.4 ± 0.1	12.0 ± 0.1
Ferulic acid (7)	20.8 ± 0.4	4.4 ± 0.1	3.9 ± 0.1	6.2 ± 0.1	4.2 ± 0.1
Isoferulic acid (8)	16.2 ± 0.2	4.4 ± 0.1	4.1 ± 0.1	6.5 ± 0.1	4.6 ± 0.1
3,4-dimethoxycinnamic acid (10)	9.9 ± 0.1	7.7 ± 0.1	7.1 ± 0.1	10.8 ± 0.1	7.7 ± 0.1
Pinocembrin (23)	-	33.4 ± 0.2	33.0 ± 0.2	49.6 ± 0.4	36.8 ± 0.3
Pinobanksin-3-acetate (27)	-	38.7 ± 0.5	38.1 ± 0.2	59.1 ± 0.5	42.8 ± 0.4
Prenyl caffeate (28)	-	20.2 ± 0.3	19.5 ± 0.2	27.4 ± 0.3	22.0 ± 0.4
Chrysin (29)	-	23.5 ± 0.2	23.1 ± 0.2	36.5 ± 0.4	27.6 ± 0.2
Phenethyl caffeate CAPE (30)	-	10.6 ± 0.2	10.4 ± 0.4	15.8 ± 0.7	11.8 ± 0.2
Galangin (31)	-	20.7 ± 0.3	20.4 ± 0.5	31.1 ± 0.8	23.5 ± 0.4
Pinostrobin (35)	-	12.9 ± 0.3	10.0 ± 0.2	8.1 ± 0.4	7.8 ± 0.3
Total	185.3 ± 1.5	193.7 ± 1.8	186.5 ± 1.8	271.6 ± 3.5	208.9 ± 2.4

-: not detected

[a]: Each value is the mean of triplicate analyses for each sample ± standard deviation

As already seen in Fig. 1.a, aqueous extract contains predominantly caffeic **2** and *p*-coumaric **6** acids with respectively 76.9 ± 0.6 and 61.4 ± 0.3 mg/g. The four others extracts (70% EtOH, MeOH, DCM and mixed solvents) exhibit the same profile (different from the aqueous one), i.e. with pinobanksin-3-acetate **27** as the major component followed by pinocembrin **23**, chrysin **29**, galangin **31**, prenyl caffeate **28** and so on. The higher total of the 12 components is obtained for the DCM extract with 271.6 ± 3.5 mg/g. The lower ones are obtained for aqueous (185.3 ± 1.5 mg/g with only 5 compounds) and the MeOH extract (186.5 ± 1.8 mg/g).

Table 2 shows the total polyphenol content (Folin-Ciocalteu assay) and the antioxidant activity using DPPH and ORAC assays, of the five extract of French propolis.

Table 2. Total polyphenol content and antioxidant activity by DPPH and ORAC assays of French propolis extracts

Extract	Solvent	Total polyphenol content[a] (mg GAE/g)	Antioxidant activity DPPH[b] (µmol TE/g)	Antioxidant activity ORAC[b] (µmol TE/g)
1	H_2O	292.1 ± 13.1	1731 ± 28	9722 ± 273
2	70% EtOH	246.3 ± 10.6	1650 ± 149	9890 ± 480
3	MeOH	238.6 ± 13.3	1386 ± 171	7769 ± 360
4	DCM	273.5 ± 6.8	1437 ± 105	9242 ± 739
5	Mixed solvents	281.0 ± 7.1	1964 ± 124	11278 ± 21
Rosemary extract (E392)		-	591 ± 20	2433 ± 88

[a] Total polyphenol contents were determined by the Folin-Ciocalteu method. The results are expressed as milligram of gallic acid equivalent par gram. Each value is the mean ± standard deviation (n=3)
[b] The DPPH and ORAC results are expressed as micromoles of Trolox equivalent per gram. For both, each value is the mean ± standard deviation (n=3).

The contents of total polyphenol are high for all extracts, in a range of 238.6 and 292.1 mg GAE/g, which is in accordance with values of propolis of poplar-type (from Europe and Asia) about 200-300 mg GAE/g.[13] The best values are obtained for the aqueous, mixed solvents and DCM extracts (respectively 292.1 ± 13.1, 281.0 ± 7.1 and 273.5 ± 6.8 mg GAE/g).

The antioxidant activity using DPPH assay of the five extracts of French propolis are 2 to 4 times higher than E392 taken as the reference, which is an ethanolic rosemary extract used as a natural antioxidant food additive in Europe (Official Journal of European Union – Directive 2010/67/UE - L 277/17). The higher activities are observed for mixed solvents, aqueous and 70% EtOH extracts (respectively 1964 ± 124, 1731 ± 28 and 1650 ± 149 µmol TE/g).

The antioxidant activity using ORAC assay of the five extracts are this time, 3 to 5 times higher than E392. The higher activities are also observed for mixed solvents, aqueous and 70% EtOH extracts (respectively 11278 ± 21, 9722 ± 273 and 9890 ± 480 µmol TE/g).

4. Conclusion

Among the five extracts of the French propolis, there are two types: the aqueous and the organic solvent types. Despite its low yield, the aqueous extract, composed mainly of caffeic and p-coumaric acids, shows high polyphenol content and antioxidant activity. Among the organic solvent type, which exhibit cinnamic acid derivatives and their esters and flavonoids, the mixed solvents extract shows the best antioxidant activity by both DPPH and ORAC assays. But it is composed of DCM, which is a toxic solvent. So two extracts could be very interesting in terms of antioxidant property in

pharmaceutical, cosmetic and food additive products: the 70% EtOH extract, obtained with a good yield (65%), and with good antioxidant activity; and the aqueous one which offers the advantage of a strong antioxidant activity combined with a "green extraction".

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