JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Article

pubs.acs.org/JAFC

Chemical Composition and Antioxidant Activity of Algerian Propolis

Anna Lisa Piccinelli,[†] Teresa Mencherini,[†] Rita Celano,[†] Zina Mouhoubi,[‡] Azeddine Tamendjari,[‡] Rita Patrizia Aquino,[†] and Luca Rastrelli*[,†]

[†]Dipartimento di Farmacia, University of Salerno, Via Ponte Don Melillo, 84084 Fisciano, Italy

[‡]Food Science Department, University of Béjaia, 24 Chemin des Cretes, Béjaia, Algeria

ABSTRACT: Chemical composition of propolis samples from north Algeria was characterized by chromatographic and spectroscopic analyses. High-performance liquid chromatorgaphy with diode-array detection (HPLC-DAD) fingerprint of the methanol extracts allowed the definition of two main types of Algerian propolis (AP) directly related to their secondary metabolite composition. Investigation of two representative types of AP by preparative chromatographic procedure and mass spectrometric (MS) and NMR techniques led to the identification of their main constituents: caffeate esters and flavonoids from an AP type rich in phenolic compounds (PAP) and labdane and clerodane diterpenes, together with a polymethoxyflavonol, from an AP type containing mainly diterpenes (DAP). Subsequently, two specific HPLC−MS/MS methods for detection of PAP and DAP markers were developed to study the chemical composition of propolis samples of different north Algerian regions. Antioxidant activity of AP samples was evaluated by 2,2-diphenyl-1-picrylhydrazyl radical (DPPH) assay, and a significant free-radical scavenging effect was observed for propolis of the PAP series rich in polyphenols.

KEYWORDS: *Algerian propolis, phenolic compounds, diterpenes, NMR spectroscopy, HPLC-PDA, HPLC−ESI-MS*

INTRODUCTION

Propolis is a resinous composite material collected by honeybees from the buds, sap flows, and barks of certain plants and trees, and this material is thought to serve as a defense substance for bee's hives.[1,2] Propolis is actually marketed by the pharmaceutical industry and health food stores for its claimed beneficial and preventive effects on human health, especially for antioxidant, antibacterial, antiviral, antifungal, anti-inflammatory, and anticancer activities.[1,2] Propolis has also been tested as a food preserver due to its bactericidal and bacteriostatic properties.[3] Furthermore, most of its components are natural constituents of food and recognized as safe substances.[3]

Normally, propolis shows an extremely variable chemical composition depending on the season and the species of bee. Furthermore, it is supposed that the vegetation at the area of collection is responsible for its chemical diversity.[4,5] In fact, remarkable differences have been observed between propolis of tropical and temperate regions.[4−6] The last possesses a similar chemical composition, the main constituents being polyphenolic compounds (flavonoids, cinnamic acids and their esters), and the main parent plant exudates those from *Populus* spp.[4,6] By contrast, because of the difference in vegetation, propolis from tropical regions shows a very different composition with prevalence of terpenoids,[7] prenylated derivatives of *p*-coumaric acids,[8] lignans,[9] isoflavonoids,[10,11] and polyisoprenylated benzophenones.[12] This variable composition of propolis may determine a broad spectrum of different biological activities and highlights the need for chemical standardization, necessary to connect a particular propolis chemical class to a specific type of biological activity.[13]

Propolis from Algeria has recently begun to be studied; therefore, information concerning to its chemical composition, phytochemical origins, and phytotherapeutic properties is still very limited. Recently, Lahouel et al.[14,15] have shown that the extract of propolis collected in northeastern Algeria (Jijel) reduces in vivo toxic effects of doxorubicin induced by oxidative stresses. The authors hypothesized that the protective effect could be due to the polyphenolic fraction of propolis. Moreover, Algerian propolis is reported to modulate matrix metalloproteinase 3 (MMP-3) expression, activation, and activity, making it an attractive candidate as a control agent of the proteolytic cascade involved in several pathological disorders (rheumatoid arthritis, periodontitis, and atherosclerosis).[16] Chicoric acid has been identified as the major bioactive component of Algerian propolis for MMP-3 inhibition.[16] Nevertheless, few studies have been accomplished on the chemical composition of Algerian propolis (AP). Flavonoids (chrysin,[17] apigenin,[17] pectolinarigenin,[17] pilosin,[17] ladanein,[17] galangin,[15] naringenin,[15] tectochrysin,[15] and methoxychrysin[15]), pinostrombin chalcone,[15] and caffeic acid derivatives[16] have been identified as constituents of a sample collected in Jijel, located in northeast Algeria, while in other AP samples, significant amounts of a hydroxyditerpenic acid have been found.[18] Therefore, a detailed insight into Algerian propolis chemical composition can aid in a better understanding of its potential.

This paper describes a comparative analysis of 14 Algerian propolis (AP) samples collected in different regions of north Algeria with the aim to investigate the chemical composition and antioxidant activity. First, the chromatographic fingerprints of AP samples were evaluated by high-performance liquid chromatography with diode-array detection (HPLC-DAD) and

Received: November 8, 2012
Revised: May 3, 2013
Accepted: May 7, 2013
Published: May 7, 2013

dx.doi.org/10.1021/jf400779w | *J. Agric. Food Chem.* 2013, 61, 5080−5088

two different propolis types, directly related to their constituents, were identified: polyphenol Algerian propolis (PAP) type, rich in polyphenolic compounds, and diterpene Algerian propolis (DAP) type, plentiful source of diterpenes. The main secondary metabolites of these two types of AP were then identified by preparative chromatographic procedures followed by NMR and mass spectrometric (MS) analyses. Afterward, two HPLC−MS/MS methods specific for PAP and DAP markers were developed to study and compare the chemical composition of AP samples collected in different regions of north Algeria. Finally, the antioxidant activity of all AP samples was evaluated by 2,2-diphenyl-1-picrylhydrazyl radical (DPPH) assay.

MATERIALS AND METHODS

Chemicals. Chloroform ($CHCl_3$), *n*-hexane, and methanol (MeOH) employed for extraction and isolation procedures were of analytical grade and were obtained from Carlo Erba (Milan, Italy). Silica gel 60 (0.040−0.063 mm) for open column chromatography separation was purchased from Carlo Erba. MeOH of HPLC grade (Carlo Erba) and ultrapure water prepared by a Milli-Q system (Millipore, Billerica, MA) were used for the HPLC−IR separations. HPLC-photodiode array (PDA)−MS analyses were performed with acetonitrile and water of HPLC super gradient quality (Romil Ltd., Cambridge, U.K.). DPPH and α-tocopherol were obtained from Sigma Aldrich (Milan, Italy).

Propolis Samples. Fourteen samples of Algerian propolis (AP1−14) were supplied by companies that use permanent beehives and standardized procedures for collecting apiaries products. These samples were collected in different regions of north Algeria from October 2008 to December 2009. The extracts were prepared according to our procedure.[11] All propolis samples were kept at 0−5 °C and protected from light. Raw materials of AP samples were frozen at −20 °C overnight and then rapidly ground in a mortar to obtain homogeneous powders. Methanol extracts of AP samples were obtained by maceration of ground sample (10 g) with methanol (100 mL, 3 times) in a closed dark bottle for 1 day at room temperature (25−30 °C). The combined extracts were filtered on paper filters, and solvent was evaporated at 40 °C under reduced pressure to obtain dry extracts.

General Experimental Procedures. HPLC-PDA analyses were performed with a HPLC system (Thermo Fisher Scientific, San Jose, CA) including a Surveyor LC pump, Surveyor autosampler, and Surveyor PDA detector equipped with Xcalibur software. Preparative HPLC separations were performed on a Waters 590 series pumping system equipped with a Waters R401 refractive index detector, using Kromasil RP-18 (250 mm × 10 mm i.d., 10 μm) or Luna C8 (250 mm × 10 mm i.d., 10 μm) columns, both from Phenomenex (Torrance, CA). Thin-layer chromatography (TLC) was performed with Macherey-Nagel precoated silica gel 60 F_{254} plates (Delchimica, Naples, Italy), and the spray reagent cerium sulfate (saturated solution in dilute H_2SO_4) and UV (254 and 366 nm) were used for the spot visualization. A Bruker DRX-600 NMR spectrometer, operating at 599.19 MHz for ^1H and at 150.86 MHz for ^{13}C, was used for NMR experiments; chemical shifts are expressed in δ (parts per million) referring to the solvent peaks δ_H 3.34 and δ_C 49.0 for CD_3OD and δ_H 7.26 and δ_C 77.0 for $CDCl_3$; coupling constants, *J*, are in hertz. Optical rotations were measured on a Jasco DIP-1000 digital polarimeter equipped with a sodium lamp (589 nm) and a 10 cm microcell in MeOH solution. Electrospray ionization mass spectrometry (ESI-MS) was performed on a LTQ XL (Thermo Fisher Scientific) linear ion trap mass spectrometer equipped with Xcalibur software. Full mass and collision-induced dissociation (CID) MS/MS spectra were acquired in both positive (PI) and negative (NI) ionization modes. Instrumental parameters were tuned for each investigated compound and specific collision energies were chosen at each fragmentation step for all the investigated compounds, and the values ranged from 15% to 33% of the instrument maximum. All compounds were dissolved in MeOH/H_2O, 1:1 v/v, at a concentration of 5 $\mu g \cdot mL^{-1}$ and infused in the ESI source with a flow rate of 5 $\mu L \cdot min^{-1}$. HPLC−MS/MS analyses were carried out with a LTQ XL (Thermo Fisher Scientific) linear ion trap mass spectrometer equipped with an Accelera 600 Pump and Accelera AutoSampler.

HPLC-PDA Analysis. For HPLC analysis, MeOH/H_2O 8:2 (v/v) solution of each extract (2 $mg \cdot mL^{-1}$) was prepared and analyzed by our method previously reported.[19] Detection by diode array was performed simultaneously at two different wavelengths, 280 and 320 nm, and the UV spectra were recorded in the range 200−600 nm.

Isolation of Compounds 1−10. Dry methanol extract of AP9 sample (0.8 g) was fractionated by open column chromatography on silica gel (60 g, 1 m × 3 cm i.d.) with *n*-hexane/$CHCl_3$ (from 100:0 to 0:100 v/v) and $CHCl_3$/MeOH (from 100:0 to 80:20 v/v) elution gradients. After TLC analysis (silica gel, $CHCl_3$/MeOH 95:5 and 8:2 v/v), fractions with similar R_f values were combined, giving six major fractions (I−VI). Each fraction was further purified by sempipreparative HPLC−IR. Fraction II (59.8 mg) was separated on a C18 column with MeOH/H_2O, 7:3 v/v, as mobile phase (flow rate of 2.5 $mL \cdot min^{-1}$) to yield pure compounds chrysin (6.9 mg) (**4**), galangin (5.6 mg) (**7**), pinobanksin (6.0 mg) (**8**), and pinobanksin 3-acetate (19.5 mg) (**9**). Fraction III (223.6 mg) was separated on C8 column with MeOH/H_2O, 7:3 v/v, as mobile phase (flow rate of 2.5 $mL \cdot min^{-1}$) to afford 3-methyl-3-butenyl (*E*)-caffeate (25.8 mg) (**1**) and phenethyl (*E*)-caffeate (29.0 mg) (**3**). Fractions IV (280.2 mg) and VI (85.3 mg) were purified on C8 and C18 columns, respectively, with the elution solvent MeOH/H_2O, 6:4 v/v (flow rate of 3.0 $mL \cdot min^{-1}$). Fraction IV yielded 2-methyl-2-butenyl (*E*)-caffeate (30.9 mg) (**2**), and fraction VI gave apigenin (1.3 mg) (**5**), kaempferol (2.2 mg) (**6**), and pinobanksin 3-(*E*)-caffeate (7.7 mg) (**10**).

Isolation of Compounds 11−20. Dry methanol extract of AP4 sample (0.8 g) was purified by silica gel column according to the procedure reported above to afford nine fractions (I−IX). The following HPLC−IR purification of these fractions allowed us to obtain isoagathotal (2.9 mg) (**15**) from fraction II (108.6 mg) (C8 column, MeOH/H_2O 8:2 v/v, 3.0 $mL \cdot min^{-1}$); torulosol (2.2 mg) (**16**), torulosal (2.1 mg) (**14**), and myricetin 3,7,4′,5′-tetramethyl ether (2.3 mg) (**20**) from fraction III (112.0 mg) (C18 column, MeOH/H_2O 85:15 v/v, 2.5 $mL \cdot min^{-1}$); cupressic acid (2.6 mg) (**11**) and cistadiol (2.5 mg) (**18**) from fraction IV (60.5 mg) (C18 column, MeOH/H_2O 74:26 v/v, 2.5 $mL \cdot min^{-1}$); agathadiol (3.0 mg) (**17**) from fraction V (90.7 mg) (C8 column, MeOH/H_2O 8:2 v/v, 3.0 $mL \cdot min^{-1}$); isocupressic acid (25.0 mg) (**12**) and imbricatoloic acid (12.0 mg) (**13**) from fraction VII (209.8 mg) (C8 column, MeOH/H_2O 8:2 v/v, 3.0 $mL \cdot min^{-1}$); and 18-hydroxy-*cis*-clerodan-3-ene-15-oic acid (3.1 mg) (**19**) from fraction VIII (86.5 mg) (C18 column, MeOH/H_2O 65:35 v/v, 2.5 $mL \cdot min^{-1}$).

Spectroscopic Data. *3-Methyl-3-butenyl (E)-Caffeate (**1**).* NMR data were consistent with those previously reported;[20] ESI/MS (NI) *m/z* 247 $[M - H]^-$; MS/MS (collision energy 25%) *m/z* 179, 135.

*2-Methyl-2-butenyl (E)-Caffeate (**2**).* NMR data were consistent with those previously reported;[21] ESI/MS (NI) *m/z* 247 $[M - H]^-$; MS/MS (collision energy 25%) *m/z* 203, 179, 135.

*Phenethyl (E)-Caffeate (**3**).* NMR data were consistent with those previously reported;[22] ESI/MS (NI) *m/z* 283 $[M - H]^-$; MS/MS (collision energy 25%) *m/z* 179, 135.

*Chrysin (**4**).* NMR data were consistent with those previously reported;[23] ESI/MS (NI) *m/z* 253 $[M - H]^-$; MS/MS (collision energy 40%) *m/z* 209, 181, 151.

*Apigenin (**5**).* NMR data were consistent with those previously reported;[23] ESI/MS (NI) *m/z* 269 $[M - H]^-$; MS/MS (collision energy 45%) *m/z* 227, 225, 201, 183, 181, 159, 151, 149.

*Kaempferol (**6**).* NMR data were consistent with those previously reported;[23] ESI/MS (NI) *m/z* 285 $[M - H]^-$; MS/MS (collision energy 45%) *m/z* 257, 243, 241, 229, 213, 151.

*Galangin (**7**).* NMR data were consistent with those previously reported;[23] ESI/MS (NI) *m/z* 269 $[M - H]^-$; MS/MS (collision energy 45%) *m/z* 227, 213, 197.

*Pinobanksin (**8**).* NMR and optical rotation data were consistent with those previously reported;[24] ESI/MS (NI) *m/z* 271 $[M - H]^-$;

dx.doi.org/10.1021/jf400779w | J. Agric. Food Chem. 2013, 61, 5080−5088

Table 1. Selected Reaction Monitoring Transitions of Compounds 1−20 in Algerian Propolis Samples

		SRM transition	
compd	t_R (min)	precursor ion (m/z)	product ion (m/z)
		PAP	
1		247, $[M - H]^-$	179, [caffeic acid − H]$^-$
2	5.7	247, $[M - H]^-$	179, [caffeic acid − H]$^-$
3	6.3	283, $[M - H]^-$	179, [caffeic acid − H]$^-$
4	6.0	253, $[M - H]^-$	181, $[M - CO_2 - CO - H]^-$
5	3.8	269, $[M - H]^-$	151, $^{1,3}A^-$;[a]
			149, $[^{1,4}B + 2H]^-$ [a]
6	4.0	285, $[M - H]^-$	151, $^{1,3}A^-$ [a]
7	6.4	269, $[M - H]^-$	213, $[M - 2CO - H]^-$;
			197, $[M - CO_2 - CO - H]^-$
8	3.6	271, $[M - H]^-$	253, $[M - H_2O - H]^-$
9	6.5	313, $[M - H]^-$	271, [pinobanksin − H]$^-$
10	6.7	433, $[M - H]^-$	271, [pinobanksin − H]$^-$
		DAP	
11	6.5	303, $[M - H_2O + H]^+$	257, $[M - H_2O - HCOOH + H]^+$
12	6.0	303, $[M - H_2O + H]^+$	257, $[M - H_2O - HCOOH + H]^+$
13	7.0	323, $[M + H]^+$	277, $[M - HCOOH + H]^+$
14	9.0	287, $[M - H_2O + H]^+$	259, $[M - H_2O - CO + H]^+$
15	8.5	287, $[M - H_2O + H]^+$	259, $[M - H_2O - CO + H]^+$
16	6.7	289, $[M - H_2O + H]^+$	271, $[M - 2H_2O + H]^+$
17	6.2	289, $[M - H_2O + H]^+$	271, $[M - 2H_2O + H]^+$
18	7.0	291, $[M - H_2O + H]^+$	163
19	6.1	305, $[M - H_2O + H]^+$	163
20	5.6	375, $[M + H]^+$	360, $[M - {}^{\circ}CH_3 + H]^+$

[a] $^{1,3}A^-$ and $^{1,4}B$ label refers to the fragment containing intact A- or B-ring in which the superscripts 1 and 3 or 4 indicate the C-ring bonds that have been broken.

MS/MS (collision energy 35%) m/z 253, 243, 241, 225, 215, 197, 185, 165, 157, 151.

*Pinobanksin 3-Acetate (**9**).* NMR[25] and optical rotation[24] data were consistent with those previously reported; ESI/MS (NI) m/z 313 $[M - H]^-$; MS/MS (collision energy 22%) m/z 271, 253.

*Pinobanksin 3-(E)-Caffeate (**10**).* NMR and optical rotation data were consistent with those previously reported;[26] ESI/MS (NI) m/z 433$[M - H]^-$; MS/MS (collision energy 20%) m/z 415, 271.

*Cupressic Acid (**11**).* NMR[27] and optical rotation[28] data were consistent with those previously reported; ESI/MS (PI) m/z 303 $[M - H_2O + H]^+$; MS/MS (collision energy 35%) m/z 285, 257, 247, 193.

*Isocupressic Acid (**12**).* NMR and optical rotation data were consistent with those previously reported;[28] ESI/MS (PI) m/z 303 $[M - H_2O + H]^+$; MS/MS (collision energy 35%) m/z 257, 247, 193.

*Imbricatoloic Acid (**13**).* NMR and optical rotation data were consistent with those previously reported;[28] ESI/MS (PI) m/z 323 $[M + H]^+$; MS/MS (collision energy 35%) m/z 305, 287, 277, 259, 181.

*Torulosal (**14**).* NMR[29] and optical rotation[28] data were consistent with those previously reported; ESI/MS (PI) m/z 287 $[M - H_2O + H]^+$; MS/MS (collision energy 35%) m/z 269, 259, 177, 163.

*Isoagathotal (**15**).* NMR and optical rotation data were consistent with those previously reported;[30] ESI/MS (PI) m/z 287 $[M - H_2O + H]^+$; MS/MS (collision energy 35%) m/z 269, 259, 163, 149.

*Torulosol (**16**).* NMR data were consistent with those previously reported;[29] ESI/MS (PI) m/z 289 $[M - H_2O + H]^+$; MS/MS (collision energy 35%) m/z 271, 243, 233, 231, 215, 201, 193, 179, 177.

*Agathadiol (**17**).* NMR and optical rotation data were consistent with those previously reported;[31] ESI/MS (PI) m/z 289 $[M - H_2O + H]^+$; MS/MS (collision energy 35%) m/z 271, 243, 231, 215, 201, 193, 179, 177, 165, 163, 161, 149, 147, 123, 109.

*Cistadiol (**18**).* NMR and optical rotation data were consistent with those previously reported;[32] ESI/MS (PI) m/z 291 $[M - H_2O + H]^+$; MS/MS (collision energy 35%) m/z 273, 235, 221, 209, 205, 203, 195, 191, 181, 177, 163, 149, 135, 127, 121, 109.

*18-Hydroxy-cis-clerodan-3-ene-15-oic Acid (**19**).* NMR and optical rotation data were consistent with those previously reported;[32] ESI/MS (PI) m/z 305 $[M - H_2O + H]^+$; MS/MS (collision energy 35%) 287, 269, 263, 249, 235, 223, 209, 195, 177, 163, 149, 135, 121, 107.

*Myricetin 3,7,4′,5′-Tetramethyl Ether (**20**).* NMR data were consistent with those previously reported;[33] ESI/MS (PI) m/z 375 $[M + H]^+$; MS/MS (collision energy 35%) m/z 360, 345, 315.

HPLC−ESI-MS Analysis of PAP Markers. Analyses were performed on a Fusion RP column (75 mm × 2.0 mm i.d., particle size 4 μm, Phenomenex), protected by C18 guard cartridge (4 × 2.0 mm i.d.), and a linear gradient (from 30% to 70% B in 10 min) of acetonitrile (B) and water (A) at flow rate of 250 $\mu L \cdot min^{-1}$, followed by column washing and re-equilibrating. The ionization conditions were as follows: ionization mode, negative; capillary temperature, 320 °C; capillary voltage, 29 V; spray voltage, 4.50 kV; sheath gas flow rate, 25 (arbitrary units); auxiliary gas flow rate 5 (arbitrary units). Selected reaction monitoring (SRM) transitions (collision energy ranged from 20% to 45%) monitored are reported in Table 1. The maximum injection time was 10 ms and the number of microscans was one. N_2 was used as the sheath and auxiliary gas.

HPLC−ESI-MS Analysis of DAP Markers. HPLC separation was accomplished on a Luna C-8 column (150 mm × 2.0 mm i.d., particle size 5 μm, Phenomenex), protected by a C8 guard cartridge (4 × 2.0 mm i.d.), and a linear gradient (from 50% to 95% B in 10 min) of water (solvent A) and acetonitrile (solvent B), both containing 0.1% (v/v) formic acid, followed by column washing and re-equilibrating. Elution was performed at a flow rate of 250 $\mu L \cdot min^{-1}$. The ionization conditions were as follows: ionization mode, positive; capillary temperature, 320 °C; capillary voltage, 6 V; spray voltage, 5 kV; sheath gas flow rate, 15 (arbitrary units); auxiliary gas flow rate, 5 (arbitrary units). SRM transitions (collision energy 25%) monitored are reported in Table 1. The maximum injection time was 10 ms and

the number of microscans was tone. N_2 was used as the sheath and auxiliary gas.

Semiquantitative Analysis. Analyses were performed with two HPLC−MS/MS methods reported above. Phenethyl (*E*)-caffeate (**3**), galangin (**7**), pinobanksin (**8**), and pinobanksin 3-acetate (**9**) were used as reference standards to quantify PAP markers, while cupressic acid (**11**) and myricetin 3,7,4′,5′-tetramethyl ether (**20**) were selected for the DAP markers. Purity of compounds used as reference standards was checked by HPLC-DAD−MS analysis and was >97%. Standard calibration curves (six concentration levels and triplicate injections for each level) were obtained in the concentration range 0.05−50.0 $\mu g \cdot mL^{-1}$ for **3**, **8**, **9**, and **20** and 0.5−200 $\mu g \cdot mL^{-1}$ for **7** and **11**. Peak areas of the external standard (at each concentration) were plotted against the corresponding standard concentrations (micrograms per milliliter) by use of weighed linear regression to generate standard curves. For the linear regression of external standards, R^2 values were >0.990. Solutions of AP samples (1 $mg \cdot mL^{-1}$) were prepared in methanol and 10 μL of each solution was injected for the analysis. The amount of the compounds was expressed in milligrams per gram of dry methanol extract, as the mean of triplicate determinations.

Free-Radical Scavenging Activity. The antiradical activity of Algerian propolis extracts was determined by the stable 2,2-diphenyl-1-picrylhydrazyl radical (DPPH) test according to our procedure previously reported.[34] Briefly 1.5 mL of DPPH solution (25 $mg \cdot L^{-1}$ in methanol, prepared daily) was added to 0.75 mL of various concentrations of each propolis sample in MeOH solution (ranged from 12 to 100 $\mu g \cdot mL^{-1}$). The mixtures were kept in the dark for 10 min at room temperature and the decrease in absorbance was measured at 517 nm against a blank consisting of an equal volume of methanol. α-Tocopherol was used as positive control. The DPPH concentration in the reaction medium was calculated from a calibration curve analyzed by linear regression, and SC_{50} (mean effective scavenging concentration) was determined by the Litchfield test as the concentration (in milligrams per milliliter) of sample necessary to decrease the initial DPPH concentration by 50%. All tests were performed in triplicate. A lower SC_{50} value indicates stronger antioxidant activity.

RESULTS AND DISCUSSION

Preliminary HPLC-PDA Analysis. Methanol extracts of 14 samples of propolis collected in different regions of north Algeria (Table 2) were analyzed by HPLC-PDA without cleanup procedures for fast screening. For this purpose an HPLC-PDA analytical procedure,[19] able to give a fingerprint of propolis and to identify a wide range of compounds with varying polarity, was applied to supply a general chemical classification of AP samples.

HPLC-PDA profiles of the 14 AP extracts and UV spectra of major peaks allowed the definition of two main types of AP directly related to their chemical composition (Figure 1). HPLC profiles of the first AP type (AP1, AP6, AP9, and AP13) exhibited a very similar chromatogram. It was extremely complex with many peaks between 4 and 65 min (Figure 1A). The UV spectra (λ_{max} 325 nm) and retention time (t_R 34.2, 35.5, and 36.0 min) of the main peaks indicated the presence of caffeic acid derivatives.[35] In addition, HPLC chromatograms of this type of propolis displayed additional peaks with UV absorption bands between 258 and 365 nm, correlated to flavone (λ_{max} 265 and 310−335 nm), flavonol (λ_{max} 355−365), and flavononol (λ_{max} 290−300 nm) derivatives.[35]

On other hand, the HPLC profile of the second AP type (AP2, AP4, and AP14) appeared more simplified, showing only a few peaks (Figure 1B). The main peaks of chromatograms of DAD samples exhibited UV spectra with low-intensity bands in the region 200−330 nm, suggesting few or no conjugated bonds and the absence of chromophores in the structure. Therefore, it can be hypothesized that they are aliphatic compounds and may be of the terpenoid class. Only peaks at 43.7 and 45.8 min showed UV spectra of higher intensity, with λ_{max} 250−265 and 350−365 nm, that could be associated to methoxylated flavonoids.[35]

HPLC profiles of the other AP samples (AP3, AP5, AP7, AP8, AP10, and AP12) showed both fingerprints, indicating that they were a mixture of two AP types.

Table 2. Free-Radical Scavenging Activities by DPPH Test of Algerian Propolis Samples Collected in Different Areas of Northern Algeria

propolis sample	area	SC_{50} (μg/mL)
AP1	Ait Ousalah	43.3 ± 1.2
AP2	Amtik Ntafat	600.0 ± 15.6
AP3	Bejaia	71.2 ± 0.7
AP4	Ibouhatmen	441.2 ± 12.3
AP5	Boulimat, Iazouen	68.5 ± 1.7
AP6	Amizour	82.5 ± 1.5
AP7	Boumerdes, Isser	42.1 ± 1.9
AP8	Boumerdes, Isser	48.7 ± 0.6
AP9	Akbou	60.4 ± 1.6
AP10	Ouadghir	79.8 ± 1.9
AP11	Tizi Ouzou, Iakouren	75.0 ± 0.9
AP12	Bejaia	208.3 ± 11.7
AP13	Bejaia	32.3 ± 1.9
AP14	Bejaia	483.9 ± 10.2
1		19.5 ± 2.8
2		12.1 ± 2.3
3		9.9 ± 1.2
6		4.2 ± 0.4
7		15.6 ± 2.7
11		120.7 ± 5.7
20		133.3 ± 3.2
α-tocopherol		10.1 ± 1.3

Isolation Procedure. From the results of HPLC-PDA analysis, AP9 and AP4 samples were selected as representative samples of the two types of Algeria propolis, and their methanol extracts were subjected to a preparative procedure with the aim to isolate their major components. Isolation of the main constituents was performed by silica gel column chromatography and reversed-phase HPLC, and the compounds were characterized by NMR and MS techniques for comparison with spectroscopic data reported in literature.

Three caffeic acid derivatives [3-methyl-3-butenyl (*E*)-caffeate (**1**), 2-methyl-2-butenyl (*E*)-caffeate (**2**), and phenethyl (*E*)-caffeate (**3**)], two flavones [chrysin (**4**) and apigenin (**5**)], two flavonols [kaempferol (**6**) and galangin (**7**)], and three flavanonols [pinobanksin (**8**), pinobanksin 3-acetate (**9**), and pinobanksin 3-(*E*)-caffeate (**10**)] were identified as main constituents of the AP9 sample (Figure 2). Compounds **1**−**9** have been previously isolated from propolis of different origins, mainly produced in temperate regions.[1,2,4,35] Moreover, **1**−**9** have also been reported in *Populus* spp, suggesting that these plants may be one of the most diffused at site of collection of AP samples.[1,2,4,35] Pinobanksin 3-(*E*)-caffeate (**10**) has never been reported in propolis; it has only been reported in *Laguncularia racemosa*.[26] Structurally similar compounds have been identified by MS procedure in propolis from Portugal, but isolation and complete characterization are lacking.[36] Except chrysin, apigenin, and galangin, the characteristic flavonoids of

dx.doi.org/10.1021/jf400779w | J. Agric. Food Chem. 2013, 61, 5080−5088

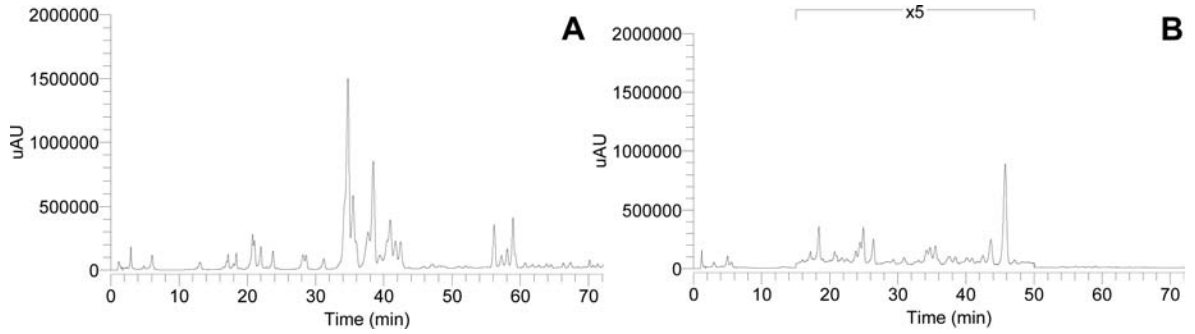

Figure 1. HPLC-PDA fingerprints (280 nm) of (A) AP9 and (B) AP4 samples, representative of two different types of AP.

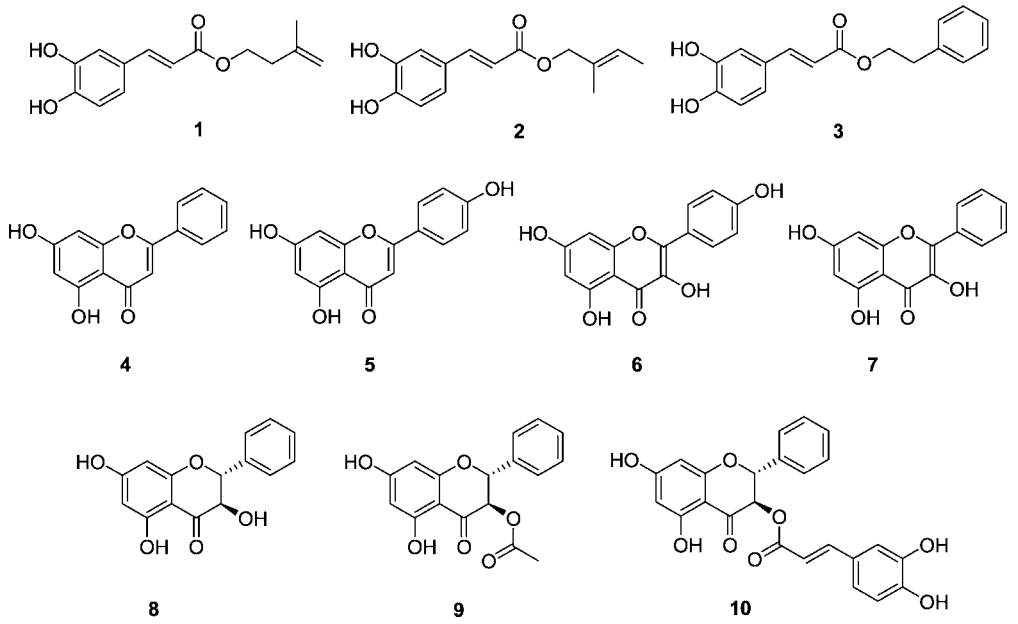

Figure 2. Main constituents of polyphenol Algerian propolis (PAP) type.

propolis from temperate regions, this representative AP sample of north Algeria showed a very different polyphenolic composition than the AP sample collected in Jijel.[15−17]

Regarding the second AP type, nine diterpenes [cupressic acid (**11**), isocupressic acid (**12**), imbricatoloic acid (**13**), torulosal (**14**), isoagathotal (**15**), torulosol (**16**), agathadiol (**17**), cistadiol (**18**), and 18-hydroxy-*cis*-clerodan-3-ene-15-oic acid (**19**)], together with myricetin 3,7,4′,5′-tetramethyl ether (**20**), were identified as main constituents of the representative sample (AP4) (Figure 3). Compounds **11**−**17** are labdane ditepenes with different oxidation at C-19 and isomers at lateral chain, whereas **18** and **19** are clerodane diterpenes. Labdane diterpenes identified in this study have been previously reported in Greek[37] and Brazilian[38,39] propolis with anti-proliferative activity. Also, clerodane diterpenoids have been previously reported in propolis,[40,41] but this is the first time that the compounds **18** and **19** have been isolated from propolis. Finally, polymethoxylated flavonol **20** has been previously reported in Tunisian propolis.[33] Literature data suggest that leaf exudate of *Cistus* spp. seems to be a plant source of compounds isolated from the second AP type.[33,38]

Results of the chemical investigation carried out on propolis representative samples of two AP types identified by HPLC-PDA indicated the presence of two different types of propolis in the north Algerian region. The former, named polyphenol Algerian propolis (PAP), showed as markers a series of polyphenolic compounds typical of propolis produced from *Populus* resins,[4,13] whereas the marker compounds of the latter (diterpene Algerian propolis, DAP) was labdane and clerodane diterpenes, characteristic of *Cistus* spp. exudates.[33,38] Both secondary metabolite classes were characteristic of propolis samples collected in the temperate regions.[37,40]

HPLC−MS/MS Analysis. Once the marker compounds of the two AP types were identified, MS/MS and HPLC−MS analyses were performed to deeply analyze all 14 propolis samples and to obtain a tool for more rapid and efficient classification of Algerian propolis. Two classes of secondary metabolites of AP (polyphenols and diterpenes) have different chromatographic and ionization behaviors related to their polarity and functional groups. Thus, two different HPLC−MS/MS methods were developed to obtain rapid and sensitive detection of two AP marker series in samples collected in different regions of the North Algeria.

Mass spectra of compounds **1**−**20** were acquired in positive (PI) or negative (NI) ionization modes by use of a linear ion trap (LIT) mass spectrometer equipped with an ESI source. For polyphenols **1**−**10** the NI mode was used due to its sensitivity and fragmentation specificity. (−)-ESI-MS spectra of compounds **1**−**10** showed $[M - H]^-$ ion as the base peak, selected for successive MS/MS experiments through collision

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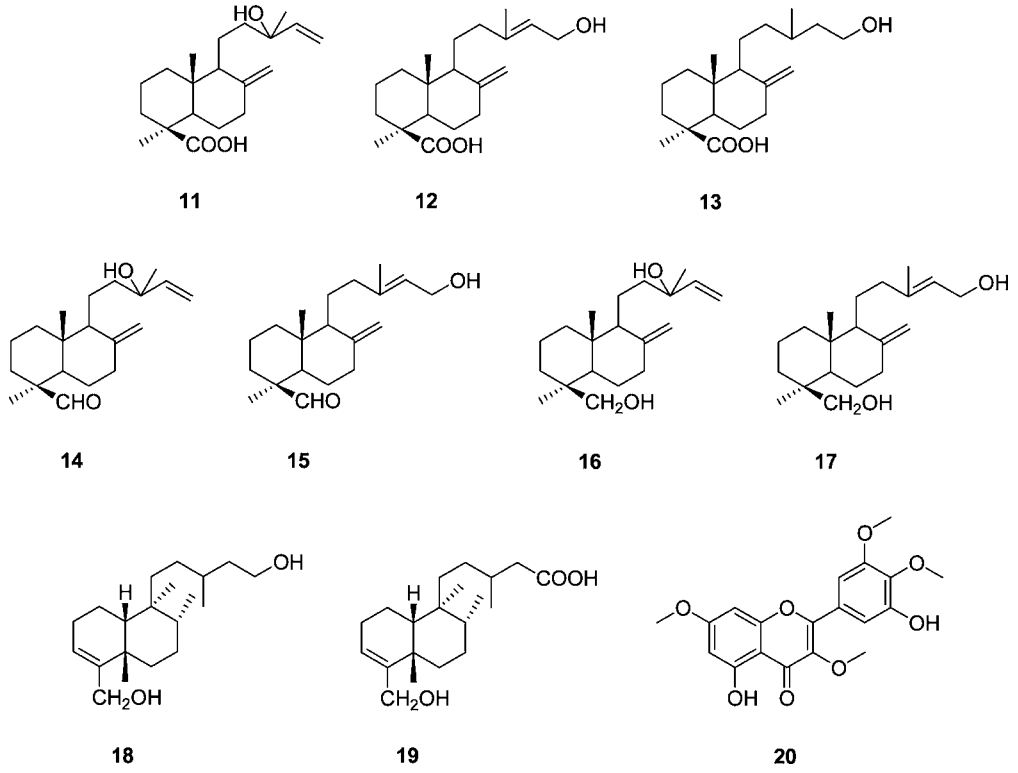

Figure 3. Main constituents of diterpene Algerian propolis (DAP) type.

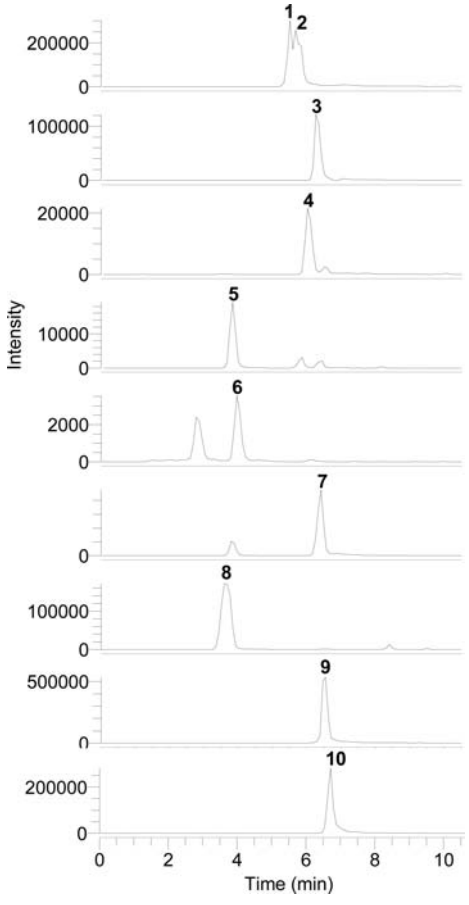

Figure 4. HPLC–ESI-MS/MS chromatogram in SRM mode of PAP type (AP9 sample).

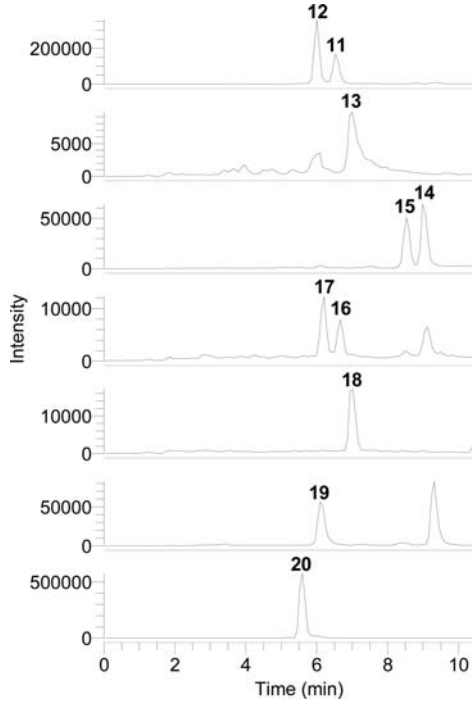

Figure 5. HPLC–ESI-MS/MS chromatogram in SRM mode of DAP type (AP4 sample).

energy ranging from 20% to 40%. Product ion spectra of caffeate esters **1**–**3** ($[M - H]^-$ at m/z 247, 247, and 283, respectively) were characterized by the fragment at m/z 179, corresponding to the deprotonated molecule of caffeic acid. (−)-MS/MS spectra of flavonoids **4**–**8** ($[M - H]^-$ at m/z 253, 269, 285, 269, and 271, respectively) presented the typical fragmentation pattern of flavonoids, whereas for pinobanksin

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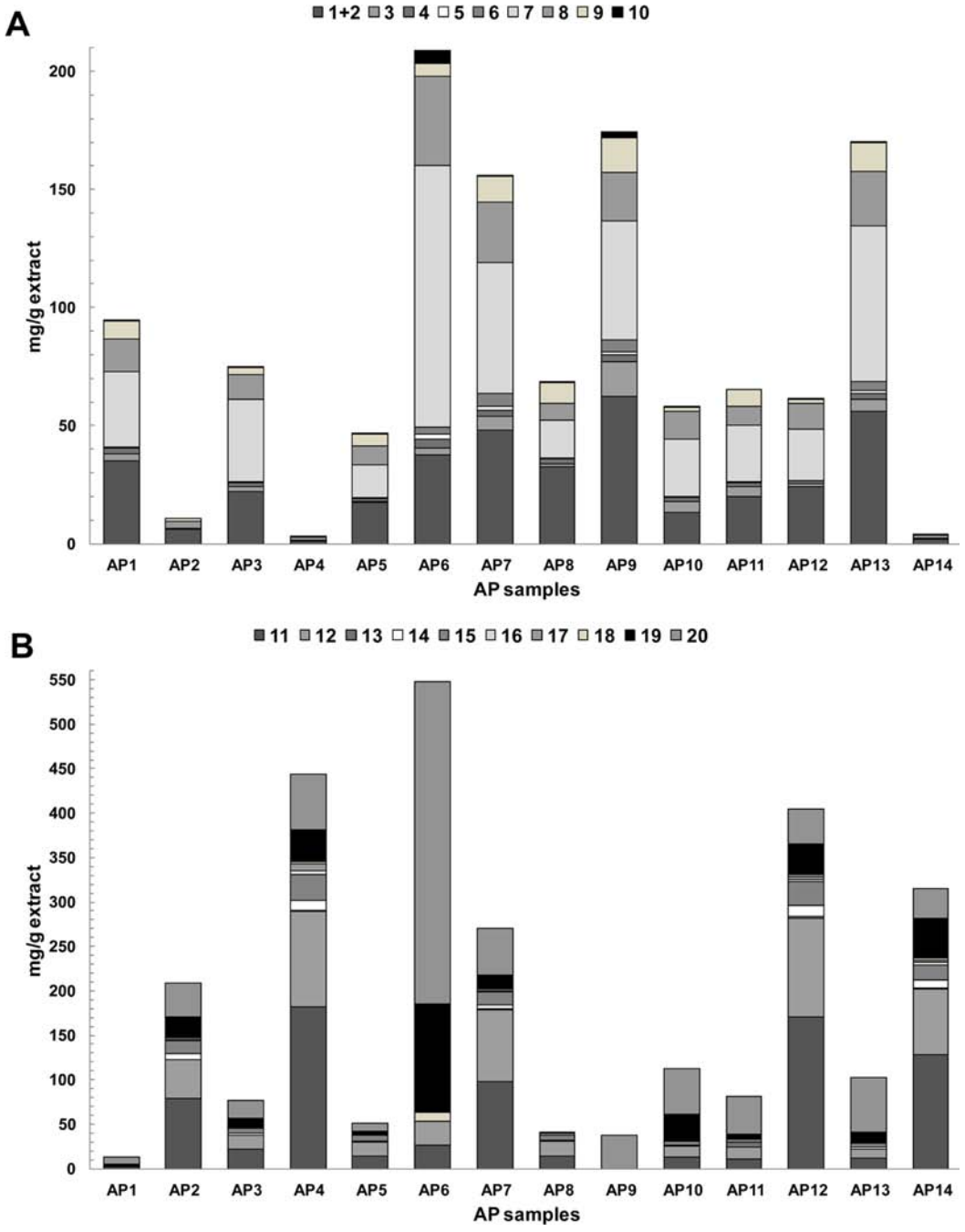

Figure 6. Distribution of compounds **1**–**10** (A) and **19**–**20** (B) in the propolis samples collected in north Algeria.

derivatives **9** and **10** ($[M - H]^-$ at m/z 313 and 433, respectively), the characteristic [pinobanksin − H]$^-$ ion at m/z 271 was observed in their (−)-MS/MS spectra. On the basis of these ESI-MS/MS data, specific precursor/product ions transitions (Table 1) were selected for compounds **1**–**10** and used in selected reaction monitoring (SRM) mode to detect PAP markers by HPLC. The selected chromatographic conditions (polar embedded C18 column, length 75 mm, and linear gradient from 30% to 70% acetonitrile) enabled the rapid separation of phenolic compounds **1**–**10** in less than 10 min. Figure 4 shows the chromatographic profile of a PAP type sample obtained by the developed HPLC–ESI-MS/MS method to analyze PAP markers.

For DAP markers (**11**–**19**), the PI mode was selected. Almost all (+)-ESI-MS spectra of diterpenes (**11**, **12**, and **14**–**19**) showed $[M - H_2O + H]^+$ ion as the base peak (m/z at 303 for **11** and **12**, at 287 for **14** and **15**, at 289 for **16** and **17**, at 291 for **18**, and at 305 for **19**). Only imbricatoloic acid (**13**) showed as the base peak the protonated molecule ($[M + H]^+$ at m/z 323) due to the absence of alcoholic group in α-position with respect to a double bond. Therefore, $[M - H_2O + H]^+$ or $[M + H]^+$ ions were selected as the base peaks in MS/MS experiments. Fragmentation patterns of labdane diterpenes (**11**–**17**) were characterized by product ions ascribable to the loss of C-19 group. In their (+)-MS/MS spectra, compounds with a carboxyl group (**11** and **13**) presented a peak due to the loss of formic acid, $[M - H_2O + H - 46]^+$; compounds with

dx.doi.org/10.1021/jf400779w | J. Agric. Food Chem. 2013, 61, 5080–5088

aldehyde group (**14** and **15**) showed a characteristic ion $[M - H_2O + H - 28]^+$ generated by the loss of CO; and those with alcoholic function (**16** and **17**) had a characteristic fragment ion corresponding to loss of water, $[M - H_2O + H - 18]^+$. Differently, (+)-MS/MS spectra of clerodane diterpenes (**18** and **19**) were characterized by ions at m/z 149 and 163. Finally, spectrum of tetramethoxyflavonol (**20**) displayed the classical loss of methyl radical ($[M + H - CH_3]^{+\bullet}$ at m/z 360) from an aromatic skeleton. For HPLC–MS/MS method, the specific precursor/product ion transitions of compounds **1**–**20**, selected for the detection of DAP markers in SRM mode, were reported in Table 1. Rapid and efficient chromatographic separation of DAP markers was obtained by use of a column employed for very hydrophobic compounds (C8 column, length 150 mm) and a linear gradient with high content of organic phase (from 50% to 95% of acetonitrile in 10 min). A typical chromatogram obtained with the developed HPLC–MS/MS method for the analysis of DAP markers is shown in Figure 5.

To correctly compare the amount of each compound in the different extracts, the suitability for semiquantitative analysis of two HPLC–MS/MS methods was assessed. Phenethyl (*E*)-caffeate, galangin, pinobanksin, and pinobanksin 3-acetate were used as reference standards to quantify PAP markers, while cupressic acid and myricetin 3,7,4′,5′-tetramethyl ether were selected for the DAP markers. Compounds were selected on the basis of their relative abundance in the extracts and chemical structure similarities. Both methods showed good linearity ($R^2 > 0.990$) in a wide concentration range. The calibration curves of phenethyl (*E*)-caffeate, galangin, pinobanksin, and pinobanksin 3-acetate were used to quantify caffeic acid derivatives (**1**–**3**), flavonols (**6** and **7**), flavones (**4** and **5**), and pinobanksin esters (**9** and **10**), respectively. Diterpenes (**11**–**19**) were quantified by use of the curve of cupressic acid.

Subsequently, two developed HPLC–ESI-MS/MS methods, specific for the PAP and DAP markers, were applied to 14 AP samples to characterize the propolis of north Algeria. HPLC–MS/MS analyses revealed that all studied AP samples showed a chemical profile superimposable on PAP and/or DAP types (Figure 6), indicating that the identified compounds are characteristic markers of propolis coming from north Algerian regions. Many AP samples showed both PAP and DAP markers (Figure 6). Therefore, these samples are a mixture of two types of AP, suggesting the contribution of different vegetal sources present at the site of collection. AP sample collected at Akbou (AP9) contained only phenolic compounds (Figure 6A), whereas samples collected at Amtik Ntafat, Ibuhatmen, and Bejaia (AP2, AP4 and AP14) seem instead to be the most representative samples of DAD type (Figure 6B).

Regarding the chemical markers, as shown in Figure 6A, 3-methyl-3-butenyl (*E*)-caffeate (**1**), 2-methyl-2-butenyl (*E*)-caffeate (**2**), galangin (**7**), and pinobanksin were the main constituent of PAP type, whereas cupressic acid (**11**), isocupressic acid (**12**), and myricetin 3,7,4′,5′-tetramethyl ether were the most abundant compounds of DAP type.

The developed HPLC–ESI-MS methods enabled us to classify the north Algerian propolis according to chemical composition and to confirm the results of the HPLC-PDA analysis. Moreover, methods here presented supply a full tool for the quality control of propolis of PAP and DAP types and derived commercial products.

Free-Radical Scavenging Activity of Algerian Propolis. Propolis possesses well-known antioxidant activity, and extracts from propolis have the ability to scavenge RL and reactive oxygen species such as superoxide and hydroxyl anions.[2]

The free-radical scavenging effect of AP samples was evaluated by DPPH test, with α-tocopherol, a strong and well-known antioxidant, as positive control.[36]The results (Table 2) showed that samples AP1, AP3, AP5–11, and AP13 possessed strong antioxidant activity (SC_{50} range 32.3–82.5 $\mu g \cdot mL^{-1}$). This should be correlated with high amounts of caffeic acid esters (compounds **1**–**3**) and flavonols [kaempferol (**6**) and galangin (**7**)]. In fact, as is well-known,[42−44] phenols with two *o*-hydroxyl groups in an aromatic ring, such as compounds **1**–**3**, possess high antioxidant properties (SC_{50} 19.5, 12.2, and 9.9 $\mu g \cdot mL^{-1}$, respectively). In the case of kaempferol (**6**) and galangin (**7**) (SC_{50} 4.2, and 15.6 $\mu g \cdot mL^{-1}$, respectively) the antioxidant properties are due to the hydroxyl group at C3 in association with the double bond C2=C3 conjugated with the B ring. On the contrary, the presence of a single hydroxyl group (apigenin) or two *m*-hydroxyl groups (chrysin) in the aromatic ring, or a hydroxyl group in C3 but not the double bond C2=C3 (pinobanksin), provides little contribution to the antioxidant properties of the molecule.[43]

On the contrary, AP2, AP4, AP12, and AP14 samples showed weak antioxidant activity (SC_{50} range 208.3–600.0 $\mu g \cdot mL^{-1}$), correlated with their low content of polyphenolic compounds and high amount of labdane diterpenes (cupressic acid **11**) and myricetin 3,7,4′,5′-tetramethyl ether (**20**) that did not strongly scavenge free radicals (Table 2).

AUTHOR INFORMATION

Corresponding Author

*Phone ++39 089 969766; fax ++39 089 969602; e-mail rastrelli@unisa.it.

Funding

The Project was funded by the Italian Ministry of the University and Research (MIUR) with PRIN Project 20098Y822F "Isolation and chemical characterization of bioactive natural products and development of nutraceutical formulations".

Notes

The authors declare no competing financial interest.

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