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RESEARCH ARTICLE

Chemodiversity of propolis samples collected in various areas of Benin and Congo: Chromatographic profiling and chemical characterization guided by ^{13}C NMR dereplication

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Abstract

Introduction: Propolis is a resinous natural substance collected by honeybees from buds and exudates of various trees and plants; it is widely accepted that the composition of propolis depends on the phytogeographic characteristics of the site of collection.

Objectives: The aim of this study was to determine the phytochemical composition of ethanolic extracts from eight propolis batches collected in different regions of Benin (north, center, and south) and Congo, Africa.

Material and methods: Characterization of propolis samples was performed by using different hyphenated chromatographic methods combined with carbon-13 nuclear magnetic resonance (^{13}C NMR) dereplication with MixONat software. Their antioxidant or anti-advanced glycation end-product (anti-AGE) activity was then evaluated by using diphenylpicrylhydrazyl and bovine serum albumin assays, respectively.

Results: Chromatographic analyses combined with ^{13}C NMR dereplication showed that two samples from the center of Benin exhibited, in addition to a huge amount of pentacyclic triterpenes, methoxylated stilbenoids or phenanthrenoids, responsible for the antioxidant activity of the extract for the first one. Among them, combretastatins might be cytotoxic. For the second one, the prenylated flavanones known in Macaranga-type propolis were responsible for its significant anti-AGE activity. The sample from Congo was composed of many triterpene derivatives belonging to *Mangifera indica* species.

Conclusion: Therefore, propolis from the center of Benin seems to be of particular interest, due to its antioxidant and anti-AGE properties. Nevertheless, as standardization of propolis is difficult in tropical zones due to its great chemodiversity, a systematic phytochemical analysis is required before promoting the use of propolis in food and health products in Africa.

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KEYWORDS

¹³C NMR dereplication, methoxylated stilbenoids or phenanthrenoids, pentacyclic triterpenes, prenylated flavanones, Propolis

1 | INTRODUCTION

Propolis is a natural resinous substance collected by bees from the buds and exudates of various trees and plants, mixed with beeswax and salivary enzymes.¹ It is used by bees to protect the entrance from intruders, plug holes, smooth internal walls, mummify dead animals (small insects) inside the hive, and balance extreme humidity or drought conditions.^{2,3} Propolis has been widely used in folk medicine since ancient times due to its wide range of therapeutic properties.⁴ Propolis is generally composed of resin (50%), wax (30%), oils (10%), pollen (5%), and additional phenolic compounds such as flavonoids.^{5,6} The chemical composition of propolis is known to be complex and varies according to plant species growing around the hive, from which bees collect exudates.⁷ It is accepted that several factors, such as the floristic composition of the area, the place and time of collection, the season, the type of collector, the availability and altitude, and the food activity developed during the exploitation of the propolis have an impact on the chemical composition of the propolis.^{8,9} There are different types of propolis, depending on the geographical area of production, botanical source, and chemical composition. The most common types of propolis are temperate, birch, tropical, Mediterranean, and Pacific.¹⁰ Propolis from temperate climatic zones, such as Europe, North America, or non-tropical regions of Asia, comes mainly from exudates of buds of *Populus* species (Salicaceae) and is therefore rich in flavonoids and phenolic acids and their esters; however, tropical propolis, originating from regions where neither poplar nor birch grows, is rich in prenylated derivatives of *p*-coumaric acids, benzophenones, or terpenoids.¹¹ Pacific propolis, typically rich in prenylated flavanones, is another important type of propolis found in Taiwan, Japan, and the Solomon Islands, and birch propolis is found specifically in Russia.¹² The study of propolis from tropical Asia has led to the discovery of *Macaranga tanarius* L. and *Mangifera indica* L. as plant sources of Indonesian propolis.¹³ On the other hand, coniferous species of the Cupressaceae family are the main botanical source of propolis in Mediterranean regions.¹⁴ Chemical analyses revealed that propolis contains more than 300 different natural products (NPs), including phenolic acid derivatives, coumarins, flavonoids, sesquiterpenes, diterpenes, triterpenes, steroids, lignans, or prenylated benzophenones.¹⁵ The biological effects of propolis have been described mainly in relation to not only their antioxidant,^{16–18} anti-AGE (i.e., inhibition of advanced glycation end-product [AGE] formation),¹⁹ anti-inflammatory,²⁰ and anti-tumor effects,^{21–23} but also their antibacterial,^{24–26} anti-fungal,¹⁵ anti-viral,²⁷ and anti-parasitic²⁸ activities. Propolis is also known to stimulate the production of antibodies, suggesting its potential use as adjuvants in vaccines.²⁹ The increasing use of this natural substance of diversified composition and therefore varying biological activities in the pharmaceutical, cosmetic, and food industries is generating particular interest in scientific research,

especially when it comes to defining its chemical composition. Most studies have been carried out on samples from Europe^{19,30–33} and Latin America, more specifically Brazil,^{20,23,34–36} while few studies were conducted in Africa.^{37–41} Information on Beninese and Congolese⁴² propolis remains scarce.

Thus, the aim of this study was to characterize major compounds from propolis samples collected in various phytogeographical zones of Benin and Congo (Africa) using ¹³C nuclear magnetic resonance (NMR) dereplication with MixONat software.^{43,44} A preliminary study using a database (DB) containing NPs previously reported from propolis together with their ¹³C predicted chemical shifts (δ_c) did not yield any usable data compared to the satisfactory results usually obtained with plant extracts.^{43–45} This can be explained by the chemical composition of propolis, which is highly dependent on the local flora, making it impossible to construct a chemotaxonomic DB. Thus, to decipher major compounds from Beninese and Congolese propolis, in the present work we performed high-performance liquid chromatography coupled with ultraviolet and evaporative light scattering detection mass spectrometry (HPLC-UV-ELSD-MS) and gas chromatography coupled with mass spectrometry (GC-MS) analyses on crude propolis extracts to identify their main structural features and build corresponding DBs of NPs suitable for MixONat software.⁴⁶ Then, after a coarse fractionation of the propolis samples selected according to their chemical profile, ¹³C NMR-based dereplication allowed to identify major NPs without further purification. Antioxidant and anti-AGE assays were also carried out.

2 | EXPERIMENTAL PROCEDURES

2.1 | Chemicals

1,1-Diphenyl-2-picrylhydrazyl (DPPH), Folin-Ciocalteu reagent, formic acid, and gallic acid, all analytical grade, were purchased from Sigma-Aldrich (St Quentin Fallavier, France). 6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox®) and 5'-caffeoylquinic acid (chlorogenic acid) were obtained from Acros Organics (Geel, Belgium).

2.2 | Propolis samples

Eight Beninese propolis samples were collected by scraping from beehives in three phytogeographic zones (Table 1 and Figure SI-1). A sample of Congolese propolis was collected in the artificial forest of acacia trees in the Bateke plateau in the center of the Republic of Congo (Table 1).

TABLE 1 Information on the location of the propolis samples.

Country/zone	Sample	Town/site	GPS location* (lat/long)
North of Benin	BN1	Peporiyakou	10.35297475/1.35438942
	BN2	Atabenou	11.27507773/2.37161035
Center of Benin	BC1	Koko	8.375579061/1.846530533
	BC2	Dassa-Zoumé	7.774161936/2.185793839
South of Benin	BS1a	Bohicon	7.172598359/2.067876393
	BS1b		
	BS2	Covè	7.219391049/2.340060799
	BS3	Dovogon	7.061627221/2.14747234
Congo	CG	Bateke plateau	3.97861111/15.3816666

*decimal degrees (latitude/longitude) in World Geodetic System 1984.

2.3 | Propolis extraction

Ethanol extracts of propolis (EPPs) were initially prepared using the following extraction protocol.¹⁹ Raw propolis was first homogenously pulverized in the presence of liquid nitrogen. For all samples, 1 g of propolis powder was macerated in 20 ml of 95% EtOH. After stirring for 2 h at room temperature, the mixture was filtered using a sintered glass disc funnel filter (16–40 µm pore size). The residue was re-extracted twice using the same steps. Then, the three gathered filtrates were maintained at −18°C overnight, filtered to remove waxes, and evaporated under reduced pressure (40°C, 10 mbar) to give the EPPs.

Amounts of 8.0 g of propolis powder of BC1 (1) and BC2 (2) or 10.3 g of CG (3) were extracted again with EtOH 95% (UAE, 4× 80 ml, 15 min) for further flash chromatography.

2.4 | Determination of total phenolic content

The total phenolic content was determined via the Folin-Ciocalteu colorimetric method as previously described,¹⁹ and recently adapted to direct use in microplates. Briefly, 10 µl of each propolis extract in MeOH (3.5 mg/ml for BC1, 5 mg/ml for BN2, BC2, and CG, 7.5 mg/ml for BN1, BS1a, BS1b, and BS2, and 10 mg/ml for BS3) was mixed with 20 µl of distilled water and 10 µl of Folin-Ciocalteu reagent in a 96-well microplate. After 3 min, 120 µl of distilled water and 40 µl of 20% aqueous sodium carbonate were added. The absorbance was measured on a TECAN® microplate spectrophotometer (V6.5) 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 and gallic acid was used to calculate the calibration curve (0.04–0.328 mg/ml; $y = 2.7241x - 0.0039$; $r^2 = 0.9982$). Each sample was analyzed in triplicate. Total phenolic content was expressed in terms of gallic acid equivalents (mg) per gram of extract (mg GAE/g).

2.5 | Preliminary chromatographical analyses

2.5.1 | Analytical TLC

Analytical thin layer chromatography (TLC) was performed on a TLC Alugram Xtra SIL G/UV₂₅₄ (Macherey-Nagel, Düren, Germany), using a mixture of cyclohexane:AcOEt as eluant. Spots in the chromatogram were visualized first under UV light (254 nm) and then by spraying with vanillin-sulfuric acid reagent (2 ml of concentrated sulfuric acid in 98 ml of a 1:99 w/v vanillin:95% ethanol solution) and heating the chromatograms to 110°C for 5 min.

2.5.2 | GC-MS procedure

GC-MS analysis was performed on non-derivatized samples using a Shimadzu Gas Chromatograph GCMS-QP2010 SE (Noisiel, France) with an ionization voltage of 70 eV (Electronic Impact). Samples were prepared in dichloromethane (DCM) at 2 mg/ml for extracts and 1 mg/ml for fractions. Separations were carried out using a Phenomenex ZB5 column (30 m × 0.250 mm internal diameter with 0.25 mm film thickness; Phenomenex, Le Pecq, France). The temperature was programmed as follows: 180°C (3 min), 180–280°C at a rate of 10°C/min, and 280°C (27 min). Helium was used as a carrier gas at a flow rate of 2.0 ml/min. Injector and detector temperatures were set at 250°C and 280°C, respectively. Metabolites were identified by comparing their retention times (R_t), nominal mass, and/or fragmentation patterns with those of authentic samples and/or those contained in the fragmentation pattern libraries of the equipment (NIST11, NIST11s, and FFNSC2).

2.5.3 | HPLC-DAD-ELSD procedure

Chromatographic analyses were carried out using a Shimadzu 2030C 3D liquid chromatograph (Noisiel, France) equipped with a diode array

detector (DAD) and an ELSD (Sedere[®]) with a Lichrospher[®] column 100 RP-18 (125 mm × 4 mm i.d., 5 µm, Merck, Darmstadt, Germany) protected with a Lichrocart[®] 4-4 guard cartridge (4 mm × 4 mm i.d.), using a flow rate of 1 ml/min. The mobile phase consisted of 0.1% formic acid in water (solvent A) and methanol (solvent B), and separation was performed by the following linear gradient: 25–100% B (0–40 min), 100% (40–45 min). UV-vis spectra were recorded in the range of 190–600 nm, and chromatograms were acquired at 254 and 280 nm. ELSD was heated at 30°C, and a gain of 4 was applied on the signal. Samples were prepared at a concentration of 10 mg/ml in MeOH and centrifuged at 13,000 *g* for 10 min prior to injection (10 µl) to remove traces of suspended materials.

2.5.4 | HPLC-UV-MS procedure

HPLC-UV-MS analyses were performed using a 2795 Waters separation module (Guyancourt, France) equipped with a Dual λ 2487 Waters detector. Column, mobile phases, and gradient were the same as described above for HPLC-DAD-ELSD. Chromatograms were acquired at 254 and 280 nm. The mass analyses were carried out on a Bruker (Bremen, Germany) electrospray ionization/atmospheric pressure chemical ionization (ESI/APCI) Ion Trap Esquire 3000 + in both positive and negative modes 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* 100–1,000. Samples were prepared at a concentration of 10 mg/ml in MeOH, centrifuged at 13,000 *g* for 10 min, and filtered through a 0.45-µm polytetrafluoroethylene (PTFE) membrane syringe filter prior to injection (20 µl) to remove traces of suspended materials.

2.6 | EEP fractionation by flash chromatography

First, 3.8 g of BC1 (1), 3.5 g of BC2 (2), or 4.0 g of CG (3) EEPs was totally dissolved in a minimal volume of DCM and acetone and mixed with silica gel (silica gel:extract ratio, 2:1). For all of them, the solvent was allowed to evaporate until a fine dry powder was obtained. For each EEP, fractionation was then performed by using a CombiFlash Teledyne ISCO apparatus (Lincoln, NE, USA) with a silica gel column (Chromabond[®] Flash RS 40 SiOH, 40 g, Macherey-Nagel, Hoerd, France) at a flow rate of 25 ml/min with cyclohexane (solvent A) and ethyl acetate (EtOAc) (solvent B) using the following gradient elution: (1) for BC1 EEP, 5% B (0–10 min), 5–20% B (10–30 min), 20–30% B (30–60 min), 30–50% B (60–85 min), and 50–100% B (85–110 min); 160 tubes of 20 ml were collected and combined into 14 fractions (BC1_F1 to BC1_F14) on the basis of their TLC chromatographic profiles (cyclohexane:EtOAc ratio, 90:10 to 50:50); (2) for BC2 EEP, 5% B (0–10 min), 5–10% B (10–20 min), 10–30% B (20–40 min), 30–40% B (40–55 min), and 40–100% B (55–70 min); 100 tubes of 20 ml were collected and combined into 8 fractions (BC2_F1 to BC2_F8) on the

basis of their TLC chromatographic profiles (cyclohexane:EtOAc ratio, 90:10 to 60:40); (3) for CG EEP, 5% B (0–10 min), 5–10% B (10–30 min), 10–20% B (30–50 min), 20–40% B (50–70 min), 40–60% B (70–80 min), and 60–100% B (80–90 min); 135 tubes of 20 ml were collected and combined into 23 fractions (CG_F1 to CG_F23) on the basis of their TLC chromatographic profiles (cyclohexane:EtOAc ratio, 80:20 to 60:40).

An additional fractionation step was performed on the BC2_F7 fraction: 200 mg was dissolved in a minimal volume of MeOH and mixed with C18-silica gel (C18-silica gel:extract ratio, 2:1). Separation was realized on a C18 column (Interchim[®] PF-C18HP, 4 g, Montluçon, France) at a flow rate of 15 ml/min with water (solvent A) and MeOH (solvent B) using the following gradient elution: 50–70% B (0–30 min) and 70–100% B (30–40 min); 80 tubes of 8 ml were collected and combined into 5 fractions (BC2_F7-1 to BC2_F7-5) on the basis of their HPLC-UV (280 nm) profiles.

2.7 | ¹H and ¹³C NMR analyses

NMR spectra (1D and 2D) of propolis fractions (7–58 mg) or sometimes NPs were recorded in deuterated chloroform (CDCl₃) or deuterated methanol (CD₃OD) (500 µl) on a JEOL NMR spectrometer at 400 MHz for ¹H and 100 MHz for ¹³C. NMR experiments (¹H NMR, ¹³C NMR, DEPT-135, DEPT-90, and 2D NMR) on fractions and pure NPs were performed at 298 K on a JEOL 400 MHz H spectrometer (JEOL Europe, Croissy-sur-Seine, France) equipped with an inverse 5-mm probe (ROYAL RO5). Chemical shifts (δ_H and δ_C) are expressed in ppm and *J* values in Hz.

For ¹³C NMR (100 MHz) spectra, a WALTZ-16 decoupling sequence was used with an acquisition time of 1.04 s (32,768 complex data points) and a relaxation delay of 2 s. Between 1,500 and 11,000 scans were collected to obtain a satisfactory S/N ratio. A 1 Hz exponential line broadening filter was applied to each free induction decay (FID) prior to the Fourier transformation. Spectra were manually phased and baseline-corrected using MestReNova software (Mestrelab Research, Santiago de Compostela, Spain) and referenced on the central resonance of the deuterated solvent at δ_C 77.16 ppm (CDCl₃) and δ_C 49.00 ppm (CD₃OD). For distortionless enhancement by polarization transfer (DEPT) experiments, between 512 and 5,500 scans were required and alignments with the ¹³C spectra were made using a given δ_C. A minimum intensity threshold was then used to manually collect positive ¹³C NMR and DEPT-90 signals and positive and negative DEPT-135 signals while avoiding potential noise artifacts.

Propolis DB1 was first built by searching for compounds described in propolis on SciFinderⁿ,⁴⁷ resulting in a DB of 1,471 NPs; δ_C values were predicted using Advanced Chemistry Development (ACD) NMR predictors (C,H). From such DBs containing NPs together with their δ_{C-SDF} values, the CTypeGen routine included in MixONat created a suitable DB: It read the spatial data file (SDF) and sorted chemical shifts by carbon type. The required c-type SDF was then created, i.e., c-type Propolis DB1.^{43,44}

Stilbenoids_Phenanthrenoids DB2 was created. A search of substances using the keywords stilbenoids and phenanthrenoids in the LOTUS DB containing 276,518 NPs⁴⁸ (LOTUS, 2022) allowed to obtain a Stilbenoids_Phenanthrenoids DB2 of 2,681 NPs as an SDF. Flavanones DB3 was then created: A search on SciFinderⁿ⁴⁷ using the 2-phenylchroman-4-one substructure allowed to select 56,679 molecules. They were further reduced to 2,893 referring to "Natural products occurrence" using an analysis of the substances with the filter "Reference role" proposed by SciFinderⁿ. After an additional refining by a filtering step based on the expected molecular weight (MW) (i.e., 340 to 424 Da), all relevant flavanones were subsequently exported as an SDF to obtain the NPs from Flavanones DB3 (684 NPs). For each NPs of DB2 and DB3, δ_{C-SDF} values were predicted using ACD NMR predictors (C,H) software as well as the methodology previously described by Nuzillard⁴⁶ to directly obtain the c-type SDF ready for use by MixONat. The latter contain for each compound of the DB the predicted δ_C values organized as methyl, methylene, methine, or quaternary carbons.

Triterpenes DB4 was built from the PNMNRP3 DB^{49,50} imported as an SDF in ACD NMR predictors (C,H) software by searching triterpenes with an MW between 100 and 500 Da (i.e., Search data: triterpenes; Search MW: 100 to 500) to directly obtain a DB of 6,623 triterpenes in the required c-type file format.

Alk(en)yl resorcinol and phenol derivatives DB5 was built from the PNMNRP3 DB^{49,50} imported as an SDF in ACD NMR predictors (C,H) software by searching NPs with an *m*-alk(en)yl phenol scaffold and classified as phenols (i.e., 3-(hexadec-8-en-1-yl)phenol Substructure search; Search Classyfire class: phenol) to directly obtain a DB of 44 NPs in the required c-type file format.

2.8 | ¹³C NMR-based dereplication using MixONat software

The peak list and intensity data obtained from each experimental spectrum (¹³C NMR, DEPT-135, and DEPT-90) were exported as a .csv file using Microsoft Excel (Microsoft 16.45) software and used as an input file in MixONat software. Such files consist of a list of δ_C values ordered in decreasing order associated with their intensities on the same line, separated by a comma.

Data were then processed using MixONat, which exploits any dataset that provides molecular structures in the previously described SDF (i.e., c-type DB1–5 file format). Based on this information, MixONat was used to compare the experimental δ_C values of the fractions to the predicted δ_{C-SDF} values from the DB and made suggestions for specific NPs potentially present in the analyzed sample. In the end, MixONat provided NP proposals with scores ranging from 0 to 1, i.e., from 0% to 100% (where 1 corresponds to a perfect match and 0 indicates no similarity for a given compound from the used DB). Acceptable values for putative identification were >0.70. Afterwards, experimental data of NPs with the best scores were compared with literature.

2.9 | Purification

2.9.1 | Preparative HPLC

Purification of fractions BC1_F6 and BC1_F8 (40 mg in 2 ml of MeOH) was carried out using a Shimadzu LC-20AP preparative liquid chromatograph equipped with a UV-vis detector SPD-40 (Noisiel, France), an injection loop of 2 ml, and a fraction collector FRC-10A, using a preparative column C18 (250 mm × 21.2 mm i.d., 5 μ m) (Pursuit XR5, Agilent, Les Ulis, France) at a flow rate of 21.24 ml/min. The mobile phase consisted of 0.1% formic acid in water (solvent A) and methanol (solvent B) and the separation was performed using the following gradient: (1) for BC1_F6, 40% B (0–5 min), 40–70% B (5–20 min), and 70–90% (20–30 min); (2) for BC1_F8, 40–70% B (0–15 min) and 70–90% (15–25 min). Chromatograms were acquired at 254 and 280 nm.

2.9.2 | Solid phase extraction (SPE)

To obtain the most polar fraction of BC2-EPP (200 mg in 5 ml of MeOH mixed with 350 mg of C18-silica gel after evaporation), purification was carried out using a C18 solid phase extraction column (2 g/15 ml, Thermo Scientific™, Villebon-sur-Yvette, France) with water:MeOH 7:3 (10 ml) and water:MeOH 6:4 (10 ml). Tubes 4–10 were combined to form the polar fraction of BC2 (BC2_SPE) on the basis of their HPLC-UV (330 nm) profiles.

2.10 | Antioxidant and anti-AGE assays

2.10.1 | Scavenging of DPPH radicals

The DPPH radical scavenging evaluation of Beninese EEPs was performed as previously described.⁵¹ Briefly, tested samples were diluted in absolute EtOH at 0.02 mg/ml from stock solutions at 1 mg/ml in dimethylsulfoxide (DMSO). Aliquots (100 μ l) of these diluted solutions were placed in 96-well plates in triplicate. About 25 μ l of freshly prepared DPPH solution (1 mM) was added to 75 μ l of absolute EtOH using the microplate reader's injector (Infinite® 200, Tecan, France) to obtain a final volume of 200 μ l per well. After 30 min in the dark at ambient temperature, the absorbance was determined at 517 nm. EtOH was used as a blank, whereas 10, 25, 50, and 75 μ M solutions of Trolox (a 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 as Trolox equivalents (micromoles of TE per g of extract).

2.10.2 | Anti-AGE assay

The effects of propolis extracts and the five major isolated naringenin derivatives on AGE formation were determined as described

previously.⁵¹ Briefly, bovine serum albumin (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 from the incubated sample in identical conditions without D-ribose was subtracted for each measurement. Pentosidine-like (excitation at 335 nm, emission at 385 nm) AGE fluorescence was measured by fluorometry.⁵² IC₅₀ values were defined as the amount of extract (μ g/ml) or positive control (μ M) required to reduce AGE formation by 50% relative to the negative control. According to the statistical validation assay,⁵³ a single analysis is sufficient for an accurate IC₅₀ determination.

3 | RESULTS AND DISCUSSION

3.1 | Chromatographic profiling

General LC profiling of the eight Beninese and the Congolese EPP using the quasi-universal ELSD detector showed that all samples contained non-polar compounds, eluted at the end of the chromatograms (Figure 1). BC1 also presented a large amount of more polar compounds, and BC2 exhibited some medium polar compounds in smaller amounts. Non-polar compounds in CG EEP appeared to be quite different from Beninese ones.

Thus, ELSD profiling led to three different and original samples whose composition was further investigated: BC1, BC2, and CG.

Firstly, GC-MS analyses were conducted, comparing the MS fragmentation with the NIST DB, to characterize the chemical classes and, when possible, to identify the constituents. Putative identification (based the percentage of match with the NIST DB) and chemical classes of compounds are presented in Table 2 (GC-total ionic current [TIC] profiling data are shown in Figure SI-2).

All Beninese EEPs presented the same profiles of non-polar compounds, labeled as letter A to F, but their amounts could vary. They were putatively identified as α - or β -amyrenone (A), β -amyrin (B), lupe none (C), α -amyrin (D), α - or β -amyrin acetate (E), and lupeol acetate (F) (cf. Figure SI-3), already described as major NPs in a Mexican propolis.⁵⁴ Zhang et al. (2014) and Tamfu et al. (2020) also described triterpenoids as amyrin/lupeol and amyrin/lupeol acetates in various African propolis samples.^{41,55} Only BC1 presented additional NPs eluted between R_t 10 and 15 min suggested as methoxylated stilbenoids or phenanthrenoids according to their fragmentation patterns. The Congolese EEP exhibited a different profile from the Beninese ones, with C₁₆ and C₁₈ acid ethyl esters as more volatile compounds associated with a good matching score of 94%, a phenol derivative at R_t 13.1 min, three resorcinol derivatives in the range of 14–17 min, and different types of triterpene derivatives after R_t 21 min.

In addition, EPPs were analyzed by HPLC-DAD-MS to visualize non-volatile NPs (cf. Figure SI-4). The different profiling showed that BC1 EEP exhibited a large amount of medium polar compounds with

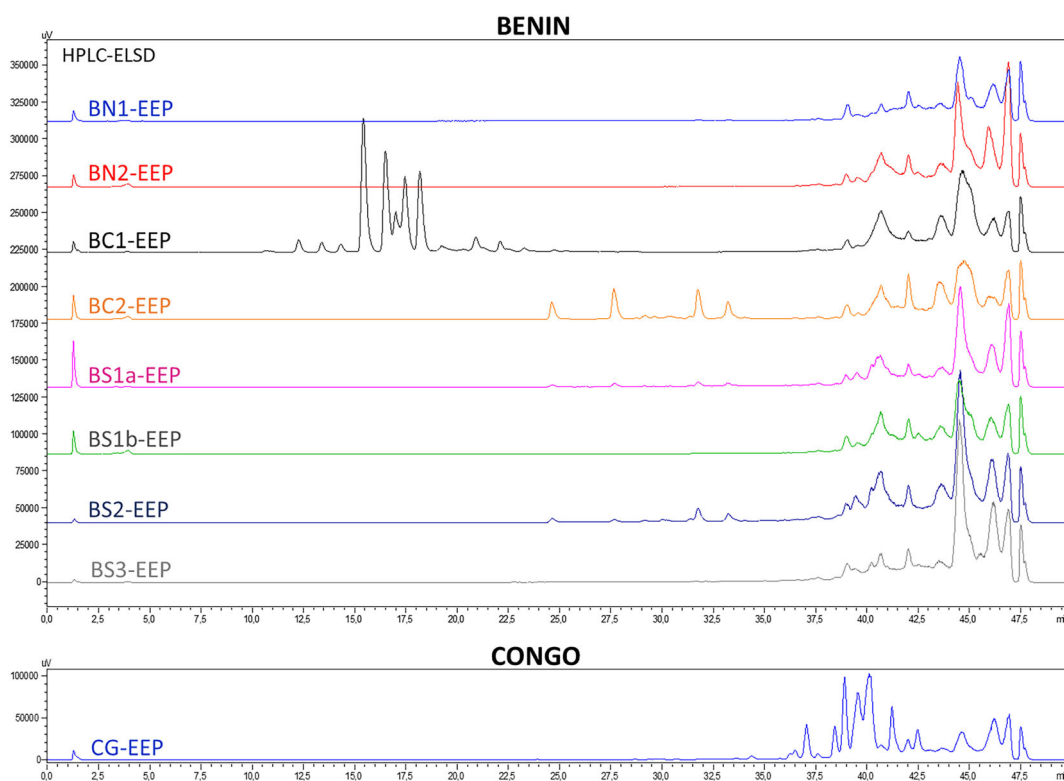


FIGURE 1 ELSD profiling of the eight Beninese and the Congolese EEP.

TABLE 2 GC-MS data of the eight Beninese and the Congolese EPP and putative identification of the constituents.

GC-MS			
R _t (min)	Target ion	Fragmentation	Compound (% match NIST) or structural class
All Beninese EEPs			
22.1	424	409, 281, 218(b), 205, 189	α/β-Amyrenone (80–91) (A)
22.7	426	411, 281, 218(b), 205, 189	β-Amyrin (88–96) (B)
23.2	424	409, 313, 245, 218, 205, 189, 109(b)	Lupenone (82–90) (C)
23.9	426	411, 218(b), 205, 189	α-Amyrin (80–86) (D)
25.2	468	218(b), 205, 189	α/β-Amyrin acetate (83–93) (E)
26.6	468	453, 393, 218(b), 205, 189	Lupeol acetate (81–91) (F)
BC1 more volatile compounds			
10.8	244	138, 107(b), 77	Phenol derivative
10.9	274	244, 167(b), 107, 77	-
12.0	304	167(b), 137, 122	-
12.1	318	181(b), 137	-
12.2	302	287, 255, 151	Methoxylated stilbenoid or phenanthrenoid
12.7	302	287, 255, 151	Methoxylated stilbenoid or phenanthrenoid
13.1	272	257, 225	Methoxylated stilbenoid or phenanthrenoid
13.9	300	285, 242, 214, 150	Methoxylated stilbenoid or phenanthrenoid
14.8	316	270(b), 255, 223, 135	Methoxylated stilbenoid or phenanthrenoid
CG			
6.7	284	241, 213, 185, 157, 115, 101, 88(b)	Palmitic acid ethyl ester (94)
8.5	310	264, 222, 180, 111, 69, 55(b)	Oleic acid ethyl ester (94)
13.1	330	234, 147, 120, 108(b)	Phenol derivative
14.0	318	222, 137, 124(b)	Resorcinol derivative
14.0	320	222, 137, 124(b)	Resorcinol derivative (pentadecyl) (92)
16.3	346	250, 137, 124(b)	Resorcinol derivative
21.8	468	424, 410, 367, 297, 205, 109(b), 95	Triterpene derivative
22.1	424	409, 281, 218(b), 203	α/β-Amyrenone (89–91) (A)
22.7	426	411, 281, 218(b), 203	β-Amyrin (89) (B)
23.2	424	409, 313, 218(b), 205, 189, 109	Lupenone (82)
23.3	424	409, 313, 286, 218, 205, 109, 95(b)	Triterpene derivative
23.6	426	393, 365, 339, 286, 271, 205, 175, 147, 109, 69(b)	Cycloartenol (89)
23.8	426	393, 315, 257, 218(b), 207, 189, 147, 109	Lupeol (79)
23.9	426	411, 393, 257, 218(b), 205, 189	α-Amyrin (77) (D)
24.7	438	423, 395, 340, 313, 207, 175, 147, 121, 109, 95(b)	Triterpene derivative
25.5	424	409, 355, 281, 205, 189, 147, 121, 95(b)	Triterpene derivative
27.9	424	355, 313, 281, 205, 189, 147, 109(b)	Triterpene derivative
28.8	426	357, 313, 281, 207, 191, 147, 109(b)	Triterpene derivative

(b) base peak

chromophores absorbing at 280 nm, as well as BC2 in smaller proportions, and CG possessed a very poor UV profile at 280 nm.

3.2 | Chemical composition of BC1 and BC2

To identify their major NPs using ¹³C NMR dereplication based on MixONat and suitable DBs, a coarse fractionation of BC1 and BC2 EEP

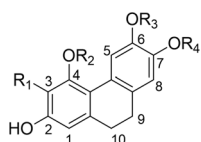
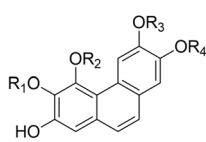
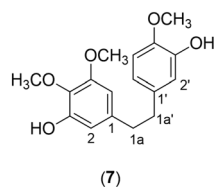
was then achieved by flash chromatography. Indeed, for BC1, GC-MS analysis revealed the presence of methoxylated stilbenoids or phenanthrenoids, which guided us to build the specific Stilbenoids_Phenanthrenoids DB2. For BC2, except for the more polar compounds at R_t 3.8 min, all compounds showed UV spectra of flavanones-dihydroflavonols, which suggested to create the Flavanones DB3.

As depicted in Table 3, dihydrophenanthrenes **1** and **3–5**, phenanthrenes **2** and **6**, and the dihydrostilbene **7** (Figure 2) were

TABLE 3 UV-MS data and ^{13}C NMR dereplication results of BC1 and BC2 EEP after fractionation.

R _t (min)	λ _{max} (nm)	ESI (-MS) [M-H] ⁻	ESI (+MS) [M+H] ⁺	MW	Present in fraction	¹³ C NMR dereplication (MixONat)		Formula	Compound/class
						Database	Results		
BC1									
12.1	277, 311	287	289	288	BC1_F6-1	DB2	Rank 2 with MW 288 filter; score 0.5	C ₁₆ H ₁₆ O ₅	2,6,7-Trihydroxy-3,4-dimethoxy- 9,10-dihydrophenanthrene (1)
13.2	257, 280sh	285	287	286	BC1_F8-1	DB2	Rank 1; score 0.86	C ₁₆ H ₁₄ O ₅	2,6,7-Trihydroxy- 3,4-dimethoxyphenanthrene (2)
15.3	276, 300	271	273	272	BC1_F5	DB2	Rank 1; score 0.75	C ₁₆ H ₁₆ O ₄	6-Methoxycelonin (3)
16.4	279, 311	301	303	302	BC1_F2	DB2	Rank 1; score 0.88	C ₁₇ H ₁₈ O ₅	2,7-Dihydroxy-3,4,6-trimethoxy- 9,10-dihydrophenanthrene (4)
16.9	278, 311	301	303	302	BC1_F2	DB2	Rank 8; score 0.76	C ₁₇ H ₁₈ O ₅	2,6-Dihydroxy-3,4,7-trimethoxy- 9,10-dihydrophenanthrene (5)
17.3	258, 280sh	299	301	300	BC1_F4	DB2	Rank 5; score 0.94 with MW 300 filter	C ₁₇ H ₁₆ O ₅	2,7-Dihydroxy- 3,4,6-trimethoxyphenanthrene (6)
18.1	268	303	305	304	BC1_F4	DB2	Rank 4; score 1 with MW 304 filter	C ₁₇ H ₂₀ O ₅	Combretastatin B-2 (7)
BC2									
3.8	299, 322	353	355	354	BC2_SPE	-	-	C ₁₆ H ₁₈ O ₉	Biflorin (8) and isobiflorin (9)
24.5	295, 338sh	339	341	340	BC2_F7-1	DB3	Rank 1; score 0.95	C ₂₀ H ₂₀ O ₅	6-Prenylnaringenin (10)
27.5	297, 338sh	339	341	340	BC2_F6	DB3	Rank 2; score 0.65	C ₂₀ H ₂₀ O ₅	6-Dimethylallylnaringenin (11)
29.5	296	423	425	424	BC2_F7-3	DB3	Rank 1; score 1	C ₂₅ H ₂₈ O ₆	6,8-Diprenylaromadendrin (12)
31.6	298, 351sh	407	409	408	BC2_F5	DB3	Rank 10; score 0.84	C ₂₅ H ₂₈ O ₅	6,8-Diprenylnaringenin (13)
33.1	295, 336sh	407	409	408	BC2_F7-5	DB3	Rank 1; score 0.84	C ₂₅ H ₂₈ O ₅	6-Geranylnaringenin (14)

DB2: Stilbenoids_Phenanthrenoids DB. DB3: Flavanones DB.

(1) $\text{R}_1=\text{OCH}_3$, $\text{R}_2=\text{CH}_3$, $\text{R}_3=\text{R}_4=\text{H}$ (3) $\text{R}_1=\text{R}_4=\text{H}$, $\text{R}_2=\text{R}_3=\text{CH}_3$ (4) $\text{R}_1=\text{OCH}_3$, $\text{R}_2=\text{R}_3=\text{CH}_3$, $\text{R}_4=\text{H}$ (5) $\text{R}_1=\text{OCH}_3$, $\text{R}_2=\text{R}_4=\text{CH}_3$, $\text{R}_3=\text{H}$ (2) $\text{R}_1=\text{R}_2=\text{CH}_3$, $\text{R}_3=\text{R}_4=\text{H}$ (6) $\text{R}_1=\text{R}_2=\text{R}_3=\text{CH}_3$, $\text{R}_4=\text{H}$ 

(7)

FIGURE 2 Structures of compounds 1–7.

suggested in BC1 EEP by MixONat and validated by comparison with literature data (cf. supporting information). When necessary, a MW filter was used based on HPLC-MS data.

More precisely, among 9,10-dihydrophenanthrene derivatives out of 2,681 NPs in DB2, MixONat suggested 6-methoxycelonin (2,7-dihydroxy-3,5-dimethoxy-9,10-dihydrophenanthrene; **3**, rank

1, score 0.75, Figures SI-13 and SI-14) in BC1-F5 and its presence was confirmed by comparison to published NMR data. It has been yet identified from Combretaceae species⁵⁶ and in the orchid *Bulbophyllum vaginatum*;⁵⁷ and 2,7-dihydroxy-3,4,6-trimethoxy-9,10-dihydrophenanthrene (**4**, rank 1, score 0.88, Figures SI-15 and SI-16) was hypothesized in BC1_F2. Compound **4** has already been

described in Senegalese propolis⁵⁸ and its presence was validated based on reported data by Pettit et al. from the African tree *Combretum caffrum*⁵⁹ and by Lu et al. from *Dioscorea nipponica* Makino.⁶⁰

The ¹³C NMR-based dereplication process also predicted 2,6-dihydroxy-3,4,7-trimethoxy-9,10-dihydrophenanthrene (**5**, rank 8, score 0.76, Figures SI-15 and SI-16) in BC1_F2 and 2,6,7-trihydroxy-3,4-dimethoxy-9,10-dihydrophenanthrene (**1**, rank 2, score 0.5, Figures SI-5 and SI-6) in BC1_F6-1 when a molecular filter at MW 288 Da was used. For **1**, the score was quite low (0.5); therefore, additional 2D NMR experiments (heteronuclear multiple-quantum correlation [HMQC], heteronuclear multiple-bond connectivity [HMBC], nuclear Overhauser effect spectroscopy [NOESY], cf. Figures SI-7–SI-10) were performed to confirm the structure; for **5**, the score was 0.76, i.e., higher than 0.70. These two NPs (**1** and **5**) were first mentioned by Letcher et al. in *Combretum* species^{61,62} and **1** has already been found in Senegalese propolis.⁵⁸

Among phenanthrene derivatives, MixONat hypothesized 2,6,7-trihydroxy-3,4-dimethoxyphenanthrene (**2**, rank 1, score 0.88, Figures SI-11 and SI-12) in BC1_F8-1, which was confirmed by comparison with its spectral data described by Letcher et al. in *Combretum apiculatum*.⁶¹ 2,7-Dihydroxy-3,4,6-trimethoxyphenanthrene (**6**, rank 5, score 0.94, Figures SI-17 and SI-18) previously isolated from *Photolida Chinensis*⁶³ was suggested in BC1_F4.

Combretastatin B-2 (**7**), a dihydrostilbene, was suggested by MixONat in first rank (score 1.0, Figures SI-17 and SI-19) in BC1 EPP when using an MW filter (MW 304 Da) and validated by comparison with NMR data described by Pettit et al. in *Combretum caffrum*.⁵⁹

Compounds **2**, **6**, and **7** have already been identified in Senegalese propolis.⁵⁸

¹H and ¹³C NMR data of **1**–**7** are available in the supporting information.

For BC2, seven compounds were identified belonging to two structural classes of polyphenols, chromone-C-glucoside and prenyl-flavanone derivatives (**8**–**14**), some of which were described for the first time in propolis (Figure 3).

The ¹³C NMR-based process using MixONat and DB1 to DB5 did not give relevant results suggesting original NPs. Two major products (**8**–**9**) were fully characterized as a mixture using mass spectra and 1D and 2D NMR data (¹H, ¹³C, HMQC, HMBC, cf. Figure SI-20–SI-23)

and identified as biflorin (**8**) and isobiflorin (**9**), two chromanone-C-glucosides newly described in propolis, previously described in *Pancreaticum biflorum*⁶⁴ and cloves of *Eugenia caryophyllata*, respectively.⁶⁵

Regarding flavanone derivatives, among 688 NPs in DB3, MixONat proposed 6-prenylnaringenin (**10**, rank 1, score 0.95, Figures SI-24 and SI-25) already described in Nigerian red propolis⁶⁶ in BC2_F7-1. 6,8-Diprenylaromadendrin (**12**, rank 1, score 1.0, Figures SI-28 and SI-29) and 6,8-diprenylnaringenin (lonchocarpol A) (**13**, rank 10, score 0.84, Figures SI-30 and SI-31), previously found in Cameroon propolis samples,⁴² were validated in BC2_F7-1 and BC2_F5, respectively. 6-Geranylnaringenin (**14**, rank 1, score 0.84, Figures SI-32 and SI-33), previously described in propolis from the Solomon Islands,⁶⁷ was hypothesized and confirmed in BC2_F7-5. Finally, 6-dimethylallylnaringenin (**11**) was suggested in second position by MixONat in BC2_F6 (rank 2, score 0.65, Figures SI-26 and SI-27). The isomer in position 8 was proposed in first position, but comparison with literature data^{68,69} showed that it is the isomer in position 6. Compound **11** was new in propolis but already described in organic extracts of *Monotes africanus*.⁷⁰ ¹H and ¹³C NMR data of **8**–**14** are available in the supporting information.

3.3 | Chemical composition of CG

For CG, the EEP from Congo, GC-MS was not effective enough to directly identify all the different constituents but allowed us to characterize several chemical classes, including fatty esters, phenol and resorcinol derivatives, and triterpenoids. This structural information prompted us to use ¹³C NMR-based dereplication using customized DBs, i.e., DB4 and DB5. Thus, flash chromatography was performed on CG EEP and fractions were gathered using TLC with sulfuric vanillin revelation (cf. Figure SI-24). ¹³C NMR dereplication with MixONat software was performed on the different fractions using DB4 and DB5, followed by comparison with literature for validation. Moreover, GC-MS data allowed us to determine the MW of major NPs, which were used with MixONat software to improve structural hypotheses (Table 4, Figure 4).

As expected, fatty esters such as palmitic acid ethyl ester (**15**) and oleic acid ethyl ester (**16**), the most volatile compounds of CG, were

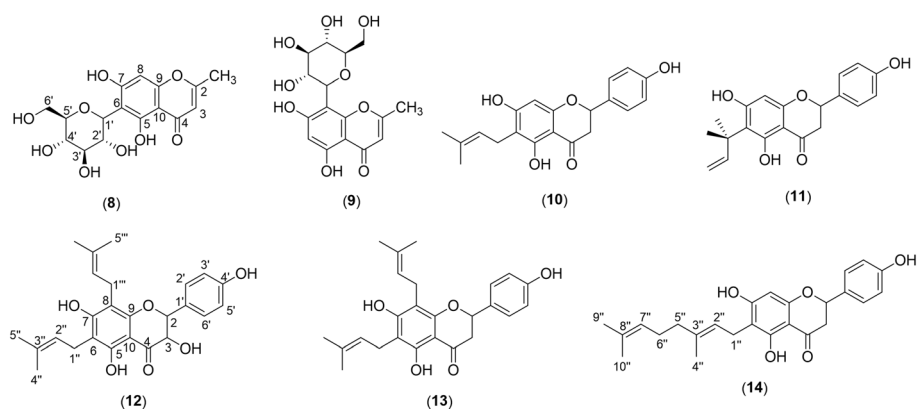


FIGURE 3 Structures of compounds **8**–**14**.

TABLE 4 Identification of CG EEP constituents by ^{13}C NMR dereplication.

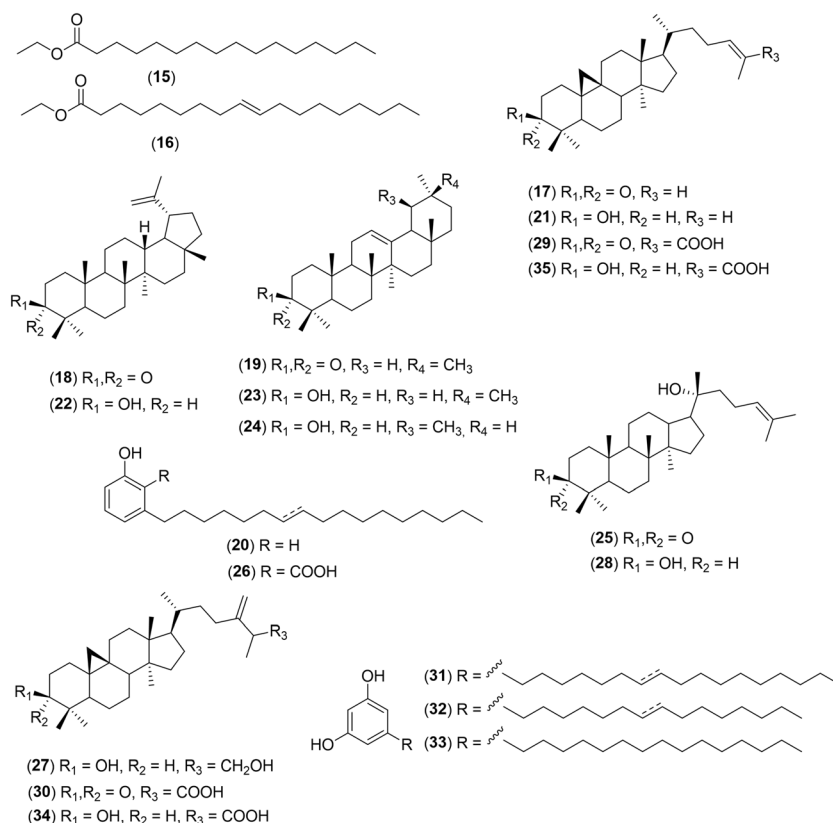
Flash chrom./TLC		^{13}C NMR dereplication (MixONat)		^1H and ^{13}C NMR dereplication/identification				
Fractions	R _f	DB	Results	Analysis	Compound/class	Formula	MW	GC-MS of fractions
CG_F2	0.85–0.96	-	-	^1H NMR	Palmitic acid ethyl ester (15)	$\text{C}_{18}\text{H}_{36}\text{O}_2$	284	R _t 6.7 min, <i>m/z</i> 284
					Oleic acid ethyl ester (16)	$\text{C}_{20}\text{H}_{38}\text{O}_2$	310	R _t 8.5 min, <i>m/z</i> 310
CG_F3	0.88	DB4	Maj: Rank 3; score 0.90 (17) Tot with MW 424 filter: Rank 1; score 0.90 (17) Rank 4; score 0.87 (18) Rank 31; score 0.83 (19)	^1H and ^{13}C NMR	Cycloartenone (17) (maj)	$\text{C}_{30}\text{H}_{48}\text{O}$	424	R _t 23.3 min, <i>m/z</i> 424
					Lupenone (18)	$\text{C}_{30}\text{H}_{48}\text{O}$	424	R _t 23.2 min, <i>m/z</i> 424
					β -Amyrenone (19)	$\text{C}_{30}\text{H}_{48}\text{O}$	424	R _t 22.1 min, <i>m/z</i> 424
CG_F5	0.69–0.85	DB5	Rank 1, score 0.87 (20)	^1H and ^{13}C NMR	<i>m</i> -Heptadecenylphenol (20)	$\text{C}_{23}\text{H}_{38}\text{O}$	330	R _t 13.1 min, <i>m/z</i> 330
CG_F8	0.44–0.67	DB4	Maj: Rank 7; score 0.90 (21) Tot with MW 426 filter: Rank 1; score 0.97 (22) Rank 34; score 0.83 (23)	^1H and ^{13}C NMR	Cycloartenol (21) (maj)	$\text{C}_{30}\text{H}_{50}\text{O}$	426	R _t 23.6 min, <i>m/z</i> 426
					Lupeol (22)	$\text{C}_{30}\text{H}_{50}\text{O}$	426	R _t 23.8 min, <i>m/z</i> 426
					β -Amyrin (23)	$\text{C}_{30}\text{H}_{50}\text{O}$	426	R _t 22.7 min, <i>m/z</i> 426
					α -Amyrin (24)	$\text{C}_{30}\text{H}_{50}\text{O}$	426	R _t 23.9 min, <i>m/z</i> 426
CG_F10	0.54–0.65	DB4	Rank 1; score 0.93 (25)	^1H and ^{13}C NMR	Dipterocarpol (25)	$\text{C}_{30}\text{H}_{50}\text{O}_2$	442	R _t 27.9 min, <i>m/z</i> 424 (442- H_2O)
		DB5	Rank 1; score 0.92: (26)		6-Heptadecenylsalicylic acid (26)	$\text{C}_{24}\text{H}_{38}\text{O}_3$	374	R _t 13.1 min, <i>m/z</i> 330 (374- CO_2)
CG_F12	0.38–0.65	DB4	Rank 20; score 0.90 with MW 442 filter (25) Rank 3; score 0.97 with MW 456 filter (27)	^1H and ^{13}C NMR	Dipterocarpol (25)	$\text{C}_{30}\text{H}_{50}\text{O}_2$	442	R _t 27.9 min, <i>m/z</i> 424 (442- H_2O)
					24-Methylene cycloartane-3 β ,26-diol (27)	$\text{C}_{31}\text{H}_{52}\text{O}_2$	456	R _t 24.7 min, <i>m/z</i> 438 (456- H_2O)
CG_F15	0.39–0.43	DB4	Rank 1; score 1 (28) Rank 10; score 0.87 with MW 454 filter (29) Rank 1; score 0.94 with MW 468 filter (30)	^1H and ^{13}C NMR	Dammarenediol II (28)	$\text{C}_{30}\text{H}_{52}\text{O}_2$	444	R _t 28.8 min, <i>m/z</i> 426 (444- H_2O)
					Mangiferonic acid (29)	$\text{C}_{30}\text{H}_{46}\text{O}_3$	454	-
					Ambonic acid (30)	$\text{C}_{31}\text{H}_{48}\text{O}_3$	468	-
CG_F16	0.26–0.43	DB5	Rank 1; score 0.91 (31)	^1H and ^{13}C NMR	Heptadecenyl resorcinol (31)	$\text{C}_{23}\text{H}_{38}\text{O}_2$	346	R _t 16.3 min, <i>m/z</i> 346
					Pentadecenyl resorcinol (32)	$\text{C}_{21}\text{H}_{34}\text{O}_2$	318	R _t 14.0 min, <i>m/z</i> 318
					Pentadecyl resorcinol (33)	$\text{C}_{21}\text{H}_{36}\text{O}_2$	320	R _t 14.0 min, <i>m/z</i> 320
CG_F18	0.13–0.35	DB4	Rank 1; score 0.87 (34) Rank 9; score 0.80 (35)	^1H and ^{13}C NMR	Ambolic acid (34)	$\text{C}_{31}\text{H}_{50}\text{O}_3$	470	-
					Mangiferolic acid (35)	$\text{C}_{30}\text{H}_{48}\text{O}_3$	456	-

DB4: Triterpenes DB. DB5: Alk(en)yl resorcinol Phenols DB.

confirmed in CG_F2 by ^1H NMR as hypothesized by GC-MS. For CG_F3, ^{13}C NMR dereplication using MixONat software with triterpenes DB4 gave more than 50 compounds with a match score higher than 0.90. ^{13}C NMR spectra of CG_F3 revealed a mixture of several triterpene derivatives (with MW at 424 for all proposed by GC-MS) including one major and two minor ones. After validation of ^{13}C NMR

data in literature, the major compound was identified as cycloartenone^{71,72} (17, rank 1, score 0.90, MW filter at 424 Da, Figures SI-35 and SI-36) and the two minor ones as lupenone^{73,74} (18, rank 4, score 0.87) and β -amyrenone⁷⁵ (19, rank 31, score 0.83, Figures SI-35 and SI-36). Similarly, in fraction CG_F8, we found a mixture of corresponding alcohols such as cycloartenol⁷⁶ (21, rank 7, score 0.90, Figures SI-

FIGURE 4 Structures of compounds 15–35.



39 and SI-40) as major NP and lupeol⁷⁷ (22) and β -amyrin⁷⁸ (23) as minor ones (rank 1, score 0.97 and rank 34, score 0.83, MW filter at 426 Da; Figures SI-41 and SI-42). The isomer of 23, α -amyrin (24), was also identified mainly based on ¹³C NMR data (comparison to literature data by Seo et al.⁷⁸ and GC-MS (MW 426) data. All these ketone and alcohol triterpene derivatives were already known in propolis.^{37,38,42,54,79} For CG_F5, as GC-MS analysis showed a major compound at 13.1 min with a phenol derivative profile (Table 1), ¹³C NMR dereplication with the alk(en)yl resorcinol_phenol DB5 rightly identified *m*-heptadecenylphenol⁸⁰ (20, rank 1, score 0.87 (Figures SI-37 and SI-38) already described in Cameroonian propolis.³⁷ In fraction CG_F10, Triterpenes DB4 accurately highlighted dipterocarpol⁸¹ (25, rank 1, score 0.93, MW 442, Figures SI-43 and SI-44) previously described in propolis from Thailand⁸² and Alk(en)yl resorcinol_phenol DB5 suggested 6-heptadecenylsalicylic acid (26, rank 1, score 0.92, MW 374, Figures SI-43 and SI-45). Regarding these two NPs, their MW did not match with those determined by GC-MS. This could be explained by the high temperature used in the GC-MS method: Compound 25 might lose a molecule of water through dehydration (m/z 424 [M – 18]) while 26 might lose a molecule of carbon dioxide through decarboxylation (m/z 330 [M-44]). For the following fraction CG_F12, the dereplication process using Triterpenes DB4 suggested a lot of compounds with high scores. Using an MW filter at 442 Da, dipterocarpol (25) already found in CG_F10 (rank 20, score 0.90, Figures SI-46 and SI-47) and 24-methylene cycloartane-3 β ,26-diol⁸³ (27, rank 3, score 0.97, MW filter at

456 Da, Figures SI-46 and SI-48) were identified. In fraction CG_F15, dammarenediol II (28) was rightly hypothesized by MixO-Nat and Triterpenes DB4 using a MW filter at 444 Da (rank 1, score 1, Figures SI-49 and SI-50; GC-MS: m/z 426 [M-18]) and confirmed by ¹³C NMR data.⁸⁴ Using the same approach, this latter fraction was shown to contain two other triterpenoids, i.e., mangiferonic acid (29, rank 10, score 0.87, MW filter at 454 Da, Figures SI-49 and SI-51) and ambonic acid (30, rank 1, score 0.94, MW filter at 468 Da, Figures SI-49 and SI-52) already found in propolis from Nigeria⁴⁰ and from Brazil.⁸⁵ These carboxylic acids were not detected by GC-MS because of their lack of volatility (intermolecular hydrogen bond due to carboxylic acid function). Likewise, in CG_F18, two other triterpenoid acids were identified, i.e., ambolic acid (34, rank 1, score 0.87, MW filter at 470 Da, Figures SI-55 and SI-56) and mangerolic acid (35, rank 9, score 0.80, MW filter at 456 Da, Figures SI-55 and SI-57) also described by Silva et al. in Brazil.⁸⁵ In the penultimate fraction CG_F16, ¹³C NMR dereplication with Alk(en)yl resorcinol_phenol DB5 accurately suggested a resorcinolic lipid, i.e., heptadecenylresorcinol⁸⁶ (31, rank 1, score 0.91, Figures SI-53 and SI-54) associated to two other resorcinol derivatives identified as pentadecenylresorcinol⁸⁷ (32, MW 318) and pentadecylresorcinol⁸⁸ (33, MW 320). All were previously isolated in Cameroonian³⁷ and Mexican propolis.⁸⁹ ¹³C NMR data of 17–35 are available in the supporting information.

For this Congolese propolis sample, most triterpenoids as well as resorcinolic lipids might originate from *M. indica*.^{83,89,90}

3.4 | Antioxidant and anti-AGE assessment

Evaluation of the antioxidant and anti-AGE activities of BC1, BC2, and CG EEPs revealed that only BC1 EEP showed a good antioxidant activity ($1,172 \pm 97 \mu\text{mol TE/g}$), twice higher than that of ethanolic rosemary extract (E392). This activity was in relation with its high total phenolic content ($297.0 \pm 15.6 \text{ mg GAE/g}$) comparable to those of poplar-type EEPs mostly found in Europe, North America, or China.¹⁹ However, the chemical analysis of BC1 EEP revealed phenanthrenoids and stilbenoid polyphenols, including combretastatin B-2 (7), a dihydrostilbene. Such derivatives have already been described by Inui et al. in 2021⁵⁸ in Senegalese propolis showing significant anti-inflammatory activity, compounds 4, 6, and 7 being the most active. The dihydrophenanthrene 6-methoxycoelonin (3) has also been described displaying cytotoxic effect against five human cancer cell lines (786-0, MCF-7, Hep2, UACC-62, and NCI/ADR-RES) with remarkable activity against UACC-62 cells ($\text{IC}_{50} 2.59 \mu\text{M}$).⁹¹ The phenanthrene 6 was also known for its moderate cytotoxic activity against KB, MCF-7, and K562 cells and its potent inhibitory activity on CDK1/cyclin B ($\text{IC}_{50} 0.07 \mu\text{M}$).⁹² Dihydrophenanthrenes from *Combretum* species such as combretastatins were also previously described as cell growth inhibitors⁵⁹: Four types of combretastatins can be distinguished, i.e., stilbene-type (combretastatin A), dihydrostilbene-type (combretastatin B), phenanthrene-type (combretastatin C), and cyclic macrolactone-type combretastatins (combretastatin D). Among them, combretastatins A (A-4, but also A-1 and A-2) exhibit the highest anti-tumor activity.^{93,94} Consequently, it seems best to use such antioxidant propolis containing phenanthrene, dihydrophenanthrene, and combretastatin derivatives with great care. It confirms the importance of the preliminary determination of the chemical composition of propolis before its use in food and health products.

Among all propolis extracts used in this study, only BC2 EEP exhibited a moderate anti-AGE activity ($\text{IC}_{50} 0.70 \text{ mg/ml}$) compared to *S. japonicum* ethanolic extract, well known for its high anti-AGE activity¹⁹ (cf. Table SI.1). As expected, all purified flavanone derivatives showed a good activity ($\text{IC}_{50} 0.20\text{--}0.26 \text{ mM}$; Table SI-1) close to the reference value (Quercetin $\text{IC}_{50} 0.20 \text{ mM}$) except for 13 ($\text{IC}_{50} 0.60 \text{ mM}$). A higher anti-AGE activity was previously reported for a French EEP ($\text{IC}_{50} 0.05 \text{ mg/ml}$) with much lower levels of triterpene derivatives and high amounts of pinobanksine derivatives.¹⁹

In conclusion, in the present work, GC-MS or HPLC-DAD-MS was first used to identify different classes of NPs and determine their MWs. Then ¹³C NMR-based dereplication using MixONat software with custom DBs allowed us to unambiguously characterize major NPs from fractions. In addition to major triterpenoids, BC1, a propolis sample originating from the center of Benin, exhibited an original composition with high levels of dihydrophenanthrene, phenanthrene, and bisbenzyl derivatives, some of which were identified for the first time in propolis. Some of these antioxidant polyphenols are probably cytotoxic and remind us of the need for systematic analysis of propolis before use in food and health products, especially for the lesser known propolis from tropical areas. Among the other extracts

belonging to *Macaranga*-type propolis, BC2, collected in the same area, contained prenyl and geranyl flavanones with anti-AGE activities. Propolis from Congo contained NPs most likely associated to the botanical source *M. indica*: cycloartane-type triterpenoids and resorcinolic lipids. Further investigation will be performed to associate the phytochemical composition of Beninese and Congolese propolis samples to the local flora and to evaluate their anti-fungal or anti-bacterial properties.

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DATA AVAILABILITY STATEMENT

All data marked SI are included in the supplementary information section.

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