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Phytochemical Analysis and Biological Evaluation of Selected African Propolis Samples from Cameroon and Congo

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The objective of this study was the chemical analysis of four selected samples of African propolis (Congo and Cameroon) and their biological evaluation. Twenty-one secondary metabolites belonging to four different chemical groups were isolated from the 70% ethanolic extracts of propolis and their structures were elucidated on the basis of spectral evidence. Three triterpenes and two diprenyl-flavonoids were identified from Congo propolis, which has been investigated for the first time, while thirteen triterpenes, three diprenyl-flavonoids, two monoterpenic alcohols and one fatty acid ester have been identified from Cameroon propolis samples. To our knowledge, the identified diprenyl-flavonoids, as well as five of the isolated and determined triterpenes, are reported for the first time in propolis. Moreover, the total polyphenol content was estimated in all extracts and the antimicrobial activities of all four extracts were studied against six Gram-positive and -negative bacteria and three pathogenic fungi, showing an interesting antibacterial profile.

Keywords: African propolis, Diprenylflavonoids, Triterpenes, Antimicrobial activity, Total polyphenol content.

Propolis (bee glue), a complex natural substance collected by honeybees from buds and exudates of certain trees and plants, possesses a wide range of biological activity due to different propolis constituents [1a, b]. It is considered responsible for the low incidence of bacteria and molds within the hive. Antimicrobial activity is an essential characteristic of propolis, and humans have used it for centuries for its pharmaceutical properties [2a, b]. It has been used in folk medicine for treatment of wounds, burns, stomach ulcers and other applications. Modern science revealed its valuable pharmacological activities: antibacterial, antimycotic, antiviral, cytotoxic, antioxidant, anti-inflammatory and immunomodulating [2b-g] due to the activities of its many and different chemical constituents. Moreover, propolis is extensively used in food and beverages to improve health and prevent diseases, as a constituent of biocosmetics and for numerous other purposes [2a,d]. The chemical composition of propolis is highly variable and its chemical diversity is primarily dependent on the local flora of the geographic region from which it is collected [3a,3b].

In the framework of our studies on bee-keeping products [1a,1b], we report in this study the chemical analyses on one propolis sample from Congo, which to our knowledge has never been studied before, and three samples from Cameroon. According to the literature, from NE Cameroon (Meiganga region) the triterpenoids lupeol, lupenone, erythrodiol,18-iso-olean-12-ene-3,11dione, 25-cyclopropyl-3β-hydroxyurs-12-ene,cycloart-3β-hydroxy-12,25(26)-diene lup20(29)-en-3β-oate and 3β-hydroxylup-20(29)-ene were isolated and structurally determined [4a,b]. Chemical investigation of NW Cameroon propolis revealed the presence of nine triterpenes α- and β-amyrin, α- and β-keto-amyrin, ursolic acid, cycloartenol, ambonic acid, magniferolic and isomagniferolic acid [4c,d]. Also, as part of a chemical profiling of African propolis by means of dereplication (LC-HRMS), a Cameroon propolis sample was proved to contain a significant amount of triterpenoids [4e].

Furthermore, the concentration of total phenolics was determined and all samples were evaluated for their antimicrobial activities as they were assayed against a panel of two human pathogenic Gram-positive bacteria, four Gram-negative bacteria and three human pathogenic fungi.

Qualitative phytochemical analyses of all samples resulted to the isolation of twenty-one secondary metabolites of which fourteen

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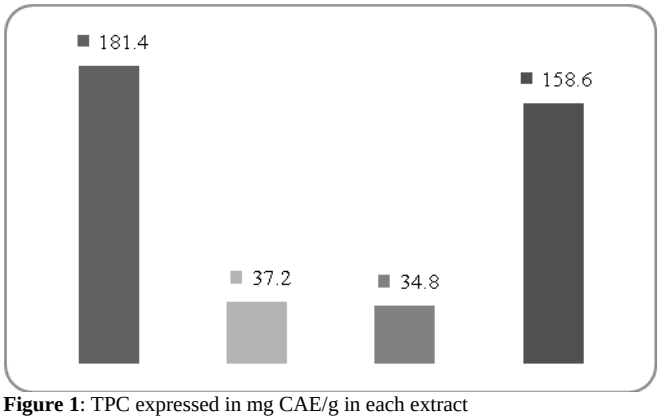

Figure 1: TPC expressed in mg CAE/g in each extract

triterpenoids, four diprenyl-flavonoids, one fatty acid ester and two monoterpene alcohols. The triterpenes lupenone (**1**), β-amyrin (**2**) and lupeol (**3**), as well as the diprenyl-flavonoids lonchocarpol A (**4**) and 6,8-diprenyl-eriodictyol (**5**), were determined from the Congo propolis sample (sample 1), which is studied for the first time.

From the three Cameroon samples thirteen triterpenoids were isolated: lupenone (**1**), β-amyrin (**2**), ψ-taraxasterol-acetate (**6**), taraxasterol acetate (**7**), lupeol acetate (**8**), lanosterol (**9**) 3α-hydroxy-olean-12-en-30-ol **(10)**, α-amyrone **(11)**, α-amyrin **(12)** β-acetoxy-amyrin (**15**), bacchara12,21dien3β-ol (**16**), betulin-aldehyde (**17**), and erythrodiol (**18**), together with two monoterpene alcohols: α-terpineol (**13**) and 1,8-terpineol **(14)**, one fatty acid ester: ethyl palmitate (**21**), and three diprenyl-flavonoids: lonchocarpol A **(4)**, 6,8-diprenyl-aromadendrin (**19**) and lespedezaflavanone C (**20**). The metabolites were identified by a combination of spectroscopic (1D and 2D NMR) and mass spectrometric techniques and comparison with literature data [6-21]. According to published studies on Cameroon propolis [4a-d] it is clearly proved that it is rich in pentacyclic triterpenes and especially in derivatives of lupeol and amyrin. The chemical constituents **4-7**, **9**, **10**, **13**, **14-17**, **19-21**, are reported for the first time in Cameroon's propolis, while **4-7**, **10**, **14**, **16**, **17** and **19-20** are reported for the first time in propolis samples according to existing literature.

Additionally, total polyphenol content (TPC) was estimated for each sample by the Folin-Ciocalteu method. The results in Figure 1 show that samples 1 & 4 were the extracts with higher TPC content. The antioxidant activity of propolis extracts can possibly be attributed to the polyphenol content as indicated by the significantly positive correlation [22].

All four samples were also studied for their antibacterial properties and samples 1 and 4 exhibited stronger activities, probably due to their higher concentration of phenolic compounds. Finally, all samples expressed minimum anti-fungal activity, which was higher in samples 1 and 4 (Table 1).

Two potential types of North African (Algeria, Tunisia, Egypt and Morocco) propolis were recently presented: poplar type (rich in flavonoids without B-rings substituents) [4e-4i] and the so called "Mediterranean type" (rich in diterpenes) [4f].

From tropical African areas (for example, Cameroon, Egypt, Gambia, Tanzania) propolis contains mainly triterpenoids, while in Cameroon samples, polyprenylated flavonoids, prenylated stilvenoids, alk(en)phenols and alk(en)-yl resorcinols were also detected [4a-e,4i].

The obtained results in this study demonstrate that Congo and Cameroon samples are propolis types rich in triterpenes and especially in derivatives of amyrin and lupeol, which is in agreement with previously published studies of African propolis [4a,4f,4i] and in diprenyl-flavonoids, which is in accordance with recent literature, where such flavonoids have been detected by means of dereplication (LC-HRMS) [4e].

Investigation of each isolated compound showed that the most melliferous plants for Congo propolis (Sample 1) belong to Leguminoseae, Fabaceae and Asteraceae families, while the most abundant plants for Cameroon propolis belong to several families, such as Asteraceae, Burceraceaea, Mimosaceae, and Moraceae.

The chemical composition of propolis depends on the availability of resinous plant materials in different geographical regions [5a,5b]. Based on the above, African propolis presents special interest for further investigation of the chemistry in order to find out the chemical variability of these propolis types, as well as its plant source.

Experimental

***General*: S**ilica 60H Merck (70-230 mesh) for vacuum liquid chromatography (VLC) and silica gel 60 Merck (230-400 mesh) for column chromatography (CC). Preparative TLC was performed on glass plates (20 cm x 20 cm silica gel F_{254} Merck). Analytical thin layer chromatography (TLC) was performed on aluminum sheets coated with silica gel 60 F_{254} Merck 0.25μm and compounds were visualized under UV light (254 nm and 366 nm) and by spraying with vanillin in sulfuric acid, followed by heating at 100°C. The total phenolic content (TPC) was measured in a UV-VIS Spectrometer (UV-1700, Shimadzu). NMR experiments were performed on Brucker DRX 400 and Brucker AC200 spectrometers [^{1}H, COSY, HMQC, HMBC (400 MHz) and ^{13}C (200MHz)]. Chemical shifts were expressed in ppm downfield from TMS. All experiments were performed by using standard Bruker microprograms.

Crude material: The propolis samples were obtained from 2 different locations in Africa (Democratic Republic of Congo and Cameroon). Sample 1: from Congo (Mampu, Plateau Bateke), Sample 2: from Oku (NW Cameroon), Sample 3: from Ngaoundal (Adamaoua region, NW Cameroon) and Sample 4: from Tekel (Adamaoua region, NE Cameroon). Crude propolis samples were frozen (−20°C) and ground in a chilled grinder.

***Extraction and isolation*:** Each propolis sample was extracted 3 times with 70% ethanol (1:10, w/v) at room temperature for 24 h. The combined aqueous ethanol solutions were concentrated under reduced pressure.

Sample (1): The ethanolic extract (1.13 g) was submitted to vacuum liquid chromatography (VLC) (gradient elution with cyclohexane/CH_2Cl_2 100:0 to 70:30 and CH_2Cl_2/MeOH gradient 100:0 to 99:1). From the chromatographic analysis, 5 known compounds were isolated, three triterpenoids, lupenone (**1**) (6.0 mg), β-amyrin (**2**), and lupeol (**3**), and two diprenyl-flavonoids, lonchocarpol A (**4**) (15.0 mg) and 6,8-diprenyl-eriodictyol (**5**) (20.0 mg). Compounds 2 and 3 were isolated as a mixture (1.2 mg).

Sample (2): The ethanolic extract (1.40 g) was submitted to chromatographic separation (VLC) (gradient elution with cyclohexane/CH_2Cl_2 100:0 to 60:40) and nine known triterpenoids were isolated: lupenone **(1)**, β-amyrin (**2**) **(**14.0 mg**),** ψ-taraxasterol-

acetate (**6**), taraxasterol acetate (**7**), 3-*O*-acetyl-lupeol (**8**) (1.7 mg), lanosterol (**9**) (3.9 mg), 3α-hydroxy-olean-12-en-30-ol (**10**) (1.0 mg), α-amyrone (**11**) and β-acetoxy-amyrin (**15**). Compounds **1** and 11 were isolated as a mixture (8 mg), as well as compounds 6 and 7 (4.7 mg).

Sample (3): The ethanolic extract (1.19 g) was submitted to CC (gradient elution with cyclohexane/CH_2Cl_2 100:0 to 0:100 and with CH_2Cl_2/MeOH 100:0 to 90:10). From the chromatographic analysis 5 compounds were isolated, three known triterpenoids: 3α-hydroxy-olean-12-en-30-ol (10) (1.2 mg), α-amyrone (11) (5.0 mg), α-amyrin (12) (5.0 mg) and two monoterpenic alcohols: α-terpineol (13) (3.0 mg) and 1,8-terpineol (14) (5.0 mg).

Sample (4): The crude extract (1.19 g) was submitted to CC (gradient elution with cyclohexane/CH_2Cl_2 100:0 to 0:100 and with CH_2Cl_2/MeOH 100:0 to 70:30). From the chromatographic analysis 11 compounds were isolated, of which 7 were triterpenoids: β-amyrin **(2)**, α-amyrone (**11**) (10.3 mg), α-amyrin (**12**) β-acetoxy-amyrin (**15**) (2.1 mg), baccharen12,21-dien-3β-ol (**16**), betulin-aldehyde (**17**), erythrodiol (**18**) (2.5 mg), three diprenyl-flavonoids: lonchocarpol A (**4**) (1.4 mg), 6,8-diprenyl-aromadendrin **(19)** (3.3 mg), lespedezaflavanone C **(20)** (3.3 mg) and one a fatty acid ester: ethyl-palmitate (**21**). Compounds **2** and **12** were isolated as a mixture (13.2 mg), as well as compounds **16** and **17** (5.0 mg) and compounds **15** and **21** (2.1 mg).

Determination of total phenol content (TPC): The concentration of TPC in the ethanolic extracts was estimated with Folin-Ciocalteu reagent [23]. Caffeic acid served as a standard for preparing the calibration curve ranging from 0-10 μg/mL assay solution. The final concentration of the tested solutions in the assay was 40 and 400 μg/mL. The TPC was expressed as caffeic acid equivalents in mg/g in each extract. The concentration of polyphenols in samples was derived from the standard curve of caffeic acid and is shown in Figure 1.

Antimicrobial bioassay: Antimicrobial activity was evaluated using the standard antibiotics netilmicin, amoxicillin, and clavulanic acid in order to control the sensitivity of the tested bacteria and 5-fluocytocine and amphotericin of the tested fungi respectively. The ethanolic extracts were dissolved in MeOH. The experiments were repeated 3 times and the results were expressed as average values. The MIC values were determined using the dilution method [1b] using 96-well plates, against two Gram-positive bacteria: *Staphylococcus aureus* (ATCC 25923) and *S. epidermis* (ATCC 13047), four Gram-negative bacteria: *Escherichia coli* (ATCC 25922), *Klebsiella pneumonia* (ATCC 13883) and *Pseudomonas aeruginosa* (ATCC 227853), as well as against three pathogenic fungi: *Candida albicans* (ATCC 10231), *C. tropicalis* (ATCC 13861) and *C. glabrata* (ATCC 28838).

Table 1: Antimicrobial activity of propolis extracts MIC (mg/mL).

Samples	*S. aureus*	*S. epidermidis*	*P. aeruginosa*	*E. cloacae*	*K. pneumoniae*	*E. coli*	*C. albicans*	*C. tropicalis*	*C. glabrata*
Sample 1	10.52	10.85	>50	21.70	21.60	>50	21.00	19.84	17.87
Sample 2	20.30	20.00	>50	12.40	20.18	>50	22.50	20.80	19.75
Sample 3	18.50	17.10	>50	12.87	21.30	>50	21.25	20.67	20.00
Sample 4	13.80	13.55	>50	19.70	22.44	>50	20.25	18.75	16.64
5-fluocytocine							$0{,}1 \cdot 10^{-3}$	$1 \cdot 10^{-3}$	$10 \cdot 10^{-3}$
amphotericin							$1 \cdot 10^{-3}$	$0{,}5 \cdot 10^{-3}$	$0{,}4 \cdot 10^{-3}$
amoxicillin	$2 \cdot 10^{-3}$	$2 \cdot 10^{-3}$	$2.4 \cdot 10^{-3}$	$2.2 \cdot 10^{-3}$	$2.8\ 2 \cdot 10^{-3}$	$2 \cdot 10^{-3}$			
netimilcin	$4 \cdot 10^{-3}$	$4 \cdot 10^{-3}$	$8.8 \cdot 10^{-3}$	$8 \cdot 10^{-3}$	$8 \cdot 10^{-3}$	$10 \cdot 10^{-3}$			
clavulanic acid	$2 \cdot 10^{-3}$	$2 \cdot 10^{-3}$	$2.4 \cdot 10^{-3}$	$2{,}2 \cdot 10^{-3}$	$2.8 \cdot 10^{-3}$	$2 \cdot 10^{-3}$			

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