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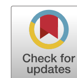
A new isoflavonol and other constituents from Cameroonian propolis and evaluation of their anti-inflammatory, antifungal and antioxidant potential



Alfred Ngenge Tamfu [a,e,*], Mathieu Sawalda [b], Maurice Tagatsing Fotsing [a], Rufin Marie Toghueo Kouipou [c], Emmanuel Talla [b,*], Godloves Fru Chi [a], Justin Jacquin Epah Epanda [b], Joseph Tanyi Mbafor [a], Tariq Ahmad Baig [d], Almas Jabeen [d], Farzana Shaheen [e,*]

[a] Department of Organic Chemistry, Faculty of Sciences, University of Yaoundé 1, Cameroon
[b] Department of Materials Engineering, School of Chemical Engineering and Mineral Industries/Faculty of Science, University of Ngaoundéré, Cameroon
[c] Department of Biochemistry, Faculty of Sciences, University of Yaoundé 1, Cameroon
[d] Dr. Panjwani Center for Molecular Medicinal & Drug Research (PCMD), International Center for Chemical and Biological Sciences (ICCBS), University of Karachi, Karachi 75270, Pakistan
[e] H.E.J. Research Institute of Chemistry, International Center for Chemical and Biological Sciences, University of Karachi, Karachi 75270, Pakistan

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ABSTRACT

Propolis is rich in diverse bioactive compounds. Propolis samples were collected from three localities of Cameroon and used in the study. Column chromatography separation of propolis MeOH:DCM (50:50) extracts yielded a new isoflavonol, 2-hydroxy-8-prenylbiochanin A (1) alongside 2′,3′-dihydroxypropylte traeicosanoate (2) and triacontyl *p*-coumarate (3) isolated from propolis for first time together with seven compounds: β-amyrine (4), oleanolic acid (5), β-amyrine acetate (6), lupeol (7), betulinic acid (8), lupeol acetate (9) and lupenone (10). These compounds were tested for their inhibitory effect on oxidative burst where intracellular reactive oxygen species (ROS) were produced from zymosan stimulated human whole blood phagocytes and on production of nitric oxide (NO) from lipopolysaccharide (LPS) stimulated J774.2 mouse macrophages. The cytotoxicity of these compounds was evaluated on NIH-3 T3 normal mouse fibroblast cells, antiradical potential on 2,2-diphenyl-1-picrylhydrazylhydrazyl (DPPH·) as well as their anti-yeast potential on four selected candida species. Compound 1 showed higher NO inhibition (IC_{50} = 23.3 ± 0.3 μg/mL) than standard compound L-NMMA (IC_{50} = 24.2 ± 0.8 μg/mL). Higher ROS inhibition was shown by compounds 6 (IC_{50} = 4.3 ± 0.3 μg/mL) and 9 (IC_{50} = 1.1 ± 0.1 μg/mL) than Ibuprofen (IC_{50} = 11.2 ± 1.9 μg/mL). Furthermore, compound **1** displayed moderate level of cytotoxicity on NIH-3 T3 cells, with IC_{50} = 5.8 ± 0.3 μg/mL compared to the cyclohexamide IC_{50} = 0.13 ± 0.02 μg/mL. Compound 3 showed lower antifungal activity on *Candida krusei* and *Candida glabrata*, MIC of 125 μg/mL on each strain compared to 50 μg/mL for fuconazole. The extracts showed low antifungal activities ranging from 250 to 500 μg/mL on *C. albicans, C. krusei* and *C. glabrata* and the values of MIC on *Candida parapsilosis* were 500 μg/mL and above. DPPH* scavenging activity was exhibited by compounds 1 (IC_{50} = 15.653 ± 0.33 5 μg/mL) and 3 (IC_{50} = 89.077 ± 24.875 μg/mL) compared to Vitamin C (IC_{50} = 3.343 ± 0.271 μg/mL) while

Abbreviations: DCM, dichloromethane; EIMS, electronic impact mass spectrometry; HREIMS, high resolution electronic impact mass spectrometry; UV, Ultraviolet; IR, infrared; m.p, melting point; TLC, Thin layer chromatography; NMR, Nuclear magnetic resonance; NO, nitric oxide; ROS, reaction oxygen species; MTT, 3-(4,5-Dimethylth iazol-2-yl)-2,5-diphenyltetrazolium bromide; MIC, Minimal inhibitory concentration; DPPH, 2,2-diphenyl-1-picrylhydrazylhydrazyl.

* Corresponding authors.
E-mail addresses: macntamfu@yahoo.co.uk (A.N. Tamfu), tallae2000@yahoo.fr (E. Talla), afnan.iccs@gmail.com (F. Shaheen).

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extracts showed moderate antiradical activities with IC_{50} values ranging from 309.31 ± 2.465 to 635.52 ± 11.05 μg/mL. These results indicate that compounds 1, 6 and 9 are potent anti-inflammatory drug candidates while 1 and 3 could be potent antioxidant drugs.

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1. Introduction

Bee products have a long-standing history of their uses by human beings throughout many civilizations as food and medicines. Besides honey which is well known by many civilizations, recent works have shown interesting biological and chemical information of other bee products such as bee venom, royal jelly and propolis which are renewable sources of therapeutic products applied in their obtained forms or as synthesized analogues (Abd El-Wahed et al., 2018; Milena et al., 2019; Tamfu et al., 2019b; Blicharska and Seidel, 2019; Zhang et al., 2019). Propolis (bee glue) is a sticky dark-colored material that honeybees (*Apis mellifera*) gather from buds and exudates of plants, combined with other bee secretions and use it to seal the walls in the hive, strengthen the borders of combs, and as a protective wall against invaders (Burdock, 1998; Bankova, 2005; Graikou et al., 2016). Propolis has been shown to possess antibacterial, antifungal, antiviral, antioxidant, anti-inflammatory, antiulcer, hepatoprotective, cytotoxic, immune-stimulating, antiprotozoal, anti-trypanosomal and so many other beneficial therapeutic effects (Bankova et al., 2000; Sforcin and Bankova, 2011; Tamfu et al., 2016). Chemical constituents and biological activities of propolis differ according to the plant sources and their geographic location and time of collection and bee species (Sforcin et al., 2005; Araujo et al., 2015). Propolis contains mainly plant resins (50%), wax (30%), oils (10%), pollen (5%), and other natural products (5%) (Marcucci, 1995). The natural product constituents of propolis include hydrocarbons, alcohols, aliphatic and aromatic acids and esters, flavonoids, terpenoids, steroids, amino acids, sugars, lignans, vitamins, phenolic compounds, pterocarpans, hydrocarbons and mineral elements (Bankova et al., 2000; Cuesta-Rubio et al., 2007). It is clear that comparing propolis samples from different regions might be the same as comparing extracts of two plants that belong to different plant families (Bankova, 2005). In order to get real application of propolis in the healthcare system, it needs standardization with reference to its chemical constituents to guarantee its efficacy, quality, and safety. Propolis of areas that have not yet been studied seems to be a promising source of bioactive molecules (Petrova et al., 2010). In Cameroon, propolis has ethnopharmacological significance in that it is used to treat tooth ache, stomach disorders, gastritis and sore throat by chewing directly while its aqueous extract is used in treating wounds, skin rashes, boils and burns (Tamfu et al., 2016).

In this study, the main objective is to isolate and characterize chemical compounds from selected Cameroonian propolis samples and evaluate their anti-inflammatory, antifungal and antioxidant potential.

2. Materials and methods

2.1. General experimental procedures

Melting points (m.p.) were recorded on a Buchi M−560 melting point apparatus equipped with a Buchi M−569 sample loader. UV spectra were recorded on a Hitachi U-3200 Spectrophotometer while IR spectra were recorded in KBr with a Shimadzu 8900 FT-IR spectrophotometer. ^{1}H NMR (500 MHz) and ^{13}C NMR (125 MHz) spectra were recorded in deuterated solvents such as $CDCl_3$ and C_5D_5N on a Bruker Avance-500. Coupling constants, J values, are given in Hertz. DEPT 135°, DEPT 90°, HSQC and HMBC NMR experiments were carried out using the conventional pulse sequences. EIMS and HREIMS were obtained with a JEOL MSRoute-600H and Thermo Finnigan MAT 95XP mass spectrometers respectively. Optical rotations were measured on a JASCO Polarimeter P-2000. Thin-layer chromatography (TLC) was performed on TLC cards pre-coated with silica gel (Merck, PF254, 20x20 cm, 0.25 mm) revealed under UV lamp UVITEC 07-22243 at 254 nm and 365 nm and also with ceric(IV)sulphate solution spray or H_2SO_4 10% spray reagent. Column chromatography was carried out on silica gel (70–230 mesh, Merck). Optical densities were measured on a Thermo Scientific Multiskan FC, Vantaa, Finland.

2.2. Propolis collection, extraction and isolation

The propolis samples were collected from three different localities in Cameroon during the month of April 2017. They were collected from the locality of Nohmedjoh, Haut-Nyong division, East Region (PR-1), from Nkambe, Donga-Mantung division of North-West Region (PR-2) and from Mbouda, Bamboutous division, West Region (PR-3).

PR-1 (250 g) was extracted by maceration in 5 L of MeOH-CH_2Cl_2 50:50 for 48 h followed by filtration and evaporation on a Rota-vapor to give a reddish-brown viscous extract. This procedure was repeated 3 times to yield 205 g of crude extract (PR-1). The crude extract (77 g) was subjected to column chromatography separation on silica gel using gradient system of increasing polarity as follows; hexane–ethyl acetate (0–100%) then ethyl acetate-MeOH (0–100%) with a gradual increase in polarity while collecting fixed volumes of 150 mL. The fractions collected where evaporated using a Rotavapor under reduced pressure and the fractions were monitored by TLC. In total 558 fractions were collected, which were further purified recrystallization and washing with a suitable solvent from which fractions 106, 132 and 193 afforded the compounds 3 (45 mg), 1 (78 mg) and 2 (13 mg) respectively.

PR-2 (200 g) was extracted with 5 L of MeOH-CH_2Cl_2 50:50 for 48 h followed by filtration and evaporation on a Rota-vapor to give a sticky brown extract. This procedure was repeated 3 times to yield 75 g of crude extract (PR-2). 25 g of this extract was subjected to column chromatography using the same procedure as above to give 301 fractions. These fractions were regrouped based on their TLC profiles into 10 pooled fractions denoted A-J. Fractions E (300 mg) was further rechromatographed on silica gel column using a gradient system of Hexane-DCM (25–50%) to give compounds 9 (240 mg) and 10 (15 mg). The fraction G (2.4 g) was rechromatographed on silica gel column using the isocratic eluent Hexane-Ethyl acetate (50:50) to afford compounds 5 (12 mg) and 8 (7.5 mg). Fraction H (5 g) was also purified by column chromatography using hexane–ethyl acetate (50–60%) and yielded the compounds 4 (62 mg) and 7 (84 mg).

PR-3 (575 g) of this sample was extracted using 5 L of MeOH-CH_2Cl_2 50:50 as described above to give 400 g of a sticky brown crude extract (PR-3). 50 g of this extract was subjected to column chromatography separation on silica gel using gradient system of

increasing polarity as follows; hexane–ethyl acetate (0–100%) then ethyl acetate-MeOH (0–100%) with a constant and gradual increase in polarity by 2.5 while collecting fixed volumes of 200 mL. The fractions collected where evaporated using a rotavapor under reduced pressure and the fractions were monitored by TLC. In total 510 fractions were collected. The fraction 87 afforded compound 6 (33.8 mg) by filtration and washing with hexane.

2.3. Oxidative burst assay

Luminol-enhanced chemiluminescence assay was performed on diluted human whole blood with three different concentrations of compounds (1, 10 and 100 μg/mL) as previously described (Erharuyi et al., 2017).

2.4. Nitric oxide assay

The nitric oxide assay was performed on mouse macrophage cell line J774.2 (European Collection of Cell Cultures, UK) using three different concentrations of compounds (1, 10 and 100 μg/mL) and with method described previously (Shah et al., 2015).

2.5. MTT cytotoxicity assay

Cytotoxicity of compounds on NIH-3 T3 fibroblast cells (ATCC, Manassas, USA) was evaluated by using the standard MTT colorimetric assay. Using three different concentrations of test compound (1, 10 and 100 μg/mL) as described previously (Shah et al., 2015).

2.6. Anti-yeast activity

The microorganisms included *Candida albicans* NR-29450, *Candida krusei* ATCC 6258, and *Candida parapsilosis* ATCC 22019, reference strains from the American Type Culture Collection and *Candida glabrata* isolate obtained from the Yaoundé Central Hospital, Cameroon. The microorganisms were maintained on agar slope at 4 °C and sub-cultured for 48 h before each experiment. The minimum inhibitory concentration (MIC) of extracts against yeasts was determined according to the Clinical Laboratory Standards Institute M27-A3 microdilution method (CLSI, 2008a) using 96-wells microtitre plates. 100 μL of two-fold diluted extracts and reference drugs in RPMI 1640 (Sigma Aldrich) were added to the wells, followed by addition of 100 μL of yeasts inoculum standardized at 2.50 × 103 cells/mL. A blank column was included for sterility control. The concentrations of extracts ranged from 0.976, 1.95, 3.90, 7.81, 15.6, 31.2, 62.5, 125, 250, and 500 μg/mL and that of fluconazole ranged from 1.25 μg/mL to 128 μg/mL. After 48 h of incubation at 37 °C, the turbidity was observed as an indication of growth. MIC was defined as the lowest concentration inhibiting the visible growth of yeast cells. All extracts were tested in triplicate.

2.7. DPPH radical scavenging activity

The DPPH was prepared in methanol at a concentration of 0.02%. For this, 20 mg of DPPH was completely dissolved in 1 L of 100% methanol. The solution was conserved in a closed bottle away from light and any heat source before usage. Initially, the compounds were diluted to final concentrations of 2000, 1000, 500, 250, 125, 62.5, 31.25, and 15.625 μg/mL in a 96 well microplate. Twenty-five microliters (25 μL) from each dilution was transferred into a new micro-plate and 75 μL of 0.02% DPPH in methanol added to obtain final concentrations of 500, 250, 125, 62.5, 31.25, 15.625, 7.8125 and 3.90625 μg/mL. The reaction mixtures were kept in the dark at room temperature for 30 mins after which the absorbances were measured at 517 nm against the blank. The positive control was made of ascorbic acid treated in the same way as the extracts with final concentrations of 25, 12.5, 6.25, 3.125, 1.5625, 0.78125, 0.390625 and 0.1953125 μg/mL. The assays were performed in triplicate. The percentage (%) radical scavenging activities of the plant extracts were calculated using the following formula below.

$$\%\text{Radical Scavenging Activity} = (Ao - As)/Ao \times 100$$

where Ao: Absorbance of the blank (DPPH + methanol); As: Absorbance of DPPH radical + compound.

From the % Radical Scavenging Activity of each compound, the IC_{50} was deduced and the antiradical power is inversely proportional to IC_{50}.

2.8. Characterization of isolated compounds

1. **2-Hydroxy-8-prenylbiochanin A:** Yellow solid; m.p. 244.0–245.7 °C; IR (KBr), υ_{max} 3320 cm^{-1}, 1627 cm^{-1}, 1603–1512 cm-1 and 1423 cm^{-1}; UV (MeOH) λ_{max} 229, 272, 323 and 371 nm; ^1H and ^{13}C NMR data see Table 1, HREIMS *m/z* 368.1260 (M^+) (calcd. for $C_{21}H_{20}O_6$, 368.1269).
2. **2′,3′-Dihydroxypropyltetraeicosanoate: m.p.** 69.5–70.9 °C. **^{13}C NMR ($CDCl_3$, 125 MHz):** 174.3 (C-1), 34.2 (C-2), 31.9 (C-3), 29.1–29.7 (C-4 to C-21), 24.9 (C-22), 22.7 (C-23), 14.1 (C-24), 65.5 (C-1′), 70.3 (C-2′), 63.34 (C-3′). **^1H NMR ($CDCl_3$, 500 MHz):** 2.32; 2.35 (H-2, t, *J* = 14 Hz), 1.62 (H-3, m), 1.23 – 1.31 (H-4 to H-21, m), 1.31 (H-22, m), 1.26 (H-23, m), 0.86 (H-24, t, *J* = 14 Hz), 4.21; 4.18 (H-1′, dd, *J* = 10 Hz, *J* = 3.5 Hz), 3.92 (H-2′, m), 3.58; 3.68 (H-3′, m), 2.43 (2′-OH), 1.97 (3′-OH).
3. **Triacontyl *p*-coumarate**: m.p. 94–95 °C. **^{13}C NMR ($CDCl_3$, 125 MHz):** 127.8 (C-1), 129.3 (C-2), 115.8 (C-3), 157.7 (C-4), 129.3 (C-5), 115.8 (C-6), 144.8 (C-7), 115.9 (C-8), 166.7 (C-9), 64.8 (C-10), 29.1 (C-11), 25.9 (C-12), 29.4 – 29.3 (C-13 to C-36), 31.9 (C-37), 22.6 (C-38), 14.1 (C-39), **^1H NMR ($CDCl_3$, 500 MHz)**: δ 7.40 (H-2), 6.80 (H-3) 6.80 (H-5), 7.41 (H-6), 7.60 (H-7), 6.30 (H-8), 4.17 (H-10), 1.53 (H-11), 1.40 (H-12), 1.37 – 1.36 (H-13 to H-36), 1.65 (H-37), 1.27 (H-38), 0.89 (H-39), 5.04 (free OH-4).
4. **β-amyrine:** m.p. 189–191 °C. **^{13}C NMR ($CDCl_3$, 125 MHz):** 38.7 (C-1), 23.6 (C-2), 79.1 (C-3), 37.2 (C-4), 55.3 (C-5),

Table 1
^1H and ^{13}C NMR data and HMBC correlations 2 of compounds 1 in $CDCl_3$.

Position	^{13}C NMR	^1H NMR	HMBC
2	135.4	–	
3	145.4	–	
4	175.5	–	
5	158.9	–	
6	99.1	6.29, s	C-6, C-5, C-7, C-8
7	104.9	–	
8	160.8	–	
9	153.8	–	
10	103.9	–	
1′	161.1	–	
2′	129.3	8.15, d, *J* = 9.0 Hz	C-1′, C-2′, C-3
3′	114.1	7.03, d, *J* = 7.0 Hz	C-3′, C-4′
4′	123.5	–	
5′	114.1	7.03, d, *J* = 7.0 Hz	C-5′, C-4′
6′	129.3	8.15, d, *J* = 9.0 Hz	C-6′, C-1′, C-3
1′′	21.9	3.60, *J* = 7.0 Hz	C-7, C-8, C-2′′
2′′	121.1	5.30, t, *J* = 13.5 Hz	C-4′′, C-1′′
3′′	135.6	–	
4′′	25.8	1.76, s	C-3′′, C-5′′, C-2′′
5′′	18.1	1.85, s	C-3′′, C-4′′, C-2′′
4′-OCH_3	55.4	3.88, s	–
2-OH	–	6.56, s	C-2, C-3, C-4
5-OH	–	11.73, s	C-5, C-6, C-10
7-OH	–	5.97, s	C-6, C-7, C-8

18.0 (C-6), 32.8 (C-7),41.5 (C-8), 47.6 (C-9), 36.8 (C-10), 28.1 (C-11), 121.8 (C-12), 145.3 (C-13), 42.1 (C-14), 26.6 (C-15), 31.1 (C-16), 40.8 (C-17), 50.5 (C-18), 28.1 (C-19), 33.7 (C-20), 39.6 (C-21), 39.7 (C-22), 28.1 (C-23), 16.7 (C-24), 15.6 (C-25), 16.8 (C-26), 23.2 (C-27), 17.5 (C-28), 18.7 (C-29), 21.3 (C-30). **^{1}H NMR ($CDCl_3$, 500 MHz)**: δ 1.91 (H-l), 1.85 (H-2), 3.24 (H-3), 0.88 (H-5), 1.54 (H-6), 1.57 (H-7), 1.67 (H-9), 1.94 (H-11), 5.18 (H-12), 2.17 (H-15), 1.94 (H-16), 1.94 (H-18), 1.38 (H-19), 1.44 (H-21), 2.06 (H-22), 0.80 (H-23), 0.91 (H-24), 0.77 (H-25), 0.94 (H-26), 1.15 (H-27), 0.81 (H-28), 1.08 (H-29), 0.84 (H-30).

5. **Oleanolic acid:** m.p. 304–305.5 °C. **^{13}C NMR ($CDCl_3$, 125 MHz):** 39.0 (C-1), 28.1 (C-2), 78.2 (C-3), 39.4 (C-4), 55.9 (C-5), 18.8 (C-6), 33.4 (C-7), 39.8 (C-8), 48.2 (C-9), 37.4 (C-10), 23.8 (C-11), 122.0 (C-12), 144.0 (C-13), 42.2 (C-14), 28.4 (C-15), 23.8 (C-16), 46.7 (C-17), 42.1 (C-18), 46.6 (C-19), 31.0 (C-20), 34.3 (C-21), 33.2 (C-22), 28.8 (C-23), 16.5 (C-24), 15.6 (C-25), 17.5 (C-26), 26.2 (C-27), 180.0 (C-28), 33.4 (C-29), 28.8 (C-30). **^{1}H NMR ($CDCl_3$, 500 MHz):** δ 1.57 (H-l), 1.93 (H-2), 3.15 (H-3), 0.88 (H-5), 1.54 (H-6), 1.37 (H-7), 1.67 (H-9), 1.96 (H-11), 5.22 (H-12), 2.18, 1.75 (H-15), 1.94 (H-16), 1.53 (H-18), 1.40 (H-19), 1.44 (H-21), 2.06 (H-22), 1.33 (H-23), 0.97 (H-24), 0.77 (H-25), 0.96 (H-26), 1.11 (H-27), 0.84 (H-29), 0.95 (H-30).
6. **β-amyrine acetate:** m.p. 225–227 °C. **^{13}C NMR ($CDCl_3$, 125 MHz):** 38.8 (C-1), 27.4 (C-2), 81.2 (C-3), 38.2 (C-4), 55.4 (C-5), 18.5 (C-6), 32.8 (C-7), 40.5 (C-8), 47.4 (C-9), 37.2 (C-10), 24.1 (C-11), 121.9 (C-12), 145.4 (C-13), 41.9 (C-14), 26.4 (C-15), 27.1 (C-16), 32.8 (C-17), 47.8 (C-18), 47.1 (C-19), 31.3 (C-20), 37.4 (C-21), 34.9 (C-22), 15.7 (C-23), 28.3 (C-24), 15.8 (C-25), 17.0 (C-26), 26.2 (C-27), 28.6 (C-28), 33.6 (C-29), 23.9 (C-30). **^{1}H NMR ($CDCl_3$, 500 MHz)**: δ 1.55, 1.49 (H-l), 1.52 (H-2), 4.48 (H-3), 0.71 (H-5), 1.53 (H-6), 1.95 (H-7), 1.95 (H-9), 1.84 (H-11), 5.16 (H-12), 2.17 (H-15), 1.94 (H-16), 1.89 (H-18), 1.58 (H-19), 1.66 (H-21), 2.06 (H-22), 0.77 (H-23), 0.98 (H-24), 0.92 (H-25), 0.94 (H-26), 1.11 (H-27), 0.81 (H-28), 0.85 (H-29), 0.84 (H-30).
7. **Lupeol**: m.p. 170–172 °C. **^{13}C NMR ($CDCl_3$, 125 MHz):** 38.8 (C-1), 27.5 (C-2), 79.0 (C-3), 39.9 (C-4), 55.3 (C-5), 19.3 (C-6), 34.2 (C-7), 41.1 (C-8), 50.5 (C-9), 37.2 (C-10), 21.2 (C-11), 25.3 (C-12), 38.5 (C-13), 42.8 (C-14), 27.2 (C-15), 35.6 (C-16), 43.0 (C-17), 48.3 (C-18), 47.8 (C-19), 150.9 (C-20), 30.1 (C-21), 40.3 (C-22), 28.4 (C-23), 16.1 (C-24), 16.0 (C-25), 15.6 (C-26), 14.5 (C-27), 18.1 (C-28), 109.3 (C-29), 20.2 (C-30). **^{1}H NMR ($CDCl_3$, 500 MHz):** δ 1.98 (H-l), 2.20 (H-2), 3.19 (H-3), 0.69 (H-5), 1.45 (H-6), 1.40 (H-7), 1.17 (H-9), 1.40 (H-11), 1.88 (H-12), 1.67 (H-13), 1.75 (H-15), 1.38 (H-16), 1.35 (H-18), 2.38 (H-19), 1.83 (H-21), 1.42 (H-22), 1.04 (H-23), 0.97 (H-24), 1.40 (H-25), 0.84 (H-26), 0.79 (H-27), 1.26 (H-28), 4.56, 4.65 (H-29), 1.69 (H-30).
8. **Betulinic acid:** m.p. 297–299 °C. **^{13}C NMR ($CDCl_3$, 125 MHz):** 38.4 (C-1), 25.4 (C-2), 76.3 (C-3), 37.5 (C-4), 50.3 (C-5), 18.2 (C-6), 34.2 (C-7), 40.9 (C-8), 49.3 (C-9), 37.3 (C-10), 22.1 (C-11), 25.5 (C-12), 38.4 (C-13), 42.5 (C-14), 32.2 (C-15), 33.3 (C-16), 56.4 (C-17), 46.9 (C-18), 49.1 (C-19), 150.4 (C-20), 29.7 (C-21), 37.1 (C-22), 28.2 (C-23), 15.9 (C-24), 16.0 (C-25), 18.2 (C-26), 14.8 (C-27), 180.6 (C-28), 109.9 (C-29), 19.4 (C-30). **^{1}H NMR ($CDCl_3$, 500 MHz):** δ 1.75 (H-l), 2.20 (H-2), 3.36 (H-3), 0.69 (H-5), 1.41 (H-6), 1.46 (H-7), 1.17 (H-9), 1.29 (H-11), 1.60 (H-12), 1.59 (H-13), 1.81 (H-15), 1.35, 1.45 (H-16), 1.38 (H-18), 2.20 (H-19), 1.83 (H-21), 1.42 (H-22), 1.01 (H-23), 0.79 (H-24), 0.81 (H-25), 1.03 (H-26), 0.91 (H-27), 4.72, 4.59 (H-29), 1.67 (H-30).
9. **Lupeol acetate**: m.p. 216 °C – 217 °C. **^{13}C NMR ($CDCl_3$, 125 MHz):** 38.4 (C-1), 23.7 (C-2), 80.9 (C-3), 37.8 (C-4), 55.4 (C-5), 18.2 (C-6), 34.2 (C-7), 40.8 (C-8), 50.5 (C-9),37.1 (C-10), 20.9 (C-11), 25.1 (C-12), 38.1 (C-13), 42.8 (C-14), 27.4 (C-15), 35.6 (C-16), 43.0 (C-17), 48.3 (C-18), 48.0 (C-19), 150.9 (C-20), 29.8 (C-21), 40.0 (C-22), 27.9 (C-23), 16.5 (C-24), 16.2 (C-25), 15.9 (C-26), 14.5 (C-27), 18.0 (C-28), 109.3 (C-29), 19.3 (C-30), 170.9 (C-1′), 21.3 (C-2′). **^{1}H NMR ($CDCl_3$, 500 MHz):** δ 1.89 (H-l), 2.30 (H-2), 4.46 (H-3), 0.69 (H-5), 1.38 (H-6), 1.41 (H-7), 1.17 (H-9), 1.35 (H-11), 1.65 (H-12), 1.67 (H-13), 1.82 (H-15), 1.36, 1.45 (H-16), 1.35 (H-18), 2.35 (H-19), 1.83 (H-21), 1.42 (H-22), 0.82 (H-23), 0.81 (H-24), 0.83 (H-25), 1.16 (H-26), 0.92 (H-27), 0.77 (H-28), 4.55, 4.85 (H-29), 1.66 (H-30), 2.03 (H-2′).
10. **Lupenone:** m.p. 170–172 °C. **^{13}C NMR ($CDCl_3$, 125 MHz):** 39.6 (C-1), 28.6 (C-2), 218.2 (C-3), 47.3 (C-4), 54.9 (C-5), 18.0 (C-6), 33.6 (C-7), 43.8 (C-8), 49.8 (C-9), 41.0 (C-10), 26.6 (C-11), 26.3 (C-12), 39.3 (C-13), 42.9 (C-14), 27.4 (C-15), 35.5 (C-16), 42.9 (C-17), 48.3 (C-18), 47.9 (C-19), 150.8 (C-20), 29.8 (C-21), 39.9 (C-22), 26.9 (C-23), 21.5 (C-24), 15.9 (C-25), 14.4 (C-26), 14.1 (C-27), 17.9 (C-28), 109.4 (C-29), 19.7 (C-30). **^{1}H NMR ($CDCl_3$, 500 MHz):** δ 1.75–1.98 (H-l), 2.20–2.40 (H-2), 0.69 (H-5), 1.45 (H-6), 1.40 (H-7), 1.17 (H-9), 1.35 (H-11), 1.07, 1.68 (H-12), 1.67 (H-13), 1.02, 1.75 (H-15), 1.38, 1.41 (H-16), 1.35 (H-18), 2.40 (H-19), 1.83 (H-21), 1.42 (H-22), 1.04 (H-23), 1.00 (H-24), 0.90 (H-25), 1.22 (H-26), 0.93 (H-27), 0.77 (H-28), 4.55, 4.85 (H-29), 1.66 (H-30).

3. Results and discussion

Chromatographic separation of studied propolis samples led to the isolation of 2-hydroxy-8-prenyl biochanin A (**1**), 2′,3′-Dihydroxypropyltetraeicosanoate (**2**) and E-triacontryl-3-(4-hydroxyphenyl) acrylate (**3**) isolated from propolis for the first time together with seven compounds: β-amyrine (**4**), oleanolic acid (**5**), β-amyrine acetate (**6**), lupeol (**7**), betulinic acid (**8**), lupeol acetate (**9**) and lupenone (**10**) whose structures are given in Fig. 1.

Compound **1** was obtained as a yellow amorphous powder and melted between 244 and 245.7 °C. The HREIMS revealed the molecular ion M^+ at *m/z* 368.1260 (calcd for $C_{21}H_{20}O_6$, 368.1269) corresponding to the molecular formula $C_{21}H_{20}O_6$. Its UV spectrum showed absorption maxima at 229, 272, 323 and 371 nm. The IR spectrum displayed the adsorption peaks of hydroxyl groups (3320 cm^{-1}), conjugated carbonyl (1627 cm^{-1}), aromatic ring (1603–1512 cm^{-1}) and alkenes (1423 cm^{-1}). Compound **1** showed the characteristic signals of isoflavones by comparison with spectroscopic data (UV, IR, NMR, EIMS) with those reported in the literature. Its structure was close to that of biochanin A (5,7-dihydroxy-4′-methoxyisoflavone) previously isolated from Cuban and Brazilian propolis (Awale et al., 2008) but with a difference in that the proton H-2 in the ^{1}H NMR spectrum of biochanin A which appears at around 8.30–8.36 ppm was absent, thus suggesting that it is substituted by an OH group. Equally, the singlet signal of the proton H-8 in biochanin A was absent and a prenyl group appeared and unambiguously ascribable to position 8 as evidenced by HMBC correlations.

The ^{1}H NMR spectrum displayed signals for three singlet methyls as follows: one aromatic methoxy group 3.88 ppm (3H, s, 4′-OCH_3), two methyl singlets attached to an olefinic carbon at 1.85 ppm (3H, s, H-5′′) and 1.76 ppm (3H, s, H-4′′). Free OH singlet signals were also observed notably the downfield chelated OH proton signal at 11.73 ppm (1H, 5-OH) together with the signals at 6.56 ppm (1H, 2-OH) and 5.97 ppm (1H, 7-OH). The free OH protons were confirmed by HSQC as they were not found to be attached to any carbon atom and their positions deduced from UV and also HMBC correlations. Aromatic proton signals appeared as singlet 6.29 ppm (1H, H-6) and the occurrence of an AA’BB’

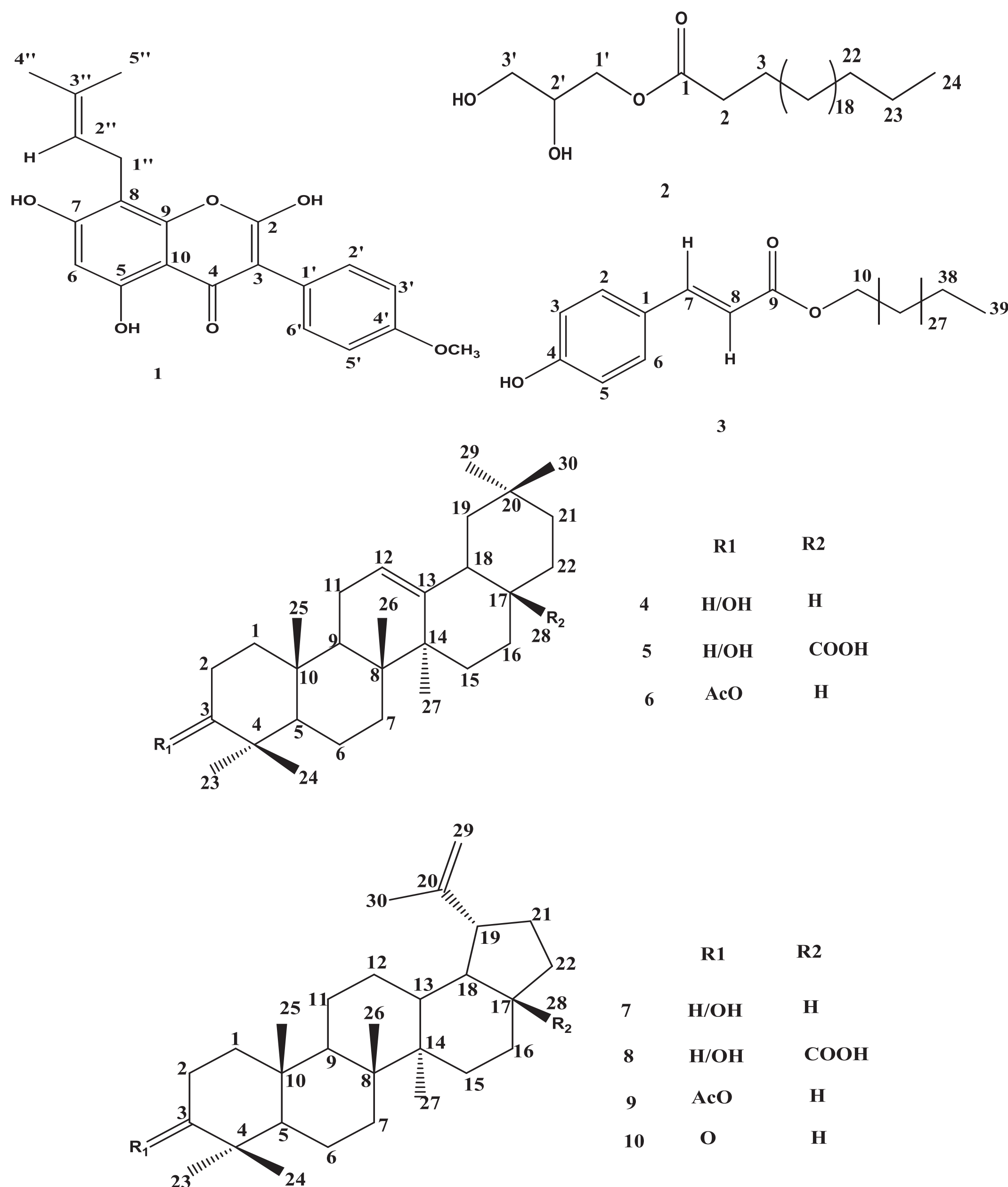

Fig. 1. Chemical compounds isolated from Cameroonian propolis.

system with signals at δ_H 7.03 (2H, d, H-3′ and 5′) and 8.15 (2H, d, H-2′ and H-6′) revealed a *para*-substituted B-ring system confirming the methoxy group at carbon 4′. The appearance of an olefinic proton at 5.30 ppm (1H, t, H-12) and an allylic methylene at 3.60 ppm (2H, d, H-11) confirmed the presence of a *C*-prenyl group suggested by the two olefinic methyl singlets mentioned above. The ^{13}C NMR broad band spectrum showed 20 carbon signals. Characteristic signals such as those at δ_C 135.6 (C-13), 121.1 (C-12), 25.8 (C-14), 21.9 (C-11) and 18.0 (C-15) ppm observed in its ^{13}C NMR spectrum confirmed the presence of one prenyl group. A methoxy carbon signal appeared at 55.4 ppm and a conjugated carbonyl signal appeared at 175.5 ppm, the low value being an indication of conjugation and substituent ring at position 3. The assignment of carbon signals was also facilitated with the use of DEPT 135 and DEPT 90 spectra. Aromatic carbon signals appeared between 99.1 and 161.1 ppm. HSQC ($^1J_{H\text{-}C}$) and HMBC ($^{1\text{-}4}J_{H\text{-}C}$) correlations between protons and carbon atoms as well as COSY cross peaks between adjacent protons allowed us to unambiguously establish their positions. The NMR data of this compound is given in table 1. The detail important HMBC and COSY correlations of compound 1 are shown on Fig. 2.

The method of extraction and isolation used in this work has an advantage that two phenolic compounds have been isolated from Cameroonian propolis, a first-time report and as new propolis constituents and one of them is a new compound. Together with the phenolic compounds are triterpenoids which are the traditional compounds previously reported from Cameroonian propolis till date especially as isolated pure compounds or by GC–MS analyses of extracts, fraction and essential oils (Carol et al., 2017; Kardar et al., 2014; Papachroni et al., 2015; Talla et al., 2017; Tamfu et al., 2019). The nature of compounds isolated have the significance in that most of them are triterpenoids as will be expected

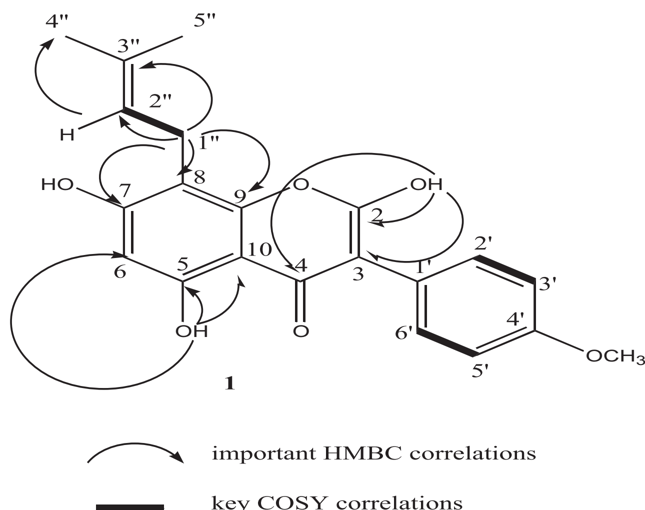

Fig. 2. Compound 1 showing some important HMBC and COSY correlations.

from tropical propolis in general and African propolis in particular (Tamfu et al., 2019b; Blicharska and Seidel, 2019).

Compounds **1**, **2**, **3**, **6** and **9** and the extracts PR-1, PR-2 and PR-3 were tested for their anti-inflammatory potential and the results shown in table 2. These compounds were tested for their inhibitory effect on oxidative burst where intracellular reactive oxygen species (ROS) were produced from zymosan stimulated human whole blood phagocytes and on production of nitric oxide (NO) from lipopolysaccharide (LPS) stimulated J774.2 mouse macrophages. The cytotoxicity of compounds was evaluated on NIH-3 T3 normal mouse fibroblast cells. The compound **6** and **9** were found to be the potent inhibitor of ROS with an IC_{50} values (4.3 ± 0.3 and 1.1 ± 0.1 µg/mL) respectively compared to the Ibuprofen (11.2 ± 1.9 µg/mL) as a standard anti-inflammatory drug as shown in table 2. The compound **1**, **2** and **3** as well as the extracts did not show activity within the tested concentrations. The compound **9** was found to be the most potent inhibitor of ROS. The results reported herein for ROS inhibition assays ascertains the high anti-inflammatory activity of these two compounds as they both showed moderate activity on NO assay.

The new compound **1** was found to be a potent inhibitor of nitric oxide (IC_{50} = 23.3 ± 0.3 µg/mL). This compound is a better NO inhibitor than the standard compound L-NMMA (IC_{50} = 24.2 ± 0.8 µg/mL) used in the study as shown in table 2. These results indicating good activity of isoflavone on nitric oxide inhibition is in conformity with some reported findings in which flavones are shown to exhibit antioxidant effects by increasing the pro-oxidant enzymes superoxide dismutase (SOD), glutathione peroxidase (GPx), and catalase (CAT) activities and decreasing lipid peroxidation and could be potent drug candidates in treatment of liver injury (El-Aarag et al., 2019). Compound **6** and **9** showed moderate level of inhibition whereas compound **2** and **3** were found to be inactive. The extracts and compounds **2**, **3**, **6** and **9** were found to be non-toxic on NIH-3 T3 cells, whereas compound **1** showed moderate level of toxicity (IC_{50} = 5.8 ± 0.3 µg/mL) compared to the cyclohexamide (IC_{50} = 0.13 ± 0.02 µg/mL) as control cytotoxic compound. Some studies have showed that antioxidant and anti-inflammatory activity of Brazilian green propolis in stimulated J774A.1 macrophages occurs though the inhibition of ROS, NO and pro-inflammatory cytokines, including TNF-α, IL-1β and IL-6 (Ewelina et al., 2013). Cameroonian propolis has been shown to possess anti-inflammatory activity and attempts have made to decipher the mechanisms and pathways involved in its anti-inflammatory action and also metabolomic studies (Abdulmalik et al., 2019). It was observed that propolis extracts exhibits an anti-inflammatory activity by inhibiting pro-inflammatory cytokines and by metabolic reprogramming of lipopolysaccharide (LPS) activity in macrophage cells, suggesting an immunomodulatory effect (Abdulmalik et al., 2019).

Most of the compounds and the extracts showed low antifungal activities mostly 10 to 20 folds less active than fuconazole on the tested strains. Compound 1 showed lowest antifungal activity for all tested strains (MIC > 500 µg/mL) while compound 3 showed highest antifungal activity, 2.5 folds less than the standard drug fuconazole, on *C. krusei* and *C. glabrata* with MIC of 125 µg/mL on each of the strains as against 50 µg/mL for fuconazole (see Table 3). Other compounds 7, 8 and 9 showed moderate antifungal activities 5 folds less than fuconazole. The extracts showed appreciable antifungal activities ranging from 250 to 500 µg/mL on *C. albicans, C. krusei* and *C. glabrata* and the values of MIC on *C. parapsilosis* were 500 µg/mL and above.

The two phenolic compounds 1 and 3 displayed highest antiradical activity on DPPH with IC_{50} of 15.653 ± 0.335 µg/mL and 89.077 ± 24.875 respectively as against Vitamin C with IC_{50} value of 3.343 ± 0.271 µg/mL as shown in Table 4. These could be justified by the fact that, in these compounds the –OH group is bonded to the benzene ring with conjugated double bond, a probable pharmacophore for antiradical activities. Other compounds however had IC_{50} values above 1000 µg/mL except compound 2 whose IC_{50} value was found to be 875.17 ± 461.500 µg/mL. The extracts showed moderate antiradical activities with IC_{50} values of 309.31 ± 2.465, 635.52 ± 11.05 and 509.78 ± 6.348 µg/mL for PR-1, PR-2 and PR-3 respectively.

Table 2
Anti-inflammatory and cytotoxic activities of some compounds.

Compound	ROS Inhibition (IC_{50} µg/mL)	NO Inhibition (IC_{50} µg/mL)	Cytotoxicity on NIH-3 T3 Cells (IC_{50} µg/mL)
1	>100	23.3 ± 0.3	5.8 ± 0.3
2	>100	>100	51.7 ± 6.3
3	>100	>100	>100
6	4.3 ± 0.3	42.7 ± 1.8	>100
9	1.1 ± 0.1	32.9 ± 0.2	23 ± 1.7
PR-1	>100	>100	>100
PR-2	>100	>100	>100
PR-3	>100	>100	>100
Ibuprofen	11.2 ± 1.9	–	–
L-NMMA	–	24.2 ± 0.8	–
Cyclohexamide	–	–	0.13 ± 0.02

L-NMMA = N^G monomethyl L-arginine acetate. – = not tested.

Table 3
Antifungal activity Minimal Inhibitory Concentrations (µg/mL).

Compound	*C. albicans*	*C. parapsilosis*	*C. krusei*	*C. glabrata*
1	>500	>500	>500	>500
2	500	500	500	500
3	500	500	125	125
5	500	500	500	500
4	500	>500	>500	500
6	500	500	500	500
7	500	500	250	500
8	500	500	250	500
9	500	500	250	500
10	500	500	500	500
PR-1	250	500	250	250
PR-2	125	>500	250	>500
PR-3	250	>500	250	250
Fluconazol	12.5	50	50	50

Table 4
DPPH radical scavenging activity.

Compound	IC_{50} μg/mL
1	15.653 ± 0.335[a]
2	875.17 ± 461.500[c]
3	89.077 ± 24.875[b]
4	>1000
5	>1000
6	>1000
7	>1000
8	>1000
9	>1000
10	>1000
PR-1	309.31 ± 2.465[b]
PR-2	635.52 ± 11.05[c]
PR-3	509.78 ± 6.348[c]
Vitamin C	3.343 ± 0.271[a]

4. Conclusion

Propolis is a highly medicinal bee product. The chromatographic separation of Cameroonian propolis samples led to the isolation of ten compounds out of which a greater majority are triterpenes as would be expected of propolis from tropical and subtropical regions. Lupeol acetate and β-amyrine acetate possessed high ROS and NO inhibition. The new isoflavone and E-triacontryl-3-(4-hydroxyphenyl) acrylate isolated from propolis for the first time had good DPPH* scavenging activity. Propolis extracts showed moderate antiradical and antifungal activities alongside some of its compounds. The ROS and NO inhibition results indicate that propolis compounds could be potential anti-inflammatory and antioxidant drug agents. It is noteworthy to mention that inflammation is involved in many illnesses. Reactive oxygen species (ROS) are implicated in a wide range of human diseases, such as atherosclerosis and certain cancers. When an imbalance between ROS generation and antioxidants occurs, oxidative damage will spread over most cell targets. Mechanisms of antioxidant action may include suppression of ROS formation, removal or inactivation of oxygen reactive species and up-regulation or protection of antioxidant defences which is beneficial to human health. To this effect, natural products and compounds from natural sources which possess anti-inflammatory and antioxidant activities are often used in traditional medicine and could be of pharmaceutical relevance.

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Declaration of Competing Interest

The authors declare that they have no competing interests.

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