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New cycloartane triterpenes from bioactive extract of propolis from Pitcairn Island

Kristina Georgieva[a], Boryana Trusheva[a,*], Veselina Uzunova[b], Tihomira Stoyanova[b], Violeta Valcheva[c,d], Milena Popova[a], Rumiana Tzoneva[b], Vassya Bankova[a]

[a] Institute of Organic Chemistry with Centre of Phytochemistry, Bulgarian Academy of Sciences, 1113 Sofia, Bulgaria
[b] Institute of Biophysics and Biomedical Engineering, Bulgarian Academy of Sciences, 1113 Sofia, Bulgaria
[c] Department of Infectious Microbiology, Institute of Microbiology, Bulgarian Academy of Sciences, 1113 Sofia, Bulgaria
[d] In vitro laboratory for evaluation of biological activity and toxicity, Sofia Tech Park, 1784 Sofia, Bulgaria

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ABSTRACT

Dichloromethane extract of propolis (DCME) originating from Pitcairn Island demonstrated potent cytotoxicity against triple-negative MDA-MB-231 human breast carcinoma cells. The results from MTT assay showed that DCME inhibits the growth of the cancer cells in a dose- and time-dependent manner and upon the cell growth inhibition propolis extract provoked apoptotic changes in the cell nuclei. A detailed chemical investigation of DCME led to the isolation of four new cycloartane triterpenes (**1**–**4**), along with 17 known compounds (**5**–**21**). The structures of the new compounds were elucidated by means of extensive analysis of their spectroscopic data and comparison with those reported for their analogues. *In vitro* antimicrobial activity of new compounds (**1**–**4**) along with the DCME against four human pathogens was evaluated. All tested constituents except compound **2** were highly active against *Escherichia coli* with MIC 64 μg/ml. Compound **1** exhibited high antifungal activity against *Candida albicans* with potency close to that of the positive control (amphotericin B). The DCME showed very good antimicrobial activity against *Staphylococcus aureus*, *Escherichia coli* and *Candida albicans*. This is the first study on propolis from Pitcairn Island.

1. Introduction

Propolis (bee glue) is a resinous hive product collected by bees from certain plant sources. The bees use it not only as a building material in their nests, but also as a chemical defence against infections. It has a long history of being used in traditional medicine and nowadays it is extensively used in functional foods and food additives to improve health and prevent diseases such as inflammation, heart disease, diabetes, and even cancer. Because of its broad spectrum of biological activities [1] there is an undying interest in the composition of propolis, because it depends on the vegetation of the area where it was collected and in different geographic regions, propolis might be of very specific chemical composition [2]. Therefore, the study of samples from areas where propolis has never been explored could reveal new propolis types and new propolis constituents of important biological activity.

There are numerous reports in the literature on the isolation and structural elucidation of bioactive phytochemicals from propolis collected in Europe, South America, Asia and the Pacific region [3], but no data exist about chemical composition and therapeutic properties of propolis from Pitcairn Islands, although it is already offered in the market and is used in the folk medicine based on empirical knowledge. Furthermore, in the last years it is of growing commercial interest, due to Pitcairn's bee population is disease free and their products are considered as clean of pollutants.

Since no previous research has been reported on propolis harvested in Pitcairn Island, we carried out a detailed chemical investigation. The work led to the isolation of 4 new cycloartane type triterpenes (**1**–**4**) (Fig. 1), together with 17 known compounds (**5**–**21**). In this paper, we report the isolation and structure elucidation of new compounds **1**–**4**, evaluation of their antimicrobial activity as well as data on the cytotoxic, apoptotic and antimicrobial activity of propolis extract.

2. Experimental

2.1. General experimental procedures

NMR spectra (^{1}H, ^{13}C, DEPT, HSQC, HMBC and NOESY) were recorded on a Bruker AVANCE II+ 600 NMR spectrometer operating at

* Corresponding author at: Institute of Organic Chemistry with Centre of Phytochemistry, Bulgarian Academy of Sciences, Acad. G. Bonchev str., Bl. 9, 1113 Sofia, Bulgaria.
E-mail address: bobi_tru@orgchm.bas.bg (B. Trusheva).

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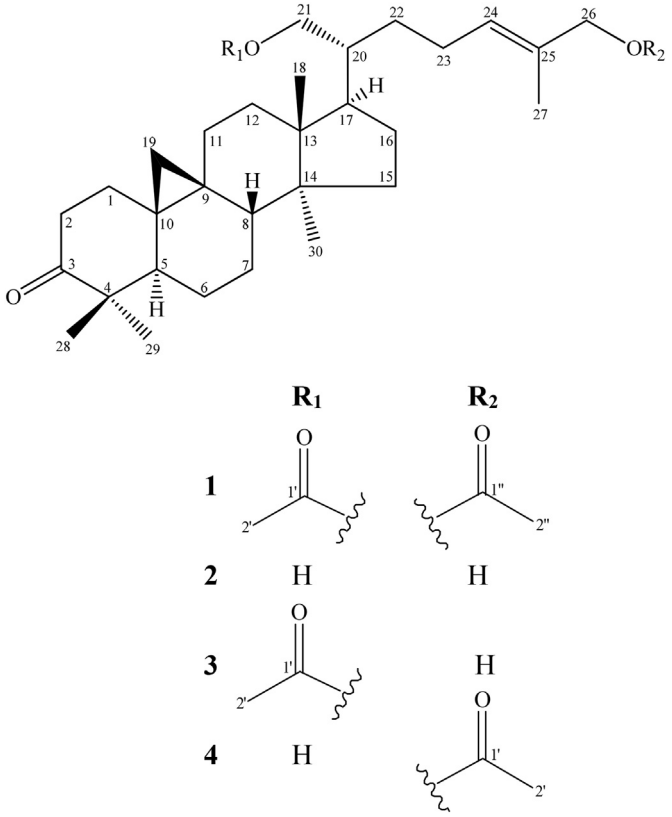

Fig. 1. New compounds in propolis from Pitcairn Island.

600 MHz (150 MHz for ^{13}C) in $CDCl_3$ with TMS as internal standard. Chemical shifts (δ) are given in ppm and coupling constants (J) in Hz. HREIMS spectra were measured on a Double- focusing High Resolution Magnetic Sector Mass Spectrometer Thermo Scientific DFS at 70 eV. Optical rotations were recorded on a Jasco P-2000 polarimeter in $CHCl_3$. IR spectra were measured on a Bruker Tensor 27 FTIR spectrometer. Vacuum liquid chromatography (VLC) was performed on Silica gel 60H (Merck, 15 μm). Column chromatography (CC) was performed on Silica gel 60 (Merck, 63–200 μm), normal phase and Sephadex LH-20 (Pharmacia Fine Chemicals, 25–100 μm). Low pressure liquid chromatography (LPLC) was carried out on silica gel using Merck Lobar column (LiChroprep® Si 60, 40–63 μm), normal phase. Preparative thin-layer chromatography (PTLC) was performed on Silica gel 60 F_{254} glass plates (Merck, 20 × 20 cm; 0.25 mm). The bands corresponding to compounds were scraped off and washed with CH_2Cl_2 and/or EtOAc. Analytical TLC was performed on Silica gel 60 F_{254} plates (Merck). Detection of the spots was achieved under UV light (254 and 366 nm) and by spraying with vanillin in sulfuric acid, followed by heating at 100 °C.

2.2. Propolis

The propolis sample originates from the Pacific Pitcairn Island and was collected from *Apis mellifera* beehives near to Adamstown in 2014 by scraping. It was stored at 4 °C out of the light until the extraction.

2.3. Extraction and isolation

The raw propolis (56.2 g) after cooling was powdered and extracted three times with CH_2Cl_2 (1:10, w/v) at room temperature for 24 h each. The combined solution after filtration was evaporated to afford 43.7 g dry residue. It was then subjected to a wax removal procedure as the residue was dissolved in hot methanol, cooled to 0 °C with ice and after removal of the precipitated wax by filtration, the resulting filtrate was evaporated to dryness to give 19.1 g wax free dichloromethane extract (DCME).

DCME (19 g) was then subjected to silica gel vacuum liquid chromatography (VLC) and eluted with gradient system of CH_2Cl_2/CH_3OH (from 1:0 to 1:1 v/v) to obtain ten fractions A-J based on TLC analysis. For further processing fractions B and J was chosen due to their less complex in comparison with other fractions relatively non polar and polar composition, respectively. Fraction B (4.5 g) was further fractionated by silica gel VLC with light petroleum ether (PE)/CH_2Cl_2 (from 1:0 to 0:1) and CH_2Cl_2/MeOH (from 1:0 to 1:1) as mobile phases. Twenty four combined fractions were obtained (B1–B24). Fraction B3 was purified by PTLC with PE/CH_2Cl_2 7:3 as eluant to yield compound **7** (1.6 mg). Fraction B7 (163.3 mg), after additional separation by LPLC using a gradient system of PE/Et_2O (from 99:1 to 0:1) afforded seventeen fractions (B7.1-B7.17). Fraction B7.3 (28 mg), eluted with PE/Et_2O 98:2 yielded compound **8**. Fraction B7.7 (26.5 mg) after additional purification by PTLC (PE/Et_2O 1:0.2) afforded an inseparable mixture of four triterpene ketones **9–12** (4.6 mg). Fractions B8 and B9 were combined (188.6 mg) and subjected to LPLC using a gradient system of PE/Et_2O (from 99:1 to 0:1). Ten fractions were obtained (B8.1-B8.10). Fraction B8.5 (6.4 mg) eluted with PE/Et_2O 98:2 yielded mixture of **13** and **14**. Fraction B13 (226.6 mg) was separated by LPLC with PE/EtOAc (from 99:1 to 1:1) as a mobile phase and eleven fractions were obtained (B13.1-B13.11). Fraction B13.4 (2.4 mg) eluted with PE/EtOAc 98:2 gave compound **5**. Fractions B13.3 (38 mg) and B13.8 (19 mg) individually were further purified by PTLC (PE/EtOAc 1:0.2) to yield compounds **15** (7.3 mg) and **6** (5.1 mg), respectively. Fraction B14 (235.7 mg) was rechromatographed by LPLC, using a gradient system of PE/EtOAc (from 98:2 to 0:1) to give fifteen fractions (B14.1-B14.15). Fractions B14.6 and B14.7 were combined (19.8 mg) and purified by PTLC with PE/EtOAc 1:0.2 to yield **16** (2.5 mg). Fraction B14.9 (17.7 mg) after additional purification by PTLC (PE/EtOAc 1:0.2) afforded **17** (7.7 mg). Fraction B14.13 (64.4 mg) was separated by PTLC with CH_2Cl_2/EtOAc 98:2 to give four fractions (B14.13.1-B14.13.4). Fraction B14.13.2 (9.9 mg) after final purification by PTLC with PE/EtOAc 1:0.2, two fold development, yielded **1** (3.3 mg). Fraction B16 (418.6 mg) was subjected to silica gel normal phase CC using a gradient system of PE/EtOAc (from 98:2 to 0:1), whereby ten fractions were obtained (B16.1-B16.10). Fraction B16.2 (60 mg) was purified by PTLC with CH_2Cl_2/MeOH 98:2 to afford **18** (4 mg). Fraction F (959 mg) was rechromatographed over Sephadex LH-20 CC (MeOH) to give four combined fractions (F1-F4). Fraction F2 (282.2 mg) was separated by LPLC with PE/EtOAc (from 90:10 to 1:1) as a mobile phase, whereby twenty seven fractions were obtained (F2.1-F2.27). Fraction F2.12 (11 mg) eluted with PE/EtOAc 85:15 yielded **19**. Fractions F2.22 (5.2 mg) and F2.25 (2.8 mg), eluted with PE/EtOAc 70:30 afforded compounds **3** and **4**, respectively. Fraction F3 (198 mg) after further separation by silica gel LPLC using a gradient system of PE/EtOAc (from 90:10 to 0:1) afforded sixteen fractions (F3.1-F3.16). Fraction F3.14 (4.9 mg), eluted with PE/EtOAc 60:40 gave compound **2**. From fraction F4 (279.2 mg), after additional purification by silica gel CC with PE/EtOAc (from 95:5 to 1:1), two inseparable mixtures of phenolic lipids **20** (23.4 mg) and **21** (18.5 mg) were obtained.

3-Oxo-cycloart-24E-en-21,26-diol-21,26-diacetate (**1**): colorless oil; $[\alpha]_D^{20}$ − 129° (c 0.23, $CHCl_3$); IR (KBr) ν_{max} 2928, 2871, 2856, 1740, 1707, 1240 cm^{-1}; ^1H NMR and ^{13}C NMR data, see Table 1; EI-MS *m/z* 540 $[M]^+$; HREIMS *m/z* 540.33206 $[M]^+$ (calcd. for $C_{34}H_{52}O_5$, 540.38166).

3-Oxo-cycloart-24E-en-21,26-diol (**2**): colorless oil; $[\alpha]_D^{20}$ − 11° (c 0.41, $CHCl_3$); IR (KBr) ν_{max} 3435, 2933, 2870, 1706, 1045 cm^{-1}; ^1H NMR and ^{13}C NMR data, see Table 1; EI-MS *m/z* 456 $[M]^+$; HREIMS m/z 456.34384 $[M]^+$ (calcd for $C_{30}H_{48}O_3$, 456.36054).

3-Oxo-cycloart-24E-en-21,26-diol-21-acetate (**3**): colorless oil; $[\alpha]_D^{20}$ − 28° (c 0.55, $CHCl_3$); IR (KBr) ν_{max} 3446, 2929, 2870, 1739, 1707, 1241 cm^{-1}; ^1H NMR and ^{13}C NMR data, see Table 1; EI-MS *m/z* 498 $[M]^+$; HREIMS m/z 498.31404 $[M]^+$ (calcd for $C_{32}H_{50}O_4$,