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New cycloartane triterpenes from bioactive extract of propolis from Pitcairn Island

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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

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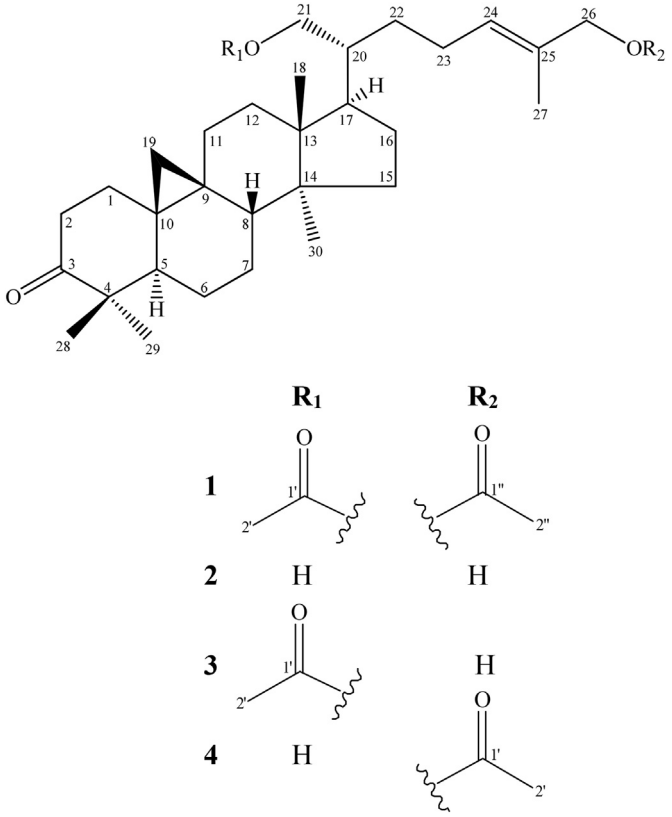


Fig. 1. New compounds in propolis from Pitcairn Island.

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 $C_{32}H_{50}O_4$,

Table 1
^{1}H NMR (600 MHz) and ^{13}C NMR (150 MHz) data of compounds **1–4** (δ in ppm, $CDCl_3$).

Position	1		2		3		4	
	δ_H (J in Hz)	δ_C	δ_H (J in Hz)	δ_C	δ_H (J in Hz)	δ_C	δ_H (J in Hz)	δ_C
1α	1.85 dt (13.2, 4.2)	33.4	1.85 dt (13.4,3.8)	33.4	1.84 dt (13.2, 4.2)	33.4	1.86 dt (13.4, 4.2)	33.4
β	1.53 m		1.54 m		1.53 m		1.54 m	
2α	2.30 ddd (13.9, 4.2, 2.5)	37.5	2.30 ddd (13.9, 4.1, 2.7)	37.4	2.30 ddd (14.0, 4.0, 2.6)	37.4	2.30 ddd (13.9, 4.2, 2.5)	37.4
β	2.71 dt (13.9, 6.4)		2.71 dt (13.8, 6.4)		2.71 dt (13.9, 6.3)		2.71 dt (13.9, 6.4)	
3	–	216.6	–	216.6	–	216.6	–	216.6
4	–	50.2	–	50.2	–	50.2	–	50.2
5	1.71 dd (12.3, 4.5)	48.3	1.71 dd (12.1, 4.6)	48.3	1.71 dd (12.3, 4.5)	48.3	1.71 dd (12.3, 4.5)	48.3
6α	1.58 m	21.4	1.57 m	21.4	1.56 m	21.4	1.57 m	21.4
β	0.95 dd (12.7, 2.3)		0.95 dd (12.6, 2.4)		0.95 dd (12.8, 2.4)		0.95 m	
7α	1.14 dd (12.7, 2.3)	25.8	1.14 dd (12.4, 2.6)	25.8	1.14 dd (12.6, 2.5)	25.8	1.15 dd (12.5, 2.6)	25.8
β	1.38 m		1.39 m		1.38 m		1.39 m	
8	1.58 m	47.8	1.59 m	47.8	1.59 m	47.8	1.59 m	47.8
9	–	20.9	–	21.0	–	20.9	–	21.0
10	–	26.0	–	26.0	–	26.0	–	26.0
11α	2.04 m	26.5	2.06 m	26.6	2.04 m	26.5	2.05 m	26.6
β	1.17 m		1.18 m		1.17 m		1.18 m	
12	1.58 m	31.7	1.72 m; 1.65 m	31.9	1.59 m	31.7	1.71 m; 1.65 m	31.9
13	–	45.1	–	45.2	–	45.1	–	45.2
14	–	48.8	–	48.8	–	48.8	–	48.8
15	1.35 m	35.4	1.35 m	35.4	1.34 m	35.4	1.35 m	35.4
16a	1.93 m	27.6	1.91 m	27.6	1.94 m	27.6	1.92 m	27.6
16b	1.35 m		1.37 m		1.36 m		1.37 m	
17	1.95 m	46.5	1.94 m	46.1	1.93 m	46.6	1.93 m	46.1
18	1.02 s	18.2	1.02 s	18.3	1.02 s	18.3	1.02 s	18.3
19a	0.78 d (4.2)	29.5	0.79 d (4.4)	29.3	0.78 d (3.9)	29.5	0.79 d (4.2)	29.1
19b	0.58 d (4.2)		0.58 d (4.3)		0.57 d (4.3)		0.58 d (4.2)	
20	1.67 m	39.5	1.51 m	42.3	1.68 m	39.5	1.50 m	42.3
21a	4.23 dd (11.3, 3.3)	64.6	3.74 dd (11.1, 2.9)	62.3	4.23 dd (11.3, 3.3)	64.7	3.73 dd (11.1, 2.9)	62.3
21b	3.94 dd (11.3, 6.1)		3.63 dd (11.1, 5.0)		3.94 dd (11.3, 6.1)		3.63 dd (11.1, 4.9)	
22a	1.51 m	29.7	1.51 m	29.5	1.52 m	29.8	1.52 m	29.5
22b	1.39 m		1.44 m		1.39 m		1.44 m	
23a	2.10 m	24.4	2.13 m	24.5	2.08 m	24.2	2.14 m	24.6
23b	1.98 m		2.01 m		1.98 m		2.03 m	
24	5.44 br. t (6.6)	129.8	5.42 br. t (7.2)	126.6	5.39 br. t (7.0)	126.2	5.48 br. t (6.6)	130.1
25	–	130.1	–	134.7	–	134.8	–	129.9
26	4.45 s	70.3	4.00 s	69.0	4.00 s	68.9	4.45 s	70.3
27	1.65 s	13.9	1.68 s	13.7	1.66 s	13.6	1.67 s	14.0
28	1.10 s	20.7	1.10 s	20.8	1.09 s	20.7	1.10 s	20.8
29	1.04 s	22.1	1.04 s	22.1	1.04 s	22.1	1.04 s	22.1
30	0.91 s	19.4	0.92 s	19.4	0.92 s	19.4	0.92 s	19.4
1′	–	171.4			–	171.4	–	171.1
2′	2.06 s	21.0			2.06 s	21.0	2.08 s	21.0
1″	–	171.0						
2″	2.08 s	21.0						

498.37110).

3-Oxo-cycloart-24E-en-21,26-diol-26-acetate (**4**): colorless oil; $[\alpha]_D^{20}$ + 34° (c 0.23, $CHCl_3$); IR (KBr) ν_{max} 3446, 2931, 2870, 1739, 1707, 1240 cm^{-1}; ^{1}H NMR and ^{13}C NMR data, see Table 1; EI-MS m/z 498 $[M]^+$; HREIMS m/z 498.39966 $[M]^+$ (calcd for $C_{32}H_{50}O_4$, 498.37110).

2.4. Evaluation of cytotoxic activity

2.4.1. In vitro cell viability assay

To observe the cytotoxicity of propolis extract from Pitcairn Island triple negative breast cancer cell line MDA-MB-231 (ATCC, Manassas, VA, USA) was used. The cells were grown and maintained in Dulbecco's Modified Eagle's Medium-High Glucose (DMEM–High Glucose), containing 10% fetal bovine serum (FCS) and supplements (L-glutamine, sodium pyruvate, antibiotic, NEAA) at 37 °C and 5% CO_2.

Cells were passaged at 80–90% confluence with 0.05% trypsin–0.02% EDTA and suspended at density of 4×10^4 cells/ml. The cytotoxicity test was performed in 96-well plates with subconfluent cell layers. The cells were incubated with the propolis extract in concentration range of 6.25–200.0 μg/ml for 24 h, 48 h or 72 h. As a control was used DMEM + DMSO (concentration corresponded to the highest concentration of DMSO in the propolis extract). MTT test (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide, Invitrogen, USA) was performed as described by Mosmann with some modifications [4]. Briefly, after the incubation period the cell medium was changed with fresh medium (200 μl/well). Then, 50 μl of MTT solution (5 mg/ml in PBS) was added. Plates were further incubated for 4 h at 37 °C, and the formed formazan crystals were dissolved by addition of 250 μl solvent (5% formic acid in 2-propanol) per well and mixing. The absorbance was recorded at 570 nm with the 96-well plate reader Tecan Infinite F200 PRO (Tecan Austria GmbH, Salzburg). Three independent experiments were performed in quadruplicate. Complete medium (200 μl), 50 μl of MTT solution (5 mg/ml in PBS) and 5% formic acid in 2-propanol (250 μl) were used as a blank solution. Cytotoxicity corresponding to cell viability after incubation with the investigated probes was presented as percentage to the cell viability of the control – DMEM + DMSO. The IC_{50} values were calculated using nonlinear regression method by GraphPad Prism 5 program. Bulgarian propolis (poplar type) was used as a positive control.

2.4.2. Morphological assessment

The nuclear morphology of MDA-MB-231 cells treated with DCME was observed with a DAPI (4′,6-diamidine-2′-phenylindole

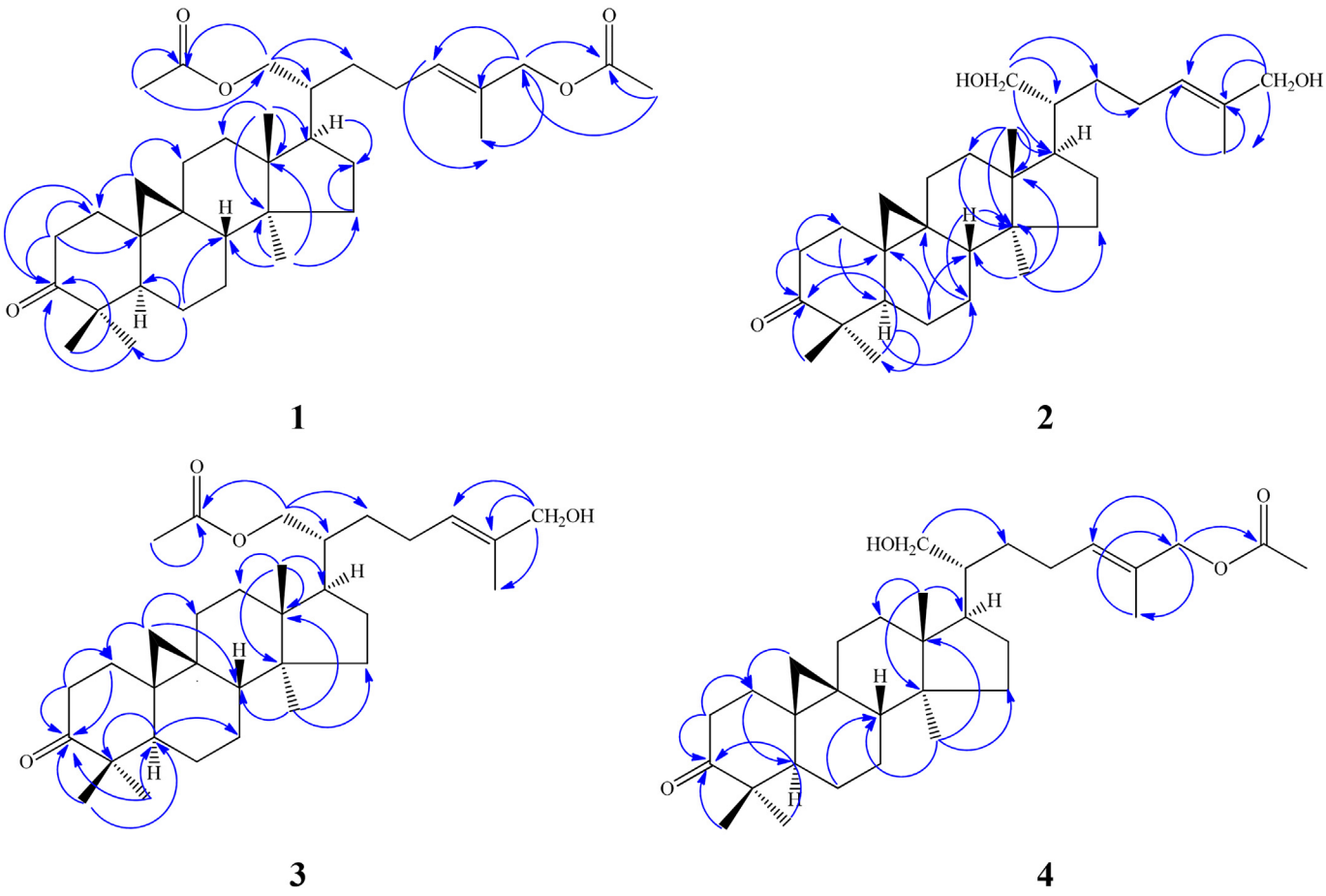


Fig. 2. Major HMBC correlations of compounds **1–4**.

dihydrochloride) staining assay. After treatment of the cells with the propolis extract in concentration of 50 or 100 μg/ml for 24 h, 48 h and 72 h the cells were rinsed with PBS and were stained with DAPI (1 μg/ml in PBS). The slides were conserved in the dark at 4 °C and were analyzed with a fluorescent microscope (Jena Lumar, Germany, with objective lens HI PLAN-40/0.50).

2.5. Antimicrobial assay

The antimicrobial activity of propolis extract and compounds **1–4** against *Staphylococcus aureus* 209, *Escherichia coli* WF+, *Pseudomonas aeruginosa* ATCC 9027 and *Candida albicans* 562 (Bulgarian Type Culture Collection, Institute for State Control of Drugs, Sofia) was evaluated *in vitro* by broth microdilution method according to Clinical and Laboratory Standards Institute (CLSI) procedures [5]. The minimal inhibitory concentration (MIC) was estimated in 96-well microplates as 50 μl of bacterial suspension in exponential phase of the growth 1×10^5 CFU/ml was added to the wells already containing 50 μl of two-fold serially diluted solutions of tested compounds in Mueller–Hinton broth (MHB). The plates were incubated at 37 °C for 24 h [6]. Experiments were performed in triplicate. MIC was determined as the lowest concentration without visible growth and was expressed as μg/ml. As positive controls were used gentamicin for bacteria and amphotericin B for fungi. DMSO was used as a negative control.

3. Results and discussion

3.1. Structural elucidation of the new cycloartane triterpenes

Compound **1** was obtained as colorless oil. Its HREIMS spectrum showed a molecular ion peak at *m/z* 540.33206 and the presence of 34 carbons in the ^{13}C NMR spectrum, suggesting a molecular formula $C_{34}H_{52}O_5$ and indicating nine degrees of unsaturation. The IR spectrum implied the presence of two sharp characteristic bands for ester carbonyl group and unconjugated keto group at 1740 and 1707 cm^{-1}, respectively. The ^{1}H NMR spectrum of **1** showed two upfield doublets at δ 0.58 and 0.78 (1H each, J = 4.2 Hz), characteristic for the non-equivalent protons of a cyclopropyl methylene group. Five singlets, three proton each, at δ 0.91, 1.02, 1.04, 1.10 and 1.65, corresponding to five tertiary methyl groups (one of which vinylic), were also observed. Slightly downfield of those signals two more sharp singlets (3H each) at δ 2.06 and 2.08 were presented, corresponding to two methyl groups attached to carbonyl carbon atoms. The ^{1}H NMR spectrum indicated the presence of two non-equivalent oxymethylene protons signals at δ 3.94 (1H, dd, J = 11.3, 6.1 Hz) and δ 4.23 (1H, dd, J = 11.3, 3.3 Hz), two oxymethylene protons signal at δ 4.45 (2H, s) and an olefinic proton signal at δ 5.44 (1H, br t, J = 6.6 Hz). The ^{13}C NMR, with the help of DEPT and HSQC, exhibited the characteristic signals for three carbonyl carbons at δ_C 216.6 (ketocarbonyl) and at δ_C 171.4 and 171.0 (ester carbonyl), thirteen methylenes, two of which oxygen-bearing (δ_C 70.3 and 64.6), five methines, one of which olefinic (δ_C 129.8), five quaternary sp^3 and a quaternary sp^2 C-atoms (δ_C 130.1). From the above data (Table 1) it was deduced that compound **1** is a diacetate of 3-oxo-9,19-cycloartane triterpenoid. The cross-peaks of H_2–1, H_2-2, H_3-28 and H_3-29 with C-3 (δ_C 216.6) in the HMBC spectrum (Fig. 2), verified that the keto group is at C-3 position. Furthermore, the HMBC correlations from H_2-21 to the C-17 (δ_C 46.5), C-20 (δ_C 39.5), C-22 (δ_C 29.7), and the ester carbonyl C-1′ (δ_C 171.4), as well as from H_2–26 to the C-24 (δ_C 129.8), C-25 (δ_C 130.1), C-27 (δ_C 13.9), and the ester carbonyl C-1″ (δ_C 171.0) confirmed the location of two acetate

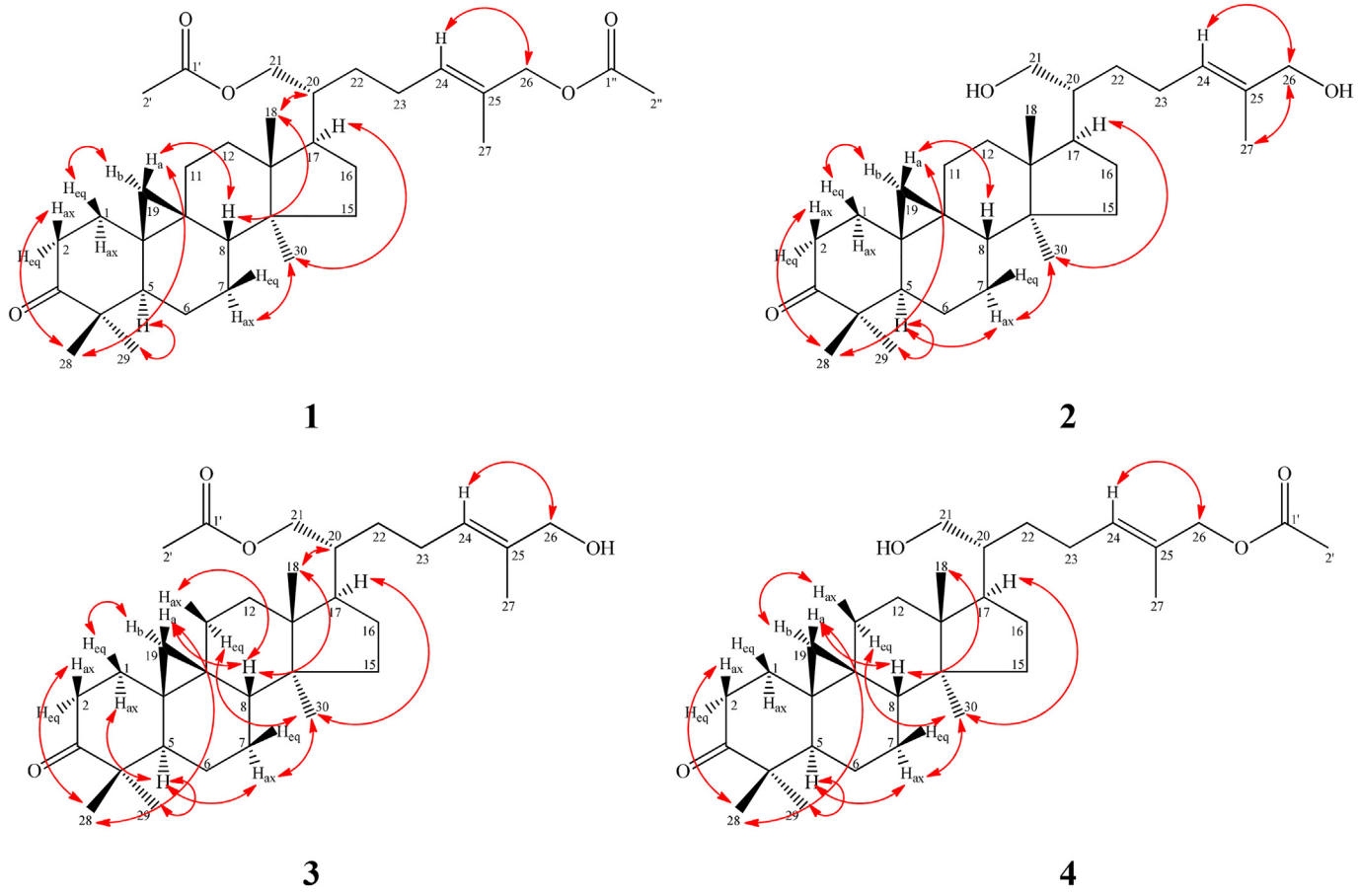


Fig. 3. Key NOESY correlations of compounds **1–4**.

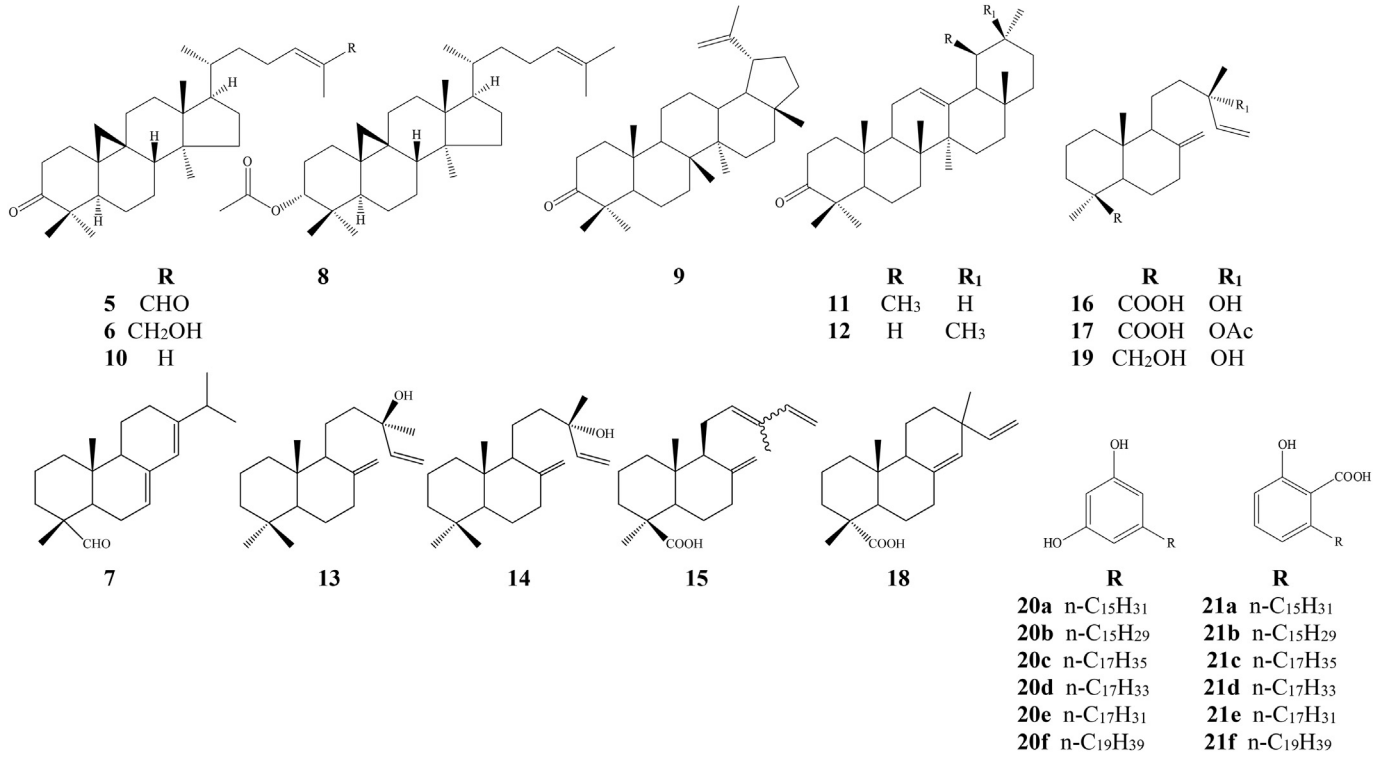


Fig. 4. Structures of the isolated compounds **5–21**.

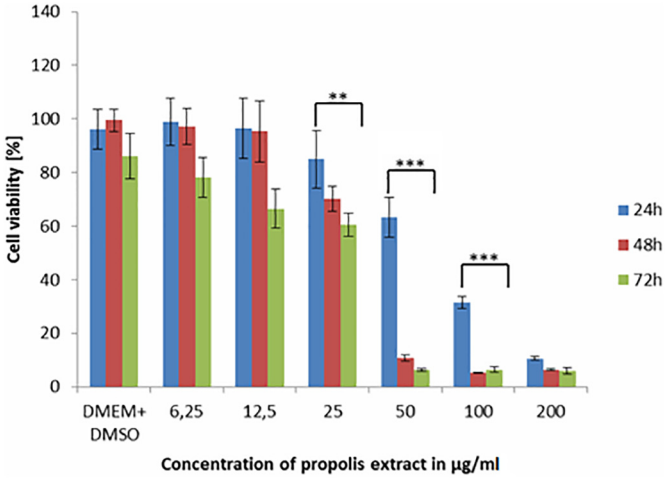


Fig. 5. Cytotoxic effect of DCME on MDA-MB-231 breast cancer cells.

fragments at C-21 and C-26, respectively. The relative configuration of **1** was established using NOESY correlations, coupling constant data, and comparison with literature data for its analogues [7,8]. The NOESY correlations of H-8 (δ_H 1.58)/H-19b (δ_H 0.78), H-8 (δ_H 1.58)/Me-18 (δ_H 1.02) and Me-18 (δ_H 1.02)/H-20 (δ_H 1.67) indicated that H-8 is β-oriented. The presence of NOESY cross-peaks of Me-30 (δ_H 0.91)/H-6α (δ_H 1.58), Me-30 (δ_H 0.91)/H-7α (δ_H 1.14) and Me-30 (δ_H 0.91)/H-17 (δ_H 1.93) confirmed the α-orientation of H-17 (Fig. 3). By analyzing the HSQC, HMBC, and NOESY spectra, all the proton and carbon signals (Table 1, Figs. 2 and 3) were assigned unambiguously and the structure of compound **1** was elucidated as 3-oxo-cycloart-24E-en-21,26-diol-21,26-diacetate (Fig. 1).

Compound **2** gave molecular ion peak at *m/z* 456.34384 in its HREIMS spectrum and the presence of 30 carbons in the ^{13}C NMR spectrum, suggesting a molecular formula $C_{30}H_{48}O_3$ and indicating seven degrees of unsaturation. The IR spectrum showed absorption bands at ν_{max} 3435 and 1706 cm^{-1}, corresponding to a hydroxy and a ketone group, respectively. It was confirmed by the resonances at δ_C 62.3, 69.0 and 216.6 in the ^{13}C NMR spectrum. The ^{1}H and ^{13}C NMR spectra of **2** were similar to those of **1**, except for an upfield shifts of two equivalent and two non-equivalent oxymethylene protons signals (**2**, δ_H 4.00, 3.74 and 3.63; **1**, δ_H 4.44, 4.23 and 3.94) and the disappearance of the two sharp signals of methyl groups attached to carbonyl C-atoms found in ^{1}H NMR of **1** (δ_H 2.06 and 2.08), as well as of their carbon signals and the signals of the ester carbonyl atoms (δ_C 21.0, 171.0 and 171.4). The absence of additional signals in ^{1}H NMR of **2** was an indication that oxymethylene protons are adjacent to free hydroxyl groups. The existence of long-range correlation between H_2-21 and C-17 (δ_C 46.1), C-20 (δ_C 42.3) and C-22 (δ_C 29.5), as well as between H_2-26 and C-24 (δ_C 126.6), C-25 (δ_C 134.7) and C-27 (δ_C 13.7) confirmed the location of two hydroxy groups at C-21 and C-26, respectively (Fig. 2). The relative stereochemistry of **2** was determined by NOESY experiment and found to be the same as **1**. The existence of NOE cross peak between olefinic proton H-24 (δ_H 5.42) and H_2-26 oxymethylene protons (δ_H 4.00) confirms *trans* configuration of $\Delta^{24,25}$ double bond. Other important NOESY correlations are presented in Fig. 3. Based on these data and by comparison with spectral data of **1** and of similar compounds [8,9] the compound **2** was undoubtedly identified as 3-oxo-cycloart-24E-en-21,26-diol (Fig. 1).

Compounds **3** and **4** were both obtained as colorless oils and showed the molecular formula $C_{32}H_{50}O_4$, as deduced from their HREIMS data, and from the presence of 32 carbons in their ^{13}C NMR spectra (Table 1). Their IR spectra showed absorption bands for hydroxyl, ester carbonyl and unconjugated keto group. The ^{1}H and ^{13}C NMR spectra of **3** resembled those of **1**, except for the absence of the signals of one acetyl group at δ_C 171.0 and 21.0 and δ_H 2.08. The upfield shift of two equivalent oxymethylene protons like it is in ^{1}H NMR of **2** (**3**, δ_H 4.00; **2**, δ_H 4.00; **1**, δ_H 4.44) was an indication that the functional group next to them is a free hydroxy group. Its location at C-26 was confirmed by the existence of long-range correlations between H_2-26 and C-24 (δ_C 126.2), C-25 (δ_C 134.8) and C-27 (δ_C 13.6) (Fig. 2). The presence of HMBC cross-peaks between H_2-21 (δ_H 3.94 and 4.23) and C-17 (δ_C 46.6), C-20 (δ_C 39.5), C-22 (δ_C 29.8) and the ester carbonyl C-1′ (δ_C 171.4) further proved attached to C-21 acetate fragment. By analyzing the HSQC, HMBC, and NOESY spectra of **3**, as well as their comparison with those of **1** and **2**, all the proton and carbon signals (Table 1, Figs. 2 and 3) were assigned unequivocally and the structure of compound **3** was elucidated as 3-oxo-cycloart-24E-en-21,26-diol-21-acetate (Fig. 1).

The NMR spectra of compound **4** were similar to those of **3** except for the differences in the chemical shifts of oxymethylene protons and carbons. In ^{1}H NMR of **4** the signals of two non-equivalent oxymethylene protons were shielded (δ_H 3.73 and 3.63) unlike the signal of equivalent ones which was deshielded (δ_H 4.45) in comparison with the respective signals in ^{1}H NMR of **3**, which suggested exchanged positions of hydroxy and acetoxy substituents. It was confirmed by long-range correlation between H_2-26 and C-24 (δ_C 130.1), C-25 (δ_C 129.9), C-27 (δ_C 14.0), and the ester carbonyl C-1′ (δ_C 171.1). The relative configuration of **4** was established by NOESY experiment (Fig. 3) and was found to be the same as others (compounds **1–3**), which once again confirmed the cycloartane skeleton. Through a detailed analysis of 1D and 2D NMR spectra of **4**, as well as their comparison with those of compounds **1–3**, the compound **4** was identified as 3-oxo-cycloart-24E-en-21,26-diol-26-acetate (Fig. 1).

3.2. Structure identification of the known compounds

The known compounds **5–21** (Fig. 4) were identified by comparing their spectroscopic data with the corresponding published values as: two cycloartanes 3-oxo-cycloart-24-en-26-al (**5**) [10] and 3-oxo-cycloart-24-en-26-ol (**6**) [9]; a diterpene aldehyde abietinal (**7**) [11], 3α-cycloartenol acetate (**8**) [12], an inseparable mixture of triterpene ketones, 20(29)-lupen-3-one (**9**), cycloartenone (**10**), α-amyrenone (**11**)

Table 2
Cell viability of MDA-MB-231 breast cancer cells and IC_{50} values obtained from the MTT assay following exposure to DCME.

Exposure time, h	DCME				BGE[a]			
	Cell viability, %[b]			IC_{50}, µg/ml[c]	Cell viability, %[b]			IC_{50}, µg/ml[c]
	50 µg/ml	100 µg/ml	200 µg/ml		50 µg/ml	100 µg/ml	200 µg/ml	
24	60	30	10	71.6 ± 12.0	80	60	60	> 200.0
48	10	5	5	29.7 ± 2.1	40	20	15	39.3 ± 4.5
72	7	7	7	26.6 ± 4.7	42	18	10	48.5 ± 5.7

[a] BGE- ethanol extract of Bulgarian propolis (poplar type) is used as a positive control.
[b] % of the control DMEM + DMSO.
[c] The values are mean ± standard deviation (SD).

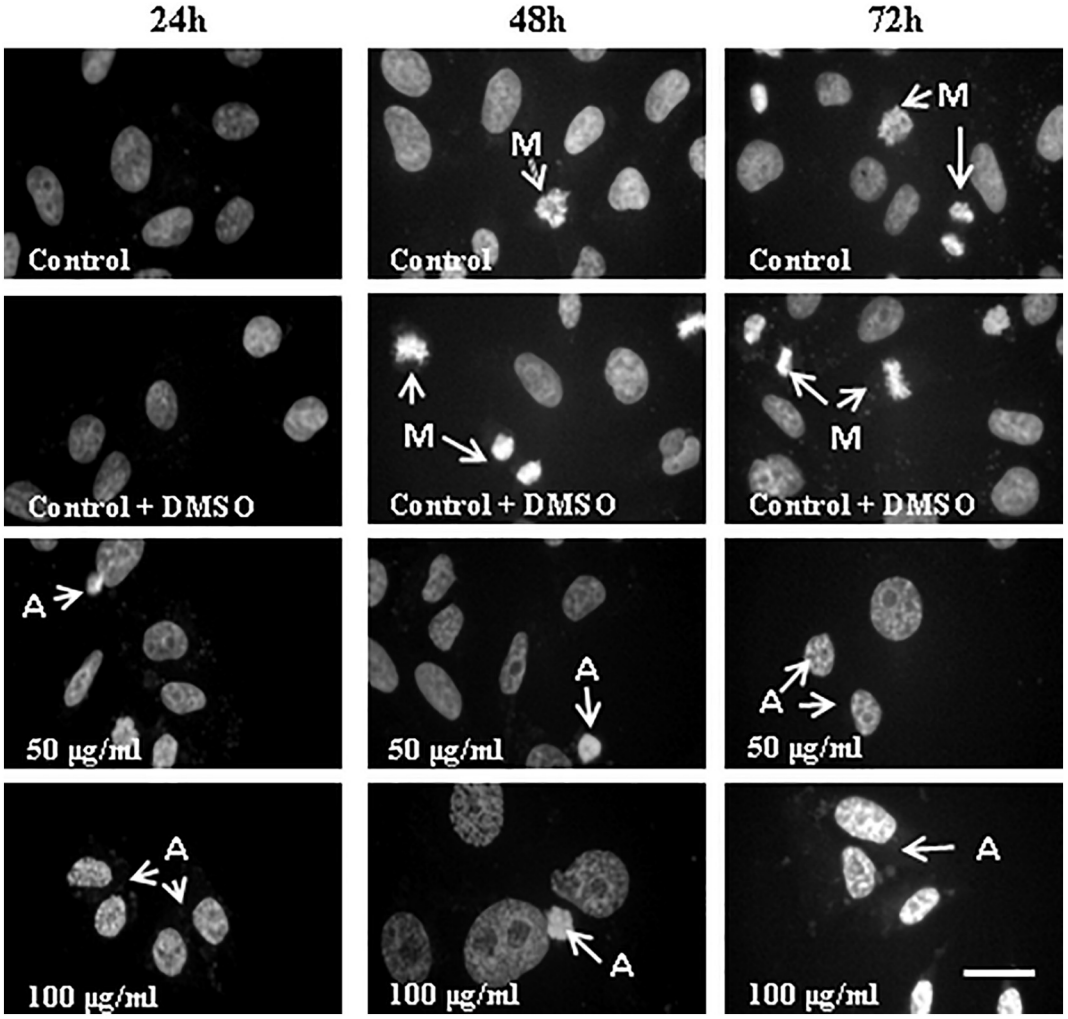


Fig. 6. Morphology of breast cancer (MDA-MB-231) cells stained with DAPI after exposure to DCME at different concentrations for 24 h, 48 h and 72 h and corresponding controls.

Table 3
Antibacterial and antifungal activities of DCME and individual compounds **1–4.**

Samples	MIC, µg/ml			
	Gram-positive bacteria	Gram-negative bacteria		Fungi
	Staphylococcus aureus 209	*Escherichia coli* WF +	*Pseudomonas aeruginosa* ATCC 9027	*Candida albicans* 562
1	64	64	128	64
2	128	256	256	128
3	128	64	128	128
4	64	64	128	128
DCME	128	128	256	128
Gentamicin[a]	32	32	64	NT
Amphotericin B[a]	NT	NT	NT	64

NT- Not tested.
[a] Positive control.

and β-amyrenone (**12**) (their structures were confirmed by GC/MS) [13], mixture of manool (**13**) and 13-epimanool (**14**) [14], *cis/trans* communic acid (**15**) in ratio 0.6:1 (based on ^{1}H NMR) [15], 13-epicupressic acid (**16**), 13-O-acetyl-13-epicupressic acid (**17**) [16], sandaracopimaric acid (**18**) [17], 13-epitorulosol (**19**) [18], two inseparable mixtures of phenolic lipids, 5-alk(en)ylresorcinols (cardols) (**20**) and 6-alk(en)ylsalicylic acids (anacardic acids) (**21**) [19,20], respectively. Compounds **5** and **7** are found for the first time in propolis. In general cycloartane type triterpenes along with phenolic lipids are typical constituents of *Mangifera indica* (mango) type propolis widespread in tropical areas and especially in the Pacific region [3,19,21]. While labdane type diterpenes (compounds **13–17** and **19**) are very characteristic for Mediterranean propolis, which plant source is *Cupressus sempervirens* and for Brazilian propolis (mostly acids) originating from *Araucaria* spp. [22–24].

3.3. Bioactivity evaluation

3.3.1. Cytotoxic activity

The cytotoxic effect of dichloromethane extract of propolis from Pitcairn Island on breast adenocarcinoma MDA-MB-231 cells was evaluated using MTT cytotoxicity assay. The cytotoxic analysis revealed that the cancer cells viability decreased depending on the concentration and time of exposure to DCME (Fig. 5). As can be seen from Table 2 the IC_{50} for 24 h is between 63.1 and 80.0 µg/ml propolis extract, and with the increasing of the incubation time, IC_{50} decreased to 28.2–31.1 µg/ml for 48 h and 23.3–29.9 µg/ml for 72 h. In addition to the antiproliferative effect the results reveal that DCME is more potent inhibitor of cancer cells growth than poplar propolis rich in phenolics (BGE), used as a positive control and shown to be highly active elsewhere [25–28]. The viability of cancer cells treated with DCME is lower in

comparison with the viability of cancer cells treated with BGE. It is probably due to the presence of cycloartane triterpenes and phenolic lipids, known for their anti-tumor properties [29–31].

DCME was further evaluated for its MDA-MB-231 cells apoptotic effect. After the treatment with different concentrations of DCME (50 or 100 μg/ml) for 24 h, 48 h and 72 h, cells are stained with DAPI and their morphology was examined by fluorescent microscopy analysis. The microscopic assessment demonstrated changes in cellular morphology. As can be seen from Fig. 6 among the population of the control cells were examined cells in mitosis. When the cells were treated with propolis extract they showed apoptotic features, such as cytoplasmic shrinkage and condensation of nuclear chromatin.

To our knowledge, this is the first evaluation of cytotoxicity of propolis extract containing mainly terpenoids (di- and triterpenes) instead of phenolics towards MDA-MB-231 cells. The results obtained are promising, which is a prerequisite for more in-depth research on the antitumor effect of this propolis type and on the possibility of its application in the fight against breast cancer.

3.3.2. Antimicrobial activity

Antimicrobial activity is the main and essential characteristic of propolis which is associated with its role in the bee hive. In this study, antibacterial and antifungal activities of DCME and the new compounds were evaluated *in vitro* by determining MIC values during incubation of samples with four human pathogens: *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa* and *Candida albicans*. Both the extract and its constituents **1**–**4** were active against *S. aureus*, as the most active compounds were **1** and **4** with MIC 64 μg/ml (Table 3). Taking into consideration, that natural products (extracts), which display MIC in the range 100–1000 μg/ml in *in vitro* susceptibility tests, can be classified as antimicrobials [32], the studied propolis extract displayed good antimicrobial activity both against gram-positive and gram-negative bacteria as well as against fungi, confirming the traditional reputation of propolis as antimicrobial agent. Unlike most propolis samples of different origin and their constituents, DCME exhibited strong antimicrobial activity against *Escherichia coli* and *Candida albicans* with MIC 128 μg/ml [33]. With the exception of 3-oxo-cycloart-24E-en-21,26-diol (**2**) other cycloartane derivatives were highly active against *Escherichia coli* more than the extract with MIC 64 μg/ml. Since it has been shown that diterpene-rich propolis (Mediterranean type) possess good antibacterial properties [24], both the isolated new cycloartanes and the contained therein diterpenes might have contribution to the propolis extract high antibacterial activity. All tested samples showed lowest antibacterial activity against the gram-negative bacteria *Pseudomonas aeruginosa* with MIC in the range 128–256 μg/ml. Among the individual compounds **2** displayed the weakest antibacterial effect against all tested bacteria. It is likely that the acetylation of hydroxy groups in the molecule of cycloartane triterpenes, especially at position 26, leads to enhanced antibacterial activity. Besides, compound **1** which has two acetylated hydroxy groups exhibited strong antifungal activity against *Candida albicans* with potency close to that of the positive control (amphotericin B).

4. Conclusions

We herein report the first chemical investigation of propolis from Pitcairn Island. As a result four new cycloartane triterpenes with good antimicrobial activity were isolated from its dichloromethane extract. The biological activity of this extract was also examined and the results showed that it inhibits human breast cancer MDA-MB-231 cells proliferation with potency higher than poplar propolis and induces changes of malignant cells' morphology, which hint to apoptotic events. Apart from this the studied propolis extract possesses strong antimicrobial activity against some of the most common pathogens causing infections in humans: *Staphylococcus aureus*, *Escherichia coli*, and *Candida albicans*. Thus, the present research provides additional data for the tropical propolis constituents, revealing the distinct character of Pitcairn propolis. Furthermore these findings once again certify the potential role of propolis as a natural chemopreventive agent and a valuable bioactive mixture for a combination therapy approach.

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Conflict of interest

The authors declare no conflict of interest.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fitote.2018.05.024.

References

[1] J.M. Sforcin, V. Bankova, Propolis: is there a potential for the development of new drugs, J. Ethnopharmacol. 133 (2011) 253–260, http://dx.doi.org/10.1016/j.jep.2010.10.032.
[2] V. Bankova, M. Popova, B. Trusheva, Plant sources of propolis: an update from a chemist's point of view, Nat. Prod. Commun. 1 (2006) 1023–1028.
[3] V. Bankova, M. Popova, B. Trusheva, Latest developments in propolis research: chemistry and biology, in: S.M. Cardoso, A.M.S. Silva (Eds.), Chemistry, Biology and Potential Applications of Honeybee Plant-Derived Products, Bentham Science Publishers, Sharjah, 2016, pp. 45–66, , http://dx.doi.org/10.2174/9781681082370116010 1.
[4] T. Mosmann, Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays, J. Immunol. Meth. 65 (1983) 55–63, http://dx.doi.org/10.1016/0022-1759(83)90303-4.
[5] CLSI, Performance standards for antimicrobial susceptibility testing, Document M100-S27, 27th ed., Clinical and Laboratory Standards Institute, Wayne, PA, 2017.
[6] M. Balouiri, M. Sadiki, S.K. Ibnsouda, Methods for *in vitro* evaluating antimicrobial activity: a review, J. Pharmaceut. Anal. 6 (2016) 71–79, http://dx.doi.org/10.1016/j.jpha.2015.11.005.
[7] F. Orsini, F. Pelizzoni, L. Verotta, Quadranguloside, a cycloartane triterpene glycoside from *Passiflora quadrangularis*, Phytochemistry 25 (1986) 191–193, http://dx.doi.org/10.1016/S0031-9422(00)94527-8.
[8] H. Achenbach, D. Frey, Cycloartanes and other terpenoids and phenylpropanoids from *Monocyclanthus vignei*, Phytochemistry 31 (1992) 4263–4274, http://dx.doi.org/10.1016/0031-9422(92)80456-O.
[9] K. Awang, X.-M. Loong, K.H. Leong, U. Supratman, M. Litaudon, M.R. Mukhtar, K. Mohamad, Triterpenes and steroids from the leaves of *Aglaia exima* (Meliaceae), Fitoterapia 83 (2012) 1391–1395, http://dx.doi.org/10.1016/j.fitote.2012.10.004.
[10] G.M. Cabrera, M. Gallo, A.M. Seldes, Cycloartane derivatives from *Tillandsia usneoides*, J. Nat. Prod. 59 (1996) 343–347, http://dx.doi.org/10.1021/np960075+.
[11] M.A. González, J. Correa-Royero, L. Agudelo, A. Mesa, L. Betancur-Galvis, Synthesis and biological evaluation of abietic acid derivatives, Eur. J. Med. Chem. 44 (2009) 2468–2472, http://dx.doi.org/10.1016/j.ejmech.2009.01.014.
[12] K. Yoshida, Y. Hirose, Y. Imai, T. Kondo, Conformational analysis of cycloartenol, 24-methylenecycloartanol and their derivatives, Agric. Biol. Chem. 53 (1989) 1901–1912, http://dx.doi.org/10.1080/00021369.1989.10869552.
[13] B. Trusheva, M. Popova, V. Bankova, S. Simova, M.C. Marcucci, P.L. Miorin, F.R. Pasin, I. Tsvetkova, Bioactive constituents of Brazilian red propolis, Evid. based Compl. Alternative Med. 3 (2006) 249–254, http://dx.doi.org/10.1093/ecam/nel006.
[14] T. Hoshino, C. Nakano, T. Ootsuka, Y. Shinohara, T. Hara, Substrate specificity of Rv3378c, an enzyme from *Mycobacterium tuberculosis*, and the inhibitory activity of the bicyclic diterpenoids against macrophage phagocytosis, Org. Biomol. Chem. 9 (2011) 2156–2165, http://dx.doi.org/10.1039/C0OB00884B.
[15] C.D. Carpenter, T. O'Neill, N. Picot, J.A. Johnson, G.A. Robichaud, D. Webster, C.A. Gray, Anti-mycobacterial natural products from the Canadian medicinal plant *Juniperus communis*, J. Ethnopharmacol. 143 (2012) 695–700, http://dx.doi.org/10.1016/j.jep.2012.07.035.
[16] E. Abdel-Sattar, A.R.A. Monem, S.M. Ezzat, A.M. El-Halawany, S.M. Mouneir, Chemical and biological investigation of *Araucaria heterophylla* Salisb resin, Z. Naturforsch. 64c (2009) 819–823, http://dx.doi.org/10.1515/znc-2009-11-1211.
[17] S.A. El Sawi, H.M. Motawe, Labdane, pimarane and abietane diterpenes from the fruits of *Juniperus phoenicea* L grown in Egypt and their activities against human liver carcinoma, Can. J. Pure Appl. Sci. 2 (2008) 115–122.
[18] W. Su, J. Fang, Y. Cheng, Labdanes from *Cryptomeria japonica*, Phytochemistry 37

(1994) 1109–1114, http://dx.doi.org/10.1016/S0031-9422(00)89538-2.
[19] B. Trusheva, M. Popova, E.B. Koendhori, I. Tsvetkova, H. Najdenski, V. Bankova, Indonesian propolis: chemical composition, biological activity and botanical origin, Nat. Prod. Res. 25 (6) (2011) 606–613, http://dx.doi.org/10.1080/14786419.2010.488235.
[20] S.M. Morais, K.A. Silva, H. Araujo, I.G.P. Vieira, D.R. Alves, R.O.S. Fontenelle, A.M.S. Silva, Anacardic acid constituents from cashew nut shell liquid: NMR characterization and the effect of unsaturation on its biological activities, Pharmaceuticals 10 (2017) 31, http://dx.doi.org/10.3390/ph10010031.
[21] B. Trusheva, K. Stancheva, N. Gajbhiye, R. Dimitrova, M. Popova, R. Saraf, V. Bankova, Two new prenylated stilbenes with an irregular sesquiterpenyl side chain from propolis from Fiji islands, Rec. Nat. Prod. 10 (4) (2016) 465–471.
[22] V. Bankova, M.C. Marcucci, S. Simova, N. Nikolova, A. Kujumgiev, S. Popov, Antibacterial diterpenic acids from Brazilian propolis, Z. Naturforsch. 51c (1996) 277–280, http://dx.doi.org/10.1515/znc-1996-5-602.
[23] M.P. Popova, K. Graikou, I. Chinou, V.S. Bankova, GC-MS profiling of diterpene compounds in Mediterranean propolis from Greece, J. Agric. Food Chem. 58 (2010) 3167–3176, http://dx.doi.org/10.1021/jf903841k.
[24] M. Popova, Structural basis of antimicrobial activity of Mediterranean propolis, in: T. Farooqui, A.A. Farooqui (Eds.), Beneficial Effects of Propolis on Human Health and Chronic Deseases, Nova Science Publishers, Inc., New York, 2013, pp. 171–188.
[25] R. Silva-Carvalho, V. Miranda-Gonçalves, A.M. Ferreira, S.M. Cardoso, A.J.F.N. Sobral, C. Almeida-Aguiar, F. Baltazar, Antitumoural and antiangiogenic activity of Portuguese propolis in *in vitro* and *in vivo* models, J. Functional Foods 11 (2014) 160–171, http://dx.doi.org/10.1016/j.jff.2014.09.009.
[26] A. Rzepecka-Stojko, A. Kabała-Dzik, A. Moździerz, R. Kubina, R.D. Wojtyczka, R. Stojko, A. Dziedzic, Ż. Jastrzębska-Stojko, M. Jurzak, E. Buszman, J. Stojko, Caffeic acid phenethyl ester and ethanol extract of propolis induce the complementary cytotoxic effect on triple-negative breast cancer cell lines, Molecules 20 (2015) 9242–9262, http://dx.doi.org/10.3390/molecules20059242.
[27] O. Milošević-Đorđević, D. Grujičić, M. Radović, N. Vuković, J. Žižić, S. Marković, *In vitro* chemoprotective and anticancer activities of propolis in human lymphocytes and breast cancer cells, Arch. Biol. Sci., Belgrade 67 (2015) 571–581, http://dx.doi.org/10.2298/ABS141013019M.
[28] H. Chang, Y. Wang, X. Yin, X. Liu, H. Xuan, Ethanol extract of propolis and its constituent caffeic acid phenethyl ester inhibit breast cancer cells proliferation in inflammatory microenvironment by inhibiting TLR4 signal pathway and inducing apoptosis and autophagy, BMC Compl. Alternative Med. 17 (2017) 471, http://dx.doi.org/10.1186/s12906-017-1984-9.
[29] M. Hemshekhar, M.S. Santhosh, K. Kemparaju, K.S. Girish, Emerging roles of anacardic acid and its derivatives: a pharmacological overview, Basic Clin. Pharmacol. Toxicol. 110 (2012) 122–132, http://dx.doi.org/10.1111/j.1742-7843.2011.00833.x.
[30] D. Teerasripreecha, P. Phuwapraisirisan, S. Puthong, K. Kimura, M. Okuyama, H. Mori, A. Kimura, C. Chanchao, *In vitro* antiproliferative/cytotoxic activity on cancer cell lines of a cardanol and a cardol enriched from Thai *Apis mellifera* propolis, BMC Compl. Alternative Med. 12 (2012) 27, http://dx.doi.org/10.1186/1472-6882-12-27.
[31] T.N. Ngo, N.M. Phan, T.D. Bui, V.S. Dang, C.L. Tran, D.T. Mai, T.P. Nguyen, Cytotoxic cycloartane triterpenoids from the leaves of *Markhamia stipulata* var. *canaense*, Phytochem. Lett. 22 (2017) 251–254, http://dx.doi.org/10.1016/j.phytol.2017.10.020.
[32] A.C. Abreu, A.J. McBain, M. Simões, Plants as sources of new antimicrobials and resistance-modifying agents, Nat. Prod. Rep. 29 (2012) 1007–1021, http://dx.doi.org/10.1039/c2np20035j.
[33] A. Kujumgiev, I. Tsvetkova, Y. Serkedjieva, V. Bankova, R. Christov, S. Popov, Antibacterial, antifungal and antiviral activity of propolis of different geographic origin, J. Ethnopharmacol. 64 (1999) 235–240, http://dx.doi.org/10.1016/S0378-8741(98)00131-7.