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Abstract

A new triterpenic diester, 3,21-dipalmitoyloxy-16β,21α-dihydroxy-β-amyrine(**1**), along with two natural cyclitols, conduritol C (**2**) and viburnitol (**3**), four known triterpenes (**4-7**), and seven known flavonoids (**8-14**) were isolated from the aerial parts of *Chrysanthemum macrocarpum*. Their structures were established on the basis of extensive 1D and 2D NMR (^{1}H, ^{13}C, COSY, HMBC, HSQC, and ROESY) and ESIMS studies. The chloroform fraction, taraxasterol (**4**) and β-sitosterol (**7**) were investigated for their antibacterial activity against *Staphylococcus aureus*, *Enterococcus faecalis*, *Escherichia coli*, *Pseudomonas aeruginosa* and *Klebsiella pneumoniae.* The chloroform fraction and taraxasterol (**4**) showed a weak antibacterial activity and were evaluated for their cytotoxic activity against human colon cancer HT-29 cells and human prostate carcinoma PC3 cells. The results indicated that both the chloroform fraction and taraxasterol (**4**) inhibited cell proliferation of both PC3 and HT-29 cells.

Keywords:

Chrysanthemum macrocarpum, Asteraceae, Triterpenic diester, Cyclitol, NMR, Antibacterial activity

1. Introduction

The *Chrysanthemum* genus (Asteraceae) is found world-wide and some species have been extensively used as food additives or in traditional medicine to treat inflammation, headache, ulcerative colitis, vertigo, eye irritation, hypertension, and respiratory diseases (Jiangsu, 1993; Yang et al., 2000; Yoshikawa et al., 2000; Matsuda et al., 2002). A series of recent studies have suggested that the *Chrysanthemum* genus possesses a strong anti-cancer and anti-human immunodeficiency virus type 1 (HIV-1) activity. (Ukiya et al., 2002; Lee et al., 2003), Anti-inflammatory (Cheon et al., 2009), antinociceptive (Shi et al., 2011) as well as anti-oxidant activities (Yan et al., 1999) have been also confirmed. The phytochemical profile of *Chrysanthemum* species has shown the presence of terpenoids (Ukiya et al., 2001), flavonoids and phenolic compounds (Lin and Harnyl, 2010). Fifteen *Chrysanthemum* species grow in Algeria (Quezeland Santa, 1963).*Chrysanthemum macrocarpum* (Sch.Bip.)Coss.&Kralik ex Batt., a synonym of *Endopappus macrocarpus* (Coss. & Kralik ex Batt. & Trabut) Sch.Bip., is an endemic species used in traditional medicine as a scabicide and to treat intestinal infections. Touareg people also use this species in food for flavouring and as an herbal tea (Boukef, 1986; Bellakhdar, 1997). We have previously analyzed the chemical composition of the essential oil of this species (Boutaghane et al., 2008). This paper reports the isolation and structural elucidation of one new triterpene fatty acid diester **(1)** and two natural cyclitols, in addition to four known triterpenes, and seven known flavonoids from the aerial parts of *C. macrocarpum* (Fig. 1). The antibacterial activity and cytotoxic activity against HT-29 and PC3 cells of the chloroform soluble fraction and taraxasterol (**4**) were also evaluated in order to obtain an insight into the beneficial effects of this plant species.

2. Results and discussion

The aerial parts of *C. macrocarpum* were extracted with 20% aq. MeOH. After MeOH removal, the crude extract in H_2O was successively partitioned with $CHCl_3$, EtOAc, and *n*-BuOH. The $CHCl_3$ soluble fraction was purified by chromatographic separations over silica gel, yielding the new triterpene fatty ester (**1**) and the known compounds identified as taraxasterol (**4**) (Reynolds et al., 1985), stigmasterol (**6**), β-sitosterol (**7**) (Kojima et al., 1990), and calenduladiol-3-*O*-myristate (**5**) (Ukiya et al., 2001) (Fig. 1). The purification of the EtOAc soluble fraction gave the natural cyclitolsconduritol C (**2**)(Hiroki et al., 1998) and viburnitol (**3**) (Kindl et al., 1966) along with seven known flavonoids: apigenin (**8**), 7-*O*-methylapigenin (**9**), apigenin-7-*O*-glucoside (**10**) (Bennini et al., 1992), kaempferol-3-*O*-glucoside (**11**), kaempferol-7-*O*-glucoside (**12**) (Kim et al., 2005), luteolin-3'-*O*-glucoside (**13**) (Moiteiro et al., 2008) and 6-*C*-glucosyl-8-*C*-arabinosylapigenin (**14**) (Krafczyk et al., 2008). Their structures were elucidated using chemical and spectroscopic methods including 1D and 2D NMR experiments, and ESIMS.

*2.1 Structure elucidation of compounds **1-3***

Compound **1** was obtained as a viscous liquid. The HRESIMS (positive ion mode) exhibited a quasimolecular ion peak at *m/z* 957.8634 $[M+Na]^+$ (calc. for $C_{62}H_{110}O_5Na$, 957.8653) suggesting a molecular formula of $C_{62}H_{110}O_5$. The positive ESIMS spectrum gave the same pseudomolecular ion peak at *m/z* 957 $[M+Na]^+$ with a ion fragment observed at *m/z* 701 $[M+Na–C_{16}H_{32}O_2]^+$ in agreement with the loss of a palmitoyloxy side chain. The presence of two acyloxy groups in compound **1** was supported by the presence in the ^{13}C NMR spectrum of two carbonyl signals at δ_C 173.3 and 173.7 and in the ^1H NMR spectra of signals due to two methylene groups bearing a carbonyl group at δ_H 2.32 (q^td, J = 7.1, 1.0 Hz, H-2') and 2.36 (q^td, J = 7.7, 2.7 Hz, H-2''), and for two

terminal methyl groups at δ_H 0.90 (6H, t, *J* = 7.4 Hz, H-16' and H-16"). The ^{1}H NMR spectra of **1** showed the presence of eight methyl singlets at δ 0.81, 0.88, 0.89, 0.91, 1.00, 1.01, 1.02 and 1.27, an olefinic proton at δ 5.33 (t, *J* = 3.5Hz, H-12), three methine protons bearing an oxygen at δ 4.43 (dd, *J* = 11.7, 4.7 Hz, H-16), 4.53 (dd, *J* = 9.3, 5.5 Hz, H-3) and 4.73 (brt, *J* = 3.4 Hz, H-21) (Table 1). The basic Δ^{12} oleanene skeleton (Mahato and Kundu, 1994) in **1** was supported by the following key HMBC correlations H-3/C-2, C-4, C-5, C-23, C-24; H-12/C-9, C-11, C-13, C-14, C-18; H-16/C-14, C-15, C-17, C-18, C-28; H-21/C-17, C-19, C-20, C-22, C-29, and C-30. The small coupling constant of H-21 (brt, *J* = 3.4 Hz, H-21) indicated an α-axial position for the hydroxyl group, and the large coupling constants of H-16 (*J* = 11.7 Hz) and H-3 (*J* = 9.3 Hz), indicated a β-equatorial position for the hydroxyl groups. In the ROESY experiment, the interaction observed between H-18/H-30 and between H-21/H-30 confirmed the β-equatorial position for H-21.The α-axial orientation of H-3 and H-16 was also confirmed by the ROEs observed between H-3 and H-5α and H-23α, while H-16 showed a ROE correlation with H-27α. Thus, the aglycone of compound **1** was determined to be 3β,16β,21α-trihydroxy-olean-12 ene. The downfield shifts of H-3 and H-21 at δ_H 4.53 and 4.73, respectively in **1** suggested the esterification of the secondary hydroxyl groups at C-3 and C-21 by two acyloxy groups. This was confirmed by the HMBC correlations observed between H-3 (δ_H 4.53) and the carbonyl carbon signal of the first acyloxy side chain (δ_C 173.7), and between H-21 (δ_H 4.73) and the second carbonyl carbon signal (δ_C 173.3). The two acyloxy groups were identified by ESIMS as being palmitic acid esters. Subtraction of the triterpene unit from the molecular formula of the ion fragment at *m/z* 701 $[M+Na–C_{16}H_{32}O_2]^+$ indicated that the second fatty acid ester was identical and correspond to a palmitic acid. Another ion fragment observed at *m/z* 685 $[M+Na–HO–C_{16}H_{32}O_2]^+$ indicated the simultaneously loss of the

palmitic acid in C-21 position and OH in C-16 position. Thus, compound **1** was identified as 3,21-dipalmitoyloxy-16β,21α-dihydroxy-β-amyrine.

Compound **2** was obtained as colourless crystals and afforded a $[M+Na]^+$ quasimolecular ion peak at *m/z* 169 suggesting the molecular formula of $C_6H_{10}O_4Na$. In the ^{13}C NMR spectrum, six carbon signals were observed at δ_C 66.6, 71.2, 72.4, 72.5, 126.6 and 132.4, and the corresponding ^1H signals were observed at δ_H3.95, 3.64, 4.20, 3.43, 5.83 and 5.75, respectively (Table 2). The ^1H–^1H COSY spectrum of **2** showed a cyclic spin system with correlations observed between the olefinic protons at δ_H 5.75 and δ_H5.83 and both protons H-4 (δ_H4.20) and H-1 (δ_H3.95). H-4 and H-1 were also correlated with H-3 (δ_H3.43) and H-2 (δ_H3.64), respectively, and H-2 with H-3, suggesting a cyclohexenetetraol. In the HMBC spectrum H-4 and H-1correlated with C-5 and C-6 indicating that the double bond was located in the C-5 (6) positions. The small coupling constant of H-4 (t, J = 4.5 Hz) indicate an α-configuration of the hydroxyl group. The large coupling constants up to 7 Hz for protons H-1, H-2 and H-3 indicate a β-configuration for the three hydroxyl groups in these positions. Therefore, **2** was deduced as being cyclohex-5-ene 1β,2β,3β,4α-tetraol, also named conduritol C previously obtained by synthesis (Balci et al., 1990). To our knowledge, this is the first time that this compound has been isolated in a natural product.

Compound **3** displayed a quasi-molecular ion peak at *m/z* 187 $[M+Na]^+$ indicating a molecular weight of 164 which is 18 mass units greater than that of **2** and suggesting a molecular formula of $C_6H_{12}O_5$. Careful comparison of the NMR spectroscopic data of **3** with that of **2** (Table 2) showed in the ^1H and ^{13}C NMR signals of **3**, the absence of the two olefinic carbon signals at δ_C126.6 and 132.4 and the occurrence of hydroxyl group (δ_H 4.02/δ_C 69.6) and methylene carbon at δ_C 37.1. Full examination of the 2D NMR

spectroscopic data further confirmed the structure of compound **3** as cyclohexa-1β,2α,3β,4α,5α-pentaol also named viburnitol (Kindl, H., et al., 1969).

2.2 Antibacterial and cytotoxic activities

The antibacterial activity of the $CHCl_3$ soluble fraction, EtOAc soluble fraction, *n*-BuOH soluble fraction, and compounds **4** and **7** was performed using the disc diffusion method against two Gram-positive (*S. aureus and E. faecalis*) and three Gram-negative (*E. coli, P. aeruginosa, K.pneumoniae*) bacteria (Table 3). The chloroform fraction and **4** and **7** were further analyzed by TLC-direct bioautography on silica gel GF_{254} to determine the number of active compounds. As observed with the disc diffusion method, the assayfor bioautography demonstrated weak inhibition zones of chloroform fraction and taraxasterol (**4**) against the growth of Gram-negative (*E. coli, P. aeruginosa, K.pneumoniae*) bacteria. The clear zones were located separately on TLC suggesting that more than one compounds at different R_f values involved in antibacterial effects. Nevertheless, no inhibition zone was observed with compound **7** at the concentration tested. Minimum inhibitory concentration (MICs) determination was performed by the serial dilution method (Table 3), ampicillin was used as a positive control in these tests. Only the $CHCl_3$ fractionof *C. macrocarpum* notably inhibited the growth of all the tested bacteria (Gram-positive and Gram negative), and was shown to possess the strongest antibacterial activity against Gram negative with the largest inhibition zones (30 and 24 mm for *P. aeruginosa* and *E. coli*, respectively) and lowest MICs 0.5 to 8 µg/ml.Compound **4** also showed relatively modest activity, most particularly against Gram negative bacteria *K. pneumonia* (32 µg/ml), *E. Coli* (32 µg/ml) and *P. aeruginosa* (16 µg/ml).Previous findings have shown that taraxasterol exhibited antibacterial properties (Singh et al., 2002; Singh and Dubey, 2001; Villarreal et al., 1994; Dai et al., 2001). The antibacterial activity of the chloroform fraction could,

in part, be associated with the triterpenes such as taraxasterol (**4**), the main component of the chloroform fraction. In fact, it was also possible that the components in lower percentage might be involved in some type of synergism with the other active compounds. Our results support the use of *C. macrocarpum* in traditional medicine for diseases caused by infective agents.

To determine the cytotoxicity of the chloroform soluble fraction of *C. macrocarpum* and taraxasterol (**4**), cell proliferation was evaluated by MTT assay on two cell lines, the human HT-29 colorectal cancer cell line and the human PC3 prostate cancer cell line. The chloroform fraction and taraxasterol inhibited cell proliferation of both HT-29 and PC3 cancer cells in a dose- and time-dependent manner. The IC_{50} were calculated on PC3 and HT-29 cells at 48h as being 69.3 and 103.1 µg/ml, respectively, for chloroform fraction and 37.1 µM and 89.7 µM, respectively, for taraxasterol (**4**) (Table 4). Taraxasterol seems to be responsible of the activity of the extract. It is known to possess antiproliferative activity against various cancer cells (Villarreal et al., 1994; Dai et al., 2001).

In conclusion, a new triterpenic diester, 3,21-dipalmitoyloxy-16β,21α-dihydroxy-β-amyrine(**1**), and two natural cyclitols, conduritol C (**2**) and viburnitol(**3**), together with four known triterpenes, and seven known flavonoids were isolated from the aerial parts of *Chrysanthemum macrocarpum*. This study clearly show that the chloroform fraction presents a good activity against Gram-negative bacteria (*P. aeruginosa, K. pneumonia, E. coli*) with MICs values of 0.5, 4 and 8 µg/ml, respectively, better than ampicilline used as control. At these concentrations, no cytotoxic activity was observed on PC3 and HT-29 cells. It can be concluded that this plant may serve as a very good source of potential antibacterial agent and justify their used in traditional medicine as scabicides and for the treatment of intestinal infections.

3. Experimental

3.1. General experimental procedures

Optical rotations were measured in MeOH or $CHCl_3$ with a Perkin-Elmer 241 polarimeter. ^{1}H and ^{13}C NMR spectra were recorded on a BrukerAvance DRX 500 NMR spectrometer (^{1}H at 500 MHz and ^{13}C at 125 MHz). 2D-NMR experiments were performed using standard Brukermicroprograms. Coupling constants were expressed in Hz, and chemical shifts were given on a δ (ppm) scale. ESIMS and HR-ESIMS experiments were performed using a Micromass Q-TOF micro instrument (Manchester, UK). The samples were introduced by direct infusion in a solution of MeOH at a rate of 5 µL min^{-1}.TLC was carried out on precoated silica gel 60 F_{254} (Merck), and spots were visualized by heating after spraying with 50% H_2SO_4. Kieselgel 60 (63-200 mesh) Merck or LiChroprep RP-18 (40-63 µm) Merck silica gel were used for Column chromatography (CC). HPLC was performed on a Dionex apparatus equipped with an ASI-100 autosampler, a P580 pump, a UVD 340S diode array detector, and Chromeleon® software.A prepacked RP-18 column (201SP510, 250 × 10 mm, 5μm, 90 Å, Dionex, Vydac, France) was used for semi preparative HPLC of the flavonoids with a binary gradient elution (solvent A: H_2O with 0.0025% TFA, solvent B: MeCN) at 25 °C and a flow rate of 3.5 mL min^{-1}; the chromatogram was monitored at 254 and 365 nm. The following reagents were used for cell culture assay: DMEM and foetal calf serum (Gibco BRL, Cergy-Pontoise, France), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), and sodium dodecyl sulfate (SDS) (Sigma Chemical Co., St. Louis, MO).

3.2. Plant material

The aerial parts of *Chrysanthemum macrocarpum*(Sch.Bip.) Coss.&Kralik ex Batt.were collected in the flowering season from Ghardaia (Algerian septentrionalsahara) in April 2005. The botanical determination was performed by Pr Gérard De Bélair from Annaba-University (Algeria). A voucher specimen has been deposited in the botanic Museum of Angers, France under reference MBAng 2005-8.

3.3 Extraction and isolation

Dried and powdered aerial parts (1380 g) of *C. macrocarpum* were macerated in 20% aq. MeOH (10 l). The hydromethanolic extract was concentrated to dryness (under low pressure). The residue was suspended in H_2O and successively partitioned with $CHCl_3$, EtOAc, and *n*-BuOH (3 ×300 ml, each), respectively, affording a $CHCl_3$ soluble fraction (20 g), an EtOAc-soluble fraction (4 g), and a *n*-BuOH-soluble fraction (20 g). A part (10 g) of the $CHCl_3$ soluble fraction was fractionated by silica gel CC, using a gradient of cyclohexane-EtOAc (1:0 to 0:1) to give 20 fractions. The fraction 6 (964 mg) eluted with cyclohexane-EtOAc (95:5) was fractionated by silica gel CC eluted with a gradient of cyclohexane-$CHCl_3$ (1:0 to8:2).Fractions 71-99 (290 mg), eluted with cyclohexane-$CHCl_3$ (9:1) were subjected to silica gel CC using 100% toluene as solvent, and then fractions 62-75 (141 mg) were further purified by TLC preparative in toluene-acetone (98:2) to give compound **1** (6.5 mg). The fraction 9 (680 mg) was submitted to silica gel CC using a gradient of toluene-acetone (1:0 to8:2) to give taraxasterol **4** (30 mg). Preparative TLC of fractions 36-50 (24 mg), eluted with toluene-acetone (98:2), developed with a mixture of toluene-acetone (96:4), give taraxasterol **4** (2.3 mg), and calenduladiol-3-*O*-myristate **5 (**8 mg).A part of fraction 12, eluted with cyclohexane-EtOAc (8:2) was subjected to silica gel vacuum liquid chromatography (VLC)using a gradient of cyclohexane-EtOAc (9:1 to 6:4). Three fractions of 150 ml were collected and monitored by TLC in cyclohexane-EtOAc (8:2).

Fraction 2 was subjected to silica gel CC using petroleum ether-EtOAc (1:0 to 8:2) to give stigmasterol **6** (15 mg) and β-sitosterol**7** (25 mg) in pure form.

The EtOAc soluble extract was fractionated by silica gel CC, using a gradient of $CHCl_3$-MeOH (1:0 to 0:1). Fractions 7-9 (150 mg), eluted with $CHCl_3$-MeOH (95:5) were purified by silica gel CC eluted with EtOAc-MeOH (99:1 to 8:2) to provided apigenin**8** (13 mg) and 7-*O*-methyl-apigenin **9** (10 mg). Fractions 13-16 (491 mg), eluted with $CHCl_3$-MeOH (9:1), were fractionated by silica gel CC using a gradient of EtOAc–MeOH (1:0 to 6:4) to afforded fifteen fractions. Fractions eluted with EtOAc-MeOH (85:15) were submitted to reversed-phase (RP-18) CC, using a gradient of MeOH-H_2O (6:4 to 1:0) as eluent, to yield conduritol C **2** (27 mg) and apigenin-7-*O*-glucoside **10** (30 mg). The purification of 25 mg of fraction 17, using the same conditions, afforded viburnitol **3** (4.4 mg). Fractions 18-21 (108 mg), eluted with $CHCl_3$-MeOH (85:15), were purified by semi-preparative HPLC, using a linear gradient (15 to 20 % B during 20 min) at 4 ml/min to give kaempferol-3-*O*-glucoside **11** (t_R 9.01 min, 3.5 mg), kaempferol-7-*O*-glucoside **12** (t_R 12.34 min, 5.5 mg), and luteolin-3'-*O*-glucoside **13** (t_R 11.24 min, 3.7 mg). Fraction 22 (25 mg) eluted with $CHCl_3$-MeOH (8:2), gave 6-C-glucosyl-8-C-arabinosylapigenin **14** (t_R 20.19 min, 7 mg), by semi-preparative HPLC using a linear gradient (10 to 20 % B during 22 min) at 4 ml/min.

*3.3.1 Compound **1***

Colourless viscous liquid; ^1H and ^{13}C NMR, see Table 1; HRESIMS (positive-ion mode) *m/z*: 957.8634 $[M+Na]^+$ (calcd for $C_{62}H_{110}O_5Na$: 957.8653); ESIMS (positive-ion mode) *m/z*: 957 $[M+Na]^+$, 701 $[M+Na–C_{16}H_{32}O_2]^+$, 685 $[M+Na–OH–C_{16}H_{32}O_2]^+$; $[\alpha]_D^{20}$+11.5° (*c* 0.76, $CHCl_3$).

*3.3.2 Conduritol C (**2**)*

Colourless crystals; ^{1}H and ^{13}C NMR, see Table 2; ESIMS (positive-ion mode) *m/z*: 169 $[M+ Na]^{+}$; $[\alpha]_D^{20}$ 209.5° (*c* 0.27, MeOH).

*3.3.3 Viburnitol (**3**)*

Colourless crystals; ^{1}H and ^{13}C NMR, see Table 2; ESIMS (positive-ion mode) *m/z*: 187 $[M+ Na]^{+}$.

3.4 Antibacterial activity

American Type of Culture Collection (ATCC) standard against namely: *Staphylococcus aureus* (ATCC 25923), *Enterococcus faecalis* (ATCC 29212) and Gram negative *Escherichia* coli (ATCC 25922), *Pseudomonas aeruginosa* (ATCC 27853) and the clinically isolated strain *Klebsiella pneumonia* were used. The reference strains were obtained from the Pasteur Institute (Algiers). The clinical strain was obtained from the laboratory of bacteriology, Benbadis Hospital, Constantine, using conventional methods (clinical isolation).

3.4.1. Antibacterial screening

The disk diffusion assay was used with some modifications (Rasoanaivo and Ratsimamanga, 1992; NCCLS, 1997) to determine the growth inhibition of bacteria by the $CHCl_3$ soluble fraction, EtOAc soluble fraction, *n*-BuOH soluble fractionand compounds **4** and **7**of *C. macrocarpum*. The bacteria tested were maintained at 4 °Con nutrient agar (NA). Base plates were prepared by pouring 10 ml Mueller–Hinton (MH) agar into sterile petri dishes (9 cm) and allowed to set. Molten MH agar held at 48 °C was inoculated with a broth culture (10^6–10^8 bacteria per ml) of the test organism and poured over the base plates forming a homogenous top layer. Paper discs (Whatman No. 3, 6mm diameter) were sterilized by autoclaving. 10 µl of the fractions or compounds **4** and **7**(128 mg/10ml) were applied to paper disc so that each disc contains 128 µg of sample. After evaporation of the solvent in a sterile atmosphere, paper discs were placed

in the top layer of the MH agar plates. The fractions were tested in triplicate, with ampicillin (10 µg ml^{-1}) and ceftazidime (30 µg $ml^{-}1$) disc as reference. The plates were evaluated after incubation at 37°C for 18 h. The results were recorded by measuring the diameter of inhibition zone at the end of 18 hours.

3.4.2. Thin-layer chromatography-direct bioautography

Thin-layer chromatography (TLC)-direct bioautographic method (Shai et al., 2008) was applied to identify and localize active compounds in the chloroform fraction against *Pseudomonas aeruginosa, Escherichia* coli (ATCC 25922), *Staphylococcus aureus* (ATCC 25923), *Enterococcus faecalis* (ATCC 29212) and *Klebsiella pneumonia*. The chloroform fraction or the compounds **4** and **7** were dissolved in chloroform at a concentration of 1 mg/ml. 100 µl of the chloroform fraction or 50 µl of **4** and **7** were spotted at 1 cm from the base of preparative Merck chromatography silica gel 60 F_{254} TLC plates. TLC plate loaded with the chloroform fraction and the pure compounds was developed in a hexane: ethyl acetate (8:2, v/v) solvent system. After migration, the solvent was evaporated. TLC plates were overlapped homogenously with 10 ml of nutrient agar (Mueller-Hinton) infected by nutrient broth containing bacteria (10^8cfu/ml). After solidification of the medium, the TLC plates were incubated for 24 h at 37 °C. Subsequently, bioautograms developed were sprayed with 2 mg/ml of *p*-iodonitrotetrazolium bromide (INT) (Sigma). Clear zones on bioautograms indicated inhibition of growth after incubating for 2 h at 37 °C.

3.4.3 Determination of the minimums inhibitory concentrations (MICs).

Tests were performed by the agar dilution method according to the NCCLS with some modifications. The $CHCl_3$ soluble fraction or compounds **4** and **7** were dissolved in ethanol to a final concentration of 512 µg/ml. This was serially diluted 2 fold with MH medium to obtain concentration of 0.5, 1, 2, 4, 8, 16, 32, 64 and 128 µg/ml. 2 ml of each

concentration was added in Petri dished containing 18 ml of MH medium previously inoculated with suspension of bacteria (10^5 CFU/ml) for testing the samples. Control plates contain the culture medium and 2 % of ethanol. Plates were incubated at 37 °C for 18hours. The minimum inhibitory concentration (MIC) was defined as the concentration at which no colony was observed after incubation. All experiments were performed in triplicates.

3.5 Cell culture and cell proliferation assay (MTT)

Human colorectal cell line HT-29 and human prostate carcinoma PC3 cells were purchased from American Type Culture Collection (LGC Standards, Middlesex, UK). Cells were seeded at $3.10^4/cm^2$ and grown in DMEM, supplemented with 10% foetal calf serum, 100 units/ml of penicillin, 100 mg/ml of streptomycin. Cells were incubated under 5% CO_2 humidified atmosphere at 37 °C. Cell viability was determined by the trypan blue dye exclusion method.

The effect of the chloroform fraction of *C. macrocarpum* and the isolated taraxasterol (**4**) on cell viability was assessed using the [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] (MTT) assay, as described previously (Mosmann, 1983). This test is based on the ability of the mitochondrial succinate deshydrogenase enzyme of living cells to reduce MTT to a purple-blue insoluble formazan precipitate. The HT-29 and PC3 cells were plated in 96-well plates at concentration of 10^4 cells/well and incubated for 24 h, then treated with various concentrations of chloroform fraction or **4** or ursolic acid used as positive control which were dissolved in dimethyl sulfoxide (DMSO) and diluted with culture medium to give the appropriate final concentrations of 20-140 µg/ml. Controls were done with the same final DMSO concentration in the medium as samples. After incubation of the cells with chloroform fraction for 24 or 48 h or with **4** for 48 hat 37 °C, 10 µl of 5 mg/ml MTT solution were added to each well

and incubated for 4 h at 37 °C. Then, 100 µl of 10% sodium dodecyl sulfate (SDS) in 0.01 M HCl was added to each well. The degree of formazan formation, an indicator of living and metabolically active cells, was determined using ELISA reader at 550 nm. Six wells per dose and time point were measured in 3 different experiments.

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Table 1. ^{1}H and ^{13}C NMR Spectroscopic Data ($CDCl_3$) of compound **1**

1					
position	δ_C	δ_H (*J* in Hz)		δ_C	δ_H (*J* in Hz)
1a 1b	38.3	1.09, m 1.65, m	Acyl in C-3		
2a 2b	23.6	1.66, m 1.92, m	1'	173.7	
3	80.5	4.53, dd (9.3, 5.5)	2'	34.9	2.32, q^{t}d (7.1, 1.0)
4	37.8		3'	25.2	1.65, m
5	55.3	0.88, m	4'- 13'	29.1-29.9	1.28, brs
6a 6b	18.2	1.44, m 1.58, m	14'	31.9	1.28, m
7a 7b	32.9	1.37, m 1.56, m	15'	22.7	1.32, m
8	39.8		16'	14.2	0.90, t (7.4)
9	46.8	1.58, m	Acyl in C-21		
10	36.8		1"	173.3	
11	23.6	1.97, m	2"	34.9	2.36, q^{t}d (7.7, 2.7)
12	122.9	5.33, brt (3.5)	3"	25.2	1.67, m
13	142.9		4"-13"	29.1-29.9	1.28, brs
14	43.9		14'	31.9	1.28, m
15a 15b	35.4	1.65, m 1.29, m	15"	22.7	1.32, m
16	68.3	4.43, dd (11.7, 4.7)	16"	14.2	0.90, t (7.4)
17	37.4				
18	48.5	2.28, dd (14.1, 4.2)			
19a 19b	42.1	1.06, m 2.11, t (13.8)			
20	34.3				
21a 21b	76.5	4.73, brt (3.4)			
22a 22b	34.1	1. 39, dd (15.7, 3.3) 2.17, dd (15.7, 3.1)			
23	28.1	0.91, s			
24	16.8	0.89, s			
25	15.6	1.00, s			
26	16.8	1.01, s			
27	26.7	1.27, s			
28	21.4	0.81, s			
29	27.4	0.88, s			
30	24.8	1.02, s			

Table 2. ^{1}H and ^{13}C NMR Spectroscopic Data (CD_3OD) of compounds **2-3**

		2		**3**
	δ_C	δ_H(*J* in Hz)	δ_C	δ_H (*J* in Hz)
1	66.6	3.95, d (7.5)	69.8	3,81, ddd (14.6, 4.8, 2.7)
2	71.2	3.64, dd (10.4, 7.7)	75.6	3.20, t (9.3)
3	72.5	3.43, dd (10.4, 4.5)	74.3	3.60, t (9.4)
4	72.4	4.20, t (4.5)	79.3	3. 42, dd (9.6, 3.0)
5	126.6	5.83, ddd (10.0, 4.5, 2.0)	69.6	4.02, d (2.6)
6	132.4	5.75, dd (10.0, 2.0)	37.1	1.50, ddd (10.0 , 2.0) 2.10 dt (14.4)

Table 3. Minimal Inhibitory Concentration (MIC) and Inhibition Zones of $CHCl_3$ fraction and compounds **4** and **7** from *Chrysanthemum macrocarpum* against Gram positive and negative bacteria.

	Inhibition Zone diameters (mm)					MIC (µg/ml)				
Microorganisms	$CHCl_3$ fraction	**4**	**7**	Ampicilline	Ceftazidime	$CHCl_3$ fraction	**4**	**7**	Ampicilline	Ceftazidime
S. aureus	24	10	12	30		128	128	128	5	
E. faecalis	30	10	10	16		128	128	128	16	
E. coli	24	18	-	18		8	32	>128	10	
P. aeruginosa	30	24	-	-	22	0.5	16	>128	-	16
K. pneumoniae	18	12	-	14		4	32	>128	32	

–, No inhibition zone

Table 4. IC_{50} of $CHCl_3$ fraction of *Chrysanthemum macrocarpum* and compound **4** on the proliferation of human PC3 prostate cancer cells line and human colon cancer HT-29 cells line at 24 and 48h

Sample	PC3 ($IC_{50,}$ µg/ml)		HT-29($IC_{50,}$ µg/ml)	
	24 h	48 h	24 h	48 h
$CHCl_3$ fraction	80.8	69.3	122.4	103.1
Taraxasterol **4**	nt	15.8 (37.1 µM)	nt	38.3 (89.7 µM)
Ursolic acid	nt	25 µM	nt	25 µM

nt : not tested at 24h

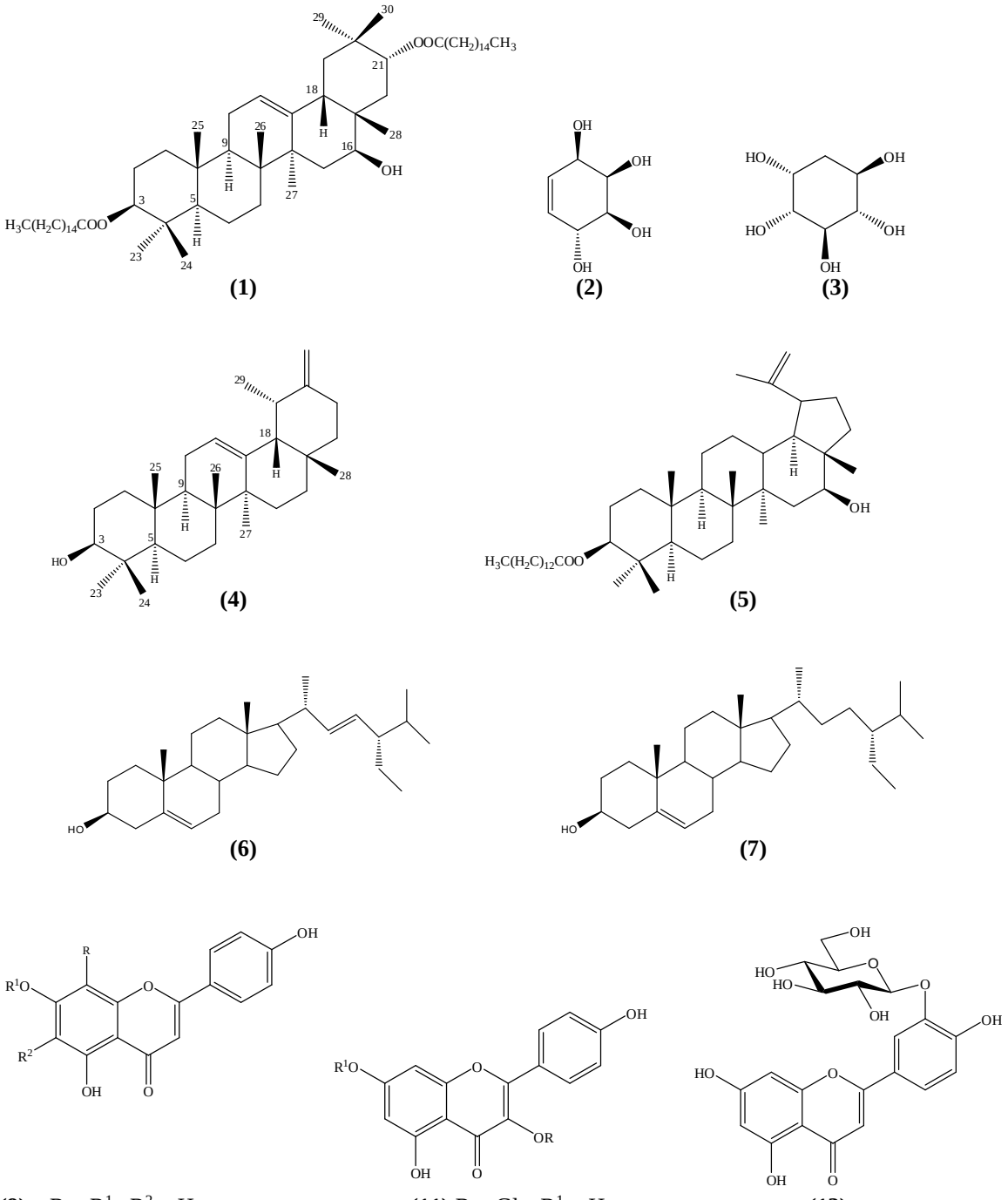

Fig. 1. Structures of compounds **1**–**14**