Non-Alkaloidal Compounds from the Bulbs of the Egyptian Plant *Pancratium maritimum*

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Phytochemical investigation of the cytotoxic fractions of fresh bulbs of *Pancratium maritimum* L. led to the isolation and structure identification of two new compounds, pancricin (**1**) and pancrichromone (**4**), together with four known compounds, including 2,4-dihydroxy-6-methoxy-3-methyl acetophenone (**2**), 5-formylfurfuryl acetate (**3**), 7-β-D-glucosyloxy-5-hydroxy-2-methylchromone (**5**), and ethyl-β-D-glucopyranoside (**6**). Their structures were established on the basis of 1D and 2D NMR spectroscopy (^{1}H, ^{13}C, COSY, HSQC, and HMBC), as well as HR mass spectral analyses. The compounds were evaluated for their antimigratory and antiproliferative activities against the highly metastatic human prostate cancer cell line (PC-3M). Compound **5** was the most active compound displaying good activity in the proliferation assay comparable to that of the positive control 4-hydroxyphenylmethylene hydantoin, while it displayed only weak antimigratory activity compared to the positive control 4-ethylmercaptophenylmethylene hydantoin.

Key words: Amaryllidaceae, *Pancratium maritimum*, Antimigratory and Antiproliferative Activities

Introduction

Amaryllidaceae have attracted attention as a source of valuable biologically active alkaloids. *Pancratium maritimum* L. is characteristic for sandy coastal habitats of the Mediterranean and exhibits antifungal, analgesic, and anticancer activities. Also, it is used as a biopesticide, emetic, hypotensive, purgative, and for treatment of spleen inflammation (El-Hadidy *et al*., 2012). Previous phytochemical studies on the bulbs led to the isolation of alkaloids (Ibrahim *et al*., 2013; Abou-Donia *et al*., 1992; Youssef and Frahm, 1998; Youssef, 1999; Berkov *et al*., 2004), chromones, flavonoids, and acetophenones (Ali *et al*., 1990; Youssef *et al*., 1998). Here, we report the isolation from fresh bulbs and structural elucidation of two new compounds, *viz.* pancricin (**1**) and pancrichromone (**4**), together with four known compounds. In addition, the antimigratory and antiproliferative activities of the compounds were evaluated.

Material and Methods

General

Melting points were determined on an Electrothermal 9100 Digital Melting Point apparatus (Electrothermal Engineering, Southend-on-Sea, Essex, UK) and are not corrected. Optical rotations were measured with a Perkin-Elmer 241 automatic polarimeter (Perkin-Elmer, Waltham, MA, USA). Electron impact (EI) mass spectra were recorded on a Finni-

gan MAT TSQ 7000 mass spectrometer (Finnigan, Waltham, MA, USA). HRFAB mass spetra were determined on a Finnigan MAT-312 instrument. UV spectra were recorded in MeOH on a Shimadzu 1601 UV/VIS spectrophotometer (Shimadzu, Kyoto, Japan). The IR spectra were measured on a Shimadzu Infrared-400 spectrophotometer. 1D and 2D NMR spectra (chemical shifts in ppm, coupling constants in Hz) were recorded on a Bruker Avance DRX 400 instrument (Bruker BioSpin, Billerica, MA, USA) using DMSO-d_6 and $CDCl_3$ as solvents; they were referenced to the solvent signals ($CDCl_3$, 7.26 ppm for ^1H and 77.0 ppm for ^{13}C; DMSO-d_6, 2.49 ppm for ^1H and 39.9 ppm for ^{13}C). Column chromatographic separations were performed on silica gel 60 (0.04 – 0.063 mm; Merck, Darmstadt, Germany), RP-18 (0.04 – 0.063 mm; Merck), and Sephadex LH-20 (0.25 – 0.1 mm; Merck). The solvent systems used for thin-layer chromatography (TLC) analyses were $CHCl_3$/MeOH (95:5, v/v; S1) and $CHCl_3$/MeOH (90:10, v/v; S2). All solvents were distilled prior to use. Spectral grade solvents were utilized for chromatographic analyses. TLC was performed on precoated silica gel 60 F_{254} plates (0.2 mm; Merck).

Plant material

Fresh bulbs of *Pancratium maritimum* L. (Amaryllidaceae) were collected in May 2010 from plants cultivated at the campus of Suez Canal University, Ismailia, Egypt. The plant material was identified and authenticated by Prof. A. Fayed, Professor of Plant Taxonomy, Faculty of Science, Assiut University, Assiut, Egypt. A voucher specimen (2010-PM1) was deposited at the herbarium of the Department of Pharmacognosy at Suez Canal University.

Extraction and isolation

Fresh bulbs (1.4 kg fresh weight) were crushed and macerated with MeOH (4 × 3 L, 72 h each) at room temperature. The combined extracts were concentrated under reduced pressure to afford a dark brown residue (15.5 g). The latter was suspended in distilled water (200 mL) and then extracted successively with *n*-hexane (4 × 500 mL), $CHCl_3$ (4 × 500 mL), and EtOAc (4 × 500 mL). Each fraction was concentrated under reduced pressure to give the *n*-hexane (fraction 1, 2.8 g), $CHCl_3$ (fraction 2, 3.82 g), EtOAc (fraction 3, 2.6 g), and aqueous extract (fraction 4, 5.5 g), respectively. Fraction 2 (2.2 g) was subjected to vacuum liquid chromatography (VLC) using an *n*-hexane/EtOAC gradient, to afford five subfractions; F2A (0.70 g, *n*-hexane, 100%), F2B (0.83 g, *n*-hexane/EtOAC, 75:25), F2C (0.46 g, *n*-hexane/EtOAC, 50:50), F2D (0.65 g, *n*-hexane/EtOAC, 25:75), and F2E (0.62 g, EtOAC, 100%). Subfraction F2B (0.83 g) was subjected to silica gel column chromatography (0.04 – 0.063 mm, 100 g, 50 cm × 3 cm) using *n*-hexane/EtOAC as an eluent to give **1** (3 mg, brown oil). Similarly, subfractions F2C (0.46 g) and F2D (0.65 g) were subjected to silica gel column chromatography (100 g, 50 cm × 3 cm) using an *n*-hexane/EtOAc gradient in order of increasing polarity to afford compounds **2** (11 mg, pale yellow needles, from F2C) and **3** (7 mg, brown amorphous residue, from F2D). Fraction 3 (2.6 g) was subjected to VLC using a $CHCl_3$/MeOH gradient to afford four subfractions; F3A (0.61 g, $CHCl_3$, 100%), F3B (0.77 g, $CHCl_3$/MeOH, 75:25), F3C (0.51 g, $CHCl_3$/MeOH, 50:50), and F3D (0.55 g, MeOH, 100%). Fraction F3C was chromatographed over a Sephadex LH-20 column (0.25 – 0.1 mm, 100 g, 50 cm × 3 cm) using MeOH/$CHCl_3$ (9:1) as an eluent; 200-mL fractions were collected and monitored by TLC to give two subfractions, F3C1 (285 mg) and F3C2 (170 mg). Subfraction F3C1 (285 mg) was loaded onto an RP-18 column (0.04 – 0.063 mm, 100 g, 50 cm × 2 cm), and elution with an MeOH/H_2O gradient gave **5** (7 mg, MeOH/H_2O, 80:20) and **4** (4 mg, MeOH/H_2O, 85:15). Repeated silica gel column chromatography of fraction F3C2 using $CHCl_3$/MeOH (95:5 to 85:15) gave **6** (17 mg).

Pancricin (**1**): Brown oil. – $R_f = 0.89$ [silica gel 60 F_{254} (S1)]. – $[\alpha]_D + 71.3°$ (*c* 0.35, $CDCl_3$). – IR (KBr): $\nu_{max} = 1336, 1296, 1702, 1050\ cm^{-1}$. – NMR: see Table I. – HRFABMS: $m/z = 249.1340$ (calcd. for $C_{11}H_{21}O_6$, 249.1338, $[M + H]^+$).

2,4-Dihydroxy-6-methoxy-3-methyl acetophenone (**2**): Pale yellow needles (MeOH/Me_2CO). – M.p. 203 – 205 °C. – $R_f = 0.83$ [silica gel 60 F_{254} (S1)]. – IR (KBr): $\nu_{max} = 3296, 1615, 1609, 1570, 1430, 896\ cm^{-1}$. – EIMS: $m/z = 196\ [M]^+$. – NMR data are in good agreement with the published data (Youssef *et al*., 1998).

5-Formylfurfuryl acetate (**3**): Brown amorphous residue. – $R_f = 0.79$ [silica gel 60 F_{254} (S1)]. – IR (KBr): $\nu_{max} = 3124, 2950, 2835, 1746, 1681, 1589, 1526, 1438, 945, 812\ cm^{-1}$. – EIMS: $m/z = 168\ [M]^+$.

Table I. NMR data (DMSO-d_6) of compounds **1** and **4**.

1				**4**			
No.	δ_C (mult.)	δ_H [mult., J (Hz)]	HMBC (C → H)	No.	δ_C (mult.)[a]	δ_H [mult., J (Hz)]	HMBC (C → H)
1	62.6 (CH_2)	3.37 m	3, 1′	2	169.5 (C)	–	3, 4, 2-CH_3
2	69.6 (CH)	3.64 m	1, 3	3	108.4 (CH)	6.26 s	2-CH_3
3	65.1 (CH_2)	4.04 dd (11.5, 3.5) 3.90 dd (11.5, 5.5)	1, 1″	4	182.4 (C)	–	–
1′	63.9 (CH)	3.71 m	1, 2′, 3′, 4′	5	152.5 (C)	–	–
2′	42.4 (CH_2)	1.48 m	3′, 4′	6	135.2 (CH)	–	8, 6-OCH_3
3′	58.6 (CH_2)	3.47 m	1′, 2′, 5′	7	157.8 (C)	–	6, 8, 1′
4′	24.3 (CH_3)	1.04 d (6.0)	2′	8	94.7 (CH)	6.85 s	6
5′	170.9 (C)	–	3′, 6′	9	155.6 (C)	–	8
6′	21.2 (CH_3)	2.02 s	–	10	106.2 (C)	–	6, 8
1″	172.0 (C)	–	3, 2″	2-CH_3	20.2 (CH_3)	2.38 s	3
2″	22.9 (CH_3)	1.77 s	–	6-OCH_3	56.7 (CH_3)	3.87 s	6
				1′	100.8 (CH)	5.10 d (7.0)	–
				2′	74.1 (CH)	3.17 – 5.51 m	–
				3′	78.0 (CH)	3.17 – 5.51 m	–
				4′	69.9 (CH)	3.17 – 5.51 m	–
				5′	77.0 (CH)	3.17 – 5.51 m	–
				6′	61.2 (CH_2)	–	–
				O*H*	–	12.95 s	–

[a] Carbon assignments were determined indirectly from the HSQC and HMBC. Carbon multiplicities were determined by HSQC experiments.

– NMR data are in a good agreement with the published data (Mehner *et al*., 2007; Khalil *et al*., 2003).

Pancrichromone (**4**): Colourless needles (MeOH/Me_2CO). – M.p. 176 – 178 °C. – $R_f = 0.77$ [silica gel 60 F_{254} (S2)]. – $[\alpha]_D - 73.9°$ (c 0.4, MeOH). – IR (KBr): $\nu_{max} = 3459$, 1665, 1625, 1594, 1020, 731 cm^{-1}. – NMR: see Table I. – HRFABMS: $m/z = 385.1137$ (calcd. for $C_{17}H_{21}O_{10}$, 385.1134, $[M+H]^+$).

7-β-D-Glucosyloxy-5-hydroxy-2-methylchromone (**5**): Amorphous pale yellow powder. – $R_f = 0.63$ [silica gel 60 F_{254} (S2)]. – $[\alpha]_D - 51.2°$ (c 0.9, MeOH). – UV (MeOH): λ_{max} (log ε) = 232 (4.19), 248 (4.26), 255 (4.29), 291 (3.82) nm. – IR (KBr): $\nu_{max} = 3459$, 1665, 1625, 1594, 1020, 731 cm^{-1}. – EIMS: $m/z = 370$ $[M]^+$ (20), 193 (100). – NMR data are in good agreement with the published data (Ghosal *et al*., 1982).

Ethyl-β-D-glucopyranoside (**6**): Yellow oil. – $R_f = 0.58$ [silica gel 60 F_{254} (S2)]. – EIMS: $m/z = 208$ $[M]^+$. – NMR data are in good agreement with the published data (Pretsch *et al*., 2000).

Wound-healing assay

The wound-healing assay is a simple method for the study of directional cell migration *in vitro* (Shaala *et al*., 2012; Rodriguez *et al*., 2005). Cells of the highly metastatic prostate cancer (PC-3M) cells derived from liver metastasis of PC-3 xenografts (Mudit *et al*., 2009) were used in this study. A 50-μM concentration of the respective tested compound was used to test its ability to inhibit PC-3M cell migration in wound healing. The cells were cultured in RPMI 1640 medium (GIBCO-Invitrogen, Grand Island, NY, USA), containing 10 mM HEPES, 4 mM L-glutamine, 10% fetal bovine serum, penicillin (100 IU/mL), and streptomycin (50 μg/mL), and grown in a 5% CO_2 atmosphere at 37 °C (Behery *et al*., 2010). Cells were plated into the wells of a sterile 24-well plate and allowed to reform a confluent cell monolayer in each well (> 95% confluence). A single wound was then inflicted to each cell monolayer across the diameter of a well using a sterile 200-μL pipette tip. Media were removed, cells were washed twice with phosphate-buffered saline (PBS), and then fresh media containing the test compounds were added to each well. Test compounds were prepared as DMSO stock solution (50 mM). The required test compound concentrations were prepared in serum-free media containing 0.5% fetal bovine serum. Initially, the ability of the compounds to inhibit the migration of the cells into the wound was recorded at 10- and 30-μM concentrations. The compounds exhibiting promising migration inhibitory activity were tested at 6 non-toxic concen-

trations prepared by serial dilution, each in triplicate, using DMSO as negative control. The incubation was carried out for 24 h, after which media were removed, and cells were fixed and stained using Diff Quick staining (Dade Behring Diagnostics, Aguada, Puerto Rico) (Mudit *et al.*, 2009; Mudit and El Sayed, 2011). The number of cells that had migrated into the scratched wound was counted under the microscope in three or more randomly selected fields (magnification, 40×). Final results were expressed as mean per 40× field.

Proliferation assay

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay allows for the measurement of cell viability and proliferation of cell populations in a quantitative colourimetric fashion by utilizing the cells' ability to reduce the MTT reagent to an insoluble purple formazan dye. Antiproliferative effects of the isolated compounds were tested in cultures of PC-3M cells using an MTT kit (Sigma-Aldrich, St. Louis, MO, USA) (Alley *et al.*, 1988; Mosmann, 1983). After 3 to 4 passages of the cells, growing cells were incubated in a 96-well plate at a density of 10^3 cells per well, and allowed to attach for 24 h. Complete growth medium was then replaced with 100 μL of serum-free RPMI medium containing various concentrations (5, 10, 20, and 50 μM) of each test compound, and culture was continued at 37 °C under 5% CO_2. After 96 h, the incubated cells were treated with MTT solution (10 μL per well) at 37 °C for 4 h. The colour reaction was stopped by the addition of solubilization/stop solution (100 μL per well), and the incubation at 37 °C continued to dissolve the formazan product completely. Absorbance of the samples was measured at 550 nm with an ELISA plate reader (PowerWave XS2; BioTek, Winooski, VT, USA). The number of cells per well was calculated against a standard curve prepared by plating various concentrations of cells, as determined by a hemocytometer, at the start of each experiment. 4-Hydroxyphenylmethylene hydantoin was used as a positive control in this assay. Each experiment was performed in triplicate, and the experiment was repeated twice.

Statistical analysis

The results were statistically evaluated by one-way ANOVA, and significance was examined by the Newman-Keuls test. A difference was considered statistically significant when $P < 0.05$.

Results and Discussion

Compound **1** (Fig. 1) was isolated as brown oil. Its molecular formula $C_{11}H_{20}O_6$ was confirmed by the HRFABMS pseudo-molecular ion peak at m/z 249.1340 ($[M+H]^+$), which required two degrees of

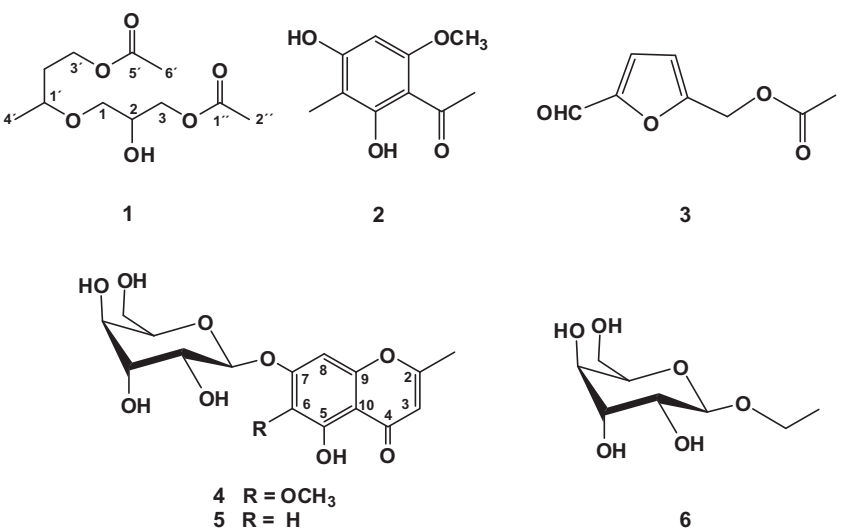


Fig. 1. Chemical structures of the isolated compounds: pancricin (**1**), 2,4-dihydroxy-6-methoxy-3-methyl acetophenone (**2**), 5-formylfurfuryl acetate (**3**), pancrichromone (**4**), 7-β-D-glucosyloxy-5-hydroxy-2-methylchromone (**5**), and ethyl-β-D-glucopyranoside (**6**).

unsaturation. The two degrees of unsaturation implied by the molecular formula was attributed, based on the ^{13}C NMR data, to two acetyl carbonyl groups at δ_C 172.0 and 170.9 ppm, respectively. The ^1H NMR spectrum showed two singlets at δ_H 1.77 (H_3-2″) and 2.02 ppm (H_3-6′) assignable to the methyl groups of the acetyl residues. The presence of a glyceryl moiety was evident from the signals at δ_H 3.37 ppm/δ_C 62.6 ppm (H_2-1/C-1), δ_H 3.64 ppm/δ_C 69.6 ppm (H-2/C-2), and δ_H 3.90, 4.04 ppm/δ_C 65.1 ppm (H_2-3/C-3), and further confirmed by the cross-peaks observed in the COSY and HMBC spectra (Table I and Fig. 2). Moreover, the ^1H NMR spectrum displayed signals at δ_H 3.71 (m, H-1′), 1.48 (m, H_2-2′), 3.47 (m, H_2-3′), and 1.04 ppm (d, $J = 6.0$ Hz, H_3-4′) corresponding to the butane-1,3-diol residue. This was corroborated by the observed correlations in the HMBC spectrum; thus H-1′, H_2-2′, H_2-3′, and H_3-4′ showed three-bond correlations to C-3′, C-4′, C-1′, and C-2′, respectively. The connectivity of the glyceryl moiety with the butane-1,3-diol moiety at C-1′ was established from the HMBC correlation of H_2-1 with C-1′. The positions of the acetyl moieties were assigned from HMBC correlations of H_2-3′ with C-5′ (δ_C 170.9 ppm) and H_2-3 with C-1″ (δ_C 172.0 ppm), respectively, confirming the assignment of **1**. On the basis of these findings, the structure of **1** was unambiguously elucidated. It was assigned as 3-(3-acetoxy-2-hydroxypropoxy)butyl acetate, for which we propose the name pancricin.

Compound **4** (Fig. 1) was isolated as colourless needles; its molecular formula $C_{17}H_{20}O_{10}$ was established by HRFABMS. The ^1H NMR data supported the presence of a chromone skeleton substituted with methyl, hydroxy, methoxy, and glucose units in positions C-2, C-5, C-6, and C-7, respectively. Its NMR data (Table I) were similar to those reported for **5** (Ghosal *et al.*, 1982), with the exception of the absence of the signal of H-6 and appearance of a new signal at δ_H 3.87 ppm/δ_C 56.7 ppm for a methoxy group in **4**. The position of the methoxy group at C-6 was evident from the observed HMBC correlations of 6-OCH_3 to C-6 and H-8 with C-6 (Fig. 2). Accordingly, the structure of **4** was unambiguously elucidated as 5-hydroxy-6-methoxy-2-methyl-7-[3,4,5-trihydroxy-6-(hydroxymethyl)tetrahydro-2*H*-pyran-2-yloxy]-4*H*-chromen-4-one and named pancrichromone.

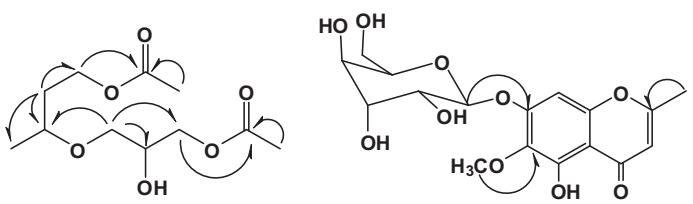
The known compounds were identified through the analysis of the spectroscopic data (1D, 2D NMR, and MS) and comparison with those in the literature; 2,4-dihydroxy-6-methoxy-3-methyl acetophenone (**2**) (Youssef *et al.*, 1998), 5-formylfurfuryl acetate (**3**) (Mehner *et al.*, 2007; Khalil *et al.*, 2003), 7-β-D-glucosyloxy-5-hydroxy-2-methylchromone (**5**) (Ghosal *et al.*, 1982), and ethyl-β-D-glucopyranoside (**6**) (Pretsch *et al.*, 2000) (Fig. 1). To the best of our knowledge, this is the first isolation of **3** from a member of the family Amaryllidaceae.

The isolated compounds were evaluated for their antimigratory and antiproliferative activities against the highly metastatic human PC-3M cells. Table II shows the effects of the compounds on cell migration. The chromone glycoside **5** displayed weak activity, allow-

Table II. Antimigratory activity of the compounds[a].

Compound	Migration (%)
DMSO	100
S-Ethyl	23.8
1	NT[b]
2	100
3	100
4	NT
5	85
6	100

[a] Tested at 50 μM against the human metastatic prostate cancer cells (PC-3M); 50 μM 4-ethylmercaptophenylmethylene hydantoin (S-Ethyl) was used as a positive drug control (Mudit *et al.*, 2009).
[b] NT, not tested.



1 **4**

Fig. 2. Selected HMBC correlations of **1** and **4**.

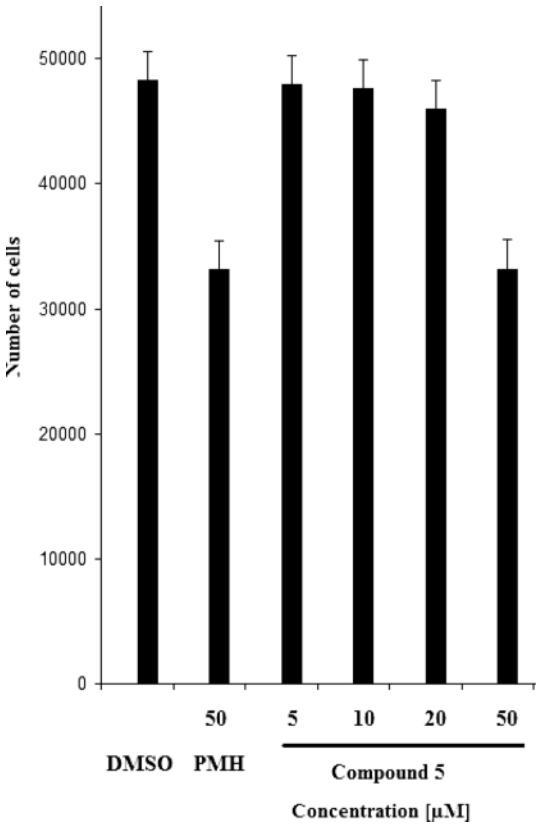


Fig. 3. Antiproliferative activity of various doses of compound **5** in the MTT assay. A dose of 50 μM of 4-hydroxyphenylmethylene hydantoin (PMH) was used as a positive drug control (Mudit *et al*., 2009).

ing 85 % of cell migration compared to 23.8 % for the positive control 4-ethylmercaptophenylmethylene hydantoin (Table II).

Other compounds were either inactive or not evaluated. In the MTT proliferation assay, compound **5** showed remarkable activity at a concentration of 50 μM comparable to that of the positive control 4-hydroxyphenylmethylene hydantoin (Fig. 3) (Mudit *et al.*, 2009; Mudit and El Sayed, 2011). This may highlight the potential of chromone glycosides as new antimigratory agents for future use in the control of metastatic prostate cancer.

Chromones are abundant in nature and possess a wide range of biological and pharmacological activities, some of these include cytotoxic, neuroprotective, HIV-inhibitory, anti-inflammatory, antimicrobial, antifungal, and antioxidant activities (Shanthi *et al*., 2013; Liu *et al*., 2012; Yoon *et al*., 2006).

Conclusions

Investigation of the $CHCl_3$ and EtOAc fractions of the methanolic extract of fresh bulbs of the Amaryllidaceous plant *P. maritimum* resulted in the identification of two new compounds, *viz.* the glycerol derivative 3-(3-acetoxy-2-hydroxypropoxy)butyl acetate (pancricin) and the 7-β-D-glucoside of the chromone 5-hydroxy-6-methoxy-2-methyl-7-[3,4,5-trihydroxy-6-(hydroxymethyl)tetrahydro-2*H*-pyran-2-yloxy]-4*H*-chromen-4-one (pancrichromone), together with four known compounds, of which 7-β-D-glucosyloxy-5-hydroxy-2-methylchromone displayed good antiproliferative and weak antimigratory activity, respectively.

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