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Lupane Triterpenoids and New Derivatives as Antiproliferative Agents Against Prostate Cancer Cells

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Abstract. *Background/Aim: This study examined the potential role of natural triterpenoids lupeol, calenduladiol and heliantriol B2, and a set of 19 derivatives, as antiproliferative and antimetastatic agents against prostate cancer cells. Materials and Methods: Natural triterpenoids were isolated from Chuqiraga erinaceae. Analogs were obtained by transformations of lupeol and calenduladiol. The effects of compounds on PC-3 and LNCaP cells were determined using the MTT assay. Compounds with half-maximal inhibitory concentration <70 μM were evaluated as antimetastatic agents by a wound-healing assay. Results: Lupeol-3β-sulfate, a new semisynthetic lupane, was the most active compound. In general, sulfated derivatives displayed higher activity than the lead against both cell lines. A new analog, calenduladiol-3β-monosulfate, inhibited the migration of PC-3 cells; heliantriol B2 and 3β-aminolupane inhibited the migration of LNCaP cells in a concentration-dependent manner. Conclusion: Our study provides novel agents with cytotoxic effects on prostate cancer cells, which may represent a potential new therapeutic approach for prostate cancer.*

Prostate cancer (PCa) is the most common malignancy in elderly men in developed countries, aside from skin cancer, and is one of the top-five types of cancer killing men worldwide (1). Although incidence rates have decreased in Western countries, increased incidence and mortality rates have been observed in several Asian countries in recent years. While there are several factors associated with PCa risk, no preventable risk factors have yet been identified for this disease (2). In general, treatment of early-stage PCa is effective and involves radiotherapy, radical prostatectomy, cryosurgery, or hormonal therapy. However, when the disease is diagnosed at an advanced stage, the current treatment options have limited efficacy and cause undesirable side-effects, leading to disease progression and metastasis. Therefore, the development of novel therapeutic modalities and preventive approaches are important in improving the survival and quality of life of patients with PCa.

Historically, nature has served as a source of medicinal products used in the treatment of many diseases, including cancer. Nowadays, naturally-occurring products continue to play a relevant role in cancer therapy with a substantial number of anticancer agents used in the clinic that are natural or derived from natural products (3).

Triterpenoids are the ubiquitous secondary metabolites that can be also found in the human diet, largely derived from cereals, fruits, vegetables and plant-derived oils. They have been widely studied due to their broad spectrum of biological activities (4). In particular, pentacyclic triterpene alcohols isolated from plants of the *Asteraceae* family have been shown to exhibit cytotoxic activity against human cancer cell lines (5-7). Among them, lupeol (**1**) [lup-20(29)-en-3β-ol], has been shown to display moderate potential in cancer treatment through inhibition of multiple intracellular signaling molecules and transcription factors involved in the initiation, progression, and promotion of tumors (8). Several lupeol derivatives have also been synthesized to improve cytotoxicity against different human cancer cell lines (9-11). During the past few decades, the *in vitro* and *in vivo*

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anticancer efficacy of lupeol against human PCa cells without cytotoxicity to non neoplastic prostate epithelial cell has been reported (12-15). Moreover, lupeol has been shown to exhibit great promise as chemopreventive agent and is considered an essential dietary agent for the prevention of PCa that could be used alone or in combination with traditional chemotherapeutic agents to prevent the occurrence of cancer, metastatic spread, or even to treat cancer (16, 17).

Chuquiraga erinacea subsp. *erinacea* (*Asteraceae*) is an endemic species widely distributed in Argentina. Its leaves are consumed locally in infusion or decoction to relieve stomach, hepatic and kidney diseases, and to strength brain and nerves (18). The phytochemical analysis of this species led to the isolation of several lupane triterpenoids, including lupeol (**1**), calenduladiol (**2**), and heliantriol B2 (**3**) (Figure 1). Both **1** and **2** were found in significant quantities (0.19% and 0.15%, respectively) in the ethanolic extract of the aerial parts of *C. erinacea*, while **3** was present in minor amounts (19). It is important to note that although lupeol is commercially available, calenduladiol, a triterpenoid with the uncommon feature of being hydroxylated at C-16, is not.

Recently, several hydroximinosteroids and sulfated analogs have been synthesized and evaluated for their cytotoxic activity against human tumor cell lines *in vitro* (20). These studies revealed that the presence of sulfate and oxime groups increase the cytotoxic activity against PC-3 and LNCaP cells, and highlighted the importance of the hydroxyl group configuration on the inhibition of these tumor cell lines. These results prompted us to synthesize lupane derivatives (**4-22**) from the natural most abundant triterpenes **1** and **2** obtained from the ethanolic extract of *C. erinacea*. To the best of our knowledge, this is the first evaluation of lupeol or calenduladiol semisynthetic derivatives as potential anticancer agents against human PCa cell lines. The *in vitro* cytotoxic activity of both natural and synthesized triterpenes was evaluated against two human prostate adenocarcinoma cell lines, PC-3 and LNCaP. Among the compounds tested, the effect of those that exhibited moderate cytotoxic activity (IC_{50} <70 μM) on the migration of PCa cells using a wound-healing assay was also examined.

Materials and Methods

Melting points were determined on a Fisher-Johns apparatus (Reichert, Austria) and are uncorrected. Nuclear magnetic resonance (NMR) measurements, including COSY, HSQC, HMBC experiments, were carried out on Bruker ARX300 spectrometer (Bruker Corp, Billerica, MA, USA). NMR spectra were recorded in CDCl3 or MeOD. Chemical shifts are given in ppm (δ) with tetramethylsilane (TMS) as an internal standard. Electrospray ionization mass spectra were recorded using an Esquire 3000 ion trap mass spectrometer (Bruker, San Diego, CA, USA) equipped with a standard ESI/APCI source. Elemental analyses (C, H) were performed with an INC CE-440 Elemental Analyzer (Exeter Analytical, Inc, North Chelmsford, USA). Microwave-assisted reactions were carried out in a CEM Discover reactor (CEM Corp, Matthews, North Carolina, USA).

Silica gel 60 (0.2-0.63 mm, Merck KGaA, Darmstadt, Germany) was used for column chromatography. Silica gel 60 (70-230 mesh; Merck KGaA, Darmstadt, Germany) was used for flash chromatography. Analytical thin layer chromatography (TLC) was performed on Silicagel 60 F254 sheets (0.2 mm thickness; Merck, Argentina). *p*-Anisaldehyde-acetic acid spray reagent (Mallinckrodt, New York, NY, USA) and UV light (254 and 366 nm) were used for detection.

Solvents were dried and distilled in accordance with standard procedure (21). Lupeol (**1**) and calenduladiol (**2**), used as starting materials for the preparation of compounds **4-22**, and heliantriol B2 (**3**) were obtained from aerial parts of *C. erinacea* subsp. *erinacea* as previously described (19, 22). Derivatives **5**, **9-11**, **14-16**, **18** and **20-22** were prepared according to the synthesis previously reported by our group (22, 23).

All derivatives were rigorously characterized by NMR spectroscopy and mass spectrometry. The NMR data of derivatives **5**, **8-11**, **14-16**, **18**, and **20-22** were identical to those previously reported (22-24). In the case of compounds **6** and **7**, we have completed the NMR data available in the literature (25, 26). Compounds **4**, **12**, **13**, **17**, and **19** are described here for the first time and bidimensional NMR spectra (COSY, HMBC, HSQC) were used for the unequivocal assignments of all carbons and representative protons.

*General procedures for the preparation of sulfated compounds **4**, **12** and **13***. A solution of $Me_3N{\bullet}SO_3$ and **1**, **10** or **11** in dry dimethylformamide (DMF) was placed in a microwave-special closed vial and the solution was irradiated for 7 min at 150°C in a microwave reactor. The reaction mixture was then cooled to room temperature and quenched with water (1 ml). After evaporation to dryness, the residue was eluted through Amberlite CG-120 (Sigma-Aldrich Chemie GmbH, Steinheim, Germany) (sodium form) with MeOH, evaporated under reduced pressure and purified by column flash chromatography on silica gel with CH_2Cl_2/MeOH mixtures as eluent to afford the sulfated compounds.

*Sodium 3β-hydroxy-lup-20(29)-ene sulfate (**4**)*. Following the general procedure, a solution of **1** (38.0 mg, 0.09 mmol) in DMF (2 ml) was treated with $Me_3N{\bullet}SO_3$ (48.8 mg, 0.36 mmol). Purification of the resulting crude by flash chromatography with CH_2Cl_2/MeOH (9:1) afforded 45.7 mg (96%) of compound **4** as a white amorphous solid, mp 142-143°C; ^{1}H-NMR (300 MHz, CD_3OD) δ 4.68 (1H, br s, H-29a), 4.55 (1H, br s, H-29b), 3.92 (1H, m, H-3α), 2.40 (1H, ddd, *J*=11.0, 11.0, 5.7 Hz, H-19), 2.08 (1H, m), 1.92 (1H, m), 1.72 (3H, s, H-30), 1.35 (22H, m), 1.06 (3H, s, H-26), 1.01 (3H, s, H-23), 0.98 (3H, s, H-27), 0.88 (3H, s, H-25), 0.81 (3H, s, H-28), 0.80 (3H, s, H-24); ^{13}C-NMR (75 MHz, CD_3OD) δ 151.9 (C-20), 110.1 (C-29), 87.8 (C-3), 57.2 (C-5), 51.8 (C-9), 49.5 (C-18), 49.4 (C-19), 44.1 (C-17), 44.0 (C-14), 42.1 (C-8), 41.0 (C-22), 39.8 (C-1), 39.6 (C-4), 39.5 (C-13), 38.2 (C-10), 36.7 (C-7), 35.5 (C-16), 30.9 (C-2), 28.7 (C-15), 28.6 (C-23), 26.4 (C-21), 25.4 (C-12), 22.1 (C-11), 19.5 (C-30), 19.4 (C-6), 18.4 (C-28), 16.8 (C-25), 16.7 (C-26), 16.6 (C-24), 15.0 (C-27); ESI–MS *m/z* 505 $[M\text{-}Na]^+$. Anal. Calcd. for $C_{30}H_{49}NaO_4S$: C 68.15; H 9.34. Found: C 68.11; H 9.39.

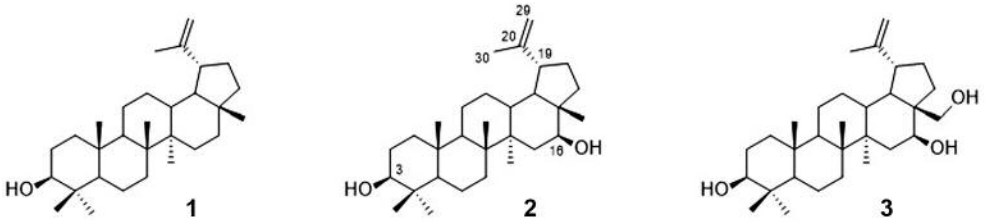

Figure 1. *Structure of lupeol (**1**), calenduladiol (**2**) and heliantriol B2 (**3**).*

*Sodium 3β-acetoxy-16β-hydroxy-lup-20(29)-ene sulfate (**12**).* Following the general procedure, a solution of **10** (10.0 mg, 0.02 mmol) in DMF (2 ml) was treated with $Me_3N{\bullet}SO_3$ (10.8 mg, 0.08 mmol). Purification of the resulting crude by flash chromatography with CH_2Cl_2/MeOH (8.5:1.5) afforded 5.3 mg (44%) of compound **12** as a white amorphous solid, mp 136-138 ˚C; ^{1}H-NMR (300 MHz, CD_3OD) δ 4.75 (1H, br s, H-29a), 4.63 (1H, brs, H-29b), 4.48 (1H, dd, *J*=10.1, 6.6 Hz, H-3α), 4.37 (1H, t, *J*=7.4 Hz, H-16α), 2.56 (1H, ddd, *J*=11.6, 11.6, 6.2 Hz, H-19), 1.76 (3H, br s), 1.74 (3H, s, H-30), 1.65 (5H, m), 1.47 (8H, m), 1.33 (3H, br s), 1.12 (3H, s, H-26), 1.10 (3H, s, H-27), 1.04 (3H, m), 0.94 (3H, s, H-25), 0.90 (3H, s, H-24), 0.90 (3H, s, H-23), 0.89 (3H, s, H-28), OAc-3 [2.05 (3H, s)]; ^{13}C-NMR (75 MHz, CD_3OD) δ 150.9 (C-20), 110.0 (C-29), 85.9 (C-16), 82.1 (C-3), 56.5 (C-5), 50.9 (C-9), 50.9 (C-19), 49.2 (C-18), 48.9 (C-17), 45.3 (C-14), 42.2 (C-8), 38.9 (C-1), 38.9 (C-15), 38.7 (C-13), 38.4 (C-4), 37.9 (C-10), 35.6 (C-22), 35.4 (C-7), 30.7 (C-21), 28.4 (C-23), 25.9 (C-12), 24.6 (C-2), 22.0 (C-11), 19.4 (C-30), 19.2 (C-6), 16.8 (C-24), 16.6 (C-25), 16.5 (C-26), 16.2 (C-27), 12.8 (C-28), OAc-3 [172.9 (s), 21.0 (c)]; ESI–MS *m/z* 563 [M-Na]$^+$. Anal. Calcd. for $C_{32}H_{51}NaO_6S$: C 65.50; H 8.76. Found: C 65.57; H 8.78.

*Sodium 3β-hydroxy-16β-acetoxy-lup-20(29)-ene sulfate (**13**).* Following the general procedure, a solution of **11** (8.0 mg, 0.02 mmol) in DMF (2 ml) was treated with $Me_3N{\bullet}SO_3$ (10.8 mg, 0.08 mmol). Purification of the resulting crude by flash chromatography with CH_2Cl_2/MeOH (8.5:1.5) afforded 3.3 mg (27%) of compound **13** as a white amorphous solid, mp 136-137˚C; ^{1}H-NMR (300 MHz, CD_3OD) δ 4.91 (1H, brs, H-16α), 4.76 (1H, br s, H-29a), 4.64 (1H, br s, H-29b), 3.96 (1H, dd, *J*=11.5, 4.4 Hz, H-3α), 2.55 (1H, ddd, *J*=11.6, 11.1, 5.6 Hz, H-19), 1.76 (2H, m), 1.74 (3H, s, H-30), 1.47 (20H, m), 1.12 (6H, s, H-24, H-27), 1.06 (3H, s, H-23), 0.93 (3H, s, H-26), 0.92 (3H, s, H-25), 0.85 (3H, s, H-28), OAc-16 [2.03 (3H, s)]; ^{13}C-NMR (75 MHz, CD_3OD) δ 151.0 (C-20), 110.7 (C-29), 87.5 (C-3), 80.7 (C-16), 57.2 (C-5), 51.3 (C-9), 50.0 (C-19), 49.2 (C-18), 48.9 (C-17), 45.3 (C-14), 42.2 (C-8), 39.8 (C-1), 39.6 (C-4), 38.8 (C-13), 38.7 (C-15), 38.1 (C-10), 35.4 (C-22), 34.6 (C-7), 30.8 (C-21), 28.7 (C-23), 26.0 (C-12), 25.4 (C-2), 22.0 (C-11), 19.5 (C-30), 19.4 (C-6), 16.7 (C-25) 16.7 (C-26), 16.6 (C-27), 16.3 (C-24), 13.1 (C-28), OAc-16 [172.6 (s), 21.2 (c)]; ESI–MS *m/z* 563 [M-Na]$^+$. Anal. Calcd. for $C_{32}H_{51}NaO_6S$: C 65.50; H 8.76. Found: C 65.54; H 8.74.

*3E-Hydroxyimino lupane (**6**).* To a solution of **5** (40.0 mg, 0.09 mmol) in EtOH (2 ml) hydroxylamine hydrochloride (18.8 mg, 0.27 mmol) and a solution of sodium acetate (11.6 mg, 0.18 mmol) in water were added. The reaction mixture was left at room temperature for 36 h, and then EtOH was evaporated. The residue was treated with water and extracted with CH_2Cl_2 (3×5 ml). The combined organic extracts were dried over $MgSO_4$, filtered, and concentrated. The solvent was removed and the residue was purified by flash chromatography on silica gel with hexane/AcOEt (9.5:0.5) afforded 12.9 mg (31%) of compound **6** as a white amorphous solid; ^{1}H-NMR (300 MHz, $CDCl_3$) δ 4.69 (1H, br s, H-29a), 4.57 (1H, br s, H-29b), 2.96 (1H, m, H-2β), 2.33 (3H, m, H-2a, H-6b, H-19), 1.66 (3H, s, H-30), 1.44 (21H, m), 1.14 (3H, s, H-23), 1.06 (3H, s, H-24), 1.04 (3H, s, H-25), 0.93 (6H, s, H-26, H-27), 0.79 (3H, s, H-28). NOH [8.26 (1H, s)]; ^{13}C-NMR (75 MHz, $CDCl_3$) δ 167.4 (C-3), 151.0 (C-20), 109.5 (C-29), 55.6 (C-5), 50.2 (C-9), 48.4 (C-18), 48.1 (C-19), 43.1 (C-17), 43.1 (C-14), 41.0 (C-8), 40.4 (C-22), 38.9 (C-1), 38.9 (C-4), 38.3 (C-13), 37.4 (C-10), 35.7 (C-16), 34.5 (C-7), 30.0 (C-21), 27.6 (C-23), 27.6 (C-2), 27.6 (C-15), 25.3 (C-12), 23.0 (C-24), 21.7 (C-11), 19.8 (C-30), 18.2 (C-6), 18.2 (C-28), 16.1 (C-26), 16.1 (C-25), 14.6 (C-27).

*Lup-20(29)-en-3β-amine (**7b**).* A solution of **6** (18.0 mg, 0.04 mmol) in dry tetrahydrofuran (THF) (5 ml) was added to a solution of $LiAlH_4$ in THF (0.40 mmol, 15.2 mg, 5 ml). The reaction mixture was refluxed for 7 h until disappearance of the starting material and then treated with a saturated solution of ammonium chloride. The aqueous phase was extracted with CH_2Cl_2 (3×5 ml). The combined organic extracts were dried over $CaCl_2$, filtered, and concentrated. The residue was purified by flash chromatography with CH_2Cl_2/MeOH (8:2) afforded 4.3 mg (25%) of compound **7b**; ^{1}H-NMR (300 MHz, CD_3OD) δ 4.72 (1H, brs, H-29a), 4.60 (1H, brs, H-29b), 2.90 (1H, dd, *J*=10.7, 4.9 Hz, H-3α), 2.44 (1H, ddd, *J*=11.0, 11.0, 5.5 Hz, H-19), 1.72 (3H, s, H-30), 1.44 (25H, m), 1.03 (3H, s, H-26), 0.98 (3H, s, H-23), 0.95 (3H, s, H-27), 0.83 (3H, s, H-25), 0.79 (3H, s, H-28), 0.76 (3H, s, H-24); ^{13}C-NMR (75 MHz, CD_3OD) δ 151.9 (C-20), 110.1 (C-29), 61.4 (C-3), 56.8 (C-5), 51.7 (C-9), 50.1 (C-18), 49.4 (C-19), 43.5 (C-17), 43.3 (C-14), 41.0 (C-8), 40.6 (C-22), 39.5 (C-1), 39.4 (C-4), 38.2 (C-13), 37.6 (C-10), 35.5 (C-16), 35.3 (C-7), 30.9 (C-2), 28.6 (C-21), 28.2 (C-15), 28.2 (C-23), 24.6 (C-12), 21.8 (C-11), 19.3 (C-30), 18.4 (C-6), 18.1 (C-28), 16.5 (C-25), 16.2 (C-26), 15.1 (C-24), 15.0 (C-27).

*Lup-20(29)-en-3α-ol (**8**).* A solution of **5** (50.0 mg, 0.12 mmol) in dry MeOH (20 ml), was treated with $NaBH_4$ (50.0 mg, 1.32 mmol). The reaction mixture was stirred at room temperature for 3 h until disappearance of the starting material verified by TLC. Then, the mixture was quenched with water (20 ml) and CH_2Cl_2 (20 ml), observing the appearance of a white solid, and filtered. The organic phase was dried over $MgSO_4$, filtered, and concentrated. The residue was purified by flash chromatography with hexane/AcOEt (9.5:0.5) afforded 2.3 mg (5%) of compound **8** and 27.0 mg (54%) of lupeol (**1**). Spectroscopic and spectrometric data of **8** were identical to those reported for epilupeol (24).

*General procedures for the preparation of oximes **17** and **19***. To a solution of **16** or **18** in EtOH, hydroxylamine hydrochloride and a solution of sodium acetate in water were added. The reaction mixture was heated to reflux for 18 h, and then EtOH was evaporated. The residue was treated with water and extracted with CH_2Cl_2 (3×5 ml). The combined organic extracts were dried over $MgSO_4$, filtered, and concentrated. The solvent was removed and the residue was purified by flash chromatography on silica gel with hexane/AcOEt mixtures as eluent to afford the oximes.

*3E, 16E-Dihydroxyimino lupane (**17**)*. Following the general procedure, to a solution of **16** (45.0 mg, 0.10 mmol) in EtOH (2 ml), hydroxylamine hydrochloride (39.8 mg, 0.60 mmol) and a solution of sodium acetate (33.8 mg, 0.40 mmol) in water were added. Purification of the resulting crude by flash chromatography with hexane/AcOEt (8.5:1.5) afforded 16.8 mg (35%) of compound **17** as a white amorphous solid, mp 179-180˚C; ^{1}H-NMR (300 MHz, $CDCl_3$) δ 4.72 (1H, br s, H-29a), 4.61 (1H, br s, H-29b), 3.16 (1H, dt, *J*=15.3, 4.7 Hz, H-2β), 2.90 (1H, d, *J*=13.4 Hz, H-15β), 2.52 (1H, ddd, *J*=10.9, 10.9, 5.6 Hz, H-19), 2.01 (4H, m), 1.79 (3H, m), 1.65 (3H, s, H-30), 1.47 (11H, m), 1.27 (2H, m), 1.12 (6H, s, H-23, H-26), 1.03 (3H, s, H-24), 1.01 (3H, s, H-28), 0.99 (3H, s, H-25), 0.89 (3H, s, H-27), NOH [9.68 (1H, s), 9.58 (1H, s)]; ^{13}C-NMR (75 MHz, $CDCl_3$) δ 167.2 (C-3), 165.2 (C-16), 149.6 (C-20), 110.5 (C-29), 56.7 (C-5), 50.1 (C-18), 50.0 (C-9), 48.8 (C-17), 47.4 (C-19), 46.1 (C-14), 41.1 (C-8), 41.1 (C-4), 39.7 (C-1), 37.9 (C-13), 37.5 (C-10), 34.5 (C-7), 33.3 (C-22), 29.6 (C-21), 27.4 (C-15), 26.4 (C-23), 24.8 (C-12), 22.6 (C-24), 20.9 (C-11), 19.2 (C-30), 18.9 (C-2), 18.9 (C-28), 17.2 (C-6), 16.6 (C-26), 15.8 (C-25), 15.1 (C-27); ESI–MS *m/z* 467 $[M-H]^+$. Anal. Calcd. for $C_{30}H_{48}N_2O_2$: C 76.87; H 10.32; N 5.98. Found: C 76.80; H 10.35; N 5.94.

*3E, 16E, 30-Trihydroxyimino lupane (**19**)*. Following the general procedure, a solution of **18** (20.0 mg, 0.04 mmol) in EtOH (2 ml), hydroxylamine hydrochloride (27.9 mg, 0.42 mmol) and a solution of sodium acetate (7.5 mg, 0.08 mmol) in water were added. Purification of the resulting crude by flash chromatography with hexane/AcOEt (7.5:2.5) afforded 6.7 mg (31%) of compound **19** as a white amorphous solid, mp 201-202˚C; ^{1}H-NMR (300 MHz, $CDCl_3$) δ 7.71(1H, s, H-30), 5.38 (1H, br s, H-29a), 5.20 (1H, br s, H-29b), 2.95 (3H, m, H-6α, H-15α, H-19), 2.07 (8H, m), 1.55 (9H, m), 1.33 (3H, s), 1.20 (3H, s, H-26), 1.17 (3H, s, H-23), 1.08 (3H, s, H-24), 1.06 (3H, s, H-28), 0.99 (3H, s, H-25), 0.92 (3H, s, H-27); ^{13}C-NMR (75 MHz, $CDCl_3$) δ 167.2 (C-3), 166.4 (C-16), 152.8 (C-30), 150.7 (C-20), 120.4 (C-29), 56.6 (C-5), 51.1 (C-18), 49.8 (C-9), 49.6 (C-19), 46.5 (C-8), 46.5 (C-17), 42.1 (C-14), 41.0 (C-4), 39.7 (C-1), 39.2 (C-13), 38.2 (C-10), 35.1 (C-7), 34.2 (C-22), 30.7 (C-12), 28.2 (C-15), 28.2 (C-23), 27.7 (C-21), 23.5 (C-24), 22.2 (C-2), 20.2 (C-11), 19.3 (C-28), 17.9 (C-6), 16.8 (C-26), 16.2 (C-25), 15.3 (C-27); ESI–MS *m/z* 497 $[M]^+$ (47), 482 (11), 480 (100), 462 (24); Anal. Calcd. for $C_{30}H_{47}N_3O_3$: C 72.40; H 9.52; N 8.44. Found: C 72.30; H 9.50; N 8.34.

Cytotoxic activity

Cell culture and reagents. Human prostate cancer cell lines PC-3 and LNCaP were obtained from the American Type Culture Collection (Manassas, VA, USA). Cells were grown in RPMI 1640 medium (EMEVE Medios, Lab MicroVet, Buenos Aires, Argentina) supplemented with 10% fetal bovine serum (FBS) (Natocor, Córdoba, Argentina) and antibiotics (Invitrogen), in a humidified atmosphere (95% air, 5% CO_2, 37˚C).

Cell viability assay. PC-3 cells (1×10^4 cells/100 μl) and LNCaP (3×10^4 cells/100 μl) were seeded into each well of a 96-well microtiter plate. After incubation for 24 h, the media were aspirated and replaced with 100 of serum-free medium containing different concentrations of each compound. Triplicate wells were prepared for each individual dose.

MTS cell viability analysis was performed 24 h post treatment using the Cell Titer 96 AQueous One Solution Proliferation Assay System (Promega Corp, Madison, WI, USA); in which viable cells convert 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS tetrazolium) into a formazan-colored product (OD490 nm). Following the manufacturer's instructions, 20 μl of MTS reagent were added to each well and cells were incubated at 37˚C for 1 h. Absorbance was detected at 490 nm on a Thermo Scientific Multiskan FC plate reader (Thermo Fisher Scientific, Waltham, USA). The IC_{50} value was defined as the concentration of tested compound resulting in a 50% reduction in cell viability compared to vehicle-treated (dimethyl sulfoxide) cells.

Wound-healing assay. The effect of compounds on cell migration was assessed in PC-3 and LNCaP cells using a wound-healing assay. PC-3 (1.0×10^4 cells/well) and LNCaP (3.0×10^4 cells/well) cells were cultured in a 96-well plate to reach 100% confluence. A vertical scratch across the well was made with a 200 μl pipette tip (time 0), followed by two washes with phosphate-buffered saline (PBS). The test compound was then added to each well. RPMI 1640 with the same corresponding percentage of dimethyl sulfoxide was used for control wells. Microscopy images were taken at 0, 18 and 23 h to assess cell migration visually. Images were captured using an inverted phase-contrast microscope (Olympus CKX-41, 4×objective; Bioanalìtica, Buenos Aires, Argentina). Each compound was added in duplicate wells, and each experiment was repeated twice. The area of wound was quantified by Image J software (http://rsb.info.nih.gov) using the polygon selection mode. The migration of cells toward the wound was expressed as the percentage of wound closure: wound closure (%)=$[(At_{0h}–At\ \Delta\ h)/At_{0h}] \times100\%$, where, At_{0h} was the area of wound measured immediately after scratching, and At Δh was the area of wound measured 18 or 23 h after scratching (27).

Statistical analysis. Statistical analysis was performed using Graph Pad Prism Software (La Jolla, CA, USA). Data are expressed as mean±standard deviation. ANOVA with Dunn's post test was employed for statistical analysis of wound-healing assay. Statistical significance was established at $p<0.05$.

Results

Natural triterpenoids **1-3** were obtained from the ethanolic extract of the aerial parts of C. *erinacea* as previously reported (Figure 1) (19). The first series of derivatives (**4-8**) was synthesized according to the reaction sequence shown in Figure 2, using lupeol (**1**) as the starting material. Treatment of **1** with 4 equivalents of trimethylamine-sulfur trioxide complex ($Me_3N{\bullet}SO_3$) for 7 min at 150˚C in a microwave reactor afforded the corresponding ammonium sulfate, which was transformed *via* ion exchange into the sodium salt **4** in excellent yield (96%), as described previously (23). The ^{1}H-NMR spectrum of **4** showed a

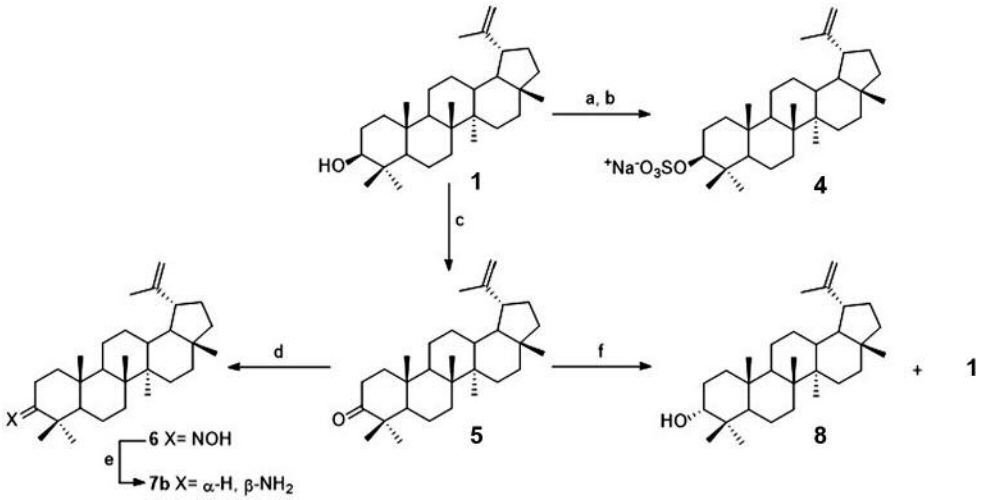

Figure 2. *Synthesis of compounds* ***4-8***. *Reagents and conditions: (a) 4 equiv.* $Me_3N{\bullet}SO_3$, *DMF, 7 min, 150˚C, microwave; (b) Amberlite CG-120 (MeOH); (c) Jones reagent, acetone, 0˚C; (d)* $NH_2OH{\bullet}HCl$, *NaOAc, EtOH/*H_2O; *(e)* $LiAlH_4$, *THF; (f)* $NaBH_4$, *MeOH*.

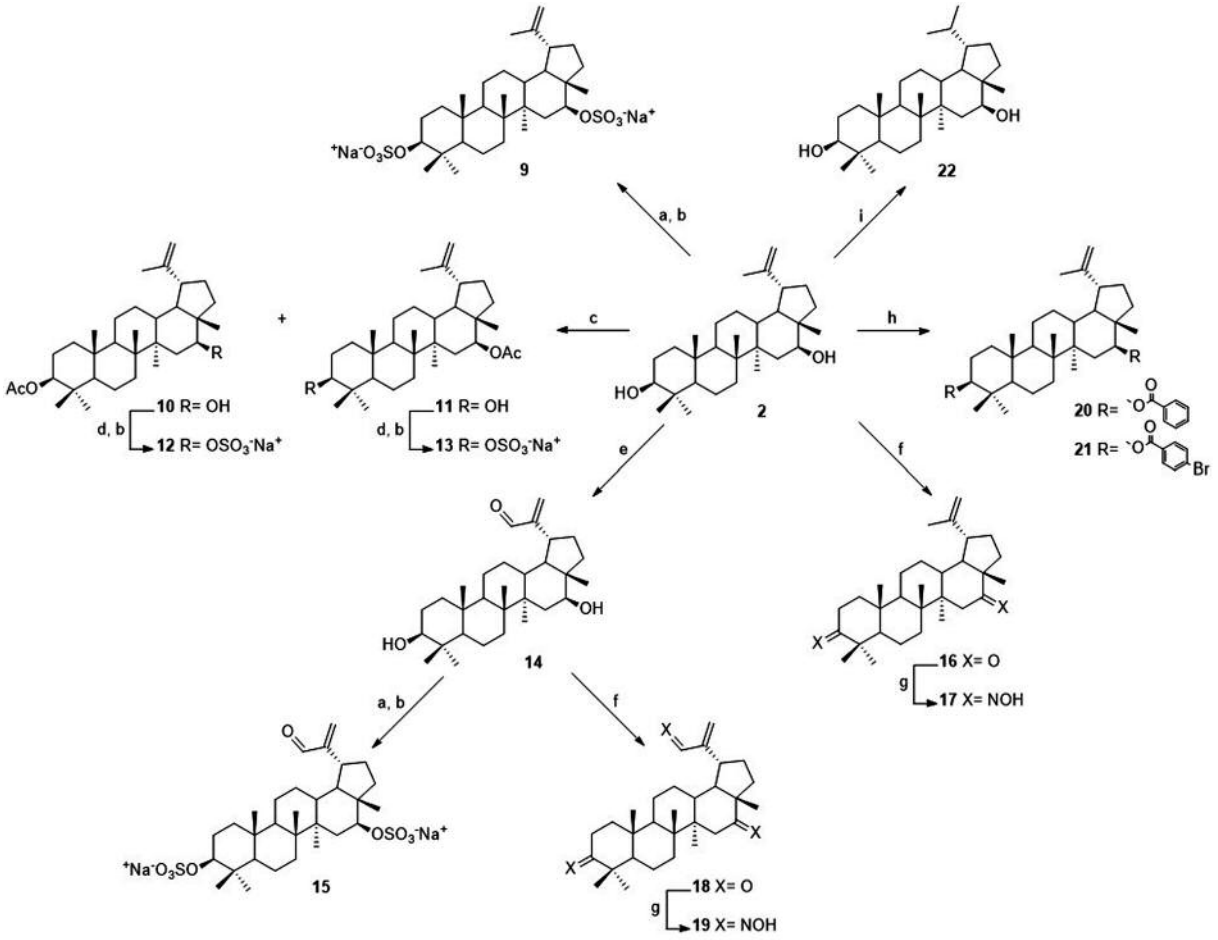

Figure 3. *Synthesis of compounds* ***9-17***. *Reagents and conditions: (a) 8 equiv.* $Me_3N.SO_3$, *DMF, 7 min, 150˚C, microwave; (b) Amberlite CG-120 (MeOH); (c) AcO, Py, DMAP, DCM; (d) 4 equiv.* $Me_3N{\bullet}SO_3$, *DMF, 7 min, 150˚C, microwave; (e)* SeO_2, *EtOH, reflux; (f) Jones reagent, acetone, 0˚C; (g)* $NH_2OH{\bullet}HCl$, *NaOAc, EtOH/*H_2O; *(h) ArCOCl, Py, DMAP, DCM; (i)* H_2, *Pd/C, EtOAc*.

multiplet at 3.96 ppm (H-3α), geminal to the sulfate group at C-3 (87.6 ppm) as determined by analysis of ^{13}C NMR and HSQC spectra, which confirmed the presence of a sulfate group at C-3 in β orientation.

Oxidation of **1** with Jones reagent in acetone rendered lupenone (**5**) in almost quantitative yield, while the reaction of **5** with hydroxylamine hydrochloride (NH_2OH•HCl) provided oxime **6** in 31% yield. From this synthesis, the *E*-isomer was obtained as the only product, as was clearly seen on TLC and confirmed by analysis of proton and carbon NMR chemical shifts. The ^1H-NMR spectrum showed a multiplet at 2.96 ppm (H-2β), which was shifted downfield due to the deshielding effect of the hydroxyl oxygen of the oxime. This assignment was confirmed by the correlation between H-2β and C-3 (167.4 ppm) in the HMBC spectra of the compound. The ^1H-NMR spectrum also showed the presence of a singlet at 8.26 ppm for the proton of the NOH group.

The reaction of **6** with $LiAlH_4$ in THF gave a mixture of amines (3α)-**7a** and (3β)-**7b** in a 27:73 ratio, which were separated by flash chromatography. The α or β orientation for the amino group attached to C-3 was established from their ^1H-NMR spectrum. However, due to the limited amount of sample, the complete spectroscopic characterization and biological activity of amine **7a** was not possible. Hydrogenation of ketone **5** using $NaBH_4$ led to a 10:90 mixture of the α- and β-epimers of lupeol (compounds **8** and **1**, respectively), according to the ^1H-NMR spectrum. Both alcohols were separated and purified by flash chromatography.

The second series of derivatives (**9-22**) were prepared from **2**, following the reaction sequence shown in Figure 3. Sulfation of **2** with 8 equivalents of Me_3N•SO_3 for 7 min at 150˚C in a microwave reactor and further transformation *via* ion exchange afforded disodium disulfate **9** in 93% yield. Esterification of diol **2** with 1 equivalent of acetic anhydride yielded a 1:1 mixture of the monoacetates **10** and **11**. Both monoacetylated derivatives were separated and purified by flash chromatography. Sulfation of **10** and **11** with 4 equivalents of Me_3N•SO_3 rendered the corresponding monosulfates **12** (44% yield) and **13** (27% yield), respectively. The ^1H-NMR spectrum of **12** showed a double doublet at 4.48 ppm corresponding to the presence of an acetoxy group at C-3 and a triplet at 4.37 ppm assignable to H-16 with a sulfate group attached to C-16. This was in accordance with the chemical shifts observed for C-3 (82.1 ppm) and C-16 (85.9 ppm), which were unequivocally assigned from the HSQC and HMBC spectra. ^1H- and ^{13}C-NMR data of compound **13** confirmed that, in this case, the sulfate group was attached to C-3 while the acetoxy group was at C-16 [δ_H 3.96 (dd, H-3α), δ_H 4.91 (br s, H-16α), δ_C 87.5 (C-3) and δ_C 80.7 (C-16)].

Allylic oxidation of **2** with 2.5 equivalents of SeO_2 was carried out for the introduction of formyl functionality at the C-30 position, yielding compound **14** in excellent yield (97%). Subsequent sulfation of **14** with 8 equivalents of Me_3N•SO_3 afforded the disulfated analog **15**. Oxidation with Jones reagent of compound **2** yielded diketone **16**, which in turn was converted in dioxime **17** (35%) by treatment with NH_2OH•HCl and heating under reflux. Analysis of proton and carbon NMR chemical shifts at C-2, C-3, C-15, and C-16 confirmed the structure of **17**. Resonances showing H-15β as a doublet at 2.90 ppm was shifted downfield due to the deshielding effect of the hydroxyl oxygen of the oxime of C-16, which was indicative of the *E* configuration. This assignment was confirmed by the correlation between H-15β and C-16 (165.2 ppm) in the HMBC spectra of compound **17**. In addition, the signals belonging to H-2β confirmed the *E* configuration of the hydroxyimino group of C-3, as described above for the oxime **6**. The ^1H-NMR spectrum also showed the presence of two broad singlets at 9.68 and 9.58 ppm for the protons of the NOH groups.

On the other hand, oxidation of compound **14** with Jones reagent rendered 3,16,30-trioxolupane **18**, and reaction of compound **18** with NH_2OH•HCl provided trioxime **19** (31% yield). Analysis of proton and carbon NMR chemical shifts confirmed the *E* configuration of the hydroxyimino groups of C-3 and C-16, as described above for the oxime **17**.

Table I. *Cytotoxicity of triterpenes **1-22** after 24 h incubation with PC-3 and LNCaP prostate cancer cells as measured with toxicity assay.*

Compound	IC_{50} (μM)	
	PC-3	LNCaP
1	>70	>70
2	>70	>70
3	46.58±1.10	42.81±1.06
4	16.13±1.10	7.43±1.32
5	>70	>70
6	>70	>70
7b	32.12±1.09	42.09±1.16
8	>70	>70
9	60.70±1.08	45.79±1.06
10	>70	>70
11	>70	>70
12	44.93±1.14	38.43±1.07
13	49.21±1.05	41.13±1.04
14	>70	>70
15	60.07±1.07	>70
16	>70	>70
17	>70	>70
18	>70	>70
19	>70	>70
20	>70	>70
21	>70	>70
22	>70	>70
Doxorubicin	1.63±0.01	1.89±0.01

IC_{50}: Concentration resulting in a 50% reduction in cell viability compared to vehicle-treated cells. Data are the mean±S.D. obtained on the basis of triplicate assays.

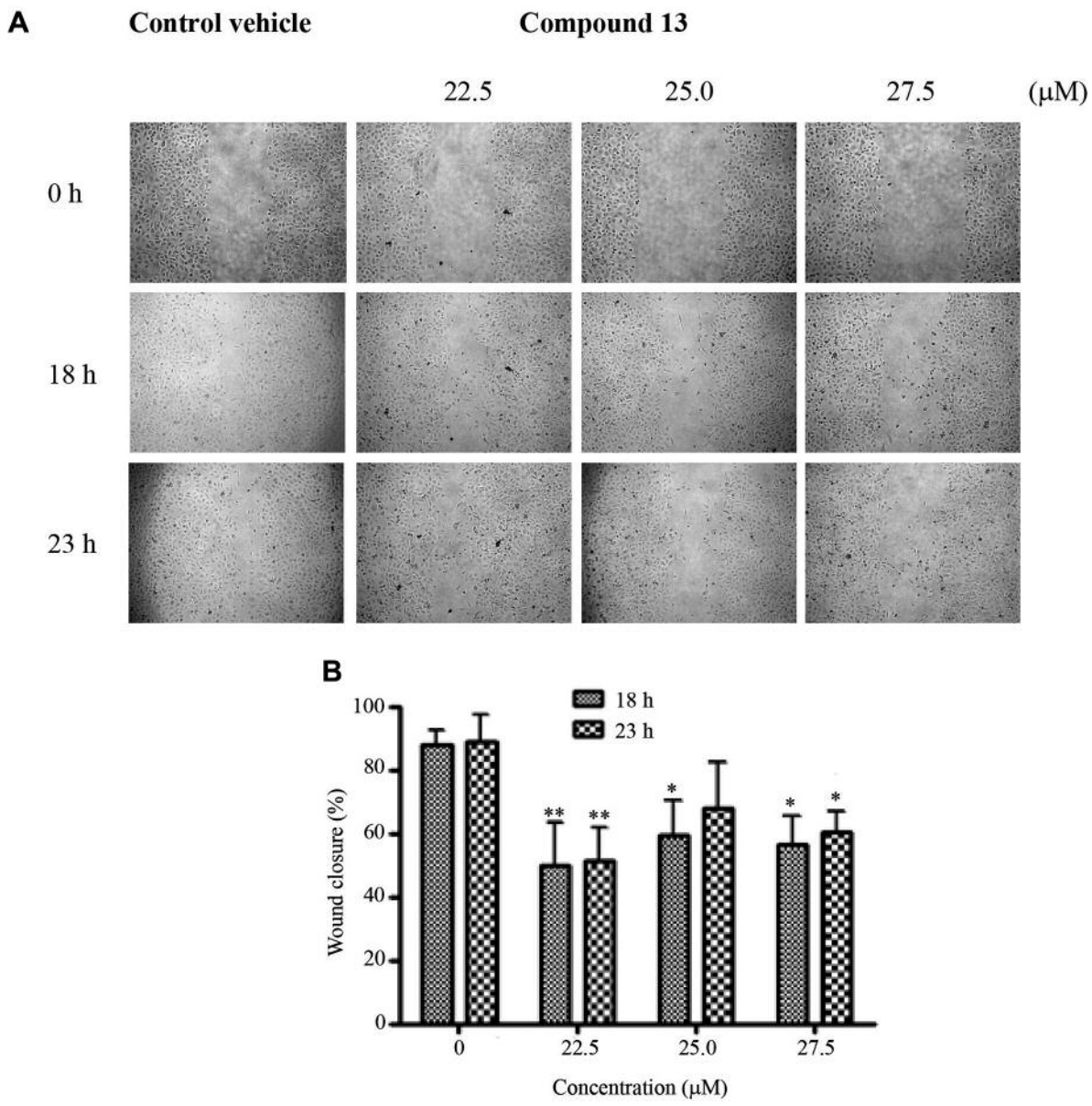

Figure 4. *Compound **13** suppressed migration of PC-3 cells in vitro. A: Representative light microscopy images of wound closure (4×). Cells were grown to confluence, wounded and then incubated with different concentrations of **13**. Images were captured at 0, 18 and 23 h after wounding. B: Quantitative analysis of wound closure was performed using the Image J software and data are expressed as the mean±standard deviation. Compound **13** significantly inhibited PC-3 cell migration at 22.5 μM compared with the control at 18 and 23 h (*p<0.05 and **p<0.001).*

Treatment of diol **2** with the corresponding acyl chlorides in pyridine and DMAP, afforded the esters **20** (41% yield) and **21** (42% yield), while reduction of the double bond of **2** was carried out by catalytic hydrogenation yielding the derivative **22** (65% yield).

The lupeol derivatives **5-8** were identified by comparison of their spectroscopic data with those reported in the literature (22, 24-26). Calenduladiol derivatives **9-11**, **14-16**, **18** and **20-22** were identified by comparison of their spectroscopic data with those reported in our previous work (22, 23). Full structural elucidation of the new lupane derivatives **4**, **12**, **13**, **17**, and **19** was achieved by NMR and mass spectroscopy, and assignments were performed based on our analysis and related literature.

Cytotoxic activity. To determine the potential application of natural triterpenoids **1-3** and their semisynthetic derivatives **4-22** for the treatment of PCa, their *in vitro* cytotoxic activity

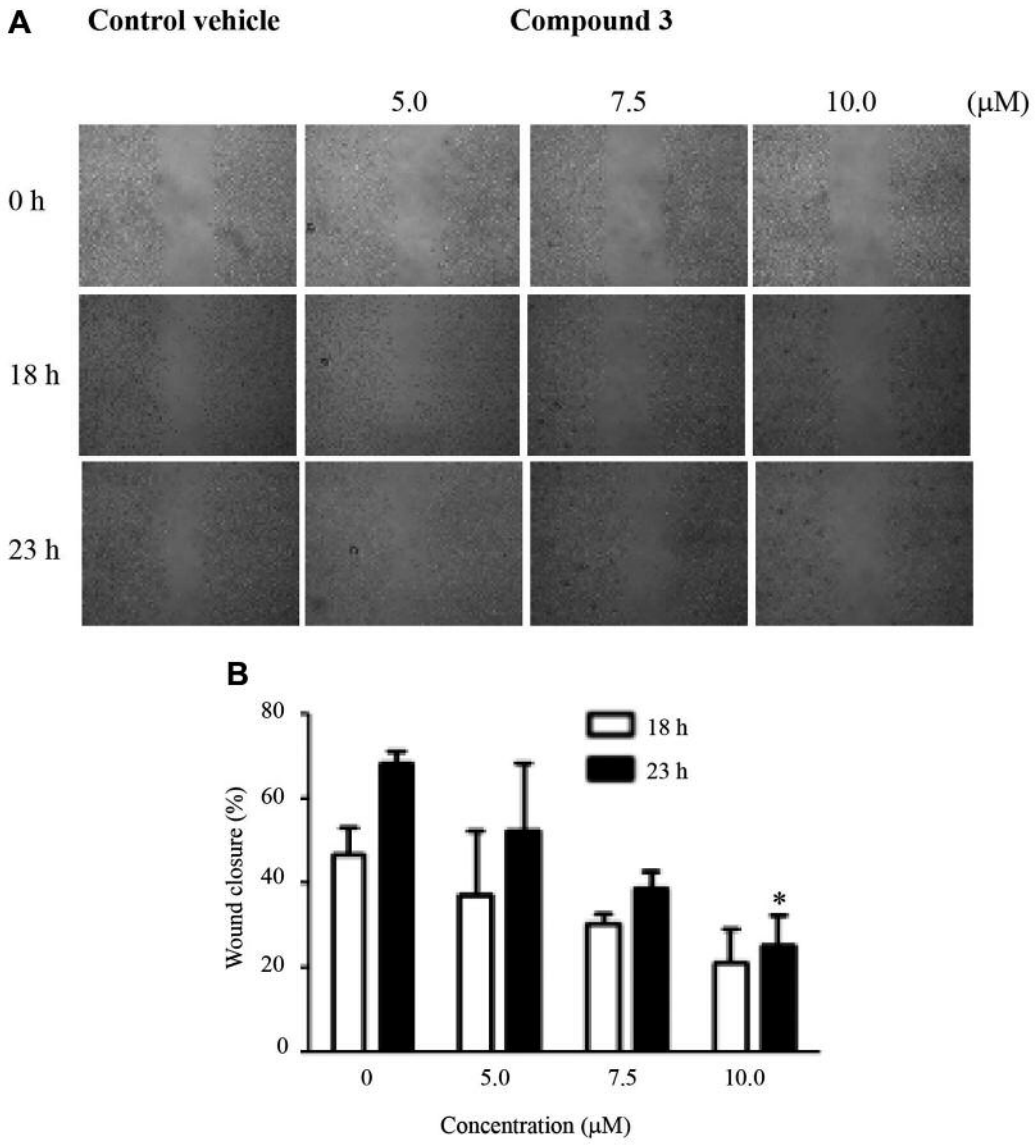

Figure 5. *Heliantriol B2 (**3**) suppressed migration of LNCaP cells in vitro. A: Representative light microscopy images of wound closure (4×). Cells were grown to confluence, wounded and then incubated with different concentrations of **3**. Images were captured at 0, 18 and 23 h after wounding. Note that the wound closure was suppressed by **3** in a dose-dependent manner. B: Quantitative analysis of wound closure was performed using Image J software and data are expressed as the mean±standard deviation. Compound **3** significantly inhibited LNCaP cell migration at 10 μM respect compared with the control at 23 h (*p<0.05).*

against two human prostate carcinoma cell lines, PC-3 (androgen-independent) and LNCaP (androgen-dependent), was evaluated. The results, expressed as IC_{50} values, are summarized in Table I. Cell lines were exposed for 24 h with the compounds at a range of 5-70 μM. MTS one-step colorimetric assay was performed to test the cytotoxic potential and to determine IC_{50} doses. Doxorubicin was used as a reference anticancer drug.

Treatment of PC-3 and LNCaP cells with natural triterpenoids **1-3** resulted in dose- and time-dependent inhibition of cell growth for both cell lines (data not shown), as previously reported for lupeol (12, 13). Among these natural triterpenoids, tri-hydroxylated triterpene heliantriol B2 (**3**) showed the greatest cytotoxic effect following 24 h of exposure. These results are consistent with our previous report of cytotoxic activity against human leukemia cell lines for this triterpene (7).

Lupeol-3β-sulfate (sodium 3β-hydroxylup-20(29)-ene sulfate, **4**), a new semisynthetic lupane, was the most active compound, with IC_{50} values of 16.13 μM (PC-3) and 7.43

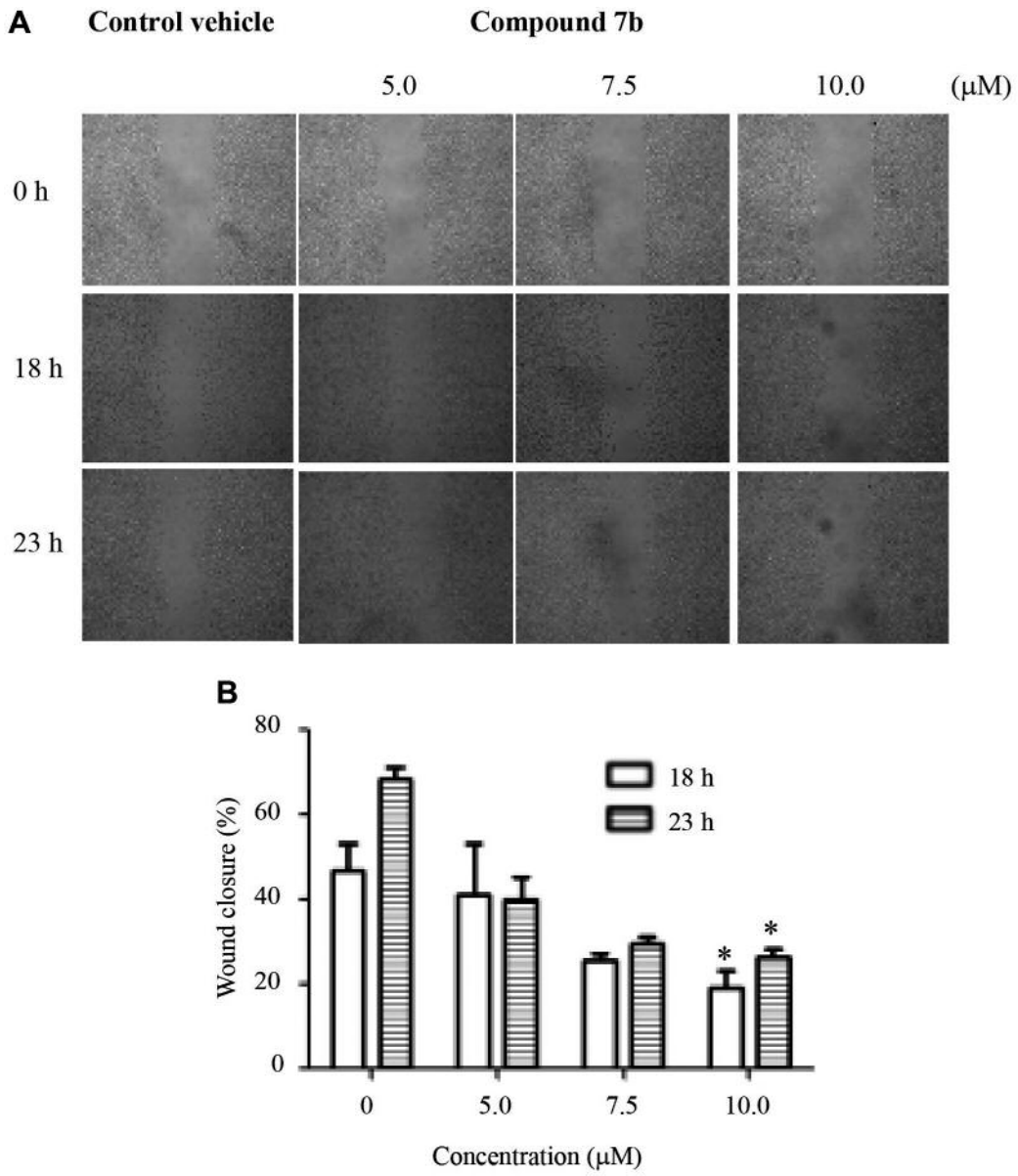

Figure 6. *Compound **7b** suppressed migration of LNCaP cells in vitro. A: Representative light microscopy images of wound closure (4×). Cells were grown to confluence, wounded and then incubated with different concentrations of **7b**. Images were captured at 0, 18 and 23 h after wounding. Note that the wound closure was suppressed by **7b** in a dose-dependent manner. B: Quantitative analysis of wound closure was performed using Image J software and data are expressed as the mean±standard deviation. Compound **7b** significantly inhibited LNCaP cell migration at 10 μM compared with the control at 18 and 23 h (*p<0.05).*

μM (LNCaP). In general, the introduction of sulfate groups at C-3 or C-16 increased cytotoxic activity against both cancer cell lines compared to the corresponding non-sulfated compounds (**4** *vs*. **1**, **9** *vs*. **2**, **12** *vs*. **10**, **13** *vs*. **11**, and **15** *vs*. **14**). This improvement in cytotoxic activity exerted by the sulfate groups has also been observed in sulfated hydroximinosteroids (20, 28, 29). On the other hand, the replacement of a hydroxyl group by a carbonyl group at C-3 or C-16 did not increase cytotoxicity (**5** *vs*. **1**, **16** *vs*. **2**, and **18** *vs*. **14**). A similar tendency was observed for derivatives **6**, **17**, and **19** with one, two or three oximes groups at C-3, C-16 or C-30, compared with ketones starting corresponding (**6** *vs*. **5**, **17** *vs*. **16**, and **19** *vs*. **18**). Surprisingly, the presence of a C-3 amino group led to a decrease in the concentration of growth inhibition of PC-3 and LNCaP, with respect to lupeol, with IC_{50} values of 32.1 and 42.1 μM, respectively

(**7b** *vs*. **1**). The introduction of formyl functionality at the C-30 position (compound **14**) was not significant for activity. In the same way, the stereochemistry of the hydroxyl group at C-3 did not seem to be relevant for activity, at the tested concentrations. This was apparent when comparing lupeol (**1**) with its epimer (compound **8**). Finally, neither the esterification of C-3 and C-16 positions with the corresponding benzoyl chlorides (compounds **20** and **21**), nor the hydrogenation of the double bond of diol **2** (compound **22**) led to an improvement in cytotoxic activity.

The identification of compounds with high cytotoxic potencies (IC_{50}<70 μM) motivated further evaluation of their ability to inhibit metastatic PCa cell migration using a wound-healing assay (30). Figure 4A shows representative light microscopy wound images of PC-3 cells, while Figures 5A and 6A correspond to LNCaP cells. For wound-healing assays, compound concentrations with no significant drug sensitivity (IC_{10}) were selected. Following exposure of PC-3 cells to derivative **13** (Figure 4A) at doses ranging from 22.5 to 27.5 μM, and of LNCaP cells to natural triterpenoid **3** (Figure 5A) and derivative **7b** (Figure 6A) at doses ranging from 5.0 to 10.0 μM, wound closure was much slower and the wound was still open at 18 or 23 h after exposure. As wound closure reflects the migratory ability of cancer cells, these results indicate that the migration of PC-3 cells was significantly inhibited by compound **13** at 22.5 μM at 18 or 23 h post-treatment (p<0.001) (Figure 4B). In addition, compound **7b** inhibited LNCaP cells motility at 10 μM at 18 or 23 h (p<0.05) (Figure 6B), while compound **3** significantly inhibited the migration of LNCaP cells only at 23 h compared with the control (p<0.05) at the same dose (Figure 5B).

Discussion

In summary, five new semisynthetic triterpenoids were obtained from **1** and **2**, and fully characterized, together with 14 known ones, in order to evaluate their cytotoxicity against prostatic cancer cell lines. Seven derivatives showed an improvement of activity compared with the natural starting compounds. In particular, the sulfation of lupeol substantially improved its antitumor activity, which means a significant decrease in the effective dose of this triterpenoid. Our results suggest that the sulfate group might be involved in the cytotoxicity of this type of structure; further investigation into the anticancer activity of this type of triterpenoid derivative may be profitable.

The formation of new tissue, invasion and tumor cell metastasis all depend on cell motility and migration (31). Therefore, cell migration constitutes an attractive target for the development of potential antitumor compounds. Herein, results obtained for the evaluated lupanes showed that the natural tri-hydroxylated triterpenoid **3** and analogs **7b** and **13** could be considered as inhibitors of cell migration after 18 and 23 h incubation, respectively, which might lead to a further decrease in cell invasion and consequently prevention of metastasis.

Conflicts of Interest

The Authors declare that there are no conflicts of interest in regard to this study.

Authors' Contributions

M.B.F., A.P.M. and J.C.C. were involved in planning and supervised the work; M.J.C performed the synthesis and characterization of compounds; V.P.C. and P.A.S. developed the assays on cells.

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References

1 Cancer: Fact Sheet no. 297. World Health Organization, Geneva, 2015. Available at http://www.who.int/en/news-room/fact-sheets/detail/cancer

2 Jemal A, Bray F, Center MM, Ferlay J, Ward E and Forma D: Global cancer statistics CA. Cancer J Clin *61*: 69-90, 2011. PMID: 21296855. DOI: 10.3322/caac.20107

3 Newman DJ and Giddings LA: Natural products as leads to antitumor drugs. Phytochem Rev *13*: 123-137, 2014. DOI: 10.1007/s11101-013-9292-6

4 Hill RA and Connolly JD: Triterpenoids. Nat Prod Rep *30*: 1028-1065, 2013. PMID: 23736383. DOI: 10.1039/c3np70032a

5 Ukiya M, Akihisa T, Tokuda H, Suzuki H, Mukainaka T, Ichiishi E, Yasukawa K, Kasahara Y and Nishino H: Constituents of Compositae plants III. Antitumor promoting effects and cytotoxic activity against human cancer cell lines of triterpene diols and triols from edible chrysanthemum flowers. Cancer Lett *177*: 7-12, 2002. PMID: 11809525.

6 García A and Delgado G: Cytotoxic cis-fused bicyclic sesquiterpenoids from *Jatropha neopauciflora*. J Nat Prod *69*: 1618-1621, 2006. PMID: 17125233. DOI: 10.1021/np060194h

7 Vela Gurovic MS, Díaz Lanza AM, Boyano Adánez MC, Estañ Omaña MC, Gañán Gómez I, Murray AP, Sancho López P: Cytotoxic effects induced by combination of heliantriol B2 and dequalinium against human leukemic cell lines. Phytother Res *25*: 603-610, 2011. PMID: 20981868. DOI: 10.1002/ptr.3310

8 Shanmugam MK, Nguyen AH, Kumar AP, Tan BKH and Sethi G: Targeted inhibition of tumor proliferation, survival, and metastasis by pentacyclic triterpenoids: Potential role in prevention and therapy of cancer. Cancer Lett *320*: 158-170, 2012. PMID: 22406826. DOI: 10.1016/j.canlet.2012.02.037

9 Hao J, Pei Y, Ji G, Li W, Feng S and Qiu S: Autophagy is induced by 3β-O-succinyl-lupeol (LD9-4) in A549 cells *via* up-regulation of Beclin 1 and down-regulation mTOR pathway. Eur J Pharmacol *670*: 29-38, 2011. PMID: 21939652. DOI: 10.1016/j.ejphar.2011.08.045.

10 Li W, Hao J and Xiao Y: Synthesis and *in vitro* antitumor activities of lupeol dicarboxylic acid monoester derivatives. Arch Pharm Res *36*: 1447-1453, 2013. PMID: 23700293. DOI: 10.1007/s12272-013-0155-x

11 Li W and Xiao Y: Synthesis and *in vitro* antitumour activities of lupeol derivatives. Nat Prod Res *32*: 48-53, 2018. PMID: 28511560. DOI: 10.1080/14786419.2017.1329729

12 Saleem M, Kweon MH, Yun JM, Adhami VM, Khan N, Syed DN and Mukhtaret H: A novel dietary triterpene lupeol induces Fas-mediated apoptotic death of androgen-sensitive prostate cancer cells and inhibits tumor growth in a xenograft model. Cancer Res *65*: 11203-11213, 2005. PMID: 16322271. DOI: 10.1158/0008-5472.CAN-05-1965

13 Prasad S, Nigam N, Kalra N and Shukla Y: Regulation of signaling pathways involved in lupeol induced inhibition of proliferation and induction of apoptosis in human prostate cancer cells. Mol Carcinog *47*: 916-924, 2008. PMID: 18404669. DOI: 10.1002/mc.20442

14 Saleem M, Murtaza I, Tarapore RS Suh Y, Adhami VM, Johnson JJ, Siddiqui IA, Khan N, Asim M, Hafeez BB, Shekhani MT, Li B and Mukhtar H: Lupeol inhibits proliferation of human prostate cancer cells by targeting beta-catenin signaling. Carcinog *30*: 808-817, 2009. PMID: 19233958. DOI: 10.1093/carcin/bgp044

15 Saleem M, Murtaza I, Witkowsky O, Kohl AM and Maddodi N: Lupeol triterpene, a novel diet-based microtubule targeting agent, disrupts survivin/cFLIP activation in prostate cancer cells. Biochem. Biophys Res Commun *388*: 576-582, 2009. PMID: 19683515. DOI: 10.1016/j.bbrc.2009.08.060

16 Khan N, Afaq F and Mukhtar H: Cancer chemoprevention through dietary antioxidants: Progress and promise. Antioxid Redox Signal *10*: 475-510, 2008. PMID: 18154485. DOI: 10.1089/ars.2007.1740.

17 Siddique HR and Saleem M: Beneficial health effects of lupeol triterpene: A review of preclinical studies. Life Sci *88*: 285-293, 2011. PMID: 21118697. DOI: 10.1016/j.lfs.2010.11.020

18 Casamiquela RM, Beeskow AM, Gavirati M, Stanganelli M and Mavrek V: Usos Tradicionales de las Plantas en la Meseta Patagónica. CENPAT – CONICET – ICBG, 1-51 2002.

19 Vela Gurovic MS, Castro MJ, Richmond V, Faraoni MB, Maier MS and Murray AP: Triterpenoids with acetylcholinesterase inhibition from *Chuquiraga erinacea* D. Don. subsp. *erinacea* (*Asteraceae*). Planta Med *76*: 607-610, 2010. PMID: 19918718. DOI: 10.1055/s-0029-1240582

20 Richmond V, Careaga VP, Sacca P, Calvo JC and Maier MS: Synthesis and cytotoxic evaluation of four new 6E-hydroximino-steroids. Steroids *84*: 7-10, 2014. PMID: 24632027. DOI: 10.1016/j.steroids.2014.03.001

21 Amarego WLF and Chai CUU: Purification of Laboratory Chemicals. Oxford, Pergamon, 1988.

22 Castro MJ, Richmond V, Faraoni MB and Murray AP: Oxidation at C-16 enhances butyrylcholinesterase inhibition in lupane triterpenoids. Bioorg Chem *79*: 301-309, 2018. PMID: 29793143. DOI: 10.1016/j.bioorg.2018.05.012

23 Castro MJ, Richmond V, Romero C, Maier MS, Estévez-Braun A, Ravelo AG, Faraoni MB and Murray AP: Preparation, anticholinesterase activity and molecular docking of new lupane derivatives. Bioorg Med Chem *22*: 3341-3350, 2014. PMID: 24835788. DOI: 10.1016/j.bmc.2014.04.050

24 Yasukawa K, Yu SY, Yamanouchi S, Takido M, Akihisa T and Tamura T: Some lupane-type triterpenes inhibit tumor promotion by 12-O-tetradecanoylphorbol-13-acetate in two-stage carcinogenesis in mouse skin. Phytomed *411*: 309-313, 1995. PMID: 23196020. DOI: 10.1016/S0944-7113(11)80008-5

25 Kumar S, Misra N, Raj K, Srivastava K and Puri SK: Novel class of hybrid natural products derived from lupeol as antimalarial agents. Nat Prod Res *22*: 305-319, 2008. PMID: 18322846. DOI: 10.1080/14786410701766349

26 Misra N, Kumar S and Raj K: Novel class of hybrid natural products derived from lupeol/lupenone. Part (II). Indian J Chem *2*: 275-283, 2011.

27 Yue PYK, Leung EPY, Mak NK and Wong RNS: A simplified method for quantifying cell migration/wound healing in 96-well plates. J Biomol Screen *15*: 427-433, 2010. PMID: 20208035. DOI: 10.1177/1087057110361772

28 Cui J, Wang H, Huang Y, Xin Y and Zhou A: Synthesis and cytotoxic analysis of some disodium 3β,6β-dihydroxysterol disulfates. Steroids *74*: 1057-1060, 2009. PMID: 19703482. DOI: 10.1016/j.steroids.2009.08.006

29 Huang Y, Cui J, Li Y, Fan L, Jiao Y and Su S: Synthesis and antiproliferative activity of some sulfated hydroximinosterols. Med Chem Res *22*: 409-414, 2013. DOI: 10.1007/s00044-012-0047-5

30 Rodríguez L, Wu X and Guan J: Wound-healing assay. Methods Mol Biol *294*: 23-29, 2005

31 Chodniewicz D and Klemke R: Guiding cell migration through directed extension and stabilization of pseudopodia. Exp Cell Res *301*: 31-37, 2004. PMID: 15501442. DOI: 10.1016/j.yexcr.2004.08.006

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