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Dehydroleucodine and dehydroparishin-B inhibit proliferation and motility of B16 melanoma cells

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ABSTRACT

Dehydroleucodine, a known sesquiterpene lactone, and dehydroparishin-B, a new guaiane type sesquiterpene acid, were isolated from aerial parts of *Artemisia douglasiana* by chloroform extraction. We identified dehydroparishin-B as (7R)-2-oxo-guaia-1(10),3(4),5(6),11(13)-tetraen-12-oic acid by MS and NMR methods. We demonstrated that both dehydroparishin-B and dehydroleucodine blocked cell proliferation of B16 melanoma cells, but not normal murine Melan-A melanocytes, in a dose-dependent manner without affecting cell viability. We also found that both dehydroparishin-B and dehydroleucodine inhibited migration of B16 melanoma cells. These results suggest that dehydroleucodine and dehydroparishin-B could represent potential candidates for the treatment of metastatic melanomas.

1. Introduction

Artemisia douglasiana Besser (Asteraceae) is a bush native to west USA, but it also grows as an adventicious plant in the west dry region of Argentina where it is popularly used as medicinal plant (Giordano et al., 1992). Extraction of *A. douglasiana* collected in Mendoza, Argentina, afforded two guaiane sesquiterpenes. One of them was identified as dehydroleucodine (**1**) (Fig. 1), a sesquiterpene lactone previously reported to occur in this species (Giordano et al., 1992). The second sesquiterpene is a new compound that was characterized as (7*R*)-2-oxo-guaia-1(10),3(4),5(6),11(13)-tetraen-12-oic acid (**2**) (Fig. 1). We named dehydroparishin-B (**2**) because of its structural relation to parishin-B, compound which differs from dehydroparishin-B (**2**) only by the saturated C_{11}–C_{13} bond. Parishin-B was previously isolated from *Artemisia tridentata* ssp. *tridentata* f. *parishii* (Gray) Beetle (Kelsey and Shafizadeh, 1979).

A. douglasiana is used as folk medicine for the treatment of chronic gastritis and peptic ulcer. It was also shown that dehydroleucodine (**1**), a major component of the plant, exhibit a powerful cytoprotective action in ethanol-induced gastric mucosal injury (Giordano et al., 1990). It also inhibits cell proliferartion of vascular smooth muscle cells (Polo et al., 2007), growth of *Trypanosoma cruzi* (Brengio et al., 2000) and differentiation of pre-adipocytes (Galvis et al., 2011). In this study we demonstrated that dehydroleucodine (**1**) and dehydroparishin-B (**2**) affect proliferation and migration of B16 melanoma cells.

2. Results and discussion

The chloroform extract of *A. douglasiana* afforded two guaiane sesquiterpenes. The major one was identified as dehydroleucodine (**1**) by comparison of its spectral data with those reported in the literature (Bohlmann and Zdero, 1972). The structure of dehydroleucodine (**1**) was also corroborated by X-ray analysis (Priestap et al., 2011). The second sesquiterpene, $C_{15}H_{16}O_3$ (HRMS-ESI-TOF), named dehydroparishin-B, is soluble in $NaHCO_3$ solution and proved to be a new compound. The UV spectrum shows absorption maxima at 239, 256 and 317 nm indicating an extended chromophore with respect to that of dehydroleucodine (**2**). Evidence of an intact carboxylic acid group in the molecule was provided by chemical behavior and formation of a methyl ester with diazomethane. The ^{1}H NMR spectrum of dehydroparishin-B (**2**) (Supplementary data, Fig. S1) shows 4 olefinic protons at δ 6.19 (s, H_B-13), 6.02 (s, H-3), 5.96 (d, H-6) and 5.37 (s, H_A-13), an aliphatic methine proton at δ 3.70 (q, H-7), two aliphatic methylenes at δ 2.33 (m, H_2-9) and 1.80 (m, H_2-8) and two

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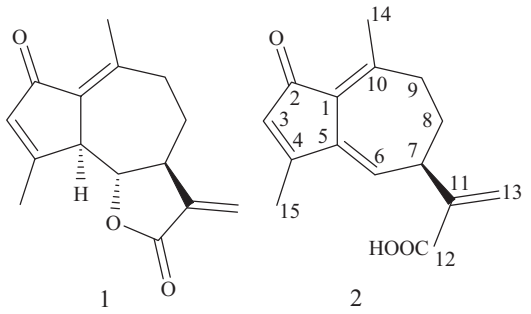

Fig. 1. Chemical structures of dehydroleucodine (**1**) and dehydroparishin-B (**2**).

methyls at δ 2.26 (s, CH_3-14) and 2.06 (s, CH_3-15). The ^{13}C NMR spectrum of dehydroparishin-B (Supplementary data, Fig. S2) displayed 15 carbon resonances; 5 signals at high fields consisting of two CH_3, two CH_2 and one CH, and 10 signals at lower fields consisting of one olefinic CH_2, two olefinic CH and 7 quaternary carbons (a ketone, a carboxyl and five olefinic). Dehydroparishin-B is then a sesquiterpene related to dehydroleucodine (**1**). It contains the oxygenated isopropenyl side chain on C-7 of dehydroleucodine but in the unlactonized form and an extra double bond in the seven-membered ring as shown in **2**. Structure **2** was corroborated by comparison of the carbon shifts with those of related compounds such as mongolicumin B (five membered ring carbons) (Shi et al., 2007), dehydroleucodine (carbons 9, 10 and 14) (de Heluani et al., 1989) and lacitemzine (carbons 11, 12 and 13) (Ghouila et al., 2008). Definitive evidence was obtained from 2D NMR experiments (^1H–^1H COSY, ^1H–^{13}C COSY and ^1H–^{13}C COLOC).

Dehydroparishin-B (**2**) has a chiral center at the C_7-position. ^1H NMR spectra of dehydroparishin-B in both DMSO-d_6 and $CDCl_3$ solution show H-7 as a quartet due to three $^3J_{HH}$ equal couplings of ca. 5 Hz to H-6 and both H-8 protons. H-7 may then be in a *syn* relationship with each of the neighboring H-8 protons indicating the α-orientation of H-7 or the R configuration at C-7. The above considerations support the structure of (7*R*)-2-oxo-guaia-1(10),3(4),5(6),11(13)-tetraen-12-oic acid (**2**) [2-((5*R*)-3,8-dimethyl-1-oxo-5,6,7-trihydroazulen-5-yl)prop-2-enoic acid; IUPAC nomenclature] for dehydroparishin-B.

Sesquiterpene lactones are a major class of plant secondary metabolites and thousands of different structures have been elucidated (Fraga, 2010). The sesquiterpene dehydroleucodine (**1**) and the related compound leucodin are known to occur in species of the Asteraceae (Bohlmann and Zdero, 1972; Martinez et al., 1988). Sesquiterpene lactones, including those of the guaianolide type, are also widely distributed in the genus *Artemisia* (Kesley and Shafizadeh, 1979). However, within this genus, dehydroleucodine (**1**) appears to be restricted to *A. tridentata* ssp. vaseyana (Rydb.) (Kesley and Shafizadeh, 1979) and to *A. douglasiana* (Giordano et al., 1990).

Despite the large number of sesquiterpenes documented for several families of plants, sesquiterpene acids are only occasionally reported (Fraga, 2010). The presence of related unlactonized sesquiterpene acids in the genus *Artemisia* seems to be restricted to parishin-B isolated from *A. tridentata* ssp. *tridentata* f. *parishii* (Gray) Beetle (Kesley and Shafizadeh, 1979; Fraga, 2010). The new sesquiterpene acid dehydroparishin-B (**2**), described in this study, may then be the second compound of this type reported to occur in the genus *Artemisia*.

Introduction of a carboxylic acid function in the isoprenyl side chain is the previous step for the formation of the sesquiterpene lactones. The involved enzymes have been described (de Kraker et al., 2001, 2002). Lactonization occurs via hydroxylation at either of the adjacent positions to the side chain by a cytochrome P450 enzyme followed by reaction between the COOH and OH groups (de Kraker et al., 2001, 2002). Dehydroleucodine (**1**) and dehydroparishin-B (**2**) may arise from a common precursor and the latter (**2**) may be formed from a 6-hydroxylated precursor of dehydroleucodine (**1**) by dehydration. The presence of dehydroparishin-B (**2**) in *A. douglasiana* may be an indication that, after introduction of an oxygen function at C-6 of a precursor, enzymatic dehydration may lead to an alternative biosynthetic pathway.

Studies on sesquiterpene lactones have shown distinctive biological actions, including anti-bacterial (Ozcelik et al., 2009), anti-viral (Efferth et al., 2008), anti-protozoal (Schmidt et al., 2009), anti-inflammatory (Khan et al., 2010) and anti-proliferative (Ghantous et al., 2010) activities, among others. Some sesquiterpene lactones have been tested for their cytotoxic effects against B6 melanoma cells (Liu et al., 2008; Vuckovic et al., 2010) and related cell lines (B16F10, B16/BL6) (Konishi et al., 2002; Taylor et al., 2008) showing a wide range of activities (IC_{50} = 3–400 μM). Here, we report for the first time the effect of dehydroleucodine (**1**) and dehydroparishin-B (**2**) on the proliferation and migration of B16 melanoma cells. As control we have used Melan-A cells, an immortalized normal human melanocytes (Bennett et al., 1987).

In Fig. 2, we show the effect of dehydroleucodine (**1**) and dehydroparishin-B (**2**) on the proliferation of B16 melanoma and Melan-A cells. Our results show that dehydroleucodine (**1**) and dehydroparishin-B (**2**) inhibited cell proliferation in B16 melanoma cells in a dose-dependent manner without affecting proliferation of Melan-A cells (Fig. 2A and B). Also, our observations show that dehydroleucodine (**1**) and dehydroparishin-B (**2**) inhibited the incorporation of [^{3}H] thymidine into DNA in B16 melanoma cells in a dose-dependent manner with an $IC_{50} = 3.4 \pm 0.3$ μM and $IC_{50} = 40 \pm 2.9$ μM for B16 cells, respectively. Both dehydroleucodine (**1**) and dehydroparishin-B (**2**) possess anti-proliferative effects, but the former compound resulted highly inhibitory and significantly more potent than dehydroparishin-B (**2**) (compare $IC_{50} = 3.4 \pm 0.3$ μM and $IC_{50} = 40 \pm 2.9$ μM for B16 cells). The trypan blue dye exclusion assay indicated that cell viability was practically unaffected by these compounds (Fig. 2C and D). As a positive control, we have used helenalin, a potent inhibitor of protein and nucleic acid synthesis (Williams et al., 1988) (compare DMSO-control: 100 ± 10 vs. 5 μM Helenalin: 46 ± 6.2).It is known that, under mild conditions (25 °C, pH 7.4), sesquiterpenes possessing the α-methylene γ-lactone structure can react with thiol groups by a Michael-type reaction (Kupchan et al., 1970). Sesquiterpene lactones may then exert their activity by interaction of the α-methylene γ-lactone moiety with thiol groups of macromolecules (Ghantous et al., 2010; Picman, 1986). Sesquiterpene lactones may inhibit the nuclear factor κB (NF-κB) that is implicated cancer development (Ghantous et al., 2010; Taylor et al., 2008). The high activity of dehydroleucodine (**1**) revealed in this investigation can be attributed to the presence in the molecule of two alkylating groups, the α-methylene γ-lactone and the α,β-unsaturated ketone (Ghantous et al., 2010). Dehydroparishin-B (**2**), compound in which only the second structure is retained, shows a diminished activity. Further studies are necessary to clarify the contribution of the different functional groups in these molecules, as well as other properties (lipophilicity, electronic features), to the activity observed against B16 melanoma cells.We also report that dehydroparishin-B (**2**) inhibits migration of B16 melanoma cells in a dose dependent manner ($IC_{50} = 72.2 \pm 3.2$ μM) (Supplementary data, Fig. S3A and C). In addition, treatment of B16 melanomas cells with 5 μM (or higher) dehydroleucodine (**1**) significantly inhibits cell migration after 16 h exposures ($IC_{50} = 5.4 \pm 0.3$ μM) (Supplementary data, Fig. S3B and D). These observations indicate an increased sensitivity of B16 melanomas cells to dehydroleucodine (**1**) as compared to dehydroparishin-B (**2**) (compare C and D). The migratory inhibitory property of dehydroleucodine (**1**) and dehydroparishin-B (**2**) on this cell line is comparable to those of staurosporine, a potent

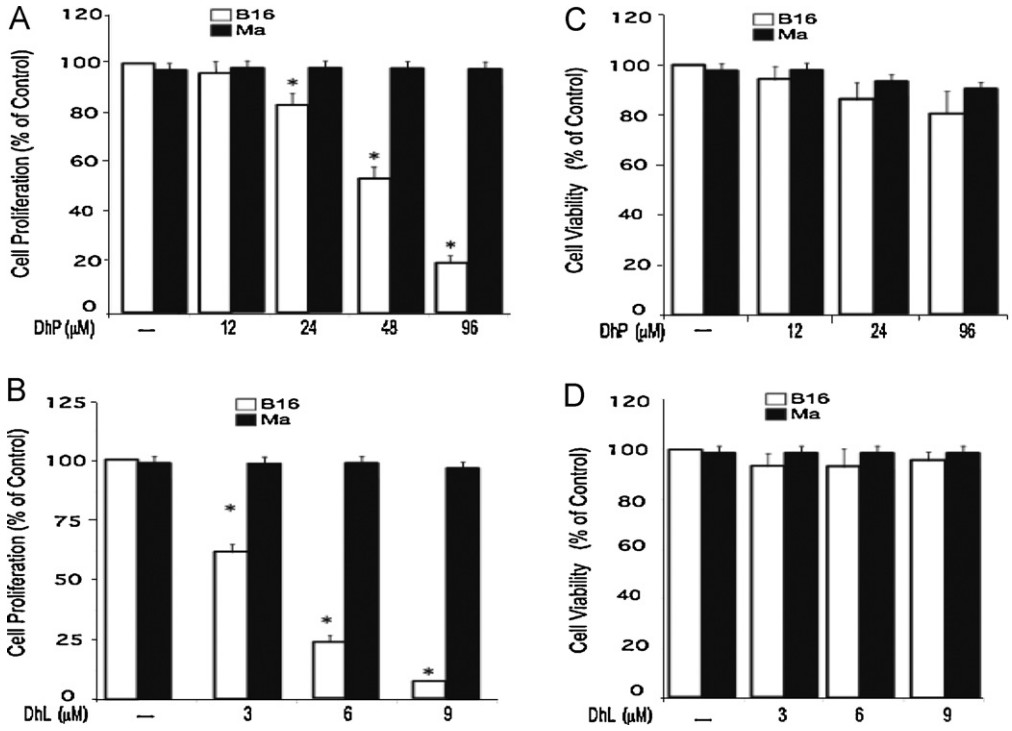

Fig. 2. Effect of dehydroleucodine (DhL) and dehydroparishin-B (DhP) on proliferation and viability of B16 melanoma (B16, □) and Melan-A (Ma, ■) cells. Cells were treated with different concentrations of DhL and DhP, and then assayed for MTT incorporation into cells (A and B) and trypan blue incorporation (C and D) as described in Section 3. Data represent the mean ± S.E.M. of two independent experiments. $^*P < 0.05$ by Student's *t*-test compared to control cells (−).

apoptotic agent (Wang et al., 2009), which was used as positive control (compare DMSO–control: 100 ± 10 vs. 100 nM Staurosporine: 50 ± 7.2). Consistent with these observations, we also found that dehydroleucodine (**1**) and dehydroparishin-B (**2**) inhibit secretion of matrix metalloprotease-2 (MMP-2), which is a known protease involved in migration and invasion of B16 cells (Hofmann et al., 2000) (Supplementary data, Fig. S4, compare DMSO–control: 100 ± 5 vs. 9 μM dehydroleucodine: 25 ± 6.2 and 96 μM dehydroparishin-B: 32.2 ± 3.1). In these assays, dehydroleucodine (**1**) was 10 times more active than dehydroparishin-B (**2**). Thus, the presence of the α-methylene γ-lactone moiety in the molecule would be required for optimal activity.

Finally our data demonstrate that dehydroleucodine (**1**) and the novel compound dehydroparishin-B (**2**) block cell proliferation and also inhibit migration of B16 melanoma cells without affecting cell viability. Future works will be required to address whether dehydroleucodine (**1**) and dehydroparishin-B (**2**) affect cell cycle progression.

3. Experimental

3.1. General experimental procedures

B16 melanoma cells were purchased from American Type Culture Collection (ATCC, Manassas, VA). Melan-A cells (immortalized normal human melanocytes, Bennett et al., 1987) were obtained from Dr. Lidia Kos (Florida International University). Dulbecco's modified Eagle's medium high glucose (DMEM), penicillin/streptomycin and L-glutamine were purchased from Mediatech, Inc. (Manassas, VA). B16 cells were maintained in DMEM supplemented with 10% fetal bovine serum (FBS), 100 Units/ml, 100 μg/ml, and 292 μg/ml at 37 °C in 5% CO_2. Melan-A (Ma) cells were maintained in the same medium supplemented with 200 nM TPA and 20 pM Cholera Toxin at 37 °C in 10% CO_2. MMP2 ELISA Kit was purchased from Abcam, Inc (Cambridge, MA). All chemicals were purchased from Sigma–Aldrich (St. Louis, MO), except otherwise indicated in the text. All chemicals utilized in this work were of analytical grade sources.

NMR spectra were recorded on a Bruker 400 MHz FT-NMR Spectrometer in DMSO-d_6 with TMS as internal standard. TLC was performed with precoated silica gel plates (PE SIL G/UV, Cat. No. 4410222, Whatman). Plates were developed with 15% ethyl acetate in chloroform. Spots were visualized with UV lamp at 254 nm (Rf: dehydroleucodine, 0.58; dehydroparishin-B, 0.29). HPLC analysis was carried out with a Thermo-Finnigan chromatograph (Thermo Electron Corporation, San Jose, CA). The chromatograph consisted of a SpectraSystem SMC1000 solvent delivery system, vacuum membrane degasser, P4000 gradient pumps and AS3000 autosampler. Column effluent was monitored at 254 nm with a SpectraSystem UV6000LP variable wavelength PDA detector and ChromQuest 4.1 software. Analytical separations were performed using a C18 RP Hypersil GOLD column (RP5, 250 mm × 4.6 mm, pore size 5 μm, Thermo Electron Corporation). The following eluting systems were used: System 1, A. 0.1% trifluoroacetic acid in acetonitrile, B. 0.1% trifluoroacetic acid in water, linear gradient 10–100% A in 30 min (dehydroleucodine, Rt 14.85 min); System 2, mobile phases A and B as in System 1, linear gradient 10–100% A in 120 min (dehydroleucodine, Rt 28.66 min); System 3, A. acetonitrile, B. 0.1 M ammonium acetate buffer pH 7.5, linear gradient 10–100% A in 30 min. Flow rate 1.0 mL/min at room temperature. GC/MS determinations were carried out in a Hewlett Packard model 6890 instrument coupled to a Q-Mass 910 quadrupole selective detector at 70 eV. A fused capillary DB-5MS column (30 m × 0.25 mm i.d.; film thickness 0.25 μm; J & W Scientific, Albany, NY) was used; injection port temperature, 200 °C; split ratio 1/20; detector temperature, 270 °C; carrier gas, helium at 1 ml/min; temperature program: 105–260 °C linear increase at 8 °C/min.

3.2. Plant material

Aerial parts of *A. douglasiana* were collected in San Carlos, Mendoza (Argentina) on March 2008 by Dr. Luis A. Lopez. A voucher specimen was deposited at Instituto Argentino de Investigaciones de las Zonas Áridas (IADIZA), Mendoza, Argentina (voucher number 9202).

3.3. Extraction and isolation

The dried crushed plant material (100 g, dry weight) was exhaustedly extracted with boiling $CHCl_3$. The $CHCl_3$ extracts were extracted with aqueous 5% $NaHCO_3$. Repeated column chromatography on silica gel (60, H70-230, Merck 7734) of the organic phase, using hexane–ethyl acetate (0–40%) mixtures as eluents, afforded dehydroleucodine (**1**) (700 mg). The aqueous $NaHCO_3$ phases containing dehydroparishin-B (**2**) were acidified with 0.1 N HCl and extracted with ethyl acetate. The organic phases were joined and evaporated to dryness to give an oily residue (280 mg), which was subjected to column chromatography on silica gel as indicated above. The fractions were monitored by TLC. Fractions containing the R_f 0.29 compound were joined and re-chromatographed in a similar way to give pure dehydroparishin-B (3.2 mg) (**2**).

3.4. (7R)-2-Oxo-guaia-1(10),3(4),5(6),11(13)-tetraen-12-oic acid, dehydroparishin-B (2)

Colorless needles (ethyl acetate); HPLC, Rt 14.92 min (system 1); Rt 31.00 min (system 2); Rt 8.49 min (system 3); UV/PDA, λ_{max} 239, 256, 317 nm; GC/MS, Rt 18.74 min; ^{1}H NMR (400 MHz, DMSO-d_6): δ = 1.80 (2H, m, 8-H_2), 2.06 (3H, s, 15-H_3), 2.26 (3H, s, 14-H_3), 2.33 (2H, m, 9-H_2), 3.70 (1H, q, J = 5.0, 7-H), 5.37 (1H, s, 13-H_A), 5.96 (1H, d, J = 5.0, 6-H), 6.02 (1H, s, 3-H), 6.19 (1H, s, 13-H_B), 12.7 (1H, br s, 12-H) (Supplementary information, Fig. S1); ^{13}C NMR (100 MHz, DMSO-d_6): δ = 13.9 (15-CH_3), 20.8 (14-CH_3), 28.5 (8-CH_2), 35.8 (9-CH_2), 41.0 (7-CH), 126.4 (13-CH_2), 126.7 (1-C), 128.9 (6-CH), 130.4 (3-CH), 137.6 (11-C), 143.1 (4-C), 153.3 (10-C), 163.3 (5-C), 167.3 (12-C), 194.6 (2-C) (Supplementary information, Fig. S2); MS (EI), m/z (rel. int.): 244 (100) $[M]^+$, 198 (35.1) $[M–CO_2–H_2]^+$, 183 (63.3), 159 (46.6), 155 (46.9), 141 (34.1), 129 (50.6), 128 (48.5), 115 (64.0), 91 (48.1), 77 (38.2); ESI-TOF-MS: m/z = 245.1162 $[M+H]^+$ (calcd for $C_{15}H_{17}O_3$, 245.1172), 267.0997 $[M+Na]^+$ (calcd for $C_{15}H_{16}O_3Na$, 267.0992), 289.0817 $[M-H+2Na]^+$ (calcd for $C_{15}H_{15}O_3Na_2$, 289.0811).

3.5. Dehydroparishin-B methyl ester

Dehydroparishin-B (**2**) was treated with ethereal diazomethane as usual to give dehydroparishin-B methyl ester. GC/MS, Rt 17.87 min; MS, m/z (rel. int.): 258 (100) $[M]^+$, 211 (27.5), 199, (35.0), 198 (65.5), 197 (30.1), 183 (78.5), 169 (25.1), 159 (48.6), 155 (57.0), 153 (26.9), 141 (33.8), 129 (41.7), 128 (54.6), 115 (57.0), 91 (32.9), 77 (20.5).

3.6. Cell proliferation assay

Cells (5×10^5) were plated in 6-well plates in Dulbecco's Modified Eagle Medium (DMEM) containing 10% fetal bovine serum (FBS). Cells were serum-starved for 12 h, and then stimulated with 10% serum for 24 h in the absence or in the presence of different concentration of dehydroparishin-B or dehydroleucodine. Cells were exposed to either 1 μCi/ml [^{3}H] thymidine or 10 μl/ml MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] for the last 4 h. For [^{3}H] thymidine incorporation, after incubation cells were washed with phosphate buffer saline (PBS) and treated first with 10% TCA at 4 °C for 30 min and then with 0.5 N NaOH/0.5% SDS. Samples were then measured in a scintillation counter. [^{3}H] thymidine incorporation was expressed as percentage of the control in the presence of 10% serum and in the absence of compound. For MTT incorporation, after incubation the medium was removed and formazan crystals were dissolved in detergent reagent following the manufacturer's instructions (ATCC). Optical density for each condition was determined at 570 nm. MTT incorporation was expressed as percentage of the control in the presence of 10% serum and in the absence of compound. Helenalin was used as a positive control.

3.7. Viability assay

The trypan blue dye exclusion assay was used. Cells (1×10^5) were plated in 6-well plates in DMEM containing 10% FBS, serum-starved for 12 h, and then stimulated with 10% serum for 24 h in the absence or in the presence of different concentration of dehydroparishin-B (8–96 μM) or dehydroleucodine (3–9 μM). After incubation, the cells were removed from the 6-well plate by treatment with trypsin. Cells suspensions were aspirated, centrifuged at 600 rpm for 5 min and washed twice with sterile PBS (pH 7.4). The cell suspension was then treated with trypan blue dye (0.4% solution) at a ratio of cell suspension:dye 4:1 and placed in heamocytometer. Live (unstained) and dead (blue stained) cells were counted using a 63× magnification in phase-contrast inverted microscope. The untreated cells (control) were also processed simultaneously under the identical conditions.

3.8. Wound-healing assay

B16 melanoma cells were cultured in 12-well plates in DMEM + 10% FBS for 24 h days. At 100% confluence, two parallel wounds of about 1 mm were made using a pipette tips. Wounds were washed with PBS to remove any floating cells and were exposed to different concentrations of dehydroparishin-B and dehydroleucodine. Images of the wound area were taken at time 0 (T = 0) and after 16 h (T = 16) of treatment. Cells exposed to DMSO were used as control. Images were taken with a Leica DM IRB microscope and a Media Cybernetics Cool Snap-Pro cf monochrome camera.

3.9. Matrix metalloproteinase ELISA assay

B16 cells (1×10^5) were cultured in the presence of 10% (v/v) serum for 24 h, washed and then incubated with different concentration of dehydroleucodine (**1**) and dehydroparishin-B (**2**) for 18 h (DhL: 3, 6 and 9 μM; DhP: 24, 48 and 96 μM). After incubation, supernatants were collected and concentrated using Centricon-30. Then, specific antibodies for mouse matrix metalloproteinase (MMP)-2 coated on 96-well microtiter plates were used to quantify the amount of MMP-2. Concentrated supernatants containing MMP-2 proteins were mixed with phosphate-buffered saline (PBS) buffer and incubated overnight at 4 °C. The wells were washed with PBS and incubated with biotinylated anti-mouse MMP-2 antibody at 25 °C for 3 h. The plates were washed with PBS and then incubated with HRP-conjugated streptavidin at 25 °C for 1 h and washed again three times with PBS. 3,3′,5,5′-Tetramethylbenzidine (TMB)-HRP substrate solution was added into wells. Plates were incubated for 2 h at 37 °C in the dark. The reaction was stopped by the addition of 1 M H_2SO_4. The absorbance was measured at 450 nm in a microplate reader (Molecular Devices). The concentration of MMP-2 in the sample was determined using a standard curve for MMP-2.

3.10. Statistical analysis

All experiments were done in duplicates and they were repeated at least two times. Data are presented as means ± S.E.M.

S.E.M. and the statistical significance was analyzed by Student's *t*-test. Results with $^{*}P < 0.05$ was considered as statistically significant.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.phytol.2012.05.018.

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