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Disintegrins extracted from totonacan rattlesnake (*Crotalus totonacus*) venom and their anti-adhesive and anti-migration effects on MDA-MB-231 and HMEC-1 cells

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ABSTRACT

Disintegrins are low molecular weight cysteine-rich proteins (4–14 kDa) that are isolated mainly from viperid snake venom. Due to their potential as lead compounds for binding and blocking integrin receptors, snake venom disintegrins have become one of the most studied venom protein families. The aim of this study was to obtain disintegrins from *C. totonacus* venom and evaluate their capability to bind and block integrin receptors. The *C. totonacus* disintegrin fraction (totonacin) represents two disintegrin isoforms obtained from *C. totonacus* venom. These disintegrins showed extracellular-matrix (ECM) protein adhesion and migration inhibitory effects on MDA-MB-231 and HMEC-1 cells. Totonacin (3 μM) inhibited MDA-MB-231 cell adhesion to the ECM proteins, fibronectin, vitronectin, and laminin by 31.2, 44.0, and 32.1, respectively. Adhesion inhibition to fibronectin, vitronectin, and laminin observed on HMEC-1 cells was 42.8, 60.8, and 51%, respectively. In addition, totonacin (3 μM) significantly inhibited MDA-MB-231 and HMEC-1 cell migration (41.4 and 48.3%, respectively). Totonacin showed more potent cell adhesion inhibitory activity toward vitronectin in both cell lines. These results suggest a major affinity of totonacin toward $\alpha_V\beta_3$, $\alpha_8\beta_1$, $\alpha_V\beta_5$, $\alpha_V\beta_1$, and $\alpha_{IIb}\beta_3$ integrins. In addition, the inhibitory effect observed on MDA-MB-231 and HMEC-1 cell migration reinforces the evidence of an interaction between these disintegrins and $\alpha_V\beta_3$ integrin, which plays a key role in migration and angiogenesis.

1. Introduction

Disintegrins are a non-enzymatic group of small molecules (4–14 kDa) found in the venom of the Viperidae snake family (Marcinkiewicz et al., 1999; Galán et al., 2008; Mackessy, 2009). They are released in venom *via* the proteolytic processing of PII snake venom metalloprotease precursors (SVMP) (Kini and Evans, 1992; Shimokawa et al., 1996). Based on their polypeptide length and the number of disulfide bonds, disintegrins are classified as long, medium, short, monomeric, and dimeric molecules (Calvete, 2005; Kim et al., 2005; Calvete, 2010). In addition, most have an arginine-glycine-aspartic acid (RGD) motif, which is responsible for binding to integrins (Huang et al., 1987; Calvete, 2005).

They inhibit various biological activities, such as platelet aggregation, angiogenesis, metastasis, and tumor growth through their interaction with various integrins (Marcinkiewicz et al., 2003; Walsh and Marcinkiewicz, 2011). Disintegrins have shown efficacy in inhibiting different tumor cells in *in vitro* and *in vivo* cancer models. Hence, these small peptides have a significant potential as anticancer agents based on their anti-angiogenic and antimetastatic effects (Selistre de Araujo et al., 2005; Tian et al., 2007; Ramos et al., 2008; Sánchez et al., 2009; Minea et al., 2010; Lucena et al., 2012).

Since tigramin was described by Huang et al. (1987), over 100 additional disintegrins have been named and studied (McLane et al., 2008). Additionally, there are currently two antiplatelet drugs derived from snake venom disintegrins, tirofiban (Chen et al., 1991; Saudek et al., 1991) and eptifibatide (Scarborough et al., 1991).

Due to their potential as lead compounds for binding and blocking integrin receptors, snake venom disintegrins have become one of the most studied venom protein families (Saviola et al., 2015).

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However, the venom of viperid snakes that are endemic to Mexico has received little interest, as only two disintegrins from rattlesnake species endemic to Mexico; basilisin from *Crotalus basiliscus* venom (Scarborough et al., 1993a) and morulustatin from *C. morulus* venom (Borja et al., 2016) have been studied. This represents ~6% of viperid species endemic to Mexico (Rivas-Mercado and Garza-Ocañas, 2017).

C. totonacus, a rattlesnake species endemic to Mexico, inhabits the states of Nuevo Leon, Tamaulipas, San Luis Potosi, Queretaro, Hidalgo, and Veracruz (Lazcano-Villarreal et al., 2010; Farr et al., 2015). To date, there have been no studies on the disintegrin content of *C. totonacus* venom and its biological activity. Therefore, the aim of this study was to obtain disintegrins from *C. totonacus* venom and evaluate their capability to bind and block integrin receptors. We assessed their ability to inhibit cell adhesion to the extracellular matrix (ECM) proteins, fibronectin, vitronectin, and laminin on the human endothelial cell line, HMEC-1, and the human breast cancer cell line, MDA-MB-231, and evaluated their inhibitory activity on cell migration.

2. Materials and methods

2.1. Snakes and venom collection

Six adult *C. totonacus* rattlesnakes were collected; four in the city of Juarez, one at Cerro de la Silla (Saddle Hill), Nuevo León state, and one in the city of Victoria, Tamaulipas state (SEMARNAT license SGPA/DGVS/05710/16). Three adult *C. totonacus* rattlesnakes were also provided from the HerpMx Herpetological Collection (SEMARNAT license SGPA/DGVS/05710/16). Venom was manually extracted from each snake using 50 mL plastic tubes (Falcon) covered with parafilm. Individual venom samples were recovered using 20 mM ammonium acetate and centrifuged (12,000 *g* at 10 °C, for 3 min) to remove cell debris and insoluble material. Samples were maintained at −70 °C and later freeze-dried and weighed. For the preparation of venom pools, aliquots of equal amounts of the dry venom of each specimen were thoroughly mixed.

2.2. Protein quantification

Protein concentrations were determined at 280 nm using an extinction coefficient of 1 mg/mL. A BioMate 3S UV–Visible spectrophotometer (Thermo Scientific, Waltham, MA, USA) was used to measure absorbance.

2.3. Disintegrin purification

2.3.1. Size exclusion chromatography (SE-LC)

A total of 160 mg of crude *C. totonacus* venom dissolved in 1.5 mL of 20 mM ammonium acetate buffer was fractionated in a Sephadex G-75 (Sigma-Aldrich, St. Louis, MO, USA) in a glass column (1.2 × 2.0 cm) and equilibrated with the same buffer at a flow rate of 14 mL/h. Fractions were collected using a Bio-Rad Model 2128 Fraction Collector (Hercules, CA, USA) at ~0.9 mL/tube, with absorbance monitored at 280 nm. An SE-LC fraction containing low molecular mass proteins evaluated by Sodium dodecyl-sulfate-plyacrylamide gel electrophoresis (SDS-PAGE) was collected and lyophilized for further purification.

2.3.2. Reverse-phase high-performance liquid chromatography (RP-HPLC)

The fraction containing low molecular mass proteins obtained from SE chromatography was further fractionated by RP-HPLC using a C_{18} column (Grace, Columbia, MD, USA, 4.6 × 250 mm; particle size: 5 μm) equilibrated with solution A (H_2O containing 0.1% trifluoroacetic acid (TFA)). Elution was performed at 1 mL/min using a gradient against solution B (CH_3CN containing 0.1% TFA) as follows: 0% B for 5 min, 0 to 15% B over 10 min, 15 to 45% B over 60 min, 45 to 70% B over 10 min, and 70% B for 9 min. Absorbance was monitored at 214 nm (Castro et al., 2013).

2.3.3. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

Samples of 15 μg SE-LC or 3 μg RP-HPLC fractions were analyzed by SDS-PAGE under reducing (β-mercaptoethanol) conditions as described by Laemmli (1970). A polyacrylamide concentration of 15% was employed. Samples were boiled (~90 °C) prior to running on the gel. Gels were stained with Coomassie Brilliant Blue G-250. Molecular weight markers were included in each electrophoretic run.

2.3.4. Inhibition of ADP-induced platelet aggregation

Inhibition of ADP-induced platelet aggregation by disintegrins was evaluated according to methods described by Angulo et al. (2014). Briefly, platelet rich plasma was prepared by centrifugation at 50 *g* for 15 min. Platelet counts were performed using a Sysmex XE automated equipment (Milton Keynes, UK) and platelet rich plasma (PRP) was diluted with platelet poor plasma (PPP), prepared by centrifuging blood at 850 *g* for 30 min to obtain a concentration of 3×10^8 platelets/mL. Aliquots of 225 μL PRP were incubated at 37 °C with various concentrations of disintegrin (37, 75, 150, and 300 nM) for 5 min with stirring at 600 rpm. Then, platelet aggregation was initiated by the addition of 25 μL of the agonist (to achieve the final concentration of 20 μM ADP) and monitored by the increase in light transmittance signal using a model AggRAM aggregometer (Helena Laboratories, TX, USA) interfaced to a chart recorder during 5 min. PPP (225 μL) alone was utilized as a blank, whereas PRP (225 μL) incubated only with ADP was utilized as a positive control for aggregation.

2.4. Protein sequencing

The fraction containing low molecular mass proteins that produced an inhibitory effect on ADP-induced platelet aggregation was used for protein sequencing. The fraction was reduced by dithiothreitol and alkylated with iodoacetamide (Weldon and Mackessy, 2009). The protein was digested using endoproteinase Lys-C (Sigma-Aldrich). Briefly, the alkylated protein was resuspended in digestion buffer (25 mM Tris/HCl + 1 mM EDTA, pH 8.5) and 10 μL of Lys-C (0.1 μg/μL) was added before incubation for 8 h at 37 °C. Digested peptides were purified by RP-HPLC and their molecular masses were obtained using a Finnigan LCQFleet mass spectrometer (Thermo Scientific, San Jose, CA, USA) with an electrospray ionization system as previously described (Guerrero-Garzón et al., 2018). The amino acid sequence of the N-terminal section of the native protein, as well as the digested peptides, was achieved using Edman degradation in a PPSQ-31A Protein Sequencer from Shimadzu Scientific Instruments, Inc. (Columbia, MD, USA). Each peptide (approximately 250 pmol) was adsorbed on a TFA-treated Glass Fiber Disk, distributed by Shimadzu. The sequence of the previously described disintegrin, tzabcanin, (Saviola et al., 2015) was used as a reference. *In silico* digestion of this sequence was performed using the Expasy PeptideMass tool (https://web.expasy.org/peptide_mass/).

2.5. Cell lines and culture conditions

The human mammary gland adenocarcinoma cell line (MDA-MB-231) and the human endothelial cell line (HMEC-1) were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). The MDA-MB-231 cells were cultured in L-15 Leibovitz medium supplemented with 10% fetal bovine serum (FBS) and incubated at 37 °C in a humidified CO_2-free air incubator. HMEC-1 cells were cultured in MCDB-131 medium supplemented with 10% FBS containing 1 μg/mL hydrocortisone and 10 ng/mL epidermal growth factor (Sigma-Aldrich) and incubated at 37 °C in a humidified atmosphere of 5% CO_2.

2.6. Cytotoxicity assay

To determine the optimal concentration of totonacin required,

MDA-MB-231 and HMEC-1 cells were exposed to totonacin and cytotoxicity was measured by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay as described by Mosmann (1983). Cells were trypsinized and resuspended in complete media at a concentration of 2.0×10^5 cells/mL and 100 µL per well of the cell suspension was seeded in 96-well plates. Cells were treated with totonacin at concentrations of 0.09, 0.18, 0.37, 0.75, 1.5, 3, 6, and 12 µM or PBS pH 7.4 (control group) and then incubated for 24 h at 37 °C. After incubation, 100 µL of MTT reagent (Sigma-Aldrich) dissolved in each cell culture medium (0.5 mg/mL) was added to the cells for a 3 h incubation at 37 °C. MTT was removed and 200 µL of isopropyl alcohol and 0.4 N HCl were added. The plate was stored at room temperature (25 °C) in the dark for 30 min and absorbance was read at 570 nm using an HTS 7000 Bio Assay Reader (Perkin Elmer, Waltham, MA, USA). Cell cytotoxicity was expressed as a percentage of the viable cell number compared to the control (non-treated cells) (Zakraoui et al., 2016). Assays at each totonacin concentration were performed in triplicate and the experiment was repeated three times.

2.7. Cell adhesion inhibition assay

Inhibition of MDA-MB-231 and HMEC-1 cells binding to fibronectin, vitronectin, and laminin induced by totonacin was measured as described by Wierzbicka-Patynowski et al. (1999), Sánchez et al. (2006), and Lima-dos-Santos et al. (2015). Triplicate wells of a 96-well plate were coated with 100 µL of either fibronectin (1 µg per well), vitronectin (1 µg per well), or laminin (1 µg per well) in 0.01 M PBS, pH 7.4, and incubated overnight at 4 °C. The ECM protein-coated wells were then blocked by adding 200 µL of specific cell culture medium containing 2% bovine serum albumin (BSA) (blocking solution). Subsequently, the plate was incubated at 37 °C for 1.5 h. Cells were harvested with 0.25% trypsin, counted, and resuspended in a medium containing 1% BSA at 5×10^5 cells/mL. Thereafter, totonacin was added to the cell suspension at various concentrations (0.09, 0.18, 0.37, 0.75, 1.5, and 3 µM) and allowed to incubate at 37 °C for 2 h. The blocking solution was aspirated and preincubated cell/totonacin aliquots (100 µL) were seeded in the fibronectin, vitronectin, and laminin-coated wells and incubated for 2 h at 37 °C. The negative control consisted of MDA-MB-231 or HMEC-1 cells incubated with PBS. In the negative control wells, cells were bound to fibronectin, vitronectin, and laminin. The wells were washed three times with culture medium containing 0.2% BSA and 200 µL of L-15 medium containing 1% BSA and 1.5 mg/mL MTT was added to the cells for a 3-h incubation at 37 °C. A total of 100 µL of dimethyl sulfoxide was added for cell lysis. The plate was gently shaken and absorbance was read at 570 nm using an HTS 7000 Bio Assay Reader (Perkin Elmer). Percent inhibition was calculated with the following formula: [(absorbance of negative control - absorbance of cell/totonacin disintegrins sample)/absorbance of negative control] × 100. Assays at each totonacin concentration for all cell lines were performed in triplicate and the experiment was repeated three times.

2.8. Wound healing assay

To measure the effect of totonacin on MDA-MB-231 and HMEC-1 cell migration, a modified wound healing assay was carried out as previously described by Ren et al. (2006) and Galán et al. (2008). Briefly, cells were plated (2×10^5 cells/mL) in a 96-well microtiter plate. After overnight incubation at 37 °C, the confluent monolayer was scratched with a sterile 200 µL pipet tip at the midline of each well. The detached cells were washed away using the culture medium, which was followed by the addition of 200 µL of new culture medium supplemented with 10% FBS. Cells received 100 µL of PBS 0.01 M, pH 7.4, which allowed cell migration to occur (control group), or 3 µM of totonacin diluted in the same PBS buffer. Cells were then incubated in a humidified chamber at 37 °C and removed from incubation for microscopic imaging at 0, 12, and 24 h. Percent migration was calculated using the following equation: [(C × E)/C)] 100, where C corresponds to the distance of cell edge (mm) at zero time of the control and E is the distance of cell edge (mm) at the final time. Images were analyzed using ImageJ 1.x software. Assays were performed in triplicate and the experiment was repeated three times.

2.9. Statistical analyses

Cytotoxicity and cell adhesion data were analyzed by analysis of variance (ANOVA) followed by Tukey's post-hoc test using GraphPad Prism 6 (GraphPad Software, La Jolla, CA, USA). Cell migration assays were analyzed with the Student's *t*-test by comparing the percent migration of treatment to the percent migration of the control at the respective time interval. P values $< .05$ were considered statistically significant.

3. Results

3.1. Disintegrin purification

The isolation of *C. totonacus* venom disintegrins was achieved by a two-step chromatographic separation. Seven fractions were collected by SE-LC and the electrophoretic protein band profile for each fraction was evaluated by SDS-PAGE. Fraction 5 had two low-molecular mass bands within the mass range for disintegrin (4–14 kDa) (Huang et al., 1987; Scarborough et al., 1993a; Marcinkiewicz et al., 1999; Zhou et al., 2000) (Fig. 1). Therefore, SE-LC fraction 5 was further fractionated by RP-HPLC. As a result, 14 fractions were obtained. Fractions 5, 6, 7, 8, and 9, eluted from 22 to 36 min, aligned with the retention times reported for snake venom disintegrins and had an SDS-PAGE single protein band of ~14 kDa (Fig. 2). RP-HPLC fractions 5, 7, and 8 inhibited ADP-induced platelet aggregation, a disintegrin activity (Fig. 3) and inhibitory concentrations (IC_{50}) of the fractions 5, 7, and 8 were 300 nM, 100 nM, and 72 nM, respectively. Fraction 8 was identified as the most potent and the most abundant. Therefore, fraction 8 was further analyzed by mass spectrometry, which revealed the presence of two protein isoforms with slightly different masses, 7437 and 7218 Da (Fig. 4).

Regarding protein sequencing, the N-terminal sequence obtained for the RP-HPLC fraction 8 was GEECDCGSPANPCCDAATCKLR. A subsequent NCBI Protein BLAST analyses indicated that the N-terminal sequence obtained belonged to the disintegrin family. The reduction of the RP-HPLC fraction 8 with endoproteinase Lys-C yielded four peptides with the following molecular masses: *P1*, 2258.4 Da; *P2*, 3485.5 Da; *P3*, 3357.1 Da; and *P4*, 2425.2 Da. Peptides P1 and P4 had molecular

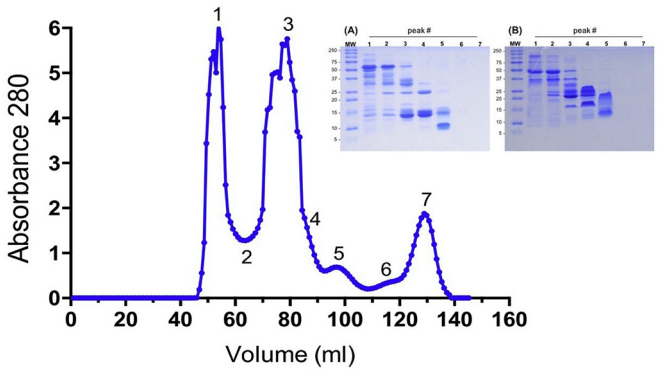

Fig. 1. Size exclusion chromatography of *C. totonacus* crude venom. A total of 160 mg of venom was fractionated on a Sephadex G-75. Fractions were analyzed by SDS-PAGE (A) reducing conditions (B) No reducing conditions. Fraction 5 showed protein bands within the disintegrin molecular mass range (4–14 kDa). Fractions 6 and 7 did not show bands.

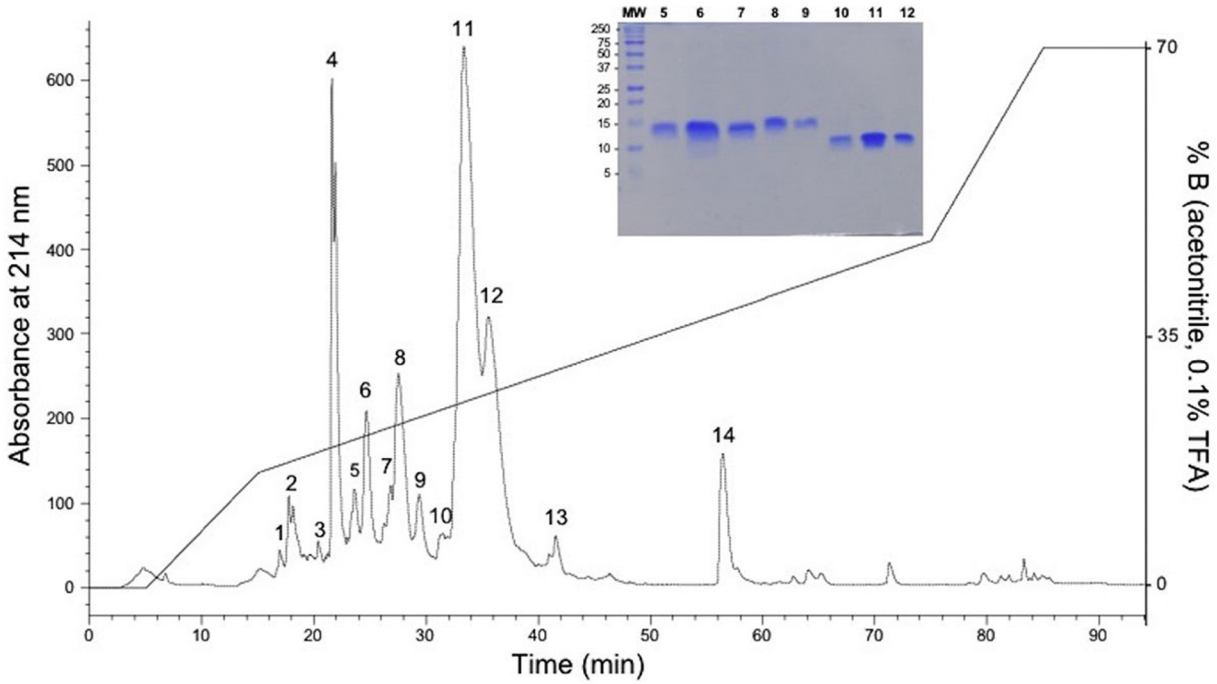

Fig. 2. RP-HPLC of SE-LC fraction 5; 1.5 mg was injected into a C_{18} Grace Vydac column; fractions collected between 22 and 38 min (fraction 5 to 12) were analyzed by SDS-PAGE under β-mercaptoethanol-reducing conditions; in addition to disintegrin activity, fraction 8 (~14 kDa) showed a more prominent peak than fractions 5 and 7.

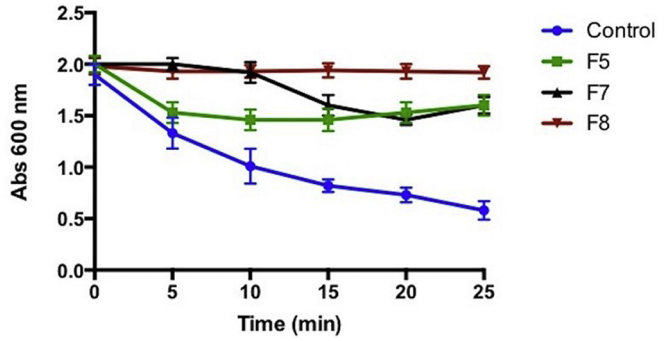

Fig. 3. Inhibition of platelet aggregation by disintegrins from *C. totonacus* venom. Only RP-HPLC fractions 5, 7, and 8 (F5, F7, and F8) showed inhibitory activity toward platelet aggregation. F8 showed more relative abundance and potency; thus, was selected for cell assays. ADP 20 μM was used as the platelet aggregation agonist. Results are presented as mean ± SD (n = 3).

masses identical to those of the theoretical peptides of tzabcanin [C0HK50]. Edman degradation of P2 and P3 yielded the following sequence, KKGTVCRPARGDWNDDTCTGQSADCPRNGLYG (Fig. 5-A). The difference in peptide masses can be explained by the initial double lysine. Complete amino acid sequence comparison using the UniProt database confirmed that this medium-size *C. totonacus* disintegrin contains the RGD binding motif (Fig. 5-B). To identify RP-HPLC fraction 8 as the RGD disintegrin obtained from *C. totonacus* venom and for study purposes, this fraction was referred to as totonacin.

3.2. Cytotoxicity

The effect of totonacin on MDA-MB-231 and HMEC-1 cells was evaluated by the MTT assay. Treating cells with totonacin (0.09, 0.18, 0.37, 0.75, 1.5, 3, 6, and 12 μM) for 24 h caused a dose-dependent decrease in cell viability. Totonacin at 6 and 12 μM decreased cell viability by 31.9 and 48.7% in MDA-MB-231 and 32.7 and 44.8% in HMEC-1 cells ($P < .05$), respectively. At 3 μM and lower

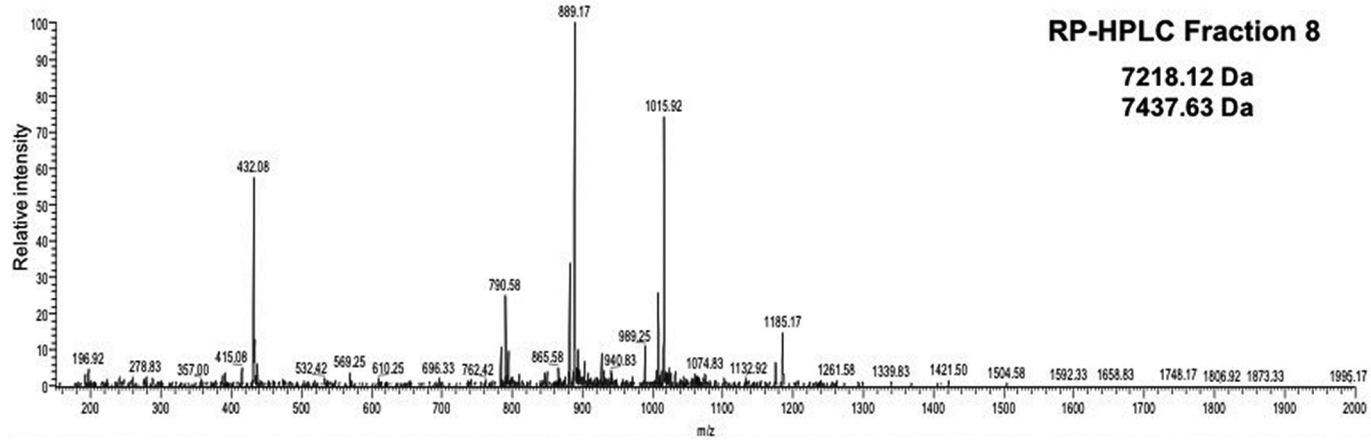

Fig. 4. Mass spectrometry analysis revealed two proteins in disintegrin mass range: 7.2 and 7.4 kDa.

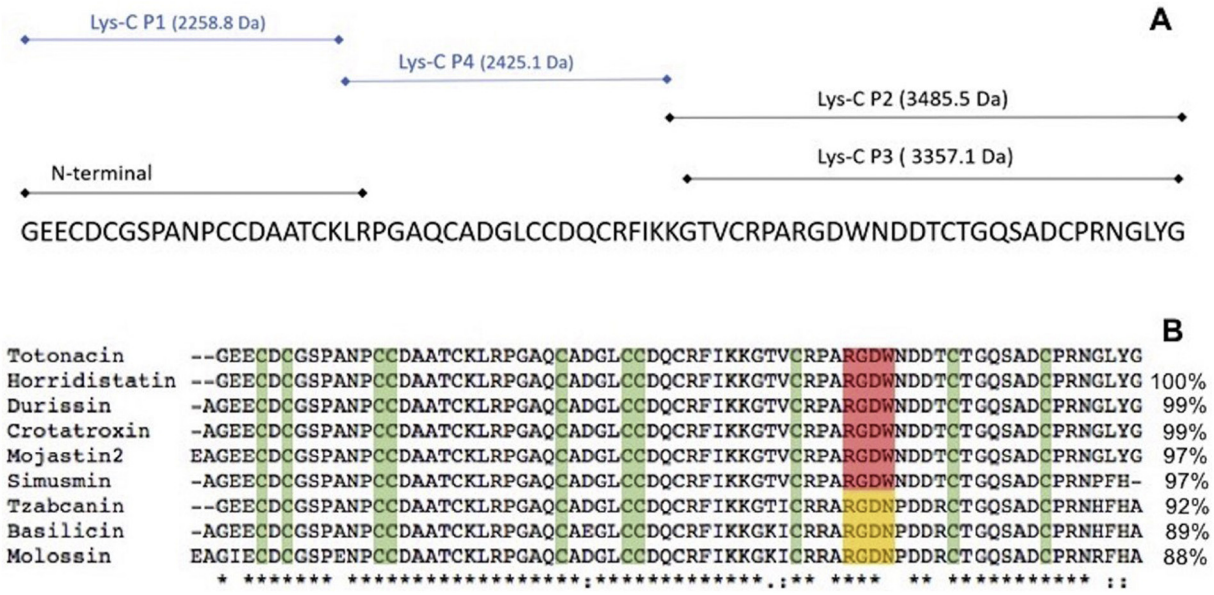

Fig. 5. (A) Amino acid sequence obtained by EDMAN degradation of RP-HPLC Fraction 8 (totonacin) after digestion with endoproteinase Lys-C and overlapping peptides. Peptides in black were completely sequenced by Edman degradation, the sequence of peptides in blue was deduced from their molecular weight, based on the sequence of tzabcanin. (B) Amino acid comparison of totonacin sequence with selected disintegrins: horridistatin, *C. horridus* (P0C7X6); durissin, *C. durissus* (P68521); crotatroxin, *C. atrox* (P68520); mojastin-2, *C. scutulatus* (P0C7X7); simusmin, *C. simus* (C0HJM4); tzabcanin, *C. tzabcan* (C0HK50); basilicin, *C. basiliscus* (P31981); and molossin, *C. m. molossus* venom (P31984). One-letter code for amino acids is used. Cysteine residues are green marked, RGDW and RGDN binding motifs are red and yellow marked, respectively. Numbers on the right side correspond to the sequence identity percentage of each selected disintegrin compared to totonacin. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

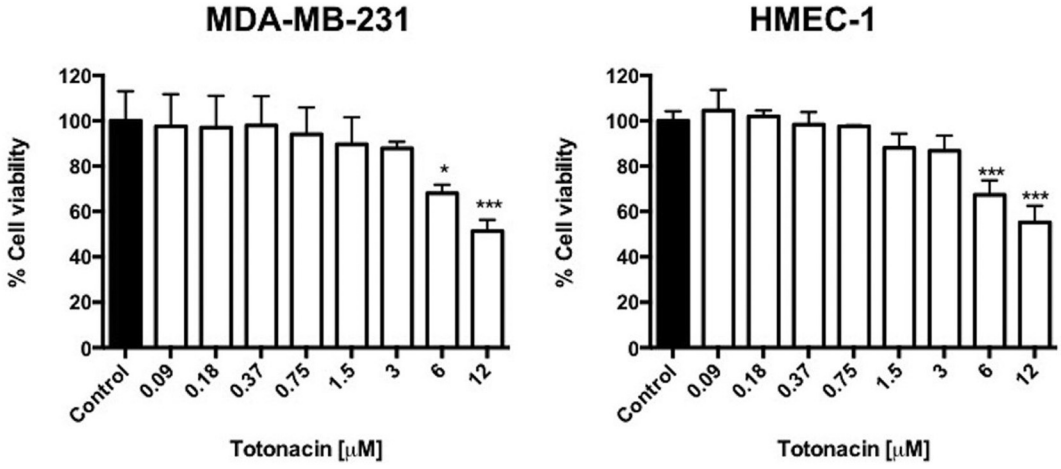

Fig. 6. Cytotoxicity effect on MDA-MB-231 and HMEC-1 cells after 24 h exposure to different totonacin concentrations. Results are presented as mean ± SD ($n = 3$). Only 6 μM ($^{*}P < .05$) and 12 μM ($^{***}p < .001$) showed significant toxicity when compared to control group. Assays at each concentration of totonacin were performed in triplicate and the experiment was repeated three times.

concentrations, totonacin resulted in a cell viability > 85% (Fig. 6). Therefore, the 3 μM concentration was selected as the highest concentration to evaluate the inhibitory activities of totonacin on fibronectin, vitronectin, and laminin cell adhesion as well as perform cell migration assays.

3.3. Cell adhesion inhibition assay

At the 3 μM concentration, totonacin inhibited MDA-MB-231 and HMEC-1 cell adhesion to vitronectin, laminin, and fibronectin ECM proteins by 44%, 32.1%, and 31.2% and 60.8%, 51.1%, and 42.8%, respectively. Significant adhesion inhibitory activity toward vitronectin was observed in both cells as shown in Fig. 7.

3.4. Wound healing assay

Cell migration was measured after scraping cells from the bottom of wells, following an *in vitro* wound healing assay. After 24 h of incubation, 3 μM totonacin significantly inhibited MDA-MB-231 and HMEC-1 cell migration by 41.4 ± 9% and 48.3 ± 5%, respectively (Fig. 8).

4. Discussion

In this study, a fraction (totonacin), of *C. totonacus* venom, containing two disintegrin isoforms, with masses of 7437 and 7218 Da, was identified from pooled venom. According to the molecular masses, amino acid sequence length, and cysteine content, totonacin was classified as a medium-size disintegrin. Totonacin shares high amino acid

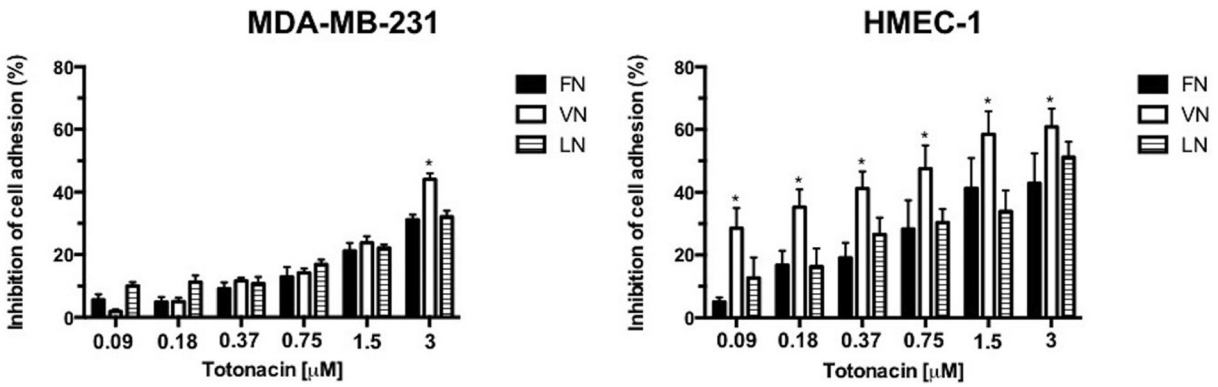

Fig. 7. Inhibition of MDA-MB-231 and HMEC-1 cell adhesion to fibronectin (FN), vitronectin (VN), and laminin (LN). Cells were treated with various concentrations of totonacin prior to seeding in 96-well microplates. The control group consisted of cells incubated with PBS buffer. Results are presented as mean ± SD ($n = 3$), all P values < .05 were considered significant.

sequence identity with disintegrins of the genus *Crotalus*. For instance, it shares a 100% amino acid identity with the disintegrin horridistatin (Galán et al., 2005), 99% with durissin and crotatroxin (Scarborough et al., 1993a), 97% with mojastin 2 (Sánchez et al., 2006) and simusmin (Angulo et al., 2014), and 92% with tzabcanin (Saviola et al., 2015) (Fig. 5-B).

All fractions obtained by the initial reverse phase chromatography were originally screened for the activity of inhibition of platelet aggregation. The purpose of this step was to target small proteins, such as disintegrins (Galán et al., 2005; Sánchez et al., 2006). Disintegrins have an important functional role in envenomated prey discrimination (Saviola et al., 2013), as well as promoting hemorrhage to allow components such as SVMP to spread through the tissues to exert its enzymatic action and disrupt in cell-cell and cell-extracellular matrix interactions (McLane et al., 1994). However, isolated disintegrins are generally non-toxic components of venoms (Walsh and Marcinkiewicz, 2011).

The RGD binding motif was observed as a part of the totonacin amino acid sequence. The biological activity of disintegrin molecules is based on the chemical interactions among the amino acids of the RGD binding motif, C-terminal domain, and cell receptor (Wierzbicka-Patynowsky et al. 1999; Calvete et al., 2005). Additionally, RGD amino acid sequences can be further classified as RGDW *i.e.*, totonacin, horridistatin, durissin, crotatroxin, mojastin-2, and simusmin or RGDN *i.e.*,

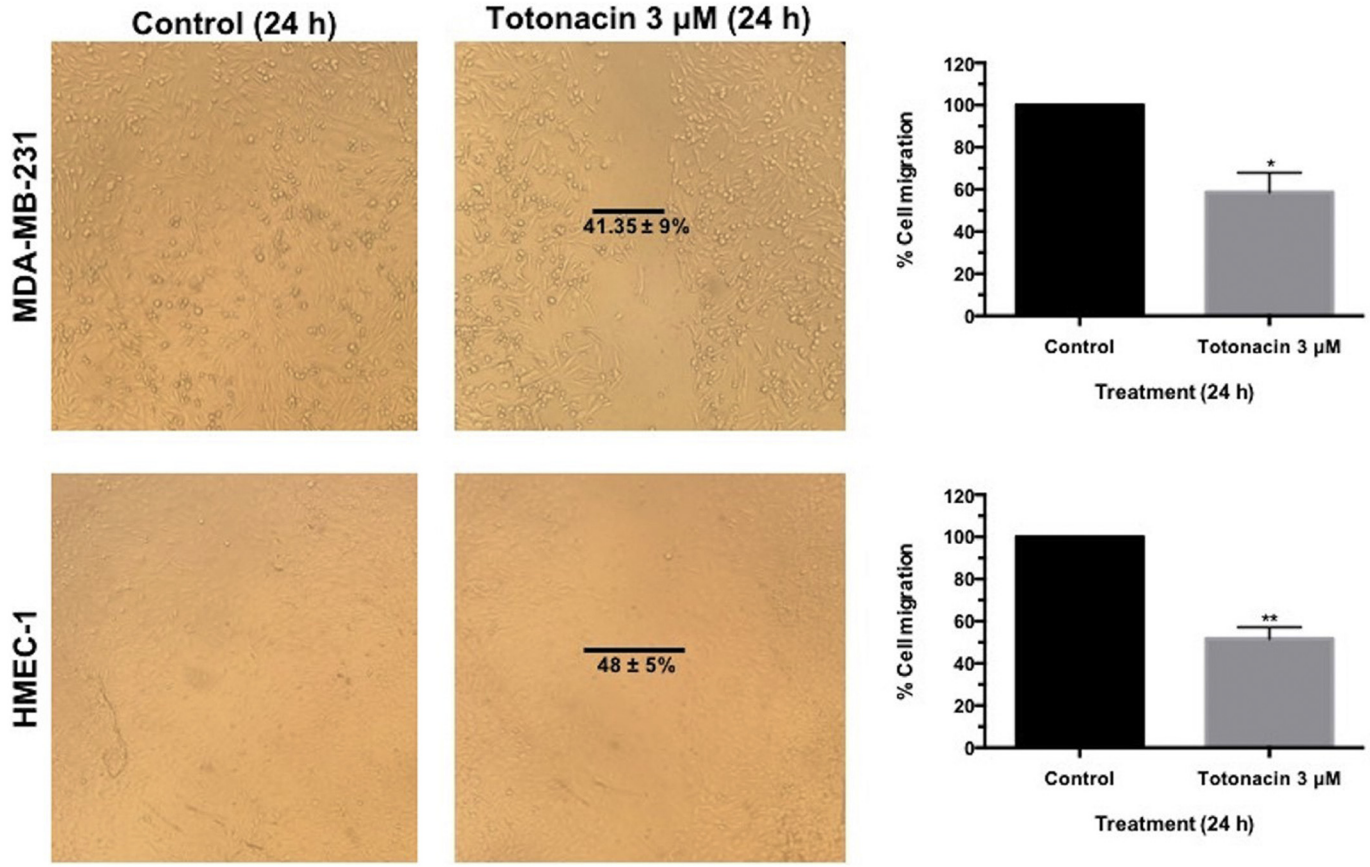

Fig. 8. Inhibition of MDA-MB-231 and HMEC-1 cell migration with 3 μM totonacin by wound healing assay. The migration percentage was quantified by multiple measurements of the width of the scrape space and images analyzed using Image J software. Results are presented as mean ± SD ($n = 3$), P values < .05 were considered significant.

tzabcanin, basilicin, and molossin (Fig. 5-B). Disintegrins expressing the RGDW domains often display high affinity to $\alpha_{IIb}\beta_3$ integrins, whereas those with RGDN display higher selectivity toward $\alpha_5\beta_1$ and $\alpha_v\beta_3$ integrins (Scarborough et al., 1993b; Marcinkiewicz et al., 1997; Juárez et al., 2008).

Because disintegrins can inhibit the adhesion of cultured cells to surfaces coated with ECM proteins (Lima-dos-Santos et al., 2015), this study evaluated the inhibitory effect of totonacin against MDA-MB-231 and HMEC-1 cell adhesion to fibronectin, vitronectin, and laminin ECM proteins. HMEC-1 cells, commonly used in angiogenesis studies, express key integrins, including α1, 2, 3, 4, 5, 6, and v, as well as β1, 3, 4, and 5. MDA-MB-231 cells, commonly used in the study of cancer, express high levels of β1 and αv integrins and β5 and $\alpha_v\beta_5$ integrins (Taherian et al., 2011; Lucena et al., 2012; Minea et al., 2012). Therefore, these cell lines have been used to evaluate the inhibitory effect of disintegrins on cell adhesion to ECM proteins, as well as cell migration inhibition by totonacin.

As totonacin showed low cytotoxicity levels at concentrations of 0.09 to 3 μM, this disintegrin concentration range was selected for biological activity assays. Totonacin had a significant inhibitory effect on the adhesion of HMEC-1 and MDA-MB-231 cells to vitronectin. Similar results were reported by Saviola et al. (2016), who found that tzabcanin from *C. tzabcan* venom inhibits cell adhesion to vitronectin (~70%) in A-375 cells. As totonacin showed major adhesion inhibition activity toward vitronectin in both MDA-MB-231 and HMEC-1 cells, and as vitronectin targets $\alpha_V\beta_3$, $\alpha_8\beta_1$, $\alpha_V\beta_5$, $\alpha_V\beta_1$, and $\alpha_{IIb}\beta_3$ integrins (Barczyk et al., 2010), totonacin antagonized one or more of the five integrin vitronectin receptors ($\alpha_V\beta_3$, $\alpha_8\beta_1$, $\alpha_V\beta_5$, $\alpha_V\beta_1$, and $\alpha_{IIb}\beta_3$). The minor cell adhesion inhibitory effect observed toward fibronectin and laminin could be explained by the greater number of integrins involved in cell adhesion to each of the ECM proteins. Fibronectin can target nine integrins: $\alpha_{IIb}\beta_3$, $\alpha_4\beta_1$, $\alpha_4\beta_7$, $\alpha_5\beta_1$, $\alpha_8\beta_1$, $\alpha_v\beta_1$, $\alpha_v\beta_3$, $\alpha_v\beta_6$, and $\alpha_v\beta_8$, whereas laminin targets seven: $\alpha_1\beta_1$, $\alpha_2\beta_1$, $\alpha_3\beta_1$, $\alpha_6\beta_1$, $\alpha_7\beta_1$, $\alpha_{10}\beta_1$, and $\alpha_6\beta_4$ (Saviola et al., 2016). Totonacin caused more inhibitory activity on cell adhesion to vitronectin than tzabcanin. *C. totonacus* disintegrins inhibited cell migration by 41.3% and 48.35% in MDA-MB-231 and HMEC-1 cells, respectively (Fig. 8), suggesting an $\alpha_v\beta_3$ integrin blockade, without excluding other possible integrins that might be targeted by totonacin, such as $\alpha_v\beta_5$, which is highly expressed in MDA-MB-231 cells and has been reported to mediate cancer cell migration (Pecheur et al., 2002; Rolli et al., 2003; Dang et al., 2006; Sloan et al., 2006; Taherian et al., 2011). Similar results on cell migration inhibition have been reported for contortrostatin, r-viridistatin 2, r-mojastin 1, vicrostatin, and crotatroxin 2 (Swenson et al., 2004; Galán et al., 2008; Minea et al., 2012; Lucena et al., 2015).

5. Conclusion

This study is the first to describe the isolation, determination of mass and amino acid sequence, and biological activities of disintegrins from *C. totonacus* venom. Overall, results obtained for *C. totonacus* disintegrin totonacin show its capacity to disrupt cell-ECM protein interaction, ability to inhibit cell migration, and potential as an anti-metastatic agent. As disintegrins in *C. totonacus* venom were obtained at concentrations below 1%, recombinant expression of this disintegrin should be continued to obtain higher amounts. In addition, evaluations of the effects of totonacin should be continued with *in vivo* models.

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Declaration of Competing Interest

The authors declare that they have no conflicts of interest.

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