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Antiproliferative and antiangiogenic effect of *Amblyomma sculptum* (Acari: Ixodidae) crude saliva in endothelial cells *in vitro*

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ABSTRACT

Neovascularization, a process that includes vasculogenesis and angiogenesis, may be a physiological or pathologic event, but in any cases the phenomenon is related to the formation of vascular net and sprouting of endothelial cells from preexisting blood vessel. The tumor environment, which counts on the tumor cell proliferation, is plenty of proangiogenic factors, such as angiogenin, TGF (α and β), FGF, VEGF, all of them playing a crucial role in angiogenesis, an important hallmark of cancer frequently related to a poor prognosis. Therefore, therapies focusing the inhibition of cancer neovasculogenesis have become an interesting strategy for the development of antitumor therapies. In this work, we investigate the effect of tick saliva on the human endothelial cells, in order to understand its inhibitory effects on angiogenesis. To this end, the HUVEC cells were used as model of angiogenesis in vitro and the anti-proliferative, anti-migratory, cytotoxicity was evaluated. Our data depicts that saliva impairs cell development by causing structural changes while precludes cell proliferation and migration, that are crucial events related to angiogenesis. Aiming the identification of the bioactive components related to antiangiogenic activity, saliva was analyzed through the Mass Spectrometry and among all molecules identified, disintegrins and cathepsin L seems to be primarily responsible for the antiangiogenic effects of saliva.

1. Introduction

Neovasculogenesis is a hallmark of cancer which is closely related to metastasis and consequently to a poor prognosis. In general, solid tumors are able to grow up to 1-2 mm^3 in the absence of neovascularization. To growth beyond this limit, cancer cells secretes proangiogenic factors such as angiogenin, TGF (α and β), FGF, VEGF which plays an important role on the initiation and proliferation of new blood vessels [1]. The modern anticancer therapy, target angiogenesis causing neoplastic "starvation" and hypoxia precluding cancer proliferation. Antiangiogenic therapies include recombinant humanized monoclonal antibody that impair angiogenesis through the inhibition of vascular endothelial growth factor-A (VEGF-A), the main inducer of angiogenesis, without directly affecting the cancerous cells [2]. In fact, antiangiogenic drugs are currently approved by FDA for clinical use in breast cancer, colorectal cancer, lung cancer and renal cell carcinoma, generally in association with other chemotherapeutic agents [3]. Although antiangiogenic therapies are quite effective in some cases, it is common for several tumors to become resistant, imposing limitations to this therapeutic scheme. This current landscape points out the need for discovery of new anticancer molecules, among them, the anti-angiogenic agents.

Previously, we described the cytotoxic effect of crude saliva from *A. sculptum* (formerly synonymy of *A. cajennense*) [4,5], on melanomas and pancreatic cancer cells *in vitro* [6]. The anticancer potential of ticks saliva has also been investigated by others [7–9], but the effect of A.

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sculptum saliva in human endothelial cells still remains to be elucidated.

Ticks are blood sucking arthropod ectoparasites of great medical and veterinary importance belonging to the class Arachnida, order Ixodida. It comprises a group containing about 900 species, distributed in two main families, Argasidae and Ixodidae [10–14].

A. sculptum occurs in all Brazilian regions constituting the main vector of *Rickettsia rickettsia*, the etiological agent of the spotted fever [15]. *A. sculptum* can remain attached to the vertebrate host for several days, by inserting and anchoring their mouthparts through the host skin. Its secretion contains a cement-like material and/or backwards pointing barbs that allow the ticks to perform the hematophagy for days or even weeks [16]. Prior to blood consumption by the tick, saliva is pumped into the feeding site and henceforth tick alternate cycles of feeding and salivation [16]. Local host responses to the injury caused by the tick may include the formation of hemostatic plug, activation of the coagulation cascade, vasoconstriction, inflammatory responses leading to wound healing and tissue remodeling, all of which would disrupt tick feeding and cause rejection of the tick, with detrimental effects on tick viability and reproduction. However, these reactions are collectively modulated by a wide range of bioactive components contained in saliva [17].

Recently our group described the effect of CS (crude saliva) from *A. sculptum* in hemostatic system and also its cytotoxic effect on melanomas and pancreatic tumor *in vitro* [6].CS consists in a complex cocktail of bioactive molecules that when inoculated in the host are able to modulate the immunologic, inflammatory and hemodynamics responses in benefit of the feeding ticks [6,17]. Francischetti et al. demonstrated for the first time the antiangiogenic effect of crude saliva or entire salivary gland of *Ixodes scapularis* and *Boophilus microplus* activity accompanied by change in cell shape and apoptosis in endothelial cells *in vitro* [18]. Interestingly, saliva from other bloodsuckers such as *Anopheles gambiae*, *An. stephensiae*, *Lutzomyia longipalpis*, *Phlebotomus papatasi*, *Aedes aegypti*, *Culex quinquefasciatus*, and *Cimex lectularius* are devoid of this effect [18]. Afterwards, the antiangiogenic properties form saliva tick was attributed to the presence of miscellaneous molecules including disintegrins, a family of small cysteine-rich polypeptides involved in a numerous biological processes especially inhibition of cell adhesion, migration and angiogenesis through the blocking the function of integrin receptors with a high degree of selectivity [19,20].

Animal secretions are well known providers of bioactive molecules and saliva tick stands out as a new potentially important source for bioprospection of new biodrugs. In the present paper we investigated the antiproliferative and antiangiogenic effect of crude saliva on HUVEC cells *in vitro*. Finally, in order to identifying the components involved on their biological effect, saliva was analyzed through the Mass Spectrometry, which allowed the identification of several bioactive molecules (Scheme 1).

2. Materials and methods

2.1. Reagents

Coomassie blue R250 was purchased from Sigma Chem. Co, (USA), RPMI 1640 medium from Gibco (Grand Island, NY – USA), streptomycin/ampicillin, fetal calf serum and Dulbecco´s Modified Eagle´s Medium was purchased from Cultilab, (São Paulo, Brazil), sodium pyruvate from Spectrum Chemical Mfg. Corp.,(New Jersey, USA). Vectashield mounting medium from Vector Laboratories, (Burlingame, CA-USA). Dimethylsulfoxide (DMSO), 3-(4,5 dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, sodium chloride, sodium monobasic phosphate, Hoechst: bisbenzimide (Hoechst 33258), L-glutamine, propidium iodide (PI), fluorescein isothiocyanate (FITC) conjugated, HEPES, proteinase K, RNase A and dopamine-3-hydroxytramine were purchased from (Sigma-Aldrich Company, St. Louis, MO, USA). Annexin-V from Life Technologies (Eugene, Oregon, USA). Matrigel was purchased from BD Biosciences, San Jose, CA, USA.

2.2. Tick maintenance and collection of saliva

The females and males of *A. sculptum* used in this work were obtained from colonies stablished on Butantan Institute's Parasitology Laboratory. All Animal experiments were carried out in accordance with protocols approved by the Ethics Committee for the Use of Animals of the Butantan Institute (CEUA N°. 3270051115).

To obtain the saliva, domestic rabbits (*Oryctolagus cuniculus*), without prior tick infestations, were fed to females (250) and males (125). Females were partially fed on New Zealand white rabbits. Around 10 to 14 days after fixation, females were removed from the host by torsion and then induced salivation according to Kaufman (1978) with modifications. Briefly, females were ventrally adhered to a double-sided tape and then, 5 to 10 µL of a dopamine solution (5% w/v in 0.15 M NaCl) was applied on the dorsum with a 30-gauge needle coupled into a micrometer Hamilton syringe. Crude saliva (CS) was collected by microcapillary tube fitted over female's hypostome. This entire apparatus was kept for 2 h at room temperature (25 °C). Collected saliva was pooled, filtered through a 0.22 µm filter (Millipore), aliquoted, frozen on dry ice and alcohol and kept at -80 °C until use. The protein concentration was determined using Bradford assay (Bio-Rad) and by absorbance at 280 nm.

2.3. Mass spectrometry

The tick saliva (30 µg) was analyzed by mass spectrometry, after in solution digestion. Briefly, sample was treated with urea 8 M at 80 °C, DTT 100 mM and IAA 200 mM for disruption of disulfide bridges. Then, it was buffered and digested with 40 ng/mL trypsin solution (ultrapure, from porcine pancreas). The reaction was carried out overnight at 37 °C and then stopped with 0.5% acetic acid. The solution was concentrated in a speed vac and applied to a C18 column (2.1 x 50 mm, 100 Å, 3 µm). Chromatography was carried out in a binary RP-HPLC (20 A Prominence, Shimadzu, Co, Japan) with elution performed by a linear gradient of B (Acetonitrile 90% containing 0.1% acetic acid) over A (0.1% acetic acid), from 5 to 40% in 25 min, under a constant flow rate of 0.2 mL.min^{-1}. The eluted content was detected by PDA, coupled to a mass spectrometry (ESI-IT-TOF, Shimadzu, Co, Japan). For LC–MS/MS analysis, the equipment was set on positive ionization mode, interface voltage at 4.5 kV, detector voltage at 1.70 kV and the capillary temperature at 200 °C. The mass range used was 100–2000 *m/z*. Instrument control, data acquisition and processing were performed by the LCMS Solution suite (Shimadzu).

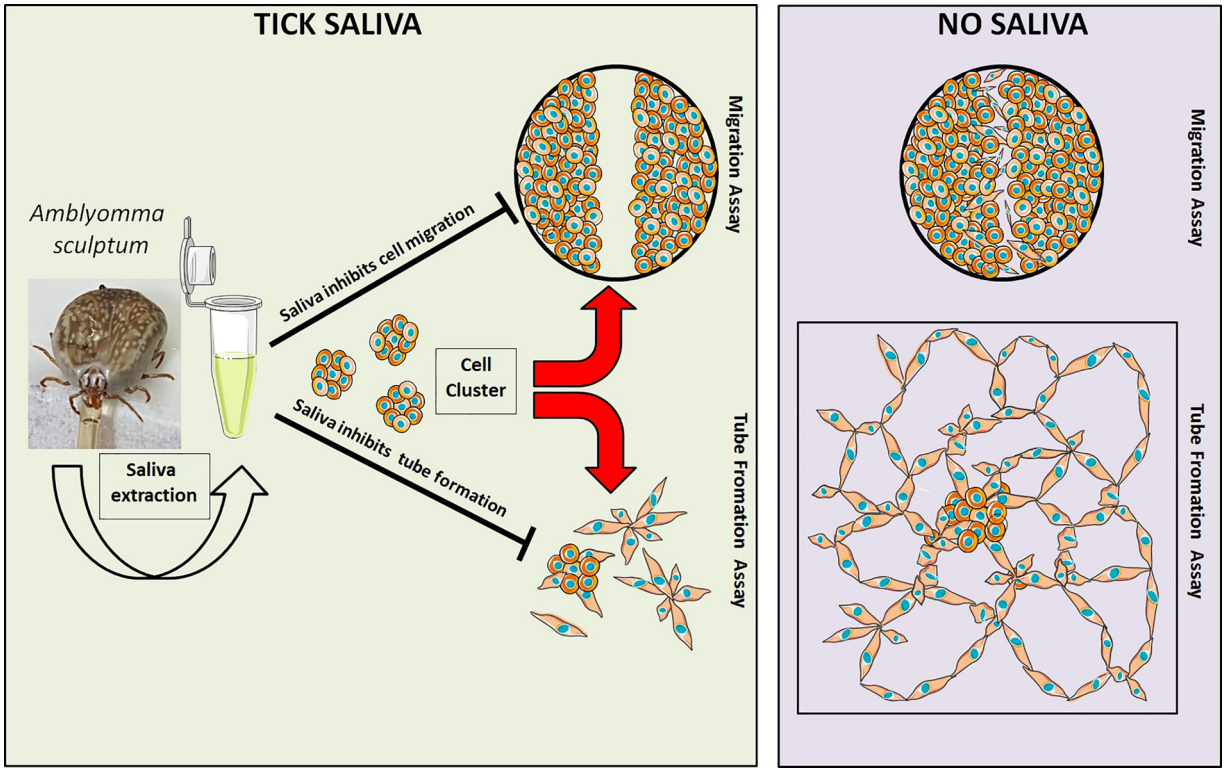
The MGF-converted MS2 profiles were analyzed by MS/MS ion search algorithms by Peaks Software 7.0 for matches with known proteins sequences deposited on the public UniProt database, constructed with proteins from *Ixodidae* family. The MS and MS/MS tolerances were fixed as 0.2 Da.

2.4. Cell culture

HUVEC was maintained in RPMI-1640 medium supplemented with 10% fetal calf serum (FCS), containing penicillin (100 units/mL) and streptomycin (100 mg/mL). The cells were cultured at 37 °C in a fully humidified incubator with 5% CO_2. All experiments described were performed at least three times using cells in the exponential growth phase.

2.5. Cell viability assay

Cells in the logarithmic growth phase were plated at a density of 10^3 cells/100 µL into 96-well plates and allowed to adhere overnight. Subsequently, culture medium was replaced by fresh medium containing 20 µg protein/mL of CS. After a 24 h treatment, cell viability was determined by the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) assay. Briefly, 10 µL of MTT solution (5 mg/



Scheme 1. CS inhibits the both events migration and formation of tubes by the HUVEC in vitro.

mL) were added to each well and incubated for 4 h at 37 °C. The supernatant was discarded after centrifugation at 2000 rpm for 10 min and 100 µL of dimethylsulfoxide were added to each well. Absorbance was measured using a multi-plate reader at 570 nm and results were normalized by the optical density of untreated control, considered as 100% viability. Each point of the test was performed in triplicate.

2.6. Wound-healing assay

HUVEC (2.5×10^5 cells) were grown to confluence in a 12-well plate, placed in medium complete for 24 h at 37 °C in an atmosphere of 5% CO_2. Upon reaching confluence, the cell layer was scratched with a sterile plastic tip and then washed twice with culture medium. Next, the cells were treated with 20 µg/mL of saliva for 24 h. Cell migration was recorded using a Nikon TE2000-E microscope system (Nikon Instrument). Migration was calculated using Image J software.

2.7. Cell cycle analysis

HUVEC were synchronized by deprivation of serum for 24 h and induced to reenter the cell cycle by the subsequent addition of serum. Cells were treated for 24 h with 20 µg/mL of saliva. Next, cells were collected and fixed with cold 70% ethanol and stored at -20 °C. The cells were washed, re-suspended in PBS and incubated at 37 °C for 45 min with 10 mg/mL RNase and 1 mg/mL propidium iodide (PI). Flow cytometric analysis was performed using a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA). Cell DNA content in the different cell cycle phases was determined using ModFit LT software (Verity Software House, Topsham, ME).

2.8. Evaluation of apoptosis by flow cytometry

For the detection and evaluation of apoptosis, HUVEC were treated with 20 µg/mL of CS for 24 h, then washed with PBS (500 µL/well), harvested (including suspension cells) and incubated with 2 mM Annexin-V and 10 mM of PI in PBS for 30 min at room temperature in the dark. The cells were immediately analyzed by flow cytometry using 488 nm excitation in a FACSCalibur flow cytometer (Scalibur-Becton Dickinson, San Jose, CA). Data from three independent experiments were analyzed using the FlowJo software.

2.9. Light and scanning electron microscopy

For light microscopy analysis, the cells were cultivated as described elsewhere. After CS treatment, cells were directly recorded using a Nikon TE2000-E microscope system (Nikon Instrument, Japan). For the electron microscopy, cells were grown on coverslips until to reach a semi confluence and, treated with saliva as described above. After, the cells were fixed in 2.5% (v/v) glutaraldehyde at 4 °C for SEM analysis. Fixed cells were rinsed in 0.1 M phosphate buffer, pH 7.2 and post-fixed with 1% osmium tetroxide. The cells were rinsed in PBS and dehydrated in a graded ethanol series of 30, 50, 70, 90, and 100% for 20 min. The cells were then critical point-dried from CO_2 in a critical point dryer (CPD- 030, BAL-TEC Co.). The dried cells were affixed to an aluminum stub with double-stick tape, coated with gold in an ionic sputter coater (SCD-005, BAL-TEC Co.) and photographed by scanning electronic microscopy (JEOL JSM-6100).

2.10. Fluorescence microscopy

The cells were seeded onto sterile glass cover slips in 24-well plates. Next, the cells were treated with CS (20 µg/mL) for 24 h and then fixed with 4% paraformaldehyde. For fluorescence microscopy, the cells were permeabilized by using a solution of 0.01% Triton X-100 diluted in PBS for 5 min. To analyze the effect of saliva on actin filaments, the cells were stained with phalloidin-fluorescein isothiocyanate (FITC) for 45 min. The nucleuses were stained with DAPI. Image analysis was performed with a fluorescence microscope (Nikon Eclipse Ni microscope Tokyo, Japan).

Table 1
proteins identified in the *A. cajanense* saliva.

Uniprot Entry	Protein name
A0A1D2AJ65	A disintegrin and metalloproteinase with thrombospondin motifs 7 like
B7PB57	Ap-3 complex subunit sigma
A0A1 × 1BE28	ATP synthase subunit beta
B7PFX9	cathepsin L
B7Q5 × 2	clathrin coat assembly protein
B7QGG6	Craniofacial development protein
A0A1D2AIW1	cysteine-rich with egf-like domain protein 2-like
B7PFG5	cytochrome P450
A0A131ZYK0	DNA excision repair protein XP-B-like protein
B7PL25	double-stranded RNA-specific editase B2
B7PNR9	ferredoxin-glutamate synthase
OBGS3051	GTP cyclohydrolase 1
A0A1D2AIN8	high density lipoprotein binding protein
B7PY36	homeobox protein
B7PN12	IK cytokine down-regulator of HLA class II
B7Q7H2	kinesin light chain
A0A1D2AJW2	kyphoscoliosis peptidase
A0A1E1XVY5	putative alpha-macroglobulin
V5HDR4	putative integral to membrane
A0A0K8RBU1	putative salivary kunitz domain protein
B7QNV1	putative uncharacterized protein
B7PNS4	putative uncharacterized protein
B7PTI0	putative uncharacterized protein
B7P7J9	putative uncharacterized protein
B7PNS4	putative uncharacterized protein
B7Q2C0	secreted metalloprotease
A0A1D2AI30	secreted salivary gland
B7QKL1	secreted salivary gland peptide
B7P3L9	serine protease inhibitor
OBGS1288	unknown hypothetical protein partial
OBGS1829	unknown hypothetical protein partial
OBGS2589	unknown hypothetical protein partial
B7P1L3	Vacuolar protein-sorting protein

2.11. In vitro angiogenesis assay

HUVECs (15×10^3 cells per well) were seeded onto Matrigel plates (containing 50 µl Matrigel), and cultured for 18 h at 37 °C in 5% CO_2. Capillary-like structures were evident and counted using a phase-contrast microscope and the networks formed by HUVECs were quantified with Image J software. Data are summarized as means ± Standard Deviation (SD).

3. Results

3.1. Proteomics of saliva

The protein composition of *A. sculptum* saliva was evaluated by shotgun proteomics techniques, which resulted in proteins identification from *Ixodidae* database. The Table 1 shows the proteins identified, according to the software criteria, after restricted set parameters.

Several secreted proteins with function on inhibition of coagulation were found, (for example salivary kunitz domain protein and alpha-macroglobulin) as well as proteins involved in homeostasis maintenance (secreted salivary gland protein, ATP synthase, GTP cyclohydrolase, etc) (Fig.1). Moreover, peptidases were also identified, along unknown and other uncharacterized tick proteins.

3.2. Microscopy analysis

The HUVEC morphological changes were evaluated after treatment with CS in comparison to the untreated control (Fig.2E). After 24 h of incubation, morphological changes, such as appearance of inner vacuoles and cytoplasmic fragmentation, were observed under the inverted phase-contrast microscope. The loss of the typical inter-cell elongations and cell adhesion was evident. At a late stage of treatment, CS induced a reduction of cell volume. Fluorescence microscopy demonstrated failure in actin polymerization/depolymerization dynamics induced by CS, compared to untreated control, leading to cell morphology changes (Fig. 3). Scanning electron microscopy depicts cytoplasmic shrinkage associated to detachment from surface. Blebs and small vessels can be observed after treatment, suggestive of apoptotic bodies (Fig. 4)

3.3. Cell viability and antiproliferative activity of CS

The antiproliferative and cytotoxicity profile of CS was evaluated by MTT colorimetric assay. The cells were exposed to CS at different concentrations for 24 h and, the IC50 values were calculated. The subsequent experiments were all performed with IC50 values (Fig.2D).

The saliva treatment exerted an important cytotoxic effect (evidenced by a sub-G0/G1 blue peak) accompanied by cell cycle arrest at G2M in comparison with control (Fig.2A-B). To corroborate this finding, the HUVEC cells were newly treated with CS for 24 h, and then doubled-stained with Annexin-V/PI, which allows the categorization between apoptotic cells (recent or late apoptosis) and necrotic cells (Fig.2C).

3.4. Cell migration and angiogenesis

To examine the effect of saliva on cells migration, we employed an *in vitro* would healing assay. Herein, we investigate whether the antiproliferative effects of CS correlate with the inhibition of cell migration (Fig. 5). Surprisingly, our major finding was that saliva inhibits the migration of endothelial cells suggesting their possible role as angiogenesis inhibitor. We found that saliva does affect the cytoskeleton organization, inducing a remarkable cytoplasm retraction, which, in turn, affects cell architecture, growth, motility and survival (Fig. 6). In addition, CS inhibits tube formation that consists in a reticular structure with elongated cells surrounding acellular areas. The cell-delimited arrangements are directly related to the angiogenic capacity of HUVEC. Treatment of HUVEC with saliva reduces their angiogenic capacity.

4. Discussion

Tick Saliva consists in a complex cocktail of bioactive compounds that when inoculated in the host are able to modulate the immunologic, inflammatory and hemodynamics responses in benefit of the feeding ticks [6,17]. The molecules present in CS depict antiplatelet, anticoagulation, vasodilatatory, anti-inflammatory and immunomodulatory properties such as prostacyclin, prostaglandin (vasoconstrictors). Ixolaris, Longistatin and Variegin are molecules involved in anticoagulation and fibrinolysis; OmCI, ISAC and SALP 20 are complement inhibitors; Sialostatin L and L2, MIF, PGE_2 present immunomodulatory properties; metalloproteases and haemangin possess anti-anti-angiogenic properties. The currently landscape summarizing all the bioactive molecules found in saliva of different ticks is elegantly discussed by [17].

Francischetti et al demonstrated for the first time that the crude saliva or entire salivary gland of *Ixodes scapularis* and *Rhipicephalus (Boophilus) microplus* exhibits a potent antiangiogenic activity accompanied by change in cell shape and apoptosis in endothelial cells *in vitro* [18]. Interestingly, saliva from other bloodsuckers, such as *Anopheles gambiae*, *An. stephensiae*, *Lutzomyia longipalpis*, *Phlebotomus papatasi*, *Aedes aegypti*, *Culex quinquefasciatus*, and *Cimex lectularius* are devoid of this effect [18].

In order to verify the antiproliferative cytotoxic effect of CS from *A. sculptum*, endothelial cells were treated with several concentrations of saliva and our findings denote a dose-dependent antiproliferative effect. Cell cycle analyses have demonstrated that saliva was able to decrease G2/M as well as inducing the sub-G0/G1 emergence, corroborating with notion that in higher concentrations saliva may be cytotoxic. Cell

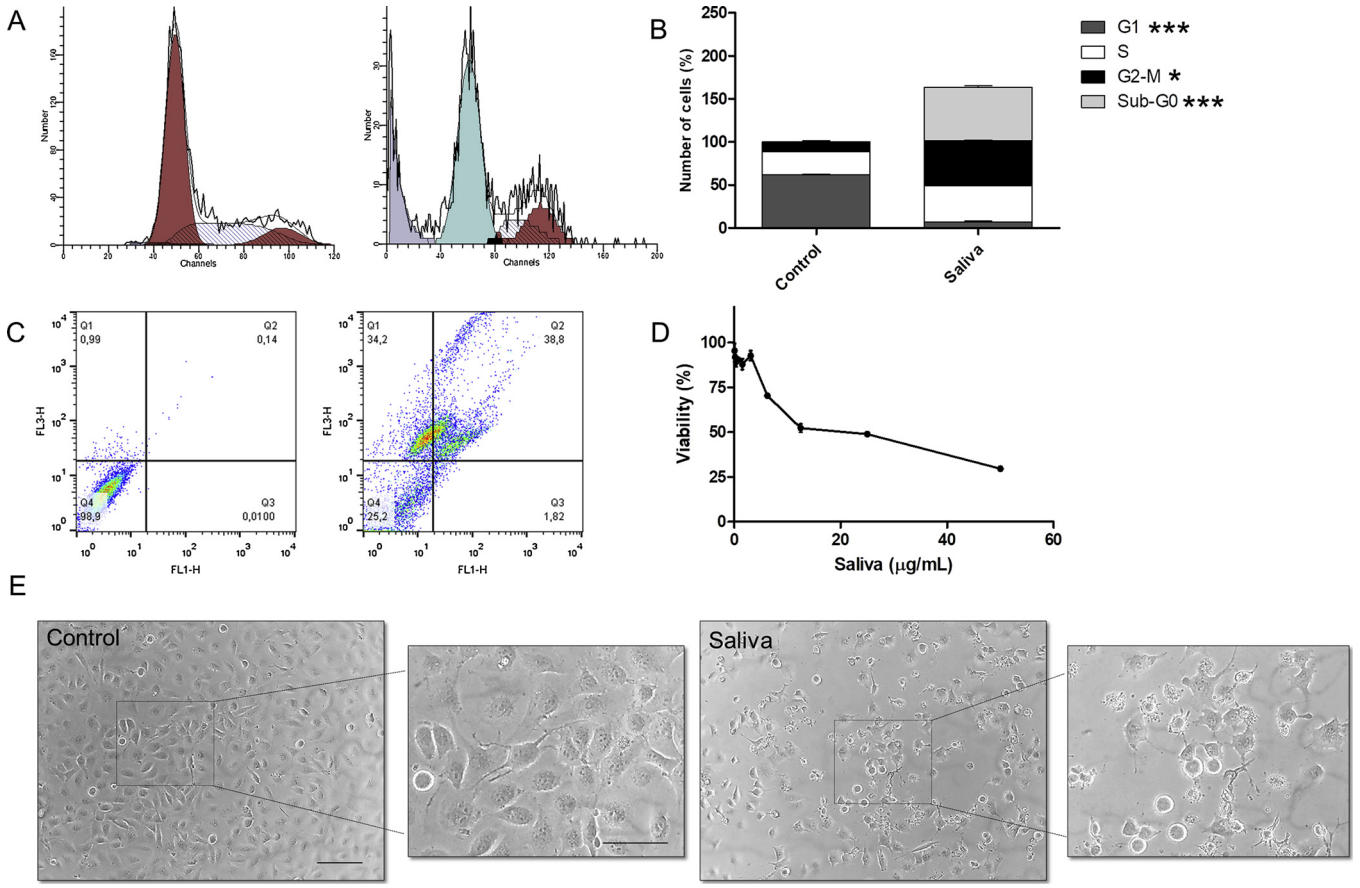


Fig. 1. Effects of Saliva on the cell cycle progression. (A) Cell cycle analyses of HUVEC cells are shown in representative histograms performed by flow cytometry. Saliva induces apoptosis, recognized as a sub-G1 (blue peak). (B) Distribution of HUVEC cells in cell-cycle phases. (C) Dot plot displays YO-PRO/PI double staining, which is representative of apoptotic and/or necrotic cells. 20% of saliva induces primarily apoptosis while high dosages, such 100 mM, induce necrosis. (D) Cytotoxic effects of saliva on HUVEC cell line. Cells were plated at a density of 5×10^5/well and treated with saliva for 24 h. Cell viability was evaluate by MTT colorimetric assay. Cell viability is expressed as the percentage of cells comparing the optical density (570 nm) of the treated cells with the optical density of the untreated cells. (E) Morphology of cells treated with Saliva. Statistical analisys was performed through Two Way ANOVA: $p < 0,05$(*); $p < 0,001$ (***).

morphology is an important criterion that may be related to cellular health and one of first observable events occurred during cell injury. The HUVEC suffered important morphological alterations after CS treatment, such as cytoplasmic shrinkage and detachment from surface, probably for interfering in the interactions of actin filaments with adhesive joints and focal adhesions in the cell-cell and cell-matrix contacts respectively [21]. More detailed analyzes performed through MEV, reveals that in addition to morphological alterations mentioned above, saliva also induced, in some cells, the formation of cytoplasmic fragments, suggestive of apoptotic bodies (Fig. 4).

Apoptosis plays a key role in cancer cell elimination, but failures in this system may favor the tumor development. Several molecular events are characteristics of apoptosis, among them, the phosphatidylserine (PS) exposition, and a membrane phospholipid that are normally distributed at the inner leaflet of cytoplasm membrane. During apoptosis, this phospholipid is exposed to outer leaflet membrane through “flip-flop” movement [22].

By using annexin-V, the exposition of PS may be quantified by flow

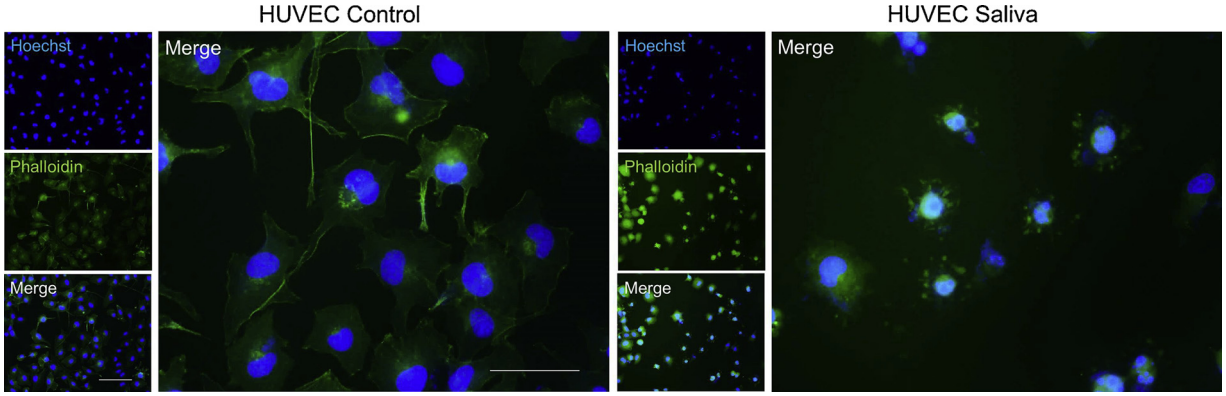


Fig. 2. Fluorescence microscopy of the cytoskeleton of saliva-treated HUVEC cells. Actin filaments are labeled in green (Phalloidin-FITC) and the nucleus in red (PI). Failure in actin polymerization/depolymerization dynamics induced by saliva, compared to control, leading to cell morphology changes.

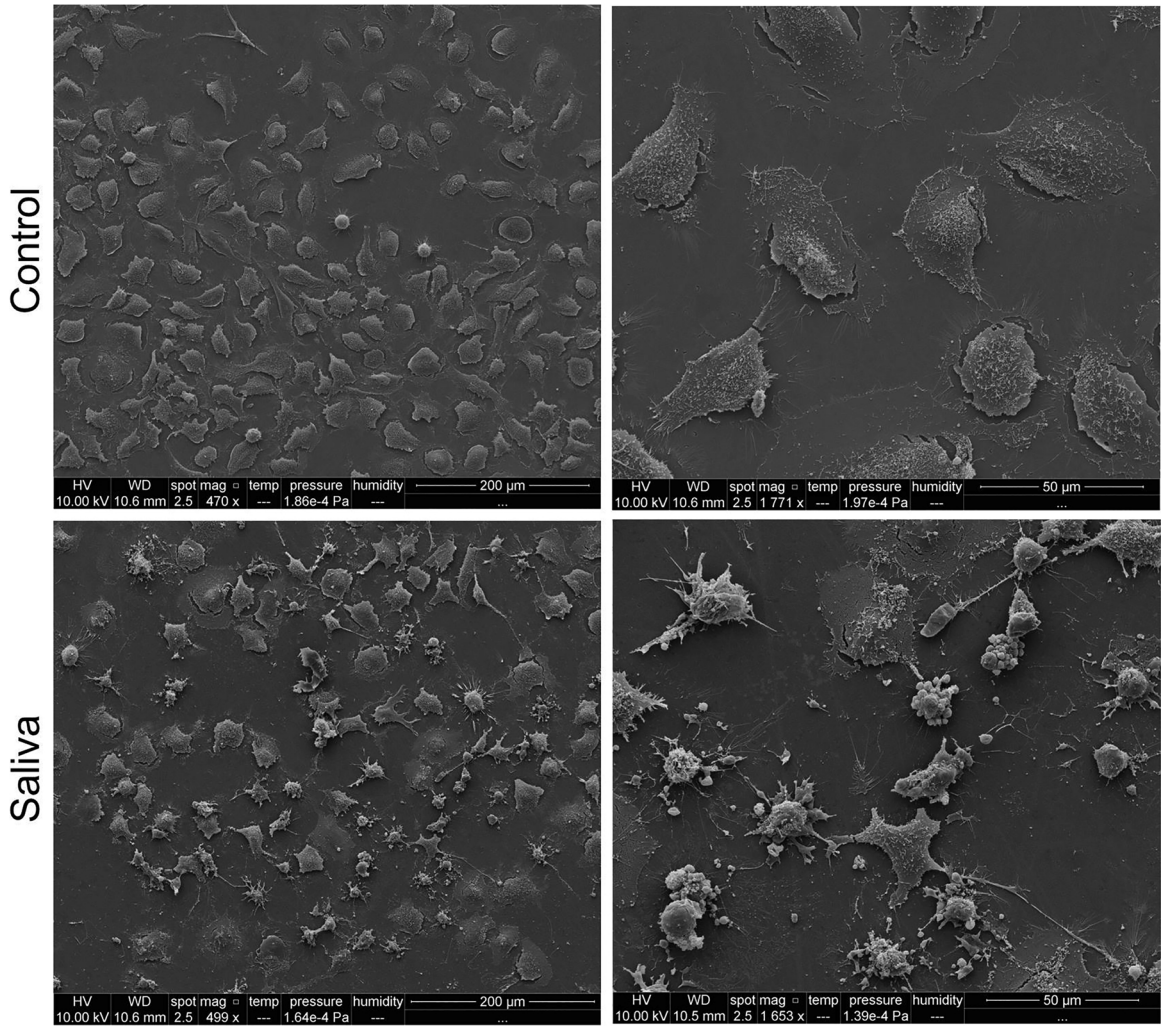


Fig. 3. Electron microscopy images demonstrating alterations of cell surfaces after treatment with Saliva.

cytometry, which in this work revealed that CS induces not only apoptosis, but also necrosis in HUVEC. In contrast to these findings, low concentration of saliva (10 µg/mL) did not cause necrosis despite drastically reducing cell proliferation (data not shown).

Endothelial cells migrate into the surrounding tissue in response to angiogenic chemokines. Cell motility is of particular interest in the development of anti-cancer therapeutics, whereas the cell migration is required for both tumor invasion and tumor angiogenesis [21]. Since the tick keeps the hematophagic wound open, especially by releasing saliva while it feeds, we speculate whether saliva could have any effect on cell motility. Indeed, the saliva tick exhibits a prominent anti-migratory effect even in low concentration. This finding is suggestive that CS is responsible for keeping the wound open for as long as the tick exerts the hematophagy, and may explain, at least in part, the fact that tick kept for so long attached to host-skin (solution of epithelial continuity, during blood feeding).

Actin is an essential cytoskeletal component for controlling angiogenesis. An intact actin cytoskeleton is required for cell motility and membrane protrusion events, which are key for mounting successful angiogenic responses [21]. Based on this, we investigated the effect of saliva in actin cellular architecture. Our results depicts that saliva tick disorganizes actin filaments in HUVEC and hence we hypothesized that saliva may preclude the cellular movement and consequently angiogenesis by deconstruct actin microfilaments.

Oliveira et al. [23] previously showed that CS of *A. sculptum* depicts a high index of inorganic components, but a detailed description of the protein components is still scarce [24]. Therefore, in order to shed new light on the knowledge of proteinaceus components present in saliva LC—MS and MS/MS analyses was performed. From this analysis, several components were identified. However, we focus our interests on disintegrin, mainly by their potent antiangiogenic effect. Disintegrins are a family of small cysteine-rich polypeptides involved in a numerous biological processes, especially inhibition of cell adhesion, migration and angiogenesis through the blocking the function of integrin receptors with a high degree of selectivity. A Disintegrin and Metalloproteinase with Thrombospondin motifs (ADAMTS) encompasses a group of shedding zinc metalloproteinases with a fairly ordered modular organization that comprises at least one thrombospondin type 1 repeat [19,20]. Analyses of the role of desintegrins in cancer model by using Cancer Hallmarks Analytics Tool (CHAT), demonstrates that this protein are involved especially in angiogenesis and has a relevant role in sustaining proliferative signaling, resisting cell death, invasion and metastasis and so on (Fig. 6).

Several disintegrins contain the RGD motif which blocks integrin functions, including $\alpha_V\beta3$, by competing with the natural RGD-containing proteins of the extracellular matrix. The active form of integrin $\alpha_V\beta3$ was found only on angiogenic endothelium and therapeutics agents targeting their blockade depict an inhibitory effect on endothelial cell adhesion, motility, invasion and tube formation [25] in both physiological and pathological conditions.

Almost half a century has passed since Judah Folkman postulated that tumors requires blood vessels in order to growth and proliferate,

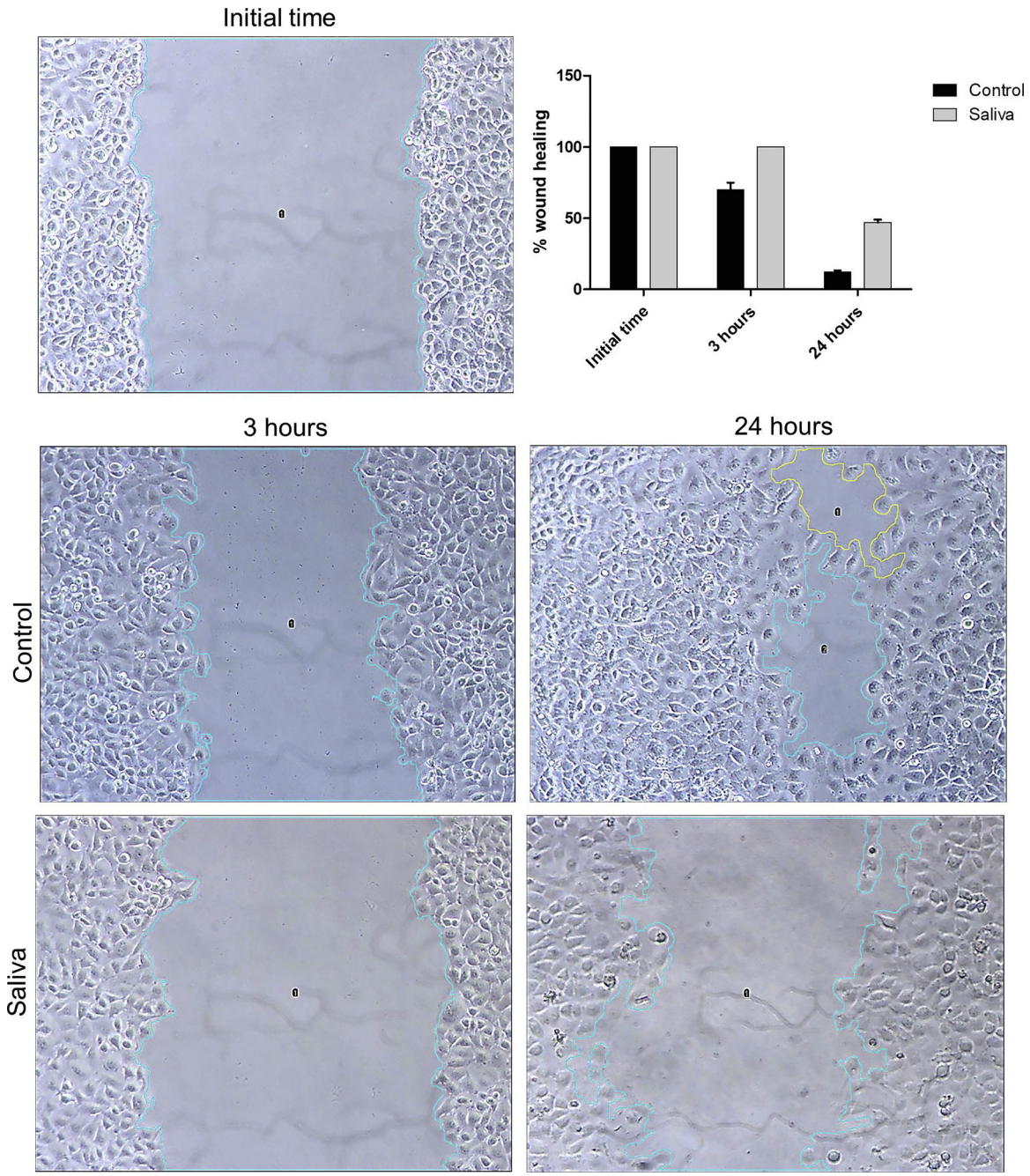


Fig. 4. Effect of saliva on HUVEC cell migration. (A) HUVEC cells were seeded in a 12-well plate, and then scraped to create a clean 1 mm wide wound area. Cells Then were treated for 24 h with saliva at concentrations 10 µg (Really 20%) and photographed and measured at 0 (t0), 3, and 24 h after wounding. The wound areas were then analyzed and calculated using Image *J* software. (B) Significant differences are indicated as: percent migration area.

and strategies to stop blood supply may cause cancer starving and remission [26]. In fact, as described by Nishida et al., [27] the tumor mass without blood circulation grew to 1–2 mm^3 in diameter and then stabilizes, but grew beyond 2 mm^3 when placed in an region where angiogenesis is possible. In the absence of vascular support, some cancerous cells may become apoptotic or even necrotic corroborating its dependence on neo-angiogenesis [27]. Therefore, therapies aiming on the reducing the blood flow for the tumor mass is an important strategy to promote tumor remission, by preventing not only its growth but also by triggering cytotoxicity in cancerous cells.

Our group have demonstrated in a previous work, the effect of saliva on the proliferation of melanoma and pancreatic adenocarcinoma cells, suggesting the existence of cytostatic or cytotoxic molecules in *A. sculptum* saliva [6]. However, its effect on angiogenesis, an essential event during tumor progression, still remains to be elucidated. Therefore, the aim of this study was investigate the effect of saliva from *A. sculptum* on the, proliferation, migration and angiogenesis of a human endothelial cell.

In conclusion, we have demonstrated the important anti-angiogenic effect of saliva of *A. sculptum in vitro* by the inhibition of HUVEC proliferation, migration and tube formation while breaking the actin stress fibers. Furthermore, this study opens new avenues on the knowledge about saliva composition and their biological properties.

Authorship

PL, DC, PM, JS, SS: Data acquisition, analysis, and interpretation, writing the first draft of manuscript, figure and table production,

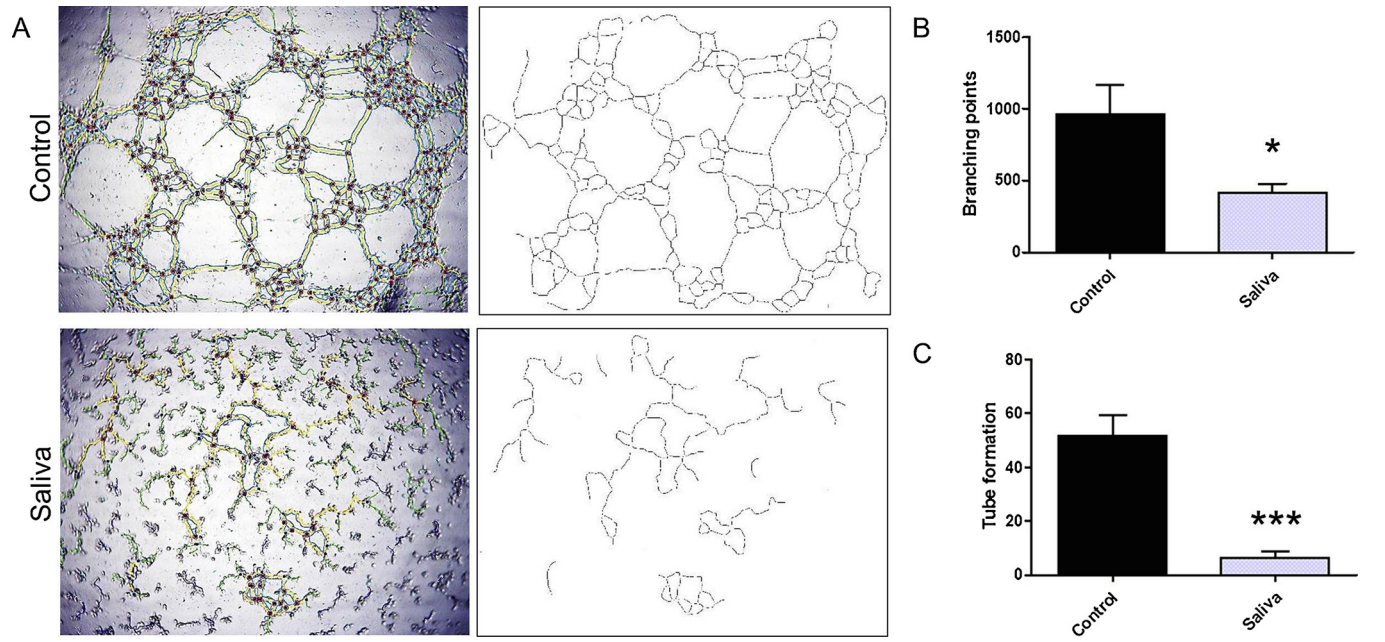


Fig. 5. Effect of saliva on angiogenesis *in vitro*. (A) Saliva-treatment inhibits the formation of capillary-like tubes. (B) CS inhibiting the Branching points comparing to untreated control. (C) CS precludes the tube formation comparing to untreated control. Statistical analisys was performed through Two Way ANOVA: $p < 005$(*); $p < 0001$ (***).

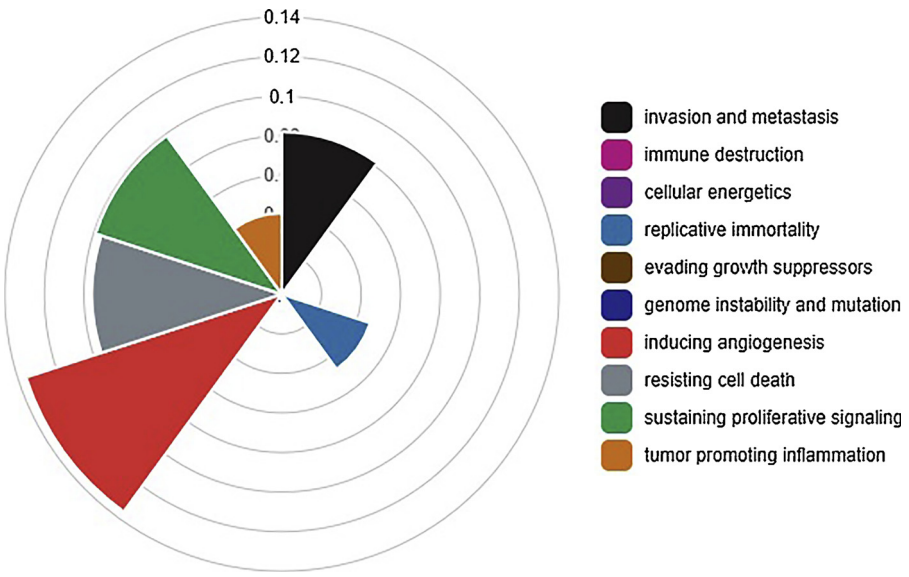


Fig. 6. Roles of Disintegrin in cancer progression. Investigations of Disintegrin in cancer research mainly focus on its crucial role in angiogenesis, migration, metastasis and so on. The data are from Cancer Hallmarks Analytics Toll. (http://chat.lionproject.net/).

references organization and manuscript revision.

AP, R, S: Contributed with reagents, materials, analysis and participation in the manuscript organization.

PL, PM, SS: Research design, analysis, and interpretation of data, writing of the manuscript, figure production, and critical revision for important intellectual content.

Competing interests

The authors declare that they have no competing interests.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.biopha.2018.11.107.

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