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Partial Characterization of Thrombin Inhibitor(s) Derived from Salivary Glands of the Tick, *Hyalomma dromedarii*, and Related Anti-Cancer Potential

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Abstract

A long-term blood feeder, like the *Hyalomma dromedarii* tick, requires extended control over all hemostatic defense mechanisms generated by the host during feeding, including blood coagulation. To overcome this, ticks have evolved numerous molecules that target proteases in the blood coagulation cascade. New insights into the role of clotting factors in the development and progression of cancer have identified anticoagulant treatment as a potential therapeutic approach. In this context, the present work assessed the anticoagulation activities of crude and fractionated salivary gland extract (SGE) prepared from semi-fed *H. dromedarii* females. Additionally, the antitumor effects of the potent anti-thrombin fractions were determined against colon cancer (Caco-2) and normal skin (HFB4) cells. Crude SGE significantly prolonged clotting time in prothrombin time (PT), activated partial thromboplastin time (aPTT) and thrombin time (TT) assays and inhibited thrombin in FII-activity assay. Using anion-exchange chromatography, the fractions that strongly inhibited thrombin (3.A4 and 3.A5) were eluted. Both fractions prolonged the aPTT and TT clotting times and reduced the activity of FII significantly. The protein profiles of both fractions indicated the presence of a single polypeptide band of about 99 kDa. Regarding anti-cancer potential of the tested fractions, Caco-2 cells showed reduced viability with obvious morphological changes, induced apoptosis and a reduced level of vascular endothelial growth factor (VEGF). G2/M cell cycle arrest was observed only in 3.A5-treated cells. No cytotoxic effects were observed in HFB4 cells. These results demon-

strated the potential of tick-derived anticoagulants, specifically thrombin inhibitors, as effective tools in colorectal cancer treatment. Further purification of the effector molecule(s) is required to fully characterize their structures and mechanisms of action.

Keywords

Hyalomma dromedarii, Salivary Glands, Thrombin Inhibitor, Apoptosis, VEGF

1. Introduction

In Egypt, the tick *Hyalomma dromedarii* is a prevalent livestock pest. It primarily parasitizes camels, mainly when it reaches the adult stage [1]. As a long-term feeder, it requires extended control over the defense mechanisms generated by the host during blood uptake. One of the host's first line of defense that must be overcome is hemostasis, which controls blood loss following vascular injury [2]. Ticks have evolved an array of anti-hemostatic agents including vasodilators, platelet aggregation inhibitors and anticoagulants [3] [4]. It is crucial for ticks to target proteases of the coagulation cascade in order to prevent clot formation and to retain blood fluidity not only in the micro-hematoma at the bite site but also in their mouthparts and midgut until repletion [5].

Blood coagulation, also referred to as secondary hemostasis, involves the sequential activation of circulating proenzymes (coagulation factors), which leads to the formation of the fibrin clot. The key enzyme within this cascade is thrombin, which plays a central role in the coordination of thrombosis and hemostasis [6]. In addition to its role in the formation of the fibrin clot, it regulates the activity of other clotting factors and stimulates platelet reactions. Other physiological processes, such as inflammation and complement activation, are also mediated by thrombin [7]. Therefore, thrombin as a target for tick compounds is an effective means for ensuring blood uptake. Large numbers of thrombin inhibitors have been identified from the salivary glands of both soft and hard ticks, reviewed in [3] [8].

Cancer and activated blood coagulation are highly connected. Cancer cells induce a hypercoagulable state through the release of procoagulants and activation of hemostatic cells, *i.e.*, endothelial cells and platelets [9]. Blood clotting factors also play an active role in the main characteristics of cancer biology, such as angiogenesis, invasion, metastasis, and modulation of innate immune responses [10]. Therefore, it has been proposed that targeting clotting proteins could serve as a therapeutic approach in cancer treatment: the antitumor effects of several anticoagulants like heparins, warfarin, factor Xa (FXa) inhibitors and direct oral anticoagulants (DOACs) have been described [11] [12] [13]. Coagulation inhibitors from ticks, specifically Ixolaris, a tissue factor (TF) inhibitor [14] and Amblyomin-X, an FXa-inhibitor [15], have also been studied in tumor

cell lines. As cancer is still one of the deadliest diseases worldwide, the need for obtaining powerful effectors, particularly from natural sources, continues to be an important aim in health research.

In the context of searching for novel anticoagulants, the present study examines the anticoagulation activities of salivary glands extract (SGE) derived from partially fed *H. dromedarii* females. It also presents the fractionation of SGE, and the partial characterization of the potential thrombin inhibitor(s). Additionally, the antitumor effects of the potential thrombin inhibitor(s) are examined on the colorectal cancer (Caco-2) and normal skin (HFB4) cells.

2. Materials and Methods

2.1. Preparation of Salivary Glands Extract (SGE)

Salivary gland crude extract was prepared from 200 semi-fed *H. dromedarii* females, collected from infested camels at Imbaba market, Giza Governorate, Egypt. Each tick was identified according to the taxonomic key described by Estrada-Peña [16]. Live semi-fed females were washed with 0.01 M PBS, pH 7.2 ± 0.2. They were dissected under a stereomicroscope (Meopta, Czech), placed in cold PBS (4˚C) and stored at −20˚C. Stored salivary glands were thawed and ultrasonicated five times, in an ice bath at 55,000 cycle/sec for 1 min each, followed by a 1 min cooling interval. The extract was cold centrifuged at 4˚C using a high-speed cooling centrifuge (Jouan-Ki-22, France) for 1 h at 14,000 rpm, according to Heller-Haupt *et al.* [17]. Supernatants were pooled together and protein concentration was determined using the micrototal protein (MT-P) Pyrogallol-Red kit (Egyptian company for Biotechnology).

2.2. Evaluation of Anticoagulation Activity

All coagulation assays were performed using normal human plasma obtained by centrifugation of blood from healthy donors (3.6 mL) and 0.11 M trisodium citrate solution (0.4 mL) at 3000 × g for 10 min.

2.2.1. Prothrombin Time (PT) Assay

Prothrombin time (PT) measures the extrinsic pathway clotting time of plasma at 37˚C in the presence of excess tissue thromboplastin and calcium chloride ($CaCl_2$) [18]. Various concentrations of SGE (0, 5, 10, 25, 50 and 100 µg/mL) in a total volume of 50 µL of 0.01 M PBS, pH 7.2 ± 0.2, were incubated with 50 µL of the citrated plasma for 6 min at 37˚C. Liquiplastin-S reagent (100 uL, pre-warmed at 37˚C) (BioMed Diagnostics) was added, and clotting time was determined using a coagulometer (BE-Thrombostat, Behnk Elektronik GmbH & Co.).

2.2.2. Activated Partial Thromboplastin Time (PT) Assay

The activated partial thromboplastin (aPTT) assay determines the intrinsic pathway clotting time of plasma at 37˚C in the presence of a platelet substitute and an activator [18]. Various concentrations of SGE (0, 5, 10, 25, 50 and 100 µg/mL) in a total volume of 50 µL of 0.01 M PBS, pH 7.2 ± 0.2, were added to the

citrated plasma (50 µL) and incubated at 37˚C for 3 min. Liquiplastin-L reagent (50 µl) (BioMed Diagnostics) was added and the mixture was incubated for 3 min at 37˚C. Finally, 50 µL of pre-warmed $CaCl_2$ (0.05 M) was added to start the reaction, and the time it took for the formation of the fibrin clot was recorded using the coagulometer.

2.2.3. Thrombin Time (PT) Assay

Thrombin time (TT) is a screening test used to detect anti-thrombin circulating anticoagulants that are naturally occurring or have been injected [19]. A mixture of 50 µL of citrated plasma and 50 µL of SGE (0, 5, 10, 25, 50 and 100 µg/mL) was incubated at 37˚C for 1 min. Pre-warmed TT reagent (50 µL) (Siemens Healthcare Diagnostics) was added and the time required for the formation of the fibrin clot was determined using the coagulometer.

2.2.4. Bio-Assay for Inhibition of FII (Prothrombin) Activity

Inhibition of FII activity was determined utilizing FII-deficient plasma in a PT-based one-stage clotting time assay [20]. A mixture of 50 µL of normal plasma and 50 µL of SGE (0, 25, 50 and 100 µg/mL) was prepared. After that, 50 µL of FII-deficient plasma (Siemens Healthcare Diagnostics) was added and the mixture was incubated at 37˚C for 1 min. Finally, 100 µl of Thromborel S reagent, pre-warmed at 37˚C, was added to determine the clotting time. Factor II content was determined from a reference curve prepared with dilutions of a standard plasma, mixed with FII-deficient plasma.

2.3. Isolation of Salivary Gland Anticoagulant Protein(s)

Crude extract from homogenizing the salivary glands of 1200 semi-fed females in 20 mM Tris-HCl (pH 8) [21] was used in this step. The supernatant was applied to a Mono Q 5/50 GL anion-exchange column (GE Healthcare Bio-Sciences AB) adapted to the AKTA Fast Performance Liquid Chromatography (FPLC) system (GE Healthcare Bio-Sciences AB) previously equilibrated with 20 mM Tris-HCl, pH 8. The adsorbed proteins were eluted with a NaCl linear gradient, ranging from 0 M to 1 M prepared in the equilibration buffer, at a flow rate of 1 mL/min. The elution profile was monitored at 280 nm. Fractions (2 mL each) were collected, dialyzed against distilled water, lyophilized, dissolved in PBS (pH 7.2) and stored at −20˚C.

All developed fractions were initially screened for anticoagulation activity using 5 µL of each fraction and 50 µL of the citrated plasma in a PTT assay. Fractions with potent anticoagulation potential (50 µg/mL) were subjected to further screening for anti-thrombin activity using TT and FII-activity assays.

2.4. Electrophoretic Characterization of Partially Purified Anticoagulant(s)

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out with 10% polyacrylamide gel under reducing conditions according to Laemmli [22]. The electrophoretic analysis was performed using the MiniPro-

tean II Dual-slab Cell (Bio-Rad, USA). Protein bands were visualized by 0.2% Coomassie Brilliant Blue R stain. Molecular weights of resolved proteins were estimated by comparing their electrophoretic mobility with those of known standard protein molecular weight markers (BLUltra Prestained Protein Ladder, GeneDirex).

2.5. Evaluation of Anti-Cancer Activity

2.5.1. Cell Culture

Two human cell lines, Caco-2 and HFB-4, were used. Normal and cancer cells were obtained from the Research & Development Sector, VACSERA, Egypt. Cancer cells were cultivated in RPMI-1640 medium, while HFB-4 cells were grown in Dulbeco's Modified Eagle's Medium (DMEM). Media were supplemented with 10% fetal bovine serum (FBS), 1 mM sodium pyruvate, 2 mM L-glutamine, 100 IU/mL penicillin and 100 µg/mL streptomycin. Media, serum, trypsin, and antibiotic were supplied by GIBCO, USA. Both cell lines were maintained at 37˚C in a humidified atmosphere with 5% CO_2. Subconfluent cultures were washed twice, either with medium or PBS, at pH 7.5. The cells were then detached with 0.05% trypsin-EDTA, spun at 1000 rpm for 5 min and resuspended in complete medium.

2.5.2. Cytotoxicity Assay

The cytotoxic effects of the SGE fractions with the strongest anti-thrombin potential on viability and proliferation of Caco-2 and HFB-4 cell lines were estimated using the MTT assay [23]. The cells were seeded (2×10^4 cells/well) in 100 µL of complete culture medium in 96-well tissue culture plates (Nunc-Denmark) and incubated for 24 h at 37˚C. Growth medium was then decanted and replaced with fresh media only, in control wells, or with fresh media containing two-fold serially diluted tested fractions in treated wells. After 48 h incubation, the treatment medium was decanted, and cells were washed using PBS. MTT solution (0.5 mg/mL) was added to each well (50 µL) and the cells were incubated for a further 4 h. The supernatant was then carefully removed, and 50 µL of dimethylsulfoxide (DMSO) was added to each well. The optical density (OD) was measured at 540 nm using a LERX-800 ELISA reader (Biotek-USA). The viability percent was calculated using the following formula:

Viability % = (OD of treated cells/OD of control cells) × 100

The half-maximal inhibitory concentrations (IC_{50} values) were calculated by fitting the survival curve, using MasterPlex 2010 software (MiraiBio, Hitachi Solutions America, Ltd.).

2.5.3. Cell Cycle Analysis

The disturbance of the cell cycle phases is one of the significant characteristics of apoptosis. Analysis of the alteration of cell cycle phases induced by the tested fractions was conducted using a Propidium Iodide Flow Cytometry Kit for cell cycle analysis (Abcam), following the manufacturer's instructions. Briefly, 1×10^6 Caco-2 cells were treated with the IC_{50} values of tested fractions for 48 h.

Subsequently, both treated and untreated cells were trypsinized, washed with cold PBS, fixed in 70% ethanol, and stored at 4˚C overnight. The cells were then washed again with cold PBS, resuspended in 200 µL of the staining solution (9.45 mL of PBS, 500 µL of 1 mg/mL propidium iodide (PI) and 50 µL of RNase A) and incubated for 30 min in the dark at room temperature (25˚C). The fractions of cells in G0/G1, S and G2/M phases were analyzed using the BD FACSCalibur™ system (BD Biosciences, USA).

2.5.4. Apoptosis Assay

The sensitivity of tumor cells to apoptosis was measured by two-color fluorescence-activated cell sorting (FACS) analysis using PI and fluorescein isothiocyanate (FITC) conjugated Annexin V. An Annexin V-FITC Apoptosis Detection Kit (BioVision) was used, according to the manufacturer's instructions. Approximately, 5×10^5 Caco-2 cells were incubated for 48 h with the IC_{50} values of tested fractions at 37˚C in a CO_2 incubator. Both treated and untreated cells were trypsinized and centrifuged at 3000 rpm for 10 min using a cooling centrifuge. After that, the cells were washed with cold PBS and re-suspended in 500 µL of binding buffer, to which 5 µL of Annexin V and 5 µL of PI were added. The mixture was then incubated at room temperature (25˚C) for 10 min in the dark. The fluorescence of the cells was immediately assessed at 488 nm, using flow cytometry.

2.5.5. Vascular Endothelial Growth Factor (VEGF)

The effect of tested fractions on the main pro-angiogenic mediator VEGF was studied using an enzyme-linked immunosorbent assay (ELISA). For this purpose, precultured Caco-2 cells (6×10^4) were treated with the IC_{50} values of test fractions and then incubated for 48 h at 37˚C. Supernatants from both treated and control cell cultures were collected, centrifuged at 3000 rpm for 10 min using a cooling centrifuge to remove cell debris and stored at −20˚C. The VEGF level in the supernatants was determined using a VEGF Human ELISA Kit (Abcam), according to the manufacturer's instructions. The intensity of the developed color was measured by reading absorbance at 450 nm, using a BioLine microplate reader.

2.6. Statistical Analysis

Data were statistically analyzed by a one-way analysis of variance (ANOVA, SAS, 2004) using general linear model (GLM) classification, followed by the Duncan Multiple Range Test to examine the significance between means. Differences were considered significant when $p < 0.05$.

3. Results

3.1. Anticoagulation Activities of SGE

The effect of SGE on the coagulability of citrated normal human plasma was studied using a number of plasma-based clotting assays (**Figure 1**). Anticoagulation

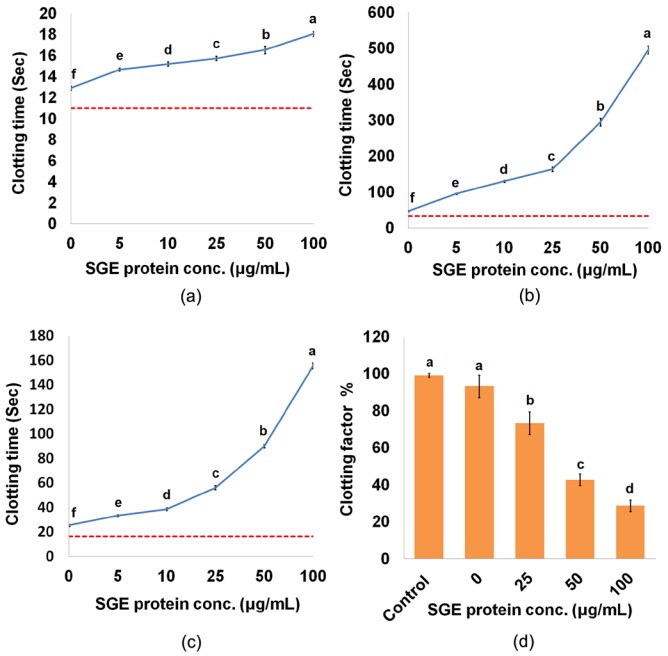

Figure 1. Effect of semi-fed *Hyalomma dromedarii* females' SGE on a number of plasma-based clotting assays. (a) PT assay; (b) aPTT assay; (c) TT assay; (d) FII activity assay. The control time in PT, aPTT and TT assays is shown as a dotted line. Results are expressed as the mean ± SD of three independent determinations. In each graph, figures followed by the same letters are statistically similar ($p > 0.05$); those followed by different letters are significantly different ($p < 0.05$ - $p < 0.001$) as determined by one-way ANOVA followed by Duncan post-Hoc test.

potential was established for both the extrinsic and intrinsic coagulation pathways using PT and aPTT assays, respectively. It was observed that the clotting time in both assays increased in a concentration-dependent manner (**Figure 1(a)** & **Figure 1(b)**). However, the PT assay was less sensitive than the aPTT assay: a 1.6-fold increase ($p < 0.05$) in the clotting time was recorded using 100 µg/mL of SGE in the PT assay, whereas in the aPTT assay, 5 µg/mL SGE was able to significantly extend clotting time 2.89-fold ($p < 0.01$).

The TT data showed a significant concentration-dependent increase in plasma clotting time compared to the control ($p < 0.01$), indicating inhibition of thrombin function (**Figure 1(c)**). This result was emphasized by the data from FII activity assay, which showed a significant inhibition ($p < 0.01$) of FII activity in a concentration-dependent manner (**Figure 1(d)**). These results suggest the presence of potent thrombin inhibitor(s).

3.2. Isolation of Salivary Gland Anticoagulant Protein(s)

To purify the one or more coagulation inhibitors that are present in the salivary glands of semi-fed *H. dromedarii* females, SGE was subjected to fractionation by means of anion exchange chromatography (**Figure 2**). The collected fractions were named 1.A (1 - 5), 1.B (1 - 5), 1.C (1 - 5), 3.A (1 - 5), 3.B (1 - 5) and 3.C (1 - 5). Protein peaks present in the eluted fraction 3.A (1 - 5) were found to have the

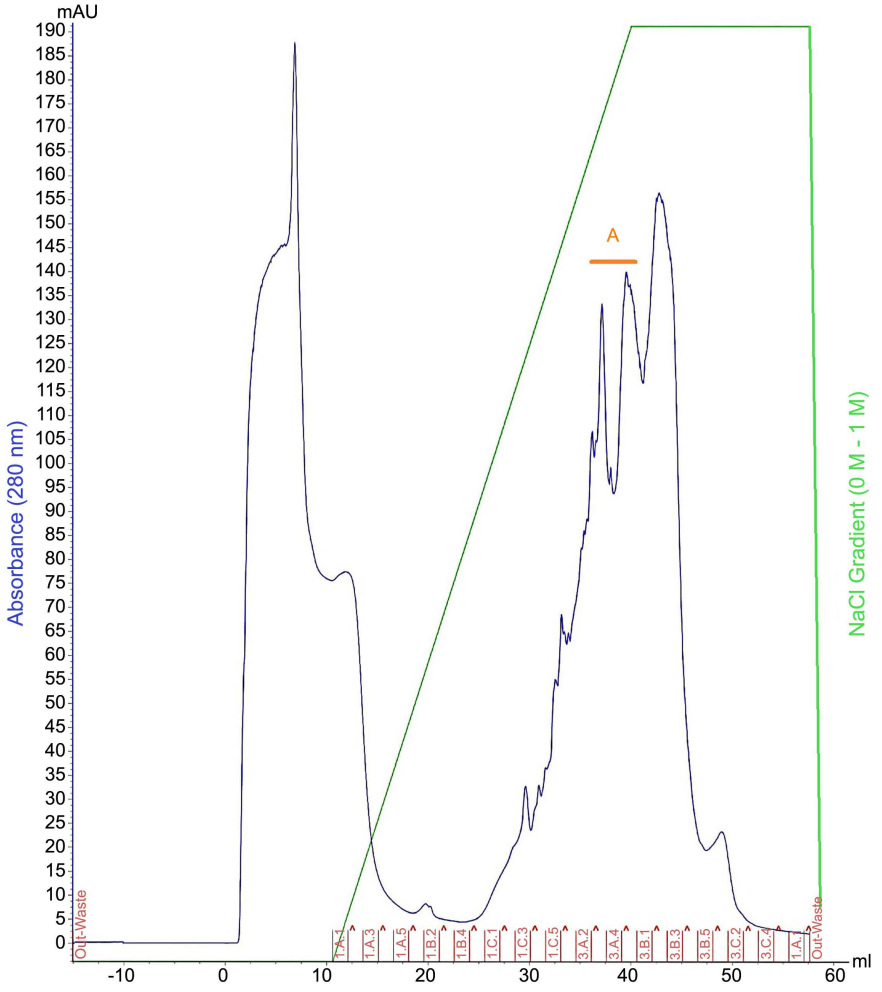

Figure 2. Anion-exchange chromatography of SGE on Mono Q 5/50 GL column. A: activity with respect to inhibition of plasma coagulability using coagulation assays.

strongest anticoagulant activity, as demonstrated by the aPTT assay (**Figure 3(a)**).

Further verification of the anticoagulation potency of these fractions was performed using the TT and FII-activity assays (**Figure 3(b)** & **Figure 3(c)**). Results of all three assays revealed that the most potent anticoagulant activity was present in the fraction 3.A5, followed by 3.A4, which were eluted using ~0.8 M - 0.9 M NaCl.

3.3. SDS-PAGE

The electrophoretic profile of SGE and fractions 3.A (1 - 5) is presented in **Figure 4**. Approximately nine polypeptide bands were identified from the SGE with respective molecular weights of approximately 252, 99, 66, 54, 51, 41, 36, 32 and 30 kDa. Single polypeptide band (~66 kDa) was resolved from the 3.A1, 3.A2 and 3.A3 fractions. The electrophoretic pattern of 3.A4 and 3.A5 indicated the presence of a single polypeptide band with a molecular weight of approximately 99 kDa.

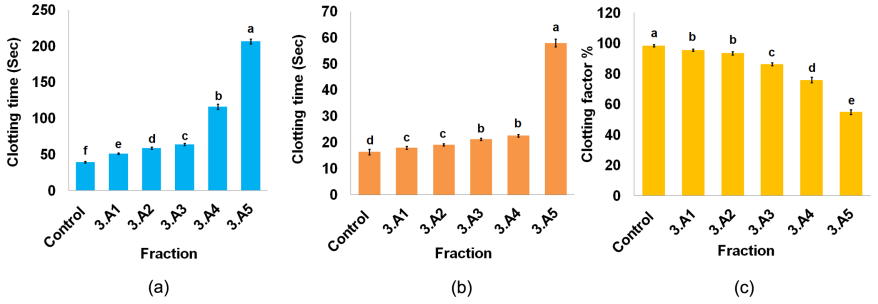

Figure 3. Effect of Mono Q-chromatographic fractions 3.A (1 - 5) on different blood coagulation assays. (a) aPTT assay; (b) TT assay; (c) FII activity assay. Results are expressed as the mean ± SD of three independent determinations. In each graph, figures followed by the same letters are statistically similar ($p > 0.05$); those followed by different letters are significantly different ($p < 0.05$ - $p < 0.001$) as determined by one-way ANOVA followed by Duncan post-Hoc test.

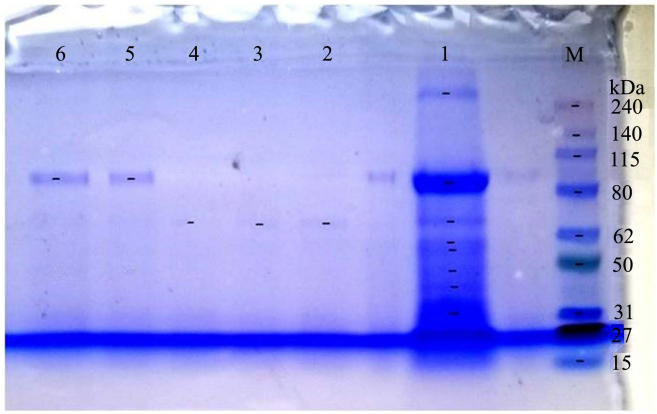

Figure 4. SDS-PAGE electrophoretic profile of SGE and its chromatographic fractions having anticoagulant activity under reducing conditions. (M) Marker, (1) Crude SGE, (2) 3.A1 fraction, (3) 3.A2 fraction, (4) 3.A3 fraction, (5) 3.A4 fraction, and (6) 3.A5 fraction. A dash indicates the polypeptide bands.

3.4. Anti-Cancer Activity

Anticoagulant fractions (3.A4 & 3.A5) were examined for their cytotoxic activity against Caco-2 and HFB-4 cells *in vitro*. MTT assay results showed that the survival of Caco-2 cells was significantly decreased by both fractions, in a concentration-dependent manner at 48 h post-treatment ($p < 0.01$) (**Figure 5(a)**). The cytotoxicity of the tested fractions recorded IC_{50} values in the order of 66 µg/mL and 54 µg/mL for the fractions 3.A4 and 3.A5, respectively. On the contrary, the survival and viability of normal HFB-4 cells were not affected by the examined fractions (**Figure 5(b)**).

The percentage of Caco-2 cells in the G0/G1 phase decreased, and those in both the S and G2/M phases increased 48 h post-treatment with the IC_{50} value of 3.A4, compared with untreated cells (**Figure 6(a)**). Cell cycle analysis of 3.A5-treated cells also recorded higher number of cells in the G2/M phase, but a decreased number of cells in both G0/G1 and S phases. These results showed

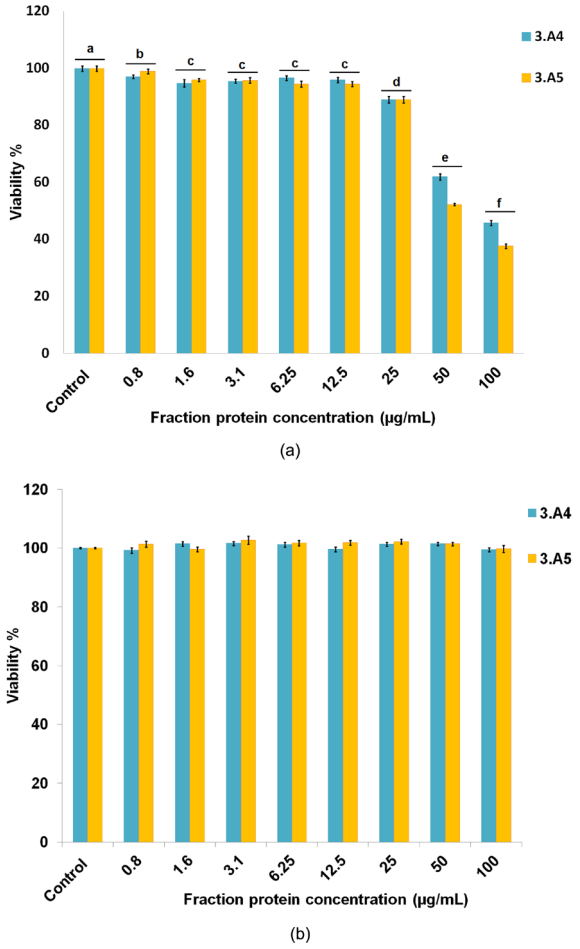

Figure 5. Anti-proliferative effect of anticoagulant fractions (3.A4 and 3.A5) against different cell lines treated with different concentrations of each fraction for 48 h as determined by the MTT assay. (a) Caco-2 cell line; (b) HFB4 cell line. Results are expressed as the mean ± SD of three independent determinations. In each graph, figures followed by the same letters are statistically similar ($p > 0.05$); those followed by different letters are significantly different ($p < 0.05$ - $p < 0.001$) as determined by two-way ANOVA followed by Duncan post-Hoc test.

that cytotoxicity of the 3.A5 fraction was accompanied by cell cycle arrest at the G2/M phase, whereas 3.A4 has no effect on any specific checkpoint of the cell cycle. However, presence of the sub G0/G1 blue peak in both 3.A4- and 3.A5-treated cells indicated a pro-apoptotic effect induced by both fractions (**Figure 6(a)**). Further investigation using double-staining with Annexin-V/PI allowed categorization of apoptotic (early or late apoptosis) and necrotic cells. The dot-blot analysis of 3.A4-treated Caco-2 cells showed approximately 8.76% total cell death, of which the proportion of apoptotic cells was 7.45%, whereas necrotic cells comprised only 1.31% (**Figure 6(b)**). In 3.A5-treated Caco-2 cells, total cell death was found to be 19.56% (**Figure 6(b)**). Also, apoptotic and necrotic indices of the untreated cells increased from 1.25% to 17.7%, and 1% to 1.86%, respectively, 48 h after incubation with the IC_{50} value of the tested fraction. Both fractions led to an apoptosis-dependent cell death in Caco-2 cells.

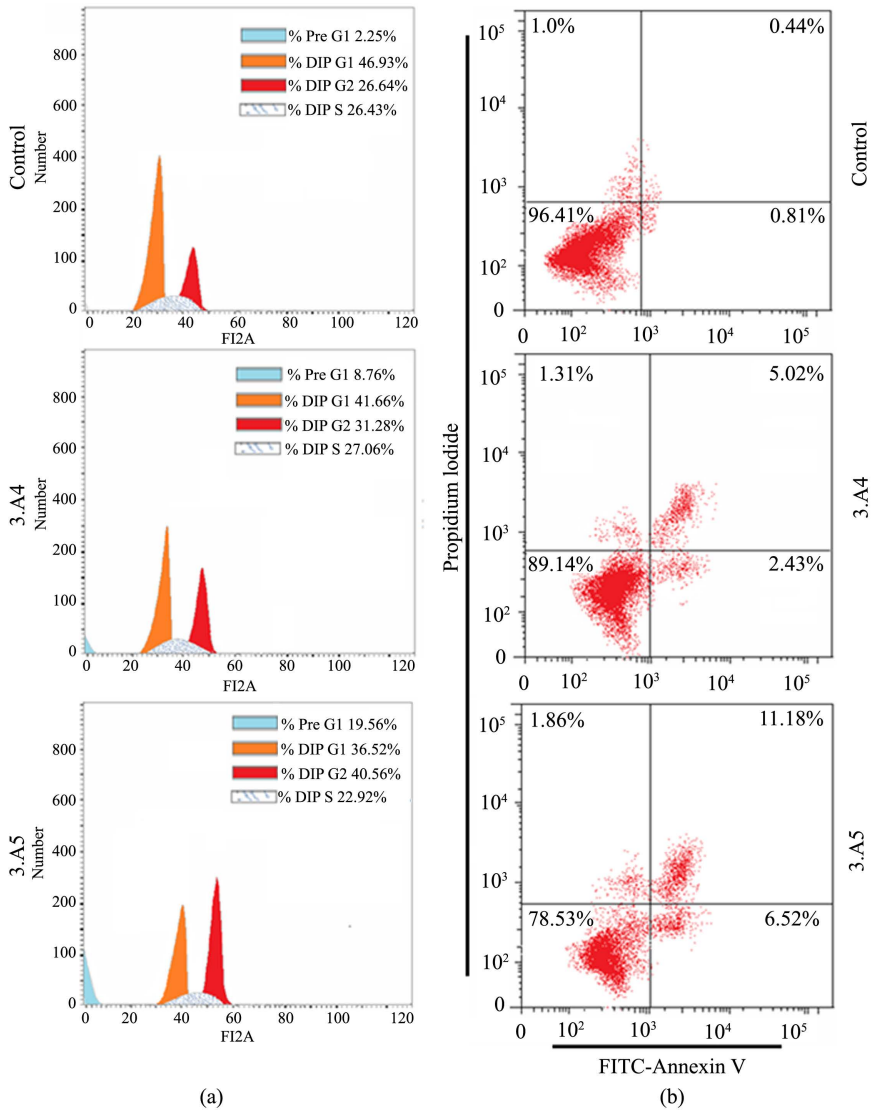

Figure 6. Flow cytometric analysis of Caco-2 cells 48 h post-treatment with the IC_{50} values of anticoagulant fractions (3.A4 and 3.A5). (a) Cell cycle progression; (b) Apoptosis induced in treated cells. Quadrants: bottom left—viable cells; bottom right—early apoptotic cells; top right—late apoptotic cells; top left—necrotic cells.

The effect of anticoagulants from both fractions on the level of VEGF was assessed in Caco-2 cells (**Figure 7**). There was a significant decrease in the levels of VEGF from 143.53 ± 5.31 pg/mL (in the control) to 80.61 ± 2.97 pg/mL and 64.05 ± 2.46 pg/mL, 48 h post-treatment with the IC_{50} values of 3.A4 and 3.A5, respectively ($p < 0.01$).

4. Discussion

After Ixodid ticks are hematophagous animals that access the blood via the wound made by their highly developed mouthparts. They target components of the hemostatic system to facilitate blood uptake for relatively long periods of time [24]. Among the targeted molecules are proteases of the blood coagulation

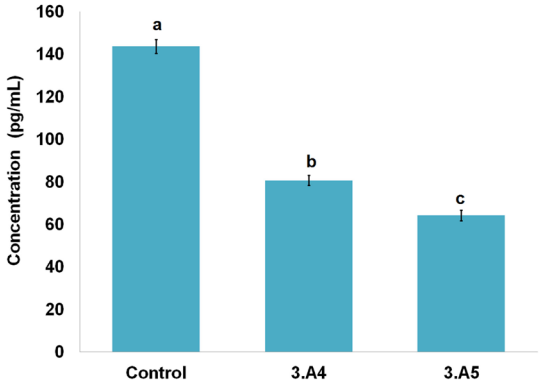

Figure 7. Level of the pro-angiogenic mediator VEGF in Caco-2 cell culture supernatants 48 h post-treatment with the IC_{50} values of anticoagulant fractions (3.A4 and 3.A5). Results are expressed as the mean ± SD of three independent determinations. In each graph, figures followed by the different letters significantly different ($p < 0.05$ - $p < 0.001$) as determined by one-way ANOVA followed by Duncan post-Hoc test.

cascade, thus the blood remains incoagulable within their mouthparts and gut during the long feeding period, until repletion [5]. The anticoagulation properties of tick saliva, therefore, represent an excellent target for research into potential pharmaceuticals for human diseases associated with hemostatic disorders [3] [25].

The blood coagulation cascade can be subdivided into three systems: the extrinsic, the intrinsic and the final stage of thrombogenesis. The extrinsic system involves factors I, II, V, VII and X; the intrinsic comprises factors I, II, V, VIII, IX, X, XI and XII; and the final stage utilizes factors I and II [26]. These systems can be evaluated in vitro, separately, using PT, aPTT and TT assays, respectively, via the addition of different coagulation activators. Our study shows that crude SGE derived from semi-fed *H. dromedarii* females effectively prolonged the time required for the formation of the fibrin clot in all three systems, with the intrinsic pathway and final stage of thrombogenesis being more affected than the extrinsic pathway. As the TT assay measures thrombin activity downstream from FXa, it is insensitive to FXa inhibitors [27]. Thus, although excessive concentrations of any anticoagulant can prolong both PT and aPTT, the TT may help to identify the type of anticoagulant present, *i.e.*, a prolonged TT indicates a thrombin inhibitor, whereas normal TT is indicative of an FXa inhibitor [28]. Consequently, this study suggests that SGE is a promising source of potential thrombin inhibitor(s). This was emphasized by the result of the FII activity assay which recorded concentration-dependent reductions in the amount of FII, by up to 70%. Anticoagulant activity against other clotting factors cannot be excluded by this study, but the anti-thrombin activity described here appears to predominate. Joubert *et al.* [29] found that SGE prepared from pre-fed *H. truncatum* females exerted an inhibitory effect on the intrinsic and extrinsic coagulation pathways, however, in this study FXa was the target of the anticoagulants, with almost 90% of FXa activity inhibited.

Thrombin plays multiple roles in blood coagulation, either as a procoagulant or an anticoagulant [30]. As a procoagulant, it converts fibrinogen to fibrin, which is further stabilized through cross-linking driven by thrombin-activated FXIII. Additionally, thrombin catalyzes its own generation through the activation of FXI, FVIII and FV, leading to the thrombin burst. It also stimulates platelet aggregation via interaction with protease-activated receptors (PARs) 1, 3 and 4 [31] [32]. Thus, choosing thrombin as a target for novel anticoagulants is logical as its inhibition not only blocks fibrin formation, but also inhibits other thrombin-mediated pathways in the clotting process [33].

In the present study, crude SGE was subjected to fractionation by means of anion-exchange chromatography, and the fractions that strongly inhibited thrombin (3.A4 and 3.A5) were eluted. Both fractions exerted a significant inhibitory effect on the intrinsic pathway and the terminal stage of the clot formation, however, the 3.A5 fraction exerted the greatest activity. The protein profiles of both fractions indicated the presence of a single polypeptide band with molecular weight of about 99 kDa. This molecular weight suggested that it belongs to protease inhibitor proteins of the type serpin which are one of the most important serine protease inhibitors that ticks rely on to control host hemostasis and immunity [34]. Seven significantly overexpressed serpins were identified in the salivary gland transcriptome of partially fed *H. dromedarii* females, from which only five proteins were identified in the proteomic analysis [35] [36]. Identification of these serine protease inhibitors from salivary glands of the semi-fed females suggests their crucial role in tick blood feeding success. Therefore, raising antibodies against them in the host may block their anti-hemostatic functions and interfere with the proper tick feeding. Further characterization of these anticoagulant molecule(s) is required in order to determine if they are the same protein or different ones with the same molecular weight. Recently, five anticoagulants that prolong the clotting time of the aPTT assay were extracted from salivary glands of *H. dromedarii* engorged females [37]. Among these proteins, P5 was found to be a potent competitive thrombin inhibitor with a molecular weight of 36 kDa. Hyalomin-1, identified from the salivary gland transcriptome of the related species, *H. marginatum rufipes* [38], is a selective inhibitor of thrombin, and competitively binds its active site, as well as the thrombin's fibrinogen-binding site, exosit-I [39]. It also blocks thrombin-mediated activation of FXI and FV and the aggregation of platelets [39].

Besides the role of thrombin in promoting hypercoagulability in cancer patients [40], circulating prothrombin and several downstream thrombin targets (*i.e.*, platelets, fibrinogen, FXIII) have been shown to strongly promote the metastatic potential of tumor cells [41] [42]. Colorectal cancer represents an important exception in that thrombin-mediated proteolysis drives both primary tumor growth and the formation of metastases [43]. Vossen *et al.*, [44] found that the susceptibility of homozygous carriers of the prothrombotic FV Leiden mutation to develop colon cancer is almost six times higher than non-carriers, signifying the importance of thrombin generation in colon tumorigenesis. Tur-

pin *et al.* [45] found that a 50% reduction in circulating prothrombin significantly, and consistently, decreased the formation of colitis-associated colonic adenomas, and reduced prothrombin expression is associated with lower mitotic indices in colon cancer models [43]. These studies demonstrate the important role of prothrombin in colonic adenoma formation. Therefore, the present study evaluated the anti-tumoral effects of the anti-thrombin fractions (3.A4 and 3.A5) against colorectal adenocarcinoma (Caco-2) cells.

The proliferation of Caco-2 cells was inhibited in a dose-dependent manner, with up to 54.29% and 62.43% reductions in cell viability recorded after incubation with 3.A4 and 3.A5 fractions, respectively. In addition, a disturbance in the cell cycle profile was recorded, with an obvious indication of cell cycle arrest at the G2/M phase in 3.A5-treated cells, and induced apoptosis in both 3.A4- and 3.A5-treated cells. No cytotoxic activity was observed in the normal cells. These findings are encouraging since an agent with the potential to target cell cycle checkpoints and induce apoptosis is a pursued aim in anticancer drug discovery [46]. Previous studies concerning tick-derived anticoagulants have demonstrated their cytotoxic and pro-apoptotic effects against different human cancer cell lines, including renal cell carcinoma, pancreatic cell carcinoma, and melanoma [47] [48], though these studies targeted other clotting factors, *i.e.*, FXa.

Tumor angiogenesis is a key step in the growth, metastatic spread and regrowth of colorectal cancer [49]. Among growth factors that have been identified as regulating angiogenesis in colorectal cancer, VEGF is the most important [50]. Its expression has been observed in primary and metastatic colon cancers, as well as in human colorectal cancer cell lines [51] [52]. Moreover, VEGF expression is the most significant prognostic indicator in advanced colorectal carcinoma [53]. Our study therefore examined the effect of both fractions on the level of VEGF in Caco-2 cell cultures: the inhibition of Caco-2 cell growth by both fractions was accompanied by a significant decrease in the VEGF level, suggesting the anti-angiogenic potential of the identified thrombin inhibitor(s).

Thrombin is a multifunctional molecule, with multiple identified substrates [54]. It can influence the pathogenesis of colon cancer through various mechanisms. Adams *et al.* [43] established that thrombin downstream targets (PAR-1 and fibrinogen) contribute to the pathogenesis of colon adenocarcinoma by influencing tumor growth, local invasion, and metastasis. Previous in vitro studies have also suggested that activation of tumor cell-associated PAR-1 promotes colon cancer proliferation and invasion [55]. Furthermore, a study by Chang *et al.* [56] showed, for the first time, that thrombin, acting through PAR-1, activated the hypoxia-inducible factor (HIF)-1α signaling pathway. This, in turn, initiated Twist and N-cadherin expression, and finally induced cell motility in colon cancer HCT116 cells. Sangole and Majumdar [57] found that treatment with dabigatran etexilate, an oral direct thrombin inhibitor, significantly reduced levels of VEGF and ERK/MAPK, and significantly reduced N-cadherin, Twist and mTOR expression in a preclinical model of colon carcinogenesis. Taking these studies into account, the effects demonstrated by the two fractions extracted in this

study, may result from decreased activation of PAR-1 and the subsequent signal transduction cascades in Caco-2 cells. The exact mechanism remains to be assessed.

In conclusion, this study demonstrates that thrombin, and consequently thrombin-mediated functions, are the main target for coagulation inhibitor(s) present in the salivary glands of partially-fed *H. dromedarii* females. Moreover, tick-derived thrombin inhibitors have the potential to be effective therapeutic tools in the treatment of colorectal cancer. This potentially results from their ability to inhibit proliferation and angiogenesis, as well as inducing apoptosis, thus interfering with the main components that contribute to tumor growth and metastasis. This study opens perspectives for characterization of naturally-derived effective molecules that could be used in developing treatments for colorectal cancer. Full characterization of the identified effector(s) appears to be a worthwhile step for determining structure and other mechanisms of action as well as exploring potential as anti-tick vaccine candidate(s).

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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