Research Article

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Screening of cytotoxic, anti-angiogenic, anti-tumorogenic and antimicrobial activities of Anatolian *Vipera ammodytes* (Nose-horned viper) venom

Anadolu'da yayılış gösteren *Vipera ammodytes* (Burunlu Engerek) zehrinin sitotoksik, anti-anjiyogenik, anti-tümör ve antimikrobiyal aktivitelerinin taranması

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Abstract

Objective: In the present study, we aimed to screen the cytotoxic, antimicrobial, anti-angiogenic and anti-tumorogenic activities of Anatolian *Vipera ammodytes* (Nose-horned Viper) crude venom.
Material and methods: The cytotoxicity was screened against PC3, HeLa, CaCo-2, U-87MG, MCF-7 and Vero cells by using MTT assay. The antimicrobial activity on *Escherichia coli* ATCC 25922, *E. coli* 0157:H7, *Enterococcus faecalis* 29212, *Enterococcus faecium* DSM 13590, *Staphylococcus aureus* ATCC 25923, *Staphylococcus epidermidis* ATCC 12228, *Salmonella typhimirium* CCM 5445, *Proteus vulgaris* ATCC 6957, *Bacillus cereus* ATCC 7064 and *Candida albicans* ATCC 10239 was assayed by determining the minimum inhibitory concentration using the broth dilution method. Anti-angiogenic and anti-tumorogenic activity was assessed by using chick chorioallantoic membrane (CAM) assay.
Results: The IC_{50} value of *V. ammodytes* venom on cultured cells varied from 1.8 to 7.0 µg/mL after 48 h treatment. Venom showed antimicrobial activity on *P. vulgaris*, *S. aureus*, *S. epidermidis*, *E. faecium* and *C. albicans* (the highest activity). The venom exhibited dose-dependent anti-angiogenic activity on CAM model at 2 and 10 µg/mL doses with scores of 1.1 and 2.0, respectively.
Conclusion: The results of the present study contributed to the knowledge of the biological activities of Anatolian *V. ammodytes* venom and showed its potential for further bioactivity guided characterization studies.

Keywords: Anti-angiogenic effect; antimicrobial activity; cytotoxicity; snake venom; *Vipera ammodytes*.

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Özet

Amaç: Bu çalışmada, *Vipera ammodytes* (Burunlu Engerek) ham zehrinin sitotoksik, antimikrobiyal, anti-anjiyojenik ve anti-tümör aktivitelerinin taranmasını amaçlanmıştır.
Metod: PC3, HeLa, CaCo-2, U-87MG ve MCF-7 kanser hücre hatları ve bir normal hücre hattı (Vero) kullanılarak MTT testi ile sitotoksite taraması yapılmıştır. Antimikrobiyal aktivite broth seyreltme metodu kullanılarak hesaplanan

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minimum inhibitör konsantrasyon ile değerlendirilmiştir. *Escherichia coli* ATCC 25922, *E. coli* 0157:H7, *Enterococcus faecalis* 29212, *Enterococcus faecium* DSM 13590, *Staphylococcus aureus* ATCC 25923, *Staphylococcus epidermidis* ATCC 12228, *Salmonella typhimirium* CCM 5445, *Proteus vulgaris* ATCC 6957, *Bacillus cereus* ATCC 7064 ve *Candida albicans* ATCC 10239 türleri kullanılmıştır. Anti-anjiyogenik anti-tümör aktivite civciv koryoallantoik membran (CAM) modeli ile değerlendirilmiştir.

Bulgular: *V. ammodytes* zehrinin 48 saat sonunda hücreler üzerindeki IC_{50} değeri, 1.8 ve 7.0 µg/mL arasında değişmiştir. Zehir *P. vulgaris*, *S. aureus*, *S. epidermidis* ve *C. albicans* (en yüksek etki) üzerinde antimikrobiyal etki göstermiştir. CAM deney modelinde 2 ve 10 µg/mL zehir dozlarında (sırasıyla 1.1 ve 2.0 skorları ile) anjiyogenezde doza bağlı baskılanma görülmüştür.

Sonuç: Bu çalışmanın sonuçları Anadolu'da bulunan *V. ammodytes* zehrinin sahip olduğu biyolojik aktiviteleri ortaya koymuş ve ileride yapılacak biyoaktivite rehberli karakterizasyon çalışmaları için sahip olduğu potansiyeli göstermiştir.

Anahtar Kelimeler: Anti-anjiyogenik etki; antimikrobiyal aktivite; sitotoksisite; yılan zehri; *Vipera ammodytes*.

Introduction

Snake venom is a complex mixture mainly consisting of proteins and peptides. Since their historical use in folk medicine for different pathophysiological cases, many bioactive proteins and peptides have been purified and identified from snake venoms, some of which have potential therapeutic value [1–3]. Major protein families found in snake venoms can be grouped as metalloproteinases, serine proteinases, L-amino acid oxidases (LAAO), hyaluronidases, phosphodiesterases, 5′-nucleotidases, arginine ester hydrolases, acetylcholinesterases and phospholipases A_2 (PLA_2) as enzymes; C-type lectins (CLP), disintegrins, cysteine-rich secretory proteins (CRISP), myotoxins, neurotoxins, nerve growth factors (NGF), vascular endothelial growth factors (VEGF), kunitz-type proteinase inhibitors, natriuretic and bradykinin potentiating peptides (BPP) as non-enzymatic proteins/peptides. Venoms from different venomous snake families vary in the composition and abundance of these proteins and also show variation at specific level [4].

Cancer is one of the leading causes of human death worldwide and its treatment is still an important problem. Natural bioactive molecule sources such as plants and animals are being extensively explored for new anti-cancer agents with lower side effects. Snake venom is one of the important natural resource for biologically active proteins and peptides expressing anticancer activities [1–3].

Bacterial and fungal infections are also a worldwide problem threatening human health and antibiotic resistance resulted in the urgent need for new antibiotics. Recently, snake venom studies also focused on their antimicrobial activity in order to assess their ability to inhibit the growth of various microorganisms, which may lead to discover novel antibiotic agents [5].

The Nose-Horned Viper, *Vipera ammodytes* (Linnaeus, 1758) has a distribution from southern Europe, through the Balkans to Turkey and Georgia with different subspecies [6, 7]. Northern/central Anatolian and Georgian populations were separated as a full species (*V. transcaucasiana*) by some of the authors, but recent molecular phylogenetic studies suggest that this taxon still should be considered as a subspecies of *V. ammodytes* [7].

The first studies related to snake venoms in Turkey intended to make taxonomical comparisons [8], and studies aiming to make biological and proteomic characterization of various Turkish viper venoms have been done recently [9–13]. General bioactivity screening is the initial step in natural product research, in the way of discovering novel bioactive molecules. Snake venoms are considered as one of the most valuable natural sources for the peptide based drug discovery studies. In the present study, we aimed to screen anticancer, anti-angiogenic and antimicrobial effects of Anatolian *V. ammodytes* crude venom in order to assess its potential as a source of novel bioactive peptides and proteins.

Materials and methods

Snake venoms

All tests were performed with pooled venom extracted from two adult *V. ammodytes* individuals that were collected in Istanbul and Kocaeli provinces in Turkey. After extraction, venom sample was centrifuged 10 min at $2000 \times g$ at 4°C and lyophilized by freeze-drying. The lyophilized venom sample was diluted (1 mg/mL) in physiological saline, centrifuged for 5 min at $600 \times g$ and then filtered through a 0.22 µm cellulose acetate syringe filter before using for tests. Venom was extracted following the appropriate ethical procedures for venom sampling, without applying pressure to venom glands [14].

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Determination of protein concentration

Protein concentration of the diluted venom sample was assayed in triplicate by Bradford method reading at 595 nm using UV-visible spectrophotometer (VersaMax, Molecular Devices, CA, USA). Bovine serum albumin was used as a standard [15].

Cell culture, in vitro cytotoxicity assay and calculation of the half-maximal inhibitory concentration (IC_{50})

Human prostate carcinoma (PC3), human cervix adenocarcinoma (HeLa), human colorectal adenocarcinoma (CaCo-2), human glioblastoma–astrocytoma (U-87 MG) and human breast adenocarcinoma (MCF-7) cells were used as cancer cell lines. A kidney epithelial cell from an African green monkey (Vero) was used as a non-cancerous cell line. The PC3 cell line was a gift from Dr. K. Korkmaz (Ege University, Bioengineering Department, Izmir/Turkey). Other cell lines were purchased from ATCC. Cells were maintained in Dulbecco's modified Eagle's medium F12 (DMEM F12), supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 100 U/mL of penicillin and 100 µg/mL of streptomycin (Biochrom AG, Berlin, Germany). The cells were incubated at 37°C in a humidified atmosphere of 5% CO_2.

Cytotoxicity of crude venom was determined by using a modified 3-(4, 5- Dimethyl-2-thiazolyl) -2, 5-diphenyl-2H-tetrazolium bromide) (MTT) assay (the assay was miniaturized in terms of MTT concentration and incubation time) [16]. Dimethyl sulfoxide was added to the wells to dissolve formazan crystals. The optical density (OD) was measured at 570 nm (with a reference at 690 nm) by UV-visible spectrophotometer (VersaMax, Molecular Devices, CA, USA) in triplicates. All cell lines were cultivated for 24 h in 96-well cell culture plates with an initial concentration of 1×10^5 cells/mL. Then, the cultured cells were treated with different doses of venom (0–77 µg/mL) and parthenolide (as a positive control, 0.25–25 µg/mL) and incubated for additional 48 h at 37°C. The % viability was determined as formulated below:

% Viable cells = [(The absorbance of the treated cells) – (the absorbance of the blank)]/[(The absorbance of the control) – (the absorbance of the blank)] × 100

Inhibition of growth 50% (IC_{50}), which is the concentration of crude venom causing 50% inhibition of cell growth compared to untreated controls, calculated by using OD values of controls and venom doses as described previously [9, 12]. Control values were set at 0% cytotoxicity. IC_{50} was calculated by fitting the data to a sigmoidal curve and using a four parameter logistic model using GraphPad Prism 5 software (San Diego, USA) and presented as an average of three independent measurements.

Chick chorioallantoic membrane (CAM) assay for anti-angiogenesis

Zero-day-old chick embryos were purchased from Has Tavuk (Bursa, Turkey) and incubated at 37°C with 55% relative humidity. The fertilized chick eggs were incubated at 37°C for 7 days (post-fertilization). Chick chorioallantoic membrane (CAM) assay was performed as described previously [17]. Briefly, a hypodermic needle was used to open a small hole in the blunt end of the egg shell and a second hole is made on the broad side of the egg over an avascular portion of the embryonic membrane. With a mild suction applied to the first hole, the air sac displaced so that the CAM dropped away from the shell. Using a Dremel drill (Racine, WI), a 1.0 cm^2 window was cut in the shell over the false air sac, allowing access to the CAM. No. 1 Whatman sterile filter paper disks pretreated with 3 mg/mL cortisone acetate and 1 mM propylthiouracil were air dried under sterile conditions. Filter disks were soaked in PBS alone (control), 3 µg/mL of fibroblast growth factor 2 (FGF2) (Millipore, CA, USA) or 2 or 10 µg/mL of crude venom in the presence of FGF2 and placed on the prepared CAMs. After incubation for 3 days, each CAM beneath the filter disk was separated from the disk and rinsed with PBS. Each membrane was placed in a 35 mm Petri dish and examined under the stereomicroscope (Carl Zeiss, Stemi SV6) at 50x magnification.

Images from 8 to 10 CAM preparations were analyzed for each treatment condition. The anti-angiogenic activity was evaluated by using a score system according to Bürgermeister et al. [18]. Each CAM was scored from 0 (no effect/difference) to 2 (strong effect, the area lacking capillary vessels was at least double the size of the pellet). 0.5 refers to very weak effect (capillary density was decreased but still no capillary free area was observed around the pellet) and 1 refers to weak-medium effect (certain areas with reduced density of capillaries was observed but not larger than double the pellet size). FGF2 treated membranes were considered as controls (score 0) and other CAM preparations with venom treatment were scored by comparing with the control samples. After scoring, the following equation [19] was used for the determination of the average mean score for each venom concentration:

Average score = [Number of eggs (Score 2) × 2 + Number of eggs (Score 1) × 1]/[Total number of eggs (Score 0, 0.5, 1, 2)].

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The final average score was presented as follows: Average score < 0.50 = no anti-angiogenic effect, 0.50–0.75 = weak, 0.75–1.00 = good, and score > 1.00 = very good/strong anti-angiogenic effect [19]. The resulting scores were presented as the mean ± standard error of the mean (SEM).

Tumor growth in the CAM cancer implant model

The effect of *V. ammodytes* venom on tumor angiogenesis and tumor growth was evaluated using CAM Matrigel® cancer implant model as described previously [20]. Seven days-old fertilized chick eggs were prepared as described above. The Matrigel® was thawed overnight at 4°C and placed on ice. PC3 and human pancreatic cancer (MPanc-96) cells in exponential growth phase were harvested by trypsinization, washed, and suspended in the medium. Approximately 1×10^6 cells in 20 µL of medium mixed with the same volume (20 µL) of Matrigel® and 10 µL (7 µg) of venom were planted on the CAM. Effect of the venom was determined on day 8 after tumor cell implantation. Results are presented as mean tumor weight (mg) per treatment group ± SEM, n = 8 eggs per group. Significance was tested using the Student t-test.

Hemoglobin content of the tumor mass was measured to evaluate the vascularity of the tumor. For this purpose, each tumor mass was placed into a 0.5 mL tube filled with ultra pure water and homogenized for 5–10 min. The samples were then centrifuged at 2500 × *g* for 10 min, and the supernatants were collected for hemoglobin measurement. The supernatant was mixed with Drabkin's reagent (1 : 1 ratio) and incubated at room temperature for 15–30 min; 100 µL of this mixture was then placed in a 96-well plate, and absorbance was measured at 540 nm with a Microplate reader (BioRad Laboratories, CA, USA). Hemoglobin concentration was determined by comparison with a standard curve in g/dL [21].

Microorganisms and antimicrobial assay

Gram-positive and gram-negative bacteria and yeast were used for antimicrobial activity studies. The Gram-negative bacteria used in this study were *Escherichia coli* ATCC 25922, *E. coli* 0157:H7, *Proteus vulgaris* ATCC 6957 and *Salmonella thyphimirium* CCM 5445. The Gram-positive bacteria used were *Bacillus cereus* ATCC 7064, *Enterococcus faecalis* ATCC 29212, *Enterococcus faecium* DSM 13590, *Staphylococcus aureus* ATCC 25923, and *Staphylococcus epidermidis* ATCC 12228. *Candida albicans* ATCC 10239 was used as a yeast species. The lyophilized bacteria and yeast were obtained from Ege University, Faculty of Science, Department of Basic and Industrial Microbiology.

The antimicrobial activity of the venom was determined by broth micro-dilution technique. Microorganisms were grown in MH broth for 5 h (exponential phase) and adjusted to 0.5 McFarland turbidity standard ($A_{600} = 1.0$), corresponding to 1.5×10^6 colony forming unit (CFU)/mL. Minimum inhibitory concentration (MIC) values were determined according to the Clinical and Laboratory Standards Institute (CLSI) guide [22]. Serial dilutions of venom (0.9–500 µg/mL) were prepared in 96-well microtiter plates, at a final volume of 80 µL. Then, 20 µL of the adjusted microbial inocula (1.5×10^5 CFU/mL) were added to each well and incubated at 37°C for 24 h. Inhibition of microbial growth was determined by visual observation. MIC was defined as the lowest concentration of the venom required to inhibit microbial growth after 24 h. Each dilution series included control wells. Ampicillin (0.25–128 µg/mL) and flucytosine (0.25–128 µg/mL) were used as standard antimicrobial agents for bacteria and yeast, respectively as a positive control. All assays were run in 3 replicates.

Results

In vitro cytotoxic effect of *V. ammodytes* venom

Protein content of *V. ammodytes* venom (1 mg/mL in saline) was calculated as 771 µg/mL. According to MTT assay results in our experimental conditions, venom of *V. ammodytes* showed cytotoxic activity against CaCo-2, MCF-7, U-87 MG and PC3 cancer cell lines in a dose-dependent manner at different levels, with a most potent activity against CaCo-2 (Supplementary Figure 1, Table 1). Venom did not show a significant cytotoxic effect on HeLa and Vero cells at the chosen doses. The calculated IC_{50} values of *V. ammodytes* venom for all cell lines after 48 h following venom treatment varied from 1.8 to 7.0 µg/mL (Table 1). Microphotographs (Supplementary Figure 1) showed parallel results in comparison with the results of MTT assay. Although we used six cell lines derived from different origins, we observed rounded, detached or aggregated cells in all cell lines -except HeLa and Vero- after venom treatment, as a general effect of venom on cell morphologies (Supplementary Figure 1).

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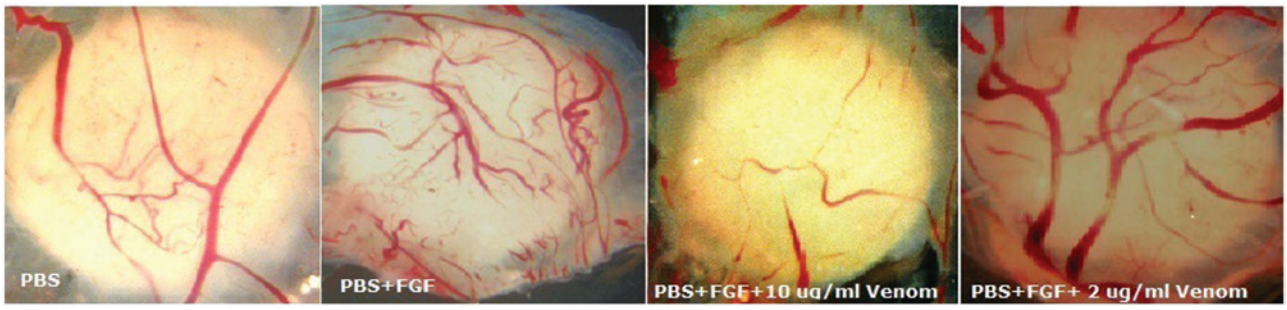


Figure 1: Anti-angiogenic activity of *V. ammodytes* venom monitored by chicken CAM assay. 2 and 10 μg/mL were applied in the presence of FGF2. PBS+FGF is considered as normal embryo with the score of 0 and treatment conditions were scored by comparing with the normal samples. The scores were calculated as 1.1 and 2.0 for 2 and 10 μg/mL venom treatments, respectively. The image shows the significant dose-dependent anti-angiogenic effect of *V. ammodytes* venom.

Table 1: IC_{50} values of *V. ammodytes* crude venom and parthenolide (positive control) for different cell lines following 48 h treatment.

Cell lines	IC_{50} of venom, μg/mL	IC_{50} of parthenolide, μg/mL
CaCo-2	1.8 ± 0.1	1.10 ± 0.07
MCF-7	4.1 ± 0.0	1.10 ± 0.08
U-87 MG	4.9 ± 0.1	4.19 ± 0.05
PC3	7.0 ± 0.1	1.50 ± 0.05
HeLa	–	0.99 ± 0.03
Vero	–	1.02 ± 0.08

–, Not detected.

Anti-angiogenic effect of *V. ammodytes* venom

We evaluated venom-induced anti-angiogenic response by CAM assay, in the presence of FGF2. Filter disk pretreated with FGF2 induced blood vessel branch formation compared to PBS treated membranes (Figure 1). We calculated the scores for each venom concentration as described in Materials and Methods section. The scores were calculated as 1.10 ± 0.05 and 2.00 ± 0.07 for 2 and 10 μg/mL venom treatment, respectively which indicates good and strong anti-angiogenic effect (Figure 1). Our results clearly showed that *V. ammodytes* venom inhibited the proangiogenic response of FGF2 in a dose-dependent manner.

V. ammodytes venom induced tumor growth and neoangiogenesis inhibition

We also evaluated tumor growth inhibition and tumor-related neoangiogenesis using the CAM cancer implant assay with PC3 and MPanc-96 cells. The tumor growth inhibition in the CAM cancer implant model was found remarkable compared to controls in terms of tumor weight (mg) ($p < 0.01$) (Figure 2). Similarly, hemoglobin levels decreased significantly in tumor masses of both cell lines ($p < 0.01$) (Figure 3), indicating the inhibition of the tumor mediated neoangiogenesis.

Antimicrobial effect of *V. ammodytes* venom

MIC values of *V. ammodytes* venom against selected microorganisms are summarized in Table 2. In our study, the values of MIC for *S. epidermidis*, *E. faecium* and *P. vulgaris* (MIC = 250 μg/mL) were higher than the MIC for *S. aureus* (MIC = 125 μg/mL). The results showed that the venom acts as a more powerful antifungal against *C. albicans* (3.9 μg/mL) in comparison to the reference agent Flucytosine.

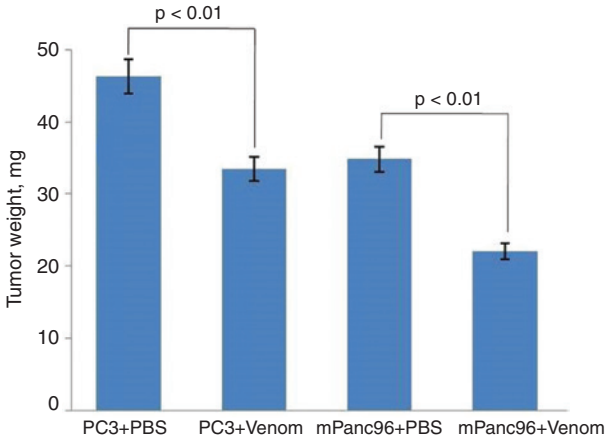


Figure 2: Demonstration of tumor growth in the CAM cancer implant model by using Matrigel® with PC3 and mPanc-96 cells. 7 μg venom or PBS (control) was applied. Results are presented as mean tumor weight (mg) per treatment group ± SEM, n = 8 eggs per group. Decreases in tumor weights of the venom treated groups, compared to controls are statistically significant.

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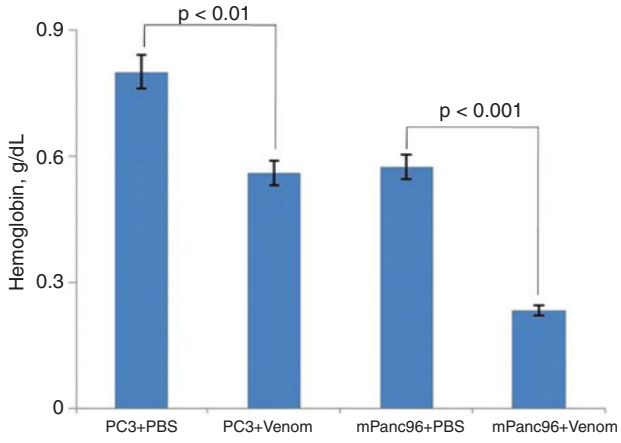


Figure 3: Demonstration of hemoglobin levels (g/dL) of Matrigel® plugs with PC3 and mPanc-96 cells, expressing neovessel formation. 7 µg venom or PBS (control) was applied. Decreases in hemoglobin levels of the venom treated groups, compared to controls are statistically significant and indicates the inhibition of the angiogenesis.

Table 2: MIC values of *V. ammodytes* crude venom for selected microorganisms.

Microorganisms	Venom, µg/mL	Ampicillin, µg/mL	Flucytosine, µg/mL
E. coli ATCC 25922	–	1.9	–
E. coli O157 : H7	–	3.9	–
P. vulgaris ATCC 6957	250	3.9	–
S. typhimirium CCM 5445	–	3.9	–
S. aureus ATCC 6538P	125	3.9	–
B. cereus ATCC 7064	–	7.8	–
E. faecalis ATCC 29212	–	7.8	–
E. faecium DSM 13590	250	3.9	–
S. epidermidis ATCC 12228	250	1.9	–
C. albicans ATCC 10239	3.9	–	7.8

–, Not detected.

Discussion

Few published reports have shown selected biological activities of crude venoms or purified proteins of different subspecies of *V. ammodytes* [23–26]. The present study is the first report on bioactivity screening of crude Anatolian *V. ammodytes* venom, including anticancer, anti-angiogenic and antimicrobial activities.

Georgieva et al. [27] identified LAAO, PLA_2, metalloproteinase, serine proteinase, CRISP, NGF, VEGF and disintegrin protein families in the venom of *V. ammodytes ammodytes* and *V. ammodytes meridionalis* distributed in Bulgaria by mass spectrometry-based bottom-up proteomics approach. We may expect a similar composition in our venom sample and one or more of these proteins may be responsible for the biological activities we report in the present study which are discussed below.

The cytotoxic effect of viper venoms on various cancer and non-cancerous cell lines have been reported previously [9, 12, 28, 29], some of which includes the cell lines used in the present study. Yalcin et al. [9] reported a cytotoxic activity of *Montivipera xanthina* viper venom on MCF-7 cells ($IC_{50} = 4.1$), while they observed no significant effect against Vero cells. In a similar study, the cytotoxic effect of Anatolian *Macrovipera lebetina obtusa* venom against U-87 MG, MCF-7, CaCo-2, A549, HeLa and Vero cells was investigated and Vero cell line was found to be the most sensitive with an IC_{50} value of 1.18 [12]. In the present study, Vero and HeLa cells resisted to maximum *V. ammodytes* venom dose and did not reach IC_{50}, but higher doses may result in the cytotoxicity. These data suggest that snake venoms show species- and cell-specific selectivity regarding to their cytotoxic activity. Species-specific activity is a result of qualitative and quantitative inter-specific variations in the composition of the venom proteins. Moreover, it is known that venom proteins have the ability of inhibiting cell proliferation and promoting cell death interfering with the wide range of metabolic activities [1]. Cell type-specific activity of snake venoms may be related to the variation in the molecular pathways that control cell proliferation and metabolism in different cancer types. The selectivity of snake venoms between normal and cancer cells as well as different cancer cell types is important for targeted cancer therapy and can provide new perspectives.

Various mechanisms have been involved for in vitro cytotoxic actions of snake venoms such as necrosis, apoptosis induction, up/down regulation of cell cycle proteins and deterioration of cell membrane integrity. Apoptosis-inducing activities of crude venoms or purified proteins such as metalloproteinase, disintegrin, PLA_2 and LAAO from different viper species have been reported on various cancer and non-cancerous cells [1, 30, 31]. Induction of apoptosis may be one of the events underlying the cytotoxic effect of *V. ammodytes* venom observed in the present study. Since many of the chemotherapeutic agents used in cancer treatment have been shown to induce apoptosis in cancer cells [32], detailed studies using functional assays must be carried out with a special emphasis to apoptosis in order to elucidate the mechanisms of action of *V. ammodytes* venom proteins in cells.

In the present study, our results of CAM assay clearly showed that the venom of *V. ammodytes* blocked neovascularization in a dose-dependent manner. The effect of *V. ammodytes* venom in the CAM tumor implant model

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suggests that venom acted primarily to limit vessel growth stimulated by the tumor cells resulting in reduced expansion of the tumor mass. Anti-angiogenic effects of various proteins purified from different viper venoms such as CLPs, disintegrins and PLA_2s have been published in the literature [33–37]. It was also reported that solid tumor sizes and angiogenesis had been reduced in in vivo cancer models with treatment of lower doses of proteins from viper venoms [1, 35, 38, 39]. Integrins play important roles in cancer progression and its treatment as well, by their activities on tumor cell proliferation, angiogenesis, invasion, and metastasis. Previous studies have shown that several snake venom proteins such as disintegrins, metalloproteinases, PLA_2s and CLPs specifically antagonize the integrin receptors and prevent tumor progression [1]. The inhibition of angiogenesis is considered as one of the important targets for the treatment of cancer [40] and snake venom proteins represent a library of molecules with different structures, potencies, and specificities as prototype candidates for developing anti-angiogenic therapeutics. To the best of our knowledge, we report anti-angiogenic activity of *V. ammodytes* crude venom in standard CAM and CAM cancer implant model for the first time in the literature and showed its potential as a source for anti-angiogenic molecules. One or more of the proteins mentioned above may be responsible for the anti-angiogenic and anti-tumorogenic activity of *V. ammodytes* venom. More detailed studies are needed with purified proteins to elucidate the mechanisms underlying its anti-angiogenic activity and its relation to reduced tumor size.

According to the results of the present study, *V. ammodytes* venom shows species-specific antimicrobial activity. The antimicrobial activity of crude extracts is generally considered to be significant if the MIC value is 100 μg/mL or lower, moderate if $100 < MIC \leq 625$ μg/mL and weak if the MIC value is higher than 625 μg/mL [41]. The potency of an antibiotic product is described as the concentration that causes similar growth inhibition of the test microorganism compared to the dose of the reference antimicrobials. Based on these criteria, *V. ammodytes* venom showed moderate activity against *S. aureus*, *S. epidermidis*, *E. faecium* and *P. vulgaris*, while it was more potent on *C. albicans* with a strong significant activity (Table 2). We also reported significant antifungal activities of other viper venoms of *Montivipera xanthina* [9] and *Macrovipera lebetina obtusa* [12] against *C. albicans* in our previous studies, which are pointing out a possible specific antifungal activity of viper venoms. These species-specific results may be due to the differences in the outer membrane of bacteria or composition and cell disruption mechanism of the venom [42]. Several snake venoms have been investigated for their antimicrobial activity and number of purified proteins were shown to inhibit the microbial growth, such as LAAO and PLA_2 [5, 9, 42, 43]. Studies with purified proteins will help to understand the specific antimicrobial activity of *V. ammodytes* venom which may present novel antibiotic candidates.

Snake venoms contain proteins and peptides with distinct pharmacological activities which have therapeutic potential against various diseases such as cancer, infections (bacterial or fungal), cardiovascular diseases or arthritis. Additionally, viper venoms contain proteins, especially interfering with coagulation cascade and platelet aggregation, which led to the design of approved drugs and diagnostic tests [1–3]. In conclusion, we present the broad bioactivity screening of Anatolian *V. ammodytes* crude venom as a combination of in vitro anticancer and antimicrobial and in vivo anti-angiogenic activities. Since snake venoms may show intra-species geographical variation, it is important to assess the activity of local populations. We have also ongoing studies aiming to fractionate and make a proteomic characterization of *V. ammodytes* venom in Turkey by a chromatography and mass spectrometry-based approach. As an initial screening study, our results on the crude venom of Anatolian *V. ammodytes* contribute to the knowledge of biological activities of snake venoms and have the potential to direct further investigations aiming to find novel bioactive molecules as anticancer and antibiotic molecule prototypes.

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Conflict of interest statement: The authors have no conflict of interest.

Ethical considerations: NI and BG have ethical permission from Ege University Animal Experiments Ethics Committee (2010–43) for venom collection from vipers. Also, they have permission from the Republic of Turkey Ministry of Forest and Water Affairs to collect vipers in the nature for venom sampling.

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