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Screening of cytotoxic and antimicrobial activity potential of Anatolian *Macrovipera lebetina obtusa* (Ophidia: Viperidae) crude venom

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The effects of snake venoms have been well known since ancient times. They contain a variety of biologically active proteins which have therapeutic potential. This study investigated the cytotoxic and antimicrobial activities of Anatolian *Macrovipera lebetina obtusa* venom against various cancer cells, Gram-negative and Gram-positive bacteria, and a fungal species. A549, HeLa, CaCo-2, U-87 MG and MCF-7 cancer cell lines and a normal cell line (Vero) were screened by the 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay. The antimicrobial activity was evaluated by determining the minimum inhibitory concentration (MIC) using the broth dilution method. The species included were *Escherichia coli* ATCC 25922, *E. coli* 0157:H7, *Enterococcus faecalis*, *Enterococcus faecium* DSM 13590, *Staphylococcus aureus* ATCC 25923, *Staphylococcus epidermidis* ATCC 12228, *Salmonella typhimurium* CCM 5445, *Proteus vulgaris* ATCC 6957, *Bacillus cereus* ATCC 7064 and *Candida albicans* ATCC 10239. Half-maximal inhibitory concentration (IC_{50}) values of *M. l. obtusa* venom on cultured cells varied from 1.18 ± 0.11 to 12.80 ± 0.22 µg/ml, with the most potent activities against Vero, U-87 MG, MCF-7 and CaCo-2 cells. Venom showed moderate antifungal activity against *C. albicans*, with an MIC of 62.50 µg/ml. In short, the venom of Anatolian *M. l. obtusa* showed promising results as a potential source of alternative therapeutics, cytotoxic and antifungal agent prototypes.

Keywords: snake venom; *Macrovipera lebetina obtusa*; biological activity; cytotoxicity; antimicrobial

Introduction

Snake venom is a mixture that is secreted from specialized venom glands and consists mainly of proteins and peptides. Proteins identified from snake venoms can be grouped into major protein families such as enzymes, e.g. metalloproteinase, serine proteinase, phospholipase A_2 (PLA_2), L-amino acid oxidase (LAAO), hyaluronidase, 5′-nucleotidase, phosphodiesterase, arginine ester hydrolase and acetylcholinesterase; and as non-enzymatic proteins, e.g. disintegrin, C-type lectin (CLP), cysteine-rich secretory protein (CRISP), natriuretic peptides, bradykinin potentiating peptides (BPPs), myotoxins, neurotoxins, vascular endothelial growth factor (VEGF), nerve growth factor (NGF) and kunitz-type proteinase inhibitors (Edstrom 1992; Tu 1996; Chippaux 2006; Mackessy 2010).

In the context of searching natural products to find bioactive molecules that have therapeutic potential, snake venom is a popular source for peptide/protein-based drug discovery. Many bioactive proteins and peptides with distinct pharmacological and biochemical properties have been purified and identified from snake venom, and a number of them (e.g. metalloproteinase, serine proteinase, disintegrin, PLA_2, LAAO, CLP, BPP) have been shown to have therapeutic value, including anticancer and antibiotic potential (Lewis & Garcia 2003; Koh et al. 2006; Fox & Serrano 2007; Gomes et al. 2010; Samy et al. 2013; Vyas et al. 2013; Calderon et al. 2014).

Blunt-nosed viper, *Macrovipera lebetina* (Linnaeus, 1758) (Viperidae), has a distribution from northern Africa to Pakistan and from the Gulf of Oman to the Caspian Sea and Dagestan (Russia), with different subspecies. The subspecies *Macrovipera lebetina obtusa* (Dwigubsky, 1832) occurs in Turkey, with a distribution from Anamur to south-eastern, southern and north-eastern Anatolia (Mallow et al. 2003; Budak & Göçmen 2008).

Previous studies on snake venom in Turkey aimed to make taxonomical comparisons (Arikan et al. 2005, 2008). Recent studies on the biological and proteomic characterization of various viper venoms in Turkey have been carried out (Igci & Demiralp 2012; Nalbantsoy et al. 2012, 2013; Topyıldız & Hayretdağ 2012; Yalcin et al. 2014). As a contribution to the authors' ongoing studies on Turkish venomous snakes, the aim of this study was to screen the cytotoxic and antimicrobial activities of Anatolian *M.*

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l. obtusa crude venom on various cancer cells and microorganisms, to assess its potential as a source of bioactive peptides/proteins which may have therapeutic value.

Material and methods

Snake venoms

All tests were performed using pooled venom extracted from three adult *M. l. obtusa* collected in the wild, in Osmaniye province (Turkey), during field trips in May 2012. A quantity of 100–150 µl venom was extracted from each individual. After extraction, venom samples were lyophilized by freeze-drying. The lyophilized venom samples were diluted (1 mg/ml) in physiological saline, centrifuged for 5 min at 600 × *g* and then filtered through a 0.22 µm cellulose acetate syringe filter before being used in the tests.

Venoms were extracted following the appropriate procedures for venom sampling. NI and BG have ethical permission from Ege University Animal Experiments Ethics Committee (2010-43) for venom collection from vipers. They also have permission from the Republic of Turkey Ministry of Forest and Water Affairs to collect vipers in the wild for venom research.

Determination of protein concentration

Protein concentration was assayed in triplicate by the Bradford method (Bradford 1976) at 595 nm using an ultraviolet (UV)-visible spectrophotometer (VersaMax; Molecular Devices, CA, USA) for diluted venom samples in saline. Bovine serum albumin was used as a standard.

Cell culture and in vitro cytotoxicity assay

Human lung adenocarcinoma (A549), human cervix adenocarcinoma (HeLa), human colorectal adenocarcinoma (CaCo-2), human glioblastoma–astrocytoma (U-87MG) and human breast adenocarcinoma (MCF-7) cells were used as cancer cell lines. Kidney epithelial cells from an African green monkey (Vero) were used as a non-cancerous cell line. Cell lines were purchased from Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ, Braunschweig, Germany). All the cells were maintained in Dulbecco's modified Eagle's medium, supplemented with 10% foetal bovine serum, 2 mM L-glutamine, 100 U/ml of penicillin and 100 µg/ml of streptomycin (Biochrom, Berlin, Germany). The cells were incubated at 37°C in a humidified atmosphere of 5% carbon dioxide.

The cytotoxicity of crude venom was determined by the general procedure based on cell viability using a modified 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay (Mosmann 1983), which measures colorimetrically the mitochondrial reductase activity of viable cells. The optical density (OD) was measured at 570 nm (reference: 690 nm) by a UV-visible spectrophotometer (VersaMax; Molecular Devices, CA, USA) in triplicate (as technical replicates in the same plate). All cell lines were cultivated for 24 h in 96-well microplates with an initial concentration of 2×10^5 cells/ml. Then, the cultured cells were treated with different concentrations of venom (0, 1.25, 2.5, 5, 10, 20 and 40 µg/ml) and incubated for 48 h at 37°C. Doses were selected based on previous studies on the cytotoxic effects of snake venom. The percentages of surviving cells in each culture after treatment with venom was determined.

Determination of IC_{50} and statistics

The half-maximal inhibitory concentration (IC_{50}), which is the concentration of venom causing 50% inhibition of cell growth compared to untreated controls, was calculated using OD values. Cytotoxicity was expressed as a decrease in the mean percentage of cell viability relative to the unexposed control ± standard deviation (SD). Control values were set at 0% cytotoxicity. The percentage viability was determined as formulated below:

$$\%\text{Viable cells} = \frac{\begin{array}{c}(\text{Absorbance of treated cells})\\ -(\text{Absorbance of blank})\end{array}}{\begin{array}{c}(\text{Absorbance of control})-\\ (\text{Absorbance of blank})\end{array}} \times 100$$

IC_{50} was calculated by fitting the data to a sigmoidal curve, using a four-parameter logistic model, and presented as an average of three independent measurements. The IC_{50} values were reported with 95% confidence intervals and calculations were performed using GraphPad Prism software (San Diego, CA, USA). The values of the blank wells were subtracted from each well of treated and control cells, and IC_{50} was calculated in comparison with untreated controls.

Microorganisms and antimicrobial assay

Gram-positive and Gram-negative bacteria and yeast were used for antimicrobial activity studies. Gram-negative bacteria used in the study were *Escherichia coli* ATCC 25922, *E. coli* 0157:H7, *Proteus vulgaris* ATCC 6957 and *Salmonella typhimurium* CCM 5445. 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 the yeast species. Lyophilized bacteria and yeast cultures were obtained from Ege University, Faculty of Science, Department of Basic and Industrial Microbiology (Izmir, Turkey).

The antimicrobial activity of the venom sample was assayed using a microdilution susceptibility test, and the minimum inhibitory concentration (MIC) was calculated. Microorganisms included in this study 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 units (CFU)/ml. MICs were determined according to the Clinical and Laboratory Standards Institute (CLSI) guide (CLSI 2009). Serial dilutions of *M. l. obtusa* venom (0.9–500 µg/ml) were prepared in 96-well microtitre trays, at a final volume of 80 µl. Then, 20 µl of the adjusted bacterial inocula (1.5 × 10^5 CFU/ml) was added to each well and incubated at 37°C for 24 h. Inhibition of bacterial growth was assessed by visual observation. The MIC was defined as the lowest concentration of venom required to inhibit microbial growth. Ampicillin and flucytosine were used as standard antimicrobial drugs for bacteria and yeast, respectively, as a positive control.

Results

The protein concentration of *M. l. obtusa* venom sample (1 mg/ml in saline) determined by the Bradford assay was 652 µg/ml. The effect of *M. l. obtusa* crude venom on cancer (A549, HeLa, CaCo-2, U-87 MG, MCF-7) and non-cancerous (Vero) cell proliferation was evaluated using the MTT assay after treatment with different concentrations of venom for 48 h. The estimated IC_{50} values of venom for all cell lines 48 h after venom treatment are given in Table 1 and varied between 1.18 ± 0.11 and 12.80 ± 0.22 µg/ml. Under the experimental conditions, the MTT assay results showed that *M. l. obtusa* venom inhibits cell proliferation, with the most potent activity against Vero, U-87 MG, MCF-7 and CaCo-2 cells with IC_{50} values of 1.18 ± 0.11, 1.90 ± 0.33, 3.85 ± 0.19 and 4.75 ± 0.21 µg/ml, respectively (Figure 1, Table 1). The observed inhibitory activity was dose dependent for A549, MCF-7, CaCo-2 and Vero

Table 1. Anatolian *Macrovipera lebetina obtusa* venom half-maximal inhibitory concentration (IC_{50}) values for cell lines following 48 h crude venom treatment (µg/ml).

Cell line	IC_{50} value
Vero	1.18 ± 0.11
U-87 MG	1.90 ± 0.33
MCF-7	3.85 ± 0.19
CaCo-2	4.75 ± 0.21
A549	9.70 ± 0.38
HeLa	12.80 ± 0.22

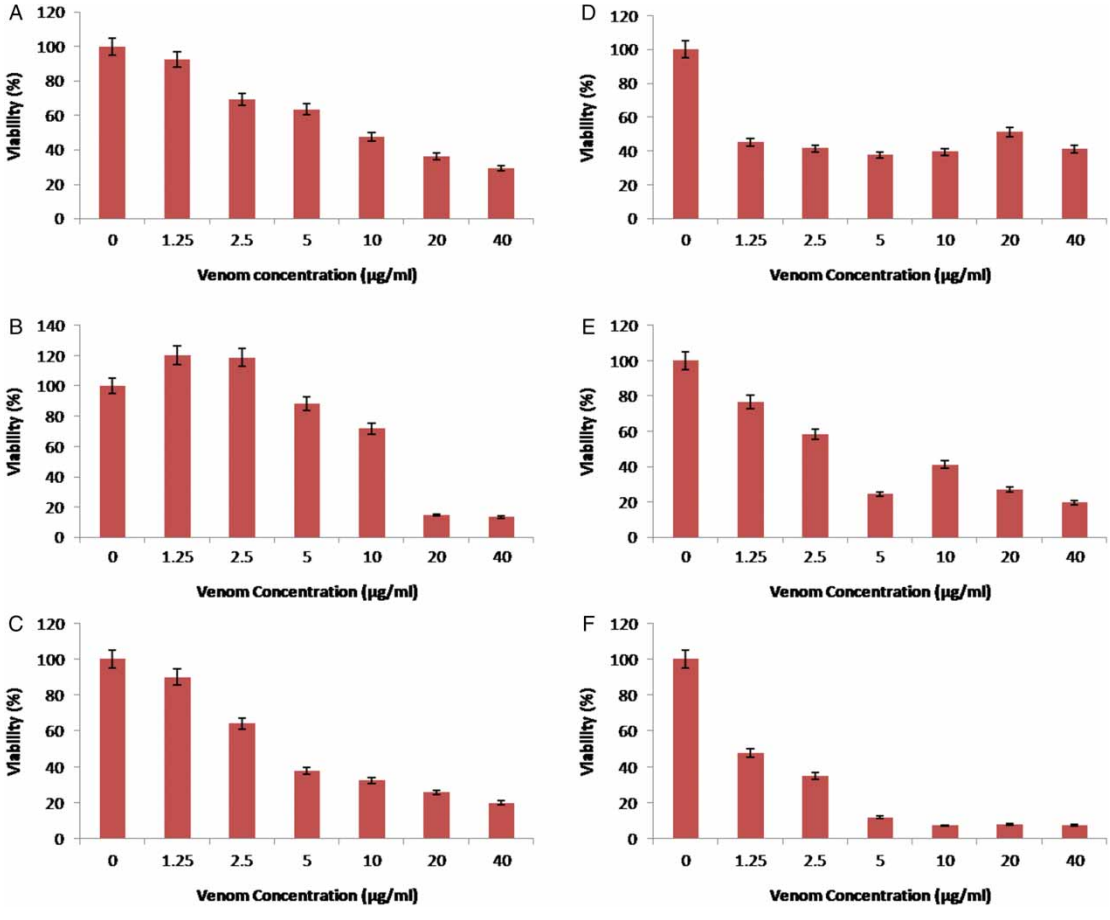

Figure 1. Effects of different concentrations of Anatolian *Macrovipera lebetina obtusa* crude venom on cell viability after 48 h exposure determined by the 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay: (A) A549, (B) HeLa, (C) CaCo-2, (D) U-87MG, (E) MCF-7, and (F) Vero.

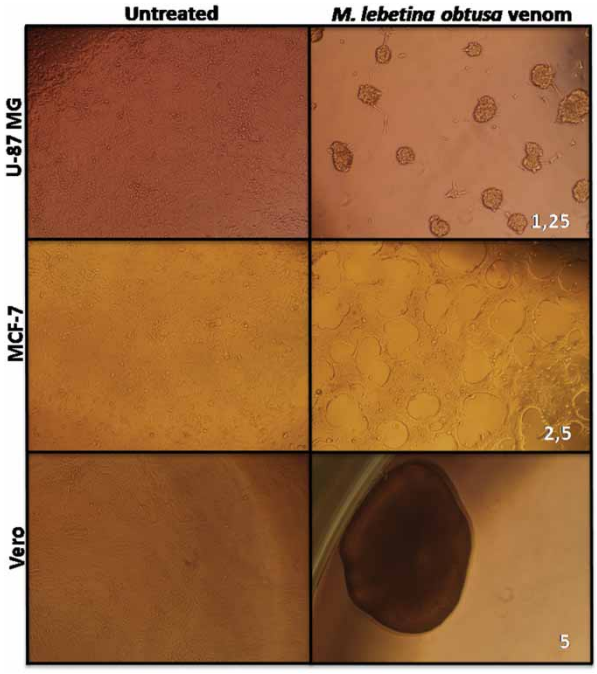

Figure 2. *Macrovipera lebetina obtusa* venom-induced morphological changes in U-87 MG, MCF-7 and Vero cells viewed by an inverted microscope. Cells were exposed to crude venom for 48 h. Numbers on the lower right corner of microphotographs indicate the concentration of venom used (μg/ml).

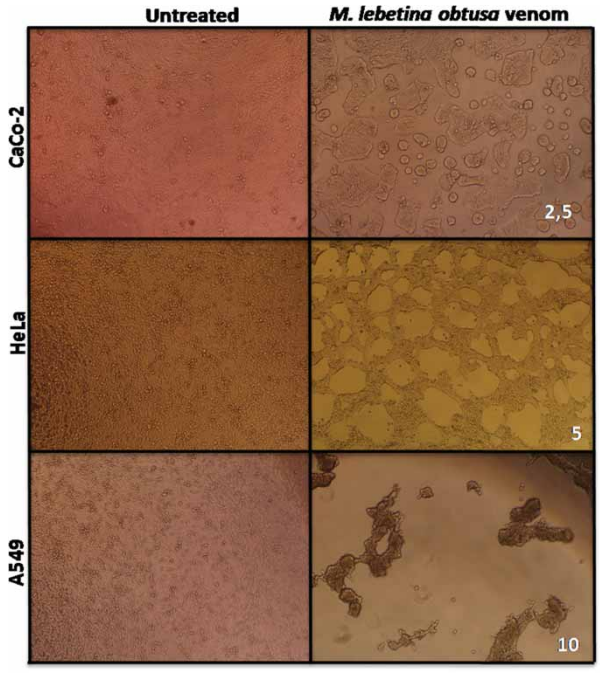

Figure 3. *Macrovipera lebetina obtusa* venom-induced morphological changes in A549, HeLa and CaCo-2 cells viewed by an inverted microscope. Cells were exposed to crude venom for 48 h. Numbers on the lower right corner of microphotographs indicate the concentration of venom used (μg/ml).

cell lines. The effect of venom on the morphology of the cells observed by light microscopy after 48 h venom exposure is demonstrated in Figures 2 and 3. The venom increased the viability of HeLa cells at lower doses (1.25 and 2.5 μg/ml). This effect can be observed for snake venoms and also scorpion venoms because of proteins, peptides and other organic compounds that may promote cell proliferation at lower concentrations (Mesquita-Ferrari et al. 2009; Erdeş et al. 2014; Yalcin et al. 2014).

According to the results, HeLa (IC_{50} = 12.80 μg/ml) is the most resistant cell line against all venom doses, while Vero and U-87 MG cell lines seem to be the most sensitive of the tested cell lines. However, high venom concentrations (20 and 40 μg/ml) were potent against HeLa cells, as well as the other cell lines (Figure 1). The venom of *M. l. obtusa* enhanced the viability of HeLa cells at lower concentrations (1.25 and 2.5 μg/ml).

Microphotography (Figures 2 and 3) after incubation of the cells with various venom concentrations showed parallel results in comparison with the MTT assay. The morphological changes observed throughout the 48 h period following venom exposure varied depending on the origin of the cell lines. Increasing venom concentrations resulted in augmented rounding up and detachment of cells, and somewhat multicellular aggregate formation (Figures 2 and 3). In addition, cell disorganization and large areas without cells were observed with increasing concentrations of venom. Untreated cells were homogeneously distributed in wells and showed their normal phenotype (Figures 2 and 3).

In vitro antimicrobial activity against Gram-positive and Gram-negative bacteria and yeast was determined using the broth microdilution technique. The MIC values of the investigated crude venom are summarized in Table 2. According to the MIC values determined against microorganisms, *M. l. obtusa* venom showed slight activity against *S. aureus*, *S. epidermidis*, *E. faecalis* and *E. faecium* (MIC = 250 μg/ml), and moderate antifungal activity against *C. albicans* (MIC = 62.5 μg/ml).

Discussion

Studies on the use of and search for bioactive molecules from natural resources, such as microorganisms, plants and animals, include screening their cytotoxic and antimicrobial effects for potential therapeutic applications. Snake venoms are a well-known natural source in drug development and discovery studies (Lewis & Garcia 2003; Koh et al. 2006; Fox & Serrano 2007; Vyas et al. 2013). Although there are published reports showing selected biological activities of crude venom or purified proteins of different subspecies of *M. lebetina* (Tõnismägi et al. 2006; Son et al. 2007; Bazaa et al. 2009; Nalbantsoy et al. 2012; Park et al. 2012; Shebl et al. 2012a; Morjen et al. 2013), the combination of cancer cell lines and microorganisms included for screening in the present study has not been

Table 2. Minimum inhibitory concentration (MIC) values of Anatolian *Macrovipera lebetina obtusa* venom for microorganisms following crude venom exposure (µg/ml).

Microorganism	Venom	Ampicillin	Flucytosine
Escherichia coli ATCC 25922	–	1.9	–
Escherichia coli O157:H7	–	3.9	–
Proteus vulgaris ATCC 6957	–	3.9	–
Salmonella typhimurium CCM 5445	–	3.9	–
Staphylococcus aureus ATCC 6538P	250	3.9	–
Bacillus cereus ATCC 7064	–	7.8	–
Enterococcus faecalis ATCC 29212	250	7.8	–
Enterococcus faecium DSM 13590	250	3.9	–
Staphylococcus epidermidis ATCC 12228	250	1.9	–
Candida albicans ATCC 10239	62.5	–	7.4

Note: – = not detected.

chosen in earlier studies, particularly on *M. l. obtusa* subspecies venom. The present study investigated cytotoxic effects on cancer and non-cancerous cells and the antimicrobial properties of Anatolian *M. l. obtusa* crude venom in order to assess its potential for further bioactivity-guided characterization studies.

Cancer is one of the leading causes of disease-related death worldwide, accounting for 8.2 million deaths in 2012. It has been projected that the number of cancer cases will increase to 25 million over the next two decades (Stewart & Wild 2014). Microbial infection is another important health problem and, owing to antimicrobial resistance, the need for new antibiotic agents has increased recently (Coates et al. 2002). In natural product research, it is important to perform general screening and biological characterization of the crude substance at the beginning of the discovery phase in order to determine possible targets.

With regard to the cytotoxicity results of this study, the crude venom of *M. l. obtusa* showed dose-dependent cytotoxic effects at various levels on some of the selected cell lines, corroborating previous reports which show cytotoxic effects of different crude snake venoms or purified venomic proteins/peptides on various cancer and non-cancerous cells (Bustillo et al. 2009; Jamunaa et al. 2012; Yalcin et al. 2014). Under the experimental conditions in this study, *M. l. obtusa* venom was more potent against Vero, U-87 MG, MCF-7 and CaCo-2 cell lines, compared to HeLa and A549 cells. According to the results, the HeLa cell line is the most resistant cell line against all venom doses, while Vero and CaCo-2 seem to be the most sensitive of the tested cell lines. The lowest IC_{50} value was calculated as 1.18 µg/ml for the Vero cell line in the present study, while the same cell line was not affected by the venom of Ottoman viper, *Montivipera xanthina* (Gray, 1849) (Viperidae) even at the highest concentration tested, according to the results of a previous study (Yalcin et al. 2014). Yalcin et al. (2014) reported an IC_{50} value of 4.1 of *M. xanthina* venom against MCF-7 cells, which is close to the value calculated in the present study.

Based on the aforementioned results of this study and those in the literature, one can conclude that snake venoms express specific distinct selectivity between different cell lines, which is possibly due to the unique properties of individual venom components in different species. Selectivity is one of the most important issues in the treatment of cancer. Although it is not possible to draw conclusions on the specific effects of venom components without purifying the active proteins/peptides, it may be stated that Anatolian *M. l. obtusa* venom shows selective activity among different cancer cell lines, based on the results in the present study. On the other hand, cytotoxicity was also observed against non-cancerous Vero cells in this study. More detailed studies using purified proteins are needed to elucidate the mechanism of action and selectivity of the venom.

There are several possible mechanisms of the *in vitro* cytotoxic effect of snake venoms. These include necrosis, induction of apoptosis, overregulation or downregulation of cell cycle proteins and deterioration of cell membrane integrity. Apoptosis-inducing cytotoxic activities of crude venoms or purified proteins such as metalloproteinases and LAAO from different subspecies of *M. lebetina* have been reported on various cancer and non-cancerous cells (Trummal et al. 2005; Son et al. 2007; Park et al. 2009, 2012; Shebl et al. 2012a; Samel et al. 2012, 2013). Induction of apoptosis may be one of the mechanisms responsible for the cytotoxic effect of Anatolian *M. l. obtusa* venom observed in the present study. This possibility deserves more attention since many of the chemotherapeutic agents used in cancer treatment have been shown to induce apoptosis in cancer cells (Gerl & Vaux 2005).

Regarding the antimicrobial activity results, *M. l. obtusa* venom showed moderate antifungal activity against the yeast strain *C. albicans*. Samel et al. (2013) investigated the antimicrobial activity of PLA_2 purified from the venom of *M. lebetina* against *E. coli*, *Vibrio fischeri*, *Bacillus subtilis* and *S. aureus*. Although they observed slight activity only against *V. fischeri* and *B. subtilis*, they did not find significant antimicrobial activity under their experimental conditions. LAAO from *M. lebetina turanica* venom has been shown to have antibacterial effects on *E. coli* and *B. subtilis* (Tõnismägi et al. 2006). Moreover, Shebl et al. (2012b) observed antimicrobial effects of crude

M. lebetina venom against *S. aureus* and *E. coli*. No strong inhibition was observed of the Gram-negative and Gram-positive bacteria included in the present study. This difference may be due to variation in the venom composition of protein activities between different subspecies. However, the antifungal activity on *C. albicans* observed in this study may be related to snake venom proteins such as LAAO or PLA_2. Antifungal effects of different snake venoms and purified proteins (e.g. LAAO, PLA_2) against *C. albicans* have been reported in the literature (Costa et al. 2008; Torres et al. 2010; Yalcin et al. 2014).

Similar to the case for cytotoxicity, *M. l. obtusa* venom shows species-specific antimicrobial activity, according to the present results, corroborating the previous literature (Ahmadi et al. 2010; San et al. 2010; Ferreira et al. 2011; Shebl et al. 2012b; Yalcin et al. 2014). The species-specific results obtained may arise from structural differences in the outer membrane of bacteria, composition and composition-related operation mechanisms of the venom (San et al. 2010). Many factors have been implicated, including the charge density and structure of lipopolysaccharides in the case of Gram-negative bacteria, or the lipid composition of the cytoplasmic membrane and the electrostatic potential across the membrane in Gram-positive bacteria. Consequently, snake venom proteins may interact selectively with specific molecules of different microorganisms (Ahmadi et al. 2010; San et al. 2010). This result is also important from the point of view of the specific antimicrobial activity of venom. Contamination events such as hospital-acquired infections need to be solved by hospital management systems in both developing and developed countries. Increasing resistance against clinically important antimicrobial agents creates an attractive area of research for drug discovery studies (Coates et al. 2002). Furthermore, after applying fractionation and isolation methodology to crude venom, antimicrobial activity can be increased as a result of exposure to more concentrated active compounds.

Proteins including disintegrins, serine proteinase, metalloproteinase, CLP, PLA_2 and LAAO have been purified and identified in the venom of different subspecies of *M. lebetina* (Marcinkiewicz et al. 2003; Tõnismägi et al. 2006; Sarray et al. 2007; Park et al. 2012; Samel et al. 2012, 2013). Sanz et al. (2008) identified PLA_2, LAAO, metalloproteinase, serine proteinase, natriuretic peptide, disintegrin, BPP and CRISP protein families in the venom of *M. l. obtusa* from Armenia by a mass spectrometry-based proteomics approach. Igci and Demiralp (2012) reported similar protein content with additions of NGF, VEGF and protease inhibitor for Anatolian *M. l. obtusa*. The present cytotoxic and antimicrobial activity observations may have resulted from the various interactions of these proteins (e.g. disintegrin, LAAO, PLA_2) affecting different molecular pathways in the cell.

Most snake venoms include a mixture of enzymes, growth factors and toxins. Purification and isolation of the target molecules is very important to progress from general screening to targeted studies. The present study may be considered as a preliminary *in vitro* screening of the anticancer and antimicrobial potential of Anatolian *M. l. obtusa* venom. These results demonstrate that further studies are needed to thoroughly investigate and characterize pure peptides/proteins possessing selective biological activities, and the authors have begun extended studies in this context.

In conclusion, cytotoxic and antimicrobial activities of Anatolian *M. l. obtusa* venom were screened for the first time as a contribution to knowledge on the bioactivities of snake venoms. Although there are some published reports on the proteomic and biochemical characterization of different subspecies of *M. lebetina*, different populations may have modified or differentiated proteins according to intraspecific population variations in the venom composition (Chippaux et al. 1991; Igci & Demiralp 2012). From this point of view, the results of the present study showed that the Anatolian population of *M. l. obtusa* could provide a source of novel bioactive protein prototypes with anticancer and antifungal activities. Further research is needed to purify and characterize the active molecules in the venom.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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