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Research Article

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Major biological activities and protein profiles of skin secretions of *Lissotriton vulgaris* and *Triturus ivanbureschi*

Lissotriton vulgaris ve *Triturus ivanbureschi* deri salgılarının başlıca biyolojik aktiviteleri ve protein profilleri

Abstract

Objective: The aim of this study was to determine the total protein amounts, protein profiles, in vitro cytotoxicities, antimicrobial activities and hemolytic effects of skin secretions of the *Lissotriton vulgaris* and *Triturus ivanbureschi*.
Methods: Skin secretions were obtained, clarified, supernatants snap-frozen then lyophilized. Total protein amounts were determined by BCA assay kit. Protein profiles were revealed by the SDS-PAGE. The cytotoxicity and antimicrobial activity were determined by using MTT assay and minimum inhibitory concentration (MIC) method. Hemolytic effects were measured on rabbit red blood cells.
Results: *Lissotriton vulgaris* and *T. ivanbureschi* skin secretions have totally 18 and 20 protein fractions. IC_{50} values were detected between 1.40 and 40.28 μg/mL. The MIC results were found between 7.8 and 250 μg/mL. *Lissotriton*
vulgaris skin secretion showed low hemolytic effect while *T. ivanbureschi* skin secretion showed high hemolytic effect.
Conclusion: This study is the first report showing the potential of *L. vulgaris* and *T. ivanbureschi* skin secretions for cytotoxicity, antimicrobial and hemolytic activity as an alternative therapeutic approach for traditional uses. Further studies need to focus on purification of the active components from these skin secretions and mode of action on cancer cell lines and microorganisms as anti-agents.

Keywords: Amphibian skin secretion; Cytotoxicity; Antimicrobial activity; Hemolytic activity; Protein profile.

Özet

Amaç: Bu çalışmanın amacı, *Lissotriton vulgaris* ve *Triturus ivanbureschi* deri salgılarının total protein miktarlarını, protein profillerini, in vitro sitotoksisitelerini, antimikrobiyal aktivitelerini ve hemolitik etkilerini belirlemektir.
Metod: Ham deri salgıları, sağıldı, arındırıldı, supernatant kısımlar donduruldu ardından liyofilize edildi. Deri salgılarının total protein miktarları BCA kiti ile belirlendi. Protein profilleri SDS-PAGE ile ortaya koyuldu. Sitotoksik ve antimikrobiyal etkiler MTT testi ve MIC metodu kullanılarak belirlendi. Hemolitik etkiler, tavşan kırmızı kan hücreleri üzerinde hesaplandı.
Bulgular: *Lissotriton vulgaris* deri salgısında 18, *T. ivanbureschi* deri salgısında 20 protein fraksiyonu bulunmaktadır. IC_{50} değerleri 1.40–40.28 μg/mL arasında tespit edildi. MIC sonuçları 7.8–250 μg/mL arasında bulundu. *Lissotriton vulgaris* deri salgısı tavşan kırmızı kan hücreleri üzerinde düşük hemolitik etki gösterirken, *T. ivanbureschi* deri salgısı yüksek hemolitik etki gösterdi.

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Sonuç: Bu çalışma, *L. vulgaris* ve *T. ivanbureschi* deri salgılarının geleneksel kullanımdaki alternatif terapötik potansiyellerini sitotoksisite, antimikrobiyal ve hemolitik aktivite bakımından ortaya koyan ilk rapor niteliğindedir. Gelecek çalışmalarda deri salgılarının anti-ajanlar olarak kullanılabilmesi için içeriğindeki aktif bileşenlerin saptanması, hücre hatları ve mikroorganizmalar üzerindeki etki mekanizmasının belirlenmesine odaklanılmalıdır.

Anahtar Kelimeler: Amfibi deri salgısı; Sitotoksisite; Antimikrobiyal aktivite; Hemolitik aktivite; Protein profili.

Introduction

Skin secretions from many species of amphibians contain a wide range of compounds with biological activity in very high concentration that has excited interest because of their potential for novel drug development [1]. Also researches have shown that skin secretions from many species of Anura are containing high concentrations of peptides with cytotoxic activities against prokaryotic and eukaryotic cells [2]. Amphibian skin/parotoid gland secretions are rich sources of antimicrobial activity against strains of antibiotic-resistant microorganisms such as bacteria and fungi, also highly cytotoxic effects on cancer cell lines [3]. Besides, it is known that dermal glands indicate physiological functions on skin and play several roles of protection from predators [4, 5].

The secreted materials of amphibian skin have been used extensively by several cultures. Chansu and Cinobufacini (Huachansu) are traditional Chinese medicines that have been used for centuries as therapy for inflammation, anesthesia and infections in China and other Asian countries [6]. Studies on bufadienolidic steroids led to isolation of marinobufagin, bufalin, telocinobufagin, and cinobufagin. These bufadienolidic steroids have shown efficiency against cancer cells in animal models and in migration of colon cancer cells (Colon 26-L5) [7, 8], human leukemia cells (K562, U937, ML1 and HL-60) [9] and breast tumor cells (MCF-7) [10]. In Telocinobufagin studies, bufadienolid was found active against *Staphylococcus aureus* and *Escherichia coli* [11]. Three components of Chansu (Bufalin, Telocinobufagin and Cinobufagin) demonstrate a collection of biological activities, including cardiotonic, anesthetic, antineoplastic activity and blood pressure stimulation [10, 12].

Natural products of amphibian skin and their proficiency are not limited with bufodienolidic steroids. Amphibian skin secretion studies have showed that Anura families such as *Ranidae, Hylidae, Bombinatoridae, Pipidae, Alytidae, Ascaphidae* and *Leptodactylidae* have

various therapeutic potentials as anti-diabetic agents, immunomodulatory agents, anti-viral and anti-cancer agents [13]. Approximately 30 years have passed since the discovery of the Magainins in the skin of the African clawed frog, *Xenopus laevis* in the family *Pipidae* [14]. Magainin from *X. laevis* show potential as anti-cancer agents displaying tumoricidal activity against human small cell lung cancer cell lines [15], the RT4, 647V, and 486P bladder cancer cell lines [16], and against suspension cultures of a wide range of hematopoietic cell lines [17]. Another biologically active compound of amphibian skin is steroidal samandarine from salamanders. Studies show that samandarine have high toxicity is likely due to its potent local anesthetic activity [18].

To sum up, there are a lot of researches related to amphibian skin secretions that have been carried out on frogs and toads. In salamanders and newts, the studies have been carried out only on *Andrias davidianus* (Chinese giant salamander) [19], *Taricha granulosa* (rough-skinned newt) [20], *Tylototriton verrucosus* (crocodile newt, Himalayan newt) [21], *Plethodon cinereus* (red-backed salamander) [22], and *Salamandra salamandra terrestis* (European fire salamander) [23].

The smooth newt, *Lissotriton vulgaris* and Balkan-Anatolian crested newt, *Triturus ivanbureschi* that are studied in this survey have similar distribution range in western and European part of Turkey. The main purpose of this study was to investigate cytotoxic, antimicrobial and hemolytic effects of *L. vulgaris* and *T. ivanbureschi* skin secretions on various cancer and non-cancerous cells, microorganisms and rabbit red blood cells to form an estimate of their potential use in medicine as therapeutic agents for the first time.

Materials and methods

Field studies

Smooth newt – *Lissotriton vulgaris* specimens (five males, five females) were collected during the field excursion in Urla/Izmir province, western Turkey in March 2016. Balkan-Anatolian crested newt – *Triturus ivanbureschi* specimens (five males, five females) were collected during the field excursion in Uzunköprü/Edirne province, Turkish Thrace in April 2013. These two species can be easily distinguished from each other. *Lissotriton vulgaris* is an obviously smaller newt species than *T. ivanbureschi*. Besides, male specimens of *L. vulgaris* have continuous dorsal fin in reproduction period, while dorsal fins of the

male specimens of *T. ivanbureschi* have an interruption at the base of the tail.

Collection of skin secretions

Skin secretions obtained by mild electrical stimulation (5–10 V) by stimulator (C.F. Palmer, London) according to Tyler et al. [24]. Each individual was rinsed with ultra-pure water into the tubes. Skin secretions clarified by centrifugation (5635×*g* for 10 min.), supernatants were snap-frozen by liquid nitrogen then lyophilized and stored at +4°C until the bioactivity assays are set up. Secretion harvesting was performed in the field, and then newts were released to their natural habitats, unharmed.

Determination of the protein profiles by the SDS-PAGE and protein concentration

Protein content was assayed triplicate for each diluted skin secretion (2 mg/mL) samples in ultra-pure water, using bovine serum albumin as a standard by BCA assay kit (Thermo Scientific, MA, USA). The protein content was calculated with using a UV/Vis spectrophotometer (Thermo Multiskan Spectrum, Bremen, Germany) at 562 nm.

Electrophoretic processes were performed with sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) using a discontinuous buffer system according to Laemmli [25]. The running process was executed on Bio-Rad Mini Protein Tetra Cell electrophoresis device. TGX Stain Free (Bio-Rad, CA, USA) gel was used which does not need any staining processes. The Thermo PageRuler Plus Protein Standard (10, 15, 25, 35, 55, 70, 100, 130 and 250 kD) was used as a marker. In protein profile studies, samples were repeated three times on gel. Approximate molecular weights of the protein fractions were calculated and photographed with using a software program (Image Lab, Bio-Rad, CA, USA) of the gel imaging system ChemiDoc (Bio-Rad, CA, USA).

Cell culture, in vitro cytotoxicity assay and determination of half maximal inhibitory concentration (IC$_{50}$)

HeLa (human cervix adenocarcinoma), A549 (human alveolar adenocarcinoma), Caco-2 (human colon colorectal adenocarcinoma), MPanc-96 (human pancreas adenocarcinoma), PC-3 (human prostate adenocarcinoma), U-87 MG (human glioblastoma-astrocytoma), MDA-MB-231 (human mammary gland adenocarcinoma) cancer cells and as

a non-cancerous cell lines, HEK-293 (human embryonic kidney) were used for determination of the cytotoxicity. Cell lines were purchased from ATCC (Manassas, VA, USA). All cells were cultivated 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 (Lonza, Visp, Switzerland). The cells were incubated at 37°C in a humidified atmosphere of 5% CO$_2$. The cells that actively proliferate in the logarithmic phase were used in the tests.

Cytotoxicity of crude skin secretions were determined by colorimetric MTT [3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide] assay [26]. The optical density (OD) was measured at 570 nm (with a reference wavelength 630 nm) by UV/Vis spectrophotometry (Thermo Multiskan Spectrum, Bremen, Germany). Cytotoxicity was assayed triplicate. All cell lines were cultivated in 96-well microplates for 24 h with an initial concentration of 1×10^5 cells/mL. Subsequently, the cultured cells were treated with different doses of skin-secretions and incubated at 37°C for 48 h. The plant-derived sesquiterpene-lactone (parthenolide) was used as positive cytotoxic control agent. The viability (%) was determined by the following formula:

%Viable cells = [(absorbance of treated cells)

  − (absorbance of blank)]/[(absorbance of control)

  − (absorbance of blank)] × 100.

In cell culture studies for untreated cell lines (negative controls) cytotoxicity was set to 0%. The IC$_{50}$ values were calculated by fitting the data to a sigmoidal curve and using a four parameter logistic model and presented as an average of three independent measurements. The IC$_{50}$ values were reported at 95% confidence interval and calculations were performed using Prism 5 software (Graph-Pad5, San Diego, CA, USA). The values of the blank wells were subtracted from each well of treated and control cells and half maximal inhibition of growth (IC$_{50}$) were calculated in comparison to untreated controls.

Microorganisms and antimicrobial assay

Following microorganisms were used for antimicrobial assays: The Gram-negative bacteria strains: enteropathogenic *Escherichia coli* 0157:H7 (RSKK 234), non-pathogenic *E. coli* ATCC 25922, *Klebsiella pneumoniae* ATCC 10031 and *Salmonella thyphimurium* CCM 5445. The Gram-positive bacteria strains: *Bacillus cereus* ATCC 7064, *Enterococcus fecalis* ATCC 29212, vancomycin-resistant *E. faecium* DSM 13590, methicillin-resistant

Staphylococcus aureus ATCC 43300, and *S. epidermidis* ATCC 12228. *Candida albicans* ATCC 10239 and *C. tropicalis* RSKK 2412 were used as yeasts. The lyophilized bacteria and yeast strains were obtained from Ege University, Faculty of Science, Department of Basic and Industrial Microbiology.

Antimicrobial effects of skin secretions were determined by broth micro-dilution method. Test 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. MICs were determined according to the Clinical and Laboratory Standard Institute [27]. Serial dilutions of skin secretions (0.9–500 µg/mL) were prepared in 96-well microtiter trays, at a final volume of 80 µL. Then, 20 µL of the adjusted bacterial and fungal inocula (1.5×10^5 CFU/mL) were added to each well and incubated at 37°C for 24 h. Inhibition of microorganisms' growth was determined by visual observation. The minimum inhibitory concentration (MIC) was defined as the lowest concentration of skin secretions required to inhibit microbial growth. Each dilution series included control wells, which consisted of 80 µL of it and 80 µL of Mueller Hinton broth. Ampicillin and flucytosine were used as standard drugs for comparison. All assays were studied three replicates.

Hemolytic activity assay

The hemolytic effect of crude *L. vulgaris* and *T. ivanbureschi* skin secretions were measured according to the modified method of [28]. Red blood cells were obtained from healthy New Zealand rabbit (Bornova Veterinary Control and Research Institute, Izmir, Turkey). Blood was collected with BD Vacutainer TM (NH 143 I.U., Belliver Industrial Estate, Plymouth, UK). Aliquots of 7 mL of blood were washed three times with sterile saline solution (0.89%, w/v NaCl, pyrogen free) by centrifugation at $2000 \times g$ for 5 min. The cell suspension was prepared by finally diluting the pellet to 0.5% in saline solution. A volume of 0.05 mL of the cell suspension was mixed in U bottom 96-well microplate with 0.05 mL diluents containing 50, 5 and 0.5 µg/mL concentration of crude *L. vulgaris* and *T. ivanbureschi* skin secretions in saline solutions. The mixtures were incubated for 30 min at 37°C and centrifuged at $800 \times g$ for 10 min. The free hemoglobin in the supernatants was measured spectrophotometrically at 412 nm. Saline and distilled water were included as minimal and maximal hemolytic controls. The hemolytic percent developed by the saline control was subtracted from all groups. Each experiment included triplicates at each concentration.

Results

Protein contents and electrophoretic protein profiles

The total protein and peptide concentrations were determined by BCA assay for *L. vulgaris* and *T. ivanbureschi* as 1775 and 1470 µg/mL, respectively.

The electrophoretic profiles of the skin secretions revealed numerous proteins with a wide spectrum of molecular masses (Figure 1). In *L. vulgaris* skin secretion, totally 18 protein fractions were determined. The approximate molecular weights of these protein fractions were calculated as 265, 252, 115, 83, 69, 60, 53, 47, 40, 34, 28, 23, 21, 18, 16, 13, 11 and 9 kDa. In *T. ivanbureschi* skin secretion, totally 20 protein fractions were found. These are calculated as 272, 263, 200, 109, 89, 78, 68, 64, 54, 50, 47, 43, 38, 30, 27, 22, 20, 16, 11 and 9 kDa, respectively.

Cytotoxicity screening

The cytotoxicity of crude skin secretions is measured against the following cell lines: HeLa, A549, Caco-2,
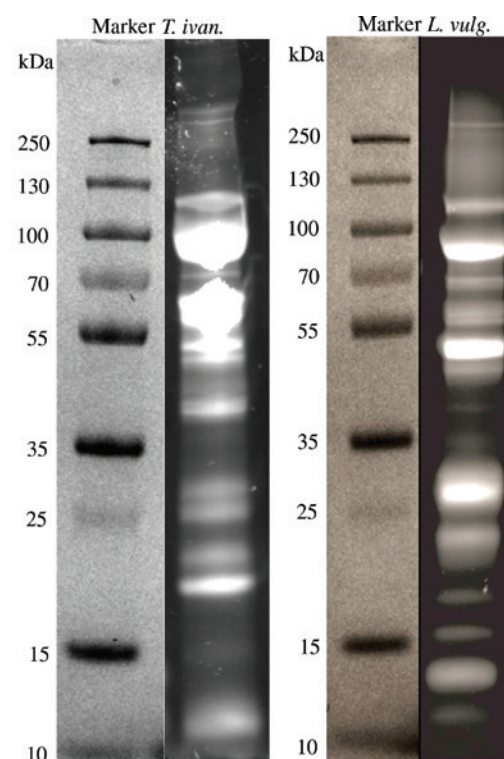

Figure 1: SDS-PAGE fractionation of proteins in *T. ivanbureschi* and *L. vulgaris* skin secretions.
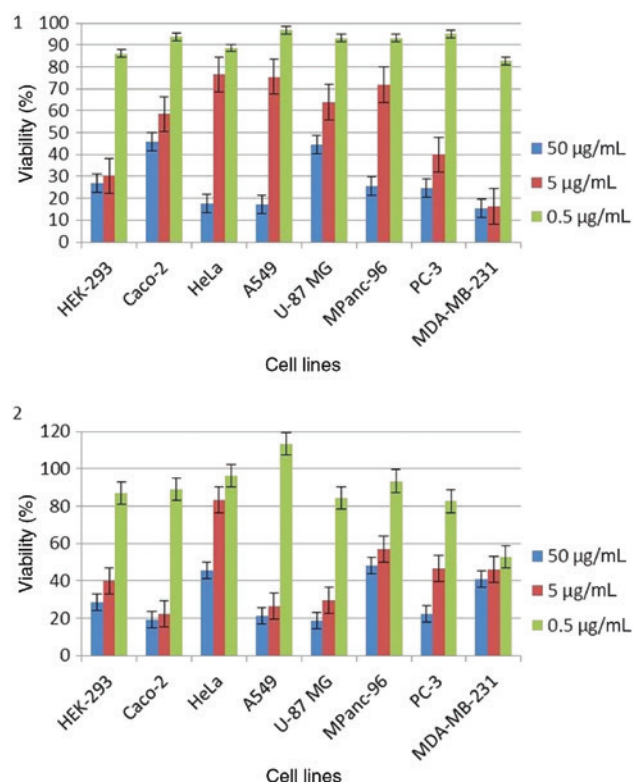
Figure 2: Viability of cancer and non-cancerous cell lines following different crude skin secretion concentrations treatment for 48 h. Cell viability was determined by MTT assay, control was exposed to vehicle only which was taken as 100% viability. 1: *Lissotriton vulgaris*, 2: *Triturus ivanbureschi*.

MPanc-96, PC-3, U-87 MG, MDA-MB-231 cancer cells and as a non-cancerous cell line, HEK-293. Crude skin secretions of the *L. vulgaris* and *T. ivanbureschi* inhibit cell viability in a dose-dependent manner (Figure 2). The IC_{50} values of skin secretions for treated cell lines are shown in Table 1.

Crude *L. vulgaris* and *T. ivanbureschi* skin secretions were showed high cytotoxic effects on all cancer and non-cancerous cell lines with IC_{50} values varying between 1.58–26.15 and 1.40–40.28 µg/mL, respectively. Additionally, we found the highest anticancer activity for both secretion samples on MDA-MB-231 cell line (IC_{50}: 1.58

and 1.40 µg/mL). These results are higher than plant-derived sesquiterpene lactone parthenolide, remarkably. The lowest cytotoxic effect observed on human cervix adenocarcimoma (HeLa) by *T. ivanbureschi* crude skin secretion (IC_{50}: 40.28 µg/mL). Morphological changes were observed after 48 h exposure to different doses of skin secretions. Increasing concentrations resulted in increased number of rounded cells, growth inhibition and the rate of various morphological abnormalities with larger areas devoid of cells when compared with the untreated control cells.

Antimicrobial activities

MIC was determined using broth dilution method (0.9–500 µg/mL). The MIC values of skin secretions against various Gram-negative and Gram-positive bacterial strains and yeasts are presented in Table 2.

The values indicate that skin secretion have MIC values varying from 3.9 to 250 µg/mL. Skin secretion of *L. vulgaris* showed the most potent activity against entero-pathogenic *E. coli* O157:H7 RSKK234 and *C. albicans* ATCC 10239 with a MIC value of 7.8 µg/mL. Especially, antifungal activity against *C. albicans* ATCC 10239 has a higher MIC value than positive control drug flucytosine, remarkably. Also, skin secretion of *L. vulgaris* showed moderate antibacterial activity against *E. faecalis* ATCC 29212, *S. epidermidis* ATCC 12228, *S. aureus* ATCC 6538P, vancomycin-resistant *E. faecium* DSM 13590 and *C. tropicalis* RSKK 2412 (MIC = 15.6 and 31.25 µg/mL). Skin secretion of *T. ivanbureschi* exhibited the most potent activity against *E. faecalis* ATCC 29212 and *C. albicans* ATCC 10239 with a MIC value of 15.6 µg/mL.

Hemolytic effects

The hemolytic effects of crude *L. vulgaris* and *T. ivanbureschi* skin secretions on rabbit red blood cells were shown in Table 3. Hemolytic activities were observed at

Table 1: The IC_{50} values for cell lines following crude skin secretion exposure by MTT assay.

Cell lines IC_{50} (µg/mL)	HEK-293 (non-cancerous kidney)	Caco-2 (colon)	HeLa (cervical)	A549 (lung)	U-87 MG (glioblastoma)	MPanc-96 (pancreas)	PC-3 (prostate)	MDA-MB-231 (breast)
Sample ID								
Parthenolide	0.55	1.65	0.98	0.26	3.33	0.91	1.24	2.78
L. vulgaris	5.27	24.40	13.25	13.04	26.15	14.57	4.99	1.58
T. ivanbureschi	5.02	5.60	40.28	11.27	6.58	27.87	5.01	1.40

Parthenolide was used as positive control.

Table 2: MIC values of *L. vulgaris* and *T. ivanbureschi* crude skin secretions and reference antibiotics against test organisms by using broth dilution method.

Microorganisms	MIC (µg/mL) values for skin secretion and antimicrobial agents			
	L. vulgaris	*T. ivanbureschi*	Ampicillin	Flucytosine
E. coli O157:H7 RSKK234	7.8	125	4.6	–
E. coli ATCC 25922	125	–	4.6	–
S. aureus ATCC 43300	31.25	250	4.6	–
S.epidermidis ATCC 12228	15.6	125	2.3	–
E. faecalis ATCC 29212	15.6	15.6	9.3	–
E. faecium DSM 13590	31.25	31.25	4.6	–
K. pneumonia ATCC 10031	125	–	9.3	–
S. thyphimurium CCM 5445	125	125	4.6	–
B. cereus ATCC 7064	125	125	9.3	–
C. albicans ATCC 10239	7.8	15.6	–	9.3
C. tropicalis RSKK 2412	31.25	–	–	9.3

Ampicillin and flucytosine were used as positive controls. –, Not detected.

Table 3: Hemolytic activities of crude *L. vulgaris* and *T. ivanbureschi* skin secretions.

Controls and samples	Concentration (µg/mL)	Absorbance value OD (412 nm)	Hemolytic percent (%)
Distilled water		0.733 ± 0.023	100 ± 3.138
Saline		0.128 ± 0.012	0 ± 1.637
L. vulgaris	50	0.1 ± 0.019	13.643 ± 2.592
	5	–	–
	0.5	–	–
T. ivanbureschi	50	0.844 ± 0.094	115.143 ± 12.824
	5	0.76 ± 0.086	103.683 ± 11.733
	0.5	0.087 ± 0.044	11.869 ± 6.003

Hemolytic percent of saline and distilled water were included as minimal and maximal hemolytic control, respectively. All values represent the mean ± SD (n = 3 test). –, Not detected.

concentrations between 0.5 and 50 µg/mL. Hemolytic effect of *L. vulgaris* skin secretion was found only in the highest concentration (50 µg/mL) with 13.6% hemolytic percent. No hemolytic activity was observed in other concentrations of the *L. vulgaris* skin secretion. Hemolytic activities were seen at all concentrations of the *T. ivanbureschi* skin secretions in dose dependent manner. It showed very high hemolytic activity at 50 and 5 µg/mL concentrations which were more than the positive control distilled water (hemolytic percents: 115.1% and 103.7%).

Discussion

The skin secretions of amphibians show differentiation across different species with biologically active compounds such as peptides/proteins, steroids, alkaloids, biogenic amines and enzymes [29]. They might be useful as pharmacological implements in drug research and potential drug design models. In this study, cytotoxic,

antimicrobial and hemolytic properties of crude *L. vulgaris* and *T. ivanbureschi* skin secretions were determined. Also, protein profiles of these crude skin secretions were revealed.

The antimicrobial and cytotoxic activities are results of the active peptide inducing alterations in the hydrophobic–hydrophilic seal of the cell membrane, effecting lysis of the bacterial or cancer cells [30].

Salamanders and newts are known to secrete toxic and noxious compounds, such as neurotoxic tetrodotoxin [31]. Nevertheless, the exact mode of action of the salamander alkaloids is almost unknown. They have local anesthetic effects by nerve-blocking activity. Respiratory paralysis was found as the reason of death according to in vivo studies [32]. Moreover, salamander alkaloids, especially samandarone, exhibit distinct antimicrobial activities, although being less potent than most antibiotics [22, 33]. Also, several peptides such as corticotropin-releasing peptides and vasoactive intestinal peptides were isolated from the newt, *Cynops pyrrhogaster* by Teranishi et al. [34].

Protein profiles of crude skin secretions of salamanders and newts are almost unknown. Up to now, only crude skin secretion of *A. davidianus* is determined. The molecular masses of the proteins in the skin secretions of the smooth newt and Balkan-Anatolian crested newt are distributed over a wide range between 9 and 272 kDa, while those of the *A. davidianus* skin secretion are above 66 kDa and between 14 and 66 kDa [19]. There is no further study on bioactivity of skin secretions of newts.

However, some comparable bioactivity studies on skin secretion of frogs and toads have been conducted. Cunha Filho et al. [11] indicated that *B. rubescens* skin-parotoid gland secretions have antimicrobial activity on *S. aureus* (MIC: 128 μg/mL) and *E. coli* (MIC: 16–64 μg/mL). We found higher effect on *E. coli* (MIC: 7.8 μg/mL) and *S. aureus* (MIC: 31.25 μg/mL) for *L. vulgaris* skin secretion when compared to the study of Cunha Filho et al. [11]. Zare-Zardini et al. [35] determined the antimicrobial and hemolytic effect of maximin peptide from *B. kavirensis* skin secretion with MIC values for *B. cereus* (18.5 μg/mL), *S. aureus* (16.3 μg/mL), *K. pneumonia* (8.9 μg/mL), *E. coli* (8.1 μg/mL), *C. albicans* (32.1 μg/mL). Also, they found 2.5% hemolytic effect with 60 μg/mL maximin on human erythrocytes. Our results on same microbial strains for *L. vulgaris* and *T. ivanbureschi* skin secretions with MIC values for *B. cereus* (both 125 μg/mL), *S. aureus* (31.25 and 250 μg/mL), *K. pneumonia* (125 μg/mL and ineffective), *E. coli* (7.8 and 125 μg/mL), *C. albicans* (7.8 and 15.6 μg/mL), respectively. Also, we found higher hemolytic effects on rabbit erythrocytes with 50 μg/mL skin secretion, 13.6% for *L. vulgaris* and 115.1% for *T. ivanbureschi*. van Zoggel et al. [36] studied the cytotoxicity of dermaseptin peptides from *P. bicolor* skin secretion on human prostate adenocarcinoma (PC-3) and found 75% inhibition with 10 μg/mL concentration. We found 60% inhibition on the same cell line with 5 μg/mL skin secretion of *L. vulgaris* and 50% viability 5 μg/mL skin secretion treatment of *T. ivanbureschi*. Cunha Filho et al. [37] performed the cytotoxicity of *R. schneideri* macrogland secretion derivatives on HCT-8 cell line and they found the IC$_{50}$ values up to 1.20 μM with no hemolytic activity. We found the IC$_{50}$ values on Caco-2 as 24.40 μg/mL for *L. vulgaris* and 5.60 μg/mL for *T. ivanbureschi* skin secretion with high hemolytic activity. These results are demonstrating potential for cancer and antimicrobial treatment.

In conclusion, biological activities and protein profiles of skin secretions of the smooth newt, *L. vulgaris* and Balkan-Anatolian crested newt, *T. ivanbureschi* were revealed for the first time. Besides, the results indicated that these crude skin secretions have a high inhibition on cancer cell lines and microorganisms with relatively high hemolytic activity and wide range molecular masses of proteins. Further studies need to focus on purification of the active components from these skin secretions and mode of action on cancer cell lines and microorganisms as anti-agents.

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