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Screening the antimicrobial, cytotoxic and hemolytic effects of skin-parotoid gland secretions of 13 taxa from six species and one hybrid population of Anatolian endemic Lycian salamanders (Genus: *Lyciasalamandra*)

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Abstract

Background and purpose: *The diverse bioactive content in the skin and parotoid glands of amphibians makes them unique and highly potential sources for pharmacological developments. The main aim of the study is to determine the total protein amounts,* in vitro *cytotoxicities, antimicrobial activities and hemolytic effects of skin-parotoid secretions of these endemic Lycian salamanders (Genus:* Lyciasalamandra*).*

Materials and methods: *Lycian salamander specimens of 13 taxa from six species and one hybrid population are collected from southwestern Anatolia. Skin-parotoid secretions were obtained, clarified, supernatants snap-frozen then lyophilized. Total protein amounts were determined by BCA assay kit. The cytotoxicity was determined on eight different cell lines (one healthy and seven cancerous) using MTT assay. The antimicrobial activity was assesed using 11 different microorganisms (nine bacteria and two yeast) from six genus by MIC method. Hemolytic effects were measured on rabbit red blood cells.*

Results: *Lycian salamanders' skin-parotoid gland secretions showed variable cytotoxic effects on all cell lines with IC_{50} values from 5.41 ± 0.17 µg/ml to ineffective. MIC results of antimicrobial activity tests were also variable and from 3.9 µg/mL to non-effective. Studied skin-parotoid secretions have no hemolytic activities on rabbit red blood cells at concentrations of 0.5, 5 and 50 µg/mL.*

Conclusions: *Skin-parotoid gland secretions of Anatolian endemic Lycian salamanders have sufficient and remarkable therapeutic potential to be candidate sources of natural and biologically active substances for novel drug discoveries.*

INTRODUCTION

Natural products and their derivatives have been known as sources of therapeutic agents. However, the development of new technics in biotechnology has improved the screening pharmaceutical potential of natural products for discovering new drugs. These compounds are significant bioactive substances for drug development, especially in the

anticancer, antihypertensive, anti-infectives, immunosuppression, and neurological disease therapeutic areas *(1)*.

The amphibian skin and its secretions have multiple important functions associated with survival and a great number of biological and pharmacological properties have been confirmed to be present on the skin *(2, 3)*. The diversity of chemical compounds in the skin and parotoid glands in amphibians makes them unique and highly potential sources for pharmacological developments.

Many studies confirmed that the amphibians are producing biogenic amines, proteins/peptides, alkaloids, and steroids *(4, 5)*. The cocktail arsenal of amphibian skin secretions has anticancer, antibacterial, antifungal, antiparasitic, antidiabetic, antiviral, wound healing, analgesic, immunomodulative, antioxidant etc. activities *(6)*. Studies on the bioactivities of skin secretions of frogs and toads are well known, but the urodeles were neglected. Newts (subfamily: *Pleurodelinae*) have the largest number of species regarding the biological activity of crude skin secretions including 15 species from 10 different genera, but only two species from two genera of the true salamanders (subfamily: *Salamandrinae*) within the family *Salamandridae* have been studied *(7)*.

These two abovementioned true salamanders (subfamily *Salamandrinae*) species, *Mertensiella caucasica* and *Salamandra salamandra,* were explored regarding some experimental effects of skin secretion. Caucasian salamander *Mertensiella caucasica* releases secretion on the dorsal part of the tail, which causes sensation of dry mouth and bitter taste with no burning sensation when tasted *(8)*. The fire salamander *Salamandra salamandra* sprays its toxic skin secretion, released by its granular glands, causing neurotoxicity that occasionally leads to death by respiratory paralysis as an anti-predator defense system *(9)*. *Salamandra salamandra* skin secretion has antimicrobial activity against both Gram-positive and Gram-negative bacteria, and the fungi *Geotrichum candidum*, *Saccharomyces cerevisiae*, *Candida krusei*, and *Trichoderma viride* which may affect microorganisms due to the presence of alkaloids in their skin secretions *(10)*. Plácido et al. *(11)* discovered a potential antioxidant peptide, Salamandrin-I in the skin secretion of *Salamandra salamandra*. Byern et al. *(12)* showed that the skin secretion of *Salamandra salamandra terrestris* has cytotoxic effect on C2C12, L929, NHDF, HUVEC and HAC cell lines. Vences et al. *(13)* revealed that *Lyciasalamandra fazilae* and *L. billae* from unknown localities and from captivity have two steroidal alkaloids in their skin, Samandarine and Samandarone without any bioactivity screening. There are no further or more detailed studies on the biological activities of skin secretions of Salamandrinae subfamily. Anatolian endemic Lycian salamanders (Genus: *Lyciasalamandra*) are also belonging to the subfamily *Salamandrinae* and bioactivities of their skin-parotoid gland secretions are completely unknown.

The main purpose of this study was to investigate for the first time the cytotoxic, antimicrobial, and hemolytic effects of skin-parotoid gland secretions of endemic Lycian salamanders on various cancer and non-cancerous cells, microorganisms, and rabbit red blood cells to evaluate their potential use in medicine as therapeutic agents.

MATERIALS AND METHODS

Field studies and collection of skin-parotoid gland secretions

Lycian salamanders were collected during the field excursions in Antalya and Muğla provinces of Turkey in south-western Anatolia. The detailed data on the salamanders are given in Table 1. The abbreviations of the taxa are provided in the second column in Table 1 for easier tracking in the upcoming tables. The authors were received special permission for the fieldworks from the Republic of Turkey, Ministry of Forestry and Water Affairs, Directorate of Nature Conservation and National Parks (permit number: 2014-51946).

Skin secretions were obtained by mild electrical stimulation (5-8 V) by stimulator (C.F. Palmer, London), while parotoid gland secretions were obtained by manual compressing. Each three individual samples were rinsed with ca. 15 mL ultra-pure water into the falcon tubes *(14)*. Skin secretions and parotoid gland secretions were pooled for each taxon, clarified by centrifugation (6000 x rpm for 10 min.), supernatants were snap-frozen by liquid nitrogen then lyophilized and stored at +4 °C until the bioactivity assays were set up. Secretion harvesting was performed in the field, then salamanders were released into their natural habitats, unharmed. The authors received ethical permission for the skin secretion milking procedures from Ege University Animal Experiments Ethics Committee (permit number: 2014-002).

Protein content determination

Protein content was assayed in triplicate for each diluted skin secretion by BCA assay kit (Thermo Scientific, USA). For this purpose, a 4 mg skin-parotoid secretion sample was dissolved in 1 mL ultra-pure water (4 mg/mL). Bovine serum albumin (BSA) was used as protein standard. The protein content was calculated using a UV/Vis spectrophotometer (Thermo Multiskan Spectrum, Bremen, Germany) at 562 nm.

Microorganisms

Gram-positive and gram-negative bacteria and yeasts were used for antimicrobial activity studies. The Gram-negative bacteria, enteropathogenic *Escherichia coli* 0157:H7 (RSKK 234), *Escherichia coli* ATCC 25922 and *Salmonella thyphimurium* CCM 5445; multi-drug resistant *Klebsiella pneumoniae* ATCC 10031 were used. The

Table 1. *The taxa, number of specimens, date, locality, and sexes for skin secretion collections are listed below.*

Taxa	Abbreviations of taxa	Number of specimens	Date	Locality	Number of specimens
L. flavimembris flavimembris	Lff	20	28.02.2015	Çiçekliköy, Marmaris, Muğla	7♂♂, 12♀♀, 1 juv.
L. flavimembris ilgazi	Lfi	29	28.02.2015	Kötekli, Kuyucak, Akyaka, Thera, Muğla	6♂♂, 19♀♀, 4 juv.
L. fazilae ulfetae	Lfu	9	28.02.2015	Gökbel, Kapıkargın, Muğla	3♂♂, 5♀♀, 1 juv.
L. billae billae	Lbb	4	01.03.2015	Küçükçaltıcak, Antalya	2♂♂, 2♀♀
L. billae arikani	Lba	15	01.03.2015	Dağdibi, Ulupınar, Antalya	6♂♂, 7♀♀, 2 juv.
L. billae irfani	Lbi	7	02.03.2015	Göynük Canyon, Antalya	3♂♂, 3♀♀, 1 juv.
L. billae yehudahi	Lby	2	02.03.2015	Tahtalı Mountain, Antalya	1♂, 1♀
L. billae eikeae	Lbe	13	08.03.2015	Geyikbayırı, Antalya	3♂♂, 8♀♀, 2 juv.
L. antalyana gocmeni	Lag	10	06.03.2015	Güllük Mountain, Antalya	2♂♂, 5♀♀, 3 juv.
L. antalyana antalyana	Laa	6	03.03.2015	Hurma, Antalya	3♂♂, 3♀♀
L. atifi veithi	Lav	16	07.03.2015	Ürünlü, Antalya	1♂, 10♀♀, 5 juv.
L. luschani luschani	Lll	5	13.03.2015	Dodurga, Muğla	1♂, 3♀♀, 1 juv.
L. luschani finikensis	Llf	6	09.03.2015	Finike, Antalya	3♂♂, 3♀♀
L. luschani basoglui X *L. luschani finikensis*	(hybrid)	5	11.03.2015	Demre, Antalya	2♂♂, 3♀♀

Gram-positive bacteria: enteropathogenic *Bacillus cereus* ATCC 7064, multi-drug resistant *Enterococcus faecalis* ATCC 29212, vancomycin-resistant *Enterococcus faecium* DSM 13590, methicillin-resistant *Staphylococcus aureus* ATCC 43300, *Staphylococcus epidermidis* ATCC 12228 were used. Also, pathogenic *Candida albicans* ATCC 10239 and *Candida tropicalis* RSKK 2412 were used as yeasts. The lyophilized bacteria and yeast were obtained from Ege University, Faculty of Science, Department of Basic and Industrial Microbiology.

Minimum inhibitory concentration (MIC) by micro-dilution susceptibility test

MIC values for skin secretions were determined by broth micro-dilution technique suggested by Clinical and Laboratory Standards Institute, CLSI *(15)*. Test microorganisms were grown in MH broth for 5 h (exponential phase) and adjusted to 0.5 McFarland turbidity standard (A600=1.0), corresponding to1.5×10^6 CFU/mL. 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 inoculate (1.5×10^5 CFU/mL) were added to each well and incubated at 37 °C for 24 h. Inhibition of microorganism's growth was determined by visual observation. 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 sample and 80 µL of Mueller Hinton broth. MIC was determined using broth dilution method (0.9–500 µg/mL). Ampicillin and flucytosine were used as standard antibacterial and antifungal agents, respectively, as a positive control. All assays were performed triplicates.

Cell culture and in vitro cytotoxicity assay

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 (pleural effusion of human mammary gland adenocarcinoma) cancer cells and as a non-cancerous cell line, HEK-293 (human embryonic kidney) were used for determination of cytotoxicity. Cell lines were purchased from ATCC (Manassas, VA, USA). All 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 (Lonza, Visp, Switzerland). The cells were incubated at 37 °C in a humidified atmosphere supplemented with 5% CO_2. Cytotoxicity of crude skin and parotoid gland secretions were determined by following the general procedure based on cell viability using a modified colorimetric MTT [3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide)] assay *(16)*. The optical density (OD) was measured in triplicates at 570 nm (with a reference wavelength 690 nm) by UV/Vis spectrophotometry (Thermo, Bremen, Germany). All cell lines were cultivated for 24h in 96-well cell culture

plates with an initial concentration of $1x10^5$ cells/mL. Subsequently, the cultured cells were treated with different concentrations (0.5, 5 and 50 µg/mL) of skin-secretions and incubated for 48h at 37°C. The plant-derived compound parthenolide was used as a positive cytotoxic control agent at concentrations 0.125, 1.25 and 12.5 µg/mL). Percentages of surviving cells in each culture were determined after incubation with skin secretions. The viability (%) was determined by the following formula:

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

Determination of half maximal inhibitory concentration (IC_{50})

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 (GraphPad5, 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}) was calculated in comparison to untreated controls.

Hemolytic activity assay

The hemolytic activity of crude skin-parotoid gland secretions of Lycian salamanders was measured according to the modified method of Yang et al. *(17)*. Red blood cells were obtained from healthy New Zealand rabbit (Bornova Veterinary Control and Research Institute, Izmir, Turkey). The authors received ethical permission for the collection of blood samples from Ege University Animal Experiments Ethics Committee (permit number: 2014-002). 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 × g for 5 min. The cell suspension was prepared by diluting the pellet to 0.5% in saline solution. A volume of 0.05 mL of the cell suspension was mixed in U button 96-well microplate with 0.05 mL diluents containing 50, 5 and 0.5 µg/mL concentrations of skin-parotoid gland secretions in saline solutions. The mixtures were incubated for 30 min. at 37 °C and centrifuged at 800 × 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 percentage was developed subtracting the saline control from all groups. Each experiment included triplicates of each concentration.

RESULTS

Protein content

The total protein and peptide concentrations were determined and ranged from 280-960 µg/mL by BCA assay. Total protein amounts of skin secretions of all the taxa are presented in Table 2.

Antimicrobial activities

The values of MIC on Gram-negative and Gram-positive bacteria and yeasts (Table 3.) indicate that skin secretions of Lycian salamanders vary from 3.9 µg/mL to ineffective. The highest antifungal activity (more than positive control drug Flucytosine) was found for *L. l. finikensis* and *L. a. gocmeni* skin secretions with MIC value of 3.9 µg/mL, *L. b. eikeae* skin secretion with MIC value of 7.8 µg/mL on *C. albicans* ATCC 10239 which are notably higher than positive control drug Flucytosine. Skin secretions of *L. f. ulfetae* and the hybrid population (*L. l. finikensis* X *L. l. basoglui*) have moderate antifungal effects on *C. albicans* ATCC 10239 with a MIC value of 15.6 µg/mL. The most potent antibacterial activity determined by the skin secretion of *L. b. irfani* against vancomycin-resistant *E. faecium* DSM 13590 and *E. faecium* ATCC 29212 with a MIC value of 7.8 µg/mL which is considerably more than the positive control of the drug Ampicillin. Skin secretions of *L. a. gocmeni* and *L. f. ilgazi* showed moderate antibacterial activity against *E. faecalis* ATCC 29212 and vancomycin-resistant *E. faecium* DSM

Table 2. *Total protein amounts of the Lycian salamander skin secretions.*

Taxa	Protein amount (µg/mL)	Taxa	Protein amount (µg/mL)
L. flavimembris flavimembris	960±65	*L. billae billae*	600±15
L. f. ilgazi	620±15	*L. b. arikani*	680±50
L. fazilae ulfetae	500±10	*L. b. yehudahi*	640±35
L. luschani luschani	940±45	*L. b. irfani*	580±15
L. l. finikensis	280±5	*L. b. eikeae*	720±20
L. l. basoglui X *L. l. finikensis* (hybrid)	750±30	*L. antalyana antalyana*	820±40
L. atifi veithi	830±40	*L. a. gocmeni*	880±25

Table 3. *MIC values of each skin secretions and standard antimicrobial agents (Ampicillin, Flucytosine) as positive controls. Full names of taxa abbreviations are given in Table 1.*

Microorganisms	MIC (µg/mL) values for skin secretion and antimicrobial agents													
	L.a.v.	L.f.f	L.a.a	L.f.u	Hybrid	L.b.a	L.f.i	L.b.b	L.l.l	L.b.y	L.l.f	L.a.g	L.b.e	Lb.i	Amp.	Flu.
E. coli O157:H7 (RSKK 234)	125	–	62.5	62.5	62.5	–	125	250	–	–	–	–	250	125	4.6	–
E. coli ATCC 25922	62.5	125	125	125	–	–	125	250	–	–	CNE	CNE	250	125	4.6	–
S. aureus ATCC 25923	62.5	–	250	62.5	125	62.5	62.5	62.5	125	–	62.5	–	–	250	4.6	–
S.epidermidis ATCC 12228	125	62.5	250	125	125	31.25	31.25	31.25	125	125	–	–	125	15.6	2.3	–
E. faecalis ATCC 29212	31.25	62.5	31.25	62.5	31.25	31.25	15.6	31.25	62.5	–	62.5	15.6	15.6	7.8	9.3	–
E. faecium DSM 13590	31.25	62.5	31.25	31.25	31.25	31.25	15.6	31.25	62.5	62.5	15.6	15.6	31.25	7.8	9.3	–
K. pneumoniae ATCC 10031	62.5	–	250	125	125	62.5	125	–	–	–	CNE	CNE	–	62.5	9.3	–
S. thyphimurium CCM 5445	125	125	125	62.5	62.5	250	62.5	–	–	–	CNE	CNE	250	125	4.6	–
B. cereus ATCC 7064	–	–	–	250	125	–	62.5	–	62.5	125	CNE	CNE	–	–	9.3	–
C.albicans ATCC 10239	31.25	62.5	125	15.6	15.6	125	31.25	62.5	31.25	125	3.9	3.9	7.8	–	–	9.3
C. tropicalis RSKK 2412	62.5	62.5	250	62.5	31.25	250	62.5	125	125	125	62.5	–	–	125	–	9.3

-: Not Detected; CNE: microorganisms that "Could Not Evaluated", because of insufficient amount of skin secretion samples

13590 (MIC=15.6 µg/mL). Skin secretion of *L. b. eikeae* exhibited moderate antibacterial activity against *E. faecalis* ATCC 29212 with a MIC value of 15.6 µg/mL.

Cytotoxic effects

The cytotoxic effects of crude skin-parotoid gland secretions were measured against the following cell lines: HeLa, A549, Caco-2, MPanc-96, PC-3, U-87 MG, MDA-MB-231 cancer cells and non-cancerous cell line, HEK-293. Skin-parotoid secretions of the Lycian salamanders inhibit cell viability in a concentration-dependent manner. The IC_{50} values of skin secretions for treating cell lines are shown in Table 4.

Crude Lycian salamander skin-parotoid gland secretions showed highly potent toxic effects on all cancer and non-cancerous cell lines. The highest cytotoxic effect is found on A549 cell line with the skin secretion of *L. l. luschani* with an IC_{50} value 5.41±0.17 µg/mL. *L. l. luschani* skin secretion also showed high cytotoxic activity (IC_{50} value 9.44±0.31 µg/mL) on MPanc-96 cells. Skin-parotid gland secretion of *L. b. irfani* and *L. l. finikensis* are found to be highly cytotoxic for Caco-2 cells with IC_{50} values 11.61±0.43 µg/mL and 25.62±0.66 µg/mL respectively. Many of the other Lycian salamander skin secretions inhibit cell proliferation in a moderate or sufficient level.

Hemolytic activities

The hemolytic activities of crude skin-parotoid gland secretions of Lycian salamanders on rabbit red blood cells were analyzed. None of the skin-parotoid gland secretion samples of Lycian salamanders showed hemolytic effects. Hemolytic activity is not observed even at the highest concentration of 50 µg/mL. Absorbance of each concentration of the skin-parotoid gland secretions were found lower than absorbance of negative control saline (<0,128).

DISCUSSION

Secretions from the skin-parotoid glands of amphibians have been used for centuries for treating infections, bites, heart disorders, hemorrhages, allergies, inflammation, pain, and even cancer and AIDS *(18)*. Amphibians have a large variety of substances in their skin-parotoid secretion with main bioactive compounds such as peptides/proteins, steroids, alkaloids, biogenic amines, and enzymes *(19)*. There are many studies on the effects of the anuran skin secretions but only a few on urodelans, terrestrial salamanders being almost neglected.

Urodelans have toxic and noxious compounds in their skin secretions such as neurotoxic tetrodotoxin/tarichatoxin *(20)*. The exact mode of action of the salamander alkaloids has not been well described in literature. These alkaloids have nerve-blocking activity resulting in local anesthetic effects or respiratory paralysis causing death in *in vivo* studies. Salamander alkaloids, especially saman-

Table 4. *The IC50 values for cell lines following skin secretion exposure. Parthenolide was used as a positive cytotoxic control drug.*

Cell Lines IC_{50} (µg/mL) / Sample ID	HEK-293 (noncancerous kidney)	Caco-2 (colon)	HeLa (cervical)	A549 (lung)	U-87 MG (glioblastoma)	MPanc-96 (pancreas)	PC-3 (prostate)	MDA-MB-231 (breast)
Parthenolide	0.55 ± 0.02	1.65 ± 0.10	0.98 ± 0.01	0.26 ± 0.01	3.33 ± 0.19	0.91 ± 0.02	1.24 ± 0.09	2.78 ± 0.14
L. flavimembris flavimembris	46.94 ± 1.71	>50	–	–	>50	>50	>50	–
L. f. ilgazi	32.33 ± 1.23	35.87 ± 1.29	32.29 ± 1.21	32.71± 1.17	>50	48.07 ± 1.83	>50	46.10 ± 1.93
L. billae billae	49.10 ± 1.67	39.20 ± 1.42	>50	36.21 ± 1.31	>50	>50	>50	–
L. b. arikani	>50	44.31 ± 1.08	–	–	>50	>50	>50	>50
L. b. yebudabi	32.08 ± 0.97	>50	>50	>50	–	>50	>50	>50
L. b. irfani	35.78 ± 1.02	11.61 ± 0.43	–	>50	>50	>50	>50	58.95 ± 2.09
L. b. eikeae	38.04 ± 1.17	>50	54.38 ± 2.01	46.38 ± 1.23	>50	>50	>50	>50
L. luschani luschani	11.50 ± 0.39	>50	33.80 ± 1.02	5.41 ± 0.17	36.54 ± 1.49	9.44 ± 0.31	>50	31.77 ± 0.91
L. l. finikensis	>50	25.62 ± 0.66	>50	>50	–	>50	>50	>50
L. l. finikensis x *L. l. basoglui* (hybrid)	>50	>50	–	>50	>50	>50	>50	–
L. antalyana antalyana	>50	43.91 ± 1.54	–	>50	>50	>50	>50	>50
L. a. gocmeni	>50	>50	>50	>50	>50	>50	>50	>50
L. fazilae ulfetae	49.90 ± 1.87	49.54 ± 1.78	49.97 ± 1.89	46.85± 1.69	>50	55.83 ± 1.99	>50	44.30 ± 1.51
L. atifi veithi	47.73 ± 1.57	39.33± 0.95	–	>50	>50	>50	–	>50

-: Not Detected; >50: low calculated cytotoxic values which need much more than 50 µg/mL for IC50

darone, exhibit distinct antimicrobial activities, although being less potent than most antibiotics *(21, 22)*. Salamandrids, including Lycian salamanders are the unique natural sources of a class of steroidal alkaloids such as Samandarines, and can be found only in the skin secretion of these family members *(13)*.

Habermehl and Preusser *(10)* have stated that the crude cutaneous gland secretion of the fire salamander *Salamandra salamandra* inhibited several strains of gram-positive and -negative bacteria and fungi. Protein amounts of skin secretions of Lycian salamanders are found relatively lower than in the amphibians according to the literature. Protein amount of three bufonid toads (*B. bufo, B. verrucosissimus and B. viridis*) are determined as 3100, 3300 and 3480 µg/mL respectively *(23)*. In another recent study, the skin secretions from the smooth newt *Lissotriton vulgaris* and the Balkan crested newt *Triturus ivanbureschi* were found to be highly bioactive towards microorganisms and cancer cell lines *(24)*. Lycian salamander skin-parotoid secretions clearly contain lower amounts of protein. The two taxa (*L. l. luschani* and *L. f. flavimembris*) with highest scores 940 and 960 µg/mL, respectively, are about half the amount of the *L. vulgaris* and *T. ivanbureschi* (1775 and 1470 µg/mL). Besides, *L. a. gocmeni* and *L. l. finikensis* skin secretions showed higher antifungal effect (3.9 µg/mL) on *C. albicans* ATCC 10239 than *L. vulgaris* and *T. ivanbureschi* (7.8 and 15.6 µg/mL). Also, *L. b. irfani* has a higher antibacterial effect (7.8 µg/mL) on *E. faecalis* ATCC 29212 and *E. faecium* DSM 13590 than *L. vulgaris* and *T. ivanbureschi* (15.6-31.25 µg/mL). *L. vulgaris* and *T. ivanbureschi* skin secretions inhibited growth of lung and pancreas cancer cell lines as follows: A549 (13.04 and 11.27 µg/mL) and MPanc-96 (14.57 and 27.87 µg/mL); while *L. l. luschani* skin secretion was found more potent and highly effective on the same cancer cell lines with IC_{50} values of 5.41 and 9.44 µg/mL, respectively. *L. l. luschani* skin secretion showed higher cytotoxicity (33.80 µg/mL) on HeLa (cervix) cancer cell line than *T. ivanbureschi* skin secretion (40.28 µg/mL). *L. b. irfani* skin secretion showed a higher cytotoxic effect (11.61 µg/mL) on Caco-2 (colon) cancer cell line than *L. vulgaris* (24.40 µg/mL). These abovementioned comparisons are supporting and showing the potential of skin secretions of Lycian salamanders.

According to results, the most potent antifungal activity was found for *L. l. finikensis* and *L. a. gocmeni* skin secretions with a MIC value of 3.9 µg/mL and *L. b. eikeae* skin secretion with a MIC value of 7.8 µg/mL on *C. albicans* ATCC 10239 which are notably higher than the positive control drug Flucytosine. The most potent antibacterial activity was determined for the skin secretion of *L. b. irfani* against vancomycin-resistant *E. faecium* DSM 13590 and *E. faecium* ATCC 29212 with a MIC value of 7.8 µg/mL which is more

than the positive control drug, Ampicillin, remarkably. The most potent cytotoxic effect was found on A549 (human alveolar adenocarcinoma) cell line and MPanc-96 (human pancreas adenocarcinoma) using the skin-parotoid secretion of *L. l. luschani* with IC_{50} values 5.41 µg/mL, 9.44 µg/mL respectively. Also, *L. b. irfani* skin-parotoid secretion was detected equally potent on Caco-2 (human colon colorectal adenocarcinoma) cells with an IC_{50} value of 11.61 µg/mL.

Skin-parotoid secretion of terrestrial amphibians displayed no hemolytic effects described by previous studies *(23)*. Lycian salamanders are, also, terrestrial amphibians and their secretions have no hemolytic effects which was confirmed by our research. This is an important parameter for the systemic usage of a natural products as therapeutic agents/drugs. The absence of lysis of blood cells is highly increasing the therapeutic potential of a natural product likewise Lycian salamanders (*Lyciasalamandra* sp.) skin-parotoid secretions.

In conclusion, this study represents for the first time a bioactivity screening for potential pharmaceutical usage of skin-parotoid gland secretions of Anatolian endemic terrestrial salamander genus, *Lyciasalamandra*. Our results demonstrated that there are remarkable *in vitro* antimicrobial and cytotoxic effects of skin-parotoid secretions obtained from Lycian salamanders without hemolytic effects. According to these results, we will be focusing our future studies on the purification and characterization of the active components from selected species' skin-parotoid gland secretions and investigating of the possible mode of action of skin secretion-induced cytotoxicity and antimicrobial activity to obtain a better understanding of their potential use as anticancer and antimicrobial agents.

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