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CYTOTOXIC EFFECTS OF NON-HEMOLYTIC SKIN-PAROTOID GLAND SECRETIONS OF THE BUFONID TOADS FROM TURKEY

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Object: Skin-parotoid secretions of amphibians contain a large number of biologically active compounds which are thought to play several roles, either in the regulation of the physiological functions of the skin or in defense mechanisms against predators or microorganisms. Especially, the biodiversity of biochemical compounds in the auricular and skin glands of toads makes them unique sources for new therapeutic agents.

Material and Method: Common Toad-Bufo bufo, Caucasian Toad-Bufo verrucosissimus and Variable Green Toad-Bufotes variabilis were collected in field, then skin secretions obtained by stimulator, while parotoid gland secretions obtained by manual compressing. Each individual was rinsed with ultra-pure water into the tubes, then snap-frozen by liquid nitrogen and then lyophilized. Protein content was determined by BCA assay kit. Cytotoxic effects was determined against HeLa, A549, Caco-2, MPanc-96, PC-3, MDA-MB-231 cancer cells and HEK-293 as a non-cancerous cell line by MTT assay. Parthenolide was used as a positive cytotoxic control agent. Percentages of surviving cells and IC50 values in each cells were calculated after incubation with secretions using GraphPadPrism 5. Hemolytic activity of secretions was also determined on rabbit red-blood cells.

Results: Protein concentrations of B. bufo, B. verrucosissimus and B. variabilis secretions were calculated as 3100 µg/ml, 3300 µg/ml, 3480 µg/ml, respectively. Crude skin-parotoid gland secretions of all taxa were showed strong cytotoxic effect on all cancer and non-cancerous cells with an IC50 values varying between <0.1-6.02 µg/ml. No hemolytic activities at concentrations between 0.5-50 µg/ml were observed.

Conclusion: Further investigations need to focus on to purify the active components from these skin-parotoid secretions and determine the possible mode of action of secretion-induced cytotoxicity to obtain a better understanding of their potential use as anticancer agents.

Keywords: Toad, skin-parotoid gland secretion, cytotoxicity, hemolytic activity.

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Object: Toad glandular secretions and skin extractions contain numerous natural agents which may provide unique resources for novel drug development. Especially the skin-parotoid gland secretions of toads from genus Bufo contain as many as 86 different types of active compounds, each with the potential of becoming a potent drug. In the present study, crude skin-parotoid gland secretions from *Bufo bufo, Bufo verrucosissimus* and *Bufotes variabilis* from Turkey were screened against various cancer cells together with normal cells using MTT assay. Hemolytic activity of each skin-secretion was also estimated for evaluating pharmaceutical potential. Both skin-parotoid gland secretions showed high cytotoxic effect on all cancerous and non-cancerous cell lines with IC_{50} values varying between <0.1 µg/ml and 6.02 µg/ml. No hemolytic activities on rabbit red blood cells at concentrations between 0.5 µg/ml and 50 µg/ml were observed. In conclusion, skin-parotoid secretions of bufonid toads might be remarkable candidates for anti-cancer agents without hemolytic activities.

Figure 1. *Bufo bufo, Bufo verrucosissimus* and *Bufotes variabilis* from Turkey

Material and Methods

Field studies and collection of skin-parotoid gland secretions: A Common Toad *B. bufo* specimen was collected during the field excursion in Geyikbayırı, Konyaaltı/Antalya province, southwestern Turkey in March-2015. The Caucasian Toad *B. verrucosissimus* specimen was collected from Güzelyalı, Fındıklı/Rize and the Variable Green Toad *B. variabilis* specimen was collected from Bork, Hanak/Ardahan during field studies in northeastern Anatolia in June-2014. The authors received special permission for the field studies from the Republic of Turkey, Ministry of Forestry and Water Affairs, Directorate of Nature Conservation and National Parks (permit number: 2014-51946). Skin secretions obtained by mild electrical stimulation (5–10 V) by stimulator (C.F. Palmer, London), while parotoid gland secretions obtained by manual compressing. Skin secretions and parotoid gland secretions were pooled for each species, clarified by centrifugation (6000 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 The ethical permission received for the milking procedures from Ege University Animal Experiments Ethics Committee (with approval number of 2014-002).

Protein content determination: Protein content was assayed for each diluted skin secretion (2 mg/ml) samples in ultra-pure wate by BCA assay kit (Thermo Scientific).

Cell culture and *in vitro* cytotoxicity assay: The following cell lines were used for determination of cytotoxicity: HeLa, A549, Caco-2, MPanc-96, PC-3, MDA-MB-231 cancer cells and as a non-cancerous cell line, HEK-293. Parthenolide was used as a positive cytotoxic control agent. Cell lines were purchased from ATCC and cultivated in DMEM/F12, supplemented with 10% FBS, 2 mM L- glutamine, 100 U/ml of penicillin and 100 µg/ml of streptomycin (Lonza). Cytotoxicity of crude skin and parotoid gland secretions were determined by following the general procedure based on cell viability using a modified colorimetric MTT assay. The cells were incubated at 37 °C in a humidified atmosphere of 5% CO_2 for 24h in 96-well microplates with an initial concentration of $1x10^5$ cells/ml. Subsequently, the cultured cells were treated with different doses of skin-secretions (50, 5 and 0.5 µg/ml) and incubated for 48 h at 37 °C. Percentages of surviving cells and half maximal inhibitory concentration (IC_{50}) in each culture were calculated after incubation with secretions. The IC_{50} values were estimated using GraphPadPrism 5 software (CA, USA).

Hemolytic activity assay: Red blood cells were obtained from healthy New Zealand rabbits. 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 button 96-well microplate with 0.05 ml diluents containing 50, 5 and 0.5 µg/ml concentrations of crude *B. bufo, B. verrucosissimus* and *B. variabilis* skin-parotoid gland secretions in saline solutions. The mixtures were incubated for 30 min at 37 °C and centrifuged at 2000 rpm 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.

Results: The total protein and peptide concentrations were determined by BCA assay for *B. bufo, B. verrucosissimus* and *B. variabilis* as 3100 µg/ml, 3300 µg/ml, 3480 µg/ml, respectively. In Figure 2 it can be seen that crude secretions of the *B. bufo, B. verrucosissimus* and *B. variabilis* inhibits cell viability in a dose-dependent manner. The IC_{50} values for all affected cell lines are shown in Table 1. No hemolytic activities at concentrations between 0,5-50 µg/ml were observed, Table 2.

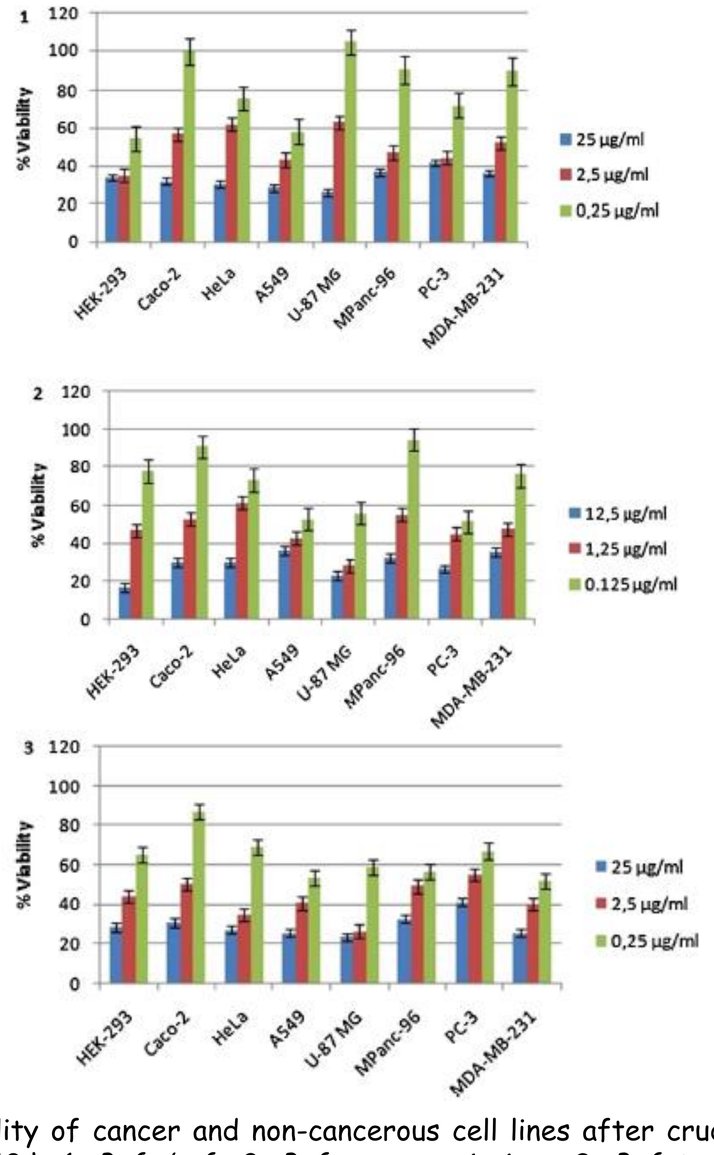

Figure 2. Viability of cancer and non-cancerous cell lines after crude skin secretion treatment for 48 h. 1: *Bufo bufo,* 2: *Bufo verrucosissimus*, 3*: Bufotes variabilis*.

Table 1
The IC_{50} values for tumor cells and normal human cells following *crude B. bufo, B. verrucosissimus* and *B. variabilis* skin secretions exposure by MTT assay. Parthenolide was used as positive control. Standard deviation (SD) was calculated from three independent samples, mean ± SD.

Cell Lines IC_{50} µg/ml SampleID	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 ± 0.010	0.26 ± 0.01	3.33 ± 0.19	0.91 ± 0.020	1.24 ± 0.09	2.78 ± 0.14
Bufo bufo	0.35 ± 0.01	5.99 ± 0.18	4.92 ± 0.34	0.85 ± 0.09	6.02 ± 0.38	4.74 ± 0.22	3.53 ± 0.20	5.56 ± 0.16
B. verrucosissimus	0.99 ± 0.02	2.26 ± 0.24	2.33 ± 0.22	0.80 ± 0.01	<0.1	2.78 ± 0.33	0.81 ± 0.05	1.70 ± 0.07
Bufotes variabilis	1.46 ± 0.03	4.06 ± 0.18	1.15 ± 0.14	0.47 ± 0.05	1.26 ± 0.10	1.20 ± 0.27	5.71 ± 0.16	0.38 ± 0.02

Table 2
Hemolytic activities of crude *B. bufo*, *B. verrucosissimus* and *B. variabilis* skin-parotoid gland secretions. Hemolytic percent of saline and distilled water were included as minimal and maximal hemolytic control. All values represent the mean ± standard deviation (n = 3 test).

Controls	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
Samples			
Bufo bufo	50	0.125 ± 0.009	–
	5	0.121 ± 0.007	–
	0.5	0.119 ± 0.006	–
Bufo verrucosissimus	50	0.124 ± 0.010	–
	5	0.122 ± 0.008	–
	0.5	0.120 ± 0.007	–
Bufotes variabilis	50	0.126 ± 0.011	–
	5	0.123 ± 0.008	–
	0.5	0.121 ± 0.005	–

–Not Detected.

Conclusion: Further investigations need to focus on to purify the active components from these skin-parotoid secretions and determine the possible mode of action of secretion-induced cytotoxicity to obtain a better understanding of their potential use as anticancer and agents.

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