Biomedicine & Pharmacotherapy 80 (2016) 298–303

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Biological activities of skin and parotoid gland secretions of bufonid toads (*Bufo bufo, Bufo verrucosissimus* and *Bufotes variabilis*) from Turkey

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ARTICLE INFO

Article history:
Received 9 November 2015
Received in revised form 8 March 2016
Accepted 9 March 2016

Keywords:
Amphibian skin secretion
Cytotoxicity
Antimicrobial activity
Hemolytic activity

ABSTRACT

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. Furthermore, the antimicrobial properties of skin secretions were tested on selected bacterial and fungal species for assessing the possible medical applications. Antimicrobial activity of skin secretions was studied by determining minimal inhibitory concentration (MIC) in broth dilution method. 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. MIC results of antimicrobial activity tests were found to be between 3.9 μg/ml and 250 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 and antimicrobial agents without hemolytic activities.

1. Introduction

Natural products have been used extensively in the treatment of many diseases [1,2]. Anti-inflammatory and anticancer drugs derived from naturally-occurring substances have received significant attention throughout the world [3]. Skin secretions of amphibians contain large numbers of biologically-active compounds, which are thought to play several roles, including the regulation of the physiological functions of the skin, or as defense mechanisms against predators or microorganisms. The diversity of chemical compounds in the auricular and skin glands of toads makes them especially important sources, from which new therapeutic agents can be developed [4]. Additionally, toad glandular secretions have been used for treating infection and inflammation for centuries in Traditional Chinese Medicine (TCM), in China and East/Southern East Asian countries. “Chan Su” and “Cinobufacini” (Huachansu) have been important medicines in TCM in China and other Asian countries, and have been used to treat a number of diseases, including sore throat, edema, pain, heart failure, skin problems, and cancers. They have also been used as an anodyne, cardiotonic, antimicrobial, local anesthetic and antineoplastic agents [5–7]. Recent studies indicate that toad glandular secretions and skin extractions can have anti-inflammatory and anticancer properties. Therefore, these types of natural products may provide a potential new strategy as combinational and/or complimentary therapies for cancer treatment by targeting key NF-κB signaling molecules and their pathways [8]. Telocinobufagin is also one of the major active monomers isolated from Chan Su and has been shown to have an anticancer effect on the liver carcinoma cell line, PLC/PRF/5 [9]. Besides, inhibition of the cyclooxygenase (COX) may provide relief from symptoms of inflammation and pain. Jiang et al. [10] indicated that bufalin

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http://dx.doi.org/10.1016/j.biopha.2016.03.034
0753-3322/

reduced the expression level of COX-2 protein in A549 cell line. Ko et al. [11] also showed that Chan Su could inhibit of the COX-2 level in T24 cell line. Bufonid skin and auricular gland secretions inhibited lung carcinoma (A549) cells by induction of apoptosis. Apoptosis of A549 cells was accomplished trough a signaling cascade of death receptor-mediated extrinsic and mitochondria-mediated intrinsic caspase pathways [12].

Increasing of resistance to the antibiotics currently employed in clinical practice is a continual stimulus for further research aiming the identification of novel antimicrobial compounds. This provides a new perspective on the skin secretions of amphibians [4,13,14]. Antimicrobial peptides (AMPs) found in animal secretions. They are components of host-innate immune responses and have survived eons of pathogen evolution. Thus, they are likely to be active against pathogens and even those that are resistant to conventional drugs. Many peptides have been isolated and shown to be effective against multi-drug-resistant pathogens. More than 500 AMPs have been identified from amphibians [14,15]. The abundance of AMPs in frog skin is remarkable and constitutes a rich resource for the design of novel pharmaceutical molecules [15]. Skin-secretions contain four types of compounds: biogenic amines, bufadienolides, alkaloid steroids, peptides and proteins. These are produced in the holocrinetype serous glands in the integument, where they are stored as granules in the lumen of the cells and rereleased upon stimulation [6,16,17]. These compounds are thought to play different roles, either in the regulation of physiological functions of the skin, or in defense against predators or microorganisms. Antimicrobial peptides are considered as the effector molecules of innate immunity, acting as a first line of defense against bacterial infections, by perturbing the phospholipid bilayer of the target cell membrane.

In brief, bulk of research relating to amphibian antimicrobial secretions has been carried out on frogs. Bufadienolides and their conjugates may be found in free and conjugated forms in the tissues and body fluids of toads of the genus *Bufo* [14]. In toads, such research has been carried out only on *Bufo rubescens*, *Bombina orientalis*, *Bufo arenarum, Bufo bufo gargarizans* and *Bufo melanostictus* [14,17–19]. Common Toad-*Bufo bufo* and Variable Green Toad—*B. variabilis* have wide distribution in Turkey. Caucasian Toad—*B. verrucosissimus* has limited distribution range in northwestern Anatolia in Turkey.

Based on our continuing studies on skin secretion of amphibian species of Turkey, the main purpose of this study was to investigate cytotoxic, antimicrobial and hemolytic effects of *B. bufo, B. verrucosissimus* and *B. variabilis* skin-secretions on various cancerous and non-cancerous cells, microorganisms and rabbit red blood cells to evaluate their potential use in medicine as a therapeutic agent.

2. Materials and methods

2.1. 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. Each individual was rinsed with ultra-pure water [20]. 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. Secretion harvesting was performed in the field; the toads were then released to their natural habitats, unharmed. The authors received ethical permission for the milking procedures from Ege University Animal Experiments Ethics Committee (with approval number of 2014-002).

2.2. Protein content determination

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

2.3. Cell culture and in vitro cytotoxicity assay

The following cell lines were used for determination of cytotoxicity: HeLa (human cervix adenocarcinoma), A549 (human alveolar adenocarcinoma), Caco-2 (human colon colorectal adenocarcinoma), MPanc-96 (human pancreas adenocarcinoma), PC-3 (human prostate adenocarcinoma), U87MG (human glioblastoma-astrocytoma), MDA-MB-231 (human mammary gland adenocarcinoma) cancer cells and HEK-293 (human embryonic kidney) as non-cancerous cell line. 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. 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 [21,22]. The optical density (OD) was measured in triplicates at 570 nm (with a reference wavelength 630 nm) by UV/Vis spectrophotometry (Thermo, Bremen, Germany). All cell lines were cultivated for 24 h in 96-well microplates with an initial concentration of 1×10^5 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. The plant-derived compound parthenolide was used as a positive cytotoxic control agent. Percentages of surviving cells in each culture were determined after incubation with skin secretions. The viability (%) was determined by the following formula:

$$\%\ \text{Viable cells} = [(\text{absorbance of treated cells}) - (\text{absorbance of blank})]/[(\text{absorbance of control}) - (\text{absorbance of blank})] \times 100$$

2.4. 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}) were calculated in comparison to untreated controls.

2.5. Microorganisms

Gram-positive and gram-negative bacteria and yeast were used for antimicrobial activity studies. The Gram-negative bacteria used were enteropathogenic *Escherichia coli* 0157:H7 (RSKK 234) and *Salmonella thyphimurium* CCM 5445. The Gram-positive bacteria used were *Enterococcus fecalis* ATCC 29212, vancomycin-resistant *Enterococcus faecium* DSM 13590, methicillin-resistant *Staphylococcus aureus* ATCC 43300, and *Staphylococcus epidermidis* ATCC 12228 and *Candida albicans* ATCC 10239 was used as yeast. The lyophilized bacteria and yeast were obtained from Ege University, Faculty of Science, Department of Basic and Industrial Microbiology.

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

MIC values for skin secretion were determined by broth micro-dilution technique [23,24]. Test microorganisms were grown in MH broth for 5 h (exponential phase) and adjusted to 0.5 McFarland turbidity standard (A600 = 1.0), corresponding to 1.5×10^6 CFU/ml. MICs were determined according to the National Committee for Clinical Laboratory Standards (NCCLS, 2009). Serial dilutions of skin-secretions (0.9–500 μg/ml) were prepared in 96-well plate, at a final volume of 80 μl. Then, 20 μl of the adjusted bacterial 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 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 antibacterial and antifungal agents, respectively, as a positive control (0.5–256 μg/ml). All assays were run using 3 replicates.

2.7. Hemolytic activity assay

The hemolytic activity of crude *B. bufo*, *B. verrucosissimus* and *B. variabilis* skin-parotoid gland secretions were measured according to the modified method of [25,26]. Red blood cells were obtained from healthy New Zealand rabbits (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 rpm 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 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. The hemolytic percent developed by the saline control was subtracted from all groups. Each experiment included triplicates at each concentration.

3. Results

3.1. Protein contents

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.

3.2. Cytotoxicity screening

We assessed the cytotoxicity of bufonids crude skin and parotoid gland secretions against the following cell lines: HeLa, A549, Caco-2, MPanc-96, PC-3, U-87 MG, MDA-MB-231 cancer cells and as a non-cancerous cell line, HEK-293. In Fig. 1 it can be seen that crude secretions of the *Bufo bufo*, *B. verrucosissimus* and *Bufotes variabilis* inhibits cell viability in a dose-dependent manner. The IC_{50} values for all affected cell lines are shown in Table 1.

Crude *B. bufo*, *B. verrucosissimus* and *B. variabilis* skin-parotoid gland secretions displayed high toxic effects on all cancerous and non-cancerous cell lines with IC_{50} values varying between <0.1 μg/ml and 6.02 μg/ml. Remarkably, all skin-parotoid gland secretion samples displayed cytotoxicity against tested cell lines as plant-derived sesquiterpene lactone parthenolide. Moreover, we found the highest anticancer activity on the A549 cell line (IC_{50}: 0.26–0.80 μg/ml). Throughout the post-treatment, morphological changes were observed with skin-parotoid gland secretions of each

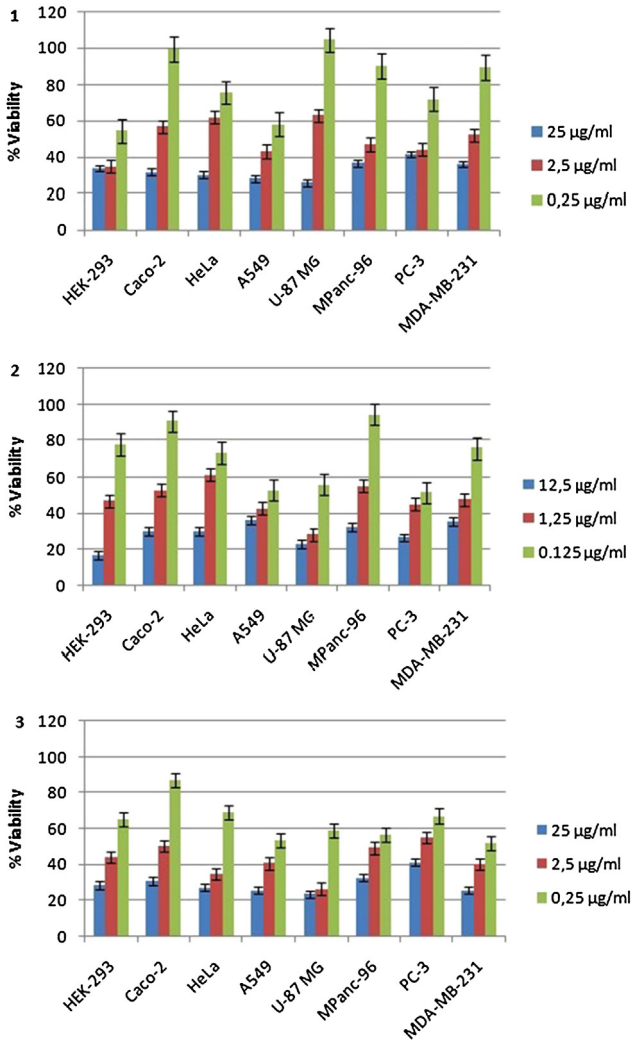

Fig. 1. Viability of cancer and non-cancerous cell lines following different crude skin secretion concentrations treatment for 48 h. Cell viability was determined by MTT assay, control was exposed to vehicle only which was taken as 100% viability. 1: *Bufo bufo*, 2: *Bufo verrucosissimus*, 3: *Bufotes variabilis*. HeLa (human cervix adenocarcinoma), A549 (human alveolar adenocarcinoma), Caco-2 (human colon colorectal adenocarcinoma), MPanc-96 (human pancreas adenocarcinoma), PC-3 (human prostate adenocarcinoma), U87MG (human glioblastoma-astrocytoma), MDA-MB-231 (human mammary gland adenocarcinoma) cancer cells and HEK-293 (human embryonic kidney) as non-cancerous cell line.

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

species, 48 h after exposure. Increasing concentrations resulted in increased number of rounded cells, growth inhibition and the incidence of various morphological abnormalities with larger areas devoid of cells when compared to untreated control cells.

3.3. 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 the yeast are presented in Table 2. The values indicate that skin secretion have MIC values varying from 3.9 μg/ml to 250 μg/ml. Skin secretions exhibited the most potent activity against *E. faecalis* ATCC 29212, *E. faecium* DSM 13590 and *S. epidermidis* ATCC 12228. Skin secretions of *B. bufo* and *B. verrucosissimus* showed moderate antibacterial activity against *S. aureus* ATCC 6538 P (MIC = 62.5 μg/ml).

3.4. Hemolytic activity

The hemolytic activities of crude *B. bufo*, *B. verrucosissimus* and *B. varibilis* skin-parotoid gland secretions on rabbit red blood cells were shown in Table 3. No hemolytic activities at concentrations between 0.5 μg/ml and 50 μg/ml were observed. Absorbance rates of each concentration of the skin and auricular gland secretions were found to be lower than negative control saline (<0.128).

4. Discussion and conclusion

The skin secretions of amphibians show differentiation across different species with biologically active content such as peptides/proteins, steroids, alkaloids, biogenic amines and enzymes [6,16,17]. They can be useful and valuable as pharmacological tools in drug research and potential drug design templates. In this study, cytotoxic, antimicrobial and hemolytic properties of crude *B. bufo, B. verrucosissimus* and *B. variabilis* skin-parotid gland secretions were determined.

Table 3
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.

Many amphibian wide-spectrum antibiotic peptides also exhibit anticancer activity. The antibiotic and anticancer activities are the result of the active peptide-inducing alterations in the hydrophobic–hydrophilic seal of the cell membrane, affecting the lysis of bacterial or cancer cells [27].

Cunha-Filho et al. [14] 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). Our MIC results were not similar on *E. coli* (no activity determined). However, we found similar effects on *S. aureus* for *B. variabilis* with MIC value of 125 μg/ml and higher inhibition with *B. bufo* and *B. verrucosissimus* (MIC: 62.5 μg/ml).

In this study, the effects of *B. bufo, B. verrucosissimus* and *B. variabilis* crude skin-parotid gland secretions were analyzed on cancer and normal cells by MTT assay. The results displayed significant cytotoxic activity against HeLa, A549, Caco-2, MPanc-96, PC-3, U-87 MG, MDA-MB-231 cancer cells and as a non-

Table 2
MIC values of *B. bufo, B. verrucosissimus and B. varibilis* crude skin secretions and reference antibiotics against test organisms using broth dilution method. All values represent the mean ± standard deviation (n = 3).

Microorganisms	MIC (μg/ml) values for skin secretion and antimicrobial agents				
	B.b	B.ver	B.var	Ampicillin	Flucytosine
E. coli O157:H7	–	–	–	4.0	–
S. aureus ATCC 43300	62.5	62.5	125	4.0	–
E. faecalis ATCC 29212	3.9	3.9	3.9	8.0	–
E. faecium DSM 13590	3.9	3.9	3.9	4.0	–
S. epidermidis ATCC 12228	3.9	3.9	7.8	2.0	–
S. thyphimurium CCM 5445	250	–	125	4.0	–
C. albicans ATCC 10239	250	125	125	–	8.0

B.b: *Bufo bufo;* B.ver: *Bufo verrucosissimus;* B.var: *Bufotes variabilis.*

cancerous cell line, HEK-293 with an IC_{50} varying between <0.1 μg/ml and 6.02 μg/ml after 48 h treatment, in a time-and-dose-dependent manner, showing potential for further development of therapeutic agents.

Toad glandular secretions and skin extractions can be made to different types of medicine, including oral solutions, ointments, injections, and coating agents. One of the most widely-used commercial preparations is Huachansu (Cinobufacini), which is a sterilized hot water extract of dried toad skin secretions [28]. Since 1991, Cinobufacini has been officially approved by the Chinese Food and Drug Administration as a regimen for treating patients with Hepatitis B virus and several types of cancer including liver, lung, colon, and pancreatic cancers [29]. An *in vivo* assay showed that the Cinobufacini injection inhibits the growing of mouse Lewis lung cancer cells with the response rate of 45–50% and prolongs their life [30]. Clinical trials of Cinobufacini injection have been conducted since the 1970s in China. The results have demonstrated the anticancer effect of Cinobufacini injection in advanced hepatocellular carcinoma and lung cancer patients with a total response rate of 10% and 16%, respectively [31]. A Phase I clinical trial conducted by Meng et al. [32] further examined the tolerable toxicity in patients. The result showed that Cinobufacini injection could be tolerated up to 8 times higher than normal administrated does. Up to 40% of lung and liver cancer patients had tumors stabilized in this trial. Additionally, Cinobufacini injections promoted the efficacy of the conventional therapies while lowering their toxicity when used in combination with chemotherapy or radiotherapy [29,33].

Jiang et al. [10] found that bufalin at the concentration of 2.5–10 μM reduced expression level of COX-2 protein and cytotoxic in A549 cells. In addition, they also found that bufalin suppressed the phosphorylation and expression of NF-κB. The study results also exhibited significant cytotoxic effects on A549 cell line with IC_{50} values 0.85, 0.80 and 0.47 μg/ml for *B. bufo*, *B. verrucosissimus*, *B. variabilis*, respectively. Chen et al. [34] demonstrated that bufalin inhibits migration and invasion in human hepatocellular carcinoma SK-Hep1 cells at least partly by the inhibition of the NF-κB signaling pathway. Ko et al. [11] indicated that Chansu inhibited the level of COX-2 mRNA and protein expression and also the PGE2 synthesis without significant influence on the level of COX-1 in human bladder carcinoma T24 cells. Cunha-Filho et al. [35] performed the cytotoxicity of *Rhinella schneideri* macrogland secretion derivatives on ileocecal colorectal adenocarcinoma (HCT-8) and they found IC_{50} values up to 1.20 μM with no hemolytic activity. We found IC_{50} values on colorectal adenocarcinoma, Caco-2 IC_{50} value as 5.99, 2.26 and 4.06 μg/ml for *B. bufo*, *B. verrucosissimus and B. variabilis*, respectively with no hemolytic activities. Yun et al. [12] determined 5 μg/ml of IC_{50} value for A549 cells for Chan Su following 24 h of incubation. Our findings against cancer cells include the same cell line IC_{50} value of 0.85, 0.80 and 0.47 μg/ml for *B. bufo*, *B. verrucosissimus*, *B. variabilis*, respectively for 48 h incubation with crude secretions. The results mentioned above support our cytotoxicity results, demonstrating potential for cancer treatment.

In conclusion, the results indicated considerable higher inhibition on cancer cell lines and microorganisms with no hemolytic activities and growth inhibition on microorganisms. Further studies are needed to focus on the purification the active component from these skin secretions and on the possible mode of action of skin secretion-induced cytotoxicity and antimicrobial activity in order to obtain a better understanding of their potential use as anticancer and antimicrobial agents.

Conflicts of interest

There are no known conflicts of interest.

Financial source

This work was supported by the Scientific and Technical Research Council of Turkey (TÜBİTAK) under Grant 114Z599, Ege University Scientific Research Project Commission (Project No: 15-FEN-004) and Ege University Science and Technology Centre (EBİLTEM) under Grant 15-BIL-006. We also thank Mehmet Kosem for editing the manuscript.

Acknowledgements

We would like to extend our thanks to AREL (Research and Education Laboratory, Ege University School of Medicine) for their laboratory facilities during our study.

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