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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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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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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.