RESEARCH ARTICLE

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Cytotoxic Activity of Parotid Gland Toxins from *Ingerophrynus philippinicus* (Philippine Toad) Against Human Colorectal Carcinoma (HCT-116)

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Abstract

Background: Colorectal cancer is the third most prevalent cancer and the second leading cause of cancer-related deaths worldwide. Limitations of current treatments include adverse effects and drug resistance, highlighting the need for new chemotherapeutic agents. This study evaluated the cytotoxic activity of crude methanolic extract of the parotid gland secretions of *Ingerophrynus philippinicus* (PGSE), a toad endemic to the Philippines, against human colorectal carcinoma cell line (HCT-116) and identified its bioactive compounds. **Methods:** Crude methanolic extract of parotid gland secretions was prepared and evaluated for antiproliferative activity using the MTT assay. An Annexin V/PI assay was used to assess apoptosis and necrosis. The presence of bufadienolides was determined using Ultra Performance Liquid Chromatography-Quadrupole Time-of-Flight Double Tandem Mass Spectrometry (UPLC-QTOF-MS/MS). **Results:** The extract exhibited cytotoxic activity against HCT-116 cells in a dose-dependent manner with an IC_{50} of 4.462 ± 2.141 µg/mL. The Annexin V/PI assay revealed that treated cells predominantly underwent late apoptosis (89.52% ± 2.25%), with smaller proportions of early apoptosis (6.16% ± 2.49%), and necrosis (4.32% ± 0.29%). The UPLC-QTOF-MS/MS analysis identified three bufadienolides (bufalin, bufotalin, and bufotalinin) in the PGSE. **Conclusion:** PGSE demonstrates potent cytotoxic activity against colorectal cancer cells, primarily through apoptosis, and contains bufadienolides with known antineoplastic activity. These findings support its potential as a source of bioactive compounds for anticancer research.

Keywords: Bufadienolides, MTT assay, Apoptosis, IC_{50}, UPLC-QTOF-MS/MS, *Ingerophrynus philippinicus*

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Introduction

In 2022, colorectal cancer (CRC) was the third most prevalent cancer and the second leading cause of cancer-related deaths globally, with over 1.9 million new cases and more than 900,000 deaths [1]. The International Agency for Research on Cancer (IARC) reported increasing CRC incidence in several Asian countries, whereas incidence declined in North America, Europe, and Oceania [2]. Current treatment options include surgery, chemotherapy, targeted therapy, and immunotherapy. However, prolonged chemotherapy can lead to systemic toxicity and the development of drug resistance, which limits treatment efficacy [3]. These limitations highlight the need for new chemotherapeutic agents.

Natural products have historically served as an important source of anticancer agents [4]. Specifically, toad secretions contain bioactive compounds with pharmacological activities, including antitumor, anti-infective, analgesic, and cardiotonic effects [5]. Among these, bufadienolides are the primary active compounds, demonstrating cytotoxic activity against several cancer cell lines [4]. Studies have shown the anticancer activity of bufadienolides from species such as Rhinella marina, Rhinella schneideri, and Rhinella ornata [6, 7]. Bufadienolides exert anticancer effects by reversing drug resistance, inhibiting cancer cell growth, inducing apoptosis and autophagy, suppressing metastasis, and regulating cell cycle progression [4].

The composition of bufadienolides in parotid gland secretions (PGS) varies across species, habitats, and collection methods. In some species, such as Melanophryniscus spp., bufadienolides are absent [5, 8]. A previous study on the Philippine toad Bufo biporcatus

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philippinicus Inger (currently recognized as *Ingerophrynus philippinicus*) reported the presence of biogenic amines and bufadienolides in skin and parotid gland secretions using thin-layer chromatography, high-performance liquid chromatography, and infrared spectroscopy [9]. The study suggested the presence of bufadienolides such as bufalin, gammabufotalin, and resibufogenin; however, detailed compound characterization and cytotoxic evaluation were not performed, leaving the anticancer effects of *I. philippinicus* insufficiently explored.

Six endemic toad species in the Philippines belong to the family Bufonidae. *I. philippinicus*, which is endemic to the Palawan Island Group, remains understudied compared with other Philippine bufonids [10, 11]. The International Union for Conservation of Nature (IUCN) lists *I. philippinicus* as Least Concern due to its stable population and adaptability to environmental changes [12]. This status supports its suitability for study, as collecting parotid gland secretions can be performed without posing a significant threat to the species' population. Therefore, given its endemic status and limited prior investigation, *I. philippinicus* was selected for this study.

This study evaluated the anticancer potential of crude methanolic extract of the parotid gland secretions of *I. philippinicus* (PGSE) against HCT-116. Specifically, it aimed to (1) determine the half-maximal inhibitory concentration (IC_{50}) of PGSE using the MTT assay, (2) assess apoptosis and necrosis via Annexin V/PI assay, and (3) identify bufadienolides through UPLC-QTOF-MS/MS.

Materials and Methods

Experimental Workflow

Experimental procedures were conducted across collaborating laboratories. Preparation of crude extracts from parotid gland secretions was performed at the Biotechnology and Biomedical Research Laboratory, West Visayas State University College of Medicine (WVSU-COM), Iloilo City, Philippines. MTT and Annexin V/PI assays were conducted at the Mammalian Cell Culture Laboratory (MCCL), Institute of Biology, University of the Philippines Diliman, Quezon City, Philippines. Chemical profiling using UPLC-QTOF-MS/MS was conducted at the Applied Chemistry Laboratory, Regional Research Center (RRC), University of the Philippines Visayas, Miagao, Iloilo, Philippines. All experiments were performed in accordance with the standard operating procedures of each laboratory. Raw experimental data were provided by the laboratories and independently analyzed by the authors. Detailed experimental procedures are described in the following subsections.

Chemicals and Reagents

Analytical-grade methanol was used for PGS extraction. Dimethyl sulfoxide (DMSO), MTT reagent (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide), and doxorubicin hydrochloride were utilized for cytotoxicity assays.

For cell culture experiments, McCoy's 5A medium, fetal bovine serum (FBS), antibiotic–antimycotic solution, and Dulbecco's phosphate-buffered saline (DPBS) were used. Apoptosis assays utilized the Dead Cell Apoptosis Kit with Annexin V Alexa Fluor™ 488 and Propidium Iodide (Thermo Fisher Scientific, USA), with DAPI used for nuclear staining. Cell culture reagents were supplied by the MCCL.

For chemical profiling, LC–MS grade acetonitrile and methanol (Merck, Germany), formic acid (Honeywell Fluka, USA), and ultrapure water generated using a Milli-Q Integral 3 purification system (Merck Millipore, Germany) were used. Samples were filtered through 0.22 µm nylon membrane syringe filters (Waters, USA) and transferred into LC–MS glass vials (Waters, USA). Sodium iodide calibration solution and leucine enkephalin (Waters, USA) were used as the calibration and reference standards, respectively.

Sample Collection

Seventy-five individuals of *I. philippinicus* were collected in Brgy. Irawan, Puerto Princesa City, Palawan Island, Philippines (Figure 1). Specimens were identified

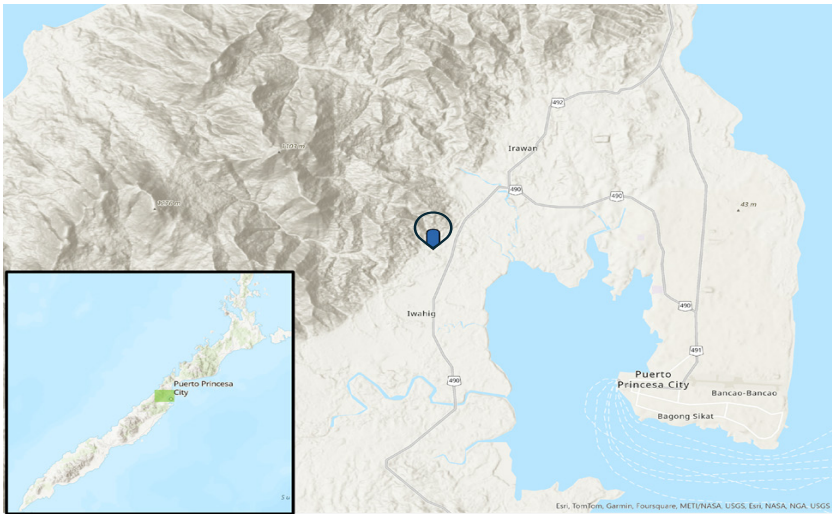

Figure 1. Map Showing the Sampling Site at Brgy. Irawan, Puerto Princesa City, Palawan Island, Philippines (blue marker).

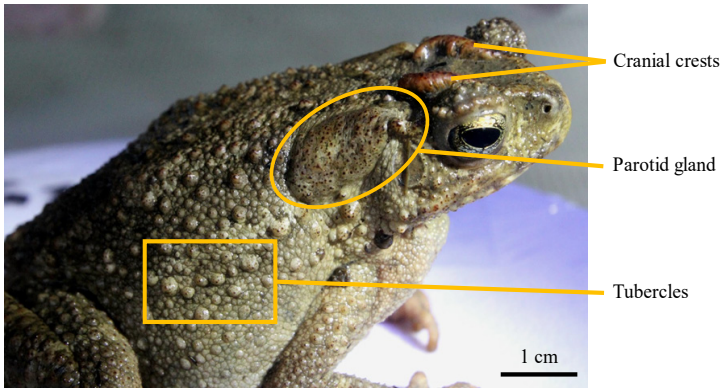

Figure 2. Identifying Morphologic Characteristics of *I. philippinicus* (Cranial Crests, Parotid Gland, and Skin Tubercles) [11].

based on morphological characteristics described by Alcala and Brown (1998) (Figure 2) [13]. Overhead and lateral images with corresponding measurements were submitted to the wildlife biologist for taxonomic confirmation. Two individuals with visible anatomical defects were excluded, resulting in 73 samples.

Parotid gland secretions (PGS) were obtained by gently pressing the parotid glands following the procedure by Schmeda-Hirschmann et al. [6] with minor modifications (Figure 3a). Secretions were collected in separate sterile microcentrifuge tubes for each individual and labeled accordingly (Figure 3b). All toads were released at the capture site immediately after PGS collection.

Animal handling procedures were conducted in accordance with local wildlife regulations and approved collection permits. Collection of specimens was authorized under the Wildlife Gratuitous Permit (GP No. 2023-20) and specimen transport under Local Transport Permit No. PPC-LTP-AO-12-2023-1150 issued by the Palawan Council for Sustainable Development (PCSD). Samples were transported to the laboratory and stored at -20°C until further processing.

Preparation of Crude Methanolic Extract of PGS

PGS stored in 73 individual microcentrifuge tubes were extracted using 500 µL methanol per vial, ultrasonicated for 10 minutes, and centrifuged at 5,500 rpm for 5 minutes [14]. The supernatants were pooled and air-dried under ambient laboratory conditions (22–24 °C) for 8 days to allow gradual solvent evaporation while minimizing thermal degradation. The dried residue was frozen at -80°C for 24 hours, then lyophilized at –55°C to complete dryness. The lyophilized extract was stored at –80°C until further use.

Cell Line Source and Authentication

The HCT-116 cell line (ATCC CCL-247) was obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). Cells were cultured in McCoy's 5A medium (Gibco, Thermo Fisher Scientific) supplemented with 10% fetal bovine serum and maintained at 37 °C in a humidified incubator with 5% CO_2. Cell lines obtained from ATCC are authenticated by short tandem repeat (STR) profiling and tested for contamination prior to distribution. The cell line was used within six months post-revival and was not further authenticated by STR

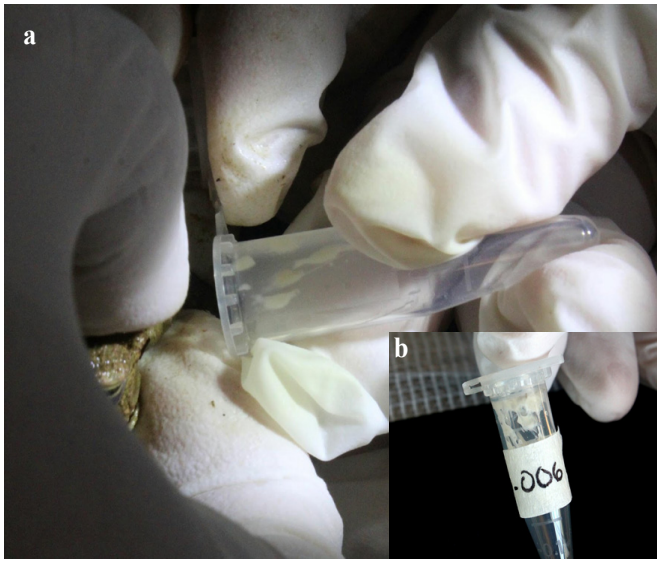

Figure 3. (a) Manual expression of PGS by gentle squeezing of the parotid gland of the toad. (b) Microcentrifuge tube containing PGS expressed from a single toad.

profiling.

MTT Assay

The antiproliferative activity of crude methanolic extract of the parotid gland secretions of *I. philippinicus* (PGSE) against HCT-116 cells was evaluated using the MTT assay based on Mosmann [15] with minor modifications. HCT-116 cells were seeded in sterile 96-well plates at a density of 8,000 cells per well and incubated overnight at 37 °C in a humidified 5% CO_2 atmosphere to allow cell attachment.

Eight serial dilution concentrations of PGSE prepared in DMSO were tested, ranging from 100 µg/mL to 0.78125 µg/mL. Doxorubicin served as the positive control at concentrations ranging from 6.25 µg/mL to 0.048828 µg/mL. DMSO (1% v/v in culture medium) served as the negative control and solvent blank. Cells were treated for 72 hours.

After treatment, MTT reagent (0.5 mg/mL) was added to each well and incubated for 3 hours. The formazan crystals were dissolved using DMSO, and absorbance was measured at 570 nm using a microplate reader. This procedure was performed with three biological replicates using cells derived from three independent culture flasks.

The IC_{50} values were calculated using GraphPad Prism version 10.0.2 with non-linear regression (log[inhibitor] vs. response). Samples with IC_{50} values <10 µg/mL were considered highly active, 10–29.99 µg/mL active, 30–100 µg/mL moderately active, and >100 µg/mL weak to inactive [16]. Percent inhibition was computed using the following formula: Percent inhibition (%) = [1 − (OD_{570} sample / OD_{570} control)] × 100.

Annexin V/propidium iodide (PI) apoptosis Assay

Apoptotic effects of PGSE were evaluated using the Annexin V/PI assay with the Invitrogen™ Dead Cell Apoptosis Kit (Annexin V Alexa Fluor™ 488 and PI; Cat. No. V13241), following established protocols [17, 18]. HCT-116 cells were cultured in McCoy's 5A medium supplemented with 10% fetal bovine serum (FBS) and 1% antibiotic-antimycotic solution and seeded in sterile 96-well plates at a density of 8,000 cells per well. Cells were incubated overnight at 37 °C in a humidified 5% CO_2 atmosphere to allow cell attachment.

Cells were treated with PGSE at 100 µg/mL for 24 hours. Doxorubicin (5 µg/mL) served as the positive control, while DMSO served as the negative control. After incubation, cells were washed with cold calcium-free DPBS and resuspended in 1× Annexin-binding buffer. Annexin V Alexa Fluor™ 488 and PI were added to a final concentration of 5:100 and 1 µg/ml, respectively. The cells were incubated for 15 minutes at room temperature in the dark.

DAPI (4′,6-diamidino-2-phenylindole) was added at a final dilution of 1:2000 to visualize and quantify the total cell population. Stained cells were visualized using a Carl Zeiss Axiovert A.1 FL-LED inverted fluorescence microscope. Three independent trials were performed, with four microscopic fields analyzed per treatment.

Cells negative for both Annexin V and PI (Annexin V^-/PI^-) were considered viable, Annexin V^+/PI^- cells were early apoptotic, Annexin V^+/PI^+ cells were late apoptotic, and Annexin V^-/PI^+ cells were necrotic. The percentage of early apoptotic, late apoptotic, and necrotic cells was collectively classified as "dead or dying" cells for statistical analysis [14].

Mass Spectrometric Analysis of PGSE

UPLC-QTOF-MS/MS analysis was performed using a Waters ACQUITY I-Class UPLC system equipped with a Binary Solvent Manager (BSM) and a Sample Manager–Flow Through Needle (SM-FTN), coupled to a Waters Xevo G2-XS QTOF mass spectrometer controlled by the UNIFI Scientific Information System software. The column used was an ACQUITY UPLC® HSS T3 (1.7 µm, 2.1 × 100 mm, C18) maintained at 30°C, while samples were kept at 4°C.

The mobile phases consisted of (A) water containing 0.1% formic acid and (B) acetonitrile containing 0.1% formic acid. All solvents were LC-MS grade, and water was ultrapure Milli-Q water with a resistivity of 18.2 MΩ·cm. Samples were dissolved in H_2O/MeCN (3:7, v/v) at a concentration of 0.5 mg/mL. Injection volumes of 0.1 µL and 1.0 µL were used. Mass spectrometric detection was performed in electrospray ionization positive mode (ESI+), with a mass scan range of m/z 100–1500. Data were acquired in MSE mode using ramped collision energy from 15 to 40 eV.

Compound annotation was based on analysis of high-resolution mass spectrometry (HRMS) and MS/MS fragmentation data. Measured retention times and accurate mass values were compared with published literature and an in-house spectral library (Traditional Medicine Library, UNIFI system). Compound assignments were supported by low mass error (ppm), isotope pattern matching (iFit score), adduct ion detection, and characteristic fragmentation patterns. Automated library matches generated by UNIFI were manually validated to minimize false-positive identifications.

Statistical analysis

Statistical analyses were performed using the IBM SPSS Statistics version 29. The level of significance was set at $\alpha = 0.05$. For the MTT assay, IC_{50} values were calculated in GraphPad Prism version 10.0.2 using nonlinear regression (log[inhibitor] vs. response). For apoptosis analysis, the raw counts of early apoptotic, late apoptotic, and necrotic cells were combined and classified as "dead or dying cells". The Kruskal-Wallis test for independent samples was utilized to analyze the differences among treatment groups. Additionally, when significant differences were detected, Dunn's post-hoc test was performed to compare the proportion of dead or dying cells between PGSE-treated cells and the negative control. P-values ≤ 0.05 were considered statistically significant.

Results

Antiproliferative effects of I. philippinicus extract on HCT-116 cells

The dose-response curves for crude methanolic extract of the parotid gland secretions of *I. philippinicus*

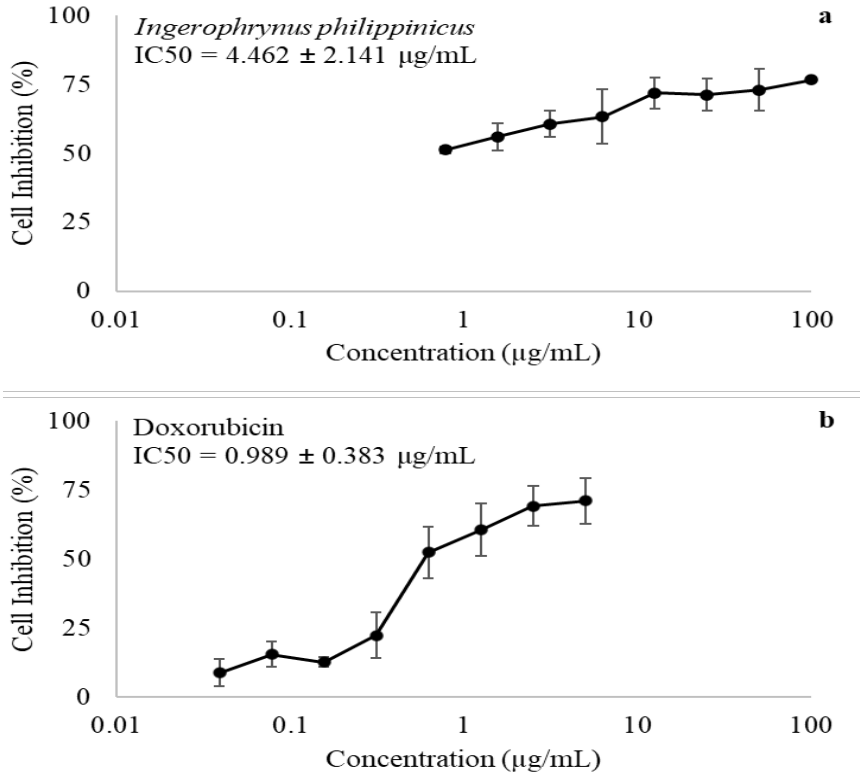

Figure 4. Inhibition of HCT-116 Cells by (a) PGSE and (b) doxorubicin. The dose-response curves represent mean values ± standard deviation of three trials each with two measurements.

(PGSE) (Figure 4a) and doxorubicin (Figure 4b) showed inhibition of HCT-116 cells after 72 hours of treatment. PGSE exhibited a dose-dependent antiproliferative effect with an IC_{50} of 4.462 ± 2.141 µg/mL, while doxorubicin had an IC_{50} of 0.989 ± 0.383 µg/mL (Figure 5).

Apoptotic and necrotic effects of I. philippinicus extract on HCT-116 cells

Figure 6 shows the percentages of HCT-116 cells that were viable (Annexin V-/PI-), early apoptotic (Annexin V+/PI-), late apoptotic (Annexin V+/PI+), and necrotic (Annexin V-/PI+). Cells treated with DMSO (negative control) exhibited complete viability. Cells treated with doxorubicin were predominantly necrotic (71.19% ± 10.84%) and partially late apoptotic (28.81% ± 10.84%). In contrast, PGSE-treated cells primarily underwent late apoptosis (89.52% ± 2.25%), with smaller proportions of early apoptosis (6.16% ± 2.49%) and necrosis (4.32% ± 0.29%).

The Kruskal–Wallis test showed a statistically significant difference among treatment groups ($p < 0.001$). Dunn's post-hoc test demonstrated significant differences between PGSE and DMSO (negative control) and between doxorubicin and DMSO (negative control) ($p < 0.001$). No significant difference was observed between PGSE and doxorubicin ($p = 0.797$).

Identification of bioactive components in the I. philippinicus extract

The total ion chromatogram (TIC) of PGSE obtained from UPLC-QTOF-MS/MS analysis is shown in Figure 7. Three bufadienolides were identified in the PGSE (Table 1;

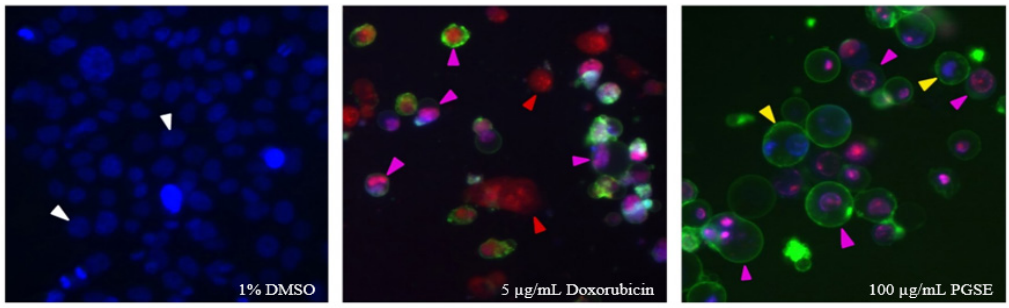

Figure 5. Cytotoxic activity of 100 µg/mL crude methanolic extract of the parotid gland secretions of *I. philippinicus* (PGSE) against HCT-116 Cells Evaluated Using Annexin V/PI Apoptosis Assay. Shown are representative images of HCT-116 cells at 24 h after treatment with DMSO, doxorubicin, and PGSE; stained with DAPI, FITC, and PI; visualized at 100X magnification. Legend: white arrowhead - living cells; yellow arrowhead - early apoptotic; pink arrowhead - late apoptotic; red arrowhead - necrotic.

Table 1. Identification of bufadienolide components of PGSE by UPLC-QTOF-MS/MS

No	Rt (min)	[M+H]+ Measured	[M+H]+ Theoretical	Error (ppm)	MS/MS Fragments	Identification
1	6.56	387.2	387.253[a]	0.5	311.158	Bufalin
2	5.95	445.2	445.2585[b]	2.1	296.2111	Bufotalin
					337.2076	
					214.1386	
					347.1793	
					365.202	
					335.1912	
					285.2098	
3	5.23	415.2	415.2115[a]	-0.3	181.089	Bufotalinin
					401.2257	
					325.109	
					339.1213	
					241.0923	
					180.0765	

Rt, retention time; MS, mass spectrometry; [a], Based on data from Barros et al. [29]; [b], Based on data from Chen et al. [31].

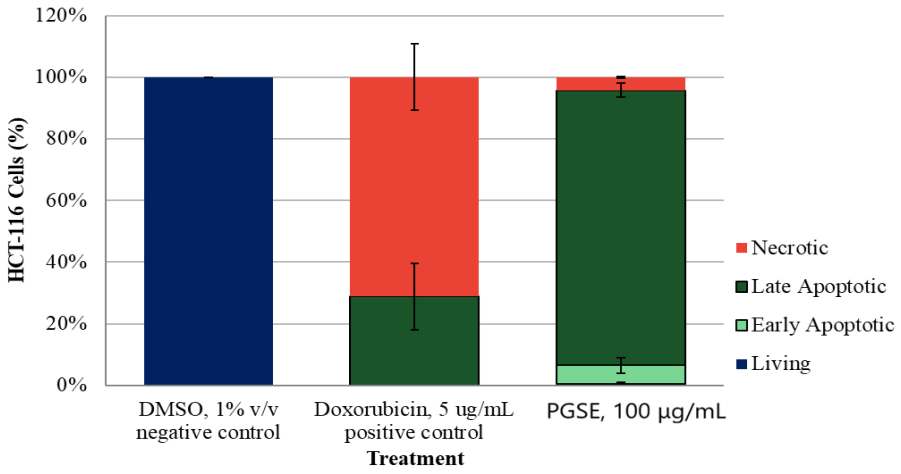

Figure 6. Cytotoxic Activity of 100 µg/mL crude methanolic extract of the parotid gland secretions of *I. philippinicus* (PGSE)" for consistency. against HCT-116 Cells Evaluated Using Annexin V/PI Apoptosis Assay. Results represent mean values ± standard deviation of three trials, each with four replicates.

Figure 8) through their retention times, observed $[M+H]^+$ mass-to-charge (m/z) values, and MS/MS fragmentation patterns, which were consistent with published literature and in-house reference libraries. The molecular structures

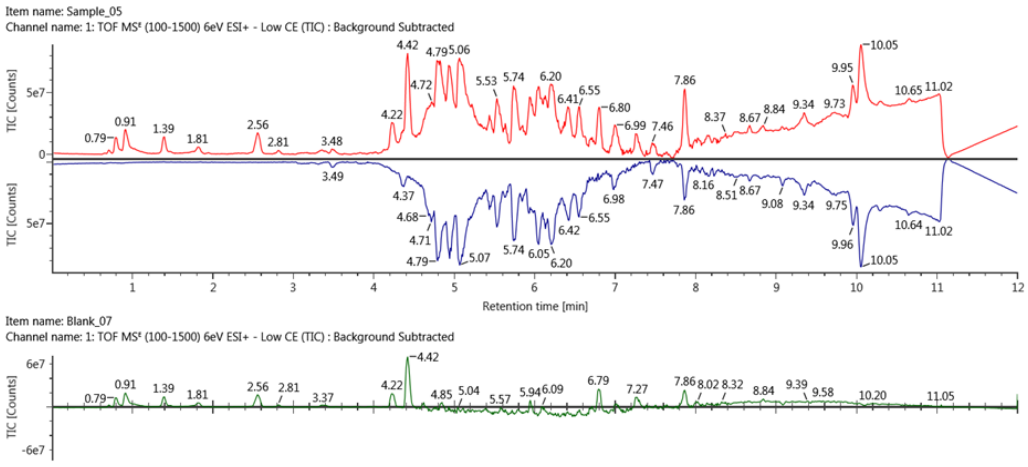

Figure 7. Total Ion Chromatogram of the Sample (Red) with the Blank (Blue) Subtracted

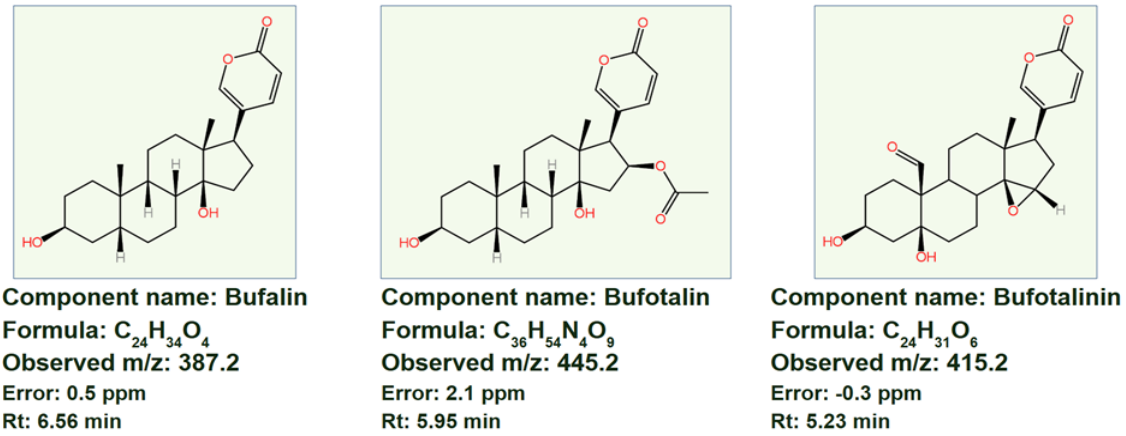

Figure 8. Bufadienolides in PGSE that were Identified through UPLC-QToF-MS/MS.

of the identified compounds were generated using Waters UNIFI software for compound annotation and visualization.

Discussion

The crude methanolic extract of the parotid gland secretions of *I. philippinicus* (PGSE) exhibited high cytotoxic activity against HCT-116 cells in a dose-dependent manner, with an IC_{50} of 4.462 ± 2.141 µg/mL, which is below the 20 µg/mL threshold at 72 h, as defined by the American National Cancer Institute (NCI) for crude extracts [19]. This suggests that PGSE possesses significant antiproliferative activity against colorectal cancer cells. However, this cytotoxic activity was evaluated in vitro using a single cell line; therefore, further studies using additional cell lines are needed to assess selectivity and broader anticancer potential.

The Annexin V/PI assay revealed that PGSE-induced cytotoxicity occurred primarily through apoptosis, with a smaller proportion of necrosis. Apoptosis is an important therapeutic mechanism in cancer treatment because it targets a fundamental hallmark of cancer, evasion of programmed cell death, as described by Hanahan and Weinberg [20]. Resistance to apoptosis is a feature of CRC progression associated with dysregulation of apoptotic signaling pathways that permits malignant cells to survive despite cellular damage. The predominance of apoptosis observed suggests that PGSE may interfere with cellular survival mechanisms that contribute to CRC's resistance to apoptosis [21].

Apoptosis is a regulated form of cell death that eliminates irreparably injured cells without triggering a host-mediated reaction, thereby limiting possible collateral tissue damage. Necrosis, on the other hand, involves the denaturation and leakage of cellular components through damaged membranes, resulting in cytokine-mediated inflammation and phagocytosis of cellular debris [22]. While necrosis can cause damage to healthy tissues, therapy-induced necrosis may also contribute to antitumor immunity through the release of damage-associated molecular patterns (DAMPs), such as HMG1, which promote dendritic cell activation and T cell responses [23, 24]. Thus, both apoptotic and necrotic mechanisms may contribute to the overall anticancer activity of therapeutic agents depending on the biological context.

In this study, PGSE caused cell death primarily through apoptosis, whereas doxorubicin predominantly induced necrosis, suggesting different mechanisms of action. This aligns with the study by Dong et al. [25], which reported dose-dependent apoptotic effects of Bufo viridis extract against various cancer cell lines (HeLa, HT-29, and MCF7). Similar findings were observed by Abdelfatah et al. [14], where toad toxins from Rhinella marina and R. schneideri induced apoptosis in the CCRF-CEM cell line. These findings are consistent with the present study and support the potential anticancer properties of compounds derived from bufonid secretions.

The cytotoxic activity of PGSE may be attributed to the bufadienolides (bufalin, bufotalin, and bufotalinin) identified through UPLC-QTOF-MS/MS analysis. Bufadienolides are steroidal cardiac glycosides that inhibit Na+/K+-ATPase, resulting in disruption of ion homeostasis and activation of cell death pathways [7, 26]. Bufalin has been reported to induce apoptosis, autophagy, and cell cycle arrest, as well as enhance sensitivity to chemotherapeutic agents such as osimertinib and cisplatin by reversing multidrug resistance [4]. Bufotalin and bufotalinin have been shown to induce apoptosis via mitochondrial and Fas-mediated pathways. Furthermore, bufotalin induces cytoplasmic shrinkage, membrane blebbing, apoptotic body formation, chromatin margination, and G2/M cell cycle arrest [27]. Lan et al. [28] reported that bufotalin and bufotalinin exhibit tumor growth inhibition and apoptosis in various cell lines. Bufotalinin has also been shown to induce p53-mediated apoptosis in human malignant melanoma cells and inhibit the growth of multidrug-resistant liver cancer cells via G2/M cell cycle arrest, suggesting its potential to overcome chemotherapy resistance. However, the use of crude extract in this study precludes quantitative determination of the individual mechanism of action of each compound. Further studies involving compound isolation and mechanistic assays are required to clarify their specific actions.

The bufadienolides detected in this study are consistent

with those reported in other bufonid species. Observed m/z values, adduct forms ($[M+H]^+$ and $[M+Na]^+$), and MS/MS fragmentation patterns align with UPLC-QTOF-MS/MS studies of bufonid PGS, including Rhinella jimi [29]. Previous studies on the Philippine toad Bufo biporcatus philippinicus Inger (currently recognized as *I. philippinicus*) reported the presence of biogenic amines and bufadienolides in skin and PGS using chromatographic and spectroscopic techniques [9]. This study expands on these findings by applying high-resolution LC-MS/MS-based metabolite profiling to characterize bufadienolide components in *I. philippinicus*. Compound annotation was supported by accurate mass measurement, isotope pattern fitting, adduct detection, and MS/MS fragmentation analysis using Waters UNIFI software and an integrated spectral library; however, confirmation using authentic reference standards was not performed.

Despite the cytotoxic properties of bufadienolides, their therapeutic application requires careful consideration, as these compounds can be cardiotoxic at higher concentrations due to inhibition of the cardiac Na^+/K^+-ATPase [30]. Future research should focus on dose optimization, toxicity profiling, and the development of delivery systems that maximize anticancer efficacy while minimizing potential systemic toxicity.

In conclusion, the PGSE exhibited high cytotoxic activity against HCT-116 cells, primarily through apoptosis, with a smaller proportion of necrosis. Bioactive compounds (bufadienolides) identified in the extract likely contribute to this activity. These findings support the potential of *I. philippinicus* as a source of bioactive compounds for anticancer research. Future studies should focus on (1) isolation and characterization of bioactive compounds and (2) investigation of their molecular mechanisms of action in cancer models.

Author Contribution Statement

H.J.U.Y. conceived the study, and J.B.F. directed the project. J.B.F., H.J.U.Y., and S.G.S. designed the methodology. N.F.L. managed resources. J.B.F. processed permits and coordinated with collaborating laboratories. Fieldwork and sample collection were conducted by J.B.F., H.J.U.Y., A.G.G.T., N.F.L., G.J.P.R., B.A.T.C., and J.D.F., with E.L.G.L. receiving samples. Sample preparation and processing were performed by the research team. Data analysis and visualization were conducted by A.G.G.T. and H.J.U.Y. J.B.F. drafted the manuscript, and all authors contributed to revision and approved the final version.

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Approval

This study was conducted as part of the Doctor of Medicine program requirements at West Visayas State University.

Ethical Approval

This study was approved by the West Visayas State University Ethics Review Committee (WVSU-URERC) and the Institutional Animal Care and Use Committee (IACUC-0001). Collection of PGS from *I. philippinicus* was authorized under Gratuitous Permit No. 2023-20 issued by the Palawan Council for Sustainable Development (PCSD). All procedures followed institutional and national guidelines. No animal mortalities occurred during sample collection.

Data Availability

The datasets generated and/or analyzed during the current study are available from the corresponding author upon reasonable request.

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