Cytotoxic Effect of Puya chilensis Collected in Central Chile

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Abstract
This study sought to evaluate the pharmacological activity of metabolites isolated from the dried and lyophilized ethanol extracts as well as other solvent fractions of the currently endangered Puya chilensis Molina (Chagual) by analyzing their effects on a human hepatocellular carcinoma (HCC) cell line. We identified several active metabolites from Chagual extracts and two, in particular, carnosol, were found in all the prepared fractions. In addition, Chagual exhibited considerable cytotoxicity against the cancer cell line used in this study, with a half-maximal inhibitory concentration (IC₅₀) of 0.44 ± 0.11 and 0.27 ± 0.04 after a 72-hour treatment and, therefore, has the potential for further investigation as a source of candidate therapeutic agents.

Keywords
human hepatocellular carcinoma, metabolites, Puya chilensis, bioactive nutrients

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Introduction
The Bromeliaceae family members have a high diversity of taxa in the neotropical region with 58 genera and 3172 species, and Puya is a large genus with close to 200 species.¹² Puya species are present in the mountain regions of Central America, in the mid to high elevations of the Andes, and south to lower elevations of Central Chile.³ The Puya species have considerable morphological variations due to the diverse climatic conditions where they grow, which range from wet to arid habitats. In Chile, the genus Puya grows in the Mediterranean area, and Puya chilensis Molina (Chagual) is a characteristic plant of the stony ground near the region that stretches from Coquimbo to Valparaiso. The inflorescence is a pyramidal scape with numerous and reflexed bracts, which are bipinnate where the lower half is densely covered with large greenish yellow flowers.³

“Chagual” is the local traditional name of P. chilensis and its conservation status conferred by the International Union for Conservation of Nature (IUCN) criteria has qualified it as of “least concern (LC)”. However, Chagual has been endangered by the decimation of the native forest area, change in soil use, and changes in global climatic conditions. In addition, the cultural use of this plant as a food item in Chile, the only Puya species that is eaten as a salad is Puya chilensis (Schmeda).

Over the last years, the bioactivities of some Bromeliaceae species have been demonstrated such as the pineapple stem (Ananas comosus L., family Bromeliaceae), which has proteases that have shown antiproliferative and proapoptotic effects in colon carcinoma.⁵ In addition, other Bromeliaceae genera have been reported to exhibit antioxidant, photoprotective, and anti-nociceptive effects.¹²,⁶ However, the specific bioactivity or phytochemical properties of the Chilean Puya are not fully known. Therefore, the aim of this study was to determine the bioactivity of some metabolites isolated from Chagual and their effect on human hepatocellular carcinoma (HCC).

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Materials and Methods

Plant material and extraction

Fresh *P. chilensis* [identified by Aly Valderrama] stems were purchased from a local collector in Melipilla (Metropolitana, Region, Chile) who sells *P. chilensis* as fresh salad. The plant material, stored at −20 °C, was cut into 2 portions and chopped; one portion was dried at room temperature until constant weight while the other was lyophilized. Both samples were macerated separately in 1 L of ethanol, diethyl ether, and ethyl acetate (analytical grade) [10% w/v] in the dark at room temperature with constant agitation for 7 days. Then, the total extract was concentrated using a rotary evaporator at 40 °C and stored at −20 °C until the analysis (Table 1).

General experimental procedures

A 6490 triple quadrupole liquid chromatography/mass spectrometry (LC/MS) system equipped with an electrospray ionization (ESI) source (Agilent Technologies, Palo Alto, CA, USA) was used. All determinations were performed under negative ionization mode at a capillary voltage of 3000 V. Nitrogen was used as the nebulizer (35 psi) and drying gas at 10 L/min at a temperature of 300 °C. The PMT, fragmenter, and skimmer were set at 850, 100, and 60 V, respectively. A full scan mass spectrum was acquired from *m/z* 100 to 1300. Data acquisition and processing were carried out using MassHunter MS Optimizer software (Agilent Technologies, Palo Alto, CA, USA).

The separation was performed using a C18 column; 150 × 4.6 mm I.D.; 5 µm particle size (Agilent Technologies, Palo Alto, CA, USA). The mobile phase consisted of water with 0.1% formic acid (phase a) and acetonitrile with 0.1% formic acid (phase b) at a flow rate of 0.6 mL/min. The linear elution gradient was from 80% (a) to 60% (a) in 20 min. Each run was followed by a 5-minute wash with 100% (b) and an equilibration period of 11 min with 80% (a)/20% (b). The total run time for the analysis was 20 min. Then, 10 µL of the sample was injected and the peaks were assigned based on the mass of the standard compounds (Sigma Aldrich, Munich, Germany).

Metabolite identification was performed using the METLIN database according to the ionization patterns of triple quad MS/MS spectra obtained for each peak (https://metlin.scripps.edu/index.php, Scripps Center for Metabolomics). Databases analyze the match between reference spectrum and experimental data; identification regarding >95% of probability of match and retention times (polarity) was used to determine the plausibility of correct identification (double control: polarity in elution and mass spectrum) [Supplementary Figure S1].

Cell culture

Human HCC HepG2 cells (American Type Culture Collection, ATCC, HB-8065) were grown in a monolayer culture in Dulbecco’s modified Eagle’s Medium (DMEM) with 10% fetal bovine serum (FBS) and antibiotic-antimycotic agents (all Gibco, NY, USA) at 37 °C in a humidified 5% CO₂ incubator.

Cytotoxicity assay

HepG2 cells were seeded in a 96-well plate at an initial density of 5 × 103 cells/well. Twenty-four hours later, the cells were treated with either the control (Milli-Q water) or various concentrations (0.5-1 mg/mL) of lyophilized and dried ethanolic extracts of the Chagual for (A and C) 48 and (B and D) 72 h. Cell viability was measured using the 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay. Insert contains half-maximal inhibitory concentration (IC₅₀) values ± standard deviation (SD). Data are mean ± SD of 3 independent experiments each performed in triplicate.

| Metabolites          | Dried plant material | Lyophilized plant material |
|----------------------|----------------------|-----------------------------|
| 3, 4-dihydroxybenzoic acid (DHBA) | Diethyl ether and ethyl acetate fractions | Ethyl acetate fraction |
| Gallic acid (GA)     | Diethyl ether and ethanol fractions | Ethyl acetate fraction |
| Shikimic acid        | Diethyl ether and ethanol fractions | Ethyl acetate fraction |
| Hesperetin           | Diethyl ether and ethanol fractions | Ethyl acetate fraction |
| 8-C-glycosylkaempferol | Diethyl ether and ethanol fractions | Ethyl acetate fraction |
| Carnosol             | Diethyl ether and ethanol fractions | Ethyl acetate fraction |
concentrations of Chagual for 48 and 72 h. The 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay was used to determine the cytotoxicity, as previously described. The absorbance of the dissolved formazan crystals was measured at 540 nm using a microplate reader (Tecnan infinite® F50, Grodig, Austria).

**Results**

**Isolation of compounds**

Diverse metabolites were isolated from the different fractions analyzed, and at least 2 metabolites were found in all the fractions. Carnosol, a potent antitumor polyphenol, was confirmed as a metabolite by comparing its spectral peak to standard spectra in the databases. The second metabolite found in all the fractions was determined to be similar to quinic acid by comparing its spectrum with a reference spectrum in the databases. The second metabolite identified were 3,4-dihydroxybenzoic acid (DHBA), 3,4,5-trihydroxybenzoic acid (gallic acid, GA), shikimic acid, hesperetin, and 8-C-glycosykaempferol.

**Cytotoxicity assay**

Exposure of tumor cells to increasing concentrations (0.5-1 mg/mL) of Chagual extracts resulted in a dose-dependent decrease in cell viability at 48 and 72 h (Figure 1). The in vitro antitumor activity analysis of ethanolic extracts of the lyophilized (Figure 1A and B) and dried (Figure 1C and D), Chagual plant material revealed that they were highly cytotoxic with a half-maximal inhibitory concentration (IC50) of 0.44 ± 0.11 and 0.27 ± 0.04, the latter only after a 72-hour treatment (Figure 1B and D, respectively).

**Discussion and Conclusions**

The compounds isolated in this study have been reported in other plants; however, it was interesting to discover their presence in the *Puya* genus. Carnosol is an ortho-diphenolic diterpene (Figure 1). Carnosol has been reported to possess antitumor properties. Recently, it was discovered to have antiproliferative activity against several human cancer cells, as well as the ability to induce the intrinsic apoptosis pathway in MCF-7 cells.

In the present work, compounds tentatively identified in the extracts of *P. chilenais* are different from other works. We also present for the first-time information on the antitumor properties of Chagual.

**Declaration of Conflicting Interests**

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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**Author’s Contribution**

Conceptualization, R.R.T. and C.E.E.; Methodology, M.O. and C.E.E.; Validation, G.Z., R.C., and C.E.E.; Formal Analysis, R.C. and C.E.E.; Investigation, R.R.T.; Resources, C.E.E.; Writing—Original Draft Preparation, R.R.T.; Writing—Review and Editing, A.V.V.; Visualization, C.E.E.; Supervision, R.R.T.; Project Administration, L.A.S.
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Not applicable, because this article does not contain any studies with human or animal subjects.

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Not applicable, because this article does not contain any clinical trials.

**Supplemental material**
Supplemental material for this article is available online.

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