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Morphological evaluation and quantification of caspases in lung cancer cells treated with a hederagenin-rich extract of *Chenopodium quinoa* Willd

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Abstract*– This study evaluated the influence of an extract rich in hederagenin obtained from Chenopodium Quinoa Willd. on cell morphology and caspase gene response in the A549 lung cancer cell line. Quinoa is notable for its high content of secondary metabolites with biological activity, with triterpene saponins being the most prominent and relevant in the field of oncology. In order to release the aglycones of interest, an initial extraction was performed with ethanol:water (1:1) followed by fractionation with n-butanol and controlled acid hydrolysis (pH 1, 80°C), purifying the final product with ethyl acetate. Quantification by high-performance liquid chromatography (HPLC) confirmed the presence of hederagenin at a concentration of 92.1 mg/L in the extract. Biological activity was determined using the MTT cell viability assay and analysis of morphological changes by hematoxylin-eosin staining. Finally, quantification by RT-qPCR revealed that there was no significant relative overexpression of caspase 3 or 7 levels, and the results of morphological analysis show that A549 cells treated with the extract generated autophagic vacuoles. These results demonstrate that the hederagenin-rich extract, under the experimental conditions evaluated, exerts its cytotoxic effect through cell death mechanisms independent of caspase activation.

Keywords*-- Quinoa, Hederagenin, Lung cancer, Caspases, Cytotoxicity.

I. Introduction

Cancer is a disease that is classified as a major public health problem with global impact. According to estimates by the World Health Organization (WHO) in 2019, cancer is the leading or second leading cause of death before the age of 70 in 112 countries worldwide. According to global data and statistics compiled in 2020 by GLOBOCAN, lung cancer is the second most common type of cancer worldwide, affecting mostly the male population. It has been established that lung cancer mortality rates will increase globally due to the rise in tobacco consumption worldwide [1–3].

Lung cancer is classified into two main types, small cell and non-small cell, with the latter being the most common, accounting for about 85% of cases. In most patients, non-small cell lung cancer (NSCLC) is not diagnosed until the disease is at an advanced stage. The most common symptom, present in 50% to 75% of cases, is coughing, followed by coughing up blood, chest pain, and shortness of breath. The diagnosis requires a biopsy for histological confirmation. Similarly, determining the extent of the tumor is considered to define the stage, which will ultimately provide information for establishing treatment options. Among the alternatives that have been developed to treat NSCLC are surgery, where a lung lobe is partially or totally removed, neoadjuvant chemotherapy, where early treatment of micrometastases is performed, and the staging of the tumor is reduced for complete removal, and adjuvant chemotherapy, where combined regimens using cisplatin are applied. Other treatments include immunotherapy or the use of specific marker inhibitors [4]. However, despite continuous and significant advances in these conventional therapies, treatment resistance and associated adverse effects remain recurring challenges in the management of the disease.

For this reason, the search for alternative therapies, especially those derived from natural sources, could offer selective toxicity and cause fewer side effects. It has been reported that a large proportion of effective antitumor drugs come directly or indirectly from natural products. This implies that the evaluation and study of various natural sources continue to be a promising strategy for finding compounds with anticancer effects that have relatively better safety profiles or novel mechanisms of action compared to synthetic compounds [5].

In this context, traditional medicine and botany are becoming increasingly important as sources of bioactive compounds with anticancer properties, as various studies have identified plants with the ability to inhibit growth and induce apoptosis in cancer cells. The WHO reports that some countries still use herbal medicines as their primary treatment, and developing countries are taking advantage of the benefits of plant-based compounds to apply them as therapy. Drugs obtained from plants for the treatment of cancer are preferred because of the availability of natural sources, ease of oral

administration through dietary intake, and because, as natural compounds, most of them have characteristics that make them more tolerable and less toxic to normal cells. If these bioactive compounds are selective in research, being non-toxic to normal cell lines and generating cytotoxicity in cancer cell lines, they are established as candidates for clinical studies, leading to further therapeutic development [6].

Among the various natural sources that have been researched for containing various compounds with anticancer activity is quinoa, a plant that has become very important in recent years. Quinoa is an amaranth plant, a pseudocereal of Andean origin that has been cultivated since ancient times in the high Andean regions of Peru. This plant is a very appropriate example of a "functional food," as it has functional properties that originate from its minerals, vitamins, fatty acids, and antioxidants that can contribute to nutrition. Additionally, it contains phytochemical compounds as non-nutritive substances that naturally contribute to the protection of the plant, but whose composition has shown cytotoxic properties [7]. One example is saponins, a complex mixture of triterpene glycosides derived from oleanolic acid, hederagenin, or phytolaccagenic acid, as well as other similar compounds, which have been shown to possess anticancer activity in different cell lines [8,9].

Hederagenin showed antitumor effects in vitro and in vivo trials. It has been shown that its mechanism of action is related to the induction of apoptosis in various types of cancerous cells, an ideal death process given that it is a controlled pathway mediated by the activation of caspases, a family of proteases responsible for executing the apoptotic cascade [10,11]. The evaluation of the gene expression of these proteases is essential for assessing the molecular mechanisms that hederagenin-rich extracts exert on cancer cells.

Therefore, this study focuses on evaluating morphological changes and quantifying caspase expression in A549 lung adenocarcinoma cells treated with a hederagenin-rich extract obtained from Chenopodium Quinoa Willd. cultivated in Peru.

II. Materials and Methods

A. Reagents and Equipment

To obtain the extract, 96° ethanol, HPLC-grade n-butanol (Merck), PA hydrochloric acid (HCl), sodium hydroxide (NaOH), and HPLC-grade ethyl acetate (Merck) were used. For quantification, a hederagenin standard (Merck) and an HPLC system (HITACHI) equipped with a diode array detector were used. For cytotoxicity analyses, the A549 ATCC: CCL-185 cell line, DMEM culture medium, fetal bovine serum (FBS), and MTT reagent [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium] were used. Readings were taken using a microplate reader (Varioskan LUX Multimode Microplate Reader). To evaluate gene expression, a real-time thermocycler (CFX Opus 96 Real-Time PCR System), SsoAdvanced Universal SYBR Green Supermix reagent, and specific primers were used. Finally, to evaluate morphological changes in the cells, Hematoxylin and Eosin staining was used, in addition to an inverted microscope.

B. Sample collection

Chenopodium quinoa Willd. seeds were collected in Puno in the district of Ayaviri, province of Melgar, one of the regions with the highest quinoa production in Peru (latitude: -14.848299, longitude: -70.647408).

The samples were stored at room temperature away from moisture and under optimal conditions for subsequent analysis.

Figure 1. Geographic map showing the location of Puno, Peru.

C. Extract collection

To obtain the sapogenin-rich extract, 100 g of crushed quinoa sample was weighed and suspended in an ethanol:water solution (1:1). The mixture was kept under constant agitation at 450 rpm for 3 hours to ensure complete homogenization. Maceration was then carried out for 24 hours, avoiding direct contact with light [12]. The extract obtained was filtered and concentrated in a rotary evaporator at 60°C. The resulting residue was dissolved in distilled water and subjected to liquid-liquid separation with n-butanol, retaining the butanolic phase. The previous solution was subjected to acid hydrolysis using 2N HCl at pH 1, maintaining constant agitation at 80 °C for 3 hours until a white precipitation formed. The mixture was then neutralized with 4N NaOH until a pH of 7 was reached. Finally, the aglycones were separated from the sapogenin-rich extract by liquid-liquid separation with ethyl acetate, concentrated by rotary evaporation, and stored for further analysis [13].

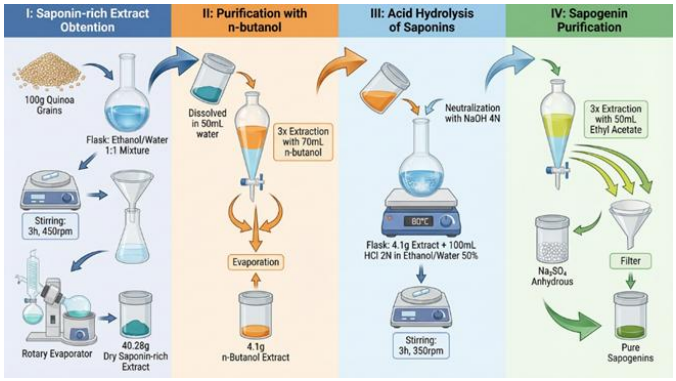

Figure 2. Process for obtaining the hederagenin-rich extract

D. Quantification of hederagenin

The concentration of hederagenin in the extract was determined using high-performance liquid chromatography (HPLC). For quantification, a calibration curve was constructed using a hederagenin standard prepared at concentrations of 2, 4, 8, 10, and 12 mg/L, ensuring the linearity of the system in that range. The final extract samples were diluted in a 1:10 ratio in HPLC-grade methanol to ensure that the readings fell within the limits of the constructed curve [14].

The chromatographic conditions consisted of the use of a C18 reverse-phase column, and an isocratic mobile phase composed of a mixture of methanol and water acidified with 0.8% phosphoric acid (88:12). Detection was performed at a wavelength of 210 nm. The concentration of hederagenin in the extract was calculated using the linear regression equation derived from the calibration curve, and the results were expressed in milligrams per litre (mg/L) [15].

E. Cell viability assay with MTT

The A549 cell line was cultured in 96-well plates at a cell density of $3x10^4$ cells/ml in DMEM medium supplemented with 10% FBS and 1% antibiotic/antifungal agent. The cells were treated with different concentrations of the hederagenin-rich extract (serial dilution starting at 2000 µg/ml) for 24 and 48 hours.

After incubation, to evaluate the cytotoxicity of the extract, the MTT reagent was added and the formazan crystals produced were solubilized with DMSO to measure the absorbance at 550 nm.

F. Hematoxylin and Eosin (H&E) staining

For morphological analysis, A549 cells were seeded in 6-well plates at a cell density of 4×10^5 cells/ml and treated with a concentration equivalent to the IC50 of the hederagenin-rich extract for 24 and 48 hours. After treatment, the cells were fixed and stained with hematoxylin and eosin. Structural changes were observed using optical microscopy, evaluating signs of cell death such as apoptosis (nuclear condensation or pyknosis, nuclear fragmentation or karyorrhexis, cytoplasmic retraction, and formation of apoptotic bodies)[16–18], necrosis (cytoplasmic hypereosinophilia, plasma membrane rupture, and cell disruption) [19,20] and autophagy (formation of cytoplasmic vacuoles and autophagic granules) [21,22].

F. Analysis of caspase 3 and 7 gene expressions using RT-qPCR

Total RNA from treated and untreated A549 cells was extracted using TRIzol™ reagent. The purity and concentration of the RNA were determined by nanodrop spectrophotometry. Subsequently, cDNA synthesis was performed by reverse transcription. The relative expression levels of caspase 3 and 7 mRNA were quantified by real-time PCR using specific primers for each gene, as shown in Table 1. The GAPDH reference gene was used for data normalization, and the $2^{-\Delta\Delta Ct}$ method was applied.

Table 1.
List of primer sequences used

Primer	Nucleotide Secuence
GADPH F	GAGAAGGCTGGGGCTCATTT
GADPH R	AGTGATGGCATGGACTGTGG
Caspase 3 F	ACATGGAAGCGAATCAATGGACTC
Caspase 3 R	AAGGACTCAAATTCTGTTGCCACC
Caspase 7 F	CGGAACAGACAAAGATGCCGAG
Caspase 7 R	AGGCGGCATTTGTATGGTCCTC

III. Results and Discussion

A. Hederagenin Quantification

The hydrolysis and purification process from 100 g of Chenopodium quinoa Willd. yielded 200 mg of dry extract. The chromatographic profile revealed a peak at a retention time (t_R) of 5.2 min, coinciding with the hederagenin standard.

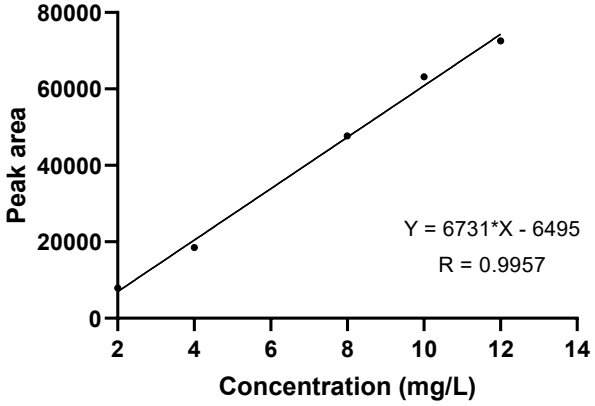

Figure 3. Calibration curve for hederagenin obtained by HPLC.

The established linear regression equation is shown in Equation 1.

$$Y = 6731x - 6495 \qquad (1)$$

The concentration of hederagenin in the extract was determined by calculating "X," which corresponds to milligrams of hederagenin per liter (mg/L), and considering that "Y" is the peak area found at 5.2 min. The quantification of

hederagenin was performed using the calibration curve (R^2=0.9957) and considering the relevant dilutions, which determined a concentration of 92.1 mg/L of hederagenin.

B. Determination of cell viability

The cytotoxic activity of the sapogenin-rich extract on the A549 cell line was evaluated using the MTT assay at 24 and 48 hours of exposure. For treatment, the dry extract was solubilized at a concentration of 40 mg/mL, from which the experimental dilutions were prepared.

The results revealed that, after 24 hours of incubation, the extract did not achieve a 50% inhibition of the cell population in the range of concentrations evaluated, as shown in Figure 4, suggesting an initial resistance of the A549 cells or a slow action of the extract components during this period.

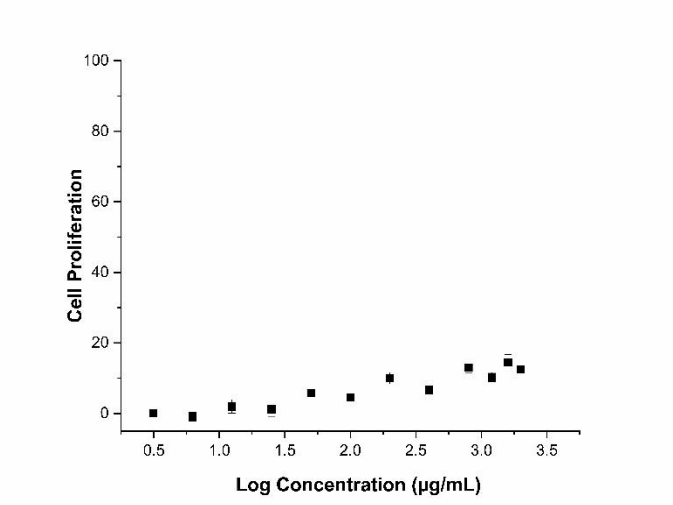

Figure 4. Determination of the IC50 of the hederagenin-rich extract at 24 hours

However, after 48 hours of exposure, a significant and dose-dependent reduction in cell viability was observed. Thus, through nonlinear regression analysis, a mean inhibitory concentration (IC50) of 798.6 µg/mL was determined. This finding indicates that the cytotoxicity of the hederagenin-rich extract requires prolonged interaction time to effectively compromise the metabolism of lung adenocarcinoma cells. The IC50 value obtained in this study is considerably higher than that reported by Huize Zhang et al., who reported a value of 39 µM. This can be attributed to several factors. First, the extract contains multiple components in addition to hederagenin, which dilutes the effective concentration of this metabolite in the final extract. Second, the presence of other secondary metabolites may interfere with the bioavailability or cellular absorption of active sapogenins under experimental conditions, and third, the degree of purity of hederagenin in the extract is also a factor that must be considered [23].

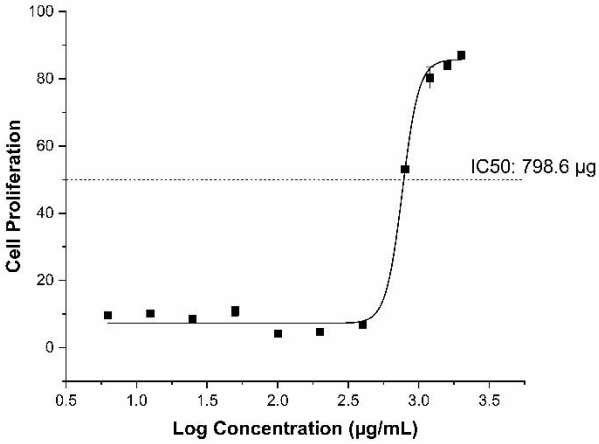

Figure 5. Determination of the IC50 of the hederagenin-rich extract at 48 hours**.**

C. Gene expression analysis of Caspases 3 and 7 using RT-qPCR

To determine whether the observed cytotoxicity of the hederagenin-rich extract (HRE) was mediated by the classical caspase-dependent apoptosis pathway, the mRNA expression levels of caspases 3 and 7 were evaluated by RT-qPCR. The results indicated that treatment with HRE did not induce significant changes in the expression of caspase 3 (Figure 1A; $p > 0.05$) or caspase 7 (Figure 1B; $p > 0.05$) compared to the control group.

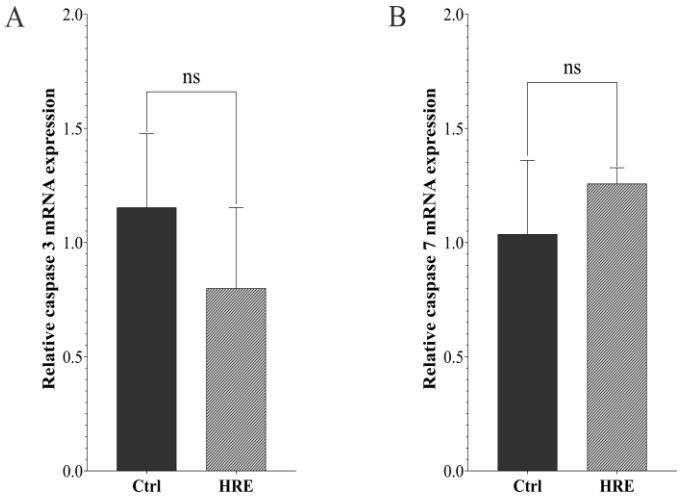

Figure 6. Relative expression of (A) caspase 3 and (B) caspase 7 genes is shown.

Hederagenin has been classified as an apoptosis inducer; however, a study by Doonan et al. found that natural triterpenoids can facilitate access to unconventional cell death pathways. The lack of positive regulation other than the control

of effector caspases suggests that the cell death observed is not carried out by the classic apoptosis cascade, but rather that HRE could compromise viability through direct metabolic or mitochondrial collapse [24].

Additionally, an article published by Potze et al. points out that the cellular response to triterpenoids can be dynamic. During the 48 hours, it is likely that the surviving cells have overcome the transcriptional activation phase of death genes or that the RNA synthesis machinery has been degraded due to the advanced progress of cell death. When apoptosis occurs, mRNA expression peaks unfold in early stages, between 6 and 24 hours, so at 48 hours caspase levels may have returned to baseline or are undetectable in a damaged cell population [25].

For apoptosis to occur, cleavage of existing pro-caspases must take place, but not the de novo synthesis of transcripts. According to Potze et al., hederagenin negatively affects mitochondrial membrane integrity, meaning that cell death could occur due to proteins present in the cytoplasm, leading to cytotoxicity without gene overexpression that can be detected by real-time PCR [25].

D. Hematoxylin and Eosin Staining

Microscopic analysis revealed that treatment with the hederagenin-rich extract induces severe morphological damage compared to the control groups. While the negative and positive control cells (Figures 7A and 7B) exhibited healthy morphology, characterized by well-spread, adherent cells with normal-appearing nuclei, those exposed to treatment (Figure 7C) showed unmistakable signs of cytotoxicity.

Marked pyknosis or nuclear condensation was observed (Arrows 1 and 2), a process indicative of DNA fragmentation. Additionally, the treatment caused an increase in cytoplasmic granularity and the formation of vacuoles, signs of cellular stress and damage to organelles. These alterations were complemented by the loss of the characteristic polygonal shape and cell retraction (Arrow 3), suggesting destabilization of the cytoskeleton before detachment from the substrate.

Microscopic analysis revealed that treatment with the hederagenin-rich extract induces severe morphological damage compared to the control groups. Figure 7A: negative control (untreated cells) shows a healthy cell with a prominent, oval-shaped nucleus and evenly distributed chromatin. The cytoplasm is continuous and has a homogeneous stain, with no signs of vacuolization or cell detachment. Regarding Figure 7B: positive control (cells treated with doxorubicin), morphological alterations indicating programmed cell death (apoptosis) are evident. In addition, pyknotic nuclei are observed, with a condensed and darker appearance; the cells take on a rounded shape and leave gaps between them. Finally, in Figure 7C: treatment (cells treated with HRE for 48 hours), marked cytoplasmic vacuolization is observed, as evidenced by small spots in the cell, indicating autophagic activity or granular stress; the size of the nucleus is similar to the control, but with cytoplasmic disorganization.

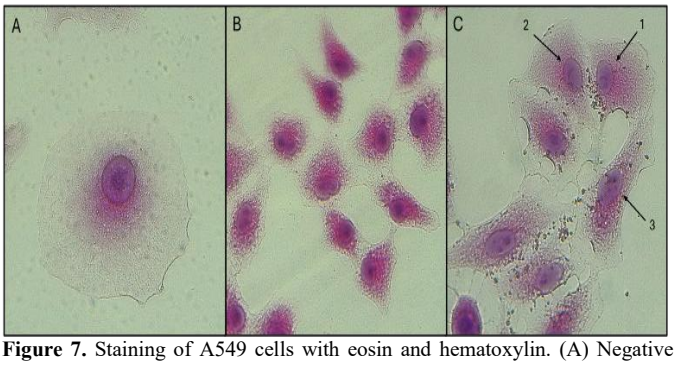

Figure 7. Staining of A549 cells with eosin and hematoxylin. (A) Negative control, (B) positive control – doxorubicin, and (C) treatment with HRE for 48 hours.

The most notable feature in Figure 7C is the presence of cytoplasmic vacuoles and a loss of cell density. According to Potze et al., triterpenoids induce severe mitochondrial stress that is counteracted by autophagy, a process that seeks to degrade damaged components to maintain cellular homeostasis, but the stress generated by the extract is continuous [25]. In the results of this research, no apoptotic bodies or extreme nuclear fragmentation were observed, which is related to the absence of caspases 3 and 7 in RT-qPCR. It has been determined that the extract rich in hederagenin does not lead to conventional apoptosis but rather induces structural disorganization that leads to death by metabolic exhaustion, a finding that coincides with that reported by Doonan et al. on the application of triterpenoids to activate antitumor autophagy [24].

IV. Conclusions

The results obtained indicate that the hederagenin-rich extract exerts a cytotoxic effect on the A549 cell line with an IC50 of 798.6 µg/mL obtained after 48 hours of treatment. However, the absence of significant regulation in the expression of Cas-3 and Cas-7 genes by RT-qPCR, in addition to the morphology with cytoplasmic vacuoles observed in hematoxylin and eosin staining, demonstrates that induced cell death does not follow a caspase-dependent apoptotic pathway now.

Evidence suggests that cytotoxicity is mediated by an alternative death mechanism, consistent with cytotoxic autophagy or direct mitochondrial collapse. According to the literature consulted, hederagenin prioritizes autophagy over apoptosis. Consequently, it is concluded that the extract effectively compromises the viability of A549 cells, but through a signaling cascade independent of effector caspases, highlighting the potential of this compound to evade apoptosis resistance in tumor cells.

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