

Citotoxic and apoptotic activity of extracts from leaves and juice of *passiflora edulis*

Abstract

Liver cancer has become as the second cause of cancer related death worldwide, with hepatocellular carcinoma being the most frequent type of liver cancer; this is caused by hepatocyte transformation, being common in patients with cirrhosis, hepatitis, metabolic syndrome such as obesity or dyslipidemia, which has favored an increase in their incidence and mortality rates. Some phytochemicals derived from different parts of plants have shown antiproliferative and proapoptotic activity; however, few advances in research on promising bioactive substances has been reported. In the present work, it was evaluated the effect of leaf and juice extracts of *P. edulis* var. *flavicarpa* on the viability, cytotoxicity and induction of apoptosis, in an *in vitro* model of liver cancer (HepG2 cell). Phytochemical analysis of the extracts was based on the quantification of polyphenolic and polysaccharide content, metabolites to which antitumor properties have been attributed.

In this way, it was found that the leaf extract had higher polyphenolic content, whereas in the juice extract more polysaccharide content was observed. On the other hand, a significant decrease in viability was observed with juice extract at 400µg/ml and a significant increase in cytotoxicity by leaf extract at 25µg/ml; finally, both extracts significantly increased proapoptotic activity. The results suggest that *P. edulis* is a potential source of phytochemical compounds with anticancer properties in the cellular model evaluated.

Keywords: *passiflora*, liver cancer, cytotoxicity, apoptosis

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Introduction

Liver cancer is the fifth most frequent neoplasm in men and the ninth in women, with 782,000 new cases of cancer diagnosed in 2012, is the second cause of death associated with cancer in the world, with 746,000 deaths in 2012.¹ Hepatocellular carcinoma (HCC) is the most common type of liver cancer. It is produced by the transformation of hepatocytes and is common in patients with cirrhosis, considered the pre-neoplastic state.² Among the main risk factors are infection with hepatitis B virus (HBV), hepatitis C virus (HCV), alcohol intake, dietary exposure to Aflatoxin B1 (AFB1) and tobacco, among others³ Likewise, in recent years it has been shown that diabetes mellitus and other factors associated with the metabolic syndrome such as obesity or dyslipidemias, favor the increase in the incidence and mortality rates of HCC.⁴ del CHC.

Throughout history, traditional medicine has been used for the treatment of cancer; practice adopted due to the advantages they have shown in terms of efficiency, few side effects, easy availability and improvement in quality of life. Numerous investigations have identified a diversity of components in these medicinal plants with chemopreventive and or chemotherapeutic potential, being the family of polyphenols a group of great interest.⁵

Among the best characterized polyphenols are the flavonoids, which are a group of secondary metabolites, with variable phenolic structure and found in different parts of the plant; More than 5,000 flavonoids have been extracted from medicinal plants and their chemical structures have been elucidated. Some of these have been reported with biological activity for the treatment of various conditions such as cardiovascular diseases,⁶ diabetes,⁷ cancer,⁸⁻¹⁰ viral

infections, as well as their protective potential against liver damage.⁵

The species *Passiflora edulis* known with the common name of passion fruit, passion fruit, or passion fruit, is cultivated in tropical and subtropical countries; the variety *P. edulis* Sims var. *flavicarpa*, has yellow fruits and grows from sea level to 1,000 masl,¹¹ different parts of the plant have been used in traditional medicine for the treatment of nervous, cardiovascular, muscle relaxant and cancer (intestinal tumors).¹²⁻¹⁴ Additionally, different studies have demonstrated the ability of their extracts to inhibit MMP-2 and MMP-9 proteins, two metallo-proteases involved in tumor invasion, metastasis and angiogenesis,¹⁵ ethanolic and aqueous extracts of *P. edulis* have shown antioxidant activity, and its effect on the reduction of the viability of colon cancer cell lines was demonstrated.¹⁶ This plant is promising for the search of bioactive compounds for the prevention and control of carcinogenic processes. In the present work, the cytotoxic and apoptotic activity of extracts of *P. edulis* Sims var. *Flavicarpa* on the line of HepG2 hepatocellular carcinoma.

Methodology

Obtaining the samples

Leaves and fresh fruits of *Passiflora edulis* F. *flavicarpa* were collected in the Municipality of Caicedonia, Valle del Cauca, Colombia (4.2424 °N, 75.5649 °W at 1100msnm). The specimens were identified in the Herbarium of the University of Quindío (Collection: 18063).

Obtaining extracts of *P. edulis*

The ethanolic extract of leaves was obtained by leaching with

96% ethanol for 8 days. Then a chlorophyll separation was carried out. The extract of the juice was obtained from fruits with a degree of maturation for human consumption. A filtration of the fruit pulp was performed and then centrifuged at 2400rpm for 1 minute. Both extracts were concentrated in a rotary evaporator (HeidolpTM) until dry, to be stored protected from light at -20 °C until use.¹⁶

Determination of the total phenolic content

The total phenolic content of both extracts was determined through the Folin-Ciocalteu method.¹⁷ The absorbance of the assays was measured at 765nm, using as a target a 2% Na₂CO₃ solution. The results were expressed as mg equivalents of gallic acid per gram of extract (mg equiv/g extract), according to a calibration curve previously made, taking as a standard the gallic acid.

Determination of the total polysaccharide content

The phenol-sulfuric acid method was used to measure the total polysaccharide content.¹⁸ Absorbance was measured at 490nm. A calibration curve was previously made with glucose; the results are expressed as mg equivalents of saccharide per gram of extract.

Cell culture

HepG2 liver cancer cell line was used for the assays. Cells were cultured in DMEM medium (Gibco) supplemented with 10% fetal bovine serum, 100U/ml penicillin, 100ug/ml streptomycin (Life Technologies), maintained at 37 °C in a humidified atmosphere with 5% CO₂. The cells were cultured in 96-well dishes (1x10⁴ cells/well) and settled for 24 hours before starting treatments.

Determination of cell viability: MTT assay

The evaluation of the cytotoxicity of the extracts was evaluated with the MTT assay [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] (Sigma Aldrich), following the methodology of Ramírez.¹⁹ After 24 hours of cell establishment, treatments were started with different concentrations of the extracts (25µg/ml, 50µg/ml, 100µg/ml, 200µg/ml and 400µg/ml), for 24, 48 and 72 hours. At the end of the time, 20µl of MTT (5mg/ml in PBS) was added and incubated for 4 hours at 37°C in the dark. Finally, the absorbance at 560nm was measured using the GlomaxTM multidetection system (Promega). Cells without treatment were used as a negative control. The assays were performed in quintuplicate for each of the concentrations evaluated.

The viability percentage was determined with the following equation: % viability = (DOt/DOc) x 100

Where, DOt is the optical density of the cells treated with the extracts and DOc is the optical density of the cells without treatment.

Evaluación del efecto de los tratamientos sobre viabilidad, citotoxicidad y apoptosis

To determine the viability, cytotoxicity and apoptosis induced by the treatments, the ApoTox-GloTM Triplex Assay commercial kit (Promega) was used following the manufacturer's recommendations. For the assay, 1 x 10⁴ cells were seeded per well, with the extracts at concentrations of 25µg/ml, 100µg/ml and 400µg/ml and incubated at 37°C for 72 hours. All measurements were made in triplicate in the Glomax multidetection system (Promega) and the results were presented as relative fluorescence units (URF) and as relative units of luminescence (URL).

Statistic analysis

For all trials, a completely randomized experimental design was used. The means and the comparison of the differences of the means between treated and untreated cells, with confidence intervals of 95%±SD were obtained using Graph Pad Prism® 6.0 (Graph Pad Software Inc., La Jolla, United States). The two-tailed Student's t test was used for unpaired analysis to compare the results between treated and untreated cells. Likewise, a one-way analysis of variance (ANOVA) was performed. Values p<0.05 were considered statistically significant. All experiments were carried out at least in triplicate.

Results

The yield percentages of each of the extracts of *P. edulis* were calculated, finding that for the leaf extract the yield was 2% and for the Juice extract of 39%. Regarding the polyphenolic content, the leaf extract presented a higher content with 150.3 mg equiv acid/g of extract, while for the polysaccharide content it was the Juice extract which had the highest proportion with 365 mg equiv/g of extract (Table 1).

Table 1 Polyphenolic and polysaccharide content of the extracts

Extract	Polyphenolic Content	Polysaccharide Content
	(mg equiv. gallic/g of extract)	(mg equiv./g of extract)
Etanolic Leaves	150,3±22,5	154,7±16,9
Aqueous Juice	28,2±12,4	365,5±18,8

With the MTT assay it was observed that for the ethanolic extract of leaves, as the concentration of the extract was increased, cell viability increased; however, by increasing the exposure time in all the concentrations evaluated, viability decreased (Figure 1). It is important to note that none of the concentrations evaluated presented a viability lower than 92%.

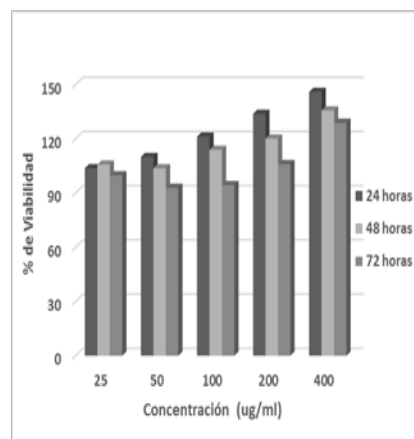


Figure 1 Cell viability with the ethanolic extract of *P. edulis* leaves.

For the extract of Juice of *P. edulis* the behavior was different, since no significant changes were obtained in the viability percentages in the first 48 hours of treatment. However, after 72 hours, a reduction in viability was observed for the first three concentrations (25µg/ml, 50µg/ml and 100µg/ml) (Figure 2). The greatest decrease in viability at 81% was with the concentration of 100µg/ml at 72.

When evaluating the viability of the HepG2 cells against the extracts with the ApoTox-GloTM Kit, it was found that for the leaf

extract the viability increased to the concentrations of 25 and 100 µg/ml, this increase being significant with respect to the control (Figure 3). On the side of the Juice extract, a decrease in viability was observed at 400 µg/ml ($p \leq 0.001$) (Figure 4).

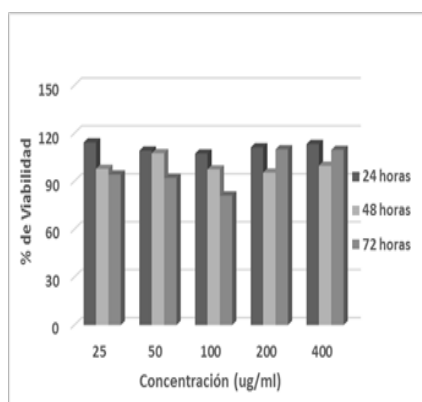


Figure 2 Cell viability with Juice extract of *P. edulis*.

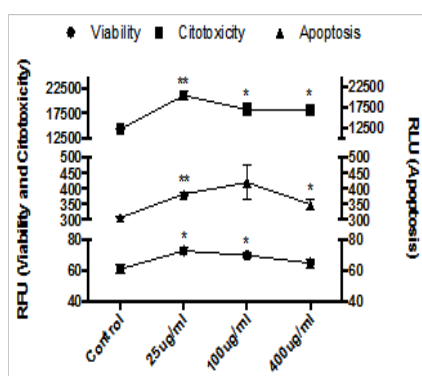


Figure 3 Feasibility, cytotoxicity and cellular apoptosis with the leaf extract of *P. edulis*.

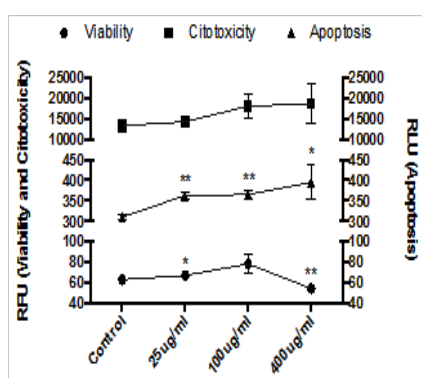


Figure 4 Viability, cytotoxicity and cellular apoptosis with Juice extract of *P. edulis*.

On the other hand, the cytotoxicity caused by the leaf extract increased significantly compared to the control, being the concentration of 25 µg/ml the one that caused the greatest toxicity (Figure 3). In the case of the Juice extract, although the levels of cytotoxicity increased as the concentration of the extract increased, this increase was not statistically significant (Figure 4). Finally, the results of the apoptosis inducing effect indicate that both the leaf extract and the Juice extract significantly increase the activity of caspases 3 and 7, with the effect of the Juice extract causing the most proapoptotic activity (Figure 3 (Figure 4).

Discussion

The development of cancer depends to a great extent on the alteration in the mechanisms that regulate cell proliferation, as well as on the components involved in the suppression of apoptosis; therefore, these cellular processes represent obvious objectives for intervention in any type of therapeutic strategy against cancer. In this sense, several studies have focused on the cytotoxic and or antiproliferative properties of natural extracts, with high polyphenolic or polysaccharide contents, demonstrating their significant potential as anticancer agents.²⁰

At present, polysaccharides are considered a group of great interest because of their potential to inhibit tumor growth or to induce apoptosis, as has been demonstrated in *in vitro* studies and in animal models;^{21,22} In addition, it has been observed that polysaccharides derived from plants are relatively safe compared to other immunomodulators due to their low toxicity. Likewise, polyphenols are another group of compounds, which have been shown in various studies to have biological activity, associated with the prevention of different types of cancer.^{9,23,24} The chemopreventive effect of polyphenols is due to the potential to regulate the cell cycle, ability to induce apoptosis, antioxidant activity, and induction of enzymatic detoxification, regulation of the immune system, anti-inflammatory activity and effects on cell signaling.²⁵ In the specific context of liver cancer, its antimutagenic and anticancer activity has been validated.^{9,20,26} For these reasons, it can be said that the effects observed on the part of the extracts of both leaves and Juice of *P. edulis*, may be due to the high content of both polysaccharides and polyphenols.

If a comparison of the observed results with leaf and juice extracts is made in other cell lines (SW480, SW620, Caco-2, Vero), a difference with respect to the cytotoxic response and in the induction of apoptosis is presented.^{16,27,28} These differences may be due to the intrinsic variation linked to the origin of each cell line, which raises the need to evaluate the extracts in the greatest diversity of cell lines possible, since the cellular response is mediated by several factors such as the metabolism of each cell. Cell line, the presence of specific membrane receptors, genomic instability and the diversity of cellular phenotypes.

In comparison with other cell lines, HepG2 cells have shown greater resistance to the biological activity of both polyphenols and polysaccharides.²² For example, in the study by Kuete et al.²⁹ the cytotoxic effect of the juice extract of *P. edulis* on CCRF-CEM leukemia cells presented an LD50 of 8.2 µg/ml, whereas for HepG2 cells the maximum concentration evaluated (80 µg/ml) did not generate toxicity. Similarly, Ramos³⁰ reported a low cytotoxic effect of passion fruit juice on MCF-7 breast cancer cells, since the LD50 was > 500 µg/ml; However, this same author reports that the type of extraction by which the extract is obtained will determine the purity of the metabolites and therefore their cytotoxic effect.

One of the possible explanations of the effect on apoptosis observed in our results, could be given by the action of the polysaccharides of the extracts on the signal transducer and activator of transcription 3 (STAT3), which is an important factor of survival in the regulation of cell proliferation and the development of cancer. Shen et al.³¹ suggest that the inactivation of STAT3, mediated by the polysaccharides present in the fruits of *Curcubita moschata*, is responsible for the apoptosis-inducing effect.

On the other hand, in all parts of *P. edulis*, with the exception of

the roots, the presence of β -carbonyl alkaloids has been found.³² This type of metabolites has been shown to have anticancer activity due to its ability to inhibit topoisomerase I and II, telomerase and CDKs (cyclin dependent kinases), among others, which was evidenced when this type of alkaloids was evaluated in HepG2 cells, demonstrating its potent cytotoxic capacity on this cellular model of liver cancer.³³

Similar to that reported by Rowe,³⁴ who evaluated the antitumor activity of *P. edulis* Juice, we can point out that the phytochemical components present in both extracts have activity on the regulation of the activation of apoptosis, specifically on the activity of the caspases, but little is its effect on the reduction in cell proliferation. Likewise, in experiments carried out with polysaccharides extracted from *P. edulis*, inhibition of tumor growth has been demonstrated, which is not related to the inhibition of cell proliferation; however, it was observed that this inhibition would be related to necrotic processes, according to the histopathological analysis.³⁵

Conclusion

This study showed that both leaf extracts and juice of *P. edulis* have proapoptotic activity in the *in vitro* model of liver cancer evaluated. In addition, the results suggest that due to its high content of secondary metabolites such as polyphenols and polysaccharides, *P. edulis* can be considered as a promising species for the search for compounds that allow the chemoprevention of this type of cancer.

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Conflict of interest

There is no conflict of interest.

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