



Cytotoxic and Apoptotic Activities of the Aqueous Fruit Extract of *Passiflora edulis Sims var flavicarpa* in an *In vitro* Model of Human Colon Cancer

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ABSTRACT

Colorectal Cancer (CRC) is the fourth leading cause of cancer death in the world. Epidemiological studies have shown an association between CRC incidence/mortality with unhealthy dietary habits, which suggests the need to promote and protect health through the adoption of sustainable measures that allow the reduction of morbidity and mortality rates, such as the consumption of products of plant origin. Some phytochemicals derived from fruits have shown antiproliferative activity and high antioxidant capacity; however, few research advances have been obtained regarding the complex mixture of phytochemicals of certain fruits that could contain promising bioactive substances. For these reasons, in the present work we evaluated the cytotoxic activity and the pro-apoptotic potential of the aqueous extract of *P. edulis Sims var. Flavicarpa* on an *in vitro* CRC model. Chemical analysis of the extract was based on a preliminary phytochemical screening in which were identified metabolites such as phenolic compounds and sterols, which have displayed antitumoral properties. The extracts exerted cytotoxic and proapoptotic potential, demonstrated by the reduction on cell viability, increase of the SubG0/G1 cell populations and the positive staining with Annexin-V of treated cells. Our results suggest that *P. edulis* is a potential source of phytochemical compounds with cytotoxic and apoptotic potential over colon cancer cell lines.

Keywords: *Passiflora edulis*; Colon cancer; Natural extracts; Phytochemicals

INTRODUCTION

Colorectal cancer (CRC) is the third most diagnosed cancer in men and the second in women in the world, with nearly 693.900 deaths and 1.4 million new cases diagnosed in 2012, [1] it is important to note that more than 50% of the cases were diagnosed in developed countries [2]. The appearance and progression of the disease are related to environmental and genetic factors; one of these is dietary habits, which has been associated with more than 50% of mortality cases. This can be explained due to presence of carcinogenic substances in the food, high fat consumption, carbohydrates and alcohol, and also for the low consumption of food with chemoprotective agents such as fruits and vegetables [3,4]. Several studies have identified a relationship between the consumption of fruits, vegetables, legumes and tea, and a decrease in the incidence of some cancer types; effects that have been associated with high content of bioactive compounds (phytochemicals) in the diet [5]. Chemoprotective features are associated to the consumption of dietary fiber, some micronutrients (A, E, C, and D vitamins, folate and carotenoids, minerals, selenium and calcium) and polyphenols [6], with antioxidant, antigenotoxic, anti-inflammatory and antiproliferative potential, which can prevent the initiation of colorectal cancer and reverse or delay the progression process [3,7].

The species *Passiflora edulis*, known as Maracuya, parchita, or passion fruit, is cultivated in tropical and subtropical countries. The *P. edulis* Sims var. *flavicarpa* variety has yellow fruits and grows from sea level to 1.000 m [8]. Different parts of the plant have been used in traditional medicine to treat neurological disorders (insomnia, epilepsy, hypnosis and sedation), heart disease (hypertension, diuretics, cholesterol and reduction of triglyceride levels), muscle relaxants (antispasmodics, stomachache relievers), which can be explained by the bioactive properties reported for this fruit [9,10]. Also, it has been demonstrated the capacity of *P. edulis* to inhibit the activity of MMP-2 and MMP-9 proteins, two metalloproteinases involved in tumor invasion, metastasis and angiogenesis [11]. Similarly, ethanol and aqueous extracts displayed antioxidant activity and its effect to reduce cell viability of colon cancer cell lines has been shown [12]. For these reasons, this plant is promising to be investigated in search of bioactive compounds to control and prevent carcinogenic processes. In the present study, it was evaluated the potential of an aqueous extract obtained from the fruit of *P. edulis* Sims var. *flavicarpa* plant, to regulate cell cycle and also to test its cytotoxic and pro-apoptotic potential on an *in vitro* CRC model.

METHODOLOGY

Extraction and Characterization

The aqueous extracts were obtained from *P. edulis* Sims var. *flavicarpa* fruits, collected in La Tebaida, Colombia; the specimens were identified by the Herbarium of Universidad del Quindío (collection number: 33974). The edible part was macerated and filtered, then left for 96 hours at 60°C and finally dried and stored at 4°C (protected from light). For the chemical characterization, a preliminary phytochemical screening was performed according to Bilbao, M [13].

Cell Culture

SW480 cell line established from human colon adenocarcinoma and SW620 cell line established from lymph node metastases of the same patient [14], were obtained from the American Type Culture Collection (ATCC, Manassas, USA). Cells were cultivated according to the procedure used by Maldonado et al. [15], the propagation was done with DMEM (Gibco) medium with 25 mM glucose and 2 mM L-glutamine, supplemented with 10% horse serum (HS), 100 UI/ml penicillin, 100 µg/ml streptomycin and 1% of non-essential amino acids. The cell cultures were maintained at 37°C and 5% CO₂. During the experiments, serum was reduced to 3%, and the medium was supplemented with insulin (10 µg/ml), transferrin (5 µg/ml) and Selenium (5 ng/ml). The treatments were added in volume/volume ratios in all cases.

Cytotoxicity Assessment

Cell cultures with viability higher than 95% were seeded in a density of 5,000 cells/well for 48 hours. Afterwards, cells were treated at the following concentrations of the extract: 10% (264 µg/ml), 7% (184.8 µg/ml), 5% (132 µg/ml), 2% (52.8 µg/ml), and 1% (26.4 µg/ml); the negative control were untreated cells in culture medium (C-), the positive control were cells with turmeric extract at 100 µg/ml (C+). After 24 hours, 20 µl of MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) were added, (5mg/ml per well), and incubated at 37°C and 5% CO₂ for 4 hours. Then, 100 µl of acid isopropanol (Triton X-100 10%, HCl 0.8%) were added per well, and kept in darkness for 30 minutes at room temperature. Measurements of absorbance were performed using Glomax™ multidetection system (Promega) at 560 nm, and the percentage of viability was estimated as: Viability % = (ODt/ODc) × 100, where ODt=optic density of treated cells, and ODc=optic density of control cells (untreated cultures). Seven replicates per treatment were performed.

Effects on Cell Cycle

Cultures were treated with 444 µg/mL and 415 µg/mL of the extract as previously described [12]. After treatments, cells were collected and fixed with 1ml Methanol-PBS 9:1 (v/v) proportion, and stored at -20°C for 2 hours. Thereafter, cells were centrifuged at 2500 rpm for 5 minutes and the excess of methanol was removed. Finally, cells were resuspended in 300 µl PBS with 250 µg/ml RNase A and 10 µg/ml propidium Iodide and incubated at room temperature for 30 minutes in darkness. The samples were analyzed in FACS Canto II flow cytometer.

Apoptosis Induction Effect

3 × 10⁵ SW480 and SW620 cells were seeded in 2 ml of culture medium and incubated for 48 hours. Then, cells were treated as described in the previous section. At the end of the experiment, supernatant was collected and cells were detached and centrifuged at 2500 rpm for 5 minutes. Thereafter, 1 × 10⁶ cells were collected in 1ml of Annexin V Kit

binding buffer (10 mM Hepes; 0.14 M NaCl; 2.5 mM CaCl₂; pH 7.4) and 4 µl of Annexin V-FITC (Annexin-V-FLUOS staining kit) and 10 µL of propidium iodide (1mg/mL) were added. The samples were incubated for 15 minutes at 4°C in darkness and 1 ml of PBS was added prior to centrifugation in order to remove the supernatant. The samples were analyzed in FACS Canto II flow cytometer.

Statistical Analysis

The data was analyzed with the program GraphPad Prism 6.0 (GraphPad Software Inc., San Diego, CA) using One Way Analysis of Variance (ANOVA) and Duncan test to compare each treatment with the negative control. In every case, statistically significant differences were considered at $p \leq 0.05$.

RESULTS AND DISCUSSION

Preliminary Phytochemical Screening

Table 1 shows the secondary metabolites identified in *P. edulis Sims* var. *Flavicarpa* fruits, like flavonoids and quinones, which have been shown to possess biological activity associated with the prevention of different types of cancer [15,16]. The chemopreventive properties of these polyphenols are related to its anti-proliferative, antioxidant and anti-inflammatory activity, effects on cell cycle and apoptosis, enzyme detoxification induction, immune system regulation and the modulation of different cell signaling pathways [17]; particularly, in CRC it has been widely validated its anti-mutagenic and anti-carcinogenic activity [15,18-23]. Flavonoids are particularly relevant, since the phenolic rings they possess have the ability to accept an electron to form relatively stable phenoxy radicals that protect the cell from damage produced by oxidative reactions. Likewise, they have shown to exert cytotoxic effects against cancer cells, through cell cycle arrest and apoptosis induction [24,25].

Table 1: Qualitative evaluation of metabolites present in extracts of *P. edulis Sims* f. *Flavicarpa*

Metabolite	Presence(+)/ Absence(-)
Tannis	-
Flavonoids	+
Quinones	+
Sterols	+
Saponines	-
Cardiac glycosides	+
Terpene lactones	-
Coumarins	-
Alkaloides	+
Deoxisugars	+
Reducing sugars	+

Regarding sterols present in the extract, increasing evidence points out their relationship with a lower risk of developing cancer and their application in the therapeutic field [26,27]. For *Pasiflora* genus, different studies have reported the presence of bioactive compounds [9,28,29], as well as antioxidant activity of leaf [12] and seeds extracts [30]. Overall, extracts from different parts of *P. edulis* plant contain different flavonoids, quinones and sterols, which display high antioxidant activity [28].

Effect of Aqueous *P. edulis* Extract on Cell Viability in SW620 and SW480 Cell Lines

In order to estimate the decrease on cell viability within 24 hours of treatment, we performed an MTT Assay. In this manner, it was observed that SW480 cells displayed significant differences between treated and non-treated cells ($p < 0.0001$), being the treatment at 10% of extract (264 µg/ml), the one that showed the highest reduction on cell viability (16.98%) (Figure 1) however, the regression analysis did not show a lineal correlation between extract concentration and viability percentage ($R^2 = 0.2352$). Likewise, for SW620 cells significant differences were observed between treated and non-treated cells ($p < 0.0001$), being the highest reduction on cell viability (18.50%) at the same concentration that was found for SW480 cells. Nevertheless, the regression analysis did not show an effect of extract concentration on SW620 viability percentage ($R^2 = 0.2352$). SW480 and SW620 cell lines have been widely studied given their isogenic features for which they have been validated as an *in vitro* model for colon cancer [31]. Cytotoxic activity of the extract displayed a dose-dependent response in both cell lines, but the concentration required to reach the half maximal inhibitory concentration (IC₅₀) must be higher than 264 µg/ml. Our results are

similar to IC₅₀ dose reported by Aguilon et al. who tested *P. edulis* juice and found an IC₅₀ of 444 µg/ml and 415 µg/ml for SW480 and SW620, respectively [12]. It is important to note that these concentrations are considerably higher than 30 µg/ml, which is the reference concentration established by the National Cancer Institute (NCI) in United States, to consider an extract with promising bioactivity [32]; because of this, the extracts tested on this assay could be considered low activity extracts. However, other authors, such as Neira, have found different fractions of *P. edulis* juice (an ethanolic fraction and a hydro soluble fraction with carotenoids and polyphenols) which inhibits growth of MOLT-4 human T cells at concentrations under 30 µg/ml [33]. Silva et al. tested an aqueous fraction rich in polysaccharides in the human colon adenocarcinoma cell line HCT-8, in which they found an IC₅₀ of 0.36 µg/ml and in the human leukemia cell line HL- 60 they found an IC₅₀ of 12.59 µg/ml [34]. As a whole, this data suggest that the cytotoxic effect of *P. edulis* extracts depends on the part of the plant used, the extraction method and the cell line tested.

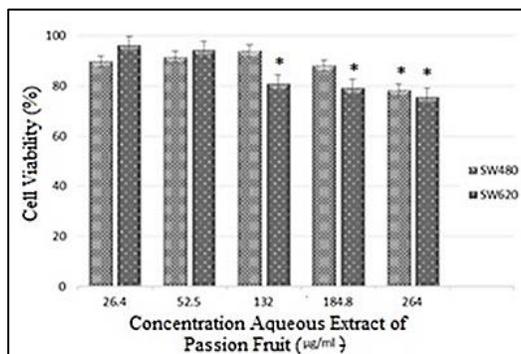


Figure 1: Cytotoxic effect of the aqueous extract of the *P. edulis* fruit on SW480 and SW620 cell lines after 24 hours of treatment

Effect of Aqueous Extract Obtained from *P. edulis* Fruit over Cell Cycle on SW620 and SW480 Cell Lines

Figure 2 presents the cell cycle distribution after treatments for 48 hours at 444 µg/mL and 415 µg/mL of the aqueous extract, for SW480 and SW620 cell lines, respectively. A significant increase in the percentage of hypodiploid cells (SubG0/G1 cell population) and a significant reduction of G2/M population was observed in cells treated with *P. edulis* aqueous extract and with turmeric extract. These results suggest that both extract possess phytochemicals with growth inhibitory properties that may be related to cell death mechanisms. Even though this approach allows to determine the amount of dead or dying cells in a population but gives no information on the cell death process (apoptosis or necrosis), we hypothesize that the mechanism induced is apoptosis as described for polyphenols like epigallocatechin gallate (EGCG) from green tea [35]. Theaflavin and Tearubigin from black tea [36] and polyphenols from olive leaf extract (hydroxytyrosol and oleuropein) [37-39].

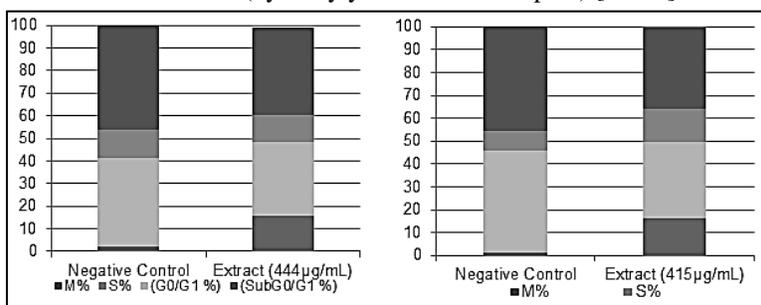


Figure 2: Cell cycle distribution in SW480 cells (right) and SW620 cells (left), treated with *Passiflora edulis* F. flavicarpa aqueous extract

Apoptosis Induction of *P. edulis* Aqueous Extract over SW620 and SW480 Cell Lines

Table 2 shows the results of the apoptosis induction by *P. edulis* fruit over SW480 and SW620 cell lines. For both cell lines, significant apoptosis levels were observed, being higher than 45% in SW480 cells and higher than 49% in SW620 cells. These results confirm that the SubG0/G1 cell population observed in the cell cycle distribution experiments (Figure 3) correspond to apoptotic cells.

Table 2: Apoptosis induction by *P. edulis* aqueous extract on SW480 and SW620 cell lines

Cell line	Population	% Viable cells	% Necrotic cells	% Cells in early apoptosis	% of cells in late apoptosis
SW480	C-	96.8 ± 0.7	0.3 ± 1.4	1.4 ± 1.3	1.5 ± 0.7
	C+	36.8 ± 2.9	5.1 ± 0.3	24.8 ± 0.6*	33.3 ± 1.7*
	<i>P. edulis</i> aqueous extract (444 µg/mL)	51.0 ± 0.1	3.7 ± 0.7	10.4 ± 2.6*	34.9 ± 0.2*
SW620	C-	96.4 ± 0.8	0.7 ± 0.5	1.2 ± 2.1	1.7 ± 0.5
	C+	27.5 ± 1.1	10.1 ± 1.5	17.2 ± 2.2*	45.2 ± 1.4*
	<i>P. edulis</i> aqueous extract (415 µg/mL)	46.6 ± 0.1	3.5 ± 2.1	11.8 ± 1.8*	38.1 ± 0.3*

(* significant difference)

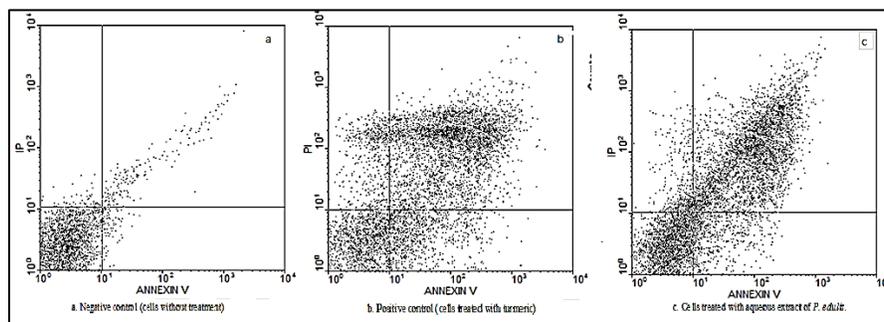


Figure 3: Scatter plot of the apoptosis inducing effect of the aqueous extract of passion fruit on SW480 cells

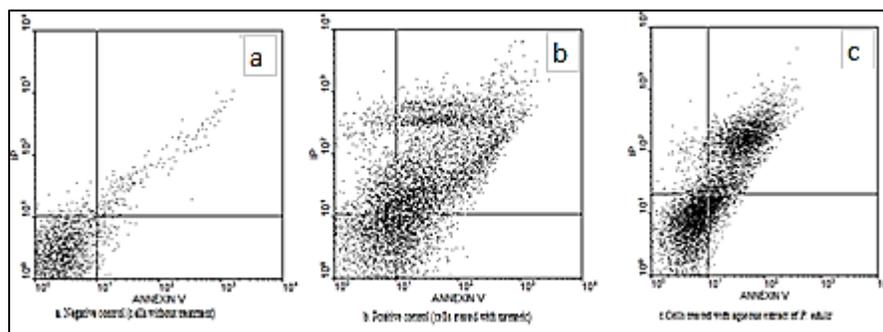


Figure 4: Scatter plot of the apoptosis inducing effect of the aqueous extract of passion fruit on SW620 cells exposed for 48 hours

This increase in the apoptotic levels can be related to the presence of flavonoids, quinones, sterols, and cardiac glycosides in the extract (Figure 4), as previously demonstrated on *in vitro* cancer models [40]. In the case of flavonoids, it has been demonstrated its capacity to modulate several signaling pathways involved in cell proliferation, migration and survival, like NFK β , MAPK, Wnt/ β -catenin and also the regulation of cell death mechanisms, like the induction of caspases proteins [41,42].

CONCLUSION

This study demonstrates that aqueous extract of Passion fruit (*P. edulis*) has proapoptotic and cytotoxic potential on an *in vitro* colon cancer model. For these reasons, along with the presence of several phytochemicals like polyphenols, sterols and cardiac glycosides in the extract, *P. edulis* can be considered as a promissory species for the search for phytotherapeutic or phytopreventive compounds for the management of colon cancer.

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