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Research Article

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Effect of the Ethanolic Extract of *Passiflora edulis F. Flavicarpa* Leaves on Viability, Cytotoxicity and Apoptosis of Colon Cancer Cell Lines

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ABSTRACT

Colorectal cancer is one of the main causes of morbidity and death in the world. This neoplasm is the second most frequent type of cancer and the fourth cause of cancer-related deaths worldwide; trends that may worsen in the coming decades. This fact has motivated the search for alternatives to prevent this type of cancer, such as promoting the use of food phytochemicals capable of blocking, delaying or reversing the process of carcinogenesis, as many of these substances have been identified in fruits and have been classified as cancer initiation blockers or suppressors of tumor promotion/progression phases. In this study, phytochemicals from ethanolic leaf extracts of P.edulis F. Flavicarpa, were identified, and their biological activity on cell viability, cytotoxicity and pro-apoptotic potential were determined over SW480 and Caco-2 human cancer cell lines. Compounds with chemopreventive potential such as flavonoids, cardiotonic glycosides, alkaloids, among others, were found in the extract. The results show that these compounds have a dose-dependent effect on cell viability at concentrations higher than 5% (v/v%) and considerable proapoptotic potential due to an increase in activity levels of caspase 3. These results suggest a potential chemopreventive effect of the extract at an in vitro level on colon cancer cell lines.

Keywords: Passifloraceae; Colon cancer; Citotoxicity; Natural extracts; Phytochemicals

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INTRODUCTION

The International Agency for Research on Cancer (IARC), estimated that approximately 14 million new cancer cases were diagnosed in 2012 and that 8.2 million cancer related deaths were reported in the same year; statistics that may worsen in the coming decades [1]. Colorectal cancer (CRC) is the third most common cancer in men and the second in women; likewise, is the fourth cause of cancer-related death worldwide [2]. More than 80% of CRC cases occur sporadically, that is, without a family history of this type of cancer, emerging as a disease linked to exogenous factors that potentiate events involved in cellular transformation mechanisms. The development of CRC consists of three stages: initiation, promotion and progression to a metastatic state. Initiation is a fast process, which includes exposure to a carcinogen and the absence of protective factors in the colon mucosa, and is phenotypically characterized by the appearance of an aberrant crypt [3]. Subsequently, in a period of about 10-20 years, a small proportion of the aberrant crypts foci form noninvasive adenomatous polyps (promotion), due to the accumulation of pre-neoplastic cells with altered cell division regulation mechanisms. This phase is followed by tumor growth and is characterized by the accumulation of genetic and epigenetic alterations that favor the acquisition of metastatic properties (progression from 1-5 years) [4].

Advances in biomedicine have allowed important progress in the knowledge of different antitumor mechanisms, which help develop cancer prevention strategies. One of these strategies is to promote the use of food phytochemicals with potential to block, delay or reverse a carcinogenesis process; many of these substances have been identified in fruits, vegetables and legumes, showing important properties as initiation phase blockers or suppressors of the promotion/progression phases [5-7]. It has been estimated that in a single portion of vegetables such as garlic, soy, ginger, onion, turmeric, tomato, broccoli, among others, numerous phytochemicals with chemopreventive properties can be obtained [8]; those phytochemicals differ in molecular structure, stability, solubility, metabolism, cellular uptake, and are subject to a competitive and/or synergetic activity that enhance their chemopreventive capacity [6]. Some of the phytochemicals, studied for their remarkable biological activity as chemopreventive agents, are present in plants of the Passifloraceae family. This taxonomic group of considerable diversity comprises 18 genera with more than 500 species [9]. The species *Passiflora edulis F. Flavicarpa* belongs to this family, and several phytochemicals such as glycosides (passiflorin and luteolin-6-C-kodososide), triterpenes (anthocyanins, eugenol, γ-lactones, carotenes, L-ascorbic acid), esters, volatile oils, amino acids, carbohydrates, thiamine, riboflavin, nicotinic acid, calcium, iron and phosphorus, have been found in its extracts [10]. In addition, different studies have shown the ability of these extracts to inhibit MMP-2 (matrix metallopeptidase 2) and MMP-9 (matrix metallopeptidase 9) proteins, two metalloproteinases involved in tumor invasion, metastasis and angiogenesis [11]. Likewise, the ethanolic and aqueous extracts of *P. edulis* have been shown to have antioxidant potential and have also displayed antitumoral properties, reducing the viability of colon cancer cell lines [12,13]. Therefore, this plant is of interest for its potential use in the prevention and control of carcinogenic processes. The aim of this study is to evaluate the effects of the ethanolic extract of *Passiflora edulis F. Flavicarpa* leaves on the viability, cytotoxicity and apoptosis over two cell lines derived from colon adenocarcinoma.

METHODOLOGY

Fresh leaves of *P. edulis F. flavicarpa* were collected in the municipality of La Tebaida, Quindío, Colombia (4.4376°N, 75.8489°W at 1165 masl). The specimens were identified by the Herbarium of Quindío University (collection number: 33974).

Isolation and Characterization of the Extract

P. edulis leaves were washed with water, dried at 40°C, and pulverized; the powder was leached for 8 days in 96% ethanol. Then, the chlorophyll was separated from the extract by a liquid-liquid extraction with ethanol-water (1:1) according to Aguillon J et al. [12] and filtered. The ethanol was evaporated under reduced pressure (60 mbar) at <40°C and the resulting solid was resuspended in a 20% solution of Dimethylsulfoxide (DMSO) and filtered again. The extract obtained was stored protected from light at 4°C until use. To the ethanolic fraction, a phytochemical content characterization was performed in order to identify secondary metabolites as described by Bilbao M [14]. Likewise, qualitative tests were used to evaluate the presence or absence of compounds such as tannins, flavonoids, quinones, sterols, saponins, cardiotonic glycosides, terpene lactones, coumarins, alkaloids and quaternary amines.

Cell Lines and Culture Conditions

The SW480 and Caco-2 cell lines (ATCC, Manassas, USA) were used in the study. Both cell lines were maintained and propagated in DMEM medium (Gibco) with glucose 25 mM, L-glutamine 2 mM, supplemented with 10% horse serum, 100 IU/ml penicillin, 100 µg/ml streptomycin and 1% non-essential amino acids. Cultures were maintained in a humidified incubator at 37°C, 5% of CO_2 and medium was changed every 48 h. For all experiments, the serum was reduced to 3%, and the medium supplemented with 10 µg/ml of insulin, 5 µg/ml of transferrin, and 5 ng/ml of selenium (ITS medium).

Cell Viability, Cytotoxicity and Apoptosis Assay

Cell suspensions of SW480 and Caco-2 were obtained for the assessment of cell viability by exclusion of trypan blue dye (0.4%) (GIBCO). Cell cultures with viability greater than 90% were used, seeding 3000 cells/well into 96-well plates; 24 hours later the medium was removed and the cells were treated with different concentrations of the extract during 24 hours. The biological activity of the extract was determined by the ApoTox-Glo™ Triplex Assay KIT (Promega) [15,16], following the manufacturer´s instructions. All measurements were performed using the microplate reader Glomax multidetection system (Promega) and the results are presented as relative fluorescence units (RFU) and as relative luminescence units (RLU), compared to non-treated controls. All the experiments were performed in triplicate. Different concentrations of the extract (v/v %), corresponding to 5% (114.53 µg/ml), 5.5%

(125.9 µg / ml) and 7% (160.3 µg / ml), were evaluated. As positive control (C+), cells were treated with H_2O_2 (250 µM), untreated cells were used as negative control (C-) and cells exposed to 0.2% of dimethylsulfoxide (DMSO) were used as vehicle control (CS) (which is the maximum concentration of the solvent in dilutions of treatments).

Statistical Analysis

Statistical analyses were performed using SAS software (Version 9.1) for completely randomized models. One-way analysis of variance (ANOVA) and Duncan's contrast test between each treatment were performed on the negative control. Statistically significant differences between treatments were considered when p-values less than 0.05 were obtained.

RESULTS AND DISCUSSION

Extract Characterization

Table 1 shows the results obtained for the characterization of phytochemical content in the ethanolic extract. The presence of different polyphenols, like tannins, flavonoids and quinones was identified, which agrees with the results of previous studies that report the presence of these compounds in the ethanolic leaf extract of *P. edulis* [17]. Polyphenols have been widely studied as potential chemopreventive molecules in different *in vitro* cancer models [18], showing diverse mechanisms of action, like anti-proliferative activity, apoptosis induction, cell cycle modulation and regulation of signaling pathways and pro-carcinogens metabolism [18,19].

Table 1: Ethanolic extract characterization *(P. edulis F. Flavicarpa)*

Metabolite	Presence(+)/ Absence(-)
Tannin	+
Flavonoids	+
Quinones	+
Esterols	-
Saponins	-
Cardiac Glucosides	+
Terpene lactones	-
Coumarins	-
Alkaloids	+
Deoxy-Sugars	+
Reducing Sugars	+
Carotenoids	+
Carbohydrates	+

Cell Viability, Cytotoxicity and Apoptosis

As shown in Figure 1, changes in viability levels were statistically significant for *P. edulis* treated cells ($p < 0.05$), when compared to untreated cells; with a stronger effect of the extract over Caco-2 cell line. Likewise, a cytotoxic effect of the extract was found at different concentrations evaluated over the two cell lines ($p<0.05$), when compared to untreated cells. In this test, we found that SW480 cells were more susceptible to treatment than Caco-2 cells, at the three concentrations evaluated (Figure 2).

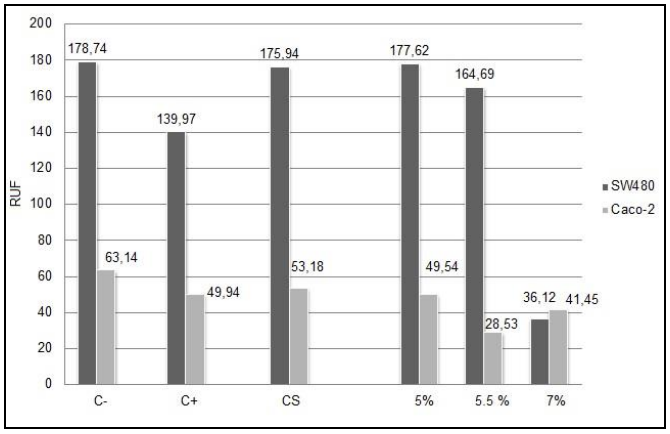

Figure 1: Cell viability assay. Effect of the ethanolic extract of leaves of *P. edulis F. Flavicarpa* on cells derived from colorectal cancer SW480 and Caco-2. The average values obtained from three independent trials are shown

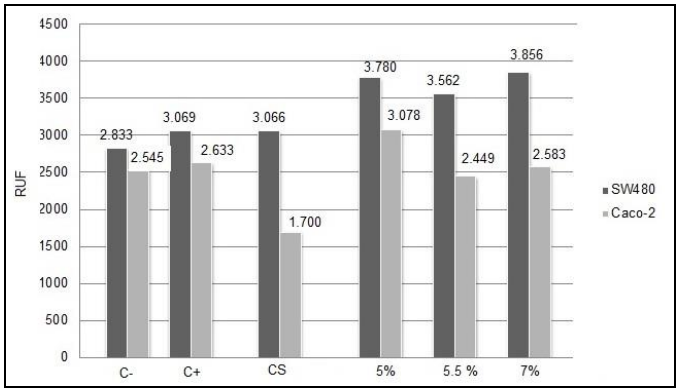

Figure 2: Cytotoxicity assay. Effect of the ethanolic extract of leaves of *P. edulis F. Flavicarpa* on cells derived from colorectal cancer SW480 and Caco-2. The average values obtained from three independent trials are shown

Regarding the apoptosis-inducing effect by activation of caspases 3 and 7, a dose-dependent effect for all treatments was observed in both cell lines, when compared to the negative control ($p<0.05$). Like in the cytotoxic analysis, SW480 cells were more susceptible to the apoptotic induction than Caco-2 cells (Figure 3).

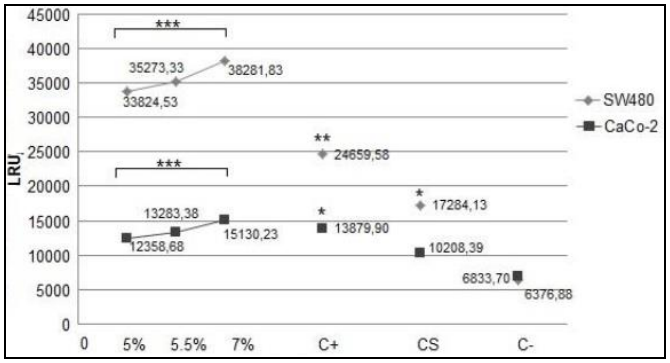

Figure 3: Apoptosis assay. Effect of the ethanolic extract of leaves of *P. edulis F. Flavicarpa* on cells derived from colorectal cancer SW480 and Caco-2. The average values obtained from three independent trials are shown

All together, these results suggest that viability of both cell lines was affected by the treatments and it was also evidenced that the cytotoxicity observed, is associated with increased activity of caspases 3 and 7. Previous work published by different researchers, has shown that inhibition and/or evasion of apoptosis is one of the main events that occur in transformation of healthy colorectal epithelium into adenocarcinoma and carcinoma [20]. This is important, because there are at least three different ways of regulating apoptosis, that are altered in the cancerous process: inactivation of *TP53* gene by genetic and epigenetic mechanisms, increased levels of *BCL2* and *c-MYC* genes, and defects in the COX-2 and Prostaglandin (PGE2) pathways [21]; highlighting that apoptosis plays an important role in the development of this type of cancer, for which the development of therapies based on mechanism of induction of apoptosis are of great importance [22]. Our results are in accordance with those reported by other authors, who evaluated the pro-apoptotic potential of phytochemicals, like polyphenols and carotenoids, present in *P.edulis* [23].

CONCLUSION

In conclusion, the results suggest that a decrease in viability and an increased activity of caspases 3 and 7 in the colon cancer cell lines SW480 and $Caco_2$ were induced by phytochemicals present in the ethanolic extract of *P. edulis*. For this reason, further research needs to be conducted, to study the chemopreventive properties of *P. edulis* for the treatment of colorectal cancer.

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