Chapter 4

Chemical, Antioxidant, and Cytotoxic Properties of Native Blue Corn Extract

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Abstract

In recent years, natural products such as dietary phytoconstituents have been the focus of scientific studies for cancer prevention. Among these are polyphenols, which have shown anticancer properties. Pigmented cereals such as blue maize are a rich source of polyphenols such as anthocyanins. Therefore, the aim of this work is to determine the chemical composition and cytotoxic activity of blue maize extract in several cancer cell lines. The total polyphenol content, total anthocyanins, and antioxidant activity of 16 blue corn samples from the Mixteco race were analyzed. From these, the sample with the highest content of polyphenols, anthocyanins, and antioxidant activity was selected and its anthocyanin fraction was isolated using an amberlite column and analyzed by means of HPLC-ESI-MS. The total polyphenol content ranged from 142.8 to 203.2 mg GAE/100g. The total anthocyanin contents varied between 19.02 and 66.92 mg C3G/100g. The antioxidant activity ranged from 18.5 to 27.8 μmol TE/g. The anthocyanin profile showed eight different compounds, mainly acylated anthocyanins. Cytotoxicity of blue corn extract on cancer cell lines was determined at concentrations of 100 and 500 μg/mL using the SRB assay. A cytotoxic effect was mainly observed on SKLU-1 and HTC-15 cell lines.

Keywords: blue corn extract, dietary phytoconstituents, anthocyanin profile, cancer cell lines, cytotoxic activity

1. Introduction

Molecules derived from natural sources, such as plants, marine organisms, and microorganisms, have become important sources of active compounds in the development of drugs for the treatment of human chronic diseases. In recent years, natural products such as dietary phytoconstituents have been the focus of scientific studies for cancer prevention [1]. Epidemiological and preclinical research indicates that dietary compounds possess chemopreventive properties, for example, garlic consumption has been associated with a lower risk of cancer [2–4]. In addition, supplementation of dietary phytochemicals for chemoprevention is gaining increased attention due to their chemical diversity, biological activity, and good availability.

Currently, more than 1000 dietary compounds belonging to different chemical classes have shown potential chemopreventive activities [5]. Among dietary constituents, polyphenols such as anthocyanins have demonstrated to exert many biological activities including anticancer properties [6]. From the chemical standpoint, anthocyanins are phenolic substances that belong to the group of flavonoids derived from the 2-phenylbenzopyrilic cation found in nature in a glycosylated or acylated form [7]. These compounds are particularly abundant in pigmented cereals such as red, purple, and black rice, black sorghum, and red, blue, or purple maize [8–10].

Mexico is the center of origin and biodiversity of maize (*Zea mays* L.). Species have an extensive genetic diversity, with 59 different races described with different shapes and colors ranging from white to yellow, red, purple, and blue [11]. Pigmented maize genotypes are used in the production of tortillas, tamales, atoles, and other traditional Mexican foods. These maize varieties have been the focus of scientific studies because they are a rich source of polyphenols such as anthocyanins. Recent data indicate that blue maize contains monomeric anthocyanins as well as acylated anthocyanins [12, 13].

Even though blue maize is an important part of the Mexican diet, there is little scientific information regarding its anthocyanin profile and anticancer properties. Chemical composition is a factor that must be considered in the selection of blue maize genotypes due to its impact on biological activity, and thus its potential applications for the treatment of disease such as cancer. For this reason, prior to embarking on cancer phytochemical trials, it is important to carry out a preclinical research in order to evaluate the potential application of phytochemicals from blue maize. It is well known that *in vitro* studies examine preliminary efficacy of phytochemicals for cancer prevention or therapy [14].

Given the above, the aim of this work is to evaluate the total content of polyphenols, anthocyanins, and the antioxidant activity of blue corn from the Mixteco race, and to determine its anthocyanin profile and the cytotoxic activity of the anthocyanin fraction in several cancer cell lines.

2. Research methods

2.1. Plant material

Sixteen samples of blue maize from the Mixteco race (**Figure 1**) were donated by the Interdisciplinary Research Center for Integral Regional Development (CIDIIR as per the

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Figure 1. Grains of blue maize from Mixteco race.

Spanish acronym) of the National Polytechnic Institute, Oaxaca Unit in Mexico. Maize kernels were grounded and placed in amber bottles for analysis.

2.2. Blue corn extracts

Ground blue corn kernels (1:5 p:v) were homogenized for 20 min with ethanol acidified with citric acid 1M (85:15 v:v). This was performed using an ultrasonic homogenizer at a frequency of 20 kHz and 750 W power (Cole-Palmer Instrument Company, VCX-750, USA) with a tip diameter of 13 mm at an amplitude of 25 μm with a pulse of 5 s in the 'On' position and 5 s in the 'Off' position. The sample was placed under refrigeration for 24 h and centrifuged at 4000 rpm for 15 min at a temperature of 5°C. The process was repeated twice and the extract was concentrated using a rotary evaporator under vacuum. The conditions of extraction have been included in a patent request, MX/A/20131011202.

2.3. Total phenolic content

For analytical purposes, total polyphenols were evaluated using the colorimetric method previously described by Folin-Ciocalteu and modified by Singleton and Rossi [15]. In this study,

0.2 mL of the extract was mixed with 3.0 mL of distilled water and 0.2 mL of Folin-Ciocalteu reagent. Next, a calcium carbonate saturated solution of 0.75 mL was added. Then the mixture was incubated for 60 min at 37°C in darkness; absorbance was read at 750 nm. This measurement was compared to a standard curve prepared with a gallic acid solution (20–120 mg/L) (Sigma Chemical). The total phenolic content was expressed as milligram equivalents of gallic acid/100 g of fresh weight (mg GAE/100g).

2.4. Total monomeric anthocyanin content

Monomeric anthocyanin content was evaluated using the differential pH method [16]. Absorbance was measured in a UV-VIS spectrophotometer (Perkin Elmer, Inc., Shelton, CT, USA). For the analysis, samples were diluted in potassium chloride buffer (pH 1.0) and sodium acetate buffer (pH 4.5). The difference in absorption at 510 and 700 nm was determined in buffers at pH 1.0 and 4.5. The monomeric anthocyanin content was expressed as cyanidin 3-glucoside mg/100g.

2.5. Antioxidant activity

The antioxidant assay was performed according to the DPPH (2,2-Diphenyl-1-picrylhydrazyl) method [17]. Trolox was used to make a calibration curve (100–800 μmol). About 2.9 mL of DPPH solution was mixed with 0.1 mL of blue corn extract, and then kept in the dark for 1 h. The sample was incubated for 30 min and it absorbance was read at 517 nm. The result was expressed as μmol eq. trolox/g of sample.

2.6. Isolation and chromatographic analysis of anthocyanins

For the isolation of anthocyanins, a column was packed with amberlite XAD-7 preconditioned with 5% acetic acid [18]. Then, 1 mL of the blue corn concentrated extract was placed into the column and eluted with acidified ethanol (5% acetic acid). The eluate was then concentrated to dryness using a Buchi rotary evaporator (Heidolph Digital Laborota pump 4011) coupled to Pimo Vacum Buchi V-700. Anthocyanins were analyzed by HPLC-ESI-MS. The HPLC system was coupled to a Brüker MicrOTOF II spectrometer. The column was C-18 ZORBAX eclipse plus column with 100 mm × 2.1 mm, 3.5 μm. The isocratic elution was done with a mix of methanol:water (2:8 v:v). Mass spectra analysis was carried out in negative ion mode, scan range: 50–3000 amu, capillary voltage 3.8 kV, dry gas flow at 4.0 L/min.

2.7. Cell culture and assay for cytotoxic activity

Prostate cancer cell lines (PC-3), neoplastic myelogenous leukemic cell lines (K-562), human colon cancer cell lines (HCT-15), human breast cancer cell lines (MCF-7), and lung cancer cell lines were provided by the National Cancer Institute (NCI), USA, and the Center of HIV/AIDS Services Center in Mexico City. Cytotoxicity of blue corn extract on tumor cells was determined at different concentrations (50, 100, and 500 μg/mL), using the protein-binding dye sulforhodamine B (SRB) assay in microculture to determine cell growth [19]. Cell lines were cultured in RPMI-1640 (Sigma Chemical Co., Ltd., St. Louis, MO, USA), supplemented

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with 10% of fetal bovine serum purchased from Invitrogen Corporation, 2 mM L-glutamine, 100 IU/mL, penicillin G, 100 mg/mL streptomycin sulfate, and 0.25 mg/mL amphotericin B (Gibco). They were maintained at 37°C in a 5% CO_2 atmosphere and 95% humidity. For the assay, the following suspensions were prepared: 5×10^4 cell mL (K-562, MCF-7), 7.5×10^4 cell/mL (PC-3), and 10×10^4 cell/mL (HCT-15, SKLU-1); 100 μL of these cells in suspension were seeded in 96-well micro-titer plates and incubated in order to achieve cell attachment to the plates. After 24 h of incubation, 100 μL of each test compound and positive substances (Cisplatin) were added to each well. After 48 h of incubation, adherent cell cultures were fixed '*in situ*' by adding 50 mL of cold 50% (wt/vol) trichloroacetic acid (TCA) and incubated for 60 min. at 4°C. The supernatant was discarded and the plates were washed three times with water and then air-dried. Cultures fixed with TCA were stained for 30 min. with 100 mL of 0.4% SRB solution. Protein-bound dye was extracted with 10 μmol of unbuffered tris base and the optical densities were measured by an Ultra Microplate Reader (Elx808, BIO-TEK Instruments, Inc.) with a test wavelength of 515 nm.

3. Results and discussion

3.1. Total content of polyphenols, monomeric anthocyanins, and antioxidant activity

The first aim of this research is to evaluate the total content of polyphenols, anthocyanins, and the antioxidant activity of blue corn extracts. Ethanol acidified with citric acid was used in the preparation of the extracts, since organic acids decrease the decomposition of anthocyanins during the following concentration step [20].

The total polyphenol content was observed in the range of 143–203 mg equivalent of gallic acid/100 g sample (**Table 1**), while the concentration of monomeric anthocyanins varied from 21 to 69 mg cyanidin-3-glucoside/100 g sample. In this study, the total polyphenol and anthocyanin levels were lower than values previously reported for American and Mexican blue corn [21]. Antioxidant activity evaluated with the DPPH method showed values between 18.5 and 26.8 μmol/100 g.

Results for the total content of polyphenols, monomeric anthocyanins, and antioxidant activity were plotted in a polygons graph in order to identify the sample with the best characteristics. **Figure 2** shows that sample CIIDIR-125 had the largest content of anthocyanins and antioxidant activity; therefore, it was selected to undergo the anthocyanin profile analysis and biological tests.

3.2. Anthocyanin profile of blue corn extract

Figure 3 shows the profile of anthocyanins isolated from blue corn using amberlite XAD-resin. The MS data analysis for blue corn anthocyanins is summarized in **Table 2**. It shows ions at m/z = 287 and 271, suggesting that anthocyanins are derived mainly from cyanidin and pelargonidin. Eight anthocyanins were identified such as: cyanidin-3-(3″,6″-dimalonyl-glucoside), pelargonidin-3-glucoside dimalonate, pelargonidin-3-(sinapoyl glucoside)-5-glucoside,

Sample	Total polyphenols[1]	Monomeric anthocyanins[2]	Antioxidant activity[3]
CIIDIR-02	154.4^{f,g,h,i}	32.5^{de2}	18.5^{e1}
CIIDIR-12	176.7^{c,d}	48.5^{b}	21.2^{d,e}
CIIDIR-54	158.4^{f,g}	31.2^{e}	18.6^{e}
CIIDIR-107	173.3^{d,e}	53.4^{b}	26.7^{a,b,c}
CIIDIR-112	142.8^{j}	30.7^{e}	18.5^{e}
CIIDIR-125	203.2^{a}	66.9^{a}	24.4^{ab,c,d}
CIIDIR-129	164.3^{e,f}	47.5^{b,c}	23.6^{b,c,d}
CIIDIR-131	173.4^{d,e}	53.4^{b}	27.8^{a}
CIIDIR-167	187.1^{b,c}	32.3^{e}	24.4^{a,b,c,d}
CIIDIR-172	147.4^{g,h,i}	28.6^{ef}	22.7^{c,d}
CIIDIR-179	162.1^{f}	30.7^{e}	21.5^{d,e}
CIIDIR-184	146.5^{i,j}	21.4^{f}	20.6^{d},^{e}
CIIDIR-185	192.9^{a,b}	35.1^{de}	26.8^{a,b}
CIIDIR-189	157.7^{f,g,h}	40.3^{cd}	22.8^{c,d}
CIIDIR-190	148.1^{g,h,i}	33.1^{de}	23.5^{b,c,d}
CIIDIR-197	175.3^{d}	35de	26.7^{a,b,c}

Samples with the same letters are not significant statistically ($p < 0.05$).
[1]mg GAE/100g.
[2]mg C3G/100 g.
[3]μmol TE/g.

Table 1. Content of total polyphenols, monomeric anthocyanins and antioxidant activity in blue corn samples.

pelargonidin 3-(3″,6‴-dimalonylglucoside), pelargonidin 3-(6″-malonyl glucoside)-5-(6″ acetyl glucoside), pelargonidin 3-glucoside-5-(6″-acetyl-glucoside), pelargonidin 3-(6″-malonylglucoside), and pelargonidin 3,5-diacetylglucoside. The data indicates that only acylated anthocyanins are present in blue corn from the Mixteco race. This could be due to genetic factors, farming practices, weather conditions, and soil type, which have an influence on the chemical composition of maize varieties.

3.3. Cytotoxic activity of the anthocyanin fraction from blue corn

In this study, the SRB assay was used to evaluate cytotoxic activity, and it was selected in order to avoid any interference of anthocyanins in the final reading. The effect of the blue corn extract at different concentrations on the prostate cancer cell line (PC3), neoplastic myelogenous leukemic cell line (K562), human colon cancer cell line (HCT-15), human breast cancer cell line (MCF-7), and lung cancer (SKLU-1) are shown in **Figure 4**. Generally speaking, it was observed that for blue maize extract, the percentage of growth inhibition of cancer cell lines improved with increased concentration; the analysis indicates that the blue corn extract causes growth inhibition in all cancer cell lines in a dose-dependent manner (**Figure 4**).

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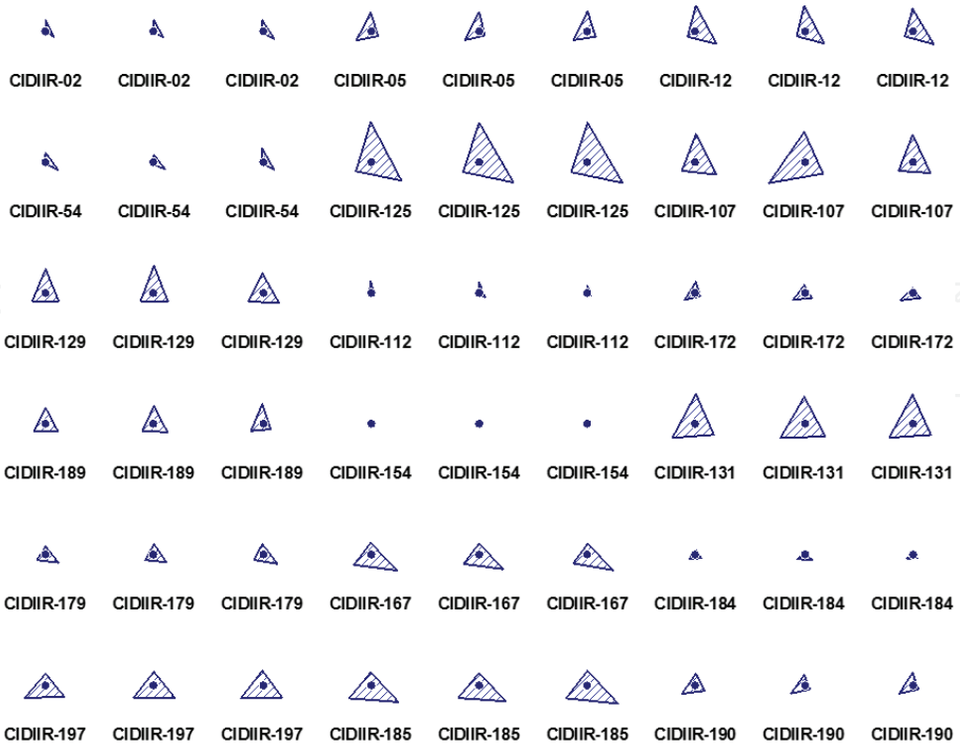

Figure 2. Polygon graph of total polyphenols, monomeric anthocyanins and antioxidant activity.

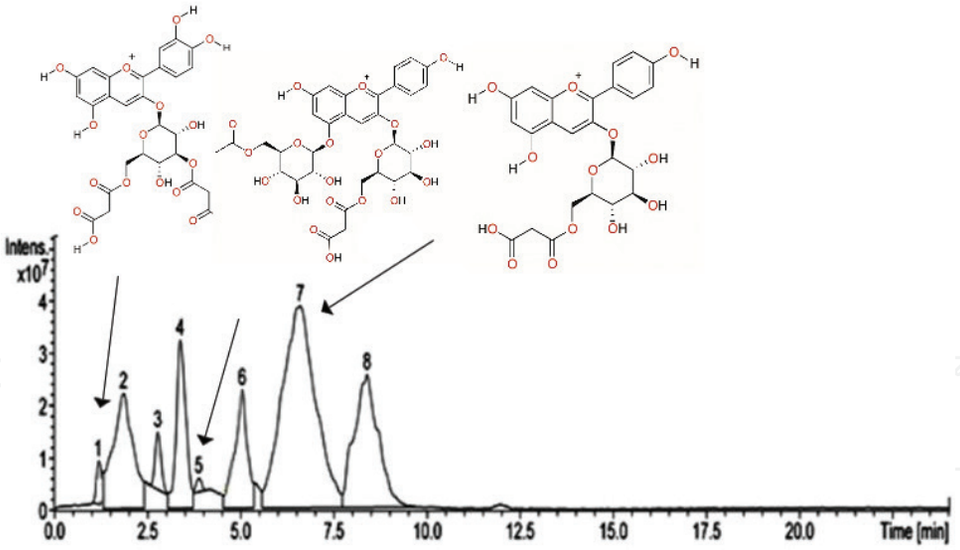

Figure 3. Anthocyanin profile of blue corn.

PC3 cells were selected due to their highly aggressive nature. Data showed 2.43% inhibition on prostate cancer cells at 500 μg/mL. Previous studies have analyzed the anticancer properties of the anthocyanin fraction from potato extracts in prostate cancer cells (PC-3) showing cytotoxicity [22]. It has been reported that the anthocyanin profile has an effect on anticancer activity. For example, pomegranate extract has an abundance of delphinidin derivatives, a compound with anticancer activity on human prostate [23]. In the present

Peak	Retention time (min)	*m/z*	Tentative identification
1	1.2	621–287	Cyanidin-3-(3″, 6″-dimalonyl-glucoside)
2	1.9	358–271	Pelargonidin-3-glucoside dimalonate
3	2.8	639–271	Pelargonidin-3-(sinapoyl glucoside)-5-glucoside
4	3.4	605–271	Pelargonidin 3-(3″,6‴-dimalonylglucoside)
5	3.9	740–519–433–271	Pelargonidin 3-(6″-malonyl glucoside)-5-(6″ acetyl glucoside)
6	5.0	654–595–434–271	Pelargonidin 3-glucoside-5-(6″-acetyl-glucoside)
7	6.6	519–271	Pelargonidin 3-(6″-malonylglucoside)
8	8.4	595–271	Pelargonidin 3,5-diacetylglucoside

Table 2. Chromatographic and mass spectral data of anthocyanins.

research, the anthocyanin profile of blue corn was composed only of cyanidin and pelargonidin derivatives; delphinidin was not detected.

In addition, the analysis indicates that the blue corn extract showed higher inhibition of cellular growth in MCF-7 cancer cells than SKLU-1 (**Figure 4**) at the same concentration

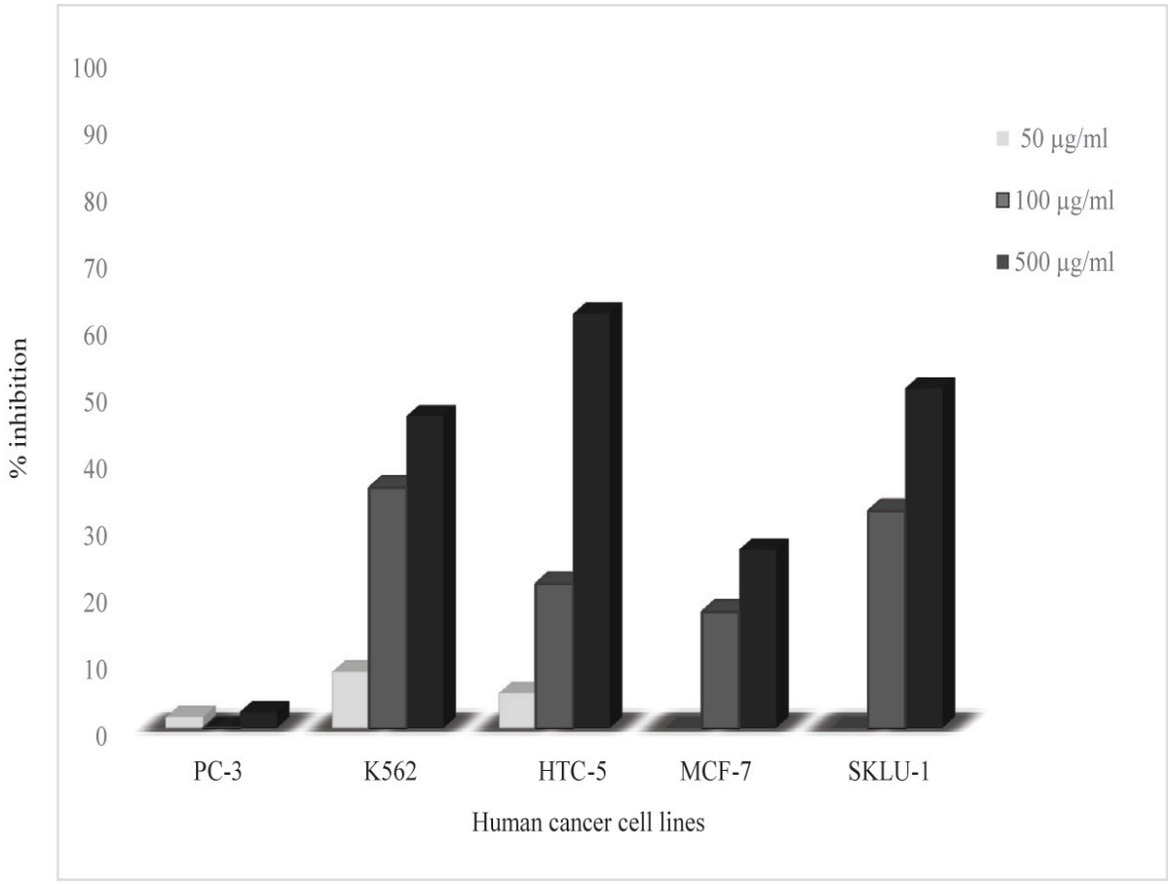

Figure 4. *In vitro* cytotoxicity of blue corn extract on several human cancer lines.

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(500 μg/mL). Anthocyanin-rich extracts of cereals such as black rice and black sorghum have also showed the anticancer effects on MCF-7 cells. On the other hand, reports on the effects of anthocyanins on SKLU-1 cells are scarce; a study performed on kenaf seed extract showed cytotoxic activity toward SKLU-1 [24].

Interestingly, blue corn extract was able to inhibit 50.9% of lung cancer cells at 500 μg/mL, which suggests a potential for application in lung cancer treatment, one of the five cancer types most frequently diagnosed in male population worldwide; however, studies *in vivo* (animal experiments) and clinical trials are needed. In this regard, studies on blueberries report the presence of anthocyanins in lung tissue of mice fed with this anthocyanin-rich fruit (5% w/w) for 10 days, suggesting that fruits, vegetables, and cereals such as blue corn may be an important source of chemopreventive dietary components [25].

Likewise, blue corn extract also inhibited the growth of neoplastic myelogenous leukemic cells (K562) and colon cancer (HCT-15) cell lines on 46.7 and 62 % at 500 μg/mL, respectively. Given the above, the blue corn extract was more effective on the growth inhibitory activity on HCT-15 colon cancer cell lines as compared to other cancer cell lines. Current statistics indicate that in 2012 colorectal cancer was the third most common cancer in the world. For this reason, there is an increasing interest for chemoprevention as a cancer prevention strategy. Dietary agents such as anthocyanins have been explored for their chemopreventive effects against colon cancer [26]. *In vitro* data obtained in this research provides information for the future application of blue corn extract as a chemopreventive agent in colon cancer.

In summary, blue corn possesses antioxidant properties and its anthocyanin profile is constituted solely by acylated anthocyanins. These results are particularly important since corn is the basis of the Mexican diet; they suggest that corn anthocyanins may have anticancer activity. Further research is necessary to obtain deeper knowledge on specific molecular targets of blue corn and to ensure the safe use of these active compounds as therapeutic agents on lung and colon cancer.

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