




# Cytotoxic and genotoxic activity of protein isolate of ayocote beans and anticancer activity of their protein fractions

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## Abstract

We evaluated the cytotoxic and genotoxic effects of protein isolates of ayocote beans on adult male CD1<sup>+</sup> mice and the anticancer activity of their protein fractions using SiHa Cells. The mice that received protein isolate showed a Polychromatic Erythrocytes (PCE) increase at 48 h; a significant PCE decrease was observed in mice administered the protein isolate-Daunorubicin (isolate-DAU) mix. A micronucleated erythrocytes (MNE) increase was observed in mice that received the mix in all times tested. All protein fractions of ayocote beans (black and purple) showed inhibition against SiHa cells proliferation at doses of 3000 or 5000 µg/mL. The maximal inhibitory concentration (IC<sub>50</sub>) of each protein fraction was reached at a concentration of 5000 µg/mL. A synergistic effect of isolate-DAU was observed in the in vivo test; the effect of the protein fractions on SiHa cells proliferation depended of the ayocote bean variety used.

**Keywords** Ayocote bean · Cytotoxic · Genotoxic · Protein fractions · SiHa cells

## Introduction

A low incidence of cancer is associated with the consumption of compounds from vegetables and fruits [1], principally associated with the consumption of nutraceutical foods. In recent years, an increasing interest in the use of dietary botanical supplements for the prevention and adjuvant therapy on cancer treatment has been observed. Generally, these botanical supplements (fruits and vegetables) are rich

in phenolic compounds, carotenoids, chlorophyll, vitamins, fiber and proteins, which have antimutagenic and/or anticarcinogenic properties. Several studies have demonstrated the potential of proteins for cancer prevention [2, 3] being the leguminous, an important source of vegetable protein due to their high protein content (20 to 50%) [4]. Other studies have demonstrated that the protein hydrolysates and peptides of the leguminous have a high biological potential [3, 5, 6]. The *Phaseolus* genus is the most important food legume consumed in the countries of Central America, South America, Central Africa and East Africa [7] because they contain a high concentration of starch and protein. In particular, the ayocote bean (*Phaseolus coccineus*), is a cultivated specie of the genus *Phaseolus*, with exceptional characteristics for several reasons. First, it is a perennial species and can live up to 10 years; however, in Mexico and Central America, it usually cultivated as an annual crop in areas that experience harsh winters. Second, it readily produces seeds for future crops due that their flowers are easily fertilized by hummingbirds [8]. In particular, evidence has shown that the *Phaseolus* genus is a promissory source of nutraceutical proteins and peptides to inhibit several types of cancer [9, 10]. Ayocote bean (*Phaseolus coccineus*) is a legume with a high nutraceutical potential due to its high content of protein (≈ 30%) [11]; however, few studies have researched

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its nutraceutical properties, reporting several activities as antineoplastic and antifungal of lectins [12], antifungal, antiproliferative and antibacterial activity of protein isolates [13]. However, the genotoxic and cytotoxic activities of protein isolates and peptides obtained from *Phaseolus coccineus* have not been reported. For this reason, in order to generate potentially-functional peptides from the ayocote bean protein isolate, a gastrointestinal digestive system was simulated to generate peptides similar to those released in a physiological digestion process, using proteinases obtained from animal tissue (pancreatin and trypsin) evaluating the anticancer activity of the protein fractions obtained and the cytotoxic and genotoxic activities of protein isolates from two varieties of ayocote beans (black and purple).

## Materials and methods

### Vegetal material

Two varieties of ayocote beans (black and purple) were obtained from Zacatlan Puebla, Mexico, with a moisture value of  $9.37 \pm 0.208\%$  and  $9.62 \pm 0.113\%$ , respectively. The seeds were selected, any extra material was removed and then the beans were grounded by a domestic coffee grinder. The dried ayocote bean powder was packed in PVC bags and stored in a LG Model GR-452SH refrigerator (LG electronics, México) at 4 °C until use.

### Preparation of protein isolates

Protein isolates from the defatted ayocote bean powder samples were prepared by the method as described by Bernardino-Nicanor et al. [14]. The defatted powder was dispersed in distilled water (1:20) and homogenized by magnetic stirring, adjusting the pH to 11.8 with NaOH (0.1 N); then the solution was centrifuged at 6000 rpm for 30 min at a temperature of 4 °C; and the supernatant was collected. Then, the pH of the collected supernatant was adjusted to pH 4 (HCl; 0.1 N), the precipitated protein was recovered by centrifugation at 3000×g for 30 min, and then the recovered protein was dried using a forced convection-drying oven (Binder, Model FD115-UL, USA) at a temperature of 50 °C for approximately 6 h.

### Enzymatic hydrolysis

The protein isolates were hydrolyzed by sequential treatment with pepsin (P7012, Sigma) and pancreatin (P1750, Sigma), according to the method of Mora-Escobedo et al. [15]. The protein isolate was suspended in distilled water to prepare the protein substrate (44 mg/mL). The protein solution was adjusted by the addition of 1 N of HCl to a pH of 2

and a temperature of 37 °C for 60 min. A measured amount of pre-suspended pepsin in distilled water and adjusted to hydrolysis pH conditions was then added to the substrate in order to obtain an enzyme/protein ratio of approximately 4.5% (AU/w). Later, the solution was adjusted by the addition of 0.9 M of NaHCO<sub>3</sub> to a pH of 5.3, then a solution of pancreatin, with an enzyme/protein ratio approximately 4.5% (AU/w), was added, the mix was gently homogenized and then the pH was adjusted to 7.5 using 1 N of NaOH and a temperature of 37 °C; the reaction mixture was maintained for 120 min. The hydrolysis reaction was stopped by heat treatment of the reaction mixture to 90 °C for 10 min. All procedures were carried out in a 100 mL glass reactor.

### Determination of the degree of hydrolysis (DH)

The degree of hydrolysis was obtained as protein solubility in trichloroacetic acid (TCA), according to Kim et al. [16]. An aliquot of 10 mL of hydrolysate was solubilized in 10% TCA solution, and after 15 min, centrifuged at 12,000×g for 15 min. The nitrogen content of the hydrolysate and the supernatant of the sample treated with TCA were analyzed by the Kjeldahl method [17]. The calculation of the degree of hydrolysis (DH) was conducted as follows:

$$\text{DH (\%)} = \frac{\text{Soluble N}_2 \text{ in TCA (10\%)}}{\text{Total N}_2 \text{ in the sample}} \times 100$$

where DH is degree of hydrolysis; N<sub>2</sub> is nitrogen; TCA is trichloroacetic acid.

### Peptide fractionation by ultrafiltration

The protein hydrolysate was further fractionated by ultrafiltration with a stirred cell and disc membrane system (Millipore Amicon, Model 8050). The protein hydrolysate solution was first separated by a 30-kDa molecular weight cutoff (MWCO) membrane in the cell at 75 psi and 4 °C. The separation generated two fractions, the streams-permeate (designated P30) and retentate 1 (R30). R30 was dialyzed against deionized water at 4 °C for 1 h and lyophilized, and then P30 was separated using a 10-kDa MWCO membrane to obtain the two fractions, the stream-permeate (designated P10) and retentate 10 (R10). R10 was dialyzed against deionized water at 4 °C for 1 h and lyophilized; P10 was separated using a 5-kDa MWCO membrane to obtain the two fractions, the stream-permeate (designated P5) and retentate 5 (R5). R5 was dialyzed against deionized water at 4 °C for 1 h and lyophilized; P5 was separated using a 3-kDa MWCO membrane to obtain the two fractions, the stream-permeate (designated P3) and retentate 3 (R3). Finally, P3 was separated by a 1-kDa MWCO membrane and generated a permeate (designated P1) and a final retentate (R1). For

satisfactory separation, each retentate was passed through the same membrane twice before being separated with the next membrane. The resulting six fractions (R30, R10, R5, R3, R1 and P1) were each freeze-dried, and the resulting powder was milled in a mortar, sealed in glass bottles, and stored at 4 °C until use.

## In vivo cytotoxic and genotoxic assays

### Animals

All experiments followed the Guidelines on Ethical Standards for Investigation of Experimental Pain in Animals.

Adult male CD1<sup>+</sup> mice (body-weight range, 20–25 g) were obtained from the vivarium of Health Sciences Institute (Pachuca Hidalgo, México). Mice were housed under controlled temperature (23 ± 2 °C) with a 12 h light/dark cycle and humidity (55 ± 10%) and were habituated to the environment for at least 1 week before experiments. Animals were provided with food and water ad libitum. Twelve hours before experiments, only food was withheld. Immediately after the experiments, all animals were euthanized in a CO<sub>2</sub> chamber.

### Experimental procedure for cytotoxic/genotoxic assays

Cytotoxic/genotoxicity assays were performed by subchronic administration. Mice were grouped into eight groups with five individuals each: a control group was administered saline solution (0.9%), and the second group was treated with 1 mg/kg of the cytotoxic drug, daunorubicin (DAU). The third group was administered with protein isolate of black ayocote beans (430 mg/kg) (BAB-430). The fourth group was administered with protein isolate of black ayocote beans (860 mg/kg) (BAB-860). The fifth group was administered with a mix of BAB-860-DAU (MIDB). The sixth group was administered with protein isolate of purple ayocote beans (430 mg/kg) (PAB 430). The seventh group was administered with protein isolate of black ayocote beans (860 mg/kg) (PAB 860). The eighth group was administered with a mix of PAB-860-DAU (MIDP).

Subchronic testing was conducted during a 5-day period. For the intragastric administration, all samples were diluted in isotonic solution (0.9%), and the volume administered was according to the mice weight. After the testing period, the mice were maintained in a recuperation period for 10 days.

### Blood sample preparation

The samples of peripheral blood of mice were taken at intervals of 48 h during all experimental procedures (360 h). A blood sample from the tail of each mouse was obtained

before the isolate and/or daunorubicin administration (0 h) and at 48, 96, 144, 192, 240, 288 and 360 h post-administration. Each sample was smeared onto ethanol-cleaned glass slides. Cells were fixed for 3 min with methanol and stained for 15 min with 5% Giemsa solution diluted in phosphate buffer (pH 6.8). Then, glass slides were washed in tap water, dried and observed at a magnification of × 1000 under a microscope (Model CX22LEDRFS1; Olympus, Tokyo, Japan).

### Genotoxic and cytotoxic potential

The genotoxic and cytotoxic potential of the protein isolates of ayocote beans was determined by scoring the number of micronucleated polychromatic erythrocytes (PCMNE) in 1000 polychromatic erythrocytes (PCE), as well as the ratio of PCE in 1000 erythrocytes per mouse, respectively, according to the description by Schmid [18] and Mac Gregor et al. [19].

### Determination of anticancer activity

#### Cell culture

SiHa cells (origin: uterus; histopathology: squamous cell carcinoma) were obtained from the Cancer National Institute (Mexico City, Mexico). SiHa cells were cultured in DMEM medium supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 U/mL of penicillin and 100 µg/mL of streptomycin in a 5% CO<sub>2</sub> humid atmosphere at 37 °C. Cells were washed with DMEM medium containing 1% FBS 24 h before experiments and replated onto 96-well plates.

#### Anticancer activity assay

Cell viability was measured by using the MTT assay, which is based on the conversion of MTT to formazan crystals by mitochondrial dehydrogenases [20]. SiHa cells (25 cells/µL) were treated with various concentrations of peptides from purple or black ayocote beans (1000–5000 mg/mL) at different contact times (3, 6, 12, 24, 48 and 72 h) at 37 °C. Then, the medium was incubated with 10 µL of 5 mg × mL<sup>-1</sup> MTT solution for 3 h. After the culture medium was removed by vacuum aspiration, 100 µL of dimethyl sulfoxide was added to each well to dissolve the formazan. Absorbance was measured at 540 nm using a microplate reader (EPOCHH, Biotek, Bio tek Instruments Inc. USA). Cell viability was expressed as a percentage of the value in the control cultures.

### Statistical analysis

The quantitative data are expressed as the mean ± standard deviation, and the analysis of variance (ANOVA) was

performed, followed by Tukey's test. SAS software was used for the data analysis, and all experimental determinations were performed in triplicate.

## Results and discussion

### Protein isolate yield

The yield of ayocote bean protein isolate was in the range of 12.1–12.3% of the seeds on a dry basis. No significant differences ( $p < 0.05$ ) were found in the yield of protein isolates among the studied variety (black and purple), principally due to that the samples were obtained of the same locality, and similar agricultural labors (fertilizer application, cultural management practices and double-crops production) [21].

Variations in the total protein yield by the isoelectric point method (53.7 and 55.35% for the black and purple ayocote beans respectively) is due to the traits such as protein concentration that usually are quantitatively inherited and influenced substantially by non genetic factors, making it difficult to evaluate plant materials, for this reason in this study variations in the protein content of the isolated product was observed (86.60 and 90.80 for the purple and black ayocote beans respectively) [22].

The results of the present study are in agreement with previous studies that reported a protein content of protein isolates from nine varieties of *Phaseolus vulgaris* between 83.96 and 89.25% [23], 81.0% for *Phaseolus aureus* [24], and 79.96 to 83.96% for kidney beans, the differences in the protein content might be attributed to salt formation of proteins with alkali or acids used in isoelectric precipitation of proteins [25]. On the other hand the recovery yield is similar to those obtained by Qayyum et al. [26] for kidney beans with a 14.60% recovery of protein.

### Degree of hydrolysis (DH)

When comparing the DH of protein isolates from purple ayocote beans (PIPAB) and black ayocote beans (PIBAB), both hydrolysates possessed a similar DH (approximately 73%). The high hydrolysis degree obtained is due to the characteristics exhibited by the enzymes used, the trypsin is a endopeptidase that have preferential release of N-terminal Arg and Lys at P1 position. While the pepsin (also an endopeptidase) has a preferential cleavage of amino acids hydrophobic, preferably aromatic residues [27]. This result was in accordance with Mora-Escobedo et al. [15] who found that the protein hydrolysates produced from soy had a similar DH (73.5%) in similar conditions and the same enzymatic system (pepsin–pancreatin). On the other hand, Dikshit and Ghadle [28] used a different enzymatic system (pepsin-trypsin) and obtained a DH of 73% for soy protein. However, the DH of the protein hydrolysate of ayocote beans was higher than the DH of soy hydrolysates, using alcalase or flavorzyme. It has been observed that the DH efficiency in black bean protein hydrolysates is higher using pepsin than that of alcalase, probably due to the low pH (2.0) of the pepsin treatment [29]. On the other hand, Tavano [27] indicated that DH is dependent not only on the enzymes used (with endo/exo activity) but also on the treatment conditions; for this reason, the reaction time of 180 min was used expecting structural and functional changes of the protein that may improve its functionality, considering that Wang et al. [30] mentioned that both the enzymatic mix and adequate enzymatic conditions could generate peptides with high antiproliferative activity.

### In vivo cytotoxic and genotoxic index

The results regarding the frequency of PCE with relation to 1000 ET of mice are shown in Table 1. The percentage of PCE among 1000 scored erythrocytes (PCE/1000 ET)

**Table 1** Induction of PCE in male mice following a subchronic test

Sample	PCE/1000 TE Time (h)					
	0	48	96	144	264	360
Control	15.8 ± 1.48 <sup>a</sup>	18.2 ± 1.92 <sup>b</sup>	20.6 ± 1.67 <sup>c,d</sup>	23 ± 0.70 <sup>b,c</sup>	24.4 ± 1.14 <sup>d</sup>	25.2 ± 3.11 <sup>b,c</sup>
DAU	16 ± 2.54 <sup>a</sup>	8 ± 2.54 <sup>c</sup>	15.25 ± 2.86 <sup>d</sup>	25.5 ± 1.50 <sup>b</sup>	39.33 ± 0.86 <sup>a</sup>	26 ± 0 <sup>b</sup>
BAB-430	15.8 ± 1.48 <sup>a</sup>	44 ± 4.00 <sup>a</sup>	34 ± 2.00 <sup>a</sup>	25.5 ± 1.80 <sup>b</sup>	28.5 ± 1.11 <sup>b,c,d</sup>	28.75 ± 1.47 <sup>b</sup>
BAB-860	15.8 ± 1.48 <sup>a</sup>	44.4 ± 1.14 <sup>a</sup>	32.8 ± 3.11 <sup>a</sup>	27.4 ± 2.07 <sup>b</sup>	28.8 ± 1.094 <sup>b,c</sup>	28.5 ± 1.80 <sup>b</sup>
PAB-430	15.8 ± 1.48 <sup>a</sup>	41.5 ± 2.16 <sup>a</sup>	29.2 ± 1.92 <sup>a,b</sup>	27 ± 2.12 <sup>b</sup>	28.25 ± 0.86 <sup>b,c,d</sup>	39.25 ± 3.89 <sup>a</sup>
PAB-860	15.8 ± 1.48 <sup>a</sup>	42.8 ± 2.68 <sup>a</sup>	29 ± 3.24 <sup>a,b</sup>	24.75 ± 0.82 <sup>b</sup>	30.5 ± 1.65 <sup>b</sup>	35.75 ± 2.48 <sup>a</sup>
MIDB	15.8 ± 1.48 <sup>a</sup>	17.24 ± 5.30 <sup>b</sup>	29 ± 5.33 <sup>a,b</sup>	40.5 ± 5.67 <sup>a</sup>	ND	ND
MIDP	15.8 ± 1.48 <sup>a</sup>	6.8 ± 1.92 <sup>c</sup>	23.6 ± 1.67 <sup>b,c</sup>	19.4 ± 1.14 <sup>c</sup>	26 ± 4.94 <sup>c,d</sup>	21.5 ± 1.35 <sup>c</sup>

Different letters in each row indicate significant differences ( $p \leq 0.05$ ). The values are means ± SD of triplicate measurements

varied between 0 and 44.4. However, considering that the proportion of PCE in the human and animal blood circulation is constant, a significantly higher frequency of PCE was observed in the blood of mice exposed to the protein isolate in the first 48 h than in the mice from the control group. On the other hand, a lower proportion of PCE was observed in the mice administered with MIDP than in mice of the control group.

The increase in the frequency of the PCE indicates that the increase in the number of the hemopoietic cells is probably associated with the interaction of the protein isolate, mainly with cells of the innate immune system that promote the cell-mediated immune response against tumors [31]. On the other hand, according to Araujo-Espino et al. [32], when a decrease in the proportion of PCE is produced, there is a connection with the cytotoxic capacity of the compound administered. Some authors indicate that the biological potential of bean proteins depends on the cultivar [3], and the apparently chemoprotective effects observed in the group administered with MIDB are due to antioxidant activity, as suggested by other authors [33]. It has been reported that the proteins have a high antioxidant activity, and in some cases, the antioxidant activity of vegetable proteins is higher than animal proteins [34].

After 48 h in the mice administered only with ayocote bean isolate, the number of PCE decreased, apparently by a bone marrow cell depletion originated probably from the lectin present in the isolate; other authors have indicated that lectins are capable of interacting with saccharides in the cell membrane originating from cell perturbation and homeostatic destabilization [35]. The differences observed in the number of PCE between the two samples of ayocote bean isolates (black or purple) apparently are due to that the cultivar significantly influenced the biological activities [36].

The results obtained in the proportion of PCMNE in 1000 TE are shown in Table 2, where it was observed that the group that received DAU (positive control) showed a significant increase in the number of PCMNE at 48, 144 and 360 h in relation to the initial count (0 h). The groups administered with each one of the two treatments of black ayocote bean protein isolates (BAB-430, BAB-860) exhibited a significant increase in the number of PCMNE in relation to the initial count at 360 h. While that with the purple ayocote bean protein isolate, only the PAB-860 showed a significant increase in the number of PCMNE at 264 h, and similar results were observed with the MIDP mix. The number of PCMNE in mice administered with all treatments of ayocote bean protein isolates (alone and mix) was lower than in the mice administered with only DAU for most times; however, an increase in the number of PCMNE at 264 h was observed with MIDP.

The animals treated with different doses of ayocote bean protein isolates showed a significant reduction in the frequency of MNE compared to the animals treated with DAU at 48, 144 and 360 h; however, a synergistic effect of ayocote bean protein isolate-DAU was observed in the mice administered with MIDB and MIDP (Table 3), which reached approximately 12-fold more MNE at 360 h than the control and threefold more than in the mice administered with DAU.

The increment in the micronucleated erythrocytes (MNE) was a result of chromosome acentric fragments or an incomplete migration and for this reason has been excluded from the main core. We observed that both ayocote bean isolates (black and purple) had moderate genotoxic effects in mice polychromatic erythrocytes at both doses (430 and 860), and the number of micronucleated erythrocytes increased significantly after administration of the mix (MIDB or MIDP). No significant differences by effects of variety and the isolate concentration administered to the mice were observed.

**Table 2** PCMNE in blood of male mice following a subchronic test

Sample	PCMNE/1000 TE					
	Time (h)					
	0	48	96	144	264	360
Control	1.5 ± 0.5 <sup>d</sup>	7.4 ± 1.140 <sup>a</sup>	2.656 ± 0.408 <sup>c,d</sup>	7.4 ± 1.341 <sup>a</sup>	5.8 ± 0.836 <sup>b</sup>	3 ± 1.000 <sup>c</sup>
DAU	1.4 ± 0.547 <sup>c</sup>	10 ± 1.224 <sup>b</sup>	2.8 ± 1.483 <sup>c</sup>	11.75 ± 2.046 <sup>a,b</sup>	3 ± 1.224 <sup>c</sup>	12 ± 0 <sup>a</sup>
BAB-430	1.5 ± 0.500 <sup>b</sup>	0 <sup>c</sup>	2.332 ± 1.080 <sup>a,b</sup>	1.5 ± 0.866 <sup>b</sup>	2.0 ± 1.089 <sup>a,b</sup>	3.0 ± 2.345 <sup>a</sup>
BAB-860	1.5 ± 0.500 <sup>b</sup>	1.7 ± 2.345 <sup>b</sup>	2.0 ± 1.304 <sup>b</sup>	2.3 ± 2.345 <sup>b</sup>	2.0 ± 2.049 <sup>b</sup>	5.0 ± 0.433 <sup>a</sup>
PAB-430	1.5 ± 0.5 <sup>a,b</sup>	2.5 ± 0.500 <sup>a</sup>	1.0 ± 0.836 <sup>b</sup>	2.0 ± 1.000 <sup>a,b</sup>	1.75 ± 1.299 <sup>a,b</sup>	2.75 ± 1.996 <sup>a</sup>
PAB-860	1.5 ± 0.5 <sup>b</sup>	1.0 ± 2.073 <sup>b</sup>	1.0 ± 1.000 <sup>b</sup>	2.0 ± 1.118 <sup>a,b</sup>	3.0 ± 0.866 <sup>a</sup>	2.3 ± 0.433 <sup>a,b</sup>
MIDB	1.5 ± 0.5 <sup>a</sup>	1.0 ± 3.000 <sup>b</sup>	0 <sup>c</sup>	1.0 ± 1.802 <sup>b</sup>	0 <sup>c</sup>	ND
MIDP	1.5 ± 0.5 <sup>b</sup>	0 <sup>c</sup>	2.0 ± 1.224 <sup>b</sup>	1.3 ± 1.643 <sup>b</sup>	4.6 ± 0.707 <sup>a</sup>	0 <sup>c</sup>

Different letters in each row indicate significant differences ( $P \leq 0.05$ ). The values are means ± SD of triplicate measurements

**Table 3** MNE in blood of male mice following a subchronic test

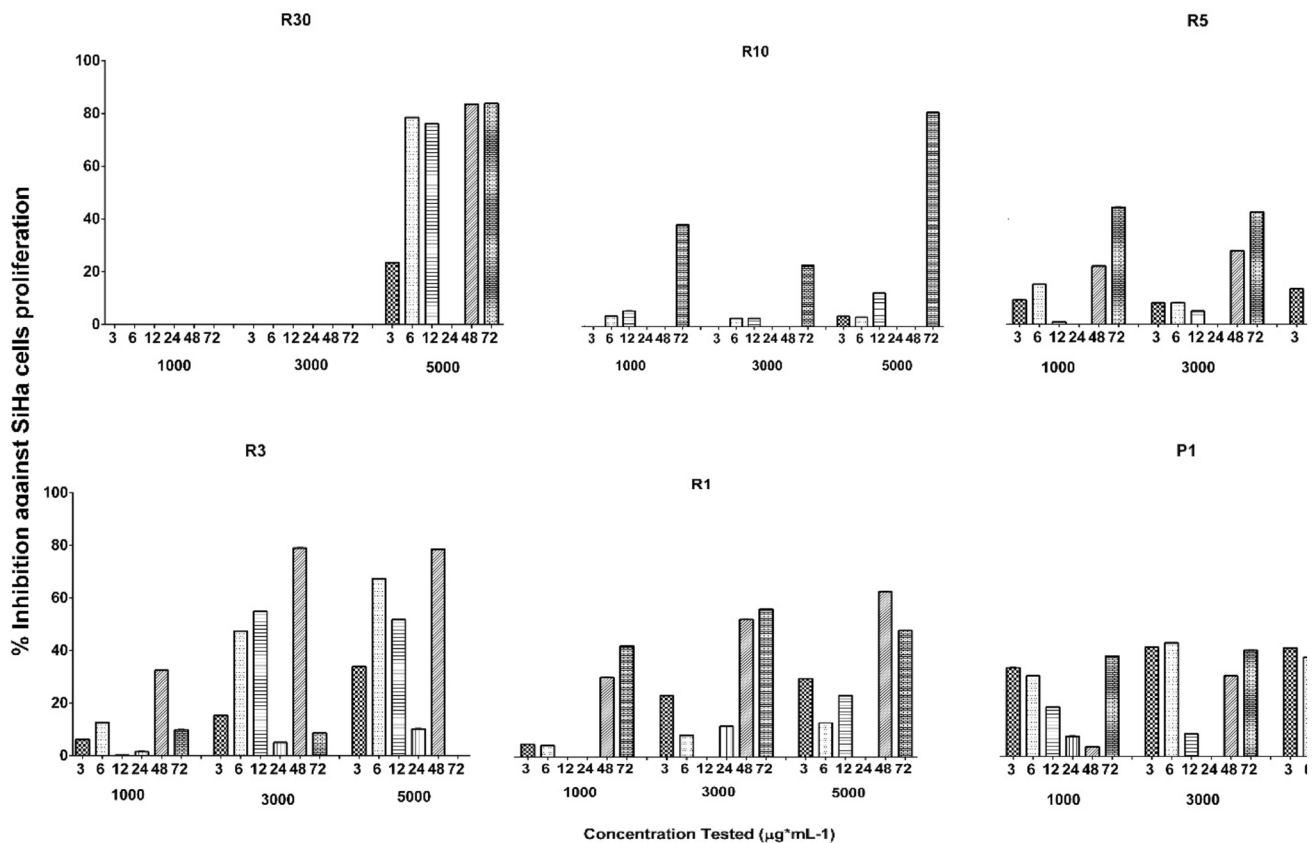
Extract	MNE/1000 ET Time (h)					
	0	48	96	144	264	360
Control	1.20 ± 0.84 <sup>e</sup>	8.20 ± 4.15 <sup>b,c</sup>	5.80 ± 0.84 <sup>c,d</sup>	13.20 ± 1.48 <sup>a</sup>	10.60 ± 0.55 <sup>a,b</sup>	2.80 ± 0.84 <sup>d,e</sup>
DAU	1.20 ± 0.84 <sup>b</sup>	9.00 ± 2.55 <sup>a</sup>	2.80 ± 1.48 <sup>b</sup>	10.20 ± 4.02 <sup>a</sup>	3.00 ± 1.22 <sup>b</sup>	12.00 ± 0.00 <sup>a</sup>
BAB-430	1.20 ± 0.84 <sup>d</sup>	0 <sup>d</sup>	6.80 ± 1.30 <sup>a,b</sup>	5.50 ± 0.87 <sup>b</sup>	7.75 ± 1.09 <sup>a</sup>	3.75 ± 0.43 <sup>c</sup>
BAB-860	1.20 ± 0.84 <sup>b</sup>	6.00 ± 2.35 <sup>a</sup>	5.60 ± 1.14 <sup>a</sup>	7.00 ± 2.35 <sup>a</sup>	6.80 ± 2.05 <sup>a</sup>	4.75 ± 0.43 <sup>a</sup>
PAB-430	1.20 ± 0.84 <sup>b</sup>	6.50 ± 0.50 <sup>a</sup>	5.20 ± 0.84 <sup>a</sup>	5.00 ± 1.00 <sup>a</sup>	7.25 ± 1.30 <sup>a</sup>	5.35 ± 2.00 <sup>a</sup>
PAB-860	1.20 ± 0.84 <sup>d</sup>	7.40 ± 2.07 <sup>a,b</sup>	5.00 ± 1.00 <sup>c</sup>	8.50 ± 1.12 <sup>a</sup>	5.50 ± 0.87 <sup>b,c</sup>	3.25 ± 0.43 <sup>c,d</sup>
MIDB	1.20 ± 0.84 <sup>d</sup>	21.00 ± 3.00 <sup>c</sup>	25.50 ± 0.35 <sup>c</sup>	32.50 ± 1.8 <sup>b</sup>	66.5 ± 4.60 <sup>a</sup>	ND
MIDP	1.20 ± 0.84 <sup>e</sup>	24.40 ± 0.89 <sup>b</sup>	17.00 ± 1.22 <sup>d</sup>	20.20 ± 1.64 <sup>c</sup>	33.00 ± 0.71 <sup>a</sup>	33.50 ± 0.35 <sup>a</sup>

Different letters in each row indicate significant differences ( $p \leq 0.05$ ). The values are means ± SD of triplicate measurements

**Effect of the ayocote bean variety and exposition time on cell proliferation**

The results of the inhibition of SiHa cells proliferation by the protein fractions from black ayocote bean protein

hydrolysate are showed in the Fig. 1. It was observed that the R5, R3, R1 and P1 protein fractions showed lower inhibition of SiHa cells proliferation than the R30 and R10 fractions at doses of 5000 µg/mL and 72 h of exposition. Similar results were observed with the protein fractions of



**Fig. 1** Anti-proliferative activity of different protein fractions from black ayocote bean protein hydrolysate against SiHa cell line. R30, protein fraction with molecular weight > 30 kDa; R10, protein fraction with molecular weight ≤ 30 kDa and > 10 kDa; R5, protein frac-

tion with molecular weight ≤ 10 kDa and > 5 kDa; R3, protein fraction with molecular weight ≤ 5 kDa and > 3 kDa; R1, protein fraction with molecular weight ≤ 3 kDa and > 1 kDa; and P1, protein fraction with molecular weight ≤ 1 kDa

the purple ayocote bean protein hydrolysate (Fig. 2). Like the protein fractions of the black ayocote bean protein, the higher inhibition of SiHa cells proliferation was observed at the higher concentration tested (5000  $\mu\text{g}/\text{mL}$ ). The R30 and R10 protein fractions of both purple and black ayocote bean hydrolysate do not show effects on SiHa cells proliferation at any concentration and these times and 24 h of exposition time. Only protein fractions of the purple ayocote bean hydrolysate showed action against SiHa cells proliferation in all time periods at doses of 1000 and 3000  $\mu\text{g}/\text{mL}$ .

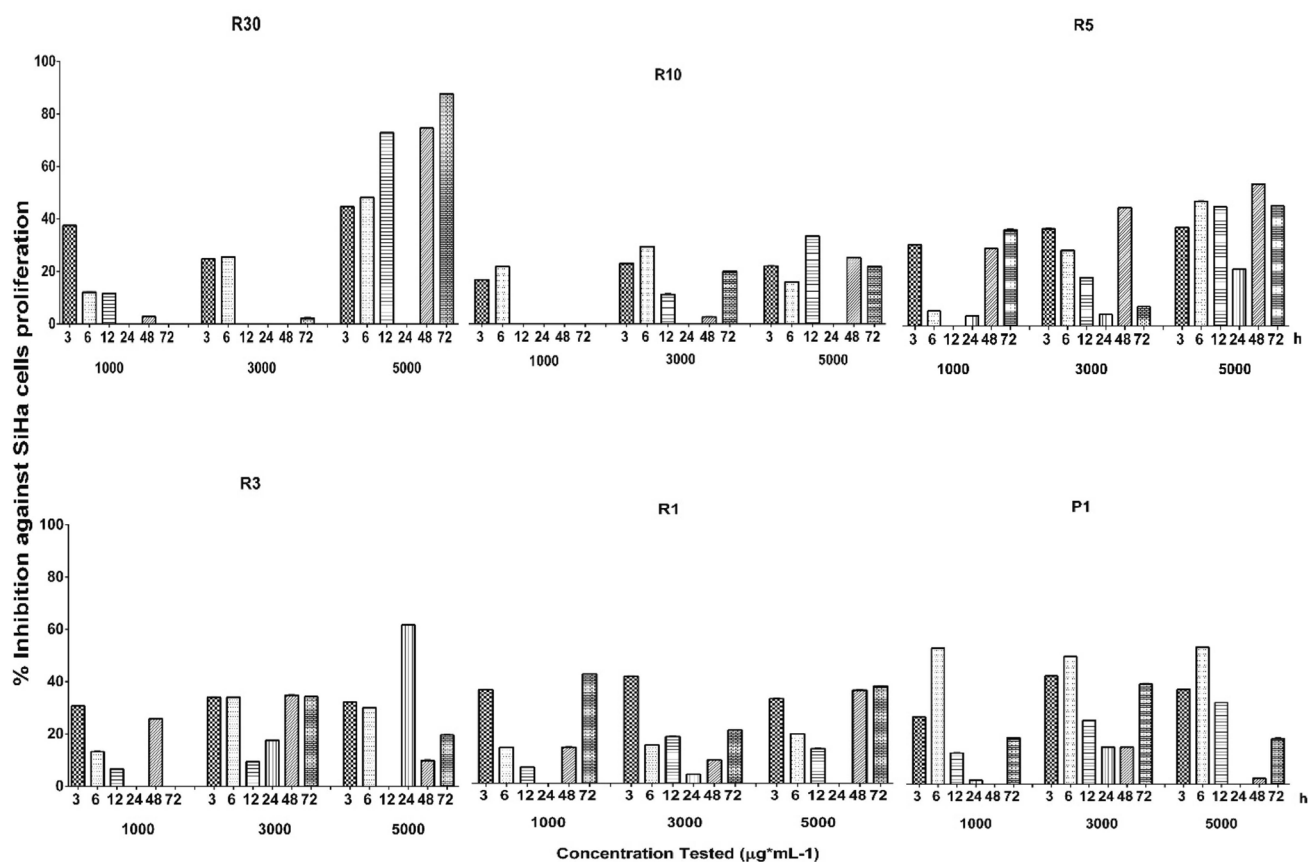
These results are in accordance with those obtained by González-Montoya et al., [37] from protein fractions of germinated soybeans in breast and cervical cancer cell lines; on the other hand, it has been reported that the anti-proliferative activity of donkey milk protein fractions of molecular mass > 10 kDa is higher than the fractions of molecular mass lower than 10 kDa on A549 human lung cancer cells [38]. It has been observed that the effectiveness against cancer cells of the bean peptides varies depending on the cultivar [3]; for this reason, the results obtained showed that the purple and

black ayocote bean protein fractions have different inhibition capacities on SiHa cells proliferation.

The maximal inhibitory concentration ( $\text{IC}_{50}$ ) of each protein fraction is shown in Table 4. Five protein fractions of the black ayocote bean protein hydrolysate reached the  $\text{IC}_{50}$  at different exposition times at the same concentration (5000  $\mu\text{g}/\text{mL}$ ), showing that these five fractions of the

**Table 4** The  $\text{IC}_{50}$  values ( $\mu\text{g}/\text{mL}$ ) of the ayocote bean protein fractions in MTT test of the SiHa cell line

Protein Fraction	Black Ayocote Bean		Purple Ayocote Bean	
	Concentration ( $\mu\text{g}/\text{mL}$ )	Exposition Time (h)	Concentration ( $\mu\text{g}/\text{mL}$ )	Exposition Time (h)
P1	5000	48	1000	6
R1	5000	72	3000	3
R3	5000	6	4400	3
R5	5000	48	3000	48
R10	3600	72	–	–
R30	5000	6	5000	6



**Fig. 2** Anti-proliferative activity of different protein fractions from purple ayocote bean protein hydrolysate against SiHa cell line. R30, protein fraction with molecular weight > 30 kDa; R10, protein fraction with molecular weight  $\leq 30$  kDa and > 10 kDa; R5, protein frac-

tion with molecular weight  $\leq 10$  kDa and > 5 kDa; R3, protein fraction with molecular weight  $\leq 5$  kDa and > 3 kDa; R1, protein fraction with molecular weight  $\leq 3$  kDa and > 1 kDa; and P1, protein fraction with molecular weight  $\leq 1$  kDa

black ayocote bean protein (R30, R5, R3, R1 and P1) have time-dependent inhibition on SiHa cells, while that of the R10 fraction reached the IC<sub>50</sub> with a lower concentration at 72 h of exposition.

The exposition time and concentration required to reach the IC<sub>50</sub> of the protein fractions of the purple ayocote bean were lower than the exposition time and concentration required for protein fractions of the black ayocote bean. The IC<sub>50</sub> values were principally reached in doses less than (5000 µg/mL) the four protein fractions (R5, R3, R1 and P1), observing that the inhibition of SiHa cells is not dependent on the exposition time, but the molecular weight is the determinant for the inhibition of SiHa cells. With the R10 fraction, the IC<sub>50</sub> at any concentration and time tested was not reached.

The MW of the protein fractions is the determinant for the activity against cancer cell lines. Previous reports indicated that the protein fraction obtained from soy with MW > 10 kDa was the most active against HeLa cells [37]. On the other hand, the protein fractions obtained from ayocote bean protein hydrolysates showed high potential as functional components against SiHa cells, considering that the R1 fraction of the purple ayocote bean protein hydrolysate showed an IC<sub>50</sub> of 1000 µg/mL against SiHa cells lower than the 1830 µg/mL of soy protein fractions required to reach the IC<sub>50</sub> against HeLa cells reported by other authors [39].

## Conclusions

Differences in the cytotoxic activity were observed between the two varieties of ayocote beans (black and purple), while that of the genotoxic activity had no significant differences observed by the variety effect. The molecular mass of protein fractions influences the effects on cancer cells, and synergistic effects were observed with the mix protein isolate-DAU, increasing the cytotoxic capacity. The ayocote bean isolates (black and purple) showed moderate genotoxic effects at doses of 430 and 830 mg. The protein fractions obtained from the purple and black isolate protein of ayocote beans have inhibition capacity against SiHa cells. The time and concentration required for effects against SiHa cells are influenced by the variety and molecular mass of the protein fractions.

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## Compliance with ethical standards

**Conflict of interest** All the authors declare that there are no conflicts of interest.

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