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Antiproliferative Effect of *Sechium edule* (Jacq.) Sw., cv. Madre Negra Extracts on Breast Cancer In Vitro

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Abstract: *Sechium edule* shows a wide biological diversity. Hybrids and varieties of *Sechium edule* have been created, producing fruits of different shapes, sizes, colors, tastes, and textures. These hybrids and varieties have been used as food and in traditional medicine. In this research, the antiproliferative activity of the Madre Negra™ genotype of *Sechium edule var. amarus silvestrys* on the MCF7 breast cancer cell line was assessed. Different extracts in hexane, methanol, and dichloromethane were obtained to perform a bio-guided study. The dichloromethane extract showed the largest significant inhibitory activity ($p < 0.05$). This extract was separated into 92 fractions, which were reduced to eight fractions by chromogenic identity. Of the eight fractions, two recorded significant antiproliferative activity ($p < 0.05$) on MCF7, an even higher activity than the total extract. The active metabolites were identified as flavonoids, tannins, and terpenes, of which cucurbitacins I, B, D, and E stood out. The present paper can be considered as preliminary results of our research work.

Keywords: chayote; *Sechium edule*; breast cancer; methanol extracts; hybrids

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1. Introduction

Plants have secondary metabolites with important applications in modern medicine. As a result, plants are considered a direct source of therapeutic agents, or prime materials for obtaining more complex synthetic drugs [1]. The World Health Organization (WHO) has defined plants as any vegetal species containing substances which can be used for therapeutic purposes or whose active principles can serve as precursors for the synthesis of new drugs [2]. In this respect, breast cancer is a very common condition among women worldwide, representing 16% of all female cancers [3]. In Mexico in 2010, the main malignant tumors among adult women (20 years old and older) hospitalized with this diagnosis were breast cancer, affecting 24.3% of adult women, with a mortality rate of 13.8% [4]. There are several treatments for breast cancer. Unfortunately, these have several unpleasant secondary effects on patients [5]. To date, the design of cancer treatment and research has been spectacular, and numerous drugs with the potential to fight the proliferation of cancer cells have been developed. However, these advancements have been insufficient, and they are costly for families with a medium to low socioeconomic status [6].

Sechium P. Br. (Cucurbitaceae) is a plant genus with 10 confirmed species, out of which *S. edule* comprises the largest number of genotypes, which can be attributed to a domestication and adaptive specialization process [7,8]. Its principal value is as food. Today, the genotypes *S. edule var. virens levis* and *var. nigrum spinosum* are vegetables exported to

USA and Canada in such a volume that makes Mexico the largest producer and exporter worldwide [9]. However, intangible values of compounds have been detected in the chemical composition of the fruit, such as peroxidases, sterols, alkaloids, saponins, phenols, polyphenols, flavonoids and cucurbitacins, related to antiallergenic, anti-inflammatory and antiviral activity, as well as antitumoral effects [10–12]. However, some authors [13–16] have recently recorded cytotoxic activity in extracts from some of the *S. edule* genotypes which have shown anti-tumoral activity on the cell lines WEHI-3, HeLa, L929, P388, and J774 [17]. Sechium, as member of Cucurbitaceae family, shares some properties with other members of this family, e.g., strong bitter taste, and this is associated with the presence of cucurbitacins [18]. Although this is beneficial, some of the evaluated genotypes act as complete extracts, and obtaining a genotype rich in metabolites, specificity toward a malignant cell line is desirable. It is not known if the genotype *S. edule var. amarus sylvestris cv. Madre Negra* shows an inhibitory effect on the MCF7 cell line (breast cancer), or whether one or the sum of the active compounds is responsible for that activity.

2. Materials and Methods

The fruits of *S. edule cv. Madre Negra* collected at horticultural maturity come from a genotype with a bitter taste, obtained through infraspecific selection, denominated *S. edule var. amarus sylvestris*. This varietal group is characterized by dark green lines in young and adult individuals, low pubescence in the internodes and medium pubescence in the nodes, and foliar polymorphism, with leaves of a trisected shape defined as permanently angular. The fruit is 7–9 cm long (8 cm on average). Its equator width is 4.5–8 cm (6.2 on average). The fruit is 4–7 cm in depth (5.5 on average). Its shape is that of an elongated berry, with a dark green color (Pantone 575c, 575c, and 576c), and the predominance of a ribless elongated cone. It shows a light basal ridge, a peduncle with medium pubescence that is dark green in color, and a dark green mesocarp with a strongly bitter taste. It shows weak spine lines, distributed in a longitudinal way, of a dark green color. The seed is 1.8–5 cm long (3.2 on average), with an equatorial width of 1.2–3.8 cm (2.1 on average) and a depth of 0.3–1.3 cm (0.80 on average). The seed is strongly adhered to the mesocarp, with the presence of fibers. It is creamy in color, with conspicuous ornamentation on the surface and a strong bitter taste. It is of late reproduction. The fruit starts flowering in September and ends in February as long as no frosts, which kill the aerial part, occur (Figure 1).

Figure 1. Fruit of *Sechium edule* cv. Madre Negra in horticultural maturity (22 ± 2 days after anthesis).

The evaluated fruits were harvested at horticultural maturity. All fruits were provided by the National Gene Bank of *S. edule* in Mexico (BANGESe) (19°08′48″ N and 97°57′00″ W), with vegetation from the Mountainous Mesophyll Forest (cloud forest) at 1340 m altitude with an annual mean temperature of 19 °C, relative humidity of 85%, and annual precipitation of 2250 mm. The Mountainous Mesophyll Forest is home to Vitric Luvisol soils rich in nutrients, of moderate fertility, thick texture, fragments of volcanic glass and a slightly acid to acid pH (4.3–6.5), high content of organic matter, iron, manganese, zinc, and low levels of calcium [19]. The plants of this cultivar were kept in the gene bank for 7 years, and their morpho-biochemical characteristics have been shown to be stable. Figure 2 shows the flowchart of methods performed in the experimentation.

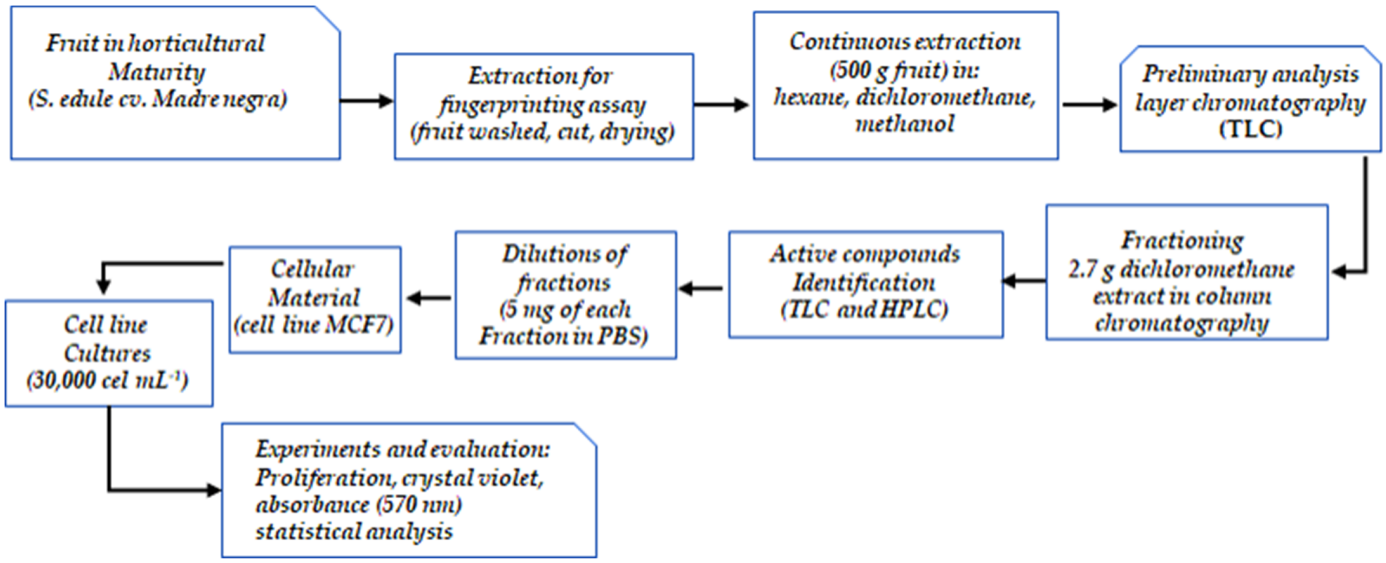

Figure 2. Flowchart of methods performed in the experimentation.

2.1. Extraction for Fingerprinting Assay

The fruits were washed, cut, weighed, and placed in an oven (BLUE-M, Electronic Company, Blue Island, IL, USA) with an air flow of 45 °C for 72 h. Then, the whole fruits were ground (exocarp, mesocarp, and seed) with a mill (Hamilton Beach, Glen Allen, VA, USA). Once the dry and ground material was recovered, a continuous extraction was performed with several solvents [13,15]. The dried material of the fruits (500 g) was extracted selectively first with hexane, then with dichloromethane, and finally with methanol as follows: The plant material was covered with the solvent and left to extract for 48 h at room temperature (22 ± 2 °C). Then, the solvent was decanted off, the process was repeated 10 times for each solvent, and the organic extract was concentrated in a rotary evaporator (BUCHI R-114, Flawil, Switzerland) at a temperature of 45 °C and pressure of 45 mm Hg. The yield percentage of the extracts was determined to obtain the extracts in hexane, dichloromethane, and methanol, respectively. Then, a preliminary analysis was performed with thin-layer chromatography (TLC), specifically for the cucurbitacins (they are the target compounds, based on the comments described before). For this preliminary assay, nine samples (came from the separation of the dichloromethane extract) were applied by dotting of four standards of cucurbitacins in silica gel plates of 20 × 20 cm (Merck, Millipore, Darmstadt, Germany).

2.2. Separation

In total, 2.7 g of the dichloromethane extract of the Madre Negra genotype was fractioned by column chromatography at low pressure. The extract was diluted with a few drops of the solvent and Kiesegel gel 60 (0.063–0.200 mm) Merck. The column was packed with 60 GF_{254} Merck silica gel and eluted with different proportions of ethyl

acetate/hexane (10:0, 9:1, 8:2, 7:3, 6:4, 5:5, 4:6, 3:7, 2:8, 1:9, 0:10). Finally, the column was eluted with methanol to completely exhaust the column. In total, 92 fractions were obtained, which were reduced to eight fractions by chromogenic identity.

2.3. Active Compounds Analysis

The purification of active compounds was realized through preparative thin-layer chromatography (TLC), whereas their identification was done by high-performance liquid chromatography (HPLC), with the support of standards of cucurbitacins. The purification by TLC involved the separation of active fractions number three and five due to the richness in their composition. During TLC, 20 × 20 preparative plates of Kieselgel 60 GF silica gel were used, and each one was applied in a stripe of 20 mg in weight. Ethyl acetate/hexane (10:90) was used as a separation system. The composition was detected with 300 nm UV light, and the stripes of interest were scratched out according to their retention factor. Finally, ethyl acetate/hexane (1:1) was added to each one of the stripes and left to agitate for 1 h, centrifuged at 4000 rpm for 5 min, and cleaned of silica residues, followed by a filtering of the supernatant and the evaporation of the solvent. Finally, once the solvent evaporated and its yield was obtained, another TLC analysis of the resulting fractions was performed. Standards of cucurbitacin I, E, B, and D were placed in the plate. The mobile phase consisted of ethyl acetate/hexane (10:90), and the derivatization was made with vanillin + H_2SO_4 at 110 °C for 5 min.

For the HPLC identification, 2 mg of fractions number three and five from the dichloromethane extract and the same amount of cucurbitacin standards I, B, E and D were weighed and placed in 2 mL Eppendorf tubes. These standards were used to construct the calibration curves. Then, 1 mL of ethyl acetate/hexane (9:1) was added, and the resulting mix was vigorously stirred in the vortex and filtered through Nylon Acrodiscs of 25 mm with a pore size of 0.45 µm. The samples were analyzed in a Hewlett-Packard liquid chromatograph, mod-1100 provided with a diode array detector and an Agilent Technologies automatic injector mod. 1200. The column was a Symmetry Shield RP 18 (4.4 × 250 mm), 5 µm. The analysis was run in isocratic mode. The mobile phase was H_2O, methanol, and acetonitrile in proportions 50:30:20. At a flow rate of 1 mL min^{-1} and a pressure of 179 bars, temperature 25 °C, 20 µL of each sample was injected. The readings were performed at 235 nm.

2.4. Cellular Material

The first human breast cancer cell line was developed in 1958 [20]. Many other lines have been established since then. The breast cancer cell line par excellence, MCF7, was developed in 1973 from a pleural spill of a human breast carcinoma [21]. The cell line was obtained from the ATCC (American Type Culture Collection). It was cultured with IMDM (Iscove's Modified Dulbeco´s Medium) (Gibco BRL, New York, NY, USA) and supplemented with deactivated bovine fetal serum (SFB) (Gibco BRL, Grand Island, NY, USA) at 10% under the conditions of 37 °C, 5% CO_2, and saturated humidity, with reseeding every 48 h.

2.5. Dilution of Fractions

A sample of 5 mg of each fraction was used. The samples were diluted at a maximum level of 300 µL of ethanol, calibrated at 1 mL with phosphate-buffered saline solution (PBS, pH 7.4), homogenized in a vortex (Scientific Industries, Bohemia, NY, USA), and centrifuged at 2000 rpm for 5 min (Hermle Labortechnik, Wehingen, Germany). Finally, the supernatant was retrieved.

2.6. Cell Line Cultures

A density of 30,000 cells mL^{-1} of MCF7 was used. The cells were kept under culture for 72 h in 96-well plates with flat bottoms (Corning, New York, NY, USA). The cells were stimulated at different concentrations depending on the extract or the fraction, employing

the addition of a vehicle (PBS:ethanol, 7:3) as a control, which was used to dilute the extracts. For the fractions, a pool of fractions and the initial dichloromethane extract was added, as well as 5 ng/mL of doxorubicin as a negative control for the inhibition of proliferation.

The cells were kept under culture for 72 h in the abovementioned conditions. Their proliferation was evaluated with the crystal violet technique [22]. This technique involved a colorant which adhered to the nuclear proteins of the cell. First, the colorant was fixed to the cells with 50 µL/well of glutaraldehyde 1%, left to rest for 1 h, and decanted. Then, 50 µL/well of crystal violet was added, and the excess colorant was removed with distilled water. Acetic acid 10% was then added, and the mix was kept in agitation for 20 min to solubilize the colorant incorporated to the cells. Finally, the absorbance was read at 570 nm with a plate spectrophotometer (Tecan Spectra, Grödig, Austria). The obtained values were used to calculate the proliferation percentage.

2.7. Statistical Analysis

The experiments were conducted in quadruplicate three independent times, and the data were submitted to an analysis of variance (ANOVA, two way) with a Tukey and Dunnett test ($p < 0.05$) using the IBM SPSS Statistics (version 26; Armonk, NY, USA), software version 11.0.

3. Results

Extracts of *Sechium edule* var. Madre Negra were obtained by discontinuous extraction starting with hexane, dichloromethane, and methanol, each of which was added to the culture of the human breast cancer cell line MCF7 in increasing concentrations from 0 to 10 µg mL^{-1}, and the effect was compared with respect to the control (0) or with 5 ng/mL of doxorubicin as a positive control for the inhibition of proliferation.

In the evaluation of cell proliferation, the hexane extracts showed significant antiproliferative effect, with respect to both controls, starting from a concentration of 0.6 µg mL^{-1}. On the contrary, in the dichloromethane extracts, a significant effect was observed starting from the concentration of 0.07 µg mL^{-1}, which is a low dose, similar to the values recorded with the antineoplastic doxorubicin, while the methanol extract showed significance with respect to both controls at a high dose of 1.2 µg mL^{-1} (Figure 3). The dichloromethane extract showed a lower IC_{50} value of only 0.58 µg mL^{-1}, unlike the hexane and methanol extracts (Table 1).

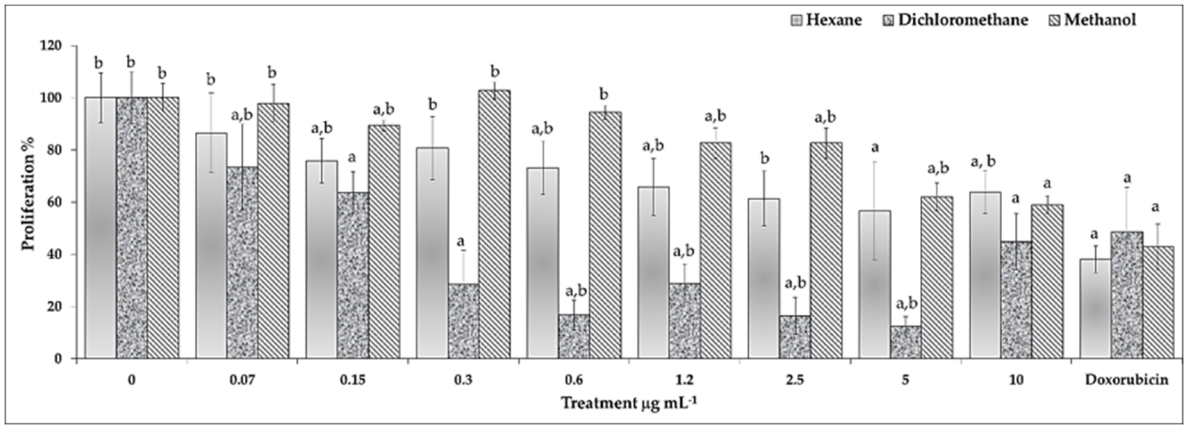

Figure 3. Antiproliferative activity of the extracts *S. edule* cv. Madre Negra depending on the solvent used: hexane, dichloromethane, or methanol. a: significant difference versus control (0) ($p < 0.05$), b: significant difference versus positive control for the inhibition of proliferation (5 ng/mL of doxorubicin) ($p < 0.05$) (ANOVA with a Tukey test). The bars indicate ± standard deviation.

Table 1. Cell inhibition values (IC_{50}) of the *S. edule* cv. Madre Negra depending on the type of solvent used.

Solvent	Cell Inhibition (IC_{50} μg mL^{-1})
Hexane	5.53 ± 0.79
Dichloromethane	0.58 ± 0.14
Methanol	10.50 ± 1.53

The strong antiproliferative effect of the dichloromethane extract guided us to conduct its separation by column chromatography, through which 92 fractions were obtained, which were reduced to eight fractions by chromatographic similarity. The eight fractions obtained were tested on the proliferation of the tumor line MCF7 at a dose of 0.58 μg mL^{-1} corresponding to the IC50 of the discontinuous extract of dichloromethane and its effect was compared with the control 0, the positive control doxorubicin, and the complete dichloromethane extract. Fractions three, four, and five significantly reduced proliferation by more than 50%, but fractions three and five have greater inhibitory potential compared to the three comparators, so it is suggested that the bioactive components of the extract obtained could be present in the dichloromethane extract from *Sechium edule* var. Madre Negra (Figure 4).

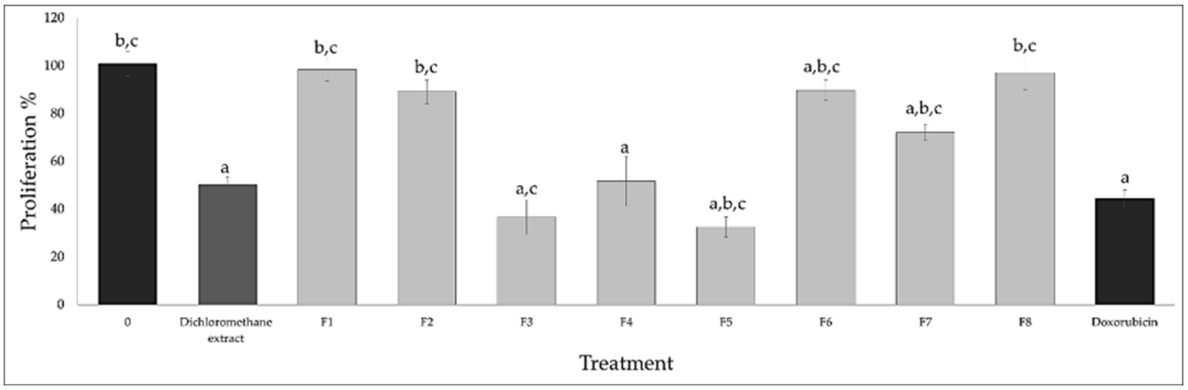

Figure 4. Antiproliferative activity of the IC_{50} of extracts *S. edule cv.* Madre Negra of the complete extract in dichloromethane and the fractions F1-F8. The letters mean: "a": significant difference versus control (0) ($p < 0.05$), "b": significant difference versus positive control for the inhibition of proliferation (5 ng/mL of doxorubicin) ($p < 0.05$), "c": significant difference versus the complete dichloromethane extract, (0.58μg/mL) ($p < 0.05$) (ANOVA with a Tukey test). The bars indicate ± standard deviation.

The pharmacological results of the eight fractions showed a remarkable significance with respect to the initial extract. In particular, significance was found among fractions number three and five, which were fractioned again by the TLC technique. This analysis showed that fraction number three had three compounds, while in fraction number five, four compounds were recorded (Figure 5). In fraction number three, the compounds showed a close identity relationship when compared to the cucurbitacin E, B, D, and I standards and compounds with retention index values of (rf) 0.73, 0.63, and 0.56. In fraction number five, the similarity was toward the components, at 0.7, 0.45, and 0.39 (Table 2).

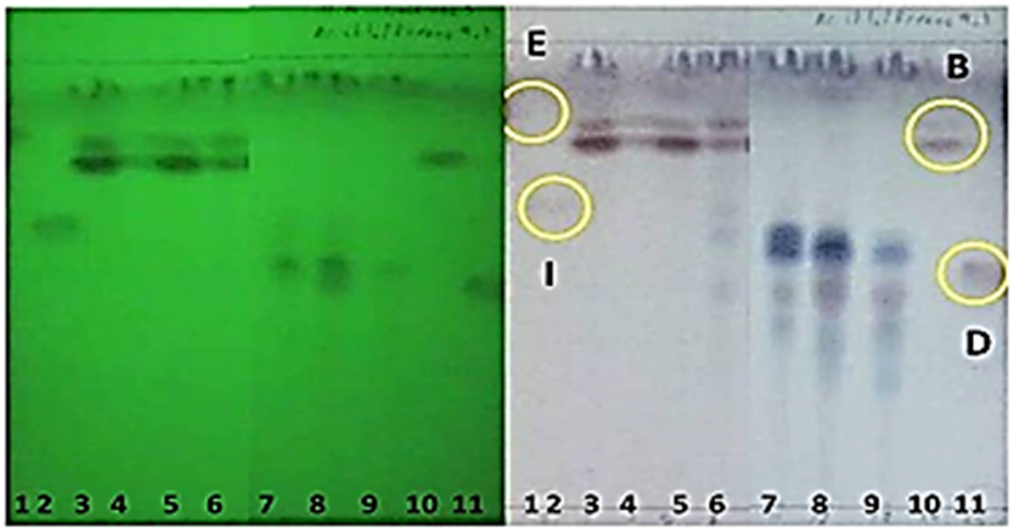

Figure 5. Preliminary identification of cucurbitacins by thin-layer chromatography (TLC) on the extract of *S. edule cv.* Madre Negra. (The fractions came from the separation/purification of the DCM extract). Lanes 1 and 2 are cucurbitacin E and D. Lanes 3–9 correspond to the fractions indicated in Table 2. Lanes 9 and 10 are cucurbitacins B and I.

Table 2. Relation of the cucurbitacin standards E, B, D, and I with fraction three (rf: 0.73, 0.63, and 0.56) and fraction five (rf: 0.7, 0.45, 0.39) of the extract of *S. edule cv.* Madre Negra.

Sample	Fraction Number	% Yield	rf	Color
Cucurbitacin E			0.65	Lilac
Cucurbitacin D			0.37	lilac
F3 (A)	45–60	1.12	0.73	Pale purple
F3 (B)			0.63	Pale brown
F3 (C)			0.56	lilac
F5 (A)	58–79	1.0561	0.7	Pale purple
F5 (B)			0.45	Pale purple
F5 (C)			0.39	Purple
F5 (D)			0.33	Brown
Cucurbitacin B			0.75	Purple
			0.6	Brown
			0.56	lilac
Cucurbitacin I			0.5	lilac

The result of the TLC analysis led us to perform a liquid chromatography analysis, which allowed us to verify the identity of the active fractions through the use of reference compounds and spectral pattern. In fraction three, cucurbitacins D, I, B, and E were identified, while in fraction five, cucurbitacins I, D, and B were identified (Figure 6).

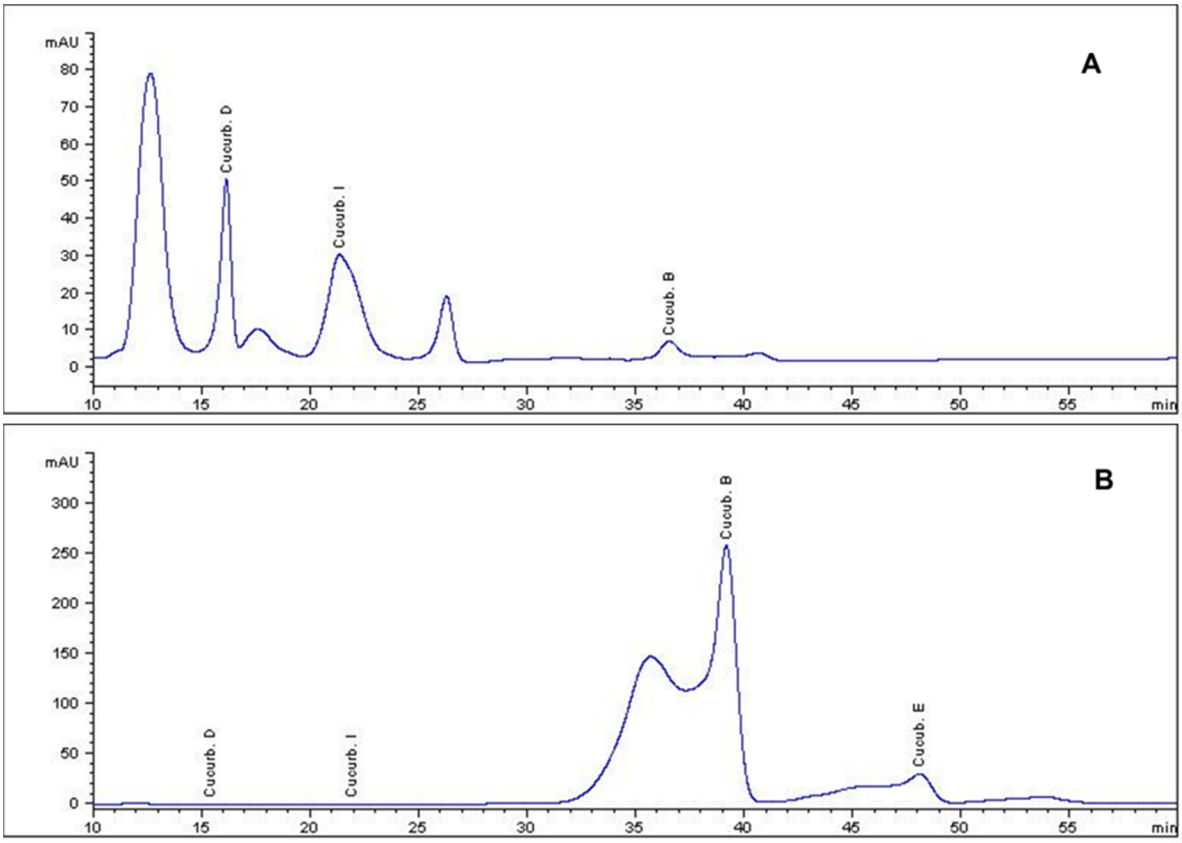

Figure 6. HPLC chromatograms for the identification of cucurbitacins in the fruit extract of *Sechium edule* cv. Madre Negra in dichloromethane extract. (**A**) fraction three, (**B**) fraction five.

4. Discussion

The antiproliferative activity of Madre Negra could be added to the group of genotypes belonging to *S. edule* that show such biological activity. It is remarkable that, among the genotypes of this species, different behaviors for the inhibition of cell proliferation have been recorded. With respect to the genotype in use, previous studies with crude extracts obtained from eight varietals of *S. edule* found an effect on lines L929, P388, and HeLa [13]. However, in the evaluation of the fractioning of the extract from the hybrid H387-07, the extract did not show any specific active compound, which indicates that its biological activity depends on the set of compounds present in the extract [23].

With respect to Madre Negra, the results are relevant when considering the comparison with the antiproliferative evaluation performed on the dichloromethane extract fractions. The results indicate that fractions number three and five show significant inhibitory activity, attributed to cucurbitacins (based in their retention time and UV properties, and compared with the standards). In addition, it has been described that the four cucurbitacins detected in this fruit (although from different sources) exert antiproliferative activity on the MCF-7 tumor line [24]. On the other hand, it has been found that these tetracyclic triterpenes show cytotoxicity against a great diversity of malignant tumors in several cell lines [25–27].

In addition, different cucurbitacins have been obtained from plant extracts with antineoplastic activity, thus cucurbitacins B, D, and E present in *Cucurbita andreana* inhibited the cell proliferation of a colorectal human carcinoma (HCT-116) and breast cancer (MCF7) and caused a decrease of the proliferation of human lung cancer cells (NCL-H460), as well as a reduction of human glioblastoma (SF268)10. Previous reports have also shown that the cucurbitacin F obtained from *Elaeocarpus dolichostylus* and cucurbitacin Q from *Bradegea bigelovii* inhibit cell proliferation [28,29]. These data support the idea that the cucurbitacins of the different factions from Madre Negra are responsible for the inhibitory effect of proliferation, which is close to that achieved by doxorubicin, but it is important to

consider the participation of other cucurbitacins that appear in the chromatogram, and it would be desirable to identify it in the future.

The data found in this work regarding the presence of four cucurbitacins and their toxicities answer the question asked in the beginning, in the sense that there was no information about the inhibitory effect of the cv. Madre Negra on the cell line MCF7 or the compounds responsible for said activity. It is prudent to point out the importance of this vegetal variety as a source of specific metabolites to treat a condition of public interest, namely breast cancer. The cv. Madre Negra can translate into a valuable therapeutic low-cost alternative under future formulation studies, since the biomass level obtained in fruits per hectare of Madre Negra in Genebank conditions exceeds 45 t year^{-1}.

This study leads us to propose that in the cv. Madre Negra, there is a source of active principles of natural origin that can widen the pharmacologic options. These active principles can be a component of alternative drugs or used as diet supplements. They can be used in the composition of nutraceutical drinks and foods due to their antioxidant capacity [30,31].

Another important condition is that, because of the strong bitter taste of its fruits, it does not have any current interest, which creates a disadvantage regarding its conservation in the rural environment compared with the edible genotypes. This is relevant if we consider the premise that an unused genetic resource is not conserved, which affects the genetic heritage of the country that contains it [17].

Various studies have reported the biological activity of *S. edule* as antineoplastic and antioxidant; however, most of these studies do not identify the genotype, including the morphotype, hybrid, or variety evaluated, generating confusion and null reproducibility [11]. Studies [14,15] show the effect of these metabolites on leukemic cell lines and induction of apoptosis by the hybrid H387-07, as well as another variety *S. edule var. nigrum spinosum*. It is relevant to highlight that the cv. Madre Negra is one of the parents that gives rise to the hybrid H387-07. Other studies [16,32] indicate that cv. Perla Negra from *S. edule* has an antiproliferative effect on malignant cell lines (HeLa), without affecting lymphocytes, even after microencapsulating the extract. The analysis of the above, showed in the content of the microspheres the cucurbitacins B, D, E, I, as well as the flavonoids Rutin, Myricetin, Quercetin, Naringenin, Phloretin, Galangin, and Apigenin. It is possible that cucurbitacins and flavonoids are involved because both exert antitumor activity [12,13,29,33] and both are present in *S. edule*.

5. Conclusions

The genotype *S. edule var. amarus sylvestris cv. Madre Negra* shows an inhibitory effect on the MCF7 cell line (breast cancer), and fraction three and five of the dichloromethane extract are responsible for that activity.

Author Contributions: Conceptualization: M.S.-H. and J.C.-I.; methodology: E.S.-O.; investigation: M.T.U.-A. and I.A.-S.; validation: L.d.M.R.-P.; formal analysis writing—original draft preparation: M.S.-H., M.T.U.-A., and J.C.-I.; J.C.-I. and I.A.-S. contributed equally to this work. All authors have read and agreed to the published version of the manuscript.

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