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Cytotoxicity and *in vitro* activity of chard *(Beta vulgaris* L. var *Cicla)* extracts on porcine pancreatic islets¤

Citotoxicidad y actividad in vitro de extractos de acelga (Beta vulgaris L. var Cicla) en islotes pacreáticos porcinos

Citotocidae e atividade in vitro de extratos de acelga (Beta vulgaris L. var Cicla) em ilhotas pancreáticas porcinas

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Summary

Background: reports from traditional medicine suggest that chard (*Beta vulgaris* L. var *Cicla*) can have remarkable effects in diabetes therapy. **Objective:** to evaluate the cytotoxic activity of chard extracts in cell lines and determine the viability of cultured porcine pancreatic islets added with or without chard extracts. **Methods:** cytotoxic activity of chard extracts was assessed in non-tumor and tumor cell lines using the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] technique, and the ability of extracts to maintain porcine pancreatic islets viability and regeneration *in vitro* was tested. **Results:** the 50% cytotoxic concentration (CC_{50}) of extracts for non-tumor cell lines was above 1,000 µg/mL, while it was 825 µg/mL, 283 µg/mL, 136 µg/mL and 380 µg/mL, for hexane, ethyl acetate, ethanol and water extracts in the tumor cell line, respectively. The CC_{50} ratio between cell lines indicates that ethanol extract is 7.5 times more toxic to tumor than non-tumor cell lines. There was an increase in viability of porcine pancreatic islets cultured with aqueous, ethyl acetate, and ethanol extracts compared with standard media (CMRL1066) and Cyclosporine A (CsA) control groups. Furthermore, a greater than one regeneration index of islets cultured with ethanol extract at 1,000 µg/mL and 500 µg/mL concentrations during 15 days was observed, which remained constant and was significantly higher than CsA group. **Conclusions:** these results suggest that chard metabolites should be researched to develop antitumor therapies and human pancreatic islets recovery in diabetes treatment.

Keywords: *beta cells, chard, diabetes mellitus, insulin.*

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Resumen

Antecedentes: reportes de medicina tradicional sugieren que la planta acelga (*Beta vulgaris* L. var *Cicla*) es importante en el tratamiento de enfermedades como la diabetes. **Objetivo:** evaluar la citotoxicidad de concentraciones de extractos de acelga en líneas celulares y determinar la viabilidad de islotes pancreáticos porcinos cultivados con y sin extracto de acelga. **Método:** se evaluó la actividad citotóxica en líneas celulares tumorales y no tumorales, con la técnica del MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide]. Específicamente se hicieron ensayos para comprobar si los extractos de acelga tienen la capacidad de mantener la viabilidad de islotes pancreáticos porcinos aislados e influir en su regeneración *in vitro*. **Resultados:** la concentración citotóxica al 50% (CC_{50}) de los extractos en líneas no tumorales fue mayor de 1.000 µg/mL, mientras que para los extractos en hexano, acetato de etilo, etanol y agua fue de 825 µg/mL, 283 µg/mL, 136 µg/mL y 380 µg/mL, respectivamente, en líneas tumorales. La proporción CC_{50} encontrada indica que el extracto en etanol es 7,5 veces más tóxico para las líneas celulares tumorales que para las no tumorales. Igualmente encontramos un aumento en la viabilidad de los islotes pancreáticos porcinos cultivados con extracto acuoso, de acetato en etilo y etanol en comparación con el medio de cultivo estándar (CMRL1066) y un control inhibidor que contenía medio con Ciclosporina A (CsA). Además, se encontró que el índice de regeneración era mayor de uno en los islotes cultivados con el extracto en etanol a concentraciones de 1.000 µg/mL y 500 µg/mL durante 15 días, que se mantuvo constante y fue significativamente mayor en comparación con el grupo de CsA. **Conclusión:** estos resultados sugieren que los metabolitos de la acelga podrían ser utilizados en la investigación de nuevos fármacos para el desarrollo de terapias antitumorales y recuperación de islotes pancreáticos en el tratamiento de la diabetes.

Palabras clave: *acelga, células beta, diabetes mellitus, insulina.*

Resumo

Antecedentes: relatos encontrados em medicina sugerem que a planta acelga (*Beta vulgaris* L. var *Cicla*) tem un papel importante no tratamento das doenças como a diabetes. **Objetivo:** avaliar a citotoxicidade de concentrações de extratos em linhagens celulares e determinar a viabilidade de ilhotas pancreáticas de porcos cultivadas com e sem extrato de acelga. **Métodos**: neste trabalho foi avaliada a atividade citotóxica dos extratos da acelga em linhagens celulares tumorais e não tumorais, usando a técnica do MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide]; além disso, foram feitos ensaios para verificar a capacidade que têm os extratos para manter a viabilidade das ilhotas pancreáticas isoladas de porcos e a influência em sua regeneração *in vitro*. **Resultados:** a concentração citotóxica ao 50% (CC50) dos extratos em linhagens não tumorais está acima de 1000 µg/mL, enquanto para os extratos de hexano, acetato de etilo, etanol y água é de 825 µg/mL, 283 µg/mL, 136 µg/mL y 380 µg/mL, respectivamente, em linhagens tumorais. A proporção CC50 entre a célula indica que o extrato de etanol é 7,5 vezes mais tóxico para as linhas celulares tumorais que para as linhas não tumorais. Houve um aumento na viabilidade dos isolados pancreáticos de porcos cultivados com extrato aquoso, de acetato de etilo y etanol, em comparação com o meio de cultura padrão (CMRL 1066) e um controle inibitório contendo meio com Ciclosporina A (CsA). Encontrou-se também uma taxa de regeneração maior do que um em ilhotas cultivadas com concentrações de 1000 µg/mL e 500 µg/mL durante 15 días, que se manteve constante e foi significativamente mais elevada em comparação com a CsA. **Conclusões**: estes resultados sugerem que os metabolitos da acelga poderiam ser usados para a pesquisa de novas drogas para o desenvolvimento de terapias antitumorais e recuperação de ilhotas pancreáticas para o tratamento da diabetes.

Palavras chave: *acelga, células beta, diabetes mellitus, insulina.*

Introduction

Allogeneic transplant of pancreatic islets has become an attractive treatment alternative for diabetes due to islets ability to restore insulin independence and the possibility of successive transplants in the same patient. However, islet transplantation is limited by the low donation of organs, difficulties with the islet isolation technique and decreased cell viability and low regeneration of beta cells (Sakuma *et al.,* 2008; Emamaullee *et al.,* 2007). A solution could be to use plant extracts to increase cell viability and number of islets.

Chard is a plant with many traditional medicinal applications. It has been used for treatment of organ

and tissue inflammation, ulcers, and haemorroids (Youssef, 2013; Rodríguez, 2011; Parekh and Chanda, 2008). Because of its emollient properties and magnesium content, it has been used on skin diseases and burns, as a laxative in constipation, and as a diuretic for kidney cleansing (Menale *et al.,* 2006). It has also been used in cardiac, blood, metabolism, vision disorders, cramps in calves, cancer, body weakness, depression, fetal development, headaches, bone formation, influenza, injuries, pregnancy or lactation, migraine, obesity, osteoporosis, colds, and to increase immunity (Sener *et al.,* 2002; Ceuterick *et al*., 2011; Rao *et al*., 2010).

In vivo and *in vitro* studies have been conducted with this plant due to its antidiabetic use in traditional medicine. Reports have shown that chard extracts administered in rats decrease symptoms and alterations of compromised organs and tissues due to diabetes, and improve the function of pancreatic beta cells (Bolkent, 2000; Sener *et al.*, 2002; Yanardağ *et al.*, 2002; Ozsoy-Sacan *et al.*, 2004). Chard extracts could help regenerating pancreatic islets *in vitro.*

In this study, cytotoxicity of chard was evaluated using the MTT technique to determine a possible selective effect on growth inhibition of tumor cell lines. Cytotoxicity assays are the starting point to evaluate the effects on diverse tumor cells by finding non-toxic extracts that can be later used for bioassays in insulin producing cells.

Materials and methods

Collection of plant material

Vegetable material was obtained from a single supplier in Medellin (Colombia). Chard is commonly sold on the market without any sexual taxonomic structure that could facilitate its identification. Accordingly, an expert professional from the Gabriel Gutierrez Villegas herbarium (Universidad Nacional de Colombia, Medellín campus) identified the plant material. According to the seller, the plants came from seeds known in Colombia as white chard stalk, which corresponds to *Beta vulgaris* L. var *Cicla.*

Preparation of plant extracts

5,000 g of chard were washed with distilled water and dried at 45 °C for 8 h. Then the plant material was pulverized using a blade mill and percolated for 24 h with 1,000 mL of hexane, ethyl acetate, ethanol (JT Baker, Xalostoc, Mexico) or water. Extracts were evaporated to dryness and stored at -20 ºC until used. To test biological activity, dried raw extracts were dissolved in dimethyl sulfoxide (DMSO; Sigma-Aldrich, St. Louis, MO, USA) to 40 mg/mL. Two-fold dilutions were prepared starting from 1,000 µg/ml extract concentration until 32.5 µg/mL.

Cell cultures

Tumor cell line HeLa and non-tumor cell line CHO were obtained from the Cell Repository of Grupo de Inmunovirología (Immunovirology Research Group) at the Universidad de Antioquia (Medellín, Colombia). The cell lines were grown and maintained in the Roswell Park Memorial Institute (RPMI) medium, supplemented with 10% FBS (Fetal Bovine Serum), 1% penicillin/streptomycin (Sigma-Aldrich, St. Louis, MO, USA), and incubated at 37 °C in 5% CO_2.

Colorimetric MTT assay for cell toxicity

The cytotoxic effect at different chard concentrations was evaluated by quantifying cell viability using the MTT technique (Betancur-Galvis *et al.*, 2002). Briefly, HeLa and CHO cell lines were seeded in 96 well plates. After 24 h incubation and 90% confluence, extract dilutions were doubled (from 1,000 µg/mL to 31.24 µg/ml), then added to each cell line and incubated 48 h at 37 °C in 5% CO_2. After this time, the supernatant was discarded and 28 µl of MTT solution 2 mg/mL in Phosphate Buffered Saline (PBS, Sigma-Aldrich, St-Louis, MO, USA) were added. The plates were incubated two hours at 37 °C and 130 µl of DMSO were added to dissolve the MTT crystals. The plates were shaken for 15 minutes and the optical density was measured in a spectrophotometer at 550 nm (DO_{550}) wavelength. Untreated and treated cells with the fractions solvent (1:80 dilution of DMSO) were used as control. The extract`s cytotoxicity was calculated as a percentage with the obtained values of optical density (OD), as follows:

Cytotoxicity percentage= [(A−B)/A]*100

Where A and B are the OD_{550} of untreated and treated cells, respectively. The CC_{50} of each extract (concentration which reduces viability of treated cells by 50% compared with control cells) was extrapolated from dose-response curves of concentration vs cytotoxicity percentage.

Selection criteria for active extracts

The selection criteria used to define active extracts was the Selectivity Index (SI) adapted from Lindholm *et al*. (2002), and Cos *et al*. (2006), based on no-tumor and tumor cytotoxicity ratio CHO (CC_{50})/ HeLa (CC_{50}).

Isolation of porcine pancreatic islets

The protocol was approved by Hospital Universitario de San Vicente Fundación research ethics committee on June 25th of 2009, minute 13. Pig pancreases for islet isolation were obtained from slaughtered animals at La Central Ganadera S.A abattoir (Medellín, Colombia). The islet isolation included: 1) pancreas cleaning to remove connective tissue, lymphoid tissue, fat and nearby organs, 2) pancreas cannulation through the pancreatic duct with FR6 Nelaton probe (SHERLEG Laboratories, Bogotá, Colombia), 3) pancreas manual distention with cold enzyme solution, 4) pancreas digestion with an enzyme combination (collagenase, thermolysin and neutral protease), 5) digest dilution and collection, 6) assessment of size, structure, integrity and presence of islets embedded in acinar tissue and free islets, and 7) initial islet culture in standard culture medium (CMRL1066; Cellgro, Mediatech Inc., Manasas, VA, USA) (Ricordi *et al.*, 1988). Yield was defined as islet equivalents per gram (IEQ/g) of processed pancreas. Islet equivalent is the number of islets in a dilution multiplied by a conversion factor given by islet size.

Evaluation of non-toxic concentrations of four chard extracts in a cell line and porcine pancreatic islets

Porcine pancreatic islets were cultured in three different groups: I) in 24-well plates by triplicate with four smaller than CC_{50} decreasing concentrations (1,000 µg/mL to 125 µg/mL) for each chard extract; II) with supplemented standard culture medium, CMRL1066, and III) with concentrations of 100, 50, 25 and 12.5 mM of CsA, diluted in CMRL1066 as an islet regeneration inhibitory substance.

Viability of pancreatic islets and regeneration index

Islets were cultured with different extract concentrations. Viability assays were carried out on days 1, 3, 5, 8, 13 and 15 in culture to evaluate the regeneration ability of extracts according to culture conditions (Groups I, II and III). Viability was determined using the inclusion and exclusion fluorochrome combination of fluorescein diacetate (FDA) (Sigma-Aldrich, St-Louis, MO, USA) and propidium iodide (PI) (Sigma-Aldrich, St-Louis, MO,USA) to differentiate membrane integrity of viable and nonviable cells (Barnett *et al.*, 2004). Viable (green) and non-viable (red/orange) islets were counted under a fluorescence microscope (Swanson *et al.*, 2001). Each sample`s viability percentage was determined according to the following equation:

$$\text{Viability } \% = \frac{\text{viable cells}}{\text{total cells}} * 100$$

With the viability values, islets regeneration was determined by calculating the "regeneration index" with the equation:

$$IR = \frac{V_{n+1}}{V_n}$$

IR means Regeneration Index, V_n means n period viability, and V_{n+1} is the viability of 1 plus period n.

Statistical analysis

The linear regression analysis for extract concentrations which produce a cell culture`s CC_{50} viability reduction in doses-response curves, were performed using GraphPad Prism version 5.0 for Windows (San Diego, CA, USA). Each experiment was repeated three times and all data are presented as mean ± standard deviation (SD).

Evaluation and comparison of optimal concentration at which pancreatic islets are regenerated were calculated using descriptive statistical analysis. Absolute and relative frequency distributions of

each extract between the different study groups are presented with scatter plots graphics. One-way analysis of variance (ANOVA) was conducted to find significant differences in viability and regeneration index, with $p<0.05$. Analysis was performed using the STATGRAPHICS Centurion 16 statistical package.

Results

Cytotoxicity assays

A dose-response effect between extract concentrations and cytotoxicity on CHO (Figure 1A) and HeLa cells (Figure 1B) was observed. Direct proportionality between cytotoxic action of the extracts and their concentrations is evident as cytotoxic percentage increases with higher extract concentration. Results show that chard extracts have more than 60% cytotoxicity against tumor cells, with higher doses of water, ethanol and ethyl acetate extracts (Figures 1A and 1B). The ethanol extract has the highest selectivity index of all the extracts tested; the CHO CC_{50} and HeLa CC_{50} ratio is 7.3, indicating that this extract is seven times more toxic to tumor cells compared to non-tumor cells (Table 1).

Effect of B. vulgaris extracts in viability and regeneration of porcine islets

The yield of isolated porcine islets was 419.5 IEQ/g of pancreas, and a mean of 3284 IEQ isolated were cultured with different extract concentrations. Porcine islets cultured with different concentration of aqueous, ethyl acetate and ethanol extracts had the highest viability percent when compared with the hexane extracts and control groups; however, only porcine islets cultured with 500 and 250 µg/mL showed significant difference with a 95% confidence interval ($p<0.05$)- with respect to the control groups II and III (Figure 2).

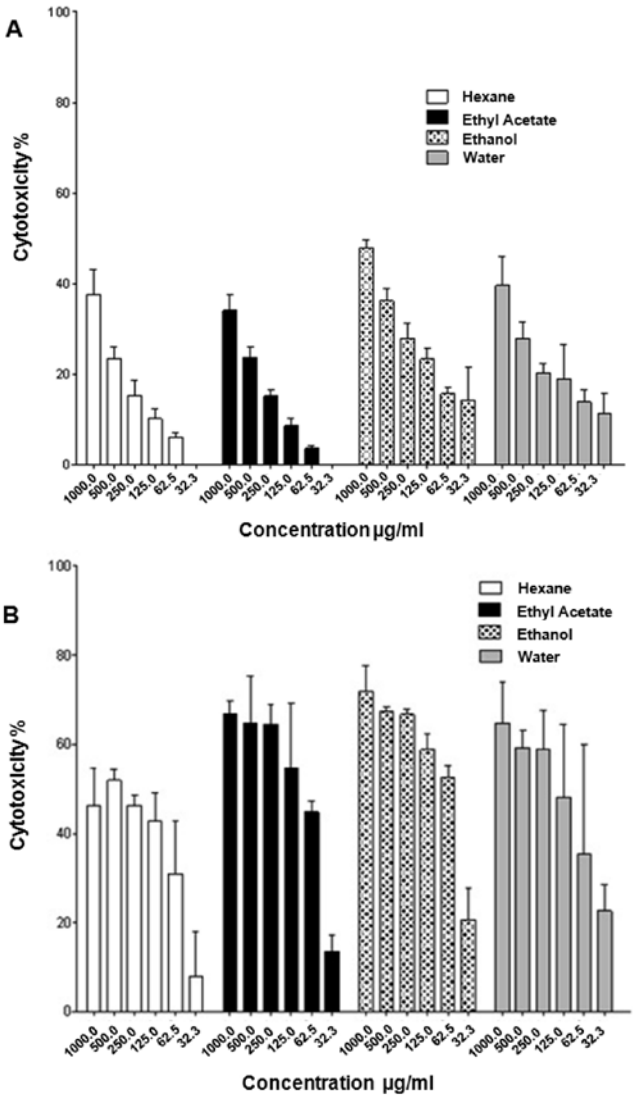

Figure 1. Cytotoxic activity of chard extracts. Cytotoxicity was quantified by MTT method after 48 h post exposition of serial dilution of four extracts on A: non-tumor (CHO) and B: tumor (HeLa) cell lines. Each bar represents the mean and ± SD of three independent experiments.

Table 1. Cytotoxic concentration 50 ratio between non-tumor and tumor cells.

Solvent	Cytotoxic Concentration 50 CC_{50} (µg/mL)		CHO CC_{50}/ HeLa CC_{50} (SI*)
	CHO	*HeLa*	
Hexane	1,298.816	824.89	1.7
Ethyl acetate	1,393.744	283.36	4.9
Ethanol	997.9142	135.79	7.3
Water	1,344.885	420.37	3.2

*SI = Selective index

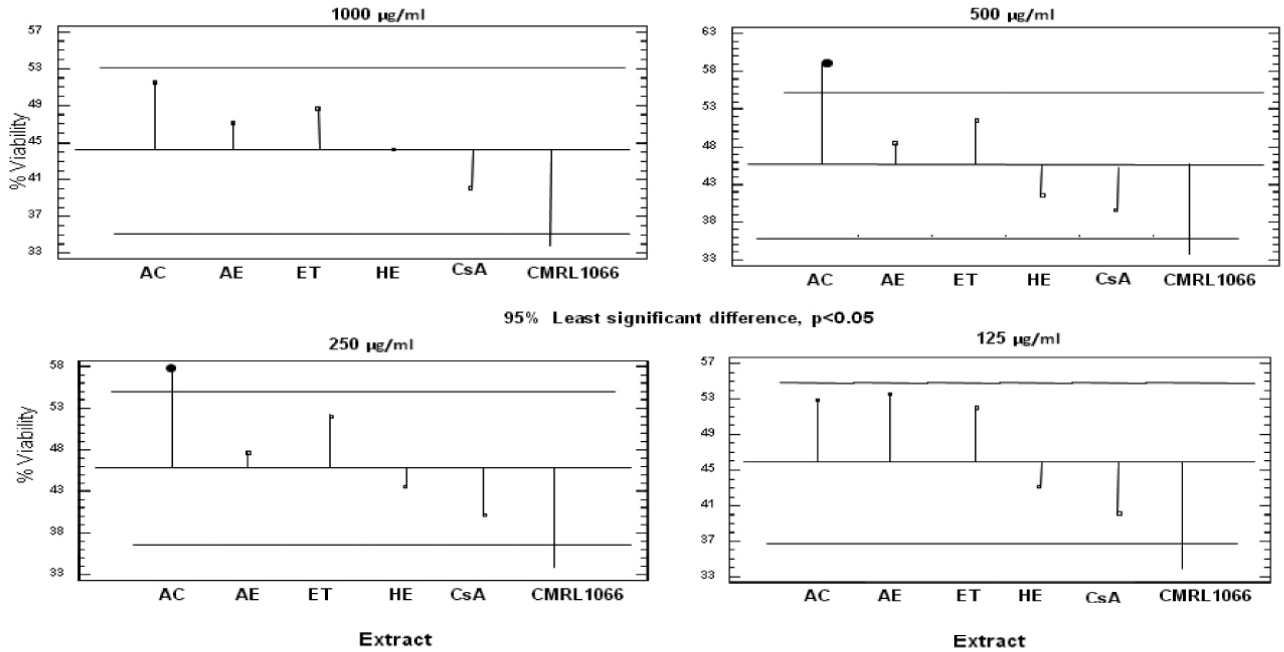

Figure 2. Pancreatic islet viability at different extract concentrations on days 1, 3, 5, 8, 13 and 15 in culture. Black circle indicates significant difference between extracts islets viability and islets cultured in CMRL1066 (p<0.05).

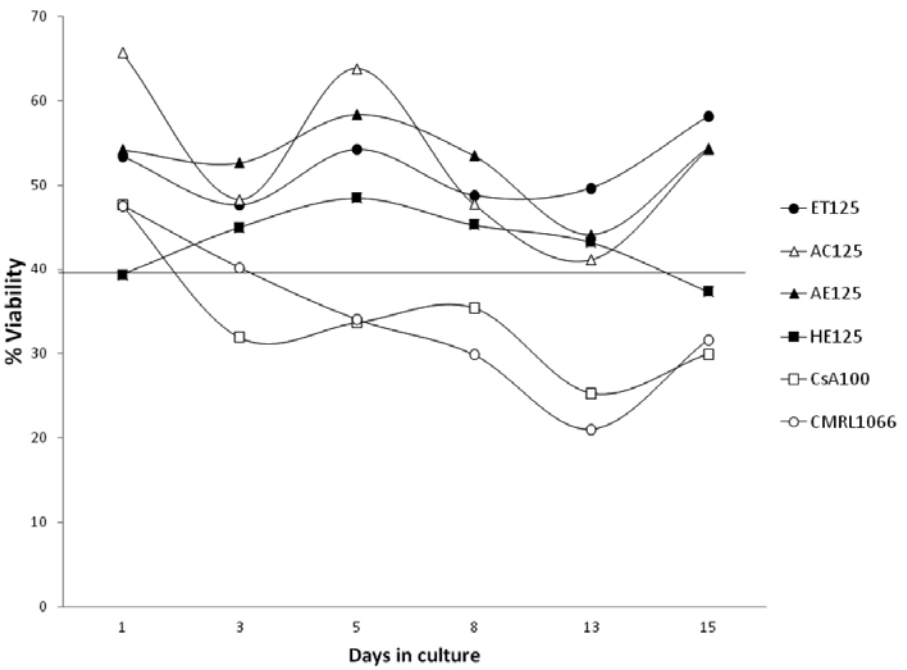

Figure 3. Islet viability percentage vs days in culture. White circles = pancreatic islets cultured in CMRL1066 without extract, white squares = pancreatic islets cultured with CsA 100 mM, black squares = islets cultured with hexane extract at 125 µg/mL, black circles = islets cultured in ethanol extract at 125 µg/mL, black triangle = islets culture in ethyl acetate extract at 125 µg/ml, white triangle = islets cultured in aqueous extract at 125 µg/mL.

Viability of islets cultured with standard medium and CsA decreased by 50% in the first 5 days while it increased in islets cultured with low concentrations of extracts. Viability percentage was kept stable from incubation days 5 through 13, with a slight increase from days 13 through 15 in culture at all extract concentrations, except those of hexane and CsA (Figure 3).

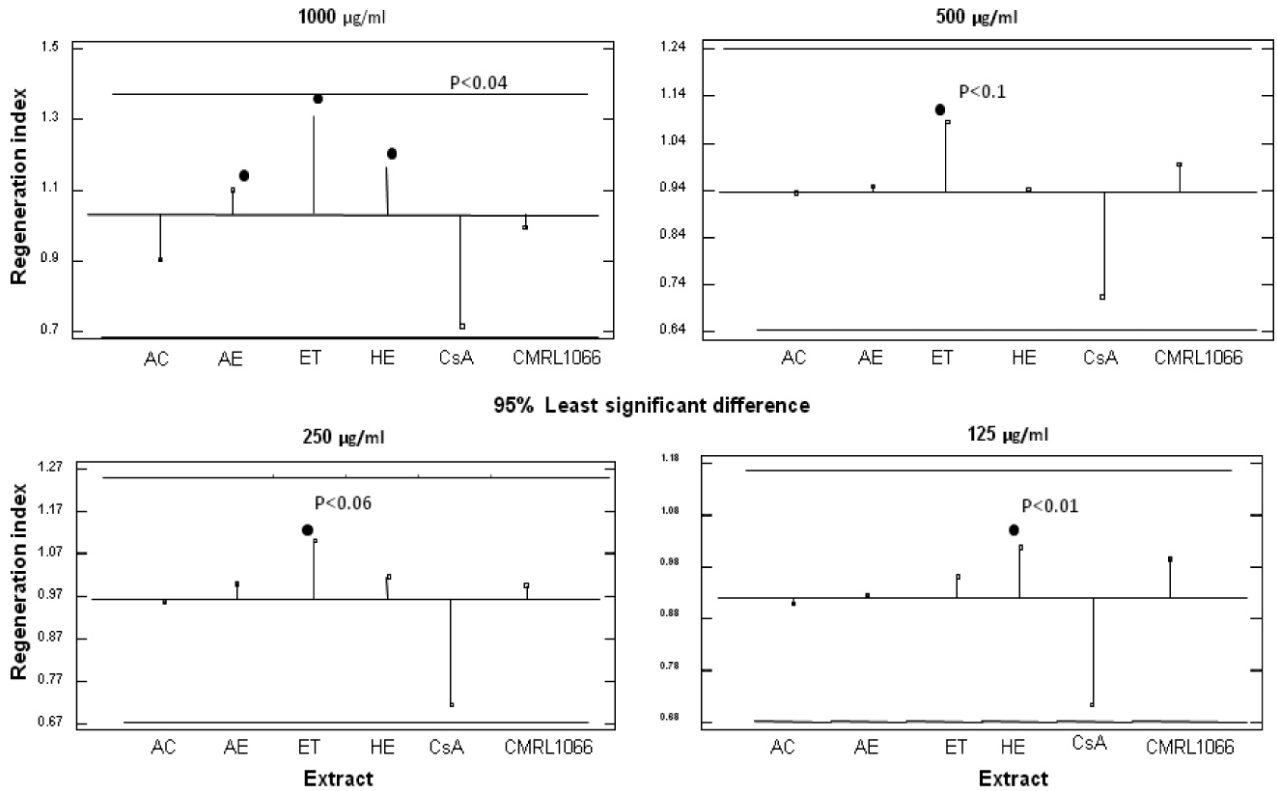

Figure 4. Pancreatic islet regeneration index at different extracts concentration. Black circle indicates significant difference between extracts islets regeneration and islets cultured in CMRL1066 (p<0.05).

Islet regeneration occurs when the regeneration index (IR) is greater than 1. Ethanol extract at 250 µg/mL or higher concentration had the most significant differences in islets regeneration compared to CsA. Islets cultured with hexane had a good IR but viability was lower compared with other extracts according to the multiple range test (p<0.05) (Figure 4). The aqueous extract had the highest viabilities, but only a significant IR at 250 µg/mL, with an 85% CI (p<0.15; data not shown).

Islets initially cultured within the control group conditions of CsA at 12.5 to 25 mM concentrations had viability and regeneration indices similar to hexane and ethyl acetate extracts in 125 µg/mL and 250 µg/mL concentrations respectively. For that reason, data shown in this article were obtained with experiments conducted with 100 mM CsA as the control group III.

Discussion

The evaluated chard extracts showed cytotoxic activity in tumor cells while being less toxic to non-tumor lines, similar to a report in which ethyl acetate extracts of chard seeds inhibited proliferation of lung cancer cells (Gennari *et al.*, 2011). This plant is commonly consumed in Latin American countries and could be ingested as a dietary supplement by patients undergoing cancer therapy.

Diabetes mellitus Type 1 occurs when there is a beta cell massive loss, insulin activity deficiency, and/or pancreas destruction (ACE/ADA, 2006). Pancreatic islet transplantation is a treatment option for select patients with this disease, but the limited islets supply from human donors and poor islet yield and quality impede progress in human islet transplantation. Although, islets can be regenerated *in vivo* in response

to tissue damage or metabolic demands (Banerjee *et al.*, 2005; Trucco *et al.*, 2005; Martin-Pagola *et al.*, 2008), the number of beta cells that carry out division is only 0.5 to 2% (Banerjee *et al.*, 2005; Swenne., 1984; Narang *et al.*, 2006; Battie *et al.*, 2002). Therefore, we looked for islet regeneration by biomolecules and natural agents such plant extracts.

Turkish researchers have used chard extracts to proof their beneficial effects on pancreatic beta cells (Tunali *et al.*, 1988; Sener *et al.*, 2002; Ozsoy-Sacan *et al.*, 2004). The present study evaluated the regenerative potential of chard extract in cultured porcine pancreatic islets. Results show that greater than 50% viability of islets cultured with different concentrations of the extracts is maintained after 15 days in culture. Taking into account that significant cytotoxic activity of plant extracts refers to CC_{50} values below 100 µg/mL, the results show that chard extracts require higher concentrations to kill 50% of the cell population, indicating that their cytotoxic effect is very low. However, it is important to test the viability and regeneration index with extract concentrations lower than 125 µg/mL due to the similar viability and regeneration found for all the concentrations used during the experiments, in order to look for the minimal extract concentration required to maintain islet viability.

CsA is an immunosuppressant drug that induces glucose intolerance by toxic effect on pancreatic endocrine tissue. Toxic CsA doses cause morphological and functional changes by decreasing islet diameter and reducing insulin production (Ajabnoor *et al.*, 2007). Islets were initially cultured with different CsA concentrations, and the ones cultured with low doses behaved similar to some of the extracts. The highest CsA concentration (100 mM) was used as an inhibitory substance in the control group.

The use of porcine islets holds great promise for large-scale application of islet transplantation. The long-term diabetes reversal after porcine islet xenotransplantation in an animal model demonstrated its potential for islet xenotransplantation in humans (Hering *et al.,* 2006; Cardona *et al.,* 2006). In our study, we found that porcine islets cultured with chard extracts have similar behavior to some reported human islets. Human islets viability between 21.63 and 59.87% was reported on day 1 in culture (Nikolić *et al.*, 2010). The porcine islet viability observed in our study was between 31 and 66%. Similar to report by Nikolić *et al.* (2010), the greatest viability percentage was obtained on the third day of cultivation. Those results show that it is possible to obtain porcine islets and maintain them morphologically intact and viable during culture with chard extracts (Nikolić *et al.,* 2010). This aspect makes the option of using pigs as xenogeneic organ donors a possibility. The use of complete pancreas from adult pigs increases the possibility of extracting about 1 million IEQ from a single pig, an essential requirement for xenotransplant from pigs to humans with Type 1 diabetes (Brandhorst *et al.,* 1999). However, in this study one of the major problems in porcine islet isolation was the marked fragility of the islets and their rapid dissociation into single cells during the isolation procedure.

Conflicts of interest

The authors declare they have no conflicts of interest with regard to the work presented in this report.

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