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Stevia rebaudiana ethanolic extract exerts better antioxidant properties and antiproliferative effects in tumour cells than its diterpene glycoside stevioside

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ABSTRACT

Steviol glycosides are currently being used as natural sweeteners by the food industry and *Stevia rebaudiana* has long been used as a sweet plant in South America for patients suffering from diabetes. In this study, a *Stevia rebaudiana* ethanolic extract (SREE) was prepared, analysed and tested for antioxidant activity in terms of free radical scavenging properties and antiproliferative effects in cervix (HeLa), pancreatic (MiaPaCa-2) and colonic (HCT116) cancer cells. The antiproliferative mechanism was confirmed by testing the effects on cyclin D1-CDK4. Bioassays were also performed for the diterpene glycoside stevioside. Our results demonstrate that the extract acts as an antioxidant being able to scavenge free radicals, but this activity was not produced by stevioside. The extract also induced cell death in the three cell lines, being more active against cervix cancer cells (HeLa); however, the concentration of stevioside needed to produce antiproliferative effects was higher than the amount of steviol glycosides found in the lower dose of extract inducing cell death. In addition, the extract clearly inhibited CDK4 whereas stevioside did not, concluding that the antiproliferative activity of stevia may be due to inhibition of cyclin-dependent kinases performed by other compounds of the extract.

Keywords: *Stevia rebaudiana*, stevioside, steviol glycosides, antioxidant, cytotoxicity, HeLa, CDK4.

1. Introduction

Stevia (*Stevia rebaudiana* Bertoni) is a South American herbaceous perennial shrub from the Asteraceae family that has traditionally been used as a medicinal plant for centuries in Paraguay and Peru (1). The plant became known when the herbalist Bertoni reported its use in the 19th century and became widely used in Paraguay by other herbalists. This plant has been used due to its characteristic sweet taste provided by non-caloric molecules named steviol glycosides. Apart from its sweetener capacity, the plant has traditionally been used for glucose regulation as an anti-diabetic herbal remedy and to prevent hypertension.

Although there are no official monographs regarding the medicinal use of this plant provided by WHO (World Health Organization), ESCOP (European Scientific Cooperative on Phytotherapy) or EMA (European Medicines Agency), several preclinical studies have been carried out and some clinical studies regarding anti-hypertensive or anti-antidiabetic properties have been conducted (2-4).

The use of this plant is nowadays rising up due to the fact that certain companies are using stevia extracts as a natural sweetener in substitution to sucrose or classical artificial sweeteners such as cyclamate, aspartame or acesulfame. In addition to this, the European Food Safety Authority (EFSA) published a scientific opinion on the safety of steviol glycosides for the proposed uses as food additives establishing an ADI (average daily intake) of 4mg/kg day (5).

The sweetener capacity of this plant is due to steviol glycosides. These compounds are ent-kaurane diterpene glycosides found in the leaves of the plant, being stevioside, rebaudoside A-C, dulcoside A and steviolbioside the most important and representatives (6). Stevioside is one of main compounds found in the stevia extracts with sweetener capacity that was estimated to be from 10 to 270 times higher than sucrose. Stevioside is formed by three molecules of glucose attached to an aglycone of steviol. Besides containing steviol glycosides, chemical studies have revealed the presence of triterpenes and sterols, phenolics and alkaloids (7, 8)

One of most interesting aspects about the use of this plant may be its benefits in diabetes and glucose regulation (4). The current first-line treatment for type 1 diabetes is subcutaneous insulin injection whereas type 2-diabetes management consists of lifestyle

modifications (diet, weight control and physical activity) and oral antidiabetics like metformin. Some studies confirm the properties and benefits of the stevia plant in diabetic patients due to its sweetener capacity without increasing glycemia and some authors have demostrated glucose captation enhanced by stevioside and rebaudioside (9). Owing to this, stevia extracts and steviol glycosides represent and interesting source of natural sweeteners.

The aim of this study is to investigate other bioactive properties of a dried stevia ethanolic extract in terms of antioxidant activity and antiproliferative effects on tumour cells in comparison with stevioside, the first and one of the main steviol glycosides reported in *Stevia rebaudiana* leaves.

2. Materials and methods

2.1. Reagents and chemicals

All chemical reagents including standards were acquired through Sigma-Aldrich; cultura media, antibiotics and serum were acquired at Pan Biotech. Acetonitrile and water, both HPLC grade, were acquired from Panreac. Dried *Stevia rebaudiana* leaves were bought at Plantarom (Linyola, Spain).

2.2. Stevia rebaudiana ethanolic extract (SREE)

75 g of stevia leaves were split in three Erlenmeyer, 500 ml of ethanol were added in each one and extraction was performed as 24 h maceration at 4 ºC. After 24h, the extract was filtered and the solvent was removed until dryness at 35 degrees using a rotatory evaporator. This process was done 3 times in order to successively extract the same plant material with ethanol. The extract was dried in the rotatory evaporator until we obtained a powder and was kept in glass vials at -20 ºC until use for experiments and bioassays. 3.8 g of dry ethanolic extract were obtained from 75 g of stevia leaves (yield of 5.1 %). The organoleptic properties revealed an intense green, sweet fine powder with a final liquorice taste.

2.3. Phytochemical analysis of the extract

The phytochemical analyses of our extract and the detection of steviol glycosides (stevioside and rebaudioside A) in our sample were done by HPLC-PDA using an Agilent 1260 Infinity LC following a described procedure (10) and by Thin Layer Chromatography (11). Steviol glycosides were identified according to their elution order, retention times and UV-vis spectra of standards. The quantification of steviol glycosides was done by comparing area values obtained for the components of our stevia ethanolic extract and the peak area of the selected standard.

Polyphenol content was analysed by the Folin-Ciocalteu method. Briefly, 9 µl of our sample was mixed with 201.5 µl of Folin-Ciocalteu reagent. After 5 min incubation at room temperature, 89.5 µl of 15 % sodium carbonate solution was added and incubated in the dark at room temperature for 45 min. Absorbance was measured at 752 nm. Gallic acid was used as standard (12)

2.4. Brine shrimp toxicity assay

General toxicity of the extract was tested by the brine shrimp (*Artemia salina*) lethality assay (13, 14). Commercial dried cysts of brine shrimp were hatched in seawater with aeration for 72 hours. The extract and stevioside were dissolved in seawater and transferred to 6-well plates to obtain concentrations of 2, 20, 200 µg/ml in 5 ml sea water with 10 nauplii in each well. Control test wells were filled with 5ml of seawater and 10 nauplii. After 24 h incubation at 25 °C, the number of viable nauplii was counted. The percentage of mortality was calculated. Standard drug colchicine was used as positive control.

2.5. Antioxidant activity

2.5.1. DPPH radical scavenging capacity

The capacity of the extract and stevioside to scavenge DPPH free radicals was measured by a colorimetric method (15). 150 µl of a DPPH methanolic solution (0.04 mg/ml) were added to 150 µl of our samples of extract or stevioside dissolved in ethanol at different concentrations. Absorbance was measured at 517 nm after 30 min of reaction at room temperature in a microplate reader. Controls contained all the reaction reagents except the samples. Ascorbic acid was used as positive control. Background

interferences from solvents were deducted from the activities prior to calculating radical scavenging capacity as follows: RSC(%)= [($Abs_{control}$-Abs_{sample})/$Abs_{control}$]x100

2.5.2. Superoxide radical scavenging

Superoxide radicals were generated by the xanthine/ xanthine oxidase (X/XO) system following a described procedure with some modifications (16). The reaction mixture contained: 240 µl of the following mixture (90 µM xanthine, 16 mM Na_2CO_3, 22.8 µM NBT in phosphate buffer pH 7.0) was mixed with 30 µl sample. The reaction was initiated by the addition of the enzyme and the mixture was incubated for 2 min at 37 °C. Antioxidant activity was determined by monitoring the effect of the extracts on the reduction of NBT to the blue chromogen formazan by the superoxide radical ($O_2^{\cdot-}$) at 560 nm: RSC(%)= [($Abs_{control}$-Abs_{sample})/$Abs_{control}$]x100

2.5.3. Antioxidant activity in HepG2 cells

Cells were grown in DMEM supplemented with 10 % foetal bovine serum and 1 % penicillin-streptomycin at 37 ºC and 5% CO_2. A general cytotoxicity assay was first performed in order to test only the antioxidant potential of non-cytotoxic concentrations of SREE. Concentrations under 31.25 µg/ml were considered non-toxic in HepG2. Then, cells were seeded in 96-well microplates at a density of 7 $x10^3$ cells per well. After 48 h, cells were treated with non-cytotoxic concentrations of SREE (3.9, 7.8 and 15.6 µg/ml) for 24 h. HepG2 cells were then treated with a 500 µM H_2O_2 PBS solution for 1 hour. After 24 hours, the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was performed and % of cell survival was calculated (17).

2.6. Antiproliferative activity in cancer cells

The cytotoxic and antiproliferative effects of the extract and stevioside were screened through the MTT assay using cervix (HeLa), colonic (HCT116) and pancreatic (MiaPaCa-2) cancer cells. Cultures were grown in DMEM supplemented with 10% foetal bovine serum and 1% penicillin-streptomycin-glutamine. Cultures were incubated in the presence of 5% CO_2 at 37 ºC and 100% relative humidified atmosphere. Cells were seeded in 96-well microplates at a density of 7 x 10^3 cells/well and grown for 24 h

at 37 ºC. Cells were then treated with various concentrations of products (1-250 µg/ml) for 72 h and a MTT solution was added and incubated for 4 h at 37 ºC. Cell survival was measured as reduction of MTT into formazan at 550 nm in a microplate reader. Three experiments were performed to screen the cytotoxicity of the extract.

2.7. Inhibition of human CDK4 (cycD1)

The effects of SREE and stevioside on the activity of the human CDK4 was quantified by measuring the phosphorylation of the substrate Ulight-CFFKNIVTPRTPPPSQGK-amide (MBP) using a human recombinant enzyme expressed in Sf9 cells and the LANCE® detection method (18). Briefly, The samples were mixed with the enzyme (16.4 ng) in a buffer containing 40 mM Hepes/Tris(pH 7.4), 0.8 mM EGTA/Tris, 8 mM $MgCl_2$, 1.6 mM DTT and 0.008% Tween 20. Thereafter, the reaction is initiated by adding 100 nM of the substrate Ulight-CFFKNIVTPRTPPPSQGK-amide (MBP) and 100 µM ATP, and the mixture is incubated for 60 min at room temperature. The reaction is stopped by adding 13 mM EDTA. After 5 min, the anti-phospho-MBP antibody labeled with europium chelate is added. After 60 min, the fluorescence is measured using a microplate reader. Stausporine was used as positive control drug.

2.8. Statistical analysis

Results are expressed as mean ± standard error of experiments performed at least in triplicates in different weeks. Data analysis was performed using GraphPad Prism version 5. ANOVA or t test were run with data depending on the type of experiments.

3. Results

3.1. Phytochemical analysis of the extract

Chromatogram of SREE at 205 nm is shown in Fig. 1. Stevioside and rebaudioside A were detected by HPLC as the main peaks at 205 nm by comparing fingerprints and retention times of the extract with standards acquired from Sigma. Steviol glycosides

were also detected by thin layer chromatography (data not shown). According to our calculations, 1 mg of our SREE contains approximately 0.28 mg (28 % mass concentration) of steviol glycosides expressed as stevioside. The proportion of polyphenols in the extract was 2.2 % (mass concentration) calculated by Folin-Ciocalteu method.

3.2. Brine shrimp toxicity assay

The viability of *Artemia salina* was not affected by treatments with SREE or stevioside at tested concentrations (0-200 μg/ml) within 24 hours (data not shown).

3.3. Antioxidant activity

The antioxidant activity of our samples was measured in terms of free radical scavenging properties using two different radicals and methods. SREE was able to scavenge both DPPH and superoxide radicals in a dose dependent manner (Fig. 2) whereas stevioside did not show radical scavenging capacity. The ability to scavenge DPPH radicals was compared with that of ascorbic acid (vitamin C); however the activity displayed by stevia was not superior to the reference ascorbic acid. SREE was also able to scavenge superoxide radicals generated by the xanthine oxidase enzymatic assay. SREE was also assayed in HepG2 cells exposed to hydrogen peroxide and results in Fig. 3 indicate a moderate antioxidant activity with significant differences for the higher tested concentration (15.62 μg/ml). When cells were exposed to 500 μM hydrogen peroxide, cell survival was reduced up to 57.4 % whereas pre-treatments with SREE at a concentration of 15.62 μg/ml increased cell survival to an average value of 68.3 %. Higher doses were not assayed because they produced cytotoxicity in the HepG2 cells.

3.4. Antiproliferative activity in cancer cells and effects in CDK4

The antiproliferative effects and cytotoxicity profile of SREE and stevioside can be seen in Fig. 4. Note that log C has been represented in the X axis. Data at 2.39 in the X axis refer to the cell viability at the maximum tested concentration of the products (= 250 μg/ml). SREE clearly shows a dose-dependent antiproliferative activity in all cancer cell lines. However, cervix cancer cells (HeLa) seem to be more sensitive to SREE than the

other cell lines. Colonic cells (HCT116) seem to be more resistant when exposed to SREE for 72 hours.

Stevioside also showed a dose dependent effect in all cell lines. But in this case, according to IC_{50} values (Table 1), the order was HeLa< HCT116< MiaPaca-2. As HeLa cells were the most sensitive to SREE and stevioside treatments, cytotoxicity in HeLa cells was also studied for the aglycon steviol, which is the diterpene base structure in all steviol glycosides. Steviol was included in this experiment in order to confirm that the antiproliferative effects are in relation with this part of the molecule that is presented in all steviol glycosides (Figure 5).

In order to determine the possible mechanism by which stevia or stevioside induce cytotoxicity in tumour cells, the effect on cyclin-dependent kinase 4 (CDK4) was studied. Cyclin-dependent kinases play a crucial role in cell cycle and proliferation; SREE was able to inhibit CDK4 at the same concentrations that were cytotoxic in the different cell lines (Fig.6) whereas stevioside was not able to inhibit CDK4 at those concentrations. Stausporine was used as positive CDK4 inhibitor with an IC_{50} value of 2.3×10^{-7}M.

4. Discussion

Stevia rebaudiana is a medicinal and food plant with increasing interest due to its health benefits, especially in diabetes and metabolic related disorders. This plant has been used in traditional medicine for centuries in people suffering from diabetes and it is nowadays an approved sweetener internationally and widely used by the food industry. Although there are some scientific works reporting benefits and properties of stevia extracts, few works report bioactivities of stevia extracts in comparison to its main steviol glycosides.

Oxidative stress is one of mechanisms involved in cellular ageing and disorders such as diabetes. Proliferation of tumour cells is also responsible for development of cancer and although artificial sweeteners are approved and supposed to be safe for human use (19), certain authors have proposed controversial health effects (20, 21).

Here in this work, we have prepared - analysed an *Stevia rebaudiana* ethanolic extract (SREE) and tested for antioxidant and antiproliferative activities in different

experiments which have been also performed with stevioside, one of the main steviol glycosides found in stevia leaves. According to the data, our extract contains stevioside and rebaudioside A as main steviol glycosides (detected and quantified by HPLC) but also a significant proportion of polyphenols (quantified by the Folin-Ciocalteu method).

In the bioassays, SREE showed a great antiradical activity involving DPPH or superoxide radicals and the activity was confirmed using HepG2 cells. This activity was not detected for stevioside due to the fact that steviol glycosides do not contain phenyl aromatic rings with capacity to scavenge free radicals. This finding suggests that the antioxidant activity of stevia is displayed by other types of compounds such as polyphenols. Karaköse and co-workers have reported the presence of chlorogenic acid derivatives and flavonoid glycosides in stevia leaves (22), which may be responsible for the antioxidant and free radical scavenging properties of stevia extracts. Bender et al. (23) have also recently reported caffeoyl-quinic acid derivatives in stevia leaf infusions; the authors demonstrated the antioxidant capacity of stevia infusions by the ORAC and cell-based methods using HepG2 as a model. However, our study differs from theirs in different aspects; we have used an ethanolic extract instead of aqueous infusions, the antioxidant activity was confirmed by different methodologies and we report antiproliferative effects in different tumour cells as well as a pharmacological mechanism inducing cell death where CDKs are involved.

According to the antiproliferative effects in cervix, colonic and pancreatic tumour cells, SREE also showed significant cytotoxic properties, in some cases higher than the effect displayed by the standard stevioside. For instance, the IC_{50} value in HeLa cells for the extract was lower than the value for stevioside which means that our extract seems to be more cytotoxic than the isolated compound. Both the extract and the isolated standard did not display toxicity in the brine shrimp assay, which may mean that these products exert some kind of selectivity towards cancer cells. In addition, considering that the amount of steviol glycosides in the extract is approximately 28 %, the cytotoxicity and antiproliferative effects may be mainly due to polyphenols and other compounds presented in stevia leaves. High doses of stevioside are needed to induce cytotoxicity whereas 48 µg/ml of SREE is sufficient to decrease cell survival by 50% in HeLa cells. At that dose of SREE, it is estimated that only 13.4 µg/ml may be the concentration of steviol glycosides; however neither stevioside nor steviol were cytotoxic at 15.625

μg/ml (Fig.5). Other authors have performed cytotoxicity studies with stevia extracts or steviol glycosides (24, 25) but here in this manuscript it is the first time that stevia and stevioside have been tested on MiaPaCa-2 or HCT116 cells. In addition, Vaško et al., (25) did not find cytotoxic effects for stevia extract; however, the authors do not specify what kind of extract they used in the study, which may differs in composition for ours as ethanol is a better solvent than water in order to extract certain polyphenols such as flavonoids (23) In relation with mechanisms inducing cell death, a relatively new approach to find and understand antiproliferative and cytotoxic substances consists of studying cyclin-dependent kinases (CDK), which are key proteins in cell cycle regulation and proliferation. The CDK family are proteins that bind a regulatory protein called cyclin. The CDK-cyclin complex is an active kinase that regulates the cell cycle of eukaryotic cells. In particular, the complex cyclin D1-CDK4 is a critical step for the S-phase entry (26). Inhibiting CDK4 implies inhibition of DNA replication in the cell, which occurs in the S phase. In this paper, we report for the first time that stevia is able to inhibit CDK4 in contrast to stevioside, which did not show this activity. This inhibitory effect explains in part why SREE showed antiproliferative activity in HeLa, HCT116 or MiaPaCa-2 cells. The CDK4 inhibitory properties could be attributed to polyphenols, as previous works on plant extracts reveal effects of these compounds in the CDK family (27-30)

These findings suggest that *Stevia rebaudiana* ethanolic extract (SREE) exerts interesting antioxidant and antiproliferative activities compared to stevioside and that both activities might be mediated by other compounds like polyphenols alone or in synergy with steviol glycosides. These results may be interesting for the food and pharmaceutical industry as may justify the use of bioactive stevia extracts instead of purified or isolated steviol glycosides.

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Conflict of interests

Authors declare no conflict of interest

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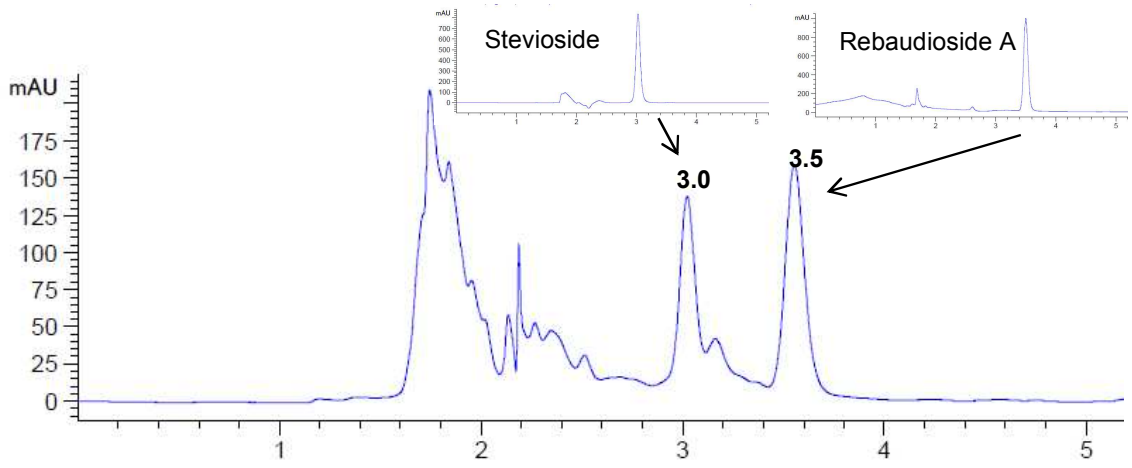

Figure 1. HPLC Chromatogram of *Stevia rebaudiana* leaves ethanolic extract (SREE) at 205 nm. Stevioside detected at retention time 3.0 min and rebaudioside A at retention time 3.5 min.

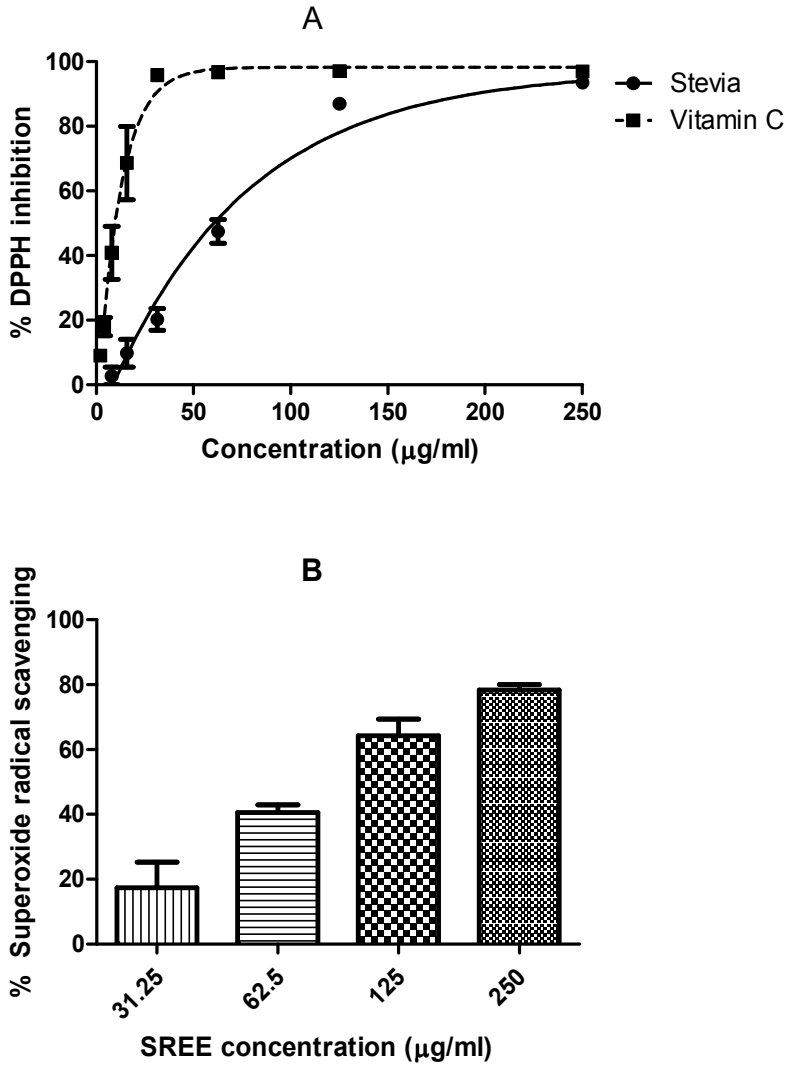

Figure 2. Antioxidant activity of SREE by the DPPH method (A) and xanthine-xanthine oxidase system (B). Stevioside was not able to scavenge free radicals (data not shown). At least three different experiments were performed in triplicates.

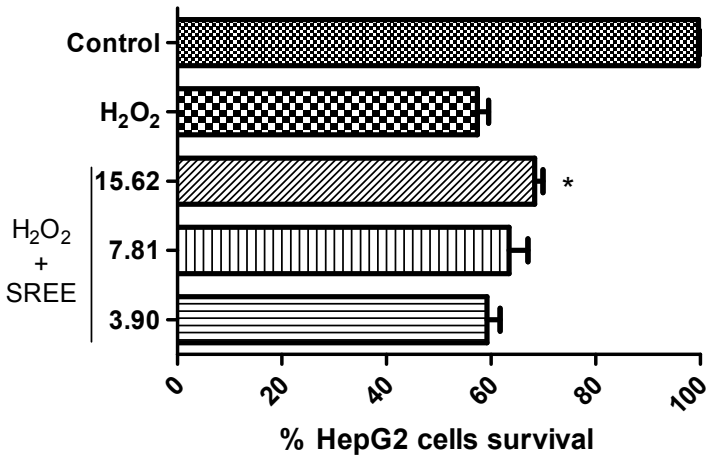

Figure 3. Antioxidant activity of SREE in HepG2 cells. Control cells were exposed to DMEM medium, H_2O_2 cells were exposed to 500 μM. * indicates a p value < 0.05 versus hydrogen peroxide treated cells using one way ANOVA and Dunnett´s multiple comparison test. Concentrations of extract are expressed in μg/ml. Values are mean ± standard error of three different experiments performed in triplicates.

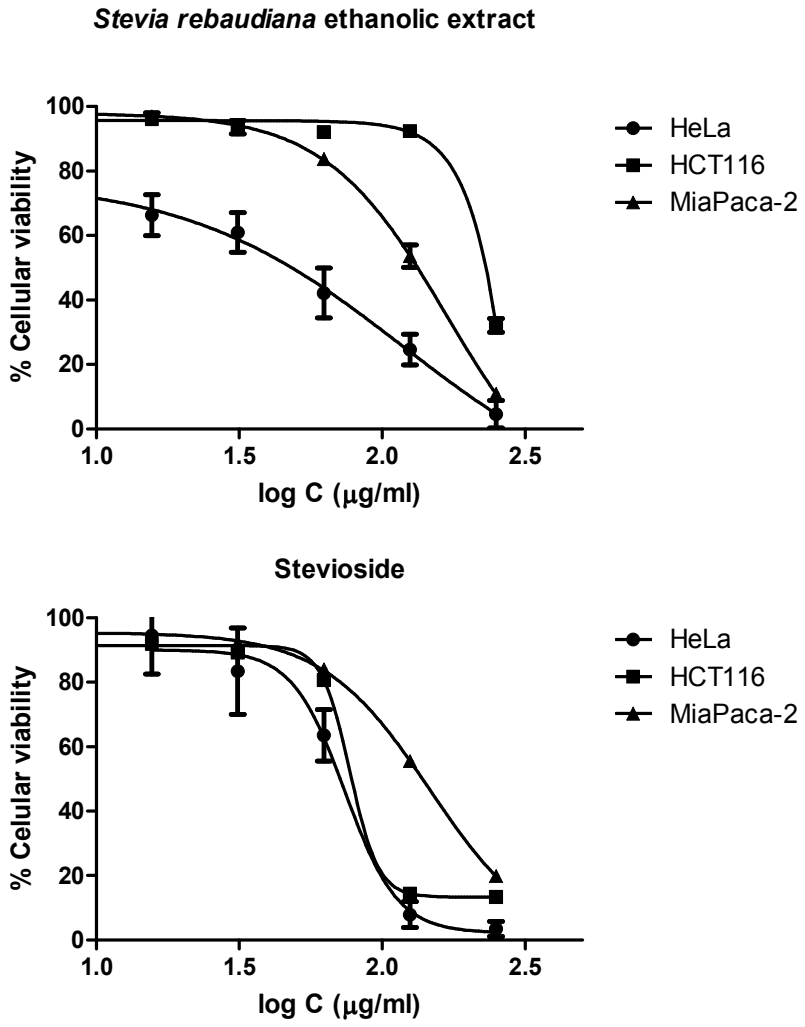

Figure 4. Antiproliferative effects of *Stevia rebaudiana* ethanolic extract (SREE) and stevioside in HeLa (cervix), HCT116 (colon) and MiaPaCa-2 (pancreatic) cells. Values are mean ± standard error of at least three experiments performed in triplicates.

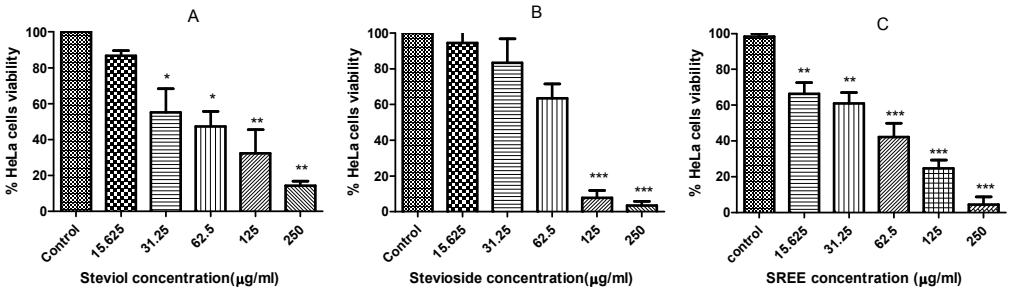

Figure 5. Cytotoxicity profile of steviol (A), stevioside (B) and *Stevia rebaudiana* ethanolic extract (C). Values are mean ± standard error of three different experiments performed in triplicates. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ versus control (non-treated cells). Significant differences were calculated by ANOVA and Dunnett´s multiple comparison test.

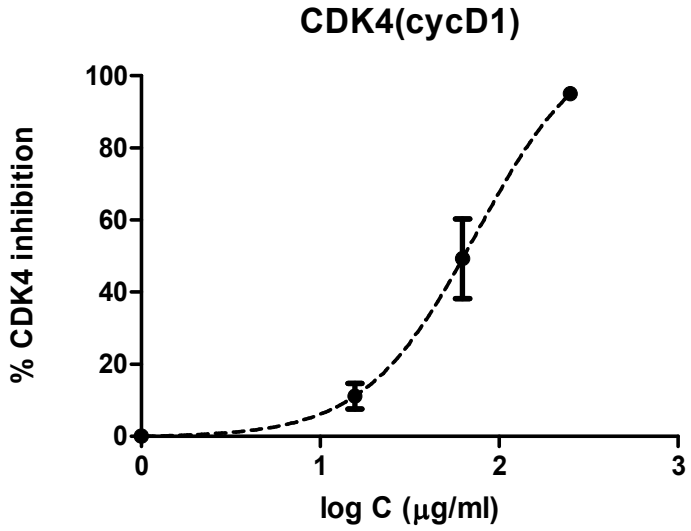

Figure 6. Effect of *Stevia rebaudiana* ethanolic extract (SREE) on CDK4. The extract was tested at 3 different concentrations (15.62, 62.50 and 250 µg/ml) in duplicate. Stevioside was not a CDK4 inhibitor as percentages of inhibition were - 1, 1 and - 4 at the same concentrations. Stausporine was used as positive control with an IC_{50} value of 2.3×10^{-7}M.

Table 1. IC_{50} values (μg/ml) of *Stevia* r*ebaudiana* ethanolic extract (SREE) and stevioside in cytotoxic and antioxidant experiments

Experiments	SREE	Stevioside
HeLa cells	48.3	71.3
MiaPaCa-2 cells	139.2	138.2
HCT116 cells	230.7	78.1
CDK4	63.68	n.a
Brine shrimp toxicity assay	n.t.	n.t.
DPPH free radical	60.4	n.a.
Superoxide radical	81.4	n.a.

n.a.: no activity at tested concentrations

n.t.: no toxicity at tested concentrations

GRAPHICAL ABSTRACT