

Effects of *Stevia rebaudiana* Glycoside on Growth and
Differentiation of Rat Osteosarcoma Cells

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Abstract: In order to determine whether stevia extract has any biochemical effect on the behavior of cancer cells, rat osteosarcoma cells (ROS 17/2.8) were treated with varying concentrations of the active glycoside of the sweetener, steviol, for various times over the course of 9 days. The treated cultures were assayed for cell density and for the osteoblastic marker enzyme alkaline phosphatase via spectrophotometry. These two measurements can give insight into the effect of steviol on cell growth and osteoblastic differentiation respectively. Cell density was observed to increase with exposure to greater concentrations of steviol, especially with increased longevity of exposure of 9 days ($p= 0.0002$) which indicates the cells engaged in proliferative activity. However, alkaline phosphatase levels were found to significantly decrease with increased exposure, with the greatest cell responsivity to steviol treatment being observed on day 6 ($p=5.38*10^{-24}$). This inverse relationship between cell proliferation and a differentiation marker is not surprising given that proliferation needs to decrease in order for cells to differentiate. However, further studies are needed to investigate why this common sweetener appears to increase the proliferation of tumor-derived cells.

Introduction: *Stevia rebaudiana* is an herbaceous plant in the sunflower family native to the grassland biomes of Brazil and Paraguay. The plant has a long history of use for its leaves which have a very potent sweetness to them, often described as having 300-fold the sweetness of cane sugar. However, the human body is unable to metabolize the glycosides responsible for the sweetness of the leaves during digestion; therefore, the sweeteners derived from stevia have no caloric value, making them an appealing option for diabetics and others that are health conscious looking for an anti-hyperglycemic sweetener¹. In 2008, the Food and Drug Administration approved the use of stevia extract as a sweetener, categorizing it: ‘generally recognized as safe.’

Amidst the increased interest and simultaneous skepticism of naturopathy, compounds hailed as ‘natural’ have been exploding in their use while being scrutinized by researchers to thoroughly examine their biochemical impacts. Most research has not only further validated the safety of the sweetener but has also discovered some potential health benefits of consuming stevia tied to its anti-cariogenic, antioxidant, and anti-inflammatory properties². During a study testing toxicological safety of long-term consumption, researchers observed the germline cells and performed micronuclei assays on the bone marrow of mice fed steviol—the active glycoside in stevia leaves—in search of genotoxicity and carcinogenic properties of the extracts. Micronuclei assays quantify the amount of chromosomal damage in cells and no notable results were found linking stevia to any mutagenic behavior based on the assays³. This result is in line with the FDA’s classification of stevia, but some studies go as far as to suggest that steviol has cancer suppression properties. One study was performed to look for antiproliferative effects of varying types of cancer cells including cervical cancer cells, colon cancer cells, and pancreatic cancer cells. The study not only observed a treatment effect, the researchers also proposed a potential mechanism for the cytotoxicity of the stevia extract on the cancer cells theorizing that the extracts have CDK4 inhibitory properties. CDK4, a protein that regulates cell division, can decrease proliferation when in underabundance. Furthermore, CDK4 has been observed by several studies to be reduced in the presence of polyphenols which exist in stevia extracts leading to the suggestion that the polyphenols in the sweetener were responsible for suppressing cell division in the cancer cells⁴. While these findings support the notion that the natural sweetener is not only safe for consumption, but also has curative properties, a very small but noteworthy fraction of the literature negates these discoveries. A paper published in the Department of Medicinal Chemistry and Pharmacognosy found that metabolically active steviol was actually

mutagenic to the liver cells of Aroclor 1254-pretreated rats⁵. As aforementioned, the property of stevia as a zero-calorie sweetener can be attributed to the fact that steviol cannot actually be metabolized into energy by most digestive systems. Therefore, while the results of the study are not entirely relevant in the context of stevia consumption, it does warrant further insight into the genotoxicity of a potentially mutagenic compound.

The objective of the following study is to attempt to discover any effects of the glycoside of stevia extract, steviol, on the differentiation of rat osteosarcoma cells. The cells in media will be exposed to varying doses of steviol over a several days and observed at various points during the incubation period. Alkaline phosphatase and general protein assays were performed on the cells to look an indicators of cell health. The cells were also fixed in methanol and stained with crystal violet to estimate cell number and compare to the assays to gauge correspondence of cell density and protein levels. Ultimately, this study will serve to provide clearer insight into the genotoxicity of such a widely used sweetener.

Materials and Methods:

Trial 1: Store bought stevia extract packets were purchased and a single packet of 0.8g of solid stevia was dissolved—heated and vortexed—in 10mL phosphate buffered saline. Stock of rat osteosarcoma cells in 10% fetal bovine serum were trypsinized while the approximate number of cells were counted in order to distribute 10,000 cells/cm² in three 24-well trays and three 12-well trays. Each tray had three conditions of stevia exposure at approximate food grade concentrations of 13μM, 80μM, and 130μ labeled ‘low,’ ‘medium,’ and ‘high’ concentrations respectively. Each well was filled with 10,000 cells/cm² and the amount of MEMα with 10% fetal bovine serum (as a media) and stevia solution to create the aforementioned concentrations

along with a vehicle condition with 130 μ M saline instead of stevia. The trays were then allowed to incubate while being fed with fresh media and stevia solution every 3 days while one 24-well and one 12-well tray was harvested every six days. When harvested, both trays were dumped, and the cells were rinsed with phosphate-buffered saline. Cold methanol was poured into the 12-well trays and allowed to sit ~10 minutes before being dumped and allowed to air-dry until ready to analyze. 250 μ L of alkaline phosphatase lysis buffer was added to each of the wells in the 24-well trays and left to sit for 20 minutes before being placed in a -70 $^{\circ}$ C freezer until ready to analyze. After harvesting all six trays, 500 μ L of Hardy's crystal violet was added to each well of the 12-well trays and allowed to sit on a rocking platform for at least 15 minutes to stain the Ros cells before being dumped and rinsed thoroughly with deionized water and left to dry overnight. Subsequently, 500 μ L methanol was added to each well of stained cells and allowed on rocking platform for at least an hour. On a 96-well tray, 50 μ L of the solution from the vehicle, low, medium, and high conditions of the 12-well trays were micropipetted, sorted by day of harvest (day 6 or 12) along with a column of 100 μ L pure methanol serving as a blank. The absorbance of the tray was read under a spectrophotometer at 570nm and the results were recorded. The 24-well trays were removed from the -70 $^{\circ}$ C freezer allowed to thaw inside a 37 $^{\circ}$ C incubator. On a new 96-well tray, 50 μ L of AP lysis buffer was added to the wells corresponding to different treatment conditions of the 24-well trays and on a separate column representing blanks for the spectrophotometer. 5 μ L of each treatment condition—vehicle, low, medium, and high—was added to the 96-well tray along with 50 μ L para-nitrophenylphosphate (PNPP) substrate (onto treatment conditions and blanks). The tray was allowed to sit for a minute to let the reaction proceed and for the solution to turn a light-yellow color before being placed in the spectrophotometer and having the absorbance read at 405nm; the results were recorded. A

protein assay was also performed on the 24-well tray using the BioRad DC Protein Assay Kit. 10 μ L of the treated media from the tray was placed on a new 96-well tray. A series of protein solutions of known concentrations of 0.3mg/mL, 0.6mg/mL, 0.9mg/mL, 1.2mg/mL, and 1.5mg/mL was placed down a column on the 96-well tray to create a standard curve. In a vial, 1mL of reagent 'A' was mixed with 20 μ L of reagent 'S' in a vial and 25 μ L of this solution was placed with the wells with either the standard curve or treatment conditions. Finally, 200 μ L of reagent 'B' was placed in all of the wells; the reaction was given time to take place and turn into a light-purple color. The absorbance was read in a spectrophotometer at 700nm; the results were recorded.

Trial 2: A second round of experiments was performed on a fresh batch of rat osteosarcoma cells except with steviol—the active glycoside in stevia extract. 5mg of steviol was dissolved in ethanol to create a 10mM solution. This vial was used as a stock for the remaining trials. The solution was poured into three separate vials and further diluted to 0.1mM, 1mM, and 10mM to create the low, medium, and high concentrations respectively. The concentrations were further diluted in cells in media to create overall concentrations of 0.1 μ M, 1 μ M, and 10 μ M; a vehicle condition of 10 μ M ethanol was also created. The procedure of the steviol trial was otherwise identical to the aforementioned stevia extract trial with two 12-well trays and two 24-well trays fed every 3 days and harvested every 6 days for cell number, alkaline phosphatase activity assay, and protein assay. Absorbances were recorded.

Trial 3: Conditions were shifted in the third trial to add a 'very high' concentration. Ten-fold the volume of the 'high' condition was placed in the 'very high' condition making the addition of a second vehicle with ten-fold the volume of ethanol obligatory. The frequency of harvest was also changed to create a more diligent schedule of monitoring biochemical activity of the cells.

Therefore, for this trial there were six 12-well trays and six 24-well trays with two of each harvested every three days while the remaining trays were fed with fresh steviol in media for a total of nine days. The remaining procedure was identical to the last trial and all the pertinent assays were performed and absorbances were read and recorded.

Trial 4: A final trial was performed completely identical to the last to confirm reproducibility of results.

Results:

Averages were taken for all of the absorbance readings for each separate day and treatment condition. These averages were then graphed out for all of the trials but are presented below for the fourth trial (Figures 1-6):

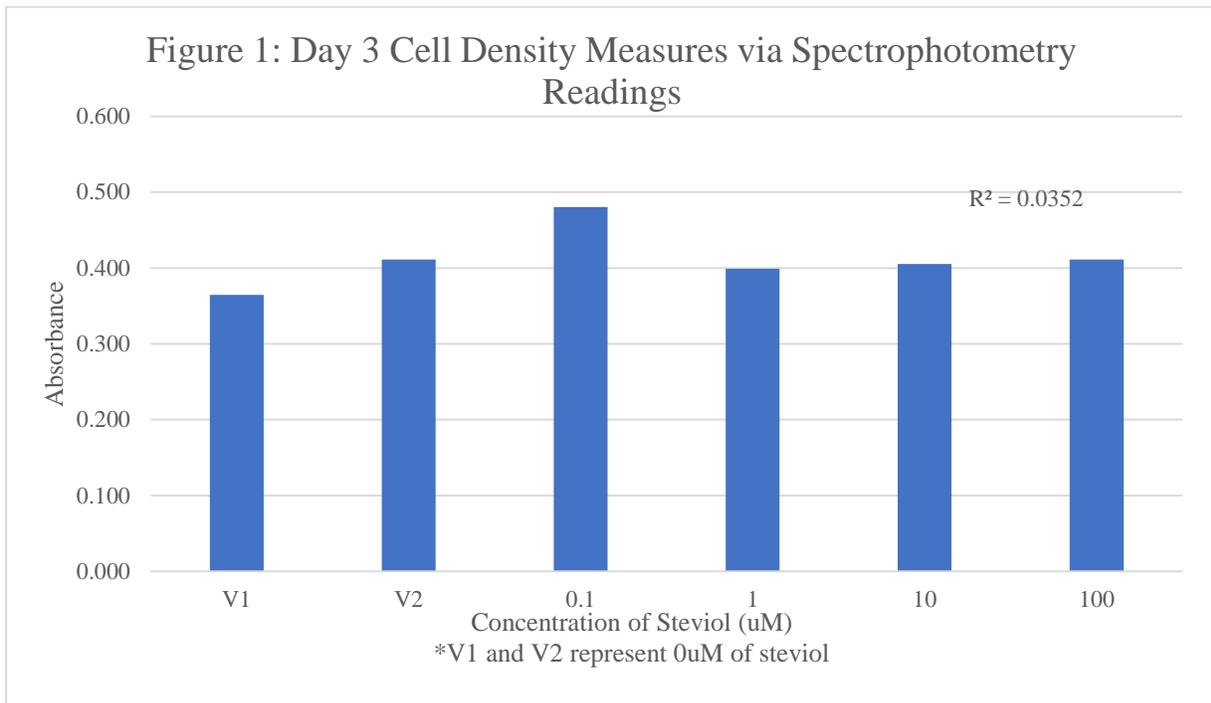


Figure 2: Day 6 Cell Density Measures via Spectrophotometry Readings

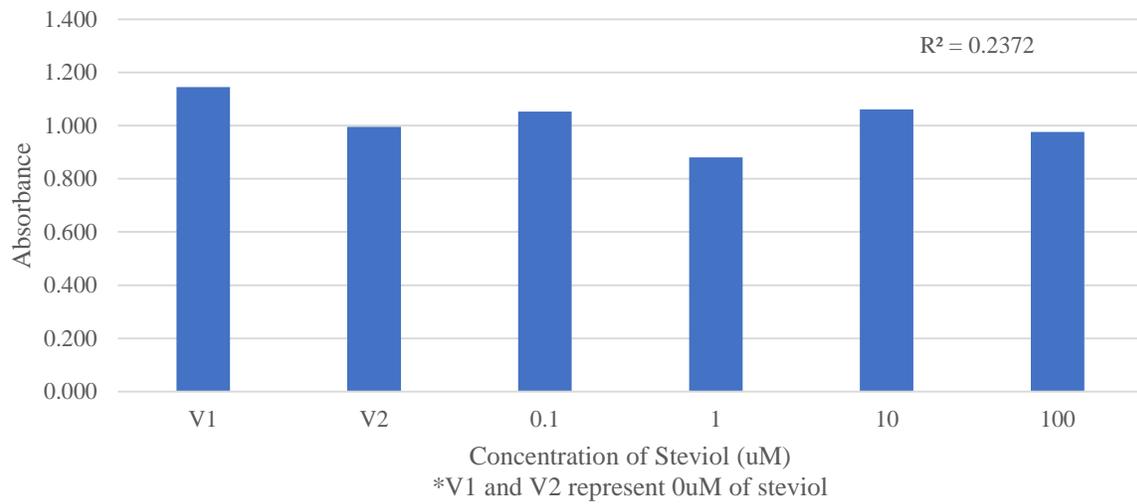


Figure 3: Day 9 Cell Density Measures via Spectrophotometry Readings

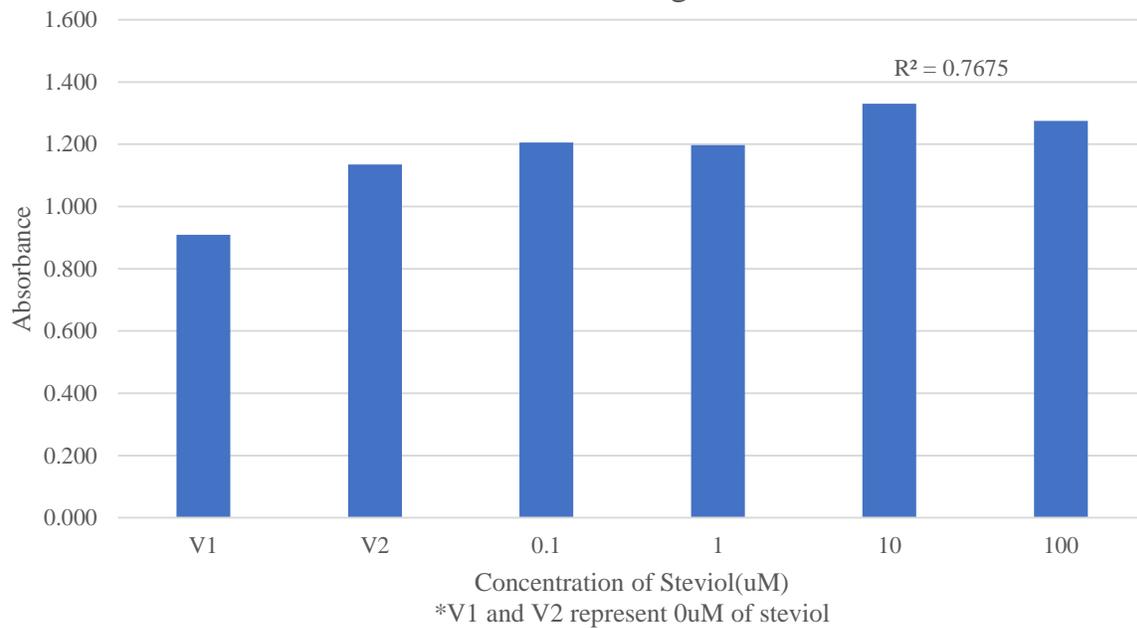


Figure 4: Day 3 Alkaline Phosphatase Concentrations

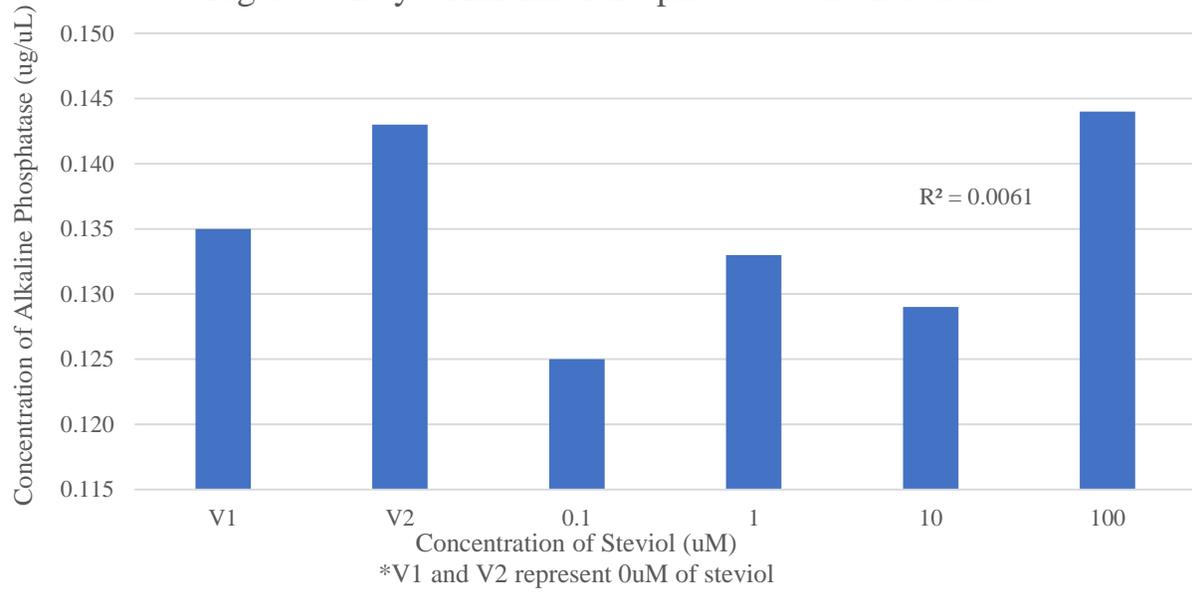
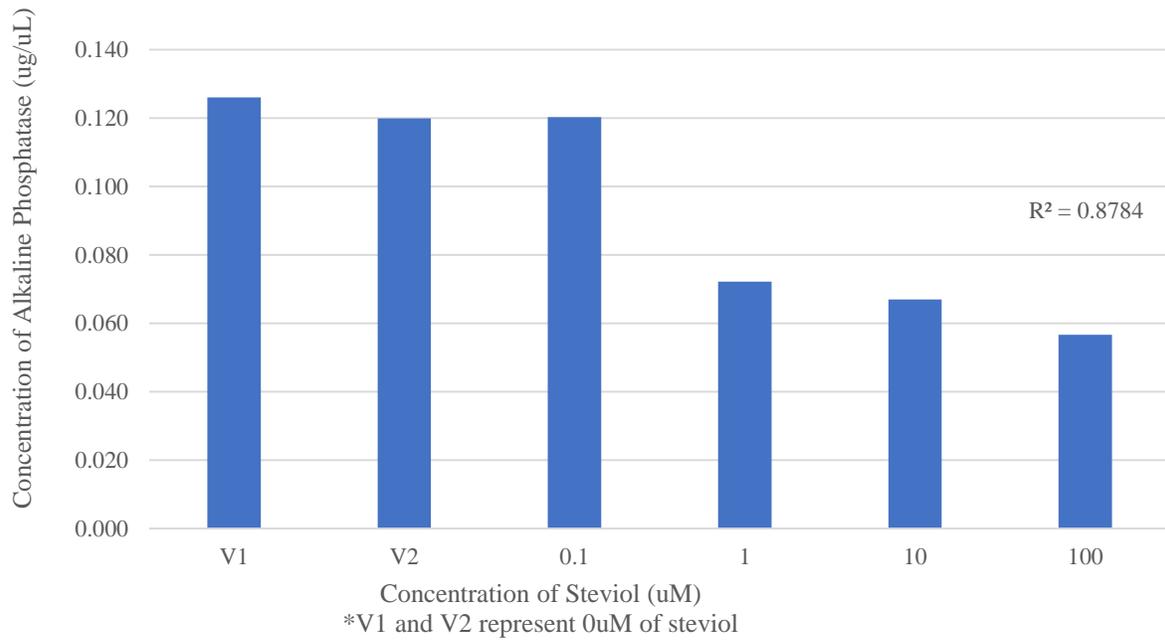
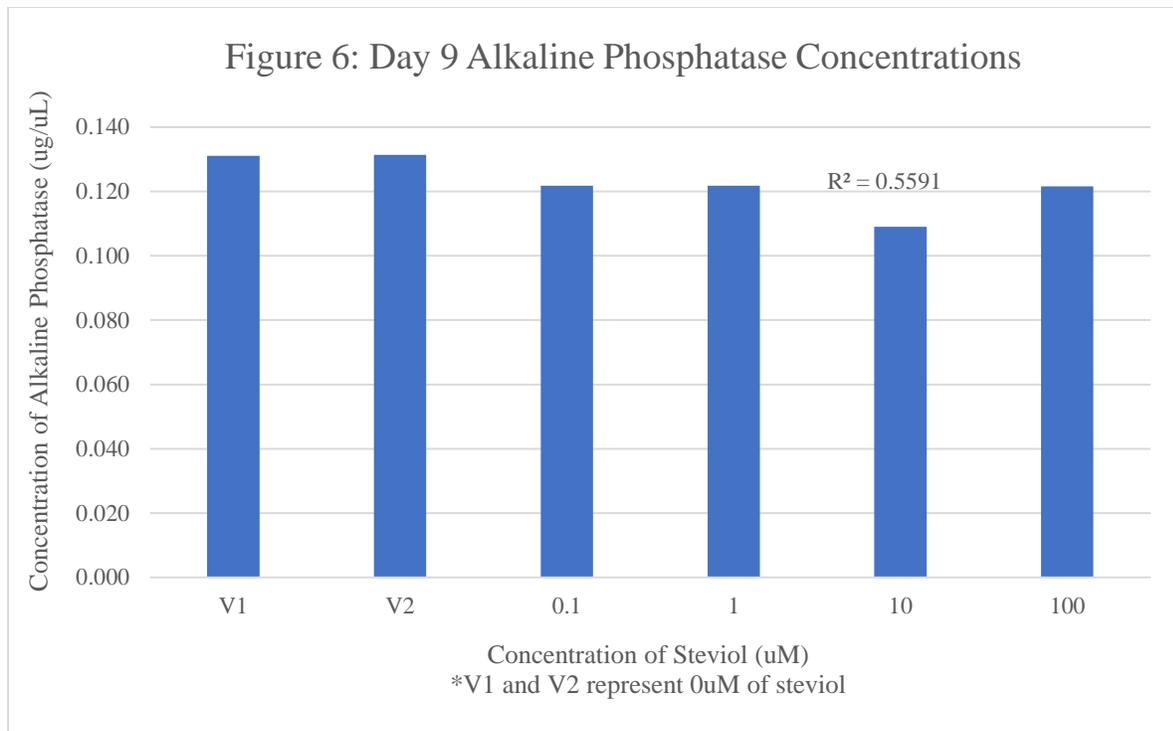


Figure 5: Day 6 Alkaline Phosphatase Concentrations





The crystal violet staining and cell density readings (Figures 1-3) exemplify inconsistent data that suggests that steviol did not have significant proliferative effect on the osteosarcoma cells. Figure 3 for the cell density of the wells at day 9 did, however, demonstrate a visible trend of increasing cell number with increasing concentration but the correlation was slight ($R^2=0.7675$) but worth noting as several other trials showed the same vague trend. The reading for cell density on the second trial is especially noteworthy as the data obtained correlation values of $R^2=0.9643$, $R^2=0.9041$, $R^2=0.7643$, for day 3, 6, and 9 respectively—all relatively high values and suggestive of a treatment effect. However, these results were inconsistent and not reproducible in other trials which mitigates any observable trends in trial 2. For the protein assay, a standard curve of samples with known concentrations allowed the absorbances of the assay to be converted to actual protein concentrations. The alkaline phosphatase spectrophotometry readings were divided by its corresponding concentration from the obtained protein assay to calculate a

measure of alkaline phosphatase concentration per total protein concentration. These alkaline phosphatase levels were additionally averaged out for individual days and treatment conditions and graphed out for Figures 4-6. The day 3 alkaline phosphatase levels varied considerably from each treatment condition in no particular direction. However, day 6 presents more stable variability which a noticeable decrease in enzyme levels with concentrations indicating a possibility of a negatively correlated treatment effect ($R^2 = 0.88$).

To further analyze whether differences in alkaline phosphatase concentration and cell density between the varying steviol concentrations were significant enough to support the notion of a treatment effect, an analysis of variance was performed. The differences between the two controls were not statistically significant enough to warrant reason to believe that the tenfold ethanol concentration in 'very high' concentration is enough to cause a treatment effect independent of the steviol. This allows for appropriate comparison of all the treatment conditions to each other and to any of the controls. Cell density in the wells of the trays naturally increased with the progression of days but an actual change within treatment groups was sparsely found throughout each individual day and was more prominent on day 9. Nonetheless, the data was significant enough ($p = 2.0 \times 10^{-4}$) in day 9 to provide evidence that counters Lopez et al. and his findings on the antiproliferative effects of steviol. Variance between treatment groups in the alkaline phosphatase assays were much more notable with day 6 demonstrating a treatment effect that negates the aforementioned the results of steviol on cell density—a decrease in alkaline phosphatase ($p = 5.38 \times 10^{-24}$). Results were inconsistent among the day 3, 6, and 9 but do not severely contradict the evidence that steviol could be associated with increased alkaline phosphatase concentrations.

Discussion: Given that the studied cells are bone tumor cells, alkaline phosphatase serves as an important indicator of their health and activity. This enzyme is particularly prominent in bone cells as it is released when osteoblasts differentiate into osteoclasts; therefore, it is associated with bone cell differentiation and proliferation⁶. It would be anticipated that the osteosarcoma cells would observe relatively higher levels of alkaline phosphatase and the control group of the experiment—vehicles 1 and 2—serve to simulate a baseline of the culture's enzyme levels. A statistically significant lowering of alkaline phosphatase levels was observed in the cells after 6 day of treatment. On the other hand, the results for day 3 is unsurprisingly insignificant which can be explained by the lack of longevity of exposure to steviol. On day 9, the data was still statistically significant but less exaggerated than the day 6 results perhaps because the cells became less responsive with continued exposure. There is an ultimate decrease of alkaline phosphatase levels with an increase in concentration of steviol which partly corroborates the research of Lopez et al. which theorized that steviol selectively inhibits CDK4 activity in cancer cells, reducing their division. If such a mechanism was true, then the lowering of alkaline phosphatase in cells exposed to stevia extract and its glycosides would be anticipated. However, this biochemical mechanism comes to question when addressing the results for the amount of cell density in the wells, which appeared to increase with higher exposure to steviol by day 9. Given the low p value of the variance between the control and the treatment groups, the results are hard to ignore and though they do not completely negate the possibility of steviol's cytotoxicity to cancer cells, they do negate the suggested mechanism of Lopez et al. Since the results suggest that steviol slowed the differentiation of the osteosarcoma cells, the drug might have some role in slowing mutations that would otherwise further increase mutation rates of cancer cells. This might be due to the fact that stevia extract has theorized by many studies to

have antioxidant properties². Antioxidants reduce the presence of free radicals from the body and since free radicals have the potential to damage DNA, they also contain the ability to disrupt gene expression in ways that increase abnormal differentiation of cancer cells. Therefore, the steviol may have reduced the alkaline phosphatase levels of the osteosarcoma cells by eliminating free radicals that were stimulating rampant differentiation in the control group. However, several studies have recently come out stating that antioxidants might play a role in increasing cancer metastasis—that is the growth and spreading of cancer cells⁷. The mechanism that was posed to explain this was that the free radicals removed by antioxidants may also be creating an environment of oxidative stress that inhibits their growth⁸. Consequently, antioxidants are beneficial to the healthy but can be fatal to those who already have tumors. Therefore, the effects of steviol on the rat osteosarcoma can be more readily explained by its antioxidant properties as opposed to the suggested CDK4 inhibitor properties not only because it explains the seemingly contradictory effects but also because steviol is more widely recognized as an antioxidant than it is an a CDK4 inhibitor. Furthermore, attributing the results to antioxidant properties provides an explanation as to how the osteosarcoma cells proliferated without differentiating much; eliminating free radicals reduced genetic damage of cells resulting in less differentiating but also reduced the oxidative stress necessary to suppress the cancerous cells. Essentially, the cells proliferated with less differentiation than what is normally anticipated in cancer cells. However, it is important to note that this study was done on a microscopic scale on rat cells—though the concentrations were chosen to simulate exposure to steviol through normal consumption, no definitive conclusions can be made regarding this study on humans or even live rats. The sweetener has been widely used after its classification as safe in 2008 by the FDA and there has been no adverse effects of its large-scale use. Nonetheless, these results

should be enough to urge other researchers to attempt to reproduce the results of the study through varying conditions to attempt to isolate the effects of steviol on cancer cells. Whether the change can be attributed to antioxidant properties, CDK4 inhibition, or any other potential mechanism, any compound that can alter the biochemical activity of cancer cells is worth exploring when there is a high demand for research in the field of cancer treatment.

Conclusion: There is no reason to believe that the results from the exposure of rat osteosarcoma cells to the glycoside of stevia extract should discourage or encourage consumption of the sweetener. The sweetener is a putative non-glycemic natural sweetener that does not have any of the appetite-stimulating effects as artificial sweeteners such as aspartame and erythritol do. Therefore, it provides the 9% of Americans living with type 2 diabetes to carry out their diets with only a simple substitution. In the meantime, studies on stevia extract and steviol warrant inclusion into the field of medical research pertaining to cancer treatments. Being that 38.4% of the entire population will get cancer at some point in their lives, this affliction is an epidemic that is difficult and currently expensive to treat. Though natural remedies often hold stigmas, there is no reason not to explore an option even if its effects are minimal. If steviol can decrease differentiation of cancer cells—as demonstrated by the reduced alkaline phosphatase in the osteosarcoma cells—than it can slow down the progression and damage that can be done by cancer cells in those already diagnosed. Furthermore, to combat its proliferative effects, the natural compound could be synthetically altered to reduce differentiation without encouraging further division of cells. Whether or not these options hold any merit, it nonetheless opens the discussion up to future researchers in hopes of controlling the outcomes of one of the most rampant and merciless diseases.

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