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 ($p = 0.0002$

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 properties[2]. 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 assays[3]. 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 cells[4]. 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 rats

protein assay was also performed on the 24-well tray using the BioRad DC Protein Assay Kit. 10 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 L of reagent ‘S’ in a vial and 25 L of this solution was placed with the wells with either the standard curve or treatment conditions.

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 resultswere pe[re)7(sult)ty of

Figure 2: Day 6 Cell Density Measures via Spectrophotometry Readings

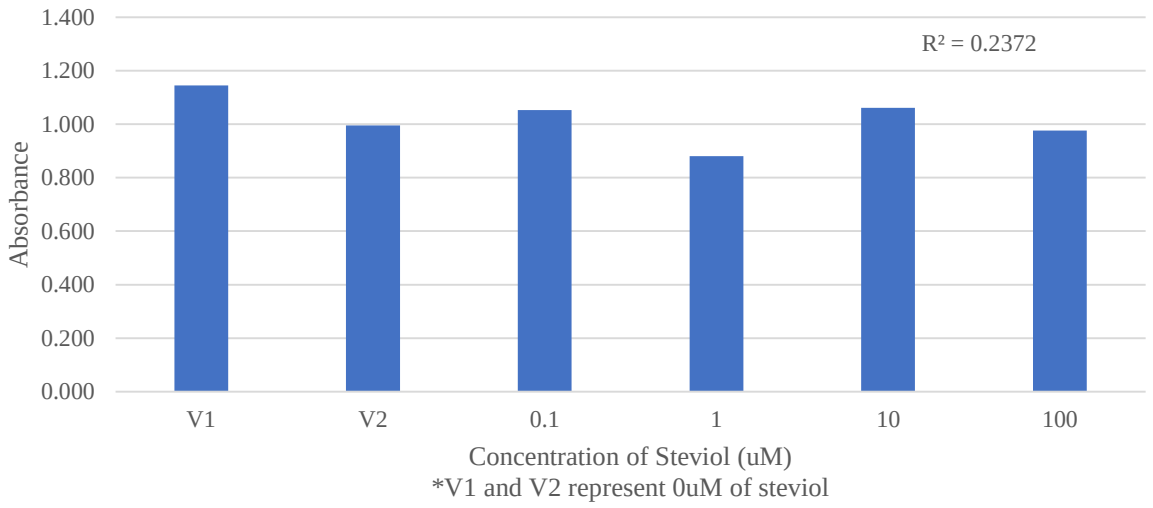


Figure 3: Day 9 Cell Density Measures via Spectrophotometry Readings

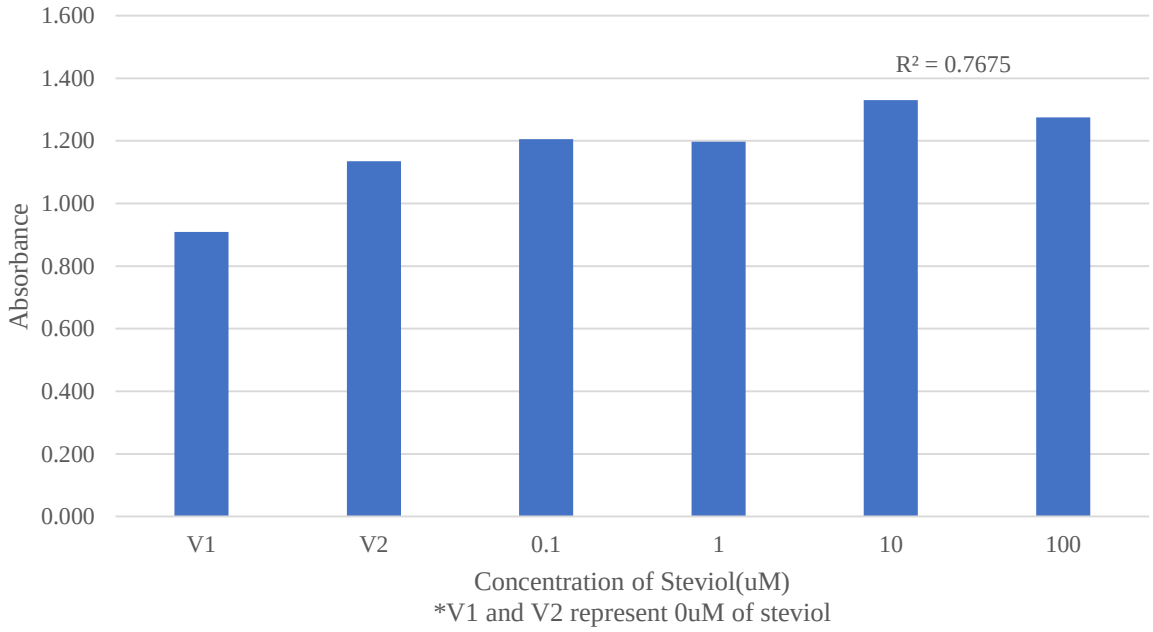


Figure 4: Day 3 Alkaline Phosphatase Concentrations

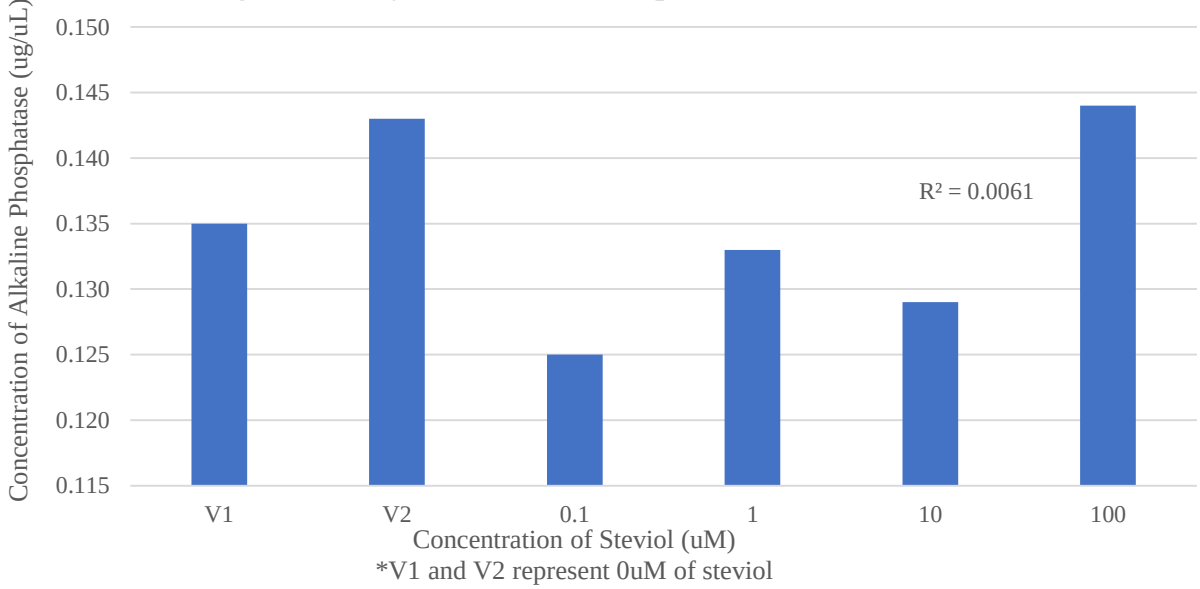
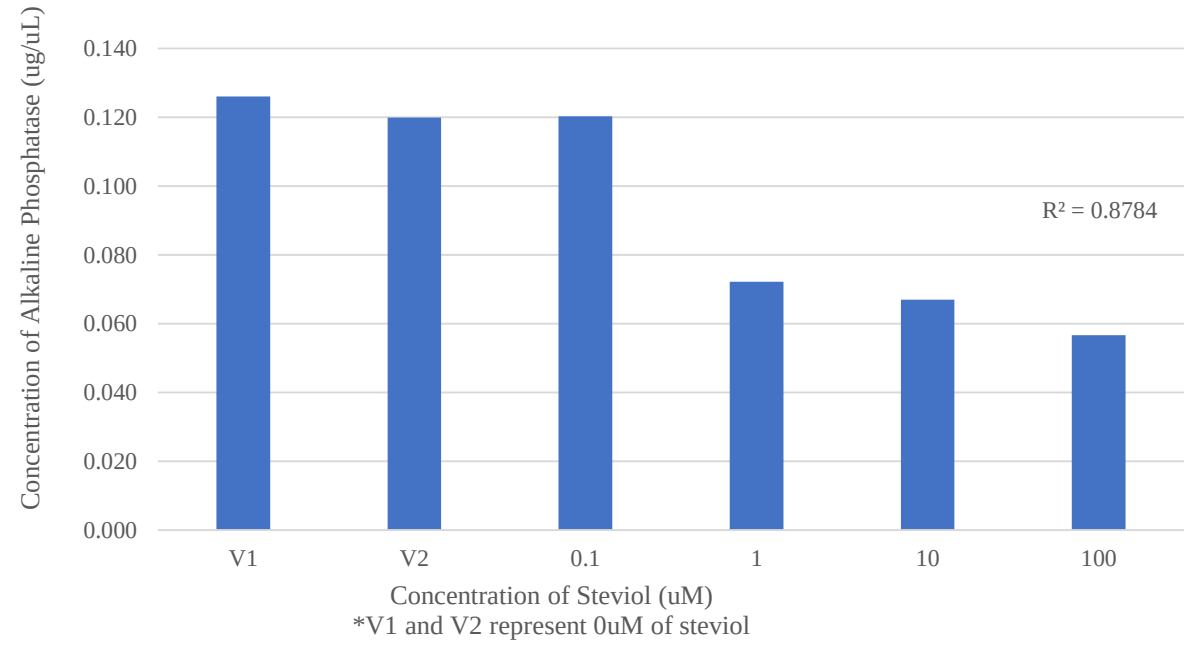
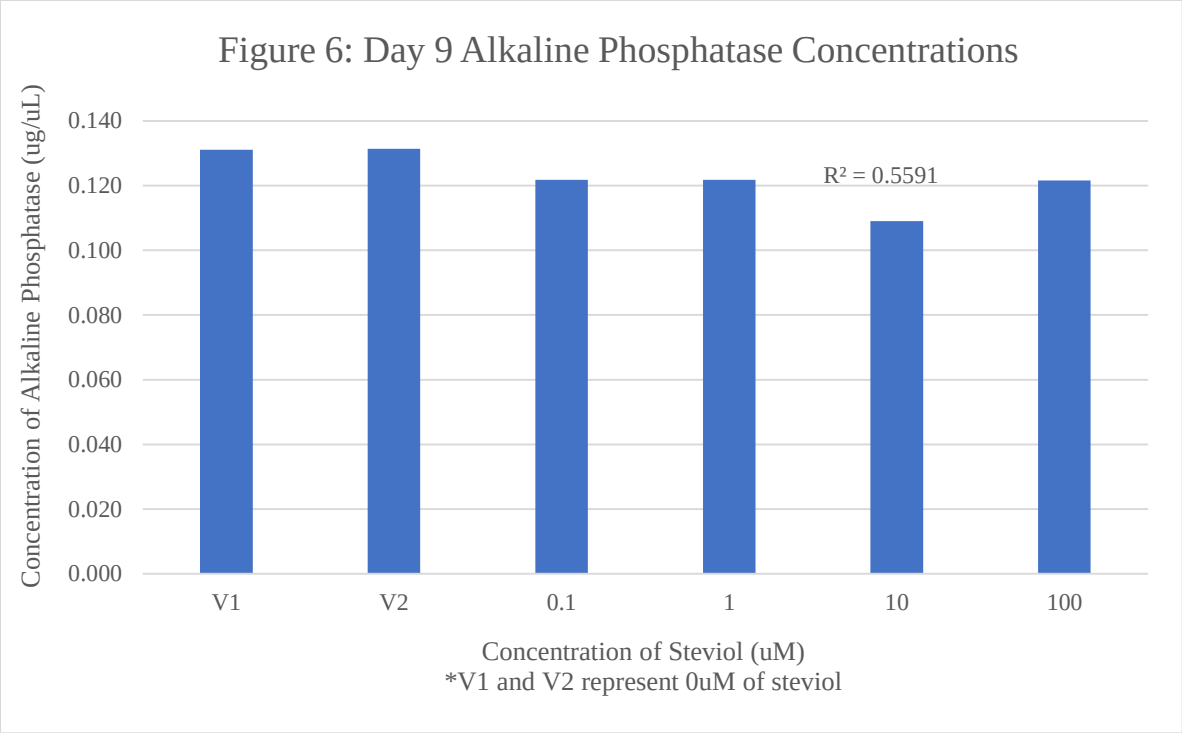


Figure 5: Day 6 Alkaline Phosphatase Concentrations





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

References:

[1] Abdullateef, R. A.; Osman, M. Studies on Effects of Pruning on Vegetative Traits in *Stevia rebaudiana* Bertoni (Compositae). *International Journal of Biology* 2011,