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

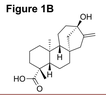
Eman Al-Yousefy & Thomas Owen (Faculty)
Ramapo College of New Jersey, Mahwah, NJ, 07430

## *Stevia rebaudiana*: Characteristics and Use

- Herbaceous plant in the sunflower family; leaves excrete glycosides with potent sweetness
- Its inability to be metabolized by humans makes it a desirable sweetener for the hyperglycemic
- Categorized by the FDA as 'generally recognized as safe'
- Nonetheless, stevia has only recently been researched rigorously

**Figure 1A**

**Figure 1B**

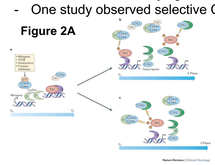



*Stevia rebaudiana* leaves (A) from which, steviol (B), the glycoside that is responsible for leaves' sweetness, resides

## Potential Effects of Stevia Extract and Steviol on the Biochemistry of Cancer Cells

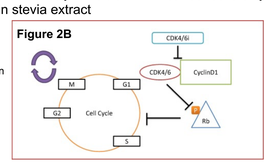
- Antioxidant effects strongly linked to polyphenol-rich content of stevia extract
- Polyphenols have been speculated to be cytotoxic to cancer cells
- Polyphenols can often demonstrate CDK4 inhibitory effects
- Current research is trying to find a compound that can selectively inhibit CDK4 in cancer cells only
- One study observed selective CDK4 inhibitory effect in stevia extract

**Figure 2A**



CDK4: protein that regulates G1 and S phase of cell cycle—less CDK4 means less cell proliferation
Selective CDK4 inhibitors have been sought after for treatment of cancer cells because they can demonstrate cytotoxicity in cancer cells

**Figure 2B**

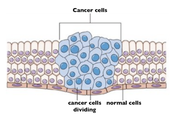



- Research is promising but results are inconsistent among numerous studies
- Otherwise, most literature on stevia extract and steviol support notion of its safety for consumption

## Objective:

- To use alternative methods of screening cancer cells and attempt to discover any effects of steviol on the growth differentiation of rat osteosarcoma cells
- Cell proliferation: cancer cells tend to proliferate at faster rate than normal cells; a measure of quantifying and comparing cell growth in cells treated with steviol will provide further insight into the merit of previous findings of antiproliferative effects in steviol-treated cells
- Cell growth and differentiation: cancer cells exhibit abnormal growth and differentiation; in bone cells, alkaline phosphatase enzyme levels act as an indicator of bone health as the enzyme is released when osteoblasts differentiate

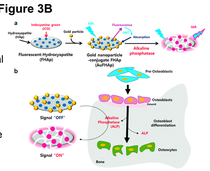
**Figure 3A**




(A) Cancer cells often contain mutations that prevent the cell cycle from stopping after several rounds of division. Mutations also result is abnormal differentiation which can damage surrounding cells

(B) In bone cells, alkaline phosphatase is released when osteoblasts differentiate into osteocytes. Normal cells release typical quantities of the enzyme but when afflicted with cancer, they release copious amounts

**Figure 3B**




## Methods and Materials

- Cultured rat osteosarcoma cells were examined in twelve 24-well trays over 9 days with varying exposure to steviol dissolved in ethanol
- Six conditions, two control groups*:
  - V1: 10 μM EtOH
  - V2: 100 μM EtOH
  - L: 0.1μM steviol
  - M: 1μM steviol
  - H: 10μM steviol
  - VH: 100μM steviol

  *Final concentrations in MEM alpha with 10% fetal bovine serum
- Trays from each condition were harvested on days 3, 6, and 9 and assayed for overall protein and alkaline phosphatase, and quantified via spectrophotometry (405nm for ALP and 700nm for protein)
- Protein assay and alkaline phosphatase values used to quantify ALP relative to remaining proteins
- Trays to be measured for cell density were fixed in methanol, stained with crystal violet; absorbance was read at 570nm to quantify relative cell density between treatment groups.

## Results: Cell Density

Figure 4A: Day 3 Cell Density Measures via Spectrophotometry Readings

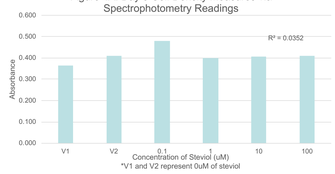



**Figure 4A**: cell density does not appear to be responsive to higher concentrations of steviol with low correlation value (Pearson's $R^2$)

**Figure 4B**: Anti-proliferative effects cannot be observed in data set for Day 6. Results suggest that the Ros cells may have required extended exposure to exhibit a treatment effect, if any

Figure 4B: Day 6 Cell Density Measures via Spectrophotometry Readings

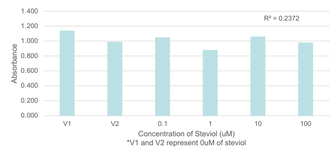



Figure 4C: Day 9 Cell Density Measures via Spectrophotometry Readings

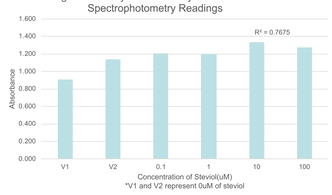



**Figure 4C:** Notable positive correlation ($R^2$=0.77) between concentration of steviol and cell density that is statistically significant (p=0.0002). Suggests proliferative effect of steviol on cancer cells but data is inconsistent with previous literature and alkaline phosphatase assay results

## Results: Alkaline Phosphatase Concentrations

Figure 5A: Day 3 Alkaline Phosphatase Concentrations

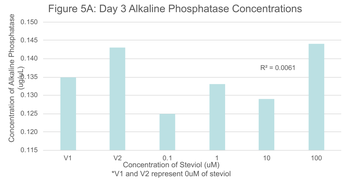



**Figure 5A:** cells exhibit response to treatment in no particular trend.
Low correlational ($R^2$) values and p>0.05; could be attributed to high responsivity of cells early in treatment that has little to do with drug treatment

**Figure 5B:** Strong response to treatment with alkaline phosphatase concentrations exhibiting a decrease in response to increasing steviol concentrations
High correlation and p=5.38*10$^{-24}$ suggest strong evidence of treatment effect

Figure 5B: Day 6 Alkaline Phosphatase Concentrations

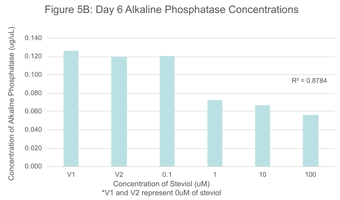



Figure 5C: Day 9 Alkaline Phosphatase Concentrations

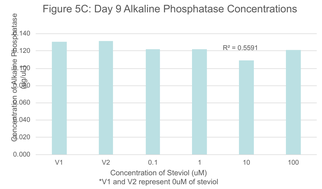



**Figure 5C:** Cells are barely responsive to treatment at the end of observation period; perhaps due to decreased responsiveness of cells with longevity of exposure

## Discussion: Does Steviol have an Effect On the Growth and Differentiation of Cancer Cells?

- Results are not surprising, with alkaline phosphatase assay suggesting less differentiation while measures of cell density suggest proliferation of osteosarcoma cells
- Differentiation needs to decrease in order for cells to proliferate, hence the inverse relationship between alkaline phosphatase and cell density
- An interesting observation was also made in the first two trials for cell density as pictured in Figure 6

**Figure 6**

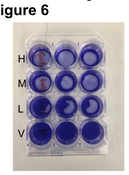



Figure 6: shown on left is a photo of an earlier trial, with only four treatment conditions (vehicle, low, medium, high concentrations).
Day 9 cells following fixation in methanol and staining with crystal violet
Cells appeared to 'slough' off the cell culture wells with no evidence of contamination—effect was more prominent in cells treated with higher concentrations of steviol
Observation was dismissed as potential error but worth noting given the visible trend in steviol concentration and remaining presence of cells
*Note that this effect was not observed in trial for which the data is presented

- A statistically significant treatment effect between higher concentrations of steviol and the lowering of alkaline phosphatase is consistent with the theory that the polyphenols in stevia extract inhibits selective CDK4 activity resulting in cytotoxicity to cancerous cells
  - Unfortunately, the aforementioned cell density data makes it difficult to make any definitive statements in support of this theory because it does not fully explain the results of this research
- Another possible mechanism to explain the results is from the antioxidant properties of steviol

**Figure 7**

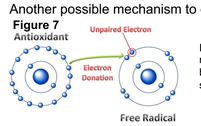



Figure 7 shows how antioxidants can reduce free radicals in cellular systems by donating single electrons to stabilize an unpaired electron

- Free radicals can damage the DNA of cells causing mutations that may turn into cancerous cells that grow and differentiate abnormally
- If the antioxidant behavior of steviol reduced the free radicals that were stimulating rampant differentiation in the control group, it can explain the reduction of alkaline phosphatase in the cells exposed to higher concentrations of steviol
- Antioxidants have also been found by previous studies to increase metastasis of cancer cells by reducing the oxidative stress that typically suppresses cancer growth
- Therefore, eliminating free radicals can reduce any genetic damage on the cells resulting in less differentiation while reducing the oxidative stress necessary to suppress the cancerous cells

## What does this mean for the industry?

- It is important to remember the microscopic scale of this study:
  - no definitive conclusions can be made regarding this study on humans or even live rats.
- Growing epidemic of type two diabetes—9% of Americans—linked to sugar consumption
  - Stevia extract is just one of the ways this issue can be mitigated
- However, rigorous research has revealed hidden side effects of artificial sweeteners
  - Aspartame and erythritol have been linked to increased appetite which can further increase risk factors of diet-related afflictions associated with over-consumption
- Results of study should not cause concern: stevia extract is a natural non-glycemic sweetener but consumption should be kept in moderation as research in this industry is still lacking

## Citations and Acknowledgments

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Antioxidant
Unpaired Electron
Electron Donation
Free Radical