Evaluation of anti-cancer effects of root and leaf extracts of Beet (Beta vulgaris L.) in cervical cancer cells (HeLa)

Stefhani A. Romero[1], Isadora C. B. Pavan[2], Ana Paula Morelli[1], Luiz Guilherme S. da Silva[1], Isabella Fagundes[1], Cayo H. R. Silva[1], Luis Gustavo Saboia[1], Mariana Camargo Silva Mancini[1], Rosângela M. N. Bezerra[1], Fernando M. Simabuco[1].

[1]Multidisciplinary Laboratory of Food and Health (LabMAS), School of Applied Sciences (FCA), University of Campinas (UNICAMP), Limeira, São Paulo, Brazil;

[2]Laboratory of Signal Mechanisms, School of Pharmaceutical Sciences (FCF), University of Campinas (UNICAMP), Campinas, São Paulo, Brazil;

Stefhani Andrioli Romero: CPF: 349.536.898-10 / Email: stefhani.andrioli.r@gmail.com ou teti-romero@hotmail.com /Telefone: (19)98103-3615 / Instituição: Faculdade de Ciências Aplicadas – UNICAMP / ORCID Stefhani Andrioli Romero: https://orcid.org/0000-0003-1729-0852

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1. INTRODUCTION

Cancer was described as the cause of about 9.2 million deaths worldwide in 2018[1] and represents in Brazil the second largest cause of death. Among the cancers, cervical cancer is ranked third with 8.1 % incidence among cancer cases that affect women in 2018[2]. Beetroot contains in its composition bioactive compounds capable of inhibiting the advance of different types of cancer cells, such as apigenin and its derivatives, through the regulation of the cell cycle, decrease in the growth, and induction of apoptosis in some cell lines[3,4]. The present study analyzed the possible functional effects of extracts of leaves and roots of beet and apigenin as anti-tumor agents in cultures of HeLa cervical cancer cells.

Literature demands more information regarding the effects of beet leaf and root on cancer cells, here we explored the anticancer potential present in the leaf and root extracts derived from beet in cervical cancer cells.

2. RESULTS AND DISCUSSIONS

2.1. Cell viability assay (MTT) and Clonogenic assay

The results demonstrated decreased cell viability of cervical cancer cells (HeLa) when treated with leaf and beetroot extracts. The reduction was evidenced in the concentrations of 10ppm and 100ppm (A and B). The viability after treatment with extracts at 10 ppm combined with anti-cancer drugs rapamycin and cisplatin was also analyzed, obtaining a greater decrease in viability in the combined extracts compared to cells treated only with the drugs (C and D). In the analysis of colony formation (Figure 2), the cells treated with concentrations of 1 and 10 ppm did not present a statistical difference regarding quantity, but a difference in the diameter of the colonies was observed, demonstrating the decrease of the proliferation.

Figure 1. **Cell viability analysis of cervical cancer cells after beet derived extracts treatment.** HeLa cells were seed at a density of $8x10^3$ in 96-well plates and treated with the extracts at 1, 10 and 100ppm concentration for 24 and 48 hours. (MTT; n = 3); the bars represent the mean ± SEM.

Significantly different from the one-way Anova, post-test Turkey vs. control (* p <0.05; ** p <0.01; *** p <0.001; **** p <0001). All treatments in figures 1C and 1D decreased compared to the control, except for cisplatin for 24 hours.

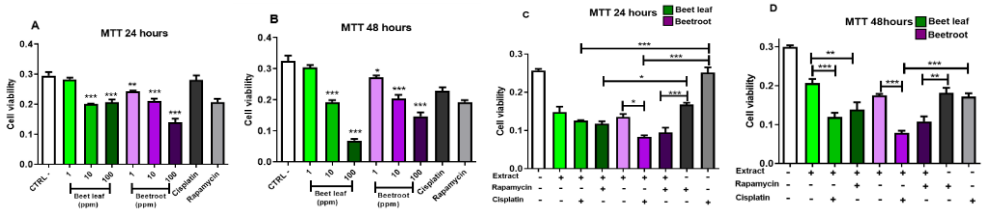
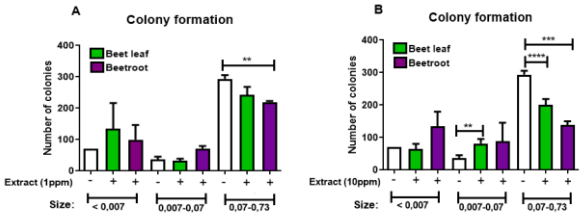


Figure 2. Analysis of colony formation. HeLa cells were seeded at a density of 5×10^2 in p-60 plates and treated with the extracts at 1 and 10 ppm concentration for 10 days, treating and after 2 days, aspirating and treating again until 10 days.(n = 3); the bars represent the mean ± SEM. Significantly different from that shown by the one-way ANOVA, post-test Multiple comparisons versus control (* p <0.05; ** p <0.01; *** p <0.001; **** p <0001) 10 days with a concentration of 1 ppm and 10 ppm.

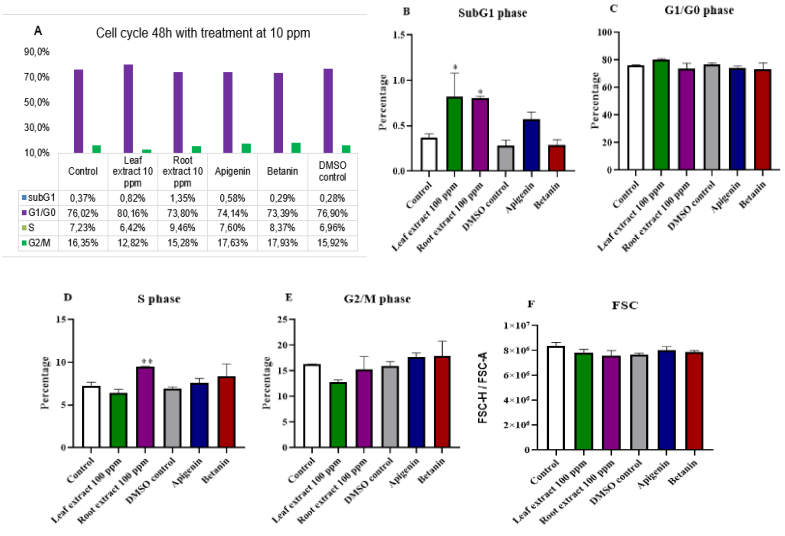


2.2. Cell cycle analysis

Cells were treated with 10 and 100 ppm of beet leaf or root extracts, apigenin and betanin were used at a corresponding concentration from each extract respectively. Here we reported that 10 ppm of beetroot extract accumulated cells in the SubG1 phase (figure 3B), characterizing apoptotic cells with fragmented DNA, besides cell cycle arrest in S phase (figure 3D). Similarly, apigenin led to S and G2/M arrest. Furthermore, 100 ppm of root and leaf extracts increased SubG1 cells (figure 4B), while decreased G1/G0, S, and G2/M cells (Figure 4 C, D, and E).

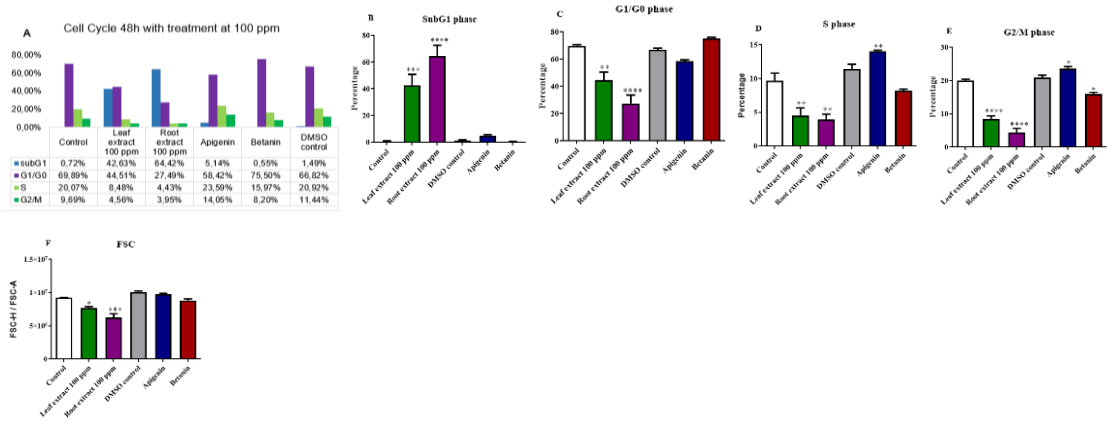
A decrease in FSC (decrease in cell size) in treatments with leaf and root extracts at 100 ppm (figure 4F) was also observed, which, according to a study by Petit et al., (1995)[5], correlates with the increase in apoptotic cells, as detected in the SubG1 and S.

Figure 3. Analysis of cell cycle after Beetroot and leaf extracts treatments at 10 ppm, as well as the isolated compounds apigenin and betalain. Hela cells were seeded at a density of 3×10^5 in 6-well plates and treated with the extracts at 10 ppm concentration for 48 hours (n = 3), or isolated apigenin and betanin in the concentrations present in the leaf and root extracts control DMSO. Cell cycle phases are represented in graphs A (all phases), B (subG1), C (G1 / G0), D (S), and E (G2 / M). Results regarding cell size are presented in graphs F (FSC).



	Control	Leaf extract 10 ppm	Root extract 10 ppm	Apigenin	Betanin	DMSO control
subG1	0,37%	0,82%	1,35%	0,58%	0,29%	0,28%
G1/G0	76,02%	80,16%	73,80%	74,14%	73,39%	76,90%
S	7,23%	6,42%	9,46%	7,60%	8,37%	6,96%
G2/M	16,35%	12,82%	15,28%	17,63%	17,93%	15,92%

Figure 4. Analysis of cell cycle after Beet root and leaf extracts treatment at 100 ppm, as well as the isolated compounds apigenin and betalain. Hela cells were seeded at a density of 3×10^5 in 6-well plates and treated with the extracts at 100 ppm concentration, apigenin 0.78 ppm, and betanin 10,900 ppm, for 48 hours (n = 3), or isolated apigenin and betanin with respectively concentrations present in the leaf and root extracts. Cell cycle phases are represented in graphs A (all phases), B (subG1), C (G1 / G0), D (S), and E (G2 / M). Results regarding cell size, after the treatments mentioned are presented in graphs F (FSC).

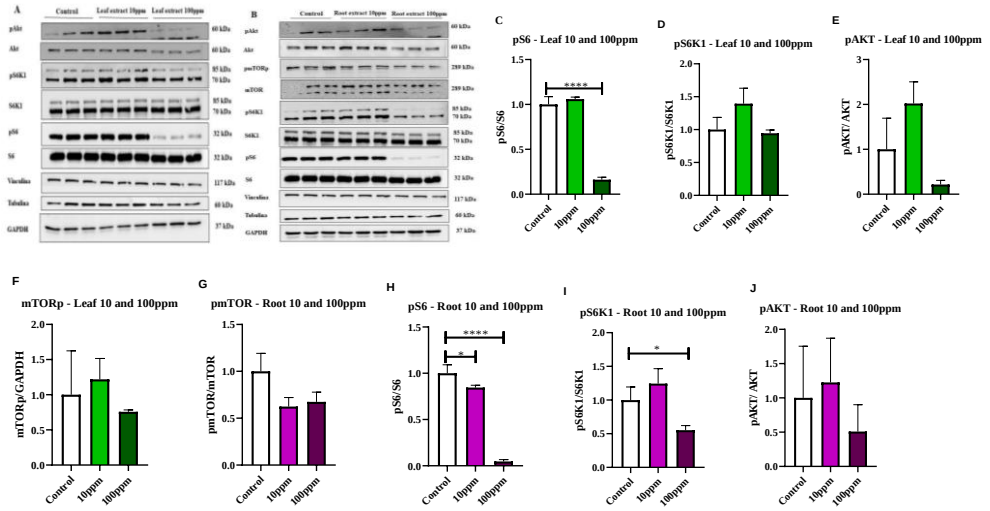


2.4. Analysis of signaling pathways by *Western blotting*

The treatment of leaf at a concentration of 100 ppm decreased S6 phosphorylation (figures 5A and C), as long as beetroot at 10 and 100 ppm. Furthermore, 100 ppm of the root extract decreased S6K1 phosphorylation (figures 5B, H, and I). We observed a trend in reducing phosphorylation of mTOR (figures 5 F and G) and Akt with both extracts, however, there was no statistical difference.

Schwarz et al. (2012)[6] showed that the activation of the PI3K/Akt pathway, proteins upstream to mTOR, S6K, and S6, is associated with the inefficient metabolic response of cervical cancer to chemoradiation, suggesting that inhibition of this pathway may improve therapeutic treatment for this type of cancer.

Figure 5. Western blotting analysis of cell growth signaling pathways. Hela cells were seeded at a density of 6×10^5 in 6-well plates and treated with the extracts at 10 and 100 ppm concentration for 24 hours (n = 3).



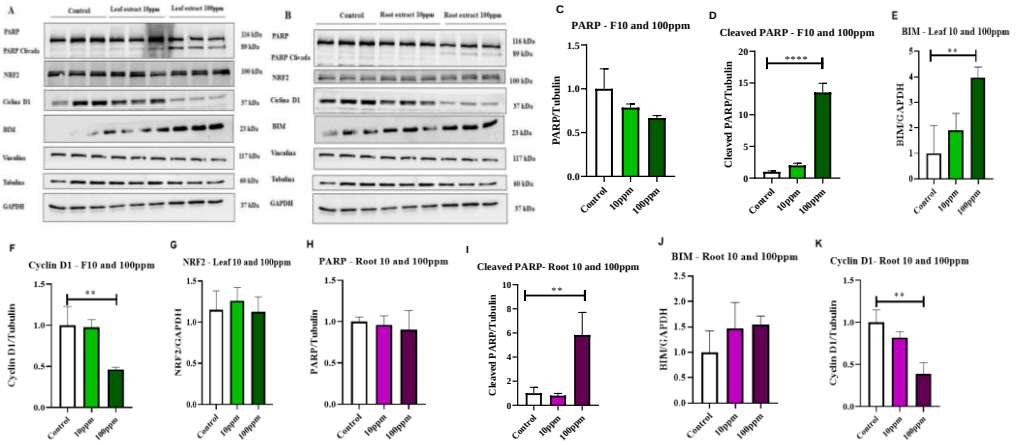
Other cell signaling pathways were evaluated, such as pathways related to apoptosis and cell survival, oxidative stress, and the cell cycle (figure 6). Both extracts at a concentration of 100 ppm were able to increase the cleavage of the PARP1 protein (figure 6 A, B, D,

and I). A study showed that the cleavage of PARP1 lead to anti-migration and anti-invasion properties in cervical cancer cells, increasing cell death regardless of anchorage and inducing anoikis (Prasad et al., 2017)[7].

There was also an increase in the content of the pro-apoptotic Bim protein under treatment with beet leaf on 100ppm concentration (figures 6 A and E). A tendency towards an increase in Bim was observed in the treatment of 100 ppm of the root extract, however, there were no statistical differences.

In addition, we also observed a reduction in cyclin D1 expression, a key regulator of cell cycle progression, in the treatment of 100 ppm of leaf and root extract, indicating cell cycle arrest (figures 6 A, B, F, and K). In both treatments, no changes were observed in NRF2 protein expression.

Figure 6. Western blotting analysis of apoptosis, cell cycle, and oxidative stress pathways. Hela cells were seed at a density of 6×10^5 in 6-well plates and treated with the extracts at 10 and 100 ppm concentration for 24 hours (n = 3).



3. CONCLUSIONS

This study claims to the effectiveness of bioactive compounds present in extracts against cancer cells, reducing cell viability in MTT assay, and decreasing cell colonies' diameter in the clonogenic capacity assay. Moreover, changes in the cell cycle were detected, indicating cell cycle arrest and apoptosis. At the molecular level, changes in signaling pathways were observed changes in proteins of the mTOR signaling pathway, which included reduced phosphorylation of S6 and S6K1. Furthermore, increased cleavage of PARP1 and increased levels of BIM were detected, meaning apoptosis induction, and decrease of cyclin D1, which regulates the progression of the cycle. This study may contribute to new therapeutic and adjuvant approaches for cervical cancer treatment using beet derived compounds and extracts.

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