Research Article

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Ruta Graveolens in Homeopathic Dilution Has *In Vitro* Antitumor Activity in Osteosarcoma Cells (U2OS) and Low Effect on Canine Mesenchymal Stem Cells (CTM)

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ABSTRACT

Ruta graveolens belongs to the family Rutaceae, and it is used to treat inflammatory diseases and some cases of cancer due to its anti-inflammatory and analgesic properties. Osteosarcoma (OS) is a bone cancer that mainly affects children and adolescents and has a high index in dogs. The treatment of this cancer consists of high doses of chemotherapy, and resection of the area affected by cancer, thus making it a painful and invasive treatment for patients. Under this perspective, it is necessary to search for new forms of treatment that are less invasive and, at the same time, effective, considering that homeopathy is used as a complementary therapy and benefits the patient's quality of life. This study aimed to compare the *in vitro* action of the homeopathic medicine Ruta D35 on U2OS osteosarcoma cells and canine mesenchymal stem cells (MSC). Our results demonstrate that Ruta D35 has a toxic effect on U2OS cells (IC50 16.38µL/mL), reducing cell viability compared to MSC (IC_{50} 67.38µL/mL). Therefore, Ruta D35 showed positive results in treating the OS U2OS line, demonstrating a potential activity in treating this cancer.

Keywords: Cancer, Homoeopathy, Cell Viability.

INTRODUCTION

Osteosarcoma (OS) is a bone cancer that affects mainly children and adolescents, reaching a second peak of occurrence in the elderly above 60 years [1,2]. Although the origin of this cancer is unknown, it is believed that mutations in mesenchymal stem cells play a crucial role in the development of OS since these cells are related to bone, cartilage, and muscle development [3].

In dogs, OS is the most common bone tumor, with risk factors related to race, sex, trauma, and castration of these animals. Its characteristics include swelling and pain in the affected region and micrometastases [4]. Some similarities of OS in humans and dogs include high heterogeneity of tumor cells, tumor development mainly in the metaphyseal regions of long bones, a higher incidence in males than females, form of treatment, and metastasis capacity [5].

The treatment of OS in both humans and dogs is accomplished with high doses of chemotherapy. Resection of the area affected by cancer is often performed, making it a painful and invasive treatment for patients [4,6,7]. Given the high resistance rate in the OS due to treatment [8], the search for new cancer drugs becomes essential for treating the disease [9].

Homeopathic therapy can be given along with chemotherapy and works as a complementary therapy. Homeopathic treatment benefits the patient's quality of life and minimizes the side effects of conventional drugs used to treat the disease [10]. *Ruta graveolens* is among these commonly used homeopathic medicines.

R. graveolens belongs to the family Rutaceae and is distributed in the tropical and temperate regions. Due to its anti-inflammatory, antiandrogenic, and analgesic properties, this plant is commonly used to treat dermatological pathologies

[11,12], inflammatory diseases, and some cancer cases, in which *R. graveolens* has shown antiproliferative effect [13]. Psoralens are one of the chemical compounds found in this plant. They are furanocoumarins capable of cross-linking DNA that can lead to changes in cell metabolism and generate cell death [13].

Since *R. graveolens* may exhibit toxic effects on tumor cells and be used in cancer treatments, the objective of this study was to evaluate and compare the *in vitro* behavior of ultra-diluted *R. graveolens* (Ruta D35) in healthy canine cells (mesenchymal stem cells) and OS cells.

MATERIALS AND METHODS

Cell culture

The cell line osteosarcoma used in these tests, U2OS (ATCC® HTB-96™), was donated by the Biotechnology and Genomic Sciences laboratory from the Catholic University of Brasilia (purchased by the ATCC and grown according to the protocol). The cells were cultured with Dulbecco's Modified Eagle Medium (DMEM) added with 10% fetal bovine serum and 0.02% amikacin (all products from the Sigma-Aldrich® brand).

Canine mesenchymal stem cells (CTM) were obtained from the Bio cell laboratory and cultivated with DMEM low glucose added with 10% fetal bovine serum and 0.02% amikacin (all products from the Sigma-Aldrich® brand).

Preparation of Ruta D35

The Mother Tincture was used as the starting point for preparing the tested substance (Ruta D35). As described in the Brazilian Homeopathic Pharmacopoeia, the Hahnemannian Decimal Method was used. Using a sterile isotonic solution, 1 part of the active ingredient was mixed with 9 parts of the inert ingredient and succussed 100 times, yielding Ruta D1 (1×10^{-1}). Then, 1 part of Ruta D1 was used with 9 parts of the inert ingredient and succussed 100 times, yielding *R. graveolens* D2 (1×10^{-2}). The successive dilution continued till *Ruta* D35 was obtained. These products were then bottled in 1.1 mL ampoules.

Cytotoxicity (MTT assay)

Cells were plated in 75cm^2 culture flasks and incubated in a controlled atmosphere cell culture oven (37ºC, 5%CO_2) until they reached approximately 80% confluence. Cells were then trypsinized with the enzyme TryPLE™ (Thermo-fisher), counted, and plated in 96-well plates at a concentration of 2.5 x 10^4 cells/mL culture medium.

After 24 hours, the medium was replaced with a medium containing Ruta D35 at five concentrations (10, 20, 30, 40, and 50 µmL/mL), remaining for 48 hours in culture. This procedure was performed in triplicate of wells and plates. The control treatment utilized the same water used in the dynamizations, at the same five concentrations from 10 to 50 µmL of water per mL of medium. Also, control wells were used only with a culture medium, without adding any other product to confirm cell growth. After 48 hours, the MTT colorimetric assay was performed by adding the reagent 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (Catalog: Sigma N. M2128) at 0.5 mg/mL, incubation for 4 hours at 37ºC and 5% CO_2. Subsequently, the following steps were performed: dilution of the formazan crystals with DMSO (dimethyl-sufoxide) (Sigma Aldrich); plate absorbance reading at 570nm in a spectrophotometer (Model DR-200BS Kasuaki); and calculation of the mean inhibitory concentration (IC_{50}) of Ruta D35 in each cell line, i.e., a concentration that inhibits the growth of 50% of the cells in culture.

Statistical analysis

From the result obtained in the MTT assay, the mean of the triplicates referring to the inhibitory concentrations of 50% of the cells (IC_{50}) of each cell line was calculated. This result was analyzed by the T-Test using the GraphPad Prism® 7.04.

RESULTS

U2OS and CTM cells were treated with different concentrations of Ruta D35 (10 - 50µL/mL), and the results showed that cell behavior was different at all concentrations tested. The OS cells showed higher sensitivity to the medicine since even the concentration of 10µL/mL caused cell damage and reduced the viability of approximately 50% of the cells. At the highest concentration (50µL/mL), cancer cells presented a low cell viability index, whereas MSC cells remained with a high percentage of viable cells (Table 1). The two cell types had a dose-response effect. However, the highest concentration tested was not sufficient to disable most MSC cells, and approximately 60% of the cells were alive. On the other hand, at this same concentration of 50µL/mL, only 20% of the tumor cells remained viable.

Table 1: Mean U2OS and MSC cell viability after MTT assay with Ruta D35 treatment at concentrations of 10µL/mL - 50µL/mL.

Ruta D35 Concentration	% Cell viabillity U2OS (Mean ± DP)	% Cell viabillity CTM (Mean ± DP)
0	100	100
10µL/mL	54.934 ± 3.33	98.45 ± 5.677
20µL/mL	46.208 ± 1.439	88.20 ± 5.551
30µL/mL	41.168 ± 1.174	80.03 ± 5.686
40µL/mL	32.704 ± 2.061	70.63 ± 11.576
50µL/mL	21.524 ± 4.095	60.29 ± 10.393

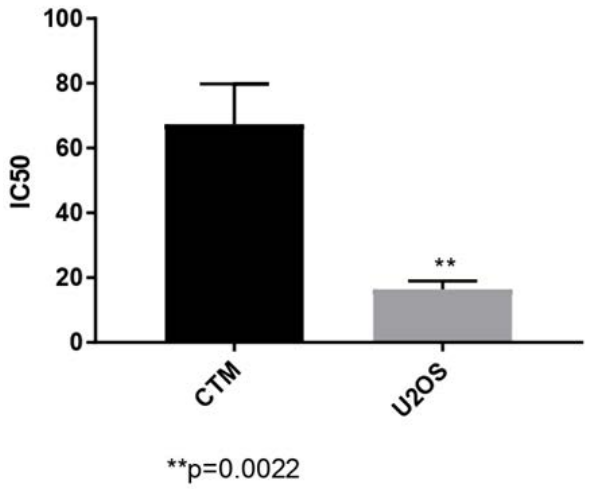

Figure 1. Inhibitory concentration of 50% of U2OS cells (osteosarcoma) and canine MSC (mesenchymal stem cells) after treatment with *Ruta graveolens*. The x axle represents the cell group, black bar – MSC; gray bar – U2OS. The y axle is the IC_{50} corresponding value. The IC_{50} in U2OS was 16.38, while in MSC was 67.38. Significant difference of p = 0.0022 (**)

From the results presented in Figure 1, it can be observed that there was a significant difference (**) in the IC_{50} value of tumor cells compared to healthy cells since the IC_{50} values of U2OS and MSC were 16.38µL/mL and 67.38µL/mL, respectively.

DISCUSSION

Homeopathy has become a complementary treatment option for some diseases, including cancer, due to its ability to improve the patient's quality of life and be less invasive. *R. graveolens* is a plant with analgesic and anti-inflammatory properties that has already been associated with antiproliferative effects on tumor cells [11–13].

This study observed that ultra-diluted Ruta at the D35 potency decreased the viability of OS cells, indicating a possible antitumor effect. The antitumor ability of the *R. graveolens* extract was also demonstrated by Fadalla et al. (2011). The authors recorded a dose-response effect in the prostate, colorectal, and breast cancer cells, indicating that these tumor cells were sensitive to the treatment and the proliferation capacity also decreased after the treatment with *R. graveolens*. This reduction in proliferation occurred due to cell cycle arrest, in which the cells treated with the plant extract had their cell cycle stopped in the S and G2/M phases. Besides that, the *R. graveolens* extracts efficiently activate p53-regulated pathways in tumor cells, thus generating cell cycle arrest [13]. Therefore, this study agrees with our findings since we also observed a dose-response effect in which the U2OS cell viability rate decreased as treatment concentration increased.

In patients with non-small cell lung cancer, it has been reported that the homeopathic treatment in conjunction with conventional treatment improved the quality of life and prolonged survival of patients [14]. The *R. graveolens* cytotoxic activity has also been observed in hematopoietic tumor cells. Similar to the other lines mentioned above, the treatment with *R. graveolens* extract was effective against the proliferation of these cells, with a higher sensitivity of tumor cells under this treatment compared to normal blood cells that did not suffer from cytotoxic effects [15].

As a result, the MTT test of the present study showed that the *in vitro* treatment of cells with Ruta D35 was more effective in OS cells and that it did not cause high cytotoxicity in MSC, thus indicating that the U2OS line was significantly more sensitive to the homeopathic treatment and presented positive results. However, further studies are needed to better understand how Ruta D35 acts on these tumor cells and what other effects this treatment can generate on these cells.

It is crucial to understand and evaluate the effects of Ruta D35 on cancer because, in addition to being a plant that has active ingredients capable of acting in various pathologies, homeopathy has become a new form of treatment for cancer, often combined with chemotherapy. Studies demonstrating the potential of this treatment modality in the multiple types of cancer are still lacking. *In vivo* studies provide an idea of how treatment with various homeopathic agents can contribute to the reduction of tumor inhibition [16]. However, it is still necessary to deepen *in vitro* and *in vivo* studies to better understand the capacity of this medicine.

CONCLUSION

This study leads to a first understanding of the Ruta D35 action on the U2OS line of osteosarcoma. The results show that the *in vitro* activity of this homeopathic medicine is stronger in tumor cells than in healthy cells. Therefore, it may be a first indication of the possibility of using such medicine as an adjunct in treating bone tumors.

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