

Original Article

Homoeopathic *Viscum album* (10⁻³) is more Cytotoxic to *in vitro* Culture of Human Breast Cancer Cell Line than to Human Mesenchymal Stem Cells

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Abstract

Background Breast cancer has been considered a public health problem and homeopathic treatments are becoming increasingly recommended due to its ways of action and absence of adverse effects. MCF-7 is an adenocarcinoma of human breast cell line useful as preclinical model to screen therapeutic agents such as ultra-diluted *Viscum album*, an European plant which extract is commonly used in cancer therapy. AIMS MCF-7 and mesenchymal stem cells (MSC) were used to evaluate the *in vitro* cytotoxicity of homoeopathic *Viscum album* 1x10⁻³ (VA3X). **Methods** cells were cultured for 24 hours in controlled environment (37.5°C and 5% CO₂) in 96-well plates. After this time, VA3X was added to the culture medium in concentrations varying from 10 to 100 µL/mL. A control group was maintained with culture medium only. Cells were cultivated for 48 hours in these conditions for evaluation of cell viability by MTT assay. **Results** Higher cytotoxicity was observed in MCF-7 when compared to MSC, as the lower concentration of VA3X was capable of inducing tumor cell death and not healthy cell death. The MTT assay results were that 42 µL/mL of VA3X reduced MCF-7 cells viability to 50% and 62 µL/mL reduced MSC cells to the same percentage, what means that tumor cells are more sensible to VA3X than healthy cells. **Conclusion** *Viscum album* presented higher cytotoxic action on human breast cancer cell line culture than on mesenchymal stem cells. This medicine is extensively used against cancer, and the use of the homoeopathic form of it brings new possibilities as no or fewer adverse effects would be present.

Keywords: cell culture; homoeopathy, mammary adenocarcinoma cells, injectable *Viscum* D3, MTT assay

Introduction

Breast cancer is the most common malignancy disease among women and the second most frequent in the world population. It has been considered a public health problem, and ways of action that lead to a reduction in the number of deaths as well as to an improvement in diagnosis and treatment are becoming increasingly necessary [1].

Among these, studies related to the investigation of effective treatments that cause less damage and fewer side effects should be performed, including the use of homoeopathic medicines, such as those based on *Viscum album* (VA), which extract is already widely used for several types of cancer [2].

Viscum album, a semi-parasitic mistletoe plant which extracts can be obtained from several host trees [3], has a mechanism of action that varies according to its composition and has been of growing



interest since its cytotoxic and immunomodulatory properties were documented [4]. Mistletoe preparations have been dimensionally used in various treatment situations for centuries, showing two kinds of activities in the treatment of cancer. Firstly, it affects the quality of life of cancer patients by the improvement of fatigue, sleep, exhaustion, nausea, vomiting, appetite, depression, anxiety, pain and side effects of traditional treatment [5,6]. Secondly, it shows antitumor activity by cytotoxicity, induction of apoptosis [7,8] and inhibition of angiogenesis [9].

The current application as an injection preparation for tumor patients is based on the first suggestions of Rudolf Steiner, founder of anthroposophy, over 100 years ago [10]. Even though the intrinsic cellular mechanisms involving the antitumor action of VA are still poorly understood, intense use of this complementary therapy is being made in the last decades [11].

Viscum album L. preparations can be divided into phytotherapeutic extracts standardized on a certain lectin level and anthroposophical/ homeopathically produced extracts [12]. The varied pharmaceutical applications of the medicines based on VA results from the rich chemical composition of this plant. The main active compounds are lectins, viscotoxins, flavonoids, phenolic acids, sterols, lignans, terpenoids, phenylpropanoids, alkaloids and fatty acids [13].

The characterization of cytotoxicity *in vitro* is a common practice in the biological evaluation of health products and medicines and is fundamental for the initial analysis of their efficacy. By exposing a cell culture to a specific medicine of interest, it is possible to characterize the resulting adverse cytotoxicity reactions [14], and mesenchymal stem cells (MSC) are a well consolidated cell lineage model to evaluate the safety of products by evaluating the cytotoxicity. Another cell line used as preclinical model to screen for therapeutic agents is MCF-7, one of several breast adenocarcinoma cell lines that are widely used as a model for breast cancer study, as it is resistant to a variety of allopathic drugs [15]. It is possible to carry out *in vitro* tests with the application of the homeopathic medicine in cell cultures and analyze cell viability.

Thus, the aim of the present study was to compare the cytotoxic effects of the ultra-diluted medicine VA 10⁻³ potency (VA3X) on mesenchymal stem cells and human breast cancer – MCF-7 cell lines.

Material and Methods

This study did without an ethical committee approval, as the cells being used are commercial cell lines, and no animal or human samples were used.

Preparation of VA3X

For the preparation of injectable *Viscum* 3X (Injectcenter®), the basic dosage form Mother Tincture (MT) is taken as a starting point. The preparation follows using the Hahnemannian Decimal Method, described in the Brazilian Homeopathic Pharmacopoeia, that is, 1 part of the active ingredient is taken, for 9 parts of the inert ingredient, usually using the sterile isotonic solution, succumbing 100 times and obtaining thus *Viscum* 1X (1X10⁻¹). Then, 1 part of the preparation *Viscum* 1X is taken for 9 parts of the inert ingredient, succumbing 100 times and obtaining *Viscum* 2X (1X10⁻²) and then 1 part of the *Viscum* 2X preparation is taken for 9 parts of the inert ingredient, succumbing 100 times and thus obtaining *Viscum* 3X (1X10⁻³). This is packaged in 1.1 mL ampoules, in a classified and certified area. The process is validated, thus guaranteeing the sterility and quality of the final product. The method of preparing the ultra-diluted injectable follows the International Compendium, the German Pharmacopoeia (German Pharmacopoeia).

Cytotoxicity with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT)

MCF-7 (ATCC® HTB-22™) and MSC obtained from commercial cell bank were cultivated in triplicate in 96-well plates at a concentration of 2.5×10^4 cells/mL of Roswell Park Memorial Institute 1640 culture medium (Sigma-Aldrich®) – RPMI – and Dulbecco's Modified Eagle Medium – DMEM – respectively. Both mediums were added with 10% of fetal bovine serum (FBS) and 0.02% of amikacin for the adhesion and sterility of cells, respectively. The plates were incubated at 37.5 °C, CO₂ at 5%, and saturated humidity. After 24 hours, the cells were observed and the medium was replaced by RPMI or DMEM added with VA3X for achieving the 13 final concentrations in cell culture: 10, 12.1, 14.7, 17.8, 21.5, 26.1, 31.6, 38.3, 46.4, 56.2, 68.1, 82.5 and 100 µL/mL. With the same methodology and at the same time, the control was carried out with the addition of the same water used in the dynamizations in the 13 concentrations, and negative control wells were used only with culture medium, without addition of any other product, to confirm cell growth. After 48 hours, the MTT colorimetric test – Thermo Fisher Scientific® – was performed with the substitution of the culture medium for 100µL of the MTT reagent and incubation of the plates for 4 hours at 37.5 °C protected from light. Subsequently, the supernatant was removed, and 100 µL of dimethyl sulfoxide (DMSO) were added to each well, homogenized for dilution of formazan crystals and absorbance was recorded at 570 nm on a microplate spectrophotometer (Elisa Plate Reader Kazuaki®) for identification of the viable cells.

Cell counting

The MCF-7 and MSC cell lines were cultured in triplicate in a 96-well plate for 24 hours with its respective culture medium at 37,5°C and 5%CO₂ and saturated humidity. After this period, this medium was replaced by medium containing VA3X in the concentrations of 10, 20 and 30 µL/mL. Cells were also cultured only with culture medium as negative control. These plates were kept in culture for 48 hours, then cells were harvested by trypsinization using Trypsin/EDTA solution and centrifuged for elimination of death cells. The supernatant was discarded and the pellet was resuspended in 1 mL of culture medium. Then, 10 µL of this cell suspension was transferred to the Neubauer chamber and cells were counted. The same was performed with the control group to compare the results.

Analysis

The MTT test and the cell counting assessed a ratio between live and dead cells in the culture. The mean inhibitory concentration (IC₅₀) of VA3X was then calculated based on the results of MTT assay, that is, a concentration that inhibits the growth of 50% of the cells in culture. The analysis of the results of the MTT tests was performed in the Graph Prism 7.04 program using the Tukey test for multiple comparisons.

Results

This study evaluated the difference of cytotoxicity of ultra-diluted VA3X in breast cancer and mesenchymal stem cells. After 24 hours of culture without any treatment, both cell lineages presented a morphology consistent to the expected for a normal cell growth, with adherence to the plastic, MSC with fibroblastic shape and MCF-7 with islands in monolayers (Figure 1). After treatment, in the MTT assay, the difference of viable cells between samples in contact with the homeopathic *Viscum album* – VA3X was compared to that of the control sample, which was not in



contact with the medicine. In the tumor cell line, MCF-7 (Figure 2), it was verified that higher concentrations of VA3X were able to significantly reduce the cell viability with a lower formation of formazan (with p value of <0.0001 in all analyzed concentrations), starting from the lower treatment dose of 10 $\mu\text{L}/\text{mL}$. A concentration-response result was also observed in MSC culture treated with VA3X, but only higher concentrations were able to induce cell death, only from 21,5 $\mu\text{L}/\text{mL}$ a significant difference between treatment and control could be observed (Figure 3).

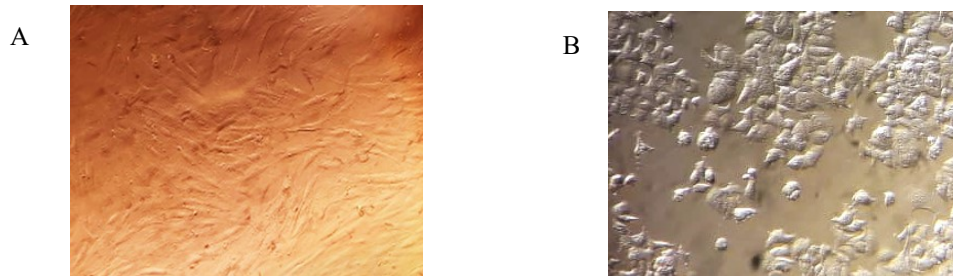


Figure 1- Morphology of cells cultured previously to the treatment with VA3X. (A) MSC: adherence to the plastic and fibroblastic shape; (B) MCF-7 cell line: islands in monolayers.

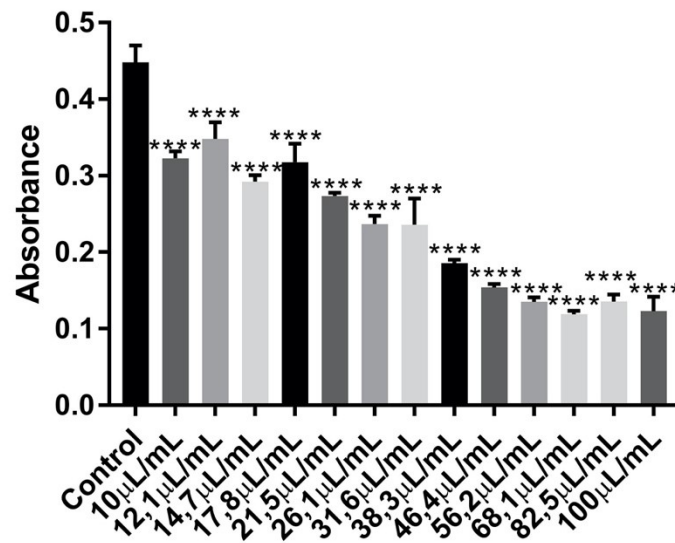


Figure 2: Absorbance media in MTT assay of all concentrations analyzed of the homeopathic VA3X in MCF-7 cell line. ****indicates difference between treatment and control group ($p < 0,0001$).

Also using the MTT assay results, it was possible to calculate cell viability and the IC_{50} cytotoxicity index, a value that indicates the concentration capable of reducing the cell population by 50%. As shown in Table 1, the IC_{50} calculated for MCF-7 cancer cells was 42 $\mu\text{L}/\text{mL}$ of VA3X and for MSC this value was 62.57 $\mu\text{L}/\text{mL}$ (Table 1). Consequently, this result indicates that VA3X is more efficacious in reducing cell viability in the tumor cell line than in a healthy one.

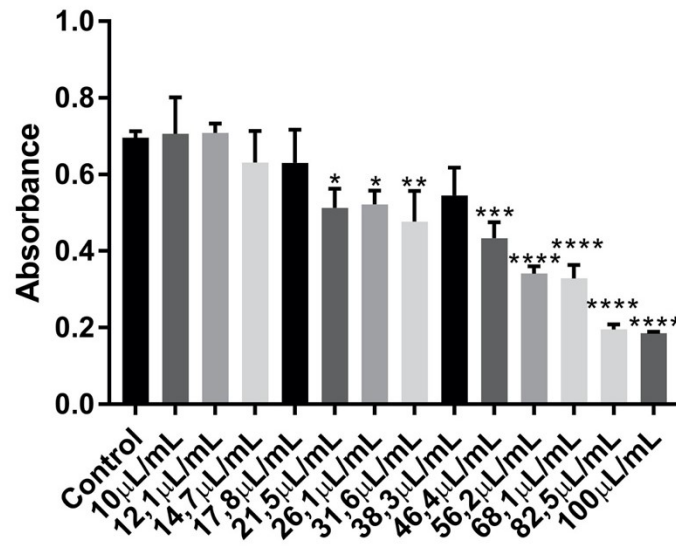


Figure 3 - Absorbance media in MTT assay of all concentrations analyzed of the homeopathic VA3X in MSC cell line. *indicates difference between treatment and control group: (*p<0,1; **p<0,01; ***p<0,001; ****p<0,0001).

Table 1 - Descriptive data of cell viability by MTT assay in each concentration tested as well as the cytotoxicity value by concentration-response (IC50) with VA3X.

Concentration of VA3X µL/mL	MCF-7 with VA3X	MSC with VA3X
0	100	100
10	71.97	101.34
12.1	77.69	101.77
14.7	65.20	90.62
17.8	70.85	90.53
21.5	60.96	73.64
26.1	52.78	74.98
31.6	52.63	68.42
38.3	41.41	78.23
46.4	34.34	62.20
56.2	30.11	49.04
68.1	26.54	47.18
82.5	30.26	28.04
100	27.43	26.65
IC ₅₀	42	62.57

The counting process also demonstrated the effectiveness of the VA3X in inducing cell death in tumor cells more than in mesenchymal stem cells (Table 2). In this test, the culture with 30 μ L/mL of *Viscum album* caused death in 86% of tumor cells and 77% of healthy cells.

Table 2 - Percentage of cell viability and mortality of MCF-7 and MSC cell lines after cultured with VA3X.

	MCF-7			MSC		
	Medium Score	% of Viability	% of Mortality	Medium Score	% of Viability	% of Mortality
Control	50000	100	0	201667	100	0.00
10 μ L/mL	36667	73.33	26.67	158333	78.51	21.49
20 μ L/mL	16667	33.33	66.67	115000	57.02	42.98
30 μ L/mL	6667	13.33	86.67	45000	22.31	77.69

Discussion

Studies evaluating the *in vitro* antitumor effects of the *Viscum album* extract have already been carried out, providing evidence on the effectiveness of this medication in promoting tumor cell death response [4]. Researchers have demonstrated the cytotoxic potential of VA extract in breast tumor cell lines [16] and have found an IC₅₀ of 172 mg/L when analyzing the action of VA extract on breast cancer cells [17], demonstrating the cytotoxic effect of this extract on tumors.

Thus, it is well-known fact that there is an action of the components of the VA extract on cancer cells [11,18,19]. However, the literature still does not present data about the *in vitro* evaluation of homeopathic VA, with only 7 articles in Pubmed database using the terms “*Viscum album* and homeopathic” (in May-2020) which are of especial relevance due to the low frequency of occurrence of side effects produced by homeopathic formulations.

Previous studies have been performed with homeopathic medicines and results of cytotoxicity have been found, such as the action of the *Terminalia chebula* against breast cancer cell lines (MDA-MB-231 and MCF-7) reporting its anticancer activity [20]. The homeopathic *Psorinum* triggered apoptosis against pulmonary adenocarcinoma cells [21] and *Lycopodium clavatum* against cervical cancer cell line (HeLa cells) causing the induction of DNA fragmentation [22], all of them tested *in vitro*.

Araro *et al* (2013) identified the potential as anticancer agents of homeopathic medicines of *Sarsaparilla*, *Phytolacca decandra* and *Ruta graveolens* can be attributed in part to its inhibition of proliferation and apoptosis induction [23]. The same antitumor activity was found with *Ruta graveolens* extract, but in this case, it was not concentration dependent. When the higher concentration was used, the activity was found to be reduced than that of the lower concentration [24]. In our study, VA3X was capable of acting on human breast cancer as well as on healthy cells (mesenchymal stem cells), but its cytotoxicity was better observed in the MCF-7 cell line, since a lower concentration of ultra-diluted VA was able to reduce cancer cells viability. This is a relevant result for cancer researchers who want to explore and develop this new approach to the treatment of breast cancer. Once more showing the importance of *in vitro* studies for the preliminary evaluation of the homeopathic medicine.

Homeopathic medicines have been to gain ground in the routine of the integrative clinic, however, it is only recently that complete *in vitro* studies evaluating their real mechanism of action and their experimental validation have started to be made [22].

In vitro product safety assessment studies involve cytotoxicity analysis, in which the main cell models used are primary cell cultures and strains [25,26]. In our study, a higher concentration was needed to provoke cytotoxicity of MSC when compared to MCF-7, for tumor cells in all tested concentrations there was a toxicity effect provoked by the homeopathic medicine based on VA, and for MSC, this toxicity started from higher concentrations, what indicates that this medicine is safe, as health cells are not damaged in concentrations that causes death of tumor cells.

Even though our study achieved results showing the *in vitro* cytotoxicity of homeopathic VA against breast adenocarcinoma cells, further work will be necessary to clarify the pathway of action of ultra-high diluted homeopathic medication.

Conclusion

In the present research, the toxic action of the homeopathic *Viscum album* medicine was observed at the potency 3X in human breast cancer cell line (MCF-7). The medicine in higher concentration was also cytotoxic to health cells (MSC), but the comparison showed this medicine is much more cytotoxic to the tumor cell line. This is a promising result since breast cancer is a high incidence type of cancer for which the available treatments can cause unwanted side effects. In this regard, we present responses observed with the homeopathic medication that may indicate effectiveness and that should be evaluated in more details by researching into the molecular mechanisms involved and the way of inducing cell death. Therefore, the homeopathic VA3X can be considered to be an important therapeutic source for cases of human breast cancer.

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