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Analysis of the performance of the ultra-diluted *Viscum album* in cultivation of mesenchymal stem cells and mammary adenocarcinoma cells pmc-42 and mcf-7

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Abstract
Adenocarcinoma in breast can be of several types and PMC-42 and MCF-7 cell lines are a well-established cell lines used in *in vitro* studies retaining much of the breast's native phenotype. *Viscum album*, a plant which extract has cytotoxic effects, is included in the brand-new class of cancer therapies and its ultra-diluted version is a promising area of interest. The MTT assay was realized to evaluate the cytotoxic potential and calculate the inhibitory concentration of different concentrations of ultra-diluted *Viscum album* D30 (VAD30). The results indicated that 60µL/mL, 37µL/mL and 35µl/mL of VAD30 were cytotoxic to 50% of mesenchymal stem cell, MCF-7 and PMC-42 cells respectively. This result shows the better potency of action of VAD30 against tumor cells, as a lower concentration inhibits the growing of this cell line in comparison to health cells, demonstrating that this medicine has the potential to be used in cancer therapy.

Keywords: *Viscum album*, homeopathy, cell viability, tumor cells

1. Introduction
Several *in vitro* methods using cell cultures have been standardized for the evaluation and/or prediction of toxicity, as well as for predictive evaluation of substance efficacy, and have favored the refinement, reduction or replacement of tests using animals in experimentation [1]. Aiming to achieve the basic principles of the 3Rs: Replacement, Reduction and Refinement; proposed since 1959 by Russell and Burch [2] and reviewed extensively by Kandárová *et al.* [3, 4], where significant progress has been made using the screening procedure, through the integration of *in vitro* tests by cell cultures and computer systems (*in silico*) to reduce or even replace the use of animals [5]. Thus, modern study guides (guidelines) have been proposed for the use of alternative methods in the process of basic and applied research of substances with the purpose of replacing, refining or reducing the use of animals [6].
Among the therapies that have been gaining space in the routine of the integrative clinic are homeopathic medicine, which are routinely used, however, *in vitro* studies involving them are rare, which promotes persistence in relation to concerns regarding their experimental validation. and mechanism of action [7,8]. Therefore, with the possibility of evaluations that can be performed today, ultra-diluted medicine can show efficacy and safety in *in vitro* culture conditions, being possible to evaluate the possible variation of their action according to the dilution [7]. Homeopathic medicine has been used for centuries as a medical alternative, highlighting the use of *Viscum album* in integrative oncology therapy [9–11]. However, safety trials to validate the efficacy and reproducibility of the actions of ultra-diluted medicine are necessary [12, 13].
The toxicology field has been challenged by the exponential discovery of new substances. Public pressure for not using animals in research and experimentation and the need to develop new methodologies that involve different toxicological outcomes for safety assessment, as well as the validation of these methods it is becoming more and more necessary [14]. This is also because animal toxicity tests are not always applicable to human health due to interspecies variations. Therefore, alternative methods to the use of animals have been considered efficient alternatives, either with the use of the species-specific cell or tissue platform or even *in chemico* or *in silico* assessments to predict toxicity [15–20].

In vitro product safety assessment studies involve cytotoxicity analysis, in which the main cell models used are primary cell cultures and strains, sometimes immortalized [21, 22]. Thus, an immense range of cell types can be studied in relation to cytotoxicity, and a cell type with high potential for these assessments is the mesenchymal stem cell (MSC), due to its capacity for self-renewal and differentiation potential [23].

Previous studies have evaluated bone marrow MSCs as a good model for predicting toxicological class by the neutral red uptake assay when comparing with already validated 3T3 NHKs and murine fibroblasts cells where 12 chemical reference reagents were tested for this validation [24]. These results are promising, however stem cells derived from bone marrow are difficult to obtain, which made it interesting to evaluate this same potential of mesenchymal stem cells obtained from adipose tissue (MSC) [23] as they are a group of easy availability, reproducibility and scalability to predict toxicity in *in vitro* tests [25, 26]. In this context, Abud and collaborators concluded that MSC represent an interesting cellular model for alternative tests to animal tests with great relevance for the prediction of toxicity that represent an important potential for industrial applications and for regulatory purposes. MSCs can be used to assess the cytotoxicity of chemical compounds, including products that are already established or are still under development [23].

In this study, immortalized adenocarcinoma lineage cells such as PMC-42 and MCF-7 were also evaluated. PMC-42 is a lineage of breast carcinoma established from a pleural effusion of a patient with metastatic breast cancer [27]. It is remarkably similar to normal breast epithelial tissue, suggesting that, unlike the most common breast cancer cell lines resulting from luminal or myoepithelial / basal cell tumors, PMC42 is a rarer cell line, originating from a stem cell of the breast, maintaining much of the native breast phenotype [28–31].

MCF-7 is a human breast cancer strain with estrogen, progesterone and glucocorticoid receptors. It is derived from the pleural effusion of a 69-year-old Caucasian metastatic breast cancer (adenocarcinoma), isolated more than 40 years ago, by Dr. Soule, of the Michigan Cancer Foundation, Detroit, Michigan in 1970 [32,33]. MCF-7 cells are useful for *in vitro* breast studies because they maintained several ideal characteristics specific to the mammary epithelium, such as estrogen processing, in the form of estradiol, through estrogen receptors (ER) in the cell cytoplasm [34,35].

Thus, the objective of the present study was to evaluate the performance of the ultra-diluted Viscum D30 (VAD30) in culturing mesenchymal stem cells derived from human adipose tissue and to compare it with the performance of this medication in culturing PMC-42 breast adenocarcinoma cells and MCF-7.

2. Material and Methods

2.1 Obtaining and cultivating cells

The mesenchymal stem cells derived from human adipose tissue (MSC) were provided by the BioCell (Cell Therapy Laboratory) and the breast cancer tumor lines PMC-42 and MCF-7 (ATCC® HTB-22 ™) by the Biotechnology and Genomic Sciences laboratory from the Catholic University of Brasilia (purchased by the ATCC and grown according to the protocol). MSCs were grown in Dubellco's Modified Eagle medium (DMEM) and tumor lines in Roswell Park Memorial Institute (RPMI) 1640 medium, both from the Sigma-Aldrich® brand according to the manufacturers' recommendations.

2.2 Experimental groups

To evaluate the cytotoxicity of the ultra-diluted drug VAD30, CTM, PMC-42 and MCF-7 cells were cultured *in vitro* for 48 hours in the following experimental groups: Control (cells with the respective culture medium) and VAD30 in different concentrations (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 per mL of culture medium). VAD30 ampoules (1.1mL) were obtained from the company Injectcenter® (Ribeirão Preto, Brazil), which produced the drug according to the German and French pharmacopoeias.

2.3 Cell viability assay (MTT and IC_{50})

The cytotoxicity of the drug was determined in the respective cell lines by means of a colorimetric assay that measures the reduction of {[3- (4,5-dimethylthiazol-2yl) -2,5-diphenyl tetrazolium]} (MTT) by mitochondrial activity. For this cell viability assay, cells were initially cultured in triplicate in 96-well plates containing 1 x 10^4 cells/mL of the respective culture medium under an incubator at 37.5 °C, 5% CO_2 for 24 hours for stabilization and cell adhesion. After this period, the *in vitro* culture medium was replaced and the evaluation factor containing the VAD30 in different concentrations (10 to 100 µL/mL) was added to each group and the cells were cultured for another 48 hours. After this period, the culture medium was removed and 100 µL of the solution containing 3- (4,5-dimethylthiazol-2-yl) -2,5-diphenyl tetrazolium bromide (Catalog: Sigma N. M2128) at 0.5mg/mL was added to each well and incubated for 4 hours at 37.5 °C and 5% CO_2 protected from light. Then, the supernatant was removed and 100µL of DMSO was added to each well, homogenized and evaluated in a microplate spectrophotometer for capturing absorbance in the 570nm spectrum (Molecular Devices, Sunnyvale, CA, USA) to identify viable cell density. Finally, after obtaining the data, the inhibitory concentration for 50% of the cells (IC_{50}) of each cell type was calculated, that is, the response concentration by which cell viability is reduced to 50%.

2.4 Statistical analysis

The analysis of the results of the MTT tests were performed in the Graph Prism 7.04 program by the Tukey test for multiple comparisons. Where (*): P value ≤ 0.05; (**): P value ≤ 0.01; (***): P value ≤ 0.001; and (****): P value ≤ 0.0001.

3. Results and Discusion

Firstly, the impact of VAD30 concentrations on the viability of MSC cells was analyzed after 48 hours of *in vitro* culture (Figure 1), MSC were subjected to *in vitro* culture medium with the addition of VAD30 in the different concentrations tested (10µL/mL-100 µL/mL) and for the control group were grown without the addition of VAD30. The mean absorbance variation measured were significant in relation to the control sample that had no contact with the homeopathic, mainly from the tested concentration of 26.1 µL/mL where a p Value ≤ 0.0001 is observed. The results (Table 1) demonstrated a maintenance of the quantity of cells after *in vitro* culture for 48 hours at the lowest concentrations tested, with a decrease in the observed

maintenance (less than 75%) from the concentration of 46.4 µL/mL (p Value of 0.0001).

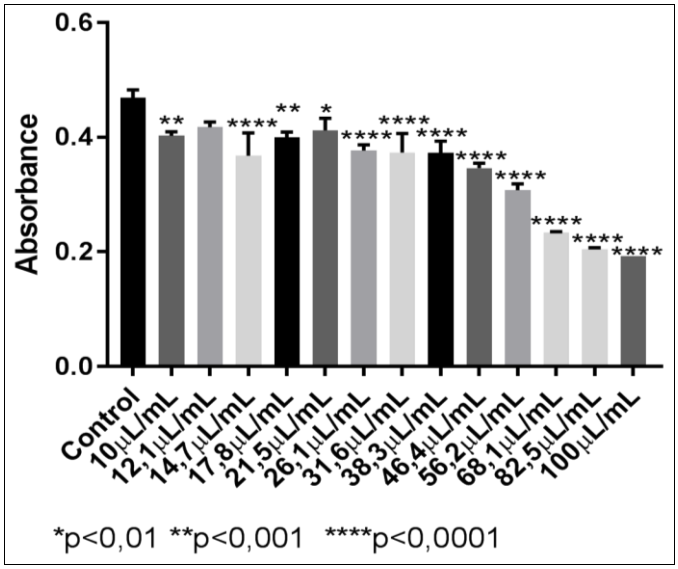

Fig 1: Absorbance measured in the cell viability assay of MSCs with ultra-diluted VAD30 at the different concentrations investigated (10 to 100 µL/ mL) after 48 h of culture

For the tumor cells PMC-42 (Figure 2) and MCF-7 (Figure 3) it can be seen that, the action of VAD30 was more effective considering that it was possible to observe that a greater efficiency of cell death occurred with the tumor cells. Separately, the PMC-42 cells showed a large variation in absorbance when comparing the samples with the homeopathic compared to the control sample, with significant action being observed at low concentrations.
The results demonstrated a reduction in the cell quantity after *in vitro* culture for 48 hours at the lowest concentrations tested, with a more significant decrease observed from the concentration of 21.5 µL/mL.

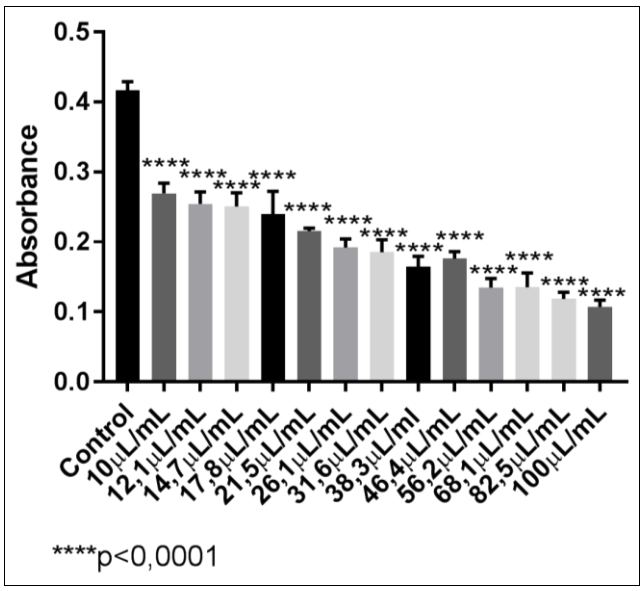

Fig 2: Percentage of cell viability after 72 hours of cultivation of PMC-42 cells with VAD30 at different concentrations.

As with the PMC-42 cells, the MCF-7 cells (Figure 3), also showed a large variation in absorbance when compared to the control sample. The results demonstrated a reduction in the cell quantity after *in vitro* culture for 48 hours at the lowest concentrations tested, with a more significant decrease observed from the concentration of 21.5 µL/mL.

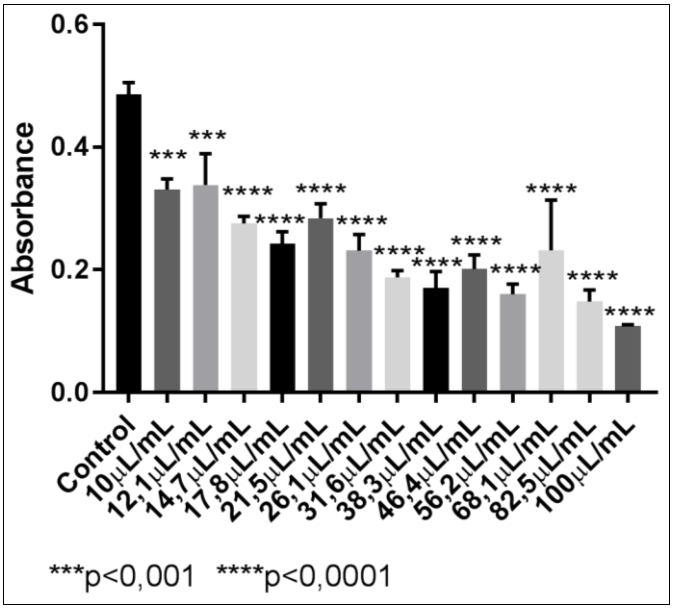

Fig 3: Percentage of cell viability after 72 hours of cultivation of MCF-7 cells with VAD30 at different concentrations.

The cell viability values obtained in each assay by the MTT test and the cytotoxicity value by concentration-response (IC_{50}) are described in Table 1.
It can be observed that VAD30 has a cytotoxic action for the tumor cells of PMC-42 and MCF-7 with IC_{50} of 35.81 and 37.5 respectively, as well as a cytotoxic action can be observed with the MSC cells, with less intensity than that observed in tumor cells (IC_{50}: 79.77). Corroborating what the results obtained by evaluating the absorbance index.

Table 1: Cell viability at test concentrations. Descriptive data with the values obtained from cell viability by MTT and the value of cytotoxicity by concentration-response (IC_{50}) for each cell type with VAD30 and control test.

Concentration of VA30 (µL/mL)	% viability MSC	% viability PMC-42	% viability MCF-7
0	100	100	100
10	85,86	64,69	68,15
12,1	89,06	61,09	69,66
14,7	78,33	60,21	56,76
17,8	85,15	57,65	50,03
21,5	87,85	51,80	58,54
26,1	80,32	46,12	47,63
31,6	79,54	44,44	38,71
38,3	79,40	39,47	35,21
46,4	73,79	42,27	41,46
56,2	65,62	32,35	33,01
68,1	49,78	32,43	47,77
82,5	43,53	28,42	30,54
100	41,12	25,78	22,37
IC_{50}	79,77	35,81	37,5

As shown in Figure 1-3 and Table 1, it is highlighted that the use of VAD30 regardless of the concentration used, the presence of the ultra-diluted *Viscum album* in the *in vitro* culture medium has deleterious effects on cell viability, in smaller proportions when using low concentrations. This data corroborates the result described in the meta-analysis

carried out by Bonamin et. al. (2017) why they showed that the *Viscum album* has a cytotoxic action against cancer cells, creating a suitable environment for a new understanding of science [9,10], this result being obtained with VAD30 already considered a safety test of the ultra-diluted medicine, as indicated by Cesar [12]. However, other safety tests and clinical tests are still needed to assess the side effects that involve the action of this ultra-diluted medicine [36].

This type of tests is important to evaluate the pattern of actions of determinate medicine, bearing in mind that some extracts present a pattern that is not dependent on concentration, like in the case of *Ruta graveolens* extract, where the same antitumor activity was found, but when the higher concentration was used, the cytotoxic activity was found to be reduced than that of the lower concentration [37].

The methodology used in this work complies with the new rules for reducing the use of animals in research and experimentation [14], being already used for a previous screening of the evaluation of safety and cytotoxicity, as well as the validation of these methods. The fact that it is carried out in human cell culture is a great advance, since animal toxicity tests are not always applicable to human health due to interspecies variations [15–18].

4. Conclusion

In this work it was demonstrated that VAD30 in different concentrations has cytotoxic action in healthy cells (MSC) only in high concentrations. Its greatest cytotoxicity was observed in tumor cells, with low concentrations in breast cancer cells (PMC-42 and MCF-7), making part of these cells unfeasible *in vitro*. This is a promising and relevant result because it shows the inhibitory potential of VAD30 in breast cancer cells. In addition, this result is relevant because it opens up new avenues to be explored also *in vivo*. The use of this medication in homeopathic form brings new possibilities for the treatment of breast cancer with no or less adverse effects present, which would be extremely important for the quality of life of patients.

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