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RESEARCH ARTICLE

Comparison of cytotoxicity caused by *Viscum album* in human mesenchymal stem cells and hepatocellular carcinoma cells

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ABSTRACT

Viscum album (VA), also known as Mistletoe, has various therapeutic properties, including analgesic, anti-inflammatory, and anticancer effects. It has been used for treating different types of cancer, exhibiting proven efficacy against breast cancer, glioblastoma, carcinoma, and other advanced-stage tumor types. Besides promoting improvements in the clinical condition, VA also helps reduce the side effects caused by conventional treatment, thereby offering patients a better quality of life. Hence, the objective of this study was to assess the effects of the homeopathic dilution of VA at the potency of D30 ($1x10^{-30}$) (VA D30) on human mesenchymal stem cells (MSCs) and hepatocellular carcinoma cells (HepG2). The cells were grown in 75 cm² flasks until they reached approximately 80% confluence. Subsequently, they were trypsinized and plated in 96-well plates at 10,000 cells/well. After 24 hours of incubation in an oven, VA was added at 30 and 40 µL/mL concentrations. The cells were further incubated for an additional 48 hours. After the treatment, the cells underwent a cell viability test using MTT. The results indicated a decrease in HepG2 viability, while no damage to normal cells (MSCs) was detected. The findings suggest that VAD30 holds promise as a potential therapeutic agent in treating hepatocellular carcinoma due to its observed cytotoxicity towards HepG2 cells while exhibiting no adverse effects on mesenchymal stem cells at the equivalent concentration.

Introduction

Viscum album (VA) has been used for medicinal purposes for over 2000 years owing to its various therapeutic properties, including antidiabetic, analgesic, anti-inflammatory, antiarrhythmic, and hypotensive effects[1]. Since the Middle Ages, VA has been documented for treating multiple diseases. Hippocrates recommended its use to treat spleen diseases and alleviate menstruation-related complaints. Paracelsus advocated its use to treat epilepsy. Additionally, Hildegard von Bingen supported the utilization of VA in addressing spleen and liver diseases[2].

In 1920, Rudolf Steiner introduced the use of VA for cancer treatment using an extract derived from the plant through a complex extraction process[3]. Since then, its use to treat various types of cancer has been increasing, and both its extracts and medicines can be employed in homeopathic dilutions. Its effect has already been evaluated in cases of breast cancer[4], glioblastoma[5], carcinoma *in situ* (CIS)[6], and other types of cancers in advanced stages[7].

The activity of VA against various types of cancer is attributed to the presence of specific bioactive compounds that have been extracted and studied[8,9]. Lectins, viscotoxins, flavonoids, phenolic acids, sterols, lignans, terpenoids, phenylpropanoids, alkaloids, and fatty acids are among these compounds[10]. Viscotoxins and lectins have been recognized for their direct ability to induce cytotoxic effects on tumor cells[11]. Additionally, triterpenes enhance the activity of natural killer (NK) cells through their antitumor and apoptotic properties[12-14].

Another advantage associated with utilizing VA concerns enhancing patients' quality of life. VA can potentially ameliorate fatigue, sleep disturbances, nausea, and other persistent side effects commonly arising from conventional chemotherapy treatments or directly caused by the cancer condition itself[15].

In vitro studies of Mistletoe activity have validated its capacity to modulate various pathways, thereby assuming significant roles in tumor proliferation, including the MAPK[16] and PI3K/AKT (phosphatidylinositol 3-kinase/protein kinase B) pathways[17]. Furthermore, Mistletoe can cause cell cycle arrest[18], loss of mitochondrial membrane permeability (MMP)[19], activating caspases and regulating pro- and anti-apoptotic proteins[20].

In this context, it is important to evaluate the therapeutic potential of VA across a wide range of cancer types that may impact patients. Cancer persists as a significant global health challenge. Despite the considerable strides in treatment modalities, a perpetual need exists for novel clinical approaches capable of enhancing patients' health outcomes and quality of life[21,22].

Hepatocellular carcinoma is a highly prevalent form of cancer. Early-stage cases can be effectively managed through surgical intervention. However, in most cases, the diagnosis occurs during the advanced stages of the disease, leading to complexities in the treatment approach and a notable reduction in patients' overall survival rates[23].

Therefore, this study aimed to assess the cytotoxic effects of a homeopathic medicine derived from VA on human mesenchymal stem cells (MSCs) and hepatocellular carcinoma cells (HepG2) through *in vitro* experiments. The objective was to determine the viability of utilizing this medicine as part of

Integrative Medicine, thereby exploring its potential therapeutic application.

Method

CELL CULTURE

HepG2 and MSC cells were grown in 75 cm² flasks and maintained in an incubator at 37°C with a 5% CO_2 atmosphere until they achieved approximately 80% confluence. Subsequently, the cells were trypsinized and plated in 96-well plates, considering 10,000 cells per well to initiate the experimental tests.

CELL VIABILITY

Once the cells were plated in 96-well plates and incubated in an oven for 24 hours, the appropriate treatments were introduced to the respective wells. For this purpose, the homeopathic VA D30 was diluted at 30 and 40 µL/mL concentrations, and triplicate doses of each concentration were added to the wells for both cell types. Untreated cells (control group) were also included in the experimental setup for testing purposes. The plates were incubated for an additional 48 hours in an oven.

Following the treatment period, the supernatant from each well was collected, and then diluted MTT was added to the culture medium. The plates were subsequently incubated for an additional 4 hours. After the 4-hour incubation with MTT, the plates were subjected to absorbance readings using a quantitative spectrophotometer to assess cell viability.

STATISTICAL ANALYSIS

The absorbance data obtained were transformed into cell viability percentages (%) relative to the control group and subjected to statistical analysis using the Student's T-test in GraphPad Prism Version 9.5.1 (528).

Results

Cells were cultured and displayed the expected morphology and growth characteristics. Hepatocellular carcinoma cells exhibited an epithelioid appearance with adherence to the plastic surface, while mesenchymal stem cells demonstrated adherence to plastic and a fusiform appearance, as depicted in Figure 1. Consequently, the cell cultures proved conducive for conducting the *in vitro* tests to analyze cell viability.

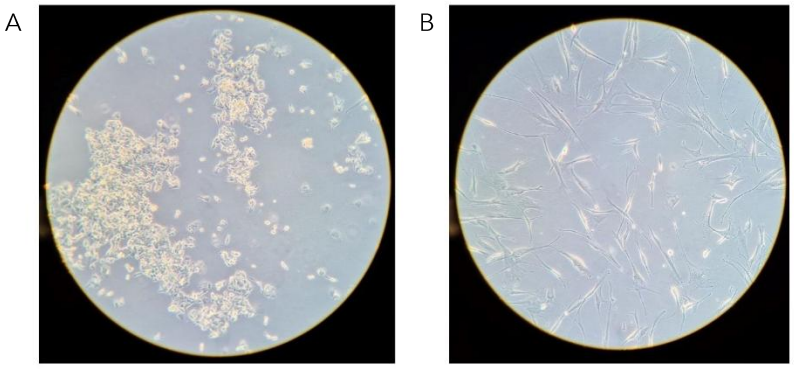

Figure 1. (A) Hepatocellular carcinoma (HepG2) and (B) human mesenchymal stem cells (MSCs) in cell culture. The images were captured using an inverted microscope at a magnification of 100x.

Following the treatment of MSCs and HepG2 cells with VA D30 at a concentration of 30 µL/mL, the average cell viability was found to be 69.3% for MSCs and 69% for HepG2 (Table 1). Importantly, no statistically significant difference in cell viability was observed between the two cell types, as depicted in Figure 2. However, upon increasing the concentration of the medicine in cell cultures to 40 µL/mL, a substantial reduction in cell viability was observed in tumor cells (45%). In contrast, a moderate decrease in viability was noted in stem cells (53.3%), as indicated in Table 1.

Table 1. Cell viability of MSC and HepG2 cells after treatment with VA D30 at two different concentrations. (v1: viability 1; v2: viability 2; v3: viability 3).

	Cell viability (%)			
	MSC		HepG2	
	30µL/mL	40µL/mL	30µL/mL	40µL/mL
V1	67	55	73	44
V2	68	53	71	46
V3	73	52	63	44
Mean	69	53	69	45

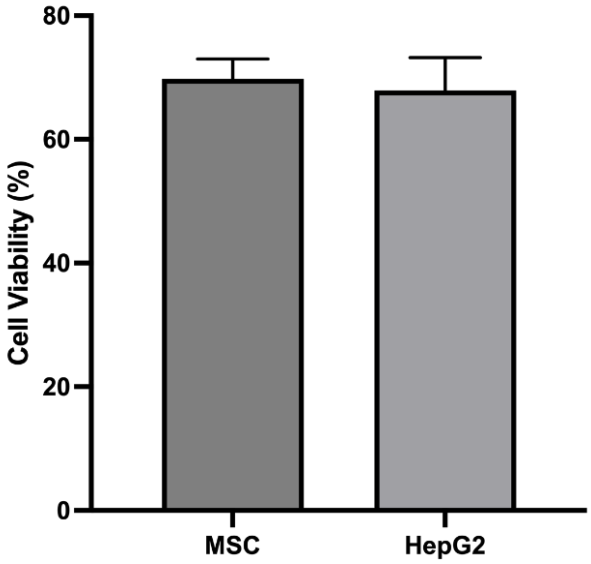

Figure 2. Cell viability of MSC (human mesenchymal stem cells) and HepG2 (hepatocellular carcinoma cells) after a 48h treatment with VA D30 at 30 µL/mL concentration.

In the treatment performed with 40 µL/mL of VA D30, cell viabilities were approximately 53.3% (MSC) and 45% (HepG2). Notably, a statistically significant difference ($p<0.005$) between the results of the two cell types was observed, as illustrated in Figure 3.

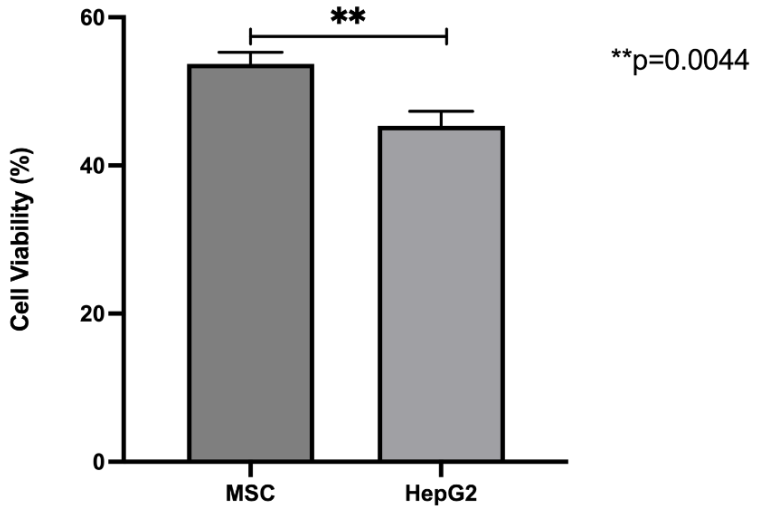

Figure 3. MSC (human mesenchymal stem cells) and HepG2 (hepatocellular carcinoma cells) viabilities after a 48h treatment with VA D30 at 40 µL/mL concentration.

The viability data obtained were also compared statistically between cell types (Figure 4), revealing a more substantial statistical difference in the treatment effects on HepG2 compared to MSCs.

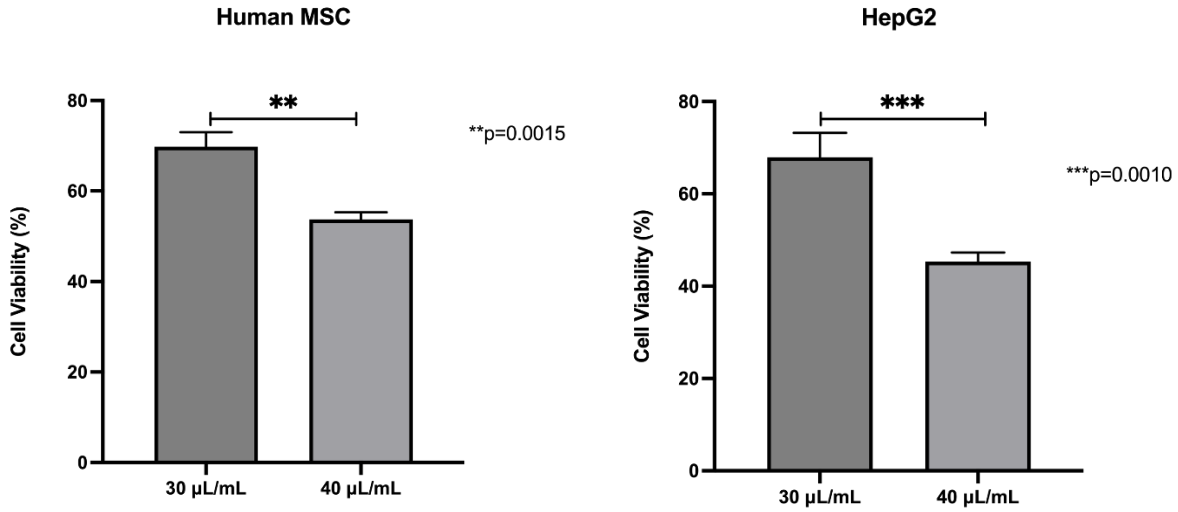

Figure 4. MSC (human mesenchymal stem cells) and HepG2 (hepatocellular carcinoma) cell viabilities after a 48h treatment with VA D30 at both concentrations (30 and 40 µL/mL).

Discussion

In vitro tests using cell culture to evaluate the effects of medicines are becoming increasingly prevalent in scientific research. VA in homeopathic dilutions has been extensively recommended in the context of cancer treatment, and the current study compared its effects on hepatocellular carcinoma cells with those on healthy cells. When exposed to the medicine, the results demonstrated a significant dose-response effect on hepatocellular carcinoma cells, which was not observed in mesenchymal stem cells. These findings indicate that, when

administered in homeopathic dilution, VA was more effective against tumor cells exhibiting minimal harm to normal cells (MSCs), showcasing its discerning selectivity towards tumor cells.

The utilization of VA originated as a therapeutic modality within the framework of anthroposophic medicine. It is recommended for cancer patients, whether in conjunction with conventional treatments such as surgery, radiotherapy, chemotherapy, or hormone therapy, or independently[24]. VA may be administered preceding conventional therapies to mitigate adverse reactions or following them to enhance the patient's quality of life[25]. In vitro studies demonstrated that the action of VA is based on multimodal therapy, with a direct effect on tumor cells and modulation of supplementary pathways[26]. Improving patient health can be aided by modulating the immune system through the stimulation of macrophages and by suppressing carcinogenic effects due to antiangiogenic activity[27].

Furthermore, MSCs can serve as a control group in *in vitro* experiments to determine the optimal dose of VA. Its utilization in cytotoxicity tests represents an alternative method by which it becomes feasible to anticipate the initial doses for usage, as prescribed by international regulatory authorities[28]. In addition, MSCs can be used as a control treatment for *in vitro* tests to determine the optimal dose of VA since it was possible to compare the effect of the homeopathic medicine on these normal and cancer cells. In this study, it was observed that at a dose of 40 µL/mL VA was capable of causing damage to hepatocellular carcinoma cells (HepG2), and according to statistical analysis, this effect was not observed in MSCs. Valle et al. (2023) used canine mesenchymal stem cells to evaluate the cytotoxicity of VA (VA D1D2). Their study demonstrated that well-established *in vitro* tests can aid in determining the optimal therapeutic dose, ensuring minimal harm to normal cells while effectively targeting cancerous cells[29].

The application of the homeopathic medicine at a concentration of 40 µL/mL exhibited remarkable cytotoxic effects on hepatocellular carcinoma cells (HepG2), leading to a significant reduction in cell viability. Tumor cells have mechanisms to evade the immune system, enabling their unimpeded proliferation. One such mechanism involves the secretion of immunosuppressive substances, which hinder the maturation process of dendritic cells. An *in vitro* study conducted with VA revealed that the extract derived from this plant effectively counteracted the immunosuppressive effects induced by tumor cells, a phenomenon likely linked to the presence of lectins within the plant extract[30].

Another mechanism of action employed by tumor cells involves disrupting the glycolysis cycle, increasing glucose uptake and aerobic glycolysis, even in the presence of oxygen. The conversion of glucose to lactic acid in the presence of oxygen was described by Warburg in 1920 and is known as the "Warburg effect"[31]. In a study conducted by Melo et al. (2022) using breast cancer cells, it was demonstrated that VA effectively inhibited the activation of the glycolytic pathway in tumor cells. This finding suggests an additional mechanism of action for the extracts derived from this plant[32].

Since tumor cells have different proliferation mechanisms, inducing apoptosis in these cells is a desirable treatment strategy, as it can impede tumor growth effectively. Indeed, this effect was observed when treating HepG2 cells with VA D30, as it decreased cell viability. This finding suggests that the homeopathic preparation of VA D30 may have induced apoptosis in the HepG2 cells, potentially hindering their growth. Similarly, treating breast cancer cells (HeLa) with extracts obtained from Visco resulted in a comparable effect, where plant proteins were identified as the causative factor behind caspase-3 activation and the elevation of reactive oxygen species levels[33].

In another study carried out on breast cancer cells, it was also observed that VA could effectively inhibit the phosphorylation of Src and STAT3 (a transcription factor that promotes the survival, proliferation, and immune evasion of cancer cells). The inhibition of STAT3 phosphorylation and the subsequent activation of apoptotic markers underline the involvement of the STAT3 pathway in the anticancer mechanisms of VA[34]. Thus, like breast cancer, overexpression and constitutive activation of STAT3 have been frequently found in hepatocellular carcinoma and associated with a poor prognosis[35].

Conclusion

Based on the results obtained and other studies conducted with VA, it is reasonable to conclude that VA may offer a promising approach for treating hepatocellular carcinoma and potentially be applicable to the treatment of various other types of cancer mentioned earlier. The effects observed on cell viability of HepG2 show that VA D30 was cytotoxic to tumor cells without causing harm to healthy cells (MSCs), demonstrating the selectivity of this medicine towards tumor cells and highlighting its preference for targeting tumor cells over normal cells. The observed effects suggest that VA's anticancer properties warrant further investigation for its therapeutic potential in combating diverse cancer types. However, more comprehensive research and clinical trials are needed to validate its efficacy and safety as a potential treatment option for these malignancies. Additional tests could be conducted to assess various concentrations of this medicine and explore the potential of achieving reduced viability of tumor cells at higher doses while safeguarding the growth of MSCs. These investigations would offer valuable insights into optimizing the therapeutic application of the medicine, striking an optimal balance between its antitumor effects and minimal impact on the growth and function of normal cells.

Conflict of Interest:

None.

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