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Research Article

Anti-Inflammatory Activity of *Viscum album* D6 Preparations in Eye Drops Formulation

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Abstract

Viscum album is widely recognized in complementary medicine for its antidiabetic, analgesic, anti-inflammatory, and antitumor properties. This plant contains various bioactive compounds, allowing for the observation of diverse pharmacological effects. The present study aimed to evaluate, by *in vitro* testing, the effect of the *Viscum album* 6DH eye drops on ocular inflammation using human keratinocytes (HaCaT) and human mesenchymal stem cells (MSCs). HaCaT cells were utilized for the MTT cytotoxicity assay, which determined the optimal concentration of the eye drops (8 µL/mL) for subsequent experiments. In the scratch assay, treatment with the eye drops resulted in significantly enhanced scratch closure compared to the control group. In parallel, MSCs were employed for cytokine analysis. Inflammation was induced using lipopolysaccharides (LPS) prior to treatment with the eye drops. Results indicated a reduction in the pro-inflammatory cytokines IL-8 and IL-12p70 following treatment. These findings suggest that the anti-inflammatory properties of *Viscum album* can be effectively harnessed in this pharmaceutical form (homeopathic eye drops), offering potential therapeutic applications for the treatment of ocular inflammation.

Keywords

Anti-inflammatory, Scratch Closure, Therapeutic Application

1. Introduction

Viscum album L., commonly known as European mistletoe or simply mistletoe, is a plant found mainly in Europe and Asia and has been studied for over 2,000 years due to its application in complementary medicine [1]. This plant is recognized for its antidiabetic, analgesic, anti-inflammatory, antiarrhythmic, and hypotensive properties, first explored in both traditional and popular medicine in ancient times [2].

Studying bioactive compounds in *Viscum album* (VA) contributes to understanding the plant's diverse pharmacological effects. Flavonoids, a class of polyphenolic compounds with antioxidant properties, are found in mistletoe leaves, while lectins, a group of glycoproteins, interact with cancer cell surface receptors, inducing apoptosis and arresting the cell cycle. Viscotoxins, also found in mistletoe, exhibit antitumor properties and play a role in immunomodulation [3].

Viscum album is also known for its anti-inflammatory

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properties. Inflammation is a basal physiological phenomenon in which innate and adaptive immune cells, as well as non-immune cells, induce the production of inflammatory mediators (chemokines, cytokines, and others) to act on various target tissues. Prostaglandin E2 (PGE2) and cyclooxygenase (COX-1 and COX-2) are involved in these processes, with PGE2 being synthesized by COX-1 and COX-2. COX-2, in particular, is upregulated in response to pro-inflammatory cytokines such as IL-1B, IFN-gamma, and TNF-alpha [4].

Interleukins are critical indicators of inflammation, and their quantification can provide insights into whether inflammation has increased or decreased. IL-8, for instance, is a marker for various diseases, including ocular inflammations, where elevated levels contribute to angiogenesis in the conjunctiva, cornea, iris, retina, and orbit, leading to inflammation [5]. IL-12p70 is another significant interleukin, often found in high concentrations in ocular inflammations such as keratoconjunctivitis sicca [6].

Given the therapeutic potential of *Viscum album* and its long-standing use in complementary medicine, this study aimed to evaluate the safety and efficacy of a homeopathic eye drops formulation (Viscum 6DH) by *in vitro* testing using human keratinocytes (HaCaT). The goal was to explore a novel pharmaceutical application of this plant.

2. Methods

2.1. Preparation of *Viscum album* 6DH

The Mother Tincture served as the starting material to prepare the tested substance (Viscum album 6DH). The Hahnemannian Decimal Method was used, as described in the Brazilian Homeopathic Pharmacopoeia. One part of the active ingredient was mixed with nine parts of the inert ingredient, using a sterile isotonic solution, and succussed 100 times to produce Viscum D1 (1×10^{-1}). Subsequently, one part of Viscum D1 was combined with nine parts of the inert ingredient and succussed 100 times, resulting in Viscum D2 (1×10^{-2}). This process of successive dilutions was repeated until Viscum 6DH was obtained. The final product was then packaged in 1.1 mL ampoules.

2.2. Cell Culture

Human keratinocytes (HaCat) were cultured in 75 cm^2 culture flasks with Dulbecco's Modified Eagle Medium (DMEM) with high glucose concentration, supplemented with fetal bovine serum (FBS). The culture flasks were incubated at 37 ℃ with 5% CO_2 in an incubator. The culture medium was exchanged every 48 hours until the cells reached a confluence between 70-80%.

2.3. Product Cytotoxicity Assessment

Cell viability was assessed using the MTT assay (3-4,5-dimethyl-thiazol-2-yl-2,5-diphenyltetrazolium bromide) to evaluate the cytotoxicity of the eye drops. HaCat cells were then plated in 96-well plates (10,000 cells/well), and the effects of the eye drops at a concentration of 8 µL/mL were evaluated. After 24h of treatment, MTT was added, and absorbance was measured at 570 nm using a spectrophotometer.

2.4. Scratch Assay

In addition to the MTT cytotoxicity assay, a scratch assay was performed to evaluate cell migration after making a scratch in the cell layer. HaCaT cells were plated in 12-well plates at a density of 500,000 cells/well and incubated for 24h at 37 ℃ and 5% CO_2 in an oven. After incubation, a mechanical scratch was created in the confluent cell monolayer. Cell migration from the edge of the lesion towards the discontinuity was then assessed to evaluate wound closure, following the method described by Cory (2011). A comparison was made between a control group and another group that was treated with the eye drops. Scratches were created using sterile P1000 pipette tips at a 90 ° angle in straight, single lines across each well. The wells were washed twice with 1X phosphate-buffered saline (PBS) without serum to remove detached cells from the supernatant. After creating the scratches, marks were made on the bottom of the plate with a permanent marker perpendicular to the lesion. The treatment was applied (photographs were taken at this time for later comparison). The treatment group received the eye drops at a concentration of 8 µL/mL, while the control group did not receive any treatment.

After an additional 24 hours of incubation, new images were captured in the same regions as the initial scratch. The images were analyzed using ImageJ software, and the percentage of scratch closure was calculated.

Statistical analysis was performed by GraphPrisma Version 9.5.0. Normality was assessed using the Shapiro-Wilk test, followed by the Student's T-test to compare the results between groups.

2.5. Cytokine Dosing

After reaching cell confluence, cultured human mesenchymal stem cells were subjected to trypsinization and plated in 96-well plates to induce inflammation with LPS. After 24 hours of incubation at 37 ℃ with 5% CO_2, the culture medium was removed, and the wells were washed with PBS. Subsequently, 200 µg/mL of LPS (lipopolysaccharides from *Escherichia coli* O55: B5 – Sigma Aldrich), diluted in an antibiotic-free medium, was added. A subsequent incubation was performed for an additional 24 hours.

Following the induction period of cellular inflammation with LPS, the medium was removed from the plate, and the wells were washed with PBS. The medicine was added at a final concentration of 8 µL/mL. The plate was then incubated for 24 hours under the previously described conditions.

After the treatment period, the supernatant was removed, and serum-free culture medium was added for 24 hours. The supernatant was collected for analysis of the released cytokines.

The assay was conducted using two controls: one with the addition of LPS and another without LPS.

Cytokine dosing was done with human mesenchymal stem cells by the BD™ Cytometric Bead Array (CBA) flow cytometry method that allows quantifying multiple proteins simultaneously. The samples were subjected to analysis according to the manufacturer's protocol. The cytokines dosed were IL8 and IL12p70.

3. Results

3.1. Cell Viability

After conducting the MTT cell viability assay, the treated group (8 µL/mL) exhibited 102% cell viability. In contrast, the control group, which did not receive treatment, demonstrated only 102 ± 1.7% cell viability, as depicted in Figure 1. These findings suggest that the eye drops were not cytotoxic under the evaluated conditions.

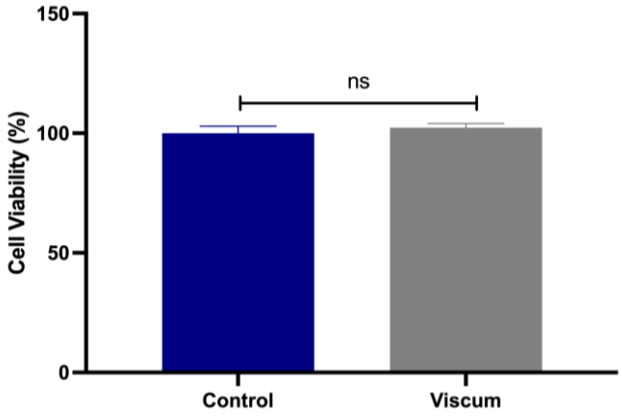

Figure 1. *Cell viability of the Viscum 6DH eye drops in HaCat cells after a 24-hour treatment. Control – cells that did not receive any treatment. Ns: no statistical difference.*

3.2. Scratch Assay

The HaCat cells were subjected to the cell proliferation evaluation assay after a scratch mimicking an eye lesion. Photographs were taken immediately after the scratch and again 24 hours post-treatment, as shown in Figure 2.

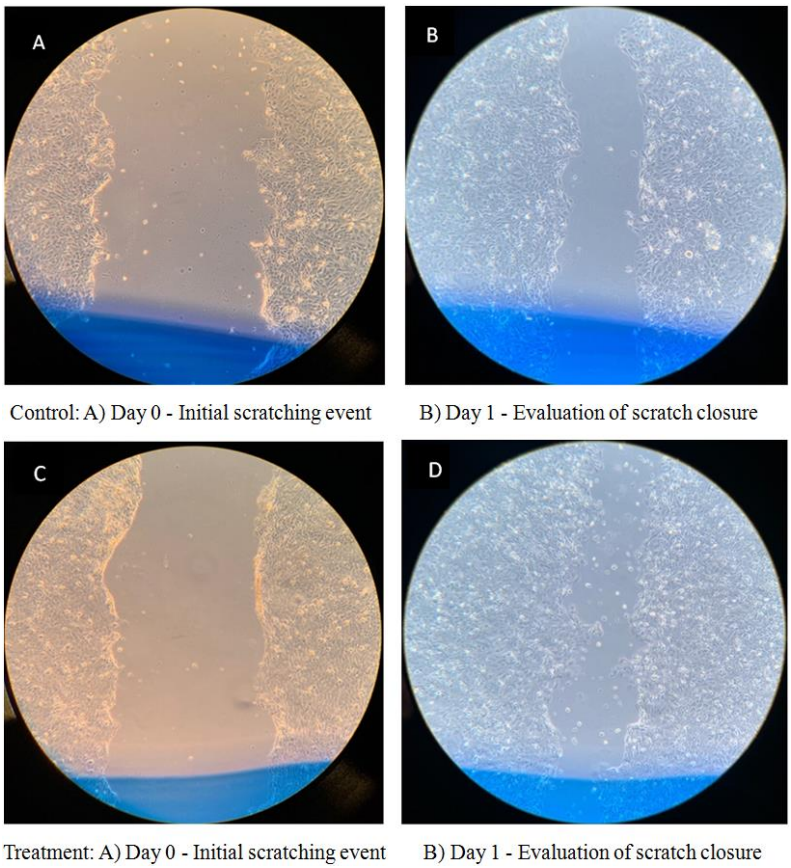

Figure 2. *Scratch assay performed on HaCat cells. HaCaT cells were incubated in 12-well plates for 24 hours in a CO_2 incubator. Afterward, a scratch was made in the cell monolayer, followed by treatment with the Viscum 6DH eye drops. After 24 hours of treatment, new photos were taken to evaluate cell migration. Control – cells that did not receive any treatment. Day 0 – scratching and treatment addition; Day 1 – 24h after incubation with the eye drops.*

Image analysis using ImageJ software revealed a significantly higher percentage of scratch closure in all triplicates treated with the eye drops compared to the control group, which received only the culture medium (Table 1). This result indicates that the eye drops facilitated the scratch closure process.

The data were considered normal (parametric), and statistical analysis, as illustrated in Figure 3, showed that the *Viscum* 6DH eye drops promoted more significant area closure (p=0.0071).

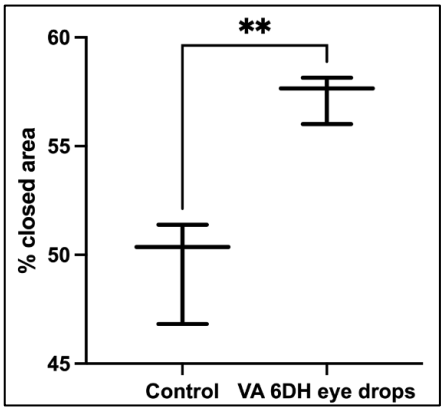

Figure 3. *Evaluation of the percentage of area closure in the control group (which did not receive the eye drops) compared to the group treated with the Viscum 6DH eye drops.*

Table 1. *Percentage of area closure in control and treatment with Viscum 6DH eye drops.*

Control (% area closure)	VA 6DH eye drops (% area closure)
47	58
51	58
50	56

3.3. Cytokine Dosage

LPS induced an inflammatory process, stimulating the production of IL-8 and IL12p70 in the cytokine dosage performed in human mesenchymal stem cells cultured with the *Viscum* 6DH eye drops. Upon treatment with the *Viscum* 6DH eye drops following inflammation induction, a substantial reduction in the production of these cytokines was observed, indicating the anti-inflammatory potential of the eye drops (Figure 4).

Notably, IL-8 levels in the treated group were even lower than those in the control group, which did not undergo inflammation induction. IL-12p70 levels in the treated group matched those of the control group. The normality analysis confirmed that the data were parametric.

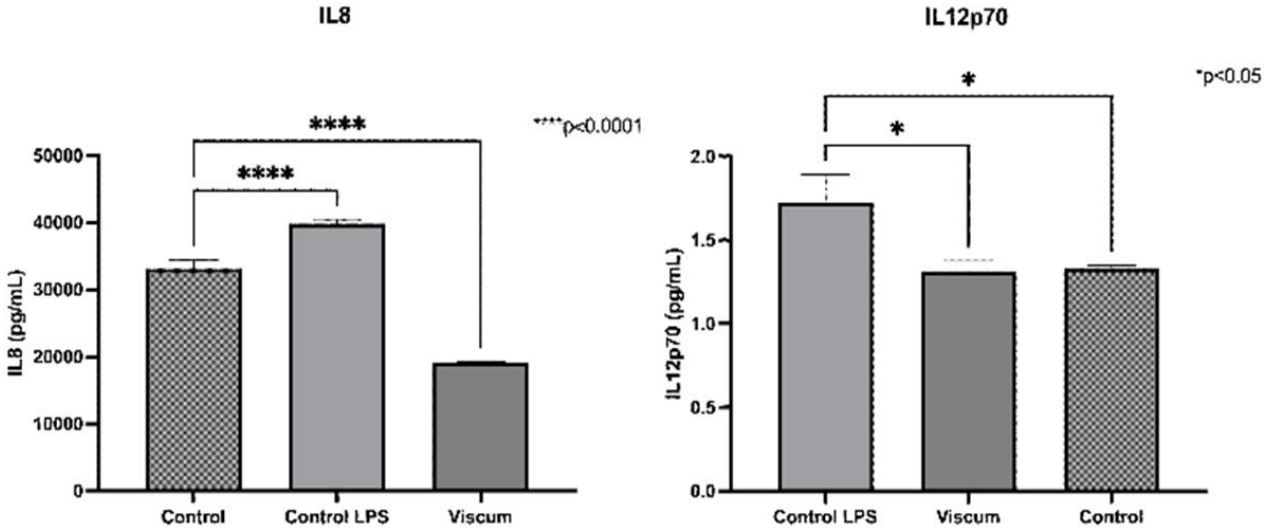

Figure 4. *Cytokine quantification in human mesenchymal stem cells after LPS-induced inflammation and treatment with the Viscum 6DH eye drops. LPS control – cells subjected to inflammation but without the eye drops treatment. Control – cells not subjected to inflammation or treatment.*

4. Discussion

In vitro tests play a fundamental role in research as they allow for the evaluation of a medicine's safety and efficacy using internationally accepted methodologies that do not involve animal testing. This approach has gained traction since 1959 when Russel and Burch introduced the theory of the 3Rs (Reduction, Refinement, and Replacement) [7].

Using *in vitro* methods to assess new eye drops is particularly important because it eliminates the need for tests like the Draize eye test in rabbits, which can cause severe eye damage [8]. Instead, human keratinocytes, such as HaCaT cells, can be cultured and used in 2D and 3D assays [9]. These cells closely resemble corneal cells, making them suitable for evaluating eye treatments.

In this study, HaCaT cells were cultured, followed by the MTT and scratch assays. The results demonstrated that the *Viscum album* 6DH eye drops were not cytotoxic to the cells. Additionally, treatment with the eye drops significantly enhanced scratch closure compared to the control group. Furthermore, in the cytokine dosage assay using human mesenchymal stem cells subjected to inflammation induction and treated with the eye drops, a marked reduction in IL-8 and IL-12p70 levels was observed after treatment.

Mistletoe species have a complex chemical profile that reflects different pharmacological activities. These activities include antiseptic, emetic, purgative, anti-inflammatory, antiarrhythmic, antispasmodic, antipsychotic, and antiepileptic properties [10].

While VA's therapeutic use is often associated with its antitumor effects, it is also known that cancer typically develops in an environment of heightened inflammation, suggesting that VA may act as an immunomodulator. Previous studies have demonstrated VA's immunomodulatory properties, including increased leukocytes, eosinophils, and granulocyte counts, specific antibody production, enhanced cytokine secretion, and improved cellular and humoral immune response, among others [11].

IL-8 is produced by various types of cells, including monocytes, lymphocytes, granulocytes, fibroblasts, endothelial cells, and epithelial cells, and is released only under inflammatory conditions [12]. The increased IL-8 production observed in the LPS-induced control group (Figure 4) confirmed the successful simulation of an inflammatory environment. The subsequent reduction in IL-8 levels after treatment with the *Viscum album* eye drops suggests its potential therapeutic role in managing inflammatory eye diseases.

Similarly, the pro-inflammatory cytokine IL-12p70, primarily secreted by activated macrophages [13], showed reduced levels following treatment with the *Viscum* 6DH eye drops (Figure 4), further supporting its anti-inflammatory effects.

Given that this medicine has been extensively studied as an immune system modulator with potential applications in cancer treatment [14], the approach presented in this study is both novel and promising.

5. Conclusions

The findings from this study present a novel application for *Viscum album*, providing *in vitro* evidence that this plant-based medicine can be formulated as homeopathic eye drops for ophthalmic use. The study indicates that the *Viscum album* 6DH eye drops could be a viable treatment option for ocular inflammation, potentially expanding the medicinal use of this plant. Further research is needed to elucidate the mechanism of action of this medicine, as well as to conduct studies in animals and humans to evaluate its in vivo response.

Abbreviations

HaCat	Human Keratinocytes
VA	Viscum Album
MTT	(3-4,5-dimethyl-thiazol-2-yl-2,5-diphenyltetrazolium Bromide)

Conflicts of Interest

The authors declare no conflicts of interest.

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