

Short Communication

Mistletoe Preparations as an Option for Treatment of Equine Sarcoids – Results of an *In vitro* Investigation on Cell Proliferation in 2D And 3D Design

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- Mistletoe lectin

Abstract

It is difficult to deal with the most common skin tumor in horses: the equine sarcoid. Its recurrence rate is very high and no single universally effective treatment without serious side effects is yet available.

Aqueous fermented mistletoe extracts of eight different host trees (pine tree, fir tree, hawthorn, apple tree, poplar tree, lime tree, oak tree, willow tree) were tested for their impact on the proliferation of the equine sarcoid cell line E42/O2 in a 2D and 3D design.

All mistletoe preparations inhibit the proliferation of the equine sarcoid cells *in vitro*. The order of the IC₅₀ concentrations was in accordance with the content of mistletoe lectin of the respective *Viscum album* preparations. The extract from the pine tree (Pini) showed the lowest potency to reduce the cell growth. The strongest inhibition of cell proliferation was obtained with the mistletoe extract of the willow tree (Salicis) with an IC50 concentration of 11 µg/ml in the 2D Alamar Blue assay and 1.2 µg/ml in the 3D Soft Agar assay. In addition to the high content of mistletoe lectin in the extracts from the mistletoe growing on willow tree, further components need to be taken into consideration to assess the full potential, such as the various acids exhibiting anti-tumoral action as well as the anti-phlogistic salicin.

These results indicate that the treatment with mistletoe preparations presents an effective therapy for horses suffering from equine sarcoids. The preparation from the willow tree mistletoe offers both cytotoxic and anti-inflammatory activity which awaits to be proven in an *in vivo* design.

ABBREVIATIONS

IC₅₀: Inhibitory concentration of 50%; BVP: Bovine papillomavirus; BCG: Bacillus Calmette Guérin; DNA: Deoxyribonucleic acid; RNA: Ribonucleic acid; ML: Mistletoe lectin; VT: Viscotoxin

INTRODUCTION

The equine sarcoid is the most prevalent skin tumor and can affect all equidae [1]. Equine sarcoids are caused by infection with BVP types 1 and 2 transmitted through insects [2]. Sarcoids are local aggressive tumors without a tendency to build metastasis and occur at the whole body of horses. Sarcoids are by no means merely a cosmetic problem, but may result in an uselessness or a loss of the horse. According to specific appearance five types of the equine sarcoid can be differentiated: occult, verrucose, nodular, fibroblastic and malignant [3]. The occult type features alopecia, skin thickening and scaling. The verrucose type has a warty or verrucose appearance (Figure 1A). The nodular equine sarcoids are sessile or pedunculated, solid, subcutaneous, spherical

tumors (Figure 1B). The malignant form is an aggressive locally invasive form. Fibroblastic sarcoids represent the most aggressive type and exhibit a fleshy fibrovascular appearance; either with thin pedicle or a wide flat base that commonly bleed easily. They may have a wet, haemorrhagic surface (Figure 1C).

The great variety of treatment methods of sarcoids ranging from chemotherapy to homeopathy shows that an universally effective therapy is often not possible. High tendency to recurrence, for treatment often unfavorable localization and the capacity of sarcoids to develop quickly from a milder form to an aggressive one after even slight damage make a successful treatment difficult. Many veterinarians use a combination of different treatment methods to reduce the relapse risk. Classical surgery is often used but has a high failure rate due to tumor relapse, especially if it used as single therapy [4-6]. In clinical practice further treatment options including cryosurgery, laser surgery, BCG immunotherapy, radiotherapy, intratumoral chemotherapy and topical formulations including zinc chloride, bloodroot extract, imiquimod or aciclovir are used. These

therapeutic options often imply undesirable side effects, e. g. necrosis, oedematous swelling or paralysis of nervus facialis after treatments at the head [1,7]. Another treatment option without serious side effects are injections based on mistletoe preparations. In the human tumor therapy the anti-tumoral, anti-angiogenic, immunomodulating and apoptosis inducing effects of the mistletoe therapy were verified in many clinical and preclinical trials [8-16]. In a study with horses suffering from equine sarcoids complete or partial remission without serious side effects and a low recurrence rate could be achieved by *Iscador® P* (Iscador AG, Arlesheim, Schweiz), a mistletoe preparation from pine tree (Figure 2A) [17]. The following investigations cover systematic

assessment of the impact of mistletoe extracts from different host trees on the growth of equine sarcoid cells. An earlier publication of these investigations was dedicated to therapeutic potential of the mistletoe extracts [18].

MATERIALS AND METHODS

Aqueous fermented extracts from mistletoe (*Viscum album*) from eight different host trees (fir tree, hawthorn, apple tree, pine tree, poplar tree, oak, willow and lime tree) produced according to German Homeopathic Pharmacopoeia, version 38 by WALA Heilmittel GmbH for the manufacture of the mistletoe preparations *Iscucin® Abietis*, *Iscucin® Crataegi*, *Iscucin® Mali*,



Figure 1: (A)- Equine sarcoid from verrucose type at 11 years old Haflinger mare
(B)- Nodular equine sarcoid with ulcerate surface at 7 years old Arabian mare (grey)
(C)- Fibroblastic equine sarcoid at 18 years old warmblood horse (gelding). 5 years after colic surgery at poorly healing wound suture.



Figure 2: (A) - Mistletoe from pine tree; (B) - Mistletoe from willow tree.

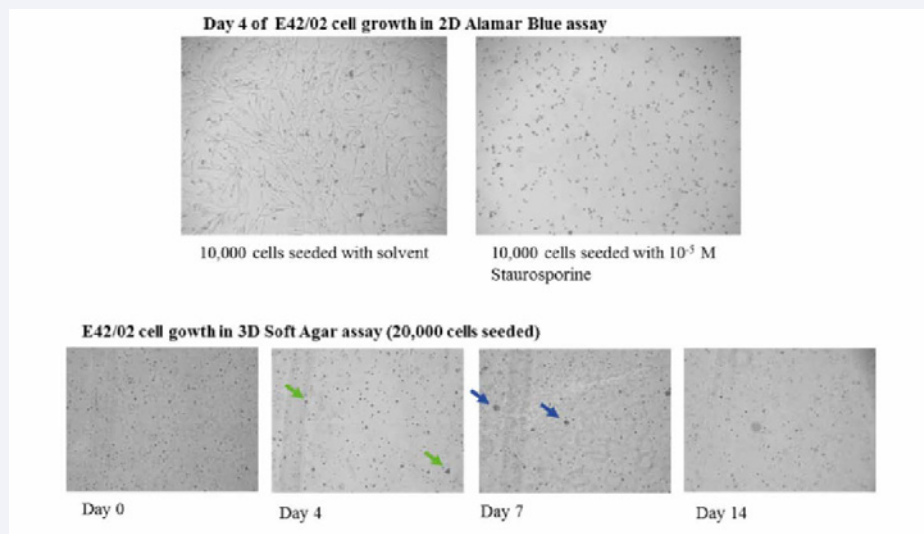


Figure 3: The cells showed a clear growth on day 0 to 4 in both assays. After day 4 individual cell colonies kept growing in the 3D Soft Agar assay. The optimal density of seeding the equine sarcoid cells was 10,000 cells per well in the 2D Alamar Blue assay. The cells incubated with 10⁻⁵ M Staurosporine (low control) showed no growth, but cells incubated with solvent (high control) proliferated. The optimal density of the equine sarcoid cells was 20,000 cells per well in the 3D Soft Agar assay.

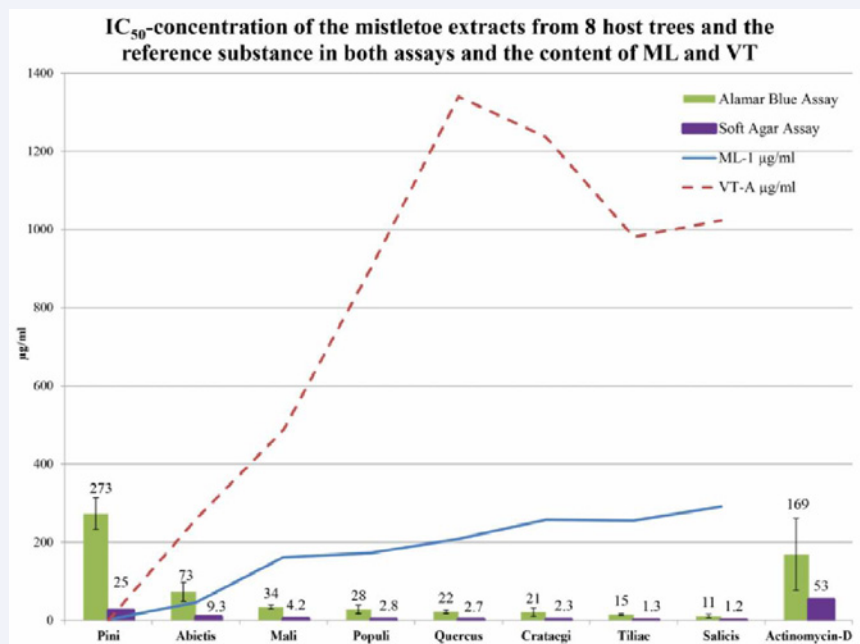


Figure 4: Cells of equine cell line E42/02 were treated with aqueous mistletoe extracts from eight host trees and with the reference substance, incubated 72 hours in the 2D Alamar Blue assay and incubated 144 hours in the 3D Soft Agar assay. The Figure shows the IC₅₀ concentrations in descending order and the respective contents of ML and VT. The tests were conducted in triplicate in the 2D Alamar Blue assay and in singlicate in the 3D Soft Agar assay.

Iscucin® Pini, *Iscucin® Populi*, *Iscucin® Quercus*, *Iscucin® Salicis* and *Iscucin® Tiliae* (Table 1) were tested for their impact to inhibit cell proliferation of the equine sarcoid cell line E42/02 (fibroblastic type) provided by Friedrich-Loeffler-Institute (Greifswald-Insel Riems, Greifswald).

Cellular proliferation assays conducted and modified by ProQinase GmbH (Freiburg, Germany). They are widely used

and accepted in oncology for analysis of the impact of anticancer drugs. The most common cellular phenotypic assay is the 2D Alamar Blue assay [19], which is generally regarded as a pretest to more sophisticated assays such as apoptosis or the 3D Soft Agar assay [20]. Anchorage-independent cell growth measured in the 3D Soft Agar assay is one of the hallmark characteristics of cellular transformation and uncontrolled cell growth, with normal cells typically not capable to proliferate in semisolid

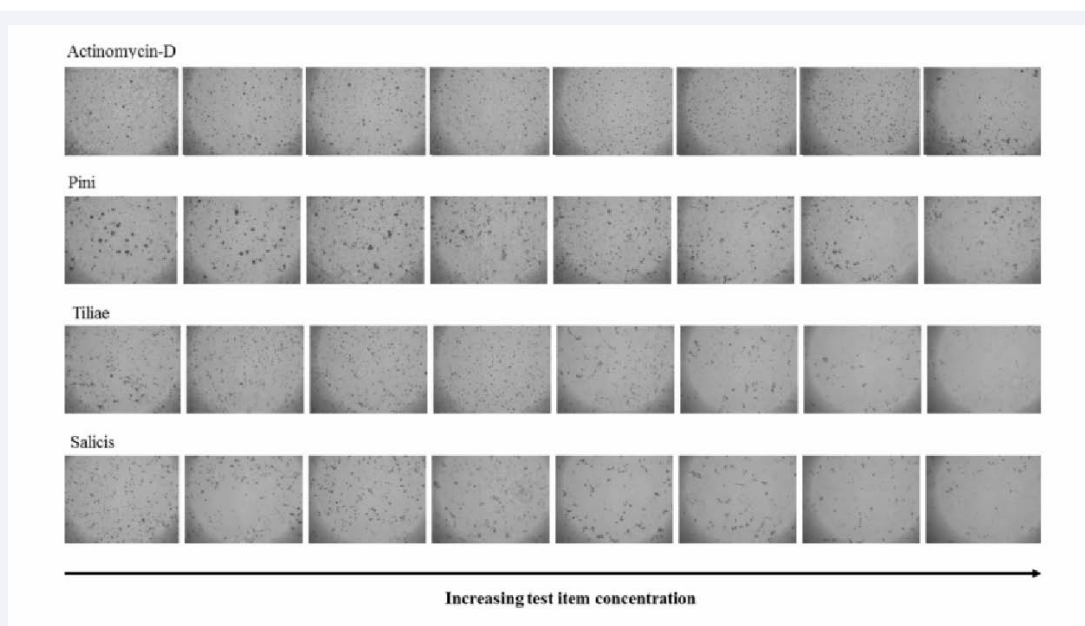


Figure 5: The picture shows the cell line in culture in the 3D Soft Agar assay treated with eight increasing concentrations (from left to right) of the reference substance Actinomycin-C and mistletoe extracts from three host trees (pine (Pini), lime (Tiliae) and willow (Salicis) tree). At the higher concentration of Salicis and Tiliae the equine sarcoïd cells are strongly minimized and slightly with Pini. For Actinomycin-C only at the highest concentration a reduction of the cells could be observed.

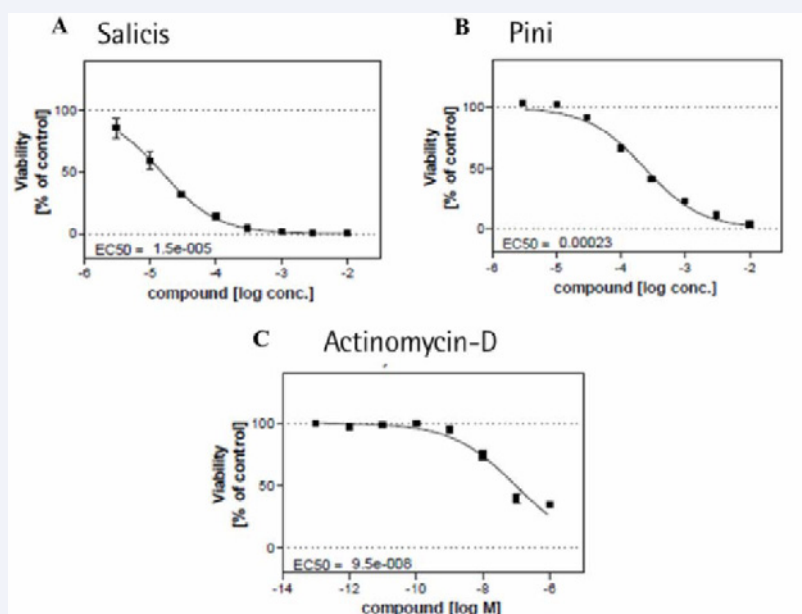


Figure 6: (A)- IC_{50} of mistletoe extract Salicis (willow) in the 2D Alamar Blue assay: 11 $\mu\text{g/ml}$, (B)- IC_{50} of mistletoe extract Pini (pine tree) in the 2D Alamar Blue Assay: 273 $\mu\text{g/ml}$, (C)- IC_{50} of reference substance Actinomycin-D in the 2D Alamar Blue assay: 169 $\mu\text{g/ml}$; Actinomycin-D was not able to inhibit the proliferation completely at the highest tested concentration.

matrices. 3D Cell growth is more similar to the *in vivo* cellular environment. Both, the 2D Alamar Blue and the 3D Soft Agar assays, are based on the quantification of the population of living cells after compound incubation using a fluorescent cell viability dye. Living cells reduce blue resazurin into the pink-colored and highly red fluorescent resorufin [21]. Raw data (fluorescence intensity) were converted into percent cell viability relative to

high controls (solvent) and low controls (10^{-5} M Staurosporine),

Which were set to 100% and 0%, respectively? IC_{50} calculation was performed using GraphPad Prism 5 software.

At the beginning the conditions and the optimal density of the E 42/02 cells were determined. Both in the 2D Alamar Blue Assay and the 3D Soft Agar assay, the cells showed a clear growth on

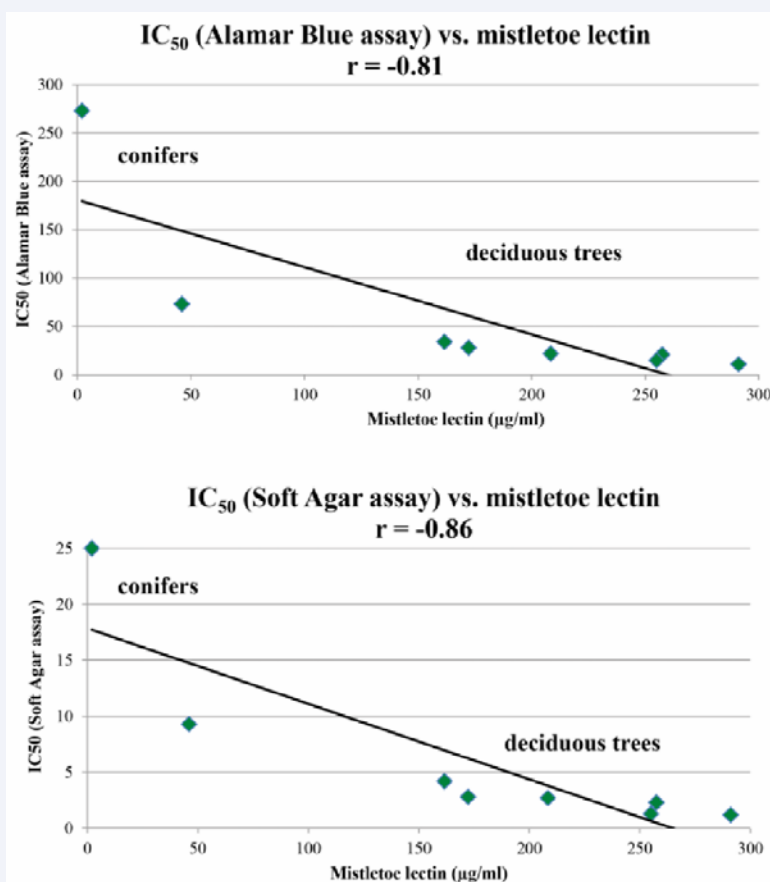


Figure 7: The correlation analysis showed that the mistletoe lectin contents and the IC₅₀ concentrations correlate with each other. There is a negative correlation. The mistletoes of the deciduous trees match well together. The mistletoes of the conifers drop a little bit out of the linear correlation.

Table 1: Overview of the mistletoe preparations (*Iscucin*[®]) from different host trees with their contents of the two substantial ingredients mistletoe lectin and viscotoxin.

Preparation name	Host tree	Content of ML	Content of VT
<i>Iscucin</i> [®] <i>Abietis</i>	fir tree	low	low
<i>Iscucin</i> [®] <i>Crataegi</i>	hawthorn	high	high
<i>Iscucin</i> [®] <i>Mali</i>	apple tree	middle	low
<i>Iscucin</i> [®] <i>Pini</i>	pine tree	low	low
<i>Iscucin</i> [®] <i>Populi</i>	poplar tree	middle	middle
<i>Iscucin</i> [®] <i>Quercus</i>	oak	middle	high
<i>Iscucin</i> [®] <i>Salicis</i>	willow	high	middle
<i>Iscucin</i> [®] <i>Tiliae</i>	lime tree	high	middle

Abbreviations: ML: mistletoe lectin; VT: viscotoxin; Low ML: 10,000 to ≤ 100,000 ng/ml; Middle ML: > 100,000 to ≤ 200,000 ng/ml; High ML: > 200,000 to ≤ 300,000 ng/ml; Low VT: 0 to ≤ 500 µg/ml; Middle VT: > 500 to ≤ 1,000 µg/ml; High VT: > 1,000 to ≤ 1,500 µg/ml

day 0 to 4. In the Alamar Blue assay the cells did not proliferate after day 4, but in the Soft Agar assay individual cell colonies grew further. The optimal density of the equine sarcoid cells was 10,000 cells per well in the Alamar Blue assay and 20,000 cells in the Soft Agar assay (Figure. 3). A very good correlation between cell number and reduction of resazurin at 5 hours was analysed by fluorescence according to an earlier report [22]. On the basis of these results the incubation time was calculated. The incubation time of the test items was 72 hours in the 2D

Alamar Blue assay and 144 hours in the 3D Soft Agar assay. The final assay concentration of the mistletoe extracts started at a dilution of 1:100 with further semi-logarithmic dilution steps in the Alamar Blue assay. In the 3D model the extracts of *Viscum album* from the coniferous trees were diluted in the same way, but the dilution of the extracts of *Viscum album* from deciduous trees began at 1:10,000. The antineoplastic agent Actinomycin-D serves as reference substance. It belongs to the group of cytotoxic antibiotics, binds to DNA and can inhibit RNA-synthesis. In

Alamar Blue Assay each sample was tested in three independent experiments.

RESULTS AND DISCUSSION

All tested mistletoe extracts were able to inhibit the proliferation of the equine sarcoid cell line E 42/02 in the analysed concentration range in both test systems. The anti-proliferative potential was higher in the 3D model than in the 2D cell layer (Figure. 4). Possible reasons might be that the cells are more sensitive in the 3D system than in the cell layer or / and that the incubation time was longer in the Soft Agar assay. The order of the IC₅₀ concentrations of the different mistletoe extracts was equal in both tests. The Salicis (willow) preparation showed the highest potency in the assays (Alamar Blue assay: IC₅₀: 11 µg/ml; Soft Agar assay: IC₅₀: 1.2 µg/ml), followed by Tiliae (lime tree) (Alamar Blue assay: IC₅₀: 15 µg/ml; Soft Agar assay: IC₅₀: 1.3 µg/ml). Pini (mistletoe preparation from pine tree) had the lowest potency to inhibit the growth of the equine sarcoid cells (Alamar Blue assay: IC₅₀: 273 µg/ml; Soft Agar assay: IC₅₀: 25 µg/ml) (Figure. 5). The reference substance Actinomycin-D (IC₅₀: 169 µg/ml) was more effective than Pini although Actinomycin-D was not able to inhibit the proliferation totally at the highest concentration tested in the Alamar Blue assay (Figure. 6). Interestingly in the 3D model that most likely compares to the *in vivo* situation, Actinomycin-D (IC₅₀: 53 µg/ml) was only half of potency than Pini.

The order of the IC₅₀ concentrations of the mistletoe extracts from different host trees followed the order of the mistletoe lectin contents of the respective *Viscum album* preparations. The calculated correlation coefficient after Pearson (r) amounts for IC₅₀ (Alamar Blue assay) vs. mistletoe lectin -0.81 and for IC₅₀ (Soft Agar assay) vs. mistletoe lectin -0.86, respectively. The Figure showed a good correlation between the mistletoes with higher lectin content. The mistletoes of the conifers (Pini and Abietis) drop a little bit out of the linear correlation (Figure. 7). Compared to other preparations, the one from willow tree showed both a high content of mistletoe lectins and an average level of viscotoxins (Figure. 2B), but also additional features such as high contents of nucleosides, protocatechuic acid, ferulic acid, chlorogenic acid and caffeic acid [23,24]. These substances also are associated with anti-cancer characteristics [24-28]. Moreover, specifically salicin has been found in willow mistletoe extract. Salicin is known for its pain killing as well anti-phlogistic effects [29,30].

CONCLUSION

The results obtained in the *in vitro* cell proliferation assay with mistletoe extracts from different host trees using an equine sarcoid cell line are promising. *Viscum album* preparations (*Iscucin*[®]) inhibit the cell proliferation of the equine sarcoid cell line more effectively than the reference substance Actinomycin-D in 2D and 3D systems. The anti-proliferative effect of mistletoe extracts on the equine sarcoid cell line E 42/02 *in vitro* pre Figure an effective systemic treatment or local therapy option of the equine sarcoid without severe side effects. In the previous published study the mistletoe injections were well tolerated by the horses. Only mild edema at injection sites occurred, which disappeared within 2-3 days without treatment. No systemic

signs were reported [17]. The median lethal dose at mice after parenteral administration amounts > 32 mg/kg. Mistletoe extracts showed no genotoxic, immunotoxic or carcinogenic activity [31]. At humans, low side effect, such as local reaction with swelling < 5 cm and erythema, are desirable at the anthroposophic medicine. Other adverse drug reactions (e. g. fever, headache) were mostly mild and self-limiting [13,32-35]. The mistletoe extract from the willow tree (Salicis) containing high mistletoe lectins and an average level of viscotoxins as well other specific ingredients with anti-tumoral actions showed a strong potential to inhibit the growth of fibroblastic equine sarcoid cells. Because of its level of salicin it might an additional role in the treatment of inflammatory processes correlated with equine sarcoid diseases. Continuing investigations *in vivo* are underway to corroborate these findings and to analyse the practical application as well as differentiation between the host trees.

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