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# Antitumor effects of *Viscum album* L. on Ehrlich Ascites Carcinoma *In vivo*

**Orsolya SÁRPATAKI [1)], Bogdan SEVASTRE [1)], Neli Kinga OLAH[2)], Daniela HANGANU[3)], Marian TAULESCU[1)], Radu MĂNĂLĂCHIOAE[1)], Cornel CĂTOI[1)], Ioan MARCUS[1)]**

[1)]University of Agricultural Science and Veterinary Medicine, Faculty of Veterinary Medicine, Manastur Street. 3-5, 400372, Cluj-Napoca, Romania, orsisarpataki@gmail.com
[2)]SC PlantExtrakt SRL, 407059 Rădaia, Cluj-Napoca,
[3)] University of Medicine and Pharmacy "Iuliu Hateganu", Faculty of Pharmacy, Emil Isaac Street 13. Cluj-Napoca, Romania

**Abstract.** *Viscum album* L. is a parasitic plant that grows on various trees, commonly known as European mistletoe. In the present study, the antitumor effects of mistletoe have been assessed. The model of choice was Ehrlich Ascites Carcinoma (EAC), which was inoculated in Swiss female mice. The *Viscum album* (VA) therapy was applied before and after EAC inoculation, and the results were both compared to EAC inoculated alone and with doxorubicin treated groups. In EAC injected animals, VA significantly increased the WBC count and the granulocytes number; while in the animals receiving VA before EAC administration, the plant extract seems to provide a lesser effect. The antiproliferative effect of VA was stronger in pretreated group, manifested by a lower cellular concentration, and a higher percentage on non-viable tumor cells.

**Keywords**: *Viscum album*, tumor cells, mice, mistletoe

## INTRODUCTION

*Viscum album* L. is a parasitic plant that grows on various trees. It is commonly known as European mistletoe. *Viscum album* preparations are used as a complementary medicine in cancer therapy. Although the beneficial properties of European mistletoe have been documented throughout history, its therapeutic application has been changing with the development of science.

Recently, many *in vitro* and *in vivo* studies have examined the antitumor properties of *Viscum album* extracts or certain constituents isolated from these extracts (Cebovic *et al.*, 2008, Khil *et al.*, 2007). It is thought that the molecular basis of the antitumoral activity of mistletoe lies in two distinct bioactivities. First, its lectin content is responsible for direct toxicity to tumor cells (Khil *et al.*, 2007). Secondly, the *Viscum album* rhamnogalacturonan oligosaccharide favours bridging of natural killer tumor cell conjugates, enhancing the cytotoxic efficiency. Moreover, it has been found that the antitumoral human cytotoxic T lymphocytes with CD T cell receptor are selectively activated by mistletoe ligands of phosphoantigen structure (Tabiasco *et al.*, 2002).

The aim of the present study is to assess the antitumor properties of local harvested mistletoe, before and after tumor cells implantation.

## MATERIALS AND METHODS

*Plant materials*: The *Viscum album* (plant raised from the apple tree – *Malus communis*) was harvested from Cluj area, in November – December 2009. The vegetal products were dried and grounded to a fine powder.

*Preparation of tinctures:* The *Viscum album* L. was prepared according to European Pharmacopoeias, method 2a, by cold extraction (maceration). 100 g of fresh *Viscum album* L. plant was cut to a pasta consistency (moisture 70 %). To cut plant material was adding 70 g 90 % vol. ethanol. The plant-ethanol mixture was macerate 10 days with periodical mixing and then pressed and filtered. The extraction ratio was 1:1 plant to extract (mother tincture). The obtained mother tincture has 8.5 % dry residue.

To prevent toxic effect of the alcohol, often more toxic than plant compounds dissolved in it, alcoholic solution was maintained in a water bath until 3/4 of the content evaporates, than filed with sterile saline solution up to 0.5 ml / animal. The aqueous solution was administrated i.p., immediately, in order to prevent the bacterial and fungus contamination.

*The animals* were caged in groups of 8 per cage, at controlled temperature of 21-22ºC, humidity (40-60%) and reversed 12/12h light/dark cycle (light off at 10 a.m.). Standard lab chow, provided by National Institute for Research and Development "Cantacuzino" Bucharest (Batch no. 2 / 26.03.2010), and water were freely available. The animal tests and experiments were allowed by the Bioethical Board of the Faculty of Veterinary Medicine Cluj-Napoca.

The experiment was carried out on 48 white Swiss female mice, 35.65 ±0.54 g body weight. The animals were divided in six equal experimental groups. The first group was the Control, the second one received *Viscum album* (VA) extract i.p. in the day 1, 3 and 6 - 50 mg d.s./kg b.w. (*Viscum album* 1:1 plant to extract in ethylic alcohol 70°). The third received Ehrlich Ascitic Carcinoma (EAC), and the fourth was inoculated with EAC and received treatment with Doxorubicin (EAC + D). The last two groups were also inoculated with EAC, one was treated with VA three times at 6, 3 and 1 day before the inoculation; the other one received VA in the days 1, 3 and 6 after, in the same dose as the VA group.

EAC groups received $10^6$ ascitic cells/animal intra peritoneal, in the day 0, and the experiment spanned for 14 days. Ehrlich ascitic carcinoma (EAC) was a generous gift from the Oncology Institute "I. Chiricuţă" Cluj Napoca. Body weight was measured at the beginning, and at the end of experiment.

The Control group received 0.5 ml alcohol 70°, i.p (after evaporation in water bath in the same way like the plant extracts) and the EAC+D group received i.p. Doxorubicin chloride 2.5 mg/ kg b.w. (Adriablastina 10 mg – Pfizer) in day1 and 6 of the experiment.

In the end, the blood was harvested from the retro orbitary sinus under diethyl ether anaesthesia and the euthanasia was made by prolonged ether narcosis. Total amount of ascitic fluid was measured, and viable tumor cell concentration was counted in a Burker camera (liquid diluted 1:100). Cell viability was assessed by tripan blue staining (Olinescu, 1992).

*Blood hematology* was investigated with Abacus Junior Vet, automatic analyzer Diatron Messtechnik, Budapest, Hungary.

*Statistics* - the data were expressed as the mean and standard error of the mean (SEM). T Student multiple range test from Excel Windows Software was used to assess the differences among groups. Differences at $p<0.05$ and $p<0.01$ were considered significant and respectively distinct significant.

## RESULTS AND DISCUSSION

The development of EAC was responsible for anemia in tumor bearing mice. The differences were seen mainly in RBC total count and HCT (Tab.1), the anemia was responsible for increased anisocytosis despite the fact that the RBC indices remained in normal range (Tab.2). Injected alone or after the EAC inoculation, the *Viscum album* L. (VA) extract was unable to influence the RBC count, HGB or HCT (Tab.1), however injected 6 days before EAC inoculation was responsible for the significant decrease of the MCHC (Tab.2).

Tab.1.

The effect of *Viscum album* alcoholic extract on the values of the red blood cells (RBCs), hemoglobin (HGB) and hematocrit (HCT) (mean ± S.E.M)

| | **RBC $10^{12}$/l** | **HGB g/l** | **HCT %** |
|---|---|---|---|
| **Control** | 8.00±0.47 | 128.83±7.95 | 38.58±1.98 |
| **EAC** | 6.52±0.49 | 107.80±7.86 | 31.78±1.91* |
| **EAC + Doxorubicin** | 7.14±0.59 | 110.38±9.64 | 33.51±2.75 |
| **EAC + *V. album*** | 6.91±0.25 | 113.70±4.43 | 32.71±1.49 |
| ***V. album* + EAC** | 5.67±0.66 | 90.62±11.8 | 27.46±3.12 |
| ***V. album*** | 8.16±0.22 | 133.87±3.42 | 39.13±0.80 |

**EAC** – Ehrlich ascites carcinoma inoculated group, **EAC + Doxorubicin** - Ehrlich ascites carcinoma inoculated group treated with doxorubicin chloride, **EAC + *V. album*** - Ehrlich ascites carcinoma inoculated group treated with *Viscum album* alcoholic extract after EAC inoculation, ***V. album* + EAC** - Ehrlich ascites carcinoma inoculated group treated with *Viscum album* alcoholic extract before EAC inoculation, ***V. album*** - group treated with *Viscum album* alcoholic extract,

*= $p<0.05$ as compared to Control group

Normal values:

RBC 7-12.5 $10^{12}$/l, HGB 102-180 g/L, HCT 36-49 % MCV 53.6-56 fl MCH 48.1-50 pg MCHC 31.3-33.2 g/dl

WBC 6-15 $10^{9}$/l (Uray Z., 1992)

The EAC inoculation was associated with significant leukocytosis, the granulocytes were in a very high number (neutrophilia), and middle cells were also increased. Doxorubicin administration down-regulates the leukocytosis throughout reduction of granulocytes synthesis. Injected alone, VA was not able to influence the WBC profile as compared to control. In EAC injected animals, VA (injected after EAC inoculation) significantly increased the WBC count and consequently the granulocytes number but not in significant manner. In the animals receiving VA 6 days before EAC administration, the plant extract seems to provide a lesser effect; WBCs count and neutrophils remains unchanged, but middle cells were significantly increased as compared to EAC group. Lymphocytes were also increased but not in significant manner. The significance remains obscure probably due to an enhanced antitumor specific response.

Tab. 2

The effect of *Viscum album* alcoholic extract on the values of mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC) and red blood cells distribution width (RDW) (mean ± S.E.M)

| | **MCV fl** | **MCH pg** | **MCHC g/dl** | **RDWs fl** |
|---|---|---|---|---|
| **Control** | 48.33±0.49 | 16.08±0.19 | 332.00±5.07 | 17.08±0.16 |
| **EAC** | 49.75±1.90 | 16.62±0.38 | 339.75±6.86 | 19.53±0.63** |
| **EAC + Doxorubicin** | 47.38±1.14 | 15.5±0.47† | 328.00±5.03 | 19.84±0.72 |
| **EAC + *V. album*** | 47.33±0.88 | 16.43±0.12 | 347.00±5.24 | 17.98±0.64 |
| ***V. album* + EAC** | 49.00±1.50 | 15.85±0.62 | 309.75±14.3† | 18.99±0.96 |
| ***V. album*** | 47.86±0.93 | 16.38±0.25 | 341.37± 2.63 | 16.78±0.18 |

**EAC** – Ehrlich ascites carcinoma inoculated group, **EAC + Doxorubicin** - Ehrlich ascites carcinoma inoculated group treated with doxorubicin chloride, **EAC + *V. album*** - Ehrlich ascites carcinoma inoculated group treated with *Viscum album* alcoholic extract after EAC inoculation, ***V. album* + EAC** - Ehrlich ascites carcinoma inoculated group treated with *Viscum album* alcoholic extract before EAC inoculation, ***V. album*** - group treated with *Viscum album* alcoholic extract,

*= $p<0.05$, **= $p<0.01$, as compared to Control group

†= $p<0.05$ as compared to EAC group

Normal values:

RBC 7-12.5 $10^{12}$/l, HGB 102-180 g/L, HCT 36-49 % MCV 53.6-56 fl MCH 48.1-50 pg MCHC 31.3-33.2 g/dl, WBC 6-15 $10^{9}$/l (Uray Z., 1992)

Tab. 3

The effect of *Viscum album* alcoholic extract on the WBC count and differential count (mean ± S.E.M.)

| | **WBC $10^9$/l** | **LYM $10^9$/l** | **MID $10^9$/l** | **GRA $10^9$/l** |
|---|---|---|---|---|
| **Control** | 6.85±0.74 | 5.08±0.62 | 0.18±0.02 | 1.59±0.17 |
| **EAC** | 21.98±5.19* | 5.73±0.94 | 0.61±0.14* | 15.62±4.79* |
| **EAC + Doxorubicin** | 12.32±5.09† | 4.38±0.82 | 0.35±0.10 | 7.59±4.29† |
| **EAC + *V. album*** | 55.7±19.54 | 8.40±2.82 | 0.90±0.38 | 29.54±11.49 |
| ***V. album* + EAC** | 23.39±2.66 | 7.43±1.15 | 1.09±0.11† | 14.83±2.07 |
| ***V. album*** | 6.80±0.76 | 4.82±0.55 | 0.23±0.10 | 1.75±0.19 |

**EAC** – Ehrlich ascites carcinoma inoculated group, **EAC + Doxorubicin** - Ehrlich ascites carcinoma inoculated group treated with doxorubicin chloride, **EAC + *V. album*** - Ehrlich ascites carcinoma inoculated group treated with *Viscum album* alcoholic extract after EAC inoculation, ***V. album* + EAC** - Ehrlich ascites carcinoma inoculated group treated with *Viscum album* alcoholic extract before EAC inoculation, ***V. album*** - group treated with *Viscum album* alcoholic extract

*= $p<0.05$ as compared to Control group

†= $p<0.05$ as compared to EAC group

Normal values:

RBC 7-12.5 $10^{12}$/l, HGB 102-180 g/L, HCT 36-49 % MCV 53.6-56 fl MCH 48.1-50 pg MCHC 31.3-33.2 g/dl, WBC 6-15 $10^{9}$/l (Uray Z., 1992)

The previous studies confirm that mistletoe extract improves the immune response in EAC inoculated mice not due to peritoneal irritation, but only if it is administrated after the EAC inoculation (Sevastre *et al*., 2010), no studies regarding the influence of *Viscum album* L. on WBCs before EAC inoculation were found.

The EAC development was as expected, followed by the increase in body weight, while doxorubicin provided a strong protective effect (Tab.4). VA, administrated before and after EAC inoculation, did not influence the variation of body weight in EAC inoculated mice (Tab.4). However previous studies suggest some antiproliferative effect of VA extract, the treated animals revealed lower difference in body weight as compared to untreated ones (Sevastre *et al*., 2010).

Tab. 4

The effect of *Viscum album* alcoholic extract on body weight changes in EAC inoculated mice (mean ± S.E.M).

| | **Initial b.w. (g)** | **Final b.w. (g)** | **dif. (g)** | **dif. (%)** |
|---|---|---|---|---|
| **Control** | 36.81±0.61 | 34.12±0.79 | -2.68±0.87 | -7.19±2.32 |
| **EAC** | 29.75±0.40 | 39.93±1.79 | 10.19±1.61*** | 34.12±5.21*** |
| **EAC+Doxorubicin** | 30.38±0.81 | 33.00±1.01 | 2.62±1.00†† | 8.96±3.69†† |
| **EAC+*V. album*** | 33.58±0.69 | 45.00±4.67 | 11.42±2.27 | 34.51±7.05 |
| ***V. album* + EAC** | 26.88±0.48 | 39.25±1.37 | 12.37±1.64 | 46.68±6.88 |

**EAC** – Ehrlich ascites carcinoma inoculated group, **EAC + Doxorubicin** - Ehrlich ascites carcinoma inoculated group treated with doxorubicin chloride, **EAC + *V. album*** - Ehrlich ascites carcinoma inoculated group treated with *Viscum album* alcoholic extract after EAC inoculation, ***V. album* + EAC** - Ehrlich ascites carcinoma inoculated group treated with *Viscum album* alcoholic extract before EAC inoculation, ***V. album*** - group treated with *Viscum album* alcoholic extract,
*** = $p<0.001$ as compared to Control group
†† = $p<0.01$ as compared to EAC group

The ascitic volume followed the trend of the body weight variation, therefore the doxorubicin treated groups shown very few amounts of ascitic fluid. The administration of VA has no obvious influence on the ascitic volume (Fig.1), despite the fact that literature data report the reduction of the ascitic fluid in a relevant manner following administration of mistletoe extract before EAC inoculation (Cebovic *et al*, 2008). The difference might be caused by the different methods of extraction used.

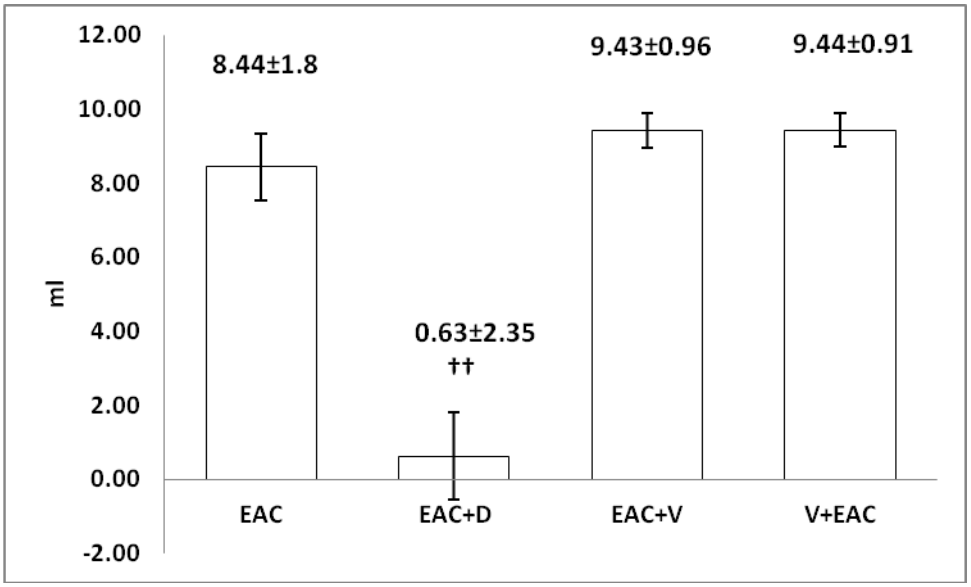


Fig. 1. The effect of *Viscum album* L alcoholic extract on the variation of the ascitic volume among experimental groups (mean ± S.E.M.) (ml)

**EAC** – Ehrlich ascites carcinoma inoculated group, **EAC + D** - Ehrlich ascites carcinoma inoculated group treated with doxorubicin chloride, **EAC + V** - Ehrlich ascites carcinoma inoculated group treated with *Viscum album* alcoholic extract after EAC inoculation, **V + EAC** - Ehrlich ascites carcinoma inoculated group treated with *Viscum album* alcoholic extract before EAC inoculation

†† = $p<0.01$ as compared to EAC group

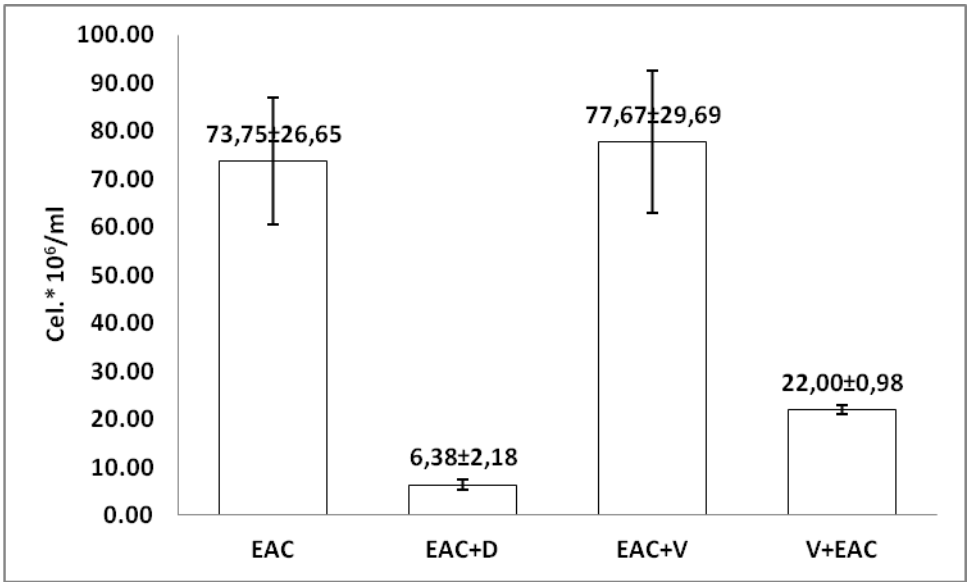


Fig. 2. The effect of *Viscum album* L alcoholic extract on the cellular concentration within ascitic fluid (mean ± S.E.M.) ($10^6$/ml)

**EAC** – Ehrlich ascites carcinoma inoculated group, **EAC + D** - Ehrlich ascites carcinoma inoculated group treated with doxorubicin chloride, **EAC + V** - Ehrlich ascites carcinoma inoculated group treated with *Viscum album* alcoholic extract after EAC inoculation, **V + EAC** - Ehrlich ascites carcinoma inoculated group treated with *Viscum album* alcoholic extract before EAC inoculation

The antiproliferative effect of VA was obvious in the cellular concentration is ascitic fluid, but this effect was found only in the group pre-treated with VA (Fig.2). Similar results were reported by Cebovic *et al* (2008), the previous administration of mistletoe extracts proved a significant protective effect in both males and females. Interestingly the post-treated animals showed higher cellular concentration in ascitic fluid similar to those found in EAC non-treated groups (Cebovic *et al*, 2008; Sevastre *et al*., 2010).

The percentage of non-viable cells was in inverse proportion with the cellular concentration, and reached the highest levels in the VA pre-treated group, even though the ascitic volume was very close to the EAC untreated group (Fig.3). The present findings were confirmed by bibliographical data, which reports, in female groups, highly increased percentage of non-viable cells in pre-treated animals and almost unchanged percent, as compared to EAC group, in post-treated animals. Notable, a significant difference in non-viable cell concentration between female groups and male groups may occur, but the explanation remains obscure (Cebovic *et al*, 2008).

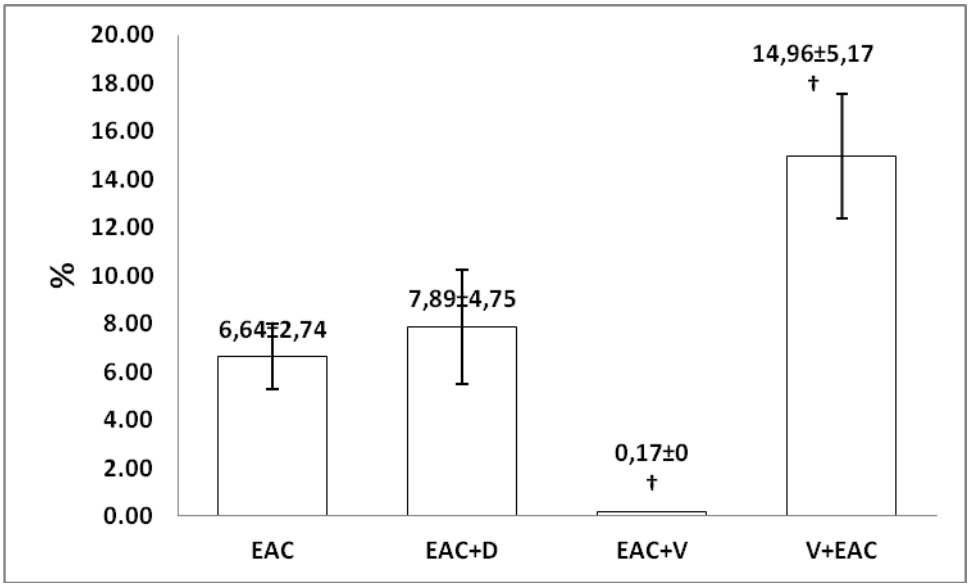


Fig. 3. The effect of *Viscum album* L alcoholic extract on the percentage of non viable cells (mean ± S.E.M.) (%)

**EAC** – Ehrlich ascites carcinoma inoculated group, **EAC + D** - Ehrlich ascites carcinoma inoculated group treated with doxorubicin chloride, **EAC + V** - Ehrlich ascites carcinoma inoculated group treated with *Viscum album* alcoholic extract after EAC inoculation, **V + EAC** - Ehrlich ascites carcinoma inoculated group treated with *Viscum album* alcoholic extract before EAC inoculation

†= $p<0.05$ as compared to EAC group

## CONCLUSIONS

In conclusion, the administration of VA extracts have a strong influence on WBC differential count mainly at the granulocytes level by up regulating the neutrophils reaction in EAC inoculated mice, suggesting an immunostimulatory effect. When VA was injected previously to EAC inoculation, the unspecific cellular immune response effect was less obvious, but an increased middle cells number. This before mentioned aspect might suggest a specific anticancer response.

Despite the fact that VA had no influence in the variation of the body weight, injected before EAC, the cytotoxic and antiproliferative effects were still ascertained by significant increase in the percentage of non-viable cells and the decrease of the cellular concentration in ascitic fluid.

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