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Viscum album L. Alcoholic Extract Enhance the Effect of Doxorubicin in Ehrlich Carcinoma Tumor Cells

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Abstract

Viscum album L. is a parasitic plant that grows on various trees, in our days, mistletoe extracts are widely used in addition to the treatment of cancer. The aim of the present study is to asses the efficiency of mistletoe extract in combination therapy with doxorubicin chloride, a widely used chemotherapeutic agent. The Viscum album (plant raised from the apple tree) was harvested from Cluj area, then dried and grounded to a fine powder. The tincture was prepared according to European Pharmacopoeias. Unexpectedly, in tumor bearing mice, mistletoe extract therapy does not provide the up regulation of the inflammatory cells; moreover even in healthy animals, the association between Viscum album and doxorubicin was responsible for the significant leucopenia, mainly because of the reduction of granulocytes synthesis. On the other hand, in doxorubicin combinative therapy, Viscum album alcoholic extract seems to enhance the anti proliferative effect against experimental inoculated cancer cells, while no cytotoxicity was seen.

Keywords: *Viscum album*, Ehrlich Ascites Carcinoma, doxorubicin

Introduction

Viscum album L. is a parasitic plant that grows on various trees. It is commonly known as European mistletoe.

Chemotherapy is the treatment of choice for many cancer patients, although, despite its certain benefits, important side effects like bone marrow suppression, hepatotoxicity, nephrotoxicity, and cardiotoxicity are commonly described. Therefore, finding new remedies with less toxicity or new therapies able to reduce the chemotherapy side effect is a highly active research domain.

In our days, *Viscum album* extracts are widely used in along chemotherapy in cancer treatment (1). Recently, many *in vitro* and *in vivo* studies have examined the antitumor properties of *Viscum album* extracts or certain constituents isolated from these extracts (2, 3), and various clinical studies revealed that mistletoe based therapies can improve the life quality, and survival time span in different cancer patients (1). It is thought that the molecular basis of the mistletoe antitumor activity lies in two distinct bioactivities. First, its lectin content is responsible for direct toxicity against tumor cells (3), secondly, the *Viscum album* rhamnogalacturonan oligosaccharide favors bridging of natural killer tumor cell conjugates,

enhancing the cytotoxic efficiency. Moreover, it has been found that the antitumor human cytotoxic T lymphocytes with CD T cell receptor are selectively activated by mistletoe ligands of phosphoantigen structure (4).

The aim of the present study is to asses the therapeutic efficiency of *Viscum album* L. alcoholic extract in combination therapy with doxorubicin chloride, a widely used chemotherapeutic agent.

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* tincture was prepared according to European Pharmacopoeias (5), method 2a, by cold extraction (maceration). An amount of 100 g of fresh *Viscum album* 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 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 at 50°C, 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 Control group received 0.5 ml alcohol 70°, i.p. (after evaporation in water bath similar to plant extracts).

Animal care and experimental procedures followed requirements put forth in the Guide for the Care and Use of Laboratory Animals (Department of Health and Education, and Welfare, National Institute of Health, 1996). The animal tests and experiments were allowed by the Bioethical Board of the Faculty of Veterinary Medicine Cluj-Napoca. The animals were caged in groups of 8 per cage, at controlled temperature of 21-22ºC, humidity (40-60%) and 12/12h light/dark cycle. 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 experiment was carried out on 64 white Swiss female mice, 37.84 ± 0.48 g body weights.

EAC was injected in 32 mice, each one received 10^6 ascitic cells intra peritoneally, in the day 0. The experiment was carried out 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.

Eight animals (previously inoculated with EAC) received *Viscum album* (VA) extract i.p. in the day 1, 3 and 6 - 50 mg dry substance (d.s.) /kg body weight (b.w.) (*Viscum album* 1:1 plant to extract in ethylic alcohol 70°) (EAC+VA). Other 8 were treated i.p. with Doxorubicin chloride 2.5 mg/ kg b.w. (Adriablastina 10 mg – Pfizer) in days 1 and 6 of the experiment representing the Ehrlich Ascitic Carcinoma with Doxorubicin (EAC + D). Another group (8 animals) received both treatments (EAC+VA+D) in the same way like the two previous group. The other 8 remaining animals were let untreated.

To assess the biological effects of substances in study, other 32 animals were subject to the same treatment like the previous groups, but without EAC inoculation.

In the end, the blood was harvested from the retro orbitary sinus under diethyl ether anesthesia 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 1:10). Cell viability was assessed by tripan blue staining (6).

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

Statistics - the data were expressed as the mean ± S.E.M., 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 Discussions

Blood count and red blood cells indices

The development of EAC was associated with a mild anemia, the red blood cells (RBCs) and HCT were significant below control (Tab. 1), but the red blood cells indices were not affected (Tab. 2).

The VA effects on blood count were inconstant. Alone, it does not influence the blood count, but associated with doxorubicin, VA was responsible for anemia.

Conversely, in EAC inoculated animals, VA does not seem to have the same anemia inducing effect; moreover, along doxorubicin, it seems to maintain the RBCs level significantly higher than those found in EAC group. The explanation of this paradox is yet to be ascertained; however, the protective effect seems to be rather indirect than a direct action on RBCs, the degree of anemia is related to tumor development, so by preventing the EAC growth, the association between VA and Doxorubicin prevents also the progression of anemia (Tab. 1). The RBCs indices, mainly, remained within normal limits (Tab.2).

White blood cells count and differential count

Inoculated alone, neither VA nor Doxorubicin was able to influence the white blood cells (WBCs) total count and differential, but together, surprisingly, they seem to have an immunosuppressive effect. The association between VA and Doxorubicin was responsible for leucopenia, mainly because of the down regulation of the lymphocytes.

EAC development was followed by significant leukocytosis; mainly due to increased granulocytes number, but middle cells were also increased in a significant manner. In EAC inoculated groups, VA raises even more the WBCs count but not in a significant manner, while doxorubicin therapy lowers WBCs count as compared to untreated EAC group, but the value remains much higher than in control.

The association between VA and doxorubicin in EAC inoculated group was responsible for a significant reduction of WBCs as compared to EAC inoculated group, all leucocytes categories were equally involved (Tab. 3). Outstandingly, all values were within the normal range and very similar to those found in control group! The association between VA and doxorubicin may have an immunosuppressive effect, but the prevention of leukocytosis, also, might be due to the reduction of tumor growth, but, in our point of view is too early to conclude.

The hematological toxicity of doxorubicin, manifested by anemia, leucopenia with neutropenia is widely known, but the effect of *Viscum album* compounds was unexpected. Well documented studies proved that mistletoe lectins (and other compounds) are able to improve the immune reaction throughout various mechanisms, including activation of macrophages and NK cells, but also by increasing leukocytes synthesis (4). They proved to be effective in cancer patients, subject of chemotherapy, but even more effective in healthy subjects (1).

The present study does not provide evidence for immune response stimulation, but, in our point of view, it does not prove adverse effect of mistletoe therapy either. Further study about the influence of this specific mistletoe extract on cell mediated immune response should be done.

Table 1. The effect of association between mistletoe extract and doxorubicin chloride on the blood count (mean ± S.E.M) red blood cells (RBCs), hemoglobin (HGB) and hematocrit HCT-

	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†
Doxorubicin + *V. album*	6.47±0.94	76.43±18.24*	30.50±4.49
Doxorubicin	8.29±0.14	135.70±4.12	39.77±1.15
V. album	8.16±0.22	133.87±3.42	39.13±0.80
EAC+Doxo+*V. album*	7.97±0.13†	126.00±5.34	37.14±1.13†

Table 2. The effect of association between mistletoe extract and doxorubicin chloride on the RBCs indices (mean ± S.E.M) mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC) and RBC volume distribution width (RDW)

	MCV fl	**MCH pg**	**MCHC g/l**	**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	15.50±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
Doxorubicin+ *V.album*	47.37±1.06	14.78±0.76	306.50±12.31	18.28±0.94
Doxorubicin	48.12±0.91	16.36±0.37	340.57±2.08	19.07±0.53**
V. album	47.86±0.93	16.38±0.25	341.37±2.63	16.78±0.18
EAC+Doxo+*V. album*	46.57±1.46	15.81±0.66	338.14±4.8	20.70±1.04

Table 3. The effect of association between mistletoe extract and doxorubicin chloride on the effect of association between mistletoe extract and doxorubicin chloride on the WBC count and differential (mean ± S.E.M.)
WBC-white blood cell count, LYM-lymphocytes, MID-middle cells, GRA-granulocytes

	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±0.1	7.59±4.29†
EAC + *V. album*	55.70±19.54	8.40±2.82	0.90±0.38	29.54±11.49
Doxorubicin + *V. album*	2.99±0.73**	2.02±0.44**	0.10±0.04	0.82±0.29
Doxorubicin	6.33±0.40	3.23±0.18*	0.22±0.11	.85±0.34**
V. album	6.80±0.76	4.82±0.55	0.23±0.10	1.75±0.19
EAC+Doxo+*V. album*	5.11±0.61†	3.00±0.41†	0.19±0.06†	1.91±0.32†

Table 4. The effect of association between mistletoe extract and doxorubicin chloride on the variation of body weight (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 + Doxo	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±1.91	11.42±2.27	34.51±7.05
Doxo+*V. album*	33.93±0.80	35.85±0.81	1.93±0.89†††	5.94±2.87†††

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, **D** – doxorubicin chloride treated group (whiteout EAC inoculation), **V** - group treated with *Viscum album* alcoholic extract, **D +V** – group treated with both doxorubicin chloride and *Viscum album* alcoholic extract, **EAC + D +V** - Ehrlich ascites carcinoma inoculated group treated with both doxorubicin chloride and *Viscum album* alcoholic extract.

*= p<0.05, **= p<0.01, *** = p<0.001 as compared to Control group
†= p<0.05, †† = p<0.01 †††= p<0.001 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 (7)

Influence on EAC development.

The EAC development was followed by the increasing in body weight, due to the accumulation of ascites; tumor development was prevented by the doxorubicin therapy in a significant manner. Injected alone, VA fails to prevent the EAC development, however the association between doxorubicin and VA seems to provide a better anti proliferative effect as compared to doxorubicin alone; in fact, all the values that reflects the tumor development (difference in body weight, EAC volume and cellular concentration) were improved in a significant manner (Tab. 4. and Fig. 1, 2 and 3).

The mechanism of doxorubicin is still unclear, but is widely accepted an antiproliferative mechanism based on intercalation on DNA molecule, and consequently by blocking the DNA biosynthesis, while *Viscum album* seems to act throughout an apoptotic mechanisms. Khil *et al.*, (3) found that *Viscum album* agglutinin induced apoptosis of colon cancer cells is due to the activation of caspases and inhibition of anti-apoptotic proteins partly through the TNFR1 signaling pathway. Even at low dose, purified mistletoe lectins reduced also melanoma growth and number of metastases in a xenograft model. The enhancement of infiltration and apoptosis induction in the melanoma cells seem to play the key role for these observed effects (8).

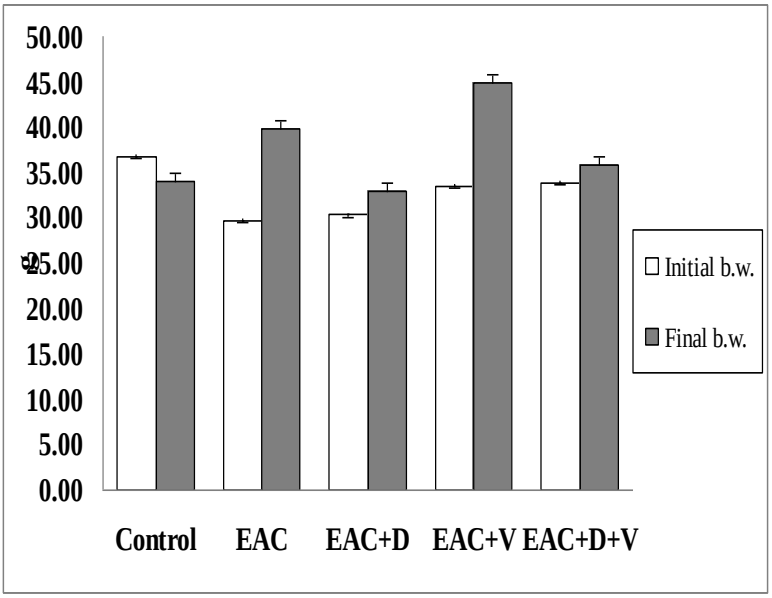

Figure 1. The effect of association between mistletoe extract and doxorubicin chloride on body weight in EAC inoculated animals (mean ± S.E.M.) (g)

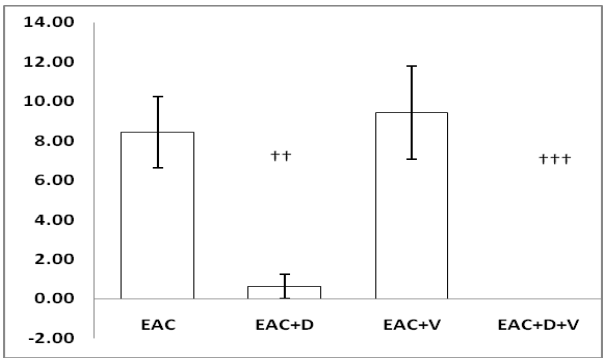

Figure 2. The effect of association between mistletoe extract and doxorubicin chloride on ascitic volume (mean ± S.E.M.) (ml)

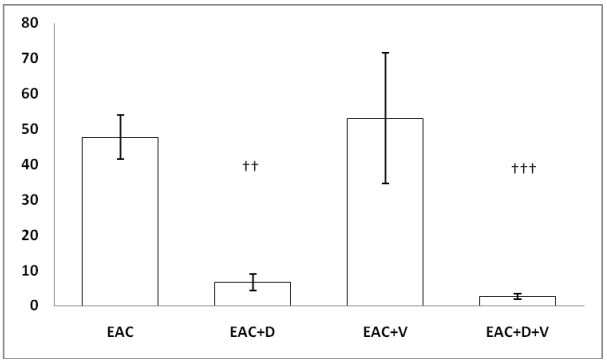

Figure. 3. The effect of association between mistletoe extract and doxorubicin chloride on the cellular concentration in the ascitic fluid (mean ± S.E.M.) (10^6/ml)

†= $p<0.05$, †† = $p<0.01$ ††† = $p<0.001$ as compared to EAC group

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, **D** – doxorubicin chloride treated group (whiteout EAC inoculation), **V** - group treated with *Viscum album* alcoholic extract, **D +V** – group treated with both doxorubicin chloride and *Viscum album* alcoholic extract, **EAC + D +V** - Ehrlich ascites carcinoma inoculated group treated with both doxorubicin chloride and *Viscum album* alcoholic extract.

Conclusions

The association between *Viscum album* L alcoholic extract and doxorubicin chloride, unexpectedly does not provide the up regulation of the inflammatory cells, moreover in healthy animals the association between these two was responsible for the significant leucopenia, mainly because of the reduction of granulocytes synthesis. In ascites inoculated animals, the combination chemotherapy maintains the inflammatory cells within normal range, whether this finding is beneficial for the animals or not, is a question for further studies.

Outstandingly, mistletoe extract enhances the anti proliferative effect of doxorubicin.

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