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Comparison of Antitumor Effect in Two *Viscum album* L. Extracts

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Abstract. *Viscum album* L. is a parasitic plant, widely used in addition to the treatment of cancer. Mistletoe alcoholic extract and glycerine macerate were inoculated in 40 white Swiss male mice, previously inoculated with Ehrlich Ascitic Carcinoma (EAC). Animals were divided in four equal groups; first group received i.p. in the day 1, 4 and 7 - 50 mg d.s./kg b.w. *Viscum album* 1:1 alcoholic extract. The second group received i.p. in the day 1, 4 and 7 – 20 ml/kg b.w. *Viscum album* 1 to 100 glycerine extract. The third group received i.p. Doxorubicin chloride 2.5 mg/ kg b.w. in day 6 of experiment, while the last group received a placebo. Other 40 animals were injected with the same plant extracts, placebo and Doxorubicin chloride, without EAC. In EAC group only 8 from 10 animals survived in the end of the study, in the other groups all animals survived to the end. *V. album* alcoholic extract increased the white blood cells (WBC) as compared to control, due to granulocytes (GRA), but the most important both mistletoe extracts provided increase WBC as compared to Doxorubicin injected group. Mistletoe extracts elevated the platelets total count (PLT) in non EAC inoculated groups. The *Viscum album* alcoholic extract prevented the development of ascitic fluid, but it was less effective in reduction of EAC cell concentration. In conclusion, *Viscum album* alcoholic tincture, and glycerine macerate provided an anticancer effect; they stimulate the immune mechanisms and inhibit the tumor cells proliferation.

Keywords: *Viscum album*, Ehrlich Ascitic Carcinoma, Mice

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, *Viscum album* L., has been documented throughout history, its therapeutic application has been changing with the development of science (Ostermann *et al.*, 2009). In our days, *Viscum album* extracts are widely used in addition to the treatment of cancer (Ostermann *et al.*, 2009). Recently, many *in vitro* and *in vivo* studies have examined the antitumoral properties of *Viscum album* extracts or certain constituents isolated from these extracts (Cebovic *et al.*, 2008, Khil *et al.*, 2007), and various clinical studies revealed that mistletoe extract preparations can improve the quality of life, and survival time span in different cancer patients (Ostermann *et al.*, 2009). 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 favors bridging of natural killer tumor cell conjugates, enhancing the citotoxic efficiency. Moreover, it has been found that the antitumoral human citotoxic T lymphocytes with CD T cell receptor are selectively activated by mistletoe ligands of phosphoantigen structure (Tabiasco *et al.*, 2002).

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* mother tincture (MT) was prepared according to German Homoeopathic and European Pharmacopoeias, method 2a, by cold extraction (maceration). 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 than pressed and filtered. The extraction ratio was 1:1 plant to extract (mother tincture). The obtained mother tincture has 8,5 % dry residue.

The *Viscum album* young shoots extract – glycerine macerate (GM) was prepared according to French Pharmacopoeia using fresh plant material. The extraction was made by maceration using as extraction solvent a mixture of 96 % vol. ethanol and glycerol. The dry plant extraction solvent ratio was 1 to 20. The fresh plant material was cut to pasta consistency and that was adding the extraction solvent. The plant-extraction solvent mixture was macerate 20 days with periodical mixing and than pressed and filtered. The obtained extract was diluted 1 to 100 with a mixture of ethanol, glycerol and water, having an ethanol content of 18 % vol.

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 10 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 40 white Swiss male mice, 35.83 ± 5.61 g body weight; each animal received 10^6 ascitic cells intra peritoneal, in the day 0. The experiment last for 14 days, in the Department of Pathophysiology, University of Agricultural Science and Veterinary Medicine Cluj-Napoca. Ehrlich ascitic carcinoma (EAC) was a generous gift from the Oncology Institute "I. Chiricuta" Cluj Napoca. Body weight was measured at the beginning, and at the end of experiment.

Animals were divided in four equal experimental groups; first group mother tincture extract (MT) received i.p. in the day 1, 4 and 7 - 50 mg d.s./kg b.w. *Viscum album* 1:1 plant to extract in ethylic alcohol 70°. The second group young shoots extract (GM) was injected intraperitoneal in the day 1, 4 and 7 – 20 ml/kg b.w. *Viscum album* 1 to 100 with a mixture of ethanol, glycerol and water (ethanol 18 % vol.). 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 last group received i.p. Doxorubicin chloride 2.5 mg/ kg b.w. (Adriablastina 10 mg – Pfizer) in day 6 of experiment.

To assess the biological effects of substances in study, other 40 animals were subject of administration of plant extracts, control and Doxorubicin chloride alone in the same doses, at the same time.

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 1:100). Cell viability was assessed by tripan blue staining (Olinescu, 1992).

Blood haematology was investigated with Abacus Junior Vet, automatic analyzer Diatron Messtechnik, Budapest, Hungary. Blood chemistry (urea and creatinine) was measured using screen point semiautomatic analyzer, STAT – FAX 1904 Plus, Global Medical Instrumentation, Inc. 6511 Bunker Lake Blvd. Ramsey Minnesota, 55303 USA, by using special determination kits in the Department of Pathophysiology, University of Agricultural Science and Veterinary Medicine Cluj-Napoca.

Statistics - the data were expressed as the mean and standard deviation. 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

No important changes in red blood cells has been found among experimental groups, the total red blood cells count, hemoglobin, hematocrit and red cells indices were within normal limit. However, increased hemoglobin and hematocrit level were found in mistletoe treated groups (Tab. 1); due to hypercromy in red blood cells (suggested by increased medium cellular hemoglobin MCH, and medium cellular hemoglobin concentration MCHC) but the significance remains obscure (Tab. 2).

Tab. 1.

The hematological profile (mean ± S.D.)

	RBC 10^{12}/l	HGB g/dl	HCT %
EAC	6.48±1.21	9.46±1.95	28.73±5.63
EAC + Doxorubicin	7.04±0.90	11.75±1.60	35.99±4.67
EAC + MT	6.23±0.93	10.24±1.84	30.23±5.06
EAC + GM	6.57±0.20	10.59±0.76	30.83±1.78
GM	7.60±0.58	12.96±0.93*	38.08±2.40*
MT	8.28±0.22	13.50±0.40*	39.81±0.42
Doxorubicin	7.33±0.98	11.26±1.29	34.35±5.26
Control	6.67±1.40	10.26±2.22	31.74±6.05

*= statistically significant at $p<0.05$ as compared to Control group

Normal values: RBC 7-12.5 10^{12}/l HGB 10.2-18 g/dl HCT 36-49 % (Uray, 1992)

Tab. 2.

The Red Cell Indices (mean ± S.D.)

	MCV fl	MCH pg	MCHC g/dl	RDWs fl
EAC	44.20±3.35	14.56±0.83	33.00±1.61	42.06±5.26
EAC + Doxorubicin	51.00±1.82	16.72±0.48	32.70±0.36	37.30±1.92
EAC + MT	48.14±1.21	16.40±0.75	38.89±1.12	33.02±0.76
EAC + GM	46.86±3.18	16.14±1.32	34.36±0.94	37.26±3.71
GM	50.00±1.22	17.02±0.30***	33.96±0.40**	34.20±4.04
MT	48.33±1.53	16.33±0.90*	33.90±0.96	34.13±1.22
Doxorubicin	46.80±3.08	15.31±1.32	32.66±1.13	39.06±3.60*
Control	47.78±2.11	15.34±0.59	32.13±1.025	34.03±4.03

*= statistically significant at p<0.05 as compared to Control group
**= statistically distinct significant at p<0.01 as compared to Control group
***= highly statistically significant at p<0.001 as compared to Control group
Normal values: MCV 53.6-56 fl, MCH 48.1-50 pg, MCHC 31.3-33.2 g/dl (Uray, 1992)

Doxorubicin, as expected, induce a significant leucopenia as compared to control group, and interestingly was responsible for reducing the total white blood cell count (WBC) in EAC injected groups (EAC inoculation was related to elevated WBC concentration). The lymphocytes were the most affected category in non EAC inoculated animals, while in EAC inoculated ones the leucopenia was induced by decreasing of the granulocyte level.

V. album alcoholic extract (MT) provides the increase in WBC as compared to control due to granulocytes (GRA) suggesting a neutrophilia, but the most important both mistletoe extracts provided increase WBC as compared to Doxorubicin group. The difference was also made by granulocytes (Tab. 3). Some peritoneal local reaction do to i.p. injection of plant extracts was excluded by gross evaluation and cytology of peritoneal fluid made at 1, 2, 6, and 24 hours after injection, in preliminary studies (data no shown).

Tab. 3.

The leukogram (mean ± S.D.)

	WBC 10^9/l	LYM 10^9/l	MID 10^9/l	GRA 10^9/l
EAC	62.32±45.82	9.15±6.61	2.58±3.58	50.56±44.44
EAC + Doxorubicin	15.01±9.46	8.93±3.014	0.34±0.39	5.64±6.37
EAC + MT	36.81±20.45	7.84±1.99	2.50±3.38	26.47±15.80
EAC + GM	32.88±6.84	8.70±2.77	1.63±0.41	22.56±4.91
GM	5.58±2.85	5.20±2.70	0.19±0.26	0.20±0.17
MT	7.37±1.35	6.06±0.94	0.14±0.05	1.17±0.72*
Doxorubicin	2.48±1.42**	1.74±0.94***	0.11±0.10	0.64±0.49
Control	4.87±1.83	4.29±1.69	0.11±0.07	0.45±0.26

*= statistically significant at p<0.05 as compared to Control group
**= statistically distinct significant at p<0.01 as compared to Control group
***= highly statistically significant at p<0.001 as compared to Control group
Normal values WBC 6-15 10^9/l (Uray, 1992)

Mistletoe extracts, induced elevated platelets total count (PLT) in non EAC inoculated groups, but other platelets characteristics remain unchanged (Tab. 4). The alcoholic extract (MT), seems to be more active than glycerin extract (GM). Trombocitosis was mainly related to unspecific cellular immunity; therefore, it suggested once again an immune stimulation.

Tab. 4.

The platelets (mean ± S.D.)

	PLT 10^9/l	PCT %	MPV fl	PDWs fl
EAC	648.80±205.39	0.40±0.21	6.20±0.38	6.70±0.60
EAC + Doxorubicin	498.50±110.03	0.32±0.08	6.38±0.31	6.40±0.24
EAC + MT	649.14±248.18	0.44±0.18	6.69±0.30	7.09±0.41
EAC + GM	749.57±126.34	0.47±0.09	6.29±0.23	6.60±0.56
GM	723.60±61.39**	0.45±0.08**	6.20±0.56	6.06±0.61
MT	662.33±133.72*	0.41±0.09*	6.17±0.15	6.13±0.60
Doxorubicin	643.70±190.93	0.38±0.11*	5.91±0.17	5.88±0.42
Control	389.33±181.25	0.23±0.11	5.97±0.19	5.84±0.43

*= statistically significant at p<0.05 as compared to Control group
**= statistically distinct significant at p<0.01 as compared to Control group
Normal values PLT 160-410 10^9/l (Uray, 1992)

In EAC group only 8 from 10 animals survived in the end of the study, in the other three experimental groups all animals survived to the end; development of EAC was expectedly followed by a significant increasing in body weight. Doxorubicin revealed a strong protective effect; in treated animals, the body weight was slightly decreased. The *Viscum album* mother tincture (MT) alcoholic extract provides also a strong protective effect comparable to doxorubicin. The *Viscum album* young shoots extract – glycerine macerate (GM) failed to strop the ascitic development (Fig. 1). However, the other parameters, total amount of ascitic fluid and cellular ascitic concentration were not influenced by the mistletoe extracts in statically significant manner (Fig. 2, 3), even lover values were found in both experimental groups. This findings should be elucidated in further studies. No notable changes were found in plasma biochemistry, gross evaluation and histopathology (data no shown).

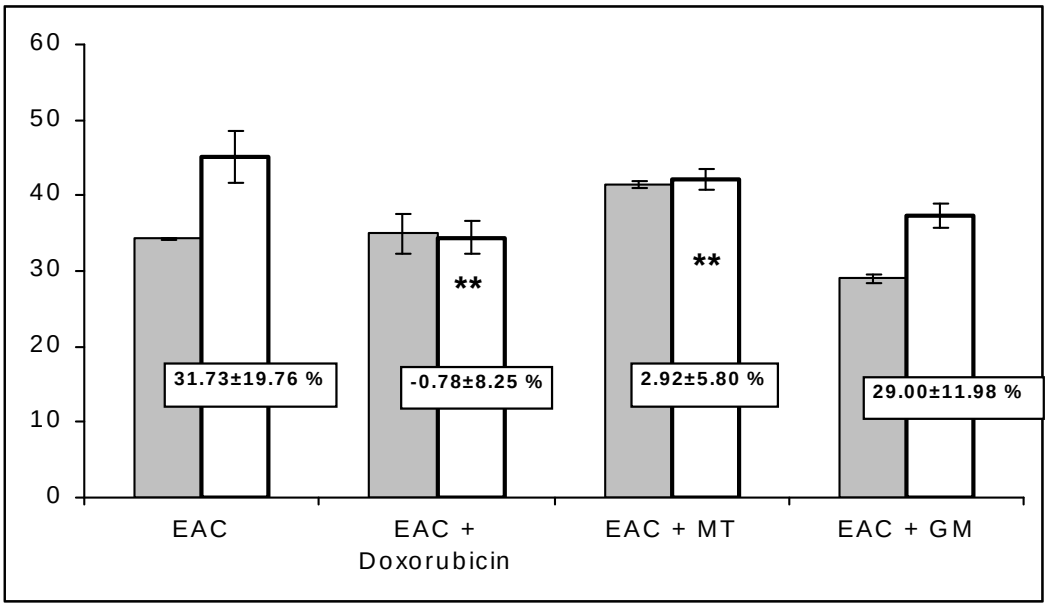

Fig. 1. The variation on body weight among experimental groups (mean ± S.D.) (g)
**= statistically distinct significant at p<0.01 as compared to Control group

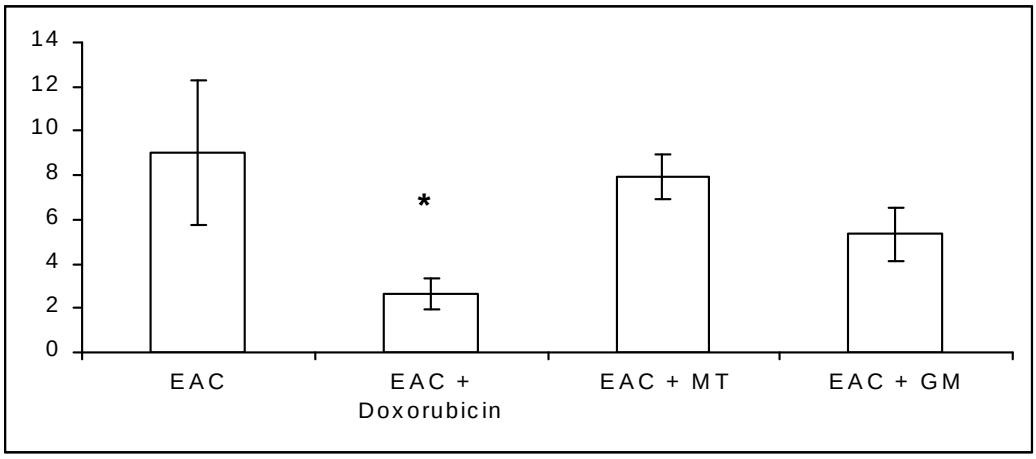

Fig. 2. The ascitic volume (mean ± S.D.) (ml)
*= statistically significant at $p<0.05$ as compared to Control group

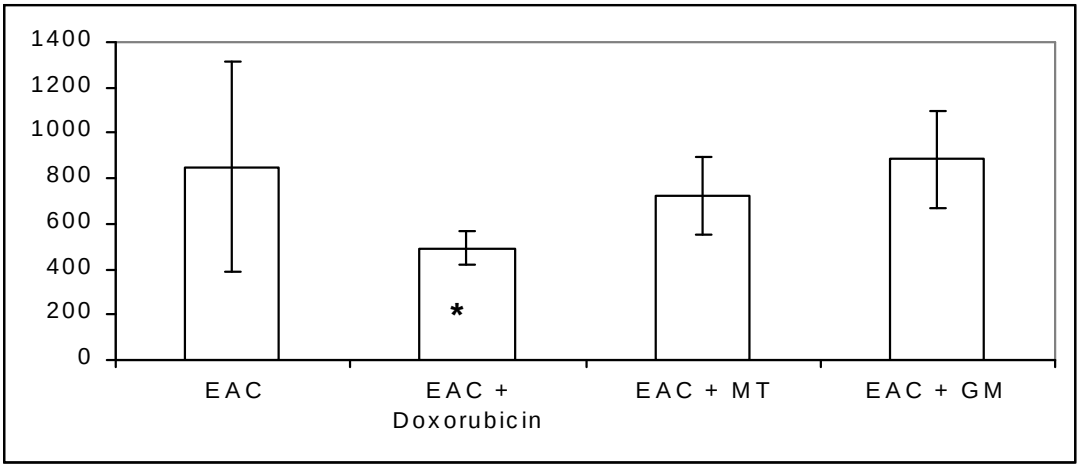

Fig. 3. The cellular concentration within ascitic fluid (mean ± S.D.) (10^6/ml)
*= statistically significant at $p<0.05$ as compared to Control group

In the present study, even the number of cells was lower in treated groups; the most of them were viable (99%). Other studies found that the administration of *Viscum album* extract against EAC was followed by a reduction in cell number and viability (Cebovic *et al.*, 2008). In some cases, especially when the mice were pretreated with the *Viscum album* extract, a total absence of EAC cells was observed. Post treatment also led to a reduction in the cell number and viability although to a lesser extent than pretreatment. These findings are in accordance with a lower degree of oxidative stress in these EAC cells. The citotoxic mechanism of action of the *Viscum album* extract on EAC development may be due to the induction of oxidative stress in the EAC cells without simultaneously affecting the antioxidant status of other tissues (Cebovic *et al.*, 2008).

However, the molecular apoptotic mechanisms in tumor cells by *Viscum album* extract have not been clearly elucidated. Khil *et al.*, (2007) 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 signalling 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 (Thies *et al.*, 2008).

Cytolysis activity against tumors involves many types of effectors cells; these include not only T-lymphocytes, but also granulocytes, activated macrophages, NK cells, and lymphokine-activated killer cells. 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 (Tabiasco *et al.*, 2002). They proved to be effective in cancer patients, subject of chemotherapy, but even more effective in healthy subjects (Ostermann *et al.*, 2009).

CONCLUSIONS

The *Viscum album* alcoholic tincture, and glycerine macerate proved a trustworthy anticancer effect, by upholding the immune mechanisms, and, also, throughout inhibition of tumor cells proliferation. Outstandingly they seem to increase the survival rate, and health condition in animal models. This might be the fist step for the development of new original anticancer remedies.

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