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ANTITUMOR EFFECT OF *VERBASCUM PHLOMOIDES L.* ON EHRLICH CARCINOMA TUMOR CELLS

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Abstract: Mullein is a plant widespread in the wild flora of Romania, but less used in traditional medicine and with less demonstrated effects. The aim of the present study was to demonstrate the in vivo antitumor activity of *Verbascum phlomoides* L. harvested from Romania and its ability to achieve protection against the side effects caused by the tumor growth. We carried out the study on 32 Mus musculus female mice, over a two week period. The tumor model was Ehrlich Ascites Carcinoma (EAC). Animals were divided in four equal groups of 8 mice: control, tumor control, EAC + doxorubicin and EAC + *Verbascum phlomoides* L. Doxorubicin was used as antitumor reference. Difference in body weight, EAC volume and tumor cell concentration were improved in significant manner. Hematological and biochemistry parameters determination were performed and no cytotoxicity was found. Therefore *Verbascum phlomoides* L. is recommended for further studies, in other to find new remedies in complementary cancer therapy.

Keywords: antitumor, Ehrlich Ascitic Carcinoma, mice, *Verbascum phlomoides* L., Doxorubicin

INTRODUCTION

Cancer as a genetic disorder is the main cause of death in economically developed countries and the second one in emerging countries (Meybodi et al., 2017). The reports indicate that about 13% of total death (7.6 million) are induced by cancer and its global burden behaviors, is increased largely regarding both the aging and growth of the world population besides the growing of cancer inducing especially smoking (Jemal et al., 2011). Mortality caused by cancers is increasing throughout the world, and it is predicted that more than 13.1 million death will occur due to cancer worldwide 2030 (Kooti et al., 2017).

The development of cancer registries throughout the world has led to a search for novel drugs that are toxic to the cancer cells while having no harmful effect on normal cells. The anticancer drugs used previously exhibited relatively high toxicity not only on the tumour cells, but also to the normal cells of the body part in which the cancer had developed. The increase in the incidence of various types of cancer creates a need for new anticancer drugs (Lichota et al., 2018). The idea that simple plants and foods can have anticancer effects is sometimes a controversial subject. The National Cancer Institute (NCI) has screened approximately 35.000 plant species for potential anticancer activities. For every person who believes that plants can slow or even kill cancer cells, there is another who will only believe in merits of chemotherapy (Desai AG et al., 2008).

The genus *Verbascum*, commonly known as "mullein", is a widespread genus of the family Scrophulariaceae, which comprises more than 2500 species worldwide (Kahraman et al., 2012). Mullein is a plant widespread in the wild flora of Romania, but less used in traditional medicine and with less demonstrated effects. It is a biennial herbaceous plant. In the first year, it forms a rosette of basal leaves and in the second year flowering stems. Type five flowers are yellow and grouped under bracts. It is commonly spread on fallow lands being deprived of pollutants and herbicides substances. It blooms in the summer between June and August and even in September (Marian et al., 2018). *Verbascum* species contain biologically active compounds, such as flavonoids, phenylethanoid and neolignan glycosides, saponins, iridoid and monoterpene glycosides (Tatli et al., 2006). Phenolic compounds exert multiple biological effects, including antioxidant and free radical-scavenging abilities. Previous studies have demonstrated that gallic acid exerts activity against several types of tumor cells (Subramanian et al., 2015). Quercetine has also an antioxidant and anticancer effect, being therefore reported as an efficient free radical scavenging (Baghel et al., 2012).

Anticancer is a broad word that can be broken down into three parts: cytotoxic – shown to fight tumor in laboratory cell cultures (in vitro); antitumor – shown to be toxic to tumors in animal studies and anticancer – shown to fight tumors in humans (Pawar et al., 2018). In this context, our group of researchers has previously reported several studies in which we demonstrated the in vitro / in vivo antioxidant and antitumor activity for Viscum album (Sarpataki et al., 2014; Stan et al., 2013;

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Sarpataki et al., 2015) , Euonymus europaeus (Sevastre et al., 2017; Sarpataki et al., 2016; Sevastre et al., 2014), Salvia officinalis (Marian et al., 2018), Centaurea cyanus and Calendula officinalis (Marian et al., 2017).

The current study is a follow up of an in vitro study performed also on *Verbascum phlomoides* L. when we demonstrated its antioxidant and antitumor properties (Marian et al., 2018). In the present study we aim to demonstrate the in vivo antitumor activity of *Verbascum phlomoides* L. harvested from Romania and the ability of *Verbascum phlomoides* L. to achieve protection against the side effects caused by the tumor growth.

MATERIALS AND METHODS

Plant material preparation

The flowers without calyx (*Verbascum* flos) were harvested gradually deppending on the degree of opening, from June to August, from lowlands and hills of western Romania region, from crops untreated with herbicides substances. After harvesting plant products were dried at the room temperature (20 - 25° C), away from light.

Preparation of the extract

Fluid extracts of *Verbascum* flos were obtained using as solvent a 70° hydroalcoholic mixture, according to Romanian Pharmacopoeia 10th edition. Hydroalcoholic extracts were centrifuged and supernatants were evaporated to dryness under vacuum in a rotavapor. The dried extract was transferred to a vessel with 10 ml of distilled water and frozen at the temperature of -25° C. The lyophilized extracts were weighed and transferred to sample ampoules.

Animal care and experimental procedures

The experiments on animals were performed according to the Directive 2010/63/EU and National low no. 43/11.04.2014. The study was performed into the Establishment for the breeding and use for laboratory animals of the University of Medicine and Pharmacy "Iuliu Hatieganu" Cluj-Napoca; the experiment was approved by The Bioethical Board of UMF "Iuliu-Hațieganu" Cluj-Napoca, and authorized by state veterinary authority. The animals were caged in 4 polycarbonate cages, at controlled temperature of 21-22°C, humidity 40-60% and 12/12h light/dark cycle. Standard lab chow, provided by the 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 32 Mus musculus female mice with a body weight about 26.11 ± 1.40 g. The mice were divided in four equal experimental groups of eight mice.

The first group was the control group, received *Verbascum phlomoides* L. 50 mg d.s. *Verbascum phlomoides* L. / b.w. was administrated intraperitoneally in the 1st, 3rd and 6th day, in 0.5 ml sterile saline solution / mice.

The second group was the tumor control group (EAC). EAC group received 106 ascitic cells / animal i.p., in the day 0, and then placebo therapy intraperitoneally (0.5 ml saline physiological solution / mice, i.p).

The third group received EAC and then doxorubicin, i.p. Accordingly to manufacturer specification, the LD50 for doxorubicin chloride, in mice, in intraperitoneally administration, is 21.9 mg/kg. The normal therapeutically dose being approximately 10 times lower, during the present experiment, mice received 2.5 mg doxorubicin chloride/b.w. (Adriblastina 10 mg, Pfizer) dissolved in sterile saline solution, up to 0.5 ml per animal. The mentioned doses were administered in the 1st and 6th day of the experiment.

The last group was also inoculated with EAC and then received *Verbascum phlomoides* L. 50 mg d.s. *Verbascum phlomoides* L. / b.w. was administrated intraperitoneally in the 1st, 3rd and 6th day, in 0.5 ml sterile saline solution / mice. The dose has been established following preliminary toxicity studies.

14 days after EAC implantation, the blood was harvested from the retro orbitary sinus under diethyl ether anesthesia and the euthanasia was made by prolonged ether narcosis.

Blood hematology (complete blood count) was investigated with Abacus Junior Vet, Diatron, 3 Diff Messtechnik, Hungary.

Blood biochemistry (urea, creatinine and transaminase) was measured using screen point semiautomatic analyzer, STAT - FAX 1904 Plus, Global Medical Instrumentation, Inc. 6511 Bunker Lake Blvd. Ramsey Minesota, 55303 USA by using special determination kits (Diagnosticum Zrt. Hungary).

The volume of ascitic fluid from the peritoneal cavity was collected and measured with a syringe, immediately after euthanasia, and transferred in phosphate buffer solution (pH 7.4, at 4°C). Then, the samples were subjected to repeated centrifugations (at 4°C), first at 4.500 rpm for 5 min, then at 12.000 rpm for 3 min, in order to obtain a dense cell suspension, which was also stored at deep freezer until further use. The tumor cell concentration was counted in a Burker chamber (dilution 1:10) and the cell viability was assessed by Tripan blue staining (0.4% in PBS).

Body weight was measured at the beginning, in the days 3, 6, 9, 12 and at the end of the study (Stan et al., 2013; Sevastre et al., 2014; Sarpataki et al., 2016).

Statistical analysis

All data are reported as the mean ± SEM. The Gaussian distribution was checked by the Shapiro-Wilk normality test. One-way analysis of variance ANOVA, followed by post hoc Dunnett's range test procedure was performed for pair-wise comparisons between the volume of ascitic fluid and variation of viable cells concentration, while the two-way ANOVA followed by the Bonferroni post-test was the choice for the variation of body weight. Statistical significance was at $p<0.05$ (95% confidence interval). Statistical values and figures were obtained using GraphPad Prism version 5.0 for Windows, GraphPad Software, San Diego California USA.

RESULTS AND DISCUSSION

The objective of the study was to demonstrate the in vivo antitumor activity of *Verbascum phlomoides* L. harvested from Romania and the ability of *Verbascum phlomoides* L. to achieve protection against the side effects caused by the tumor growth.

To demonstrate the in vivo antitumor activity of *Verbascum phlomoides* L., the body weight, the ascitic volume and the tumor cell concentration were evaluated. At the end of the study the survival rate was 100%.

The development of EAC was expectedly followed by a significant increasing in body weight. The body weight, as a measure of Ehrlich ascites accumulation in the peritoneal cavity is shown in Fig.1. The doxorubicin and *Verbascum phlomoides* L. therapies prevents body weight gain. Doxorubicin revealed a strong protective effect, in treated animals, the body weight was decreased more significant. The differences were visible starting from the third day of the study (Fig.1). Even if it is not as effective as doxorubicin, *Verbascum phlomoides* L extract provides a relevant protective effect.

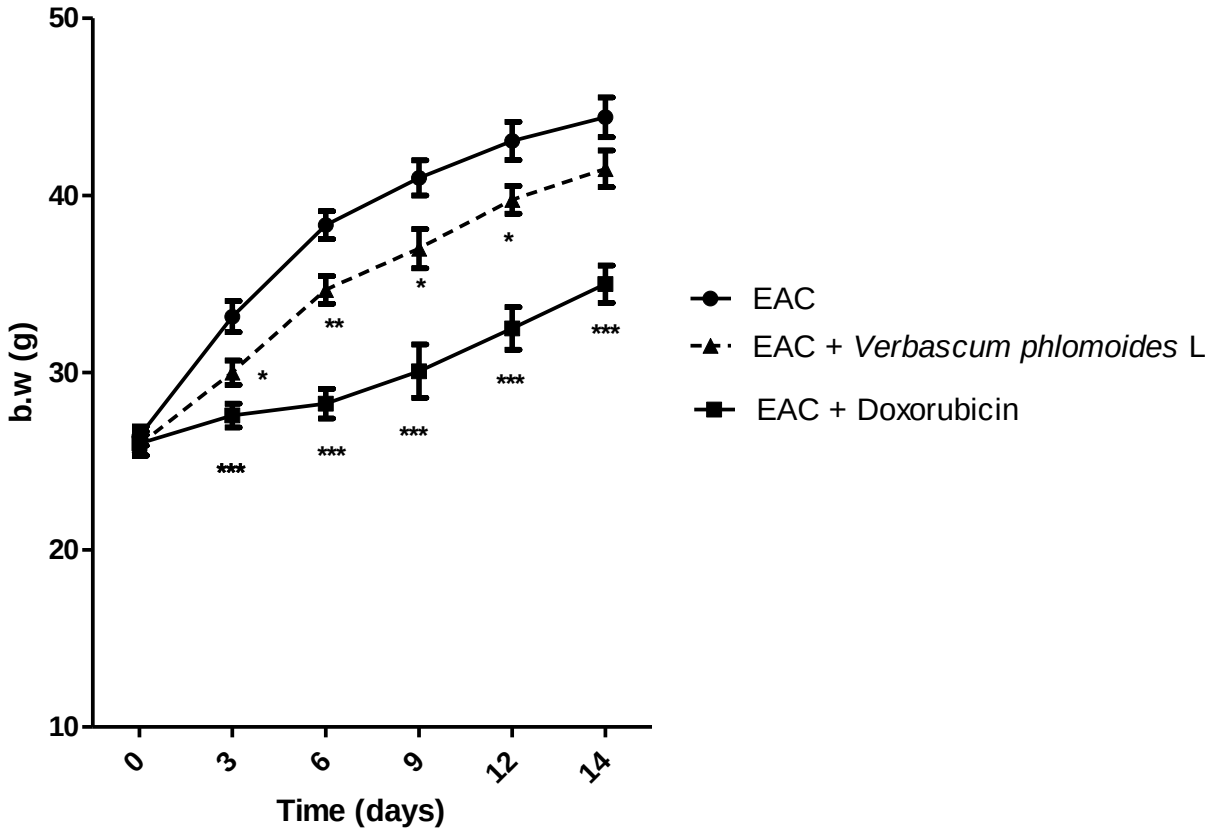

Fig.1. The effect of doxorubicin and of *Verbascum phlomoides* L. extract on body weight gain in EAC inoculated mice (* = p<0.05: ** = p<0.01; *** = p<0.001 as compared to EAC group) (Mean ± SD) (8 animals/group)

The amount of ascitic fluid has varied proportionally with the body weight gain. We found a significant decrease in the ascitic volume (Fig.2A) and in the tumor cell concentration (Fig.2B) for EAC + doxorubicin as compared with the tumor control group. A decrease exists also for EAC + *Verbascum phlomoides* L. but the decrease is not statistically significant.

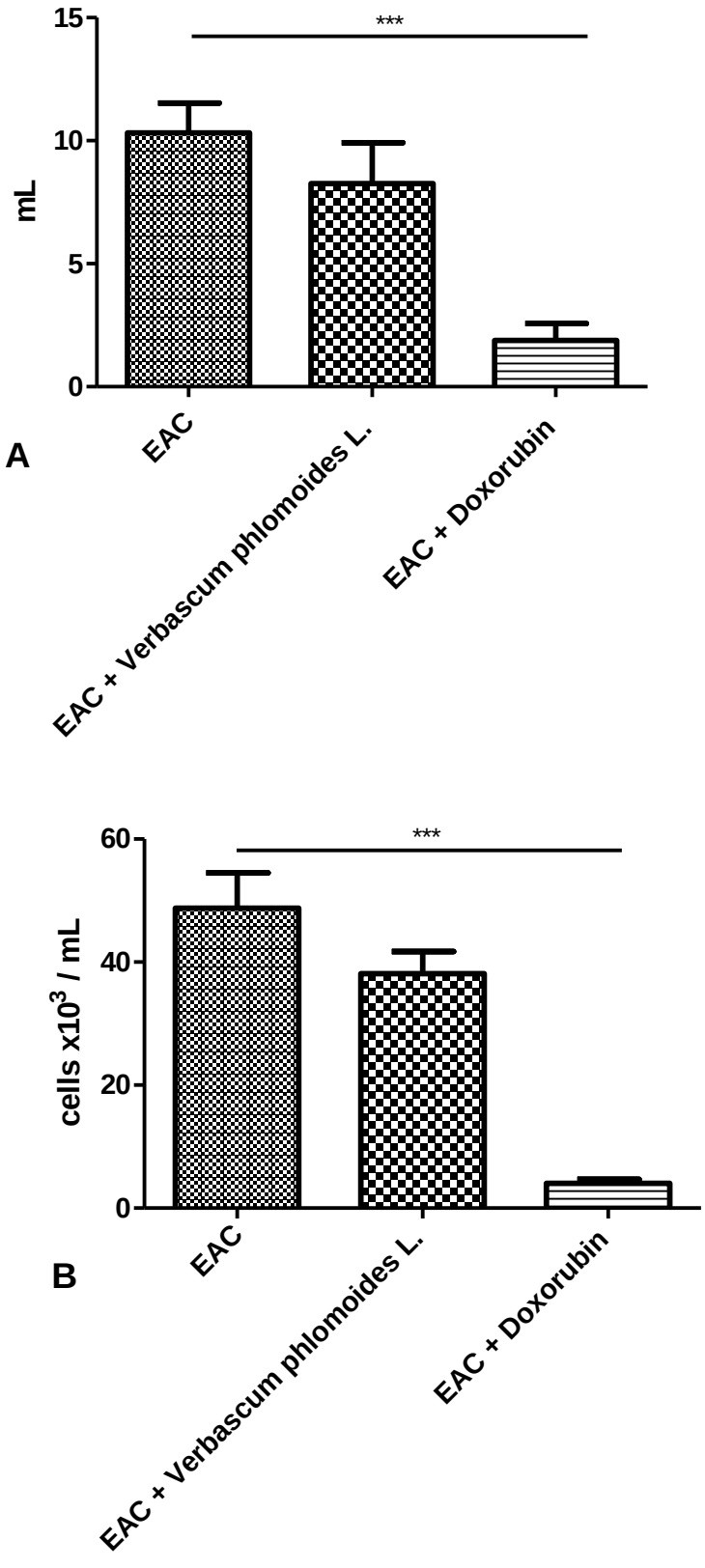

Fig.2. The effect of doxorubicin and of *Verbascum phlomoides* L. extract on ascitic volume (ml) (A) and tumor cell concentration (cells x 103 / ml) (B)
(*** = p<0.001 as compared to EAC group) (Mean ± SD) (8 animals/group)

Although tumor development was prevented by doxorubicin therapy more effectively than *Verbascum phlomoides* L extract, the extract proved also notable antitumor activity. Thus, in the future we intend to study if the association between doxorubicin and *Verbascum phlomoides* L extract is more active than Doxorubicin alone.

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Very important is the fact that, both, doxorubicin and *Verbascum phlomoides* L extract provide cytostatic effects, and no citotoxic effect, because tripan blue staining revealed almost 100% viable cells in ascitic fluid.

The mechanism of action for doxorubicin is still unclear, but is widely accepted an anti-proliferative mechanism based on intercalation on DNA molecule, and consequently by blocking the DNA biosynthesis (Thorn et al., 2011).

The antitumor mechanism of action for *Verbascum* sp. is still unknown. A strong inhibitory effect of the aqueous extract of *Verbascum* sp. on protein biosynthesis was demonstrated in isolated rat liver ribosomes. The saponin fraction was shown to be responsible for this activity and it was compared to commercial glycyrrhizic acid and its aglycon as the reference drug. It was found that these compounds strongly inhibited the incorporation of [14C] leucine into proteins in vitro and that the target site for inhibition was the ribosome fraction from rat liver cells (Tatli et al., 2006).

Some plants have long been used in folk medicine as sources of antitumor remedies. Their effect on protein biosynthesis in vitro have been examined and described. The separation features of the peptide elongation system, isolated from tumor cells, have been demonstrated. Some elongation factors or ribosomes have been shown to be a target site for the inhibition of protein biosynthesis caused by the substances isolated from various sources. Saponin glycoside and its aglycon, isolated from *Verbascum* sp flowers inactivated ribosomes. It may be supposed that the plant inhibitors of protein biosynthesis could be utilized for searching specific antitumoral preparations (Kahraman et al., 2012).

To check the ability of *Verbascum phlomoides* L. to achieve protection against the side effects caused by the tumor growth, blood hematology and blood biochemistry was performed.

Determinations of biochemical parameters (creatinine, urea, transaminase) didn't show significant statistical changes compared to control during the current study (14 days). So, in this way we demonstrated the protective effect of *Verbascum phlomoides* L and Doxorubicin administration after EAC inoculation and the fact that they are not toxic.

EAC development was responsible for anemia in tumor bearing mice. The red blood cells and the hematocrite were significant below control and under the normal values (Tab.1), but the red cells indices were in normal limits.

Tab.1.

The effect of *Verbascum phlomoides* L. extract on the values of the red blood cells (RBC), hemoglobin (HGB) and hematocrite (HCT) (Mean ± SD)

Group	RBC 1012/l	HGB g/dl	HCT %
Control	8.19±0.40	12.56±1.32	37.21±1.05
EAC	6.41±0.37*	10.46±1.05	30.73±1.15*
EAC + doxorubicin	7.13±0.68	10.78±0.80	33.46±2.54
EAC + *Verbascum phlomoides* L.	6.99±0.50	10.15±0.33	33.12±1.42

Normal values: RBC 7-12.5 1012/l HGB 10.2-18 g/dl HCT 36-49 % (Uray, 1992)
(*=p<0.05 as compared to control group)

The administration of doxorubicin and *Verbascum phlomoides* L. at EAC inoculated mice maintain the RBCs level significant higher than the value found in EAC group. The RBC level for EAC + *Verbascum phlomoides* L. group is inferior to EAC + doxorubicin group. The protective effect seems to be rather indirect than a direct action on RBCs. It seems that the degree of anemia is related to the tumor development, so by preventing the EAC growth, the administration of doxorubicin and of *Verbascum phlomoides* L. prevents also the progression of anemia (Tab.1).

EAC development was responsible for significant leukocytosis. Granulocytes and middle cells were also increased in a significant manner. In EAC inoculated group, doxorubicin therapy lowers WBC count as compared to untreated EAC group, but the value remains much higher than the control. In EAC inoculated group, *Verbascum phlomoides* L. therapy lowers also WBC count as compared to untreated EAC group, the value remains also much higher than the control, but is superior as compared to EAC + doxorubicin group, that means that doxorubicin administration is more protective. All leucocyte categories were involved in the same way (Tab.2). The prevention of leukocytosis might be due to the reduction of tumor growth after the administration of doxorubicin and *Verbascum phlomoides* L.

Tab. 2.

The effect of *Verbascum phlomoides* L. extract on the values of the white blood cells (WBC), lymphocytes (LYM), middle cells (MID) and granulocytes (GRA) (Mean ± SD)

Group	WBC 109/l	LYM 109/l	MID 109/l	GRA 109/l
Control	8.62±1.83	4.29±0.65	0.19±0.07	1.47±0.26
EAC	27.22±2.32*	5.17±0.97	0.76±0.15*	15.05±2.79*

EAC + doxorubicin	17.01±2.16a	4.83±0.88	0.37±0.11	7.61±2.20a
EAC + *Verbascum phlomoides* L.	18.27±1.22a	4.90±0.16	0.44±0.15	8.01±0.13a

Normal values WBC 6-15 109/l (Uray, 1992)
(*=p<0.05 as compared to control group; a=p<0.05 as compared to EAC group)

The total platelet count (PLT), platelet hematocrit (PCT), mean platelet volume (MPV) and distribution width (PDWs) do not undergo significant statistical changes compared to control and EAC group and there was no evidence of toxicity.

Therefore, after the determinations of hematological parameters we demonstrated the protective effect of *Verbascum phlomoides* L and Doxorubicin administration after EAC inoculation on all blood cell lines.

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 effects is a highly active and challenging research domain.

CONCLUSIONS

Verbascum phlomoides L extract revealed a considerable antitumor activity, along with the safety profile. Therefore *Verbascum phlomoides* L is recommended for further studies in order to find new remedies in complementary cancer therapy. Our group of researchers intends to study if the association of *Verbascum phlomoides* L extract to doxorubicin increase the antitumor effect of doxorubicin administrated alone. The results are also promising in terms of *Verbascum phlomoides* L extract use in cancer prevention, because it is known that cancer usually takes years to develop, so prevention is preferable to any treatment.

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DECLARATION OF INTERESTS

The authors report no conflict of interest.

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