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Testing of Antitumor Effects of *Hypericum Perforatum* L. and *Hypericum Maculatum* C. in Ehrlich Ascite in Swiss Mice

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Abstract *Hypericum perforatum* L or St. John's wort is a medicinal plant with long history use in traditional. Because Romanian and European Pharmacopoeia accept as officinal only *H. perforatum* L, the present study compare the characteristics of the *H. perforatum* and *H. maculatum*. The experiment was carried out on 28 white Swiss male mice, for 14 days long; each animal received 10^6 Ehrlich Ascitic Carcinoma cells i.p., in the day 0. Animals were divided in four equal experimental groups, First group (HM) received 172 mg/kg b.w. *H. maculatum*, the second group (HP) 172 mg/kg b.w. *H. perforatum*, the control group, and the last group Doxorubicin cloride 2.5 mg/ kg b.w in day 6 of experiment. Both groups HM and HP received intra peritoneal injections in days 1, 4, and 7 of experiment. White blood cells and the platelets were increase in *Hypericum* treated groups; leucocytosis was more evident in *H. perforatum*, mainly because of the granulocytes. Doxorubicin prevents the elevation of granulocytes levels, no similar effect was found in *Hypericum* treated animals. Even no so effective like Doxorubicin, alcoholic extracts provides a protective effect, but statistical significance was found only for *H. maculatum.* In conclusion, both *Hypericum* sp. (*H. maculatum* and *H. perforatum*), have shown anticancer effect, through inhibition of tumor cells development and elevating the white blood cells level. This might be the basis of new pharmaceutical remedies using *H.maculatum* a wide spread species in mountain regions of Romania.

Key words: *Hypericum sp.*, Ehrlich Ascitic Carcinoma, antiproliferative effect.

INTRODUCTION

Hypericum perforatum L or St. John's wort is a medicinal plant with a long history of use in traditional medicine all over Europe. In Romania's spontaneous flora are mentioned 12 species of *Hypericum* (Stănescu *et. al.* 2004). The most widely spread, and used species are *H. perforatum* L. (in the hills regions) and *H. maculatum* Crantz. (in the mountain regions) Tămaş, *et. al.* (2001); *H. maculatum* is also harvested in order to obtain *Hyperici herb* (Ciulei, *et. al.* 1993).

Both Romanian Pharmacopoeia (R.Ph. 1993) and European Pharmacopoeia (E. Ph. 2008) accept as officinal only *H. perforatum* L, therefore the aim of the present study is to compare the botanical and physiochemical characteristics of the *H. perforatum* and *H. maculatum*, the most widely spread species from Romania.

Aerial parts of *Hypericum species* contain the following active substances: naphtodiantrons 0.1-0.5% (hypericin, pseudohypericin and their isomers), flavonoids 4-5% (hyperoside, rutoside, quercitroside, biapigenin), tannins 10-20%, hyperforin,

proanthocyanins, caffeic acid derivatives, xanthons, essential oil 0.1-1% etc (Herman, and Gheorgiu, 1961)

Ehrlich ascitic carcinoma (EAC) is a convenient model for investigation of tumour proliferation and side effects (Olinescu, 1992). EAC, induce significant plasma biochemical changes, including antioxidant systems; transplantable tumors have been successfully used to prove the antioxidant and anti malignant properties for beta carotene (Marcus, *et. al.* 1998). Significant antioxidant effects were also provided by *Peucedanum oreoselinum* even no obvious antiproliferative effect was found (Sevastre, *et al*. 2008).

MATERIALS AND METHODS

Plant materials: the aerial parts of *H. perforatum* were harvested from Chiribiş (Bihor county) and the aerial parts of *H. maculatum* were harvested from Ciucea (Cluj county) in the blossom period. The vegetal products were dried and grounded to a fine powder (sieve VI - R. Ph. 1993), (Istudor,1998).

*Preparation of tinctures***:** 10g of powdered aerial parts of vegetal product were extracted with 100g of ethanol 70° at room temperature, as described in Romanian Pharmacopoeia X^{th} Edition.

In E. Ph. 2008 it is mentioned as minimum content in total hypericins for the medicinal drug *Hyperici herb* 0.08%, determined by a spectrophotometer method and a qualitative analysis by TLC (York, *et. al.* 1990) in order to identify the flavonoids and hypericin.

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 to 0.5 ml for each animal. The sterile solution was administrated in the same day in order to prevent bacterial and fungus contamination.

To assess the biological effects of substances in study, other 28 animals were subject of administration of plant extracts, control and Doxorubicin chloride alone in the same doses, at the same time.

The animals were caged in groups of seven 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 permission for the animal tests and experiments has been given by the Bioethical Board of the Faculty of Veterinary Medicine Cluj-Napoca.

For assessing the antiproliferative effect, the experiment was carried out on 28 white Swiss male mice, 34.5±3.5g body weight; each animal received 10^6 ascitic cells intra peritoneal, in the day 0 of the experiment. 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.

Animals were divided in four equal experimental groups; all inoculated with Ehrlich Ascitic Carcinoma. Body weight was measured at the beginning, and at the end of experiment.

First group (HM) received 172 mg/kg b.w., *H. maculatum*, the second group (HP) received 172 mg/kg b.w. *H. prerforatum*. Both groups HM and HP received intra peritoneal injections in days 1, 4, and 7 of experiment. The control group received only EAC, and the

last group received intra peritoneal injections with Doxorubicin cloride 2.5 mg/ kg b.w. (Adriablastina 10 mg – Phizer) in days 1 and 6 of experiment.

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 1:200). 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 creatinin) 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.

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 DISCUSSIONS

Body weight. 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. Even no so effective like Doxorubicin, alcoholic extracts provides a protective effect, but statistical significance was found only for *H. maculatum.*

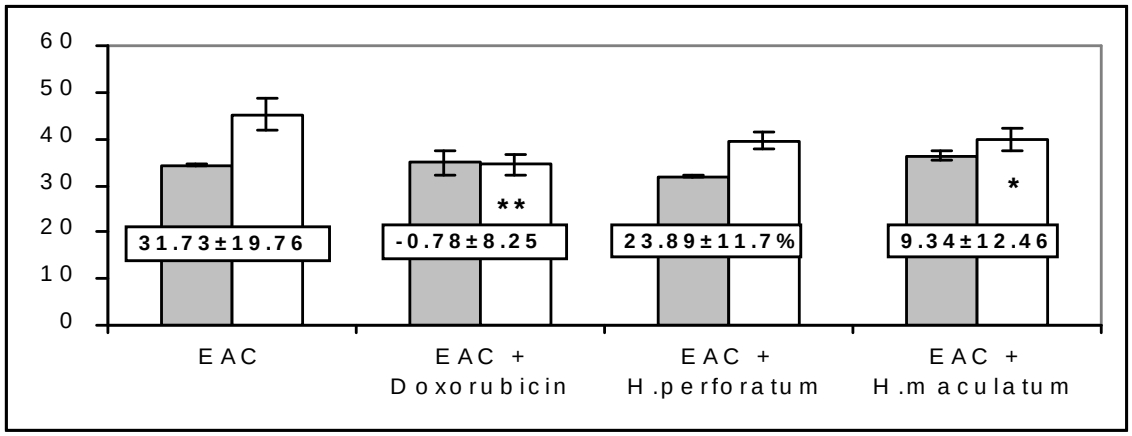

Fig. 1. The variation on body weight among experimental groups (mean ± S.D.) (g)

Ascitic volume was highly decreased in Doxorubicin treated group; *H. perforatum* provides more protection comparative to *H. maculatum*, but not statistically significant (Fig. 2). However, this cytostatic effect should not be excluded, at higher dosage. Important both substances provide cytostatic effects, and no citotoxic ones, because tripan blue staining revealed almost 100% viable cells in ascitic fluid. Plasma biochemistry, gross evaluation and histopathological exam reveal no significant lesion for *Hypericum maculatum* and *Hypericum perforatum* alcoholic extracts.

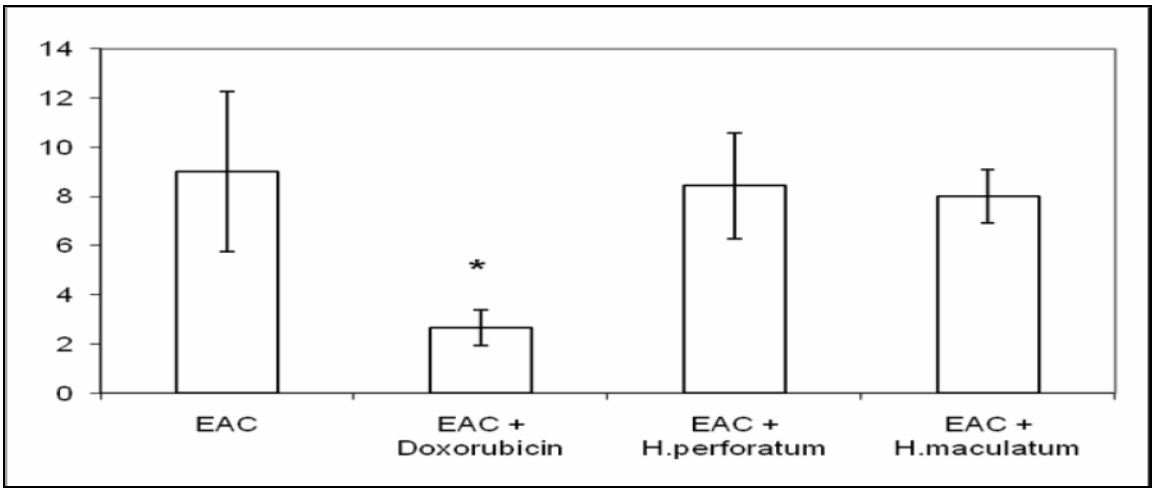

Fig. 2 The ascitic volume (mean ± S.D.) (ml)

Alcoholic solutions inhibited the increase in body weight due to tumor burden when compared to the EAC control group. Hence, it may be concluded that alcoholics tinctures of *H. maculatum and H. perforatum* by decreasing the nutritional fluid volume and arresting the tumor growth.

Red blood cells count (RBC) count and hematocrit (HCT), shown normal values only in Doxorubicine treated groups and in HM group, in the others groups the values wore slightly decreased without statistical significance. (Table 1)

White blood cells (WBC) were increase in *Hypericum* treated groups, more evident in *H. perforatum*. The increasing of WBC level was done mainly on granulocytes. On EAC inoculated animals, doxorubicin prevents the elevation of granulocytes levels, no similar effect was found in *Hypericum* treated animals.

A decrease in hemoglobin and the number of erythrocytes and a significant increase in total WBC (Table 1, 2) in the tumor-bearing mice are known. Anaemia is found frequently in cancer patients (De Vita VT Jr *et. all*. 1993). Similar results were observed in the present study.

Tab. 1.

The haematological profile (erythrocytes values) (mean ± S.D.)

	RBC 10^{12}/l	**HGB g/dl**	**HCT %**	**MCV fl**	**MCH pg**	**MCHC g/dl**	**RDWs fl**
EAC	6.48±1.21	9.46±1.95	28.73±5.63	44.20±3.35	14.56±0.83	33.00±1.61	42.06±5.26
EAC+ Doxo	7.04±0.90	11.75±1.60	35.99±4.67	51.00±1.82	16.72±0.48	32.70±0.36	37.30±1.92
EAC+ HP	6.36±0.63	10.87±0.90	30.34±1.95	47.85±2.61	17.16±1.24	34.81±1.67	34.08±2.31
EAC+ HM	6.70±0.88	10.70±1.20	31.28±4.04	46.71±1.80	16.02±0.75	34.27±0.77	33.27±1.98
HM	7.58±0.81	12.08±1.31	36.08±3.01	47.60±1.34	15.96±0.19*	33.50±0.98	33.79±1.04
HP	6.89±1.19	11.12±1.59	33.36±3.96	49.00±3.32	16.02±0.88*	33.24±0.83	35.46±1.88
Doxo	7.33±0.98	11.26±1.29	34.35±5.26	46.80±3.08	15.31±1.32	32.66±1.13	39.06±3.60*
M	6.67±1.40	10.26±2.22	31.74±6.05	47.78±2.11	15.34±0.59	32.13±1.025	34.03±4.03

Normal values: RBC 7-12.5*10^{12}/l HGB 10.2-18 g/dl HCT 36-49 % MCV 53.6-56 fl
MCH 48.1-50 pg MCHC 31.3-33.2 g/dl (Uray, 1992)

The Ehrlich Ascitic Tumor implantation induces *per se* a local inflammatory reaction, with increasing vascular permeability, which results in an intense edema formation, cellular migration, and a progressive ascitic fluid formation (Fecchio D. *et. al* 1990).

Tab. 2.

The leucogram (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 + Doxo	15.01±9.46	8.93±3.014	0.34±0.39	5.64±6.37
EAC + HP	57.83±28.76	8.65±4.28	6.20±4.56	42.97±20.39
EAC + HM	22.92±16.29	5.40±4.00	1.19±0.92	16.32±12.28
HM	7.02±2.93	4.49±2.14	0.13±0.04	2.41±0.91***
HP	7.65±2.77*	3.80±1.46	0.29±0.19*	3.55±2.21**
Doxo	2.48±1.42**	1.74±0.94***	0.11±0.10	0.64±0.49
M	4.87±1.83	4.29±1.69	0.11±0.07	0.45±0.26

Normal values WBC 6-15 10^9/l (Uray, 1992)

On the healthy animals as well as on tumor inoculated animals both alcoholic extracts induces a significant increasing of the platelets as compared to control group that may suggest an imunostimulatory effect (Table 3)

Tab. 3/

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 + Doxo	498.50±110.03	0.32±0.08	6.38±0.31	6.40±0.24
EAC + HP	674.57±196.02	0.43±0.14	6.24±0.40	6.33±0.94
EAC + HM	757.00±287.97	0.49±0.20	6.41±0.32	6.44±0.59
HM	905.60±149.11***	0.59±0.09***	6.56±0.23	7.04±0.23
HP	941.60±302.34***	0.68±0.19***	7.36±0.72***	8.78±1.76***
Doxo	643.70±190.93	0.38±0.11*	5.91±0.17	5.88±0.42
M	389.33±181.25	0.23±0.11	5.97±0.19	5.84±0.43

Normal values PLT 160-410 10^9/l (Uray, 1992)

*= 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

In previous studies on *Hypericum* spp. extracts, despite previous scientific rapports, no antiproliferative effect in oral administration has been found (Prodan *et all*. 2009). However, now, the intra peritoneal administration, proved to be far more effective against tumor cell development, and, also, were able to induce hematological changes.

In the development of new cancer therapies, significant antitumor activity without toxicity is the goal. In the case of plant-derived antitumor agents, an abundant supply of the plant is also a prerequisite. St. John's Wort appears to satisfy both criteria, as it grows abundantly in Europe, North America, North Africa, and Asia, and has an excellent safety profile with minimal side effects. Schempp *et all*. (2002) found hyperforin dose dependently inhibited the growth *in vitro* of rat and human mammary cancer, as well as squamous cell carcinoma, malignant melanoma, and lymphoma.

CONCLUSIONS

In the actual study, both Hypericum sp. (*H. maculatum* and *H. perforatum*), have shown anticancer effect, through inhibition of tumor cells development and elevating the white blood cells level. The antitumor effectiveness of *H. perforatum* is already well documented, but remarkably *H. maculatum* was not only effective, but it was even more active than *H. perforatum*. This might be the basis of new pharmaceutical remedies using *H.maculatum* a wide spread species in mountain regions of Romania.

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