ANTI-TUMOR EFFECT OF *EUONYMUS EUROPAEUS* ON EHRLICH TUMOR CELLS *IN VIVO*

BOGDAN SEVASTRE[1*], ORSOLYA SÁRPATAKI[1], NELI K. OLAH[2], ROXANA L. STAN[3], MARIAN TAULESCU[1], IOAN MARCUS[1], CORNEL CĂTOI[1]

[1] University of Agricultural Science and Veterinary Medicine, Faculty of Veterinary Medicine, 3/5 Manastur Street, 400372, Cluj-Napoca, Romania,
[2] PlantExtrakt Ltd, 407059 Rădaia, Cluj-Napoca, Romania and Department of Pharmaceutical Industry, Faculty of Pharmacy, "Vasile Goldis" West University of Arad, 1 Feleacului Street, 300041, Romania
[3] University of Medicine and Pharmacy "Iuliu Haţieganu", Faculty of Pharmacy, Department of Pharmaceutical Biochemistry and Clinical Laboratory, 4/6 Pasteur Street, 400349, Cluj-Napoca, Romania
** corresponding author: sevastre_b@mail.com*

Abstract

Euonymus europaeus L., (EE) is the sole representative of *Celastraceae* family in Europe. The aim of the present study was to investigate EE anticancer effect on an *in vivo* model. The ethanolic extract was administered in different doses both orally and intraperitoneally in Swiss female mice intraperitoneally implanted with Ehrlich ascitic tumour cells. The EE extract showed a significant antiproliferative effect in a dose dependent manner, the highest dose of 50 mg/kg b.w., reduced the body weight gain (by up to 5%), accumulation of ascitic fluid (by up to 65%), and the tumour cell concentration (by up to 43%) ($p<0.05$). The inhibitory effect was positively correlated with the concentration of the inflammatory cells into the peritoneal exudate.

Rezumat

Euonymus europaeus L., (EE) este singurul membru al familiei *Celastraceae* din Europa. Scopul acestui studiu a fost investigarea efectului antitumoral al extractului *in vivo*. Extractul etanolic a fost administrat în doze diferite atât pe cale orală cât şi intraperitoneal, la şoareci din linia Swiss, femele, injectate în prealabil cu celule tumorale ascitogene Ehrlich. EE a dovedit un semnificativ efect antiproliferativ doză dependent. Cea mai mare doză administrată, 50mg/kgc a redus semnificativ ($p<0,05$) creşterea masei corporale (la 5%), acumularea lichidului ascitic (la 65%), precum şi concentraţia celulelor tumorale în lichidul ascitic (la 43%). Reducerea dezvoltării tumorii ascitogene Ehrlich a fost corelată pozitiv cu concentraţia celulelor inflamatorii din exsudatul peritoneal.

Keywords: *Euonymus europaeus*, antiproliferative, haematology, cytology.

Introduction

Euonymus europaeus L., (EE) also known as spindle tree, is the sole representative of *Celastraceae* family in Europe. It is a deciduous small tree with poisonous fruits, until now, with limited use in phytotherapy. However,

plants from Celastraceae family have been used from centuries in South America, China and Africa in traditional medicine for the treatment of various diseases including cancer [1]. *E. europaeus* (EE) contains a wide range of active compounds including sesquiterpenes, alkaloids [1], lectins [7]. Very few studies regarding the pharmacological effects of European spindle tree were done, but other *Euonymus* species were intensively studied. One of the most studied was *E. alatus*, a species native from southeastern Asia. It exhibits antitumor properties throughout apoptosis *via* mitochondrial pathway [4] and, by inhibition of the tumor invasion [3].

The aim of the present study was to investigate the possible anticancer effect of EE on Ehrlich ascites carcinoma model.

Materials and Methods

Plant materials:

Fruits of EE were harvested in September 2011, from the forests near Cluj, Romania. The fruits were botanically identified by the Quality Control laboratory of TC PlantExtrakt Ltd, Radaia, Cluj County, Romania; a voucher specimen was filed and kept in the company archives (B. no. 063811, QoA 4817/20.09.2011).

The fresh fruits were used to prepare a hydro alcoholic extract. The extract was obtained according to European Pharmacopoeia, method 1.1.3 (monograph 2371, European Pharmacopoeia 7.2), corresponding to the method 2a from the German Homeopathic Pharmacopoeia. The extraction was performed by cold maceration of the fresh plant material in 90% vol. ethanol, daily mixing the plant-solvent mixture for 17 days long. The extraction ratio was 1:1.5 (dry plant – solvent). Due to the processing of the vegetal material in fresh state, the final alcohol content into the extract was reduced by half. Following decantation and pressing, the extract was kept for 5 days at maximum of 25°C and then filtered [9].

The extract was characterized by determining the aspect, relative density, dry residue, ethanol content, and identity by TLC (thin layer chromatography) and the cardiotonic compounds level by UV-Vis spectrophotometry. The relative density was determined using an Anton Paar DMA 35 digital densimeter. The residue was determined by evaporation in oven, at 105°C, for 2 hours. The ethanol content was determined according to the European Pharmacopoeia (2.9.10), by distillation [9].

The identification was performed by TLC analysis, according to the German Homeopathic Pharmacopoeia (GHP) using silica gel plate, a mixture of ethyl acetate : methanol : water (81:11:8, v/v) as mobile phase

and digitoxin (Merck) 0.5 mg/mL in methanol respectively lanatoside C (Merck) 0.5 mg/mL in methanol as standards. 10 mL of extract were mixed with 20 mL water and 10 mL of 9.5% lead acetate solution. The obtained mixture was extracted with 2 x 15 mL of chloroform : iso-propanol (3:2 v/v). The separated organic phase was concentrated by draying; then, the residue was re-dissolved with 0.5 mL of methanol. Samples of 40 µL and 10 µL were applied from each standard. The visualization of the plate was performed in fluorescence after spraying with a mixture of 3% chloramine T solution and 25% trichloroacetic acid solution (2:8, v/v) and heating at 110°C [10].

The cardiotonic compounds level was measured by UV-Vis spectrophotometry using as reagent the basic sodium picrate and as standard a 50 µg/mL digitoxine solution in 96% vol. ethanol. The determination was performed at 495 nm [10].

Before inoculation, the alcoholic solution was maintained in a rotary evaporator 40°C, until 3/4 of the content evaporated, then filled with sterile saline solution up to 0.5 mL/animal. The aqueous solution was immediately administrated in order to prevent the bacterial and fungal contamination. The control group received *a placebo*, 0.5 mL alcohol 70°C, previously evaporated, similarly to plant extract method. The animals received the therapy in different doses, *per os* or intraperitoneally according to the protocols described below.

Animal care and experimental procedures

The experiments and animals Welfare were in accordance to the *Guide for the Care and Use of Laboratory Animals* (Department of Health Education, and Welfare, National Institute of Health, 1996), and followed the guidelines of European Communities Council Directive (86/609/1986) and Ordinance No. 37 of the Romanian Government from 2nd February 2002. The animal tests and experiments were aproved by the Bioethical Board of the Faculty of Veterinary Medicine Cluj-Napoca. The animals were housed in polycarbonate cages, at a controlled temperature of 21-22ºC, humidity (40-60%) and 12/12h light/dark cycle. Standard laboratory chow, provided by the National Institute for Research and Development Cantacuzino Bucharest, and water were freely available. The experiment was carried out on white Swiss female mice, 34.13±3.61 g body weight.

Preliminary toxicity tests

The toxicity of EE was evaluated according to Udeanu *et al*. [8] modified protocol. Swiss female mice were divided equally into six groups of five animals each with weight as limited factor. Three groups were inoculated intraperitoneally (i.p.), while in other two groups the extract was

administrated *per os* and one group, the control, was i.p. injected with the solvent only. The doses injected i.p. were 1500 mg/kg b.w., 3000 mg/kg b.w. and respectively 4500mg/kg b.w., and *per os* the EE extract was administrated in doses of 2000 mg/kg b.w. and 6000mg/kg b.w. The animals were housed one by one during the first 48 h after dosing, and they were observed at 30 min, 2, 4, 8, 24, and 48 hours to detect the onset of toxicological signs. Following the 48h observation period, all animals returned to their original groups of five for the remainder of the 14 days observation period. In the end the blood was harvested under deep narcosis, and then animals were euthanized, subjected to macroscopic examination and histological examination of liver and kidney. Blood samples were used for haematological assessment and plasma biochemistry.

Experimental design

Animals were divided into six equal experimental groups of 6 mice, under the following treatments:

I. mice implanted with Ehrlich ascites carcinoma (EAC) cells, 10^6 ascitic cells each, on day 0, as described above, and received *placebo* therapy intraperitoneally;

II. EAC implanted mice, receiving the EE extract, 50 mg/kg b.w. i.p.;

III. EAC implanted mice, receiving the EE extract, 25 mg/kg b.w. i.p.;

IV. EAC implanted mice, receiving the EE extract, 12.5 mg/kg b.w. i.p.;

V. mice, receiving the EE extract, 50 mg/kg b.w. i.p., without EAC implantation;

VI. EAC implanted mice, receiving the EE extract, 50mg/kg b.w. orally; the extract was prepared in the same way as for the rest of the groups.

The EE ethanolic extract was administrated on the 1^{st}, 3^{rd} and 6^{th} day. Body weight was measured at the beginning and at the end of experiment. The variation in body weight was calculated individually, according to formula:

Variation b.w. (%) = (final b.w. – initial b.w.) x 100 / initial b.w.

Blood automatic haematology (WBCc (white blood cells count), RBCs (red blood cells count) parameters) was investigated with Abacus Junior Vet, automatic analyser Diatron Messtechnik, Budapest, Hungary.

Ehrlich ascitic fluid was implanted, 10^6 ascitic cells to each mice, on day 0 of the experiment. Fourteen days after EAC implantation, blood was collected from the retro orbitary sinus under anaesthesia; the abdominal ascitic fluid was measured using a syringe, and then transferred in phosphate buffer solution (4°C, pH 7.4). The aspect of the abdominal cavity was evaluated by two independent observers without knowledge of the

experimental design. The fibrin content was quantified as absent = 0, low = 1, high = 2, very high = 3. The congestion on the abdominal wall was classified as absent = 0, low = 1, and high = 2.

The cell viability was assessed by Tripan blue staining (0.4% in PBS (phosphate buffer saline)), the total and viable tumour cell concentration was counted in a haemocytometer (dilution 1:10). Smears were made from peritoneal fluid and fresh blood, and then they were fixed in acetone, and stained by *Dia Quick Panoptic method.* The cells were differentiated into tumour cells, neutrophils, macrophages, lymphocytes by using classic morphological patterns, counting 200 cells for each sample. Evaluation was performed by an experimented observer without knowledge about the experimental groups. The same method was used for blood smears. The concentration of each type of cell (made from venous blood or peritoneal fluid) was calculated based on WBCs count (on automatic analyser) and total tumour cells count (on Burker camera), according to formulas:

$$\text{Cells}\,(10^6/\text{mL})\,(\text{in peritoneal fluid}) = \text{Cells}\,(\%) \times \text{EAC cells}\,(10^6/\text{mL}) / \text{EAC cells}\,(\%)$$

$$\text{Cells}\,(10^6/\text{mL})\,(\text{in blood}) = \text{Cells}\,(\%) \times \text{WBCs}\,(10^6/\text{mL}) / 100\,(\%)$$

Statistical analysis

All data were reported as the mean ± SEM. To assume Gaussian distribution normality distribution was checked by Shapiro-Wilk test of normality.

One-way analysis of variance ANOVA, followed by post hoc Tukey's range test procedure was done for pair-wise comparisons and Pearson test analysed the correlation between normally distributed values (ascitic fluid, viable cells percentage, non-viable cells percentage, total cells concentration in ascitic fluid, and haematology data).

For the values, which were not normally distributed (the intensity of congestion in the abdominal wall and fibrin content in the ascitic fluid), multiple pare-wise comparison was performed by one-way analysis of variance Kruskal-Walis, followed by post-test Dunns. Spearman's correlation was the test of choice.

Pearson's correlation was used in order to assess the correlation between normally distributed variables; interpretation was done according to Colton scale.

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

Quality control of the EE hydroalcoholic extract

The obtained hydroalcoholic extract (mother tincture) is a clear, brown-orange liquid.

TLC analysis identified 2 yellow bands; one at the lanatoside C level and one below the digitoxine level, the standards had blue band for lanatoside C and yellow band for digitoxine. The bands corresponded to the chromatogram description from the GHP (German Homeopathic Pharmacopoeia). The TLC conditions and the position on chromatogram relative to the used standards indicated that the bands corresponded to sterol structure compounds [11]. The quality parameters of the extract, in comparison with the admissibility range from German Homeopathic Pharmacopoeia (GHP) are detailed in the table below (Table I).

Table I

Quality parameters of the extract in comparison with the admissibility range from German Homeopathic Pharmacopoeia

Parameter	Admissibility range (GHP) [10]	Results on the extract
Relative density	0.935 – 0.955	0.953
Dry residue	Min. 4%	5.76%
Ethanol content	40 – 50% vol.	45% vol.
Identity	According to GHP	Corresponding
Cardiotonic compounds level	Max. 0.01%	0.0028 %

All the results were within the admissibility range; therefore the obtained extract corresponded qualitatively to the GHP conditions.

Preliminary toxicity tests

All the mice subjected to toxicity test remained alive until the end of the study. However, at 4500 mg/kg b.w., in the first 90 minutes following intraperitoneally administration the mice showed lethargy, spasms, abdominal tenderness, but no other clinical signs were observed afterwards. The body weight increased not significantly in a similar manner to control group. The necropsy did not reveal abnormalities in any of the organs. Serum biochemistry reflected normal kidney and liver function aspect confirmed by histopathology studies. The Complete Blood Count showed normal values, similar to those found in control group. These findings suggest that the EE extract is safe even at very high doses; the clinical signs are more likely to be induced by local irritating effect than systemic toxicity.

The effect of EE on haematological parameters of EAC inoculated mice

Red blood cells, haemoglobin and haematocrit showed no significant changes associated to EAC development; therefore, a possible protective

effect could not be established (Tables II and III). Development of transplantable tumours, like Dalton's lymphoma, was associated with severe anaemia [2], but previous studies conducted on EAC model showed that the tumour development is not associated with significant changes in RBCs [6].

Table II

The effect of *E. europaeus* alcoholic extract on the values of the red blood cells (RBCs), haemoglobin (HGB) and haematocrit (HCT) (mean ± S.E.M.)

Group	RBCs 10^{12} / L	HGB g / L	HCT %
I	5.77±0.32	99±1.42	32.1±0.74
II	6.60±0.18	113±1.05	34.79±0.48
III	5.86±0.36	98.33±1.55	30.61±0.82
IV	6.58±0.15	105.75±0.53	33.07±0.31
V	6.22±0.13	95.12±1.12	32.45±0.21
VI	7.67±0.25	81±0.88	40.01±0.59

S.E.M = Standard Error of the Mean

Table III

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

Group	MCV fL	MCH pg	MCHC g / dL	RDWs fL
I	55.75±0.24	17±0.22	305.5±1.03	39.65±0.28
II	52.75±0.31	17.05±0.28	323.5±1.07	35.92±0.34
III	52.33±0.29	16.43±0.29	313.33±1.36	38.55±0.29
IV	50.5±0.18	16.075±0.16	319.75±0.61	35.72±0.24
V	52.4±0.15	14.21±0.21	315.2±0.25	36.67±0.28
VI	52.25±0.32	10.77±0.41	208.25±1.90	38.3±0.31

S.E.M = Standard Error of the Mean

On day 15th, tenfold increase in WBCs count was observed in EAC inoculated mice. The leucocytosis was mainly based on the neutrophils, but other categories like eosinophils, lymphocytes and monocytes were also increased ($p<0.05$). A reduction of WBCs count was observed in mice treated with EE in a dose dependent manner; i.p. administration was, by far, more effective than the oral therapy. The neutrophils and eosinophils were decreased ($p<0.05$), while the monocytes and lymphocytes were less affected (Table IV).

The blood neutrophils levels were positively correlated with those of lymphocytes ($r = 0.49$, $p<0.05$). Neutrophils showed also a good positive

correlation with fibrin content of the ascitic fluid ($r = 0.62$, $p<0.05$) and congestion of the abdominal wall ($r = 0.67$, $p<0.05$).

Table IV

The effect of *E. europaeus* alcoholic extract, on the WBC count and differential count (mean ± S.E.M.) (10^9cells / L)

Group	WBCs	N	E	B	L	M
I	3.79±0.27	1.03±0.19	0.16±0.09	0.02±0.04	2.32±0.22	0.25±0.12
II	31.07±1.01	24.47±0.91	0.69±0.20	0.04±0.07	5.09±0.38	0.76±0.12
III	7.78±0.55 [a]	4.09±0.48 [a]	0.11±0.08 [a]	0±0	3.19±0.34	0.19±0.11
IV	20.8±0.75	17.13±0.72	0.25±0.13 [a]	0.02±0.05	3.35±0.23	0.06±0.08
V	18.8±0.83	15.34±0.64	0.14±0.08 [a]	0±0	2.85±0.35	0.22±0.14
VI	14.98±0,76	12.06±0.75	0.13±0.1 [a]	0±0	2.34±0.26	0.45±0.17

S.E.M = Standard Error of the Mean, WBCs = White Blood Cells, N = neutrophils, E = eosinophils, B = basophils, L = lymphocytes, M = monocytes, [a] = $p<0.05$, as compared to EAC group

The effect of EE on the development of EAC and the composition of ascitic fluid

The weight of mice as a measure of proliferation of Ehrlich ascites tumour cells in the peritoneal cavity of mice is showed in Figure 1. The maximum gain of body weight (18%) was observed in the EAC group.

The EE therapy reduced significantly the body weight gain at the dose of 50 mg/kg b.w. intraperitoneally, while lower doses or oral administration showed no significant protection. The amount of ascitic fluid varied in a similar manner with body weight gain (Figure 1).

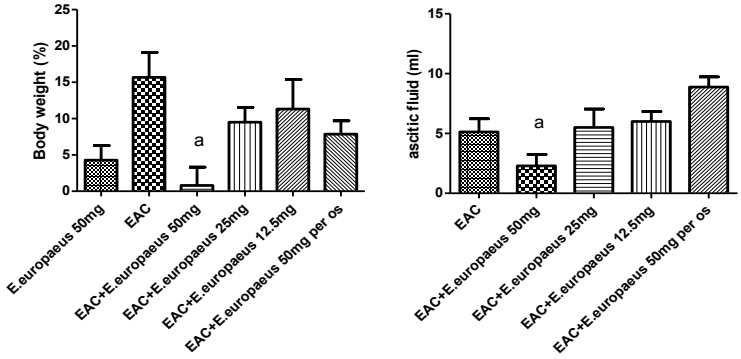
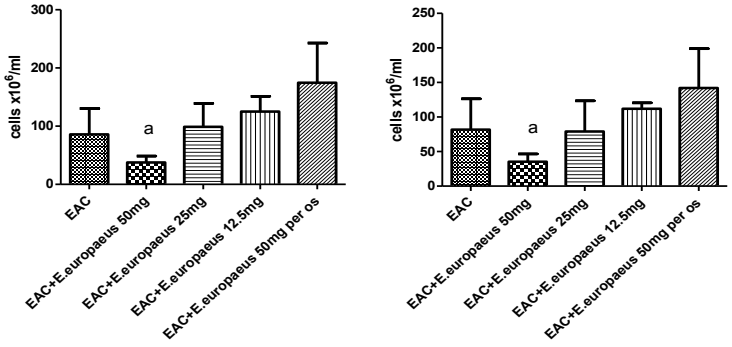


Figure 1.

Effects of *Euonymus europaeus* extract on the body weight gain (%) and accumulation of the ascitic fluid (mL) (mean ± SEM)
([a] = $p>0.05$ as compared to EAC group)

Both total tumour cells and the viable tumour cells, presented in the peritoneal cavity of mice, decreased as a consequence of EE therapy in the higher doses ($p<0.05$), while other doses showed no obvious effect (Figure 2). The amount of ascitic fluid was positively correlated with total ascitic tumour cells concentration ($r = 0.47$, $p<0.05$) and with concentration of viable tumour cells ($r = 0.5$, $p<0.05$).

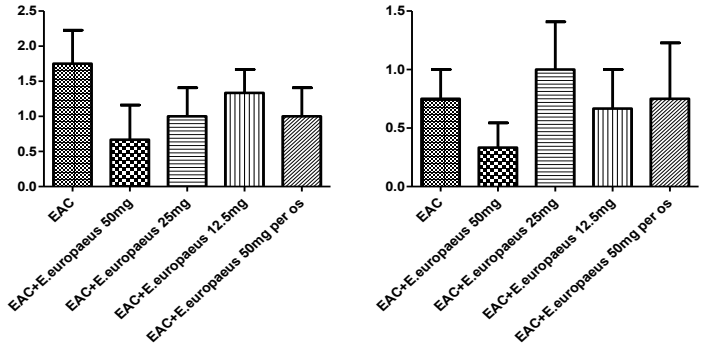


A B

Figure 2.

Effects of *Euonymus europaeus* extract on Ehrlich ascites tumour cells concentration (A - total tumour cells concentration, B - viable tumour cells concentration) (mean ± SEM) (^{a} = $p>0.05$ as compared to EAC group)

The morphological aspects of tumour related inflammation like the fibrin content of the ascitic fluid and congestion of the abdominal wall were not influenced in a significant manner (Figure 3).



A B

Figure 3.

Effects of *Euonymus europaeus* extract on inflammatory parameters of the ascitic fluid (A- fibrin content of the peritoneal fluid B - congestion in the abdominal wall) (mean ± SEM)

The concentration of the neutrophils and lymphocytes in the ascitic fluid were several times higher in EE treated mice, in doses of 50mg/kg b.w. i.p. (Table V).

These findings suggest that the inflammatory cells in the ascitic fluid provided some protective effect against ascites development, while blood leucocytes did not. The correlation test confirmed this observation, while ascitic lymphocytes showed a negative correlation with fibrin content of the ascitic fluid ($r = -0.45$, $p<0.05$) and congestion of the abdominal wall ($r = 0.61$, $p<0.05$), blood neutrophils were positively correlated with these two parameters. The correlation of the ascitic lymphocytes and other inflammatory cells with EAC associated parameters provided no significant values.

Table V

The effect of *E. europaeus* alcoholic extract on the cytology of the peritoneal fluid (mean ± S.E.M.) (10^9 cells / L)

Group	EAC cells	N	L	M
I	85.75±2.36	17.91±0.710	10.08±0.65	0±0
II	37.5±0.87 [a]	109.21±1.83 [a]	98.58±2.45 [a]	1.5±0.32
III	98.75±2.24	29.86±1.34	12.10±0.82	0±0
IV	82.21±2.32	25.57±1.83	10.14±1.12	0±0
VI	174.5±2.92	19.00±0.80	15.03±0.96	0±0

S.E.M = Standard Error of the Mean, [a] = $p<0.05$, as compared to EAC group

The present data showed that EE extract significantly ($p<0.05$) reduced the ascitic tumour development, and inhibited the tumour cell proliferation in a dose dependent manner. Antiproliferative effect may be due to induction of apoptosis or cell cycle arrest, but the enhancement of local immune activity suggested by the increased concentration of lymphocytes, cannot be excluded either.

Well-documented studies shown that various plant extracts proved a significant antiproliferative activity both *in vitro* [2, 5] and *in vivo* [2, 6]. Commonly, the phytotherapeutic extracts responsible for tumour inhibition contain more than one antitumor compound; therefore all the mechanisms described above should be considered in future studies.

Little is known about the pharmacological properties of EE, but several studies found in this plant various active compounds, some of them with possible anticancer effect, like sesquiterpene, alkaloids [1], lectins [7]. On the other hand, other *Euonymus* species, *E. alatus* already proved antitumor properties throughout caspase-3 activation followed by apoptosis

via mitochondrial pathway [4]. Other studies suggest that 5-caffeoylquinic acid found in *E. alatus* might prevent the tumour invasion by inhibition of the MMP-9 (matrix metalloproteinase – 9) activity [3].

Conclusions

In the present study, we proved that EE extract was not toxic; it significantly reduced the ascitic tumour development, and inhibited the tumour cell proliferation in a dose dependent manner. The inhibition was positively correlated with the amplitude of local inflammatory response in the peritoneal fluid.

Acknowledgements

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