Anticancer Activity of *Euonymus europaeus* Fruit Extract on Transplantable Mouse Tumor Model

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Abstract

*Euonymus europaeus* L. (EE), is the only European representative of the *Celastraceae* family, with limited use in phytotherapy. In the present study, we investigated the anticancer effects of EE hydro alcoholic extract prepared from fresh fruits. HPLC analysis revealed the presence of evodiamine in concentration of 0.404 g/ml. Antitumor effect was assessed in vivo on Swiss mice. Mice implanted with transplantable tumor cells were treated with 25 or 50 mg/kg EE extract, by IP injection on the 1st, 3rd and 6th day, the study lasted for 14 days long. A parallel study assesses the median survival rate. EE therapy, at the higher dose, prevented the body weight gain (p<0.001), reduced the volume of ascitic fluid by 71.88% (p<0.001) and increased the median survival rate by 28.57% (p<0.05).

Keywords: antitumor, Ehrlich ascites carcinoma, evodiamine, medicinal plants

INTRODUCTION

Research for new anticancer drugs is a very active domain, as long as natural products represent an important source of new drugs. Nowadays, more than 60% of the new therapeutic compounds are either isolated from natural products, or incorporate themselves synthetic compounds, which have similar chemical structure of some natural products (Chabner and Roberts, 2005).

Plants from the *Celastraceae* family have been used for centuries in traditional medicine, in South America, China, and Africa (Descoins *et al.*, 2002). Of all, *Euonymus alatus* (Thunb) *Celastraceae* (EA), a species native to Southeastern Asia, is commonly recommended as medicinal plant, inclusive in cancer treatment (Xu, 2015). Various authors described several pathways responsible for its remarkable anticancer properties; two of them are of main importance. According to Kim *et al.* (2006), it exhibits antitumor properties by inducing apoptosis via mitochondrial pathway. Other authors showed that EA inhibits tumor invasion, mainly by suppression of the matrix metalloproteinase-9 (MMP-9) activity (Cha *et al.* 2003, Jin *et al.* 2005), effect provided by dihydroxycinnamic acid (caffeic acid) (Park *et al.*, 2005). The inhibition of MMP-2 and MMP-9 seems to be mediated by NK-kappaB pathway (Chung *et al.*, 2004).

*Euonymus europaeus* L., (EE) known also as the spindle tree, is the only European represen-
tative of the Celastraceae family. It is a small deciduous tree with poisonous fruits, with very limited use in phytotherapy until now. Few phytochemical analysis performed on EE revealed some active compounds including sesquiterpene polysters belonging to the alatol, 3-deoxymatol, 3,4-dideoxymaytol families, evonimate alkaloids, dihydro-ß-agarofuran polysters (Descoins et al., 2002), and lectins (Teneberg et al., 2003) with erythrocyte agglutination properties (Agostino et al. 2015).

The aim of the present study is to investigate the anticancer activity of EE alcoholic extract using a transplantable tumor models in vivo.

MATERIALS AND METHODS

Plant materials and extraction

Fresh fruits of EE, harvested in September 2011, from the forests near Cluj, Romania, were used to prepare the hydro alcoholic extract. The collecting of fruits was performed according to the GACP rules, from an unpolluted area, at a minimum of 3 km distance from any circulated roads. The fruits were identified by the Quality Control laboratory of PlantExtrakt TC Ltd, Rădaia, Cluj County, Romania. A voucher specimen was filed and kept in the company archives (B. no. 063811, CoA 4817/20.09.2011).

The moisture in the fresh vegetal material is 70 %, as described in previous studies (Sevastre et al., 2014). Briefly, the freshly cut vegetal material was mixed with 90 % vol. ethanol, at 1:0.7 ratio. The mixture was kept at room temperature for ten days long, and then, the mixture was pressed and filtered. All steps were performed on an API-GMP certified production flow at SC Plant Extrait SRL in Rădaia, Cluj County, Romania, according to method 2a from the German Homeopathic Pharmacopoeia.

Before use, the alcoholic solution was processed in a rotary evaporator at 40°C, until 3/4 of the content was evaporated, then refilled with sterile saline solution up to 0.5ml/animal. The aqueous solution was sterilized by transferring throughout syringe filters, and was immediately administered. The control group received a placebo, 0.5ml alcohol 70°, previously evaporated, similarly to the plant extract method.

Standardization of mother tincture

The standardization procedures were already presented in previous studies (Sevastre et al., 2014), however the methods were being briefly described in the paragraphs below.

Determination of aspect, relative density, dry residue and ethanol content

The countenance, color and smell of mother tincture was determined by observation, additionally relative density, dry residue and the ethanol content were determined

Thin-layer chromatography (TLC)

The Thin-layer chromatography (TLC) determination was performed according to the German Homeopathic Pharmacopoeia. 10 ml of EE extract was mixed for five minutes with 20 ml water and 10 ml 9.5 % lead acetate. The extraction was made with 2 x 15 ml mixture of chloroform – iso-propanol (3:2, v/v). The organic phase was separated and dried at maximum 50°C. The dry residue was dissolved in 0.5 ml methanol. 40 µl of prepared sample and 20 µl of standard solutions, respectively, were applied on TLC plate. The TLC determination was performed on Silicagel F254 plates, using as mobile phase a mixture of ethyl acetate, methanol and water (B1:11:8, v/v). Digitoxin 0.5 mg/ml and lanatosid C 0.5 mg/ml, respectively, were used as standards. The evaluation of the plate was made after spraying it with a mixture of 3 % trichloramine T and 25 % trichloracetatic acid (2:8, v/v), exposing it to heating for 5-10 minutes, at 105-110°C and visualizing it afterwards in fluorescence at 365 nm.

Determination of evodiamine content

The evodiamine content was determined by HPLC, using a method provided by Phytolab and adapted in the view of getting a better separation of compounds from mother tincture, as a start. The determination was performed using a Varian ProStar HPLC system. A Phenomenex Luna C18 silicagel-C18 column was used, 150 mm x 4.6 µm with a precolumn of 5 mm x 4.6 µm, both having particles of 5 µm. As a mobile phase, a solvent gradient with phosphoric acid pH=2.5, water and acetonitrile, with a 1 ml/min flow rate were used. The detection was performed with a DAD detector at 228 nm. The UV-Vis spectra were recorded from 200 nm to 600 nm.

Volumes of 100 µl of EE extract from each concentration of standard evodiamine solution were injected. For the quantitative determination, a calibration curve with concentrations of evodiamine ranging between 50 µg/ml and 500 µg/ml was built. The calibration curve’s equation
is $A = 60110x C - 478977$ and the correlation factor is 0.9807. The identification was made based on the comparison between the retention time and UV-Vis spectra of compounds separated from EE extract and the standard evodiamine. The absorption maximum of evodiamine is at 228 nm.

**Animal care and experimental procedures**

The experiments on animals were performed according to the Directive 2010/63/EU of the European Parliament and of the Council and Low no. 43/11.04.2014 of the Romanian Parliament, following the guidelines of *Guide for the Care and Use of Laboratory Animals* (Department of Health Education, and Welfare, National Institute of Health, 1996). The Bioethical Board of the Faculty of Veterinary Medicine, Cluj-Napoca, authorized the experiment. The animals were housed in polycarbonate cages, at a controlled temperature (21-22°C), humidity (40-60%) and 12/12h light/dark cycle. Standard lab chow, provided by National Institute for Research and Development Cantacuzino Bucharest, and water were freely available. Albino Swiss female mice were used in these experiments, having a body weight about 33.75±3.52 g.

In the present study, we performed two independent experiments: the first one was designed to assess the antiproliferative effect of the extract and the ability to reduce the side effect of tumor growth, while the second one was dedicated to survival analysis.

For the first experiment, the animals were organized in three equal groups of eight mice, and treated as follow:

I. - mice implanted with Ehrlich ascites carcinoma (EAC) cells, $10^6$ ascitic cells each, on day 0, and placebo intraperitoneally;
II. - EAC implanted mice, receiving 50 mg/kg b.w.;
    EE extract, IP
III. - EAC implanted mice, receiving 25 mg/kg b.w
    EE extract, IP;
All animals received the EE ethanolic extract on the 1st, 3rd and 6th day. The body weight was measured on a daily basis, until the end of the study.

*Ehrlich ascitic fluid* was implanted ($10^6$ tumor cells/mouse), on day 0 of the experiment. Fourteen days after EAC implantation, blood was collected from the retro orbital sinus under anesthesia and the animal were subsequently euthanized. Complete blood count (CBC) was performed using Abacus Junior Vet, automatic analyzer (Diatron Messtechnik, Budapest, Hungary).

The volume of ascitic fluid was measured with a syringe, and then transferred in a phosphate buffer solution (4°C, pH 7.4). The cell viability was assessed by Trypan blue staining (0.4% in PBS), the total and viable tumor cell concentration was counted by using a hemocytometer (dilution 1:10). Smears were made from peritoneal fluid and stained by *Dia Quick Panoptic* method. The cells were then differentiated on a 100x field objective, by an observer unaware of the experimental design. The cells were differentiated into tumor cells, neutrophils, macrophages, lymphocytes using classic morphological patterns, counting 200 cells for each sample, based on classical morphological criteria (Jain, 1993).

For the second experiment, we use only the higher dose of *E. europaeus* (50mg/kg). Sixteen animals were organized in two equal groups: both implanted with Ehrlich ascites carcinoma (EAC) cells, one group was treated and the other one not. In this study we performed two experiments: one in which the animals were allowed to live for 2 weeks long and then euthanized to assess the amount of ascitic fluid and cellular content of ascitic fluid (all of them survived to the end of 2 weeks), and the second experiment in which the animals were not killed but observed until the day they die, by a survival test. The results were then analyzed by Long Rank test and survival curves were generated.

**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. Pearson’s correlation was used in order to assess the correlation between normally distributed variables; this interpretation was made according to the Colton scale. Statistical significance was at $p<0.05$ (95% confidence interval). The comparison between survival curves was evaluated throughout Long-rank (Mantel-Cox) Test. Statistical values and figures were obtained.
RESULTS AND DISCUSSION

Standardization of the studied mother tincture

The obtained hydro alcoholic extract (mother tincture) was a clear, brown-orange liquid. The quality parameters of the extract were within the admissibility range of German Homeopathic Pharmacopoeia. TLC chromatogram of the EE extract showed the presence of lanatoside C, a cardiotonic glycoside. Hence, only insignificant amount of digitoxin was also found. HPLC chromatogram revealed the presence of evodiamine in concentration of 0.404 g/ml, while the recorded UV-vis spectra further proved the presence of evodiamine in the extract (Sevastre et al., 2014)

Antiproliferative effect in vivo

In EAC inoculated mice, the WBCs count was four times over the normal maximum range. The blood leukocytosis was mainly based on neutrophilia (p<0.001), but the lymphocytes number was also significantly increased (p<0.001). A significant decrease in WBCs count was observed in mice treated with EE alcoholic extract, both neutrophils and lymphocytes being influenced (Tab. 1.).

The blood neutrophils were positively correlated with the lymphocytes (r=0.55, p<0.01), but not with other leucocytes. Neutrophils also showed a good positive correlation with fibrin content of the ascitic fluid (r=0.52, p=0.05) and congestion of the abdominal wall (r=0.43, p=0.05). There was also a positive correlation between the number of neutrophils and the medium platelet volume (r=0.63, p<0.01), which suggested a common inflammatory mechanism beneath these two hematological changes.

The body weight, as a measure of Ehrlich ascites accumulation in the peritoneal cavity is shown in figure 1. As expected, the ascites development was followed by 32.90 ± 7.98% increase in the body weight. The EE therapy prevents body weight gain. The differences were visible starting from the third day of the study: both doses proved to provide significant protection, but the effect was more visible in the group treated with the higher dose.

The amount of ascitic fluid varied proportionally with the body weight gain. 50 mg/kg EE administered reduced by 71.88% the medium volume of ascites fluid, but the lower dose (25 mg/kg) was also effective: it reduced the accumulation of ascitic fluid by up to 39.06% (EAC 10.67±2.42 ml, EAC+50 mg/kg 3.06±0.75 ml, EAC+25 mg/kg 6.50±1.44 ml, p<0.05) (Fig 2 A).

The total viable tumor cell concentration in the peritoneal fluid significantly decreased in the groups treated with EE. The extract administered in dose of 50 mg/kg and 25 mg/kg reduced the tumor cell concentration by up to 67.93% and 66.30% (p<0.01) (Fig 2 B).

The correlation test showed a positive correlation between the percentage of body weight gain and the amount of ascitic fluid (r=0.54 p<0.05), but no relation between the amount of ascitic fluid and the tumor cell concentration could be proven (r=-0.044, p=0.89). Furthermore, the cellularity of the ascitic fluid was positively correlated with the blood neutrophils count (r=0.803, p<0.01), but again, no correlation with the ascitic volume could be proven (r=0.12, p=0.72).

The evaluation of ascitic hemorrhage showed that EE therapy provided some protection, but the semiquantitive analysis showed no statistically significant data. The hemorrhagic aspect of the ascitic fluid is due to extensive leaking of red blood cells.

Tab. 1. The effect of *E. europaeus* alcoholic extract on the WBC count and differential count (mean ± S.E.M.) (10³ cells/L)

<table>
<thead>
<tr>
<th>Group</th>
<th>WBCs</th>
<th>N</th>
<th>E</th>
<th>B</th>
<th>L</th>
<th>M</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>46.79±2.05</td>
<td>31.72±2.82</td>
<td>0.30±0.14</td>
<td>0.07±0.06</td>
<td>12.81±1.96</td>
<td>1.88±0.34</td>
</tr>
<tr>
<td>II</td>
<td>11.59±2.40***</td>
<td>6.04±1.39***</td>
<td>1.00±0.68</td>
<td>0.01±0.01</td>
<td>2.84±0.43***</td>
<td>1.69±0.59</td>
</tr>
<tr>
<td>III</td>
<td>19.58±2.39***</td>
<td>12.73±1.53***</td>
<td>0.31±0.09</td>
<td>0.06±0.04</td>
<td>5.19±0.68**</td>
<td>1.29±0.20</td>
</tr>
</tbody>
</table>

(*** = p<0.01, ** = p<0.001 as compared to EAC group)

S.E.M = Standard Error of the Mean (8 animals / group)

WBCs – White blood cells, N – Neutrophils, E – Eosinophils, B – Basophiles, L – Lymphocytes, M – Monocytes

Normal values: WBC 2.61-10.05 10³ cells/µL, N 0.4-2.0 10³ cells/µL, L 1.27-8.44 10³/µL, M 0-0.29 10³ cells/µL, E 0-0.17 10³ cells/µL, B 0-0.02 10³ cells/µL (Jain, 1993)
blood cells into the peritoneal cavity and it is a malignant side effect of EAC development (Mayer, 1966). Many ascitic samples rich in red blood cells also showed a high fibrin content, probably also related to the increased vascular permeability in the abdominal wall capillaries. The vascular changes of the abdominal wall were less evident in groups subjected to therapy, but the differences were not statistically sustained.

The percentage of white blood cells in the ascitic fluid was several times higher in EE treated mice, while the tumor cell percentage decreased. Neutrophils were the most prevalent category, but lymphocytes were also found (tab. 2). These findings suggest that the inflammatory cells in the ascitic fluid provided some protection against ascites development, while blood leucocytes did not. The correlation test confirms this observation, while ascitic lymphocytes showed a negative correlation with fibrin content in the ascitic fluid (r=−0.52, p<0.05) and congestion of the abdominal wall (r=0.51, p<0.05); neutrophils found in the ascitic fluid were also negatively correlated with these two parameters.

Blood neutrophilic leukocytosis is a side effect of tumor cell proliferation in response to tumor inflammatory cytokines (Liao et al., 2005, Sarpataki et al., 2011), but the efficiency of this reactive response seems to be limited because the circulating inflammatory cells fail to pass through capillary walls and migrate into the peritoneal fluid. Consequently, the ascitic fluid in control EAC implanted mice is rich in tumor cells, but the inflammatory cells are sparsely distributed. As a result of therapy, the number of tumor cells decreased, but the percentage of inflammatory cells in the ascitic fluid was elevated, in inverse correlation with the circulating blood leukocytes.

The EE anticancer effect was further proved within the survival experiment carried out. The therapy increased by 28.57% the median survival of EAC-bearing mice (EAC median, 14 days, EAC + EE 50 mg/kg b.w, 18 days; p<0.05) (Fig 3).
Initially, phytochemical investigation searched for phytotherapeutic compounds already known to be found in the EE extract, such as cardiac glycosides as digitoxin and lanatoside c. However, cardiac glucosides alone seems to be unable to explain this potent anticancer effect on their own, thus, we searched for more compounds, and, eventually, evodiamine was found.

In vivo studies were performed on a classic transplantable tumor model – Ehrlich ascites carcinoma inoculated in mice. This study provided valuable information regarding the antiproliferative potential of the EE extract, but they also implied important limitations related to kinetics, bioavailability, tissue distribution, route of systemic circulation, catabolism and secretion. Therefore, we consider that further studies need to be carried out in order to establish its applicative value in finding new molecules that can be used in cancer therapy.

Little is known about the pharmacological properties of *E. europaeus*, but several studies found various active compounds in this plant, some of them with possible anticancer effect, like sesquiterpenes, alkaloids (Descoins *et al.*, 2002), lectins (Teneberg *et al.*, 2003). On the other hand, the other members of *Euonymus* species *E. alatus* have well documented anticancer properties. An antiproliferative mechanism was proposed for mammary and genital tumors, respectively the inhibition of aromatase activity. Aromatase or estrogen synthetase is an enzyme responsible for estrogen synthesis. A large proportion of breast cancer expresses their own aromatase; therefore, the cell proliferation is enhanced by an autocrine stimulating mechanism. *E. alatus* was highly effective in inhibiting the intracellular aromatase

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**Fig. 2.** Effects of *Euonymus europaeus* extract on ascitic fluid accumulation (ml) (A) and viable Ehrlich tumor cells concentration (B).

(‘’ = p>0.05; **’’ = p>0.01; as compared to EAC group). (mean ± SEM) (8 animals / group)

**Tab. 2.** The effect of *E. europaeus* alcoholic extract on the cytology of the peritoneal fluid (mean ± S.E.M.) (%)

<table>
<thead>
<tr>
<th>Group</th>
<th>EAC cells</th>
<th>N</th>
<th>E</th>
<th>L</th>
<th>M</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>65.00±8.44</td>
<td>23.25±6.26</td>
<td>0.00±0.00</td>
<td>11.75±3.57</td>
<td>0.00±0.00</td>
</tr>
<tr>
<td>II</td>
<td>28.50±9.58</td>
<td>45.50±11.44</td>
<td>0.33±0.21</td>
<td>25.50±14.18</td>
<td>0.17±0.17</td>
</tr>
<tr>
<td>III</td>
<td>56.25±5.86</td>
<td>35.50±4.21</td>
<td>0.00±0.00</td>
<td>08.25±1.79</td>
<td>0.00±0.00</td>
</tr>
</tbody>
</table>

S.E.M = Standard Error of the Mean (8 animals / group)

in myometrial and leiomyomal cells, in dose-dependent manner, consequently inhibiting the estrogen synthesis in tumor cells (Lee et al. 2004).

The toxicity of EE on mice was evaluated in a previous study (Sevastre et al. 2014). All the mice subjected to toxicity test remained alive until the end of the study; therefore, LD 50 could not be established. Gross necropsy and histopathology did not reveal abnormalities in any of the organs. Serum biochemistry and complete blood count showed normal values, therefore we concluded that findings suggest that the EE extract is safe even at very high dose (Sevastre et al. 2014).

The EAC is a transplantable tumor of epithelial origin descended from a murine mammary gland adenocarcinoma. Not only it provides an appropriate model to assess the antiproliferative effects of various compounds, but it is also suitable to investigate the side effects of tumor growth and the interaction between tumor cells and the immune system. The Ehrlich tumor cells development in the abdominal cavity results in accumulating of abundant exudate, rich in tumor cells. Mediators produced by tumor cells are responsible for the increased vascular permeability, which leads to the extensive red blood cells leakage into abdominal cavity, resulting in anemia. This is widely accepted as malignant consequence of the EAC. Hemorrhage is secondary to extensive extravasations of red blood cells in peritoneal cavity, which leads to degradation of peritoneal capillaries (Mayer, 1966). In our current findings, supported by previous studies performed on Ehrlich ascitic tumor cells, decreased numbers of inflammatory cells were present, even in highly hemorrhagic samples, but they can increase significantly in response to therapy. Furthermore, we proved that the concentration of inflammatory cells in the ascitic fluid is negative correlated to the quantity of ascitic fluid and tumor cell concentration (Sarpataki et al., 2011). The inflammatory cells found in the ascitic fluid seem to provide some protective effect, but the blood leukocytes had no influence on tumor cells development. WBCs reach elevated concentrations in response to tumor cell proliferation, more likely because of having been stimulated by inflammatory cytokines produced by tumor cells. However, circulating white blood cells seem to provide little protection, because they are in negative correlation with the inflammatory cell population in ascitic fluid.

The current data, as described above, shows that EE extract significantly (p<0.05) reduced ascitic fluid accumulation and inhibited tumor cell proliferation in a dose-dependent manner. The anticancer mechanism is likely to be provided by more than one mechanism. The EE extract clearly reduced the intensity of ascitic hemorrhage, but whether it was a specific protective effect on the vascular walls or a simple side effect of reduced proliferation, remains to be established. The

![Graph showing effects of Euonymus europaeus 50 mg/kg on survival curves of EAC bearing mice.](image)

**Fig. 3.** Effects of *Euonymus europaeus* 50 mg/kg on survival curves of EAC bearing mice. (mean ± SEM) (p<0.05) (8 animals / group)
antiproliferative effect of EE was undoubtedly proven, by reduced body weight gain, by decreased ascitic fluid volume and decreased viable tumor cells. Furthermore, the survival experiment proved that EE therapy was able to improve the survival rate in a significant manner. The antiproliferative effect may be due to the induction of apoptosis or cell cycle arrest, but the enhancement of local immune activity cannot be excluded either. As support to the last hypothesis, there is an elevated number of neutrophils and lymphocytes found in the ascitic fluid as a response to therapy.

In the present study, we proved that the EE alcoholic extract inhibits the development of Ehrlich tumor cells on mice model. The inhibition was positively correlated with the amplitude of the local inflammatory response in the peritoneal fluid and negatively correlated with the systemic inflammatory response, which suggest that the enhancement of cell-mediated immunity might be part of the tumoricidal properties of EE.

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Declaration of interests: The authors report no declaration of interest.

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