ANTICANCER ACTIVITY OF EUONYMUS EUROPAEUS FRUITS EXTRACT ON HUMAN MELANOMA CELLS

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Abstract

Euonymus europaeus L. (Celastraceae) (EE), known as spindle tree, is a deciduous shrub commonly found all over European continent, used in traditional medicine for the treatment of ectoparasites. The present study investigates the in vitro anticancer effects of EE hydro-alcoholic extract prepared from fresh fruits. Qualitative and quantitative analysis of the EE extract content where made by high-performance liquid chromatography coupled with mass spectrometry (HPLC-MS). Cell Proliferation Assay and Anexin V-fluorescein isothiocyanate apoptosis detection where performed on both human melanoma cell line and normal human fibroblasts, in serial doses ranging from 0.05 µg/mL up to 50 µg/mL, at 6 and 24 hours exposure. Evodiamine, a potent anticancer compound was found in significant amounts. In vitro melanoma cells were inhibited in a concentration-dependent manner (p < 0.001), (IC₅₀ = 6.76 µg/mL), and they were almost ten-fold more sensitive than fibroblasts (IC₅₀ = 53.08 µg/mL). EE had a significant and selective antitumor activity.

Keywords: antitumor, Euonymus europaeus, evodiamine, cell viability

Introduction

Despite some progress, cancer remains a major research challenge. Research for new anticancer drugs is a very active domain; natural products represent an important source of new drugs. Actually, more than 60% of the new therapeutic compounds are either isolated from natural products or incorporate synthetic compounds based on the chemical structure of natural products [1, 5, 18]. Plants from the Celastraceae family have been used for centuries in the field of traditional medicine, in South America, China, and Africa, for the treatment of various diseases, including cancer [7, 21]. One of the most investigated was Euonymus alatus (Thunb) Celastraceae (EA), a native species from South-Eastern Asia, traditionally used for cancer treatment. There are several pathways responsible for its remarkable anticancer properties, but two of them are of main importance. First, it exhibits antitumor properties by inducing apoptosis via mitochondrial pathway [10, 19]. Second, EA inhibits tumour invasion, mainly by suppression of the matrix metalloprotease-9 (MMP-9) activity [4, 9], effect provided by dihydroxycinnamic acid (caffeic acid) [13]. The inhibition of MMP-2 and MMP-9 is mediated by NK-kappaB pathway [6]. Euonymus europaeus L., (Celastraceae) (EE) also known as the spindle tree, is the only European representative of the Celastraceae family. The dried fruits and seeds are used against external parasites as scabies, ticks and lice, while the seeds...
are emetic and purgative [2]. Relatively few phytochemical analysis on EE were performed, but they revealed active compounds including sesquiterpene polyesters belonging to the alatol, 3-deoxymatol, 3,4-dideoxymaytol families, evonimate alkaloids, dihydro-β-agarofoor polyesters [7], and lectins [17]. Evodiamine is a quinazolinecarbolone alkaloid initially isolated from Chinese herb Evodia rutaecarpa, represent a promising chemotherapeutic agent in the multiple-drug resistant cancer cells [12]. Evodiamine has been shown to exhibit anticancer properties by various mechanisms as cell cycle arrest, induction of apoptosis [19].

In the present study, we investigated the anticancer activity of EE. To differentiate the general cytotoxicity from a selective antitumor effect, we tested the inhibitory potential on both normal fibroblastic line and on melanoma cell line and then we investigated apoptosis in EE treated cultures.

**Materials and Methods**

**Plant materials and extraction.** Fresh fruits of EE, harvested in September 2011, from a forest near Cluj-Napoca, Romania, were used to prepare the hydro alcoholic extract. The harvest of fruits was performed according to the Good Agricultural and Collection Practices rules, from an unpolluted area, at a minimum of 3 km distance from any circulated road. The fruits were botanically identified by Quality Control Laboratory of PlantExtrakt TC Ltd, Râdaia, Cluj County, Romania. A voucher specimen was filed and kept in the company’s archives (B. no. 063811, CoA 4817/20.09.2011).

The sampling of the vegetable material was performed according to the European Pharmacopoeia, and the botanic identification, according to the German Homeopathic Pharmacopoeia. The moisture of the fresh vegetal material was 70%.

The freshly cut fruit was mixed with 90% vol. ethanol. The extraction ratio was 1 part fruit to 0.7 parts solvent. The extraction was performed at room temperature, by maceration and periodical mixing (minimum 10 min x 2 times a day x 10 days). After 10 days, the relative density and the dry residue were evaluated. The mixture of plant and solvent was subsequently pressed, left at maximum 30°C for 5 days and then filtered. The resulting extract was the tincture used for the further experiments. All preparation steps were performed on an API-GMP certified production flow at SC PlantExtrakt SRL in Râdaia, Cluj County, Romania, according to method 2a from the German Homoeopathic Pharmacopoeia [20].

Before use, the alcoholic solution was processed in a rotary evaporator at 40°C, until 3/4 of the content evaporated, then refilled with sterile saline solution to initial volume for use on cell culture.

**Standardization of the tincture.** The aspect of the tincture was determined by observation of countenance, colour and smell. The relative density was measured using an Anton Paar 35 DMA digital densitometer. The result is the average of 3 independent determinations. The dry residue was determined by drying at 110°C, for 3 hours. The result is the average of 3 independent determinations. Firstly, the ethanol content was determined by distillation, and secondly by the relative density of the distillate solution correlated with the values from the alcohoreometric tables.

**Determination of evodiamine content.** The evodiamine content was determined by HPLC, using a method provided by Phytolab and adapted. The determination was performed using a Varian ProStar HPLC system. We used a Phenomenex Luna C18 silicagel-C18 column, 150 mm x 4.6 μm with a pre-column 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 [22].

100 μL of EE extract from each concentration of standard evodiamine solution were injected. Evodiamine was used as the standard. For the quantitative determination, a calibration curve with concentration between 50 and 500 μg/mL was built. The calibration curve’s equation is:

\[ A = 60110 \times C - 478977 \]

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 maximum absorption of evodiamine is at 228 nm.

**Cell culture.** The assessment of antiproliferative effect was performed both on normal human dermal fibroblasts (HDFa - Invitrogen, Willow Creek, USA) and on a human radial growth phase (RGP) melanoma cell line (WM35). Fibroblasts were cultivated in a Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% fetal calf serum, 50 μg/mL gentamicin and 5 ng/mL amphotericin (Biochrom AG, Berlin, Germany). Melanoma cells (obtained from M. Herlyn, the Wistar Institute, Philadelphia, PA, USA) [8] were maintained in RPMI medium supplemented with 5% fetal calf serum, 50 μg/mL gentamicin and 5 ng/mL amphotericin (Biochrom AG, Berlin, Germany). Both cultures were incubated in a humid atmosphere at 37°C and 5% CO₂ [16].

**Cell Proliferation Assay.** The cell cytotoxicity assay was performed using the CellTiter 96® Aqueous Non-Radioactive Cell Proliferation Assay (Promega Corporation Madison, USA) as specified by the manufacturer. The cells were seeded at a density of
10^4 cells/well in ELISA 96-well micro titration flat bottom plates and allowed to accommodate for 24 h in normal growth conditions. The cultures were then exposed to EE extract (prepared as described above) in increasing concentrations, ranging from 0.05 to 50 µg/mL for 6 and 24 hours. Each experiment was carried out in triplicate. Cell cultures treated only with medium where used as controls.

After each individual time point, the E. europaeus treated cells and their counterpart controls were incubated for 2 hours with 20 µL of 3-(4,5-di-methylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphenyl)-2H-tetrazolium MTS/phenazine methosulfate (PMS) mixture in 100 µL culture medium [3]. The absorbance of each sample was read at 490 nm using an ELISA plate reader (Tecan, Männedorf, Switzerland). The viability was evaluated based on the comparison with control cells. The IC₅₀ values representing the extract concentration required to inhibit 50% of cell proliferation were calculated from the calibration curve by linear regression using Microsoft Excel.

**Evaluation of apoptosis.** The Annexin V-fluorescein isothiocyanate (FITC) apoptosis detection kit was used in conjunction with the vital dye propidium iodide (PI) (BD Biosciences, San Jose, CA, USA). The induction of apoptosis was investigated by assessing positive cells (%) for Annexin V-FITC and/or PI. The Annexin V-FITC positive cells were detected by green fluorescence, while the presence of red fluorescence was the indicator for PI. The viable cells (showing no apoptosis) were identified as Annexin V (-)/PI (-); the apoptotic cells were identified as Annexin V (+)/PI (-) (early apoptosis), and Annexin V (+)/PI (+) (late apoptosis). The differentiation among these three-cell type populations was made by flow cytometric detection.

WM35 and fibroblast cells were seeded on Petri glass dishes (Nalgene, Nunc, USA) at a density of 5 x 10^4/cm² for 24 hours in medium, then exposed to Euonymus europaeus extract in concentrations of 0.5 and 1 µg/mL for 6 and 24 hours, followed by FITC Annexin V and PI staining, according to the manufacturer’s instructions.

The flow cytometric analyses were performed at room temperature with a BD FACSCanto II flow cytometer (Becton Dickinson & Company, Franklin Lakes, NJ, USA) equipped with two lasers as excitation sources: blue (488 nm, air cooled, 20 mW solid state) and red (633 nm, 17 mW HeNe). The cytometer setup was performed using the BD Cytometer Setup & Tracking beads (CS&T beads), while the compensation was done using compensation beads (BD Comp-Bead). The data was analysed using the BD FACSDiva Software (Becton Dickinson). A total number of 10,000 events were recorded for each sample. The fluorescence spectrum of Annexin V and PI were detected using a 530/30 nm and a 575/26nm band-pass (BP) filter, respectively.

**Statistical analysis.** All data are reported as the mean ± SEM. The Gaussian distribution was checked using the Shapiro-Wilk normality test. 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 values and figures were obtained using GraphPad Prism version 5.0 for Windows, GraphPad Software, San Diego California, USA.

**Results and Discussion**

**Standardization of the hydro alcoholic extract.** The obtained hydro alcoholic extract was a clear, brown-orange liquid. The quality parameters of the extract were within the admissibility range of German Homeopathic Pharmacopoeia, as follows: relative density 0.953 (0.935 - 0.955), dry residue 5.76% (Min. 4%), ethanol content 45% vol. (40 - 50% vol.), digitoxin content of 0.0028% (Max. 0.01%). Evodiamine content was 0.404 g/mL [20].

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**Figure 1.**

HPLC chromatograms at 150 nm of standard evodiamine (A) and the compounds isolated from the ethanolic extract of Euonymus europaeus (B)
Evodiamine presence in the EE was shown by HPLC chromatogram (Figure 1), while the recorded UV-VIS spectra proved the presence of evodiamine in the extract (Figure 2).

**Figure 2.**
UV-Vis spectra of standard evodiamine (A) and the compound isolated from the ethanolic extract of *Euonymus europaeus* (B)

*In vitro cytotoxicity.* Human melanoma cell line (WM35) was compared to normal human dermal fibroblasts for sensitivity to EE. The differences of absorption percentage among different concentrations were statistically covered for both fibroblasts and VM35 cells \( (p < 0.001) \).

The dose-response curves showed that cancer cells were, by far, more sensitive to EE treatment than normal fibroblasts, the IC\(_{50}\) at 6h was almost ten-fold higher. The IC\(_{50}\) values for VM35 cells were 6.76 ± 1.08 µg/mL after 6 h and 5.89 ± 0.93 µg/mL after 24 h, while IC\(_{50}\) of the fibroblasts were 53.08 ± 5.47 µg/mL and respectively 25.04 ± 3.73 µg/mL (Figure 3).

**Figure 3.**
Comparative cytotoxicity of *Euonymus europaeus* extract on fibroblasts and VM35 cultures after 6 hours (A) and 24 hours (B) of exposure at a different dose versus untreated cells (mean ± SEM) \( (n = 3) \)

The fibroblasts inhibition showed a very different pattern, suffering a gradual dose-dependent and time-dependent inhibition of viability, already visible after 6 h, and enhanced after 24 h, a dose dependent reduction of cell concentration was clearly visible.
Apoptosis/necrosis assessment. To determine whether the growth inhibition is associated with cell death, Annexin V Propidium Iodide double staining of VM35 cells and fibroblasts followed by flow cytometric analysis were performed. As shown in Figure 4 in the upper left quadrant, the fluorescein isothiocyanate conjugate of Annexin V (FITC-A) staining in propidium iodide (PE-A) negative cells - early apoptotic cells - represented less than 0.5% of cells in all treatment conditions. This suggests that apoptosis was insignificant, therefore all cells positive for PE-A, positive or negative for FITC-A, confined in the right side of the panel, were considered as necrotic cells.

In tumour cells cultures, the percentage of necrotic cells increased from 3.6% in control cells up to 18.6% at 1.0 µg/mL in 24 h. Fibroblasts were even more sensitive. The control cells showed 8.3% percentage of necrosis and 24 h later, the necrosis in the fibroblast subjected to a dose of 1.0 µg/mL, increased up to 72.1%.

Apoptosis was measured using flow cytometric analysis. Figure 5 represents scatter plots of Annexin V-fluorescein isothiocyanate (FITC-A) (y - axis) versus vital dye propidium iodide (PE-A) labelling (x - axes). Lower left quadrants (absence of both markers) indicate viable cells; upper left quadrants (FITC-A positive, PE-A negative) indicate the apoptotic cells (early stage apoptosis). In the present study, the necrotic cells are represented by the right side of the panel (PE-A staining alone or together with FITC-A). Fibroblasts and VM35 cells were treated with Euonymus europaeus extract in doses of 0.5 µg/mL and 1.0 µg/mL, after 6 hours and 24 hours while untreated cells were used as controls.

The present study investigates the underlying mechanisms of tumoricidal properties of EE extract, already observed in the preliminary study [15]. The in vitro investigations were performed using a human melanoma cell line VM35 and, in order to check the specificity of inhibition against tumour cells, we conducted parallel studies on a human fibroblasts cell line. As far as we know, no other studies focusing on tumouricidal properties of EE were ever published by other research groups. Despite the fact that no studies about EE are available, other Euonymus species, for example...
Euonymus alatus, already proved to have antitumor properties. Euonymus alatus exhibits antitumour properties by induction of apoptosis via mitochondrial pathway [10] and inhibits tumour invasion by inducing the inhibition of matrix metalloprotease-9 (MMP-9) activity [9]. Comparative studies on MMP-9 inhibition showed that Euonymus alatus was among the most effective extracts providing an inhibitory activity of up to 90% [14]. Another antiproliferative mechanism was discovered for mammary and genital tumours, respectively the inhibition of aromatase activity. Aromatase is an enzyme responsible for oestrogen synthesis. A large proportion of breast cancers express their own aromatase. Euonymus alatus was highly effective in inhibiting the intracellar aromatase in myometrial and leiomyomalous cells, in a dose-dependent manner [11]. This is the first study showing the presence of evodiamine in EE. Evodiamine was initially isolated from a Chinese herb named Evodia rutaecarpa and represents a promising chemotherapeutic agent in the multiple-drug resistant cancer cells [12]. Therefore, in the present study, the phytochemical investigation was focused on revealing the potent and selective anticancer effect of EE plant extract. Our in vitro studies proved that EE has a strong dose-dependent inhibitory effect on human melanoma cell line VM35. The effect was present in relatively small concentrations of plant extract, respectively 2.5 µg/mL. Remarkable, when a similar concentration was added in normal human fibroblasts, it had very low effect on cell viability. In very high doses, the EE extract was able to induce necrosis, but the apoptosis was insignificant. This finding suggests that the apoptotic or necrotic mechanism is not the main mechanism involved in EE tumouricidal activity, but it might have other targets e.g. cell cycle control, down regulation of protein synthesis, enhancement of oxidative stress etc. Commonly, the phytotherapeutic extracts responsible for tumour inhibition contain more than one antitumour compound. Therefore, all mechanisms described above should be considered in further studies in order to establish their applicative value in cancer therapy.

Conclusions

The present study proves that the EE alcoholic extract reduces the proliferation of VM35 melanoma cell line. The induction of apoptosis is unlikely, but other mechanisms, such as cell cycle control, are more probably involved. Therefore, we consider that further studies need to be carried out in order to establish the applicative value of EE extract in cancer therapy. Our future directions include to performing in vivo antiproliferative studies and survival analysis on a classic transplantable tumour model - Ehrlich ascites carcinoma inoculated in mice. This transplantable tumour model will give us also the possibility to investigate the side effect of tumour growth and the interaction between tumour cells and the immune system.

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Declaration of interests

The authors report no declaration of interest.

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