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Biological Activity of Orally Given Ethyl Acetate Extract from *Cotinus coggygria* in Albino Mice with Solid and Ascites Forms of Ehrlich's Tumor

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Biological activity of ethyl acetate extract from the leaves of *Cotinus coggygria* Scop. (smoke tree) was studied in an *in vivo* model of mice both healthy and developing solid or ascites form of Ehrlich's mammary gland carcinoma. Thus, the toxicity of the extract, applied per os, as well as its possible antitumor activity were evaluated. Clinical and pathomorphological studies were carried out. According to the results, no signs of overall or organ-specific toxicity were found. The extract did not prevent the development of Ehrlich's tumor but reduced the solid tumor grade by enhancing the cells differentiation. Additionally, the herb was shown to possess a mild tissue-protective activity expressed by less pronounced pathological changes in the internal organs. Another beneficial effect of the extract application was the prolonged life expectancy of treated mice.

Key words: Cotinus coggygria Scop., plant extracts, Erlich's breast cancer, mouse model, pathomorphology

Introduction

To date the interest in the replacement of conventional chemotherapeutic drugs used as antitumor agents, with natural compounds is growing exponentially, mostly to avoid

unfavorable side effects. Different plants, especially traditionally used over the centuries by mankind, are a good source of active substances with beneficial effects in tumor diseases. *Cotinus coggygria* Scop. (syn. *Rhus cotinus*, European smoketree, Venetian sumach) is a beautiful flowering plant from the family *Anacardiaceae*, widely spread in Europe, including our country, and commonly used in folk and modern medicine. Chemical analyses of the extracts from *C. coggygria* have shown that the plant is rich in polyphenols, glycosylated flavonols, gallic acid and gallotannins [8, 10]. *C. coggygria* extracts were reported to possess antiseptic, anti-inflammatory, hepatoprotective, antioxidant and antitumor activities [4, 8]. Our *in vitro* studies have shown that ethyl acetate extract from *C. coggygria* leaves has a selective effect on the cell viability in different tumor and not tumorigenic human cell lines [5, 6]. On the other hand, the plant is considered poisonous due to the presence of strong allergens in the essential oil.

The mouse model of Ehrlich's mammary gland carcinoma (ascites and solid forms) has proven to be convenient for the *in vivo* studies of therapeutic potential of different preparations[1, 2]. It represents a transplantable malignant tumor exhibiting aggressive behavior and poor differentiation, which can be developed in practically all strains of mice.

The aim of the present study was to evaluatethe possible toxicity of the ethyl acetate extract from *C. coggygria* leaves given *per os,* and to characterize its effecton the organs of laboratory mice developing ascites and solid forms of Ehrlich's breast carcinoma.

Materials and Methods

Ethyl acetate extract of *C. coggygria* leaves was obtained exactly as described in our earlier studies [5, 6].

For the study, 42 male albino mice, 20 g body weight (b.w.), were randomly allocated to seven groups (6 mice each), as follows:

Group 1 – not-treated controls;
Group 2 – treated daily *per os* with DMSO solution;
Group 3 – toxicity controls receiving daily a dissolved extract of *C. coggygria per os*;
Group 4 – mice inoculated with Ehrlich's cells s.c in the hind leg and developing solid tumor;
Group 5 – like group 4 but receiving daily the extract of *C. coggygria per os*;
Group 6 – mice inoculated with Ehrlich's cells i. p. and developing ascites carcinoma;
Group 7 – like group 6 but receiving daily the extract of *C. coggygria per os.*

All the animals were fed and watered *ad libitum* during the whole testing period (20 days). Control group 2was treated only with the solvent, i.e. 0.2 ml 0.1 % DMSO in Phosphate Buffered Saline (PBS, Sigma) for 20 days. Toxicity controls (group 3) received daily 30mg/kg b.w. extract of *C. coggygria* dissolved in 0.2 ml 0.1% solution of DMSO in PBS for 20 days. The same treatment was applied also in groups 5 and 7. For the development of Ehrlich's carcinoma, animals were inoculated with $1x10^6$ cells of the line EAC (Ehrlich's Ascites Carcinoma, maintained by *in vivo* culturing in albino

mice) in PBS s.c.in the hind leg (groups 4 and 5) or i.p. (groups 6 and 7) on the 10th day of the experiment.

The experiments were carried out in the Institute of Experimental Morphology, Pathology and Anthropology with Museum, Bulgarian Academy of Sciences (Permission Nr 11 30 127) in accordance with the national regulation Nr 20/01.11.2012 regarding laboratory animals and animal welfare and European directive 2010/63/EU of the European Parliament.

Observations of the animals were performed daily, regarding the specific signs and symptoms of the tumor disease. Animals' monitoring included also: general condition, appetite, water intake, signs of intoxication, body posture and movements, ataxia, paresis, scratching, etc.

The animals from all groups were sacrificed by cervical dislocation on the 20th day and tissue samples from solid tumors, liver, kidneys, lung, small intestine, brain, spleen and pancreas were extracted, stained with H&E according to the standard methods of histology and examined microscopically (Leica DM 5000B, Wetzlar, Germany) for the presence of morphological signs of toxicity, necrosis or destruction, tumor metastases and inflammatory reactions. Ascites' smears were stained with DiaPath May-Grünwald Giemsa Fast Method and examined under the microscope as above.

Results and Discussion

Ehrlich Ascites Carcinoma (EAC) cell line has been isolated from a spontaneously developed mammary adenocarcinoma in a mouse. It can be maintained by *in vivo* culturing through i.p. injections in virtually every mouse species [reviewed in 9]. Inoculation of experimental animals can be performed both i.p. and s.c. to develop ascites or solid form of the tumor respectively. Ehrlich's carcinoma is a poorly differentiated highly transplantable and rapidly developing tumor, resembling the most sensitive to chemotherapy human breast cancers [9]. It has been widely used to study the possible therapeutic effects of substances of both natural and synthetic origin [e.g. 1. 2].

In our experiments, we chose to apply the ethyl acetate extract of *C. coggygria* leaves orally to evaluate its overall and organ toxicity upon ingestion, since this is the most common way of uptake through teas or tinctures. Actually, we did not expect the extract to be particularly toxic as all the allergens are in the essential oil, which evaporates during the extraction process. Additionally, previous studies showed safe doses of 25-50 mg/kg b.w of *C. coggygria* total flavonoids orally given to mice [11]. In the present study we use a dose of 30 mg/kg b.w.

Clinical observations. All animals monitored during the study were observed for both normal and abnormal behavior and parameters mentioned above. As expected, groups 1 and 2 did not show any abnormal reactions or toxicity signs.The same applied also for group 3-treated with the *C. coggygria* extract. Tumors developed very quickly in other groups (2nd to 3rd day after inoculation), as noted by palpation (solid form) or abdominal circumference (ascites form). The animals from those groups showed characteristic signs of fatigue, lameness with the injected leg, enlarged abdominal circumference (ascites form of the tumor), difficulty in movement, reduced tone and

a significant decrease in activity and general condition. It should be noted that the animals in groups treated with *C. coggygria* extract (5 and 7) showed a preserved food intake and prolonged life expectancy in the range of the experiment, whereas two of the animals of the group 6 and one of the 4th group died before the end of the testing time.

Macroscopically, solid tumors in the groups inoculated s.c. (4 and 5) varied in size independently of whether the mice received extract or not. The same applied also for the volume of ascites in the animals, inoculated i.p. (groups 6 and 7). The internal organs seemed unchanged except for the pancreas which was enlarged in all the animals with developing ascites tumors.

Pathomorphology. No clinical signs of general toxicity or pathohistological evidences for organ toxicity were observed in mice treated only with the extract (group 3) during the 20 days period of the experiment. All the examined organs of those animals did not show any differences from the control groups 1 and 2. Histological studies of the solid tumors (group 4) revealed a pronounced cell anaplasia, cells in different phases of mitosis, rarely pyknotic nuclei and cytoplasm vacuolization, pointing out a low level of apoptosis (**Fig. 1A**). Tumor masses of the animals treated orally with the extract exhibited signs of cell differentiation and maturation in the tumor periphery (**Fig. 1C and D**). Also, cells with karyorrhectic or pyknotic nuclei as well as a substantial

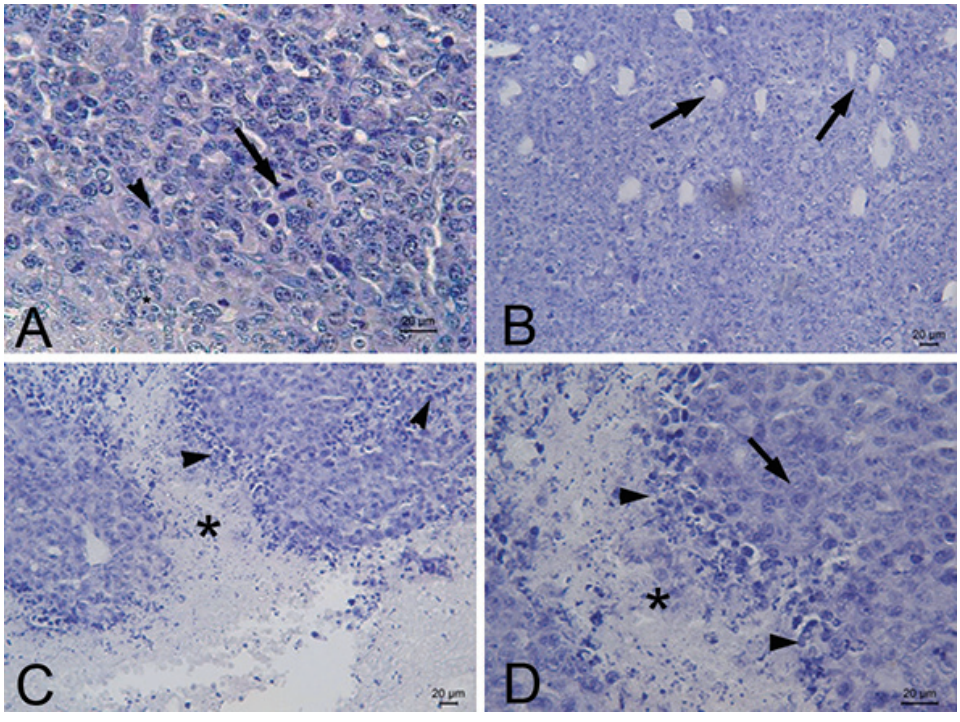

Fig. 1. Solid form of Ehrlich's carcinoma. Mice not treated with *C. coggygria* (A) – anaplasia, cells in different phases of mitosis (arrow), low number of pyknotic nuclei (arrowhead); Mice treated with the extract (B, C, D) – abundant vasculature in the tumor periphery (arrows) (B); C and D – formation of necrotic foci (asterisk) in the central tumor masses, lots of cells with pyknotic or karyorrhectic (apoptotic) nuclei (arrowheads), visible cell differentiation (arrow) in the tumor periphery (D). H&E, scale bar = 20 µm.

vacuolation of the cells were observed (**Fig. 1C and D**), which can be assumed as an indication of apoptotic processes. Necrotic areas in the tumor center were also observed (**Fig. 1C and D**). Neovascularization was visible in the peripheral area with the lumen of the vessels filled with plasma proteins content (**Fig. 1B**). In nonvascular regions formation of hypoxic areas and focal necroses were noticed.

Cell smears of the animals with ascites form of Ehrlich's carcinoma showed mitotic figures and formation of multinucleated giant carcinoma cells. Pyknosis or vacuolation were rare (**Fig. 2A, B**). Visibly, the number of giant cells in the ascites from animals treated with the extract was lower. Leukocytes and mononuclear cells were also presented.

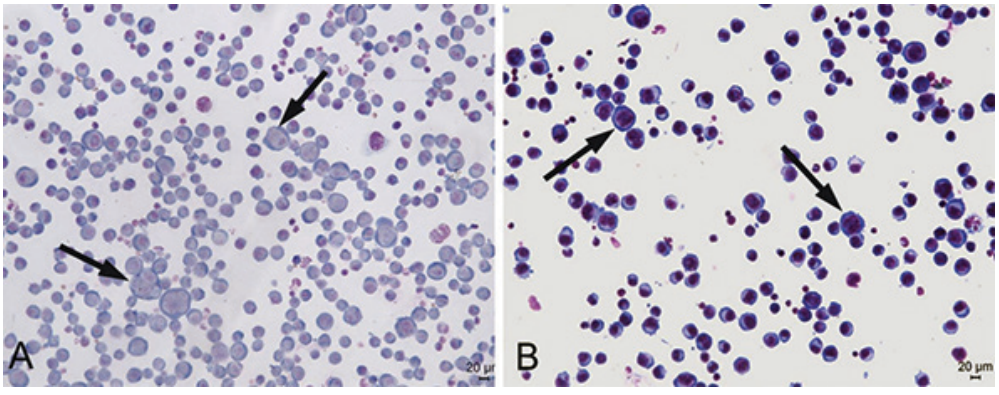

Fig. 2. Ascites form of Ehrlich's carcinoma. Mice not treated with *C. coggygria* (A). Mice treated with the extract (B). Giant carcinoma cells (arrows). May-GrunwaldGiemsa, scale bar = 20 µm.

Ehrlich's mammary gland carcinoma in mice has been shown to metastasize in the liver, kidney and spleen and different methods have been tested to reduce the tumor spread [e.g. 3]. In our experiment, no metastases were found in the animals with solid form of the tumor (groups 4 and 5), whereas tumor cells infiltrations were found in the kidney and pancreas of mice with ascites form of the tumor (group 6). Liver and spleen also showed pathological changes in the animals not-receiving *C. coggygria* extract. In the liver, enlarged sinusoids with immune cell content but invisible (not enlarged) perisinusoidal spaces (Disse spaces) were observed. Groups of cells with pyknotic nuclei were also seen in the liver lobules (**Fig. 3B**). Otherwise, mice treated with the extract (groups 5 and 7) showed a normal architecture of the liver lobules (**Fig. 3A**). In the spleen, the megakaryocytes population was increased with both normal and dwarf cells, some of which having endomytotic figures (**Fig. 3F**). The animals treated with *C. coggygria* had a lower number of megakaryocytes with normal appearance (**Fig. 3E**). Kidneys of some ofthe animals with ascites form of the tumor had metastatic tumor infiltrations (**Fig. 3D)**. In certain animals enlarged periglomerular spaces due to a shrinkage of the glomeruli were seen as well as disrupted tubules organization (**Fig. 3D**). Those findings should be connected with the aggressive tumor growth and organism exhaustion. No metastases were observed in the animals receiving *C. coggygria* and the structure of kidney parenchyma in those animals appeared preserved (**Fig. 3C**).

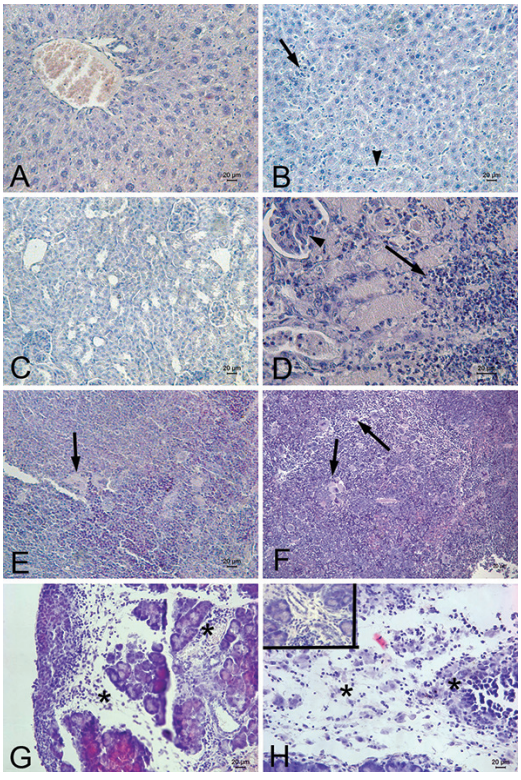

Fig. 3. Pathomorphology of the internal organs of mice, developing ascites Ehrlich's carcinoma. Left (A, C, E, G) – treated with C. coggygria extract; Right (B, D, F, H) – not treated with the extract. A – Normal appearance of a hepatic lobule; B – groups of cells with pyknotic nuclei (arrow), enlarged sinusoid with immune cells content (arrowhead). C – Preserved structure of the kidney cortex; D – tumor cells and inflammatory cells infiltrations (arrow) in mice of group 6, renal corpuscles (arrowhead); E – Increased number of megakaryocytes with normal appearance (arrows); F – a large number of normal and dwarf megakaryocytes (arrows), some of which with mitotic nuclei. G and H– Leukocytes and metastatic tumor cells infiltrations in the fibrovascular capsule and pancreatic septa (asterisks); H inclusion – cell spreading in the perivascular spaces and pancreatic ducts in mice of group 6. H&E, scale bar = 20 μm.

Metastatic Ehrlich's tumor infiltrations were found in pancreatic peripheral exocrine parenchyma more distinct in two mice from the group 6 (**Fig. 3H**). Similar but less pronounced infiltrations were seen also in mice, treated with the extract (**Fig. 3G**).

In general, it can be said that the herb extract mitigates the pathological changes resulting from the tumor development in parenchymal organs. Earlier studies point out the large content of gallic acid and gallotanins in the preparations of *C. coggygria* [8, 10]. Gallic acid (free or liberated from gallotanins by intestinal enzymes) is readily absorbed by the intestinal mucosa and spread with the blood to all organs, forming a major depot in the liver [12]. Biological activity of this compound includes inhibition of tumor cells proliferation and common tissue-protective effects [reviewed in 7]. It can be assumed that the tissue protective activity of the *C. coggygria* extract on the parenchymal organs in our study is due at least in part to gallic acid.

In conclusion, ethyl acetate extract from *C. coggygria* leaves does not show any signs of toxicity by application *per os* in mice at least at a dose of 30 mg/kg b.w. The extract cannot prevent the development and growth of Ehrlich's mammary gland carcinoma but can reduce the solid tumor grade by enhancing the cells differentiation. Additionally, the herb possesses a mild by the less pronounced pathological changes of the internal organs. Also, the lack of lethal cases for the 10 days post-inoculation period in the groups treated with the extract could be attributed to the herb's protective effect on parenchymal organs.

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