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Exploring the Possibility of a Synergistic Effect of the Extracts from Two Medicinal Plants in a Mouse Model of Ehrlich's Breast Carcinoma

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A combination of two extracts: ethyl acetate /water from the leaves of *Cotinus coggygria* Scop. and 80 % aqueous ethanol from the roots of *Geranium sanguineum* L., was tested for a possible synergistic antitumor effect in a mouse model of the solid form of Ehrlich's breast carcinoma. The effects of the combined extracts were compared to those of the chemotherapeutic 5-fluorouracil applied in the same *in vivo* model. Histopathological and blood analyses were performed. The obtained results showed similar activity of the two preparations (combined extracts and 5-fluorouracil). They both provoked apoptosis in tumor masses thus decreasing the degree of tumor aggression. However, they also caused a severe chronic inflammation probably due to the intra-peritoneal way of administration. We concluded that the combined extracts can act as a substitute of the standard chemotherapeutic 5-fluorouracil however, applied in a more suitable way.

Key words: plant extracts, *Cotinus coggygria* Scop., *Geranium sanguineum* L., 5-fluorouracil, Ehrlich's carcinoma

Introduction

With the development of personalized medicine, it becomes increasingly clear that the use of the same drugs in different patients not only might not lead to a cure, but could also cause undesirable side effects in some cases. Therefore, the search for a variety of antitumor drugs is essential to improve the efficiency of treatment. It is well known that plant-based preparations are suitable for use as adjuncts in a number of diseases, including cancer, due to their better biological tolerance, easy digestibility and biodegradability, and above all, because of their lower toxicity compared to synthetic drugs.

Recently, we obtained extracts from a number of medicinal plants endemic to Bulgaria using both mono- and biphasic organic solvent systems. The resulting extracts were tested for antitumor activity on a panel of human tumor cell lines of different origin compared to a non-tumorigenic human cell line [4, 11]. As expected, each of the tested extracts showed a positive selective action in different types of tumor diseases. Some preliminary studies have shown that the antitumor effects of individual preparations are based on different mechanisms such as high antioxidant potential, marked genotoxicity to tumor cells, proapoptotic activity, cell cycle arrest or several of those effects (unpublished results). Thus, the question arises whether a combination of two extracts with different mechanisms of antitumor action could lead to an enhancement of the beneficial effect. On the other hand, it is necessary to establish whether administration other than oral (as with standard chemotherapeutics) would be safe. For the present study, we chose two preparations – ethyl acetate / water (pH 3.0) extract from the leaves of *Cotinus coggygria* Scop. (smoke tree) and 80 % ethanol extract from the roots of *Geranium sanguineum* L. (bloody geranium). Bloody geranium is known as one of the best antioxidants in nature [1, 10] whereas smoke tree has a widely used anti-inflammatory action [8, 9]. Since both inflammation and oxidative stress are prerequisites of cancer, it is motivating to investigate the effect of such combination *in vivo*.

The aim of the present study is to explore the possibility of a synergistic effect of a combination of the above two extracts in a mouse model of Ehrlich's breast carcinoma.

Materials and Methods

Leaves from *C. coggygria* and roots from *G. sanguineum* in the form of dried crashed materials were purchased from Dikrasin Ltd (Bulgaria) which is specialized in cultivation and distribution of medicinal plants in accordance with international legislation and the Law on the Protection of Biological Diversity in Bulgaria.

Extracts. The two extracts were obtained exactly as described before [2, 5].

G. sanguineum extract (GSE). In brief, dried and crushed roots of *G. sanguineum* were subjected to solid-liquid extraction in 80 % aqueous ethanol at a ratio of 1g : 10 mL initially for 3 hours, after which the solid residue was re-extracted for 3 hours and finally, overnight. The combined filtrates were concentrated *in vacuo*, acetonitrile was added and then evaporated. Then, the residue was treated with diisopropyl ether, filtered and dried *in vacuo* [2].

C. coggygria extract (CCA). Briefly, dried and powdered leaves of *C. coggygria* were extracted with the biphasic system ethyl acetate / water, acidified to pH 3.0 using hydrochloric acid, (at a ratio 1g herb : 8 mL solvent system) with stirring for 2 hours at room temperature. After that, the solid residue was re-extracted with the same biphasic system. The collected filtrates were concentrated on a vacuum evaporator, filtered, the precipitates were washed with diisopropyl ether and dried under vacuum [5].

In vivo experiment. Sixteen mature albino mice (20g b.w.) of the ICR breed were used in the experiment. The animals were kept in plastic cages (each group in a single cage) in the licensed vivarium of the Institute of Experimental Morphology, Pathology and Anthropology with Museum - Bulgarian Academy of Sciences (Permition 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. Before the experiment they were fed and watered *ad libitum*. The animals were inoculated with Ehrlich's ascites carcinoma cells (EAC cell line) by a single subcutaneous (*s.c.*) injection of 0.2 mL suspension of 1.10^6 cells/mL into the hind leg to develop a solid form of Ehrlich's mammary gland carcinoma. The test started after a palpation of a tumour masses. Then, the animals were randomly divided into three groups, as follows:

Group 1: 4 animals bearing solid form of Ehrlich's mammary gland carcinoma (positive controls).

Group 2: 8 mice, treated daily for six days by *i.p.* injections with a combination of GSE and CCA. The single dose was composed of 15 mg/kg b.w. from each extract, dissolved together in 0.2 mL PBS (the CCA was pre-dissolved in a minimal amount of DMSO).

Group 3: 4 animals, treated daily for six days with 0.2 ml solution of 15 mg/kg b.w. 5-fluorouracil (5FU – a standard therapeutic) in PBS.

Two days after the end of experiment, the animals were euthanized by decapitation under sedation with 2% xylazine. Blood for testing was collected from the neck. During the autopsy, tissue samples of the tumour mass, liver, kidney and other organs were collected. The tissue pieces were fixed in 10% neutral buffered formalin (Diapath, Italy) and processed according to the standard procedure. The sections were stained with H&E (haematoxylin-eosin). Histopathological observations were made under a microscope Leica DM 5000B (Germany). Blood counts were performed on a Mindray BC 2800 Vet analyzer (Mindray, China) and included the following hematological parameters: leukocytes (WBC), erythrocytes (RBC), hemoglobin (HGB), platelets (PLT) and leucocyte cell counts.

Statistical analyses. The results of the blood tests were expressed as mean values ± SD of the experiments from the four animals. Statistical analyses were performed by the one-way analysis of variance (ANOVA) test using GraphPad Prism 8.0 software; results were denoted as $*p<0.05$, $**p<0.01$ and $***p<0.001$ depending on the statistical significance.

Results and Discussion

Chemical compositions of the two extracts used in the present study were preliminary analyzed by LC-HRMS (Liquid Chromatography – High Resolution Mass Spectrometry) in negative ion mode. Compounds were identified by MS and MS/MS data analysis and in comparison to data from previous studies. It was shown that the ethyl acetate / water extract from the leaves of *C. coggygria* (CCA) contains mainly hydrolysable gallotannins in the form of oligo-O-galloylglucoses with a number of galloyl residues from 5 to 9. Additionally, small amounts of glycosylated flavonols such as quercetin-O-glucoside and myricetin-O-rhamnoside were also found [5]. Those substances all contain a large number of hydroxyl groups, which makes the extract a good antioxidant. On the other hand, the anti-inflammatory properties of aqueous solutions

and different extracts of this herb were well known and widely used in both traditional and modern medicine [8, 9]. The 80 % ethanol extract from the roots of *G. sanguineum* was analyzed similarly to show that the main components are flavan-3-ols (catechin, epicatechin, ellagocatechin and epiellagocatechin), as well as condensed tannins based on them – proanthocyanidins (unpublished results). These compounds also possess a large quantity of hydroxyl groups, which explains the powerful antioxidant activity of the herb reported previously by other authors [1. 10].

Furthermore, the two extracts were tested separately by us in the same *in vivo* model of Ehrlich's breast carcinoma in mice for their antitumor activity [2, 12]. However, in the previous study [12], CCA was administered *per os*. Obviously, the oral application does not preserve the main compounds of the extracts and the noticed mild antitumor and pronounced organ-protective activities may not be due to the extracts' compounds but to their metabolites. In confirmation of this assumption, a previous study [6] showed a high diversity of hydrolytic processes taking place in gut both by the enzymes of intestinal enterocyte and the microbiota, which brake down gallotanins and other plants' secondary metabolites. On the other hand, the *in vitro* experiments on tumor cell lines, which usually precede the *in vivo* tests, involve the herbs' compounds as preserved molecules. That is why we decided to administer the extracts *i.p.* and test the effect of the whole conserved active compounds. The *i.p.* application of GSE [2] did not show any changes in mice behavior, appetite, water consumption, etc. and so, it was considered to be harmless. Also, we performed a pilot study to test the *i.p.* administration of CCA extract in a couple of healthy mice for possible adverse effects. Unfortunately, this led to a mild dysfunction of the hind legs and we decided to lower the dose. In the present study, no visible harmful effects of the combined preparation were noticed during the six days application.

In this study, we used two types of controls – a positive control of mice bearing solid carcinomas, not-treated with any preparation and negative controls of mice with solid tumor, treated with the standard therapeutic 5-fluorouracil (5FU), which can be used in breast carcinoma including in mouse models [3].

The histopathological results from the present experiment are given in **Fig. 1.**

It should be noted that we took pieces of other organs as well (spleen, pancreas, lung and small intestine), but no differences between the groups were noticed. So, we show here only the livers, kidneys and tumor masses.

In the solid tumor mass of non-treated (positive) controls (**Fig. 1A**), liquefactive necrotic masses with cellular debris were observed, as well as a small number of apoptotic cells, surrounded by cellular debris. Additionally, neoplastic multinucleation of many giant tumor cells, mitotic figures and cells with prominent nucleoli could also be seen. All of these findings are in agreement with the usual progression of solid tumors in the Ehrlich's model of breast carcinoma observed by us and in previous experiments. Solid tumors of mice, treated with the extracts preparation (**Fig. 1B**) also showed necrotic masses and cellular debris. In addition, a number of neutrophil granulocytes were seen, as well as abundance of tumor cells with pyknotic nuclei, pointing out to a development of apoptotic processes. Similar results were observed in mice, treated with 5FU (negative controls), in which the number of apoptotic cells was even larger (**Fig. 1C**). It should be pointed out that the solid tumors of several animals infiltrated the nearby subcutaneous adipose tissue and focal islets of adipocytes in the tumor periphery were seen. Such contact between breast cancer cells and fatty tissue is

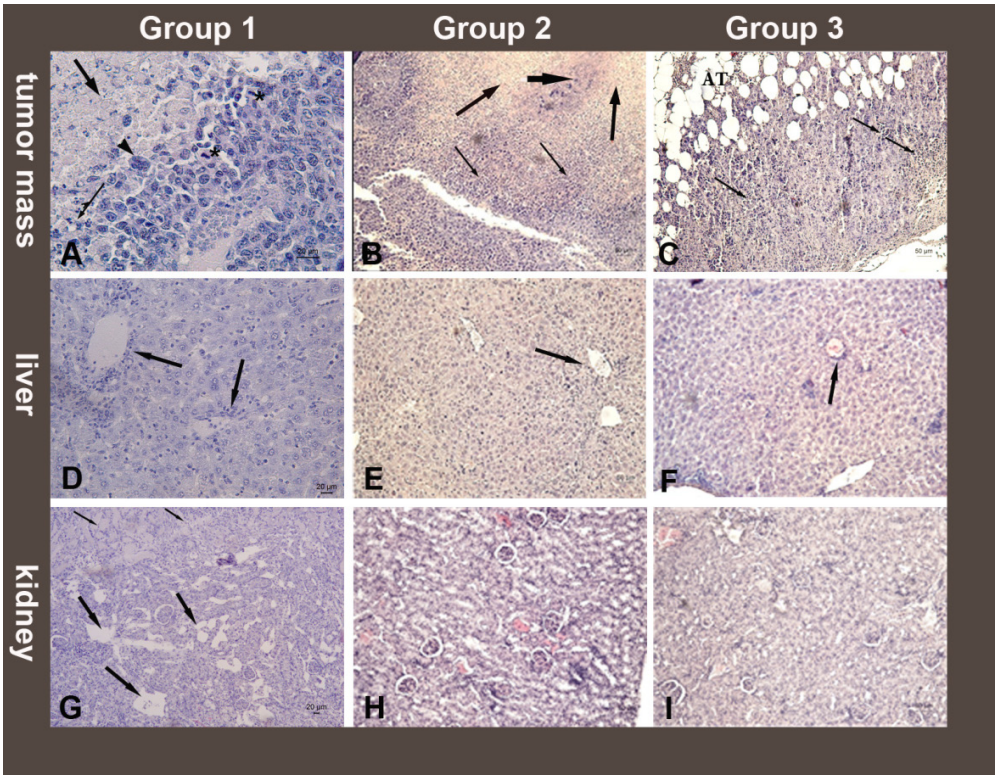

Fig. 1. Histopathological findings (H&E). Left column: Group 1 (positive control, non-treated); middle column: Group 2 (treated with CCA and GSE); right column: Group 3 (treated with 5FU). Upper row: *tumor tissues*: liquifactive necrotic masses (thick arrows), neoplastic multinucliation (arrowhead), apoptotic cells and cell debris (thin arrows), mitotic figures (asterisk), neutrophil granulocytes (short arrow), AT – adipose tissue. Middle row: *liver*: active immune cells (thick arrows). Bottom row: *kidney*: hyaline casts in tubules (thin arrows), dilatation and wall rupture of renal tubules (thick arrows). 200×, Bar = 20 μm.

of a great risk of prompting the tumor growth, due to the stimulation of the proliferation and invasion by secreted proteases, pro-inflammatory cytokines and by modulating cancer cell metabolism [7]. It can be concluded that both 5FU and combined extracts intensify visually the apoptosis of tumor cells.

The liver tissues of all the groups of animals did not show substantial pathological changes except for the elevated number of activated immune cells (mainly leukocytes – some granulocytes and macrophages) observed around the central vein and also, within the blood vessels (**Fig. 1D, E, F**). This effect was more pronounced in non-treated controls (**Fig. 1D**). The kidneys of the positive (non-treated) controls however, showed hyaline casts in tubules and the walls of some renal tubules were ruptured (**Fig. 1G**). The kidney morphology of all the treated animals from groups 2 and 3 was normal (**Fig. 1H, I**). Thus, a mild protective effect of the two types of drugs was assumed.

Results from the blood analyses are given in **Table 1**.

Table 1. Results from the blood analysis. Results are given as mean ± SD from 4 independent counts of four animals. Statistical significance was: *p<0.05, **p<0.01 and ***p<0.001 in comparison to the referent values.

Treatment	Parameters						
	WBC (10^9/L)	RBC (10^{12}/L)	HGB (g/L)	PLT (10^9/L)	Leucocyte cell count (%)		
					Lym.	Mon.	Gran.
Referent values	0.8 - 6.8	6.36 – 9.42	110 – 143	450 – 1590	55.8 – 90.6	1.8 – 6.0	8.6 – 38.9
Tumor control (Group 1)	19.4 ± 0.14***	8.64 ± 0.7	117 ± 1.03	1060 ± 6.7	50.1 ± 0.7	5.8 ± 0.7	44.1 ± 0.2**
CCA+GSA (Group 2)	41.5 ± 0.9***	8.9 ± 0.3	108 ± 1.4*	1317 ± 1.8	12.6 ± 0.2***	3.7 ± 0.3	83.7 ± 0.5***
5FU (Group 3)	27.8 ± 2.29***	7.97 ± 1.63	110 ± 9	1548±28.7	18.9 ± 6.2***	3.0 ± 1.5	78.1 ± 7.5***

From the table, a pronounced leukocytosis is seen in all the experimental animals, which is high in 5FU-treated animals, but especially prominent in the mice treated with the combined extracts. From leukocyte cell count it becomes clear that the leukocytosis is due to the very big number of granulocytes, again especially high in the extracts-treated animals. This result coincides with the morphological findings of a substantial number of neutrophils in the tumor tissue (Fig.1B). On the other hand, the number of lymphocytes in the two treated groups is lower than the one in tumor controls and the referent values. Both results of high granulocytes and low lymphocytes can be a sign of a chronic inflammation. Obviously, both 5FU and plant extracts increase the inflammation to a higher extent than the tumor itself. This result most probably is due to the *i.p.* application of those substances. Of course, this is not the common way to apply a chemotherapeutic of any kind. However, in a mouse model it would be very difficult to perform an intravenous application. On the other hand, RBC remains within the reference ranges. The HGB of extract treated animals is slightly lower than the reference and tumor values but not enough to consider anemia. Thus, the results from the blood analysis can be considered as ambiguous.

Conclusions

From the above studies it becomes obvious that *i.p.* application is not proper neither for combined extracts nor for the standard chemotherapeutic 5FU. Since from our previous studies such application mode proved to be safe for GSE, we rationalized

that the hydrolysable gallotannins in CCA may cause a local irritation, and the dose of 5FU most probably is higher than the safety one. We can also conclude that CCA and GSE have not a detectable synergistic effect. On the other hand, the combined preparation has the same effect in the solid Ehrlich's breast carcinoma as 5FU which is proved from both the blood and morphological analyses. Thus, it would be reasonable to assume that the extracts can be usable instead of 5FU however, by a more suitable way of application.

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