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CYTOSTATIC AND CYTOTOXIC EFFECTS OF *TRIGONELLA FOENUM GRAECUM* (FENUGREEK) SEED EXTRACT

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ABSTRACT. The antineoplastic effect of *Trigonella foenum graecum* seed extract has been evaluated in the Ehrlich ascites carcinoma (EAC) model on mice. Intra-peritoneal administration of the plant extract both before and after inoculation of EAC cell in mice produced alterations of ascites cells and inhibition of tumor growth compared to the control. The cytostatic effects on Ehrlich ascites are nuclear and cytoplasmic oedema, nucleoli hypertrophy, expansion of mitochondria and rough endoplasmic reticulum, ribosome detachment, diminishment of smooth endoplasmic reticulum and finally the inhibition of protein and enzymes synthesis. In this study, after the analysis of ultrastructural modifications we observed that the blockage is a reversible process, compatible with the survival of cells; although these cells observed even after 3-4 weeks of treatment that they have lost the malignity property and the ability of entering into mitosis. The alterations observed in the cytoplasm are similar to those we have found in literature: expansion of mitochondria, depletion of ribosomes, increase of lysosomes and lipid drops and picnosis. This effect of blocking the malignity without killing the cells is characterised by ultrastructural mechanisms inducing a rapid senescence. The nuclear inter-membrane space enlarged, the chromatin distribution changes and the cell organelles undergo modifications.

Keywords: *Trigonella foenum graecum*, fenugreek, Ehrlich ascites cells, cytostatic, cytotoxic

INTRODUCTION

Trigonella foenum graecum known as fenugreek is an annual herb that belongs to the family Fabaceae. It has a long history as both a culinary and medicinal herb. The seeds of *Trigonella* are commonly used as a spice in food preparations due to the strong flavour and aroma. The seeds are reported to have restorative and nutritive properties (Pribac et al., 2008). *Trigonella* seeds are used in remedies for diabetes and hypercholesterolemia in Indian, Arabic and Chinese medicine. Its utility has been proved experimentally in diabetic humans (Pribac et al. 2007).

Rosin et al. (1994) reported that tumor promoters recruit inflammatory cells to the application site and cancer development may also act by aggravating inflammation in the tissue and vice versa. Rosin et al. (1994) also reported that inflammatory cells are capable of inducing genotoxic effects. So it is likely that anti-inflammatory agents may possess antitumor activity and vice versa. This background and our previous research lead us to look for the antitumour effect of fenugreek seeds, and in particular, the antitumor effect of *Trigonella foenum graecum* seed extract against Ehrlich ascites carcinoma (EAC) in mice (Sur P. et al., 2001).

Ehrlich tumor carcinoma is a transplantable, poorly differentiated malignant tumor which appeared originally as a spontaneous breast carcinoma in a mouse. It grows in both solid and ascitic forms.

MATERIALS AND METHODS

Animals. The experiments were carried out in A2G male mice, weighing 20-25 g. They were given standard laboratory diet and water *ad libitum* and were maintained under 12 h light/dark cycle at 25°±2°C. Animals (n = 10 each group) were inoculated (i.p.) with 4 x 10^6 EAC cells/mouse on day 0 and treatment was started with fenugreek extract (alcohol) 24 h after inoculation at a dose of 100 and 200 mg/kg i.p. for 2 days. The control group was treated with the same volume of 0.9% saline. All treatments were continued for 2 days. The animals were killed on day 3 after transplantation and tumor cells were collected by repeated intraperitoneal wash with 0.9% saline.

Tumor cells. Ehrlich ascites carcinoma (EAC) cells were used. EAC cells were maintained by weekly intraperitoneal (i.p.) inoculation of 4 x 10^6 cells / mouse. Ascites cells were collected after 1, 3, 24 and 48 hours and examined using the electron microscope. Intraperitoneal cells and macrophages were counted using a haemocytometer (Hudson L. and Hay F.C. 1989; Thakur A.M. et al., 1994).

The ascitic fluid was collected from 12 days control ascites. The collected material was placed in a test tube and we added approximately 10% ascitic fluid, 3% glutaraldehyde on phosphate buffer 0.15 M, 7.2 pH mixed and maintained for 15-20 minutes on room

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temperature. The mix was then centrifuged at 1,000 rotations / minute for 5 minutes. After the removal of the supernatant, the cells were fixed in 2.5% glutaraldehyde on phosphate buffer 0.15 M, pH 7.2, for 1 hour at 4°C. The solution was then dehydrated using acetone and fixed using cu Vestopal W. Fixed sections were then obtained with a type LKB-III ultramicrotome and contrasted with uranyl acetate and lead citrate and then examined using an electron microscope.

Extraction of fenugreek seed. The fenugreek seeds were purchased from Natura Plant (Bucharest, Romania) and the seeds were germinated. Finely powdered seeds of fenugreek (1 kg) were defatted with light petrol and then exhaustively extracted by percolation with 95% ethanol (5 x 5 L) for 10 days. The alcohol extract was pooled and the solvent was evaporated under reduced pressure at 40°-50°C to furnish a dark brown residue (35 g), stored at 4°C until used (Sur P. et al., 2001).

Statistics. At least six experiments were carried out to determine the effect of a single dose of the test compound. The Student's t-test and a probability level of $p < 0.05$ were chosen as the criteria of statistical significance. Values reported are mean. As for the statistical software we used SPSS version 17.0.

RESULTS AND DISCUSSIONS

Mice treated with the alcohol extract of fenugreek (200 mg/kg i.p.) did not show a toxic effect regarding body weight or general appearance. No pathological changes in vital organs could be observed. When the mice were treated with the fenugreek extract (100 mg/kg and 200 mg/kg i.p. daily for 5 days) prior to tumor inoculation, a 85% and 95% inhibition respectively, of tumor cell growth with respect to the control, were observed.

On day 3 after transplantation, the intraperitoneal ascites cells count (control) was 1.58×10^7 cells / mouse. Fenugreek treatment for 3 days resulted in about 70% inhibition of tumor cell growth in the fenugreek treated group. The average number of peritoneal exudate cells per normal mouse was found to be 0.87×10^7. Fenugreek treatment increased the number of peritoneal cells significantly ($p < 0.01$). Similarly fenugreek treatment was also able to enhance the number of macrophages.

Ehrlich ascites cells from control group (fig. 1) presented a spherical shape, with a diameter of 8-12 μm. There were also bigger cells, with an oval shape, with a diameter of 10-14 μm and multinucleated, or smaller cells with a diameter of 6 μm, in the case of old cells.

Ascites cells presented short microvilli-like prolongations, in reduced number, or bud-like cytoplasmic evaginations. The nucleus is elongated with a irregular contour and a peripheral position. Some nuclei were fragmented or multi-fragmented. The nucleus contains a large nucleolus adherent to the nuclear membrane, meaning an intense rRNA synthesis and an active ribosomal transfer into the cytoplasm. The nuclei are rich in chromatin, especially euchromatin. The heterochromatin is dispersed as agglomerations of various sizes.

The cytoplasm of Ehrlich ascites cells is electro-dense due to the abundance of ribosomes. The endoplasmic reticulum is represented especially by rough endoplasmic reticulum. This consists of short and narrow profiles, intensively electro-dense and in a network-fashion.

The smooth endoplasmic reticulum is weakly developed and can be found as vesicles in few ascites cells. The mitochondria are various in shape and electro-density. In some cells these are elongated, with an electro-dense mitochondrial matrix and prominent cristae. There are also mitochondria with cristae arranged in a longitudinal fashion from one side to the other of the mitochondrium (fig. 2).

The Golgi apparatus is rarely well structured, usually having 5-7 flattened sacks, slightly vacuolated. This organelle is positioned usually in the concave area of the nucleus where it can be observed together with other cell formations arranged radially around the centrioli.

At the periphery of the cells there are lipid drops, slightly electro-dense (fig. 1). In senescent cells these are larger and more numerous and sometimes it may fuse. During this physiologic involution, the protein synthesis decreases and lipid synthesis increases. In these cells, cell organelles are present in a low number, proving a decrease of metabolic activity.

After 1 hour of treatment (fig. 3) we observed some alterations compared with the control group; inter-membrane space of the nucleus is wider, the heterochromatin decreases and the nucleus' capacity of fragmentation is diminished. Cell divisions are rare, even stagnate or just one division is still possible. Electro-density of the cytoplasm is diminished due to the decrease of ribosome's and mitochondria number. Consequently, the endoplasmic reticulum becomes less obvious or in some cells may totally disappear.

The activity of Golgi apparatus is intensified and the formation of numerous lisosomes takes place. These are arranged radially around the centrioli. The general aspect of cells shows a diminishment of the metabolic function.

After 3 hours of treatment (fig. 4) the aspect is similar. Some cells have dilated mitochondria with deformed cristae. After 24 hours of treatment (Fig. 5 and 6) the nuclei of Ehrlich ascites cells appear dilated with reduced peripheral heterochromatin. The nucleoli are small, rarely in contact with nuclear membrane. From these observations we can assume that the transfer processes between the nucleus and the protoplasm are low (Fig. 5).

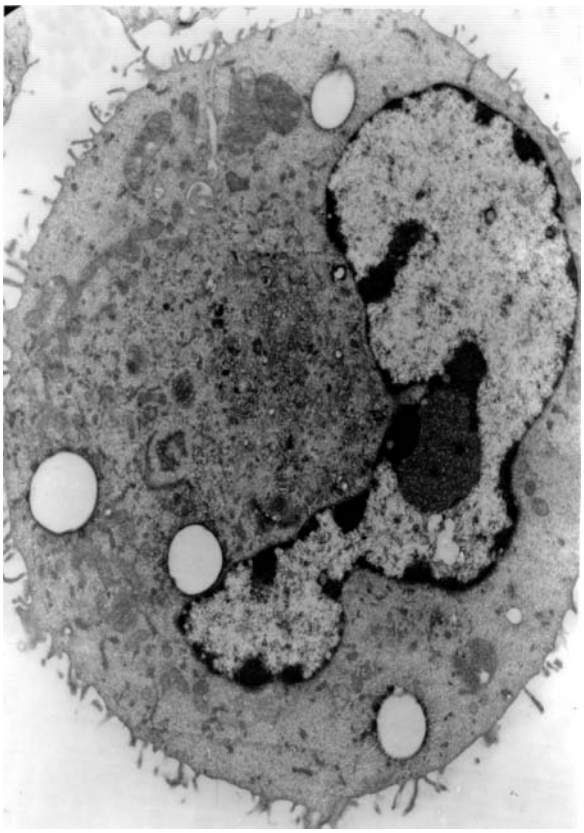

Fig. 1 Ehrlich ascites cells in control group, x12,000

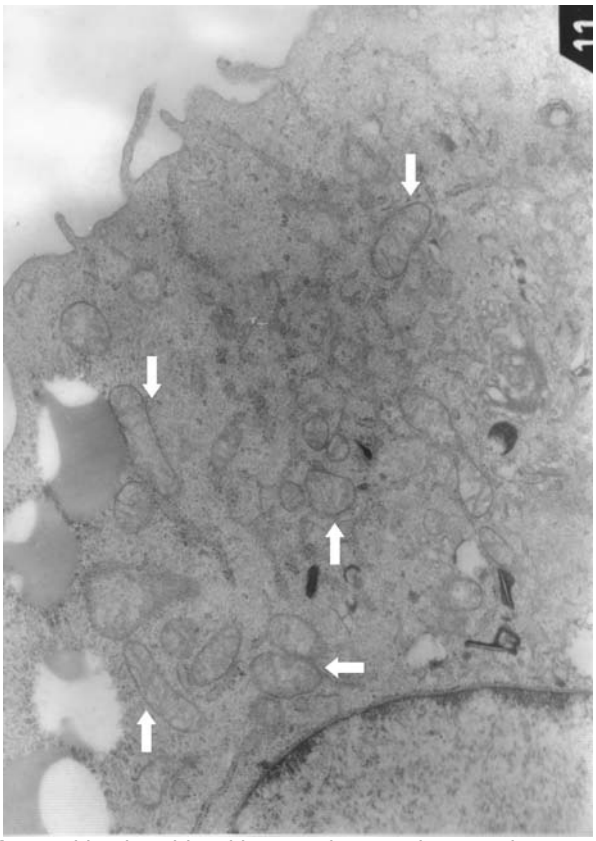

Fig. 2 Mithochondria with normal aspect in control group (white arrows), x24,000

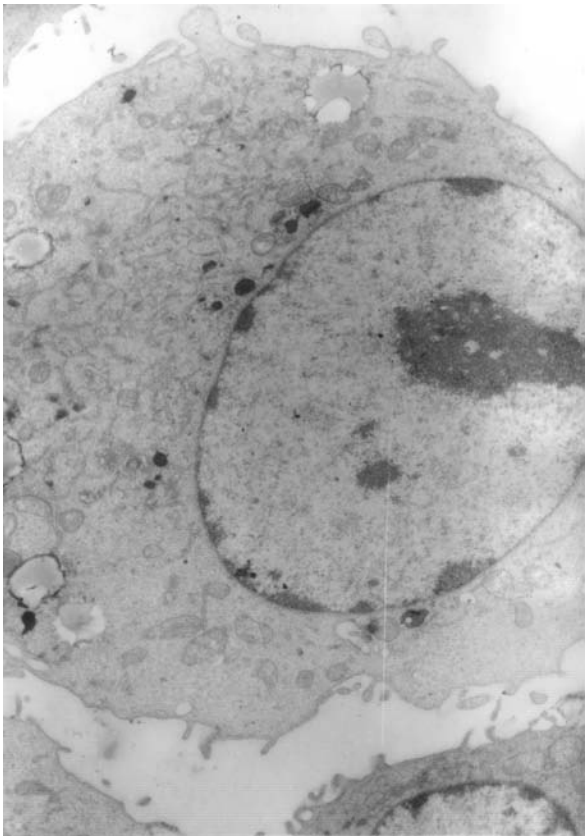

Fig. 3 Ehrlich cell with rarefied heterocromatine and low electrodensity of cytoplasm at 1 hour after treatment, x12,000

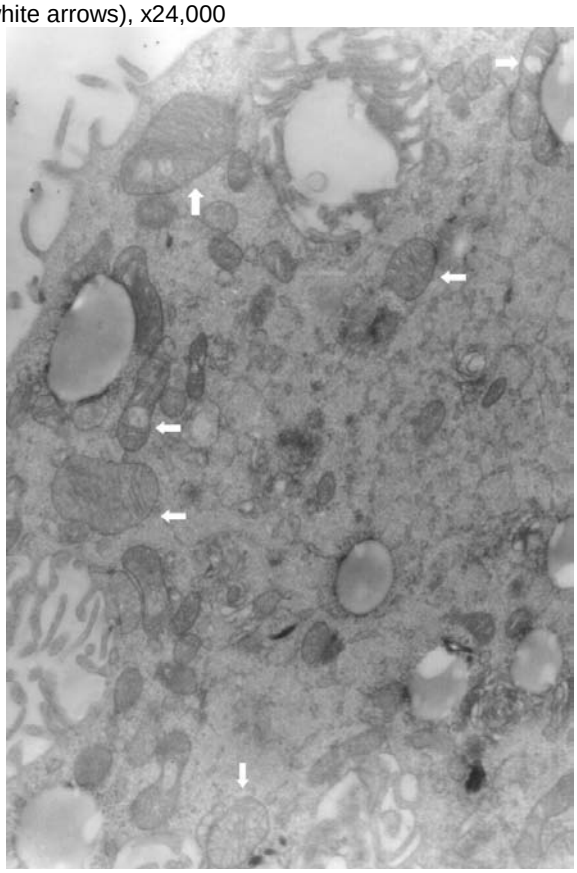

Fig. 4 Dilated mithochondria, with deformed cristae, after 3 hours of treatment (white arrows), x12,000

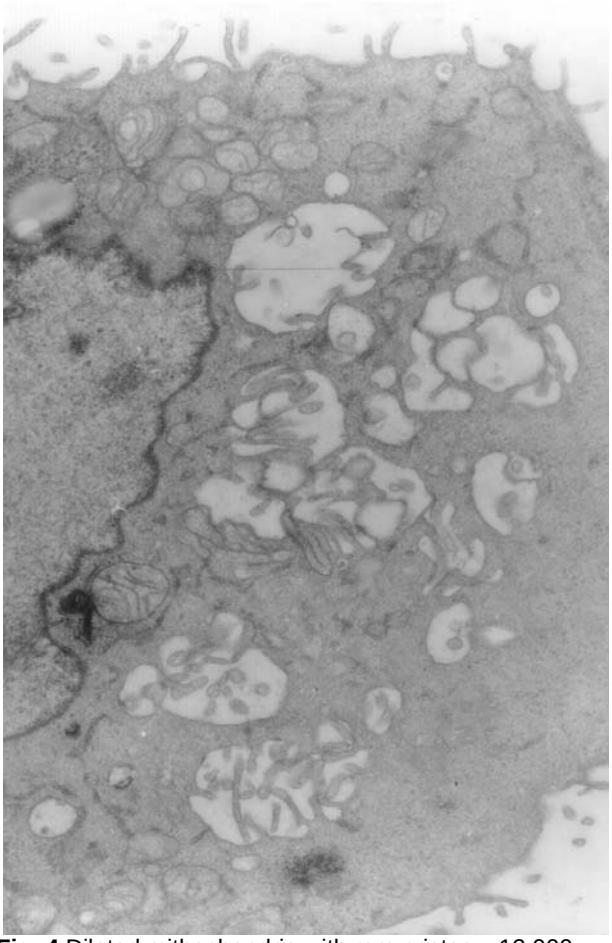

Fig. 4 Dilated mithochondria with rare cristae, x12,000

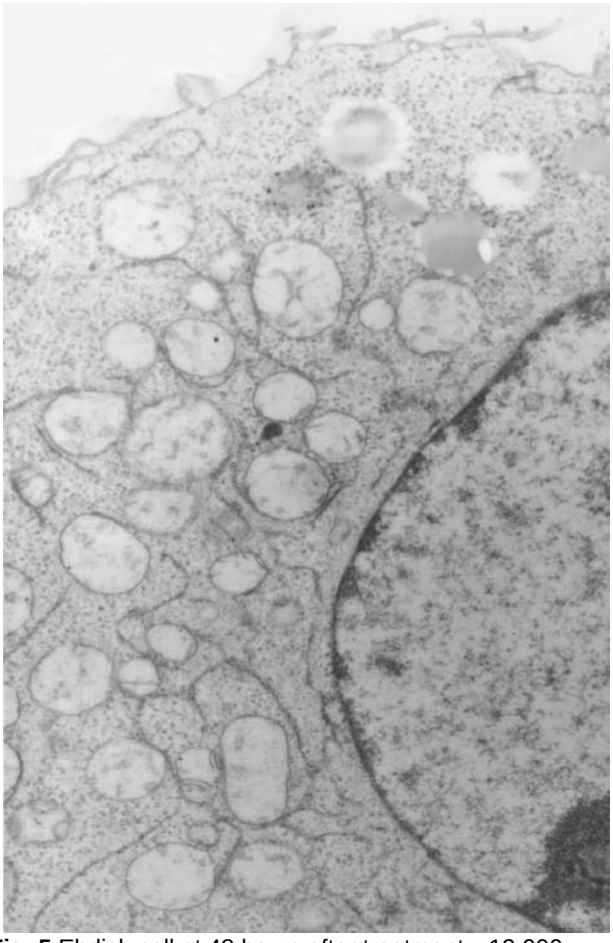

Fig. 5 Ehrlich cell at 48 hours after treatment, x12,000

The quantity of rough endoplasmic reticulum and ribosomes decreases dramatically. Frequently we observed that the membranes of the endoplasmic reticulum suffer a ribosomal depletion. The mitochondria are fewer, dilated, with a weak electro-dense matrix with rare and slightly obvious cristae (Fig. 5). These modifications are evolving in such a manner, that we could observe mitochondria with dilated cristae, transformed into vesicles giving the mitochondrium an aspect of multi-vesicular bodies.

The Golgi apparatus is present as multi-vesicular agglomerations. In numerous cells, the Golgi apparatus presents all the elements dilated, suggesting a diminished activity. In a large number of cells there is a rich formation of lisosomes. After 48 hours, the aspect of ascites cells is very similar with those after 24 hours (fig. 6).

CONCLUSIONS

Morphologically, the blockage appears after the first hours of treatment, especially through nuclear alterations. These suggest isolation between the nucleus and the cytoplasm. Often, these modifications are on a long-term. The other modifications are specific for the phenomenon of senescence.

The cytostatic effects on Ehrlich ascites (Razin et al. 2001, Tarillion et al. 1997) are nuclear and cytoplasmic oedema, nucleoli hypertrophy, expansion of mitochondria and rough endoplasmic reticulum, ribosome detachment, diminishment of smooth endoplasmic reticulum and finally the inhibition of protein and enzymes synthesis. In this study, after the analysis of ultrastructural modifications we observed that the blockage is a reversible process, compatible with the survival of cells; although these cells observed even after 3-4 weeks of treatment that they have lost the malignity property and the ability of entering into mitosis.

The alterations observed in the cytoplasm are similar to those we have found in literature: expansion of mitochondria, depletion of ribosomes, increase of lysosomes and lipid drops and picnosis.

Treatment with the extract was found to enhance both the peritoneal exudate cell and macrophage cell counts. When these extract treated normal animals underwent i.p. inoculation with EAC cells, tumor cell growth was found to be significantly inhibited. These results demonstrated the indirect effect of fenugreek seeds on EAC cells, probably mediated through enhancement and activation of macrophages.

The findings also indicate the preventive effect of fenugreek extract against EAC. The data in the present study showed the possibility of developing fenugreek

extract as a potential agent in the area of cancer chemotherapy.

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