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EXPERIMENTAL STUDIES OF FENUGREEK SEED TREATMENT ON RATS INTOXICATED WITH ETHANOL

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ABSTRACT. Our paper was done using an *in vivo* experimental model, in which animals were administered two differents doses of grounded seeds, on the background of ethanol intoxication. Because of their content in polyphenolic flavonoids, *Trigonella foenum graecum* seeds have antioxidant and membrane-protective effects, as reported by several Indian research teams. In this geographical area, fenugreek is used not only for its curative effects, but also as a flavoring food supplement. Most studies focused on the hypoglycemiant and antidiabetic properties of fenugreek seeds; there are also a few evidences regarding their utilization in preventing alcoholic liver pathology. Animals were adult male Wistar rats weighing 180-200 g, divided into four groups: a control (C) group, which received a standard diet and water *ad libitum*; an ethanol-treated group (EtOH) which received the same standard diet and 10% (v/v) ethanol in the water; two groups which had the same ethanol solution instead of drinking water, and their diet contained 5% and 10%, respectively, fenugreek flour (EtOHTr5 and EtOHTr10). The alcohol and fenugreek flour were administered daily, for 30 days. At the end of the treatment period, animals were killed and liver samples were collected, for histological and ultrastructural investigations. The liver of EtOH animals showed macro- and microvesicular fat infiltrations, as well as inflammation in the periportal regions, which are the first areas subjected to neutrophil invasion. Ethanol induced major modifications in hepatocyte nuclei, which had an irregular outline and large heterochromatin areas. Hepatocytes fatty infiltration was accompanied by dilatation of sinusoids, altered function of Ito cells and proliferation of smooth endoplasmic reticulum (SER). Mitochondria became condensed, electrondense, with dilated cristae. In EtOHTr5 and EtOHTr10 groups, which received fenugreek flour and ethanol, the structural and ultrastructural modifications caused by alcohol intoxication were much attenuated, better results being obtained with 5% fenugreek. SER proliferation was substantially reduced and the appearance of mitochondria was similar to the one in control animals. The lipid droplets followed the normal transit from parenchimal cells to Ito cells, which preserved their function as lipocytes. Periportal inflammation was also diminished. The majorities of parenchymal cells nuclei preserved their spherical shape and were predominantly euchromatic, with little, evenly dispersed heterochromatin. Our results plead for the utilization of *Trigonella* seeds as a dietary supplement, to prevent cellular alteration and the onset of steatosis and fibrosis, in subjects with liver conditions produced by excessive drinking.

Key words: hepatoprotective effects - *Trigonella* seeds - hepatocyte ultrastructure

INTRODUCTION

Trigonella foenum graecum, (sicklefruit fenugreek) is an annual herb belonging to the family *Leguminosae*; its seeds are used from ancient times in Oriental gastronomy, as a flavor and spice. Because of their rich content in polyphenolic flavonoids, *Trigonella foenum graecum* seeds have antioxidant and membrane-protective effects, as reported by several Indian research teams. In this geographical area, fenugreek is intensely used. Most investigations focused on the hypoglicemiant and antidiabetic properties of fenugreek seeds; there are only two studies (one on cell lines and one *in vivo)* regarding their utilisation in preventing alcoholic liver pathology.

Lately, plant extracts are intensely used as an alternative to drug therapy. The possibility to obtain and use plants and plant extracts at a low cost, and with a low toxic potential, is one reason that contributed to the development of phytotherapy.

Hepatobiliary and renal disorders are presently increasing, being favored by environmental pollution, alcohol and synthetic drugs abuse and by viral infections (Rusu *et al*, 2005). Plenty of plant extracts can be already found in drugstores, for treating liver and kidney diseases, to neutralize the negative effects of certain xenobiotics (Tămaş *et al*., 1995). Thus, the hydroalcoholic extract obtained from tetterwort (*Chelidonium majus*), is lately used in chronic gallbladder disease and as a hepatoprotective. It is to be mentioned that, for most plant extracts, their mechanism of action at cellular and subcellular level have not yet been studied (Adam, 1984; Adam *et al*., 1979; Ciulei *et al*., 1993; Hrişcu *et al*., 1979; Vahlensieck *et al*., 1995). Certain reference works in this area have been published by Craciun *et al.* (1985, 1989 a

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and b, 1994, 1995 a and b, 2001, 2003), Ciobanu *et al.,* (2003), and Rosioru *et al.* (1999).

Using plants to treat diseases have become long ago a tradition, „the nature's pharmacy" being an important source for therapy (phytotherapy). Presently, the properties of medicinal plants are reevaluated due to the progress made in chemical, pharmacological and clinical research of plants and vegetal pharmaceuticals. Phytotherapy has major advantages: lack of, or very reduced toxicity, lack of undesired side effects, low producing costs as compared to synthetic drugs.

Investigation of medicinal plants and natural plant extracts, as potential factors involved in drug development programs, or as potential adjuvants for animal species of economic interest and for humans, needs extensive and complex studies: the introduction of new, efficient biotechnologies, focused on studying cellular processes, assessing of enzyme activities, membrane transport, receptor-related properties etc.

It is known that 2% to 10% of the ingested alcohol is eliminated unchanged through kidneils and lungs, while the rest of it is oxidized in the liver. Ethanol molecules are small and hydrophilic, therefore they are readily absorbed in the digestive tract and in the alveolae; a small amount is already absorbed in the oral cavity (Tutunaru *et al.,* 2007). Ethanol also passes easily through the skin and placenta. Chronic ethanol intoxication of laboratory animals causes neurological disregulations and disfunctions of digestive tract, liver, kidney, heart, pancreas and alterations in fetuses (Matsumoto and Matsumoto, 2008). Ethanol intoxication is considered, in 80% of cases, the main cause of chronic pancreatitis (Gullo, 2005).

Many investigations demonstrated that both acute and chronic ethanol intoxications induce excess lipid accumulation in hepatocytes and development of fatty liver (Pinzani, 1995; Naveau, 1997; Ikejima, 1998; Craciun *et al.,* 1985, 1995 a and b.; Roşioru *et al.*, 1999).

It is considered that ethanol intoxication produces an increased mobilization of fatty acids from adipose tissue stores and their excessive accumulation in the liver (Zhang, 1997). It has also been demonstrated that a high $NADH_2$ concentration in hepatocytes also contributes to fat accumulation in the liver, by favoring triglyceride synthesis. Moreover, alcohol disrupts lipids export from hepatocytes, causing their storage in these cells. Other authors (Tutunaru *et al.,* 2007) reported a decreased catalase activity in the alcoholic liver, which overomes cell's capability to oppose oxidative stress.

Tousands of studies made on animal and human liver in normal, pathological and experimental conditions have been published in numerous papers and books (Bacon *et al.,* 2006; Kaplowitz and DeLeve, 2007; Dufour and Clavan, 2010; Friedman and Keeffe, 2004; Kuntz and Kuntz, 2006; Arias, 2009; Boyer *et al.,* 2006). In this context, this is the first ultrastructural study on the effects of fenugreek seed flour, at cellular and subcellular level, on hepatocytes from ethanol intoxicated rats.

MATERIALS AND METHODS

Our experiments have been carried out on male, adult Wistar rats, weighing 180-200 g. Animals concomitantly received ethanol in the drinking water and fenugreek flour in the diet. Rats were divided in 4 groups of 10 individuals each, as follows:

- The control group (C), which received the standard diet and tap water *ad libitum*;
- The ethanol treated group (EtOH), which received ethanol 10% (v/v), in the drinking water, and had the same standard diet;
- The group that received ethanol as the previous one and had a diet with 5% flour from fenugreek seeds (EtOHTr5);
- The group that was treated with ethanol (as above) and had 5% flour from fenugreek seeds in the diet (EtOHTr10).

The experiment lasted for 30 days; in the end the animals were sacrificed, liver samples were collected and properly processed for structural and ultrastructural investigations.

For ultrastructural investigations, pieces of hepatic tissue harvested immediately after killing the animal were prefixed at room temperature, for 2 hours, in a 2.7% glutaraldehyde solution in phosphate buffer 0.1M, pH 7.2. Samples were then washed in 4 succesive baths, at 1 hour intervals, in 0.15M phosphate buffer, at 4°C. Pieces were further postfixed in 1% osmic acid (OsO_4) in 0.15M phosphate buffer, pH 7.2, for 90 min. at 4°C, and then washed 3 times, for 15 min., in the same phosphate buffer. Liver tissue fragments were then submitted to a dehydration process, by keeping them in acetone baths with succesively increasing concentrations (30%, 50%, 70%, 80%, 90% and three times 100%), 30 min in each bath. Samples were gradually infiltrated with synthetic resin Epon 812, in mixtures of 1:3, 1:1 and 3:1 epoxidic resin:acetone, and finally included in special capsules. The capsules were kept in a thermostat at 50°C for 72 hours, to let the resin polymerize. The resin blocks containing the samples were then shaped. For electron microscopy, ultrathin sections of 30-60 nm were obtained with a Leica UC6 ultramicrotome, with a Diatome Ultra 35° diamond knife, and then collected on electrolytic grids with 100-200 Mesh, and double-contrasted with a 50% alcoholic solution of saturated uranyl acetate, and lead citrate in distilled water, pH 12. Finally, the sections were examined under an FEI Tecnai G2 Spirit TWIN/BioTWIN electron microscope, at International Center of Electron Microscopy of „Vasile Goldis" Western University, Arad. Images were captured with a Mega Wiew III camera.

These methodologies are consistent with the classical methods widely used in transmission electron microscopy (TEM) (Ploaie and Petre, 1979; Weakley, 1981; Hayat, 2000; Pavelka and Roth, 2005).

RESULTS AND DISCUSSIONS

ULTRASTRUCTURE OF THE CONTROL LIVER (FIGS. 1-4)

Examination of normal liver tissue revealed a parenchymal structure with hepatocytes arranged in regular cords. Each hepatocyte had one (Figs. 1 and 3), or occasionally two (Fig. 2) spheric nuclei, with a diameter of 5-7 μm and a uniform outline (Figs. 1-3).

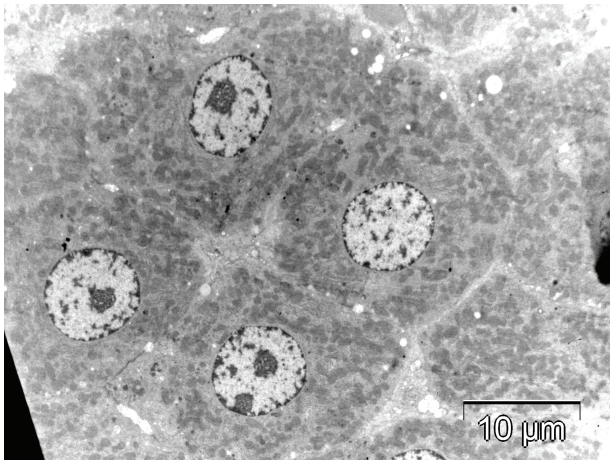

Fig. 1. Control group (C)

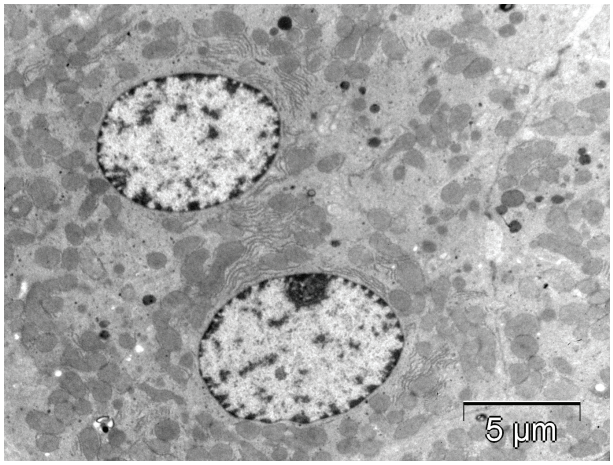

Fig. 2. Control group (C)

Nuclei were predominantly euchromatic, with the heterochromatin dispersed in karyolymph as a network of small electrondense blocks, in a fine layer along the inner leaflet of the nuclear anvelope (Figs. 1-3). Nuclear pores, having a lower electrondensity than the neighboring heterochromatin, appeared clearly delimited.

The best represented organelle in hepatocytes is the mitochondrion. In control liver, mitochondria were very numerous, had a medium electrondensity and were spheric or slightly elongated in shape (Figs. 1-3). They did not contain paracrystalline formations and their cristae were arranged transversely. The granular endoplasmic reticulum (GER) was well represented as thin, parallel profiles, or arranged between and around mitochondria (Fig. 2).

There were numerous ribosomes attached to the reticulum, which indicate a normal protein synthesis. The smooth endoplasmic reticulum (SER) was poorly represented, as small vesicles uniformly dispersed in the cytoplasm.

The cytoplasmic matrix had a high electrondensity, due to the presence of free ribosomes and of glycogen microparticles, arranged in groups between mitochondria or uniformly spread in all the cytoplasm, and visible at high magnifications.

The Golgi complex was discrete, practically unobservable, and noted only in the proximity of bile canaliculi, where its presence was signaled by few electrondense lysosomal peribiliary bodies (Fig. 2).

Bile canaliculi had a normal structure, with a small lumen delimited by many microvilli (Figs. 2 and 3). The plasma membranes of the adjacent cells were connected, in the proximity of bile canaliculi, by intercellular tight junctions (*zona occludens*) (Feldman *et al.,* 1978).

Lipid droplets were very rare in the cytoplasm of hepatocytes, visible as small non-electrondense granules; this suggests that lipids were exported as they were synthesized (Figs. 1 and 2). Few lipid droplets were located at the vascular pole of hepatocytes, which indicates their transit towards the lipid storing Ito cells (Fig. 3) (Ito and Shibasaki, 1968).

The perisinusoidal (Disse) spaces were narrow, containing the protruded microvilli of the vascular pole of nearby hepatocytes (Fig. 4). There was no collagen stored in these spaces. The sinusoid capillaries were narrow, with erythrocytes in their lumen; Kupffer cells had a normal activity, with few materials accumulated by phagocytosis (Fig. 4).

Inter-hepatocitary spaces were normal, with constant dimensions (approx. 15 nm), without dilatations. Because only molecules or particles with a molecular weight lower than 10 kDa can pass through tight junctions, through these small canaliculi can leak only electrolytes, water, glucose and biliary pigments (584 Da) – all hydrosoluble substances.

These straight, without dilatations intercellular spaces ensure a normal filtration of sinusoidal fluid, thus easing the reabsorption of water, electrolytes, glucose and amino acids (Fig. 1). This occlusive system on the pathway of interhepatocitary spaces is very sensitive to even minimal physiological irritants which may penetrate together with the normal sinusoidal filtrate. The metabolic rate of hepatocytes depends on the proper functioning of sinusoid system: the space of Disse, interhepatocitary spaces and bile canaliculi.

All these functional gates form a physiological unit that works with rhythmic periodicity, ensuring the normal secretory activity of the liver.

The above presented observations on the ultrastructure of control liver are consistent with previously reported data by Bruni and Porter (1965), Bloom and Fawcett (1975), Crăciun (1985), Crăciun *et al.* (1988, 1992, 1995), Bacon *et al.* (2006); Arias (2009).

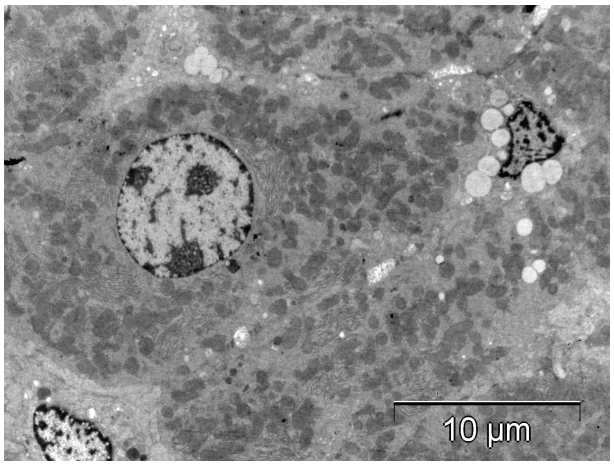

Fig. 3. Control group (C)

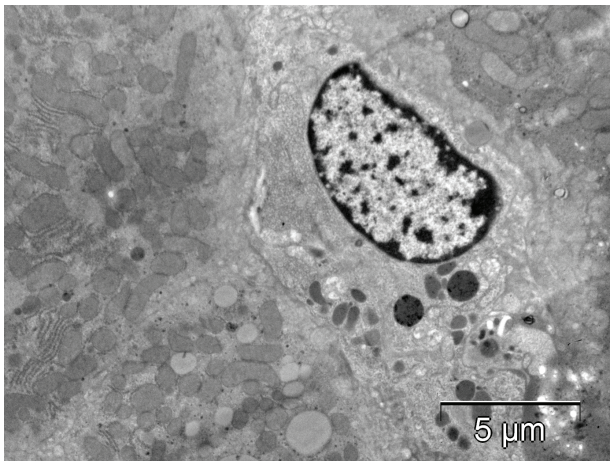

Fig. 4. Control group (C)

ULTRASTRUCTURE OF THE ALCOHOLIC LIVER (ETOH – FIGS. 5 – 10)

Electron microscopy examination confirmed the modifications observed with optical mycroscope and provided more details on the effects of ethanol at subcellular level.

An increased presence of lipid droplets was noticed in most images; droplets were located predominantly in the periphery of hepatocytes, or spread all over the cell (Figs. 5, 7 and 8). The absence of droplets in Ito cells (Fig. 8) clearly suggests the influence of ethanol, which impaired lipid transport outside the hepatocytes, leading to their accumulation inside the cells (Figs. 7 si 8).

Another effect of ethanol concerns the form and shape of nuclei: many of them lost their spherical form, and their outline in irregular (Figs. 6 and 8). In the cytoplasm of hepatocytes in the centrolobular area, mitochondria had a weak electrondense, tenuous matrix, suggesting a poor participation to energetic metabolism. As a defense reaction against the toxicity of ethanol, a proliferation of SER was noticed. GER had dilated canaliculi; the perinuclear space was also dilated, which indicated a decrease of protein synthesis (Fig. 6). It is notable that ethanol induced a depletion of glycogen granules, which were almost absent in hepatocytes.

Dilatations of sinusoid capillaries (Figs. 5 and 10), were seen close to the centrolobular area; interhepatocitary spaces were also dilated, which in some cases produced the partially detaching of some hepatocytes from the cellular cords, with destruction of the Disse space and of the sinusoid capillary endothelium.

In some cases, the bile canaliculi were dilated and almost without microvilli in their lumen (Fig. 8), while in other cases the bile canaliculus was obstructed, preventing the removement of bile; this is also suggested by the agglomeration of lisosomal peribiliary bodies in the proximity. Kupffer cells, liver's macrophages, had a relatively intense activity, to capture and destroy the structures altered by ethanol (Fig. 9).

Neutrophils were noticed in the lumen of sinusoid capillaries; their presence clearly indicates a hepatic inflammatory process (Fig. 10).

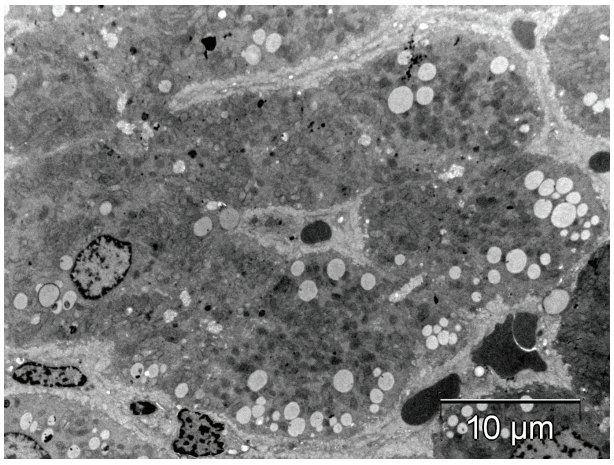

Fig. 5. Ethanol treated group (EtOH)

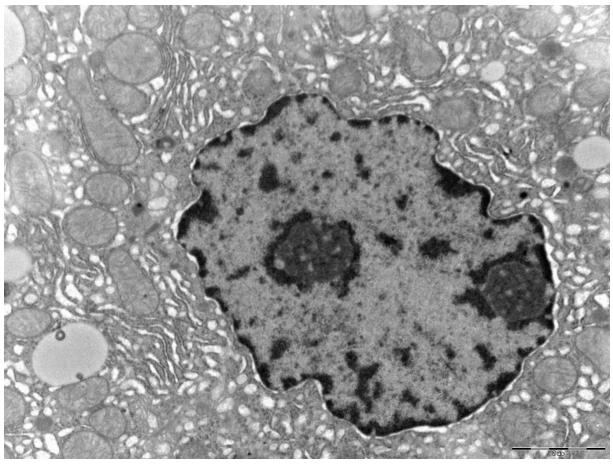

Fig. 6. Ethanol treated group (EtOH)

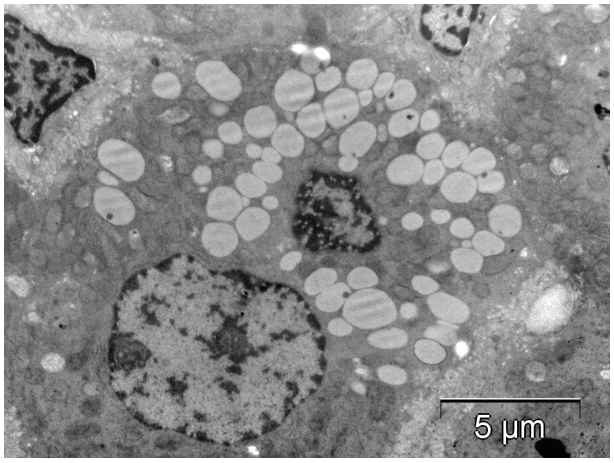

Fig. 7. Ethanol treated group (EtOH)

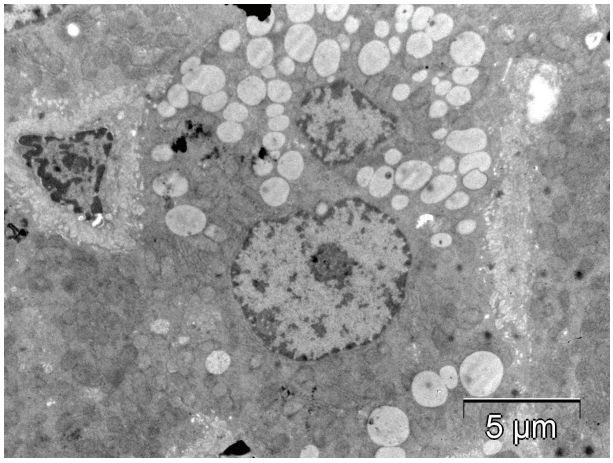

Fig. 8. Ethanol treated group (EtOH)

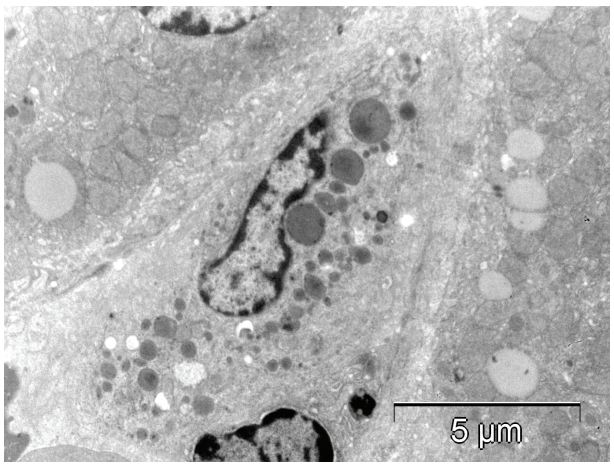

Fig. 9. Ethanol treated group (EtOH)

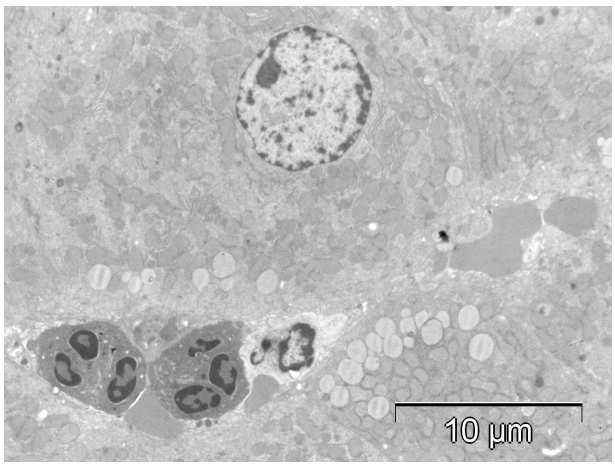

Fig. 10. Ethanol treated group (EtOH)

ULTRASTRUCTURE OF RAT LIVER SIMULTANEOUSLY TREATED WITH ETHANOL AND FENUGREEK SEED FLOUR IN A DOSE OF 5% (ETOHTR5 – FIGS. 11 – 13)

Electron microscopy studies entirely confirm the results of investigations performed on semithin sections with optical microscope. Most ultrastructural features described in the control liver were retrieved in the livers of EtOHTr5 group, proving the hepatoprotective action of fenugreek seed flour against the alterations induced in liver cells by ethanol.

Thus, we noticed that the majority of nuclei were spheric and with an uniform outline (Figs. 11 and 12). In a few cases, the nuclear outline was slightly irregular. The presence, in some nuclei, a 3 nucleoli, and the arrangement of GER in narrow, parallel profiles (Fig. 11) indicates an enhancement of protein synthesis.

The number of double-nucleated hepatocytes was higher than in the control group, suggesting that fenugreek flour stimulated liver tissue regeneration, as a reaction to the toxic effects of ethanol (Fig. 11).

In most hepatocytes lipid droplets were in small number (Figs. 11 si 12); where they were more numerous, droplets were located in the periphery of cells (Fig. 12), as indicating a transit process towards sinusoids or Ito cells (Fig. 13).

This aspect shows a normal lipid metabolism, comparable with the on in the control liver. Fenugreek seed flour also protected glycogen stores (Fig. 12).

As a result of the protective action of fenugreek, SER had a discrete appearance, and other organelles (mitochondria, GER) and bile canaliculi emphasized a normal structure.

It was obvious that *Trigonella* seed flour, administered to rats in a dose of 5%, had a protective effect on the alcoholic liver.

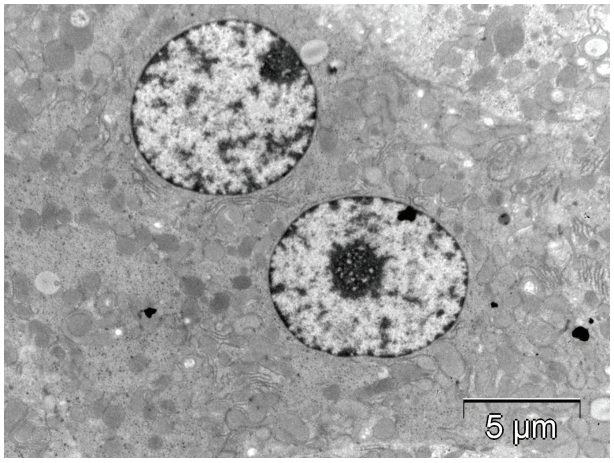

Fig. 11. Ethanol and fenugreek seed flour in a dose of 5% (EtOHTr5)

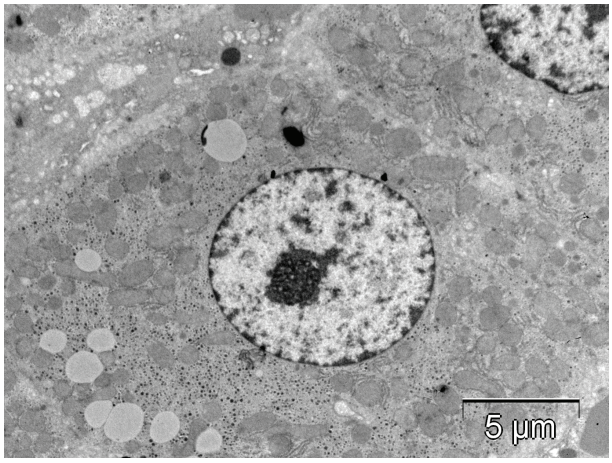

Fig. 12. Ethanol and fenugreek seed flour in a dose of 5% (EtOHTr5)

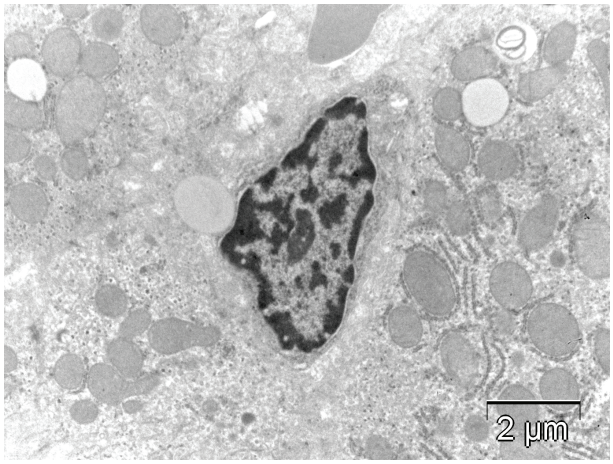

Fig. 13. Ethanol and fenugreek seed flour in a dose of 5% (EtOHTr5)

ULTRASTRUCTURE OF THE ALCOHOLIC LIVER CONCOMITANTLY TREATED WITH ETHANOL AND *TRIGONELLA* IN A DOSE OF 10% (ETOHTR10 – FIGS. 14 - 19)

Electron microscopy studies abundantly proved the hepatoprotective action of high fenugreek dose, although its effects were less pronounced than those of 5% dose.

In this group we also noticed that most of the cells had a normal structure, with spheric nuclei, regular outline and structured nucleoli (Figs. 14 and 15). However, some cells had nuclei with slightly irregular outline and altered shape (Fig. 17 si 18). Double-nucleated hepatocytes were more numerous than in the livers of the control group, provinf once more stimulation by *Trigonella* of the regenerative capability of the liver (Fig. 14).

Mitochondria had a normal structure in all hepatocytes (Figs. 16 - 18). GER showed narrow profiles with parallel arrangement, suitable for a good protein synthesis (Figs. 15 and 16). SER could be seen in only few cells. The presence of glycogen microparticles was evident, in some cells on large areas (Fig. 15).

Bile canaliculi had a small diameter and many microvilli in their lumen, similar to those in the control liver: they were surrounded by peribiliary bodies (Figs. 14 and 16).

Lipid droplets were in a small number and could be seen only in a few hepatocytes (Figs. 14, 16 and 18). The droplets were accumulated in Ito cells (Fig. 14), which suggests that their metabolisation is less efficient than in the case of rats that received 5% fenugreek. Kupffer cells were very active, overloaded with engulfed cellular debris, showing intense digestive processes in numerous lysosomes (Fig. 19); the defensive reaction against the toxic action of alcohol seemed to be very intense.

Our data indicate the hepatoprotective actions of 10% fenugreek flour on alcohol intoxicated liver.

CONCLUSION

We may conclude that both fenugreek seed flour doses had an obvious hepatoprotective effect against structural alterations induced by alcohol in hepatocytes. However, the small fenugreek dose (5%) seemed to be more effective than the dose of 10%.

ACKNOWLEDGEMENTS

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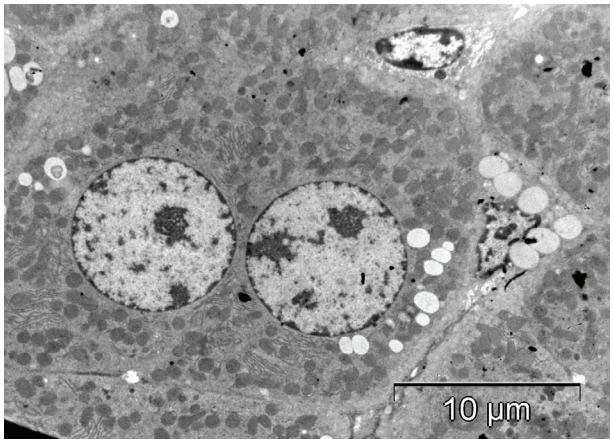

Fig. 14. Ethanol and fenugreek seed flour in a dose of 10% (EtOHTr10)

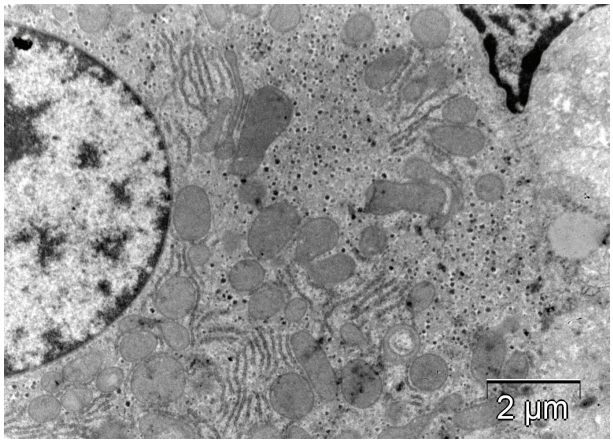

Fig. 15. Ethanol and fenugreek seed flour in a dose of 10% (EtOHTr10)

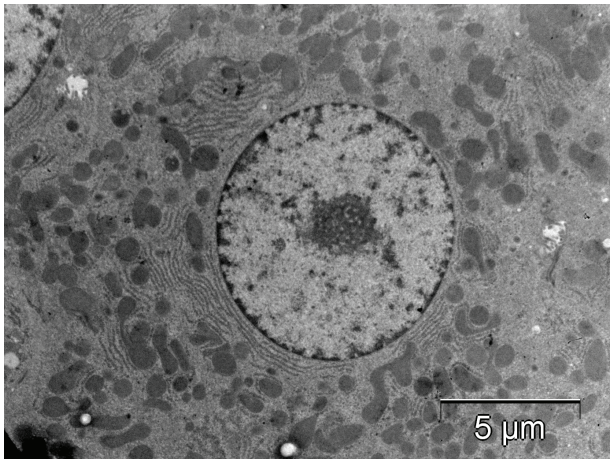

Fig. 16. Ethanol and fenugreek seed flour in a dose of 10% (EtOHTr10)

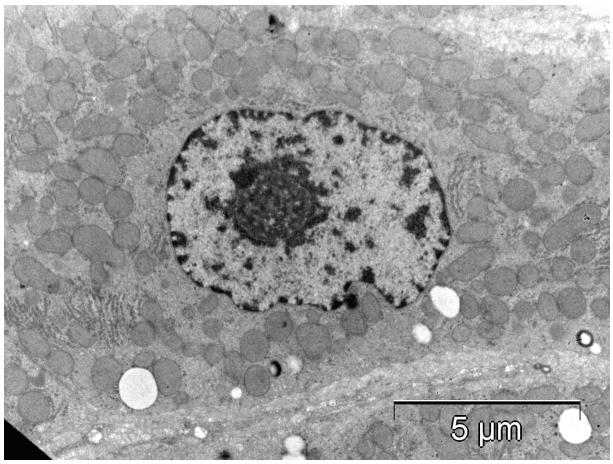

Fig. 17. Ethanol and fenugreek seed flour in a dose of 10% (EtOHTr10)

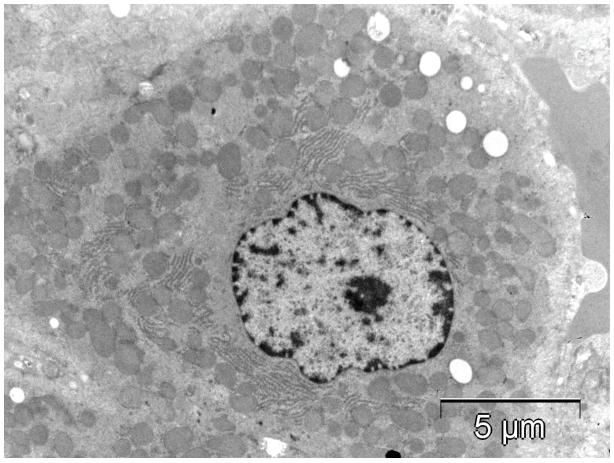

Fig. 18. Ethanol and fenugreek seed flour in a dose of 10% (EtOHTr10)

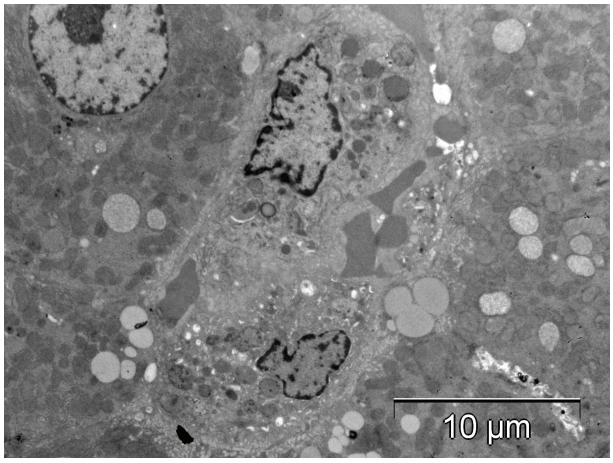

Fig. 19. Ethanol and fenugreek seed flour in a dose of 10% (EtOHTr10)

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