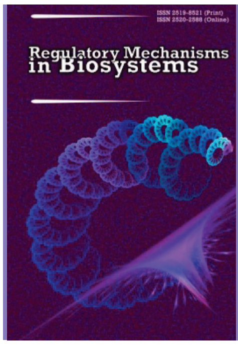

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Ultrastructural changes caused by *Datura innoxia* seeds extract in the liver of rats

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The main purpose of the investigation is to determine the toxic effects of the alkaloid-rich extract of the seeds of *Datura innoxia* Mill. on the liver tissue of white laboratory rats. *Datura innoxia* is an annual herb belonging to the Solanaceae family and widely distributed in the territory of the Republic of Azerbaijan. *Datura* species are known as a source of tropane alkaloids, which have toxic and therapeutic effects. The primary symptoms of *Datura* poisoning, related to the anticholinergic effects of tropane alkaloids, include hallucinations, mydriasis, dry skin, dizziness, tachycardia, reduced urinary retention, etc. The various pathological changes in living organisms due to the toxicity of tropane alkaloids were detected by different groups of researchers. In the presented study alkaloid-rich extract of the plant seeds was prepared using the acid-base extraction method and dissolved in 0.9% physiological saline. Animals were subjected to oral administration of the alkaloid-rich extract for a period of 30 days at a dose of 5 mg/kg body weight, once daily. At the end of experimental study, liver samples were obtained from control and experimental groups. Araldite-Epon blocks were prepared following established protocols for electron microscopy, semi-thin and ultrathin sections were obtained using a Leica EM UC7 ultramicrotome. The sections were stained and examined under Primo Star light microscope and JEM-1400 transmission electron microscope (TEM). The results revealed increased vascular permeability due to damage to the endothelial cells of the central veins and sinusoids. Edema formation was observed in the periendothelial and perivascular spaces. Stagnation in the sinusoidal lumen and the presence of bridge-like connections among the majority of sinusoids were identified. Necrosis was observed in the perivascular spaces of veins. The membranes of hepatocytes, which constitute the parenchyma of the liver, were damaged, and cytoplasmic organelles migrated to the intercellular and Disse spaces. Glycogen in the cytoplasm of hepatocytes transformed into an amorphous form, with certain nuclei of hepatocytes experiencing dystrophy, the tight junction of the bile canaliculus was disrupted, and sometimes not visible. The identified pathological changes indicate that the utilization of the alkaloid-rich extract at a dose of 5 mg/kg over 30 days resulted in toxic effects on the white laboratory rats.

Keywords: TEM; tropane alkaloids; hepatotoxicity; pathological changes.

Introduction

The biological active substances of medicinal plants are utilized both in therapeutic agents and in the production of pharmaceutical preparations (Cinelli & Jones, 2021). The examination of the toxic effects is one of the current issues, acknowledgeing that plant-derived medicinal preparations can elicit damaging effects in living organisms, in addition to the positive effects (El-Darier et al., 2023). There are many reported cases of poisoning associated with *Datura* species from the Solanaceae family (Arefi et al., 2016; Fatur et al., 2020; Khoshnam-Rad et al., 2022). *Datura innoxia* Mill. (also known as downy thorn-apple) also one of the poisonous plant from the family.The plant is widely distributed in Central and South America, Asia, Australia, Europe (Maslo & Šarić, 2019), and also in the Republic of Azerbaijan (Zernov & Mirzoeva, 2021). *Datura innoxia* contains tropane alkaloids, with the dominance of scopolamine (Mardare et al., 2022). For therapeutic purposes, *Datura* species are primarily utilized for their leaves, flowers, and seeds (Shama et al., 2014; Islam et al., 2023). Besides alkaloids, flavonoids, phenolic compounds, the withanolide group of steroids and tannins were identified in the content of *Datura* species (Sharma et al., 2021; Wu et al., 2022). The chemical compounds of D. innoxia from Azerbaijan have been investigated in our previous study (Valiyeva et al., 2023).

Datura species are used for the treatment of asthma, coughs, rheumatism, inflammation, muscle pains, headache, hemorrhoids, etc. (Benítez et al., 2018; Lian et al., 2022; Murtala et al., 2023). Moreover, it is known that the different extracts of *Datura* species exhibit positive antimicrobial (against *Staphylococcus aureus*, *Proteus vulgaris*, *Pseudomonas aeruginosa*, *Escherichia coli*, *Aspergillus niger*) and antifungal activity (Benouadah et al., 2016; El-Darier et al., 2023). Studies have shown that, different extracts of *Datura* species have antioxidant, anti-inflammatory, analgesic, cytotoxic and anticancer effects (against leukemia) (Nasir et al., 2020; Baig et al., 2021; Maldonado et al., 2021). Despite positive therapeutic effects, the usage of plants from the *Datura* genus and their derived biological active substances can lead to various pathological issues in different organisms, depending on the dosage and duration of exposure. Several poisoning cases, involving agricultural animals (ruminants, poultry, dogs, horses, etc.) as well as humans, have been documented in the references, resulting from the ingestion of the Datura species (Papoutsis et al., 2010; Ogunmoyole et al., 2019; Fatihu et al., 2023).

As a vital organ, the liver is responsible for eliminating and detoxifying different metabolic products. While detoxifying, some chemicals or their metabolites could be harmful to hepatocytes (Alam et al., 2021; Eltayeib & Matter, 2021). Toxicity of aqueous and methanolic extracts of seeds and leaves of *D. innoxia* in white rats experimentally investigated by histological methods and pathological changes has been detected in the liver, kidney and brain tissues. The pathological changes arising from the toxic effects of plant extracts have resulted in dysfunction of vital organs (Shama et al., 2014; Eltayeib & Matter, 2021).

There are references about the investigation of the toxicity of *Datura stramonium* by histological methods (Adekomi et al., 2011; Fatihu et al., 2023; Murtala et al., 2023). Various experiments on laboratory animals (mice and rats) have revealed changes in various organs (liver, kidney, brain, etc.) depending on the dosage and duration of exposure to extracts, alkaloid-rich mixtures, or different biological active combinations and substances obtained from various organs of the plant (Benouadah et al., 2016; Imo et al., 2019; Musa et al., 2020).

The review of the literature reveals that studies on the toxicity of *Datura* species have been limited to histological methods (Ekanem et al., 2016; Ogunmoyole et al., 2019; Alam et al., 2021). However, ultrastructural changes in the liver of white rats due to biologically active substances extracted from *D. innoxia* are not found.

In the presented study light and electron microscopic methods were used to determine the ultrastructural changes in the liver of white laboratory rats which ingested an alkaloid mixture of *D. innoxia* seeds.

Materials and methods

Ethical considerations. The experiments have been conducted in accordance with the Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes of the Council of Europe (18.03.1986, Strasbourg).

Collection of plant material. Datura innoxia samples were collected near Baku city (Ramana district, H. B. Zardabi Street. 40°27′23″ N, 49°58′57″ E) in October 2022. The identification of the *D. innoxia* samples was conducted at The Institute of Botany of the National Academy of Sciences of Azerbaijan. The collected plant specimens were verified to be *D. innoxia*. The seeds of the plant were dried under controlled conditions in well-ventilated rooms.

Extraction of alkaloid-rich extract. For the following experiments, 300 g of ground seeds were macerated with 2 L of 95% ethanol for 24 hours, extraction repeated for five times, with each repetition utilizing a fresh solvent. After the solvent evaporated, a dark brown, semi-solid oily residue was obtained. The semi-solid extract was dissolved with 200 mL of water and 200 mL of hexane. The mixture was carefully mixed in a separating funnel. The hexane layer was removed, and the aqueous extract was washed with hexane twice. 1 N sulfuric acid solution was added to the aqueous phase, leading to transfer of alkaloids to the salt form. The acidic aqueous solution was washed with chloroform three times. The pH of the aqueous extract was adjusted to 11 by using 25% ammonia (on the ice bath), and 500 mL of chloroform was added to extract the base form of alkaloids. The resulting mixture was filtered through an anhydrous Na_2SO_4 on filter paper. The filtrate was concentrated by evaporation and 500 mg of dry residue was obtained. The final residue was dissolved in 2.5 mL of 95% ethanol and diluted with 0.9% NaCl solution to 500 mL for achieving a concentration of 1 mg/mL alkaloid-rich extract.

Phytochemical profiling. The chemical compounds of the alkaloid-rich extract used in the experiments was analyzed using GC-MS (Gas Chromatography-Mass Spectrometry). The "Shimadzu QP-2010 Ultra" GC-MS system from Japan was applied with a 70 eV electron impact ionization during the experiments (El Bazaoui, et al. 2011). The alkaloid-rich extract of *D. innoxia* seeds was constituted of 65% alkaloids (scopolamine 32%, atropine 2.69%, apoatropine 5.61%, 3-phenylacetoxy-6,7-epoxytropane 3.27%, methylscopolamine 2.96%, dihydroaposcopolamine 0.33%, 3β-phenylacetoxytropane 5.12 %, scopoline 0.23%, scopine 0.10%, 3α-tropine 0.01%).

Description of the experiment. The experiments were carried out with 18 white rats, which a weight range of 180–220 g. The animals were divided into two groups, control (8 rats) and experimental (10 rats), who were housed under standard conditions at a temperature of 22 ± 2 °C, provided with unlimited access to food and water. Animals were orally administered the physiological solution containing the alkaloid-rich extract at a dose of 1 mg/mL, corresponding to 5 mg/kg body weight, once daily for 30 days. The control group was given the same volume of physiological solution without alkaloidal-rich extract. Metabolic parameters such as changes in skin color and structure, food and water intake were monitored using metabolic cages. At the end of the research, both control and experimental animals were decapitated, and liver samples were collected from the animals.

Light and electron microscopic investigations. Liver samples obtained from experimental and control groups were fixed in a solution containing 2% paraformaldehyde, 2% glutaraldehyde, and 0.1% picric acid in 0.1 M phosphate buffer (pH 7.4). After at least 24 hours of remaining in this fixation, samples were post-fixed in 1% osmium tetroxide in 0.1 M phosphate buffer (pH 7.4) for two hours. According to established protocols in electron microscopy, Araldite-Epon blocks were prepared from the material (Kuo, 2014). Semi-thin sections (1–2 µm) obtained from the blocks using a Leica EM UC7 (Germany) ultramicrotome were stained with methylene blue, azure II, and basic fuchsine or toluidine blue (Morikawa et al., 2018). The relevant sections were examined under a Primo Star microscope (Zeiss, Germany) and images of the required areas were captured with an EOS D650 digital camera (Canon). Ultra-thin sections, 50–70 nm in thickness, obtained from the same blocks, were initially treated with a 2% uranyl acetate solution, followed by staining with 0.6% lead citrate prepared in a 0.1 N NaOH solution. Ultra-thin sections were examined using a JEM-1400 (Joel, Japan) transmission electron microscope under a tension of 80–120 kV and electronograms were obtained. The morphometric analysis of the images was conducted using the software program (The TEM imaging platform) developed by the German company "Olympus Soft Imaging Solutions GmbH," on electronograms captured in TIF format (Rzayev et al., 2022; Hajiyeva et al., 2024).

Results

In the presented study the toxicity of the alkaloid-rich extract of *D. innoxia* seeds was tested on white laboratory rats compared with a control group. Liver samples of animals were monitored through light and electron microscopic methods at the ultrastructural level. In Figure 1, images obtained from preparations of the liver of a white rat, prepared from semi-thin sections (1 µm) under light microscope (Fig. 1a), and from ultra-thin sections (50–70 nm) under electron microscope (Fig. 1b), are presented.

In Figure 1a, hepatocytes (1), which constitute the structural elements of the liver, are clearly visible, along with their nuclei (2) located at the center of their cytoplasm, and sinusoids (3) apparent between the hepatocytes. In Figure 1b, the presented electronogram illustrates hepatocytes (1) with a magnified view, revealing mitochondria in their cytoplasm, glycogen (4), a large centrally located nucleus (2), erythrocytes (5) in the lumen of the sinusoid (6), endothelial cells (7) forming its wall, the space of Disse (8) between the endothelium and hepatocytes, and bile canaliculus (9) between two hepatocytes. Pathologies were not observed in either the light or electron microscopic images (Fig. 1a and 1b).

Liver samples obtained 30 days after the administration of the alkaloid-rich extract of *D. innoxia* seeds to white rats were investigated using both light and electron microscopic methods. It is essential to mention in advance that during the investigation of the structural elements of the liver, significant pathologies were identified in both semi-thin and ultra-thin sections. Figures 2a and 2b illustrate the venous (1) vessels (Fig. 2a) in the liver, where erythrocytes (2) and monocytes (3) are observed in the lumen. The coalescence of erythrocytes in the lumen of the vessel results in the emergence of the blood "sludge" phenomenon, which is noteworthy. Furthermore, a significant presence of structurally altered cells, indicative of necrosis (4), is observed in the perivascular space (Fig. 2a).

In Figure 2b, disruptions in the integrity of the wall are observed in various parts along the course of the venous vessel wall. Therefore, the occurrence of edema in the mentioned areas occurred by passing through the plasma membrane into the perivascular and intercellular spaces (Fig. 2a and 2b). In Figure 2c, alongside the enlargement of sinusoids (5) situated between hepatocytes (6), stagnation is observed in their lumen. Additionally, in the images obtained under light microscopy, bridge-like connections are observed between the majorities of sinusoids. Destruction is observed in the nuclei of endothelial cells that constitute the walls of sinusoids (Fig. 2c). Figure 2d presents the general appearance of hepatocytes, which constitute the main part of the liver parenchyma. In addition to the absence of distinct boundaries between hepatocytes in the semi-thin sections, nuclei in their cytoplasm either underwent alteration or were generally not identified (Fig. 2d). Numerous fuchsinophilic structures in the cytoplasm of hepatocytes were found (Fig. 2c and 2d). Furthermore, glycogen in the cytoplasm not only exhibited unequal distribution but also underwent changes in its structure.

The impact of the alkaloid-rich extract on the liver of white rats has been investigated at the ultrastructural level using transmission electron microscopy (Fig. 3). In Figures 3a, 3b, and 3c, the lumen and the structural elements forming the wall of the central vein (1) in the liver are presented. In Figure 3a, the general appearance of the vein and its lumen with erythrocytes (2), lymphocytes, and other structural elements is demonstrated. In Figure 3b, a fragment of figure 3a, fragmentation of the endothelial cell (3) forming the wall of the vein is observed.

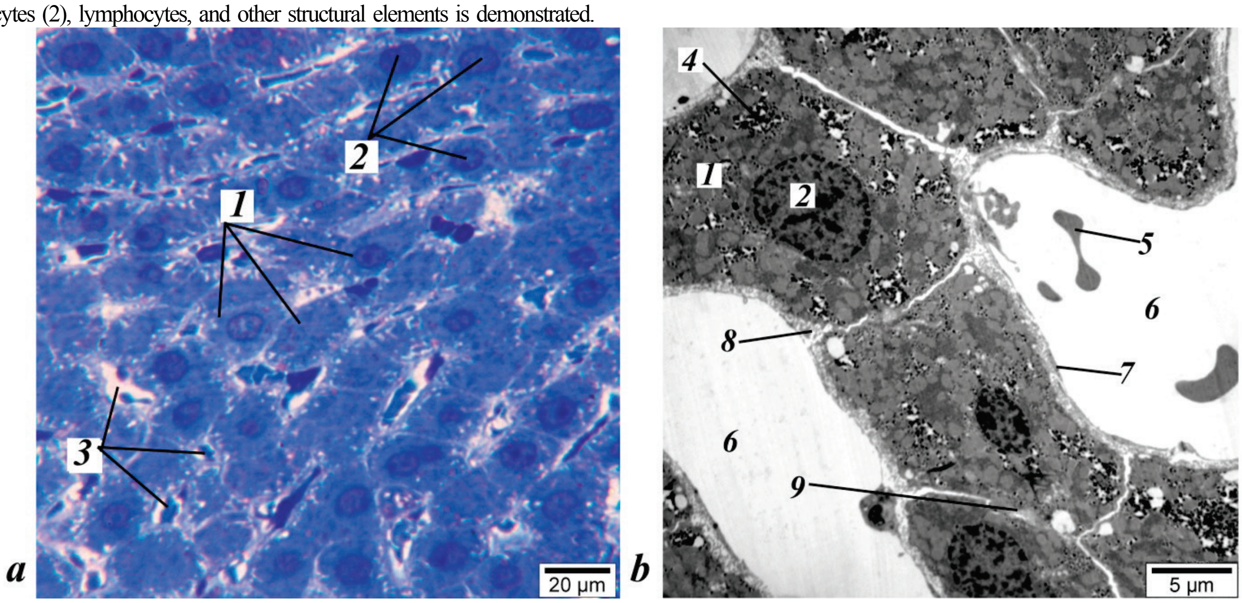

Fig. 1. General view of rat (control) liver under light (*a*) and electron (*b*) microscopes: semi-thin section (1 μm), method of one-step staining (Morikawa et al., 2018); ultrathin sections (50–70 nm), staining: uranyl-acetate and Pb citrate; designations: *1* – hepatocyte, *2* – nucleus of hepatocyte, *3* – sinusoid, *4* – glycogen, *5* – erythrocyte, *6* – lumen of sinusoid, *7* – endothelium, *8* – Disse space, *9* – bile canaliculus (explanation is given in the text)

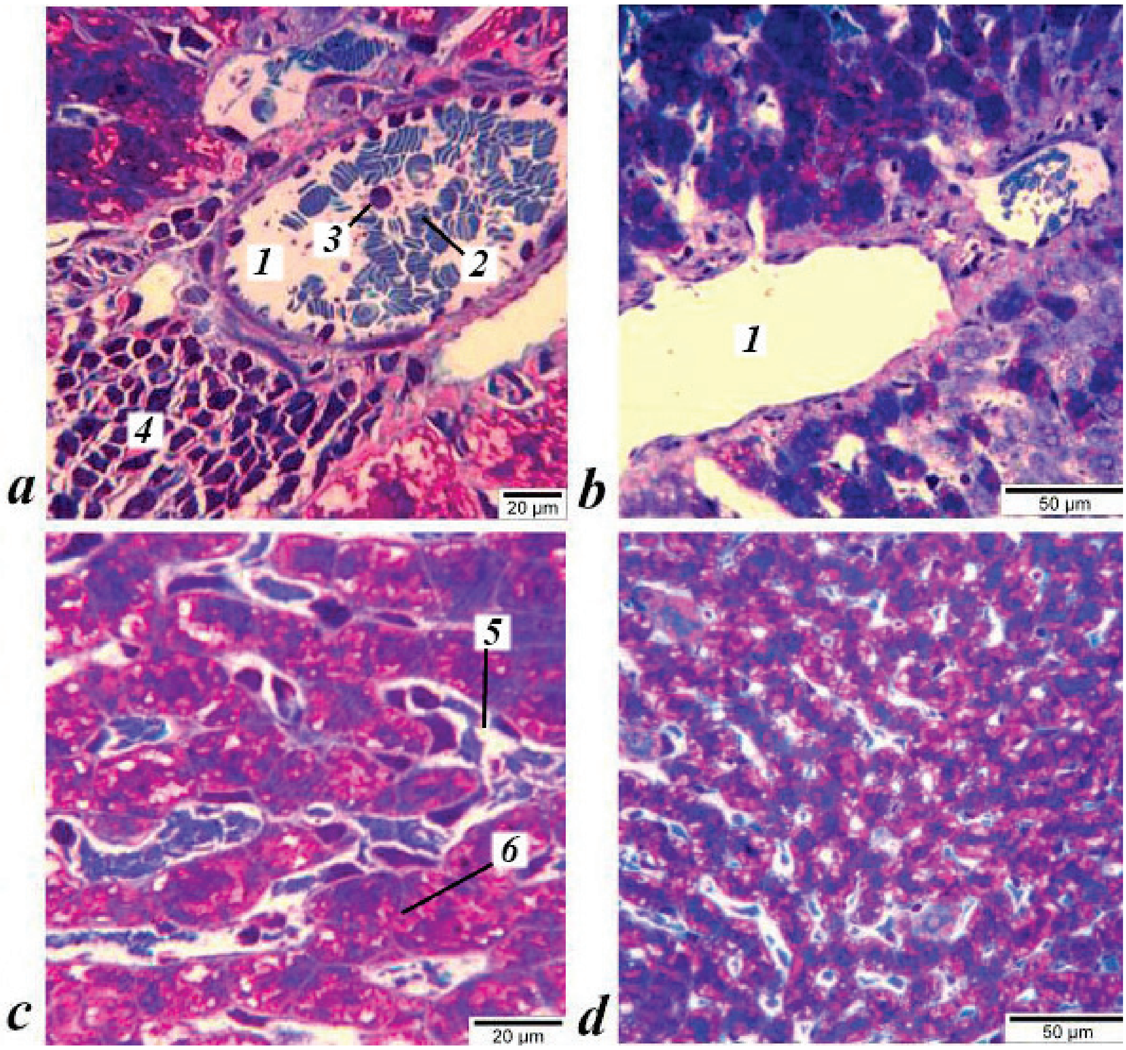

Fig. 2. Light microscope images of the changes in the liver of rats under the influence of alkaloid-rich extract obtained from the seeds of *D. innoxia*: semi-thin section (1 μm), Morikawa et al. (2018) method of one-step staining; designations: *1* – venous vessels, *2* – erythrocytes, *3* – monocytes, *4* – necrosis, *5* – sinusoid, *6* – hepatocyte (explanation is given in the text)

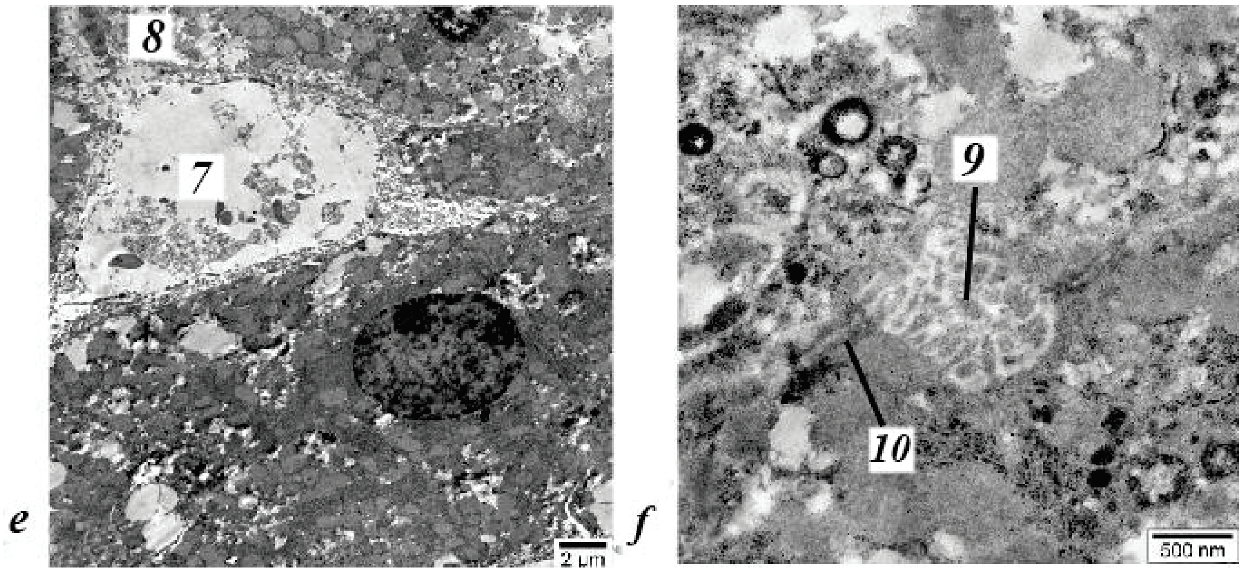

Fig. 3. Electron microscope (TEM) images of the changes in the liver of rats under the influence of alkaloid-rich extract obtained from the seeds of *D. innoxia*: ultrathin sections (50–70 nm), staining: uranyl-acetate and Pb citrate; designations: *1* – central vein, *2* – erythrocytes, *3* – endothelial cell, *4* – nuclei of the endothelial cell, *5* – edematous fluid, *6* – collagen fiber bundles, *7* – sinusoid, *8* – Disse space, *9* – bile canaliculus, *10* – tight junctions (explanation is given in the text)

Endothelial cells (3) in some parts it becomes thinner, and in others, it is destroyed. The nuclei (4) of the endothelial cells have undergone deformation. Edematous fluid (5) has accumulated in periendothelial space (Fig. 3b). In Figure 3c, an enlarged view of the wall of the vein and the perivascular space is presented. Here, it is seen that the increased endothelial permeability due to the influence of the alkaloid-rich extract leads to the accumulation of edematous fluid (5) in the periendothelial and perivascular spaces and along with the numerous collagen fiber (6) bundles (Fig. 3c). The impact of the alkaloid-rich extract has resulted in the observation of severe pathologies in the ultrastructure of both veins and sinusoids (7). Electronograms related to sinusoids (7) are provided in figures 3d and 3e. Here, both in Figure 3d and 3e, the complete destruction of the endothelial cell forming the wall of the sinusoid are observed. In addition to the dilatation of the sinusoidal lumen, fragmented structures of endothelium are also observed here. Therefore, the amount of edematous fluid in the Disse space (8) has increased, and this space has thickened (Fig. 3d and 3e). The tight junctions (10) of the bile canaliculus (9) of hepatocytes have been disrupted, and the bile canaliculus are obstructed (Fig. 3f).

The pathological changes in hepatocytes and their cytoplasmic elements resulting from the influence of the alkaloid-rich extract on white rats have been determined through transmission electron microscopy (TEM). It is noteworthy that the membranes of hepatocytes are damaged, while in different areas, they are entirely destroyed. Consequently, the cytoplasmic organelles of hepatocytes (1) have migrated to the intercellular spaces and the lumen of sinusoides (2) (Fig. 4a). In hepatocytes which have undamaged membranes, the cristae in mitochondria were not detected, and certain nuclei (3) underwent deformation (Fig. 4b).

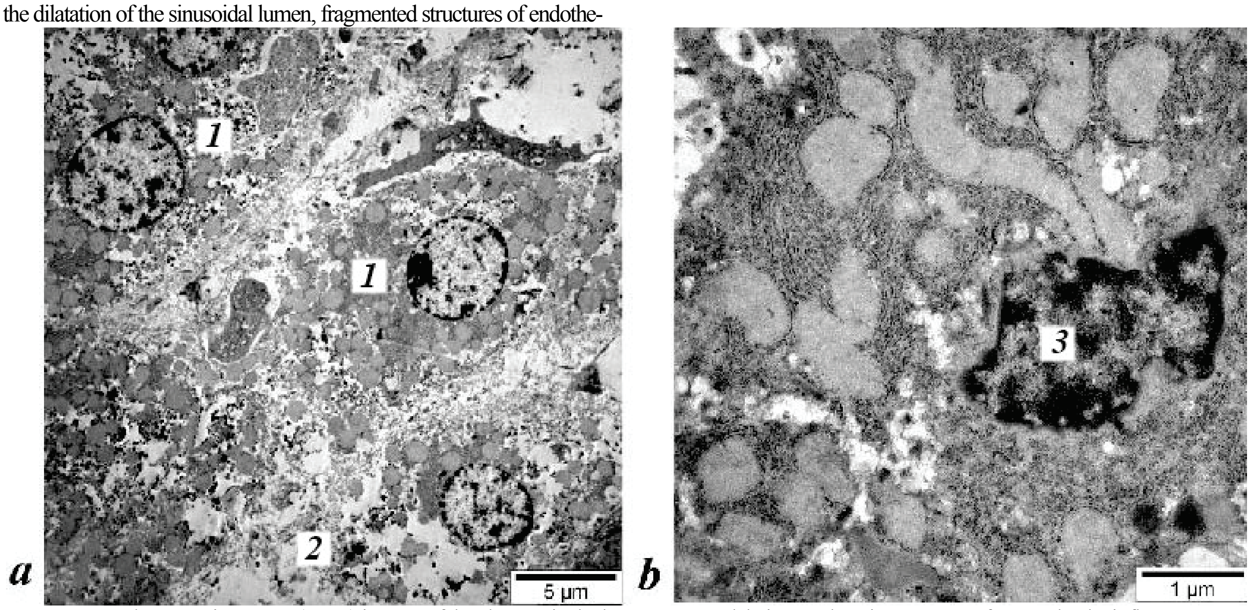

Fig. 4. Electron microscope (TEM) images of the changes in the hepatocytes and their cytoplasmic structures of rats under the influence of alkaloid-rich extract obtained from the seeds of *D. innoxia*: ultrathin sections (50–70 nm); staining: uranyl-acetate and Pb citrate; designations: *1* – hepathocyte, *2* – sinusoid, *3* – nuclei (explanation is given in the text)

Fat granules, which are considered a sign of fibrosis in the cytoplasm of hepatocytes, were observed (Fig. 3e, 3f). Furthermore, it was seen that the volume of glycogen, existing in an amorphous form, in the cytoplasm of hepatocytes has increased when compared to the control. This case has been recognized in both lights and electron microscopic images (Fig. 2c, 4b).

Discussion

Several references are available regarding the histological determination of the toxic effects of *Datura* species (Adekomi et al., 2011; Benouadah et al., 2016), including studies on *D. innoxia* (Shama et al., 2014;

Eltayeib & Matter, 2021). The analysis of available references has revealed that in experiments on laboratory animals (rats, mice), the composition, dosages, forms, and the duration of usage of these components have exhibited significant variation. In this regard, histological investigations were conducted on the liver, kidneys, and brain tissues, following the administration of aqueous and alcoholic extracts prepared from the leaves and seeds of *D. innoxia* at dosages of 40, 60, and 80 mg/kg over the period of 30 days. It has been determined that aqueous and alcoholic extracts exhibit similar toxic effects. The increase in dosages has resulted in a direct correlation with the exacerbation of pathologies occurring in liver toxicity. The impact of the extract has been observed to induce vasodilation, stagnation, edema, segmentation, necrosis in the perivascular space, cellular degeneration, and other pathologies (Eltayeib & Matter, 2021). In another investigation, changes in the liver tissues of rats were examined through the utilization of aqueous and alcoholic extracts prepared from the seeds of *D. innoxia*, administered at doses of 100 and 300 mg/kg over a period of 21 days. In conclusion, an increase in dosage also increases the pathologies occurring in the liver. The occurrence of necrosis and lymphocytic infiltration in the liver due to the impact of the extract has been detected (Shama et al., 2014). Changes such as significant stagnation in the blood vessels of the rat liver and degeneration of hepatocytes have been documented at a dose of 60 mg/kg with alkaloids extracted from *D. stramonium* (Benouadah et al., 2016). The analysis of the results obtained from the abovementioned study has revealed that, as a consequence of the presence of bioactive substances in the plant extract and the increase in dosage, pathological occurrences in the livers of experimental animals consistently occur in an ascending direction. Similar pathologies to those observed in other studies have been identified in the results of our conducted experiments. However, it is essential to mention that the results have only been compared with works conducted using histological methods. In this context, references related to the study of changes occurring at the ultrastructural level of the liver with the assistance of electron microscopic methods has not been detected. Therefore, we provide a comparative analysis of the liver investigated at the ultrastructural level in relation to the norm. Initially, it is essential to emphasize that, as a consequence of the increased permeability of the hepatic venous vessel walls compared to the control group under the impact of alkaloids, a fraction of the interstitial plasma transitioned into periendothelial and perivascular regions, resulting in an augmentation of edema formation and the occurrence of endothelial fragmentation (Fig. 3a, 3b, 3c). Almost identical changes occurred in the sinusoids (Fig. 3d and 3e). Serious pathologies have been observed in hepatocytes and cytoplasmic structures as well. In comparison with the control group, the integrity of hepatocyte membranes has been compromised under the influence of alkaloids, with organelles distributing throughout the intercellular and Disse spaces. Cristae of mitochondria were not observed (Fig. 4a, 4b). The tight junctions of bile canaliculus were disrupted, and in some instances, they have not been identified at all (Fig. 3f). The ultrastructural changes examined through electron microscopic methodology have been identified for the first time in our study. Another noteworthy occurrence involves the identified increase in glycogen within the cytoplasm of hepatocytes, displaying a non-equal distribution, along with the observed changing in its structure through both light and electron microscopy techniques (Fig. 2c, 2d, and 4a). In accordance with reference materials, hepatocytes exposed to the influence of toxic substances, under conditions of stress, begin to utilize glycogen intensively, by undergoing a change in its structure (glycogen content depletion), transparent areas are observed in the cytoplasm instead (Almansour et al., 2015).

Conclusion

The application of light and transmission electron microscopic techniques revealed significant pathological changes in the hepatic parenchyma of experimental animals, particularly in hepatocytes, sinusoids, bile canaliculus, and venous vessels, following the administration of an alkaloid-rich extract (5 mg/kg) of *D. innoxia* seeds over a period of 30 days. As a result of the toxic effect of the alkaloid mixture, the permeability of the vessels in the liver increased, with damage observed in numerous parts of their walls. Edema occurred in perivascular and periendothelial areas, hepatocyte membranes were compromised, and there were changes in glycogen form in the cytoplasm.

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The authors declare that there are no conflicts of interest.

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