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Histological Changes Resulting From Infection With The Entamoeba Histolytica In Mice And The Effect Of Treatment With Extracts Of The Aloe Barbadensis Plant

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ABSTRACT

The current study was designed to investigate the effect of aqueous and alcoholic extracts of Aloe barbadensis on the tissues of 4-8-week-old male Balb/c mice weighing 22-28 grams, collected from the beginning of September 2025 until October 2025. These mice were taken from the animal house of the College of Veterinary Medicine, Tikrit University, and placed in special cages in the animal house, which were bedded with wood shavings. Care was taken to keep the cages clean. Microscopic examination of tissue sections taken from mice infected with the Entamoeba histolytica parasite revealed pathological histological changes in the large intestine (colon) and liver. Specifically, necrosis of the intestinal mucosa was observed in the lamina propria, along with degeneration of several cells lining the intestinal glands. Large numbers of white blood cells were also found infiltrating the lamina propria between the glands, compared to the control group. The intestinal glands appeared tubular in shape with cavities containing mucous droplets. Between the bases and walls of the glands, mucous membranes were also found. In the liver of the positive (infected) control group, the portal vein appeared longitudinally wide and contained a mass of red blood cells and some white blood cells, with thickening of its wall adjacent to the bile duct, compared to the negative (uninfected) control group. Microscopic examination of histological sections taken from the intestines of mice infected and treated with an aqueous extract of Aloe barbadensis showed that the intestinal mucosa contained epithelial cells lining the intestinal lumen, with a large number of mucosal goblet cells distributed among the epithelial cells. In the infected liver treated with the aqueous extract, the liver cells appeared in long rows, each row containing normal-shaped hepatocytes with a polygonal appearance and a large, spherical nucleus. In the intestines of mice infected and treated with an alcoholic extract of Aloe barbadensis the intestinal mucosa showed numerous mucosal goblet cells and mucosal droplets in the cytoplasm of these cells, in addition to columnar epithelial cells facing the lumen. In the intestines and in the infected liver treated with the alcoholic extract, the liver tissue contained hepatocytes arranged in interlocking rows similar to honeycomb cells with large spherical nuclei, indicating the efficiency of the extracts in repairing damaged tissues.

Keywords: ***E. histolytica, Aloe barbadensis, Histological changes***

1. INTRODUCTION

The *Entamoeba histolytica* parasite is a protozoan that infects humans, causing amoebic dysentery. Clinical symptoms include colitis and bloody diarrhea. Infection can be asymptomatic or lead to severe illness (1). In some cases, amoebic abscesses develop, where *Entamoeba histolytica* invades other organs such as the liver and lungs and can even reach the brain and form abscesses (2). According to the World Health Organization, it is the third leading cause of death worldwide after *schistosomiasis* and *malaria* (3). The *Entamoeba histolytica* parasite is transmitted through the ingestion of food and water contaminated with the cyst stage (4). It can also be transmitted from person to person and through swimming in water contaminated with the parasite (5). Amoebic dysentery is endemic in subtropical and tropical regions and is associated with poor sanitation and socioeconomic conditions (6). The parasite is found in the human large intestine and appears in feces in several forms: the

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trophozoite stage, the pre-cystic stage, and the oocyst stage. The cyst, which is the infectious stage, causes symptoms such as intestinal colic, spasms, fatigue, fever, and bloody diarrhea (7). The damage to the epithelial cell layer lining the intestines includes the destruction of tissue cells and blood vessels, thus causing bloody diarrhea (8). *Aloe barbadensis* is a plant known for its medicinal properties. It contains a large number of biologically active compounds, such as vitamins, enzymes, amino acids, and polysaccharides. These components contribute to anti-inflammatory, antioxidant, and immunomodulatory properties (9). Important compounds in aloe include terpenoids, sterols, resins, polymannans, anthrones, lectins, flavonoids, saponins, and tannins (10).

2. MATERIALS AND METHODS

Laboratory mice

The mice used in this study were male white mice of the Balb/c type, aged 4-8 weeks and weighing 22-28 grams, which were taken from the animal house of the College of Veterinary Medicine/Tikrit University. These mice were placed in special cages in the animal house that were lined with wood shavings, with care taken to keep the cages clean. The mice were placed throughout the study under suitable laboratory conditions in terms of the availability of suitable lighting, temperature, and ventilation and were provided with water and the prepared feed until the end of the experiment.

Parasite Isolation

The *Entamoeba histolytica* parasite was isolated from stool samples obtained from individuals with diarrhea who were diagnosed with the parasite by direct smear microscopy as follows:

1. Five grams of stool were taken, distilled water was added, and the sample was filtered through six layers of gauze. The filtrate was then collected, and distilled water was added to it.
2. The tubes were centrifuged at 5000 rpm for 5 minutes.
3. The filtrate was discarded, and the sediment was collected. The process was repeated until the filtrate became clear.
4. Two milliliters of zinc sulfate were added to the sediment. The tubes were then centrifuged at 5000 rpm for 5 minutes. The supernatant was discarded, and the sediment was collected.
5. Two milliliters of zinc sulfate was added to the sediment, and the mixture was stored at -20°C.

A 0.2 ml drop of the filtrate was taken and placed on a hemocytometer slide. The number of parasite cysts in one drop was counted in order to determine the dose of infection according to the following equation: 200 x (number of cysts)/(0.2). The amoebic suspension was kept at -20°C until the mice were inoculated (11).

Dosing the mice:

Mice were inoculated orally with 5000 amoeba-containing saline solution using a curved needle. The needle was inserted orally into the esophagus and then the stomach, and the fluid containing the saline was pushed directly into the stomach. Negative control mice were inoculated with physiological saline in the same manner. For 10 days after infection, the mice were placed in clean cages. The presence of *Entamoeba histolytica* parasite cysts was investigated in the feces of infected mice by preparing several fecal smears on a glass slide and examining them under a microscope. After confirming infection, the mice were inoculated with aqueous and alcoholic extracts for 14 days. After the specified experimental period, the mice were anesthetized with ether for a few seconds, and the abdomen was opened longitudinally

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using dissecting scissors. The organs (liver and large intestine) were isolated and placed in dishes containing physiological saline to remove fatty tissue and any adhering ligaments. They were then fixed in 10% diluted formalin solution for 24 hours and preserved in 70% alcohol for histological examination. During the period of infection, which lasted for two months.

Collecting *Aloe barbadensis* samples

Aloe barbadensis:

The *Aloe barbadensis* leaves were collected from local markets, the plants were cleaned of dust, washed thoroughly with water, dried at room temperature, and sorted based on (12).

Preparation of *Aloe barbadensis* Extracts

1- Crude *Aloe barbadensis* gel Extract

The crude extract of *Aloe barbadensis* gel was obtained from the leaves as follows:

Aloe barbadensis leaves were collected, washed with distilled water, dried, and cut lengthwise into two equal halves. The gel was then extracted using a tablespoon and placed in an electric blender for two minutes. It was then filtered through four layers of cheesecloth, and the sediment was collected while the filtrate was discarded.

2- Preparation of the aqueous and alcoholic extract of *Aloe barbadensis*

500 grams of *Aloe barbadensis* gel were collected as in the previous step, then the gel was mixed with 1000 ml of distilled water (aqueous extract) or 100 ml of 70% ethyl alcohol (alcoholic extract) using an electric mixer for two minutes. The mixture was left for 12 hours with continuous stirring using an electric shaker, then placed in an incubator at 37°C until it dried completely and became a dry powder. The powder was then collected in opaque bottles and stored in the refrigerator until it was used to administer to mice (13).

Design of Experiment:

The mice were divided into six groups, each consisting of eight mice of similar weights, as follows:

1- (Group 1: Negative Control Group (G1)): This group included eight mice treated with a 0.9% normal physiological saline solution.
2- (Group 2: Positive Control Group (G2)): This group included eight mice treated with an amoebic slug for 10 days.
3- (Group 3: Infected and Treated Group (G3)): This group included eight mice infected with *Entamoeba histolytica* and treated with an aqueous extract at a dose of 0.02 mg/kg daily for 14 days.
4- (Group 4: Infected and Treated Group (G4)): This group included eight mice infected with *Entamoeba histolyticus* and treated with an alcoholic extract of *aloe barbadensis* at a dose of 0.02 mg/kg daily for 14 days.
5- (Group 5 (G5)): This group included 8 uninfected mice that were treated with an aqueous extract of *aloe barbadensis* at a dose of 0.02 mg/kg for 14 days.
6- (Group 6 (G6)): This group included 8 uninfected mice that were treated with an alcoholic extract of *aloe barbadensis* at a dose of 0.02 mg/kg for 14 days.

Histological study:

The tissue sections were prepared using Method (14):

1- **Fixation**: The liver and large intestine of the experimental groups were fixed in 10% formalin to prevent tissue autolysis for 24 hours. Afterward, the organs were placed in glass vials containing the aforementioned medium.
2- **Washing**: The samples were washed with running tap water for half an hour to remove any remaining fixative.
3- **Dehydration:** The organs were passed through increasing concentrations of ethyl alcohol (50%, 70%, 90%, and 100%) for one hour at each concentration to dehydrate the samples and prepare them for the next stage, clarification.

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4- **Clearing**: Xylene was used due to its ability to remove alcohol from tissues and make them more transparent. The organs were placed in xylene for 30 minutes, depending on the tissue type.
5- Infiltration: The samples were immersed in paraffin wax with a melting point of 80°C for one hour to saturate the tissues.
6- **Embedding**: The samples were embedded in paraffin wax by pouring a small amount of wax gently into special L-shaped molds. The tissue was then secured in the mold using hot forceps before use. The tissue was then fully immersed in the wax by pouring it all at once to avoid air bubbles. The molds were then left to solidify at room temperature and subsequently refrigerated until sectioning using a rotary microtome.
7- **Tissue Sectioning**: The tissue sections were sectioned using a rotary microtome into strips 5 micrometers thick. The sections were then transferred to a water bath at 40°C for a few minutes to spread them out. Finally, they were transferred to glass slides and left to dry on a hot plate at 37°C.

Hematoxylin and Eosin Staining:

The sections were stained with hematoxylin-eosin according to method (15):

1- Paraffin wax was removed from the sections by immersing them in xylene for 15 minutes to remove any remaining wax.
2- The sections were hydrogenated by immersing them in ethyl alcohol at decreasing concentrations (100%, 90%, and 70%) for 5 minutes at each concentration.
3- The sections were stained by immersing them in hematoxylin for 5 minutes.
4- The sections were rinsed with tap water.
5- The sections were immersed twice in acid alcohol solution to differentiate them.
6- The sections were rinsed with running tap water until the nucleus turned blue.
7- The sections were stained with eosin for 15 seconds (two quick coats).
8- The sections were perfused with increasing concentrations of 70%, 90%, and 100% ethyl alcohol for 30 minutes at each concentration to remove water from the tissue.
9- The sections were clarified with xylene for 5 minutes to remove the alcohol from the tissue. This was done by transferring the sample to the xylene twice, for half an hour each time, to ensure clarification.
10- The glass slide cover was sealed by placing drops of D.P.X. (Distrene plastizer xylene) on the sample with slight pressure and allowing it to dry. Examination under a light microscope revealed blue staining of the nucleus and pink staining of the cytoplasm. Imaging and Diagnosis: The tissue sections were examined using a light microscope to detect changes in the tissue organs of the studied samples and all experimental groups, and these were compared with the negative control group.

3. RESULTS AND DISCUSSION

After examining the prepared sections, pathological changes were observed in the group of mice injected with the histolytic amoeba parasite after the tenth day of infection through direct microscopic examination of the mice's feces. It was found that the histolytic amoeba parasite had caused changes in the tissues of the mice's organs in both the large intestine (colon) and liver. When comparing the histological sections in the negative control group (Figure 1) and

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the positive control group (infected with the parasite) (Figure 2), the results in the positive control group showed necrosis of the intestinal lamina and degeneration of a number of cells lining the intestinal glands, with the infiltration of large numbers of white blood cells in the lamina between the glands, along with the breakdown of some smooth muscle fibers in the intestinal wall. The results of the current study were consistent with the results of study (16), as significant superficial epithelial changes were observed, such as breakdown and degeneration of the mucous layer, ulceration of the apical surface, infiltration of inflammatory cells into different areas, and extension to the deep mucosa, causing structural changes. Mild these are the features of amoebiasis and are consistent with the results of (17), who explained that the parasite weakens the mucous barrier and stimulates an immune response involving neutrophils, and together they lead to necrosis, compared to the negative control group, as the intestinal glands appeared tubular in shape with cavities containing mucous droplets, and between the bases of the glands and their walls, an infiltration of a number of white blood cells was found. The muscular layer of the intestinal wall contained a portion of the internal smooth muscle fibers in a circular direction.

These pathological histological changes are attributed to the fact that the tissue-hemolytic amoeba, after reaching the intestines, begins to divide and multiply its number, then adheres to the mucous membrane and breaks down the tissue by means of the enzymes possessed by the trophozoite phase, cysteine proteinase. This leads to an inflammatory condition, as during the penetration process the parasite kills and engulfs epithelial cells and red blood cells. The parasite possesses myosin IB in the pseudopodium of the vesicles and in the plasma membrane of the active phase, where this substance plays an important role in the engulfment process, as it surrounds the material to be engulfed and adapts the shape of engulfment around the engulfed body. This is consistent with what was indicated by (18). As for the liver in the negative control group, it is shown in Figure (3) that the central vein in the liver lobe is surrounded by some white blood cells, in addition to the hepatocytes, which were found in long rows extending towards the periphery of the vein, and they are surrounded by blood sinuses and contain some Cowper cells. In the liver of the positive control group, as shown in Figure (4), the portal vein appeared longitudinally wide and contained a mass of red blood cells and some white blood cells. Thickening of its wall was observed adjacent to the bile duct and lymphatic vessel, where numerous white blood cells infiltrated the portal region. Externally, these blood and bile vessels were surrounded by a mass of enlarged, tightly packed hepatocytes, with narrow sinuses containing a limited number of phagocytic Kupffer cells. The results of the current study are consistent with (19), which indicated histological changes resulting from tissue infection by the *Entamoeba histolytica* parasite. Hepatocytes are among the most vulnerable cells because they are the first cells to receive substances via the portal circulation from the inferior vena cava, which forms part of the gastrointestinal tract (20), thus making them particularly susceptible to damage.

Microscopic examination of tissue sections taken from the intestines of infected mice treated with an aqueous extract of *Aloe barbadensis,* as shown in Figure (5), revealed that the columnar epithelial cells lining the intestinal wall appeared tightly packed together. The underlying

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lamina was filled with mucous-secreting intestinal glands, with white blood cells distributed sub-epithelially and between the glands. The sub mucosa contained loose fibrous tissue with small blood vessels and some white blood cells. The muscularis appeared as bundles of smooth muscle fibers. These results are consistent with those of study (21), which showed that the intestinal villi appeared finger-shaped, lined with simple columnar epithelial cells, with some goblet cells present. The villi medulla contained multiple white blood cells when the group infected with the tissue-hemolytic amoeba was treated with an aqueous extract of pumpkin seeds.

As shown in Figure (6), the liver of a mouse infected and treated with the aqueous extract of the *Aloe barbadensis* plant appeared in long rows, and each row contained normal-shaped hepatocytes with a polygonal appearance and a large spherical nucleus. The blood sinuses appeared as long-directed channels containing Cowper cells. The results of the study are consistent with (22) through his study of the effect of the aqueous extract of the nut grass plant on the *Entamoeba histolytica* parasite, where he observed that the intestinal villi appeared intact with the observation of a lymphoid lymphatic pool within the main lamina of the intestine. The microscopic examination of the tissue sections taken from the liver showed that the liver parenchyma contained well-defined hepatocytes.

Figures (7)(8) show a section of the intestine of an infected mouse treated with an alcoholic extract of *Aloe barbadensis*. The intestinal mucosa contains numerous goblet cells and mucoid droplets in their cytoplasm, in addition to the columnar epithelial cells facing the intestinal lumen. The basement membrane is filled with inflammatory leukocytes around the loosened intestinal glands, where a few goblet cells are visible. Leukocytes have spread into the epithelial layer and the sub mucosa below. The mucosa contains numerous villi lined with epithelial cells, including goblet cells. The nuclei of the epithelial cells appear as a dark row at the basement membrane, i.e., at the cell bases. The villus medulla contains an infiltration of large numbers of diffuse leukocytes along with some fibroblasts. Figures (9)(10) show a section of the liver of an infected mouse treated with an alcoholic extract of *Aloe barbadensis* The liver lobe contains a large, central vein with an abundance of red blood cells, and around it are rows of cells. The liver cells are radially arranged, each cell has a spherical nucleus, and each nucleus has more than one nucleolus. Between the liver cells, blood sinuses were found, containing a number of Kupffer cells. The portal region contained an excessively large and hyper congested portal vein, along with branches of the bile duct and a branch of the hepatic artery. There was an infiltration of numerous white blood cells, and numerous hepatocytes were present, exhibiting hypertrophy, with rupture in the cytoplasm and thickening of some nuclei. This may be due to the effective therapeutic effect of *Aloe barbadensis* extracts, which contain active substances such as glycosides, phenols, saponins, alkaloids, tannins, proteins, carbohydrates, fats, amino acids, minerals, and vitamins (23). These substances weaken the *Entamoeba histolytica* parasite within the intestines, thus preventing *Entamoeba histolytica* parasite from dividing and multiplying within the intestines, and consequently preventing the parasite from continuing to cause pathogens.

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The results showed no side effects from the aqueous and alcoholic extracts of *Aloe barbadensis* at a concentration of 0.02 mg/kg. Figure (11) shows the intestine of a mouse treated with the aqueous extract of *Aloe barbadensis*. The base of the lamina propria shows intestinal glands surrounded by numerous white blood cells in the interstitial tissue, with some white blood cells also present in the sub mucosa. The muscular layer consists of bundles of smooth muscle fibers, circular in direction and longitudinally in direction, where some stenosis is observed. Figure (12) shows the liver of a mouse treated with the aqueous extract of *Aloe barbadensis*. The hepatic artery in the portal region shows a blood clot with numerous white blood cells. Its wall is surrounded by an infiltration of white blood cells from the outside. These cells also surround the bile duct, which is lined with simple cuboidal cells. The hepatocytes around the portal region appear as compact masses with a radial arrangement. Figures (13) and (14) show the intestine of a mouse treated with the alcoholic extract of *Aloe barbadensis*. The intestinal mucosa contains a large number of cells. The mucous secretion, where hyperplasia occurred within the intestinal tract beneath the epithelial surface, with homogeneous inflammatory edema between the mucous droplets and the interstitial tissue. Large numbers of white blood cells, phagocytes, and intestinal gland bases infiltrated, extending to the mucosa separating the sub mucosa. The intestinal mucosa surface contained numerous mucous droplets, and the deep layers of the mucous glands showed hyperplasia of yeast-secreting cells, where the cells appeared blue and surrounded by large numbers of inflammatory white blood cells between the gland walls and at their bases, extending to the muscularis layer. The image (15)(16) shows a liver treated with the alcoholic extract. The liver tissue contains hepatocytes arranged in interlocking rows similar to honeycomb cells with large spherical nuclei. The blood sinuses are found as a network of blood channels between the hepatocytes, filled with hypertrophic Kupffer cells. The central vein at the middle of the hepatocyte lobe is wide-lubrilized and contains a limited fibrous thrombus. The walls of the vein are attached to the wide blood sinuses containing numerous hypertrophic Kupffer cells. These blood sinuses surround the rows of hepatocytes that are uniform in shape and arranged radially around the central vein.

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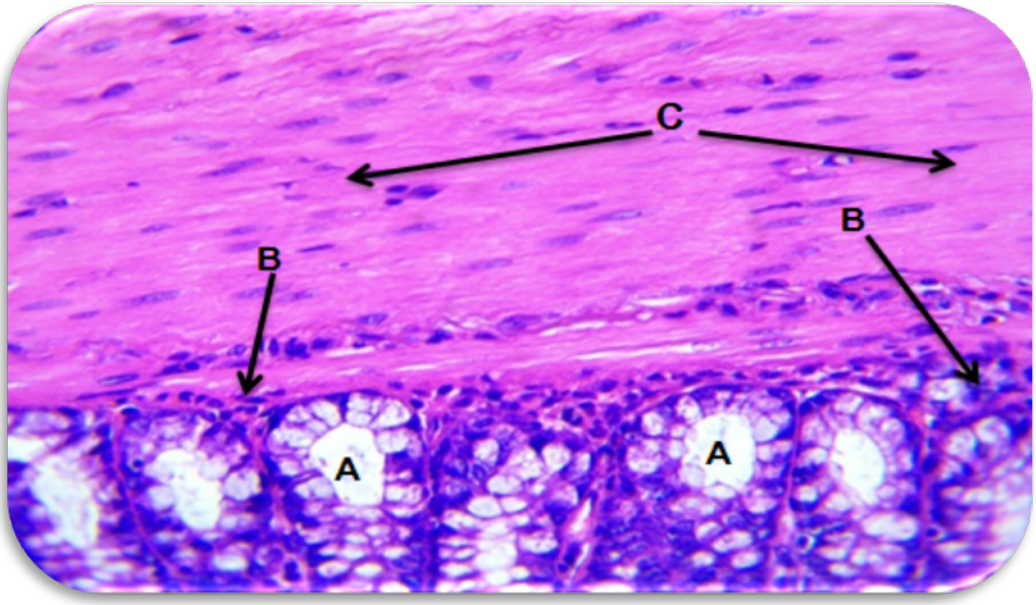

Figure (1): Section of the intestine of an uninfected mouse showing the deep layer of the lamina propria containing the bases of the intestinal glands (A), white blood cells between the glands (B), and smooth muscle layer (C) (400H&E×).

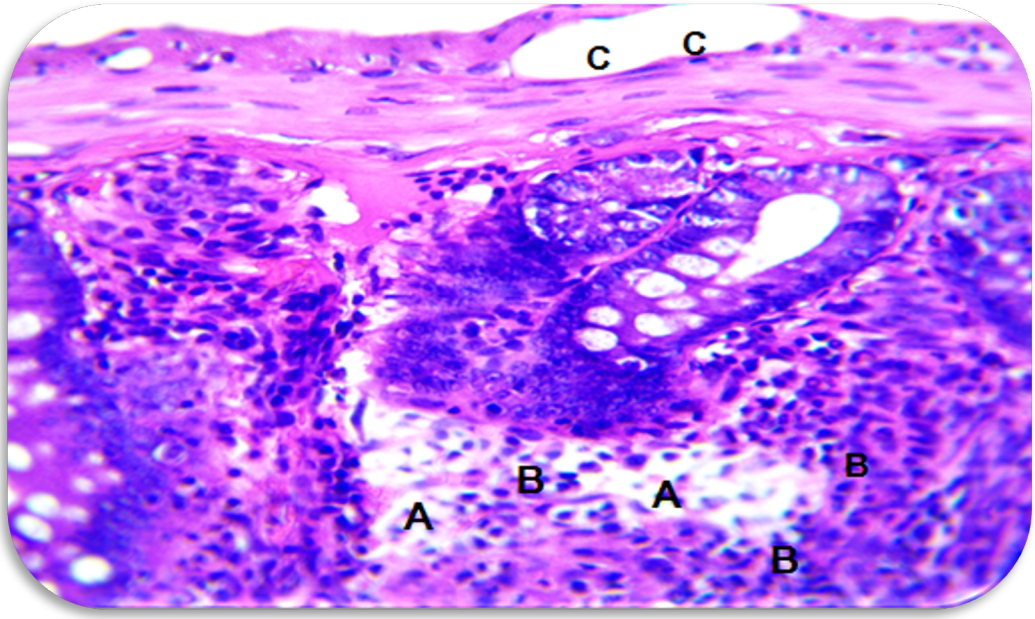

Figure (2): Section of the intestine of a mouse injected with the tissue-lysing amoeba parasite showing necrosis of the basic lamina of the intestinal mucosa (A), diffusion of white blood cells between the glands (B), and disintegration of some smooth muscle fibers in the intestinal wall smooth muscle layer (C) (400H&E×).

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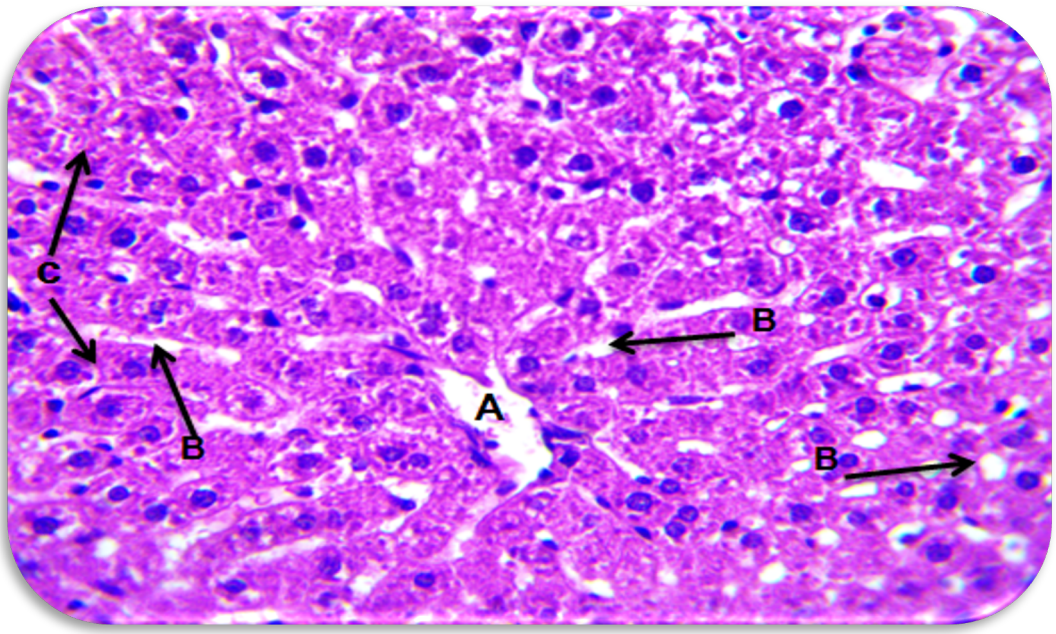

Figure (3): Section of an uninfected mouse liver showing the hepatic lobule, central vein (A), rows of hepatocytes (B), and blood sinuses containing a coffer (C) (400H&E×)

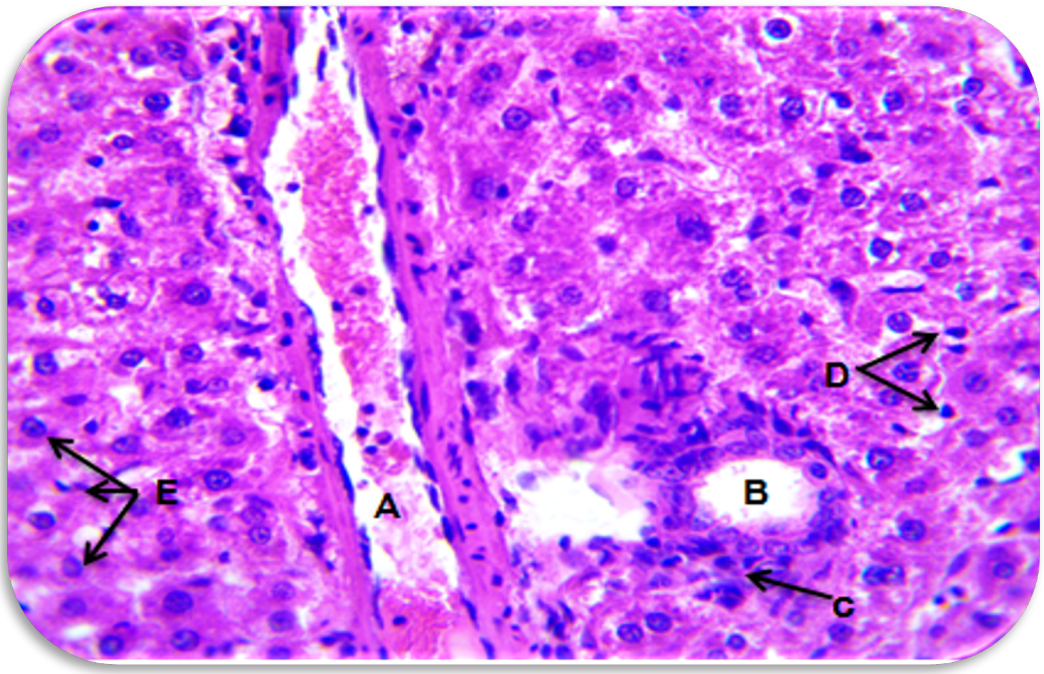

Figure (4): Section of a mouse liver injected with the *Entamoeba histolytica* parasite showing the portal region of the liver, the portal vein with a blood clot (A), the bile duct (B), infiltration of white blood cells (C), Kupffer cells (D), and hepatocytes (E) (400H&E×).

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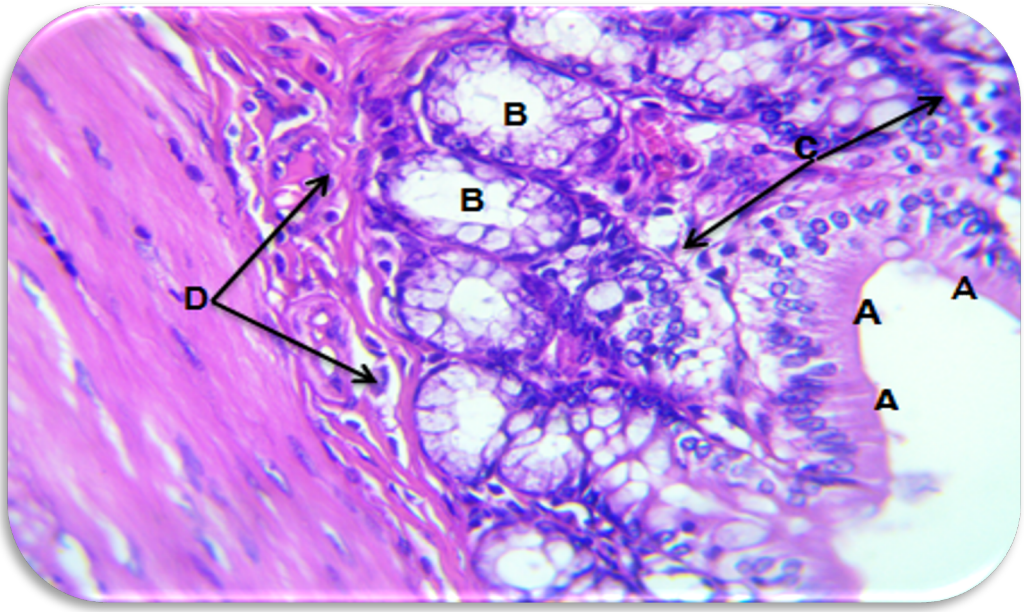
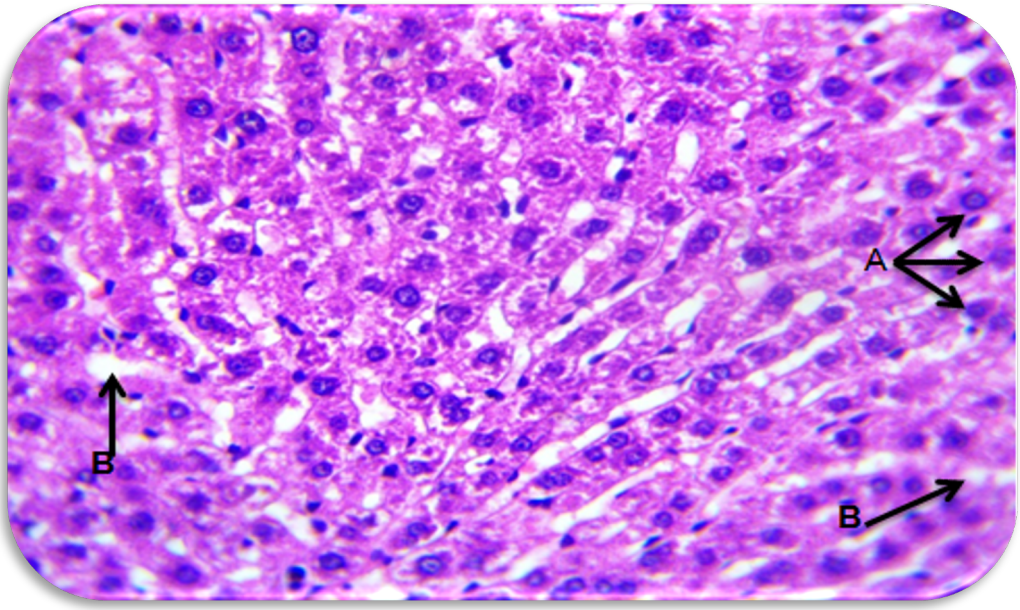


Figure (5): Section of the intestine of a mouse injected with the *Entamoeba histolytica* parasite and treated with an aqueous extract of *Aloe barbadensis.,* showing the simple intestinal epithelium of the intestinal lining (A), intestinal mucous glands (B) in the lamina propria, white blood cells between the glands (C), and loosened lymphoid tissue in the sub mucosa (D) (400H&E×).



Figure(6): Section of a liver injected with the parasite and treated with an aqueous extract of the *Aloe barbadensis.* plant showing the rows of hepatocytes in a radial pattern (A) and blood sinuses (B) (400H&E×)

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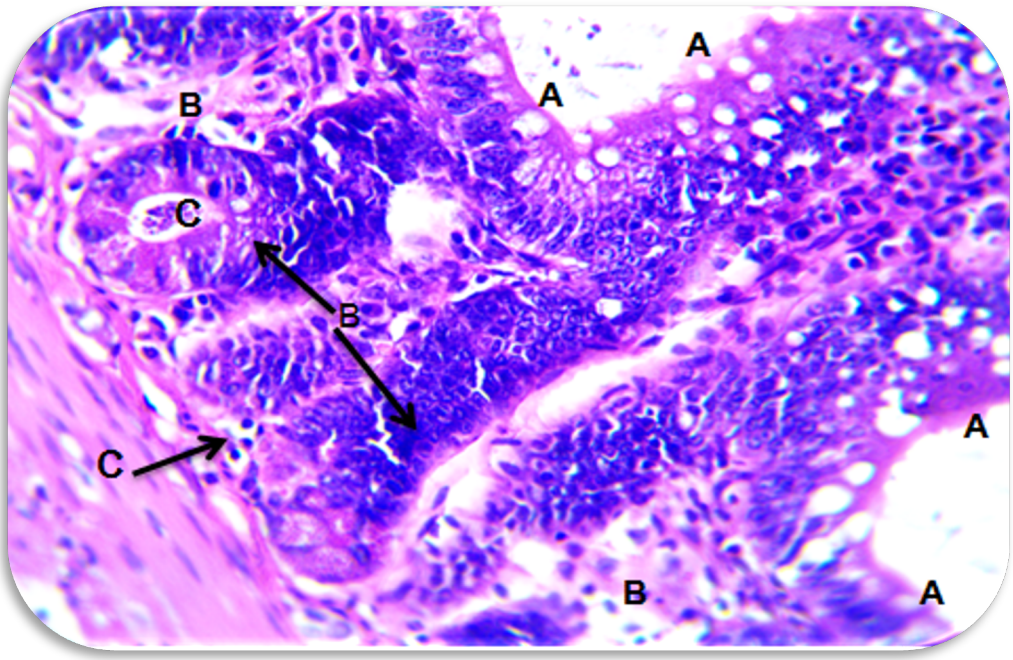

Figure (7): Section of intestine infected with the tissue-healing amoeba parasite and treated with the alcoholic extract of the *Aloe barbadensis* plant. It shows the intestinal mucosa with mucous droplets spread over the entire epithelial surface (A), disintegrated intestinal glands (B), and white blood cell spread (C) in the lamina propria and sub mucosa (400H&E×).

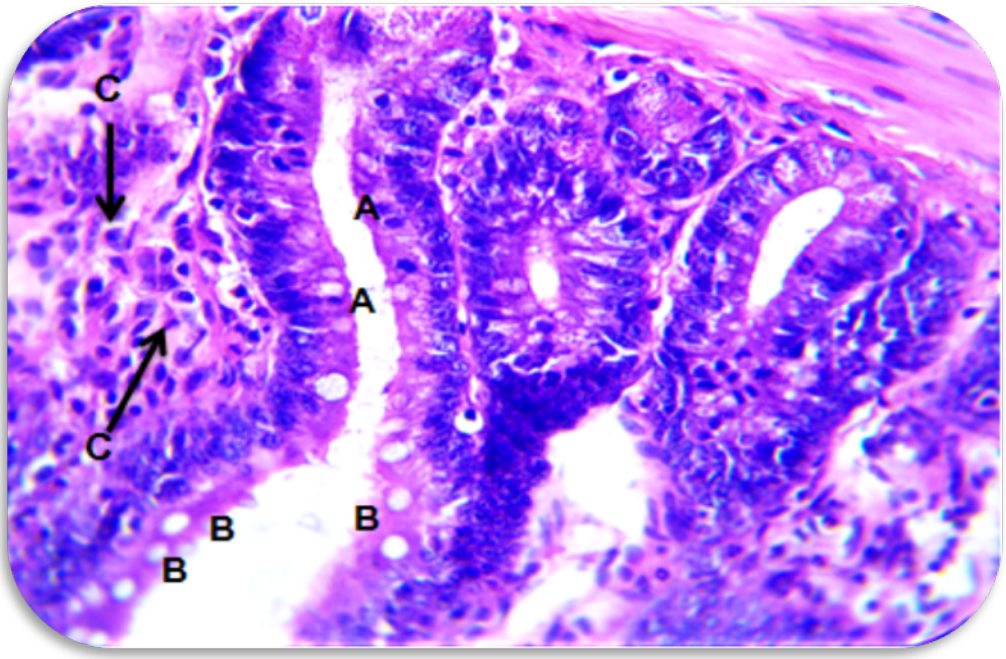

Figure (8): Section of intestines inoculated with the tissue-lysing amoeba parasite and treated with the alcoholic extract of the *Aoe barbadensis* plant, showing the intestinal villi lined with simple columnar cells (A), goblet cells (B), villus medulla with infiltration of white blood cells (C) with fibroblasts (400H&E×).

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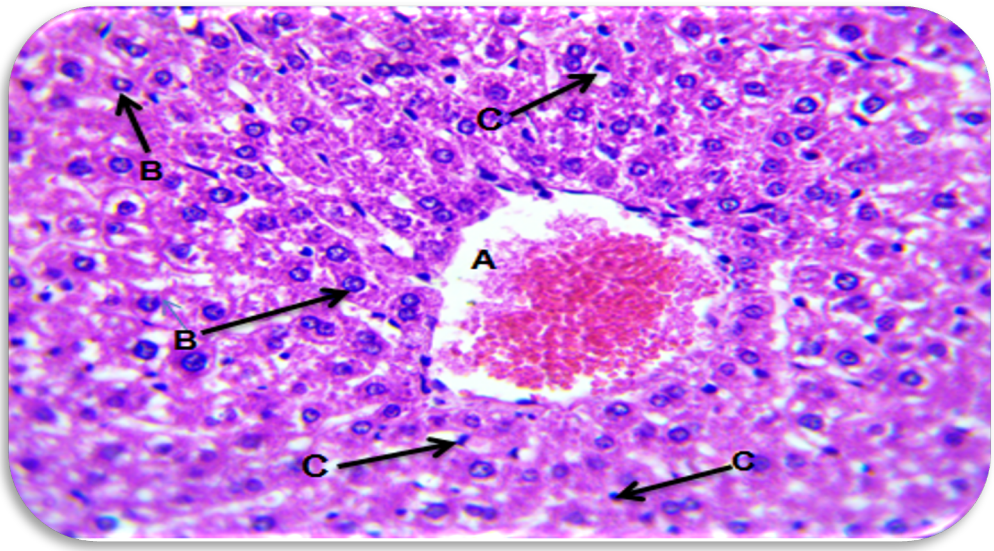
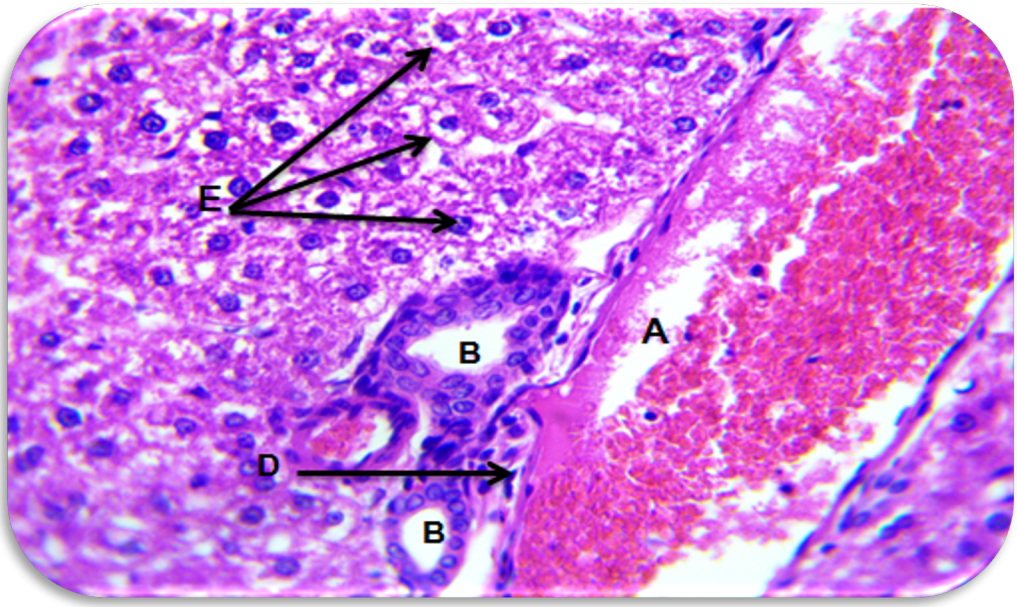


Figure(9): Section of a mouse liver injected with the parasite and treated with the alcoholic extract of the *Aloe barbadensis* plant, showing the central vein containing red blood cells (A), rows of hepatocytes (B), and blood sinuses containing kupffer cells (C) (400H&E×).



Figure(10): Section of a mouse liver injected with the parasite and treated with the alcoholic extract of the *Aloe barbadensis* plant, showing the portal region of the liver, the hypertrophied portal vein with blood congestion (A), branches of the bile duct (B), a branch of the hepatic artery, enlargement of the hepatocytes and thickening of their nuclei (C), and white blood cells (D) (400H&E×).

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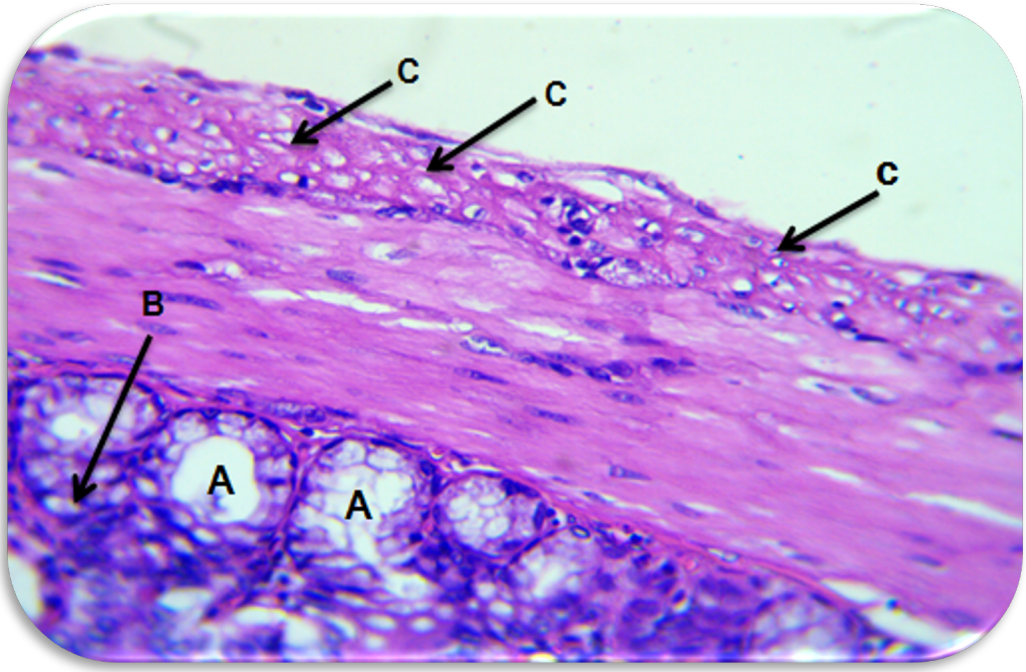

Figure (11): Section of the intestine of a mouse treated with an aqueous extract of the *Aloe barbadensis* plant showing the bases of the intestinal glands in the main lamina of the intestine (A) White blood cells between the glands (B) and fluctuation of smooth muscle fiber cells in the outer part of the muscular layer (C) (400H&E×)

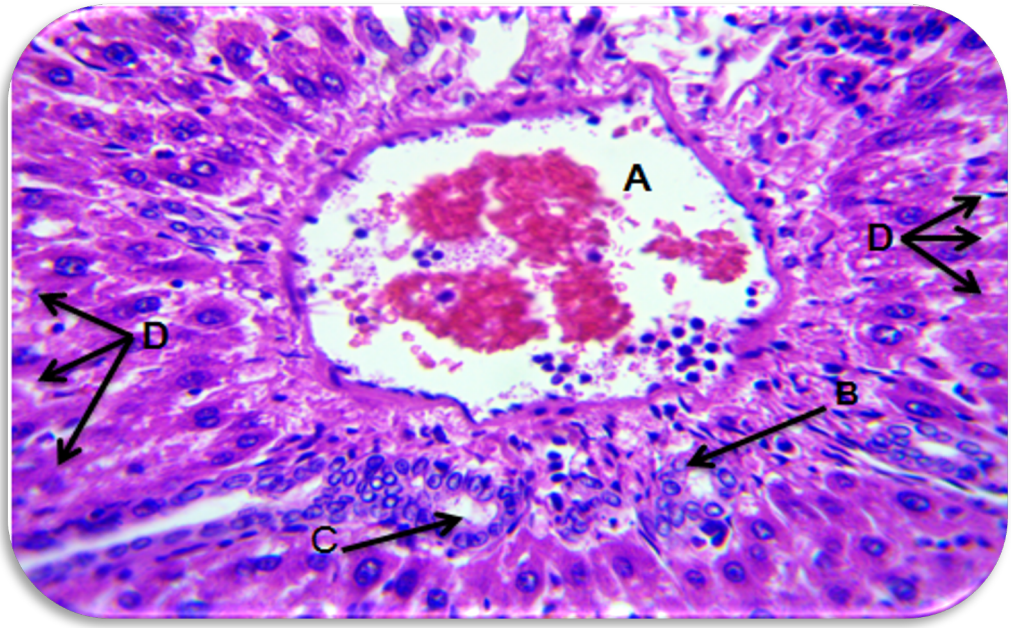

Figure (12): Section of a mouse liver treated with the aqueous extract showing a branch of the hepatic artery in the portal region with a blood clot (A), white blood cell infiltration around the artery (B), bile duct (C), and radially packed hepatocytes (D) (400H&E×)

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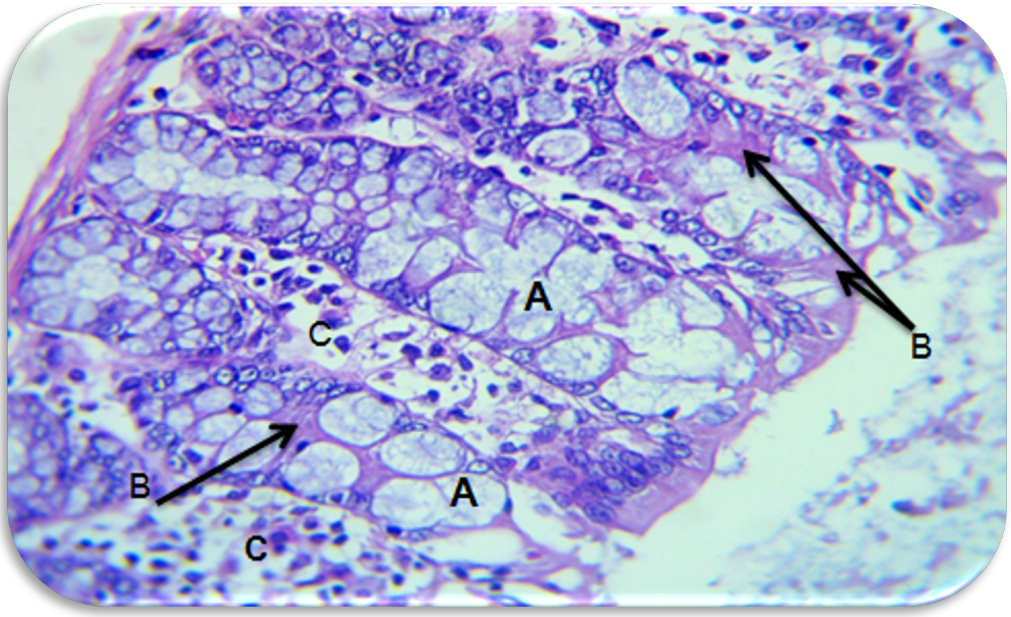
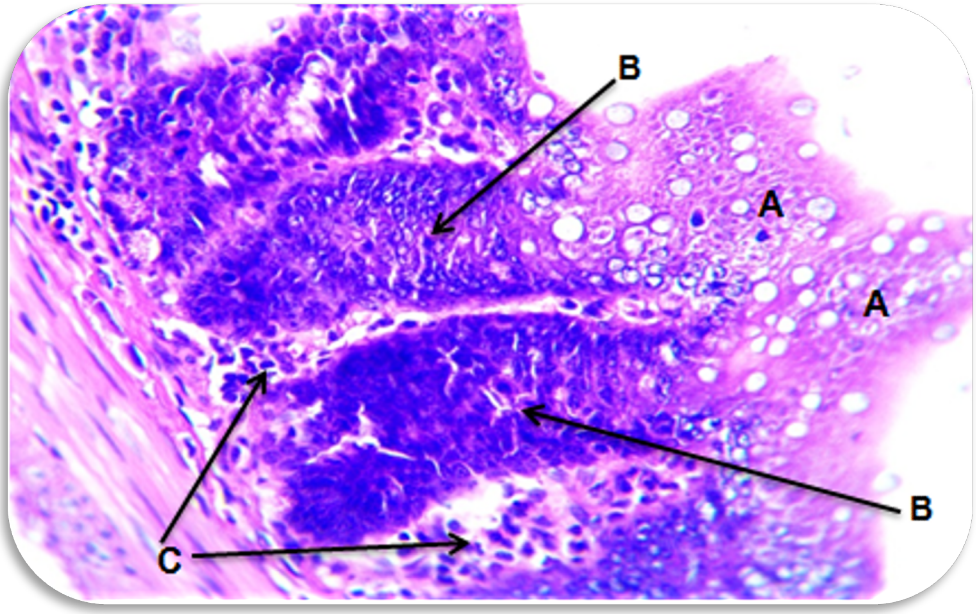


Figure (13): A section of the intestine of a mouse treated with the alcoholic extract of the *Aloe barbadensis* plant, showing the intestinal mucosa, in which there is hypertrophy of the mucus-secreting intestinal gland cells (A), inflammatory edema (B), and infiltration of a number of white blood cells into the crescent tissue of the lamina propria (C) (400H&E×).



Figure(14): Section of intestine treated with the alcoholic extract of the *Aloe barbadensis* plant showing the surface of the intestinal mucosa, in which there is an accumulation of many mucous droplets (A), hyperplasia of yeast-secreting cells (B) in the intestinal glands, and infiltration of white blood cells between the glands (C) (400H&E×)

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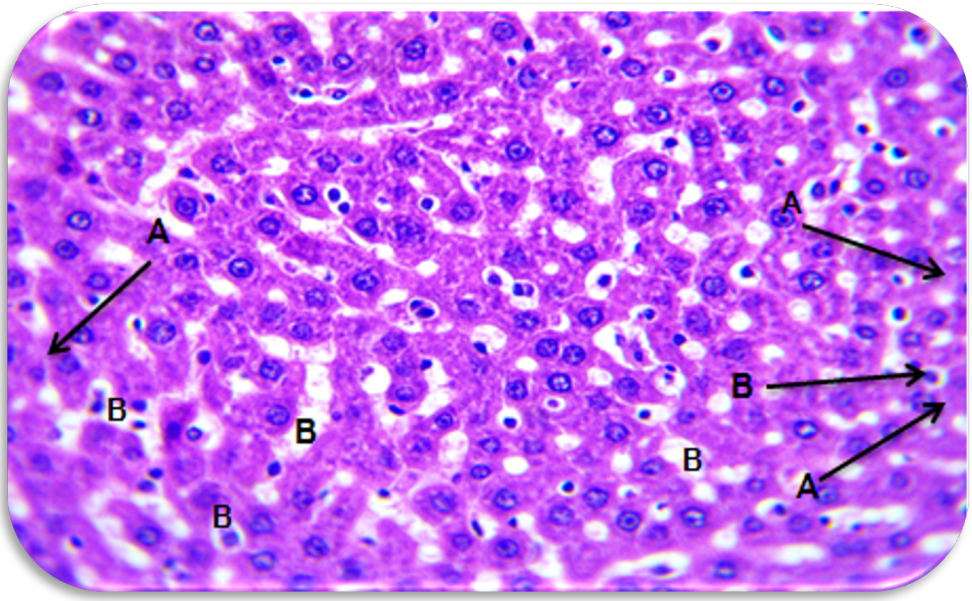
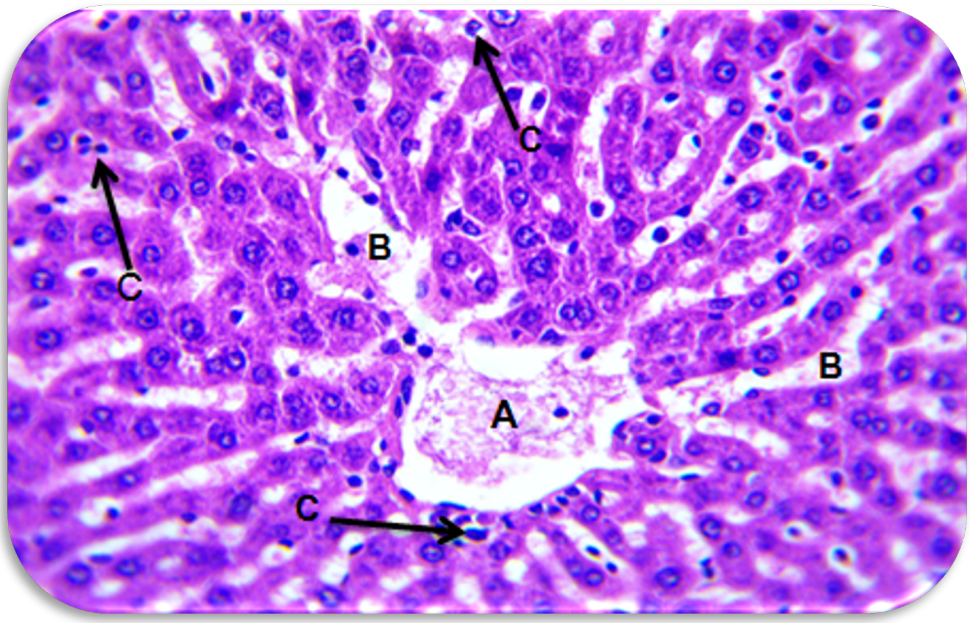


Figure (15): A section of a mouse liver treated with the alcoholic extract of the *Aloe barbadensis* plant showing the liver tissue, rows of hepatocytes in the form of a honeycomb (A) surrounded by a network of blood sinuses and containing hypertrophic kupffer cells (B) (400H&E×).



Figure(16): Section of a mouse liver treated with the alcoholic extract of the *Aloe barbadensis* plant showing the wide-lubricated central vein with a fibrous thrombus (A), blood sinuses connected to the central vein (B), and kupffer cells (C) (400H&E×).

4. CONCLUSION

Adding a concentration of 0.02 mg/kg of the aqueous and alcoholic extract of the ***Aloe barbadensis*** plant had a positive effect in reducing the number of parasite cysts excreted with feces in mice and repairing damaged tissues compared to the positive (infected) control group, which indicates the efficiency of the aqueous and alcoholic extract in repairing damaged tissues.

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