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HISTOLOGICAL STUDY OF EXTRACTION OF LIPOPEPTIDE BIOSURFACTANT FROM *BACILLUS SUBTILIS* IN OIL SPILLED SOIL ON WHITE RATS TISSUE

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ABSTRACT

Thirty bacterial isolates were obtained from 20 oil contaminated soil samples from various automobile work shops, cars fuel stations, of three different areas in Al-Najaf city. About 18 isolate suspected to belong to genus *Bacillus*. All these isolates were subjected to microscopic examination and cultural characteristics, about 12 isolate belonged to genus *Bacillus subtilis*. 2 isolate were selected for the purpose of knowing their ability to produce biosurfactant. All these isolates were screened for their ability for biosurfactant production through blood haemolysis activity on blood agar indicated that all isolates were β -haemolysis, the safety of the extract was studied on white rats tissues including liver, kidney, spleen and intestine. The results showed no damage to tissue compared with control treatment.

KEYWORDS: *Bacillus subtilis*, Biosurfactant, Albino Rats.

INTRODUCTION

Naturally surface-active compounds derived from microorganisms are named biosurfactants.These are amphiphilic biological compounds produced from many bacteria, filamentousfungi and yeast of the cell membrane or extra-cellularly[1] The major features of surfactants is bility to decrease the surface tension. Biosurfactants were performed many purposes such as polymers, lubricants and solvents, in detergents shampoo toothpaste, oil additives, and in many purchaser and industrial merchandises. The production of surfactant over-all has exceedede ″2.5 million tons in 2002″.[2] Biosurfactants are cover chemical parts such as fatty acids, phospholipids, glycolipids, antibiotics, lipopeptides and peptides. Biosurfactants lead to an increasing interest on these microbial products as alternatives to chemical surfactants.[3] Many reports on the production of surfactants by using microorganisms and water-soluble compounds such as ethanol, glucose, glycerol and sucrose as substrates.[4]

Aims of study

1. Isolation of *Bacillus* spp. from different localities contaminated with hydrocarbons.
2. Morphological and Biochemical identification of *B.subtilis*.
3. Production of biosurfactant from efficient isolates that screening for their ability to produce biosurfactant.
4. Study the histological changes of crude extract of biosurfactant against rats tissue.

MATERALS AND METHODS

Culture media

The following media were used in this study

- Nutrient agar and Nutrient broth.
- Simmon's citrate agar.
- Motility test agar.
- Blood agar.
- Brain heart infusion broth.

Reagents and Solutions

1. Catalase Test Reagent

Use aconcentration of H_2O_2 (3%), prepared by dissolving 3 ml (H_2O_2) in 97 ml D.W. to prove thr bacter is ability to the ability of bacteria to secrete the catalase enzyme.[5]

2. Oxidase Test Reagent

Attended by dissolving 1 g of material ″Tetra-Methyl-P-phenylene diamine-dihydrochloride″, in D.W., then put in a bottle and kepping in at 4ºC (for a maximum of one week) to find out the capacity of bacteria to secrete the enzyme oxidase.

3. Gram Stain Solution

This stain was consist of the following solutions prepared by[6]

- Crystal Violet
- Sufranin
- Iodin
- Alcohol

4. Labrotary animals

Male albino rats used in this study,age between 12-15 week weights ranged between (200-250g), appropriate conditions have been created in terms of nutrition, ventilation and suitable lighting, the animals were housed at 22±3ºC temperature and left to acclimatize for 1-2 week before the experiments.[7]

5. Soil samples collection

Thirty soil samples were collected from different locations of Kufa and Najaf governorates. Samples were taken from the auto-mobile, work-shop, oil spilled area of the, below the surface of contaminated soils of machine, hydrocarbons soil beneath cars fuel stations, oil refineries and other contaminated sites from September to October 2018.

6. Isolation of Bacteria

Isolation of *Bacillus* spp. was performed by by adding (1) gm from each sample of soil to[9] ml of sterile D.W., mixed well and then heated in awater bath at 80ºC for 20 min, with gentle agitation. This high temperature result in total of vegetative micro-organisms in the sample of soil died and spore of *bacillus* stayed the bacillus spores, then left to cool at room temperature, after cooling, (0.1) ml of soil sample solutions was taken from each sample and spreaded on a nutrient agar medium place followed by aerobically incubated to 24 hrs. at ″30°C″. After incubation, colonies appeared with different sizes and shapes were selected for identification.[8]

7. Identification of bacterial isolates

Diagnosed bacterial isolates depending on the culture, microscopic, biochemical and molecular characteristics are as follows:

1. Morphological and cultural characteristics

The shape, size, color and edge and appearance of bacterial colonies were studied on nutrient agar plates after 24 hr of incubation.[9]

2. Microscopical examinations

- **Gram's Stain**

Single colony of each bacterial isolate was transferred to a clean slide and fixed by flame, the smear was stained with Gram stain to study its Gram reaction and spore formation under the light compound microscope.[10]

- **Endospore features**

Cultures were used for detection of endospores present, endospores position with in sporangium.[8]

3. Biochemical Tests

1. Catalase Test

Put the amount of bacterial culture 24 hr on aclean slide and then add one drops of H_2O_2 concentration (3%) on culture, formation of air bubbles is evidence of the positive result.[5]

2. Oxidase Test

Filter paper was saturated with the substrate (Tetra-Methyl-P-phenylene diamine - dihydrochloride), and directly add bacterial colony to be tested age 24 hr on the filter paper by asterile wooden stick, the color change to dark purple through[2-10] seconds indicated apositive examination.[11]

3. Citrate Utilization

This test was an indicater for utilization of citrate by bacteria as asole carbon source, in which aslant of simmon's citrate was inoculated with ayoung colonies and incubated at 30ºC for 3 days, the formation of deep blue color indicated on positive result.[12]

4. Blood haemolysis test

Blood agar medium was inoculated by bacterial culture using streaking methods, then place were put in an incubater at ″37 ºC for 24 hrs″, the appearance of adefinite clear zone (β-haemolysis) around the colonies indicated the concerned *Bacillus* colonies were selected.[13]

5. Motility Test

Prepared aserial of tubes containing motility test agar and then inoculated with bacterial culture by the needle which enter and cut through the agar, then tubes were incubated at 37ºC for 24 hrs. Your organism is non-motile if distribution in one place or direction, if the dispersal on the all media the organism is motile, in the other word the growth will often look fuzzy.

8. Screening of biosurfactant production by local isolates of *Bacillus* spp

1-Preparation of inoculums

Take single colony of each bacterial culture that was isolates and selected for biosurfactant production and put ″10 ml″ of nutrient broth media, then put in shaker incubator (180 rpm) for 18 hr.at 30ºC, after this10 µl of fresh culture was added to 10 ml of the nutrient broth which incubated to the mid logarithmic phase at the same conditions.

2-Biosurfactant production

A portion of 1ml of the inoculum preparation above was taken about 100 ml of it, in triplicate, then aerobically incubated in shaker incubater at 180 rpm at 30ºC for three periods (24, 48 and 72 hr.). Each culture was then centrifuged at 4ºC, 10.000 rpm. for 20 min, after centrifugation of broth, cell-free supernatant was taken which contains biosurfactant.

3-Qualitative screening

Qualitative screening for the ability of local isolates of *Bacillus* spp. of biosurfactants production were achieved by

4-Detection of hemolytic activity

Haemolytic activity was detected by streaking each local isolate on blood agar medium, after inoculated the plates was incubation to ″48 hrs. at 30ºC″, then dishes examined for the formation of clear zones surround the colonies bacteria.[14]

5-Estimation of biosurfactant dry weight

Cell-free supernatants of each isolate were treated to acid precipitation by adding drops of 6N HCl continuously until to reach to pH of 2,after this allowing the supernatants for precipitate over night at 4ºC. Precipitation was collected by centrifugation with10.000 rpm at 4ºC for 20min.,then pellets were resuspended by 2 ml of D.W. then adjusted to pH 7 with agitation in order

to complete dissolving and finally lyophilized by lyophilizer and weighed.
″Dry weight of biosurfactants =weight the plate after drying -weight of the empty plate″

9. Preparing of compounds solutions for testing
One mg of extract was dissolved in water,then prepared (100, 250 and 500 µg/ml) concentrations.

10. Sterality of Crude extract test
Sterility was determinated by culturing of crude extract on blood and nutrient agar.

11. Safety of crude extract Test
To detect safety injected the intraperitionally three rats with 1 ml of crude extract for each concentration that mentioned above for 1 week and monitoring clinical feature in rats.

12. Preparation of histological sections
The histological sections were prepared in laboratory histological sections in the faculty of science/biological department followed the method of Bancroft and Steven in 1982 by placing models of the first experiment in formation for 24 hours and then carried out the steps dehydration, clearing, infiltration, embedding, sectioning, staining and mounting.

RESULTS AND DISCUSSION

Isolation of biosurfactant – producing *Bacillus* species
For isolate *Bacillus* producing biosurfactant, 20 samples of heavily oil contaminated soil were collected from automobile work shop, cars fuel stations and surface beneath electrical machine of different area from which 30 bacteria were isolate. The obtained isolates were examined to microscopic examination with gram staining, morphological and cultural characteristics on nutrient agar according to[9], these results observed that 16 isolates were suspected to be *Bacillus subtilis*, high percentage of this genus were expected because this genus is ability to production of biosurfactants is well documented.

Identification of Biosurfactant producing *Bacillus* species
The collected isolates were identified depending on many tests as the following:

Morphological characteristics of the *Bacillus* species
Through laboratory tests for the growth of bacterial isolates on nutrient agar colonies most isolates appeared to be acircular, colony very in size but more are relatively large, smooth, opaque, with a round edge quickly turn to the edge of lobed with growth, less whitish or cream colored colony and these features match to culture character for colonies of genus *B.subtilis* that pointed by.[10,15]

Microscopic characteristics of *Bacillus* species
Microscopic examination of the results of the bacterial colonies isolates installed on slides and dyed with gram stain showed that *Bacillus* isolates they were gram positive, cells rods in shape (figure 1).

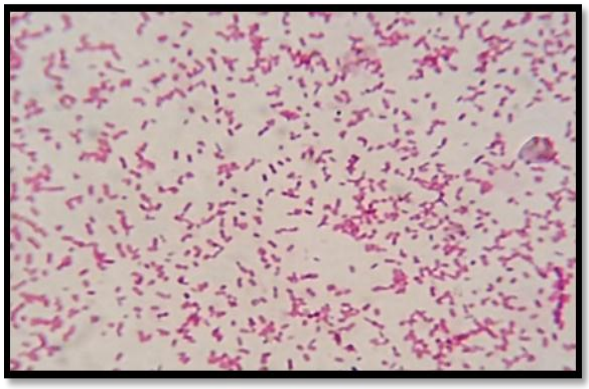


Figure (1): *Bacillus subtilis* cells under an optical microscope magnification X100 strongly after incubated 24 hours at 37 ºC.

Some occur singly,others arranged in pairs or chains, endospore formation, spores were ellipsoidal in shape, central in position, these results were in agreement with (5 and 9) on the microscopic forms of *B.subtilis* cells and the nature of regularity.

Biochemical Test of *Bacillus* species
The results of biochemical tests that recorded in (table1), were considered as a complementary of the initial identification of *Bacillus* isolates belonged to the species *B.subtilis*.

Table (1): Results of biochemical tests of *Bacillus subtilis.*

NO.	Biochemical Tests	Results
1	Catalase	+
2	Oxidase	variable
3	Haemolysis	β-haemolysis
4	Motility	+
5	Citrate Utilization	+

Screening of biosurfactant producing isolates
The first criteria for separation of biosurfctant was haemolysis. Haemolytic activity was regarded as indicator for production of surfactant, it is performed spead way for screening of bacteria,the results indicated that all 30 bacterial isolates were capable to haemolyse blood. The dishes lined by culture expound the ″β-haemolytic activity″. It is indicate to synthesis bio surfactant when produsedhemolysis. It lined with the studies of.[16] Which mentioned in their research that the blood haemolysis test is indicated for examining biosurfactant synthesis organisms.

Extraction of biosurfactants
All selected isolates of *B.subtilis* were grown separately in nutrient broth medium. The results showed (figure 2),

all isola[B]ave the lowest production of biosurfactant after 24 [illegible]s of incubation, followed by an increase, but at a low rate in production with increasing time resulting in proportional increase in the number of bacteria-producing biosurfactant in the broth medium and thus increase the bacterial consumption of carbon-source in the medium. The highest percentage of the production of the surface active agents after 72 hours of incubation in the medium. Also it was found that the highly produced biosurfactant reach to (0.025) g/l yield after 72 hours of incubation.

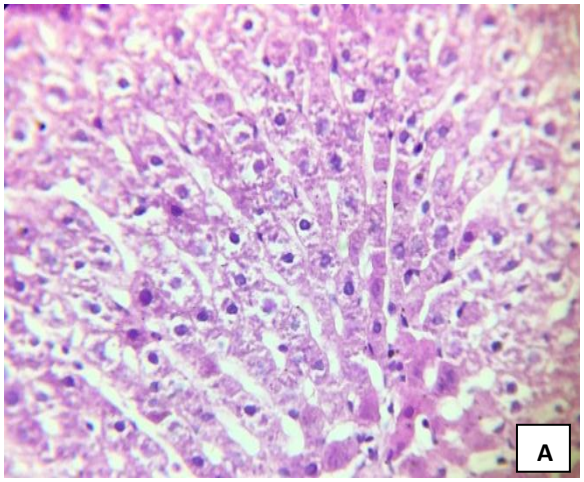
Figure (2): Biosurfactant Extraction before and after drying.

Sterality test of biosurfactant

The crude culturing on blood and nutrient agar then examind in plates after seven days The results showed no presence any growth of microorganism indicated sterality.

Safety of Crude Extract of biosurfactant

Rats injected with bacterial extract in the intraperitionally, and monitoring rats for 7 days to ensure the safety of extract used in the experiment. The results showed no pathological signs appear in the organ tissue of those rats when compared with a control treatment indicated to the safety of biosurfactant, figure (3),(4),(5) and (6).



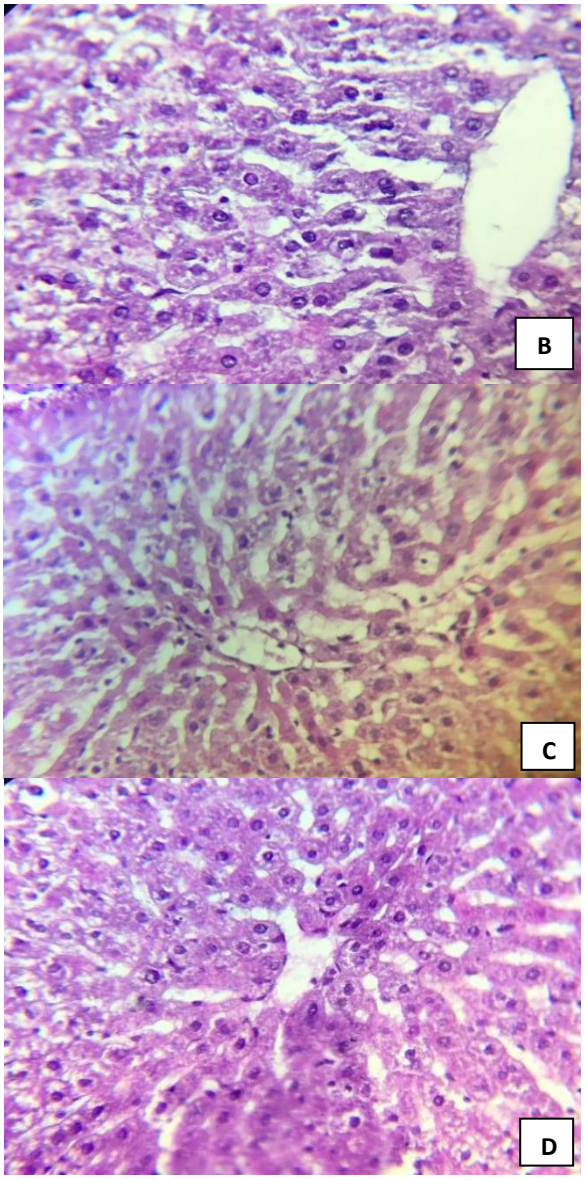
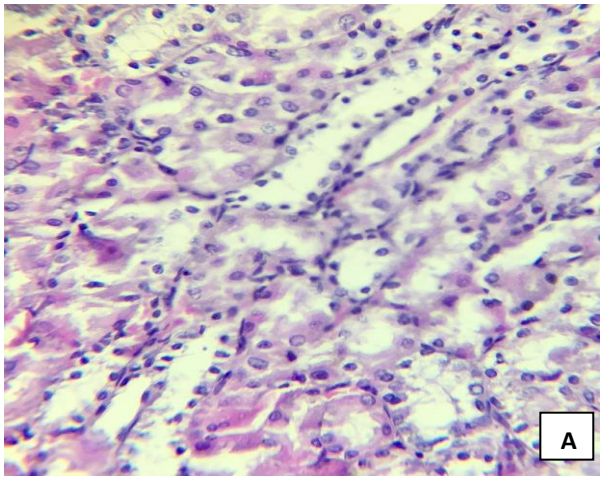


Figure (3): The histological sections of liver tissue in different concentration after 7 days, 100x magnification, A- control treatment, B-″100 mg/kg, C-250 mg/kg, D- 500 mg/kg.



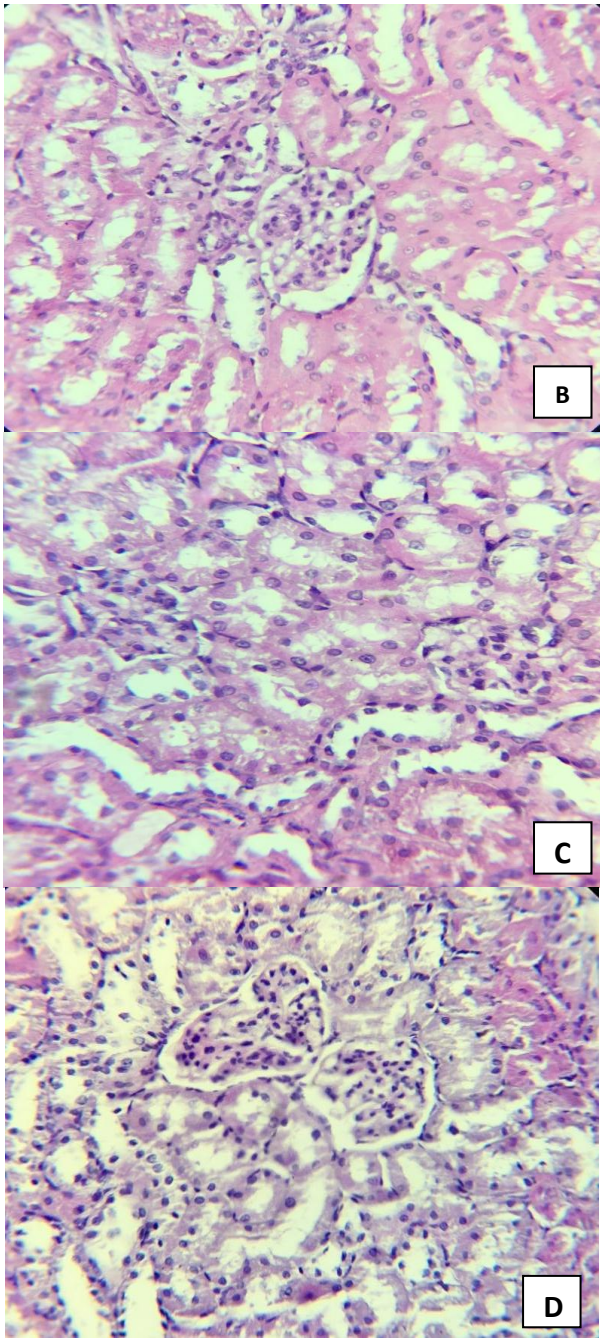


Figure (4): The histological sections of kidney cortex tissue in different concentration after 7 days, 100x magnification, A- control treatment, B-″100 mg/kg, C-250 mg/kg, D- 500 mg/kg″.

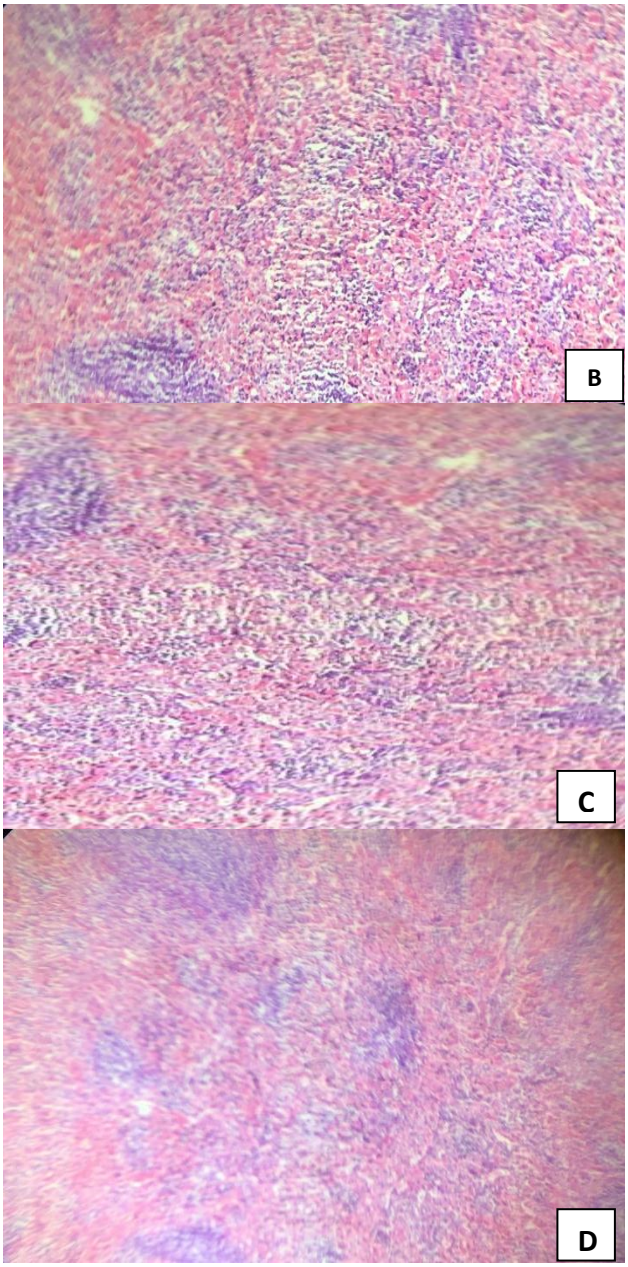
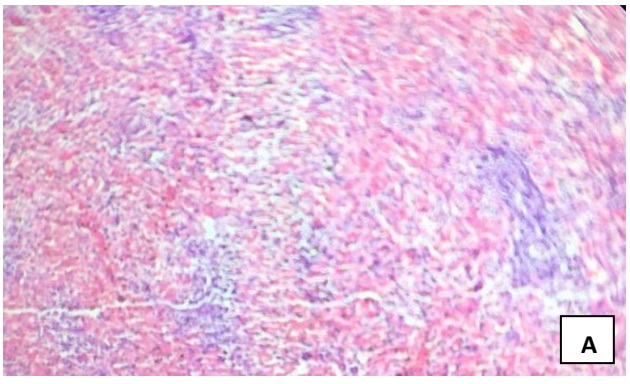
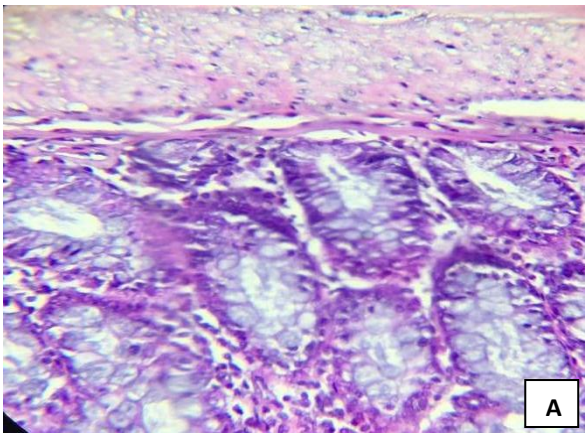


Figure (5): The histological sections of spleen tissue in different concentration after 7 days, 100x magnification, A- control treatment, B-″100 mg/kg, C-250 mg/kg, D-500mg/kg″.





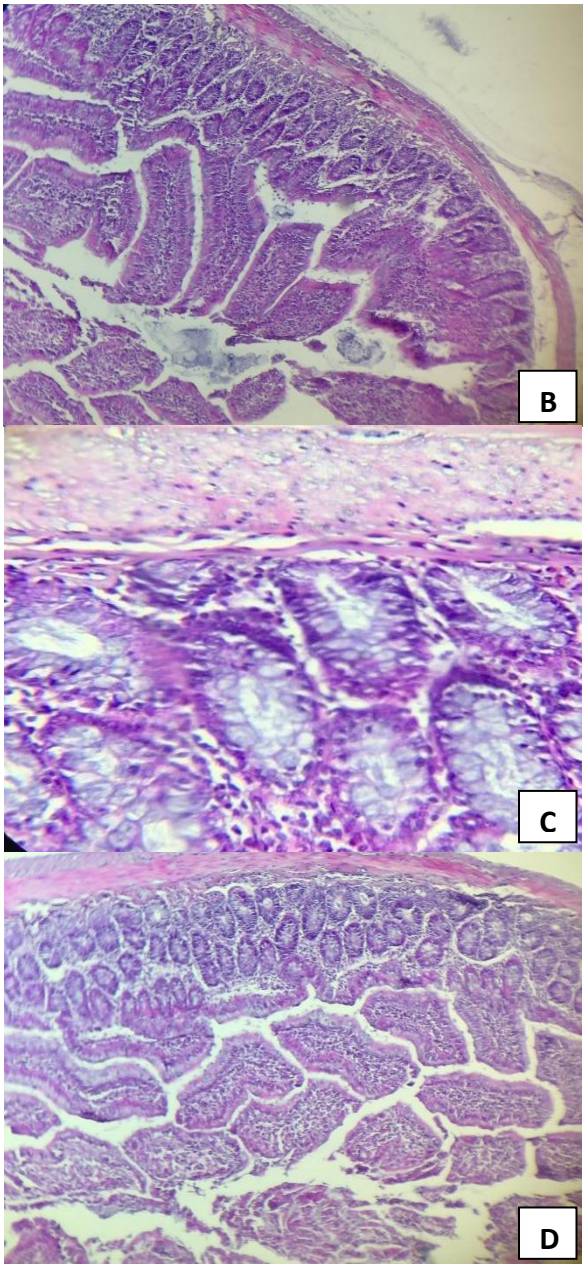


Figure (6): The histological section of duodenum tissue in different concentration after 7 days, 100 x magnification, A- control treatment, B-″100 mg/kg, C-250 mg/kg, D-500 mg/kg″.

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