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Biochemical and histological study of effect of black *Punica granatum* extracts on *Giardia lamblia* in mice

Shatha Khudair, Saad A. Al-Jashaami and Mustafa A.H. Ahmed*

Dep. of Biology, College of Science, Al-Mustansiryia University, Baghdad, Iraq
*Corresponding author: mustafaalzubaydi534@gmail.com

Abstract

The study aimed to introduce the effect of *Punica granatum* extracts to treatment *Giardia lamblia* infection in mice compared with metronidazole and some biochemical and histological changes. This study included the collected 100 feces samples and 120 Swedish mice. Best effect was full killed of flagyl to parasite on the 6th day, followed by an Water extract and oil extract full killed on the 7th day and less influence of alcohol extract full killed on the 8th day was compared with the control of positive. Total protein decrease in oil extract, Water extract, alcohol extract and flagyl the rate which were 58±1.2, 62.81±1.1, 72.5±2.9 and 54.24±1.9g/l respectively compared with Control negative which were 85.87± 0.8g/l and increase in all groups compared with control positive group the rate was 43.14± 0.9g/l. The level of glucose increasing in oil extract, Water extract, alcohol extract, flagyl and control negative groups which were 163.9±1.3, 152.8±1.8, 183.1±4.8, 125.6±0.3 and 154.6±0.8mg/dl respectively compared with control positive group which was 117.8±0.9mg/dl. The level of the enzymes (GOT and GPT) were high in all groups compared with a control negative. The cholesterol level in serum decreased in all groups compared with control negative. Triglycerides increased in all groups compared with control negative. Creatinine value increased in water extract compared with other groups.

Keywords: *Giardia lamblia*, *Punica granatum*, Metronidazole.

Introduction

Giardia lamblia parasite is a flagellated protozoan which lives in the small intestine of human and variety of mammals, amphibians and birds (Stuart *et al.,* 2003), *Giardia* has two life stage: environmentally resistant cysts and intestinal dwelling replication trophozoite (Faubert, 2000). Trophozoites can be seen in the duodenum and fasting in the stool in the case of diarrhea but frequently it's not found in the toughen stool which is not resistant to external environmental conditions on the opposing cysts that are more resistant and may remain in the water for three months at least, it is also the basis for the injuries of human where swallowing cysts lead to *Giardia lamblia* infection (Roberts and Janovy, 2000).Giardiasis is wide spread in the world where that affected people of all ages but most peoples that have this disease are children (Rajesh wari *et al.,* 1996), The infection happen through contamination hands with the cyst of *Giardia lamblia* in stool or through contact between infected and non-infected person (Peterson *et al.,* 1988), The specific mechanism of *Giardia* pathogenesis lead to diarrhea (Khudier, 2011), and causing symptoms that are associated with diarrhea including dehydration, vomiting , malabsorption and malnutrition (Botero-Garcès *et al.,* 2009).

Materials and Methods

Preparation of the cold water extract, cold alcohol extract and oil extract of *Punica granatum*: Used black *Punica granatum* imported from Iran and dried before use, water extract prepared according to (Ratheesh and Helen, 2007), alcohol extract prepared according to (Harbone, 1984). Oil prepared from the fresh black *Punica granatum* seed according to (Al-Kaisey *et al.,* 2002).

Stool collection: This study included the collected 100 feces sample in Fatema-El-Zahraa-hospita, Ibn Al-balady hospital and Al-Kadimiyah Teaching Hospital in Baghdad from children and patients, stool samples from each patients was collected in clean, dry, tight fit cover and then transferred to histology and genetics laboratory in the College of Science, University of Al-Mustansiriya for examination the samples to detect *Giardia lamblia*.

Direct stool smear examination: Feces samples was examined from people infected with parasite *Giardia lamblia* in accordance with the method of direct wet film preparation using normal saline and lugol's iodine (Cheesbrough and McArthur, 1976).

Purification of parasite: Method was used to isolate the parasite then (Cysts and Trophozoites) were suspended in phosphate buffer saline (PBS-7.2) and the final concentration was attended by rate 1×10^3 cell/0.1 ml (Robert-Thompson *et al.,* 1976).

Preparation of laboratory animals: Using (120) mouse from white Swiss mice (Males and Females) were obtained from National Center for Research and Drug Control and an average age of between 5-12 weeks and weight 16-22gm and provided sterile water for drinking by special bottles with the provision of temperature and proper ventilation and put a private room at home, feces of mice were examined before the start of the experiment examined stool to ensure that they are free from infection, and by placing a small amount of stool on a glass slide and mixed with a little iodine and then covered with a cover slide was examined under a microscope.

Experiment design: 120 mice given (1×10^3) cell/0.1 ml after 48hrs the stool of all mice examined and after sure infected by the parasite divided into 6 groups each group contain 20 mice then inoculated as follows:

Group 1: 0.1ml from metronidazole orally at a single dose per day (Savas-Erdevc *et al.,* 2000).

Group 2: 0.1ml from water extract of *Punica granatum* in concentration of 29.314mg /0.1ml /mice once day.

Group 3: 0.1ml from alcohol extract of *Punica granatum* in concentration of 41.786mg/0.1ml /mice once day.

Group 4: 0.1ml from oil extract of *Punica granatum* (Mahmoud *et al.,* 2002).

Group 5: 0.1 ml from normal saline and consider as control positive.

Then the last group leave without infection and consider as control negative and given 0.1 ml of normal saline.

Enumeration of *Giardia lamblia*: (cysts and trophozoite) in faces were enumerated as (Shukla *et al.,* 2008) briefly mice feces were collected first four groups daily from each mouse, one gram of fecal sample was dissolved in 10 ml of normal saline, homogenized then counted every day by suing hemocytometer.

Biochemical test: The all following tests were performed by using biochemical instrument (Roch Cobas c 111) autoanalyzer:

Measurement of (Total Protein, Glucose, Glutamate–Pyruvate Transaminase (GPT), Glutamate–Oxaloacetate Transaminase (GOT), Cholesterol, Creatinine, Triglyceride): All tests was measured in serum according to method of kit.

Histological examination: The mice were killed and extracted the organs (small intestine and liver) and fixed in 10% buffered formalin processed stained with hematoxylin and eosin for study histopathological changes.

Statistical analysis: Data are reported as mean± standard deviation and the inter group variation performed by t-test (SAS, 2012).

Results and Discussion

Enumeration of parasite: Table (1) showed the number of cysts raised in mice during different time periods in accordance with the duration of treatment compared with the positive control group.

In this study the use of oil extracted in the treatment of *Giardia lamblia* is the first in Iraq and the world. The results of present study in Table (1) show that therapeutic substances caused all effective in the treatment, but they differed in the length of time needed to bring about the complete treatment and ending injury. We conclude from the above table that the best effect was full killed of flagyl to parasite on the sixth day, followed by an Water extract and oil extract full killed on the seventh day and less influence of alcohol extract full killed on the eighth day was compared with the control of positive. Cysts continue to decline until it reaches zero on the sixth day, and the seventh and eighth day after treatment with flagyl, water and oil extract, and alcoholic extract of pomegranate, respectively. The results were compatible with the current reported Al-Samarrai (2006) where the drug metronidazole took less time (to treat *Giardia* in mice) than in the fungi *Agaricus bisporus*, Where took 10 and 14 days, respectively. The present study results coincided with the results of (Al-Kubaisi, 2007) in some aspects, noting that the period it takes to cure mice infected with *Giardia lamblia* treated with drug metronidazole was 5days.The present study results did not correspond to the study done by (Al-Kubaisi, 2007) of where the warm aqueous extract for pomegranate fruit peels give the therapeutic efficiency of 100% at the 3day after treatment, took less time than the drug to treat the infection with *Giardia*. While the present study coincides with the study of Sanad and Al-Ghabban (2011) in the fact that the drug metronidazole more efficient than aqueous extract of garlic which give the efficiency of the amount of 70% after 10 days of treatment. May be it attributes the

difference in the length of time it takes to treat the infection with *Giardia lamblia* treated by metronidazole to the existence of different strains of the parasite and thus differing resist these strains to treatment by drug metronidazole.

Rossignol (2010) has pointed to the evolution of resistance some strains of the parasite *Giardia lamblia* against the drug metronidazole. Also Gardner and Hill (2001) point out that the genetic variation between *Giardia* parasite strains might reflect on parasite resistance and virulence. The drug-resistant parasite is produced from the parasite's ability to reduce the drug into force across membrane , or may be produce from its ability to bring complex changes of genes responsible for encryption parasite membrane proteins (Buret, 2005). But in general, remain mechanical resistance the parasite to drug metronidazole unclear and incomprehensible (Upcroft *et al.,* 2006; Al-Masoudi, 2001) has indicated that the substance tannin in pomegranate are responsible for primarily for the pharmaceutical qualities of pomegranates.

Table (1): The number of cysts raised in mice during different time periods

Groups	Days								LSD value
	Mean ± SD								
	1	2	3	4	5	6	7	8	
Control +ve	38.60 ±0.37 a *	40.10 ±0.41 a*	41.80 ±0.35 a*	43.80 ±0.32 a*	46.00 ±0.29 a*	46.60 ±0.37 a*	47.10 ±0.23 a*	48.00 ±0.00 a*	3.19 *
Flagyl	36.90 ±0.60 a*	25.80 ±1.38 b**	14.50 ±1.26 c **	7.10 ±0.67 d**	2.40 ±0.31 d**	0.00 ±0.00	0.00 ±0.00	0.00 ±0.00	5.07 *
Water extract	37.60 ±0.34 a*	29.30 ±0.57 b**	20.90 ±0.37 c***	12.60 ±0.60 d**	5.70 ±0.36 e**	2.40 ±0.16 e**	0.00 ±0.00	0.00 ±0.00	5.63 *
Alcohol extract	39.70 ±0.70 a*	34.90 ±0.37 a**	28.80 ±0.42 b****	22.30 ±0.52 c***	14.40 ±0.54 d***	8.40 ±0.54 e**	3.10 ±0.34 e**	0.00 ±0.00	5.49 *
Oil extract	39.90 ±0.43 a*	34.70 ±0.30 a**	25.90 ±0.37 b****	16.60 ±0.47 c***	9.10 ±0.31 d***	3.90 ±0.31 d**	0.00 ±0.00	0.00 ±0.00	6.71 *
LSD value	4.022NS	5.619 *	4.985 *	5.722 *	7.413 *	6.314 *	5.883 *	5.256 *	---

* (P<0.05).

LSD was calculated at equal or less than 0.05 LSD FOR DAY NUMBERS: raws Expressed as (a, b ,c ,d ,e) LSD FOR TREATMENT columns expressed as (*,**,***,****).

Level of total protein in the serum: The Total protein results in the blood serum of infected children with *Giardia lamblia*, and Total protein level in the table (2) shown a marked decrease where it was 43.14 g/L in infected mice compared with Control/- ve group 85.87 g/L, this is agreement with the study of (Sencer *et al.,* 2004). This study agreement with the study of (Mallakh, 2015). Reported that value of total protein are compared to control/-ve, the relationship is significant statistically this means that, although *Giardia lamblia* lacks synthesis of amino acids and depends on scavenging them from the intestinal milieu in which trophozoite replicates, and it has been found that kinetics of amino sugar phosphate synthesis in encysting *Giardia lamblia* favors the path that supports synthesis of cyst wall (Adel *et al.,* 2001).

Table (2): compare between different groups in Total protein

Groups	Total protein g/l (Mean ± SD)
Control/- ve	85.87 ± 0.8 a
Alcohol extract	72.5 ± 2.9 b
Water extract	62.81 ± 1.1 c
Oil extract	58 ± 1.2 d
Flagyl	54.24 ± 1.9 d
Control/+ ve	43.14±0.9 e
LSD	6.35

Similar letters mean the absence of significant differences and different letters mean the presence of significant differences at the level of probability (P<0.01).

Level of Glucose in the serum: The level of glucose in the blood serum of infected mice and described in the table (3) shown decrease where it was

117.8mg/dl in infected mice compared with Control/- ve group 154.6mg/dl, The decrease is due that glucose is an necessary substance for growth of *Giardia lamblia* trophozoites. Experimentally found when glucose concentration is reduced the replication rate of *Giardia lamblia* is reduced (Schofield *et al.,* 2002). The result of Glucose agreement with the study of (Mallakh, 2015) on mice infected with *Giardia lamblia*.

Table (3): compare between different group in glucose

Groups	Glucose mg/dl Mean ± SD
Alcohol extract	183.1 ± 4.8a
Oil extract	163.9 ± 1.3b
Control/-ve	154.6 ± 0.8c
Water extract	152.8 ± 1.8c
Flagyl	125.6 ± 0.3d
Control/+ve	117.8 ± 0.9e
LSD	9.3

Similar letters mean the absence of significant differences and different letters mean the presence of significant differences at the level of probability (P<0. 01).

Level of Glutamate–Oxaloacetate Transaminase (GOT) and Level of Glutamate–Oxaloacetate Pyruvate Transaminase (GPT) in the serum: There is a relationship between the occurrence of diarrhea with the level of (GOT and GPT), the reason is due to diarrhea leads to necrosis and damage of some the liver cells leading to the permeability membranes of these cells and then these enzymes are leak to circulation and its levels will raisein the blood (Doudarka and Kruft,1995). This raise may be alter the metabolic functions of the liver during a stage of diarrhea resulting from inflammation of intestinal that leading to malabsorption and malnutrition, which leads to disorder in the function of liver (Behrman *et al.,* 1992). The result of GPT and GOT show in Table (4) agreed with the study done by (Abbass,2012) on patients infected with *Giardia lamblia* in Al-Kadimiyah Teaching Hospital in Iraq.

Levels of cholesterol and triglycerides in serum: The result of Cholesterol Agreement with that reported by Bansal *et al.* (2005), Ma'ani and Dhuha (2013). The result shows that the cholesterol levels were decreased in infected mice because *Giardia lamblia* consumed the cholesterol of the host in the biosynthesis of the cell, because the parasite is unable to synthesize cholesterol by itself. The association of steatorrhea with the infection of *Giardia lamblia* may be observed on the basis of damaging the intestinal mucosa, causing functional derangements , reducing brush border enzymes (Friedewald *et al.,* 1972). Recent studies show that Cysteine rich proteins (CRPs) which is produced by *Giardia lamblia* can bind to heavy metals like zinc in the small intestine and as this binding inhibit the enzymes of small intestine (Field *et al.,* 1990). Thus prevent lipids metabolism .*Giardia* may consumed only cholesterol and neglect the other lipids (Hague *et al.,* 2007) who showed that cholesterol starvation consider a trigger for trophozoite differentiation into cyst. *Giardia lamblia* trophozoites may inhibit lipolysis (the process of lipids degradation) and the degree of inhibition increased with longer duration of lipase exposure to trophozoites (Field *et al*., 1990), this result agree with the study done by (Thomson *et al.,* 1993). Increase in Triglycerides may be associated with risk of coronary heart diseases (Hussein *et al.,* 2013). The results of Triglycerides obtained in this test agreed with what it says (Abdrabo *et al.,* 2014) were noted increase in level of Triglycerides in infected school children compared with healthy children.

Level of creatinine in the serum: A molecule of creatinine plays a role as a store of energy in muscles and floates kidney glomerules (Kaddurah-Daouk and Wyss.2000). The result is within normal range for its concentration in serum and this was agreement with (Abraham *et al.,*2005), Creatinine is an test of indicative signs to check function of kidney because its metabolic products are ineffective which exist through the kidney outside, and any rise in the concentration of creatinine in serum on natural border reflects the functional status of the kidney (Cohen *et al.,* 2000).

Histological study of the small intestines: Histological results in Control/-ve showing the normal structural appearance which consist the intestinal villi as in Figure (1) and showing with prominent goblet cells and its mucine secretion as in Figure (2), Histological results for infected animals with *Giardia* control+ve showing degeneration and shortening of intestinal villi as in Figure (3), while the results of treated by Water extract showing slight degree of still shortness of intestinal villi as in Figure (4) and showing inflammatory cells inside the villi as in Figure (5).

Table (4): compare between different groups in the GPT and GOT

Group	Concentration rate of GPT u/l Mean ± SD	Concentration rate of GOT u/l Mean ± SD
Water extract	73.16±4.6a	413.11±0.6a
Alcohol extract	35.11±4.5c	187.08±0.6c
Oil extract	34.83±6.5c	117.17±0.5e
Flagyl	42.11±0.8b	160.29±0.2d
Control/+ ve	42.43±1.1b	262.11±0.7b
Control/- ve	25.01±0.6d	108.97±0.3e
LSD	9.85	8.36

Similar letters mean the absence of significant differences and different letters mean presence of significant differences at the level of probability (P<0.01).

Table (5): compare between different groups in the Cholesterol and Triglycerides.

Group	Concentration rate of cholesterol mg/dl Mean ± SD	Concentration rate of triglycerides mg/dl Mean ± SD
Water extract	87.49±3.3d	84.74±0.5d
Alcohol extract	135.11±1.2b	138.41±1.1b
Oil extract	102.41±0.6c	196.74±0.6a
Flagyl	102±0.6c	112.94±0.5c
Control/+ ve	107±22.3c	83.32±1.2d
Control/- ve	159.52±16a	75.2±0.4e
LSD	9.25	6.4

Similar letters mean the absence of significant differences and different letters mean the presence of significant differences at the level of probability (P<0. 01).

Table (6): compare between different groups in the Creatinine

Group	Concentration rate of Creatinine mg/dl Mean ± SD
Water extract	0.285±0.04a
Alcohol extract	0.2±0 b
Oil extract	0.2±0 b
Flagyl	0.2±0 b
Control/+ ve	0.2±0 b
Control/- ve	0.2±0.03b
LSD	0.01

Similar letters mean the absence of significant differences and different letters mean presence of significant differences at the level of probability (P<0.01).

Histologicial result in infected mice treated by Alcohol extract showing the histological structure look-like near the normal as the length of intestinal villi and goblet cells presence as in figure(6),treated by Oil extract showing shortening some intestinal villi and other like normal as in figure (7) .The study the effectiveness of Flagyl shows a decrease in the number of *Giardia lamblia* when examine the stool microscopically in the fifth day of giving the drug, microscopic examination of tissue section showing return some intestinal villi into looks-like normal while other area still these was shortening and become flat surface structure as in figure (8). The presence of *Giardia lamblia* in the intestines causes inflammation cellular infiltration who appeared in the present study and penetration of trophozoites for lining of intestinal where the parasite led to mucus tissue decomposition and then penetrate it leading to damage the tissue. This inturn leads to prevalence inflammatory cells and as a result scratch of the parasite goblet cells stimulated mucus secretion, and through penetration process the *Giardia* parasite kill and consume immune cells and epithelial cells (William and Sodeman, 2000).

Figure (1): A Section of small intestine control – ve showing the normal structural appearance which consist the intestinal villi. (H&E) (200X).

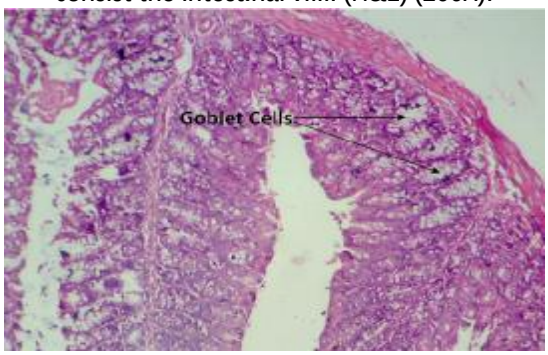

Figure (2): A Section of Small intestine control – ve showing the normal structural appearance with prominent goblet cells and its mucine secretion .(H&E) (400X).

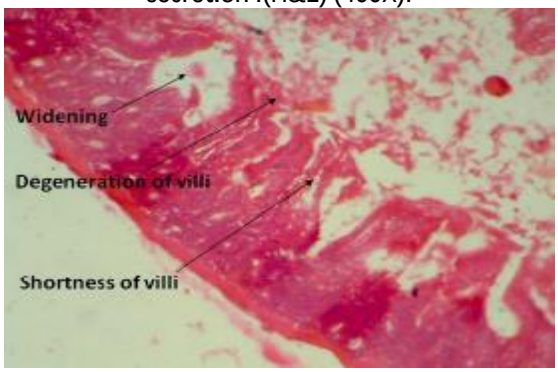

Figure (3): A Section of small intestine control + ve showing degeneration and shortening of intestinal villi. (H&E) (400X).

Figure (4): A Section of Small intestine treated by Water extract showing slight degree of still shortness of intestinal villi. (H&E) (200X).

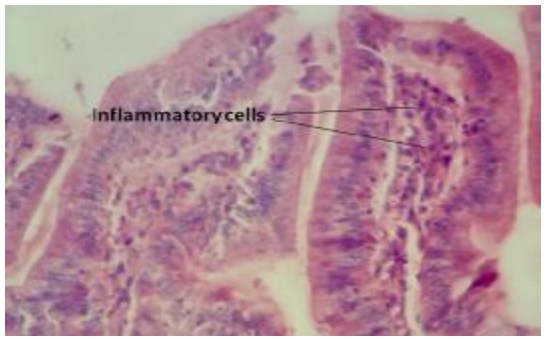

Figure (5): A Section of Small intestine treated by Water extract showing inflammatory cells inside the villi. (H&E) (400X).

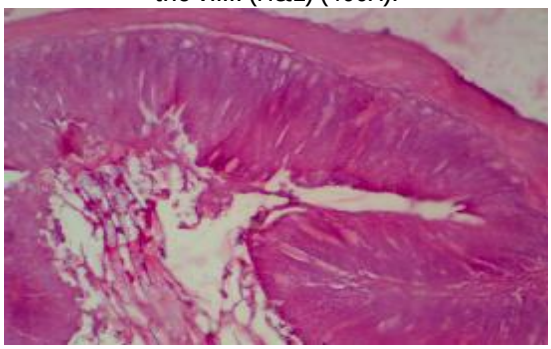

Figure (6): A Section of Small intestine treated by Alcohol extract showing near A normal as the length of intestinal villi and goblet cells presence. (H&E) (400X).

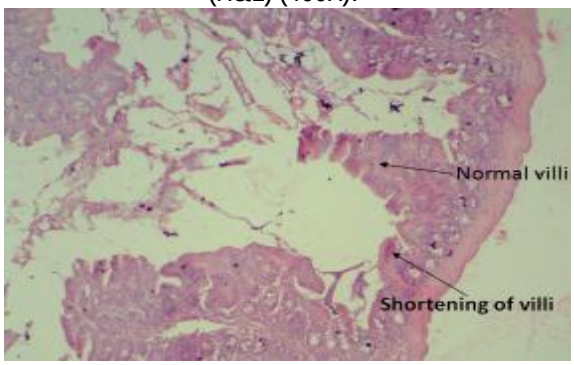

Figure (7): A Section of Small intestine treated by Oil extract showing shortening some intestinal villi and other like normal. (H&E) (200X).

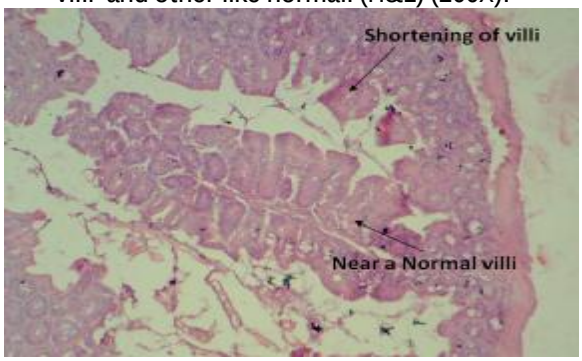

Figure (8): A section of small intestine treated by Flagyl showing return some intestinal villi into looks-like normal while other area still shortening and become flat surface structure. (H&E) (400X).

Histological study of the liver: Histological section of the liver for infected animals in figure (10) showed Clear changes when compared with liver tissue for animal is uninfected in figure (9) , as was observed presence of beginning necrosis in some of its parts with the infiltration of monocyte inflammatory cells in figure (10) and after taking treatment (Flagyl) appear bloody congestion with simple necrosis in liver cells in figure (14) and observed appeared acute necrosis of the liver cells with accumulate of lipids and also observed infiltration of inflammatory cells near the pylori area with hyperplasia in figure (11),(12) and (13)These changes were close to the changes reached by (Jumah ,2000), where the infection lead to hyperplasia for Kupfer cells as a result of the presence of the parasite inside its and also the incidence of infiltration in monocyte inflammatory cells as a immune response to the liver tissue (Salata *et al.,*1989). Collect these lymphatic cells around Kupffer cells lead to form granular tumors (Mc-Elrath *et al*., 1988). Also observed fatty drops inside liver cells, that resulted from the disruption in liver function, altered from building of lipoproteins which led to accumulate of lipids within cells(Hallberg *et al.,*1984).

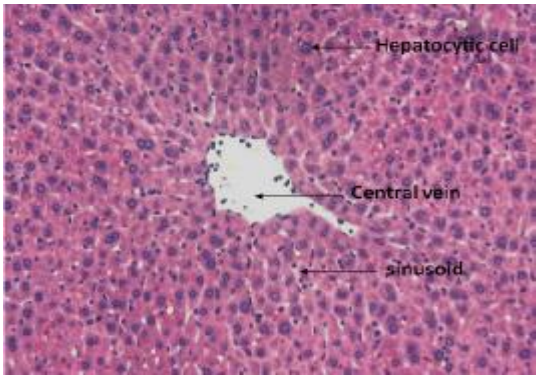

Figure (9): A Section of liver control –ve showing the normal structure which consisting central vein surrounding by hepatocytic cells. (H&E) (200X).

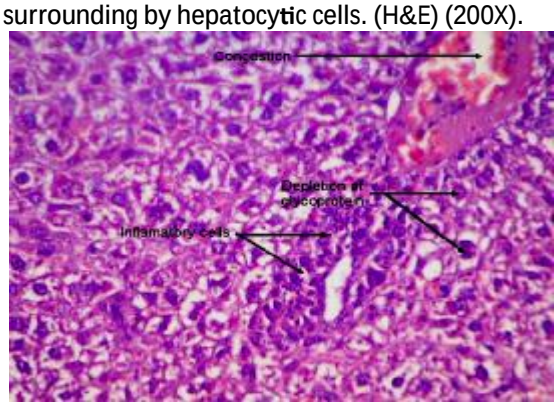

Figure (10): A Section of liver control + ve showing congestion with foci of necrotic hepatocyte and mild inflammatory cells infiltration around portal area, and slight depletion of glycoprotein inside the hepatocyte cells. (H&E) (200X).

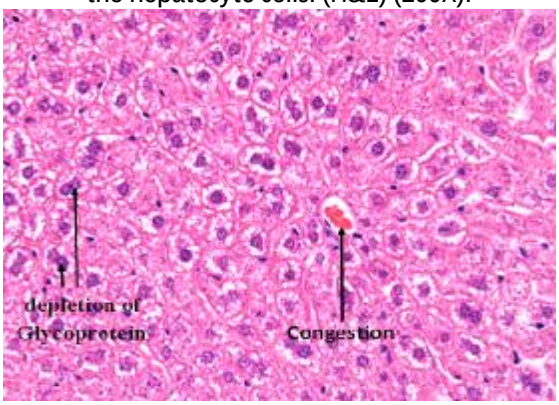

Figure (11): A section of liver treated by Water extract showing slight congestion and depletion of Glycoprotein granules with few apoptotic cells. (H&E) (200X).

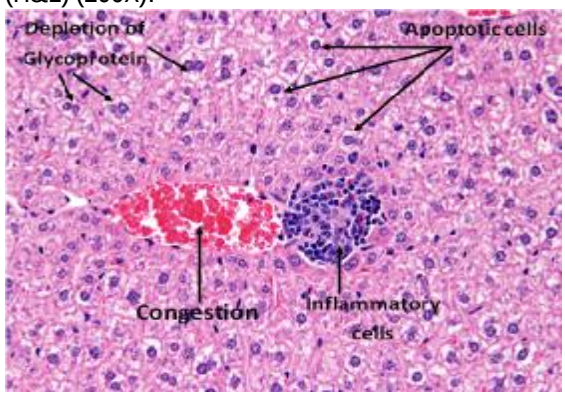

Figure (12) A Section of liver treated by Alcohol extract showing presence congestion and inflammatory cells infiltration and apoptotic cells and depletion of glycoprotein and number of kupffer cells.(H&E)(200X).

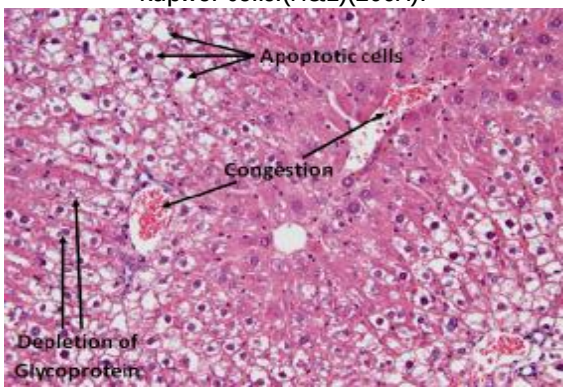

Figure (13) A section of the liver treated by oil extract showing mild congestion, apoptotic cells, depletion of glycoprotein granules.(H&E) (200X).

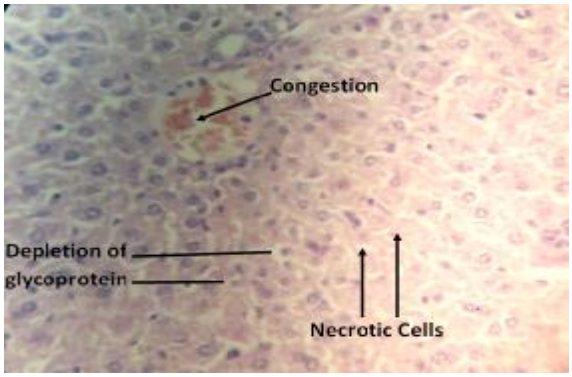

Figure (14) A Section of the liver treated by flagyl and showing congestion with simple necrotic hepatocyte cells, depletion of glycoprotein .(H&E) (200X).

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