Effect of saffron on liver development in mouse embryo

AmalAyedh and Fatma Al-Qudsi

Biology Department, Science Faculty, King AbdulAziz University, Jeddah, Saudi Arabia
P.O. Box 42650, Jeddah 21551, Saudi Arabia
falqudsi@kau.edu.sa

Abstract: Saffron is internationally used as a food additive it is also used as an herbal medicine. The objective of this study was to assess the effect of everyday used doses of aqueous saffron extract on liver development in mice embryos. Forty five pregnant Swiss white Rodeless mice were divided into three groups. Group 1 received 10ml/kg body weight double distilled wateras control, group 2 received a high dose of aqueous saffron extract 100 mg saffron / kg body weight and group 3 received a low dose of aqueous saffron extract 2.5 mg saffron / kg body weight. Doses were administered orally for 5 days during the first and second weeks of gestation and for four days during the third week of gestation. Embryos were extracted on day 14, 18 of gestation and day 1 neonates. LPO, GSH and total protein content of liver at day 18 of gestation were measured. Histological structure of embryonic liver was studied. Significant decrease in LPO, significant increase in the GSH and a non-significant decrease in the total protein content of 18 day livers of embryos of treated mothers was seen compared to the control group. On the histological and ultra-structural level many deterioration were seen in the liver of embryos of treated mothers such as dilation and congestion of central veins and portal veins. It was concluded that oral administration of both doses of saffron caused deterioration of embryonic liver tissue.
[AmalAyedh andFatma Al-Qudsi. **Effect of saffron on liver development in mouse embryo.** *Life Sci J* 2013;10(1):1480-1495] (ISSN:1097-8135). http://www.lifesciencesite.com. 219

Key words: Saffron, mouse embryo liver development, liver histology, apoptosis, hepatocytes.

1.Introduction

Saffron (*Crocus sativus* L. stigma) has been used since ancient times (Deo, 2003). Saffron is very usable in kitchen because of its color, taste and aroma. It is used for garnishing and coloring all kinds of rice and chicken, soup, seafood, desserts, drinks and home-baked sweets. In Arab countries, it is popular to make special tea and coffee with saffron. In India, a dish called Biryani is prepared with Saffron. In Italy and Switzerland, it is common to use saffron to cook rice. Cake is made with saffron in Germany and England, as well as many foods that are made with saffron from Spanish paella to French bouillabaisse **(Abdullaev 1993, 2002;** Negbi **1999; McGee 2004; Dharmananda 2005; Al-Mofleh*et al*. 2006;** Çavusoglu, *et al.,* 2009; Verma and Middha 2010).

Saffron hasbeen used in folklore medicine,as well as in modern pharmacy. Saffron has been reputed to beuseful in the treatment of numerous human illnesses **(Duke 1987**;**Zargari 1993**; **Abdullaev 2002).** It can also be used topically to help clear up and cure sores and to reduce the discomfort of teething in infants. Furthermore, saffron contributes as an excellent stomach ailment and an antispasmodic as well. It helps digestion and increases appetite. It also relieves renal colic, reduces stomachaches and relieves tension. It's essential oil is relaxant and could be useful in insomnia of nervous origin. Saffron as a medical plant is considered as an excellent medicine in chronic bronchitis and lung diseases. In South Asia saffron is widely used for kidney, liver, vesica disease and for medication of cholera. External application of saffron tincture is useful for dermal disease such as impetigo (**Abdullaev 1993; Khorasani*et al*. 2008**). Saffron has positive effects in lowering blood cholesterol and triglycerides (Arasteh*et al*., 2010). The therapeutic function of saffron, included treatment of certain cancers, as in 1990's scientist reported for the first time the antitumor effect of *Crocus sativus* L. in mice.As well as cures for cerebrovascular and cardiovascular diseases has been well established (Nair *et al.,*1991; Negbi 1999; Abdullaev, 2002). Saffron is also considered as an anticonvulsant, antioxidant, anti-inflammatory , and is used to cure burns and treat depression (Salomi, 1991; Abdullaev, 2002; Hosseinzadeh and Younesi, 2002). Moreover, *Crocus sativus* is applicable for treatment of nervous disorders, anxiolytic, spasms, asthma (Abdullaev, 2002; Hosseinzadeh*et al*. ,2005; Mohajeri*et al*., 2007; Hosseinzadeh and Noraei, 2009).

In modern pharmacy saffron has the reputation to be useful in treatment of many diseases including diabetes. In a study of diabetic male rats it was found that 50 mg/kg of hydromethanolic extract of saffron injected intraperitoneally decreased serum glucose, cholesterol levels and increased serum insulin

(Alarcon-Aguilara*et al.*, 1998; Mohajery *et al.,*2009; Arastehe*t al*., 2010).

Saffron is used as an essential and natural colorant in industrial factories Dairy factories use saffron in making varied kinds of butter, cheese and ice cream. Products of drink, jelly, candy, cake and cookie factories benefit from saffron. The use of saffron in the cosmetic industry is now fairly widespread with the trend to use natural products and owing to its active substances. Saffron is also used as a perfume ingredient in many famous internationally brands and also as a dye or ink(Estilai, 1978; Basker and Negbi, 1983; Abdullaev, 2002; Hill, 2004; Aytekin and Acikgoz, 2008). Saffron extract given by oral administration to mice infected with cancer can inhibit the growth of cancer. It also increased the life of the treated mice (Chermahini*et al.*,2010).

Adult rats having alcoholic and carbon tetrachloride (CCl_4) liver toxicity were given saffronby intragastric feedingtubeto determine the therapeutic effectofsaffron. It was noted that saffron was usefulin the prevention ofliverinjury by alcoholic and CCl_4, where the serum level of ALT decreased significantly in saffron groups as compared with rats treated with alcoholic and CCl_4 only. However it was also notedthat saffron groups had obvious pathological changes (Anlin*et al.*, 2000).

Adult liver metabolizes both beneficial and harmful substances. It stores nutrients and other useful substances, as well as detoxifying or breaking down harmful compounds. The liver regulates carbohydrate, lipid and protein metabolism and synthesis. Most of the water soluble minerals, vitamins and nutrients absorbed by the small intestine are transferred into the liver through the portal blood (Tso and McGill, 2003; Van Tellingen, 2003). Also liver also plays an important role in the storage and metabolism of iron and vitamins such as A, D, B12, synthesizes prothrombin, fibrinogen and clotting factors. Aged RBCs are destroyed primarily in spleen and liver. The liver also excrete calcium via the bile, breaks down toxic substances (Tso and McGill, 2003; Van Tellingen, 2003). The liver can be taken as the major source of hematopoietic cells during gestation at the age from 11.5 to 12.5 days (Sasaki and Matsumura 1986). Macrophages surround the erythroblasts to function as nurse cells during erythropoiesis and to phagocytize the expelled erythrocytic nuclei (Chasis, 2006; Isern*et al.*, 2008). At the age of 12 days of gestation, the most abundant protein (albumin) is synthesized by mature hepatocytes and increases until adulthood (Tilghman and Belayew, 1982). The hepatocytes are involved in bile acids synthesis, bile formation, and absorption of nutrients and xenobiotics. Also they are able to maintain the homeostasis of glucose, amino acid, ammonia and bicarbonate in the body(Tso and McGill, 2003). From age 12 to age 14 days of gestation stellate cells contain distinct fatty droplets in the cytoplasm that contain vitamin A. Megakaryocytes are found in the liver in large numbers at 13.5 day of gestation and produce platelets and store glycogen (Gibb and Stowell, 1949; Crawford, *et al.*, 2010). Kupffer cells play an essential role in removing bacteria, virus particles and fibrin-fibrinogen complexes from the circulation. Kupffer cells in the liver can remove damaged RBCs. The RBCs are digested by secondary lysosomes in kupffer cells to release heme (Tso and McGill, 2003; Naito *et al.*, 2004).

The usages of saffron in pregnant mice can have some complications on the embryo as giving rise to absorbed and abnormal embryos (Tafazoli *et al.,*2004). A study showed that oral administration of high and low doses of aqueous saffron extract to mice during pregnancy caused significant decrease in embryonic growth parameters and caused congenital malformations (AlQudsi and Ayedh, 2012). Studies on the effect of normally consumed saffron amounts on liver embryonic development are scarce and keeping in mind the importance of the liver during embryonic development and adult life therefore came the urgency to do such research.

2.Materials and Methods

All experimental procedure was approved by the Biology department at King Abdulaziz University. Forty five (45) mature female and 45 mature male SWR (Swiss White Rodeless mice) from King Saud University Animal house, were used. Weighing 25-30 grams. They were maintained at 22± 2ºC on 12 hours light: 12 hours dark daily in plastic cages, and fed on corn cob pellets with water bottle. Saffron, the dried stigmasof *Crocus sativus* flower were taken from Al-alawy market in Jeddah, and was identified by our Botany taxonomist, in the Biology Department at the Faculty of Science, King Abdulaziz University.

The estrus stage was determined according to the method described by (Walmer*et al.* 1992; Caligioni 2009). A male and a female (in estrus stage) were placed in one cage and left overnight. The presence of the vaginal plug, next morning was considered as fertilization evidence, and this was considered to be the first day of pregnancy therefore sexes were separated.

In this research two doses were used. (100 mg/kg body weight) as high dose (HD) taken from (Premkumar*et al.* 2003a). While the other dose (2.5 mg/kg body weight) as low dose (LD) used in everyday life, taken from (Al-Qudsi and Ayedh, 2012). The aqueous extract of saffron was prepared

according to the method described by (Premkumar*et al.*, 2003a, Al-Qudsi and Ayedh, 2012).

Pregnant mice were divided into three groups control (C), high dose treated group (HD) and low dose treated group (LD) each groupconsisted of 15 pregnant mice. group (C) was given (10 ml / kg body weight) of double distilled water, group (HD)was given (10 ml / kg body weight from the saffron aqueous extract concentration 100 mg / kg body weight) (Premkumar*et al.,* 2003) and group (LD) was given (7.3 ml / kg body weight from the saffron aqueous extract concentration (2.5 mg / kg body weight) (Al-Qudsi and Ayedh, 2012). Saffron solution or distilled water doses were orally administrated to pregnant mice using oral gavage that was cleaned with distilled water after administration of each experimental group. Doses were administered for 5 days during each of the first and second weeks and for four days during the third week of gestation. A pilot study was done to see the effect of giving double distilled water to pregnant females 10 ml / kg and 7.3 ml / kg as no significant difference was seen with the outcome. It was decided to keep one control group with 10 ml / kg double distilled water dose.

Sample collection

Embryos were collected from all groups on day 14, 18 of gestation and day 1 neonates. Then embryos were washed in a Petri dish containing saline solution (0.99 grams of sodium chloride in 100 ml of distilled water). Embryos were then preserved in formalin 10%. The complete process of sample collection was completely photographed (Sony camera model DSC-T900) for each dissected mother including the photos of each embryo the camera had a fixed zoom and fixed distance from the specimen this was done to record and review all data available during sample collections.

For biochemical assays 18 days embryo's livers were frozen immediately after extraction from embryos. Livers were shopped to piecesof 0.1 mg weight for each batch.

Morphometric studies

All collected embryos of all ages and experimental groups were patted dry and photographed by a dissecting microscope model (Olympus SZX10) connected with a camera model (Olympus DP25) at king Abdulaziz University girl's section Faculty of Science Central Laboratory. Morphometric measurements were performed using DP2-BSW software. The morphometric measurements studied were embryo'sexternal visible liver area (Figure 1) for day 14 of gestation only (we were not able to take the liver external area for 18 days embryos and day 1 neonates as it was not clear).

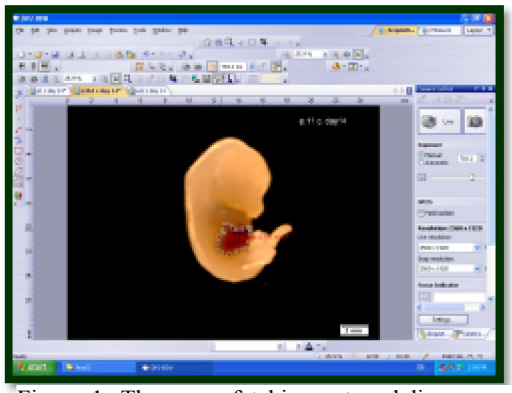


Figure 1: The way of taking external liver area of embryos on day 14 using DP2-BSW software.

Preparation of samples for histological studies

Samples were prepared for light microscope histological study as follows: Extracted Embryos were fixed in formalin 10% solution. Samples were dehydrated by passing in a series of ascending grades of alcohols of 70% up to 90%,for clearing samples were passed in Xylene. Then samples were passed in two changes of melted paraffin wax. Wax blocks were cut into cross, longitudinal and sagittal serial sections of 4 micron-thick. Tissue sections were mounted on a glass microscope slide. Sections werethen hydrated and stained with Haematoxyline and Eosin(H&E) (Drury and Wallington, 1980) and covered by Canada balsam.

Slide photographing

Tissue sections were examined and photographed using a compound microscope (Olympus BX51) connected to a camera model (Olympus DP72) at king Abdulaziz University girl's sections Faculty of Science Central Laboratory.

Preparation of samples for electron microscopy

Liver samples were extracted from mice neonates at birth (day 1 neonate) from all groups. Liver samples were then cut into very small parts of 1mm. Samples were placed in a fixative solution consisting of (5% glutaraldehyde in 5.1 M sodium cacodylate buffer pH 7.3) for 4 hours (Mercer and Birbeck, 1972). Samples were washed twice in distilled water each time about 15 minutes. Then were put in 1% Osmium tetroxide buffer dissolved in sodium cacodylate buffer pH 7.3 for one hour for re-fixation.Samples were then washed twice in distilled water each time for about 15 minutes. The samples were dehydrated in ascending series of ethanol 70% and 95% each concentration for 15 minutes, then 100 % ethanol for 10 minutes three times. At the stage of clearing, samples were passed in two changes of a solution of propylene oxide for 8 minutes for each change. In Infiltration process samples were transferred to a mixture of propylene oxide with epon resin (812) in equal volumes 1:1 for 30-45 minutes.

The samples were then embedded in pure epon resin and left for 30-45 minutes,then embedded in pure resin into blocks at 60ºC overnight. Samples were sectioned for light microscope by LKB ultramicrotome with glass knives to produce semi-thin sections (0.5 micron). Samples were stained for light microscope, by Toludin blue to determine the place to be examined by electron microscope. Samples were then cut into ultrathin sections of 50 nm by ultra-microtome with glass knives. Ultrathin sections were placed on mesh naked copper grids. For staining a drop of dye was put on the copper grid using uranil acetate for 30 minutes and lead citrate for 15 minutes (Reynold 1963, Mercer and Birbeck, 1972). Sections were examined and photographed by transmission electron microscope at King Fhad Medical Research Center in Jeddah.

Lipid peroxidation (LPO) assay

The extent of LPO was estimated as the concentration of thiobarbituric acid (TBA) reactive product MDA by using the method of Ohkawa*et al.,*(1979). Two hundred fifty microliters of liver tissue homogenate were added to 1.5 ml of 1% phosphoric acid (pH 2.0) and 1 ml of 0.6% of TBA in air–light tubes and were placed in a boiling water bath for 25 min. After incubation, the sample was cooled to room temperature and MDATBA was extracted with 2.5 ml of butanol. Organic phase was separated by centrifugation for 5 min at 2000g and measured at 532 nm. MDA concentrations were determined using 1,1,3,3-tetraethoxypropane as standard and expressed as l mol/g liver tissue.

Reduced glutathione (GSH) assay

Reduced GSH estimation was performed by the method of (Beutler *et al.,*1963). Livers were homogenized in 1 ml of 1.1% KCl cooled, then homogenate (100 µl) was mixed with 750 µl of precipitate solution (1.67 g of glacial metaphosphoric acid, 0.2 g of EDTA and 30 g of NaCl in 100 ml of distilled water) and 900 ml of distilled water. Homogenated tissues were centrifuged at 2000g for 15 min to precipitate proteins. Protein-free supernatant (250 ml) was added to 1 ml of Na^2 HPO4 (0.3 M) solution and the reaction was initiated by adding 125 ml of DTNB (6 mM) and the absorbance of 5-thio-2-nitrobenzoic acid (TNB) formed was measured at 412 nm. The level of GSH was obtained by standard curve and expressed as mmole per g liver tissue.

Determination of total proteins content

Principle

At an alkaline pH, the protein reacts with the copper in the biuret reagent causing an increase in absorbance. The increase in absorbance at 550 nm due to the formation of the colored complex is directly proportional to the concentration of protein in the reaction (Gornall*et al.,*1949).

Protein + Cu^{++} blue-violet complex

Reagents

(1) Standard albumin (5 g/dL).

(2) Biuret reagent consists of cupric sulfate (6 mmol/L), sodium potassium tartrate (21 mmol/L), sodium hydroxide (750 mmol/L) and potassium iodide (6 mmol/L).

Procedure

1-The following volumes (mL) were pipetted into cuvettes and were mixed well:

Sample (mL)	Standard (mL)	Blank (mL)	
-	0.025	-	Standard
0.025	-	-	Sample
1.0	1.0	1.0	Biuret reagent

2- All tubes were incubated at 37 °C for 10 min.

3- The absorbance of the sample (A_{sample}) and standard ($A_{standard}$) against reagent blank was read at 550 nm.

4- Color stable for an hour, linearity up to 10 g/dL

5- The values were derived by the following equation:

Total protein concentration (g/dL) = (A_{sample}/ $A_{standard}$)x 5.

Statistical analysis

Data was analyzed using SPSS 16. The test used with normal distribution was Anova, Student-Neuman Keul test. In case of abnormal distribution Man-Whiteney U test was used from the non-parametrictest. Significance (*) was at $p<0.05$.

3.Results:

Effect of saffron on external liver area: A non-significant decrease in the outer seen liver area of treated embryos (HD) and (LD) was seen on day 14 compared the control group.

Effect of saffron on liver weight: A slight non-significant decrease in liver weight of treated (HD) and (LD) embryos was seen on day 18 compared the control group.

Effect of saffron on liver peroxidation:

A significant decrease in liver LPO of treated embryos (HD) and (LD) was seen on day 18 of gestation compared to the control group $p= 0.046$ for (HD) and $p=0.041$ for (LD)(Figure 2).

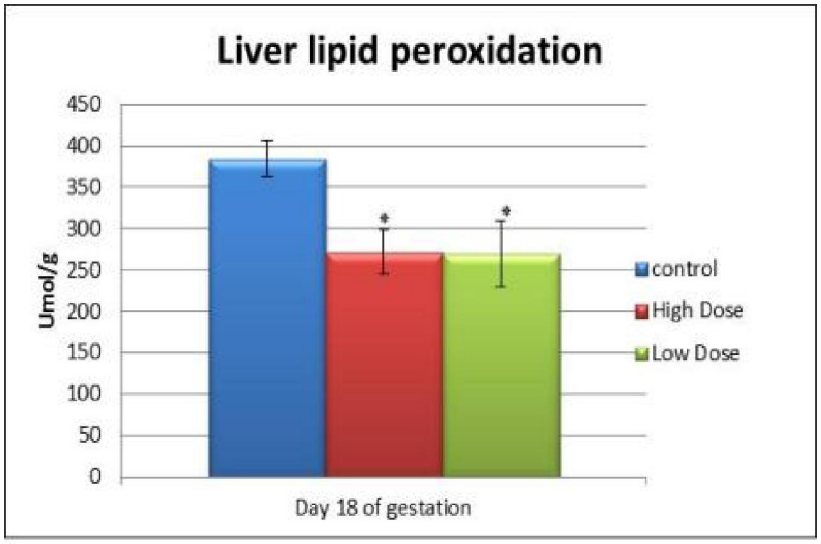


Figure 2 Graph showing the effect of saffron on embryo's liver lipid peroxidation at day 18 of gestation. Values are M±SE taken from 15 samples for each age treatment (*) $P< 0.05$.

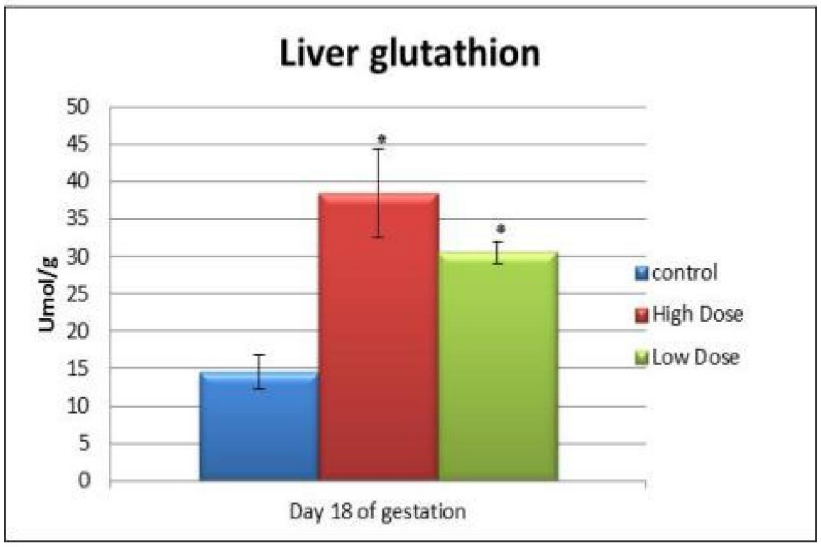


Figure 3 Graph showing the effect of saffron on embryo's liver glutathione at day 18 of gestation. Values are M±SE taken from 15 samples for each age treatment (*) $P< 0.05$.

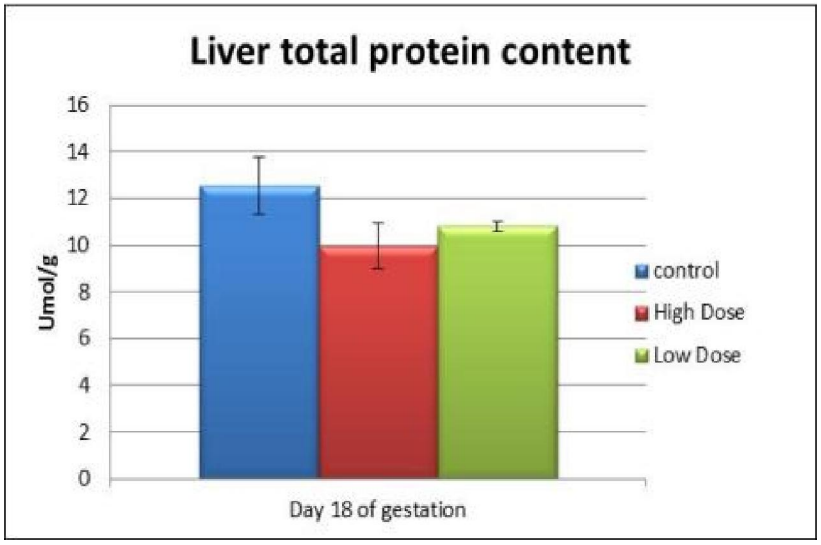


Figure 4 Graph showing the effect of saffron on embryo's liver total protein content at day 18 of gestation. Values are M±SE taken from 15 samples for each age treatment (*) $P< 0.05$.

Effect of saffron on liver glutathione: A Significant increase in the GSH of liver of treated embryos (HD) and (LD) was seen on day 18 of gestation compared to the control group, p= 0.001 for (HD) and p= 0.023 for (LD) (Figure 3).

Effect of saffron on liver total protein content: A non-significant decrease in the total protein content of (HD) and (LD) liver was seen on day 18 of gestation compared to the control group (Figure 4).

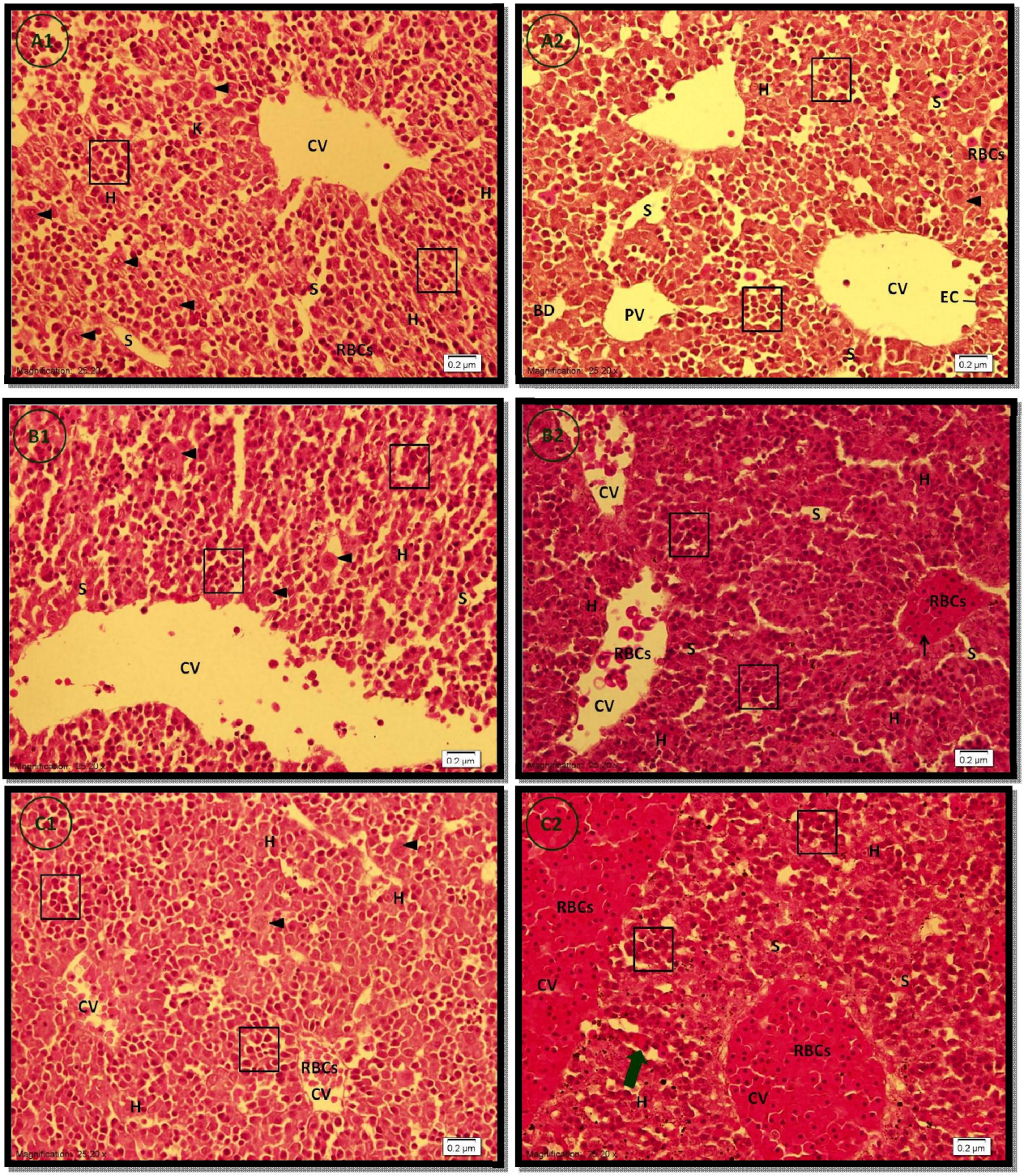
Effect of saffron on hepatic tissue in mice at day 14 of gestation:
Both HD and LD treatments caused the liver of 14 day embryo to have dilated central veins, dilated blood sinusoid with hemolysis, and caused a declination in the seen quantity of megakaryocytes compared to the controls (Figure 5).

Effect of saffron on hepatic tissue in mice at day 18 of gestation:
When comparing the general histological structures of HD group mice liver at 18 day of gestation to controls the following changes were seen the appearance of numerous dilated congested vessels outside the capsule and within liver parenchyma .The lumen of central vein was full with blood (congestion). There was marked damage of endothelial lining. Near portal area showed marked dilated congested portal vein. Bile ducts showed damaged endothelial lining. Hepatocytes showed vacuolated unstained cytoplasm with small dark nuclei. Megakaryocytes seemed to be present in large amount compared to the controls. Hematopoietic cell numbers seemed to be less than controls. Leucocytes were seen under the capsule and aggregation was also seen near portal area (Figure 6).
Histological structures of LDgroup mice liver at 18 day of gestation showed the appearance of numerous dilated congested vessels. Congested central vein, blood sinusoid and portal vein. Hepatocytes had unstained vacuolated cytoplasm with small dark nucleus. Blood sinusoids showed leucocyte infiltration. Leucocytes aggregates were seen near the portal vein. Megakaryocyte numbers seemed to increase compared to the controls (Figure 6).

Effect of saffron on hepatic tissue in mice at day 1 neonates:
Liver histological structures of day 1 neonates HD group showed the appearance of numerous dilated congested vessels. Both portal area and hepatic sinusoid showed mononuclear cellular infiltration and leucocyte invasion. Congestion and dilation were observed in central vein, blood vessels and the portal vein with hemolysis. Focal mononuclear cell infiltration was observed in periportal connective tissues and hepatic sinusoid. Bile ducts showed damaged endothelial lining.Many megakaryocytes were seen in HD group.Liver histological structures of day 1 neonates LDgroup showed changes similar to HD group. In addition to the appearance of numerous dilated congested vessels. The presence of marked dilated and congested central vein and portal vein were seen. Mononuclear cell infiltration around central vein and portal vein was less than the HD group. Many lipid droplets appeared in the cytoplasm of hepatocytes. Many megakaryocytes were also seen (Figures 7& 8).

Effect of saffron on the ultrastructure of hepatic tissue in mice day 1 neonates:
When comparing treated (HD) neonate mice liver electron micrographs with the control neonate liver micrographs. It was noticed that several morphological distortions were seen. These were as follows. Numerous mitochondria were noticed within cells compared to the controls while a severe decline in the endoplasmic reticulum which appeared fragmented and decomposed was noticed. Glycogen granules appeared in smaller quantities. Big amounts of lipid droplets of varying size were seen in the hepatocytes. Some nuclei appeared to be shrunken (pyknosis) and had irregular nuclear membrane. Chromatin condensation on the nuclear membrane was seen in some cells. Peroxisomes were seen as small electron-dense granules surrounded by a membrane. Bile canaliculi were seen with degenerated microvilli. Lack of intercellular space connecting cells with each other and degenerated microvilli was noticed (Figures 9-11).

Also several morphological distortions were seen in the ultrastructure of hepatocytes from (LD) treated neonate mice. The membranes of the hepatocytes had more elaborated microvilli compared to the controls. Hepatocytes appeared with an increase in the amount of lipid droplets compared to the controls. Bile canaliculi appeared wider and dilated with degenerated microvilli. Cell death type I was seen as shrinkage in cell size and the nucleus that seems to be totally dissolved within the cytoplasm (type I cell death). Blood sinusoids contained anucleated red blood cells, lymphocytes and polymorphonuclear leucocytes which appeared numerous. Invading of leucocytes to the liver parenchyma was seen. Glycogen granules were very much reduced compared to controls (Figures 9- 11).


A1
K
CV
H
H
S
H
S
RBCs
A2
H
S
RBCs
S
CV
EC
BD
PV
S
B1
H
S
S
CV
B2
CV
H
S
H
RBCs
RBCs
S
S
CV
H
H
C1
H
H
CV
RBCs
CV
H
C2
H
RBCs
S
CV
S
RBCs
H
CV

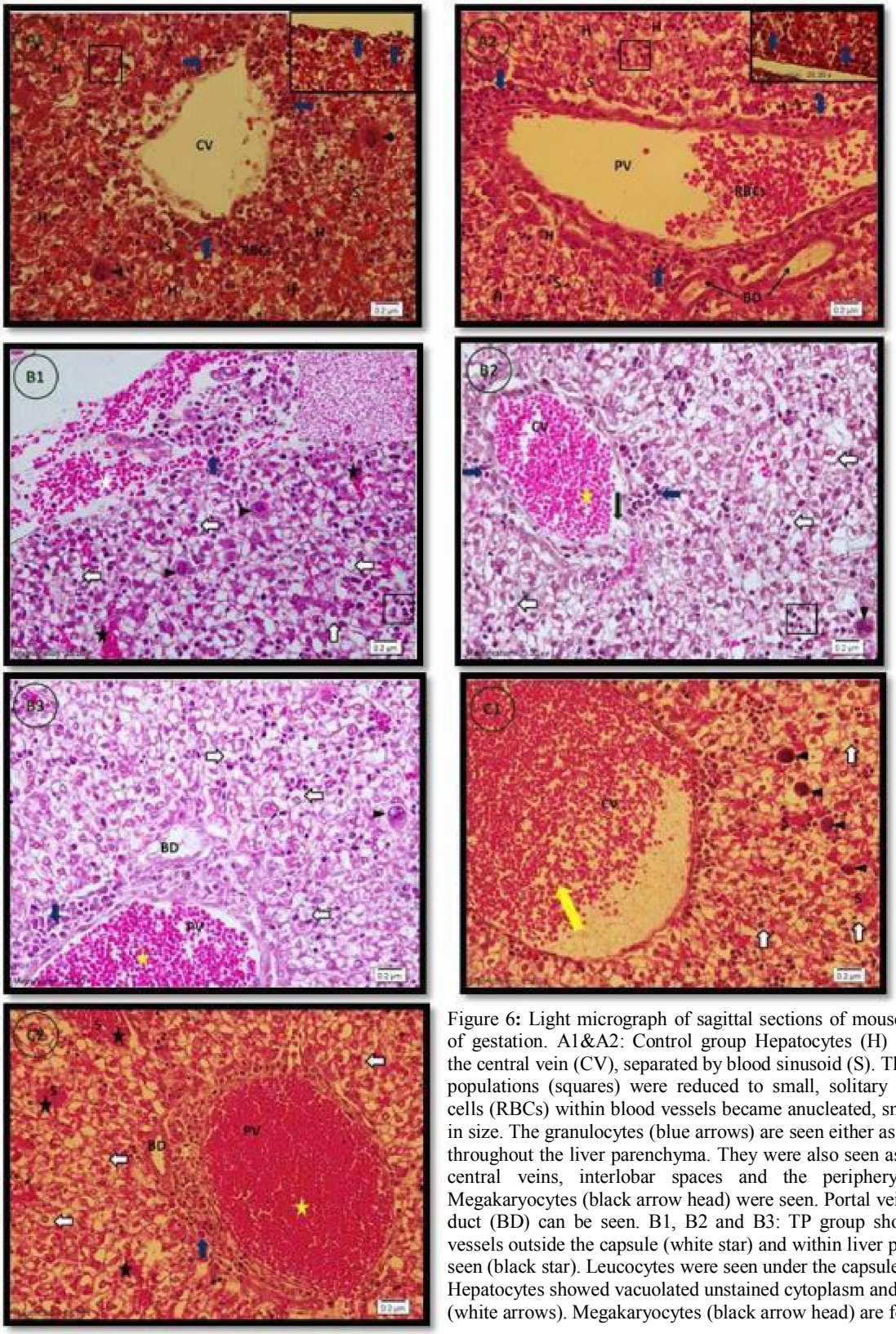


Figure 6**:** Light micrograph of sagittal sections of mouse liver at day 18 of gestation. A1&A2: Control group Hepatocytes (H) arranged around the central vein (CV), separated by blood sinusoid (S). The hematopoietic populations (squares) were reduced to small, solitary foci. Red blood cells (RBCs) within blood vessels became anucleated, small and uniform in size. The granulocytes (blue arrows) are seen either as foci or scattered throughout the liver parenchyma. They were also seen as clusters around central veins, interlobar spaces and the periphery of the liver. Megakaryocytes (black arrow head) were seen. Portal vein (PV) with bile duct (BD) can be seen. B1, B2 and B3: TP group showing congested vessels outside the capsule (white star) and within liver parenchyma were seen (black star). Leucocytes were seen under the capsule (blue arrows). Hepatocytes showed vacuolated unstained cytoplasm and dark nuclei (white arrows). Megakaryocytes (black arrow head) are found. Hematopoietic cells were decreased (squares). The lumen of central vein (CV) was full with blood (congestion) (yellow star). There was marked damage of endothelial lining (green arrow). Near portal area showing marked dilated congested (yellow star) portal vein (PV). Bile ducts (BD) showing damaged endothelial lining. Leucocytes aggregation was seen near portal area (blue arrow). C1&C2: TQ group showing congested central vein (CV) and blood sinusoid (S) were seen. Hepatocytes have unstained vacuolated cytoplasm (white arrows) with small dark nucleus. Blood sinusoids showed leucocytic infiltration (black star). Portal vein (PV) seemed congested (yellow star). Leucocytes (blue arrow) aggregates were seen near the portal vein (H/E, x 400).

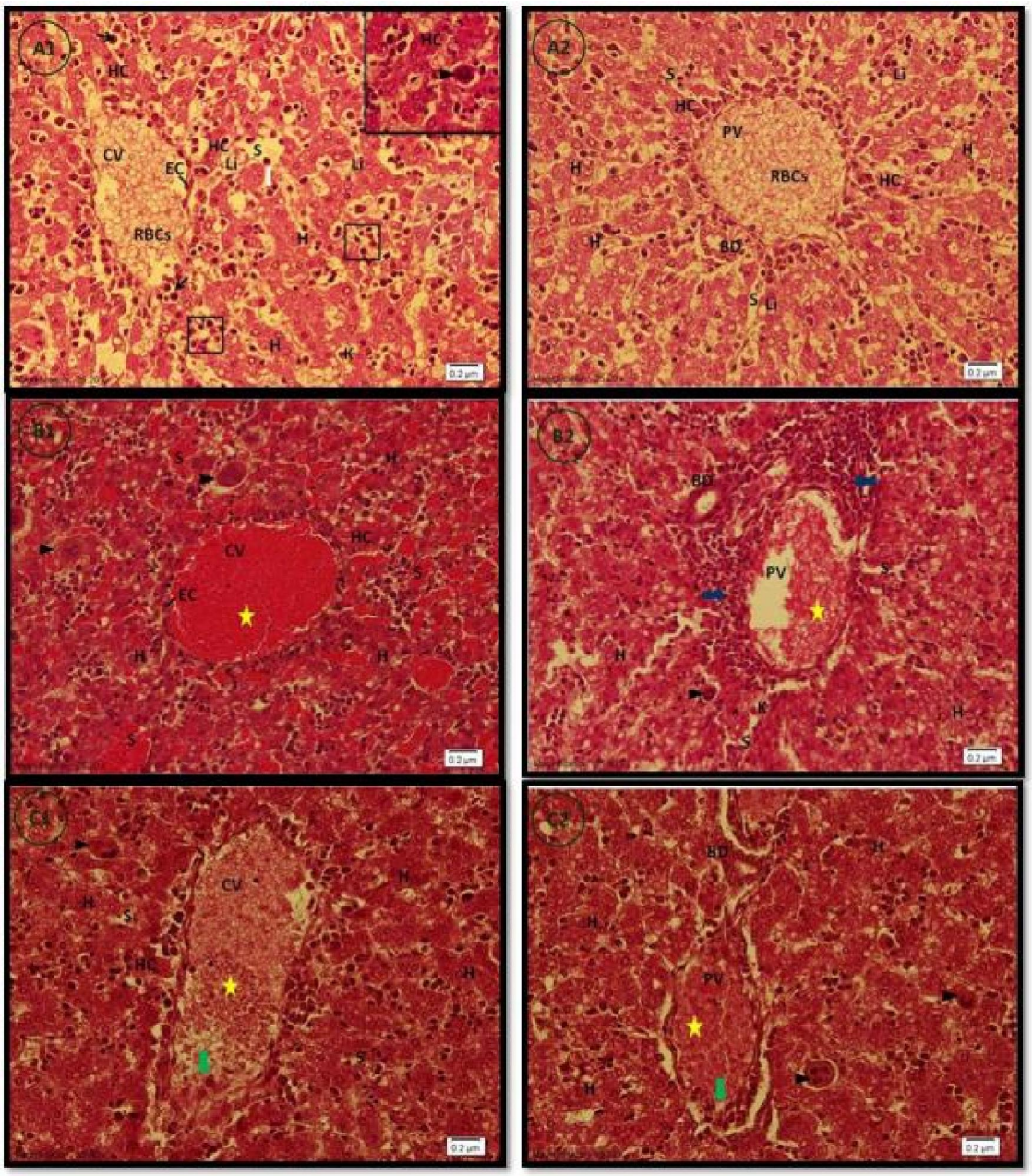


Figure 7**:** Light micrograph of sagittal sections of mouse liver of day 1 neonates. A1, A2: Control group showing central vein (CV) lined with epithelial cells (EC) and containing anucleated red blood cells (RBCs). Hepatocytes (H) can be seen linked in such a manner that they form hepatic cords (HC) and have small round nuclei densely stained. The hepatic cords are separated from each other by blood sinusoids (S). Blood sinusoids are lined with endothelial cells (EC) and Kupffer cells (K). Hematopoietic cells (squares) seem more differentiated and fewer. Nucleated RBCs (white arrow) are still seen. Lymphocytes can be distinguished (black arrows). Megakaryocyte (black arrow head) can be also found as isolated cells surrounded by hepatocytes. Lipid droplets (Li) can be seen in the cytoplasm of some hepatocytes. Portal vein (PV) present in portal triad and bile duct (BD), lined with single columnar epithelial cells. B1, B2: HD group showing congested dilated central vein (CV), blood vessels (S) and the portal vein (PV) with hemolyzed blood (yellow star). Focal mononuclear cell infiltration was observed in periportal connective tissues and hepatic sinusoids (blue arrow). Bile ducts (BD) showed damaged endothelial lining. Note, many megakaryocytes (black arrow head) were seen in TP group. Hepatic cord can be seen degenerated. C1, C2: LD group showing the presence of marked dilated and congested central vein (CV) and portal vein (PV) with hemolyzed blood (yellow star). Both central vein and portal area showed mononuclear cellular infiltrate leucocyte invasion (green arrows). Many of lipid droplets (Li) appeared in the cytoplasm of hepatocytes. Note, many megakaryocytes (black arrow head) also were seen.(H&E), (x 400).

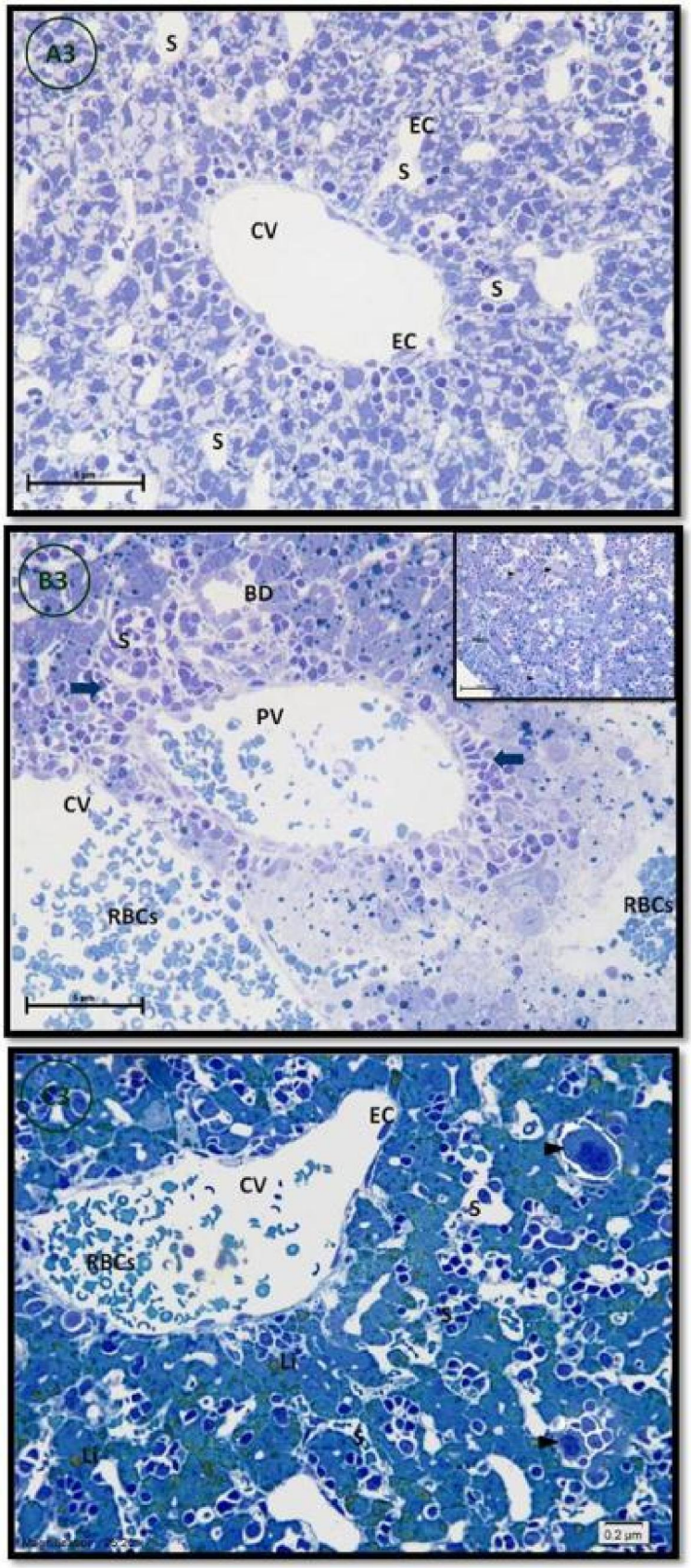


Figure 8: Light micrograph of sagittal sections of mouse liver of day 1 neonates. A3: Control group showing central vein (CV) lined with epithelial cells (EC) The hepatic cords are separated from each other by blood sinusoids (S). Blood sinusoids are lined with endothelial cells (EC). B3: HD group showing congested dilated central vein (CV), blood vessels (S) and the portal vein (PV) with hemolyzed blood. Focal mononuclear cell infiltration was observed inperiportal connective tissues and hepatic sinusoids (blue arrow). Bile ducts (BD) showed damaged endothelial lining. Note, many megakaryocytes (black arrow head) were seen in HD group. Hepatic cord can be seen degenerated. C3: LD group showing the presence of marked dilated and congested central vein (CV) Many of lipid droplets (Li) appeared in the cytoplasm of hepatocytes. Note, many megakaryocytes (black arrow head) were seen.(A3, B3 and C3, Semi-thin section by T.B.) (x 400).

4. Discussion

The liver is the largest organ in the body exhibiting both endocrine and exocrine properties. The liver can be taken as the major source of hematopoietic cells during gestation at the age from 11.5 to 12.5 days (Sasaki and Matsumura, 1986).

In this study aqueous saffron extract (HD) 100 mg/kg, (LD) 2.5 mg/kg given to pregnant mothers for five daysinthefirsttrimesterofpregnancy, five days in the second trimester of pregnancy and two days in the third trimester of pregnancy caused a significant decrease in liver LPO of treated fetuses (HD) and (LD) and a non-significant decrease in the total protein content of liver. However, it caused a significant increase in liver GSH of treated embryos. The most important signs that indicate the presence of oxidative damage are the presence of LPO. It was found that LPO has an important role in the toxicity of many xenobiotics (Anane and Creppy,2001; Ali *et al.,* 2010). It has been also reported that aqueous saffron extract decreased free radicals levels in mouse liver and has an antioxidant activity (Ali *et al.,* 2010). Aqueous saffron extracts (40 and 80 mg/kg) given for five consecutive days protected mice from genotoxins-induced oxidative stress (Premkumar*et al.,* 2003b). In this study, we observed that LPO in the embryos liver significantly decreased, while GSH significantly increased. Throughout the body oxidative stress might increase GSH synthesis rates (Cantin*et al.,* 2007). It seems that saffron induced oxidative stress which increased GSH synthesis. Saffron plays an important role as an anti-cancer. Saffron also works on the inhibition of many cellular enzymes and for this reason it may lead to disabling the functions of these enzymes thus works as an antitumor (Chermahini*et al.,* 2010). Crocin an active constituents of saffron can induce apoptosis (Thatte*et al.,*2000). This study showed that aqueous saffron extract caused a non-significant decrease in liver weight of treated embryos at 18 day of gestation in (HD) and (LD).

A study reported that an increase in the numbers of leukocytesmay bearesult of the reaction of inflammation in thedamagedtissuein theliver aftertreated withsaffron (Mohajery*et al.,* 2009; Khayatnouri*et al.,* 2011). Results of this study showed intensive invasion of monocytes and leucocytes in embryonic liver. In this study the aqueous saffron extractmight have been transferred from the treated mothers to the embryos throughtheplacentaandthatmight haveledtoaninflammation of the embryonic liver tissue. Mohajery*et al.* (2009) have reported that saffron induced normochromic-normocytic anemia and this may bedue tobonemarrow suppression. It was also reported that saffron has caused a significant increase ALT and AST that might cause damage to theliver tissue. Histopathological studies proved that the extractof saffron caused damagein the liver and kidneys tissues in miceasit causednecrosisand degeneration in thecentral areaof the hepatocytes. Thismaybedueto the reduction in the amount of oxygenreceived in this area(Mohajery*et al.,* 2009; Khayatnouri*et al.,* 2011).Mohajeri*et al.* (2007) as well reported that giving rat (0.35 g/kg saffron extract) led to dilation and congestion of central veins, dilated sinusoids, necrosis and loss of the usual arrangement of hepatocytes. Similar results were seen in this study regarding embryonic liver sections where dilation and congestion was seen in the hepatic central and portal veins. When using (0.70 and 0.35 g/kg saffron extract) necrosis was seen in the central portions and infiltrations of lymphocytes and granulocytes in the portal areas. But when mice were given (1.05 g/kg saffron extract) more necrosis and degeneration of periportal area was seen. In addition to accumulation of Kupffer's cells with active phagocytosis in the periportal regions, bridging of inflamed portal tracts between different lobules, wide spread lytic necrosis and hemorrhages (Mohajeri*et al.,* 2007). Similar results were seen in this study where apoptosis was also seen in hepatocytes. Necrosis and degeneration of hepatocytes occurs in the centrilobular when it receives less amount of oxygenized blood. As for necrosis and degeneration of periportalithappenedasaresultofexposuretotoxins from ethanolic extract of saffron (*Crocus sativus*) (Cullen, 2007; Mohajeri*et al.,* 2007).

This study indicated that oral administration of everyday used doses of saffron aqueous extract (2.5 mg saffron / kg body weight) to pregnant mice might have caused deterioration of the histological structure of 14 and 18 day embryonic liver and ultrastructure of the liver of day 1 neonate. It also caused a significant increase of LGH, significant decrease of LPO of 18 day embryo liver compared to the controls. More studies should be performed to assess the effect of low saffron doses on other embryonic organ development.

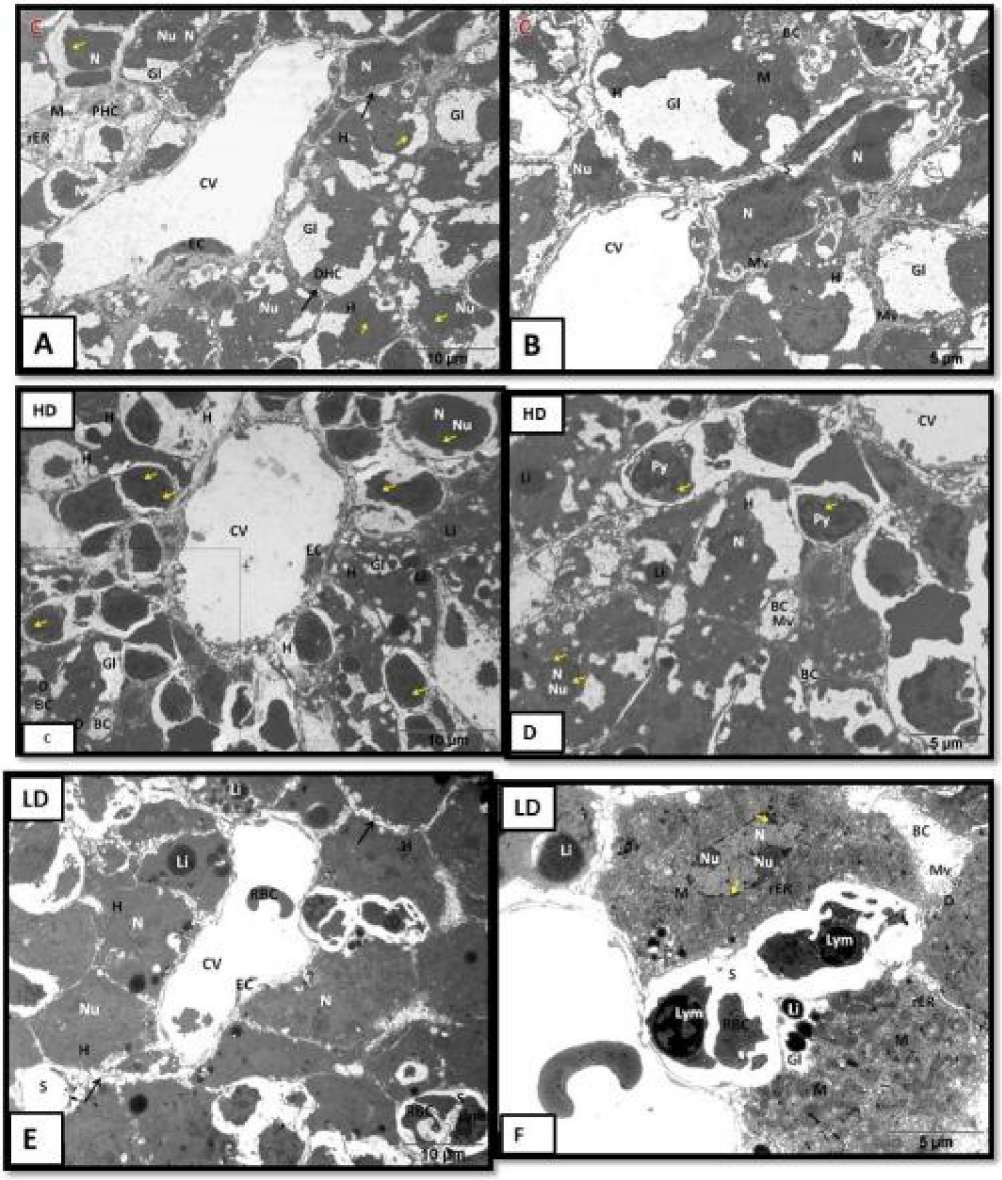


Figure 9: (A) TEM micrograph showing the ultrastructure of control neonate mouse liver (day 1). Normal hepatocytes (H) appeared polygonal and had distinct intercellular space (black arrows), they appeared arranged radially around the central vein (CV), that is lined by endothelial cells (EC). Hepatocytes appeared dark (DHC) and pale (PHC). The hepatocytesappearedcontaining a nucleus (N), with a nucleolus (Nu) or two nucleoli having euchromatin (yellow arrows). Rough endoplasmic reticulum (rER) could be seen throughout the cytoplasm intermixed with mitochondria (M). The normal abundance of glycogen rosettes (Gl) was also seen. **(B)** Enlarged portion of (A) shows hepatocytes (H) with euchromatic nuclei (N) and prominent nucleoli (Nu). The cytoplasm contains mitochondria (M) and glycogen (Gl). Large central vein (CV) can be seen. (S): a blood sinusoid. The blood vessels are separated from the hepatocytes by narrow spaces containing microvilli (Mv). Bile canaliculus (BC) can be seen between hepatocytes. **(C)** TEM micrograph showing the ultrastructure of (HD) neonate mouse liver (day 1). Hepatocytes (H) appeared around the central vein (CV) containing nuclei (N) of different sizes with dense chromatin (yellow arrows). Central vein lined by endothelial cells (EC). Lipid droplets (Li) could be seen in the cytoplasm. A narrow bile canaliculus (BC) could be seen between adjacent hepatocytesbounded by desmosome (D). Glycogen rosettes (Gl) appeared in smaller quantities compared to the control. **(D):** Enlarged portion of (C) where the hepatocytes (H) appeared containing a nucleus (N), nucleolus (Nu). Some hepatocytes appeared with pyknosis (Py) nuclei. Note chromatin (yellow arrows) condensation on the nuclear envelope. canaliculus (BC) can be seen with degenerated microvilli (Mv).**(E):**TEM micrograph showing the ultrastructure of (LD) neonate mouse liver (day 1). Hepatocytes (H) appear arranged radially around the central vein (CV). That is lined by endothelial cells (EC). Red blood cell (RBC) can be seen in the central vein. The hepatocytesappearedcontaining a nucleus (N), nucleolus (Nu). Elaborated microvilli can be seen between some hepatocytes (black arrows). Blood sinusoids (S) could be seen between the hepatocytes containing anucleated red blood cell (RBC) and lymphocyte (Lym).**(F):**TEM micrograph showing the ultrastructure of (LD) neonate mouse liver (day 1). The hepatocytesappearedcontaining a nucleus (N), nucleolus (Nu)with euchromatin (yellow arrows), mitochondria (M) and rough endoplasmic reticulum (rER). Glycogen (Gl) granules are very much reduced compared to control. Hepatocytes appeared to have an increase in the number of lipid droplets (Li). Bile canaliculi (BC) became much dilated with degenerated microvilli (Mv). Desmosome (De) was also seen. Blood sinusoids (S) can be seen containing lymphocyte (Lym).

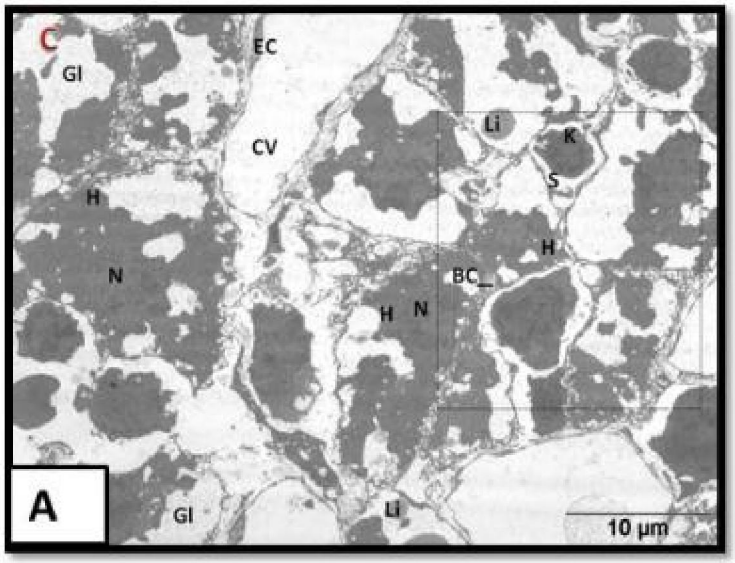
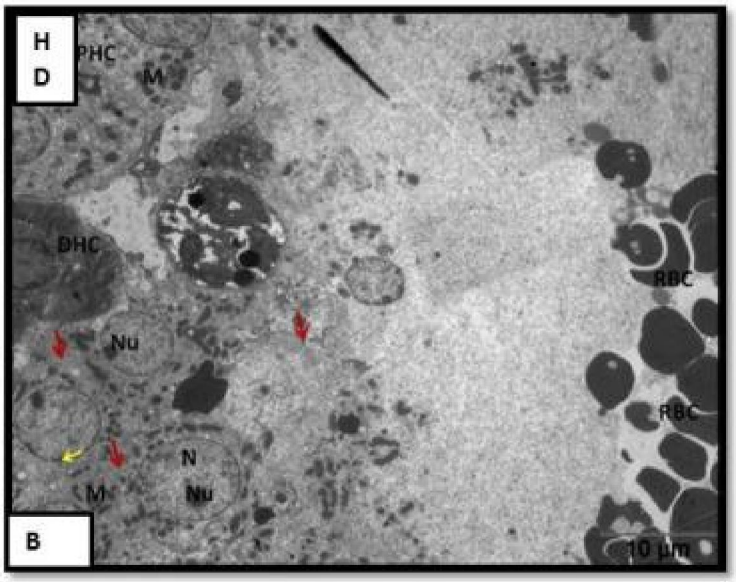
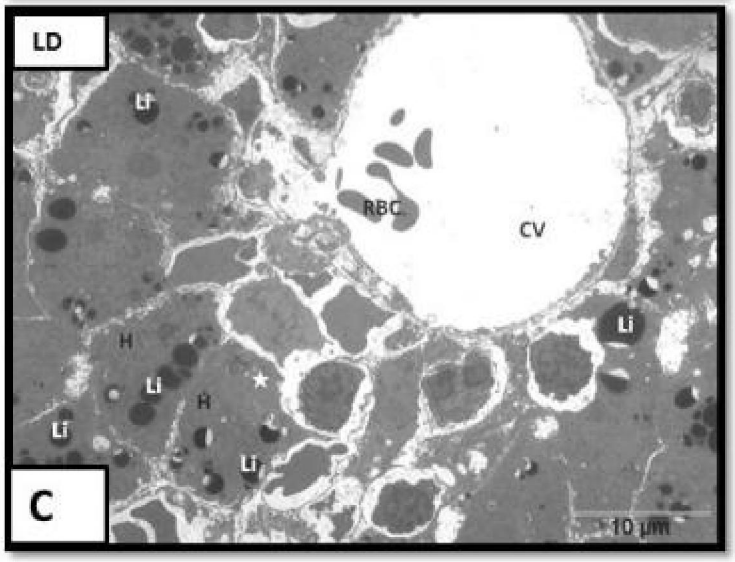


Figure 10**:**(A) TEM micrograph showing the ultrastructure of control neonate mouse liver (day 1). Hepatocytes (H) appear around central vein (CV) with euchromatic nuclei (N). Central vein is lined by endothelial cell (EC). Glycogen (Gl) appearing as rosette shape in different cells. Lipid droplets (Li) can be seen in the cytoplasm. A narrow bile canaliculus (BC) can be seen between adjacent hepatocytes. The blood sinusoid (S) is separated from the hepatocytes by narrow spaces and contains Kupffer cell (K) (Uranayl acetate – lead citrate – 1800x).



(B) TEM micrograph showing the ultrastructure of (HD) neonate mouse liver (day 1). Shows hepatocytes with no clear plasma membrane (red arrows) only the nucleus (N) and nucleolus (Nu) are clear. However we can see very clear pale hepatocytes (PHC) with very clear plasma membrane and near it in the upper middle very clear dark hepatocyte (DHC). Small mitochondria (M) can be seen. Red blood cells can be seen free within the tissue (Uranayl acetate – lead citrate – 1400x).



(C) TEM micrograph showing the ultrastructure of LD neonate mouse liver (day 1). Hepatocytes (H) with cell death type I can be seen. Type I cell death shrinkage in cell size and the nucleus might be totally dissolved within the cytoplasm (white star).Central vein (Cv). Red blood cell (RBC).Lipid droplets (Li) (Uranayl acetate – lead citrate – 1800x).

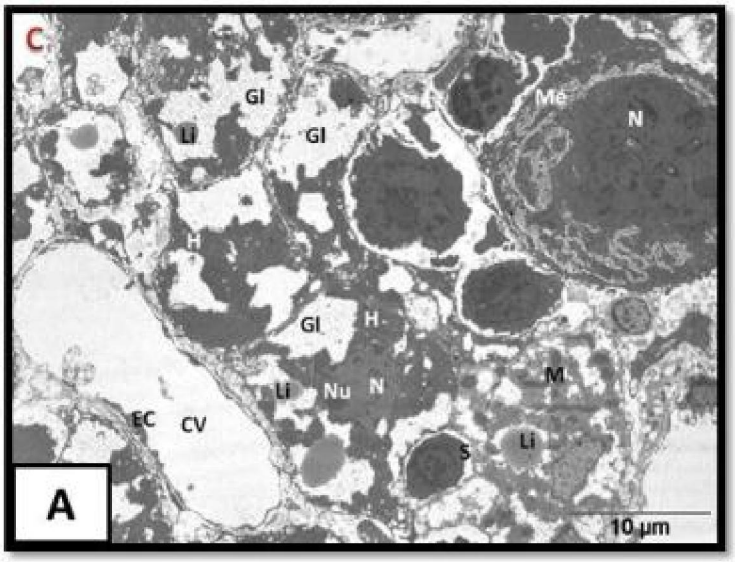
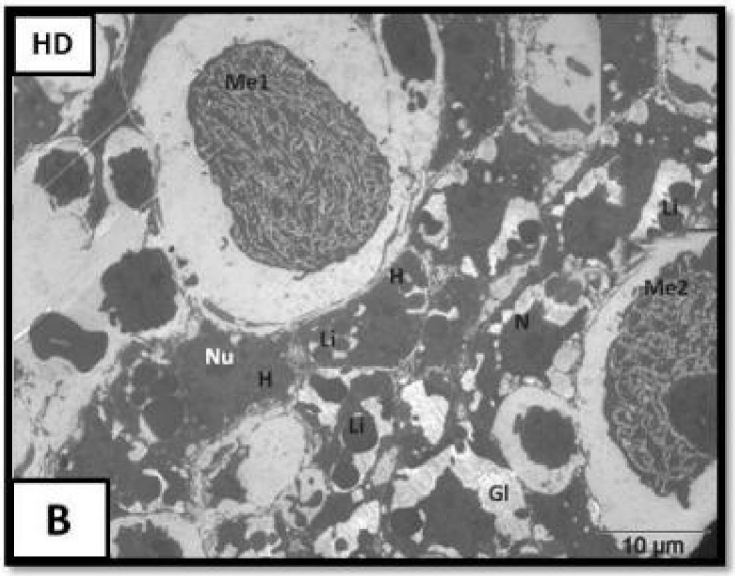
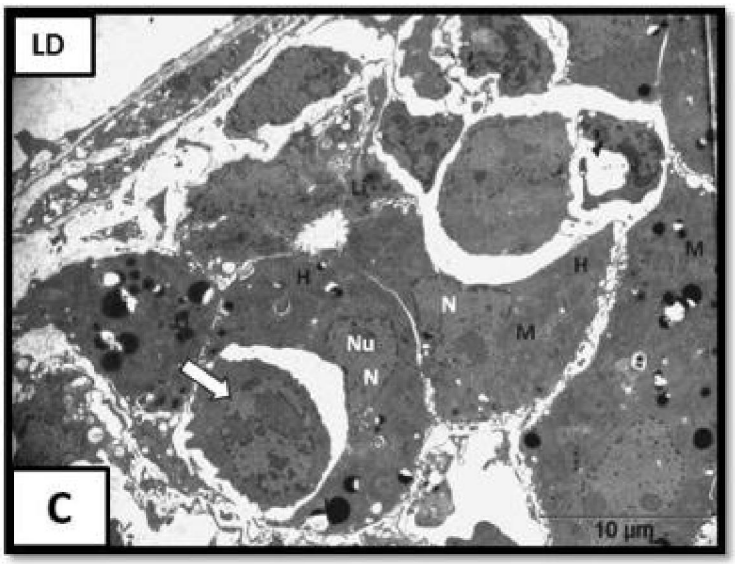


Figure 11: (A)TEM micrograph showing the ultrastructure of control neonate mouse liver (day 1). Polygonal hepatocytes (H) arranged radially around the central vein (CV) that is lined by endothelial cells (EC). The hepatocytes appeared containing a nucleus (N), nucleolus (Nu), glycogen rosettes (Gl) and lipid droplets (Li). The blood sinusoid (S) can be seen between the hepatocytes. The megakaryocytes (Me) can be observed with large and lobulated nucleus (N) (Uranayl acetate – lead citrate – 1800x).



(B) TEM micrograph showing the ultrastructure of (HD) neonate mouse liver (day 1). Shows the change in the shape of hepatocytes (H) from its natural form with euchromatic nucleus (N), nucleolus (Nu). Small amount of glycogen (Gl) can be seen. Increasing numbers of lipid droplets in cytoplasm (Li). Two megakaryocytes can be observed (Me1) necrotic megakaryocyte with pyknotic nucleus, (Me2) with large nucleus (N) (Uranayl acetate – lead citrate – 1400x).



(C) TEM micrograph showing the ultrastructure of (LD) neonate mouse liver (day 1). Hepatocytes (H) had a crescent shape were pushed by invading leucocytes (white arrow). The hepatocytes appeared containing a nucleus (N), nucleolus (Nu), mitochondria (M) and an increase in the numerous of lipid droplets (Li) (Uranayl acetate – lead citrate – 2200x).

Acknowledgement

The authors would like to thank King Abdulaziz City for science and technology for funding this research, and King abdulaziz University for providing labs and apparatus. Special thanks to Dr. Najia Alzanbagi for providing the animal house in the girl's campus at KAU.Dr. SawsanJalalah for helping with TEM, Dr. Suad Shaker for help with histology sections, Dr. Nahla El-shenawi for help with LPO, GSH and total protein essays.

Corresponding author:
Fatma Al-Qudsi,
Biology Department, Science Faculty, KingAbdulaziz University, Jeddah, Saudi Arabia P.O. Box,42650, Jeddah 21551, Saudi Arabia
falqudsi@kau.edu.sa

References

1. Abdullaev, F. I. (1993) Biological effects of saffron, *Biofactors*, vol. 4(2):83–86.
2. Abdullaev, F. I. (2002) Cancer chemopreventive and tumoricidal properties of saffron (*Crocus sativus*L.), *Experimental Biology and Medicine*, vol. 227(1):20-25.
3. Alarcon-Aguilara, F. J., Roman-Ramos, R., Perez-Gutierrez, S., Aguilar- Contreras, A., Contreras-Weber, C. C. and Flores-Saenz, J. L. (1998) Study of the anti- hyperglycemic effect of plants used as antidiabetics, *Journal Ethnopharmacology*, vol.61(2):101-110.
4. Ali, A., Shati, M. S. C., Saad, A. and Alamri, M. S. C. (2010) Role of saffron (*Crocus sativus L.*) and honey syrup on aluminum-induced hepatotoxicity, *Saudi Medical Journal*, vol. 31 (10): 1106-1113.
5. Al-Mofleh, L. A., Alhaider, A. A., Mosssa, J. S., Al-Sohaibani, M. O., Qureshi, S. and Rafatullah, S. (2006) Antigastric ulcer studies on 'saffron' Crocus sativus L. in rat, *Pakistan Journal of Biological Sciences*,vol. 9 (6):1009-1013.
6. Anane, R. and Creppy, E. E. (2001) Lipid peroxidation as pathway of aluminium cytotoxicity in human skin fibroblast cultures: prevention by superoxide dismutase+ catalase and vitamins E and C., *Human & Experimental Toxicology*, vol. 20: 477.
7. Anlin, M. A., Tieyong, W. U., Enyu, D. and *et al.* (2000) Prevention and Treatmentof Saffron on Experimental Alcoholic and CCI_4 Liver Injury in Wistar Rats, *Chinese Journal of Integrated Traditional and Western Medicine on Liver Diseases*, 06-019.
8. Arasteh, A., Aliyev, A., Khamnei, S., Delazar, A., Mesgari, M. and Mehmannavaz,Aytekin, A. and Acikgoz, A. O. (2008) Hormone and microorganism treatments in the cultivation of saffron (*Crocus sativus* L.) plants, *Molecules*, vol. 13: 1135-1146.
9. Basker, D. and Negbi, M. (1983) Uses of saffron Crocus sativus, *Econ. Biol.*, vol. 37(2): 228-236.
10. Beutler, E., Duron, O. and Kefly, B. M. (1963) Improved method for determination of blood glutathione, *Journal Laboratory and Clinical Med*icine, 61: 882-888.
11. Caligioni, C., (2009) Assessing reproductive status/stages in mice, *Current Protocols in Neuroscienc* A. 4I, 1–8.
12. Cantin, A. M., White, T. B., Cross, C. E., Forman d, H. J., Sokol, R. J. and Borowitz, D. (2007) Antioxidants in cystic fibrosis Conclusions from the CF Antioxidant Workshop, Bethesda, Maryland,*Free Radical Biology & Medicine*, vol. 42: 15 –31.
13. Çavusoglu, A., Erkel, I. E. and Sülüsoglu, M. (2009) Saffron (*Crocus sativus* L.) Studies with Two Mother Corm Dimensions on Yield and Harvest Period Under Greenhouse Condition, *American-Eurasian Journal of Sustainable Agriculture*, vol. 3(2): 126-129.
14. Chasis, J. A. (2006) Erythroblastic islands: Specialized microenvironmental niches for erythropoiesis, *CurrOpinHematol*, vol. 13: 137–41.
15. Chermahini, S. H., Abd. Majid, F. A., Sarmidi, M. R., Taghizadeh, E. and Salehnezhad, E. (2010) Impact of saffron as an anti-cancer and anti-tumor herb, *African Journal of Pharmacy and Pharmacology*,vol. 4(11), pp. 834-840.
16. Crawford, L. W., Foley, J. F. and Elmore, S. A. (2010) Histology atlas of the developing mouse hepatobiliary system with emphasis on embryonic days 9.5-18.5, *Toxicologic Pathology*, vol. 38: 872-906.
17. Cullen, J. M. (2007) *Liver, Biliary system and Exocrine pancreas*. In: Pathologic Basis of Veterinary Disease, 4th Edn., McGavin, M.D. and J.F. Zachary (Eds.). Mosby, London, pp: 403-406. http://www.elsevier.com/wps/find/bookdescription.
18. Deo, B. (2003) Growing saffron-the world's most expensive spice, *Crop & Food Research Ltd*, vol. 20: 11-23.
19. Dharmananda, S. (2005) Saffron: an anti-depressant herb, *Institute for Traditional Medicine*, from: http://www.itmonline.org/articles/saffron/ saffron.htm.
20. Drury, R. A. B. and Wallington, E. A. (1980) *Carleton's histolgical technique*, 5thm edition, Oxford,UK: Oxford University Press.
21. Duke, J. A. (1987) *Handbook of medicinal herbs*, KorasanJahadeKeshavarzi organization: CRC press.
22. Estilai, A. (1978) Variability in saffron (*Crocus sativus* L.), *Experientia*, vol. 34: 725.
23. Fatma Al-Qudsi and AmalAyedh. (2012) Effect of saffron on mouse embryo development**.** *J Am Sci*2012;8(12):1554-1568].(ISSN:1545-1003). http://www.jofamericanscience.org.
24. Gibb, R. P. and Stowell, R. E. (1949) Glycogen in human blood cells, *Blood*, vol. 4: 569– 79.
25. Gornall, A. G., Bardawill, C. J. and David, M. M. (1949) Determination of serum proteins by means of the biuret reaction,*The Journal of Biological Chemistry*, vol. 177(2): 751-766.
26. Hill, T. (2004) *The contemporary encyclopedia of herbs and spices: Seasonings forthe global kitchen*, *274*, New Jersey: Wiley.

27. Hosseinzadeh, H. and Noraei, N. B. (2009) Anxiolytic and hypnotic effect of *Crocus sativus* aqueous extract and its constituents, crocin and safranal, in Mice, *Phytother.Res.*, vol. 23: 768-774.
28. Hosseinzadeh, H., and Younesi, H. (2002) Antinociceptive and anti- inflammatory effects of *Crocus sativus* L. stigma and petal extracts in mice, *BioMed Central Pharmacology*, vol. 2: 7.
29. Hosseinzadeh, H., Sadeghnia, H. S., Ziaee, T. and Danaee, A. (2005) Protective effect of aqueous saffron extract (*Crocus sativus* L.) andcrocin, ischemia-reperfusion- induced oxidative damage in rats, *Journal of Pharmacy & Pharmaceutical Sciences*, vol. 8(3):387-393.
30. Isern, J., Fraser, S. T., He, Z., and Baron, M. H. (2008) The fetal liver is a niche for maturation of primitive erythroid cells, *ProcNatlAcadSci U S A*, vol. 105: 6662–6666.
31. Khayatnouri, M., Safavi, S. E., Safarmashaei, S. Babazadeh, D. and Mikailpourardabili, B (2011) The effect of Saffron Orally Administration on Spermatogenesis Index in Rat, *Advances in Environmental Biology*, vol. 5(7): 1514-1521.
32. Khorasani, G., Hosseinimehr, S. J. ,Zamzni, P. , Ghasemi, M. , and Ahmadi, A. (2008) The Effect of Saffron (*Crocus sativus*) Extract for healing of second- degree burn wounds in rat, *Keio Journal Med.*, vol. 57 (4):190-195.
33. McGee, H. (2004) *On food and cooking: The science and lore of the kitchen*, *422*, New York: Scribner.
34. Mercer, E. H. and Birbeck, M. S. (1972) *Electron microscopy; Microscopy, Electron; Technique*, 3rd edition, Blacwell Scientific (Oxford): United Kingdom.
35. Mohajeri D., MesgariAbbasi M., Delazar A., Doustar Y., Mousavi, Gh. and Amouoghli Tabrizi B (2009) Histopathological study of subacute toxicity of *Crocus sativus* L. (Saffron) stigma total extract on liver and kidney tissues in the rat, *Pharmaceutical Sciences Journal*, 15(2): 115-124.
36. Mohajeri, D. ,Mousavi, G., Mesgari, M., Doustar, Y. and Nouri, M. H. K. (2007) Subacute toxicity of *Crocus sativus* L. (saffron) stigma ethanolic extract in rat, *American Journal Of Pharmacology and Toxicology*, vol. 2 (4):189-193.
37. Mohajery, D., Mousavi, G. and Doustar, Y. (2009) Antihyperglycemic and pancreas protective effects of *Crocus sativus* L. (saffron) stigma ethanolic extract on rats with alloxan-induce diabetes, *Journal of Biological Sciences*, vol. 9(4): 302-310.
38. Nair, S. C., Pannikar, B. and Panikkar K. R. (1991) Antitimour activity of saffron (*Crocus sativus)*, *Cancer Letters*, vol. 57(2):109-114.
39. Naito, M., Hasegawa, G., Ebe, Y. andYamamoto, T. (2004) Differentiation an function of Kupffer cells, *Med Electron Microsc*, vol. 37(1):16-28.
40. Negbi, M. (1999) *Saffron Crocus sativus L.*, 1-59, Amesterdam: Harwood academic publishers.
41. Ohkawa, H., Ohishi, N. and Yagi, K. (1979) Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction, Analytical Biochemistry, vol. 95(2): 351–358.
42. Premkumar, K. , Abraham, S. K. , Santhiya, S. T. and Ramesh, A. (2003a) Inhibitory effects of aqueous crude extract of saffron (*Crocus sativus* L.) on chemical-induced genotoxicity in mice, *Asia Pacific Journal of Clinical Nutrition*,12 (4):474-476.
43. Premkumar, K., Abraham, S. K., Santhiya, S. T. and Ramesh, A. (2003b) Protective effects of saffron (*Crocus sativus*L.)on genotoxins-induced oxidative stress in Swiss albino mice, *Phytotherapy Research*, 17:614-617.
44. Reynold, E. S. (1963) Theuse of lead citrate at high phase an electronopaque stain in electron microscopy, *Journal Cell Biology*, (17): 208-212.
45. Salomi, M. J. (1991) Inhibitory effects of *Nigella sativa and saffron* (*Crocus sativus*) on chemical carcinogenesis in mice, *Nutr. Cancer*, vol. 16(1):67-72.
46. Sasaki, K. and Matsumura, G. (1986) Haemopoietic cells of yolk sac and liver in the mouse embryo: A light and electron microscopical study, *J Anat*, vol. 148: 87–97.
47. Tafazoli, M. , Kermani, T. and Sadat Jou, S. A. R. (2004) Effect of saffron on abnormal and its side effect on mice balb/c, *Ofogh-E-Danesh Fall*, vol. 10(3):53-56.
48. Thatte, U., Bagadey, S. and Dahanukar, S. (2000) Modulation of programmed cell death by medicinal plants, Cellular and Molecular Biology (Noisy-le-grand), vol. 46:199-214.
49. Tilghman, S. M., and Belayew, A. (1982) Transcriptional control of the murine albumin/alpha-fetoprotein locus during development, *Proc. Natl Acad. Sci. USA*, vol. 79: 5254–5257.
50. Tso, P. and McGill, J. (2003) "The Physiology of the Liver", In, A. George, Tanner and A. Rodney Rhoades *Medical Physiology*, Second edition, Lippincott, Williams & Wilkins: (in press), 514-523.
51. Walmer, D. K., Wrona, M. A., Hughes, C. L., Nelson, K. G. (1992) Lactoferrin expression in the mouse reproductive tract during the natural estrous cycle: correlation with circulating estradiol and progesterone, *Endocrinology*, 131:1458–1466.
52. Van Tellingen, C. (2003*) Physiology: organ physiology from a phenomenological point of view*, *28-38*, Driebergen: Louis Bolk Institute.
53. Verma, R. S. and Middha, D. (2010) Analysis of Saffron (*Crocus sativus* L. Stigma) Components by LC–MS–MS, *Chromatographia*, vol. 71: 117–123.
54. Zargari, A. (1993) *Medicinal plants*, 574–578, Tehran: Tehran University Press.

12/22/2012