Revista de Chimie
https://revistadechimie.ro
https://doi.org/10.37358/Rev.Chim.1949

Detraction of Diclofenac-Associated Hepatotoxicity by Fresh Beet Root Juice in Male Albino Mice

TAHANI MOHAMAD ALHAZANI[1], BADR ABDULLAH ALDAHMASH[1], DOAA MOHAMED EL-NAGAR[1,2], KHALID ELFAKI IBRAHIM[1], SAHEED OLAIDE ANIFOWOSE[1], AHMED MOSTAFA RADY[1*]
[1]King Saud University, College of Science, Zoology Department, P.O. Box 2455, Riyadh 11451, Saudi Arabia
[2]Zoology Department, Faculty of Women for Arts, Science and Education, Ain Shams University, Cairo, Egypt

Abstract: *The beet root as dietary supplement hepatoprotective ability has gained interest in recent days. The present study was designed to determine the potential hepatoprotective effect of beet root juice as anti-inflammatory and anti-oxidant agent to eliminate the hepatotoxic effect of diclofenac as wide spread analgesic agent. Male albino mice were divided randomly into 4 groups, the 1st group served as control group, the 2nd group received 8 ml/kg of freshly prepared beet root juice, the 3rd group received oral administration 20 mg/kg of diclofenac and the 4th group pre-treated with beet root before one-hour diclofenac administration for 30 days. Biochemical results revealed sharp significant raised levels of liver enzymes level (AST, ALT, ALP and GGT) in the 3rd group that received diclofenac, besides to marked pathological changes manifested by high pathological scoring system such as hepatocytes degeneration, ballooning, infiltration and fibrosis. Immunohistochemical analysis elucidated massive incidence of MDA as an indicator of oxidative stress, moreover great number of neutrophils were seen as main component of inflammation. Whereas, pre-treatment of beet root juice one hour before diclofenac resulted in significant decrease of liver enzymes, clear attenuation of pathological features, decrease of pathological score. A great reduction of MDA in liver tissue and number of neutrophils stained histochemically. It was concluded that beet root juice possessed beneficial hepatoprotective role against diclofenac, as significant anti-oxidant and anti-inflammatory effect.*

Keywords**:** *Hepatotoxicity, diclofenac, beet root, inflammation, oxidative stress*

1.Introduction

Diclofenac is a phenylacetic acid derivative used as anti-inflammatory and analgesic drug to treat many diseases as inflammatory disorders, fevers and rheumatoid arthritis [1]. Many studies proved that long term administration of diclofenac like non-steroidal anti-inflammatory drugs resulted in significant incidence of liver injury, that Liver is an important target for drugs such as chemicals and xenobiotics, caused damage that it regulates metabolic functions [5,19]. Hepatotoxicity induced by diclofenac may be attributed to oxidative stress production and hepatocytes apoptosis [11, 13]. Diclofenac is considered one of the drugs accompanied with idiosyncratic hepatotoxicity that unrelated to its pharmacology but depend on duration of exposure [27].

Some herbs, fruits and vegetables achieved an impact role in liver problems improvement [25]. Beetroot is an edible vegetable rich with antioxidants as betalains that including red betacyanins mainly betanin [14]. Betanin possess high antioxidant effect because of its high ability to donate electrons and then its free-radical scavaging capacity [20]. Some experiments proved that betanin had preventive effect against lung and skin carcinogenesis [2, 15], besides to reduction of liver injury induced by carbon tetra chloride [18]. Betanin could regulate ROS production and DNA injury, also improved enzymic antioxidant profiles [16, 29]. Betanin seemed to be radioprotective against gamma irradiation, besides to reduction of heme decomposition and production of peroxides [14].

As such, the present study investigated the potential anti-oxidant and anti-inflammatory effect of beet root juice as dietary supplement to detract the hepatotoxicity diclofenac- induced.

*email: rady_gad1983@yahoo.com

2.Materıals and methods

2.1. Animals and Experimental design

Male albino mice (25±5 g) were obtained from King Saud University animal house were kept at 22±2ºC with free access of food and clean water, exposed to 12h light-dark cycle. After 1 week adaptation period, mice were randomly divided into four groups, the first group served as control, the second group administered daily oral dose of 8 mL/kg of freshly prepared beet root juice, the third group administered daily oral dose of 20 mg/kg of diclofenac, the fourth group administered daily oral combined dose of beet root juice one-hour pre-administration to diclofenac. The duration of experiment was 30 days; all mice were sacrificed one-day post to the last dose. All procedures were carried out by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) and the National Institutes of Health (NIH) in compliance with the requirements set out in the Guidelines for the treatment and use of experimental animals.

2.2. Liver index

All mice and its livers were weighed; liver index was calculated by dividing the liver weight of the mouse over its body weight multiplied by 100. Data were subjected to statistical analysis.

2.3. Biochemical analysis

The collected sera samples subjected to measurement of alanine aminotransferase (ALT), asparate aminotransferase (AST), alkaline phosphatase (ALP), and gamma-glutamyl transferase (GGT) levels via UV-VIS Spectrophotometer.

2.4. Histopathological Analysis

Liver was cut into small cubic pieces and fixed in 10% buffered neutral formaldehyde, embedded in wax, sections were cut at thickness of 5µm, stained with Hematoxylin-Eosin, Masson's trichrome, examined and microphotos were taken by Motic-2000 microscope.

2.5. Pathological scoring System

For the microscopic analysis, the liver fragment slides were stained with hematoxylin and eosin (HE). The scoring system was recommended by the Pathology Committee of NASH Clinical Research Network. Steatosis, normal (<5%)= 0, 5–33%=1, 33–66%= 2 and >66% =3, Inflammation, none = 0, <2 foci per 200 field = 1, 2–4 foci per 200 field = 2, >4 foci per 200 field = 3, Ballooning, none= 0, few balloon cells= 1, many cells= 2, prominent balloon cells= 3, Fibrosis, none=0 and 1=fibrosis, the overall results represented as follows 0 =normal, 1 = mild, 2 =moderate and 3 = severe (17).

2.6. Immunohistochemistry

Liver paraffin sections were cut at 5 µm, then mount on electrostatic glass slides, after deparaffinization and rehydration sections were masked in heated citrate buffer (*p*H 6) in microwave for 5 min. Sections washed with PBS buffer, then incubated in peroxidase blocking solution for 10 min, followed by primary antibody incubation (Anti-NIMP-R14 (ab2557) specific for neutrophils & Anti-MDA (ab 94671) specific for oxidative stress) over night at 4˚C, continued by secondary antibody incubation for 30 min, then followed by avidin-biotin complex incubation for 30 min, then DAB incubation for 10 min , followed by Mayer's haematoxylin counter stain . Sections were dehydrated and cleared, then covered with slip based on DPX mount. Liver sections were examined for immune reactivity and filming. Sections stained against (Anti-NIMP-R14) subjected to (IHC Tool Box – Image J system) for nuclei stained with H-DAB for neutrophils counting in 20 field x 200 mag per each group, then results analyzed statistically by SPSS.

2.7. Statistical analysis

Data expressed as mean±SEM, data was subjected to ANOVA test by (SPSS 17), values are considered significant at $p \leq 0.05$.

3. Results and discussions

3.1. Units, Abbreviations and, Acronyms

The liver is a vital organ concerned with main functions of the body as metabolism and detoxification, so it may be prone to damage as a result of drugs [7]. Diclofenac is an example of more widely used non-steroidal drugs all over the world that caused hepatotoxicity [27]. In the present study, liver index showed no significant difference between all experimental groups against control group (Table 1). Mice group received diclofenac showed very high liver enzymes levels (195, 79, 290 and 20.6 respectively). Daily oral administration of diclofenac for 30 days' lead to sharp rising of liver enzymes AST, ALT, ALP, and GGT in the blood serum that was coincided with [1, 19, 21]. This hepatocellular damage caused leaking of cellular enzymes into the plasma due to transport functions disturbance of inured hepatocytes resulted in increased levels of liver enzymes (AST, ALT, ALP and GGT) in the blood serum [21].

In the current study, histological examination of liver section of mice received oral dose of diclofenac for 30 days displayed marked pathological changes manifested by dilated vein congested with hemorrhage and edema, besides to moderate inflammation scored [2] with abundant migrated neutrophils, severe ballooning of hepatocytes scored [3] (Figure 1c), and moderate fibrosis scored [2] consisted of blue stained collagenous fibers surrounded dilated vein (Figure 2c). Diclofenac administration resulted in severe pathological changes reflected by loss of characteristic configuration of liver manifested by severe ballooning of hepatocytes, inflammation, dilatation and congestion of portal vessels besides to fibrosis incidence. These results were agreed with many studies findings, who observed significant pathological changes of liver characterized by focal areas of necrosis, mononuclear cells infiltration, bile ducts proliferation, dilatation of vessels and small pyknotic nuclei as signs of apoptosis [4, 19]. Diclofenac undergoes some chain reactions as glicuronidation by cytochrome P_{450} to form unstable diclofenac acyl glucuronides which might be oxidized by CYP2C8 to form 5-hydroxyldiclofenac, when diclofenac acyl glucuronide and metabolites transported to biliary canaliculi and accumulated, generate oxidative stress and mitochondrial injury leading to hepatocellular damage [23]. The present work proved that diclofenac administration lead to accumulation of collagenous fibers in the liver tissue that followed hepatocellular damage due to the activation of stellate cells in the sinusoids after hepatic cells damage that characterized by proliferation and contractility and then hyper production of extracellular matrix and collagen fibers leading to fibrosis, that exactly coincided with Taha et al., findings who declared that diclofena receiving caused hepatocellular damage , increased depositions of collagenous fibers around portal tracts and central veins [27].

Some investigations declared that diclofenac administration induced marked increase of MDA content as lipid peroxidation [3, 9], which harmonized with the present findings, that liver section of mice received diclofenac showed intense dark brown color as strong immune response to MDA (Figure 3c) as an evidence to strong oxidative stress incidence, also great number of abundant inflammatory neutrophils stained dark brown (count 133) were seen as positive response against NIMP-R14 antibodies (Figure 4c) ensured heavy incidence of inflammation. Administration of diclofenac for 30 days resulted in confluent incidence of MDA in liver tissue as a result of lipid peroxidation of oxidative stress. Diclofenac administration caused acute hepatitis-like hepatotoxicity associated with focal necrosis and replacement of degenerated hepatocytes with leukocytic inflammatory infiltration consisted mainly of neutrophils and lymphocytes [6]. The present work agreed with the previous results that diclofenac administration resulted in massive accumulation of inflammatory cells mainly neutrophils that reached high record.

Betalains considered the main constituent of beet root which are vacuolar N-heterocyclic pigments known as betalamic acid that oxidized many times to form violent betacyanins and yellow betaxanthins

which finally accumulated in the beet root plant [16]. A number of studies emphasized that betalains possessed antioxidant activity, in vitro bioactivity and clinical efficacy in animal models [12, 22]. Liver enzymes (AST, ALT, ALP and GGT) of mice group received beet root juice displayed no significant difference (67, 36, 227 and 3.8 respectively) against control group (66, 35, 226 and 3.6 respectively). Moreover, mice group treated with beetroot juice one-hour prior to diclofenac dose showed significant decrease in the enzymes levels versus mice group received diclofenac only (95, 43, 260 and 10.8 respectively) (Table 1). The present work proved that pre-treatment with beetroot juice one-hour before diclofenac administration resulted in decreasing of liver enzymes levels against mice group received only diclofenac that due to the healthy hepatic cells because of the protection of beetroot juice to liver against diclofenac.

Table 1. Liver index, liver enzymes (AST, ALT, ALP and GGT) between mice groups received beetroot, diclofenac and the group treated with beet root one hour prior to diclofenac

Sample	Parameter				
	Liver index	AST	ALT	ALP	GGT
Control	7.3±0.2	66±0.2	35±0.2	226±0.4	3.6±0.2
Beet root	7±0.3	67±0.2	36±0.2	227±0.5	3.8±0.2
Diclofenac	7±0.3	195±0.3^{*a}	79±0.4^{*a}	290±0.5^{*a}	20.6±0.6^{*a}
Diclofenac + beetroot	7.5±0.2	95±0.5^{*a,b}	43±0.6^{*a,b}	260±0.8^{*a,b}	10.8±0.3^{*a,b}

Data=mean±SEM, *a=significant difference against control group, *b=significant difference against diclofenac group

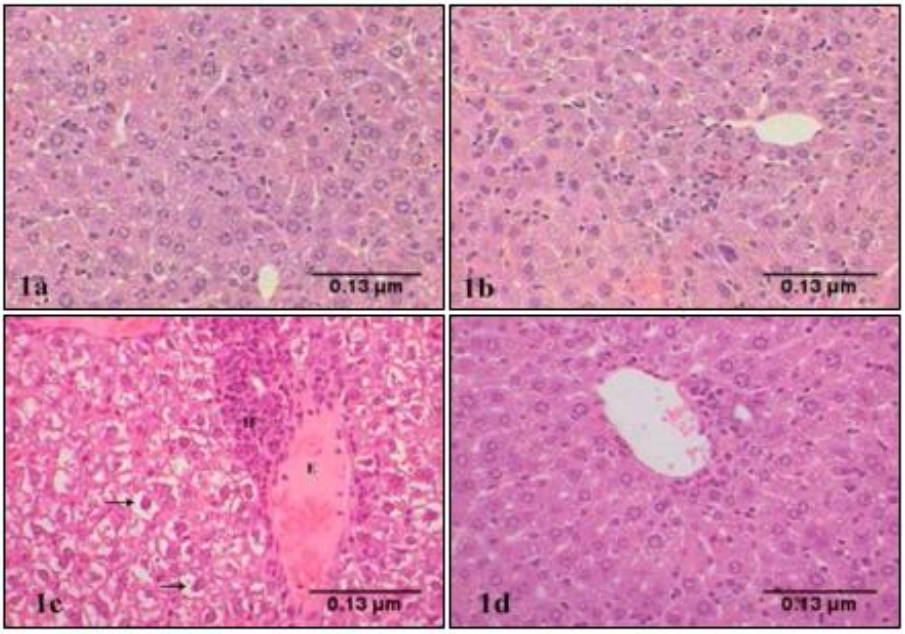

Figure 1. Hx&E-400 staining of liver sections. 1**a**: Photomicrograph of control mice liver showing normal liver structure. **1b**: Photomicrograph of mice liver received 8 mL/kg of beet root juice showing healthy liver tissue. 1**c**: Photomicrograph of mice liver Received 20 mg/kg of diclofenac showing congested vein with edema (E) surrounded by aggregations of inflammatory cells (IF), cytoplasmic degeneration and ballooning of hepatocytes (arrows). **1d**: Photomicrograph of mice liver pre-treated with beet root juice one hour before diclofenac administration showing healthy hepatocytes and a few number of inflammatory cells

Rats feed on betalain rich red beet extract showed mitigated hepatic toxicity caused by NDEA, CCl_4, and DMBA was recorded through many investigations [18, 26, 30]. control and mice group received freshly prepared beetroot juice showed normal liver consisted of central veins, strands of hepatocytes and blood sinusoids in between, pathological score revealed (0) that no pathological signs were seen (Figures 1a, 1b, 2a and 2b). Moreover, liver section of mice pretreated with beetroot juice one-hour

before diclofenac administration revealed improved healthy section characterized by relative healthy hepatocytes with some bi-nucleated cells, no steatosis was seen scored (0), a few number of inflammatory cells were abundant that inflammation scored (1), ballooning scored (1) (Table 2) that a few number of hepatocytes showed ballooning (Figure 1d), also no fibrosis was detected scored (0) (Figure 2d). The present study proved that pre-treatment of beet root juice before diclofenac resulted in potent decrease of pathological signs in liver that caused by diclofenac which manifested by healthy hepatocytes, less ballooning, inflammation and fibrosis.

Table 2. Showing the liver pathological score and count of neutrophils in control and experimental groups

Sample	Parameter				
	Steatosis	Inflammation	Ballooning	Fibrosis	Count of neutrophils
control	0	0	0	0	0±0
beetroot	0	0	0	0	0±0
diclofenac	0	2	3	1	133±0.3^{*a}
Beetroot and diclofenac	0	1	1	0	10±0.2^{*a,b}

*a=significant difference against control group,
*b=significant difference against diclofenac group

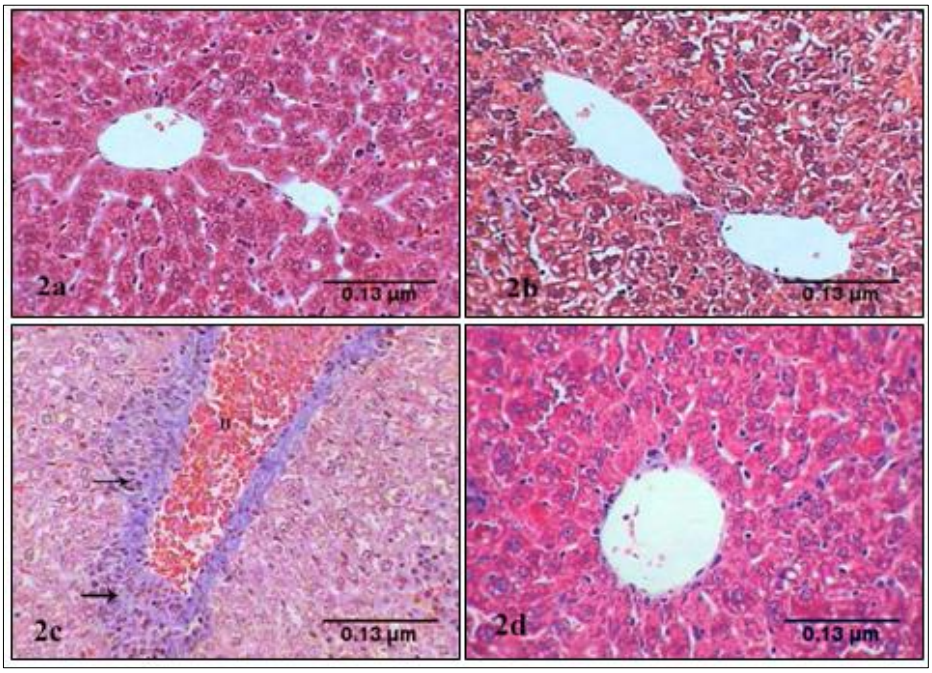

Figure 2. Photomicrograph of the liver sections using M. tr.-400. 2a: Photomicrograph of control mice liver showing no fibrosis. 2b: Photomicrograph of mice liver received 8 mL/kg of beet root juice showing no fibrosis. 2c: Photomicrograph of mice liver received 20 mg/kg of diclofenac showing dilated vein congested with hemorrhage (H) and collagenous fibers mixed with leukocytes (arrows). 2d: Photomicrograph of mice liver pre-treated with beet root juice one hour before diclofenac administration showing no collagenous fibers. (M.tr.-400)

Many studies displayed that betalain possess radical-scavenging activity which is the ability to delay or prevent oxidation of carbohydrates, proteins, lipids and even DNA caused by reactive nitrogenous species and reactive oxygen species [8, 28]. Preliminary evidence came from some studies ensured that betalain is considered one of the effective inhibitors of lipid peroxidation and DNA damage induced by many factors as H_2O_2 and peroxynitrite, not only by its reactive scavenging activity but also by

transactivation of paraoxonase 1, which is an antioxidant enzyme produced in the liver [10]. Liver of control and mice group received beetroot juice showed negative immune response against MDA (Figure 3a,3b) and NIMP-R14 antibodies (Figure 4a, 4b), that no oxidative stress and neutrophils as main component of inflammatory cells were detected. Moreover, liver section of mice treated with beetroot juice one-hour before diclofenac administration revealed weak immune reactivity against MDA (Figure 3d) and a few number of neutrophils (count 10) (Figure 4d) as evidence to less oxidative stress and less inflammation. The present work coincided with the previous results, that the present results declared that pre-treatment of beet root juice one hour before diclofenac administration resulted in clear decreasing of MDA which is the main product of lipid peroxidation, as an evidence that beet root juice could decrease oxidative stress. Beet root contains many compounds such as betalain besides to carotenoids, glycine, saponins, polyphenols and flavonoids are responsible for free radical scavenging anti-oxidant activity that promotes highly significant anti-inflammatory effect [24]. The present work proved that beet root juice was significantly attenuated leukocytic inflammation in liver diclofenac- induced manifested by sharp decrease of neutrophils number

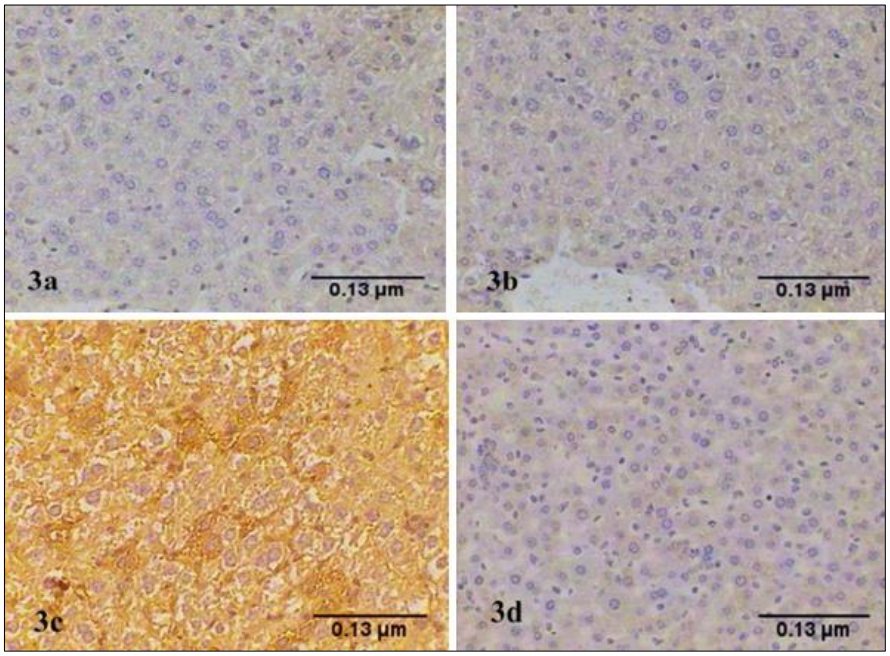

Figure 3. Photomicrograph of liver sections using (Avidin-Biotin Complex- 400)
3a: Photomicrograph of control mice liver showing negative immune response against MDA. **3b:** Photomicrograph of mice liver received 8 mL/kg of beet root juice showing no immune response against MDA. **3c:** Photomicrograph of mice liver received 20 mg/kg of diclofenac showing intense immune reactivity against MDA. **3d:** Photomicrograph of mice liver pre-treated with beet root juice one hour before diclofenac administration showing weak immune response against MDA. (ABC- 400)

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juice one-hour before diclofenac administration revealed weak immune reactivity against MDA (Figure 3d) and a few number of neutrophils (count 10) (Figure 4d) as evidence to less oxidative stress and less inflammation. The present work coincided with the previous results, that the present results declared that pre-treatment of beet root juice one hour before diclofenac administration resulted in clear decreasing of MDA which is the main product of lipid peroxidation, as an evidence that beet root juice could decrease oxidative stress. Beet root contains many compounds such as betalain besides to carotenoids, glycine, saponins, polyphenols and flavonoids are responsible for free radical scavenging anti-oxidant activity that promotes highly significant anti-inflammatory effect [24]. The present work proved that beet root juice was significantly attenuated leukocytic inflammation in liver diclofenac- induced manifested by sharp decrease of neutrophils number.

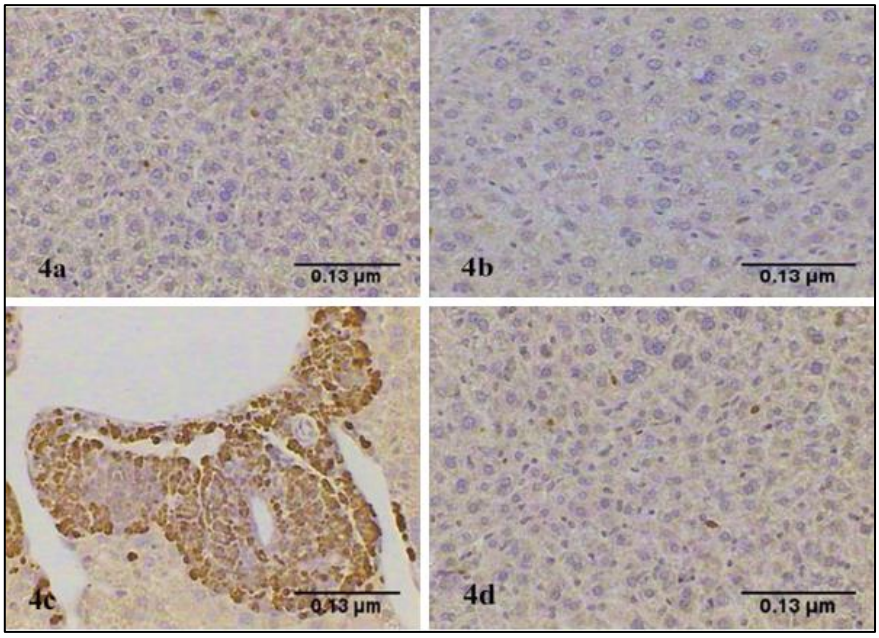

Figure 4. Photomicrograph of liver sections using (Avidin-Biotin Complex- 400).
4a: Photomicrograph of control mice liver showing no neutrophils.**4b**: Photomicrograph of mice liver received 8 mL/kg of beet root juice showing no neutrophils. **4c**: Photomicrograph of mice liver received 20 mg/kg of diclofenac showing a great number of neutrophils.
4d: Photomicrograph of mice liver pre-treated with beet root juice one hour before diclofenac administration showing a few number of neutrophils. (ABC- 400)

4.Conclusıons

It was concluded that beet root juice possessed beneficial hepatoprotective role against diclofenac, as significant anti-oxidant and anti-inflammatory effect.

Acknowledgement: This article is funded by King Saud University, Riyadh, Saudi Arabia, Researchers Support Project (RSP-2020/214).

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Mnauscript received: 8.12.2021