

Anti-fibrotic and antioxidant ameliorative effect of Naringenin against thioacetamide induced liver fibrosis

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Abstract

This work conducted to evaluate the anti-fibrotic and antioxidant role of naringenin (NAR) toward thioacetamide (TAA) induced liver fibrosis. Fifty adult male albino rats randomly divided into 5 groups (10 each); first group kept as control one; the second treated I/P by 200 mg/kg TAA twice a weeks for 8 weeks; the third was gavaged daily with 50 mg /kg/ b.wt of NAR; the fourth was co-treated by TAA and NAR while the fifth was treated with TAA for 8 w then gavaged daily by NAR for 1 month. TAA administration significantly increases the hepatic cell enzymes (ALT, AST, ALP and GGT) in the serum referring to hepatic cell destruction with an increase in hepatic MDA with a reduction in GSH concentrations, antioxidant enzyme activities as well as down regulation of their expression levels. NAR administration either with or after TAA ameliorates this effect suggesting its antioxidant ability. In the fibrotic pathway, TAA treatment up-regulates the expression levels of fibrogenic biomarkers (TGF- β , collagen 1 α and fibronectin) genes while NAR down-regulates these genes suggesting its anti-fibrotic ability. Histopathological analysis confirms the biochemical results. In conclusion, NAR ameliorates the deleterious effect of TAA through its antioxidant and anti-fibrotic abilities.

Introduction

Liver is the main metabolic regulatory and detoxifying organ in the body (Dutta et al 2021). Liver diseases including hepatitis and liver cancers representing a global health problems, threatening lives with great loses in health and money (Asrani et al 2019).

Fibrosis of liver cells pathologically is a process leading to hepatic cell failure, cirrhosis with a tendency to develop hepatocarcinoma (HCC). Liver fibrosis is characterized primarily by increasing the extracellular matrix proteins deposition such as collagen in hepatic cells leading to hepatic architecture distortion with nodular formation, alteration of blood flow leading to portal hypertension and finally hepatic failure (Sharma and Nagalli 2020; Akdemir et al 2016). The major fibrotic biomarkers are collagen, fibronectin, nidogen, laminin and transforming growth factor beta-1 (TGF- β 1) (Moustafa and Hussein 2016).

Most of chronic hepatic diseases such as viral hepatitis, alcoholic abuse, metabolic diseases, biliary diseases, and parasitic diseases such as Schistosomiasis, chronic drugs and toxins are associated with hepatic cell fibrosis and characterized pathologically by inflammation with parenchymal damage. (Mormone et al 2011). Till now no satisfactory anti-fibrotic substances can be used for treatment or prevention of liver fibrosis despite a great progress in the mechanisms and treatment of liver fibrosis.

Experimentally, the classical hepatotoxic agent Thioacetamide (TAA) is usually used for hepatic fibrosis and cirrhosis induction in rats. TAA in acute applications results in centrilobular damage and liver disease (Al-Attar and Al-Rethea 2017; Dođru-Abbasođlu et al 2001) while in chronic applications results in liver cirrhosis (Natarajan et al 2006; Liedtke et al 2013) through the induction of oxidative stress that resulted in membrane damage with lipid droplets accumulation in the cytoplasm of hepatocyte which enhance the induction of liver injury and inflammation in rats (Ipsen et al 2018).

Till now, medicinal plants still representing a part of human culture and many of human population depends on it as a primary aspect of health care especially as antioxidant protecting the body from oxidative stress and reactive oxygen species (Ali et al 2014). The components of natural plants have been extensively studied and its role against many illness have been approved including liver diseases due to their important role in prevention of fibrosis and thereafter in cirrhosis (Casas-Grajales and Muriel 2015). Recently, the interest in natural antioxidants has expanded extensively as a result of their valuable impacts of avoidance and hazard decrease (Siger et al 2012). It is approved that, antioxidants consumption is related to a decreased danger of a few sicknesses, for example, cardiovascular, cancer and liver diseases (Pandey and Syed 2009).

Naringenin (NAR) is a bioflavonoid (4', 5, 7-trihydroxy flavanone) present in citrus fruits (oranges, lemons, grapefruit, tangerines) tomato, cherries, grapefruit and coca (Hussein et al 2015; Yen et al 2009). Some reports has been demonstrated the NAR use for prevention of experimentally induced acute liver damage by carbon tetrachloride (CCl₄), heavy metals, alcohol or lipopolysaccharide (Pinho-Ribeiro et al 2016; Ozkaya et al 2016). Also, there is some other reports associated NAR with anticancer and anti-fibrotic properties (Arul and Subramanian 2013; Mershiba et al 2013). NAR was reported to have the ability to prevent deposition of collagen in rat's haptic cells treated by diethyl-nitrosamine (DEN) but with no clear explained mechanism (Lee et al 2004). Du et al., reported that, NAR can induce anti-fibrotic effect in lung tissues due to down-regulation of TGF- β activity (Du et al 2009). However, according to our knowledge, there is no studies on the anti-fibrotic effect of NAR against liver fibrosis. Therefore, the main purpose of our interest was directed to investigate the possible protective effects of NAR and its mechanism of protection against TAA induced liver fibrosis

Material And Methods

Chemicals and reagents

TAA, Naringenin and all chemicals used were purchased from Sigma-Aldrich Chemical Co., (St. Louis, MO, USA).

Animal selection and housing

Fifty male adult albino rats, within 6 months age and average weighting (120 ± 15) gm were purchased from the breeding unit of laboratory animal farm (Helwan, Egypt) and housed in a standard cages in groups of 5 rats per cage under temperature ($24 \pm 2^\circ\text{C}$) with a relative humidity between (50–60%) and light/dark cycle (12:12hr) in the faculty of Vet. Med. Zagazig University, Egypt at animal House. The animals were kept on a standard diet with water ad libitum.

Experimental design and animals grouping

After acclimatization period, the animals were randomly divided into 5 groups (10 each).

Group 1 (control group): didn't receive any treatment all over the experiment

Group 2 (TAA treated group): treated I/P with 200 mg/kg thioacetamide (TAA) twice a week for 8 w according to Bruck, et al 2001

Group 3 (NAR treated group): gavaged daily with 50 mg /kg/ b.wt NAR according to Mershiba et al 2013

Group 4 (preventive group): co-treated by TAA and NAR by the previous mechanisms for 8 w

Group 5 (treated group): treated with TAA for 8 w then gavaged daily by NAR for 1 month.

Sampling

After the end of the experiment, the animals were decapitated, blood samples were collected and centrifuged at 3.000 rpm for 10 min. to obtain serum samples needed for determination of serum ALT, AST, ALP and GGT. The serum were stored at -20°C until used.

Hepatic tissues were also collected rapidly after decapitation, washed in normal saline and separated into 3 parts for Molecular, biochemical, and hitopathological evaluations.

The first part was taken, weighted and kept in liquid nitrogen to follow up the changes of gene expression of TGF- β 1, Collagen 1 α , Fibronectin, CAT, SOD and GPx

The second part accurately weighed and homogenized in Potter–Elvehjem tissue homogenizer for antioxidant status evaluation.

The third part was taken and kept in 10% formalin for histopathological examination.

Biochemical determination

Serum hepatic enzymes were determined using their corresponding commercial kits according to the methods of Breuer 1996 for ALT and AST and Moss et al 1987 for ALP and GGT.

Malonyldialdehyde (MDA) the lipid peroxidation marker was determined in accordance to the method of Buege and Aust 1978. GSH (reduced glutathione) was also determined according to the method of Ellman 1959.

Glutathione peroxidase GPx, Glutathione reductase, SOD, and CAT were also determined in accordance to the methods that described in Hussein et al 2015. All biochemical parameters were measured using spectrophotometer (Shimadzu UV 120-02).

Molecular determination

Hepatic total RNA of all groups was isolated using RNeasy Mini kit (Qiagen) (Cat. No. 74104) and following the manufacture instructions.

Checking the quantity and purity of isolated RNAs performed OD260/OD280 using ND-1000 Spectrophotometer NanoDrop®. RNA samples which have a purity of 1.8 or more only were used for the

synthesis of cDNA using RevertAid™ H Minus (Fermentas, USA).

One µl of cDNA with 1 µl of each primer (10 pmol/µl), 9.5 µl of RNase-free water and 12.5 µl of SYBR Green Master Mix to reach a total volume of 25 µl was used for Semi-quantitative RT-PCR using a Rotor-Gene Q cycler (Qiagen) using a BioRad® SYBR® Green PCR Kit.

The primer sequences and thermal cycler profile for TGF-β1, Collagen 1α, Fibronectin, CAT, SOD, GPx and β-actin were listed in Table 1. The relative fold changes ($2^{-\Delta\Delta C_t}$) were calculated in accordance to the method of Livak and Schmittgen 2001.

Table 1
The designed forward and reverse primer sequences and RT-PCR conditions

Genes	Primer sequence	Annealing temp./ number of cycles	Accession number
TGF-β1	F 5'- GGGCTACCATGCCAACTTCTG - 3' R 5'- GAGGGCAAGGACCTTGCTGTA-3'	60°C / 30 cycles	NM_021578.2
Collagen 1 α	F 5'- GACATGTTTCAGCTTTGTGGACCTC- 3' R 5'- GGGACCCTTAGGCCATTGTGTA-3'	59°C/ 60 s (40 cycles)	NM_053304.1
Fibronectin	F 5'-TGGCTGCCTTCAACTTCTC-3' R 5'-AGTCCTTTAGGGCGGTCAAT-3'	58°C/ 60 s (40 cycles)	NM_019143.2
CAT	F 5'-GTCCGATTCTCCACAGTCGC-3' R 5'- CGCTGAACAAGAAAGTAACCTG-3'	58°C/ 60 s (30 cycles)	S50336.1
SOD	F AAGCATGGCGATGAAGG-3'-5' R GAGACTCAGACCACATAGGGA- 3'-5'	55°C/ 60 s (25 cycles)	NM_017050.1
GPx	F 5'-CACAGTCCACCGTGTATGCC-3' R 5'-AAGTTGGGCTCGAACCCACC- 3'	55°C- 60 s (28 cycles)	Z21917.1
β-actin	F 5'- ACCACAGCTGAGAGGGAAATCG-3' R 5'- AGAGGTCTTTACGGATGTCAACG- 3'	59°C/ 60 s (30 cycles)	BC063166.1

Statistical analysis

The obtained results were analyzed using SPSS version 22 Software and expressed as the mean \pm standard error (SE) using one-way ANOVA for data analysis and followed by Post hoc Duncan's tests for comparison of groups using p-value < 0.05 to show a significant statistical difference.

Histopathological Examination

According to Bancroft and Gamble 2008, hepatic tissues were collected, washed in normal saline and fixed in 10% buffered formalin solution. The samples were then dehydrated in ethanol (70–100%), cleared in xylene, and embedded in paraffin. 5 micron thick paraffin sections were prepared, stained with hematoxylin and eosin (HE) dyes and then microscopically examined.

Results

Biochemical investigation

Effect of TAA and/ or Naringenin (NAR) on some serum and hepatic tissue parameters.

TAA treatment resulted in hepatic cell dysfunction which indicated by a significant increase in the serum hepatic function indicative enzymes (ALT, AST, ALP and GGT); whereas NAR administration for a healthy rats didn't show any significant change in the hepatic enzymes serum levels. Co-treatments of NAR and TAA minimized the deleterious effects of TAA treatment by decreasing the serum levels of hepatic enzymes. Treatment with NAR after TAA treatment resulted in reduction of the hepatic enzymes serum levels but in a lesser extent than performed in the fourth protective group (Table 2). TAA treatment resulted also in a significant increase in the hepatic lipid peroxidation biomarker MDA with a reduction in the hepatic levels of GSH and other antioxidant enzymes, GPx, GR, SOD and CAT. NAR treatment either with TAA treatment or after TAA treatment success to improve the hepatic antioxidant status by decreasing MDA and increasing antioxidant substance levels and enzymes activities (Table 2). The degree of improvement was higher when NAR administrated with TAA as a co-treated substance.

Table 2
Effect of TAA and/ or Naringenin (NAR) on some serum and hepatic tissue parameters.

	Control	TAA group	NAR group	TAA&NAR co-treated group	TAA then NAR treated group
Serum ALT (U/L)	34.32 ± 3.62 ^d	96.36 ± 4.56 ^a	32.2 ± 2.25 ^d	52.85 ± 3.15 ^c	69.7 ± 3.8 ^b
Serum AST (U/L)	62.25 ± 4.46 ^d	128.75 ± 4.62 ^a	58.43 ± 2.68 ^d	73.92 ± 5.82 ^c	95.45 ± 5.85 ^b
Serum ALP (U/L)	88.42 ± 3.52 ^d	156.2 ± 4.5 ^a	86.53 ± 3.65 ^d	96.75 ± 6.22 ^c	117.32 ± 6.65 ^b
Serum GGT (U/L)	22.46 ± 1.6 ^d	67.45 ± 3.54 ^a	21 ± 2.72 ^d	37.22 ± 3.62 ^c	51.56 ± 3.36 ^b
Hepatic MDA (µmol/L)	9.2 ± 0.76 ^d	24.6 ± 2.55 ^a	7.14 ± 0.54 ^e	14.35 ± 1.14 ^c	20.66 ± 1.53 ^b
Hepatic GSH (mg/gm tissue)	24.52 ± 1.55 ^b	10.63 ± 1.68 ^e	27.27 ± 2.25 ^a	18.14 ± 2.1 ^c	14.4 ± 1.69 ^d
Hepatic GPx (µmol NADPH /mg protein)	62.4 ± 5.2 ^a	25.06 ± 2.42 ^d	61.4 ± 2.6 ^a	48.54 ± 2.67 ^b	35.94 ± 3.01 ^c
Hepatic GR (U/ gm tissue)	22.32 ± 1.68 ^a	9.85 ± 0.88 ^d	23.28 ± 1.16 ^a	17.25 ± 1.23 ^{bc}	13.28 ± 1.02 ^c
Hepatic SOD (eu/mg protein)	44.35 ± 2.07 ^a	23.52 ± 2.12 ^d	42.83 ± 4.33 ^a	36.95 ± 1.15 ^b	29.84 ± 1.76 ^c
Hepatic CAT (µmol H ₂ O ₂ decomposed/ gm tissue)	88.1 ± 4.32 ^a	45.6 ± 3.65 ^d	90.4 ± 2.44 ^a	77.7 ± 2.1 ^b	62.3 ± 2.85 ^c
<i>Means ± SE within the same rows and carrying different superscripts are significantly different at (P < 0.05).</i>					

Molecular investigation

Treatment with TAA up-regulated the expression levels of TGF-β, collagen 1 α and fibronectin genes with a down-regulation in the expression levels of antioxidant enzymes (CAT, SOD and GPx) genes. NAR administration alone didn't significantly affects the expression levels of the above mentioned genes whereas it improved the deleterious effects resulted from TAA administration when it administrated either with or after its treatment. The co-treated effect of NAR was higher and better than the treatment after induction of liver cell fibrosis.

Histopathological findings

The control group showed normal hepatic parenchyma including central vein, hepatic cords, and sinusoids. (Figs. A). Hepatic tissue treated with TAA showed multi-portal fibrous strands extended to interlobular septa with focal interstitial inflammatory cells with collagen fibrous deposits (Figs. B1). With high power Focal interstitial inflammatory cells aggregations were also seen beside Hepaticellular necrosis replaced by round cells infiltrations with disorganized hepatic parenchyma by collagen fibrous deposits (Fig B2).

The liver sections from rats received only NAR revealed normal hepatic parenchyma without any collagen fibers deposits. (Figs. C)

The liver sections from rats received with TAA with NAR as a preventive to liver fibrosis didn't show any fibrosis and appeared nearly normal hepatocytes, with mild centro-lobular ballooning degeneration of a few cells as seen (Figs. D).

The liver sections from rats received TAA then treated by NAR showed congested blood vessels with moderate portal fibrosis and diffuse ballooning degeneration (Fig. E).

Discussion

Fibrosis of hepatic cells representing a dangerous medical problem due to its morbidity and mortalities as it is the main leader to cirrhosis, liver failure and hepatocellular carcinoma (Renner DA 2009). Experimentally, TAA is ideally used as a model for liver fibrosis for testing antifibrotic effect of different drugs or natural products in rodents (Kornek et al (2006). Decreasing the risk of liver fibrosis or preventing it is a great hope of many scientists. Many trials about substance that decreasing the risk of hepatic cell fibrosis by discovering compounds either natural or synthetic was performed by many researchers (Zhang et al 2013). The direction towards natural antioxidants to minimize the deleterious effects was started many years ago (Atta et al 2017). In our research we used TAA (the potent toxin and carcinogens) for induction of hepatic cell fibrosis as well as NAR as a potent antioxidant flavonoid to study its potential anti-fibrotic effects.

The results obtained in our experiment after TAA treatment confirmed its ability to induced hepatic cell fibrosis as it results firstly in hepatic cell dysfunction showed in serum increase of hepatic enzymes ALT, AST, ALP and GGT with induction of hepatic cell lipid peroxidation observed in increase of MDA levels (Table 2). At the same time it destroyed the antioxidant system by decreasing GSH levels and decreasing the mRNA expression levels of GPx, CAT and SOD with decreasing their enzyme activities. This creates a defect in free radical/ antioxidant status in the cell.

The pathogenesis produced by TAA for induction of hepatic cell fibrosis based mainly on induction of inflammation in hepatic cell through up-regulating the inflammatory cytokines (Pellicoro et al 2014; Kisseleva et al 2006; Gressner et al 2002; Mi et al 2019). Our results confirmed this hypothesis as showed

in Fig. 1 which clarify the up-regulatory action of TAA on inflammatory and fibrotic markers gene expression (TGF- β , collagen 1 α and fibronectin). For more confirmation, histopathological examination of hepatic cells was performed showing that, fibrotic strands from interlobular septa with collagen fibrous deposits in low power, after zooming in the collagen fibrous deposits were very clear (Fig. 2).

NAR, the natural flavonoid was reported for its antioxidant power especially against hepatic illness (Hernández-Aquino and Murie 2018); its ability to improve hepatic status were examined by its administration as a protective within TAA treatment and as a treatment after induction of hepatic fibrosis by TAA. The obtained results showed that, NAR alone didn't affect the fibrotic or antioxidant status in normal non treated rats which suggests its safety as natural safe product; after its administration with or after TAA treatment it improves the hepatic cell function firstly by decreasing the serum hepatic enzymes levels (ALT, AST, ALP and GGT) together with improving the antioxidant status through lowering the marker of hepatic lipid peroxidation (MDA) levels as well as increasing the levels of GSH with increasing both antioxidant enzymes activities and gene expression. The ability of NAR to improve hepatic function may be due to its ability in prevention of hepatic cell membrane damage due to its antioxidant property (Peterson 2017). NAR has the ability to prevent lipid peroxidation via its hydroxyl groups that facilitates its adherence to polar groups of lipid bilayer as well as interacting with hydrophobic tail of phospholipids by its nonpolar nucleus to reduce damaging effect of free radicals on membranes (Xylina et al 2018). On the other hand, TAA treatment decreased GSH levels; however, NAR preserved normal hepatic GSH levels; this may be due to its up-regulation effect on the expression of GR gene, which catalyzes the reduction of oxidized glutathione to the reduced form (Ursini and Maiorino 2013) and GPx that detoxifies H₂O₂ utilizing two molecules of GSH (Liu et al 2006). As well as its effect on their activities.

The new here its role in down regulation of expression levels of inflammatory and fibrotic biomarkers (TGF- β , collagen 1 α and fibronectin) that plays a significant role in minimizing the deleterious effect of TAA treatment. Liu et al 2006 previously also examined NAR for its effect on TGF-1 for first time suggesting its role in decreasing the expression levels of TGF-1 gene via disruption of TGF-1-Smad3 signaling pathway. Down regulation of NAR to fibrotic markers plays the main role in its antifibrotic effect against the fibrosis induced by TAA in this experiment.

Histopathological examination of hepatic cells supports the obtained biochemical results and confirmed the role of NAR in improvement of hepatic cell status by decreasing the collagen deposition caused by TAA treatment (Fig. 2)

Conclusion

From all of the above mention we can concluded that, NAR flavonoid may have hepatic ameliorative effect against TAA treatment, and can improve the hepatic cell function through its antioxidant and anti-fibrotic effect.

Declarations

Acknowledgments

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Ethical Approval

The experiment was applied under the instructions of National Institutes of Health Guide for the Care and Use of Laboratory animals (NIH Publications No. 8023, revised 1978) that approved by the faculty of Vet. Med. Zagazig University.

Consent to participate

Not applicable

Consent to publish

All authors have read and agreed to the published version of the manuscript.

Author Contributions

Haytham Ali and Mohamed Afifi contributed to the approaches assessment; writing—original draft preparation and writing—review and editing.

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Conflicts of interest

The authors declare no conflict of interest.

Data Availability Statement

Supporting data presented in this paper are available on request from the corresponding author

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Figures

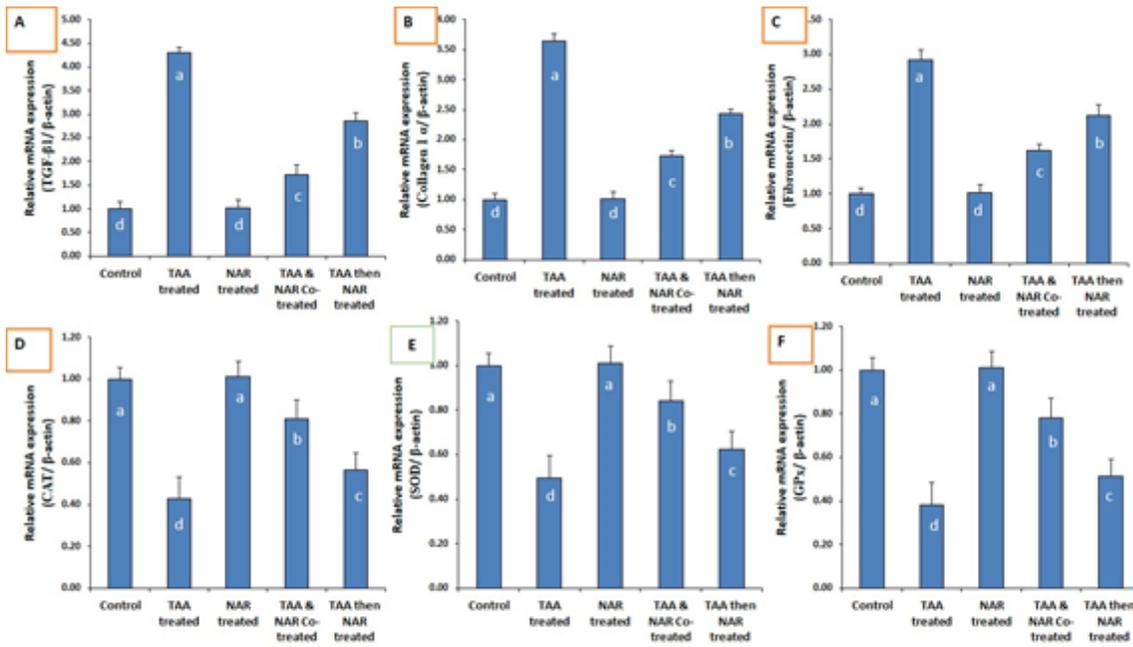


Figure 1

Effect of TAA and/or NAR administration on mRNA expression levels of TGF-β1, collagen 1α, Fibronectin, CAT, SOD and GPx genes in hepatic tissues. Different letters (a, b, and c) by bars indicate significant differences ($p < 0.05$).

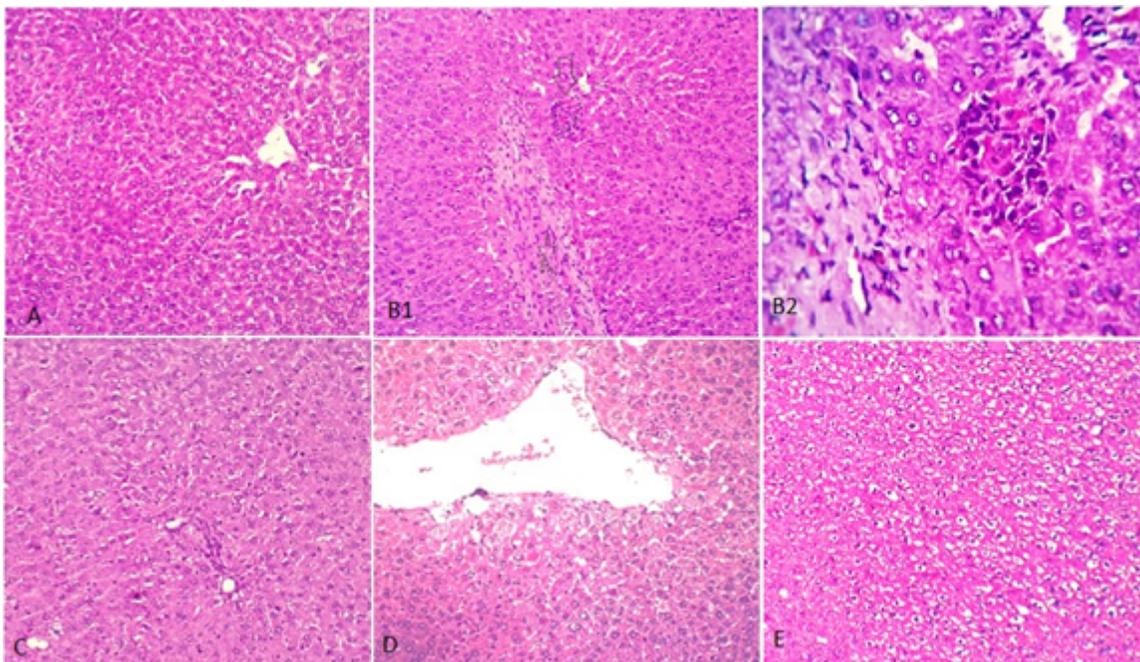


Figure 2

Hisopathological examination of hepatic tissue of rat by H&E stain at scale bar 100µm. A) Control group that showed normal hepatic parenchyma without any fibrosis or any lesions.

B1) TAA treated group and showed fibrous strands extended to interlobular septa with thickening and fibrosis (star) beside inflammatory cells aggregation (arrow). With high power (Scale bare 50µm), hepatic cells showed interstitial necrosis with round cell infiltrations beside disorganized hepatic parenchyma and the cells were replaced by collagen fibrous deposits (B2).

C) The group treated with naringinin only, the hepatic cells were normal without any lesions, the same as control group.

D) The preventive group treated with TAA & naringenin at the same time, hepatic cells showed apparently normal hepatic parenchyma with mild centro-lobular ballooning degeneration of a few cells.

E) The naringinin treated group after induction of liver fibrosis by TAA, the hepatic cells showed diffuse ballooning degeneration with congested blood vessels.