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ANTI-FIBROTIC AND ANTIOXIDANT AMELIORATIVE EFFECTS OF NARINGENIN AGAINST THIOACETAMIDE INDUCED LIVER FIBROSIS

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Abstract: Liver fibrosis still life-threatening problem and searching out ameliorative products motives many scientists, so this work evaluated the anti-fibrotic and antioxidant role of naringenin (NAR) against thioacetamide (TAA) induced liver fibrosis. Fifty adult male albino rats randomly divided into 5 groups (10 each); the first kept as control; the second treated I/P by 200 mg/kg TAA twice a week for 8 weeks; the third was gavaged daily with 50 mg /kg/ b.wt of NAR for 8 weeks; 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 1α 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.

Key words: naringenin; thioacetamide; liver fibrosis; fibrogenic markers

Introduction

Liver is the main metabolic regulatory and detoxifying organ in the body (1). Liver diseases including hepatitis and liver cancers representing a global health problems, threatening lives with great loses in health and money (2).

Fibrosis of the hepatic cells is a common pathological process leading to hepatic cell failure, cirrhosis with a high risk for developing hepatocarcinoma (HCC). Liver fibrosis is characterized primarily by increasing the deposition of extracellular matrix proteins such as collagen in hepatic cells leading to hepatic architecture distortion with nodular formation, alteration of blood flow, portal hypertension, HCC, and ultimately liver failure (3, 4). The major fibrotic biomarkers are collagen, fibronectin, nidogen, laminin and transforming growth factor beta-1 (TGF-β1) (5).

Hepatic cell fibrosis is commonly associated with the most of chronic hepatic diseases and pathologically characterized by inflammation with parenchymal damage. The common causes of liver fibrosis are viral hepatitis especially hepatitis C, alcoholic abuse, metabolic diseases such as Wilson's disease and hemochromatosis, obesity, biliary diseases, parasitic diseases such as Schistosomiasis and chronic drug, toxin and chemical exposure (6). Till now no satisfactory

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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, TAA is considered a classical hepatotoxic reagent used for induction of hepatic fibrosis and cirrhosis in rats. TAA can results in liver diseases and centrilobular damage in acute applications (7,8) while in chronic applications results in liver cirrhosis (9, 10) through the induction of membrane damage, oxidative stress and accumulation of lipid droplets in the hepatocyte cytoplasm to enhance inflammation and liver injury in rodents (11).

Medicinal plants have always been a part of human culture, and up to 80% of the world population relies on this system for some aspects of primary health care especially as antioxidant protecting the body from oxidative stress and reactive oxygen species (12). The natural compounds have been studied extensively and are relevant to many illnesses including liver diseases as they play an important role in the prevention of fibrosis and thereafter in cirrhosis (13). Recently, the interest in natural antioxidants has expanded extensively as a result of their valuable impacts of avoidance and hazard decrease (14). It is well known that consumption of antioxidants is related to a decreased danger of a few sicknesses, for example, cardiovascular, cancer and liver diseases (15).

NAR, 4', 5, 7-trihydroxy flavanone is a bioflavonoid present in tomato, citrus fruits (oranges, lemons, grapefruit, tangerines), cherries, grapefruit and coca (16, 17). Some reports has been demonstrated the NAR use for a prevention of experimentally induced acute liver damage by alcohol, carbon tetrachloride (CCl4), heavy metals or lipopolysaccharide (18,19). Also, there is some other repots associated NAR with anticancer properties and anti-fibrotic properties of NAR. (20, 21). NAR was reported to have the ability to prevent deposition of collagen in rat's haptic cells treated by diethyl-nitrosamine (DENA) but with no clear explained mechanism (22). Du et al., reported the anti-fibrotic effect of NAR in lung tissue and connected it with down-regulation of TGF-β activity. However, according to our knowledge, there is no studies on the anti-fibrotic effect of NAR against liver fibrosis (23). 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.

Materials and methods

Chemicals and reagents

TAA and NAR were purchased from Sigma-Aldrich Chemical Co., (St. Louis, MO, USA), while all other chemicals used in this experiment were purchased from analytical grade Co., India.

Study design

Fifty male adult albino rats, with 6 months age and average weighting at the beginning of the experiment (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 in animal House of the faculty of Vet. Med. Zagazig University, Egypt under controlled conditions (temperature 23±1°C, a 12:12 light/dark cycle), and relative humidity (50–60%) and were given a standard diet and water ad libitum.

Animals were kept for two weeks under control conditions for acclimatization before the beginning of the experiment.

Rats were randomly divided into 5 groups, 10 rats each; the first group kept as a control group and didn't receive any treatment all over the experiment; the second group treated I/P with 200 mg/kg b.wt. TAA twice a weeks for 8 weeks according to Bruck, et al., (24); the third group was gavaged daily with NAR at dose of 50 mg /kg/ b.wt according to Mershiba et al., (21); the fourth group was co-treated by TAA and NAR by the previous mechanisms for 8 weeks as a preventive group; the fifth group was treated with TAA for 8 w then gavaged daily by NAR for 1 month as a treated group.

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, revised1978) that approved by the faculty of Vet. Med. Zagazig University.

Sampling

At the end of the experimental period, the rats were subjected to overnight fasting then decapitated, blood samples were collected and centrifuged at 3.000 rpm for 10 min. for serum separation for determination of serum ALT, AST, ALP and GGT. The serum was stored at -20°C until used.

Hepatic tissues were collected rapidly after decapitation, washed in normal saline and divided into 3 parts for biochemical, molecular and hitopathological examinations. The first part accurately weighed and homogenized in chilled potassium chloride (1.17%) using tissue homogenizer (Potter–Elvehjem) for determination of antioxidant status. The second part was taken, weighted and kept in liquid nitrogen to follow up the changes of gene expression of TGF-β1, Collagen 1α, Fibronectin, CAT, SOD and GPx. The third part was taken and kept in 10 % formalin for histopathological examination.

Biochemical investigations

Serum ALT (EC 2.6.1.2), AST (EC 2.6.1.1), ALP (EC 3.1.3.1) and GGT (EC 2.3.2.2) were determined using the commercial kits according to the methods described by Breuer (25) for ALT and AST and Moss et al. (26) for ALP and GGT. Malonyldialdehyde (MDA) was determined as a marker for lipid peroxidation according to the method described by Buege and Aust, (27). GSH (reduced glutathione) was also determined according to the method described by Ellman (28). Glutathion peroxidase (GPx) (EC 1.11.1.9), Glutathione reductase (GRD) (EC 1.8.1.7), superoxide dismutase (SOD) (EC 1.15.1.1), and Catalase (CAT) (EC 1.11.1.6), were also determined according to the methods described by Hussein et al., (16). All pervious parameters were measured by Shimadzu spectrophotometer (UV 120-02).

Molecular investigation

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 RevertAidTM 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-ΔΔCt) were calculated in accordance to the method of Livak and Schmittgen (29) by determining the $2^{-\Delta\Delta Ct}$.

Table 1: Oligonucleotide primer sequences and real-time PCR conditions

Gene name	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’-GACATGTTCAGCTTTGTGGACCTC-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 ± 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

Hepatic tissues were collected, washed in normal saline and fixed in 10% buffered neutral formalin solution, dehydrated in gradual ethanol (70-100%), cleared in xylene, and embedded in paraffin. Five-micron thick paraffin sections were prepared and then routinely stained with hematoxylin and eosin (HE) dyes (30) and then examined microscopically.

Results

Biochemical investigation

The effects of TAA treatment and/ or NAR on the serum and hepatic tissue parameters were reported in table 2. 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.

Molecular investigation

The results showed in figure 1 represented that, TAA treatment increases the expression levels TGF-β, collagen 1 α and fibronectin genes with a reduction in the expression levels of antioxidant enzymes genes (CAT, SOD and GPx). NAR administration alone didn't significantly affects the expression levels of the above mentioned

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_2O_2 decomposed/ gm tissue	88.9±3.51^{a}	45.3±2.65^{d}	90.4±2.44^{a}	77.7±2.1^{b}	62.3±2.85^{c}

Means within the same columns and bearing different superscripts are significantly different at (P< 0.05).

genes whereas it improved the deleterious effects resulted due to TAA administration when it administrated either with or after its treatment. The co-treated effect of NAR was higher and better that 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 (31). Experimentally, TAA is ideally used as a model for liver fibrosis for testing antifibrotic effect of different drugs or natural products in rodents (32). 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 (33). The direction towards natural antioxidants to minimize the deleterious effects was started many years ago (34). 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 antifibrotic effects.

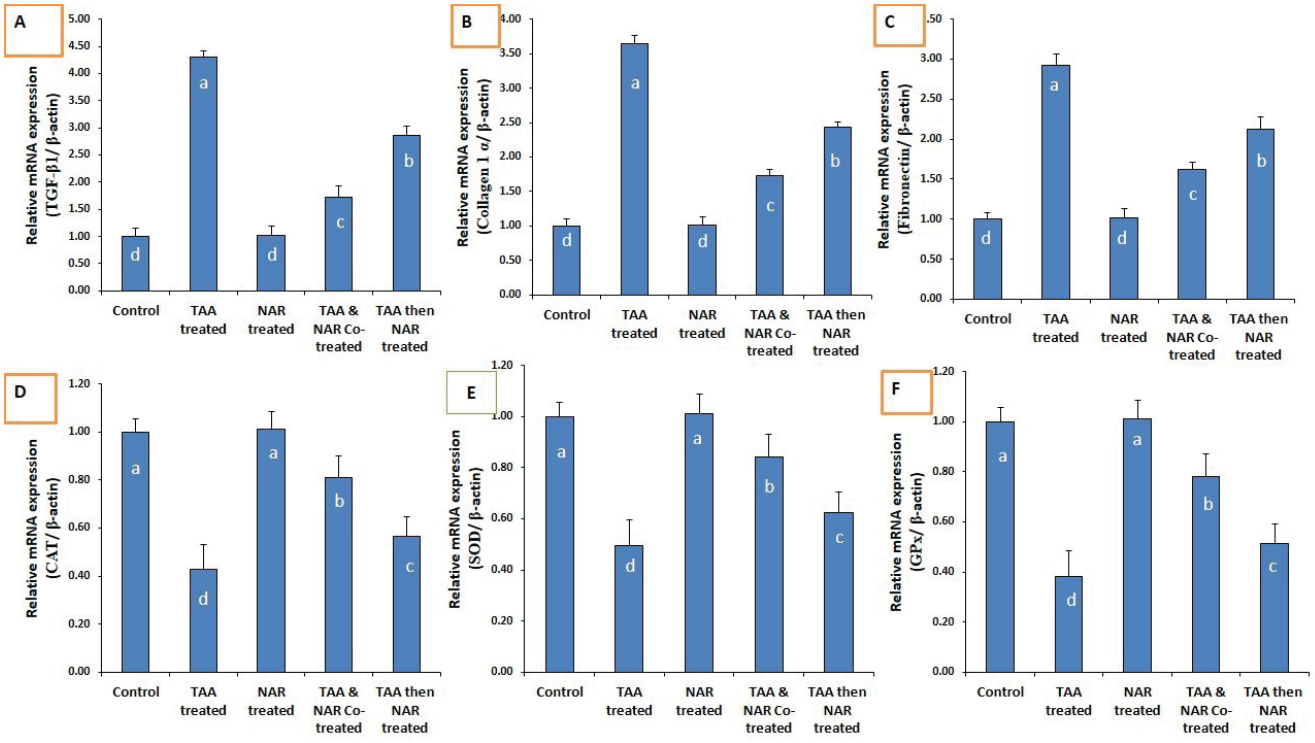

Figure 1: Effect of TAA and/or NAR administration on mRNA expression levels of TGF-β1, collagen 1α, Fibronectin, CAT, SOD and GPx genes in hepatic tissues Different letters (a, b, and c) by bars indicate significant differences ($p < 0.05$).

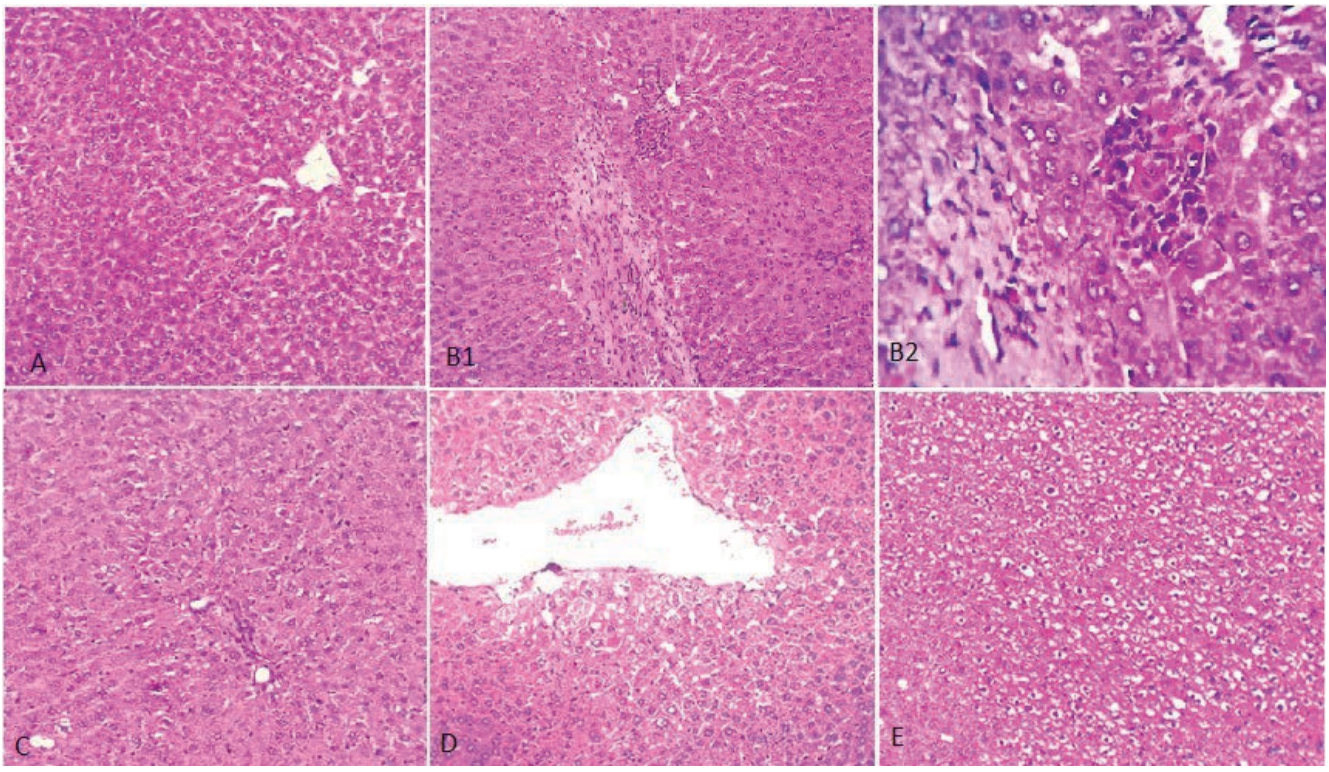

Figure 2: Hisopathological examination of hepatic tissue of rat by H&E stain at scale bar 100µm

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 inflamma-tory cells aggregation (arrow). With high power (Scale bare 50µm), hepatic cells showed interstitial necrosis with round cell infiltrations beside disor-ganized hepatic parenchyma and the cells were replaced by collagen fibrous de-posits (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 ap-parently normal hepatic parenchyma with mild centro-lobular ballooning degeneration of a few cells. E) The nar-inginin treated group after induction of liver fibrosis by TAA, the hepatic cells showed diffuse ballooning degenera-tion with congested blood vessels.

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 (35-38) which also confirmed by our results represented in figure 1 that showed the up-regulatory effect of TAA on inflammatory and fibrotic markers gene expression (TGF-β, collagen 1α 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 (figure 2).

NAR, the natural flavonoid was reported for its antioxidant power especially against hepatic illness (39). Its ability to improve hepatic status were examined by its administration as a protective agent with TAA treatment and as a treatment agent 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 decreasing the hepatic lipid peroxidation

marker MDA and increasing the GSH levels with increasing both antioxidant enzymes activities and gene expression. The ability of NAR to improve hepatic function may be due to its ability to prevent membrane damage due to its antioxidant properties (41). 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 reducing the deleterious effects of free radicals on membranes (42). 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 glutathione reductase, which catalyzes the reduction of oxidized glutathione to the reduced form (43) and GPx that detoxifies H_2O_2 utilizing two molecules of GSH (44). 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 1α and fibronectin) that plays a significant role in minimizing the deleterious effect of TAA treatment. Liu et al., (44) 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.

Hitopathological 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 (figure 2).

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.

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The authors declare no conflict of interest.

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