Benha Journal of Applied Sciences (BJAS) **print : ISSN 2356–9751**
Vol.(2) Issue(2) Nov.(2017), 65-73 **online : ISSN 2356–976x**
http:// bjas.bu.edu.eg

Chemopreventive Effect of Silymarin in N-Nitrosodiethylamine Induced Hepatocellular Carcinoma in Rats Via Modulation of Inflammatory Mediators, Caspase -3, Oxidative Damage and Antioxidant Status In Liver Tissues

S.A.Hussein[1], M.M.Karousa[2] , A.E.Elmashad[3] and F.S.M.Elashtokhy[4]
[1]Biochemistry Dept., Faculty of Veterinary Medicine, Moshtohor, Benha Univ., Egypt
[2]Animal hygiene behavior &management Dept., Faculty of Veterinary Medicine, Moshtohor, Benha Univ., Egypt
[3]Pathology Dept., Faculty of Veterinary Medicine, Moshtohor, Benha Univ., Egypt
E-mail: Samyaziza@yahoo.com

Abstract

Liver cancer, predominantly hepatocellular carcinoma (HCC), represents a complex and fatal malignancy driven primarily by oxidative stress and inflammation. The chemopreventive effect of silymarin on inflammatory markers and oxidative damage, caspase-3 and antioxidant status as well as the histopathological alterations in hepatic tissue in N-nitrosodiethylamine (DEN)-induced hepatic carcinogenesis in male albino rats was investigated. To induce hepato cellular carcinoma, rats were given DEN injections (i.p, 200 mg/kg b.wt.) three times at a 15 days interval. Seventy five rats divided into five equal groups. Group I :(Control group): received no drugs. Group II :(DEN group). Group III :(DEN + silymarin protected group): orally received silymarin (37.8mg/kg b. wt/day) one week before DEN injection and continued to be 13 weeks. Group IV :(DEN + silymarin treated group): Injected with DEN then orally treated with silymarin from the 8th week till the end of the experiment (13th week). Group V: (normal –silymarin group): received silymarin. Blood samples and liver tissues were collected from all experimental groups at the end of experiment on 13th week. The obtained results showed that, DEN-induced hepatic carcinogenesis significantly decreased super oxide dismutase (SOD) and Catalase (CAT) activities in liver tissue. On the other hand, a marked increase in liver tissue L-Malondialdhyde (L-MDA), DNA fragmentation percent, caspase-3 and nuclear factor -kappa beta (NF-κB) and in serum AFP, IL-6 and TNF-α levels were observed in DEN injected rats. silymarin was able to mitigate liver tissue damage induced by DEN through increasing of SOD and CAT activities in addition to decreasing DNA fragmentation percent, L-MDA, caspase-3 and NF-κB and nuclear factor kappa B P65. These data suggest that silymarin exhibited significant protection against DEN-induced hepatocarcinogenesis, which might be related with the enhancement of the antioxidant activity and the induction of apoptosis.

Keywords: Hepatocarcinogenesis, Silymarin, Inflammatory mediators, Oxidative damage, Histopathology.

1. Introduction

Liver disease is one of the most common causes of hospitalization worldwide[1]. Hepatocellular carcinoma (HCC) is the most common primary malignancy of the liver. HCC is a fatal disease with rising incidence in the world [2]. More than half a million individuals per year are diagnosed with hepatocellular carcinoma. From a global perspective it ranks as the fifth most common cancer in men and the seventh in women. Worldwide it ranks third in cancer mortality behind lung and gastric cancer [3].

Diethylnitrosamine (DEN), which is present in tobacco smoke, cured and fried meals, cosmetics and pharmaceutical agents, has been established to be a powerful hepatocarcinogen in rats. The proposed mechanisms of DEN-induced hepatocarcinogenesis include the alteration of DNA structure, the formation of alkyl DNA adducts and the induction of chromosomal aberrations and micronuclei in the liver. The sequential administration of DEN for several weeks has been demonstrated to induce HCC in rodents [4] .

Silymarin is a polyphenolic mixture of flavonoligands derived from the seeds of the milk thistle plant (*Silybum marianum*). Silymarin has been clinically applied for the treatment of liver diseases as an encapsulated, standardized extract, since it is not water-soluble[4] .

Silymarin has been described to possess antioxidant, immunomodulatory, antiproliferative, antifibrotic, and antiviral activities. Intravenous administration of silibinin, composed of a 1:1 mixture of silybin A and silybin B, causes dose-dependent reduction of viral load in patients with chronic hepatitis C .Intravenous administration of silibinin, composed of a 1:1 mixture of silybin A and silybin B, causes dose-dependent reduction of viral load in patients with chronic hepatitis C [5].

Also, Silymarin treatment exerted important anticarcinogenic effects, including the activation of death receptor apoptotic signaling pathway in Hep-55.1 [6].

Accordingly, The chemopreventive effect of silymarin on N-nitrosodiethylamine (DEN)-induced hepatic carcinogenesis in male albino rats was investigated through evaluation of some serum inflammatory cytokines and liver biomarkers, oxidant and antioxidant status of hepatic tissues, caspase-3, nuclear factor -kappa beta (NF-κB) and DNA fragmentation in addition to histopathological examination of liver samples were also investigated.

2. Materials and methods

2.1 Experimental animals

Seventy five white male albino rats of 4-5 weeks old and weighting 100-150 gm were used in the experimental investigation of this study. The rats were obtained from the Laboratory Animals Research Center, Faculty of Veterinary Medicine, Benha University. Rats were housed in separated wire mesh cages and kept at constant environmental and nutritional conditions throughout the period of experiment. The animals were fed on constant ration and fresh, clean drinking water was supplied ad- libitum. The animals were left ٧ days for acclimatization before the beginning of the experiment.

2.2 Silymarin

Physical properties: silymarin manufactured by South Egypt Drug Industries Co. (SEDICO), 6 October City Egypt. silymarin present in sachet/capsule form with 140 mg concentration in each capsule.

Preparation and dosage of silymarin: silymarin were dissolved in water and were administered orally in a daily dose of 37.8 mg/kg body weight[7].

2.3 Induction of hepatic carcinogenesis

Physical properties: N-nitrosodiethylamine presents in a clear yellow liquid form with the molecular formula $C_4H_{10}N_2O$ and molecular weight 102.14 g/mol. It was kept in room temperature. DEN was purchased from Sigma Aldrich Company for Trading Chemicals, Medicines and Medical Appliances, Egypt.

Preparation and dosage of DEN: DEN was freshly prepared in normal saline [8] , and injected to rats at a dose of 200 mg/kg b.wt (i.p) three times at an interval of 15 days [9] .

2.4 Experimental design

Rats were randomly divided into five main equal groups, 15 rats each, placed in individual cages and classified as follow:-

Group 1: Control Normal group: received no drugs, served as control non-treated for all experimental groups.

Group (2): DEN (Positive control) group: Rats considered as the carcinogen control injected with DEN at a dose of (200 mg/kg body weight i.p) three times at an interval of 15 days at experimental weeks 2, 4 and 6.

Group (3): DEN + silymarin protected (co-treated) group: Rats were pretreated with silymarin at a dose of (37.8mg/kg b. wt, daily) (beginning from the 2nd week) till the end of experiment. Rats injected with DEN (200mg/Kg body weight) at experimental weeks 6,8,10.

Group (4): DEN +silymarin treated (Post-treated) group: Rats injected with DEN (200mg/Kg b.wt, i.p) at experimental weeks 2,4 and 6 similar to group 2 and subsequently treated with silymarin at a dose of (37.8mg/kg b.wt, orally) from the 8th week (beginning 2 week after the last dose of DEN)till the end of the experiment.

Group (5): silymarin normal treated group: Rats were administrated silymarin (37.8mg/kg b.wt, daily, orally) starting from the 2nd week till the end of experiment.

2.5 Sampling

Blood samples and tissue specimens (liver tissues) were collected at the end of experiment on 13th week for all groups (control and experimental groups).

2.5.1 Blood samples

Blood samples for serum separation were collected by ocular vein puncture at the end of each experimental period in dry, clean, and screw capped tubes and serum were separated by centrifugation at 3000 r.p.m for 15 minutes .The clean, clear serum was separated by automatic pipette and received in dry sterile samples tube and kept in a deep freeze at -20°C until used for subsequent biochemical analysis.All sera were analyzed for Alpha-Fetoprotein, Interleukin- 6 and tumor necrosis factor alpha .

2.5.2 Liver tissue samples

2.5.3 For biochemical analysis

At the end of the experiment, rats of each group were sacrificed by cervical decapitation. The abdomen was opened and the liver specimen was quickly removed and opened gently using a scrapper, cleaned by rinsing with ice-cold isotonic saline to remove any blood cells, clots and scraps of food, then blotted between 2 filter papers and quickly stored in a deep freezer at (-20 °C) for subsequent biochemical analysis. Briefly, liver tissues were divided into appropriate portions, homogenized with a glass homogenizer in 9 volume of ice-cold 0.05 mM potassium phosphate buffer to make 10% homogenates. The homogenates were centrifuged at 6000 r.p.m for 15 minutes at 4°C then the resultant supernatant were used for the determination of the following parameters: SOD, CAT, L-MDA, DNA fragmentation percent, caspase-3 gene and nuclear factor kappa B P65.

2.5.4 For Histopathological examination

Liver specimen of rats was carefully examined by naked eyes for detection of any abnormalities. Small liver specimens were taken from different parts. The specimens were preserved in 10% neutral buffered formalin solution and subjected for histo-pathological examination [10].

2.6 Biochemical analysis

Serum Alpha-Fetoprotein, Interleukin-6, tumor necrosis factor alpha and liver tissue SOD, CAT, L-MDA, DNA fragmentation percent, caspase-3 gene and NF-κB were analyzed by Rat ELISA Kit For Alpha-Fetoprotein (Designed by Cloud-Clone Corp., assembled by Uscn Life Science Inc. ISO9001:2008: ISO13485:2003, Catalog Number: MBS724171 according to the manufacturer's instruction) [11-16] ; mice Caspase- 3 (Casp-3) ELISA Kit (CUSABIO BIOTECH CO., LTD) Cat.No.CSB-E08857r); Elisa Kit (Cat.No.MBS814487), respectively.

2.7 Statistical analysis

The obtained data were analyzed and graphically represented using the statistical package for social science (SPSS, 13.0 software, 2009), for obtaining mean and standard deviation and error. The data were analyzed using one-way ANOVA to determine the statistical significance of differences among groups. Duncan's test was used for making a multiple comparisons among the groups for testing the inter-grouping homogeneity.

3. Results

3.1 Protective and treatment effect of silymarin on serum AFP, IL-6 and TNF-α concentrations in N-nitrosodiethylamine -induced hepatocarcinogenesis in rats.

The obtained results in table (1) revealed that, a significant increase in serum AFP, IL-6 and TNF-α levels were observed in liver cancer induced rats. Pretreatment and treatment with silymarin in DEN-induced liver cancer in rats resulted in a significant decrease in serum AFP, IL-6 and TNF-α levels when compared with liver cancer non treated group.

3.2 Protective and treatment effect of silymarin on liver tissues SOD,CAT, L-MDA, DNA fragmentation, Caspase-3 and NF-kB P65 in N-nitrosodiethylamine -induced hepatocarcinogenesis in rats.

The obtained results presented in table (2) revealed that, a significant decrease in liver tissue super oxide dismutase (SOD) and catalase (CAT) activities were observed in liver cancer induced rats. On the other hand, a significant increase in liver tissue, L-Malondialdhyde (L-MDA), DNA fragmentation percent, caspase-3 gene and NF-κB P65 were observed in liver cancer induced rats when compared with control group. Pretreatment with silymarin in DEN-induced liver cancer in rats resulted in non-significant increase in liver tissue super oxide dismutase (SOD) and a significant increase in catalase (CAT) activities. Meanwhile, the value of liver tissue L-Malondialdhyde (L-MDA), DNA fragmentation percent, caspase-3 gene and NF-κB P65 were significantly decreased when compared with liver cancer non treated group.

3.3 Histopathological examination

The microscopical examination of liver of rats injected with diethylnitrosamine (DEN) revealed marked congestion of the sinusoidal spaces, central veins and portal blood vessels Fig (1A**)**. Moreover, severe thickening and hyalinization of wall of the portal blood vessels was seen. Abnormal architecture of hepatocytes with extensive degree of degenerative changes in the form of marked diffuse hepatocellular vacuolation with multiple small foci of telangiectasis and the presence of cysts. The hepatocytes showing the presence of clear vacuoles in their cytoplasm which squeezed the nucleus to over side giving it the shape of signet-ring Fig (1B). Moreover, the hepatocytes were suffering from necrotic changes in the form of pyknotic nuclei and more eosinophilic cytoplasm either scattered in the hepatic parenchyma Fig (1C**)** or in the portal area Fig (1D**)**. Interestingly, most of hepatocytes showing the criteria of neoplasia that represented in pleomorphism. The nucleus of most of hepatocytes showing hyperchromacia and numerous mitotic figures with bi-nucleated hepatocytes Fig (2A&B). Moreover, hyperplasia of the biliary epithelium with periductual fibrosis in association with newly formed bile ductile was also demonstrated Fig (2C**)**. Additionally, peri-neoplastic changes in the lining epithelium of bile duct was also detected in some examined cases Fig (2D).

Meanwhile, pretreatment of rats with silymarin (Daily dose =38.7mg/kg body weight) from the 2nd wk) till the end of experiment (13 weeks), with injection of rats with DEN (200mg/Kg body weight) at experimental weeks 6, 8,10 (Group 3) revealed mild reduction in the neoplastic changes that was observed in DEN injected group (Group 2) as dilatation and congestion of hepatic blood sinusoids was detected Fig (3A) as well as marked congestion of hepatic blood vessels with clear vacuoles in the cytoplasm of hepatocytes Fig (3B), only few hepatocytes containing marked large vacuoles. Additionally, normal structure of the portal area was also seen.In addition, individual coagulative necrosis of hepatocytes in which more eosinophilic cytoplasm of hepatocytes with pyknotic nuclei was observed Interestingly, the antihepatocarcinogenic effect of cellamerin in treated group (group 4) was more pronounced. Treatment of rats with silymarin throughout (38.7mg/kg b.w.) one week after DEN administration till the end of the experiment (13 weeks) (Group 4) improved the hepatocellular architecture with more regular and less altered hepatocytes when compared to Group 2. The liver showed mild congestion of the hepatic and portal

blood vessels. Mild degenerative changes in the cytoplasm of hepatocytes in the form of vacuolar degeneration were seendemonstrated Fig (3C**)**. Furthermore, the liver cells were mostly mononucleated with regular sized nuclei. Additionally, the portal area was markedly improved in comparison to group 2 as mild peri-portal fibrous connective tissue proliferation was observed Fig (3D**)**.

However, the hepatic parenchyma of rats received silymarinonly (group 5) showed dilatation of central vein with minute vacuolation of hepatocytes with activation of Vonkupfer´s cells was seen only in few cases Fig (3E**)**.

Table (1) Protective and treatment effect of silymarin on serum AFP, IL-6 and TNF-α concentrations in N-nitrosodiethylamine -induced hepatocarcinogenesis in rats.

Parameters Exp. groups	AFP pg/ml	IL-6 pg/ml	TNF- α g/dl
Control	35.67±10.82c	51.82±6.07b	14.06±2.06c
DEN(Positive control)	119..21±12..59a	225.78±38.18a	66.64±8.27a
Silymarin + DEN (Protected)	73.60±6.62b	136.07±7.30b	36.99±5.48b
DEN +silymarin (Treated)	50.01±4.86bc	98.05±18.81bc	26.67±6.87bc
silymarin	30.77±5.09c	31.31±6.45d	8.07±0.74d

Data are presented as (Mean ± S.E). S.E = Standard error.
Mean values with different superscript letters in the same column are significantly different at (*P≤0.05*)

.

Table (2) Protective and treatment effect of silymarin on liver tissues SOD, CAT, L-MDA, DNA fragmentation, Caspase-3 and NF-kB P65 in N-nitrosodiethylamine -induced hepatocarcinogenesis in rats.

Parameters Exp.groups	SOD U/g tissue	CAT mmol/g. tissue	L-MDA mmol/g. tissue	DNA fragmentation cell/g. tissue	Caspase-3 ng/g.tissue	NF-kB P65 ng/g.tissue
Control	44.78±4.74a	65.87±3.21a	26.72±7.12c	100.54±44.25b	0.62±0.09c	5.14±0.59c
DEN	13.39±1.80c	31.45±1.64c	193.52±17.20 a	3464.38±1411.46a	2.83±0.25a	12.63±0.68a
silymarin+ DEN	20.89±3.66c	35.09±3.58b	125.31±14.64b	789.32±76.90b	2.25±0.10b	8.31±0.60b
DEN +silymarin	34.57±5.09b	51.63±6.13a	61.64±10.80c	789.32±76.90b	1.62±0.15c	6.78±0.36bc
silymarin	50.87±2.70a	64.11±6.80a	62.23±11.62c	263.01±72.74b	1.18±0.07c	5.05±0.51c

Data are presented as (Mean ± S.E). S.E = Standard error.
Mean values with different superscript letters in the same column are significantly different at (*P≤0.05*).

4. Discussion

N-Nitrosodiethylamine (DEN) causes a wide range of tumors in all animal species and such compounds are hazardous to human health. The formation of reactive oxygen species (ROS) is apparent during the metabolic biotransformation of DEN resulting in oxidative stress. Oxidative stress leads to carcinogenesis by several mechanisms including DNA, lipid and protein damage, change in intracellular signaling pathways and even changes in gene expression[17].

In the present study, the obtained data revealed a significant increase in serum AFP. AFP serum levels are often elevated in HCC, but this is not always the case. AFP levels may be elevated initially in the early stages of HCC and then drop or even normalize before rising again as disease progression occurs. Moreover, AFP elevation has also been recognized in the presence of acute and chronic viral hepatitis as well as in patients with cirrhosis caused by hepatitis C. Given the multiple indications that present with elevated AFP levels, it is necessary to evaluate the significance of serum concentrations. Several studies that used DEN for HCC induction in albino rats give similar serum AFP elevation [18-20].

In the current study, a significant increase in serum TNF-α and IL-6 levels was determined. High levels of pro-inflammatory TNF-α have been associated with carcinogenesis[21].

Moreover, TNF-α can also be generated as a consequence of stimulation of a wide range of pro-inflammatory cytokines, including TNF-α itself. Tumor necrosis factor-alpha is also a well-known inducer of the inflammatory response and a regulator of immunity. Its inflammatory properties are classically mediated by means of a wide variety of pro-inflammatory cytokines, including IL (interleukin)-1, IL-2, IL-4, IL-6, IL-10, IL-12, IFN-γ (interferon-γ) and TGF-β (transforming growth factor-β), generated mainly through NF-κB (nuclear factor κB) activation and autocrine IL-6 is crucial for the initiation and progression of HCC [22].

Moreover, TNF-α expression was elevated in HCC patients, especially those with recurrence. An increased level of TNF-α was also shown to

correlate with hepatic inflammation, necrosis, and hepatic failure [23] . These findings are clarified the obtained pathological changes in the liver of rats treated with DEN. In patients with chronic liver inflammation, serum IL-6 levels are elevated including alcoholic hepatitis, HCV, HBV infections, and steatohepatitis. Many studies indicated a big role for IL-6 in the process of liver damage and carcinogenesis [24].

Furthermore, a significant decrease in tissue SOD and CAT activities was detected in the current work in group 2. It has been proposed that oxidative stress plays an important role in the progress and development of various cancer types, including liver cancer. Free oxygen radicals are primarily removed by various enzymatic antioxidants, such as SOD, CAT, GP_X and GST and by various non-enzymatic antioxidants, such as glutathione (GSH), α-tocopherol and vitamin C [25].

A loss in catalase activity during cancer development is associated with tumor formation and metastasis. ROS may downregulate catalase through the methylation of promoter during the development of HCC [26].

The obtained data presented in table (2) revealed that, a significant increase in tissue MDA activity. Many studies reported that serum MDA levels of primary and metastatic liver cancer patients are significantly higher than the normal group. The high lipid peroxidation in liver cancer may be due to excessive ROS production. Most of the hepatotoxic chemicals damage liver cells mainly by inducing lipid peroxidation and other oxidative damages in liver. Enhanced lipid peroxidation produced during the liver microsomal metabolism of ethanol may result in hepatitis and cirrhosis [27].

The obtained data presented in table (2) revealed that, a significant increase in tissue DNA fragmentation, caspase-3 gene and NF-κB. DNA fragmentation and caspase-3 gene are markers of cell apoptosis. In particular, the balance between cell proliferation and cell death may play an important role in hepato-carcinogenesis. NF-κB is found in almost all animal cell types and Nuclear factor-kappaB is a ubiquitous transcription factor that is implicated in the activation of many genes including those involved in alcoholic liver injury[28]. It is a dimeric protein complex made of subunits of both p50/p65 [29]. In unstimulated cells, NF-κB exists in cytoplasm in an inactive form associated with regulatory proteins called IκB. When these cells are stimulated, it is translocated to the nuclei and bound to decameric DNA sequences, and activates transcription of target gene. Nuclear factor-kappaB can be activated by lesion-induced oxidative stress, bacterial endotoxin, or cytokines and subsequently transactivate the expression of many cytokines and adhesion molecules[30] .

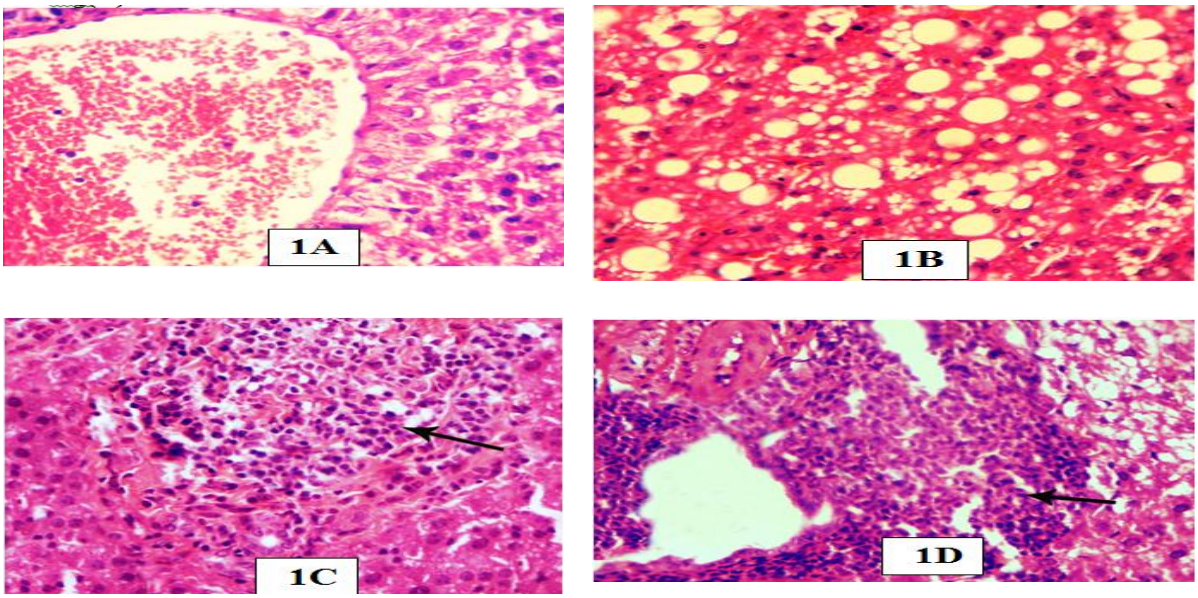
However, the histopathological examination of liver obtained from rats injected with DEN revealed criteria of neoplasia in hepatic parenchyma. These results could be attributed to the ability of DEN treatment in rat to causes enzymatic and non-enzymatic antioxidant defence depletion in hepatic tissue resulting in neoplastic changes in rat with a substantial increase of mitochondrial ROS.

In this study, the protective effect of silymarin against DEN-induced hepato-carcinogenesis in albino rats was proven. silymarin significantly lowered the serum levels of AFP, IL-6 and TNF-α concentration and activity of liver MDA, caspase-3 gene, NF-kB P65 and DNA fragmentation percent. They found that silymarin treatment increased the levels of GSH and the activities of antioxidant enzymes in liver. Pretreatment of mice with silymarin significant increase GSH and CAT levels[31] .

Silymarin is an active extract from the plant milk thistle seeds which contains approximately 65-80% silymarin flavonolignans (Silymarin complex) with small amounts of flavonoids and approximately 20-35% fatty acids and other polyphenolic compounds. Silybin, silibinin, is the major component of Silymarin [32] .

Silymarin may be used alone or in combination with traditional chemotherapeutic agents to prevent the occurrence of cancer, their metastatic spread, or even to treat cancer[33-34].

Silymarin and silibinin exert antioxidant activity and support redox homeostasis in several *in vitro* and *in vivo* models. Silymarin inhibits MDA formation in epidermal microsomes, and also inhibits TPA-and benzoyl peroxide (BPO)-caused lipid peroxidation in mouse skin epidermis, which shows its strong *in vivo* antioxidant activity [35]**.** With the notion that the antioxidant properties and free radical scavenging of silymarin could prevent or reduce the onset and progression of chemotherapy-induced toxicity, oral administration of Siliphos for treatmenet at acute has exerted protective effect on chemotherapy-induced hepatotoxicity [36]. In the present work, histopathological analysis confirmed the pathological improvement in the hepatic tissue of rats either pre-treated or treated groups (group 3 and 4).



Fig(1) H&E stained sections of liver tissue taken from rats received 3 doses DEN 200mg/kg body weight inter-peritonealy at 15 days intervals at experimental weeks 2, 4 and 6 (group 2), showing (A) severe congestion and dilatation of central vein. Notice also, degenerative changes in hepatocytes (x400), (B) clear variable sized vacuoles in the cytoplasm of hepatocytes with squeezing the nucleus to over side (x200), (C) focal areas of coagulative necrosis in the hepatic parenchyma (arrow, x200), (D) marked necrosis in portal area with hyalinization of portal blood vessels (arrow, x200).

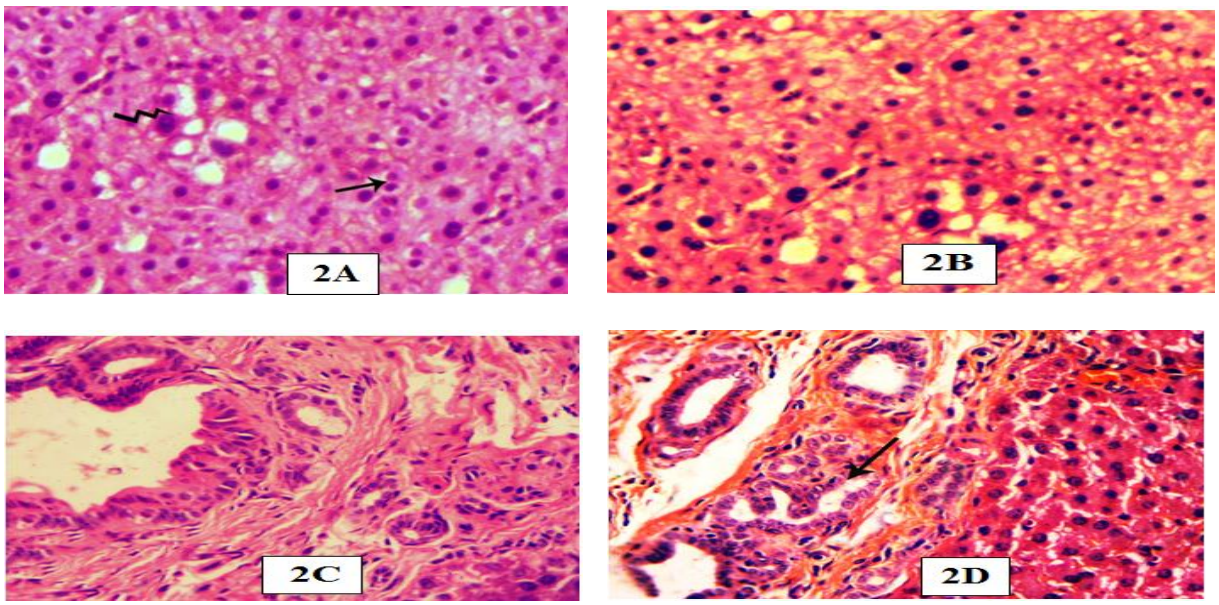


Fig (2) H&E stained sections of liver tissue taken from rats received 3 doses DEN 200mg/kg body weight inter-peritonealy at 15 days intervals at experimental weeks 2, 4 and 6 (group 2), showing (A) criteria of malignancy, hyperchromacia and poleomorphism (zigzag arrow). Notice also, bi-nucleated hepatocytes (arrow, x400), (B) hyperchromacia of the nucleus of hepatocytes and poleomorphism (x400), (C) hyperplasia of the biliary epithelium with periductual fibrosis. Notice also, newly formed bile ductule. (H & E, x400), (D) peri-neoplastic changes in the lining epithelium of bile duct (arrow, 400).

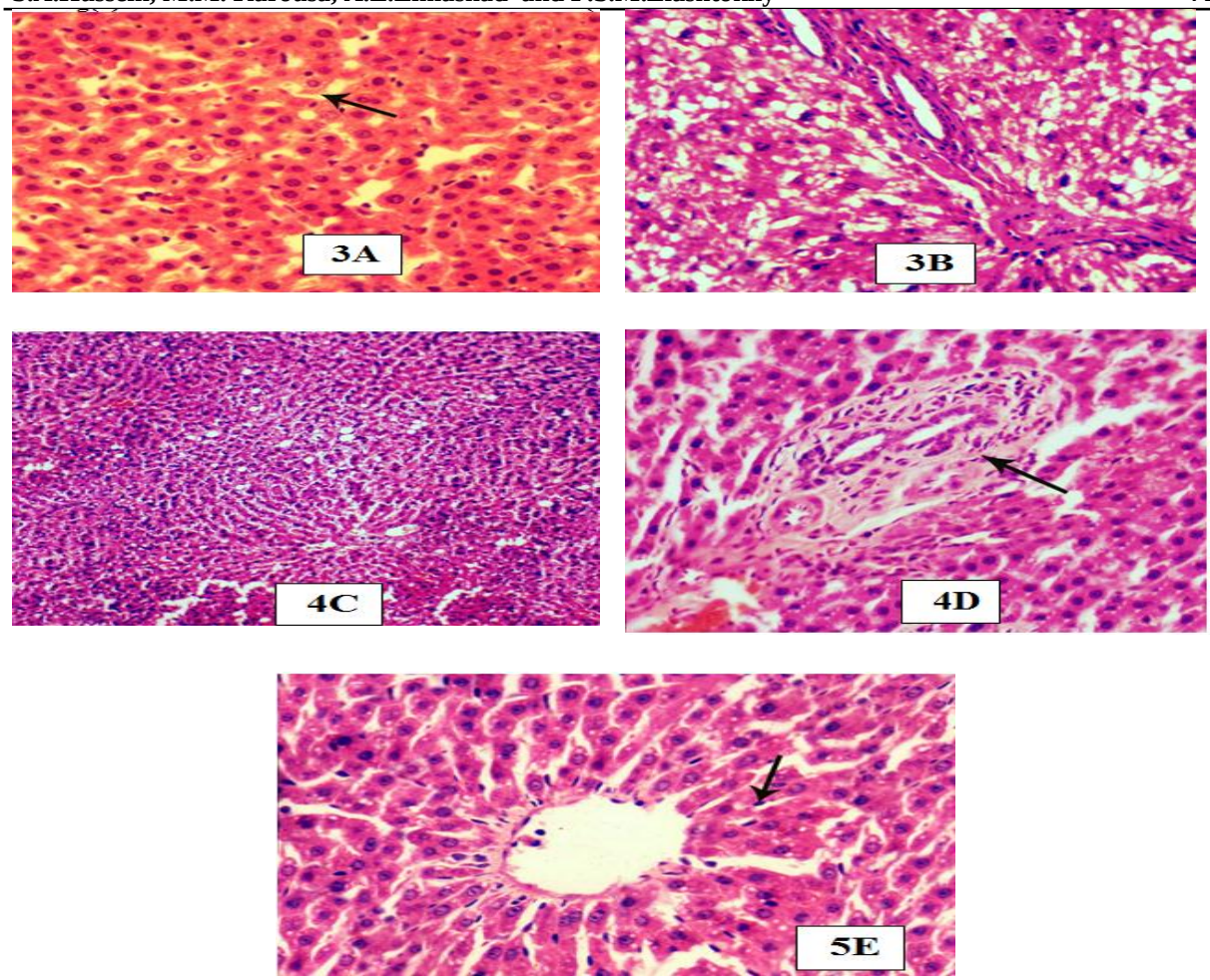


Fig (3) H&E stained sections of liver tissue (A&B) taken from rats pretreated with silymarin (Daily dose =38.7mg/kg body weight) (beginning from the 2nd wk) till the end of experiment (13 weeks) , and rats were injected with DEN (200mg/Kg body weight) at experimental weeks 6, 8, 10, (group 3), (C&D) taken from rats received silymarin after DEN (group 4) and (E) taken from rats received silymarin only(group5), showing (A) dilatation of hepatic blood sinusoids (arrow, x200), (B) vacuolization of hepatocytes with nearly normal structure of the portal area (x200), (C) mild degree of vacuolation in the cytoplasm of the affected hepatocytes (x100), (D) mild fibrous connective tissue proliferation around the portal area (arrow, x200), (E) minute vacuoles in hepatocyts with activation of Vonkupfer´s cells (x 400).

5. Conclusion & recommendations

In conclusion, the present study demonstrated that silymarin administration provided an effective protection and treatment against hepatic carcinogenesis and oxidative damage in liver tissue induced by DEN in rats, indicated the potential and beneficial role of silymarin in preventing oxidative stress-mediated damage and strengthening antioxidant defense mechanism increased antioxidant status of animalsand highly promising agent for protecting hepatic tissue against oxidative damage and in preventing hepatic injury and dysfunction. since silymarin was able to ameliorate serum biochemical parameters, enzymatic and non-enzymatic antioxidant defense system in liver tissue. Also,we recommended that supplementation of diet rich in the natural siymarinis extremely essential for protection of different body organs especially liver tissue, against oxidative stress or even inflammation or cancer.

References

[1] S.Vitor, R.T.Marinho, J.Gíria, J. Velosa, *An observational study of the direct costs related to hospital admissions, mortality and premature death associated with liver disease in Portugal,* BMC Res Notes, vol. 9, p. 62, 2016.

[2] T.Hato, A.X. Zhu, D.G. Duda, *Rationally combining anti-VEGF therapy with checkpoint inhibitors in hepatocellular carcinoma,* Immunotherapy, vol. 3, pp. 299-313,2016.

[3] X.Mu, R.Español-Suñer, I.Mederacke, S.Affò, R.Manco, C.Sempoux, F.P. Lemaigre, A.Adili, D.Yuan, A.Weber, K.Unger,

M.Heikenwälder, I.A.Leclercq, R.F.Schwabe, *Hepatocellular carcinoma originates from hepatocytes and not from the progenitor/biliary compartment*, J Clin Invest; vol.125, pp. 3891-3903, 2015.

[4] R.Imamoto, J.I..Okano, S.Sawada, Y.Fujise, R.Abe, Y.Murawaki, *Null anticarcinogenic effect of silymarin on diethylnitrosamine-induced hepatocarcinogenesis in rats*, Exp Ther Med., vol. 7, pp. 31–38, 2014.

[5] S.J.Polyak, C.Morishima, V.Lohmann, S.Pal, D.Y.W.Lee, Y.Liu, T.N.Graf, N.H.Oberliesf, *Identification of hepatoprotective flavonolignans from silymarin*, Proc Natl Acad Sci U S A., vol. 107,pp. 5995–5999, 2010.

[6] S.Bousserouel, G.Bour, H.Kauntz, F.Gossé, J.Marescaux, F.Raul, *Silibinin inhibits tumor growth in a murine orthotopic hepatocarcinoma model and activates the TRAIL apoptotic signaling pathway*, Anticancer Res., vol.32, pp.2455-62, 2012.

[7] G.Ramakrishnan, H.R.Raghavendran, R. Vinodhkumar, T.Devaki, *Suppression of N-nitrosodiethylamine induced hepatocarcinogenesis by silymarin in rats*, Chem Biol Interact., vol. 161, pp.104-114, 2006.

[8] N.B.Singh, R.B.Singh, B.K.Sarmaa, H.B.Singh, *Potential chemoprevention of N-nitrosodiethylamine-induced hepatocarcinogenesis by polyphenolics from Acacia nilotica bark*, Chem Biol Interact, vol.181, pp.20-28, 2009.

[9] M.S.Khan, H.Devaraj, N.Devaraj, *Chrysin abrogates early hepatocarcinogenesis and induces apoptosis in N-nitrosodiethylamine-induced preneoplastic nodules in rats*, Toxicol Appl Pharmacol., vol. 251, pp. 85-94, 2011.

[10] J.D.Bancroft, A. Stevens, *Theory and Practice of Histological Techniques*, Edinburgh: Churchill Livingstone, vol.4, p. 766,1996.

[11] D.W.Chan, N.T. Perlstein, *Immunology: A practical Guide Eds*, Academic: Press: New York, p71,1987.

[12] R. Beyaert, W. Fiers, *Tumor Necrosis Factor and Lymphotoxin. In Cytokines*, A.R.M.-S. a. R. Thorpe, eds. Academic Press, San Diego, pp. 335-360,1998.

[13] M.Nishikimi, N.Appaji, K.Yagi,*The occurrence of superoxide anion in the reaction of reduced phenazine methosulfate and molecular oxygen*, Biochem Biophys Res Commun, vol. 46, pp. 849-854, 1972.

[14] H.Aebi, *Catalase in vitro assay methods. Methods in Enzimology*, vol. 105, pp.121-126,1984.

[15] L.Mesbah, B.Soraya, S.Narimane, P.F. Jean, *Protective effect of flavonides against the toxicity of vinblastine cyclophosphamide and paracetamol by inhibition of lipid – peroxydation and increase of liver glutathione*, Haematol., vol. 7, pp. 59-67, 2004.

[16] L.Shi, S.Mai, S.Israels, K.Browne, J.A.Trapani, A.H.Greenberg, *Granzyme B (GraB) autonomously crosses the cell membrane and perforin initiates apoptosis and GraB nuclear localization*, J. Exp. Med., vol. 185, pp. 855–866, 1997.

[17] K.Balamurugan, J.Karthikeyan, *Evaluation of Luteolin in the Prevention of N-nitrosodiethylamine-induced Hepatocellular Carcinoma Using Animal Model System*, Indian J Clin Biochem., vol. 27, pp. 157-163, 2012.

[18] N.S.Metwally, T.E.Kholeif, K.Z.Ghanem, A. R.Farrag, N.M. Ammar, A.H.Abdel-Hamid,*The protective effects of fish oil and artichoke on hepatocellular carcinoma in rats*, Eur Rev Med Pharmacol Sci.vol. 15, pp. 1429-1444, 2011.

[19] D.Rajasekaran, J.Elavarasan, M.Sivalingam, E.Ganapathy, A.Kumar, K. Kalpana, D.Sakthisekaran, *Resveratrol interferes with N-nitrosodiethylamine-induced hepatocellular carcinoma at early and advanced stages in male Wistar rats*, Mol Med Rep., vol. 4, pp. 1211-1217, 2011.

[20] B.Chen, M.Ning, G.Yang, *Effect of paeonol on antioxidant and immune regulatory activity in hepatocellular carcinoma rats*. Molecules,vol.17, pp.4672-4683,2012.

[21] B.B. Aggarwal, *Signalling pathways of the TNF superfamily: a double-edged sword*, Nat Rev Immunol., vol.3, pp.745-756, 2003.

[22] G.He, D.Dhar, H.Nakagawa, J.Font-Burgada, H.Ogata, Y.Jiang, S.Shalapour, E.Seki, SE.Yost, K.Jepsen, K.A.Frazer, O.Harismendy, M.Hatziapostolou, D.Iliopoulos, A.Suetsugu, R.M.Hoffman, R.Tateishi, K.Koike, M. Karin, *Identification of liver cancer progenitors whose malignant progression depends on autocrine IL-6 signaling*, Cell., vol. 155, pp.384-396, 2013.

[23] A. Budhu, X.W.Wang, *The role of cytokines in hepatocellular carcinoma*, J Leukoc Biol., vol. 80, pp.1197-1213,2006.

[24] M.S.Othman, A.M.Aref, A.A.Mohamed, W.A.Ibrahim, *Serum Levels of Interleukin-6 and Interleukin-10 as Biomarkers for Hepatocellular Carcinoma in Egyptian Patients*, ISRN Hepatology, vol. 2013, p.9, 2012.

[25] A. Arslan, H. Demir, M.F. Ozbay, H. , Arslan, *Evaluation of Lipid Peroxidation and Some Antioxidant Activities in Patients with Primary and Metastatic Liver Cancer*, Journal of Cancer Therapy, vol. 5, pp.192-197, 2014.

[26] J.Y.Min, S.O.Lim, G.Jung, *Downregulation of catalase by reactive oxygen species via hypermethylation of CpG island II on the*

catalase promoter, FEBS Letters, vol.584, pp,201.2427-2432,2010.

[27] V. Breinholt, S.T. Lauridsen, B. Daneshvar, J. Jakobsen, *Dose-response effects of lycopene on selected drug-metabolizing and antioxidant enzymes in the rat*, Cancer Lett.,vol. 154, pp.201-210, 2000.

[28] P.J. Barnes, M. Karin, *Nuclear factor-kappaB: a pivotal transcription factor in chronic inflammatory diseases*, N Engl J Med., vol. 336, pp.1066-1071,1997.

[29] H.Lu, W.Ouyang, C.Huang, *Inflammation.; a key event in cancer development*, Mol Cancer Res., vol. 4, pp. 221-233, 2006.

[30] F. Chen, V. Castranova, X. Shi, L.M. Demers, New insights into the role of nuclear factor-kappaB.; a ubiquitous transcription factor in the initiation of diseases, Clin Chem.vol. 45, pp.7-17, 1999.

[31] C.Girish, B.C.Koner, S.Jayanthi, K.; Rajesh, B.Ramachandra Rao, S.C.Pradhan, *Hepatoprotective activity of picroliv.; curcumin and ellagic acid compared to silymarin on paracetamol induced liver toxicity in mice*,Fundam Clin Pharmacol., vol. 23, pp.735-745, 2009.

[32] D.J.Kroll, H.S.Shaw, N.H.Oberlies, *Milk thistle nomenclature: why it matters in cancer research and pharmacokinetic studies*, Integr Cancer Ther.,vol.6, pp.110-119,2007.

[33] J.K.Prasain, S.Barnes, *Metabolism and bioavailability of flavonoids in chemoprevention: current analytical strategies and future prospectus*, Mol Pharm.; vol. 4, pp.846-864, 2007.

[34] H.Nishino, *Prevention of hepatocellular carcinoma in chronic viral hepatitis patients with cirrhosis by carotenoid mixture*. Recent Results Cancer Res., vol.174, pp.67-71, 2007.

[35] R.Agarwal, C.Agarwal, H.Ichikawa, R.P.Singh, B.B.Aggarwal, *Anticancer potential of silymarin: from bench to bed side*. Anticancer Res., vol. 26, pp.4457-4498, 2006.

[36] K.Ramasamy, R.Agarwal, *Multitargeted therapy of cancer by silymarin*. Cancer Lett.,vol.269,pp.352-362,2008.