Improved anti-inflammatory effect of Silymarin in rats induced liver carcinogenesis

Ali Alkaladi[1] and Aser Mohamed Abdelazim[2,3]

[1] Department of Biological Sciences, Faculty of Science, King Abdulaziz University, Saudi Arabia.
[2]Department of medical Science, Faculty of Art and Science, King Khalid University, Saudi Arabia.
[3]Department of Biochemistry, Faculty of Vet. Medicine, Zagazig University, Zagazig, Egypt.
alkaladi@kau.edu.sa

Abstract: In the present work the protective role of silymarin in rats-induced liver carcinogenesis was studied. Twenty eight male albino rats were randomly assigned to five groups: Group A ; served as control, (Group B) ; HCC-induced group, Group C ; HCC-induced group and treated with silymarin for 2 weeks and (Group D); HCC-induced group and treated with silymarin 2 weeks before induction the carcinogenesis and 2 weeks after induction of carcinogenesis. HCC –induced rats were treated with an oral dose of 20 mg/kg of DENA. Rats treated with silymarin received a daily single dose of (40 mg/kg body weight) suspended in saline by gavages. Blood samples were collected for determination of ALT,AST, bilirubin, AFP, IL-2 and IL-6 in serum, liver samples were collected for studying the gene expression of IL-2, IL-6 and GAPDH and histopathological examination. Our results demonstrated that ALT, AST, bilirubin levels were significantly lower in silymarin treated groups if compared with non treated HCC-induced rats. Circulating AFP, IL-2&6 were significantly low in silymarin treated groups if compared with HCC-induced rats. The expression level of IL-6 showed the highest expression level in non treated HCC-induced rats with high expression level of IL-2 in control groups. In conclusion silymarin improve the anti-inflammatory status in HCC-induced rats.

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1. Introduction

Primary liver cancer (or hepatocellular carcinoma, HCC) is the sixth most common cancer worldwide in terms of numbers of cases of 626,000, and the third most common cause of death from cancer (598,000 deaths annually) **[1].** Since over 80% of deaths are in developing countries, liver cancer has been a major public health problem in these parts of the world. The rate of hepatocellular carcinoma (HCC) has been increasing in Egypt with a doubling in the incidence rate in the past few years. This has been attributed to several biological (e.g. hepatitis B and C virus infection) and environmental factors (e.g. aflatoxin, AF). Other factors such as cigarette smoking, occupational exposure to chemicals such as pesticides, and endemic infections in the community, such as schistosomiasis, may have additional roles in the etiology or progression of the disease [**2**]**.** The major etiologies and risk factors for the development of HCC are well defined, and 1–6% of cirrhotic patients will develop HCC per year depending on etiology, activity and duration of underlying liver disease HCC has a high prevalence in Africa and Asia mainly due to endemic hepatitis B virus (HBV) infection and additional aflatoxin B1 intake [**3**]**.** Furthermore, the prevention of the progression of existing chronic liver disease by the treatment of inherited metabolic and autoimmune liver disorders, and particularly by antiviral therapy of HBV- and HCV-infected patients which may prevent progression to end-stage liver disease and cirrhosis **[4].** In general, more experience with gene expressing profiles, proteomic approaches and recent progress in metabonomics will produce a large number of potential biomarkers. These approaches will allow the identification of patients at high risk for the development of HCC [5]**.** One of the most frequently used tumour markers for HCC surveillance is alpha-fetoprotein (AFP), generally used associated with ultrasounds. The plasma level of this marker is proportional to the size and evolutionary stage of the tumour [**6**]**.** Silymarin, a flavonolignan isolated from *Silybum marianum*, has also been used for centuries as a natural remedy for liver diseases and now reported to have cancer preventive and therapeutic effects. Results of studies in experimental animal models suggest that silymarin has a broad spectrum of hepatoprotective effects. A number of studies suggest that factors related to the inflammations e.g. of the ovarian surface epithelium (OSE) such as ovulation, In particular, inflammatory mediators and several cytokines produced by activated innate immune cells such as tumour necrosis factor (TNF)-a, and IL-6 have been shown to promote tissue growth and progression **[7].**

2. Materials and Methods:

Protocol and experimental design

Twenty eight male albino rats with average weight and age at beginning of the experiment equals 120±10 grams and 4-5 months respectively, were housed in groups of sevens in stainless steel cages in room with temperature 23 °C±2°C and relative humidity of 55% ± 5%, with a light-dark phase of 12 hours with free access to basal diets and water. All animals from all experimental groups were left to free access to water and designed basal diet all over the experimental period. After acclimatization for 7 days, all rats were randomly assigned to five groups: Group A (n = 7); served as control they did not receive any type of treatments during the experiment, (Group B) (n = 7); HCC-induced group; they did not receive any treatment, Group C (n= 7); HCC-induced group and treated with silymarin for 2 weeks and (Group D) (n = 7); HCC-induced group and treated with silymarin 2 weeks before induction the carcinogenesis and 2 weeks after induction of carcinogenesis.

Induction of carcinogenesis and silymarin treatment

HCC –induced rats were treated with an oral dose of 20 mg/kg of DENA (Sigma-Aldrich Co., St. Louis, Missouri, USA) for 9 weeks (5 days/week) followed by another oral dose of 10 mg/kg of NDEA for 6 weeks (5 days/week) according to the protocol cited by Seufi *et al.* **[8].** Then rats were received three times a week for 17 weeks 0.04 cc of a 40 percent solution of CCl_4 (Sigma, St. Louis, MO, USA) in olive oil by oral gavages. Rats treated with silymarin (Sigma, St. Louis, MO, USA); received a daily single dose of (40 mg/kg body weight) suspended in saline by gavages. Rats were subsequently sacrificed at the indicated times after a one-week washout to eliminate acute effects of CCl4, According to the protocol cited by Fujii *et al.*[**9].**

Blood and tissue sampling

Blood samples were collected from rats, kept for a time, centrifuged at 3000 r.p.m. for 15 minutes, the resulting serum were collected and used for biochemical determinations. The liver was sectioned and fixed in phosphate-buffered 10% formaldehyde for histological analysis. The remaining portions of the liver were collected in RNase-free tubes and snap-frozen in liquid nitrogen for gene expression. Samples for histopathological examinations were immediately fixed in 10% formalin until examination.

Biochemical determinations

ALT, AST Total Bilirubin concentrations (Vitro scient kinetic) were determined in all rats serum. While the quantitative measurement of serum AFP, IL-2 and IL-6 concentration was performed using enzyme immunoassay method (Diagnostic System was determined by Reverse transcriptase polymerase chain reaction (RT-PCR), First strand cDNA was synthesized using two steps Superscript II kit (Invitrogen, Carlsbad, CA). RT-PCR was performed using IL-2 and IL-6 specific oligoneuclotide primers pair, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as housekeeping gene. The primer pairs for amplification were designed as the following, IL-2 according to Zhaia *et al.* [**10**] were forward5'-TGCCTGAAAATGAACTCGG-3' and reverse 5'-CTGGCTCATCATCGAATTGG-3', IL-6 primers according to Klein *et al* **[11]** were; forward 5'GATGCTACCAAACTGGATATAATC-3', reverse 5'-GGTCCTTAGCCACTCCTTCTGTG-3' and for and for GAPDH, forward, 5`-CCCGTAGACAAAATGGTGAAGGTC-3`and reverse, 5`-GCCAAAGTTGTCATGGATGACC-3`with product sizes 164, 249, and 215 respectively. The amplification was performed using thermal cycler (Takara MP, Japan) PCR for was performed with the following cycling conditions; 28-30 cycles at 94°C denaturation for 90 s, 50 and 60 °C annealing temperature respectively for 60 s, and 72 °C elongation temperature and chilled in ice for 5 minutes. Then the amplified PCR products were electrophorised on 1.5% Agarose gel in 1X Tris acetate EDTA running buffer (1 x TAE) with condition of 100 Voltage/ 40 min as described by Uchida *et al.* **[12].**

Statistical analysis

The data was processed using the statistical package for social science (SPSS Inc., Chicago, IL, version 13, USA). All results are expressed as mean ± SD. Comparison among groups was made by Student's t-test (unpaired), One-way analysis of variance (ANOVA). Duncan's test was used for testing the inter-grouping homogeneity. Statistical significance was set $P \leq 0.05$.

3. Results and Discution:

As shown in table (1); the results were expressed in mean ±SE indicated the effect of silymarin on HCC-induced rats as the following

Liver function tests

There is significant decline in hepatic AST and ALT and Bilirubin in silymarin treated HCC-induced groups if compared with non treated HCC-induced groups at ($P \leq 0.05$). **Alpha- feto protein concentration**

AFP as a marker for hepatic carcinoma; show highest level in HCC-induced groups. The level significantly declined in silymarin treated groups if compared with their control.

Cytokines levels

IL-2 and IL-6 were used to evaluate the effect of silymarin on HCC-induced rats; there is a

significant decrease in the level of IL-2 and IL-6 in HCC-induced rats serum after their treatment with silymarin if compared with their control.

Table 1: Illustrates Effect of Silymarin on hepatic enzymes, bilirubin, AFP, IL-2 and IL-6 in Hepatocellular carcinoma induced rats.

Mean ±SE	ALT (µU/ml)	AST (µU/ml)	Bilirubin (mg/dl)	AFP (ng/ml)	IL-2 (Pg/ml)	IL-6 (Pg/ml)
Control	22.2±0.5^{a}	52.24±3.4^{a}	1.4±0.2^{a}	5.9±0.36^{d}	126.4±2.9^{b}	38.04±0.87^{d}
HCC	54.2±3.8^{c}	155.4±1.4^{c}	6.02±0.3^{c}	22.6±0.16^{a}	162.8±12.2^{a}	67.2±0.83^{a}
HCC+ Silymarin after carcinogenesis	32.3±3.6^{b}	58.13±4.2^{ab}	2.5±0.9^{ab}	12.9±0.46^{b}	147.7±1.9^{ab}	56.3±0.56^{b}
HCC+ Silymarin before and after carcinogenesis	22.5±0.8^{a}	52.97±5.4^{a}	2.30±0.2^{b}	9.7±0.27^{c}	140.4±4.3^{ab}	51.9±0.76^{c}

Means within the same column carrying different superscripts are significant at (P ≤0.05).

IL-2 and IL-6 mRNA expression level in rats liver tissues

Figure (1); IL-2 show the highest expression level in control group if compared with other experimental groups while IL-6 show highest expression in HCC-induced groups, the expression level of both IL-2 and IL-6 was equal in both silymarin treated HCC-induced rats.

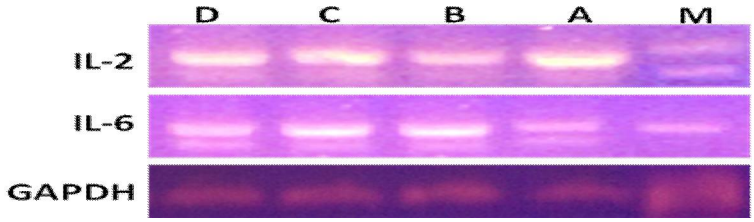

Fig. 1: the mRNA expression level of IL-2 and IL-6 compared to GAPDH in HCC-induced rats liver tissues treated/non treated with silymarin. M; molecular marker, A; control group, B; non treted HCC-induced group, C; Silymarin treated group after induction of carcinogenesis and D; Silymarin treated group after and before induction of carcinogenesis.

Histopathological examination

As shown in figure (2); Normal central vein and liver cords were appeared in control group, while in non treated HCC-induced group there was a focal area of HCC with the rest of liver tissue showing moderate dysplasia, micro vesicular steatosis was appeared in silymarin treated group after induction of carcinogenesis. Silymarin treated group after and before induction of carcinogenesis showing lobular inflammation with aggregates of chronic non-specific inflammatory cells.

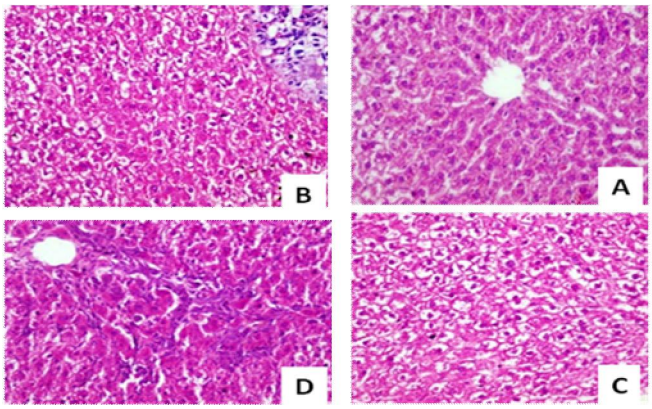

Fig. 2: Photomicrograph of rat liver (H&E, original magnification × 400), A; control group, B; non treated HCC-induced group, C; Silymarin treated group after induction of carcinogenesis and D; Silymarin treated group after and before induction of carcinogenesis.

In the present study we tended to evaluate the effect of silymarin as one of flavonolignan which has a potent effect as cancer treatment with other medical importance **[13]** on the HCC-induced rats. In our study the hepatocelluar carcinoma was confirmed by the high serum level of hepatic enzymes, bilirubin and alpha-fetoprotein *(Table 1)* and histopathological examination *(Figure 2)* in DENA treated groups. Cytokines were determined to evaluate the effect of silymarin on the inflammatory/immune mediators in HCC-induced rats. In general the role of inflammation in cancer has been the focus of extensive research **[14].** Determining whether an association between cytokines likes IL-2 or IL-6, and cancer exists, are important because such knowledge could inform preventative strategies or help in the development of methods for early diagnosis of cancer **[15].** Most studies on the role of inflammation have focused on IL-6 signalling which seems to play the main role **[16].** IL-6 is one of the major immunoregulatory cytokines present in the body cells. Both cancer cells and associated macrophages produce IL-6, and high serum levels of IL-6 are known to be associated with specific immune and metabolic alterations that finally lead to cancer cachexia, one of the main causes of death in cancer patients. IL-6 has been demonstrated to be involved in the autocrine growth of many cancer cells most likely by increasing their capacity to secrete matrix metalloproteinase (MMP)-9 **[17].** On the other hand IL-6 plays an important role in the development of ascites as well as the spread of many cancers like ovarian cancer through its induction of tumor angiogenesis, thus leading to rapid progression and short survival **[18].** IL-2 as one of main factors of the cell-mediated immune response with IL-6, which acts as a second signal for the production of IL-2 and induces the expression of the IL-2 receptor (RIL-2) on cytotoxic T lymphocytes. IL-2 is the key cytokine in the regulation of the antineoplastic immunity. The activity of IL-2 is the synergistic effect of IL-2 and other cytokines deriving from the activated immune system may play an active role in the anti-tumor cytotoxic attack by counteracting neoplastic cell growth. However, some cytokines IL-6 **[19].** Recent studies have demonstrated that a high serum level of IL-6 may be considered an indicator of the inflammatory and oxidative status of patients with carncer **[20]** these data come in the same line of our observation; the expression level and serum concentration of IL-6 in our experinamtal HCC-induced rats were significantly higher than in other experimental groups other in the same line demonstrated that Cancer patients' IL-6 concentrations were higher than healthy controls' in most studies, but the results of investigations comparing IL-6 in cancer patients and individuals with benign diseases were less consistent **[15].** The paracrine effects of IL-6 have also been demonstrated. High levels of IL-6 induce an immune suppressive status in the tumor microenvironment by inhibiting IL-2 synthesis, T cell activation and proliferation, and by promoting lymphocyte apoptosis **[21]** this explains the low mRNA expression level of IL-2 in HCC-induced experimental group in our study. On the other hand silymarin protects animals against multiple types of experimental liver injury such as acetaminophen, carbon tetrachloride, ethanol, iron overload, bile duct obstruction and amanita mushroom poisoning **[13].** Some positive results have been reported in humans; indeed, silymarin has been claimed for clinical applications in the treatment of viral hepatitis, fatty liver, cirrhosis, and radiation toxicity due to its antioxidative, anti-lipid-peroxidative, antifibrotic, immunomodulating, anti-inflammatory, and even liver regenerating effects **[22].** Our results approved that silymarin improve the anti-inflammatory status in HCC-induced rats this appeared through the low level of IL-2 and IL-6 observed in experimental rats treated with silymarin, our results demonstrated also the protective role of silymarin before induction of carcinogenesis. In the same line of our data; many studies have demonstrated that silymarin can suppress the proliferation of a variety of tumor cells such as prostate, breast, ovary, colon, lung, and bladder, through cell cycle arrest at the G1/S-phase **[23].**

4. Conclusion

Silymarin has the ability to improve the anti-inflammatory status in HCC-induced rats through decrease circulating IL-2 and IL-6. Also it has the ability to reduce their gene expression level in livers it can be used as a potent protective and therapeutic agent in liver induced carcinogenic patients.

Corresponding author:
Dr. Ali Alkaladi
Department of Biological Sciences, Faculty of Science, King Abdulaziz University, North Campus, PO Box 11508, Jeddah, 21463, Saudi Arabia.
Email: alkaladi@kau.edu.sa

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