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Ginger potentiates the effects of silymarin on liver fibrosis induced by CCL4: the role of galectin-8

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Abstract. – OBJECTIVE: The liver is an important organ that is actively involved in metabolic functions and targeted by a number of toxicants. Galectin-8 (Gal-8) is downregulated in liver fibrosis. Reduced Gal-8 expression correlates with inflammation and metastasis. Therefore, this study aimed to further investigate the benefits of combined administration of silymarin and ginger for CCl4-induced liver injuries in mice. We also investigated the mechanisms underlying the hepatoprotective activity of these herbal drugs and evaluated the role of Gal-8 and apoptosis in liver fibrosis.

MATERIALS AND METHODS: Eighty male albino mice were used in this study. Animals were divided into the following groups: control group, fibrotic group, silymarin and ginger group. The CCL4 model was used for the induction of liver fibrosis.

RESULTS: Gal-8 expression was reduced in the fibrotic group, while Gal-8 expression was increased in the ginger group and silymarin and ginger group. Tissue levels of nitric oxide (NO) and malondialdehyde (MDA) were markedly increased in the fibrotic group but decreased in the silymarin and ginger group. Additionally, tissue caspase-3 activity and antioxidant markers were decreased in the fibrotic group. However, these markers were increased in the silymarin and ginger group.

CONCLUSIONS: Gal-8 is a diagnostic and/or prognostic glycoprotein for liver fibrosis. The combination of silymarin and ginger has protective liver action and reduces the severity and incidence of liver fibrosis.

Key Words
Ginger, Silymarin, Liver fibrosis, CCl4, Galectin-8.

Introduction

Liver fibrosis is considered a major cause of morbidity and mortality worldwide due to fatty liver disease and chronic viral hepatitis. The critical event in fibrosis is the activation of hepatic stellate cells because these cells become the primary source of extracellular matrix in the liver upon injury. As fibrogenesis increases, the key challenge will be translating new advances into the development of antifibrotic drugs for patients with chronic liver disease[1]. Carbon tetrachloride eventually induces fatty liver and liver fibrosis in rats and mice following repeated administration over ten weeks. In contrast, intra-gastric administration of a single dose of CCl_4 was located in the endoplasmic reticulum in hepatocytes, and membrane-bound polysomes on the endoplasmic reticulum dissociated after one hour of administration[2]. Herbal products such as silymarin are widely used for hepatoprotective effects. In addition, silymarin alone or in combination may represent a new strategy for treating liver fibrosis in humans[3]. Moreover, in ancient medical practice, ginger was used for treatment of various disorders such as rheumatoid arthritis, neurodegenerative diseases, inflammation and asthma[4]. Ginger is the root of *Zingiber officinale* and one of the most used spices in many countries. Ginger extract also exhibits antioxidant activity and reduces the levels of proinflammatory biomarkers[5]. Galectins are proteins defined by at least one carbohydrate recognition domain (CRD) with affinity for β-galactosides and conserved sequence motifs[6]. Galectin-8 (Gal-8) is a tandem-repeat type of lectin that possesses two CRDs; thus, it behaves as a bivalent molecule and is downregulated in tumour hepatocytes[7]. The role of Gal-8 has been mostly investigated in relation to tumour malignancy in a variety of tumours with different origins[8]. Caspases belong to a family of highly conserved cysteine-dependent aspartate-specific acid proteases that share stringent specificity for cleaving their substrates after aspartic acid residues in target proteins[9]. Caspase 3, a prodeath caspase, plays a central role in the apoptotic machinery. The balance between proapoptotic and proinflammatory caspase activity related to

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liver pathology, as well as in the protective effects of pancaspase inhibitors, remains incompletely understood. Given its central role in apoptosis, blocking caspase activity is now widely used as a therapeutic and diagnostic approach for several diseases, including non-alcoholic steatohepatitis[10]. Similarly, based on experimental and clinical data, oxidative stress (OS) mediates the progression of fibrosis, and OS-related molecules may act as mediators of molecular and cellular events implicated in liver fibrosis. The generation of reactive oxygen species (ROS) plays an important role in producing liver damage and initiating hepatic fibrogenesis[11]. In our study, we aimed to further investigate the benefits of combined administration of silymarin and ginger for CCl_4-induced liver injuries in mice. In addition, we aimed to further investigate the mechanisms underlying the hepatoprotective activity of these herbal drugs and evaluate the role of Gal-8 and apoptosis in liver fibrosis.

Material and Methods

Treatment and Sampling

Male albino mice (n= 80) (30 g body weight) were purchased from the animal house of Assiut University (Assiut, Egypt). The experimental procedures were carried out according to the National Institutes of Health Guidelines for Animal Care and approved by the Ethics Committee of the Faculty of Pharmacy, Damanhour University, with Approval No. 518PB6. Mice were divided into four groups (twenty mice in each group); the first control group was injected intraperitoneally (IP) with corn oil (0.25 ml/kg) twice weekly for 12 weeks. The second group, the fibrotic group, was injected IP with CCl_4 (0.5 ml/kg, 1:1 mixed with corn oil) twice weekly for 12 weeks[12]. CCL_4 was purchased from Sigma-Aldrich Company (St. Louis, MO, USA) (20 mg/kg). The LD_{50} value of CCl_4 is 2.8 ml/kg [13]. The third group, the silymarin and ginger group, was injected IP with CCl_4 (0.5 ml/kg, 1:1 mixed with corn oil) twice weekly for 12 weeks and received silymarin (100 mg/kg) and ginger orally (25 mg/kg) suspended in 1% Tween 80. The fourth group, the ginger-treated group, was injected IP with CCl_4 (0.5 ml/kg, 1:1 mixed with corn oil) twice weekly for 12 weeks and received ginger orally (25 mg/kg, suspended in 1% Tween 80) according to Kuhad's modified method[14].

Samples were collected (serum and liver tissues) and used for nucleic acid preparation, assessment of OS markers, antioxidants and caspase-3 activity. The protein content of liver homogenate was determined using Bradford reagent. Samples were aliquoted and stored at -40°C until use.

Primer Design

The National Center for Biotechnology Information (NCBI) reference sequence was used for designing primers for mice Gal-8, caspase-3 and β-actin.

Gal-8 forward Tm= 60°C:
5'-GTT GTC CTT AAA CAA CCT ACA G-3'
Gal-8 reverse Tm= 60°C:
5'-TAA CGA CGA CAG TTC GTC CAG-3'
Caspase-3 forward Tm= 62°C:
5'-GAC CAT GGA GAA CAA CAA AC-3'
Caspase-3 reverse Tm= 62°C:
5'-GGC AGG CCT GAA TGA TGA AG-3'
β-actin forward Tm= 60°C:
5'-CAT GGA TGA CGA TAT CGC TG-3'
β-actin reverse Tm= 60°C:
5'-CAT AGA TGG GCA CAG TGT GG-3'

Ribonucleic Acid (RNA) Preparation and Reverse Transcription Polymerase Chain Reaction (RT-PCR)

A Total RNA Kit (Omega Bio-Tek, Winooski, VT, USA) provided rapid isolation of total RNA (Bioron RT/PCR preMix kit (Cat No.: 122020-105)). PCR was performed using Tag master/high yield (Jena Bioscience, Jena, Germany). A deoxyribonucleic acid (DNA) ladder was performed using a low-range DNA ladder 50-1000 bp linear scale (Jena Bioscience, Jena, Germany). Total RNA fractions were prepared using a total RNA extraction kit. First strand cDNA was synthesized according to the instruction manual of a Revert Aid TM First strand cDNA synthesis kit (Fermentas, Burlington, ON, Canada) from mouse liver total RNA. PCR was performed using Tag master/high yield (Jena Bioscience, Jena, Germany) with the following conditions: pre-denaturing for 5 min at 94°C, denaturing at 94°C for 30 s, annealing at 55°C and extension at 72°C for 1 minute. The amplification was carried out in 28 cycles using a Biometra cycler (Göttingen, Germany).

Assessment of Serum Oxidative Stress, Caspase-3 and Antioxidant Parameters

Blood samples were collected and centrifuged. Sera were isolated for serological studies. Liver enzymes (alanine aminotransferase (ALT) and aspartate aminotransferase (AST)) and total

tissue proteins were estimated using spectrophotometry. OS markers included nitrite and MDA. Nitrite was determined according the method described by Van Bezooijen et al[15], and MDA was determined according the method described by Buege et al[16]. We also used the apoptosis marker caspase-3. Caspase-3 proteolytic activity was determined using a modified procedure described by Kim et al[17]. The activity of some antioxidants, including reduced glutathione (GSH) and superoxide dismutase (SOD), was analysed. GSH content in liver homogenate was determined using Ellman's reagent according to the method described by Ellman[18], and SOD was determined using the method described by Marklund[19]. Both GSH and SOD were estimated by using commercially available kits according to the manufacturer's instructions (Biodiagnostic, Cairo, Egypt).

Histopathological Study

The liver was dissected and washed with 0.9% sterile saline solution. The liver was cut into small parts and kept in 10% formalin solution. Formalin-fixed liver specimens were transferred to 70% ethanol and embedded in paraffin. Tissue sections were stained with haematoxylin and eosin (HE) and examined under an optical microscope to detect pathological changes.

Statistical Analysis

Statistical analysis was performed using GraphPad InStat software (GraphPad Inc., Program, version 4.0, La Jolla, CA, USA). Data are presented as the mean ± standard deviation (SD), and the levels of significance were accepted at $p < 0.001$. Multiple comparisons were performed using one-way analysis of variance (ANOVA) followed by the Tukey-Kramer test as a multiple comparison post-ANOVA test.

Results

Galectin-8 Expression

Gal-8 expression was markedly increased in the groups treated with silymarin and ginger. Conversely, Gal-8 expression was markedly reduced in the fibrotic group, as shown in Figure 1.

Caspase-3 Expression

Figure 2 shows a marked increase in caspase-3 expression in the groups treated with silymarin and ginger. However, caspase-3 expression was markedly reduced in the fibrotic group.

Serum Levels of ALT and AST

The serum levels of ALT and AST were significantly higher in the fibrotic group than in the other groups. By contrast, the serum levels of ALT and AST gradually decreased in the group treated with silymarin and ginger and the group treated with ginger only (Table I).

Total Proteins, MDA, NO, Caspase-3 Activity, GSH and SOD Activity in Liver Tissue

Compared with the control group, the fibrotic group, the group treated with silymarin and ginger

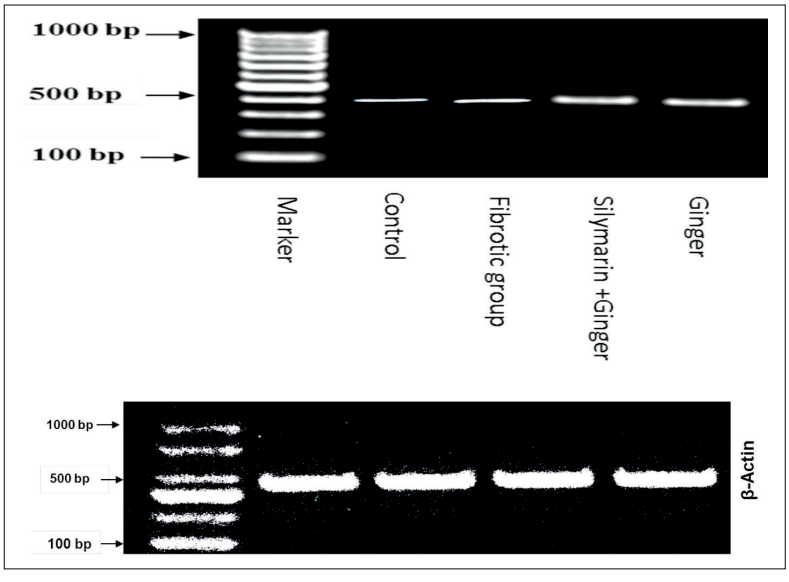

Figure 1. Galectin-8 expression determined by RT-PCR in all experimental groups (control, Fibrotic, silymarin and ginger, and ginger only).

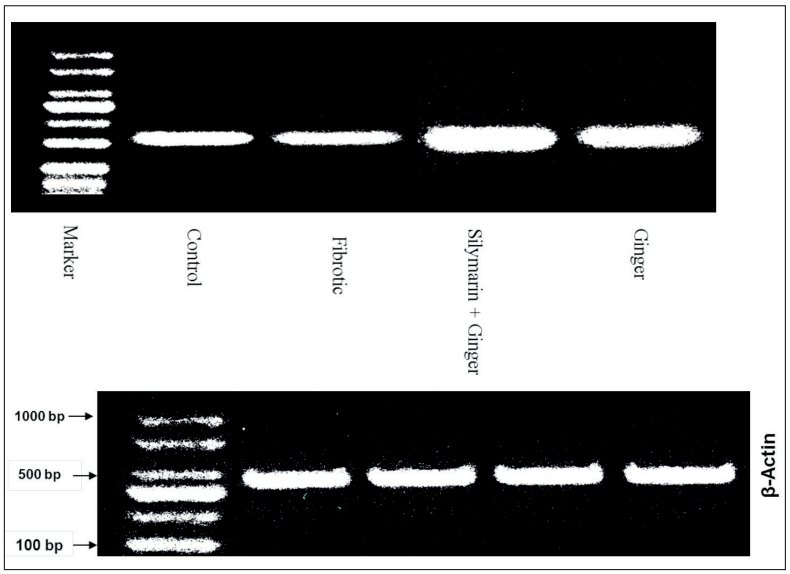

Figure 2. Caspase-3 expression determined by RT-PCR in all experimental groups (control, Fibrotic, silymarin and ginger, and ginger only).

and the group treated with ginger only showed significant differences in the tissue levels of OS, apoptosis, and antioxidants (Table I). The tissue levels of total tissue proteins, caspase-3 activity, GSH and SOD activity were significantly decreased in the fibrotic group, but gradually increased in the group treated with silymarin and ginger and the group treated with ginger only. In contrast, the tissue levels of MDA and NO were significantly increased in the fibrotic group, while these levels increased in the group treated with silymarin and ginger and the group treated with ginger only, but did not reach the levels in the control group (Table I).

Histopathological Examinations

In the control group, normal hepatocytes were observed surrounding the central vein; these cells were polygonal with pale vesicular nuclei and prominent nucleoli, as shown in photomicrographs (1 and 2). Additionally, these micrographs show the portal vein tributaries and the branch of the bile duct (Figure 3).

Photomicrographs (3 and 4) show vacuolar changes in the hepatocytes, and many of the hepatocytes have two nuclei as a result of fibrosis in the liver. These images also show a dilated and congested portal vein. Moreover, the portal tract containing the portal vein and bile duct showed inflammatory cell infiltration in the liver and fibrotic cells (Figure 3). Photomicrographs (5 and 6) show intact hepatic architecture with a mild degree of vacuolation and many hepatocytes with normal cytoplasmic appearance in the liver as a result of using silymarin and ginger (Figure 3). Finally, photomicrographs (7 and 8) show intact hepatic architecture with no inflammatory cells

Table I. Levels of serum ALT and AST in mice and levels of total proteins, MDA, NO, Caspas-3 activity, GSH and SOD activity in the liver tissue homogenate of the studied mice.

	Control group	Fibrotic group	Ginger + Silymarin	Ginger group
ALT (IU/L)	39.38±4.52	154.42±6.41* p<0.001	81.98±3.01$^{*\#}$ p<0.001	93.8±4.91$^{*\#}$ p<0.001
AST (IU/L)	57.72±4.88	208.05±6.22* p<0.001	118.95±4.47$^{*\#}$ p<0.001	132.88±6.09$^{*\#}$ p<0.001
Total tissue proteins (gm/dl)	7.02±0.77	1.91±0.59* p<0.001	4.38±0.42$^{*\#}$ p<0.001	3.48±0.81$^{*\#}$ p<0.001
MDA (µM/g wet tissue)	1.73±0.775	5.92±0.848* p<0.001	3.01±0.892$^{*\#}$ p<0.001	3.85±0.554$^{*\#}$ p<0.001
NO (µM/g wet tissue)	31.92±2.113	58.84±3.945* p<0.001	42.55±3.311$^{*\#}$ p<0.001	49.13±2.925$^{*\#}$ p<0.001
Caspas-3 (U/mg protein)	16.98 ± 0.972	8.82 ± 1.008* p<0.001	13.36± 0.843$^{*\#}$ p<0.001	14.54± 1.188$^{*\#}$ p<0.001
GSH (U/mg protein)	15.72±1.102	8.34±1.421* p<0.001	13.17±1.618$^{*\#}$ p<0.001	10.1916±1.083$^{*\#}$ p<0.001
SOD (U/mg protein)	26.34±2.092	11.22±2.003* p<0.001	16.96±1.877$^{*\#}$ p<0.001	12.94±2.910* p<0.001

$^{*}p$<0.001 compared to control group. $^{\#}p$<0.001 compared to fibrotic group.

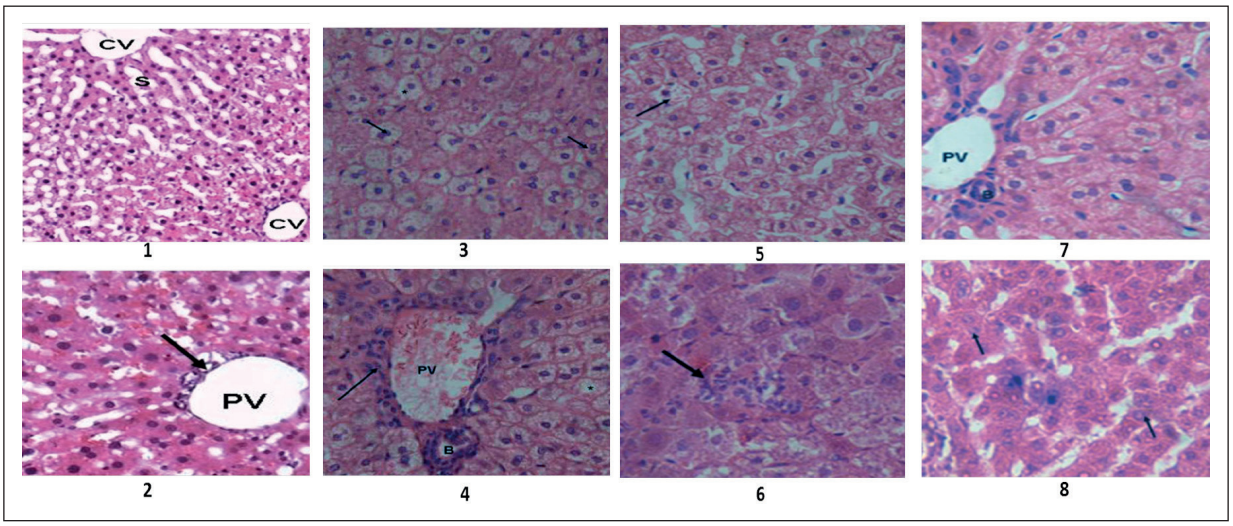

Figure 3. Histopathological changes in liver tissues; control group (photomicrographs 1&2), fibrotic group (photomicrographs 3&4), silymarin and ginger group (photomicrographs 5&6) and ginger group (photomicrographs 7&8). Slides were examined using H&E stain with magnification X400.

in the portal area and prominent connective tissue stroma between the hepatic lobules in the liver. Hepatocytes exhibit a normal nuclear and cytoplasmic appearance. Moreover, few degenerated cells appeared in the liver as a result of ginger treatment (Figure 3).

Discussion

Liver fibrosis is one of the most common chronic liver diseases. The main pathogenesis of this disease is viral infections and toxin exposure. Among these toxins is CCl_4, which causes liver fibrosis. Liver fibrosis is the main cause of hepatocellular carcinoma. These results agree with those of Rahimlou et al[20] who reported that liver diseases, which include a wide variety of liver disorders such as hepatic fibrosis and cirrhosis, are the most common chronic diseases worldwide. Additionally, Meng et al[21] reported that chemicals such as alcohol and pollutants cause liver diseases, which could consequently develop into fibrosis, cirrhosis and liver failure or even hepatocellular carcinoma. Thus, liver injuries have remained a serious public health problem worldwide. Currently, natural products play an important role in the prevention, limitation and treatment of liver fibrosis. The use of natural products as a good option for the treatment of liver fibrosis has been generally accepted. Ginger and silymarin are among the natural products with antioxidant effects. These findings are similar to those of previous studies in which numerous natural products and their bioactive components showed protective action against liver injuries, such as blueberry, cactus fruits and silymarin[21]. To our knowledge, the current study is one of the few to examine the combined molecular effects of ginger and silymarin on the prevention and/or treatment of liver fibrosis. The combination of ginger and silymarin exerts a synergistic effect. The anti-tumour and anti-inflammatory effects of ginger and silymarin manifested through increased expression of gal-8 in mice injected with ginger and silymarin. Gal-8 is a glycoprotein whose expression is markedly reduced in the presence of inflammation. Therefore, the present study shows a reduction in gal-8 levels in the fibrotic group. In contrast, Gal-8 levels were increased in mice that were treated with silymarin and ginger. In addition, investigating the roles of Gal-8 is necessary to determine whether Gal-8 downregulation is associated with poor prognosis of HCC. Overall, one of the strengths of this study is that we demonstrated effects on liver enzymes, inflammatory markers, apoptotic markers, OS, antioxidants and hepatic fibrosis scores in all experimental stages. Apoptosis is an important mechanism and pathway that explains the incidence and reduction in liver fibrosis. Caspase-3 is an apoptotic agent that exhibits decreased expression during inflammation and fibrosis. In mice treated with silymarin and ginger, caspase-3 activity was increased. CCl_4 is a novel toxin used to establish liver fibrosis

models. CCl_4 is a xenobiotic that increases OS markers and damage to hepatocytes. This toxin is metabolized into trichloromethyl, which initiates liver damage and fibrosis. Thus, the estimation of OS markers and antioxidants is considered the main molecular mechanism for explaining the therapeutic effect of silymarin and ginger. A previous study showed a significant decrease in ALT and AST liver enzymes and OS markers NO and MDA. According to the present study, ginger supplementation reduces apoptotic markers such as caspase-3 activity. In agreement with these findings, Matthews et al[22] reported similar results. The results of the present study revealed a marked increase in OS markers NO and MDA in the fibrotic group. In contrast, OS levels were decreased in the groups treated with silymarin and ginger. In a similar manner, the levels of antioxidants such as SOD and GSH activity were markedly reduced in the fibrotic group, whereas marked increases were observed in the groups treated with silymarin and ginger. These findings are in agreement with a previous study in which CCl_4, as a xenobiotic, caused OS and may injure hepatic cells. Many studies have established that CCl4 is metabolized in the liver into a highly reactive substance, trichloromethyl, which initiates free radicals that mediate lipid peroxidation. For this reason, antioxidation is an extremely significant activity that can be used as a preventive agent against diseases[23]. According to numerous observational studies and animal studies, the active compounds in ginger can enhance the antioxidant defence systems, such as glutathione peroxidase and glutathione S-transferase and reduce levels of MDA and hepatic steatosis. One of the main reasons for this protective feature appears to be the effect of ginger on the expression of proliferating cell nuclear antigen[24]. Using a histopathology technique to determine the pathological state of the liver, score liver fibrosis, we showed the grades of disease were one of the strengths of this study. Histopathological examination revealed that the state of the liver was generally improved in mice that were injected with silymarin and ginger.

Conclusions

We found that Gal-8 is a diagnostic and/or prognostic glycoprotein for liver fibrosis. The combination of silymarin and ginger has protective liver action and reduces the severity and incidence of liver fibrosis by increasing apoptotic pathways through elevating caspase activity. In addition, these combinations increase the expression of Gal-8, which is included in the mechanisms underlying liver protection against fibrosis.

Conflict of Interests

The Authors declare that they have no conflict of interests.

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