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Effects of emodin and silymarin on the resolution of liver fibrosis induced by carbon tetrachloride in mice

Nabila Zein [a]*, Mariam Sh. Saad [a] and Atef M. Amer [b]

a Biochemistry Division, Chemistry Department, Faculty of Science, Zagazig University, Zagazig 44519, Egypt
b Organic Chemistry Division, Chemistry Department, Faculty of Science, Zagazig University, Zagazig 44519, Egypt

Abstract

Liver fibrosis is currently recognized as a critical mechanism of the pathogenesis of the chronic liver disease. Both Emodin, traditional Chinese medicine, and Silymarin, herbal medicine, have hepato-therapeutic potential. However, the antifibrotic mechanism of Emodin and Silymarin combination on liver fibrosis is still unclear. We aimed to investigate if the combination of Emodin and Silymarin could effectively reduce fibrosis of the liver to develop a new treatment strategy. A total of 50 Swiss albino mice were categorized into five groups; one served as a negative control, while the other four received carbon tetrachloride (CCl4) twice weekly for 4 weeks, then the mice were partitioned into a control positive untreated group, emodin treated group, Silymarin treated group and a combination group of emodin and Silymarin, all groups were kept for another 3 weeks. Biochemical liver function tests ALT, AST, ɣ-GGT, and AFP, liver histological analysis and proliferative capacity (proliferating cell nuclear antigen)were evaluated. As per our findings, Emodin and Silymarin may be able to reduce hepatic fibrosis by lowering liver function assessments and improving the degree of liver fibrosis by minimizing Hepatic Stellate Cell stimulation and extracellular matrix formation. This study is a novel outcome suggested that the combination of Emodin and Silymarin may decrease liver fibrosis.

Keywords: silymarin; emodin; carbontetrachloride;PCNA;liver fibrosis ;hepatic stellate cells.

INTRODUCTION

Liver fibrosis is one of the most serious health problems in the world, and there is no effective treatment[1]. Hepatic fibrosis is both an energy process and a wound healing process that can be reversed. When triggered, dormant hepatic stellate cells (HSCs) proliferate first, then transdifferentiate into myofibroblasts. These cells cause the extracellular matrix (ECM) to be deposited and the wound to proceed in the liver. The early signs of liver fibrosis are subtle, allowing you to modify and rewrite your content swiftly and effectively[2].

Chronic indigestion, loss of appetite, chronic gastritis, and bleeding are clinical markers of hepatic fibrosis, which can progress to debilitating cirrhosis or hepatitis. Liver fibrosis is caused by damage to the liver caused by a range of factors, including alcoholism, autoimmune illnesses, congenital abnormalities, viral hepatitis, excessive copper or iron deposition, insulin resistance, and steatosis.[3, 4].

The most successful treatment for liver disease is liver transplantation. While the high cost and a limited number of accessible liver donors make it difficult for every patient to receive. Accordingly an effective treatment for liver fibrosis is required [5].

There are a slew of drugs being developed to treat liver fibrosis. Several studies published in the last few years have shown that traditional Chinese medicine has a wide range of therapeutic characteristics for liver problems[6]. Emodin and silymarin are two natural chemicals with antifibrotic effects. Several natural compounds and their bioactive components, such as silymarin, cactus fruits, and blueberries, have been found to protect against liver damage[7].

Emodin (systematic nomenclature: 1,3,8-trihydroxy-6-methylanthraquinone) it is a primary active monomer found in traditional Chinese medicines like Giant knotweed and rhubarb root. Emodin has been shown to have anticancer, antiviral, anti-inflammatory, and immunomodulatory properties in the lab and has been used in Asia for centuries to

*Corresponding author e-mail: dr.nabila.zein@gmail.com, Scopus Author ID 56556306000, https://orcid.org/0000-0003-0921-6919
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treat gastrointestinal, lung, inflammatory, and liver ailments. Many studies have demonstrated that emodin is beneficial in reducing liver fibrosis induced by carbon tetrachloride (CCl4 -) in rats, but further research is needed to determine the optimal dosage and mechanism[8, 9].

Silymarin (Si) is a herbal medicine derived from the seed of the Silybum marianum plant, it ia a flavonoid complex including silybin, isosilybin, silydianin, and silychristin, as well as the entire therapeutic components of Silybum marianum. Silymarin has a wide range of biological activities, including antioxidant, immunomodulatory, and anti-inflammatory activity. Due to its low toxicity, lack of adverse effects, and particular efficacy, silymarin is utilized as a traditional liver-protecting medication. The role of silymarin in protecting liver cells and reducing hepatic fibrosis has been proven in several in vitro and in vivo studies[10].

As a result, the purpose of this study was to assess further the potential of concurrent dosing of combination of Emodin and Silymarin for CCl4 stimulated liver injury in mice. Additionally, to elucidate the mechanisms behind these herbal medications therapeutic efficacy and assess apoptosis associated with liver fibrosis in mice.

2.Materials and Methods

2.1Animal model

A total of fifty adults of male Swiss albino mice with (25 ± 5g) average weight were acquired from the Laboratory Animal Center of the Scientific and Medicinal Research center (Zagazig University, Egypt). The animals were housed in a temperature-controlled breeding chamber. With a constant temperature (22 ± 2°C) and humidity level (60 - 80 percent). The breeding room was illuminated by an electric light cycle that alternated between 12 hours of lighting and 12 hours of darkness each day and was regularly sanitized. Mice were fed a standard laboratory diet and were given water. All animal research was done in compliance with the ethical standards established by Zagazig University, Faculty of Science Ethical Committee and authorized by the Institutional Animal Care and Use Committee (ZU-IACUC) using the Ethics Reference Number (ZU-IACUC/1/F/39/2019).

2.2 Reagents and Antibody

Emodin was kindly provide by (Dr.Atef M.Amer)[11],Highly pure carbon tetrachloride (ccl4), phosphate buffer slain (PBS) were purchased from El-Gomhouria Co. (El-Gomhouria, Cairo, Egypt). Silymarin was was obtained from Sedico Pharmaceutical Co. (SEDICO, Cairo, Egypt). Olive oil was purchased from Dr. Olive Co. for agricultural production and processing (Dr. Olive, Cairo, Egypt). Aspartate aminotransferase (AST), Alanine aminotransferase (ALT), and colorimeter testing kit were obtained from Teco Diagnostic Co. (Anaheim, USA). Gamma-glutamyl transferase (GGT) was obtained from Xpress Bio Life Science Co. (Xpress Bio, USA), Anti-PCNA antibody (ab18197).

2.3 Experimental design and fibrosis induction

Five groups of ten mice each were randomly assigned; the negative control group got no medication. For the positive control group, mice were injected intraperitoneally with CCl4 at a concentration of 1 mg/kg (diluted 1:1 in olive oil) twice weekly for four weeks in order to induce liver fibrosis [12] then left for another 21 days without any treatment. Emodin treated group; mice were treated with emodin; after induction of fibrosis, emodin was injected intraperitoneally in a dose of (5mg/kg) every day for 21 days [13]. Silymarin treated group; mice received silymarin after induction of fibrosis orally every day in a dose of 100mg/kg [14] for 21 days. Combination treated group, mice injected intraperitoneally with emodin in a dose of (2.5 mg/kg) and received silymarin orally in a dosage of (50 mg/kg) after induction of fibrosis every day for 21 days.

At the end of the experiment, each group of mice was anesthetized with sodium pentobarbital and necropsied completely. Blood samples obtained from the inferior vena cava of each mouse and collected in non-heparinized glass tube, and the serum was maintained at -20°C for biochemical examination. Liver tissue samples from the same location in each group were dissected out and subjected to histopathological examination through the Hematoxylin and Eosin stain, and immunohistochemistry stain for proliferating cell nuclear antigen (PCNA).

2.4 *Hepatic function assessment*

We used an automated biochemical analyzer to determine serum (ALT) and (AST) (Teco Diagnostic Co., Anaheim, USA). Its principal procedure was based on reflectance spectrophotometer, (AFP) and (γ-GGT), which were evaluated by quantitative sandwich ELISA kits (My Bio Source Co., My BioSource, USA-Xpress Bio Life Science Co., Xpress Bio, USA); and the method was applied as per the manufacturer's instructions.

2.5 *histological examination*

The liver lobes were washed with normal 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.

2.6 *Immunohistochemical examination of PCNA*

Immunostaining was performed on serial sections of paraffin blocks, 4 μm thickness cut. The tissue

sections were deparaffinized in xylene and rehydrated in graded ethanol. Deparaffinized tissue sections were treated with hydrogen peroxide for 10 min to block nonspecific peroxidase reaction. Microwave antigen retrieval was performed for 20 min in citrate buffer 0.01 M (pH 6.0). After washing with phosphate buffer saline (PBS), the slides were incubated for 60 min at room temperature with rabbit monoclonal; Anti-PCNA antibody (ab18197). Binding site of primary antibodies was visualized by using the DakoEnVision ™ kit (Dako, Copenhagen, Denmark). The peroxidase reaction was visualized by incubating the sections with diaminobenzidine (DAB) for 15 min. The sections were counter stained with Mayer's hematoxylin.

2.7 *Statistical analysis*

Data were entered, verified, and analyzed using SPSS version 23 for data processing (SPSS Inc., Chicago, IL, USA). The following statistical methods were used to analyze the present study results: Data were expressed as for qualitative variables, using numbers and percentages, while for quantitative variables, using mean ± standard deviation (SD). The one-way ANOVA test (F-test) was used to determine the difference between quantitative variables in more than two groups, accompanied by the LSD (least significant difference). A P-value of ≤0.05 indicates a significant difference, and a P-value of ≤ 0.001 demonstrates a greatly significant difference.

3.Results and Discussion

3.1 Emodin and silymarin inhibited CCl4- induced liver fibrosis and improved liver function test

Chronic liver damage from a variety of causes, such as liver disease, hepatitis B and C, fatty alcohol use, autoimmune hepatitis, and cholestasis, has resulted in hepatic fibrosis.[15]. Although hepatic fibrosis usually reverses after the termination of damage, it may be developed into cirrhosis if the causal disease is not treated effectually, at which point it is, in general irreversible[16]. Contrary to popular belief, cirrhosis is not an irreversible condition. There is strong proof that fibrosis, even cirrhosis, may be reversed[9, 17] In animal models, carbon tetrachloride (CCl4) can cause hepatic fibrosis and hepatocyte apoptosis[18]. The damage reactions induced by injecting CCl4 in mice and rats are similar to those seen in people. CCl4 is a well-known hepatotoxicity model that can cause liver damage and can be used in clinical trials to assess hepatoprotective and anti-hepatotoxic treatment [19]. For liver fibrosis, there is a shortage of effective therapeutic treatment modalities. [15]. Natural medicine and traditional Chinese may be viable options [20].

Previous investigations have shown that emodin has a preventive effect in hepatic fibrosis[21]. Several animal and clinical investigations have shown that silymarin protects liver cells while also reducing hepatic fibrosis[22].So, in this study, it is used as a standard drug for the medication of liver fibrosis.

According to our knowledge, this is the first study to investigate the combined therapeutic effects of Emodin and Silymarin in the treatment of liver fibrosis. Emodin and Silymarin have a synergistic effect when used together.In this study, to detect the effect of Emodin and Silymarin in liver fibrosis either alone or in combination, the liver function tests such as ALT, AST, AFP and γ-GGT;) were investigated and studied in details. In addition, liver histological studyand immunohistochemistry study for PCNA was evaluated.

Statistical analysis of liver enzymes biomarkers, as shown in Table 1 revealed that there was a substantial difference among the five different groups in aspartate aminotransferase (AST), with the highest level in the positive group (202.7 ± 18.9) and the lowest level in the negative group (40.2 ± 0.95) ($p < 0.001$). At the same time, the combination-treated group showed significantly lower levels among the treated groups(49.3 ± 1.5) ($p < 0.001$) compared to the positive control group (Fig. 1A). Also, alanine aminotransferase (ALT) enzyme results marked a significant difference between the five different groups with the highest level in the positive group (151.7 ± 16.9) in comparison with the negative group (25.5 ± 1.3) ($p < 0.001$) while the combination-treated group had the least level among the treated groups (34.3 ± 1.5)($p < 0.001$) (Fig. 1B).

For the gamma-glutamyl transferase (γ-GGT) enzyme, as revealed from Table 2, data recorded a substantial difference between the five different groups with the highest level in the positive group (213 ± 19.2) and the lowest level in the negative group (44.5 ± 1.3) ($p < 0.001$) while the combination-treated group had the least level among the treated groups (61 ± 1.1) followed the Silymarin group and the Emodin group (Fig. 1C). Correspondingly, results highlighted a significant difference between the five different groups in alpha-fetoprotein (AFP) with the highest level in the positive group (17.5 ± 1.1) and the lowest level in the negative group (0.5 ± 0.08) ($p < 0.001$) while the combination-treated group had the least level among the treated groups (2.6 ± 0.15) following silymarine and Emodin groups respectively Silymarin group and the Emodin group (Fig. 1D) contrasted to the positive group with p-value less than 0.001 this result is agree with[23]

Table (1):Effect of Emodin, Silymarin,and combined therapy (Emodin and Silymarin) on liver function tests (ALT,AST,ɣ-GGT, and AFP), All values are presented as mean ± SD. Statistical significance was defined asa P-value of ≤0.05 indicating significant difference and a P-value of ≤0.001 indicating a highly significant difference. (*) indicate a highly significant difference between positive group and negative group. (+) indicate a highly significant difference between positive group and treatment groups.

Groups	***1st group*** Positive	***2nd group*** Negative	***3rd group***Silymarin & emodin	***4th group*** Silymarin	***5th group*** Emodin
***Serum AST* mean ± SD**	202.7±18.9*	40.2±0.95	49.3±1.5+	68±2.6+	97.2±9.5+
Serum ALT mean ± SD	151.7±16.9*	25.5±1.3	34.3±1.5+	34.5±2.9+	67.8±8.3+
GGT- mean ± SD	213±19.2*	44.5±1.3	61±1.1+	78.7±3.8+	109.6±9.48+
AFP level mean ± SD	17.5±1.1*	0.5±0.08	2.6±0.15+	6.0±0.2+	10.6±0.248+

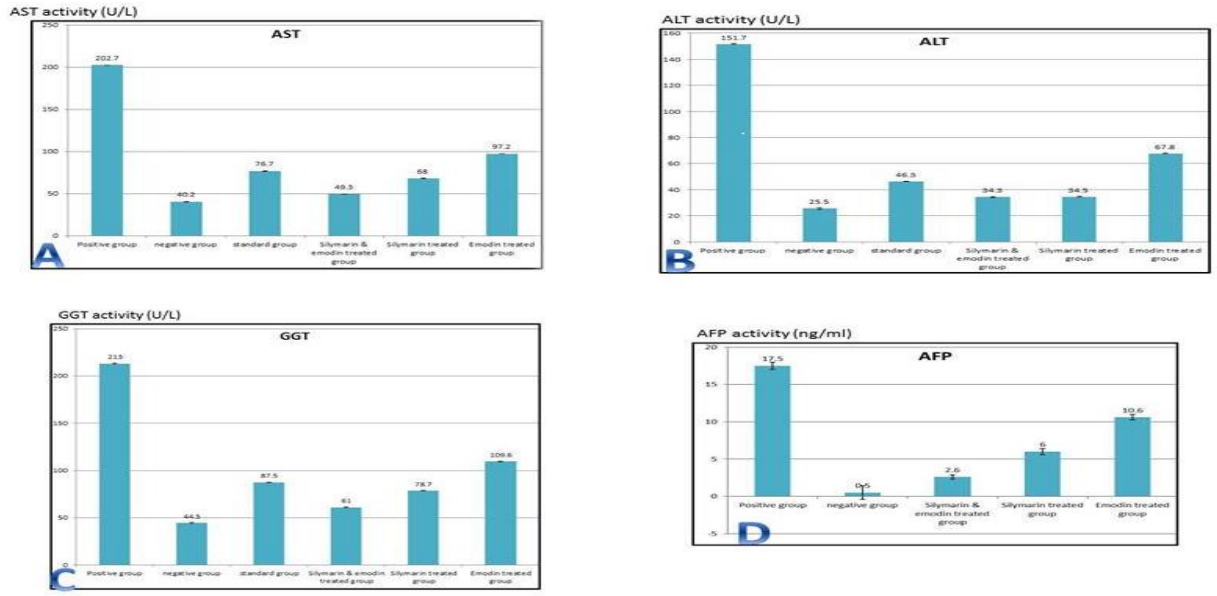

Figure (1):Graphs clarified the level of liver enzymes biomarkers in all tested groups as a bar chart for (A) AST, (B)ALT, (C)GGT, and (D) AFP.

We demonstrated the grades of disease using a histology approach to identify the pathological state of the liver and score hepatic fibrosis, which was one of the study's strengths. In mice given a combination of silymarin and emodin, histopathological analysis revealed that the status of the liver was typically improved. Microscopically , emodin and silymarin were displayed therapeutic effect compared to ccl4-induced liver fibrosis. Ccl4 provokes liver fibrosis in mice[7]. In a normal control group, the liver has smooth and plane surface, whereas ccl4 groups, the liver seemed rough and nodular through the development of micronodules. Besides, histopathology study of liver in the negative 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 (A). Additionally, these micrographs show the portal vein tributaries and the branch of the bile duct (Fig2). Photomicrographs (B) 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 (Fig2). Photomicrographs (C,d) show intact hepatic manner with a mild degree of vacuolation and many hepatocytes with normal cytoplasmic appearance in the liver as a result of using Emodin and silymarin individual (Fig2). Finally, photomicrographs (e) show intact hepatic architecture with no inflammatory cells 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 combination of emodin and silymarin treatment (Fig 2).

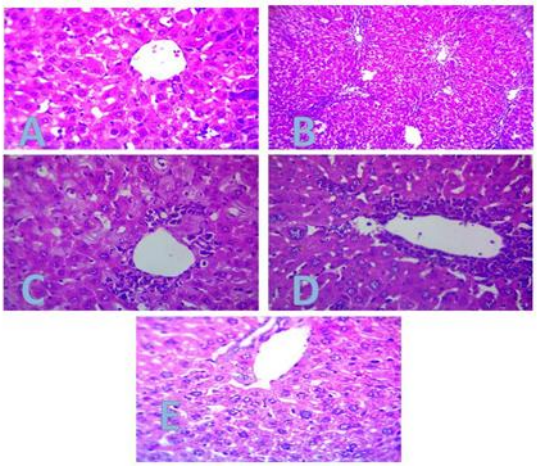

Figure (2): Histopathological changes in liver tissues; negative control group (photomicrographs A), fibrotic group (photomicrographs B), silymarin group (photomicrographs C) and emodin group (photomicrographs D) combination group(photomicrographsE). Slides were examined using H&E stain with magnification X400.

3.3 Effects of Silymarin and emodin on cellular proliferation

PCNA was used as a marker of hepatocyte proliferation (fig 3). CCl4 administration significantly increased the number of PCNA-positive cells as compared with the negative control group , emodin group , silymarin group and combination group .Silymarin ,emodin and their combination administration, significantly reduced the number of PCNA-positive hepatocytes in the experimental groups as compared with the CCl4 group. These results were in agreement with Herna´ndez-Mun˜ozet et al 2001 [24], Who found an increased PCNA after the discontinuation of the toxin, but without effective proliferative capacity of the liver. In our experiment, Silymarin and emodin were capable of restoring the normal proliferative capacity of hepatocytes, with no significant difference be-tween the doses. It could be a crucial step for the beneficial effect of Silymarin and emodin upon the carcinogenic potential of chronic hepatopathies evolution. In experimental models ,silymarin and emodin reduces cellular proliferation and inhibits the progression of metastasis[8, 25].

6. Conclusion

Our findings provide novel information about the anti-hepatic fibrosis effects of emodin, suggesting that these antifibrotic effects are probably mediated by decreasing enzymes checked for liver function test compared with the positive control group, which was further elucidated by the decrease of proliferating cell nuclear antigen, this novel study suggest that combination of emodin and silymarin exerts a synergistic effect as therapeutic agents for treatment of hepatic fibrosis after hepatotoxic injury.

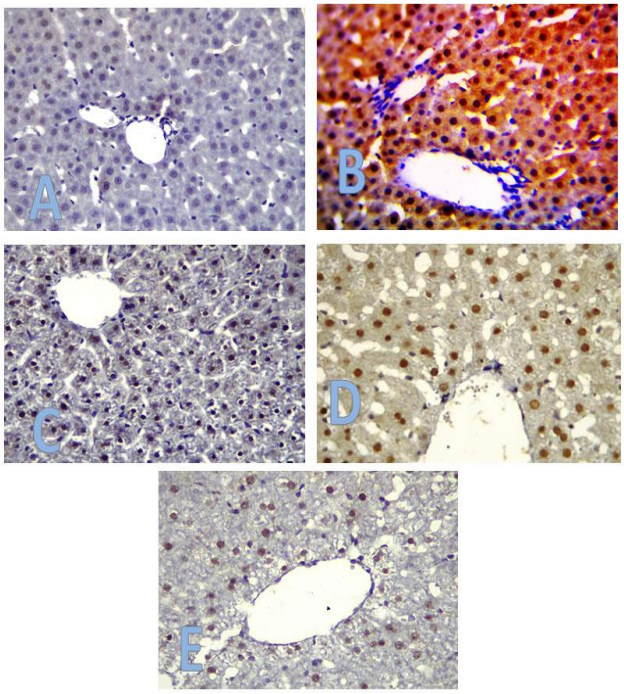

Figure (3):Influence of emodin and silymarin and their combination on PCNA in histology of liver slices. (A) negative controls show normal liver construction and no PCNA staining. (B) ccl4 controls cluster have numerous PCNA-positive hepatic nucleus (C) silymarin section of mice liver showed moderate PCNA expression. (D) emodin section of mice liver showed moderate PCNA expression.n. (E) combination group section of (combination group) liver mice stained by anti-PCNA antibody showed mild nuclear stain . (Magnification 400X)

7.Conflicts of interest

There is no conflict of interest, according to the authors.

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