Biochemical study on effect of Emodin and silymarin as a treatment on ccl4 induced hepatic fibrosis in mice

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\textbf{ABSTRACT}: A natural supplement with potential for hepatoprotection, renowned due to the fact that it has anti-inflammatory, anti-fibrotic, and immunomodulating properties, is known as silymarin (Si) and emodin. We recently proved that early liver fibrosis can be stopped in its tracks by using the typical therapeutic doses of silymarin and emodin. We used CCl\textsubscript{4} to cause liver damage in mice in order to further investigate the advantages of silymarin, emodin, and their combination administration upon liver modifications following the cessation of hepato-toxin. Carbon tetrachloride administration induces inflammation and liver fibrosis. Fifty Swiss albino mice were apportioned among 5 groups at random (\(n=10\)). The negative control group received olive oil twice a week for 4 weeks. The positive control (CCl\textsubscript{4} group) received CCl\textsubscript{4} in olive oil, by intraperitoneal injection, twice a week for four weeks. The emodin-treated group, after induction of fibrosis, was injected (i.p) with emodin daily for 3 weeks. After induction of fibrosis, the silymarin treated group received silymarin orally every day for 3 weeks. After induction of fibrosis, the combination treated group received Emodin (i.p) and silymarin orally every day for 3 weeks. We evaluated indicators of hepatic stellate cell activation (alpha-SMA expression) and liver function tests 4 weeks after the experiment had started. The novel outcome of this study proposed that emodin, silymarin and their combination could reduce liver inflammation. The effects of emodin mixed with silymarin were more efficient than those of emodin or silymarin alone.

\textbf{KEYWORDS}: Emodin; Silymarin; Liver fibrosis;alpha-SMA; HSCs.

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\section{I. INTRODUCTION}

Hepatic fibrosis is one of the most serious health issues in the world that has no effective cure. Hepatic stellate cells (HSCs), which are dormant and are activated, proliferate, and transdifferentiate into myofibroblasts, cause the production and deposition of extracellular matrix (ECM) and the progression of the wound in the liver, resulting in hepatic fibrosis. This process of wound healing is reversible. Early signs of liver fibrosis are not always apparent. (Wang et al., 2019).

Although hepatic fibrosis might progress to debilitating cirrhosis or hepatitis, the clinical signs of these diseases include bleeding, persistent gastritis, indigestion, and appetite loss. Liver fibrosis develops as a result of liver damage brought on by a range of factors, such as excessive alcohol use, autoimmune diseases, congenital abnormalities, viral hepatitis, excessive copper or iron deposition, insulin resistance, and steatosis(Seki & Brenner, 2015). A specific approach does not reveal hepatic fibrosis. Therefore, treating hepatic fibrosis is
challenging. The most effective way for healing liver damage is through liver transplantation. While the high cost and limited supply of donor livers make it difficult for every patient to get. Finding a medication or drug that effectively combats liver fibrosis is therefore necessary. According to our earlier published findings, growing numbers of substances are being discovered to possess the capacity to suppress hepatic fibrosis (Liu, Zhang, Qian, Wu, & Ma, 2018; Wang, Wang, Song, Li, & Yuan, 2018).

Numerous studies have discovered that Chinese herbal medicine offers special advantages in the healing of liver illnesses, and many prospective small-molecule substances, including curcumin and quercetin, have been discovered (Olivas-Aguirre et al., 2017; Wei et al., 2018). Herbal products such as emodin or silymarin are widely used for hepatoprotective effects. In addition, emodin alone or in combination may present a new strategy for treating liver fibrosis.

For generations, Asians have utilised emodin (1,3,8-trihydroxy-6-methylanthraquinone), an extract of giant knotweed and rhubarb root, to treat gastrointestinal, lung, inflammatory, and liver problems. Giant Knotweed Rhizome contains the main active component, emodin, which has a variety of potent pharmacological actions, including anticancer, anti-inflammatory, and antibacterial activities. According to numerous studies, emodin is beneficial in decreasing liver fibrosis. The fundamental mechanisms, however, still need to be clarified. Although emodin has been demonstrated to reduce the effects of CCl4, further research is still needed to determine the optimal dosage and the precise mechanism of CCl4 induced hepatic fibrosis in rats (Hu, Liu, Xue, & Li, 2020).

A medicinal plant called silymarin (Si) contains all of the silybummarianum's therapeutic elements. It consists of silybin, silydianin, isosilybin, and silychristin, a unique flavonoid complex. Silymarin has a wide range of biological actions, including anti-inflammatory, immunomodulatory, and antioxidant effects. Since silymarin has low toxicity, no side effects, and specific efficacy, it is utilised as a traditional liver-protecting medication. Numerous in vitro and in vivo experiments have demonstrated the protective effects of silymarin on liver cells as well as its ability to reduce hepatic fibrosis. But the main mechanism is still a mystery (Tighe, Akhtar, Iqbal, & Ahmed, 2020).

Therefore, in this work, we sought to learn more about the advantages of silymarin and emodin administration in treating the liver damage caused by CCl4 in mice.

II. EXPERIMENTAL DESIGN: ANIMAL HANDLING AND EXPERIMENTAL PROTOCOL.

Animal

All animal-related work was done in compliance with the institutional animal care and use committee's (ZU-IACUC) ethical reference number (ZU-IACUC/1/F/39/2019) guidelines for animal studies, which were published by the faculty of science at Zagazige College.

Male albino mice weighing 25–5 g were obtained from the laboratory animal centre of the scientific and medicinal research center. The animals were grown in a breeding chamber with a regulated environment (temperature: 22°C and above; humidity: 60–80%). A 12-hour cycle of artificial light and darkness was used to illuminate the breeding room, which was also routinely cleaned and disinfected. Water and food were available to the animal at all times. The Centre for Scientific and Medical Research provided the standard mice meal.

Reagents and Antibody

Emodin was kindly provided by Dr. Atef M. Amer. Highly pure carbon tetrachloride (CCL4) and phosphate buffer slain (PBS) were purchased from El-GomhouriaCo. (El-Gomhouria, Cairo, Egypt). Silymarin 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). The Alanine aminotransferase (ALT), aspartate aminotransferase (AST), and colorimeter testing kits were obtained from...
Teco Diagnostic Co. (Anaheim, USA). Anti-PCNA antibody (ab18197) from Xpress Bio Life Science Co. (Xpress Bio, USA) was used to obtain GGT.

Experimental protocol

A total of 50 mice were at random divided into four groups (10 mice per group).
1-Negative control group: healthy mice fed a normal chow diet and receiving no drug received olive oil twice a week for 4 weeks.
2-Positive control group: in which mice were injected intraperitoneally with CCl4 at a dose of 1 mg/kg (diluted in the olive oil 1:1) twice weekly for 4 weeks to induce liver fibrosis.
3-Emodin treated group: mice were treated with emodin. After induction of fibrosis, emodin was injected intraperitoneally in a dose (5mg/kg) every day for 21 days.
4-Silymarin treated group: in which mice received silymarin after induction of fibrosis orally every day at a dose of 100mg/kg for 21 days.

In the 5-combination treated group, mice were injected intraperitoneally with emodin in a dose of (2.5 mg/kg) and received silymarin orally in a dose of (50 mg/kg) after induction of fibrosis every day for 21 days.

Finally, experiment animals were euthanized with sodium pentobarbital intraperitoneal as well as subjected to a complete necropsy. Blood samples were obtained from the inferior vena cava of each mouse and collected in non-heparinized glass tubes for measuring biochemical markers. Each group's liver tissue samples came from the same area and were preserved in neutral formalin (10).

Hepatic function assessment

The automatic biochemical analyzer was used to measure the major markers of liver function, serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) (Teco Diagnostic Co., Anaheim, USA). Its primary method relied on a reflectance spectrophotometer, and quantitative sandwich ELISA kits were used to assess alpha-fetoprotein (AFP) and gamma-glutamyl transferase (GGT), two significant serum liver function markers. The technique was applied in accordance with the manufacturer's instructions (My Bio Source Co., My Bio Source, USA-Xpress Bio Life Science Co., xpressBio, USA).

Immunohistochemical examination of α-SMA

Serial portions of paraffin blocks with a 4 m thickness cut were immunostained. The tissue samples were washed thoroughly in graded ethanol after being deparaffinized in xylene. To stop an unintended peroxidase reaction, deparaffinized tissue pieces were exposed to hydrogen peroxide for 10 minutes, 20 minutes of microwave antigen retrieval were spent in citrate buffer 0.01 M (pH 6.0). The Alpha-Smooth Muscle Actin Monoclonal Antibody was applied to the slides and incubated for 60 minutes at room temperature after washing with phosphate buffer saline (PBS) (M0851, Dako, USA). r's instructions

Statistical analysis

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III. RESULTS

Emodin and silymarin attenuated CCL4 induced liver fibrosis and improve liver function markers.
The amount of aminotransferase in the blood is the most often used marker of liver impairment in medicine. The liver's ALT, AST, AFP, and -GGT enzymes were therefore tested for in the serum from peripheral blood of the mice from each group during the in vivo experiment using blood taken from the abdominal aorta of each animal. The amounts of the AST, ALT, AFP, and -GGT markers were all statistically greatly increased in the positive control group than in the negative control group (P < 0.05), as shown in Figure 1 and Table 1, but they were statistically significantly lower in the emodin treated group and the silymarin treated group when compared to the positive control group. The current investigation discovered that these indicators were considerably less in the combination group when compared to the separate therapy groups. In mice exposed to CCl4, silymarin and emodin can effectively slow the development of liver fibrosis; however, the efficacy of the treatment was dose dependent for both emodin and silymarin, as evidenced by these divergent test results. This shows that emodin and silymarin may be effective drugs to prevent liver damage.

The effects of emodin and silymarin on alpha -SMA expression in hepatic fibrosis brought on by CCL4

α-SMA is a crucial indicator of activated HSCs. No other cells in the liver, outside the activated HSCs and vascular smooth muscle cells, exhibit high cytoplasmic alpha-SMA staining. Activated HSCs with the myofibroblastic phenotype can be identified by their alpha-SMA expression, and liver tissue can be examined for alpha-SMA expression levels to measure the degree of liver injury.

Immunohistochemical results of liver sections highlighted positive expressions of alpha-smooth muscle actin antibody (α-SMA) in all tested groups. Sections of the negative control group revealed a normal positive reaction to α- SMA surrounding hepatic sinusoids (Fig. 2A). Conversely, the positive ccl4 group exhibited a strong reaction to SMA through all hepatic tissue (Fig. 2B). The liver from the emodin treated group (Fig. 2C) as well as the silymarin treated group (Fig. 2D) displayed a moderate expression of α-SMA. On the other hand, liver sections from the combination treated group exhibited a normal reaction to α-SMA, which was nearly identical to the negative control group (Fig. 2E). Intensity analysis of α-SMA confirmed the photographic sections and cleared a high significant variation between the negative control group and positive ccl4, emodin, as well as silymarin treated groups. Meanwhile, no significant difference was recorded between the negative control group and the combination treated group. These results encourage the potential effect of the combination of emodin and silymarin in the treatment of liver fibrosis (Fig. 2F).

IV. DISCUSSION

Hepatic fibrosis is the result of severe liver disease of different etiologies including hepatitis B and C, fatty liver disease, alcohol intake, autoimmune hepatitis, and cholestasis. (Zhou et al., 2020). Although hepatic fibrosis usually reverses after termination of damage, it may develop into cirrhosis if the causal disease is not treated effectively, at which point it is in general irreversible. (Hu et al., 2020). Contrary to conventional wisdom, which holds that cirrhosis is an irreversible condition, there is compelling evidence that fibrosis, including cirrhosis, may be curable. (Hu et al., 2020; Luedde & Schwabe, 2011; Seki & Brenner, 2015).

In animal models, carbon tetrachloride (CCl4) can cause hepatic fibrosis and hepatocyte apoptosis. Damage reactions prompted by ccl4 injection in mouse and rat models and human damage responses are similar. One of the best models for hepatotoxicity is Ccl4, and because it is able to cause liver damage, it can be utilised in clinical trials to assess potential hepatoprotective and anti-hepatotoxic therapies. (Guo et al., 2013; Hu et al., 2020). There are currently no proven clinical treatments for liver fibrosis. (Zhao et al., 2018; Zhou et al., 2020). Natural and traditional Chinese medicine might be a promising option. (X. Dong et al., 2020; Zhou et al., 2020).

While hepatocytes undergo apoptosis, hepatic stellate cells (HSCs), which are the main source of activated myofibroblasts, migrate to the site of damage to engulf the apoptotic bodies. This engulfment induces activation of these cells, and in their activated state, these cells encourage the synthesis and insertion of extracellular matrix (ECM) and wound progress in the liver (Fehér & Lengyel, 2012; Han, Wu, Yang, & Cao, 2020; Hu et al., 2020; Seki & Brenner, 2015).
There is no established treatment for hepatic fibrosis (Duval, Moreno-Cuevas, González-Garza, Rodríguez-Montalvo, & Cruz-Vega, 2014; Seki & Brenner, 2015). Even though it is known that preventing liver injury events, such as stopping alcohol or successfully treating viral hepatitis, helps to regulate the process. Nevertheless, in the vast majority of patients, these acts do not seem to be adequate to prevent progression to cirrhosis.

(Henderson & Iredale, 2007), Our knowledge of the pathogenesis of hepatic fibrosis has significantly improved during the past 20 years, but there are still significant gaps in our ability to translate this fundamental knowledge into potent antifibrotic medications. Strategies for treating liver fibrosis should address all of the relevant cell lines, beginning with HSC and hepatocytes, and should take into account the aetiology of the disease's variable manifestations. Due to their safety, affordability, and adaptability, medicinal herbs are used more frequently as anti-fibrotic medicines. We've already looked at how herbal remedies can inhibit HSC activation and lessen ECM buildup in the liver, which can diminish liver fibrosis (Duval et al., 2014). However, this activity may be clarified by other antifibrotic processes, such as the regulation of the different cell lines' apoptosis.

This study concentrates on two additional ways that bioactive substances inhibit apoptosis to reduce hepatic fibrosis: HSC protection against apoptosis and activation of apoptosis in HSC (Duval et al., 2014). Giant knotweed and rhubarb root are two traditional Chinese remedies that contain the major active component emodin. The pharmacological effects of emodin have been shown to include anticancer, antiviral, anti-inflammatory, and immunomodulatory action. According to a prior study, emodin has a preventive effect on hepatic fibrosis (Hu et al., 2020; Liu et al., 2018).

Silymarin (Si) is a medicinal plant. It is extracted from the seeds of Silybummarianum (Si), mainly containing silybin, isosilybin, silydianin, and silychristin. Because of its very low toxicity and efficiency, it is widely used in the treatment of hepatic fibrosis. Several animal studies and clinical trials have established that silymarin has the role of protecting liver cells and releasing hepatic fibrosis (Okda, Abd-Alhaseeb, Barka, & Ragab, 2019; Zhao et al., 2017). In this study it is used as a standard drug for treatment hepatic fibrosis. To our understanding, this research is one of the few to assess the combined molecular effects of emodin and silymarin on the treatment of liver fibrosis. The combination of emodin and silymarin exerts a synergistic effect. When liver cells suffer damage or necrosis, the release of AST, ALT, -GGT, and AFP enzymes into the serum reveals the degree and type of liver injury based on the enzyme levels (Lu, Yang, Huang, Feng, & Li, 2017; Woo et al., 2017).

In contrast to other human tissues, ALT is established in larger amounts in the kidneys, muscles, heart, and liver. Regardless of the connection between significantly elevated ALT levels and its specificity for hepatocellular disorders, higher than normal levels may suggest bile duct or liver damage or injury. However, the absolute ALT elevation peak does not correspond with the degree of damage to liver cells (Kruse & Gu, 2009). AST catalyses the transamination process. The cytoplasmic and mitochondrial forms of the isoenzyme AST are genetically distinct from one another. The heart contains the largest quantities of AST when compared to other body tissues such as the liver, skeletal muscle, and kidney. (Gowda et al., 2009). Severe tissue necrosis, chronic liver illnesses such as necrosis and liver tissue degeneration, and myocardial infarction all cause elevated mitochondrial AST levels. (Thapa & Walia, 2007). Patients with cirrhosis and other liver diseases usually have elevated AST levels in addition to their common elevated ALT levels (Gowda et al., 2009).

AFP is found in small intestinal mucosal epithelia, proximal convoluted kidney, liver, bone, and placenta tubules. Intestinal lipid transfer and calcification in the bone were carried out. High AFP levels may also be caused by hepatic and bony metastasis. An increase in AFP can be caused by other diseases, including infiltrative liver diseases, abscesses, granulomatous liver disease, and amyloidosis. In cirrhosis hepatitis and congestive heart failure, moderately elevated levels of AFP can be observed (Gowda et al., 2009).
Hepatocytes, biliary epithelial cells, renal tubules, the pancreas, and the gut all contain the microsomal enzyme GGT. Additionally, it is a component of the cell membrane, where it functions to transport peptides into the cell and aid in glutathione metabolism. While renal tissue exhibits higher concentrations, the hepatobiliary system is primarily responsible for producing serum GGT. Increased levels may be a sign of liver or bile duct disease (Gowda et al., 2009). The biochemical index recorded in the current study revealed that the CCl4 group's liver function was significantly impaired, as indicated by indices of liver damage, despite the fact that ALT, AST, AFP, and GGT were dramatically decreased after treatment with emodin and silymarin. These results are in line with previous studies of (Liu et al., 2018), (Tsai et al., 2008) and (Hu et al., 2020). According to our study results, the combination of silymarin and emodin applies a synergistic effect on the liver enzymes decreasing compared to each individual group in mice treated with emodin and silymarin.

After liver damage, quiescent HSCs are stimulated into fibrogenic and proliferative cells. Hepatic fibrosis is still primarily caused by this stimulation. It makes hepatic stellate cells a primary target for a treatment for fibrosis (Han et al., 2020; Hu et al., 2020; Wang et al., 2019).

Alpha-SMA is the most significant factor used for detecting liver fibrosis. It is an effective biomarker for detecting myofibroblast-like cells in both rats and people (Hu et al., 2020; Nouchi, Tanaka, Tsukada, Sato, & Marumo, 1991; Tsai et al., 2008). Additionally, a reliable indicator of activated hepatic stellate cells that occur prior to the deposition of fibrous tissue (Carpino et al., 2005; Wang et al., 2019; Zhou et al., 2020). As a result, it is useful for evaluating the effectiveness of treatment. The current data demonstrated the liver tissues from the positive control group had considerably more -SMA than those from the normal group. These alterations were extensively reversed in the therapy group by silymarin and emodin. According to reports, the quantity of hepatic stellate cells that are active decreases along with the decline in -SMA (Parsons et al., 2004). Liver fibrosis is reversed through the death of the activated hepatic stellate cells by apoptosis (Hu et al., 2020; Iredale et al., 1998). As a result, we propose that silymarin and emodin's ameliorative effects on the resolution of liver fibrosis may at least be achieved by encouraging the apoptosis of the activated hepatic stellate cells. Our findings are consistent with (Tsai et al., 2008), (Guo et al., 2013) and (M.-X. Dong et al., 2009) and (Clichici et al., 2016). Our research's discovery that the combination of emodin and silymarin decreased alpha-SMA expression in vivo further supports the theory that the two compounds work together to limit CCL4-induced liver fibrosis by blocking HSC activation.

Compliances and ethics

The author(s) certify that no conflicts of interest exist. All animal research was done in compliance with the ethical standards established by Zagazig University, Faculty of Science, Ethical Committee and authorised by the Institutional Animal Care.

REFERENCES


Tables and figures legends:

Table 1: Effect of Emodin, Silymarin, and combined therapy (Emodin and Silymarin) on liver function tests (ALT, AST, \(\gamma\)-GGT, and AFP). All values are presented as mean \(\pm\) SD. Statistical significance was defined as a P-value of \(\leq 0.05\) indicating significant difference and a P-value of \(\leq 0.001\) 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.

<table>
<thead>
<tr>
<th>Groups</th>
<th>1st group Positive</th>
<th>2nd group Negative</th>
<th>3rd group Silymarin &amp; emodin</th>
<th>4th group Silymarin</th>
<th>5th group Emodin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum AST mean (\pm) SD</td>
<td>202.7(\pm)18.9*</td>
<td>40.2(\pm)0.95</td>
<td>49.3(\pm)1.5*</td>
<td>68(\pm)2.6*</td>
<td>97.2(\pm)9.5*</td>
</tr>
<tr>
<td>Serum ALT mean (\pm) SD</td>
<td>151.7(\pm)16.9*</td>
<td>25.5(\pm)1.3</td>
<td>34.3(\pm)1.5*</td>
<td>34.5(\pm)2.9*</td>
<td>67.8(\pm)8.3*</td>
</tr>
<tr>
<td>GGT mean (\pm) SD</td>
<td>213(\pm)19.2*</td>
<td>44.5(\pm)1.3</td>
<td>61(\pm)1.1*</td>
<td>78.7(\pm)3.8*</td>
<td>109.6(\pm)9.48*</td>
</tr>
<tr>
<td>AFP level mean (\pm) SD</td>
<td>17.5(\pm)1.1*</td>
<td>0.5(\pm)0.08</td>
<td>2.6(\pm)0.15*</td>
<td>6.0(\pm)0.2*</td>
<td>10.6(\pm)0.248*</td>
</tr>
</tbody>
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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.

Figure 2: Photomicrographs of mice liver demonstrated the reaction of smooth muscle to α-SMA antibody in all tested groups: (A) Liver section from negative control group presented normal reaction to α-SMA (arrow). (B) Section of the positive CCl₄-group, liver mice showed strong expression of α-SMA (arrow) in liver sinusoids and surrounding central vein (CV). (C) The Emodin-treated group exhibited moderate cytoplasmic expressions inside hepatocytes, liver sinusoids (arrow), and around the central vein (CV). (D) Section of liver mice from the silymarin treated group displayed moderate expression of α-SMA around the central vein and portal tract (arrow). (E) Section of the combination-treated group showed mild expression of α-SMA around the central vein and in liver sinusoids. (F) Graph clarified that there is no significant variation among the negative and the combination group. Meanwhile, there is a significant difference between the negative group, emodin, and silymarin groups compared with the positive group.