ISSN: 0975 -8542

Journal of Global Pharma Technology

Available Online at: www.jgpt.co.in

RESEARCH ARTICLE

Effects of Emodin on CCl_4 Induced Liver Fibrosis in Mice Model

Mohannad Falah Hassan[1*], Haitham Mahmood Kadhim[2], Enass Jawad Kadhim[3]

1. *Al-Nahrain University/Medical college, Baghdad, Iraq*
2. *Pharmacology Department / College of Pharmacy, Al-Nahrain University, Baghdad, Iraq.*
3. *Pharmacognosy Department, College of Pharmacy, University of Baghdad, Baghdad, Iraq.*

***Corresponding Author: Mohannad Falah Hassan**

Abstract

Many compounds demonstrated an anti-fibrotic activity, but none have been used in the clinics. The current study aims to assess the *in-vivo* emodin anti-fibrotic activity in liver fibrosis. Thirty-two mice were divided into four groups; the first group is composed of eight healthy mice to be a negative control group, while the remaining twenty-four mice had received intraperitoneal carbon tetrachloride 1ml/kg twice weekly for six weeks. Then, eight of these mice were sacrificed to be a positive control group, while the remaining sixteen mice were divided into two groups; one received six intraperitoneal doses every other day of emodin 40 mg/kg and the other received silymarin 20 mg/kg. Mice livers have taken and examined histopathologically to confirm the diagnosis of liver fibrosis and to assess the antifibrotic effects of emodin, then immunohistochemical evaluation was done for collagen III and Interleukin-4 secretion. Statistics used are ANOVA test followed by Tukey's post HOC analysis. Emodin reduces fibrosis significantly in histopathologic sections, collagen content and Interleukin-4 content (in which $p<0.0001$ was significant). There is no significant difference between emodin and silymarin in lowering fibrosis grade ($p=0.1411$), collagen III level ($p=0.958729$), and interleukin-4 level ($p=0.144028$). Emodin anti-fibrotic effects is related to lowering nuclear factor-κB leading to IL-4 lowering, decrease of epithelial mesenchymal transition by increasing E-cadherin, suppression of TGF-ß1 Smad signaling, Up-regulation of metastasis- associated gene 3, inhibits the Toll-like receptor-4 (TLR-4) pathway, and blocking the migration and proliferation of HSCs induced by platelet derived growth factor. Emodins have anti-fibrotic activity comparable to that of silymarin and can alleviate liver cirrhosis.

Keywords: *Liver fibrosis, Collagen III, Emodin, Interleukin-4, Silymarin.*

Introduction

This study was aimed to assess the *in vivo* antifibrotic activity of emodin in liver fibrosis. Carbon tetrachloride (CCl_4) was used to induce liver fibrosis in the current study. CCl_4 is an industrial solvent widely used for dissolving non-polar compounds. Once CCl_4 has been injected, extensive metabolism via liver CYP2E1 can produce many types of free radicals like trichloromethyl ($CCl_3\cdot$), trichloro-methylperoxy ($OOCCl_3\cdot$), and chloride ($Cl\cdot$) [1, 2]. Free radicals have very high affinity for electrons; seeking biological tissues ones. It causes protein peroxidation and enzyme and DNA distortion and initiates lipid peroxidation process [3, 4].

These free radicals can affect different tissues and organs [5, 6], detailed as follows:

- The long-term oral exposure to CCl_4 causes marked hepatotoxicity with resulting fibrosis, bile duct proliferation, cirrhosis and even hepatocellular carcinoma, CCl_4 is the most commonly used liver fibrosis induction model [2].The mechanisms responsible for CCl_4-induced liver fibrosis may include retinol metabolism, peroxisome proliferator agonist receptor (PPAR) signaling pathway, arachidonic acid metabolism, glycolysis/gluconeogenesis, and glycerolipid metabolism by regulating targets such as CYP4A3,ALDH7A1 and $ALDH_2$ [7]. Portal hypertension and decompensated liver also develop when CCl_4 administration is used for a sufficient period [8].

- CCl_4 causes bowman capsule damage, renal tubular degeneration and loss of border brush and increased serum creatinine, urea and uric acid levels. There is a significant rise in serum markers of oxidative stress and the lipid peroxidation product malondialdehyde along with the reduction of antioxidant enzymes such as superoxide dismutase, catalase and glutathion peroxidase [9].

- CCl_4 can affect the liver and kidneys simultaneously, causing hepatorenal syndrome [10] by peroxidizing cell proteins thereby activating the inflammatory pathway [11].

- The brain can be affected also. Even a single dose of CCl_4 can induce brain toxicity. A marked increase in thiobarbituric acid reactive substances (TBARS) in the brain, to an extent higher than that of liver. Being lipophilic, CCl_4 easily crosses cell membranes and gets distributed in tissues like the brain and generates free radical which leads to membrane lipid peroxidation [12].

- In bone, CCl_4 induce osteoporosis by oxidative stress and apoptosis. CCl_4 dose given by intramuscular injections showed that CCl_4 cause muscle tissue atrophy, decrease in muscle fibers diameter, cartilage diameter and bone tissue collagen [13].

- CCl_4 caused germ cells loss, meiosis interruption, abnormally shaped sperms, abnormally germinative epithelium, abnormally fibroblast and inflammatory cells, atrophy of somniferous tubules [6], and necrosis in spermatogoneal cells which line seminiferous tubules [1].

- Increased reactive oxygen species (ROS) are responsible for lung carcinoma, pulmonary fibrosis, chronic bronchitis, emphysema, and pleural diseases by intraperitoneal application of 1 ml/kg of CCl_4 for 10 days [14].A single high intraperitoneal dose of CCl_4 (2 ml/kg) causes increased TNF-α, malondialdehyde (MDA), and nitric oxide(NO) [15].

- Hematological parameters such as white blood cells count, red blood cells count, mean corpuscular hemoglobin concentration and blood platelets count has also decreased by CCl_4 [16].

- Single dose of CCl_4 caused cardiotoxicity observed by a high rise in plasma aspartate aminotransferase, lactate dehydrogenase, troponine I and creatine kinase activities [17]. After eight weeks of CCl_4 administration, tissue ischemia and myocardial infarction was observed by increased levels of cardiac marker enzymes creatine kinase (CK), cardiac creatine kinase-MB fraction (CK-MB).The integrity of cardiac cell membrane gets disturbed as a consequence of peroxidation of membrane by oxygen-derived free radicals [18].

Emodin (1, 3, 8- trihydroxy- 6-methylanthraquinone) is a naturally occurring poly-phenolic anthraquinone with a possible antifibrotic effect (figure 1). Emodin has *in vitro* antifibrotic activity in both total collagen accumulation and nodule formation assays [19]. In the current study, emodin is extracted from *Aloe Barbadensis*, a plant authenticated by Dr. Zainab Jalil Awwad /department of Pharmacognosy/College of pharmacy/Baghdad University.

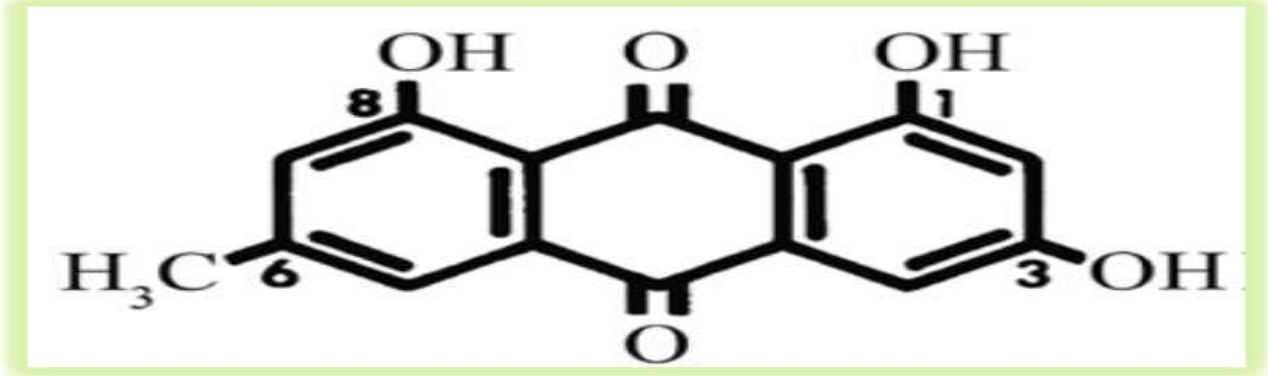

Figure 1: Emodin structure

Emodin is converted to an aglycone active part by large intestine flora, exerting laxative effect by disrupting epithelial cells,acting on cystic fibrosis trans-membrane receptor (CFTR) chloride channels [20]. Hydroxyl, methyl and carbonyl groups are the key determinants of biological activities besides than laxative effect [21], which are summarized as follows:

- *Antifibrotic action* involving regulating gene expression of epidermal growth factor (EGF),transforming growth factor beta-1 (TGF-ß1) ,and platelet derived growth factor (PDGF).The protective effect on hepatocytes alleviate cholestatic hepatitis by antagonizing pro-inflammatory cytokines and mediators, inhibiting oxidative damage, improving hepatic microcirculation, reducing impairment signals, and controlling neutrophils infiltration [21].Emodin may have hepatoprotective effects by its reactive oxygen scavenging effect [22].

- *Antimicrobial action*: which is related to inhibiting electron flow in the respiratory chain especially at ubiquinone and cytochrome b level, dissipation of the proton motive force [23], and by generation of superoxide after the reduction to semiquinone [24]?

- *antinflammatory actions*: which may be related to inhibition of nitric oxide production, reduction of prostaglandin synthesis, or even by its inhibitory effect against superoxide production [25], Decrease of IL-1, IL-2, IL-4 and TNF-α cytokine production and Hydrogen peroxide generated from semiquinone and suppression of NFκB [26]. Other mechanisms involve modulation of Th1/Th2/Th17 cytokines in spleenocytes by inhibiting the PI3K/Akt signaling pathway [27, 28]. Emodin could be a therapeutic potential against allergic bronchial asthma by exhibiting anti-inflammatory effects in the airway inflammation [29].

- *Anticancer actions*: related to cell cycle arrest at G2/M at HepG2/C3A cells via up regulation of p53 and p21 genes [30].It reports that emodin cause down regulation of the TCF/LEF transcriptional activity in human colorectal cancer cells (SW480 and SW620), interfering in the Wnt-signaling pathway .The quinone structure of emodin is converted to semiquinone during metabolism generating ROS, which contributes to mitochondrial injury, reduction of mitochondrial trans-membrane potential, cytochrome C and Smac release, and subsequent caspase activation resulting induction of apoptosis as another mode of emodin anticancer effect [31].

- *Antiangiogenic property*: which is related to down regulation of the transcription factor Runx2 and inhibition of matrix metalloproteinases and vascular endothelial growth factor receptor-2 (VEGFR-2) [32].

- *Cardiovascular* protective effect is related to increasing atrial natriuretic peptide (ANP) secretion via activating ATP-linked K^+ channels and inhibition of L-type Ca2+ channels [33].

- *Neuroprotective* effects are related to inhibition of cortical neurons apoptosis and prevent ß-amyloid-induced neuronal death in vitro [34].

- There is a promise in early-stages diabetes *nephropathy* observed by inhibiting p38 MAPK pathway and fibronectin down regulation. Emodin also had protective role against cisplatin-induced nephrotoxicity in rats [35].

Liver fibrosis is the continued accumulation of normal extracellular matrix (ECM) macromolecules ,it is a dynamic process involving a progression and resolution steps [36].Cirrhosis is the end stage sequela of fibrosis ,in which each liver lobule appear as a distinct nodule [37]. Liver vascular architecture can be severely distorted when collagen, glycoproteins and proteoglycans are present in high contents ending in complete organ failure [38]. The ECM also acts as a reservoir for pro-inflammatory and pro-fibrogenic mediators [36]. Fibrosis involves participation of parenchymal and non-parenchymal liver cells, as well as infiltrating immune cells [39].

Continued hepatocyte death causes oxygen free radical production and damage-associated molecular patterns (DAMPs) leading to continued hepatic stellate cell (HSCs) activation and impaired regenerative capacity of the liver [40]. HSCs are the fibrogenic effector cell, hepatic resident macrophages (Kupffer cells) and lymphocytes can also activate HSCs [41].

Kupffer cells release pro-inflammatory factors such as TNFα and Interlekin-6 (IL-6), and pro-fibrogenic factors, especially TGF-ß and connective tissue growth factor(CTGF) [41] .What increase the complexity of fibrosis process is the vicious cycle between these factors, for example, TGF-ß stimulate CTGF synthesis while CTGF acts as a mediator to enhance the TGF-ß fibrogenic actions [42].Another example for complexity is the activation of toll-like receptor 4 (TLR4) which further enhances TGF-ß dependent HSC activation [43].

Many compounds demonstrated an antifibrotic activity, but none have used in the clinics. Food and Drug Administration (FDA) have not approved any medical treatments to treat liver fibrosis patients. Specific therapies for liver disease have primarily been etiology-driven by eliminating or ameliorating the causative agent of chronic liver diseases (CLD) [44, 45].

Materials and Methods

Materials

The chemicals are supplied by the sources in brackets as follows: Silymarin and chloroform (Sigma Aldrich-USA),carbon tetrachloride and dimethyl sulphoxide "DMSO"(Alpha Chemika-India),collagen 3α1 polyclonal antibody (My Bio Source-USA), interleukin-4 antibody (Santa Cruz biotechnology-USA),emodin standard (Chengdu biopurify phytochemicals-China), eosin yellow (Thomas Baker -India), formaldehyde solution (37-38%) (Panreac-Spain), hematoxylin stain (Fluka-Germany), methanol for preparative high pressure liquid chromatography" preparative HPLC' (Romil-UK), olive oil (pure 100%) (Oilex-Madrid/Spain), paraffin wax blocks (BDH-England), staining kit (abcam-UK).

Instruments

Distillator (Boeco-Germany), chiller Ultratemp 2000 (Buchi-Germany), electronic sensitive balance (Sartorious-Germany), hitocenter (Shandon-China), light microscope supplied with camera (Genix-England), micropipette 100 to 1000 µland 20 to 200µl (Huawei-China), micropipette 5 to 50µl (Slamed-Germany), microtome (Sakura-Japan),plain microscope slide and antibody containing immunohistochemical slide (EC Isolab-Germany) , preparative HPLC device with UV detector(Jas.co-Japan),and rotary evaporator(Stuart-UK).

Extraction of emodin from Aloe Barbadensis

Coarsely shade dried seeds and areal parts of the plants were defatted with hexane for 24 hour then allowed to dry at room temperature. The defatted plant material was extracted with 80% ethanol in soxhlet apparatus until complete exhaustion. The alcoholic extract was evaporated under reduced pressure at a temperature not exceeding 40º to give a dark greenish-yellow residue designated as a crude fraction [46].

One gram (1 gm) of extract obtained from plant was dissolved in a minimum quantity of chloroform and injected into preparative HPLC device using Methanol: water (65:35) as a mobile phase, mediterraneaC18, 5 µm 15 X 2.12 as a column, the flow rate: 3 ml / min and the injection volume was one ml and the UV Detector was at λ 366 nm [47].

Preparative HPLC was used in this study to isolate in a very pure and high quantity emodin from *aloe* plant. Preparative HPLC is associated with large columns and high flow rates and can receive large injected volumes (1 ml as compared to the microliters in analytical one).Chromatogram gave three peaks which represent three different compounds, one of them (E2) is a major peak, which represent emodin. The solution of the highest peak is dried to get the powder. Figure 2 illustrate Aloe extract peaks in preparative HPLC recorder [46]. **Animals**

Under the agreement of institutional review board of medical college/AL-Nahrain University, thirty-two BALB-c mice weighing about 25 to 28 gm and aged about 1.5 to 3 months supplied from vaccines and sera institute/Iraqi Ministry of health, were housed in cages at the period between 26 January 2018 to 7 April 2018 in a good ventilated room and suitable temperature (20° to 25°), all were enabled to receive water and normal mice diet (the standard pellets). After completing the study, all animals were anesthetized by chloroform and humanely killed to get their livers. *Experimental design*

Thirty-two mice were divided into four groups, each one contains eight mice.

Group I: Negative control (apparently healthy mice that will not receive any treatment).

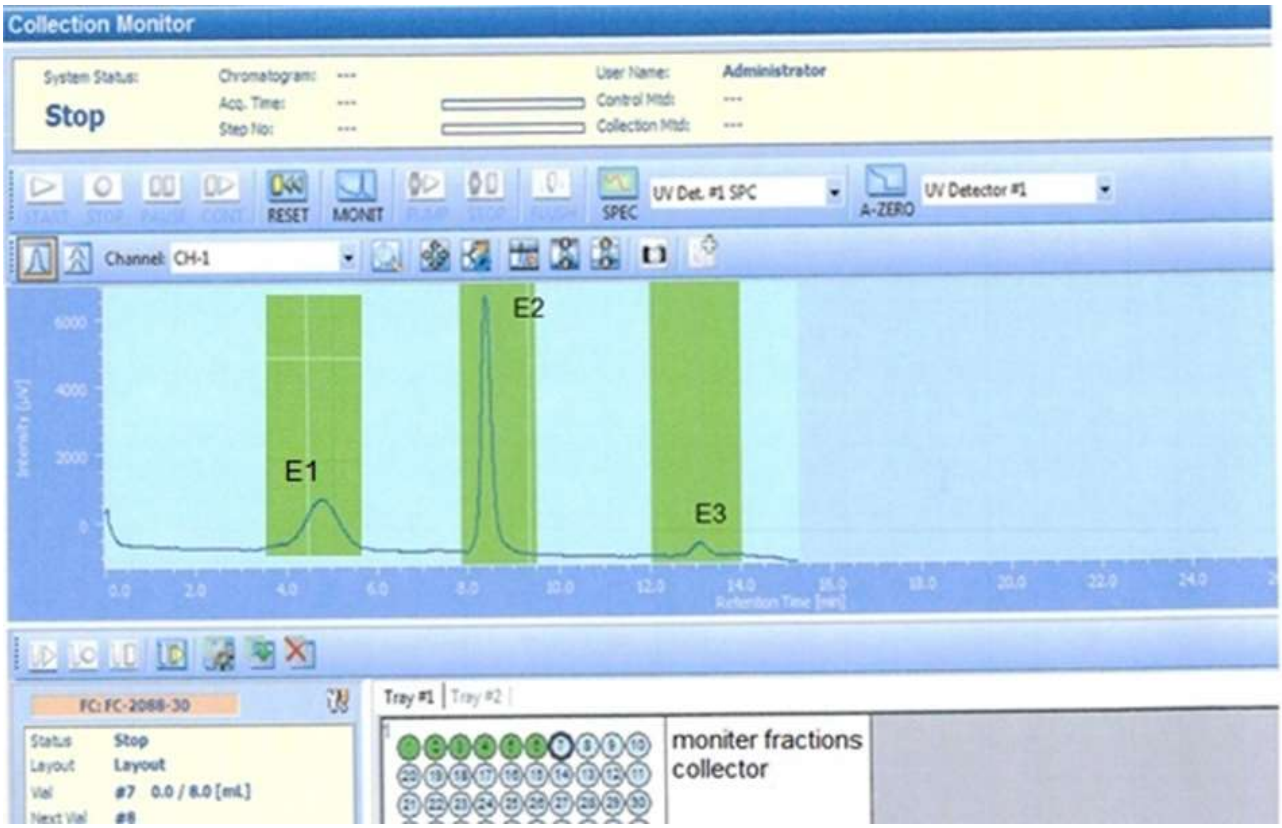

Figure 2: Aloe extract peaks in preparative HPLC recorder

Group II: Positive control (liver disease group induced by CCl_4 that will be untreated by any drug). The CCl_4 is given twice weekly. All mice were intraperitoneally injected by 1 ml/kg body weight of 100 % (v/v) carbon tetrachloride (CCl_4) diluted in ten times volumes of olive oil [7].

Group III: Emodin group: after finishing the CCl_4 doses, they received six doses of emodin 40 mg / kg every other day [48].

Group IV: Silymarin group: after finishing the CCl_4 doses, they received six doses of silymarin 20 mg/kg, the standard protective treatment for liver fibrosis, every other day schedule [49].

Mice liver were examined histopathologically under light microscope, and a small piece of these mice liver are treated with anti-collagen III, anti-interleukin 4 to be analyzed immunohistochemically.

Preparing of injections: Pure 100% concentrated CCl_4 was diluted in a 1:10 ratio with olive oil, while emodin and silymarin were diluted in DMSO, in which 10 mg of each one was dissolved in 1ml. The injected volume was 0.1 ml for the all three materials. Emodin and silymarin were insoluble in water.

Tissue Preparation

The entirely taken liver will be kept immediately in a 15% recently formalin solution until the time of tissue slice formation. Liver tissues were sectioned into 5 mm slices and transferred into formalin (10%); Fixative volume was 20 times that of tissue on a weight per volume, tissue was fixed for a minimum 48 hours at room temperature.

These tissues were undergone gentle agitation using ethanol 70% ,80%,90%, then 100% ,each one for 2 hours, then xylene for 2 hours, for two times, separated by 24 hours, and finally were Embedded in paraffin for 2 hours at 58°C for two times, separated by 24 hour. The paraffin-embedded tissues should be stored as paraffin blocks to be used at any time for preparing further slides according the required number of tests [50].

Slide Preparation

Serial tissue sections (3-5 µm) thicknesses were obtained using microtome, from each

tissue paraffin block, three slides were prepared. Sections were mounted on ordinary slides (to be used for Haematoxylin and Eosin staining system) or positively charged slide (to be used for immunohistochemistry) using a water bath of 45C° to prevent tissues sections folding during mounting procedure [50].

Histopathological Assessment

The Metavir scoring system is used to assess drug effects on the liver [51], Table 1.

Table 1: Metavir scoring system

Description	Score
No scarring	**0**
Minimal scarring	**1**
Scarring has occurred and extends outside the areas in the liver that contains blood vessels	**2**
Bridging fibrosis is spreading and connecting to other areas that contain fibrosis	**3**
Cirrhosis or advanced scarring of the liver	**4**

Immunohistochemical Assessment

Immunohistochemistry (IHC) is a method for detecting cellular tissue antigens via antigen-antibody reactions by means of optical microscopy. Two Immunohistochemical markers are used in the current study (collagen III and interlukin-4 to detect a specific antibody bound to an antigen in the tissue section. The secondary antibody-enzyme complex is then visualized with an appropriate substrate/chromogen using ordinary microscope [52].

Collagen Assessment using Anti-collagen III Antibody

Rabbit polyclonal antibody to collagen III can only recognizes 3D epitopes with negligible cross-reactivity with Type I, II, IV, V or VI collagens. Non-specific cross-reaction of anti-collagen antibodies with other human serum proteins or non-collagen extracellular matrix protein is negligible. Polyclonal rabbit antibody provided in liquid form, shipped at 4°C, PH 8 [52].

Collagen should be assessed in terms of intensity and distribution outside the cells since it is an extracellular protein, Staining intensity was graded visually by the pathologist as Negative, weak, moderate, and strong. They are given the scores 0, 1, 2, and 3 respectively. While distribution was graded, as patchy, given the score "1" if 50% or less of the portal areas is stained by collagen or as diffuse, given the score "2" if more than 50% of the portal areas stained. The final score was calculated as the product of intensity and distribution.

Collagen is chosen in this study as a marker for changes in fibrosis stages. There is a close relationship between collagen III secretion and fibrosis grade [53].

Immunohistochemical Assessment of Interleukin-4(IL-4)

While activated myofibroblasts and HSC are the major producers of the fibrotic scar, their fibrogenic activation and proliferation depends on a complex interplay with other resident or recruited cells (such as the immune cells) and their secreted factors. Immune cells, which promote or attenuate fibrogenesis, have become targets of anti-fibrotic treatments. An intensity score was assigned, representing the average intensity of positive cells (0, none; 1, weak; 2, intermediate; and 3, strong).

A proportion score was assigned, which represented the estimated proportion of positive-staining cells (0, <5%; 1, 5%-25%; 2, 6%-%; 3, 51%-75%; and 4,>75%). The proportion and intensity scores were then added to obtain a total score, which ranged from 0 to 8.The total score was further divided into the score as follows: <2, (-); 2-3, (+); 4-5, (++); 6-7, (+++) [54].

Statistical Analysis

Results were expressed as Mean ± SD calculated using Microsoft excel calculator. All statistical comparisons of histopathological and immunohistochemical observations were made by one-way ANOVA test followed by Tukey's post HOC analysis using an on-line calculators in which *P*

values less than 0.05 were considered statistically significant and those *P* values less than 0.01 is considered highly significant [55].

Results

Liver Histopathology

According to Metavir analysis, liver tissue scoring for the study groups are listed below (Table.2) while the statistical comparisons between these groups are illustrated in Table.3.

Table 2: Histopatological scores for the four groups of the study

Fibrosis score	Frequency of grades from each group			
	Gp I (Healthy)	Gp II (CCl_4)	Gp III (Emodin)	Gp IV (Silymarin)
0	8	…	…	…
1	…	…	4	8
2	…	…	4	…
3	…	4	…	…
4	…	4	…	…
Mean	0	3.5	1.5	1
STEDVA	0	0.5477	0.5477	0

Table 3: Statistical comparison for liver histopathologic scoring

N	Comparison between	*P* value calculated by Tukey's post HOC test
1	CCl_4 vs. emodin group	0.0000863
2	CCl_4 vs. silymarin group	0.000000567
3	CCl_4vs healthy group	0.0000000232
4	Emodin vs. silymarin group	0.1411
5	Emodin vs. healthy group	0.0000531
6	Silymarin vs. healthy group	0.000537

Fibrosis scoring was higher in the diseased group "Gp II" (mean and standard deviatin=3.5±0.5477) while the scoring was lowered in the group treated by emodin (1.5±0.4577) and silymarin gave better scoring (mean is 1.0)

No significant difference between the effects of emodin as compared with the standard therapy for liver disease (silymarin) (p>0.05).On the other hand, there was a significant lowering in the histopathological scores in emodin or silymarin group as compared to the CCl_4 group. The liver histopathologic sections were pictured in two powers under light microscope. The 10x power (Fig.3) illustrate two grades of liver fibrosis after using CCl_4.The 20x power sections (Fig.4) illustrate the intensity of fiber bands and a liver histopathological view of the four groups .Fibrosis is appeared as thick fibrous bands connecting separated lobules.

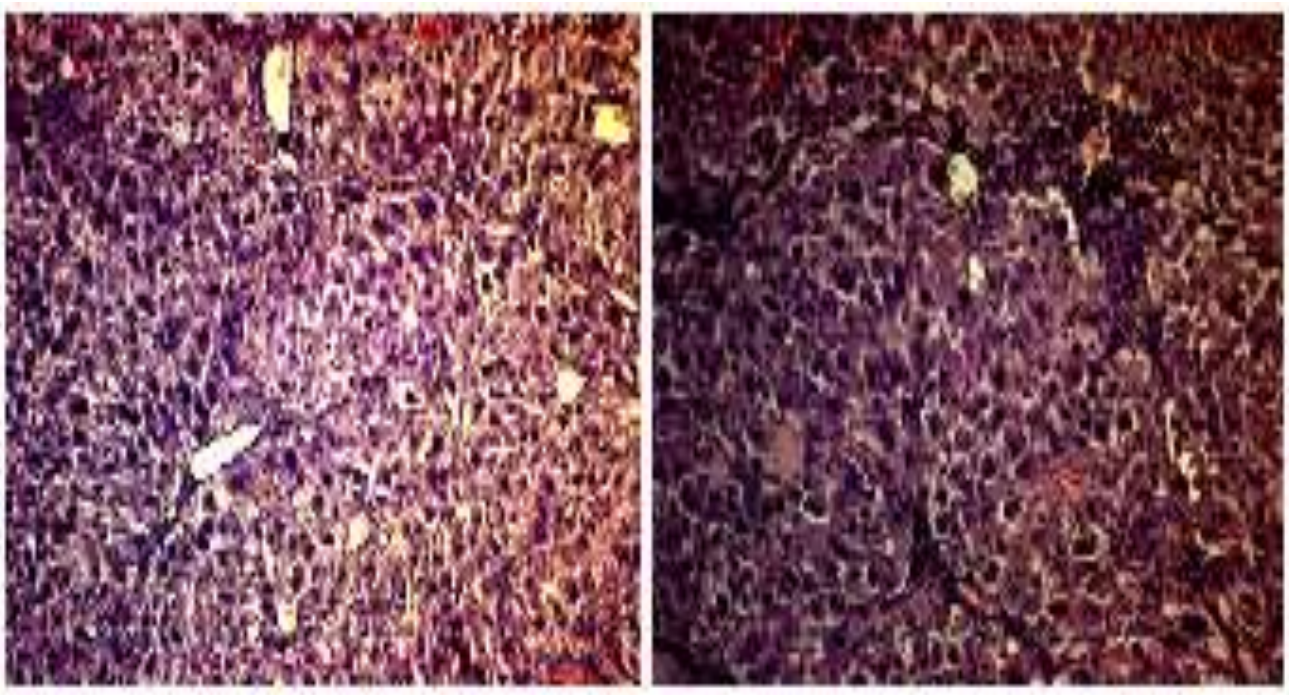

Figure 3: Fibrosis grade 4 (left), grade 3 (right)

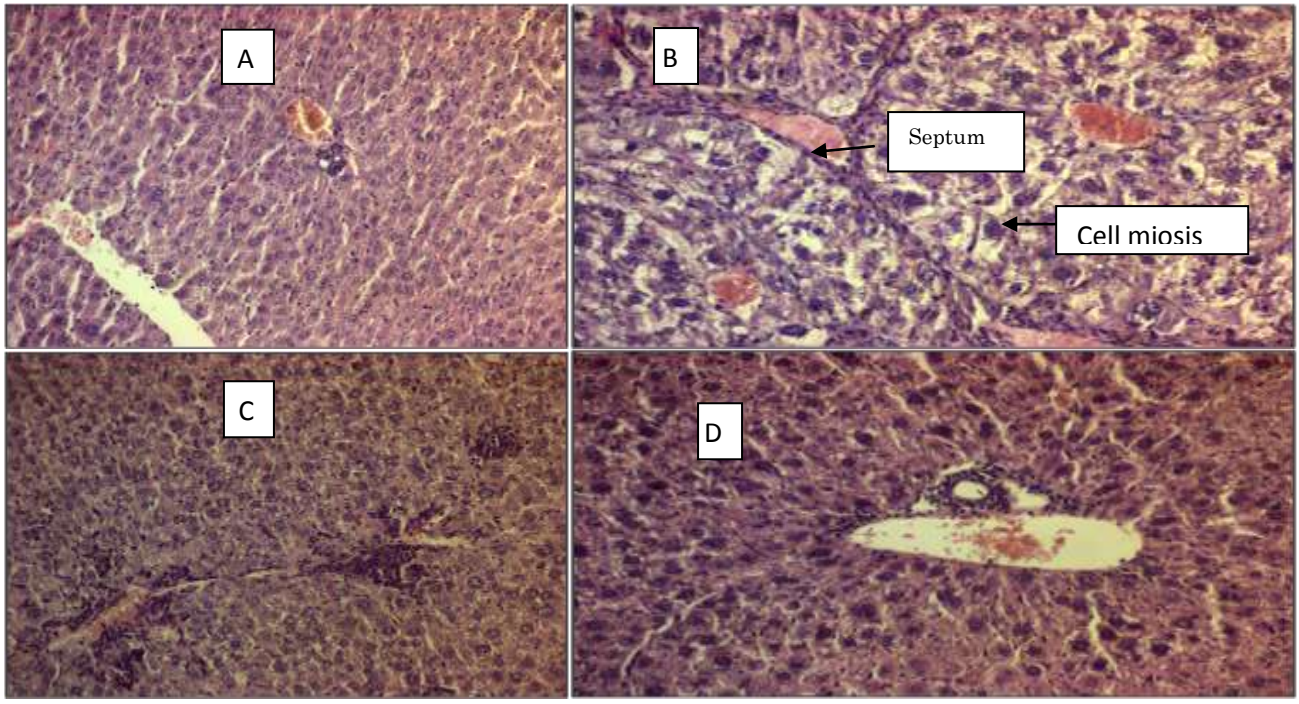

Figure 4: Histopathological view of the four groups. A. group I (negative control (Healthy), b. group II (positive control (CCl_4), c. Group III (emodin), d. group IV (silymarin)

Immunohistopathology of Liver Collagen

The intensity of staining was graded visually by the pathologist as negative, weak, moderate, and strong, given the scores 0, 1, 2 and 3, respectively. The distribution of staining was graded as patchy (score 1) or diffuse (score 2). The final score was calculated as the product of intensity and distribution. Means of IHC scores of IL-4 is illustrated in Table.4 while Table.5 illustrates statistical comparisons between the study groups.

Table 4: Mean and STEDVA of IHC scores of collagen for the four groups of the study

Collagen score	Gp I(Healthy)	Gp II(CCl_4)	Gp III (Emodin)	Gp IV(Silymarin)
Mean	0.333	5.667	1.833	1.8
STEDVA	0.516	0.816	0.753	1.304

Table 5: Statistical comparison between the study groups for collagen IHC scoring

N	Comparison between	*P* value calculated by Tukey's post HOC test
1	CCl_4 vs. emodin group	0.000007
2	CCl_4 vs. silymarin group	0.000198
3	CCl_4 vs. healthy group	<0.000001
4	Emodin and silymarin group	0.958729
5	Emodin vs. healthy group	0.031872
6	Silymarin vs. healthy group	0.162852

There was a highly significant lowering in emodin and in silymarin groups as compared to the CCl_4 group, and also between the healthy and the diseased CCl_4 group (p <0.0001).There is no significant difference in the collagen lowering ability between emodin and silymarin group, and between silymarin and healthy group(p is >0.05)

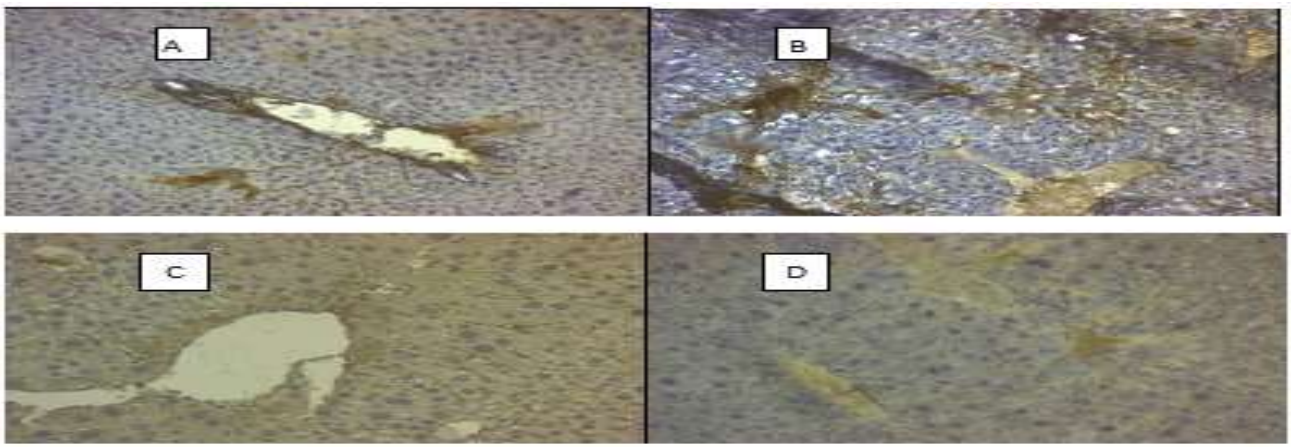

Figure 5: Collagen immunostaining under 20x power. The scoring is presenting as int "intensity 0,1,2 and 3" and pat "patchy" or dif "diffuse".CCl_4 group contain the more intense slide as compared to other groups. a. negative control (int1,pat),b. CCl_4 (int 3, dif) , c. emodin (int 1,pat), d. silymarin (int 1, pat)

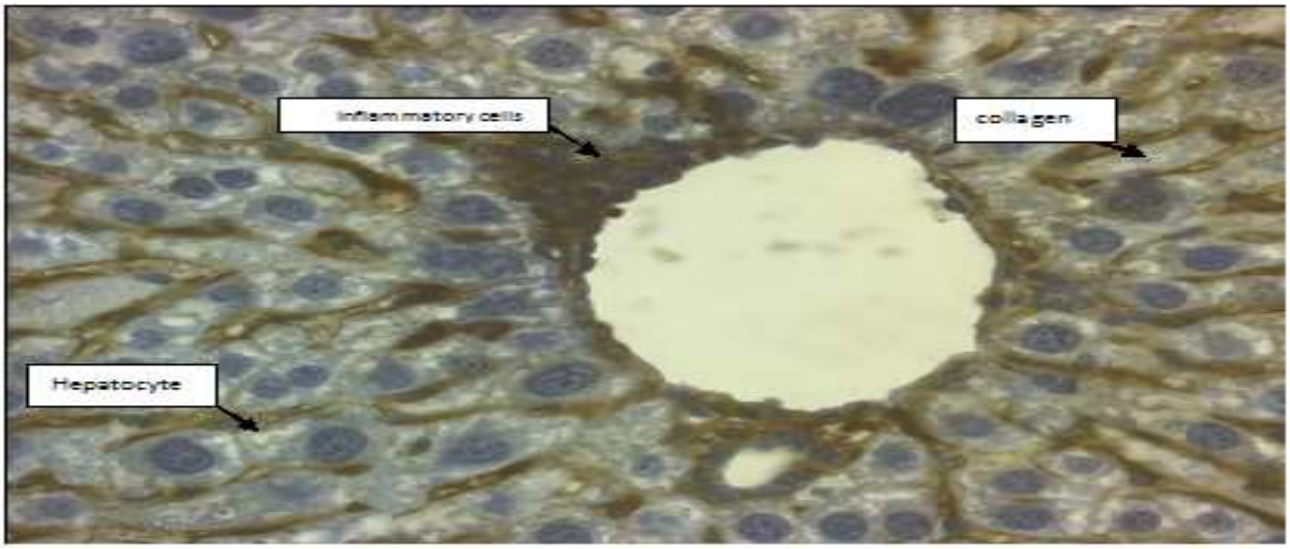

Figure 6: Illustrates hepatocytes, endothelial cells and inflammatory cells response in a slide taken from emodin group under the 40x power

Interleukin-4 IHC Results

IL-4 assessment was performed in terms of intensity and number of cells stained. The intensity score refer to the degree of the inflammatory marker "IL-4" in the cells while the proportion score indicate the estimated proportion of cells which has been stained and elucidating the staining in that assessed field. The staining intensity and proportion was graded visually by the pathologist as negative, weak, moderate, and strong. They were given the scores 0, 1, 2, and 3 respectively. While proportion was graded as 0,1,2,3,4 representing cells proportion which take the stain as <5%,5-25%,26-50%,51-75%,>75%, respectively.

The final score was calculated as the summation of intensity and proportion scores. Table.6 demonstrates the means of IL-4 IHC scores for the four groups of the study while Table.7 shows statistical differences between the four study groups.Figure.7 illustrates how the interleukin intensity appeared in the small inflammatory cells while it is absent in the hepatocytes. This figure was taken from silymarin group under the 40x power.

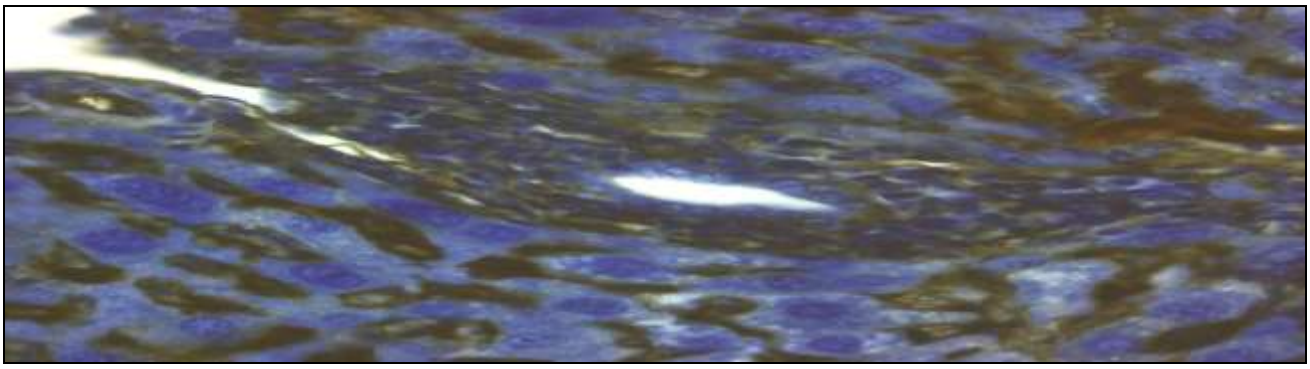

Fig.7: Interleukin staining in the inflammatory cells at the middle

Table 6: Mean and STEDVA of Interleukin-4 IHC scores for the five study groups

Interleukin score	Gp I(Health)	Gp II(CCl_4)	Gp III (Emodin)	Gp IV (Silymarin)
Total score	6	42	18	14
Mean	1	7	3	2.333

Table 7: Statistical comparison between the study groups for interleukin-4 IHC scoring

N	Comparison between	*P* value calculated by Tukey's post HOC test
1	C CCl_4 vs. emodin group	0.000001
2	CCl_4 vs. silymarin group	0.000001
3	CCl_4 vs. healthy group	0.000001
4	Emodin vs. silymarin group	0.144028
5	Emodin vs. healthy group	0.000034

There was a highly significant lowering in emodin and in silymarin group as compared to the CCl_4 group, and also between the healthy and the diseased CCl_4 group (p <0.0001).There is no significant difference in the interleukin-4 lowering ability between emodin and silymarin group and between silymarin with healthy group (p is >0.05) .Table.7 illustrate the statistical comparisons in IL-4 in the four groups of study

Figure 8 illustrate an immunohistochemical comparison under the 10x power of light microscope between the all study groups. Figure 8 demonstrate IL4 distribution between cells.

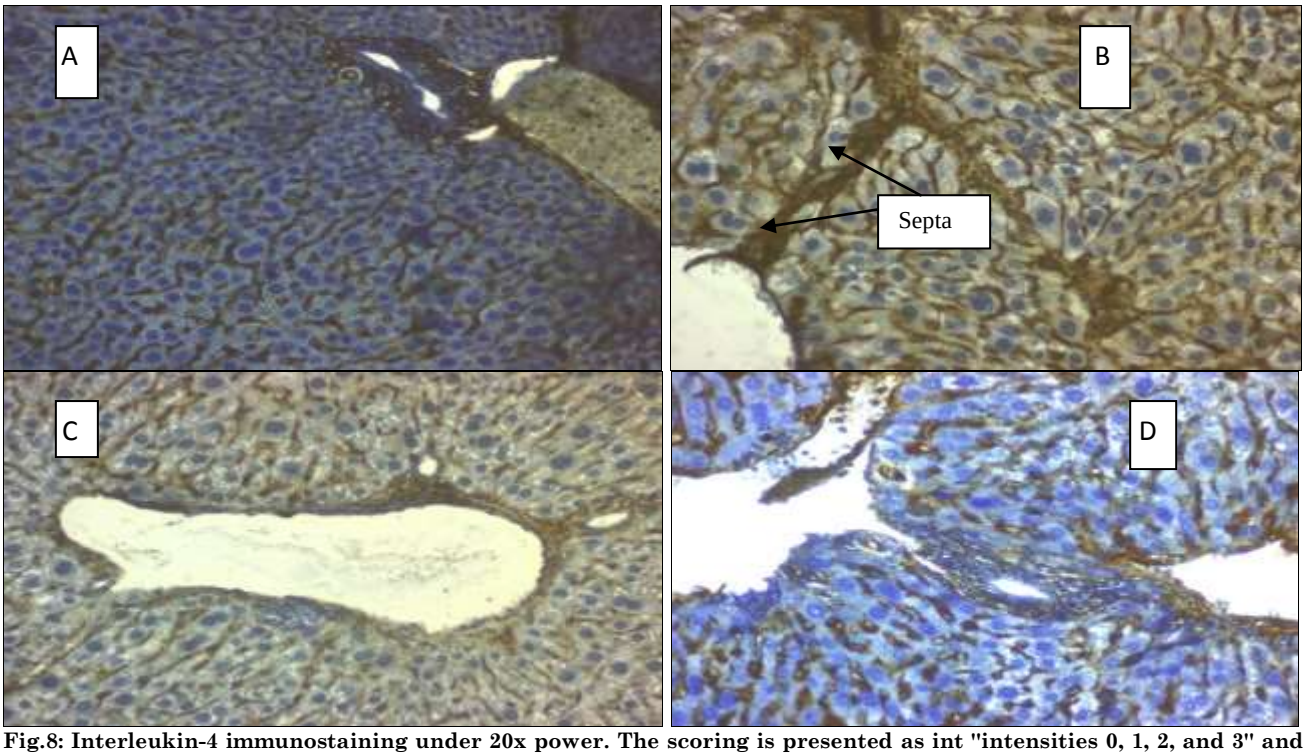

Fig.8: Interleukin-4 immunostaining under 20x power. The scoring is presented as int "intensities 0, 1, 2, and 3" and prop "proportion score" .a. Negative control (Healthy) (int 1, prop 0), b. Positive control (CCl_4) (int3, prop4) c. Emodin group (int3.prop1) d. Silymarin group (int1, prop2)

Discussion

Assessment of .Emodin Antibrotic Effects on Liver

Low dose CCl_4 for long period achieved two grades of liver fibrosis (Fig.3), while collagen assessment using IHC confirmed the diagnosis semi quantitatively. Emodin lowered fibrosis grade and collagen secretion. Matrix proteins are many types like collagen III, IV, V, VI and vitronectin, but only Collagen III and vitronectin showed the most gradual increase in the different stages of liver fibrosis [53], so there is a close relationship between collagen III secretion and fibrosis grade. The present study demonstrated that emodin dose of 40 mg/kg had decreased the collagen deposition significantly and in a better way in the histopathological sections and in the Immunohistochemical analysis.

Different mechanisms may contribute to emodin anti-fibrotic effects as follows:

- Increase the expression of E-cadherin leading to suppression of epithelial-mesenchymal transition (EMT), Our study is in agreement with Liu *et al* study [45] in which 40 mg/kg dose produced the greater anti-fibrotic effects. Hepatocytes and chol-angiocytes damage causes N- and E-cadherin loss leading to hepatic epithelial cells transformation into fibroblasts, myofibroblasts or mesenchymal cells, with the concomitant expression of markers; including vimentin and α- smooth muscle actin [56].Emodin increases the expression of E-cadherin and decrease that of vimentin so emodin suppress EMT [45].

- Suppression of TGF-ß/Smad signaling [45]. TGF-ß1 signaling through Smad molecules is a known mechanism of liver fibrosis [57].TGF-ß1 transmits intracellular signals by Smad2 and Smad3 activation and phosphorylation.$Smad_{2/3}$ and $Smad_{1/5/8}$ form a heteropolymer with $Smad_4$.At the last, regulation of gene transcription will occur after $Smad_4$ translocation to the nucleus which interacts directly with DNA or via coenzyme factors [58,59].

- Emodin inhibits the Toll-like receptor-4 (TLR-4) pathway. TLR-4 mediates antiapoptotic and inflammatory effects in HSCs leading to cirrhosis [60].emodin attenuate TLR-4 signaling pathways [61].

- Up-regulation of metastasis- associated gene 3 (MTA3) MTA3 is decreased in liver and heart fibrosis [62, 63]. MTA3 binds to the promoter regions the of E-cadherin (E-cad) transcription activator and the loss of E-cad leads to EMT [62]. Emodin up-regulate MTA3 [63].

- Blocking the migration and proliferation of HSCs induced by PDGF [64].

Effects of emodin on Interleukin -4 intensity and Expressing Cells in Liver Tissue

IL-4 is a cytokine that is secreted by T-helper cells(Th2),γδ T cells, NKT cells, T-follicular helper cells, basophils, eosinophils, mast cells and type-2 innate lymphoid cells (ILC2) [65, 66].IL-4 and IL-13 are related cytokines in which IL-4 production is calcineurin-dependent while the production of IL-13 is only partially calcineurin-dependent [66].

Upon an appropriate stimulation of the cells, the locus control region (LCR) of the RAD 50 gene allow excess transcription factors to the DNA and the subsequent transcription of IL-4 [65]. The cytokine-binding receptor chain for IL-4 is the widely expressed receptor IL-4 Rα. Most cells carry at least low numbers of this receptor chain.IL-4/IL Rα-complex will bind a secondary receptor chain of two types; IL-2Rγc presented more in hematopoietic cells and IL-13Rα1presented more in non-hematopoietic cells such as fibroblasts and endothelial cells. IL-4utilize STAT6 transcription factor uniquely to perform specific functions on different cell types.

Receptors signal through JAK family kinases, which phosphorylate and create docking sites for the transcription factor STAT6 that is phosphorylated and translocates to the nucleus upon ligand binding [66]. IL4 have different roles in liver fibrosis depending on the receptor type and depending on the liver disease stage [67].

IL-4Rα on macrophages promotes both hepatic inflammation and fibrosis during progression, while it permits unrestricted fibrosis resolution during spontaneous reversal. Macrophage IL-4Rα signaling can promote liver fibrosis regression via STAT6-dependent transcriptional activation of Mmp12, and that pharmacological inhibition of IL-4Rα recapitulated the functional effects of genetic IL-4Rα deletion [68]. IL-4 has a profibrotic role [69].

It is a potent stimulator of collagen biosynthesis in fibroblasts [70] and a fibroblast proliferation enhancer [71].Also IL-4 prevent myofibroblasts apoptosis in organs that undergo fibrosis such as liver by stimulating macrophages to secrete insulin-like growth favtor-1 (IGF-1) [72].

Macrophages and T-cells are the most significant cells that respond toIL-4 in liver fibrosis and have and inflammation [73]. The Blood monocytes, the precursors of macrophages and dendritic cells, are brought to liver by copiously presented cytokines in chronic liver diseases and cirrhosis like CCL2, CCL3 and CCL6 [74]. These infiltrating cells affect liver fibrosis by two ways:

- By producing potent cytokines and chemokines which have pro-inflammatory or direct fibrogenic effects on HSCs and myofibroblasts [75].

- These cells can be converted to a more specialized cell, for example, monocytes are converted to M1 and the M2 macrophages. While M1 is a pro-inflammatory and potentially antifibrotic cell. [76], M2 are five subtypes [77].IL-4 has a role in favoringM1- towards M2-type macrophage [74].

IL-4 cause's tissue macrophage accumulation [78].Tissue macrophages metabolize arginine and proline, which are essential amino acids for collagen biosynthesis [21].

This study ensure with that Emodin inhibits IL-4 [79]. One of the possible mechanisms by which emodin acid can lowers IL-4 is by lowering nuclear factor κB (NF-κB) [80]. NF-κB activation plays an important role in the IL-4-induced protection from apoptosis [81]. IL-4 prevents myofibroblasts apoptosis [72].

Conclusions

Emodins have antifibrotic activity comparable to that of the standard therapy by silymarin ensured by lowering collagen secretion, which is the marker for fibrosis. Emodin anti-fibrotic effects is related to lowering nuclear factor-κB leading to IL-4 lowering, decrease of epithelial mesenchymal transition by increasing E-cadherin,

suppression of TGF-ß1 Smad signaling, Up-regulation of metastasis- associated gene 3, inhibits the Toll-like receptor-4 (TLR-4) pathway, and blocking the migration and proliferation of HSCs induced by platelet derived growth factor. Emodin can alleviate liver cirrhosis.

Acknowledgements

Grateful thanks to Pharmacognosy departments at Baghdad University/ college of pharmacy, pathology department and pharmacology laboratory at Al-Nahrain University, and Ibtihaj Hani Al-Saffar veterinary clinic. This study has no government financial support.

References

1. Rehan MR, Zedan AMG, Hashash SA, Farid MA, Shafie GA (2016) Protective effects of some fruit juices with low-fat diet on rat testis damaged by carbon tetrachloride: A genetic and histological study. African Journal of Biotechnology, 1977-1986.
2. Scholten D, Trebicka J, Liedtke C, and Weiskirchen R (2015) The carbon tetrachloride model in mice. Laboratory Animals, 49(S1):4-11.
3. Ogeturk M, Kus I, Colakoglu N, Zararsiz I, Ilhan N, Sarsilmaz M (2005) Caffeic induced nephrotoxicity and protective effect of betaine in Sprague Dawleyrats. Urology, 62: 353-356.
4. Balahoroğlu R, Dülger H, Özbek H, Bayram I, Şekeroğ MR (2008) Protective effects of antioxidants on the experimental liver and kidney toxicity in mice. Eur. J. Gen. Med., 5(3):157-164.
5. Abraham P, Wilfred G, Catharine SP (1999) Oxidative damage to the lipids and proteins of the lungs, testis and kidney of rats during carbon tetrachloride intoxication. Clin Chim Acta, 289: 177-9.
6. Khan RA (2012) Protective effects of Launae aprocumbens on rat testis damage by CCl_4. Lipids Health Dis., 11: 103.
7. Dong S, Cheng OL, Song YN, Sun Y, Wei B, Li XY et al (2016) Mechanisms of CCl_4-induced liver fibrosis with combined transcriptomic and proteomic analysis. The journal of toxicological sciences, 41 (4): 561-572.
8. Fortea JI, Fernández-Mena C, Puerto M, Ripoll C, Almagro J, Bañares J et al (2018) Comparison of Two protocols of carbon tetrachloride-induced cirrhosis in rats-improving yield and reproducibility. Scientific reports, 8: 9163.
9. Haghi ME, Dehghan G, Banihabib N, Zare S, Mikaili P, Panahi F (2014) Protective effects of Cornus mas fruit extract on carbontetrachloride induced nephrotoxicity in rats. Indian Journal of Nephrology, 24: 5.
10. Obinna OK, Siminialay IM, Orisakwe OE (2017) Carbon tetrachloride induced hepatorenal toxicity in rats: possible protective effects of wild Pleurotus tuber-regium. Clinical Phytoscience, 3: 2.
11. Wong WL, Abdulla MA, Chua KH, Kuppusamy UR, Tan YS, Sabaratnam V (2012) Hepatoprotective effects of Panus giganteus (Berk.) Corner against thioacetamide-(TAA-) induced liver injury in rats. Evid Based Complementary Altern. Med., Article ID 170303: 10.
12. Ritesh KR, Suganya A, Dileepkumar HV, Rajashekara Y, Shivanandappa T (2015) A single acute hepatotoxic dose of CCl_4 causes oxidative stress in the rat brain. Toxicology Reports, 2: 891-895.
13. Kuntana YP, Balia R, Hidayat YA (2016) The effects of carbon tetrachloride inducing osteoporosis to white rats (Ratus Norvegicus) judging from the muculoskeltal tissue damage. Animal Science Series, 66: 161-165.
14. Ahmad B, Khan MR, Shah NA (2015) Amelioration of carbon tetrachloride-induced pulmonary. Toxicity with Oxalis corniculata. Toxicol. Ind. Health, 31: 1243-1251.
15. Kurt A, Tumkaya L, Yuce S, Turut H, Cure MC, Sehitoglu I, et al (2016) The protective effect of infliximab against carbon tetrachloride-induced acute lung injury. Iran J. Basic. Med. Sci., 19 (6):685-691.
16. Rahmoni F, Rabai T (2017) Protective effects of Teucrium Pollium aqueous extract and ascorbic acid on hematological and some biological parameters against CCl_4 toxicity in rats. Biomedicine and Pharmacotherapy, 91: 43-48.

17. Hamed H, Gargouri M, Bellasoued K, Ghannoudi Z, Elfeki A, Gargouri A (2017) Cardioprotective effects of camel milk against CCl_4 induced oxidative stress. The journal of metabolic diseases, 24 (3): 253-260.

18. Sahreen S, Khan MR, Khan RA, Alkreathy HM (2014) Cardioprotective role of leaves extracts of Carissaopaca against CCl_4 induced toxicity in rats.BMC Research Notes, 7: 224.

19. Hu Q, Noor M, Wong YF, Hylands PJ, Simmonds MS, Xu Q, Jiang D, et al (2009) In vitro anti-fibrotic activities of herbal compounds and herbs. Nephrol. Dial. Transplant 24: 3033-3041.

20. Dong X, Fu J, Yin X, Cao S, Li X, et al (2016) Emodin: A review of its pharmacology, toxicity and pharmacokinetics. Phytother Res., 30 (8): 1207-1218.

21. Sharma R, Tiku AB, Giri A (2017) Pharmacological properties of emodin anthraquinone derivatives. J. Nat. Prod. Resour., 3 (1): 97-101.

22. Li N, Wang JB, Zhao YL, Zhang L, Ma XB, Xiao-Fei Li (2017) Liver protective and reactive oxygen Species Scavenging Effects of Emodin in Lipopolysaccharide/Bacillus CalmetteGuerin-injured Mice by Optical Molecular Imaging. Int. J. Pharmacol., 13: 175-182.

23. Ubbink-Kok T, Anderson JA, Konings WN (1986) Inhibition of electron transfer and uncoupling effects byemodin and emodinanthrone in Escherichia coli. Antimicrob. Agents Chemother, 30 (1): 147-151.

24. Rahimipour S, Bilkis I, Peron V, Gescheidt G, Barbosa F, Mazur Y, et al (2001) Generation of free radicals by emodic acid and its [D-Lys6] GnRH-conjugate. Photochem Photobiol., 74 (2): 226-236.

25. Chen RF, Shen YC, Huang HS, Liao JF, Ho LK, Chou YC, Wang, et al (2004) Evaluation of the antinflammatory and cytotoxic effects of anthraquinones and anthracenes derivatives in human leucocytes. J. Pharm. Pharmacol., 56 (7): 915-919.

26. Zhang X, Zhang R, Lv P, Yang J, Deng Y, Xu J, et al (2014) Emodin up-regulates glucose metabolism, decreases lipolysis, and attenuates inflammation in vitro, J. Diabetes, l7 (3): 360-368.

27. Sharma R, Tiku AB (2014) Emodin, an anthraquinone derivative, protects against gamma radiation-induced toxicity by inhibiting DNA damage and oxidative stress. Int. J. Radiat. Biol., 9 (4): 275-283.

28. Liu C (2015) Inhibition of mechanical stress-induced hypertrophic scar inflammation by emodin, Mol. Med. Rep., 11 (6): 4087-4092.

29. Wang T, Zhong XG, Li YH, Jia X, Zhang SJ, Gao YS, et al (2015) Protective effect of emodin against airway inflammation in the ovalbumin-induced mouse model. Chin J. Int. Med., 21 (6): 431-437.

30. Shieh DE, Chen YY, Yen MH, Chiang LC, Lin CC (2004) Emodin-induced apoptosis through p53-dependent pathway in human hepatoma cells. Life Sci., 74: 2279-2288.

31. Ismail S, Haris K, AbdulGhany AR, Abdullah JM, Johan MF, Mohamed Yusoff AA (2013) Enhanced induction of cell cycle arrest and apoptosis via the mitochondrial membrane potential disruption in human U87 malignant glioma cells by aloe emodin. J. Asian Nat. Res.

32. Ma J, Lu H, Wang S, Chen B, Liu Z, et al (2015) s The anthraquinone derivative emodin inhibits angiogenesis and metastasis through down-regulating Runx2 activity in breast cancer. Int. J. Oncol., 46 (4):1619-1628.

33. Zhou GH, Zhang F, Wang XN, Kwon OJ, Kang DG, Kang DG, Lee HS, et al (2014) Emodin accentuates atrial natriuretic peptide secretion in cardiac atria. Eur. J. Pharmacol., 735: 44-51.

34. Jackson TC, Verrier JD, Kochanek PM (2013) Anthraquinone-2-sulfonic acid (AQ2S) is a novel neurotherapeutic agent. Cell Death Dis., 4: 1-24.

35. Ali BH, Al-Salam S, Al Husseini IS, Al-LawatiI, Waly M, et al (2013) Abrogation of cisplatin-induced nephrotoxicity by emodin in rats. Fundam Clin Pharmacol., 27 (2): 192-200.

36. Wynn T, Ramalingam TR (2012) Mechanisms of fibrosis: therapeutic transition for fibrotic disease. Nat. Med., 18 (7): 1028-1040.

37. Schuppan D, Kim YO (2013) Evolving therapies for liver fibrosis. The Journal of Clinical Investigation, 123 (5): 1887-1901.

38. Rosenbloom J, Mendoza FA, Jimenez SA (2013) Strategies for anti-fibrotic therapies, Biochimica ET Biophysica Acta, 1832: 1088-1103.

39. Pinzani M, Barragan JM (2010) Update on the pathophysiology of liver fibrosis. Expert Rev Gastroenterology Hepatol., 4: 459-472.

40. Tsuchida T, Friedman SL (2017) Mechanisms of hepatic stellate cell activation. Nature Reviews Gastroenterology and Hepatology, 14: 397-411.

41. Liu C, Chen X, Yang L, Kisseleva T, Brenner DA, Seki E (2014) Transcriptional repression of the transforming growth factor b (TGF-b) Pseudoreceptor BMP and activin membrane-bound inhibitor (BAMBI) by Nuclear Factor jB (NFjB) p50 enhances TGF-b signaling in hepatic stellate cells, 289: 7082-7091.

42. Igarashi A, Nashiro K, Kikuchi K, Sato S, Ihn H, Grotendorst GR, Takehara K (1995) Significant correlation between connective tissue growth factor gene expression and skin sclerosis in tissue sections from patients with systemic sclerosis. J. Invest Dermatol., 105: 280-284.

43. Seki E, Minicis S, Osterreicher CH, Kluwe J, Osawa Y, Brenner DA, et al (2007) TLR4 enhances TGF-beta signaling and hepatic fibrosis. Nat. Med., 13: 1324-1332.

44. Trautwein C, Friedman SL, Schuppan D, Pinzani M (2015) Hepatic fibrosis: Concept to treatment. Journal of Hepatology, 62: S15-S24.

45. Liu F, Zhang J, Qian J, Wu G, Ma Z (2018) Salvianolic acid alleviates CCl4 induced liver fibrosis by suppressing epithelial mesenchymal transition and transforming growth factor ß1 in rats. J. Molecular Medicine Reports, 18: 3262-3270.

46. Harborne JB (1973) Phytochemical Methods, A Guide to Modern Techniques of Plant Analysis.1st ed. London: Chapman and Hall; New York.

47. Choche T, Shende S, Kadu P (2014) Extraction and identification of bioactive components from Aloe barbadensis Miller. Research & Reviews Journal of Pharmacognosy and Phytochemistry, 2: 14-23.

48. Wang CH, Gao ZQ, Ye B, Cai JT, Xie CG, Qian KD, Du Q (2007) Effect of emodin on pancreatic fibrosis in rats; World J. Gastroenterol., 13 (3): 378-382.

49. Sabzevarizadeh and Najafzadeh (2012) Comparison effect of silymarin and vitamin C on liver function in myoglobinuric status in rats. World Applied Sciences Journal, 17 (2): 228-232.

50. Kristie CA (2013) Preparation of formalin-fixed paraffin- tissue embedded for immunohistochemistry. Methods in enzymology, 533: 255-233.

51. Theise ND (2007) Liver biopsy assessment in chronic viral hepatitis, A personal, practical approach. Article in Modern Pathology, 1: S3-14.

52. Tilg H, Moschen AR (2006) Adipocytokines: mediators linking adipose tissue, inflammation and immunity. Nat. Rev. Immunol., 6 (10): 772-783.

53. Chen W, Jonathan B, Rock, Yearsley MM, Ferrell LD, Frankel WL (2014) Different collagen types show distinct rates of increase from early to late stages of hepatitis C-related liver fibrosis. Human Pathology, 45: 160-165.

54. Du WJ, Zhen JH, Zeng ZQ, Zheng ZM, Yan Xu, Qin LY et al (2013) Expression of Interleukin-17 associated with disease progression and liver fibrosis with hepatitis B virus infection: IL-17 in HBV infection. Diagnostic Pathology, 8 (40): 7.

55. Mehrzadi S, Iman Fatemi I, Esmaeilizadeh M, Ghaznavie H, Hadi Kalantar H, Goudarzi M (2018) Hepatoprotective effect of berberine against methotrexate induced liver toxicity in rats. Biomedicine& Pharmacotherapy, 97: 233-239.

56. Zhao YL, Zhu RT and Sun YL (2016) Epithelial-mesenchymal transition in liver fibrosis. Biomed Rep., 4: 269-274.

57. Hayashida T (2010) Integrins modulate cellular fibrogenesis at multiple levels; Regulation of TGF-ß signaling. Endocr. Metab Immune Disord. Drug Targets, 10: 302-319.

58. Blank U, Karlsson S (2011) The role of S mad signaling in hematopoiesis and translational hematology. Leukemia, 25: 1379-1388.

59. Xu F, Liu C, Zhou D, Zhang L (2016) TGF-ß/SMAD pathway and its regulation in hepatic fibrosis. J. Histochem Cytochem, 64: 157-167.

60. Guo J, Friedman SL (2010) Toll-like receptor 4 signaling in liver injury and hepatic fibrogenesis. Fibrogenesis & Tissue Repair, 3: 21.

61. Ding Y, Liu P, Chen Z-L, Zhang S-J, Wang Y-Q, Cai X, Luo L, Zhou X, Zhao L (2018) Emodin attenuates lipopolysaccharide-induced acute liver injury via inhibiting the TLR4 signaling pathway in vitro and in vivo. Front in Pharmacol., 9: 962. doi: 10.3389/fphar.2018.00962.

62. Li Q, Li H, Lin Y , Che H, Hu Y, Kang X, et al (2019) High glucose promotes hepatic fibrosis via miR-32/MTA3-mediated epithelial-to-mesenchymal transition. Molecular Medicine Reports, 19: 3190-3200.

63. Xiao D, Zhang Y, Wang R, Fu Y, Zhou T, Diao H, Wang et al (2019) Emodin alleviates cardiac fibrosis by suppressing activation of cardiac fibroblasts via up-regulating metastasis associated protein. Chinese Pharmaceutical Association.

64. Imanishi Y, Maeda N, Otogawa K, Seki S, Matsui H, Kawada N, Arakawa T (2004) Herb medicine Inchin-ko-to (TJ-135) regulates PDGF-BB-dependent signaling pathways of hepatic stellate cells in primary culture and attenuates development of liver fibrosis induced by thioacetamide administration in rats. J. Hepatol., 41: 242-250.

65. Zhu J (2015) T helper 2 cell differentiation, type-2 innate lymphoid cells (ILC2) development and regulation of interleukin-4 (IL-4) and IL-13 production. Cytokine, 75: 14-24.

66. Junttilla IS (2018) Tuning the cytokine responses: An update on interleukin-4 (IL-4) and IL-13 receptor complexes. Frontiers in pharmacology, 9. Article 883.

67. Gadani SP, Cronk JC, Norris GT, Kipnis J (2012) Interleukin-4: A Cytokine to Remember. J. Immunol., 1: 189(9):4213-4219.

68. Zhan YT, Liu B, Li DG, Bi CS (2014) Mechanism of emodin for antifibrosis of liver. Clical. J. Hepatol., 12: 245-246.

69. Kanellakis P, Ditiatkovski M, Kostolias G, Bobik A (2012) A pro-fibrotic role for Interleukin-4 in cardiac pressure overload. Cardiovascular Research, 95: 77-85.

70. Aoudjehane L, Pissaia A Jr, Scatton O, Podevin P, Massault PP, Chouzenous S, et al (2008) Inetrleukin-4 induces the activation and collagen production of cultured human intrahepatic fibroblasts via the STAT-6 pathway. Lab Interest, 88: 973-985.

71. Sommer M, Eismann U, Gerth J, Stein G (2002) Inetrleukin-4 co-stimulates the PDGF-BB and bFGF- mediated proliferation of mesangial cells and myofibroblasts. Nephron, 92:868-880.

72. Wynes MW (2004) Frankel SK, Riches DW. IL-4-induced macrophage-derived IGF-1 protects myofibrobalsts from apoptosis following frowth factor withdrawal. J. Leukoc. Biol., 76: 1019-1027.

73. Weng SY, Wang X, Vijayan S, Tang Y, Kima Y, Padberg K, et al (2018) IL-4 Receptor Alpha Signaling through Macrophages Differentially Regulates Liver Fibrosis Progression and Reversal. E Bio Medicine, 29: 92-103.

74. Karlmark KR, Weiskirchen R, Zimmermann HW, Gassler N, Ginhoux F, Weber C, et al (2009) Hepatic recruitment of the inflammatory Gr1+ monocyte subset upon liver injury promotes hepatic fibrosis. Hepatology, 50: 261-274.

75. Mehal WZ, Schuppan D (2015) Anti-fibrotic therapies in the liver. Semin Liver Dis., 35:184-198.

76. Egawa M, Mukai K, Yoshikawa S, Iki M, Mukaida N, Kawano Y, et al (2013) Inflammatory monocytes recruited to allergic skin acquire an anti-inflammatory M2 phenotype via basophil-derived interleukin-4. Immunity., 38: 570-580.

77. Gundra UM, Girgis NM, Gonzalez MA, San Tang TM, Van der Zande HJP, Lin JD, et al (2015) Vitamin A mediates conversion on monocyte-derived macrophages into tissue-resident macrophages during alternative activation. Nat. Immunol., 18: 642-653.

78. Wheling-Henricks M, Jordan MC, Gotoh T, Grody WW, Roos KP, Tidball JG (2010)

Arginine metabolism by macrophages promotes cardiac and muscle fibrosis in mdx muscular dustrophy .PLos One, 5: e10763.

79. Jenkins SJ, Rucker lD, Cook PC, Jones LH, Finkelman FD, Van Rooijen N, et al (2011) Local macrophages proliferation, rather than recruitment from blood, is a signature of Th2 inflammation. Science, 332: 1284-1288.

80. Xie R, Liu M, Li S (2019) Emodin weakens liver inflammatory injury triggered by lipopolysaccharide through elevating microRNA-145 in vitro and in vivo. Artificial Cells, Nanomedicine, and Biotechnology, 47 (1): 1877-1887.

81. Zamorano J, Mora AL, Boothby M, Achsah DK (2001) NF-κB activation plays an important role in the IL-4-induced protection from apoptosis. International Immunology, 13 (12):1479-1487.

