RESEARCH ARTICLE

Effects of Emodin and Salvianolic Acid on Carbon Tetrachloride (CCl4)-induced Lung Fibrosis in Mice Model

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ABSTRACT

Pulmonary fibrosis (PF) is a chronic, progressive illness associated with poor prognosis and morbidity, defined by an abnormal buildup of fibrotic tissue in the lungs parenchyma. The current study aims to assess the *in-vivo* emodin and salvianolic acid antifibrotic activity on lung fibrosis. Forty mice were divided into five groups; The first group is composed of eight healthy mice to be a negative control group, while the remaining thirty-two mice had received 1-mL/kg intraperitoneal carbon tetrachloride (CCl_4) twice weekly for six weeks. Then, eight of these mice were sacrificed to be a positive control group, while the remaining twenty-four mice were divided into three groups, the first group received six every other day intraperitoneal doses of emodin 40 mg/kg and the second group received silymarin 20 mg/kg while the third group received salvianolic acid by the same route. Mice lungs were taken and examined histopathologically to assess the antifibrotic effects.

Fibrosis is reduced significantly in histopathologic sections in the emodin and salvianolic acid groups.

Antifibrotic effects of emodin can be due to nuclear factor-κb lowering, decrease of epithelial mesenchymal transition, TGF-ß1 Smad signaling suppression, Toll-like receptor-4 (TLR-4) pathway inhibition, and hematopoietic stem cells (HSCs) inhibition. The antifibrotic effects of Salvianolic acid can be due natural killer cells activation, nuclear factor erythriod-derived 2-like 2 up-regulation, transforming growth factor-β1 inhibition. Both agents (Emodin and salvianolic acid) have a comparable antifibrotic activity to that of silymarin, alleviating lung fibrosis.

Keywords: Emodin, Lung fibrosis, Salvianolic acid, Silymarin.

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INTRODUCTION

This study was aimed to assess the *in vivo* antifibrotic activity of emodin and salvianolic acid on lung fibrosis. Carbon tetrachloride (CCl_4) was used to induce lung 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 lung CYP2E1 can produce many types of free radicals like trichloromethyl ($CCl_3^{\cdot}$**)**, trichloro-methyl peroxy ($OOCCl_3^{\cdot}$), and chloride (Cl).[1] Free radicals have very high affinity for electrons; seeking biological tissues. It causes protein peroxidation and enzyme and DNA distortion and initiates lipid peroxidation process. An increased species of reactive oxygen (ROS) is responsible for 10 days of intraperitoneal administration of 1-mL/kg CCl4 for lung carcinoma, pulmonary fibrosis, chronic bronchitis, and emphysema, as well as for pleural diseases.[2] Increased TNF-α, malondialdehyde (MDA), and nitric oxide (NO) are caused by a single large intraperitoneal dose of CCl_4 (2 mL/kg).[3]

Emodin(1,3,8-trihydroxy-6-methyl anthraquinone) is a naturally occurring poly-phenolic anthraquinone with a possible antifibrotic effect. Emodin has *in vitro* antifibrotic activity in both total collagen accumulation and nodule formation assays.[4] In the current study, emodin is extracted from *Aloe barbadensis*.

Salvianolic acids are a group of phenolic acids. They can be extracted from different species of salvia genus such as *Salvia miltiorrhiza* (Danshen) and *Salvia officinalis* (sage).[5] Sage's treatments include seizure, ulcers, gout, rheumatism, inflammation, dizziness, tremor, paralysis, diarrhea, and hyperglycemia.[6] In the current study, sage was used to get salvianolic acid mixture A and B**.**

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. Hydroxyl, methyl, and carbonyl groups are the key determinants of biological activities besides than laxative effect. The antifibrotic effect involves the regulation

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expression of the gene of epidermal growth factor (EGF), transforming growth factor beta-1 (TGF-β1), and platelet-derived growth factor (PDGF). Hepatocytes' protective function reduces cholestatic hepatitis through antagonism between pro-inflammatory cytokines and mediators, inhibits oxidative damage, improves microcirculation of the hepatic, reduces signs of impoverishment and controls the infiltration of neutrophil.[7] By its reactive oxygen scavenging action, Emodin can be hepatoprotective.[8]

Salvianolic acid antifibrotic effects can be related to induction of Micro RNAs (miR-152), leading to the silencing of DNA methyltransferase 1 (DNMT1) and Patched1 (PTCH1) demethylation which inhibits the hedgehog (Hh) pathway and contributes to the suppression of epithelial-mesenchymal transition (EMT) in activated hepatic stellate cells (HSCs). PTCH1 is a negative regulatory factor of the Hh signaling pathway.[9]

Many substances are antifibrotic but none in clinics have been utilized. No medicinal therapies for treating individuals with lung fibrosis are authorized by the Food and Drug Administration (FDA). The primary etiological effect of certain treatments for pulmonary illness is the elimination or improvement of chronic lung disease causative agents (CLD).[10,11]

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), emodin standard (Chengdu biopurify phytochemical-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).

Instruments

Distillator (Boeco, Germany), chillerUltratemp 2000 (Buchi-Germany), electronic sensitive balance (Sartorious, Germany), histocenter (Shandon-China), light microscope supplied with camera (Genix-England), micropipette 100 to 1000 µL and 20 to 200 µL (Huawei-China), micropipette 5 to 50 µL (Slammed, Germany), microtome (Sakura-Japan), plain microscope slide, preparative HPLC device with UV detector (Jas.co, Japan), and rotary evaporator (Stuart-UK).

Extraction of Emodin from A. barbadensis

During the 24 hours, hexane was deformed to dry coarsely shaded dried seeds and areal portions of plant at room temperature. With 80% ethanol in the soxhlet device, defatted plant components were extracted to full exhaustion. At a temperature not exceeding 40°C, the alcoholic extract evaporated under decreased pressure to yield a dark greenish-yellow residue known as a crude fraction.[12] One gram of plant extract was diluted in a little amount of chloroform and injected into a preparative HPLC system using Methanol: water (65:35) as a mobile phase, Mediterranea C18, 5 m 152.12 as a column, flow rate of 3 mL/min, injection volume of one ml, and UV Detector at λ366 nm.[13]

Preparative HPLC was used in this study to isolate in a very pure and high quantity emodin from *aloe* plants. Large columns and high flow rates are connected with preparative HPLC, which may receive large injected volumes (1-mL as compared to the microliters in the analytical one). The chromatogram revealed three peaks, each of which represents a distinct chemical; one of them (E2) is a significant peak, indicating emodin. The powder is made by drying the solution of the highest peak. Figure 2 illustrates Aloe extract peaks in preparative HPLC recorder.[12]

Extraction of Salvianolic Acid Mixture A and B from S. Officinalis

S. officinalis cultivated in the north of Iraq, has been brought to pharmacy college of Baghdad University, Iraq. The whole plant aerial parts are left to be dried for one week, then is crushed using an electric house grinder. Salvia extract underwent decantation to get the pure extract then sent to rotary and chiller for 1-hour then the pure extract sent to preparative HPLC with UV detector.

The 280 nm wavelength was utilized to separate salicylic acid (Sal A) and salvianolic acid (Sal B) in preparative HPLC, with a limit of detection of 0.0080.160 g/mL.

The chromatographic separation was done using a UV detector, and all separations were done on a Mediterranea column C18, 5 m 15 X 2.12-Japan).

The separation was carried out using a linear gradient elution of Eluents A (0.5 percent (v/v) aqueous formic acid) and B (0.5 percent (v/v) formic acid in acetonitrile).

The elution procedure was carefully planned and carried out as follows:

The first linear gradient was 5–20% Eluent B in the range of 0–10 minutes, the second was 20–25% Eluent B in the range of 10–17 minutes, and the third was 25–55% Eluent B in the range of 17–35 minutes. After 5 minutes, the system was returned to its original state.

The flow rate of the solvent was 1.0 mL/min, the injection volume was 20 µL, and the column temperature was 30°C. The chromatograms were taken at 280 nm for the first 13 minutes, 326 nm for the next 1323.5 minutes, and 286 nm for the next 23.535 minutes.[12]

Animals

Under the agreement of the 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 26th January 2018 to 7th April 2018 in a good ventilated room and suitable temperature (20 to 25°C), 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 lungs.

Experimental Design

Forty mice were divided into four groups, each one containing eight mice.

- *Group I:* Negative control(apparently healthy mice that will not receive any treatment).
- *Group II:* Positive control (Lung disease group). These mice received 1-mL/kg intraperitoneal CCl_4 twice weekly of 100% (v/v) carbon tetrachloride (CCl_4) diluted in ten times volumes of olive oil.[14]
- *Group III:* Emodin group: after finishing the CCl_4 doses, they received six doses of emodin 40 mg/kg every other day.[15]
- *Group IV:* Salvianolic acid group: after finishing the CCl_4 doses, they received six doses of salvianolic acid 40 mg/kg every other day.[16]
- *Group V:* Silymarin group: after finishing the CCl_4 doses, they received six doses of silymarin 20 mg/kg, the standard protective treatment for fibrosis, every other day schedule.[17]

Mice lungs were examined histopathologically under light microscope.

Preparing of Injections

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

Tissue Preparation

The entirely taken lung will be kept immediately in a 15% recent formalin solution until the time of tissue slice formation. Lung tissues were cut into 5 mm slices and placed in formalin (10%) to preserve them. Tissue was fixed for a minimum of 48 hours at room temperature using a fixative volume 20 times that of the tissue on a weight-per-volume basis. These tissues were undergone gentle agitation using ethanol 70, 80, 90, and 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 2 times, separated by 24 hours. The paraffin-embedded tissues should be stored as paraffin blocks to be used at any time for preparing further slides according to the required number of tests.[18]

Slide Preparation

Using a microtome, serial tissue slices of 3 to 5 m thickness were produced, and three slides were made from each tissue paraffin block. To prevent tissue slices from folding during the mounting technique, sections were mounted on conventional slides (for Haematoxylin and Eosin staining) or positively charged slides (for immunohistochemistry) using a 45ºC water bath.[18]

Histopathological Assessment

The Ascroft lung score system[19] in Table 1, is used to evaluate medication effects on the lungs. The average microscope field ratings was used to grade the samples on a scale of 0 to 8.

RESULTS

Our study observed that using emodin or salvianolic acid restore the normal architecture of the lung and resolves the fibrosis (Figures 1 and 2).

a. Severe distortion of structure and large fibrous areas in the untreated lung fibrosis group.
b. Minimal fibrous thickening of alveolar or bronchial wallsin the emodin group.
c. In the salvianolic acid group, moderate wall thickening without apparent damage.
d. In the Silymarin group, moderate wall thickening without apparent lung injury.

DISCUSSION

CCl_4 in low doses for a prolonged period produces grade 7 of pulmonary fibrosis (Figures 1 and 2). Cuboidal type 2 (AEC2) and long thin type 1 (AEC1) cells make up the alveolar epithelium. Repeated lesions to the alveolar epithelium trigger an aberrative healing process marked by AEC2 apoptosis, proliferation, and epithelial-mesenchymal cross-talk, followed by fibroblast migration, myofibroblast proliferation, and extracellular matrix buildup.

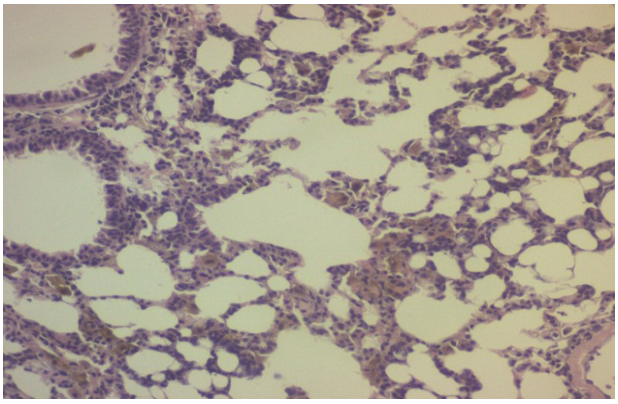
Fibroblast growth factor (FGF), plasminogen activator inhibitor 1 and 2 (PAI-1 and 2), platelet-derived growth factor (PDGF), transforming growth factor-beta 1 (TGF-β1); tumor necrosis factor-alpha (TNF-α); vascular endothelial growth factor (VEGF) all play a part in the fibrosis process.[20]

The current study aids Liu *et al* study.[21] Our previous work observed that 40 mg/kg dose produced the greater antifibrotic effects of emodin.[22]

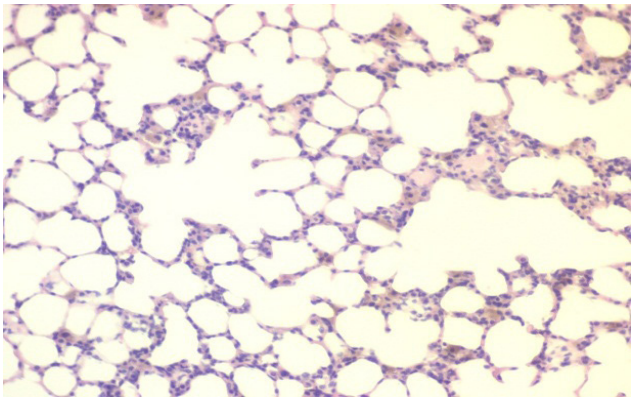
TGF-$_\beta$1 signaling through Smad molecules is a well-known fibrosis mechanism.[23] TGF-β1 activates and phosphorylates $Smad_2$ and $Smad_3$ to send intracellular signals. With $Smad_4$, $Smad_{2/3}$ and $Smad_{1/5/8}$ create a heteropolymer. At the last, regulation of gene transcription will occur after $Smad_4$translocation to the nucleus which interacts directly with DNA or via coenzyme factors.[24,25]

Table 1: Lung scoring system

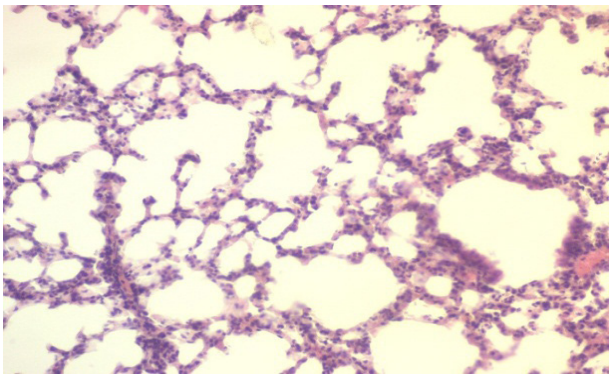
Grade of fibrosis	*Histological features*
0	Lung function is normal
1	Alveolar or bronchial walls with little fiber thickening
2 and 3	Moderate wall thickening without apparent lung injury
4 and 5	Increased fibrous with evident lung structural damage and the development of fibrous bands or a tiny fibrous mass
6 and 7	Large fibrous regions and severe structural deformation (honi-comb lung)
8	The field has been completely obliterated by fibrous obliteration.



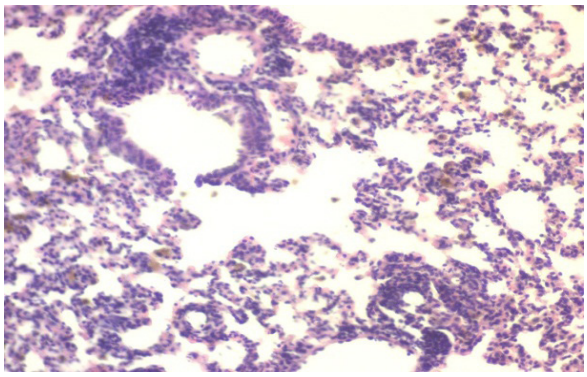
a. Untreated lung fibrosis group (Grade 7 fibrosis)



b. Emodin group (Grade 1 fibrosis)

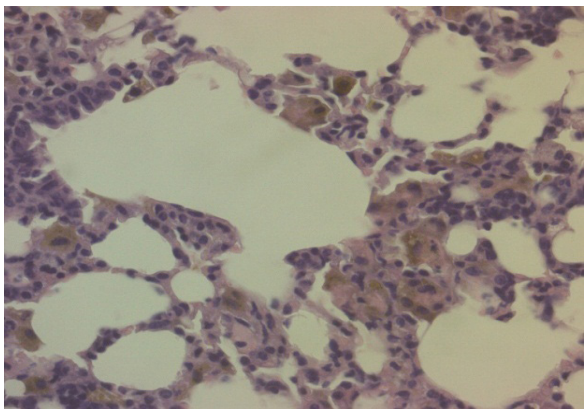


c. Salvianolic acid group (Grade 2)

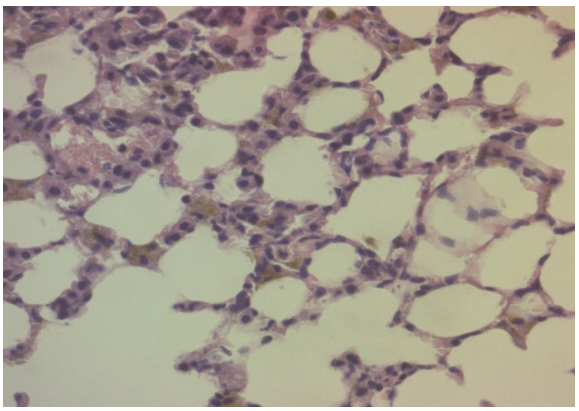


d. Silymarin group (Grade 3)

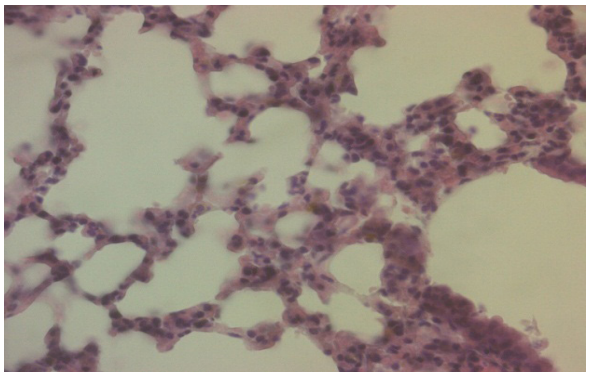
Figure 1: Lung histopathology under 20x power of microscope for the groups of study



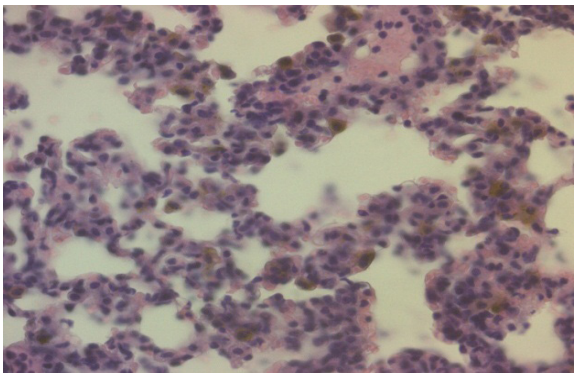
Untreated lung fibrosis group (Grade 7)



Emodin group (Grade 1)



Salvianolic acid group (Grade 2)



Silymarin group (Grade 3)

Figure 2: Lung histopathology under 40x power of microscope for the groups of study

Emodin had shown to inhibit the TLR-4 pathway, TLR-4 mediates anti-apoptotic and inflammatory effects in fibrosis leading.[26] Emodin attenuates TLR-4 signaling pathways.[27] IL-4 has a profibrotic role.[28] It is a potent stimulator of collagen biosynthesis in fibroblasts,[29] and also IL-4 prevents myofibroblasts apoptosis in organs that undergo fibrosis such as lung by stimulating macrophages to secrete insulin-like growth favtor-1 (IGF-1).[30] One of the possible mechanisms by which emodin can lower IL-4 is by lowering nuclear factor κB (NF-κB).[31] The activation of NF-κB is crucial for IL-4-induced apoptosis protection.[32] IL-4 prevents myofibroblasts apoptosis.[30]

Lung parenchymal cell death, drugs, and toxins may contribute to fibroblast activation during chronic lung injury. Lung fibroblasts differentiate into fibrogenic, proliferative, and contractile myofibroblasts that produce ECM proteins such as collagen.[33] On the surface of target cells, several types of receptors are expressed, and based on these receptors, NK cells will kill or not kill the targeted cells.[34]

NKG_2D, NKp46, NKp30, and NKp44 are NK cell stimulatory receptors that were up-regulated by *S. miltiorrhiza* therapy, probably triggering NK killing.[35] Both *S. miltiorrhiza* and *S. officinalis* contain salvianolic acids.[36,37] The above-mentioned stimulatory receptors interact with stimulatory ligands expressed on target cell surface like RAE-1 promoting NKcell activities. To decrease NK cell activity, inhibitory receptors such as Ly49A and CD94/NKG2 interact with inhibitory ligands (e.g., MHC-I) expressed on target cells.[35]

Salvianolic acid up-regulate nuclear factor erythriod-derived 2-like 2 (Nrf2) at both the protein and mRNA levels inhibits myofibro-blasts trans-differentiation.[38]

TFG-β1 is the most potent inducer of collagen production and ECM accumulation and the most essential event in lung fibrosis. The activated fibro-blasts secretes TFG-β1 and up-regulates the metalloproteinase inhibitors, leading to matrix stabilization.[39,40]

CONCLUSION

Both Emodin and Salvianolic acid has nearly potent fibrosis fighting activity to that of silymarin. Emodin antifibrotic activity can be due to different mechanisms like nuclear factor-κbinhibition, inhibition of epithelial mesenchymal transition, TGF-ß1 Smad signaling suppression, suppressed TLR-4 pathway, and HSCs inhibition. Salvianolic acid promotes NK cell activities by up-regulating the NKG_2D, NKp46, NKp30, and NKp44 receptors. Salvianolic acid also up-regulate nuclear factor erythriod-derived 2-like 2 (Nrf2) at both the protein and mRNA levels inhibits myofibroblasts transdifferentiation. Salvianolic acid can also inhibits (TFG-β1).

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