I.O.D.A.S. - "VASILE GOLDIS" Western University of Arad
DOCTORAL SCHOOL OF BIOLOGY

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SUMMARY
DOCTORAL THESIS

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PhD THESIS

Therapeutic strategies of liver fibrosis with high bioavailability bioactive natural compounds

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TEZA DE DOCTORAT

Strategii terapeutice ale fibrozei hepatice cu compuși naturali biodisponibili

Doctorand: **Sami T. H. Gharbia**

Conducător de doctorat: **Prof.univ.dr. Anca Hermenean**

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INTRODUCTION

Epidemiological data indicate that approximately 180 million of patients worldwide were affected by a form of chronic liver disease. More than one million deaths (representing approx. 2.0% of the total), and approximately of 31,000,000 hepatic injuries had occurred a disability in years-life due to liver cirrhosis. Alcoholic liver diseases alone are responsible for 493,000 deaths [1].

Liver fibrosis can be described as a process of overdoing in the progression of fibrogenesis and deficiency in the regression pathway of fibrinolysis that leads to accumulation of the collagen and reduced remodeling of extracellular matrix [2]. There are several relevant issues that need to be investigated and evaluated. The monitoring of the fibrotic progression through chronic liver diseases highlights biological role of growth factors/cytokines and their intracellular signal pathways and effectiveness of the currently proposed treatments.

Many current treatments and research results are mentioned in the literatures. One of the most important and useful hepatoprotective natural compounds is silymarin, extracted from the seeds of *Silybum marianum*.

Drug delivery to target tissues or cells is the main tool of medical and biological research by using special prepared nanoparticles. Nanoparticle "loaded" with the therapeutic agent is the aim to prolong the effect and made resistant to digestive juice, to increase solubility and absorption at intestinal level. Therefore, the active substances are able to arrive at the targeted organ or specific cells through the circulation system.

Cyclodextrins have been utilized as drug delivery system to improve solubility, dissolution, and bioavailability, for reducing the side effect of drugs, prevention of drug-drug interaction, alleviation of gastrointestinal or ocular irritation and unpleasant taste and smell [3,4].

Our aim was to investigate the protective capacity of encapsulated silymarin in two types of cyclodextrins (silymarin / HPBCD and silymarin / RAMEB) in CCl_4 – induced liver fibrosis model in mice.

CURRENT STATE OF KNOWLEDGE

CHAPTER 1. LIVER PATHOLOGY AND RECOVERY

1.1. Liver morphophysiology

Liver has a capacity to regenerate by itself after injury, and adjust its own size to match its recipients.

Liver regeneration after hepatectomy is a process similar to wound healing, but the signaling pathway triggered during this process is stronger, and occur over the entire liver.

1.2. The structure and function of liver cells

Liver tissue consists of many types of cells. Hepatocytes represents approximately 80% of the liver tissue and they perform the most metabolic and synthetic functions in the liver. Other types are Kupffer cells, Pit cells, stellate cells and endothelial cells [5].

Stellate cells, also called Ito cells, are located between the sinusoids and liver hepatocytes (in the space of Disse), and store fat and vitamin A. These cells are the major type of cells involved in liver fibrosis, consisting in scar tissue formation, as response to liver damage. Dendritic cells (DCs) are situated in the parenchymal tissue on portal region, and have an important role in binding innate and adaptive immune system. The DCs capture, identify, uptake and process those pathogens brought by blood through the hepatic artery, –further being presented as antigens to T cells. In addition, they produce interferons and interleukin - 10(IL-10) [6].

1.3. Liver pathology

Many liver diseases are acquired from infection and exposed to toxic substances, such as alcohol or drugs. The most common liver diseases are Hepatitis A, B and C and cirrhosis.

The liver fibrosis is a serious condition where healthy tissue in the liver is destroyed and ruined after that replaced by scar tissue, which starts to block the flow of blood through the liver and alter the normal hepatic functions.

1.4. Oxidative stress as a crucial factor in the liver diseases

Oxidative stress plays a critical role in liver inflammation and fibrogenesis, as well as in HSCs activation. The damaged cells generate ROS (superoxide and hydrogen peroxide) as a response to NOX expression and induce proliferation and transformation of HSCs to myofibroblasts. Different types of enzymes participate in ROS production: CYP2E1, NO synthetases and NOX. In normal condition, superoxide dismutase (SOD) and catalase (CAT), as antioxidant enzyme system, eliminate the ROS products in healthy liver cells. In the case of chronic liver diseases, the activity of antioxidants is decreased and production of ROS increased. Generally, NOX is important in regulating cell signal and host defense [7].

The overexpression of ROS inhibits the activities of antioxidant enzyme, as superoxide dismutase (SOD), minimize glutathione (GSH) and stimulates HSCs activation, proliferation, migration and collagen production. In addition, the active HSCs also express ROS compared to quiescent HSCs, as a mechanism of apoptotic protection [8].

1.5. The dynamics of liver regeneration

The hepatic regeneration is a compensatory hyperplasia. It means the proliferation of all cells within the liver includes hepatocytes, epithelial cells and endothelial cells with the help of DNA synthesis. The process is associated with cascades of signaling and involving growth factors, cytokines and ECM rearrangement.

There are two steps in liver regeneration process [9]:

1. The transition of the quiescent hepatocyte into the cell cycle under control of TNF and IL-6 family cytokines;
2. The progression beyond the G0 point in the G1 cycle by growth factors HGF and TGF-α.

Hepatocytes are the first cells which enter into the cell cycle. They receive signals to exit from G0 and to initiate the expression of a set of genes required for regeneration and undergo to mitosis [10], followed by the proliferation of hepatic stellate cells, Kupffer cells and biliary epithelial cells, endothelial cells and angiogenesis progression.

The proliferation begins in the portal region (around the portal triads) and goes toward the centers of the lobules by the formation of clumps, then transforms to plates.

HSCs and portal fibroblast are involved in liver regeneration by secretion of mitogenic, anti-apoptotic and growth factors (insulin-like growth factor 1 (IGF-I), HGF, neurotrophins, IL-6 and Wnt ligant) [11]. The mechanisms of the liver regeneration ending by several negative regulatory signals, include TGF-β1, P53, p21 and C/EBPα. Mice lacking p21, p53, and C/EBPα showed continuous hepatocyte turnover and hyperproliferation of hepatocytes [12], whereas plasminogen-

activator inhibitor (PAI) blocked the transforming of pro-HGF into active form of HGF, suppressor of cytokine signaling-3 (SOCS-3) and IL-6 signal ending [13].

CHAPTER 2. CELLULAR AND MOLECULAR PATHOGENESIS OF HEPATIC FIBROSIS

Fibrosis is characterized by the presence of parenchymal "scar" lesions in response to a variety of acute and/or chronic stimuli: ethanol, virus infection, drugs and toxins, cholestatic and metabolic liver diseases. The evolution of liver fibrosis, by increasing the synthesis and deposition of fibrillar collagen, leads to cirrhosis, is a condition characterized by altered structural-functional liver formation of septa and nodules, altered blood flow, portal hypertension and in some conditions by liver failure [14,15].

Liver fibrosis is an imbalance appearing in the process of fibrogenesis and fibrolysis, leading to scar formation. Actually, it occurs as a result of the trans-differentiation of (HSCs) into myofibroblasts and is associated with an increased expression and activation of TGF-β1.

Liver fibrosis is characterized as a degradation of the parenchyma and substitution with a collagen-rich tissue, where the hepatic stellate cells (HSCs) are the main collagen-producing cells in the liver. The mechanism starts when the liver tries to heal the hepatic injury. If the liver injury persists, the mechanism of healing will turn to another pathway where the hepatocytes are substituted with extracellular matrix (ECM) [16,15].

TGF-β1 is a critical factor in the progression of hepatic alcoholic disease (BHA) in patients with steatosis and steatohepatitis. Acetaldehyde does not change Smad3 and Smad4 protein concentration, but selectively induce phosphorylation of Smad3, but not of Smad2. Weng et al. [17] identified a significant correlation between phosphorylation of Smad2 and stage of

fibrosis or inflammation score. The TGF-β signaling is a very important pathway for HSCs activation, that stimulates cytoplasmic Smad proteins and modulates the transcription of those target genes encoding ECM (eg. procollagen I and III) [18].

2.1. Cells involved in the pathogenesis of hepatic fibrosis

The process of fibrosis is driven by a heterogeneous cells population (myofibroblast-like cells), that migrate, differentiate and proliferate through hepatogenic factors. Chronic liver disease has a pattern of interactions between all hepatic cells and fibrogenesis factors up-regulation (e.g.: growth factors, cytokines, chemokines and ROS). The following types of cells are involved in the pathogenesis of hepatic fibrosis:

Hepatic stellate cells

Hepatic stellate cells (HSCs) are found in the Disse space between hepatocytes and sinusoidal endothelial cells. HSCs responsibilities are characterized by a significant expression of cytoskeletal proteins (desmin and glial acidic fibrillary proteins), followed by desmin and vitamin A (and lipid droplets) storage. As a result of hepatic injury, HSC loses their vitamin A content (vitamin A is used as energy source during trans-differentiation) [19,15], it intensifies the expression of alpha smooth muscle actin (α-SMA), increases the expression of GFs, induces high levels of collagen-I and acquires a phenotype resembling of myofibroblasts by transforming into a star shape. Activated HSCs proliferate, become mobile and pro-fibrogenic, contractile and show abundant rough endoplasmic reticulum [20].

Portal fibroblasts

Portal connective tissue of healthy liver is surrounded by portal fibroblasts, which is a second cell population that may be involved in portal liver fibrosis. Portal fibroblasts are derived from small portal vessels and they express distinct markers of HSC (for example, elastin). The proliferation of biliary cells is often accompanied with proliferation of portal fibroblast, which form lamellar configurations around the structure of biliary cells and they acquire a phenotype resembling myofibroblasts, which therefore are involved in storing of early extracellular matrix (ECM) in the portal zone [21].

Mesenchymal stem cells derived from bone marrow and Fibrocytes

Several studies have shown that the mesenchymal stem cells derived from bone marrow may be a source of multi-lineage cells to different organs. They have an ability to differentiate into hepatocytes, biliary epithelial cells, sinusoidal endothelial cells and Kupffer cells, even in the presence of a suitable hepatic microenvironment [22]. There is an increasing evidence to suggest that stem cells derived from bone marrow are recruited during the progression and regression of liver fibrosis. During regression of liver fibrosis induced by CCl_4, mesenchymal stem cells derived from bone marrow migrate into the injured liver area, where they can express MMP9 and MMP13 [23].

Epithelial cells and Hepatocytes

Several studies have described the involvement of epithelial cells and hepatocytes in the epithelial–mesenchymal transition (EMT) of hepatic fibrogenesis process. In primary biliary cirrhosis, was demonstrated that cells of bile duct expressed the protein-1 specific fibroblasts and vimentin, early markers of fibroblasts [24]. Both of epithelial and cholangiocyte

cells exposed to TGF-β leads to trans-differentiating to myofibroblasts and expressed the fibroblast-specific protein-1 (FSP-1) [25].

In vitro studies with human biliary epithelial cells confirmed these clinical observations. In this regard, EMT may be considered the essential mechanism involved in the pathogenesis of the chronic cholestatic liver disease [26].

Myofibroblast cells

Myofibroblast cells (MFs) are characterized by high expression of α-SMA in all chronic liver disease. In addition, most inflammatory and fibrogenic factors are expressed in other hepatic cells, which is an indication that MFs are originated and trans-differentiated from any type of hepatic cell, but mainly from HSCs and portal fibroblast cells [19,15].

The main properties of myofibroblasts are proliferation, migration and accumulation in the injured livers, as response to fibrogenic factors like growth factors (GFs), cytokines, chemokines and production of fibrillar collagens, low level of MMPs and high level of TIMPs, all of these factors leading to up-regulation and down-regulation of the fibrogenesis progression [25].

2.2. TGF-β signaling and the fibrotic response

The fibrotic disease is characterized by excessive production of the ECM due to a failure of the normal healing response. After injury, new connective tissue and mesenchymal fibroblasts (stellate cells) become activated and migrate into the damaged region and synthesize an elevated level of matrix proteins, including collagen and fibronectin but in fibrotic disease the regenerative pathways are different. These proteins,

including profibrotic proteins TGF-β, CTGF and the anti-fibrotic proteins TNF-α and IFN-γ, play a critical role in a normal process. The TGF-β induces fibroblasts to synthesize and contract ECM. This cytokine has been long time considered a central mediator of the fibrotic response.

The TGF-β is synthesized as latent precursors complexed with latent TGF-β binding proteins (LTBP). TGF-β is activated when LTBP is removed extracellularly by proteolytic cleavage (MMP-2 and -9, thrombospondin-1, integrin αvβ6) [27,28]**.** When TGF-β is activated and bound with the receptor transforming growth factor beta receptor (TGF-βR), the complex up-regulates signaling cascade transcription within the cell by Smad family and translocates into the nucleus to bind target gene and start matrix synthesis [27].

The induction of collagen through TGF-β occurs by requiring the action of the Smad's. The Smad 3 is essential for the induction of profibrotic matrix genes such as collagen type I and CTGF [29]. The TGF-β causes matrix deposition in mesenchymal cells (stellate cells) by promoting expression of ECM genes and suppressing the activity of genes, such as MMP's genes.

2.3. Role of the metalloproteinases (MMPs) and TIMPs

Extracellular matrix (ECM) is an extremely dynamic environment, undergoing constant remodeling, whereby the synthesis of the new components takes place simultaneously with degenerative processes. Imbalances occur in pathological conditions of these processes.

Matrix-metalloproteinases (MMPs) are zinc-dependent endopeptidases and generally are calcium-dependent. MMPs are the major enzymes responsible for the degradation of ECM.

Tissue inhibitor of metalloproteinases (TIMPs) has the ability to inhibit MMPs [30]. Therefore, adjusting the balance MMP - TIMP is crucial for efficient ECM remodeling. The report MMP - TIMP can be unbalanced due to multiple pro-fibrogenic injuries. Activated HSC not only synthesize, but also secretes ECM proteins, such as collagen type I and III, but also produces MMP-1 [31] and MMP-13 [32]. However, the expression MMP-1 and -13 decreases with advancing activation of HSC, while other activities of MMP remains relatively constant, except MMP-2 and MMP-9 [33]. The increased activity of MMP-2 is associated with a distortion of normal lobular architecture, which further activates HSC. Moreover, activated HSC amplifies the expression and synthesis of TIMP-1 and TIMP-2 [31]. TIMP-1 not only prevents the degradation of the ECM by blocking the rapid increase of MMP, but also inhibits apoptosis of activated HSC [34]. The net result is deposition of mature collagen in the Disse space and therefore the scarring of hepatic tissue occurs. As result, the inhibition of the effect of TIMP-1 could be an important factor in the regression of fibrosis. Several factors can activate TIMP-1, including leptin, angiotensin II and sphingosine-1-phosphate [35]. The controlling on these signaling pathways is important for the successfully TIMP-1inhibition, besides increasing and activation of MMP, hepatic expression can also lead to regression of fibrosis [36]. Stellate cells express MMP-2, MMP-9 and MMP-1, as main collagen-I degradation enzyme. In the case of progressive fibrosis and start forming scars, stellate cells express high level of TIMP-1 and TIMP-2 as indication of ongoing fibrogenesis; therefore, they downregulate the activity of MMPs for degradation of unmodulated accumulated matrix. The expression of plasminogen activator inhibitor 1 (PAI-1) leads to

inhibition of MMPs through the inhibition of plasminogen that is important for activities of MMPs [37,38,33].

MMP-2 and -9 are significantly found in vascular regions. MMP-2 is activated by TGF-β and that leads to modulate fibrogenic factors (e.g: IL-1β and TNF-α), while MMP-1 modulates MCP-1. Overexpression of MMP-1, -8 and -13 is associated in reducing liver fibrosis. MMP-9 is expressed in the early stage of fibrogenesis, and also activates TGF-β and enhances HSCs apoptosis in case of low expression of TIMP-2 [39]. MMP-2 suppresses expression but it does not degrade the collagen-I, whereas MMP-19 has a critical role in fibrogenesis by activating TGF-β.

CHAPTER 3. PLANT BIOACTIVES FOR THE PREVENTION AND TREATMENT OF THE LIVER DISEASES

3.2. Silymarin and its formulations for the prevention and therapy of the liver diseases

Silymarin is a mixture of flavonolignans (silymarin complex) extracted from *Silybum marianum* dried seeds, that contain 60-80% of flavonolignans silymarin complex (silibini 50-60%, silichristin 20%, silidianin 10% and isosilibinin 5%). Other complexes include dehydrosilybin, deoxysilycistin, deoxysilydianin, silandrin, silybinome, silyhermin and neosilyhermin, in addition to flavonoids include toxifolin, apigenin, silybonol, myristic, oleic palmitin and stearin acids [40,41].

Pharmacokinetic studies showed that the silymarin has poor absorption, rapid metabolism and lower bioavailability properties due to the conjugation and metabolism and low permeability across intestinal epithelial cell [42]. Silymarin is lipophobic and poorly water souble and was mention that it is low percent absorbed (20-50%) from the gastrointestinal tract. Silymarin is metabolized in the liver by CYP450-2C8 and undergoes to phase I and II. In phase I it is metabolized into O-demethylated-silybin, mono- and dihydroxy-silybin, whereas in phase II it is metabolized and conjugated with glucuronide or sulfate and forming silybin-monoglucuronide, diglucuronide, monosulfate and silybin glucuronide sulfate. The free silybin and conjugated forms are excreted rapidly from the body in bile and urine [43,44].

There are some research results with the benefits of silymarin administration against liver disorders. Jia et al. [45] revealed that silymarin reduces hepatic collagen deposits by 35% in rats with secondary biliary cirrhosis after 6 weeks of bile duct occlusion. According with this study, silymarin downregulated elevated procollagen α1(I), TIMP-1 and TGF-β1 mRNA levels by 40–60%.

In other study, prophylactic use of silymarin in combination with verapamil improved anti-fibrotic effects in rat-induced liver fibrosis [46]. Other combined tratment of silybin–phosphatidylcholine–Vitamin E complex administrated orally was able to prevent the dimethylnitrosamine-induced loss in liver weight, as well as to reduce the degree of liver injury, as determined by necroinflammatory score and reduced hepatic stellate cells proliferation both after 1 and 5 weeks of treatment [47].

Many approaches have been investigated to improve bioavailability, including some modification, insertion or enzymatically reformulation, or performing complexes with solid dispersion, microparticles and nanoparticles, self-microemulsifing drug delivery system, micelles, liposomes and phytosomes [48]. All these formulations were done in order to facilitate its passage across the gastrointestinal mucosa, increased absorption, lowering the therapeutic dose, and better stability.

PERSONAL CONTRIBUTION

Hepatic fibrosis is a pathological consequence of chronic liver diseases and results in excessive scar tissue, due to an incorrect wound healing response to liver injury. This pathology is characterized by parenchymal cells death and their replacement with extracellular matrix (ECM) enriched in types I and III fibrillar collagens [49]. Accumulation of ECM proteins change the liver architecture and finally leads to cirrhosis, characterized by presence of fibrotic septa, surrounding regenerating nodules [50].

Recent research results show that fibrogenesis is a dynamic process which can be modulated by arresting progression and/or promoting fibrosis resolution [51].

1. AIMS AND GENERAL OBJECTIVES

Silymarin, a complex a mixture of flavonolignans and taxifolin, is extracted from the seeds of *Silybum marianum L*. (milk thistle). The active constituents of silymarin are: silibinin, isosilybinin, silydianin, and silychristin, of which silibinin is the major and most active component, representing about 60–70% of flavonoids [52].

It is well known the silymarin ability to treat chronic liver diseases, as non-alcoholic fatty liver disease, through nicotinamide adenine dinucleotide (NADC) level restoration [53]. Also, silymarin has shown the ability to protect livers against hepatotoxicity induced by ethanol [54], carbon tetrachloride [55,56,57], cisplatin [58,59], arsenic [60,61], anti-tuberculosis drugs [62], thioacetamide [63] and acetaminophen [64].

One of the therapeutic effect limitations of silymarin is the poor water solubility and oral-bioavailability. Its oral absorption is only about 23–47% [65], generating an oral bioavailability of 0.73% [66]. According with this concerning aspects, one of the research challenges regarding the new silymarin pharmaceutical products is to find the proper drug-delivery system in order to increase their solubility, oral-bioavailability, biological properties which will increase anti-fibrotic effects.

In our experiment, we used two types of β-cyclodextrin: 2-Hydroxypropyl-β-cyclodextrin (HPBCD) and randomly methylated β-cyclodextrin (RAMEB) in order to increase the solubility of pure silymarin, to enhance the bioavailability at oral administration and to have a better anti-fibrotic effect than pure flavonoid.

The first objective of the thesis was to obtain inclusion complexes of sylimarin into β-cyclodextrins: silymarin/ hydroxypropyl (HPBCD) and randomly methylated (RAMEB) β-cyclodextrins complexes, with increased solubility compared to non-formulated silymarin, designed to enhance the anti-fibrotic properties of pure flavonoid in a mouse model of liver fibrosis.

The second objective investigated the oxidative stress and inflammatory responses during fibrogenesis induced by CCl_4 i.p. administration to mice and the ability of Sy/HPBCD and Sy/RAMEB complexes to enhance resolution of fibrosis, through anti-oxidant effects, as well as the process after inflammation that leads to liver fibrosis in relatively earlier stages of fibrosis pathways. Also, we aimed to investigate the connection between inflammation, reaction, and fibrosis in the liver, compared to non-formulated silymarin. In this respect, we planned to measure the lipid peroxidation products - MDA, carbonyl groups and AOPP, alongside with glutathione (GSH) level. Liver

inflammation was evaluated by verifying the gene expression of NF-kB p50, the main nuclear transcription factor which activated inflammatory cytokines, and by TNF-a, and IL-6 genes expression analysis.

The third objective of the study evaluated HPBCD/Sy and RAMEB/Sy effects on TGF-β -mediated HSCs activation caused by carbon tetrachloride (CCl_4) in a mouse model of liver fibrosis. According to this objective, we aimed to perform histopathology of the livers, evaluation of parenchymal collagen deposits, α-SMA mRNA expression and liver deposition, the balance of between TGF-β1 and Smad family (2, 3 and 7), which are involved in up- and down-regulation of the main fibrogenetic pathway.

The fourth objective of the study refers to the ability of HPBCD/Sy and RAMEB/Sy to down-regulate Col-1 genes and decrease expression and distribution of these proteins in hepatic tissue. In this respect, we set ourselves up to investigating Col-1, Timp-1, MMP-1, MMP-2, MMP-2, MMP-9 expressions and protein levels and how they are balanced during fibrosis resolution induced by silymarin - β-cyclodextrin inclusion complexes.

2. MATERIALS AND METHODS

2.1. Preparation of silymarin/cyclodextrin complexes

Silymarin/hydroxypropyl-β-cyclodextrin (Sy-HPβCD) and silymarin/randomly methylated-β-cyclodextrin (Sy-RAMEB) complexes were prepared by ratio (1:20) within the University of Debrecen – Faculty of Pharmacy, Hungary.

Complexes were prepared by kneading method.

2.2. Phase-solubility study

Complexation efficiencies were calculated from the slope of the phase-solubility diagrams according to the equation: CE = S0 · K1:1 = slope/(1-slope)

2.3. HPLC analysis

The samples were analysed by a high-performance liquid chromatography (HPLC).

2.4. Scanning electron microscopy (SEM)

The morphology of solid particles of cyclodextrin, silymarin, silymarin-cyclodextrin physical mixtures and silymarin-cyclodextrin complexes were investigated by Hitachi S-4300 CFE SEM (Scanning Electron Microscope) using 15 kV accelerating voltage.

2.5. Animals and experimental design

The experiments were performed on white male CD1 mice (5-6 weeks old, 26±3g) from the Institute of Life Sciences within Vasile Goldis Western University of Arad.

Group 1 (control group): mice were intraperitoneally (i.p.) injected 2 times/week for 7 weeks with normal saline

solution (2 ml/kg) and then treated orally by gavage (P.O) (**Fig. 10. b**) with 0.1 ml carboxymethyl cellulose (CMC) 0.7%, for 2 weeks;

Group 2 (CCl_4 group): mice were intraperitoneally (i.p.) injected 2 times/week for 7 weeks with 2 ml/kg CCl4 (dissolved in olive oil 20%, v/v) and euthanized 72 hours after the last i.p. injection;

Group 3 (CCl_4 control group): mice were intraperitoneally (i.p.) injected 2 times/week for 7 weeks with 2 ml/kg CCl4 (dissolved in olive oil 20%, v/v) and then treated orally by gavage (P.O) with 0.1 ml carboxymethyl cellulose (CMC) 0.7%, for 2 weeks. This group represent self-recovery group;

Group 4 (CCl_4/Sy-RAMEB group): mice were intraperitoneally (i.p.) injected 2 times/week for 7 weeks with 2 ml/kg CCl4 (dissolved in olive oil 20%, v/v) and then treated orally by gavage (P.O) with 50 mg/kg Sy-RAMEB for 2 weeks;

Group 5 (CCl4/Sy-HPBCD group): mice were intraperitoneally (i.p.) injected 2 times/week for 7 weeks with 2 ml/kg CCl4 (dissolved in olive oil 20%, v/v) and then treated orally by gavage (P.O) with 50 mg/kg Sy-HPBCD for 2 weeks;

Group 6 (CCl4/Sy group): mice were intraperitoneally (i.p.) injected 2 times/week for 7 weeks with 2 ml/kg CCl4 (dissolved in olive oil 20%, v/v) and then treated orally by gavage (P.O) with 50 mg/kg of pure silymarin for 2 weeks;

2.6. Histology technique

Liver samples were processed by histological paraffin in order to evidence structural changes in hepatic tissue (H&E stain) and collagen accumulation (Fouchet van Gieson Trichrome stain)

2.7. Immunohistochemistry – Paraffin technique (IHC-P)

In our study we used Novocastra kits (Leica Biosystems, Germania) for IHC, according with manufacture's instructions. The primary antibodies used: rabbit polyclonal Anti-MMP-2 (ab97779), Anti-MMP-9 (ab38898), Anti-Collagen I (ab34710), NF-Kb P65 (A) (SC-109), IL-6 (M-19)-R (SC-1265-R), TGF-β1 (sc-146), Smad2/3 (sc-8332) and mouse monoclonal Anti-MMP-1 (NBP2-22123), α-SMA (sc-53142) .

2.8. Transmission Electron Microscopy Technique (TEM)

For transmission electron microscopy, the glutaraldehydefixed liver samples were washed with 0.1 M phosphate buffer and postfixed in 2% osmic acid (Sigma-Aldrich, St Louis, Missouri) in 0.15 M phosphate buffer. Dehydration was performed in acetone and embedded in the epoxy embedding resin (. Sections of 60 nm were made on Leica EM UC7 ultramicrotome and analyzed with a Tecnai 12 Biotwin transmission electron microscope.

2.9. Quantitative gene expression analysis by RT-PCR technique

Messenger RNA expressions of Samd2, 3, 7, α-SMA, TGF-β1, TIMP-1, MMP-1, -2, -3, -9, Col-1, TNF-α, IL-6, NF-kB50, and NF-Kb65 were determined using real-time quantitative polymerase chain reaction (qPCR).

2.10. Western-blot assay

In our study we used primary antibody rabbit polyclonal TIMP-1 (GTX53226), Anti-MMP-2 (ab97779) and Anti-MMP-9

(ab38898) that were diluted with blocking buffer 1:50n or 1:100 according to manufacturer's instructions.

2.11. Specific biochemistry tests

2.11.1. Measurement of lipid peroxidation

The content of hepatic malondialdehyde (MDA) was measured in order to estimate lipid peroxidation. The fluorimetric technique was based on the reaction of MDA with thiobarbituric acid. Relative fluorescence units read at FP-6300 JASCO spectrofluorometer (lex = 520 nm; lem = 549 nm) were transformed to nmol MDA using 1,1,3,3-tetramethoxypropane as standard. The values were normalized to the protein concentration and shown as percentages of control.

2.11.2. Assessment of reduced glutathione (GSH) level

The cell lysates were deproteinized with 5% sulfosalicylic acid and the GSH content was spectrophotometrically measured, using the commercial glutathione assay kit (Sigma-Aldrich), based on the reduction of 5,50-dithiobis-2-nitrobenzoic acid (DTNB) into 5-thio-2- nitrobenzoic acid (TNB). The absorbance was recorded at 405 nm using the FlexStation 3 multi-mode microplate reader and the concentration was calculated in nmoles/mg protein, presented as a percentage of control.

2.11.3. Determination of advanced oxidation protein products (AOPP) concentration

The AOPP concentration was assessed as previously described. A volume of 200 mL protein extract was incubated with 10 mL of 1.16 M potassium iodide and 20 mL of glacial acetic acid. The optical densities were read at 340 nm in a 96-well plate using the FlexStation 3 multi-mode microplate reader and the AOPP levels were calculated using a chloramine-T standard curve and reported to the protein concentration.

2.11.4. Measurement of carbonyl groups content

The concentration of carbonyl groups was assessed. Briefly, the diluted total protein extract was incubated with an equal volume of 10 mM 2,4-dinitrophenylhydrazine for 1 h at room temperature, and after that, 20% TCA was added in a volume equal to previous solution. The mixture was incubated for 30 min on ice and centrifuged for 3 min at 13,000 rpm at room temperature in order to obtain the pellet which was washed with ethanol: ethyl acetate (1:1) solution. Finally, the pellet was rendered soluble in 500 mL of 1MNaOH, and the absorbance was read at 370 nm and reported to the protein concentration of each sample.

2.12. Statistical analysis

Statistical analysis was conducted with one-way ANOVA using Stata 13 software (StataCorp LP, Texas, USA). A value of $p<0.05$ was considered to be statistically significant.

3. THE PREPARATION AND CHARACTERIZATION OF SILYMARIN/ HPBCD AND RAMEB β-CYCLODEXTRINS COMPLEXES

3.1. Phase-solubility analysis of silymarin/ β-cyclodextrins complexes

Phase-solubility profiles were determined to characterize the effect of cyclodextrins on silymarin solubility. **Fig. 1** shows the solubility profiles of silymarin in the presence of HP-β-CD and RAMEB. Both cyclodextrin derivatives were able to improve the solubility of silymarin with increasing cyclodextrin concentrations. According to the solubility enhancement, RAMEB and HPBCD have similar ability to improve silymarin solubility in water.

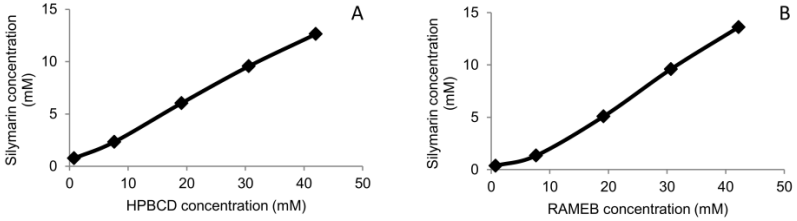

Fig. 1: Phase-solubility diagrams of Sy-HPBCD (A) and Sy-RAMEB (B).

3.2. Characterization of silymarin/ β-cyclodextrins complexes by SEM analysis

After the complexation process the original cyclodextrin and silymarin particles cannot be identified, but aggregates containing smaller particles reveal the interaction between silymarin and cyclodextrins. The newly formed structure contributes to the higher solubility and improved bioavailability of silymarin (**Fig. 2**)

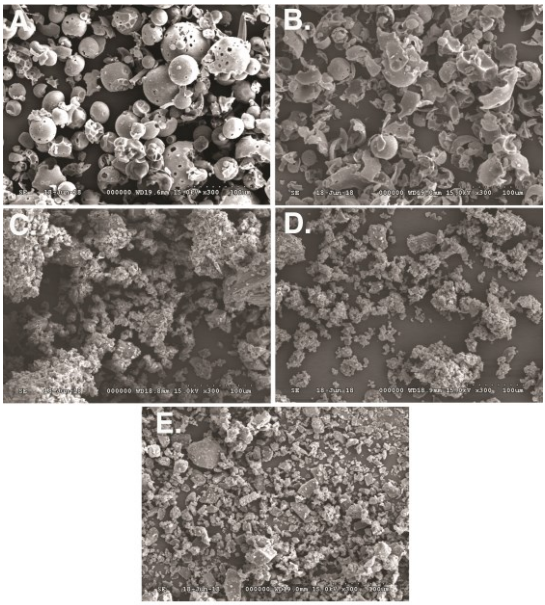

Fig. 2: Scanning electron microscopy (SEM) images of HPBCD (A), RAMEB (B), silymarin-HPBCD complex (C), silymarin-RAMEB complex (D), free silymarin (E).

4. HPBCD AND RAMEB ENHANCES ANTI-FIBROTIC EFFECTS OF SILYMARIN THROUGH ANTI-OXIDANT ACTIVITY AND INFLAMMATORY PATHWAYS DOWN-REGULATION, IN A MOUSE MODEL OF LIVER FIBROSIS

4.1. Sy-HPBCD and Sy-RAMEB complexes reduce oxidative injury and increase antioxidant enzymes activities

In this study, we found that treatment with silymarin complexation with cyclodextrin (Sy-HPBCD or Sy-RAMEB complexes) improved the anti-oxidant efficacy of silymarin against CCl4-induced liver fibrosis in mice.

Table 1. The values of MDA, GSH, carbonyl groups and AOPP concentrations after the treatment with CCl4 and various forms of silymarine. Data are expressed as mean ± SD (n = 5). **p<0.01 and ***p<0.001 versus control; #p<0.05, ##p<0.01 and ###p<0.001 versus CCl4.

Animal group	Control	CCl_4	CCl_4 control	CCl_4/ Sy-RAMEB	CCl_4/ Sy-HPBCD	CCl_4/ Sy
MDA (nmoles/ mg protein)	0.036 ± 0.012	0.099 ± 0.022 ***	0.065 ± 0.025 #	0.039 ± 0.021 ###	0.036 ± 0.014 ###	0.057 ± 0.015 ##
GSH (nmoles/ mg protein)	1.59 ± 0.46	0.81 ± 0.25 **	1.13 ± 0.19 ##	1.37 ± 0.34 ##	1.67 ± 0.49 #	1.20 ± 0.26 #
Carbonyl groups (nmoles/ mg protein)	9.55 ± 0.59	13.10 ± 0.90 ***	9.70 ± 1.68 ###	9.4 ± 0.99 ###	9.46 ± 1.83 ###	10.33 ± 0.72 ###
AOPP (nmoles/ mg protein)	55.88 ± 34.3	119.1 ± 25.0 **	76.80 ± 57.8 #	61.42 ± 39.3 ##	57.54 ± 26.6 ###	64.91 ± 19. ##

4.2. Sy-HPBCD and Sy-RAMEB complexes down-regulate NF-kB Signaling and inflammatory cytokines expression

Significant increase in NF-kB p50, NF-kB p65, TNF-a and IL-6 mRNA expressions were detected in CCl_4-induced liver fibrosis in mice, compared to control (**Fig. 3A,D,E and F**). Fourteen days of daily Sy-HPBCD or Sy-RAMEB oral administration induces significant down-regulation of all genes compared to CCl4 group ($p < 0.001$). The anti-inflammatory activity of Sy-HPBCD has been more highlighted.

Therefore we tracked activation of NF-kB p65 in livers by immunohistochemistry analysis (**Fig. 3B**). In CCl_4 and CCl_4 control groups the p65 protein concentrated in the nucleus (**Fig. 3C**), whereas in silymarin-cyclodextrin treated groups, the expression decreased.

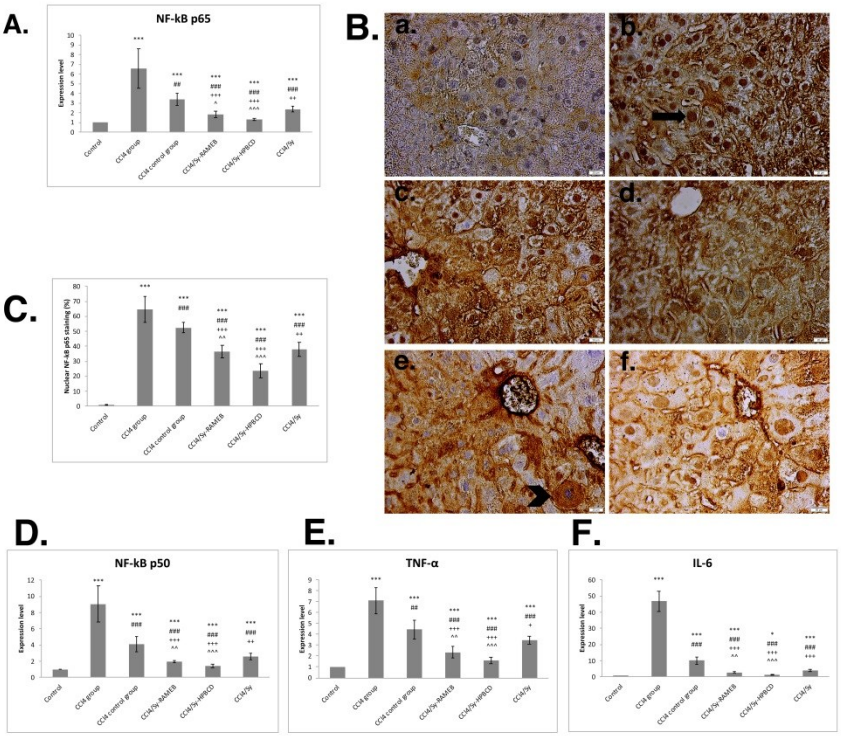

Fig. 3: Effects of Sy-HPBCD and Sy-RAMEB complexes on nuclear translocation of NF-kB in hepatocytes and inflammatory cytokines down-regulation in liver fibrosis. mRNA expression of NF-kB p65 (A), NF-kB p50 (D), TNF-a (E) and IL-6 (F); Immunohistochemical staining of NF-kB p65 (B); arrow – hepatocytes with only nuclear NF-kB p50 staining; arrowhead hepatocytes with only cytoplasmic NF-kB p50 staining. The percentage of hepatocytes with only nuclear NF-kB p65 staining out of the total number of hepatocytes was calculated (C). Data are expressed as the arithmetic mean _ standard deviation (SD) of five mice per group; $p < 0.001$ compared to control; ###$p < 0.001$ compared to CCl4 group; ##$p < 0.01$ compared to CCl4 group; CCl4< 0.001 compared to CCl4 control group; CCp <0.01 compared to CCl4 control group; ^^$p < 0.01$ compared to CCl4/Sy group; ^^^$p < 0.001$ compared to CCl4/Sy group. Cp < 0.05 compared to CCl4 group; $p < 0.05$ compared to control.

5. HPBCD AND RAMEB ENHANCE ANTI-FIBROTIC EFFECTS OF SILYMARIN THROUGH DOWN-REGULATION OF TGF-BETA PATHWAY AND INHIBITION OF HEPATIC STELLATE CELLS (HSC) IN A MOUSE MODEL OF LIVER FIBROSIS

5.1. Silymarin/cyclodextrin complexes alleviate CCl_4-induced structural changes in fibrotic livers

Our histopathological analysis suggest that, after 7 weeks of fibrosis induction, daily oral administration of both silymarin/cyclodextrin complexes for two weeks improves the state of steatosis and also suppresses hepatic fibrogenesis by reducing the thickness of bridging fibrotic septa. Silymarin/cyclodextrin complexes decrease the areas of hepatic fibrosis.

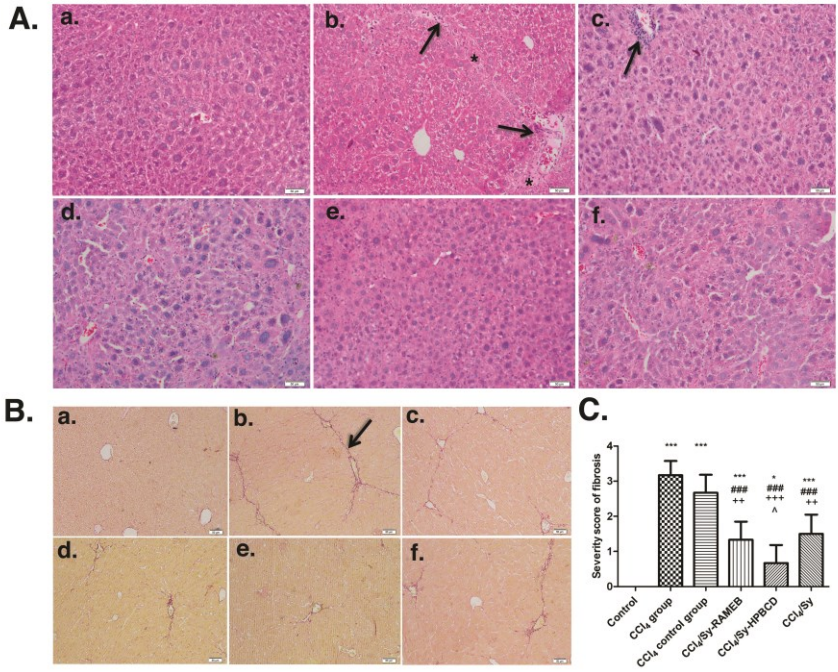

Fig 4: Effect induced by Sy-HPBCD and Sy-RAMEB complexes on the histological changes in liver of CCl4-treated mice. (A) H&E. (B) Fouchet van Gieson trichrome. (C) Histogram showing the percentage area of Fouchet van Gieson staining of collagen. $*p < 0.05$ compared to control; $***p < 0.001$ compared to control; $###p < 0.001$ compared to CCl4 group; $++p < 0.01$ compared to CCl4 control group; $+++p < 0.001$ compared to CCl4 control group; $\hat{}p < 0.05$ compared to CCl4/Sy group.

5.2. Sy/HPBCD and Sy/RAMEB inclusion complexes decrease the expression of α-SMA

CCl_4 stimulated HSCs activation in our mouse model, demonstrated by a significant increase of α-SMA immunoreactivity and mRNA up-regulation. Both HPBCD/Sy and RAMEB/Sy complexes down-regulated the expression of α-SMA, thus providing evidence for the deactivation of the HSCs as key cells in the progression of liver fibrosis.

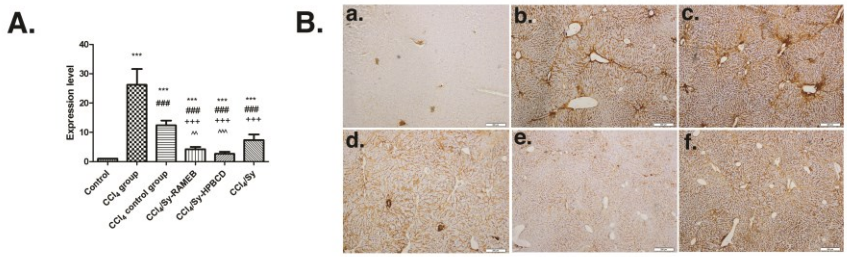

Fig. 5: The mRNA expression and specific tissue distribution of α-SMA. (A) RT-PCR analysis of α-SMA gene level. ***p < 0.001 compared to control; ###p < 0.001 compared to CCl4 group; +++p < 0.001 compared to CCl4 control group; ^^p < 0.01 compared to CCl4/Sy group; ^^^p < 0.001 compared with CCl4/Sy group. (B) Immunohistochemical expression of α-SMA in experimental livers. (a) Control group, (b) CCl4-treated group, (c) CCl4-control group, (d) CCl4 and Sy-RAMEB co-treated group, (e) CCl4 and Sy-HPBCD co-treated group, (f) CCl4 and free Sy co-treated group.

5.3. Sy/HPBCD and Sy/RAMEB inclusion complexes down-regulate TGF-β1/Smad signaling pathway

Our results showed a significant increase of gene expression and TGF–β1 immunohistochemical expression in CCl_4 group ($p<0.001$) that remained at the highest value after cessation of CCl_4 administration (CCl_4 control group). HPBCD/Sy and RAMEB/Sy administration for 14 days down-regulated the TGF- β1 and Smad 2 and 3 gene expression and up-regulated the Smad7 significantly, compared to free silymarin group. These data suggest that Silymarin complexes reduced activated HSCs and rebalanced TGF-β/Smad signaling.

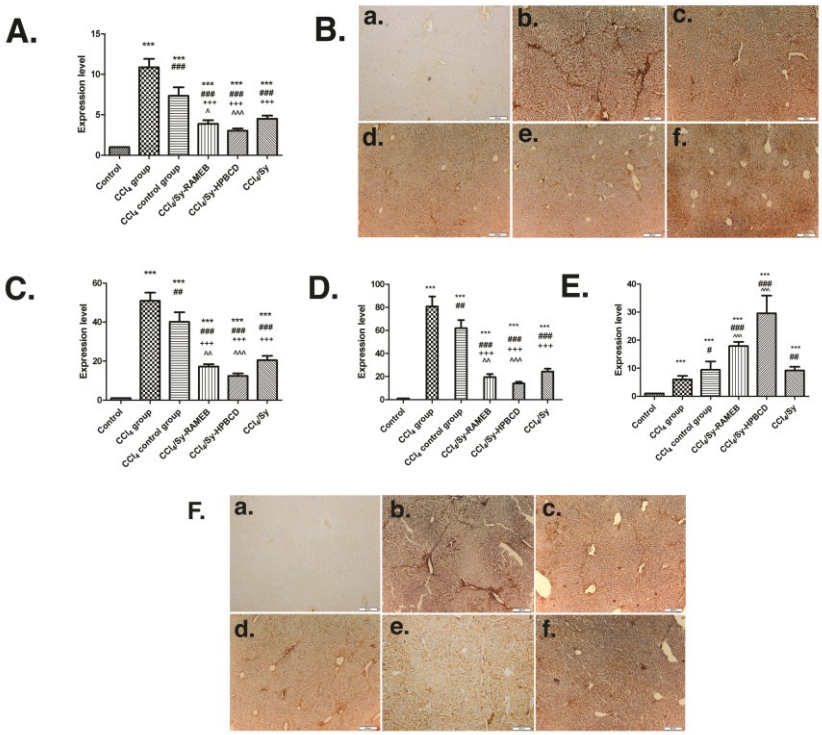

Fig. 6: Effects of Sy-HPBCD and Sy-RAMEB complexes on TGF-β1/Smad signaling pathway. (A) RT-PCR analysis of TGF-β1 gene level. ***p < 0.001 compared to control; ###p < 0.001 compared to CCl4 group; +++p < 0.001 compared to CCl4 control group; ^p < 0.05 compared to CCl4/Sy group; ^^^p < 0.001 compared to CCl4/Sy group. (B) Immunohistochemical expression of TGF-β1 in experimental livers. (a) Control group, (b) CCl4-treated group, (c) CCl4-control group, (d) CCl4 and Sy-RAMEB co-treated group, (e) CCl4 and Sy-HPBCD co-treated group, (f) CCl4 and free Sy co-treated group; RT-PCR analysis of Smad 2 (C), Smad 3 (D), and Smad 7 (E) gene levels. ***p < 0.001 compared to control; #p < 0.05 compared to CCl4 group; ##p < 0.01 compared to CCl4 group; ###p < 0.001 compared to CCl4 group; +++p < 0.001 compared to CCl4 control group; ^^p < 0.01 compared to CCl4/Sy group; ^^^p < 0.001 compared to CCl4/Sy group. (F) Immunohistochemical expression of Smad 2/3 in experimental livers. (a) Control group, (b) CCl4-treated group, (c) CCl4-control group, (d) CCl4 and Sy-RAMEB co-treated group, (e) CCl4 and Sy-HPBCD co-treated group, (f) CCl4 and free Sy co-treated group.

6. HPBCD AND RAMEB ENHANCE ANTI-FIBROTIC EFFECTS OF SILYMARIN THROUGH REGULATION OF EXTRACELLULAR MATRIX REMODELING

6.1. Sy/HPBCD and Sy/RAMEB complexes down-regulate Col-1 and decrease expression and distribution of these proteins in hepatic tissue

CCl_4-induced liver fibrosis was associated with a marked up-regulation of Col 1 gene expression ($p<0.001$). Compared to CCl_4 group, the mRNA Col 1 levels for Sy-HPBCD or Sy-RAMEB groups were significantly reduced by about 18,65 fold, respectively 11,59 fold compared to control, whereas, in the CCl_4 control group (group 3), its level was found significantly higher compared to all flavonoid co-treated groups ($p<0.001$). The protective response of Sy-HPBCD and Sy-RAMEB groups was higher with 35.87%, respectively 57.87% than free Sy co-treatment (**Fig. 7**).

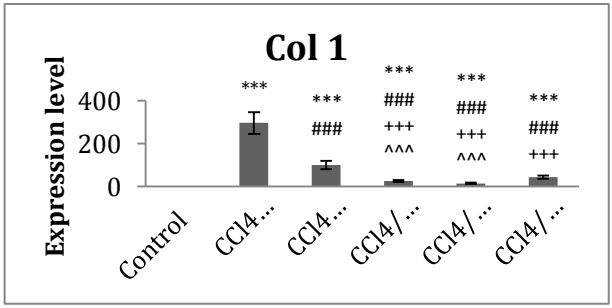

Fig. 7: mRNA levels of Col-1 in liver tissue of experimental mice. ***p<0.001 compared to control; ### p<0.001 compared to CCl4 group; +++p<0.001 compared to CCl4 control group; ^^^p<0.001 compared to CCl4/Sy group

Immunohistochemical expression of Col 1 in the control group was detectable mainly in perilobular areas (**Fig. 8-a**). The CCl_4 administration significantly induced Col 1 immunoreactivity within the fibrotic septa (**Fig. 8-b**). The same pattern was

observed in CCl_4 group after two weeks since the interruption of the treatment (**Fig. 8-c**). Expression was almost withdrawn after 50 mg/kg Sy-HPBCD or Sy-RAMEB administration for 2 weeks (**Fig. 8-e and d**). Free silymarin treatment significantly reduced hepatic Col I expression (**Fig. 8-f**), but not at the level of cyclodextrin complexes.

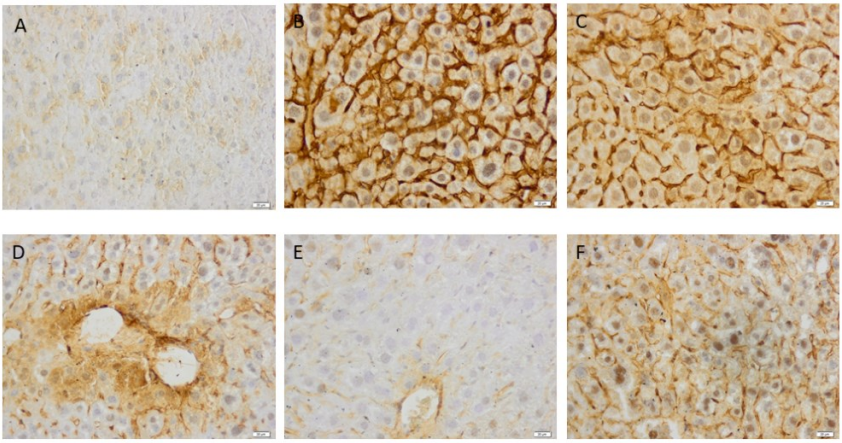

Fig. 8: Immunohistochemical expression of Col 1 in experimental livers. (a) Control group, (b) CCl4-treated group, (c) CCl4-control group, (d) CCl4 and Sy-RAMEB co-treated group, (e) CCl4 and Sy-HPBCD co-treated group, (f) CCl4 and free Sy co-treated group.

The efficiency of silymarin was increased when included in cyclodextrin (Sy/HPBCD and Sy/RAMED complexes). The administration of HPBCD/Sy and RAMED/Sy for two weeks down-regulated the collagen I gene expression compared to free silymarin, and this is evidence that HPBCD and RMBCD are able to improve silymarin efficiency and to control collagen deposition. The expressions of collagen I genes in free silymarin group was significantly reduced but still higher than those observed in control mice and Sy/HPBCD and Sy/RAMED treated groups.

6.2. Sy/HPBCD and Sy/RAMEB complexes down-regulate TIMP-1 and MMP-2, -3, and -9 and decrease expression and distribution of those proteins in hepatic tissue

In CCl_4 group, the mRNA levels of TIMP-1 was elevated significantly compared to control group ($p<0.001$). After two weeks of CCl_4-treatment cessation, TIMP-1 mRNA expression was reduced by about 2.97 fold compared to CCl4 group, while Sy-HPBCD and Sy-RAMEB treated groups reveled decreased levels by about 18.65 fold respectively 11.9 fold compared to CCl_4 group. Free silymarin administration induced a reduction by about 5.82 fold compared to CCl_4 group (**Fig. 9 A and B**).

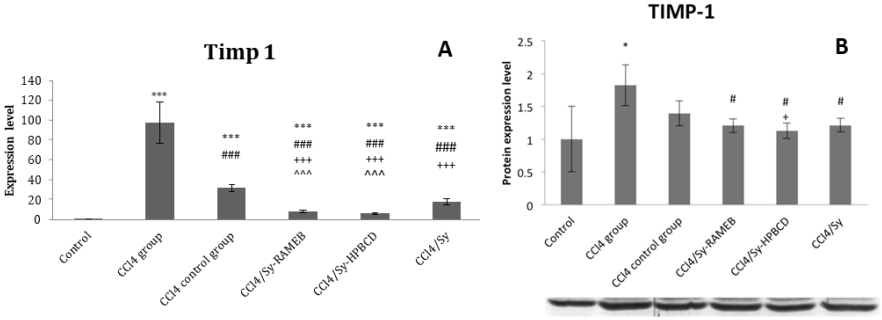

Fig. 9: Effects of Sy-HPBCD and Sy-RAMEB complexes on TIMP-1 in liver tissue of experimental mice. (**A**) mRNA levels of TIMP-1 in liver tissue of experimental mice. ***p<0.001 compared to control; ### p<0.001 compared to CCl_4 group; +++p<0.001 compared to CCl_4 control group; ^^^p<0.001 compared to CCl4/Sy group. (**B**) Western Blot analysis of TIMP-1 protein expression levels. *p<0.05 compared to control; # p<0.05 compared to CCl_4 control group; +p<0.05 compared to CCl4/Sy group

Sy-HPBCD and Sy-RAMEB treated groups down-regulate MMP-2 significantly ($p<0.001$) compared to CCl4-treated group. The decrease was by about 0.7 fold for Sy-HPBCD group, respectively 0.46 compared to free silymarin group (**Figs. 10 A, B and C**).

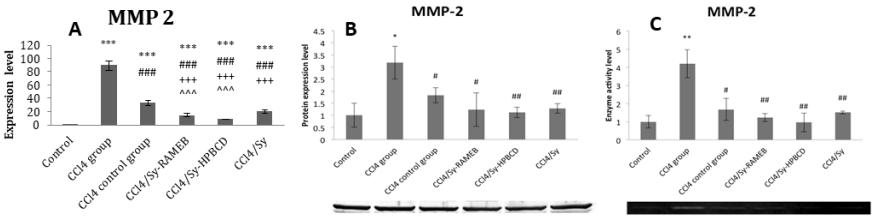

Fig. 10: Effects of Sy-HPBCD and Sy-RAMEB complexes on MMP-2 in liver tissue of experimental mice. (**A**) mRNA levels of MMP-2 in liver tissue of experimental mice. ***p<0.001 compared to control; ### p<0.001 compared to CCl_4 group; +++p<0.001 compared to CCl_4 control group; ^^^p<0.001 compared to CCl4/Sy group. **(B)** Western Blot analysis of MMP-2 protein expression levels. *p<0.05 compared to control; ## p<0.01 compared to CCl_4 group; # p<0.05 compared to CCl_4 group. **(C)** Zymography analysis of MMP-2 enzyme activity levels. **p<0.01 compared to control; ## p<0.01 compared to CCl_4 group; # p<0.05 compared to CCl_4 group

The effects for MMP-3 followed the same pattern as for MMP-2. Sy-HPBCD and Sy-RAMEB treated groups down-regulate MMP-2 significantly (p<0.001) compared to CCl4-treated group. The decrease was about 1.75 fold for Sy-HPBCD group, respectively 1.24 compared to free silymarin group (**Fig. 11**).

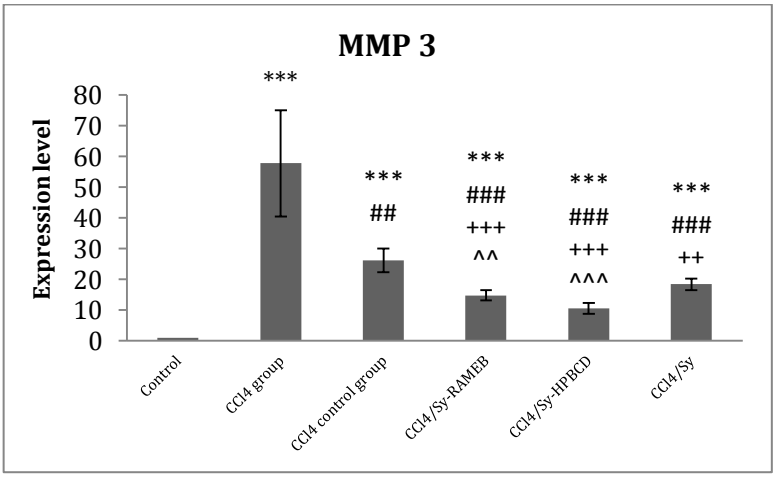

Fig. 11: mRNA levels of MMP-3 in liver tissue of experimental mice. ***p<0.001 compared to control; ### p<0.001 compared to CCl_4 group; ## p<0.01 compared to CCl_4 group; +++p<0.001 compared to CCl_4 control group; ++p<0.01 compared to CCl_4 control group; ^^^p<0.001 compared to CCl4/Sy group; ^^p<0.01 compared to CCl4/Sy group

In CCl_4 group, the mRNA MMP-9 was up-regulated significantly compared to control group ($p<0.001$). After two weeks of CCl_4-treatment cessation, MMP-9 mRNA expression was reduced by about 1,53 fold compared to CCl4 group, while Sy-HPBCD and Sy-RAMEB treated groups were down-regulated by about 4.18 fold respectively 3.27 fold compared to CCl_4 group. Free silymarin administration induced a reduction by about 2.35 fold compared to CCl_4 group (**Figs. 12 A, B and C**).

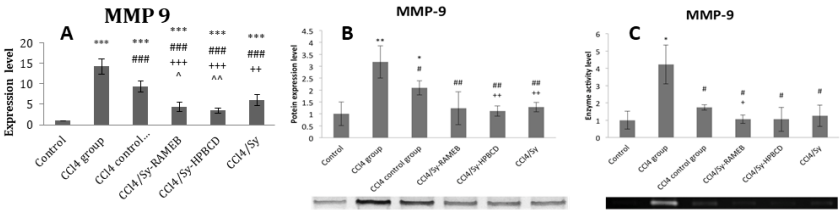

Fig. 12: Effects of Sy-HPBCD and Sy-RAMEB complexes on MMP-9 in liver tissue of experimental mice. (**A**) mRNA levels of MMP-9 in liver tissue of experimental mice. ***p<0.001 compared to control; ### p<0.001 compared to CCl_4 group; +++p<0.001 compared to CCl_4 control group; ++p<0.01 compared to CCl_4 control group; ^^p<0.01 compared to CCl4/Sy group; ^p<0.05 compared to CCl4/Sy group. **(B)** Western Blot analysis of MMP-9 protein expression levels. **p<0.01 compared to control; *p<0.05 compared to control; ## p<0.01 compared to CCl_4 group; # p<0.05 compared to CCl_4 group; ++p<0.01 compared to CCl_4 control group. **(C)** Zymography analysis of MMP-9 enzyme activity levels. *p<0.05 compared to control; #p<0.05 compared to CCl_4 group; +p<0.05 compared to CCl_4 control group

6.3. Sy/HPBCD and Sy/RAMEB complexes up-regulated MMP-1 and decrease expression and distribution of protein in hepatic tissue

Sy-HPBCD and Sy-RAMEB treated groups up-regulate gene expression of MMP1 by about 411.13%, respectively 251.09% compared to CCl_4 group. Free silymarin administration induced an increase by about 209, 15% compared to CCl_4 group (**Fig. 13**).

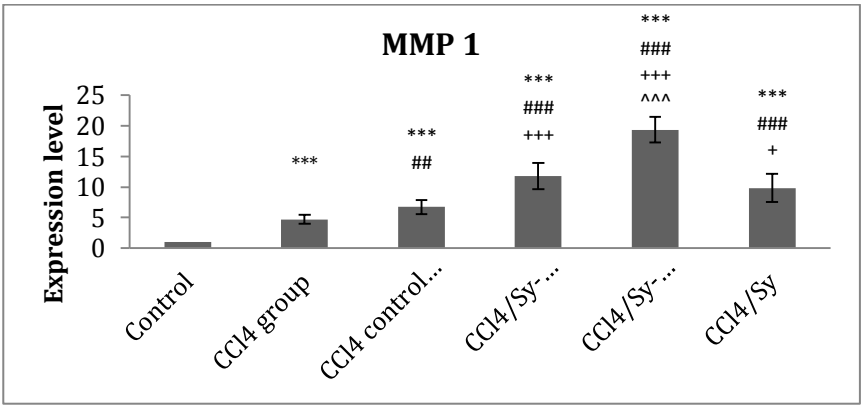

Fig. 13: mRNA levels of MMP-1 in liver tissue of experimental mice. ***p<0.001 compared to control; ### p<0.001 compared to CCl_4 group; ## p<0.01 compared to CCl_4 group; +++p<0.001 compared to CCl_4 control group; +p<0.05 compared to CCl_4 control group; ^^^p<0.01 compared to CCl4/Sy group

Immunohistochemical expression of MMP-1 in the control group was poorly detectable (**Fig. 14-a**). The CCl_4 administration significantly induced MMP-1 immunoreactivity within the fibrotic septa (**Fig. 14-b**). The same pattern was observed in CCl_4 group after two weeks since treatment cessation (**Fig. 14-c**). Expression was almost withdrawn on 50 mg/kg Sy-HPBCD or Sy-RAMEB administration for 2 weeks (**Fig. 14-e and d**). Free silymarin treatment significantly reduced hepatic MMP-1 expression (**Fig. 14-f**), but not at the level of cyclodextrin complexes.

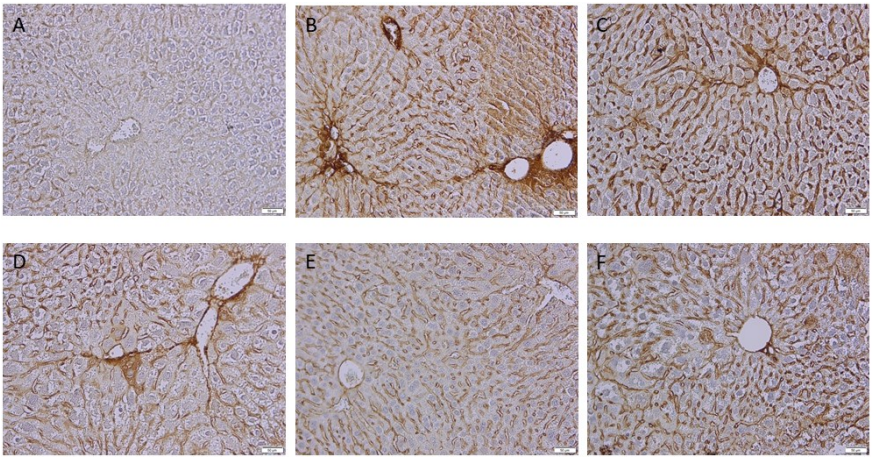

Fig. 14: Immunohistochemical expression of MMP-1 in experimental livers. (a) Control group, (b) CCl4-treated group, (c) CCl4-control group, (d) CCl4 and Sy-RAMEB co-treated group, (e) CCl4 and Sy-HPBCD co-treated group, (f) CCl4 and free Sy co-treated group.

In our study, mRNA expression of Col I and collagen accumulation were significantly increased in CCl_4-treated mice, whereas Sy/HPBCD and Sy/RAMEB treatment significantly down-regulated Col I. Both of Sy/HPBCD and Sy/RAMEB complexes can prevent collagen accumulation caused by the chronic- induced injury and alleviate the development of liver fibrosis.

Our results showed down-regulation of TIMP-1 and an up-regulation of MMP-1 during fibrosis resolution, and further stimulated cleavage of the fibrillar collagens, especially Col-I, by regulating the extracellular balance via TIMP-1/MMP-1 components.

The genes expressions remained at the highest value after cessation of CCl_4 administration (CCl_4 control group) and compared to the control group. Sy/HPBCD and Sy/RAMED administration for two weeks down-regulated MMP-2,-3,-9 and TIMP-I genes expressions, as well as significantly up-regulated

the MMP-1 and it was higher compared to free silymarin group. We demonstrated that Silymarin/Cyclodextrin complexes reduce the activity of the HSCs and fibroblast cells and therefore there were progresses registered in the remodeling process through the expression of genes that have a role in hepatic fibrosis such as MMP-1 by western blotting and RT-PCR.

7. Sy/HPBCD and Sy/RAMEB ALLEVIATES ULTRASTRUCTURE OF FIBROTIC LIVERS

The ultrastructure of hepatocytes has a normal aspect in the control group, including nuclear shape and rER's profiles, normal glycogen deposits and few lipid droplets into cytoplasm (**Fig. 15-a**).

Electron microscopy micrographs of fibrotic group (CCl4 group) highlight dense bundle of collagen fibers proliferating in the parenchyma, in the space of Disse and between swollen profiles of a sinusoid endothelial cell (**Fig. 15-b**), and being maintained in self-recovery group (CCl4-control group) (**Fig. 15-c**). Proliferation of smooth endoplasmic vesicles was also present. The ultrastructure of livers was alleviated on Sy-HPBCD, Sy-RAMEB and Sy-treated livers (**Fig. 15-e,d and f**).

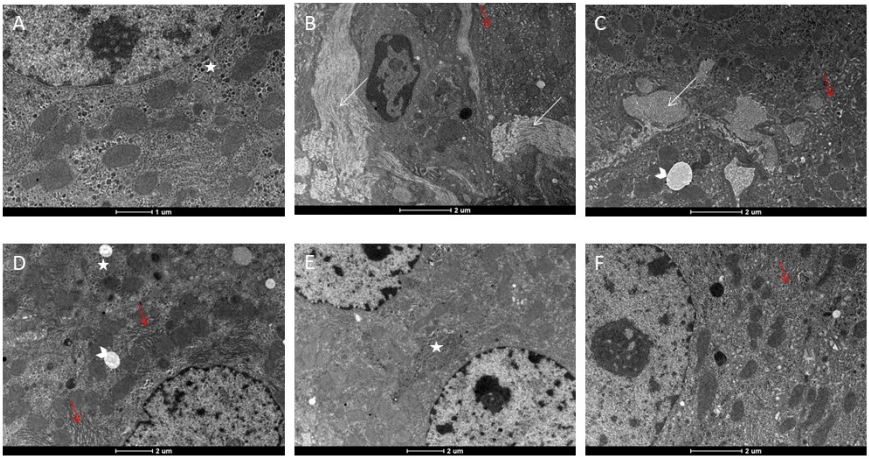

Fig. 15: Effects of Sy-HPBCD and Sy-RAMEB complexes on collagen deposition in experimental livers by electron microscopy. (a) Control group, (b) CCl4-treated group, (c) CCl4-control group, (d) CCl4 and Sy-RAMEB co-treated group, (e) CCl4 and Sy-HPBCD co-treated group, (f) CCl4 and free Sy co-treated group; Accumulated collagen (arrow), glycogen (astrix), Lipid globules (arrowhead) and developed rER (red arrow).

One of the important functions of the liver is glycogen and lipid storage, as a source of energy. The administration of CCl_4 in mice for 7 weeks led to progression the liver fibrosis, alongside with the depletion of glycogen and lipid droplets from stellate cells. The treatment with silymarin recovered glycogen level after the administration of CCl_4.

In our study, liver ultrastructure has shown that Sy/HPBCD and Sy/RAMED complexes are able to alleviate ultrastructural changes caused by the administration of CCl_4. HPBCD/Sy and RAMED/Sy administration for two weeks markedly reduced the content of collagen and restore the glycogen to the normal conditions compared to the free silymarin. Free silymarin-treated mice have shown remarkable improvement and normalized architecture and these observations supporting as non-toxic flavonoid even at higher physiological doses and can be a good candidate for treating liver diseases securely.

CONCLUSIONS

Fibrosis is a reversible scarring response that occurs in almost all patients with chronic liver injury. In the last stages, hepatic fibrosis leads to cirrhosis, associated with nodule formation and organ contraction. While cirrhosis is considered irreversible, fibrosis can be modulated by arresting progression and/or by promoting fibrogenesis resolution [51]. The mechanism of live fibrosis resolution involves cessation of chronic damage, shifting the intrahepatic balance from inflammation to restoration, deactivation and elimination of myofibroblasts and extracellular matrix degradation [51].

Considering the ability of silymarin to treat liver chronic diseases and taking into acount their poor water solubility and oral-bioavailability, we have proposed to establish a new drug-delivery system proper for this flavonoid, in order to increase their solubility, oral-bioavailability and to enhance their biological properties regarding anti-fibrotic effects.

Silymarin was included successfully (1:1) in two types of drug delivery systems: 2-hydroxypropyl-β-cyclodextrin (HPBCD) and randomly methylated β-cyclodextrin (RAMEB), proved by complexation efficiency (CE), stability constant of 1:1 complex of silymarin formed with HPBCD and RAMEB ($K_{1:1}$) recording. The newly-formed structure contributes to the higher solubility and improved bioavailability of silymarin, proving to have improvement of dissolution calculated from the phase-solubility curves of silymarin complexed with HPBCD and RAMEB. SEM analysis revealed that after the complexation process, the original cyclodextrin and silymarin particles cannot be identified, but aggregates containing smaller particles highlights the

interaction between silymarin and cyclodextrins could be notices.

The complexes have proven better anti-fibrotic properties in fibrotic livers than pure sylimarin. We concluded that the curative effects are due to the following mechanisms involved in the reversal of hepatic fibrosis:

1. Anti-oxidant properties. It is well known that oxidative stress and succesive damage contributing to lipid peroxidation, represent one of the main key factors involved in the genesis and progression of liver fibrosis or cirrhosis.

The oxidative stress induced by CCl_4 in the liver was shown by the increased levels of MDA, carbonyl groups and AOPP, which were significantly higher compared to control group. In addition, a reduction by half of GSH content confirmed the CCl_4- liver toxicity. A period of 2 weeks of self recovery without any external intervention after the CCl_4 cessation, did not succeed to shift the values of oxidative markers near to those of control group. Sy-HPBCD formulation had the best ability to inhibit the negative action of CCl_4, the contents of protein and lipid oxidation products and levels of GSH being similar to control mice.

2. **Anti-inflammatory properties.** Inflammation is a physiological defence mechanism of the body against injury stimuli, essential to eliminate harmful stimuli and to facilitate tissue healing after injury. Failure to resolve, leads to chronic inflammation, extended tissue destruction and progressive fibrosis. Inflammation and fibrosis can thus be viewed as a continuum of events within the framework of tissue defence, repair and regeneration.

Our results showed a clearly resolution of liver inflammation after two weeks of Sy-HPBCD/RAMEB oral

treatment, through transcription factors inhibition and inflammatory cytokines down-regulation. These events stop amplifying the inflammatory response due to regulatory activities of hepatic stellate cells and portal myofibroblasts in the injured liver, by promoting recruitment of inflammatory cells and promotion of inflammatory cytokine release. Therefore, indirectly, anti-inflammatory effects noticed for both of silymarin/ β-cyclodextrins contribute to fibrosis resolution in hepatic parenchyma.

3. **Inhibition of hepatic stellate cells (HSCs).** Hepatic stellate cells (HSC) (or Ito cells) play a key role in the pathogenesis of fibrosis. Activated HSCs increase secretion of fibrillar collagen (collagen I and III) and show intense α-SMA immunoreactivity. Hence, α-SMA is an important marker of hepatic fibrosis used to identify activated HSCs, that show a myofibroblastic phenotype.

As expected, CCl_4 stimulated HSCs activation in our mouse model, demonstrated by a significant increase of α-SMA immunoreactivity and mRNA up-regulation. Both of HPBCD/Sy and RAMEB/Sy complexes down-regulated the expression of α-SMA, which is an evidence of deactivation of the HSCs, the key cells in the progression of liver fibrosis.

4. **Down-regulation of TGF-β1/Smad signaling pathway.** Because TGF-β/Smad signaling is a key pathway leading to liver fibrosis, we investigated the effects of HPBCD/Sy and RAMEB/Sy complexes oral administration in CCl_4-induced liver fibrosis resolution. Our study showed that Smad 2/3 is stimulatory as suggested by higher levels of Smad 2 and 3 in hepatic tissue and up-regulated expression of target genes in mice with CCl_4-induced liver fibrosis. Both of Sy/ β - cyclodextrin complexes down-regulated the TGF- β1, Smad 2 and Smad 3 gene

expression and up-regulated the Smad7 significantly, compared to free silymarin group. These data suggest that Silymarin complexes rebalance TGF- β/Smad signaling and block the main pro-fibrogenic pathway.

5. Regulation of extracellular matrix remodeling. In healthy liver, extracellular matrix homeostasis is sustained by a balance regulation of MMPs enzymes and their specific inhibitors, TIMPs. Upon chronic damage of liver tissue, HSCs become activated and differentiate into a myofibroblast-like phenotype. In activated HSCs, expression of TIMP-1 is especially upregulated, leading to the inhibition of MMPs activity and subsequent induced accumulation of matrix proteins in the extracellular space. A substantial change in ECM composition is the deposition of collagens, mainly fibril-forming types -I, -III, and -IV which increase in fibrotic extracellular matrix up to tenfold [67]. Therefore, in liver matrix, MMPs and their specific inhibitors TIMPs, plays a central role in both, fibrogenesis and fibrolysis processes [68].

Our data showed up-regulation of Col I and MMPs expressions, which led to an altered extracellular matrix enriched in collagen after 7 weeks of CCl_4 toxic administration, confirmed as well by trichrome staining and electron microscopy analysis.

This study showed that both Sy-HPBCD and Sy-RAMEB were able to up-regulate mRNA and protein MMP-1 (interstitial collagenase) expression and further stimulate cleavage of the native fibrillar Col I, by regulating the EMC balance via TIMP-1/MMP-1 components. We also found that Sy-complexes abrogated CCl_4-induced MMP-2 and -9 up-regulation. The complexation of sylimarin with β -CD clearly contributed to the

attenuation of CCl_4- induced liver fibrosis in mice, as the free silymarin administration was not so efficient.

In conclusion we showed that Sy-HPBCD and Sy-RAMEB complexes decreased extracellular matrix accumulation by inhibiting HSC activation, reducing the oxidative damage and increased antioxidant defense system. Considering the mechanics, this might have occurred via the inhibition of the TGF-β1/Smad signal transduction, and MMPs/TIMP rebalance, by blocking the synthesis of Col I and decreasing the collagen deposition. These results suggest that the complexation of silymarin with HPBCD or RAMEB are viable options for its oral delivery, as a potential therapeutic candidate with applications in the treatment of liver fibrosis.

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LIST OF PUBLICATIONS
Articles published *in extenso* as a result of doctoral research

I. **Scientific articles published in indexed journals ISI Thomson Web of Science (Clarivate Analytics)**

Gharbia, S., Balta, C., Herman, H., Rosu, M., Varadi, J., Bacskay, I., Vecsernyes, M., Fenyvesi, F., Voicu, S.N., Stan, M., Cristian, R.E., Dinischiotu, A., Hermenean, A. 2018. Enhancement of silymarin anti-fibrotic effects by complexation with hydroxypropyl (HPBCD) and randomly methylated (RAMEB) β-cyclodextrins in a mouse model of liver fibrosis, *Frontiers in Pharmacology*, section Ethnopharmacology, Volume:9, Article Number: 883 , doi:10.3389/fphar.2018.00883, IF=3.831

Hermenean, A., Smeu, C., **Gharbia, S.**, Krizbai, I.A., Ardelean, A. 2016. Plant-Derived Biomolecules and Drug Delivery Systems in the Treatment of Liver and Kidney Diseases. *Current Pharmaceutical Design*, 22(35): 5415-5441, IF=3,052

II. **Papers presented at national and international conferences published in volumes of abstracts**

Gharbia, S., Balta, C., Herman, H., Ardelean, A., Fenyvesi, F., Hermenean, A., Antioxidant silymarin/cyclodextrin incusion complex against liver fibrosis, 8th CASEE Conference, May 14-16, 2017, Warsaw, Poland, Book of Abstracts, pp.35.

Gharbia, S., Balta, C., Herman, H., Anca Hermenean, A., Ferenc Fenyvesi, F., Silymarin-hydroxypropyl (HPBCD)/or -randomly methylated (RAMEB) β-cyclodextrin complexes attenuates liver fibrosis through TGF- β /Smad signaling pathway, 9th National Congress with International Participation and 35th Annual

Scientific Session of the Romanian Society for Cell Biology, June 7-11 2017, Iasi, Romania, Bulletin of Romanian Society for Cell Biology, no.45, pp. 25, 2017, Editura SEDCOM LIBRIS, ISSN:2392-7933.

Gharbia, S., Cornel Balta, C., Hildegard Herman, H., Rosu, M., Váradi, J., Ildikó Bácskay, I., Vecsernyés, M., FFenyvesi, F., Voicu, S.N., Stan, M.S., Cristian, R.E., Dinischiotu, A., Hermenean, A., Hydroxypropyl (HPBCD) and randomly methylated (RAMEB) β-cyclodextrin inclusion complexes enhances anti-fibrotic effects of silymarin in a mouse model of liver fibrosis, 10th National Congress with International Participation and 36th Annual Scientific Session of the Romanian Society for Cell Biology, June 6-9 2018, Craiova, Romania, Bulletin of Romanian Society for Cell Biology, no.46, pp. 26-27, 2018, Vasile Goldis University Press, ISSN:1584-5532.

Gharbia, S., Balta, C., Herman, H., Rosu, M., Voicu, S.N., Stan, M.S., Cristian, R.E.., Dinischiotu, A., Hermenean A., Silymarin/ β-cyclodextrins are potentially biomolecules for the alleviation of the liver fibrosis, 20th DKMT Euroregion Conference on Environment and Health, Sept 7-8 2018, Arad, Romania, Book of Abstract, pp.18, ISBN: 979-606-8875-39-2