Therapeutic effects of the traditional medicinal plant Ipomoea stolonifera for the treatment of liver diseases
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Chapter 1

General introduction

Traditional Chinese medicine for treatment of liver diseases

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The liver is the largest internal organ involved in metabolism and detoxification in humans. It metabolizes and stores nutrients (protein, glycogen, vitamins, lipids, cholesterol), it is responsible for the synthesis of bile acids from cholesterol and the synthesis of many plasma proteins and it detoxifies xenobiotics, such as drugs and alcohol [1, 2]. These activities can be grouped into (a) synthetic functions, (b) metabolic functions, (c) detoxification. Not surprisingly, impairment of the normal function of the liver can lead to serious disruption of homeostasis and liver diseases are therefore characterized by high morbidity and mortality. In traditional Chinese medicine, the heart is considered the ‘King’ or ‘Supreme Commander’ over body functions, whereas the liver is considered as the ‘General’ or ‘Long-range Planner’. These terms are paralleled in Western physiology, e.g. the liver plays a critical role in maintaining body energy (‘Qi’) and the balance between ‘Yin’ and ‘Yang’ [3, 4]. The ‘planning’ capacity of the liver can be translated to maintaining blood quality (detoxification, plasma protein synthesis), digestion and metabolism of carbohydrates (glucose), and storage of important nutrients (vitamins, lipids, glycogen). Sometimes it is difficult to translate the terminology from Chinese traditional medicine to Western physiology. In traditional Chinese medicine, the liver ‘has its opening in the eyes’. In ‘Neijing’, it is stated ‘liver qi communicates with the eyes’, suggesting that the eyes are closely linked to the liver [5]. This could be related to the fact that the eyes are nourished by liver (blood) and that the liver is the storage organ for vitamin A, a crucial vitamin in vision, e.g. vitamin A deficiency leads to (night) blindness. Furthermore, the eye is an indicator for liver function: yellow colorization of the eyes may reflect jaundice. Additionally, according to traditional Chinese medicine, the liver plays a central role in harmonizing the emotional flow and governing mental and spiritual functions [6]. Likewise, intense emotions like chronic rage, complaining, despair, jealousy often adversely affects liver function. Thus, a healthy liver is indispensable for normal body function, both from the Western and the Eastern perspective.

In humans, the liver is composed of two liver lobes and has a dual blood supply: oxygen-rich blood is supplied by the hepatic artery that accounts for 25% of the liver blood supply, whereas 75% of the blood supply comes from the gut via the portal vein [7]. The blood from the portal vein contains nutrients absorbed from the intestinal lumen, but also potentially harmful substances absorbed from the gut, e.g. toxins, pathogens, xenobiotics and gut-derived endotoxins. The liver is therefore the first organ exposed to toxic substances from the gut and therefore susceptible to damage induced by these noxious compounds. Fortunately, the liver is well-equipped to deal with these challenges: the liver is the primary site of detoxification of many toxic compounds and the clearance of pathogens and gut-derived endotoxins. Furthermore, the liver has a remarkable capacity to adapt and withstand various forms of injury through regenerative repair. The equilibrium between cell death, proliferation and differentiation is crucial for the maintenance of tissue homeostasis throughout life. Within the liver, various phenotypically distinct cell types are responsible for the maintenance of this delicate equilibrium. These cell
types include parenchymal cells (mainly hepatocytes and cholangiocytes) and non-parenchymal (mesenchymal) cells, including endothelial cells, stellate cells, Kupffer cells and lymphocytes (Fig. 1).

Figure 1. The location and structure of various cell types in the liver, which contains hepatocytes, liver sinusoidal endothelial cells, hepatic stellate cells, and resident hepatic macrophages (Kupffer cells), B and T cells, NK and NKT cells, hepatic dendritic cells (DCs). Some of cells participate in inflammatory and immune responses. Reprinted with permission from Steven E. Shoelson © 2006 American Society for Clinical Investigation [8].

First, we introduce these cell types and their functions in the liver.

**Parenchymal cells (hepatocytes and cholangiocytes)**

Within the liver there are two types of polarized epithelial cells: hepatocytes and cholangiocytes (also known as biliary epithelial cells). In terms of mass and number, the hepatocyte is the predominant liver cell type, comprising around 70% of the total number of cells in the liver and approximately 80% of the total liver mass [2, 9, 10]. Cholangiocytes line the bile ducts and modulate bile flow [11]. Both cell types, hepatocytes and cholangiocytes, are crucial for liver function and homeostasis. Damage to one of these cell types is the cause of most liver diseases leading to inflammation and fibrosis [12]. The hepatocytes are responsible for many liver-specific functions, such as intermediary metabolism, detoxification of toxic compounds, synthesis of bile acids and many plasma proteins, and generation of bile acid-dependent bile flow. In the healthy liver, very few of the hepatocytes are progressing through the cell cycle, the vast majority remains in a quiescent state (G0) [13]. However, hepatocytes retain the ability to re-enter the cell cycle in response to a liver insult, e.g. after partial resection of the liver [14] or severe liver injury [15]. In most liver diseases, in particular inflammatory liver diseases, it are mostly the hepatocytes that perish. Loss of hepatocytes occurs by cell death. The major types of cell death are apoptosis and necrosis although alternative forms of cell death also occur, e.g. autophagy. Although apoptosis and necrosis have clear definitions, it is
increasingly recognized that they represent two extremes of a continuum. Indeed, intermediate forms of cell death like necro-apoptosis (necroptosis) displaying features of both apoptosis and necrosis have been described [16]. Apoptosis, or programmed cell death, is characterized by cell shrinking, membrane blebbing, DNA condensation, nuclear fragmentation, and finally the formation of apoptotic bodies. Mitochondrial morphology remains intact and the cellular content remains confined and does not spill into the circulation [17, 18]. The morphological events are accompanied by the activation of specific proteases, called caspases, in particular the downstream effector caspases-3, -6 and -7, which cleave cellular structures and proteins resulting in cell death. In contrast, necrosis, or ‘passive’ cell death, is characterized by cell swelling, rupture of the plasma membrane with subsequent spilling of cellular content into the circulation, resulting in inflammation and mitochondrial swelling. Nuclear morphology remains intact [19]. Autophagy, or cellular self-digestion (Greek for “self-eating”), is cell death mediated by the lysosomal degradation of cellular components. Three subtypes of autophagy are distinguished: macroautophagy, microautophagy, and chaperone-mediated autophagy [20, 21]. At present, the exact causal role of autophagy to liver damage is not clear. For example, autophagy could act both as an agonist and as an antagonist of cell death depending on the experimental context. Although apoptosis is crucial for embryonic development and normal tissue homeostasis, including liver homeostasis, excessive apoptosis in liver diseases is detrimental. The predominant mode of hepatocyte cell death is dependent on the liver disease, e.g. necrotic cell death is the predominant mode of cell death in acute acetaminophen intoxication and many chronic liver diseases, whereas apoptotic cell death is observed in acute (viral) hepatitis and nonalcoholic steatohepatitis (NASH). Most liver diseases, however, display a mixed phenotype of cell death [22, 23].

Non-parenchymal cells

Macrophages

In the liver, macrophages can be broadly defined as either resident macrophages (Kupffer cells) or monocyte-derived macrophages [12], which are responsible for the elimination of pathogens (bacteria) and clearance of foreign compounds, e.g. apoptotic bodies by phagocytosis through a variety of receptors. Kupffer cells reside in the hepatic sinusoids, in close contact with other circulating immune cells, so they participate in local immune responses and are well-positioned to eliminate invading pathogens and remove obsolete cells (erythrocytes) or cell fragments (apoptotic bodies). Macrophages are also the driving force of inflammation in many liver diseases. Upon activation by various triggers (e.g. bacterial products) the macrophages, including the Kupffer cells, release pro-inflammatory cytokines (Tumor necrosis factor (TNF)-α and IL-1β), chemokines (CCL2 and CCL5), other inflammatory mediators (prostaglandins and leukotrienes) and reactive oxygen species (ROS). These secreted products influence not only the hepatocytes, but also stellate cells, portal myofibroblasts and other immune cells. In chronic liver diseases,
the continuous inflammatory state leads to a fibrogenic response, since the products released from Kupffer cells contribute to the activation, proliferation and survival of stellate cells and myofibroblasts, in part via the activation of the transcription factor Nuclear Factor-κB (NF-κB) in HSCs and myofibroblasts [24]. On the other hand, macrophages also possess a latent capacity to revive damaged tissue by secreting matrix metalloproteinases (MMPs) that degrade newly synthesized scar matrix. The protease activity of MMPs is inhibited by concurrent production of tissue inhibitors of metalloproteinases (TIMPs) by myofibroblasts and macrophages.

Hepatic stellate cells (HSCs)

Hepatic stellate cells (HSCs) account for 5%–8% of the cells in the liver and are located in the space of Disse, the space between the sinusoidal endothelial cells and the basolateral surface of hepatocytes [25, 26]. In the healthy liver, HSC contain large quantities of retinyl-esters packed in big lipid droplets that serve as the main storage site of vitamin A in the body. In liver fibrogenesis, the key event is the transdifferentiation of these “quiescent”, vitamin A-containing HSCs into proliferative myofibroblasts that progressively lose their vitamin A content. Liver myofibroblasts are derived mainly from the activation of quiescent HSCs, but may also arise from other sources, e.g. portal fibroblasts [27, 28] and bone marrow-derived cells [29, 30]. Although the exact initial events in the activation of HSCs are still not completely elucidated, various cytokines, growth factors and reactive oxygen species (ROS) are involved. Upon activation, the HSCs lose their vitamin A content and demonstrate increased proliferation, contractility, matrix production and pro-inflammatory signaling. The accumulation and enhanced density and cross-linking of hepatic extracellular matrix (ECM) components produced by myofibroblasts leads to fibrosis and is characterized by increased matrix stiffness, hampered blood flow through the liver, portal hypertension and complications like variceal bleeding. Regression of liver fibrosis can be achieved when the disease-causing factor(s) can be eradicated. Activated HSCs either undergo apoptosis or revert to a quiescent phenotype characterized by downregulation of markers of fibrosis such as type I collagen (col1a1) and alpha-smooth muscle actin (α-SMA) [31, 32]. Subsequently, other cell types in the liver contribute to the clearance of HSCs, e.g. apoptotic HSCs are phagocytized by macrophages and senescent HSCs are removed by natural killer cells [33-35]. Therefore, removal and/or “de-activation” of HSCs/myofibroblasts is increasingly recognized as an essential step towards the resolution of liver fibrosis.

Natural Killer (NK) cells and Natural Killer T-cells (NKT cells)

The immune system plays an important role in maintaining tissue homeostasis and dysregulation of this system may contribute to several liver diseases. In the context of liver diseases, NK cells and NKT cells are important. NK and NKT cells both contain a characteristic set of markers for NK cells (mouse: DX5 or NK1.1; rat: NKR-P1A; human: CD56 ) , while NKT cell, in addition, express T cell markers (like
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CD1) [7, 36, 37]. In acute or chronic liver injury, both cell types play an important role in the first-line innate defense against viral infection and tumor transformation, which is associated with many liver diseases [38, 39]. The distribution of NK and NKT cells is different in the livers of mice, rats and humans. NKT cells account for 30–40% of total lymphocytes in mouse liver and only 5-10% of total lymphocytes in rat and human liver [40]. Hepatic NK and NKT cells have many similar functions after activation, such as the production of pro-inflammatory cytokines (IFN-γ and IL-4) and the killing of virus-infected cells and tumor cells [7]. The major mechanism by which NK cells lead to hepatocyte cell death and liver injury is via the release of TRAIL and granzyme B, whereas NKT cells release predominantly FasL [41]. NK cells also appear to have a negative regulatory effect on fibrogenesis and have been reported to directly kill early-activated or senescent hepatic stellate cells [35, 42, 43]. This is probably mediated by IFN-γ, released by activated NK cells, that inhibits stellate cell activation and amplifies NK cell cytotoxicity against stellate cells [44-46]. On the other hand, IFN-γ is also a proinflammatory cytokine that induces hepatocyte cell-cycle arrest and apoptosis [47, 48]. Therefore, inhibition of the production of IFN-γ by NK cells and NKT cells could be beneficial for the survival of hepatocytes and allow hepatic regeneration, but could also promote fibrogenesis. Interestingly, experimental evidence suggests that IFN-γ that is specifically targeted to stellate cells suppresses liver fibrosis in mice [49].

Liver sinusoidal endothelial cells (LSEC)

Liver sinusoidal endothelial cells (LSEC) line the hepatic sinusoids. They have a unique morphological phenotype that is characterized by open (non-diaphragmed) fenestrae and a lack of basement membrane [50]. This morphology facilitates an optimal exchange of nutrients, oxygen and waste products between the sinusoidal blood and space of Disse. LSECs are also responsible for removing soluble macromolecular and colloidal waste products (smaller than 100 nm) [9, 51]. Furthermore, LSEC have the appropriate location, together with Kupffer cells, to play a role in the clearance of pathogens and viruses. Giugliano et al [52] reported the release of antiviral exosomes from the LSECs that amplify the antiviral activity of interferons in hepatocytes. Additionally, differentiated LSECs that maintain their normal fenestration and function prevent HSC activation. In cocultures of LSECs and HSCs, the presence of LSECs maintains the quiescent state of HSC [53]. Once LSEC differentiation is lost, they promote HSC activation and liver fibrosis. Therefore, differentiated LSECs have a function as gatekeeper in the fibrotic process [50].

Liver diseases

A number of different conditions (inflammatory disorders and metabolic disorders) affect liver functions, which are associated to multiple cell types discussed above. Traditionally, liver diseases are categorized into acute liver failure (ALF) and chronic liver failure (CLF) [54, 55]. The essential feature of ALF is the abrupt loss of hepatic function due to the loss of large numbers of hepatocytes [56]. This rare but sudden
syndrome in a patient with no history of liver disease, but causes severe complications (i.e. jaundice, coagulopathy, hepatic encephalopathy, and even multi-organ failure) [57]. Viral hepatitis remains the cause of a high proportion of cases of ALF, which is common caused by infections of virus A, B, C and E, and leads a high rate of death worldwide. And virus hepatitis predominates in developing countries more than in developed countries [58]. It is not optimistic that of the 350 million hepatitis B virus (HBV) carriers worldwide, one-third reside in China [59]. That means 780,000 people die every year due to complications of hepatitis B [60], the number in China is nearly a half reported by National Health and Family Planning Commission of China. Besides virus, toxins, drugs, immunological attacks and various chemicals could also cause hepatitis, and each of these stimulations could be possible to result in CLF.

CLF, a progressive and slow deterioration, often occurs in people with incurable chronic liver disease or stable cirrhosis. CLF leads to metabolic disorders of various toxins and presents irreversible chronic loss of liver function, which usually is accompanied by continuous inflammation [61]. Alcohol consumption has been globally identified as a major risk factor in liver diseases, such as alcoholic fatty liver disease (AFLD) and ASH [62]. The characteristic of non-alcoholic fatty liver disease (NAFLD) and AFLD are fat accumulation in the liver (steatosis) and with alcohol consumption 20-30 g/day (AFLD) and without any evidence of chronic liver diseases and lower alcohol drink (NAFLD). Furthermore, advanced stages of these two liver diseases are NASH and ASH [63]. The consequence and final stage of various CLF are generally liver fibrosis and liver cirrhosis [64]. Actually, fibrosis is the liver's protective response to injury, and this intrinsic reaction will protect the liver against further damage from the structure and functions of differential cell types. So the liver can be reversed by itself from fibrosis even cirrhosis to normal architecture when the insults removed or the causes treated. The successful reversion of fibrosis and cirrhosis should include the degradation of excess ECM [64], and matrix-metalloproteinases (MMPs) associated with tissue inhibitors of metalloproteinases (TIMPs) make main contributions to degrade excess ECM. But the objective situations usually do not look good. Accumulation of deposited extracellular matrix (types I and type IV fibrillar collagens), server liver injury and uncontrolled immune responses to inflammatory, will finally cause irreversible liver fibrosis characterized by cellular activation of HSCs (see above). And therefore, this is one main topic focused in this thesis. Cirrhosis is defined as fibrous scarring of the hepatic parenchyma resulting in nodule formation and collapse of liver structures. Now it is fourth cause of morbidity and mortality in central Europe [65]. Collectively, these are among the key initiating risk factors for two types of primary liver cancer: hepatocellular carcinoma (HCC) and cholangiocarcinoma (CCA) [66].

Additionally, in 1995, a third type of liver failure acute-on-chronic liver failure (ACLF) was described for the first time [67]. The definition of this new entity is based on patients with 3 key syndromes: liver cirrhosis presenting with acute decompensation, a high rate of organ failure and a high short –term of mortality [68,
Unfortunately, the classification of ACLF is still not universal defined so far. However, the further improvement concerning of ALF according to its prognosis will be beneficial to clinical managements on these patients.

Signal transduction in hepatic injury

Although the liver has a high regenerative capacity, the loss of viable and functional hepatocytes may lead to liver failure, depending on the type and severity of the injury. The massive and acute loss of a large proportion of functional hepatocytes, as in acute liver failure or fulminant hepatitis, may not be compensated by hepatocyte regeneration because of an inability of the hepatocytes to regenerate in these conditions or because the loss of hepatocytes is too extensive. On the other hand, in chronic liver failure there is a continuous, but more moderate loss of hepatocytes, which can be compensated by regeneration. However, in chronic liver injury, the continuous presence of an insult may result in sustained inflammation and fibrogenesis, eventually exceeding the regenerative capacity of hepatocytes and leading to liver failure. In addition, the continuous regenerative trigger may lead to hepatocellular carcinoma (HCC). To understand the pathogenesis of liver diseases we will now first review some of the key signaling mechanisms involved in liver inflammation and liver fibrosis.

Inflammatory cytokines, interferons and chemokines

In acute and/or chronic liver injury, the cellular wound-healing response is accompanied by the production of inflammatory cytokines and chemokines. The release of these soluble factors contributes to the wound healing response and the clearance of foreign substances and cell debris and is therefore beneficial. However, excessive and/or prolonged production of these factors may be detrimental to hepatocytes and also lead to uncontrolled activation and proliferation of stellate cells. In fact, some of the released cytokines, such as TNF-α, interleukin (IL)-1, and IL-6 play a role in the proliferative response of hepatocytes and induce the production of so-called acute phase proteins that are important in maintenance of homeostasis. Other cytokines, like FasL, TNF-α and other ligands of the so-called “death receptor family”, can induce apoptotic cell death in hepatocytes. Hence, TNF-α plays a crucial role in the inflammatory response of the liver, since it has both proliferative and cell protective effects by activating the transcription factor NF-κB and it has pro-apoptotic properties (when cells are unable to activate NF-kB) by activating death receptor pathways. Furthermore, TNF-α controls the production of many other cytokines. The biological effects of TNF-α are mediated by its receptors TNF receptor 1 (TNF-R1) and TNF-R2. Signaling by TNF-R1 and TNF-R2 is achieved by recruitment of adaptor proteins that bind to the cytosolic domains of the receptors (like MyD88) and initiate signal transduction events [70]. IL-1β is another potent inflammatory cytokine that signals through the IL-1 receptor 1 (IL-1R1), leading to an inflammatory cascade. Increased levels of IL-1β contribute to the progression of several chronic inflammatory liver diseases, including NASH and Alcoholic
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steatohepatitis (ASH).

Interferons (IFNs, IFN-α/β and IFN-γ) comprise a family of proteins, which interact with cells via distinct cellular receptors. Type II IFN (IFN-γ) is produced mainly by macrophages, NK cells, and T lymphocytes [71], while type I IFN is produced by fibroblasts, epithelial cells and hepatocytes [72]. IFNs have been tested as anti-fibrotic agents in patients with moderate fibrosis. It was demonstrated that IFN-γ displays anti-fibrotic effects and suppresses proliferation and α-SMA expression in HSCs. Furthermore, IFN-γ activates NK cells that in turn induce death of HSCs [73]. Additionally, IFN-β can inhibit the repeated hepatocellular injury and reduce liver fibrosis induced by Con A [74].

Chemokines are small proteins (8–13 kDa) and are categorized into 4 different families: CC, CXC, CX3C, C) [75]. It has been shown that chemokines and their receptors play a seminal role in the pathogenesis of various acute and chronic liver diseases. Chemokines, by virtue of their capacity to attract inflammatory and immune cells to sites of inflammation, not only drive inflammation and immune responses, but also participate in fibrogenesis and cancer [76]. CCL2 (monocyte chemotactic protein-1, MCP-1) is one of the best characterized chemokines. In the chronically inflamed liver, CCL2 is secreted by many different cell types [77, 78], but mainly by Kupffer cells and HSCs, which contribute to the recruitment and accumulation of macrophages and monocytes into the liver. During development of non-alcoholic fatty liver disease (NAFLD) and NASH, CCL2 and its receptor are upregulated in the liver, where it promotes hepatic and systemic inflammation related to metabolic disorders [79-81].

Transcription factor Nuclear Factor-κB (NF-κB)

NF-κB is a ubiquitous transcription factor that is activated by a variety of stimuli, including cytokines (TNF-α, Transforming growth factor (TGF)-β) and reactive oxygen species (ROS). NF-κB is a key regulator of many cellular responses involved in inflammation and stress in chronic liver diseases (e.g. viral hepatitis, liver fibrosis and NAFLD) [82]. The ‘classical’ NF-κB is a heterodimer of the subunits p50 (NF-κB -1) and p65 (Rel-A). Additional subunits exist giving rise to alternative homo- and heterodimers. Normally, NF-κB is retained in an inactive form in the cytoplasm through association with one of the IκB inhibitory proteins, including IKK-α or IKK-β, and this interaction blocks the ability of NF-κB to translocate to nucleus. Upon stimulation of appropriate receptors, e.g. binding of TNF-α to its receptor, the IκB kinase (IKK) complex is phosphorylated and activated. The IKK complex is composed of a regulatory subunit (IKK-γ) and two kinase subunits (IKK-α, IKK-β) that are responsible for the phosphorylation of IκB. Phosphorylation of IκB leads to its poly-ubiquitination and subsequent proteolytic degradation. These events allow the translocation of NF-κB from the cytoplasm to the nucleus where NF-κB acts by inducing the transcription of target genes [83]. The target genes of NF-κB include inflammation-related genes, like cytokines, including TNF-α itself and chemokines,
but also anti-apoptotic genes. Therefore, NF-κB has a pivotal role in the inflammatory response. Therefore, there is a tremendous, ongoing effort to identify and develop drugs that target NF-κB activity. However, since NF-κB is associated with pro-survival activity, the continuous activation of this transcription factor in inflammatory disorders might also predispose for an increased risk for hepatocellular carcinoma.

**c-Jun N-terminal kinase (JNK)**

C-Jun N-terminal kinases (JNKs), members of the mitogen-activated protein kinase (MAPK) family, are involved in multiple signaling cascades that lead to hepatocellular cell death. It is a pro-apoptotic kinase and is involved in hepatocellular injury in inflammatory, metabolic and fibrotic liver diseases. The liver expresses two JNK isoforms, JNK1 and JNK2, but not JNK3 [84]. Cross-talk exists between JNK and TGF-β and platelet-derived growth factor PDGF-mediated Smad 2/3 signaling [85]. In addition, the interplay between NF-κB and JNK has been extensively investigated and reviewed [70, 86, 87]. Importantly, it has been demonstrated that NF-κB generates its survival signals in part via inhibition of the prolonged activation of JNK by TNF-α [70], and in turn, activation of JNK is prolonged in NF-kB-deficient cells [88].

**Toll-Like Receptors (TLRs)**

Toll-Like Receptors (TLRs) are essential receptors in the host defense against pathogens in the early innate immune response. TLRs have the ability to recognize highly conserved structural motifs known as pathogen-associated microbial patterns (PAMPs), which include various bacterial cell wall components, such as lipopolysaccharide (LPS), peptidoglycan (PGN) and lipopeptides. LPS, the TLR4 ligand, induces inflammatory signals in multiple cell types in the liver. The activation of TLR4 on Kupffer cells leads to the production of many proinflammatory cytokines, including TNF-α, IL-1β, CCL2 and CCL20 [89-91]. In addition, TLR4 signaling also modulates TGF-β-mediated HSC activation in liver fibrosis [92, 93]. Several therapeutic agents targeting the TLRs are now under pre-clinical and clinical evaluation. However, given the complexity of signaling and the multitude of TLRs, interventions in these pathways may act as double-edged swords either promoting or inhibiting disease progression.
Liver injury leads to necrotic and/or apoptotic cell death of parenchymal cells. Phagocytosed cell debris leads to activation of macrophages and HSCs, which is amplified by the release of cytokines and reactive oxygen species. These pro-inflammatory mediators also recruit T cells, neutrophils and lymphocytes, amplifying the inflammatory response. Increased gut permeability, e.g. during acute inflammation, leads to increased translocation of gut-derived endotoxins, via the portal blood, to the liver. In the liver, endotoxins and other bacterial products bind to CD14 and TLR4 receptors on Kupffer cells, triggering an inflammatory response, including the activation of stress-activated and mitogen-activated protein kinase (p38, JNK), and NF-κB [94]. Among the secreted pro-inflammatory and pro-fibrotic mediators is TGF-β, one of the key factors driving fibrogenesis. TGF-β is mainly

Figure 2. Cascade of signals following liver injury CCL2 and CCR2: CC-chemokine ligand 2; (CCL2)–CC-chemokine receptor 2. IL, interleukin; TNF, tumor necrosis factor. Reprinted with permission from Antonella Pellicoro © 2014 Nature Publishing Group [12].
produced by inflammatory and immune cells and directly promotes liver fibrosis via stimulation of HSC transdifferentiation into myofibroblasts, a process characterized by increased expression of \( \alpha \)-SMA and collagen type I. The growth factor PDGF also stimulates proliferation of myofibroblasts. Reversal of matrix deposition and scar accumulation is regulated by MMPs and its natural inhibitors (TIMPs). Upon removal or termination of the underlying triggers for liver injury (alcohol, fat, hepatitis viruses), the liver is, to some extent, capable to regenerate and resolve fibrosis [95-97].

**Therapeutic drugs for liver diseases**

Liver diseases are difficult to treat by medication. Yet, several drugs are now prescribed for various liver diseases: for NASH and NAFLD, statins, such as pravastatin and atorvastatin, are the most prescribed drugs. They act by inhibiting 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase and thereby reduce cholesterol synthesis. These drugs improve liver histology and serum markers in patients and are in general safe, with only rare examples of toxic side-effects [98]. In recent years, the anti-diabetic drug metformin is increasingly prescribed for NASH/NAFLD patients with good results [99]. In the treatment of HCC, sorafenib is commonly used for unresectable/non-ablatable or advanced-stage HCC. It is an inhibitor of various tumor growth-promoting tyrosine kinases (raf-, VEGF receptor, and PDGF receptor) [100]. Ursodeoxycholic acid is used in the treatment of cholestatic diseases [101]. Lamivudine, a deoxycytidine analogue that has a high oral bioavailability is active against hepatitis B virus (HBV) [102]. The major mechanism of lamivudine is blocking viral DNA synthesis and inhibiting HBV replication. Finally, hepatitis C virus infection is treated by a cocktail of drugs that inhibit various HCV proteins, including the HCV polymerase and protease. Unfortunately, many of the currently available therapies are not very effective (with the exception of the novel treatment for HCV [103]). Furthermore, most of these therapeutic interventions target the agents that cause the disease, but not target the already ongoing inflammation, cell death and/or fibrogenesis. In fact, there are currently no drugs available that directly target liver fibrosis, while more general anti-fibrotic drugs have a significant risk for side effects [104], for instance pirfenidone that may lead to gastrointestinal syndromes[15] (inhibiting TGF-\( \beta \) expression and activation) [105, 106], and Imatinib (targeting the non-receptor tyrosine kinase cAbl) [107]. For end stage liver failure, the only effective therapeutic option is liver transplantation. Since the supply of donor organs is inadequate to meet the growing demand, this is also not an optimal solution for many patients. Therefore, complementary and alternative medicines have gained increasing attention for the treatment of liver diseases.

**Herbal products for the treatment of liver disease**

An important group of complementary and alternative medicines are herbal products. For thousands of years, herbal products have been used by indigenous people worldwide to treat a plethora of illnesses. In the past few decades, these
natural products also caught attention in Western countries. Many cultures have used traditional medicine for the treatment of diseases, e.g. in China (traditional Chinese medicine, TCM), Japan (Kampō medicine), Korea (traditional Korean medicine), Indonesia (Jamu), India (Ayurvedic medicine), North America (phytotherapy), and Europe (herbalism) [108]. The medical application of herbal products has fostered research into the chemical and biological analyses of numerous prescriptions [109-112]. Simultaneously, it has initiated a successful approach to novel drug identification and development through the isolation and purification of active ingredients from herbal products [113] [114]. A few examples of herbal products that have been used in the treatment of liver diseases will now be discussed.

**Silymarin (Milk thistle)**

Silymarin, the extract of the Milk thistle (*Silybum marianum*) contains four flavonoids: isosilybin, silybin, silychristin and silydianin (Fig. 2). The major component silybin (also called silybin) accounts for 90% of the herb’s flavonoid content in most preparations. Silymarin is found commonly throughout Europe, Asia and North America. Its extracts were already used as early as the 4th century B.C. and became a single-herb remedy for liver disease and jaundice in the 1960s [115, 116]. One of the reasons why silymarin became so popular is because of its postulated mechanisms of actions that include antioxidant activity, anti-inflammatory activities and hepatoprotective actions with little or no toxicity [117-119]. Studies in cell culture and animal models demonstrated that silymarin enhances the activity of ribosomal RNA polymerase in hepatocytes and decreases hepatic injury by inhibiting glutathione depletion in hepatocytes and inhibiting the production of leukotrienes, prostaglandins and TNF-α by Kupffer cells [120]. Furthermore, sylimarin has been shown to block proliferation of HSCs [121] and to inhibit TGF-β1-induced collagen secretion [122]. Several clinical trials have shown the beneficial effects of silymarin in liver diseases, including liver cirrhosis, non-alcoholic fatty liver disease, hepatitis B/C virus infection and liver cancer [123, 124]. The beneficial effects of silymarin on parameters of liver injury (AST, ALT) are thought to be caused by modulation of immune responses and improvement of anti-oxidant defenses by increasing superoxide dismutase (SOD) activity and increasing glutathione levels. Although silymarin shows low intestinal absorption after oral administration, dosages of 420 mg/day are well-tolerated and have shown significant therapeutic effects in the treatment of liver cirrhosis and viral hepatitis. Low doses of silybin (20 to 48 mg/kg/day) are used as an antidote for acute *Amanita phalloides* (deathcap mushroom) poisoning [118], which occurs frequently in Europe, especially in Germany. The main mechanism of this protective action is repressing the uptake of amatoxin (the toxin of *Amanita phalloides*) via competitive inhibition of the transporter OATP1B3 and thereby decreasing the concentration of amatoxin in the enterohepatic circulation [125]. Although the value of silymarin in the treatment of chronic liver failure is still unclear because of the lack of well-controlled clinical trials, it remains the best currently available therapy for *A. phalloides* poisoning.
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Figure 3. The milk thistle (*Silybum marianum*) and the chemical structures of its four major flavonoid constituents. Taken from: [http://www.besmartstayhealthy.com/milk-thistle.html](http://www.besmartstayhealthy.com/milk-thistle.html)

**Glycyrrhizin (licorice root extract)**

Glycyrrhiza glabra (licorice root), is a perennial herb that is widely cultivated in Southeast Europe and Western Asia (Fig. 3). It has been used for centuries in traditional medicine to treat cough, bronchitis, gastritis and liver inflammation [126, 127]. It is available as over-the-counter medication in liquid, powder and pill forms in the United States. Glycyrrhizin is the aqueous extract of licorice root. It is the conjugate of two molecules of glucuronic acid and one molecule of 18β-glycyrrhetic acid (GA) [128]. Other components of the aqueous extract are several flavonoids, isoflavonoids, hydroxy-coumarins and β-sitosterols [129]. Glycyrrhizin prevents or attenuates liver injury in several animal models of liver disease, as well as in in liver disease patients in clinical trials. Glycyrrhizin targets the hepatocyte membrane [130, 131] and this property is being exploited by developing hepatocyte-specific delivery systems incorporating glycyrrhizin [132, 133]. In Japan, Neominophagen C, a more potent formulation that contains glycyrrhizin, cysteine and glycine, is administered parenterally to treat acute and chronic hepatitis [134, 135]. The use of glycyrrhizin in acute and chronic hepatitis is based on its hepatoprotective, immunomodulatory and anti-inflammatory effects. In animal models, glycyrrhizin has been demonstrated to suppress the inflammatory response via PI3K-mediated inhibition of NF-κB activation, resulting in diminished production of inflammatory cytokines like TNF-α, IL-4, IL-6 and IL-1β and inflammatory mediators like nitric oxide, reactive oxygen species and prostaglandin E2 [136]. Glycyrrhizin inhibits infiltration of inflammatory cells into the liver and reduced liver injury in the concanavalin A (ConA) model [137]. In the model of D(+)-galactosamine (GalN)/lipopolysaccharide (LPS)-induced fulminant hepatitis, glycyrrhizin prevented High-Mobility Group Box 1 (HMGB1)-dependent hepatocyte apoptosis [138] and glycyrrhizin also reduced ischemia/reperfusion (I/R)-induced liver injury [139]. Glycyrrhizin also inhibits ConA-induced proliferation of splenic CD4(+)T and enhances IFN-γ and IL-10 expression in these cells via JNK, ERK and PI3K/Akt-dependent mechanisms. Moreover, glycyrrhizin inhibited ConA-induced phosphorylation of JNK, ERK and PI3K/Akt in this study, suggesting that glycyrrhizin attenuates liver injury and fibrosis via modulation of the CD4(+)T cell response [140]. Of note, some studies
demonstrated that several nuclear receptors, e.g. glucocorticoid receptor, pregnane X receptor (PXR), estrogen receptor and peroxisome proliferator-activated receptor gamma (PPAR-γ), are regulated by glycyrrhizin, which may also be pharmaceutical targets of glycyrrhizin. Moreover, glycyrrhizin was shown to lower AST and ALT levels in a long-term clinical study of HCV patients in Japan and the Netherlands [145, 146]. In addition, glycyrrhizin exerts anti-inflammatory effects on SAH-induced vasospasm and attenuates the expression of PPARs, especially PPAR-γ, which corresponds to the severity of SAH-related inflammation.


Traditional Chinese Medicine

Traditional Chinese medicine (TCM) is an ancient, holistic treatment system established through empirical evaluation. It aims to restore energy (Qi) and balance (Yin and Yang) through the use of complex medicinal plants, fungi, animal products and minerals. TCM typically contains many components and therefore appears quite different from the therapeutic approaches of Western medicine. TCM is a holistic approach, using individualized herbal remedies to target complex syndromes and to help the body regain balance and harmony [147]. Thus, in TCM, the entire human body is treated as a single unit and the selected herbs are able to act in a complementary fashion. Supporting evidence for TCM was documented already in 16 B.C. in the book Pen Tsao, describing more than 300 herbs for medical treatment. The most important basic theory of TCM is the Jun-Chen-Zuo-Shi principle of combining different medicinal compounds in a specific manner when preparing TCM formulations (Fufang). Fufang means that each component in a TCM formulation has its own biological target and the combined action of all components on different biological targets is the principle of Fufang. For instance, most herbal mixtures comprise 4 to 5 herbs of which 1 or 2 are pharmacologically active compounds present in high doses. The herb that targets the major symptom of the disease is called Jun (“Emperor” or “King”). The remaining components, Chen (“Minister”), Zuo (“Assistant”) and Shi (“Courier”), have supporting functions, such as strengthening Jun’s therapeutic effects, eliminating possible adverse or toxic effects of the Jun and/or Chen components or working synergistically with Jun [148].
It has been demonstrated that at least 42% of patients with liver diseases use some form of traditional medicine, 20% of whom use herbal preparations [134]. The following section of the introduction describes some therapeutic examples of complex herbal medicines that are currently being investigated in clinical trials.

**Xiao-Chai-Hu-Tang (TJ-9)**

Xiao-Chai-Hu-Tang (TJ-9, Sho-saiko-to in Japanese), a classic herbal composite formula, is widely used in China and in Japan to treat liver diseases. It consists of 7 herbal components: bupleurum root, pinellia tuber, scutellaria root, jujube fruit, ginger rhizome, ginseng root and glycyrrhiza root. The six purified components of TJ-9 are baicalein, baicalin, Saikosaponins, ginsenosides, wogonin, and gingerol (Fig. 4). It is administered orally in doses of up to 7.5 g to patients with chronic viral liver diseases and the concentration of each active component is regulated [149]. In 1995, Oka et al [150] performed a prospective, randomized, non-blind controlled study in 260 patients, which demonstrated a significant beneficial preventive effect of TJ-9 on HCC development, in particular in patients that were HBs-Ag negative [119]. In addition, an anti-fibrotic effect of TJ-9 in two rat models of liver fibrosis (diethylnitrosamine- and pig serum-induced liver fibrosis) was demonstrated [151, 152]. More recently, Takahashi et al. reported their findings of TJ-9 treatment in non-alcoholic steatohepatitis (NASH) [153]. TJ-9 significantly alleviated necroinflammation and fibrosis in the liver in this mouse model of NASH. The beneficial effects of TJ-9 were (partly) caused by increased expression of peroxisome proliferator-activated receptor-gamma (PPARγ) and reduced expression of TNF-α and IL-6. Among the components of TJ-9, saikosaponin-C and saikosaponin-D, the isomers of saikosaponin, induced apoptosis of lymphocytes, partly by increasing levels of c-myc and p53 mRNA and decreasing levels of Bcl-2 mRNA [154]. Thus, TJ-9 may hold great promise for the prevention and/or treatment of complex liver diseases.

**Figure 5.** The seven herbal components of Kampo TJ 9 include bupleurum root (at the bottom), jujube fruit (left bottom), pinellia tuber (bottom right), ginger rhizome (on the top), glycyrrhiza root (up left), scutellaria root (top right), and ginseng root (on the left). Taken form: [http://kampo.ca/herbs-formulas/formulas/shosaikoto/](http://kampo.ca/herbs-formulas/formulas/shosaikoto/)
Fuzheng Huayu (FZHY)

Similar to TJ-9, Fuzheng Huayu (FZHY) is a complex preparation of Chinese herbal medicine consisting of six other medicinal herbs (Fig. 6): Radix Salvia Miltiorrhizae, Cordyceps, Semen Persicae, Gynostemma Pentaphyllammak, Pollen Pini and Fructus Schisandrae Chinensis [155]. It is approved by the State Food and Drug Administration (SFDA) of China as an antifibrotic medicine [156] and it has also been reported to promote blood flow and reduce markers of liver injury and cirrhosis (Child-Pugh score) [157]. In clinical trials, FZHY was shown to significantly improve clinical symptoms and liver function, to reverse hepatic fibrosis and to decrease portal pressure in patients with chronic hepatitis B and liver cirrhosis [158]. In animal models of liver fibrosis, FZHY decreased α-SMA expression, attenuated ECM deposition and inhibited the TGF-β1 signal transduction pathway [159, 160]. FZHY also induced apoptosis in rat HSC-T6 cells by activating p38 and inhibiting SAPK/JNK [161, 162], which is opposite to its effect on hepatocytes. In addition, FZHY has been shown to have antifibrotic properties by increasing the production of IFN-γ by hepatic NK cells [163] and to have anti-oxidant and anti-inflammatory properties by reducing the expression of cytochrome P450 2E1 and TNF-R1 [164]. FZHY has successfully completed phase 2 clinical trials in the United States [147, 165].
Figure 6. The product information of Fuzheng Huayu capsules of the Institute of Liver Diseases, Shanghai University of Traditional Chinese Medicine. The constituents of FZHY, clockwise from the top, are Cordyceps sinensis mycelium (tonifying spirit), Semen Persicae (Zuo: ‘assistant’, supports the function of Salvia), Fructus Schisandraceae Chinensis (prevents liver injury), Gynostemma Pentaphyllamman (removing heat and toxicity), Pollen Pini (nourishes the liver) and Radix Salvia Miltiorrhizae (prevents blood stasis).
Figure 7. Members of the Convolvulaceae family. *Ipomoea*, a large genus of more than 500 species, full of rich colors, grows in the tropical and subtropical zones throughout the world. Most of these species can adapt to harsh environments, such as salty and dry coastal areas. Taken from http://toptropicals.com/

**Ipomoea stolonifera** (beach morning-glory)

*Ipomoea stolonifera* (IS) is a coastal herb belonging to the Convolvulaceae (morning glory) family (Fig. 6). Like other herbal products, the exact composition of this herb can vary with location, season and even the altitude where the herbs grow [166, 167]. Even within one genus, extracts of flowers with different colors may vary in their medicinal properties [168, 169]. *I. stolonifera* normally occurs in tropical and sub-tropical regions, along sandy dunes and beaches, e.g. the Chaoshan area in Guangdong [170]. Traditionally, it has been used for the treatment of sunstroke, colitis, dysentery and fish puncture wounds, but IS has also been used for the treatment of inflammatory disorders, like rheumatoid arthritis [171]. The actual effective constituents of *I. stolonifera* are beginning to be elucidated, which is part of the work described in the thesis. In previous research, the n-butanol extract of *I. stolonifera* (BE-IS) was prepared and characterized [171]. This extract showed strong anti-inflammatory activity in the carrageenan-induced paw edema test in rats. In
order to identify novel and active compounds, the BE-IS was subjected to chromatographic analysis and five major constituents were identified based on nuclear magnetic resonance (NMR) spectrum and mass spectrometry (MS) analysis: the coumarins scopoletin, esculetin and umbelliferone and the flavonoids hesperetin and curcumin. Multiple activities for these compounds have been described, including antioxidant and anti-inflammatory properties, but the therapeutic effectiveness of BE-IS and its five purified components in liver injury has not been elucidated yet.

Summary

Traditional medicine holds great promise: the multicomponent nature of traditional medicines has great advantages in the therapy of many diseases. However, there are also challenges: reconstitution of complex mixtures from synthetic, purified compounds may not always reflect the original natural components and minor components may be ‘missed’. On the other hand, processing of original preparation to obtain extracts that can be administered easily (e.g. preparing extracts), may change the composition of the original preparation or alter the chemical structure of the active ingredients. In addition, there may be batch-to-batch variation between different preparations depending on e.g. season or geographical location. A major challenge will be to identify the optimal composition of complex mixtures. It is often not clear which are the most potent active ingredients or which combination has major therapeutic effects. Therefore the complexity and reproducible preparations of natural products still provides significant scientific challenges [172]. The strict regulation and standardization of all aspects of medicinal plant preparation by the Food and Drug Administration (FDA) and the European Medicines Evaluation Agency (EMEA) hampers the registration and manufacture of herbal products.

Another challenge is bridging the gap between experimental studies and clinical application. Although the past few decades have witnessed an enormous increase in knowledge from experimental studies, this has not been translated in an increase in clinical studies. The reasons for this ‘translational gap’ are mentioned above: the complexity of natural products and the difficulty in manufacturing specified and reproducible preparations of natural products. Nevertheless, the prospects for successfully treating patients with liver diseases with validated preparations of natural products have never been brighter.
Scope of the thesis

The aim of this thesis is to investigate the butanol-extract (BE-IS) form *Ipomoea stolonifera* and five purified compounds of this extract on acute and chronic liver diseases, in particular inflammatory liver diseases and liver fibrosis *in vivo* and *in vitro*.

In Chapter 1, we provide an overview of the most important liver cell types and signaling cascades involved in inflammatory liver diseases and an overview of traditional medicine. In Chapter 2, we evaluated the hepatoprotective effect of BE-IS and its five individual components on the inflammatory response and bile acid-induced cell death in primary hepatocytes *in vitro*. We demonstrate that each component of BE-IS displayed differential properties. Following this initial screen, hesperetin was selected for detailed investigation. In Chapter 3, we evaluated the therapeutic properties of hesperetin in two mouse models of fulminant hepatitis: the concanavalin A (Con A) model and the D-galactosamine /lipopolysaccharide (D-GalN/LPS) model. We demonstrate a profound therapeutic effect of hesperetin in these models of fulminant hepatitis. Following promising effects of the BE-IS component esculetin on hepatic myofibroblasts *in vitro*, in Chapter 4 we evaluated esculetin for anti-fibrotic effects in a mouse model of liver fibrosis (CCl4-induced hepatotoxicity). In Chapter 5, a follow-up study of the anti-fibrotic effect of esculetin is presented, in which the optimal route of administration and duration of treatment for esculetin were determined. In Chapter 6, the results of this thesis are discussed focusing on strategies for the treatment of inflammatory liver disorders and hepatic fibrosis and providing an outlook for the application of our findings in the treatment of human liver diseases.
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Chapter 1