Journal of Microbiology, Immunology and Infection 55 (2022) 314—322

Available online at www.sciencedirect.com

ScienceDirect

journal homepage: www.e-jmii.com

Original Article

Antifibrotic and anthelminthic effect of casticin on *Schistosoma mansoni*-infected BALB/c mice

Ho Yin Pekkle Lam [a,b,1], Ting-Ruei Liang [a,c,1], Yi-Chia Lan [d], Kai-Chih Chang [d], Po-Ching Cheng [e,f], Shih-Yi Peng [a,b,c,*]

[a] *Department of Biochemistry, School of Medicine, Tzu Chi University, Hualien, 97004, Taiwan*
[b] *Institute of Medical Sciences, Tzu Chi University, Hualien, 97004, Taiwan*
[c] *Ph.D. Program in Pharmacology and Toxicology, School of Medicine, Tzu Chi University, Hualien, 97004, Taiwan*
[d] *Institute of Laboratory Medicine and Biotechnology, College of Medicine, Tzu Chi University, Hualien, 97004, Taiwan*
[e] *Department of Molecular Parasitology and Tropical Diseases, School of Medicine, Taipei Medical University, Taipei, 110, Taiwan*
[f] *Research Center of International Tropical Medicine, College of Medicine, Taipei Medical University, Taipei, 110, Taiwan*

Received 7 October 2020; received in revised form 22 February 2021; accepted 25 March 2021
Available online 12 June 2021

KEYWORDS
Casticin;
Fibrosis;
Schistosoma mansoni;
Schistosomiasis

Abstract *Background/Purpose:* Schistosomiasis is an important tropical disease caused by *Schistosoma*. Although the pathogenesis of liver fibrosis has been intensively studied, the choice of effective treatment is still inadequate. In this study, we aimed to investigate the potential of using Casticin to treat *Schistosoma mansoni*-induced liver fibrosis.
Methods: BALB/c mice were divided into three groups — control, infection, and treatment group. The infection and treatment group were percutaneously infected with 100—120 cercariae. Mice from the treatment group were treated with 20 mg/kg/day Casticin for 14 consecutive days to investigate the potential protective effects of Casticin. Mice were sacrificed and were used for histological, RNA, protein, and parasite burden analysis.
Results: Our results showed that hepatic fibrosis was significantly attenuated, as indicated by histology and reduction of fibrotic markers such as collagen AI, transforming growth factor β (TGF-β), and α-smooth muscle actin (α-SMA). Furthermore, Casticin treatment significantly reduced worm burden. Anthelmintic effect of Casticin was also observed by scanning electron microscopy.

* Corresponding author. Department of Biochemistry, College of Medicine, Tzu Chi University, No. 701, Zhongyang Rd., Sec 3, Hualien, 97004, Taiwan. Fax: +886 3 857 8387.
E-mail address: pengsy@mail.tcu.edu.tw (S.-Y. Peng).
[1] Both authors contributed equally to this work.

https://doi.org/10.1016/j.jmii.2021.03.017

Conclusion: Collectively, our study suggested that Casticin may be a beneficial candidate in treating *S. mansoni* infection.

Introduction

Schistosoma haematobium (*S. haematobium*), *S. mansoni*, and *S. japonicum* are three common species that cause schistosomiasis in humans.[1] The disease affects more than 250 million people globally, of which more than 200 million people in Africa.[2,3]

After the cercariae penetrate the human skin, *S. mansoni* matures into schistosomula in the skin and then adults in the mesenteries of intestines to produce eggs. While some of the eggs are excreted through the stool, some of them are not excreted and are trapped in the liver. The trapped eggs in the liver can induce host immune responses and cause inflammation. In addition, chronic inflammation can result in granulomas formation and eventually, liver fibrosis.[3]

The fibrotic liver is characterized by the aberrant accumulation of activated hepatic stellate cells (HSCs), which produce a great amount of extracellular matrix (ECM), leading to the development of myofibroblast and result in liver fibrosis.[4,5] The activation of HSCs can be divided into three different stages: the initiation stage, the perpetuation stage, and the resolution stage. The initiation stage is characterized by which the quiescent HSCs are activated by liver injuries and cytokines. The perpetuation stage involves the accumulation of ECM released by activated HSCs; therefore, causing liver fibrosis. The resolution stage, at last, resolves liver injuries by inducing apoptosis.[5—7] Several fibrogenic proteins such as platelet-derived growth factor (PDGF) and transforming growth factor β (TGF-β) have been shown to promote the process of liver fibrosis.[8,9] Also, tumor-necrosis factor α (TNF-α) and tissue inhibitor of metalloproteinase 1 (TIMP-1) promote liver fibrosis by inhibiting HSCs apoptosis.[10—12]

Numerous traditional Chinese herbs or medicines have been shown to have anti-fibrotic effects.[13—16] *Vitex* species is one of the Chinese herbs that have been used for many years in treating headaches, female hormone conditions, inflammatory diseases, and cancers.[17] It had also been shown to have anti-trypanosoma effects.[18] Casticin, a flavonoid isolated from *Vitex rotundifolia* and *Vitex trifolia*, had been shown to have anti-inflammatory, anti-cancer, anti-fibrotic effects in various studies.[16,19—21] Since Casticin protected against thioacetamide (TAA)-induced, carbon tetrachloride (CCL4)-induced, and bile duct ligation (BDL)-induced liver fibrosis,[16,22] we hypothesized that Casticin also attenuates liver fibrosis caused by *S. mansoni*. Therefore, in this study we aimed to evaluate the effects of Casticin on *S. mansoni*-induced hepatic fibrosis.

Materials and methods

Parasite, animals, and animal welfare

Puerto Rico strain of *S. mansoni* was obtained from the Biomedical Research Institute, Rockville, MD 20852, USA and was maintained in our laboratory. The freshwater snail *Biomphalaria glabrata* was used as the intermediate host and male BABL/c mice were used as the final host. BALB/c mice were purchased from the National Laboratory Animal Center, Taipei and were housed in an animal facility under a 25 °C ± 2 °C and a 12-h light/dark cycle condition with free access to water and food.

All protocols involving animals were approved by the regulation of the Institutional Animal Care and Use Committees (IACUC) of Tzu Chi University (No. 106055). All experimental procedures were carried out under approved guidelines of the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals (DHHS publication No. NIH 85—23, revised 1996).

Casticin

Casticin ($C_{19}H_{18}O_8$, molecular weight 374.3 kDa, isolated from *V. trifolia* by HPLC, 98% purity) was purchased from Chengdu Biopurify Phytochemicals Ltd (Chengdu, China). To prepare one single dose (200 μL) of casticin for one mouse, 0.5 mg Casticin powder (assuming mice weight were 25 g) was dissolved in 10 mL 0.25% Tween 80 to yields a concentration of 20 mg/kg.[16]

Animal infection and treatment

Thirty-six eight-week-old male BALB/c mice were equally and randomly divided into three groups — one control, one infection, and one treatment group. Each mouse of the infection and treatment group was percutaneously infected with 100—120 cercariae via the tail, and the control group was treated with normal saline. Five mice in each group were used for RNA, protein, and histological studies; whereas seven mice in each group were used for worm burden reduction and egg reduction analysis. Mice from the infection group were treated with water by oral gavage for 14 consecutive days, starting from week eight post-infection; whereas mice from the treatment group were treated with 20 mg/kg/day Casticin for 14 consecutive days. All mice were euthanized at week ten post-infection Upon mice dissection, liver and spleen were examined for any pathological changes and liver specimens were

obtained. Six additional mice were equally divided into the control and casticin control group to exclude any adverse effects of Casticin on mice. While the control group was treated with water; the casticin control group was treated with 20 mg/kg/day casticin for 14 consecutive days. Experimental results from these groups can be found in Supplementary Figs. 1 and 2.

RNA isolation and cDNA synthesis

Total RNA was isolated from liver tissues using TRIzol reagent (Thermo Scientific, Rockford, IL, USA), according to the manufacturer's instructions. 5 μg RNA was used for cDNA synthesis in a 20 μL reaction mixture using the RevertAid First Strand cDNA Synthesis Kit (Fermentas International Inc., Ontario, Canada). RT-PCR was performed at 50 °C for 1 h, followed by extension for 15 min at 70 °C. The synthesized cDNA was stored at −20 °C.

Real-time quantitative PCR (qPCR)

qPCR was performed with the LabStar SYBR qPCR Kit (Bioline, London, UK) using a Roche LightCycler 480 System. The qPCR conditions were as follows: 10 min pre-incubation (95 °C); 45 cycles of denaturation at 90 °C for 10 s, annealing at 60 °C for 20 s, and extension at 72 °C for 10 s. The primers used in this study are shown in Table 1. The relative gene expression was calculated using the ΔΔCt method and gene expression levels were normalized to *GAPDH*.

Protein extraction

Liver tissues were washed with PBS, and 500 μL RIPA buffer (Thermo Scientific, Rockford, IL, USA) was added. Tissues were homogenized with a glass Dounce homogenizer on ice. Cell lysates were agitated for 30 min at 4 °C and centrifuged at 8100×*g* for 15 min at 4 °C. The supernatant containing the soluble protein was transferred to a new Eppendorf for further analysis.

Western blot analysis

Proteins were separated on 10% or 12% SDS-PAGE along with the Precision Plus Protein Standard Ladder (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The gels were then transferred to a polyvinylidene difluoride (PVDF) membrane (EMD Millipore, Burlington, MA, USA). Membranes were blocked with 5% non-fat milk prior to overnight incubation at 4 °C with agitation with the following primary antibodies individually: α-tubulin (GTX628802, GeneTex, Irvine, CA, USA); collagen AI (GTX112731, GeneTex), α-SMA (14395-1-AP, Proteintech, Chicago, IL, USA), TGF-β (#3711, Cell signaling technology, Danvers, MA, USA), TNF-α (GTX110520, GeneTex), and IL-1β (#12242, Cell signaling). The membranes were incubated with HRP-conjugated goat anti-mouse IgG (EMD Millipore) or goat anti-rabbit IgG (EMD Millipore) secondary antibodies for 1 h, following which the membranes were developed using ECL detection reagent (EMD Millipore). Relative protein levels were quantified using Image J software (Version 1.46, National Institute of Health, Bethesda, MD, USA), and protein densitometries were expressed relative to that of α-tubulin.

Hematoxylin and eosin staining

Before proceeding with the staining protocol, the slides were deparaffinized and rehydrated with Sub-X (Leica Biosystem Richmond, IL, USA), 100, 95, 75, and 50% ethanol, and finally rinsed with distilled water. The rehydrated sections were stained as follows: hematoxylin solution (3 min), tap water (1 min), 70% ethanol with 1% HCl (5 s), tap water (1 min), eosin solution (3 min), 95% ethanol (3 × 5 min), 100% ethanol (2 × 5 min), and Sub-X (3 × 15 min).

Masson's trichrome stain

Masson's trichrome stain was performed using a Trichrome stain kit (ScyTek Laboratories, Inc., West Logan, USA), according to the manufacturer's instructions. Briefly, the deparaffinized and rehydrated slides were incubated with pre-heated Bouin's fluid for 60 min and rinsed with distilled water. Slides were then stained as follows: Weigert's

Table 1 Primer pairs of candidate genes used in real-time quantitative PCR.

Gene name	Primer	Melting Tm (°C)
GAPDH	Forward 5-AGGTCGGTGTGAACGGATTTG-3 Reverse 5-GGGGTCGTTGATGGCAACA-3	60
Collagen AI	Forward 5- GCGGTAACGATGGTGCTGTT -3 Reverse 5- CTTCACCCTTAGCACCAAC -3	60
α-SMA	Forward 5- ATTGAACATGGCATCATCACC -3 Reverse 5- GCAGCTCATAGCTCTTCTCC -3	60
TGF-β1	Forward 5- CAACGCCATCTATGAGA-3 Reverse 5- AAGCCCTGTATTCCGTCTCC-3	60
TNF-α	Forward 5- GCTCCCTCTCATCAGTTCTAT -3 Reverse 5- TTTGCTACGADCTGGGCTA -3	60
IL-1β	Forward 5- CAACCAACAAGTGTATTCTCCAT -3 Reverse 5- GTGTGCCGTCTTTCATTA -3	60

solution (10 min), Biebrich Scarlet/Acid Fuchsin Solution (15 min), Phosphomolybdic/Phosphotungstic acid solution (15 min), 1% Aniline Blue solution (5 min), 95% ethanol (3 × 5 min), 100% ethanol (2 × 5 min), and Sub-X (3 × 15 min).

Scanning electron microscopy (SEM)

Adult worms from the mice were isolated from the hepatic portal vein and mesenteric veins by portal perfusion method as described.[23] The adult worm was rinsed in PBS and fixed in 2.5% glutaraldehyde for 60 min at 4 °C. Worms were then washed twice with 5% sucrose and incubated with 1% osmium tetroxide for 60 min at room temperature. Increasing concentrations of ethanol (50%, 70%, 80%, 90%, and 100%) were used to dehydrate the worms. Worms were then critically point-dried, and sputter-coated with gold. Samples were visualized using a HITACHI S-4700 field emission scanning electron microscope (Hitachi Ltd, Tokyo, Japan).

Worm burden and hepatic eggs count

Adult worms were isolated by portal perfusion method.[23] The number of worms was counted under a dissecting microscope. Hepatic eggs were counted using the Kato-Katz technique directly performed on liver tissues.[24] Hepatic egg burdens were expressed as eggs per gram of liver. The reduction rate of worms or eggs was calculated as:

$$\frac{Worm/eggs\ recovered\ from\ infected\ group - Worm/eggs\ recovered\ from\ treatment\ group}{Worm/eggs\ recovered\ from\ infected\ group} \times 100\%$$

Liver function test

Levels of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were used as a parameter of liver function. Whole blood was obtained by cardiac puncture and was centrifugate at 600×*g* for 10 min to obtain the serum. Serum was then analyzed for AST and ALT using Hitachi 7080 Chemistry Analyzer (Hitachi Ltd, Tokyo, Japan).

Statistical analysis

All experimental data were analyzed using GraphPad Prism 6.01 software (GraphPad Software, San Diego, CA, USA). Data were presented as the mean ± S.D. One-way analysis of variance (ANOVA) was used, followed by a Tukey's post-hoc test, to determine differences between groups. The symbol "*" indicates a significant difference at the 0.05 level (*P*-value < 0.05).

Results

Casticin is protective against *S. mansoni* induced hepatic fibrosis

Mice were infected with 100–120 cercariae percutaneously. After eight weeks post-infection, the mice were treated with Casticin at 20 mg/kg/day for 14 days. Mice were euthanized and observed for pathological changes. As expected, mice infected with *S. mansoni* showed hepatosplenomegaly. Granuloma nodules were observed on the liver surface, as indicated by the white spots (Fig. 1A-B). After Casticin treatment, hepatosplenomegaly was improved and fewer hepatic granuloma nodules were observed (Fig. 1C). Along with the observation of gross appearance, liver fibrosis was confirmed with H&E and Masson trichrome stain (Fig. 1D-E). Similarly, fibrotic areas of the liver were much severer in the infected mice, compared with the control mice. In contrast, mice treated with Casticin showed improved liver morphology and reduced fibrotic areas. Therefore, Casticin can improve *S. mansoni* induced hepatic fibrosis.

Casticin improves liver function in *S. mansoni* infected mice

We then measure the serum markers for liver function, respectively ALT and AST. Serum ALT and AST increased significantly in the infected mice and decreased after mice treated with Casticin (Fig. 2A-B). In addition, Casticin treatment to normal mice showed no significant change in ALT and AST (Fig. 2A-B), suggesting that Casticin does not have a toxic effect on livers and is, therefore, safe to use (see also Supp Fig. 1).

Casticin resolves hepatic fibrosis by downregulating fibrotic and inflammatory genes and proteins expression

To further investigate the role of Casticin on *S. mansoni*-induced liver fibrosis, we investigated the expression of some fibrotic- and inflammatory-related genes and proteins. qPCR and Western blot analysis showed that expression levels of collagen AI significantly increased in *S. mansoni*-infected liver, but downregulated in mice treated with Casticin (Fig. 3A, F, and G). α-SMA, the protein involved in HSC activation, was also found to increase in expression during *S. mansoni* infection and decreased upon treatment with Casticin (Fig. 3B, F, and H). A similar pattern was observed for TGF-β (Fig. 3C, F,

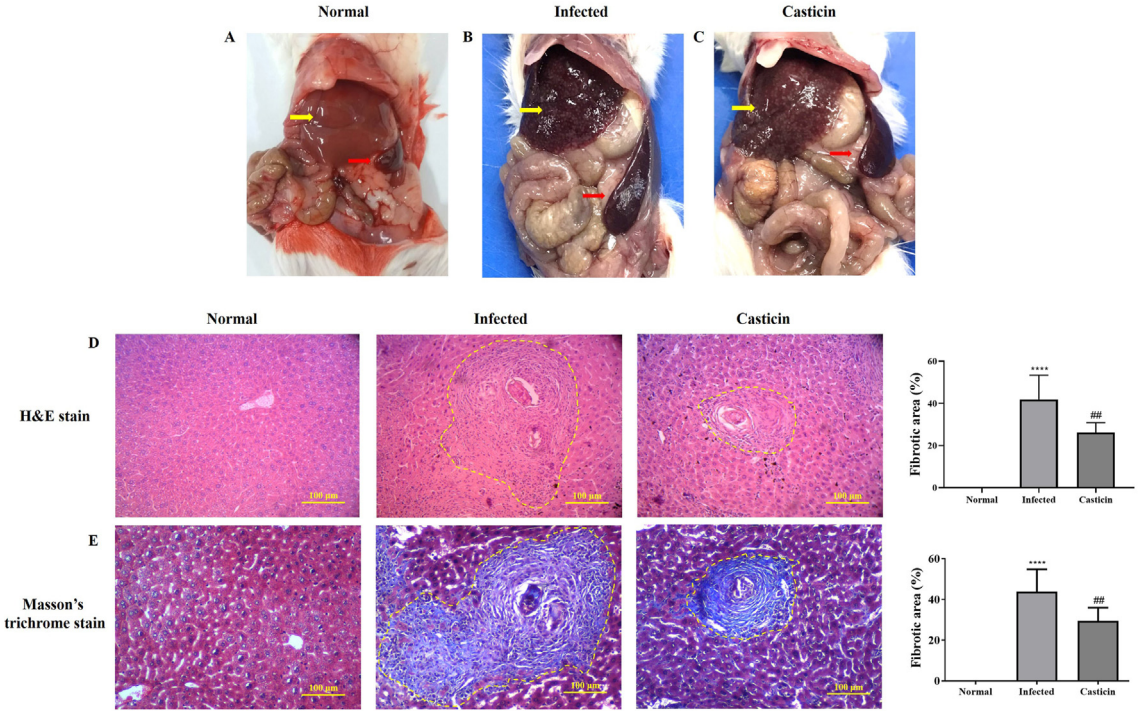

Figure 1. Casticin treatment ameliorates hepatosplenomegaly and reduces liver fibrosis in *S. mansoni*-infected mice (A—C) Representative images showing gross pathology of mice uninfected or infected with *S. mansoni*. Hepatosplenomegaly was observed in infected mice (liver, yellow arrows; spleen, red arrows). White spots seen on the surface of liver indicate granuloma nodules. Mice treated with Casticin showed reduced size of liver and spleen, compared with the infected mice (D) Representative images showing H&E staining of liver sections of the mice. Uninfected mice showed normal liver cell integrity with no fibrosis. Liver fibrosis (encircled by a yellow dotted line) was observed in the infected mice, which are reduced in size upon Casticin treatment (E) Representative images of Masson's trichrome staining on liver sections of the mice. Collagens were stained as blue (encircled by a yellow dotted line). Quantification was performed on five slides in each group. Ten microscopic fields were counted on each slide. **** *P*-value < 0.0001 compared with normal group; ## *P*-value < 0.01 compared with infected group.

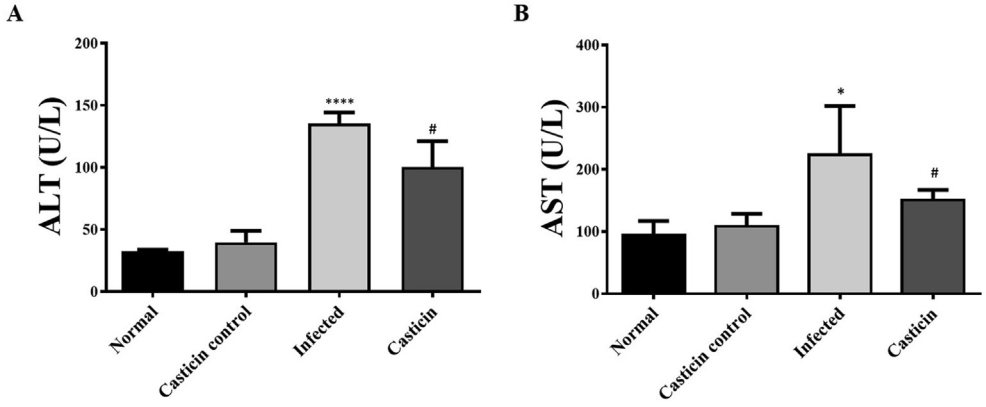

Figure 2. Casticin treatment improves liver function of *S. mansoni*-infected mice (A) ALT and (B) AST levels measured in the serum, shown as mean $\pm$ S.D. (n $=$ 5). Infected mice showed increased ALT and AST levels, suggesting an impaired liver function. Casticin treatment reduced ALT and AST levels. * *P*-value < 0.05, **** *P*-value < 0.0001 compared with normal group; # *P*-value $<$ 0.05 compared with infected group.

and I). Two inflammatory cytokines, TNF-α and IL-1β, were also increased during *S. mansoni* infection and decreased after Casticin treatment (Fig. 3D—F, and J-K). Of note, Casticin treated to normal mice does not affect the expression of these proteins (Supp Fig. 2). These results suggested that Casticin treatment alleviates inflammation and resolves liver fibrosis during *S. mansoni* infection.

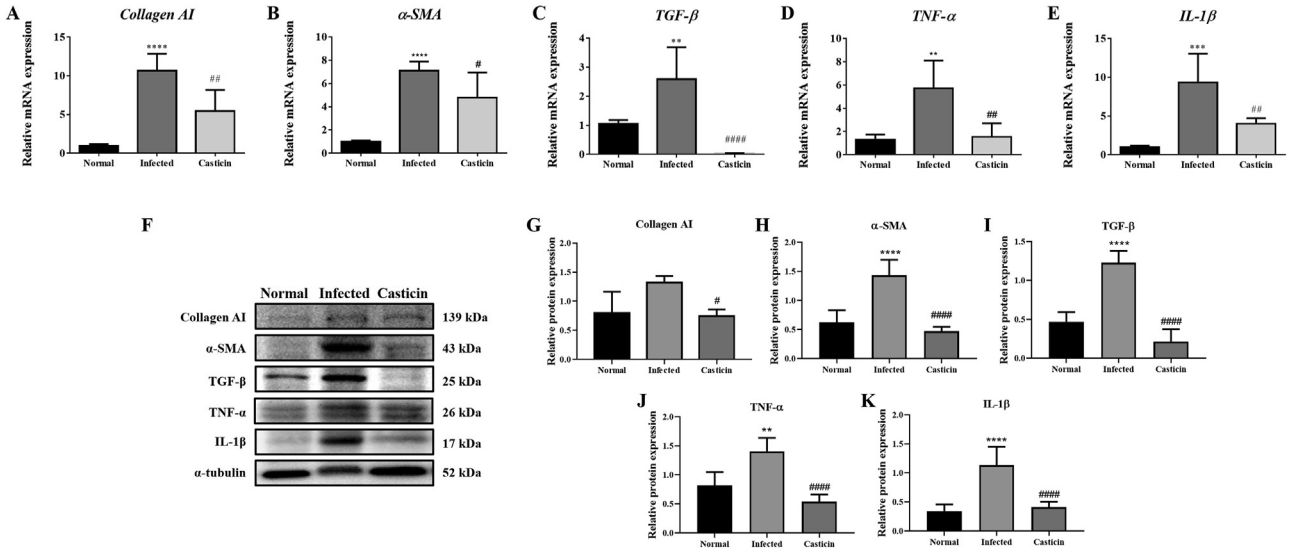

Figure 3. Casticin downregulates fibrotic and inflammatory genes and protein expression. (A—E) RNA transcription levels of fibrotic and inflammatory markers including *collagen AI*, *α-SMA*, *TGF-β*, *TNF-α*, and *IL-1β*, measured by qPCR. Data are presented as mean ± S.D. (n = 5) (F) Representative Western blot images showing protein levels of fibrotic and inflammatory markers. (G—K) Bar graphs showing protein expression levels relative to that of α-tubulin. Results are presented as mean ± S.D. (n = 3). ** *P*-value < 0.01, *** *P*-value < 0.001, and **** *P*-value < 0.0001 compared with normal group; # *P*-value < 0.005, ## *P*-value < 0.01, #### *P*-value < 0.0001 compared with infected group.

Casticin has an antischistosomal activity

The number of adult worms recovered was significantly lower in the Casticin treatment group, compared with the infection group (Fig. 4A). However, there were no statistically significant differences in eggs burden between the infection and Casticin treatment group (Fig. 4B). Under SEM, we observed that both male and female worms isolated from Casticin-treated mice showed ultrastructural damage to the tegument surface (Fig. 4C—F). Hole-shaped erosions were observed along the body of the male worm (Fig. 4D-E), while surface sloughing and erosion were observed on the female worm (Fig. 4G-H).

Discussion

Schistosomiasis is affecting more than 200 million people worldwide and has caused severe liver fibrosis among these patients.[3] At around five weeks post-infection of the host, female *S. mansoni* adult lay eggs in the mesenteric venules of the host, following that the eggs will circulate and trap in the liver of hosts. It has been shown that the trapped eggs can induce hepatic fibrosis at and after eight weeks post-infection.[3] Currently, praziquantel remains the choice of treatment for schistosomiasis. Although praziquantel effectively kills the worm, it cannot resolve liver damages and prevent re-infection. Moreover, resistance of praziquantel has been suggested in vivo and in vitro studies.[25,26] For this reason, we examined the effects of Casticin, which has been shown to have anti-fibrotic properties, on *S. mansoni*—induced hepatic fibrosis.

In the current study, we first confirmed that Casticin does not have toxic effects on livers (Supp Fig. 1). We then found that symptoms and functions of the liver were much improved after *S. mansoni*-infected mice treated with Casticin. Also, Casticin treatment significantly reduced granuloma sizes. These liver-protective effects of Casticin have also been confirmed by another study which showed that high dose Casticin will not alter the level of glutathione (GST), another marker of liver function.[27] The protective effects against *Schistosoma*-induced hepatic fibrosis corroborated that Casticin protects against bile duct ligation (BDL) induced- and carbon tetrachloride (CCL4) induced-hepatic fibrosis.[16]

Casticin resolves *S. mansoni*-induced hepatic fibrosis by downregulating certain fibrotic proteins including collagen AI and α-SMA. Interestingly, in the casticin group, the correlation of these two markers differs between the transcript levels and protein levels. A decrease in mRNA expression is supposedly observed (Fig. 3A-B); however, protein expression decreases more evidently, even lower than that of the control (Fig. 3G-H). The differences between the transcript levels and protein levels can be explained by the aspects of transcriptional and translational regulation.[28,29] For example, translation rates can be modulated by the binding of regulatory proteins or micro RNAs on the transcript.[30] In this case, transcript and proteins may vary a lot in their expression levels. Also, in the casticin-treated group, collagen AI was reduced to a protein level even lower than control (Fig. 3G); however, in the same group, Masson's trichrome staining showed levels higher than control (Fig. 1E). Masson's trichrome specifically stains collagen type I.[31] Since collagen type I composed of two subtypes, collagen A1 and A2.[32] Measuring only the level of collagen A1 may not be able to represent the total collagen type I levels, making the Western blot results not corroborate to the Masson's trichrome staining.

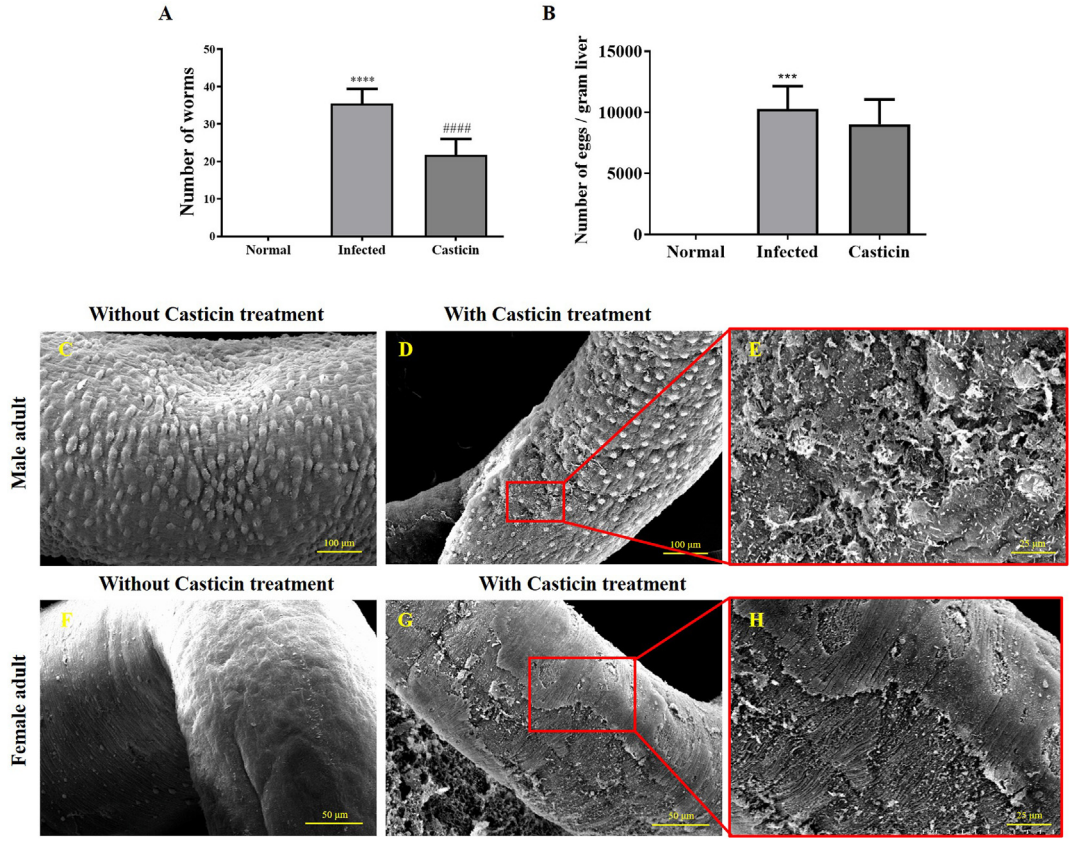

Figure 4. Antischistosomal activity of Casticin (A) Number of worms recovered from the mice. Casticin treatment reduced the number of worms isolated from the mice. (B) Number of eggs counted per gram of liver. Data are presented as mean ± S.D. (n = 5—7). *** *P*-value < 0.001, and **** *P*-value < 0.0001 compared with normal group; #### *P*-value < 0.0001 compared with infected group (C—H) Representative SEM images showing the ultrastructural surface of male and female worms isolated from the mice. (D—E) Erosions were seen on the body of male worms isolated from Casticin-treated mice. (G—H) Surface sloughing and erosions were observed on female worms isolated from Casticin-treated mice.

TGF-β is a multifunctional cytokine that takes part in fibrosis, anti-inflammation, and inhibition of the host immune system.[33,34] It has been shown that TGF-β involved in all stages of liver disease progression, including liver inflammation, fibrosis, and cancers.[35,36] TGF-β has long been recognized as a profibrogenic cytokine as it stimulates HSCs activation and promotes liver cell apoptosis.[37] Moreover, TGF-β takes part in the fibrogenesis during *Schistosoma* infection.[38,39] In this study, the increased TGF-β expression during S. *mansoni* infection was reduced by Casticin treatment. Our results here corroborate with the results in BDL induced- and CCL4 induced-hepatic fibrosis.[16] One study also found involvement of TGF-β during *Clonorchis sinensis*, a liver fluke, infection.[40] The reduction of TGF-β takes the edge off the fibrogenesis,[41] we therefore emphasized that Casticin protects liver fibrosis by down-regulating TGF-β.

Inflammation has been positively related to liver fibrosis.[42] Several proinflammatory cytokines in the liver could induce hepatic-inflammation and cause liver fibrosis. The two cytokines of high importance in causing liver fibrosis, TNF-α and IL-1β,[42—44] were also found to decrease in our study after Casticin treatment to S. *mansoni*-infected mice. TNF-α is a pleiotropic cytokine and can trigger multiple signaling pathways that contribute to the pathogenesis of liver fibrosis.[43] TNF-α, in addition to causing inflammation, can augment the survival of HSCs. Inflammation-induced hepatocyte apoptosis results in enhanced production of apoptotic bodies and TNF-α. Once the apoptotic bodies being engulfed by HSCs, it increases their profibrogenic responses. In addition, TNF-α engulfed by macrophages can stimulate more hepatocyte apoptosis and produce more TNF-α.[43] Macrophage-derived TNF-α contributes to HSCs survival through the NF-κB pathway, thereby promoting liver fibrosis.[12] On the other hand, IL-1β has been involved in causing liver fibrosis in hypercholesterolemic[45] and TAA-injected mice.[46] Mice deficient from IL-1 receptors were protected against liver fibrosis,[46] suggesting IL-1β serves a signal in fibrogenesis. IL-1β signaling has also been shown to activates matrix metallopeptidases 9 (MMP-9) and induces liver fibrosis.[46—48] The results we observed from this study suggested that the liver protective effects of Casticin also involved in lowering inflammatory responses.

Since Casticin causes a significant reduction in the worm count, we investigate the effect of Casticin on S. *mansoni* itself. SEM was performed to observe the adult worm isolated from the mice. It can be seen that Casticin treatment

causes damage to the integument of the worm. These results all suggested that Casticin can have an anthelmintic effect by damaging the surface of the adult worm. Tasdemir and Tierney et al. have shown that methoxylated flavonol compounds, including Casticin, have the ability to kill protozoan parasites.[49] We believe that the two mechanisms that kill the worm are similar. However, Casticin has no effect on clearing the eggs trapped in the liver. Since eggs were produced and trapped in the liver at a later stage of the infection, treating the infected individuals with Casticin at early stage may be beneficial. Furthermore, combination therapy using praziquantel and Casticin may offer synergistic efficacy for *Schistosoma* treatment.

Collectively, our current findings provide evidence that Casticin has both the effect of killing adult *S. mansoni* and improves *S. mansoni*-induced liver fibrosis. Therefore, Casticin has the potential to replace or to use in combination with praziquantel in treating schistosomiasis.

Author contributions

S.Y.P designed the study. H.Y.P.L, T.R.L, and Y.C.L conducted the study. P.C.C and K.C.C provided the laboratory techniques and analyzed the data. H.Y.P.L wrote the manuscript with input from all the co-authors.

Declaration of competing interest

All authors declare that they have no conflicts of interest related to this article.

Acknowledgements

We thank the Electron Microscopy Laboratory of Tzu Chi University for their help with the SEM evaluations. This work was supported by the Tzu Chi University [grant number: TCMRC-P-107014].

References

1. Gryseels B, Polman K, Clerinx J, Kestens L. Human schistosomiasis. *Lancet* 2006;**368**(9541):1106—18.
2. Hotez PJ, Alvarado M, Basanez MG, Bolliger I, Bourne R, Boussinesq M, et al. The global burden of disease study 2010: interpretation and implications for the neglected tropical diseases. *PLoS Neglected Trop Dis* 2014;**8**(7):e2865.
3. Colley DG, Bustinduy AL, Secor WE, King CH. Human schistosomiasis. *Lancet* 2014;**383**(9936):2253—64.
4. Zhang CY, Yuan WG, He P, Lei JH, Wang CX. Liver fibrosis and hepatic stellate cells: etiology, pathological hallmarks and therapeutic targets. *World J Gastroenterol* 2016;**22**(48):10512—22.
5. Friedman SL. Mechanisms of hepatic fibrogenesis. *Gastroenterology* 2008;**134**(6):1655—69.
6. Suk KT, Kim DJ. Staging of liver fibrosis or cirrhosis: the role of hepatic venous pressure gradient measurement. *World J Hepatol* 2015;**7**(3):607—15.
7. Canbay A, Friedman S, Gores GJ. Apoptosis: the nexus of liver injury and fibrosis. *Hepatology* 2004;**39**(2):273—8.
8. Ying HZ, Chen Q, Zhang WY, Zhang HH, Ma Y, Zhang SZ, et al. PDGF signaling pathway in hepatic fibrosis pathogenesis and therapeutics (Review). *Mol Med Rep* 2017;**16**(6):7879—89.
9. Meng XM, Nikolic-Paterson DJ, Lan HY. TGF-beta: the master regulator of fibrosis. *Nat Rev Nephrol* 2016;**12**(6):325—38.
10. Murphy FR, Issa R, Zhou X, Ratnarajah S, Nagase H, Arthur MJ, et al. Inhibition of apoptosis of activated hepatic stellate cells by tissue inhibitor of metalloproteinase-1 is mediated via effects on matrix metalloproteinase inhibition: implications for reversibility of liver fibrosis. *J Biol Chem* 2002;**277**(13):11069—76.
11. Osawa Y, Hoshi M, Yasuda I, Saibara T, Moriwaki H, Kozawa O. Tumor necrosis factor-α promotes cholestasis-induced liver fibrosis in the mouse through tissue inhibitor of metalloproteinase-1 production in hepatic stellate cells. *PloS One* 2013;**8**(6):e65251.
12. Pradere JP, Kluwe J, De Minicis S, Jiao JJ, Gwak GY, Dapito DH, et al. Hepatic macrophages but not dendritic cells contribute to liver fibrosis by promoting the survival of activated hepatic stellate cells in mice. *Hepatology* 2013;**58**(4):1461—73.
13. Feng Y, Cheung KF, Wang N, Liu P, Nagamatsu T, Tong Y. Chinese medicines as a resource for liver fibrosis treatment. *Chin Med* 2009;**4**:16.
14. Li LC, Kan LD. Traditional Chinese medicine for pulmonary fibrosis therapy: progress and future prospects. *J Ethnopharmacol* 2017;**198**:45—63.
15. Hu Q, Noor M, Wong YF, Hylands PJ, Simmonds MS, Xu Q, et al. In vitro anti-fibrotic activities of herbal compounds and herbs. *Nephrol Dial Transplant* 2009;**24**(10):3033—41.
16. Zhou L, Dong X, Wang L, Shan L, Li T, Xu W, et al. Casticin attenuates liver fibrosis and hepatic stellate cell activation by blocking TGF-beta/Smad signaling pathway. *Oncotarget* 2017;**8**(34):56267—80.
17. Rani A, Sharma A. The genus Vitex: a review. *Phcog Rev* 2013;**7**(14):188—98.
18. Kiuchi F, Matsuo K, Ito M, Qui TK, Honda G. New norditerpenoids with trypanocidal activity from Vitex trifolia. *Chem Pharm Bull (Tokyo)* 2004;**52**(12):1492—4.
19. Chan EWC, Wong SK, Chan HT. Casticin from Vitex species: a short review on its anticancer and anti-inflammatory properties. *J Integr Med* 2018;**16**(3):147—52.
20. Prasad EM, Mopuri R, Islam MS, Kodidhela LD. Cardioprotective effect of Vitex negundo on isoproterenol-induced myocardial necrosis in wistar rats: a dual approach study. *Biomed Pharmacother* 2017;**85**:601—10.
21. Rasul A, Zhao BJ, Liu J, Liu B, Sun JX, Li J, et al. Molecular mechanisms of casticin action: an update on its antitumor functions. *Asian Pac J Cancer Prev APJCP* 2014;**15**(21):9049—58.
22. Kadir FA, Kassim NM, Abdulla MA, Yehye WA. Hepatoprotective role of ethanolic extract of Vitex negundo in thioacetamide-induced liver fibrosis in male rats. *Evid Based Complement Alternat Med* 2013;**2013**:739850.
23. Duvall RH, DeWitt WB. An improved perfusion technique for recovering adult schistosomes from laboratory animals. *Am J Trop Med Hyg* 1967;**16**(4):483—6.
24. Katz N, Coelho PM, Pellegrino J. Evaluation of Kato's quantitative method through the recovery of Schistosoma mansoni eggs added to human feces. *J Parasitol* 1970;**56**(5):1032—3.
25. Vale N, Gouveia MJ, Rinaldi G, Brindley PJ, Gärtner F, Correia da Costa JM. Praziquantel for schistosomiasis: single-drug metabolism revisited, mode of action, and resistance. *Antimicrob Agents Chemother* 2017;**61**(5).
26. Wang W, Wang L, Liang YS. Susceptibility or resistance of praziquantel in human schistosomiasis: a review. *Parasitol Res* 2012;**111**(5):1871—7.
27. Yuan L, Kaplowitz N. Glutathione in liver diseases and hepatotoxicity. *Mol Aspect Med* 2009;**30**(1—2):29—41.

28. Liu Y, Beyer A, Aebersold R. On the dependency of cellular protein levels on mRNA abundance. *Cell* 2016;**165**(3):535—50.
29. Vogel C, Marcotte EM. Insights into the regulation of protein abundance from proteomic and transcriptomic analyses. *Nat Rev Genet* 2012;**13**(4):227—32.
30. Barrett LW, Fletcher S, Wilton SD. Regulation of eukaryotic gene expression by the untranslated gene regions and other non-coding elements. *Cell Mol Life Sci* 2012;**69**(21): 3613—34.
31. Calvi ENdC, Nahas FX, Barbosa MV, Calil JA, Ihara SSM, Silva MdS, et al. An experimental model for the study of collagen fibers in skeletal muscle %. *J Acta Cirúrgica Brasileira* 2012;**27**:681—6.
32. Ricard-Blum S. The collagen family. *Cold Spring Harbor perspectives in biology* 2011;**3**(1):a004978.
33. Schon HT, Weiskirchen R. Immunomodulatory effects of transforming growth factor-β in the liver. *Hepatobiliary Surg Nutr* 2014;**3**(6):386—406.
34. Borthwick LA, Wynn TA, Fisher AJ. Cytokine mediated tissue fibrosis. *Biochim Biophys Acta* 2013;**1832**(7):1049—60.
35. Fabregat I, Moreno-Càceres J, Sánchez A, Dooley S, Dewidar B, Giannelli G, et al. TGF-β signalling and liver disease. *FEBS J* 2016;**283**(12):2219—32.
36. Dewidar B, Meyer C, Dooley S, Meindl-Beinker AN. TGF-β in hepatic stellate cell activation and liver fibrogenesis-updated 2019. *Cells* 2019;**8**(11).
37. Hernandez-Gea V, Friedman SL. Pathogenesis of liver fibrosis. *Annu Rev Pathol* 2011;**6**(1):425—56.
38. Farah IO, Mola PW, Kariuki TM, Nyindo M, Blanton RE, King CL. Repeated exposure induces periportal fibrosis in Schistosoma mansoni-Infected baboons: role of TGF-β and IL-4. *J Immunol* 2000;**164**(10):5337—43.
39. Herbert DR, Orekov T, Perkins C, Finkelman FD. IL-10 and TGF-beta redundantly protect against severe liver injury and mortality during acute schistosomiasis. *J Immunol* 2008;**181**(10): 7214—20.
40. Jin Y, Wi HJ, Choi MH, Hong ST, Bae YM. Regulation of anti-inflammatory cytokines IL-10 and TGF-β in mouse dendritic cells through treatment with Clonorchis sinensis crude antigen. *Exp Mol Med* 2014;**46**(1):e74.
41. Dooley S, ten Dijke P. TGF-β in progression of liver disease. *Cell Tissue Res* 2012;**347**(1):245—56.
42. Koyama Y, Brenner DA. Liver inflammation and fibrosis. *J Clin Invest* 2017;**127**(1):55—64.
43. Yang YM, Seki E. TNFα in liver fibrosis. *Curr Pathobiol Rep* 2015;**3**(4):253—61.
44. Meier RPH, Meyer J, Montanari E, Lacotte S, Balaphas A, Muller YD, et al. Interleukin-1 receptor antagonist modulates liver inflammation and fibrosis in mice in a model-dependent manner. *Int J Mol Sci* 2019;**20**(6).
45. Kamari Y, Shaish A, Vax E, Shemesh S, Kandel-Kfir M, Arbel Y, et al. Lack of interleukin-1α or interleukin-1β inhibits transformation of steatosis to steatohepatitis and liver fibrosis in hypercholesterolemic mice. *J Hepatol* 2011;**55**(5):1086—94.
46. Gieling RG, Wallace K, Han YP. Interleukin-1 participates in the progression from liver injury to fibrosis. *Am J Physiol Gastrointest Liver Physiol* 2009;**296**(6):G1324—31.
47. Esnault S, Kelly EA, Johnson SH, DeLain LP, Haedt MJ, Noll AL, et al. Matrix metalloproteinase-9-dependent release of IL-1β by human eosinophils. *Mediat Inflamm* 2019:7479107. 2019.
48. Yan C, Zhou L, Han YP. Contribution of hepatic stellate cells and matrix metalloproteinase 9 in acute liver failure. *Liver Int* 2008;**28**(7):959—71.
49. Tasdemir D, Tierney M, Sen R, Bergonzi MC, Demirci B, Bilia AR, et al. Antiprotozoal effect of artemisia indica extracts and essential oil. *Planta Med* 2015;**81**(12—13):1029—37.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jmii.2021.03.017.