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Complementary Efficacy of *Antrodia cinnamomea* Mycelia on Patients with Chronic Hepatitis C Virus Infection: A Randomized Controlled Pilot Clinical Study

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Abstract **Objectives:** Hepatitis C virus (HCV) can cause liver inflammation. The peginterferon and ribavirin (pegRiba) therapy are used but is still unsatisfactory by side effects. Many studies indicated that *Antrodia cinnamomea* mycelia (ACM) have beneficial effects on liver function. However, the effect of ACM in chronic hepatitis C (CHC) patients remains unclear. Thus, this study aims to investigate the effects of oral supplementation ACM in chronic hepatitis C patients with pegRiba therapy. **Design and interventions:** Sixty CHC subjects were randomly collected and were randomly assigned to pegRiba group (30 subjects), or combined treatment with oral ACM (4.68 g/day) (pegRiba +ACM, 30 subjects) for 24-48 weeks. **Main outcome measures:** The blood samples were taken to analysis levels of biochemical data, viral response, heavy metals, immune function, cytokines and antioxidant status. **Results:** The results of biochemical data, viral response, immune function, inflammatory cytokines and antioxidant status were not significantly different ($p<0.05$) between the pegRiba and pegRiba +ACM groups. However, the zinc level of the pegRiba +ACM group was increased significantly after the 12th week compared with baseline ($p<0.05$). In addition, lower aluminum, mercury and arsenic levels of the pegRiba +ACM group were presented when compared with baseline ($p<0.05$). **Conclusion:** This first pilot study of ACM in CHC patients indicates that the complementary effects of ACM did not induce adverse events during therapy. The oral supplementation ACM could reduce levels of heavy metals and lead to increased zinc level. Increased zinc level is negatively correlated with side effects during the therapy in CHC patients.

Keywords: *chronic hepatitis C, Antrodia cinnamomea, trace elements, heavy metals*

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1. Introduction

Hepatitis C is a well-noted infectious disease worldwide, marking the importance of exploring its therapeutic options. The prevalence of hepatitis C is 4.4%, with 420,000 people in Taiwan having been diagnosed from 1996 to 2005. As for the different counties with significant differences in its prevalence, areas with the highest prevalence of hepatitis C included Mioli at 10.5% and Chiayi at 10.3% and the lowest prevalence was in Kinmen at 0.4%. Patients infected with the hepatitis C virus may progress toward chronic hepatitis, liver cirrhosis, and liver cancer [1,2].

Hepatitis C virus (HCV) infection often progresses to liver cirrhosis. Patients infected with HCV may also progress toward chronic hepatitis, liver cirrhosis, and liver cancer [3,4]. The mechanisms underlying the progression of chronic hepatitis C (CHC) to liver cirrhosis and liver cancer are complex. Many studies indicate that there are higher oxidative stress and lower antioxidative substances, such as retinol, alpha-tocopherol, gamma-tocopherol, beta-cryptoxanthin, lycopene, alpha-carotene, beta-carotene, glutathione and selenium, in CHC patients compared to healthy individuals [5]. The changes in cellular immunity in CHC patients are one of the important reasons for these differences [6,7], leading to poor immunological response or dysfunction [8,9,10]. Presently, the treatment of hepatitis C, which involves immunotherapy, uses pegylated interferon –α combined with Ribavirin treatment. Hepatitis C patients who underwent combined treatment had a sustained virological response (SVR) of 50-70% [11], but noticeable side effects were noted with the interferon/ ribavirin therapy.

Antrodia cinnamomea (AC, synonym: *Antrodia camphorata*), inhabiting the rotten wood of *Cinnamomum*

kanehirai, is a rare and expensive traditional medicinal fungus in Taiwan. *A. cinnamomea* has been reported to have therapeutic and pharmacological activities, including antitumor [12], anti-inflammatory [13,14,15], antioxidative [16,17], immunomodulatory [18] and hepatoprotective effects [14,19,20]. The well-known hepatoprotective effects of AC have been found to enact beneficial effects in reducing serum AST and ALT levels from the liver, inhibiting lipid peroxidation, and increasing catalase and SOD activity [19]. Furthermore, polysaccharides from AC mycelia have been found to show anti-hepatitis B virus activity in the cell line model [21]. More importantly, metabolites from AC showed potent inhibitory activity on HCV protease. However, the clinical effect of *A. cinnamomea* in hepatitis C infection is still not clear. Thus, this clinical study aims to investigate the effects of oral supplementation *A. cinnamomea* mycelia (ACM) in chronic hepatitis C patients with peginterferon α-2a and ribavirin therapy. This is the first clinical study to investigate the effects of ACM. Study findings suggest that oral supplementation of ACM was safe for CHC subjects. In addition, the supplementation of ACM could reduce levels of heavy metals and increase zinc levels.

2. Materials and Methods

2.1. Materials

A. cinnamomea mycelia preparation and proximate composition analysis

The liquid fermented ACM product was produced as described previously [13]. In brief, the fungi species of *A. cinnamomea* BCRC 35398, purchased from the Bioresources Collection and Research Center in Food Industry Research and Development Institute (Hsinchu, Taiwan), was cultured in the broth consisting of the following ingredients (g/L): glucose, 1.0; soybean powder, 0.5; peptone, 0.5; $MgSO_4$, 0.01. The pH was adjusted to 4.0 with 1 N HCl and was incubated at 28°C, with continuous agitation at 90 rpm and aeration at 60 L/min for 12 days. The filtered mycelia were freeze-dried and stored at −20 °C for further use. The AOAC official procedures [methods 950.46.B, 984.13, 43.275, 968.08, and 991.43] were applied for the determination of moisture, crude protein, total crude lipid, ash and fiber contents of the freeze-dried mycelia powder. The previous method [13] in determining the water-soluble polysaccharide content in ACM was followed. In brief, the AC freeze-dried powder (10 g) was refluxed repeatedly at 90°C with deionized water (100 mL) and the extraction was performed in triplicate. The filtrates were combined and concentrated *in vacuo* to give 10 mL of residue. Three-fold ethanol (95%) was added to the concentrated filtrate in order to precipitate the polysaccharides overnight. The precipitate was then lyophilized to obtain the ACM water-soluble polysaccharide, and afterwards, the polysaccharide was determined by the phenol-H_2SO_4 method.

Patient selection

The study was comprised of 60 patients (28 women and 32 men) with CHC recruited from 2011 to 2013 in Kuang-Tien General Hospital. Patients with other forms of liver diseases such as active hepatitis A or B virus infection, liver cirrhosis, hepatocellular carcinoma, hemochromatosis, alcoholic liver diseases, and toxin exposures were excluded. All patients included in the study were tested positive for anti-HCV antibody and were not classified as acute cases of hepatitis, and they all began their treatment with peginterferon α-2a (Pegasys) and ribavirin (Robatrol). The protocol was approved by the Kuang Tein General Hospital Institute Review Board, and every patient signed an informed consent form before inclusion.

Study design

This study was approved by the institutional review board (IRB) of Kuang-Tien General Hospital (No: 10015). Participants followed a personalized treatment of peginterferon alfa-2a (Pegasys®) and ribavirin (Robatrol®). Affected individuals are orally given 180ug of peginteferon a-2a taken once a week and a daily dose of 1000 to 1200mg ribavirin as the treatment plan. They were instructed not to take any non-prescribed medication or antioxidant supplements. The investigator decided random allocation sequence but did not assign participants to interventions. The subjects were randomly (simple randomization) divided into two groups: (1) pegRiba group (n=30): subjects were only treated with peginterferon alfa-2a and ribavirin; (2) pegRiba + ACM group (n=30): subjects were not only treated with peginterferon alfa-2a and ribavirin but also had oral intake of ACM (4.68g/day; from Biotechnology Center, Grape King Inc., Taoyuan City, Taiwan). Prior to the treatment, blood samples were taken to test for hepatitis C virus genotype and the participants' baseline values. During the 4^{th}, 12^{th}, and 24^{th} week of treatment as well as post-treatment, the blood samples were tested for albumin, triglyceride, cholesterol, AST, ALT, ferritin, creatinine, complete blood count (WBC, RBC and platelet), hemoglobin, antioxidant status, inflammatory cytokines and heavy metals. Lastly, a final hepatitis C RNA viral test was required after the 24-week treatment. The participants, care providers, those assessing outcomes were blinded after assignment to interventions and only those who provide ACM know. Collectively, they were then statistically analyzed (Figure 1).

Outcome definitions

Treatment responses were defined as follows: rapid virological response (RVR), < 15 IU/ml after 4 weeks of therapy; early virological response (EVR), a greater than 2 log10 decrease in HCV RNA load from baseline after 12 weeks of therapy; and end-of-treatment virological response (ETVR), < 15 IU/ml at the end of treatment. SVR was defined as HCV RNA load < 15 IU/ml at least 24 weeks after the completion of the therapy. Non-SVR outcomes included non-response, viral breakthrough, and relapse. Non-response was defined as viral load > 15 IU/ml throughout the duration of therapy. Viral breakthrough was defined as HCV RNA load > 15 IU/ml after an initial response (HCV RNA load < 15 IU/ml) during treatment. Virologic relapse was defined as HCV RNA load < 15 IU/ml at the end of treatment but > 15 IU/ml during follow-up.

Biochemical determination and HCV-RNA analysis

Serum alanine aminotransferase (ALT), aspartate aminotransferase (AST), complete blood count, plasma

total cholesterol (TC), and triglyceride (TG) were determined by SE-900 Automatic Analyzer. Plasma albumin was determined by Hitachi 7060 Automatic Analyzer. Plasma ferritins, serum iron, Total Iron Binding Capacity (TIBC) were measured by Beckman coulter synchron LX20 Automatic Analyzer. HCV RNA was analyzed by Roche LightCycler. HCV genotype was determined by Roche LightCycler and Amersham Pharmacia Biosciences ABI 3730 XL DNA automatic sequencer. HCV RNA levels were evaluated by a real-time polymerase chain reaction assay (COBAS AmpliPrep HCV Monitor/COBAS TaqMan HCV test, Roche, Grenzach, Germany) with a lower limit of quantification of 15 IU/ml.

Immune function assay

Peripheral blood mononuclear cells (PBMCs) were isolated from heparinized blood by density gradient centrifugation using Ficoll-Hypaque (Sigma Chemical Co., St Louis, MO, USA). Expression surface markers of T cells were analyzed by multi-parameter flow cytometry on a Beckman Coulter EPICS XL flow cytometer (Beckman Coulter.) using the System II software and the tetraONE System (version 1.0; Beckman Coulter). The antibodies used in this study were: anti-CD3- FITC, anti-CD4- PE, anti-CD8- FITC, anti-CD19- FITC and anti-CD16- FITC (BD Pharmingen, San Diego, CA, USA). Study data were analyzed using the FlowJo software (Tree Star, Inc., San Carlos, CA).

The levels of cytokines assay

The supernatants from the PBMC stimulation assay were harvested and frozen at -20 °C until their use. Samples and serum were assayed for IFN-γ, IL-2, TNF-α, IL-6 and IL-10 using ELISA kits (eBioscience, San Diego, CA, USA) according to the manufacturer's instructions. All assays were performed in duplicate.

The Catalase status and liver lipid peroxidation assay

According to the method by Cohen and colleagues, 25 µL of serum was mixed with 975 µL of 6 mM H_2O_2/50mM potassium phosphate at pH 7.0. The decrease of A240nm in 2 min was recorded. The enzyme activity was calculated by E240 = 43.6 $M^{-1}cm^{-1}$. Following the protocol in Buege et al, a colorimetric method that measures the reaction product of thiobarbituric acid (TBA) with aldehydes such as malondialdehyde (MDA) was performed and showed a pink color compound that could be measured at 535 nm. The value was named TBARS.

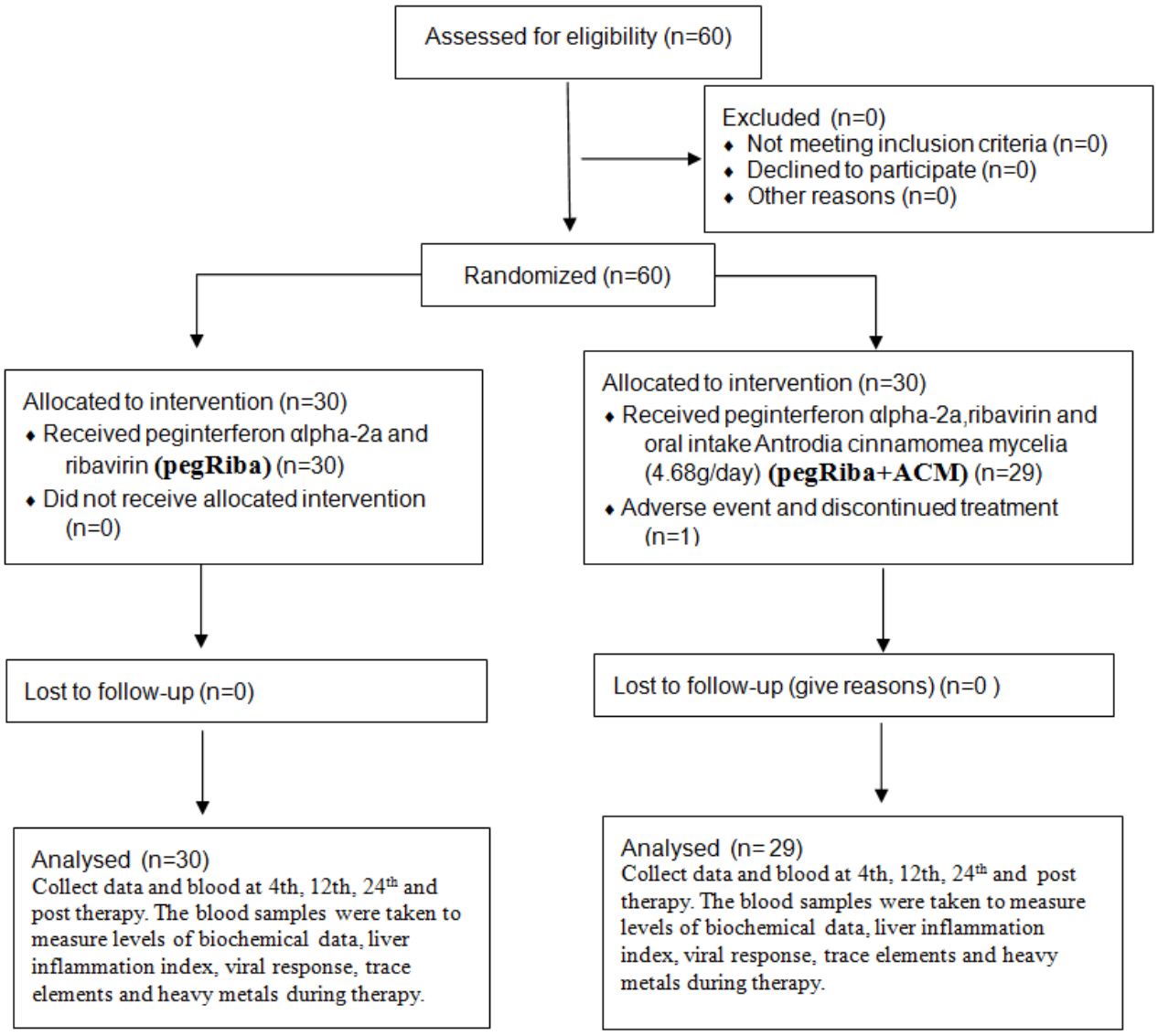

Figure 1. Flow Diagram on chronic hepatitis C patients with peginterferon α-2a and ribavirin therapy (pegRiba), and combined treatment of *A. cinnamomea* mycelia (pegRiba+ACM)

The trace elements and heavy metals assay

Trace elements and heavy metals in patient plasma were determined as described in a previous report (Langford-Smith et al., 2016) using inductively coupled plasma mass spectrometry (ICP-MS; 7900, Agilent Technologies, Santa Clara, CA, USA) according to the internal standard method and was expressed in μg/mL (ppm).

Statistical analysis

All data were expressed as mean ± SD. Significant differences between the two groups were determined by Student's t-test. Significant differences ($p<0.05$) at every time point were revealed by one-way ANOVA and unpair-*t* test. All calculations were performed with SPSS version 12 (SPSS Inc., Chicago, IL, USA).

3. Results

The main compositions of A. cinnamomea mycelia

The freeze-dried ACM powder used in the study has been determined with the highest amount of total carbohydrate (39.27%), the crude protein (32.01%) in the second, the crude fiber (10.07%), the crude fat (6.67%) and the lowest amount of crude ash (3.22%) as shown in our previous report (Chen et al., 2007). The Phenol-sulfuric acid method on the quantitative percentage of water-soluble polysaccharide of ACM was 2.92%. However, the water-insoluble residues after extraction might contain a relatively high percentage of polysaccharides, which would be effective on the contribution of immunomodulatory function.

Table 1. Baseline characteristics for patients before treatment with peginterferon and ribavirin

	Total	pegRiba[1]	pegRiba+ACM[2]
Sex (male/female)	32/28	15/15	16/13
Age	56 ±11	55±1.8	56.92.2
BMI (kg/m^2)	25±4.1	25.2±0.8	24.9±0.7
Hb (g/dL)	14.4±1.6	14.4±1.6	14.7±1.8
WBC(μL)	5874±1656	5985±1862	5760±1435
Lymphocyte (%)	37.6±9.1	36.2±9.6	39±8.4
Neutrophil (%)	51±12	53.2±12	48.7±11.5
Platelet (*10^3/μL)	178±51	181.3±52.3	174.5±50.7
ALT (U/L)	111.7±63.6	105.3±50.9	118.4±74.8
AST (U/L)	75.6±34.3	70.9±29.9	80.5±38.1
AC Sugar (mg/dL)	111.6±33.9	116.8±40.4	106.9±26.2
Creatinine (mg/dL)	0.79±0.19	0.97±0.18	0.78±0.03
TG (mg/dL)	159±311	156.14±303	163.4±324
Cholesterol (mg/dL)	175.5±32.8	177.7±28.4	173.2±37.4
HDL (mg/dL)	48.9±14.5	49.5±12.1	48.3±13.9
LDL (mg/dL)	99±33.2	99.3±31.3	98.6±35.5
Ferritin (ng/mL)	287.9±235.6	261±200	314±267
Serum iron (μg/dl)	121.1±53.2	127.8±57.8	111.8±47
HCV RNA (IU/ml)	$6.12\times10^6\pm1.3\times10^7$	$5.66\times10^6\pm1.60\times10^7$	$6.60\times10^6\pm1.04\times10^7$

[1, 2]Values are expressed as mean ± SD with n=30 and n=29, respectively.

Table 2. The viral responses and genotype in CHC patients treated with peginterferon and ribavirin (pegRiba) and combined with ACM therapy

	pegRiba[a]				pegRiba+ACM[a]			
	N	RVR[b]	EVR[c]	SVR	N	RVR[b]	EVR[c]	SVR[d]
Total subjects	30	25(83%)	5(17%)	25(83%)	29	22(75.9%)	7(24.1%)	22(75.9%)
Genotype 1a	2	1(50%)	1(50%)	2(100%)	0	-	-	-
Genotype 1b	8	5(63%)	3(38%)	5(63%)	9	7(77.8%)	2(22.2%)	5(55.6%)
Genotype 1a+2b	0	-	-	-	1	1(100%)	-	1(100%)
Genotype 1b+2a	2	2(100%)	0	2(100%)	1	1(100%)	-	1(100%)
Genotype 2a	9	9(100%)	0	8(89%)	8	5(62.5%)	3(37.5%)	6(75%)
Genotype 2b	2	1(50%)	1(50%)	1(50%)	2	2(100%)	0	2(100%)
Genotype 3a	0	-	-	-	1	1(100%)	0	1(100%)
Genotype 6	0	-	-	-	1	0	1(100%)	1(100%)
others	7	7(100%)	0	7(100%)	6	5(83.3%)	1(16.7%)	5(83.3%)

[a]Data included number of subjects and percentage.
[b]**RVR:** rapid virological response, HCV-RNA detect <50 IU/ml plasma at 4th week.
[c]**EVR:** early virological response, HCV-RNA detect <50 IU/ml plasma at 12th week.
[d]**SVR:** sustained virological response, HCV-RNA detect <50 IU/ml plasma at post-therapy 24th week.

The characteristics and clinical data of HCV patients before receiving pegylated interferon-α with ribavirin therapy

This study recruited 60 subjects (32 males and 28 females) and had 59 subjects completed study. The baseline characteristics and clinical data of the two groups' patients were summarized in Table 1. Both groups of patients had higher BMI, AST, ALT, TG and ferritin than normal values.

Out of the 59 participants, 12 patients were infected with HCV 1a or 1b genotype (32.2%) and 16 patients were infected with subtypes 2a or 2b (35.6%). Total of 79.7% of subjects had RVR (rapid virological response) while100% of patients had EVR (end of therapy virological response). There were 26 patients (79.7%) who had SVR (sustained virological response). However, there were 12 patients (20.3%) who had non-SVR or relapse, or was non-responsive (Table 2). The characteristics and clinical data of the two groups were summarized in Table 3. Throughout the 48-week intervention, safety of the ACM treatment was assessed using biochemical characteristics during the peginterferon with ribavirin therapy. Liver and kidney functions were not affected; however, the AMC group had higher AST and ALT than the control group during the 12^{th} and 24^{th} week of therapy but remained within normal reference limits. There were no adverse events nor any other complications reported.

Table 3. Impact of biochemical parameters of patient during the treatment of pegRiba and pegRiba combined with ACM

Items	Groups	Baseline	12^{th} week	24^{th} week	End point
Hb (g/dL)	pegRiba [1]	14.4±1.6	11.5±1.5	11.3±1.3	11.7±1.3
	pegRiba+ACM [2]	14.7±1.8	11.6±1.4	12.6±2.2	11.9±1.5
WBC(µL)	pegRiba	5985±1862	3472±1172	3221±1041	3768±1633
	pegRiba+ACM	5760±1435	3273±825	3426±959	5501±900
Lymphocyte (%)	pegRiba	36.2±9.6	38.4±10.6	35.8±6.8	31.8±6.6
	pegRiba+ACM	39±8.4	43±10.4	34.5±11	33.6±9.7
Neutrophil (%)	pegRiba	53.2±12	51.6±10.9	54.4±7	57.9±8.8
	pegRiba+ACM	48.7±11.5	47.1±10.7	55.2±9.8	56.2±10.6
Platelet ($*10^3$/µL)	pegRiba	181.3±52.3	135.7±46.8	138.9±44.9	147±60.5
	pegRiba+ACM	174.5±50.7	130.3±52.8	140.25±46	134.1±43.6
ALT (U/L)	pegRiba	105.3±50.9	30.9±19.5	31.9±23.3	27.3±18.6
	pegRiba+ACM	118.4±74.8	47.3±42.8*	54.7±51.7*	51.8±62.7*
AST (U/L)	pegRiba	70.9±29.9	33.7±15.9	34.5±20.9	31.8±16.8
	pegRiba+ACM	80.5±38.1	49.2±35*	57±57*	51.5±45.7*
AC Sugar (mg/dL)	pegRiba	116.8±40.4	107.8±21.2	102.5±10.1	121.7±53.6
	pegRiba+ACM	106.9±26.2	100.3±13	116±32.9	107.8±24
Creatinine (mg/dL)	pegRiba	0.97±0.18	0.92±0.27	0.9±0.3	0.82±0.39
	pegRiba+ACM	0.78±0.03	0.78±0.03	0.72±0.03	0.7±0.03
TG (mg/dL)	pegRiba	156.14±303	165.9±115	209±171	153.5±81.5
	pegRiba+ACM	163.4±324	195±272	178.1±104	130±59
Cholesterol (mg/dL)	pegRiba	177.7±28.4	147.3±27.1	154.8±15.2	153.6±33.8
	pegRiba+ACM	173.2±37.4	159±36.5	171.2±30	162±32
HDL (mg/dL)	pegRiba	49.5±12.1	39.8±10.5	42.9±10.7	37.9±8.5
	pegRiba+ACM	48.3±13.9	41.7±10	40±12.8	44.6±15.6
LDL (mg/dL)	pegRiba	99.3±31.3	83.5±24.5	84.7±20.2	84.8±28.8
	pegRiba+ACM	98.6±35.5	85±27	100±18.9	94.6±24.7
Ferritin (ng/mL)	pegRiba	261±200	479±276	542±357	779±1665
	pegRiba+ACM	314±267	1023±883	503±171	826±903
Serum iron (µg/dL)	pegRiba	127.8±57.8	125.1±50.1	94.1±29.4	136.6±99.9
	pegRiba+ACM	111.8±47	119.4±28	124.3±41.4	117.5±39.8

[1, 2] Values are expressed as mean ± SD with n=30 and n=29, respectively. Mean in the row with different superscript symbols are significantly different (p<0.05) by ANOVA and least significantly difference (LSD). *: compared with baseline.

Table 4. Impact on the immunity, cytokine and antioxidant status of HCV patients during pegRiba and pegRiba combined ACM therapy

	pegRiba [1]					
	0w	**4w**	**12w**	**24w**	**48w**	**End point**
Immune function (%)						
Total T	34.8±35.0	39.1±41.6	39.3±42.5	22.2±24.2$^{\#}$	6.3±19.6$^{*\#\&\blacksquare}$	48.3±43.1$^{\blacksquare\square}$
Total B	25.8±28.5	27.5±31.6	32.4±36.5	17.4±23.2$^{\&}$	6.6±20.0$^{*\#\&\blacksquare}$	48.0±41.2$^{*\#\blacksquare\square}$
CD3CD4	18.6±19.5	23.5±21.0	23.5±24.2	10.9±12.5$^{*\#\&}$	4.0±12.6$^{*\#\&\blacksquare}$	38.4±39.5$^{*\#\&\blacksquare\square}$
CD3CD8	16.2±17.9	16.4±21.1	20.3±22.8	11.3±14.4	2.3±7.8$^{*\#\&\blacksquare}$	36.3±39.7$^{*\#\blacksquare}$
NK	31.9±33.4	35.5±34.8	39.2±39.3	33.5±29.6	6.9±22.5$^{*\#\blacksquare}$	53.3±40.6$^{*\#\blacksquare\square}$
Cytokines (pg/ml)						
IFN-γ	53.6±40.2	49.5±39.2	39.7±44.3	21.8±43.2*$^{\#}$	2.0±5.8*$^{\#\&\blacksquare}$	50.2±121.1$^{\square}$
IL-2	29.4±19.0	30.3±26.7	32.9±34.6	16.7±19.3*$^{\#\&}$	2.5±9.0*$^{\#\&\blacksquare}$	14.1±20.7*$^{\#\&\square}$
TNF-α	20.2±16.4	14.2±10.6	8.9±7.6*$^{\#}$	3.7±3.5*$^{\#\&}$	0.6±1.9*$^{\#\&\blacksquare}$	5.1±3.8*$^{\#\&\square}$
IL-6	50.3±46.6	44.1±39.9	30.6±33.5*	13.4±19.9*$^{\#\&}$	1.7±5.5*$^{\#\&\blacksquare}$	16.3±16.6*$^{\&\#\square}$
IL-10	42.7±32.8	30.4±22.2*	23.3±18.8*	8.1±8.9$^{\#\&}$	1.8±5.3*$^{\#\&\blacksquare}$	7.4±9.0*$^{\#\&\square}$
Antioxidant status (μM)						
Catalase	47.3±32.6	39.5±17.9	39.1±26.9*	26.0±27.4$^{*\#\&\blacksquare}$	4.3±11.5$^{\square}$	36.8±31.6
TBARS	18.4±13.3	16.9±10.3	14.4±9.9	7.0±6.0$^{*\#\&}$	3.8±10.2$^{*\#\&}$	13.0±9.4$^{*\blacksquare\square}$
	pegRiba+ACM [2]					
	0w	**4w**	**12w**	**24w**	**48w**	**End point**
Immune function (%)						
Total T	26.9±24.6	37.7±37.5	37.8±45.3	20.5±22.4	7.1±25.5$^{*\#\&\blacksquare}$	36.6±41.0$^{\square}$
Total B	19.4±24.1	26.9±30.1	33.6±37.7*	17.9±24.3$^{\&}$	6.6±19.7$^{*\#\&\blacksquare}$	33.7±42.2$^{\blacksquare\square}$
CD3CD4	15.8±15.5	20.7±18.7	20.8±24.0	13.1±16.6	5.6±14.5$^{*\#\&\blacksquare}$	29.8±38.8$^{*\blacksquare\square}$
CD3CD8	11.2±11.6	17.8±19.7	17.0±22.2	7.7±8.9$^{\#}$	3.3±12.3$^{*\#\&\blacksquare}$	29.0±38.3$^{*\blacksquare\square}$
NK	28.3±29.1	35.6±32.2	42.8±39.1	35.1±29.8	6.9±22.0$^{*\#\blacksquare}$	40.9±43.7$^{\square}$
Cytokines (pg/ml)						
IFN-γ	42.1±34.3	40.5±33.1	34.0±38.6	30.4±95.8	4.0±10.0*$^{\#\&}$	40.3±121.6
IL-2	27.8±13.4	32.4±18.7	28.7±21.1	17.7±19.7*$^{\#\&}$	3.5±10.5*$^{\#\&\blacksquare}$	10.2±13.4*$^{\#\&\blacksquare\square}$
TNF-α	21.2±18.1	16.4±12.2*	9.5±7.4*$^{\#}$	4.4±4.0*$^{\#\&}$	1.6±3.9*$^{\#\&\blacksquare}$	5.3±5.9*$^{\#\&\square}$
IL-6	48.7±44.7	43.9±38.1	30.4±34.9*	7.0±9.1*$^{\#\&}$	11.5±37.4*$^{\#\&}$	15.7±17.7*$^{\#\&\blacksquare}$
IL-10	40.2±29.9	31.6±21.6	24.2±21.2*	10.2±13.5*$^{\#\&}$	1.8±4.4*$^{\#\&\blacksquare}$	6.3±10.4*$^{\#\&\square}$
Antioxidant status (μM)						
Catalase	49.3±33.4	47.4±23.2	34.8±22.5$^{*\#}$	26.2±31.6$^{*\#}$	14.0±48.8$^{*\#\&}$	26.9±30.0$^{*\#}$
TBARS	18.0±10.7	20.8±16.2*	14.4±10.0$^{*\#}$	7.1±6.2$^{*\#\&}$	2.6±6.8$^{*\#\&\blacksquare}$	12.6±12.0$^{*\#\blacksquare\square}$

[1, 2]Values are expressed as mean ± SD with pegRiba (n=30) and pegRiba+ACM (n=29) groups, respectively. Mean in the row with different superscript symbols are significantly different (p<0.05) by ANOVA and least significantly difference (LSD). *: compared with 0 week, $^{\#}$: compared with 4th week, $^{\&}$: compared with 12th week, $^{\blacksquare}$: compared with 24th week, $^{\square}$: compared with 48th week, $^{\$}$: compared with control and ACM groups.

The cytokines, antioxidant and immunologic status of HCV patients during therapy between control group and ACM group

In terms of the levels of cytokines, the levels of IFN-γ, IL-2, TNF-α, IL-6 and IL-10 were increased. Regarding antioxidant status, there was a higher catalase level in the control group, and there was a lower level of TBARS at the 24th and 48th week during therapy compared to the baseline (p<0.05) in the pegRiba + ACM group. There was no significant difference between the pegRiba and pegRiba +ACM groups (Table 4). As for the immunologic status, the proportion of total T cells, total B cells, CD4^{+} cells, CD8^{+} cells and NK cells were determined where they were increased baseline in both groups compared to their respective baselines during treatment. However, these parameters were not significantly different between the pegRiba and pegRiba +ACM groups during therapy.

The heavy metal and mineral levels of HCV patients during therapy in ACM group

During the therapy, there were decreased levels of chromium (Cr),aluminum (Al), mercury (Hg), arsenic (As), barium (Ba) and increased levels of zinc (Zn) in the pegRiba +ACM group compared to its baseline (Table 5).

Table 5. Impact on heavy metals and trace elements levels of HCV patients during pegRiba and pegRiba combined ACM therapy

	Group	Baseline (ppm)	4th week (ppm)	12th week (ppm)	24th week (ppm)	End point (ppm)
Se	pegRiba [1]	0.08±0.08	0.07± 0.06	0.09± 0.12	0.06± 0.06	0.07± 0.07
	pegRiba+ACM [2]	0.07±0.05	0.07± 0.05	0.08± 0.05	0.07± 0.07	0.06± 0.06
Zn	pegRiba	3.0± 1.02	3.07± 1.64	3.38± 1.47	3.45± 2.06	3.4± 2.0
	pegRiba+ACM	2.5± 0.74	2.73± 0.93	3.58± 1.30*	3.37± 1.67*	3.2± 1.77*
Cu	pegRiba	1.05± 0.26	1.10± 0.25	1.06± 0.26	1.17± 0.26	1.23± 0.38*
	pegRiba+ACM	1.10± 0.27	1.05± 0.19	1.09± 0.23	1.09± 0.23	1.23± 0.31
Cr	pegRiba	0.19± 0.28	0.19± 0.14	0.19± 0.17	0.09± 0.09	0.12± 0.12
	pegRiba+ACM	0.16± 0.18	0.17±0.17	0.17±0.12	0.09±0.08	0.06±0.06*#&
Mn	pegRiba	0.11±0.22	0.10±0.14	0.10±0.10	0.15±0.16	0.12±0.15
	pegRiba+ACM	0.1±0.16	0.11±0.15	0.10±0.11	0.13±0.09	0.14±0.09
Al	pegRiba	0.74±0.88	0.57±0.68	0.48±0.47	0.21±0.28*	0.33±0.32*
	pegRiba+ACM	0.70±0.71	0.44±0.49	0.54±0.52	0.29±0.29*	0.26±0.31*
Fe	pegRiba	3.08±2.06	4.77±2.07*	4.69±1.77*	3.91±1.72*	3.56±0.62
	pegRiba+ACM	4.3±1.54	5.1±1.78	4.97±1.89	3.97±2.17	3.56±1.08
Hg	pegRiba	0.5±1.24	0.37±1.22	0.16±0.73	0.24±1.09	0.03±0.07*
	pegRiba+ACM	0.55±1.60	0.32±1.01	0.01±0.03*	0.03±0.13*	0.01±0.01*
As	pegRiba	0.002±0.011	0.007±0.03	0.009±0.004	0.003±0.008	0.008±0.02
	pegRiba+ACM	0.004±0.02	0*	0.003±0.01	0*	0.002±0.01
Cd	pegRiba	0.008±0.01	0.013±0.02	0.01±0.01	0.011±0.015	0.003±0.009
	pegRiba+ACM	0.007±0.01	0.007±0.013	0.01±0.015	0.008±0.012	0.006±0.01
Pb	pegRiba	0.04±0.09	0.02±0.07	0.05±0.1	0.02±0.07	0.02±0.05
	pegRiba+ACM	0.03±0.08	0.03±0.07	0.01±0.03	0.01±0.03	0.01±0.04
Ni	pegRiba	0.11±0.22	0.10±0.09	0.07±0.08	0.06±0.08	0.07±0.08
	pegRiba+ACM	0.09±0.10	0.07±0.09	0.08±0.10	0.08±0.08	0.05±0.07
Co	pegRiba	0.005±0.013	0.003±0.01	0.01±0.02	0.009±0.014	0.006±0.016
	pegRiba+ACM	0.003±0.008	0.005±0.019	0.006±0.014	0.007±0.014	0.006±0.018
Ba	pegRiba	0.27±0.25	0.27±0.23	0.26±0.25	0.18±0.11	0.14±0.08*
	pegRiba+ACM	0.29±0.26	0.31±0.35	0.30±0.36	0.18±0.11	0.15±0.09*#

[1, 2] Values are expressed as mean ± SD with n=30 and n=29, respectively. Mean in the row with different superscript symbols are significantly different (p<0.05) by ANOVA and least significant difference (LSD). *: compared with baseline, #: compared with 4th week, &: compared with 12th week.

4. Discussion

This is the first double-blinded, randomized controlled clinical trial to investigate the effects and safety of 24-48 weeks of treatment with ACM (4.68 g/day). *A. cinnamomea* (AC) is a native mushroom parasitic on the endemic perennial tree, *Cinnamomum kanehirai* Hay in Taiwan [22]. Many studies have shown that ACM is composed of neutral monosaccharides, including mannose, glucose and xylose which are linked by a β-D-glucan chain, and has biological activity that can be used as an adjuvant of drugs and functional foods [23,24,25]. Regarding the safety of ACM, a 90-day subchronic toxicity test showed no systemic toxicity, and another study showed no teratogenic effects in pregnant female rats to ACM administration [26]. In addition, this ACM product had been obtained through FDA NDI No. 833. Throughout an 8-week intervention in hypertension subjects in a previous study, the safety of ACM treatment was assessed where liver and kidney functions as well as other biochemistry data were not affected and there were no adverse events nor any other complications reported [41]. In this study, the safety of ACM in 30 hepatitis C patients who had oral ACM (4.68g/day) during the 24-48 weeks of treatment was assessed where there were no adverse events or any abnormal biochemistry data. Oral supplement of ACM was used in conjunction with therapeutic drugs for HCV, and the treatment rate was similar to the control group, suggesting that there may be no opposing food-drug interaction.

When hepatocytes are damaged, the AST and ALT in the liver are released into the serum. Therefore, the AST and ALT levels are the most commonly used biochemical markers for assessing liver injury [27]. According to previous studies, there were decreased serum AST and ALT levels after ACM usage, which indicated that ACM had good liver-protective effects [19,28]. In addition, the polysaccharide-antrodan, isolated from ACM has been proved with beneficial hepatoprotective effects against lipopolysaccharide-induced acute hepatic damages [19]. In a previous clinical study, ACM could reduce serum AST, but serum ALT was not affected in hypertension subjects with 8 weeks ACM treatment [41]. As for the current study, we found that CHC subjects had decreased serum

AST and ALT levels during combined therapy. In the pegRiba +ACM group, there was also decreasing AST and ALT levels, but there were no significant differences between the ACM and control groups. The phenomenon of higher AST and ALT levels in pegRiba +ACM group than those in control group was also observed in our study with hepatitis B (data not shown). Therapy with ACM may trigger the immune system to attack the hidden HCV in liver cells, leading to induce higher AST and ALT levels.

In this study, we found that the pegRiba +ACM group of CHC subjects had a higher level of serum zinc than the control group. CHC patients were previously found to have lower zinc levels [29], and low zinc levels were correlated with hepatic disorders (viral hepatitis and cirrhosis) [30,31]. Zinc is an essential trace element, which is ubiquitously distributed in mammalian cells and is involved in various metabolic processes. Zinc can also reduce oxidative stress to protect liver cells [32,33]. Increase zinc levels of CHC patients can reduce some adverse side effects caused by INF-α/ribavirin therapy, but it has no effect on the treatment outcome results [34]. Of the constituents of ACM, the essential amino acids such as leucine, valine, isoleucine, phenylalanine and alanine of the five major components [42], were supposed to involve in the synthesis of endogenous antioxidant enzymes, i.e. GSH, SOD and CAT. Ameliorative effects of ACM on hepatic damages induced by inflammatory factors might be attributed from the enhancing effects on antioxidant activity. Furthermore, some trace elements such as zinc (25 ppm), manganese (36 ppm) and copper (3 ppm) present in ACM but not in placebo group (data not shown) might contribute in the antioxidant activity by acting as cofactors to antioxidant enzymes. Previous study has indicated that the zinc supplementation may increase cell cellular components of innate immunity (i.e. phagocytosis by macrophages and neutrophils, NK cell activity), the numbers of cytotoxic CD8 T-cells and antibody responses [43]. The decreased level of TBARS in serums after oral ACM might be attributed from the enhancement of antioxidant enzymes activity (Table 4). In addition, the increased levels of cytokine IFN-γ during the combined treatment of ACM described that the increasing of zinc levels in serum might modulate the immunity and is beneficial to the treatment of pegRiba on chronic hepatitis C patients. Regarding other heavy metals, CHC patients have higher Copper (Cu), Iron (Fe), Lead (Pb), Cadmium (Cd), and Aluminum (Al) [35]. It has been found to play an important role in inducing abnormal liver function [36]. The oxidative stress can also be produced by exposure to these metals and then induce liver inflammation to cause injury [37,38]. In addition, Mercury also induce ROS production and inhibit activity of antioxidant enzyme to cause oxidative stress and liver injury [39]. In animal model, mercury induce liver injury and ACM supplementation reduced mercury- induced oxidative stress and liver injury [40]. In this study, the first clinical study, we also found that supplementation of ACM could reduce levels of serum Chromium (Cr), Aluminum (Al), Barium (Ba), Arsenic (As), and especially Mercury (Hg), and increased zinc, which may play an important role of inducing protective liver function. Regarding the other heavy metals, the clinical study results indicated that the add-on oral supplementation of ACM to pegRiba could reduce levels of serum chromium (Cr), aluminum (Al), barium (Ba), arsenic (As), and especially mercury (Hg) (Table 5). A limitation of this study relies is the small number of subjects recruited. In future studies, we will include more subjects to confirm the levels of these markers.

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Author Contributions

All authors contributed extensively to the work presented in this paper.

Conflicts of Interest

All authors declare that they have no conflicts of interest, financial or otherwise.

This study was approved by the institutional review board (IRB) of Kuang-Tien General Hospital (No: 10015).

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